Measuring biomarkers in wastewater as a new source of epidemiological information: current state and future perspectives

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ABSTRACT

The information obtained from the chemical analysis of specific human excretion products (biomarkers) in urban wastewater can be used to estimate the exposure or consumption of the population under investigation to a defined substance. A proper biomarker can provide relevant information about lifestyle habits, health and wellbeing, but its selection is not an easy task as it should fulfil several specific requirements in order to be successfully employed. This paper aims to summarize the current knowledge related to the most relevant biomarkers used so far. In addition, some potential wastewater biomarkers that could be used for future applications were evaluated. For this purpose, representative chemical classes have been chosen and grouped in four main categories: (i) those that provide estimates of lifestyle factors and substance use, (ii) those used to estimate the exposure to toxicants present in the environment and food, (iii) those that have the potential to provide information about public health and illness and (iv) those used to estimate the population size. To facilitate the evaluation of the eligibility of a compound as a biomarker, information, when available, on stability in urine and wastewater and pharmacokinetic data (*i.e.* metabolism and urinary excretion profile) has been reviewed. Finally, several needs and recommendations for future research are proposed.

Key words

Wastewater; Epidemiology; Biomarker; Consumption; Exposure; Population

INTRODUCTION

Relevant epidemiological information about lifestyle habits, public health and wellbeing can be obtained from the chemical analysis of urban wastewater. This approach, called *wastewater-based epidemiology* (WBE), is based on the analysis of specific human metabolic excretion products (biomarkers) in wastewater as indicators of consumption or exposure of the population served by the sewer network under investigation to different substances. WBE has been successfully applied as a suitable approach for the estimation of illicit drugs consumption (Ort et al., 2014; Thomaidis et al., 2016; Thomas et al., 2012; van Nuijs et al., 2011a; Zuccato et al., 2008), but it has also recently been employed to assess other lifestyle-related factors such as alcohol (Rodríguez-Álvarez et al., 2015; Ryu et al., 2016), nicotine (Castiglioni et al., 2015b; Lopes et al., 2014; Rodríguez-Álvarez et al., 2014b), caffeine (Senta et al., 2015a) and new psychoactive substances (NPS) (Kinyua et al., 2015; Reid et al., 2014a; van Nuijs et al., 2014). WBE has also been applied to verify community-wide exposure to endocrine disruptors and antimicrobial agents in personal care and household products (O'Brien et al., 2015; Rydevik et al., 2015). The broad range of information that can be gathered from wastewater opens up the possibility of expanding WBE to other human biomarkers providing clues about diet, health, diseases and exposure to contaminants. For example by linking exposure to environmental or food contaminants with health outcomes such as diabetes or cancer.

In general, a human biomarker can be an endogenous compound (produced naturally in the body) or a metabolite of a xenobiotic/exogenous substance (produced through metabolic processes after intentional consumption of a substance, accidental exposure to environmental contaminants, as well as through diet or ingestion of a substance). Biomarkers can be classified on the basis of their function as biomarkers of exposure (compounds that give information about substances consumed or ingested) and biomarkers of effect (indicators of measurable changes or alterations in an organism that can be associated with health problems or wellbeing) and on the basis of biological

nature (e.g. metabolites, hormones), or of the disease they can indicate (e.g. cardiovascular biomarkers, obesity biomarkers) (Pischon, 2009).

The selection of a specific biomarker is not an easy task, as it needs to satisfy different criteria **(Figure 1)** (Castiglioni and Gracia-Lor, 2015; Gracia-Lor et al., 2016). From a WBE perspective, a suitable biomarker must be excreted mainly via urine and concentration levels in urine should be at least in the µg/L range to ensure its detection in raw wastewater after dilution (Chen et al., 2014).

A biomarker should also be sufficiently stable in wastewater during the transport (in-sewer stability) from the input (i.e. toilet) to the sampling point and during sampling, storage and analysis (in-sample stability) (McCall et al., 2016a). In wastewater biomarkers can undergo further transformation due to microbial activity (Mardal and Meyer, 2014) and/or sorption to particulate matter (Baker and Kasprzyk-Hordern, 2011; Daughton, 2012a; McCall et al., 2016a). The fate of biomarkers in the sewer can be also predicted by using mathematical models to simulate physicochemical and microbial processes (Bisceglia and Lippa, 2014; McCall et al., 2016b; Ramin et al., 2016). It is important to note that biomarker transformation pathways in the sewer might be different from human metabolic pathways.

Furthermore, a biomarker should preferably be specific to the compound under investigation and unique to human metabolism, thus ensuring that its presence only derives from human excretion and not from exogenous sources (Daughton, 2012b). Therefore, pharmacokinetic data on human metabolism are necessary but unfortunately this information is not always feasible as for many substances it is very limited or do not even exist. This information, however, is highly relevant not only to back-calculate the consumption/exposure of/to a certain substance by a community, but also to distinguish the amount of a substance originating from human metabolism or other sources. Unfortunately, pharmacokinetic studies are time-consuming and have to fulfil strict ethical rules. Alternative approaches, which allow for the identification and selection of

appropriate biomarkers, are therefore required; for example, *in-vitro* studies using liver enzymes, which metabolize the parent compound, help in the elucidation of the chemical structure of the metabolites formed (i.e. possible biomarkers) formed (Mardal et al., 2016). Computer-based *in-silico* modelling also allow the prediction of pharmacokinetics (Reid et al., 2014a). However these alternatives provide qualitative information on metabolism, but not data regarding excretion rates of parent substances and their metabolites (Gracia-Lor et al., 2016).

The present manuscript emerges within the framework of the pan-European inter-disciplinary network (Sewage analysis CORE group-SCORE), which brings together experts from different disciplines interested in standardizing the WBE approach and in coordinating international studies (http://score-cost.eu/). The aim of this review is to describe the criteria for selecting suitable biomarkers and to give an overview of relevant human (urinary) metabolites and potential wastewater biomarkers. Biomarkers have been grouped in four sections: (i) those that provide estimates of lifestyle factors and substance use, (ii) those used to estimate the exposure to toxicants present in the environment and food, (iii) those giving information about public health and (iv) those used to estimate the population size. For each group and biomarker, a thorough review of the available pharmacokinetic data (*i.e.* metabolism and excretion profile) and stability in urine and wastewater (if known) is provided. This information can be used to evaluate their suitability according to the criteria described above. Finally, potential gaps or limitations are discussed and future research directions are proposed.

2. LIFESTYLE AND SUBSTANCE USE BIOMARKERS

Initially, WBE was applied to evaluate lifestyle, in particular illicit drug use within a community. Its ability to deliver objective and near-real-time data on drug use, being able to detect changes over time and local patterns of use, suggests that this method can be used as a complementary and extended data source to existing epidemiological tools. WBE has been well

established for monitoring the use of cocaine, cannabis, amphetamine, methamphetamine and MDMA (3,4-methylenedioxymethamphetamine).

Additional applications to estimate consumption of other substances, such as alcohol, tobacco, caffeine and NPS, have been employed more recently. Alcohol and nicotine (tobacco) are probably the most popular and accepted recreational drugs. However, many negative social, economic and health aspects have been linked to their use, causing millions of deaths every year (World Health Organization, 2015, 2014). It is therefore important and of particular interest for policy makers to obtain continuous monitoring data on consumption levels and patterns of use, in order to reduce the disease burden related to alcohol and tobacco use. Caffeine use has been limitedly investigated, although it is one of the most extensively used legal stimulants, found in widely-consumed products, such as coffee, tea, soft and "energy" drinks. Besides monitoring its consumption, caffeine has also been proposed as a human biomarker for assessing the size and dynamics of the population served (see section 5.3) by a particular wastewater treatment plant (WWTP) (Senta et al., 2015a). NPS are emerging narcotic or psychotropic substances which may pose similar threats to public health such as classical illicit drugs (European Union, 2005; Papaseit et al., 2014). Due to the delay between their appearance on the market and their addition to the list of banned (or controlled) substances, many NPS can be legally purchased, thus promoting their proliferation worldwide. Furthermore, new substances appear continuously on the market (Bijlsma et al., 2016; EMCDDA, 2015a). WBE has been proposed as a tool for providing useful information on temporal and regional trends in the use of NPS.

Current state and some new features of WBE, with regard to lifestyle and substance use are presented in this chapter. Furthermore, specific biomarkers of each lifestyle factor are suggested (**Table S1**) and conceptual approaches for dealing with NPSs using biomarkers in wastewater are proposed.

2.1. Illicit drugs

Among the available epidemiological indicators, general population surveys have been traditionally used to assess illicit drug use at the population level. Yet, due to their inherent biases, complementary and real-time approaches are needed. The determination of illicit drug consumption through wastewater was first theorized by Daughton (Daughton, 2001) and implanted by Zuccato *et al.* using cocaine as an example (Zuccato et al., 2005). Since then, WBE has been widened to include other illicit drugs (Asimakopoulos and Kannan, 2016; Castiglioni et al., 2008; Hernández et al., 2016; van Nuijs et al., 2011a).

The biomarkers currently used are either the illicit drug itself (i.e. amphetamine, methamphetamine, and 3,4-methylenedioxy-methamphetamine-MDMA) or one of its metabolites (i.e. benzoylecgonine (BEG) for cocaine, 11-nor-9-carboxy-delta9-tetrahydrocannabinol (THC-COOH) for cannabis and morphine or 6-acethylmorphine for heroin).

Cocaine, the first substance studied in WBE, is considered unstable in wastewater; however, its unique and stable metabolite (BEG) makes back-calculation to drug consumption more straightforward. It must be noted that significant degradation of BEG from cocaine in sewage is also reported (Plósz et al., 2013), which could result in over estimation of cocaine consumption if this formation is neglected. Considering human excretion rates, a cocaine: BEG ratio around 0.1 or lower can indicate consumption, and any value higher (between 0.1 and 0.7) could indicate other sources of cocaine, such as direct disposal (Castiglioni et al., 2011a). However, more research is needed in this regard (Bijlsma et al., 2012; Postigo et al., 2010; Van Nuijs et al., 2009).

Δ9-tetrahydrocannabinol (THC), the active ingredient of cannabis, is metabolized to more than 20 metabolites after consumption, with 11-nor-Δ9-carboxy-THC (THC-COOH) and 11-hydroxy-THC (THC-OH) being those primarily excreted. THC-COOH has been shown to be highly stable and is thus normally used to estimate cannabis consumption, albeit with some analytical

difficulties arising in multi-residue methods resulting from its non-polarity compared to other illicit drugs (Bijlsma et al., 2014; Ort et al., 2014; Pedrouzo et al., 2011).

Two more recently works studied illicit drugs are ketamine and methadone. Ketamine is a dissociative anaesthetic which has been used as a recreational drug, whilst methadone is a synthetic opioid used clinically to relieve pain and also as maintenance treatment of opioid addicts (Castiglioni et al., 2011b; Preston et al., 2003). Both ketamine and its metabolite norketamine are fairly stable in wastewater (Castiglioni et al., 2015a; McCall et al., 2016a), with the parent compound generally used as a biomarker for reliable estimation of drug usage. Variable stability for methadone has, however, been reported i.e. from high (Senta et al., 2014) to low (González-Mariño et al., 2010).

Opioids use in Europe remains a central issue, reflecting the significant impact these drugs still have on mortality and morbidity (EMCDDA, 2015b). In recent years, the production of high purity heroin has been rising, thereby increasing heroin-related mortality (UNODC, 2015). In the human body, heroin is rapidly hydrolyzed to 6-monoacetylmorphine (6-MAM) by blood esterases (Bencharit et al., 2003) and further hydrolyzed to morphine in the liver (Smith, 2009). In wastewater, heroin shows low stability (González-Mariño et al., 2010). Although 6-MAM detected in urine is used as a marker of heroin consumption (Staub et al., 2001), 6-MAM is not always detected in wastewater as it is not stable in wastewater (Thai et al., 2014). Back-calculations using 6-MAM as biomarker provides inconsistent results (Been et al., 2015). Therefore, morphine is considered as an alternative biomarker for heroin. However, therapeutic consumption of morphine should be subtracted from the total measured morphine in sewage (Khan and Nicell, 2011; van Nuijs et al., 2011a; Zuccato et al., 2016), which necessitates the availability of registered prescribed morphine at the time of wastewater sampling. Morphine is also formed in the sewer due to deconjugation of morphine glucuronide and deacetylation of 6-MAM, which imposes new

challenges in back-calculation schemes. Although fractions of morphine originating from codeine can be considered negligible (Zuccato et al., 2008), more research is needed to find a drug biomarker for heroin which fulfils all the aforementioned criteria.

As shown in **Table 1**, the most frequently used illicit drug biomarkers are benzoylecgonine, amphetamine, methamphetamine, MDMA and THC-COOH (Thomas et al., 2012). Information about excretion and stability in urine and wastewater of these and other illicit drug biomarkers less frequently studied is presented in **Table S1**.One of the most current analytical challenges associated with WBE is represented by chirality. Amphetamine, methamphetamine and MDMA are among the illicit drugs that are chiral and as a result they can exist as enantiomers (one enantiomeric pair per each chiral centre). The verification of their chiral signature in wastewater (i.e. relative proportion of two enantiomers within each enantiomeric pair) allows to distinguish between illicit or licit use and direct disposal (Emke et al., 2014). It has been shown that the distinction between the consumption or the disposal of MDMA could be made by differentiating the loads of the enantiomers present in wastewater. Indeed, enantiomeric fractions (EFs) greater than 0.5 indicated illicit use, whilst EFs equal to 0.5 indicated direct disposal, when EF was calculated as follows:

$$EF = \frac{(-) - MDMA}{(-) - MDMA + (+) - MDMA}$$

Enantiomeric profiling of MDMA's metabolites were recently investigated in wastewater by Castrignanò et al., suggesting enantioselective metabolism for HMMA (Castrignanò et al., 2016). Amphetamine and methamphetamine can also be investigated at enantiomeric level, however due to both legal and illicit uses, a clear understanding between consumption and direct disposal is difficult (Emke et al., 2014; Kasprzyk-Hordern and Baker, 2012).

2.2. Alcohol

Following the consumption of alcoholic beverages, the majority of ingested ethanol is rapidly metabolized in the human body in a two-stage oxidation process, first to acetaldehyde and then to acetic acid. The remaining part is excreted unchanged in urine, sweat and exposed breath (Jones, 1990). However, a very small fraction (<0.1%) undergoes a conjugation reaction with glucuronic acid to produce ethyl glucuronide (EtG) (Dahl et al., 2002) and with 3'-phosphoadenosine 5'-phosphosulfate to produce ethyl sulphate (EtS) (Helander and Beck, 2005). These metabolites are excreted within a few hours and are detectable in urine for considerably longer times (up to 1-2 days, depending on the subject and the alcohol dose) (Helander and Beck, 2005; Høiseth et al., 2008), making them unequivocal indicators of recent alcohol consumption (Dahl et al., 2011; Dresen et al., 2004).

EtG was found to degrade ~50% after 18 hours, whereas EtS showed little or no degradation (Reid et al., 2011). In addition, no significant differences were found between its stability in sewage and in an ethanol-fortified wastewater sample (Reid et al., 2011), indicating that it is unlikely to be formed from unconsumed alcohol discarded into the sewer system. Taking into account these observations, EtS has been used by several researchers to estimate community-wide alcohol consumption through wastewater analysis (**Table 1**). Typically, its determination in this matrix is performed by direct injection, after filtration and/or centrifugation, into a liquid chromatography-mass spectrometry system. The alcohol consumption rates estimated through WBE have revealed specific drinking patterns, temporal and spatial variations. The study conducted by Reid et al. (Reid et al., 2011), for example, clearly showed the weekend elevated drinking pattern in Oslo. Furthermore, the estimated consumption rates were in good agreement with sales statistics (Reid et al., 2011). The increase in alcohol consumption during the weekend was also found in three Spanish cities, eight Belgian cities an done Italian city (Andrés-Costa et al., 2016; Boogaerts et al., 2016; Mastroianni et al., 2014; Rodríguez-Álvarez et al., 2015, 2014a; Ryu et al., 2016). However, a different consumption pattern was observed during a special event in Valencia, where an increased

alcohol use was noticeable, reaching the maximum rate on Wednesday, which corresponded to the last day of the "Fallas" festivities (Andrés-Costa et al., 2016). Co-consumption of alcohol and cocaine was also evaluated through WBE by analyzing cocaethylene, a specific biomarker excreted when the two substances are consumed together (Mastroianni et al., 2014; Rodríguez-Álvarez et al., 2015). In the studies carried out in Belgium (Boogaerts et al., 2016) and Greece (Gatidou et al., 2016) higher alcohol consumption in urbanized cities than in smaller villages was evidenced. Although all these studies highlight the potential of EtS as a reliable biomarker for estimating alcohol consumption in relative terms, the main limitation is the uncertainty associated with its percentage of excretion, which might lead to inaccurate back-calculations in absolute amounts. Until now, there have been insufficient pharmacokinetic studies evaluating this percentage to provide a unique, representative figure (Halter et al., 2008; Høiseth et al., 2008; Lostia et al., 2013; Schneider and Glatt, 2004; Wurst et al., 2006). In the aforementioned WBE studies, the range 0.010-0.016% (on molar basis) was used by (Andrés-Costa et al., 2016; Reid et al., 2011); the median value of the excretion rates provided by Høiseth et al. (Høiseth et al., 2008), 0.011%, was used by (Mastroianni et al., 2014; Rodríguez-Álvarez et al., 2014a). Finally, four studies (Boogaerts et al., 2016; Gatidou et al., 2016; Rodríguez-Álvarez et al., 2015; Ryu et al., 2016), employed a people-weighted value of 0.012%, based on the data provided by (Høiseth et al., 2008) and (Wurst et al., 2006).

2.3. Tobacco

Nicotine is the principal alkaloid found in tobacco and, although not being directly associated with diseases, its addictiveness is the major cause of continued use of tobacco products (Hukkanen, 2005). Nicotine is extensively metabolized in humans, with 70-80% of the initial dose being converted to cotinine (Benowitz and Jacob, 1994), which is then further metabolized into various compounds, the most abundant being *trans*-3'-hydroxycotinine (Byrd et al., 1992). Nicotine

and its major metabolites are also excreted as glucuronides. Globally, nicotine is excreted unchanged at rates between 8 and 10%, whilst its glucuronide makes up for 3-5% of the initial dose (Byrd et al., 1992). Cotinine and its glucuronide are excreted at rates between 10-15% and 12-17%, respectively, while *trans*-3'-hydroxycotinine and its glucuronide make up for 33-40% and 7-9% of the initial dose, respectively (Hukkanen, 2005).

Nicotine and its metabolites, cotinine and *trans*-3'-hydroxycotinine, have been analyzed in wastewater as biomarkers (**Table S1**) to estimate tobacco use in various communities (Castiglioni et al., 2015b; Lopes et al., 2014; Mackuľak et al., 2015; Rodríguez-Álvarez et al., 2014b; Senta et al., 2015a). The three compounds were shown to be stable in wastewater samples stored at 4° C and 20° C during 24 h (Chen et al., 2014; Rodríguez-Álvarez et al., 2014b; Senta et al., 2015a). However, the concentration of the glucuronide of *trans*-3'-hydroxycotinine was shown to decrease even in refrigerated samples (i.e., 35% decrease over 8 h at 4° C). The authors of the study thus suggested to enzymatically deconjungate the compounds prior to extraction and analysis (Rodríguez-Álvarez et al., 2014b).

The amounts of these compounds in wastewater range from 0.1 to 7 µg/L (Buerge et al., 2008; Mackuľak et al., 2015; Rodríguez-Álvarez et al., 2014b; Senta et al., 2015a), and the levels of cotinine and *trans*-3'-hydroxycotinine reflected the excretion profiles expected from pharmacokinetic studies, whilst nicotine was found at higher levels (Rodríguez-Álvarez et al., 2014b; Senta et al., 2015a). The contribution from ashes and cigarettes butts has been advanced as a possible explanation for this observation (Castiglioni et al., 2015b; Rodríguez-Álvarez et al., 2014b; Senta et al., 2015a). In fact, higher nicotine levels have been reported during rain events, supporting the hypothesis that ashes and cigarette butts found on streets eventually contribute to measured nicotine loads (Senta et al., 2015a). Thus, cotinine and *trans*-3'-hydroxycotinine were used as biomarkers to estimate the amount of nicotine used per capita in a population, as indicated in **Table**

1 (Castiglioni et al., 2015b; Mackuľak et al., 2015; Rodríguez-Álvarez et al., 2014b; Senta et al., 2015a).

In some studies, figures were corrected to account for the portion of nicotine absorbed during smoking (Castiglioni et al., 2015b; Mackuľak et al., 2015), thus providing estimates of the gross amount of number of cigarettes. Additionally, Mackul'ak and co-workers (Mackuľak et al., 2015) included a factor to account for losses due to degradation, based on the mean residence time of wastewater in sewers. From the estimated nicotine consumption, the number of cigarettes smoked per capita was also calculated using as reference value 0.8 mg of nicotine per cigarette (Gorrod and Wahren, 1993; Lopes et al., 2014; Rodríguez-Álvarez et al., 2014b) or 1.25 mg of nicotine (Castiglioni et al., 2015b). The obtained figures highlighted substantial differences in consumption within the same country. For example, researchers from Italy found significant differences between the north, centre and south of the country (Castiglioni et al., 2015b; Senta et al., 2015a). These results were in agreement with epidemiological data, which suggested a higher prevalence of tobacco use in the south (Castiglioni et al., 2015b). Similarly, important differences were found in cities in Slovakia and Spain (Mackuľak et al., 2015; Rodríguez-Álvarez et al., 2014b). In Portugal, estimates of nicotine consumption derived from wastewater analysis were in line with findings from a European survey (Lopes et al., 2014).

Mass loads measured in wastewater were also used to investigate weekly consumption patterns and findings suggested that this was stable throughout the week (Chen et al., 2014; Rodríguez-Álvarez et al., 2014b; Senta et al., 2015a). Public holidays and specific touristic locations, attracting larger crowds, were the only exceptions (Lopes et al., 2014; Mackuľak et al., 2015).

The results obtained show that the measurement of nicotine metabolites is a useful tool which could potentially be used to complete current knowledge about the prevalence of tobacco use.

2.4. Caffeine

Caffeine (1,3,7-trimethylxanthine) is the world's most widely consumed stimulating agent (Garattini, 1993). It is found in many globally popular products, including tea and cola drinks, as well as in some medications and dietary supplements, but the most important source of this alkaloid is coffee.

Caffeine metabolism is extensive (Baselt, 2004), with at least 17 urinary metabolites identified in humans (Garattini, 1993). The major metabolites include 1-methyluric acid (excretion rate 12-25%), 1-methylxanthine (9-18%), 7-methylxanthine (2-8%), paraxanthine (1,7-dimethylxanthine; 4-7%), 1,7-dimethyluric acid (5-8%) and unstable product 5-acetylamino-6-formylamino-3-methyluracil (4-15%), with a small percentage (1-4%) of the initial dose excreted as the parent compound (Carrillo and Benitez, 1994; Garattini, 1993). The list of caffeine metabolites identified in humans, together with the excretion rates can be found in **Table S1**. Besides being complex, caffeine metabolism is also rather variable, with the different excretion rates observed not only in different studies, but also between individuals within the same studies (Carrillo and Benitez, 1994; Grant et al., 1983). These variations can be related with genetic differences (Blanchard et al., 1985; Grant et al., 1983) or influenced by other factors, such as age (Blanchard et al., 1985; Grant et al., 1983), pregnancy ((Carrillo and Benitez, 1994; Garattini, 1993) or medications (Callahan et al., 1983). However, certain metabolites, such as paraxanthine, 1,7-dimethyluric acid and 1-methylxanthine were found to be less affected by the genetic background compared to the parent compound and they were, therefore, suggested as potential biomarkers for caffeine dietary intake (Crews et al., 2001). Furthermore, most of the pharmacokinetic data on caffeine metabolism in humans are quite old (Blanchard et al., 1985; Grant et al., 1983) and some of them include a relatively low number of subjects (Blanchard et al., 1985).

385 Due to its wide usage in modern societies, caffeine is among the most ubiquitous wastewater
386 micro-contaminants, usually detected at relatively high concentration levels (μg/L) in untreated
387 wastewater (Martínez-Bueno et al., 2011; Rosal et al., 2010; Santos et al., 2009). Due to this,
388 caffeine was proposed as anthropogenic marker to indicate the discharge of domestic wastewater in
389 rivers and lakes (Buerge et al., 2003), but so far has been rarely used as a biomarker in a WBE
390 approach. Caffeine has also been proposed as a human biomarker for assessing population size and
391 the dynamics of people served by a particular WWTP (Daughton, 2012b) (see section 5.3).
392 However, with the exception of paraxanthine, data on the occurrence of caffeine metabolites
393 in wastewater are still very scarce. In fact, the first comprehensive study which included most of the
394 major caffeine metabolites (1-methylxanthine, 7-methylxanthine and paraxanthine) was published
395 just recently (Senta et al., 2015a). Concentrations of these metabolites found in Italian wastewater
396 were similar to those of the parent compound, i.e. in the μg/L range. In the same work temporal and
397 spatial patterns of use were also studied and the mean mass loads of caffeine and its major
398 metabolites revealed to be slightly lower during the weekend, probably due to the lower
399 consumption of coffee. Similar findings for caffeine was reported by Rico et al. (Rico et al., 2016;
400 Senta et al., 2015a). On the other hand, no clear geographical trends could be observed. Besides
401 being easily detectable, caffeine, 1-methylxanthine, 7-methylxanthine and paraxanthine fulfill
402 additional important requirement for an ideal biomarker - they are stable in wastewater samples
403 stored at 4 °C and 20 °C for 24 h (Senta et al., 2015a). However, it is noteworthy that more research
404 is needed in order to select the most suitable caffeine biomarker in wastewater for the correct
405 interpretation of the obtained results within the concept of WBE.
406
407 **2.5. New Psychoactive Substances**
408 The detection of NPS and the estimation of their use are especially challenging for drug
409 epidemiology, since new compounds appear continuously on the market and consumers do not

always know the composition of the drugs they take. WBE can shed some light and provide additional information, but it is also affected by important challenges. First, pharmacokinetic data are essentially non-existent for most NPS, making it extremely difficult to define appropriate biomarkers. Second, the prevalence of abuse of a single substance is generally low, leading to very low concentrations in wastewater. Finally, their stability in this matrix is largely unknown (EMCDDA, 2016; Reid and Thomas, 2016). Based on the limited information available, this section attempts to present a selection of potential biomarkers, to be used in WBE studies, for the most common classes of NPSs: synthetic cannabinoids, synthetic cathinones, phenethylamines, piperazines, tryptamines, arylcycloalkylamines and benzodiazepines (EMCDDA, 2015a). The two first groups constitute the largest categories and also account for the majority of seizures in Europe (EMCDDA, 2015a).

Synthetic cannabinoids include a broad range of structurally different compounds sharing affinity for the cannabinoid receptors in the brain (Pertwee, 2008). Due to their recent increased popularity, their human metabolism is a growing area of research. Several *in vitro* and *in vivo* experiments have been performed over the past few years and, although individual pharmacokinetic profiles remain to be elucidated for many of them, it is generally thought that synthetic cannabinoids are extensively oxidized in the human body and excreted as a complex mixture of phase I and phase II metabolites (Fantegrossi et al., 2014; Seely et al., 2012). JWH-type cannabinoids are the most popular drugs within this class. Monohydroxylation, either at the N-alkyl side chain, the naphthyl moiety or the indole moiety (followed by the corresponding glucuronidation) has been identified as their major metabolic pathway and, in fact, monohydroxylated metabolites have been detected in urine from JWH-type cannabinoids consumers (Hutter et al., 2012; Ozturk et al., 2015; Wohlfarth et al., 2013). However, the lack of rigorous pharmacokinetic data, essential to calculate excretion rates, prevents from extrapolating these analyses to whole communities by the WBE approach. Another important limitation concerns

their instability in wastewater: the scarce literature available suggests that some synthetic cannabinoids and their metabolites are highly labile and tend to get adsorbed to particle matter, hindering their determination and sub-estimating the potentially derived abuse calculations (Reid et al., 2014a, 2014b). As a reflection of these intrinsic difficulties, to the best of our knowledge only the metabolite JWH 018 N-5-hydroxypentyl and the parent compounds JWH-210 and JWH-122, have been positively detected in wastewater in two out of all the studies dealing with NPS in this matrix (Borova et al., 2015; Reid et al., 2014b) (see **Table S1**).

Synthetic cathinones are known to have been abused for approximately 15 years and the synthesis of cathinone derivatives has been reported since the late 1920s (Hyde and Adams, 1928; Prosser and Nelson, 2012). They all refer to cathinone ((S)-2-amino-1-phenyl-1-propanone), a naturally occurring stimulant found in the leaves of Catha edulis (Khat) (Prosser and Nelson, 2012). In general, the drugs are in part extensively metabolized in humans. However, some of the synthetic cathinones are also excreted unchanged in urine (Uralets et al., 2014). Details on the metabolism and detectability of synthetic cathinones can be found in original articles and are summarized in several review articles (Ellefsen et al., 2015; Helfer et al., 2007; Meyer et al., 2014, 2012, 2010a, 2010b; Meyer and Maurer, 2010; Pawlik et al., 2012; Pozo et al., 2014; Shima et al., 2014; Staack and Maurer, 2005; Uralets et al., 2014; Welter-Luedeke and Maurer, 2015). Also, data on the stability, especially under storage conditions, were published (Senta et al., 2015b) and highlighted the possible instability of the parent compounds under alkaline conditions (Johnson and Botch-Jones, 2013; Tsujikawa et al., 2012). However, detailed and comprehensive studies are missing on their chemical stability in wastewater and also biotransformation in the sewer or wastewater should be considered (McCall et al., 2016a). Several studies were published on the analysis of synthetic cathinones in wastewater samples, with mephedrone, methylenedioxypyrovalerone, methcathinone, methylone and α-pyrrolidinovalerophenone (α-PVP) being the most frequently detected (Borova et

al., 2015; Chen et al., 2013; González-Mariño et al., 2016a, 2016b; Kinyua et al., 2015; Mwenesongole et al., 2013; Ocaña-González et al., 2015; Thai et al., 2016; Tscharke et al., 2016).

Phenylethylamines are a class of substances related to amphetamine and methamphetamine, possessing psychoactive and stimulant effects; however, modification of these compounds can lead to potent hallucinogens (Zaitsu et al., 2011; Zawilska and Andrzejczak, 2015). They include amphetamine derivatives such as MDMA, 2C and 'D' series drugs. However, the phenethylamine core is shared among several compounds including cathinones and catecholamines. Several metabolism studies have been conducted in an effort to understand their metabolic profiles (Ewald et al., 2008, 2006; Lai et al., 2015b; Staack et al., 2003) but more information is needed.

Piperazine-like compounds include the original member 1-benzylpiperazine (BZP), its methylenedioxy analogue and several phenylpiperazines. They are mainly known to bind to serotonin receptors, with BZP additionally producing amphetamine-like stimulant effects (Bye et al., 1973; De Boer et al., 2001). A summary with details on the metabolism of piperazines can be found in some articles (Maurer et al., 2004; Staack et al., 2001; Staack and Maurer, 2005); furthermore, one study showed the detection of metabolites in human urine (Tsutsumi et al., 2005). Some examples are shown in **Table S1**.

Tryptamine is a primary amine alkaloid found widely in nature in both the plant and animal kingdoms and known for its hallucinogenic effects (Collins, 2011). Metabolism of some synthetic tryptamines has been studied (Kamata et al., 2006; Michely et al., 2015; Narimatsu et al., 2008).

Arylcycloalkylamines, which include the ketamine derivative methoxetamine (MXE) and phencyclidine derivatives, have emerged as legal alternatives to ketamine (Roth et al., 2013). MXE, which has gained popularity in several European countries (EMCDDA, 2014), is extensively metabolized (Meyer et al., 2013) but it was detected as parent MXE in wastewater from Belgium and Switzerland (Kinyua et al., 2015).

Benzodiazepines are psychoactive substances whose core structure is a benzene ring fused to a diazepine ring. 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 – even to the extent of being used as "date rape" drugs (Schwartz et al., 2000). From now on we will refer to those benzodiazepines used illegally as design benzodiazepines. Designer benzodiazepines have become a rapidly growing class of drugs on the NPS online market, since a medical prescription is not needed. Since designer benzodiazepines have increased in popularity, studies have been conducted characterizing their human metabolism (Huppertz et al., 2015; Moosmann et al., 2013).

Up to now, no designer phenethylamines, tryptamines or designer benzodiazepines and metabolites have been detected in wastewater and only two studies has reported the stability of some phenylethylamines in wastewater (Bade et al., 2016; Senta et al., 2015b).

Although the interpretation of quantitative results should be done carefully for NPS due to the lack of metabolic information, the qualitative monitoring could lead to a better understanding of the frequency of use and could identify changes in consumption.

3. EXPOSURE BIOMARKERS FROM ENVIRONMENT AND FOOD

Two important exposure pathways for potentially harmful compounds are the dietary intake and the exposure from the surrounding daily environment. The monitoring of various classes of compounds for which exposure commonly occurs through these routes is necessary to safeguard public health. Representative chemical classes have been chosen as examples for this paper. Pesticides, mycotoxins and parabens are three classes of compounds for which exposure occurs through the intake of contaminated food or absorption through the skin and adverse health effects can be foreseen for humans (Błędzka et al., 2014; Heyndrickx et al., 2015; Rizzati et al., 2016; Warth et al., 2013). Exposure through the indoor environment (furniture, electronics, packaging and

personal care products (PCPs)) is characteristic for UV-filters, plasticizers and brominated flame retardants.

This section reviews the specific biomarkers of each of the above mentioned chemical classes which could be measured in wastewater in order to assess the overall exposure to these compounds through a WBE approach. When relevant, we have also included the metabolites of these chemicals to be explored as a suitable biomarker. The suggested biomarkers are reported in **Table S2** including also metabolites, whenever such information is available.

3.1 Pesticides

Pesticides are chemicals commonly used for control of harmful organisms, such as fungi, insects and weeds. They are mostly used for crop protection, but can also be used for livestock protection, as well as for other industrial and household purposes, such as termite prevention. The general population is exposed to pesticides mainly through diet (Ntzani et al., 2013), but also through household use (Trunnelle et al., 2013) and inhalation of polluted air - particularly in agricultural areas where aerial spraying of pesticides occurs (Coscollà et al., 2010). Exposure to pesticides is of public concern as they may cause health effects such as elevated rates of chronic diseases, like cancer or diabetes, as well as neurodegenerative disorders such as Parkinson disease, birth defects and reproductive diseases (Rizzati et al., 2016). Young children are the most susceptible to be at risk (European Food Safety Authority, 2013).

There are several types of pesticides and they are generally classified by their chemical structure: carbamate, organophosphate or triazine pesticides (**Table S2**). They may also be classified by the type of pest they control, such as herbicides, which are intended to kill weeds and other unwanted plants, and insecticides, which kill insects and other arthropods. Pesticides are mostly formulated as mixtures with individual components which may act independently of each other, interact or have dose-addition effects (Hernández et al., 2013).

Until now, there are only two WBE studies (Rousis et al., 2016a, 2016b) published on human exposure to pesticides. The first work (Rousis et al., 2016a) proposed for the first time a new application for pesticides, where pyrethroid, triazine and organophosphate metabolites were monitored in influent wastewater of seven Italian cities. The most frequently detected compounds were the specific metabolite of chlorpyrifos and chlorpyrifos-methyl, 3,5,6-trichloro-2-pyridinol (TCPY), the metabolite of diazinon (2-isopropyl-6-methyl-4-pyrimidinol, IMPY), the pyrethroid metabolites 3-phenoxybenzoic acid (3-PBA, common metabolite of about 20 pyrethroids), 3-(2,2-dichlorovinyl)-2,2-dimethyl-(1-cyclopropane)carboxylic acid (DCCA, common metabolite of permetrin, cypermetrin and cyflutrin) and two alkyl phosphate metabolites. The second work (Rousis et al., 2016b) applied the novel WBE approach to assess further exposure to pyrethroids, concretely 3-PBA, cis-DCCA and trans-DCCA. The obtained results were in agreement with the Human Biomonitoring (HBM) profiles in urine samples of the general population, reported in the literature.

Yusa et al. 2015 reviewed analytical methods for HBM of pesticides and found that the most commonly biomonitored ones are carbamates, herbicides, neonicotinoids, organophosphates, pyrethroids and sulfonylurea herbicides – all of which can be monitored in urine samples and they can be good potential biomarkers for WBE. However, some other pesticide classes, such as organochlorines, are probably not suited to WBE due to their non-polar characteristics and their poor excretion in urine (Yusa et al., 2015).

As described previously for other substances, the metabolites of pesticides rather than the parent substances should be measured in wastewater to avoid contributions from sources other than human metabolism. It has to be emphasized that some pesticide metabolites are also formed in the environment (i.e. atrazine undergoes dealkylation in water systems forming human metabolites) and therefore more research is needed. Moreover, there are some common metabolites produced by

different classes of compounds, such as organophosphate pesticides, organophosphate plasticizers and flame retardants, and this should be taken into account in a WBE approach. The novel method developed by Rousis et al. is considered as a valuable tool for obtaining objective, direct information on pesticide exposure levels and could provide complementary information for HBM studies. **Table S2** presents the main potential biomarkers of exposure to pesticides selected by considering the detection frequency in urine, and the concentration levels (Barr, 2008; Yusa et al., 2015).

3.2 Mycotoxins

Mycotoxins are toxic fungal metabolites that can be found in food and feed which are intended for human and animal consumption (i.e. cereals such as rice, maize and wheat). There is huge concern of human health risks related to the ingestion of these substances, since they are stable in food processing and cooking. Maximum tolerable levels in food commodities were therefore legally established in many countries (*Comission Regulation 1881/2006*, 2006). While, nowadays, approximately 400 compounds belong to this group, only 10-15 are considered to be priority mycotoxins, due to higher occurrence and toxicity. These latter compounds belong to the groups of aflatoxins, ochratoxins, patulin and fusarium toxins (tricothecenes, fumonisins, zearalenone and zearalenone derivatives) (Anfossi et al., 2016; Turner et al., 2015). HBM studies performed on general population have shown that the most studied mycotoxin biomarkers in urine samples are aflatoxin M1 (AFM1), ochratoxin A (OTA), deoxynivalenol (DON), nivalenol (NIV), fumonisin B1 (FB1) and zearalenone (ZON) (H Fromme et al., 2016; Heyndrickx et al., 2015). If mycotoxin contaminations are going to be increased in the near future due to higher global food demand and global climate and environment changes, new methods are needed to evaluate the human exposure

to mycotoxins (Marroquín-Cardona et al., 2014). Thus, a novel approach such as the WBE can be useful to provide complementary information to existing methods.

Few studies dealing with the determination of mycotoxins in wastewater have been published. The studied analytes were detected at very low concentrations (few ng/L), but at high detection frequency. In addition to parent compounds, some human metabolites were also investigated. The detected mycotoxins were DON, beauvericin (BEA), 3-Acetyldeoxynivalenol (3-AcDON), NIV, ZON, α-zearalenol (α-ZOL) and β-zearalenol (β-ZOL) (Kolpin et al., 2014; Laganà et al., 2004; Schenzel et al., 2012, 2010; Wettstein and Bucheli, 2010). None of these studies attempted to apply the WBE approach to these substances; they had only a monitoring scope. In the present paper a selection of mycotoxins and their related potential biomarkers for a WBE approach were reported for the first time (**Table S2)**.

3.3 Parabens

Parabens are a group of chemicals that is drawing a lot of interest in the current discussion given their potential endocrine disrupting properties, since studies have shown that they have potential adverse health effects (Hu et al., 2013; Kim et al., 2015; Zhang et al., 2013). This has raised concern considering their widespread use. Parabens are used as preservatives in many different products, such as cosmetics, PCPs and foods, and can be commonly found in household products.

Some studies also investigated the occurrence and fate of parabens in wastewater (González-Mariño et al., 2009; Gracia-Lor et al., 2012a; Kasprzyk-Hordern et al., 2008), but not from a WBE perspective. Therefore, a list of known urinary biomarkers for paraben exposure is reported in **Table S2.** Future research should be addressed in order to explore paraben biomarkers for WBE.

3.4. UV-Filters

Overexposure to ultraviolet (UV) radiation has been associated with skin disorders, such as cancer (Ramos et al., 2016). This led to the widespread usage of UV filters in a variety of personal care products to protect against UV radiation, i.e., sunscreen, cosmetics, beauty creams, body lotions, hair sprays and shampoos (Brausch and Rand, 2011). UV filters are also used in food packages, plastics and textiles to prevent polymer degradation. Hence, human exposure occurs through multiple routes such as dermal absorption, ingestion of contaminated food and tap water (Valle-Sistac et al., 2016). Two major types of UV filters are currently available; organic UV filters are used to absorb UVA and/or UVB radiation, whereas inorganic UV filters mainly reflect the radiation. Given the high photostability and lipophilicity, many UV filters can enter biological membranes and bioaccumulate in the body, including in the placental tissues (Valle-Sistac et al., 2016). However, it is important to note that most UV-Filters are released into the sewers without going through the body (Daughton and Ruhoy, 2009; Ruhoy and Daughton, 2008). This fact would contribute to a large uncertainty in its estimation.

Urinary analysis has frequently detected UV filters at various levels, demonstrating human exposure (Dewalque et al., 2014; Louis et al., 2015). Despite their widespread use, between 2010 and 2015 only 20 studies have been published in peer reviewed journals dealing with UV filters detection in wastewater (Ramos et al., 2016). Yet, available data indicates that major UV filters groups, i.e. benzophenone derivatives, p-aminobenzoic acid derivatives, camphor derivatives, benzotriazole derivatives, salicylate derivatives, benzimidazole derivatives, triazine derivatives, cinnamate derivatives, crylene derivatives, and dibenzoyl methane derivatives, are ubiquitous in wastewater with concentrations ranging from the ng/L to the mg/L level (Gago-Ferrero et al., 2011; Rodil et al., 2012). Evidence from mammalian studies indicate that various UV filters are endocrine disruptors, acting as estrogenic, antiestrogenic, antiandrogenic or antithyroid (Louis et al., 2014). These results find support in recent epidemiologic studies reporting an association between human urinary levels of certain UV filters and couples fecundity, i.e. BP-2 (Louis et al., 2014), and

630 decrease semen quality, i.e. BP-3 and BP-8. Therefore, (Louis et al., 2015) highlighted the
631 importance of further studies exploring human exposure to UV filters. Despite the presence of UV
632 filters has been reported in wastewater (Ramos et al., 2016; Tsui et al., 2014) no WBE approaches
633 have been yet tested to evaluate human exposure to these substances. However, the high stability of
634 these compounds and the indication of particular metabolite signatures (Le Fol et al., 2015) suggest
635 potential biomarkers for UV filters in wastewater based biomarkers to support epidemiological
636 studies **(Table 1 and S2).**
637
638 **3.5. Plasticizers**
639 Plastics are very versatile materials typically consisting of organic polymers of high
640 molecular mass, which may contain other substances. Manufacturers often add different chemicals
641 to plastics to give them specific characteristics, such as flexibility, resilience and pliability. These
642 plasticizers mainly include phthalates and adipates, and because of their environmental persistence
643 and their widespread use, it is unsurprising that they can be found in wastewater and in the
644 receiving environment (Barnabé et al., 2008; Gao and Wen, 2016; Olofsson et al., 2013; Zolfaghari
645 et al., 2014). Some of these chemicals and/or their derivatives interfere with endogenous hormone
646 signalization in animals and humans, raising concerns about their potential to cause long-term
647 diseases (Joint Fao Oms Expert Committee On Food Additives, 2010). In particular phthalates (e.g.
648 bis(2-ethylhexyl) phthalate and, dibutyl phthalate) were associated with the disruption of
649 hormonally-mediated pathways, as well as increased risk for cancer ("Toxicological profile for
650 di(2-ethylhexyl)phthalate (DEHP)," 2002, "Toxicological profile for Di-n-butyl-Phthalate," 2001).
651 Furthermore, epidemiological observational studies suggest that there is a consistent association of
652 blood and urine concentrations of phthalates, and some effects, such as those mentioned above
653 (Joint Fao Oms Expert Committee On Food Additives, 2010; Kim et al., 2015; Wang et al., 2016).
654 Due to a better toxicological profile (Bhat et al., 2014) and a better blood compatibility (Zhong et

al., 2013), other plasticizers, such as di-isononyl cyclohexane-1,2-dicarboxylate (DINCH), have been increasingly used in recent years as alternatives in PVC films and medical devices. Metabolites of phthalates, adipates, and DINCH have been found in urine (Fromme et al., 2016; Guo et al., 2011; Herrero et al., 2015; Loftus et al., 1993; Silva et al., 2007), but their presence in wastewater has never been investigated. For a list of known biomarkers in urine see **Table S2.**

3.6 Flame retardants

Flame retardants (FRs) are chemical additives for manufactured materials, such as plastics and textiles, to inhibit, suppress, or delay the production of flames to prevent the spread of fire. Brominated flame retardants (BFRs) and organophosphorus flame retardants (PFRs) are the most used classes of organic FRs. Due to their high log K_{ow}, BFRs are lipophilic and preferentially retained in the human body, e.g. in the blood or adipose tissue. They are only slowly metabolized to hydroxylated metabolites (e.g. HO-PBDEs), which are also retained in the body and thus not excreted in the urine. The presence of BFRs in the sewer system is largely due to direct input from the indoor environment, following washing out of dust and being associated with particles. PFRs are less persistent and rapidly metabolized in the human body (Van den Eede et al., 2013), they have been measured in municipal wastewater in Europe (Loos et al., 2012; Marklund et al., 2005), Australia (O'Brien et al., 2014) and United States (Schreder and La Guardia, 2014). PFRs metabolites are excreted via urine and they are thus suitable biomarkers to assess human exposure to PFRs (Van den Eede et al., 2015); however, there are no reports on the presence of PFR metabolites in wastewater and no studies testing them in a WBE approach **(Table S2).**

4. HEALTH BIOMARKERS

Community health programs play an essential role for public health agencies to monitor and evaluate the present status of health in a community and measure the success of programs aimed at

improving it. Current challenges mainly consist of the quick and reliable evaluation of the overall health of a population, and detect possible health and illness threats such as pandemics or higher prevalence of diabetes or cancer.

The quantitative measurement of specific exogenous and endogenous biomarkers related to these diseases in wastewater has the potential to provide rapid information on different factors related to public health and illness. Specific classes of pharmaceuticals such as antibiotics and benzodiazepines and their metabolites are exogenous compounds, which can be related to their use for specific illnesses or diseases, whereas endogenous compounds, such as α-fetoprotein, chroriogonadotropin (hCG) and isoprostanes, are more directly related to cancer or stress.

In this section, both exogeneous and endogenous specific biomarkers are presented and suggested to monitor health issues (**Table S3**) through the WBE approach. In addition, DNA-based approaches, currently applied in the field of WBE, have been reviewed.

4.1 Pharmaceuticals

4.1.1 Antibiotics

Antibiotics (ABs) can be suitable biomarkers for representing human health status associated with bacterial infections. The determination of reliable data on their consumption is of interest as AB use is one of the main factors responsible for AB resistance (Euro-CDC, 2012). WBE may give a better understanding of real time use and misuse of ABs at the population level, by supporting for example prescription data from official sources and annual sales.

Many ABs are excreted unchanged in urine (Castiglioni et al., 2006; Huang et al., 2011), hence, parent drugs are generally targeted as biomarkers **(Table S3**). However, the selection of a significant AB biomarker should not be limited to the parent drug only; in fact, the investigation of specific metabolites is adding specificity to the analysis avoiding biases coming from the direct disposal of the AB. This is particularly relevant for ABs widely used for veterinary treatments. The

most targeted classes of ABs are β-lactams, quinolones and fluoroquinolones, sulphonamides, tetracyclines and macrolides. Apart from β-lactams that undergo easy hydrolysis, sulphonamides and macrolides are very persistent, and are therefore also detected in treated wastewater (Jelic et al., 2012). Stability of the ABs metabolites in wastewater is less understood.

The occurrence of ABs in influent wastewater has been widely investigated in several countries (Gracia-Lor et al., 2012b; Kümmerer, 2009; Verlicchi et al., 2012). Seasonal variability of population-normalized mass loads was observed by Castiglioni et al. 2006, using the WBE approach, showing a difference in percentage from winter to summer of 47, 77 and 100 for ciprofloxacin, ofloxacin and sulphamethoxazole, respectively (Castiglioni et al., 2006). Temporal monitoring of ABs at several time scales showed a higher variability monthly/hourly than daily/weekly along with seasonality in mass fluxes for ciprofloxacin, ofloxacin and clindamycin (Coutu et al., 2013). Deconjugation during in-sewer transport may influence the influent loading of sulfamethoxazole (Snip et al., 2016) depending on the type and size of the served catchment (Polesel et al., 2016). Application of WBE helped in determining the usage of ABs in areas where consumption data were scarce or a proper regulation was missing, revealing an excessive use in China (Yuan et al., 2015).

4.1.2 Benzodiazepines

Benzodiazepines are used therapeutically for a considerable number of applications, including anxiety and sleep disorders. Their primary mode of action is an enhancement of the action of the neurotransmitter gamma-aminobutyric acid which may result in anticonvulsant, anxiolytic, hypnotic, muscle relaxant and sedative effects. Benzodiazepines and benzodiazepine analogs are commonly prescribed; however, they are also among the most frequently abused prescription medications (Button, 2015). Despite the risk for abuse, approximately 5.2% of US adults between

729 18 and 80 years of age used benzodiazepines in 2008, with a double prevalence for women than
730 men (Olfson et al., 2015). As such, monitoring of benzodiazepines is of public concern.

731 Monitoring benzodiazepines in populations could be achievable via WBE as they are
732 normally halogenated and hence resistant to biodegradation (Kosjek et al., 2012). Multiple studies
733 have already identified both parent benzodiazepines and their urinary metabolites in wastewater
734 influent (Baker et al., 2014; Borova et al., 2014; Castrignanò et al., 2016; Fernández et al., 2014;
735 Hummel et al., 2006; Kosjek et al., 2012; Racamonde et al., 2015, 2014). Differences in the
736 behavior of benzodiazepines are associated with differences in functional substituent groups, and
737 mainly the hydroxylated tranquilizers, oxazepam, and temazepam, were reported to be present in
738 influent and effluent wastewater (Bijlsma et al., 2012; Hummel et al., 2006; Löffler et al., 2005).

739 A summary of the most commonly prescribed and detected benzodiazepine parent
740 compounds and their metabolites, which have been identified in urine, in addition to identification
741 in wastewater and stability data, when available, are presented in **Table S3.**

742

743 **4.1.3 Other pharmaceuticals**

744 Even if many works have analysed the presence of pharmaceuticals in urban wastewater,
745 only a few studies investigated these chemicals as WBE biomarkers. Some examples can be found
746 in **Table 1**. Furthermore, a list of proposed pharmaceuticals is given in **Table S3** with their
747 excretion rates.

748

749 **4.1.4. Chiral pharmaceuticals**

750 More than 50% of pharmaceuticals currently used are chiral although they are usually
751 manufactured as racemic mixtures (Petrie et al., 2015; Vazquez-Roig et al., 2014). Human
752 metabolism and microbial processes during wastewater treatment can result in the enrichment of
753 one specific enantiomer. Thus, the analysis of chiral compounds in wastewater allows to distinguish

754 between usage of pharmaceuticals due to intentional human ingestion and from accidental release
755 (direct disposal). For instance, enantioselective analysis was used by (Vazquez-Roig et al., 2014) to
756 tentatively propose direct disposal of atenolol where a moderate higher average daily load was
757 observed. Recently, (Petrie et al., 2016) identified direct disposal of the antidepressant fluoxetine
758 via the sewer network using wastewater analysis.
759

760 **4.2. Endogenous compounds**

761 Endogenous chemicals are produced by biological processes associated with stress or
762 normal metabolism. Changes in biological mechanisms may result in alterations of the endogenous
763 compound production and, therefore, measurement of such compounds can be used as indicator of
764 health status and disease (Daughton, 2012b; Group, 2001; Hagger et al., 2006). Endogenous
765 biomarker analysis has been extensively studied as diagnostic or prognostic tools in clinical
766 medicine, and can be further applied to the field of WBE (Daughton, 2012b). Thus far, the
767 investigation of endogenous biomarkers has been more focused on diseases such as cancer, diabetes
768 and cardiovascular disorder than on the overall health status. However, the number of biomarkers
769 validated for routine clinical practice is rather limited (Poste, 2011; Rifai et al., 2006), which falls
770 into even smaller numbers of biomarkers for WBE when considering only those excreted into urine.
771 Nevertheless, a range of endogenous compounds have been suggested as wastewater biomarkers of
772 effect including cancer (prostate specific antigen, α-fetoprotein) (Thomas and Reid, 2011; Yang et
773 al., 2015c), oxidative stress (isoprostanes) (Daughton, 2012b; Ryu et al., 2015; Thomas and Reid,
774 2011) and health (anti-inflammatory eicosanoids) (Daughton, 2012b). To date, studies conducted on
775 candidate endogenous biomarkers in wastewater are based on targeted analysis of specific markers
776 such as isoprostanes (Ryu et al., 2015) and cancer biomarkers (Yang et al., 2015c). However, it is
777 important to note that omics approaches also hold promising and important roles in future

778 developments and applications of endogenous biomarkers analysis in WBE (Rice et al., 2015). The
779 added value of analyzing these compounds would reside mainly in relative comparisons, both intra-
780 and inter- communities (Daughton, 2012b). Compared to the interpretation of the exogenous
781 biomarkers, where absolute values are emphasized, the use of endogenous biomarkers is more
782 focused on detecting changes over time or between communities. Such data can reveal emerging
783 trends (i.e., early warning system) and health disparities caused by various factors (e.g., exposure,
784 lifestyle).
785
786 **4.3. DNA**
787 The demand for sensitive, low-cost and high-throughput methods to characterize DNA/RNA
788 sequences has driven the development of molecular biology techniques and bioinformatics, i.e.,
789 PCR-based approaches and next generation sequencing (NGS) (Ryoo et al., 2013). Massive
790 sequencing is nowadays possible, owing to the development of different NGS platform that allows
791 an entire genome to be sequenced in less than one week. These technical advances led to a rapid
792 increase in new applications, including DNA-based health biomarkers. During the last decade an
793 increasing number of studies took advantage of these developments, and applied them to the field of
794 WBE. Several examples highlight the potential of the approach. In the field of virological
795 surveillance, wastewater screening has been used to identify the viral strains that are circulating in
796 the community, supporting epidemiological studies of the related viral infections and working as an
797 early warning tool (Hellmér et al., 2014; Kokkinos et al., 2011; Mclellan et al., 2013; Zhou et al.,
798 2014). Hellmér et al. 2014 investigated the presence of eight pathogenic viruses (norovirus,
799 astrovirus, rotavirus, adenovirus, Aichi virus, parechovirus, hepatitis A virus [HAV], and hepatitis E
800 virus) in wastewater from Sweden to explore whether their identification could be used as an early
801 warning of outbreaks. Results show that two strains were involved in an ongoing outbreak in

802 Scandinavia and were also identified in samples from patients with acute hepatitis A in Gothenburg
803 during spring of 2013.

804 A similar framework has been applied in other areas such as the study of the epidemiology
805 of the emerging human pathogens (Mclellan et al., 2013; Webb et al., 2015), and antibiotic
806 resistance patterns of populations (Colomer-Lluch et al., 2014; Kumaraswamy et al., 2014;
807 McLellan and Eren, 2014). One of the most recent applications has been in the field of human
808 metabolic disorders. With the obesity epidemic reaching alarming levels, there is a need to set
809 biomarkers to identify populations or sub-populations at risk (Lyssimachou et al., 2015). Recently,
810 a good correlation has been established between the gut microbiome and obesity. In fact, only a few
811 bacterial species are sufficient to distinguish between lean and obese individuals (Le Chatelier et al.,
812 2013). These findings prompted a large study in the US using oligotyping of high-throughput 16S
813 rRNA gene sequence data to screen wastewater from 71 cities. It was demonstrated that cities could
814 be differentiated by their sewage bacterial communities, and the community structures were good
815 predictors of a city's estimated level of obesity (Newton et al., 2015). This example illustrates that
816 once specific biomarkers are identified, DNA-based analysis in wastewater can work as a powerful
817 tool to support epidemiological studies
818

819 **5. POPULATION BIOMARKERS**

820 Accurate estimation of population size is necessary to normalize WBE data to the per capita
821 level, which allows for temporal and spatial comparisons to be made (van Nuijs et al., 2011b). A
822 review of all uncertainties associated with WBE found that there is a direct relationship between the
823 uncertainty in measuring the population size and the uncertainty in the calculated daily loads of
824 drugs (Castiglioni et al., 2013; Lai et al., 2015a). Therefore, accurate data on population size are
825 needed to make decisions involved with planning and forecasting, assessing services and

826 infrastructure, policy making, informing legislation and resource allocation at the level of
827 neighborhood, city, province or country.

828 Current methodologies to estimate population size are based on public surveys (such as
829 census taking), complemented with a wide array of demographic statistics, such as tourism and
830 potential commuters. Census, however, can become increasingly outdated and cannot be easily
831 updated to accommodate change such as births, deaths, and migration (movement). Ideally, the
832 census should be able to estimate both the *de jure* and the *de facto* population. The *de jure*
833 population comprises all "usual" residents, mainly those with formal residences. The *de facto*
834 population comprises all those who are present, regardless of the location of their formal or usual
835 residence (Daughton, 2012a). A *de facto* population therefore includes all non-residents (e.g.,
836 commuters, visitors, tourists) and excludes all permanent residents who are absent. However, the
837 census approach acquires a static snapshot estimate and usually succeeds in only capturing a portion
838 of the population. Population size can also be estimated from hydrochemical parameters that are
839 routinely determined in the WWTPs, including chemical oxygen demand (COD), biological oxygen
840 demand (BOD) and total nitrogen and phosphorus. However, these parameters are highly influenced
841 by wastewater composition (i.e. industrial, domestic or mixed).

842 Addressing the population uncertainty and identifying suitable markers for the population
843 size markers is thus an important aspect of WBE (Been et al., 2014; Brewer et al., 2012; Lai et al.,
844 2011; O'Brien et al., 2014). Many compounds can be considered as biomarkers for population size.
845 Possible candidates are both naturally occurring and synthetic xenobiotics (and their metabolites or
846 formulation impurities), as well as products of endogenous metabolism. A variety of chemicals
847 have been studied as biomarkers of population, including drugs (e.g., carbamazepine (Gasser et al.,
848 2010)), biocides (e.g., triclosan (Singh et al., 2010)), chemicals in household cleaning agents, e.g.,
849 fluorescent whiteners, trialkylamines (Managaki et al., 2006; Valls et al., 1989), and food additives,
850 e.g., sucralose (Oppenheimer et al., 2011). An essential characteristic for a biomarker to be useful

for measuring population size is, in addition to the general requirements for a biomarker, to have a low variance in the per capita daily excretion (Daughton, 2012a); the knowledge of quantities excreted daily ensures that diurnal variations (e.g., resulting from circadian biorhythms) are fully accommodated. Another requisite for these groups of biomarkers is that daily per capita excretion should not be affected by variables such as season, weather and geographic location.

To date, none of the population size markers proposed have yet met all necessary criteria mentioned above and additional characteristics described before for a WBE biomarker should also be considered. Some specific applications are listed below.

5.1 Artificial sweeteners

The most popular artificial sweeteners used in foodstuffs include acesulfame (ACE), alitame (ALI), aspartame (ASP), cyclamate (CYC), neotame (NEO), neohesperidin dihydrochalcone (NHDC), saccharin (SAC) and sucralose (SUC) (**Table S4**) (Kokotou et al., 2012; Lange et al., 2012). All of them, except NEO and ALI, are allowed to be used as additives in food by the European Union (EPCD, 2003), whereas five of them, ACE, ASP, NEO, SAC and SUC are approved to be used in the United States (USFDA, 2006).

After ingestion, ACE, CYC and SAC are unaffected by the human metabolism, and thus largely eliminated from human bodies mainly unchanged in urine (Fermin and Vallvey, 2004; Lange et al., 2012; Renwick, 1985; Roberts et al., 2000; Sardesai and Waldshan, 1991). Studies have shown that, due to variations in individual metabolism, CYC could be metabolized to cyclohexylamine and excreted in urine (Renwick et al., 2004). For ALI, 7–22% is excreted unchanged in feces, while the rest, about 78–93% is hydrolyzed to aspartic acid and alanine amide (Fermin and Vallvey, 2004). The glucuronide conjugates of ALI metabolites are the major urinary metabolites in the first 24 hours. ASP is largely broken down in human gut to aspartic acid, phenylalanine and methanol (Fermin and Vallvey, 2004; Lange et al., 2012). NEO and its

metabolites are excreted in urine and feces (WHO Food Additive Series No. 52, 2004). Less than 2% is excreted unchanged, but it is extensively metabolized in humans via de-esterification to *N*-[*N*-(3,3-dimethylbutyl)-L-*alpha*-aspartyl]-L-phenylalanine (WHO Food Additive Series No. 52, 2004). Minor metabolites of NEO include *N*-(3,3-dimethylbutyl)-L-aspartic acid, 3,3-dimethylbutanoic acid and the carnitine conjugate and glucuronide conjugate of 3,3-dimethylbutanoic acid (WHO Food Additive Series No. 52, 2004). NHDC is hydrolyzed in humans to isoferulic acid, 3-hydroxyphenylpropionic acid, and 3-hydroxycinnamic acid (Fermin and Vallvey, 2004; Lange et al., 2012). SUC is mainly excreted unchanged in human feces, while 8-22% was excreted in urine unchanged together with its glucuronide conjugates (Roberts et al., 2000).

ACE, CYC, SAC, and SUC were found highly stable in raw wastewater at 4°C and room temperature over four days (Ordóñez et al., 2012). Under these conditions, only 20-30% of ASP remained after one day and none left after two days. Similarly, the amount of NHDC was found less than 10% in the raw wastewater at 4°C after one day and linearly decreased at room temperature over three days. Similar results were also reported in another study, in which ACE, CYC, SAC and SUC remained stable in raw wastewater at 4°C over three weeks, whereas ASP and NHDC were degraded within a day (Tran et al., 2013).

Since they are exclusively non-metabolized in humans and highly stable in wastewater, the parent compounds ACE, CYC, SAC and SUC can be measured for the WBE approach. However, the analysis of the metabolites of ALI, ASP, NEO and NHDC, rather than of the parent compounds, is required, since these artificial sweeteners are largely metabolized in humans. Stability tests for the metabolites in raw wastewater are also necessary for future studies. The use of artificial sweeteners has been shown to be highly related to human activities (Buerge et al., 2009) and, therefore, human consumption is considered as the major source of these substances in raw wastewater; however, other sources, such as animal feedings, agriculture farms and industries, can contribute to their presence in sewage systems (Kokotou et al., 2012).

Certain artificial sweeteners also showed a specific weekly pattern: in general higher loads in influents (i.e. consumption) were observed during weekdays than during weekends (Kokotou et al., 2013). This could be associated with more commuters during the weekday than the weekend in the studied catchment. These previous studies together suggested that measuring artificial sweeteners could be useful for the WBE approach to understand the population flow in a given catchment. This concept of using human consumed chemicals, such as the artificial sweetener ACE, to back-estimate the population size from a given wastewater sample was firstly attempted and discussed by (Lai et al., 2011) and further refined using wastewater samples collected on the census day and applying a Bayesian model (O'Brien et al., 2014). Importantly, with chemical-derived population estimates, the robustness of the WBE data was improved, since the total methodological uncertainty of the approach was reduced (Lai et al., 2015a, 2011).

5.2. Nicotine

Currently, nicotine and its metabolites have been used as population markers on two occasions (Chen et al., 2014; Senta et al., 2015a). In the first case, the authors focused solely on cotinine, whose loads varied only limitedly over one week and showed good correlation with the size of the investigated populations (i.e., correlation coefficient = 0.981) (Chen et al., 2014). However, geographical/cultural differences in tobacco use or fluctuations in the number of users have been raised as potential flaws to the use of cotinine as population marker (Chen et al., 2014). Moreover, consumption of tobacco could change due to tax and other tobacco-related policies, which could affect the potential of nicotine and its metabolites as population markers. In the second study (Senta et al., 2015a), cotinine and *trans*-3'-hydroxycotinine loads were used to estimate the number of individuals contributing to the collected wastewater samples. Good agreement was found between nicotine metabolite load population estimates and census data, suggesting that the method is a viable approach to estimate the size of a population.

926

927 ### 5.3. Caffeine

928 Caffeine and some of its major metabolites were recently tested as a population biomarkers.
929 Caffeine was one of the compounds included in the exploratory study to estimate population size
930 using samples collected on the census day and applying a Bayesian model (O'Brien et al., 2014). A
931 strong correlation between caffeine mass loads and population size was observed. In the second
932 study, generally good agreement between caffeine loads and hydrochemical parameters routinely
933 determined at the WWTPs was found (Rico et al., 2016). In another recent study, three major
934 caffeine metabolites: 1-methylxanthine, 7-methylxanthine and paraxanthine were tested together
935 with caffeine as possible population biomarkers (Senta et al., 2015a). These compounds fulfilled
936 some of the major requirements for an ideal biomarker - they are easily detectable and stable in
937 wastewater samples. However, their mass loads in wastewater did not completely reflect the human
938 excretion profile of caffeine, probably due to biases in caffeine pharmacokinetic data (see section
939 2.4 and **Table S2**) and additional sources of some metabolites and unconsumed caffeine. This
940 makes the possibility of using caffeine and/or its metabolites as biomarkers for population size
941 assessment rather difficult, at least without additional studies.

942

943 ### 5.4. Pharmaceuticals

944 Concentrations and mass loads of pharmaceuticals in wastewater were used in the WBE field
945 for the estimation of population size only on three occasions (Lai et al., 2011; O'Brien et al., 2014;
946 Rico et al., 2016). The investigated compounds by Lai et al. (Lai et al., 2011) were atenolol (beta-
947 blocker), gabapentin (anti-convulsant), hydrochlorothiazide (diuretic), and venlafaxine (anti-
948 depressant). Atenolol was concluded to be the best option for this aim for the specific catchment. In
949 addition to the compounds selected by Lai et al., the same group also investigated carbamazepine
950 (antiepileptic), codeine, ibuprofen, paracetamol (analgesics), furosemide (diuretic), iopromide

951 (contrast medium), naproxen (anti-inflammatory) and salicylic acid (metabolite of acetylsalicylic
952 acid) and the measured loads were used in a collective model for the estimation of the population
953 size (O'Brien et al., 2014). By cross validating the data, the authors demonstrated that large
954 populations sizes could be estimated fairly accurately using the information of multiple chemical
955 mass loads. However, it could not be improved for small populations. In the work published by
956 (Rico et al., 2016) twelve human urine biomarkers were tested to estimate population size, six of
957 them being pharmaceuticals (hydrochlorothiazide, carbamazepine, codeine, naproxen, salicylic acid
958 and atenolol). However, by using these compounds, the population was under or overestimated
959 compared to the hydrochemical population, but they have good prospects if the appropriate data
960 sales are available.
961
962 **5.5. Endogenous compounds**
963 An alternative for estimating the population size in the catchment area of a WWTP relies on
964 monitoring influent wastewater for a biomarker linked to human metabolism. Chemicals involved
965 in endogenous metabolism avoid many of the problems encountered with xenobiotics, since their
966 association with human activities has a higher fidelity. Yet, their main problem is excessive intra-
967 and inter-individual variation in excretion. Biomarkers of endogenous origin derive from human
968 biochemical processes and undergo continuous urinary or fecal excretion. Several endogenous
969 biomarkers, which have been considered in the past or which have the potential to estimate the
970 population size more accurately **(Table S4)**, are further discussed.
971 An important endogenous biomarker, widely used in clinical chemistry and with detailed
972 knowledge about its excretion, is creatinine (CR). A small portion of creatine (and
973 phosphocreatine), which is stored predominately in skeletal muscle, is continually converted to
974 form the endogenous anhydride, CR (a nitrogenous waste product cleared via the kidney); the rate
975 of conversion, in males for example, is about 1.6–1.7% per day. The major factors involved with

variability in CR output have been summarized by (Ryan et al., 2011). However, intra- and inter-day CR excretion is not constant and daily excreted quantities can have high variance, being strongly influenced by diet composition. In addition, CR is being increasingly used as a food and nutritional supplement, adding yet another source of potential variation to CR excretion rates. Although CR has been used in WBE studies as population marker (Brewer et al., 2012; Chiaia et al., 2008), it was shown to be unstable in wastewater (completely decomposed within 24 h) (Chen et al., 2014).

Another potential biomarker is coprostanol (CoP) that originates from gut microbial metabolism, making up roughly 60% of the overall sterol content in human feces. CoP is poorly absorbed from the gut (it does not undergo enterohepatic circulation) and is therefore fully excreted in the feces. Since the 2000s, CoP has been used as anthropogenic marker in wastewater and to gauge the degree of dilution of raw or treated wastewater in receiving surface water (Takada and Eganhouse, 1998). However, CoP is excreted by various vertebrates in differing absolute and relative quantities and it is sometimes difficult to distinguish between human and animal contamination (Bull et al., 2002). Furthermore, CoP adsorbs substantially onto particulate matter found in wastewater and was thus discarded as potential population marker (Chen et al., 2014). Similar results were obtained for cholesterol (Chen et al., 2014); cortisol and androstenedione were investigated, but rapidly degraded in wastewater (Chen et al., 2014).

Another example of biomarker relatively unique to human metabolism is 1-aminopropan-2-one (1-aminopropanone: APR; 1-aminoketone). Through 1-aminopropan-2-ol, APR serves as a precursor to vitamin B-12 (Fitzsimons and Belt, 2005). It is very water soluble and it is excreted via urine, but in much lower daily quantities than CoP. However, it is sometimes found in wastewater at levels higher than in urine, implicating potential *de novo* microbial formation in sewage (Fitzsimons and Belt, 2005), whilst it could not be detected on other occasions (Singh and Gardinali, 2006).

5-hydroxyindoleacetic acid (5-HIAA), a metabolite of serotonin, has also been investigated. Its excretion might be altered due to diseases (e.g., carcinoid tumors (Zuetenhorst, 2004)) and diet (i.e., some fruits and nuts (Feldman and Lee, 1985) and salt intake (Sharma et al., 1993). Furthermore, intra- and inter-individual variability in excretion has also been highlighted (Curtin et al., 1996). Results from wastewater analysis showed good correlation with census data and the authors considered it as a promising marker (Chen et al., 2014).

Ammonium (NH_4^+) represents the major form in which ammonia (NH_3) is found in wastewater and originates from the breakdown of urea (Udert et al., 2006). It is mainly introduced via toilets (Butler et al., 1995) and it is routinely measured by WWTP as a water quality parameter. It is supposedly less affected by non-human sources compared to conventional parameters (e.g., chemical or biological oxygen demand, total phosphorous) (van Nuijs et al., 2011b) and can potentially be measured online using ion-selective electrodes. Fluctuations in ammonium loads have been shown to link well to population dynamics (Been et al., 2014). Yet, its use to estimate absolute figures of the size of the *de facto* population might be undermined in rural areas due to the contribution of agricultural sources.

5.6. DNA

Deoxyribonucleic acid (DNA) is a nucleic acid that carries most of the genetic instructions from all known living organisms and many viruses. DNA can be naturally shed into the environment through urine, feces, exudates or tissue residues. Compared to most of chemical compounds as a candidate of population biomarkers, DNA is much more stable and able to persist in the environment from month to hundred years depending on species (Prüfer et al., 2014; Thomsen and Willerslev, 2015). DNA biomarkers have been widely used in the field of medical diagnostics and biomedicine (Altintas and Tothill, 2013; Liu et al., 2011; Ralla et al., 2014; Wang et al., 2012). For WBE, DNA has a great potential to act as a population biomarker, not only because

of its little affinity to other species in wastewater and constant excretion by humans, but also for its extreme stability and the possibility of being quantifiable Those robotic characteristics well meet the proposed criteria of a proper population biomarker candidate (Dejean et al., 2011; Thomsen and Willerslev, 2015).

Typically, the changes of DNA component and structure such as DNA damage, repair and mutation could be used as biomarkers. Recently, a H2AX histone phosphorylation assay was developed as DNA damage biomarker for human population study, as it represents an early event in the cellular response against DNA double-strand breaks (Sánchez-Flores et al., 2015). However, to select a population biomarker for WBE uses, one of the crucial criteria is to screen human specific DNA. Wastewater is a complex matrix, which may contain DNA from various species such as plants, animals, and viruses. A recent study by Yang *et al* (Yang et al., 2015a, 2015b) has proposed to use community sewage sensors to identify human-specific mitochondrial DNA as a potential population biomarkers. In this study, human specific mitochondrial DNA associated with disease biomarkers (Liu et al., 2011; Tipirisetti et al., 2014) was amplified from wastewater by a specifically designed primer using quantitative real-time polymerase chain reaction (PCR) (Yang et al., 2015a). More importantly, the amplicons were detectable by an electrochemical biosensor based on a custom synthesized ferrocence intercalator as a signal transducer. The developed biosensors allow for the detection of single nucleotide variation and enable the potential of portable sensors for rapid identification of specific human biomarkers in wastewater.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

WBE is a rapidly developing scientific discipline with a strong transdisciplinary character. It has shown great progress, and opens up many possibilities for expanding its application to provide relevant information about lifestyle and public health.

This review has outlined potential wastewater biomarkers of exposure or effect that could be used for future applications associated with lifestyle and wellbeing studies. However, it has also discussed limitations and highlighted that more research is needed, for various proposed biomarkers, before WBE can appropriately be applied. Moreover, several trends, needs and recommendations are indicated:

- Human pharmacokinetic data (metabolism and urinary profile of excretion) are necessary to ensure that the candidate biomarker is formed in the body in a high proportion and is excreted mainly via urine. This information is highly relevant not only to back-calculate the consumption/exposure of a certain substance by a community, but also to distinguish the amount of a substance coming from human or other sources.
- In-sample and in-sewer stability studies are needed for a better application in WBE. Stability tests are often performed in the laboratory, trying to reproduce the real conditions of temperature and sewage composition or in-sewer conditions. An alternative would be the use of *in-silico* tools to predict the stability of a compound in wastewater treatment processes. These models do not guarantee the formation of a biotransformation product, so it may be used as an indicator or a guide about the in-sewer stability of a residue and its potential adsorption (Reid 2014). Sorption onto the solid particulate or the conjugation of the biomarkers must also be taken into account when assessing stability.
- Source identification is needed to ensure that discharges from exogenous sources that might cause overestimation of the real amounts consumed are considered.

- Cross validation of data (e.g. concentrations of pharmaceuticals in wastewater with bench-top sales) is recommended for all applications.
- Multiple biomarkers for estimating the population size need to be set to allow for the normalization of the data. The development of portable biosensors may allow rapid estimation of the population contributing to the wastewater samples in the near future.
- Regular monitoring of sewage for viruses based on similar DNA biosensors may give an early warning of a possible upcoming outbreak.
- Omics approaches also hold promising and important roles in future developments and applications of endogenous biomarkers analysis in WBE.

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1104 **TABLES**

1105

1106 **Table 1.** Overview of the most relevant biomarkers used so far and potential biomarkers (for more
1107 details, please read the corresponding text and/or supporting information).

1108

Class	Parent compound	Biomarker/potential biomarker	WBE application	Reference
Illicit drugs	Cocaine	Benzoylecgonine	YES	(Castiglioni and Gracia-Lor, 2015; Gracia-Lor et al., 2016)
	Amphetamine	Amphetamine	YES	(Castiglioni and Gracia-Lor, 2015; Gracia-Lor et al., 2016)
	Methamphetamine	Methamphetamine	YES	(Castiglioni and Gracia-Lor, 2015; Gracia-Lor et al., 2016)
	MDMA	MDMA	YES	(Castiglioni and Gracia-Lor, 2015; Gracia-Lor et al., 2016)
	THC/Cannabis	THC-COOH	YES	(Castiglioni and Gracia-Lor, 2015; Gracia-Lor et al., 2016)
Alcohol	Ethanol	Ethyl sulfate	YES	(Rodríguez-Álvarez et al., 2015)
Tobacco	Nicotine	Cotinine + trans-3'-hydroxycotinine	YES	(Castiglioni et al., 2015b)
Caffeine	Caffeine	See Table S1	NO	
NPS		See Table S1	NO	
Pesticides	20 pyrethroids	3-PBA	YES	(Rousis et al., 2016b)
	Permetrin, cypermetrin, cyflutrin	cis-DCCA	YES	(Rousis et al., 2016b)
	Permetrin, cypermetrin, cyflutrin	trans-DCCA	YES	(Rousis et al., 2016b)
Mycotoxines		See Table S2	NO	
Parabens		See Table S2	NO	
UV-filters		See Table S2	NO	
Plasticizers		See Table S2	NO	
Flame		See Table S2	NO	

retardants				
Pharmaceuticals	Atenolol	Atenolol	YES	(Baz-Lomba et al., 2016; van Nuijs et al., 2015)
	Citalopram	Citalopram	YES	(Baz-Lomba et al., 2016; van Nuijs et al., 2015)
	Carbamazepine	Carbamazepine	YES	(Baz-Lomba et al., 2016; van Nuijs et al., 2015)
	Diclofenac	Diclofenac	YES	(Baz-Lomba et al., 2016)
	Metformin	Metformin	YES	(van Nuijs et al., 2015)
	Valsartan	Valsartan	YES	(van Nuijs et al., 2015)
Benzodiazepines	Oxazepam	Oxazepam	YES	(Baz-Lomba et al., 2016)
Artificial sweeteners	Acesulfame	Acesulfame	YES	(Lai et al., 2015a)
Endogenous Compounds	Serotonin	5-HIAA	YES	(Rico et al., 2016)
	Ammonia	Ammonium	YES	(Been et al., 2014)

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1110
1111

FIGURE CAPTIONS

Figure 1. Main requirements of a biomarker

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