Critical review on the stability of illicit drugs in sewers and wastewater samples

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Abstract

Wastewater-based epidemiology (WBE) applies advanced analytical methods to quantify drug residues in wastewater with the aim to estimate illicit drug use at the population level. Transformation processes during transport in sewers (chemical and biological reactors) and storage of wastewater samples before analysis are expected to change concentrations of different drugs to varying degrees. Ignoring transformation for drugs with low to medium stability will lead to an unknown degree of systematic under- or overestimation of drug use, which should be avoided. This review aims to summarize the current knowledge related to the stability of commonly investigated drugs and, furthermore, suggest a more effective approach to future experiments. From over 100 WBE studies, around 50 mentioned the importance of stability and 24 included tests in wastewater. Most focused on in-sample stability (i.e., sample preparation, preservation and storage) and some extrapolated to in-sewer stability (i.e., during transport in real sewers). While consistent results were reported for rather stable compounds (e.g., MDMA and methamphetamine), a varying range of stability under different or similar conditions was observed for other compounds (e.g., cocaine, amphetamine and morphine). Wastewater composition can vary considerably over time, and different conditions prevail in different sewer systems. In summary, this indicates that more systematic studies are needed to: i) cover the range of possible conditions in sewers and ii) compare results more objectively. To facilitate the latter, we propose a set of parameters that should be reported for in-sewer stability experiments (laboratory and full-scale). Finally, a best practice of sample collection, preservation, and preparation before analysis is suggested in order to minimize transformation during these steps.

Keywords: Transformation, sewage epidemiology, sample preservation, psychoactive substances, biodegradation.

Content

Abbreviations

6-MAM	6-monoacetyl morphine
AMP	Amphetamine
BE	Benzoylecgonine
COC	Cocaine
COCA	Cocaethylene
COD	Codeine
EDDP	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EME	Ecgonine methyl ester
KET	Ketamine
LSD	Lysergic acid diethylamide
MBDB	Methylbenzodioxolylbutanamine
MDA	3,4-methylenedioxyamphetamine
MDEA	3,4-methylenedioxy-N-ethylamphetamine
MDMA	3,4-methylenedioxy-methamphetamine
METH	Methamphetamine
MOR	Morphine
MTD	Methadone
nor-BE	Nor-benzoylecgonine
nor-COC	Nor-cocaine
SPE	Solid-phase extraction
SPM	Suspended particulate matter
THC	Δ9-tetrahydrocannabinol
THC-COOH	11-nor-9-carboxy-THC
THC-OH	11-hydroxy-THC
TSS	Total suspended solids
VSS	Volatile suspended solids
WBE	Wastewater-based epidemiology

1 Introduction

Wastewater-based epidemiology (WBE) is a recently introduced monitoring tool in drug epidemiology. It provides objective information about the levels and patterns of drug use at the population level and as such is complementary to existing survey-based methods. It has also the potential to serve as an early warning system for the use of new psychoactive substances (NPS, e.g., Kinyua et al., 2015; Reid et al., 2014) and to investigate the effectiveness of intervention programs (e.g., Burgard et al., 2014; Castiglioni et al., 2014). The principle of WBE is predicated by the fact that substances are excreted as parent compounds and/or metabolites - subsequently referred to as *biomarkers* (of illicit drugs) - after consumption and transported through the sewer network to wastewater treatment plants (Figure 1).

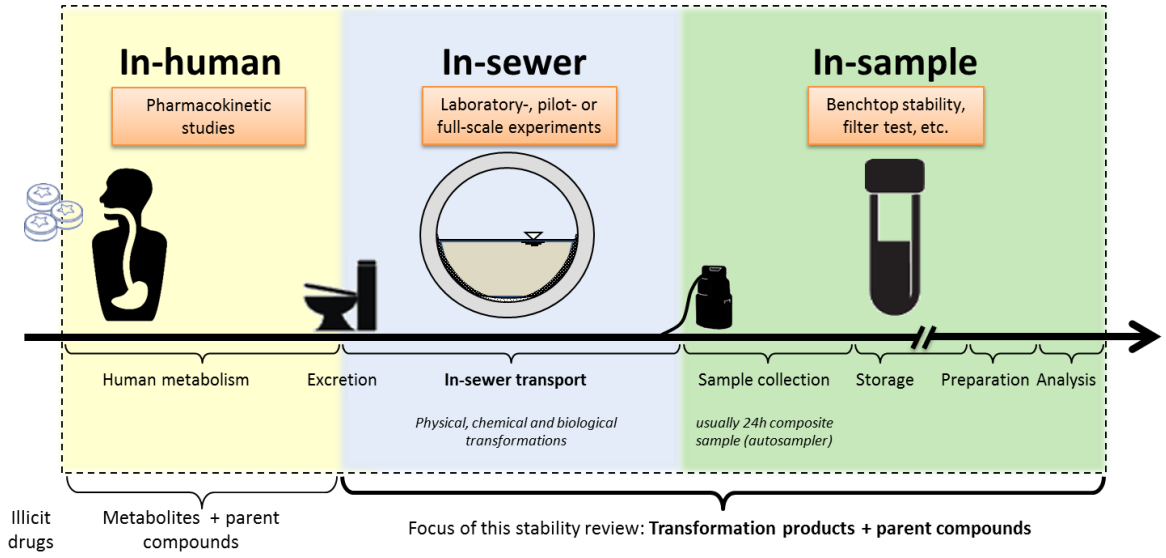

Figure 1. System boundaries used in wastewater-based epidemiology.

The concentrations of biomarkers in the wastewater (c_i) can be quantified with advanced analytical instruments, such as liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Consumption estimates are calculated according to Eq. 1:

$$drug\ use_i = \frac{Q \times C_i \times m_i}{P \times e_i \times p_i} \quad (1)$$

where Q = wastewater volume, c_i = concentration of drug i, m_i = molar mass ratio (parent to metabolite), P = population for normalization, e_i = drug-specific pharmacokinetic excretion rate (average or distribution of urinary excretion, e.g., van Nuijs et al., 2011a; Zuccato et al., 2005) and p_i = purity of

drug. In 2005, WBE was applied for the first time, back-calculating the cocaine use of communities in Italy (Zuccato et al., 2005). Since then, studies have compared temporal and spatial drug use trends by consumption differences between rural and metropolitan areas (e.g., Irvine et al., 2011; van Nuijs et al., 2009a; van Nuijs et al., 2011b), among different countries (e.g., Ort et al., 2014; Thomas et al., 2012) and during special events (festivals, holidays (e.g., Lai et al., 2013)) to name some of several applications.

Most illicit drugs may or may not have licit medical purposes, but they are produced, trafficked and/or consumed illegally on a large scale (United Nations Office of Drug and Crime, 2014). In this review, we focus on the most frequently used illicit drugs and their metabolites: cocaine, amphetamines and amphetamine-type substances, opiates, cannabinoids and selected other illicit substances, such as ketamine (KET) and lysergic acid diethylamide (LSD).

One of the main challenges in WBE is to reduce the uncertainty of each variable in the back-calculation equation (Eq. 1). High uncertainty is related to the excretion rates based on pharmacokinetic literature (Castiglioni et al., 2013) and the chemical analysis of the biomarkers in the complex wastewater matrix. Numerous efforts have focused on improving the accuracy (trueness and precision) of different analytical methods, and inter-laboratory studies were conducted in order to evaluate and harmonize the different analytical procedures being used (Castiglioni et al., 2013; Thomas et al., 2012). Uncertainties associated with the population estimates have recently been conceptually reduced (Lai et al., 2015; O'Brien et al., 2014), as well as the uncertainty related to wastewater sampling (Ort et al., 2010). Furthermore, sample collection, storage and preparation methods have been evaluated, deducing critical, substance-specific parameters: i) solvent and temperature used during the evaporation of solid-phase extraction (SPE) extracts, and ii) biomarker stability in the wastewater matrix and silanisation of glassware (Baker and Kasprzyk-Hordern 2011a). To further minimize uncertainty of WBE, a better understanding of in-sewer and in-sample stability of biomarkers is needed (Fig. 1).

Castiglioni et al. (2013) estimated that the uncertainty related to the stability of the biomarkers during in-sewer transport is less than 10%. However, they also concluded that more research is needed. Other studies urge the consideration of the stability of the illicit drug biomarkers in the back-calculation method (van Nuijs et al., 2009b; Östman et al., 2014).

While in-sample stability has been studied to some degree for most of the biomarkers (e.g., Baker and Kasprzyk-Hordern, 2011a; Chen et al., 2013; Östman et al., 2014), their in-sewer stability under the influence of varying environmental conditions is not well understood. The sewer is considered a biological and chemical reactor, influenced by physical processes (Hvitved-Jacobsen et al., 2013). Residence times of 30min to 12h (rarely up to 24h) in most catchments and potential environmental processes facilitate formation of transformation products (Heuett et al., 2015a). Consequently, the omission of biomarker-specific in-sewer transformation may add an unknown level of uncertainty. Yet, only few studies have investigated in-sewer stability of selected biomarkers under environmental conditions (Senta et al., 2014; Thai et al., 2014; van Nuijs et al., 2012) and accounted for stability in the back-calculation of drug use (Baker et al., 2014; Östman et al., 2014).

In this review, we summarize and critically evaluate the available scientific literature focusing on the stability of the most frequently used illicit drugs during i) in-sewer transport and ii) in-sample storage. Using this information, more insight is obtained regarding the uncertainty of WBE associated with stability and, additionally, suggestions for best practices in future stability studies are provided.

1.1 Environmental processes in sewer networks

In general, two major categories of processes determine the overall fate of illicit drug biomarkers in the sewer network. First, mass transfer processes that leave the structure of the chemicals unchanged, e.g., transport, mixing and transfer among different phases and/or compartments (sorption, sedimentation, and uptake by organisms). The second category includes processes that alter the structure of the compounds, e.g., chemical and/or biological transformation reactions (Schwarzenbach et al., 2003b). For the remainder of the manuscript, the term *transformation* will be used to refer to any of these three processes, although physical processes are rather transfer, not transformation, processes.

Wastewater contains a large number of soluble, colloidal, and suspended components (e.g., nutrients, metals, micropollutants and pathogenic and nonpathogenic microorganisms). Its content varies in time and space, which favors or inhibits specific environmental processes. In addition, sewer designs and operation modes influence the prevailing conditions, e.g., oxygen concentrations (redox potential), pH, temperature, flow velocities and sediments (Figure 2). The dominating processes that may influence

biomarker concentrations in the sewer are most likely residence time, abiotic and biotic transformations (e.g., hydrolysis, deconjugation, biodegradation), as well as sorption to SPM. Further, the presence of biofilms on the sewer walls should be accounted for (Hvitved-Jacobsen et al., 2013).

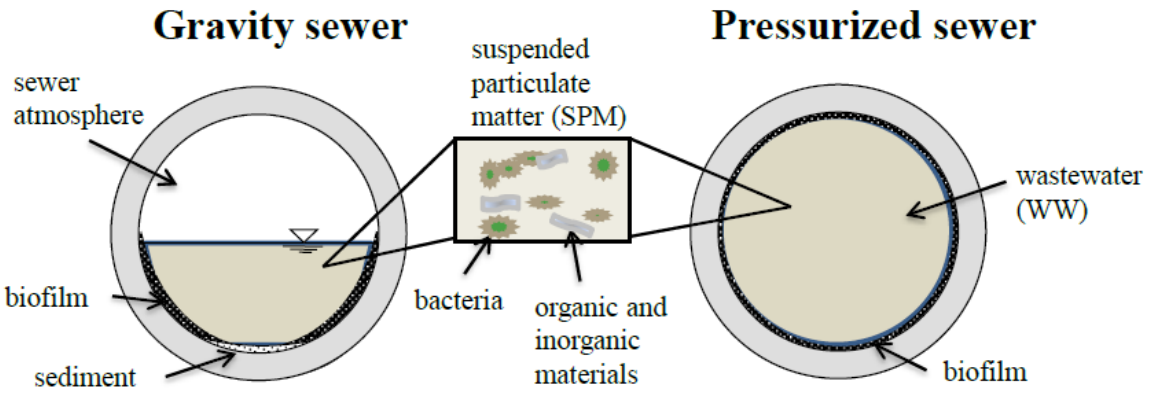

Figure 2. Cross-sections of gravity driven and pressurized sewers.

1.2 Stability of illicit drug biomarkers during wastewater treatment processes and in the environment

It is noteworthy that compounds may also be transformed through numerous processes, e.g., chlorination or ozonation, during wastewater treatment or photodegradation in the environment. Effects of these processes and resulting transformation products were beyond the scope of this review. To fully assess their fate, transport and toxicological impact on the natural environment, comprehensive monitoring and additional investigations are needed (Bijlsma et al., 2013; Heuett et al., 2015a). Several reviews are available for these pertinent topics (Bijlsma et al., 2013; Boix et al., 2014; Postigo et al., 2011a, 2011b; Rodayan et al., 2014; Heuett et al., 2015a).

2 Summary of reviewed studies

More than 50 WBE studies mentioned the importance of stability, from which 24 actually investigated in-sewer or in-sample stability to some extent. Overall, a clear distinction between in-sewer and in-sample stability is lacking in the current literature.

2.1 General setup

In-sewer stability experiments should account for all relevant processes occurring in sewer compartments: i) the bulk liquid (wastewater with suspended particulate matter (SPM)), ii) the biofilm growing on the sewer walls, iii) the sediments, and iv) the sewer atmosphere in gravity sewers. Most studies only investigated the stability in the bulk liquid. No experiments to date have investigated the effect of sewer sediments or the sewer atmosphere on biomarker transformation. Focusing on illicit drugs, only one pilot-scale sewer reactor study included the sewer wall biofilm (Thai et al., 2014). Considering the physico-chemical properties of most biomarkers (Table S1) and their hydrophilic character, precipitation and evaporation seem negligible (Ternes and Joss, 2006).

In this review, we consider all laboratory studies using unfiltered wastewater at a typical pH around 7-8, at temperatures above 10°C as in-sewer studies. Nonetheless, the experimental setups of these studies differed in complexity with different degrees of approximation. Several research groups conducted stability studies as part of a validation method, and only a few publications showcased an exclusive focus on analyte stability in wastewater. Consequently, the main study conditions are summarized in Table 1. Usually, the concentration of biomarkers in spiked, unfiltered wastewater was monitored in glass or plastic containers, in the dark, under constant pH and temperature, over periods of 12-72h. Six publications considered the fraction of biomarker that bound to SPM or sludge (in wastewater treatment plants).

Table 1. Overview of experimental conditions

Publication	in-sewer stability study	in-sample stability study	sorption to SPM study	Matrix	Screening	Spiking levels	Sampling interval	Experimental container	pH	Temperature	Redox conditions
Baker and Kasprzyk-Hordern, 2011a	x			unfiltered WW	target	1 µg/L	0, 12, 24, 72 h	amber silanized glass bottles	pH 7.4	19 °C	NA
		x		unfiltered and filtered WW		1 µg/L	0, 12, 24, 72 h		pH 7.4 and 1.8	2°C and 19°C	
		x[a]		filtered WW (filter type not reported)		1 µg/L	0, 2, 4,6 weeks		pH 2	-20°C	
Baker and Kasprzyk-Hordern, 2011b			x	unfiltered WW	target	none	not relevant	amber silanised bottles	NA	NA	NA
Bisceglia and Lippa, 2014	x			WW (coarsely filtered 11µm Whatman Nb1)	target	3-600x the background concentrations	> 10 x over 24 h	1-L glass Erlenmeyer flask	pH 7.3	9°C, 23°C, 31°C	NA
Boix et al., 2014		x		WW, surface water	suspect	1000 µg/L	0, 1, 3, 7, 10, 17 days	NA	NA	room temperature	NA
Burgard et al., 2013		x		unfiltered WW	target	1 µg/L	8 x over 72 h	glass container	NA	max. 20°C	NA
Castiglioni et al., 2011a		x		unfiltered WW	target	1-5 µg/L	0, 1, 3 days; 3 freeze-thaw cycles	glass bottles	NA	4°C, -20°C freeze-and-thaw	NA
Castiglioni et al., 2006		x		unfiltered WW	target	0.5-5 µg/L	0, 3 days	glass bottles	NA	4°C	NA
Castiglioni et al., 2015		x		unfiltered WW	target	0.1 µg/L	0, 3, 6, 24, 48 h	glass bottles	NA	4°C; room temperature	NA
	x			unfiltered WW		0.1 µg/L	0, 3, 6, 24, 48 h	glass bottles	NA	room temperature	NA

Chen et al., 2013	x			unfiltered WW	target	> 0.1 µg/L	0, 1, 2, 3, 7, 14 days	NA	NA	20°C	NA
		x		unfiltered WW, filtered WW, unfiltered WW +$Na_2S_2O_5$		> 0.1 µg/L	0, 1, 2, 3, 7, 14 days	NA	pH of WW (~7) and at pH 2	20°C, 4°C and -20°C	NA
Chiaia et al., 2008		x		unfiltered WW	target	0.2 µg/L	1, 2, 3, 4, 7, 14, 21 days	HDPE bottles	pH of WW (~7) and at pH 2	room temperature, 4°C, -20°C	NA
Gheorghe et al., 2008		x		surface water	target	0.1-0.4 µg/L	0, 1, 3, 5 days	glass bottles	pH 6 and 2	−20°C, 4°C and 20°C	NA
González-Mariño et al., 2010		x		filtered WW and filtered WW +NaN_3	target	100 µg/L	0, 1, 3, 5, 7, 14, 21, 84 days	amber glass bottles	NA	4°C and -20°C	NA
Heuett et al., 2015b		x		unfiltered WW	target	0.25 µg/L	0, 3, 7, 17, 27, and 123 days	glass bottles	NA	-20°C	NA
Jelic et al., 2014	x			unfiltered WW +sewerwall biofilm	target	no spike	0, 21 h	NA (real sewer)	pH 7.2-7.6	22°C	anaerobic
Langford et al., 2011			x	sludge	target	no spike	not relevant	silanized glass flask	NA	NA	NA
Mardal and Meyer, 2014	x[b]			unfiltered WW, unfiltered WW +rat urine and feces	suspect	100 µg/L	0, 1, 2, 3, 4, 5, 6, 7 days	amber glass bottles	NA	22°C	aerobic
Metcalfe et al., 2010			x	unfiltered WW	suspect	none	not relevant	NA	pH 3	NA	NA
Östman et al., 2014		x		filtered WW; purified water	target	0.9 µg/L	0, 24 h	polyethylene bottles (HDPE)	NA	room temperature; 4°C	NA

Plósz et al., 2013	x			unfiltered WW + activated sludge	target	no spike	0, 15, 30, 60, 90, 120 min, 4, 6, 8, 10, 16, 18 and 24 h	glass reaction vessel	pH 7.4	21°C	aerobic and anaerobic
			x	preclarified WW +mercury chloride		no spike	0, 10, 20, 30, 45, 60 min	glass reaction vessel	pH 7.4	NA	NA
Rosa Boleda et al., 2011		x		drinking water	target	none	0, 3, 5, 8 days	sterilized polypropylene bottles with sodium thiosulfate	NA	4°C and room temperature	NA
Senta et al., 2014	x			unfiltered WW	target	0.2 µg/L, cannabinioids 1 ug/L	0, 4, 6, 24, 48, 72 h	glass bottles	pH 7.5	10° and 20°	NA
			x	unfiltered WW		4 µg/L	0, 4, 6, 24, 48, 72 h	glass bottles	pH 7.5	10° and 20°	NA
		x		unfiltered WW		0.25 µg/L	0, 24 h	HD polypropylene bottles	pH 7.5 and 2	4°C	NA
Senta et al., 2013			x	unfiltered raw WW, secundary effluent WW, activated sludge	target	none	not relevant	NA	pH≈7.5	NA	NA
Subedi and Kannan, 2014			x	unfiltered raw WW, primary effluent WW, secundary effluent WW, activated sludge, sludge	target	none	not relevant	amber glass jars	NA	NA	NA
Thai et al., 2014	x			unfiltered WW in sewer pilot reactor with sewerwall biofilm	target	deuterium labelled substances 10 µg/L;	0, 0.25, 0.5, 1, 2 ,3, 6, 9 and 12 h	Perspex™	pH 7.5	20°C	gravity sewer (aerobic) and rising main sewer (anaerobic)
van Nuijs et al., 2012	x			unfiltered WW	target	0.12 - 1.6 ug/L	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 26 h	silanized glass flask	pH 7.5	20°C	NA

Wick et al., 2011	(x)[c]			unfiltered WW+activated sludge	suspect	2 µg/L	8x over 3 days	amber glass bottles	regulated at pH 7 (±0.2)	NA	aerobic

[a] longterm stability study; [b] incubation of WW with rat urine and feces to mimick human excreation; [c] Matrix was enriched with activated sludge to mimick the wastewater treatment

For most biomarkers, we found a range of transformation values, most likely as a result of different environmental conditions that were tested. Therefore, we propose to rate *in-sample stability* and *in-sewer stability* separately for each substance based on the available literature and our judgment: *low* stability (60-100% transformation), *medium* stability (20-60% transformation), *high* stability (0-20% transformation), or *variable* stability over 24h. The knowledge for the main groups of compounds is described in the subsequent sections. For other compounds and further information, see Table 2.

215 **Table 2.** Literature summary of stability of illicit drug biomarkers from in-sewer and in-wastewater samples.

In-sewer stability			**WW = wastewater** *Substances used for consumption back-calculation		
In-sample stability			The stability of each substance during in-sewer transport and in-sample is rated as: stability is low (60-100% transformation), medium (20-60% transformation), high (0-20% transformation) or variable (if not otherwise indicated over **24h**).		
Group	**Parent**	**Metabolite**	**# stability studies**	**Stability**	**References**
Cocaine and metabolites	**Cocaine (COC)**		10	**Low**: 60% transformation over 12 h @ 20°C in gravity sewer laboratory reactor --> 100% transformation expected over 24 h. In raising main laboratory reactor 45% transformation occured over 12 h; with activated sludge 100% transformation over 24 h at 21°C and pH 7.4	Baker and Kasprzyk-Hordern, 2011a; Bisceglia and Lippa, 2014; Chen et al., 2013; Plósz et al., 2013; Senta et al., 2013, 2014; Subedi and Kannan, 2014; Thai et al., 2014; van Nuijs et al., 2012; Wick et al., 2011
			12	**Low**: at neutral pH: at 4°C, 9°C, 19°C, 20°C, 23°C & 31°C over 1-3 d; filtered/unfiltered WW at 2°C & 19°C over 3 d (slightly higher for unfiltered WW at 20°C over 3 d but lower afterwards); after two freeze-and-thaw cycles within 3 d. **High**: *neutral pH*: at -20°C over 3 weeks; at 2°C over 3 d; *low pH*: at 2°C & 19°C - 20°C over 3 d; at -20°C over 3 weeks; at 37°C over 3.5 h; in Milli-Q water at 4°C & 25°C for 24 h; with $Na_2S_2O_2$ at 20°C for 2 weeks; absorbed on SPE cartridges (HLB) over 12 weeks	Baker and Kasprzyk-Hordern, 2011a, 2011b; Castiglioni et al., 2006, 2011a; Chen et al., 2013; Chiaia et al., 2008; Gheorghe et al., 2008; González-Mariño et al., 2010; Heuett et al., 2015b; Metcalfe etal., 2010; Östman et al., 2014; Senta et al., 2014
		benzoylecgonine* (BE)	10	**Medium**: 14% transformation over 12 h @ 20°C in gravity sewer laboratory reactor --> ca. 28% transformation expected over 24 h. In raising main sewer 8% transformation occured over 12 h; with activated sludge 80% transformation over 24 h at 21°C and pH 7.4	Baker and Kasprzyk-Hordern, 2011a; Bisceglia and Lippa, 2014; Chen et al., 2013; Plósz et al., 2013; Senta et al., 2013, 2014; Subedi and Kannan, 2014; Thai et al., 2014; van Nuijs et al., 2012; Wick et al., 2011
			12	**High**: at low pH: at 2°C & 19°C - 20°C over 3 d; at -20°C over 3 weeks; at 37°C over 3.5 h; at neutral pH: at -20°C over 3 weeks; at 2°C, 4°C, 9°C, 19°C, 20°C, 23°C & 31°C over 1-3 d, 7 d; filtered / unfiltered WW at 2°C & 19°C over 3 d (higher for filtered WW at 20°C over 14 d); in Milli-Q water at 4°C & 25°C for 24 h; after two freeze-and-thaw cycles within 3 d; with $Na_2S_2O_2$ at 20°C for 2 weeks; absorbed on SPE cartridges (HLB) over 12 weeks	Baker and Kasprzyk-Hordern, 2011a; Castiglioni et al., 2006, 2011a; Chen et al., 2013; Chiaia et al., 2008; Gheorghe et al., 2008; González-Mariño et al., 2010; Heuett et al., 2015b; Metcalfe etal., 2010; Östman et al., 2014; Senta et al., 2014
		ecgonine methyl ester (EME)	3	**Medium**: 20-40% loss in unfiltered WW (pH 7.5, 20-23°C) and and surface water (pH 6, 20°C); **Low**: with activated sludge >80% transformation over 24 h at 21°C, pH 7.4	Bisceglia and Lippa, 2014; Plósz et al., 2013; van Nuijs et al., 2012
			3	**Low:** at neutral pH at 4°C, 9°C, 20°C, 23°C & 31°C over 1-3 d; after two freeze-and-thaw cycles within 3 d	Castiglioni et al., 2011a; Gheorghe et al., 2008; González-Mariño et al., 2010
			1	<5% change in unfiltered WW (pH 7.4, 19°C)	Baker and Kasprzyk-Hordern, 2011a

		nor-benzoylecgonine (nor-BE)	4	**High:** at low pH: at 2°C & 19°C over 3 d; at -20°C over 3 weeks; at neutral pH: at -20°C over 3 weeks; at 2°C, 4°C & 19°C over 1 - 3 d; filtered / unfiltered WW at 2°C & 19°C over 3 d (similar); after two freeze-and-thaw cycles within 3 d	Baker and Kasprzyk-Hordern, 2011a; Castiglioni et al., 2006, 2011a; Chiaia et al., 2008; González-Mariño et al., 2012
		nor-cocaine (nor-COC)	1	<30% change in unfiltered WW (pH 7.4, 19°C)	Baker and Kasprzyk-Hordern, 2011a
			5	**High:** at 2°C, 4°C & 19°C over 1-3 d; in filtered > unfiltered WW at 2°C & 19°C over 3 d; at low pH: at 2°C & 19°C over 3 d; -20°C over 3 weeks; at neutral pH: at -20°C over 3 weeks; **Low**: after two freeze-and-thaw cycles within 3 d	Baker and Kasprzyk-Hordern, 2011a; Castiglioni et al., 2006, 2011a; Chiaia et al., 2008; González-Mariño et al., 2012; Subedi and Kannan 2012
		cocaethylene (COCA)	2	**Medium**: 20-50% change in unfiltered WW (pH 7.4, 19-23°C)	Baker and Kasprzyk-Hordern, 2011a; Bisceglia and Lippa, 2014
			5	**Variable: High**: at low pH at 2°C & 19°C over 3 d; at neutral pH at 2°C, 9°C and 19°C over 1-3 d; in filtered > unfiltered WW at 2 & 19°C over 3 d; **Low**: at neutral pH at 23°C & 31°C over 1-3 d; after two freeze-and-thaw cycles within 3 d.	Baker and Kasprzyk-Hordern, 2011a; Castiglioni et al., 2006, 2011a; Chiaia et al., 2008; González-Mariño et al., 2012; Heuett et al., 2015; Subedi and Kannan 2012
Amphetamine-type substances	**amphetamine* (AMP)**		3	**Variable**: -40% in unfiltered WW (pH 7.4, 19°C), <20% loss (pH 7, 20°C)	Baker and Kasprzyk-Hordern, 2011a; Chen et al., 2013; Senta et al., 2014
			9	**Variable:** most studies found <10% transformation at 4°C and 20°C up to 24 h; one study measured increase of 26-73% at 2°C and room temperature over 24h (may be formed from other substances); over 3 d at 13°C AMP decreased (38% transformation); H**igh** stability at -20°C in unfiltered WW for up to 123 d; stable over 72 h at 37°C in urine	Baker and Kasprzyk-Hordern, 2011a; Burgard et al., 2013; Castiglioni et al., 2006; Chen et al., 2013; Chiaia et al., 2008; González-Mariño et al., 2010; Heuett et al., 2015b; Östman et al., 2014; Senta et al., 2014
	methamphetamine* (METH)		4	**High**: 5% transformation over 12 h @ 20°C in gravity sewer laboratory reactor --> ca. 10% transformation expected over 24 h. In raising main sewer 0% transformation occured over 12 h.	Baker and Kasprzyk-Hordern, 2011a; Chen et al., 2013; Senta et al., 2014; Thai et al., 2014
			9	**High:** transformation <10% up to 24 h at 4°C and 20°C; <10% degradation at -20°C over 123 d	Baker and Kasprzyk-Hordern, 2011a; Burgard et al., 2013; Castiglioni et al., 2006; Chen et al., 2013; Chiaia et al., 2008; González-Mariño et al., 2010; Heuett et al., 2015b; Östman et al., 2014; Senta et al., 2014
	3,4-methylene-dioxymeth-amphetamine* (MDMA)		4	**High**: <10% transformation over 12 h @ 20°C in gravity sewer laboratory reactor. In raising main sewer <10% transformation occured over 12 h.	Baker and Kasprzyk-Hordern, 2011a; Chen et al., 2013; Senta et al., 2014; Thai et al., 2014
			9	**High**: transformation <10% up to 24 h at 4°C and 20°C; <20% degradation at -20°C over 123 d	Baker and Kasprzyk-Hordern, 2011a; Burgard et al., 2013; Castiglioni et al., 2006; Chen et al., 2013; Chiaia et al., 2008; González-Mariño et al., 2010; Heuett et al., 2015b; Östman et al., 2014; Senta et al., 2014
		3,4-methylene-dioxyamphetamine (MDA)	2	**High**: <10% transformation in unfiltered WW 19/20°C at pH 7.4	Baker and Kasprzyk-Hordern, 2011a, Chen et al., 2013
			6	**High:** transformation <10% up to 72 h at 4°C and 20°C; <30% transformation at -20°C over 123 d	Baker and Kasprzyk-Hordern, 2011a; Castiglioni et al., 2006; Chen et al., 2013; Chiaia et al., 2008; González-Mariño et al., 2010; Heuett et al., 2015b; Östman et al., 2014

		3,4-methylene-dioxy-Nethyl-amphetamine (MDEA)	1	<10% transformation in unfiltered WW 19°C at pH 7.4	Baker and Kasprzyk-Hordern, 2011a
			6	**High:** transformation <10% up to 24 h at 4°C and 20°C; <20% degradation at -20°C over 123 d	Baker and Kasprzyk-Hordern, 2011a; Castiglioni et al., 2006; Chiaia et al., 2008; González-Mariño et al., 2010; Heuett et al., 2015b; Östman et al., 2014
	methylbenzodioxolyl-butanamine (MBDB)		1	<20% transformation in unfiltered WW 19°C at pH 7.4	Baker and Kasprzyk-Hordern, 2011a
			2	**High**: transformation <10% up to 24 h at 4°C and 20°C	Baker and Kasprzyk-Hordern, 2011a; Östman et al., 2014
	methylenedioxypyrovalerone* (MDPV)		1	in unfiltered WW no transformation at 22°C	Mardal et al. 2014
			1	transformation <10% after 24 h at 22°C in wastewater; in urine stable over 14 d at room temperature, 4°C and -20°C	Mardal et al. 2014
	methylphenidate* (ritalin)		0	NA	NA
			1	36% transformation at 4°C and 88% transformation at room temperature over 24 h in wastewater; in milliQ <10% transformation	Östman et al., 2014
		ritalinic acid*	0	NA	NA
			1	<10% transformation after 72 h in wastewater at 20°C	Burgard et al., 2013
	mephedrone*		0	NA	NA
			1	in wastewater <10% transformation during 24 h at room temperature and at 4 °C	Östman et al., 2014
Opiates	**heroin**		1	in unfiltered WW >90% transformation at 19°C and pH 7.4	Baker and Kasprzyk-Hordern, 2011a
			3	**Low**: transformed 66% after 12 h at 2°C and 79% at 19°C; 50% degradation at -20°C over 7 d and >90% over 123 d	Baker and Kasprzyk-Hordern, 2011a; González-Mariño et al., 2010; Heuett et al., 2015b
		6-mono-acetyl-morphine* (6-MAM)	3	**Low**: 88% transformation over 12 h @ 20°C in gravity sewer laboratory reactor --> ca. 100% transformation expected over 24 h. In raising main sewer 87% transformation occured over 12 h.	Baker and Kasprzyk-Hordern, 2011a; Senta et al., 2014; Thai et al., 2014
			5	**Low**: degraded quickly in WW (6% transformation at 20°C over 12 h); but relatively stable in milliQ; **High:** <20% degradation at -20°C over 3, 7, 17, 27 d	Baker and Kasprzyk-Hordern, 2011a; Castiglioni et al., 2006; Heuett et al., 2015b; Östman et al., 2014; Senta et al., 2014
	methadone* (MTD)		3	**Variable:** in unfiltered WW <10% loss at 19/20°C and pH 7.4; +10% at 20°C in unfiltered WW pH 7.5; may be prone to sorption	Baker and Kasprzyk-Hordern, 2011a; Senta et al., 2014; van Nuijs et al., 2012
			8	**Variable:** <20% difference at room temperature and 4°C; may be prone to sorption; >40% degradation at -20°C over 123 d	Baker and Kasprzyk-Hordern, 2011a; Castiglioni et al., 2006; Rosa Boleda et al., 2011; Chiaia et al., 2008; González-Mariño et al., 2010; Heuett et al., 2015b; Östman et al., 2014; Senta et al., 2014
		2-ethylidene-1,5-dimethyl-3,3-	1	in unfiltered WW ca.20% loss at 19°C and pH 7.4; may be prone to sorption	Baker and Kasprzyk-Hordern, 2011a

		diphenyl-pyrrolidine* (EDDP)	5	**Variable**: less than 15% difference after 24 h; may be prone to sorption; ca. 40% degradation at -20°C over 3, 7 and 123 d	Baker and Kasprzyk-Hordern, 2011a; Rosa Boleda et al., 2011; Castiglioni et al., 2006; Heuett et al., 2015b; Östman et al., 2014
	morphine* (MOR)		2	**Variable:** in unfiltered WW up to 50% loss (19°C, pH 7.4); up to 20% increase (20°C, pH 7.5) (transformation product of other substances)	Baker and Kasprzyk-Hordern, 2011a; Senta et al., 2014
			5	**Variable:** difficult to judge because of the generation of morphine from other drugs; generally relativ **high** stablity at 4°C in unfiltered WW over 24 h; <20% degradation at -20°C over 3, 7, 17, 27 d	Baker and Kasprzyk-Hordern, 2011a; Castiglioni et al., 2006; Heuett et al., 2015b; Östman et al., 2014; Senta et al., 2014
		morphine-3β-D-glucuronide	1	complete transformation in unfiltered WW pH 7.5 20°C	Senta et al., 2014
			2	**Low:** at pH 7.5 over 24 h at 20°C >80% transformation in WW; 96% transformation in WW at 4°C over 3 d; **high**: in WW at pH 2 over 78 h at 20°C	Castiglioni et al., 2006; Senta et al., 2014
	oxycodone*		1	<10% transformation in unfiltered WW at 19°C and pH 7.4	Baker and Kasprzyk-Hordern, 2011a
			4	**High**: stable (<20% transformation) at 19°C and pH 7.4; <10% degradation at -20°C over 3, 7, 17, 27, 123 d	Baker and Kasprzyk-Hordern, 2011a; Castiglioni et al., 2006; Heuett et al., 2015b; Östman et al., 2014
	fentanyl*		1	<20% transformation in unfiltered WW at 19°C and pH 7.4; may be prone to sorption	Baker and Kasprzyk-Hordern, 2011a
			3	**Variable/medium:** <10% degradation in filtered WW at room temperature; <20% loss in unfiltered WW 19°C and pH 7.4 but 62% loss after 72h under same conditions; may be prone to sorption **high:** in milliQ	Baker and Kasprzyk-Hordern, 2011a; Rosa Boleda et al., 2011; Östman et al., 2014
	buprenorphine*		1	<10% transformation in unfiltered WW at 19°C and pH 7.4	Baker and Kasprzyk-Hordern, 2011a
			1	**Variable:** ca.30% formation in filtered WW at room temperature; <10% degradation in unfiltered WW at 4°C and 19°C and pH 7.4; **high**: in milliQ <10% transformation	Baker and Kasprzyk-Hordern, 2011a; Östman et al., 2014
	codeine* (COD)		1	**High:** <20% transformation/formed in unfiltered WW at 19°C and pH 7.4	Baker and Kasprzyk-Hordern, 2011a
			4	**Variable: High**: In filtered/unfiltered WW <20% transformation over 24 h at 4°C, 19°C and room temperature; may be formed from other substances; <10% degradation at -20°C over 3, 7, 17, 27, 123 d; **Low**: 80% transformed in diluted activated sludge pH 7	Baker and Kasprzyk-Hordern, 2011a; Heuett et al., 2015b; Östman et al., 2014; Wick et al., 2011
Cannabinoids	**tetrahydro-cannabinol (THC)**		1	<20% transformation in unfiltered WW pH 7.5 20°C; may be lost due to sorption	Senta et al., 2014
			1	**NA** - almost no THC is excreted in human urine (Karch and Jenkins, 2006; Postigo et al., 2011; Lai et al., 2011); in spiked unfiltered WW stored at -20°C 50% degradation over 7 days and >90% after 123 d	Heuett et al., 2015b
		THC-COOH*	1	<20% transformation in unfiltered WW pH 7.5 20°C; may be lost due to sorption	Senta et al., 2014

			4	**Variable:** may be lost due to sorption; **High** in WW at 4°C and 20°C over 72 h; high on SPE cartridges over three weeks at -20°C; high at -20°C over 3, 7, 17, 27, 123 d; **Low** at pH 2 over 24 h	Boix et al., 2014; Castiglioni et al., 2006; González-Mariño et al., 2010; Heuett et al., 2015b; Senta et al., 2014
		THC-OH	1	<20% transformation in unfiltered WW pH 7.5 20°C	Senta et al., 2014
			1	<20% transformation at pH 7.4, pH 2, 10°C and 20°C in unfiltered WW	Senta et al., 2014
Other substances	**ketamine* (KET)**		2	**High**: <10% transformation in unfiltered WW pH 7.5, 20°C	Baker and Kasprzyk-Hordern, 2011a; Castiglioni et al., 2015
			3	**High**: at 4°C and room temperature at WW pH and acidified to pH 4 and in milliQ water at 4°C and room temperature	Baker and Kasprzyk-Hordern, 2011a; Castiglioni et al., 2015; Östman et al., 2014
		norketamine (norKET)	2	**High**: <10% transformation in unfiltered WW pH 7.5, 20°C	Baker and Kasprzyk-Hordern, 2011a; Castiglioni et al., 2015
			3	**High**: at 4°C and room temperature at WW pH and acidified to pH 4 and in milliQ water at 4°C and room temperature	Baker and Kasprzyk-Hordern, 2011a; Castiglioni et al., 2015; Östman et al., 2014
	lysergic acid diethylamide* (LSD)		1	<10% transformation in unfiltered WW pH 7.5, 20°C	Baker and Kasprzyk-Hordern, 2011a
			4	**High:** in WW pH and acidified to pH 2 at room temperature and at 4°C; **Medium**: in WW at room temperature up to 24% transformation; in milliQ water at 4°C and room temperature (40% transformation); over 3, 7, 17, 27, 123 d at -20°C >20 and <50% degradation	Baker and Kasprzyk-Hordern, 2011a; Chiaia et al., 2008; Heuett et al., 2015b; Östman et al., 2014
		2-oxo-3-hydroxy-LSD	1	<20% transformation in unfiltered WW pH 7.5, 20°C	Baker and Kasprzyk-Hordern, 2011a
			3	**High:** at WW pH and acidified to pH 2 at room temperature and at 4°C; in milliQ water at 4°C and room temperature	Baker and Kasprzyk-Hordern, 2011a; Chiaia et al., 2008; Östman et al., 2014

2.2 Cocaine and metabolites

In-sample stability of cocaine (COC) and its metabolites has been widely studied over a range of different conditions. These studies focused mostly on in-sample stability of COC and its main metabolite, benzoylecgonine (BE), which is used for back-calculation. Stability of COC was generally *low* under all tested conditions (Table 2). Hydrolysis of COC seems pH-dependent (Warner and Norman, 2000), and acidification of the sample can preserve COC concentrations at low and high temperatures over at least three days (Table 2). Transformation of COC can also be prevented using preservatives (e.g., sodium metabisulfite ($Na_2S_2O_5$), sodium azide (NaN_3)), and COC concentration changes seemed negligible after extraction onto SPE cartridges (HLB) (Chen et al., 2013; González-Mariño et al., 2010).

The wastewater matrix seems to have an influence on the extent of transformation (Castiglioni et al., 2006, 2011a; Gheorghe et al., 2008). COC concentrations remained slightly higher over three days in unfiltered samples compared to filtered wastewater (20°C, pH 7) (Chen et al., 2013). At -20°C and pH 7, COC concentrations in unfiltered wastewater only slightly decreased over three weeks (Chiaia et al., 2008), and another study reported that COC can be stable over 24h in Milli-Q water at 4°C and 25°C (Östman et al., 2014). Furthermore, it is important to note that sorption to SPM of COC and its metabolites seems to be negligible (Baker and Kasprzyk-Hordern, 2011b), but COC concentrations in wastewater samples decreased during freeze-and-thaw cycles (Castiglioni et al., 2011).

In-sewer stability of COC was relatively *low* over 12h at pH 7.1-7.5 and 20°C in a study considering aerobic and anaerobic sewer biofilms, in which transformation of COC appeared to be stronger under aerobic conditions, compared to anaerobic in-sewer conditions (Thai et al., 2014).

Unlike COC, its main metabolite BE showed *high* in-sample stability under various conditions (Table 2). In-sewer stability of BE with aerobic and anaerobic biofilms has been shown to be *high* at 20°C over 12h (Thai et al., 2014), whereas one study revealed a decrease of BE under aerobic and anaerobic conditions with activated sludge biomass at 21°C over 24h (Plósz et al., 2013). Information on other COC metabolites is listed in Table 2.

2.3 Amphetamine and amphetamine-type substances

This group encompasses compounds with chemical structures similar to that of amphetamine (AMP). It includes methamphetamine (METH), 3,4-methylenedioxymethamphetamine (MDMA) and its metabolite 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxy-N-ethylamphetamine (MDEA), methylbenzodioxolylbutanamine (MBDB), as well as, the novel synthetic cathinones, cathinones HCl, methylenedioxypyrovalerone (MDPV), mephedrone, methylphenidate (ritalin) and its metabolite, ritalinic acid.

In-sample stability of AMP in unfiltered wastewater has been consistently shown to be *high* at pH 7 at 4°C and 20°C for 24h in most studies (Table 2). However, Baker et al. 2011a reported an increase (26%) of AMP concentrations at 2°C and pH 7 and a 73% increase in AMP at room temperature over 24h. AMP is also a metabolite of METH, and the pharmaceuticals, selegiline and dextroamphetamine (Kraemer and Maurer, 2002; Heuett et al., 2015a). However, in-sample stability of METH is *high* in unfiltered wastewater at pH 7 at 4°C and room temperature (Table 2) and substantial in-sewer transformation of METH to AMP is thus unlikely. AMP and METH were reported as *highly* stable under all tested conditions, particularly after addition of NaN_3 to the samples over three weeks, instant freezing (-20°C) over 123 days, and after acidification over three weeks (Table 2).

MDMA, MDA and MDEA showed *high* in-sample stability in unfiltered wastewater at pH 7 and at 4°C and 20°C for 24h (Table 2). In addition, they were stable after instant freezing (-20°C) and acidification for 24h up to three weeks. One study investigating the stability of MBDB in unfiltered wastewater at 2°C and at 20°C, both at pH 7, reported a *medium* stability with lower concentration after 12-24h (Baker and Kasprzyk-Hordern, 2011a).

The transformation of MDPV and 12 metabolites – three of them previously reported as human metabolites – was investigated in wastewater at 22°C, and no significant decrease of MDPV (*high* in-sewer and in-sample stability) was observed in a 10-day experiment (Mardal and Meyer, 2014). Further experiments demonstrated the glucuronidase activity in wastewater, since the signal of four glucuronide phase II metabolites decreased by more than 99% after one day (Mardal and Meyer, 2014).

One study in unfiltered wastewater found that in-sample stability of mephedrone is *high* at 4°C and room temperature over 24h (Östman et al., 2014). Similarly, mephedrone was stable in urine at 4°C, room temperature and -20°C over at least 2 days (Johnson and Botch-Jones, 2013). Methylphenidate's in-sample stability in wastewater ranged from *medium* (after 24h at 4°C) to *low* (at room temperature, Burgard et al., 2013; Östman et al., 2014). In Milli-Q water, methylphenidate was stable for 24h both at 4°C and at room temperature (Östman et al., 2014). Ritalinic acid had a *high* in-sample stability in wastewater for 72h at 20°C (Burgard et al., 2013).

Few studies have investigated the influence of sewer biofilm or SPM on stability. The in-sewer stability study conducted by Thai et al. (2014) found a non-significant increase of METH (<5% after 12h) in the presence of aerobic biofilm. Subedi et al. (2014) showed no sorption for METH, MDMA and MDEA and a medium loss (30 – 40%) due to sorption to SPM for MDA and AMP. In contrast, Baker et al. (2011b) found <10% of AMP sorbed to SPM (Baker and Kasprzyk-Hordern, 2011b).

In general, the in-sample stability of AMP, METH, MDMA, MDA and MDEA in unfiltered and filtered wastewater samples at different temperatures have yielded similar results, demonstrating that these compounds had a *high* stability with the exception of AMP, for which a higher change in concentration was reported in some experiments (>20%) (Baker and Kasprzyk-Hordern, 2011a; Östman et al., 2014) (Table 2).

2.4 Opiates

Heroin use had been estimated by measuring its metabolite 6-monoacetyl morphine (6-MAM), however, 6-MAM itself has *low* in-sample stability and can transform quickly to morphine (MOR) in the wastewater matrix. A wastewater sample can lose up to 42% 6-MAM after 24h at 19°C (Baker and Kasprzyk-Hordern, 2011a). Similarly, in-sewer stability is *low*, since up to 90% of 6-MAM was lost at 20°C after 12h in the study with sewer biofilms, performed by Thai et al. (2014). A reliable unbiased biomarker for heroin has not yet been found.

Although the stability of morphine (MOR) is *high* in wastewater samples, the estimation of use of heroin from MOR concentrations is difficult, because it is used itself as a pharmaceutical and is a metabolite of other opiates (e.g., ethyl morphine, 6-MAM, codeine (COD)). The associated glucuronides of MOR,

morphine-3β-D-glucuronide and morphine-6β-D-glucuronide, can be measured in wastewater, but they have a *low* stability and quickly deconjugate to MOR (Table 2).

Most of a COD dose is excreted with urine, either as unchanged COD or as a conjugate. COD is *highly* stable in wastewater samples (Table 2), and its consumption can, therefore, be estimated by measuring the load of COD in wastewater. Jelic et al. (2015) also reported *high* in-sewer stability. However, COD exhibited *low* stability in batch experiments with diluted activated sludge from wastewater treatment plants (Wick et al., 2011).

Most wastewater studies to date measured both methadone (MTD) and its main metabolite EDDP, but the consumption of MTD was only estimated using the parent compound. Both MTD and EDDP seem to be stable in wastewater both under in-sample storage conditions (at 4°C, Östman et al., 2014) and simulated in-sewer conditions (at 19°C and pH 7, van Nuijs et al., 2012). However, a portion of MTD and EDDP can adsorb to SPM in wastewater. Baker and Kasprzyk-Hordern (2011a) observed a significant reduction of spiked MTD (23%) and EDDP (72%) in unfiltered wastewater after 72h at 19°C, most likely due to sorption processes. Overall, the stability of MTD and EDDP is *variable* depending on the conditions and SPM/biofilm content.

Buprenorphine has a *variable* stability in wastewater samples (Table 2). It is a relatively hydrophobic compound and eliminated primarily via feces as free drug with low concentrations occurring in urine. Oxycodone and fentanyl are therapeutic opiates that receive increasing attention as drugs of abuse. To date, all wastewater studies used the parent compounds as biomarkers, although each drug has specific metabolites (e.g., noroxycodone and norfentanyl, Baselt, 2008). Östman et al. (2014) found both compounds stable under storage condition, but Baker and Kasprzyk-Hordern (2011) observed significant degradation of fentanyl in unfiltered wastewater (62%) after 72h at 19°C and pH 7.4. This again may be attributed to adsorption to SPM, since only 6% of fentanyl was lost during the same period in filtered wastewater. Based on the reviewed studies, fentanyl had a *medium* in-sample stability, whereas oxycodone was *highly* stable.

2.5 Cannabinoids

Cannabis's primary active compound is Δ9-tetrahydrocannabinol (THC), which after consumption is metabolized to more than 20 metabolites, the two main ones being 11-nor-9-carboxy-THC (THC-COOH) and 11-hydroxy-THC (THC-OH) (Karch and Jenkins, 2006). These metabolites are excreted as glucuronide conjugates, however in wastewater, they are hydrolyzed/deconjugated to the parent metabolite (Castiglioni et al., 2008). For this reason, THC-COOH is normally used to estimate cannabis consumption in WBE studies (Castiglioni et al., 2011b; Lai et al., 2011). However, there are analytical difficulties especially associated with the sample treatment and detection of THC-COOH due to its higher lipophilicity compared to other illicit drugs (Vazquez-Roig et al., 2013). This sometimes may hamper the inclusion of THC-COOH in analytical methods for routine multi-class determination of illicit drugs.

In-sample stability tests with raw wastewater (pH 7-8) showed *high* stability over 72h with minimal impact of temperature (Table 2). However, after longer storage times at 4°C, degradation became more significant after seven days (González-Mariño et al., 2010). Frozen samples were stable up to 4 months (Heuett et al., 2015b). Acidification of samples to pH 2 (with H_3PO_4) increased the transformation of THC-COOH and THC-OH in wastewater (Khan and Nicell, 2012; Senta et al., 2014). At pH 2, THC-COOH was found to have enhanced adsorption (loss of 54%), compared to only 10% loss at unadjusted pH 7.4 (Senta et al., 2013). This was also found in a similar study where only 1.3% of THC-COOH was expected to have adsorbed to SPM at environmental pH conditions (pH ≈ 7.5) (Khan and Nicell, 2012).

2.6 Other substances

The stability of lysergic acid diethylamide (LSD) in wastewater at pH 7.4 and acidified to pH 2 at room temperature and at 4°C after 24h was *high* (Table 2). The metabolite 2-oxo-3-hydroxy-LSD also exhibits *high* stability and only slightly decreased (10-20%) in wastewater pH 7.4 at room temperature after 24h, while acidification and/or lowering the temperature prevented this (Baker and Kasprzyk-Hordern, 2011a; Chiaia et al., 2008).

The in-sample stability of ketamine (KET) and its metabolite norKET was studied for different conditions: temperature (4°C, room temperature), pH (2, 7.4), and time (12-72h) (Table 2). Both KET and norKET had a *high* stability in all analyzed conditions.

3 Discussion

The reviewed studies clearly show that concentrations of several substances decreased in unfiltered wastewater under different conditions. In order to explain discrepancies for a given biomarker among different studies with variable wastewater matrices, the effects of chemical, biological and physical processes need to be considered.

Biological and chemical transformation processes

In the absence of appropriate abiotic controls, it is difficult to differentiate between chemical and biological transformations. Several of the investigated biomarkers, e.g., COC and 6-MAM, have chemical structures (esters) that are prone to abiotic or biotic hydrolysis in wastewater. Abiotic control experiments were only carried out in three studies (Wick et al., 2011; Mardal and Meyer, 2014; Senta et al., 2014).

Other important, chemically or biologically mediated, processes are conjugation and deconjugation. THC, morphine and MDPV are excreted in conjugated form (e.g., as glucuronides and sulfates) and tend to deconjugate during in-sewer transport (Boleda et al., 2007; D'Ascenzo et al., 2003; Evgenidou, 2015, Mardal and Meyer, 2014).

Studies of biological transformation (biotransformation) mechanisms related to biomarkers in sewers are scarce (Mardal and Meyer, 2014). Biotransformation can occur under aerobic and anaerobic conditions, whereby illicit drugs demonstrate affinity for bacterial enzymes and serve as co-metabolic (non-growth) substrates (Siegrist and Joss, 2012). In most of the reviewed stability studies, the redox conditions (aerobic/anaerobic) were not measured or monitored, even though the redox potential influences bacterial activity, and biotransformation is higher under aerobic than anaerobic conditions (Thai et al., 2014). The extent of biotransformation, therefore, depends on the type and amount of active biomass in the sewer, which may vary among different networks (Roth and Lemmer, 1994).

It is still not well understood which species of bacteria are responsible for biological transformation of organic micropollutants in wastewater treatment processes (Siegrist and Joss, 2012). Further, the microbial community in wastewater treatment plants deviates from sewer communities (Hvitved-Jacobsen et al., 2013). The conditions in the activated sludge process in wastewater treatment are selected to favor growth of specific microorganisms, such as nitrifying and phosphorous-accumulating bacteria (Henze et al., 2002). Under the conditions prevailing in sewers, fast-growing heterotrophic bacteria outcompete the slower growing organisms, such as nitrifying bacteria (Hvitved-Jacobsen et al., 2013). Therefore, the use of activated sludge to mimic in-sewer transformation may not be representative for the active microbial community in sewers. Only one study that investigated the stability of illicit drug biomarkers included sewer wall biofilm (Thai et al., 2014), and the results implied that biofilm is an important parameter that needs to be taken into account.

Another factor that may explain some of the variability in stability is the influence of other organic and inorganic constituents of SPM, such as feces and toilet paper (natural polymers, cellulose). These factors have not yet been investigated for their potential effect on transformations.

Two of the key environmental variables influencing chemical and biological reactions are temperature and pH. Most studies reported or investigated the effect of these variables on the stability. Wastewater temperatures in sewers can vary from 10°C in winter up to 30°C in summer (Tchobanoglous and Burton, 1991). The reviewed transformation studies were conducted at constant temperature, either at low temperature (2-10°C) or at room temperature (19-23°C). Results show a temperature dependence of the transformation rates for COC, 6-MAM and morphine-3β-D-glucuronide (Senta et al., 2014). Similarly, (chemical and biological) hydrolysis rates of COC in wastewater were higher at 31°C compared to 23°C and 9°C. The transformation rate coefficients were larger than those reported in deionized water at similar pH and temperatures, confirming that biologically mediated hydrolysis can play an important role in wastewater (Bisceglia, 2007; Bisceglia and Lippa, 2014).

The reviewed studies typically state one pH value, omitting whether pH was recorded at the beginning or end of the experiment or frequently monitored. A recent study found that biotransformation is pH-dependent, where the neutral fraction of ionizable substances (e.g., amines) induced a higher microbial uptake (Gulde et al., 2014). During the transport of illicit drug biomarkers in sewers or over the course

of an experiment, transformations of macronutrients in the wastewater can change the pH (Sharma et al., 2013), which may change the bioavailability of biomarkers with pKas close to the wastewater pH.

Spiking concentration levels

All studies conducted multi-target analysis, which can make the interpretation of results difficult for some substances, if both parent compounds and metabolites were spiked together. To avoid this, one study spiked mass-labelled analogues to be able to differentiate between parent compound and metabolite transformations in separate batch experiments (Thai et al., 2014).

Another limitation may arise if the selected excreted human biomarker is also a transformation product formed in the sewer (Heuett et al. 2015a). For example, the stability of an excreted metabolite may be low, but as a result of in-sewer transformation from the parent compound, a stability study may nonetheless exhibit constant concentration levels over the investigated period (as in the case of COC/BE). One approach to tackle this problem, then, is to use several biomarkers of one parent-compound to more reliably back-calculate the consumption (Heuett et al., 2015a; Baker et al., 2014). This method is, however, only feasible when multiple metabolites are available and stable in the sewer.

Spiking levels in the studies spanned from zero, i.e., relying on the concentrations already present in the wastewater, up to 100 $\mu g L^{-1}$. It is necessary to distinguish between pathway investigation studies, where high spikes seem appropriate, and studies to monitor biomarker stability at environmentally relevant concentrations. Testing guidelines (e.g., Organization for Economic Cooperation and Development (OECD)) recommend high concentrations mostly to reduce analytical constraints. However, at high concentrations, microorganisms may need time to adapt before starting to transform illicit drugs (Mardal and Meyer, 2014). Thai et al. (2014) found no impact of high spiking levels on the transformation of COC, BE, MDMA and 6-MAM in their in-sewer stability study. Whether high levels of other illicit drugs may even inhibit biotransformation was not investigated and may require further comparative studies.

Another, so far overlooked, aspect is the effect of spiking analytes dissolved in organic solvents. The typical solvent methanol is a potential substrate for microorganisms and may inhibit or enhance the co-metabolic transformation of organic micropollutants (Plósz et al., 2010). A previous study found that

the transformation of COC, BE and EME seems to be unaffected by the readily biodegradable substrate content present in pre-clarified sewage (Plósz et al., 2013).

Physical processes

The overall findings for sorption to SPM in the bulk liquid (i.e., excluding biofilm on sewer walls) were similar in most studies. A detailed description of the methods applied to conduct these control experiments can be found in the supporting information. Sorption has been shown to play a limited role for COC, BE, AMP, METH, MDMA and COD, whereas some sorption was observed for EDDP, fentanyl and MTD (Baker and Kasprzyk-Hordern, 2011a, 2011b; Bisceglia and Lippa, 2014; Langford et al., 2011; Senta et al., 2014). Even more so, THC-COOH tends to sorb to SPM with increased sorption at low pH values (Khan and Nicell, 2012; Senta et al., 2014, 2013). No studies have specifically investigated the sorption of illicit drugs to the sewer biofilm. Substances that already tend to sorb to SPM in wastewater, e.g., THC-COOH, MTD and potentially LSD, may also sorb to sewer wall biofilms. Generally, since sorption is biomass-specific, extrapolating results from studies with SPM in wastewater to real systems with biofilm may not be adequate.

4 Recommendations for future in-sewer experiments

While it would be ideal to carry out in-sewer transformation studies in real sewers, there are several factors making full-scale experiments often (too) laborious to obtain accurate results, e.g., limited access to confined space, numerous confluents that would require monitoring and methods to experimentally account for unknown in- or exfiltration to close mass balances etc.,. Furthermore, due to varying environmental conditions that cannot be controlled during the experiments, several studies would be necessary. Based on the gaps identified in this review, we, therefore, propose the subsequent recommendations focusing on systematic laboratory and pilot-scale experiments to cover the wide range of realistic environmental conditions. This will facilitate interpretation and comparison of individual experiments, and allow to estimate the actual transformation potential in full-scale sewers.

Biofilm

Generally, if in-sewer experiments are performed in the laboratory, all sewer compartments should be considered, including biofilms growing on the sewer walls. Since recent in-sewer studies showed that biofilm could enhance transformation, future studies should consider realistic types and amounts of sewer biofilm. The result is an increase of the total biomass in the system that can influence transformation rates.

Reproducibility and monitoring

A major challenge of using real, fresh wastewater is the reproducibility of results. Its composition is likely to differ from experiment to experiment; therefore, a meticulous approach to the assessment of its composition and monitoring of the environmental conditions is necessary. We propose the following minimum set of parameters to be included: total suspended solids (TSS), volatile suspended solids (VSS) and the main process variables, dissolved oxygen, temperature, pH, conductivity, dissolved inorganic sulfide/methane and soluble and total chemical oxygen demand.

Spiking concentration levels

The present illicit drug biomarker concentrations in grab samples or 24h composite samples may be too low to conduct meaningful experiments. Degradation pathways and relevant processes must be considered carefully when deciding to spike substances, including using a mix of analytes in one experiment or multiple experiments with individual analytes. Further, the aim of the study (pathway investigation or stability monitoring) influences the decision on concentration levels, and willingness for sample preparation (effort for SPE). For kinetic stability studies, spiking may be necessary to guarantee levels of substances to be sufficiently above the Limit of Quantification (LOQ). In some cases, the use of deuterated or ^{13}C-labelled compounds might be needed, since illicit drugs may already be present in the wastewater matrix. For pathway investigation studies, the spike of usually only one compound at high concentrations may be necessary. Ideally, the spike should be done without organic solvent.

Controls

To facilitate interpretation and meaningful comparison of experiments carried out at different points in time, in different laboratories and under different conditions, appropriate controls must be included. This

can be done by analyzing substances with transformation behavior known from numerous studies, e.g., caffeine, nicotine (positive control) or carbamazepine (negative control). This may be helpful to compare different experiments and to confirm typical behavior in the setup.

Abiotic controls without active biomass and enzymes present are necessary to account for losses due to volatilization and abiotic transformations, such as chemical hydrolysis. We recommend to filter (<1 µm) and potentially autoclave the abiotic control reactor content (Gulde et al., 2014; Helbling et al., 2010).

For certain substances, sorption controls are necessary to interpret the transformation data correctly and distinguish between losses due to biotransformation or sorption.

Experimental design and reporting of results

In most stability studies, the stability is represented as percentage decrease or increase from the initial concentration, determined from a sample at time t_0=0 and t=t_{end}. In studies of micropollutant removal in wastewater treatment (pharmaceuticals, personal care products etc.), it is common to describe, if applicable, the exponential decrease in concentration over time with first-order kinetics (Schwarzenbach et al., 2003a). The resulting rate constants (k_{bio}) are usually normalized to TSS or VSS, which facilitates comparison among different studies (i.e., different amount or activity of present biomass). We suggest performing in-sewer experiments over meaningful timeframes, which appears to be a relevant maximum hydraulic residence time in most sewer systems. To calculate rate constants, it is recommended to regularly collect samples, e.g., at the following points in time, related to the initial spike t($c_{background}$)= -5 min, t(c_0)= 2 – 5 min after spike, 30 min, 1 h, 2 h, 4 h, 8 h, 16 h, 24 h. The results should then be presented in conjunction with a suitable transformation model. For most substances, this may be (pseudo) first-order kinetics (Bisceglia and Lippa, 2014; Plósz et al., 2013).

Sampling

In kinetic studies, terminating the transformation processes at exact time points is crucial. After collecting the sample, the activity of the microorganisms can be stopped by adding a microorganism deactivating substance (see Controls); however, this will not stop abiotic processes. Guidelines suggest flash freezing (using solid CO_2 (dry ice)/acetone or liquid nitrogen bath), followed by lyophilization and

extraction, with alternative centrifugation (and filtration) and separate extraction of the liquid and solid phase (OECD, 2008). Sample preservation and storage recommendations are provided in Section 5.

5 Recommendations for preserving illicit drugs in wastewater samples

In previous sections, stability of each specific compound has been discussed based on the available literature. However, most laboratories store and prepare samples for multi-class analysis. Therefore, in this section, recommendations on preserving illicit drugs are provided for multi-class methods. Key points associated with in-sample stability are: termination of transformation processes, filtration, preservation and long-term storage.

In general, samples should be filtered prior to storage. Filtration is mainly carried out to stop potential biotransformation and prevent clogging when performing SPE. A variety of membrane and glass fiber filters, with different pore sizes as low as 0.1µm, are available and have been used (Baker and Kasprzyk-Hordern, 2011a; Östman et al., 2014). Smaller pore sizes are able to filter out suspended bacteria, but are more readily blocked, leading to an increase in preparation time (Chen et al., 2013). It is important to evaluate for possible losses of the analytes during the filtration process. The addition of mass-labelled internal standards prior to filtration and storage is recommended, in order to compensate for losses and transformation (Bijlsma et al., 2014; Castiglioni et al., 2013). Due to time constraints, wastewater samples are often frozen without previous filtration or the addition of labelled internal standards. In those cases, it is important to immediately add labelled internal standards after the sample has thawed. This can be justified by conducting freeze and thaw stability studies for the targeted analytes and accounting for the potential losses.

The most important aspects relating to in-sample stability is storage. Addition of preservatives, pH adjustment and storage at different temperatures has been studied in order to determine their impact. However, in a multi-class method, a compromise must be made for the optimal storage conditions. For example, lowering the pH increases the stability of several illicit drugs, except for THC-COOH (Baker and Kasprzyk-Hordern, 2011c; Chen et al., 2013; González-Mariño et al., 2010; Senta et al., 2014). If it is not possible to analyze the samples immediately, they can be stored upon arrival at -20°C without any

adjustments. Alternatively, samples could be extracted on SPE cartridges and stored at -20°C. Using these approaches, analytes are stable for up to 3 to 6 weeks, respectively (Baker and Kasprzyk-Hordern, 2011a; Chiaia et al., 2008; González-Mariño et al., 2010).

The stability of illicit drugs continues to be an issue during the subsequent sample preparation. Although this relates more to the analytical methodology and, therefore, is out of the scope of this review, it is worth mentioning that issues, such as the use of silanized glassware and evaporation temperature for reconstitution of sample extracts, should be addressed (Baker and Kasprzyk-Hordern, 2011a).

For in-sample stability (in a closed container), the following variable parameters are recommended to be measured at least at the beginning and end of the experiment: pH, temperature, conductivity, TSS, VSS and soluble and total chemical oxygen demand. This will allow a more effective and reliable comparison of results among different experiments and conditions.

6 Conclusions

In wastewater-based epidemiology, the back-calculation currently used to estimate drug consumption at the population level does not account for potential in-sewer transformation of the targeted drug residues. This increases the uncertainty of estimates to an unknown degree.

Since most experiments were conducted with different grab wastewater samples of unknown composition, the occurring transformation processes in the studies might have varied. In addition, each study reported a different level of detail about these wastewater components and the experimental conditions, complicating the interpretation and generalization of compound behavior. Several illicit drugs, such as MDMA, KET and MDPV, seem to have a *high* stability in different wastewater studies at neutral pH and temperatures up to 20°C. Also BE and AMP are most likely substances with high stability in those wastewater conditions; however, they may be formed as transformation products of other substances. Accordingly, the low stability of COC and 6-MAM seems to be well investigated. More research is needed for the drugs with variable behavior or few performed studies, such as THC-COOH, fentanyl, mephedrone and cathinones.

- The few studies to-date show that in-sewer transformation is compound-specific, influenced by the prevailing environmental conditions in sewers (temperature, sewer type). There is a lack of studies systematically investigating the influence of the different environmental conditions (pH, suspended particulate matter, biofilm) on the transformation of illicit drugs.

- In order to compare different studies and environmental conditions, a reproducible experimental approach with quality controls for in-sewer transformation studies is needed. Therefore, this review recommends a best-practice approach for future in-sewer stability studies.

- Further, we summarize the best strategies to assure good in-sample stability of illicit drugs in the field of wastewater-based epidemiology. In multi-compound studies, most illicit drugs had a high stability at neutral pH and -20°C for at least three weeks. Alternatively, acidification of the sample preserved most drugs, except for THC and metabolites.

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