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Title: Assessment of stability of drug biomarkers in municipal wastewater as a factor influencing the estimation of drug consumption using sewage epidemiology

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Abstract: Stability of the selected urinary biomarkers of six illicit drugs and two therapeutic opioids in municipal wastewater was studied in order to determine errors associated with their possible transformation in the sewer. The stability was assessed in experiments conducted at 10 °C and 20 °C in order to simulate typical winter and summer temperature conditions in the sewer system. Among fourteen substances tested, the most unstable compounds were morphine-3-β-D glucuronide (MG), 6-acetyl morphine (6-AM), cocaine (COC) and 6-acetyl codeine (6-AC), while all other investigated compounds appeared to be highly stable over a period of 72 hours. The transformation of all degradable compounds followed pseudo-first order kinetics with significantly longer half-times (t1/2) at winter conditions. At 20 °C, t1/2 of MG, 6-AM, COC and 6-AC was 7 h, 77 h, 35 h and 58 h, respectively, while the corresponding t1/2 values at 10 °C were 18 h, 139 h, 173 h and 87 h. The main transformation mechanism of MG, 6-AM and 6-AC was most probably their enzymatic hydrolysis to morphine (MOR), while COC transformation to benzoylecgonine (BE) was primarily governed by chemical hydrolysis. The results from this study indicate that the observed degradation of COC and 6-AM would not significantly affect the estimates of COC and heroin consumption if the in-sewer hydraulic retention time is lower than 12 h. Acidification of the wastewater samples proved to be the good way to stabilise the wastewater samples for the analysis of all selected compounds, except for 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH). This finding should be taken into account when selecting the preservation technique for multiresidual analyses of different groups of illicit drugs.

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Dear Editor,

please find enclosed our manuscript on *Assessment of stability of drug biomarkers in municipal wastewater as a factor influencing the estimation of drug consumption using sewage epidemiology*.
We hope that you will find this study suitable for publication in the special issue of the Science of the Total Environment.
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Sincerely yours,

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Highlights

- Stability of fourteen urinary drug biomarkers in municipal wastewater was evaluated
- The most unstable compounds were cocaine, morphine glucuronide and acetyl morphine
- The degradation followed pseudo first order kinetics and was temperature dependant
- In-sewer changes of common drugs do not strongly affect their consumption estimates

Assessment of stability of drug biomarkers in municipal wastewater as a factor influencing the estimation of drug consumption using sewage epidemiology

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Abstract

Stability of the selected urinary biomarkers of six illicit drugs and two therapeutic opioids in municipal wastewater was studied in order to determine errors associated with their possible transformation in the sewer. The stability was assessed in experiments conducted at 10 °C and 20 °C in order to simulate typical winter and summer temperature conditions in the sewer system. Among fourteen substances tested, the most unstable compounds were morphine-3-β-D glucuronide (MG), 6-acetyl morphine (6-AM), cocaine (COC) and 6-acetyl codeine (6-AC), while all other investigated compounds appeared to be highly stable over a period of 72 hours. The transformation of all degradable compounds followed pseudo-first order kinetics with significantly longer half-times ($t_{1/2}$) at winter conditions. At 20 °C, $t_{1/2}$ of MG, 6-AM, COC and 6-AC was 7 h, 77 h, 35 h and 58 h, respectively, while the corresponding $t_{1/2}$ values at 10 °C were 18 h, 139 h, 173 h and 87 h. The main transformation mechanism of MG, 6-AM and 6-AC was most probably their enzymatic hydrolysis to morphine (MOR), while COC transformation to benzoylecgonine (BE) was primarily governed by chemical hydrolysis. The results from this study indicate that the observed degradation of COC and 6-AM would not significantly affect the estimates of COC and heroin consumption if the in-sewer hydraulic retention time is lower than 12 h. Acidification of the wastewater samples proved to be the good way to stabilise the wastewater samples for the analysis of all selected compounds, except for 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH). This finding should be taken into account when selecting the preservation technique for multiresidual analyses of different groups of illicit drugs.

Keywords: illicit drugs; stability; urinary biomarkers, degradation, transformation, municipal wastewater, LC-MS-MS

1. **Introduction**

Municipal wastewater effluents are usually regarded as one of the main sources of input of different types of contaminants into the environment. However, the analysis of municipal wastewater has been recently increasingly used as a valuable source of information about a given community, including the estimation of collective drug abuse (Karolak et al., 2010; Kasprzyk-Hordern et al., 2009; Postigo et al., 2010; Terzic et al., 2010; Thomas et al., 2012; van Nuijs et al., 2011; Zuccato et al., 2005; Zuccato et al., 2008). Namely, municipal wastewater contains a very large number of versatile compounds excreted by humans after the consumption of different illegal and legal drugs. Having the data on the metabolic pathways of selected drugs of abuse and wastewater flow, the concentrations of selected urinary drug biomarkers could be used to estimate collective drug consumption. This innovative approach has a potential to become a rather useful complementary tool to the existing epidemiological methods, although further evaluation and standardisation is needed. The reliability of the consumption estimates is not dependent only on the accuracy of the chemical measurements but, among other things, on the stability of the selected urinary biomarker in the sewer system, as well as during the sample collection and storage (Castiglioni et al., 2013). Stability of the selected urinary drug biomarkers in the wastewater has already been assessed by several research groups (Baker and Kasprzyk-Hordern, 2011; Castiglioni et al., 2006, 2011; Chiaia et al., 2008; Gonzalez-Marino et al., 2010; Plosz et al., 2013; van Nuijs et al., 2012). The setup of these experiments was rather different in terms of temperature and pH conditions used, number and type of compounds studied, as well as the duration of the experiment. Most of the published stability experiments covered time scales from 12 to 72 h. In most of the cases, the samples were analysed only at the beginning and at the end of the experiment, while only two studies included sampling at multiple shorter time intervals (van Nuijs et al., 2012, Plosz et al., 2013). Furthermore, the experiments were focused mainly on the stability of wastewater samples during the collection and storage (Castiglioni et al., 2006, 2011; Gonzalez-Marino et al., 2010), while only limited number of them was performed applying the temperature and pH conditions typical for sewer systems. (Baker and Kasprzyk-Hordern, 2011; Plosz et

al., 2013; van Nuijs et al., 2012). The compilation of the literature data obtained in different stability studies (van Nuijs et al., 2012, Castiglioni,. 2013) show that the results are not always consistent and additional data are needed in order to better understand the fate of drug target residues in the sewer system. For instance, Baker and Kasprzyk-Hordern (2011) reported the significant increase of amphetamine concentration (≈50%) after 12 h-experimental period (19 °C, pH 7.4), while no significant change in the concentration of this compound was observed in the experiments performed by Castiglioni et al. (2006) and van Nuijs et al. (2012) at fairly similar experimental conditions (20 °C, pH 7.4-7.5). Furthermore, Gonzalez-Marino et al. (2010) reported a complete loss of methadone after 72 h at 4 °C, while other studies reported its high stability in the wastewater (Castiglioni et al., 2006; Baker and Kasprzyk-Hordern 2011; van Nuijs et al., 2012). Besides that, all experiments mimicking the sewer conditions were performed at the typical summer temperature conditions (19 °C or 20 °C).

The aim of this paper was to study the stability of fourteen selected urinary biomarker compounds excreted after the consumption of six illicit and two licit drugs at the typical winter and summer in-sewer temperatures and to assess the impact of their potential degradation/formation on the estimation of drug consumption based on the wastewater analysis. Additionally, the stability of the selected drugs during the collection of the 24-h composite wastewater samples was also assessed as a possible source of error in the estimation of drug abuse.

2. Experimental

2.1. Selection of analytes

The stability experiments encompassed 13 substances that are excreted after consumption of 6 illegal drugs and 2 therapeutic opioids. The target analytes included morphine (MOR), 6-acetyl morphine (6-AM) and morphine-3-β-D glucuronide (MG) as principal heroin-derived substances, while 6-acetyl codeine (6-AC) was selected as a structural analogue of

6-AM. Cocaine (COC) and its main metabolite benzoylecgonine (BE) were selected as the main urinary biomarkers of COC. The amphetamine-type drugs included amphetamine (AMP), 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) and methamphetamine (MAMP), while 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (THC-COOH) and 11-hydroxy-Δ^9-tetrahydrocannabinol (THC-OH) were selected as biomarkers of cannabis consumption. Methadone (MTHD) and its metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) were monitored as representatives of therapeutic drugs used in the treatment of heroin addicts. The list of all investigated compounds is presented in the Electronic supplementary material (Table S1).

2.2. Chemicals and materials

Standard solutions of all target analytes and their deuterated analogues (Table 1) were purchased from Lipomed AG (Switzerland) at concentration of 1 mg/mL and 0.1 mg/mL, respectively. Mixed standard solutions of the analytes and their deuterated analogues were prepared in methanol (MeOH) at the concentration level of 10 µg/mL and 2 µg/mL, respectively, and kept in the dark at -20 °C. MeOH (J.T.Baker, Deventer, the Netherlands) and acetic acid (Fluka, Switzerland) were LC-MS grade. Aqueous ammonia (NH_3) solution (25%), phosphoric acid (H_3PO_4) and acetic acid (CH_3COOH) were also supplied by Fluka (Fluka, Switzerland). Water was purified using Elix-Milli-Q system (Millipore, Bedford, USA).

Oasis HLB (200 mg/6 mL) and Oasis MCX (150 mg/6 mL) cartridges were purchased from Waters (Milford, MA, USA), while Strata NH_2 cartridges (200 mg/3 mL) were delivered by Phenomenex (Torrance, California, USA). HPLC columns, used in this study, were manufactured by Phenomenex. Chromatographic separation of the basic drugs was achieved on Synergy 4µ POLAR-RP 80 Å 150 x 3 mm column, while Kinetex 2.6 µm PFP 100 Å 11 x 2.1 mm was used for the analyses of cannabinoids.

2.3. Experimental setup

Stability of the selected drugs in the sewer system was assessed using a series of laboratory die-away experiments. The experiments were performed at 10 °C and 20 °C, respectively, in order to simulate typical winter and summer in-sewer temperatures. Wastewater samples (2L), collected from the main sewer system of the city of Zagreb at the entrance to WWTP, were spiked with a mixture of all selected drugs at the environmentally relevant concentrations of 200 ng/L of each individual compound, with the exception of cannabinoid compounds, which were spiked at 1 μg/L. Municipal wastewater of the city of Zagreb contains residues of all investigated drugs (Terzic et al., 2010) but some of them are present at very low levels, so that for the purpose of this experiment, additional spiking of original wastewater sample was necessary. The experiment was carried out at the original pH of the wastewater (7.5). In addition, control experiments were performed at pH 2. The samples for the control experiments were prepared by spiking 2 L of wastewater acidified with H_3PO_4 with the same amounts of drugs. After spiking, both sets of wastewater samples were well homogenised by shaking and divided into 7 identical aliquots of 250 mL. The aliquots were placed in the glass bottles (300 mL), and capped with cotton plugs. The die-away experiments were performed in the dark using a thermostated cabinet. The aliquots of the initial samples were processed immediately after spiking, while extraction of other aliquots was performed in the time-intervals of 2, 4, 6, 24, 48 and 72 hours. Surrogate standards were spiked to the filtered samples just prior to the extraction.

A separate experiment was designed to assess the stability of MOR and COD and to explain the mechanisms responsible for the degradation of COC. In this experiment, municipal wastewater of the city of Zagreb (pH 7.5) was spiked with an enhanced concentration of MOR, COD and COC (4 μg/L of each) in order to minimise the potential interfering effects of drug residues already present in the original wastewater (MG: 4.7 ng/L; 6-AM: 3.1 ng/L; 6-AC: 1 ng/L; BE: 125 ng/L), on the results. In this experiment, two different types of control samples were prepared: the sample acidified to pH 2 (H_3PO_4) and the sample poisoned with mercury chloride ($HgCl_2$; 50 mg/L). All prepared samples were well homogenised by shaking, divided into aliquotes of 250 mL and processed in the identical way as described above for the first two experiments.

To evaluate the stability of the investigated compounds during the collection of 24-hour composite wastewater samples, 500 mL of raw wastewater (RW) and secondary effluent (SE) from WWTP of the city of Zagreb were spiked with 500 ng of each analyte. The spiked samples were homogenised and divided in 2 subsamples of 250 mL. One of each paralel sample was then acidified to pH 2 by the addition of H_3PO_4. Acidified and nonacidified samples were further divided in 40 mL aliquots, which were placed in HD polypropylene bottles and placed at the thermostated dark place at 4 °C. In this experiment, the samples were analysed at the beginning and at the end of 24-hour experimental period. All analyses in this experiment were conducted in duplicate.

2.4. Sample preparation and analyses

After selected time-intervals the samples were immediately filtered and prepared for the analyses. The sample preparation and liquid chromatography-tandem mass spectrometry (LC-MS-MS) analyses were performed identically as described for the dissolved phase analyses by Senta et al.(2013).

Briefly, the samples were filtered through glass-fiber filters (Whatman, GF/D). The dissolved fraction was acidified to pH 2 and spiked with 30 ng of individual deuterated surrogate standards. The samples were enriched using preconditioned Oasis MCX cartridges. Before elution of adsorbed analytes, the cartridges were washed with 6 mL of ultrapure water and subsequently dried with N_2 (30 min). The elution of the enriched drugs was performed by 6 mL of MeOH (cannabinoid fraction) followed by 6 mL of 0.5% NH_3 in MeOH (basic drug fraction). Before the analysis, the cannabinoid fraction was additionally cleaned up using Strata NH_2 cartridges. The extracts were evaporated to dryness under N_2 using a TurboVap evaporator (Caliper Life Sciences, Hopkinton, MA, USA) The dry residues of basic drug fraction and cannabinoid fraction were redissolved in 0.5 mL of H_2O/MeOH (8/2; v/v), containing 0.1% of acetic acid and 0.5 mL of H_2O/MeOH (3/7; v/v), respectively, and analysed by LC-MS-MS.

All LC-MS-MS analyses were performed on a Thermo Electron HPLC system, equipped with an autosampler (Surveyor, Thermo Electron, USA) and HPLC pump (MS Pump, Thermo Electron, USA) interfaced to a triple-quadrupole mass spectrometer (Quantum AM, Thermo Electron, USA), equipped with an electrospray ionisation source. The chromatographic separation of basic drugs was achieved on Phenomenex Synergy 4µ POLAR-RP column (Phenomenex, 150 x 3 mm), while Kinetex PFP (Phenomenex, 100 x 2.1 mm) was used for the analyses of cannabionoid compounds.

The analysis of basic drugs was performed in positive ionisation polarity (PI), while cannabinoids were analysed under negative ionisation conditions (NI). The capillary voltage under PI and NI conditions were 3500 V and 3000 V, respectively. For both ionisation modes, the capillary temperature was 350 ^{o}C. The desolvatation (40 arbitrary units) and auxiliary (10 arbitrary units) gas was N_2, while Ar was applied as a collision gas. The collision energy and tube lens offset were optimised for each analyte and surrogate separately. Identification and quantification was performed using two characteristic transitions for the analysed compounds (MRM mode). Quantification of all analytes was performed using corresponding deuterated internal standards.

3. Results and discussion

The stability of fourteen target compounds in municipal wastewater at typical summer (20 ºC) and winter (10 ºC) temperatures is presented in the Fig 1. Since no significant differences were obtained between the control experiments (pH 2) performed at different temperatures, for sake of clarity, the results from the control experiments at 10 ºC were omitted from the Fig 1. The measurements were fitted by die-away curves assuming pseudo-first order kinetics as follows:

$$c = c_0 * e^{-kt} \qquad (I)$$

were c and c_0 represent concentrations at times t and $t_{0,}$ respectively, and k is the degradation rate constant.

Consequently, the die-away half-life ($t_{1/2}$) was calculated from the

$$t_{1/2} = \ln 2/k \qquad \text{(II)}$$

Similar model of degradation kinetics was applied by Plosz et al.. (2013) in their study of cocaine stability. The stability of the individual compounds in our study highly varied depending on the compound type and the applied temperature conditions (Fig 1). It should be pointed out that in all control experiments drug biomarker concentrations were rather stable, indicating only negligible changes during the period of 72 hours in acidified samples. Die-away curves of amphetamine-type drugs (AMP, MAMP, MDMA), cannabinoid compounds (THC-COOH, THC-OH) as well as of MTHD and EDDP were not significantly different from the stability curves for the control experiments (pH 2). In fact, the estimated degradation half-lives of these drug biomarkers were rather long (>> 200 h) at both temperatures examined, which indicated that these compounds could be considered virtually stable in a typical sewer system. This is in a good agreement with the results reported by van Nuijs at al. (2012). In contrast to amphetamines, cannabinoids and therapeutic opioids, significant changes were observed for MG, 6-AM, 6-AC,COC, BE, MOR and COD. The die away curves for MG, 6-AM, 6-AC and COC followed the first order kinetics with pronounced temperature dependence (Table 1). The $t_{1/2}$ of MG, 6-AM, 6-AC and COC at 20 °C was 7 h, 77 h, 58 h and 35 h, respectively, while the corresponding $t_{1/2}$ values at 10 °C were 18 h, 139 h, 87 h and 173 h, respectively. These results indicated a pronounced seasonal variability of the MG, 6-AM, 6-AC and COC stability in the sewer. However, the significance of the seasonal differences in drug stability on the reliability of drug consumption estimates strongly depends on in-sewer hydraulic retention time. In the sewer systems, like the one in the city of Zagreb (Croatia), having relatively short average residence times (4 hours), the seasonal impact on in-sewer losses becomes significant only for MG (33% and 16%, in summer and winter, respectively). Nevertheless, for the systems having retention times longer than 12 h, the seasonal differences would become more prominent for all four compounds.

As opposed to MG, 6-AM, 6-AC and COC, the stability curves of BE, MOR and COD showed an increasing trend indicating that significant transformations of COC to BE, 6-AM and MG to MOR as well as of 6-AC to COD. Obviously, these transformation processes

were faster than the possible degradation of BE, MOR and COD themselves. Analogous behaviour of 6-AM and 6-AC further indicated that the hydrolysis of acetyl group on the 6 position, was probably the common transformation mechanism of these compounds in the wastewater. On the other hand, MG was most probably transformed to MOR by glucuronidase enzymes of the bacteria present in the wastewater (e.g. Ternes 1998). This transformation was efficiently prevented in the control experiment by lowering pH to 2.

3.1. Stability of cocaine biomarkers and their impact on cocaine consumption estimates

Stability of COC and its potential transformation to BE is an important issue in sewage epidemiology since BE, as the main human metabolite of COC, has been most frequently used for the estimation of COC consumption (e.g. Postigo et al., 2010; Terzic et al., 2010; Thomas et al., 2012; van Nuijs et al., 2011; Zuccato et al., 2005, 2008). Several reports from the literature suggested that COC could be partially transformed to BE during the passage through the sewer system (for example Gheorghe et al.. 2008; van Nuijs et al., 2012). The results from our study are in a full agreement with these findings (Fig 1). In order to clarify the mechanisms leading to the degradation of COC, an additional experiment, in which the wastewater sample was spiked with enhanced concentration of COC (4 µg/l) was performed (see Experimental part for the details). To calculate the amount of newly-formed BE, the initial BE concentration was subtracted from the BE concentrations measured for each sampling point. The results of this experiment are presented in Table 2 and Fig S1-A. The die-away curve of COC (Fig S1-A) was rather similar to the corresponding curve obtained in the first experiment (Fig 1). Furthermore, the degradation curves of COC in the non-preserved sample (pH 7.5) and the control sample preserved with mercury chloride to prevent biological activity (pH 7.5; $HgCl_2$) were quite similar (see Fig S1-A) and showed gradual decrease of COC. This suggested that the main mechanism governing the degradation of COC in our experiments was not biodegradation. On the contrary, the concentration of COC in the sample acidified to pH 2 was virtually stable during the whole experiment, which indicated that COC degradation was caused

almost exclusively by chemical hydrolysis. In contrast, recent study by Plosz et al. (2013) reported on a significant biodegradability of COC and BE at 21 °C, both under aerobic and anaerobic conditions. This discrepancy possibly suggests that biodegradability of COC depends on specific conditions in wastewater, including the composition and pre-adaptation of the present microbial consortium, as well as the total heterotrophic biomass. These authors concluded that the biotransformation of COC must be taken into account when estimating the COC consumption based on BE determination, especially during the festival periods, characterised by enhanced relative concentrations of COC. On the other hand, van Nuijs et al.. (2012) suggested that the influence of formation of BE in sewage epidemiology back-calculations was supposed to be low, even for in-sewer residence times longer than 12 hours. Therefore, further research is needed to clarify these issues.

As to the transformation products formed, our experiment suggested that COC was transformed almost exclusively into BE (Table 2). The relative increase in BE concentration, estimated from our experiments at summer conditions and considering BE/COC ratio of 1.2 and the hydraulic wastewater retention time of 2-12 hours, was 1 to 6%. However, in the real wastewater, which is generally characterised by higher BE/COC concentration ratio (3.3±0.2; Terzic et al.. 2010) than in our spiked samples, the impact of COC hydrolysis on the BE levels would be even lower than the one estimated above.

Besides the stability of COC, potential instability of BE might have an important impact on the accuracy of the COC consumption estimates. The die-away curves of BE represent a combined result of its formation and possible further degradation. Nevertheless, the accumulation of BE (Table 2, Fig 1), indicated its relatively high stability in the wastewater for at least 72 hours. Two experiments showed virtually quantitative transformation of COC into BE, while the mass balance analysis of one of the experiments, conducted at 20 °C (Table 2) showed that the increase of BE after 72 hours was 19% lower than the amount of transformed COC. This indicated either simultaneous formation of other COC transformation products or further transformation of BE. Plosz et al.. (2013) showed that BE could be biotransformed in the sewer, however biotransformation was much slower than its in-sewer formation from COC.

3.2. Stability of heroin biomarkers and their impact on heroin consumption estimates

As can be seen in Fig. 1, clear decreasing temporal trends of MG and 6-AM were followed by a concomitant increase in MOR concentration. This suggested that the transformation of MG and 6-AM to MOR was faster than MOR degradation (Fig 1). In fact, additional experiment, which was performed with wastewater sample spiked solely with MOR, showed that MOR itself was relatively stable in the wastewater over the entire investigated time-period of 72 h (Fig S1). However, a detailed mass balance analysis of the experiments (Table 2) showed that the loss of MG and 6-AM was slightly greater than the amount of newly formed MOR. After 72 hours the difference was equivalent to about 14-16% of the theoretically expected concentration of MOR, which might be due to its further biotransformation. However, UPLC-QTOF screening of the extracts by UPLC/Q-TOF MS did not confirm any detectable concentrations of known MOR transformation products (Wick et al.. 2011).

The transformation of both MG and 6-AM was efficiently prevented by the acidification (pH 2) of the wastewater sample, suggesting enzymatic hydrolysis as the main mechanism for both biomarkers.

MG is one of the major heroin metabolites (Baselt, 2008). However, it is generally assumed that such conjugate compounds are readily re-transformed to the parent compounds in the municipal wastewater due to the presence of β-glucuronidase enzymes of the fecal bacteria (e.g. Ternes, 1998). However, kinetic models derived from our experiments showed that, assuming the hydraulic sewer residence time of 2 and 12 h, approximately 63 to 92% of non-transformed MG would remain in the sewer at winter temperature conditions. In summer, these percentages decrease to 30 and 82%, but remain significant. The remaining MG could be interpreted as one of the factors leading to underestimation of heroin if the estimation is based on morphine measurements. Further deconjugation of MG would probably occur during the composite sample collection and sample preparation. Nevertheless, our results suggest that MG should be measured and summed up with the corresponding MOR concentration in order to avoid underestimation of heroin consumption based on MOR measurements.

The alternative way to estimate heroin consumption is based on 6-AM measurements. Unlike MG and MOR, 6-AM is a minor, but exclusive metabolite of heroin. However, the reliability of the heroin estimation strongly depends on 6-AM stability in the wastewater. According to our results, the residual concentration of 6-AM after 2 to12 h in the sewer would be 98 to 91% and 99 to 94% of its initial concentration, assuming summer and winter temperature conditions, respectively. Consequently, the errors in estimations of the heroin consumption using 6-AM can become significant only at higher sewer hydraulic retention times (>12 h) under summer temperature conditions.

3.3. Stability of the drug biomarkers during the collection of 24-h composite samples

Besides the stability in the sewer, sampling over a prolonged period is also a potential source of error. In this study, we examined the stability of drug biomarkers during the collection of the 24-hour composite samples. The experiment was performed at 4 °C, since this is a typical temperature applied during the sample collection in automatic samplers. The results obtained for both raw wastewater (RW) and secondary effluent (SE) are presented in the Fig. 2. Most of the investigated compounds exhibited rather high stability at the applied experimental conditions in both matrices. For most of the compounds the concentration changes after 24 hours were within the error margins of the analytical method and cannot be considered significant. The compound losses for the samples kept at original pH (Fig. 2) were similar to the losses in the control samples kept at pH 2 (Fig. S2). A significant difference between the two sample types was obtained for THC-COOH, which residual percentage was much lower in the control samples (46% at pH 2) than in the non-acidified samples (90% at pH 7.4). This result indicates most probably the enhanced adsorption of THC-COOH at pH 2 as compared to the environmental pH. Namely, according to Khan and Nicell (2012) only 1.3% of THC-COOH is expected to be adsorbed on sewer-borne solids at environmental pH conditions (pH ≈ 7.5), while its adsorption at pH 2 was estimated to be much higher (56.3%).

4. Conclusion

Most of the illicit drug biomarkers examined in this study, including 6 illicit drugs and 2 therapeutic opioids, proved to be rather stable in model experiments, simulating in-sewer degradation. The most unstable biomarkers were MG, 6-AM, 6-AC and COC and the errors associated with their changes should be carefully taken into account when estimating the collective drug consumption. Their degradation followed pseudo-first order kinetics that was much faster at summer (20 °C) than at winter temperatures (10 °C). COC degradation was caused predominately by chemical hydrolysis to BE. However it was estimated that this process would significantly affect the accuracy of BE-based COC consumption estimates only if the in-sewer hydraulic retention time is very long (> 12 h) and/or when COC/BE ratio is unusually high.

The heroin consumption estimates, based on MOR measurements, could be significantly underestimated if MG is not measured and summed-up with MOR measurements. This would be more pronounced at winter temperature conditions due to the much slower transformation of MG to MOR. On the other hand, the heroin consumption estimates based on 6-AM measurements are less prone to the errors due to the in-sewer transformations and could become significant only at very high hydraulic retention times (>12 hours).

Acidification proved to be a good way to stabilise wastewater samples for the analysis of the drug biomarkers, except for THC-COOH, most probably due to the adsorption losses. This finding should be taken into account when selecting the preservation technique for multiresidual analyses of different groups of illicit drugs.

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Figure Captions

Figure 1. Stability of illicit drugs and their metabolites in the wastewater at winter (10 °C) and summer (20 °C) sewer temperature conditions. (COC-cocaine; BE-benzoylecgonine; MOR-morphine; 6-AM-6-acetylmorphine; MG- morphine-3-β-D glucuronide; 6-AC- 6-acetyl codeine; MDMA-3,4-methylenedioxymethamphetamine; AMP-amphetamine; MAMP- methamphetamine; MTHD-methadone; EDDP-2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; THC-COOH-11-nor-9-carboxy-Δ^9-tetrahydrocannabinol; THC-OH-11-hydroxy-Δ^9-tetrahydrocannabinol)

Figure 2. Twenty-four-hour stability of different types of urinary biomarkers in the raw wastewater (RW) and secondary effluent (SE) at 4 °C. (COC-cocaine; BE-benzoylecgonine; MOR-morphine; 6-AM-6-acetylmorphine; MG- morphine-3-β-D glucuronide; MDMA-3,4-methylenedioxymethamphetamine; AMP-amphetamine; MAMP-methamphetamine; MTHD-methadone; EDDP-2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; THC-COOH-11-nor-9-carboxy-Δ^9-tetrahydrocannabinol; THC-OH-11-hydroxy-Δ^9-tetrahydrocannabinol; COD-codeine)

Table 1
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Table 1. Kinetic parameters determined for degradable compounds at two different temperature conditions.

	10 °C; pH 7.5				20 °C; pH 7.5			
	equation	r^2	k (h^{-1})	$t_{1/2}$	equation	r^2	k (h^{-1})	$t_{1/2}$
COC	$y = e^{-0.004x}$	0.85010	0.004	173	$y = e^{-0.02x}$	0.9897	0.020	35
6-AM	$y = e^{-0.005x}$	0.8695	0.005	139	$y = e^{-0.008x}$	0.9540	0.008	87
MG	$y = e^{-0.039x}$	0.9899	0.039	18	$y = e^{-0.1x}$	0.9335	0.100	7
6-AC	$y = e^{-0.008x}$	0.9587	0.008	87	$y = e^{-0.012x}$	0.9880	0.012	58

COC-cocaine; 6-AM-6-acetyl morphine; MG- morphine-3-β-D glucuronide; 6-AC-6-acetyl codeine

Table 2
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Table 2. Mass balance of cocaine (COC), benzoylecgonine (BE), morphine-3-β-D glucuronide (MG), 6-acetyl morphine (6-AM) and morphine (MOR) at the end of the performed 72-hour die-away experiments.

	Δc_{COC} (nmol/L)	Δc_{BE} (nmol/L)	$\Delta c_{MG} + \Delta c_{6\text{-}AM}$ (nmol/L)	Δc_{MOR} (nmol/L)
10 °C, pH 7.5; 0.2 µg/L*	-0,18	+0.18	-0.51	+0.28
20 °C, pH 7.5; 0.2 µg/L*	-0,62	+0.50	-0.70	+0.48
20 °C, pH 7.5; 4 µg/L*	-7.74	+7.80	NA	NA
20 °C, pH 7.5; $HgCl_2$, 4 µg/L*	-7.05	+6.85	NA	NA
20 °C, pH 2; 4 µg/L*	+0.09	0.03	NA	NA

NA-not applicable; * spiking level (note that a real initial concentration of individual compounds was a sum of the spiked concentration and the concentration of each biomarker already present in the wastewater sample); Δc represents the difference between the final and initial concentration (e.g. $\Delta c_{MOR} = c(MOR_{t72h}) - c(MOR_{t0})$)

Figure 1

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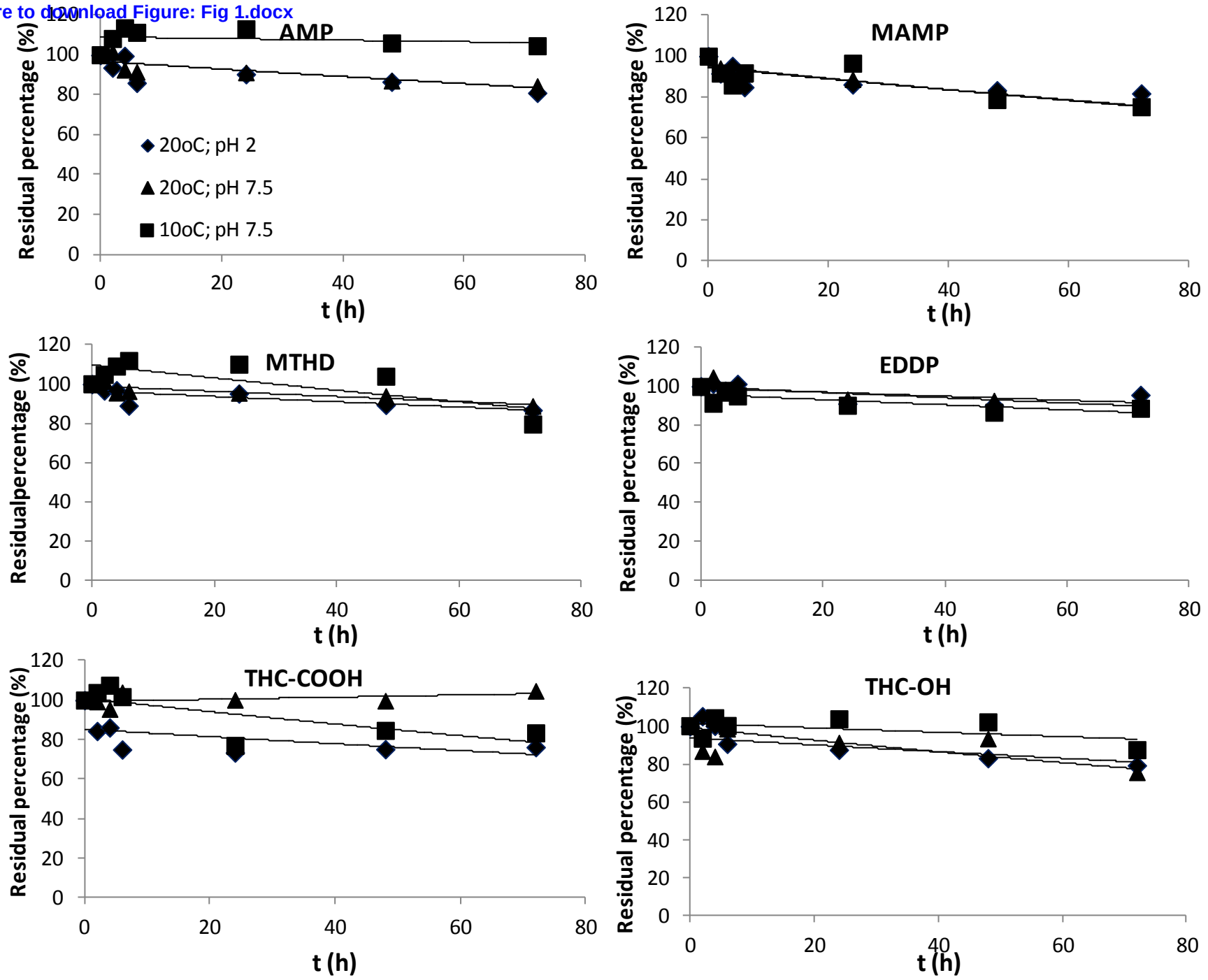
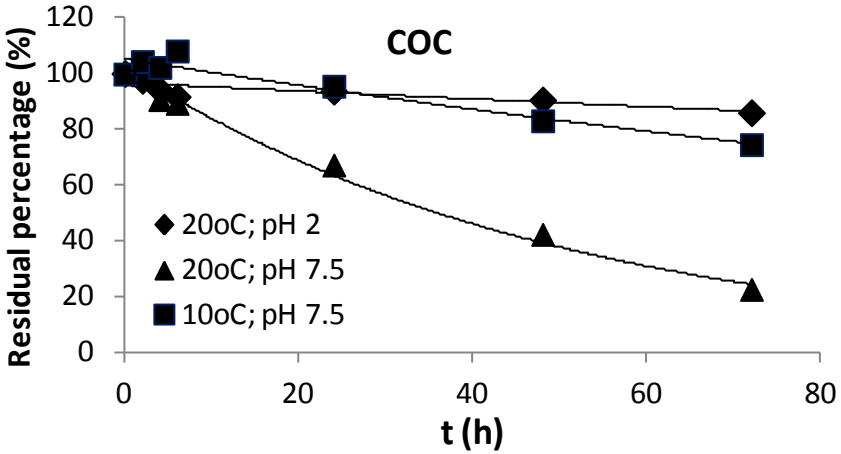
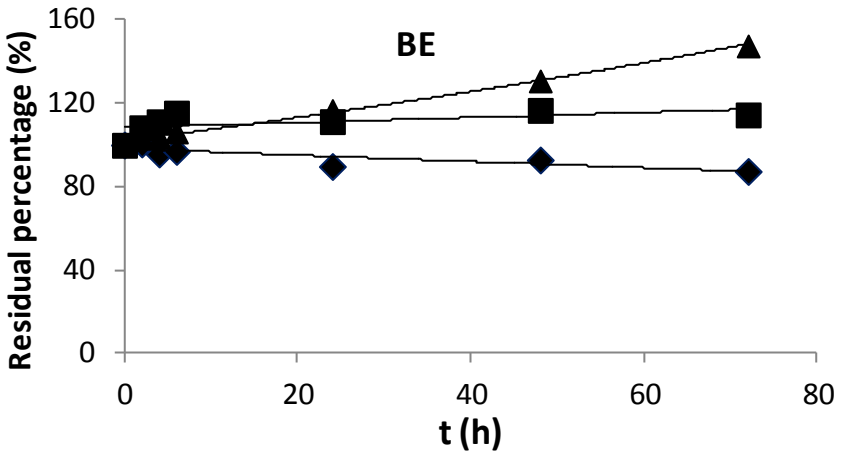
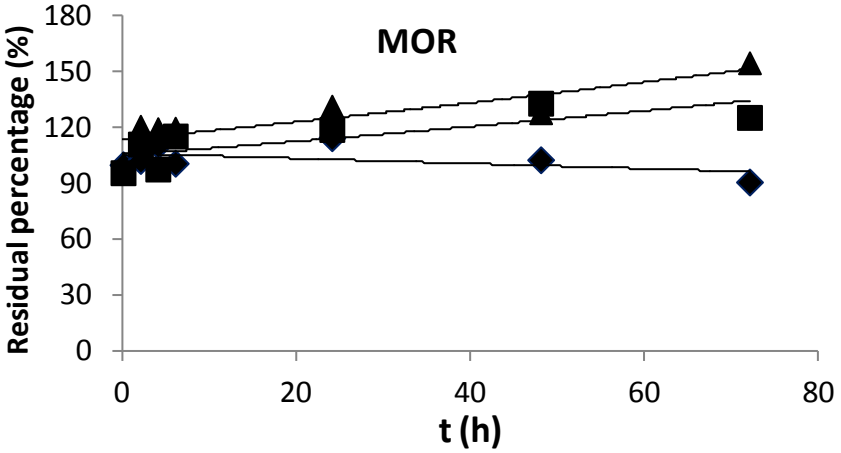
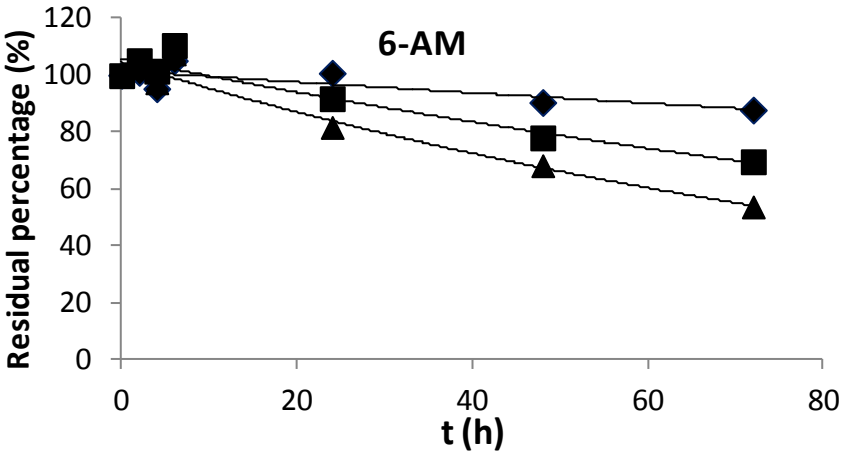
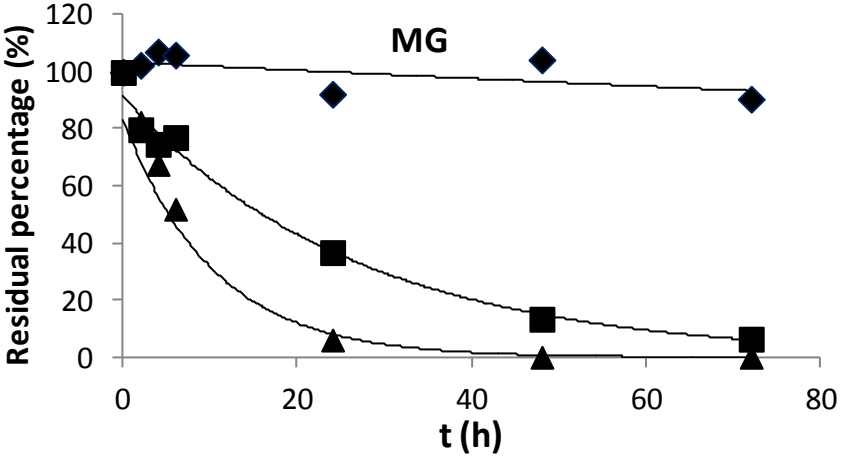


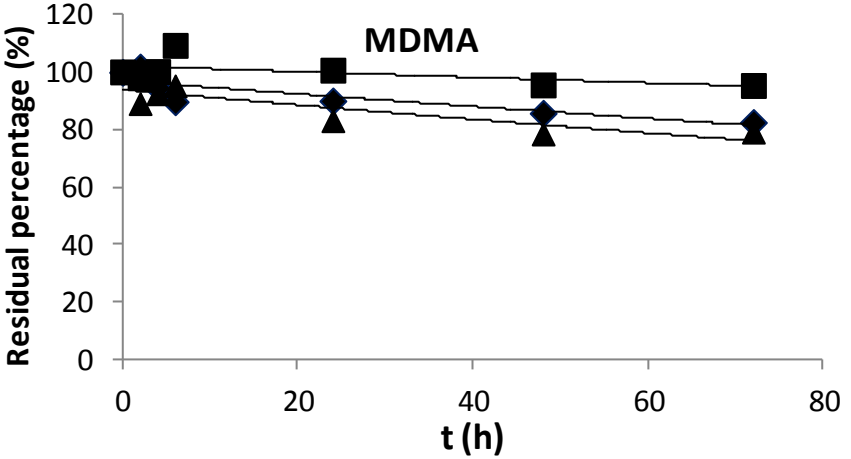

COC
Residual percentage (%)
t (h)
20oC; pH 2
20oC; pH 7.5
10oC; pH 7.5


BE
Residual percentage (%)
t (h)


MOR
Residual percentage (%)
t (h)


6-AM
Residual percentage (%)
t (h)


MG
Residual percentage (%)
t (h)


MDMA
Residual percentage (%)
t (h)

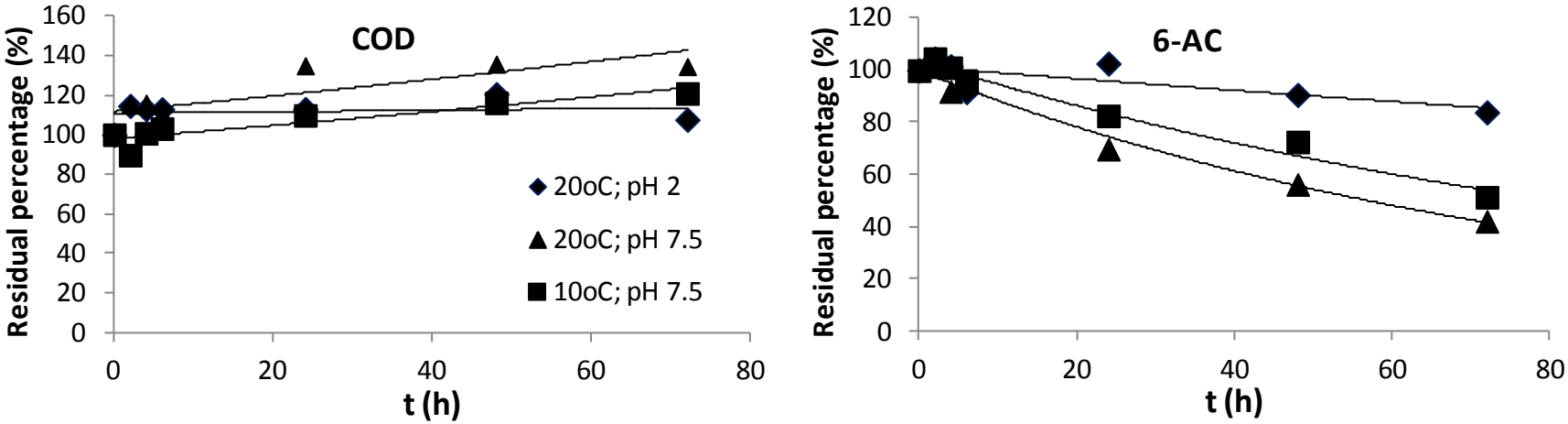

COD
Residual percentage (%)
t (h)
20oC; pH 2
20oC; pH 7.5
10oC; pH 7.5
6-AC
Residual percentage (%)
t (h)

Fig. 1

Figure 2
Click here to download Figure: Fig 2.xlsx

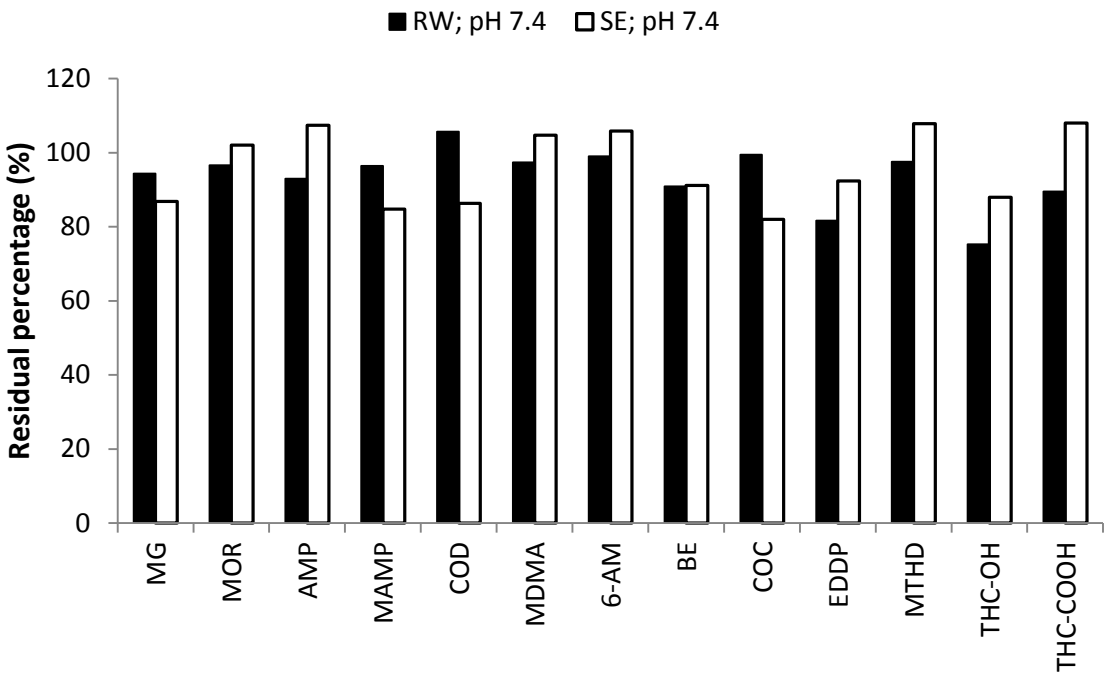


Figure 2

Supplementary Material

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