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Highlights

- In-sewer loss of drug biomarkers vary under different sewer conditions
- Biofilm plays an important role in the transformation of biomarkers
- Conjugated compounds can be de-conjugated in-sewer
- In-sewer loss can increase uncertainty for consumption estimation
- Understanding the sewer systems is important for comparing WBE data between catchments

ABSTRACT:

Wastewater-based epidemiology (WBE) is considered as a useful tool to monitor chemical consumption in the population. However, the lack of information on potential transformation of biomarkers in the sewer system can compromise the accuracy of the consumption estimation. The present study contributes to addressing this issue by investigating the in-sewer stability of biomarkers of a number of commonly used drugs using laboratory sewer reactors that can mimic different sewer conditions. A stable and an unstable chemical (carbamazepine and caffeine) were also used as benchmarking chemicals to reflect the chemical degradation potential of different sewer conditions. The results suggested that ketamine and norketamine were unstable in gravity and rising main sewer, ketamine was unstable in bulk liquid while norketamine was stable with less than 5% transformation in the control reactor. Similarly, mephedrone and methylone were unstable in sewer conditions with considerable deviation. Significant loss of buprenorphine, methadone, oxycodone and codeine was observed in rising main sewer. Morphine and codeine were found to be deconjugated from glucuronides quickly in the presence of biofilms. This study indicates that it is important to evaluate the stability of biomarkers in the sewer system before using them in WBE for estimating consumption/exposure to reduce uncertainties.

Keywords: Benchmarking chemicals; Biofilm; In-sewer degradation; New Psychoactive Substances; Wastewater-based epidemiology;

1. INTRODUCTION

According to the recent report of the United Nations Office on Drugs and Crime and other authorities on drug control, in addition to the abuse of traditional illicit drugs such as heroin, cocaine, and amphetamines, people also illegally consumed large amount of new psychoactive substances (NPS) and prescription drugs (Heikman et al., 2016; UNODC, 2015). Ketamine and phencyclidine-type substances, synthetic cathinones and synthetic cannabinoids are the predominant groups of NPS identified in the global market (UNODC, 2013). Designing adequate policy responses to drug problems would require better data on the prevalence of different types of illicit drug use (Degenhardt et al., 2011). However, obtaining the temporal and spatial consumption patterns of many illicit drugs remains challenging to authorities.

Wastewater-based epidemiology (WBE) is a potent complementary approach to estimate chemical consumption in the population (van Nuijs et al., 2015). A number of studies have utilized WBE to monitor the level of illicit drug consumption in the population (EMCDDA, 2016b; Lai et al., 2016; Li et al., 2014). WBE demonstrated some advantages such as the capability to provide quick and objective estimation of illicit drug consumption in different temporal and geographical scales (Castiglioni et al., 2015; Postigo et al., 2011; Thomas et al., 2012; Tscharke et al., 2015). Recently, research on WBE has been focused on the evaluation of uncertainties of the approach to improve the accuracy of the consumption estimates (Castiglioni et al., 2013; EMCDDA, 2016a). Besides optimization of sampling protocols, refining chemical-specific excretion factors and improvement of the catchment population estimation (Gracia-Lor et al., 2016; Thai et al., 2016a), information about the fate of biomarkers during in-sewer transport is essential for providing more accurate consumption estimates of drugs in WBE applications (McCall et al., 2016b; Ramin et al., 2016; Thai et al., 2014a) because the in-sewer loss of biomarkers could lead to considerable underestimation of drug consumption (Castiglioni et al., 2013; Senta et al., 2014).

Initially, studies on the stability of drug biomarkers in sewers only included wastewater with or without suspended solids (Senta et al., 2014; van Nuijs et al., 2012). After sewer biofilms were demonstrated to have the important role in transforming biomarkers of major illicit drugs such as cocaine and 6-acetylmorphine (heroin metabolite) (Thai et al., 2014a), the review on in-sample and in-sewer stability of biomarkers of drugs of abuse by McCall et al. (2016a) has recommended further stability studies to include the biofilms in the experimental designs. Since then, there have been two studies that investigated the in-sewer transformation of biomarkers by biofilms (McCall et al., 2016b) and suspended solids (Ramin et al. 2016), which again indicated the importance of understanding the in-sewer interactions of biomarkers under different sewer conditions.

Some chemicals can undergo intensive metabolism including glucuronidation in the human body before being excreted to the sewer system. The stability of glucuronide-conjugated compounds in sewer can also contribute to the uncertainty in WBE back-estimations because the deconjugation to their free forms (Hedgespeth et al., 2012; Langford and Thomas, 2009; Lishman et al., 2006). To what extent the human conjugates are converted to free biomarkers in the sewers is not well studied. Only two studies by Senta et al. (2014) and Ramin et al. (2016) have investigated the transformation of morphine glucuronide in wastewater and thus it is important to continue and expand the research in this aspect of WBE.

In this study, we aim to evaluate the in-sewer transformation of biomarkers of a suite of pharmaceuticals, new psychoactive substances and prescription opioids that are prone to abuse as

well as two chemicals that we propose could be used as benchmarking chemicals in stability studies. Two glucuronide conjugated metabolites were also included.

2. MATERIALS AND METHODS

The experimental approach employed in this study has been used previously for several conventional illicit drugs (Thai et al., 2014a). The illicit drugs investigated in this study include ketamine and its metabolite norketamine, methylone, mephedrone; the prescription opioids include methadone, codeine, oxycodone, buprenorphine, and two glucuronide conjugates, morphine-glucuronide and codeine-glucuronide. Two substances, carbamazepine and caffeine, were selected as benchmarking chemicals to reflect the activity of the sewer reactors regarding transformation of chemicals and to facilitate comparisons with other studies (McCall et al., 2016a; McLachlan et al., 2017).

2.1 Chemicals and Reagents

Deuterated labelled standards of ketamine, norketamine, methadone, codeine, buprenorphine, morphine-glucuronide and codeine-glucuronide were used in this experiment to enable the monitoring of the possible formation of degradation products as deuterated chemicals prevent the interference of the native drugs in the wastewater. Methylone, mephedrone and oxycodone were spiked as native compounds because their metabolites were not monitored. The properties of selected biomarkers are presented in Table S1. All the deuterated and native standards were purchased from Cerilliant (Texas, US). Spiking solutions of deuterated labelled and native standards were prepared in methanol and spiked to fresh wastewater as shown in Table S2. LCMS grade methanol was purchased from Merck, Germany. Deionized water was produced by a MilliQ system (Millipore, 0.22 μm filter, 18.2 mΩ●cm^{-1}).

2.2 Laboratory-scale sewer reactors

The experiment was carried out with laboratory-scale sewer reactors, which have previously demonstrated the capability of mimicking typical sewer conditions (Jiang et al., 2011; Thai et al., 2014a,b). Three reactors were employed, namely a rising main (RM), a gravity (GS) and a control (CR) sewer reactor. The reactors were made of Perspex^{TM} with a volume of 750 mL (diameter of 80 mm and a height of 149 mm) (Jiang et al., 2009). Plastic carriers (Anox Kaldnes, Norway) of 1 cm diameter were clustered on four stainless-steel rods inside the reactor to provide additional

surfaces for biofilm growth and provide similar area/volume ratio as actual sewers in RM and GS. The total surface area on the reactor walls and carriers supporting biofilm growth is estimated to be 544 cm^2 for the RM reactor. The GS reactor had the same dimensions but was only partially filled with wastewater, allowing a gas phase at the top of the reactor. The total surface area for biofilm growth is estimated to be 322 cm^2 for the GS reactor. The gas phase had free air exchange to the atmosphere in the GS reactor. A mixture of aerobic and anaerobic biofilm had been previously developed in the GS reactor. The control reactor (CR) is a clean reactor identical to the GS and RM with no biofilm present on the reactor. Thus, CR reactor is essentially a container of wastewater similar to that used in other stability studies (Senta et al., 2014; van Nuijs et al., 2012) and is able to determine if a chemical is stable in-sample during and after collection.

2.3 Batch tests for the transformation of biomarkers

Three batch tests were conducted with the different reactors described above. Information about chemicals investigated in each batch test is presented in Table S2. Separate batches were used to avoid the interference of potential transformation between parent drug and its metabolite (e.g. ketamine and norketamine) to the stability evaluation.

Three replicates were performed for each batch test. Fresh wastewater was collected prior to each batch test and stored at 4 ℃. Before each test, wastewater was warmed to 20 ℃ and spiked with biomarkers at relevant concentrations as shown in Table S2. Continuous mixing was maintained in each reactor with magnetic stirrers at 250 rpm (Heidolph MR3000) for the duration of the tests. Wastewater samples were taken at time 0, 0.25, 0.5, 1, 2, 3, 6, 9 and 12 hours after the experiment started. The experiment was terminated after 12 hours considering the majority of real sewer systems have retention times less than 12 hours. For each time point 1mL of wastewater was filtered into a vials using 0.45 mm syringe filter (Phenomenex, Australia) with 8 µl of 2 M HCl to adjust each of the samples to pH 2. The acidified samples were then frozen at -20 ℃ until analysis.

2.4 Chemical analysis

The chemical analysis in this study was based on a previously developed analytical method (Lai et al., 2011; van Dyken et al., 2016). Additional compounds with optimised mass spectrometry parameters were also included (Table S3). Briefly, analysis was performed using liquid chromatography (Shimadzu Prominence) coupled with tandem mass spectrometer (AB-SCIEX 5500® QTrap) with electrospray ionisation source in positive mode. Chemical separation was performed on a Luna C18 analytical column (Phenomenex, 150x2.1 mm, 3 µm) with the mobile

phase of (A) 1% acetonitrile and 99% Milli-Q water and (B) 95% acetonitrile and 5% Milli-Q water; both with 0.1% formic acid, at the gradient: 8% B, 0-1 min; 35% B at 3.5 min; 100% B at 11 min for 4 min; 8% B at 15.1 min for 5 min. However, for morphine-3-*β*-D-glucuronide-D3 and codeine-6-*β*-D-glucuronide-D3, a Kinetex Biphenyl column (Phenomenex, 50x2.1 mm, 2.6 µm) was used for their retention and separation with the mobile phase of (A) 1% methanol and 99% Milli-Q water and (B) 95% methanol and 5% Milli-Q water; both with 0.1% acetic acid, at the gradient: 5% B, 0-1 min; 100% B at 7.5 min for 3 min; 5% B at 9.6 min for 3.4 min. The flow rate was set at 0.3 mL/min and the injection volume was 8 µL. The MS was operated in multiple reaction monitoring (MRM) mode for data acquisition. The MS parameters for each MRM transition of the target chemical were optimised (Table S3). Chemical concentrations in the samples were analysed and quantified together with a six point calibration standard. Three deuterated compounds including norfloxacin-D_5, acetyl sulfamethoxazole-D_4 and caffeine-$^3C_{13}$ were spiked (10 ng each) to the samples to check the instrumental stability over the analysis. The intraday variation (CV% of chromatographic peak area; n=81) was 7.58% for norfloxacin-D5, 7.11% for acetyl sulfamethoxazole-D4 and 6.94% for caffeine-D3. The interday variation (CV% of chromatographic peak area; across 3 days) was 10.6% for norfloxacin-D5, 8.73% for acetyl sulfamethoxazole-D4 and 8.97% for caffeine-D3. The instrumental variation was minimal for adequate sample analyses (as shown in Table S4). Similar methodology has been applied in our previous study (Thai et al. 2014a).

For dissolved sulphide, samples were analysed within 24 h of sampling using an ion chromatograph with a UV and conductivity detector (Dionex ICS-2000). For methane analysis, BD vacuum tubes were allowed to reach gas/liquid equilibrium overnight. Methane in the gas phase was measured by gas chromatography (Shimadzu GC-9A) equipped with a flame ionization detector. Concentrations of methane in wastewater were calculated using mass balance and Henry's law.

2.5 Benchmarking chemicals

In order to make inter-study and/or cross-catchment comparisons of biomarker degradation, it is favourable to have stable and unstable benchmarking chemicals that can reflect chemical degradation potential in different sewer conditions and catchment characteristics. Carbamazepine is reported stable in wastewater, surface water, and even different treatment process (Clara et al., 2004; Weigel et al., 2002; Zhang et al., 2008; Zuccato et al., 2005) and thus was selected as a stable benchmarking chemical. Caffeine is reportedly unstable (Buerge et al., 2003; Thomas and Foster, 2005; O'Brien et al., 2017) in wastewater and was selected as an unstable benchmarking chemical.

2.6 Data processing

Average concentration of chemicals at time 0, 0.25 and 0.5 hour is treated as initial concentration (100%) since we observed some fluctuation of concentrations in the first half an hour possibly due to mixing and sorption equilibrium. All the concentrations during the 12 hour test were normalised to a percentage relative to the initial concentrations. The production of transformation products was normalized to the molar percentage of the parent chemicals. Zero-order and first-order kinetic models were tested, and the model with higher correlation value was selected for the chemical under the tested sewer conditions. Half-life was calculated in pseudo first-order model (Prism 7, GraphPad software, Inc.).

3. RESULTS AND DISCUSSION

3.1 Bioactivity in the sewer reactor

Before the batch test, biofilms were cultivated in the RM and GS reactors with real wastewater pumping scheme of every 6 hours. Seven days before the experiment, the methane and sulfide production in each reactor was stable. In these batch tests, the methane and sulfide profile is comparable with a previous study by Thai et al (2014b). The RM reactor had much higher methane and sulfide production than the GS reactor during the 12 hours experiment while the CR reactor showed no significant biological activity as it did not contain sewer biofilms. Dissolved oxygen in the GS was below 0.33 mg/L despite continuous stirring and contact between the liquid phase and sewer atmosphere, which indicates rapid consumption of oxygen by aerobic activity in the reactor. It is also expected that anaerobic microbes could live in the deep biofilm where oxygen cannot reach. Activities of sulphate-reducing bacteria and methanogenic archaea in the RM reactor were measured at 5.59 ± 0.75 mg·S L^{-1} h^{-1} and 12.07 ± 0.39 mg·COD L^{-1} h^{-1} respectively which is similar to previously reported values for both real and laboratory-scale sewers (Guisasola et al., 2008; Jiang et al., 2011; Thai et al., 2014a,b).

3.2 Benchmarking chemicals under different sewer conditions

In this study, carbamazepine was observed to be stable throughout 12 hours in all the sewer reactors as expected while caffeine was observed to have undergone higher degradation in RM and GS than in CR (Fig. 1). Faster degradation of caffeine was observed in RM with higher A/V ratio than GS. In GS, about 50% of caffeine was left after 12 hours while in RM less than 5% of the initial caffeine remained after 12 hours. This result confirmed that the sewer reactors with biofilms can greatly enhance the degradation of selected chemicals in wastewater as reported

previously (Thai et al., 2014b) but has no effect on persistent chemicals (Thai et al., 2014a).

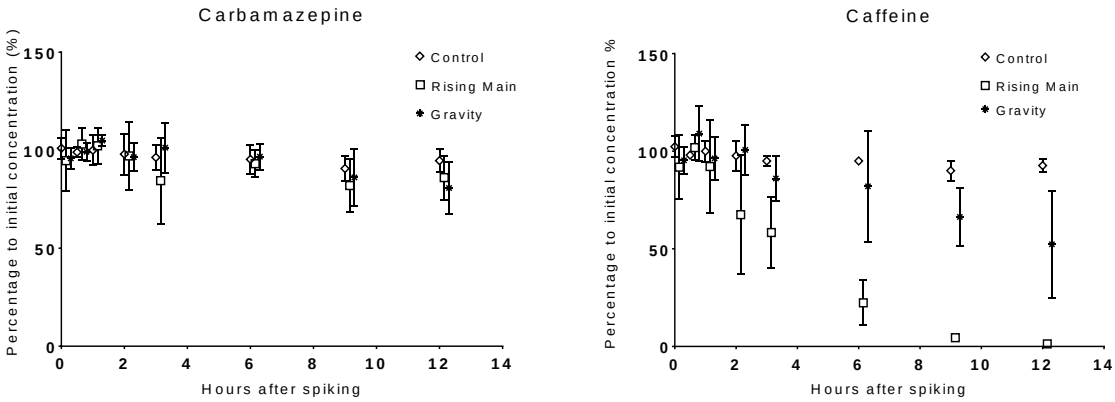


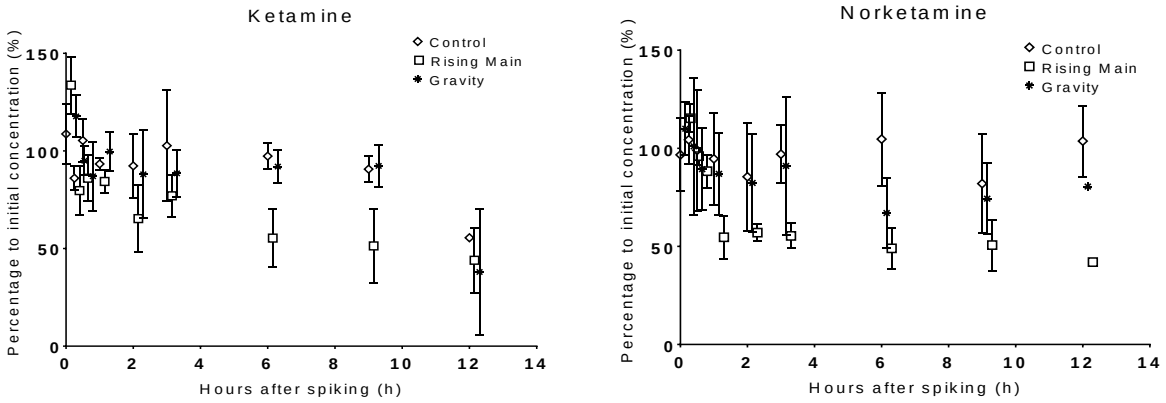
Fig. 1 Stability of two benchmarking chemicals (stable and unstable) in the sewer reactors (error bars are the standard deviation of triplicates)

3.3 Transformation of biomarkers of drugs of abuse

3.3.1 Ketamine, norketamine

Ketamine (in form of ketamine-D4) was relatively unstable in different sewer conditions with 45±1%, 62±32% and 56±17% transformation in CR, GS and RM over 12 hours respectively (Fig. 2). The transformation of ketamine in RM is significant higher than CR but no significant than GS (p=0.0391 and 0.0938, two tail t test). Computer modelling of ketamine degradation also suggests that ketamine could have some biodegradation (Reid et al., 2014). However, Castiglioni et al (2015) and Baker and Kasprzyk-Horden (2011) reported that ketamine was stable up to 72 hours in wastewater without biofilm. It indicated that both the biofilm and other sewer conditions could contribute to the transformation of ketamine. Norketamine-D4 was monitored in the same sample set and it was not detected in any samples. This indicates that ketamine is unlikely to be demethylated to norketamine in the sewer.

Norketamine (in form of norketamine-D4) was stable in CR with less than 5% loss after 12 hours, similar to other studies conducted by Castiglioni et al. (2015) and McCall et al. (2016b) under similar conditions (Table 1). Twelve hours after spiking, about 20% norketamine was lost in GS reactor and more than 50% was lost in RM reactor. The transformation of norketamine in RM is significant higher than CR and GS (p=0.0195 and 0.0195, two tail t test). This could possibly be because the GS has lower A/V ratio and also less biofilm mass. Meanwhile, in the study of McCall et al. (2016b), norketamine was observed to be stable with less than 10% loss with suspended gravity biofilms. It suggests that under GS conditions, biomarker degradation could vary. It is also interesting to notice that the loss of norketamine in RM mostly happened in the first hour.



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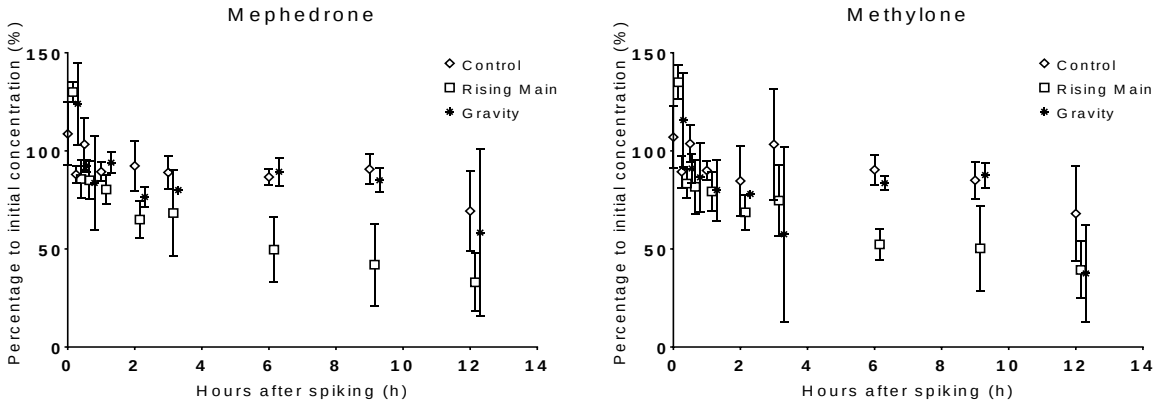

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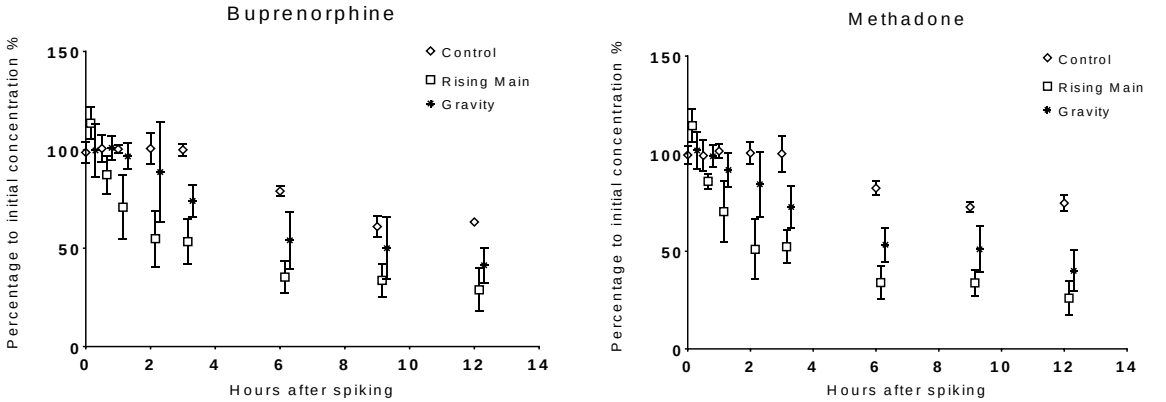

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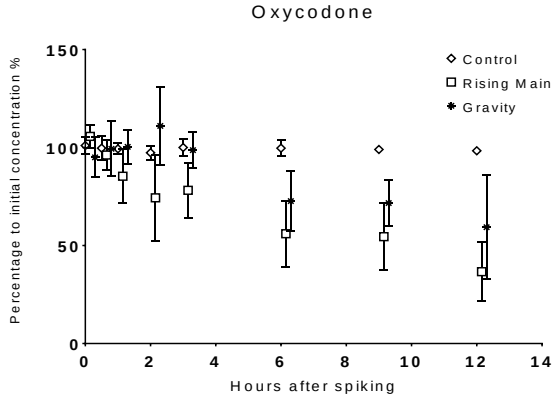

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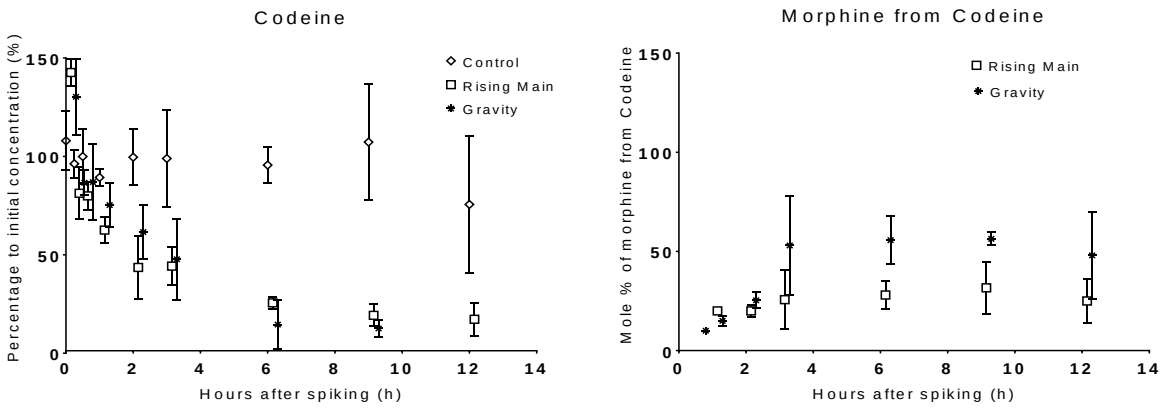


Fig. 2 The transformation/formation of selected biomarkers in the sewer reactors (error bars are the standard deviation of triplicates)

3.3.2 Methylone and Mephedrone

The stability profiles of mephedrone and methylone are very similar (Fig. 2) probably because they are from the cathinone group and have similar molecular structures (Table S1). Both mephedrone and methylone had considerable in-sewer degradation in the RM and GS reactors (Table 1 & Fig 2). Mephedrone was observed 30±20%, up to 40% and 67±15% in CR, GS and RM respectively by 12 hours (Table 1). The transformation of mephedrone in RM is significant higher than CR and GS (p=0.0391 and 0.0195, two tail t test). Mephedrone was reported to be stable for 48 hours in urine samples at room temperature (Johnson and Botch-Jones, 2013). Up to 80% loss mephedrone was observed after 24 hours in the presence of resuspended gravity biofilms from McCall et al. (2016b). More than 70% loss of mephedrone within 24 hours was observed under aerobic condition and about 30% loss under anaerobic condition investigated by Ramin et al. (2016). In CR, 30±20% loss was observed for mephedrone, while Ostman et al. (2014) reported less than 5% transformation of mephedrone during 24 hours under room temperature without biofilms while Bade et al. (2017) reported approximately 50% loss in filtered wastewater under natural pH and 20 °C in 24 hours . This demonstrated that different wastewater composition could lead to different transformation rate of mephedrone.

Only one study investigated the stability of methylone in filtered wastewater in natural pH 20 °C, by 24 hours, approximately 20% of methylone was lost. The results of this study indicate that methylone is unstable with up to 30%, 60% and 60% loss in CR, GS and RM respectively. The transformation of methylone in RM is significant higher than CR but no significant than GS (p=0.0391 and 0.4258, two tail t test). The instability of methylone has been demonstrated previously in urine samples (Concheiro et al., 2013). Therefore, care should be taken when interpret the data of methylone from WBE.

3.3.3 Buprenorphine, methadone, oxycodone and codeine

Noticeable loss of buprenorphine, methadone, oxycodone and codeine was observed in RM and GS reactors (Table 1, Fig. 2). The highest loss of methadone was observed in RM followed by GS and CR. By 12 hours, 25±4%, 40±10% and 76±9% methadone was lost in RM, GS and CR respectively. Formation of 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) was not observed following the significant degradation of methadone in RM and GS, suggesting that unlike in-human metabolism, there are other transformation pathways for methadone in the sewer. Similarly, Ramin et al (2016) also observed independent transformation pathways for methadone do not include EDDP as a metabolite. However, Ramin et al observed significantly faster transformation of methadone under aerobic (gravity) conditions than anaerobic conditions (rising main). This discrepancy may be attributed to the different biomass/wastewater ratio, the anaerobic biomass is higher than aerobic biomass in the present study. Unlike van Nuijs et al (2012) and Castiglioni et al (2006), methadone in CR had about 20% loss in this study while the previous studies reported no loss or even some formation of methadone under similar conditions. The continuous stirring in the present study could have introduced air/oxygen to the wastewater and potentially enhanced the transformation of chemicals compared with previous studies.

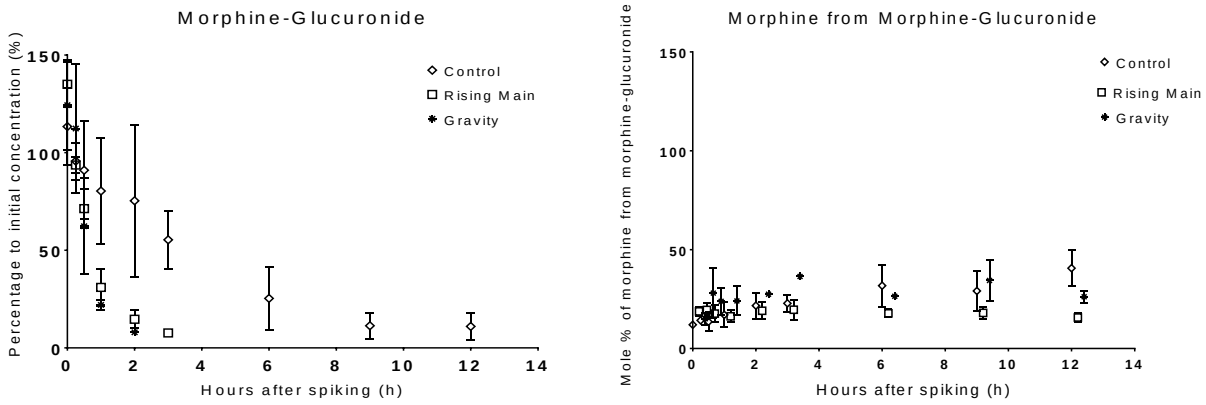
Twelve hours after spiking buprenorphine was observed 59±9% and 37±2% loss in GS and CR. While 71±11% was observed in RM. There is only one study investigated the stability of buprenorphine (Ostman et al., 2014), and less than 5% was observed under conditions similar to CR in the present study. This may be caused by the different wastewater composition and the microbes in the suspend solids. Oxycodone had the highest degradation in RM with 63±15% loss followed by 41±26% in GS during 12 hours, CR had minor loss of 2±1.5%. High stability of oxycodone in bulk liquid phase was also observed by two other studies under similar conditions as CR (Baker and Kasprzyk-Hordern, 2011; Ostman et al., 2014).

Codeine had significant degradation in both RM and GS but was relatively stable in CR (up to 25% transformation)(Fig. 2). It is noticeable that, in GS up to 50% of codeine was transformed to morphine within 12 hours while the overall loss was higher than 95%. While in RM, 30% of codeine transformed to morphine with more than 80% loss indicating multiple transformation pathways in GS and RM. This may be attributed to the transformation pathways of codeine differing in GS and RM due to their different microbial communities. It is also possible that morphine is not as stable in RM as in GS. Codeine was observed with high stability in bulk liquid or resuspended gravity biofilms (Baker and Kasprzyk-Hordern, 2011; Chen et al., 2013; McCall et al., 2016b), the discrepancy could be attributed to the microbe composition difference in

suspended solids and biofilms.

3.3.4 Morphine-glucuronide and codeine-glucuronide

Both morphine-glucuronide and codeine-glucuronide were not stable under GS and RM conditions. (Fig. 3). More than 80% of both compounds were degraded after 2 hours in RM and GS, and almost 100% were lost after 6 hours. The degradation rate was slower in the CR but by 12 hours, more than 80% morphine-glucuronide and about 20% codeine-glucuronide have degraded in CR, respectively. In GS and CR reactors, approximately 25% and 40% of morphine-glucuronide was transformed to morphine, while in the RM, the morphine from morphine-glucuronide is about 15% after 12 hours, this indicates that there could be other morphine-glucuronide transformation products in RM. Limited net formation of morphine from morphine-glucuronide under both aerobic and anaerobic conditions was also observed by (Ramin et al., 2016) and the authors suspected that there are more transformation pathways for morphine-glucuronide. The different degradation rate of morphine (as transformation product of morphine-glucuronide) in the three reactors could also contribute to the observation. Morphine-glucuronide and codeine-glucuronide was reported as stable (less than 10% loss) in human urine samples at 24 ℃ within 20 hours (Murphy and Huestis, 2005). This result indicates that abiotic chemical degradation of these two glucuronides is limited. Also microbes in the sewer play an important role in the transformation. Similarly, morphine-glucuronide was also observed with low stability with more than 95% loss within 24 hours under conditions similar as CR (Baker and Kasprzyk-Hordern, 2011; Ramin et al., 2016; Senta et al., 2014).



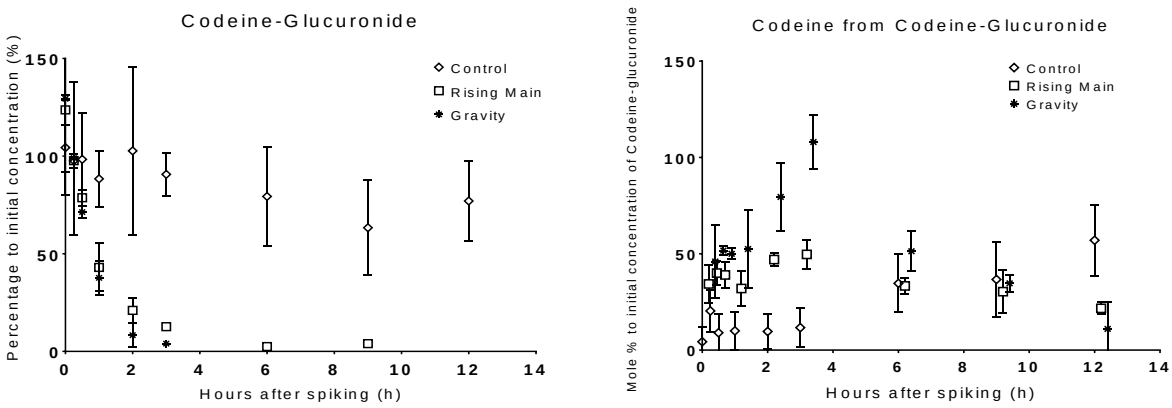


Fig. 3 In-sewer transformation of human glucuronides and the formation of free compounds (error bars are the standard deviation of triplicates)

3.4 Transformation kinetics of biomarkers

Linear regression (zero-order) and pseudo first-order regression was applied for the data acquired from the batch tests. As shown in Table 2, most of the R^2 for both kinetic models is less than 0.95, we selected the model with better R^2. It indicated that there are certain deviations of the observed degradation to the theoretical kinetic model. It may cause by the complexity of the bioactivity in the reactors. In CR reactor, most of the biomarkers investigated fits better with zero-order kinetics. In the RM, all the biomarkers fit better with first-order except oxycodone and caffeine. Morphine release from morphine-3-β-D-glucuronide and codeine release from codeine-6-β-D-glucuronide had poor R^2 and hence neither model was selected for these two transformation products. In GS, only codeine-6-β-D-glucuronide and codeine were suitable for first-order reaction with slightly better R^2 values (0.85 vs 0.83 and 0.86 vs 0.80), all the other markers fit better in zero-order model except mephedrone, ketamine, codeine from codeine-6-β-D-glucuronide with poor R^2.

3.5 Implication of these results to other WBE studies

Our study suggests that a stable and an unstable benchmarking chemical (carbamazepine and caffeine) could be used in biomarker stability studies. The benchmarking chemicals can reflect the chemical transformation potential under different sewer conditions and potentially can be used as a tool to normalise results from different studies.

The half-life of morphine-glucuronide and codeine-glucuronide in the GS and RM are quite short, with less than one hour in RM, indicating the release of morphine and codeine from their glucuronides could be considerably quick in the sewer. This shows that the glucuronide conjugation is unlikely to have any impact on the back-calculation if the excretion factor used has considered the free form and conjugates.

In the RM, the half-life of first-order chemicals (mephedrone, methylone, ketamine, norketamine, codeine, buprenorphine and methadone) were all less than 1.5 hours (as shown in Table 2). Considering the average retention time of sewage in the rising main pipes, caution should be taken to interpret data from catchments with considerable proportion of rising mains and with long hydraulic retention time. Alternatively, investigation could be done to identify more stable biomarkers for back-calculation.

The present study provided objective evidence on the transformation of 11 biomarkers under different sewer conditions. It is evident that both gravity and rising main biofilms enhanced the transformation of unstable biomarkers. To evaluate substance consumption status in population through WBE and further investigate the temporal and geographical behaviour, caution should be taken to systematically evaluate the associated uncertainties and the possible bias. Detailed catchment investigation (population characteristics, sewer infrastructures and wastewater profile) should be carried out for better interpretation of the chemical consumption behaviour. The different transformation rate of some biomarkers in different studies highlighted the need to develop a systematic tool to evaluate the in sewer loss of biomarkers taking the chemical property, catchment infrastructure and sewer conditions into account to reflect the possible sorption and transformation of biomarkers.

3.6 Limitations

Although the laboratory scale sewer reactor can mimic the real sewer conditions with controllable parameters, real world sewer infrastructure is far more complex. The A/V ratio of RM and GS used in this study is estimated to be 72.5 and 50 m^2/m^3 that is the typical ratio of small diameter pipes, large diameter pipelines especially large diameter gravity sewers are not reflected in the study. In addition, we cannot quantitatively measure the total biofilm mass in GS and RM reactors, we cannot provide transformation rate relative to the biomass mass for the comparison of GS and RM. Furthermore, the real sewer could have more complex active biomass and enzymes, more fluctuated redox potentials and dynamic flow rate that the current study did not consider due to practical reasons. All these factors can potentially contribute to the transformation of biomarkers in the real sewer and need further investigation.

Generally speaking, compounds with $\log K_{ow}$ values lower than 3.0 are not expected to be sorbed to the particles (Behera et al., 2011). Another study carried out by McCall et al (2016b) also pointed out that considering the real sewer A/V ratio, the sorption of biomarkers to suspended

solids and biofilm is negligible. Due to the low vapour pressure and hydrophilic property of selected biomarkers in the present study, the in sewer loss of these chemicals could be mostly due to chemical and biochemical transformation rather than volatilisation and adsorption (Baker et al., 2012). However, opioids with relatively high $\log K_{ow}$ values could have considerable sorption to particular matter and biofilms (Baker et al., 2012; Subedi and Kannan, 2014). This study cannot differentiate sorption and degradation since it only monitored the aqueous biomarker concentrations. A well designed sorption study would provide more insight into the loss of biomarkers under different sewer conditions.

4. CONCLUSION

In-sewer conditions can transform certain chemicals that were used as biomarkers in WBE. But the transformation of biomarkers is compound specific, and dependant on sewer conditions. Therefore, estimation of chemical consumption in the population by WBE should consider the possible in-sewer degradation of biomarkers to avoid underestimation or in some cases overestimation if the biomarkers were formed during the sewer transportation. Interpretation of geographical pattern of chemical consumption should take the catchment characteristics and the associated in-sewer transformation of biomarkers into account to achieve better understanding. Further study with a mathematical modelling approach to evaluate the in-sewer loss of biomarkers could provide information for more accurate back-calculation.

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