Potential impact of the sewer system on the applicability of alcohol and tobacco biomarkers in wastewater-based epidemiology

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ABSTRACT

Understanding the actual consumption of alcohol and tobacco in the population is important for forming public health policy. For this purpose, wastewater-based epidemiology has been applied as a complementary method to estimate the overall alcohol and tobacco consumption in different communities. However, the stability of their consumption biomarkers, ethyl sulfate, ethyl glucuronide, cotinine and trans-3'-hydroxycotinine, in the sewer system has not yet been assessed. This study aimed to conduct such assessment using sewer reactors mimicking conditions of rising main, gravity sewer and wastewater alone, over a 12-hour period. The results show that cotinine and trans-3'-hydroxycotinine are relatively stable under all sewer conditions while ethyl sulfate was only stable in wastewater alone and gradually degraded in rising main and gravity sewer conditions. Ethyl glucuronide quickly degraded in all reactors. These findings suggest that cotinine and trans-3'-hydroxycotinine are good biomarkers to estimate tobacco consumption; ethyl sulfate may be used as a biomarker to estimate alcohol consumption but its in-sewer loss should be accounted for in the calculation of consumption estimates. Ethyl glucuronide, and probably most of glucuronide compounds, are not suitable biomarkers to be used in wastewater-based epidemiology due to their in-sewer instability.

Keywords: sewer reactor; stability; degradation; sewer biofilms; wastewater analysis; biomarkers;

1. INTRODUCTION

Alcohol (ethanol) and tobacco (nicotine) are the two most frequently used recreational substances in the world. The use of both substances have been associated with a significant increase in disease burden, including lung cancer from tobacco smoke and liver disease from alcohol consumption [1, 2]. Excessive alcohol consumption also causes a wide range of other harms including road and other accidents, domestic and public violence. Therefore, monitoring the consumption of these two substances in the population would help provide evidence for future policies to estimate health risks that arise from their consumption.

Traditionally survey methods and sales data have been used to monitor tobacco and alcohol consumption but recently wastewater analysis or wastewater-based epidemiology (WBE) has been used as an alternative method to estimate the consumption of chemicals at the population level [3]. There are already some WBE studies to monitor the consumption of alcohol and tobacco in different populations, from multiple cities to music festivals [4-7]. These studies were based on the measurement of the unique metabolites of ethanol, ethyl sulfate (EtS) and ethyl glucuronide (EtG), and the metabolites of nicotine, cotinine (Cot) and trans-3'-hydroxycotinine (HCot). In WBE, the use of metabolites like EtG, EtS, Cot and HCot (Fig. 1) as biomarkers are preferable to the use of parent compounds because it avoids the uncertainty caused by the potential direct disposal of alcohol and tobacco, cigarette stubs or nicotine products into the sewer. However, until now, most studies have estimated the total consumption of chemicals including ethanol and nicotine without considering the uncertainty incurred due to the potential degradation of the chemicals during the sewer passage.

A number of studies have recognised and attempted to address the issue of the stability of these biomarkers in wastewater [4, 8-12]. The findings from these studies indicated that in wastewater samples these biomarkers are relatively stable. However, in-sewer degradation is not only

associated with the chemical being in wastewater but may also be influenced by the biofilms present on the sewer walls [13, 14]. In recognition of this, McCall et al. [15] recommended to use laboratory or pilot-scale experiments that cover realistic conditions of the sewers including the involvement of sewer biofilm and wastewater.

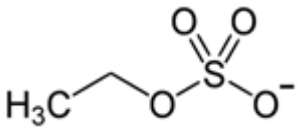
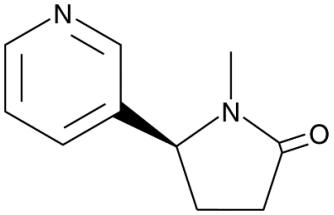
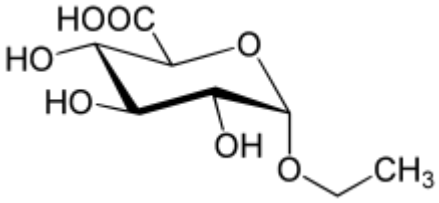
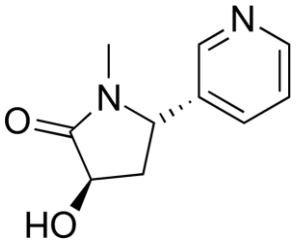
Ethanol (alcohol) biomarkers	**Nicotine (tobacco) biomarkers**
	
Ethyl sulfate (EtS)	Cotinine (COT)
	
Ethyl glucuronide (EtG)	trans-3'-Hydroxycotinine (HCot)

Fig. 1. Structure of metabolites of ethanol and nicotine as potential biomarkers in WBE.

Such recommendation is consistent with the fact that the actual sewer system typically comprises fully anaerobic pipes (rising main) and mixed aerobic/anaerobic pipes (gravity sewer), which are usually covered by biofilms on the inner pipe walls [16]. Biofilms are capable of transforming/degrading various chemical compounds more strongly than the suspended microbes in wastewater alone [13, 14]. The capacity of sewer biofilms to enhance degradation of certain chemicals including cocaine, 6-acetylmorphine (heroin metabolite), creatinine, and paracetamol has been reported [13, 14, 17, 18].

Therefore, in this study, we aimed to investigate the stability of EtG, EtS, Cot and HCot under different sewer conditions using laboratory-scale sewer reactors and to assess the impact of in-sewer degradation of these biomarkers to their applicability in WBE studies including in consumption estimation and population estimation purposes.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Analytical grade hydrochloric acid 32% was purchased from Univar (Ingleburn, Australia). Analytical standards, EtG, EtS, Cot, HCot, acesulfame-D4, atenolol-D7 were purchased from Sigma Aldrich (Castle Hill, Australia). Cocaine was purchased from Cerilliant (Texas, US). Deionised water was produced by a MilliQ system (Millipore, 0.22 μm filter, 18.2 MΩ cm^{-1}). LCMS grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany).

2.2. Laboratory-scale sewer reactors

This study employed laboratory-scale reactors to simulate typical sewer conditions, which have been reported in our previous studies [13, 17]. Three different sewer reactors were employed, which included a rising main (RM) sewer reactor, a gravity sewer (GS) reactor and a control (CR) sewer reactor without biofilms. Detailed description of the reactors can be found in Jiang et al. [19].

Sewer biofilms in RM and GS reactors have been cultivated for over 8 years using real wastewater before the experiments. Domestic wastewater, collected weekly from a local pumping station in Brisbane (Australia), was used as the feed. The sewage (pH around 7.2) typically contained sulfide at concentrations of <3 mg-S/L, sulfate at 10-25 mg-S/L, total COD and soluble COD at 450-600 mg/L and 260-450 mg/L, respectively, with the latter including volatile fatty acids at 50-120 mg-COD/L. The sewage was stored at 4 °C and heated up to 20 °C before being pumped into the reactors. The reactors were fed with sewage through a peristaltic pump (Masterflex 7520-47) every 6 hours, which is similar to ~~an~~ the average sewage hydraulic retention time in a sewer catchment ~~(~~estimated from data of 50 WWTP reported by Ort et al. [20]~~)~~. Every feed pumping event lasted for 2 minutes, delivering one reactor volume (0.75 L) of sewage into each reactor. To ensure homogeneous distribution in reactors, gentle mixing was provided with magnetic stirrers at 250 rpm (Heidolph MR3000).

During the batch tests described in Section 2.3, the biological activities of sewer biofilms, i.e. sulfate reduction and methanogenesis, in the sewer reactors were determined. Wastewater samples were taken at 0, 20, 40, and 60 minutes after feeding for the analysis of dissolved inorganic sulfur and methane. The analytical methods for those parameters were described previously in Jiang et al. [21].

2.3. Batch tests for the biomarkers

Batch tests in triplicate were conducted to investigate the degradation of the biomarkers under different sewer conditions created by different sewer reactors. For each replicate of the batch test, newly collected wastewater was warmed to 20^{o}C and its pH was measured and adjusted if necessary to be 7.2. The temperature and pH were comparable with OECD guideline No. 314 [22] and with other studies on the stability of chemicals in wastewater [23].

To ensure that the concentrations of EtS, EtG, Cot and HCot in the sewer reactors could be measured by direct injection LCMS method, prior to each batch test, the wastewater in the reactors was spiked with a standard mixture of EtS, EtG, Cot and HCot to increase the biomarker concentrations to approximately 10, 5, 10 and 5 ng/mL, respectively. These initial concentrations were still within the range of concentrations of these compounds measured in the actual wastewater samples from previous studies [9, 24-26].

Continuous mixing was maintained ~~for~~ in each reactor with magnetic stirrers at 250 rpm (Heidolph MR3000) during all the batch tests. During the batch tests the 6 hour feeding events normally used to cycle though the sewer reactors were turned off. Samples of the wastewater in the sewer reactors were taken at 0, 0.25, 0.5, 1, 2, 3, 6, 9 and 12 hours after spiking. Samples were immediately processed by filtering 1 ml of wastewater through a 0.20 μm PTFE syringe filters (Phenomenex, Australia) into a vial where 15 μL of 2 M HCl was added to acidify the sample to approximately pH 2 and then frozen at -20 °C until analysis.

2.4. Chemical analysis of biomarkers in wastewater

The filtered samples were analysed by direct injection onto the liquid chromatography (Shimadzu Nexera LC system) coupled with tandem mass spectrometry (ABSciex 5500®QTRAP) (LC-MS/MS). The electrospray ionisation (ESI) interface of the MS was operated at negative mode for EtS and EtG and positive mode for Cot and HCot, resulting in two injections per sample for analysis. Chemical separation was achieved on a Synergi™ Polar-RP analytical column (Phenomenex®, 100x2 mm, 2.5 µm) at 40 ^{o}C. For the negative ESI analysis, the mobile phase of (A) 100% Milli-Q water and (B) 95% acetonitrile; both with 0.1% formic acid, was used and at the gradient: 0% B, 0-1.9 min; 60% B at 2.4 min for 3 min; 0% B at 5.5 min for 3.5 min. For the positive ESI analysis, 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% formic acid and 5 mM ~~ammonia~~ammonium formate, was used and at the gradient: 0% B, 0-1 min; 40% B at 3 min; 60% B at 3.1 min for 2.9 min; 0% B at 6.1 min for 3.9 min. The flow rate was set at 0.3 mL/min and the injection volume was 2 µL.

The MS/MS employed a multiple reaction monitoring (MRM) as the data acquisition method. Different MS/MS parameters were optimised for the MRM transitions of each analyte (Table S1). The two most abundant MRM transitions were used for quantification and confirmation of the analyte in samples. Two deuterated compounds (-ve ESI: acesulfame-D4 and +ve ESI: atenolol-D7; each 10 ng) were spiked into the samples and used for assessing and correcting potential instrumental variabilities over the analysis. The retention time of these two deuterated compounds was similar to that of our studied analytes; also, they have been routinely analysed in our previous studies [27, 28]. The average recovery of acesulfame-D4 in the samples was 104% and that of atenolol-D7 was 105%. The intraday (CV%; n=97) and interday variations (CV%; across 2 days) were 7.5% and 11% respectively for acesulfame-D4 and 8.0% and 11% for atenolol-D7. Analyte concentrations in the samples were measured together with the calibration standards (six points, 0.5-100 ng/mL). The limit of detection and quantification for the target compounds (Table S1) was

estimated using the low calibration standard with the signal-to-noise ratio of 3 and 10, respectively. MilliQ water was used as pProcedural blank samples ~~were included~~ during sample preparation and instrumental analysis. In these samples the target analytes were not detected.

2.5. Data processing

The concentrations of biomarkers measured during the 12-hour tests were normalised to the percentage relative to the initial spiked concentrations. Linear regression (zero order) and pseudo first order regression were applied to the data obtained from batch tests in order to identify the best fit degradation kinetics. Graphs and statistical tests (non parametric t-test) were performed using Prism 7 (GraphPad software, Inc.).

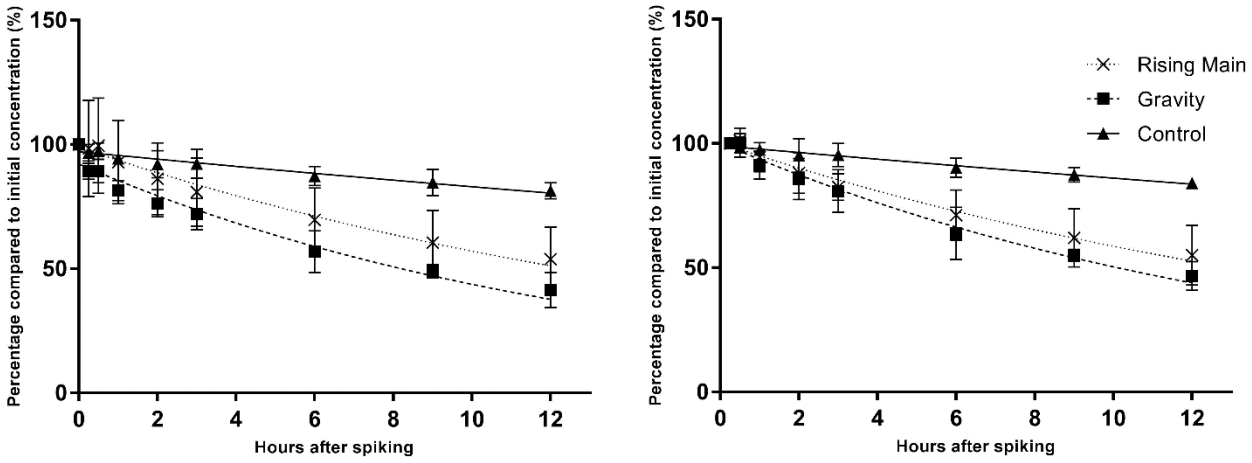
3. RESULTS

3.1. Biological activities in sewer reactors.

The biological activity of the RM and GS reactors used in this experiment was found to represent the biological activity and associated degradation of chemicals within actual sewers. The control reactor showed no methane or sulfide production (change of concentrations with the natural variations observed for the sample storage within 12 h) as it does not contain the sewer biofilms compartment. The RM reactor had higher sulfide and methane production rate than the GS reactor indicating that it was under stronger anaerobic conditions. Activities of sulfate-reducing bacteria and methanogenic archaea in the RM reactor were measured at 7.21 ± 0.74 mg S~~/L h~~ $L^{-1}h^{-1}$ and 29.7~~3~~ ± 0.6~~3~~ mg COD $L^{-1}h^{-1}$~~/L h~~ respectively which is similar to previously reported values for both real and laboratory-scale sewers [29, 30] confirming that the laboratory setup can mimic actual conditions of sewer pipes. Dissolved oxygen in the GS reactor was measured below 0.33 mg/L despite continuous aeration which indicates aerobic activity consuming oxygen. It is also expected that anaerobic conditions may be present at the bottom of the reactor where oxygen could not reach.

This is supported by the low reduction of sulfate (4.21 ± 0.77 mg S/L-h) and the low production of methane (14.8 ± 2.31 mg COD/L-h). This is primarily due to the aerobic conditions, and also partly due to the continuous dissipation of gases through the air-water interface. Detailed discussion can be found in Thai et al. [13] where it demonstrated that the biological activities observed in the laboratory-scale reactors coincide well with those in real sewer systems.

Additionally we also used cocaine, an illicit drug that was tested in this reactor system previously [13], as an indicator for the performance of the reactors. The degradation of cocaine followed similar patterns as observed in previous study (Fig. 2). The similarity of cocaine degradation pattern between the two studies confirmed the comparability of the systems and thus the bioactivities of the sewers used in this study.



Previous study (Thai et al. [13]) This study

Fig. 2. Degradation of cocaine in two studies as an indicator for reactors' performance.

3.2. Degradation of biomarkers of alcohol consumption.

The two biomarkers of alcohol consumption, EtS and EtG, showed different patterns of degradation in the three different sewer reactors (Fig. 3a, b). During the monitoring period, EtS remained stable in the CR but not in the other two sewer reactors (Fig. 3a). EtS remained stable longer in the GS than in the RM, from 100% to less than 20% in 9 hours vs. 3 hours. The degradation profile of EtS

in the GS and RM followed the first-order kinetic model, with $t_{1/2}$ of 3.77 and 1.27 hours respectively (Table 1).

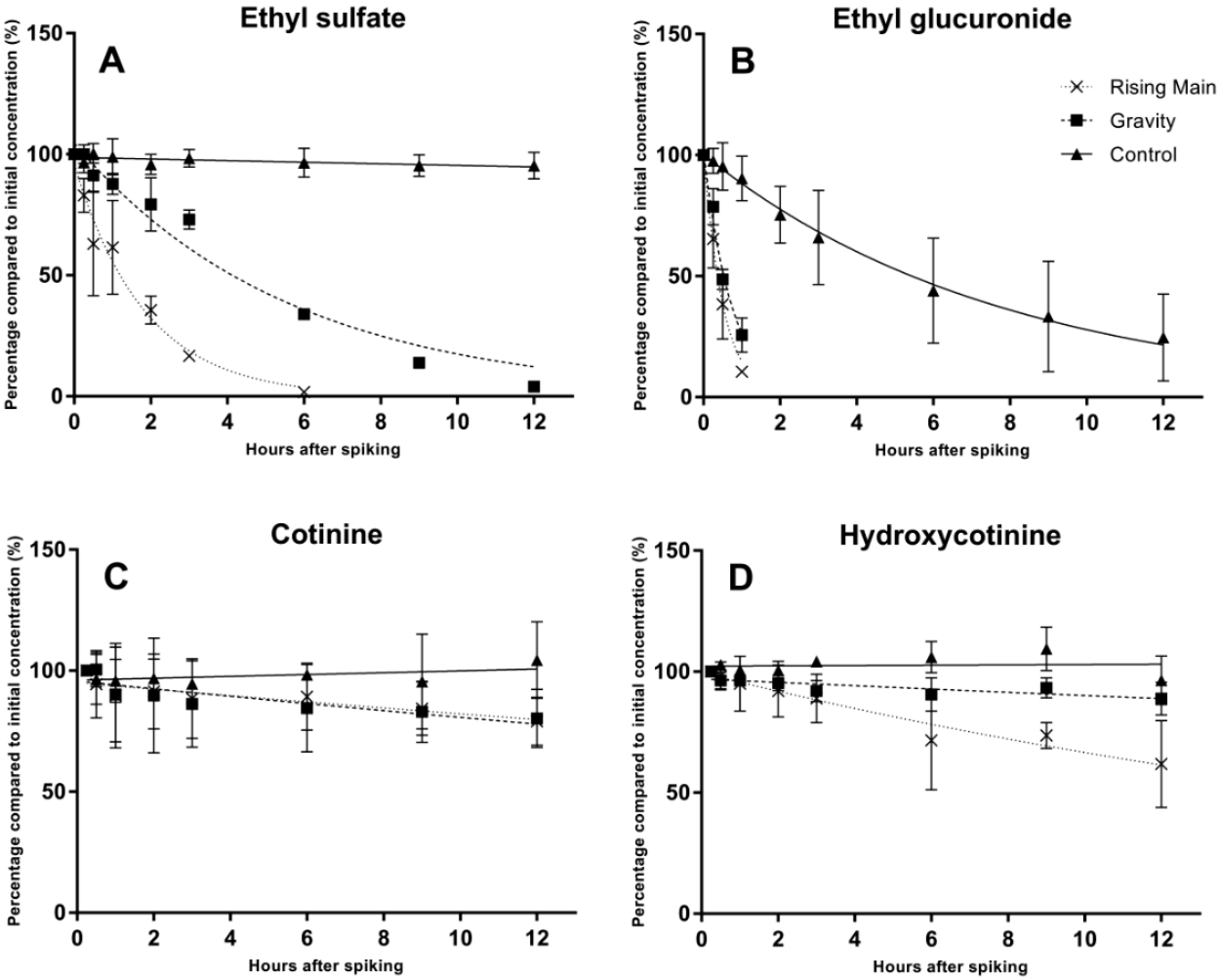


Fig. 3. Degradation profiles of alcohol and tobacco biomarkers under different sewer conditions. Curves were used for the best fit model selected as showed in Table 2. Error bars represent the standard deviation of 3 replicates.

Compared to EtS, the stability of EtG was relatively low in the three sewer conditions tested (Fig. 3b). Only 35% of EtG left in the CR after 12 hours. The degradation of EtG in the CR followed zero order kinetics with the slope of -6.6 %/h (Table 1).

The degradation of EtG was even more rapid in both the GS and RM, from 100% to 25% and to 11% respectively, within an hour. The degradation of EtG was not significantly different between the GS and RM ($p = 0.065$). Same as EtS, the degradation profile of EtG in the two active sewer reactors

followed the first-order kinetic model, with $t_{1/2}$ of 0.51 hours in the GS, and 0.36 hours in the RM (Table 1).

Table 1. Selection of kinetics models for studied biomarkers in different sewer reactors. Model fitting with higher R^2 value was selected to fit the data shown in Fig. 3.

		Linear regression		First-order kinetics	
		Slope (%/h)	R^2	Half-life (h)	R^2
Control (CR reactor)	EtS	ns		ns	
	EtG	**-6.6 (-5.1 to -8.0)**	**0.942**	12.5 (9.89 to 16.9)	0.830
	Cot	ns		ns	
	HCot	ns		ns	
Gravity sewer (GS reactor)	EtS	-8.6 (-9.3 to -7.9)	0.960	**3.77 (3.30 to 4.41)**	**0.964**
	EtG	-75.0 (-89.1 to -60.9)	0.934	**0.51 (0.43 to 0.62)**	**0.960**
	Cot	**-1.4(-0.5 to -2.4)**	**0.711**	93.4 (46.5 to ∞)	0.169
	HCot	**-0.7 (-0.2 to -1.2)**	**0.669**	217 (132 to 630)	0.319
Rising main (RM reactor)	EtS	-15.2 (-19.2 to -11.3)	0.774	**1.27 (1.00 to 1.75)**	**0.904**
	EtG	-90.1 (-114 to -66.0)	0.889	**0.36 (0.28 to 0.49)**	**0.935**
	Cot	**-1.3 (-0.6 to -1.9)**	**0.788**	109 (53.9 to ∞)	0.163
	HCot	**-3.2 (-2.4 to -4.0)**	**0.940**	39.7 (28.8 to 63.5)	0.618

n.s. not significantly deviated from zero

Bold: models were selected for the degradation of the chemicals

The data overall suggested that the presence of sewer biofilms substantially increased the degradation of EtS and EtG (Fig. 3a, b). For EtS, such effect appeared more pronounced in the RM than GS since the $t_{1/2}$ of EtS in RM was three time shorter than that in the GS (Table 1). This may also mean that the microbial activity of the anaerobic biofilms enhanced the degradation of EtS in a faster pace in a sealed system than an open system. For EtG, biofilm activities in both the RM and GS reduced its stability in very similar ways (Fig. 3b). The results obtained from the CR indicated that suspended microbes could also affect the stability of EtG, but not that of EtS, in the wastewater alone over the studied experimental period, a ~~phenomena~~ phenomenon that was reported recently by Ramin et al. [31].

3.3. Degradation of biomarkers of tobacco consumption

Cot and HCot, the two biomarkers for nicotine use, showed a difference in their degradation patterns over the 12-hour monitoring period (Fig. 3c, d). Cot remained stable in the CR (Fig. 3c) with no significant degradation over the monitoring period. Cot showed a similar degradation in both the RM and GS, with 56 and 59% remaining after 12-hours respectively. The degradation pattern of Cot in the RM and GS followed a linear regression model with a loss of 1.3 and 1.4%/h, respectively (Table 1). HCot remained stable over 12-hours in the CR with no significant degradation measured in this study. However, it degraded in the RM and GS with only 89 and 55% of the original HCot remained after 12 hours, both fitting a linear regression model with rates of loss of 0.7 and 3.2%/h, respectively (Table 1).

The data overall suggests that both Cot and HCot are stable in wastewater but are prone to degradation in sewers with the presence of biofilms. For Cot, similar rates of loss between the RM and GS show that the microbial activity in both the anaerobic and aerobic biofilms similarly degrades cotinine in the sewer. Meanwhile, HCot was less affected by aerobic biofilms in the GS but was strongly transformed by anaerobic biofilms in the RM. The results from the CR reactor showed that the microbes suspended in wastewater do not play a significant role in the degradation of Cot or HCot in wastewater.

4. DISCUSSION

4.1. Implication for the use of those biomarkers in future WBE studies

The biomarkers for alcohol and tobacco consumption investigated in this study have been used in clinical and forensic settings to detect the consumption of alcohol and tobacco through urinalysis, which led to their application in WBE to estimate population consumption of those recreational products [4, 9, 32].

The stability of those biomarkers is important for an accurate outcome of WBE as it is estimated to contribute ~10% of uncertainty to the final estimates [33]. Understanding the in-sewer stability of WBE biomarkers is also urgently needed in order to minimise the overall uncertainty of WBE outcomes [33]. Consequently, there have been several studies investigating the stability of EtS, EtG, Cot, and HCot as shown in Tables 2 & 3. However, the conditions of those stability experiments were far from in-sewer conditions defined by McCall et al. [15] for WBE approach because they only used wastewater in flasks [34] or small tubes [10, 11, 35, 36] without or minimal mixing (e.g. 3 gentle stirs per day in Chen et al. [33]). Bisceglia [37] applied adequate stirring (180 rpm) but used coarsely filtered wastewater, which could considerably reduce the microbial population of the wastewater medium. It means that the previously reported data may underestimate the impact of in-sewer activities to the stability of alcohol and tobacco biomarkers.

In this study, we successfully mimicked the in-sewer conditions (RM and GS reactors), which were confirmed by the gas production activities of microorganisms and the degradation profile of a studied chemical (i.e. cocaine). The in-sewer conditions used in this study have enhanced the degradation rate of both alcohol and tobacco biomarkers compared to that in the wastewater alone (in-sample activity, CR sewer), most significantly for alcohol biomarkers. The faster degradation is probably due to the presence of biofilms on the walls of RM and GS. These results are in agreement with previous studies where selected chemicals degraded faster in the presence of sewer biofilms or suspended than in wastewater [13, 14, 18, 31].

- *Impact ~~t~~on~~o~~ consumption estimation of alcohol and tobacco*

The potential rapid degradation of EtS and EtG, especially EtG (Fig. 3a,b), in the sewer system may limit their applicability as biomarkers for the estimation of alcohol consumption in the population although both of them are still currently used in urinalysis to detect alcohol consumption in individuals [38]. With a complete loss after only 2 hours in both RM and GS reactors, EtG is not

277 recommended to be used to estimate alcohol consumption in WBE studies, which supports the
278 practice in all WBE studies related to alcohol [7, 39-45] after the initial assessment of Reid et al. [9]
279 (see also Table 2). In general, glucuronide compounds are very quickly transformed in wastewater
280 ([9, 11, 46, 47] and this study) and hence are not suitable to be used as biomarkers in WBE.
281
282 EtS is more persistent than EtG, especially in wastewater only. However, there were still
283 considerable losses of EtS in RM and GS reactors. Evidence of the degradability of EtS has been
284 reported previously in the wastewater treatment processes [39, 48]. Interestingly, Peeters et al. [48]
285 also reported that the biodegradation of EtS required specialised microbes. We observe that the
286 degradation profile of EtS is similar to that of codeine and 6-acetyl morphine (the biomarker of
287 heroin consumption). Codeine was initially reported as stable in wastewater only [15, 47] but later
288 found degradable in wastewater with the presence of biofilm [17, 18] with half-lives of 3.8 h and
289 2.1 h in RM and GS conditions, respectively [17]. Similarly, 6-acetyl morphine was relatively stable
290 in wastewater alone but degraded quickly in RM and GS conditions with half-lives of 4.23 h and
291 4.26 h, respectively [13]. The combined effect of rapid degradation, small excretion rate (~0.5%) of
292 6-acetyl morphine, and low consumption volume of heroin made it very difficult to detect 6-acetyl
293 morphine in wastewater samples. Consequently, there is hardly any study to monitor heroin
294 consumption by WBE with 6-acetyl morphine as biomarker.
295 The potential in-sewer degradation of EtS reported in this study, together with its low excretion
296 factor (mean = 0.012%), could affect the feasibility of WBE to monitor alcohol consumption. But in
297 contrast to the low consumption volume of illicit heroin, the average daily consumption of alcohol,
298 a legal and popular psychoactive substance, is much higher and thus makes it possible to be
299 measured in influent wastewater samples even with direct injection LCMS method (e.g. Reid et al.
300 [9]). Nevertheless, the uncertainty due to in-sewer degradation and excretion factor of EtS may have
301 contributed to the discrepancy between WBE and surveys' estimates [7, 45]. Therefore, it is

important to i) have field experiments to determine the realistic extent of degradation of EtS and ii) consider the impact of degradation in future WBE estimates for alcohol consumption.

Cot and HCot are much more stable than EtS and EtG although there was still a measurable loss of Cot and HCot in-sewer with the presence of aerobic and aerobic biofilm (Fig. 3c,d). The degradation rates of Cot and HCot found in this study (Table 1) are relatively low, which would keep >80% of the excreted amount of Cot and HCot in the influent wastewater after an average sewer retention time of 6 hours (estimated from data of 50 wastewater treatment plants in Ort et al., 2014). Therefore, the uncertainty caused by in-sewer stability of Cot and HCot is considered acceptable for WBE studies because it is equal or less than those from chemical analysis [33] and both biomarkers can be used to estimate consumption of tobacco in the population. This finding supported the selection of many studies to use Cot and HCot as biomarkers for tobacco consumption (e.g. [4, 44, 49]).

- *Impact ~~to~~on the applicability as population markers*

Due to its popular consumption and high level found in wastewater (high excretion rates), Cot and HCot have been proposed and investigated to be used as population markers [12, 24]. Both studies found Cot and HCot stable in wastewater only experiments and concluded that Cot is a suitable population marker. Senta et al. [12] also recognised HCot potential as population marker. Considering the potential degradation of Cot and HCot in the sewer system, we applied the criteria recommended by O'Brien et al. [17] to assess whether they are "Best Practice" population markers. Cot satisfies this set of 4 criteria to be used for population estimation purpose while HCot does not meet the average 10% loss threshold. The caveat for Cot here is that it should not be used as a single population marker as there are differences in tobacco consumption among regions [12] or countries [40]. Moreover, a recent study by Tscharke et al. [50] also suggested to monitor two alkaloids – anabasine and anatabine – which are specific to dried tobacco in addition to nicotine and

cotinine to obtain a better picture of the tobacco consumption situation. But anabasine and anatabine will need to be tested in the sewer reactors to ensure that they are stable under in-sewer conditions.

4.2. Limitations

However, we acknowledge that the sewer reactors used in this study only represent a certain type of real sewers because they are designed with a fixed area/volume (A/V) ratio, and are operated at a fixed hydraulic retention time. These factors should be considered while extending the results obtained in this study to various sewer systems, with different pipe diameters and pumping patterns. The A/V ratio of the RM and GS reactors (72.5 and 50 m^2/m^3, respectively) in this study is considered similar to small diameter pipes. Large diameter pipes which are typical in the actual sewer system have a smaller A/V ratio and are not represented in this study. We hypothese that a higher A/V ratio would provide more bacteria and more surface for contact between chemicals and biofilms that lead to more degradation of chemicals in the sewer system. However, McCall et al. [18] did not observe any significant difference in the level of chemical degradation among increasing values of A/V ratios. Additionally, the reaction processes in the sewer is likely to be the result of the combined effects of the A/V ratio and the hydraulic retention time of wastewater in the sewer [16]. Therefore, knowing the characteristics of a sewer system would help better estimate the extent of degradation rate of each chemicals and consequently help to improve the accuracy of the consumption estimation.

Table 2. Comparing results of different stability studies of biomarkers of alcohol in wastewater.

(Values are the percentage loss at the end of the experiment)

	Halter *et al*. [36] (OECD 301 test)		Reid *et al.* [9]	Rodríguez-Álvarez *et al.* [11]	This study		
Experimental conditions	Closed Bottle Test* 20ºC; 28 d	Manometric Respiratory Test*; 28 d	Wastewater Room temp. 18 h.	Wastewater 20 °C; 1 week.	Wastewater pH = 7; 20°C; 12 h.	Gravity sewer pH = 7; 20°C; 12 h.	Rising main pH = 7; 20°C; 12 h.
EtS	Stable	Degrade after 6 d	up to -10	Stable	Stable	-96 ± 0.4	Complete loss after 9 hours
EtG			-50		-75.3 ± 18.1	Complete loss after 2 hours	Complete loss after 2 hours

*inoculated with effluent from WWTP

Table 3. Comparing results of different stability studies of biomarkers of tobacco in wastewater.

(Values are the percentage loss at the end of the experiment)

	Chen *et al.*, [34]	Bisceglia [37]	Baker and Kasprzyk-Hordern [35]	Rodríguez-Álvarez *et al.*, [11]	Castiglioni *et al.*, [4]	Senta *et al.*, [12]	Rico *et al.*, [24]	This study		
Experimental conditions	Wastewater pH = 7; 20°C; 24 h.	Wastewater pH = 7; 31°C /20°C; 24 h.	Wastewater pH = 7.4; 19 °C; 12h/24h	Wastewater 20 °C; 24 h.	Wastewater 20 °C; 24 h.	Wastewater pH = ~7; 22 °C; 24 h.	Wastewater 26 °C; 18h/24h/36h.	Wastewater pH = 7; 20°C; 12 h.	Gravity sewer pH = 7; 20°C; 12 h.	Rising main pH = 7; 20°C; 12 h.
Cot	9.8 ± 4.3	Stable	56.5 / 61.4	Stable	Stable	Stable	Stable	Stable	-19.7 ± 12.0	-20.9 ± 9.9
HCot				Stable	Stable	Stable	Stable	Stable	-11.1 ± 6.8	-38.2 ± 17.9

5. CONCLUSIONS

This study evaluated the stability of common biomarkers of alcohol and nicotine consumption under different sewer conditions using sewer reactors. It also discussed the applicability of these biomarkers for WBE studies when their in-sewer stability is taken into consideration. The main conclusions are:

1. EtG is unstable and is not suitable to be used as biomarker to estimate alcohol consumption through WBE approach.
2. EtS is stable in wastewater only but the simulated sewer conditions (RM and GS) significantly enhanced its degradation. Field study might be required to determine the actual extent of EtS degradation in a sewer catchment to improve the accuracy of alcohol consumption estimates by WBE.
3. Both Cot and HCot are relatively stable and could be used in WBE to estimate tobacco consumption. Cot also meets all the criteria to be used as a population marker but it is suggested that it should be used with other population markers to minimize the uncertainty raised by tobacco consumption practices in different cultures.

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