Interaction of pharmaceutical & personal care products (PPCPs) and endocrine disrupting contaminants (EDCs) with microbial communities in South African wastewater treatment works and environmental waters

by

Edward Archer

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Faculty of Science

Stellenbosch University

Supervisor: Prof. Gideon M. Wolfaardt

Department of Microbiology, Faculty of Science, Stellenbosch University,

Private Bag X1, Matieland, 7602, Stellenbosch, South Africa

Co-supervisors:

Prof. Johannes H. Van Wyk

Department of Botany and Zoology, Faculty of Science, Stellenbosch University

Private Bag X1, Matieland, 7602, Stellenbosch, South Africa

Dr. Barbara Kasprzyk-Hordern

Department of Chemistry, Faculty of Science, University of Bath, Claverton Down, Bath,

BA2 7AY, United Kingdom

March 2018

DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

Global surface waters are increasingly shown to be contaminated by anthropogenic chemical pollutants which, in turn exert potential lethal- and sub-lethal toxicity risks to the aquatic environment and humans. In particular, pollutants which are able to modulate endocrine system pathways, known as endocrine disrupting contaminants (EDCs) are of an emerging global concern. Treated wastewater discharge is a major contributing source of this pollution, with the recalcitrance and passage of various contaminants through wastewater treatment posing a risk to water security. This is highlighted as a critically important global challenge which need to be further addressed, especially for developing countries that are subjected to increased demands for clean water and sanitation services due to rapid population growth and urbanisation. Furthermore, routine monitoring and refinement of analytical methodologies for risk assessment are largely limited in the country, which points to the needed to assess the harmful impact of priority micro-pollutants in surface water systems. One of the aims of the present study was to assess the presence and fate of EDCs and other emerging contaminants (ECs) within a selection of South African wastewater treatment works (WWTWs) and associated environmental waters in order to refine the monitoring tools and methodology used for risk assessment approaches.

Endocrine-disrupting activities generated by *in vitro* steroid hormone receptor binding assays, namely the yeast (anti)estrogen screen (YES/YAES), highlighted the complexity when dealing with environmental samples containing a mixture of analytes. Even though notable reductions of estrogenicity by the WWTWs were measured, some remaining loads in effluent receiving river waters remained above risk-based trigger values, therefore potentially compromising human- and aquatic health. Estimation of the potential toxic masking by analytes with anti-estrogenic effect/activity highlighted further refinement that will be needed evaluating potential endocrine disrupting activity when applying bioassays for risk assessment. Both diurnal as well as seasonal variation in endocrine disrupting activities were recorded and discussed. Also, treated wastewater effluent served as a diluting medium to lower estrogenicity within recipient river waters at some study sites, and highlighted the contribution of alternative pollution sources that may significantly impact the quality of river systems.

Although EDCs are mostly assumed to be associated with steroid hormones, in the present study I conducted scoping studies at selected WWTWs and showed the extent of regularly-used pharmaceuticals & personal care products (PPCPs) and drugs of abuse (DOA) present

within wastewater and surface waters - having variable degradation profiles during wastewater treatment. In particular, ECs which were highlighted as priority micro-pollutants, such as anti-epileptics, non-steroidal anti-inflammatory drugs (NSAIDs), opioids and anti-depressants showed moderate- to negative removal during wastewater treatment, even during advanced activated sludge treatment processes. Although all of these pollutants are known to undergo biological degradation, the present study recommended further refinement of current treatment processes to improve on the removal of such persistent ECs. The need to define the environmental impact of EC breakdown-products were also discussed, as their potential health risks are largely unknown.

The dissertation also showed the value of urban water profiling to report on the use and abuse of licit and illicit DOA within communities connected to sewer networks at two study sites. Several prescription and over-the-counter (OTC) medications were detected within wastewater originating from domestic sewage, in particular opioids, an anaesthetic and anti-depressant drug – all of which are reported to be abused in South Africa, although limited statistics exist. For illicit DOA, the loads of cocaine, 3,4-methylenedioxymethamphetamine (MDMA), methamphetamine, heroin and the new psychoactive substance (NPS) mephedrone confirmed their consumption within the communities connected to the WWTWs, which were enriched by including the detection of their metabolic breakdown products, as well as enantiomeric profiling of the chiral drugs.

The present study encapsulated the benefit of urban water profiling to address current- and emerging global challenges for environmental- and human sustainability. Incorporation of the research outputs from the current study during refinement of risk-based approaches in South Africa may greatly improve water reclamation and management strategies to ultimately safeguard this valuable commodity for driving community- and environmental resilience.

OPSOMMING

Oppervlak water regoor die wêreld word toenemend besoedel deur menslike chemiese besoedelstowwe, wat verder getoon word om verskeie dodelike en sub-dodelike toksisteitsrisikos te veroorsaak in die akwatiese omgewing en mense. Besoedelstowwe wat in staat is om endokriene stelsels te belemmer, wat bekend staan as endokrien-versteurende verbindings (EVVs) is veral wêreldwyd van ‘n opkomende besorgdheid. ‘n Hoof bydraende bron van sulke besoedelstowwe is afkomstig van behandelde afvalwater wat na die omgewing lei, waar stowwe wat nie voldoende afgebreek word nie ‘n groot risiko vir water sekuriteit bied. Hierdie is ‘n krities-belangrike internasionale uitdaging wat verder aangespreek moet word, veral vir ontwikkelende lande wat onderworpe is aan verhoogde eise vir skoon water en sanitêre dienste as gevolg van vinnige bevolkingsgroei en verstedeliking. Roetiene monitering en verfyning van analitiese metodes om risikobepaling te doen is ook grootliks beperk in die land, wat dus die behoefde uitwys om die skadelike impak van prioriteit besoedelstowwe in oppervlakswaters te assesseer. Een doel van die studie was dus om die teenwoordigheid en lot van EVVs en ander opkomende kontaminante (OKs) te assesseer in verskeie Suid-Afrikaanse afvalwaterbehandelingswerke en die geaassosieerde omgewingswaters om ten einde die moniterinsinstrumente en metodes wat gebruik word vir risikobepaling te verfyn.

Endokriene ontwrigsaktiwiteiete wat gemeet was deur ‘n *in vitro* steroïedhormoon reseptorbindingstoets genaamd die gis (anti)oestrogeen toets (YES/YAES) het die kompleksiteit beklemtoon wanneer omgewingsmonsters, wat ‘n mengsel van komponente besit, gehanteer word. Alhoewel 'n noemenswaardige vermindering van oestrogenisiteit gemeet was gedurende afvalwaterbehandeling het sommige oorblywende vragte in uitvloeiselwater bo risiko-gebaseerde snellerwaardes gebly, wat verwys word om potensieël die mens- en watergesondheid te beïnvloed. Bepaling van die potensiële toksiese maskering van anti-oestrogeniese effekte/aktiwiteite het die nood van verdere verfyning gemerk as sulke toetse gebruik word vir risikobepaling. Beide seisoenale, sowel as daaglikse variasies in endokriene ontwrigtingsaktiwiteite by die verskillende studie terreine was ook bespreek. Dit was ook gewys dat behandelde afvalwater soms dien as 'n verdunningsmiddel om oestrogenisiteit in ontvanklike rivierwater te verminder. Die bydrae van alternatiewe besoedelingbronne wat die kwaliteit van rivierstelsels aansienlik kan beïnvloed was ook uitgelig.

Alhoewel EVVs meestal geassosieer word met steroïed hormone het ek verder omvangsbepalingstudies by geselekteerde afvalwaterbehandelingswerke gedoen. Die omvang van gereeld-gebruikte farmaseutiese- en persoonlike versorgingsprodukte asook dwelmmiddels was aangetoon. Hierdie stowwe het baie variasie in hul afbrekingsprofiele gewys binne die verskeie afvalwaterbehandelingswerke. OKs wat veral uitgelig was as prioriteits kontaminante, insluitend anti-epileptiese stowwe, nie-steroïdale anti-inflammatoriese middels (NSAIMs), opioïede en anti-depressante het matige- tot negatiewe verwydering getoon tydens afvalwaterbehandeling, selfs vir werke wat gevorderde geaktiveerde slykbehandeling prosesse gebruik. Alhoewel al hierdie besoedelstowwe bekend is dat hulle biologies afgebreek kan word, dui die studie daarop dat verdere verfyning van huidige behandelingsprosesse gedoen kan word om ten einde die behandeling van OKs te verbeter. Die behoefte om die omgewingsimpak van OK-afbreekprodukte te ondersoek was ook bespreek, aangesien hul potensiële gesondheidsrisiko's grootliks onbekend is.

Die verhandeling het ook die waarde van stedelike waterprofiel studies gewys om te rapporteer op die gebruik en misbruik van beide wettige en onwettige dwelmmiddels binne gemeenskappe wat aan rioolsisteme verbind is in twee studie areas. Verskeie voorskrif- en oor-die-toonbank (ODT) medikasies was opgespoor in afvalwater wat afkomstig is van huishoudelike rioolwater, veral opioïede, 'n narkose- en anti-depressante middel – waar al hierdie stowwe uitgelig word vir hul misbruik in Suid Afrika, alhoewel beperkte statistieke bekombaar is. Die teenwoordigheid van die onwettige dwelmmiddels kokaïen, 3,4-metielendioxymetamfetamien (MDMA), metamfetamien, heroïen en die nuwe psigoaktiewe stof (NPS) mephedrone in afvalwater het die gebruik van hierdie dwelms in die gemeenskap bevestig, wat verder verryk was deur die teenwoordigheid van hul metaboliese afbreekprodukte, sowel as enantiomeriese profilering van die chirale middels te ondersoek.

Die huidige studie het die samevattende voordele van stedelike waterprofiel studies uitgelig om huidige- en ontluikende globale uitdagings vir omgewings- en menslike volhoubaarheid aan te spreek. Deur die verfyning van risiko-gebaseerde benaderings te oorweeg wat uitgelig was in die huidige studie kan dus tot verbeterde waterherwinning en bestuurstrategieë lei om hierdie waardevolle kommoditeit te beskerm wat grootliks verantwoordelik is vir gemeenskaps- en omgewingsveerkragtigheid.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

11-KT 11-ketotestosterone
17α-MT 17-alpha-methyltestosterone
4-MBC ..... Benzedrone
ACTH..... Adrenocorticotropic hormone
ADHD ..... Attention Deficit Hyperactivity Disorder
AMPH ..... Amphetamine
AMR ..... Anti-microbial resistance
ANOVA ..... Analysis of Variance
AOP..... Adverse outcome pathway
ARV ..... Antiretroviral
AS ..... Activated sludge
ATS ..... Amphetamine-type stimulant
BC ..... Biological clarification
BEG..... Benzoylecgonine
BF..... Biological filtration
BNR ..... Biological nutrient removal
CBZ..... Carbamazepine
CBZ-*ep*..... Carbamazepine epoxide
CF..... Correction factor
CE ..... Cocaethylene
CHO ..... Chinese hamster ovary
clar ..... Clarification
COD ..... Chemical oxygen demand
CRH ..... Corticotrophin-releasing hormone
CRR..... Cumulative risk ratio
CYP..... Cytochrome P450
DDE ..... Dichlorodiphenyldichloroethylene
DDT ..... Dichlorodiphenyltrichloroethane
DHT ..... Dihydrotestosterone
*dh-h*CBZ ..... Dihydro-hydroxycarbamazepine
dio3 ..... Thyroxine 5-deiodinase
DMDCS ..... Dimethyl-dichlorosilane
DMV ..... Desmethylvenlafaxine
DMX ..... Dimethylxanthine
DOA..... Drugs of abuse
DST..... Department of Science and Technology
DTR..... Drug target residue
DWS..... Department of Water and Sanitation
E_1..... Oestrone
E_2..... Oestradiol
EC ..... Emerging contaminant
EC_{50}..... Effect concentration for 50% of test organisms
EDC..... Endocrine disrupting contaminant
EDSTAC ..... Endocrine Disruptor Screening and Testing Advisory Committee
EDTA..... Endocrine disrupters testing and assessment
EE_2 ..... Ethynyl-oestradiol
EF..... Enantiomeric fraction
eff..... Effluent

EMCDDA ..... European Monitoring Centre for Drugs and Drug Addiction
EPH ..... Ephedrine
EPS..... Extracellular polymeric substance
ERα ..... Estrogen receptor alpha
ERA..... Environmental risk assessment
ERDC..... U.S. Army Engineer Research and Development Centre
ERWAT ..... East Rand Water Care Company
ESI..... Electron spray ionisation
ETU..... Ethylenethiourea
FR..... Flow rate
FSH ..... Follicle-stimulating hormone
GD..... Green Drop
GnRH ..... Gonadotropin-releasing hormone
GWRC..... Global Water Research Coalition
hAR..... Human androgen receptor
hER ..... Human estrogen receptor
HMA 4-hydroxy-3-methoxyamphetamine
HMMA..... 4-hydroxy-3-methoxymethamphetamine
HPA..... Hypothalamic-pituitary adrenal
HPG..... Hypothalamic-pituitary gonadal
HPT..... Hypothalamic-pituitary thyroid
HRT..... Hormone replacement therapy
inf ..... Influent
JRC..... European Commission Joint Research Centre
KE ..... Key event
KER..... Key event relationships
K_{ow}..... Octanol-water coefficient
LC-MS ..... Liquid chromatography – mass spectrometry
LD_{50}..... Lethal dose for 50% of test organisms
LH ..... Luteinizing hormone
LOEC ..... Lowest observed effect concentration
MDMA..... 3,4-methylenedioxymethamphetamine
MEC..... Measured environmental concentration
MeOH ..... Methanol
METH ..... Methamphetamine
MIE ..... Molecular initiating event
ML..... Megalitre
MOA ..... Mode of action
MP..... Maturation ponds
MRM..... Multiple reaction monitoring
mRNA ..... Messenger ribonucleic acid
NDP..... National Development Plan
NDT ..... *N*-desmethyltramadol
NOEC..... No-observed effect concentration
NP ..... Nonylphenol
NPS ..... New psychoactive substance
NSAID ..... Non-steroidal anti-inflammatory drug
NTMP ..... National Toxicity Monitoring Programme
O-6-MAM *O*-6-monoacetylmorphine
ODT ..... *O*-desmethyltramadol

OECD..... Organisation for economic co-operation and development
OMC ..... Octyl-methoxycinnamate
OTC..... Over-the-counter
P ..... Progesterone
PE..... Population estimate
PEC ..... Predicted environmental concentration
*p*EPH..... Pseudoephedrine
PNEC ..... Predicted no-effect concentration
POP ..... Persistent organic pollutants
PPCP ..... Pharmaceuticals and personal care products
PTFE ..... Polytetrafluoroethylene
QC..... Quality control
RQ..... Risk quotient
rw ..... River water
SACENDU..... South African Community Epidemiology Network on Drug Use
SAID ..... Steroidal anti-inflammatory drug
SCORE..... Sewage CORE analysis group
SDG..... Sustainable development goals
SHBG..... Steroid hormone binding globulin
SPE..... Solid phase extraction
STW ..... Sewage treatment works
T ..... Testosterone
T_3..... Triiodothyronine
T_4..... Thyroxine
TCC..... Triclorocarbanilide
TCS ..... Triclosan
thrβ..... Thyroid hormone receptor beta
TR ..... Thyroid hormone receptor
TRH..... Thyrotropin-releasing hormone
TSH..... Thyroid-stimulating hormone
UNODC ..... United Nations Office on Drugs and Crime
USEPA..... United States Environmental Protection Agency
UV..... Ultraviolet
VTG ..... Vitellogenin
WBE..... Wastewater-based epidemiology
WHO..... World Health Organisation
WWTW..... Wastewater treatment works

CHAPTER 1: GENERAL INTRODUCTION

1.1. Rationale of the Thesis

Safe- and clean freshwater resources have become a generally scarce commodity on a global scale due to increasing pressures associated with human population growth and related agricultural and industrial development, which contribute towards organic- and inorganic pollution. The contribution of treated- and/or untreated wastewater are of the utmost relevance as a primary pollution source, whereby a huge variety of pollutants are simultaneously discharged, often without necessarily being regulated or efficiently removed during treatment (Bolong et al., 2009; Petrie et al., 2014; Verlicchi et al., 2012). Despite the aim to treat wastewater to comply with national standards, the presence and fate of micro-pollutants that may exert toxic effects on ecosystems and human health are regularly overlooked – collectively known as 'emerging contaminants' (ECs). Such toxicity potentials are not restricted to compounds showing lethal toxicity (a conventional parameter for risk management), but also include chemical substances which can exert sub-lethal toxic effects by disrupting endocrine system pathways of wildlife and humans over extended periods of exposure at environmental concentrations – collectively known as endocrine-disrupting contaminants (EDCs). In developing countries such as South Africa, these problems are exacerbated by a combination of high population growth and urbanization rates. Little has been done to date to mandate the inclusion of priority ECs into water quality legislation, largely due to limited studies showing the extent to which various micro-pollutants may impact both short- and long-term health outcomes. For this reason, it is vital to assess the sources of recalcitrant ECs within environmental waters, such as discharge from wastewater treatment works (WWTWs).

A WWTW may be seen as a series of interconnected ecosystems (modules) harbouring a diversity of microorganisms that are responsive to the varying abiotic conditions and the organic-rich influent wastewater medium. As a result, a vast array of metabolic processes will be at play for the biodegradation of organics. In WWTWs using activated sludge (AS) during secondary treatment, the increased contact to microbial aggregates and particulate matter facilitates sorption into the sludge, thereby lowering the loads within the aqueous phase which may then be subjected to further treatment. Such sorption is influenced by various factors, including the chemistry of the pollutant, redox conditions and other physical factors (temperature, pH, etc.) (Luo et al., 2014). For example, persistent compounds such as the anti-epileptic drug carbamazepine and the non-steroidal anti-inflammatory drug diclofenac are

known to have weak partitioning into solid material (Baker and Kasprzyk-Hordern, 2011; Radjenović et al., 2009), and as an effect may pass untreated or only partially treated. On the other hand, pharmaceuticals such as the opioid drug tramadol and antidepressant venlafaxine have high tendencies to be absorbed within sludge (Baalbaki et al., 2017; Boix et al., 2016; Falås et al., 2016), although the recalcitrance of these compounds in treated effluent still remain a concern. Furthermore, compounds such as the plasticizer Bisphenol-A show rapid sorption and desorption kinetics in AS (Banihashemi and Droste, 2014), highlighting the potential of such compounds to rapidly dissolve back into newly-introduced wastewater. Predicting their environmental risk is further complicated by the large variety of enzymes produced by the heterogeneous microbial communities in the different components of WWTPs, which may favour different metabolic pathways leading towards transformation into stable by-products instead of complete mineralization. Clearly, improved risk assessment and management of water resources require a comprehensive understanding of the link between the fate of organic micro-pollutants during wastewater treatment and microbiological interactions that are responsible for their degradation. Relevant tests and analytical procedures, combined with novel approaches that are responsive to new challenges presented in various combinations, are essential for the development of such an expertise base.

A promising approach for improved water management and risk strategies include the implementation of urban water profiling, also known as wastewater-based epidemiology (WBE; Castiglioni et al., 2016), which is increasingly also being adopted to estimate drug use and abuse through the detection of drug target residues (DTRs) in wastewater originating from communities connected to a sewer system network. As highlighted by the United Nations Office on Drugs and Crime (UNODC), the increase in substance abuse and distribution creates several pitfalls to sustainable social development, spanning from increased crime and health risks to higher rates of poverty and unemployment, especially in rural areas. Several social studies in South Africa have reported on the rising drug use problem in all parts of the country (Dada et al., 2017; Parry et al., 2017; Weich et al., 2017). There is also not sufficient information to accurately assess the extent of drug use and abuse within the country, especially for easily-obtainable prescription medications such as opiates and other new psychoactive substances (NPS).

1.2. Research Aims and Objectives

The overall goal of the research was to apply wastewater profiling as a strategy for improved risk assessment concerning surface water pollution, with emphasis on the recalcitrance of priority ECs. The chapters are presented in the dissertation in a journal article style, with specific key outcomes addressed in each chapter through case/scoping studies conducted in two provinces of South Africa. The study sites included multiple wastewater treatment works (WWTWs) in Gauteng Province, and one WWTW in the Western Cape Province, as well as environmental waters up- and downstream from the WWTPs. The specific objectives were:

- To compile a comprehensive literature overview to address the current knowledge regarding the presence of pharmaceuticals and personal care products (PPCPs) within South African surface waters, along with their associated risk to cause potential modulation of vertebrate endocrine systems – *Chapter 2*
- To expand the knowledge base regarding the fate of endocrine-disrupting activities during wastewater treatment and refinement of a cost-effective bioassay using effect-based risk assessment approaches – *Chapter 3*
- Monitoring of multiple DTRs to assess the fate of PPCPs and drugs of abuse (DOA) during wastewater treatment and within environmental surface waters, and to evaluated the result following conventional risk assessment approaches (ERA) and adverse outcome pathways (AOPs) – *Chapter 4 and 5*
- To introduce urban water profiling through wastewater-based epidemiology (WBE) in an African case study - *Chapter 6*
- To establish an analytical capacity to serve as a platform for multi-stakeholder partnerships between local- and international academic institutions and industry, which will set benchmarks for treatment plants' efficiency, aimed at enhancing biodegradation of ECs.

CHAPTER 2: A REVIEW ON PHARMACEUTICAL AND PERSONAL CARE PRODUCTS (PPCPs) IN SOUTH AFRICAN SURFACE WATERS, THEIR ASSOCIATED ENDOCRINE-DISRUPTING EFFECTS AND 21ST CENTURY RISK ASSESSMENT

Article:

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Declaration by the candidate

With regard to chapter 2, the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution
Manuscript writing, corresponding author	70%

The following co-authors have contributed to chapter 2:

Name	Email and affiliation	Nature of contribution	Extent of contribution
Prof. Gideon M. Wolfaardt	gmw@sun.ac.za Department of Microbiology, Stellenbosch University	Manuscript editing	30%
Prof. Johannes H. van Wyk	jhvw@sun.ac.za Department of Botany and Zoology, Stellenbosch University	Manuscript editing	

Declaration with signature in possession of candidate and supervisor

Abstract

Globally, water resources are under constant threat of being polluted by a diverse range of man-made chemicals, and South Africa is no exception. These contaminants can have detrimental effects on both human and wildlife health. It is increasingly evident that several chemicals may modulate endocrine system pathways in vertebrate species, and these are collectively referred to as endocrine disrupting contaminants (EDCs). Although the endocrine-disrupting effect of water pollutants has been mainly linked to agricultural pesticides and industrial effluents, other pollutants such as pharmaceuticals and personal care products (PPCPs) are largely unnoticed, but also pose a potentially significant threat. Here we present for the first time in a South African context, a summarised list of PPCPs and other EDCs detected to date within South African water systems, as well as their possible endocrine-disrupting effect *in vitro* and *in vivo*. This review addresses other factors which should be investigated in future studies, including endocrine disruption, PPCP metabolites, environmental toxicology, and antibiotic resistance. The challenges of removing EDCs and other pollutants at South African wastewater treatment works (WWTWs) are also highlighted. The need for focused research involving both *in vitro* and *in vivo* studies to detect PPCPs in water systems, and to delineate adverse outcome pathways (AOPs) of priority PPCPs to aid in environmental impact assessment (EIA), are discussed.

2.1. Introduction

Fresh water is an essential resource for the survival of all life on earth. It is globally recognised that humans are creating great pressure on the quality of our water resources by means of anthropogenic (man-made) pollutants entering freshwater systems (WHO, 2012). The major sources of freshwater pollutants typically originate from industry, domestic practices, and/or agriculture (Genthe et al., 2013). These practices introduce either non-degradable and/or harmful chemicals into water systems, thereby creating health risks to both wildlife and humans. Reductions in fertility, increases in the incidence of several cancers, spontaneous abortions, and a range of in-utero physiological disorders and birth defects have been linked to contaminants found in freshwater (Robins et al., 2011; Soto and Sonnenschein, 2010).

South Africa is a developing country, with a mid-year estimated population of 56.5 million people for 2017 (http://www.statssa.gov.za/). During the last formal 2011 census, the population was estimated at 51.8 million, of which 77.7% (40.3 million) are living in formal settlements, 7.9% (4.1 million) in traditional settlements, and 13.6% (7.1 million) in informal settlements (http://www.statssa.gov.za/). More recent statistics for 2016 showed an increase in formal housing (79.2%), and an increase in households having access to clean water supplies (from 70.9% in 2011 to 83.5% in 2016; http://www.statssa.gov.za/). These statistics therefore not only show the rapid increase in the country's population, but also highlight the rapid rate of urbanisation within the country, both of which are directly associated with increased demand for water and sanitation services.

Apart from the provision of clean water to the South African public, water treatment facilities are faced with increased pressures for the provision of improved sanitation services. Efficient operation of wastewater treatment works (WWTWs) is therefore important to remove pathogens and pollutants from surface waters, which might impact the health of both wildlife and human ecosystems. The performance of the WWTWs to remove pathogens and pollutants depends on several factors, such as the type of deployed treatment technologies, capacity, hydraulic retention time, as well as stakeholder requirements of the plant. However, the general target factor for all water treatment facilities is to improve on the quality of the water resource, and therefore ensure the health of the populations dependent on these resources. By extension, access to clean water supplies and proper sanitation services is therefore dependent on the performance of these facilities in order to adhere to water quality standards. As with many

countries worldwide, although most treatment processes are developed to successfully eliminate or lower the levels of pathogens and chemical pollutants to safe levels, this is not always the case in South African water treatment facilities. In a survey of 986 WWTWs in South Africa, it was shown that 50% of the plants are receiving less than 0.5 megalitres (ML) per day, 32% between 0.5 and 10 ML per day, and 17% more than 10 ML per day (Snyman et al., 2006). A suggested explanation for the occurrence of inefficient pathogen and micro-pollutant removal is the inadequate human resources for maintenance and operation of the plants. To assess the South African situation, the South African Department of Water and Sanitation (DWS) launched a Green Drop (GD) certification programme in 2008, to evaluate the performance of the country's wastewater works. This initiative was mandated to improve the quality of discharged effluent from wastewater treatment operations by awarding the operating bodies with a GD status if they comply with the DWS criteria for quality wastewater treatment (Ntombela et al., 2016). This initiative aimed to provide annual assessment reports on the operating efficiency of the plants, by awarding a cumulative risk rating (CRR) based on the design capacities (and hydraulic loading into receiving waters), operational flow relative to plant capacity, compliance/non-compliance of effluent quality being discharged into receiving waters, and compliance/non-compliance of technical skills utilised at the WWTWs (DWS, 2012, 2013). In 2012, the GD report has shown that of the 831 WWTWs assessed nationwide, 323 of these plants (39%) did not comply with the DWS standards, and 153 to 212 (18–26%) of all WWTWs received a critical and high-risk rating (Ntombela et al., 2016). Furthermore, some of the plants were reported to have unknown design capacities and/or not measure the plant influent at the required frequency, creating difficulties in reporting on the water quality these plants are treating. Although the 2013 GD report has shown an improvement in the overall CCRs of the assessed WWTWs for the 2012/13 year, it was still estimated that 49.6% of the WWTWs are still below 50% compliance (DWS, 2013). Furthermore, the DWS also awarded a Purple Drop status (critical state) to the 30.1% of the assessed WWTWs that achieved < 30% compliance (DWS, 2013). Taken from these reports, the high percentage of non-compliance with water quality and service delivery criteria therefore increases the risk for higher pathogen and harmful chemical loads in environmental waters. Although it is reported that the assessed WWTWs receiving a Purple Drop status in the 2013 GD report will be placed under regulatory surveillance (under the Water Services Act, Act 108 of 1997), the ongoing non-compliance of these treatment facilities creates great pressure on general surface water quality. This emphasizes the need to conduct environmental risk assessments (ERAs) to monitor both influents and effluents of water treatment facilities. Apart from the problem that

some WWTWs in South Africa do not comply with water quality standards and service delivery, another problem exists in that untreated river water is also not subjected to such water quality guideline initiatives, as this is not regarded as a drinking water resource in South Africa (Genthe et al., 2013). However, several rural communities depend on water taken directly from rivers for general daily activities, such as washing, cooking and consumption, as well as for agricultural purposes.

Due to the complexity and sheer volume of pollutants potentially present in natural water systems, global regulating bodies, such as the United States Environmental Protection Agency (USEPA) and the World Health Organisation (WHO), have set out a framework to investigate and identify environmental pollutants in freshwater systems (USEPA, 1997; WHO, 2012). These approaches consist of four main steps to be followed when doing impact assessment of water pollutants: (i) identifying the hazard to the environment, (ii) conducting dose-response assessments, (iii) exposure assessment of the pollutants to non-target organisms, and (iv) implementing risk characterisation for possible pollutants entering freshwater ecosystems (Fig. 2.1).

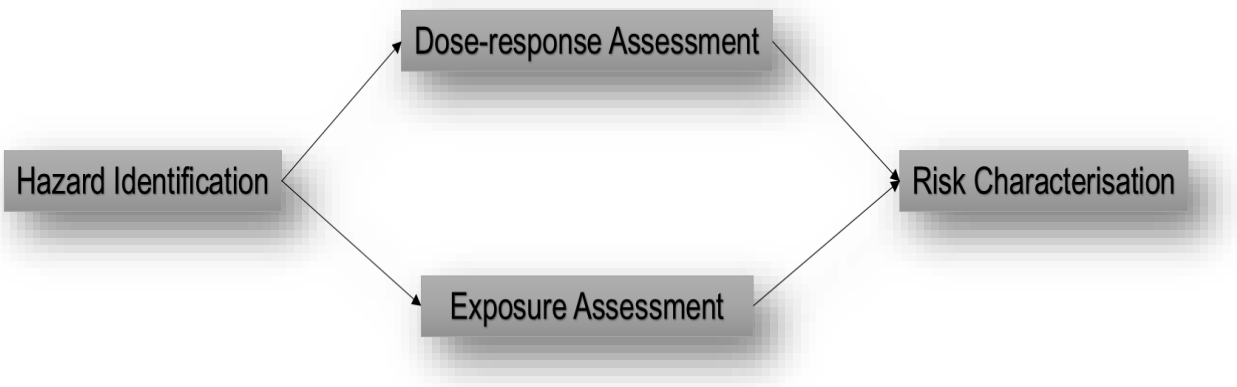

Figure 2.1: Framework for the identification and regulation of environmental pollutants in freshwater systems, as set out by the United States Environmental Protection Agency

By adhering to these approaches, the first line of investigation should include hazard identification of environmental pollutants entering freshwater systems. It is evident from literature that the identification of problematic areas in South Africa where water systems may be subjected to various pollutants coming from the abovementioned human sources (households, industry, and agriculture) is much needed. Also, water treatment facilities need to be a focus point for monitoring freshwater pollutants, especially in a developing country such

as South Africa, as these facilities can provide information regarding the origin of freshwater pollutants in areas of interest. In regard to the pollution of our natural water resources by human activities, some insight can be obtained by observing the wellbeing (health status) of wildlife populations within contaminated waters. Wildlife species inhabiting polluted freshwater supplies are in first-line contact with environmental pollutants and can provide useful information on the presence of pathogens in environmental waters and long-term exposure effects. Such sentinel species therefore serve as a valuable tool for hazard identification.

2.2. Hazard Identification

2.2.1. Endocrine disruptors and impacts on wildlife

Several micro-pollutants, or emerging contaminants (ECs), found in environmental waters have been linked to potentially causing a large variety of health effects in both invertebrates and vertebrates (Bolong et al., 2009; Daughton and Ternes, 1999; Mckinlay et al., 2008). In particular, selected pollutants have been suggested to interact with endocrine system pathways of vertebrates, and are collectively referred to as endocrine-disrupting contaminants (EDCs). The USEPA defines an EDC as: 'An exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behaviour' (USEPA, 1997). Man-made compounds most frequently implicated as EDCs include pesticides, pharmaceuticals, personal care products, and industrial by-products. Classic examples of environmental endocrine disruption include studies showing the feminisation of male fish and widespread occurrence of anti-androgenic ligands within UK rivers which receive effluent from connected WWTWs (Liney et al., 2006; Jobling et al., 2009). Guillette and co-workers published a series of accounts confirming the disruption of the male reproductive system in juvenile male alligators in several lakes (especially Lake Apopka) situated within Florida, USA (Guillette et al., 1999, 1996). Reproductive deformities, ranging from reduced penis size to altered plasma testosterone (T) levels were associated with extensive agricultural use of the insecticide DDT and other persistent organic pollutants (POP) leading towards non-point source pollution in water systems flowing into the lakes (Guillette et al., 1999, 1996). Along with the concerns about the general disruption of human reproductive systems leading to various detrimental effects such as ovarian cancer, breast cancer and declined sperm quality, international concerns were voiced regarding the potential subtle disruption of the endocrine systems of humans and wildlife during the organisational window

during development (Colborn et al., 1993). The documented reports on the occurrence of endocrine disruption within natural wildlife populations have raised international awareness of the harmful effects which man-made pollutants can exert on surface water quality for reuse.

EDCs are known to modulate either one of the three major axes of the endocrine system, namely the hypothalamus-pituitary-gonad (HPG), hypothalamus-pituitary-thyroid (HPT), and hypothalamus-pituitary-adrenal (HPA) axes (Fig. 2.2). Within these pathways, several hormones, metabolic enzymes and receptors are responsible for the dispersal, activity and function of various physiological traits in vertebrates. Due to the vast cross-talk between endocrine system axes, disruption of a particular component within one endocrine axis may also cause modulation of other endocrine systems. It is therefore evident that a cocktail of EDCs present in the environment can have a range of negative effects on vertebrate health through modulating various endocrine system pathways.

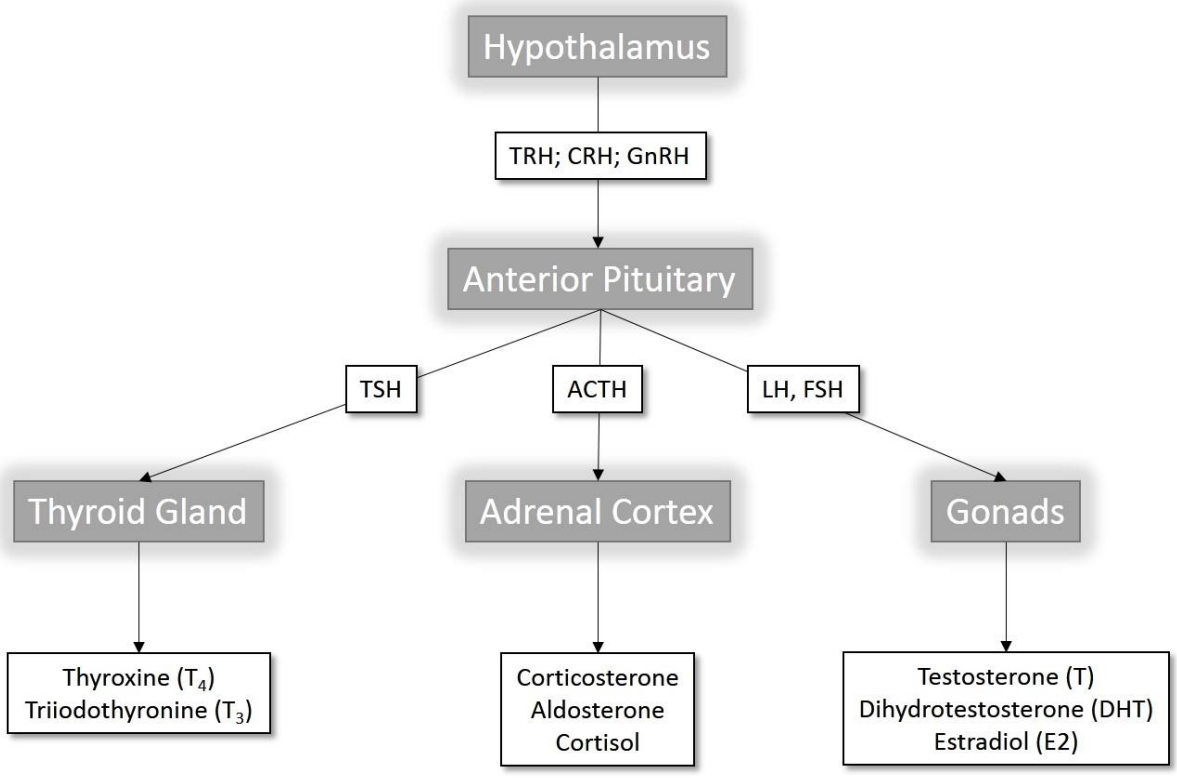

Figure 2.2: Basic representation of the three major endocrine system axes in humans mediated by hormonal signalling from the hypothalamus and anterior pituitary gland in the brain. TRH – thyrotropin-releasing hormone; CRH – corticotropin-releasing hormone; GnRH – gonadotropin-releasing hormone; TSH – thyroid-stimulating hormone; ACTH – adrenocorticotropic hormone; LH – luteinising hormone; FSH – follicle-stimulating hormone.

Environmental contaminants causing disruption of the reproductive endocrine system have been the focus of many EDC studies around the world, including South Africa, where varying concentrations of contaminants having known estrogenic endocrine-disrupting effects have been found in surface waters (Aneck-Hahn et al., 2009; Barnhoorn et al., 2004; Bornman and Bouwman, 2012; Genthe et al., 2013). Such studies may only be the tip of the iceberg, as increasing number of ECs are shown to have endocrine-disrupting activities. Although termed 'emerging contaminants', many of these contaminants have only recently been screened for their presence in the environment, despite being used for years; therefore, the full extent of their presence and associated risk is not fully understood.

Studies on gonadal abnormalities in wildlife living within polluted water systems have been done in South Africa, similar to those done at Lake Apopka in the United States. The presence of intersex in Sharptooth Catfish (*Clarias gariepinus*) has been observed at two impoundments at the Rietvlei Nature Reserve in the Gauteng Province (Barnhoorn et al., 2004; Kruger et al., 2013). Among the intersex fish, the presence of testicular oocytes was observed, in which the possible cause was linked to the presence of an industrial pollutant, p-nonylphenol (NP), in the water. The endocrine-disrupting activity of NP has been linked to its lipophilic properties and persistence in the environment (Folmar et al., 2002; Lech et al., 1996). Since the detection of endocrine disruption in freshwater fish, as well as the presence of POPs in the Rietvlei Nature Reserve, this area has been identified as a national priority area to monitor the presence of EDCs (Bornman and Bouwman, 2012). Further examples include populations of *C. gariepinus* in the Hartebeespoort Dam in the Gauteng province, where testicular abnormalities in male fish have been linked to the presence of POPs detected in the dam (Wagenaar et al., 2012). Intersex fish were also found in Mozambique Tilapia populations (*Oreochromis mossambicus*) at three impoundments in the Limpopo Province, which are also situated within an area that is intensively sprayed with DDT to combat malaria transmission (Barnhoorn et al., 2010). Sampling of *O. mossambicus* in the Loskop Dam (Mpumalanga province), which receives water from the Olifants River (a highly polluted river system), showed elevated plasma thyroxine (T_3) hormone levels and enlarged thyroid gland follicles, indicating potential thyroid-modulating EDCs in the water. In African Clawed Frog populations (*Xenopus laevis*), the presence of testicular ovarian follicles was observed in male frogs caught in the north-eastern region of South Africa, which are situated in areas of high agricultural pesticide usage (Du Preez et al., 2005). Male *X. laevis* frogs collected within impoundments in the Western Cape also situated near agricultural practices also showed modulation of testicular spermatogenic

development and altered plasma steroid- and thyroid hormone levels (Van Wyk et al., 2014). As these studies only aimed to link the presence of endocrine disruption in wildlife to pesticide contamination in water systems, the presence of other contaminants, such as pharmaceuticals and personal care products (PPCPs) or synthetic steroid hormones was most probably overlooked.

Although the harmful effects of man-made pollutants on wildlife species are well documented (Aneck-Hahn et al., 2009; Barnhoorn et al., 2004; Kruger et al., 2013; Wagenaar et al., 2012), no national monitoring programmes or water quality guidelines have been implemented in South Africa to assess and monitor the occurrence and frequency of pollutants affecting endocrine pathways of non-target organisms (Jooste et al., 2008). The clinical implications of EDC contamination in surface waters have also received little attention in South Africa, and the importance of using sentinel species as bio-indicators of water pollution is regularly overlooked, especially by assessing the health of these organisms up- and downstream of water treatment processes.

It is evident that most studies link the endocrine-disrupting effect observed in wildlife and water sources to the usage of agricultural pesticides. This assumption is supported by the fact that agriculture comprises a large percentage of a country's gross produce, and therefore also utilises large quantities of available surface water. Because of the notable dependence of food production on pesticides, various point (identifiable) or non-point (diffuse) pollution sources for surface and groundwater are anticipated. However, the presence of PPCPs is regularly overlooked. These chemicals are used on a daily basis for improved healthcare, personal hygiene and/or as daily supplements. It can therefore only be assumed that the presence of PPCPs in environmental waters may contribute even more towards EDC pollution in freshwater resources than pesticides used in households or agriculture. Although it is globally recognised and recorded that several classes of PPCPs are present in environmental waters (Blair et al., 2015; Kasprzyk-Hordern et al., 2009a; Petrie et al., 2014), not many studies have been done on PPCP pollutants present in South African waters (especially PPCPs acting as EDCs), and this therefore needs to be addressed in future studies.

2.3. Risk characterisation

2.3.1. Sources and emission routes of pharmaceuticals into the environment

Pharmaceuticals can enter the environment by various routes, including wastewater/sewage effluents and sludge from water treatment facilities, improper disposal of unused pharmaceuticals, in faeces and urine from livestock feedlots, and from waste products in PPCP-producing industries (GWRC, 2003). The two main sources of pharmaceuticals entering the environment are sewage from urbanised areas, ending up in sewage treatment works (STWs), and livestock feedlots using pharmaceuticals for growth promotion and disease control (Maletz et al., 2012). Through these pathways of exposure, several types of PPCPs (such as anti-inflammatory drugs, antibiotics, anti-epileptics, anti-depressants, skin care products, disinfectants, etc.) eventually end up at water treatment facilities with the hope that these chemicals are effectively removed before being discharged into rivers and impoundments downstream. Although the amount of PPCP waste products from producers is relatively low (GWRC, 2003), several other industries, such as hospitals and clinics, can contribute greatly to the discharge of PPCPs through sewage and wastewater into the environment (Al Aukidy et al., 2014; Maletz et al., 2012). One way of estimating the levels of PPCPs in the environment is to gather information regarding the usage of PPCPs by the general public in the area of concern.

2.3.2. Human pharmaceutical use in South Africa

In South Africa, as with many developing countries, the information about the presence of pharmaceuticals in environmental and drinking waters is limited to a few studies. These studies have been restricted to certain regions in the country, without multiple studies confirming the occurrence of PPCPs in the same areas. A national survey of pharmaceutical compounds present in South African waters has therefore not yet been conducted. However, the limited amount of studies done on the presence of pharmaceuticals in environmental and drinking water provides a good indication on the type of compounds present in water bodies, and also gives an indication of priority PPCPs for future screening.

Pharmaceutical usage may vary in the ratio of prescription and over-the-counter medication issued by the private vs. public health sectors. A study by Osunmakinde et al. (2013) listed 50 of the most prescribed pharmaceuticals in both the public and private health sectors of South Africa. From these lists, the analgesic paracetamol (acetaminophen) is shown to be the most prescribed drug in both sectors. Other pharmaceutical compounds included in the list are antibiotics amoxicillin, ampicillin, ceftriaxone, chloramphenicol, trimethoprim and

sulfamethoxazole, the beta-blocker atenolol, and contraceptives containing levonorgestrel and the synthetic estrogen ethynyl-oestradiol (EE_2) (Osunmakinde et al., 2013). In the private health sector, analgesics are the most prescribed, followed by antihistamines, bronchodilators, and antibiotics at second, third and fourth, respectively (Osunmakinde et al., 2013). In the public health sector, analgesics are also the most prescribed, followed by hypotensives, antiretrovirals (ARVs), and antibiotics at second, third and fourth, respectively (Osunmakinde et al., 2013). For both the public and private health sectors, it is shown that hypertension medication, analgesics, ARVs, antibiotics, non-steroidal anti-inflammatory drugs (NSAIDs), antidiabetics and antihistamines are the most common prescribed medications in South Africa. Therefore, it can be expected that water systems may contain a large amount of different types of pharmaceutical compounds in South Africa.

2.3.3. Pharmaceuticals and steroid hormones detected in South African waters

Initial detection studies of EDCs in South Africa consisted of steroid hormone detection (especially estrogens) in water systems (Table 2.1). This is due to the ubiquitous usage of synthetic estrogens as contraceptives and hormone replacement therapy (HRT) by a large percentage of the population. These hormones were shown to originate from human excretions and improper disposal of pharmaceuticals into sewage (Manickum et al., 2011; Manickum and John, 2014; Swart and Pool, 2007). However, it is increasingly becoming known that several types of PPCPs are also accumulating in water systems to the same extent as contraceptive medications. These compounds can serve as EDCs and are not completely removed during water treatment (Ncube et al., 2012). From these contaminants, pharmaceuticals stand out as one of the sources which might potentially cause endocrine-disrupting activities in non-target organisms. Although it has been globally recognised that pharmaceutical compounds do enter surface waters, the detection of PPCPs in water systems has only recently been done in the country (Table 2.1). To our knowledge, this summarised table is novel on both a local- and African scale by depicting the current knowledge and research to date regarding trace levels of PPCPs and hormones in South African surface waters. These detections provide valuable information regarding the presence of pharmaceutical drugs in South African waters.

Table 2.1: List of pharmaceuticals and steroid hormone concentrations (in µg.L^{-1}) detected in South African WWTWs and surface waters

Pharmaceutical group / Active Ingredient	Concentration (µg.L^{-1})	Location (Province)	Source	Reference
NSAIDs				
Acetaminophen	5.8 – 58.7	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2014
	5.8	KwaZulu-Natal	WWTW influent	Matongo et al., 2015
	1.0 – 1.7	KwaZulu-Natal	Surface water	Matongo et al., 2015
	136.9 – 343.6	Gauteng	WWTW influent	Archer et al., 2017a
	0.04 – 0.2	Gauteng	WWTW effluent	Archer et al., 2017a
	0.02 – 0.2	Gauteng	Surface water	Archer et al., 2017a
Aspirin	2.2 – 10.0	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2014
	13.7 - 25.4	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2016
Diclofenac	1.1 - 15.6	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2014
	222.7	KwaZulu-Natal	WWTW influent	Agunbiade and Moodley, 2016
	123.7	KwaZulu-Natal	WWTW effluent	Agunbiade and Moodley, 2016
	0.6 – 8.2	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2016
	2.7 – 5.6	Gauteng	WWTW influent	Archer et al., 2017a
	2.2 – 2.5	Gauteng	WWTW effluent	Archer et al., 2017a
	0.3 – 2.2	Gauteng	Surface water	Archer et al., 2017a
Ibuprofen	0.8 – 18.9	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2014
	39.8	Gauteng	WWTW influent	Amdany et al., 2014
	12.6	Gauteng	WWTW effluent	Amdany et al., 2014b

Table 2.1 (continued)

	111.9	Gauteng	WWTW influent	Amdany et al., 2014b
	24.6	Gauteng	WWTW effluent	Amdany et al., 2014b
	0.02	Gauteng	WWTW influent	Osunmakinde et al., 2013
	1.2	KwaZulu-Natal	WWTW influent	Agunbiade and Moodley, 2016
	1.1	KwaZulu-Natal	WWTW effluent	Agunbiade and Moodley, 2016
	0.4 – 0.7	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2016
	62.8	KwaZulu-Natal	WWTW influent	Matongo et al., 2015
	58.7	KwaZulu-Natal	WWTW effluent	Matongo et al., 2015
	0.5 – 8.5	KwaZulu-Natal	Surface water	Matongo et al., 2015
	9.1 – 15.8	Gauteng	WWTW influent	Archer et al., 2017a
	0.3 – 1.2	Gauteng	WWTW effluent	Archer et al., 2017a
	0.1 – 0.6	Gauteng	Surface water	Archer et al., 2017a
Ketoprofen	0.4 – 8.2	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2014
	1.1 – 2.0	KwaZulu-Natal	Surface water	Madikizela et al., 2014
	1.7 – 6.4	KwaZulu-Natal	WWTW influent	Madikizela et al., 2014
	1.2 – 4.3	KwaZulu-Natal	WWTW effluent	Madikizela et al., 2014
	0.02	Gauteng	WWTW influent	Osunmakinde et al., 2013
	0.0001	Gauteng	WWTW effluent	Osunmakinde et al., 2013
	3.2	KwaZulu-Natal	WWTW influent	Agunbiade and Moodley, 2016
	0.4	KwaZulu-Natal	WWTW effluent	Agunbiade and Moodley, 2016

Table 2.1 (continued)

	0.4 – 0.7	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2016
	0.4 – 5.6	Gauteng	WWTW influent	Archer et al., 2017a
	0.2 – 0.7	Gauteng	WWTW effluent	Archer et al., 2017a
	0.01 – 0.8	Gauteng	Surface water	Archer et al., 2017a
Naproxen	55.0	Gauteng	WWTW influent	Amdany et al., 2014b
	13.5	Gauteng	WWTW effluent	Amdany et al., 2014b
	52.3	Gauteng	WWTW influent	Amdany et al., 2014b
	20.4	Gauteng	WWTW effluent	Amdany et al., 2014b
	2.9 – 5.5	Gauteng	WWTW influent	Archer et al., 2017a
	1.8 – 2.9	Gauteng	WWTW effluent	Archer et al., 2017a
	0.2 – 1.9	Gauteng	Surface water	Archer et al., 2017a
Antibiotics/Biocides				
Ampicillin	2.5 – 14.5	KwaZulu-Natal	River water	Agunbiade and Moodley, 2014
	6.6	KwaZulu-Natal	WWTW influent	Agunbiade and Moodley, 2016
	8.9	KwaZulu-Natal	WWTW effluent	Agunbiade and Moodley, 2016
	3.2 – 5.5	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2016
Chloramphenicol	0.5 – 10.7	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2014
Erythromycin	0.6 – 22.6	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2014
	0.6	KwaZulu-Natal	WWTW influent	Matongo et al., 2015
	0.2	KwaZulu-Natal	WWTW effluent	Matongo et al., 2015

Table 2.1 (continued)

	0.1 – 0.2	KwaZulu-Natal	Surface water	Matongo et al., 2015
Fluoroquinolones	0.09 – 0.1	Western Cape	WWTW influent	Hendricks and Pool, 2012
	0.07 – 0.09	Western Cape	STW effluent	Hendricks and Pool, 2012
	0.7 – 16.9	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2014
	27.1	KwaZulu-Natal	WWTW influent	Agunbiade and Moodley, 2016
	20.5	KwaZulu-Natal	WWTW effluent	Agunbiade and Moodley, 2016
	2.4 – 14.3	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2016
Nalidixic acid	1.7 – 30.8	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2014
	29.9	KwaZulu-Natal	WWTW influent	Agunbiade and Moodley, 2016
	25.2	KwaZulu-Natal	WWTW effluent	Agunbiade and Moodley, 2016
	12.4 – 23.5	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2016
Streptomycin	0.8 – 8.4	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2014
Sulfamethoxazole	3.68	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2014
	0.1 – 0.2	Western Cape	WWTW influent	Hendricks and Pool, 2012
	0.08 – 0.1	Western Cape	STW effluent	Hendricks and Pool, 2012
	34.5	KwaZulu-Natal	WWTW influent	Matongo et al., 2015
	1.2 – 5.3	KwaZulu-Natal	Surface water	Matongo et al., 2015
	0.6 – 2.6	Gauteng	WWTW influent	Archer et al., 2017a
	1.2 – 1.6	Gauteng	WWTW effluent	Archer et al., 2017a
	0.6 – 1.4	Gauteng	Surface water	Archer et al., 2017a

Table 2.1 (continued)

Tetracycline	0.6 – 5.7	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2014
Trimethoprim	0.3	KwaZulu-Natal	Surface water	Matongo et al., 2015
	4.5 – 11.1	Gauteng	WWTW influent	Archer et al., 2017a
	1.2 – 1.6	Gauteng	WWTW effluent	Archer et al., 2017a
	0.3 – 1.1	Gauteng	Surface water	Archer et al., 2017a
Tylosin	0.2 – 22.0	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2014
Biocide				
Triclosan	78.4	Gauteng	WWTW influent	Amdany et al., 2014b
	10.7	Gauteng	WWTW effluent	Amdany et al., 2014b
	127.7	Gauteng	WWTW influent	Amdany et al., 2014b
	22.9	Gauteng	WWTW effluent	Amdany et al., 2014b
	0.4 – 0.9	KwaZulu-Natal	Surface water	Madikizela et al., 2014
	2.1 – 9.0	KwaZulu-Natal	WWTW influent	Madikizela et al., 2014
	1.3 – 6.4	KwaZulu-Natal	WWTW effluent	Madikizela et al., 2014
Beta-blockers				
Atenolol	1.0 – 39.1	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2014
	1.6 – 2.5	Gauteng	WWTW influent	Archer et al., 2017a
	0.4 – 0.7	Gauteng	WWTW effluent	Archer et al., 2017a
	0.1 – 0.5	Gauteng	Surface water	Archer et al., 2017a
Pindolol	0.03	Gauteng	WWTW influent	Osunmakinde et al., 2013

Table 2.1 (continued)

	0.00003	Gauteng	WWTW effluent	Osunmakinde et al., 2013
Anti-epileptics				
Carbamazepine	0.02 – 0.3	Free State	Drinking water	Patterton, 2013
	0.01 – 0.02	KwaZulu-Natal	Drinking water	Patterton, 2013
	0.01	Gauteng	Drinking water	Patterton, 2013
	0.03 – 0.1	Gauteng	Drinking water	Patterton, 2013
	0.01	Gauteng	WWTW influent	Osunmakinde et al., 2013
	2.2	KwaZulu-Natal	WWTW influent	Matongo et al., 2015
	0.9	KwaZulu-Natal	WWTW effluent	Matongo et al., 2015
	0.1 – 3.2	KwaZulu-Natal	Surface water	Matongo et al., 2015
	0.3 – 0.6	Gauteng	WWTW influent	Archer et al., 2017a
	0.4	Gauteng	WWTW effluent	Archer et al., 2017a
	0.2 – 0.3	Gauteng	Surface water	Archer et al., 2017a
Anti-psychotic				
Clozapine	8.6	KwaZulu-Natal	WWTW influent	Matongo et al., 2015
	9.6	KwaZulu-Natal	WWTW effluent	Matongo et al., 2015
	2.2 – 8.9	KwaZulu-Natal	Surface water	Matongo et al., 2015
Lipid regulators				
Bezafibrate	0.8 – 8.7	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2014
	0.2	KwaZulu-Natal	WWTW influent	Agunbiade and Moodley, 2015

Table 2.1 (continued)

	0.03	KwaZulu-Natal	WWTW effluent	Agunbiade and Moodley, 2016
	0.003 – 0.2	KwaZulu-Natal	Surface water	Agunbiade and Moodley, 2016
	1.4 – 3.0	Gauteng	WWTW influent	Archer et al., 2017a
	0.3 – 0.7	Gauteng	WWTW effluent	Archer et al., 2017a
	0.05 – 0.4	Gauteng	Surface water	Archer et al., 2017a
Antivirals				
Ribavirin	0.02	Gauteng	WWTW influent	Osunmakinde et al., 2013
	0.00004	Gauteng	WWTW effluent	Osunmakinde et al., 2013
Famciclovir (Famvir)	0.02	Gauteng	WWTW influent	Osunmakinde et al., 2013
	0.00006	Gauteng	WWTW effluent	Osunmakinde et al., 2013
Tenofovir	0.25	Gauteng	Surface water	Wood et al., 2015
	0.16 – 0.19	Free State	Surface water	Wood et al., 2015
Zalcitabine	0.07	Free State	Surface water	Wood et al., 2015
	0.03	Gauteng	Surface water	Wood et al., 2015
	0.008	Gauteng	Tap water	Wood et al., 2015
Lamivudine	0.09 – 0.24	Gauteng	Surface water	Wood et al., 2015
Didanosine	0.05	Free State	Surface water	Wood et al., 2015
Stavudine	0.41 – 0.78	Gauteng	Surface water	Wood et al., 2015
Zidovudine	0.22 – 0.62	Gauteng	Surface water	Wood et al., 2015
	0.45 – 0.97	Gauteng	WWTW effluent	Wood et al., 2015

Table 2.1 (continued)

	0.05	Gauteng/Free State	Surface water	Wood et al., 2015
	0.07	Gauteng	Tap water	Wood et al., 2015
Nevirapine	0.24 – 1.48	Gauteng	Surface water	Wood et al., 2015
Lopinavir	0.28 – 0.31	Gauteng	Surface water	Wood et al., 2015
	0.13	Gauteng	WWTW effluent	Wood et al., 2015
Human indicators				
Caffeine	4.5	KwaZulu-Natal	WWTW influent	Matongo et al., 2015
	0.6	KwaZulu-Natal	WWTW effluent	Matongo et al., 2015
	0.1 – 3.3	KwaZulu-Natal	Surface water	Matongo et al., 2015
	5.1 – 1214.4	Gauteng	WWTW influent	Archer et al., 2017a
	0.5 – 3.8	Gauteng	WWTW effluent	Archer et al., 2017a
	0.6 – 6.6	Gauteng	Surface water	Archer et al., 2017a
Steroid hormones				
Oestrone (E_1)	0.001 – 0.03	KwaZulu-Natal	WWTW downstream	Manickum and John, 2014
	0.01 – 0.02	Western Cape	STW downstream	Swart et al., 2011
	0.009 – 0.011	Western Cape	STW efffluent	Swart and Pool, 2007
	0.01	Western Cape	STW efffluent	Swart and Pool, 2007
	0.003 – 0.02	KwaZulu-Natal	STW effluent	Manickum et al., 2011
	0.01 – 0.35	KwaZulu-Natal	WWTW influent	Manickum and John, 2014
	0.003 – 0.08	KwaZulu-Natal	WWTW effluent	Manickum and John, 2014

Table 2.1 (continued)

	0.02 – 0.02	Western Cape	STW influent	Swart et al., 2011
	0.01 – 0.02	Western Cape	STW downstream	Swart et al., 2011
	0.002 – 0.004	Gauteng	Drinking water	Van Zijl et al., 2017
	0.0004 – 0.001	Western Cape	Drinking water	Van Zijl et al., 2017
Oestradiol (E_2)	0.001 – 0.03	KwaZulu-Natal	WWTW upstream	Manickum and John, 2014
	0.002 – 0.07	KwaZulu-Natal	WWTW downstream	Manickum and John, 2014
	0.001	Western Cape	STW effluent	Swart and Pool, 2007
	0.005	Western Cape	STW effluent	Swart and Pool, 2007
	0.01 – 0.02	KwaZulu-Natal	STW effluent	Manickum et al., 2011
	0.02 – 0.20	KwaZulu-Natal	WWTW influent	Manickum and John, 2014
	0.004 – 0.11	KwaZulu-Natal	WWTW effluent	Manickum and John, 2014
	0.001 – 0.03	Mpumalanga	Surface water	Van Wyk et al., 2014
	0.04 – 0.37	Gauteng	Drinking water	De Jager et al., 2013
	0.05 – 0.37	Western Cape	Drinking water	De Jager et al., 2013
	0.00003	Gauteng	Drinking water	Van Zijl et al., 2017
	0.00002 – 0.00005	Western Cape	Drinking water	Van Zijl et al., 2017
Ethynyl-oestradiol (EE_2)	0.003	KwaZulu-Natal	WWTW upstream	Manickum and John, 2014
	0.001 – 0.004	KwaZulu-Natal	WWTW downstream	Manickum and John, 2014
	0.01 – 0.095	KwaZulu-Natal	WWTW influent	Manickum and John, 2014
	0.001 – 0.008	KwaZulu-Natal	WWTW effluent	Manickum and John, 2014

Table 2.1 (continued)

	0.001 – 0.01	Mpumalanga	Surface water	Van Wyk et al., 2014
	0.00002	Gauteng	Drinking water	Van Zijl et al., 2017
Progesterone (P)	0.01	KwaZulu-Natal	WWTW upstream	Manickum and John, 2014
	0.06	KwaZulu-Natal	WWTW downstream	Manickum and John, 2014
	0.16 – 0.90	KwaZulu-Natal	WWTW influent	Manickum and John, 2014
	0.03	KwaZulu-Natal	WWTW effluent	Manickum and John, 2014
Testosterone (T)	0.005 – 0.02	KwaZulu-Natal	WWTW upstream	Manickum and John, 2014
	0.003 – 0.02	KwaZulu-Natal	WWTW downstream	Manickum and John, 2014
	0.12 – 0.64	KwaZulu-Natal	WWTW influent	Manickum and John, 2014
	0.03	KwaZulu-Natal	WWTW effluent	Manickum and John, 2014

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Although there has been limited information linking these contaminants to wildlife and human health disorders locally, the mechanism of physiological action is well established for all of these compounds. A worrying factor from these studies is that these contaminants are still being detected after wastewater treatment, as well as within environmental waters in rivers. Such trends of persistence of priority emerging contaminants after wastewater treatment are also recorded globally for PPCPs (Blair et al., 2015; Causanilles et al., 2017; Deo, 2014; Falås et al., 2016; Kasprzyk-Hordern et al., 2009a; Petrie et al., 2014). Contaminants which are not removed from water treatment processes are therefore destined to end up back in the environment (therefore affecting wildlife), or might possibly end up in drinking water (De Jager et al., 2013; Patterton, 2013). This emphasises the need to further conduct comprehensive monitoring studies in South African surface water systems to report on the fate of priority ECs to assist with environmental risk assessment.

2.3.4. Environmental Risk Assessment (ERA) and Adverse Outcome Pathways (AOPs)

Although the presence and recalcitrance of several PPCPs has already been demonstrated within surface water systems on a global scale, the implementation of such monitoring studies to predict environmental risk needs more scrutiny. Conventional methods for environmental risk assessment (ERA) are based on acute and/or chronic toxicity studies which assess toxicity towards the most sensitive organisms within ecosystems. These test organisms include several trophic levels, such as bacteria, algae, crustaceans, and vertebrate species. Acute toxicity data is based on short-term toxicity effects (< 24 h) which are expressed as EC_{50} (concentration which shows an effect in 50% of the experimental population) or LD_{50} (lethal dose at 50% of the experimental population), whereas chronic toxicity data are based on long-term toxicity effects (> 24 h) which are expressed as NOEC (concentration which shows no effect in the experimental population) or LOEC (lowest concentration which shows an effect in the experimental population). These toxicity endpoints are then corrected by an assessment factor to calculate the predicted no-effect concentration (PNEC) of the EC of interest. This PNEC is then compared to either predicted environmental concentrations (PEC) or measured environmental concentrations (MEC) of the EC to obtain a risk quotient (RQ). A RQ value calculated larger or equal to 1 then reflects the EC to be of environmental concern. Although such ERAs using RQ estimation for pollutants are valuable to assess toxicity risks in the environment, there are several limitations to this conventional calculation of ERA, namely:

- The relevance for selecting only specific test organisms to calculate PNEC in the environment is an underestimation of the total ecological impacts of the ECs
- Using animal models is timely and not cost-effective to calculate risk for the vast majority of ECs on the market and in environmental waters
- Ethical concerns using animal bio-indicators for toxicity testing
- Sub-lethal and chronic (long-term; multigenerational) toxicity (such as endocrine disruption, behavioural effects and other physiological modulation) are not estimated
- The prediction of environmental risk is also unknown for pollutants in highly complex chemical mixtures in the environment, which leads to the need to establish more defined ERAs which are not chemical-specific

Due to these constraints that are shown for conventional ERA, it is necessary to construct more accurate and thorough models for risk assessment, constituting both lethal and sub-lethal toxicity. *In vitro* screening assays, molecular screening technologies and bioinformatics are just a few examples to be included in the decision-making process in predictive ecotoxicology. To add to the understanding of evolving 21st century toxicity testing and risk assessment, an adverse outcome pathway (AOP) framework has been proposed (Edwards et al., 2016; Villeneuve et al., 2014). This framework is structured upon existing knowledge based on the relationships between physiological pathways, spanning from molecular initiating events (MIEs), which in turn causes a perturbation in normal biological functioning, therefore impairing a sequence of measurable key events (KEs), ranging from cellular- to organism level (Edwards et al., 2016). Each KE is further linked with key event relationships (KERs) based on a weight-of-evidence approach. This downstream series of KEs are then coupled with a particular adverse outcome (AO) on a population level, which can be used for regulatory decision-making (Fig. 2.3). The AOP framework is well described by several authors (Edwards et al., 2016; Villeneuve et al., 2014), highlighting the advances made since its establishment by Ankley and colleagues (2010). Advancements of the AOP framework are still ongoing, which includes the broader AOP Knowledge Base (AOP-KB; https://aopkb.org/), containing the AOP Wiki (https://aopwiki.org/wiki/index.php/Main_Page). This initiative is led by several global regulating bodies, namely the USEPA, the Organisation for Economic Co-operation and Development (OECD), the European Commission Joint Research Centre (JRC), and the US Army Engineer Research and Development Centre (ERDC).

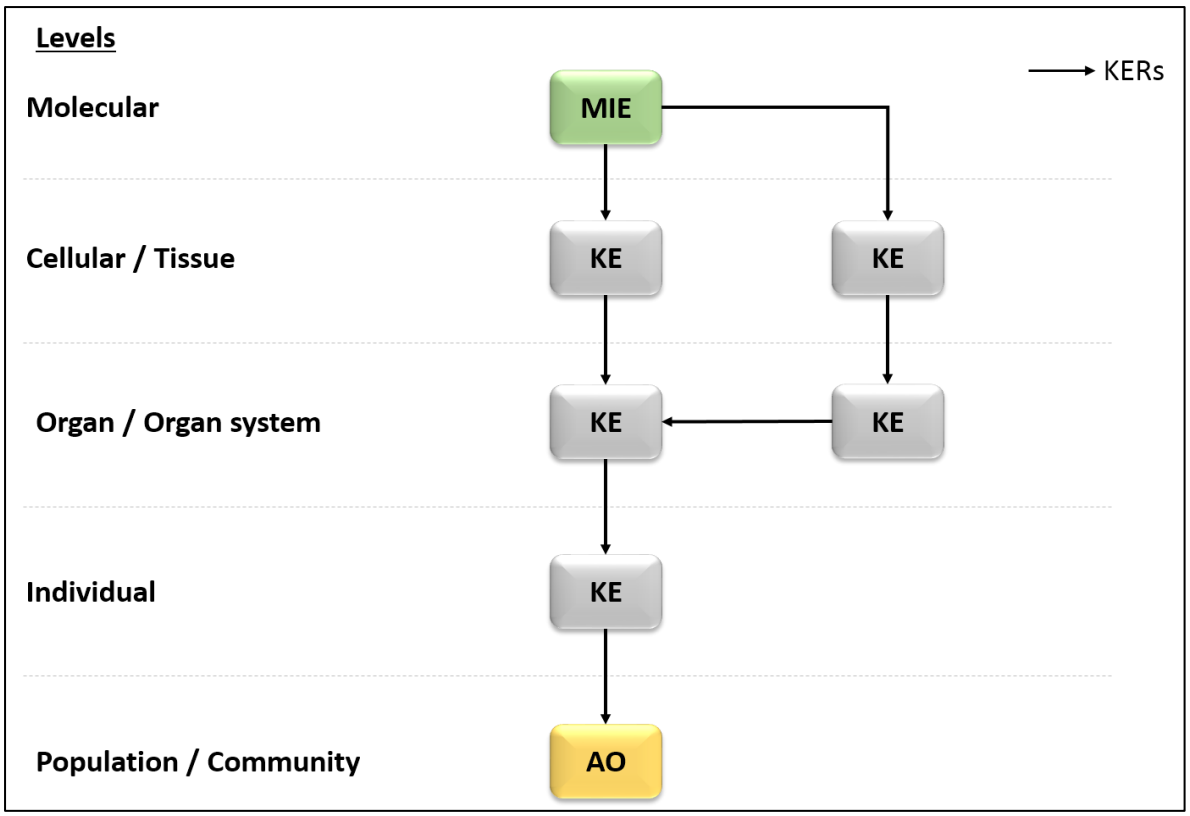

Figure 2.3: Basic representation of an adverse outcome pathway (AOP). A specific molecular initiating event (MIE) is linked to several key events (KE) by means of key event relationships (KERs) from a cellular to organism level, leading towards an adverse outcome (AO) observed within a population/community. Ultimately, several AOPs can be connected into an AOP network through several MIEs and KEs leading towards the same AO. Diagram based on the template from AOP-Wiki (https://aopwiki.org/wiki/index.php/Main_Page)

Although the AOP framework is not considered to be part of risk assessment, nor constructed to show chemical-specific outcomes, it helps with the extrapolation of possible AOs which might occur when specific MIEs or KEs are altered. Moreover, this initiative can assist with the understanding of further downstream pathways which could be modulated when a specific KE is shown to be altered by micro-pollutants. Studies indicating the environmental risk caused by micro-pollutants is still lacking in South Africa. Incorporating ERAs and the AOP framework in a local context will aid in the re-establishment of the National Toxicity Monitoring Programme (NTMP), which is only concentrated on a few pollutants and does not address more recent 'emerging contaminants' such as PPCPs (Jooste, 2008). In order to contribute to a more thorough national programme, it is clear that more information is needed on the chronic, sub-lethal level of toxicity using wildlife species and several other bio-markers for more accurate ERA analysis. This can be achieved by adopting a tiered screening approach to quantify and organise/categorise both lethal and sub-lethal toxicity data on both *in vitro* and *in vivo* screening approaches.

2.3.5. Endocrine disruption of detected pharmaceutical contaminants

Although studies indicating the monitoring of PPCPs within South African waters are on the increase (Table 2.1), there is still a lack of research effort towards correlating these levels of micro-pollutants with potential sub-lethal toxicological endpoints (such as endocrine disruption) for risk assessment. As discussed before, the potential for correlating MECs with known disruptions of KEs within an AOP framework can assist in demonstrating potential disruption of vertebrate endocrine systems, therefore serving as early-warning biomarkers of environmental health. Pollutants such as PPCPs are regularly shown to be present in water systems, either as their breakdown products, or to persist as their active ingredient, depending on several environmental factors and physiochemical properties of the compounds. Furthermore, several *in vitro* and *in vivo* toxicological studies have shown the potential of PPCPs to alter endocrine system pathways (Table 2.2), and many of these compounds have been detected within South African environmental waters and WWTW effluents (Table 2.1). Even more alarming, the levels of certain PPCPs detected within South African waters are well within, or close to, the levels reported to disrupt specific endocrine system pathways (as shown in Table 2.2).

Table 2.2: Endocrine-disrupting effects of commonly-detected and priority pharmaceuticals and personal care products (PPCPs), which are also referred to as key events (KEs) being disrupted within an adverse outcome pathway (AOP). Abbreviations: *LOEC* – Lowest Observed Effect Concentration for endocrine disruption in aquatic organisms; *NSAIDs* – non-steroidal anti-inflammatory drugs; *SHBG* – steroid hormone binding globulin; *VTG* – yolk precursor protein vitellogenin; *CYP* – cytochrome P450 enzyme; *hAR* – human androgen receptor; *hER* – human estrogen receptor; *SAID* – steroidal anti-inflammatory drug; T_3 – triiodothyrosine, T_4 - thyroxine, *mRNA* – messenger ribonucleic acid

Active ingredient	Key event (KE) / endocrine disruption	Assay / test species	LOEC ($\mu g.L^{-1}$)	Reference
Anti-epileptic				
Carbamazepine	• Reduced steroid hormone levels, elevated SHBG levels in male and female patients	Human patients	-	Herzog et al., 2005; Svalheim et al., 2009
	• Decrease 11-ketotestosterone levels in male fish	Zebrafish (*Danio rerio*)	0.5	Galus et al., 2013
NSAIDs				
Diclofenac	• Elevated levels of the enzyme cytochrome P450 and VTG protein; estrogenic effects	Japanese medaka (*Oryzias latipes*)	1.0	Hong et al., 2007
	• Decreased thyroid hormone levels in male and female patients	Human patients	-	Bishnoi et al., 1994
	• Decreased thyroid hormone levels in fish	Indian major carp (*Cirrhinus mrigala)*	1.0	Saravanan et al., 2014
Ibuprofen	• Increased VTG production in male fish	Japanese medaka (*Oryzias latipes*)	1 000	Han et al., 2010
	• Reduced reproduction behaviour in fish	Japanese medaka (*Oryzias latipes*)	10	Han et al., 2010
	• Increased estradiol hormone levels and aromatase enzyme activity; decreased testosterone hormone levels	Human adenocarcinoma cell line (H295R)	2 000	Han et al., 2010
	• Decreased egg fertilisation in fish.	Florida flagfish (*Jordanella floridae*)	0.1	Nesbitt et al., 2011
	• Disruption of thyroid hormone-mediated reprogramming in tadpoles	North American bullfrog (*Rana catesbeiana*)	1.5	Veldhoen et al., 2014
Naproxen	• Decreased thyroid hormone levels in male and female patients	Human patients	-	Bishnoi et al., 1994
	• Decreased egg fertilisation in fish	Florida flagfish (*Jordanella floridae*)	0.1	Nesbitt et al., 2011

Table 2.2 (continued)

Anti-depressant				
Fluoxetine	• Increased uterine weight in female rats	Wistar rats	-	Müller et al., 2012
	• Estrogenic response, proliferation of cells	MCF-7 breast cancer cells	-	Müller et al., 2012
	• Upregulation of CYP11β2 gene expression	Human adenocarcinoma cell line (H295R)	-	Gracia et al., 2007
	• Modulation of testicular structure, induced VTG production in male fish	Fathead minnows (*Pimephales promelas*)	0.03	Schultz et al., 2011
Biocides				
Triclosan	• Decreased T_4 hormone levels in female rats	Long-Evans rats	-	Crofton et al., 2007
	• Increased VTG gene expression, decreased sperm counts in male fish	Western Mosquitofish (*Gambusia affinis*)	101.3	Raut and Angus, 2010
	• Decreased T_3 hormone levels and other thyroid-related gene expressions in tadpoles	North American bullfrog (*Rana catesbeiana*)	0.03	Veldhoen et al., 2006
	• Increased hepatic VTG in male fish	Japanese medaka (*Oryzias latipes*)	20.0	Ishibashi et al., 2004
	• Decreased hatchability and time of hatching of fertilised eggs in fish	Japanese medaka (*Oryzias latipes*)	313.0	Ishibashi et al., 2004
	• Antagonist for steroid ER/AR-responsive gene expression	Recombinant human ovarian cancer cells (BG1Luc4E2, ERα–positive), recombinant human cells (T47D-ARE)	-	Ahn et al., 2008
	• Enhance testosterone-dependent activation of AR-responsive gene expression	MDA-kb2 breast cancer cell line	-	Christen et al., 2010
Triclocarban	• Induction of CYP2B6 and CYP1B1 mRNA expression; activate estrogen receptor target genes in female ovaries	ERα positive MCF7 breast cancer cells, humanised UGT1 mice	-	Yueh et al., 2012
	• Enhance testosterone action through interaction with the AR	Castrated male rats, cell-based human AR-mediated bioassay	-	Chen et al., 2008
	• Agonist of ER/AR-responsive gene expression	Recombinant human ovarian cancer cells (BG1Luc4E2, ERα–positive), recombinant human cells (T47D-ARE), MDA-kb2 breast cancer cell line	-	Ahn et al., 2008; Christen et al., 2010

Table 2.2 (continued)

Antibiotics				
Amoxicillin	• Upregulation of CYP19 and CYP17 gene expression and estradiol hormone levels	Human adenocarcinoma cell line (H295R)	-	Gracia et al., 2007
Erythromycin	• Upregulation of CYP11β2 gene expression and progesterone/estradiol hormone levels; downregulation of testosterone hormone levels.	Human adenocarcinoma cell line (H295R)	-	Gracia et al., 2007
Cephalexin	• Upregulation of CYP19 gene expression; downregulation of testosterone hormone levels	Human adenocarcinoma cell line (H295R)	-	Gracia et al., 2007
Oxytetracycline	• Upregulation of CYP19 and 3βHSD2 gene expression; increased estradiol hormone levels and aromatase enzyme activity	Human adenocarcinoma cell line (H295R)	-	Gracia et al., 2007 Ji et al., 2010
Sulfathiazole	• Upregulation of CYP17 and CYP19 gene expression • Increased estradiol hormone levels and aromatase enzyme activity, increased estradiol hormone levels in male fish	Human adenocarcinoma cell line (H295R), Japanese medaka (*Oryzias latipes*)	-	Ji et al., 2010
Doxycycline	• Upregulation of CYP19 gene expression	Human adenocarcinoma cell line (H295R)	-	Gracia et al., 2007
Tylosin	• Upregulation of CYP11β2 gene expression, downregulation of testosterone and estradiol hormone levels	Human adenocarcinoma cell line (H295R)	-	Gracia et al., 2007
SAID				
Dexamethasone	• Upregulation of CYP11β2 gene expression, downregulation of testosterone hormone levels	Human adenocarcinoma cell line (H295R)	-	Gracia et al., 2007
Growth promoter				
Trenbolone	• Upregulation of CYP19 gene expression, downregulation of testosterone hormone levels	Human adenocarcinoma cell line (H295R)	-	Gracia et al., 2007

Table 2.2 (continued)

Painkiller				
Acetaminophen	• Upregulation of CYP11β2 gene expression and progesterone hormone levels	Human adenocarcinoma cell line (H295R)	-	Gracia et al., 2007
Bronchodilator				
Salbutamol	• Upregulation of CYP17 gene expression, downregulation of estradiol hormone levels	Human adenocarcinoma cell line (H295R)	-	Gracia et al., 2007
Lipid regulator				
Bezafibrate	• Decrease in plasma 11-ketotestosterone levels in fish	Zebrafish (*Danio rerio*)	-	Velasco-Santamaría et al., 2011
Clofibrate	• Upregulation of CYP11β2 gene expression, downregulation of testosterone hormone levels	Human adenocarcinoma cell line (H295R)	-	Gracia et al., 2007
Preservatives				
Parabens	• Estrogenic and anti-androgenic effects *in vitro* and *in vivo*	Rat uterus receptor binding assay, MCF-7 breast cancer cells, Transfected Chines hamster ovary (CHO-K1) cells, Recombinant yeast screens	-	Boberg et al., 2010
	• Hepatic necrosis, testicular fibrosis, induction of hepatic VTG in male fish	Common carp (*Cyprinus carpio*)	840	Barse et al., 2010
	• Increase in plasma VTG concentrations, and increase in mRNA expression of VTG subtypes and estrogen receptor (ERα) in the liver of male fish	Japanese medaka (*Oryzias latipes*)	9 900	Inui et al., 2003

Table 2.2 (continued)

UV screens				
Benzophenones 4-MBC OMC	• Agonistic binding to the human estrogen receptor (hER), induced breast cancer cell proliferation, increased uterine weight female rats	MCF-7 breast cancer cells and female Long-Evans rats	-	Schlumpf et al., 2001
	• Increase in plasma VTG concentrations, and increase in mRNA expression of VTG subtypes and estrogen receptor (ERα) in the liver of male fish	Japanese medaka (*Oryzias latipes*)	9 900	Inui et al., 2003

It is apparent that several PPCPs can exert a range of MIEs and KEs according to the *in vitro* and *in vivo* studies shown in Table 2.2. The potential of these commonly-detected PPCPs in environmental waters to exert endocrine-disrupting activities therefore raise concerns for their impact upon environmental and human health. A further concern is that these compounds have been detected in broader environmental water systems, such as direct point sources of drinking water for human consumption. A study by Patterton and colleagues (2013) detected pharmaceutical compounds in drinking water from taps in Johannesburg (Gauteng Province) and Bloemfontein (Free State Province), South Africa. In particular, the anticonvulsant drug carbamazepine was detected in 63% of tap water tested in these regions (Table 2.1; Patterton, 2013). Anticonvulsant drugs, such as carbamazepine, levetiracetam, lamotrigine and valproate, have been shown to cause several reproductive endocrine system side-effects in men and women suffering from epilepsy (Table 2.2; Rättyä et al., 2001; Svalheim et al., 2009; Harden et al., 2010), as well as in fish species exposed to carbamazepine (Galus et al., 2013). In men using levetiracetam and valproate as treatment, it has been shown that these drugs can lead to increased T and steroid hormone-binding globulin (SHBG) levels, which is responsible for the transport of steroid hormones in blood plasma (Rättyä et al., 2001; Harden et al., 2010). In the same studies, it was shown that men treated with carbamazepine also evidenced increased levels of SHBG, pituitary FSH and LH (Herzog et al., 2005; Svalheim et al., 2009). Therefore, it might be possible that anticonvulsant compounds found in drinking water resources can lead to altered steroidogenesis in men. Alternatively, carbamazepine treatment in women has been shown to lead to higher SHBP levels and lower levels of P and T steroid hormones (Löfgren et al., 2006; Svalheim et al., 2009). These endocrine-disrupting effects of anticonvulsant drugs were shown in wildlife as well, including modulation of steroidogenesis and ovarian malformations in ovarian follicular cells (Svalheim et al., 2009).

Another group of pharmaceuticals that are frequently prescribed and also detected in South African waters are NSAIDs. A study by Amdany and colleagues (2014) detected varying levels of naproxen and ibuprofen in the influents and effluents of two WWTWs in the Gauteng province of South Africa (Table 2.1). These compounds have been shown to alter endocrine systems in non-target vertebrate species. In a full life-cycle study, exposing Japanese Medaka Fish (*Oryzias latipes*) to ibuprofen concentrations as low as 0.1 µg.L^{-1} resulted in delayed hatching success, while a concentration of 1 mg.L^{-1} resulted in increased blood plasma levels of the glycoprotein vitellogenin (VTG) (Table 2.2; Han et al., 2010). This protein molecule is the precursor for egg yolk, and has been validated as a biomarker to express estrogenic

endocrine disruption in egg-laying vertebrate species. In the same study, the exposure of ibuprofen to a human adrenocortical carcinoma cell line (H295R) resulted in an increase in E_2 hormone levels at concentrations of 2 and 20 $mg.L^{-1}$, and also increased aromatase enzyme activity at concentrations of 0.2 and 2 $mg.L^{-1}$ (Table 2.2; Han et al., 2010). Aromatase is the enzyme responsible for the metabolism of T to E_2 in steroidogenic pathways. Apart from the possible gonadal endocrine-disrupting activity of ibuprofen, exposure of *X. laevis* larvae to concentrations ranging between 30.7 and 39.9 $mg.L^{-1}$ leads to malformations in the development of these larvae, indicating teratogenic effects of ibuprofen as well (Richards and Cole, 2006). Another NSAID that has been investigated for its endocrine-disrupting effect is diclofenac. In South Africa, diclofenac has been detected in a KwaZulu-Natal Province river system at concentrations varying between 1.1 $\mu g.L^{-1}$ and 15.6 $\mu g.L^{-1}$ (Table 2.1; Agunbiade and Moodley, 2014). The exposure of *X. laevis* embryos to diclofenac has been shown to cause teratogenicity at a concentration of 4 $mg.L^{-1}$ (Chae et al., 2015). Furthermore, diclofenac exposure in male *O. latipes* fish showed that concentrations as low as 1 $\mu g.L^{-1}$ can increase the gene expression for VTG in the liver, thereby showing estrogenic effects (Hong et al., 2007). Furthermore, assessment of patients using diclofenac as an NSAID has shown a reduction in serum T_3 levels (Table 2.2; Bishnoi et al., 1994), which is the more active thyroid hormone responsible for growth, development and metabolism in the body. The other NSAID that has also been found in South African waters, naproxen, has also been shown to cause a reduction in serum T_3 levels in patients taking this medication (Bishnoi et al., 1994). However, according to our knowledge, little is known about the endocrine-disrupting activity of naproxen pollution into the environment, and the effects of this compound on non-target organisms. The dose-dependent response of thyroid disruption by naproxen exposure still needs to be assessed in future studies. Although some of the endocrine-disrupting effects shown above may only occur at high levels of exposure to these NSAIDs, it is important to note that a mixture of different pharmaceuticals and other contaminants might accumulate in the water system. The presence of NSAIDs such as ibuprofen, naproxen and diclofenac may therefore contribute to endocrine disruption caused by other water pollutants as well. Furthermore, these compounds have been confirmed to be present in South African surface waters (Table 2.1), showing that they are not completely removed from the water system after treatment. The above-mentioned studies imply that NSAIDs, such as ibuprofen, naproxen and diclofenac, have the possibility to alter both gonadal and thyroid endocrine system pathways, and also possibly cause teratogenicity at environmentally relevant concentrations.

The PPCPs which are the most frequently detected in surface waters worldwide are antibiotics and biocides. Regularly-prescribed antibiotic pharmaceuticals, such as ampicillin, chloramphenicol, ciprofloxacin, erythromycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline, and tylosin, have all been detected in South African river systems (Table 2.1; Agunbiade and Moodley, 2014). These compounds have all been shown to have endocrine-disrupting effects. The semi-synthetic macrolide antibiotic tylosin, which is used in veterinary medicine, has been shown to increase the expression of the aldosteronogenic gene (CYP11β2), and decrease the production of T and E_2 at a concentration of 3 $mg.L^{-1}$ in an H295R steroidogenic assay, showing that this chemical can serve as both an anti-estrogenic and anti-androgenic EDC (Table 2; Gracia et al., 2007). In the same study, another macrolide antibiotic, erythromycin, showed an increase in the expression of CYP11β2 and a reduction in T production at a concentration of 3 $mg.L^{-1}$, but caused increased production of E_2 and P in the assay (Table 2.2; Gracia et al., 2007). Exposure of erythromycin in a recombinant yeast estrogen screen (YES) showed that this compound may be a minor mimic of E_2 in binding to the estrogen receptor in a dose-dependent manner (Archer *et al*. unpublished). This shows that, although tylosin and erythromycin share the same macrolide ring in their chemical composition, the endocrine-disrupting effect differs between these two compounds, and therefore complicates environmental endocrine disruption studies if, for example, both these two types of chemicals are present in environmental samples. A study by Garcia and colleagues (2007) also showed that tetracyclines, exposed at a concentration of 81 $\mu g.L^{-1}$ to H295R cells, can increase the expression of CYP19 enzymes and 3βHSD2 genes (Table 2), which are responsible for T-E_2 metabolism and the production of P, respectively. Although these antibiotics were not detected in the range which showed endocrine system modulation in an *in vitro* assay, their effect on wildlife through long-term exposure within environmental waters is currently unknown. Furthermore, due to the extensive usage of antibiotics in both humans and livestock, the expected concentrations of these chemicals in the environment may be underestimated, and may also have a cumulative endocrine-disrupting effect in the water if they accumulate in mixtures with other pollutants. It is therefore evident that antibiotic chemical pollutants should receive high priority in environmental screening in water systems and water treatment facilities in South Africa.

Apart from the regularly-prescribed antibiotic pharmaceuticals detected in environmental waters, it is shown that compounds in personal care products can also have endocrine-disrupting properties. One of the most well documented compounds is the biocide triclosan

(TCS), which is used as a disinfectant in soaps, detergents, toothpastes, mouthwash, and more (Raut and Angus, 2010). This compound also shows a high partition coefficient (K_{ow}) value (Log K_{ow} 4.66; KOWWIN v. 1.67, EPI Suite), which indicates that TCS is highly lipid-soluble and does not readily dissolve in water. For this reason, TCS can be regarded as a POP, which can accumulate in the fat tissue of exposed organisms, and can also be transported in water bodies over great distances. This has been shown in a study demonstrating high levels of TCS in blood plasma (163 times more) and breast milk (12 times more) in pregnant women compared to unexposed individuals (Allmyr et al., 2006). Although the use of TCS has been phased out in several personal care products in developed countries, it is still found in South African consumer products, and therefore detected in surface waters (Amdany et al., 2014b; Madikizela et al., 2014). Amdany and colleagues (2014) showed varying levels of TCS in influents and effluents from two WWTWs in the Gauteng province of South Africa. These levels ranged from 78.4 to 127.7 $\mu g.L^{-1}$ in influent samples, and 10.7 to 22.9 $\mu g.L^{-1}$ in effluent samples (Amdany et al., 2014a). Although the concentrations of TCS are significantly reduced after water treatment, these levels are still high if sub-lethal effects are taken into account. Exposure of North American bullfrog tadpoles (*Rana catesbeiana*) to TCS showed that concentrations as low as 0.3 $\mu g.L^{-1}$ can significantly lower tadpole body mass and decrease thyroid hormone receptor (TR) gene expression (Table 2.2; Veldhoen et al., 2006). Exposure of TCS at 20 $\mu g.L^{-1}$ has also been shown to induce hepatic VTG levels in male Japanese Medaka (*Oryzias latipes*) (Table 2.2; Ishibashi et al., 2004). Exposure of mature male Western Mosquitofish (*Gambusia affinis*) to TCS at 101.3 $\mu g.L^{-1}$ can cause decreased sperm counts, and also elevate VTG gene expression (Table 2.2; Raut and Angus, 2010). TCS exposure in MDA-kb2 breast cancer cells showed that a concentration of 289 $\mu g.L^{-1}$ significantly induces cell proliferation, and a concentration as low as 290 $ng.L^{-1}$ caused an elevated androgenic response when treated along with dihydrotestosterone (DHT), which is a more metabolically active androgen than testosterone (Table 2.2; Christin et al., 2012). These results show that TCS serves as an androgen agonist by binding to the androgen receptor in a human cell-based bioassay (Christin et al., 2012). These concentrations of endocrine disruption are either equivalent or lower than levels observed in South African waters (Table 2.1). Therefore, wildlife species living either upstream or downstream of WWTWs may be affected by levels of TCS in the environment. Bearing these studies in mind, it is possible that exposure to low concentrations of TCS over a long period of time (chronic exposure) may modulate both gonadal and thyroid endocrine systems in humans and other wildlife species at concentrations currently being detected in environmental waters.

Due to the regular detection and known endocrine-disrupting effect of TCS, it is also important to investigate other compounds found in personal care products and detected in South African waters. Based on chemical analyses and endocrine disruption studies done elsewhere in the world, it is evident that compounds used as preservatives, disinfectants and UV filters have not received much attention as priority environmental pollutants and EDCs in South Africa. Preservatives such as parabens (methylparaben, propylparaben, octylparaben), other biocides such as trichlorocarban (TCC), and UV filters in sunscreens (4-MBC, OMC) have all been shown to accumulate in wastewater systems and cause potential endocrine disruption. Several paraben compounds, as well as their metabolites, have been shown to have both estrogenic and anti-androgenic effects *in vitro* and *in vivo* (Table 2.2). These studies imply that contaminants such as parabens can affect multiple endocrine pathways, and are therefore of environmental concern. Biocides such as TCC are regularly included in several cosmetic and personal care products to deter microbial organisms. Although the endocrine disrupting effect of the biocide TCS has been well documented, and also found at high concentrations in the environment (Table 2.1, Halden and Paull, 2005), limited data are available on the endocrine-disrupting effect of TCC. Exposure to TTC in human cell-based bioassays, and exposure of rodents to TTC, indicated that the biocide does not have endocrine-disrupting activity on its own, but rather enhances the action and binding affinity of steroid hormones (Table 2.2) (Ahn et al., 2008; Christen et al., 2010; Yueh et al., 2012). This shows a potentiating mechanism of endocrine disruption, as well as an alternative mode of endocrine disruption other than direct modulation of endocrine pathways. Several compounds used as UV filters in sunscreens, such as benzophenones, benzedrone (4-MBC), and octyl methoxycinnamate (OMC) have been shown to agonistically bind to the human estrogen receptor (hER) in human cell-based bioassays, and to increase VTG production in female rats and male fish species (Table 2.2). These compounds are therefore regarded as estrogenic contaminants, which might persist for long periods of time in the environment due to their low water solubility. The abovementioned compounds are all used as either ‘wash-off’ or ‘application’ personal care products. Therefore, it can be assumed that products containing biocides, UV screens, and preservatives will either be washed down in drain water, or will be absorbed through the skin after application. These compounds thus have multiple routes of exposure to either humans or other non-target organisms in water. Also, due to the fact that these chemicals are regularly used in personal care products, and the fact that these compounds have low solubility in water, their presence and persistence in the environment can be high. Paraben concentrations as high as 11 $mg.L^{-1}$

have been detected in a UK river system, with concentrations as high as 30 $mg.L^{-1}$ in wastewater influents (Kasprzyk-Hordern et al., 2009b). TCC concentrations of 6 $\mu g.L^{-1}$ have been documented in a US river system (Halden and Paull, 2005). Environmental concentrations of UV filters as high as 13 $mg.L^{-1}$ have been reported in wastewater influents (Kasprzyk-Hordern et al., 2009b), with 6 $mg.L^{-1}$ in wastewater effluents (Kasprzyk-Hordern et al., 2009b), 266 $ng.L^{-1}$ in swimming pools (Cuderman and Heath, 2007), and in seawater at concentrations of 3.3 $\mu g.L^{-1}$ (Sánchez Rodríguez et al., 2015). These compounds have not been screened for their presence in South African water systems, which therefore highlights the importance of screening for these chemicals to evaluate their fate within water treatment facilities.

2.4. Future perspectives

Monitoring studies focusing on the presence and fate of EDCs and PPCPs in surface waters have been comprehensive internationally. However, based on the available information on South African toxicological studies, there are several aspects which still need to be addressed. Apart from conventional EDC investigations, there are also a large variety of other topics which need to be addressed. A few of these topics are mentioned below, and will provide a significant contribution towards the understanding of the fate and presence of chemical pathogens in environmental waters. Such interdisciplinary studies should receive high priority for future research, as they are all interlinked into the larger scope of environmental water pollution investigations in South Africa.

2.4.1. Pharmaceutical metabolites and conjugates in WWTWs

Most pharmaceutical detection studies concentrate on the detection of parent pharmaceutically active ingredients. However, it is known that some pharmaceutical compounds are rapidly metabolised in the body after consumption, resulting in their breakdown products being the predominant component of wastewater. Also, after their excretion and/or discharge into wastewater, some PPCPs may be further transformed through biotic (microbial metabolism) and/or abiotic (photodegradation, etc) factors which could affect the drug's stability and fate. It is therefore possible that some toxicologically active compounds may be overlooked when screening for pharmaceutical residues in water bodies, due to the metabolic processing of the parent compound. For some pharmaceuticals, it is known that it is rather the major metabolite products from the parent compound which exert the physiological effect. For example, the analgesic compound tramadol will undergo hepatic metabolism by desmethylation to produce

the primary metabolite *O*-desmethyltramadol, which is a more potent and persistent opioid than tramadol itself. The anti-epileptic compound carbamazepine is also almost completely metabolised in the liver to produce the more potent carbamazepine-10,11-epoxide. These metabolites are then excreted at higher levels than the parent compounds. Regardless, the metabolites of pharmaceutical compounds are regularly ignored in environmental screening of water systems and need to be included in future studies. The environmental consequences of pharmaceutical metabolites in water systems may be even more detrimental than the presence of their parent compounds. Several metabolites of parent compounds of other types of environmental pollutants (mostly pesticides) have been shown to have more severe endocrine-disrupting effects on non-target organisms than their parent compounds. For example, the dithiocarbamate fungicide mancozeb is shown to cause thyroid-modulating effects, but it is rather its metabolite, ethylene thiourea (ETU) which exerts this thyroid-modulating, and possible carcinogenic, activity (Opitz et al., 2006). The dicarboximide fungicide vinclozolin is also shown to be an anti-androgenic EDC, but it is rather its metabolites that have the greater half-lives and mobility in water necessary to cause endocrine-disrupting effects (Bayley et al., 2003). Furthermore, metabolites from parent EDCs may modulate other endocrine system pathways as well, such as the organochloride insecticide DDT, which is a known oestogenic EDC, but its metabolite *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE) is shown to rather have anti-androgenic activity (Mills et al., 2001). Also, although some breakdown products of EDCs may not have any endocrine-disrupting activity, these components might contribute towards an elevated pathogenic effect of another EDC (i.e. potentiating mixture interactions) in water bodies, as a large mixture of different EDCs might be present in water bodies. The potential of parent compounds and metabolites (breakdown products) having endocrine-disrupting activities might therefore cause several toxicological mixture interactions in environmental waters (Hendricks and Pool, 2012).

Apart from the potential of pharmaceutical metabolites to exert higher health impacts if they are present in the environment, the occurrence of negative mass balances for pharmaceuticals and their metabolites has also been detected at several WWTWs globally (Blair et al., 2015; Kasprzyk-Hordern et al., 2009b; Subedi and Kannan, 2014). This trend in negative mass balances is defined as higher concentrations of ECs being detected in WWTW effluents compared to raw wastewater entering the plant. One possibility is that the parent compounds of ECs are not detected within raw sewage, as the metabolite form of these compounds is more prevalent. It may be possible that these metabolites might then be re-transformed to their parent

compounds by the microbial communities present in the treatment plant, or by abiotic factors such as photolytic processes (Aris et al., 2014; Blair et al., 2015; Bonvin et al., 2012). This may therefore indicate that some compounds may bio-accumulate or transform within WWTWs, either through enzymatic metabolism or other abiotic pathways, and then be discharged into environmental waters. In particular, it is therefore important to further the knowledge regarding the metabolic capabilities of microbial communities to consume or transform xenobiotics in water treatment facilities.

2.4.2. Mixture interactions of environmental pollutants

It is recognised on a global scale that numerous xenobiotic chemicals accumulate in complex mixtures in the environment. Although the concentrations of pollutants range from $mg.L^{-1}$ to $ng.L^{-1}$, the chemical interaction between pollutants can be great (Carvalho et al., 2014). It is regularly found that chemical mixture studies do not always conform to conventional predicted ecotoxicological mixture interactions. These mixture interactions are largely dependent on the individual chemical's general mode of action (MOA). The MOA of chemicals having gonadal endocrine-disrupting activities, for example, are grouped as being estrogenic, anti-estrogenic, androgenic, and anti-androgenic (Behrends et al., 2010). In ideal mixture interactions of environmental pollutants, it is generally assumed that compounds having the same MOA (e.g. estrogenic + estrogenic) will generate additive mixture interactions, meaning that the chemical mixture acts jointly to generate a larger physiological or toxicological response than their individual counterparts. This is generally known as the additivity null hypothesis (Christiansen et al., 2009). In contrast, chemicals having dissimilar MOAs (e.g. estrogenic + androgenic) are proposed to act independently from one another in regard to a measured physiological or toxicological endpoint. This is referred to as independent action (Christiansen et al., 2009). However, it is not as simple as grouping chemicals according to a general endocrine outcome. Chemicals having the same MOA (e.g. anti-estogenic) may have dissimilar mechanisms exerting the same MOA, for example, modulating steroid receptor binding or inhibiting steroidogenic enzyme functions. Both of these mechanisms have the same MOA, but act in a dissimilar manner, which can cause complex mixture interactions. This complexity in mixture interactions has been highlighted (Archer and Wyk, 2015; Kjaerstad et al., 2010). Therefore, recent mixture interaction studies refer not to the general outcome of the MOAs (estrogenic, androgenic, etc.), but rather to their mechanisms of action (steroid receptor agonism/antagonism, steroidogenesis inhibition/stimulation, enzyme inhibition/modulation). Bearing in mind that a vast majority of xenobiotic compounds from agriculture, industries and

domestic waste accumulate in water systems, it is expected that a large variety of compounds having both similar and dissimilar MOAs are present in the water matrix. This opens up the possibility of other ecotoxicological mixture interactions to occur, such as potentiation, synergism and antagonism. Furthermore, several compounds are known for having multiple MOAs for a large variety of physiological and toxicological endpoints, therefore creating further complications in mixture interaction studies. Regardless, from the retrospective information present to date, along with continuing research being done on this topic, the knowledge regarding mixture interactions of environmental pollutants is complex and needs to be addressed in future environmental studies.

2.4.3. Antibiotic resistance

Apart from the harmful endocrine-disrupting effects of several pharmaceutical contaminants in wildlife and human populations, the presence of pharmaceuticals, especially antibacterial agents, may influence the type and persistence of bacterial communities (harmless or pathogenic) in the environment and in societies. The most common types of water-related infectious diseases include gastroenteritis, amoebiasis, salmonellosis, dysentery, cholera, typhoid fever, hepatitis-A and diarrhoea, whereby the spread of such infected disease are associated with poor health and sanitation.

Pathogenic bacteria are renowned for developing resistance to antimicrobial compounds (Levy, 1998). Bacterial pathogens can come from several sources, depending on the type of species. Some strains are waterborne, coming from human and animal faecal matter (Gerba and Smith, 2005). Other multidrug-resistant bacteria are known for their specific occurrence in hospitals. Due to the fact that most pathogenic bacteria are prone to infect immune-compromised individuals, these micro-organisms are not only responsible for their associated illnesses, but have also been shown to increase the death rates in patients with other communicable diseases (Levy et al., 1998). The control of pathogenic bacterial colonisation in public healthcare institutions, such as hospitals, is therefore vital to improve the health of the population. Resistance generated by pathogenic bacteria can also lead to insufficient response to antibacterial therapy, and to the implementation of further alternative drugs which might also eventually create resistance. In a South African study, Essack and colleagues (2005) identified 24 pathogenic bacterial strains in 16 hospitals (2 tertiary, 9 regional and 5 district) in the KwaZulu-Natal province. Of the 1 270 bacterial isolates retrieved from patients at the different hospitals, 3% were sensitive to all 24 antibiotics tested, and 91% were resistant to multiple

antibiotics (Essack et al., 2005). However, less resistance was observed in isolates treated with cephalosporins and fluoroquinolones, which are regarded as 'newly developed' antibiotics. Fluoroquinolones have also been detected in a WWTW effluent in the Western Cape Province (Table 2.1; Hendricks and Pool, 2012) and, although this pharmaceutical has high sensitivity as an antibacterial treatment, its increased occurrence in water systems might also lead to bacterial adaptation and future resistance.

With the known occurrence of pharmaceutical contaminants in water systems, it is also possible that the presence of low levels of these contaminants may improve the antibiotic resistance of undesired and/or pathogenic bacterial communities and/or influence the structure and occurrence of bacterial pathogens in the water system, therefore making it more difficult to eradicate these pathogens from the waters. These problems are the reason for increased pressure on water treatment facilities to remove both pathogens and toxins from the water system. Although water treatment facilities assure that bacterial pathogens are removed from the water, it is important to note that not all water treatment facilities operate at their expected levels (DWS, 2013). Although some WWTW facilities in South Africa comply with the recommended limits of physiochemical parameters in the effluent, they fail to adhere to other target standards (Odjadjare et al., 2012). A study by Odjadjare et al. (2012) detected several antibiotic-resistant *Pseudomonas* species in South African WWTW effluents. These strains did not only show resistance to antibiotics, but also to chemical treatments in the water treatment process, such as chlorination. It has also been noted that chlorination may increase the resistance of bacteria to the antibiotics ampicillin, cephalotin, sulfanilimide and tetracycline (Murray et al., 1984) through various potential mechanisms. Also, informal settlements situated above water treatment facilities might utilise water resources directly from the source, therefore prior to treatment. Although the levels of antibiotics in environmental waters might be below their concentrations to exert an antibiotic effect, these pollutants might serve as a 'primer' for further anti-microbial resistance (AMR) development. Taking all these factors into consideration, the development of AMR needs to be investigated further, by incorporating the data generated from environmental pharmaceutical chemical analyses, to assess whether low concentrations of antibiotics detected in environmental waters might induce further antibiotic resistance in pathogenic bacteria.

2.4.4. Environmental biofilms (epilithon) as biomarkers of micro-pollutants

In addition to the development of AMR in bacteria caused by selective evolutionary pressure (such as exposure to antibiotics), morphological and physical characteristics of microbial communities also play a role. It is known that microorganisms can adhere to solid surfaces where they deviate from their planktonic state, and grow and multiply to form biofilms. These biofilms typically constitute a community of multiple types of microorganisms, potentially including bacteria, fungi, and algae (Edwards and Kjellerup, 2013). The adherence of microbial communities to surfaces is assisted by the formation of extracellular polymeric substances (EPS) which keep the biofilm community intact. This facilitates a niche for the microorganisms to proliferate and colonise new surfaces by either single cell detachment, or multiple cell detachment, which is transported within the liquid environment (Ghadakpour et al., 2014). Biofilms may contain both harmless and pathogenic micro-organisms, and are challenging to eradicate in both the environment as well as WWTWs (Flemming et al., 2016). Therefore, pathogenic microbes may proliferate in the environment if favourable conditions are present for them to form biofilms, making them hard to remove at water treatment plants.

Due to several obligate and opportunistic pathogens that have developed resistance to antibiotic chemicals, it is becoming even more important to prevent the formation of pathogenic biofilms in the environment. Such pathogenic microorganisms do not only proliferate in contaminated environmental waters, but can also spread to areas where humans are in direct contact with these pathogens. This has been highlighted by Hota et al. (2009), who investigated the incidence of 36 patients infected with a multidrug-resistant strain of *P. aeruginosa* in a Canadian hospital during 2004 and 2006. The research showed that a biofilm containing the pathogenic strain was lined around sink piping situated close to patient beds. Although the sinks were treated with antibacterial agents for disinfection, this did not inhibit the dispersal of the bacteria when accidental splashing from the sink occurred (Hota et al., 2009). Such incidences are very likely to occur in South African hospitals as well, as most public sector hospitals do not meet basic sanitation and hygiene requirements. The outbreak studied by Hota et al. (2009) highlights the importance of controlling and limiting the possible source points of adaptive pressure enabling these bacterial pathogens to develop resistance. If antibacterial pharmaceuticals are not effectively removed from drinking water supplies, these chemicals might accumulate and increase the antimicrobial resistance of pathogenic bacterial biofilms. Also, the continued usage of certain antibiotic compounds leads to the development of resistance by the pathogens against that specific compound, and therefore leads to the

development or usage of alternative antibiotic compound/s. These are, however, short-term and cyclic solutions, since they ultimately increase the suite of genetic adaptation mechanisms available to the microbial community. This is of particular concern in freshwater drinking sources, as it is found on a daily basis that more and more antibiotic pollutants are found in our freshwater systems, even after water treatment processes.

Despite the detrimental effects of pathogenic biofilm communities, the presence of biofilms in environments is not always harmful. Biofilm communities can be found on streambed sediment surfaces in environmental waters, and are a critical element of the chemical and nutrient cycling in aquatic systems (Writer et al., 2011). Biofilms and microbial flocs are one of the primary means of carbon and nitrogen removal in wastewater treatment plants, responsible for facilitating clean water and preventing eutrophication of our water bodies (Sheng et al., 2010). Biofilms are also used for bioremediation and biotransformation of toxic compounds at water treatment facilities (see Edwards and Kjellerup, 2013 for a summary of biofilm-based treatment techniques). Therefore, biofilms may be used as a screening tool to investigate the accumulation and removal of harmful chemicals (including PPCPs and other EDCs) in surface waters. It is shown that steroid hormones and alkylphenols (such as NP) can partition very rapidly into the organic matter of biofilms (Writer et al., 2011). Biofilms can also contribute to the oxidation of organic material, such as converting E_2 to E_1 (Writer et al., 2011), thereby changing the chemical profiles in the water system. Thus, biofilms can serve as a bio-indicator of EDC presence in the environment, as organic compounds can be retained within the biofilm community and undergo transformation processes (Edwards and Kjellerup, 2013). However, other environmental factors may also influence the retention capacity of organic compounds in biofilms, such as temperature, light, competing carbon substrates and oxygen concentrations. Regardless, biofilm communities in environmental waters may serve as a valuable tool for EDC detection studies. Several aspects of biofilm interaction with environmental EDCs can be investigated in the future. Pharmaceuticals in environmental waters might exert pressure on the structure and composition (physical and community) of microbial biofilms in the environment, due to possible antimicrobial resistance or other chemical interactions with the microbial communities. This might have beneficial or detrimental effects on the fate and persistence of EDCs in the environment, as biofilms may assist in the bioaccumulation and/or biodegradation of organic pollutants, either making them less harmful or changing them into more biologically active metabolites. Thus, both antibiotics and broader pharmaceutical compounds will have a

modulating effect on the microbiological ecology of both wastewater treatment works and freshwater systems, as well as the endocrine-disrupting effects discussed above.

2.4.5. A tiered approach to endocrine disruption of PPCPs of environmental concern

Taking all environmental and socio-economic factors mentioned in the current review into consideration, as well as research to date on the accumulation of EDCs and other ECs in surface waters, and their implications towards the health of humans and wildlife (AMR and EDCs), it is important to implement a tiered approach towards identifying and categorising possible pathogens and routes of exposure in environmental and treated water systems.

Several global regulatory bodies such as the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) managed by the USEPA, and a task force on Endocrine Disrupters Testing and Assessment (EDTA) by the OECD, have mandated the development and validation of testing (screening) methods for standardised assessments. The tiered approach suggested by USEPA was accepted globally and includes a battery of assays to screen or test for endocrine interactions and to identify and evaluate the potential of contaminants serving as EDCs (EDSTAC, 1998). First-tier assays were chosen to act as high-throughput screens to identify and prioritise ECs for second-tier testing, which aids in the understanding of the specific physiological MOA which are modulated by the EC of interest (EDSTAC, 1998). Second-tier screens aim to evaluate the results obtained from the first-tier screens in multi-generational or long-term *in vivo* studies to gain further support for a compound or mixture of contaminants to serve as environmental EDCs (EDSTAC, 1998). First- and second-tier screening can therefore provide biologically relevant information, which can be used to support ERAs and to establish AOPs for more detailed eco-toxicological assessment.

2.5. Conclusions

Based on the current status of micro-pollutant understanding and analyses described in this review, it is evident that there is a large source of information available which can aid in the prioritisation of emerging contaminants for environmental risk assessment. Several key points were discussed and should therefore receive priority in future studies to ensure sustainability of our freshwater resources, namely:

- Further reports on the occurrences of PPCPs and their metabolites in surface waters

- Establishing the possible endocrine-disrupting effects of commonly-detected PPCPs and other micro-pollutants through a tiered eco-toxicological approach
- Investigating the contribution of environmental micro-pollutants towards the global epidemic of AMR
- Report on the effectiveness of WWTWs to remove priority micro-pollutants (such as EDCs), as well as biological pathogens
- Raising public awareness of the consequences of liberal and irresponsible PPCP use and disposal
- Establish and/or improve initiatives such as the National Toxicity Monitoring Programme (NTMP) to assist with environmental risk assessment through the use of AOP networks
- Developing more effective water treatment technologies to eradicate persistent micro-pollutants from the water system in order to deem the system safe for reuse

CHAPTER 3: SEASONAL AND DAILY VARIATIONS IN ESTROGENIC AND ANTI-ESTROGENIC ACTIVITY AT WASTEWATER TREATMENT WORKS AND RECEIVING WATERS: IMPLICATION FOR RELIABLE MONITORING

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Abstract

The potential of natural- and man-made pollutants to modulate reproductive endocrine system pathways are well known. Natural/synthetic steroid hormones and many organic pollutants persisting through wastewater treatment works (WWTWs) are of particular concern for their endocrine-disrupting activities observed in receiving surface waters. Apart from the demonstrated presence of estrogen- and estrogen-mimicking compounds in surface waters, antagonistic (anti-estrogenic) responses originating from wastewater effluent are reported, but less known. Estrogenicity and anti-estrogenicity were assessed using recombinant yeast estrogen receptor binding assays (YES/YAES) at ten WWTWs and receiving rivers. Raw influent and treated effluent, as well as river water upstream and downstream of the various WWTWs were sampled for one week during two sampling campaigns within summer 2015/16 and 2016/17 (December), and one sampling campaign during winter 2016 (June). Estrogenicity was notably reduced at most of the WWTWs. Estradiol-equivalent (EEQs) estimations in raw wastewater varied between 4.4 to 34.4 $ng.L^{-1}$ during summer periods, and 6.6 to 31.5 $ng.L^{-1}$ during winter, whereas treated wastewater effluent varied between 0.3 to 6.9 $ng.L^{-1}$ during summer and 0.2 to 4.9 $ng.L^{-1}$ during winter. From these results, EEQs from several treated effluent and river water samples were above effect-based trigger values posing endocrine-disruption risk for aquatic organisms and/or potential adverse health risks for humans. Furthermore, estrogenicity recorded in samples collected upstream from WWTW discharge also exceeded predicted no-effect concentrations (PNEC) and trigger values as shown for estradiol (E_2), which highlights the impact of alternative pollution sources contributing towards endocrine disruption in the environment. The YAES showed variable tamoxifen-equivalent concentrations (TAM-EQ) in treated wastewater during two sampling campaigns, which varied between 3.7 to 78.7 $\mu g.L^{-1}$ during winter 2016, and 0.7 to 10.0 $\mu g.L^{-1}$ during summer 2016/17 for selected WWTWs. The current study served as both scoping- and case study to highlight the variety of factors that will affect bioassay outcomes and conclusions drawn from the results for risk decision making. The experienced mismatch between estrogenic and anti-estrogenic activity suggested a potential masking effect WWTW effluents, which highlights the complexity of environmental monitoring of endocrine disruption in samples containing a large mixture of both estrogenic and anti-estrogenic properties.

3.1. Introduction

Wastewater originating from domestic, industrial and agricultural practices are reported to create severe pressure on wastewater treatment works (WWTWs) to eliminate a large variety of organic micro-pollutants from the surface water system. However, several recalcitrant pollutants and their associated metabolites (breakdown products) are indeed not completely removed within treatment systems before discharged into recipient environmental waters, or re-used for potable- and/or non-potable purposes. Causes for of such incomplete removal include a variation of biotic- and abiotic factors, such as the physiochemical properties of the pollutants, the treatment technologies and capacity of the water works, environmental conditions (climate, pH, hydrology, geology, etc.), and the ratio of domestic and industrial contribution to the influent. These factors lead to the large variety in removal efficiencies of organic pollutant WWTWs, reported in studies around the globe (Bolong et al., 2009; Ort et al., 2010; Petrie et al., 2014). There is a continuous effort to advance water treatment technologies; concurrent development of analytical techniques with increasing sensitivity, coupled with expanding databases should offer new avenues to assess the efficiency of WWTWs to mitigate harmful discharge into environmental waters.

Apart from the potential toxicological risk and lethal effects which organic pollutants may pose upon wildlife and human health, it has become evident that several micro-pollutants such as pharmaceuticals, consumer products, pesticides and industrial by-products are also able to interact with endocrine systems, in one way or another, at concentrations regularly detected in surface waters (Archer et al., 2017b; Mckinlay et al., 2008). In particular, compounds interfering with gonadal endocrine system pathways have been of interest in surface water monitoring studies during the past few decades (Barnhoorn et al., 2004; Gracia et al., 2007; Guillette et al., 1996; Mckinlay et al., 2008; Mills et al., 2001; Löfgren et al., 2006). The presence of these endocrine-disrupting contaminants (EDCs) in surface waters have been indirectly linked to various developmental- and reproductive disorders observed in wildlife. The fact that the endocrine system among vertebrates (including humans) is conserved, it has been proposed that similar long-term effects might be observed in humans sharing the same environment. Non-communicable diseases, such as infertility, *in utero* developmental disorders, compromised immune systems, and several cancer-types have been suggested to be linked to EDC exposures (Colborn et al., 1993). However, it remains difficult to extrapolate the possible effect which many untested environmental pollutants may have on wildlife and

humans, as these compounds do not impose an immediate- or acute toxic outcome. It appears possible that a mixture of organic compounds, as well as their breakdown products (or metabolites) in water bodies may yield several mixture interactions that are not yet fully understood. *In vitro* biomarker assays and *in vivo* bio-indicator organisms offer a valuable approach to assess the net endocrine-modulation effect or functionality of these compounds. Disadvantages associated with *in vivo* experimentation, such as logistics, cost and time, warrants the consideration of using *in vitro* bioassays to show potential risk prior to *in vivo* assessment. Although bioassays are widely applied to assist decision-making by weight-of-evidence, the possibility of toxic masking within bioassay endpoints (leading to false negatives) may be a reality to be considered (König et al., 2017; Petrovic et al., 2004).

This study was conducted in South Africa, a relatively arid country facing rapid urbanisation and population growth that outpace infrastructure development, thus offers a suitable proxy for other developing countries facing similar challenges. A consequence of such population dynamics are increased risk of surface water pollution due to insufficient sanitation services, along with a higher demand for treated water, food and health services – all being negatively impacted by pollution. For instance, the higher demand for food production leads towards an increased pesticide- and pharmaceutical use on crops and livestock, respectively, while the unhealthy living conditions in the rapidly growing informal or low-cost housing schemes further lead to an increased reliance on pharmaceuticals. Rapid population growth also directly leads towards an increased presence of natural steroid hormones within sewage. These factors, combined with the demonstrated recalcitrance of organic- and inorganic micro-pollutants place severe strain on WWTWs, especially in countries without sufficient financial resources to upgrade or expand existing systems.

The aim of this study was to use an *in vitro* recombinant yeast receptor binding assay to assess the level and persistence of estrogenicity at several South African WWTWs that are compliant with the Department of Water and Sanitation (DWS) Green Drop accreditation (http://dwa.gov.za). The seasonal and daily variation of estrogenicity within WWTW influent and effluent, as well as the associated river waters upstream and downstream of the plants were compared and considered in the context of potential adverse health risks associated with the measured estrogenic concentrations. The influence of compounds which may antagonise estrogen-mediated receptor binding (anti-estrogens) in the bioassay used for the present study

was also investigated in order to further elaborate on the potential endocrine disrupting risk which treated wastewater may pose on surface waters spanning over multiple modes of action.

3.2. Materials and Methods

3.2.1. Study Site and Sampling Procedure

The study site was situated in the East Rand district of the Gauteng Province, South Africa. This region of the country experience its rainfall during the summer period (December - February), whereas a colder but drier climate is experienced winter (June - August). Ten WWTWs were selected for the study, which vary in operating capacities, treatment processes and sources of the wastewater (Table 3.1). The ten WWTWs were all sampled during a summer (2015/16) and winter (2016) period for five consecutive days (Monday to Friday). Four WWTWs were then selected and further sampled during the following summer period (2016/17) for seven consecutive days (Monday to Sunday). No rainfall was recorded at any of the study sites during the summer 2015/16 and winter 2016 sampling campaigns, whereas high rainfall was recorded for the whole study region during summer 2016/17. For the summer 2015/16 and winter 2016 sampling, grab samples (200 ml) were collected daily from both the influent (after grit screens) and effluent (after chlorination), acidified to pH 3 using Hydrochloric acid (HCl) and stored at 4°C. These daily samples were subsequently topped up until the final sampling day (Friday) when analyte extraction took place, giving a week-long composite sample for each WWTW. For the summer 2016/17 sampling, grab samples (200 ml) of both influent- and effluent wastewater were collected each morning, followed by immediate filtration and sample processing, giving a daily grab sample for each of the four WWTWs. For all the sampling campaigns (Dec 2015/16; 2016/17 and Jun 2016), grab samples of surface water (200 ml) in receiving rivers were also taken at locations upstream (50 m) and downstream (50 m) from the respective points of discharge of the WWTWs. Daily sampling was not possible due to logistical constrains. Therefore, samples were collected on a single day during the week, acidified (pH 3) using HCl and kept cold (± 4^0C) during transportation and storage until analyte extraction.

Table 3.1: Information of the sample sites during the current study.

	Load (Domestic:Industrial)	Capacity (ML/day)	Treatment	Population estimate [#]
WWTW1	70:30	105.0	BF, AS, BNR (Bardenpho)	528 942
WWTW2	60:40	55.0	AS (2-stage), BNR (Phostrip)	260 448
WWTW3	100:0	0.4	AS	4 516
WWTW4	100:0	16.0	BF, AS (BNR 3-stage)	23 273
WWTW5	90:10	35.0	AS (Phoredox), *chlor*	275 929
WWTW6	60:40	32.0	BF, *chlor*	229 674
WWTW7	83:17	36.0	BF, AS, *chlor*	73 574
WWTW8	96:4	83.0	BF (1-3), AS (Bardenpho), *chlor*, MP	330 473
WWTW9	100:0	10.0	AS (BNR 3-stage), *chlor*	35 397
WWTW10	96:4	155.0	BT, AS, *chlor*	1 092 297

[#] based on chemical oxygen demand (COD) measurements generated from raw sewage influent from each WWTW
BF – Biological filtration; AS – Activated sludge; BNR – Biological nutrient removal; BC – Biological clarification; *chlor* – Chlorination; MP – Maturation ponds; n.a. – not available

3.2.2. Extraction Procedure

Water samples were filtered using glass fibre filters (1.2µm pore size, Munktell) using a glass vacuum filtering system (Millipore) to get rid of solids in the samples. The filtrate was then extracted through solid-phase extraction (SPE) with Oasis HLB cartridges (6cc, 200 mg; Waters, Microsep, Johannesburg, South Africa). The cartridges were conditioned with 4 mL methanol, followed by 4 mL of ultrapure water (Millipore) and allowed to pass through the column by gravity. After conditioning, the water samples (200 mL) were passed through using a manifold (Supelco Visiprep) at a flow rate of 5 $mL.min^{-1}$ and allowed to run dry for a minimum period of 30 minutes. The dried cartridges were eluted with 5 mL MeOH (HPLC-grade; Sigma) by gravity and then dried under a gentle stream of nitrogen. The evaporated samples were then re-suspended in 400 µL MeOH, giving a 500x concentrated water sample. All samples were stored in 2 mL amber glass vials (CNW Technologies, Stargate Scientific) and stored at -20ºC until the bioassays were done.

3.2.3. Yeast Estrogen Screen (YES)

The yeast-based screen followed the protocol described by Sohoni and Sumpter (1998). Briefly, *Saccharomyces cerevisiae* transfected with the human oestrogen receptor (hER) gene and a plasmid containing an estrogen response element-linked *lac*-Z gene was used. Successful binding of ligands in the water samples (steroids and other EDCs) to the receptors in the yeast cells initiates the expression of the *lac*-Z reporter gene which encode for the enzyme β–galactosidase in the assay. The β–galactosidase then metabolises chlorophenol red galactopyranoside (CPRG), which results in a colour change of the assay medium, indicating a dose-dependent activity of the ligands to bind to the estrogen receptor.

The assay medium was prepared as described by Sohoni and Sumpter (1998). The yeast were incubated in assay medium containing no CPRG for 48 hours under 26ºC on an orbital shaker. The concentrated wastewater extracts (500x) were serially diluted and 10 µL was spiked into the 96-well sterile flat-bottomed plates with low evaporation lids (Costar, 3370, Sigma). The previously incubated yeast culture was then included into new assay medium containing CPRG at a concentration of approximately $8x10^5$ cells/mL. The seeded assay medium was then added at 200 µL/well into the assay plate to provide a final concentrations of the water extracts ranging from a 50x to a 1.56x. A concentration of 1x was depicted as an un-concentrated water sample. For the raw wastewater samples, serial dilutions of the samples were made with MeOH to obtain a concentration range of each sample ranging from 12.5x to 0.39x in the assay due to cytotoxicity observed in the 50x and 25x concentrated sample. For the final effluent and river water samples, serial dilutions of the samples were made with MeOH to obtain a concentration range of each sample ranging from 50x to 6.25x due to the lower observed estrogenicity in these samples compared to raw wastewater samples. All samples were analysed in triplicate in the same assay plate, and each assay was repeated twice. A standard curve for the steroid hormone 17β-estradiol (E_2; CAS 50-28-2; Sigma) was included for each assay plate in 12 serial dilutions, ranging from 1.0 to 2700.0 $ng.L^{-1}$. Blank wells were also included in each assay plate containing only assay medium and 10 µL of evaporated MeOH without any hormone spike or water sample extracts. The assay plates were then allowed to incubate on a shaker for 72 hours at 30ºC under dark conditions.

3.2.4. Yeast anti-estrogen screen (YAES)

The YAES was performed for the treated wastewater effluent samples during two sampling campaigns (winter 2016 and summer 2016/17) in the same manner as described for the YES, with minor modifications. Each well in the 96-well assay plate was spiked with a submaximal E_2 concentration of 450 ng/L prior to addition of the concentrated water extracts (final concentration of 75x, 50x and 25x). Apart from an E_2 standard curve as used in the YES, the YAES contained a positive control of the estrogen receptor antagonist tamoxifen (TAM; CAS 10540-29-1; Sigma) in 12 serial dilutions (0.91 to 1857.6 $mg.L^{-1}$ in the assay). The blank wells were separated into two sets; one set containing assay medium and yeast with a submaximal E2 spike (6 wells) and one set containing blank wells with only the assay medium and yeast (6 wells). The assay plates were then allowed to incubate on a shaker for 72 hours at 30ºC under dark conditions.

3.2.5. Calculations

Upon the 72 hours of incubation, the YES and YEAS assay plates were measured for colour change using a spectrophotometer. The absorbance was measured at 570 nm for colour change of CPRG caused by steroid hormone-mediated β–galactosidase production, and 620 nm for turbidity change and cytotoxicity. The turbidity change calculations (620 nm) were necessary to assess potential false negative- or positive results generated for the colour change calculations (570 nm), as a loss in turbidity (caused by cytotoxic analytes in the sample) leads to less viable yeast cells in the assay to produce β–galactosidase. The threshold for cytotoxicity in the samples were determined using equation 1. Samples which were below this threshold were excluded from further calculations.

$$\text{Cytotoxicity} = \text{Median Blank}_{620nm} - (3*\text{stdev of Blank}_{620nm}) \quad [1]$$

In order to correct for turbidity in the wells, a corrected absorbance (CA) was calculated for each sample in the assay using equation 2. This calculation compensated for background absorbance from the yeast suspension and allowed for more accurate measurement of the colour change in the assay medium.

$$\text{Corrected absorbance (CA)} = (OD_{570nm} - [OD_{620nm} - \text{Blank}_{620nm}]) \quad [2]$$

where OD_{570nm} and OD_{620nm} refers to the optical density of the sample measured at 570nm and 620nm respectively, and Blank_{620nm} refers to the median optical density measured for the blank wells in each assay plate at 620nm. Water samples were only considered for further analysis if the corrected absorbance was above a detection threshold using equation 3:

$$\text{Detection} = \text{Median blank}_{CA} + (3*\text{stdev of Blank}_{CA}) \quad [3]$$

For the YES, the CA of water samples above the detection threshold of the assay were then log-transformed and expressed as a percentage of the maximum log-absorbance value calculated in the E_2 standard curve using equation 4:

$$\text{Log \% max } E_2 \text{ (sample/standard curve)} = (\text{log-CA}_{sample} / \text{log-CA}_{E2\ max}) * 100 \quad [4]$$

A non-linear calibration curve was then constructed for the E_2 standard curve of each individual assay plate by plotting the calculated log % max E_2 of the E_2 dilution series against its known concentration (in $ng.L^{-1}$). An E_2-equivalent concentration (EEQ; ng/L) for each water sample was then calculated from the generated trend-line of the calibration curve, and corrected for their dilution factors to obtain a final EEQ concentration of each water sample (in $ng.L^{-1}$).

The calculated EEQ values were then used to estimate their mass loads within raw wastewater and treated effluent in order to compensate for the variation in flow rates of the various treatment plants between the daily and seasonal sampling campaigns using equation 5:

$$\text{Mass load } (g.day^{-1}) = EEQ_{inf/eff} * FR_{inf/eff} * 1/1000 \quad [5]$$

where $EEQ_{inf/eff}$ refers to the EEQ concentration (in $ng.L^{-1}$) of the samples in the YES for influent and effluent wastewater samples, and $FR_{inf/eff}$ refers to the flow rate ($ML.day^{-1}$) of the influent and effluent wastewater for each WWTW during the time period of the sampling campaigns.

For estimation of the removal efficiency of estrogenicity at the WWTWs, removal (%) from each raw wastewater sample and the corresponding treated effluent sample was calculated using equation 6:

$$\text{Removal } (\%) = (ML_{inf} - ML_{eff}) / ML_{inf} * 100 \quad [6]$$

where $ML_{inf/eff}$ refers to the mass loads (in $g.day^{-1}$) of the samples calculated from EQ. 5 for influent and effluent wastewater samples at the various WWTWs.

For the YAES, the CA of the water extracts were compared with the CA measured for the blank wells containing the submaximal E_2 spike in order to evaluate a percentage change of the sample extracts from the submaximal E_2 spike. Samples which were above the E_2 spike threshold (above 100%) were considered to have a masking effect of inert estrogenicity supressing the anti-estrogen response, whereas samples below the E_2 spike threshold (below 100%) were considered to contain analytes which significantly supress the binding of E_2 to the hER in the assay, therefore showing an anti-estrogenic response. The samples that successfully supressed E2-mediated receptor binding were then considered for quantification of a

tamoxifen-equivalent concentration (TAM-EQ, $\mu g.L^{-1}$) as calculated in a similar manner as calculated for the EEQ calculations in the YES.

3.2.6. Statistical analysis

All statistical analyses were performed using GraphPad Prism (v. 5.00). Variation between individual samples were assessed using an unpaired t-test. For the determination of significant variation between sampling days and study sites, a one-way analysis of variance (ANOVA) with a Tukey's multiple comparisons *post hoc* test was performed. Significant variance was achieved with $P < 0.05$.

3.3. Results and Discussion

The results presented below relate to three interrelated themes, namely 1) the incidence of estrogenicity within raw wastewaters sourced from areas having heterogenous demographics, 2) the transfer and fate of (anti)estrogenicity from treated wastewater to receiving surface waters, and 3) refining bioassay outcomes for risk assessment, with particular reference to effect-based trigger values and observed endocrine disrupting outcomes in sentinel aquatic organisms. The study highlights the complexity associated with interpreting bioassay outcomes, such as the YES, when multiple potential biotic- and abiotic factors may influence the results. In particular, the variation in climatic factors between seasons (rainfall, temperature, etc.), as well as WWTW having variable capacities, treatment technologies, sources of wastewater, and *de novo / de facto* population may all have a significant impact on the contribution of estrogenic EDCs within wastewater and their associated environmental waters. Furthermore, the complex composition of environmental samples containing both inorganic- and organic micro-pollutants poses challenges when endpoints such as total estrogenicity and anti-estrogenicity are assessed using a single bioassay, where masking of agonistic and antagonistic substances by one-another, as well as other complex chemical mixture interactions are proposed. In light of the global need to improve on the quality of surface water resources, as set out by the United Nations Sustainable Development Goals for 2030, and the associated benefit of these goals towards human- and environmental resilience warrants the refinement of cost-effective bioassays such as the YES to improve on current risk assessment approaches.

3.3.1. Influent wastewater

Raw influent wastewater showed varying levels of estrogenic activity during the various sampling campaigns, which ranged from 4.4 – 45.4 ng.L^{-1} EEQ during summer periods (2015/16 and 2016/17; APPENDIX Table A1 and A2) and 6.6 – 31.5 ng.L^{-1} EEQ during the winter 2016 sampling (APPENDIX Table A1). WWTW2, 7 and 10 showed the highest levels of estrogenicity during summer 2015/16 (APPENDIX Table A1), and WWTW1, 3 and 5 during winter 2016 (APPENDIX Table A2). However, by taking the variations of plant flow rates into consideration, EEQ mass loads showed that WWTW1, 2, 8 and 10 had highest levels during summer 2015/16, compared to WWTW1, 2, 5 and 10 during winter 2016 (Fig. 3.1). As it is expected that the estrogenic response in the YES will most likely be from natural- and synthetic steroids (associated to domestic wastewater), the WWTWs which received the largest estrogenic loads were those that predominantly receives domestic sewage, apart from WWTW2, which receive wastewater from surrounding industries and also from a nearby airport.

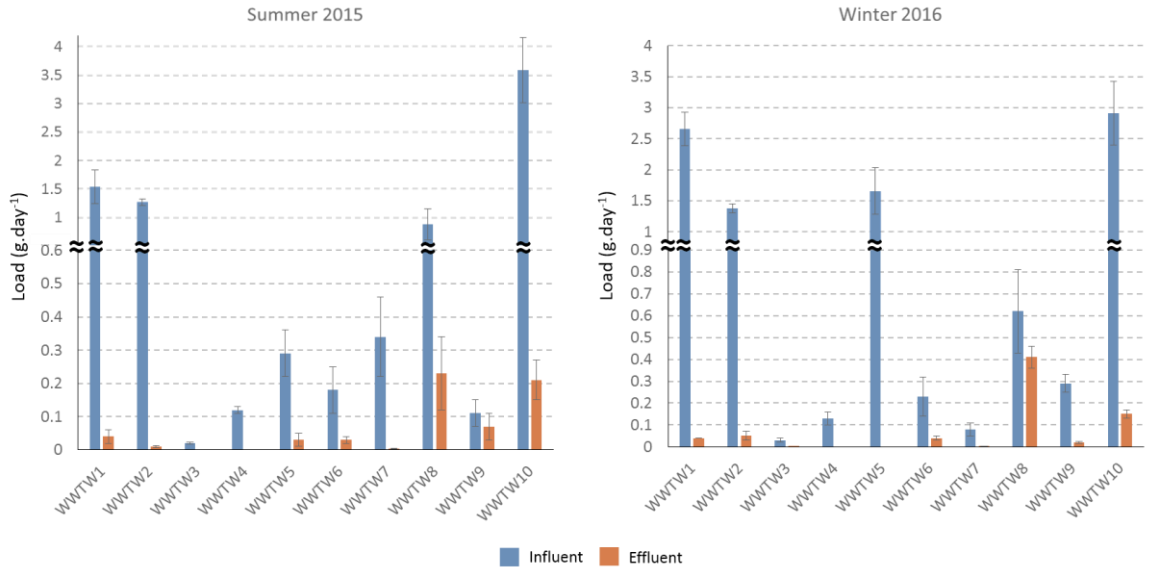

Figure 3.1: Mass load (g.day^{-1}) of estrogen equivalent concentrations (EEQ) measured using the yeast estrogen screen (YES) for the various WWTWs during sampling campaigns in summer 2015/16 (December) and winter 2016 (June).

For the selected WWTWs screened during summer 2016/17, the EEQ estimations within raw wastewater did not differ significantly between sampling days for either WWTW2, 9 or 10 (ANOVA, $P > 0.05$; APPENDIX Table A2). However, EEQ values for WWTW8 showed a significant variation in estrogenicity between sampling days (ANOVA, $P < 0.05$), with values on Tuesday and Wednesday being significantly lower than weekend samples (Tukey, P <

0.001). The variation in daily flow rates could also impact the variation of net estrogenicity between sampling days. For this reason, EEQ mass loads were instead used to compare between sampling days. For WWTW2, significant variation was only calculated between the Tuesday and Sunday sample (ANOVA, $P = 0.033$; Tukey, $P < 0.05$; Fig. 3.2; APPENDIX Table A3). For WWTW8, a significant increase in estrogenic loads were calculated over the weekend period (Friday to Sunday), as well for the following Monday (ANOVA, $P < 0.0001$; Tukey, $P < 0.05$; Figure 3.2; APPENDIX Table A3). For WWTW9, estrogenic loads decreased as the week continued, and increased significantly from Friday to the weekend period (ANOVA, $P < 0.0001$; Tukey, $P < 0.05$; Figure 3.2; APPENDIX Table A3). For WWTW10, the same trend was observed as for WWTW9, with a more pronounced decrease during the week, followed by an increase over the weekend period (ANOVA, $P < 0.0001$; Tukey, $P < 0.01$; Figure 3.2; APPENDIX Table A3). Again, WWTW8, 9 and 10 almost exclusively receives domestic wastewater, whereas WWTW2 receives a large proportion of industrial wastewater (Table 3.1). Furthermore, the level of estimated EEQ mass loads calculated for these WWTWs (WWTW10 > WWTW8 > WWTW2 > WWTW9; Fig 3.2) were in association with each plant's estimated population size (Table 3.1). These results further suggest that estrogenicity are predominantly influenced by the *de facto* and *de novo* population being served by the sewage plants. As shown by the current study, the daily variation for estrogenicity in raw wastewater do not follow the same trend for all WWTWs (Fig. 3.2), but highlight the fact that wastewater treatment monitoring for estrogenicity should not just be a periodic event, as significant variation in estrogenic compounds entering WWTWs may occur over time.

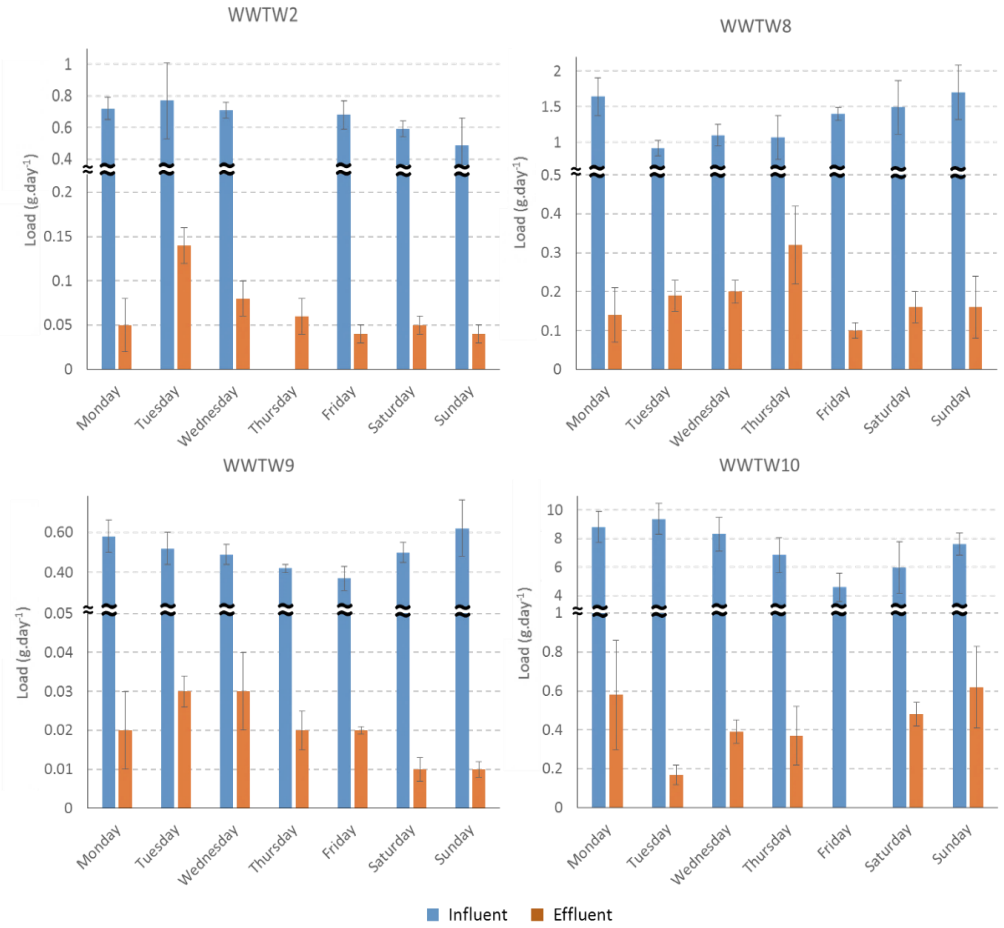

Figure 3.2: Daily variation in mass loads (g.day^{-1}) of estrogen equivalent concentrations (EEQ) estimated for the various WWTWs during the summer 2016/17 sampling campaign (December).

The two summer sampling campaigns (2015/16 and 2016/17) allowed for comparisons between similar seasons experiencing varying climatic events, such as rainfall. The EEQ mass loads in raw wastewater influent between the two sampling campaigns did not differ significantly for WWTW8 (t-test, $p > 0.05$), but were significantly different for WWTW2, WWTW9 and WWTW10 (t-test, $p < 0.05$; Table 3.2). Higher loads were estimated during summer 2015/16 for WWTW2 as opposed to higher loads which were recorded during summer 2016/17 for WWTW 9 and WWTW10. To further demonstrate the complex nature of these analyses, it is worth to consider the high rainfall pattern which was experienced during summer 2016/17 sampling, which subsequently led to higher flow rate operations from the WWTWs (APPENDIX Table A1). In particular, the flow rates for both raw influent and treated effluent at WWTW10 increased more than two fold (APPENDIX Table A1). Due to the large treatment capacity of WWTW10, raw wastewater destined for WWTW7 and WWTW8 are normally diverted to this plant during large runoff surges, such as rainfall events. The estimated EEQs (ng.L^{-1}) in raw wastewater for this plant between the two summer sampling campaigns did not

differ significantly (t-test, $p > 0.05$; APPENDIX Table A1 & A2), whereas EEQ mass loads ($g.day^{-1}$) increased by a factor of 2.1 from summer 2015/16 to summer 2016/17 (Table 3.2). As EEQ estimates were relatively similar between summer 2015/16 and 2016/17, the 2-fold increase in EEQ mass loads are merely due to a larger percentage of wastewater being treated, but not necessarily diluting the total estrogenic concentrations.

Estimating the exact source of estrogenicity within environmental samples may prove difficult, which was the reason why chemical analysis of known estrogenic micro-pollutants were not considered during the present study. Several pesticides, pharmaceuticals, personal care products and industrial by-products have been established as estrogenic EDCs (Archer et al., 2017b; Mckinlay et al., 2008). However, estrogenicity within waste- and surface waters are primarily attributed to the presence of synthetic- and natural estrogen hormones (Tanaka et al., 2001), merely due to such steroid hormones being more potent ER agonists than the estrogen-mimicking EDCs. For example, plastisizers such as bisphenol-A (BPA) are known to bind to the hER in a dose-dependent manner, although at far lower potencies than E_2 and EE_2 (Bistan et al., 2012). Regardless, this warrants the potential of such known estrogen agonists to exert an additive mixture effect towards a net estrogenic response in bioassays such as the YES.

Table 3.2: Mass loads (g.day^{-1}) estimated using EEQ concentrations in the YES for both raw- and effluent wastewater samples at the various WWTWs.

Site	December 2015 (Summer)			June 2016 (Winter)			December 2016 (Summer)		
	Influent	Effluent	Removal (%)	Influent	Effluent	Removal (%)	Influent	Effluent	Removal (%)
WWTW1	1.54 ± 0.29	0.04 ± 0.02	97	2.66 ± 0.27	0.04 ± 0.001	98	-	-	-
WWTW2	1.27 ± 0.06	0.01 ± 0.004	99	1.38 ± 0.07	0.05 ± 0.02	96	0.66 ± 0.1	0.07 ± 0.04	89
WWTW3	0.02 ± 0.003	0.0004 ± 0.0001	98	0.03 ± 0.01	0.001 ± 0.0002	98	-	-	-
WWTW4	0.12 ± 0.01	-	-	0.13 ± 0.03	-	-	-	-	-
WWTW5	0.29 ± 0.07	0.03 ± 0.02	90	1.66 ± 0.37	-	-	-	-	-
WWTW6	0.18 ± 0.07	0.03 ± 0.01	83	0.23 ± 0.09	0.04 ± 0.01	83	-	-	-
WWTW7	0.34 ± 0.12	0.003 ± 0.001	99	0.08 ± 0.03	0.002 ± 0.0001	98	-	-	-
WWTW8	0.88 ± 0.27	0.23 ± 0.11	74	0.62 ± 0.19	0.41 ± 0.05	34	1.33 ± 0.3	0.18 ± 0.1	86
WWTW9	0.11 ± 0.04	0.07 ± 0.04	36	0.29 ± 0.04	0.02 ± 0.005	93	0.50 ± 0.09	0.02 ± 0.01	96
WWTW10	3.59 ± 0.57	0.21 ± 0.06	94	2.91 ± 0.51	0.15 ± 0.02	95	7.36 ± 1.69	0.37 ± 0.2	95

3.3.2. Treated Effluent

EEQ estimations in treated wastewater effluents from the various WWTWs varied between 0.3 to 6.9 ng.L^{-1} during summer 2015/16 (APPENDIX Table A1), 0.2 to 4.9 ng.L^{-1} during winter 2016 (APPENDIX Table A1), and 0.2 to 3.0 ng.L^{-1} during summer 2016/17 (APPENDIX Table A2). Mass loads of the estimated EEQs, which compensated for flow variations between the WWTWs, ranged between 0.0004 to 0.23 g.day^{-1} during summer 2015/16, and 0.001 to 0.41 g.day^{-1} during winter 2016 (Fig. 3.1; APPENDIX Table A3), and 0.01 to 0.58 g.day^{-1} during summer 2016/17 (Fig. 3.2; Table 3.2). The estimated EEQs from the current study correlates well to similar monitoring studies done elsewhere in the world. EEQs estimated in two Canadian WWTW effluents ranged from 1.0 – 24 ng/L (Arlos et al., 2018), 0.6-1.7 ng/L in WWTW effluent from the Netherlands (Avberšek et al., 2011), and 0.5-18 ng.L^{-1} in WWTW effluent from 75 European WWTWs (Jarošová et al., 2014b). Although high EEQs were estimated in the study by Jarošová et al. (2014b), it should be noted that final effluents of only two of the 75 WWTWs sampled showed EEQs above 10 ng.L^{-1}, most ranging between 1 and 4 ng.L^{-1} (Jarošová et al., 2014b).

Some studies have aimed at correlating the concentrations of steroid hormones with observed EEQs generated from assays such as the YES. A study by Truter et al. (2016) compared levels of E_2 and EE_2 with estimated EEQ values using the YES. Within a particular surface water sampling site, concentrations of E_2 ranged between 3.9 – 30.8 ng.L^{-1}, for EE_2 between 1.36 – 10.83 ng.L^{-1}, and for EEQ in the YES between 10.15 – 43.01 ng.L^{-1}. From these results, the combined load of the major estrogen analytes (E_2 and EE_2) correlated well with the EEQs generated by the YES in the same samples. However, the same was not observed for comparisons between the concentrations of known steroid hormones and EEQ estimates in various WWTW effluents, in which the EEQs were much lower than the concentrations of the known estrogenic micro-pollutants (Aerni et al., 2004). The current study did not analyse the levels of steroid hormones. However, concentrations of E_2 from WWTW effluents have been shown to range from 1 – 20 ng.L^{-1} in South African monitoring studies (Manickum et al., 2011; Swart and Pool, 2007). Although these maximum levels of E_2 detected in WWTW influent and effluent are higher than shown for total estrogenicity in the current study (APPENDIX Tables A1 & A2), it should be noted that the current assay deals with a mixture of micro-pollutants in the water sample. Therefore, analytes that do not necessarily bind to steroid hormone receptors, but rather interfere with steroid receptor binding (such as anti-estrogens) may influence the

results from the assay (Gehrmann et al., 2016). For this reason, the presence of anti-estrogenicity was measured in treated wastewater to verify such a possible masking effect.

The estimated TAM-EQ concentrations ranged from 3.7 to 78.7 ug.L^{-1} in the treated wastewater from the ten WWTWs during the winter 2016 sampling campaign (APPENDIX Table A6), and 0.7 to 10.0 ug.L^{-1} for four WWTWs during the summer 2016/17 sampling campaign (APPENDIX Table A7). The results from the YAES indicated a possible masking effect by estrogenic compounds present in the water samples, whereby estrogenicity in the YAES decreased in the concentrated water extracts in a dose-dependent manner (APPENDIX Figs. A1 & A2). This is represented by a significant deviation above 100% of the E2 spike in the YAES for a 75x concentrated sample of WWTW2, 6, 9 and 10, whereby the deviation was less within a 50x and 25x concentrated sample respectively (APPENDIX Fig. A1). This may be attributed to the estrogenicity calculated for these WWTWs in the YES during the same sampling periods (APPENDIX Figs. A1 & A2). Higher estrogenicity was associated with lower anti-estrogenicity for most of the WWTW effluent samples (Fig. 3.3 & 3.4), as further pointed out in *Appendix A.*

Pollutants contributing to anti-estrogenicity may vary between the locations of the WWTWs, which are influenced by e.g., the types of industry in the area, as well as different agricultural practices and types of crops necessitating different pesticide applications. Recalcitrant pollutants with the ability to antagonise ER binding may potentially persist throughout wastewater treatment processes. For example, the bisphenol antiseptic hexachlorophene (HCP), the synthetic vitamin menadione (K3) and the pesticide/disinfectant pentachlorophenol (PCP) have all shown affinity to antagonise ER binding and associated transcriptional activity by several *in vitro* assays (Jung et al., 2004). Anti-estrogenicity has also been identified for the fungicides propiconazole and myclobutanil, the herbicides dicamba, mecroprop, terbutryn, diuron and irgarol, and the neonicotinoid thiacloprid in a dose-dependent manner (Westlund and Yargeau, 2017). Fragrances such as tonalide and galaxolide, and UV filters such as BP3, 4-MBC, octocrylene and EHMC are all known anti-estrogens, all with demonstrated recalcitrance to biodegradation (Hernandez Leal et al., 2010). Furthermore, degradation processes may create undesired by-products which may lead to anti-estrogenicity in treated wastewater. In particular, chlorination is known to increase anti-estrogenicity in wastewater by means of dissolved organic matter creating various disinfection by-products (Wu et al., 2009).

Focused studies at WWTWs that apply chlorination as final disinfection step prior to- and after the chlorination step should provide valuable information in this regard.

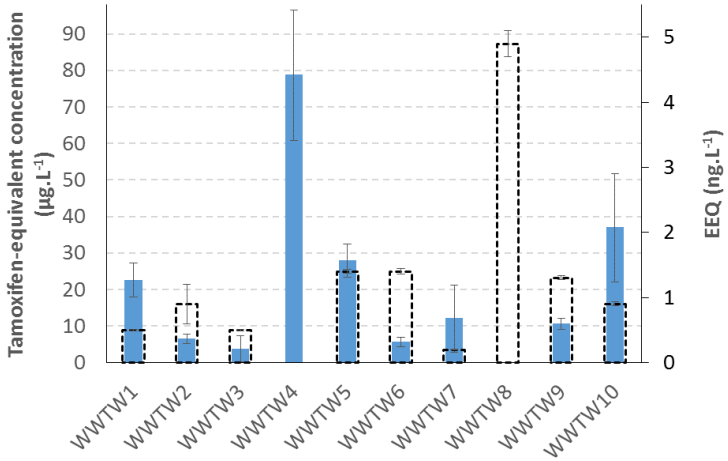

Figure 3.3: Anti-estrogenicity calculated as tamoxifen-equivalent concentrations (1º y-axis; µg.L^{-1}) for treated wastewater effluent at the various WWTWs during the winter 2016 sampling campaign (solid bar). The calculated EEQ concentrations (ng.L^{-1}; 2º y-axis) are represented by the dashed bar for the same WWTWs.

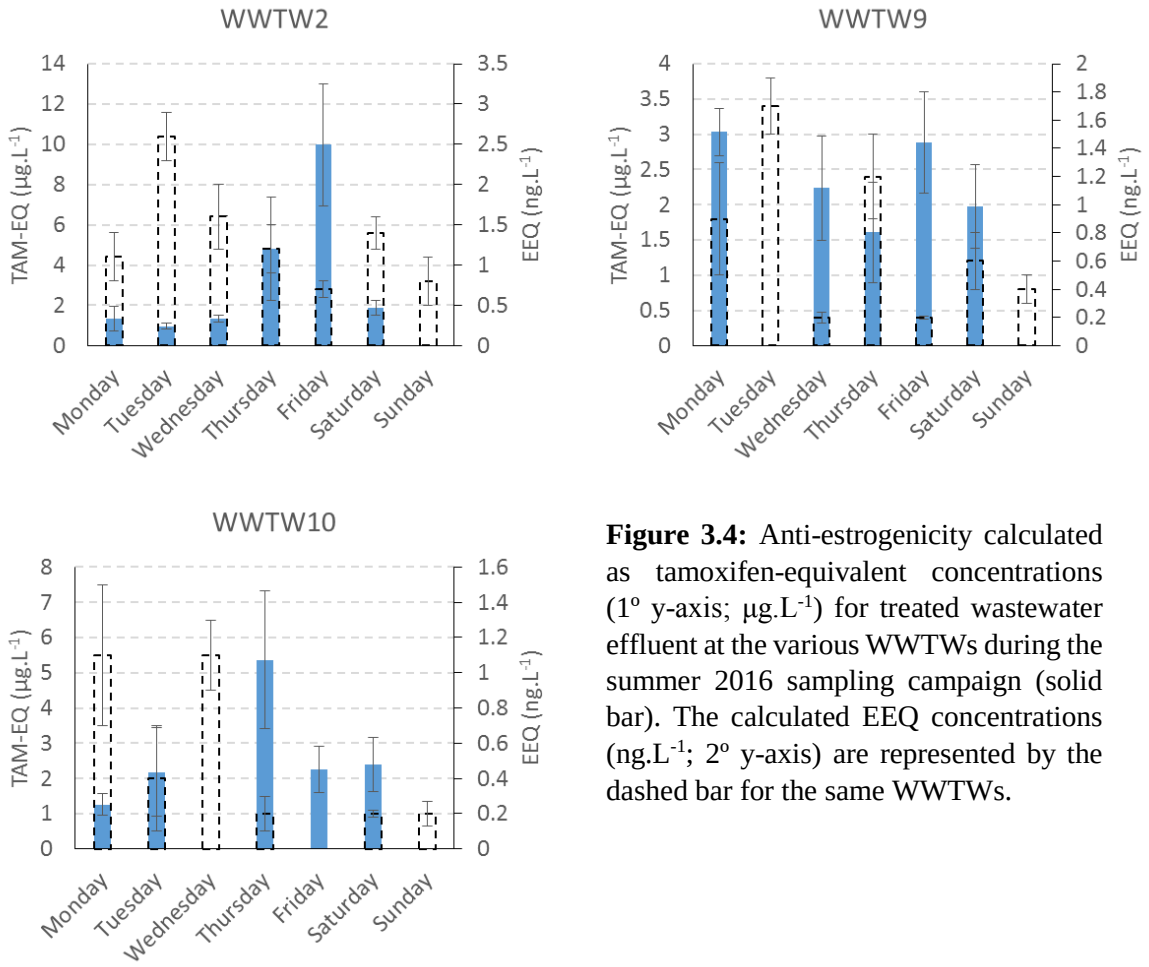

Figure 3.4: Anti-estrogenicity calculated as tamoxifen-equivalent concentrations (1º y-axis; µg.L^{-1}) for treated wastewater effluent at the various WWTWs during the summer 2016 sampling campaign (solid bar). The calculated EEQ concentrations (ng.L^{-1}; 2º y-axis) are represented by the dashed bar for the same WWTWs.

3.3.3. Removal of estrogenicity during wastewater treatment

The EEQ mass load estimates ($g.day^{-1}$) were considered as the most useful to show the removal of estrogenicity at the various WWTWs and between sampling campaigns, rather than using raw EEQ concentrations ($ng.L^{-1}$). For example, EEQ estimations ($ng.L^{-1}$) for raw- and treated effluent wastewater for WWTW9 showed a removal of 13% during the summer 2015/16, and a removal of 26% for WWTW8 during winter 2016 (APPENDIX Table A1). By rather using EEQ mass load estimations, removal was 36% for WWTW9 and 34% for WWTW8 respectively (Table 3.2). EEQ loads showed moderate- to high removal in all the WWTWs during the sampling periods (APPENDIX Tables A1 & A2), with the exception of the previously mentioned WWTWs. In particular, WWTW8 showed moderate removal during summer 2015/16 and low removal during winter 2016 (APPENDIX Table A1), as well as variable removal between sampling days during summer 2016/17 (APPENDIX Table A2).

It should be stated that the hydraulic retention time of the WWTWs were not included for removal calculations. Therefore, such removal estimations are also considered semi-quantitative, as a raw influent- and treated effluent sample taken during the same day does not reflect the time taken for the treatment of the influent sample before its effluent product was analysed in parallel. Regardless, the removal estimates from the current study shows good agreement with similar studies using the YES (Murk et al., 2002), and for known estrogenic compounds during conventional wastewater treatment (Arlos et al., 2018; Avberšek et al., 2011; Manickum and John, 2014). For example, concentrations of E_2 has been estimated to range between 20 – 200 $ng.L^{-1}$ in a WWTW influent situated in Kwazulu Natal, with effluent levels ranging from 4 – 110 $ng.L^{-1}$ in the same plant (Manickum and John, 2014). Taken these upper and lower limits of detection, the removal may range between 45% and 80%. Although a moderate- to significant removal of estrogenic compounds during wastewater treatment are reported, the levels which will still be discharged into recipient waters may still pose an environmental risk or may add to the pollutants which are present within the surface water system.

3.3.4. River water

Although the source of estrogenicity in surface waters are often proposed to primarily originate from WWTW effluent discharge, the EEQ estimates for river water located upstream from seven of the 10 WWTWs showed higher levels than downstream water samples (APPENDIX

Table A1). This was particularly shown for WWTW9, where the average upstream EEQ value during winter 2016 sampling was 18.9 (±0.2) ng.L^{-1} as opposed to a calculated EEQ value of 15.1 (± 0.9) ng.L^{-1} for the raw influent of the plant (APPENDIX Table A1). This river system has been reported to experience extreme pollution pressures, mainly form peri-urban communities located upstream from the WWTW, including direct sewage disposal into the river system. The river system associated with WWTW1 passes through informal settlements and industrial areas. The high loads of esterogenicity within upstream river waters samples are therefore indicative of alternative pollution sources. The river system associated with WWTW2 serves as a main feed into a reservoir in a nature preserve, providing approximately 8% of the drinking water to a city with a population exceeding 2.1 million. This drinking water resource has been previously shown to be highly impacted by industrial pollutants and other known EDCs (Aneck-Hahn et al., 2009; Barnhoorn et al., 2015, 2004). Alarmingly, the estimated EEQs from the downstream river sample was higher than EEQs estimated for WWTW2 effluent during the summer 2015/16 sampling campaign (APPENDIX Table A1). We previously reported similar results for this river, showing an extensive list of pharmaceuticals and personal care products at higher concentrations in downstream river water compared to treated wastewater discharge (Archer et al., 2017b). The same trend was shown in the current study for WWTW1, 3, 6, 7 and 9 during summer 2015/16, and WWTW1, 3, 6 and 9 during winter 2016 sampling.

The YES received wide recognition as a bioassay to estimate net estrogenicity in surface waters, with estimated EEQ values ranging between 0.33-4.5 ng.L^{-1} in France (Cargouët et al., 2004), up to 10 and 26.5 ng.L^{-1} in Belgium and the UK respectively (Matthiessen et al., 2006), 0.4-6.3 ng.L^{-1} in South Korea (Ra et al., 2011), and 0.07-6.4 ng.L^{-1} in China (Jiang et al., 2012; Xiao et al., 2017). The higher levels of estrogenicity of the current study are in agreement with estimated EEQs in another South African river system receiving domestic wastewater discharge and surrounded by agriculture and mining activities, ranging between 10.2 ng.L^{-1} during winter, 14.2 ng.L^{-1} during spring, and 43.0 ng.L^{-1} during summer (Truter et al., 2016). The reliance on these rivers for potable water at various scales – from small communities to large cities, highlights the need to establish the environmental risk through a refined weight-of-evidence approach.

3.3.5. Risk assessment

Despite the moderate- to efficient removal of estrogenicity by wastewater treatment (Table 3.2), the measured EEQ values still pose a potential adverse health risk. As conventional risk assessment approaches are focussed on acute- or chronic toxicity endpoints, the use of predicted no-effect concentrations (PNEC) and no-observed effect concentrations (NOEC) are mostly incorporated to assess potential lethal toxicity in aquatic wildlife (Hernando et al., 2006). However, such an approach is largely focussed on the toxicity of individual chemicals, and therefore does not consider the complex mixture interactions of environmental pollutants as a whole within a water system. The YES offers a viable option that indicate the net estrogenic potential of a water sample to modulate hormone receptor binding, with the estimated EEQs providing a semi-quantitative assessment of all compounds which may mimic an estrogenic response in a similar manner as E_2. It is therefore possible to compare such EEQ values to other toxicological studies. For example, it has been shown that a concentration of 5 $ng.L^{-1}$ E_2 may induce the production of vitellogenin (VTG) in male fish (Brion et al., 2004). This protein is a precursor of egg yolk in oviparous animals, and is considered as an established biomarker of endocrine disruption, as its production is mediated by plasma steroid hormones (Aerni et al., 2004). Any pollutant in the water samples which mimics an E_2 mechanism of action (expressed as EEQs in the YES) may therefore create the same effect. As expected, nearly all of the EEQ estimates of raw influent WWTW samples were above the 5 $ng.L^{-1}$ threshold (APPENDIX Tables A1 & A2), as several micro-pollutants are known to mimic an estrogen response similar to E_2 (Archer et al., 2017c; Mckinlay et al., 2008). On the other hand, only one WWTW effluent sample showed an EEQ estimate above the 5 $ng.L^{-1}$ threshold during the summer 2015/16 sampling campaign (Fig. 5; APPENDIX Table A1), with both its river water samples located upstream and downstream of the discharge to also surpass this 5 $ng.L^{-1}$ level during both the summer and winter sampling campaigns (Fig. 7; APPENDIX Table A1). Overall, the estimated river water EEQs showed more pronounced environmental risks than treated wastewater effluent, whereby EEQ estimates for up- and downstream river water at WWTW1 and WWTW9 exceeded, or were close to this threshold during summer 2015/16 and winter 2016 (Fig. 3.7; APPENDIX Table A1).

Apart from the observed estrogenic limit which shows a definite modulation of fish endocrine systems using an *in vivo* model, a predicted no-effect concentration (PNEC) of 2.0 $ng.L^{-1}$ E_2 has also been established as a baseline limit which may modulate fish reproduction (Caldwell et al., 2012). The EEQ estimations for treated effluent samples showed that WWTW8 exceeded

this threshold during both the summer 2015/16 and winter 2016 sampling campaigns (Fig. 3.5; APPENDIX Table A1) and on one day during summer 2016/17 (Fig. 3.6; APPENDIX Table A2). Furthermore, only a single sampling day for treated effluent at WWTW2 and 10 showed EEQs above this threshold (Fig. 3.6; APPENDIX Table A2). For river water, upstream and/or downstream sampling locations from WWTW1, WWTW7 and WWTW9 were above this PNEC during summer 2015/16 and for WWTW1, WWTW8 and WWTW9 during winter 2016 (Fig. 3.7; APPENDIX Table A1). Using such risk indicator concentrations for the YES therefore implicates whether further *in vivo* studies should proceed, as such studies are timely and costly, but still necessary to confirm areas under severe risk. On the other hand, effect-based trigger values have also been proposed to serve as a marker for a potential risk of adverse health outcomes. An estimated E_2 trigger value of 0.7 $ng.L^{-1}$ for drinking water standards have been proposed (Genthe et al., 2013), above which further monitoring should be considered to establish the identify and origin of the compounds. Jarosova et al (2014a) proposed a lower effect-based EEQ trigger value of 0.4 $ng.L^{-1}$ for long-term exposure to effluent-impacted surface waters. Several WWTW effluent and river water samples from both the summer 2015/16 and 2016/17, as well as the winter 2016 sampling campaigns were above these thresholds (Fig. 3.6, 3.7 & 3.8; APPENDIX Tables A1 & A2).

It is clear from the current study that the interpreting bioassay outcomes, such as the YES, are complex, given the multiple biotic- and abiotic factors which may influence the results. In particular, the variation in climatic factors between seasons (rainfall, temperature, etc.), as well as WWTW having variable capacities, treatment technologies, sources of wastewater, and *de novo / de facto* population may all have a significant impact on the contribution of estrogenic EDCs within wastewater and their associated environmental waters. Furthermore, the complex composition of environmental samples containing both inorganic- and organic micro-pollutants poses challenges when endpoints such as total estrogenicity and anti-estrogenicity are assessed using a single bioassay, where masking of agonistic and antagonistic substances by one-another, as well as other complex chemical mixture interactions are proposed. In light of the global need to improve on the quality of surface water resources, as set out by the United Nations Sustainable Development Goals for 2030 (UN-SDG), and the associated benefit of these goals towards addressing human- and environmental resilience warrants the refinement of cost-effective bioassays such as the YES to improve on current risk assessment approaches.

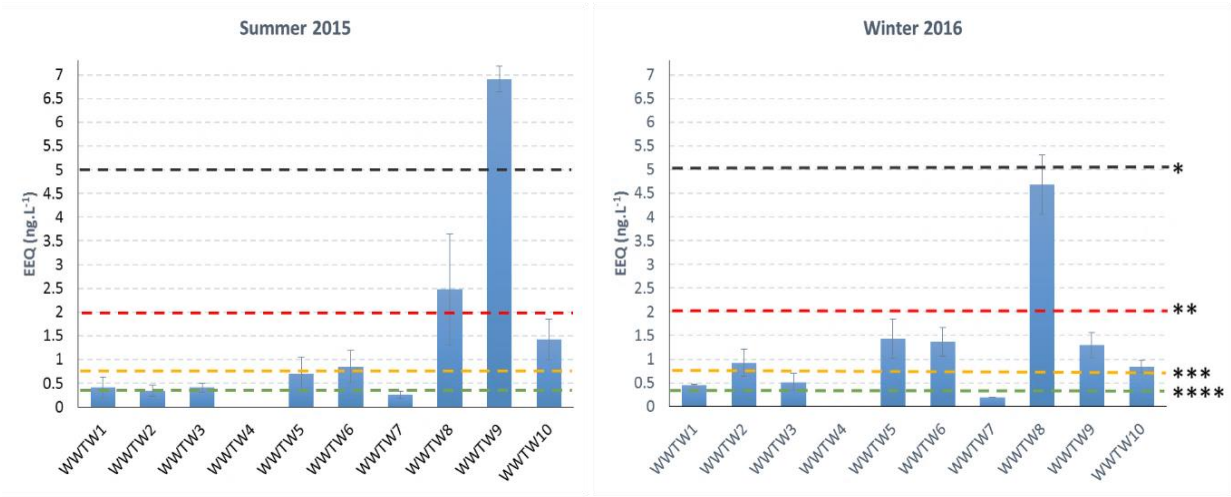

Figure 3.5: E_2-equivalent concentrations (EEQ; ng.L^{-1}) measured at the treated effluent samples from the 10 WWTWs during December 2015 (summer) and June 2016 (winter). * Concentration of E_2 (5 ng.L^{-1}) showing increased VTG production in fish (Brion et al., 2004). ** Predicted no-effect concentration (PNEC, 2 ng.L^{-1}) to modulate fish reproduction (Caldwell et al., 2012). *** Estimated trigger value (0.7 ng.L^{-1}) for risk in drinking water (Genthe et al., 2013). **** EEQ trigger value (0.4 ng.L^{-1}) of effluent on long-term fish exposure (Jarošová et al., 2014a).

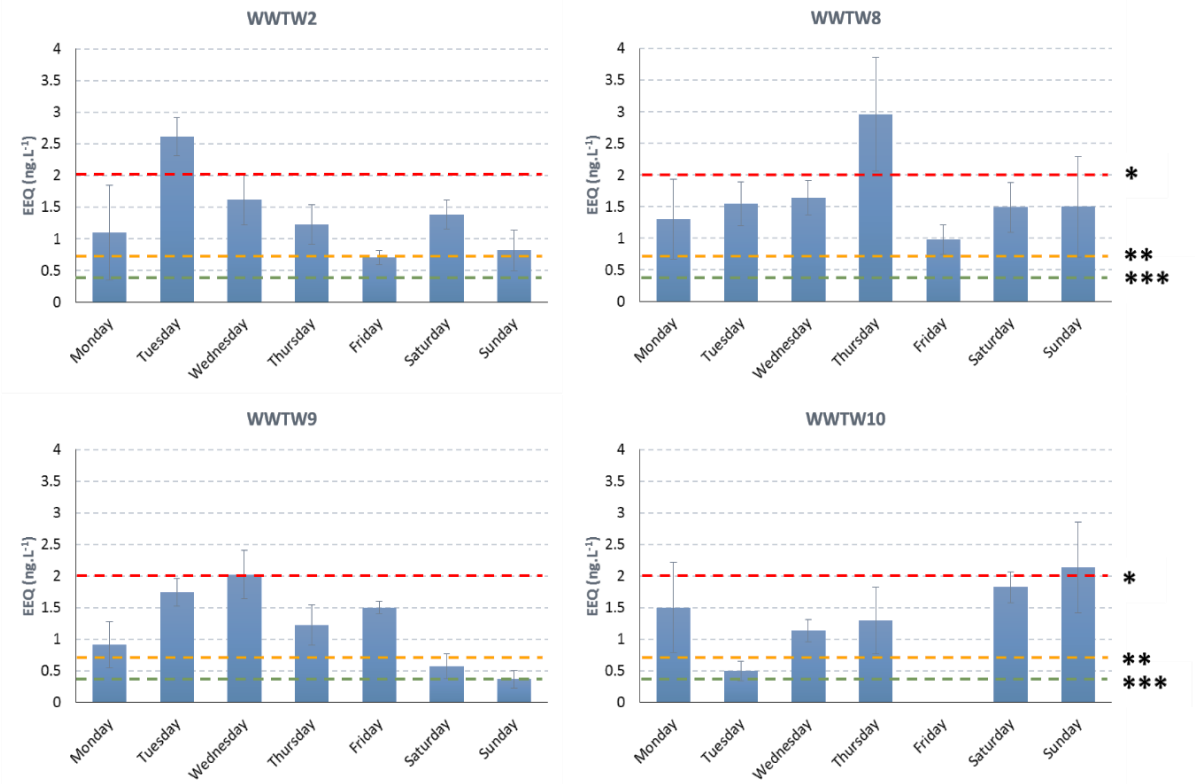

Figure 3.6: Daily E_2-equivalent concentrations (EEQ; ng.L^{-1}) measured at the treated effluent samples from the four WWTWs during December 2016 (summer). * Predicted no-effect concentration (PNEC, 2 ng.L^{-1}) to modulate fish reproduction (Caldwell et al., 2012). ** Estimated trigger value (0.7 ng.L^{-1}) for risk in drinking water (Genthe et al., 2013). *** EEQ trigger value (0.4 ng.L^{-1}) of effluent on long-term fish exposure (Jarošová et al., 2014a).

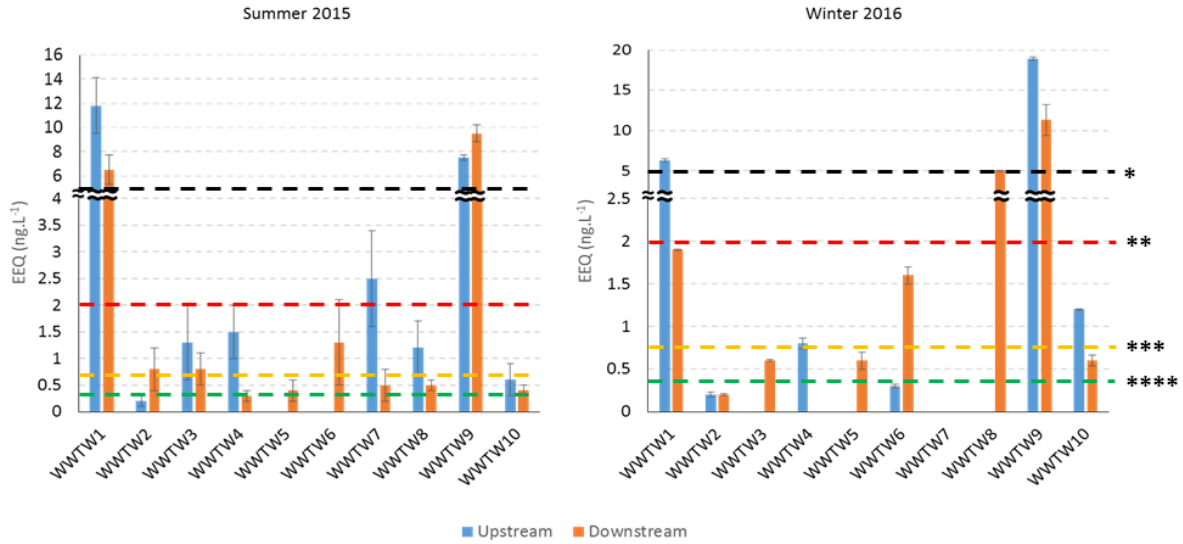

Figure 3.7: E_2-equivalent concentrations (EEQ; ng.L^{-1}) measured for river water samples located upstream and downstream of the WWTW discharges during December (summer 2015/16) and June (winter 2016). * Concentration of E_2 (5 ng.L^{-1}) showing increased VTG production in fish (Brion et al., 2004). ** Predicted no-effect concentration (PNEC, 2 ng.L^{-1}) to modulate fish reproduction (Caldwell et al., 2012). *** Estimated trigger value (0.7 ng.L^{-1}) for risk in drinking water (Genthe et al., 2013). **** EEQ trigger value (0.4 ng.L^{-1}) of estrogenicity on long-term fish exposure (Jarošová et al., 2014a).

3.4. Conclusions

The results presented here highlight the complex range of factors that may influence the fate of estrogenic EDCs during wastewater treatment, and the complexity when total estrogenicity and anti-estrogenicity are assessed using a single bioassay. The calculated EEQ and TAM-EQ values for total (anti)estrogenicity revealed by YES and YAES, respectively correlate well with existing literature and highlight the value of flow-proportional EEQ mass load estimations as an approach to compare WWTWs with variable treatment capacities. This offers a more refined estimation of removal estimations at WWTWs, and the subsequent potential negative impact caused by discharge. The yeast estrogen and anti-estrogen screens performed in this study further highlighted the complexity of environmental samples where a mixture of organic- and inorganic pollutants are present. The potential masking of both outcomes, however, should be considered when using such *in vitro* assays for risk decision making, as several mechanisms other than agonistic/antagonistic receptor binding may also influence the results. Regardless of whether environmental waters contain both estrogenic and anti-estrogenic analytes, the combined response generated from the YES and YAES still provide a semi-quantitative outcome, as these assays reflects a net (anti)estrogenic response.

Even though a notable reduction of estrogenicity by the WWTPs was measured, the effluent EEQs reported here remain a concern because of the risk for adverse outcomes to the aquatic environment and, by extension, humans' health. Furthermore, despite the evidence implicating WWTPs as a major source for estrogenic endocrine disruption, our data suggest additional sources. In many parts of the developing world, alternative pollution sources include greywater from informal settlements and leakage from aging sewage systems, in addition to those also found in the developed world; amongst others, pesticide run-off. Clearly, the relative contribution of these alternative sources will show great regional variation, and with building evidence of the possible negative effect of other micro-pollutants not discussed here, including micro-plastics, it is imperative to further develop/refine techniques such as YES and YAES to become affordable tools to provide realistic indication of health risks, even in remote areas. This is especially relevant to the third UN-SDG for '*Good Health and Well-Being'* calling for strengthened capacity for early warning and risk reduction – especially within developing countries. Reliable early detection of EDCs in water may indeed greatly facilitate improved universal healthcare through prevention rather than reactive treatment.

Acknowledgements

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CHAPTER 4: THE FATE OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS (PPCPS), ENDOCRINE DISRUPTING CONTAMINANTS (EDCS), METABOLITES AND ILLICIT DRUGS IN A WWTW AND ENVIRONMENTAL WATERS

Article:

Archer, E., Petrie, B., Kasprzyk-Hordern, B., Wolfaardt, G. M. 2017. The fate of pharmaceuticals and personal care products (PPCPs), endocrine disrupting contaminants (EDCs), metabolites and illicit drugs in a WWTW and environmental waters. *Chemosphere*, 174, 437-446.

Declaration by the candidate

With regard to chapter 4, the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution
Sampling and sample processing, data processing, manuscript writing, corresponding author	70%

The following co-authors have contributed to chapter 4:

Name	Email and affiliation	Nature of contribution	Extent of contribution
Dr. Bruce R. Petrie	b.r.petrie@rgu.ac.uk *previous address*: Department of Chemistry, University of Bath, United Kingdom *current address*: Robert Gordon University, Aberdeen, United Kingdom	LC-MS method development and technical assistance, manuscript editing	20%
Dr. Barbara Kasprzyk-Hordern	b.kasprzyk-hordern@bath.ac.uk Department of Chemistry, University of Bath, United Kingdom	Manuscript editing	10%
Prof. Gideon M. Wolfaardt	gmw@sun.ac.za Department of Microbiology, Stellenbosch University	Manuscript editing	

Declaration with signature in possession of candidate and supervisor

Abstract

A large number of emerging contaminants (ECs) are known to persist in surface waters, and create pressure on wastewater treatment works (WWTW) for their effective removal. Although a large database for the levels of these pollutants in water systems exist globally, there is still a lack in the correlation of the levels of these pollutants with possible long-term adverse health effects in wildlife and humans, such as endocrine disruption. The current study detected a total of 55 ECs in WWTW influent surface water, 41 ECs in effluent, and 40 ECs in environmental waters located upstream and downstream of the plant. A list of ECs persisted through the WWTW process, with 28% of all detected ECs removed by less than 50%, and 18% of all ECs were removed by less than 25%. Negative mass balances of some pharmaceuticals and metabolites were observed within the WWTW, suggesting possible back-transformation of ECs during wastewater treatment. Three parent illicit drug compounds were detected within the influent of the WWTW, with concentrations ranging between 27.6-147.0 $ng.L^{-1}$ for cocaine, 35.6-120.6 $ng.L^{-1}$ for mephedrone, and 270.9-450.2 $ng.L^{-1}$ for methamphetamine. The related environmental risks are also discussed for some ECs, with particular reference to their ability to disrupt endocrine systems. The current study propose the potential of the pharmaceuticals carbamazepine, naproxen, diclofenac and ibuprofen to be regarded as priority ECs for environmental monitoring due to their regular detection and persistence in environmental waters and their possible contribution towards adverse health effects in humans and wildlife.

4.1. Introduction

There is growing evidence that a variety of pharmaceuticals and personal care products (PPCPs) persist in natural freshwater resources (Blair et al., 2015; Kasprzyk-Hordern et al., 2009b; Petrie et al., 2014). These organic pollutants are considered a part of emerging contaminants (ECs), which enter water systems from various sources, such as human excretion (sewage), wrongful disposal, leeching from landfill, drain water, or from industries. Even though it has been reported that these ECs are typically present at low environmental concentrations (ng.L^{-1} to µg.L^{-1} range), it is still unclear whether the levels of these compounds present in environmental waters can cause undesired physiological effects in wildlife and humans.

Research has shown that several regularly-used PPCPs may mimic or alter different vertebrate endocrine system pathways, which are collectively referred to as endocrine-disrupting contaminants (EDCs) (Boberg et al., 2010; Schlumpf et al., 2001; Veldhoen et al., 2014). Several PPCPs have been shown to be persistent or pseudo-persistent during wastewater treatment, thereby posing potential risk when discharged in environmental waters (Al Aukidy et al., 2014; Kasprzyk-Hordern et al., 2009b; Petrie et al., 2014). The prioritisation of these ECs for risk assessment is difficult, because concentrations in environmental waters show huge variation (Matongo et al., 2015; Petrie et al., 2014), and their effects at sub-lethal concentrations are not yet well established. Studies reporting on the detection of PPCPs in South African water systems have increased in the past few years (Agunbiade and Moodley, 2016, 2014; Amdany et al., 2014b; Madikizela et al., 2014; Matongo et al., 2015). These studies focussed on the detection of several classes of PPCPs and the plasticizer bisphenol-A, with concentrations regularly surpassing the µg.L^{-1} level in WWTW effluent and environmental waters. However, with the vast majority of ECs shown to be present in wastewater and environmental waters on a global scale, their fate and environmental effects within WWTWs and environmental waters are still poorly described.

Apart from the ubiquitous detection of PPCPs in South African surface waters, the detection of illicit drugs and other drugs of abuse are poorly investigated. Although illicit drug usage is shown to be on the rise in South Africa (Dada et al., 2017), the sources of information for drug abuse in the country are largely limited to law enforcement and treatment centre data. However,

these sources may underestimate the extent of drug abuse. A promising approach to estimate illicit drug use include wastewater-based epidemiology (WBE), which estimates drug consumption through the detection of illicit drugs and their metabolites in wastewater (Baker et al., 2014; Castiglioni et al., 2016).

The present study focused on the daily loads and fate of ECs and metabolites in the aqueous phase of a WWTW influent and effluent, as well as in surface waters in a river system located upstream and downstream of the plant. This study is a first to verify the detection and fate of a large list of PPCPs, metabolites, and illicit drugs at a South African WWTW. A further aim was to correlate the persistence of selected ECs detected during the study with data showing modulation of wildlife reproductive and thyroid systems, with reference to established adverse outcome pathway (AOP) frameworks.

4.2. Materials and Methods

4.2.1. Chemicals and Materials

The study included the screening for 90 ECs including 38 deuterated internal standards for the method development. All the reference standards were supplied by the Department of Chemistry at the University of Bath (Bath, UK; see Petrie et al., 2016a for further details). All chemicals were prepared at either concentrations of 0.1 or 1 $mg.mL^{-1}$ in the relevant solvents and stored in the dark at $-20^{0}C$. All glassware were deactivated using dimethylchlorosilane (DMDCS) in toluene (5% v/v) to limit the sorption of basic chemicals to glass surfaces. Both the MeOH and toluene used for experimentation were obtained from Sigma-Aldrich (99%, HPLC grade).

4.2.2. Study Site and Sampling Procedures

The site of study was at a WWTW situated in the Gauteng Province of South Africa (Fig. 4.1; APPENDIX Fig. B1). Treated wastewater effluent is discharged into a nearby river, which joins other streams that eventually feeds into a major dam that supplies approximately 6% of the total municipal drinking water to the surrounding communities.

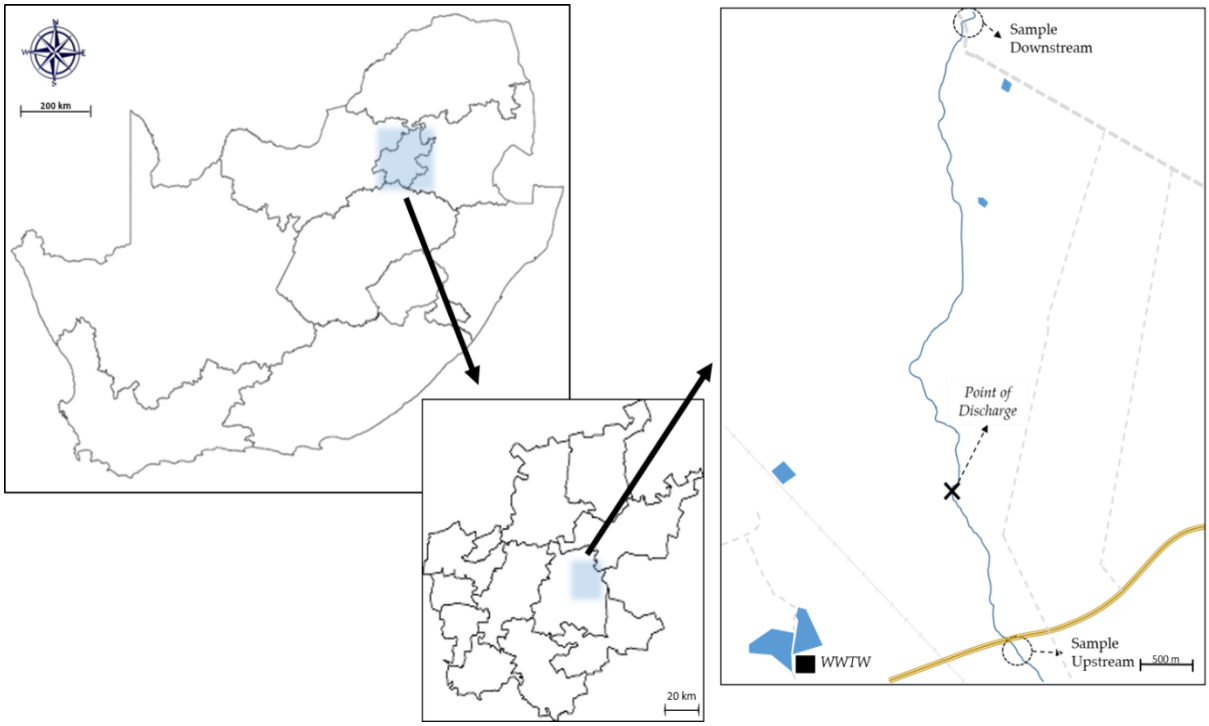

Figure 4.1: Location map of the WWTW, point of discharge into the receiving waters, and sampling locations upstream and downstream from the plant.

Sampling was done over five consecutive days during the month of July 2015 (Monday to Friday). Influent and effluent samples were taken each day at 60 min intervals (100 $mL.h^{-1}$) from the WWTW influent (after grit screens) and effluent (after chlorination), upon which a final composite sample (100 mL) was obtained for each sampling location per day. The influent and effluent samples were taken concurrently and not matched to the hydraulic retention time of the WWTW. Grab samples of river surface water (100 mL) were taken at a location upstream (100 m) and downstream (3.5 km from the point of discharge) of the WWTW (Fig. 4.1). All samples were transported on ice from the sampling sites to the laboratory and were kept cold (± 4 ^{0}C) and in the dark until analyte extraction, which was done within a maximum time of 10 hours.

4.2.3. Extraction Procedure

The collected water samples were adjusted to a pH of 7 (± 0.2) and filtered using 0.45µm pore size PTFE filters prior to solid-phase extraction (SPE). Each 100 mL water sample from the various locations were split into two 50 mL samples to allow for duplicate extraction of each locality and between each day of sampling. Each sample included 50 ng of each of the deuterated PPCP internal standard and was mixed well before extraction. The water samples were extracted using Oasis® HLB (3cc, 60 mg) SPE cartridges (Waters; Microsep,

Johannesburg, South Africa). The cartridges were conditioned with 2 mL methanol, followed by 2 mL of ultrapure water (Millipore) at a flow rate of 1 mL min^{-1}. After conditioning, the water samples (50 mL) were passed through the SPE cartridges at a flowrate of 5 $mL.min^{-1}$ and allowed to run dry for a minimum period of 15 min. The dried cartridges were kept frozen (-20^{0}C) until all sampling were completed, from which the cartridges were then sent to the University of Bath (United Kingdom) for chemical analysis within 10 days of extraction. Each cartridge was individually wrapped with foil and placed frozen in a polystyrene pack lined with ice packs. Upon arrival at the University of Bath, the cartridges were dried and eluted with 4 mL MeOH using a manifold at a flow rate of 1 $mL.min^{-1}$. The extracts were dried under a gentle stream of nitrogen using a TurboVap evaporator (Caliper, UK, 40^{0}C, N_2, <5psi) and the evaporated samples were re-suspended in 500 µL of a H_2O:MeOH (80:20) solvent, giving a 100x concentrated sample, and transferred to polypropylene MS vials (Waters, Manchester, UK) for chromatography.

4.2.4. Liquid Chromatography – Mass Spectrometry

A Waters Acquity UPLC system coupled to a Xevo Triple Quadrupole Mass Spectrometer (UPLC/TQD-MS; Waters, Manchester, UK) was used following the method described by Petrie et al. (2016). Two separate chromatography methods were used for the quantification of acidic and basic compounds, as further described in *Appendix B* (Figure B2). In essence, both methods used a reversed-phase BEH C18 column (150 x 1.0 mm, particle size – 1.7 µm; Waters, Manchester, UK) coupled with a 0.2 µm (2.1 mm) in-line column filter. Mobile phase flow rates and injection volumes were maintained at 0.04 $mL.min^{-1}$ and 15 µL respectively for both methods. Argon was used as the collision gas, and nitrogen as the desolvation and nebulising gas. Solvent blanks containing H_2O:MeOH (80:20) were inserted after each ten samples, and two solvent blanks after quality control (QC) samples of the internal standards. The corrected recoveries of the analysed compounds are shown in *Appendix B* (Table B1).

4.2.5. Calculations

The mass loads of the target analytes in the aqueous phase at both the influent and effluent of the WWTW during the sampling period ($g.day^{-1}$) were determined using Equation 1:

Mass Load ($g.day^{-1}$) = *(Inf or Eff) * FR * 1/1000* [Eq. 1]

Where *Inf* and *Eff* refers to the concentration (in $ng.L^{-1}$) of the analytes detected at the influent and effluent wastewater samples, and *FR* refers to the mean flow rate of the plant ($ML.day^{-1}$) during each sampling day. The ability of the WWTW to remove the detected compounds in the aqueous phase were determined by calculating the percentage removal efficiency (RE %) between influent and effluent wastewater during the sampling period using Equation 2:

$$RE\ (\%) = ((Inf - Eff) / Inf) * 100 \qquad \text{[Eq. 2]}$$

Where *Inf* refers to the mass loads ($g.day^{-1}$) of the analytes detected at the influent sample site of the plant, and *Eff* refers to the mass loads ($g.day^{-1}$) of the analytes detected at the effluent sample site of the plant.

4.2.6. Statistical analysis

Statistical analyses were performed using Statistica (version 13.0). Concentrations of the ECs detected at the WWTW influent and effluent, as well as water samples located upstream and downstream of the plant during the sampling period were compared using a repeated measure mixed-model ANOVA with the sampling day as a random factor. Significant differences were recorded as $p < 0.05$.

4.3. Results and Discussion

4.3.1. Analysis of WWTW samples

A total of 55 ECs were detected in wastewater influent, and 41 ECs in effluent samples, which represented 19 classes of PPCPs, human indicators, illicit drugs and metabolites (Fig. 4.2; APPENDIX Table B2). The human indicators contained chemicals which are associated with endogenous products of human metabolism or which are used for population equivalent estimates. Although flow data ($ML.day^{-1}$) from each day was incorporated to calculate the mass loads of the ECs within the WWTW during the sampling period, the variation in the mass loads of the ECs between sampling days may be attributed to several factors, such as the variation in the daily human usage of these compounds, pollution events from surface water sources leading towards the plant, the retention time of wastewater within the plant, or the overall performance of the treatment processes within the sampling period.

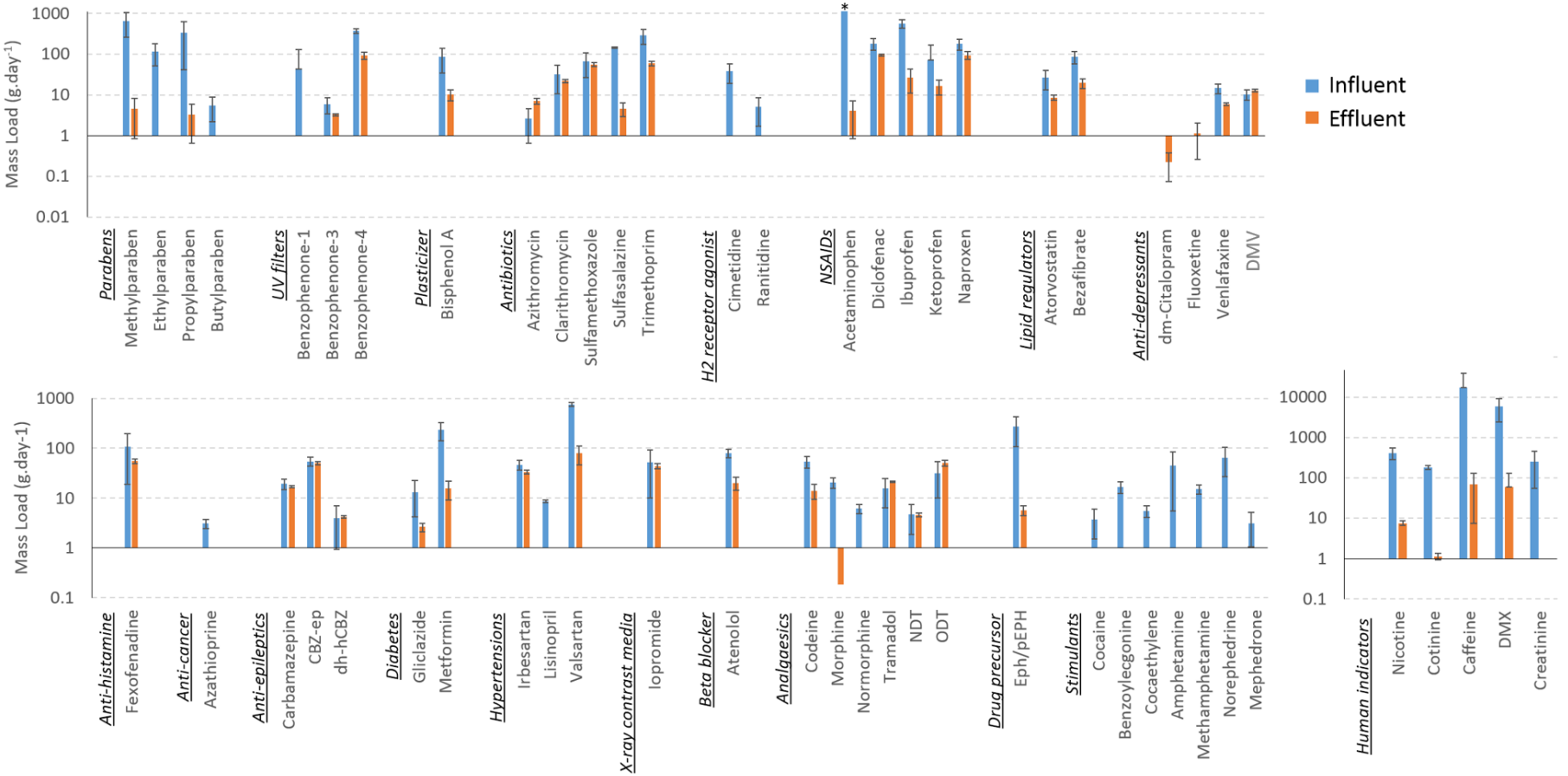

Figure 4.2: Mass loads (g.day^{-1}) for the detected compounds at the WWTW during the sampling period. Values are expressed on a logarithmic scale. The standard deviations shows variation between sampling days. *NSAIDs*: non-steroidal anti-inflammatory drugs, *EPH/pEPH*: ephedrine/pseudoephedrine, *dh-hCBZ*: 10,11-dihydro-10-hydroxycarbamazepine, *CBZ-ep*: carbamazepine-10,11-epoxide, *DMX*: 1,7-dimethylxantine, *NDT*: *N*-desmethyltramadol, *ODT*: *O*-desmethyltramadol, DMV; desmethylvenlafaxine. * Mass load of acetaminophen = 10530 (± 3216) g.day^{-1}.

The WWTW sampled in the current study showed varying removal efficiencies of the detected ECs (APPENDIX Table B3) and conforms to similar removal data of PPCPs reported in other studies (Verlicchi et al., 2012; Bahlmann et al., 2014; Petrie et al., 2014 & 2016). By comparing the average mass loads of the ECs at the influent and effluent, it was calculated that 28% of all detected ECs were removed by less than 50%, and 18% of all ECs were removed by less than 25% (Fig. 4.3). A significant increase in final effluent concentration of the pharmaceutical metabolite desmethylvenlafaxine (DMV; from venlafaxine; $p = 0.039$), were measured compared to influent wastewater (negative mass balance). Also, a significant average negative mass balance was observed for the antibiotic azithromycin ($p = 0.001$; Fig. 4.3). Negative mass balances were also calculated based on the average mass load concentrations for the pharmaceutical metabolites 10,11-dihydro-10-hydroxycarbamazepine and *O*-desmethyltramadol, as well as the parent compound tramadol (Fig. 3). However, statistical analysis deemed these negative mass balances non-significant due to the variation between sampling days ($p>0.05$).

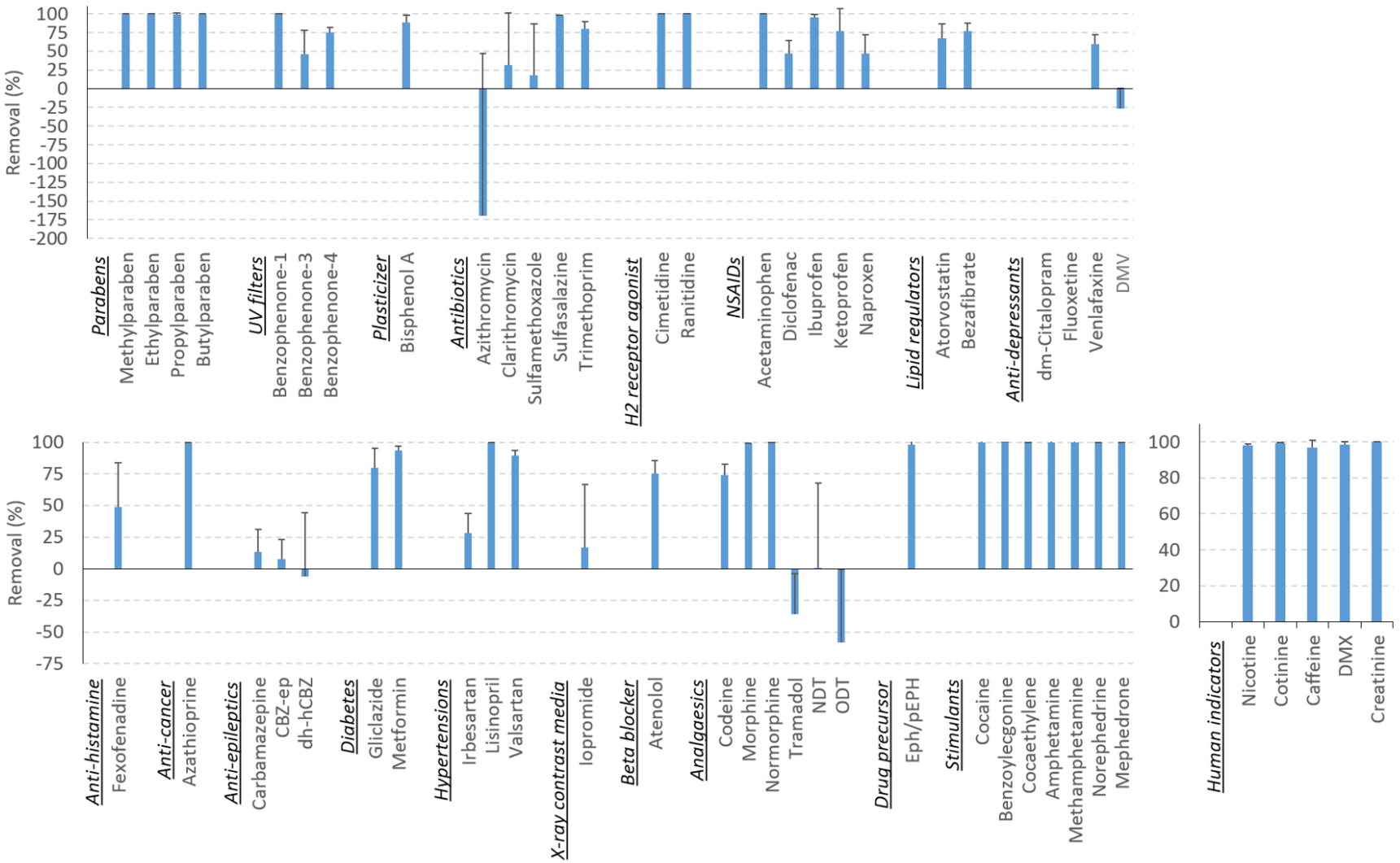

Figure 4.3: Removal efficiencies (%) of the detected compounds at the WWTW during the sampling period. Standard deviations indicate variation in between sampling days. *NSAIDs*: non-steroidal anti-inflammatory drugs, *EPH/pEPH*: ephedrine/pseudoephedrine, *dh-hCBZ*: 10,11-dihydro-10-hydroxycarbamazepine, *CBZ-ep*: carbamazepine-10,11-epoxide, *DMX*: 1,7-dimethylxantine, *NDT*: *N*-desmethyltramadol, *ODT*: *O*-desmethyltramadol, DMV; desmethylvenlafaxine.

Two potential explanations for the occurrence of negative mass balances for ECs in WWTWs have been postulated, namely: i) persistent ECs accumulate in aggregates, leading to subsequent dissolution through biotic or abiotic processes, and/or ii), metabolites and/or conjugate forms of ECs are not detected at the WWTW influent and are subsequently back-transformed or de-conjugated into parent compounds through either biotic or abiotic processes within the WWTW (Blair et al., 2015; Verlicchi et al., 2012). For example, conjugate forms of ethynyl-estradiol (EE_2), carbamazepine and diclofenac have been shown to be de-conjugated by bacterial cultures (Aris et al., 2014; Lee et al., 2012; Vieno et al., 2007), while metabolites of the antibiotic sulfamethoxazole were shown to be transformed to its parent compound by photolytic processes (Bonvin et al., 2012). Although not all metabolites and conjugate forms of ECs can be regarded to be easily transformed within wastewater, it is possible that the perceived recalcitrance of some compounds in the current study at the WWTW (such as carbamazepine, tramadol, sulfamethoxazole and NSAIDs) may partly be caused by a combination of biotic and/or abiotic events leading towards the transformation of parent compounds and their metabolites.

The recalcitrance of certain ECs (such as tramadol) is well-known. Tramadol, is rapidly metabolised in the liver by desmethylation enzymatic activities encoded by the Cytochrome P450 gene (CYP2D6), which give rise to its two primary metabolites *O*-desmethyltramadol (ODT) and *N*-desmethyltramadol (NDT) (Ardakani and Rouini, 2007). Only 10-30% of the parent tramadol is excreted in sewage, and hence, a large amount of the primary or secondary metabolites will also be prevalent in wastewater (Ardakani and Rouini, 2007). It is also noteworthy to mention that levels of the enzyme CYP2D6 may vary significantly between human individuals and sex. Taken that the WWTW screened during the current study receives wastewater from public, domestic and industrial sources, the daily levels of ECs (such as tramadol and its primary metabolites) in wastewater may vary greatly according to the *de facto* population contributing sewage of the WWTW. Partition coefficients for tramadol (log *K*ow 3.01) and its metabolites ODT and NDT (log *K*ow 2.45; EPI Suite v4.11, KOWWIN, v1.68 estimate) are indicative of a moderate tendency towards sorption onto organic constituents within soil, sediment and/or sludge. Such association with microbial aggregates and solids may protect these compounds from degradation until release. Floc breakup in the sludge and other forms of decay may then lead to association-disassociation events that may explain the large variation in metabolite levels.

Apart from the possible transformation of parent and/or metabolite ECs in wastewater, a number of factors could also potentially contribute to the attenuation and biodegradation of ECs in WWTWs. These include climatic conditions, physiochemical properties of the ECs, the hydrological retention time of the WWTW, and the microbial activity within the plant during the sampling period. The latter will include the properties of the extracellular polymeric substances (EPS) produced by microbial biofilms to absorb organic and inorganic pollutants (Petrie et al., 2014; Writer et al., 2011). It is possible that, unless some of these ECs are fortuitously co-metabolized, the energy that can be gained by microorganisms via degrading them at the low, environmentally relevant concentrations would not warrant the energy input required for enzyme production for further biodegradation. Furthermore, it was shown that environmentally relevant concentrations of the antibiotics phenazone, amoxicillin, and erythromycin can affect the initial adhesion of bacteria onto surfaces (Schreiber and Szewzyk, 2008), and that a specialist degradative strain of yeast had a lesser habitat range than non-degradative strains in soil (Barratt et al., 2003). Admittedly speculative, it is possible that such a shift, together with the availability of labile nutrients at relatively high concentrations may favour generalists to dominate the microbial community at the expense of specialist degraders, causing ECs to pass through unaltered.

4.3.2. Detection of illicit drugs at the WWTW

During the current study, eight ECs classified as illicit drugs, their metabolites, and a drug precursor were identified in raw wastewater (Fig. 4.2). An increase in the loads of breakdown products and precursors of amphetamine-type stimulants (ATS) were observed at the WWTW (pseudoephedrine > norephedrine > amphetamine > methamphetamine) (Fig. 4.2). The new psychoactive substance (NPS) mephedrone was detected only at influent samples, ranging from 36 – 121 $ng.L^{-1}$ and calculated at an average load of 3.1 $g.day^{-1}$ during the sampling period. Similar to the ATS drugs, higher levels in the breakdown products of cocaine was observed in the wastewater influent (benzoylecgonine > cocaethylene > cocaine) (Fig. 4.2). The drug-precursor and nasal decongestant, (pseudo)ephedrine, was detected at an average concentration of 6321 $ng.L^{-1}$ (269.4 $g.day^{-1}$) (APPENDIX Table B3). Norephedrine was detected at an average concentration of 1519 $ng.L^{-1}$ (65.1 $g.day^{-1}$). In comparison, the levels of ephedrine and norephedrine ranged between 8.7 – 1979.5 $ng.L^{-1}$ (median load of 16.5 $g.day^{-1}$) and 15.0 – 99.9 $ng.L^{-1}$ respectively at the influent of six WWTWs in the UK (Baker and Kasprzyk-Hordern, 2013). These levels were noticeably lower than reported in the current study. Although (pseudo)ephedrine is generally used as an over-the-counter nasal decongestant, a report by the

South African Department of Social Development mentioned that South Africa is one of the largest importers of (pseudo)ephedrine in the world (CDA, https://www.cda.gov.za), which explains the high levels of the compound detected in the current study.

It is worthy to mention that the sources of ATS drugs in surface waters might vary. Methamphetamine is shown to be metabolised to amphetamine by de-methylation enzymes from the CYP2D6 gene (De La Torre et al., 1991), whereas amphetamine is also an active ingredient in medication for the treatment of attention deficit hyperactivity disorder (ADHD). Therefore, the presence of certain compounds could not be attributed to a specific source of exposure or drug abuse. The levels detected for methamphetamine (271-450 $ng.L^{-1}$; 15.2 $g.day^{-1}$), cocaethylene (186-225 $ng.L^{-1}$; 8.8 $g.day^{-1}$), and (pseudo)ephedrine (283-16640 $ng.L^{-1}$; 269.4 $g.day^{-1}$) in the WWTW influent (APPENDIX Tables B2 & B3) were detected at much higher levels than reported for UK WWTWs (Petrie et al., 2014). The current study therefore highlights the usage of sewage samples to indicate the trends of drug abuse within a community, and may therefore serve as a valuable tool in epidemiological studies.

4.3.3. ECs in Environmental Waters

A total of 40 ECs were detected in surface waters located upstream and downstream of the plant during the sampling period (Table 4.1). The levels of diclofenac, ibubrofen, ketoprofen, sulfamethoxazole, and bezafibrate were also analysed in other South African surface waters, and were detected at higher concentrations than in the current study (Agunbiade and Moodley, 2016). However, the average concentrations of the measured ECs in surface water during the current study showed that 30 of the 40 detected ECs (75%) were higher than reported in UK surface waters (Petrie et al., 2016a, 2014). Although methylparaben, bisphenol-A, nicotine, cotinine, caffeine, and 1,7-dimethylxantine were removed with moderate to high efficiency by the WWTW (Fig. 4.2), the average concentrations of these compounds were calculated to be higher in downstream samples compared to the WWTW effluent (APPENDIX Table B2). However, only the levels of nicotine were found to be significantly higher in downstream samples compared to WWTW effluent samples during the sampling period ($p < 0.05$), mainly due to large variations of the other EC levels during the sampling period and also due to composite samples which were taken at the WWTW effluent and grab samples taken at the downstream site. In contrast, by comparing the average levels of ECs at the upstream and downstream sampling sites, it was shown that 26 out of the 40 detected ECs in surface waters (65%) were found to be two-fold or higher in downstream samples, with codeine detected

higher than 10 fold (Table 4.1). The concentrations of ECs in surface waters are known to not only fluctuate on a seasonal or daily basis, but also in distance from the plant (Vieno et al., 2005). The higher levels of some PPCPs detected at downstream samples may therefore largely be attributed to the distance between the WWTW effluent and downstream sampling points (3500 m from the point of discharge), and fluctuations in the concentrations of the detected compounds between sampling days could also be attributed to variations in river water flow rates between sampling days.

Table 4.1: Mean concentrations (ng.L^{-1}) of detected PPCPs, metabolites, illicit drugs and human indicator compounds at sampling localities located upstream and downstream of the WWTW. Standard deviation indicate variation between sampling days for the compounds. Abbreviations: NSAIDs: non-steroidal anti-inflammatory drugs, EPH/*p*EPH: ephedrine/pseudoephedrine, *dh*-10-*h*CBZ: 10,11-dihydro-10-hydroxycarbamazepine, CBZ-*ep*: carbamazepine-10,11-epoxide, DMX: 1,7-dimethylxantine, NDT: *N*-desmethyltramadol, ODT: *O*-desmethyltramadol.

	Upstream		Downstream				Upstream		Downstream		
	Average	Stdev	Average	Stdev	Fold change		Average	Stdev	Average	Stdev	Fold change
Parabens						*Anti-epileptic*					
Methylparaben	58.7	29.2	146.1	107.3	2.5	Carbamazepine	157.1	11.5	279.5	24.1	1.8
Propylparaben	31.8	17.4	136.7	76.8	4.3	CBZ-ep	398.8	27.9	752.2	69.4	1.9
						dh-hCBZ	22.7	1.58	56.9	8.9	2.5
UV filters											
Benzophenone-3	56.2	1.7	64.3	6.0	1.1	*Diabetes*					
Benzophenone-4	441.1	23.8	1076.5	389.6	2.4	Gliclazide	43.2	2.4	53.9	22.3	1.3
						Metformin	73.3	7.2	174.6	81.7	2.4
Plasticizer											
Bisphenol-A	239.0	72.1	396.4	208.1	1.7	*Hypertensions*					
						Irbesartan	311.1	28.7	554.4	120.0	1.8
Antibiotics						Valsartan	263.7	24.6	924.7	50.2	3.5
Azithromycin	24.6	0	6.4	3.4	0.3						
Clarithromycin	76.2	13.4	235.5	66.1	3.1	*Anti-depressants*					
Sulfamethoxazole	757.4	83.2	1013.2	294.2	1.3	Fluoxetine	34.4	22.1	109.2	125.6	3.2
Sulfasalazine	37.6	3.4	53.0	13.0	1.4	Venlafaxine	35.4	3.7	94.6	19.6	2.7
Trimethoprim	383.0	42.2	898.7	303.0	2.4	Desmethylvenlafaxine	50.0	7.5	174.9	53.8	3.5
NSAIDs						*Analgaesics*					
Acetaminophen	20.8	4.5	63.7	76.1	3.1	Codeine	11.3	6.7	128.9	65.4	11.5
Diclofenac	467.4	176.2	1461.5	508.7	3.1	Tramadol	97.7	11.2	299.9	73.2	3.1
Ibuprofen	153.3	39.5	312.1	204.6	2.0	NDT	16.0	9.9	74.0	8.1	4.6
Ketoprofen	642.2	0	330.3	319.0	0.5	ODT	207.6	32.2	577.3	149.8	2.8
Naproxen	224.3	31.1	1112.8	518.3	5.0						

Table 4.1 (continued)

Lipid regulators						*Drug precursor*				
Atorvostatin	74.0	5.2	150.6	55.7	2.0	Eph/pEPH	38.8	8.0	80.4	28.4	2.1
Bezafibrate	54.9	8.3	234.4	116.8	4.3						
						Stimulants					
Antihistamine						Amphetamine	27.1	22.6	37.0	22.6	1.4
Fexofenadine	368.4	36.7	887.0	172.0	2.4						
						Human indicators					
X-ray contrast media						Nicotine	154.3	78.7	245.5	67.6	1.6
Iopromide	265.8	11	598.3	235.4	2.3	Cotinine	25.5	3.3	31.7	11.7	1.2
						Caffeine	812.2	146.3	2077.5	259.7	2.6
Beta-blockers						DMX	479.4	357.6	957.6	728.6	2.0
Atenolol	156.2	34.43	272.0	154.6	1.7						

The fact that human indicator compounds (such as nicotine) were detected at higher average levels in the downstream samples relative to the WWTW effluent samples (Table S2) indicates human activities further downstream from the WWTW, which could have re-introduced some ECs (such as methylparaben, codeine and bisphenol-A) back into the water system after the levels of the pollutants were lowered at the plant. The direct discharge and/or illegal dumping of sewage and other waste products may have been a contributing factor to these observations. This might also explain the high fold-increase in codeine further downstream of the plant (Table 1), which is regarded as the most abused over-the-counter drug in the country. However, the difference in mode of sampling should also be pointed out; composite samples were taken at the WWTW, whereas grab samples were taken for the river water, which should be corrected in future studies. Some industrial (such as a brick manufactory) and agricultural practices (poultry and other livestock) are present between the point of discharge of the WWTW and the downstream sample taken in the study. The presence of PPCPs and some metabolites point to other waste sources and human activities further downstream from the WWTW.

4.3.4. Environmental risk of the detected pollutants

The demonstrated presence of a vast mixture of ECs in WWTW effluent and river water in the current study emphasises the need to consider potential associated environmental risks. Conventional methods for environmental risk assessment (ERA) includes acute- and/or chronic toxicity data based on the most sensitive organism or combination of organisms within a given ecosystem to determine a predicted no-effect concentration (PNEC) of an environmental pollutant. This value is then compared to predicted- or measured environmental concentrations (PEC or MEC respectively) to obtain a risk quotient (RQ) of the EC of interest. An RQ value exceeding 1.0 is then regarded as an environmental risk. Table B4 in appendix B reports on RQs determined for some ECs which were detected in WWTW effluent and river water during the current study. The MECs show that 5 out of the 41 detected ECs in WWTW effluent, and 4 of the 40 detected ECs in environmental surface waters posed an environmental risk (RQ > 1; APPENDIX Table B4). These ECs included diclofenac, sulfamethoxazole, clarithromycin, codeine, and nicotine, with clarithromycin only showing an environmental risk for WWTW effluent water (RQ > 1), although still of environmental relevance (RQ = 0.8; Table S4).

Although conventional ERA is valuable to show toxicity risks of ECs found in environmental waters, a few limitations exist. These models only consider lethal toxicity on an *in vivo* level as a risk endpoint and hence, the consequences of pollutants triggering sub-lethal toxicity on

physiological pathways (from molecular to cellular level) are highly underestimated. The possibility of PPCPs to modulate molecular and/or cellular pathways (such as those involved in endocrine system function) at concentrations well below lethal toxicity therefore makes such outcomes more ecologically relevant for risk assessment. To assist with the understanding of toxicity mechanisms leading towards an observed adverse outcome on a population level, an adverse outcome pathway (AOP) framework has been proposed (Ankley et al., 2010). This conceptual framework is aimed towards using existing toxicological knowledge to establish a relationship between biological events on cellular- to organism level (termed key events, KEs) through key event relationships (KERs), which is initiated by a molecular initiating event (MIE) (Margiotta-Casaluci et al., 2016; Villeneuve et al., 2014). The KERs are dependent on a weigh-of-evidence approach to show the relationship between established KEs. The downstream KEs through biological complexity subsequently lead towards an observed adverse outcome (AO) which can be used for environmental regulatory decision-making (Ankley et al., 2010). Although the AOP framework is not directly constructed to be chemical specific, nor to serve as a risk assessment tool, the correlation of established AOP networks to EC monitoring studies (such as the current study) can therefore show the possibility of ECs in environmental waters to modulate certain MIEs or KEs. As a result, RQ values can be generated for certain MIEs and KEs (RQ_{MIE}/RQ_{KE}) in a similar way as conventional ERA. Such risk predictions can therefore serve as early warning systems to prioritise ECs for their potential to contribute towards possible detrimental health effects in the environment.

Several studies have suggested that organic pollutants can modulate endocrine system pathways of vertebrate species at low concentrations which are regularly detected in the environment. *In vitro* studies have shown, for example, that the plasticizers bisphenol-A, disinfection products (such as parabens), and UV filters can induce MIEs such as agonistic binding to the human estrogen receptor (hER), which has further been shown to lead towards the proliferation of breast cancer cells (Boberg et al., 2010; Schlumpf et al., 2001). Exposure of fish to paraben and UV filter compounds have been reported to induce MIEs and KEs such as increased expression of mRNA transcripts of the estrogen receptor-α (ERα) and protein vitellogenin (VTG) in the liver of male fish, which can be linked to further downstream KEs such as increased levels of plasma VTG in the body (Barse et al., 2010; Inui et al., 2003). The protein VTG is a precursor of egg yolk in oviparous animals, and serves as a common biomarker to show estrogenic endocrine disruption in aquatic organisms due to its direct KERs with circulating androgen- and estrogen hormone levels (Jones et al., 2000)(AOP-Wiki, KE219

& KE285). The relevant MIEs and KERs leading towards increased VTG production has been well documented to be directly linked with KEs on organismal level such as fecundity and spawning, which can eventually have an effect on population trajectories (AOP-Wiki, AOP25). Although it has been shown in the current study that parabens and UV filters are effectively- to moderately removed in the WWTW (Fig. 4.2), methylparaben, benzophenone-4, and benzophenone-3 were still detected in river water downstream of the plant (Table 4.1), albeit well-below reported levels to modulate KEs as shown in laboratory studies (Barse et al., 2010; Inui et al., 2003) (RQ_{KE} << 1; APPENDIX Table B4).

Exposure of other pharmaceuticals, such as the NSAIDs diclofenac and ibuprofen have also been shown to modulate KEs such as elevated plasma estradiol levels and induction of VTG in male fish, either through upregulation of upstream MIEs (such as increased aromatase activity) or other molecular targets (APPENDIX Table B4). Although the concentrations to modulate these observed estrogenic endpoints are higher than the environmental concentrations for ibuprofen in the current study, the concentration of diclofenac in WWTW effluent and river water samples regularly exceeded the 1000 $ng.L^{-1}$ value previously reported to modulate VTG production in fish (Hong et al., 2007; RQ_{KE} > 1; APPENDIX Table B4). Although an increase in VTG production has been shown for diclofenac at 1000 $ng.L^{-1}$, Gröner and colleagues (2017) mentioned that such a concentration, however, does not impair population-relevant endpoints such as survival and hatching success. On the contrary, both ibuprofen and naproxen have been reported to cause decreased egg fertilisation in fish at a concentration of 100 $ng.L^{-1}$ (Nesbitt et al., 2011), which therefore reflects upon modulation of an established KE of fecundity and spawning (AOP-Wiki, KE78). The concentrations of ibuprofen and naproxen within WWTW effluent and river water during the current study were well above the threshold to potentially modulate such a KE (RQ_{KE} > 1; APPENDIX Table B5), and therefore indicates a potential environmental risk to impair fecundity and spawning, which can ultimately lead to a decline in fish populations (AOP-Wiki, AO360). Whether the concentrations of these NSAIDs is sufficient to induce further downstream KEs, or can be extrapolated to other vertebrate species need further investigation.

Apart from NSAIDs showing reproductive endocrine disruption in aquatic organisms, *in vivo* exposure of 500 $ng.L^{-1}$ carbamazepine in water has been shown to cause significant reduction in plasma 11-ketotestosterone (11-KT) concentrations in male fish (Galus et al., 2013b), of which the upstream MIE causing this endpoint is still unknown. 11-KT is the primary teleost

androgen necessary for normal reproductive functioning, and hence, also influence the reproductive success of fish populations. The average concentration of carbamazepine in the WWTW effluent of the current study was close to the 500 $ng.L^{-1}$ threshold which can cause such a reduction in fish steroid hormone levels (APPENDIX Table B4). Although the modulation of circulating 11-KT is not shown to lead towards altered estradiol hormone synthesis through aromatase enzyme activities (such as the case with circulating testosterone levels), the modulation of circulating 11-KT levels in fish can still be regarded as a potential KE to lead towards reproductive dysfunction in male fish. Furthermore, no studies have been conducted to report on the potential endocrine disrupting effect of the primary metabolites of carbamazepine, which was shown to be detected at higher concentrations within WWTW effluent and river water (Fig. 4.3; Table 4.1), and removed to a lower extent in the WWTW (Fig. 4.2). We therefore propose that this compound and its metabolites need further monitoring for its potential to cause detrimental effects in aquatic vertebrates, especially due to its demonstrated recalcitrance and potential to modulate androgen-controlled endocrine pathways.

Apart from the potential of ECs to modulate reproductive endocrine system pathways, studies showing modulation of the thyroid endocrine system have also been reported. Exposure of the NSAID ibuprofen to tadpoles of the American bullfrog (*Rana catesbeiana*) at concentrations ranging from 1500 – 15 000 $ng.L^{-1}$ have been shown to potentiate triiodothyronine (T_3)-induced mRNA transcription of thyroid hormone receptors (Veldhoen et al., 2014). In the same study, exposure to ibuprofen alone resulted in increased mRNA transcripts for enzymes such as thyroxine 5-deiodinase (*dio3*) in a tail fin tissue assay, which is necessary to regulate thyroid hormone homeostasis (Veldhoen et al., 2014). Although the levels of ibuprofen observed in the present study was below these concentrations in both the WWTW effluent and river water, the levels of other NSAIDs, such as naproxen (average concentration of 2296 $ng.L^{-1}$ in WWTW effluent; APPENDIX Table B2) and diclofenac (average concentration of 2326 $ng.L^{-1}$ in WWTW effluent; APPENDIX Table B2), fell within the concentration range to possibly alter such MIEs, given that these NSAIDs yield the same mechanism of endocrine disruption as ibuprofen. It has been shown that diclofenac (but not naproxen) can antagonistically bind to the thyroid hormone receptor-ß (thrß) in a human reporter assay, and also inhibit T_3-induced vasodilation of rat mesenteric arteries (Zloh et al., 2016). Also, it is important to note that cross-talk between endocrine system pathways exist. For example, Nelson and Habibi (2016) demonstrated an increased production of VTG and upregulation of ERα following a treatment

of T_3 in female goldfish (*Carassius auratus*). Therefore, the potential of NSAIDs to alter both thyroid- and gonadal endocrine system pathways can be linked to several KEs and KERs, given that the concentrations and bioaccumulation of these pollutants are sufficient within the organisms to exert such effects. However, relatively little has been done to assess the impact of NSAIDs on whole-life cycles within aquatic organisms, and should receive more attention in future studies, especially due to their ease of purchase, their regular usage, and moderate persistence in the aqueous phase at WWTWs as shown in the current study. These results therefore indicate the importance to regard NSAIDs as priority environmental EDCs in future studies.

Another complication which arise in establishing ECs as an environmental risk is the complex range of interactions that are found in environmental waters. Several ECs generally accumulate in complex mixtures of varying concentrations (including other types of organic and inorganic pollutants), in which the modulating effects of such mixtures may differ from observed effects of the individual pollutants. For example, a study by Galus and colleagues (2013a) showed a mixture of environmentally relevant concentrations of acetaminophen, carbamazepine, gemfibrozil, and venlafaxine (500 $ng.L^{-1}$ for each compound) significantly altered embryo production, oocyte development, and fecundity in female zebrafish (*Danio rerio*), while the individual compounds exposed to the fish species at the same concentrations did not yield the same results (Galus et al., 2013b). Although investigations of PPCP mixture effects generate a more ecologically relevant scenario, it is more feasible to identify the KEs and KERs which are modulated by individual compounds, which may ultimately lead to a better understanding of the health risks in environmental waters. The fact that some ECs in the current study showed low removal from the WWTW, as well as the possibility to cause adverse effects on vertebrate endocrine system pathways (such as carbamazepine, UV filters, plasticizers, parabens and NSAIDs) highlights the importance to further monitor these priority ECs to limit their impact on both the aquatic ecosystem and contamination to drinking water resources.

4.4. Conclusions

The current study aimed to provide a link between the monitoring of ECs in WWTWs and environmental waters with possible adverse health consequences in wildlife. Although most ECs were shown to be notably reduced in WWTW effluent, some persisted, and were even

detected at higher concentrations in effluent (as shown for some pharmaceutical metabolites and parent compounds). It is therefore important to report on the fate of both parent ECs as well as their metabolites and/or conjugate forms in surface waters to elucidate the possible negative mass balances observed in WWTWs and recalcitrance of pollutants in environmental waters. Drawing definite conclusions regarding the health impact which these pollutants may cause when entering environmental waters is no simple task, considering that these pollutants are present in complex mixtures with varying physiochemical properties, as well as their varying affinities to modulate a range of molecular and cellular pathways in wildlife species. It is therefore clear that there is a need for more eco-toxicological assessment on the sub-lethal effects of ECs and polluted water systems into identifying MIEs, KEs and KERs which certain ECs can modulate to advance current risk assessment approaches.

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CHAPTER 5: ENANTIOMERIC PROFILING AND THE FATE OF DRUGS OF ABUSE (DOA) DURING WASTEWATER TREATMENT AND WITHIN ENVIRONMENTAL WATERS

Abstract

Contamination of surface waters with organic micro-pollutants is a growing concern. In particular, the persistence of pharmaceuticals that result in their passage through wastewater treatment works (WWTWs) before being discharged into environmental waters or reused for non-potable purposes pose a severe threat to global freshwater security. There are numerous reports on the recalcitrance of several emerging contaminants (ECs) during wastewater treatment, including contaminants which show high tendencies to be absorbed into solid particulate matter, therefore posing an environmental risk. The current study included a weeklong monitoring campaign at two South African WWTWs. Influent, treated effluent, as well as the liquid- and solid phase of return activated sludge (RAS) and environmental surface waters sampled upstream and downstream of the plants were screened for the presence of drugs of abuse (DOA), known recalcitrant pharmaceuticals, and their associated metabolites. Most compounds analysed for were removed with high efficiency at both treatment plants, with the exception of the opioid drug tramadol (TRAM), the anti-depressant venlafaxine (VEN), their metabolites, as well as the anaesthetic drug ketamine. Methamphetamine (METH) was shown to be enriched with the *R*-enantiomer in wastewater effluent due to stereoselective biodegradation favouring the *S*-enantiomer. The DOA TRAM, codeine, morphine, VEN and *S*-(+)-METH were detected in environmental waters not necessarily associated with wastewater discharge, suggesting alternative pollution sources. The study highlights the impact of recalcitrant micro-pollutants on environmental and human health, and point to the need for expanded monitoring to trace the origin of ECs in environmental waters, especially for rural and peri-urban areas which are not connected to the sewage system.

5.1. Introduction

Contamination of surface waters by organic micro-pollutants is globally shown as a growing concern, with treated wastewater discharge being considered a major source. Bioprocesses, including activated sludge (AS) employed in wastewater treatment play an important part in reducing the organic load discharged to surface waters. However, conventional wastewater treatment works (WWTW) have been shown to be inefficient to eradicate a number of recalcitrant ECs (Petrie et al., 2014; Yang et al., 2017). This is partly the result of rapid urbanisation and population increase pushing facilities beyond their design capacity, as well as increased loads of pharmaceuticals, personal care products, pesticides and industrial by-products being directly discharged and/or excreted in surface waters and sewage respectively. These ECs are therefore considered pseudo-persistent due to their regular occurrence within wastewater influent due to their partial degradation, as well as their continued release into receiving waters (Evans and Kasprzyk-Hordern, 2014), from which the environmental risk assessment are largely limited.

Removal efficiency of EC's during wastewater treatment depends on several factors, such as their physio-chemical properties, the treatment process and associated residence time, as well as the source input of the pollutants (domestic/industrial). Compared to removal of parent compounds, the presence and fate of their breakdown products received less attention. For pharmaceuticals, several studies suggested a negative mass balance of some compounds, by which a larger concentration will be detected in the treated effluent compared to influent samples (Archer and Wyk, 2015; Baalbaki et al., 2017; Blair et al., 2015; Subedi and Kannan, 2014). Furthermore, compared to the aqueous phase, the sorption onto suspended particulate matter such as AS has received less attention (Boix et al., 2016). While the increased contact time with microbial aggregates in activated sludge processes generally enhance removal efficiency (Kasprzyk-Hordern and Baker, 2012a), the recalcitrance of pharmaceuticals such as the opioid drug tramadol (TRAM) and antidepressant drug venlafaxine (VEN) has been ascribed for their tendency to be absorbed into solid particulate matter (SPM) during the AS treatment process (Baalbaki et al., 2017; Boix et al., 2016; Gasser et al., 2012). Re-circulation of AS (through return activated sludge; RAS) allows for floc maturity, as well as increased contact time to SPM. Compounds showing attenuation onto SPM, but not necessarily degraded may therefore ultimately be desorbed into the aqueous phase of treated wastewater or be discharged in waste sludge that are regularly destined for the use of agricultural fertilisers.

As the list of ECs being detected in surface waters are ever-increasing, it is vital to prioritise their monitoring following a weigh-of-evidence (WOE) approach to promote improved risk assessment. This warrants frequent assessment of their presence/concentrations in surface waters, establishing their fate throughout wastewater treatment, delineating the mechanisms for their persistence, as well as their potential risk to cause adverse health effects in both wildlife and potentially - humans.

Several prescription- and over-the-counter (OTC) medications such as the opioids TRAM and codeine are addictive. Along with the known abuse of illicit drugs, the rise in both licit- and illicit drugs of abuse (DOA) is an ongoing concern globally. Furthermore, substance abuse data is limited for developing countries, whereby most information are largely generated from abuse treatment centres (Dada et al., 2017). This may lead to a significant underestimation of substance abuse, as only a limited number of abusers will admit to their drug use or be willing to be admitted for rehabilitation. For this reason, a wastewater-based epidemiology (WBE) approach, based on water profiling of defined sewage-collections systems, has been proposed to assist with drug use estimates in a region (Castiglioni et al., 2016; Castrignanò et al., 2016; Causanilles et al., 2017).

The current study focussed on the fate of several licit and illicit drugs of abuse (DOA) and known recalcitrant pharmaceuticals during wastewater treatment and associated environmental waters, followed by conventional risk assessment approaches for the study sites. The attenuation of ECs within return activated sludge (RAS) was also investigated to assess the partitioning of recalcitrant compounds into the solid vs. the aqueous phases, in search for explanations for their persistence during passage through treatment plants.

5.2. Materials and Methods

5.2.1. Sampling locations

Two WWTWs were selected for a consecutive 7-day sampling campaign during the study: WWTW1 is located in the Northern interior (Gauteng Province), and WWTW2 located near the coastline in the Western Cape Province of South Africa (Fig. 5.1). The information for the plants are shown in Table 5.1. WWTW1 is situated 3 km from a lifestyle estate and 4 km from the nearest populated area, whereas WWTW2 is situated within a close proximity of a

populated area. For WWTW1 the total treated effluent is directly discharged into the receiving river system after disinfection, whereas WWTW2 diverts its treated effluent into either maturation ponds or the receiving river system after chemical disinfection.

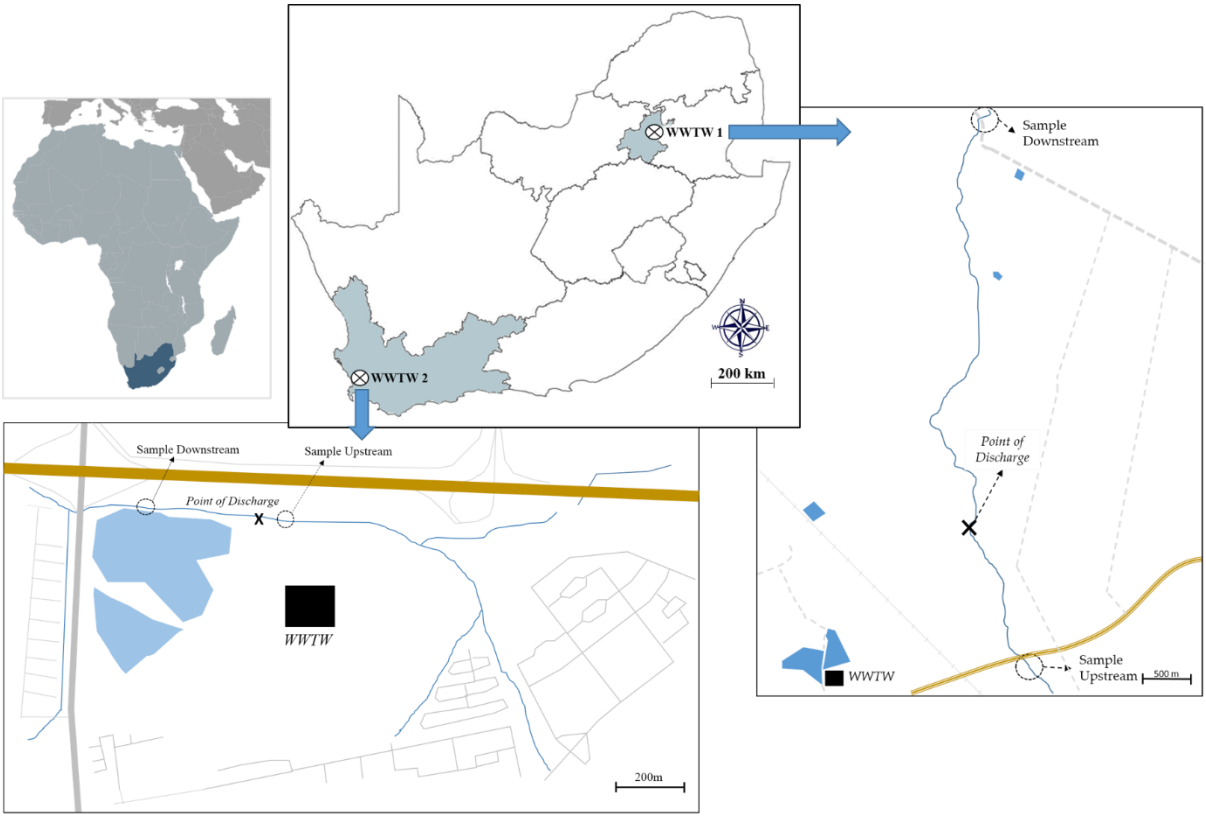

Figure 5.1: Map showing the sampling locations of WWTW 1 (Gauteng Province) and WWTW 2 (Western Cape Province).

Table 5.1: Plant information of the two WWTWs during the study.

	Capacity ($ML.day^{-1}$)	Domestic: Industrial	HRT[a] (hours)	Population Estimate[b]
WWTW1	63.0	60:40	24	200 000
WWTW2	105.0	80:20	24	470 000

[a] Hydraulic retention time (in hours) of wastewater within the treatment process.
[b] *de facto* population estimate for 2017 based on projected census data from the last formal census in 2011.

5.2.2. Chemicals and consumables

The study included the multi-residue quantification for a selected list of DOA using analytical methods described elsewhere (Castrignanò et al., 2016). The following internal standards were included in the water samples to enable quantification: cocaine-d3, benzoylecgonine-d8, amphetamine-d5, methamphetamine-d5, mephedrone-d3, MDA-d5, MDMA-d5, cotinine-d3, EDDP-d3, heroin-d9, codeine-d6, oxycodone-d6, hydrocodone-d6, methadone-d9, ketamine-

d4, norketamine-d4 and 1S,2R-(+)-ephedrine-d3. Hyper-grade methanol (MeOH, 98%) and ultra-pure water (Millipore) were used for cleaning glassware and for solid phase extraction. All glassware were deactivated using 5% dimethyldichlorosilane (DMDCS) in toluene, followed by two wash steps in toluene and three wash steps in MeOH.

5.2.3. Sample collection and preparation

Sampling was done in 7 consecutive days at the WWTWs, which included samples from the raw sewage after grit screens, final effluent after chlorination, return activated sludge (RAS), and river water collected upstream and downstream from the plants. For WWTW2, the downstream sampling point was located 3.5 km from the point of discharge due to inaccessibility closer to the point of discharge. Time-proportional composite samples (100 ml) was taken every 10 minutes from raw sewage and final effluent, and then combined to obtain a 24 hour sample of the treatment steps. Grab samples (250 ml) were taken for the RAS and river water samples. The samples were kept cold during sampling and transport to the laboratory, from which further sample filtration and extraction were completed immediately. The samples were filtered using 0.7 µm GF/F filters using a vacuum manifold, except for the RAS samples.

The focus of this report is on analyses performed on liquid samples. RAS samples were centrifuged, from which the supernatant was collected to separate the liquid phase of the RAS (RAS_{liquid}), and the centrifuged pellet was placed into a glass jar for lyophilisation to obtain the solid phase of the RAS samples (RAS_{solid}) for follow-op work. The freeze-dried RAS_{solid} was subjected to a standard protocol of microwave-assisted extraction (MAE) (Evans et al., 2015), with slight modifications. Briefly, a 0.25 g dried sludge sample were spiked with the internal standard mixture (5 µl of a 1 $\mu g.ml^{-1}$ concentration) and left for 30 mins. The samples were then suspended in 30 ml of a 50:50 mixture of MeOH and ultrapure water. The suspension was transferred to the MAE tubes and extracted using a Mars5 MAE machine (CEM Corporation). The temperature was allowed to increase over 9 minutes, and held at 121°C for 30 minutes. The samples were then allowed to cool down to room temperature and then filtered through GF/F filters (Whatmann). The filtered samples were diluted in 270 ml ultrapure water to obtain a concentration of MeOH less than 5%, followed by solid phase extraction (SPE).

5.2.4. Solid phase extraction (SPE)

All aqueous samples were extracted using Oasis® HLB cartridges (Waters; 3cc, 60 mg), and Oasis® MAX cartridges (Waters; 3cc, 60 mg) for the RAS_{solid} samples using the following protocol: The Oasis® HLB cartridges were conditioned with 2 ml MeOH, followed by 2 mL of ultrapure water, and the Oasis® MAX cartridges with 4 mL MeOH and 4 mL ultrapure water, where the solvents were allowed to pass by gravity. Each sample was then divided into duplicates, which included a 50 mL sample for raw sewage, final effluent, and RAS_{liquid}, 100 mL for river water upstream and downstream, and 300 mL for the RAS_{solid}. The extraction was carried out using a vacuum manifold (Supelco Visiprep). The samples were allowed to pass through the cartridges at a rate of 6 $mL.min^{-1}$ and allowed to run dry for at least 30 minutes. The dried cartridges were then transported refrigerated for elution and analysis. Upon arrival, the cartridges were eluted with 4 mL MeOH into 5 mL salinized glass vials and dried under a gentle stream of nitrogen (5-10 psi, 40^0C) using a TurboVap evaporator (Caliper, UK). The dried samples were then reconstituted in 0.5 mL of the mobile phase used during LC/MS (1 mM ammonium acetate:methanol, 85:15, v/v), where after the suspended samples were vortexed and filtered through 0.2 µm PTFE filters (Whatman, Puradisc, 13 mm) using 3 mL syringes. The filtered samples were then placed in polypropylene plastic vials fitted with pre-slit PTFE/Silicone septa (Waters, UK) for chemical analysis.

5.2.5. Chiral liquid chromatography coupled with tandem mass spectrometry

The method parameters and conditions used are described elsewhere (Castrignanò et al., 2016) (APPENDIX C, Table C1). Briefly, the analytes in the processed samples were separated using a Waters ACQUITY UPLC® system (Waters, Manchester, UK) equipped with a CHIRALPAK® CBH HPLC column, 5 µm particle size, L × I.D. 10 cm × 2.0 mm (Chiral Technologies, France) and a Chiral-CBH guard column 10 × 2.0 mm, 5 µm particle size (Chiral Technologies, France). The ACQUITY UPLC™ autosampler was kept at 4^0C, and the column temperature was set at 25^0C. All samples were injected at 20 µL. The mobile phase (1 mM ammonium acetate / methanol, 85:15, v/v) was injected at 0.1 $ml.min^{-1}$ under isocratic conditions. The separated analytes were quantified using a triple quadrupole mass spectrometer (Xevo TQD, Waters, Manchester, UK) with an electrospray ionisation (ESI) source, which was managed in the multiple reaction monitoring (MRM) mode. Two- to three MRM transitions were created for each compound, which assisted with confirmation and quantification. Spiked quality control (QC) standards containing the deuterated and non-deuterated analytes were

incorporated throughout the analytical procedure. The quality and quantification of the analytes in the samples were determined using the method detection limits (MDL) and method quantification limits (MQL) as set out in Castrignanó et al. (2016), as well as quality control criteria according to the European Commission Council Directive 2002/657/EC (European Commission, 2002).

5.2.6. Calculations

For each target analyte within raw wastewater and final effluent, a mass load ($g.day^{-1}$) was calculated to compensate for the variation in daily WWTW flow rates at the sewage inlet (influent) and outlet (effluent). This was achieved by multiplying the estimated concentration of the compounds ($ng.l^{-1}$) generated during LC-MS with the daily flow rate measurement ($Ml.day^{-1}$) at the WWTW influent or effluent. This estimate was then used to calculate the removal of the analytes during wastewater treatment. For removal estimates, the following equation was used:

$$\text{Removal (\%)} = [(ML_{influent} - ML_{effluent})/ML_{influent}]*100$$

where $ML_{influent}$ and $ML_{effluent}$ refers to the daily mass load (in $g.day^{-1}$) measured for each analyte within raw wastewater and treated effluent. For each removal estimation, the hydraulic retention time (HRT) was considered. Both WWTWs are recorded to have a HRT of 24 hours (Table 5.1), which led to removal estimates using the ML of influent wastewater for the one day (for example Monday) and the ML of the effluent wastewater recorded for the next day (for example Tuesday).

The enantiomeric fraction (EF) for the chiral drugs was calculated to assess whether the chiral drugs are present in a racemic mixture or rather enantiomerically enriched within the various water matrices. This was achieved using the following equation:

$$\text{Enantiomeric Fraction (EF)} = \frac{(+)}{[(+) + (-)]} \quad or \quad \frac{E1}{[E1 + E2]}$$

5.3. Results and Discussion

5.3.1. Wastewater

A total of 38 DTRs, including illicit stimulants and hallucinogens, precursor drugs, opioids, antidepressants, anaesthetics, and four human chemical markers were screened for in the aqueous phase of wastewater. In total, 31 of the target compounds were quantified within raw wastewater at WWTW1 (APPENDIX, Table C1), and 35 of the compounds at WWTW2 (APPENDIX, Table C2). These results confirm the use of illicit drugs, as well as other OTCs and prescription DOA by the surrounding population. An extensive discussion on the estimation of illicit drug use estimates will be shown in *Chapter 6* using a wastewater-based epidemiology (WBE) approach (Castiglioni et al., 2016). Overall, the majority of the DTRs were detected at higher concentrations in WWTW2, largely due to its wastewater source having a larger proportion of domestic sewage, as well as its larger holding capacity and estimated population being 1.7-fold and 2.4-fold higher respectively than recorded for WWTW1 (Table 5.1).

Mass load ratios of cocaine (COC) compared to its primary metabolite, benzoylecgonine (BEG), in raw wastewater ranged from 1.8 - 4.0 BEG/COC at WWTW1, and 1.9 - 3.8 BEG/COC at WWTW2. These ratios suggest that a large proportion of COC is present within sewage in its un-metabolised form, suggesting possible disposal rather than consumption (BEG/COC < 5)(Karolak et al., 2010). However, the overall higher loads of BEG compared to COC in the raw samples does indeed confirm hepatic metabolism of the drug, thereby confirming its consumption within the domestic community connected to the sewage system. The enantiomeric signature of methamphetamine (METH) in raw wastewater suggests an illicit origin (Castrignanò et al., 2017b; Xu et al., 2017), as all raw wastewater samples were predominantly enriched with the *S*-enantiomer (APPENDIX, Table C1 and C2). The new psychoactive substance (NPS) mephedrone was detected only in raw wastewater from WWTW2 (APPENDIX, Table C2). Although data showing mephedrone abuse in the country is limited, the current study suggest its use within the community connected to WWTW2, which is known for high rates of substance abuse (Dada et al., 2017). The same was shown for MDMA, whereby the loads were more predominant at WWTW2 (APPENDIX, Table C2), but suggest that this drug is only used as a secondary DOA on a recreational basis. Heroin, which is classified as one of the primary DOA in South Africa (Dada et al., 2017), was not detected at any raw or treated effluent samples during the current study, largely due to its low stability leading to rapid metabolism after administration and within wastewater. However, a minor, yet exclusive metabolite of heroin, *O*-6-monoacetylmorphine (*O*-6-MAM) was detected in raw

influent of WWTW2 only, confirming the presence of heroin use within the communities served by WWTW2. Ketamine, which is mostly used as a veterinary anaesthetic but also a licit DOA, was detected in raw wastewater at loads ranging from 0.1 to 17.2 g.day^{-1} at WWTW1 (APPENDIX, Table C3), and 0.4 to 1.4 g.day^{-1} at WWTW2 (APPENDIX, Table C4). Although it is reported that the metabolite norketamine would rather be excreted during its consumption, the current study only detected ketamine. This result is in agreement with similar studies (Bijlsma et al., 2016; Castiglioni et al., 2015), which point to the need for further refinement of ketamine's excretion profile in sewage in order to estimate its abuse.

All of the illicit compounds and their associated metabolites were removed with high efficiency at both WWTWs (Fig. 5.2A). Both WWTWs utilise advanced AS process for improved biological nutrient removal (BNR), which is known to cause a higher rate of stereoselective reduction of chiral drugs compared to other types of treatment systems (Kasprzyk-Hordern and Baker, 2012a). Removal estimates for (±)-METH was shown to be stereo-selective at both WWTWs, whereby *R*-(-)-METH was less removed than *S*-(+)-METH (Fig. 5.2A). This has been proven both on micro- and macroscale biodegradation studies, confirming an enrichment of *R*-(-)-METH during biological treatment (Bagnall et al., 2013; Xu et al., 2017). The same was shown for MDMA, whereby *R*-(-)-MDMA was enriched in treated wastewater effluent at WWTW2 and therefore, less effectively removed during treatment (Fig. 5.2A). This result has been confirmed in microcosm studies where stereoselective biodegradation was shown to favour the degradation of the *S*-enantiomer (Evans et al., 2016). The reason why removal of MDMA did not show the same trend for WWTW1 can be ascribed by the limited number of detections within raw- and treated effluent wastewater during the sampling campaign (APPENDIX, Table C1). In addition, AS may also provide biological catalysts which may cause chiral inversion. This has been shown for some chiral compounds (Hashim and Khan, 2011; Neirinckx et al., 2011; Reist et al., 1998), but not for the drugs detected during the current study. Ketamine showed moderate- to negative removal during WWTW treatment at both study sites (Fig. 5.2B), which is in agreement with other studies (Baker and Kasprzyk-Hordern, 2011) and warrants this compound as a priority EC to assess its potential risk environmental risk.

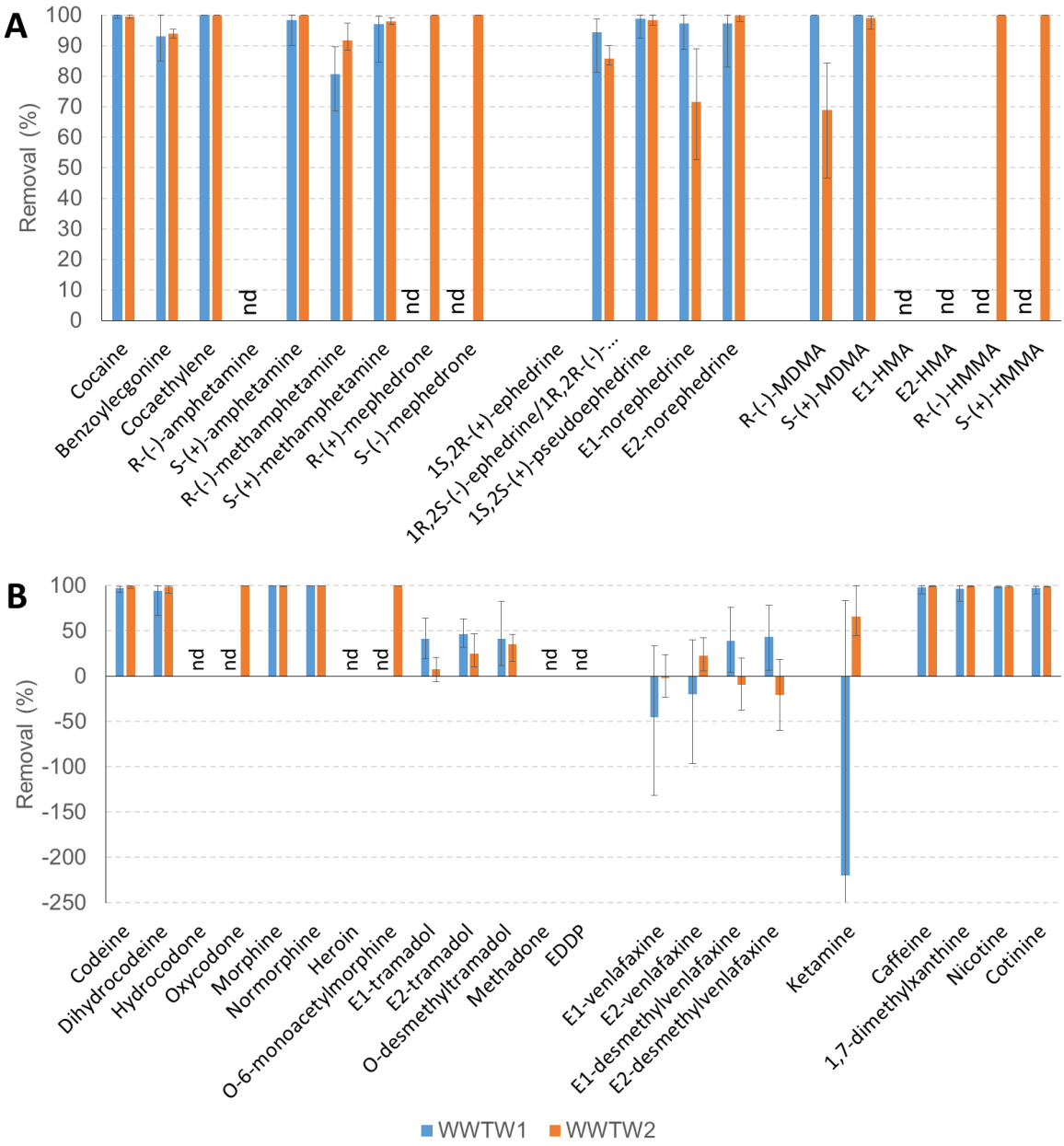

Figure 5.2: Removal (%) of illicit drugs (A) and other drugs of abuse and human indicators (B) during wastewater treatment. Error bars represent the lowest- and highest recorded removal for each compound during sampling. nd – not determined.

For the prescription and OTC drugs (including ketamine), distinctions could not be made between their general use apart from their potential abuse within the communities due to the unavailability of a unified pharmacological prescription database for the country (currently divided into the public- and private health sector). Regardless of this, influent wastewater samples revealed high concentrations of the opiates codeine and morphine up to $\mu g.L^{-1}$ levels (APPENDIX, Table C1 and C2). Codeine concentrations in raw influent wastewater for WWTW1 ranged between 1 712.2 and 2 619.5 $ng.L^{-1}$ (APPENDIX, Table C1), and for WWTW2 between 1 663.2 and 20 567.7 $ng.L^{-1}$ (APPENDIX, Table C2) compared to measured

codeine concentrations at a maximum concentration of 5 200 ng.L^{-1} in other parts of the world (*summarised in* Thai et al., 2016). These results highlight the extent of codeine use and potential abuse within the current study areas. Attempts to estimate codeine abuse through wastewater-based epidemiology (WBE) have proven troublesome, as unified and/or refined excretion- and stability correction factors are lacking for the drug, as well as the inability to distinguish from its therapeutic use (at least for the current study).

Establishing the source of opiates and their metabolites are complex for urban water profiling, as these drugs are metabolised into one another (Fig. 5.3), whereby the metabolites are also used for prescription medications. For example, heroin administration will predominantly be excreted as morphine in the body, whereas morphine may also be derived from the metabolism of codeine or through administration of itself (Fig. 5.3). Morphine concentrations in raw influent wastewater for WWTW1 ranged between 291.8 and 407.2 ng.L^{-1} (APPENDIX, Table C1), and for WWTW2 between 761.1 and 9 379.7 ng.L^{-1} (APPENDIX, Table C2), which once again, are high compared to similar case studies around the world (Baker et al., 2014; Castrignanò et al., 2016; Causanilles et al., 2017). Furthermore, during wastewater treatment, hydrocodone was detected in treated effluent wastewater, but not in raw influent at both WWTWs (APPENDIX, Table C5 and C6). Although hydrocodone serves as an active ingredient for some pharmaceutical prescriptions, the current findings suggest that this compound was rather derived from codeine metabolism driven by microbial bio-degradation within WWTW processes. For example, enzyme isolates from environmental bacterial strains, such as morphinone reductase isolated from *Pseudomonas putida*, has been described to result in the conversion of morphine and codeine to hydromorphone and hydrocodone respectively (French and Bruce, 1994), whereby such organisms are able to utilise these compounds as a sole nutrient source. Furthermore, high codeine degradation is proposed to be driven by ammonia-oxidising bacteria under anaerobic conditions (Falås et al., 2016). Within the aqueous phase of RAS samples in the current study, the concentrations of hydrocodone were higher than for codeine, ranging from 40.8 to 60.2 ng.L^{-1} at WWTW1 and from 89.7 to 318.2 ng.L^{-1} at WWTW2, as opposed to average concentrations of codeine calculated at 2.8-274.6 ng.L^{-1} and 6.6-15.1 ng.L^{-1} at WWTW1 and WWTW2 respectively (APPENDIX, Tables C9 and C10). The single high concentration for codeine on the Sunday sample at WWTW1 was just after the plant experienced a treatment downtime on the Saturday (further discussed below), which is therefore proposed to have led to inefficient degradation of codeine (APPENDIX, Table C9). Interestingly, the concentration of hydrocodone was not lower during the same day as expected.

It should be highlighted that the concentrations of codeine within raw sewage was also at its highest for the Sunday sample (APPENDIX, Table C1), which suggest that the degradation of codeine and eventual formation of hydrocodone are influenced by 'fresh' compounds entering the plant, as well as low levels of codeine being re-circulated during AS treatment (from RAS). Regardless of the various metabolic pathways which opiates may follow, both parent- and their respective metabolites were removed with high efficiency at both WWTWs (Fig. 5.2B). It should be noted, however, that such removal estimations are based on the comparisons of the drug levels in raw- and treated wastewater effluent, whereby the risk of the residual load after treatment should still be determined. More discussion on this will follow in the *risk assessment* section below.

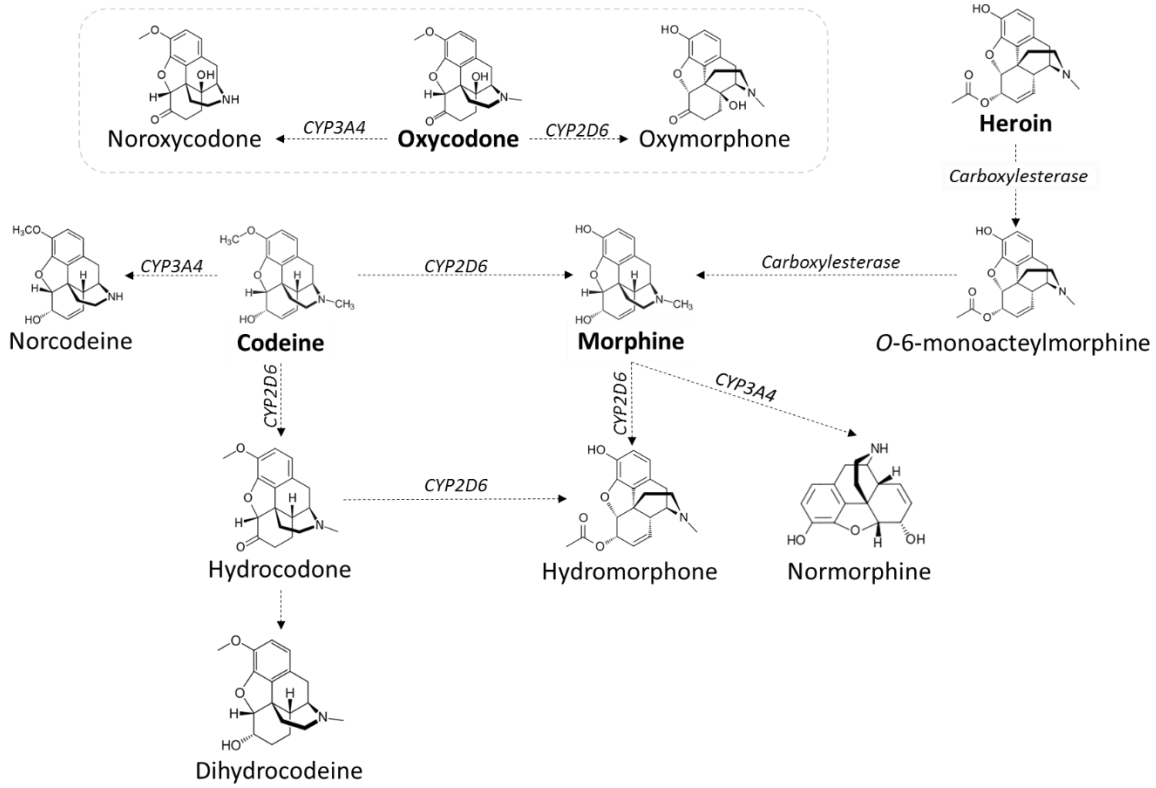

Figure 5.3: Metabolic pathways for the three main opiates and oxycodone and the associated enzymes driving their metabolism in humans. CYP – cytochrome-P450.

The two pharmaceutical compounds which ranged from moderate- to negative removal during the current study include the opioid drug TRAM, the anti-depressant drug VEN and their primary metabolites (Fig. 5.2B). TRAM and its primary metabolite, *O*-desmethyltramadol (*O*-DMT) were moderately removed at WWTW1, whereas the removal at WWTW2 for TRAM was stereo-selective, but generated the same removal profile for *O*-DMT between the study sites (Fig. 5.2B). VEN showed an overall negative mass balance during treatment at WWTW1,

whereas its metabolite, desmethylvenlafaxine (DMV) was moderately removed (Fig. 5.2B). Removal of VEN and DMV at WWTW2 showed an opposite result, whereby VEN removal was slightly negative- to low, and DMV was negatively removed on average during the sampling campaign (Fig. 5.2B). Negative removal has been largely attributed to two contributing factors; 1) the high tendency of both compounds to sorb onto particulate matter, from which desorption into the aqueous phase may occur periodically during primary clarification, and 2) biological de-conjugation or re-methylation processes during wastewater treatment, leading towards a build-up of the parent compound at the treated effluent (Blair et al., 2015; Subedi and Kannan, 2014). The current study further suggests that such recalcitrant compounds which are known to have a strong association with solids are constantly recirculated within RAS, from which desorption from the solid matrix and increased contact time with anaerobic digestion may largely contribute towards its pseudo-persistence throughout sampling days.

Both TRAM and VEN undergo desmethylation metabolism into their primary metabolites, which are shown to be predominantly driven by anaerobic digestion, and not aerobic, in AS treatment (Baalbaki et al., 2017; Falås et al., 2016; Gasser et al., 2012; Kasprzyk-Hordern and Baker, 2012a). However, significant degradation under anaerobic conditions mostly require long residence times (14 days), which are not necessarily feasible for WWTW treatment. The average negative removal of VEN during WWTW1 treatment may well be due to a difference in sludge maturity and residence times. Also, a municipal power outage (which will be further discussed later) could also have impacted the overall performance of the plant. As de-methylation does in fact lead to the degradation of VEN, it may inversely lead to the build-up of DMV during treatment, which is shown by the negative mass balances of DMV estimated for WWTW2 (Fig. 1B). However, as both VEN and TRAM remain within the wastewater treatment system (within RAS), their pseudo-persistence may compromise daily estimations of removal. Enantiomeric profiling of VEN and DMV within the aqueous phase of RAS showed a racemic mixture for VEN (average EF = 0.5 ±0.02; Fig. 5.4C & D), and slight stereoselectivity for DMV (average EF = 0.6 ±0.01; Fig. 5.4C & D) at both WWTWs. On average, the concentrations of a racemic mixture of DMV compared to VEN were 3.9 times more in RAS_{liquid} samples for WWTW1 and 4.2 times more for WWTW2. In raw wastewater samples, DMV/VEN ratios were 8.3 and 3.0 for WWTW1 and WWTW2 respectively, suggesting further desmethylation metabolic processes during AS treatment for WWTW2, but rather high possible de-methylation processes within sewage prior to treatment at WWTW1

(in-sewer degradation). This is supported by the fact that a racemic DMV concentration were similar between raw influent and RAS_{liquid} samples for WWTW1, but rather 1.8 times higher in RAS_{liquid} samples than raw influent wastewater for WWTW2. These results imply that although recalcitrant compounds such as VEN and its metabolite are returned into the treatment steps (hence increasing residence time for their removal), their continued persistence and regular input into raw sewage will compromise the removal during the overall treatment process.

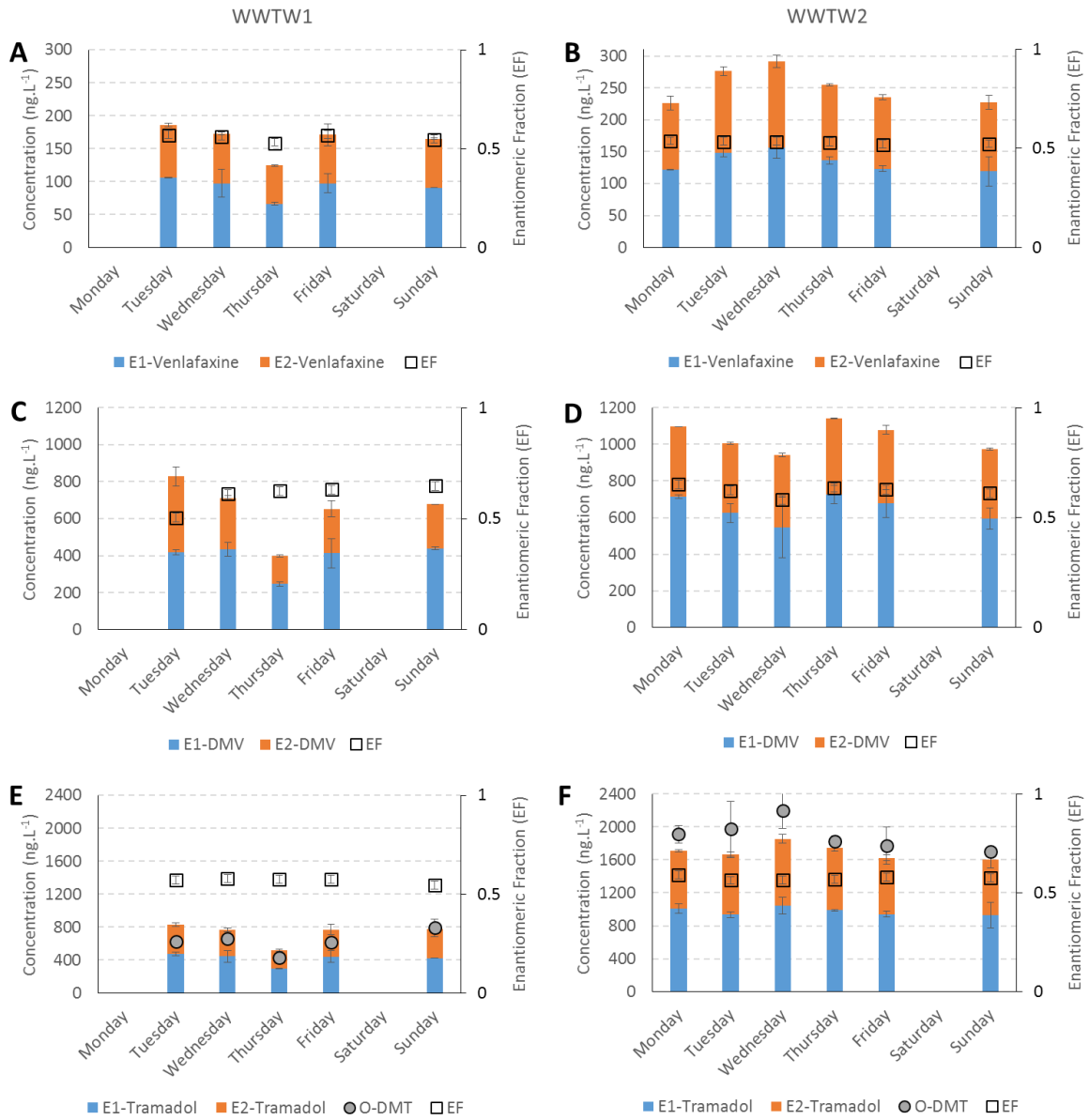

Figure 5.4: Concentrations ($ng.L^{-1}$) and enantiomeric fractions (EF) of venlafaxine (A & B), desmethylvenlafaxine (DMV; C & D) and tramadol (E & F) within the aqueous phase of return activated sludge (RAS).

It should be noted that the treatment performance of WWTW1 were compromised during the weekend period of the current sampling campaign. A clear spike in the loads of BEG, *S*-(+)-METH, ephedrines, codeine, caffeine, 1,7-dimethylxanthine and cotinine were observed on the Sunday in the treated wastewater effluent (APPENDIX, Table C5 and C7) - all compounds which are known to be effectively degraded during AS treatment. During the Saturday, the plant experienced a municipal power outage, which led to a downtime in the treatment modules. The flow from the effluent discharge was still maintained, which led to a sampling day whereby wastewater were not treated before discharged into the recipient river system. Interestingly, the loads of *O*-DMT and DMV were significantly lower during Saturday and Sunday in treated effluent compared to the other sampling days, but not for the parent TRAM and VEN (Fig. 5.5). This may suggest a decrease in desmethylation metabolic activities during treatment downtime, leading to reduced degradation of TRAM and VEN into their primary metabolites. On the other hand, both TRAM and VEN are known to have high sorption affinity to solids (Baalbaki et al., 2017; Boix et al., 2016). As a consequence of the treatment module downtime, the solid particulate matter (SPM) within AS treatment settled down, which may have retained these compounds; explaining why they were not detected at higher concentrations in treated effluent. Overall, the concentrations in treated effluent from WWTW1 were 347.0 – 687.5 $ng.L^{-1}$ for TRAM, 157.1 – 905.1 $ng.L^{-1}$ for *O*-DMT, 78.2 – 148.9 $ng.L^{-1}$ for VEN, and 167.4 – 691.0 $ng.L^{-1}$ for DMV (APPENDIX, Table C5). For WWTW2, concentrations in treated effluent were 1155.5 – 1514.0 $ng.L^{-1}$ for TRAM, 1141.0 – 1949.5 $ng.L^{-1}$ for *O*-DMT, 159.0 – 206.9 $ng.L^{-1}$ for VEN, and 513.1 – 955.2 $ng.L^{-1}$ for DMV (APPENDIX, Table C6). The chiral signature for TRAM and VEN were both racemic for influent and effluent samples at the WWTWs (Fig. 5.5), which suggest no enantio-selective degradation during treatment. The EF for DMV also did not differ between influent and effluent samples for both study sites (Fig. 5.5). However, the chiral signature for DMV showed slight stereo-selectivity, with EFs of 0.62 (±0.01) and 0.64 (± 0.01) in influent and effluent samples respectively at WWTW1, and 0.63 (±0.02) and 0.61 (± 0.02) in influent and effluent samples respectively for WWTW2 (Fig. 5.5). This suggest that DMV degradation is also not enantio-selective during wastewater treatment, but rather emphasise the need to establish its enantio-selective toxicity for refined risk assessment purposes.

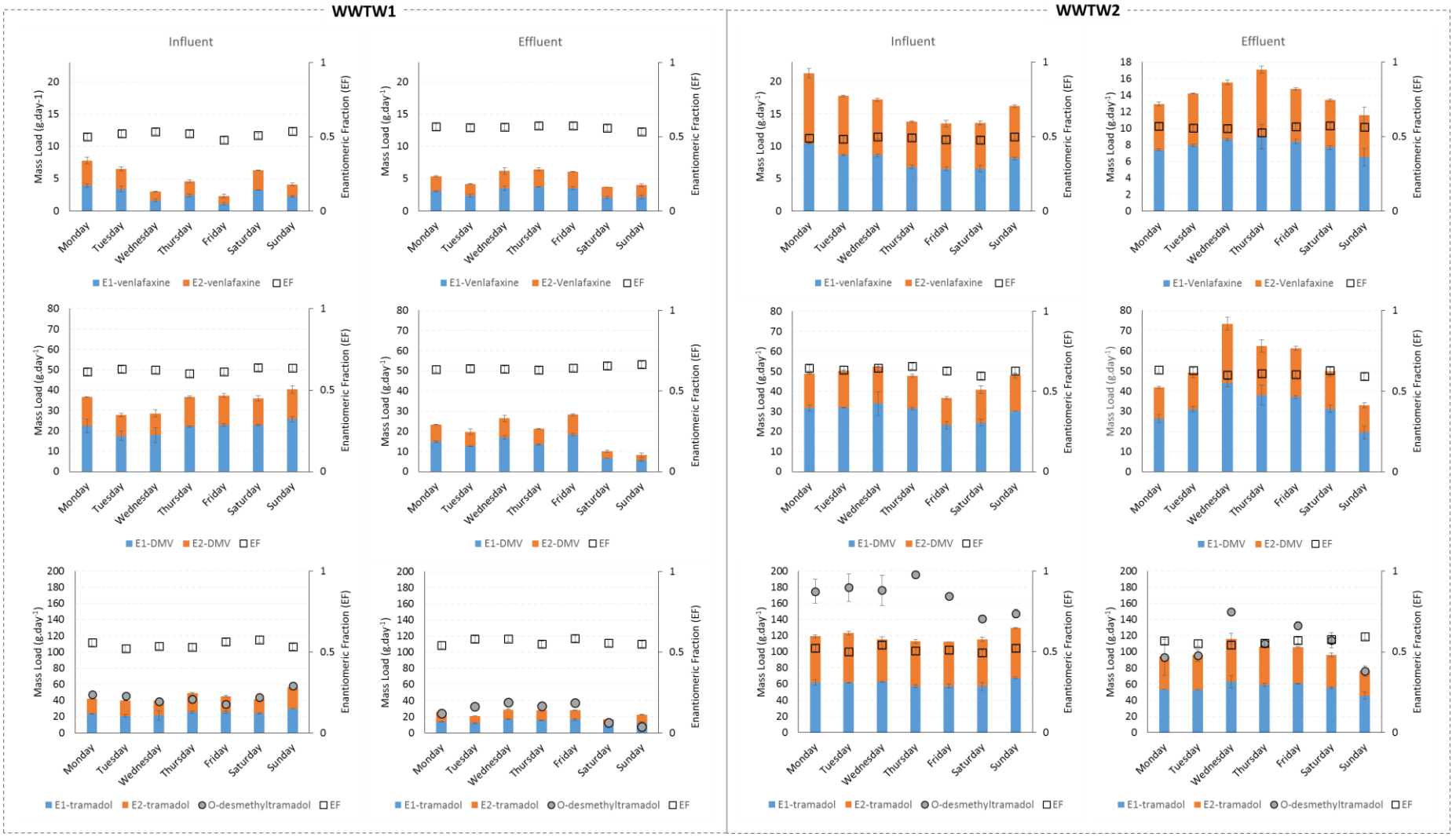

Figure 5.5: Mass loads (g.day^{-1}) and enantiomeric fractions (EF) of venlafaxine, desmethylvenlafaxine (DMV) and tramadol within raw influent- and treated effluent wastewater samples from the two study sites.

5.3.2. River water

Compounds removed with high efficiency at WWTW1 and thus discharged at low concentrations from treated wastewater, such as the human markers caffeine, nicotine and their metabolites, were overall detected at higher concentrations in downstream samples during the sampling period (APPENDIX, Table C12). This was especially true for samples collected on Monday, with a clear spike in the concentrations of COC, BEG, *S*-(+)-METH, ephedrines, codeine, caffeine, 1,7-dimethylxanthine, and cotinine compared to the rest of the sampling days (APPENDIX, Table C12). This result is in accordance to the observed spike in the same compounds within treated effluent wastewater on the previous day (Sunday) (APPENDIX, Table C5 & C7). Even though the downstream sample was taken 3.5 km from the point of discharge (Fig. 5.1), it is possible that the pollutant load originated from this WWTW discharge event. However, the average concentrations of *S*-(+)-METH in effluent wastewater was calculated at 27.5 ng.L^{-1} in comparison to 29.0 ng.L^{-1} for the downstream samples, which were not significantly different. Assuming the high loads in treated effluent was a result for the loads on the following day in the downstream sample, the fold change in *S*-(+)-METH were still 1.2 (higher in downstream). It is therefore unlikely that the discharge from wastewater would still have resulted in the high loads of *S*-(+)-METH at least in the downstream sample, taken the long distance between the sampling points (Fig. 5.1), as well as the known stereo-selective biodegradation of *S*-(+)-METH over *R*-(-)-METH (Bagnall et al., 2013). The average concentration of *S*-(+)-METH during the sampling campaign was further shown to be 18 times higher in the downstream samples than upstream river water (Table 5.2), from which *S*-(+)-METH was removed with relatively high efficiency during wastewater treatment (Fig. 5.2A). As this compound is known to be the primary enantiomer of illicit drug use, the current results suggest that this compound is either excreted or directly disposed from an additional source downstream from the WWTW. Interestingly, the same result of higher DTRs in downstream than treated effluent discharge was shown in a similar sampling campaign done at the WWTW during 2015 (Archer et al., 2017b), when the WWTW did not experience any treatment failures. Overall, 25 of the 38 target compounds screened for during the current study were detected in river water associated with WWTW1, whereby 23 of the compounds were detected at concentrations two-fold and higher in downstream samples (Table 5.2).

For river water samples associated with WWTW2, the opposite was observed than for the WWTW1 study site, whereby the concentrations of the analytes for upstream river water samples were higher than downstream water samples (Table 5.2). It is worth to mention that

the plant discharges a large percentage of its treated effluent into maturation ponds for reuse due to the ongoing droughts in the region, although the ratio of treated wastewater discharge into maturations ponds compared to river discharge could not be verified. The distance between upstream- and downstream sampling sites at WWTW2 was much shorter than for WWTW1 (300m as opposed to 4km), thus less time for degradation of the target analytes between these two sampling points. Regardless, the compounds which were detected at higher concentrations in downstream than upstream samples were *R*-(-)-METH, *R*-(-)-MDMA, VEN and DMV (Table 5.2), which were also reported to be less removed during wastewater treatment (Fig. 5.2). In particular, METH and MDMA were the two chiral drugs that were shown to undergo stereoselective degradation during wastewater treatment, leading to an enrichment of the *R*-enantiomers in treated wastewater. As opposed to these recalcitrant compounds, TRAM and *O*-DMT, which also showed low- to moderate removal during wastewater treatment were not detected at higher concentrations in downstream samples (Table 5.2), which may suggest other contributing factors that may lead to their sorption onto sediment rather than persisting in the aqueous phase of environmental waters (although the same would have been assumed for VEN and DMV). Another interesting observation was the detection of the minor metabolite of heroin, *O*-6-MAM, during two sampling days in upstream water, along with concentrations of *S*-(+)-METH which were higher than the concentrations measured for treated sewage effluent (APPENDIX, Table C13). Therefore, similar to the results observed for river water associated with WWTW1, the higher concentrations of most target analytes within upstream samples from WWTW2 suggests alternative pollution or possible dumping of sewage into the river system, as this river system passes directly through both formal- and informal domestic housing.

Table 5.2: Concentrations (ng.L^{-1}) of the DOA within river water located upstream and downstream from WWTW1 and WWTW2.

Chemical	River associated with WWTW1					River associated with WWTW2				
	Upstream		Downstream		Fold	Upstream		Downstream		Fold
	Average	Min - Max	Average	Min - Max	Change*	Average	Min - Max	Average	Min - Max	Change*
Illicit drugs										
Cocaine		ND	5.2		-	1.0	0.6 - 1.6	0.8	0.4 - 2.1	0.8
Benzoylecgonine	3.3	2.7 - 3.7	10.7	5.4 - 34.9	3.2	20.6	14.2 - 28.7	20.1	13.2 - 31.0	1.0
R-(-)-methamphetamine	4.8	3.2 - 5.8	20.0	10.7 - 33.1	4.2	14.1	6.1 - 25.9	18.5	9.6 - 28.3	1.3
S-(+)-methamphetamine	1.6	0.8 - 5.0	29.0	1.7 - 178.8	18.1	218.7	137.9 - 465.2	113.3	73.5 - 206.1	0.5
Methamphetamine-rac	6.3	4.2 - 10.8	49.0	12.4 - 210.5	7.8	232.7	144.2 - 491.1	131.8	81.3 - 234.4	0.6
R-(-)-MDMA		ND		ND	-	0.7	0.4 - 1.2	2.1	0.7 - 4.6	3.0
S-(+)-MDMA		ND		ND	-	0.3	0.2 - 0.4		ND	-
MDMA-rac		-		-	-	0.9	0.6 - 1.5		-	-
Precursors										
1S,2R-(+)-ephedrine	0.6	0.7 - 1.1	1.8	1.2 - 3.1	3.0		ND		ND	-
1R,2S-(-)-ephedrine/*1R,2R*-(-)-pseudoephedrine	19.4	16.9 - 22.9	42.6	20.1 - 142.6	2.2	28.8	26.2 - 35.9	30.9	24.3 - 35.1	1.0
1S,2S-(+)-pseudoephedrine	3.9	3.1 - 5.6	19.2	5.3 - 46.2	4.9	12.5	9.2 - 20.1	13.1	11.6 - 14.4	1.0
E1-Norephedrine		ND	3.1	1.9 - 5.4	-		ND	1.7	0.7 - 2.4	-
E2-Norephedrine		ND		ND	-		ND		ND	-
Opioids										
O-6-MAM		ND		ND	-	14.6	11.7 - 17.5		ND	-
Morphine		ND		ND	-	64.1	54.8 - 80.0	30.2	19.5 - 55.1	0.5
Codeine	5.6	4.1 - 9.4	50.3	4.3 - 217.5	9.0	42.1	29.4 - 51.4	27.6	16.3 - 35.8	0.7
Hydrocodone	4.7	4.3 - 5.2		ND	-	16.6	13.7 - 18.6		ND	-
E1-tramadol	37.6	21.3 - 54.3	128.1	55.6 - 196.2	3.4	1137.2	881.4 - 1458.9	705.7	494.7 - 974.8	0.6
E2-tramadol	30.8	15.3 - 44.6	98.6	38.6 - 149.3	3.2	829.8	594.0 - 1076.7	505.1	368.5 - 647.5	0.6
Tramadol-rac	68.4	36.6 - 98.9	226.7	94.2 - 345.5	3.3	1967.0	1475.4 - 2535.6	1210.7	863.2 - 1622.3	0.6
O-DMT	61.1	51.9 - 73.2	257.3	62.9 - 410.1	4.2	2573.5	2221.5 - 2824.7	1753.7	1394.4 - 2100.6	0.7
Anti-depressants										
E1-venlafaxine	6.2	2.5 - 8.9	27.9	14.4 - 47.9	4.5	35.0	24.7 - 46.8	61.1	37.2 - 104.5	1.7
E2-venlafaxine	5.6	2.5 - 7.8	21.7	10.9 - 37.2	3.9	41.1	31.4 - 54.6	49.4	26.9 - 83.4	1.2
Venlafaxine-rac	11.8	5.0 - 16.7	49.6	25.3 - 85.1	4.2	76.1	56.1 - 101.4	110.6	64.1 - 187.9	1.5
E1-DMV	36.5	12.1 - 52.2	107.4	24.0 - 187.3	2.9	227.5	110.9 - 347.8	256.2	153.8 - 423.4	1.1
E2-DMV	24.2	9.0 - 34.4	64.1	14.8 - 106.2	2.6	108.7	48.3 - 173.0	135.2	84.3 - 241.0	1.2
DMV-rac	60.7	21.1 - 85.7	171.5	39.5 - 293.5	2.8	336.1	159.2 - 506.9	391.3	238.1 - 664.4	1.2
Anaesthetics										
Ketamine	2.3	1.6 - 2.9	13.5	3.8 - 28.3	5.9	7.8	6.6 - 9.1	5.9	3.9 - 7.9	0.8

Table 5.2 (continued)

Human markers									
Caffeine	541.2	104.4 - 1100.0	3289.0	113.0 - 21130.9	6.1	2293.7	1313.4 - 4316.1	1105.6	187.3 - 1894.4	0.5
1.7-DMX	592.5	165.1 - 1298.5	3632.3	216.5 - 21464.9	6.1	9654.9	4196.1 - 15027.0	4089.8	1308.5 - 6130.5	0.4
Nicotine	139.1	16.6 - 312.4	241.4	60.1 - 438.2	1.7	1645.9	553.3 - 4332.2	1956.4	280.6 - 7007.5	1.2
Cotinine	28.6	14.8 - 48.5	71.6	24.0 - 268.4	2.5	293.2	169.6 - 473.4	156.2	46.3 - 297.3	0.5

*Differences in the average concentrations of the analytes between upstream and downstream samples. Fold change > 1 = higher concentration in downstream water.
Abbreviations: *O-6-MAM*, O-6-monoacetylmorphine; *O-DMT*, O-desmethyltramadol; *DMV*, desmethylvenlafaxine; *1,7-DMX*, 1,7-dimethylxanthine; *ND*, not detected

5.3.3. Risk assessment

The formulation to conduct conventional risk assessment has been previously discussed (see *Chapter 4*). Based on the generated risk quotients (RQs), a RQ < 0.1 was considered low risk, between 0.1 and 1.0 a medium risk, and above 1.0 as a high risk (Hernando et al., 2006). These RQs were determined using predicted no-effect concentrations (PNECs) generated from literature showing lethal toxicity for the most sensitive test species (either algae, cladocerans or fish) (Bergmann et al., 2011; Deo, 2014; Mendoza et al., 2014; Minguez et al., 2016). From the assessed target analytes during the current study, the compounds which showed the most pronounced lethal toxicity risk were TRAM, codeine and nicotine, having high RQs for both treated wastewater effluent and river water at both study sites (Table 5.3). For VEN, a medium risk was calculated for the surface waters and treated effluent wastewater from both study sites, whereas the other analytes were considered a low risk for lethal toxicity endpoints (Table 5.3).

Table 5.3: Environmental risk screening based on acute predicted no-effect concentration (PNEC) toxicity data on the most sensitive evaluated test species in literature (algae, cladocerans or fish). Risk quotients (RQ) were measured for the minimum and maximum measured environmental concentration (MEC; $ng.L^{-1}$; range, min and max) determined for each analyte within WWTW effluent (*eff*) and surrounding environmental waters (*rw*).

Compound	PNEC (ng/L)	Reference	WWTW1				WWTW2			
			MEC_{eff} (ng/L)	RQ_{eff}	MEC_{rw} (ng/L)[#]	RQ_{rw}	MEC_{eff} (ng/L)	RQ_{eff}	MEC_{rw} (ng/L)[#]	RQ_{rw}
Tramadol	320.0	Bergmann et al., 2011	511.7 (341.6-714.8)	1.1-2.2 ***	147.6 (36.6-345.5)	0.1-1.1 ** - ***	1308.8 (1142.8-1646.4)	3.6-5.1 ***	1588.9 (863.2-2535.6)	2.7-7.9 ***
Methamphetamine	1970.0	Mendoza et al., 2014	66.4 (28.7-212.2)	<0.1 *	27.7 (4.2-210.5)	<0.1 *	143.7 (118.3-182.5)	<0.1 *	182.2 (81.3-491.1)	<0.1 *
Venlafaxine	322.0	Minguez et al., 2016	108.1 (78.2-148.9)	0.2-0.5 **	30.7 (5.0-85.1)	<0.1-0.2 * - **	191.3 (182.4-206.9)	0.6 **	93.3 (56.1-187.9)	0.2-0.6 **
Ketamine	720.0	ECOSAR	23.9 (6.0-63.6)	<0.1 *	7.9 (1.6-28.3)	<0.1 *	3.7 (2.1-6.0)	<0.1 *	6.8 (3.9-9.1)	<0.1 *
Morphine	93.0	Mendoza et al., 2014	-	-	-	-	0.1	<0.1 *	47.1 (19.5-80.0)	0.2-0.9 **
Cocaine	2280.0	Mendoza et al., 2014	0.6	<0.1 *	5.4	<0.1 *	1.1 (0.6-2.7)	<0.1 *	0.9 (0.4-2.1)	<0.1 *
Benzoylecgonine	4900.0	ECOSAR	19.0 (9.0-54.0)	<0.1 *	7.6 (2.7-34.9)	<0.1 *	40.1 (27.7-57.8)	<0.1 *	20.4 (13.2-31.0)	<0.1 *
Codeine	60.0	ECOSAR	61.4 (8.4-150.6)	0.1-2.5 ** - ***	30.0 (4.1-217.5)	0.1-3.6 ** - ***	53.6 (14.3-96.3)	0.2-1.6 ** - ***	34.8 (16.3-51.4)	0.3-0.9 **
Nicotine	14.0	Bergmann et al., 2011	340.1 (73.3-715.3)	5.2-51.1 ***	190.2 (16.6-438.2)	1.2-31.3 ***	932.5 (246.5-1759.5)	17.6-125.7 ***	1801.2 (280.6-7007.5)	20.0-500.5 ***
MDMA	216.0	Mendoza et al., 2014	0.3	<0.1 *	-	-	5.8 (2.8-11.0)	<0.1 *	1.5 (0.6-4.6)	<0.1 *
Caffeine	490 000.0	Deo, 2014	3837.2 (96.0-23732.5)	<0.1 *	1915.1 (104.4-21330.9)	<0.1 *	393.4 (171.0-864.4)	<0.1 *	1699.7 (187.3-4316.1)	<0.1 *
Cotinine	520 000.0	Deo, 2014	92.5 (27.6-331.0)	<0.1 *	50.1 (14.8-268.4)	<0.1 *	56.5 (27.5-104.3)	<0.1 *	224.7 (46.3-473.4)	<0.1 *
Ephedrine	3620.0	Mendoza et al., 2014	41.8 4.2-217.5)	<0.1 *	14.6 (0.7-142.6)	<0.1 *	40.3 (11.1-63.8)	<0.1 *	21.3 (9.2-35.9)	<0.1 *
O-6-MAM	1340.0	Mendoza et al., 2014	-	-	-	-	-	-	14.6 (11.7-17.5)	<0.1 *

* Low risk, ** median risk, *** high risk based on RQ values (Hernando et al., 2006)

[#] Average measured concentration of the compounds in river water samples (upstream and downstream from the WWTW)

Apart from reports showing lethal toxicity risks, the potential of the target analytes to exert sub-lethal adverse health effects are discussed below. These outcomes are more representative on the potential long-term health effects of the pollutants through continued daily exposure rather than sporadic events which may lead to acute and/or chronic lethal toxicity. As an effect sub-lethal toxicity endpoints are also more useful to represent toxicity for higher vertebrates other than using invertebrate and/or microbial test organisms.

Toxicity of illicit drugs

Illicit substances are not generally regarded as priority ECs for risk assessment. However, some studies included cocaine (COC) in toxicological studies. COC concentrations as low as 40 $ng.L^{-1}$ showed a significant decrease in lysosomal stability and increased DNA damage in hemocytes of the Zebra mussel, *Dreissena polymorpha* (Binelli et al., 2012). Furthermore, higher concentrations during the same study (220 and 10 000 $ng.L^{-1}$) led to a further increase in cytotoxicity and genotoxicity. During the current study, no wastewater effluent or environmental surface water samples reached these concentrations for possible toxicity. It should be noted that only a portion of consumed COC will be excreted unmetabolised in sewage (1-9%), while the majority will be found as primary metabolites such as BEG (Baselt, 2004). BEG was frequently detected above 40 $ng.L^{-1}$ in wastewater effluent from both study sites (up to 57.5 $ng.L^{-1}$; Table 3). Similar to the study for COC, BEG at concentrations of 500 $ng.L^{-1}$ showed an increase in oxidative stress, as well as increased cytotoxicity of hemocytes in *D. polymorpha* (Parolini et al., 2013). More recently, BEG exposure as low as 11.5 $ng.L^{-1}$ showed DNA damage and slight oxidative stress in Zebrafish embryos (*Danio rerio*), as well as other cyto-genotoxic endpoints at concentrations of 115 $ng.L^{-1}$ (Parolini et al., 2017). Furthermore, the authors concluded that BEG pose a higher cyto-genotoxicity risk than COC in their combined analysis with the *D. rerio* embryos. Even though the information is still relatively limited, there is thus increasing evidence that metabolic breakdown products have significant adverse health effects apart from their parent counterparts, emphasising the need to also include them for risk assessment.

The modulation of neuroendocrine system has also been reported for laboratory animals and aquatic vertebrates. Exposure of methamphetamine to rats under laboratory conditions resulted in increased corticosterone release, as well as decreased dopamine levels in the brain of exposed animals compared to controls (Herring et al., 2010). Methamphetamine has further been shown to induce mating behaviour in male sailfin molly fish (*Poecilia latipinna*), from which such

elevated sexual behaviour was attributed to the modulation of monoamine levels in the brain and disruption of other dopaminergic pathways (Ghazilou and Ghazilou, 2011). The influence of monoamines to control gonadotropin release in the vertebrate brain are well known (Waye & Trudeau, 2011). As an effect, the modulation of neurotransmitter release (including monoamines) through environmental exposure of psychoactive stimulants such as methamphetamine may not only affect physiological pathways associated with cognitive function and behaviour, but also potentially modulate the early onset of the control in gonadal endocrine system pathways.

Toxicity of Opioids

Although conventional risk assessment shows a low environmental risk for morphine, based on lethal toxicity endpoints (Table 5.3), sub-lethal toxicity endpoints have been ascribed for morphine exposure. A strong association with observed endocrine modulations in laboratory animals and humans have been drawn following opioid administration, especially for morphine (Vuong et al., 2010). In particular, morphine exposure to female rats caused a polycystic morphology of ovaries, decreased intact brain neurons (Karimi et al., 2017), as well as other undesired endocrine system modulations, including altered growth- and thyroid-stimulating hormone levels, and decreased steroid hormone levels (Vuong et al., 2010). Even though endocrine-disrupting endpoints on aquatic wildlife have received little attention, the potential genotoxicity which could be caused by opioids should also not be ignored. For example, morphine exposure in mice has been linked to increased incidence of micro-nucleated bone marrow erythrocytes in a dose-dependent manner (Puli and Patil, 2007). From these results, the potential of opiates, such as morphine, to modulate endocrine systems and to cause genotoxicity in exposed aquatic surface waters should receive priority. Taken that metabolism of codeine and heroin use also lead to the formation of morphine and its derivatives highlights the need for combined mixture risk assessment, as the combination of these opiates may have synergistic, potentiating or additive mixture interactions. This warrants the need to include these compounds as priority ECs for risk assessment monitoring, especially due to their regular detection within surface waters as highlighted within the current study. Apart from their potential adverse risks to wildlife and human health exposed to contaminated surface waters, the abuse of these substances in communities are also of a socio-economic concern. In particular, the abuse of common over-the-counter (OTC) medications, such as codeine, are globally well reported to be on the rise, with South Africa being no exception (Parry et al., 2017). However, substance abuse data is limited to treatment centre data in the country, which

may be subjective. Therefore, the use of urban water profiling may well assist with opioid use estimations, given that more sufficient prescription databases become available for the country.

The widespread use of TRAM for pain relief, as well as its reported abuse necessitates the continued monitoring for its presence and fate in surface waters. TRAM has also been associated with variable toxicological risks to aquatic ecosystems. Acute exposure to *Danio rerio* eggs at a concentration of 10 $\mu g.L^{-1}$ has been shown to cause hatching retardation, but with no mortality (Sehonova et al., 2016). Chronic exposure (21 days) of common carp (*Cyprinus carpio*) larvae to TRAM at 10 $\mu g.L^{-1}$ lead to a retardation in total body length development (Sehonova et al., 2016). The authors concluded that the lowest-observed effect concentration (LOEC) of 10 $\mu g.L^{-1}$ can significantly influence development of fish during early ontogeny (Sehonova et al., 2016). However, monitoring studies have shown that this compound is not detected at such high concentrations in environmental surface waters, including the current study. Although TRAM itself does not lead to significant high levels in surface waters to affect aquatic vertebrate development, as shown by Sehonova and colleagues (2016), it should also be pointed out that its primary metabolites are also regularly detected in wastewater effluent and surface waters. It is still unclear though, whether these metabolites may pose an additive toxicological effect with its parent counterpart. However, it is reported that *O*-DMT has higher affinity for opiate receptors than TRAM itself (de Jongh et al., 2012), making this a much more potent EC than the parent compound. The recalcitrance of TRAM and *O*-DMT during wastewater treatment, consequently leading to their pseudo-persistence in discharged effluent, warrants the need for future investigation into the possible mixture interaction of such a chemical with other recalcitrant pollutants with similar physiological modes of action for aquatic wildlife.

Concerning human health risks, a study by de Jongh and colleagues (2012) aimed at establishing a provisional drinking water guideline value (pGLV) for a combined mixture of TRAM and *O*-DMT by considering daily therapeutic doses, acceptable daily intake (ADI) and/or tolerable daily intake (TDI) estimations. The authors concluded that a threshold of 6 $\mu g.L^{-1}$ should be considered for a combined parent/metabolite risk for human health. During the current study, the highest combined TRAM/*O*-DMT concentrations were 1.35 and 3.46 $\mu g.L^{-1}$ in treated wastewater effluent from WWTW1 and WWTW2 respectively, whereas highest concentrations in river water was recorded at 0.76 and 5.3 $\mu g.L^{-1}$ at the respected study sites. This implicates that river water from at least one study site were close to this threshold to

potentially impact human health. As with the opiates, the risk of TRAM has not only been associated with aquatic toxicology, but is also highlighted as a licit DOA. Urban water profiling for TRAM will therefore not only provide further information to show the extent of drug abuse within communities associated with the sewage network, but also for source tracking to show alternative pollution other than wastewater discharge due to its recalcitrance within surface waters.

Toxicity of venlafaxine

Studies showing potential sub-lethal toxicity of VEN in aquatic vertebrates generated much lower effect-concentrations than reported for TRAM. Chronic exposure of VEN to male fathead minnows (*Pimephales promelas*) at a concentration of 305 $ng.L^{-1}$ has been shown to result in 40% mortality of the test organisms (Schultz et al., 2011). Exposure of VEN to rainbow trout (*Oncorhynchus mykiss*) at a concentration as low as 260 $ng.L^{-1}$ led to a significant reduction in the dopamine metabolism within the brain, whereas a concentration of 1 020 $ng.L^{-1}$ significantly modulate brain neuroendocrine pathways (Melnyk-Lamont et al., 2014). In *Danio rerio*, VEN exposure of 500 $ng.L^{-1}$ showed a reduction in plasma estradiol (E_2) concentrations in female fish and a reduction in plasma 11-ketotestosterone concentrations in male fish (Galus et al., 2013b). These results highlight the possibility of VEN to not only modulate neuroendocrine responses in aquatic species at environmentally relevant concentrations, but also pose a risk towards the modulation of reproductive endocrine systems. Such adverse health risks may be extrapolated to higher vertebrates, including humans, subjected to chronic exposure to water resources containing low levels of the drug, as VEN may bio-accumulate within tissues. During the current study, daily detected concentrations for a racemic mixture of venlafaxine within both upstream and downstream water samples ranged between 5.0 to 85.1 $ng.L^{-1}$ at WWTW1, and 56.1 to 187.9 $ng.L^{-1}$ at WWTW2 (Table 5.3). These concentrations are well below the lethal toxicity levels of 305 $ng.L^{-1}$ for *P. promelas*, but still close to reported levels studied by Melnyk-Lamont and colleagues (2014) to modulate the levels of monoamine neurotransmitters in other fish species.

Considering that metabolism of VEN leads to the formation of a pharmacologically active metabolite (DMV), it would be feasible to consider DMV for risk assessment as well. Also, DMV showed a slight enrichment of one enantiomer in its chiral signature in treated wastewater (EF = 0.61 – 0.64 ±0.02) and environmental waters (EF = 0.61 – 0.67 ±0.02) at both study sites, which warrants the need to establish its enantio-specific toxicity on aquatic wildlife and/or

human health. However, such toxicity data are highly limited for most chiral compounds, although such stereo-specific toxicities are known for other anti-depressants such as fluoxetine (Evans et al., 2017). An additional consideration is the potential added mixture effect that VEN and DMV may have on aquatic- and human health, as DMV was shown to be present at higher concentrations in surface waters compared to VEN (Table 5.3). Global monitoring studies have shown a similar result in that DMV are present more than two-fold in surface waters compared to VEN (Evans et al., 2017). During the current study, concentrations of a racemic mixture of DMV ranged between 21.1 to 293.5 $ng.L^{-1}$ at WWTW1, and 159.2 to 664.4 $ng.L^{-1}$ at WWTW2 (Table 5.3). These levels are close to- or within the reported lethal toxicity levels shown for VEN, and also within the range showing neuroendocrine and steroidogenesis modulation in fish. The toxicity of DMV has not been established in a similar manner than for its parent compound in vertebrates. However, given the fact that several pharmaceutical metabolites are primarily responsible for the desired physiological effect, the presence of both pharmaceutical parent compounds and metabolites in surface water systems should be considered for risk assessment approaches (de Jongh et al., 2012). As for TRAM risk assessment for human health, estimated pGLV for a VEN and DMV mixture was calculated at 19 $\mu g.L^{-1}$ (de Jongh et al., 2012). However, this value is much higher than any combined concentrations for either treated effluent- or surface waters at both study sites, which implicates a low risk for human health.

5.4. Conclusions

The current study included a multi-disciplinary approach through environmental micro-pollutant monitoring by investigating 1) the presence and fate of organic micro-pollutants during wastewater treatment, 2) the impact of RAS on the recalcitrance of priority ECs, 3) urban water profiling to show the use of licit- and illicit DOA within communities, and 4) evaluating the lethal- and sub-lethal health risks associated with the detected DTRs. The results demonstrate the recalcitrance of ECs (such as VEN and TRAM), even with the use of advanced AS processes for wastewater treatment. Although effective removal was reported for most of the target analytes, enantiomeric profiling showed stereoselective degradation of some compounds during wastewater treatment. Although the physiological potency of most chiral drugs are shown to be stereo-specific, their associated toxicity risks still need to be evaluated in future studies to establish whether the residual enantiomers pose a significant environmental risk. Also, the results further highlighted the need for the monitoring of metabolic by-products, as removal of parent compounds does not necessarily imply that their potential environmental

risks are completely mitigated. The lack in sufficient risk assessment parameters for pharmaceutical breakdown products should therefore receive more attention in the future.

As the database and knowledge of ECs within surface waters are growing on global scale, it would appear inevitable that the discharge of priority ECs would be incorporated into legislation or at least refined for risk assessment guidelines and/or directives due to increasing information regarding their pronounced risks on wildlife and human health. Although such legislation will be more likely for developed- than developing countries, the influence on more strict export quality standards, for example in agricultural produce, will also greatly impact international trade for developing countries if reclaimed water and digested sludge are used for irrigation and fertilisation purposes.

CHAPTER 6: WASTEWATER-BASED EPIDEMIOLOGY (WBE) AND ENANTIOMERIC PROFILING OF ILLICIT DRUGS OF ABUSE (DOA) IN SOUTH AFRICAN WASTEWATER: APPLICATION IN AN AFRICAN CONTEXT

Article:

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Declaration by the candidate

With regard to chapter 5, the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution
Sampling and sample processing, data processing, manuscript writing	70%

The following co-authors have contributed to chapter 6:

Name	Email and affiliation	Nature of contribution	Extent of contribution
Dr. Erika Castrignanó	e.castrignano@bath.ac.uk Department of Chemistry, University of Bath, United Kingdom	LC-MS method development and technical assistance, manuscript editing	20%
Dr. Barbara Kasprzyk-Hordern	b.kasprzyk-hordern@bath.ac.uk Department of Chemistry, University of Bath, United Kingdom	Manuscript editing	10%
Prof. Gideon M. Wolfaardt	gmw@sun.ac.za Department of Microbiology, Stellenbosch University, South Africa	Manuscript editing	

Declaration with signature in possession of candidate and supervisor

Abstract

The current study is aimed to introduce a wastewater-based epidemiology (WBE) approach for the first time on the African continent where substance abuse data is limited. The study included the quantification of several drugs of abuse (DOA) in raw wastewater samples. Quantification of urinary metabolites as drug target residues (DTR), as well as enantiomeric profiling of chiral DOA were performed to distinguish between consumption and direct disposal into sewage. Monitoring campaigns were undertaken at two South African wastewater treatment works (WWTWs) located within two provinces of the country. The presence of non-racemic 3,4-methylenedioxymethamphetamine (MDMA) and methamphetamine, as well as the metabolite of cocaine, benzoylecgonine (BEG), confirmed their consumption within the areas investigated. Enantiomeric profiling further pointed to the abuse of methamphetamine as the primary DOA with use estimates calculated between 181.9 and 1184.8 $mg.day^{-1}.1000\ inhabitants^{-1}$. Population-normalised mass loads for MDMA and cocaine confirmed their status as secondary DOA within the study sites. Use estimates for the new psychoactive substance (NPS) mephedrone were performed for one WWTW. The minor metabolite of heroin, *O*-6-monoacetylmorphine (*O*-6-MAM), was also detected at one WWTW and served as a qualitative indicator for heroin abuse within the area. These findings provide a novel comparison of the WBE approach in a developing-country with other global studies, with the aim to strengthen this approach as a tool to inform drug prevention strategies in countries where substance abuse data is limited due to financial constraints and lack of government structures to facilitate conventional monitoring.

6.1. Introduction

Drug use and abuse have notable socio-economic consequences that globally impede sustainable development amongst communities (UNODC, 2016). Such drugs of abuse (DOA) are not only limited to illicit substances, but also include prescription and over-the-counter (OTC) medications, which have the potential to cause addiction through their designed physiological mechanisms of action. However, the methods to collate information on drug use are largely limited to substance abuse treatment centres and law enforcement reports, which may lead to inaccurate or underestimations of drug abuse. Wastewater-based epidemiology (WBE) has shown great promise to assist with such constraints by providing a near-real time profile of substance abuse (Castiglioni et al., 2016).

As with any consumed product, DOAs are excreted in sewage either as its parent form, or as primary and secondary metabolites, depending on their metabolic pathways in the body. These substances are then transported through the connected sewage network to wastewater treatment works (WWTWs). Apart from their role in the degradation of a large variety of organic pollutants, WWTWs may thus also serve as composite sampling sites for the chemical profiling of wastewater as a non-intrusive tool to estimate drug use and abuse within the communities connected to the sewer system (Castiglioni et al., 2014; Daughton, 2001). However, several discrepancies to this approach have been discussed (Castiglioni et al., 2016), which include the fate of the parent drug in wastewater, as well as the distinction between drug consumption and direct disposal into the recipient waters (Kasprzyk-Hordern and Baker, 2012a). For this reason, the inclusion of metabolic breakdown products as drug target residues (DTR) were proposed to address this limitation by serving as more stable DTRs for consumption estimates, as well as confirming whether the drug has undergone metabolic breakdown due to consumption (Petrie et al., 2016b).

Apart from the benefits of establishing DOA metabolite loads in wastewater, the enantiomeric profiling of chiral DOA may also be used to distinguish between direct disposal, consumption, and manufacturing (Camacho-muñoz et al., 2016; Emke et al., 2014; Kasprzyk-Hordern and Baker, 2012a, 2012b; Petrie et al., 2016b). Some DOA, such as 3,4-methylenedioxymethamphetamine (MDMA) and mephedrone are manufactured in their racemic form, from which the enantiomers will follow different metabolic pathways and excretion patterns within the body, which leads to a non-racemic mixture in sewage (Castrignanò et al., 2017a/2017b; Kasprzyk-Hordern and Baker, 2012a). Therefore, if the

enantiomeric composition of the drug is racemic in the wastewater sample, it might indicate direct disposal of the drug rather than its consumption (Emke et al., 2014). In contrast, the manufacturing of some chiral illicit drugs, such as methamphetamine, is primarily enantioselective (Castrignanò et al., 2017b; Xu et al., 2017), as the potency and desired physiological effects differ between the chiral isoforms. For methamphetamine, the *S*-enantiomer is the predominant form to represent an illicit origin, which has also been confirmed during WBE (Castrignanò et al., 2017b; Xu et al., 2017). However, it has been reported that both enantiomers may also be associated with illicit methamphetamine use, depending on the method of synthesis and trafficking. For example, a racemic mixture of methamphetamine was detected in Norwegian wastewater samples in contrast to other European countries where wastewater was predominantly enriched with the S-enantiomer (Castrignanò et al., 2017b). The authors highlighted that this occurrence was due to the known differences in manufacturing and trafficking of the drug between countries. Establishing the enantiomeric signature of chiral DOA in wastewater therefore provide an added value to WBE for improved drug enforcement strategies, substance abuse estimates, and information to social services.

WBE has been applied in many countries to date (Castiglioni et al., 2014; Devault et al., 2017; Emke et al., 2014; Evans et al., 2016; Lai et al., 2017; Ort et al., 2014; Petrie et al., 2016b; Subedi and Kannan, 2014; Xu et al., 2017). Ironically, the value of implementing such an overarching approach to monitor drug abuse in African countries are lacking, where it may arguably provide an effective means to fill a void left by a chronic shortages in funding and human capacity. Given the current state of substance abuse within developing countries, as well as limited drug use statistics, assessment tools such as WBE are needed to assist with future drug use prevention strategies. Recent reports have highlighted an increase in the abuse of illicit drugs in South Africa (Dada et al., 2017; USDS, 2017), with these substances shown to be present in wastewater (Archer et al., 2017b). The aim of the current study was therefore (i) to monitor the loads of common illicit drugs (cocaine, methamphetamine, MDMA and heroin), as well as the new psychoactive substance (NPS) mephedrone at two South African WWTWs in order to estimate the drug use patterns within communities serviced by the sewage systems, (ii) to lay a foundation for local drug monitoring programmes and (iii) to adopt this approach that should facilitate a common ‘language’ with nascent programmes in developed countries.

6.2. Materials and Methods

6.2.1. Sampling locations

Two WWTWs were selected for a consecutive 7-day sampling campaign during 2017 (Fig. 6.1). WWTW1 is Gauteng Province serving one city in the East Rand district adjacent to the city of Johannesburg, and WWTW2 located in the Western Cape Province of South Africa serving several suburbs around the city of Cape Town (Fig. 6.1). The information for the plants are shown in the supplementary information (APPENDIX, Table D1). The current *de facto* population estimate (PE) for the WWTWs were estimated from population growth projections since the last national census campaign in 2011, which resulted in a PE of 200 000 for WWTW1 and 470 000 for WWTW2.

Figure 6.1: Map showing the sampling locations of WWTW 1 (Gauteng Province) and WWTW 2 (Western Cape Province).

6.2.2. Chemicals and consumables

The study included the multi-residue quantification for 16 DTRs (cocaine, benzoylecgonine, cocaethylene, (±)-amphetamine, (±)-methamphetamine, (±)-mephedrone, (±)-ephedrine, (±)-pseudoephedrine, norephedrine, (±)-MDMA, (±)-HMMA, (±)-HMA, heroin, *O*-6-monoacetylmorphine, morphine and normorphine) using analytical methods described elsewhere (Castrignanò et al., 2016), which is summarised in the supplementary information (Fig. S1). The following internal standards were included in the water samples to assist with

quantification: cocaine-d3, benzoylecgonine-d8, cocaethylene-d3, amphetamine-d5, methamphetamine-d5, mephedrone-d3, MDMA-d5, heroin-d9, *1S,2R*-(+)-ephedrine-d3, morphine-d6 and PCP-d5. Methanol (MeOH, HPLC-grade; Sigma) and ultra-pure water (Millipore) were used for cleaning glassware and for solid phase extraction (SPE). All glassware were deactivated using 5% dimethyldichlorosilane (DMDCS) in toluene, followed by two wash steps in toluene, and three wash steps in MeOH. Acetonitrile, DMDCS, MeOH and ammonium acetate were all purchased from Sigma-Aldrich.

6.2.3. Sample collection and preparation

Raw wastewater samples from the two study sites were taken over a period of seven consecutive days during the month of March 2017. Briefly, composite samples (100 ml every 10 mins) were taken over a 24 hour period (9am to 9am) using a time-and-volume-proportional composite sampler (Aquacell, Aquamatic Ltd, UK) at the raw sewage inlet after the grit screens. The samples were kept cold during sampling and transportation to the laboratory, from which sample filtration and extraction were completed upon arrival. Duplicate raw wastewater samples (50 mL each) from each sampling locality and day were filtered using 0.7 µm glass microfiber filters (grade GF/F; Whatman®, Sigma-Aldrich) using a vacuum manifold, whereby all aqueous samples were extracted using Oasis HLB cartridges (3cc, 60 mg). The cartridges were conditioned with 2 ml MeOH, followed by 2 mL of ultrapure water. The samples were then allowed to pass through the cartridges at a rate of 6 $mL.min^{-1}$, washed with 3 mL ultrapure water, and allowed to run dry for at least 30 minutes. The dried cartridges were frozen and then transported on ice to the University of Bath (UK) for elution and analysis. Upon arrival, the cartridges were eluted with 4 mL MeOH into 5 mL silanized glass tubes and dried under a gentle stream of nitrogen (5-10 psi, 40^0C) using a TurboVap evaporator (Caliper, UK). A mixture of the ISs were added to each dried sample and reconstituted in 0.5 mL of the mobile phase used for chiral LC-MS/MS analysis (1 mM ammonium acetate:methanol, 85:15, v/v) to give a 100x concentrated sample containing a final concentration of $1 \mu g.L^{-1}$ IS mix per 50 mL water sample. The suspended samples were filtered through 0.2 µm PTFE filters (Whatman®, Puradisc, 13 mm) and placed in polypropylene plastic vials bonded with a pre-slit PTFE/Silicone septa (Waters, UK).

6.2.4. Chiral liquid chromatography coupled with tandem mass spectrometry

A chiral method for the detection of a list of chiral and achiral DOA was used as developed by Castrignanò et al. (2016). Briefly, the analytes in the processed samples were separated using a

Waters ACQUITY UPLC® system (Waters, Manchester, UK) equipped with a CHIRALPAK® CBH HPLC column (5 µm particle size, L × I.D. 10 cm × 2.0 mm; Chiral Technologies, France) and a Chiral-CBH guard column (10 × 2.0 mm, 5 µm particle size; Chiral Technologies, France). The ACQUITY UPLC™ autosampler was kept at 4^0C, and the column temperature was set at 25^0C. All samples were injected at 20 µL. The flow rate of the mobile phase was set at 0.1 ml.min^{-1} under isocratic conditions. The separated analytes were identified and quantified using a triple quadrupole mass spectrometer (Xevo TQD, Waters, Manchester, UK), equipped with an electrospray ionisation (ESI) source, which was managed in the multiple reaction monitoring (MRM) mode. Quality controls (QC) spiked with standards and deuterated compounds were added throughout the run of the batch for ensuring good instrumental performance. The quality and quantification of the analytes in the measured samples followed the criteria set by the European Commission Council Directive 2002/657/EC (European Commission, 2002).

6.2.5. Daily loads and drug use estimate calculations

In order to normalise for the variation in daily wastewater flow rates of the plants, the daily mass loads of the target analytes (g.day^{-1}) at each WWTW were calculated by multiplying the measured concentration of the DTR from LC-MS/MS analysis (in ng.L^{-1}) in the wastewater samples with the flow volume (L.day^{-1}) of wastewater entering the plant over 24 hrs. Population-normalised drug loads (mg.day^{-1}.1000 inhabitants^{-1}) of the selected target analytes were then calculated using equation 1:

$$\text{Population-normalised drug loads (mg.day}^{-1}\text{.1000 inhabitants}^{-1}\text{)} = \frac{Load_{inf}\,(\text{g.day}^{-1}) * CF * 1\text{x}10^6}{Pop.} \qquad \text{Eq. 1}$$

where $Load_{inf}$ refers to the calculated mass loads (g.day^{-1}) of the DTRs, *CF* refers to the correction factor calculated by dividing the most recent excretion rates of each DTR by the molar mass ratios between the parent drug and DTR (Table S2), and *Pop.* refers to the population estimate (*de facto* number of individuals) for each WWTW (Table S1).

In order to distinguish between the illicit or pharmaceutical origin of the chiral drugs, or to assist with distinction between direct disposal and consumption of the DTRs within the study

areas, the enantiomeric fraction (EF) of the selected chiral compounds in wastewater were calculated using equation 2:

$$\text{Enantiomeric Fraction (EF)} = \frac{(+)}{[(+) + (-)]} \quad or \quad \frac{E1}{[E1 + E2]} \qquad \text{Eq. 2}$$

where (+) and (-) are referred to the concentrations ($ng.L^{-1}$) of the enantiomers in wastewater influent or E1 and E2 as the mass load concentrations ($g.day^{-1}$) of the first- and second eluted enantiomer respectively. An EF equal to 1 or 0 represents an enantiomerically pure substance, whereas an EF equal to 0.5 represents a racemic mixture of the drug.

6.3. Results and Discussion

The concentrations (ng.L^{-1}) of the DOA that were quantified in this study can be found in the supplementary information (Table S3 and S4), from which the calculations for mass loads (g.day^{-1}; Table S5 and S6) and population-normalised mass loads (mg.day^{-1}.1000 inhabitants^{-1}; Table 1) were done.

6.3.1. Cocaine

The mass loads calculated for cocaine in the aqueous phase of raw wastewater showed a slight increase during the weekend period (Saturday and Sunday) for WWTW2, but not WWTW1 (Fig. 6.2; APPENDIX, Table D5 & D6). However, both the metabolites of cocaine, benzoylecgonine (BEG) and cocaethylene (CE) showed a significant increase in their loads within raw wastewater during the weekend period for both WWTWs (ANOVA, $P < 0.05$; Fig. 6.2; APPENDIX, Table D5 & D6). This can be attributed to the known recreational use of this drug. BEG loads ranged from 5.59 to 17.26 g.day^{-1} at WWTW1 (Fig. 6.2; APPENDIX, Table D5), and between 33.59 to 76.98 g.day^{-1} at WWTW2 (Fig. 6.2; APPENDIX, Table D6), whereas cocaine loads ranged between 2.12 and 7.23 g.day^{-1} at WWTW1 (Fig. 6.2; APPENDIX, Table D5), and between 8.84 to 39.97 g.day^{-1} at WWTW2 (Fig. 6.2; APPENDIX, Table D6). The observed higher mass loads for BEG compared to cocaine in raw wastewater samples therefore assumes its consumption rather than direct disposal into wastewater. For WWTW1, the cocaine/BEG ratio varied between 0.2 and 0.6 (median 0.3), and for WWTW2 varied between 0.3 and 0.5 (median 0.4), which is below the suggested cut-off ratio of 0.75 that is indicative of human consumption (Van Nuijs et al., 2009). However, these ratios were higher than suggested in a more recent study to assume *in vivo* cocaine metabolism, which was set at 0.1 or lower (Castiglioni et al., 2011). This may then suggest some direct disposal of the drug or possibly other factors affecting these ratios, such as the route of administration of street drugs containing cocaine and co-administration with other substances.

The detection of CE in wastewater highlights a few considerations which should be addressed when WBE is applied for cocaine consumption estimates. This metabolite is formed when cocaine is co-administered with alcohol, which is shown to lead towards a decrease in hepatic metabolism of the parent drug (Parker and Laizure, 2010). It has been shown that the percentage of excretion products during co-administration of cocaine and alcohol over a 24 hour period was 4.6% for cocaine, 21.1% for BEG and 0.7% for CE (De La Torre et al., 1991). Although

only eight subjects for the study were used, these excretion values are much different from case studies where cocaine was administered alone (Khan and Nicell, 2011). Although cocaine is shown to be less stable in wastewater than BEG (Castiglioni et al., 2016), the increased levels of the parent drug can therefore not be considered to be a sole result of direct disposal, but may also be as a result of co-administration with alcohol. Co-administration of cocaine with alcohol may thus supress hepatic metabolism to BEG and instead cause increased excretion of the parent drug. This implies that re-adjustment of the correction factors should be considered in future studies that will include refinement of parent/metabolite ratios in order to compensate for simultaneous alcohol intake by users.

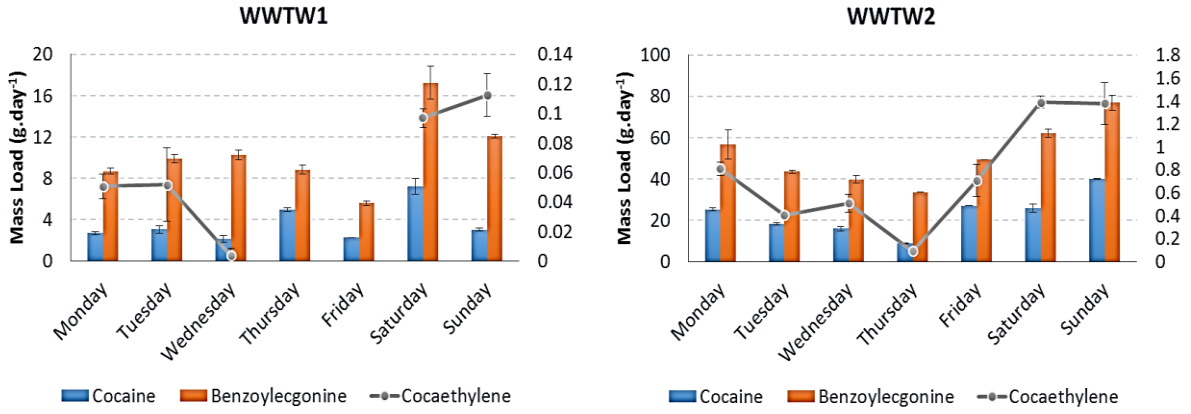

Figure 6.2: Daily mass loads (g.day^{-1}) estimated for cocaine, BEG (1º y-axis) and cocaethylene (2º y-axis) within raw wastewater entering WWTW1 and WWTW2.

Average population-normalised mass loads for cocaine (using BEG) for WWTW1 was estimated at 155.8 mg.day^{-1}.1000 inhabitants^{-1} during weekdays (Monday to Friday), and 263.8 mg.day^{-1}.1000 inhabitants^{-1} over the weekend (Saturday and Sunday) (Table 6.1). For WWTW2, mean consumption of cocaine during the week was estimated at 342.0 mg.day^{-1}.1000 inhabitants^{-1} and 533.0 mg.day^{-1}.1000 inhabitants^{-1} over the weekend (Table 6.1). Although it is reported that cocaine is considered as a secondary DOA in South Africa (Dada et al., 2017), the use estimates fell within the range of cocaine consumption estimated at several European cities during a 2016 monitoring campaign (Table 6.1), but were lower than estimates in South American studies and some European countries such as Belgium and England (EMCDDA, 2016; APPENDIX, Table D7).

6.3.2. MDMA

The loads of MDMA in South African wastewater have not been reported previously. As with cocaine, this drug is normally shown to be used for recreational purposes and is reported to be a secondary DOA in the country according to substance abuse treatment data (Dada et al., 2017). During the current sampling campaign, mass loads of ±-MDMA ranged between 0.10 to 0.23 g.day^{-1} at WWTW1 (Fig. 6.3; APPENDIX, Table D5), and between 0.94 to 6.52 g.day^{-1} at WWTW2 (Fig. 6.3; APPENDIX, Table D6). This estimate is low compared to similar studies done for European cities (Baker et al., 2014; EMCDDA, 2016), which highlights its status as a secondary DOA in the country. Regardless of the low mass loads detected in the current study, a significant increase in MDMA loads in raw wastewater for both WWTWs on the Sunday was observed compared to the rest of the sampling days (ANOVA, $P < 0.05$; Fig. 6.3; APPENDIX, Table D5 & D6), confirming its recreational use. The consumption of MDMA was further confirmed by the presence of the metabolite 4-hydroxy-3-methoxymethamphetamine (HMMA) within raw wastewater at both WWTWs, with ±-HMMA loads varying from 0.25 to 0.42 g.day^{-1} at WWTW1 (APPENDIX, Table D5), and 0.72 to 2.49 g.day^{-1} at WWTW2 (APPENDIX, Table D6). Another metabolite of MDMA, namely 4-hydroxy-3-methoxyamphetamine (HMA), was not detected at any of the sampling sites during the current study. The use of HMMA as DTR has also been recently proposed (Castrignanò et al., 2017b), as it is indicative of consumed MDMA rather than possible direct disposal into wastewater.

Both MDMA and HMMA are chiral, with conventional methods for clandestine manufacturing producing racemic MDMA (Castiglioni et al., 2016). Therefore, a racemic mixture of MDMA in a sample indicates direct disposal (EF = 0.5), where an EF lower than 0.5 will indicate its consumption due to the drug's stereoselective metabolism in the body (Emke et al., 2014; Petrie et al., 2016b). In the current study, average EF values for MDMA in raw wastewater samples were 0.36 (± 0.09) for WWTW1, and 0.33 (± 0.05) for WWTW2, which indicates a clear enrichment of *R*-(-)-MDMA at both WWTWs. This finding conforms to similar studies showing an enrichment of *R*-(-)-MDMA in wastewater (Castrignanò et al., 2016; Emke et al., 2014; Kasprzyk-Hordern et al., 2010). Other potential factors leading towards a shift in EFs also need further investigation, such as biological processes in wastewater (in-sewer degradation) which may favour the degradation of *S*-(+)-MDMA, leading to a further enrichment of *R*-(-)-MDMA in the water sample (Evans et al., 2016, 2015). Nevertheless, enantiomeric profiling of both MDMA and HMMA has been shown to further confirm consumption rather than disposal. It has been proposed that if MDMA is found to be enriched

with *R*-(-)-MDMA (as with the current study), an enrichment of *S*-(+)-HMMA will further verify consumption of the drug (Castrignanò et al., 2017b). A similar trend was found for WWTW2, as the enantiomeric signature of HMMA showed an average EF value above 0.5, indicating an enrichment of *S*-(+)-HMMA (APPENDIX, Table D6). A similar trend was not detected in WWTW1, which may be attributed to lower loads of parent MDMA in the raw wastewater samples (Fig. 6.3; APPENDIX, Table D5) and may therefore merely reflect a lower use of the drug within the communities.

Population-normalised MDMA load estimates ranged between 2.2 and 4.9 mg.day^{-1}.1000 inhabitants^{-1} at WWTW1 (Table 6.1), and 9.0 to 61.6 mg.day^{-1}.1000 inhabitants^{-1} at WWTW2 (Table 6.1). The highest consumption estimate for WWTW1 (5.0 mg.day^{-1}.1000 inh^{-1}, Table 6.1) was similar to monitoring campaigns in Italy, Spain and Finland, whereas the highest consumption estimate for WWTW2 (61.6 mg.day^{-1}.1000 inhabitants^{-1}, Table 1) was similar to monitoring campaigns in the United Kingdom and Switzerland (EMCDDA, 2016). Recent statistics on drug use and abuse in South Africa show that although MDMA abuse are reported, the true extent of its abuse in the country is largely unknown (Dada et al., 2017). Again, most statistics are limited to individuals being treated for drug use, and therefore does not reflect on the total number of drug users in an area, especially for such a recreational substance. The current study demonstrated the presence of both MDMA and its metabolite in wastewater, thus expanding the scope of investigation beyond individuals that received treatment.

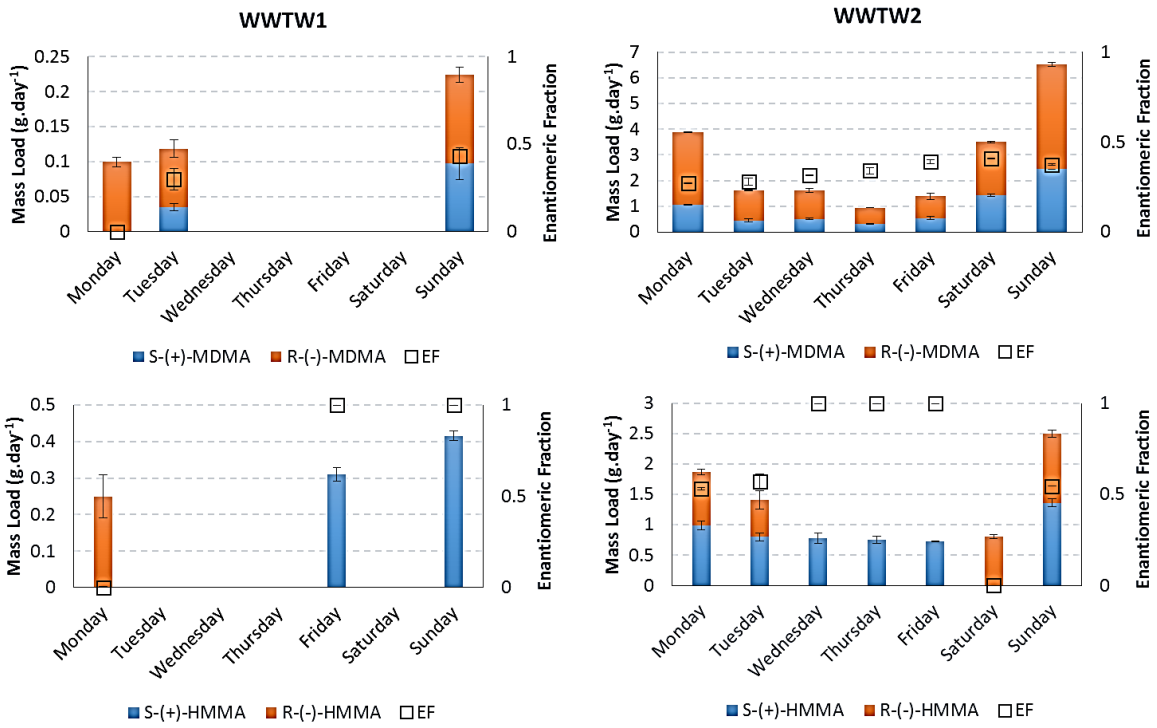

Figure 6.3: Daily mass loads (g.day^{-1}) estimated for MDMA and HMMA within raw wastewater entering WWTW1 and WWTW2. EF = Enantiomeric fraction.

6.3.3. Amphetamine and methamphetamine

Mass loads of methamphetamine calculated is the current study were the highest when compared to other detected illicit drugs (APPENDIX, Table D5 & D6), highlighting its use as a primary abused substance in the study areas. The chiral signature for methamphetamine showed, almost exclusively, the presence of *S*-(+)-methamphetamine at both WWTWs (EF 0.8 – 1.0; Fig. 6.4), which is similar to monitoring studies in Europe (Castrignanò et al., 2017b; Evans et al., 2016) and China (Xu et al., 2017). At WWTW1, mass loads varied between 2.90 to 6.30 g.day^{-1} for *R*-(-)-methamphetamine, and 12.92 to 40.01 g.day^{-1} for *S*-(+)-methamphetamine (APPENDIX, Table D5). At WWTW2, mass loads were higher for both enantiomers, which varied between 7.77 to 11.94 g.day^{-1} for *R*-(-)-methamphetamine, and 130.17 to 229.07 g.day^{-1} for *S*-(+)-methamphetamine (APPENDIX, Table D6). The mass loads for the racemic methamphetamine mixture also increased significantly during the weekend period at WWTW2 (ANOVA, $P < 0.05$; Fig. 4), which is not surprizing, as this plant is located in an area where adolescent and recreational use of the drug is high (Asante and Lentoor, 2017; Pluddemann et al., 2010).

Clandestine manufacturing of illicit methamphetamine usually aims at the synthesis of the *S*-enantiomer due to its more desired physiological effect over the *R*-enantiomer (Xu et al., 2017). The chiral signature for methamphetamine during the current study therefore suggests an illicit origin rather than a resultant breakdown from licit pharmaceuticals, given the history of high methamphetamine abuse in the country (Arfer et al., 2017; Asante and Lentoor, 2017; Dada et al., 2017; Pluddemann et al., 2010). Although *R*-(-)-methamphetamine loads were lower than *S*-(+)-methamphetamine in raw wastewater for both WWTWs, the loads were not negligible and could possibly have been derived from licit pharmaceuticals, for example Parkinson's disease treatments containing selegiline. However, a lack of national prescription data limits the possible profiling to distinguish between the licit- or illicit origin of the *R*-enantiomer. In contrast, no enantiomeric study has been conducted in the country to verify the chiral signature and purity of 'street' methamphetamine, which are largely synthesised using ephedrine precursors and may yield variable enantiomerically pure drugs between various clandestine laboratories and/or imported drugs. For this reason, the racemic loads of methamphetamine were still deemed feasible to be used for consumption estimates. The population-normalised methamphetamine loads ranged from 181.9 to 532.5 $mg.day^{-1}.1000\ inhabitants^{-1}$ at WWTW1, and 675.0 to 1184.8 $mg.day^{-1}.1000\ inhabitants^{-1}$ at WWTW2 (Table 6.1). These estimates correlate well with reports in other countries where high methamphetamine use is reported, such as Slovakia and the Czech Republic (EMCDDA, 2016), China (Xu et al., 2017), Australia (Thai et al., 2016) and New Zealand (Lai et al., 2017) and highlights the need for further profiling to more accurately estimate its consumption and manufacturing within the country.

For amphetamine, it has been highlighted that the presence of the drug in wastewater cannot solely be ascribed to abuse, nor can it be pinpointed to originate from a single source. Amphetamine may originate from several prescription medications, which may either include the *S*- and/or *R*-enantiomer or a mixture thereof. In South Africa, amphetamine-type stimulants (ATS) are registered to be used in several prescription medications, including attention-deficit hyperactivity disorder (ADHD) medications, non-steroidal anti-inflammatory drugs (NSAIDs), Parkinson's disease treatment, and appetite suppressants. However, prescription data in the country is not available, along with few reports on the abuse of ATS medications. In the current study, amphetamine was detected exclusively as *S*-(+)-amphetamine in raw sewage (EF = 1), which ranged between 0.71 to 1.04 $g.day^{-1}$ at WWTW1 (Fig. 6.4; APPENDIX, Table D5), and 4.17 to 9.15 $g.day^{-1}$ at WWTW2 (Fig. 6.4; APPENDIX, Table D6). This finding differs from monitoring studies where amphetamine in raw wastewater was rather detected to be enriched

with *R-(-)*-amphetamine, which was concluded to have originated from the use of amphetamine itself rather than as a breakdown product form other compounds (Kasprzyk-Hordern and Baker, 2012a, 2012b). In contrast, other studies have also detected an enrichment of *S*-(+)-amphetamine in raw sewage (Evans et al., 2016; Xu et al., 2017). Several factors led to the conclusion that the presence of *S*-(+)-amphetamine in the wastewater samples analysed during the current study were derived from the reduction of methamphetamine rather than amphetamine use. First, the use and abuse of amphetamine itself may be low in the country due to access to other more easily-available illicit substances. Second, given the more rapid metabolism of *S*-(+)-amphetamine over *R*-(-)-amphetamine during human metabolism and in wastewater (Kasprzyk-Hordern and Baker, 2012a), it would be assumed that raw wastewater samples would have at least contained traces of *R*-(-)-amphetamine, or mostly be enriched with this more stable enantiomer. Third, the breakdown of *S*-(+)-methamphetamine will exclusively lead to the formation of *S*-(+)-amphetamine (Bagnall et al., 2013). The higher load of *S*-(+)-methamphetamine in wastewater (EF = 0.9 ±0.1) at both study sites may therefore lead to a higher fraction of breakdown of the *S*-enantiomer than the *R*-enantiomer. The low levels of *R*-(-)-methamphetamine in wastewater then simply leads to a lower fraction of *R*-(-)-amphetamine, which was below detection.

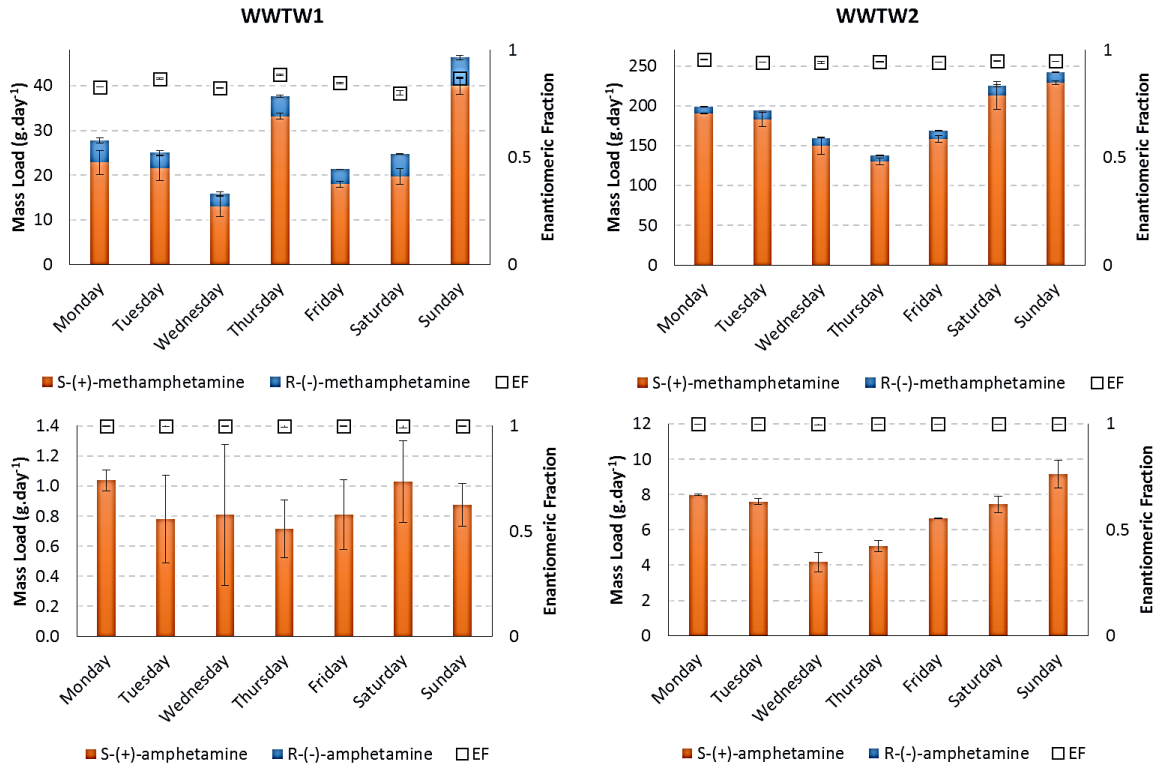

Figure 6.4: Daily mass loads (g.day^{-1}) methamphetamine and *S*-(+)-amphetamine in raw wastewater entering WWTW1 and WWTW2. EF = Enantiomeric fraction.

Due to the probable origin of *S*-(+)-amphetamine coming from *S*-(+)-methamphetamine degradation during the current study, the distinction between methamphetamine consumption and disposal were assessed by considering the amphetamine and methamphetamine load ratios of the *S*-enantiomers (AMP/METH). Estimated AMP/METH ratios in urine samples of abusers show considerable variation ranging from 0.025 to 0.208, depending on the post-dose time of sampling and the amount of the consumed dose (Valentine et al., 1995; Xu et al., 2017). The AMP/METH ratios for influent wastewater during the current study were estimated at 0.041 (± 0.02) for WWTW1, and 0.038 (± 0.005) for WWTW2, which are also within the range that was reported in several Chinese wastewater influents (0.017-0.076) where the *S*-enantiomer for both analytes was predominantly or exclusively detected (Xu et al., 2017). Dumping rather than consumption of methamphetamine would be sporadic and therefore yield spikes in methamphetamine between sampling days, along with a clear shift in AMP/METH ratios compared to the other sampling days. For WWTW2, no such trends were observed during the study. However, the clear spike in methamphetamine loads on the Thursday and Sunday samples at WWTW1 (Fig. 4) also resulted in AMP/METH ratios of 0.02 compared to an average AMP/METH ratio of 0.05 (± 0.01) if these sampling days were excluded. This also conforms to a similar finding by Xu et al. (2017), where an AMP/METH ratio of 0.017 was considered as a dumping event due to this value which deviated significantly from other wastewater samples. The two sampling days during the current study thus suggest possible discharge of methamphetamine during manufacturing operations or attempted seizures rather than consumption, but further suggest an overall consumption of the drug rather than disposal at the two sampling sites.

6.3.4. Drug precursors

The loads of ephedrine, pseudoephedrine and the metabolite norephedrine were investigated due to their possible association with methamphetamine use and manufacturing. The production of methamphetamine from phenyl-2-propanone (P2P) are less common in Africa, where the conventional use of ephedrine precursors are reported to be common practice (USDS, 2017). Due to ephedrine having two chiral centres, four isomers can be established. Both *1R,2S*-(-)-ephedrine and *1S,2S*-(+)-pseudoephedrine are known to be used in clandestine manufacturing of methamphetamine using the Nagai method, which exclusively generates *S*-(+)-methamphetamine (Xu et al., 2017). However, as several OTC medications also contain ephedrine diastereomers, along with unknown prescription data for the country, the use of

ephedrine for clandestine manufacturing could not be distinguished from its medicinal use. This constraint may be addressed by screening for additional compounds used during clandestine manufacturing in future sampling campaigns. The chiral LC-MS method used for the current study did not allow for the distinction between *1R,2S*-(-)-ephedrine and *1R,2R*-(-)-pseudoephedrine, and hence is reported as a combined load. Mass loads of *1R,2S*-(-)-ephedrine/*1R,2R*-(-)-pseudoephedrine were calculated to range from 41.52 to 95.18 g.day^{-1} at WWTW1 (APPENDIX, Table D5), and 22.47 to 32.74 g.day^{-1} at WWTW2 (APPENDIX, Table D6), whereas *1S,2S*-(+)-pseudoephedrine loads ranged from 40.61 to 68.78 g.day^{-1} at WWTW1 (APPENDIX, Table D5), and 35.29 to 60.32 g.day^{-1} at WWTW2 (APPENDIX, Table D6). From these estimations, the loads of *1S,2S*-(+)-pseudoephedrine are similar for both sites, whereas loads for *1R,2S*-(-)-ephedrine/*1R,2R*-(-)-pseudoephedrine were higher at WWTW1 than WWTW2. Norephedrine is proposed to form through several sources, such as demethylation of ephedrine and/or pseudoephedrine, but also from methamphetamine and other OTC medications. During the current study, mass loads of E1/E2-norephedrine were calculated to range between 5.89 and 8.34 g.day^{-1} at WWTW1 (APPENDIX, Table D5), and between 2.03 to 6.37 g.day^{-1} at WWTW2 (APPENDIX, Table D6). Average EF values were 0.39 (± 0.02) for WWTW1, and 0.33 (± 0.07) for WWTW2. The higher load in norephedrine at WWTW1 than WWTW2 shows a similar trend as for ephedrine loads at the two WWTWs, suggesting that the source of norephedrine is most likely from ephedrine/pseudoephedrine metabolism rather than from other sources.

Although methamphetamine abuse is shown to predominate in the region where WWTW2 is situated (Dada et al., 2017), manufacturing is presumed to be higher in the province where WWTW1 is situated (USDS, 2017). The mass load estimates for ephedrine partly support this hypothesis (APPENDIX, Table D5 and D6). The combined average loads of ephedrines were 114.5 (± 27.4) g.day^{-1} at WWTW1, and 78.5 (± 11.8) g.day^{-1} at WWTW2, whereas the methamphetamine loads were significantly higher at WWTW2 than WWTW1. Interestingly, a clear spike in *1R,2S*-(-)-ephedrine/*1R,2R*-(-)-pseudoephedrine loads was detected for the Thursday samples at WWTW1 (APPENDIX, Table D5), which correlates to the clear spike shown for *S*-(+)-methamphetamine loads on the same day as well (Fig. 6.4; APPENDIX, Table D5). Additionally, a clear spike in hydro-chemical parameters (COD, PO_4, NH_3, suspended solids and conductivity), as well as a spike in mass loads of caffeine were also measured for the Thursday samples at WWTW1 (data not shown). This may suggest an alternative discharge

event during this day or even an alternative source of sewage effluent being discharged into the WWTW inlet as previously recorded for this WWTW.

6.3.5. Mephedrone

The NPS drug mephedrone was only detected in raw wastewater for WWTW2, with only two days of quantitative data on the Friday and Sunday sampling days (APPENDIX, Table D6). Mass loads of ±-mephedrone during these two sampling days ranged between 3.80 and 5.58 $g.day^{-1}$ for Friday and Sunday respectively (APPENDIX, Table D6). No clear trend in weekday/weekend use could be seen for this drug as shown for the recreational drugs cocaine and MDMA, suggesting low use of this drug within the study area. As for MDMA, mephedrone is also generally manufactured as a racemic mixture (EMCDDA, 2011). Therefore, a non-racemic mixture in wastewater will further point to its consumption. Enantiomeric fractions for both sampling days revealed a racemic mixture (EF = 0.52 ±0.01), which contradicts studies showing an enrichment of *R*-(+)-mephedrone in wastewater (Castrignanò et al., 2017a, 2016). Although mephedrone is listed as an illegal substance in South Africa, its abuse is also poorly reported. Mephedrone was also detected in South Africa, in a WWTW monitoring campaign during 2015. No chiral signature of mephedrone was determined then (Archer et al., 2017a). These findings indicate its possible use in the country, although lower compared to other DOA. Population-normalised mephedrone loads were estimated to range between 7.6 to 10.4 $mg.day^{-1}.1000\ inhabitants^{-1}$ (Table 6.1). This estimate is still close to a UK study where population-normalised mass loads were shown to range between 7.6 and 26.3 $mg.day^{-1}.1000\ inhabitants^{-1}$ during a 2014 sampling campaign, but lower than estimates during the following year, ranging from 14.9 to 47.7 $mg.day^{-1}.1000\ inhabitants^{-1}$ (Castrignanò et al., 2017a). The current results suggest low use of this drug in the study area, but still confirm its presence in wastewater. For this reason, only semi-quantitative consumption estimates were suggested for the present study. More sensitive analytical methods will be required to verify actual mephedrone use in the country.

6.3.6. Heroin

Heroin is considered the most commonly-abused opioid in South Africa, with its abuse has increased more than 10-fold between 1997 and 2011 in the province where WWTW2 is situated (Weich et al., 2017), where it is often used in combination with other drugs such as low-grade cannabis and cocaine (Weich et al., 2008). As a consequence, heroin is typically smoked rather than conventional intravenous route of administration - also reported in

Colombia (Bijlsma et al., 2016). Despite its popularity as a primary DOA in South Africa, heroin was not detected at any of the WWTWs during the current study (APPENDIX, Table D5 and D6), which has been largely attributed to its low recovery and stability during sample preparation (Baker and Kasprzyk-Hordern, 2011), as well as its low excretion and rapid metabolism in the body after consumption (Castiglioni et al., 2016). WBE thus offer an accurate estimation of heroin consumption, as its metabolism in the body predominantly lead to the formation of morphine, which then undergo further metabolism into its conjugated form and nor-morphine. During the current study, morphine was detected in raw wastewater, ranging from 12.56 to 20.93 g.day^{-1} at WWTW1 (APPENDIX, Table D5), and 54.03 to 769.13 g.day^{-1} at WWTW2 (APPENDIX, Table D6), whereas nor-morphine loads ranged from 4.62 to 7.89 g.day^{-1} at WWTW1 (APPENDIX, Table D5), and 10.71 to 14.60 g.day^{-1} at WWTW2 (APPENDIX, Table D6). Both morphine and nor-morphine mass loads were similar throughout the sampling period at WWTW1 (APPENDIX, Table D5), whereas morphine loads at WWTW2 were much higher during the week than for the weekend period (APPENDIX, Table D6). Apart from the detection of morphine and its metabolite within the wastewater samples, a minor metabolite of heroin, *O*-6-monoacetylmorphine (*O*-6-MAM), was also detected at WWTW2, with loads ranging from 2.09 to 5.54 g.day^{-1} (APPENDIX, Table D6). The detection of this metabolite confirms the use of heroin within the region, as this metabolite is formed exclusively from heroin consumption. However, population-normalised mass loads for heroin consumption could not be calculated, as *O*-6-MAM is a minor, relatively unstable metabolite, which can lead to underestimation (Castiglioni et al., 2016).

Table 6.1: Drug use estimates (mg.day^{-1}.1000 inhabitants^{-1}; ±stdev) of selected illicit drugs at WWTW1 and WWTW2 based on the detected loads of parent- and metabolite compounds in raw wastewater.

WWTW1								
Drug	DTR	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Cocaine	BEG	156.4 ± 5.1	178.4 ± 77.5	184.9 ± 7.9	158.9 ± 8.0	100.6 ± 3.5	310.7 ± 28.7	216.9 ± 3.2
MDMA	MDMA	2.2 ± 0.2	2.6 ± 0.2	-	-	-	-	4.9 ± 0.7
	HMMA	6.2 ± 1.5	-	-	-	7.7 ± 0.5	-	10.4 ± 0.3
Methamphetamine	Methamphetamine	319.4 ± 38.1	287.5 ± 37.7	181.9 ± 31.3	433.1 ± 10.9	245.2 ± 8.9	284.7 ± 21.5	532.5 ± 28.6
Mephedrone	Mephedrone	-	-	-	-	-	-	-
WWTW2								
Drug	DTR	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Cocaine	BEG	435.6 ± 54.3	333.1 ± 6.7	305.1 ± 13.4	257.3 ± 0.6	378.7 ± 0.1	476.3 ± 15.9	589.6 ± 27.5
MDMA	MDMA	36.7 ± 0.04	15.2 ± 0.6	15.3 ± 1.1	9.0 ± 0.1	13.1 ± 1.7	33.1 ± 0.7	61.6 ± 0.7
	HMMA	19.9 ± 1.2	15.0 ± 2.3	8.3 ± 0.9	7.9 ± 0.6	7.7 ± 0.1	8.5 ± 0.3	26.6 ± 1.4
Methamphetamine	Methamphetamine	975.0 ± 5.4	948.4 ± 45.4	781.5 ± 52.9	675.0 ± 20.5	825.6 ± 22.6	1100.3 ± 93.4	1184.8 ± 13.5
Mephedrone	Mephedrone	1.6 ± 0.6	4.3 ± 0.7	1.7 ± 0.1	-	7.6 ± 0.3	2.6 ± 0.2	10.4 ± 0.9
EMCDDA-SCORE 2016*								
Cocaine	**113.8** (*Munich, GER*), **138.4** (*Oslo, NOR*), **169.6** (*Paris, FRA*), **390.4** (*Bristol, UK*), **409.6** (*Antwerp, BEL*), **484.7** (*Geneva, SWI*), **699.1** (*Barcelona, SPA*)							
Methamphetamine	**58.3** (*Oslo, NOR*), **83.4** (*Helsinki, FIN*), **89.5** (*Espoo, FIN*), **136.7** (*Dresden, GER*), **261.9** (*Budweis, CR*), **310.2** (*Piestany, SLO*), **671.8** (*Bratislava, SLO*)							
MDMA	**2.5** (*Athens, GRE*), **4.5** (*Milan, ITA*), **10.8** (*Porto, POR*), **17.2** (*Paris, FRA*), **34.2** (*Helsinki, FIN*), **51.2** (*Bristol, UK*), **59.3** (*Zurich, SWI*)							

* Examples of daily means of population-normalised mass loads estimated for several European cities as reported by the EMCDDA-SCORE initiative for 2016. A more detailed list can be found in the supplementary information (Table S7).

6.4. Conclusions

Methamphetamine was confirmed to be the primary DOA detected within the raw sewage, with enantiomeric profiling (EF > 0.5) confirming an enrichment of *S*-(+)-methamphetamine and suggesting an illicit origin of the drug. Similarly, quantification of the loads and enantiomeric profile of MDMA, and subsequent enantiomeric profiling of the metabolite HMMA point to consumption over direct disposal of the drug in the study areas. Cocaine use estimates was comparable to some use estimates in European countries. Although BEG was predominantly detected in both WWTWs, the cocaine/BEG ratios suggest possible direct discharge of the drug in wastewater. Mephedrone was also detected at one WWTW where substance abuse is of a growing concern.

Finally, the results highlight the under-estimation of drug use when substance abuse treatment data is exclusively considered, as this data only represent a fraction of people admitting to their drug abuse. Further refinement of PEs for WBE should consider the demographic age of active substance abusers, as conventional PE may not be a true reflection of inhabitants contributing to substance abuse within a region. Also, more distinction need to be placed between the *de facto* and *de novo* population contributing waste to the WWTW, as the use of recreational drugs, for example cocaine, MDMA and mephedrone, may not reflect on the actual population connected to the sewer system. This will include the advances of using chemical markers (e.g. cotinine, caffeine) for population estimates. However, more studies are needed to refine statistical data on caffeine and tobacco use within South African communities in order to use such markers to generate sufficient estimations. We encourage the use of WBE to serve as a supplement to drug use statistics in a country where substance abuse data is limited, with the aim to extend this approach to other African countries.

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CHAPTER 7: GENERAL CONCLUSIONS

It is evident that a daunting task lies ahead concerning the quality of surface waters on a global scale. This is emphasized by the increased knowledge regarding possible health effects of a growing list of emerging contaminants (ECs) being detected in surface waters. Most anthropogenic substances are perceived needed to sustain human development, support industry and enhance agricultural output. However, it is vital to regulate their exposure to surface waters in order to decrease their negative impacts on the aquatic environment. This will consequently lead to innovative approaches to manage potential environmental health risks, which inevitably reflects back to community resilience.

At the forefront of the need to limit human impact on the environment shared by wildlife lies the sufficient treatment of wastewater and the need for increased restoration of surface water resources. From the scoping studies presented in this dissertation, it must be emphasised that prioritisation of micro-pollutants will be dependent on the specific problem that need to be addressed, being for general water quality legislation, antimicrobial resistance (AMR) development, or to report on the occurrence of long-term adverse health outcomes associated with endocrine disruption. Although current legislation does not mandate the regulation of ECs, this should change in the near future. Furthermore, it became clear that the understanding of both biotic- and abiotic degradation of emerging micro-pollutants during wastewater treatment processes need further scrutiny, especially in view of incomplete degradation of compounds in WWTPs that are indeed biodegradable in microcosm studies. Furthermore, the impact of additional pollution sources on surface water quality, other than WWTW discharge, need more investigation. This will include more refined sampling and analytical approaches to identify such ‘non-WWTW pollution hotspots’. This is especially important for developing countries undergoing rapid urbanisation, industrialization and agricultural growth, where current infrastructure does not facilitate increased water provision, sanitation or recycling services.

The databases compiled in *Chapter 2* summarized the current knowledge concerning a vast quantity of ECs (known- or suggested to occur) in South African surface waters, along with a reflection of their potential negative impacts on aquatic ecosystems and human health. The literature review showed that several compounds regularly detected in surface waters may indeed be capable of causing various adverse health effects, considering the potential mixture interactions of these substances in the contaminated waters. More refined research should

therefore focus on prioritising these contaminants on a weight-of-evidence and outcome-specific approach, by considering their persistence in surface waters, as well as their lethal- and/or sub-lethal health effects. The development and implementation of a routine first-tier risk assessment approach is therefore needed for a developing country where data are limited, which therefore require the use of sensitive- and cost-effective bioassays to address specific molecular- and/or key-event outcomes leading to adverse health effects.

The use of the *in vitro* estrogen receptor binding assays (YES/YAES) in *Chapter 3* screened for pollutants in surface waters which interfere with steroid hormone receptor binding (estrogenic and anti-estrogenic activities). The results highlighted the challenges faced when multiple risk-based outcomes are addressed, as well as the need for continuous risk assessment among study sites. In particular, the impact of climatic conditions, such as rainfall, as well as other seasonal and diurnal changes in both biotic- and abiotic conditions warrants further investigation, which are highly site-specific. Even though the WWTWs that were used for the present case studies showed compliance with current South African water treatment standards, additional parameters, including the fate of EDCs and other ECs within treatment discharges and recipient surface waters need further scrutiny for their inclusion in water quality legislation. The presence of anti-estrogenic activity in treated wastewater effluent also warrants further investigation, as the anthropogenic origin of compounds which exert this endocrine-disrupting response and their fate during wastewater treatment are less known. Also, the high microbial metabolic activities associated with wastewater treatment processes, as well as final disinfection steps, such as chlorination and UV treatment, may potentiate the loads of anti-estrogenicity in treated wastewater, which further impacts other endocrine system outcomes through toxic masking. However, the YES bioassay screened for the collective endocrine-disrupting effect in water bodies without the need to establish the micro-pollutant chemistry of the sample. As an effect, net-estrogenicity in the water samples (calculated in EEQs) which were recorded above effect-based trigger values points to focus areas where further risk assessment should be conducted. In particular, estrogenicity in river waters not necessarily associated with WWTW discharge were shown surpass these trigger values, showing the need to monitor further upstream as well. This increased health risk reported for environmental waters further highlighted the need to include analytical chemistry to identify potential EDCs and persistent substances. In particular, establishing the presence and fate of metabolic by-products during wastewater treatment need further attention, as their eco-toxicological impact are less known compared to their parent counterparts.

The monitoring studies reported on in *Chapters 4 and 5* included the detection and quantification of various pharmaceuticals and personal care products (PPCPs) as well as drugs of abuse (DOA) in WWTWs utilising activated sludge treatment processes. In agreement with literature, these studies revealed a range of low- to high removal rates of various pollutants, but also confirmed negative mass balances of some compounds (higher loads in treated effluent than raw influent). From the list of PPCPs selected for the scoping study reported in *Chapter 4*, the compounds which stood out for both their persistence and potential adverse health risks were the antibiotics azithromycin, clarithromycin and sulfamethoxazole, the non-steroidal anti-inflammatory drugs, diclofenac and naproxen, the antidepressant, venlafaxine and its primary metabolite, the anti-epileptic drug, carbamazepine and its metabolites, as well as the opioid drug, tramadol and its metabolites. The persistence of these highlighted antibiotics raises concern regarding their potentiating effect on multi-drug AMR development. The other classes of pharmaceuticals were further shown to potentially modulate endocrine system pathways through various molecular- and cellular pathways. The association of compounds such as venlafaxine and tramadol within solid particulate matter during activated sludge treatment processes were further highlighted in *Chapter 5* to contribute to their recalcitrance in WWTWs. Longer retention time associated with the matured activated sludge (RAS) process did not necessarily lead to improved removal of these compounds and their metabolites. Although these compounds were degraded to some extent, the loads of their metabolic by-products in treated wastewater effluent also need routine monitoring, as the toxicological mixture interactions between parent drugs and metabolites are still elusive. Also, the novelty in establishing enantio-selective degradation profiles of chiral pharmaceuticals and illicit substances for risk assessment were outlined. Although it is known that various chiral pharmaceuticals have variable potencies on a pharmacological level, their stereo-selective toxicities on the aquatic environment and human health are hardly investigated. Future considerations will therefore be to enrich the database of enantio-specific toxicities of chiral drugs which are shown to persist during wastewater treatment in order to improve on the value of chiral profiling for risk assessment purposes.

The study during *Chapter 6* introduced wastewater-based epidemiology (WBE) for the first time in an African country. This approach showed promise to serve as an additional approach for substance abuse estimates in a country with limited drug use statistics. Detections and quantification of the illicit substances benzoylecgonine (for cocaine), MDMA,

methamphetamine, O-6-MAM (for heroin) and mephedrone in raw wastewater samples highlighted the extent of their abuse in the study areas. In particular, use estimates for methamphetamine were calculated to be high, even compared to other countries worldwide. The detection of the metabolic breakdown products and the enantiomeric profiling of the chiral illicit drugs was further shown to refine such consumption estimates. The results presented in this chapter emphasised the need to extend such an approach to other African countries by also addressing the estimation of licit pharmaceutical abuse. However, the study was limited to inadequate prescription data of licit DOA such as codeine, morphine and tramadol to draw adequate conclusions whether these substances are indeed misused. Future refinement may also include to establish the age demographics of particular abused substances in study areas in order to lower underestimations in drug use estimates. The inclusion of communities which are not directly connected to sewer systems also need attention, as these sources may indirectly contribute towards the sewage load at WWTWs trough septic tank sewage disposal. The latter is of particular importance in developing countries where substance abuse is of an ongoing concern in informal- and peri-urban settlements.

Another consideration for future sampling strategies include the consideration of an extended sampling period and a fractionated sampling approach as mentioned by others (Baalbaki et al., 2016; Ort et al., 2010). As highlighted by Ort and colleagues (2010), several experimental and analytical uncertainties arise during wastewater sampling. Such uncertainties are attributed to several variable factors which may influence micro-pollutant distributions within sewage systems, including, but not limited to, diurnal variations in the flow parameters of influent- and effluent wastewater, the source distribution of wastewater (domestic/industrial), amount of ‘flushing events’, and residence time distributions (RTD) of treatment units (Baalbaki et al., 2016; Ort et al., 2010). Taking these considerations into account may further refine the investigation on the fate of emerging contaminants during wastewater treatment, which further include the analysis of analyte concentrations within solid particulate matter (SPM) of receiving wastewater and especially accounting for RTDs of treatment units for more accurate removal estimates. The results reported in *Chapter 5* further highlighted the influence of return activated sludge (RAS) which may contribute towards elongated retention of pollutants during the activated sludge maturation process, and therefore may also impact removal estimates. During the course of the experimental chapters for the current dissertation, a series of sampling methodologies were used, ranging from single grab sampling of river water, to high interval, time-proportional composite samples for wastewater influent and effluent. It is indeed

acknowledged that removal estimates from the various chapters would have benefitted by considering SPM concentrations and the influence of wastewater treatment RTDs, and should therefore receive priority for future sampling campaigns.

Scientific significance of the thesis

With freshwater resources being a valuable- and scarce commodity, the impact of pollution raise several socio-economic concerns, such as affecting human health and increasing environmental risk by limiting access to reliable and safe water resources. The use of urban water profiling for risk assessment and drug-use estimation addresses several global socio-economic challenges, as outlined by the United Nations Sustainable Development Goals (SDG) for 2030 (http://www.un.org), in particular, the target set out by SDG-12 (Responsible Consumption and Production):

"By 2020, a*chieve the environmentally sound management of chemicals and all wastes throughout their life cycle, in accordance with agreed international frameworks, and significantly reduce their release to air, water and soil in order to minimize their adverse impacts on human health and the environment*" – United Nations Sustainable Development Goals for 2030

Other targets set out by SDG-3 (Good Health and Well-being) and SDG-6 (Clean Water and Sanitation) can therefore be considered as the foundation which largely contribute towards environmental- and community resilience. Risk management and WBE approaches should be further refined to address other key global challenges, such as the development of antimicrobial resistance (AMR), which are of an emerging global concern.

From a local perspective, the present study showed alignment with the South African Government National Development Plan (NDP) for 2030 by addressing specific targets, such as (1) 'conducting research on critical issues affecting long-term development', and (2) addressing 'interventions to ensure environmental sustainability and resilience to future shocks'. International research has highlighted the possible risk of *in utero* exposure to contaminants, therefore placing emphasis on current issues associated with water pollutants that translate into possible health consequences over generations. Especially in a country such as South Africa, where rapid population growth and urbanisation leads to an increased demand for potable water, numerous communities still rely on environmental surface waters as a

drinking water source. Advancing the knowledge and education on the possible health consequences associated with freshwater pollutants will contribute to enhanced community resilience. A further alignment with the NDP include the issue to 'reduce crime by strengthening criminal justice and improving community environments', whereby WBE may provide an innovative approach to estimate substance abuse and their manufacturing in countries where statistics are largely limited. The study further showed alignment with the Department of Science and Technology's (DST) Ten-Year Innovation Plan, especially Grand Challenge #4 regarding water scarcity. Although the extent of water pollution is evident in South African surface waters, the risks associated with these emerging contaminants need to be clearly defined, especially in view of chronic water scarcity that leads to increased reliance on water reuse. This requires improved monitoring and risk assessment approaches, which will support wastewater management, advance treatment and reuse initiatives, and ultimately lead to improved access to clean and safe water and sanitation services as set out by the DST Grand Challenge #5.

In summary, the research described here (1) refined approaches and considerations to use cost-effective bioassays for risk assessment, (2) identified emerging micro-pollutants and their associated by-products that should receive priority based on their associated risk, and (3) highlighted other socio-economic challenges such as substance abuse. These case studies may prove valuable for decision-making in water quality management by identifying their associated risk to cause adverse human- and environmental health consequences. By investigating the fate of these priority micro-pollutants in conventional WWTWs may provide a valuable database for the industry on the decision-making to improve current wastewater treatment infrastructure. This is of particular importance to provide safe reusable water supplies to communities and industry – relieving the pressure for access to a commodity which is already almost 100% allocated, with no surplus to accommodate the projected rates of population growth and urbanization.

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APPENDIX A: SUPPLEMENTARY INFORMATION FOR CHAPTER 3

Seasonal and daily variations in estrogenic and anti-estrogenic activity at wastewater treatment works and receiving waters: Implication for reliable monitoring

Table A1: E_2-equivalent concentrations (EEQs; ng.L^{-1} ±stdev) measured within the various water matrices from the WWTWs and environmental waters during summer (December 2015) and winter (June 2016).

Site	Summer					Winter				
	Influent	Effluent	Removal (%)	Upstream	Downstream	Influent	Effluent	Removal (%)	Upstream	Downstream
WWTW1	16.8 ± 4.1****	0.4 ± 0.2*	98	11.8 ± 2.3****	6.5 ± 1.2****	30.1 ± 4.9****	0.5 ± 0.01*	99	6.3 ± 0.2****	1.9 ± 0.01**
WWTW2	34.1 ± 0.4****	0.3 ± 0.1	99	0.2 ± 0.1	0.8 ± 0.4**	23.5 ± 2.1****	0.9 ± 0.3**	96	0.2 ± 0.03	0.2 ± 0.01
WWTW3	14.9 ± 3.4****	0.4 ± 0.1*	97	1.3 ± 0.7**	0.8 ± 0.3**	31.5 ± 2.8****	0.5 ± 0.01	98	-	0.6 ± 0.01*
WWTW4	11.8 ± 1.3****	-	-	1.5 ± 0.5**	0.3 ± 0.1	13.5 ± 0.1****	-	-	0.8 ± 0.06**	-
WWTW5	4.4 ± 1.1***	0.7 ± 0.4**	84	-	0.4 ± 0.2*	29.4 ± 5.4****	1.4 ± 0.03**	95	-	0.6 ± 0.1*
WWTW6	5.3 ± 2.2****	0.9 ± 0.3**	84	-	1.3 ± 0.8*	7.0 ± 1.6****	1.4 ± 0.04**	81	0.3 ± 0.02	1.6 ± 0.1**
WWTW7	29.4 ± 6.3****	0.3 ± 0.1	99	2.5 ± 0.9***	0.5 ± 0.3*	11.4 ± 2.0****	0.2 ± 0.01	98	-	-
WWTW8	8.8 ± 2.7****	2.6 ± 1.3***	70	1.2 ± 0.5**	0.5 ± 0.1*	6.6 ± 0.7****	4.9 ± 0.2***	26	-	5.0 ± 0.1****
WWTW9	7.8 ± 2.5****	6.9 ± 0.3****	13	7.5 ± 0.2****	9.5 ± 0.7****	15.1 ± 0.9****	1.3 ± 0.03**	91	18.9 ± 0.2****	11.3 ± 1.9****
WWTW10	22.8 ± 4.9****	1.1 ± 0.04**	95	0.6 ± 0.3*	0.4 ± 0.1*	15.3 ± 3.8****	0.9 ± 0.03**	94	1.2 ± 0.01**	0.6 ± 0.06*

* EEQ trigger value (0.4 ng.L^{-1}) of effluent estrogenicity on long-term fish exposure (Jarošová et al., 2014a).

** Estimated trigger value (0.7 ng.L^{-1}) for E_2 in humans (Genthe et al., 2013)

*** Predicted no-effect concentration (PNEC, 2 ng.L^{-1}) for E_2 to modulate fish reproduction (Caldwell et al., 2012)

**** Concentration of E_2 (5 ng.L^{-1}) showing increased VTG production in adult male zebrafish (Brion et al., 2004)

Additional remarks on river water

Estimated EEQs for upstream river samples ranged from 0.2 – 11.8 ng.L^{-1} during summer 2015/16, and 0.2 – 18.9 ng.L^{-1} during winter 2016, whereas estimated EEQs for downstream river samples ranged from 0.3 – 6.5 ng.L^{-1} and 0.2 – 11.3 ng.L^{-1} during summer 2015/16 and winter 2016 respectively (Table S1). Four upstream water sample locations were significantly higher than their associated downstream locations during both summer 2015/16 and winter 2016 (ANOVA, $P < 0.05$; Table S1). Furthermore, the average EEQ value for the water samples collected upstream from WWTW9 during the winter sampling was measured at 18.9 ng.L^{-1}, which was at an even higher concentration than estimated for the raw influent of the plant (Table S1).

Table A2: Daily variation in E_2-equivalent concentrations (EEQs; ng.L^{-1}) using the yeast estrogen screen (YES) for the various WWTWs December 2016 (summer).

Day	WWTW2			WWTW8			WWTW9			WWTW10		
	Influent	Effluent	Removal	Influent	Effluent	Removal	Influent	Effluent	Removal	Influent	Effluent	Removal
Monday	15.7 ± 1.6****	1.1 ± 0.3**	93	14.3 ± 2.3****	1.3 ± 0.6**	91	28.9 ± 4.0****	0.9 ±0.4**	97	21.0 ± 2.6****	1.5 ± 0.4**	95
Tuesday	13.9 ± 4.3****	2.6 ± 0.3***	81	6.9 ± 0.9****	1.5 ± 0.3**	78	27.9 ± 3.3****	1.7 ±0.2**	94	45.4 ± 3.0****	0.5 ± 0.3*	99
Wednesday	13.5 ± 0.9****	1.6 ± 0.4**	88	8.2 ± 1.1****	1.6 ± 0.3**	80	27.7 ± 2.8****	1.9 ±0.4**	93	22.3 ± 3.2****	1.1 ± 0.2**	95
Thursday	-	1.2 ± 0.3**	-	9.2 ± 2.6****	3.0 ± 0.9***	67	23.8 ± 1.2****	1.2 ±0.3**	94	22.1 ± 3.9****	1.3 ± 0.1**	99
Friday	10.8 ± 2.2****	0.7 ± 0.1**	94	12.7 ± 0.8****	1.0 ± 0.2**	92	21.7 ± 3.3****	1.5 ±0.01*	92	21.2 ± 4.8****	-	-
Saturday	14.3 ± 1.5****	1.4 ± 0.2**	90	13.0 ± 3.3****	1.5 ± 0.4**	88	25.4 ± 2.5****	0.6 ±0.2*	98	21.2 ± 6.4****	1.8 ± 0.2**	99
Sunday	10.6 ± 3.6****	0.8 ± 0.3**	93	15.1 ± 3.4****	1.5 ± 0.8**	90	24.7 ± 11.9****	0.4 ±0.1*	98	24.4 ± 2.5****	2.1 ± 0.7***	99
Average	**13.1 ± 2.0******	**1.3 ± 0.6****	**90**	**11.3 ± 3.2******	**1.6 ±0.6****	**84**	**26.8 ± 3.5******	**0.7 ±0.6***	**97**	**25.4 ±8.9******	**1.4 ± 0.4****	**98**

* EEQ trigger value (0.4 ng.L^{-1}) of effluent estrogenicity on long-term fish exposure (Jarošová et al., 2014)

** Estimated trigger value (0.7 ng.L^{-1}) for E_2 in humans (Genthe et al., 2013)

*** Predicted no-effect concentration (PNEC, 2 ng.L^{-1}) for E_2 to modulate fish reproduction (Caldwell et al., 2012)

**** Concentration of E_2 (5 ng.L^{-1}) showing increased VTG production in adult male zebrafish (Brion et al., 2004)

Table A3: Mass loads (g.day^{-1}) estimated using EEQ concentrations in the YES for both raw- and effluent wastewater samples at the various WWTWs during the December 2016 sampling campaign.

Day	WWTW2			WWTW8			WWTW9			WWTW10		
	Influent	Effluent	Removal (%)	Influent	Effluent	Removal (%)	Influent	Effluent	Removal (%)	Influent	Effluent	Removal (%)
Monday	0.72 ± 0.07	0.05 ± 0.03	93	1.64 ± 0.26	0.14 ± 0.07	92	0.58 ± 0.08	0.02 ± 0.01	97	8.81 ± 1.08	0.58 ± 0.28	93
Tuesday	0.77 ± 0.24	0.14 ± 0.02	82	0.92 ± 0.11	0.19 ± 0.04	79	0.52 ± 0.08	0.03 ± 0.004	94	9.38 ± 1.10	0.17 ± 0.05	98
Wednesday	0.71 ± 0.05	0.08 ± 0.02	89	1.10 ± 0.15	0.20 ± 0.03	82	0.49 ± 0.05	0.03 ± 0.01	94	8.32 ± 1.18	0.39 ± 0.06	95
Thursday	-	0.06 ± 0.02	-	1.07 ± 0.31	0.32 ± 0.10	70	0.42 ± 0.02	0.02 ± 0.005	96	6.84 ± 1.21	0.37 ± 0.15	95
Friday	0.68 ± 0.09	0.04 ± 0.01	94	1.40 ± 0.09	0.10 ± 0.02	93	0.37 ± 0.06	0.02 ± 0.001	94	4.58 ± 1.01	-	-
Saturday	0.59 ± 0.05	0.05 ± 0.01	91	1.49 ± 0.38	0.16 ± 0.04	89	0.50 ± 0.05	0.01 ± 0.003	98	5.96 ± 1.80	0.48 ± 0.06	92
Sunday	0.49 ± 0.17	0.04 ± 0.01	93	1.70 ± 0.38	0.16 ± 0.08	91	0.62 ± 0.14	0.01 ± 0.002	99	7.62 ± 0.77	0.62 ± 0.21	92

Table A4: Hydrochemical parameters and flow rates measured for selected WWTWs during the December 2016 (summer) sampling campaign.

WWTW #	Capacity	December 2015				June 2016				December 2016				Pop. Eq. COD	Pop. Eq. NH3
		COD (mg/L)	NH_3 (mg/L)	FR_{raw} (ML/day)	$FR_{effluent}$ (ML/day)	COD (mg/L)	NH_3 (mg/L)	FR_{raw} (ML/day)	$FR_{effluent}$ (ML/day)	COD (mg/L)	NH_3 (mg/L)	FR_{raw} (ML/day)	$FR_{effluent}$ (ML/day)		
WWTW1	105.0	1092.1	39.2	92.6	86.7	619.7	39.2	83.4	78.1	583.7	39.2	87.7	82.1	528942	499144
WWTW2	55.0	797.6	29.7	37.1	35.2	559.0	17.4	59.4	56.3	726.7	13.1	50.8	48.3	260448	135241
WWTW3	0.4	693.9	41.8	1.1	1.0	348.1	36.5	1.2	1.1	-	-	-	-	4516	6297
WWTW4	16.0	340.4	16.7	10.0	9.3	287.3	27.9	8.8	8.1	-	-	-	-	23273	30265
WWTW5	35.0	455.7	17.6	66.1	44.8	605.3	18.6	66.8	-	-	-	-	-	275929	174313
WWTW6	32.0	995.0	5.9	33.3	33.3	819.9	22.0	31.8	31.9	-	-	-	-	229674	65284
WWTW7	36.0	973.4	25.8	13.1	12.9	738.7	20.4	8.3	8.6	-	-	-	-	73574	37392
WWTW8	83.0	480.6	31.2	100.2	92.6	334.4	24.9	94.3	87.2	398.0	20.5	119.6	110.6	330473	380454
WWTW9	10.0	324.9	18.4	14.3	10.0	187.4	16.5	19.3	18.0	290.7	22.4	18.8	16.3	35397	48160
WWTW10	155.0	714.7	30.5	158.2	146.3	583.6	23.3	187.4	176.9	627.6	20.4	325.1	300.7	1092297	763990

Table A5: Hydrochemical parameters and flow rates measured for selected WWTWs during the December 2016 (summer) sampling campaign.

WWTW #	Hydrochemical parameters	Tuesday 06/12/2016	Wednesday 07/12/2016	Thursday 08/12/2016	Friday 09/12/2016	Saturday 10/12/2016	Sunday 11/12/2016	Monday 12/12/2016
WWTW2	COD (mg/L)	711.0	786.0	621.0	890.0	529.0	634.0	916.0
	NH_3 (mg/L)	6.4	15.0	13.6	14.7	16.2	13.2	12.6
	$Flow_{raw}$ (ML/day)	55.5	53.0	54.1	59.9	41.6	45.6	46.0
	$Flow_{effluent}$ (ML/day)	52.8	50.4	51.4	56.9	39.5	43.3	43.7
WWTW8	COD (mg/L)	323.0	450.0	969.0	187.0	276.0	231.0	350.0
	NH_3 (mg/L)	15.6	15.5	17.8	21.8	24.6	26.7	21.3
	$Flow_{raw}$ (ML/day)	133.4	133.6	116.9	110.4	114.8	112.6	115.2
	$Flow_{effluent}$ (ML/day)	123.4	123.6	108.2	102.1	106.2	104.1	106.9
WWTW9	COD (mg/L)	282.0	361.0	485.0	303.0	178.0	269.0	157.0
	NH_3 (mg/L)	28.5	17.1	17.2	17.2	21.1	31.5	24.1
	$Flow_{raw}$ (ML/day)	19.9	17.7	17.5	17.1	19.8	19.2	20.2
	$Flow_{effluent}$ (ML/day)	17.3	15.4	15.2	14.9	17.3	16.8	17.5
WWTW10	COD (mg/L)	576.0	362.0	1121.0	718.0	674.0	461.0	481.0
	NH_3 (mg/L)	24.3	13.3	17.3	20.0	25.1	21.4	21.2
	$Flow_{raw}$ (ML/day)	370.1	373.1	308.7	210.6	281.9	311.7	419.6
	$Flow_{effluent}$ (ML/day)	342.3	345.1	285.6	194.8	260.8	288.3	388.1

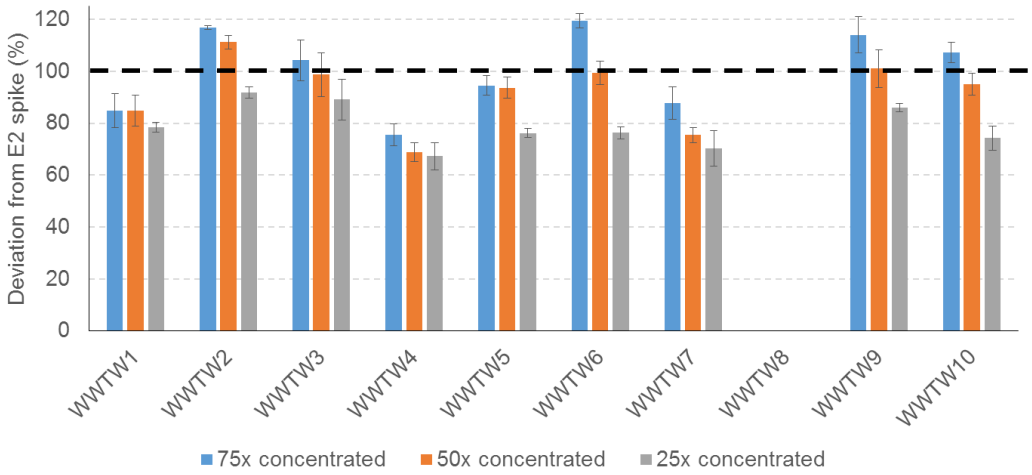

Figure A1: Deviation (%) from the submaximal E_2 spike in the YAES for treated wastewater effluent samples from the various WWTWs during the winter 2016 sampling campaign.

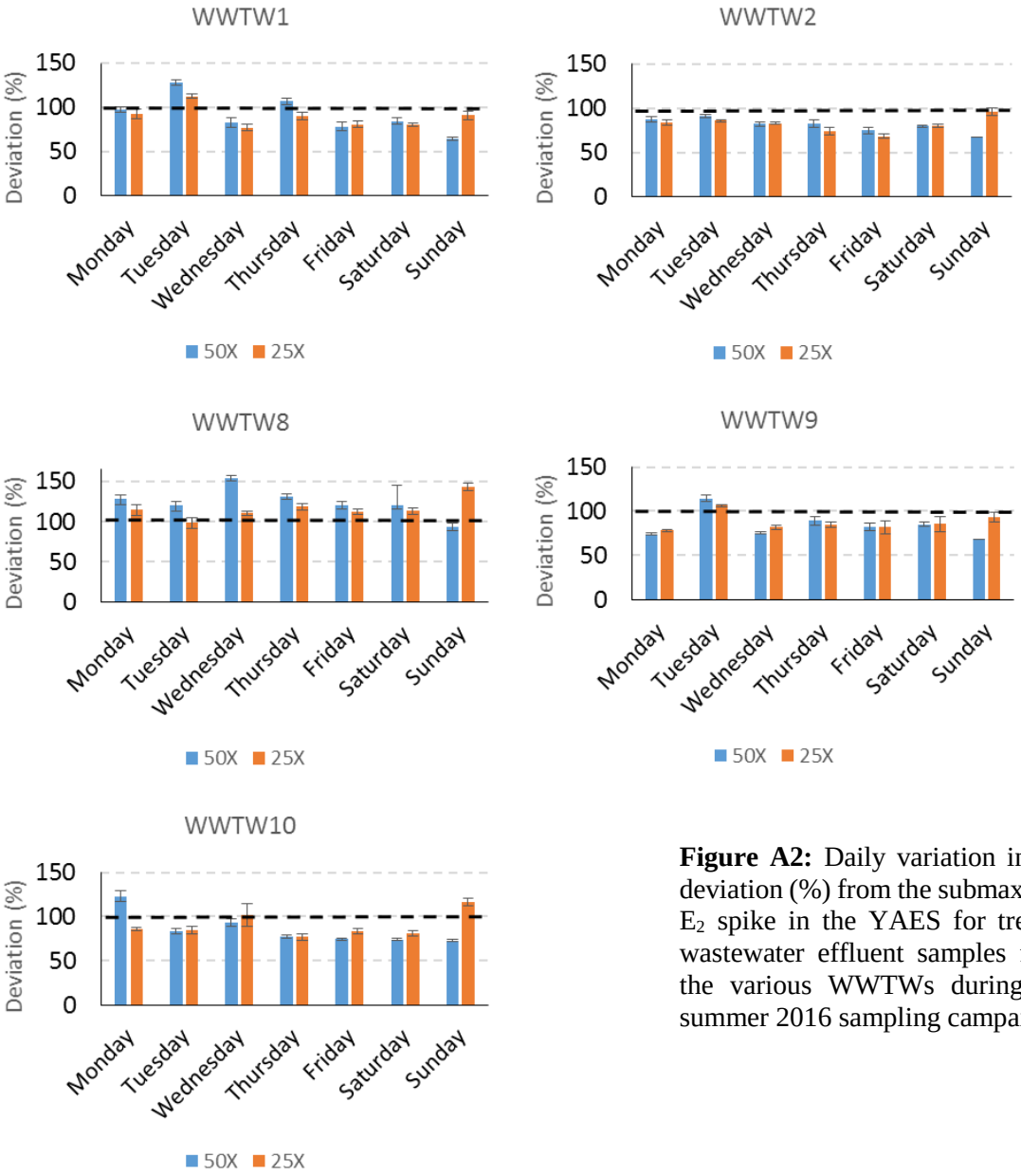

Figure A2: Daily variation in the deviation (%) from the submaximal E_2 spike in the YAES for treated wastewater effluent samples from the various WWTWs during the summer 2016 sampling campaign.

Table A6: Tamoxifen-equivalent concentrations (TAM-EQ; µg.L^{-1}) estimated for treated effluent water samples from the various WWTWs during the winter 2016 sampling campaign.

Site	TAM-EQ (ug.L^{-1})
WWTW1	22.5 ± 4.6
WWTW2	6.5 ± 1.2
WWTW3	3.7 ± 3.6
WWTW4	78.7 ± 17.9
WWTW5	28.0 ± 4.6
WWTW6	5.6 ± 1.2
WWTW7	12.0 ± 9.2
WWTW8	-
WWTW9	10.7 ± 1.5
WWTW10	36.9 ± 14.8

Table A7: Daily variation in tamoxifen-equivalent concentrations (TAM-EQ; µg.L^{-1}) estimated for treated effluent water samples from selected WWTWs during the summer 2016 sampling campaign.

Sampling day	WWTW1	WWTW2	WWTW8	WWTW9	WWTW10
Monday	-	1.4 ± 0.6	-	3.0 ± 0.3	1.3 ± 0.3
Tuesday	-	1.0 ± 0.2	-	-	2.2 ± 1.3
Wednesday	3.8 ± 2.2	1.3 ± 0.2	-	2.2 ± 0.7	-
Thursday	-	4.8 ± 2.6	-	1.6 ± 0.7	5.4 ± 2.0
Friday	2.3 ± 1.3	10.0 ± 3.0	-	2.9 ± 0.7	2.2 ± 0.7
Saturday	2.2 ± 0.6	1.9 ± 0.4	-	2.0 ± 0.6	2.4 ± 0.7
Sunday	0.7 ± 0.3	-	-	-	-
Average	**2.3 ± 1.3**	**3.4 ± 3.5**	**-**	**2.3 ± 0.6**	**2.7 ± 1.6**

Additional remarks on anti-estrogenicity

High estrogenicity was shown for WWTW8 during the same sampling period where the YAES showed cytotoxicity within the assay (Fig. 3). On the other hand, estrogenicity at WWTW4 was below detection in the YES, but showed the highest anti-estrogenicity from all WWTW samples in the YAES (Fig. 3). During the summer 2016/17 sampling campaign, WWTW2, 9 and 10 showed an observable lower concentration of EEQs during certain sampling days, whereas TAM-EQ concentrations were shown to be higher, and *vice versa* (Fig. 4). However, not all the results showed such a trend. For example, similar EEQ values were recorded for WWTW5 and WWTW6 during the winter 2016 sampling campaign, but TAM-EQ concentrations differed significantly between these two sampling sites (t-test, $p < 0.05$; Fig. 4). These two plants have similar treatment capacity, but different treatment processes (Table 1), with WWTW5 primarily receiving domestic sewage, and WWTW6 receiving wastewater from both domestic sewage and industry (including mining wastewater). Although WWTW5 and WWTW6 showed the same level of estrogenicity in treated effluent samples, a dose-dependent deviation above 100% of the E_2 spike was not observed for WWTW5 in the YAES, as was the

case for WWTW6, which may be explained by the higher concentrations of anti-estrogenicity calculated for WWTW5 compared to WWTW6, resulting in less masking. The presence of higher anti-estrogenicity at WWTW5 may therefore be due to analytes originating from domestic sewage rather than industrical by-products. On the other hand, the different treatment technologies being utilised by the plants may degrade or possibly create anti-estrogenicity differently than for estrogenic compounds, or may contain different composition of estrogenic- and anti-estrogenic compounds in their wastewater.

APPENDIX B: SUPPLEMENTARY INFORMATION FOR CHAPTER 4

The fate of pharmaceuticals and personal care products (PPCPs), endocrine disrupting contaminants (EDCs), metabolites and illicit drugs in a WWTW and environmental waters

Acidic compounds

Column: Reversed-phase BEH C18 column (150 x 1.0 mm, particle size – 1.7 µm)

Separation:

- 80:20 H_20:MeOH + 1 mM NH_4F (mobile phase A)
- 5:95 H_20:MeOH + 1 mM NH_4F (mobile phase B)

Gradient:

100:0 % (A:B) for 0.5 mins (starting)
40:60 % (A:B) for 2 mins
0:100 % (A:B) for 5.5 mins
0:100 % (A:B) for 6 mins
100:0 % (A:B) for 0.1 mins
100:0 % (A:B) for 8.4 mins

Total run time: 22.5 mins

ESI- mode: CV = 3.2 kV

Flow rate: 0.04 mL.min^{-1}

Cone gas flow: 100 L.h^{-1}(Argon)

Desolvation gas flow: 550 L.h^{-1} (Nitrogen)

Column temp.: 25 °C

Starting column pressure: ≈ 8500 psi

Sample manager: 4 °C

Injection volume: 15 µL

Basic compounds

Column: Reversed-phase BEH C18 column (150 x 1.0 mm, particle size – 1.7 µm)

Separation:

- 80:20 H_20:MeOH + 5 mM NH_4OAc + 0.3% ethanoic acid (mobile phase A)
- 100% MeOH (mobile phase B)

Gradient:

100:0 % (A:B) (starting)
10:90 % (A:B) over 20 mins
10:90 % (A:B) for 6 mins
100:0 % (A:B) for 0.5 mins
100:0 % (A:B) for 7.5 mins.

Total run time: 34 mins

ESI+ mode: CV = 3.00 kV

Flow rate: 0.04 mL.min^{-1}

Cone gas flow: 100 L.h^{-1} (Argon)

Desolvation gas flow: 550 L.h^{-1} (Nitrogen)

Column temp.: 25 °C

Starting column pressure: ≈ 8000 psi

Sample manager: 4 °C

Injection volume: 15 µL

Figure B1: Liquid chromatography-Mass spectrometry (UPLC/TQD-MS) methods for acidic and basic compound detection.

Table B1: List of corrected recoveries of target ECs within WWTW influent/effluent and river water upstream and downstream samples.

Chemical Class	Chemical	Corrected recovery (%)			
		WWTW		River water	
		Effluent	Influent	Upstream	Downstream
UV filters	Benzophenone-1	45.7	39.3	63.4	64.5
	Benzophenone-3	164.6	41.9	92.7	98.3
	Benzophenone-4	145.7	121.9	136.2	128.1
Parabens	Methylparaben	97.1	83.9	94.2	93.5
	Ethylparaben	62.8	50.6	56.9	60.6
	Propylparaben	106.1	83.7	149.1	170.7
	Butylparaben	95.1	81.8	135.6	156.9
Plasticizer	Bisphenol-A	119.5	87.7	125.8	119.0
Antibiotics	Sulfasalazine	118.9	177.6	165.5	140.1
	Clarithromycin	158.2	56.2	97.6	104.8
	Azithromycin	51.3	30.6	59.6	76.5
	Trimethoprim	158.2	165.2	149.3	164.1
	Sulfamethoxazole	135.4	130.7	107.0	110.0
H2 receptor agonists	Ranitidine	66.5	57.3	63.7	68.7
	Cimetidine	73.7	93.8	117.8	119.5
	Ketoprofen	107.6	113.7	108.5	110.8
	Ibuprofen	87.1	81.9	110.4	110.3
NSAIDs	Naproxen	85.5	86.8	101.9	103.6
	Diclofenac	191.8	208.7	190.9	176.5
	Acetaminophen	120.7	122.6	104.8	103.2
Lipid regulators	Bezafibrate	90.4	95.2	102.6	106.5
	Atorvastatin	229.4	270.2	159.2	116.8
	Venlafaxine	71.1	50.5	85.2	76.5
Anti-depressants	Desmethylvenlafaxine	88.0	88.5	107.1	101.9
	Fluoxetine	107.5	108.3	117.1	120.7
	Desmethylcitalopram	95.6	114.4	88.2	79.6
Antihistamines	Fexofenadine	66.1	73.7	61.1	47.4
Anti-cancer	Azathioprine	145.8	165.3	104.1	104.1
	Carbamazepine	95.5	100.6	99.0	100.2
Anti-epileptic	Carbamazepine10,11-epoxide	128.9	139.2	126.8	135.2
	10,11-Dihydro-10-hydroxycarbamazepine	131.9	154.3	115.8	124.1
Diabetes	Metformin	113.6	122.6	123.0	118.1
	Gliclazide	125.5	56.5	101.5	93.7
	Valsartan	185.0	195.6	165.7	145.1
Hypertension	Irbesartan	143.6	137.9	195.4	203.0
	Lisinopril	67.0	47.9	39.4	37.1
X-ray contrast media	Iopromide	137.8	147.0	167.6	174.5
Beta-blocker	Atenolol	91.7	86.8	97.4	102.4
	Morphine	91.4	111.3	104.6	102.3
	Normorphine	94.6	106.5	93.5	94.6
Analgaesics	Codeine	100.6	80.0	103.7	98.0
	Tramadol	83.5	83.6	100.2	94.1
	N-desmethyltramadol	67.6	67.0	74.8	74.9
	O-desmethyltramadol	101.2	118.1	104.4	99.4
Drug precursor	Ephedrine/pseudoephedrine	69.0	60.9	77.5	87.0
	Amphetamine	106.1	103.3	101.1	106.3
	Methamphetamine	98.1	107.8	93.4	92.2
Stimulants	Cocaine	102.8	101.8	100.9	102.0
	Benzoylecgonine	121.6	116.9	112.8	119.9
	Cocaethylene	101.8	103.8	103.6	101.4
	Mephedrone	99.9	103.3	105.3	99.9
	Creatinine	67.3	82.2	71.0	66.8
Human indicators	Nicotine	80.2	108.3	77.6	82.4
	Caffeine	132.0	124.4	92.7	90.3

Cotinine	98.9	83.1	100.7	100.5
1,7 dimethylxantine	122.2	114.6	67.3	60.7

1

Table B2: Quantitative concentrations (ng.L^{-1}) of the detected ECs in samples obtained from influent and effluent WWTW, and river water samples located upstream and downstream of the plant. Abbreviations: BEG – benzoylecgonine; *DMX* - 1,7-dimethylxantine; *NDT* – *N*-desmethyltramadol; *ODT* – *O*-desmethyltramadol; *CBZ-ep* – carbamazepine epoxide; *dh-h-CBZ* – 10,11-dihydro-10-hydroxycarbamazepine; *DMV* – desmethylvenlafaxine; *dm-citalopram* – desmethylcitalopram; METH – methamphetamine; PSE/EPH – (pseudo)ephedrine; SMX - sulfamethoxazole.

	Concentration (ng.L^{-1}, ± stdev)																			
	06/07/2015 (Monday)				07/07/2015 (Tuesday)				08/07/2015 (Wednesday)				09/07/2015 (Thursday)				10/07/2015 (Friday)			
	US	Inf.	Eff.	DS	US	Inf.	Eff.	DS	US	Inf.	Eff.	DS	US	Inf.	Eff.	DS	US	Inf.	Eff.	DS
Chemicals in ESI- mode																				
Atorvostatin	82 ±0.4	278 ±8.6	206 ±13.4	76 ±4.4	77 ±0.7	570 ±5.3	175 ±4.7	146 ±4.9	71 ±0.4	635 ±3.2	184 ±1.6	152 ±13.2	70 ±0.1	447 ±8.6	233 ±6.6	233 ±8.5	71 ±0.8	1101 ±12.3	252 ±4.9	146 ±1.8
Benzophenone-1	-	4552 ±2909.2	-	-	-	172 ±2.1	-	-	-	67 ±4.9	-	-	-	183 ±1.7	-	-	-	121 ±1.7	-	-
Benzophenone-4	443 ±8.5	9085 ±49.5	2869 ±9.5	498 ±1.4	411 ±2.6	7280 ±70.7	2549 ±3.1	1584 ±0.9	448 ±7.4	7505 ±233.4	1978 ±3.3	1022 ±5.2	475 ±4.2	9710 ±70.7	1961 ±5.3	1184 ±7.8	429 ±0.6	9440 ±113.1	1904 ±4.4	1094 ±8.6
Bezafibrate	46 ±1.3	1611 ±3.6	540 ±12.0	133 ±8.6	47 ±3.3	1952 ±5.6	664 ±3.7	427 ±2.5	56 ±4.9	1403 ±8.6	463 ±6.2	249 ±0.1	66 ±5.0	2998 ±10.3	405 ±2.1	206 ±1.1	59 ±0.9	1999 ±5.7	317 ±5.2	157 ±0.4
Bisphenol A	167 ±0.1	944 ±39.5	301 ±0.8	614 ±95.5	360 ±184.9	4043 ±273.9	223 ±7.4	616 ±452.3	213 ±3.1	1567 ±23.8	148 ±0.6	167 ±42.4	233 ±41.9	1640 ±121.1	225 ±0.6	338 ±18.9	223 ±8.7	1742 ±7.6	337 ±67.5	247 ±39.6
Butylparaben	-	-	-	-	-	-	-	-	-	39 ±2.3	-	-	-	165 ±1.6	-	-	-	173 ±4.5	-	-
Diclofenac	280 ±6.1	5579 ±231.5	2525 ±3.1	758 ±58.9	647 ±388.3	4130 ±314.8	2285 ±4.3	2182 ±233.3	354 ±4.9	2734 ±9.5	2152 ±2.0	1570 ±161.6	393 ±0.1	2910 ±1.3	2283 ±8.8	1424 ±9.5	663 ±1.6	5647 ±0.5	2385 ±106.1	1374 ±83.5
Ethylparaben	-	885 ±5.9	-	-	-	4649 ±9.3	-	-	-	1983 ±7.9	-	-	-	3279 ±17.1	-	-	-	2291 ±14.6	-	-
Fexofenadine	413 ±3.4	-	1384 ±2.1	591 ±14.9	347 ±2.5	0.8 ±1.1	1198 ±10.4	1025 ±3.4	351 ±1.3	4114 ±63.2	1314 ±7.3	959 ±11.7	402 ±8.1	156 ±220.4	1216 ±3.1	891 ±6.8	330 ±5.4	3331 ±28.7	1613 ±6.9	968 ±0.8
Ibuprofen	129 ±3.8	15780 ±410.1	1151 ±1.8	233 ±2.0	175 ±0.4	9055 ±7.1	1031 ±3.0	608 ±1.6	213 ±1.6	10865 ±431.3	444 ±4.8	145 ±0.7	125 ±1.5	14810 ±155.6	375 ±4.3	436 ±3.5	125 ±0.6	15250 ±240.4	304 ±0.5	138 ±1.5
Irbesartan	305 ±4.1	1224 ±6.8	872 ±7.4	489 ±2.3	347 ±98.3	1133 ±2.2	908 ±2.8	759 ±45.8	273 ±2.8	897 ±1.6	772 ±3.5	563 ±2.2	299 ±10.7	1333 ±9.3	737 ±4.5	498 ±3.1	332 ±3.5	837 ±2.3	823 ±28.7	463 ±4.2
Ketoprofen	-	353 ±19.1	296 ±4.9	12 ±16.8	-	353 ±4.5	244 ±3.9	-	-	500 ±6.9	394 ±3.4	341 ±1.5	-	1490 ±2.9	435 ±2.7	205 ±5.5	642 ±1.9	5586 ±4.1	665 ±4.5	764 ±2.9
Methylparaben	26 ±0.3	6110 ±551.5	44 ±2.5	61 ±0.4	38 ±9.8	30050 ±480.8	46 ±4.5	41 ±0.9	65 ±1.3	14455 ±49.5	132 ±48.9	117 ±49.7	102 ±0.2	12030 ±806.1	252 ±4.0	295 ±20.5	62 ±3.2	12800 ±961.7	76 ±10.7	216 ±18.0
Naproxen	209 ±2.1	3675 ±21.2	2882 ±0.3	471 ±9.1	184 ±0.8	2925 ±21.2	2799 ±8.4	1899 ±64.9	221 ±10.1	3205 ±21.2	2058 ±2.7	1070 ±10.7	243 ±4.9	5455 ±21.2	1993 ±7.3	1199 ±2.2	265 ±2.3	5295 ±49.5	1751 ±12.9	924 ±3.7
Propylparaben	-	-	-	-	14 ±19.4	10950 ±509.1	-	-	21 ±0.2	15830 ±56.6	72 ±49.9	96 ±64.6	51 ±9.1	2835 ±162.6	144 ±19.9	225 ±13.9	41 ±51.1	1305 ±431.3	22 ±27.4	89 ±0.7
Sulfasalazine	38 ±1.9	3330 ±0.0	163 ±1.4	36 ±1.8	43 ±11.5	3330 ±14.1	131 ±16.2	44 ±5.2	37 ±3.0	3335 ±7.1	67 ±15.2	67 ±23.7	35 ±1.8	3350 ±14.1	122 ±3.6	63 ±37.9	35 ±0.5	3440 ±99.0	79 ±22.5	55 ±28.9
Valsartan	233 ±5.7	19660 ±919.2	2692 ±4.0	674 ±6.5	253 ±7.7	17535 ±728.3	2834 ±3.0	1768 ±2.5	300 ±6.2	15620 ±452.6	1630 ±3.4	605 ±5.8	264 ±0.2	17510 ±664.7	1338 ±3.1	1020 ±6.9	269 ±2.1	17825 ±558.6	1131 ±4.7	556 ±0.8
Chemicals in ESI+ mode																				
Acetaminophen	-	232760 ±11229	61 ±5.4	27 ±4.0	24 ±2.4	282675 ±8619	44 ±8.7	39 ±3.6	26 ±0.6	343620 ±25343	41 ±4.1	26 ±10.0	17 ±3.0	228893 ±15737	216 ±23.7	200 ±11.6	17 ±2.6	136887 ±680	123 ±10.8	27 ±1.6
Amphetamine	18 ±0.3	1110 ±290.1	60 ±0.1	76 ±8.1	20 ±3.0	2591 ±332.6	184 ±2.5	38 ±4.8	67 ±2.3	535 ±74.2	94 ±2.4	29 ±6.7	17 ±2.8	760 ±61.2	73 ±2.7	21 ±2.0	14 ±0.1	256 ±12.0	54 ±5.8	22 ±2.8
Atenolol	202 ±0.5	2541 ±13.1	392 ±26.0	193 ±10.0	158 ±15.5	1593 ±18.1	712 ±70.5	544 ±6.1	161 ±5.1	1745 ±18.6	537 ±17.8	241 ±22.6	155 ±7.9	1839 ±110.4	457 ±19.9	216 ±11.2	105 ±2.1	1627 ±101.5	364 ±12.1	166 ±1.4
Azathioprine	-	89 ±2.2	-	-	-	65 ±2.4	-	-	-	57 ±7.4	-	-	-	83 ±3.8	-	-	-	65 ±0.9	-	-
Azithromycin	-	32 ±2.8	157 ±12.1	3 ±1.0	-	23 ±1.8	173 ±3.5	7 ±2.5	25 ±0.6	139 ±15.6	213 ±7.2	11 ±1.8	-	65 ±4.5	178 ±1.5	5 ±1.1	-	45 ±3.3	138 ±24.5	-
Benzophenone-3	54 ±0.5	173 ±9.3	79 ±0.2	58 ±1.8	55 ±0.4	211 ±3.7	75 ±3.5	62 ±6.4	57 ±0.1	103 ±8.1	87 ±9.5	60 ±5.9	59 ±0.5	137 ±1.3	77 ±3.3	71 ±3.8	56 ±1.3	63 ±4.1	76 ±6.7	70 ±5.2
BEG	-	514 ±0.9	7 ±0.8	-	-	478 ±10.8	-	-	-	298 ±8.7	-	-	-	276 ±12.2	-	-	-	380 ±29.4	-	-
Carbamazepine	172 ±8.3	403 ±6.2	426 ±20.2	241 ±23.3	153 ±10.3	459 ±9.5	411 ±18.3	304 ±14.9	152 ±3.1	332 ±7.7	390 ±13.9	281 ±16.8	165 ±4.8	458 ±20.0	422 ±14.5	294 ±6.2	144 ±7.0	610 ±24.8	414 ±3.5	277 ±5.7
CBZ-ep	421 ±3.3	1119 ±26.2	1239 ±20.0	647 ±37.4	367 ±24.7	1183 ±44.3	1363 ±1.8	834 ±40.0	398 ±11.6	1094 ±60.7	1159 ±9.6	733 ±26.5	432 ±10.1	1666 ±82.4	1235 ±4.2	765 ±21.4	376 ±8.9	1269 ±12.2	1184 ±30.6	783 ±20.9

Caffeine	573 ±171.6	15125 ±559.3	799 ±60.9	1039 ±77.8	784 ±117.9	5094 ±61.9	470 ±22.8	356 ±62.1	950 ±77.9	347155 ±30568.0	718 ±97.5	787 ±268.6	862 ±97.7	1214375 ±21291.0	3824 ±102.9	6646 ±122.1	891 ±257.6	389460 ±29090.0	2557 ±26.1	1559 ±41.3
Cimetidine	-	731 ±3.8	-	-	-	499 ±2.3	-	-	-	1337 ±86.3	-	-	-	454 ±23.5	-	-	-	1378 ±8.1	-	-
Clarithromycin	91 ±0.5	491 ±6.3	488 ±0.2	126 ±3.0	59 ±4.1	497 ±12.9	516 ±5.9	265 ±2.0	87 ±2.5	1541 ±2.4	603 ±0.4	235 ±5.0	67 ±4.7	953 ±2.1	495 ±5.7	250 ±2.7	78 ±0.2	260 ±39.9	579 ±0.1	302 ±8.2
Cocaethylene	-	225.5 ±4.5	-	-	-	186.0 ±39.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cocaine	-	28 ±0.1	-	-	-	147 ±31.0	-	-	-	125 ±4.4	-	-	-	97 ±8.8	-	-	-	40 ±2.1	-	-
Codeine	1 ±0.4	1550 ±2.1	516 ±6.2	34 ±3.3	-	1011 ±4.8	401 ±3.0	216 ±1.8	15 ±2.8	1737 ±4.5	289 ±6.9	138 ±3.0	15 ±3.8	947 ±45.9	286 ±15.2	144 ±4.0	13 ±1.2	1070 ±22.1	235 ±4.1	112 ±1.1
Cotinine	21 ±0.9	4892 ±3.8	32 ±0.1	35 ±0.0	23 ±1.3	3333 ±21.6	25 ±0.9	17 ±0.4	27 ±0.9	4493 ±19.2	23 ±0.9	23 ±4.2	27 ±2.6	4147 ±13.9	25 ±2.0	36 ±0.3	29 ±6.0	4266 ±0.8	35 ±2.2	47 ±1.8
Creatinine	-	2849 ±33.2	-	-	-	2939 ±9.5	-	-	-	-	-	-	-	4900 ±339.4	-	-	-	12625 ±374.8	-	-
dh-h-CBZ	23 ±2.5	33 ±0.5	110 ±1.7	41 ±5.6	21 ±1.6	55 ±3.6	110 ±2.1	62 ±2.0	21 ±1.0	42 ±3.7	99 ±3.5	60 ±2.5	24 ±1.0	137 ±1.6	103 ±0.8	61 ±3.0	24 ±1.5	196 ±4.8	98 ±4.5	60 ±0.2
dm-citalopram	-	-	6 ±0.4	-	-	-	1 ±1.6	-	-	-	11 ±5.4	-	-	-	3 ±0.3	-	-	-	6 ±0.1	-
DMX	262 ±96.0	237345	22 ±31.5	471 ±551.8	24 ±33.2	163613	-	216 ±305.1	901 ±37.5	16765	716 ±1012.8	630 ±426.9	452 ±579.0	110975	3705	1641 ±1879.1	759 ±962.0	147445	1216	1830 ±2260.5
DMV	60 ±0.9	-	297 ±11.0	80 ±6.9	43 ±9.7	171 ±21.9	287 ±10.0	191 ±6.4	44 ±1.6	206 ±2.0	317 ±3.7	192 ±13.8	56 ±4.1	245 ±15.1	337 ±1.3	200 ±18.4	47 ±1.3	323 ±43.6	334 ±1.8	212 ±15.9
Gliclazide	46 ±0.3	671 ±219.1	79 ±2.3	19 ±26.7	43 ±1.1	169 ±2.5	58 ±1.3	60 ±8.8	41 ±0.4	258 ±34.9	48 ±0.8	47 ±1.1	41 ±0.1	304 ±0.5	68 ±5.6	75 ±4.4	45 ±0.6	135 ±7.1	71 ±1.3	69 ±2.7
Iopromide	271 ±4.4	386 ±3.5	944 ±3.5	256 ±3.4	247 ±7.2	622 ±18.2	1218 ±37.4	808 ±10.5	265 ±5.5	1029 ±23.2	1049 ±44.5	484 ±14.8	275 ±3.1	2762 ±232.6	1113 ±15.5	814 ±4.1	271 ±4.2	1172 ±56.8	978 ±100.9	629 ±24.4
Lisinopril	-	-	-	-	-	-	-	-	-	196 ±5.2	-	-	-	200 ±1.6	-	-	-	-	-	-
Mephedrone	-	-	-	-	-	-	-	-	-	56 ±12.8	-	-	-	121 ±0.7	-	-	-	36 ±0.5	-	-
Metformin	75 ±0.1	9228 ±280.0	499 ±7.8	126 ±0.9	67 ±0.6	4799 ±122.9	566 ±30.6	316 ±43.1	78 ±0.6	3585 ±89.0	378 ±13.1	160 ±0.9	81 ±0.4	4723 ±14.1	295 ±37.2	161 ±0.9	65 ±0.9	4740 ±7.0	167 ±1.9	111 ±1.0
METH	-	450 ±6.7	0.2 ±0.3	-	-	420 ±42.9	25 ±1.5	25 ±1.6	-	271 ±40.2	13 ±1.6	-	-	315 ±15.1	4 ±1.8	-	-	316 ±27.4	3 ±1.3	-
Morphine	-	687 ±30.6	-	-	-	414 ±5.4	-	-	-	391 ±4.4	-	-	-	474 ±40.8	-	-	-	457 ±25.2	-	-
NDT	7 ±1.3	-	111 ±1.8	65 ±5.1	8 ±0.6	145 ±12.9	95 ±5.7	69 ±4.2	29 ±4.8	149 ±11.9	121 ±10.0	84 ±21.1	24 ±12.4	-	115 ±3.0	72 ±8.3	12 ±5.8	33 ±2.8	126 ±0.4	81 ±2.8
Nicotine	95 ±4.9	4874 ±95.0	182 ±1.3	268 ±1.9	108 ±21.5	11866 ±138.5	175 ±4.7	182 ±0.2	239 ±23.9	11866 ±141.4	174 ±4.8	232 ±4.5	241 ±38.0	10365 ±35.4	217 ±2.8	350 ±3.0	89 ±16.8	8625 ±190.9	169 ±7.3	195 ±6.4
Norephedrine	-	2914 ±890.7	-	-	-	1950 ±265.7	-	-	-	1107 ±357.7	-	-	-	852 ±20.8	-	-	-	772 ±34.1	-	-
Normorphine	-	-	-	-	-	111 ±6.6	-	-	-	144 ±65.5	-	-	-	128 ±12.6	-	-	-	185 ±8.6	-	-
ODT	199 ±9.8	-	1469 ±57.8	319 ±26.3	182 ±16.5	214 ±303.0	1330 ±71.6	691 ±47.7	254 ±10.0	1283 ±33.16	1139 ±33.3	594 ±54.2	227 ±10.0	-	1136 ±16.0	671 ±29.3	177 ±16.3	-	1056 ±55.1	612 ±14.1
PSE/EPH	35 ±3.7	4885 ±77.8	177 ±3.6	61 ±1.5	30 ±4.6	4080 ±70.7	167 ±7.9	85 ±5.9	44 ±2.1	5720 ±254.6	109 ±0.7	43 ±8.6	50 ±1.8	-	113 ±14.9	102 ±1.5	36 ±3.8	16640 ±2152	138 ±7.8	111 ±5.6
Ranitidine	-	240 ±16.2	-	-	-	143 ±0.7	-	-	-	50 ±19.7	-	-	-	52 ±12.8	-	-	-	103 ±18.0	-	-
SMX	859 ±7.3	2589 ±3.2	1560 ±18.6	711 ±6.3	749 ±29.7	554 ±16.5	1229 ±2.1	1283 ±69.7	745 ±15.4	1339 ±6.0	1473 ±81.9	1351 ±43.1	801 ±56.9	806 ±4.8	1289 ±0.1	962 ±35.9	634 ±19.4	2521 ±46.1	1173 ±42.4	758 ±19.4
Tramadol	101 ±2.3	-	549 ±0.9	169 ±0.8	87 ±5.2	480 ±0.6	495 ±4.0	337 ±0.4	92 ±3.1	411 ±4.4	514 ±4.6	324 ±0.1	116 ±1.2	503 ±4.5	514 ±43.5	332 ±3.2	94 ±0.1	493 ±9.6	540 ±18.5	337 ±0.9
Trimethoprim	410 ±0.3	6249 ±34.8	1490 ±6.0	381 ±10.0	318 ±20.6	11136 ±981.5	1117 ±4.0	879 ±1.8	419 ±4.1	7250 ±53.0	1446 ±11.2	1045 ±60.8	404 ±9.9	4537 ±296.4	1501 ±7.3	1067 ±31.5	363 ±3.7	4502 ±908.1	1676 ±24.1	1121 ±24.4
Venlafaxine	34 ±0.4	-	148 ±5.3	60 ±3.2	36 ±2.2	461 ±22.0	126 ±1.0	102 ±0.6	34 ±1.3	335 ±21.2	143 ±3.2	107 ±4.7	42 ±0.0	270 ±0.0	140 ±4.5	101 ±6.3	32 ±1.1	275 ±21.2	155 ±3.6	104 ±0.0

1

Table B3: Average mass loads (g.day^{-1}) determined for the ECs at influent and final effluent water in the WWTW.

Chemical Class	Compound Name	Mass Load (g.day^{-1}, av. ± stdev)		Removal (%)
		Influent	Effluent	
UV Filters	Benzophenone-1	43.3 ±83.7	-	100
	Benzophenone-3	5.9 ±2.5	3.2 ±0.2	46
	Benzophenone-4	370.4 ±52.1	92.0 ±17.3	75
Parabens	Methylparaben	650.8 ±389.8	4.5 ±3.7	99
	Ethylparaben	113.3 ±62.8	-	100
	Propylparaben	331.2 ±289.8	3.3 ±2.6	99
	Butylparaben	5.4 ±3.3	-	100
Plastisizer	Bisphenol A	85.8 ±52.1	10.1 ±3.0	88
Antibiotics	Azithromycin	2.6 ±2.0	7.0 ±1.1	-170
	Sulfasalazine	144.4 ±3.6	4.6 ±1.6	97
	Clarithromycin	32.2 ±21.6	21.9 ±1.9	32
	Sulfamethoxazole	66.7 ±40.0	54.9 ±6.2	18
	Sulfasalazine	144.4 ±3.6	4.6 ±1.6	97
	Trimethoprim	289.6 ±118.1	59.1 ±8.2	80
Hypertensions	Irbesartan	46.8 ±9.9	33.6 ±2.8	28
	Lisinopril	8.6 ±0.4	-	100
	Valsartan	758.4 ±63.3	78.6 ±32.0	90
H_2 receptor agonists	Cimetidine	37.6 ±18.7	-	100
	Ranitidine	5.0 ±3.3	-	100
NSAIDs	Ibuprofen	565.8 ±130.1	27.0 ±16.1	95
	Ketoprofen	71.1 ±95.7	16.6 ±6.6	77
	Naproxen	177.3 ±53.3	93.8 ±20.7	47
	Diclofenac	180.2 ±58.3	95.1 ±5.6	47
	Acetaminophen	10530.2 ±3216.0	4.0 ±3.2	100
Lipid Regulators	Bezafibrate	86.1 ±28.6	19.5 ±5.4	77
	Atorvostatin	26.0 ±13.0	8.6 ±1.4	67
Diabetes	Metformin	232.6 ±92.0	15.6 ±6.5	93
	Gliclazide	13.2 ±9.0	2.6 ±0.5	80
Beta-blockers	Atenolol	80.3 ±16.2	20.1 ±5.8	75
X-ray contrast media	Iopromide	51.9 ±41.8	43.4 ±5.1	16

Anti-depressants & metabolites	Venlafaxine	14.5 ±3.8	5.8 ±0.4	60
	Desmethylvenlafaxine	10.2 ±2.8	12.9 ±1.0	-26
	Fluoxetine	-	1.1 ±0.9	-
	Desmethylcitalopram	-	0.2 ±0.1	-
Anti-epileptics & metabolites	Carbamazepine	19.5 ±4.4	16.9 ±0.8	13
	10,11-dihydro-10-hydroxycarbamazepine	4.0 ±3.1	4.2 ±0.3	-6
	Carbamazepine-10,11-epoxide	54.6 ±11.4	50.5 ±3.9	7
Antihistamine	Fexofenadine	107.5 ±88.5	54.9 ±6.2	49
Anti-cancer	Azathioprine	3.1 ±0.6	-	100
Analgaesics & metabolites	Tramadol	15.7 ±9.3	21.3 ±0.8	-36
	N-desmethyltramadol	4.7 ±2.8	4.6 ±0.5	1
	O-desmethyltramadol	21.1 ±28.9	50.1 ±6.8	-58
	EDDP	0.6 ±0.0	0.6 ±0.0	5
	Codeine	54.1 ±14.3	14.1 ±4.5	74
	Morphine	20.8 ±5.0	0.2 ± -	99
	Normorphine	6.1 ±1.3	-	100
Drug precursor	(Pseudo)ephedrine	269.4 ±260.9	5.7 ±1.2	98
Stimulants & metabolites	Amphetamine	45.4 ±39.9	3.8 ±2.2	92
	Cocaine	3.8 ±2.3	0.4 ±0.0	90
	Benzoylecgonine	16.7 ±4.5	0.3 ± -	98
	Cocaethylene	8.8 ±1.1	-	100
	Methamphetamine	15.2 ±3.3	0.4 ±0.4	98
	Norephedrine	65.1 ±38.5	-	100
	Mephedrone	3.1 ±2.0	-	100
Human indicators & metabolites	Cotinine	181.6 ±22.9	1.1 ±0.2	99
	Nicotine	410.2 ±127.5	7.5 ±1.0	98
	1,7-dimethylxantine	5818.9 ±3420.3	48.7 ±63.2	99
	Caffeine	17224.1 ±21941.2	69.1 ±61.7	100
	Creatinine	251.1 ±196.2	-	100

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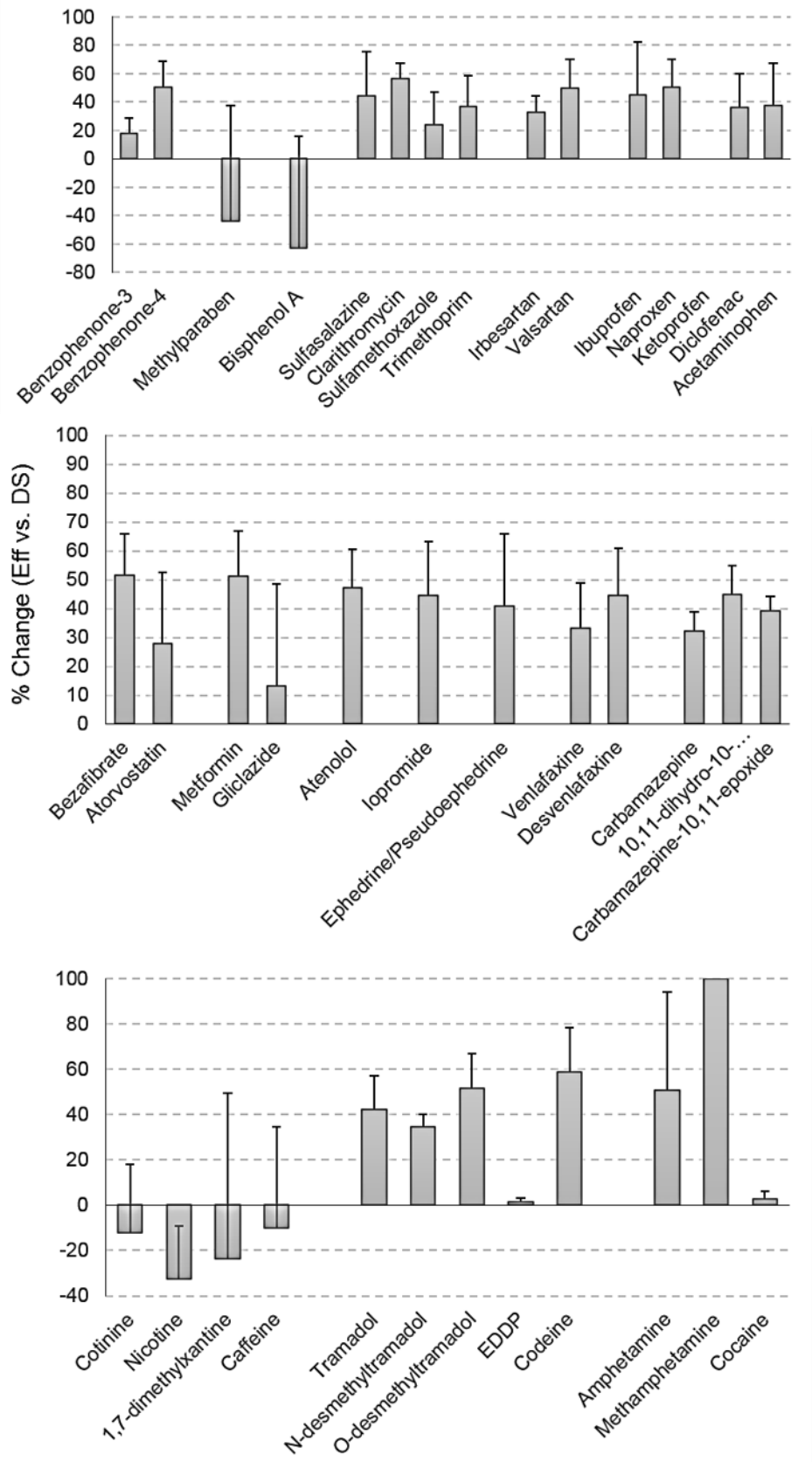

Figure B2: Percentage change of the detected ECs between final effluent water samples and river samples located downstream of the plant. Standard deviation shows variation between sampling days.

Table B4: Environmental risk calculation of the detected ECs based on conventional environmental risk assessment (ERA) and modulation of molecular initiating events (MIEs) and key events (KEs). Abbreviations: MEC – measured environmental concentration (based on concentrations in the current study); PNEC – predicted no-effect concentration (acute or chronic lethal toxicity outcomes); RQ – risk quotient ($MEC.PNEC^{-1}$ or $MEC.AOP\ conc.^{-1}$). An RQ > 1 is indicated in bold and reflects that the EC is of environmental concern. # Concentration of river water is a combination of both upstream and downstream water samples.

	MEC ($ug.L^{-1}$)		Environmental Risk Assessment (ERA)				Molecular Initiating Events (MIEs) and Key Events (KEs)				
Compound	Effluent	River Water#	PNEC ($\mu g.L^{-1}$)	Reference	RQ_{eff}	RQ_{rw}	Event	Conc. ($\mu g.L^{-1}$)	Reference	RQ_{eff}	RQ_{rw}
Diclofenac	2.31	0.96	0.10	Bergmann et al., 2011	23.1	10.0	Increased VTG gene expression in fish (MIE)	1.0	Hong et al., 2007; Gröner et al., 2017	2.31	0.96
							Decreased thyroid hormone levels in fish (KE)	1.0	Saravanan et al., 2014	2.31	0.96
Ibuprofen	0.66	0.23	7.10	Carlsson et al., 2006a	0.13	0.04	Lower thyroid-mediated mRNA transcripts in tadpoles (MIE)	1.5	Veldhoen et al., 2014	0.44	0.15
							Decreased egg fertilisation in fish (KE)	0.1	Nesbitt, 2011	6.60	2.30
Naproxen	2.30	0.67	3.30	Bergmann et al., 2011	0.7	0.2	Decreased egg fertilisation in fish (KE)	0.1	Nesbitt, 2011	23.0	6.7
Carbamazepine	0.41	0.22	2.50	Ferrari et al., 2003	0.16	0.09	Lower keto-testosterone hormone levels in fish (KE)	0.5	Galus et al., 2013	0.82	0.4
Azithromycin	0.17	0.015	4.80	Bergmann et al., 2011	0.04	0.003					
Iopromide	1.06	0.43	6800.0	Bergmann et al., 2011	0.001	0.001					
Sulfamethoxazole	1.34	0.89	0.59	Bergmann et al., 2011	2.27	1.51	Induction of VTG in fish	1000.0	Kang et al., 2006	0.001	0.001
Acetaminophen	0.10	0.04	0.24	Bergmann et al., 2011	0.42	0.17					
Clarithromycin	0.54	0.16	0.2	Bergmann et al., 2011	2.7	0.8					
Irbesartan	0.82	0.43	100.0	Minguez et al.,2016	0.01	0.004					
Valsartan	1.93	0.59	100.0	Minguez et al.,2016	0.02	0.006					
Ketoprofen	0.41	0.39	3.10	Bergmann et al., 2011	0.13	0.13					

Bezafibrate	0.48	0.14	1.20	Bergmann et al., 2011	0.4	0.12					
Tramadol	0.52	0.20	0.96	ECOSAR	0.54	0.21					
Venlafaxine	0.14	0.07	47.60	Minguez et al.,2016	0.003	0.001					
Methamphetamine	0.009	-	2.30	ECOSAR	0.004	-					
Morphine			32.0	ECOSAR							
Cocaine	0.009	-	4.90	ECOSAR	0.002	-					
Benzoylecgonine			4.90	ECOSAR							
Codeine	0.35	0.08	0.06	ECOSAR	5.83	1.33					
Benzophenone*	1.16	0.41	6.00	ECOSAR	0.19	0.07					
Methylparaben	0.11	0.11	11.2	Carlsson et al., 2006b	0.01	0.01	Increase VTG and lower GSI in fish	8400.0	Barse et al., 2010	0.001	0.001
Propylparaben	0.08	0.08					Increased VTG in fish	9.0	Scott, 2014	0.009	0.009
Chloramphenicol			0.019	Bergmann et al., 2011							
Amphetamine	0.09	0.03	0.98	Bergmann et al., 2011	0.09	0.03					
Bisphenol A	0.25	0.32	1.0	ECOSAR	0.25	0.32	Induction of VTG in fish	10.0	Villeneuve et al., 2011	0.03	0.03
Atenolol	0.49	0.21	100.0	Minguez et al.,2016	0.005	0.002					
Trimethoprim	1.45	0.64	20.0	Bergmann et al., 2011	0.07	0.03					
Nicotine	0.18	0.20	0.014	Bergmann et al., 2011	12.86	14.29					

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APPENDIX C: SUPPLEMENTARY INFORMATION FOR CHAPTER 5

Enantiomeric profiling and fate for drugs of abuse (DOA) during activated sludge treatment and environmental waters

Table C1: Concentrations (ng.L^{-1}) calculated from **Influent** wastewater at **WWTW1** over a 7-day period.

Compound	Concentration (ng.L^{-1})						
	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Stimulants							
Cocaine	52.4 ± 3.1	60.8 ± 7.7	40.9 ± 6.1	91.3 ± 3.9	52.4 ± 0.1	145.1 ± 14.8	58.6 ± 2.5
Benzoylecgonine	169.1 ± 5.5	197.6 ± 8.3	197.5 ± 8.5	162.3 ± 8.2	129.8 ± 4.5	346.3 ± 32.0	233.1 ± 3.5
R-(-)-amphetamine	ND	ND	ND	ND	ND	ND	ND
S-(+)-amphetamine	20.2 ± 1.3	15.6 ± 5.8	15.5 ± 9.0	13.1 ± 3.5	18.8 ± 5.4	20.7 ± 5.4	17.0 ± 2.7
R-(-)-methamphetamine	96.1 ± 11.0	69.1 ± 11.1	55.7 ± 8.8	83.0 ± 5.3	78.5 ± 1.3	102.7 ± 2.4	121.8 ± 9.2
S-(+)-methamphetamine	444.2 ± 53.4	429.3 ± 54.2	248.5 ± 43.6	609.4 ± 12.2	417.0 ± 16.7	394.0 ± 35.1	773.9 ± 38.9
R-(+)-mephedrone	ND	ND	ND	ND	ND	ND	ND
S-(-)-mephedrone	ND	ND	ND	ND	ND	ND	ND
Precursors							
1S,2R-(+)-ephedrine	ND	ND	ND	ND	ND	ND	ND
1R,2S-(-)-ephedrine/*1R,2R*-(-)-pseudoephedrine	1208.1 ± 28.1	965.8 ± 0.7	1472.6 ± 23.6	1749.9 ± 34.3	964.6 ± 12.1	1134.6 ± 97.3	1252.1 ± 106.4
1S,2S-(+)-pseudoephedrine	1299.7 ± 4.7	822.1 ± 9.6	864.9 ± 0.2	1264.5 ± 3.8	943.5 ± 47.1	961.6 ± 79.4	893.7 ± 15.5
E1-norephedrine	61.4 ± 3.4	47.4 ± 1.0	44.2 ± 4.0	55.2 ± 1.6	58.1 ± 0.6	55.5 ± 9.6	59.1 ± 0.2
E2-norephedrine	100.8 ± 3.6	78.6 ± 1.5	69.1 ± 1.0	79.6 ± 1.5	81.8 ± 0.3	82.4 ± 2.7	94.5 ± 3.4
Hallucinogens							
R-(-)-MDMA	1.9 ± 0.1	1.7 ± 0.3	ND	ND	ND	ND	2.5 ± 0.2
S-(+)-MDMA	ND	0.7 ± 0.1	ND	ND	ND	ND	1.9 ± 0.4
E1-HMA	ND	ND	ND	ND	ND	ND	ND
E2-HMA	ND	ND	ND	ND	ND	ND	ND
R-(-)-HMMA	4.9 ± 1.1	ND	ND	ND	ND	ND	ND
S-(+)-HMMA	ND	ND	ND	ND	7.2 ± 0.4	ND	8.0 ± 0.2
Opioids							
Codeine	1712.2 ± 40.3	1796.8 ± 115.3	1372.4 ± 8.5	1866.0 ± 74.6	1857.0 ± 145.6	1925.6 ± 9.8	2619.5 ± 233.7
Dihydrocodeine	7.7 ± 0.7	10.4 ± 0.3	6.2 ± 1.1	10.3 ± 0.1	9.1 ± 0.5	13.7 ± 1.4	10.0 ± 1.3
Hydrocodone	ND	ND	ND	ND	ND	ND	ND
Oxycodone	6.8 ± 0.6	ND	ND	4.2 ± 0.8	3.4 ± 0.7	3.2 ± 0.2	3.3 ± 0.8
Morphine	407.2 ± 6.7	355.4 ± 41.1	328.9 ± 6.3	326.6 ± 10.3	291.8 ± 31.8	380.0 ± 6.9	400.8 ± 30.8
Normorphine	150.2 ± 24.2	157.2 ± 5.7	88.9 ± 0.02	103.3 ± 8.0	105.8 ± 12.6	143.6 ± 21.4	127.3 ± 10.6
Heroin	ND	ND	ND	ND	ND	ND	ND
O-6-monoacetylmorphine	ND	ND	ND	ND	ND	ND	ND
E1-tramadol	459.9 ± 8.7	414.2 ± 38.2	143.5 ± 111.7	476.6 ± 26.6	593.4 ± 36.7	478.9 ± 15.8	577.7 ± 3.2
E2-tramadol	364.5 ± 8.8	380.8 ± 4.4	357.2 ± 29.1	422.6 ± 19.0	460.8 ± 23.1	353.8 ± 9.9	507.9 ± 30.1
O-desmethyltramadol	920.1 ± 7.2	907.4 ± 62.1	742.2 ± 17.3	768.6 ± 47.3	820.4 ± 48.6	880.2 ± 84.8	1122.3 ± 34.6

Methadone	ND	ND	ND	ND	ND	ND	ND
EDDP	ND	ND	ND	ND	ND	ND	ND
Antidepressants							
E1-venlafaxine	75.8 ± 5.2	67.4 ± 7.6	31.4 ± 4.2	44.1 ± 3.9	26.4 ± 5.1	65.1 ± 0.8	42.1 ± 2.7
E2-venlafaxine	75.1 ± 10.3	62.3 ± 5.2	27.3 ± 0.7	40.6 ± 3.4	26.9 ± 6.2	62.6 ± 0.4	36.3 ± 4.5
E1-desmethylvenlafaxine	435.3 ± 61.3	348.3 ± 43.4	342.9 ± 70.7	406.6 ± 5.4	531.5 ± 17.2	460.4 ± 6.2	497.7 ± 27.0
E2-desmethylvenlafaxine	276.0 ± 2.4	204.4 ± 14.9	206.5 ± 34.4	267.9 ± 6.9	334.4 ± 19.9	260.3 ± 25.4	285.1 ± 33.9
Anaesthetics							
Ketamine	4.7 ± 1.4	ND	1.9 ± 0.1	3.9 ± 1.6	399.0 ± 19.9	12.7 ± 1.0	5.5 ± 0.5
Human biomarkers							
Caffeine	231174.9 ± 3546.8	443400.9 ± 14578.8	230741.3 ± 6176.1	458704.1 ± 7592.3	270872.9 ± 13534.5	265864.3 ± 15780.7	133546.0 ± 2057.9
1,7-dimethylxanthine	208044.4 ± 373.6	155020.9 ± 605.5	139927.1 ± 5497.6	106914.6 ± 2428.0	114163.6 ± 16617.6	162228.5 ± 15890.6	123071.2 ± 10808.4
Nicotine	35442.8 ± 150.6	40987.4 ± 2713.4	20884.2 ± 1620.5	19262.9 ± 311.0	16939.7 ± 1187.7	26721.1 ± 2678.3	13423.7 ± 277.3
Cotinine	3876.0 ± 57.2	3773.9 ± 112.6	3418.7 ± 123.8	3177.5 ± 51.4	3265.0 ± 80.4	3480.9 ± 251.0	3556.1 ± 54.7

Table C2: Concentrations (ng.L^{-1}) calculated from **Influent** wastewater at **WWTW2** over a 7-day period.

Compound	Concentration (ng.L^{-1})						
	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Stimulants							
Cocaine	308.3 ± 7.6	217.3 ± 5.2	195.2 ± 12.4	107.8 ± 3.6	323.1 ± 1.3	349.7 ± 26.3	563.0 ± 3.3
Benzoylecgonine	693.5 ± 86.5	517.7 ± 10.4	485.8 ± 21.3	409.6 ± 0.9	588.5 ± 0.1	840.2 ± 28.0	1084.2 ± 50.5
R-(-)-amphetamine	ND	ND	ND	ND	ND	ND	ND
S-(+)-amphetamine	97.3 ± 0.6	90.5 ± 2.1	50.9 ± 6.6	62.1 ± 3.8	79.2 ± 0.1	100.6 ± 6.3	128.9 ± 11.1
R-(-)-methamphetamine	112.1 ± 6.8	135.2 ± 3.8	119.3 ± 1.4	94.7 ± 4.5	118.7 ± 3.9	161.3 ± 19.1	183.5 ± 2.3
S-(+)-methamphetamine	2317.6 ± 6.6	2171.9 ± 106.6	1828.3 ± 133.2	1587.4 ± 46.6	1889.8 ± 51.2	2877.1 ± 238.7	3226.4 ± 36.5
R-(+)-mephedrone	ND	2.0 ± 1.0	1.6 ± 0.1	ND	3.5 ± 0.1	1.6 ± 0.01	6.4 ± 0.4
S-(-)-mephedrone	1.4 ± 0.5	2.0 ± 0.3	ND	ND	3.4 ± 0.3	1.1 ± 0.2	5.7 ± 1.5
Precursors							
1S,2R-(+)-ephedrine	ND	ND	ND	ND	ND	ND	ND
1R,2S-(-)-ephedrine/*1R,2R*-(-)-pseudoephedrine	374.1 ± 22.8	384.3 ± 14.7	274.1 ± 2.4	362.9 ± 14.1	373.6 ± 1.8	415.2 ± 4.9	461.1 ± 25.0
1S,2S-(+)-pseudoephedrine	664.5 ± 38.0	718.1 ± 7.0	430.4 ± 8.8	501.0 ± 33.7	628.0 ± 27.9	580.0 ± 3.6	742.2 ± 5.1
E1-norephedrine	25.1 ± 1.7	27.7 ± 4.3	5.7 ± 0.4	26.6 ± 14.6	18.0 ± 0.1	16.7 ± 3.7	22.2 ± 1.2
E2-norephedrine	41.5 ± 0.5	48.1 ± 1.5	19.1 ± 0.6	29.7 ± 2.2	44.6 ± 1.3	39.7 ± 0.3	46.9 ± 1.1
Hallucinogens							
R-(-)-MDMA	34.5 ± 0.1	13.8 ± 0.2	13.5 ± 1.0	7.6 ± 0.1	10.0 ± 1.4	28.0 ± 0.4	57.5 ± 1.1
S-(+)-MDMA	12.8 ± 0.1	5.4 ± 0.6	6.2 ± 0.4	3.9 ± 0.2	6.4 ± 0.6	19.3 ± 0.6	34.4 ± 0.02
E1-HMA	ND	ND	ND	ND	ND	ND	ND
E2-HMA	ND	ND	ND	ND	ND	ND	ND
R-(-)-HMMA	10.7 ± 0.5	7.3 ± 1.8	ND	ND	ND	10.8 ± 0.4	16.0 ± 0.9
S-(+)-HMMA	12.1 ± 0.9	9.5 ± 0.8	9.5 ± 1.0	9.1 ± 0.7	8.6 ± 0.1	ND	19.2 ± 0.9
Opioids							
Codeine	11527.4 ± 1184.3	9856.0 ± 632.5	10426.3 ± 244.9	20567.7 ± 213.5	10402.5 ± 86.9	1987.8 ± 100.0	1663.2 ± 88.7
Dihydrocodeine	59.7 ± 1.0	30.4 ± 3.4	33.9 ± 2.2	24.7 ± 2.1	17.5 ± 0.4	10.6 ± 4.9	10.5 ± 2.2
Hydrocodone	ND	ND	ND	ND	ND	ND	ND
Oxycodone	ND	ND	ND	ND	ND	4.8 ± 1.8	6.6 ± 1.2
Morphine	9379.7 ± 1078.0	3585.8 ± 422.8	4002.8 ± 123.0	4528.2 ± 3.8	3757.4 ± 83.2	927.5 ± 193.0	761.0 ± 10.1
Normorphine	ND	173.8 ± 39.5	ND	131.8 ± 3.4	139.4 ± 25.5	ND	152.3 ± 26.3
Heroin	ND	ND	ND	ND	ND	ND	ND
O-6-monoacetylmorphine	ND	ND	29.9 ± 4.5	34.1 ± 8.8	66.0 ± 3.7	28.2 ± 0.1	29.3 ± 3.2
E1-tramadol	756.0 ± 39.6	732.1 ± 7.1	763.6 ± 4.8	695.1 ± 25.8	682.8 ± 27.5	768.6 ± 71.3	950.5 ± 15.7
E2-tramadol	694.5 ± 19.3	732.6 ± 25.3	645.7 ± 38.2	679.8 ± 27.8	651.8 ± 0.2	788.9 ± 32.6	870.4 ± 6.5
O-desmethyltramadol	2129.2 ± 180.1	2135.5 ± 204.3	2147.2 ± 229.7	2382.4 ± 5.9	2011.6 ± 28.2	1901.9 ± 58.5	2073.8 ± 77.7

Methadone	ND	ND	ND	ND	ND	ND	ND
EDDP	2.8 ± 0.8	ND	4.6 ± 1.1	0.6 ± 0.04	2.4 ± 0.1	2.9 ± 0.4	2.9 ± 0.2
Antidepressants							
E1-venlafaxine	127.3 ± 1.8	102.7 ± 1.4	105.1 ± 2.2	82.7 ± 3.5	77.0 ± 3.1	88.1 ± 6.2	114.7 ± 2.4
E2-venlafaxine	132.7 ± 8.0	109.2 ± 0.7	104.7 ± 3.0	85.6 ± 1.5	83.4 ± 5.4	95.6 ± 3.5	114.1 ± 2.3
E1-desmethylvenlafaxine	386.0 ± 17.8	380.5 ± 1.0	413.7 ± 72.5	384.0 ± 10.0	274.9 ± 21.7	329.4 ± 21.5	424.9 ± 1.2
E2-desmethylvenlafaxine	211.4 ± 4.9	219.1 ± 2.5	226.2 ± 2.6	200.4 ± 8.6	162.5 ± 8.8	223.2 ± 27.6	251.0 ± 18.3
Anaesthetics							
Ketamine	4.9 ± 0.6	17.1 ± 2.9	ND	5.5 ± 1.2	5.5 ± 0.3	ND	ND
Human biomarkers							
Caffeine	104776.3 ± 7150.1	89854.1 ± 1409.1	8717.4 ± 6253.5	90543.8 ± 34.4	93770.0 ± 3717.6	66837.7 ± 2029.0	54565.1 ± 846.4
1,7-dimethylxanthine	316160.1 ± 37977.5	271209.5 ± 3961.2	203751.1 ± 21425.9	178205.7 ± 26396.4	255945.2 ± 337.1	256005.4 ± 247.9	217148.0 ± 10490.7
Nicotine	90513.1 ± 4649.8	66786.7 ± 251.4	90344.6 ± 3118.0	64497.6 ± 2072.2	74124.6 ± 1890.5	82624.5 ± 9212.2	66578.1 ± 2832.6
Cotinine	5523.0 ± 298.5	4762.3 ± 2.4	4503.2 ± 89.8	4038.7 ± 83.1	5097.3 ± 139.1	5666.8 ± 108.5	5654.2 ± 0.8

Table C3: Mass loads (g.day^{-1}) calculated from **Influent** wastewater at **WWTW1** over a 7-day period.

Compound	Mass Load (g.day^{-1})						
	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Stimulants							
Cocaine	2.7 ± 0.2	3.0 ± 0.4	2.1 ± 0.3	5.0 ± 0.2	2.3 ± 0.01	7.2 ± 0.7	3.0 ± 0.1
Benzoylecgonine	8.7 ± 0.3	9.9 ± 0.4	10.3 ± 0.4	8.8 ± 0.4	5.6 ± 0.2	17.3 ± 1.6	12.0 ± 0.2
R-(-)-amphetamine	ND	ND	ND	ND	ND	ND	ND
S-(+)-amphetamine	1.0 ± 0.1	0.8 ± 0.3	0.8 ± 0.5	0.7 ± 0.2	0.8 ± 0.2	1.0 ± 0.3	0.9 ± 0.1
R-(-)-methamphetamine	11.4 ± 1.3	8.0 ± 1.3	6.7 ± 1.1	10.4 ± 0.7	7.8 ± 0.1	11.8 ± 0.3	14.5 ± 1.1
S-(+)-methamphetamine	54.8 ± 6.6	51.7 ± 6.5	31.0 ± 5.4	79.6 ± 1.6	43.1 ± 1.7	47.1 ± 4.2	96.0 ± 4.8
R-(+)-mephedrone	ND	ND	ND	ND	ND	ND	ND
S-(-)-mephedrone	ND	ND	ND	ND	ND	ND	ND
Precursors							
1S,2R-(+)-ephedrine	ND	ND	ND	ND	ND	ND	ND
1R,2S-(-)-ephedrine/*1R,2R*-(-)-pseudoephedrine	62.1 ± 1.4	48.4 ± 0.03	76.6 ± 1.2	95.2 ± 1.9	41.5 ± 0.5	56.5 ± 4.9	64.7 ± 5.5
1S,2S-(+)-pseudoephedrine	66.8 ± 0.2	41.2 ± 0.5	45.0 ± 0.01	68.8 ± 0.2	40.6 ± 2.0	47.9 ± 4.0	46.2 ± 0.8
E1-norephedrine	3.2 ± 0.2	2.4 ± 0.05	2.3 ± 0.2	3.0 ± 0.1	2.5 ± 0.03	2.8 ± 0.5	3.1 ± 0.01
E2-norephedrine	5.2 ± 0.2	3.9 ± 0.1	3.6 ± 0.1	4.3 ± 0.1	3.5 ± 0.01	4.1 ± 0.1	4.9 ± 0.2
Hallucinogens							
R-(-)-MDMA	0.1 ± 0.01	0.1 ± 0.01	ND	ND	ND	ND	0.1 ± 0.01
S-(+)-MDMA	ND	0.04 ± 0.005	ND	ND	ND	ND	0.1 ± 0.02
E1-HMA	ND	ND	ND	ND	ND	ND	ND
E2-HMA	ND	ND	ND	ND	ND	ND	ND
R-(-)-HMMA	0.2 ± 0.1	ND	ND	ND	ND	ND	ND
S-(+)-HMMA	ND	ND	ND	ND	0.3 ± 0.02	ND	0.4 ± 0.01
Opioids							
Codeine	88.0 ± 2.1	90.1 ± 5.8	71.4 ± 0.4	101.5 ± 4.1	79.9 ± 6.3	96.0 ± 0.5	135.4 ± 12.1
Dihydrocodeine	0.4 ± 0.03	0.5 ± 0.01	0.3 ± 0.1	0.6 ± 0.01	0.4 ± 0.02	0.7 ± 0.1	0.5 ± 0.1
Hydrocodone	ND	ND	ND	ND	ND	ND	ND
Oxycodone	0.3 ± 0.03	ND	ND	0.2 ± 0.05	0.1 ± 0.03	0.2 ± 0.01	0.2 ± 0.04
Morphine	20.9 ± 0.3	17.8 ± 2.1	17.1 ± 0.3	17.8 ± 0.6	12.6 ± 1.4	18.9 ± 0.3	20.7 ± 1.6
Normorphine	7.7 ± 1.2	7.9 ± 0.3	4.6 ± 0.001	5.6 ± 0.4	4.6 ± 0.5	7.2 ± 1.1	6.6 ± 0.5
Heroin	ND	ND	ND	ND	ND	ND	ND
O-6-monoacetylmorphine	ND	ND	ND	ND	ND	ND	ND
E1-tramadol	23.6 ± 0.4	20.8 ± 1.9	21.5 ± 5.8	25.9 ± 1.4	25.5 ± 1.6	23.9 ± 0.8	29.9 ± 0.2
E2-tramadol	18.7 ± 0.5	19.1 ± 0.2	18.6 ± 1.5	23.0 ± 1.0	19.8 ± 1.0	17.6 ± 0.5	26.3 ± 1.6
O-desmethyltramadol	47.3 ± 0.4	45.5 ± 3.1	38.6 ± 0.9	41.8 ± 2.6	35.3 ± 2.1	43.9 ± 4.2	58.0 ± 1.8
Methadone	ND	ND	ND	ND	ND	ND	ND

EDDP	ND	ND	ND	ND	ND	ND	ND
Antidepressants							
E1-venlafaxine	3.9 ± 0.3	3.4 ± 0.4	1.6 ± 0.2	2.4 ± 0.2	1.1 ± 0.2	3.2 ± 0.04	2.2 ± 0.1
E2-venlafaxine	3.9 ± 0.5	3.1 ± 0.3	1.4 ± 0.04	2.2 ± 0.2	1.2 ± 0.3	3.1 ± 0.02	1.9 ± 0.2
E1-desmethylvenlafaxine	22.4 ± 3.2	17.5 ± 2.2	17.8 ± 3.7	22.1 ± 0.3	22.9 ± 0.7	22.9 ± 0.3	25.7 ± 1.4
E2-desmethylvenlafaxine	14.2 ± 0.1	10.3 ± 0.7	10.7 ± 1.8	14.6 ± 0.4	14.4 ± 0.9	13.0 ± 1.3	14.7 ± 1.8
Anaesthetics							
Ketamine	0.2 ± 0.1	ND	0.1 ± 0.004	0.2 ± 0.1	17.2 ± 0.9	0.6 ± 0.05	0.3 ± 0.03
Human biomarkers							
Caffeine	11882.4 ± 182.3	22241.0 ± 731.3	11998.5 ± 321.2	24948.9 ± 412.9	11658.4 ± 582.5	13250.7 ± 786.5	6904.3 ± 106.4
1,7-dimethylxanthine	10693.5 ± 19.2	7775.8 ± 30.4	7276.2 ± 285.9	5815.1 ± 132.1	4913.6 ± 715.2	8085.5 ± 792.0	6362.8 ± 558.8
Nicotine	1821.8 ± 7.7	2055.9 ± 136.1	1086.0 ± 84.3	1047.7 ± 16.9	729.1 ± 51.1	1331.8 ± 133.5	694.0 ± 14.3
Cotinine	199.2 ± 2.9	189.3 ± 5.6	177.8 ± 6.4	172.8 ± 2.8	140.5 ± 3.5	173.5 ± 12.5	183.8 ± 2.8

Table C4: Mass loads (g.day^{-1}) calculated from **Influent** wastewater at **WWTW2** over a 7-day period.

Compound	Mass Load (g.day^{-1})						
	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Stimulants							
Cocaine	25.3 ± 0.6	18.3 ± 0.4	16.0 ± 1.0	8.8 ± 0.3	27.1 ± 0.1	25.9 ± 1.9	40.0 ± 0.2
Benzoylecgonine	56.9 ± 7.1	43.5 ± 0.9	39.8 ± 1.7	33.6 ± 0.1	49.4 ± 0.01	62.2 ± 2.1	77.0 ± 3.6
R-(-)-amphetamine	ND	ND	ND	ND	ND	ND	ND
S-(+)-amphetamine	8.0 ± 0.05	7.6 ± 0.2	4.2 ± 0.5	5.1 ± 0.3	6.7 ± 0.01	7.4 ± 0.5	9.2 ± 0.8
R-(-)-methamphetamine	21.1 ± 1.3	26.1 ± 0.7	25 ± 0.3	17.9 ± 0.8	22.9 ± 0.8	27.5 ± 3.2	30.0 ± 0.4
S-(+)-methamphetamine	456.1 ± 1.3	437.9 ± 21.5	359.8 ± 26.2	312.4 ± 9.2	381.0 ± 10.3	511.0 ± 42.4	549.8 ± 6.2
R-(+)-mephedrone	ND	1.1 ± 0.5	0.9 ± 0.04	ND	1.9 ± 0.03	0.8 ± 0.004	2.9 ± 0.2
S-(-)-mephedrone	0.8 ± 0.3	1.1 ± 0.2	ND	ND	1.9 ± 0.2	0.5 ± 0.1	2.6 ± 0.7
Precursors							
1S,2R-(+)-ephedrine	ND	ND	ND	ND	ND	ND	ND
1R,2S-(-)-ephedrine/*1R,2R*-(-)-pseudoephedrine	30.7 ± 1.9	32.3 ± 1.2	22.5 ± 0.2	29.8 ± 1.2	31.4 ± 0.1	30.7 ± 0.4	32.7 ± 1.8
1S,2S-(+)-pseudoephedrine	54.5 ± 3.1	60.3 ± 0.6	35.3 ± 0.7	41.1 ± 2.8	52.8 ± 2.3	42.9 ± 0.3	52.7 ± 0.4
E1-norephedrine	2.1 ± 0.2	2.3 ± 0.4	0.5 ± 0.03	2.2 ± 1.2	1.5 ± 0.01	1.2 ± 0.3	1.6 ± 0.1
E2-norephedrine	3.4 ± 0.04	4.0 ± 0.1	1.6 ± 0.1	2.4 ± 0.2	3.7 ± 0.1	2.9 ± 0.02	3.3 ± 0.1
Hallucinogens							
R-(-)-MDMA	2.8 ± 0.01	1.1 ± 0.01	1.1 ± 0.1	0.6 ± 0.01	0.8 ± 0.1	2.1 ± 0.03	4.1 ± 0.1
S-(+)-MDMA	1.1 ± 0.01	0.4 ± 0.1	0.5 ± 0.04	0.3 ± 0.02	0.5 ± 0.1	1.4 ± 0.04	2.4 ± 0.002
E1-HMA	ND	ND	ND	ND	ND	ND	ND
E2-HMA	ND	ND	ND	ND	ND	ND	ND
R-(-)-HMMA	0.9 ± 0.04	0.6 ± 0.1	ND	ND	ND	0.8 ± 0.03	1.1 ± 0.1
S-(+)-HMMA	1.0 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.01	ND	1.4 ± 0.1
Opioids							
Codeine	945.2 ± 97.1	827.9 ± 53.1	855.0 ± 20.1	1686.5 ± 17.5	873.8 ± 7.3	147.1 ± 7.4	118.1 ± 6.3
Dihydrocodeine	4.9 ± 0.1	2.6 ± 0.3	2.8 ± 0.2	2.0 ± 0.2	1.5 ± 0.04	0.8 ± 0.4	0.7 ± 0.2
Hydrocodone	ND	ND	ND	ND	ND	ND	ND
Oxycodone	ND	ND	ND	ND	ND	0.4 ± 0.1	0.5 ± 0.1
Morphine	769.1 ± 88.4	301.2 ± 35.5	328.2 ± 10.1	371.3 ± 0.3	315.6 ± 7.0	68.6 ± 14.3	54.0 ± 0.7
Normorphine	ND	14.6 ± 3.3	ND	10.8 ± 0.3	11.7 ± 2.1	ND	10.8 ± 1.9
Heroin	ND	ND	ND	ND	ND	ND	ND
O-6-monoacetylmorphine	ND	ND	2.5 ± 0.4	2.8 ± 0.7	5.5 ± 0.3	2.1 ± 0.005	2.1 ± 0.2
E1-tramadol	62.0 ± 3.2	61.5 ± 0.6	62.6 ± 0.4	57.0 ± 2.1	57.4 ± 2.3	56.9 ± 5.3	67.5 ± 1.1
E2-tramadol	57.0 ± 1.6	61.5 ± 2.1	52.9 ± 3.1	55.7 ± 2.3	54.8 ± 0.03	58.4 ± 2.4	61.8 ± 0.5
O-desmethyltramadol	174.6 ± 14.8	179.4 ± 17.2	176.1 ± 18.8	195.4 ± 0.5	169.0 ± 2.4	140.7 ± 4.3	147.2 ± 5.5
Methadone	ND	ND	ND	ND	ND	ND	ND
EDDP	0.2 ± 0.1	ND	0.4 ± 0.1	0.05 ± 0.03	0.2 ± 0.01	0.2 ± 0.03	0.2 ± 0.01

Antidepressants							
E1-venlafaxine	10.4 ± 0.1	8.6 ± 0.1	8.6 ± 0.2	6.8 ± 0.3	6.5 ± 0.3	6.5 ± 0.5	8.1 ± 0.2
E2-venlafaxine	10.9 ± 0.7	9.2 ± 0.1	8.6 ± 0.2	7.0 ± 0.1	7.0 ± 0.5	7.1 ± 0.3	8.1 ± 0.2
E1-desmethylvenlafaxine	31.6 ± 1.5	32.0 ± 0.1	33.9 ± 5.9	31.5 ± 0.8	23.1 ± 1.8	24.4 ± 1.6	30.2 ± 0.1
E2-desmethylvenlafaxine	17.3 ± 0.4	18.4 ± 0.2	18.5 ± 0.2	16.4 ± 0.7	13.7 ± 0.7	16.5 ± 2.0	17.8 ± 1.3
Anaesthetics							
Ketamine	0.4 ± 0.05	1.4 ± 0.2	ND	0.5 ± 0.1	0.5 ± 0.03	ND	ND
Human biomarkers							
Caffeine	8591.7 ± 586.3	7547.7 ± 118.4	7148.1 ± 512.8	7424.6 ± 2.8	7876.7 ± 312.3	4946.0 ± 150.1	3874.1 ± 60.1
1,7-dimethylxanthine	25925.1 ± 3114.2	22781.6 ± 332.7	16707.6 ± 1756.9	14612.9 ± 2164.5	21499.4 ± 28.3	18944.4 ± 18.3	15417.5 ± 744.8
Nicotine	7422.1 ± 381.3	5610.1 ± 21.1	7408.3 ± 255.7	5288.8 ± 169.9	6226.5 ± 158.8	6114.2 ± 681.7	4727.0 ± 201.1
Cotinine	452.9 ± 24.5	400.0 ± 0.2	369.3 ± 7.4	331.2 ± 6.8	428.2 ± 11.7	419.3 ± 8.0	401.5 ± 0.1

Table C5: Concentrations (ng.L^{-1}) calculated from **Effluent** wastewater at **WWTW1** over a 7-day period.

Compound	Concentration (ng.L^{-1})						
	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Stimulants							
Cocaine	0.6 ± 0.03	ND	ND	ND	ND	ND	ND
Benzoylecgonine	9.0 ± 0.01	12.9 ± 0.5	14.3 ± 3.0	ND	14.4 ± 0.5	11.0 ± 0.8	52.6 ± 1.9
R-(-)-amphetamine	ND	ND	ND	ND	ND	ND	ND
S-(+)-amphetamine	ND	ND	ND	ND	ND	ND	ND
R-(-)-methamphetamine	34.1 ± 3.3	26.4 ± 0.02	29.3 ± 5.0	44.8 ± 0.4	52.9 ± 4.9	35.6 ± 0.5	49.1 ± 4.0
S-(+)-methamphetamine	20.5 ± 5.1	3.3 ± 0.8	3.7 ± 1.0	2.3 ± 0.3	7.6 ± 0.1	7.0 ± 0.4	148.4 ± 16.9
R-(+)-mephedrone	ND	ND	ND	ND	ND	ND	ND
S-(-)-mephedrone	ND	ND	ND	ND	ND	ND	ND
Precursors							
1S,2R-(+)-ephedrine	ND	ND	ND	ND	ND	ND	ND
1R,2S-(-)-ephedrine/*1R,2R*-(-)-pseudoephedrine	26.8 ± 0.7	38.9 ± 0.004	41.6 ± 1.6	49.1 ± 3.6	32.2 ± 0.8	31.3 ± 0.5	217.5 ± 17.9
1S,2S-(+)-pseudoephedrine	ND	ND	ND	ND	ND	ND	73.3 ± 2.7
E1-norephedrine	0.6 ± 0.7	ND	ND	5.0 ± 1.7	2.6 ± 0.4	0.7 ± 0.4	ND
E2-norephedrine	ND	ND	ND	ND	ND	ND	10.4 ± 0.7
Hallucinogens							
R-(-)-MDMA	ND	0.3 ± 0.03	ND	ND	ND	ND	ND
S-(+)-MDMA	ND	ND	ND	ND	ND	ND	ND
E1-HMA	ND	ND	ND	ND	ND	ND	ND
E2-HMA	ND	ND	ND	ND	ND	ND	ND
R-(-)-HMMA	ND	ND	ND	ND	ND	ND	ND
S-(+)-HMMA	ND	ND	ND	ND	ND	ND	ND
Opioids							
Codeine	71.9 ± 5.6	51.8 ± 0.2	57.5 ± 2.2	11.8 ± 3.1	76.4 ± 3.9	10.9 ± 3.6	149.4 ± 1.7
Dihydrocodeine	ND	ND	ND	ND	4.2 ± 0.2	ND	ND
Hydrocodone	ND	ND	31.6 ± 3.2	21.1 ± 0.5	40.4 ± 3.3	ND	ND
Oxycodone	ND	ND	ND	ND	ND	ND	ND
Morphine	ND	ND	ND	ND	ND	ND	ND
Normorphine	ND	ND	ND	ND	ND	ND	ND
Heroin	ND	ND	ND	ND	ND	ND	ND
O-6-monoacetylmorphine	ND	ND	ND	ND	ND	ND	ND
E1-tramadol	284.4 ± 6.1	253.4 ± 12.4	339.6 ± 10.3	302.1 ± 6.6	402.1 ± 30.3	193.7 ± 1.5	252.1 ± 19.8
E2-tramadol	239.4 ± 5.0	182.3 ± 0.7	242.2 ± 10.4	246.0 ± 11.0	285.4 ± 8.2	153.3 ± 9.1	205.5 ± 10.9
O-desmethyltramadol	490.5 ± 39.9	681.6 ± 21.2	767.3 ± 28.4	638.7 ± 39.1	905.1 ± 85.3	264.4 ± 48.6	157.1 ± 3.0
Methadone	ND	ND	ND	ND	ND	ND	ND
EDDP	ND	ND	ND	ND	ND	ND	ND

Antidepressants							
E1-venlafaxine	62.5 ± 1.6	49.4 ± 4.4	70.9 ± 6.8	71.6 ± 0.4	85.7 ± 5.5	43.6 ± 3.8	43.6 ± 6.2
E2-venlafaxine	47.7 ± 2.2	38.3 ± 1.7	54.6 ± 10.2	53.2 ± 5.1	63.2 ± 1.0	34.6 ± 0.3	37.8 ± 4.5
E1-desmethylvenlafaxine	302.9 ± 8.9	263.7 ± 1.4	340.2 ± 19.1	258.8 ± 4.4	444.4 ± 14.2	138.6 ± 0.5	111.1 ± 8.9
E2-desmethylvenlafaxine	174.4 ± 3.6	148.9 ± 28.8	194.6 ± 33.4	152.3 ± 0.7	246.6 ± 11.0	72.8 ± 12.5	56.3 ± 23.3
Anaesthetics							
Ketamine	26.1 ± 3.7	9.8 ± 0.02	6.6 ± 0.8	6.8 ± 0.3	28.4 ± 0.6	61.4 ± 3.0	28.1 ± 7.1
Human biomarkers							
Caffeine	1556.5 ± 301.4	548.9 ± 17.7	658.1 ± 55.8	96.0 ± 41.2	107.1 ± 21.0	161.4 ± 17.5	23732.5 ± 864.0
1,7-dimethylxanthine	913.6 ± 464.8	927.5 ± 16.5	1913.2 ± 117.9	252.3 ± 0.9	303.9 ± 7.1	337.1 ± 43.0	27964.2 ± 1107.0
Nicotine	186.7 ± 160.4	394.9 ± 5.5	558.4 ± 117.9	200.6 ± 55.0	637.7 ± 109.7	195.6 ± 75.5	206.8 ± 36.1
Cotinine	33.9 ± 19.7	77.5 ± 0.9	88.7 ± 1.0	27.6 ± 0.1	57.1 ± 0.6	31.6 ± 3.3	331.0 ± 5.8

Table C6: Concentrations (ng.L^{-1}) calculated from **Effluent** wastewater at **WWTW2** over a 7-day period.

Compound	Concentration (ng.L^{-1})						
	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Stimulants							
Cocaine	1.1 ± 0.3	1.0 ± 0.05	1.7 ± 1.5	0.7 ± 0.2	0.8 ± 0.2	1.1 ± 0.4	ND
Benzoylecgonine	39.8 ± 12.7	34.2 ± 1.4	52.9 ± 6.9	32.9 ± 3.3	28.0 ± 0.4	39.9 ± 2.8	52.6 ± 4.4
R-(-)-amphetamine	ND	ND	ND	ND	ND	ND	ND
S-(+)-amphetamine	ND	ND	ND	ND	ND	ND	ND
R-(-)-methamphetamine	27.1 ± 2.4	29.1 ± 0.2	29.7 ± 3.2	26.8 ± 1.6	27.3 ± 1.3	30.5 ± 2.5	30.7 ± 5.3
S-(+)-methamphetamine	135.3 ± 2.2	151.9 ± 1.8	105.0 ± 16.9	104.3 ± 4.7	96.6 ± 6.6	102.8 ± 5.8	108.7 ± 19.1
R-(+)-mephedrone	ND	ND	ND	ND	ND	ND	ND
S-(-)-mephedrone	ND	ND	ND	ND	ND	ND	ND
Precursors							
1S,2R-(+)-ephedrine	ND	ND	ND	ND	ND	ND	ND
1R,2S-(-)-ephedrine/*1R,2R*-(-)-pseudoephedrine	58.0 ± 2.3	57.9 ± 1.6	62.6 ± 0.1	63.8 ± 1.4	48.2 ± 4.5	46.2 ± 5.0	46.3 ± 1.9
1S,2S-(+)-pseudoephedrine	16.7 ± 2.9	20.2 ± 2.1	ND	ND	ND	11.1 ± 0.8	12.5 ± 1.1
E1-norephedrine	4.5 ± 2.3	4.8 ± 0.3	6.1 ± 1.0	4.0 ± 0.7	5.7 ± 2.1	5.3 ± 1.3	5.1 ± 1.3
E2-norephedrine	ND	ND	ND	ND	ND	ND	ND
Hallucinogens							
R-(-)-MDMA	9.3 ± 2.3	8.7 ± 0.4	6.2 ± 0.9	4.0 ± 0.2	2.9 ± 0.04	3.1 ± 0.3	5.3 ± 1.0
S-(+)-MDMA	ND	ND	ND	ND	ND	ND	1.1 ± 0.06
E1-HMA	ND	ND	ND	ND	ND	ND	ND
E2-HMA	ND	ND	ND	ND	ND	ND	ND
R-(-)-HMMA	ND	ND	ND	ND	ND	ND	ND
S-(+)-HMMA	ND	ND	ND	ND	ND	ND	ND
Opioids							
Codeine	ND	88.6 ± 0.8	22.6 ± 1.5	42.8 ± 3.8	93.5 ± 4.0	26.4 ± 4.2	ND
Dihydrocodeine	ND	4.6 ± 0.2	ND	ND	ND	ND	ND
Hydrocodone	59.9 ± 21.0	68.3 ± 4.6	ND	ND	167.1 ± 2.3	148.5 ± 18.9	81.3 ± 7.0
Oxycodone	ND	ND	ND	ND	ND	ND	ND
Morphine	ND	ND	ND	ND	62.1 ± 3.2	ND	ND
Normorphine	ND	ND	ND	ND	ND	ND	ND
Heroin	ND	ND	ND	ND	ND	ND	ND
O-6-monoacetylmorphine	ND	ND	ND	ND	ND	ND	ND
E1-tramadol	656.0 ± 0.9	682.9 ± 1.1	821.3 ± 97.9	716.2 ± 25.5	768.2 ± 10.4	802.2 ± 16.2	731.1 ± 74.3
E2-tramadol	499.5 ± 8.5	554.5 ± 11.8	692.7 ± 89.4	574.2 ± 3.8	577.9 ± 5.1	586.5 ± 34.4	498.8 ± 48.8
O-desmethyltramadol	1141.0 ± 272.4	1228.8 ± 90.4	1949.5 ± 41.4	1336.1 ± 25.8	1683.2 ± 63.9	1654.4 ± 138.2	1225.1 ± 106.2
Methadone	ND	ND	ND	ND	ND	ND	ND

EDDP	ND	ND	ND	ND	ND	ND	ND
Antidepressants							
E1-venlafaxine	90.9 ± 1.1	102.2 ± 2.0	112.5 ± 1.8	108.9 ± 18.0	106.6 ± 3.5	111.3 ± 3.1	105.1 ± 16.9
E2-venlafaxine	68.1 ± 2.8	81.2 ± 0.7	90.3 ± 3.7	98.0 ± 5.1	81.1 ± 1.9	83.1 ± 1.9	81.1 ± 15.7
E1-desmethylvenlafaxine	324.4 ± 23.8	398.9 ± 17.3	572.8 ± 22.0	460.4 ± 58.5	469.8 ± 8.2	452.5 ± 26.8	312.6 ± 51.0
E2-desmethylvenlafaxine	188.7 ± 6.3	235.0 ± 31.1	382.4 ± 42.1	294.5 ± 36.4	307.5 ± 13.5	268.4 ± 36.6	216.7 ± 20.9
Anaesthetics							
Ketamine	ND	2.8 ± 0.4	5.4 ± 0.9	4.2 ± 0.3	3.3 ± 1.1	ND	2.8 ± 1.0
Human biomarkers							
Caffeine	409.1 ± 115.4	591.1 ± 15.7	864.4 ± 71.7	273.3 ± 24.4	145.8 ± 33.6	171.0 ± 25.7	298.9 ± 76.3
1,7-dimethylxanthine	2789.5 ± 1670.4	4250.9 ± 180.3	2905.7 ± 397.3	2103.4 ± 128.4	1441.4 ± 41.2	1232.6 ± 118.3	1505.7 ± 277.7
Nicotine	1053.6 ± 765.2	1751.9 ± 10.8	1301.1 ± 327.5	331.0 ± 119.4	282.2 ± 9.1	481.9 ± 26.8	1325.8 ± 371.7
Cotinine	66.4 ± 38.4	104.3 ± 5.1	73.1 ± 11.2	34.6 ± 1.8	27.5 ± 2.5	34.2 ± 7.3	55.1 ± 11.6

Table C7: Mass loads (g.day^{-1}) calculated from **Effluent** wastewater at **WWTW1** over a 7-day period.

Compound	Mass Load (g.day^{-1})						
	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Stimulants							
Cocaine	0.03 ± 0.002	ND	ND	ND	ND	ND	ND
Benzoylecgonine	0.4 ± 0.001	0.6 ± 0.02	0.7 ± 0.2	ND	0.6 ± 0.02	0.5 ± 0.04	2.6 ± 0.1
R-(-)-amphetamine	ND	ND	ND	ND	ND	ND	ND
S-(+)-amphetamine	ND	ND	ND	ND	ND	ND	0.1 ± 0.01
R-(-)-methamphetamine	1.5 ± 0.1	1.1 ± 0.001	1.3 ± 0.2	2.1 ± 0.02	2.0 ± 0.2	1.5 ± 0.02	2.2 ± 0.2
S-(+)-methamphetamine	1.0 ± 0.2	0.2 ± 0.04	0.2 ± 0.05	0.1 ± 0.02	0.3 ± 0.01	0.3 ± 0.02	7.3 ± 0.8
R-(+)-mephedrone	ND	ND	ND	ND	ND	ND	ND
S-(-)-mephedrone	ND	ND	ND	ND	ND	ND	ND
Precursors							
1S,2R-(+)-ephedrine	ND	ND	ND	ND	ND	ND	ND
1R,2S-(-)-ephedrine/*1R,2R*-(-)-pseudoephedrine	1.3 ± 0.03	1.9 ± 0.0002	2.1 ± 0.1	2.5 ± 0.2	1.3 ± 0.03	1.5 ± 0.02	10.7 ± 0.9
1S,2S-(+)-pseudoephedrine	ND	ND	ND	ND	ND	ND	3.6 ± 0.1
E1-norephedrine	0.03 ± 0.04	ND	ND	0.3 ± 0.09	0.1 ± 0.02	0.03 ± 0.02	ND
E2-norephedrine	ND	ND	ND	ND	ND	ND	0.5 ± 0.03
Hallucinogens							
R-(-)-MDMA	ND	0.01 ± 0.001	ND	ND	ND	ND	ND
S-(+)-MDMA	ND	ND	ND	ND	ND	ND	ND
E1-HMA	ND	ND	ND	ND	ND	ND	ND
E2-HMA	ND	ND	ND	ND	ND	ND	ND
R-(-)-HMMA	ND	ND	ND	ND	ND	ND	ND
S-(+)-HMMA	ND	ND	ND	ND	ND	ND	ND
Opioids							
Codeine	3.5 ± 0.3	2.5 ± 0.01	2.8 ± 0.1	0.6 ± 0.2	3.1 ± 0.2	0.5 ± 0.2	7.3 ± 0.1
Dihydrocodeine	ND	ND	ND	ND	0.2 ± 0.01	ND	ND
Hydrocodone	ND	ND	1.6 ± 0.2	1.1 ± 0.03	1.7 ± 0.1	ND	ND
Oxycodone	ND	ND	ND	ND	ND	ND	ND
Morphine	ND	ND	ND	ND	ND	ND	ND
Normorphine	ND	ND	ND	ND	ND	ND	ND
Heroin	ND	ND	ND	ND	ND	ND	ND
O-6-monoacetylmorphine	ND	ND	ND	ND	ND	ND	ND
E1-tramadol	13.9 ± 0.3	12.1 ± 0.6	16.8 ± 0.5	15.6 ± 0.3	16.4 ± 1.2	9.2 ± 0.1	12.4 ± 1.0

E2-tramadol	11.7 ± 0.2	8.7 ± 0.03	12.0 ± 0.5	12.7 ± 0.6	11.7 ± 0.3	7.3 ± 0.4	10.1 ± 0.5
O-desmethyltramadol	23.9 ± 1.9	32.5 ± 1.0	37.9 ± 1.4	33.0 ± 2.0	37.0 ± 3.5	12.5 ± 2.3	7.7 ± 0.1
Methadone	ND	ND	ND	ND	ND	ND	ND
EDDP	ND	ND	ND	ND	ND	ND	ND
Antidepressants							
E1-venlafaxine	3.0 ± 0.1	2.4 ± 0.2	3.5 ± 0.3	3.7 ± 0.02	3.5 ± 0.2	2.1 ± 0.2	2.1 ± 0.3
E2-venlafaxine	2.3 ± 0.1	1.8 ± 0.1	2.7 ± 0.5	2.7 ± 0.3	2.6 ± 0.04	1.6 ± 0.02	1.9 ± 0.2
E1-desmethylvenlafaxine	14.8 ± 0.4	12.6 ± 0.1	16.8 ± 0.9	13.4 ± 0.2	18.2 ± 0.6	6.6 ± 0.2	5.5 ± 0.4
E2-desmethylvenlafaxine	8.5 ± 0.2	7.1 ± 1.4	9.6 ± 1.7	7.9 ± 0.04	10.1 ± 0.5	3.4 ± 0.6	2.8 ± 1.1
Anaesthetics							
Ketamine	1.3 ± 0.2	0.5 ± 0.001	0.3 ± 0.04	0.3 ± 0.02	1.2 ± 0.02	2.9 ± 0.1	1.4 ± 0.3
Human biomarkers							
Caffeine	76.0 ± 14.7	26.2 ± 0.8	32.5 ± 2.8	5.0 ± 2.1	4.4 ± 0.9	7.6 ± 0.8	1165.5 ± 42.4
1,7-dimethylxanthine	44.6 ± 22.7	44.2 ± 0.8	94.5 ± 5.8	13.0 ± 0.05	12.4 ± 0.3	15.9 ± 0.2	1373.3 ± 54.4
Nicotine	9.1 ± 7.8	18.8 ± 0.3	27.6 ± 5.8	10.4 ± 2.8	26.1 ± 4.5	9.3 ± 3.6	10.2 ± 1.8
Cotinine	1.7 ± 1.0	3.7 ± 0.04	4.4 ± 0.05	1.4 ± 0.005	2.3 ± 0.02	1.5 ± 0.2	16.3 ± 0.3

Table C8: Mass loads (g.day^{-1}) calculated from **Effluent** wastewater at **WWTW2** over a 7-day period.

Compound	Mass loads (g.day^{-1})						
	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Stimulants							
Cocaine	0.1 ± 0.02	0.1 ± 0.005	0.1 ± 0.1	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.04	ND
Benzoylecgonine	3.2 ± 1.0	2.7 ± 0.1	4.1 ± 0.5	2.7 ± 0.3	2.2 ± 0.03	2.8 ± 0.2	3.3 ± 0.4
R-(-)-amphetamine	ND	ND	ND	ND	ND	ND	ND
S-(+)-amphetamine	ND	ND	ND	ND	ND	ND	ND
R-(-)-methamphetamine	2.0 ± 0.2	2.1 ± 0.02	2.1 ± 0.1	2.0 ± 0.1	2.0 ± 0.1	1.9 ± 0.3	1.7 ± 0.3
S-(+)-methamphetamine	11.0 ± 0.2	11.8 ± 0.1	8.1 ± 0.7	8.6 ± 0.4	7.6 ± 0.5	7.1 ± 0.4	6.8 ± 1.2
R-(+)-mephedrone	ND	ND	ND	ND	ND	ND	ND
S-(-)-mephedrone	ND	ND	ND	ND	ND	ND	ND
Precursors							
1S,2R-(+)-ephedrine	ND	ND	ND	ND	ND	ND	ND
1R,2S-(-)-ephedrine/*1R,2R*-(-)-pseudoephedrine	4.7 ± 0.2	4.5 ± 0.1	4.8 ± 0.01	5.3 ± 0.1	3.8 ± 0.4	3.2 ± 0.3	2.9 ± 0.1
1S,2S-(+)-pseudoephedrine	1.8 ± 0.3	2.0 ± 0.2	ND	ND	ND	1.0 ± 0.1	1.0 ± 0.1
E1-norephedrine	0.4 ± 0.2	0.4 ± 0.03	0.5 ± 0.04	0.3 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	0.3 ± 0.1
E2-norephedrine	ND	0.1 ± 0.1	ND	ND	ND	ND	ND
Hallucinogens							
R-(-)-MDMA	0.8 ± 0.2	0.8 ± 0.04	0.3 ± 0.04	0.3 ± 0.02	0.3 ± 0.005	0.3 ± 0.04	0.5 ± 0.1
S-(+)-MDMA	ND	ND	ND	ND	ND	ND	0.1 ± 0.01
E1-HMA	ND	ND	ND	ND	ND	ND	ND
E2-HMA	ND	ND	ND	ND	ND	ND	ND
R-(-)-HMMA	ND	ND	ND	ND	ND	ND	ND
S-(+)-HMMA	ND	ND	ND	ND	ND	ND	ND
Opioids							
Codeine	ND	6.9 ± 0.1	1.7 ± 0.1	3.5 ± 0.3	7.4 ± 0.5	1.8 ± 0.5	ND
Dihydrocodeine	ND	0.4 ± 0.02	ND	ND	ND	ND	ND
Hydrocodone	4.9 ± 1.6	5.3 ± 0.4	ND	ND	13.1 ± 0.3	10.3 ± 2.0	5.1 ± 0.7
Oxycodone	ND	ND	ND	ND	ND	ND	ND
Morphine	ND	ND	ND	ND	2.2 ± 0.4	ND	ND
Normorphine	ND	ND	ND	ND	ND	ND	ND
Heroin	ND	ND	ND	ND	ND	ND	ND
O-6-monoacetylmorphine	ND	ND	ND	ND	ND	ND	ND
E1-tramadol	53.5 ± 0.1	53.0 ± 0.1	63.1 ± 4.0	59.2 ± 2.1	60.5 ± 1.2	55.4 ± 1.7	45.5 ± 7.3

E2-tramadol	40.7 ± 0.6	43.0 ± 1.1	53.2 ± 3.6	47.4 ± 0.3	45.5 ± 0.6	40.5 ± 3.7	31.0 ± 4.8
O-desmethyltramadol	93.0 ± 20.6	95.4 ± 8.6	149.7 ± 1.7	110.4 ± 2.1	132.5 ± 5.0	114.35 ± 9.5	76.2 ± 6.6
Methadone	ND	ND	ND	ND	ND	ND	ND
EDDP	0.06 ± 0.01	ND	ND	ND	ND	ND	0.2 ± 0.003
Antidepressants							
E1-venlafaxine	7.4 ± 0.1	7.9 ± 0.2	8.6 ± 0.1	9.0 ± 1.5	8.4 ± 0.4	7.7 ± 0.3	6.5 ± 1.7
E2-venlafaxine	5.6 ± 0.2	6.3 ± 0.1	6.9 ± 0.2	8.1 ± 0.4	6.4 ± 0.2	5.7 ± 0.2	5.0 ± 1.5
E1-desmethylvenlafaxine	26.4 ± 1.9	31.0 ± 1.3	44.0 ± 1.7	38.0 ± 4.8	37.0 ± 0.6	31.3 ± 1.9	19.4 ± 3.2
E2-desmethylvenlafaxine	15.4 ± 0.5	18.2 ± 2.4	29.4 ± 3.2	24.3 ± 3.0	24.2 ± 1.1	18.5 ± 2.5	13.5 ± 1.3
Anaesthetics							
Ketamine	ND	0.2 ± 0.04	0.4 ± 0.04	0.4 ± 0.02	0.3 ± 0.1	ND	0.2 ± 0.1
Human biomarkers							
Caffeine	33.3 ± 8.7	45.9 ± 1.5	66.4 ± 2.9	22.6 ± 2.0	11.5 ± 3.8	11.8 ± 2.8	18.6 ± 7.5
1,7-dimethylxanthine	227.3 ± 126.1	329.9 ± 17.1	223.9 ± 16.2	173.7 ± 10.4	113.4 ± 4.7	85.2 ± 12.7	93.7 ± 27.4
Nicotine	85.9 ± 57.8	135.9 ± 1.0	99.9 ± 13.3	27.3 ± 9.6	22.2 ± 1.0	33.3 ± 2.9	82.5 ± 36.6
Cotinine	5.4 ± 2.9	8.1 ± 0.5	5.6 ± 0.5	2.9 ± 0.1	2.2 ± 0.3	2.4 ± 0.8	3.4 ± 1.1

Table C9: Concentrations (ng.L^{-1}) calculated from **RAS$_{liquid}$** samples at **WWTW1** over a 7-day period.

Compound	Concentration (ng.L^{-1})						
	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Stimulants							
Cocaine	ND	ND	ND	0.3 ± 0.004	ND	ND	0.4 ± 0.001
Benzoylecgonine	ND	ND	ND	ND	ND	ND	ND
R-(-)-amphetamine	ND	ND	ND	ND	ND	ND	ND
S-(+)-amphetamine	ND	ND	ND	ND	ND	ND	ND
R-(-)-methamphetamine	ND	32.9 ± 0.3	45.2 ± 4.1	35.3 ± 1.0	54.3 ± 9.0	ND	84.1 ± 1.5
S-(+)-methamphetamine	ND	2.5 ± 0.7	5.1 ± 1.2	9.4 ± 1.0	6.1 ± 0.7	ND	264.4 ± 6.9
R-(+)-mephedrone	ND	ND	ND	ND	ND	ND	ND
S-(-)-mephedrone	ND	ND	ND	ND	ND	ND	ND
Precursors							
1S,2R-(+)-ephedrine	ND	ND	5.6 ± 0.6	8.5 ± 0.7	8.3 ± 0.2	ND	8.8 ± 1.0
1R,2S-(-)-ephedrine/*1R,2R*-(-)-pseudoephedrine	ND	ND	23.5 ± 1.6	60.5 ± 1.9	55.6 ± 1.5	ND	218.0 ± 11.3
1S,2S-(+)-pseudoephedrine	ND	ND	ND	9.1 ± 0.7	ND	ND	45.2 ± 0.3
E1-norephedrine	ND	ND	ND	ND	ND	ND	ND
E2-norephedrine	ND	ND	ND	ND	ND	ND	ND
Hallucinogens							
R-(-)-MDMA	ND	ND	ND	ND	0.5 ± 0.01	ND	0.9 ± 0.04
S-(+)-MDMA	ND	ND	ND	ND	ND	ND	ND
E1-HMA	ND	ND	ND	ND	ND	ND	ND
E2-HMA	ND	ND	ND	ND	ND	ND	ND
R-(-)-HMMA	ND	ND	ND	ND	ND	ND	ND
S-(+)-HMMA	ND	ND	ND	ND	ND	ND	ND
Opioids							
Codeine	ND	5.3 ± 0.3	8.9 ± 2.0	15.1 ± 2.7	4.8 ± 0.01	ND	274.6 ± 29.9
Dihydrocodeine	ND	ND	ND	ND	ND	ND	ND
Hydrocodone	ND	40.8 ± 3.9	58.3 ± 6.2	57.6 ± 3.7	47.6 ± 1.3	ND	60.2 ± 4.7
Oxycodone	ND	ND	ND	ND	ND	ND	ND
Morphine	ND	4.9 ± 1.4	2.7 ± 0.4	5.3 ± 2.1	3.1 ± 0.01	ND	47.7 ± 5.8
Normorphine	ND	ND	ND	ND	ND	ND	ND
Heroin	ND	ND	ND	ND	ND	ND	ND
O-6-monoacetylmorphine	ND	ND	ND	ND	ND	ND	ND

E1-tramadol	ND	474.4 ± 20.4	444.6 ± 71.6	298.7 ± 3.8	442.8 ± 72.2	ND	423.7 ± 0.6
E2-tramadol	ND	355.2 ± 23.9	322.1 ± 21.6	220.4 ± 11.7	327.0 ± 60.3	ND	353.1 ± 7.4
O-desmethyltramadol	ND	625.2 ± 36.2	660.1 ± 4.0	430.9 ± 8.2	620.5 ± 46.6	ND	791.2 ± 104.9
Methadone	ND						
EDDP	ND						
Antidepressants							
E1-venlafaxine	ND	106.1 ± 0.3	97.3 ± 21.2	66.1 ± 2.1	97.5 ± 14.4	ND	90.8 ± 0.4
E2-venlafaxine	ND	79.8 ± 3.2	75.5 ± 2.0	58.1 ± 1.0	73.4 ± 16.8	ND	74.4 ± 1.6
E1-desmethylvenlafaxine	ND	418.1 ± 15.7	434.2 ± 36.5	247.7 ± 12.2	412.3 ± 79.2	ND	439.3 ± 7.6
E2-desmethylvenlafaxine	ND	410.8 ± 51.0	275.6 ± 14.0	149.3 ± 6.5	240.8 ± 42.9	ND	238.7 ± 0.8
Anaesthetics							
Ketamine	ND	13.1 ± 1.5	11.5 ± 1.8	14.0 ± 0.01	197.7 ± 37.3	ND	62.6 ± 3.5
Human biomarkers							
Caffeine	ND	234.8 ± 4.6	194.8 ± 34.3	202.9 ± 14.3	452.2 ± 35.8	ND	316.5 ± 16.2
1,7-dimethylxanthine	ND	566.0 ± 21.2	551.9 ± 157.9	640.9 ± 33.4	1545.4 ± 4.3	ND	3282.9 ± 84.9
Nicotine	ND	381.9 ± 41.5	423.8 ± 8.5	788.4 ± 27.6	825.2 ± 15.0	ND	259.0 ± 86.3
Cotinine	ND	ND	ND	ND	ND	ND	ND

Table C10: Concentrations (ng.L^{-1}) calculated from **RAS$_{liquid}$** samples at **WWTW2** over a 7-day period.

Compound	Concentration (ng.L^{-1})						
	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Stimulants							
Cocaine	0.4 ± 0.001	ND	1.0 ± 0.1	ND	ND	ND	0.3 ± 0.004
Benzoylecgonine	ND	ND	ND	ND	ND	ND	ND
R-(-)-amphetamine	ND	ND	ND	ND	ND	ND	ND
S-(+)-amphetamine	ND	ND	ND	ND	ND	ND	ND
R-(-)-methamphetamine	32.0 ± 0.1	31.8 ± 2.1	31.1 ± 4.6	25.2 ± 4.4	30.6 ± 1.8	ND	32.3 ± 3.3
S-(+)-methamphetamine	108.1 ± 6.6	130.8 ± 16.3	79.2 ± 4.2	90.7 ± 6.6	84.3 ± 13.1	ND	76.4 ± 13.0
R-(+)-mephedrone	ND	ND	ND	ND	ND	ND	ND
S-(-)-mephedrone	ND	ND	ND	ND	ND	ND	ND
Precursors							
1S,2R-(+)-ephedrine	ND	ND	ND	ND	ND	ND	ND
1R,2S-(-)-ephedrine/*1R,2R*-(-)-pseudoephedrine	ND	ND	ND	ND	ND	ND	ND
1S,2S-(+)-pseudoephedrine	9.8 ± 0.7	9.2 ± 0.1	ND	ND	7.2 ± 0.8	ND	ND
E1-norephedrine	ND	ND	ND	ND	ND	ND	ND
E2-norephedrine	ND	ND	ND	ND	ND	ND	ND
Hallucinogens							
R-(-)-MDMA	6.4 ± 0.1	8.1 ± 0.7	6.4 ± 1.3	4.1 ± 0.6	2.6 ± 0.04	ND	3.4 ± 0.7
S-(+)-MDMA	ND	1.1 ± 0.1	ND	0.4 ± 0.01	ND	ND	ND
E1-HMA	ND	ND	ND	ND	ND	ND	ND
E2-HMA	ND	ND	ND	ND	ND	ND	ND
R-(-)-HMMA	ND	ND	ND	ND	ND	ND	ND
S-(+)-HMMA	ND	ND	ND	ND	ND	ND	ND
Opioids							
Codeine	7.1 ± 1.2	8.0 ± 0.4	6.6 ± 0.3	15.1 ± 1.0	15.3 ± 0.6	ND	8.8 ± 0.8
Dihydrocodeine	ND	ND	ND	ND	ND	ND	ND
Hydrocodone	180.2 ± 14.3	184.7 ± 18.7	89.7 ± 5.2	213.5 ± 1.9	318.2 ± 12.7	ND	269.0 ± 16.5
Oxycodone	ND	ND	ND	ND	ND	ND	ND
Morphine	4.9 ± 0.5	8.2 ± 4.5	15.4 ± 1.0	14.4 ± 1.5	21.7 ± 3.4	±	8.2 ± 0.4
Normorphine	ND	ND	ND	ND	ND	ND	ND
Heroin	ND	ND	ND	ND	ND	ND	ND
O-6-monoacetylmorphine	ND	ND	ND	ND	ND	ND	ND

E1-tramadol	1005.6 ± 56.9	936.1 ± 37.1	1047.9 ± 102.5	989.3 ± 8.0	940.0 ± 34.8	ND	926.6 ± 154.7
E2-tramadol	701.3 ± 14.1	726.9 ± 37.7	806.1 ± 51.4	755.6 ± 35.6	683.4 ± 33.4	ND	679.1 ± 106.1
O-desmethyltramadol	1910.2 ± 107.8	1980.3 ± 332.8	2194.4 ± 214.3	1829.4 ± 64.0	1773.0 ± 224.9	ND	1701.8 ± 35.5
Methadone	ND	ND	ND	ND	ND	ND	ND
EDDP	ND	ND	ND	ND	ND	ND	ND
Antidepressants							
E1-venlafaxine	122.0 ± 0.7	148.2 ± 6.6	156.7 ± 17.7	136.3 ± 5.9	123.2 ± 4.7	ND	119.1 ± 22.6
E2-venlafaxine	104.3 ± 10.7	127.8 ± 7.0	134.6 ± 10.1	118.8 ± 1.5	112.1 ± 3.8	ND	108.1 ± 11.3
E1-desmethylvenlafaxine	713.8 ± 9.3	625.2 ± 51.3	547.0 ± 164.6	725.7 ± 49.8	676.4 ± 77.0	ND	594.3 ± 59.5
E2-desmethylvenlafaxine	382.6 ± 0.2	380.1 ± 5.8	393.9 ± 10.1	416.6 ± 1.8	401.8 ± 23.5	ND	378.9 ± 5.8
Anaesthetics							
Ketamine	ND	3.1 ± 0.04	3.5 ± 1.3	3.9 ± 0.7	3.8 ± 0.6	ND	4.3 ± 1.0
Human biomarkers							
Caffeine	349.1 ± 48.2	289.1 ± 280.8	362.5 ± 22.1	457.6 ± 100.6	196.9 ± 27.5	ND	1656.1 ± 458.1
1,7-dimethylxanthine	1376.8 ± 121.7	864.2 ± 111.5	684.0 ± 36.8	ND	575.7 ± 65.8	ND	5159.4 ± 352.4
Nicotine	740.8 ± 1.1	125.1 ± 54.4	418.9 ± 169.6	688.7 ± 22.4	337.6 ± 29.3	ND	4523.4 ± 4.0
Cotinine	ND	ND	ND	ND	ND	ND	ND

Table C11: Concentrations (ng.L^{-1}) calculated from **Upstream** river water at **WWTW1** over the 7-day sampling period.

Compound	Concentration (ng.L^{-1})						
	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Stimulants							
Cocaine	ND	ND	ND	ND	ND	ND	ND
Benzoylecgonine	4.7 ± 0.03	3.7 ± 0.1	2.8 ± 1.2	ND	ND	2.7 ± 0.4	2.8 ± 0.8
R-(-)-amphetamine	ND	ND	ND	ND	ND	ND	ND
S-(+)-amphetamine	ND	ND	ND	ND	ND	ND	ND
R-(-)-methamphetamine	5.8 ± 0.3	5.8 ± 1.0	3.2 ± 0.05	3.7 ± 0.3	4.4 ± 0.3	5.3 ± 0.7	5.2 ± 0.4
S-(+)-methamphetamine	1.3 ± 0.1	5.0 ± 1.0	1.0 ± 0.2	1.0 ± 0.03	0.9 ± 0.2	0.8 ± 0.2	1.0 ± 0.05
R-(+)-mephedrone	ND	ND	ND	ND	ND	ND	ND
S-(-)-mephedrone	ND	ND	ND	ND	ND	ND	ND
Precursors							
1S,2R-(+)-ephedrine	0.5 ± 0.3	0.6 ± 0.2	0.2 ± 0.02	0.5 ± 0.03	1.1 ± 0.1	0.9 ± 0.1	0.7 ± 0.1
1R,2S-(-)-ephedrine/*1R,2R*-(-)-pseudoephedrine	18.8 ± 0.1	22.9 ± 0.8	16.9 ± 0.3	18.2 ± 0.4	20.3 ± 0.3	20.0 ± 0.2	18.6 ± 0.4
1S,2S-(+)-pseudoephedrine	3.6 ± 0.03	5.6 ± 0.1	3.1 ± 0.3	3.7 ± 0.1	4.1 ± 0.1	3.8 ± 0.04	3.5 ± 0.1
E1-norephedrine	ND	ND	ND	ND	ND	ND	ND
E2-norephedrine	ND	ND	ND	ND	ND	ND	ND
Hallucinogens							
R-(-)-MDMA	ND	ND	ND	ND	ND	ND	ND
S-(+)-MDMA	ND	ND	ND	ND	ND	ND	ND
E1-HMA	ND	ND	ND	ND	ND	ND	ND
E2-HMA	ND	ND	ND	ND	ND	ND	ND
R-(-)-HMMA	ND	ND	ND	ND	ND	ND	ND
S-(+)-HMMA	ND	ND	ND	ND	ND	ND	ND
Opioids							
Codeine	5.5 ± 1.4	9.4 ± 1.3	ND	4.1 ± 0.3	ND	4.2 ± 1.5	5.0 ± 0.3
Dihydrocodeine	ND	ND	ND	ND	ND	ND	ND
Hydrocodone	ND	4.3 ± 0.03	ND	ND	ND	4.5 ± 0.3	5.2 ± 0.3
Oxycodone	ND	ND	ND	ND	ND	ND	ND
Morphine	ND	ND	ND	ND	ND	ND	ND
Normorphine	ND	ND	ND	ND	ND	ND	ND
Heroin	ND	ND	ND	ND	ND	ND	ND
O-6-monoacetylmorphine	ND	ND	ND	ND	ND	ND	ND

E1-tramadol	48.5 ± 0.4	40.9 ± 2.9	21.3 ± 1.7	21.5 ± 1.5	33.5 ± 3.1	43.0 ± 2.2	54.3 ± 1.9
E2-tramadol	41.6 ± 0.5	32.5 ± 2.4	15.3 ± 1.6	19.5 ± 0.3	29.4 ± 0.2	32.9 ± 2.2	44.6 ± 1.4
O-desmethyltramadol	73.2 ± 2.3	56.8 ± 1.1	53.3 ± 2.6	53.4 ± 1.7	51.9 ± 1.1	66.9 ± 0.4	72.1 ± 1.1
Methadone	ND	ND	ND	ND	ND	ND	ND
EDDP	ND	ND	ND	ND	ND	ND	ND
Antidepressants							
E1-venlafaxine	8.8 ± 0.5	6.5 ± 0.3	2.5 ± 0.02	3.6 ± 0.2	5.1 ± 0.2	8.1 ± 0.4	8.9 ± 0.4
E2-venlafaxine	7.3 ± 0.7	5.8 ± 0.6	2.5 ± 0.3	3.6 ± 0.2	5.3 ± 0.6	7.0 ± 0.8	7.8 ± 0.9
E1-desmethylvenlafaxine	52.2 ± 3.4	43.2 ± 0.2	12.1 ± 0.1	16.6 ± 0.2	35.9 ± 5.0	44.2 ± 7.3	51.3 ± 3.6
E2-desmethylvenlafaxine	31.0 ± 0.2	28.3 ± 0.3	9.0 ± 0.9	12.6 ± 0.2	22.4 ± 1.4	32.0 ± 1.6	34.4 ± 4.0
Anaesthetics							
Ketamine	2.9 ± 0.8	2.8 ± 0.03	1.6 ± 0.3	1.8 ± 0.2	1.7 ± 0.5	2.7 ± 0.2	2.3 ± 0.1
Human biomarkers							
Caffeine	1100.0 ± 16.1	779.1 ± 18.3	461.4 ± 40.9	405.3 ± 0.6	104.4 ± 7.7	358.5 ± 19.6	579.8 ± 12.9
1,7-dimethylxanthine	634.9 ± 32.8	1298.5 ± 31.8	505.7 ± 305.1	660.8 ± 39.0	165.1 ± 7.3	537.3 ± 6.9	345.2 ± 1.2
Nicotine	83.5 ± 0.8	108.1 ± 8.8	312.4 ± 139.7	243.1 ± 5.2	16.6 ± 2.2	69.9 ± 6.9	140.1 ± 0.3
Cotinine	22.6 ± 0.5	48.5 ± 1.3	22.8 ± 5.0	32.8 ± 1.1	14.8 ± 0.8	34.8 ± 0.9	23.7 ± 1.1

Table C12: Concentrations (ng.L^{-1}) calculated from **Downstream** river water at **WWTW1** over the 7-day sampling period.

Compound	Concentration (ng.L^{-1})						
	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Stimulants							
Cocaine	5.2 ± 0.1	ND	ND	ND	ND	ND	ND
Benzoylecgonine	34.9 ± 3.2	8.7 ± 0.6	5.4 ± 0.4	6.3 ± 0.6	6.1 ± 0.4	5.8 ± 0.3	7.4 ± 0.2
R-(-)-amphetamine	ND	ND	ND	ND	ND	ND	ND
S-(+)-amphetamine	ND	ND	ND	ND	ND	ND	ND
R-(-)-methamphetamine	31.7 ± 2.9	33.1 ± 0.2	14.0 ± 1.7	15.5 ± 2.5	24.4 ± 7.9	10.8 ± 2.0	10.7 ± 0.7
S-(+)-methamphetamine	178.8 ± 0.9	10.5 ± 0.7	2.1 ± 0.1	3.0 ± 0.7	4.8 ± 0.9	1.9 ± 0.9	1.7 ± 0.03
R-(+)-mephedrone	ND	ND	ND	ND	ND	ND	ND
S-(-)-mephedrone	ND	ND	ND	ND	ND	ND	ND
Precursors							
1S,2R-(+)-ephedrine	ND	1.7 ± 0.1	ND	1.4 ± 0.2	3.1 ± 0.03	1.6 ± 0.1	1.2 ± 0.002
1R,2S-(-)-ephedrine/*1R,2R*-(-)-pseudoephedrine	142.6 ± 14.7	30.8 ± 1.4	20.1 ± 0.3	22.4 ± 1.6	36.9 ± 0.5	23.8 ± 1.7	21.3 ± 0.1
1S,2S-(+)-pseudoephedrine	46.2 ± 6.0	5.3 ± 0.4	ND	ND	6.0 ± 0.02	ND	ND
E1-norephedrine	5.4 ± 0.2	2.0 ± 0.03	ND	ND	1.9 ± 0.1	ND	ND
E2-norephedrine	ND	ND	ND	ND	ND	ND	ND
Hallucinogens							
R-(-)-MDMA	ND	ND	ND	ND	ND	ND	ND
S-(+)-MDMA	ND	ND	ND	ND	ND	ND	ND
E1-HMA	ND	ND	ND	ND	ND	ND	ND
E2-HMA	ND	ND	ND	ND	ND	ND	ND
R-(-)-HMMA	ND	ND	ND	ND	ND	ND	ND
S-(+)-HMMA	ND	ND	ND	ND	ND	ND	ND
Opioids							
Codeine	217.5 ± 4.1	29.7 ± 0.2	11.3 ± 2.6	12.5 ± 1.0	26.5 ± 0.3	ND	4.3 ± 1.2
Dihydrocodeine	ND	ND	ND	ND	ND	ND	ND
Hydrocodone	ND	ND	ND	ND	ND	ND	ND
Oxycodone	ND	ND	ND	ND	ND	ND	ND
Morphine	ND	ND	ND	ND	ND	ND	ND
Normorphine	ND	ND	ND	ND	ND	ND	ND
Heroin	ND	ND	ND	ND	ND	ND	ND
O-6-monoacetylmorphine	ND	ND	ND	ND	ND	ND	ND

E1-tramadol	157.0 ± 0.6	196.2 ± 0.1	122.6 ± 4.9	119.5 ± 5.4	185.5 ± 40.8	60.3 ± 0.03	55.6 ± 0.4
E2-tramadol	121.2 ± 21.0	149.3 ± 10.9	94.9 ± 10.3	96.5 ± 4.7	141.6 ± 46.8	48.4 ± 1.4	38.6 ± 0.7
O-desmethyltramadol	392.5 ± 1.4	421.3 ± 10.7	251.2 ± 2.4	198.9 ± 1.9	410.1 ± 24.4	62.9 ± 1.0	64.2 ± 0.5
Methadone	ND	ND	ND	ND	ND	ND	ND
EDDP	ND	ND	ND	ND	ND	ND	ND
Antidepressants							
E1-venlafaxine	28.2 ± 1.4	47.9 ± 0.1	25.0 ± 0.9	24.9 ± 0.1	39.2 ± 8.2	16.0 ± 0.5	14.4 ± 1.7
E2-venlafaxine	22.9 ± 0.2	37.2 ± 0.4	19.3 ± 1.3	19.9 ± 0.1	28.9 ± 9.4	12.5 ± 0.5	10.9 ± 0.3
E1-desmethylvenlafaxine	144.3 ± 4.5	187.3 ± 5.3	119.1 ± 0.9	101.7 ± 4.5	149.7 ± 6.6	25.9 ± 2.3	24.0 ± 0.6
E2-desmethylvenlafaxine	76.7 ± 4.0	106.2 ± 8.0	75.4 ± 2.2	59.1 ± 2.4	101.1 ± 25.5	14.8 ± 0.4	15.5 ± 0.8
Anaesthetics							
Ketamine	22.6 ± 0.8	12.9 ± 0.04	5.6 ± 0.2	3.8 ± 0.4	4.1 ± 1.3	17.2 ± 0.5	28.3 ± 1.2
Human biomarkers							
Caffeine	21130.9 ± 1148.1	603.9 ± 0.2	652.4 ± 27.2	233.4 ± 11.9	113.0 ± 25.6	127.4 ± 30.1	161.9 ± 1.8
1,7-dimethylxanthine	21464.9 ± 2015.5	1326.7 ± 21.0	1244.3 ± 35.2	530.3 ± 54.8	216.5 ± 18.3	388.8 ± 24.3	254.9 ± 6.2
Nicotine	438.2 ± 13.9	255.2 ± 0.2	382.1 ± 0.5	349.8 ± 45.8	60.1 ± 36.8	113.6 ± 17.2	90.5 ± 8.4
Cotinine	268.4 ± 4.8	56.7 ± 2.2	55.3 ± 0.7	35.8 ± 0.01	24.0 ± 0.03	35.0 ± 0.5	25.8 ± 0.9

Table C13: Concentrations (ng.L^{-1}) calculated from **Upstream** river water at **WWTW2** over the 7-day sampling period.

Compound	Concentration (ng.L^{-1})						
	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Stimulants							
Cocaine	1.6 ± 0.3	1.1 ± 0.3	0.7 ± 0.2	0.6 ± 0.2	1.1 ± 0.9	0.6 ± 0.04	1.0 ± 0.1
Benzoylecgonine	28.7 ± 2.2	21.0 ± 0.001	18.2 ± 0.3	21.8 ± 1.9	14.2 ± 0.2	14.2 ± 1.0	26.4 ± 0.5
R-(-)-amphetamine	ND	ND	ND	ND	ND	ND	ND
S-(+)-amphetamine	ND	ND	ND	ND	ND	ND	ND
R-(-)-methamphetamine	25.9 ± 7.4	10.2 ± 1.6	13.6 ± 4.0	15.5 ± 1.0	6.1 ± 1.8	6.3 ± 0.7	20.8 ± 2.2
S-(+)-methamphetamine	465.2 ± 9.5	180.6 ± 10.4	170.3 ± 20.0	190.7 ± 4.2	139.0 ± 44.8	137.9 ± 14.4	246.9 ± 8.2
R-(+)-mephedrone	ND	ND	ND	ND	ND	ND	ND
S-(-)-mephedrone	ND	ND	ND	ND	ND	ND	ND
Precursors							
1S,2R-(+)-ephedrine	ND	ND	ND	ND	ND	ND	ND
1R,2S-(-)-ephedrine/*1R,2R*-(-)-pseudoephedrine	35.9 ± 1.1	30.6 ± 0.5	27.2 ± 0.7	29.3 ± 1.9	26.2 ± 1.5	26.4 ± 1.2	26.3 ± 1.0
1S,2S-(+)-pseudoephedrine	12.3 ± 0.05	12.1 ± 0.1	9.2 ± 0.8	20.1 ± 1.5	11.8 ± 0.6	11.9 ± 0.1	10.1 ± 1.2
E1-norephedrine	ND	ND	ND	ND	ND	ND	ND
E2-norephedrine	ND	ND	ND	ND	ND	ND	ND
Hallucinogens							
R-(-)-MDMA	1.1 ± 0.1	1.2 ± 0.1	0.8 ± 0.2	0.6 ± 0.02	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.04
S-(+)-MDMA	0.4 ± 0.04	- ±	0.3 ± 0.01	0.2 ± 0.01	0.2 ± 0.1	0.2 ± 0.02	0.2 ± 0.01
E1-HMA	ND	ND	ND	ND	ND	ND	ND
E2-HMA	ND	ND	ND	ND	ND	ND	ND
R-(-)-HMMA	ND	ND	ND	ND	ND	ND	ND
S-(+)-HMMA	ND	ND	ND	ND	ND	ND	ND
Opioids							
Codeine	51.1 ± 0.7	42.1 ± 0.03	29.4 ± 0.4	51.4 ± 0.7	48.3 ± 1.8	32.7 ± 0.6	39.4 ± 3.2
Dihydrocodeine	ND	ND	ND	ND	ND	ND	ND
Hydrocodone	17.2 ± 0.1	13.7 ± 1.0	18.6 ± 2.0	17.4 ± 0.9	15.0 ± 0.1	ND	17.4 ± 1.2
Oxycodone	ND	ND	ND	ND	ND	ND	ND
Morphine	80.0 ± 10.3	66.2 ± 1.0	56.2 ± 1.6	73.6 ± 5.5	62.1 ± 3.2	54.8 ± 3.2	55.7 ± 8.2
Normorphine	ND	ND	ND	ND	ND	ND	ND
Heroin	ND	ND	ND	ND	ND	ND	ND
O-6-monoacetylmorphine	ND	11.7 ± 0.4	ND	ND	17.5 ± 0.03	ND	ND
E1-tramadol	1458.9 ± 52.8	1408.9 ± 83.3	881.4 ± 49.6	936.3 ± 39.8	1070.4 ± 412.6	933.9 ± 43.4	1270.3 ± 9.4
E2-tramadol	1076.7 ± 0.2	1059.3 ± 45.4	594.0 ± 50.5	674.8 ± 16.7	734.9 ± 234.0	712.3 ± 47.6	956.8 ± 1.9
O-desmethyltramadol	2698.9 ± 124.5	2824.7 ± 102.1	2483.7 ± 52.7	2564.8 ± 37.5	2443.4 ± 67.1	2221.5 ± 44.8	2777.8 ± 16.8
Methadone	ND	ND	ND	ND	ND	ND	ND
EDDP	ND	ND	ND	ND	ND	ND	ND

Antidepressants							
E1-venlafaxine	46.8 ± 1.9	41.6 ± 1.2	30.1 ± 3.0	24.7 ± 1.9	29.0 ± 6.8	30.9 ± 1.5	41.8 ± 0.6
E2-venlafaxine	54.6 ± 2.6	51.4 ± 1.9	32.1 ± 1.7	31.4 ± 4.0	36.3 ± 11.4	31.8 ± 1.4	50.3 ± 0.6
E1-desmethylvenlafaxine	253.3 ± 133.6	347.8 ± 36.0	110.9 ± 34.8	185.5 ± 5.4	218.3 ± 87.5	185.7 ± 6.4	290.8 ± 4.0
E2-desmethylvenlafaxine	173.0 ± 1.0	159.1 ± 0.6	48.3 ± 38.1	102.8 ± 0.2	100.5 ± 29.4	67.2 ± 35.9	109.8 ± 38.6
Anaesthetics							
Ketamine	6.8 ± 0.1	6.6 ± 1.1	7.6 ± 0.3	7.8 ± 0.7	9.1 ± 4.9	8.8 ± 0.3	7.9 ± 1.2
Human biomarkers							
Caffeine	2276.2 ± 5.4	1529.0 ± 29.5	2449.4 ± 30.8	4316.1 ± 241.1	2480.8 ± 29.5	1313.4 ± 40.2	1691.3 ± 79.3
1,7-dimethylxanthine	12597.6 ± 172.7	8264.5 ± 17.2	6170.2 ± 59.5	15026.7 ± 1862.1	11605.7 ± 613.6	4196.1 ± 98.1	9723.8 ± 785.0
Nicotine	1359.3 ± 59.1	1184.2 ± 342.3	1121.8 ± 30.8	4332.2 ± 182.6	1966.3 ± 73.6	553.3 ± 30.9	1004.2 ± 114.1
Cotinine	399.2 ± 6.8	201.1 ± 7.8	260.4 ± 4.4	473.4 ± 8.7	280.8 ± 11.3	169.6 ± 1.1	267.7 ± 5.9

Table C14: Concentrations (ng.L^{-1}) calculated from **Downstream** river water at **WWTW2** over the 7-day sampling period.

Compound	Concentration (ng.L^{-1})						
	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Stimulants							
Cocaine	2.1 ± 0.2	0.9 ± 0.04	0.9 ± 0.2	0.4 ± 0.1	0.5 ± 0.2	0.4 ± 0.04	0.6 ± 0.3
Benzoylecgonine	31.0 ± 0.8	26.7 ± 1.3	21.3 ± 0.1	20.6 ± 0.2	13.2 ± 0.02	14.5 ± 0.4	13.5 ± 0.5
R-(-)-amphetamine	ND	ND	ND	ND	ND	ND	ND
S-(+)-amphetamine	ND	ND	ND	ND	ND	ND	ND
R-(-)-methamphetamine	28.3 ± 1.1	22.5 ± 1.6	21.5 ± 0.3	20.2 ± 1.9	9.6 ± 0.7	15.6 ± 0.9	11.8 ± 0.05
S-(+)-methamphetamine	206.1 ± 3.1	73.5 ± 5.3	119.5 ± 8.8	146.9 ± 20.3	90.8 ± 20.3	86.5 ± 0.1	69.5 ± 2.2
R-(+)-mephedrone	ND	ND	ND	ND	ND	ND	ND
S-(-)-mephedrone	ND	ND	ND	ND	ND	ND	ND
Precursors							
1S,2R-(+)-ephedrine	ND	ND	ND	ND	ND	ND	ND
1R,2S-(-)-ephedrine/*1R,2R*-(-)-pseudoephedrine	33.5 ± 0.7	35.1 ± 0.3	34.2 ± 0.8	33.2 ± 1.0	25.8 ± 2.5	30.0 ± 0.3	24.3 ± 0.2
1S,2S-(+)-pseudoephedrine	12.1 ± 0.2	14.4 ± 0.3	11.6 ± 0.1	14.4 ± 0.2	ND	ND	ND
E1-norephedrine	1.8 ± 0.2	2.4 ± 0.02	2.0 ± 0.3	2.2 ± 0.2	1.0 ± 0.3	1.9 ± 0.4	0.7 ± 0.4
E2-norephedrine	ND	ND	ND	ND	ND	ND	ND
Hallucinogens							
R-(-)-MDMA	4.6 ± 0.01	2.3 ± 0.2	3.2 ± 0.1	2.0 ± 0.1	0.9 ± 0.2	1.0 ± 0.1	0.7 ± 0.03
S-(+)-MDMA	ND	ND	ND	ND	ND	ND	ND
E1-HMA	ND	ND	ND	ND	ND	ND	ND
E2-HMA	ND	ND	ND	ND	ND	ND	ND
R-(-)-HMMA	ND	ND	ND	ND	ND	ND	ND
S-(+)-HMMA	ND	ND	ND	ND	ND	ND	ND
Opioids							
Codeine	25.3 ± 0.1	31.4 ± 1.7	16.3 ± 0.1	35.8 ± 4.2	33.6 ± 0.4	29.2 ± 1.1	21.8 ± 1.3
Dihydrocodeine	ND	ND	ND	ND	ND	ND	ND
Hydrocodone	ND	ND	ND	ND	ND	ND	ND
Oxycodone	ND	ND	ND	ND	ND	ND	ND
Morphine	27.7 ± 0.5	22.1 ± 0.1	19.5 ± 1.8	35.6 ± 3.8	55.1 ± 2.8	29.9 ± 1.3	21.2 ± 4.5
Normorphine	ND	ND	ND	ND	ND	ND	ND
Heroin	ND	ND	ND	ND	ND	ND	ND
O-6-monoacetylmorphine	ND	ND	ND	ND	ND	ND	ND
E1-tramadol	974.8 ± 18.0	711.0 ± 42.2	652.1 ± 22.7	785.0 ± 16.4	618.4 ± 33.1	703.6 ± 58.6	494.7 ± 8.0
E2-tramadol	647.5 ± 30.5	503.9 ± 7.5	470.1 ± 12.4	606.0 ± 12.0	429.2 ± 43.2	510.2 ± 3.2	368.5 ± 21.7
O-desmethyltramadol	1691.3 ± 38.1	1892.6 ± 146.6	1394.4 ± 42.1	1960.2 ± 91.5	1758.5 ± 105.5	2100.6 ± 4.9	1478.2 ± 4.7
Methadone	ND	ND	ND	ND	ND	ND	ND
EDDP	ND	ND	ND	ND	ND	ND	ND

Antidepressants							
E1-venlafaxine	81.4 ± 0.3	104.5 ± 5.7	60.2 ± 4.5	62.3 ± 10.7	37.2 ± 1.8	44.6 ± 5.3	37.7 ± 1.2
E2-venlafaxine	68.7 ± 0.6	83.4 ± 2.8	47.2 ± 2.0	51.9 ± 10.7	26.9 ± 1.1	36.3 ± 2.7	31.6 ± 1.6
E1-desmethylvenlafaxine	297.1 ± 7.4	423.4 ± 49.5	231.7 ± 11.4	308.4 ± 12.1	163.0 ± 7.4	215.7 ± 30.3	153.8 ± 3.9
E2-desmethylvenlafaxine	142.9 ± 6.2	241.0 ± 17.2	122.1 ± 5.0	159.2 ± 2.8	84.9 ± 6.5	111.7 ± 15.1	84.3 ± 4.5
Anaesthetics							
Ketamine	6.7 ± 0.1	5.0 ± 0.6	6.2 ± 0.04	5.6 ± 0.4	5.9 ± 0.4	7.9 ± 0.99	3.9 ± 0.3
Human biomarkers							
Caffeine	792.6 ± 13.6	187.3 ± 7.9	806.8 ± 122.1	1605.1 ± 36.3	1894.4 ± 31.6	697.4 ± 36.5	1755.4 ± 65.7
1,7-dimethylxanthine	4620.0 ± 168.4	1308.5 ± 41.2	2192.1 ± 63.5	6130.5 ± 395.5	5417.6 ± 710.1	3049.1 ± 366.8	5910.9 ± 156.9
Nicotine	635.8 ± 2.5	574.2 ± 3.6	492.6 ± 21.4	2176.7 ± 122.7	2527.5 ± 147.2	280.6 ± 2.3	7007.5 ± 84.6
Cotinine	154.9 ± 4.2	46.3 ± 0.5	95.5 ± 2.0	212.0 ± 0.2	189.1 ± 0.6	97.6 ± 4.7	297.9 ± 4.9

APPENDIX D: SUPPLEMENTARY INFORMATION FOR CHAPTER 6

Wastewater-Based Epidemiology and Enantiomeric Profiling for Drugs of Abuse in South African Wastewater

Table D1: Plant information of the two WWTWs during the study.

	Capacity ($ML.day^{-1}$)	Domestic: Industrial	HRT[a] (hours)	Population Estimate[b]	$Flow_{inf}$[c] ($ML.day^{-1}$)
WWTW1	63.0	60:40	24	200 000	42.6
WWTW2	105.0	80:20	24	470 000	79.9

[a] Hydraulic retention time (in hours) of wastewater within the treatment process.
[b] *de facto* population estimate based on projected census data for 2017 from the last formal census done in 2011.
[c] Average flow rate (in megalitres per day) of wastewater influent during the sampling period.

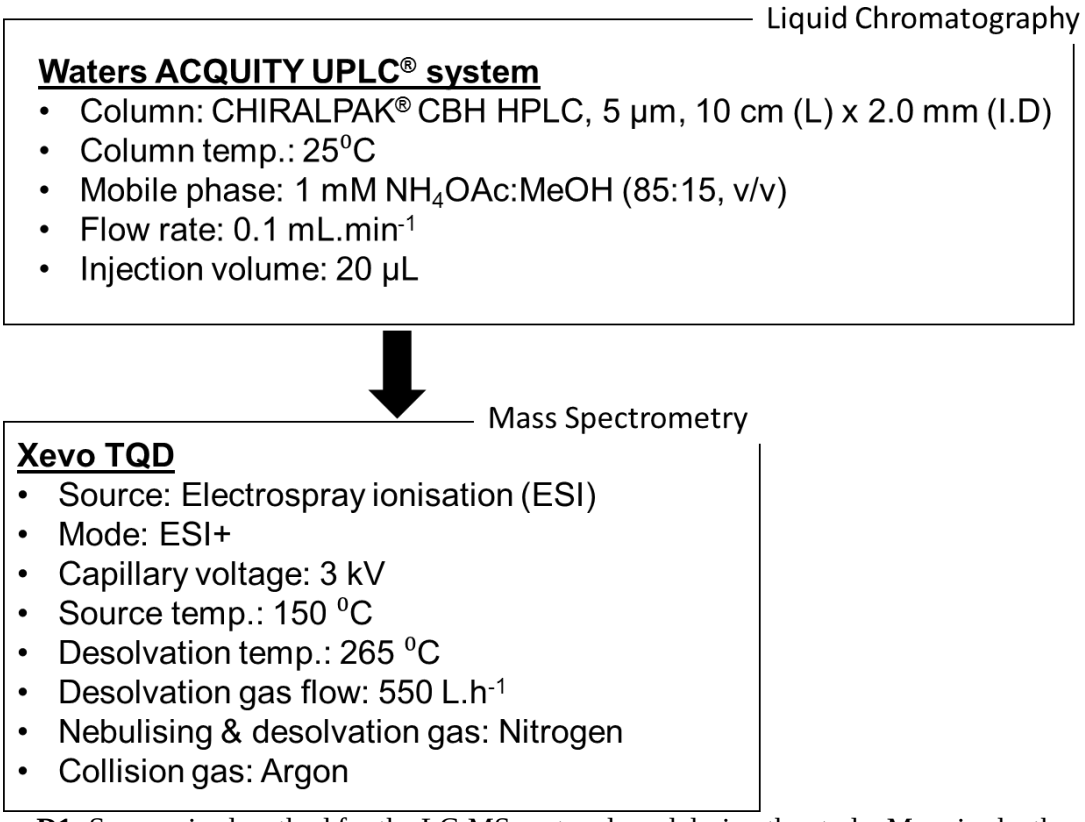

Figure D1: Summarised method for the LC-MS protocol used during the study. More in-depth method validation can be found in Castignanò et al., 2016.

Table D2: Correction factors for the excretion profiles of the DTRs in faeces/urine as well as their molecular mass ratios used for mass load and population-normalised mass load estimates.

Drug	DTR	Excretion (%)	Mw (parent/DTR)	CF
Cocaine	Benzoylecgonine	29.0[a]	1.05	3.6
Methamphetamine	Methamphetamine	43.0[a]	1.0	2.3
	S-(+)-methamphetamine	40.9[b]	1.0	2.4
	R-(-)-methamphetamine	44.2[b]	1.0	2.3
	Amphetamine	4.0-7.0[c]	1.1	20.1
	Norephedrine	5.0[c]	0.99	19.7
MDMA	MDMA	22.5[d]	1.0	4.4[g]
	R-(-)-MDMA	35.5[e]	1.0	2.8
	S-(+)-MDMA	9.8[e]	1.0	10.2
	HMMA	18.2[d]	0.99	5.0[c]
Mephedrone	Mephedrone	15.4[f]	1.0	6.5[c]

[a] Castiglioni et al., 2016
[b] Stereoselective excretion after oral administration of racemic methamphetamine (Gracia-lor et al., 2016)
[c] Castrignanò et al.,2017a
[d] Gracia-lor et al., 2016
[e] Stereoselective excretion of an oral racemic MDMA administration (Lanz et al., 1997)
[f] Bade et al., 2017
[g] González-Mariño et al., 2017

Table D3: Concentrations (ng.L^{-1}) calculated from raw wastewater at WWTW1 over a 7-day period.

Compound	Concentration (ng.L^{-1})						
	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Stimulants							
Cocaine	52.4 ± 3.1	60.8 ± 7.7	40.9 ± 6.1	91.3 ± 3.9	52.4 ± 0.1	145.1 ± 14.8	58.6 ± 2.5
Benzoylecgonine	169.1 ± 5.5	197.6 ± 8.3	197.5 ± 8.5	162.3 ± 8.2	129.8 ± 4.5	346.3 ± 32.0	233.1 ± 3.5
Cocaethylene	1.0 ± 0.2	1.0 ± 0.5	ND	ND	ND	1.9 ± 0.1	2.2 ± 0.3
R-(-)-Amphetamine	ND	ND	ND	ND	ND	ND	ND
S-(+)-Amphetamine	20.2 ± 1.3	15.6 ± 5.8	15.5 ± 9.0	13.1 ± 3.5	18.8 ± 5.4	20.7 ± 5.4	17.0 ± 2.7
R-(-)-Methamphetamine	96.1 ± 11.0	69.1 ± 11.1	55.7 ± 8.8	83.0 ± 5.3	78.5 ± 1.3	102.7 ± 2.4	121.8 ± 9.2
S-(+)-Methamphetamine	444.2 ± 53.4	429.3 ± 54.2	248.5 ± 43.6	609.4 ± 12.2	417.0 ± 16.7	394.0 ± 35.1	773.9 ± 38.9
R-(+)-Mephedrone	ND	ND	ND	ND	ND	ND	ND
S-(-)-Mephedrone	ND	ND	ND	ND	ND	ND	ND
Precursors							
1S,2R-(+)-Ephedrine	ND	ND	ND	ND	ND	ND	ND
1R,2S-(-)-Ephedrine/*1R,2R*-(-)-pseudoephedrine	1208.1 ± 28.1	965.8 ± 0.7	1472.6 ± 23.6	1749.9 ± 34.3	964.6 ± 12.1	1134.6 ± 97.3	1252.1 ± 106.4
1S,2S-(+)-Pseudoephedrine	1299.7 ± 4.7	822.1 ± 9.6	864.9 ± 0.2	1264.5 ± 3.8	943.5 ± 47.1	961.6 ± 79.4	893.7 ± 15.5
E1-Norephedrine	61.4 ± 3.4	47.4 ± 1.0	44.2 ± 4.0	55.2 ± 1.6	58.1 ± 0.6	55.5 ± 9.6	59.1 ± 0.2
E2-Norephedrine	100.8 ± 3.6	78.6 ± 1.5	69.1 ± 1.0	79.6 ± 1.5	81.8 ± 0.3	82.4 ± 2.7	94.5 ± 3.4
Hallucinogens							
R-(-)-MDMA	1.9 ± 0.1	1.7 ± 0.3	ND	ND	ND	ND	2.5 ± 0.2
S-(+)-MDMA	ND	0.7 ± 0.1	ND	ND	ND	ND	1.9 ± 0.4
E1-HMA	ND	ND	ND	ND	ND	ND	ND
E2-HMA	ND	ND	ND	ND	ND	ND	ND
R-(-)-HMMA	4.9 ± 1.1	ND	ND	ND	ND	ND	ND
S-(+)-HMMA	ND	ND	ND	ND	7.2 ± 0.4	ND	8.0 ± 0.2
Opioids							
Heroin	ND	ND	ND	ND	ND	ND	ND
O-6-MAM	ND	ND	ND	ND	ND	ND	ND
Morphine	407.2 ± 6.7	355.4 ± 41.1	328.9 ± 6.3	326.6 ± 10.3	291.8 ± 31.8	380.0 ± 6.9	400.8 ± 30.8
Normorphine	150.2 ± 24.2	157.2 ± 5.7	88.9 ± 0.02	103.3 ± 8.0	105.8 ± 12.6	143.6 ± 21.4	127.3 ± 10.6

MDMA - 3,4-methylenedioxymethamphetamine; HMA - 4-hydroxy-3-methoxyamphetamine; HMMA - 4-hydroxy-3-methoxymethamphetamine; *O*-6-MAM - O-6-monoacetylmorphine.

Table D4: Concentrations (ng.L^{-1}) calculated from raw wastewater at WWTW2 over a 7-day period.

Compound	Concentration (ng.L^{-1})						
	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Stimulants							
Cocaine	308.3 ± 7.6	217.3 ± 5.2	195.2 ± 12.4	107.8 ± 3.6	323.1 ± 1.3	349.7 ± 26.3	563.0 ± 3.3
Benzoylecgonine	693.5 ± 86.5	517.7 ± 10.4	485.8 ± 21.3	409.6 ± 0.9	588.5 ± 0.1	840.2 ± 28.0	1084.2 ± 50.5
Cocaethylene	9.9 ± 0.7	4.8 ± 0.3	6.2 ± 0.9	1.1 ± 0.03	8.4 ± 1.7	18.8 ± 0.7	19.4 ± 2.6
R-(-)-Amphetamine	ND	ND	ND	ND	ND	ND	ND
S-(+)-Amphetamine	97.3 ± 0.6	90.5 ± 2.1	50.9 ± 6.6	62.1 ± 3.8	79.2 ± 0.1	100.6 ± 6.3	128.9 ± 11.1
R-(-)-Methamphetamine	112.1 ± 6.8	135.2 ± 3.8	119.3 ± 1.4	94.7 ± 4.5	118.7 ± 3.9	161.3 ± 19.1	183.5 ± 2.3
S-(+)-Methamphetamine	2317.6 ± 6.6	2171.9 ± 106.6	1828.3 ± 133.2	1587.4 ± 46.6	1889.8 ± 51.2	2877.1 ± 238.7	3226.4 ± 36.5
R-(+)-Mephedrone	ND	2.0 ± 1.0	1.6 ± 0.1	ND	3.5 ± 0.1	1.6 ± 0.01	6.4 ± 0.4
S-(-)-Mephedrone	1.4 ± 0.5	2.0 ± 0.3	ND	ND	3.4 ± 0.3	1.1 ± 0.2	5.7 ± 1.5
Precursors							
1S,2R-(+)-Ephedrine	ND	ND	ND	ND	ND	ND	ND ND
1R,2S-(-)-Ephedrine/*1R,2R*-(-)-pseudoephedrine	374.1 ± 22.8	384.3 ± 14.7	274.1 ± 2.4	362.9 ± 14.1	373.6 ± 1.8	415.2 ± 4.9	461.1 ± 25.0
1S,2S-(+)-Pseudoephedrine	664.5 ± 38.0	718.1 ± 7.0	430.4 ± 8.8	501.0 ± 33.7	628.0 ± 27.9	580.0 ± 3.6	742.2 ± 5.1
E1-Norephedrine	25.1 ± 1.7	27.7 ± 4.3	5.7 ± 0.4	26.6 ± 14.6	18.0 ± 0.1	16.7 ± 3.7	22.2 ± 1.2
E2-Norephedrine	41.5 ± 0.5	48.1 ± 1.5	19.1 ± 0.6	29.7 ± 2.2	44.6 ± 1.3	39.7 ± 0.3	46.9 ± 1.1
Hallucinogens							
R-(-)-MDMA	34.5 ± 0.1	13.8 ± 0.2	13.5 ± 1.0	7.6 ± 0.1	10.0 ± 1.4	28.0 ± 0.4	57.5 ± 1.1
S-(+)-MDMA	12.8 ± 0.1	5.4 ± 0.6	6.2 ± 0.4	3.9 ± 0.2	6.4 ± 0.6	19.3 ± 0.6	34.4 ± 0.02
E1-HMA	ND	ND	ND	ND	ND	ND	ND
E2-HMA	ND	ND	ND	ND	ND	ND	ND
R-(-)-HMMA	10.7 ± 0.5	7.3 ± 1.8	ND	ND	ND	10.8 ± 0.4	16.0 ± 0.9
S-(+)-HMMA	12.1 ± 0.9	9.5 ± 0.8	9.5 ± 1.0	9.1 ± 0.7	8.6 ± 0.1	ND	19.2 ± 0.9
Opioids							
Heroin	ND	ND	ND	ND	ND	ND	ND
O-6-MAM	ND	ND	29.9 ± 4.5	34.1 ± 8.8	66.0 ± 3.7	28.2 ± 0.1	29.3 ± 3.2
Morphine	9379.7 ± 1078.0	3585.8 ± 422.8	4002.8 ± 123.0	4528.2 ± 3.8	3757.4 ± 83.2	927.5 ± 193.0	761.0 ± 10.1
Normorphine	ND	173.8 ± 39.5	ND	131.8 ± 3.4	139.4 ± 25.5	ND	152.3 ± 26.3

MDMA - 3,4-methylenedioxymethamphetamine; HMA - 4-hydroxy-3-methoxyamphetamine; HMMA - 4-hydroxy-3-methoxymethamphetamine; *O*-6-MAM - O-6-monoacetylmorphine.

Table D5: Drug loads (g.day^{-1}) calculated from raw wastewater at WWTW1.

Compound	Mass Load (g.day^{-1})						
	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Stimulants							
Cocaine	2.69 ± 0.2	3.05 ± 0.4	2.12 ± 0.3	4.97 ± 0.2	2.25 ± 0.01	7.23 ± 0.7	3.03 ± 0.1
Benzoylecgonine	8.69 ± 0.3	9.91 ± 0.4	10.27 ± 0.4	8.83 ± 0.4	5.59 ± 0.2	17.26 ± 1.6	12.05 ± 0.2
Cocaethylene	0.05 ± 0.01	0.05 ± 0.02	0.004 ± 0.01	ND	ND	0.10 ± 0.01	0.11 ± 0.01
R-(-)-Amphetamine	ND	ND	ND	ND	ND	ND	ND
S-(+)-Amphetamine	1.04 ± 0.1	0.78 ± 0.3	0.81 ± 0.5	0.71 ± 0.2	0.81 ± 0.2	1.03 ± 0.3	0.88 ± 0.1
R-(-)-Methamphetamine	4.94 ± 0.6	3.47 ± 0.6	2.90 ± 0.5	4.51 ± 0.3	3.38 ± 0.1	5.12 ± 0.1	6.30 ± 0.5
S-(+)-Methamphetamine	22.83 ± 2.7	21.53 ± 2.7	12.92 ± 2.3	33.15 ± 0.7	17.95 ± 0.7	19.64 ± 1.7	40.01 ± 2.0
R-(+)-Mephedrone	ND	ND	ND	ND	ND	ND	ND
S-(-)-Mephedrone	ND	ND	ND	ND	ND	ND	ND
Precursors							
1S,2R-(+)-Ephedrine	ND	ND	ND	ND	ND	ND	ND
1R,2S-(-)-Ephedrine/*1R,2R*-(-)-pseudoephedrine	62.09 ± 1.4	48.45 ± 0.03	76.57 ± 1.2	95.18 ± 1.9	41.52 ± 0.5	56.55 ± 4.9	64.74 ± 5.5
1S,2S-(+)-Pseudoephedrine	66.80 ± 0.2	41.24 ± 0.5	44.98 ± 0.01	68.78 ± 0.2	40.61 ± 2.0	47.93 ± 4.0	46.21 ± 0.8
E1-Norephedrine	3.16 ± 0.2	2.38 ± 0.05	2.30 ± 0.2	3.00 ± 0.1	2.50 ± 0.03	2.77 ± 0.5	3.06 ± 0.01
E2-Norephedrine	5.18 ± 0.2	3.94 ± 0.1	3.59 ± 0.1	4.33 ± 0.1	3.52 ± 0.01	4.10 ± 0.1	4.89 ± 0.2
Hallucinogens							
R-(-)-MDMA	0.10 ± 0.01	0.08 ± 0.01	ND	ND	ND	ND	0.13 ± 0.01
S-(+)-MDMA	ND	0.04 ± 0.005	ND	ND	ND	ND	0.10 ± 0.02
E1-HMA	ND	ND	ND	ND	ND	ND	ND
E2-HMA	ND	ND	ND	ND	ND	ND	ND
R-(-)-HMMA	0.25 ± 0.1	ND	ND	ND	ND	ND	ND
S-(+)-HMMA	ND	ND	ND	ND	0.31 ± 0.02	ND	0.42 ± 0.01
Opioids							
Heroin	ND	ND	ND	ND	ND	ND	ND
O-6-MAM	ND	ND	ND	ND	ND	ND	ND
Morphine	20.93 ± 0.3	17.83 ± 2.1	17.10 ± 0.3	17.76 ± 0.6	12.56 ± 1.4	18.94 ± 0.3	20.72 ± 1.6
Normorphine	7.72 ± 1.2	7.89 ± 0.3	4.62 ± 0.001	5.62 ± 0.4	4.55 ± 0.5	7.16 ± 1.1	6.58 ± 0.5

MDMA - 3,4-methylenedioxymethamphetamine; HMA - 4-hydroxy-3-methoxyamphetamine; HMMA - 4-hydroxy-3-methoxymethamphetamine; *O*-6-MAM - O-6-monoacetylmorphine.

Table D6: Drug loads (g.day^{-1}) calculated from raw wastewater at WWTW2.

Compound	Mass Load (g.day^{-1})						
	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Stimulants							
Cocaine	25.28 ± 0.6	18.25 ± 0.4	16.00 ± 1.0	8.84 ± 0.3	27.14 ± 0.1	25.88 ± 1.9	39.97 ± 0.2
Benzoylecgonine	56.86 ± 7.1	43.49 ± 0.9	39.84 ± 1.7	33.59 ± 0.1	49.44 ± 0.01	62.18 ± 2.1	76.98 ± 3.6
Cocaethylene	0.81 ± 0.1	0.40 ± 0.03	0.51 ± 0.1	0.09 ± 0.002	0.71 ± 0.1	1.39 ± 0.1	1.38 ± 0.2
R-(-)-Amphetamine	ND	ND	ND	ND	ND	ND	ND
S-(+)-Amphetamine	7.98 ± 0.05	7.60 ± 0.2	4.17 ± 0.5	5.09 ± 0.3	6.66 ± 0.01	7.44 ± 0.5	9.15 ± 0.8
R-(-)-Methamphetamine	9.19 ± 0.6	11.36 ± 0.3	9.78 ± 0.1	7.77 ± 0.4	9.97 ± 0.3	11.94 ± 1.4	13.03 ± 0.2
***S*-(+)-**Methamphetamine	190.05 ± 0.5	182.44 ± 9.0	149.92 ± 10.9	130.17 ± 3.8	158.75 ± 4.3	212.90 ± 17.7	229.07 ± 2.6
R-(+)-Mephedrone	ND	1.07 ± 0.5	0.87 ± 0.04	ND	1.93 ± 0.03	0.75 ± 0.004	2.95 ± 0.2
S-(-)-Mephedrone	0.75 ± 0.3	1.11 ± 0.2	ND	ND	1.87 ± 0.2	0.54 ± 0.1	2.63 ± 0.7
Precursors							
1S,2R-(+)-Ephedrine	ND	ND	ND	ND	ND	ND	ND
1R,2S-(-)-Ephedrine/*1R,2R*-(-)-pseudoephedrine	30.68 ± 1.9	32.28 ± 1.2	22.47 ± 0.2	29.76 ± 1.2	31.38 ± 0.1	30.73 ± 0.4	32.74 ± 1.8
1S,2S-(+)-Pseudoephedrine	54.49 ± 3.1	60.32 ± 0.6	35.29 ± 0.7	41.08 ± 2.8	52.75 ± 2.3	42.92 ± 0.3	52.69 ± 0.4
E1-Norephedrine	2.06 ± 0.2	2.33 ± 0.4	0.47 ± 0.03	2.18 ± 1.2	1.51 ± 0.01	1.23 ± 0.3	1.58 ± 0.1
E2-Norephedrine	3.40 ± 0.04	4.04 ± 0.1	1.56 ± 0.1	2.44 ± 0.2	3.74 ± 0.1	2.94 ± 0.02	3.33 ± 0.1
Hallucinogens							
R-(-)-MDMA	2.83 ± 0.01	1.16 ± 0.01	1.11 ± 0.1	0.62 ± 0.01	0.84 ± 0.1	2.08 ± 0.03	4.08 ± 0.1
S-(+)-MDMA	1.05 ± 0.01	0.45 ± 0.1	0.51 ± 0.04	0.32 ± 0.02	0.54 ± 0.1	1.43 ± 0.04	2.44 ± 0.002
E1-HMA	ND	ND	ND	ND	ND	ND	ND
E2-HMA	ND	ND	ND	ND	ND	ND	ND
R-(-)-HMMA	0.88 ± 0.04	0.61 ± 0.1	ND	ND	ND	0.80 ± 0.03	1.13 ± 0.1
S-(+)-HMMA	0.99 ± 0.1	0.80 ± 0.1	0.78 ± 0.1	0.75 ± 0.1	0.72 ± 0.01	ND	1.36 ± 0.1
Opioids							
Heroin	ND	ND	ND	ND	ND	ND	ND
O-6-MAM	ND	ND	2.45 ± 0.4	2.80 ± 0.7	5.54 ± 0.3	2.09 ± 0.005	2.08 ± 0.2
Morphine	769.13 ± 88.4	301.21 ± 35.5	328.23 ± 10.1	371.31 ± 0.3	315.62 ± 7.0	68.63 ± 14.3	54.03 ± 0.7
Normorphine	ND	14.60 ± 3.3	ND	10.81 ± 0.3	11.71 ± 2.1	ND	10.82 ± 1.9

MDMA - 3,4-methylenedioxymethamphetamine; HMA - 4-hydroxy-3-methoxyamphetamine; HMMA - 4-hydroxy-3-methoxymethamphetamine; *O*-6-MAM - O-6-monoacetylmorphine.

Table D7: Population-normalised mass load estimates ($mg.day^{-1}.1000inh^{-1}$) for several countries using the WBE approach.

Drug	Sampling locations	Country	Year Sampled	Load ($mg.day^{-1}.1000inh^{-1}$)	Reference
Cocaine	2 WWTWs	South Africa	2017	100.6 – 589.6	*Current study*
	49 cities	Europe	2016	0.9 – 914.8	EMCDDA, 2016
	Medellin	Colombia	2015	2747.0 – 3465.0	Bijlsma et al., 2016
	Bogotá	Colombia	2015	703.0 – 871.0	Bijlsma et al., 2016
	Auckland	Australia	2014	24.3 – 37.1	Lai et al., 2017
	Liberia	Costa Rica	2014	1880.0 – 2550.0	Causanilles et al., 2017
	El Roble	Costa Rica	2014	2390.0	Causanilles et al., 2017
	1 WWTW	Canada	2014	12.0	Palardy et al., 2015
	1 WWTW	UK	2011	1023.0 – 1767.0	Baker et al., 2014
	1 WWTW	Czech Republic	2011	115.9 – 329.2	Baker et al., 2012
	2 WWTWs	Canada	2010	390.0 – 3750.0	Yargeau et al., 2014
Methamphetamine	2 WWTWs	South Africa	2017	181.9 – 1184.8	*Current study*
	44 cities	Europe	2016	0.4 – 671.8	EMCDDA, 2016
	Oslo	Norway	2015	172.4	Castrignanò et al., 2017b
	Zurich	Switzerland	2015	20.2	Castrignanò et al., 2017b
	14 cities	China	2014 - 2015	208.6 – 1789.9	Xu et al., 2017
	1 WWTW	Canada	2014	9.0	Palardy et al., 2015
	Auckland	Australia	2014	144.0 – 1130.0	Lai et al., 2017
	5 cities	South-Korea	2012 - 2013	<2.6 – 67.9	Kim et al., 2015
	1 WWTW	Czech Republic	2011	293.3 – 626.7	Baker et al., 2012
	2 WWTWs	Canada	2010	54.0	Yargeau et al., 2014

Mephedrone	2 WWTWs	South Africa	2017	1.6 – 10.4	*Current study*
	-	UK	2015	14.9 – 47.7	Castrignanò et al., 2017a
MDMA	2 WWTWs	South Africa	2017	2.2 – 69.3	*Current study*
	53 cities	Europe	2016	1.5 – 125.7	EMCDDA, 2016
	Milan	Italy	2015	5.0 – 53.2	González-Mariño et al., 2017
	Lugano	Switzerland	2015	0.6 – 4.3	González-Mariño et al., 2017
	Porto	Portugal	2015	1.2 – 10.5	González-Mariño et al., 2017
	Castellon	Spain	2015	3.2	Castrignanò et al., 2017b
	Utrecht	Netherlands	2015	62.0	Castrignanò et al., 2017b
	Auckland	Australia	2014	45.9 – 88.5	Lai et al., 2017
	1 WWTW	Czech Republic	2011	21.2 – 173.3	Baker et al., 2012
	Brussels	Belgium	2010	13.0	van Nuijs et al., 2011
	2 WWTWs	Canada	2010	140.0	Yargeau et al., 2014
	Zagreb	Croatia	2009	3.6	Terzic et al., 2010