# Wastewater-based epidemiology, an analytical chemical approach for the investigation of human consumption of lifestyle chemicals

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Ana Causanilles


# Wastewater-based epidemiology, an analytical chemical approach for the investigation of human consumption of lifestyle chemicals

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A mis padres

*Nothing in life is to be feared, it is only to be understood.*

*Now is the time to understand more, so that we may fear less.*

*– Marie Skłodowska Curie*

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# Chapter 1

# General introduction

The general introduction describes the background to the research presented in this thesis. It elaborates on the interest of studying population's lifestyle habits (1.1), focusing on the use of illicit drugs and licit substances with potential for abuse (1.2), and the role of the results from the chemical analysis of wastewater as a complementary source of information in drug epidemiology (1.3). Next, an overview of the substances studied along the thesis is given (1.4). Finally, the general aim of the research and the specific objectives of each chapter are discussed (1.5). In addition, as this research was part of a Marie Curie International Training Network, a description of the project and the research secondments performed by the PhD candidate is provided (1.6).

# 1.1. Human health and lifestyle habits

The health and wellbeing of a society is influenced by the social, the economic and the physical environment, as well as by each person's individual characteristics and behavior. Factors such as (epi)genetics, income and social status, level of education, social support, access to safe water and clean air, healthy workplaces, safe houses, communities and roads, etc. play an important role.

In general, individuals are unable to control their context of life, and therefore, unlikely to directly control many of those determinants of their health. However, their behavior and coping skills (how we deal with life's stresses and challenges), or their lifestyle habits, have a direct impact on their condition. It is in this context that individuals can make their own choices, and consequences may affect not only themselves individually but also the community where they live in.

The choice of certain lifestyle habits may influence human health and wellbeing. Habits such as eating a balanced diet or keeping physically active will have a good impact and improve their condition. On the other hand, habits such as smoking, drinking alcohol, computer gaming, gambling… or the abuse of certain drugs, can turn into harmful addictions.

There is an intriguing controversy (that goes beyond the scope of this work) on whether these lifestyle choices that lead to addictions are truly based on the mechanism of free will or are a chronic disease of the brain (NIDA, 2008). This is because addictive substances hyper stimulate the brain's reward system, which might eventually result in brain damage, which might affect the quality of a person's choices. However, empirical studies exist that have shown a voluntary substance use reduction in some addicts (Heyman, 2017), which would undermine this "brain disease" theory. Regardless this interesting controversy, it can be agreed that drug addiction is a global problem with extensive consequences for human health and the world economy.

# 1.2. The size of the problem in numbers

The use of illicit drugs, and the abuse of licit drugs, is increasingly becoming a worldwide trend in lifestyle that is prevalent in rich and poor countries alike. According to the United Nations Office on Drugs and Crime (UNODC) 1 in 20 adults, or a quarter of a billion people between the ages of 15 and 64 years, used at least one drug in 2014 (UNODC, 2016). The number has been stable over the past years in proportion to the global population (see **Fig. 1.1.** for the available prevalence data on worldwide injecting drug use), nevertheless over 29 million suffer from drug use disorders and only 1 in 6 people with drug use disorders is in treatment.

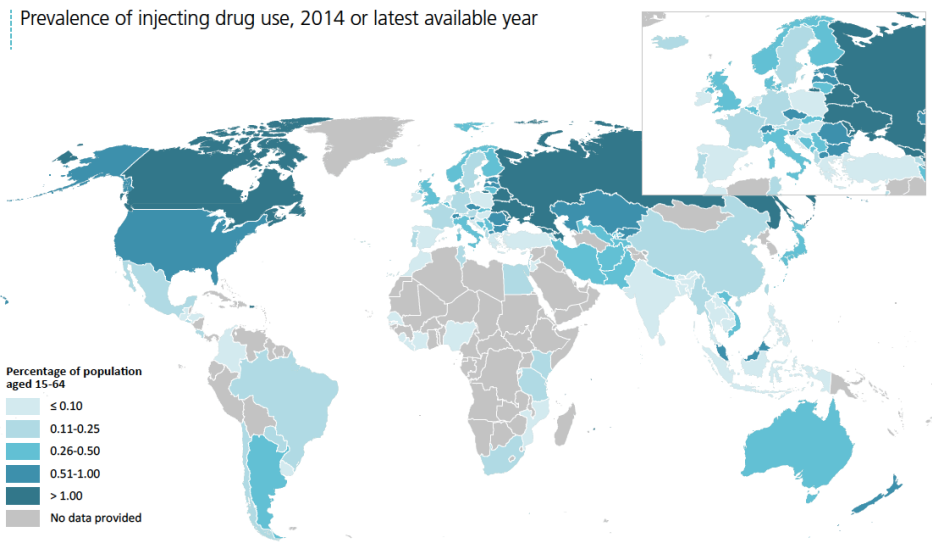


**Fig. 1.1.** Prevalence of injecting drug use, 2014 or latest available (taken from UNODC, 2016).

In addition to the use of established illicit drugs, namely cocaine, cannabis or amphetamine type stimulants, the expansion of a “new” group of substances known as new psychoactive substances (NPS, also known as designer drugs or “legal highs”), has arisen more attention in recent years. NPS are substances that are not directly controlled by international conventions (Malcolm Reid and Thomas, 2016). They are produced by introducing slight modifications to chemical structures of controlled substances and are expected to mimic the effects of illicit substances. They may pose a public health threat because very little is known about their pharmacokinetics, recommended dose, effects or safety. Furthermore, they are easily acquired through the Internet and smart shops where they are sold under various product labels with often misleading information. Every year new substances are reported to the Early Warning System (EWS) at the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). In 2015, 98 NPS were detected for the first time, bringing the number of new substances monitored to more than 560 (EMCDDA, 2016a). This trend can be seen in **Fig. 1.2**. Data on the prevalence of these NPS is still scarce due to the rapid changes in the market to avoid criminalization, that typically happen before a significant user base becomes established.

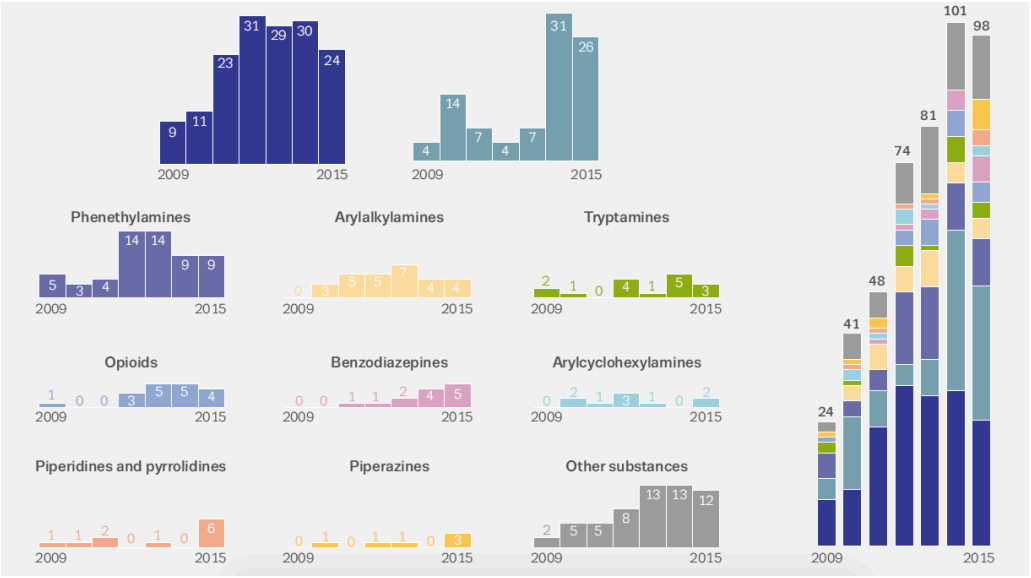


**Fig. 1.2**. Number and categories of new psychoactive substances notified to the EU Early Warning System for the first time, 2009 – 15 (taken from EMCDDA, 2016a).

Another major threat to human health derives from the use of counterfeit or sub-standard pharmaceuticals or medical products available from illegal sources, especially due to the easy access provided by the Internet and rogue online pharmacies. These products are often of sub-standard quality, and mislabeled, which might increase the risk of side effects and overdosing. These medical products might have a lifesaving or lifestyle purpose, and are typically used for healing stigmatized conditions, where users tend to hide their related drug use. Operation Pangea VIII, the largest ever Internet-based operation focusing on the illicit sale of medicines and medical devices via the Internet, reported the success that in 2015 more than 20 million units of illicit medicines were seized, 550 illicit online adverts were blocked and 2,400 illicit online pharmacies had been suspended (Interpol, 2015).

In the same line of threats, there is the specific case of the use of certain banned substances with the purpose of enhancing (athletic) performance, known as doping. This practice, besides a health concern, is considered as an unethical violation. Doping control is widely implemented at professional level (WADA, 2015), and advanced standardized methodologies already exist to detect a great variety of prohibited and controlled substances, usually in body fluids. However, for amateur athletes and the general population data are scarce, and the true prevalence is difficult to measure.

# 1.3. Drug epidemiology

The extent of the drug problem in our society is considerable, given the numbers provided in the previous section. Therefore, there is a significant interest from social scientists and professionals involved in the phenomenon of drug addiction from all its perspectives, or law enforcement officers fighting pharmaceutical crime, or policy makers evaluating anti-doping measures, to monitor the prevalence of the previously mentioned practices.

Classical methods are based on epidemiological, sociological and criminological indicators, which include and combine the direct information obtained from population surveys, with indirect information extracted from police and customs statistics about drugs seizures, offenses or criminal prosecutions and medical records (EMCDDA, 2016b). In the particular case of sports doping, sources of information include questionnaires at professional and amateur level, and laboratory-based chemical analyses in biological matrices (blood and urine) for professional athletes (de Hon et al., 2015).

These methods have the advantage of providing very detailed and sometimes also individual information, including sex, age range, dose or frequency of use. But combining all the information and processing the final results is time-consuming, and in the case of the questionnaires, there is an associated uncertainty because they rely on the willingness of users to self-report.

### 1.3.1. Wastewater-Based Epidemiology (WBE)

The innovative WBE approach, conceptualized in 2001 by Daughton (Daughton, 2001a), has proved to be successful as a complimentary source of information to the classical strategies (EMCDDA, 2016b). This approach assumes that everything that human beings eat, drink, ingest or absorb is excreted in our urine and feces partially unchanged or as transformation products or metabolites (biomarkers), and ends up in the sewage system. Untreated urban wastewater is a very complex matrix, the mixture of all the disposals from human activities, but advanced analytical techniques, e.g. liquid chromatography coupled to mass spectrometry, allow the qualitative and quantitative determination of the residues of such recreational

activities at trace level. This approach has the advantages of delivering objective and near real-time information and being able to detect changes in geographical and temporal drug use patterns. The application of the approach seems simple but it is sophisticated, and includes several steps and their corresponding uncertainties (Castiglioni et al., 2013).

The key parameters for the successful application of the WBE approach with reliable results are: (i) the biomarker selection; (ii) the collection of representative wastewater samples (sampling mode); (iii) the measurement of biomarkers in wastewater; (iv) the calculation of the normalized loads and, finally, (v) the estimation of the drug consumption per capita:

(i) The ideal biomarker can provide relevant information about lifestyle habits, health and wellbeing when it fulfills certain requirements. Its selection is, therefore, not straightforward (Emma Gracia-Lor et al., 2017). The most suitable target biomarker must be specific to the substance of interest and unique to human metabolism, it needs to be excreted via urine in a sufficient concentration (Chen et al., 2014), and remain stable in-sewer and in-sample during stages of transport and storage until chemical analysis (McCall et al., 2016a). The more pharmacokinetic data (metabolism and urinary excretion profile) and stability information in urine and in wastewater is available, the better for a successful and trustworthy application of WBE. In wastewater, biomarkers can undergo further transformation to varying degrees due to chemical, biological and physical processes (McCall et al., 2016b; Ramin et al., 2017, 2016). Ignoring these processes for compounds with low to medium stability will lead to an unknown degree of systematic under- or overestimation of substance use (McCall et al., 2016a).

(ii) The appropriate sampling mode needs to be selected to ensure the collection of representative samples of the real situation in the catchment area. The most desirable is the flow-proportional option (Ort et al., 2010), as it adjusts the frequency of sampling to the external changes in the influent flow. However, it requires an autosampler equipped with a flow meter to trigger the frequency of sample collection. The most suitable options when simpler equipment is available are the volume-proportional

mode, and ultimately time-proportional mode, ensuring a sampling frequency of 15 minutes maximum.

(iii) The accurate measurement of the concentrations of biomarkers in wastewater with advanced analytical techniques has received a lot of scientific attention due to its fundamental role within the overall WBE application (Hernández et al., 2016). It has also been the focus of this thesis. Untreated wastewater is a very complex matrix that requires extensive, and in some cases, compound specific sample treatment. In addition, the analytes are expected at very low concentrations, which explains the need of a concentration step. The best approach for the instrumental analysis of the extracts typically consists of chromatography coupled to mass spectrometry, due to its versatility, robustness, selectivity and high sensitivity. Liquid chromatography is preferred over gas chromatography because of the physic-chemical properties of the targeted compounds in wastewater (polarity, low volatility and thermolability), which would require a derivatization step to be GC compatible. For the MS part, different strategies are possible depending on the available instrument and the goal of the analysis:

a. Target screening is the most common choice as WBE has a quantification purpose. Data acquisition is done using the Selected Reaction Monitoring (SRM) mode of a triple quadrupole (QqQ) mass spectrometer. For this method, a determined number of compounds are selected in advance and at least two specific transitions of each compound are monitored. Quantification of samples is made typically with the most abundant transition, and identification of samples is based on agreement of ion intensity ratios and retention time in comparison with the reference standard. In the last years, high resolution mass spectrometry (HRMS) has been also used for this purpose.

b. Suspect screening is used with a qualitative purpose. This is done using HRMS instruments such as Orbitrap or Time of Flight (TOF). Suspect analyses investigate a list of compounds that are potentially present in samples based on a database, containing name and molecular formula (or exact mass), as a minimum requirement. The

reference standard is initially not required, and therefore compounds for which the reference standard is not available can be found with a high degree of confidence as "tentative" identification (Schymanski et al., 2014), based on fragment ions and their comparability and compatibility with literature and possible chemical structure, respectively.

c. Non-target screening is also used with a qualitative purpose. This is done using HRMS instruments such as Orbitrap or TOF. Contrary to the suspect screening strategy, there is neither prior information nor a list of chemicals. The use of databases such as ChemSpider, PubChem or NIST and other specific software such as MetFrag (Ruttkies et al., 2016) to predict fragmentation patterns, is necessary to search for the identity of the possible compound of interest. The use of this approach is a real analytical challenge, very time-consuming and it may only yield limited results. Nevertheless, non-target analysis is gaining in scientific interest.

The analytical methodology used for all measurements must be fully validated to assure the reliability of the results produced. Even though there are no official guidelines for assuring the good performance of quantitative methods in wastewater, it is suggested in the best-practice protocol to study the parameters such as linearity, limits of detection and quantification, precision intra-day and inter-day (repeatability), procedural recovery (accuracy) and matrix effects.

(iv) The calculation of the normalized load depends on two key parameters in addition to the measurement of the biomarker in wastewater (section iii). One is the measurement of the total influent flow rate during the sampling period, to calculate the load from concentration, and the other is the population equivalent, or the number of people connected to the sewer system, to normalize and allow comparison among different studied areas. An assessment of the sewer network from an engineering point of view is desirable to identify possible wastewater losses due to exfiltration of leaking sewers or infiltration of groundwater into the sewers, which would lead to under- or over estimations in the flow rate. In the case of the population equivalent, this number can be provided by

the census data or by chemical indicators determined in the wastewater. When using the census number, the assessment is important to check the number of house connections to the sewer system. Ignoring the possibility of the use of septic tanks or other disconnections to the system, as well as a high percentage of industrial waste, would lead to under- or over estimations in the population. The investigation of the population equivalent using chemical indicators such as chemical or biological oxygen demand ($COD/BOD_5$), or organic nitrogen or phosphorous content (Andreottola et al., 1994) can be used to determine the reliability of the census figure.

(v) The final step in the back-calculation to drug consumption requires knowledge of the pharmacokinetics of the selected biomarker (especially the percentage of excretion in urine) as indicated in section i, which at this stage is translated into a correction factor (Gracia-Lor et al., 2016). Corrections factors are developed by considering the mean excretion rate of a given parent substance or metabolic product targeted in wastewater and the molecular mass ratio parent drug/metabolite (Zuccato et al., 2008). However, this is not an easy task because excretion can vary depending on the route of administration, the frequency of use of a substance, the co-consumption of different substances, and other factors such as ethnicity, gender, age, dose administered and medical condition (Castiglioni and Gracia-Lor, 2016). In addition, when the final aim is to estimate the amount of drug consumed, the information regarding the original dose and the street purity is also required (EMCDDA, 2016a).

# 1.4. Substances of interest

The research presented in this thesis has targeted different illicit drugs and licit substances with potential for abuse which use is related to lifestyle habits, health and wellbeing.

In order to categorize the harm and risks of drug use, a very interesting scientific and evidence-based fashion was proposed by Nutt and colleagues (Nutt et al., 2007). In their scale, substances are assessed individually according to different categories of harm: the physical harm to the individual user caused by the drug; the tendency of the drug to induce dependence; and the effect of drug use on families, communities, and society (Nutt et al., 2007). It can be seen in **Fig. 1.3**. that the outcome in this classification does not directly correlate with the current regulatory system, as for example alcohol and tobacco rank higher than ecstasy or LSD, while the use of the last two is illegal and the first two is not.

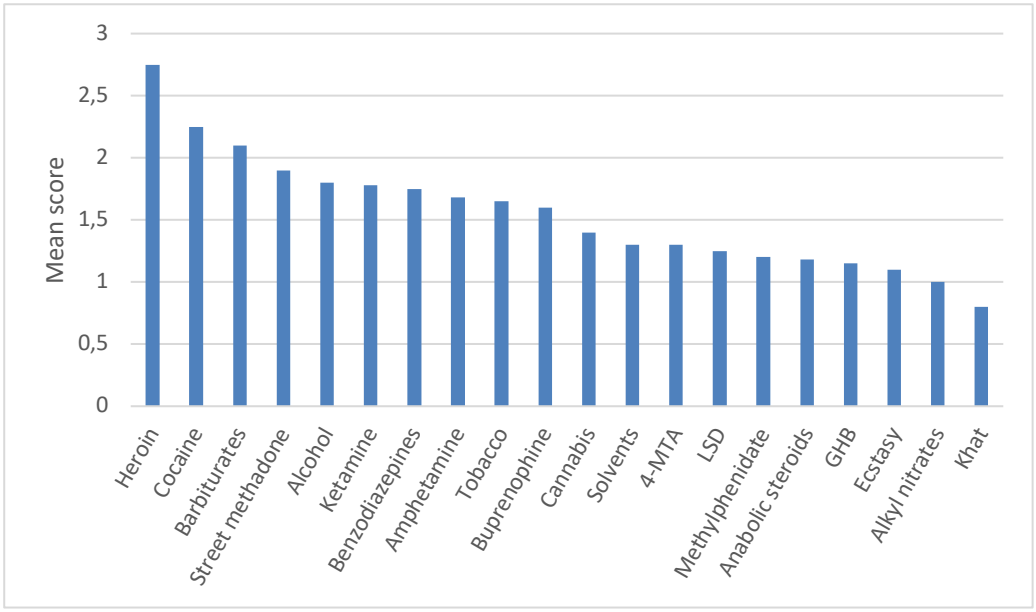


**Fig. 1.3.** Mean harm scores for 20 substances (modified from Nutt et al., 2007).

The substances studied in this thesis have been selected regardless of their legal status, but because of the epidemiological interest in monitoring their use due to the potential harm that their use may produce to the population. They have been grouped below into different classes, and briefly described:

1.4.1. Cocaine is an alkaloid that occurs naturally in the leaves of the coca plant (*Erythroxylon coca*) (Moffat et al., 2011). It is used in two chemical forms: typically snorted as a hydrochloride salt or injected as a free base (crack). After ingestion, cocaine is rapidly hydrolyzed in liver to benzoylecgonine and ecgonine methyl ester, which are present in urine at 45 % and 40 % from the administered dose respectively. It is also partially excreted unchanged (1 to 9 %, depending on the urine pH). In addition, it can also be metabolized into cocaethylene when used in combination with alcohol (ethanol). The target biomarker used in WBE to estimate cocaine consumption is its major metabolite benzoylecgonine. Unlike cocaine, benzoylecgonine has showed high stability in wastewater.

1.4.2. Cannabinoids are the group of chemicals with hallucinogenic properties found in the cannabis plant, being tetrahydrocannabinol (Δ9-THC) the primary and main responsible of its psychotropic effects (Moffat et al., 2011). There are three main types of cannabis products: herb (marijuana), resin (hashish) and oil (hashish oil). THC is a lipophilic compound with low solubility in water and therefore usually self-administered by smoking. THC is extensively metabolized in the liver to the active metabolite 11-hydroxy-THC (OH-THC), which is further oxidized producing the inactive metabolite 11-nor-9-carboxy-Δ9-THC (THC-COOH). THC-COOH is used as target biomarker in WBE. However, this compound presents some challenges from the analytical point of view, due to its specific physico-chemical properties.

1.4.3. Opioids include a group of natural, semisynthetic and synthetic alkaloid drugs derived from the juice the opium poppy (*Papaver somniferum*) (Moffat et al., 2011). The most potent natural product is morphine, from which structurally similar derivatives such as heroin, codeine and fentanyl (among others) have been synthesized. Opioids are widely abused because of the pain relieve effect through euphoria inducing properties. Their potency varies from low to high depending on the affinity on the opioid receptor. Most opioids undergo

extensive metabolism in the liver and are excreted as glucuronides. Morphine is the most abundant metabolite of heroin and codeine (therapeutic opiate use). The alternative in WBE is 6-Monoacetylmorphine (6-MAM), which is unique to heroin (Boerner et al., 1975).

1.4.4. Amphetamine-type stimulants refer to a group of low-molecular weight basic stimulants. These substances are sympathomimetic agents that release monoamines from nerve endings in the brain via the neurotransmitters noradrenaline, dopamine and serotonin (Moffat et al., 2011). The members of the group are amphetamine, methamphetamine, 3,4-Methylenedioxymethamphetamine (MDMA, or ecstasy), 3,4-Methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-N-ethylamphetamine (MDEA); ephedrine, norephedrine and methylphenidate also fall into this group.

These substances are mainly excreted in the urine in unaltered forms, and therefore one might assume that differentiating their presence in wastewater either from use or from direct disposal would be difficult. However, many of the popular psychoactive drugs contain one or more asymmetric carbon atoms. The chemical synthesis of compounds containing one asymmetric C atom will generally lead to equal amounts of the two corresponding enantiomers in the product synthesised. And metabolism of a product containing a racemic mixture of the enantiomers will change the enantiomeric ratio as a result of differences in metabolic conversion rates of the enantiomers (Emke et al., 2014). This particular case is important for amphetamines, because the enantiomers have similar physico-chemical properties but differ in their biological properties such as distribution, metabolism and excretion, as these processes (due to stereospecific interactions of enantiomers with biological systems) favor one enantiomer over the other (Kasprzyk-Hordern and Baker, 2012). This wastewater enantiomeric profiling (beyond the scope of this thesis) aims to solve the afore mentioned distinction.

1.4.5. Benzodiazepines are known as tranquilizers and are among the most commonly prescribed antidepressant medications. Although a useful pharmaceutical, there is potential for abuse due to their hypnotic and sedative effects, especially by high-risk opioid users. Despite having a

relatively low toxicity in overdose, the abuse can cause tolerance, physical dependence, substance use disorder, and benzodiazepine withdrawal syndrome. The members of the group studied in this thesis are diazepam, temazepam, oxazepam and nordazepam.

1.4.6. New psychoactive substances are synthesized by introducing slight modifications to chemical structures of controlled substances, expecting to mimic their effects (Malcolm Reid and Thomas, 2016). The families monitored by the EWS include synthetic cannabinoids, cathinones, phenethylamines, arylalkalyamines, tryptamines, opioids, benzodiazepines, arylcyclohexylamines, piperidines and pyrrolidines, piperazines, and other substances. As earlier indicated, little information is available about their pharmacokinetics, and therefore they are measured in wastewater as unchanged form. In addition, with analytical standards not available or at a high cost, the use of HRMS will become the preferred tool for the analysis of samples suspected of containing NPS because of the ability to acquire full scan MS and MS/MS that can then be retrospectively interrogated (Pasin et al., 2017). However, the lower use of NPS in comparison with traditional illicit drugs will be a limitation when investigating their presence in wastewater, and method sensitivity will be a key parameter in analysis.

1.4.7. Phosphodiesterase type V (PDE5) inhibitors, sildenafil, vardenafil and tadalafil, are the active pharmaceutical ingredients in drugs used for the treatment of erectile dysfunction (European Medicines Agency, 2009, 2008a, 2008b). They were synthesized in the late 90's and marketed patent protected until recently. They were the first effective oral treatment available for the condition, which induced their counterfeit production (Keizers et al., 2016; B J Venhuis et al., 2014c). The unchanged forms are excreted only in minor amounts in urine. In the case of sildenafil, desmethyl- and desethyl-metabolites are targeted in WBE studies (Muirhead et al., 2002b).

1.4.8. Sport doping context:

(i) Anabolic-androgenic steroids represent the greatest group of performance-enhancing substances used in sports. They are synthetic derivatives of the male sex hormone testosterone. Their use promotes masculinity by enhancing muscle growth. In this thesis, the following

compounds were included: metandienone, metenolone, mibolerone, nandrolone, and trenbolone.

(ii) Weight loss products are used to rapidly lose weight in order to perform at full potential as well as being able to participate in a certain weighing class during sport contests. This group of compounds can be classified into appetite suppressants and cerebral stimulants. In this thesis, the following compounds were included: the stimulants ephedrine and norephedrine; the sympathomimetic drug methylhexanamine; sibutramine and 2,4-dinitrophenol. Clenbuterol, a $\beta$-2 agonist with both performance enhancing and weight-loss properties, was also included.

(iii) Masking agents are hormone antagonists used to disguise the use of prohibited substances. In this thesis, the following compounds were included: clomiphene, tamoxifen, anastrozole and finasteride.

# 1.5. Objectives of the thesis

The general aim of the research presented in this thesis was to develop analytical methods capable of determining the presence of licit and illicit drugs in the aquatic environment according to the hypothesis that WBE approach can be used as an alternative and non-intrusive technique that provides information about a population's lifestyle habits and the abuse of controlled substances.

To this aim, advanced analytical methods based on liquid chromatography coupled to (high resolution) mass spectrometry were developed for specific groups of compounds that are typically used as counterfeit medicines, doping or alternatives to illicit drugs (new psychoactive substances) and for which information on human consumption is lacking. The methodology development was focused on application to a difficult aqueous matrix, namely wastewater, so as to enable a WBE approach that would provide sound estimates of a population's use of such substances from objective chemical measurements. The methodology development included improvements in analytical performance, applications to new types of samples, and testing of new workflows for new compound identification.

The outline of the thesis lists the specific objectives of each chapter:

Chapter 2 presents three sub-chapters with the work related to drugs of abuse. Chapter 2.1 presents the challenge of including the most commonly consumed illicit drug, cannabis, in WBE studies. Its determination in wastewater is done via the analysis of the urinary biomarker THC-COOH; concentrations of which seem to depend heavily on environmental factors, sample preparation and analyses, commonly resulting in an underestimation. The chapter describes the joint effort of a pan-European group of researchers to investigate, identify and diminish the source of bias when analysing THC-COOH in wastewater. Several experiments were performed to individually assess the initial aspects of the process, such as the number of freeze-thaw cycles, filtration, sorption to different container materials and in-sample stability, and the most suitable order of preparatory steps. The final purpose of the work was to translate the findings into a recommended best-practice protocol, after its validation with an inter-laboratory study organized with eight laboratories that tested the performance of the proposed procedure.

Chapter 2.2 describes the application of an in-house validated analytical methodology to samples collected at two WWTPs in Costa Rica, with different treatment technologies. The main purpose of the work described in this chapter was to obtain information about drug consumption in a Latin–American country, since very few data of this type are available for this part of the world. Moreover, wastewater treatment is often different from practice encountered in Europe and may lead to different behavior of the analytes. Samples from the untreated influent, effluent and surface water of nearby locations were included in the study to provide insight in the impact of compounds and their fate in the wastewater treatment process applied on the environmental quality of the aquatic ecosystem.

Chapter 2.3 presents the challenge of including new psychoactive substances in WBE studies, because of the reduced number of users that translates into low concentrations of residues and the limited pharmacokinetics information available, which renders the choice of target biomarker difficult. The chapter describes how a successful monitoring benefits from the sampling during special social settings, the analysis with improved analytical techniques, and data processing with specific workflows to narrow the search. The main purpose of the work described in this chapter was to obtain a snapshot of the recreational substances used during a city festival, with special attention to NPS. To do so, samples collected during a city festival, where likely users of recreational substances and consequently higher residual concentrations of used NPS were expected, were analysed using liquid chromatography coupled to high-resolution mass spectrometry. Data were processed using a qualitative screening workflow based on an in-house database containing about 2,000 entries, including NPS and transformation products.

Chapter 3 presents three sub-chapters with the work related to phosphodiesterase type V inhibitors. Chapter 3.1 describes the development and validation of an analytical methodology to determine the presence of phosphodiesterase type V inhibitors in wastewater. This simple, fast and reliable method was based on direct injection followed by liquid chromatography coupled to tandem mass spectrometry and included transformation products and analogues in the target list besides the three active pharmaceutical ingredients (sildenafil, vardenafil and tadalafil). The method performance was thoroughly investigated, including the analyte stability in wastewater and matrix effect. PDE-V inhibitors are the main active ingredients in erectile dysfunction pharmaceuticals. The final aim of this work was to evaluate the use of ED products in the Netherlands between 2013 and 2015, considering the

patent expiration at the end of 2013, and the acquisition from legal sources as well as rogue online pharmacies.

Chapter 3.2 describes the application of the in-house validated method included in chapter 3.1 to wastewater samples collected during one week at the entrance of three wastewater treatment plants serving the catchment within the cities of Amsterdam, Eindhoven and Utrecht.

Chapter 3.3 describes the application of the in-house validated method described in chapter 3.1 to wastewater samples collected during one week at the entrance of eight wastewater treatment plants serving the catchment within the cities of Bristol, Brussels, Castellon, Copenhagen, Milan, Oslo, Utrecht and Zurich.

In both cases, the main purpose of the work was to demonstrate the potential of WBE studies to track counterfeit medication and rogue online pharmacy sales. Measured concentrations in wastewater were compared to predicted environmental concentrations estimated from national prescription data.

Chapter 4 investigates the applicability of the chemical analysis of wastewater as a complimentary source of information to assess the use of doping substances by the general population and amateur athletes. Doping control is widely implemented at professional level, however, data are still scarce with few scientific articles addressing the subject so far, and with results showing a wide variance in the prevalence at both amateur and professional level. The chapter describes the development and validation of an analytical methodology to determine the presence of 15 substances from the groups of anabolic steroids, weight-loss products and masking agents in wastewater. The method is based on solid phase extraction coupled to high-resolution mass spectrometry, which turns it into a very sensitive and specific method. Samples collected at the entrance of three wastewater treatment plants and one pumping station while different sport events were taking place in the catchment area were included in the study to provide insight in the use prior and/or during specific sport events.

# 1.6. ITN SEWPROF

This work has been made as part of the European Union funded Marie Curie International Training Network (ITN) SEWPROF "A new paradigm in drug use and human health risk assessment: Sewage profiling at the community level". This ITN is multi-disciplinary and multi-institutional, covering subjects from analytical chemistry to statistics and epidemiology and includes eleven institutions in nine countries (Norway, Denmark, Germany, United Kingdom, The Netherlands, Belgium, Switzerland, Italy and Spain). Eleven Early Stage Researchers (ESRs) and four Experienced Researchers (ERs) from all over the world were recruited for this project. The main objective of this project is to "advance knowledge of the epidemiology of (illicit) drug use and to bridge gaps in the available expertise with the ultimate goal of applying this cutting edge interdisciplinary approach within epidemiological studies of societal health" (http://sewprof-itn.eu/). The interdisciplinary nature of this project is exemplified by the institutes and departments involved: from toxicologists (Toxicological Centre, University of Antwerp and Department of Experimental and Clinical Toxicology, University of Saarland) to water and environmental research institutes (KWR Watercycle Research Institute, Department of Environmental Health Sciences (Mario Negri Institute of Pharmacological Research), Ecotoxicology and Risk Assessment (Norwegian Institute for Water Research and Research Institute of Pesticides and Water (University Jaume I)), engineers (Department of Environmental Engineering (Technical University of Denmark) and Urban Water Management (Swiss Federal Institute of Aquatic Science and Technology)), epidemiologists (Centre for Drug and Addiction Research (University of Oslo), drug identification agencies (TICTAC Communications Ltd.) and pure chemists (Department of Chemistry, University of Bath). As implied from the title "International Training Network", research stays for training and research purposes are mandatory. The PhD candidate performed 3 research stays for a total of 4 months at the Research Institute of Pesticides and Water, University Jaume I, Spain; University of Bath, United Kingdom; and University of Antwerp, Belgium.

### 1.6.1. Research stays, and brief statement of work undertaken

Research Institute of Pesticides and Water: Investigation of the possible source of bias and the improvement of the accuracy of the determination of THC-COOH in influent wastewater during sample treatment, under supervision of Dr. Félix Hernández and Dr. Lubertus Bijlsma. Duration: 3 March – 4 April 2014. This work resulted in Chapter 2.1 (Causanilles et al., 2017a).

University of Bath: Chiral biomarkers and their analysis with the usage of chiral LC coupled with tandem mass spectrometry, under supervision of Dr. Barbara Kasprzyk-Hordern, and in collaboration with Dr. Erika Castrignanò. Duration: 26 May – 26 June 2015. While this work did provide some good results, further research is required before a publication can be anticipated.

University of Antwerp: Qualitative screening of new psychoactive substances (NPS) in pooled urine and wastewater samples collected from festivals by using UPLC-QTOF-MS, under supervision of Dr. Alexander van Nuijs and Dr. Adrian Covaci, and in collaboration with Dr. Juliet Kinyua. Duration: 3 September – 30 October 2015. This work resulted in Chapter 2.3 (Causanilles et al., 2017b) and a co-authored paper (Kinyua et al., 2016).

# Chapter 2

# Drugs of abuse

# 2.1.

# Improving wastewater-based epidemiology to estimate cannabis use: focus on the initial aspects of the analytical procedure

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# Abstract

Wastewater-based epidemiology is a promising and complementary tool for estimating drug use by the general population, based on the quantitative analysis of specific human metabolites of illicit drugs in urban wastewater. Cannabis is the most commonly used illicit drug and of high interest for epidemiologists. However, the inclusion of its main human urinary metabolite 11-nor-9-carboxy-$\Delta^9$-tetrahydrocannabinol (THC-COOH) in wastewater-based epidemiology has presented several challenges and concentrations seem to depend heavily on environmental factors, sample preparation and analyses, commonly resulting in an underestimation. The aim of the present study is to investigate, identify and diminish the source of bias when analysing THC-COOH in wastewater. Several experiments were performed to individually assess different aspects of THC-COOH determination in wastewater, such as the number of freeze-thaw cycles, filtration, sorption to different container materials and in-sample stability, and the most suitable order of preparatory steps. Results highlighted the filtration step and adjustment of the sample pH as the most critical parameters to take into account when analysing THC-COOH in wastewater. Furthermore, the order of these initial steps of the analytical procedure is crucial. Findings were translated into a recommended best-practice protocol and an inter-laboratory study was organized with eight laboratories that tested the performance of the proposed procedure. Results were found satisfactory with z-scores ≤ 2.

# 2.1.1. Introduction

Drug use has not only a negative impact on health and well-being of individuals and people around them, but also represents a clear threat to the stability and security of entire regions and to economic and social development. Cannabis is the most widely cultivated and trafficked illicit drug, responsible for over 75% of drug seizures in Europe (EMCDDA, 2016a). As the most commonly used illicit drug, it is of great interest from an epidemiological point of view. According to the United Nations Office on Drugs and Crime (UNODC), 3.8% of the global population used cannabis in 2014 (UNODC, 2016) and the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) estimated that 13.3% of young adults (15-34) consumed cannabis in the European Union that same year (EMCDDA, 2016b). Although the use of cannabis has remained stable worldwide over the past years, in some regions, particularly North America and Western and Central Europe, its use has recently increased (UNODC, 2016). The development and use of complementary monitoring tools is important to have a more complete understanding of cannabis use and the impact of new cannabis policies.

Estimating community drug use through the chemical analysis of specific human biomarkers in wastewater has demonstrated its potential to become a useful complementary approach to established drug monitoring tools such as epidemiological surveys, treatment demand and law enforcement data. This technique, referred to as wastewater-based epidemiology (WBE), provides near-real-time information on geographically and temporal drug use patterns, particularly relevant against the backdrop of an ever-shifting drug problem. This quantitative approach is well established to estimate the consumption of cocaine, amphetamine, methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA) (Been et al., 2016; EMCDDA, 2016b; Ort et al., 2014). However, in contrast to these substances, the estimation of cannabis using WBE is problematic (EMCDDA, 2016b).

The principal active ingredient of cannabis is $\Delta^9$-tetrahydrocannabinol (THC), but in WBE studies the urinary metabolite of THC, 11-nor-9-carboxy-$\Delta^9$-tetrahydrocannabinol (THC-COOH), is used as target biomarker [6]. THC-COOH is specific and, compared to other metabolites, shows high stability over 72 h in wastewater (McCall et al., 2016a; Senta et al., 2014). The metabolism of THC is diverse and extensive, a relatively low percentage of THC is excreted as THC-COOH (EMCDDA, 2016b; Gracia-Lor et al., 2016). One challenge is therefore the need for more research to better understand the excretion percentage of THC-COOH in order

to refine back calculations to estimate THC consumption. This challenge will not be addressed in the present paper. Another challenge is the analytical determination of THC-COOH in wastewater. Some knowledge gaps associated with physical processes were identified, such as its potential to partition on particulate matter (Khan and Nicell, 2012; Senta et al., 2013) and adsorption onto hydroxyl sites present on the surface of glassware (Baker and Kasprzyk-Hordern, 2011a). THC-COOH has different physicochemical properties compared to the other conventional illicit drugs (see **Table A1** in Annex A). At acidic pH, THC-COOH is present in its non-charged hydrophobic form, which means it may partition to particulate matter, sample containers or filter material, while at neutral pH and the basic pH of natural wastewater the molecule is negatively charged and more hydrophilic. In general, the analytical difficulties and non-instrumental factors have strongly been related to the lower polarity (high lipophilicity) of THC-COOH compared to other illicit drugs when included in multi-residue methods (Hernández et al., 2016; Pedrouzo et al., 2011; van Nuijs et al., 2011; Vazquez-Roig et al., 2013). The results of inter-laboratory exercises performed by the Sewage analysis CORe group Europe Network (SCORE COST, 2017a) corroborated the difficulties related to the chemical analysis of THC-COOH in wastewater (Ort et al., 2014). Although the laboratories involved in those exercises successfully determined THC-COOH in the methanol standards, the recoveries of THC-COOH spiked into wastewater were initially low. This observation suggested that concentrations of THC-COOH in wastewater might be underestimated, probably due to losses during some critical analytical steps.

The present manuscript is a result of studies performed by a working group established within the framework of the pan-European inter-disciplinary network (SCORE), which brings together experts from different disciplines interested in standardizing the WBE approach and in coordinating international studies (SCORE COST, 2017b). The aim of the present work is to investigate and identify the sources of possible bias when analysing THC-COOH in wastewaters and to propose best-practice protocols regarding the initial steps of the analytical procedure. The research is an important step in attempting to provide more accurate estimations of cannabis use through WBE.

# 2.1.2. Materials and methods

This paper describes a study that has been performed by a collaborative group involving 12 institutions, and 10 laboratories. A summary of in-house validated analytical methodologies of each participating laboratory is presented in **Table 2.1.1** and the full details can be accessed in **Tables A2** (Supplementary Information file, Annex A). These multi-residue methods were also applied to measure several illicit drugs in wastewater for WBE monitoring studies organized by SCORE (Ort et al., 2014).

**Reagents and materials**

Analytical standards of THC-COOH and its deuterated analogue were prepared starting from certified ampoules, purchased either from Lipomed AG (Arlesheim, Switzerland) or Cerilliant (Round Rock, TX, USA). All laboratories used THC-COOH-$d_3$ as isotope labelled internal standard (ILIS), except Lab 9 who used THCCOOH-$d_9$.

A range of different filter materials with pore sizes ranging from 0.2 to 2.7 mm were tested: glass fibre, regenerated cellulose, mixed cellulose acetate and cellulose nitrate, and polytetrafluoroethylene (PTFE), polyvinylidene fluoride (PVDF) and polyethersulfone (PES) membranes. Filters were supplied by Pall Corporation (Port Washington, NY, USA), Nalgene (Rochester, NY, USA), Phenomenex (Torrance, USA), Whatman (Dassel, Germany), Millipore (Bedford, MA, USA), VWR International (Radnor, PA, USA) and Agilent (California, USA).

The solid-phase extraction (SPE) cartridges used for sample concentration and clean-up were polymer-based: cation exchange mixed mode (Oasis MCX or Strata-XC), or neutral hydrophilic lipophilic balanced (Oasis HLB). Amino silica-based Strata $NH_2$ cartridges were used for additional extract clean up by Lab 6. Oasis and Strata cartridges were supplied by Waters (Milford, MA, USA) and Phenomenex (Torrance, USA), respectively (see **Table A2**).

During preliminary tests vials of different materials were tested: glass and polypropylene (PP).

**Table 2.1.1.** Overview of in-house methods performed by participating laboratories.

| Lab # | Lab 1 | Lab 2 | Lab 3 | Lab 4 | Lab 5 | Lab 6 | Lab 7 | Lab 8 | Lab 9 [1] | Lab 10 [1] |
|---|---|---|---|---|---|---|---|---|---|---|
| Sample volume | 50 mL | 5 mL | 100 mL of "sample" (25 mL sample + 75 mL ultrapurewater) | 50 mL of supernatant | 100 mL | 125 mL | 100 mL | 100 mL | n.a. | n.a. |
| Particulate removal | Filtration 1.6 µm glass fiber filter | Filtration 0.2 µm RC syringe filter | Dilution | Centrifugation | Filtration (1) Whatman No. 41 filter paper (2) 0.2 µm PTFE syringe filter | Filtration 2.7 µm Whatman, glass fiber filter | Filtration (1) 1.6 µm glass microfiber filter GF/A (2) 0.45 µm mixed cellulose acetate & cellulose nitrate | Filtration (1) 1 µm glass fiber filter A/E (2) 0.2 µm PES membrane filter | Filtration 0.2 µm Whatman PTFE syringe filter Primo 1 mL syringe | Filtration (1) 1.6 µm glass microfiber filter GF/A (2) 0.45 µm mixed cellulose acetate & cellulose nitrate filter |
| pH at extraction | Natural | Natural | Natural | Natural | Natural | Acid | Acid | Natural | n.a. | n.a. |
| SPE material | Oasis HLB | Strata-XC | Oasis HLB | Oasis HLB | Oasis HLB | Oasis MCX | Oasis MCX | Oasis HLB | n.a. | n.a. |
| Analytical instrument [2] | LC-QqQ | LC-QqQ | LC-QqQ | LC-QqQ | LC-QqQ | LC-QqQ | LC-QqQ | LC-LTQ-FT-Orbitrap | LC-QqQ | LC-QTOF MS |
| Ionization mode (ESI) | - | - | + | + | + | - | - | + | + | - |
| Reference | (van Nuijs et al., 2014) | Unpublished | (Bijlsma et al., 2014a) | Adaptation from (Bijlsma et al., 2014a) | Unpublished | (Senta et al., 2013) | Adaptation from (González-Mariño et al., 2012) | (Bijlsma et al., 2013) | Adaptation from (McCall et al., 2016b) | Unpublished |
| Instrumental variability[3] (Intra-day, RSD (%)) | 6% (n=6) | 2% (n=6) | 7% (n=6) | 3% (n=6) | 1% (n=5) | 5% (n=6) | 4% n=6) | 2% (n=6) | 10% (n=5) | 8% (n=6) |
| Instrumental variability[3] (Inter-day, RSD (%)) | 11% (n=6) | 3% (n=6) | 7% (n=6) | 3% (n=6) | 2% (n=5) | 7% (n=6) | 5% (n=6) | 4% (n=3) | 6% (n=3) | 7% (n=6) |

n.a. not applicable; [1] Labs 9 and 10 did not participate in the interlaboratory study but provided results in preliminary experiments; [2] QqQ: triple quadrupole; LTQ-FT Orbitrap: linear ion trap-Fourier transform Orbitrap; QTOF: quadrupole-time-of-flight; [3] Instrumental variability was performed using a standard solution of 50 ng/L in solvent

**Analytical methodology**

Instrumental analysis was performed with liquid chromatography coupled to mass spectrometry (LC-MS). In all cases, chromatographic separation was performed using reversed-phase LC columns. Eight laboratories used low resolution MS and two used high resolution MS. Electrospray ionization (ESI) was used in all cases, in either positive or negative mode. More information regarding instrumental parameters can be found in **Table A2** (SI, Annex A). Statistical analysis of results was performed with GraphPad Prism version 5.01.

**Experimental**

Preliminary experiments were set up in order to identify possible sources of bias regarding the sample preservation and treatment. In all experiments, two types of matrices were included: ultrapure water and filtered wastewater (free of solid particles). Samples were spiked at a sufficiently high concentration level (50 ng $mL^{-1}$) in order to perform analysis without further pre-treatment. The sample pH reduction was recommended as one of the WBE "best practice" requirements (Castiglioni et al., 2013) to decrease the bacterial degradation and increase the sample stability. However, a study performed by Senta and colleagues (Senta et al., 2014) indicated enhanced pre-analytical losses of THC-COOH when samples were filtered at pH 2. Therefore, we included pH adjustment as a parameter in our experiments. These preliminary experiments were performed by multiple laboratories in the consortium. Results were evaluated with the recovery, expressed as percentage (%), and defined as the relative response of THC-COOH divided by the deuterated response and compared to t = 0. In addition, laboratories were asked to evaluate their instrumental variability (expressed as relative standard deviation, RSD%) by analysing at least 5 replicates over 3 days.

**Freeze-thaw cycles**

The effect of multiple cycles of freezing and thawing of samples containing THC-COOH was evaluated by spiking 20 mL of matrix at 50 ng $mL^{-1}$ THC-COOH and distributing aliquots of 0.5 mL in 2 mL glass vials. Each vial was exposed to a different number of freeze-thaw cycles: 0, 1, 2, 5, 10 and 20 (n = 3 in every case). After all freeze-thaw cycles had been performed, the ILIS was added and the vials were analysed by direct injection into the LC-MS. Three laboratories provided results.

**In-sample stability**

The in-sample stability of THC-COOH was tested at three temperatures (20 °C, 4 °C and 20 °C) over a period of 7 days, with sampling points at 0, 1, 4, 7 days. The matrix (3 mL) was spiked at 50 ng $mL^{-1}$ of the analyte, homogenized and distributed in 3 vials of 2 mL, and each stored at one of the three temperatures. After the experiments, the ILIS was added to each vial and samples were directly injected into the LC-MS system. Four laboratories provided results.

**Filtration**

The effect of sample filtration prior to analysis was assessed at natural pH (~7.5) and acidic pH (samples adjusted to pH 2.5). From 20 mL of THC-COOH spiked matrix at 50 ng $mL^{-1}$ level, 1 mL was transferred into a glass vial for direct analysis while the rest was filtered. Different types of filters were used: (1) type GF/A glass microfiber filters + cellulose nitrate and acetate filters, (2) type A/E glass fibre filters + PES membrane filters, (3) type GF/C glass fibre filters, (4) regenerated cellulose filters + PES membrane filters. The filtered aliquots were spiked with ILIS and directly injected into the LC-MS system. The resulting recovery was compared to the nonfiltered sample, and the loss during filtration was calculated as follows:

$$1 - ((average\ recovery\ filtered)\ /\ (average\ recovery\ nonfiltered))$$

Four laboratories provided results.

**Sorption**

The potential sorption of THC-COOH to the different container surfaces was investigated by storing 1 mL of matrix spiked with THC-COOH at 50 ng $mL^{-1}$ level in vials of two different materials: glass and polypropylene (PP) (n = 3). The sample pH was considered as a second variable. Therefore, two pHs were investigated: natural pH (7.5) and acidic pH (pH adjusted to 2.5). An aliquot was taken after a determined number of days (storage at 4 °C: 0, 1, 4 and 7 days), spiked with the ILIS and directly analysed by LC-MS. Three laboratories provided results.

**Order of preparatory steps**

In addition to the preliminary experiments described above, the order of sample preparation steps, often performed prior to SPE, was evaluated. The steps were: ILIS addition, sample filtration and pH adjustment (acidification). To do so, one wastewater sample spiked at 800 ng $L^{-1}$ was divided into 4 sub-samples. The order of steps for each of the sub-samples was varied. Samples were subsequently extracted and analysed using the validated methodology of the one laboratory (Lab 6) that performed the experiment.

**Inter-laboratory study**

From the preliminary experiments, a best-practice protocol was derived stating recommendations on the pre-analytical aspects of the analysis of THC-COOH in wastewater (see below). In order to test the performance of this protocol, an inter-laboratory study was organized with eight laboratories.

40 L of wastewater collected at the entrance of the WWTP in Utrecht (The Netherlands) were used as matrix. A stainless-steel mixing tank was used to homogenize the bulk by stirring for 30 min at 400 rpm. Homogenized wastewater was distributed in four 5 L glass volumetric flasks. Wastewater test samples were prepared by KWR as followed: Sample 1, non-spiked, at natural pH (7.5); Sample 2, spiked at low level (72 ng $L^{-1}$), natural pH (7.5); Sample 3, spiked at high level (720 ng $L^{-1}$), natural pH (7.5); and Sample 4, acidified to pH 2.5 and spiked at high level (720 ng $L^{-1}$). The low level (72 ng $L^{-1}$) and high level (720 ng $L^{-1}$) were prepared by spiking 0.5 mL and 5 mL of a THC-COOH solution of 0.72 mg $L^{-1}$ (in methanol), respectively into the 5 L bottles and filling up with homogenized wastewater. Each of the prepared samples was distributed in 0.5 L PP bottles. Each bottle contained approx. 450 mL of sample. Bottles were stored in a freezer (25 °C) overnight in order to be shipped frozen the following day to the participants.

# 2.1.3. Results and discussion

Based on previous inter-laboratory exercises performed by the SCORE consortium (SCORE COST, 2017a), the study started from the premise that the instrumental procedures and multi-residue methods of the different laboratories are successful in determining THC-COOH in standard solutions in methanol in the ng $mL^{-1}$ range (SCORE COST, 2017b). Participating laboratories measured THC-COOH in negative- or positive-ESI mode and sample preparation consisted of filtration/dilution/centrifugation and off-line SPE using different types of filters and cartridges (**Table 2.1.1**). Multi-residue methods applied by 3 out of the 8 laboratories consisted in the use of cation exchange mixed mode cartridges for SPE. Although this type of sorbent is most selective towards basic compounds, THC-COOH showed acceptable recovery when interacting with the MCX sorbent through the reversed phase mechanism (González-Mariño et al., 2012; Senta et al., 2013). ILIS was used as surrogate in order to ensure the analytical quality of the results. Instrumental variability within the participating labs was <10% in all cases (**Table 2.1.1**).

**Effect of sample pre-treatment operations**

**Freeze-thaw cycles**

After 20 freeze-thaw cycles, the THC-COOH concentration showed a slight decrease (10%, RSD = 13%) from the initial concentration (see **Fig. A1.1** for wastewater matrix and **A1.2** for ultrapure water). However, the variability of the result fell within the level of accepted uncertainty of replicate analyses (Castiglioni et al., 2013) and, therefore, the decrease was considered not significant.

**In-sample stability**

The in-sample stability results were calculated relative to day 0 (as the mean recovery of each lab before freezing the sample for the first time) (Fig. **A2.1** for wastewater matrix and **A2.2** for ultrapure water). THC-COOH remained stable in wastewater up to 7 days at all temperatures tested, with relative recoveries between 80 and 120%.

These results confirm the findings reported by González-Mariño et al., 2012 and Heuett et al., 2015 who reported high stabilities up to 3 and 4 months, respectively

when stored at 20 °C. Gonzalez-Mariño also reported losses of THC-COOH when stored at 4 °C, whereas in our study no significant loss was observed at that temperature. In another study (Senta et al., 2014) that included pH as a second variable, a lower stability of THC-COOH was observed in the acidified samples (54% decrease from the original concentration at pH 2) than in the non-acidified samples (10% decrease from the original concentration at pH 7.4) when stored at 4 °C. This result can be explained by the enhanced adsorption of THC-COOH to solid particulate matter observed at pH 2 as compared to natural pH (Khan and Nicell, 2012).

**Filtration**

Details on the individual performance of each filter or filter combination at pH 7.5 and pH 2.5 can be accessed in SI, Annex A (**Table A3**). Results presented in **Table A3** clearly demonstrate that filtration has a great impact on the THC-COOH recovery, and that it is highly pH dependent. At acidic pH, THC-COOH is not charged and its lipophilicity increases (logD: 5.1 at pH 2.5 vs 2.4 at pH 7; chemicalize.com). In the case of wastewater at natural pH, the small-volume syringe filter of regenerated cellulose (RC) performed the best (no loss during filtration). However, when filtering larger volumes, the loss amounted to 27-30% independent of the filter material. In the case of acidified wastewater, results invariably showed losses during filtration >75%, which is in a good agreement with findings reported by Senta et al., 2014. As can be seen in **Fig. 2.1.1**, the average loss during filtration when sample pH was not adjusted (pH ≈ 7.5) amounted to 20% (RSD = 3%). This impact was even higher when wastewater was acidified to pH 2.5 and the loss amounted to 90% (RSD = 1%). Means differed significantly (paired t-test, p-value = 7e-4).

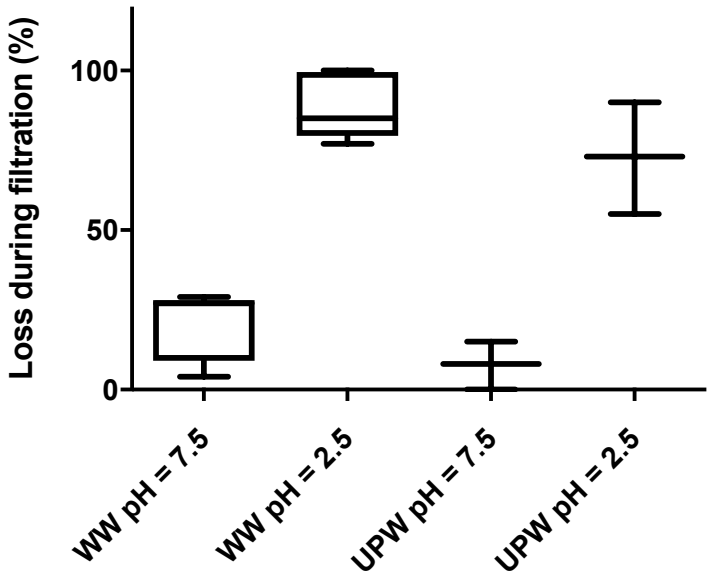


**Fig. 2.1.1**. Losses of THC-COOH during filtration and influence of matrix (WW = wastewater, UPW = ultrapure water) and different sample pH. The data are presented as box plots of grouped results (WW = 4 laboratories, 5 different filter types tested, 3 replicates each; UPW = 3 laboratories, 3 different filter types tested, 3 replicates each) and expressed as percentage of the average recovery of the filtered versus the non-filtered sample. Boxes represent the mean, 25% and 75% percentile values and the whiskers extend to the minimum and maximum values.

**Sorption**

Results from the sorption experiments are shown in **Fig. A3.1** for wastewater matrix and **A3.2** for ultrapure water). Sample pH appears to be a more important parameter than the type of sample container (glass or PP) used. Losses due to sorption to container walls occur more rapidly and to a higher extent at pH = 2.5, as the compound is in its non-charged hydrophobic form.

Altogether, the results from filtration, in-sample stability and sorption tests have identified pH as the variable having the most significant impact on the recovery of THC-COOH. This corroborated that, given the specific physico-chemical properties of THC-COOH, its behaviour is highly dependent on wastewater pH.

**Order of preparatory steps**

The order of sample preparation steps was evaluated by comparing the recovery obtained in each case. These preparatory steps are performed prior to SPE and employed to prevent the SPE material from clogging (Bijlsma et al., 2013) or to prevent and correct for in-sample degradation effects as well as matrix effects (i.e. ILIS addition). They are frequently applied when a multi-residue analysis is foreseen (Senta et al., 2014). The results for these experiments were in agreement with those assessed in the previous sections.

The conclusion is that sample acidification, if required by the selected enrichment protocols, should be performed only after the sample filtration. Ideally, ILIS should be added before filtration to correct for any potential loss. The results of the preliminary experiments highlighted the influence of pH and the importance of the correct execution order of sample preparation steps before SPE, with sample acidification being critical. When consulting the SCORE inter-laboratory exercise participant laboratories (SCORE COST, 2017b), only 5% had performed their analysis using the order of steps identified as the optimal one in this study: 1st ILIS addition 2nd filtration 3rd pH adjustment (only if needed). Therefore, it was decided to perform an inter-laboratory study within the group in order to confirm this hypothesis before making any recommendation.

**Inter-laboratory study**

An inter-laboratory study was performed using the optimal approach identified in the preliminary experiments described above. Four samples were prepared as described in previous “Inter-laboratory study” section and shipped frozen to each participant. All samples were received within 24 h in frozen conditions. Each laboratory was asked to analyse three independent replicates and report THC-COOH concentrations in ng $L^{-1}$ for each sample. The resulting data was tested for homogeneity, the presence of outliers and normality distribution, and z-scores were calculated in order to measure the performance of each laboratory with regard to the group average.

First, the homogeneity of the variances was tested to confirm the correct data comparison (Cochran test). Results showed that the variance for samples 1, 2 and 4 for laboratory 8 was too high (C = 0.738 (sample 1), 0.696 (sample 2), 0.830 (sample 4) > 0.561), therefore those data were removed from the following evaluation. The remaining data set was evaluated for outliers (Grubbs, $\alpha = 0.05$) and the Shapiro-Wilk

normality test (α = 0.05) was applied to determine if the results derived from a normal distribution. All samples passed with following p-values: sample 1, 0.22 (n = 7); sample 2, 0.26 (n = 7); sample 3, 0.34 (n = 8); sample 4, 0.29 (n = 6).

The group's mean average concentration and relative standard deviation per sample was calculated (see **Table 2.1.2**), following the ISO guidelines (ISO 13528). For more details, **Table A4** shows the mean concentration and standard deviation per laboratory and per sample. Results showed good repeatability (<10%) within laboratories, and reproducibility (≈30%, calculated as the RSD for the mean dispersion), except for sample 4. The reproducibility for samples 1 to 3 is comparable to other inter-laboratory tests (Heath et al., 2010). In contrast, the reproducibility for sample 4 was much worse (50%, initially 110% due to the outlier), due to the issues described in previous sections.

**Table 2.1.2**. Group's mean (M) per sample expressed in ng $L^{-1}$, Recovery (R) expressed in absolute value (ng $L^{-1}$) and percentage (%), and group's relative standard deviation (RSD%) in the inter-laboratory study.

| | M | R | RSD (%) | n |
|---|---|---|---|---|
| Sample 1 – WW blank | 814 [b] | - | 28% [b] | 7 [b] |
| Sample 2 – WW blank + 72 ng $L^{-1}$ | 860 [b] | 46 (64%) | 27% [b] | 7 [b] |
| Sample 3 – WW blank + 720 ng $L^{-1}$ | 1527 | 807 (112%) | 34% | 8 |
| Sample 4[a] – WW blank acidified + 720 ng $L^{-1}$ | 442 [b] | -372 (-52%) | 50% [b] | 6 [b] |

R = sample x (x=2,3,4) – sample 1 (WW blank)

[a] Modified order of analytical steps, the sample was acidified at KWR before being shipped frozen to the laboratories.

[b] after removal of laboratory 8 data

Z-scores were calculated to help in the identification of random or systematic errors. To do so, the difference between each individual lab's mean (m) and the group's mean (M) was subtracted, and then divided by the group's standard deviation. This computation provides a value that can be either positive or negative (when the mean is above or below the group's average, respectively), as a measure of the accuracy of each laboratory. The accepted cut-off value is z-score ≤ |3|, whilst a value between 2

and 3 is considered questionable, in accordance with the IUPAC (Thompson et al., 2006) terminology. Graphical results are presented in **Fig. 2.1.2**.

Z-scores were in general consistently positive or negative for each of the laboratories, which might indicate some type of systematic bias, but within the acceptance criteria. Certain laboratories seemed to be grouped systematically in the lower or higher end, however these groupings appear to be independent of extraction and analysis procedures. Laboratory 8 showed high results for all samples, particularly for samples 1, 2 and 4, as commented above. However, an unambiguous explanation could not be found for this performance.

Recoveries of THC-COOH, defined as the difference between the group's mean for the spiked samples subtracted by the blank sample (see **Table 2.1.2**), were satisfactory (64-112%), with good accuracy from the participating labs for samples 2 and 3, confirming the correct use of the recommended protocol. The mean recovery (52%) observed for the acidified sample 4 demonstrated the negative influence that acidification of the sample may have on recovery.

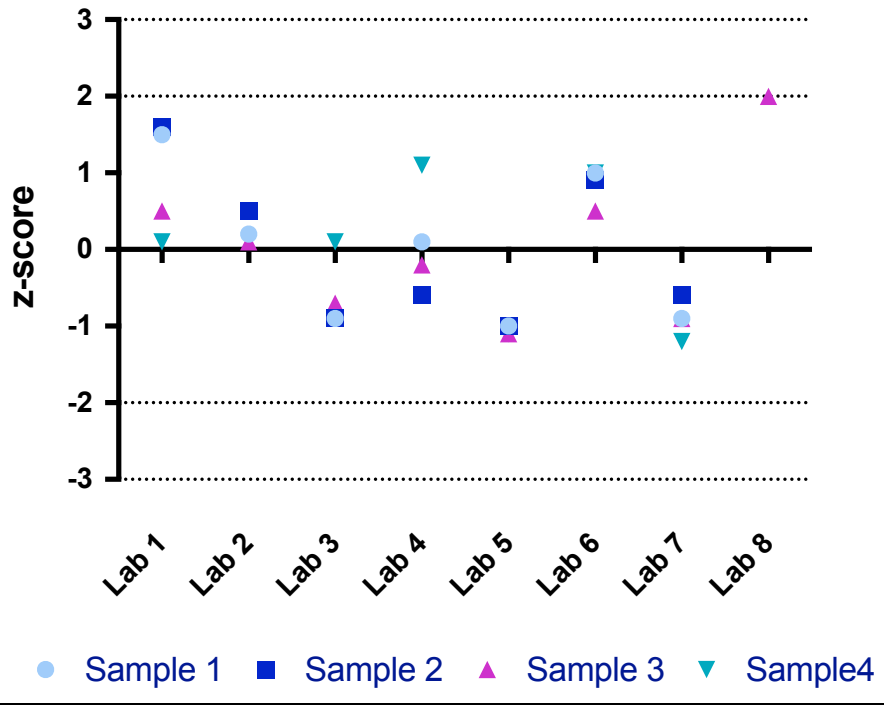


**Fig. 2.1.2**. Inter-laboratory study z-scores per laboratory and sample, calculated as the difference between each individual lab's mean (m) and the group's mean (M) divided by the group's standard deviation.

# 2.1.4. Conclusions

The estimation of cannabis use through wastewater analysis is of high interest. Previous studies have identified several important knowledge gaps as well as analytical challenges. This means that previously published results should be considered with care, as results could have been underestimated.

The results obtained in the current study can be used to define the way forward towards more accurate determination of THC-COOH in wastewater. The adjustment of pH has been identified as a critical step in sample processing. If necessary, samples should be acidified after filtration and only after the ILIS have been added to correct for possible losses. Although the results among all labs varied by approximately 30% and therefore higher than optimal, the proposed protocol was successfully tested, and can, therefore, be recommended for future WBE applications.

Studies regarding THC-COOH sorption to biofilms and solid particles during in-sewer transport would be needed (i) to further reduce uncertainties, as they have already been done for other illicit substances (McCall et al., 2016a, 2016b, Ramin et al., 2017, 2016; Senta et al., 2014), as well as (ii) to better understand the cannabis excretion profile in order to achieve a more accurate back calculation of its consumption.

# Acknowledgements

We wish to acknowledge colleagues Dr. Sara Castiglioni from Mario Negri and Prof. Dr. Alain Verstraete from Ghent University for their input in work discussions. This article is based upon work from COST Action ES1307 supported by COST (European Cooperation in Science and Technology). Ana Causanilles acknowledges the European Union for her Early Stage Researcher (ESR) contract as part of the EU-International Training Network SEWPROF (Marie Curie-PEOPLE Grant #317205). Alexander van Nuijs acknowledges the Research Foundation e Flanders (FWO) for his postdoctoral fellowship. Authors from the University of Santiago de Compostela acknowledge funding by the Spanish Research Agency (AEI) through project CTM2014-56628-C3-2-R (AEI/FEDER, EU), the Galician Council of Culture, Education and Universities (Iria Gonzalez-Mariño postdoctoral contract from the “Plan Galego I2C” and ref. GRC2013-020, cofounded by FEDER, EU). The authors are grateful for the technical support of all WWTPs included in the study.

# Annex A – Supplementary Information Chapter 2.1.

**Table A1**. Physico-chemical properties of some illicit drugs and metabolites.

| Compound | Formula | $pK_a$ | | LogP | |
|---|---|---|---|---|---|
| | | Experimental | Calculated | Experimental | Calculated |
| Amphetamine[1] | $C_9H_{13}N$ | 10.1 | 9.9 | 1.8 | 1.8 |
| Methamphetamine[1] | $C_{10}H_{15}N$ | 10.1 | 10.4 | 2.1 | 2.2 |
| MDMA[1] | $C_{11}H_{15}NO_2$ | 9.4 | 10.3 | n.a. | 2.1 |
| Cocaine[1] | $C_{17}H_{21}NO_4$ | 8.6 | 8.9 | 2.3 | 2.3 |
| Benzoylecgonine[1] | $C_{16}H_{19}NO_4$ | n.a. | 10.8, 3.3 | −1.3 | 2.3 |
| THC[2] | $C_{21}H_{30}O_2$ | n.a. | 9.3 | n.a. | 5.9 |
| THC-COOH[2] | $C_{21}H_{28}O_4$ | n.a. | 4.2 | n.a. | 5.1 |

n.a. not available

[1] (Baker and Kasprzyk-Hordern, 2011b)

[2] (ChemAxon software-calculated values)

**Table A2**. Full details of the analytical methodology used by each participant laboratory: sample treatment, LC conditions, MS parameters.

| Lab # | Lab 1 | Lab 2 | Lab 3 | Lab 4 | Lab 5 | Lab 6 | Lab 7 | Lab 8 | Lab 9 [(1)] | Lab 10 [(1)] |
|---|---|---|---|---|---|---|---|---|---|---|
| ILIS | THC-COOH-$d_3$ | THC-COOH-$d_3$ | THC-COOH-$d_3$ | THC-COOH-$d_3$ | THC-COOH-$d_3$ | THC-COOH-$d_3$ | THC-COOH-$d_3$ | THC-COOH-$d_3$ | THC-COOH-$d_9$ | THC-COOH-$d_3$ |
| Filtering material | 1.6 µm Glass fiber filter | 0.2 µm RC filter (syringe) | No filtering Sample diluted 4x | No filtering. Sample centrifuged at 3000 rpm for 10 min and the supernatant used for analysis | (1) Whatman No. 41 filter paper (2) 0.2 µm PTFE syringe filter | 2.7 µm Whatman, glass-fiber filter | (1) 1.6 µm glass microfiber filter GF/A (2) 0.45 µm mixed cellulose acetate & cellulose nitrate | (1) 1 µm glass fiber filter A/E (2) 0.2 µm PES membrane filter | 0.2 µm Whatman PTFE syringe filter Primo 1 mL syringe | (1) 1.6 µm glass microfiber filter GF/A (2) 0.45 µm mixed cellulose acetate & cellulose nitrate filter |
| pH at extraction | Natural | Natural | Natural , except the acidified sample (pH 3-4) | Natural | Natural | Acid (pH=2) | Acid (pH 4.5) | Natural | n.a. | n.a. |
| SPE material | Oasis HLB | Phenomenex Strata-XC (3cc, 60mg) | Oasis HLB (3cc, 60mg) | Oasis HLB (3cc, 60mg) | Oasis HLB (6cc, 200 mg) | Oasis MCX (6cc, 150mg), extra clean up with Strata $NH_2$ (3cc, 200mg) | Oasis MCX (6cc, 150 mg) | Oasis HLB (6cc, 150 mg) | n.a. | n.a. |
| SPE protocol: Conditining | MeOH + ultrapure water | MeOH + 25mM $NH_4CH_3CO_2$ | MeOH + ultrapure water | MeOH + ultrapure water | MeOH + ultrapure water | MeOH + ultrapure water + 25mM $H_3PO_4$ | MeOH 5% $NH_4OH$ + ultrapure water (pH 4.5) | MeOH + ultrapure water | n.a. | n.a. |
| SPE protocol: Sample load | 50 mL sample | 5 mL sample | 100 mL of "sample" (25mL sample + 75mL ultrapure water) | 50ml of supernatant | 100 mL of sample | 125mL of sample | 100 mL sample adjusted at pH 4.5 | 100 mL of sample | n.a. | n.a. |
| SPE protocol: Wash | no | 85/15 water/ acetonitrile | no | ultrapure water | ultrapure water + 50% MeOH | ultrapure water | ultrapure water pH 4.5 | ultrapure water | n.a. | n.a. |
| SPE protocol: Drying | yes | yes | yes | yes | yes | yes | yes | yes | n.a. | n.a. |
| SPE protocol: Elution | 8 mL MeOH | 2mL MeOH + 2 mL 85/15 ethyl acetate/iso propyl alcohol | 5mL MeOH | 5ml MeOH | MeOH | 6mL MeOH | 4 mL MeOH 5% NH4OH | 8mL MeOH | n.a. | n.a. |
| SPE protocol: Extra clean-up | no | no | no | no | no | Conditionin: 1% HCOOH in MeOH Loading: MCX extract (MeOH ) acidified with 60µL HCOOH) Additional elution: 4mL 1% HCOOH in MeOH | no | no | no | no |
| SPE protocol: Evaporation | to dryness, at 35 °C | to dryness, at 40 °C | to dryness, at 35 °C | to dryness, at 40 °C | to ~ 0,5 mL, at 40 °C. | to dryness, at 40 °C | to ~ 0,5 mL | to 250 µL, addition of 250 µL of ultrapure water, second evaporatio n to 250 µL. | n.a. | n.a. |
| SPE protocol: Reconstitution | 100 µL ACN + 100 µL 5 mM $NH_4CH_3CO$ in ultrapure water | 1000 µL 5mM $NH_4HCO_2$ | 100 µL MeOH + 900uL $H_2O$ | 1mL MeOH and diluted 1/10 with MeOH due to matrix effects | 1 mL with MeOH | 500 µL $H_2O$:MeOH= 8:2 with addition of 0.1% acetic acid | 1 mL with MeOH | 0.5 mL water:MeO H, 90:10 | n.a. | n.a. |
| Time between samples received and analysis | Samples received on 6/9/16; stored at -20 °C until analysis on | Samples received on 7/9/16; stored at -20°C until analysis on | Samples received on 6/9/16; stored at -20°C until analysis on | Samples received on 6/9/16; stored at -20°C until analysis on | Samples received on 7/9/16; stored at 4°C until analysis on | Samples received on 6/9/16; stored at -20 °C until analysis on | Samples received on 7/9/16; stored at -20°C until analysis on | Samples received on 6/9/ 16; stored at -20°C until analysis on | n.a. | n.a. |

| | 22/9/16 | 22/9/16 | 16/9/16 | 11/10/16 | 23/9/16 | 26/9/2016. | 22/9/16 | 18/11/16 | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Analytical instrument | Agilent 6410 (QqQ) | Agilent 1260 LC with a 6460 triple quad ms (QqQ) | Waters Xevo triplequad (QqQ) | Waters Xevo TQS Micro (QqQ) | Sciex Triple Quad 6500+ LC-MS/MS System (QqQ) | ThermoTSQ Quantum AM (QqQ) | Varian LC - Varian 320-MS (QqQ) | LTQ-FT-Orbitrap (Thermo Electron, Bremen, Germany) | Applied Biosystems 5500 QTrap linear ion trap triple quadrupole mass spectrometer (Sciex, Darmstadt/ Germany) (QqQ) | Agilent LC – Agilent 6550 iFunnel Q-TOF |
| Mobile phase composition | A: Ultrapure water 5 mM ammonium acetate; B: Acetonitrile | A: Ultrapure water 5 mM ammonium acetate; B: Methanol | A: Ultrapure water 5 mM ammonium acetate + 0.01% formic acid; B: MeOH | A: Ultrapure water 5 mM ammonium acetate + 0.01% formic acid; B: MeOH | A: Ultrapure water 5 mM ammonium formate with 0.01 % formic acid; B: Acetonitrile 0.01 % formic acid | A: Ultrpure water 0.1 % acetic acid; B: MeOH 0.1 % acetic acid | A: Ultrapure water 5 mM ammonium acetate; B: MeOH 5 mM ammonium acetate | A: Ultrapure water 0.05 % formic acid; B: MeOH 0.05 % formic acid | A: Ultrapure water 5 mM ammonium formate buffer at pH 3; B: MeOH 0.5% of a 1 M ammonium formate | A: Ultrapure water 5 mM $NH_4HCO_2$; B: Acetonitrile |
| Ionization mode | negative | negative | positive | positive | positive | negative | negative | positive | positive | negative |
| Transitions | THC-COOH Quantifier: 343>299 Qualifier: 343>245 | THC-COOH Quantifier: 343>299 Qualifier: 343>245<br><br>THC-COOH-$d_3$ Quantifier: 346>302 Qualifier: 346>248 | THC-COOH Quantifier: 345 >193 Qualifier: 345 > 299 | THC-COOH Quantifier: 345.3 >299.2 Qualifier: 345.3 > 327.3<br><br>THC-COOH-$d_3$ Quantifier: 346.1>302.1 | THC-COOH Quantifier: 345.2 >193.2 Qualifier: 345.2 > 299.2<br><br>THC-COOH-$d_3$ Quantifier: 348.2>302.2 | THC-COOH Quantifier: 343 > 245 Qualifier: 343 > 299<br><br>THC-COOH-$d_3$ Quantifier: 346 > 248 | THC-COOH Quantifier: 343.2 > 299 Qualifier: 343.2 > 245<br><br>THC-COOH-$d_3$ Quantifier: 346.2 > 302 | THC-COOH $[M+H]^+$ 345.2060 qualifiers: 345 > 327<br><br>THC-COOH-$d_3$ $[M+H]^+$ 348.2249 | THC-COOH Quantifier: 345.1 > 299.2 Qualifier: 345.1 > 193.1<br><br>THC-COOH-$d_9$ Quantifier: 354.1 > 336.2 | THC-COOH $[M+H]^+$ 343.1915 Quantifier: 299.2017 Qualifiers: 245.1547 191.1078 325,1809<br><br>THC-COOH-d3 $[M+H]^+$ 346.2103 Quantifier: 302.2205 Qualifiers: 248.1735 194.1266 |
| Reference | (van Nuijs et al., 2014) | n.p. | (Bijlsma et al., 2014a) | Adaptation from (Bijlsma et al., 2014a) | n.p. | (Senta et al., 2013) | Adaptation from (González-Mariño et al., 2012) | (Bijlsma et al., 2013) | Adaptation from (McCall et al., 2016b) | n.p. |
| Instrumental variability[3] (Intra-day, RSD (%)) | 6% (n=6) | 2% (n=6) | 7% (n=6) | 3% (n=6) | 1% (n=5) | 5% (n=6) | 4% (n=6) | 2% (n=6) | 10% (n=5) | 8% (n=6) |
| Instrumental variability[3] (Inter-day, RSD (%)) | 11% (n=6) | 3% (n=6) | 7% (n=6) | 3% (n=6) | 2% (n=5) | 7% (n=6) | 5% (n=6) | 4% (n=3) | 6% (n=3) | 7% (n=6) |

n.a. not applicable

n.p. unpublished

(1) Labs 9 and 10 did not participate in the interlaboratory study but provided results in preliminary experiments

(2) QqQ: triple quadrupole; LTQ-FT Orbitrap: linear ion trap-Fourier transform Orbitrap; QTOF: quadrupole-time-of-flight

(3) Instrumental variability was performed using a standard solution of 50 ng/L in solvent

**Table A3.** Loss during filtration (expressed as %) with standard deviation (n=3)

| | Wastewater | | | | Ultrapure water | | | |
|---|---|---|---|---|---|---|---|---|
| Filter material | pH = 7.5 | sd (n=3) | pH = 2.5 | sd (n=3) | pH = 7.5 | sd (n=3) | pH = 2.5 | sd (n=3) |
| Glass fibre + PES | 27 | 0.1 | 100 | 0.1 | -8 | 0,1 | 73 | 0,2 |
| Glass fibre+ cellulose nitrate and acetate | 30 | 0.6 | 82 | 0.1 | 15 | 0,3 | 90 | 0,1 |
| Glass fibre (45 mm) | 27 | 0.2 | 77 | 0.1 | 8 | 0,2 | 55 | 0,1 |
| RC (syringe filter) | 4 | 0.03 | 85 | 0.1 | - | | - | |
| PES syringe (syringe filter) | 14 | 0.04 | 99 | 0.2 | - | | - | |

**Table A4.** Mean (m) of replicates (expressed in ng $L^{-1}$) and standard deviation (sd) (n=3) per sample and participant laboratory in the inter-laboratory study.

| | | Lab 1 | Lab 2 | Lab 3 | Lab 4 | Lab 5 | Lab 6 | Lab 7 | Lab 8 |
|---|---|---|---|---|---|---|---|---|---|
| Sample 1 | m | 1158 | 860 | 604 | 848 | 588 | 1040 | 602 | 1210 |
| | sd | 94 | 92 | 11 | 10 | 13 | 81 | 97 | 308 |
| Sample 2 | m | 1226 | 983 | 665 | 727 | 629 | 1055 | 732 | 1434 |
| | sd | 15 | 65 | 22 | 67 | 20 | 51 | 137 | 267 |
| Sample 3 | m | 1762 | 1580 | 1148 | 1413 | 975[a] | 1759 | 1043 | 2540 |
| | sd | 56 | 79 | 32 | 19 | - | 103 | 95 | 133 |
| Sample 4 | m | 458 | N/D | 472 | 695 | 193 | 663 | 174 | 2532 |
| | sd | 77 | - | 111 | 74 | 7 | 82 | 25 | 390 |

N/D: non detected (below LOD)
[a] n=1

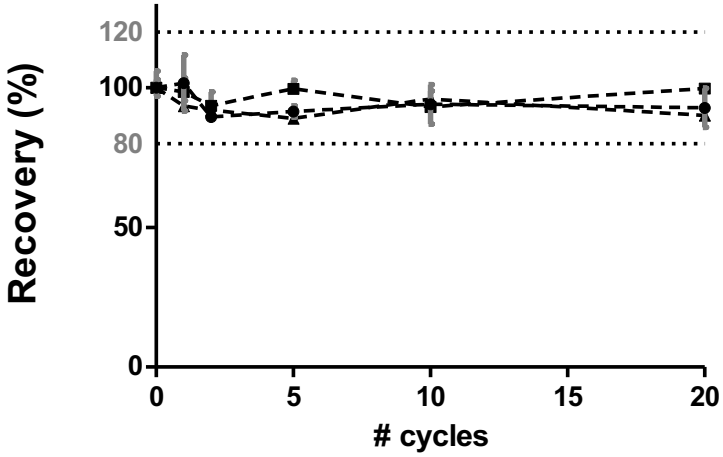


**Figure A1.1.** Average THC-COOH recovery (in wastewater matrix) after n cycles of freezing-thawing relative to the 1st cycle. Error bars represent the standard deviation (n=3). Dotted lines at y=80 and 120%. Legend: Lab 1: circle ●; Lab 2: square ■; Lab 3: triangle ▲.

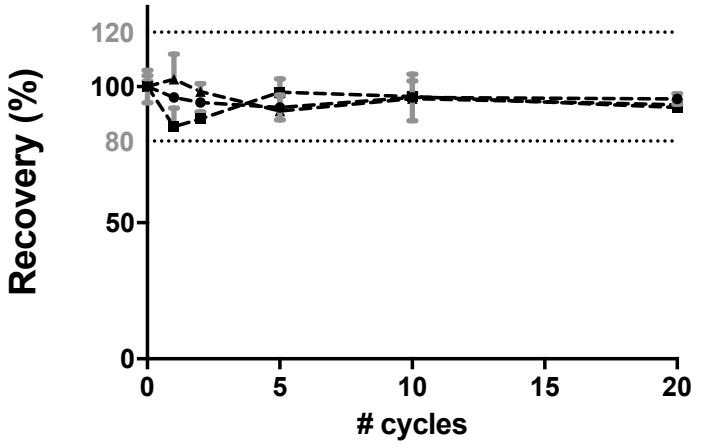


**Figure A1.2.** Average THC-COOH recovery (in ultrapure water matrix) after n cycles of freezing-thawing relative to the 1st cycle. Error bars represent the standard deviation (n=3). Dotted lines at y=80 and 120%. Legend: Lab 1: circle ●; Lab 2: square ■; Lab 3: triangle ▲.

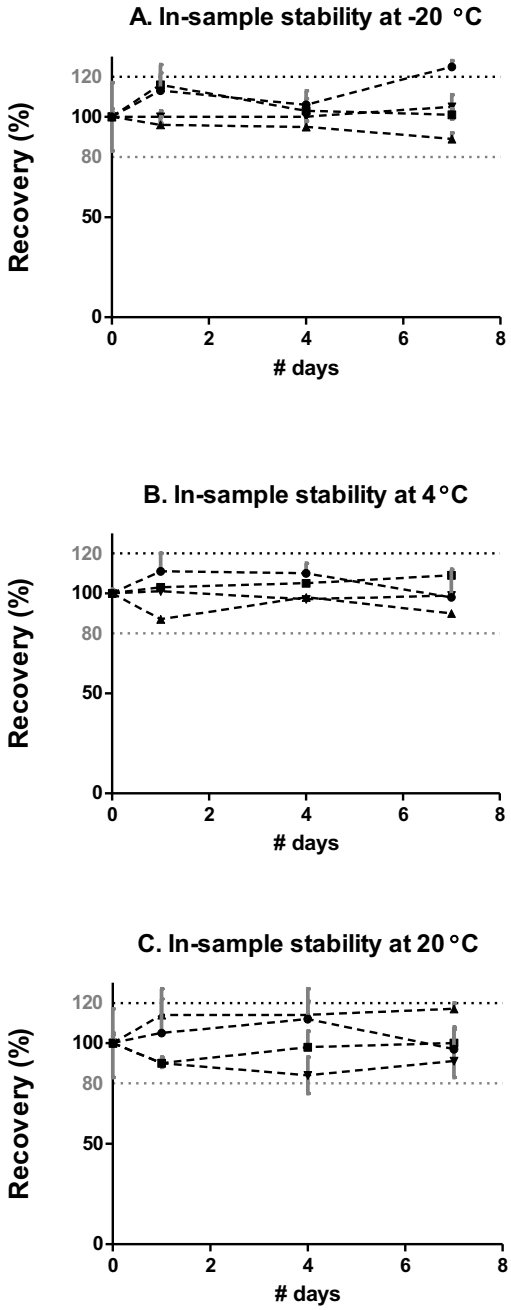


**Figure A2.1**. Stability of THC-COOH in wastewater stored at different temperatures. Data are expressed as recovery relative to day 0. A at -20°C, B at 4°C, C at 20°C. Lab 1: circle ●; Lab 2: square ■; Lab 3: triangle ▲; Lab 4: triangle upside down ▼

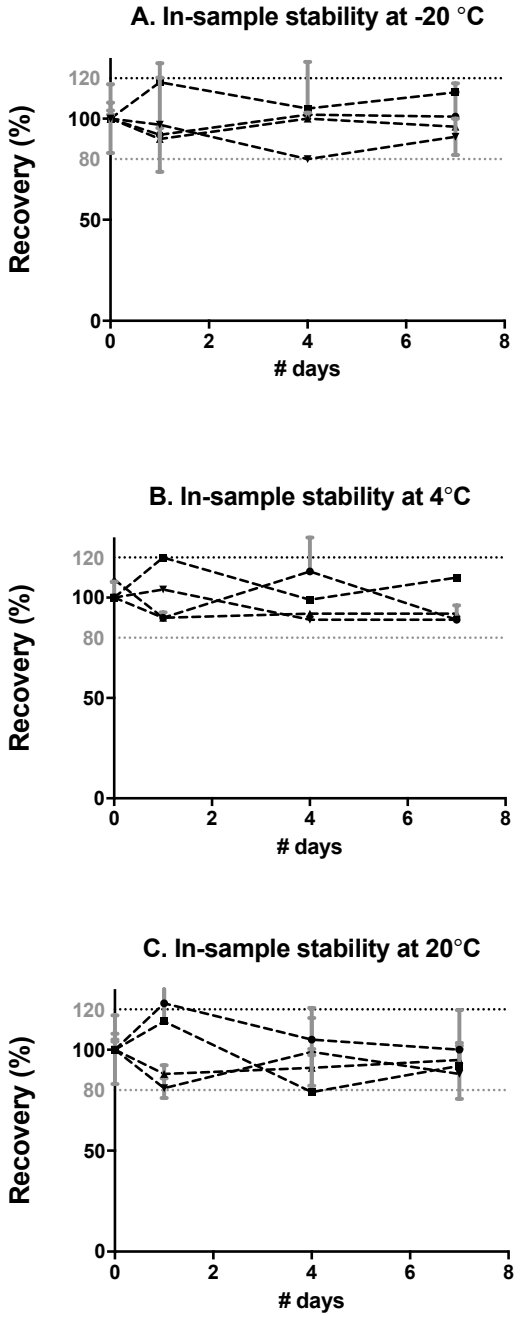


**Figure A2.2**. Stability of THC-COOH in ultrapure water stored at different temperatures. Data are expressed as recovery relative to day 0. A at -20°C, B at 4°C, C at 20°C. Lab 1: circle ●; Lab 2: square ■; Lab 3: triangle ▲; Lab 4: triangle upside down ▼

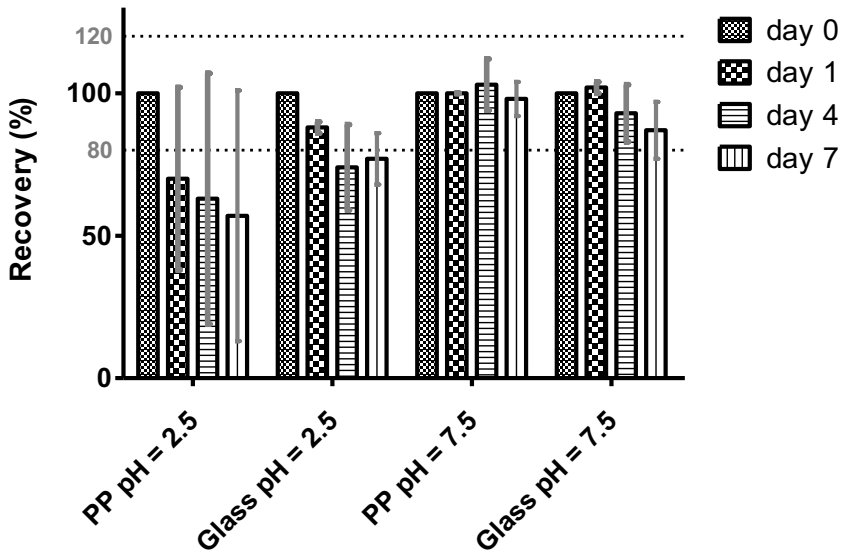


**Figure A3.1**. Influence of pH on sorption to polypropylene or glass container walls of THC-COOH spiked in wastewater. Data collected during a period of 7 days and expressed as recovery relative to day 0.

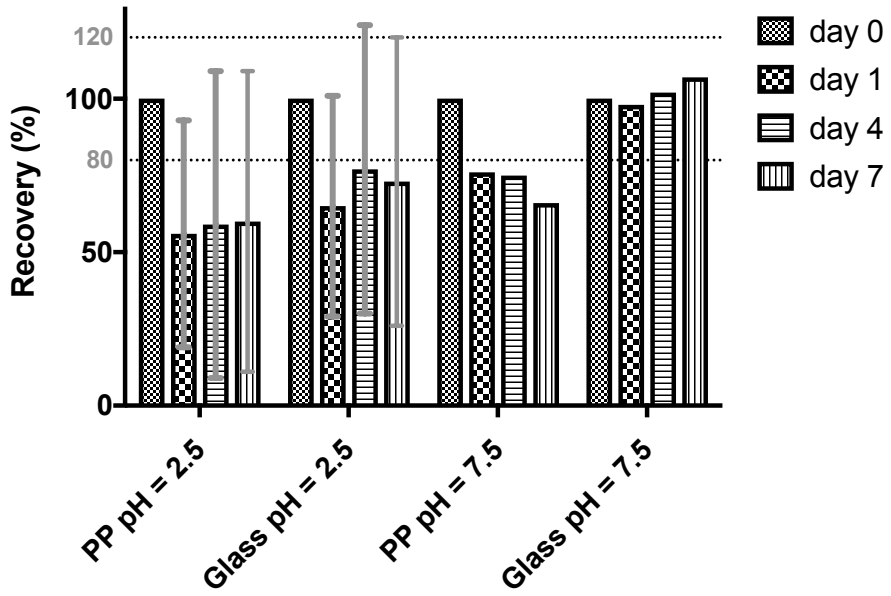


**Figure A3.2**. Influence of pH on sorption to polypropylene or glass container walls of THC-COOH spiked in ultrapure water. Data collected during a period of 7 days and expressed as recovery relative to day 0.

# 2.2.

# Occurrence and fate of illicit drugs and pharmaceuticals in wastewater from two wastewater treatment plants in Costa Rica

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Science of the Total Environment, 599 – 600 (2017) 98 – 107

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# Abstract

Chemical analysis of raw wastewater in order to assess the presence of biological markers entering a wastewater treatment plant can provide objective information about the health and lifestyle of the population connected to the sewer system. This work was performed in a tropical country of Central America, Costa Rica, with the aim of extending this knowledge to new world regions. This work is the first to report wastewater-based epidemiological data on the use of illicit drugs in this region of the world. Composite wastewater samples from the influents of two different wastewater treatment facilities and surface water samples from surrounding areas were collected applying the best practice protocol and analysed to investigate the occurrence and fate of selected illicit drugs of abuse and pharmaceuticals. Results showed the presence of chemical indicators of the classic drugs cocaine and cannabis at high concentration levels, besides the moderate presence of the opiates codeine and morphine. Neither the worldwide commonly used psychoactive substances of abuse such as synthetic phenethylamines, nor pharmaceuticals from the family of benzodiazepines were detected, demonstrating the spatial differences in drug use among different world regions. In addition, effluent wastewater samples were analysed and compared to influent concentrations in order to evaluate the decrease in concentration of the targeted analytes through two treatment technologies. As a final step, a wide-scope qualitative screening, including hundreds of suspect compounds, was applied in order to have a better knowledge on the presence of pharmaceuticals in waters and to assess the potential impact of the treated wastewater into the receiving aquatic ecosystems.

# 2.2.1. Introduction

Chemical analysis of wastewater influents entering wastewater treatment plants (WWTPs) can provide valuable information on the health and lifestyle of the community connected to the sewer system (Daughton, 2001a). In recent years several studies have been published showing the variety of licit and illicit substances which can be found in wastewater (Causanilles et al., 2016; Ort et al., 2014; Thomas et al., 2012). Loads of illicit drugs and pharmaceuticals are transported to municipal WWTPs where their concentrations can be determined by sophisticated analytical methodologies, and the results used to estimate drug use by the population. This approach, known as wastewater-based epidemiology, has allowed comparative studies between different urban centres around the world. Since the first European collaborative study in 2012 (Thomas et al., 2012), many other researchers have published their results, and the interest has been expanded to other world regions including America and Asia (Bijlsma et al., 2016; Devault et al., 2016; Du et al., 2015; Gatidou et al., 2016; Kim et al., 2015; Klupczynska et al., 2016; Lai et al., 2016). This type of studies that focus on the determination of drug residues in the raw sewage is now recognised as a complimentary tool in the assessment of drug prevalence (Amundsen and Reid, 2014; van Wel et al., 2016).

Besides this epidemiology point of view, there are also environmental implications, because the composition of raw sewage gives information that helps to decide what is the most appropriate treatment to remove or minimise those pollutants and prevent their discharge to the environment (Mara, 2003). Earlier studies in Europe (Bijlsma et al., 2012; Camacho-Muñoz et al., 2009) have shown this potential environmental threat that results from the fact that WWTPs are not specifically designed for removing illicit drug chemical indicators. Actually, some of these, such as 3,4-methylenedioxymethamphetamine (MDMA), are not removed at all by the treatment thus resulting in actual discharges into surface waters. In developing countries it is important that the treatment of municipal wastewater is efficient as much as sustainable, simple in its maintenance, and low in energy and chemicals usage (Mara, 2003). These considerations should be taken into account in order to implement the most appropriate treatment for the specific circumstances of the wastewater to be treated (which are not necessarily the same as in industrialized areas). Treatment processes can vary from wastewater stabilization ponds (WSP), where the wastewater flows at very low flow rate to enable removal by natural processes facilitated by sunlight and high temperatures during a determined

residence time, to more technologically advanced systems, more appropriate when the land area required in natural systems is not available (Mara, 2003).

The study presented in this paper was performed at two locations of Costa Rica, in Central America. The goals were to investigate the presence of 15 selected chemical residues of illicit substances and pharmaceuticals in raw wastewater entering two WWTPs with different treatment technologies. The analytical information obtained was used in an attempt to apply the wastewater-based epidemiology approach for the first time to Costa Rican communities as well as to relate the loads observed to consumption by the population connected to the sewer system. Given the different treatment processes employed in the two WWTPs included in the study, their potential to reduce loads of illicit drugs and pharmaceuticals into the aquatic environment was assessed as well. Finally, since very few data are available for the occurrence of pharmaceuticals in the aquatic environment of Central America and in order to evaluate the potential impact of the wastewaters, a wide-scope qualitative screening was applied to influent and effluent wastewater as well as to surface water down and upstream from the effluent discharge point.

# 2.2.2. Materials and methods

**Sampling sites**

Samples were collected in two areas of Costa Rica, at two different WWTPs (see map in **Fig. 2.2.1**). The first WWTP sampled is located in the northwest of the country and serves the city of Liberia (capital of Guanacaste province). The treatment process consists of 4 WSP, connected in two series. Each pond measures 265 m long, 60 m wide and 2 m deep, containing up to 30,000 $m^3$ of water. The average influent flow rate is 2,680 $m^3$ per day and the hydraulic retention time varies from 24.3 days for the primary ponds to 31.5 days for the secondary ponds, being the total retention time of 55.8 days (Abarca Garbanzo, 2000). See a diagram of the system in **Fig. B1** (SI, Annex B). The second WWTP sampled is located in the central west part of the country in the neighbourhood of El Roble and serves the city of Puntarenas. The process at this WWTP consists of primary settling and secondary treatment with Integrated Fixed-film Activated Sludge (IFAS). The influent wastewater is pumped to the entrance where it is separated into two parallel streams. The average influent flow rate is 6,650 $m^3$ per day. The two WWTPs were selected for the study based on the difference in treatment technologies and to further investigate the presence of pharmaceuticals and illicit drug residues found in a preliminary collection of grab samples.

In addition, surface waters were collected at two rivers nearby: the Liberia and Tárcoles rivers. The Liberia river flows through the city of Liberia, receiving the effluent discharged from the WSPs. It is a small tributary that discharges into the Tempisque river on its way to the Gulf of Nicoya. The river Tempisque is 144 km long. The Tárcoles river originates on the southern slopes of the Cordillera Central volcanic range and flows in a south-westerly direction to the Gulf of Nicoya. The river is 111 km long and its watershed encompasses around 50% of the country's population based at the central valley. See **Fig. 2.2.1** for information on the sampling locations.

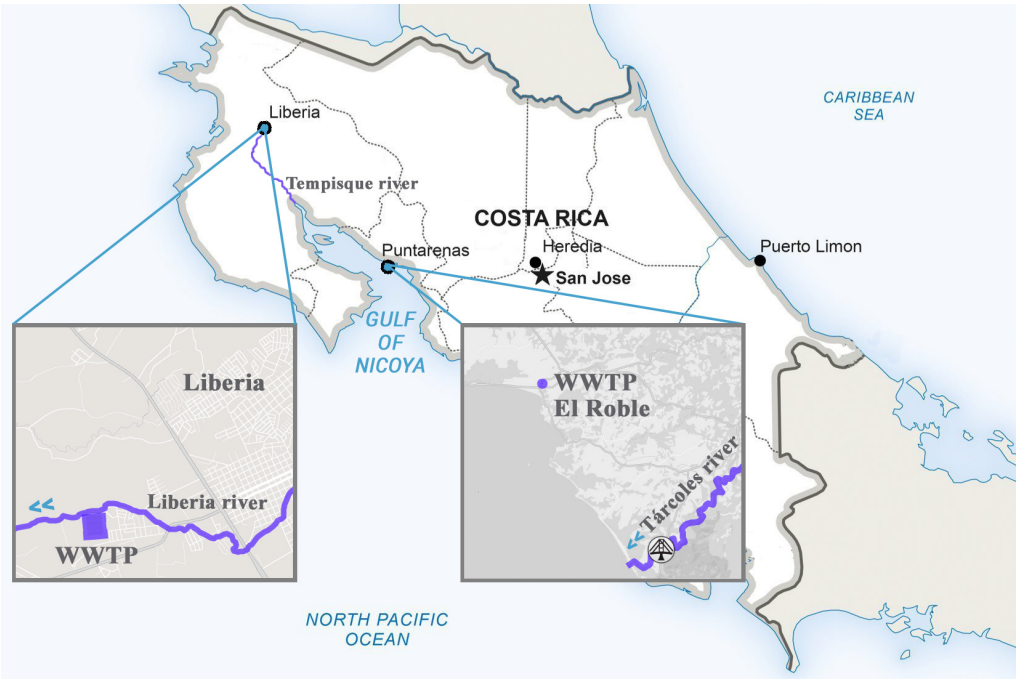


**Fig. 2.2.1.** Map of Costa Rica with detailed information on the location of the WWTPs and rivers included in the study (flow direction indicated by arrows <<).

### Sewer system characterization

The two sewer systems were characterized by means of a standardized questionnaire developed by Ort and colleagues (Castiglioni et al., 2013). The information describes relevant catchment properties, such as number of inhabitants connected to the sewer system and its basis of the estimation, the type of sewer drains, whether exfiltration is expected or not, influent flow control and flow profile or variations. Wastewater chemical properties were also measured: pH, biochemical and chemical oxygen demand ($BOD_5$ and COD) and organic nitrogen content (Nitrogen-Kjeldahl) (Andreottola et al., 1994). A summary is presented in **Table B1**. The collection of this meta-data is essential for the correct interpretation of data obtained and their normalization for further comparison.

**Sample collection**

Different types of samples were collected in order to address the different goals (see **Table B2** for details):

A. 24-h composite influent wastewater samples at both WWTPs to relate the presence of residues of illegal drugs and pharmaceuticals to their consumption by the community included in the catchment area.
B. Grab effluent wastewater samples to describe the efficiency of both treatment systems in removing those residues.
C. Grab river surface water samples to investigate whether those residues can be observed in the aquatic environment.
D. Pooled surface water samples at the different WSP of the system at Liberia to gain more knowledge on the fate of chemicals along this natural treatment process.

For the collection of the influent 24-h composite wastewater samples (A) an ISCO 6712 portable sampler (Teledyne Isco, USA) equipped with a 12-bottle (1 L) rack and a solar panel as energy source was used. The selected operation mode was time-proportional, with a 15-minute sampling time interval of 50 mL aliquots. The 12-bottle rack was surrounded by ice cubes which assured that the sampled aliquots were directly cooled down during the 24-h cycle in order to minimise degradation. Grab effluent samples (B) were collected from the two parallel treatment lines at both WWTPs and pooled and mixed directly. To sample the river Liberia (C), two grab samples were collected 200 m upstream and downstream, respectively, from the WWTP effluent discharge point. A grab sample was taken at the river Tárcoles (C), the sampling point was located at the bridge by the coastal road 34 and 33 km on the way to the city of Jacó. To better represent each WSP (D), three samples were collected with the help of a 5-metre extension stick at the short sides of each pond (represented by a star in **Fig. B1**) and then mixed into a pooled sample (represented by a blue circle, with sample number indicated inside, **Fig. B1**). In total 8 pooled samples were thus collected, one representing each short side of the 4 ponds (see diagram in **Fig. B1**).

**Analytical methodology for quantitative analysis**

Sample treatment and specific information on instrument operating conditions, both chromatographic and spectrometric, and on method validation have been described in detail elsewhere (Bijlsma et al., 2013). Details on dates of sampling, extraction, and analysis are provided in **Table B2**. Briefly, 100 mL of sample were spiked with a mix of isotope labelled internal standards (ILIS), vacuum filtered through 1 µm type A/E glass fibre filters, and then SPE extracted with Oasis HLB cartridges (150 mg, 6 $cm^3$). Two additional samples spiked at 360 ng $L^{-1}$ served as quality control (estimating the recovery). Cartridges were stored frozen at −20 °C and shipped to the laboratory at KWR Watercycle Research Institute, the Netherlands, for further sample treatment and analysis. Cartridges were eluted with methanol and extracts automatically evaporated under a gentle nitrogen stream with a Barkey Optocontrol (Germany). The final extract was reconstituted to 500 µL of 10% methanol aqueous solution.

A 20 µL sample extract aliquot was injected into a liquid chromatography coupled to a linear ion trap (LTQ) FT Orbitrap system (Thermo Electron, Bremen, Germany). Chromatographic separation of the compounds was achieved using a XBridge C18 (2.1 × 150 mm; 3.5 µm particle size) column and an optimized gradient water:methanol, both with 0.05% formic acid. The mass spectrometer was equipped with a Heated Ion Max Electrospray Ionization (HESI) and operated in positive mode. Full-scan accurate mass spectra from *m/z* 100 to 600 Da were obtained at a resolution of 30,000 at full width half maximum (FWHM) (*m/z* 400), besides when an ion exceeded a pre-set threshold and corresponded to the target mass inclusion list specified by the user, the instrument switched to product-ion scan mode ($MS^n$) in the ion trap part with nominal mass measurements. In this way, relevant information for identification and confirmation, e.g., retention time, molecular weight and fragmentation, was obtained in a single analysis. All data were acquired and processed using the software Xcalibur version 2.1 (Thermo).

Mass calibration was performed prior to every batch run using a flow injection of Polytyrosine-1,3,6 solution ($[M + H]^+$ 182.01170/508.20783 and 997.39781) at a flow rate of 10 µL $min^{-1}$. Identification and quantification of the 15 target compounds was performed using the accurate mass of the protonated molecule within a mass window of 5 ppm. For confirmation of the identity of the compounds, in addition to the accurate mass of the precursor ion, at least one nominal mass product ion was used together with its retention time and its relative abundance, and was compared

with that of the reference standards. See Supplementary material (Annex B) for further details (**Tables B3** and **B4**).

**Normalization of mass loads and estimation of drug use**

Daily mass loads in mg $day^{-1}$ 1000 $inhabitants^{-1}$ were calculated by multiplying the concentration in each sample (ng $L^{-1}$) by the corresponding wastewater flows (L $day^{-1}$) and normalizing the obtained values to 1000 inhabitants.

The normalized mass loads for benzoylecgonine (BE) and 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (THC-COOH) were then converted into an estimated pure drug level of consumption: in the case of cocaine, a correction factor of 3.59 was used in order to account for the BE/cocainemolar mass ratio (0.954) and the average molar fraction (29%) of a cocaine dose that is excreted as BE; in the case of cannabis, a correction factor of 182 was used taking into account 0.5% urinary excretion after smoking and the molecular weight ratio tetrahydrocannabinol (THC)/THC-COOH (Gracia-Lor et al., 2016).

**Qualitative screening by UHPLC-QTOF-MS**

The extracts from the pooled surface water samples collected at the WSP system in Liberia were re-analysed separately with a Waters Acquity UPLC system (Waters, Milford, MA, USA) interfaced to a hybrid quadrupole-orthogonal acceleration-TOF mass spectrometer (XEVO G2 QTOF, Waters Micromass, Manchester, UK), using a Z-Spray ESI interface operating in both positive and negative ion mode. The chromatographic separation was performed using an Acquity UPLC BEH C18 1.7 µm particle size column 100 × 2.1 mm (Waters) at a flow rate of 300 µL $min^{-1}$. The mobile phase used was water:methanol, both with 0.01% formic acid. Nitrogen was used as drying gas and nebulizing gas. The desolvation gas flow was set at 1,000 L $h^{-1}$ and the cone gas at 80 L $h^{-1}$. TOF-MS resolution was approximately 20,000 at FWHM at *m/z* 556.

MS data were acquired in centroid mode over an *m/z* range of 50–1,000 Da. Data were acquired in both positive and negative ionization modes in two separate runs. Capillary voltages of 0.7 kV and 2.5 kV were used in positive and negative ionizations modes, respectively. A cone voltage of 20 V was used. Collision gas was argon

99.995% (Praxair, Valencia, Spain). The desolvation temperature was set to 600 °C, and the source temperature to 130 °C. The column temperature was set to 40 °C.

For $MS^E$ experiments, two sequential acquisition functions with different collision energies were created. The low energy (LE) function, selecting a collision energy of 4 eV, and the high energy (HE) function, with a collision energy ramp ranging from15 to 40 eV in order to obtain a greater range of fragment ions. The scan time for both LE and HE function was 0.4 s.

Processing of MS data was done using ChromaLynx XS application manager (within MassLynx v 4.1; Waters Corporation). The following parameters were used for screening: mass window 150 ppm (for positive ID ≤5 ppm), isotope fit as well as retention time (maximum deviation of ≤2.5%) and fragmentation, when available. Software specific settings were: peak width at 5% height: 6 s, peak-to-peak baseline noise: 1,000 and threshold absolute area: 200.

A large screening of pesticides and transformation products, pharmaceuticals belonging to different therapeutic groups, veterinary drugs, X-ray agents, personal care products (PCPs) preservatives and UV filters, sweeteners, illicit drugs and a notable number of metabolites was performed. For this purpose, a homemade database with > 1,500 emerging contaminants was used.

The criteria used for communicating the identification level of the results were based on the following points:

- Detection, based on the presence of 1 accurate-mass ion (mass error ≤ 5 ppm) and retention time agreement (maximum deviation ≤ 2.5%).
- Confirmation of the identity, with at least 2 accurate-mass ions (≤5 ppm) and retention time (≤2.5%).
- Tentative identification, with at least 2 accurate-mass ions justified by literature data and/or compatibility with candidate chemical structure.

The full details of the methodology applied can be found elsewhere (Hernández et al., 2015; Pitarch et al., 2016).

# 2.2.3. Results and discussion

**Concentrations in influent wastewater**

In this work, 15 target compounds were initially selected as indicators of the use of illicit and licit drugs of abuse. Compounds selected included cocaine, cannabis, opioids, some NPS and several benzodiazepines (see **Table B3**). Results from the quantitative analysis of influent composite wastewater samples showed a relatively high concentration of metabolites from the classical illicit drugs cocaine and cannabis, and a moderate concentration of the chemical indicators from the opioids codeine and morphine. The same compounds were present in the influents of both studied WWTPs. None of the chemical indicators from other popular psychoactive substances such as the synthetic phenethylamines (namely amphetamine, MDMA, methamphetamine) or the pharmaceuticals from the family of benzodiazepines (namely oxazepam, temazepam, diazepam), or illicit drugs, such as heroine, were observed.

These results were in agreement with the Report on Drug Situation in Costa Rica that highlights cannabis as the most consumed drug in the country, closely followed by cocaine as the drug that has experienced a major increase in use (UNODC, 2013). A possible explanation is that the country is in the centre of the drug trafficking routes, with easy access to high purity drug at a reasonable price. The latest available prices reported by consumers in Costa Rica are 7 $ per gram of marijuana and 17 $ per gramof cocaine (Havocscope, 2016). Regarding the synthetic drugs prevalence, our results also agreed with data available, revealing that their use remains low and consumption is still a novel phenomenon. The use of amphetamine-type stimulants has been thus far mostly associated with music festivals and tourists coming from North America and Europe (UNODC, 2013).

Concentration data, expressed in ng $L^{-1}$, for each sample is shown in **Table 2.2.1**. Cocaine and its main human metabolite benzoylecgonine were found in the range of 525 – 1,050 and 2,280 – 3,520 ng $L^{-1}$ in the influents of El Roble, and 763 – 2,710 and 2,100 – 4,500 ng $L^{-1}$ in the influents of Liberia, respectively. According to human metabolism, the expected cocaine to benzoylecgonine ratio in raw wastewater should be 0.1 or lower (Castiglioni et al., 2013), in our study the ratios were 0.3 ± 0.1 and 0.5 ± 0.1 respectively, which is in line with other studies (Castiglioni et al., 2011; van Nuijs et al., 2012). The excretion ranges observed in the urine of single individuals in pharmacokinetic studies are variable (e.g. cocaine, range 1–14%), and since in

wastewater the urine excreted by an entire community is collected, only a general comparison is possible (Castiglioni et al., 2011). Besides, in both sewer systems the longest travel distance from toilet to WWTP is no > 4 km, which decreases the possibility of cocaine degradation to BE in the sewer.

Cannabis use was reported based on the detection of two metabolites: THC-COOH and OH-THC. The human metabolism of cannabis varies as a function of its route of administration, and so the presence in wastewater of the two metabolites will vary depending on whether the THC has been consumed by inhalation (smoked) or by oral ingestion. OH-THC is present in much higher concentration after oral administration compared with inhalation, where it is excreted as a minor metabolite. THC-COOH has been selected as the main metabolite in many studies, because of its higher concentration after inhalation and because it has a longer half-life after both routes of administration (Grotenhermen, 2003; Schwilke et al., 2009). Other wastewater-based epidemiology studies which included both cannabis metabolites reported an insignificant amount of OH-THC (Postigo et al., 2011). It is important to highlight that in our study both cannabis metabolites were detected: in El Roble both metabolites were detected at a similar concentration range, whereas in Liberia the acid metabolite presented a higher concentration range than the hydroxyl. The cannabis biomarker more commonly used to estimate consumption, THC-COOH, was present in the range of 124 – 206 ng $L^{-1}$ in the influents of El Roble, and 169 – 502 ng $L^{-1}$ at Liberia; OH-THC was present in the range of 47 – 192 ng $L^{-1}$ in the influents of El Roble, and 26 – 101 ng $L^{-1}$ at Liberia. These results may indicate that the use of cannabis in the studied locations is not only smoked but also ingested (and the oral administration more prevalent in El Roble than in Liberia).

Finally, the opioids codeine and morphine, were found in the range of 448 – 538 and 67 – 77 ng $L^{-1}$ in the influents of El Roble, and 143 – 36 and 16–77 ng $L^{-1}$ at Liberia, respectively.

**Table 2.2.1**. Concentrations in ng $L^{-1}$ of the illicit drug and pharmaceutical residues detected in the samples collected from two Costa Rican WWTPs.

| Sample name | Sample type | Cocaine (ng $L^{-1}$) | Benzoylecgonine (ng $L^{-1}$) | THC-COOH (ng $L^{-1}$) | OH-THC (ng $L^{-1}$) | Codeine (ng $L^{-1}$) | Morphine (ng $L^{-1}$) |
|---|---|---|---|---|---|---|---|
| El Roble - Friday | WW influent 24-h | 950 | 2280 | 124 | 69 | 462 | 71 |
| El Roble - Saturday | WW influent 24-h | 651 | 3520 | 187 | 192 | 460 | 72 |
| El Roble - Sunday | WW influent 24-h | 1050 | 3200 | 206 | 78 | 458 | 77 |
| El Roble - Monday | WW influent 24-h | 525 | 2700 | 158 | 47 | 538 | 75 |
| El Roble - Tuesday | WW influent 24-h | 709 | 2780 | 185 | 87 | 448 | 67 |
| El Roble - Friday | WW effluent grab (1) | 29 | 340 | 10 | 20 | 503 | 15 |
| El Roble - Tuesday | WW effluent grab (2) | 62 | 792 | 36 | < LOD | 665 | 61 |
| Liberia - Wednesday | WW influent 24-h | 1390 | 3410 | 259 | 101 | 278 | 77 |
| Liberia - Thursday | WW influent 24-h | 1210 | 3240 | 228 | 62 | 284 | 65 |
| Liberia - Friday | WW influent 24-h | 1730 | 2770 | 169 | 26 | 325 | 53 |
| Liberia - Saturday | WW influent 24-h | 1880 | 4500 | 502 | 65 | 363 | 62 |
| Liberia - Sunday | WW influent 5-h | 2710 | 4470 | 295 | < LOD | 143 | 16 |
| Liberia - Monday | WW influent 5-h | 763 | 2100 | 231 | 65 | 193 | 41 |
| Liberia WSP | WW effluent grab (1) | < LOD | 1320 | < LOD | 44 | 11 | <LOQ |
| Liberia WSP | WW effluent grab (2) | < LOD | 1260 | 35 | 102 | 29 | <LOQ |
| Liberia WSP | WW effluent grab (3) | < LOD | 1450 | 37 | 200 | 20 | <LOQ |
| Liberia WSP - 1.1 | Pooled SW | < LOD | 3440 | 127 | 75 | 230 | 33 |
| Liberia WSP - 1.2 | Pooled SW | < LOD | 2900 | 119 | 71 | 203 | 30 |
| Liberia WSP - 2.1 | Pooled SW | < LOD | 3280 | 114 | 458 | 252 | 29 |
| Liberia WSP - 2.2 | Pooled SW | < LOD | 3030 | 128 | < LOD | 218 | 36 |
| Liberia WSP - 3.1 | Pooled SW | < LOD | 1930 | 23 | 127 | <LOQ | < LOD |
| Liberia WSP - 3.2 | Pooled SW | < LOD | 1740 | < LOD | 170 | 39 | <LOQ |
| Liberia WSP - 4.1 | Pooled SW | < LOD | 763 | 29 | 133 | 19 | <LOQ |
| Liberia WSP - 4.2 | Pooled SW | < LOD | 832 | 39 | 203 | 29 | < LOD |
| River Liberia - upstream | SW grab | 10 | 4 | 4 | 18 | < LOD | < LOD |
| River Liberia - downstream | SW grab | <LOQ | 88 | < LOD | 37 | <LOQ | < LOD |
| River Tárcoles | SW grab | <LOQ | 72 | < LOD | 86 | 10 | <LOQ |

WSP: Wastewater Stabilization Ponds (see **Fig. B1**)
WW: wastewater
SW: surface water
<LOD: below limit of detection
<LOQ: below limit of quantification

**Normalization of mass loads and estimation of drug use**

Several parameters are crucial in order to calculate the consumption of illicit drugs and pharmaceuticals using the wastewater-based epidemiology approach (Castiglioni et al., 2013). These include: (i) how representative is the average concentration in the composite sample collected (depending on the selected sampling mode), (ii) how accurate are the influent flow data and (iii) the population size estimation used for normalization, (iv) how much is known for each compound about its stability in the specific conditions of the studied sewage, and (v) what is its biotransformation rate, etc. In this preliminary study, which is the first attempt to apply wastewater-based epidemiology in Costa Rica, our efforts were concentrated in gathering the required information and applying the best possible practice protocol suggested by Castiglioni et al. (2013) in order to provide an estimate of drug use.

The biggest challenge and, at the same time, key step to ensure the reliability of the results was the sampling. According to (Ort et al., 2010), the most suitable sampling mode to account for the fluctuations in wastewater quantity or quality with time is flow-proportional. However, it requires a flowmeter able to trigger the auto sampler, and such equipment was not available to us at the time of the study. As our best alternative option (Ort et al., 2010), a time-proportional mode with a high frequency sampling interval of 15 min was selected at both locations to collect the 24-h composite samples. Cooling of the sampling equipment was done to minimise in-bottle degradation in the best possible practical way but because of the high local temperatures some degradation of relatively unstable compounds (e.g. cocaine) may have occurred at the end of every 24-h sampling cycle due to melting of the ice cubes used for cooling.

In the case of the WWTP at El Roble, the sewer system is pressurized and composed of several pumping stations that pool and direct the wastewater to the entrance of the WWTP depending on the water level. With this type of sewer, it is not so important to keep a high frequency sampling because each pulse will represent the sub-catchment rather than each individual toilet flush. However, it was noticeable that during early morning, midday and evening, the pump cycle was shorter than during the rest of the day. Therefore, high frequency sampling was applied to better represent those peak moments, concurring with mealtimes.

In the case of the WWTP at Liberia, the sewer system is gravity drained, therefore a high frequency sampling is crucial to account for the expected wastewater pulses in a representative manner. The majority of the 24 h composite samples collected at the

Liberia plant were indeed obtained by time-proportional sampling every 15 min. Unfortunately, the last two composite samples collected at Liberia only account for 5 h, since the auto sampler stopped due to heavy rainfall events. These two samples only represent the midday period (from13 h to 18 h), and therefore were not used for back-calculation purposes.

Another challenge was the accurate measurement of the influent flowrate, which is key to back-calculate concentration in the composite sample to daily loads. The WWTP at Roble had a flow meter installed at the entrance, so accurate and reliable data was easily acquired for each day (see **Table B5**). The influent flow rate ranged from 7,351 to 8,669 $m^3$ $day^{-1}$ during the 5 days of sampling. In the case of Liberia, the influent flow data was not available at the time of sampling. A flow meter was later installed at the entrance of the WSP, and we could access the continuous data recorded during 15 days in a similar dry period. The influent flow rate ranged from 2,140 to 2,900 $m^3$ $day^{-1}$. This estimated range was selected and used for our normalization purposes (see **Table B6**). To the best of our knowledge the range was stable during dry period, and although we are aware of the limitation, it was our best alternative option.

Another issue was the population size estimation used for normalization of daily loads. The information provided by WWTP operators was obtained from the census of house connections to the sewer. In order to check the reliability of that figure, the inhabitant equivalents (I.E.) from $BOD_5$ data were calculated. Two factors are important in the measurement of the wastewater quality when judged by its $BOD_5$: the water consumption and the amount of organic waste produced per person per day. In industrialized countries, water consumption is generally high (350–400 L $person^{-1}$ $day^{-1}$), which results in wastewater with $BOD_5$ levels of 200–250 mg $L^{-1}$ (Mara, 2003) and it is considered that 1 person represents 60 g $O_2$ per day (Ort et al., 2014). In developing countries $BOD_5$ levels in wastewater are higher, 300–700 mg $L^{-1}$, as the water consumption is typically much lower (40–100 L $person^{-1}$ $day^{-1}$), and it is considered that 1 person represents 40 g $O_2$ per day (Mara, 2003).

According to the information available at El Roble, the number of inhabitants whose households are connected to the sewer system was estimated at 35,758 (census year 2011). The I.E. calculation from $BOD_5$ resulted in a range of 41,550 ± 2,530, for an estimation of 40 g $O_2$ $person^{-1}$ $day^{-1}$, to 27,700 ± 1,690, for 60 g $O_2$ $person^{-1}$ $day^{-1}$. Considering that the census data fell in between the calculated I.E. range, it was selected as the most reliable figure for the normalization.

In the case of Liberia, the number of inhabitants whose households are connected to the sewer system was estimated at 14,215 (census year 2014). The I.E. calculation from $BOD_5$ resulted in a range of 21,170, for 40 g $O_2$ person$^{-1}$ day$^{-1}$ and 2,900 m$^3$ flow, to 10,415 for 60 g $O_2$ person$^{-1}$ day$^{-1}$ and 2,140 m$^3$ flow. Again, the census data fell in between the calculated I.E. range, so it was selected as the most reliable figure for the normalization.

After the above considerations, daily mass loads in mg day$^{-1}$ 1000 inh$^{-1}$ were calculated as indicated in the materials and methods section. In the case of Liberia, the results are presented as a range, corresponding to the normalization taking into account the minimum and the maximum daily flow respectively. Normalized mass loads are showed in **Table 2.2.2.** The highest mass loads corresponded to benzoylecgonine: El Roble presented an average of 650 ± 145 mg day$^{-1}$ 1000 inh$^{-1}$, and at Liberia it ranged from 524 ± 110 to 710 ± 149 mg day$^{-1}$ 1000 inh$^{-1}$. The second most abundant compound was cocaine, El Roble presented an average of 172 ± 45 mg day$^{-1}$ 1000 inh$^{-1}$, and at Liberia it ranged from 234 ± 46 to 317 ± 63 mg day$^{-1}$ 1000 inh$^{-1}$. Cannabis acid presented an average mass load in mg day$^{-1}$ 1000 inh$^{-1}$ of 38 ± 8 at El Roble and a range from 44 ± 22 to 59 ± 30 at Liberia, and OHTHC presented an average mass load in mg day$^{-1}$ 1000 inh$^{-1}$ of 22 ± 14 at El Roble and a range from10 ± 5 to 13 ± 6 at Liberia. The opioid codeine presented an average mass load in mg day$^{-1}$ 1000 inh$^{-1}$ of 105 ± 11 at El Roble and a range from 47 ± 6 to 64 ± 8 at Liberia, whereas morphine presented an average mass load in mg day$^{-1}$ 1000 inh$^{-1}$ of 16 ± 1 at El Roble and a range from 10 ± 2 to 13 ± 2 at Liberia.

Normalized daily mass loads can be used as an indirect estimation of drugs use and for comparative purposes between different cities; in the particular case of cocaine and cannabis it was possible to estimate their consumption by back-calculation from the data for their main human metabolites benzoylecgonine and THCCOOH, as indicated in materials and methods section.

The estimate of pure cocaine consumption at El Roble revealed an average of 2,390 ± 520 mg day$^{-1}$ 1000 inh$^{-1}$ (n = 5 days) and at Liberia, the estimation ranged between 1,880 ± 395 to 2,550 ± 536 mg day$^{-1}$ 1000 inh$^{-1}$ (n = 4 days). These results were remarkably high, but in agreement with recently reported measurements in South America that revealed an average of 3,022 mg day$^{-1}$ 1000 inh$^{-1}$ in Medellin, Colombia (Bijlsma et al., 2016). Notwithstanding the number of samples analysed was low, and the other limitations of this exploratory study discussed above, it seems that one can conclude that the level of consumption in two Costa Rican towns was above that found elsewhere in the world (Gatidou et al., 2016; Lai et al., 2016; Ort et al., 2014).

The estimate of pure cannabis consumption at El Roble revealed an average of 7,160 ± 1,460 mg day$^{-1}$ 1000 inh$^{-1}$ (n = 5 days) and at Liberia, the estimation ranged between 7,930 ± 4,020 to 10,700 ± 5,440 mg day$^{-1}$ 1000 inh$^{-1}$ (n = 4 days). These results were again remarkably high compared to other studies that we have found in literature that reported cannabis consumption figures, even though we used an updated correction factor (Bijlsma et al., 2016; Postigo et al., 2011). In addition, this result should be carefully considered since the chemical analysis of cannabis acid metabolite has several limitations due to its hydrophobic nature and therefore an incorrect load could lead to under-estimations.

These preliminary conclusions on the estimated consumption of cocaine and cannabis should be supported with additional and more detailed studies in the near future. Unfortunately, the statistical study of the distribution of the data could not be performed because the number of observations was not sufficient for the proper significance, however when plotting the data, a weak increase in consumption during the weekend could be observed, representing the recreational use known as "weekend effect" (**Figs. 2.2.2** and **2.2.3**).

**Table 2.2.2**. Normalized mass loads of detected drugs expressed in mg day$^{-1}$ 1000 inh$^{-1}$.

| | | **Load (mg day$^{-1}$ 1000 inh$^{-1}$)** | | | | | |
|---|---|---|---|---|---|---|---|
| | | **Cocaine** | **Benzoylecgonine** | **THC-COOH** | **OH-THC** | **Codeine** | **Morphine** |
| **El Roble** | **Friday** | 195 | 469 | 26 | 14 | 95 | 15 |
| | **Saturday** | 158 | 853 | 45 | 46 | 111 | 17 |
| | **Sunday** | 236 | 721 | 46 | 18 | 103 | 17 |
| | **Monday** | 117 | 606 | 35 | 11 | 120 | 17 |
| | **Tuesday** | 153 | 600 | 40 | 19 | 97 | 14 |
| **Liberia** | **Wednesday** | 209 - 284 | 513 - 696 | 39 - 53 | 15 - 21 | 42 - 57 | 12 - 16 |
| | **Thursday** | 182 - 247 | 488 - 661 | 34 - 46 | 9 - 13 | 43 - 58 | 10 - 13 |
| | **Friday** | 260 - 353 | 417 - 565 | 25 - 34 | 4 - 5 | 49 - 66 | 8 - 11 |
| | **Saturday** | 283 - 384 | 677 - 918 | 76 - 102 | 10 - 13 | 55 - 74 | 9 - 13 |

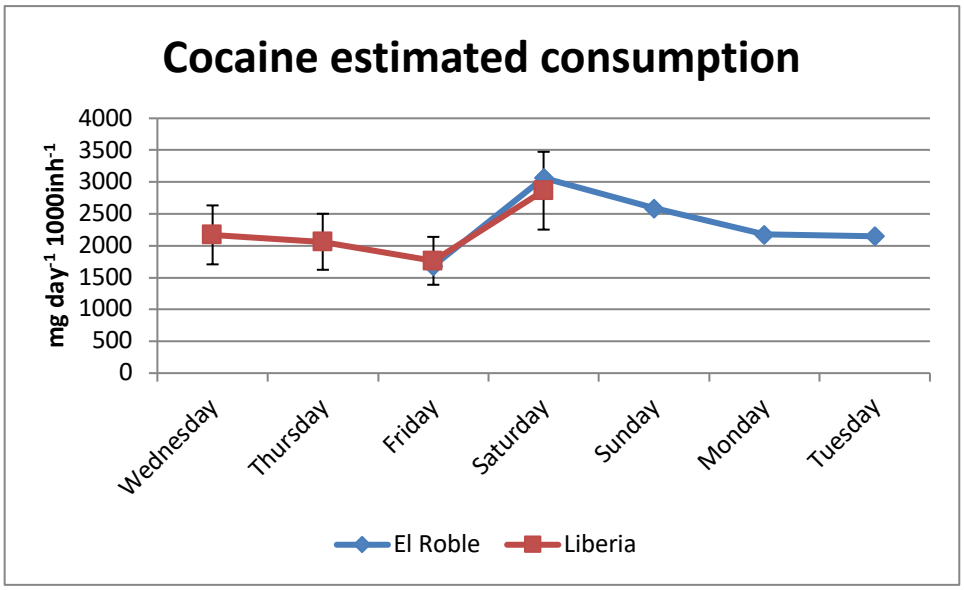


**Fig. 2.2.2**. Cocaine consumption in mg day$^{-1}$ 1000 inhabitants$^{-1}$, calculated from benzoylecgonine normalized loads in the influents of both studied locations using a correction factor of 3.59 (see text). Error bars for the results obtained from Liberia WSP correspond to the range in flow data.

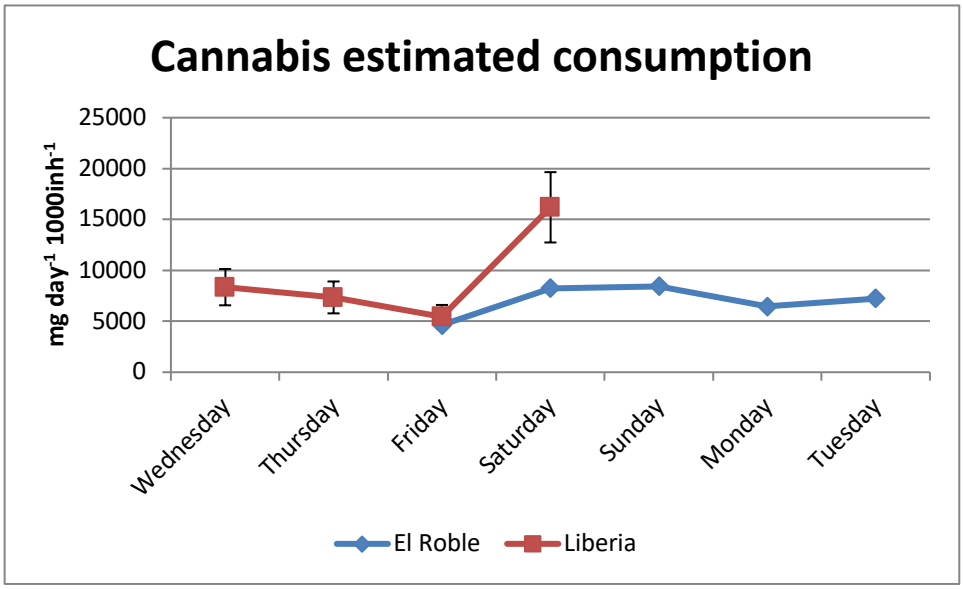


**Fig. 2.2.3**. Cannabis consumption in mg day$^{-1}$ 1000 inhabitants$^{-1}$, calculated from THC-COOH normalized loads in the influents of both studied locations using a correction factor of 182. Error bars for the results obtained from Liberia WSP correspond to the range in flow data.

**Reduction of drug concentrations from wastewater and discharge into the environment**

It is known that WWTPs are an important source of organic pollutants in the environment because the removal rates of most technologies applied nowadays to treat wastewater are not completely efficient. The main concern is the potential impact that this inefficiency might have on the aquatic ecosystem after the discharge of the polluted effluents that usually contain emerging contaminants, such as pharmaceuticals and illicit drugs (Bijlsma et al., 2014b; Gracia-Lor et al., 2012).

The removal rates of the WWTP can be evaluated by comparing the analyte concentrations in influent and their corresponding effluents. In the present study, a true comparison could not be made, since the hydraulic retention times at both WWTPs could not be taken into account when collecting the samples. Therefore, the comparison between average concentrations in influent and effluent was used as a rough estimation of the range of reduction rates, giving characteristic information on how efficient the treatment processes applied in the WWTPs seemed to be towards the detected illicit drugs and pharmaceuticals. The reduction potential was calculated as indicated below:

[1-((mean concentration in effluent)/(mean concentration in influent))]·100 (%).

Concentrations in ng $L^{-1}$ of the individual samples are shown in **Table 2.2.1,** and the graphical summary of the potential decrease in the concentrations is presented in **Fig. 2.2.4**.

Differences per location and compound could be expected because of the different nature of the two treatment technologies applied: while the treatment at the WSPs of Liberia is for the most part anaerobic, and also includes photolytic degradation, the treatment at El Roble with IFAS is for the most part aerobic. As can be seen from **Fig. 2.2.4**, data from both WWTPs were in general agreement for cocaine and benzoylecgonine with reduction efficiencies ranging between 60 and 100%. In the case of cocaine such a complete removal agrees with results of previous studies that categorize this compound as very unstable in wastewater (McCall et al., 2016a). In the WSP of Liberia, cocaine was detected in high concentration in the influent but not detected in any of the pooled surface water nor the effluent samples. This could be explained by its rapid transformation into BE, but also by the higher matrix effects affecting this type of sample (with higher LOQ, see **Table B4**). In the samples collected at the Liberia river (where a lower LOQ was obtained due to reduced matrix effects) a dilution can be observed comparing upstream to downstream. In samples from both

rivers (Liberia and Tarcoles) the residual concentration of cocaine was detectable but below LOQ. At the WWTP of El Roble, cocaine was detected in the effluent, although at lower concentration compared to the influent. The incomplete removal could be explained by the shorter retention time in this system and also because the transformation of cocaine to its metabolites has been identified to occur at higher rate under anaerobic conditions (Ramin et al., 2016) (while treatment at El Roble is mostly aerobic). In the case of benzoylecgonine, the relatively high concentration of cocaine in the influents at both locations may have caused its higher residual concentration. Besides, BE has previously been categorized as highly stable (McCall et al., 2016a). The higher reduction at El Roble can be explained because BE has shown higher transformation in an aerobic system (as opposite to cocaine) (Ramin et al., 2016). Liberia river concentration of BE was higher downstream than upstream, and concentration downstream was similar in both rivers sampled. This highlights how persistent this compound is in the aquatic environment, not getting fully eliminated by the studied wastewater treatments, and therefore discharged into the aquatic environment. This finding is in contrast with some other reports (Bijlsma et al., 2012) where elimination is more complete probably due to more sophisticated treatment.

Cannabinoids and opioids are excreted as glucuronide metabolites and hydrolyzed/deconjugated in wastewater to the parent metabolite. For THC-COOH relatively high reduction rates were observed in both WWTPs, approx. to 90%, and non-detected in river samples. This was in good agreement with other studies in which it was also removed at high rates independently of the WWTP under study (Bijlsma et al., 2012). THC-OH was moderately reduced at El Roble (80%) whereas in the WSPs of Liberia the effluent contained higher levels than the influent. Less pharmacokinetic information about levels in urine is available for this biomarker; THC-COOH and its glucuronide version are considered the main metabolites in urine. Glucuronide bonds in the THC-OH conjugate are believed to be more stable than glucuronide bonds in the THC-COOH conjugate (Schwilke et al., 2009). This might result in the different cleavage rates of the conjugated molecules present in the influents. A possible explanation is that at El Roble there is not enough time for the complete deconjugation to happen due to the relatively short residence time of the water and therefore this is translated as a good removal of non-conjugated THC-OH, while the glucuronide is present and stable and therefore we are not measuring it. In contrast in Liberia, with a long residence time of the water, there might be enough time for the THC-OH-glucuronide to deconjugate stimulated by the natural process and high temperatures.

For codeine, we observed an almost complete reduction in the WSP of Liberia, whereas in the WWTP of El Roble no reduction was observed and the compound appeared to be persistent throughout with an even somewhat higher concentration in the effluent than in the influent. McCall et al., 2016a reported the stability of morphine to be variable, whereas the morphine conjugates rapidly deconjugate. And codeine was reported to be stable in wastewater. This could explain our observations in the current study: in the Liberia system there is sufficient time for both MOR and COD to become deconjugated and subsequently removed (either by biodegradation or sorption). For the El Roble plant, with an estimated residence time of the wastewater of less than 1d, we observed a much reduced removal rate that could be explained by concomitant deconjugation of the glucuronides. The short residence time would not allow the formed deconjugated species to be transformed because of slow subsequent transformation or because the time is too short for the sorption equilibrium for COD and MOR to be reached.

To sum up, the residual low ng $L^{-1}$ concentrations in river samples of benzoylecgonine, OH-THC, and codeine highlighted their stable and persistent behaviour in the aquatic environment, and additional research is required to investigate their possible effect on the ecosystem (Mastroianni et al., 2016).

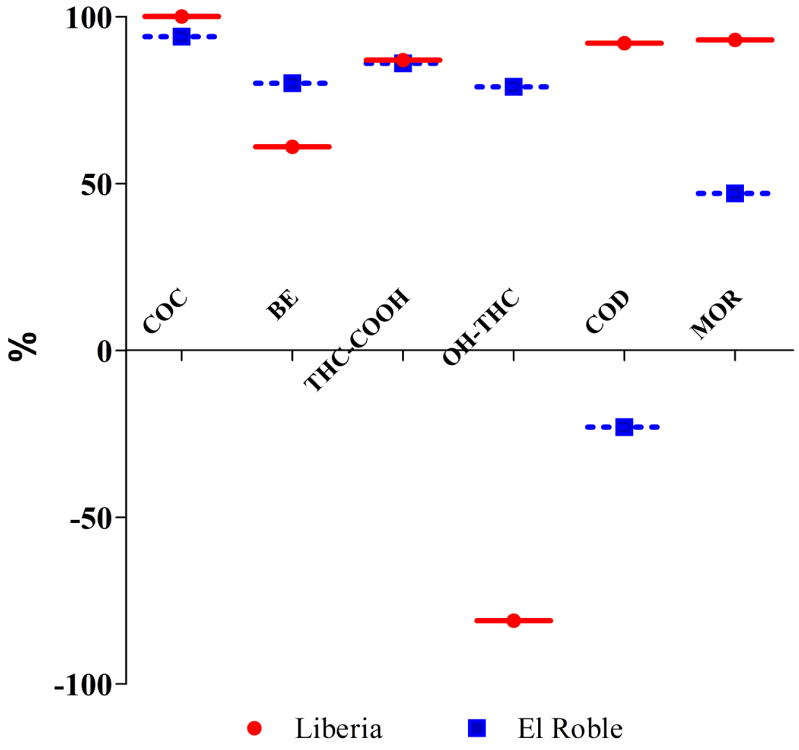


**Fig. 2.2.4**. Reduction potential (expressed in %) calculated for each compound from its mean concentrations in influent and effluent at both WWTPs. Positive values represent higher mean concentration in the influent than in the effluent, whereas negative values represent lower mean concentration in the influent than in the effluent. COC: Cocaine; BE: Benzoylecgonine; THC-COOH: carboxyl-THC; OH-THC: hydroxyl-THC; COD: Codeine; MOR: Morphine.

**Qualitative screening with UPLC-QTOF-MS**

A subsequent step of this work consisted on the application of a wide-scope screening in order to have a comprehensive overview on the presence of pharmaceuticals in the aquatic environment. The objective of these analyses was to widen the scope of the target analytical methodology applied, and to detect and identify other emerging contaminants in the samples, including mainly pharmaceuticals and personal care products. Thus, the potential impact of effluent wastewaters on the surrounding areas could be evaluated. Results from the qualitative screening performed on the pooled samples collected at the different ponds of the WSP system at Liberia are shown in **Table 2.2.3**. From the 1,500 compounds included in the suspect list a relatively small number of compounds could be identified. It should be noted that compounds not included in the suspect list might well be present in the ponds, although not identified because they were not searched for. Similarly, compounds included in the suspect list but present at very lowconcentrations could be missed.

From the identified compounds, three groups could be differentiated:

(1) Persistent compounds, detected in all samples from the river upstream, also in the influent and in the ponds, to the effluent and the river downstream.
(2) Compounds present in the influent, in the ponds, the effluent and the river downstream, but that were not present in the river upstream; therefore introduced into the aquatic environment by the inefficient treatment.
(3) Compounds present in the influent but not present in the river downstream; therefore, reduced or even removed by the treatment system.

Pharmaceuticals and their metabolites were most frequently detected. Some have been already identified as persistent in the environment in other studies. For example, acetaminophen, atenolol and ibuprofen were found to be relatively resistant to photodegradation (Yamamoto et al., 2009) and in the current study they were found to be present in all samples. In general, studies that focused on the biodegradability and transformation of human pharmaceutical active ingredients in the aquatic environment have resulted in the finding of transformation products with a considerably longer half-life DT50 compared to the parent compound (Berkner and Thierbach, 2014). This highlighted the need of including transformation products in the present assessment. The stimulant substance caffeine was also identified in all samples, which corresponds to previously reported findings in which caffeine has been identified as a distinct indicator of anthropogenic influence (Chen et al., 2002)

due to its persistence. The environmental impact of artificial sweeteners has been recently evaluated, since they are newly recognised as persistent and ubiquitous in various aquatic ecosystems (Sang et al., 2014). In the WSPs of Liberia, saccharin and sucralose were identified in the different ponds as well as in river samples and therefore categorized as persistent (group 1). Acesulfame was categorized in group 3 as it was not detected in the river samples. However, photodegradation studies showed that under prolonged exposure to intensive solar irradiation (which would be the case in the WSPs of Liberia) photodegradation products of acesulfame at least six times more persistent than the parent compound were formed (Sang et al., 2014). The UV-filter benzophenone-3 was identified in the influent and effluent. Concern about its environmental persistence may still exist since earlier studies have identified photolysis products despite its categorization as photostable (Liu et al., 2011). The pesticide imidacloprid was identified in the influent and in three ponds only. The intermittent presence could be explained by its concentration level being close to the limit of detection. Finally, the insect repellent N,N-Diethyl-3-methylbenzamide (DEET) was tentatively identified in all samples. This is in agreement with its reported incomplete removal from wastewater (Aronson et al., 2012).

**Table 2.2.3.** Compounds identified by UHPLC-QTOF-MS in different types of water samples: 1 influent and 1 effluent wastewater, 8 pooled surface waters from WSP Liberia (see **Fig. B1**), and 2 surface waters from the river Liberia: approx. 200 m upstream and downstream from the effluent discharge point.

| Substance category | Compound[a] | River Upstream | Influent | 1.1 | 1.2 | 2.1 | 2.2 | 3.1 | 3.2 | 4.1 | 4.2 | Effluent | River Downstream |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pharmaceuticals and metabolites | Acetaminophen | d | √ | d | d | √ | d | | | | | | d |
| | Atenolol | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ |
| | Cefotaxim | | √ | | √ | √ | √ | | √ | | | | |
| | Furosemide (-) | | √ | | | * | * | | | | | | |
| | Gemfibrozil (-) | | √ | √ | √ | * | * | √ | √ | √ | √ | √ | √ |
| | Irbesartan | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ |
| | Naproxen | d | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ |
| | Sulfapyridine | | √ | √ | √ | √ | √ | d | d | d | d | √ | d |
| | Sulfamethoxazole | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ |
| | Telmisartan | d | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | |
| | Salicilic acid (-) | | √ | | | * | * | | | | | d | |
| | Valsartan | | √ | √ | | √ | √ | √ | √ | √ | √ | √ | √ |
| | OM10 (1) | | ⊕ | | | | | | | | | ⊕ | |
| | ISW1b (2) | | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ |
| | IbB4 (-) (3) | ⊕ | ⊕ | ⊕ | ⊕ | * | * | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ |
| | IB5 (4) | | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ |
| | 4-acetyl aminoantipyrine (5) | | √ | | √ | √ | √ | √ | √ | √ | √ | √ | √ |
| | 4-formyl aminoantipyrine (6) | | √ | | √ | √ | √ | √ | √ | √ | √ | √ | √ |
| | Clopidogrel carboxylic acid | | √ | | √ | √ | √ | √ | √ | √ | √ | √ | |
| Stimulant substance | Caffeine | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ |
| Sweeteners | Acesulfame (-) | | d | √ | √ | * | * | √ | √ | √ | √ | √ | |
| | Saccharin (-) | ⊕ | ⊕ | ⊕ | ⊕ | * | * | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ |
| | Sucralose (-) | √ | √ | √ | √ | * | * | √ | √ | √ | √ | √ | √ |
| UV-filter | Benzophenone-3 | | √ | | | | | | | | | √ | |
| Pesticides | Imidacloprid | | √ | | d | | | | | d | d | | |
| | N,N-Diethyl-3-methylbenzamide (DEET) | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ | ⊕ |

Table legend (continues on next page):

Sample codes: primary pond Nº1: samples 1.1 and 1.2; primary pond Nº2: samples 2.1 and 2.2; secondary pond Nº3: samples 3.1 and 3.2; secondary pond Nº4: samples 4.1 and 4.2. See Fig. B1.

[a]A (-) sign denotes analysed in negative mode.

(d) Detected, not confirmed (1 accurate-mass ion <5 ppm + retention time <2.5%).
(√) Confirmed with at least two accurate-mass ions (<5ppm) and retention time (<2.5%) with reference standard.
(⊕) Tentatively identified (at least two accurate-mass ions justified by literature data and/or compatible with the candidate chemical structure).
*Samples 2.1 and 2.2 were only injected in ESI+ mode as not enough extract was available.
([1]) Elucidated metabolite of omeprazole (Boix et al., 2014)
([2]) Elucidated metabolite of irbesartan, ([3]) Elucidated metabolite of ibuprofen, ([4]) Elucidated metabolite of irbesartan (Boix et al., 2016)
([5]) 4-AAA, metabolite of metamizole or dipyrone
([6]) 4-FAA, metabolite of metamizole or dipyrone

# 2.2.4. Conclusions

Chemical analysis of wastewater performed in the present study has revealed the presence of residues of illicit drugs and pharmaceuticals in the influents of two WWTPs in Costa Rica. Results showed high concentration levels of chemical indicators of the classic drugs cocaine and cannabis, besides the moderate presence of the opiates codeine and morphine. These findings support the established drug use pattern described by classical epidemiological tools. It is noteworthy the absence of synthetic phenethylamines such as amphetamine, methamphetamine and MDMA (ecstasy), which are commonly found in wastewaters collected in other regions of the world; as well as the absence of benzodiazepines and other drugs such as heroine. In order to obtain reliable loads and estimated consumption, an assessment to apply the best practice protocol was performed.

The analysis of effluents revealed the incomplete removal of some of the compounds in the two different treatment processes applied in the WWTPs investigated in the study. Additional qualitative screening of a large number of pharmaceuticals allowed to conclude that several compounds were present in the river samples of the surrounding areas, thus showing that WWTP discharges lead to appreciable levels of emerging contaminants in fluvial waters in Costa Rica, the possible impacts of which needs further elucidation.

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# Annex B – Supplementary Information Chapter 2.2.

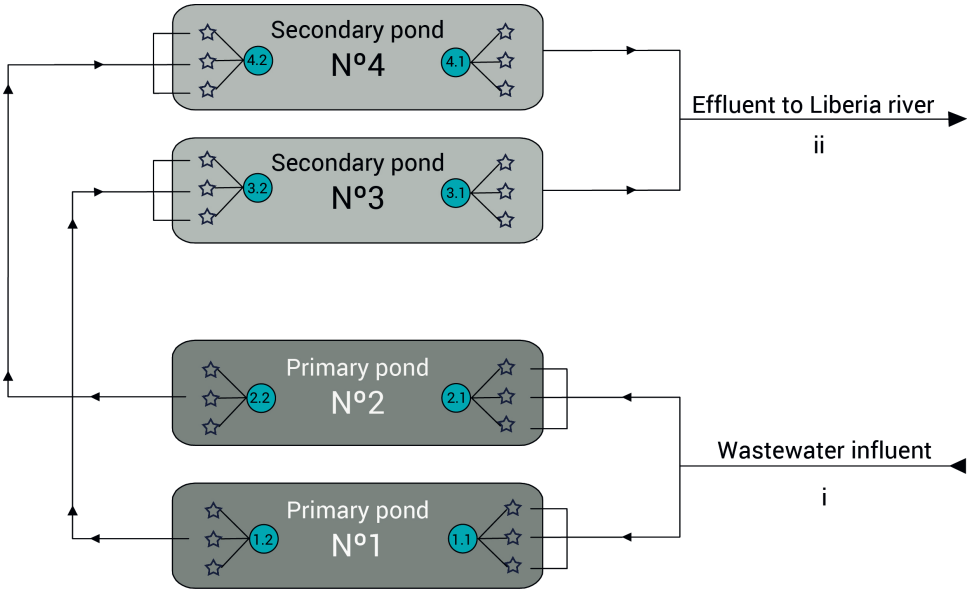


**Fig. B1.** Diagram of the Liberia WSP system. The stars represent the single grab samples collected; the 3 samples representing each short side of a lagoon were pooled into 1 final sample (blue circles, with sample number indicated inside). Other sampling points were: (i) influent (composite), (ii) effluent (grab).

**Table B1.** Summary of responses to the standardized questionnaire describing sewer system characteristics and key parameters of influent wastewater from both WWTPs.

| | El Roble - Puntarenas | Liberia |
|---|---|---|
| Number of inhabitants connected to the sewer system (estimated by house connections) | 35758 | 14215 |
| Year last estimation was made | 2011 | 2014 |
| Sewer system (type of sewer drains) | 75% gravity flow + 25% pressurized | 100% gravity flow |
| Shortest travel distance (km) | 0.2 | 1 |
| Longest travel distance (km) | 2 | 4 |
| Average travelling time from homes to WWTP | Water level dependent | 30 min |
| Flow control devices | Yes | No |
| Description of flow control | By water level | No control |
| Hydraulic retention time in the WWTP | Up to 1 day | 56 days |
| Time of day when sample bottles are changed | 12:00 | 13:00 |
| pH range | 7.55 – 7.66 | 7.03 – 7.34 |
| $BOD_5$ (mg $L^{-1}$) | 209 ± 20 (n=2) | 292 (n=1) |
| COD (mg $L^{-1}$) | 241 ± 3 (n=2) | 312 (n=1) |
| Nitrogen (Kjeldahl) (mg $L^{-1}$) | 40 ± 1 (n=2) | 43 (n=1) |

**Table B2.** Sample details for all type of samples: type, date of collection, date of extraction, reconstitution and analysis.

| Sample name | Sample type | Sample date | Day of SPE | Extract reconstitution | LC-MS |
|---|---|---|---|---|---|
| El Roble - Friday | WW influent 24-h | 28/11/2014 | 1/12/2014 | 19/02/2015 | 25/02/2015 |
| El Roble - Saturday | WW influent 24-h | 29/11/2014 | 1/12/2014 | 19/02/2015 | 25/02/2015 |
| El Roble - Sunday | WW influent 24-h | 30/11/2014 | 1/12/2014 | 19/02/2015 | 25/02/2015 |
| El Roble - Monday | WW influent 24-h | 1/12/2014 | 3/12/2014 | 19/02/2015 | 25/02/2015 |
| El Roble - Tuesday | WW influent 24-h | 2/12/2014 | 3/12/2014 | 19/02/2015 | 25/02/2015 |
| El Roble - Friday | WW effluent grab (1) | 28/11/2014 | 1/12/2014 | 19/02/2015 | 25/02/2015 |
| El Roble - Tuesday | WW effluent grab (2) | 3/12/2014 | 3/12/2014 | 19/02/2015 | 25/02/2015 |
| Liberia - Wednesday | WW influent 24-h | 19/11/2014 | 20/11/2014 | 19/02/2015 | 25/02/2015 |
| Liberia - Thursday | WW influent 24-h | 20/11/2014 | 21/11/2014 | 19/02/2015 | 25/02/2015 |
| Liberia - Friday | WW influent 24-h | 21/11/2014 | 25/11/2014 | 19/02/2015 | 25/02/2015 |
| Liberia - Saturday | WW influent 24-h | 22/11/2014 | 25/11/2014 | 19/02/2015 | 25/02/2015 |
| Liberia - Sunday | WW influent 5-h | 23/11/2014 | 25/11/2014 | 19/02/2015 | 25/02/2015 |
| Liberia - Monday | WW influent 5-h | 24/11/2014 | 25/11/2014 | 19/02/2015 | 25/02/2015 |
| Liberia WSP | WW effluent grab (1) | 22/11/2014 | 25/11/2014 | 15/12/2014 | 9/01/205 |
| Liberia WSP | WW effluent grab (2) | 22/11/2014 | 25/11/2014 | 15/12/2014 | 9/01/205 |
| Liberia WSP | WW effluent grab (3) | 22/11/2014 | 25/11/2014 | 15/12/2014 | 9/01/205 |
| Liberia WSP - 1.1 | Pooled SW | 22/11/2014 | 25/11/2014 | 15/12/2014 | 9/01/205 |
| Liberia WSP - 1.2 | Pooled SW | 22/11/2014 | 25/11/2014 | 15/12/2014 | 9/01/205 |
| Liberia WSP - 2.1 | Pooled SW | 22/11/2014 | 25/11/2014 | 15/12/2014 | 9/01/205 |
| Liberia WSP - 2.2 | Pooled SW | 22/11/2014 | 25/11/2014 | 15/12/2014 | 9/01/205 |
| Liberia WSP - 3.1 | Pooled SW | 22/11/2014 | 25/11/2014 | 15/12/2014 | 9/01/205 |
| Liberia WSP - 3.2 | Pooled SW | 22/11/2014 | 25/11/2014 | 15/12/2014 | 9/01/205 |
| Liberia WSP - 4.1 | Pooled SW | 22/11/2014 | 25/11/2014 | 15/12/2014 | 9/01/205 |
| Liberia WSP - 4.2 | Pooled SW | 22/11/2014 | 25/11/2014 | 15/12/2014 | 9/01/205 |
| River Liberia - upstream | SW grab | 22/11/2014 | 25/11/2014 | 15/12/2014 | 9/01/205 |
| River Liberia - downstream | SW grab | 22/11/2014 | 25/11/2014 | 15/12/2014 | 9/01/205 |
| River Tárcoles | SW grab | 5/02/2013 | 20/02/2013 | 20/02/2013 | 11/08/2013 |

**Table B3.** Exact masses of the protonated target drugs of abuse, nominal masses and relative abundance of product ions, together with their retention times and isotope labelled internal standards used for quantification.

| Compound | $t_R$ (min) | Precursor ion $[M+H]^+$ *m/z* | Product ion 1 *m/z* | Product ion 2 *m/z* | RA (%) | Internal standard |
|---|---|---|---|---|---|---|
| Cocaine | 9.15 | 304.15433 | 182.1 | 150.2 | 2.6 | Cocaine-$d_3$ |
| Benzoylecgonine | 8.50 | 290.13868 | 168.2 | 272.2 | 4.8 | Benzoylecgonine-$d_3$ |
| THC | 21.55 | 315.23186 | 259.2 | 193.2 | 76.7 | THC-$d_3$ |
| THC-COOH | 20.25 | 345.20604 | 327.2 | 299.3 | 6.1 | THC-COOH-$d_3$ |
| OH-THC | 19.85 | 331.22677 | 313.3 | - | | OH-THC-$d_3$ |
| Codeine | 5.30 | 300.15942 | 215.2 | 243.1 | 47.7 | 6-MAM-$d_3$ |
| Morphine | 3.45 | 286.14334 | 201.1 | 229.1 | 51.9 | Morphine-$d_3$ |
| 6-MAM | 6.35 | 328.15433 | 211.2 | 268.2 | 73.7 | 6-MAM-$d_3$ |
| MDMA | 6.91 | 194.11755 | 163.1 | 58.0 | 1.0 | MDMA-$d_5$ |
| Amphetamine | 6.45 | 136.11208 | 119.1 | 91.1 | 0.5 | Amphetamine-$d_{11}$ |
| Methamphetamine | 6.70 | 150.12773 | 119.0 | 91.1 | 9.0 | Methamphetamine-$d_5$ |
| Oxazepam | 15.00 | 287.05818 | 269.1 | 241.1 | 3.9 | Oxazepam-$d_5$ |
| Diazepam | 16.07 | 285.07892 | 257.1 | 222.2 | 30.4 | Diazepam-$d_5$ |
| Temazepam | 15.28 | 301.07383 | 283.0 | 255.2 | 9.2 | Nordazepam-$d_5$ |
| Nordazepam | 15.65 | 271.06327 | 243.1 | 208.1 | 37.7 | Nordazepam-$d_5$ |

RA: relative abundance of product ions.

**Table B4.** Theoretical limits of quantification, expressed in ng $L^{-1}$, were updated for the different type of matrices.

The LOQ can be determined by using the lowest standard visible in the calibration curve which meets all the identification criteria (typically the absence/presence of the confirmation product ion is the critical parameter) and corrected for the matrix suppression and the concentration factor. The matrix suppression was calculated by using the area of the accurate mass signal of the deuterated standard, spiked before extraction (in matrix), divided by the average area of the deuterated standard in the calibration curve (in solvent).

| Compound | Recovery (%) | LOQ (ng $L^{-1}$) per matrix type | | | |
|---|---|---|---|---|---|
| | | Influent | Effluent | River | Pooled surface water from WSPs |
| Cocaine | 105 | 21 | 14 | 9 | 51 |
| Benzoylecgonine | 100 | 6 | 4 | 3 | 5 |
| THC | 110 | 33 | 71 | 57 | 360 |
| THC-COOH | 95 | 10 | 5 | 4 | 11 |
| OH-THC | 91 | 22 | 17 | 12 | 51 |
| Codeine | 106 | 16 | 9 | 6 | 13 |
| Morphine | 99 | 11 | 8 | 10 | 9 |
| 6-MAM | 103 | 33 | 93 | 5 | 28 |
| MDMA | 103 | 180 | 6 | 5 | 360 |
| Amphetamine | 120 | 180 | 23 | 10 | 360 |
| Methamphetamine | 101 | 180 | 180 | 5 | 360 |
| Oxazepam | 98 | 10 | 5 | 3 | 10 |
| Diazepam | 101 | 7 | 6 | 4 | 8 |
| Temazepam | 102 | 9 | 5 | 14 | 9 |
| Nordazepam | 99 | 8 | 6 | 3 | 10 |

**Table B5.** Flow data WWTP El Roble.

| day | date | Flow $m^3$ $day^{-1}$ |
|---|---|---|
| Friday | 28-11-2014 | 7351 |
| Saturday | 29-11-2014 | 8669 |
| Sunday | 30-11-2014 | 8028 |
| Monday | 1-12-2014 | 7999 |
| Tuesday | 2-12-2014 | 7715 |

**Table B6.** Flow data used for deriving minimum and maximum flow rates at the WWTP Liberia.

| | Flow $m^3$ $day^{-1}$ |
|---|---|
| day 1 | 2905 |
| day 2 | 2838 |
| day 3 | 2786 |
| day 4 | 2743 |
| day 5 | 2662 |
| day 6 | 2633 |
| day 7 | 2817 |
| day 8 | 2643 |
| day 9 | 2734 |
| day 10 | 2482 |
| day 11 | 2295 |
| day 12 | 2234 |
| day 13 | 2240 |
| day 14 | 2139 |
| day 15 | 2788 |
| MIN | 2140 |
| MAX | 2900 |

# 2.3.

# Qualitative screening for new psychoactive substances in wastewater collected during a city festival using liquid chromatography coupled to high-resolution mass spectrometry

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# Abstract

The inclusion of new psychoactive substances (NPS) in the wastewater-based epidemiology approach presents challenges, such as the reduced number of users that translates into low concentrations of residues and the limited pharmacokinetics information available, which renders the choice of target biomarker difficult. The sampling during special social settings, the analysis with improved analytical techniques, and data processing with specific workflow to narrow the search, are required approaches for a successful monitoring. This work presents the application of a qualitative screening technique to wastewater samples collected during a city festival, where likely users of recreational substances gather and consequently higher residual concentrations of used NPS are expected. The analysis was performed using liquid chromatography coupled to high resolution mass spectrometry. Data were processed using an algorithm that involves the extraction of accurate masses (calculated based on molecular formula) of expected *m/z* from an in-house database containing about 2,000 entries, including NPS and transformation products. We positively identified eight NPS belonging to the classes of synthetic cathinones, phenethylamines and opioids. In addition, the presence of benzodiazepine analogues, classical drugs and other licit substances with potential for abuse was confirmed. The screening workflow based on a database search was useful in the identification of NPS biomarkers in wastewater. The findings highlight the specific classical drugs and low NPS use in the Netherlands. Additionally, *meta*-chlorophenylpiperazine (mCPP), 2,5-dimethoxy-4-bromophenethylamine (2C-B), and 4-fluoroamphetamine (FA) were identified in wastewater for the first time.

# 2.3.1. Introduction

A current trend in analytical and environmental chemistry is the chemical analysis of raw wastewater in order to identify specific biomarkers that could inform on the health and lifestyle of the population living in the catchment area under study (Daughton, 2001b). This approach, named wastewater-based epidemiology (WBE), has been successfully applied in revealing the use of illicit substances (Ort et al., 2014; Thomas et al., 2012) and other licit substances, such as pharmaceuticals (J.A. Baz-Lomba et al., 2016; Causanilles et al., 2016), alcohol (Yeonsuk Ryu et al., 2016), tobacco (Castiglioni et al., 2014), stress biomarkers (Ryu et al., 2015), and more recently new psychoactive substances (NPS) (Bade et al., 2017; Borova et al., 2015; González-Mariño et al., 2016b; Kinyua et al., 2015a; Senta et al., 2015).

NPS are psychotropic drugs that produce similar effects to those produced by illicit substances, and are not directly controlled by international conventions (M. Reid and Thomas, 2016). They may pose a public health threat, because there is no scientific evidence of their pharmacokinetics, recommended dose, effects or safety. Furthermore, they are easily acquired through the internet and smart shops where they are sold under various product labels with often misleading information. To date, more than 560 NPS have been reported (EMCDDA, 2016a). Their monitoring and control is a challenging task because the NPS market is very dynamic with analogues constantly emerging to satisfy consumers' demands and avoid criminalization.

Typically, WBE studies focus on target analysis of well-known biomarkers that are present at a sufficient concentration to be detected and quantified. In the case of NPS, several challenges may arise: the choice of target biomarker is difficult; analytical standards are high-priced and in some cases not available, particularly for metabolites. These recreational substances are mostly consumed to a lesser extent compared to popular substances (cocaine, ecstasy), which will be translated into very low ng $L^{-1}$ residue concentrations in wastewater. As such, recent WBE studies that focused on target screening of NPS with available reference standards using low and high-resolution MS have shown that extremely low levels are detected or none at all (Bade et al., 2017; Borova et al., 2015; González-Mariño et al., 2016b; Kinyua et al., 2015a; Senta et al., 2015). In contrast, studies that applied qualitative screening techniques based on high-resolution mass-spectrometry (HRMS) to biological matrices from individuals, namely blood and urine, (Andreasen et al., 2015; Kinyua et al., 2015b; Negreira et al., 2016b; Sundström et al., 2015) or collective pooled urine from festivals (Archer et al., 2014, 2013; Kinyua et al., 2016) have detected and

identified several NPS and their metabolites. The results from the previously cited works stress the importance of different sample matrices, sampling locations and the role of improved analytical techniques to track NPS use. Pooled urine from recreational areas has the advantage that levels of drug biomarkers are higher and easier to detect compared to wastewater (Kinyua et al., 2016; Mardal et al., 2017). However, the collection of pooled urine may likely miss samples from female users (Kinyua et al., 2016). Wastewater influent samples collected at a wastewater treatment plant (WWTP) are representative for populations connected to the sewer system and would provide useful information about an entire community's use of NPS within a catchment area if advanced analytical techniques are applied. However, consumption by the population, and hence concentrations of NPS in wastewater, are much lower than those of the more traditional illicit drugs and the daily wastewater levels may thus not be sufficiently high to allow detection. A modification aimed at gathering qualitative information on NPS use by targeting sampling at some social settings (festivals or events) where substance use is elevated would increase the likelihood of successfully identifying and detecting NPS (M. Reid and Thomas, 2016). Another powerful improvement which can be applied when no reference standards are available to confirm mass spectra and retention time information is the use of HRMS using the "suspect screening" approach, which involves extraction of the exact masses of expected ions $[M+H]^+$ or $[M-H]^-$ from the acquired data (Hernández et al., 2016; Kinyua et al., 2015b; Krauss et al., 2010). Fundamentally, we can cast a wider net for screening of NPS by creating a suspect list that includes all potential biomarkers of interest.

In the Netherlands, NPS appear to be used at a lower rate compared to other countries, more in particular the UK, because of the high quality, low price and availability of classical drugs such as cocaine and 3,4-methylenedioxymethamphetamine (MDMA) (Hondebrink et al., 2015). It is noteworthy to mention that amphetamine-type stimulants are easy to acquire because The Netherlands and Belgium are the most important European production areas (EMCDDA and Europol, 2016). Thus far, NPS have been found as adulterants or as a replacement of classical drugs without users' awareness. However, their use is increasing progressively in recent years as drug of choice, as data from the Poisons Information Centre show (Hondebrink et al., 2015).

The aims of the present study were to: i) analyse wastewater collected during a special social setting with a suspect screening approach; ii) elucidate the identity of NPS and provide a qualitative snapshot of recreational substances used during a

festival in the Netherlands; iii) discuss the challenges in applying a suspect screening approach to wastewater samples. For this purpose, we used liquid chromatography coupled to HRMS for the screening of eight 24-h composite raw wastewater samples collected at the WWTP serving the catchment area of Amsterdam in 2012 and 2014, during festivals that brought approximately 300,000 visitors to the city.

# 2.3.2. Materials and methods

**Sample collection**

Eight 24-h flow-dependent influent composite samples were collected after the sand trap at the main WWTP serving the city of Amsterdam, representing 769,000 inhabitants (according to census data). The sampling campaign was performed in the summer of 2012 and 2014 just prior to and during a festival that attracted ~300,000 visitors to the city. Such festivals are key sites for drug use among young people (Dilkes-Frayne, 2016). Four samples corresponding to 24-h composite samples from Thursday to Sunday were collected in both years. WWTP characteristics and sample details can be found in **Table C1 (**in Supplementary Information, Annex C).

**Sample treatment**

Samples were stored in HDPE containers and frozen immediately after collection at -20 °C until analysis. Samples were thawed at 4 °C for 24 h, and after homogenization, a 50 mL aliquot was filtered on glass microfiber filters GF/A (1.6 µm). Solid-phase extraction (SPE) was performed with Oasis HLB cartridges (150 mg, 6 cc) pre-conditioned with 8 mL of methanol and 8 mL of ultra-pure water. After sample loading, the SPE cartridge was washed with 4 mL of ultra-pure water and vacuum dried for 30 min. After elution with 8 mL methanol, the eluate was evaporated to dryness with a gentle nitrogen stream in a water bath at 35 ºC. The final extract was reconstituted to 250 µL water/methanol 90:10, v/v.

**Instrumental analysis**

Extracts were analysed twice by liquid chromatography coupled to high-resolution mass spectrometry: (i) with an Agilent LC-QTOFMS at the Toxicological Centre at the University of Antwerp, Belgium; and (ii) with a Thermo LC-LTQ-Orbitrap at the KWR Watercycle Research Institute in Nieuwegein, The Netherlands.

Chromatographic separation was achieved for both systems with a Phenomenex Biphenyl (100 mm x 2.1 mm, 2.6 µm) column fitted to a SecurityGuard ULTRA Holder for UHPLC columns (2.1 - 4.6 mm). The mobile phase was ultrapure water (A) and 80:20 acetonitrile:water (B) both with 0.04% of formic acid, and with the following

gradient: 0 min: 2% B; 2 min: 2% B; 18 min: 40% B; 25 min: 90% B; 29 min: 90% B; 29.5 min: 2% B; 33 min: 2% B. The total run time including column equilibration was 33 min. The injection volume was set to 2 µL and the flow rate was 0.4 mL $min^{-1}$.

(i) Quadrupole-time-of-flight mass spectrometry

The MS system consisted of an Agilent 6530 Accurate-Mass QTOF instrument (Agilent Technologies, Santa Clara, USA) operated with a jet stream electrospray ionisation source (Dual AJS ESI source). The source parameters were as follows: gas temperature, 325 °C; gas flow, 8 L $min^{-1}$; nebulizer gas, 40 psi; sheath gas temperature, 325 °C; sheath gas flow, 11 L $min^{-1}$; capillary voltage, 3,500 V and the nozzle voltage, 0 V. The data-independent acquisition (All-ions MS/MS) was set-up to acquire three scan segments in MS mode alternating the collision energies to 0 eV, 15 eV, and 35 eV, in only one injection. With this acquisition mode, all ions are fragmented without a specific isolation of a precursor ion in the first mass analyser. The mass accuracy (within ± 2 ppm) of the QTOFMS was calibrated before each analysis using a reference solution for scanning up to *m/z* 1,700. The scan range was set to acquire between *m/z* 50 - 1000 at a rate of 2.5 spectra/s for each scan segment. For measurements, the MS was operated in 4 GHz High Resolution mode with a typical resolution of 9,000-20,000 full width at half maximum (FWHM) for the mass range *m/z* 118.0862- 622.0289. Analyses were performed in positive ESI mode. Mass calibration of the QTOFMS system was controlled by constant infusion of a reference mass solution (acquired from Agilent Technologies) into the source of the QTOFMS system during the analysis. The ions selected for recalibrating the mass axis, ensuring the accuracy of mass assignations throughout the chromatographic run were the protonated reference ions ($[M+H]^+$ 121.0509 and 922.0098).

(ii) Linear Trap Quadrupole Orbitrap mass spectrometry

The MS system consisted of an LTQ FT Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) equipped with a Heated Ion Max Electrospray Ionization (HESI) probe and operated in the positive ion mode. The conditions were: source voltage 3,000 V, heated capillary temperature 300 °C, vaporizer temperature 350 °C, capillary voltage 24 V and tube lens 70 V. The mass spectrometer operated under data-dependent-acquisition (DDA) mode during the complete chromatographic run, in which both MS and $MS^n$ spectra were acquired simultaneously. The instrument was initially set to operate in full-scan mode with accurate mass measurements. Full-scan accurate mass spectra (mass range from 50 to 1,000 Da) were obtained at a mass resolution of 60,000 FWHM (*m/z* 400). When an ion exceeded a pre-set threshold, or

corresponded to the inclusion mass list specified by the user, the instrument switched to product-ion scan mode ($MS^n$) in the ion trap with nominal mass measurements. This inclusion list was built with the masses previously tentatively identified by the QTOFMS (see **Tables C2** and **C3** in SI, Annex C) with a retention time window of ± 2 min and collision energy of 35%. In this way, relevant information for identification and confirmation, e.g., retention time, molecular weight and fragmentation, was obtained in a single analysis. The total cycle time at the selected resolution corresponded to 0.15 s. Mass calibration was performed before each batch run by using flow injection of a Polytyrosine-1,3,6 solution ($[M+H]^+$ 182.01170, 508.20783 and 997.39781) at a flow rate of 10 µL $min^{-1}$.

**Data analysis workflow for suspect screening**

MassHunter qualitative analysis software Version B.06.00 with the personal compound database and library manager (PCDL, Version Rev. B.04.01, Agilent Technologies, Santa Clara, USA), ACD/MS Workbook Suite 2015 (MS Fragment), and Xcalibur software Version 2.1 (Thermo Fisher Scientific, Breda, The Netherlands) were used for data processing using a modification of the workflow described elsewhere (Kinyua et al., 2015b).

An updated version of our in-house spectral library was used, comprised of (il)licit drugs and pharmaceuticals of potential abuse, NPS, and their metabolites and transformation products of the different chemical families. Since wastewater can be considered a diluted urine sample, and metabolites/transformation products are expected to be more abundant (Meyer and Maurer, 2016), an effort was made to include the latter on the suspect list. To this end, the database was built upon the currently available knowledge on pharmacokinetics of NPS. This information was primarily obtained from existing literature (*in vitro* and *in vivo* studies) and the few Phase I and Phase II metabolites listed, from organizations such as European Early Warning System (EWS), European Monitoring Center for Drugs and Drug Addiction (EMCDDA), United Nations Office on Drugs and Crime (UNODC) and TICTAC Communications Limited (London), and from the section Forensic Science Products in caymanchem.com. The library currently consists of almost 2,000 entries.

The workflow is based on a 'Find by Formula (FbF)' algorithm (Agilent Technologies, Santa Clara, USA) that involves the extraction of accurate masses (calculated based on molecular formula) of expected ions $[M+H]^+$ in combination with specific parameters from the data acquired. The criteria for the proposal of a candidate list according to

these parameters is defined as follows: error in the accurate mass lower than 10 ppm for the precursor ion and 20 ppm for the product ions, retention time deviation of maximum 0.1 min, signal to noise ratio lower than 3, and isotope abundance and spacing of minimum 50%. Eventually a list of candidates with their proposed fragments is generated. See **Fig. 2.3.1** for the schematic of the followed workflow, and **Fig. 2.3.2** for the schematic workflow used to eliminate false positives and prioritize compounds for purchase.

To communicate confidence of the identifications, we used the levels described by Schymanski and colleagues (Schymanski et al., 2014). Level one was assigned after confirmation by injection of a reference standard for determination of retention time ($t_R$), MS and MS/MS spectra. For Level two (2a), a probable structure was proposed based on matching existing library and literature spectra data or using non-reported diagnostic MS/MS product ions evidence. The Level 2a confirmations were based on the in-house library spectra data available from previous experiments, and intoxication cases received at our forensic toxicology laboratory (including *in vivo* samples from individual users) (Kinyua et al., 2015b; Lai et al., 2015b; Mardal et al., 2017; Negreira et al., 2016a, 2016b, 2015). In addition, we used literature spectra from *in vitro* and *in vivo* studies on NPS. The Level 2a identification is definite, but lacks a commercial reference standard to warrant the Level 1 identification. For Level 3, a tentative candidate was proposed where the evidence existed for possible structure(s), however the exact structure remained unconfirmed.

Using MetFrag 2.2 developed by Ruttkies et al., 2016, available at (http://msbi.ipb-halle.de/MetFragBeta/), we input the neutral molecular formula of the precursor, and input the qualified measured product ion accurate masses and their abundances. Then MetFrag retrieves candidate structures from selected databases (ChemSpider, PubChem, KEGG and STOFF-IDENT). The candidates were *in silico* fragmented (two fragmentation steps) and the generated fragments were compared with the product ions in the acquired measured mass spectrum.

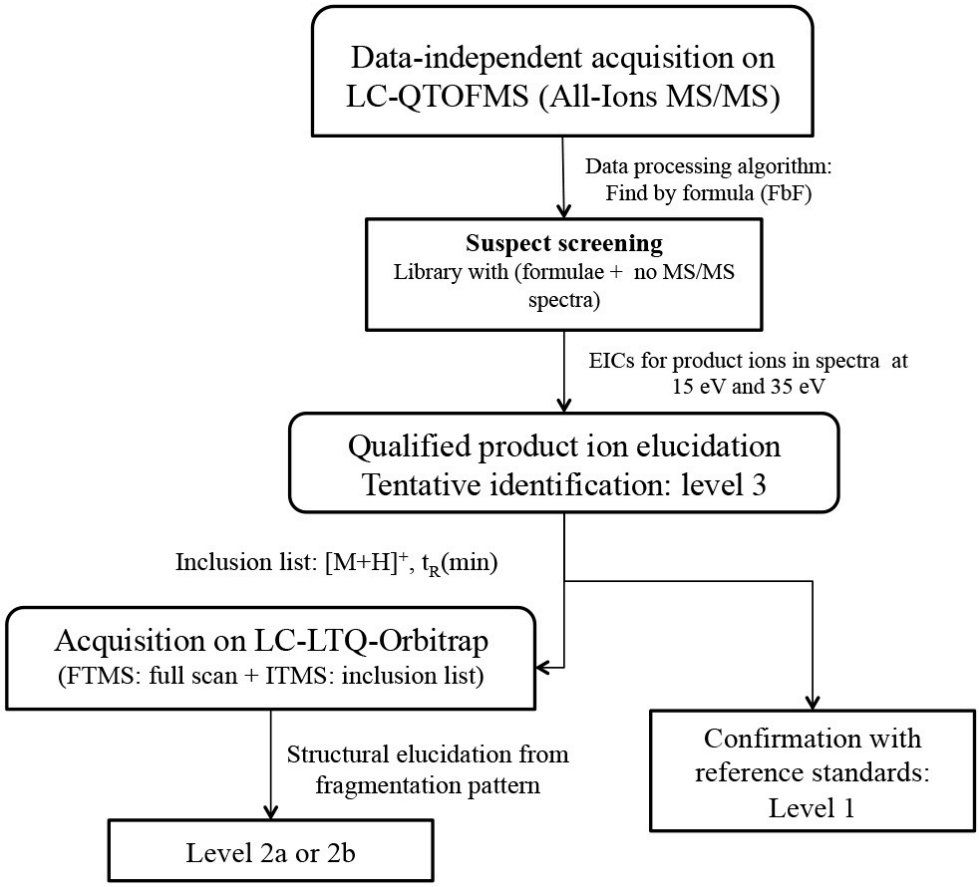


**Fig. 2.3.1**. Schematic of suspect screening workflow.

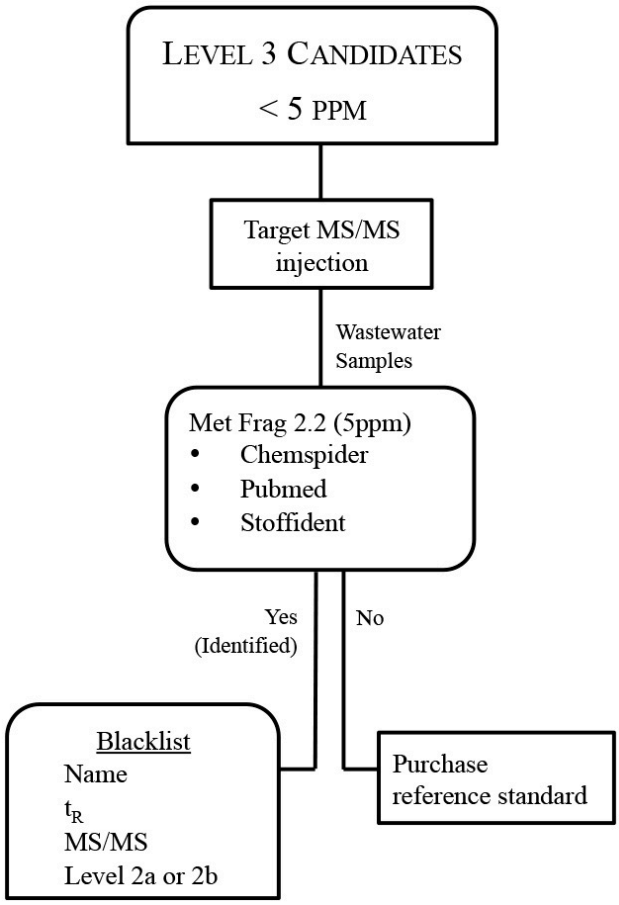


**Fig. 2.3.2.** Schematic of workflow used to eliminate false positives and prioritize compounds for purchase.

# 2.3.3. Results and discussion

**Snapshot of recreational substances in use**

The application of the suspect screening workflow was helpful in the search for NPS. The database allowed narrowing the search from a list of almost 2,000 entries, including 560 parent NPS in addition to their known metabolites and transformation products. **Table 2.3.1** shows the final list of 8 detected NPS that were identified at least at level 3. The identity of two NPS could not be confirmed at Level 1 due to the lack of reference standards. m-Chlorophenylpiperazine (mCPP), 2,5-dimethoxy-4-bromophenethylamine (2C-B), and 4-fluoroamphetamine (FA) were identified for the first time ever in wastewater samples.

Synthetic cathinones and phenethylamines were in general terms the most frequently detected families, considering the full list of tentative hits (levels 4 – 5, results not shown). They are commonly used as stimulants to substitute or complement MDMA, amphetamine and cocaine. 3-methoxy-4-methylamphetamine (MMA) was detected in 5 out of the 8 samples analysed. The detection was classified with identification level 2a thanks to the library spectrum match from its previous identification by Kinyua et al., 2016 in pooled urine samples from London. 2C-B was confirmed as Level 1 in one sample in the present study. 2C-phenethylamines became list I substances of the Opium Act in the Netherlands in the late 90s despite no health incidents occurring. FA was confirmed at Level 1, in agreement with being widely reported in the Netherlands starting sometime between 2007 and 2009 until nowadays, and also elsewhere in Europe (Al-Saffar et al., 2013; Röhrich et al., 2012). In the form of 4-FA, it is found as an adulterant in ecstasy and speed, but also as a drug of choice due to its subjective effects ranged between those of amphetamine and MDMA (Linsen et al., 2015). Its presence in urine and blood samples has been reported occasionally (Al-Saffar et al., 2013; Röhrich et al., 2012).

Piperazines are mainly used as tranquilizers or antidepressants, although some also have a recreational use as stimulants. mCPP was detected once, and confirmed at Level 1 (**Fig. 2.3.3**). Recently reported by the Drugs Information and Monitoring System in the Netherlands, mCPP was found to be one of the contents in a new liquid designer drug that appeared on the Dutch drug market called ‘Explosion’ (Bossong et al., 2005). From the opioid family, fentanyl was detected in one sample, and we confirmed its presence using a reference standard. Fentanyl is a potent, synthetic opioid analgesic known to have a rapid onset and short duration of action (Clotz and

Nahata, 1991). Fentanyl is estimated to have hundreds of times the potency of pure, pharmacy-grade heroin and about 80 times the potency of morphine (Poklis, 1995). In the Netherlands, it is a prescribed pharmaceutical, therefore its presence could be related to such approved use. Synthetic cannabinoids are the largest group of substances monitored by the EMCDDA in Europe. In the Netherlands, the ready availability of cannabis for adults through the coffee shop system conflicts with the interest in synthetic cannabinoids. Nevertheless, one of the substances from this class was detected. L-759,633 was detected (Level 3) in four samples (**Fig. C1**).

**Table 2.3.1**. NPS identified and confirmed (Level 1-3) in wastewater samples collected during Amsterdam street festivals in 2012 and 2014.

| Compound | $t_R$[a] (min) | Ion formula $[M+H]^+$ | Measured *m/z* $[M+H]^+$ | Average Δm (ppm)[b] | Qualified product ions[c] | Level[d] | Hits[e] 2012 | Hits[e] 2014 |
|---|---|---|---|---|---|---|---|---|
| 3-Methoxy-4-methylamphetamine (MMA) | 3.6 | $[C_{11}H_{18}NO]^+$ | 180.1380 | -1.7 | 77.0406, 91.0567, 121.0595 | 2a | 4 | 1 |
| Methylhexanamine | 5.5 | $[C_7H_{18}N]^+$ | 116.1433 | -0.9 | 57.0706 | 1 | 2 | |
| 4-fluoroamphetamine (4-FA) | 6.2 | $[C_9H_{13}FN]^+$ | 154.1030 | 1.9 | 83.0297, 109.0449, 137.0761 | 1 | 1 | |
| 3,4-Methylenedioxy-N-ethylamphetamine (MDEA) | 8.8 | $[C_{12}H_{18}NO_2]^+$ | 208.1341 | 4.3 | 105.0697, 135.0439, 163.0755 | 1 | 1 | |
| *meta*-Chlorophenylpiperazine (mCPP) | 10.4 | $[C_{10}H_{14}ClN_2]^+$ | 197.0840 | -2.5 | 77.0385, 91.0547,118.0649, 154.0424 | 1 | 1 | |
| 2,5-dimethoxy-4-bromophenethylamine (2C-B) | 11.1 | $[C_{10}H_{15}BrNO_2]^+$ | 260.0274 | -2.7 | 91.0573 | 1 | 1 | |
| Fentanyl | 15.5 | $[C_{22}H_{29}N_2O]^+$ | 337.2274 | 0.0 | 79.0531, 105.0686, 188.1418 | 1 | 1 | |
| L-759,633 | 19.2 | $[C_{26}H_{41}O_2]^+$ | 385.3101 | 0.0 | 253.1219, 367.2564 | 3 | | 4 |

[a] Retention time in minutes, measured on the QTOF; [b] *m/z* accurate mass measurement error as the deviation from the theoretical protonated ion; [c] DDA; [d] Identification level according to (Schymanski et al., 2014); [e] Hits = number of times detected in 4 samples

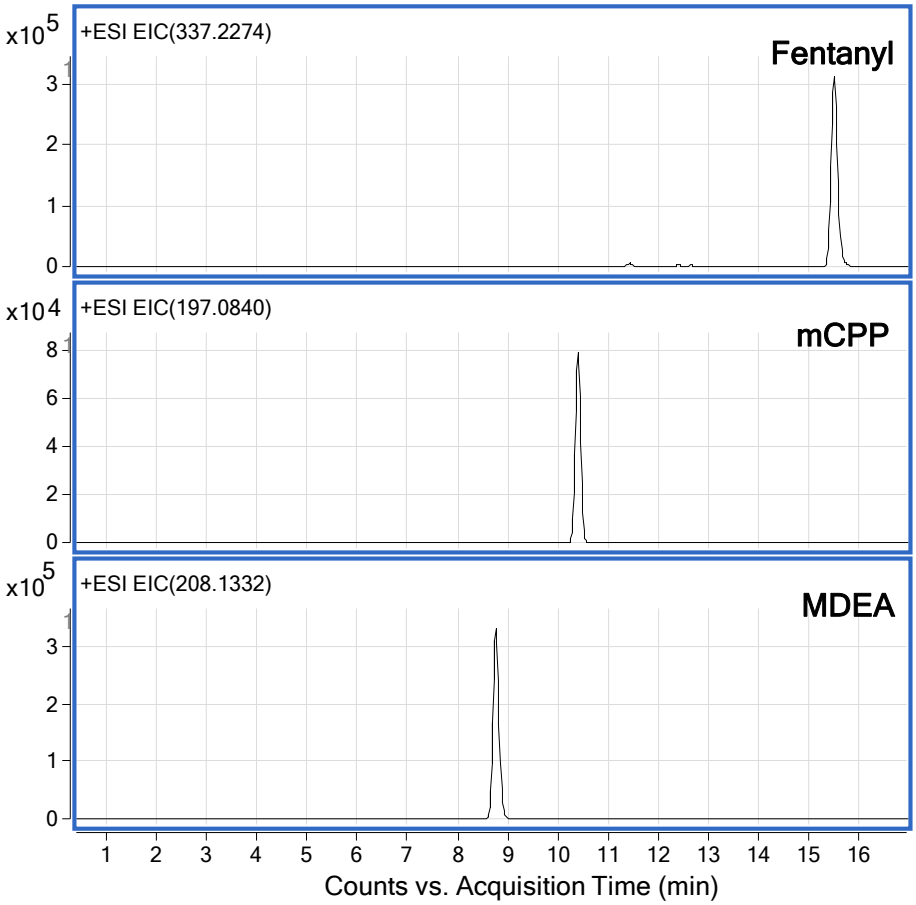


**Fig. 2.3.3.** NPS detected in a wastewater sample collected in 2012 during a festival in Amsterdam (Data from LC-QTOFMS).

Our findings partially match the information available from different information points in the Netherlands: the Drugs Information and Monitoring System from Trimbos Institute and the Dutch Poisons Information Centre from the University Medical Centre in Utrecht. According to their last Drug annual report (Trimbos Instituut, 2015), the most popular NPS are the phenethylamines: 4-FA, 2C-B and 5/6-APB; and ketamine and methoxetamine. The general conclusion provided by the aforementioned information shows that NPS use is not yet widespread in the Netherlands, although it is recently increasing. In the authors' opinion, the snapshot of recreational substances in use during the specific social setting sampled in this work highlights the wastewater chemical analysis as a complementary source of

information in the quest for knowledge on the use of NPS. Furthermore, it supports the information from the report reflecting low use of NPS in the country, and the rising concern after the positive identification of the phenethylamines 4-FA and 2C-B.

In addition, the application of the workflow led to the positive detection and identification of 12 pharmaceuticals and other licit substances associated with the potential of abuse, and 14 classical drugs and metabolites (**Table 2.3.2** and **2.3.3**). These findings might not be directly linked to festival attendees themselves, but rather to the city population in general, as they have been previously reported in WBE studies including the city of Amsterdam (Bijlsma et al., 2012; Ort et al., 2014).

The most frequently detected compounds were the antidepressant venlafaxine and the anxiolytics from the benzodiazepine family oxazepam, temazepam, diazepam and nordiazepam. The use of Ritalin was observed by the presence of its main active ingredient methylphenidate and its metabolite ritalinic acid. This pharmaceutical is used to treat attention deficit disorder (ADD), attention deficit hyperactivity disorder (ADHD), and narcolepsy. But its use is prevalent in some regions as a central nervous system stimulant (Clemow and Walker, 2014). In the Netherlands, methylphenidate prescriptions have quadrupled in recent years (Health Council of the Netherlands, 2014), and this is the first time that it has been reported in wastewater. The antipsychotic clozapine, used for the treatment of schizophrenia, was also detected. Finally, biomarkers of beer (hordenine), coffee (caffeine and paraxanthine) and tobacco (cotinine) were positively identified. The presence of hordenine, which is a controlled substance in the UK (Archer et al., 2013), has not been reported before in the Netherlands.

In the case of the classical illicit drugs and metabolites the most frequently detected one was THC-COOH, the main cannabis biomarker. This is expected in the Netherlands, since cannabis use is tolerated in this country. Cocaine use was detected by the presence of the parent compound as well as two human metabolites, benzoylecgonine and ecgonine methyl ester. Additionally, the transformation product cocaethylene, which is formed when cocaine and alcohol are consumed together, was also detected. In the case of amphetamine type stimulants, amphetamine and MDMA were widely detected, while the metabolites MDA and 4-hydroxy-3-methoxymethamphetamine (HMMA) were also present. Other recreational substance positively identified was ketamine, together with 6-MAM and EDDP, the metabolites of the opioids heroin and methadone, respectively. Finally, LSD was initially tentatively identified (Level 3). This identification was made definite (Level 1) after the injection of a reference standard. The chromatograms corresponding to the

accurate mass of the LSD protonated molecule in both the analytical standard and the sample can be consulted in **Fig. C2**.

**Table 2.3.2**. List of pharmaceuticals with potential of abuse and other licit substances identified and confirmed (Level 1) in wastewater samples collected during Amsterdam street festivals in 2012 and 2014.

| Compound | $t_R$[a] (min) | Ion formula $[M+H]^+$ | Measured *m/z* $[M+H]^+$ | Average Δm (ppm)[b] | Qualified product ions[c] | Level[d] | Hits[e] 2012 | Hits[e] 2014 |
|---|---|---|---|---|---|---|---|---|
| Cotinine | 3.1 | $[C_{10}H_{13}N_2O]^+$ | 177.1022 | -0.22 | 70.0658 | 1 | 4 | 4 |
| Hordenine | 3.1 | $[C_{10}H_{16}NO]^+$ | 166.1226 | 1.56 | 77.0387, 121.0642 | 1 | 4 | 3 |
| Paraxanthine | 6.3 | $[C_7H_9N_4O_2]^+$ | 181.0720 | 1.31 | 130.1598 | 1 | 3 | 2 |
| Caffeine | 8.0 | $[C_8H_{11}N_4O_2]^+$ | 195.0877 | 0.42 | 138.0666 | 1 | 2 | 1 |
| Ritalinic acid | 8.4 | $[C_{13}H_{18}NO_2]^+$ | 220.1332 | 1.26 | 82.0648 | 1 | 2 | 4 |
| Methylphenidate | 8.7 | $[C_{14}H_{20}NO_2]^+$ | 234.1489 | -0.24 | 84.1078 | 1 | 1 | |
| Venlafaxine | 12.2 | $[C_{17}H_{28}NO_2]^+$ | 278.2115 | 1.19 | 58.0656 | 1 | 4 | 4 |
| Clozapine | 12.9 | $[C_{18}H_{20}ClN_4]^+$ | 327.1371 | -1.43 | 221.1377 | 1 | 3 | |
| Nordiazepam | 17.3 | $[C_{15}H_{12}ClN_2O]^+$ | 271.0633 | 0.12 | 243.0174 | 1 | 1 | |
| Oxazepam | 17.3 | $[C_{15}H_{12}ClN_2O_2]^+$ | 287.0582 | -2.72 | 269.0834 | 1 | 3 | 4 |
| Temazepam | 19.4 | $[C_{16}H_{14}ClN_2O_2]^+$ | 301.0738 | -0.94 | 255.0675 | 1 | 4 | |
| Diazepam | 20.1 | $[C_{16}H_{14}ClN_2O]^+$ | 285.0789 | 0.29 | 257.1232 | 1 | 1 | |

[a] Retention time in minutes, measured on the QTOF; [b] *m/z* accurate mass measurement error as the deviation from the theoretical protonated ion; [c] DIA; [d] Identification level according to (Schymanski et al., 2014); [e] Hits = number of times detected in 4 samples

**Table 2.3.3**. List of classical illicit drugs and/or metabolites thereof identified and confirmed (Level 1) in wastewater samples collected during Amsterdam street festivals in 2012 and 2014.

| Compound | $t_R$[a] (min) | Ion formula $[M+H]^+$ | Measured *m/z* $[M+H]^+$ | Δm (average ppm)[b] | Qualified product ions[c] | Level[d] | Hits[e] 2012 | Hits[e] 2014 |
|---|---|---|---|---|---|---|---|---|
| Ecgonine Methyl Ester | 1.0 | $[C_{10}H_{18}NO_3]^+$ | 200.1281 | 0.90 | 82.0658 | 1 | 4 | 1 |
| HMMA | 4.6 | $[C_{11}H_{18}NO_2]^+$ | 196.1332 | -1.77 | 165.1389 | 1 | 1 | 4 |
| Amphetamine | 5.3 | $[C_9H_{14}N]^+$ | 136.1121 | 0.91 | 91.0542 | 1 | 4 | 3 |
| MDA | 6.8 | $[C_{10}H_{14}NO_2]^+$ | 180.1019 | 4.97 | 163.2555 | 1 | 1 | |
| MDMA | 7.8 | $[C_{11}H_{16}NO_2]^+$ | 194.1176 | 3.91 | 163.0781 | 1 | 4 | 3 |
| 6-MAM | 8.1 | $[C_{19}H_{22}NO_4]^+$ | 328.1543 | 3.25 | 211.0827, 268.1868 | 1 | 1 | |
| Benzoylecgonine | 9.4 | $[C_{16}H_{20}NO_4]^+$ | 290.1387 | 2.47 | 105.0338, 168.1023 | 1 | 3 | 3 |
| Ketamine | 9.6 | $[C_{13}H_{17}ClNO]^+$ | 238.0993 | 1.04 | 220.1161, 207.0640 | 1 | 3 | |
| Cocaine | 12.2 | $[C_{17}H_{22}NO_4]^+$ | 304.1543 | -0.44 | 182.0812 | 1 | 3 | |
| LSD | 13.3 | $[C_{20}H_{26}N_3O]^+$ | 324.2070 | -2.28 | 223.1910, 197.1519 | 1 | 1 | |
| Cocaethylene | 13.7 | $[C_{18}H_{24}NO_4]^+$ | 318.1700 | 0.15 | 196.1324, 278.1743 | 1 | | 3 |
| EDDP | 17.9 | $[C_{20}H_{24}N]^+$ | 278.1903 | -1.95 | 234.1253 | 1 | 4 | 2 |
| Methadone | 19.1 | $[C_{21}H_{28}NO]^+$ | 310.2165 | -2.17 | 265.1570, 57.0348 | 1 | 4 | |
| 11-nor-9-carboxy-THC | 22.6 | $[C_{21}H_{29}O_4]^+$ | 345.2060 | 0.06 | 327.1843, 299.3178 | 1 | 4 | 4 |

[a] Retention time in minutes, measured on the QTOF; [b] *m/z* accurate mass measurement error as the deviation from the theoretical protonated ion; [c] DIA; [d] Identification level according to (Schymanski et al., 2014); [e] Hits = number of times detected in 4 samples

**Filtering of false positives**

Wastewater is a very complex matrix containing numerous compounds like pharmaceuticals and personal care products (PPCPs), pesticides, and emerging contaminants from many sources-residential, agricultural and industrial (Kosma et al., 2014; Ort et al., 2010). These compounds are likely interferences when analysing influent wastewater, the reason why a Level 3 identification (the exact structure is unconfirmed) remains speculative and could likely be a false positive from isobaric compounds. Considering we have a matching accurate mass (<5 ppm), isotopic pattern information and qualified product ions for the tentative candidates, searching its corresponding structural formula in databases like Chemspider can still generate > 500 possibilities. Consequently, we tried to improve the identification from Level 3 to Level 2 (before purchasing standards), and considered it worthwhile to explore their likely matches in various databases.

Since LTQ Orbitrap has a superior selectivity compared to QTOF, by using the Orbitrap we could likely achieve resolution >50,000 for most compounds with which the tentative candidates could be distinguished from interferences of the same nominal *m/z*. Furthermore, we could apply MetFrag 2.2, an *in silico* fragmenter, which allows one to combine compound searches from databases with fragmentation prediction in order to compare to measured data. The databases linked could be large with millions (ChemSpider and PubChem) or smaller with several thousands (KEGG, HMDB, STOFF-IDENT) of structures and ensured an exhaustive search. Additionally, MS Fragment (ACD Labs) also provides in silico fragmentation generating possible product ions and their fragmentation pathway. On completion of this search one can either generate a 'blacklist' backed by literature (Level 2a) or diagnostic evidence from MetFrag (Level 2b); and a justified reason to purchase reference standards to confirm tR and spectra. The blacklist remains an excellentway to quickly identify false positives in future analysis.

The piperazines para-methoxyphenylpiperazine (MeOPP) and 3-trifluoromethyl-phenylpiperazine (TFMPP) were initially tentatively identified (Level 3) but finally rejected after MetFrag analysis and standard injection because of the mismatch of the retention time. This shows the challenge associated with reporting Level 3 hits in wastewater samples.

**Future of suspect screening in monitoring NPS in WBE**

In the case of NPS, the number of substances with recreational use reported for the first time to the EWS keeps steadily increasing. It seems important to adapt a common "one-step-ahead" strategy where epidemiologists, analytical chemists, policy makers, law enforcement and forensic practitioners work together. Analytical toxicologists have brought forward the suspect screening of NPS in wastewater samples collected in specific social settings as a complimentary source of information. The findings of this work have shown how certain tools available in HRMS can be applied to the data processing workflow in order to reduce false positives, which seems to be the main disadvantage thus far. The future in this application of WBE seems to be directed towards the reporting of frequency of use as opposed to quantifying. This is so because of the little pharmacokinetic information, which renders the choice of target biomarkers difficult, and the uncertainties related to estimating the number of people contributing to the sample during such festivals or events. Besides, the use of information from external sources to build stronger databases for the "suspect" screening is essential for its successful application. This relates to findings from forensic laboratories on intoxication cases and biotransformation studies. Altogether, the efforts to curb NPS use would streamline since the information will be available at a faster pace.

# 2.3.4. Conclusions

This work has shown that sampling during specific social settings, in combination with the analysis using high resolution mass spectrometry and a specific “suspect” screening processing workflow based in a database search can be very useful to narrow the quest for NPS in wastewater analysis. As a result, the snapshot of recreational substances in use during city festivals has been presented. NPS from several groups were detected within the synthetic cathinone, phenethylamine, and synthetic cannabinoid families. The screening method helped to identify the presence of mCPP, 2CB and 4-FA inwastewater for the first time. In addition, the use of in silico fragmentation tools has proven to be useful in the rejection of false positives when analytical standards are not available.

# Acknowledgements

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# Annex C – Supplementary Information Chapter 2.3.

**Table C1**. Wastewater sample collection details in 2012 and 2014.

| Sampling date | Flow $m^3$ 24-$h^{-1}$ | Sampling interval min | $BOD_5$ mg $L^{-1}$ | COD mg $L^{-1}$ | N Kjeldahl mg $L^{-1}$ | P total mg $L^{-1}$ |
|---|---|---|---|---|---|---|
| Thursday - 2012 | 302680 | 1.7 | n.a. | n.a. | n.a. | n.a. |
| Friday - 2012 | 154200 | 3.3 | n.a. | n.a. | n.a. | n.a. |
| Saturday - 2012 | 158270 | 3.2 | n.a. | n.a. | n.a. | n.a. |
| Sunday - 2012 | 148710 | 3.4 | n.a. | n.a. | n.a. | n.a. |
| Thursday - 2014 | 145768 | 3.5 | 210 | 480 | 52 | 7.0 |
| Friday - 2014 | 141565 | 3.6 | n.a. | n.a. | n.a. | n.a. |
| Saturday - 2014 | 184245 | 2.7 | n.a. | n.a. | n.a. | n.a. |
| Sunday - 2014 | 138248 | 3.6 | 230 | 500 | 53 | 6.8 |

n.a. not available

**Table C2**. Inclusion list samples 2012

| Name | Formula | $[M+H]^+$ | $t_R$ |
|---|---|---|---|
| Ephedrone/Methcathinone/Mc | $C_{10}H_{13}NO$ | 164.1075 | 2.89 |
| Ephedrine | $C_{10}H_{15}NO$ | 166.1226 | 3.15 |
| 4-MAR/Cotinine | $C_{10}H_{12}N_2O$ | 177.1022 | 3.15 |
| Ephedrine | $C_{10}H_{15}NO$ | 166.1226 | 3.18 |
| (-)-Norpseudoephedrine/Amphetamine, 4-hydroxy-/cathine/PPA/nor-ephedrine | $C_9H_{13}NO$ | 152.1068 | 3.62 |
| MMA | $C_{11}H_{17}NO$ | 180.1383 | 3.64 |
| 4-MAR/Cotinine | $C_{10}H_{12}N_2O$ | 177.1022 | 4.01 |
| MeOPP | $C_{11}H_{16}NO_2$ | 195.1254 | 4.13 |
| (-)-Norpseudoephedrine/Amphetamine, 4-hydroxy-/cathine/PPA/nor-ephedrine | $C_9H_{13}NO$ | 152.1068 | 4.89 |

| | | | |
|---|---|---|---|
| M_440_MPDV | $C_{21}H_{29}NO_9$ | 440.1908 | 4.93 |
| Ephedrine | $C_{10}H_{15}NO$ | 166.1226 | 5.20 |
| Hydroxy-Nor-Fentanyl | $C_{14}H_{20}N_2O_2$ | 249.1598 | 5.23 |
| 3,4-Ethylenedioxy-N-methylamphetamine (3,4-EDMA) | $C_{12}H_{18}NO_2$ | 209.1410 | 5.89 |
| 3,5-methoxy-4-Propoxyamphetamine (3-CP) | $C_{14}H_{23}NO_3$ | 254.1751 | 6.54 |
| 3,4-MDPA | $C_{13}H_{19}NO_2$ | 222.1489 | 6.81 |
| Ephedrine | $C_{10}H_{15}NO$ | 166.1226 | 6.83 |
| MDA-19 | $C_{21}H_{23}N_3O_2$ | 350.1863 | 6.87 |
| 1-(1,4-cyclohexadienyl)-2-methyl Aminopropane (CMP) | $C_{10}H_{17}N$ | 152.1434 | 6.91 |
| Ephedrine | $C_{10}H_{15}NO$ | 166.1226 | 7.01 |
| 4-acetoxy-DMT/O-Acetylpsilocin | $C_{14}H_{18}N_2O_2$ | 247.1435 | 7.20 |
| Propylhexedrine | $C_{10}H_{21}N$ | 156.1747 | 7.84 |
| 2,4,5-trimethoxyamphetamine | $C_{12}H_{19}NO_3$ | 226.1438 | 8.09 |
| M_250_alphaPVP/O-DT | $C_{15}H_{23}NO_2$ | 250.1802 | 8.19 |
| M_220_ethylphenidate (Ritalinic acid) | $C_{13}H_{17}NO_2$ | 220.1332 | 8.44 |
| M_262_2_MPDV | $C_{15}H_{19}NO_3$ | 262.1438 | 8.63 |
| 2-MeO-KET | $C_{14}H_{19}NO_2$ | 234.1488 | 8.71 |
| 3,4-MDMA methylene homolog | $C_{12}H_{17}NO_2$ | 208.1332 | 8.77 |
| 4-OH-DET/4-OH-MiPT/Norfentanyl | $C_{14}H_{20}N_2O$ | 233.1648 | 9.18 |
| 5-MeO-MiPT | $C_{15}H_{22}N_2O$ | 247.1805 | 9.22 |
| M_250_alphaPVP | $C_{15}H_{23}NO_2$ | 250.1802 | 10.17 |
| mCPP | $C_{10}H_{13}ClN_2$ | 197.0840 | 10.40 |
| 4-Bromo-2,5-Dimethoxy-B-Phenethylamine | $C_{10}H_{14}BrNO_2$ | 260.0281 | 11.06 |
| AB-CHMINACA metabolite M2 | $C_{20}H_{27}N_3O_3$ | 358.2125 | 11.66 |
| 4-acetoxy-DMT/O-Acetylpsilocin | $C_{14}H_{18}N_2O_2$ | 247.1435 | 11.77 |
| ADB-PINACA pentanoic acid metabolite | $C_{19}H_{26}N_4O_4$ | 375.2027 | 11.89 |
| 3,4-Ethylenedioxymethcathinone (3,4-EDMC) | $C_{12}H_{15}NO_3$ | 222.1125 | 12.07 |
| TFMPP (3-Trifluoromethylphenylpiperazine) | $C_{11}H_{13}F_3N_2$ | 231.1104 | 13.17 |
| LSD | $C_{20}H_{25}N_3O$ | 324.2070 | 13.26 |
| 3,5-methoxy-4-Propoxyamphetamine (3-CP) | $C_{14}H_{23}NO_3$ | 254.1751 | 13.40 |
| 4-acetoxy-DMT/O-Acetylpsilocin | $C_{14}H_{18}N_2O_2$ | 247.1435 | 13.63 |
| PCP | $C_{17}H_{25}N$ | 244.2060 | 14.65 |
| 4-fluoro-a-PVP | $C_{15}H_{20}FNO$ | 250.1602 | 14.69 |
| Fentanyl | $C_{22}H_{28}N_2O$ | 337.2274 | 15.55 |
| 4-methoxy-a-Pyrrolidinobutiophenone(4-MeOPBP)/Methylphenidate (Ritalin)/MPPP | $C_{15}H_{21}NO_2$ | 248.1645 | 16.02 |

| | | | |
|---|---|---|---|
| 4-methoxy-a-Pyrrolidinobutiophenone(4-MeOPBP)/MPPP | $C_{15}H_{21}NO_2$ | 248.1645 | 16.05 |
| Nordiazepam | $C_{15}H_{11}ClN_2O$ | 271.0633 | 17.32 |
| EMDP | $C_{19}H_{21}N$ | 264.1747 | 17.69 |
| 4-fluoro PV8 | $C_{17}H_{24}FNO$ | 278.1915 | 17.92 |
| THG | $C_{21}H_{28}O_2$ | 313.2162 | 18.35 |
| Cannabidiolic Acid (CBDA) (Resorcylic Acid) | $C_{22}H_{30}O_4$ | 359.2217 | 18.65 |
| THG | $C_{21}H_{28}O_2$ | 313.2162 | 18.99 |
| Tetrahydrocannabivarin (9-Tetrahydrocannabivarin) (THV) (THCV) | $C_{19}H_{26}O_2$ | 287.2006 | 19.27 |
| Temazepam | $C_{16}H_{13}ClN_2O_2$ | 301.0738 | 19.36 |
| AM2201 benzimidazole analog (BIM-2201) (BZ-2201) (FUBIMINA) (FTHJ) | $C_{23}H_{21}FN_2O$ | 361.1711 | 19.89 |
| A-796260 (LTI-258) | $C_{22}H_{30}N_2O_2$ | 355.2380 | 19.97 |
| RCS-4 | $C_{21}H_{23}NO_2$ | 322.1802 | 20.44 |
| 5-fluoro AB-PINACA N-(4-hydroxypentyl) metabolite | $C_{18}H_{25}FN_4O_3$ | 365.1983 | 20.77 |
| A-796260 (LTI-258) | $C_{22}H_{30}N_2O_2$ | 355.2380 | 20.85 |
| A-796260 (LTI-258) | $C_{22}H_{30}N_2O_2$ | 355.2380 | 20.95 |
| 8-THC | $C_{21}H_{30}O_2$ | 315.2319 | 21.16 |
| Tetrahydrocannabivarin (THV) (THCV) | $C_{19}H_{26}O_2$ | 287.2006 | 21.28 |
| VDM11 | $C_{27}H_{39}NO_2$ | 410.3054 | 22.09 |
| CP 47,497-C8-homolog C-8-hydroxy metabolite | $C_{22}H_{36}O_3$ | 349.2737 | 23.67 |
| AM-1248 | $C_{26}H_{34}N_2O$ | 391.2744 | 26.31 |

**Table C3**. Inclusion list samples 2014

| Name | Formula | $[M+H]^+$ | $t_R$ |
|---|---|---|---|
| Psilocybin | $C_{12}H_{17}N_2O_4P$ | 285.0998 | 1.40 |
| Ephedrone/Methcathinone/Mc | $C_{10}H_{13}NO$ | 164.1070 | 2.75 |
| Ephedrine | $C_{10}H_{15}NO$ | 166.1226 | 3.00 |
| 4-MAR/Cotinine | $C_{10}H_{12}N_2O$ | 177.1022 | 3.10 |
| MMA | $C_{11}H_{17}NO$ | 180.1383 | 3.49 |
| (-)-Norpseudoephedrine/Amphetamine, 4-hydroxy-/cathine/PPA/nor-ephedrine | $C_9H_{13}NO$ | 152.1068 | 3.77 |
| 4,4'-Dimethylaminorex (4,4'-DMAR) | $C_{11}H_{14}N_2O$ | 191.1184 | 4.20 |
| 2,4,5-trimethoxyamphetamine | $C_{12}H_{19}NO_3$ | 226.1438 | 4.30 |
| HMMA/Methamphetamine, 4-hydroxy-3-methoxy | $C_{11}H_{17}NO_2$ | 196.1332 | 4.57 |
| M_440_MPDV | $C_{21}H_{29}NO_9$ | 440.1908 | 4.85 |
| Ephedrine | $C_{10}H_{15}NO$ | 166.1226 | 5.10 |

| | | | |
|---|---|---|---|
| 3,4-Ethylenedioxy-N-methylamphetamine (3,4-EDMA) | $C_{12}H_{18}NO_2$ | 209.1410 | 5.89 |
| 2,3-Penylone Isomer/3,4-Methylenedioxy-5-Methyl-N-Ethyl Cathinone/Bk-Dmbdb/Dibutylone/Bk-Ebdb/Eutylone/Bk-Mbdp/Pentylone | $C_{13}H_{17}NO_3$ | 236.1281 | 6.28 |
| 3,5-methoxy-4-Propoxyamphetamine (3-CP) | $C_{14}H_{23}NO_3$ | 254.1751 | 6.46 |
| Ephedrine | $C_{10}H_{15}NO$ | 166.1226 | 6.75 |
| MDA-19 | $C_{21}H_{23}N_3O_2$ | 350.1863 | 6.81 |
| 1-(1,4-cyclohexadienyl)-2-methyl Aminopropane (CMP) | $C_{10}H_{17}N$ | 152.1434 | 6.90 |
| Ephedrine | $C_{10}H_{15}NO$ | 166.1226 | 6.93 |
| 4-acetoxy-DMT/O-Acetylpsilocin | $C_{14}H_{18}N_2O_2$ | 247.1435 | 7.06 |
| MDA-19 | $C_{21}H_{23}N_3O_2$ | 350.1863 | 7.48 |
| 2-APB | $C_{11}H_{13}NO$ | 176.1070 | 7.85 |
| M_250_alphaPVP/O-DT | $C_{15}H_{23}NO_2$ | 250.1802 | 8.14 |
| Benzylbutylbarbiturate | $C_{15}H_{18}N_2O_3$ | 275.1390 | 8.18 |
| M_220_Methylphenidate (Ritalinic acid) | $C_{13}H_{17}NO_2$ | 220.1332 | 8.36 |
| M_262_2_MPDV | $C_{15}H_{19}NO_3$ | 262.1438 | 8.55 |
| 2,3-MDMC/MDA 2 amido analog (MMDPPA) | $C_{11}H_{13}NO_3$ | 208.0968 | 8.75 |
| 5-fluoro AMB (5-fluoro AMP) | $C_{19}H_{26}FN_3O_3$ | 364.2031 | 8.79 |
| 5-MeO-MiPT | $C_{15}H_{22}N_2O$ | 247.1805 | 9.10 |
| para-Fluorobutyryl fentanyl | $C_{23}H_{29}FN_2O$ | 369.2337 | 9.45 |
| 2-MMC/5-Me-MDA/BDB | $C_{11}H_{15}NO_2$ | 194.1176 | 9.87 |
| M_250_alphaPVP | $C_{15}H_{23}NO_2$ | 250.1802 | 10.12 |
| 2-MMC/5-Me-MDA/BDB | $C_{11}H_{15}NO_2$ | 194.1176 | 10.12 |
| 4-acetoxy-DMT/O-Acetylpsilocin | $C_{14}H_{18}N_2O_2$ | 247.1435 | 11.73 |
| 3,4-Ethylenedioxymethcathinone (3,4-EDMC) | $C_{12}H_{15}NO_3$ | 222.1125 | 11.99 |
| 4-acetoxy-DMT/O-Acetylpsilocin | $C_{14}H_{18}N_2O_2$ | 247.1435 | 13.55 |
| AB-CHMINACA metabolite M6 | $C_{20}H_{26}N_4O_4$ | 387.2027 | 14.12 |
| 4-MeO-PV8 | $C_{18}H_{27}NO_2$ | 290.2115 | 15.19 |
| 4-methoxy-a-Pyrrolidinobutiophenone(4-MeOPBP)/MPPP | $C_{15}H_{21}NO_2$ | 248.1645 | 15.94 |
| PF-3845 | $C_{24}H_{23}F_3N_4O_2$ | 457.1846 | 18.11 |
| Cannabidiolic Acid (CBDA) (α-Resorcylic Acid) | $C_{22}H_{30}O_4$ | 359.2217 | 18.56 |
| L-759,633 | $C_{26}H_{40}O_2$ | 385.3101 | 19.15 |
| A-796260 (LTI-258) | $C_{22}H_{30}N_2O_2$ | 355.2380 | 19.97 |
| 5-fluoro AB-PINACA N-(4-hydroxypentyl) metabolite | $C_{18}H_{25}FN_4O_3$ | 365.1983 | 20.77 |
| A-796260 (LTI-258) | $C_{22}H_{30}N_2O_2$ | 355.2380 | 20.85 |
| 8-THC | $C_{21}H_{30}O_2$ | 315.2319 | 21.12 |
| M-144 (XLR11 2-methylindole analog) | $C_{22}H_{30}FNO$ | 344.2384 | 21.93 |
| Pristimerin | $C_{30}H_{40}O_4$ | 465.2999 | 21.95 |

| | | | |
|---|---|---|---|
| VDM11 | $C_{27}H_{39}NO_2$ | 410.3054 | 22.06 |
| CP47,497 (CP 47, 947) | $C_{21}H_{34}O_2$ | 319.2632 | 23.09 |
| 3-epiCP 47,497-C8-homolog | $C_{22}H_{36}O_2$ | 333.2788 | 23.15 |
| MADOL | $C_{20}H_{32}O$ | 289.2526 | 23.26 |
| 5-fluoro AEB | $C_{20}H_{28}FN_3O_3$ | 378.2187 | 23.34 |
| AM-1248 | $C_{26}H_{34}N_2O$ | 391.2744 | 25.69 |
| AM-1248 | $C_{26}H_{34}N_2O$ | 391.2744 | 28.56 |

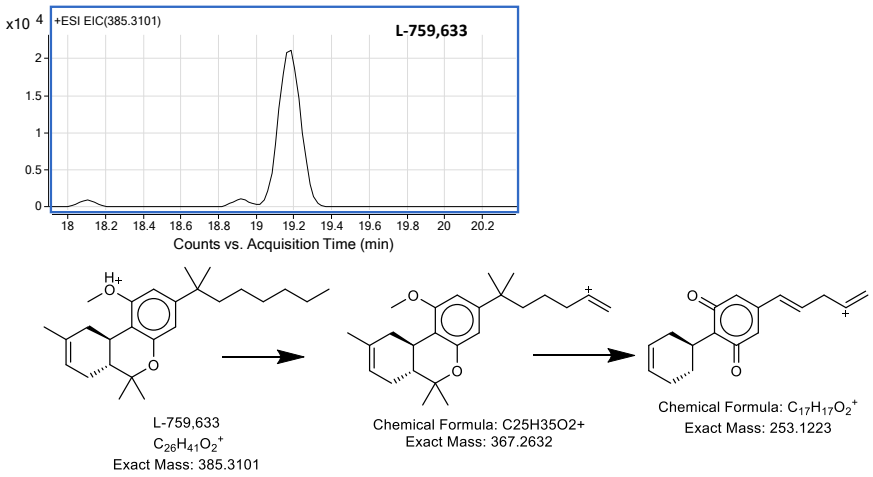


**Fig. C1.** Identification of synthetic cannabinoid L-759,633 in wastewater

# Chapter 3

# Erectile dysfunction drugs

# 3.1.

# Determination of phosphodiesterase type V inhibitors in wastewater by direct injection followed by liquid chromatography coupled to tandem mass spectrometry

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# Abstract

A simple, fast and reliable analytical method for the determination of phosphodiesterase type V inhibitors in wastewater was developed and validated. The method was based on direct injection followed by liquid chromatography coupled to tandem mass spectrometry with triple quadrupole as mass analyzer. Transformation products and analogues were included in the target list besides the three active pharmaceutical ingredients (sildenafil, vardenafil and tadalafil). The method performance was thoroughly investigated, including the analyte stability in wastewater and matrix effect. All target compounds presented linear fits between their LOD and 500 ng/L. The quantification limits ranged from 1.6 to 30 ng/L for all compounds except for n-octylnortadalafil (LOQ: 100 ng/L); precision calculated as intraday repeatability was lower than 30%; accuracy calculated as procedural recovery ranged successfully between 85 and 105% in all cases. The method was applied to samples collected during three week-long monitoring campaigns performed in 2013, 2014 and 2015 in three Dutch cities. Only sildenafil and its two metabolites, desmethyl- and desethylsildenafil, were present with normalized loads ranging from LOQ to 8.3, 11.8 and 21.6 mg/day/1000 inh, respectively. Two additional week-long sets of samples were collected in Amsterdam at the time that a festival event took place, bringing around 350,000 visitors to the city. The difference in drug usage patterns was statistically studied: “weekday” versus “weekend”, “normal” versus “atypical” week; and results discussed. The metabolite to parent drug concentration ratio evolution during consecutive years was discussed, leading to several possible explanations that should be further investigated. Finally, wastewater-based epidemiology approach was applied to back-calculate sildenafil consumption.

# 3.1.1. Introduction

Phosphodiesterase type V (PDE5) inhibitors are the active pharmaceutical ingredients (APIs) in drugs used for the treatment of erectile dysfunction (ED). Viagra® (sildenafil) was authorized for marketing throughout the European Union for the first time in 1998 (European Medicines Agency, 2008a). Cialis® (tadalafil) and Levitra® (vardenafil) were introduced a few years later (European Medicines Agency, 2009, 2008b). Since the introduction on the market of these prescription-only pharmaceuticals they have fundamentally changed the treatment of ED. Their easy uptake as an oral drug has replaced other types of invasive treatments, namely injections and implants; and they have proved to be safe, reliable and very effective (Gresser and Gleiter, 2002). However, despite medical advances, erectile dysfunction is still highly stigmatized in many societies and consequently users tend to hide their related drug use. Therefore, men may increasingly choose to purchase counterfeit medicines and adulterated products from unreliable sources on the internet outside of the official health system (Fittler et al., 2013). Thereby they avoid a visit to the doctor and save money, as the official pharmaceuticals are more expensive (Schnetzler et al., 2010). But they are not aware of the involved health risk to which they expose themselves due to the unreliable origin and quality of these products (B J Venhuis et al., 2014c).

Analytical studies with PDE5 inhibitors as target compounds have been mainly addressed to the identification of new synthetic analogues since homosildenafil was first reported (Shin et al., 2003). The commonly applied techniques for structure elucidation and confirmation are liquid chromatography coupled to mass spectrometry (MS, HRMS, and MS/MS), UV detection, IR spectroscopy and NMR. Several recent examples can be found in the literature: structure elucidation of a thiono analogue of sildenafil in an alleged herbal aphrodisiac (Venhuis et al., 2008); isolation and structural elucidation of cyclopentynafil and n-octylnortadalafil in a dietary supplement (Hasegawa et al., 2009); identification of a nitrosated prodrug of aildenafil in a dietary supplement (Venhuis et al., 2011); identification of a new tadalafil analogue, acetaminotadalafil, in a dietary supplement (Lee et al., 2013a). Besides, screening methods based on liquid chromatography coupled to tandem mass spectrometry have been validated in order to detect and quantify the presence of PDE5 inhibitors and a large list of analogues in potentially adulterated products. To date, N50 unapproved analogues have been reported as adulterants (Patel et al., 2014) and the number is still growing. Analysed samples are solid-based (pills, hard and soft capsules, bulk powders, herbal products), typically treated with a simple

liquid–liquid extraction with an organic/aqueous mix solution and after centrifugation, the supernatant is diluted and/or filtered prior to injection to prevent contamination of the LC–MS system (Patel et al., 2014). When applied to potentially adulterated or counterfeit products, sildenafil and tadalafil as well as their analogues were the most frequently detected (Lebel et al., 2014; Lee et al., 2013b), although analogues were quantified at impurity levels which might indicate that their presence is due to by-product formation from the synthesis of the main pharmaceutical ingredient.

Regarding the analysis in environmental matrices: sildenafil has been included in multi-residue methods (Baker et al., 2014; Boleda et al., 2013), but very few studies have analysed the other 2 APIs tadalafil and vardenafil (Nieto et al., 2010), or have included metabolites and transformation products (Schröder and Thevis, 2010). Recently, also photodegradation studies for the identification of new transformation products have been conducted (Eichhorn et al., 2012; Herbert et al., 2015) with special attention to those observed to be notably persistent, which might indicate a potential impact for the aquatic environment.

The aim of this work was to determine the actual use of PDE5 inhibitors in The Netherlands. To do so we used wastewater-based epidemiology (WBE) (Daughton, 2001a; Ort et al., 2014), which is a powerful and objective approach for studying the health and lifestyle of the community living in the catchment area of the wastewater treatment plant where influent samples are collected. We present an analytical method based on liquid chromatography coupled to tandem mass spectrometry for the quantification of the three APIs present in ED pharmaceuticals, including transformation products and analogues (10 target compounds in total) in wastewater. Available information on pharmacokinetics plays an important role on the modelling, since the excretion ratio % with regard to the consumed/absorbed amount has to be applied. According to literature, after a single dose of Viagra® 92% of the drug is absorbed. From that absorbed dose, 27% is excreted as metabolites desmethyl and desethylsildenafil with no detectable parent in either feces or urine (Muirhead et al., 2002a). However, the presence of sildenafil in municipal wastewater has been reported in several cities in the past (Baker et al., 2014; Boleda et al., 2013; Nieto et al., 2010; Schröder and Thevis, 2010).

The method was applied to real influent samples collected during a series of week-long monitoring campaigns in the years 2013, 2014 and 2015 in the cities of Amsterdam, Eindhoven and Utrecht, and during two festival events in Amsterdam taking place in 2012 and 2014. Besides determining the environmental loads of the

target compounds, an estimation of the sildenafil consumption was done as it has been previously done for environmental loads of other prescription pharmaceuticals (ter Laak et al., 2010) in The Netherlands (B J Venhuis et al., 2014a).

# 3.1.2. Materials and methods

**Reagents and standards**

Sildenafil citrate, desmethylsildenafil, desethylsildenafil and noracetildenafil were obtained from LGC (Luckenwalde, Germany). Vardenafil dihydrochloride, n-desethyl vardenafil, tadalafil, aminotadalafil, chloropretadalafil and n-octyl nortadalafil were obtained from TRC Toronto Research Chemicals Inc. (Ontario, Canada). Two isotopically labeled internal standards (ILIS) were used as surrogates: sildenafil-$d_8$ and desmethylsildenafil-$d_8$, supplied by TLC Pharmachem (Ontario, Canada). All the above-mentioned standards were of high purity grade (>98%).

Individual stock solutions were prepared from powdered substance in methanol at a level ranging from 100 to 500 mg/L, and stored at −20 °C. A mix stock solution was prepared at a final level of 5 mg/L in methanol. Working solutions were prepared by dilution to the desired concentration with methanol, and stored at −20 °C. Calibration curve was prepared daily by diluting with ultrapure water the appropriate mix to a final composition water:methanol (90:10, v/v).

Methanol and acetonitrile, both HPLC grade solvents, were supplied by Mallinckrodt Baker B.V. (Deventer, The Netherlands). Formic acid (50% in water) was obtained from Fluka Analytical (Sigma-Aldrich, Stenheim, Germany). The ultrapure water was obtained by purifying demineralized water in a Milli-Q system from Millipore (Bedford, MA, USA).

For the sample preparation, regenerated cellulose filters RC 0.2 µm were purchased from Phenomenex (Torrance, USA).

**Sampling and sample preparation**

Raw influent wastewater was collected after the sand trap from the WWTPs serving Amsterdam, Eindhoven and Utrecht, in The Netherlands. The sewer systems were characterized according to the questionnaire developed by Dr. Ort already used in previous WBE studies (Castiglioni et al., 2013).

For seven consecutive days 24-h volume-dependent influent composite samples (Castiglioni et al., 2013) were taken at each WWTP in 2013 ($6^{th}$–$12^{th}$ March), 2014 ($11^{th}$–$18^{th}$ March) and 2015 ($4^{th}$–$10^{th}$ March). Each of these weeks was considered to

reflect a “normal” week regarding the population's drug use, as no big event or festivity took place during the weeklong monitoring. Two extra sets of samples collected at the WWTP in Amsterdam during festival events in 2012 and 2014 were also included in the study. These latter weeks were considered to reflect atypical behaviour of the people involved regarding drug use, as amass event took place during these two sampling weeks, lasting from Thursday to Sunday that attracted ~350,000 visitors to the city. The average sampling interval in all cases was lower than 15 min. WWTP characteristics were provided by the WWTP operators and included the following parameters: biological and chemical oxygen demand ($BOD_5$ and COD), total Kjeldahl nitrogen ($N_{tot}$) and total phosphorus and phosphate ($P_{tot}$). These parameters were used to check if any deviating conditions occurred during the sampling weeks (except for influent flows due to variations in rainfall. The volume-proportional sampling accounts for these variations; example given sample time interval changes co-variantly from 6 to 2.6 min, see **Table D1** in the Supplementary Information, Annex D). The information about WWTP characteristics can be found in Annex D (**Tables D1–D5**).

The autosamplers located at the three WWTPs kept samples refrigerated at 4 °C during composite collection. Afterwards samples were stored in HDPE containers and frozen immediately after collection at −20 °C until analysis. The maximum time samples were stored prior to analysis was 2 years for the case of 2012 samples, 6 months for 2013 samples and a week for 2014 and 2015 samples. For their preparation, samples were thawed at 4 °C for 24 h. After homogenization, a 10-mL aliquot was spiked with ILIS mix at 50 ng/L (with 10 uL of a 5 µg/L solution). After filtration through 0.2 µm regenerated cellulose filter, samples were directly injected into the HPLC–MS system without further pretreatment.

**Liquid chromatography tandem mass spectrometry**

A Thermo Scientific TSQ Vantage triple quadrupole mass spectrometer provided with a heated electro spray ionisation source (Thermo Electron, Bremen, Germany) was interfaced to a Surveyor HPLC system (Thermo Electron).

The chromatographic separation was achieved on a XBridge C18 column (150 mm × 2.1 mm I.D., particle size 3.5 µm) (Waters, Etten-Leur, the Netherlands) preceded by a KrudKatcher ULTRA HPLC in-line SS filter (0.5 µm × 0.1 mm I.D.) (Phenomenex, Torrance, USA) maintained at a temperature of 21 °C. From the sample aliquot 100 µL were injected into the system, and by using an optimized ternary gradient of

ultrapure water (A), methanol (B) and acetonitrile (C), all three with 0.05% formic acid, the compounds were separated at a constant flow rate of 0.3 mL/min and introduced into the mass spectrometer.

Because 6 out of the 10 compounds studied had a log Kow value within a narrow window (1.99–2.58, see **Table 3.1.1**) a ternary gradient was needed to provide sufficient chromatographic separation. The percentage of organic solvent B was changed as follows: 0–1 min, 10%; 4 min, 45%; 14 min, 20%; 15–17 min, 100% and the percentage of organic solvent C was changed as follows: 0–1 min, 0%; 4 min, 5%; 14 min, 30%; and 15–17 min, 0%. Between consecutive runs, the analytical column was re-equilibrated for 3 min.

The system was operated in positive mode. For the ionisation nitrogen gas was used. The source voltage was set to 2.5 kV, and the capillary and vaporizer temperature to 300 °C and 350 °C, respectively. The acquisition parameters in selected reaction monitoring (SRM) mode can be found in **Table 3.1.1**. For each compound, at least two transitions of the protonated molecular ion $[M + H]^+$ were monitored. The first transition (Q) was used for quantitation and the second transition (q) was used for confirmation purposes.

Analyte concentrations were quantified using sildenafil-$d_8$ (ILIS 1) for analytes sildenafil, desethylsildenafil, noracetildenafil, tadalafil, aminotadalafil, chloropretadalafil, n-octyl nortadalafil, vardenafil and n-desethylvardenafil; and desmethylsildenafil-$d_8$ (ILIS 2) for desmethylsildenafil.

**Table 3.1.1.** Selected PDE5 inhibitors and LC-MS/MS parameters used for compounds identification.

| | CAS number | Molecular formula | Log Kow (*) | $[M+H]^+$ | Product ions (*m/z*) | Collision energy (V) | S-Lens | RT window (min) |
|---|---|---|---|---|---|---|---|---|
| Sildenafil (ILIS 1) | 171599-83-0 | $C_{22}H_{30}N_6O_4S$ | 2.30 | 475.2 | 58.2 (Q) | 36 | 118 | 8.40 – 13.40 |
| | | | | | 100.2 (q1) | 28 | | |
| | | | | | 283.2 (q2) | 36 | | |
| Desmethylsildenafil (ILIS 2) | 139755-82-1 | $C_{21}H_{28}N_6O_4S$ | 2.09 | 461.1 | 283.1 (Q) | 35 | 130 | 8.50 – 13.50 |
| | | | | | 311.1 (q) | 29 | | |
| Desethylsildenafil (ILIS 1) | 139755-91-2 | $C_{20}H_{28}O_4N_6S$ | 1.99 | 449.2 | 283.1 (Q) | 36 | 138 | 7.00 – 12.00 |
| | | | | | 311.1 (q) | 27 | | |
| Noracetildenafil (ILIS 1) | 949091-38-7 | $C_{24}H_{32}N_6O_3$ | n.a. | 453.2 | 97.1 (Q) | 31 | 148 | 6.80 – 11.80 |
| | | | | | 113.1 (q) | 31 | | |
| Tadalafil (ILIS 1) | 171596-29-5 | $C_{22}H_{19}N_3O_4$ | 0.04 | 390.0 | 204.1 (Q) | 57 | 92 | 12.00 – 17.00 |
| | | | | | 268.1 (q) | 14 | | |
| Aminotadalafil (ILIS 1) | 385769-84-6 | $C_{21}H_{18}N_4O_4$ | -1.20 | 391.0 | 204.1 (Q) | 56 | 87 | 9.70 – 14.70 |
| | | | | | 262.1 (q) | 31 | | |
| Chloropretadalafil (ILIS 1) | 171489-59-1 | $C_{22}H_{19}ClN_2O_5$ | 2.58 | 427.1 | 274.1 (Q) | 31 | 93 | 14.40 – 19.40 |
| | | | | | 135.0 (q) | 19 | | |
| N-octyl nortadalafil (ILIS 1) | 1173706-35-8 | $C_{29}H_{33}N_3O_4$ | 5.22 | 488.2 | 366.2 (Q) | 17 | 120 | 15.85 – 19.85 |
| | | | | | 169.1 (q) | 39 | | |
| Vardenafil (ILIS 1) | 224789-15-5 | $C_{23}H_{33}N_6O_4S$ | 2.79 | 489.3 | 151.1 (Q) | 41 | 159 | 7.25 – 12.25 |
| | | | | | 312.1 (q) | 39 | | |
| N-desethylvardenafil (ILIS 1) | 448184-46-1 | $C_{21}H_{28}N_6O_4S$ | 2.09 | 461.2 | 151.1 (Q) | 43 | 143 | 7.25 – 12.25 |
| | | | | | 312.2 (q) | 33 | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *ILIS 1 Sildenafil-$d_8$* | 951385-68-5 | $C_{22}H_{22}D_8N_6O_4S$ | 2.30 | 483.3 | 62.1 (Q) | 37 | 126 | 8.25 – 13.25 |
| | | | | | 108.3 (q) | 29 | | |
| *ILIS 2: Desmethylsildenafil-$d_8$* | 1185168-06-2 | $C_{21}H_{20}D_8N_6O_4S$ | 2.09 | 469.2 | 283.1 (Q) | 37 | 160 | 8.50 – 13.50 |
| | | | | | 311.1 (q) | 30 | | |

n.a.: not available

(*) Log Kow (KOWWIN estimates)

**Method performance**

The method was validated in terms of linearity, limits of detection and quantification, precision intra-day (repeatability) and inter-day, procedural recovery and matrix effects by analyzing spiked waste water collected as a grab sample at the WWTP in Utrecht.

A calibration curve was established by analyzing spiked wastewater samples with standard solutions at 6 different concentrations (0–500 ng/L) to investigate linearity. The concentration of analyte in a sample was obtained by comparing the peak area ratio of the analyte and internal standard to its corresponding ratio in the calibration curve.

Limits of detection and quantification (LOD and LOQ, respectively) were defined as the concentration that provides signal-to-noise (S/N) values of 3 and 10 for the quantifier ion of each analyte. The values were calculated at the lowest point of the calibration curve (in wastewater) correctly confirmed with qualifier ion ratio. Concentration values determined in the analysed real samples that were <LOQ were treated as follows: (1) if all values at a location for a certain compound were <LOQ, loads were set to zero; (2) if at least one value was >LOQ, values <LOQ were replaced with 0.5 × LOQ (Ort et al., 2014).

Intra-day and inter-day precision were assessed at 4 levels: 20, 50, 100, 500 ng/L; with n = 7 replicates per level, and during 3 non-consecutive days. Procedural recovery (%) was calculated as the ratio of the signal of the analyte spiked to a sample after sample filtration (C) against the signal of the analyte spiked to the same sample before filtration (B): [C/B] × 100. Matrix effect (%) was calculated as the ratio of the signal of solution B (where the signal of the native compound present in the used sample was subtracted) against the signal of the analyte spiked to ultrapure water (A): [B/A] × 100. The specificity was evaluated with two parameters, the retention time and the ratio between the quantifier (Q) and the qualifier (q): RT ± 2.5% and Q/q < 30%.

**Analyte stability**

Few studies have addressed the stability in sewage of the target compounds, metabolites and transformation products (Baker and Kasprzyk-Hordern, 2011b; van Nuijs et al., 2012). As these products are bioactive, further metabolization and transformation can occur after sample collection. Stability is therefore an important

parameter to study for the correct quantification of the drugs, thus avoiding significant under estimations.

The stability experiments consisted of following the concentration change of analytes spiked in untreated raw (and unfiltered) wastewater over a definite period of storage at a temperature of 4 °C, as these are the conditions in which sample is kept in the autosampler during sampling. The experiment was carried out at the original pH (7.5–8). For each time interval (0, 3, 6, 9, 24, 30, 48 h), one blank and three independent sample aliquots spiked at 100 ng/L with all native compounds were prepared. Time 0 was considered as control. After the selected-time interval, ILIS were spiked to all samples, which were then homogenized and immediately filtered for the analysis as explained in the sample preparation section.

# 3.1.3. Results and discussion

**Method optimization: DI-HPLC-(QqQ)-MS/MS**

Although influent wastewater is a very complex matrix that may require appropriate sample treatment for the removal of suspended matter and matrix components, direct injection is an interesting alternative because of a reduction of sample treatment steps that may lead to analyte losses and because of time saving. For direct injection, obviously the sensitivity of the method should be considered beforehand. From pilot experiments and data published in literature (Nieto et al., 2010) we expected that concentrations in Dutch wastewater influents were such that direct injection could be applied. To make sure, we evaluated the sample procedure proposed by Nieto et al. (Nieto et al., 2010) with acetone:ethylacetate for the elution of the OASIS HLB cartridge, as well as a direct injection procedure described for the determination of isothiazolinones (Speksnijder et al., 2010). Results showed similar performances (recoveries, satisfactory sensitivity, and matrix effects, results for SPE not shown here, see below for direct injection results) and therefore direct injection was selected in order to simplify the analytical methodology.

**Method performance**

Quality parameters are summarized in **Table 3.1.2**. All target compounds present a good linear fit within the studied range (LOD — 500 ng/L) with r-squared values higher than 0.999. Regarding the LOD and LOQ, compounds could be divided into three groups: sildenafil, desethylsildenafil, tadalafil and aminotadalafilwith the lowest limits, LOQs ranging between 1.6 and 7.5 ng/L. The second group with LOQ ranging between 13.3 and 30.0 ng/L: desmethylsildenafil, noracetildenafil, chloropretadalafil, vardenafil and n-desethylvardenafil. Finally, n-octylnortadalafil with highest LOQ at 100 ng/L. Intraday and inter-day repeatability is presented as RSD (%). For all compounds values were lower than 26% at the two higher levels. For the lower levels, sildenafil and its two metabolites, tadalafil, aminotadalafil, chloropretadalafil, n-octylnortadalafil and vardenafil also presented values in the same range whereas in the case of noracetildenafil and n-desethylvardenafil the RSD (%) for the lowest levels was higher than 30%. Procedural recovery ranged successfully between 85 and 105 in all cases, with RSD (%) values lower than 25%. Regarding matrix effects appeared to be concentration dependent. At lower concentrations of the spike, invariably matrix

enhancement was observed, up to 5-fold (desethylsildenafil), whereas at higher concentrations much lower enhancement and also sometimes suppression of the signal (down to 46% for noracetildenafil) was observed (see **Table 3.1.2.b.**).

**Table 3.1.2.a.** Method performance: linearity, limits of detection and quantification, intraday and interday repeatability.

| | linearity | LOD | LOQ | Intraday repeatability (RSD (%), n=7) | | | | Interday repeatability (RSD (%), n=7, d=3) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | ($r^2$) | ng/L | ng/L | 20 ng/L | 50 ng/L | 100 ng/L | 500 ng/L | 20 ng/L | 50 ng/L | 100 ng/L | 500 ng/L |
| sildenafil | 0.9997 | 1.8 | 6 | 16 | 10 | 5 | 5 | 24 | 9 | 10 | 9 |
| desmethylsildenafil | 0.9999 | 5.4 | 18 | 27 | 15 | 7 | 12 | 25 | 24 | 8 | 9 |
| desethylsildenafil | 0.9997 | 0.5 | 1.6 | 18 | 11 | 10 | 4 | 33 | 18 | 9 | 8 |
| noracetil | 0.9990 | 6 | 20 | 31 | 13 | 5 | 6 | 36 | 23 | 9 | 6 |
| tadalafil | 0.9998 | 2.3 | 7.5 | 10 | 11 | 11 | 7 | 13 | 13 | 13 | 11 |
| aminotadalafil | 0.9995 | 1.8 | 6 | 8 | 11 | 11 | 8 | 14 | 16 | 11 | 11 |
| chloropretadalafil | 0.9993 | 4 | 13.3 | 6 | 8 | 9 | 8 | 12 | 15 | 8 | 10 |
| n-octylnortadalafil | 0.9999 | 30 | 100 | 11 | 15 | 10 | 10 | 20 | 27 | 26 | 16 |
| vardenafil | 0.9998 | 7.2 | 24 | 17 | 18 | 9 | 5 | 22 | 20 | 14 | 7 |
| n-desethylvardenafil | 0.9998 | 9 | 30 | 26 | 16 | 9 | 8 | 37 | 30 | 15 | 13 |

**Table 3.1.2.b.** Method performance: procedural recovery and matrix effect.

| | Procedural Recovery ± RSD (%) | | | | Matrix Effect ± RSD (%) | | | |
|---|---|---|---|---|---|---|---|---|
| | 20 ng/L | 50 ng/L | 100 ng/L | 500 ng/L | 20 ng/L | 50 ng/L | 100 ng/L | 500 ng/L |
| sildenafil | 93.1 ± 19.7 | 102.7 ± 10.4 | 100.1 ± 11.7 | 97.5 ± 14.7 | 241.9 ± 22.6 | 247.8 ± 15.9 | 82.3 ± 10.1 | 73.6 ± 12.2 |
| desmethylsildenafil | 99.9 ± 20.2 | 100.4 ± 16.9 | 99.9 ± 12.5 | 90.8 ± 21.1 | 406.6 ± 35.0 | 437.1 ± 34.7 | 116.8 ± 12.6 | 82.0 ± 21.3 |
| desethylsildenafil | 97.2 ± 22.1 | 100.7 ± 10.4 | 102.3 ± 11.4 | 93.2 ± 19.0 | 393.4 ± 30.9 | 549.0 ± 17.1 | 156.8 ± 14.7 | 99.8 ± 13.3 |
| noracetil | 94.8 ± 57.7 | 102.7 ± 17.1 | 104.4 ± 13.9 | 99.0 ± 15.7 | 298.6 ± 85.3 | 216.1 ± 33.6 | 70.5 ± 51.0 | 46.7 ± 34.0 |
| tadalafil | 89.3 ± 21.5 | 96.5 ± 7.8 | 96.0 ± 8.6 | 97.7 ± 12.7 | 246.6 ± 23.6 | 270.1 ± 14.2 | 84.0 ± 10.3 | 72.2 ± 12.5 |
| aminotadalafil | 91.3 ± 16.5 | 100.9 ± 8.6 | 97.5 ± 8.8 | 98.2 ± 13.9 | 217.5 ± 15.7 | 251.0 ± 15.4 | 77.8 ± 10.8 | 69.1 ± 13.6 |
| chloropretadalafil | 93.4 ± 15.4 | 87.2 ± 8.4 | 91.7 ± 10.2 | 92.4 ± 11.5 | 195.0 ± 20.6 | 243.8 ± 14.2 | 73.1 ± 10.2 | 64.9 ± 13.6 |
| n-octylnortadalafil | - | - | 16.4 ± 20.5 | 27.4 ± 36.8 | 163.1 ± 19.3 | 234.0 ± 24.1 | 77.4 ± 18.8 | 75.3 ± 10.9 |
| vardenafil | 92.2 ± 23.6 | 101.3 ± 12.2 | 102.1 ± 12.5 | 96.6 ± 12.1 | 320.5 ± 32.2 | 322.7 ± 24.9 | 96.5 ± 17.2 | 83.4 ± 12.3 |
| n-desethylvardenafil | 95.4 ± 25.0 | 96.5 ± 14.4 | 98.9 ± 13.0 | 97.0 ± 16.7 | 607.0 ± 26.9 | 616.0 ± 26.0 | 152.1 ± 14.7 | 125.8 ± 13.0 |

**Analyte stability**

The results of the stability test are presented as percentage of change from the initial concentration, and plotted in **Fig. 3.1.1**. The error bars correspond to the standard deviation (n=3). The statistical evaluation of the results was performed using ANOVA in order to assess a statistically significant change of the signal along the time. For two compounds a statistically significant (p-value b0.05) decrease was observed: noracetildenafil (p = 0.001) and n-octylnortadalafil (p = 0.048) over 48 h. For both compounds, p-value below 0.05 was observed from the 24-h point meaning that stability starts decreasing within the 9 to 24 h range. All other compounds tested did not experience any statistically significant decrease and can therefore be considered stable. Given that the composite sample collection lasts 24 h, noracetildenafil and n-octylnortadalafil might experience some degradation. However, this is minimized since the samples are frozen immediately after collection.

**Analysis of wastewater influents**

Sample concentrations in WWTP influents were transformed into daily loads of analytes entering the WWTP using flow rates provided by the WWTP operators (see **Tables D1–D5**). The results were expressed in normalized loads (mg/day/1000 inh) according to **Eq. 3.1.1.,** where the concentration in the sample in ng/L is multiplied by the registered flow during the corresponding 24-h period and divided by the number of inhabitants within the catchment area. Census data were used for normalization to number of inhabitants connected to the sewer system.

**Eq. 3.1.1**: $load\ mg/day/1000\ inh = \frac{Flow\ (^{10^3 L}/_{day}) \cdot Conc\ (^{10^{-6} mg}/_{L})}{n^{\circ}\ inh \cdot 10^3}$

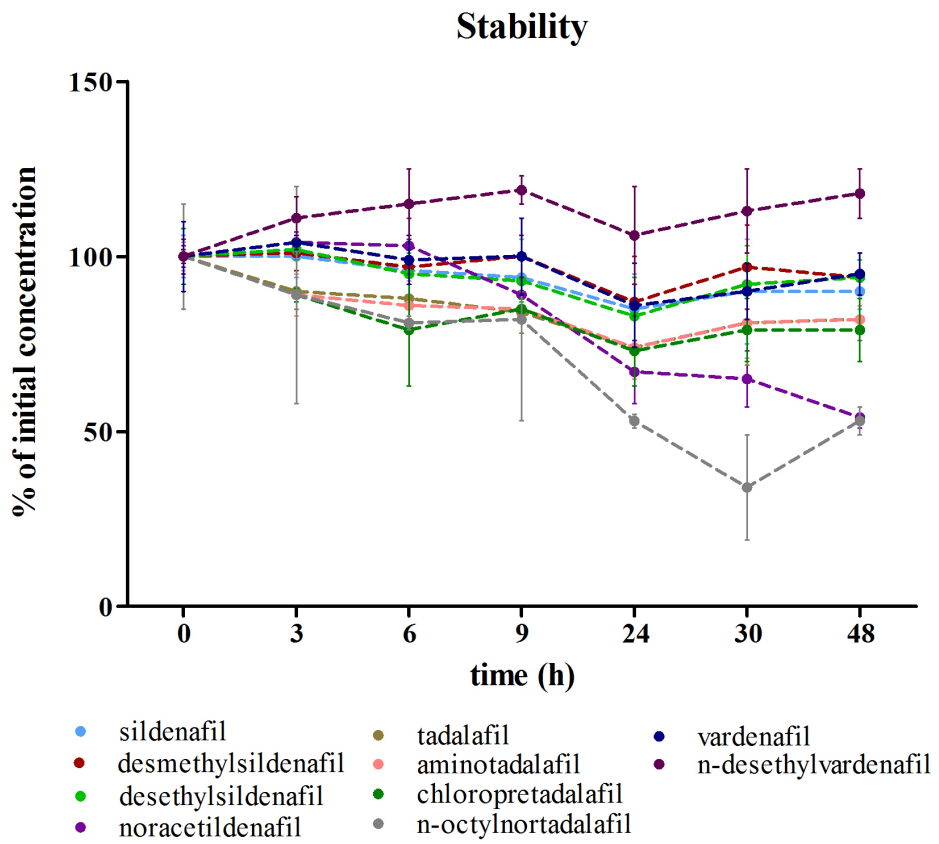


**Fig. 3.1.1.** Stability plot for PDE5 inhibitors in wastewater at natural pH and 4 ºC. Results have been normalized as percentage of initial concentration. Error bars correspond to the standard deviation of triplicates.

### Weekly variations

Sildenafil and its two transformation products desmethyl- and desethylsildenafil were the only active ingredients detected in the samples collected. This finding was in accordance with prescription data, as the three erectile dysfunction pharmaceuticals are similarly prescribed in The Netherlands but with lower daily doses: Viagra® at 50–100 mg compared to Levitra® + Cialis® at 10–20 mg. Therefore, vardenafil and tadalafil (and their corresponding analogues and transformation products) could be expected at a lower level below their respective LODs. **Tables D6–D8** present the normalized daily loads determined in the samples collected during the three week-long monitoring campaigns performed in Amsterdam, Eindhoven and Utrecht in 2013,

2014 and 2015. Sildenafil was present in all samples analysed, with loads ranging from 1.6 to 8.3 mg/day/1000 inh. Temporal trends in the city of Amsterdam showed an increase from 2013 to 2014 and 2015, from a weekly average of 3.4 to 6.3 and 6.8 mg/day/1000 inh. In the case of Eindhoven, the weekly average remained constant in 2013 and 2014, followed by a decrease in 2015: 3.8, 3.8 and 2.6 mg/day/1000 inh, respectively. In the city of Utrecht an increase was observed from 2013 to 2014, and then a decrease in 2015 with weekly averages being 2.4, 3.1 and 2.4 mg/day/1000 inh, respectively. Desethylsildenafil is the most abundant metabolite of sildenafil in urine (Muirhead et al., 2002a). It was detected in all but two samples in 2013, which represents 97% of the total number of samples analysed. Temporal trends showed an increase in Amsterdam over the years, doubling from 2013 to 2014. Values corresponded to 6.3, 15.7 and 19.2 mg/day/1000 inh respectively. In the cities of Eindhoven and Utrecht, a remarkable increase was observed from 2013 to 2014, but thereafter levels remained constant from 2014 to 2015. Values corresponded to 4.3, 11.1 and 11.1 mg/day/1000 inh in Eindhoven and 2.1, 7.8 and 8.0 mg/day/1000 inh in Utrecht. Desmethylsildenafil is the second metabolite detected, and known to be less abundant in urine than desethylsildenafil (Muirhead et al., 2002a). The time trend for this compound showed an increase over the years in Amsterdam, doubling from 2013 to 2014. Values corresponded to 3.9, 8.4 and 11.2 mg/day/1000 inh respectively. In the cities of Eindhoven and Utrecht, it could not be detected in 2013, and a decrease from 2014 to 2015 was observed. Values corresponded to 5.5 and 3.4 mg/day/1000 inh in Eindhoven and 3.6 and 2.2 mg/day/1000 inh in Utrecht. Daily variations expressed as a percentage of the total weekly load of the individual compounds are presented in **Fig. 3.1.2** (A: sildenafil; B: desmethylsildenafil; C: desethylsildenafil). The box represents the median, 25% and 75% percentile values and the whiskers extend to the minimum and maximum values. This way of presenting the results allows the rapid observation of a difference between weekdays (Mon-Fri) and weekends (Sa-Su), regarding the recreational use of the drug. Depending on the human excretion rates of individual drugs and the sewer residence time, the “weekend peak” may appear earlier or later. In the case of sildenafil and its two metabolites, an ANOVA single factor was performed to evaluate whether the difference among different days and the difference between weekdays and weekend were statistically significant. The result showed that there was no statistically significant difference, which can be interpreted as a non-recreational usage pattern.

The finding of the presence of sildenafil in wastewater, that has been reported before (Baker et al., 2014; Nieto et al., 2010; Schröder and Thevis, 2010) is contradicting results from pharmacokinetic studies, which invariably conclude that no appreciable

amounts of unchanged sildenafil are being excreted from the body after its consumption (Muirhead et al., 2002a; Walker et al., 1999). In another study, however in the urine of two individuals dosed with sildenafil some unchanged compound was found (Strano-Rossi et al., 2010). In addition, phase-II conjugation possibly leads to excretion of a conjugated form of sildenafil that may be deconjugated in the sewer.

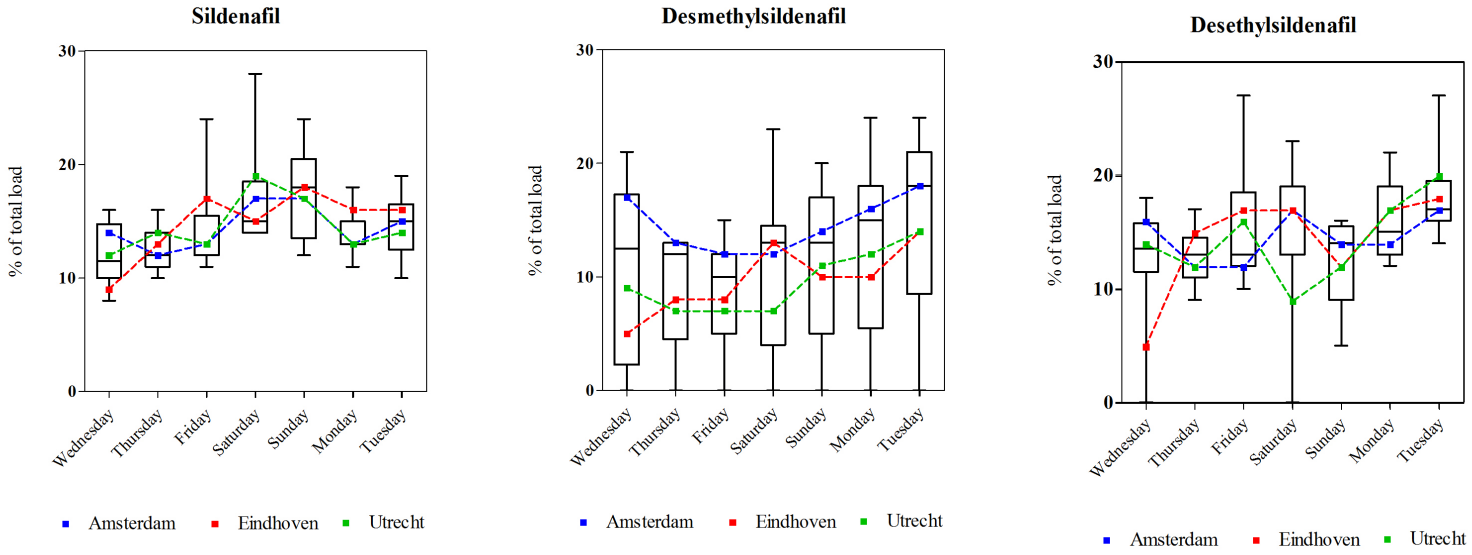


**Fig 3.1.2.** (A: sildenafil; B: desmethylsildenafil; C: desethylsildenafil). Daily variations expressed as the percentage of the total load, combined results from 2013, 2014 and 2015. The box represents the median, 25% and 75% percentile values and the whiskers extend to the minimum and maximum values. The dotted line represents the average per city

**Festival event results**

Besides the three week-long monitoring campaigns, two additional week-long sets of samples were collected in Amsterdam at the time that a festival event took place. This annually recurring event lasts from Thursday to Sunday bringing around ~350,000 visitors to the city. For these weeks, the comparison among the loads within the city were expressed in g/day, since the normalization by 1000 inh was difficult to apply. **Tables D9–D10** present the daily loads determined in the samples collected during the two week-long monitoring campaigns performed in Amsterdam in 2012 and 2014.

At this point, and in order to better evaluate the observed trends along the years, it is worth mentioning that the patent that the pharmaceutical company Pfizer held for Viagra® expired in some European countries (including The Netherlands) in June 2013. For this reason, two periods can be differentiated. The first one including the results from the sampling campaigns performed in 2012 and 2013, when the patent was still valid; and the second including the results from the sampling campaigns performed in 2014 and 2015, after the expiration of the patent. **Fig. 3.1.3** (A: sildenafil; B: desmethylsildenafil; C: desethylsildenafil) presents in box plots the daily loads during the five week-long monitoring campaigns performed in Amsterdam. To evaluate the difference between "event" weeks and "normal" weeks an ANOVA single factor was performed. Both the parent sildenafil and the transformation products showed similar temporal patterns: the daily loads in week 2013 were statistically significantly lower than those in other weeks ($p < 0.05$). Before the expiration of the event, it was more difficult to obtain the drug, and perhaps during the recreational event users took more trouble to obtain the drug (which would explain the statistical difference between the daily loads in the "normal" week in 2013 and in the event week in 2012). After the expiration, it became easier to obtain the drug, and event weeks no longer showed statistical differences with "normal" weeks. The "weekend peak" was also statistically investigated and resulted in a not significant difference of the loads between weekdays (Mon-Fri) and weekend (Sat–Sun).

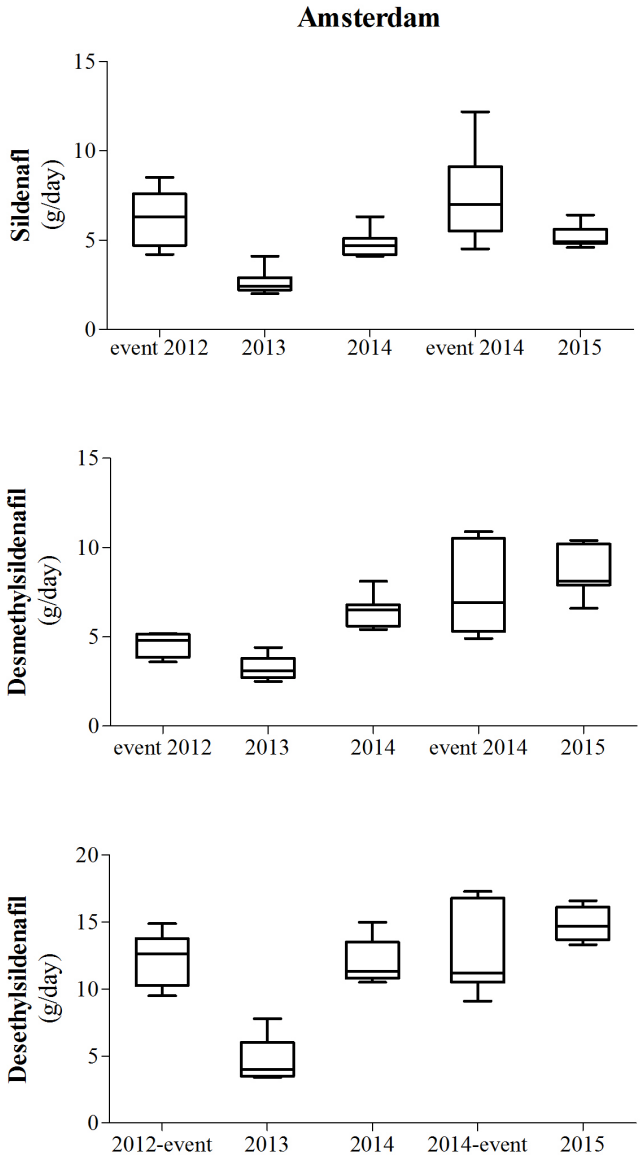


**Fig. 3.1.3.** (A: sildenafil; B: desmethylsildenafil; C: desethylsildenafil). Loads in g/day from the five weeklong monitoring campaigns performed in Amsterdam. The box represents the median, 25% and 75% percentile values and the whiskers extend to the minimum and maximum values. Number of observations n = 5 (2012), n=7 for all other weeks.

**Metabolite to parent concentration ratio**

In order to evaluate the clearance of sildenafil, the daily ratios between the main transformation product and the parent drug obtained during one week are presented in **Fig. 3.1.4.** Only the ratio desethylsildenafil/sildenafilwas evaluated, since the ratio desmethylsildenafil/sildenafil was not always available. The ratio desethylsildenafil/sildenafil showed a marked trend among cities and years. In the case of Amsterdam, the median experienced a slight increase of 1.5-fold from 1.7 in 2013, to 2.6 in 2014 and 2.7 in 2015. The increase was larger for the other two cities. In the case of Eindhoven, the median of the ratio increased from 1.1 in 2013 to 3.2 in 2014 and 4.3 in 2015, which corresponded to a 4-fold increase. And in the case of Utrecht, the ratio increased from 1.2 in 2013 to 2.5 in 2014 and 3.5 in 2015, which corresponded to a 3-fold increase.

The observed phenomenon is intriguing, since we expected the ratio to remain more or less constant along the years because of a stable transformation rate of the drug in the human body. To our knowledge, the change in the metabolite to parent drug concentration ratio has not been previously reported for PDE5 inhibitors. We propose two speculative explanations of this phenomenon.

**Sewer characteristics**. The first explanation is based on the effect of wastewater quality in the transformation of sildenafil during its residence time in the sewer (from the toilet to the WWTP entrance). One of the parameters that could have an effect is the wastewater temperature. The average temperatures in the country during the weeks preceding the sampling campaigns averaged 2.5 °C in 2013, whereas in 2014 and 2015 they averaged 8 °C and 6.5 °C respectively (see daily temperatures in **Tables D6–D8**). We evaluated the stability of the analytes in wastewater at 4 °C in the laboratory and found no change in concentration over a 48-h period for the two compounds (see **Fig. 3.1.1**), but a difference of several degrees could already promote the further transformation of sildenafil into desethylsildenafil in the sewer. Besides, it is well-known in The Netherlands that often waste dumpings from illegal drug labs (Nu.nl “Vaker dumping van drugsresten in riool Nederlandse steden”) or under the pressure of a police raid (Emke et al., 2014) occur, and that the frequency of these dumpings has increased in the period 2013–2015 (“Omroep Brabant”). The disposal of this illegal waste can be either directly into the environment or into the sewer system, the latter affecting the wastewater conditions and possibly resulting in increased formation of sildenafil metabolite. More research on the degradation pathway of sildenafil in the sewer, and the effect of the different parameters involved in wastewater quality would be required to support the finding and this explanation.

**Pharmacokinetics**. The second explanation is based on the possibility that the expiration of the patent in 2013 has widened the number of currently available ED products, and that different formulations or doses experience different excretion ratios. It could be that sildenafil synthetic analogues not formally approved, but being the active ingredient in one of the counterfeit medicines or products such as dietary supplements, undergo a metabolic pathway leading towards the formation of desethylsildenafil. Further research in the counterfeit analogue area is required, not only regarding their identification but also their pharmacokinetics. Besides, the alteration in sildenafil pharmacokinetics has been studied as an effect of different parameters such as age, renal and hepatic functions (Muirhead et al., 2002b). The study reported a slower metabolism in elderly and patients with hepatic and renal failure, who showed a higher exposure to the drug because of a slower sildenafil clearance, compared to youngmen and healthy patients. Another possible reason to induce a change in the metabolism could be the prolonged use and the dose-dependence. It is well known that sildenafil users outside the official health system auto-medicate themselves (Rao et al., 2015) and prolong the (ab)use with high doses. Further research is required to study to which extent sildenafil pharmacokinetics might change for long-term users.

**Back-calculation of sildenafil consumption**

The obtained environmental loads can be used to back-calculate sildenafil consumption by the population connected to the sewer system (WBE). This was done based on pharmacokinetic data (Muirhead et al., 2002a) and the assumption that there were no losses in sewage due to degradation and no dumping of unused drugs (B J Venhuis et al., 2014b). Normalized results are shown in **Fig. 3.1.5.** for 2013, 2014 and 2015 and for the two festival events in 2012 and 2014. The data for 2013 have been used in a separate study in order to quantify illegal sales of sildenafil (B J Venhuis et al., 2014a). The data were expressed both as mg/week/1000 inhabitants and doses/week/1000 in. assuming that the recommended dose for an adult is 50 mg. The dose data for 2013, 2014 and 2015, where an increase is visible between 2013 and 2014 for all three cities, again suggest that the expiration of the Viagra® patent end of 2013 has led to increased sales and consumption of sildenafil.

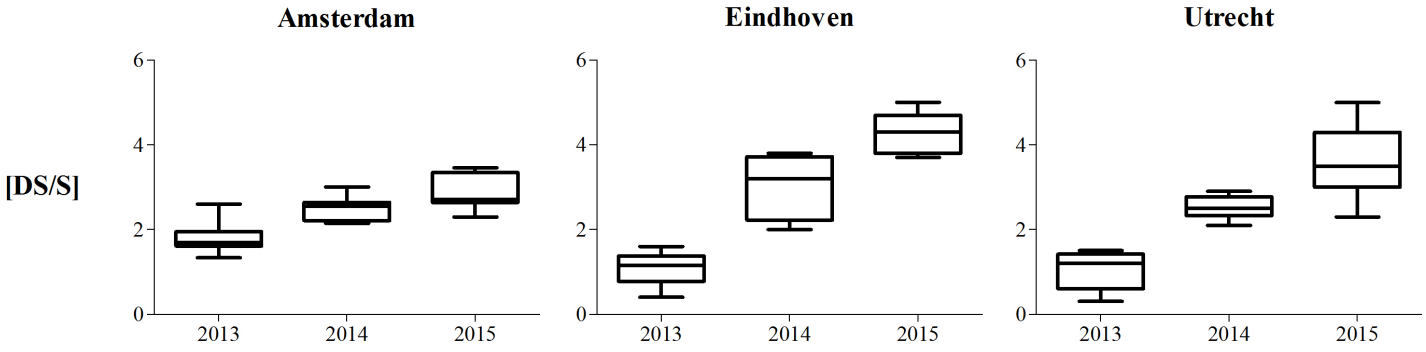




**Fig. 3.1.4.** Box plots representing the median, 25-75% percentiles, and lowest and highest ratios of desethylsildenafil/sildenafil observed during one week of sampling in three consecutive years for the cities of Amsterdam, Eindhoven and Utrecht.

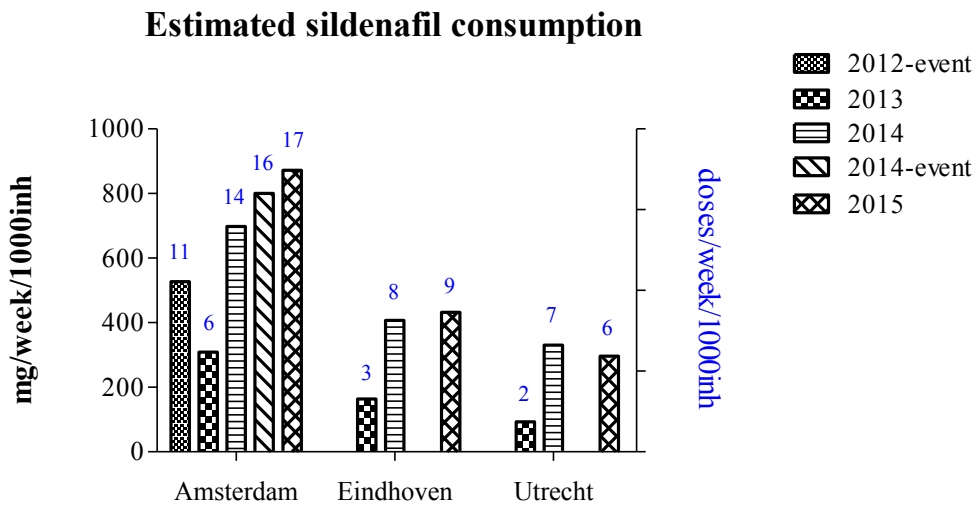


**Fig. 3.1.5.** Estimated sildenafil consumption expressed in mg/week/1000 inhabitants and doses/week/1000 inhabitants (blue figures) for the three Dutch cities included in the study.

# 3.1.4. Conclusions

An analytical method was developed and validated to determine the presence of ten PDE5 inhibitors in influent wastewater. Its application to wastewater influents collected from 2012 to 2015 in three Dutch cities resulted in the successful determination of sildenafil and its two main human metabolites. The presence of unchanged sildenafil in wastewater contradicted results from pharmacokinetic studies, whereas the other seven target analytes were not detected as expected from prescription data. Besides, sildenafil showed no recreational usage pattern, since the statistical study of the differences between weekdays (Mon-Fri) and weekend (Sat-Sun) loads was not significant. Two additional week-long sets of samples collected in Amsterdam at the time that a festival event took place were also included in the study. Only in the studied weeks before the patent expiration an increase in the load between “normal” and “event” was observed. The metabolite to parent drug concentration ratio was plotted, and showed a marked increase from 2013 to 2015, leading to several speculative explanations that open new research lines. Finally, the environmental loads of sildenafil and its two metabolites were back-calculated into the estimated sildenafil consumption.

# Acknowledgements

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# Annex D – Supplementary Information Chapter 3.1.

**Table D1**. Characteristics WWTP Amsterdam, Eindhoven and Utrecht in 2013

| City | date 24/h sample taken | | Flow $m^3$/24h | sampling interval min | BOD mg/L | COD mg/L | Ntot mg/L | Ptot mg/L |
|---|---|---|---|---|---|---|---|---|
| Amsterdam | 6-3-2013 | Wednesday | 84390 | 6.0 | 290 | 650 | 68 | 8.8 |
| Amsterdam | 7-3-2013 | Thursday | 84470 | 6.0 | 310 | 700 | 66 | n.a. |
| Amsterdam | 8-3-2013 | Friday | 84420 | 6.0 | 230 | 545 | 43 | 5.4 |
| Amsterdam | 9-3-2013 | Saturday | 194460 | 2.6 | 110 | 270 | 39 | 4.6 |
| Amsterdam | 10-3-2013 | Sunday | 125350 | 4.0 | 160 | 435 | 63 | 7.3 |
| Amsterdam | 11-3-2013 | Monday | 92200 | 5.5 | 240 | 525 | 53 | 6.4 |
| Amsterdam | 12-3-2013 | Tuesday | 88990 | 5.7 | 270 | 580 | 58 | 7.3 |
| Eindhoven | 6-3-2013 | Wednesday | 110023 | 10.5 | 160 | 442 | 59 | 10.0 |
| Eindhoven | 7-3-2013 | Thursday | 144175 | 8.0 | 220 | 472 | 62 | 11.0 |
| Eindhoven | 8-3-2013 | Friday | 321874 | 3.6 | 140 | 494 | 30 | 5.5 |
| Eindhoven | 9-3-2013 | Saturday | 158169 | 7.3 | 220 | 661 | 60 | 11.0 |
| Eindhoven | 10-3-2013 | Sunday | 114808 | 10.0 | 89 | 280 | 33 | 4.8 |
| Eindhoven | 11-3-2013 | Monday | 136339 | 8.4 | 140 | 400 | 49 | 9.2 |
| Eindhoven | 12-3-2013 | Tuesday | 128968 | 8.9 | 120 | 412 | 50 | 9.7 |
| Utrecht | 6-3-2013 | Wednesday | 45640 | 11.0 | 200 | 622 | 50 | 9.6 |
| Utrecht | 7-3-2013 | Thursday | 46500 | 10.8 | 200 | 548 | 50 | 8.0 |
| Utrecht | 8-3-2013 | Friday | 81470 | 6.2 | 190 | 491 | 46 | 7.4 |
| Utrecht | 9-3-2013 | Saturday | 234930 | 2.1 | 63 | 241 | 19 | 3.3 |
| Utrecht | 10-3-2013 | Sunday | 71490 | 7.0 | 150 | 746 | 38 | 6.0 |
| Utrecht | 11-3-2013 | Monday | 49170 | 10.3 | 160 | 512 | 46 | 7.5 |
| Utrecht | 12-3-2013 | Tuesday | 49430 | 10.2 | 160 | 489 | 52 | 9.2 |

*n.a.: not available*

**Table D2**. Characteristics WWTP Amsterdam, Eindhoven and Utrecht in 2014

| City | date 24/h sample taken | | Flow $m^3$/24h | sampling interval min | BOD mg/L | COD mg/L | Ntot mg/L | Ptot mg/L |
|---|---|---|---|---|---|---|---|---|
| Amsterdam | 12-3-2014 | Wednesday | 148310 | 3.4 | 260 | 510 | 68 | 8.4 |
| Amsterdam | 13-3-2014 | Thursday | 147750 | 3.4 | n.a. | n.a. | n.a. | n.a. |
| Amsterdam | 14-3-2014 | Friday | 149870 | 3.4 | n.a. | n.a. | n.a. | n.a. |
| Amsterdam | 15-3-2014 | Saturday | 149770 | 3.4 | 130 | 610 | 65 | 8.3 |
| Amsterdam | 16-3-2014 | Sunday | 150400 | 3.4 | n.a. | n.a. | n.a. | n.a. |
| Amsterdam | 17-3-2014 | Monday | 146400 | 3.4 | n.a. | n.a. | n.a. | n.a. |
| Amsterdam | 18-3-2014 | Tuesday | 165240 | 3.1 | 340 | 680 | 72 | 9.7 |
| Eindhoven | 11-3-2014 | Tuesday | 118281 | 9.7 | n.a. | n.a. | n.a. | n.a. |
| Eindhoven | 13-3-2014 | Thursday | 100504 | 11.5 | n.a. | n.a. | n.a. | n.a. |
| Eindhoven | 14-3-2014 | Friday | 100049 | 11.5 | n.a. | n.a. | n.a. | n.a. |
| Eindhoven | 15-3-2014 | Saturday | 97910 | 11.8 | n.a. | n.a. | n.a. | n.a. |
| Eindhoven | 16-3-2014 | Sunday | 96068 | 12.0 | n.a. | n.a. | n.a. | n.a. |
| Eindhoven | 17-3-2014 | Monday | 105452 | 10.9 | n.a. | n.a. | n.a. | n.a. |
| Utrecht | 11-3-2014 | Tuesday | 46680 | 12.3 | n.a. | 1166 | 49 | 22.5 |
| Utrecht | 12-3-2014 | Wednesday | 46430 | 12.4 | n.a. | 876 | 55 | 20.2 |
| Utrecht | 13-3-2014 | Thursday | 46460 | 12.4 | n.a. | 667 | 39 | 14.1 |
| Utrecht | 14-3-2014 | Friday | 44110 | 13.1 | n.a. | 523 | 34 | 11.0 |
| Utrecht | 15-3-2014 | Saturday | 43960 | 13.1 | n.a. | 614 | 41 | 15.8 |
| Utrecht | 16-3-2014 | Sunday | 44410 | 13.0 | n.a. | 608 | 38 | 12.1 |
| Utrecht | 17-3-2014 | Monday | 45600 | 12.6 | n.a. | 670 | 36 | 10.6 |

*n.a.: not available*

*Note: In 2014, the monitoring of Amsterdam started one day later due to malfunction of the sampler and was extended one day. For Eindhoven on day 2 a malfunction of the autosampler prevented from taking a sample. In this case no extension of the sampling period was made.*

**Table D3**. Characteristics WWTP Amsterdam, Eindhoven and Utrecht in 2015

| City | date 24/h sample taken | | Flow $m^3$/24h | sampling interval min | BOD mg/L | COD mg/L | Ntot mg/L | Ptot mg/L |
|---|---|---|---|---|---|---|---|---|
| Amsterdam | 4-3-2015 | Wednesday | 189509 | 2.7 | 250 | 600 | 59 | 7.8 |
| Amsterdam | 5-3-2015 | Thursday | 158867 | 3.2 | n.a. | n.a. | n.a. | n.a. |
| Amsterdam | 6-3-2015 | Friday | 157713 | 3.2 | n.a. | n.a. | n.a. | n.a. |
| Amsterdam | 7-3-2015 | Saturday | 154819 | 3.3 | 270 | 570 | 65 | 8.3 |
| Amsterdam | 8-3-2015 | Sunday | 154340 | 3.3 | n.a. | n.a. | n.a. | n.a. |
| Amsterdam | 9-3-2015 | Monday | 156486 | 3.2 | n.a. | n.a. | n.a. | n.a. |
| Amsterdam | 10-3-2015 | Tuesday | 156810 | 3.2 | 280 | 620 | 65 | 8.2 |
| Eindhoven | 4-3-2015 | Wednesday | 130195 | 8.8 | n.a. | n.a. | n.a. | n.a. |
| Eindhoven | 5-3-2015 | Thursday | 119362 | 9.7 | n.a. | n.a. | n.a. | n.a. |
| Eindhoven | 6-3-2015 | Friday | 116331 | 9.9 | n.a. | n.a. | n.a. | n.a. |
| Eindhoven | 7-3-2015 | Saturday | 120548 | 9.6 | n.a. | n.a. | n.a. | n.a. |
| Eindhoven | 8-3-2015 | Sunday | 122700 | 9.4 | n.a. | n.a. | n.a. | n.a. |
| Eindhoven | 9-3-2015 | Monday | 132945 | 8.7 | n.a. | 510 | 56 | 11 |
| Eindhoven | 10-3-2015 | Tuesday | 139752 | 8.2 | n.a. | n.a. | n.a. | n.a. |
| Utrecht | 4-3-2015 | Wednesday | 47740 | 12.1 | n.a. | 530 | 41 | 8.9 |
| Utrecht | 5-3-2015 | Thursday | 45030 | 12.8 | n.a. | 811 | 56 | 9.9 |
| Utrecht | 6-3-2015 | Friday | 49530 | 11.6 | n.a. | 530 | 42 | 10.3 |
| Utrecht | 7-3-2015 | Saturday | 46030 | 12.5 | n.a. | 568 | 39 | 9.2 |
| Utrecht | 8-3-2015 | Sunday | 46900 | 12.3 | n.a. | 598 | 43 | 9.7 |
| Utrecht | 9-3-2015 | Monday | 45970 | 12.5 | n.a. | 648 | 41 | 9.1 |
| Utrecht | 10-3-2015 | Tuesday | 44580 | 12.9 | n.a. | 524 | 39 | 9.7 |

*n.a.: not available*

**Table D4**. Characteristics WWTP Amsterdam in 2012, during a festival event

| City | date 24/h sample taken | | Flow $m^3$/24h | sampling interval min | BOD mg/L | COD mg/L | Ntot mg/L | Ptot mg/L |
|---|---|---|---|---|---|---|---|---|
| Amsterdam | 2-8-2012 | Thursday | 302680 | 1.7 | n.a. | n.a. | n.a. | n.a. |
| Amsterdam | 3-8-2012 | Friday | 154200 | 3.3 | n.a. | n.a. | n.a. | n.a. |
| Amsterdam | 4-8-2012 | Saturday | 158270 | 3.2 | n.a. | n.a. | n.a. | n.a. |
| Amsterdam | 5-8-2012 | Sunday | 148710 | 3.4 | n.a. | n.a. | n.a. | n.a. |
| Amsterdam | 7-8-2012 | Tuesday | 194730 | 2.6 | n.a. | n.a. | n.a. | n.a. |
| Amsterdam | 8-8-2012 | Wednesday | 139570 | 3.6 | n.a. | n.a. | n.a. | n.a. |

*n.a.: not available*

**Table D5**. Characteristics WWTP Amsterdam in 2014, during a festival event

| City | date 24/h sample taken | | Flow $m^3$/24h | sampling interval min | BOD mg/L | COD mg/L | Ntot mg/L | Ptot mg/L |
|---|---|---|---|---|---|---|---|---|
| Amsterdam | 30-7-2014 | Wednesday | 156376 | 3.2 | n.a. | n.a. | n.a. | n.a. |
| Amsterdam | 31-7-2014 | Thursday | 145768 | 3.5 | 210 | 480 | 52 | 7,0 |
| Amsterdam | 1-8-2014 | Friday | 141565 | 3.6 | n.a. | n.a. | n.a. | n.a. |
| Amsterdam | 2-8-2014 | Saturday | 184245 | 2.7 | n.a. | n.a. | n.a. | n.a. |
| Amsterdam | 3-8-2014 | Sunday | 138248 | 3.6 | 230 | 500 | 53 | 6,8 |
| Amsterdam | 4-8-2014 | Monday | 159048 | 3.2 | n.a. | n.a. | n.a. | n.a. |
| Amsterdam | 5-8-2014 | Tuesday | 134828 | 3.7 | n.a. | n.a. | n.a. | n.a. |

*n.a.: not available*

**Table D6.** Loads of sildenafil and two of its transformation products calculated for the WWTPs of the cities of Amsterdam, Eindhoven and Utrecht in 2013

| **2013** | | day | T °C | sildenafil | desmethylsildenafil | desethylsildenafil |
|---|---|---|---|---|---|---|
| | | | mean | load mg/day/1000 inh | load mg/day/1000 inh | load mg/day/1000 inh |
| | | | Schiphol | | | |
| Amsterdam | Wednesday | 6-3-2013 | 10.8 | 2.9 | 5.7 | 7.8 |
| Amsterdam | Thursday | 7-3-2013 | 8.3 | 2.5 | 3.5 | 4.4 |
| Amsterdam | Friday | 8-3-2013 | 8.1 | 2.9 | 3.3 | 4.6 |
| Amsterdam | Saturday | 9-3-2013 | 3.7 | 5.4 | 2.3 * | 10.2 |
| Amsterdam | Sunday | 10-3-2013 | 0.2 | 3.5 | 3.6 | 4.7 |
| Amsterdam | Monday | 11-3-2013 | -1.7 | 3.1 | 4.1 | 5.2 |
| Amsterdam | Tuesday | 12-3-2013 | -2.0 | 3.8 | 4.9 | 7.4 |
| | | | Eindhoven | | | |
| Eindhoven | Wednesday | 6-3-2013 | 10.7 | 2.0 | <LOD | <LOD |
| Eindhoven | Thursday | 7-3-2013 | 11.0 | 3.7 | <LOD | 4.3 |
| Eindhoven | Friday | 8-3-2013 | 11.6 | 6.4 | <LOQ | 6.9 |
| Eindhoven | Saturday | 9-3-2013 | 6.8 | 3.8 | <LOD | 6.1 |
| Eindhoven | Sunday | 10-3-2013 | 0.7 | 3.2 | <LOD | 1.3 |
| Eindhoven | Monday | 11-3-2013 | -2.1 | 3.9 | <LOD | 3.3 |
| Eindhoven | Tuesday | 12-3-2013 | -3.7 | 3.1 | <LOQ | 4.1 |
| | | | De Bilt | | | |
| Utrecht | Wednesday | 6-3-2013 | 11.2 | 1.6 | <LOD | 1.6 |
| Utrecht | Thursday | 7-3-2013 | 8.9 | 1.8 | <LOQ | 1.2 |
| Utrecht | Friday | 8-3-2013 | 9.1 | 2.0 | <LOD | 2.8 |
| Utrecht | Saturday | 9-3-2013 | 4.7 | 4.7 | <LOD | <LOD |
| Utrecht | Sunday | 10-3-2013 | -0.1 | 2.5 | <LOQ | 0.8 |
| Utrecht | Monday | 11-3-2013 | -2.1 | 1.9 | <LOQ | 2.7 |
| Utrecht | Tuesday | 12-3-2013 | -2.5 | 2.4 | <LOQ | 3.4 |

*Note: according to Ort et al 2014 <LOQ is treated as follows: (1) if all values at a location for a certain compound were <LOQ, loads were set to zero; (2) if at least one value was >LOQ, values <LOQ were replaced with 0.5 × LOQ*
*<LOQ, concentration between LOD and LOQ (1)*
**, value between LOD and LOQ, value shown was calculated as 0.5*LOQ (2).*

**Table D7.** Loads of sildenafil and two of its transformation products calculated for the WWTPs of the cities of Amsterdam, Eindhoven and Utrecht in 2014

| **2014** | | day | T °C | sildenafil | desmethylsildenafil | desethylsildenafil |
|---|---|---|---|---|---|---|
| | | | mean | load mg/day/1000 inh | load mg/day/1000 inh | load mg/day/1000 inh |
| | | | Schiphol | | | |
| Amsterdam | Wednesday | 12-3-2014 | 8.8 | 6.1 | 8.4 | 15.9 |
| Amsterdam | Thursday | 13-3-2014 | 8.6 | 5.3 | 7.0 | 14.1 |
| Amsterdam | Friday | 14-3-2014 | 6.3 | 5.4 | 7.3 | 13.7 |
| Amsterdam | Saturday | 15-3-2014 | 9.6 | 6.6 | 8.1 | 14.7 |
| Amsterdam | Sunday | 16-3-2014 | 10.2 | 8.2 | 8.8 | 17.5 |
| Amsterdam | Monday | 17-3-2014 | 10.1 | 5.7 | 8.8 | 14.6 |
| Amsterdam | Tuesday | 18-3-2014 | 9.4 | 6.5 | 10.5 | 19.6 |
| | | | Eindhoven | | | |
| Eindhoven | Tuesday | 11-3-2014 | 9.6 | 3.6 | 5.8 | 13.4 |
| Eindhoven | Thursday | 13-3-2014 | 8.9 | 2.7 | 4.3 | 10.3 |
| Eindhoven | Friday | 14-3-2014 | 7.6 | 3.5 | 4.9 | 8.2 |
| Eindhoven | Saturday | 15-3-2014 | 9.6 | 3.4 | 4.9 | 9.7 |
| Eindhoven | Sunday | 16-3-2014 | 9.9 | 5.4 | 6.7 | 10.9 |
| Eindhoven | Monday | 17-3-2014 | 9.2 | 4.0 | 6.2 | 13.9 |
| | | | De Bilt | | | |
| Utrecht | Tuesday | 11-3-2014 | 9.3 | 3.8 | 5.7 | 9.5 |
| Utrecht | Wednesday | 12-3-2014 | 8.3 | 3.2 | 4.5 | 8.6 |
| Utrecht | Thursday | 13-3-2014 | 7.9 | 3.6 | 3.0 | 7.6 |
| Utrecht | Friday | 14-3-2014 | 6.7 | 2.5 | 2.6 | 6.8 |
| Utrecht | Saturday | 15-3-2014 | 9.9 | 3.1 | 3.3 | 7.3 |
| Utrecht | Sunday | 16-3-2014 | 10.5 | 2.8 | 3.1 | 6.9 |
| Utrecht | Monday | 17-3-2014 | 10.2 | 2.7 | 2.8 | 7.9 |

**Table D8.** Loads of sildenafil and two of its transformation products calculated for the WWTPs of the cities of Amsterdam, Eindhoven and Utrecht in 2015

| **2015** | | day | T °C | sildenafil | desmethylsildenafil | desethylsildenafil |
|---|---|---|---|---|---|---|
| | | | mean | load mg/day/1000 inh | load mg/day/1000 inh | load mg/day/1000 inh |
| | | | Schiphol | | | |
| Amsterdam | Wednesday | 4-3-2015 | 5.4 | 7.3 | 11.8 | 19.7 |
| Amsterdam | Thursday | 5-3-2015 | 5.4 | 6.0 | 10.4 | 18.0 |
| Amsterdam | Friday | 6-3-2015 | 6.4 | 6.3 | 8.6 | 17.3 |
| Amsterdam | Saturday | 7-3-2015 | 7.7 | 6.8 | 10.2 | 17.9 |
| Amsterdam | Sunday | 8-3-2015 | 8.2 | 8.3 | 10.6 | 19.2 |
| Amsterdam | Monday | 9-3-2015 | 8.0 | 6.3 | 13.3 | 21.6 |
| Amsterdam | Tuesday | 10-3-2015 | 7.1 | 6.2 | 13.6 | 20.9 |
| | | | Eindhoven | | | |
| Eindhoven | Wednesday | 4-3-2015 | 4.4 | 1.8 | 2.6 * | 8.3 |
| Eindhoven | Thursday | 5-3-2015 | 4.3 | 2.1 | 2.4 * | 9.0 |
| Eindhoven | Friday | 6-3-2015 | 5.5 | 2.2 | 2.3 * | 9.9 |
| Eindhoven | Saturday | 7-3-2015 | 7.8 | 2.8 | 5.6 | 10.4 |
| Eindhoven | Sunday | 8-3-2015 | 9.9 | 3.2 | 2.5 * | 11.8 |
| Eindhoven | Monday | 9-3-2015 | 8.5 | 2.7 | 2.7 * | 13.5 |
| Eindhoven | Tuesday | 10-3-2015 | 8.4 | 3.4 | 5.8 | 14.5 |
| | | | De Bilt | | | |
| Utrecht | Wednesday | 4-3-2015 | 5.1 | 1.7 | 1.4 * | 7.5 |
| Utrecht | Thursday | 5-3-2015 | 5.7 | 2.4 | 1.4 * | 7.1 |
| Utrecht | Friday | 6-3-2015 | 6.6 | 2.5 | 1.5 * | 7.9 |
| Utrecht | Saturday | 7-3-2015 | 8.4 | 2.3 | 1.4 * | 8.2 |
| Utrecht | Sunday | 8-3-2015 | 9.3 | 3.6 | 2.9 | 8.5 |
| Utrecht | Monday | 9-3-2015 | 8.8 | 2.2 | 3.6 | 8.6 |
| Utrecht | Tuesday | 10-3-2015 | 7.6 | 1.6 | 2.9 | 8.0 |

*Note: according to Ort et al 2014 <LOQ is treated as follows: (1) if all values at a location for a certain compound were <LOQ, loads were set to zero; (2) if at least one value was >LOQ, values <LOQ were replaced with 0.5 × LOQ <LOQ, concentration between LOD and LOQ (1)*

**, value between LOD and LOQ, value shown was calculated as 0.5*LOQ (2).*

**Table D9**. Loads of sildenafil and two of its transformation products calculated for the WWTPs of the cities of Amsterdam in 2012, during a festival event

| City | day | | sildenafil | desmethylsildenafil | desethylsildenafil |
|---|---|---|---|---|---|
| | | | load g/day | load g/day | load g/day |
| Amsterdam | 2-8-2012 | Thursday | 80.4 * | <LOD | 14.9 |
| Amsterdam | 3-8-2012 | Friday | 8.5 | 3.6 | 9.5 |
| Amsterdam | 4-8-2012 | Saturday | 5.2 | 4.1 | 10.5 |
| Amsterdam | 5-8-2012 | Sunday | 6.7 | 5.2 | 12.7 |
| Amsterdam | 7-8-2012 | Tuesday | 6.3 | 5.1 | 13.4 |
| Amsterdam | 8-8-2012 | Wednesday | 4.2 | 4.8 | 12.6 |

**, outlier*

**Table D10**. Loads of sildenafil and two of its transformation products calculated for the WWTPs of the cities of Amsterdam in 2014, during a festival event

| City | day | | sildenafil | desmethylsildenafil | desethylsildenafil |
|---|---|---|---|---|---|
| | | | load g/day | load g/day | load g/day |
| Amsterdam | 30-7-2014 | Wednesday | 4.5 | 4.9 | 9.1 |
| Amsterdam | 31-7-2014 | Thursday | 5.7 | 6.9 | 10.8 |
| Amsterdam | 1-8-2014 | Friday | 5.5 | 5.3 | 10.5 |
| Amsterdam | 2-8-2014 | Saturday | 8.1 | 8.5 | 16.0 |
| Amsterdam | 3-8-2014 | Sunday | 7.0 | 6.8 | 11.2 |
| Amsterdam | 4-8-2014 | Monday | 12.2 | 10.9 | 17.3 |
| Amsterdam | 5-8-2014 | Tuesday | 9.1 | 10.5 | 16.8 |

# 3.2.

# Success of rogue online pharmacies: sewage study of sildenafil in the Netherlands

B.J. Venhuis, P. de Voogt, E. Emke, A. Causanilles, P.H.J. Keizers

BMJ 2014; 349: g4317

DOI: 10.1136/bmj.g4317

The internet continues to harbour thousands of rogue online pharmacies (Wise, 2013). We investigated the success of their practice by measuring the sewage load of the erectile dysfunction drug sildenafil in three cities in the Netherlands.

We measured concentrations of sildenafil and two metabolites at the three main sewage treatment plants serving Amsterdam, Eindhoven, and Utrecht for seven consecutive days (Nieto et al., 2010). Total sewage load was back calculated to original sildenafil consumption (Muirhead et al., 2002a). For a conservative estimate, we assumed that there were no losses in sewage due to degradation (Baker and Kasprzyk-Hordern, 2011b). We found no indication of dumping of unused drugs.

We estimated the consumption of legitimately dispensed sildenafil from the national dispensary database from 12 months before the study to three months after. We used the repetitive dispensing behaviour for each patient in the sampled area to estimate consumption during the seven days of sampling. About a quarter of the dispensed sildenafil was prescribed to treat pulmonary hypertension.

**Fig. 3.2.1**. shows that at least 60% of the sewage loads of sildenfil were not explained by legitimately prescribed sildenafil. The illicit fraction was similar for each city, despite major differences in tourism and commuting.

We conclude that the unexplained fraction of sildenafil in sewage is primarily illicit. If our results are representative of other communities, the consumption of illicit erectile dysfunction drugs might dwarf the consumption of the legitimately dispensed versions. The apparent success of rogue online pharmacies would be an important area of further inquiry.

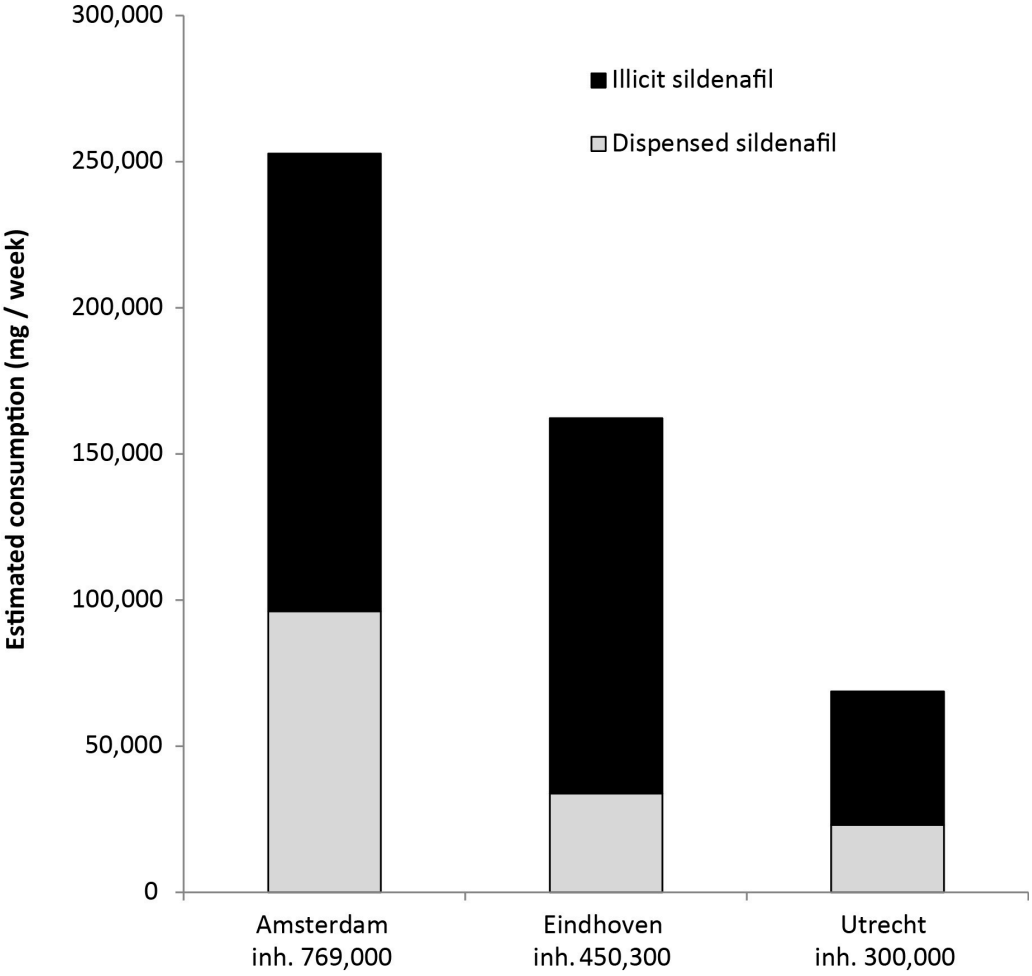


**Fig. 3.2.1.** Estimated sildenafil consumption in three cities in the Netherlands by type of prescription (illicit and dispensed)

# Annex E – Long rapid response

**Re: Record number of fake drugs are seized in crackdown**

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The record number of fake drugs seized under Operation Pangea VI is just the tip of the iceberg. At present, we don't really know the size of the European market for illicit medicines, neither do we know much about public exposure, let alone the incurred health damage. We therefore set out to estimate the actual use of illicit medicines by using sewage epidemiology. Sewage epidemiology is an established science for monitoring drugs of abuse (Castiglioni et al., 2013). It involves the back calculation of drugs and their metabolites in sewage to their corresponding original intake. When applying this methodology to illegal medicines the major difference is having to account for the fraction of legitimately dispensed medication. We selected sildenafil (Viagra®, Revatio®) as a model compound as it is the most prevalent drug in seized illicit medicines (Venhuis and de Kaste, 2012). At the time of this study, generic sildenafil was not legally available.

To estimate the legitimate use of sildenafil we used the Dutch national dispensary database that covers 97% of all legitimate sales. For each patient in the sampled areas, the milligrams of sildenafil purchased were divided by the number of days until the next purchase. When a patient would not return after a purchase his own average consumption was used for his last purchase. When a patient would not return after a first purchase, the city populations' average consumption was assumed. The individual data were summed to provide the average daily consumption from 12 months prior to the study until 3 months after. Because Viagra® is taken as needed, and mostly around the weekend, we used a 7-day average consumption for the sampling week (Wednesday – Tuesday). Revatio® is used daily and accounts for about 25% of the legitimate sildenafil consumption. The use of sildenafil in hospitals was ignored as it was negligible.

Sewage sampling was performed at the sewage treatment plants (STPs) for Amsterdam (769.000 inh.), Eindhoven (450.300 inh.) and Utrecht (300.000 inh.). Flow proportional samples were taken about every 10 minutes and were analysed as 24h-average samples. Sampling started on a Wednesday to catch the expected peak levels for sildenafil at the weekend. The STPs for Amsterdam and Utrecht serve most of the cities whereas the STP for Eindhoven serves the city as well as its surrounding communities. It takes excreta several hours to reach the sewage treatment plant depending on the sewage system (Amsterdam (±12h), Eindhoven (±3h), and Utrecht (±6h)). Sildenafil and its metabolites UK-103,320 and UK-150,564 were identified and quantified in all 21 samples.

To estimate the actual use of sildenafil, sewage loads were back calculated according to literature data (Muirhead et al., 2002a). This shows that in fasted men, about 92% of oral sildenafil is absorbed after which it is prone to rapid metabolism. About 25% of the original dose (equivalent to 27% of the absorbed dose) is excreted as UK-103,320 and UK-150,564. Although this study does not report the excretion of unchanged sildenafil, it is reported in the sewage of several cities (Nieto et al., 2010; Schröder and Thevis, 2010). As stable ratios of the three analytes ruled out significant dumping of unused medication we concluded that, in the general population, sildenafil is excreted unchanged indeed. Thus, the actual sildenafil consumption was estimated by summing the sewage load of unchanged sildenafil and the absorbed dose. The absorbed dose was back calculated from the metabolite load using the formula: [(Load UK-103,320 (moles) + Load UK-150,564 (moles)) / 0.27] * 474. For a conservative estimate, we assumed there were no losses of compounds in sewage due to degradation. The unchanged sildenafil in Amsterdam, Eindhoven and Utrecht respectively accounted for 10%, 9% and 10% of the estimated actual consumption, matching the oral absorbance reported in literature.

It is our conservative estimate that a considerable fraction of the sewage loads cannot be explained by legitimately prescribed sildenafil: Amsterdam (61%), Eindhoven (79%), and Utrecht (66%). Tourism appears to play a limited role as Amsterdam proportionally receives most tourists, yet has the lowest unexplained fraction. Commuting may have some influence as Eindhoven has the highest unexplained fraction and its STP also serves the surrounding communities where most of its commuters live. Nevertheless, the unexplained fraction of sildenafil is primarily

ascribed to the use of illicit sildenafil[1]. If this is representative of other communities, the consumption of illicit erectile dysfunction drugs might dwarf the consumption of the legitimately dispensed versions. Therefore, the apparent success of rogue online pharmacies would be an important area of further inquiry.

[1] To check the methodology, it was also applied to methylphenidate (e.g. Ritalin®). We did not expect to find an illicit fraction as illicit methylphenidate is rarely seized. This was confirmed based on the ritalinic acid levels in sewage and the estimated consumption from dispension records.

# 3.3.

# Comparison of phosphodiesterase type V inhibitors use in eight European cities through analysis of urban wastewater

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# Abstract

In this work, a step forward in investigating the use of prescription drugs, namely erectile dysfunction products, at European level was taken by applying the wastewater-based epidemiology approach. 24-h composite samples of untreated wastewater were collected at the entrance of eight wastewater treatment plants serving the catchment within the cities of Bristol, Brussels, Castellón, Copenhagen, Milan, Oslo, Utrecht and Zurich. A validated analytical procedure with direct injection of filtered aliquots by liquid chromatography-tandem mass spectrometry was applied. The target list included the three active pharmaceutical ingredients (sildenafil, tadalafil and vardenafil) together with (bio)transformation products and other analogues. Only sildenafil and its two human urinary metabolites desmethyl- and desethylsildenafil were detected in the samples with concentrations reaching 60 ng $L^{-1}$. In addition, national prescription data from five countries was gathered in the form of the number of prescribed daily doses. The data were transformed into predicted concentrations and compared to the measured concentrations in wastewater. The prediction was satisfactory in one out of the five cases for sildenafil. The back-calculation to sildenafil consumption from measured concentrations in wastewater resulted in the evidence of a different spatial trend across Europe. In Utrecht and Brussels prescription data could only partly explain the total amount found in wastewater; whereas in Bristol, the comparison was in agreement; and in Milan and Oslo a lower amount was found in wastewater than expected from the prescription data. This study demonstrated the potential of wastewater-based epidemiology to investigate the use of counterfeit medication and rogue online pharmacy sales.

# 3.3.1. Introduction

The chemical analysis of raw wastewater with advanced mass spectrometry techniques allows for the determination of human urinary biomarkers when these are excreted in sufficient concentrations and remain stable on their way along the sewer system (Castiglioni et al., 2013).The finding of specific biomarkers may reveal valuable near real-time information regarding a population's lifestyle, illness and exposure to external agents. Successful studies thus far have revealed the population's level of oxidative stress (Y. Ryu et al., 2016), its exposure to pesticides (Rousis et al., 2017), and to phthalate plasticizers (González-Mariño et al., 2017), its consumption of legal substances such as alcohol, nicotine or caffeine (J.A. Baz-Lomba et al., 2016; E. Gracia-Lor et al., 2017; Yeonsuk Ryu et al., 2016), its use of illicit drugs (Causanilles et al., 2017a, 2017c; Ort et al., 2014) and other psychoactive substances (Bade et al., 2017; Castrignanò et al., 2017; Causanilles et al., 2017b; González-Mariño et al., 2016a), and its intake of certain pharmaceuticals (Causanilles et al., 2016).

The monitoring of active pharmaceutical ingredients (APIs) and their metabolites in wastewater offers an interesting value (van Nuijs et al., 2015) because these substances have gone through clinical trials before their final usage approval. Therefore, the information regarding the absorbed dose after drug intake, the biotransformation pathway and the excretion profile and rates in biological matrices is relatively well known (Abed, 2014). This information facilitates the selection of the appropriate target urinary biomarker in the application of wastewater-based epidemiology (WBE). Concentrations in untreated wastewater, considered a collective, diluted pooled urine sample, are then obtained as measured concentration (MC) of the unchanged product and/or its metabolites, which can be converted into mass loads and then back-calculated into consumption estimates applying the appropriate correction factor. In addition, the number of dispensed pharmaceutical in the form of defined daily doses (DDD) or product quantities dispensed by pharmacies or doctors can be obtained (in most cases, depending on the pharmaceutical and the country legislation). From these data the average amount of the API that has been legally dispensed per day can be calculated and transformed into predicted concentration (PC) (Carballa et al., 2008; Verlicchi et al., 2014).

The comparison between prescription data and wastewater loads can result in three different scenarios:

(i) Consumption estimated from measured wastewater loads is lower than the load expected from the dispensed data. This would represent the case of pharmaceuticals under consumption, with a lower usage that the quantity prescribed or defined by the DDD;

(ii) Consumption estimated from measured wastewater loads is similar to the expected from dispensed data, which represents the ideal situation, where there is no misuse;

(iii) Consumption estimated from measured wastewater loads is higher than the load expected from the dispensed data;

This third scenario represents the case of pharmaceuticals that are genuine but available from parallel import or in a counterfeit or falsified form and that can be acquired from other sources such as rogue online pharmacies or black market. This was the case observed for the phosphodiesterase type V (PDE5) inhibitor sildenafil, API in erectile dysfunction pharmaceuticals, in a study performed in the Netherlands in 2013 (B J Venhuis et al., 2014a). Results showed that only one third to one half of the consumption estimated from wastewater loads could be related to the acquisition of the drug from legal sources (B J Venhuis et al., 2014a).

However, the comparison needs to be handled with care, since other sources for discrepancy can be present. They might be related to the sewer system, with the incomplete release to the sewer system or elimination processes between the consumption point and the wastewater treatment plant (WWTP), namely (bio)transformation, sorption and sedimentation (McCall et al., 2016b; Ramin et al., 2017, 2016; van Nuijs et al., 2015; Verlicchi et al., 2014). Alternatively, they could be related to other sources such as inaccurate or highly variable pharmacokinetic parameters between individuals, different applied dosages of the used API (which makes it difficult to compare it with a DDD), or no representative comparison (e.g. 1-week wastewater monitoring vs. monthly/yearly prescription data; national vs. local comparison).

Erectile dysfunction is estimated to affect 25 to 35 million men over the age of 18 in Europe, according to the European Federation of Pharmaceutical Industries and Associations (EFPIA, 2017). It is a disorder of increasing concern, since an aging population will result in higher prevalence. Despite the high number of men affected, it is still highly stigmatized, and users usually tend to hide their related drug use. Illegal trading with products from the internet and with counterfeit medicines is increasing (Chiang et al., 2017). However, the individuals purchasing medicines via the

internet are for the most part not sufficiently aware of the risks they run in doing so (Keizers et al., 2016). Concerns about the quality of these products may arise, specially towards the possible presence of impurities that may lead to poisoning if toxic, and an increased risk of side effects or overdosing.

In this work the WBE approach was applied to assess the use of PDE5 inhibitors in eight European cities accounting for almost 5 million inhabitant equivalents. 24-h composite influent wastewater samples were collected in each city for seven consecutive days and analysed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Measured concentrations in the samples were converted into mass loads and back calculated with known pharmacokinetic information to estimate consumption. In addition, available data at national level of the number of prescribed or dispensed erectile dysfunction pharmaceuticals were gathered to discuss their correlation.

# 3.3.2. Materials and methods

**Chemicals and materials**

The following analytes were selected in the study: sildenafil citrate, desmethylsildenafil, desethylsildenafil and noracetildenafil, purchased from LGC (Luckenwalde, Germany); vardenafil dihydrochloride, n-desethyl vardenafil, tadalafil, aminotadalafil, chloropretadalafil and n-octyl nortadalafil, purchased from TRC Toronto Research Chemicals Inc. (Ontario, Canada). Two isotopically labeled internal standards (ILIS) were used as surrogates: sildenafil-$d_8$ and desmethylsildenafil-$d_8$, supplied by TLC Pharmachem (Ontario, Canada). All the above-mentioned standards were of high purity grade (>98%). Individual stock and working solutions were prepared in methanol and stored at -20 °C. Calibration curve was prepared daily by diluting with ultrapure water to a final composition water:methanol (90:10, v/v).

Methanol and acetonitrile HPLC grade solvents were supplied by Avantor Performance Materials B.V (Deventer, the Netherlands). Formic acid (50% in water) was obtained from Fluka Analytical (Sigma-Aldrich, Stenheim, Germany). The ultrapure water was obtained by purifying demineralized water in an Elga Purelab Chorus ultrapure water system (High Wycombe, United Kingdom). Regenerated cellulose filters RC 0.2 µm were purchased from Phenomenex (Torrance, USA).

**Sample collection**

A week-monitoring sampling campaign was performed in March 2015 in eight European cities. For seven consecutive days 24-h influent composite samples were collected at the entrance of the WWTPs serving the cities of Bristol, England; Brussels, Belgium; Castellón, Spain; Copenhagen, Denmark; Milan, Italy; Oslo, Norway; Utrecht, the Netherlands; and Zurich, Switzerland. The number of inhabitants included in the total catchment area under study represented almost 5 million people in Europe. **Table F1** compiles detailed information about the sample collection at the different locations: date of sample collection, influent flow ($m^3$ $day^{-1}$), sampling mode and frequency, average wastewater temperature (°C), pH, biological and chemical oxygen demand ($BOD_5$ and COD), total phosphate ($P_{tot}$), and nitrogen content as Kjeldahl ($N_{tot}$) and ammonia ($NH_4$-N).

**Analytical methodology**

The analytical methodology used to perform the wastewater chemical analysis was previously validated (Causanilles et al., 2016). All samples were collected in high-density polyethylene bottles, shipped frozen to KWR in Nieuwegein (NL) and stored in the dark at –20 °C until treatment. Samples were thawed and homogenized. Then a 10 mL aliquot was spiked with deuterated analogues to act as surrogate and filtered with regenerated cellulose syringe filters (0,2 µm). With no further pre-treatment, a 100 µL aliquot of each sample was injected into the liquid chromatography coupled to triple quadruple mass spectrometer (Thermo Scientific TSQ Vantage, Thermo Electron, Bremen, Germany). Chromatographic separation was achieved with a XBridge C18 column (150 mm × 2.1 mm I.D., particle size 3.5 µm, Waters, Etten-Leur, the Netherlands) preceded by a KrudKatcher ULTRA HPLC in-line SS filter (0.5 µm × 0.1 mm I.D., Phenomenex, Torrance, USA). The mobile phase consisted of an optimized water-methanol-acetonitrile gradient at 0.3 mL $min^{-1}$ flow. The MS system operated in selected reaction monitoring (SRM) and positive ionisation mode during data acquisition. For each compound two transitions of the precursor ion $[M+H]^+$ were monitored, one for quantification and the second for confirmation purposes. Analyte concentrations were quantified using calibration with standards in solvent and the correspondent deuterated analogue. Additional details of the analytical method can be found in the Supplementary information: **Table F2** presents the specific LC-MS/MS parameters for compound identification, **Table F3** presents the quality parameters of the method's performance, and **Fig. F1** presents the resulting chromatogram.

**Calculations**

PDE5 inhibitors are the API in pharmaceutical products used to treat erectile dysfunction (ED) and as pulmonary vasodilator antihypertensive (VA). Their classification within the ATC-system (Anatomic Therapeutic Chemical) corresponds to the group of genitourinary system and sex hormones (G), urological (04B), erectile dysfunction (E). The individual codes are necessary to find the national prescription and sales data of all formulations containing them as API despite the differences in brand name. The codes of the three approved substances included in the study and their established DDDs can be found in **Table 3.3.1**. DDD is defined as the assumed average maintenance dose per day for a drug used for its main indication in adults (WHO, 2017). Sildenafil does not only have a registration as erectile stimulant, but also for pulmonary arterial hypertension. For this treatment purpose, both the DDD

and the number of prescriptions is lower. In the case of Belgium, only the prescription data for the application of sildenafil as VA was available. A similar trend in the prescription data was expected compared to the neighbouring country of the Netherlands and therefore the ratio ED/VA was extrapolated to estimate the number of prescriptions of sildenafil as erectile dysfunction drug in Belgium.

The number of DDDs prescribed in the year 2015 (see **Table 3.3.1**) were multiplied by the DDD value in mg and converted into kg $year^{-1}$. The kg of each API were multiplied by the urinary excretion factor (%) and divided by the country's population, and the L $day^{-1}$ $inh^{-1}$ estimated from the influent flow at the wastewater treatment plants included in the study (as a way of measuring the water use per inhabitant, and multiplied by 365 to estimate the yearly use) (Carballa et al., 2008; Verlicchi et al., 2014). The excretion factors used in the calculation were gathered from different pharmacokinetic studies. According to Muirhead and colleagues (Muirhead et al., 2002a) sildenafil is not excreted unchanged in urine, however in previous work it was found to account for up to a 10% in wastewater (Causanilles et al., 2016). The excretion ratios for its human urinary metabolites desmethyl- and desethylsildenafil were 3 and 22 % respectively (Muirhead et al., 2002a). In the case of tadalafil, only a minor amount is excreted unchanged in urine (Phillips et al., 2004). In the case of vardenafil, approximately 0.7 – 3 % is excreted unchanged in urine, and its major metabolite component formed by N-deethylation, up to 5 % (EMA, 2005). PCs were obtained in ng $L^{-1}$ (see **Eq. F1**).

The chemical analysis of the wastewater samples included in the study resulted in the MC of each of the analytes in ng $L^{-1}$. The ratio PC/MC was calculated to evaluate the accuracy of the predictions (Verlicchi et al., 2014).

The loads were obtained by multiplying the measured concentration in each sample in ng $L^{-1}$ by the daily influent flow rate at the WWTP in $m^3$ $day^{-1}$. Loads, expressed as mg $day^{-1}$, were normalized dividing them by the population included in the catchment area. Normalized loads were expressed as mg $day^{-1}$ per 1000 inhabitants, allowing in this way the direct comparison of results among the different communities included in the study. In the case of concentration values in real sample below limits of quantification (LOQ), values were replaced by 0.5 × LOQ when at least one day in the week had a concentration value above the LOQ. Concentration values below limits of detection (LOD), as well as concentration values lower than LOQ when all values at that location were below LOQ, were set to 0.5 × LOD (Ort et al., 2014). Sildenafil consumption was estimated from mass loads as indicated elsewhere (B J Venhuis et

al., 2014b). The calculation was based on the available pharmacokinetic data and the assumption that there were no elimination processes such as (bio)transformation or sorption between the consumption point to the WWTP or dumping of unused drugs. Further research of the biomarkers' behaviour in the sewer (see the introduction) would be required to verify this assumption. Earlier stability studies confirmed there was not a statistically significant decrease in concentration of the target compounds after 48 h storage at 4 °C (Causanilles et al., 2016). According to Lai et al., 2015, 2011, the uncertainties related to flow measurements needed for converting MC into mass loads account for less than 20% in intercity comparison studies.

Statistical analysis of the data, using ANOVA to compare differences between cities and between weekdays and weekends was performed using GraphPad Prism 5.

**Table 3.3.1**. Information of the investigated pharmaceuticals and national prescription data.

| Pharmaceutical | ATC code | DDD[a] value (use) | Total number of DDDs prescribed in 2015 | | | | |
|---|---|---|---|---|---|---|---|
| | | | Belgium[1] | England[2] | Italy[3] | the Netherlands[4] | Norway[5] |
| Sildenafil | G04BE03 | 50 mg (ED)<br>20 mg (VA) | 602,596[b] (ED)<br>106,648 (VA) | 23,572,110 (ED)<br>198,800 (VA) | 13,314,239 (ED+VA) | 2,190,688 (ED)<br>387,710 (VA) | 1,949,770 (ED+VA) |
| Tadalafil | G04BE08 | 10 mg (ED) | 85,276 | 9,120,725 | 13,314,239 | 1,570,918 | 2,203,956 |
| Vardenafil | G04BE09 | 10 mg (ED) | n.a. | 1,262,350 | n.a. | 159,520 | 338,096 |

n.a. not available

# 3.3.3. Results and discussion

**Measured and predicted concentrations**

Results from the week-monitoring sampling campaign are reported in **Table 3.3.2**, together with the LODs and LOQs. Measured concentrations per city are presented as the 7-day mean with standard deviation, expressed in ng $L^{-1}$. Sildenafil and its two human metabolites were present at levels above the LOD in all cities and could be quantified in most of the samples. The parent compound was detected at a level between LOD and LOQ in the samples from Castellón and Milan, while in the city of Oslo it was at about the LOQ level only in the Sunday sample. When sildenafil was quantifiable, its concentrations were in the range of 4 to 19 ng $L^{-1}$. Desmethylsildenafil, the less abundant sildenafil metabolite, could not be quantified in the cities of Castellón, Milan, Oslo and Zurich. In Copenhagen and Utrecht on 2 and 4 days, respectively, levels were <LOQ, and these were therefore replaced by 0.5 × LOQ for the calculation of the city's average. Values were found in the range of 14 to 36 ng $L^{-1}$. Desethylsildenafil, the most abundant metabolite of sildenafil, was quantified in all samples, with concentrations between 5 and 51 ng $L^{-1}$. Neither the other two APIs included in the study, tadalafil and vardenafil, nor their metabolites nor analogues were found above their LOD.

The metabolite to parent concentration ratio was calculated when available. The ratio of desethylsildenafil to sildenafil ranged from 1.7 to 3.6 (6 cities, 2.8 ± 0.8). These results were in line with the range of ratios observed in the Dutch cities of Amsterdam, Eindhoven and Utrecht in the years 2013 to 2015 (Causanilles et al., 2016). The ratio of desmethylsildenafil to sildenafil ranged from 0.9 to 2.3 (4 cities, 1.6 ± 0.6). These results confirm literature findings: a lower ratio is expected for desmethylsildenafil, since it is the less abundant urinary metabolite (Muirhead et al., 2002a).

The PCs obtained for the unchanged API sildenafil and its two urinary metabolites desmethyl- and desethylsildenafil are presented in **Table 3.3.3** (the yearly prescribed kg are shown in **Table F4**). The highest PC was estimated for England, followed by the Netherlands, Norway and Italy with similar values, and the lowest was estimated for Belgium. PC was not calculated for tadalafil, since the literature indicates that only a minor amount of the unchanged form was putatively identified in urine. This would result in a PC close to zero, which is below the LOD in wastewater for this compound. PCs for vardenafil and its metabolite, N-desethylvardenafil, were calculated for the

countries with prescription data available, i.e. Norway, England and the Netherlands, although their presence in the wastewater was estimated to be minimal, below 1 ng $L^{-1}$ (see **Table F5**). This predicted value is lower than the LOD in wastewater.

**Table 3.3.2**. Measured concentrations (MCs) expressed in ng $L^{-1}$ with standard deviation (± SD) for 7 sampling days, n=7.

| Compounds | LOD ng $L^{-1}$ | LOQ ng $L^{-1}$ | MC (mean ± SD), ng $L^{-1}$ | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Bristol | Brussels | Castellón | Copenhagen | Milan | Oslo | Utrecht | Zurich |
| Sildenafil | 2 | 6 | 12 ± 4 | 19 ± 3 | + | 14 ± 5 | + | 4 ± 2[a] | 15 ± 4 | 9 ± 2 |
| Desmethylsildenafil | 5 | 18 | 26 ± 7 | 36 ± 2 | + | 19 ± 8[a] | + | + | 14 ± 4[a] | + |
| Desethylsildenafil | 1 | 2 | 28 ± 8 | 33 ± 5 | 13 ± 3 | 51 ± 7 | 5 ± 1 | 8 ± 4 | 51 ± 4 | 32 ± 5 |
| Noracetildenafil | 6 | 20 | - | - | - | - | - | - | - | - |
| Tadalafil | 2 | 8 | - | - | - | - | - | - | - | - |
| Aminotadalafil | 2 | 6 | - | - | - | - | - | - | - | - |
| Chloropretadalafil | 4 | 13 | - | - | - | - | - | - | - | - |
| N-octylnortadalafil | 30 | 100 | - | - | - | - | - | - | - | - |
| Vardenafil | 7 | 24 | - | - | - | - | - | - | - | - |
| N-desethylvardenafil | 9 | 30 | - | - | - | - | - | - | - | - |

[a] At least one value out of 7 is >LOQ; then the values <LOQ are replaced by 0.5 × LOQ
- = <LOD
+ = <LOQ

**Table 3.3.3**. Predicted concentrations (PCs) in wastewater influents for sildenafil and its two metabolites, expressed in ng $L^{-1}$.

| Country | PC ng $L^{-1}$ | | |
|---|---|---|---|
| | Sildenafil | Desmethylsildenafil | Desethylsildenafil |
| Belgium | 3.0 ± 0.4 | 0.9 ± 0.1 | 7 ± 1 |
| England | 25 ± 2 | 8 ± 1 | 56 ± 5 |
| Italy | 8 ± 1 | 2.3 ± 0.3 | 17 ± 2 |
| the Netherlands | 12.2 ± 0.4 | 3.7 ± 0.1 | 27 ± 1 |
| Norway | 11 ± 1 | 3.3 ± 0.4 | 24 ± 3 |

**Comparison between predicted and measured concentrations**

The PC/MC ratio was evaluated using the arbitrary criteria given below, in order to establish the accuracy of the prediction:

- If PC/MC > 2, then the PC is relatively high, indicative of case (i)
- If 0.5 < PC/MC < 2, then the PC is within reasonable boundaries of a value of 1, indicative of case (ii)
- If PC/MC < 0.5, then the PC is relatively low, indicative of case (iii)

The results are graphically presented in **Fig. 3.3.1**. The ratios for sildenafil and desethylsildenafil were only satisfactory in Utrecht (0.9 ± 0.2 and 0.5 ± 0.1 respectively), while for desmethylsildenafil they were satisfactory in Milan (0.9 ± 0.1) and Oslo (1.3 ± 0.1), although one should note that the MC was calculated as 0.5 × LOD to facilitate the ratio calculation, because in both cities the metabolite was found at levels below the LOQ in all samples.

Two observations can be made from the evaluation of the PC/MC ratios. One is that in the case of Brussels, the PCs of sildenafil are much lower than the actual concentrations measured in wastewater. Although this may have been caused by unregistered use of sildenafil (case (iii), see introduction), one should bear in mind that for the calculation of PC in this case the estimation of prescribed DDDs was obtained by extrapolation from the Dutch ED/VA trend, because actual DDD data were lacking. The actual ED/VA ratio for Belgium may be different of course. Another

possible reason for obtaining relatively low PCs, e.g. heavy rainfall during the sampling week, was discarded, as it did not occur. The second observation corresponds to the three cities Bristol, Milan and Oslo, where both sildenafil and desethylsildenafil show a high PC/MC ratio. This translates into lower measured concentrations than what is predicted from national prescription data. This could be explained by the non-consumption of the total prescribed amount or by any of the other sources of discrepancy mentioned in the introduction such as a higher (bio)transformation or sorption of the compounds in the local sewer systems, or a less representative comparison between local and national prescription data. We currently do not have evidence to substantiate the likeliness of higher rates of in-sewer degradation in these countries. Overall, the comparison results must be handled with care since this study was performed only in one city per country in a limited time period (7 consecutive days), and therefore the extrapolation of results to the whole country will be surely biased by the specific spatial and temporal profiles of that city (versus other areas within the countries).

For tadalafil and vardenafil the ratio PC/MC could not be calculated per se because of the minimal excretion ratios and non-detects in all wastewater samples. This fact is in agreement with what was predicted based on prescription data because these PCs would also fall below the actual limits of detection.

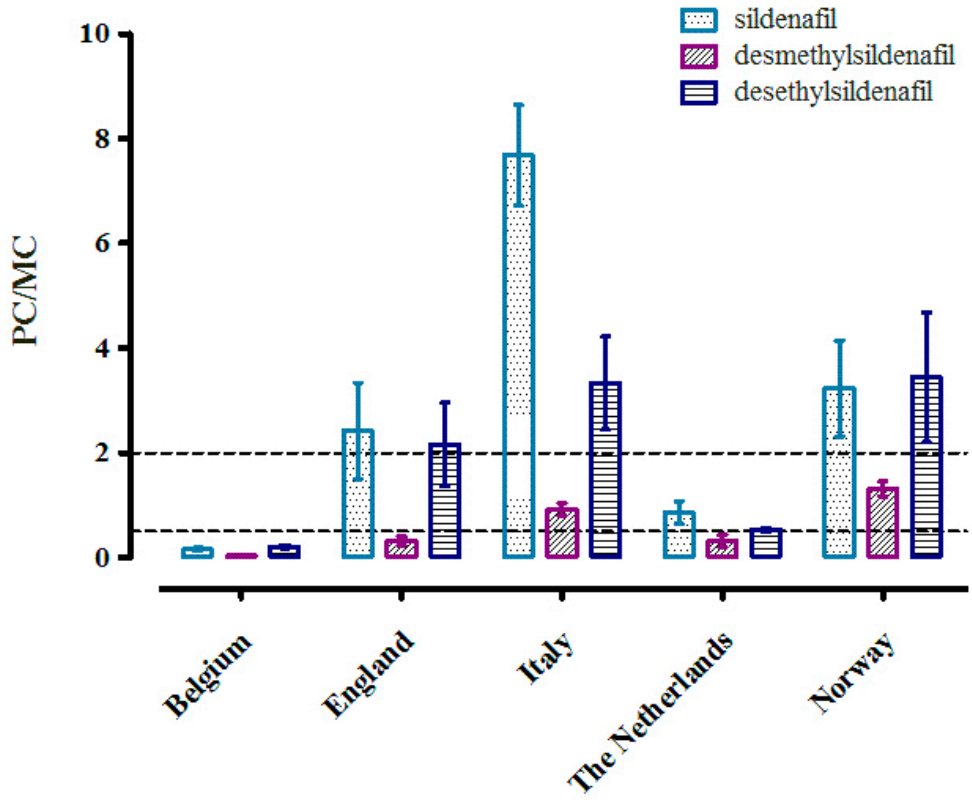


**Fig. 3.3.1**. Comparison of predicted (PC) and measured (MC) concentrations in influent wastewater, expressed as the PC/MC ratio. Dotted lines at y = 0.5 and 2 represent the criteria limits.

**Daily loads and back-calculated consumption**

MC were translated into normalized loads in mg $day^{-1}$ per 1000 inhabitants to allow a better comparison between the cities included in the study. The 7-day average data for each city together with standard deviation is presented in **Table 3.3.4**. The highest normalized sildenafil load was found in the city of Brussels closely followed by Zurich and Copenhagen. Compared to these cities, a medium load was found in Bristol and Utrecht, and the lowest levels were observed in Milan and Castellón. For the metabolites, a similar trend was found, in accordance with their excretion ratios. The daily variations are presented in **Fig. 3.3.2,** expressed as percentages of the total load. No statistically significant increase in loads was found in weekend samples compared to weekday samples, suggesting the use of sildenafil as needed and not with a clear recreational aim. The "weekend effect" is however very typical for some illicit drugs such as cocaine or ecstasy (MDMA) (Bijlsma et al., 2014b; Causanilles et al., 2017c; Salvatore et al., 2015). Interestingly, it can be seen in **Fig. 3.3.2** that in the case of sildenafil the highest load is detected on Sunday whereas for the two metabolites the maximum is detected on Monday. This could be explained by the metabolites being excreted later in time than the unchanged parent.

Considering the loads for sildenafil and its two metabolites, it is possible to back-calculate into sildenafil consumption by the population connected to the studied sewer system. This estimation was done as explained elsewhere (B J Venhuis et al., 2014b). The estimated consumption of sildenafil in mg $week^{-1}$ 1000 $inh^{-1}$ back-calculated from wastewater loads (see **Table 3.3.4**) arranged the cities in the following order from a higher to a lower estimated use (including previously published results from other Dutch cities (Causanilles et al., 2016): 1$^{st}$ Amsterdam, with 872 mg $week^{-1}$ 1000 $inh^{-1}$; 2$^{nd}$ Copenhagen; 3$^{rd}$ Brussels; 4$^{th}$ Zurich; 5$^{th}$ Eindhoven, 432 mg $week^{-1}$ 1000 $inh^{-1}$; 6$^{th}$ Bristol; 7$^{th}$ Utrecht; 8$^{th}$ Oslo; 9$^{th}$ Castellón; and 10$^{th}$ Milan.

Only in the case of Brussels (where the prescription data was estimated by extrapolating the Dutch trend) and Utrecht, the estimation of sildenafil consumption from wastewater-based approach was higher than what could be expected by the national prescription data. In the cities of Amsterdam and Eindhoven, previously reported results (Causanilles et al., 2016) showed an even higher consumption, that could not be explained by national sales data (at least 60% of the wastewater loads of sildenafil were not explained by legitimately prescribed sildenafil (B J Venhuis et al., 2014a). In Bristol the predicted and measured values were in good agreement, while in Milan and Oslo the estimated consumption from wastewater was lower than

expected by prescription data. The final evaluation of the correlation between wastewater data and prescription data was found to be non-significant by Spearman's correlation coefficient (ρ = -0.30) with p-value above 0.05 (p = 0.68) (see **Fig. 3.3.3**)

**Table 3.3.4.** Averaged normalized loads for sildenafil and its two metabolites expressed in mg $day^{-1}$ per 1000 $inh^{-1}$ with standard deviations (± SD) for 7 sampling days, and sildenafil estimated consumption from loads expressed in mg $week^{-1}$ 1000 $inh^{-1}$.

| | Loads (mean ± SD), mg $day^{-1}$ 1000 $inh^{-1}$ | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Bristol | Brussels | Castellón | Copenhagen | Milan | Oslo | Utrecht | Zurich |
| Sildenafil | 2.8 ± 1.1 | 5.1 ± 1.0 | 0.2 ± 0.1 [b] | 3.8 ± 1.2 | 0.4 ± 0.1 [b] | 1.7 ± 0.7 [a] | 2.4 ± 0.7 | 4.2 ± 1.5 |
| Desmethylsildenafil | 6.2 ± 1.7 | 9.4 ± 1.3 | 0.6 ± 0.1 [b] | 5.3 ± 1.9 [a] | 1.0 ± 0.2 [b] | 1.2 ± 0.1 [b] | 2.1 ± 0.9 [a] | 1.1 ± 0.2 [b] |
| Desethylsildenafil | 6.6 ± 2.1 | 8.5 ± 1.2 | 3.0 ± 0.6 | 13.7 ± 1.7 | 2.1 ± 0.5 | 3.7 ± 1.5 | 8.0 ± 0.5 | 13.9 ± 3.1 |
| Sildenafil estimated consumption (mg $week^{-1}$ 1000 $inh^{-1}$) | 365 | 517 | 100 | 542 | 87 | 145 | 292 | 439 |

[a] At least one value out of 7 is >LOQ then when <LOQ replaced by 0.5 × LOQ.

[b] All values <LOQ then replaced by 0.5 × LOD (SD was obtained from the different daily flow rate).

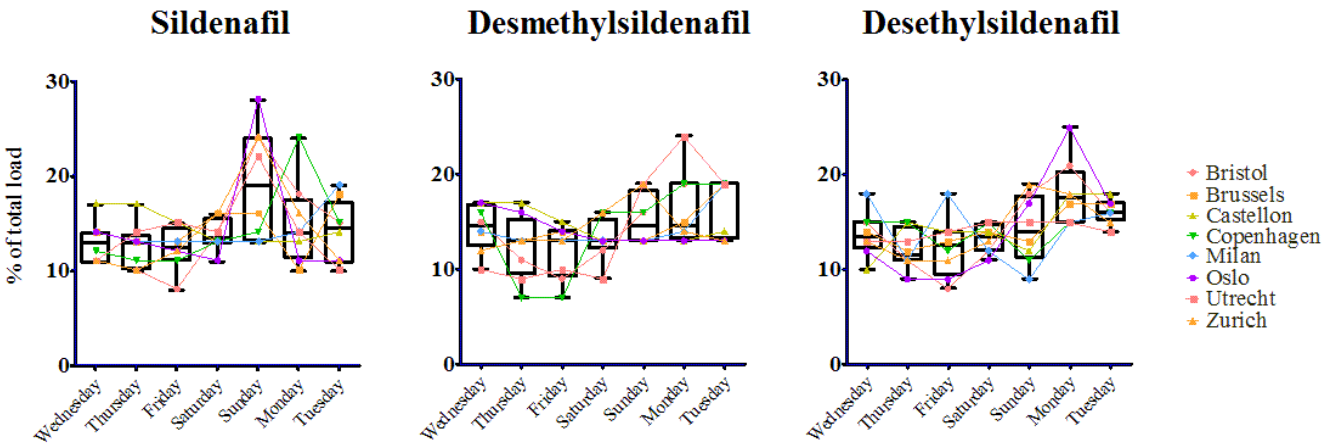


**Fig. 3.3.2**. Daily variations expressed as the percentage of the total load, combining results for the 8 cities. The box represents the median, 25% and 75% percentile values and the error bars extend to the minimum and maximum values. The coloured lines represent each of the cities.

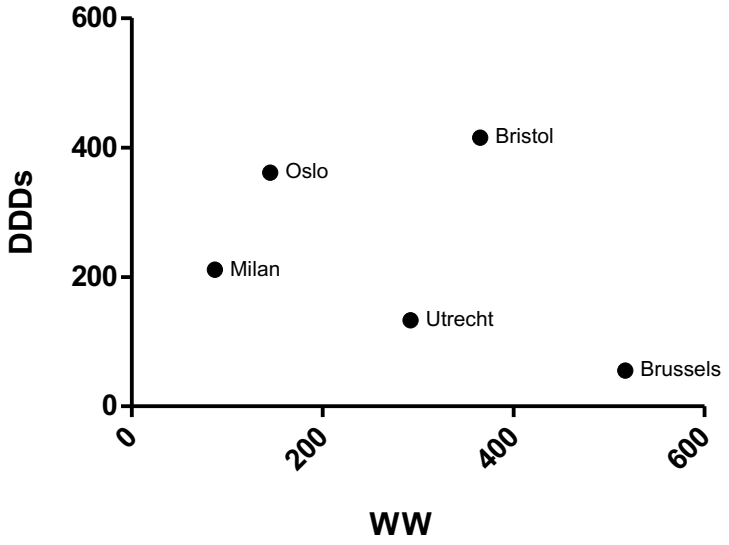


**Fig. 3.3.3**. Relationship between the estimation of sildenafil consumption calculated from the prescription data (DDDs) and from the wastewater environmental loads (WW), both expressed in mg week$^{-1}$ 1000 inh$^{-1}$. For Castellón, Copenhagen and Zurich, no prescription data was available.

# 3.3.4. Conclusions

The present study is the first to compare the use of the erectile dysfunction products in different European cities through chemical analysis of wastewater. The analysis of influents revealed the presence of sildenafil and its two human metabolites in all cities sampled with average loads varying between 0.2 and 14 mg $day^{-1}$ 1000 $inh^{-1}$. None of the other ED products analysed were observed in concentrations above the method detection limits. While it is known that sildenafil is available in products from illegal sources such as internet shops, the results of the present study show that consumption beyond prescribed doses is not common across Europe. Despite the limitations related to the assessment of both predicted and measured loads, it seems that the populations in Utrecht (and also in other cities in The Netherlands) and Brussels might be more inclined towards the use of products from illegal sources or rogue online pharmacies than in the other 3 European cities included in the study for which prescription data were available (Bristol, Milan and Oslo). After this first study illustrating the potential of wastewater-based epidemiology in this field, further research would allow to improve the application of this approach for investigating the use of rogue pharmacies and counterfeit medication.

# Acknowledgements

This work is part of the EU Marie Curie ITN SEWPROF (Marie Curie-FP7-PEOPLE, grant number 317205) and the financial support is gratefully acknowledged. The authors thank the people and agencies that assisted in the collection of the wastewater samples and the national prescription data. Alexander van Nuijs acknowledges the Research Foundation – Flanders (FWO) for his scholarship. Authors also wish to acknowledge dr. Christoph Ort from Eawag for his advice and contribution to discussions.

# Annex F – Supplementary Information Chapter 3.3.

**Table F1**. WWTPs characteristics.

| City | | Bristol | Brussels | Castellon | Copenhagen | Milan | Oslo | Utrecht | Zurich |
|---|---|---|---|---|---|---|---|---|---|
| Residential Population | | 886650 | 953987 | 180690 | 531000 | 1100000 | 580639 | 300000 | 410000 |
| Date of sample collection day 1 | dd.mm.yyyy | 16-3-2015 | 18-3-2015 | 25-3-2015 | 10-3-2015 | 10-3-2015 | 11-03-2015 | 4-3-2015 | 18-3-2015 |
| Date of sample collection day 2 | dd.mm.yyyy | 10-3-2015 | 19-3-2015 | 26-3-2015 | 11-3-2015 | 11-3-2015 | 12-03-2015 | 5-3-2015 | 19-3-2015 |
| Date of sample collection day 3 | dd.mm.yyyy | 11-3-2015 | 20-3-2015 | 27-3-2015 | 12-3-2015 | 12-3-2015 | 13-03-2015 | 6-3-2015 | 20-3-2015 |
| Date of sample collection day 4 | dd.mm.yyyy | 12-3-2015 | 21-3-2015 | 28-3-2015 | 13-3-2015 | 13-3-2015 | 14-03-2015 | 7-3-2015 | 21-3-2015 |
| Date of sample collection day 5 | dd.mm.yyyy | 13-3-2015 | 22-3-2015 | 29-3-2015 | 14-3-2015 | 14-3-2015 | 15-03-2015 | 8-3-2015 | 22-3-2015 |
| Date of sample collection day 6 | dd.mm.yyyy | 14-3-2015 | 23-3-2015 | 30-3-2015 | 15-3-2015 | 15-3-2015 | 16-03-2015 | 9-3-2015 | 23-3-2015 |
| Date of sample collection day 7 | dd.mm.yyyy | 15-3-2015 | 24-3-2015 | 31-3-2015 | 16-3-2015 | 16-3-2015 | 17-03-2015 | 10-3-2015 | 24-3-2015 |
| Total influent day 1 | $m^3$/24h | 197493 | 234264 | 50228 | 148724 | 423110 | 333480 | 47740 | 157084 |
| Total influent day 2 | $m^3$/24h | 204491 | 235442 | 49161 | 150936 | 403240 | 308279 | 45030 | 161005 |
| Total influent day 3 | $m^3$/24h | 198950 | 234906 | 43728 | 147175 | 412310 | 277450 | 49530 | 161427 |
| Total influent day 4 | $m^3$/24h | 197523 | 233096 | 38301 | 144840 | 402240 | 256766 | 46030 | 200010 |
| Total influent day 5 | $m^3$/24h | 252682 | 230375 | 37243 | 145197 | 403020 | 250384 | 46900 | 243013 |
| Total influent day 6 | $m^3$/24h | 220687 | 234774 | 37469 | 137793 | 422690 | 254570 | 45970 | 177167 |
| Total influent day 7 | $m^3$/24h | 193194 | 359951 | 40476 | 137244 | 597470 | 252722 | 44580 | 160912 |
| Sampling mode | -proportional | time | volume | time | volume | volume | volume | volume | volume |
| Sampling interval | $m^3$ or min | 15 min | 1300 $m^3$ | 15 min | 2000 $m^3$ | 3800 $m^3$ | 1500 $m^3$ | 400 $m^3$ | 900 $m^3$ |
| Sampling frequency day 1 | min | 15 | 8 | 15 | 19 | 13 | 6 | 12 | 8 |
| Sampling frequency day 2 | min | 15 | 8 | 15 | 19 | 14 | 7 | 13 | 8 |
| Sampling frequency day 3 | min | 15 | 8 | 15 | 20 | 13 | 8 | 12 | 8 |
| Sampling frequency day 4 | min | 15 | 8 | 15 | 20 | 14 | 8 | 13 | 6 |
| Sampling frequency day 5 | min | 15 | 8 | 15 | 20 | 14 | 9 | 12 | 5 |
| Sampling frequency day 6 | min | 15 | 8 | 15 | 21 | 13 | 8 | 13 | 7 |
| Sampling frequency day 7 | min | 15 | 5 | 15 | 21 | 9 | 9 | 13 | 8 |
| Average wastewater temperature day 1 | °C | n.a. | n.a. | 13.1 | n.a. | 17.5 | 7.8 | 13.5 | 14.7 |
| Average wastewater temperature day 2 | °C | n.a. | n.a. | 12.0 | n.a. | 17.6 | 8.0 | n.a. | 14.7 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Average wastewater temperature day 3 | °C | n.a. | n.a. | 16.7 | n.a. | 17.6 | 8.2 | n.a. | 14.7 |
| Average wastewater temperature day 4 | °C | n.a. | n.a. | n.a. | n.a. | 17.7 | 8.1 | 14.1 | 14.1 |
| Average wastewater temperature day 5 | °C | n.a. | n.a. | n.a. | n.a. | 17.5 | 8.2 | n.a. | 12.6 |
| Average wastewater temperature day 6 | °C | n.a. | n.a. | 17.5 | n.a. | 17.2 | 8.5 | 13.0 | 14.2 |
| Average wastewater temperature day 7 | °C | n.a. | n.a. | 19.5 | n.a. | 16.0 | 8.7 | n.a. | 14.7 |
| pH in sample day 1 | | n.a. | n.a. | 7.4 | 8.0 | 8.0 | 7.5 | 8.6 | 7.8 |
| pH in sample day 2 | | n.a. | n.a. | 6.9 | 8.0 | 7.9 | n.a. | 8.3 | 8.1 |
| pH in sample day 3 | | n.a. | n.a. | 7.6 | 8.2 | 7.7 | n.a. | 8.3 | 8.3 |
| pH in sample day 4 | | n.a. | n.a. | n.a. | 8.1 | 7.8 | n.a. | 8.0 | 8.0 |
| pH in sample day 5 | | n.a. | n.a. | n.a. | 8.0 | 7.7 | n.a. | 8.1 | 8.0 |
| pH in sample day 6 | | n.a. | n.a. | 7.5 | 8.0 | 7.7 | n.a. | 8.3 | 8.1 |
| pH in sample day 7 | | n.a. | n.a. | 7.7 | 8.1 | 7.6 | 7.4 | 8.1 | 8.0 |
| $BOD_5$ day 1 | mg/L | n.a. | n.a. | 245 | 411 | 183 | 103 | n.a. | n.a. |
| $BOD_5$ day 2 | mg/L | n.a. | n.a. | 245 | 377 | 172 | n.a. | n.a. | n.a. |
| $BOD_5$ day 3 | mg/L | n.a. | n.a. | 250 | 451 | 179 | n.a. | n.a. | n.a. |
| $BOD_5$ day 4 | mg/L | n.a. | n.a. | n.a. | 423 | n.a. | n.a. | n.a. | n.a. |
| $BOD_5$ day 5 | mg/L | n.a. | n.a. | n.a. | 456 | n.a. | n.a. | n.a. | n.a. |
| $BOD_5$ day 6 | mg/L | n.a. | n.a. | 200 | 434 | 175 | n.a. | n.a. | n.a. |
| $BOD_5$ day 7 | mg/L | n.a. | n.a. | 360 | 439 | 102 | 186 | n.a. | n.a. |
| COD day 1 | mg/L | n.a. | n.a. | 516 | 909 | 372 | 273 | 530 | n.a. |
| COD day 2 | mg/L | n.a. | n.a. | 516 | 585 | 344 | n.a. | 811 | n.a. |
| COD day 3 | mg/L | n.a. | n.a. | 498 | 664 | 303 | n.a. | 530 | n.a. |
| COD day 4 | mg/L | n.a. | n.a. | n.a. | 644 | 298 | n.a. | 568 | n.a. |
| COD day 5 | mg/L | n.a. | n.a. | n.a. | 755 | 292 | n.a. | 598 | n.a. |
| COD day 6 | mg/L | n.a. | n.a. | 677 | 693 | 385 | n.a. | 648 | n.a. |
| COD day 7 | mg/L | n.a. | n.a. | 807 | 667 | 226 | 372 | 524 | n.a. |
| Ntot day 1 | mg/L | n.a. | n.a. | 47.5 | 64.4 | 31.0 | n.a. | n.a. | n.a. |
| Ntot day 2 | mg/L | n.a. | n.a. | n.a. | 61.7 | 29.4 | n.a. | n.a. | n.a. |
| Ntot day 3 | mg/L | n.a. | n.a. | n.a. | 57.8 | 29.9 | n.a. | n.a. | n.a. |
| Ntot day 4 | mg/L | n.a. | n.a. | n.a. | 66.1 | n.a. | n.a. | n.a. | n.a. |
| Ntot day 5 | mg/L | n.a. | n.a. | n.a. | 64.3 | n.a. | n.a. | n.a. | n.a. |
| Ntot day 6 | mg/L | n.a. | n.a. | 76.0 | 63.2 | 31.6 | n.a. | n.a. | n.a. |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Ntot day 7 | mg/L | n.a. | n.a. | n.a. | 61.2 | 21.0 | n.a. | n.a. | n.a. |
| Ptot day 1 | mg/L | n.a. | n.a. | 7.4 | 9.7 | 3.6 | 3.5 | 8.9 | n.a. |
| Ptot day 2 | mg/L | n.a. | n.a. | n.a. | 8.9 | 3.5 | 3.5 | 9.9 | n.a. |
| Ptot day 3 | mg/L | n.a. | n.a. | n.a. | 8.7 | 3.5 | 3.5 | 10.3 | n.a. |
| Ptot day 4 | mg/L | n.a. | n.a. | n.a. | 9.0 | n.a. | 3.5 | 9.2 | n.a. |
| Ptot day 5 | mg/L | n.a. | n.a. | n.a. | 10.1 | n.a. | 3.5 | 9.7 | n.a. |
| Ptot day 6 | mg/L | n.a. | n.a. | 8.0 | 9.3 | 3.9 | 4.3 | 9.1 | n.a. |
| Ptot day 7 | mg/L | n.a. | n.a. | n.a. | 9.5 | 2.4 | 4.3 | 9.7 | n.a. |
| $NH_4$-N day 1 | mg/L | n.a. | n.a. | n.a. | 44.0 | n.a. | 15.9 | 40.7 | 20.9 |
| $NH_4$-N day 2 | mg/L | n.a. | n.a. | n.a. | 41.0 | n.a. | n.a. | 55.8 | 26.6 |
| $NH_4$-N day 3 | mg/L | n.a. | n.a. | n.a. | 41.0 | n.a. | n.a. | 41.9 | 23.0 |
| $NH_4$-N day 4 | mg/L | n.a. | n.a. | n.a. | 45.0 | n.a. | n.a. | 38.7 | 21.1 |
| $NH_4$-N day 5 | mg/L | n.a. | n.a. | n.a. | 44.0 | n.a. | n.a. | 43.3 | 17.8 |
| $NH_4$-N day 6 | mg/L | n.a. | n.a. | n.a. | 42.0 | n.a. | n.a. | 41.1 | 20.8 |
| $NH_4$-N day 7 | mg/L | n.a. | n.a. | n.a. | 41.0 | n.a. | 21.4 | 39.4 | 28.6 |

**Table F2**. Selected PDE5 inhibitors and LC-MS/MS parameters used for compounds identification.

| | CAS number | Molecular formula | Log Kow (*) | $[M+H]^+$ | Product ions (m/z) | Collision energy (V) | S-Lens | RT (min) |
|---|---|---|---|---|---|---|---|---|
| Sildenafil (ILIS 1) | 171599-83-0 | $C_{22}H_{30}N_6O_4S$ | 2.30 | 475.2 | 58.2 (Q) | 36 | 118 | 10.5 |
| | | | | | 100.2 (q1) | 28 | | |
| | | | | | 283.2 (q2) | 36 | | |
| Desmethylsildenafil (ILIS 2) | 139755-82-1 | $C_{21}H_{28}N_6O_4S$ | 2.09 | 461.1 | 283.1 (Q) | 35 | 130 | 9.6 |
| | | | | | 311.1 (q) | 29 | | |
| Desethylsildenafil (ILIS 1) | 139755-91-2 | $C_{20}H_{28}O_4N_6S$ | 1.99 | 449.2 | 283.1 (Q) | 36 | 138 | 9.4 |
| | | | | | 311.1 (q) | 27 | | |
| Noracetildenafil (ILIS 1) | 949091-38-7 | $C_{24}H_{32}N_6O_3$ | n.a. | 453.2 | 97.1 (Q) | 31 | 148 | 9.2 |
| | | | | | 113.1 (q) | 31 | | |
| Tadalafil (ILIS 1) | 171596-29-5 | $C_{22}H_{19}N_3O_4$ | 0.04 | 390.0 | 204.1 (Q) | 57 | 92 | 13.9 |
| | | | | | 268.1 (q) | 14 | | |
| Aminotadalafil (ILIS 1) | 385769-84-6 | $C_{21}H_{18}N_4O_4$ | -1.20 | 391.0 | 204.1 (Q) | 56 | 87 | 11.9 |
| | | | | | 262.1 (q) | 31 | | |
| Chloropretadalafil (ILIS 1) | 171489-59-1 | $C_{22}H_{19}ClN_2O_5$ | 2.58 | 427.1 | 274.1 (Q) | 31 | 93 | 16.9 |
| | | | | | 135.0 (q) | 19 | | |
| N-octyl nortadalafil (ILIS 1) | 1173706-35-8 | $C_{29}H_{33}N_3O_4$ | 5.22 | 488.2 | 366.2 (Q) | 17 | 120 | 17.8 |
| | | | | | 169.1 (q) | 39 | | |
| Vardenafil (ILIS 1) | 224789-15-5 | $C_{23}H_{33}N_6O_4S$ | 2.79 | 489.3 | 151.1 (Q) | 41 | 159 | 9.6 |
| | | | | | 312.1 (q) | 39 | | |
| N-desethylvardenafil (ILIS 1) | 448184-46-1 | $C_{21}H_{28}N_6O_4S$ | 2.09 | 461.2 | 151.1 (Q) | 43 | 143 | 9.6 |
| | | | | | 312.2 (q) | 33 | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *ILIS 1*<br><br>*Sildenafil-$d_8$* | 951385-68-5 | $C_{22}H_{22}D_8N_6O_4S$ | 2.30 | 483.3 | 62.1 (Q) | 37 | 126 | 10.5 |
| | | | | | 108.3 (q) | 29 | | |
| *ILIS 2:*<br><br>*Desmethylsildenafil-$d_8$* | 1185168-06-2 | $C_{21}H_{20}D_8N_6O_4S$ | 2.09 | 469.2 | 283.1 (Q) | 37 | 160 | 10.7 |
| | | | | | 311.1 (q) | 30 | | |

*n.a.: not available*

*(*) Log Kow (KOWWIN program estimates)*

**Table F3a.** Method performance: linearity, limits of detection and quantification, intraday and interday repeatability.

| | linearity | LOD | LOQ | Intraday repeatability (RSD (%), n=7) | | | | Interday repeatability (RSD (%), n=7, d=3) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | ($r^2$) | ng/L | ng/L | 20 ng/L | 50 ng/L | 100 ng/L | 500 ng/L | 20 ng/L | 50 ng/L | 100 ng/L | 500 ng/L |
| sildenafil | 0.9997 | 1.8 | 6 | 16 | 10 | 5 | 5 | 24 | 9 | 10 | 9 |
| desmethylsildenafil | 0.9999 | 5.4 | 18 | 27 | 15 | 7 | 12 | 25 | 24 | 8 | 9 |
| desethylsildenafil | 0.9997 | 0.5 | 1.6 | 18 | 11 | 10 | 4 | 33 | 18 | 9 | 8 |
| noracetil | 0.9990 | 6 | 20 | 31 | 13 | 5 | 6 | 36 | 23 | 9 | 6 |
| tadalafil | 0.9998 | 2.3 | 7.5 | 10 | 11 | 11 | 7 | 13 | 13 | 13 | 11 |
| aminotadalafil | 0.9995 | 1.8 | 6 | 8 | 11 | 11 | 8 | 14 | 16 | 11 | 11 |
| chloropretadalafil | 0.9993 | 4 | 13.3 | 6 | 8 | 9 | 8 | 12 | 15 | 8 | 10 |
| n-octylnortadalafil | 0.9999 | 30 | 100 | 11 | 15 | 10 | 10 | 20 | 27 | 26 | 16 |
| vardenafil | 0.9998 | 7.2 | 24 | 17 | 18 | 9 | 5 | 22 | 20 | 14 | 7 |
| n-desethylvardenafil | 0.9998 | 9 | 30 | 26 | 16 | 9 | 8 | 37 | 30 | 15 | 13 |

**Table F3b.** Method performance: procedural recovery and matrix effect.

| | Procedural Recovery ± RSD (%) | | | | Matrix Effect ± RSD (%) | | | |
|---|---|---|---|---|---|---|---|---|
| | 20 ng/L | 50 ng/L | 100 ng/L | 500 ng/L | 20 ng/L | 50 ng/L | 100 ng/L | 500 ng/L |
| sildenafil | 93.1 ± 19.7 | 102.7 ± 10.4 | 100.1 ± 11.7 | 97.5 ± 14.7 | 241.9 ± 22.6 | 247.8 ± 15.9 | 82.3 ± 10.1 | 73.6 ± 12.2 |
| desmethylsildenafil | 99.9 ± 20.2 | 100.4 ± 16.9 | 99.9 ± 12.5 | 90.8 ± 21.1 | 406.6 ± 35.0 | 437.1 ± 34.7 | 116.8 ± 12.6 | 82.0 ± 21.3 |
| desethylsildenafil | 97.2 ± 22.1 | 100.7 ± 10.4 | 102.3 ± 11.4 | 93.2 ± 19.0 | 393.4 ± 30.9 | 549.0 ± 17.1 | 156.8 ± 14.7 | 99.8 ± 13.3 |
| noracetil | 94.8 ± 57.7 | 102.7 ± 17.1 | 104.4 ± 13.9 | 99.0 ± 15.7 | 298.6 ± 85.3 | 216.1 ± 33.6 | 70.5 ± 51.0 | 46.7 ± 34.0 |
| tadalafil | 89.3 ± 21.5 | 96.5 ± 7.8 | 96.0 ± 8.6 | 97.7 ± 12.7 | 246.6 ± 23.6 | 270.1 ± 14.2 | 84.0 ± 10.3 | 72.2 ± 12.5 |
| aminotadalafil | 91.3 ± 16.5 | 100.9 ± 8.6 | 97.5 ± 8.8 | 98.2 ± 13.9 | 217.5 ± 15.7 | 251.0 ± 15.4 | 77.8 ± 10.8 | 69.1 ± 13.6 |
| chloropretadalafil | 93.4 ± 15.4 | 87.2 ± 8.4 | 91.7 ± 10.2 | 92.4 ± 11.5 | 195.0 ± 20.6 | 243.8 ± 14.2 | 73.1 ± 10.2 | 64.9 ± 13.6 |
| n-octylnortadalafil | - | - | 16.4 ± 20.5 | 27.4 ± 36.8 | 163.1 ± 19.3 | 234.0 ± 24.1 | 77.4 ± 18.8 | 75.3 ± 10.9 |
| vardenafil | 92.2 ± 23.6 | 101.3 ± 12.2 | 102.1 ± 12.5 | 96.6 ± 12.1 | 320.5 ± 32.2 | 322.7 ± 24.9 | 96.5 ± 17.2 | 83.4 ± 12.3 |
| n-desethylvardenafil | 95.4 ± 25.0 | 96.5 ± 14.4 | 98.9 ± 13.0 | 97.0 ± 16.7 | 607.0 ± 26.9 | 616.0 ± 26.0 | 152.1 ± 14.7 | 125.8 ± 13.0 |

**Table F4**. Amount of API prescribed in 2015, expressed in kg.

| Country | Prescribed kg year$^{-1}$ | | |
|---|---|---|---|
| | Sildenafil [a] | Tadalafil | Vardenafil |
| Belgium | 32[b] | 1 | n.a. |
| England | 1183 | 91 | 13 |
| Italy | 666 | 133 | n.a. |
| the Netherlands | 117 | 16 | 2 |
| Norway | 97 | 22 | 3 |

[a] total sildenafil

[b] Estimated from the ED/VA ratio observed in the Netherlands

n.a.: not available

**Table F5**. Predicted concentrations (PCs) for vardenafil and its metabolite, expressed in ng L$^{-1}$.

| Country PEC ng L$^{-1}$ | PC ng L$^{-1}$ | |
|---|---|---|
| | Vardenafil | N-desethylvardenafil |
| Belgium | n.a. | n.a. |
| England | 0.081 ± 0.007 | 0.13 ± 0.01 |
| Italy | n.a. | n.a. |
| the Netherlands | 0.050 ± 0.002 | 0.083 ± 0.003 |
| Norway | 0.11 ± 0.01 | 0.19 ± 0.02 |

n.a.: not available

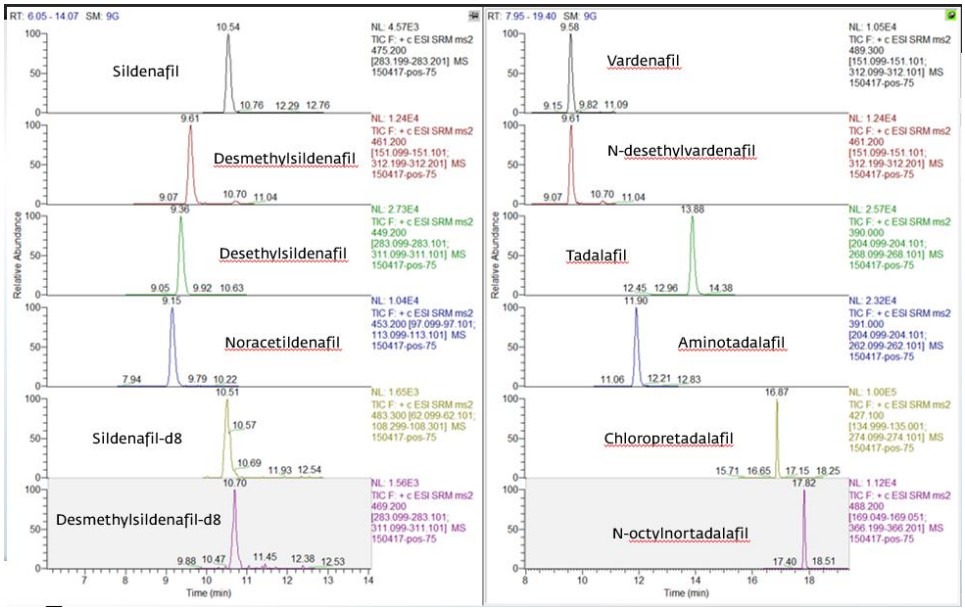


**Figure F1**. Chromatogram from a standard mixture of the selected PDE5 at 50 ng $L^{-1}$ concentration level.

**Equation F1**. Predicted concentration calculation.

$$PC = \frac{(N°DDD \cdot DDD) \cdot EF(\%)}{Pop \cdot inh\,flow\ (L\ \mathrm{day}^{-1}\ \mathrm{inh}^{-1}) \cdot 365}$$

Nº DDD = numbers of DDDs prescribed in 2015 (Table 1)

DDD = value in ng (Table 1)

EF = excretion factor (%)

Pop = Residential population (Table SI-1)

# Chapter 4

# Doping

# 4.1.

# Wastewater-based tracing of doping use by the general population and amateur athletes

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# Abstract

The present study investigates the applicability of the chemical analysis of wastewater to assess the use of doping substances by the general population and amateur athletes. To this end, an analytical methodology that can identify and quantify a list of 15 substances from the groups of anabolic steroids, weight loss products, and masking agents in wastewater has been developed. The method uses solid phase extraction to increase the detection sensitivity of the target analytes, expected to be present at very low concentrations (ng $L^{-1}$ range), and decrease possible matrix interferences. Instrumental analysis is performed by liquid chromatography coupled to high-resolution mass spectrometry, allowing data acquisition in both full scan and tandem MS mode. The method has been successfully validated at two concentration levels (50 and 200 ng $L^{-1}$) with limits of quantification ranging between 0.7 and 60 ng $L^{-1}$, intra- and inter-day precision expressed as relative standard deviation below 15%, procedural recoveries between 60 and 160% and matrix effects ranging from 45 to 121%. The stability of the analytes in wastewater was evaluated at different storage temperatures illustrating the importance of freezing the samples immediately after collection. The application of the method to 24-h composite wastewater samples collected at the entrance of three wastewater treatment plants and one pumping station while different sport events were taking place revealed the presence in wastewater, and hence the use, of the weight loss substances ephedrine, norephedrine, methylhexanamine, and 2,4-dinitrophenol. The use of these stimulants was visible just prior and during the event days and in greater amounts than anabolic steroids or masking agents.

# 4.1.1. Introduction

The prevalence of doping in sports and fitness is an issue of current concern for a healthy society, and in particular for all those involved in sports, for example for evaluating antidoping policy measures (de Hon et al., 2015; Papadopoulos et al., 2006; Stubbe et al., 2014). Its evaluation for the general population, however, is not an easy matter, and even though it is virtually impossible to uncover the exact prevalence of a prohibited activity such as doping, various methods are available to expose parts of this particular problem. These include (i) laboratory-based chemical analyses (in biological matrices such as blood and urine), (ii) questionnaires, (iii) inferences from performances, and (iv) inferences from ego documents. Doping control is widely implemented at professional level (e.g., Olympic games and world championships) and advanced standardized methodologies already exist to detect a great variety of prohibited and controlled substances. However, data are scarce and few scientific articles have addressed the subject so far, with results showing a wide variance in the prevalence of doping at both amateur and professional level. For example, a study showed a prevalence of 2.6% among tertiary education students in Europe (Papadopoulos et al., 2006), evaluated by anonymous questionnaires, and up to 8.2% in fitness centres in the Netherlands (Stubbe et al., 2014), evaluated by randomized response technique. In the case of elite sports, the range is even larger and likely to be between 14 and 39% (de Hon et al., 2015).

Recently, analytical chemical methods have been developed that may play a more important role in this topic. Wastewater contains the excreted biomarkers of human metabolism that directly reflect the exposure and stressors placed upon an entire contributing community. The quantitative measurement of these specific biomarkers in wastewater from communities allows the averaged patterns of factors related to lifestyle, disease, and environment to be used for the assessment of community health (Thomas and Reid, 2011). It has been shown that individual communities present different patterns with respect to the levels of various illicit drug residues in wastewater (Causanilles et al., 2017c; Ort et al., 2014; Thomas et al., 2012) and it is hypothesized that this will also concur for other biomarkers (Thomas and Reid, 2011). This approach, known as wastewater-based epidemiology (WBE), has been also used to determine the use of other substances such as erectile dysfunction pharmaceuticals (Causanilles et al., 2016), alcohol (Yeonsuk Ryu et al., 2016), nicotine, and caffeine (Jose Antonio Baz-Lomba et al., 2016) and the exposure of a population to pesticides (Rousis et al., 2017) or phthalate plasticizers (González-Mariño et al.,

2017). Hence, there is the clear potential to develop a wider range of innovative solutions to quantitatively assess patterns of factors related to health, fitness, and illness within populations, while also providing means of collecting data for epidemiological and socio-economic studies.

Professional athletes are tested by national and international organizations on the use of doping substances. Information on the exact amounts of doping substances that are actually being taken is mostly unknown. The same holds true for athletes visiting fitness centres. WBE can be a valuable complimentary approach for national doping authorities, as suggested by recent studies (de Hon et al., 2015; Katsoyiannis and Jones, 2011). It can give information on the usage of known compounds in a specified area or group, albeit at the (averaged) group level. The presence of substances that are commonly used either as doping/lifestyle drugs or for the treatment of diverse diseases, has already been identified in wastewaters from fitness centres and wastewater treatment plant (WWTP) influents and effluents (Schröder and Thevis, 2010), providing a deeper insight into the origin and whereabouts of these compounds.

The present study was conducted to further investigate the application of the WBE approach for assessing the use of doping substances, targeting amateur athletes and other users in the general population at specific sport events. To this aim, a sensitive analytical methodology based on solid phase extraction (SPE) followed by liquid chromatography coupled to high-resolution mass spectrometry was developed and validated. The method was applied to 24-h composite wastewater samples collected at the entrance of three WWTPs and one pumping station while different sport events were taking place within the corresponding sewer catchment area.

# 4.1.2. Materials and methods

**Reagent and standards**

The analytical standards included in the study were as follows: metandienone, metenolone, mibolerone, nandrolone, sibutramine, clomiphene, tamoxifen and anastrozole, obtained from TRC Toronto Research Chemicals Inc. (Ontario, Canada); ephedrine, norephedrine, trenbolone, clenbuterol, methylhexanamine, 2,4-dinitrophenol, and finasteride, obtained from Sigma-Aldrich (Stenheim, Germany). The chemical properties and structures of the analytes selected are compiled in **Table G1** (see the Supplementary Material in Annex G). The isotopically labeled internal standards (ILIS) used as surrogates for the quantification of their analogue native analytes were the following: methandrostenolone-$d_3$, mibolerone-$d_3$, trenbolone-$d_5$, sibutramine-$d_6$, 2,4-dinitrophenol-$d_3$, clomiphene-$d_5$, tamoxifen-$d_5$, anastrozole-$d_{12}$, and finasteride-$d_9$, purchased from TRC; ephedrine-$d_3$, norephedrine-$d_3$, clenbuterol-$d_9$, and methylhexanamine-$d_4$, purchased from Sigma-Aldrich. The purity of the ILIS was verified (> 99%) by means of the injection of increasing concentrations up to 160 µg $L^{-1}$, the result being that even in high concentrations no trace of the native signal was detected. In addition, the deuterated standard atrazine-$d_5$ was added as external standard to all vials (calibration and samples) prior to analysis in order to monitor the injection and ionization process.

Individual stock solutions were prepared from either the powdered substance or a 1 mg $mL^{-1}$ ampoule solution, in methanol, at a level ranging from 60 to 160 mg $L^{-1}$ for the native compounds and 20 mg $L^{-1}$ for the ILIS, and stored at −20 °C. Two mix stock solutions containing the native analytical standards and the ILIS separately were prepared at a final level of 1 mg $L^{-1}$ in methanol. Working solutions were prepared by dilution to the desired concentration with methanol and stored at − 20 °C. Calibration curves were prepared daily by diluting with ultrapure water the appropriate mix (containing atrazine-$d_5$ as external standard) to a final water/methanol (90:10, v/v) composition.

Methanol and acetonitrile HPLC-grade solvents, ammonium hydroxide solution (28 – 30%), and hydrochloric acid were supplied by Avantor Performance Materials B.V (Deventer, The Netherlands). Formic acid, FA (50% in water) was obtained from Fluka Analytical (Sigma-Aldrich, Stenheim, Germany). The ultrapure water was obtained by

purifying demineralized water in an Elga Purelab Chorus ultrapure water system (High Wycombe, UK).

Glass fiber filters (type A/E, 1 µm) were purchased from Pall Corporation (Port Washington, NY, USA). SPE cartridges, built of a mixed-mode, reversed-phase/strong cation exchange, water-wettable polymer (Oasis MCX, 150 mg, 6 $cm^3$) were obtained from Waters (Milford, MA, USA).

For the calibration of the Sciex TripleTOF mass spectrometer APCI positive and negative calibration solutions were obtained from AB Sciex (MA, US).

**Sample collection**

Four 15-day sampling campaigns were carried out at four different locations, targeting three different sport events. The 2-week sampling period was planned including samples from a period where no sport event was taking place to determine the background concentrations corresponding to excretions from the regular population. This allowed the comparison to the event period, where visitors and athletes would add up. The samples collected during the four sampling campaigns were stored in HDPE bottles at − 20 °C until analysis.

The targeted sport events and the sampling strategy are described below:

**Event A**

This relatively large 5-day Olympic sport event took place in a large size city (between 500,000 and 1000,000 inhabitants). More than 1000 professional athletes participated, all of who were monitored by the international anti-doping system in place as overseen by the World Anti-Doping Agency. In addition, 125,000 visitor tickets were sold to attend the different events. The sample collection was performed at the entrance of the WWTP, at a 10-km distance from the main event location. The 24-h composite samples were collected in volume proportional mode, with an average sampling frequency of 3.5 min.

**Event B**

The second was a relatively small 1-day bodybuilding event that took place in a small-size city (less than 100,000 inhabitants). It gathered 500 amateur athletes, coaches and volunteers, and more than 800 visitors. There were no anti-doping controls in place for this event. The sample collection was performed at the entrance of the

WWTP, at a 3-km distance from the event location. The 24-h composite samples were collected in volume proportional mode, with an average sampling frequency of 12 min.

**Event C**

The third was a relatively large 2-day bodybuilding event that took place in a town (less than 100,000 inhabitants) close to a medium-size city (between 100,000 and 500,000 inhabitants). Over 100 amateur athletes participated and 8000 visitors attended. There were no anti-doping controls in place for this event. The sample collection was performed at two locations, at a pumping station closer to the source (in the town) and at the entrance of the WWTP serving the larger catchment area (that included both the town and the medium-size city). The distance from the event location to the sampling collection points were 5 and 12 km, respectively. At the pumping station, the composite samples were collected in time proportional mode with a frequency of 5 min, whereas at the WWTP they were collected in volume proportional mode, with an average sampling frequency of 12 min.

## Analytical methodology

### Sample treatment

Fifty milliliters of homogenized sample were spiked at 200 ng $L^{-1}$ with ILIS to act as surrogates during the sample handling and analysis and to correct for possible analyte losses and/or matrix effects. The samples were filtered using a 1-µm type A/E glass fibre filter and acidified to pH 2 to 3 with a solution of hydrochloric acid. Next, the sample was loaded onto a mix-mode cationic polymer-based cartridge (Oasis MCX) previously conditioned with 8 mL of methanol and 8 mL of ultrapure water acidified with 2% FA. After loading the samples, the cartridges were washed with 4 mL of ultrapure water acidified with 2% FA. Next, the cartridges were vacuum dried. Prior to elution, a second washing step with 4 mL of ultrapure water with 5% acetonitrile acidified with 2% FA was performed. Elution was done in two steps, using 4 mL of acetonitrile followed by 4 mL of acetonitrile with 5% $NH_4OH$, both collected as one eluate. The eluate was evaporated to 250 µL by means of a Barkey optocontrol (Germany) with a gentle $N_2$ stream (block temperature set at 300 °C), where after 250 µL of ultrapure water were added and evaporated again to 250 µL (adjusted by weight) and reconstituted to 0.5 mL of water/methanol 90:10 (v/v) with an 80:20 solution containing atrazine-$d_5$ as external standard.

**Instrumental analysis**

Fifty milliliters of the sample extracts were injected into a UPLC (NEXERA X2 LC-30AD, Shimadzu Corporation, Kyoto, Japan) coupled to a time of flight high-resolution mass spectrometer (TripleTOF 5600+, AB Sciex, MA, US). The LC separation was performed on a XBridge BEH XP C18 column (Waters) with particle size 2.5 µm, and dimensions of 2.1 mm × 150 mm, preceded by a 2.0 mm × 2.1 mm I.D. Phenomenex SecurityGuard Ultra column (Phenomenex, Torrance, USA), at a constant flow rate of 0.250 mL $min^{-1}$. Mobile phase solvents were ultrapure water and methanol both with 0.05% FA (v/v). The percentage of organic solvent changed as follows: 0 min, 20%; 12 min, 100%; 15 min, 100%; 16 min, 20%; 20 min, 20%. Between consecutive runs, the analytical column was re-equilibrated for 4 min.

The TripleTOF was set up to acquire the full scan in the range of 50 to 800 *m/z* as well as the full scan of the product ions of the target compounds, for which the retention time and optimal collision energy were pre-set. Mass calibration was performed with every batch run just prior to the sequence start. The system operated in positive ionization mode, with the ion spray voltage at 5 kV, source temperature at 500 °C and declustering potential adjusted to 70 V. For 2,4-dinitrophenol, the system was operated in negative mode, with the ion spray voltage at − 4.5 kV, source temperature at 500 °C and declustering potential adjusted to − 70 V. Gas 1, gas 2, and curtain gas were set at 40, 50, and 25 psi, respectively.

Data processing was performed with the MultiQuant 3.0 software, version 3.0.5373.0 (AB Sciex). Analyte concentrations were quantified from the sum of the acquired product ions relative signal (native divided by the corresponding deuterated analogue, when available; when it was not available, a closely resembling deuterated was selected). The acquisition and data processing parameters can be found in **Table G2** (see the SM in Annex G). In addition, GraphPad Prism 5 was used for post statistical evaluation of the results.

Analyte concentrations were multiplied with 24-h flow rates to obtain daily loads. For comparison between different cities, daily loads were normalized to the numbers of inhabitants connected to the corresponding sewer system that were obtained from census data provided by the WWTP managers (see SM in Annex G, **Table G3**). The number of inhabitants used for the calculation was not corrected for the number of extra people attending each event, since the additional number did not change the normalized load with more than 1%, and we assumed that many visitors would be the

people living in the cities, and therefore already accounted in the number of inhabitants.

**Method validation**

The method was validated in terms of linearity, limits of detection and quantification, precision intra-day and inter-day (repeatability), procedural recovery (accuracy), and matrix effects by analyzing wastewater spiked with selected analytes.

A calibration curve was established by analyzing spiked ultrapure water and wastewater with standard solutions at 10 different concentrations, ranging from 0 to 50 μg $L^{-1}$, to investigate linearity. Limits of detection and quantification (LOD and LOQ, respectively) were defined as the concentration that provides signal-to-noise (S/N) values of 3 and 10 for the quantifier ion of each analyte. The values were calculated at the lowest point of the calibration curve. Intra-day and interday precision was assessed at two levels, 50 and 200 ng $L^{-1}$, with six replicates per level, and during three non-consecutive days. Procedural recovery (%) was calculated as the ratio of the signal of the analyte spiked to a sample after sample treatment (γ) against the signal of the analyte spiked to the same sample before treatment (β): [γ/β] × 100. Matrix effect (%) was calculated as the ratio of the signal of solution β (where the signal of the native compound present in the used sample was subtracted) against the signal of the analyte spiked to ultrapure water (α): [β/α] × 100.

**Analyte stability**

It was important to assess the stability of the target compounds in wastewater, in-sewer and in-sample conditions, since the samples were stored at − 20 °C between 1 and 8 months before analysis. Besides, samples were kept in the autosampler at 4 °C during the 24-h cycle. In addition, in the case of the automated sampling at the pumping station (event C), there was sometimes a delay and samples had to be left longer in the autosampler (max 24 h).

To evaluate the stability, wastewater samples were spiked at 400 ng $L^{-1}$ in order to assure quantification of the parent compound, even if it would degrade by tenfold. The stability test was performed in triplicate for each temperature and storage condition. The experiment was scheduled to last a month and a half, with six sampling points during that period, and using three different storage temperature conditions:

freezer (− 20 °C), fridge (4 °C), and laboratory room temperature (20 °C). Samples were prepared and stored in polypropylene tubes.

This type of experiment allows measuring the total degradation that compounds might suffer in wastewater due to transformation processes in-sewer and storage; however, it is neither possible to differentiate the type of degradation (chemical, biological, or physical) nor to identify when (during in sewer transport or storage) the degradation may have occurred (McCall et al., 2016a; Ramin et al., 2017, 2016).

# 4.1.3. Results and discussion

**Selection of target compounds**

The choice of substances was made in order to detect compounds relevant for actual doping use, such as those promoting muscle growth (anabolic steroids), increasing metabolism by burning fat (weight loss stimulants), or hiding the derived effects or preventing the detection of doping substances use (masking agents). **Table G1** (see the SM in Annex G) presents the list of compounds, which it is not all-inclusive but fit-for-purpose for this research, with a description of their type of action and licit formulation (ATC code) when available. Not all of them are mentioned on the 2017 Prohibited List as published by the World Anti-Doping Agency or known to be abused in a fitness-setting. For example, finasteride is not prohibited but mentioned as a confounding factor used to alter athlete's steroid profile and 2,4-dinitrophenol, also not listed but considered relevant due to its use being associated with fatal incidents (Grundlingh et al., 2011).

**Method development**

**Sample treatment**

Sample preparation plays an important role in the method development because wastewater is a very complex matrix and target analytes are expected at the low ng $L^{-1}$ concentration level. Preparatory steps such as sample dilution and filtration to minimize matrix interferences as well as the different parameters involved in the concentration by SPE required optimization. For the optimization of the sample treatment, a pooled wastewater reference sample was used as matrix.

First, the effect of diluting the sample was investigated at four dilution levels in triplicate (1× (no dilution), 2×, 5×, 10×). Results showed no impact on the overall recoveries (defined as the comparison between the quantified concentration and the nominal spike value); therefore, dilution of the sample was not performed during the method development. Second, the effect of sample filtrationwas evaluated for four different filter materials: type GF/F glass microfiber filter (0.7 µm, Ø 47mm, Whatman), regenerated cellulose (0.2 µm, Ø 47 mm, Satorius), type A/E glass fiber filter (1 µm, Ø 47 mm, Pall), and polyethersulfone (PES) membrane (0.2 µm, Ø 90 mm, Nalgene). Removing the particulate phase from the sample by filtering could have

implications for the calculation of the daily loads for compounds having a high log Kow (or Koc). For the compounds observed at levels above their LOQ, this holds only for sibutramine and metandienone. Their fraction sorbed could be substantial if the particle concentration would be more than 1 g $L^{-1}$. We estimated/observed particulate phase concentrations in the samples invariably below 1 g $L^{-1}$ and therefore the contribution of the compounds sorbed to the particle phase negligible. Type A/E glass fibre presented the most convenient combination of good recovery and being less affected by matrix effects and was therefore chosen.

Optimization of the SPE procedure was done with the aim of reaching good extraction recoveries for all the target analytes despite differences in physicochemical properties, and concentrating expected influent concentrations from low ng $L^{-1}$ to the µg $L^{-1}$ range. The pKa of the analytes indicated that the majority of species would be either neutral or positively charged at pH = 7 (see SM in Annex G, **Table G1**), except for 2,4-dinitrophenol which has pKa 4.09 and will therefore be almost entirely (89–100%) in its anionic form at pH 5–9. SPE cartridges Oasis HLB and MCX were selected for the optimization test because of their hydrophilic–lipophilic balance and cation exchange properties, respectively. In addition, C18 cartridges were included as they are commonly used for the analysis of doping substances in human urine. Our results showed that weight loss agents as well as anabolic steroids were hardly or not recovered with C18 cartridges. As a result, the use of the C18 sorbent was renounced. Regarding HLB and MCX, they provided satisfactory results overall, but MCX was selected due to its mixed-mode properties that allowed a better retention of weak bases as well as neutral compounds.

Once the MCX cartridge was chosen, the standard procedure recommended by the manufacturer was adjusted by optimizing the solvent composition used for the elution, evaluating the possibility of dividing the elution into two different steps, and adjusting the solvent volumes. The use of acetonitrile

rather than methanol decreased the matrix interferences in the eluate, and adding 5% of $NH_4OH$ allowed higher recovery for those compounds with lower pKa (namely ephedrines). A two-step elution was then selected, with first 4 mL of acetonitrile (best for anabolic steroids) followed by 4 mL of 5% $NH_4OH$ in acetonitrile, combining these to a final eluate of 8 mL.

Finally, the effect of washing was investigated, including up to two steps, one before and/or one after the cartridge drying, with different combinations of ultrapure water and/or 2% FA and/or 5% acetonitrile. Results for anabolic steroids and masking agents

improved when a washing step after drying the cartridge was incorporated. Therefore, the final procedure consisted of washing with 2% FA in ultrapure water before drying and another washing step with 5% acetonitrile and 2% FA in ultrapure water after drying.

**LC-MS/MS (HRMS)**

The sensitivity and selectivity of the analytical method was optimized by selecting the most appropriate LC column, the mobile phase composition and gradient, and the injection volume (for the LC part) and the product ions (for the MS part).

Three LC columns were tested: Xbridge C18 150 × 2.1 mm, 2.5 µm particle size, Kinetex 1.7 µm F5 100 A 150 × 2.1 mm, and Kinetex 1.7 µm Biphenyl 100 A 150 × 2.1 mm. The column selected was the Xbridge because it provided sufficient separation for the target compounds and its characteristics were suitable for a wide range of compounds in the case that a screening for suspects was applied. Both, acetonitrile and methanol with 0.05% FA were tested as organic solvent for the mobile phase composition. Acetonitrile provided lower backpressure in the LC column and better peak shape and sensitivity for methylhexanamine. However, the peak shape for three masking agents worsened, and clomiphene isomers were not resolved. To compromise the general peak shape methanol was selected, and to control the backpressure the flow was adjusted to 0.25 mL $min^{-1}$. The mobile phase gradient was important because all anabolic steroids were eluting within the 10–13 min range. Modifications of the gradient were tested to improve the separation, but no clear improvements were observed while the run time increased substantially. In addition, the injection volume was set to 50 µL as a compromise between a higher signal while keeping a Gaussian peak shape. For the MS part, individual analytes were injected in order to obtain the optimal collision energy and the accurate mass of at least one product ion in addition to the accurate mass of the protonated ion. As can be seen in **Table G2** (see the SM in Annex G), two transitions were acquired for all compounds, with the only exception of methylhexanamine and 2,4-dinitrophenol, for which only one product ion was used, due to the small molecule size, which made it troublesome to obtain more specific ions and/or with enough sensitivity.

**Analyte stability**

A summary of the results of the stability tests is presented in **Table 4.1.1**, which shows the time points at which the change in concentration compared to the initial concentration is larger than 20%, for each compound and temperature. **Fig. G1** (see the SM in Annex G) presents the full data evaluation. In order to identify outlying values, for each compound, a stability model was generated based on a quadratic or linear fit, as has been previously done for other illicit substances (Baker and Kasprzyk-Hordern, 2011b; van Nuijs et al., 2012). The statistical evaluation revealed that a linear fit was the preferred model in most cases except for sibutramine at 20 °C, trenbolone at −20 °C, metandienone at 20 °C, and clomiphene at 4 and −20 °C, where a quadratic fit was preferred. Outliers were identified by the best-fit model evaluation and excluded from the graphs. A variation of ± 20% was not considered significant as it was accepted as the method variability. Our results highlight the importance of immediately storing the samples in the freezer after collection (− 20 °C); otherwise, target compounds might undergo significant degradation. At − 20 °C, only for clomiphene a loss of 25% after 3 days was observed, which could be related to its low solubility in water. When stored at 4 °C, most analytes remained stable in the studied period; however, compounds such as metandienone experienced 50% decrease after 7 days, and metenolone, tamoxifen, and clomiphene suffered a severe degradation (up to 75%) after 3 days. Such degradation could also take place during the automated 24-h cycle of sample collection and might explain the non-detection of these compounds (see below). At room temperature (20 °C), compounds such as sibutramine and metandienone experienced a significant decrease of 50% after 1 week; 2,4-dinitrophenol after 3 days; and clomiphene, tamoxifen, and metenolone already after 1 day. Unexpectedly, storage at 4 or 20 °C led to higher concentrations than the nominal value for some compounds. This was the case of ephedrine or norephedrine that was found to be stable in this study but strongly degraded in another study with same temperature and pH conditions (Baker and Kasprzyk-Hordern, 2011b). A clear explanation was not found, but it could be due to the higher variability in measurements induced by changes in matrix components as a result of storage at higher temperature, or in the case of norephedrine, due to in-sample transformation from amphetamine (Caldwell et al., 1972).

**Table 4.1.1.** Results of the stability tests conducted at three different temperatures. Results are expressed as the time point (in days) where the change from the initial concentration is higher than 20% for each compound.

| | 20 °C | 4 ºC | -20 °C |
|---|---|---|---|
| Norephedrine | 30 < 45 | > 45 | > 45 |
| Ephedrine | 0 < 1 | 7 < 14 | > 45 |
| Methylhexanamine | 0 < 1 | 0 < 1 | 7 < 14 |
| Clenbuterol | 30 < 45 | 30 < 45 | > 45 |
| Anastrozole | 30 < 45 | 30 < 45 | 30 < 45 |
| Sibutramine | 1 < 3 | 14 < 30 | 30 < 45 |
| Trenbolone | 0 < 1 | 1 < 3 | 30 < 45 |
| Nandrolone | 0 < 1 | 0 < 1 | 30 < 45 |
| Metandienone | 3 < 7 | 7 < 14 | > 45 |
| Finasteride | 0 < 1 | 3 < 7 | > 45 |
| Clomiphene | 0 < 1 | 0 < 1 | 1 < 3 |
| Mibolerone | > 45 | > 45 | > 45 |
| Metenolone | 0 < 1 | 0 < 1 | > 45 |
| Tamoxifen | 0 < 1 | 0 < 1 | > 45 |
| 2,4-dinitrophenol | 1 < 3 | 7 < 14 | > 45 |

## Method performance

**Table 4.1.2** (a and b) summarizes the results of the method validation. The linearity of the response expressed by the regression coefficient (r) invariably showed a value above 0.99, without a significant difference between solutions prepared in ultrapure water and wastewater. For the further quantitative analyses, the calibration in ultrapure water was chosen. LODs ranged from 0.2 ng $L^{-1}$ for anastrozole to 20 ng $L^{-1}$ for mibolerone, trenbolone, and nandrolone. LOQs were below 30 ng $L^{-1}$ except for mibolerone, for which only the high concentration level was successfully validated, and trenbolone and nandrolone, for which, depending on the wastewater tested (see SM in Annex G, **Table G4**), the LOQs slightly varied around 50 ng $L^{-1}$. The precision, expressed as relative standard deviation (RSD%), remained between 2 and 15%, with

the exception of drostanolone (33%). Procedural recoveries were in general satisfactory and ranged from 60 to 130%, with the exception of metenolone, which presented the highest recoveries (up to 160%). For this compound, a deuterated analogue was not available, and this might be the reason for such a high value. RSDs remained below 16% in all cases, which indicated low variability. Matrix effects ranged from 43 to 121%, with RSD up to 22%. Norephedrine, anastrozole, nandrolone, metenolone, and drostanolone seemed to be suppressed by the matrix, whereas the response of ephedrine was slightly enhanced. For clomiphene and tamoxifen, the matrix effect could not be evaluated since no signal was detected in the spiked ultrapure water sample, which corresponds to 100% matrix suppression.

**Table 4.1.2.a** Method performance in terms of linearity, limits of detection and quantification, intra-day and inter-day precision, procedural recovery and matrix effects.

| | Linearity | Limits | | Precision | | | |
|---|---|---|---|---|---|---|---|
| | r | LOD | LOQ | Intra-day RSD (%) (n=6) | | Inter-day RSD (%) (n=18, d=3) | |
| | | ng $L^{-1}$ | ng $L^{-1}$ | 50 ng $L^{-1}$ | 200 ng $L^{-1}$ | 50 ng $L^{-1}$ | 200 ng $L^{-1}$ |
| Norephedrine | 0.9997 | 3 | 10 | 4 | 4 | 4 | 3 |
| Ephedrine | 0.9997 | 3 | 10 | 5 | 2 | 4 | 3 |
| Methylhexanamine[a] | 0.9989 | 7 | 25 | 9 | 6 | 8 | 8 |
| Clenbuterol | 0.9987 | 3 | 10 | 4 | 3 | 10 | 10 |
| Anastrozole | 0.9988 | 0.2 | 0.7 | 4 | 8 | 7 | 12 |
| Sibutramine | 0.9997 | 0.7 | 2 | 5 | 3 | 8 | 7 |
| Trenbolone | 0.9923 | 20 | 50 | 4 | 5 | 5 | 9 |
| Nandrolone | 0.9994 | 20 | 50 | 12 | 11 | 7 | 14 |
| Metandienone | 0.9993 | 7 | 25 | 4 | 2 | 6 | 4 |
| Finasteride | 0.9934 | 0.7 | 2 | 2 | 6 | 12 | 13 |
| Clomiphene | 0.9997 | 7 | 25 | 4 | 2 | 10 | 5 |
| Mibolerone[b] | 0.9986 | 20 | 60 | - | 3 | - | 3 |
| Metenolone | 0.9986 | 3 | 10 | 4 | 4 | 14 | 14 |
| Tamoxifen | 0.9996 | 7 | 25 | 6 | 2 | 8 | 3 |
| 2,4-dinitrophenol | 0.9995 | 9 | 30 | 6 | 5 | 13 | 7 |

LOD and LOQ obtained for pooled wastewater sample. **Table G4** presents LODs and LOQs specific for each of the sampling locations

[a] n=5

[b] Only one level was successfully validated for this compound

**Table 4.1.2.b** Method performance in terms of linearity, limits of detection and quantification, intra-day and inter-day precision, procedural recovery and matrix effects.

| | Recovery[c] | | Matrix effect[c] | |
|---|---|---|---|---|
| | [$\gamma/\beta$] ± RSD (%) (n=6) | | [$\beta/\alpha$] ± RSD (%) (n=6) | |
| | 50 ng $L^{-1}$ | 200 ng $L^{-1}$ | 50 ng $L^{-1}$ | 200 ng $L^{-1}$ |
| Norephedrine | 132 ± 7 | 84 ± 9 | 82 ± 9 | 93 ± 7 |
| Ephedrine | 125 ± 9 | 105 ± 9 | 121 ± 12 | 89 ± 5 |
| Methylhexanamine[a] | 63 ± 15 | 70 ± 4 | 92 ± 16 | 87± 12 |
| Clenbuterol | 92 ± 7 | 69 ± 15 | 96 ± 7 | 100 ± 5 |
| Anastrozole | 92 ± 8 | 59 ± 14 | 74 ± 8 | 87 ± 9 |
| Sibutramine | 130 ± 11 | 104 ± 11 | 64 ± 12 | 91 ± 22 |
| Trenbolone | 91 ± 9 | 70 ± 16 | 101 ± 7 | 96 ± 10 |
| Nandrolone | 104 ± 14 | 99 ± 15 | 89 ± 18 | 77 ± 18 |
| Metandienone | 105 ± 8 | 85 ± 11 | 95 ± 3 | 96 ± 9 |
| Finasteride | 84 ± 10 | 61 ± 14 | 107 ± 14 | 102 ± 16 |
| Clomiphene | 96 ± 10 | 73 ± 14 | n.a. | n.a. |
| Mibolerone[b] | - | 99 ± 4 | - | 111 ± 5 |
| Metenolone | 160 ± 16 | 147 ± 5 | 43 ± 10 | 45 ± 16 |
| Tamoxifen | 98 ± 9 | 69 ± 12 | n.a. | n.a. |
| 2,4-dinitrophenol | 77 ± 16 | 60 ± 20 | 65 ± 7 | 88 ± 5 |

n.a. not available, compound non-detected in ultrapure water ($\alpha$), equivalent to 100% matrix suppression

[a] n=5

[b] Only one level was successfully validated for this compound

[c] For explanation of symbols used in recovery and matrix effect calculations, see the text

**Application to wastewater samples**

**Local wastewater characteristics**

As already highlighted above, wastewater is a complex matrix. The method validation presented in the previous section corresponds to the performance obtained with an in-house reference sample made from pooled wastewater. However, the composition of wastewater is very much affected by geographical location and temporal characteristics. For this reason, the procedural recovery and the limits of detection and quantification were re-evaluated for each location sampled. To this end, with every sample batch, three samples were analysed in duplicate one being spiked at 200 ng $L^{-1}$. Results are presented in **Table G4** (see the SM in Annex G).

The variability of the recoveries remained below 25%in all but three cases. Ephedrine showed high variability in the samples from WWTPs A and C due to higher noise signals in the chromatograms. Regarding the limits of detection and quantification, in general, they were in line (similar range) with those obtained with the reference-pooled wastewater, although some differences were observed. In the case of methylhexanamine and clenbuterol, lower LOQs were observed at all locations, whereas for ephedrine, anatrozole, finasteride, mibolerone, metenolone, clomiphene, tamoxifen, and drostanolone LOQs were higher at all locations. For the remaining compounds (norephedrine, sibutramine, trenbolone, nandrolone, and metadienone) LOQs were in the same range as those previously estimated in the reference pooled wastewater.

**Results obtained for the wastewater influents**

The concentrations calculated in wastewater influents were transformed into influent loads (expressed in mg $day^{-1}$, see **Tables G5 to G8** in the SM, Annex G) by multiplying with the daily influent flow rates provided by the WWTP operators (presented in SM, Annex G, **Table G3**).

**Event A**

The results obtained for the samples collected at the entrance of the WWTP A (SM **Table G5**) showed the presence of three weight loss compounds in all the samples: ephedrine with the highest loads, ranging from 70 to 122 g $day^{-1}$, followed by norephedrine, ranging from 6.6 to 14 g $day^{-1}$, and methylhexanamine, ranging from 7

to 13 g $day^{-1}$. From the other weight loss products, sibutramine, was quantified in 4 out of the 15 samples analyzed, ranging from 776 to 2910 mg $day^{-1}$, and detected in other 4 samples < LOQ, and 2,4-dinitrophenol was quantified in 1 sample at 4300 mg $day^{-1}$ and detected in other 1 sample < LOQ. The anabolic steroid metandienone was quantified in 5 out of the 15 samples, ranging from 1480 to 2020 mg $day^{-1}$, and detected in other 8 samples < LOQ. Clenbuterol was detected in 1 sample < LOQ. None of the remaining compounds were detected (i.e., no signal or signal below LOD).

Statistical evaluation of the loads during the event and the corresponding reference weekend using two tailed Mann-Whitney test ($p < 0.05$ significant difference) showed that the medians of both periods were significantly different for norephedrine ($p = 0.03$). This suggested an enhanced use of norephedrine during the event weekend. This stimulant is most commonly used to reduce body fat before a sport event by fast increasing human metabolism. However, it is also a metabolite of the recreational substance amphetamine (Caldwell et al., 1972), and therefore, the increase could also be caused by an enhanced consumption of this illegal substance (and its degradation from wastewater (Baker and Kasprzyk-Hordern, 2011b)).

**Event B**

The results obtained for the samples collected at the entrance of the WWTP B (SM **Table G6**) showed the presence of three weight loss compounds in all the samples: ephedrine with the highest loads, ranging from 6 to 15 g $day^{-1}$, followed by norephedrine (457 to 894 mg $day^{-1}$), and methylhexanamine (254 to 710 mg $day^{-1}$). From the other weight loss products, sibutramine was quantified in 1 out of the 15 samples analyzed, at 90 mg $day^{-1}$, and was detected, albeit below LOQ, in another 10 samples, and 2,4-dinitrophenol was quantified in 3 samples ranging from 516 to 6430 mg $day^{-1}$ and present at levels between LOD and LOQ in another 6 samples. The anabolic steroid metandienone was detected in all of the samples at levels between LOD and LOQ.

An increase in loads just prior to the event weekend can be observed for ephedrine, norephedrine, methylhexanamine, and 2,4-dinitrophenol. This increase in loads might be explained by the fact that the athletes arrived already on Friday or Saturday. Statistically evaluating the use of ephedrine, norephedrine, and methylhexanamine in the two weekends (and the days prior to the weekend) using a two-tailed Mann-Whitney test, a significant difference between the medians was observed for norephedrine and methylhexanamine ($p = 0.03$ in both cases) but not for ephedrine.

In the case of 2,4-dinitrophenol, there was not sufficient data for a statistical evaluation. However, its use just prior to and during the sport event was obvious as it was found to be present at quantifiable concentrations only in those days (SM **Table G6**), while being < LOQ during the reference weekend (see also section "Normalized loads" below). We conclude that weight loss products had been used on the days prior to the sport event B. The case of 2,4-dinitrophenol was particularly noteworthy, because its use has been associated with severe adverse effects and even risk of death (Grundlingh et al., 2011).

**Event C**

The results obtained for the samples collected at the pumping station C and the entrance of the WWTP C (SM, **Tables G7 and G8**) revealed again the presence of three weight loss compounds in all of the samples. The highest loads were observed for ephedrine, ranging from 4 to 33 g $day^{-1}$ at the pumping station and 29 to 108 g $day^{-1}$ at the WWTP entrance, respectively. Norephedrine loads ranged from 341 to 1400 and 4540 to 8790 mg $day^{-1}$, respectively, and methylhexanamine loads from 594 to 2020 and 4450 to 125,000 mg $day^{-1}$, respectively. From the other weight loss products, sibutramine was quantified in 6 out of the 14 samples analyzed in wastewater from pumping station C, ranging from 22 to 335 mg $day^{-1}$, and detected in 2 more samples at a level between LOD and LOQ. In the influent of WWTP C, sibutramine was quantified in 5 out of the 16 samples analyzed, ranging from 295 to 1660 mg $day^{-1}$, and detected in 6 more samples at concentrations between LOD and LOQ. 2,4-Dinitrophenol was quantified in 7 out of the 14 samples analyzed in wastewater from pumping station C, ranging from 255 to 9740 mg $day^{-1}$, and detected in 5 more samples at a level between LOD and LOQ, and in the case of WWTP C 2,4-DNP was quantified in 6 out of the 16 influent samples analyzed, ranging from 6390 to 717,000 mg $day^{-1}$, and detected in 10 more samples at a level between LOD and LOQ. Metandienone was quantified in 4 out of 16 samples from the influent of WWTC C, ranging from 761 to 1190 mg $day^{-1}$. Clenbuterol, clomiphene, and nandrolone were just detected at levels between LOD and LOQ (clenbuterol, 1 sample at each location; clomiphene, 2 samples from pumping station C; nandrolone, 2 samples from WWTP C).

Statistical evaluation of the loads during the event and the corresponding reference weekend using two-tailed Mann-Whitney test ($p < 0.05$ significant difference) showed that the medians of both periods and both locations were not significantly different

for ephedrine, norephedrine, and methylhexanamine. Once again in the case of 2,4-dinitrophenol, there was not sufficient data for a statistical evaluation; however, its use just prior to and during the sport event was obvious (at both locations) due to the relatively high levels quantified during those days, while loads were much lower or below LOQs in the reference weekend (see also the next section).

**Normalized loads**

The comparison of the normalized loads per compound is presented in **Fig. 4.1.1**. In the case of norephedrine, ephedrine, and methylhexanamine, the trend is similar. Lower normalized loads correspond to the small-size city sampled at WWTP B. In the large and medium-size cities (A and C), the normalized loads appear to be similar. One characteristic that might support this difference between A and C against B is the relative number of students in each of the cities. Whereas in A and C, the percentage of residing students represents a 14% of the total population, in B it only represents 0.2% ("https://www.studiekeuze123.nl/steden, accessed on 30-08-2017"). In the past, many studies have revealed the use of stimulant drugs by college students for cognitive enhancement, specially the use of methylphenidate, the active pharmaceutical ingredient for the treatment of attention deficit hyperactivity disorder, but also other type of prescription and lifestyle stimulant drugs. A recent study in the Netherlands showed evidence of polydrug use with this purpose, although with lower prevalence than in other countries in Europe (1.6 versus 4.6–16%) (Schelle et al., 2015).

It is noteworthy that the normalized loads from the pumping station, which correspond to a small part (20%) of the larger city C, also match with the larger cities (A and C). This might be explained by the effect of the sport event. Whereas event A was held for professional athletes and official anti-doping controls were performed, events B and C were held for amateur athletes and no anti-doping measures were taken. Especially event C is also known among the amateur bodybuilding community, and not only the participants might use illicit substances but also the event visitors.

The normalized loads of 2,4-dinitrophenol clearly reveal an aberrantly high load during the B and C events. This compound was originally used in the manufacture of dyes, wood preservatives, and as a pesticide. However, another use was discovered as solution for rapid weight loss. Although it seems an effective solution to this end, it is highly toxic, and even small overdoses have been reported to result in death (Miranda et al., 2006; Tewari et al., 2009). The high loads found during the events

(including the day before the events start, but not before, and not after) might indicate the use as weight loss, since such a sudden increase/decrease would not be expected in any other of its known uses, for example as a pesticide. It is therefore of great concern to discover its use, and measures should be taken to inform and bring awareness to the athletes community, not only restricted to anti-doping.

Finally, an interesting finding is the relative absence of detection of analytes from the group of anabolic steroids. However, it is important to stress that the targeted analytes in this method are the parent compounds, and they might not be the best biomarkers due to excretion as conjugates or in the form of metabolites or transformation products. Temperatures of the wastewaters were such that for a few compounds some degradation may have occurred in-sewer (see **Table 1** and SM **Table G3**). Therefore, estimation of the use of these compounds in wastewater would require a more in-depth study on the most suitable biomarkers to be determined in this complex matrix.

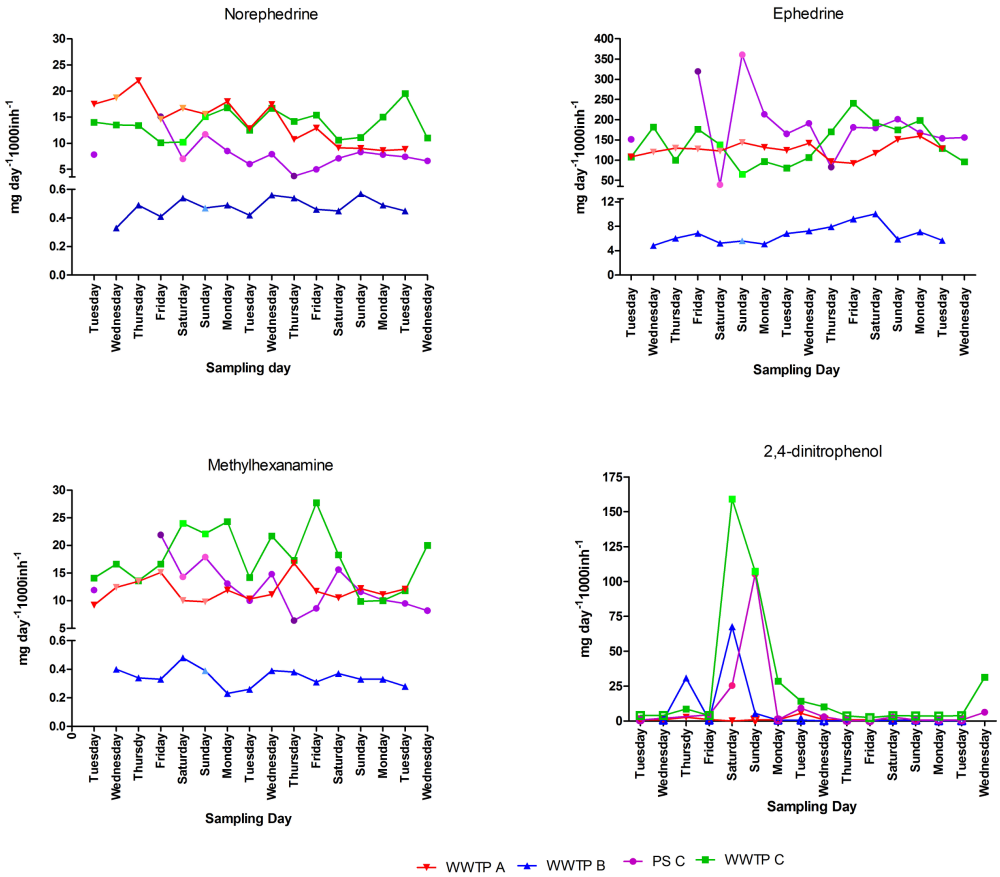


**Fig. 4.1.1.** Normalized loads (expressed in mg day$^{-1}$ 1000 inh$^{-1}$) of the most detected weight loss products (ephedrine, norephedrine, methylhexanamine, and 2,4-dinitrophenol) per sampling location

# 4.1.4. Conclusions

Chemical analysis of wastewater can reveal the use of doping agents by the general population and during sport events. The results of the present study provided valuable information that can be of interest for anti-doping authorities. The analytical methodology developed in this work, based on the use of LCMS/MS, allowed the detection and quantification in wastewater of doping substances used by the general population and amateur athletes attending the targeted events. Weight loss stimulants, namely ephedrine, norephedrine, and methylhexanamine, were found in high amounts. In addition, the detection of 2,4-dinitrophenol is a major concern due to its known adverse effects. The results suggested the increase in loads of some substances possibly during the monitored sport events.

Further refinement of analysis to include metabolites and transformation products would provide valuable information, especially in the cases of compounds rapidly metabolized by the human body and therefore not present in the urine and wastewater (or present in minor quantities).

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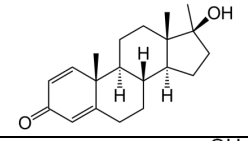
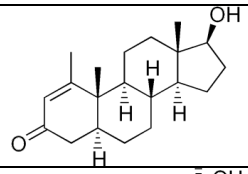
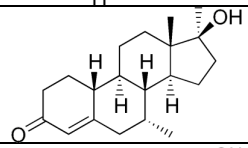
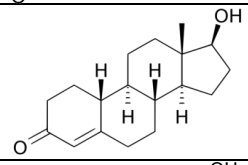
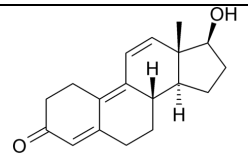
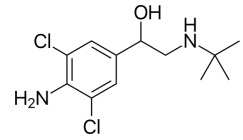
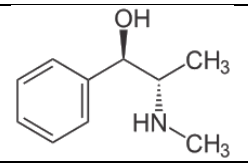
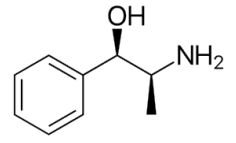
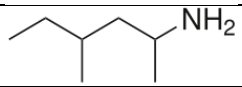
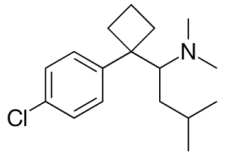
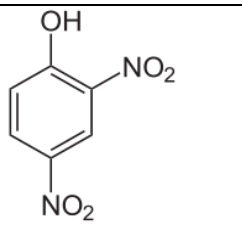
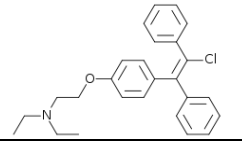
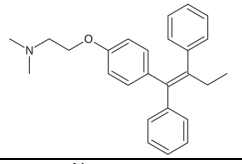
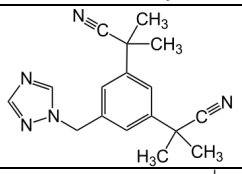
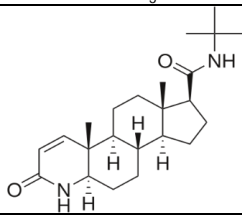
# Annex G - Supplementary Information Chapter 4.1.

**Table G1.** List of the selected target compounds with their chemical properties description.

| Name | Type of action | ATC code | CAS number | Formula | Log Kow | pKa[1] | Structure |
|---|---|---|---|---|---|---|---|
| Metandienone | anabolic-androgenic steroid | A14AA03 | 72-63-9 | $C_{20}H_{28}O_2$ | 3.51 | -0.53 - 14.53 |  |
| Metenolone | anabolic-androgenic steroid | A14AA04 | 153-00-4 | $C_{20}H_{30}O_2$ | 3.69 | -0.88 – 19.38 |  |
| Mibolerone | anabolic-androgenic steroid | None | 3704-09-4 | $C_{20}H_{30}O_2$ | 3.19 | -0.53 - n.a. |  |
| Nandrolone | anabolic-androgenic steroid | A14AB01 S01XA11 | 434-22-0 | $C_{18}H_{26}O_2$ | 2.62 | -0.88 – 19.28 |  |
| Trenbolone | anabolic-androgenic steroid | none | 10161-33-8 | $C_{18}H_{22}O_2$ | 2.65 | -0.89 – n.a. |  |
| Clenbuterol | β2 agonist – performance-enhancing drug, also weight-loss | R03AC14 R03CC13 | 37148-27-9 | $C_{12}H_{18}Cl_2N_2O$ | 2.00 | 9.63 – 14.06 |  |
| Ephedrine | Weight-loss – substitited phenethylamine stimulant | C01CA26 | 299-42-3 | $C_{10}H_{15}NO$ | 1.13 | 9.52 – 13.89 |  |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Norephedrine | Weight-loss – substitited phenethylamine stimulant | none | 14838-15-4 | $C_9H_{13}NO$ | 0.67 | 9.37 – 13.90 |  |
| Methylhexaneamine | Weight-loss - sympathomimetic drug | none | 105-41-9 | $C_7H_{17}N$ | 2.16 | 10.54 – n.a. |  |
| Sibutramine | Weight-loss – serotonin-norepinephrine reuptake inhibitor | A08AA10 | 106650-56-0 | $C_{17}H_{26}NCl$ | 5.73 | 9.77 – n.a. |  |
| 2,4-dinitrophenol | Weight-loss | none | 51-28-5 | $C_6H_4N_2O_5$ | 1.67 | -7.80 – 4.04 |  |
| Clomiphene | hormone antagonist - antiestrogen | G03GB02 | 911-45-5 | $C_{26}H_{28}ClNO$ | 6.74 | 9.31 – n.a. |  |
| Tamoxifen | hormone antagonist - antiestrogen | L02BA01 | 10540-29-1 | $C_{26}H_{29}NO$ | 6.30 | 8.76 – n.a. |  |
| Anastrozole | hormone antagonist - antiestrogen | L02BG03 | 120511-73-1 | $C_{17}H_{19}N_5$ | 2.37 | 2.25 – n.a. |  |
| Finasteride | hormone antagonist - 5-α-reductase-inhibitor | D11AX10 G04CB01 | 98319-26-7 | $C_{23}H_{36}N_2O_3$ | 3.03 | 2.22 – 14.53 |  |

[1] Strongest basic – strongest acidic (ChemAxon)

None: not available or not existing

**Table G2**. LC-HRMS acquisition parameters: $t_R$, $[M+H]^+$ (*except $[M-H]^-$ for 2,4-dinitrophenol), optimal collision energy (CE), and accurate mass for at least one product ion.

| | $t_R$ (min) | $[M+H]^+$ | CE | $[M+H]^+$ (product ion1) | $[M+H]^+$ (product ion2) | ILIS used |
|---|---|---|---|---|---|---|
| Norephedrine | 4.1 | 152.1070 | 40 | 91.0539 | 115.0536 | Norephedrine-$d_3$ |
| Ephedrine | 4.5 | 166.1226 | 50 | 91.0538 | 115.0532 | Ephedrine-$d_3$ |
| Methylhexanamine | 6.3 | 116.1434 | 15 | 57.0699 | — | Methylhexanamine-$d_4$ |
| Clenbuterol | 6.6 | 277.0869 | 30 | 203,0132 | 168,0441 | Clenbuterol-$d_9$ |
| Anastrozole | 8.8 | 294.1713 | 25 | 225,1388 | 210.1150 | Anastrozole-$d_{12}$ |
| Sibutramine | 10.5 | 280.1827 | 25 | 125.0145 | 139.0301 | Sibutramine-d6 |
| Trenbolone | 11.2 | 271.1693 | 25 | 199.1115 | 253.1594 | Trenbolone-$d_5$ |
| Nandrolone | 11.5 | 275.2006 | 32 | 109.0635 | 145.0997 | Methandrostenolone-$d_3$ |
| Metandienone | 11.7 | 301.2162 | 25 | 121.0632 | 149.1308 | Methandrostenolone-$d_3$ |
| Finasteride | 11.9 | 373.2850 | 32 | 317.2237 | 305.2598 | Finasteride-$d_9$ |
| Clomiphene | 12.0 | 406.1932 | 30 | 100.1113 | 297.1278 | Clomiphene-$d_5$ |
| Mibolerone | 12.3 | 303.2319 | 30 | 285.2197 | 121.0992 | Mibolerone-$d_3$ |
| Metenolone | 12.5 | 303.2319 | 30 | 187.1472 | 83.0482 | Mibolerone-$d_3$ |
| Tamoxifen | 12.4 | 372.2322 | 35 | 72.0807 | 129.0690 | Tamoxifen-$d_5$ |
| 2,4-dinitrophenol | 8.7 | 183.0047* | -30 | 123.0440* | — | 2,4-dinitrophenol-$d_3$ |

**Table G2 (continued)**. LC-HRMS acquisition parameters: $t_R$, $[M+H]^+$ (*except $[M-H]^-$ for 2,4-dinitrophenol), optimal collision energy (CE), and accurate mass for at least one product ion.

| ILIS | $t_R$ (min) | $[M+H]^+$ | CE | $[M+H]^+$ (product ion 1) | $[M+H]^+$ (product ion 2) |
|---|---|---|---|---|---|
| Norephedrine-$d_3$ | 4.1 | 155.1258 | 35 | 93.0663 | 117.0662 |
| Ephedrine-$d_3$ | 4.5 | 169.1415 | 30 | 136.1067 | 117.0692 |
| Methylhexanamine-$d_4$ | 6.2 | 120.1685 | 15 | — | — |
| Clenbuterol-$d_9$ | 6.5 | 286.1434 | 30 | 204.0178 | 169.0487 |
| Anastrozole-$d_{12}$ | 8.7 | 306.2466 | 25 | 237.2123 | — |
| Sibutramine-$d_6$ | 10.5 | 286.2203 | 25 | 179.0602 | 153.0446 |
| Trenbolone-$d_5$ | 11.1 | 276.2006 | 25 | 258.1879 | 204.1406 |
| Methandrostenolone-$d_3$ | 11.7 | 304.2350 | 25 | 121.0628 | 152.1491 |
| Finasteride-$d_9$ | 11.8 | 382.3414 | 32 | 317.2237 | 305.2598 |
| Clomiphene-$d_5$ | 12.0 | 411.2246 | 30 | 100.1103 | 302.1567 |
| Mibolerone-$d_3$ | 12.2 | 306.2507 | 30 | 288.2385 | 124.1180 |
| Tamoxifen-$d_5$ | 12.4 | 377.2636 | 35 | 72.0805 | 70.0645 |
| 2,4-dinitrophenol-$d_3$ | 8.6 | 186.0236* | -30 | 126.0270* | — |

**Table G3**. Sample weekday and corresponding daily influent flow rates at the different sampling locations.

| | | Flow $m^3 d^{-1}$ | | | |
|---|---|---|---|---|---|
| | | WWTP A | WWTP B | Event C | |
| | | | | Pumping station[3] | WWTP C |
| Number of inhabitants | | 769,000 | 95,000 | 92,500 | 450,300 |
| Range of temperatures | | 18 – 25 °C | 2 – 10 °C | 5 – 12 °C | 5 – 12 °C |
| Sample day | Tuesday | 155546 | - | 14439 | 96823 |
| | Wednesday | **155708** | 11590 | 14633[2] | 98325 |
| | Thursday | **148062** | 23512 | 15047[2] | 100873 |
| | Friday | **156895** | n.a.[1] | 27527 | 97194 |
| | Saturday | **144113** | 22759 | **14040** | **94546** |
| | Sunday | **142268** | **14933** | **24811** | **136543** |
| | Monday | 144487 | 12194 | 17032 | 114614 |
| | Tuesday | 150896 | 11415 | 14283 | 100593 |
| | Wednesday | 151886 | 11439 | 20023 | 126486 |
| | Thursday | 140841 | 11400 | 7900 | 90095 |
| | Friday | 141184 | 11360 | 11473 | 105499 |
| | Saturday | 134028 | 11833 | 14699 | 94348 |
| | Sunday | 135930 | 10810 | 14341 | 89267 |
| | Monday | 135967 | 10478 | 13606 | 87453 |
| | Tuesday | 134850 | 12282 | 14563 | 94966 |
| | Wednesday | - | 12239 | 14033 | 96754 |

**Values** in bold correspond to the event days; [1] n.a. not available due to pump failure; [2] Autosampler failed and no sample was collected; [3] At the pumping station flow rate measurements are most reliable when between 7,000 and 15,000 $m^3$ (dry weather days). At higher flow rates, caused by heavy rainfall, a bypass is switched on to handle the incoming influent. Flow rates from these days may therefore be underestimated. Notwithstanding, the actual flow rates given by the WWTP operators were used, as no other flow data were available and will provide minimum loads. As the number of days with such events was only minor (3) this will be of little influence on the total picture.

**Table G4**. Results from the re-evaluation of the method performance with spiked wastewater samples from each sampling location in terms of recovery, R (%) and relative standard deviation (RSD, %), limits of detection (LOD, ng $L^{-1}$) and quantification (LOQ, ng $L^{-1}$).

| | WWTP A | | | WWTP B | | | Pumping station C | | | WWTP C | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R ± RSD (%)[1] | LOD | LOQ | R (%)[2] | LOD | LOQ | R ± RSD (%)[1] | LOD | LOQ | R ± RSD (%)[1] | LOD | LOQ |
| Norephedrine | 98 ± 14 | 4 | 14 | 106 | 3 | 9 | 98 ± 2 | 2 | 6 | 94 ± 15 | 1 | 4 |
| Ephedrine | 43 ± 94 | 4 | 12 | 28 | 15 | 49 | 160 ± 19 | 5 | 18 | 20 ± 54 | 5 | 16 |
| Methylhexanamine | 100 ± 5 | 4 | 14 | 102 | 5 | 16 | 85 ± 10 | 4 | 14 | 131 ± 4 | 7 | 22 |
| Clenbuterol | 89 ± 6 | 2 | 8 | 96 | 2 | 6 | 81 ± 2 | 2 | 7 | 87 ± 3 | 2 | 6 |
| Anastrozole | 89 ± 7 | 1 | 4 | 78 | 0 | 1 | 86 ± 12 | 1 | 4 | 90 ± 3 | 1 | 4 |
| Sibutramine | 88 ± 7 | 1 | 3 | 133 | 1 | 5 | 98 ± 12 | 0.5 | 2 | 89 ± 3 | 1 | 4 |
| Trenbolone | 112 ± 7 | 16 | 54 | 106 | 11 | 36 | 135 ± 2 | 13 | 44 | 102 ± 2 | 20 | 68 |
| Nandrolone | 29 ± 11 | 3 | 12 | 66 | 40 | 135 | 18 ± 20 | 12 | 41 | 17 ± 4 | 22 | 72 |
| Metadienone | 97 ± 9 | 3 | 10 | 104 | 12 | 42 | 105 ± 6 | 3 | 10 | 107 ± 3 | 3 | 8 |
| Finasteride | 104 ± 17 | 25 | 82 | 112 | 2 | 6 | 125 ± 22 | 19 | 64 | 170 ± 4 | 19 | 62 |
| Clomiphene | 98 ± 5 | 18 | 61 | 110 | 37 | 128 | 84 ± 8 | 26 | 85 | 92 ± 15 | 25 | 84 |
| Mibolerone | 110 ± 11 | 33 | 111 | 116 | 48 | 160 | 112 ± 4 | 55 | 182 | 127 ± 3 | 49 | 165 |
| Metenolone | 13 ± 21 | 6 | 20 | 83 | 23 | 78 | 12 ± 28 | 11 | 36 | 12 ± 22 | 15 | 51 |
| Tamoxifen | 103 ± 7 | 31 | 103 | 106 | 24 | 81 | 88 ± 14 | 37 | 124 | 94 ± 22 | 48 | 161 |
| 2,4-dinitrophenol[3] | 95 | 9 | 28 | 88 | 3 | 9 | 95 | 3 | 9 | 117 | 11 | 36 |

[1] n=3

[2] n=1, no RSD(%)

[3] n=1, no RSD(%)

**Table G5**. Loads of doping substances expressed in mg $d^{-1}$ quantified in the influent samples collected at the WWTP A.

| Sample day | Norephedrine | Ephedrine | Methylhexanamine | Clenbuterol | Anastrozole | Sibutramine | Trenbolone | Nandrolone |
|---|---|---|---|---|---|---|---|---|
| Tuesday | 13400 | 83200 | 7040 | — | — | 1090 | — | — |
| **Wednesday** | **14400** | **92400** | **9530** | **+** | **—** | **2910** | **—** | **—** |
| **Thursday** | **15400** | **99600** | **10400** | **—** | **—** | **+** | **—** | **—** |
| **Friday** | **11300** | **98000** | **11600** | **—** | **—** | **—** | **—** | **—** |
| **Saturday** | **12900** | **94200** | **7680** | **—** | **—** | **+** | **—** | **—** |
| **Sunday** | **12000** | **111000** | **7560** | **—** | **—** | **—** | **—** | **—** |
| Monday | 13900 | 101000 | 9160 | — | — | — | — | — |
| Tuesday | 9850 | 95500 | 7930 | — | — | — | — | — |
| Wednesday | 13400 | 109000 | 8520 | — | — | 776 | — | — |
| Thursday | 8260 | 74200 | 12900 | — | — | + | — | — |
| Friday | 9890 | 70700 | 9020 | — | — | + | — | — |
| Saturday | 7000 | 90100 | 8050 | — | — | — | — | — |
| Sunday | 6910 | 116000 | 9360 | — | — | — | — | — |
| Monday | 6610 | 122000 | 8530 | — | — | — | — | — |
| Tuesday | 6740 | 98700 | 9310 | — | — | 1270 | — | — |

— below LOD

+ below LOQ

**Values** in bold correspond to the event days.

**Table G5 (continued)**. Loads of doping substances expressed in mg $d^{-1}$ quantified in the influent samples collected at the WWTP A.

| Sample day | Metandienone | Finasteride | Clomiphene | Mibolerone | Metenolone | Tamoxifen | 2,4-dinitrophenol |
|---|---|---|---|---|---|---|---|
| Tuesday | + | — | — | — | — | — | — |
| **Wednesday** | **2020** | **—** | **—** | **—** | **—** | **—** | **—** |
| **Thursday** | **+** | **—** | **—** | **—** | **—** | **—** | **+** |
| **Friday** | **+** | **—** | **—** | **—** | **—** | **—** | **—** |
| **Saturday** | **1480** | **—** | **—** | **—** | **—** | **—** | **—** |
| **Sunday** | **1700** | **—** | **—** | **—** | **—** | **—** | **—** |
| Monday | 1600 | — | — | — | — | — | — |
| Tuesday | — | — | — | — | — | — | 4300 |
| Wednesday | — | — | — | — | — | — | — |
| Thursday | + | — | — | — | — | — | — |
| Friday | 1490 | — | — | — | — | — | — |
| Saturday | + | — | — | — | — | — | — |
| Sunday | + | — | — | — | — | — | — |
| Monday | + | — | — | — | — | — | — |
| Tuesday | + | — | — | — | — | — | — |

— below LOD

+ below LOQ

**Values** in bold correspond to the event days.

**Table G6.** Loads of doping substances expressed in mg $d^{-1}$ quantified in the influent samples collected at the WWTP B.

| Sample day | Norephedrine | Ephedrine | Methylhexanamine | Clenbuterol | Anastrozole | Sibutramine | Trenbolone | Nandrolone |
|---|---|---|---|---|---|---|---|---|
| Wednesday | 474 | 6530 | 478 | — | — | + | — | — |
| Thursday | 730 | 10800 | 904 | — | — | + | — | — |
| Friday | 658 | 8130 | 453 | — | — | + | — | — |
| Saturday | 894 | 14800 | 710 | — | — | + | — | — |
| **Sunday** | **764** | **7400** | **676** | **—** | **—** | **+** | **—** | **—** |
| Monday | 543 | 6480 | 455 | — | — | + | — | — |
| Tuesday | 526 | 5520 | 254 | — | — | — | — | — |
| Wednesday | 462 | 7410 | 279 | — | — | — | — | — |
| Thursday | 609 | 7860 | 421 | — | — | 90 | — | — |
| Friday | 580 | 8550 | 412 | — | — | + | — | — |
| Saturday | 519 | 10400 | 352 | — | — | + | — | — |
| Sunday | 457 | 10300 | 384 | — | — | + | — | — |
| Monday | 565 | 5850 | 332 | — | — | + | — | — |
| Tuesday | 567 | 8240 | 384 | — | — | — | — | — |
| Wednesday | 474 | 6530 | 478 | — | — | — | — | — |

— below LOD

+ below LOQ

**Values** in bold correspond to the event days.

**Table G6 (continued).** Loads of doping substances expressed in mg $d^{-1}$ quantified in the influent samples collected at the WWTP B.

| Sample day | Metandienone | Finasteride | Clomiphene | Mibolerone | Metenolone | Tamoxifen | 2,4-dinitrophenol |
|---|---|---|---|---|---|---|---|
| Wednesday | + | — | — | — | — | — | + |
| Thursday | + | — | — | — | — | — | 2930 |
| Friday | + | — | — | — | — | — | + |
| Saturday | + | — | — | — | — | — | 6430 |
| **Sunday** | **+** | **—** | **—** | **—** | **—** | **—** | **516** |
| Monday | + | — | — | — | — | — | + |
| Tuesday | + | — | — | — | — | — | + |
| Wednesday | + | — | — | — | — | — | — |
| Thursday | + | — | — | — | — | — | — |
| Friday | + | — | — | — | — | — | — |
| Saturday | + | — | — | — | — | — | + |
| Sunday | + | — | — | — | — | — | + |
| Monday | + | — | — | — | — | — | — |
| Tuesday | + | — | — | — | — | — | — |
| Wednesday | + | — | — | — | — | — | — |

— below LOD

+ below LOQ

**Values** in bold correspond to the event days.

**Table G7.** Loads of doping substances expressed in mg $d^{-1}$ quantified in the influent samples collected at the pumping station C.

| | Norephedrine | Ephedrine | Methylhexanamine | Clenbuterol | Anastrozole | Sibutramine | Trenbolone | Nandrolone |
|---|---|---|---|---|---|---|---|---|
| Tuesday | 721 | 14000 | 1100 | + | — | 335 | — | — |
| Friday | 1400 | 29500 | 2020 | — | — | 70 | — | — |
| Saturday | 651 | 3630 | 1320 | — | — | 22 | — | — |
| Sunday | 1080 | 33300 | 1660 | — | — | + | — | — |
| Monday | 791 | 19700 | 1210 | — | — | 35 | — | — |
| Tuesday | 553 | 15300 | 926 | — | — | — | — | — |
| Wednesday | 727 | 17700 | 1370 | — | — | + | — | — |
| Thursday | 341 | 7650 | 594 | — | — | — | — | — |
| Friday | 463 | 16700 | 796 | — | — | — | — | — |
| Saturday | 655 | 16600 | 1440 | — | — | — | — | — |
| Sunday | 767 | 18600 | 1070 | — | — | — | — | — |
| Monday | 722 | 15500 | 936 | — | — | — | — | — |
| Tuesday | 685 | 14200 | 880 | — | — | 41 | — | — |
| Wednesday | 609 | 14400 | 755 | — | — | 78 | — | — |

— below LOD

+ below LOQ

**Values** in bold correspond to the event days.

**Table G7 (continued).** Loads of doping substances expressed in mg $d^{-1}$ quantified in the influent samples collected at the pumping station C.

| | Metandienone | Finasteride | Clomiphene | Mibolerone | Metenolone | Tamoxifen | 2,4-dinitrophenol |
|---|---|---|---|---|---|---|---|
| Tuesday | — | — | + | — | — | — | + |
| Friday | — | — | — | — | — | — | 397 |
| **Saturday** | **—** | **—** | **—** | **—** | **—** | **—** | **2350** |
| **Sunday** | **—** | **—** | **—** | **—** | **—** | **—** | **9740** |
| Monday | — | — | — | — | — | — | + |
| Tuesday | — | — | — | — | — | — | 849 |
| Wednesday | — | — | — | — | — | — | 273 |
| Thursday | — | — | — | — | — | — | + |
| Friday | — | — | — | — | — | — | — |
| Saturday | — | — | — | — | — | — | 255 |
| Sunday | — | — | — | — | — | — | — |
| Monday | — | — | — | — | — | — | + |
| Tuesday | — | — | — | — | — | — | + |
| Wednesday | — | — | + | — | — | — | 581 |

— below LOD

+ below LOQ

**Values** in bold correspond to the event days.

**Table G8.** Loads of doping substances expressed in mg $d^{-1}$ quantified in the influent samples collected at the WWTP C.

| Sample day | Norephedrine | Ephedrine | Methylhexanamine | Clenbuterol | Anastrozole | Sibutramine | Trenbolone | Nandrolone |
|---|---|---|---|---|---|---|---|---|
| Tuesday | 6320 | 48300 | 6340 | — | — | 468 | — | — |
| Wednesday | 6090 | 81800 | 7460 | — | — | 295 | — | — |
| Thursday | 6050 | 44800 | 6110 | — | — | + | — | — |
| Friday | 4540 | 79400 | 7480 | — | — | + | — | — |
| **Saturday** | **4590** | **62100** | **10800** | **—** | **—** | **+** | **—** | **—** |
| **Sunday** | **6820** | **29300** | **9960** | **—** | **—** | **+** | **—** | **+** |
| Monday | 7550 | 43500 | 11000 | — | — | + | — | + |
| Tuesday | 5610 | 36200 | 6380 | — | — | — | — | — |
| Wednesday | 7540 | 47800 | 9760 | — | — | — | — | — |
| Thursday | 6400 | 76600 | 7780 | — | — | + | — | — |
| Friday | 6930 | 108000 | 12500 | — | — | — | — | — |
| Saturday | 4760 | 86600 | 8250 | — | — | — | — | — |
| Sunday | 4980 | 78700 | 4450 | — | — | — | — | — |
| Monday | 6750 | 89300 | 4520 | — | — | 776 | — | — |
| Tuesday | 8790 | 58100 | 5330 | + | — | 1660 | — | — |
| Wednesday | 4960 | 43100 | 9020 | — | — | 355 | — | — |

— below LOD

+ below LOQ

**Values** in bold correspond to the event days.

**Table G8 (continued).** Loads of doping substances expressed in mg $d^{-1}$ quantified in the influent samples collected at the WWTP C.

| Sample day | Metandienone | Finasteride | Clomiphene | Mibolerone | Metenolone | Tamoxifen | 2,4-dinitrophenol |
|---|---|---|---|---|---|---|---|
| Tuesday | 1190 | — | — | — | — | — | + |
| Wednesday | + | — | — | — | — | — | + |
| Thursday | 761 | — | — | — | — | — | 3870 |
| Friday | + | — | — | — | — | — | + |
| **Saturday** | **+** | **—** | **—** | **—** | **—** | **—** | **71700** |
| **Sunday** | **+** | **—** | **—** | **—** | **—** | **—** | **48300** |
| Monday | + | — | — | — | — | — | 12800 |
| Tuesday | — | — | — | — | — | — | 6390 |
| Wednesday | 1050 | — | — | — | — | — | + |
| Thursday | + | — | — | — | — | — | + |
| Friday | — | — | — | — | — | — | — |
| Saturday | — | — | — | — | — | — | + |
| Sunday | — | — | — | — | — | — | + |
| Monday | — | — | — | — | — | — | + |
| Tuesday | 1130 | — | — | — | — | — | + |
| Wednesday | — | — | — | — | — | — | 14100 |

— below LOD
+ below LOQ
**Values** in bold correspond to the event days.

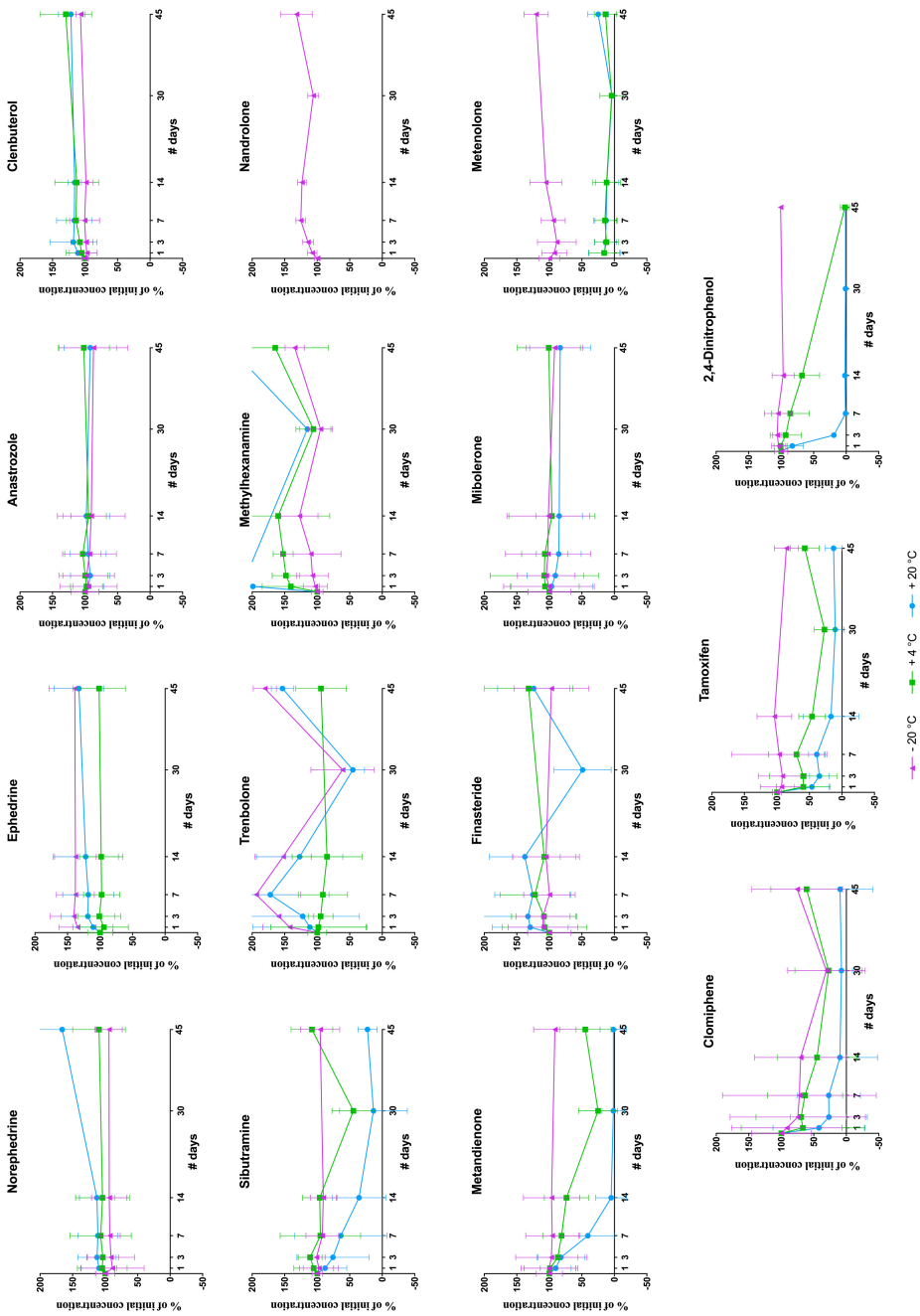



**Fig. G1**. Stability plots for 15 of the studied compounds in wastewater at natural pH and 3 different temperatures. Results have been normalized as percentage of initial concentration. Error bars correspond to the standard deviation of triplicates.

# Chapter 5

# Synthesis

Wastewater-based epidemiology was at its infancy when this PhD project started back in March 2013. Until then, the idea that the chemical analysis of raw wastewater could provide objective information regarding drug use had been introduced as an extension of the existing methods that monitored pharmaceuticals in surface waters such as antibiotics and other products from human activities including personal care products (Daughton, 2001a, 2001b). Researchers working in this newly born field joined forces to propose a best practice protocol that would ensure the production of reliable results. With that ultimate goal in mind, critical uncertainties and knowledge gaps were identified (Castiglioni et al., 2013).

The International Training Network SEWPROF, funded by the Marie Curie Actions of the European Union Seventh Framework Programme, aimed at the development of interdisciplinary and cross-sectoral research capability for the next generation of scientists working in this newly-emerging field of wastewater-based epidemiology. The major outcomes of the project have been the development of robust sampling approaches, the investigation towards a better understanding of biomarkers transformation in-sewer and in wastewater, the validation of new methodologies utilizing hyphenated (mass spectrometry-based) techniques for targeted analysis, screening of unknowns and retrospective analysis of wastewater samples, and the standardization of the WBE approach for novel applications to assess community-wide health and lifestyle, namely levels of oxidative stress, exposure to environmental agents such as pesticides or phthalates, or alcohol use.

The work described in this thesis has contributed to strengthen the role of analytical chemistry within the WBE approach, and has resulted in analytical methods and workflows for new compounds and in new data that reflect the consumption of lifestyle chemicals by human populations. The selection of the substances of interest was inspired by the need of expanding the applicability of WBE to different fields as proposed initially by (Thomas and Reid, 2011).

**From multi-residue to ad hoc analytical methods**

The general approach in analytical methods development based on chromatography coupled to mass spectrometry has usually aimed at targeted multi-residue methods, taking advantage of the combined resolution, selectivity and sensitivity of this hyphenated technique that allows the inclusion of a notable number of compounds in one single analysis (Hernández et al., 2014). Nevertheless, this multi-residue approach might fail when specific compounds within a targeted group have varying

physicochemical properties or are expected at very different concentration levels, as was the case for two types of illicit drugs studied in this thesis, namely for cannabis and NPS.

The work presented in this thesis contributed to identify these compounds for which previous attempts to be incorporated in WBE had not been successful. The identification of the nature of these substances in wastewater and the need of an ad hoc strategy were the key. In the case of the cannabis biomarker, carboxy-THC, it was the hydrophobic nature of the compound compared to other illicit drugs, which required special attention during the analytical treatment. In the case of NPS, the challenge came from the broad possibility of substances from different chemical families and the low concentrations encountered in wastewater due to their use only by a minority of the population. The selection of the suspect screening strategy, as an added feature offered by high-resolution mass spectrometry (Hernández et al., 2016) was crucial here. In both examples, a maturation of the WBE approach was achieved by taking this step from multi-residue to ad hoc methods.

For the future development of analytical methods within the WBE approach it will be important to bear in mind the necessity of including a concentration step in order to increase method sensitivity, and selecting the appropriate sample treatment to that end that will accommodate acid, basic and neutral compounds with different solubility ranges. Furthermore, the development of robust HRMS methods and workflows, incorporating the use of specific software, will be an advantage to process the acquired data and reduce the workload.

These newly available analytical methods to monitor the prevalence of illicit drugs and other lifestyle substances will enable us to evaluate changes in trends and spatial and temporal differences within the population in a rapid and low-cost manner. This will be particularly important as urbanization, population growth and climate change continue to have an impact on public health.

**New data in different contexts**

From a social perspective, the new data that has been obtained from the different samples and situations described in the chapters of this thesis has helped to objectively understand certain habits of the population in the communities monitored. In general terms, the WBE approach, by measuring endogenous and

exogenous biomarkers pooled in urban wastewater, allows the development of a public health early warning system.

As was foreseen by Thomas and Reid et al (2011), it is now possible to monitor the success of specific actions of law enforcement against drug use. This is also possible for the case of anti-doping or anti online rogue pharmacies policies, as illustrated in this thesis. Furthermore, other applications with great potential will be developed. For example, in the field of forensics, by allowing the comparison of the epidemiological data with crime statistics (Been et al., 2016) or being able to trace the production facilities of illicit drugs by monitoring the precursors and by-products from the synthesis waste that is discharged into sewers until the source (Emke et al., 2018).

Another interesting novel application of WBE is the possibility of monitoring the stress level within a community (Y. Ryu et al., 2016) or the exposure of the population to environmental threats such as plasticizers or pesticides (González-Mariño et al., 2017; Rousis et al., 2017).

In the future, research must continue to study which new biomarkers will give the appropriate information in the interest of public health diagnostics. The determination in wastewater of endogenous biomarkers from the most pressing illnesses from our current times, namely cancer or Alzheimer, will provide valuable information that might help to predict and mitigate future crises before they become manifest and expand towards an increasing number of the population, or even more severe, turn into pandemics.

**Environmental context**

Apart from the societal implications that the work described in this thesis can have, the environmental context of the work deserves further discussion. Municipal wastewater treatment plants, even the more sophisticated ones, have not been designed to get rid of lifestyle chemicals and pharmaceuticals present in influent wastewater. Consequently, several of the lifestyle chemicals that are flushed down the toilets, in particular the more polar ones, will not or incompletely be removed in the treatment plant and thus end up in the effluent and the receiving waters. Several studies have shown that e.g. some of the drugs of abuse considered in the present thesis are passing the wastewater treatment plants almost unhindered, e.g. ketamine, methadone and MDMA (Andrés-Costa et al., 2014; Bijlsma et al., 2012).

Thus far there is not sufficient data on the ecotoxicological effects of the concentration levels of chemicals released to the environment in aquatic species. Further investigations into which removal mechanisms can eliminate DOA from influents and how these can be implemented in existing wastewater treatment technologies are necessary if we would like to protect our environment for future generations.

In addition, knowing removal rates in wastewater treatment processes would allow one to estimate drug loads emitted to receiving waters and modelling the resulting concentrations, so that a proper environmental exposure assessment can be made. These model results could then be combined with no effect concentrations of the compounds, tested with different aquatic species, in order to allow a complete risk assessment to be made.

To finalise, further research would be very interesting towards a better conversion of wastewater into reusable/drinkable water. The reason is obvious as water scarcity is a worldwide pressing matter, a consequence of our exponentially growing population and urbanisation. And considering that only a small percentage of the water resources in planet Earth is fresh and able to be used for drinking, a better (waste)water treatment becomes crucial.

# Summary

The research presented in this thesis focuses on the essential role of analytical chemistry to develop advanced methodology for the reliable determination of illicit drugs and also other licit and illicit substances in the aquatic environment, to support the hypothesis that WBE approach can be used as an alternative and non-intrusive technique that provides information about a population's health and lifestyle habits. Liquid chromatography hyphenated to different mass spectrometers, employing both low and high resolution, and using target and suspect acquisition strategies, has been investigated to this end. Chapter 1 provided the background to the research, an overview of the substances included in the study and the specific objectives.

In Chapter 2, different challenges affecting the monitoring of illicit drugs were tackled. The first challenge was the inclusion of cannabis in WBE studies. In general, drugs and their biomarkers are small and hydrophilic compounds, and therefore their determination in the aquatic environment via LC-MS is straight forward. However, carboxy-THC, which is the urinary biomarker of cannabis, is a lipophilic molecule (log $D_{ow}$ ~4) which has the potential to partition to particulate matter and adsorb onto hydroxyl sites present on the surface of glassware. These specific physico-chemical properties prevented its correct determination using multi-residue methods. The results obtained in the study presented in chapter 2.1 should be used to define the way forward towards more accurate determination of carboxy-THC in wastewater. The adjustment of pH was identified as a critical step in sample processing. And although the results among all labs participating in the inter laboratory study varied by approximately 30% and therefore were higher than optimal, the proposed protocol was successfully tested, and should be therefore recommended for future WBE applications.

The second challenge was the lack of WBE data from different world regions. At the start of the thesis work, WBE had been applied mainly in Europe (Ort et al., 2014; Thomas et al., 2012). In recent years, many studies have been conducted in North America (Banta-Green et al., 2009; Burgard et al., 2013), Australia (Lai et al., 2016; Tscharke et al., 2015) and Asia (Kim et al., 2015). The work performed in Chapter 2.2, providing results from a sampling campaign performed in Costa Rica, contributed to fill this gap and extend the global knowledge derived from the chemical analysis of wastewater to Central America. Our results showed high concentration levels of the biomarkers of the classic drugs cocaine and cannabis, and moderate presence of the opiates codeine and morphine. These findings supported the established drug use

pattern described by classical epidemiological tools. The absence of synthetic phenethylamines, such as amphetamine, methamphetamine and MDMA (ecstasy), which are commonly found in wastewaters collected in other regions of the world, was noteworthy; as well as the absence of benzodiazepines and other drugs such as heroine. Geographical differences in drugs use were thus identified. In addition, not only the social context of drug use but also the environmental context was evaluated. The analysis of surface waters from nearby locations to two WWTPs revealed that the application of more or less sophisticated treatment technologies did not or only partly eliminate drugs residues, and these were eventually released to the environment, although at relatively low concentrations.

The third topic was the inclusion of NPSs in WBE studies. The main challenges for these substances are the reduced number of users that translates into low concentrations of their residues in wastewater, and the limited pharmacokinetics information available, which renders the choice of target biomarker difficult. Chapter 2.3 showed how sampling during specific social events where users are likely to gather, in combination with the analysis using high resolution mass spectrometry and a specific "suspect" screening processing workflow based on a database search, was very useful to narrow the quest for NPS in wastewater analysis.

The work performed in Chapter 2 contributed to the maturation of WBE as a complimentary epidemiological source of information, so that authorities such as the EMCDDA can have a better picture of the prevalence of illicit drugs.

In chapters 3 and 4, the aim was to go a little further and extrapolate the WBE approach from monitoring mainly illicit drugs to including also other pharmaceuticals with potential for abuse, such as counterfeit medicines. In these chapters erectile dysfunction products, and (il)licit substances used in the sport doping context were investigated. To this aim, two new analytical methods were developed, validated and applied to wastewater samples.

Chapter 3 focused on PDE5 inhibitors, active pharmaceutical ingredients in erectile dysfunction products. A simple, but at the same time sensitive method, was validated, consisting of the filtration of the samples followed by direct analysis with liquid chromatography coupled to tandem mass spectrometry with triple quadrupole analyser. The method was able to quantify sildenafil and its two human metabolites at the very low ng $L^{-1}$ range. In addition to analysing real wastewater samples, sales data from pharmacies and medical prescriptions were gathered in order to enable a comparison of prescription-derived concentration levels with actual ones. This was

done at the national Dutch level in chapters 3.1 and 3.2, and at European level in Chapter 3.3. Despite the limitations of the comparison, the WBE approach appeared to be successful for tracking rogue pharmacies and counterfeit medication. This was specially the case in the Netherlands, where the unexplained fraction of sildenafil in wastewater accounted for at least 60% of the total loads observed.

Chapter 4 focused on (il)licit substances used in the sport doping context. The analytical method based on SPE followed by UPLC-MS/MS allowed the detection and quantification in wastewater of doping substances used by the general population and amateur athletes attending targeted events. Weight-loss stimulants, namely ephedrine, norephedrine and methylhexanamine, were found in high amounts. In addition, the detection of 2,4-dinitrophenol was of concern due to its known adverse effects. The results suggested an increase in loads of some substances during the monitored sport events. WBE was thus shown to be a valuable complimentary approach for national doping authorities, as it was able to provide information on the usage of known compounds in a specified area or group, albeit at the (averaged) group level.

Finally, the synthesis presented in Chapter 5 discussed the contribution of this thesis in strengthening the role of analytical chemistry in WBE, and the implications of the obtained data in a social and environmental context.

# Samenvatting

Het onderzoek dat beschreven is in dit proefschrift had als voornaamste doel het ontwikkelen van analytische meetmethoden om zowel verboden als toegelaten drugs en geneesmiddelen in water te kunnen bepalen. Rioolwaterepidemiologie (RWE) – de chemische analyse van residuen van o.a. drugs en geneesmiddelen in rioolwater - is een nieuwe en alternatieve aanpak om informatie over de gezondheid en levensstijl van populaties te verkrijgen. In dit proefschrift is onderzocht in hoeverre vloeistof-chromatografie gekoppeld aan diverse soorten massaspectrometers (LC-MS) voor dit soort analyses gebruikt kan worden.

Hoofdstuk 1 beschrijft de aanleiding en achtergronden voor het onderzoek, geeft een overzicht van de stoffen waarnaar is gekeken en presenteert de doelen van het onderzoek.

In hoofdstuk twee zijn enkele uitdagingen behandeld die zich aandienen bij het monitoren van verdovende middelen. De eerste betrof het opnemen van cannabis in RWE. Over het algemeen zijn drugs en de biomarkers daarvan (dat zijn residuen die met de urine worden uitgescheiden) kleine moleculen met een hydrofiel karakter, en is de analyse daarvan met LC-MS niet met veel problemen behept. Voor carboxy-THC, wat de belangrijkste biomarker van cannabis is, geldt echter dat het een lipofiel molecule is (log Dow ~4) dat makkelijk adsobeert aan deeltjes of aan de wand van materialen. Deze eigenschappen van carboxy-THC maakten het opnemen ervan in multi-residuemethoden erg lastig. De resultaten die in hoofdstuk 2.1 zijn beschreven laten zien dat de keuze van de pH bij de monsterbehandeling van doorslaggevend belang is voor een nauwkeurigere en betrouwbaardere bepaling van cannabisgehalten in rioolwater. In een ringonderzoek, waarin de aanbevelingen uit dit hoofdstuk werden gehanteerd, werd een variatie van 30% tussen de deelnemende laboratoria gevonden, hetgeen een acceptabele variatie is wanneer dit wordt vergeleken met eerdere metingen. Alhoewel dit getal nog wel voor verdere verbetering vatbaar is, laat het tevens zien dat de aanbevelingen opgevolgd moeten worden om toekomstige resultaten van THC die met RWE gemeten worden voldoende betrouwbaar te laten zijn.

De tweede uitdaging werd gevormd door het gebrek aan RWE meetgegevens uit sommige delen van de wereld. Bij de aanvang van het promotieonderzoek was RWE voornamelijk in Europa toegepast. Inmiddels zijn er ook vele studies in Noord-Amerika, Australië en Azië uitgevoerd. Het onderzoek beschreven in hoofdstuk 2.2

betrof metingen van monstercampagnes verricht in Costa Rica. Op deze manier werden gegevens uit een van de niet eerder onderzochte continenten gegenereerd waarmee de dataset over de globale verspreiding van drugs is uitgebreid. De resultaten lieten zien dat er relatief hoge concentraties van de biomarkers van cocaïne en cannabis voorkomen in rioolwater, en dat ook codeïne en morfine aanwezig waren. Deze resultaten zijn in overeenstemming met gegevens over drugsgebruik aldaar afkomstig van ander (sociaal-)epidemiologische onderzoek. Opmerkelijk was de bevinding dat er in het rioolwater geen sporen werden aangetroffen van de synthetische phenetylamines zoals amfetamine, metamfetamine of MDMA (ecstasy), drugs die in andere delen van de wereld meestal wel in rioolwater aanwezig zijn. Benzodiazepines of heroïne leken evenmin aanwezig. Er zijn dus duidelijke geografische verschillen in het gebruik van drugs. Van de twee afvalwaterzuiveringsinstallaties die in Costa Rica werden onderzocht werden ook effluenten bemonsterd, waaruit bleek dat deze installaties niet of maar in beperkte mate de drugsrestanten verwijderen. Inderdaad werden er benedenstrooms in oppervlaktewater, weliswaar lage, concentraties van de drugs gemeten, zodat we kunnen concluderen dat deze stoffen daadwerkelijk in het milieu terechtkomen.

De derde uitdaging was om RWE ook voor nieuwe psychoactieve stoffen (NPS) te kunnen gebruiken. Hierbij spelen twee zaken een rol: NPS worden slechts door een beperkt aantal gebruikers ingenomen, zodat de residueconcentraties in het rioolwater laag zijn. Bovendien is er maar weinig farmacokinetische kennis beschikbaar over deze stoffen, zodat het lastig is om een geschikte biomarker te vinden. Hoofdstuk 2.3 beschrijft hoe monstername tijdens speciale evenementen waarbij veel gebruikers worden verwacht, in combinatie met hoge resolutie massapectrometrische analyse, waarbij een speciale workflow voor suspect screening gebaseerd op database zoekfuncties werd gebruikt, met succes kon worden toegepast bij de zoektocht naar NPS in rioolwater.

De resultaten van de studies beschreven in hoofdstuk twee hebben bijgedragen aan de verdere ontwikkeling van RWE als een complementair stuk gereedschap voor het vergaren van informatie over drugsgebruik en –verspreiding, dat gebruikt kan worden door de autoriteiten, zoals het Europees Agentschap voor Drugs en Drugsverslaving (EMCDDA).

In hoofdstuk 3 en 4 is onderzocht of RWE ook gebruikt kan worden bij het opsporen van gebruik of misbruik van (vervalste) geneesmiddelen. Het onderzoek was gericht op middelen tegen erectiestoornissen en stoffen die als doping worden gebruikt.

Hiervoor werden twee nieuwe analytische methoden ontwikkeld, gevalideerd en toegepast op rioolwatermonsters.

Een relatief eenvoudige, maar tegelijkertijd gevoelige methode voor de bepaling van PDE5 inhibitoren, werd ontwikkeld en is beschreven in hoofdstuk 3. PDE5 inhibitoren zijn de actieve farmaceutische ingrediënt in middelen tegen erectiestoornissen. De ontwikkelde methode bestaat uit het filtreren van de monsters, direct gevolgd door analyse met LC-triplequad-MS/MS (hoofdstuk 3.1). Met deze methode bleek het mogelijk om sildenafil en twee metabolieten, die na gebruik van sildenafil worden gevormd in het menselijk lichaam, te meten in het lage $ng.L^{-1}$ gebied. Vervolgens werden rioolwatermonsters geanalyseerd, en de zo verkregen concentraties werden vergeleken met te verwachten concentraties die waren berekend met gegevens over via recepten voorgeschreven en verkochte hoeveelheden van het middel, zowel voor Nederland (hoofdstukken 3.2) als voor enkele Europese steden (hoofdstuk 3.3). Ondanks een aantal beperkingen van de studie bleek de RWE methode in staat om aan te tonen dat er in Nederland op grote schaal (meer dan 60% gebruik dat niet uit voorgeschreven recepten is te verklaren) van niet gecontroleerde internetapotheken gebruikt gemaakt wordt voor het verkrijgen van sildenafil.

Hoofdstuk 4 beschrijft het onderzoek naar middelen die sporters als doping, afslankmiddel of voor het maskeren van doping gebruiken. Met de ontwikkelde analysemethode, die gebaseerd is op SPE gevolgd door UPLC-MS/MS, bleek het mogelijk om dergelijke middelen te identificeren en te kwantificeren in rioolwater. De aanwezigheid van achtergrondconcentraties van deze middelen in rioolwater wijst op dopinggebruik door de bevolking, terwijl uit monsters verzameld tijdens sportevenementen, waarin verhoogde concentraties van enkele afslankmiddelen werden aangetoond, bleek dat amateursporters en/of bezoekers van dergelijke evenementen efedrine, norefedrine en methylhexanamine gebruiken. Ook werd tijdens drie sportevenementen een verhoogde concentratie van 2,4-dinitrofenol aangetoond, een vetverbrander waarvan het gebruik tot ernstige gezondheidseffecten kan leiden. Met dit onderzoek is aangetoond dat RWE een waardevolle aanvulling voor de anti-doping autoriteiten kan zijn.

In het afsluitende hoofdstuk 5 zijn de resultaten van het onderzoek in een breder kader geplaatst. Het onderzoek versterkt enerzijds de rol van analytische chemie in de rioolwaterepidemiologie. Anderzijds vormen de bevindingen van het in dit proefschrift beschreven onderzoek samen met die van de andere promovendi die deel uitmaakten van het International Training Network SEWPROF, dat het onderhavige onderzoek mogelijk maakte, de basis voor een volwaardige rol van RWE

in toekomstig onderzoek gericht op zowel het vergroten van kennis over gezondheid en levensstijl van populaties, als op forensische- en milieubeschermingsdoeleinden.

# Resumen

La investigación que se presenta en esta tesis se centra en el papel esencial de la química analítica para el desarrollo de metodología avanzada capaz de determinar drogas ilícitas y otras sustancias (lícitas e ilícitas) en el medio acuático de manera fehaciente, respaldando la hipótesis de que la epidemiología de aguas residuales se puede utilizar como medio alternativo y no intrusivo que proporciona información sobre la salud de la población y sus hábitos de vida. Con este fin, se ha investigado el uso de cromatografía líquida acoplada a diferentes analizadores de espectrometría de masas, de baja y alta resolución, y el uso de estrategias de adquisición de datos (dirigida y de cribado basado en base de datos).

El capítulo 1 proporciona los antecedentes de la investigación, una visión general de las sustancias estudiadas, y enumera los objetivos específicos.

En el capítulo 2, se abordan diferentes retos analíticos que afectan a la monitorización de las drogas ilícitas y sus biomarcadores en aguas residuales. El primer reto consiste en la inclusión del cannabis en este tipo de estudios epidemiológicos. En general, las drogas y sus biomarcadores son compuestos químicos pequeños e hidrofílicos, y por tanto su determinación en el agua vía LC-MS es sencilla. Sin embargo, el carboxi-THC, que es el metabolito en orina del cannabis, es una molécula lipofílica (log Dow ~4) que tiene el potencial de adherirse a la materia particulada y adherirse a los huecos hidroxilados presentes en la superficie del vidrio. Esta propiedad fisicoquímica impide su correcta determinación cuando se utilizan métodos multi-residuo. Los resultados que se presentan en el capítulo 2.1 muestran el camino a seguir hacia una determinación más precisa del biomarcador carboxi-THC en aguas residuales. El ajuste de pH se identificó como el paso crítico durante el tratamiento de muestra. Y aunque los resultados entre los laboratorios participantes en el estudio inter laboratorio mostraron una variación de un 30% aproximadamente (y por tanto más elevada de lo habitual), el protocolo presentado se comprobó satisfactoriamente y debería ser recomendado para aplicaciones futuras de la epidemiología de aguas residuales.

El segundo reto consiste en la falta de datos de diferentes regiones del mundo. Al inicio de este trabajo, la epidemiología de aguas residuales se había aplicado principalmente en Europa. En los últimos años, muchos estudios se han realizado en EE. UU., Australia y Asia. La investigación realizada en el capítulo 2.2, que presenta resultados de una campaña de muestreo realizada en Costa Rica, contribuye a llenar

este hueco y así extender el conocimiento derivado del análisis químico de las aguas residuales a Centro América. Los resultados muestran un nivel de concentración alto de las drogas cocaína y cannabis, y una presencia moderada de los opiáceos codeína y morfina. Estos hallazgos respaldan el patrón de uso de drogas descrito por las herramientas epidemiológicas tradicionales. La ausencia de fenetilaminas sintéticas, como la anfetamina, metanfetamina o MDMA (éxtasis), que se encuentran habitualmente en las aguas residuales de otras regiones, fue digna de mención; así como la ausencia de benzodiacepinas y otras drogas como la heroína. Por tanto, se identificaron diferencias geográficas. Además, no sólo se evaluó el contexto social, sino también el medioambiental. El análisis de aguas superficiales de la zona cercana a las plantas de tratamiento de agua residual muestreadas reveló que la aplicación de un tratamiento más o menos sofisticado sólo elimina los residuos parcialmente, y por tanto éstos son liberados al medio ambiente, aunque en concentraciones relativamente bajas.

El tercer reto es la inclusión de las nuevas sustancias psicoactivas (NPS) en este tipo de estudios epidemiológicos. El principal desafío es el número reducido de usuarios, que se traduce en concentraciones bajas de sus residuos en agua residual, y la limitada información farmacocinética disponible, que dificulta la elección de biomarcador. El capítulo 2.3 muestra cómo el muestreo durante eventos sociales específicos donde los usuarios se reúnen, en combinación con el análisis con espectrometría de masas de alta resolución y un flujo de trabajo de procesado de datos de cribado basado en la búsqueda en base de datos, fue muy útil a la hora de estrechar la búsqueda de NPS en el análisis de aguas residuales.

La investigación que se presenta en el capítulo 2 ha contribuido a la madurez de la epidemiología de aguas residuales como una fuente de información alternativa, para que las autoridades como el Observatorio Europeo de las Drogas y las Toxicomanías (EMCDDA) puedan obtener una mejor visión de la prevalencia de las drogas ilícitas.

En los capítulos 3 y 4, el objetivo fue el de ir un poco más lejos y extrapolar la epidemiología de aguas residuales de la evaluación del uso de drogas a la inclusión de otros fármacos con potencial de abuso, como por ejemplo los medicamentos falsificados. En estos capítulos se investigaron productos de disfunción eréctil y sustancia ilícitas utilizadas en contexto deportivo. Para esto, se desarrollaron y validaron dos nuevos métodos analíticos, y con ellos se analizaron aguas residuales.

El capítulo 3 se centra en inhibidores de la fosfodiesterasa-5, que son los ingredientes activos en productos contra la disfunción eréctil. Se validó un método simple pero

muy sensible, que consiste en el filtrado de la muestra seguido de su análisis directo por cromatografía liquida acoplada a espectrometría de masas tándem (analizador triple cuadrupolo). El método es capaz de cuantificar sildenafil y sus dos metabolitos a una concentración muy baja en el orden de ng $L^{-1}$. Además de analizar muestras de agua residual, se recogieron datos de venta en farmacias y prescripciones médicas para comparar los niveles derivados de estos datos con los encontrados en las aguas residuales. Esto se realizó con datos nacionales de los Países Bajos, en los capítulos 3.1 y 3.2, y a nivel europeo en el capítulo 3.3. A pesar de la limitación de esta comparativa, los resultados obtenidos por epidemiología de aguas residuales se intuyeron satisfactorios en el rastreo de farmacias fraudulentas y medicación falsificada. Este fue el caso especialmente en los Países Bajos, donde la fracción de sildenafil inexplicable en agua residual representó al menos el 60% de lo observado.

El capítulo 4 se centró en las sustancias ilícitas utilizadas en el contexto del dopaje en el deporte. El método analítico basado en extracción en fase sólida de la muestra seguido del análisis por UPLC-MS/MS permitió la detección y cuantificación en agua residual de sustancias dopantes utilizadas por la población y deportistas aficionados que asistieron a los eventos muestreados. Estimulantes para perder peso, como la efedrina, norefedrina y metilhexanamina, se encontraron en altas concentraciones. Además, la detección de dinitrofenol es preocupante debido a sus efectos adversos para la salud. Los resultados sugieren el aumento de cantidad de algunas de estas sustancias durante los eventos deportivos monitorizados. Por tanto, la epidemiología de aguas residuales mostró ser una técnica complementaria capaz de aportar información de valor para autoridades nacionales antidopaje, aportando información de uso de compuestos conocidos en grupos y áreas específicas, a nivel de promedio de grupo.

Finalmente, la síntesis presentada en el capítulo 5 comenta la contribución de esta tesis en fortalecer el papel de la química analítica en la epidemiologia de aguas residuales, y las implicaciones de los resultados obtenidos en un contexto social y medioambiental.

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**2018**

**A. Causanilles**, D. Rojas Cantillano, E. Emke, R. Bade, J.A. Baz-Lomba, S. Castiglioni, E. Castrignanò, E. Gracia-Lor, F. Hernández, B. Kasprzyk-Hordern, J. Kinyua, A.K. McCall, A.L.N. van Nuijs, B.G. Plósz, P. Ramin, N.I. Rousis, Y. Ryu, K.V. Thomas, P. de Voogt "Comparison of phosphodiesterase type V inhibitors use in eight European cities through analysis of urban wastewater" Environment International 115 (2018) 279 – 284 (*doi: 10.1016/j.envint.2018.03.039*)

**A. Causanilles**, V. Nordmann, D. Vughs, E. Emke, O. de Hon, F. Hernández, P. de Voogt "Wastewater-based tracing of doping use by the general population and amateur athletes" *Analytical Bioanalytical Chemistry (2018) 410(6), 1793-1803 (doi: 10.1007/s00216-017-0835-3)*

E. Castrignano, Z. Yang, R. Bade, J.A. Baz-Lomba, S. Castiglioni, **A. Causanilles**, A. Covaci, E. Gracia-Lor, F. Hernandez, J. Kinyua, A.K. McCall, A.L.N. van Nuijs, C. Ort, B.G. Plósz; P. Ramin, N.I. Rousis, Y. Ryu, K.V. Thomas, P. de Voogt, E. Zuccato, B. Kasprzyk-Hordern "Enantiomeric profiling of chiral illicit drugs in a pan-European study" *Water Research 130 (2018) 151 – 160 (doi: 10.1016/j.watres.2017.11.051)*

**2017**

**A. Causanilles**, J.A. Baz-Lomba, D. A. Burgard, E. Emke, I. González-Mariño, I. Krizman, A. Li, A.S.C. Löve, A.K. McCall, R.M. Montes, A. L.N. van Nuijs, C. Ort, J. Benito Quintana, I. Senta, S. Terzic, F. Hernández, P. de Voogt, L. Bijlsma "Improving wastewater-based epidemiology to estimate cannabis use: focus on the initial aspects of the analytical procedure" *Analytica Chimica Acta 988 (2017) 27-33 (doi: 10.1016/j.aca.2017.08.011)*

E. Gracia-Lor, N.I. Rousis, E. Zuccato, R. Bade, J.A. Baz-Lomba, E. Castrignanò, **A. Causanilles**, F. Hernández, B. Kasprzyk-Hordern, J. Kinyua, A.K. McCall, A.L.N. van Nuijs, B.G. Plósz, P. Ramin, Y. Ryu, M.M. Santos, K. Thomas, P. de Voogt, Z. Yang, S. Castiglioni "Estimation of caffeine intake from analysis of caffeine metabolites in wastewater" *Science of the total Environment 609 (2017) 1582-1588 (doi: 10.1016/j.scitotenv.2017.07.258)*

P. Ramin, A.L. Brock, **A. Causanilles**, B. Valverde-Pérez, E. Emke, P. de Voogt, F. Polesel, B.G. Plósz "Transformation and sorption of illicit drug biomarkers in sewer biofilms" *Environmental Science & Technology, 2016, 50 (24), pp 13397–13408 (doi: 10.1021/acs.est.6b06277)*

**A. Causanilles**, J. Kinyua, C. Ruttkies, A.L.N. van Nuijs, E. Emke, A. Covaci, P. de Voogt "Qualitative screening for new psychoactive substances in wastewater collected during a city festival using liquid chromatography coupled to high-resolution mass spectrometry" *Chemosphere 184 (2017) 1186-1193 (doi: 10.1016/j.chemosphere.2017.06.101)*

N.I. Rousis, E. Gracia-Lor, E. Zuccato, R. Bade, J.A. Baz-Lomba, E. Castrignanò, **A. Causanilles**, A. Covaci, P. de Voogt, F. Hernández, B. Kasprzyk-Hordern, J. Kinyua, A.K. McCall, C. Ort, B.G. Plósz, P. Ramin, Y. Ryu, K.V. Thomas, Z. Yang, S. Castiglioni "Wastewater-based epidemiology to assess pan-European pesticide exposure" *Water Research 121 (2017) 270-279 (doi: 10.1016/j.watres.2017.05.044)*

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R. Bade; L. Bijlsma; J.V Sancho; J.A. Baz-Lomba; S. Castiglioni; E. Castrignanò; **A. Causanilles**; E. Gracia-Lor; B. Kasprzyk-Hordern ; J. Kinyua; A.K. McCall; A.L.N. van Nuijs; C. Ort; B.G. Plósz; P. Ramin; N.I. Rousis; Y. Ryu; K.V. Thomas; P. de Voogt; E. Zuccato; F. Hernandez "Liquid chromatography-tandem mass spectrometry determination of synthetic cathinones and phenethylamines in influent wastewater of eight European cities" *Chemosphere 168 (2017) 1032 – 1041 (doi: 10.1016/j.chemosphere.2016.10.107)*

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Y. Ryu, E. Garcia-Lor, R. Bade, J.A. Baz-Lomba, J.G. Bramness, S. Castiglioni, E. Castrignanò, **A. Causanilles**, A. Covaci, P. de Voogt, F. Hernandez, B. Kasprzyk-Hordern, J. Kinyua, A.K. McCall, C. Ort, B.G. Plósz, P. Ramin, N.I. Rousis, M.J. Reid, and K.V. Thomas "Increased levels of the oxidative stress biomarker 8-iso-prostaglandin F2α in wastewater associated with tobacco use" *Scientific Reports 6 (2016) 39055 (doi: 10.1038/srep39055)*

J.A. Baz-Lomba, S. Salvatore, E. Gracia-Lor, R. Bade, S. Castiglioni,E. Castrignanò, **A. Causanilles**, F. Hernandez, B. Kasprzyk-Hordern, J. Kinyua,A.K. McCall, A.L.N. van Nuijs, C. Ort, B.G. Plósz, P. Ramin, M. Reid, N.I. Rousis, Y. Ryu, P. de Voogt, J. Bramness and K.V. Thomas "Comparison of pharmaceutical, illicit drug, alcohol, nicotine and caffeine levels in wastewater with sale, seizure and consumption data for 8 European cities" *BMC Public Health (2016) 16:1035 (doi: 10.1186/s12889-016-3686-5)*

P. Ramin, A.L. Brock, F. Polesel, **A. Causanilles**, E. Emke, P. de Voogt, B.G. Plósz "Transformation and sorption of illicit drug biomarkers in sewer systems: understanding the role of suspended solids in raw wastewater" *Environmental Science & Technology 2016, 50 (24), 13397–13408 (doi: 10.1021/acs.est.6b03049)*

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B.J. Venhuis, P. de Voogt, E. Emke, **A. Causanilles**, P.H.J. Keizers. Letter "Success of rogue online pharmacies: sewage study of sildenafil in the Netherlands" *BMJ 2014;349:g4317 (doi: 10.1136/bmj.g4317)*

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# List of author's contributions

- Chapter 2.1: Causanilles et al. (2017) ACA

AC, JABL, DB, IGM, IKM, AL, ASCL, AKM, RM, AvN, IS and LB performed wastewater analysis. AC organised the interlaboratory study. CO and JB performed statistical analysis. AC drafted the manuscript with significant contributions from LB and PdV. All authors read and approved the final manuscript.

- Chapter 2.2: Causanilles et al. (2017) STOTEN

AC, CR, EE and PdV collected the samples. AC and MI performed wastewater analysis. AC drafted the manuscript with significant contributions from all authors. All authors read and approved the final manuscript.

- Chapter 2.3: Causanilles et al. (2017) Chemosphere

AC and JK contributed equally to the research. AC and JK performed wastewater analysis. AC, JK and CR processed the data. AC and JK drafted the manuscript. All authors read and approved the final manuscript.

- Chapter 3.1: Causanilles et al. (2016) STOTEN

AC developed the method and performed wastewater analysis. EE organised the collection of the wastewater samplers. AC drafted the manuscript with significant contributions from EE and PdV.

- Chapter 3.2: Venhuis et al. (2014) BMJ

AC validated the analytical method and performed wastewater analysis, EE and PdV organised wastewater collection, BJV coordinated the study drafted the manuscript with significant contributions from EE and PdV, PK estimated the actual and legitimate consumption of sildenafil and prepared the figure. All authors read and approved the final manuscript.

- Chapter 3.3: Causanilles et al. (2018) Environment International

AC and DRC performed wastewater analysis. AC drafted the manuscript with significant contributions from FH and PdV. AC, RB, JABL, SC, EC, EGL, FH, BKH, JK, AKM, AvN, BGP, PR, NIR, YR and KT organised the collection of the wastewater

samples and provided relevant data for WBE calculations and national prescription data.

- Chapter 4.1: Causanilles et al. (2018) ABC

AC and EE organized the sample collection. AC and VN validated the analytical method and performed wastewater analysis. AC drafted the manuscript with significant contributions from FH and PdV. All authors read and approved the final manuscript.

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