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Research Paper

Effects of sewer biofilm on the degradation of drugs in sewage: A microcosm study

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ABSTRACT

A thorough understanding of the in-sewer stability of chemical biomarkers is critical in applying wastewater-based surveillance of community drug use. In this study, we examined the effects of sewer biofilm on the degradation of commonly abused drugs, namely, morphine, fentanyl, cocaine, and amphetamine, in wastewater using 48-h batch degradation tests. The experiments were designed to distinguish among abiotic, biochemical, and physical degradation processes, and used mature biofilm obtained from an actual sewer line. Parallel microcosm tests were conducted using wastewater with and without suspended biofilm. Results indicate that first order kinetics describe the degradation of the drugs in both wastewater and wastewater-biofilm microcosms. Amphetamine was most stable in all microcosms, with a maximum removal of only 34% after 48 h. Abiotic chemical transformation played a major role in the degradation of morphine (k_{ab} = 0.018 h^{-1}), fentanyl (k_{ab} = 0.022 h^{-1}) and cocaine (k_{ab} = 0.049 h^{-1}) in wastewater. Fentanyl removal from wastewater was also influenced by the presence of biofilm (k_f = 0.015 h^{-1}). This study is the first to report on the effect of sewer biofilm on fentanyl degradation, and highlights the need to account for in-sewer drug stability in wastewater-based drug use estimation, particularly for chemicals with high affinity for organics.

1. Introduction

Wastewater-based epidemiology (WBE) is an increasingly popular approach for community drug use surveillance where the concentrations of drugs of interest are measured in sewage, and the measurements are used to back calculate community drug consumption. This consumption estimation method relies on a solid understanding of the metabolic and in-sewer transformation kinetics of drugs (Castiglioni et al., 2013). Uncertainties in WBE model estimates are due to several components, including laboratory methods for analyte extraction and detection, estimation of sewershed population, and drug stability in sewers and during wastewater transport and storage. Analytical methods for drug detection have been in development for over a decade and are now well established (European Monitoring Centre for Drugs and Drug Addiction, 2016; Wang et al., 2020). Recent advances in stochastic WBE modeling focus on improving estimates of real time mobile populations (ORourke and Subedi, 2020; Thomas et al., 2017) and sewage residence times (Li et al., 2019). However, the need for a better understanding of in-sewer drug stability has been underscored (McCall et al., 2016a). Most available WBE mass load estimation models account for the metabolic pathways of drugs in humans but data on in-sewer drug kinetics are limited (McCall et al., 2016a).

For many drugs of abuse, the degradation and removal rates in sewage have been studied for wastewater treatment plant (WWTP) settings; in contrast, there are few studies of in-sewer drug degradation (McCall et al., 2016a). Furthermore, the microbial community structure in sewage can significantly differ between sewers and WWTPs (LaMartina et al., 2021), which may have implications on drug transformation. Previous research has evaluated the degradation of select drugs in rising mains (Thai et al., 2014), which are generally anaerobic. There are comparatively fewer studies for gravity sewers, which can be characterized by an aerobic zone at the top liquid layer, and increasingly anaerobic zones at the bottom layers. In addition, the majority of studies have focused on degradation in the bulk liquid phase, but there is a need to evaluate the potential effects of sewer biofilms on the transformation of drugs in wastewater (Thai et al., 2014; Ramin et al., 2017; Gao et al.,

Abbreviations: AMP, Amphetamines; COC, Cocaine; FEN, Fentanyl; MOR, Morphine; MTD, Methadone; SPE, Solid-phase extraction; WBE, Wastewater-based Epidemiology; WWTP, Wastewater Treatment Plant.

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2017; McCall et al., 2016b).

The present study examined the effects of sewer biofilm on the degradation of priority drugs of abuse in sewage using batch microcosm experiments consisting of wastewater and sewer biofilm. The target drugs were selected on the basis of their frequency of detection in sewage and their reported high human consumption (European Monitoring Centre for Drugs and Drug Addiction, 2016). These analytes include two opioids: morphine (MOR) and fentanyl (FEN); and two stimulants: cocaine (COC) and amphetamine (AMP). The microcosm experiments were set-up to mimic aerobic degradation and differentiate among abiotic, biochemical, and sorption processes. The findings of this study contribute to the limited literature on in-sewer drug stability and help increase confidence in WBE model estimates.

2. Experimental

2.1. Chemicals and reagents

High purity reagent chemicals (HPLC-grade methanol, formic acid, ammonium hydroxide) were purchased from Sigma Aldrich (St. Louis, MO) while solutions of isotopically-labeled drug analogs (100 ug/mL in methanol or acetonitrile) were sourced from Cerilliant. Mixed analyte standard solutions were prepared in ultrapure water on the day of use in the experiments. Ultrapure water was prepared on-site in our laboratory (Barnstead E-pure, Thermo Scientific) as needed.

2.2. Drugs of interest

The structure and chemical properties of the target drug analytes are summarized in Table S1, Supporting information (SI). Morphine is a prescription medication for severe pain. Fentanyl is a highly potent opioid analgesic that is also the leading cause of drug-related overdose mortality in the United States (National Institute on Drug Abuse). Cocaine is a commonly abused recreational drug derived from the coca plant while amphetamine is used in treating attention deficit hyperactivity disorder. These drugs are highly soluble in water, except for fentanyl which has high affinity for organics as indicated by its high octanol-water coefficient. To counter the effects of background concentrations in wastewater, only the isotopically-labeled analogs of the target drugs were used as chemical spikes in the microcosm experiments.

2.3. Biofilm and wastewater sampling

Sewer biofilm was collected from the gravity-flow influent intake line of a wastewater treatment facility servicing a generally residential rural town with a population of 40,000. Using a long-handle metal scraper, biofilm was scraped from the inner sewer wall about 0.1–0.5 m below the wastewater surface. The wet biofilm was transported in plastic containers on ice and suspended in batch reactors within 3 h of sampling. During biofilm sampling, 20 L of influent wastewater were also collected at the influent line where the biofilm was taken from for use in setting up the microcosm experiments. In the laboratory, the wastewater was equilibrated to room temperature, shaken vigorously to ensure a homogenous mixture, and filtered (1.0 μm GFC A/E; VWR) to remove large particles. After filtration, 10 L were separated for autoclaving to sterilize the wastewater.

2.4. Microcosm set-up

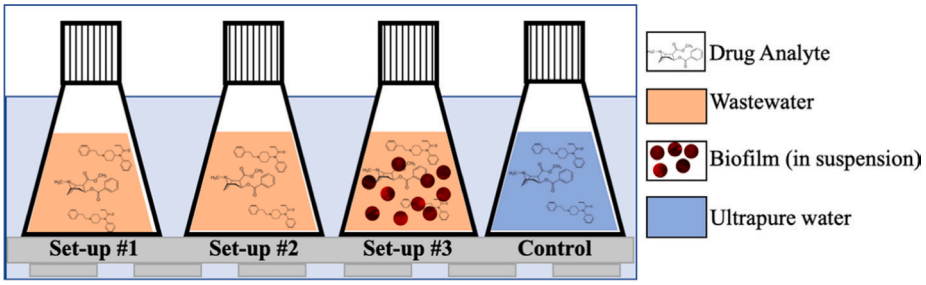
The experimental design for the degradation tests was adapted from a previous method (McCall et al., 2016b) with modifications to accommodate limitations in lab equipment and field sampling logistics (sewer sampling at WWTP). The microcosms were set-up to capture relevant degradation processes, namely: hydrolysis, abiotic chemical degradation in bulk wastewater (chemical transformation in the absence of biological influence), biochemical degradation, and sorption (effect of sewer biofilm). Parallel batch degradation experiments were conducted as follows (Fig. 1): Set-up #1: use of filtered and autoclaved raw wastewater as matrix to study abiotic chemical degradation, Set-up #2: use of filtered raw wastewater without sterilization to study biochemical degradation without the influence of suspended particulates, and Set-up #3: use of filtered raw wastewater plus sewer biofilm to study combined physical (e.g., sorption) and biochemical processes. Each set-up comprised of 16 replicate samples at the beginning of the experiments. Each replicate consisted of a 100-mL wastewater sample placed in a 300-mL glass bottle, covered with air-permeable seals, and shaken continuously in a shaker bath at 70 RPM at 25 °C throughout the experiment. The bulk biofilm from the WWTP was weighed, homogenized, and divided into 16 equal parts (5 g wet mass/L) into each bottle of Set-up #3. A negative control (ultrapure water) was included for comparison purposes as well as to account for pure water hydrolysis. At the start of the experiment, each bottle was spiked with a mixture of labeled drug standards of MOR-D_3, FEN-D_5, COC-D_3, and AMP-D_6 to an in-solution concentration of 200, 100, 500, and 1000 ng/L, respectively (C_0). These initial concentrations were based on measured levels in environmental samples reported in our previous work (Pagsuyoin et al., 2019) and in other studies (Centazzo et al., 2019). Background drug concentrations in the microcosms were not monitored, though our separate WBE study indicated that the average background drug concentrations in the influent during the week of biofilm sampling were as follows: 146 ng/L, 5.2 ng/L, 434 ng/L, and 525 ng/L for morphine, fentanyl, cocaine, and amphetamine, respectively. To evaluate drug degradation, the concentrations of the labeled drugs in solution were measured over seven-time intervals (1, 2, 4, 8, 12, 24 and 48 h) by removing 2 replicate microcosm bottles at each time interval (i.e., duplicate sampling), and performing solid phase extraction and chemical quantification via LC-MS/MS analysis. As this research aimed to mimic sewers conditions, photolysis was excluded from the experiments by conducting the experiments under dark conditions (no light).

2.5. Analyte extraction and quantification

The extraction and quantification method for labeled drug analytes was adapted from our previous work (Pagsuyoin et al., 2019) with modification. Briefly, wastewater samples were filtered (47 mm, 1.0 μm GFC type A/E; VWR) and passed through solid phase extraction in a 24-position vacuum manifold (15 in. Hg) using Phenomenex Strata-X (200 mg/6 mL) cartridges. Each cartridge was pre-conditioned with 10 mL methanol and equilibrated with 10 mL ultrapure water prior to sample loading (2 mL/min), washed with 10 mL 5% methanol in water, sequentially eluted with solvents (5 mL methanol with 2% formic acid; 2 mL ethyl acetate: isopropanol (85:15), and 3 mL methanol with 5% ammonium hydroxide), dried to dryness in a vacuum oven, and reconstituted in 5 mL methanol:water (1:9, v/v). Drug analyte concentrations were quantified using a Shimadzu ultra-fast liquid chromatograph interfaced to a triple-quadrupole mass spectrometer (AB SCIEX API 4000) under positive electrospray ionization. Chromatographic separation was achieved in a C18 column (Kinetex® 2.6 μm, 100 mm × 4.6 mm) using a gradient program with an aqueous mobile phase of 0.1% formic acid in water and organic mobile phase of 0.1% formic acid in methanol (see Table S2, SI). Sample injection volume was 10 μL. Quantitation was performed in the instrument software *Analyst* (ver. 1.7, AB SCIEX). Method detection limits and recoveries are summarized in Table S3 (SI).

2.6. Modeling degradation kinetics

We followed a NAFTA standard procedure for screening kinetic models that best describe the degradation of chemicals in environmental media (Bohaty et al., 2015). This preliminary screening step indicated that a first-order model best fits our degradation data, which is



Set-up #1: Filtered and autoclaved raw wastewater to evaluate abiotic chemical degradation
Set-up #2: Filtered wastewater to evaluate biochemical degradation
Set-up #3: Filtered wastewater and sewer biofilm to study combined physical and biochemical degradation.
Negative Control: ultrapure water as matrix to account for hydrolysis

Fig. 1. Microcosm Set-up for Batch Degradation Studies. 100 mL of aqueous samples in glass bottles covered with air-permeable seals were spiked with labeled drug analytes and placed in a shaker bath to observe drug degradation over 48 h.

consistent with prior findings on drug degradation in sewage (McCall et al., 2016b; Bisceglia and Lippa, 2014; Devault et al., 2017; Plósz et al., 2013; Senta et al., 2014; van Nuijs et al., 2012). Based on the assumption that the effect of each degradation process on overall degradation is additive, the first-order degradation equation for drug *i* can be expressed as:

$$C_i(t) = C_0 \quad exp^{-(k_h+k_{ab}+k_b+k_f)t} \tag{1}$$

where $C_i(t)$ is the concentration of the drug analyte at time *t*, C_0 is the initial concentration at time = 0, k_h is the hydrolysis constant in DI water, k_{ab} is the abiotic chemical degradation constant, k_b is the wastewater biodegradation rate constant, and k_f is the biofilm rate constant. k_h can be determined from the slope of the plot of ln (C_i/C_o) vs *t* in the Control set-up. The slope of the ln (C_i/C_o) vs *t* plot for Set-up #1 yields combined k_h and k_{ab}; k_{ab} can be calculated by difference. The slope of the ln (C_i/C_o) vs *t* plot for Set-up #2 will yield combined k_h, k_{ab}, and k_b; k_b can be calculated by difference. The slope of the ln (C_i/C_o) vs *t* plot for Set-up #3 will yield combined k_h, k_{ab}, k_b and k_f; k_f can be subsequently calculated by difference.

2.7. Microbial community characterization

The microbial consortia in the microcosms were characterized for taxa and relative abundance using molecular techniques at the onset of the experiment (0 h) and after the biodegradation tests (48 h). Although this is a relatively short period of time to observe major shifts in microbial composition, environmental bacteria have been shown to display rapid succession within 48 h (Datta et al., 2016). Our intention was to capture acute taxonomic changes in response to the introduction of drugs and in relation to the time period for biodegradation. The entire content of each sampled microcosm bottle was filtered through a 0.45 μm sterile mixed cellulose ester membrane. DNA in the filter residue was extracted using a NucleoSpin DNA kit (Macherey-Nagel, PA) following manufacturer instructions. Gene amplification of the 16S rRNA gene, as well as quality assessment, sequence library preparation, and sequencing were performed at the UMass Lowell Core Laboratory facilities. The 16S V3–V4 region was amplified using the primers 5'-CCTACGGGNGGCWGCAG-3' and 5'-GACTACHVGGGTATCTAATCC-3' for paired-end sequencing of 150 bp reads (Illumina iSeq). Raw reads were trimmed for adapters and quality using default settings in Cutadapt (Martin, 2011), paired reads were joined together, and processed in QIIME 2 for denoising and taxonomic assignment using the classify-sklearn tool and the pre-compiled SILVA database (version 138-99) (Bolyen et al., 2019). Microbial community compositions were compared among samples using proportional counts of taxa at the phylum and family levels in R (R Core Team, 2018), and using diversity metrics as calculated with the vegan package (Oksanen et al., 2020). Low abundance taxa that were found below a proportional threshold of 0.01 (1% across all samples) were combined into one "Other" category for visualization purposes.

3. Results and discussion

3.1. Wastewater sampling conditions and sewage characteristics

Our wastewater samples were obtained from a small wastewater treatment facility that has been operational for over four decades and receives sewage from a largely school-age population demographic. The influent sewer line where the biofilm was taken is normally never disturbed and was only accessed to remove the biofilm during our sampling day. Facility records indicate that the average daily influent suspended solids and carbonaceous biological oxygen demand are approximately 240 mg/L and 160 mg/L, respectively, which are typical values for domestic wastewater (Butler et al., 1995). Our sewer sampling occurred in the fall season when influent flow characteristics approximate these averages. The influent pH and temperature were 7.5 and 15.5 °C, respectively. Influent alkalinity, NH_3-N, and total Kjeldahl N were 169 mg/L, 27.5 mg/L and 33.8 mg/L, respectively. Furthermore, sampling was conducted within our 2-year surveillance of drugs in sewage in the same facility, where we also detected all target drugs in the present study at 100% frequency in the influent (Pagsuyoin et al., 2019).

3.2. Effects of degradation processes

3.2.1. Hydrolysis

The plot of the relative aqueous drug concentrations in the microcosm systems indicate distinct degradation trends for the four target drugs (Fig. 2). Hydrolysis (in ultrapure water) played a minimal role in drug degradation; after 48 h, aqueous drug concentrations were reduced from 2.8% (for amphetamine) to 12.7% (for fentanyl). Except for cocaine, the acid dissociation constants (pKa) of the drugs are higher than neutral pH (Table S1) so that hydrolysis effects on these drugs are expected to be minimal. The pKa of cocaine (7.43, (National Institutes of Health Department of Health and Human Services, 2021)) is close to neutral pH such that hydrolysis may occur but its effect on degradation was not found to be significant ($p = 0.455$). The high stability of cocaine in ultrapure water (pH 6.4) (McCall et al., 2016b) and milli-Q water (pH 5.7) (González-Mariño et al., 2012) has been previously reported, though hydrolysis in tap water (pH 7.6) has been noted (McCall et al., 2016b) due to contributions of other chemicals present in the matrix (Boleda et al., 2011).

3.2.2. Abiotic degradation

Abiotic chemical degradation played an important role in the

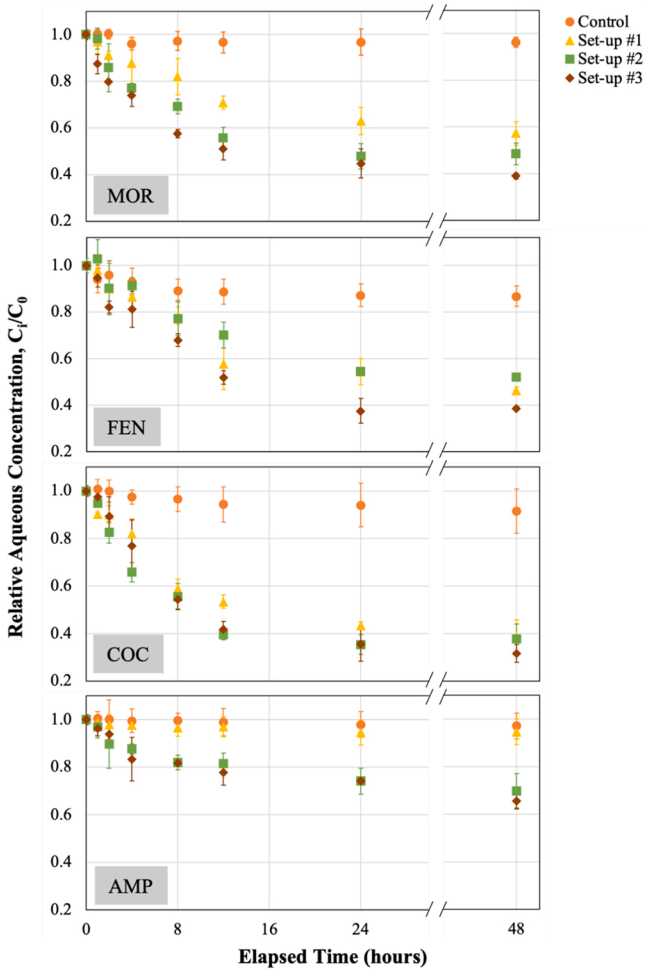


Fig. 2. Plot of remaining drug concentrations relative to initial concentration (C/C_0) over time in the aqueous phase in each microcosm set-up. Markers and error bars represent average and min-max range, respectively, of replicates.

attenuation of cocaine, morphine, and fentanyl in wastewater. In particular, 60.9% abiotic reduction of cocaine in the aqueous phase was observed after 48 h, increasing only up to 62.1% with biochemical degradation, and 68.3% when biofilm contribution was included (Fig. 2). For morphine, 42.8% abiotic reduction was observed after 48 h, and increasing to 60.9% when combined biodegradation and biofilm contribution were included. For fentanyl, abiotic reduction was the main degradation process, where 45.8% of fentanyl was removed after 24 h, rising to 53.1% after 48 h.

3.2.3. Biochemical degradation

Morphine showed significant degradation in wastewater even in the absence of biofilm. Likewise, the minimal degradation of amphetamine, which is relatively stable in all wastewater microcosms with and without biofilm, is mostly due to biochemical transformation. Generally, morphine and amphetamine were rapidly biochemically degraded in the first 12–24 h and slowed thereafter. For morphine, 44.3% was removed via biochemical degradation in the first 12 h, and 52.0% after 24 h and marginally thereafter. For amphetamine, 18.6% was removed via biochemical degradation in the first 12 h, rising only to 25.9% after 24 h, and 30.1% after 48 h.

3.2.4. Biofilm contribution

Sewer biofilms are a complex microbiome made up mostly of heterotrophic bacteria (Lemmer et al., 1994) that may contribute substantially to in-sewer drug transformation, mainly via sorption and biotic transformation (Ramin et al., 2017) (i.e., active biomass). In this study, transformation processes due to the biofilm are lumped together in the k_h term in Eq. (1). The effect of biofilm addition was greatest in fentanyl where up to 61.2% removal was achieved after 48 h in the wastewater-biofilm microcosm compared to 47.5% removal in the wastewater-only microcosm (an increase of 13.7 ± 1.9%). The effect of biofilm addition was lesser in morphine (9.3 ± 15.7%), cocaine (6.2 ± 7.3%), and amphetamine (4.3 ± 8.1%), indicating that the presence of biofilm in the microcosms mostly likely increased drug degradation via sorption. These observed differences in removal rates is expected given the high affinity of fentanyl to organics, as indicated by its significantly higher octanol-water coefficient (Table S1) compared to the other target drugs in this study.

3.3. Modeling degradation kinetics

The fitted first order degradation models for the four analyte drugs in the microcosms are shown in Fig. 3. Pairwise covariance analysis indicated statistically significant differences ($p \leq 0.001$) among the regression slopes of the different microcosms. The corresponding calculated first-order degradation rate constants are summarized in Table 1. These results reflect the relative importance of the different degradation processes to each drug analyte (e.g., negligible effect of biofilm addition to overall cocaine degradation).

3.3.1. Morphine

Hydrolysis and biofilm sorption have minimal influence on the removal of morphine from the aqueous wastewater phase, as indicated by their low first-order rate constants (Table 1) and corresponding half-lives of 483 and 696 h, respectively. In contrast, abiotic and biotic degradation are important, accounting for 55% and 37% of the overall removal observed after 48 h in the aqueous phase. In the absence of biofilm, the calculated half-life of morphine in filtered wastewater was 22 h. Senta et al. (2014) determined lower half-lives (7–18 h) for morphine conjugates in raw wastewater over a range of temperatures lower than the one used in this study (25 °C). However, their experimental set-up involved unfiltered wastewater so that sorption to suspended particulate matter may have been a factor in their observed faster degradation in these lower temperature environments.

3.3.2. Fentanyl

Compared to morphine, fentanyl is prescribed and consumed at much lower doses (Mitchell Jr, 2017). Thus, while it is frequently abused and is the leading cause of overdose deaths (National Institute on Drug Abuse), it has been detected less frequently and at much lower concentrations in wastewater (ORourke and Subedi, 2020; Centazzo et al., 2019). Fentanyl is stable in DI water (Östman et al., 2014) during short-term storage, while biodegradation was reported in unfiltered raw wastewater after 72-h storage at 19 °C (Baker and Kasprzyk-Hordern, 2011a). To our knowledge, the present study is the first to report on the effect of sewer biofilm on the stability of fentanyl in wastewater. Abiotic degradation and biofilm sorption were the primary contributors to fentanyl degradation in wastewater, accounting for 55% and 36%, respectively, of the overall fentanyl removal. The rate of abiotic degradation was greater in fentanyl ($k_{ab} = 0.0223\ h^{-1}$) than in morphine ($k_{ab} = 0.0179\ h^{-1}$) but lower than in cocaine ($k_{ab} = 0.0490\ h^{-1}$).

3.3.3. Cocaine

Cocaine is a frequently detected recreational drug in wastewater that has been measured at relatively high concentrations (European Monitoring Centre for Drugs and Drug Addiction, 2016). It has low stability in aqueous media and rapidly decomposes into its main metabolite, benzoylecgonine, which in contrast exhibits high stability in wastewater matrices (European Monitoring Centre for Drugs and Drug Addiction, 2016). In the present study, cocaine stability was mostly affected by abiotic and biochemical degradation, accounting for 65% and 28%,

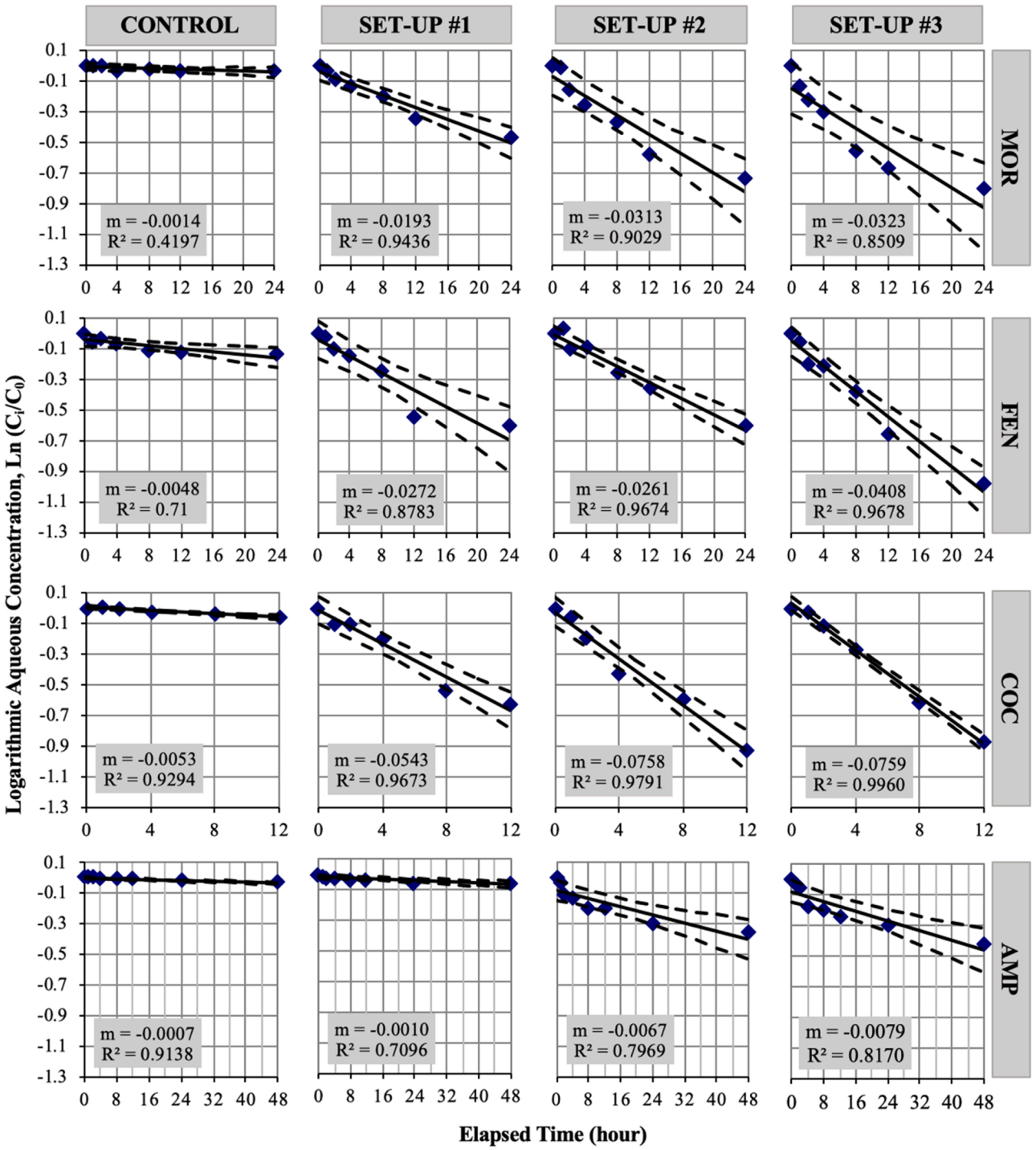


Fig. 3. Fitted first order models to degradation data from the microcosm studies. Dots represent relative concentration of drug remaining in aqueous phase (average of replicate samples), solid lines represent model fits, and dashed lines represent 95% CI (upper and lower bound) for the fitted models. The slope (m) and goodness of fit (R^2) of the fitted models are also indicated.

Table 1
Summary of first order rate constants for different degradation processes.

Drug analyte (CODE)	First-order degradation rate constant (h^{-1})			
	Hydrolysis (k_h)	Abiotic chemical degradation (k_{ab})	Biodegradation (k_b)	Biofilm (k_f)
Morphine (MOR)	0.0014 ± 0.0008	0.0179 ± 0.0022	0.0119 ± 0.0051	0.0010 ± 0.0076
Fentanyl (FEN)	0.0048 ± 0.0014	0.0223 ± 0.0047	-0.0011 ± 0.0050	0.0147 ± 0.0040
Cocaine (COC)	0.0053 ± 0.0007	0.0490 ± 0.0050	0.0215 ± 0.0075	0.0002 ± 0.0060
Amphetamine (AMP)	0.0007 ± 0.0001	0.0003 ± 0.0003	0.0057 ± 0.0014	0.0012 ± 0.0021

respectively, of the overall cocaine removal in the aqueous phase. Correspondingly, the half-lives for abiotic and biochemical degradation were 14 and 32 h, respectively. Our findings are consistent with previous results that indicate significant contributions of abiotic and biochemical transformations to cocaine stability in sewage (Ramin et al., 2017).

3.3.4. Amphetamine

Of the four drugs in this study, amphetamine was the most resistant to degradation regardless of microcosm set-up. After 48 h, relatively minimal amphetamine was degraded via abiotic biochemical removal (5.4%, set-up #1 in Fig. 2) or combined sorption and biochemical transformation (34.4%, set-up #3). This minimal removal was mainly due to biodegradation in wastewater, as indicated by the relatively higher biodegradation rate constant ($k_b = 0.0057\ h^{-1}$) for amphetamine (compared to other degradation processes, Table 2). These observations are consistent with prior findings of high amphetamine stability in various wastewater matrices (van Nuijs et al., 2012).

3.4. Drug degradation half-lives

The calculated half-lives of the four drugs in the different microcosms are summarized in Table 2. These half-life values corroborate literature values, where prior data for similar microcosm studies are available. As can be inferred from the range of half-life values ($1–10^2$ h), there is no singular trend for drug stability across all drugs in different wastewater microcosms. Certain degradation processes have more pronounced effects on the stability of one drug than in others. For example, based on half-life values from this study, cocaine will degrade three times faster than fentanyl and eleven times faster than amphetamine in wastewater that has low suspended particulate matter.

Table 2 also highlights the lack of available kinetic data on the in-sewer stability of drugs of abuse, particularly opioids. The stability of cocaine and amphetamine in microcosms mimicking sewer conditions (McCall et al., 2016b) have been previously investigated but less is known about the stability of morphine and fentanyl in wastewater matrices. These two prescription analgesic opioids are commonly abused and are often detected in wastewater systems. Morphine is also a metabolite of codeine, another commonly abused opioid, and has been measured at high concentrations ($1–10^3$ ng/L) in influents. Fentanyl is the leading cause of drug overdose deaths in the United States and its detection in wastewater has of late been increasingly reported (ORourke and Subedi, 2020). The present study contributes new information to the limited literature on the stability of these frequently detected opioids in sewage.

3.5. Microbial community characteristics

Amplicon sequencing of microbial communities indicated that at the phylum level, *Proteobacteria* were the most abundant in all wastewater microcosms with or without sewer biofilm (Fig. 4). Comparisons of microbial communities across degradation experiments revealed a small but general decrease in abundant taxa after 48 h. The community composition of wastewater with (Set-up #1) and without (Set-up # 2) autoclave sterilization was highly similar with a significant positive correlation in phylum abundance changes over the course of the experiment ($p < 0.001$). Nonetheless, these temporal changes in abundance were small, suggesting that the live microbial communities did not significantly change in response to the biochemical degradation of drugs within 48 h. In contrast, the addition of sewer biofilm (Set-up #3)

Table 2
Drug degradation half-lives determined from this study and from previous studies.

Drug analyte	This study		Previous studies		
	Microcosm Set-up	Half-life, $t_{1/2}$ (h)	Half-life, $t_{1/2}$ (h)	Experimental condition (pH; Temp, °C)	Ref.
Morphine* **(MOR)**	CONTROL: hydrolysis in DI water	482.7	–	–	–
	SET-UP #1: abiotic chemical deg.	35.8	–	–	–
	SET-UP #2: biochemical deg.	22.1	–	–	–
	SET-UP #3: biochemical deg. + sorption	21.5	–	–	–
Fentanyl (FEN)	CONTROL: hydrolysis in DI water	143.4	–	–	–
	SET-UP #1: abiotic chemical deg.	25.5	–	–	–
	SET-UP #2: biochemical deg.	26.6	–	–	–
	SET-UP #3: biochemical deg. + sorption	17.0	–	–	–
Cocaine (COC)	CONTROL: hydrolysis in DI water	130.6	866	6.5; 22	(McCall et al., 2016b)
			72	7.5; 19	(Bisceglia and Lippa, 2014)
	SET-UP #1: abiotic chemical deg.	12.8	20	9; 22	(McCall et al., 2016b)
			39[a]	7.5; 23	(Li et al., 2018)
	SET-UP #2: biochemical deg.	9.1	12	7.4; 23	(Bisceglia and Lippa, 2014)
	SET-UP #3: biochemical deg. + sorption	9.1	20–198[c]	8–8.9; 22	(McCall et al., 2016b)
			35–137[c]	7.5; 10–20	(Senta et al., 2014)
			10[b]	7.5; 20	(Thai et al., 2014)
Amphetamine** **(AMP)**	CONTROL: hydrolysis in DI water	1003.4	866	6.5; 22	(McCall et al., 2016b)
	SET-UP #1: abiotic chemical deg.	693.4	151	9; 22	
	SET-UP #2: biochemical deg.	102.8	–	–	–
	SET-UP #3: biochemical deg. + sorption	87.5	43[c]-173[c]	8–8.9; 22	(McCall et al., 2016b)
			55[b]	8.2–8.4; 22	

* Most available literature on morphine degradation kinetics pertain to the conjugated metabolite (e.g., morphine glucuronide with $t_{1/2}$ of 7–18 h in unfiltered wastewater at pH 7.5 and 10–20 °C (Senta et al., 2014)). 24-h stability test for unconjugated morphine in filtered wastewater (pH 7.4, 19 °C) has been reported (as % C_{24}/C_0) by Baker and Kasprzyk-Hordern (2011b), 49 ± 2.4.

** High stability for amphetamine in unfiltered wastewater stored at cold temperatures (4 °C) has been reported (as % C_{24}/C_0) by Xu et al. (2017), 93 ± 0.4 after 48 h, and by Senta et al. (2015), 92.4–94 after 24 h.

[a] Used autoclaved unfiltered wastewater.

[b] Both sewer biofilm and suspended matters in bulk wastewater were included in the kinetics study.

[c] Sewer biofilm was not added to the microcosms but suspended particulate matter in wastewater was included.

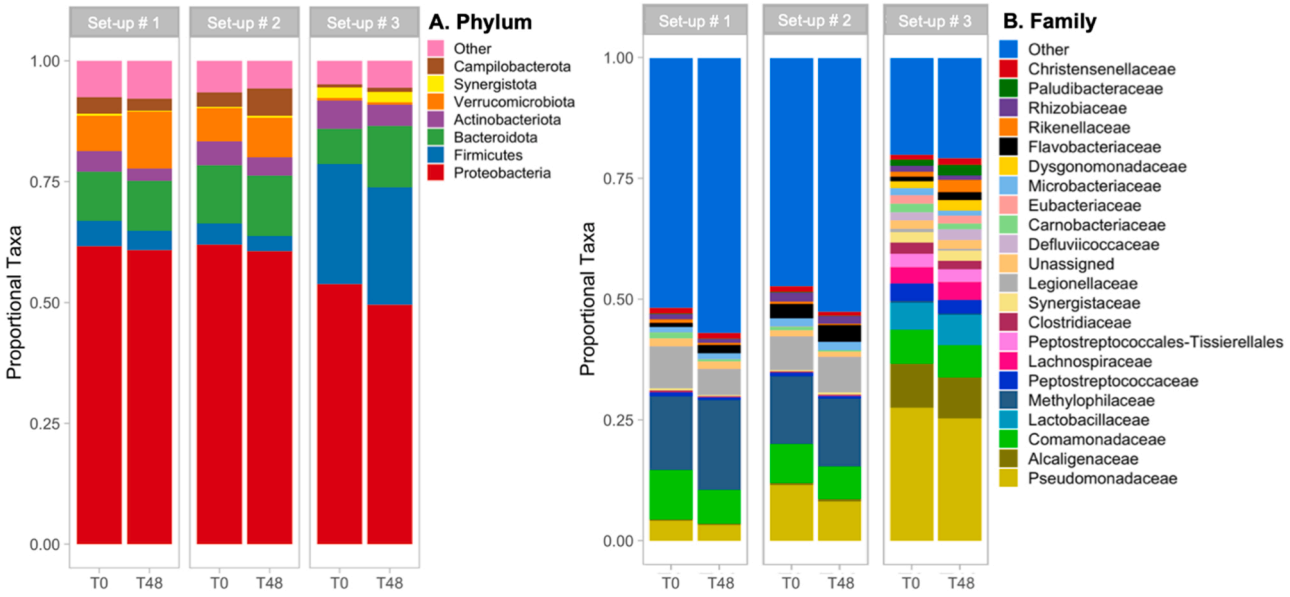


Fig. 4. Stacked bar charts displaying taxonomic composition of microcosms at the start (T0) and end (T48) of the experiments. Microbial communities are represented as proportions of (A) Phyla and (B) Families among the microcosm Set-ups, showing all taxonomic groups represented by at least 1% of their respective communities (all taxa below 1% are grouped together as "Other" for ease of visualization). (For interpretation of the references to color in this figure text, the reader is referred to the web version of this article.).

contributed to slight shifts in taxa; at the phylum level, biofilm led to highly abundant Firmicutes and to a lesser extent Synergistota relative to non-biofilm microcosms, with a decreased proportion of Verrucomicrobiota and Campilobacterota, as well as Proteobacteria (Fig. 4A). Over the course of 48 h, the phyla with the largest proportional changes in Set-up #3 were Bacteroidota (5% increase compared to < 1% increase in Set-up #2), Fusobacteriota (1% increase compared to being absent in Set-up #2), Proteobacteria (4% decrease compared to 1% decrease in Set-up #2), and Actinobaceriota (1% decrease in both Set-up #2 and #3) (Table S4, SI). The phylum-level alpha diversity (as measured by the Inverse Simpson Diversity) was only slightly higher in the biofilm microcosms compared to non-biofilm microcosms (mean Inverse Simpson 2.9 vs 2.5). At the family level however, there were substantial differences in taxonomic composition in the biofilm microcosms, with notably only 25% of taxa found at low proportions (below 1%) compared to 50% of taxa among non-biofilm microcosms (Fig. 4B – "Other" in blue). This is reflected in the lower family-level alpha diversity in biofilms (mean Inverse Simpson 10.7 vs 19.3). Over the course of 48 h, the families with the largest proportional changes in Set-up #3 tended to show opposite direction or no difference in abundance changes in Set-up #2 (Table S5, SI), however, these changes in proportional abundance tended to be small (< 2%).

In wastewater systems, differences in microbial community structure are thought to be modulated by several factors, including wastewater source and environmental/treatment conditions (e.g., temperature, retention time) (Nascimento et al., 2018; Yu and Zhang, 2012). Proteobacteria were found to be dominant in aerobic municipal WWTPs whereas Bacteroidota (formerly Bacteroidetes) were dominant in anaerobic systems (Hu et al., 2012). In a study of in-sewer drug degradation, McCall et al. (2016b) also found Proteobacteria and Firmicutes, and to a much lesser degree Bacteroidetes, Actinobacteria, and Opisthokonta as the dominant phyla in sewer biofilms obtained from different sewer sites; higher biomass diversity was associated with greater biotransformation of drugs in the sewage. The microbial community structure in sewage can differ significantly in sewers and in WWTPs (in both influent and treatment train) (LaMartina et al., 2021), and this can have implications on drug transformations in sewage. In our study, there was greater microbial diversity in the presence of biofilm, but this did not necessarily translate to increased degradation for all drug analytes.

3.6. Implications to WBE modeling

The present study is the first to report on the effect of sewer biofilm on fentanyl degradation, and highlights the need to account for in-sewer drug stability in wastewater-based drug use estimation, particularly for chemicals with high affinity for organics. Lipophilic drug analytes such as fentanyl, methadone, and cannabinoids can sorb onto sewer biofilm and suspended organic particulates in sewage while in transit in sewers, leading to their underestimation in WBE mass load modeling when sorption is neglected.

Our study findings also underscore the varying drug stabilities in sewage and the significance of individual biodegradation processes in in-sewer drug transformation. While wastewater characteristics and sewer conditions vary geographically and temporally (Krithika et al., 2017; Di et al., 2019), our findings provide insights for identifying which commonly used drugs may be underestimated or overestimated in currently available WBE models. In particular, the values of the drug degradation half-life provide context on drug stability in relation to the in-sewer residence times of sewage as it is transported to wastewater treatment facilities. Where drug half-lives are low compared to in-sewer residence times, significant underestimation can occur when only pharmacokinetic (i.e., metabolic) pathways (McCall et al., 2016a) are accounted for in mass load models. When drugs are highly unstable (i.e., short half-life) in sewage, it may be necessary to choose alternative drug biomarkers, for example, benzoylecgonine is a highly stable by-product and biomarker for cocaine. Conversely, higher drug half-life values indicate drug stability and increase confidence in the estimates from existing pharmacokinetics-based WBE models. In most catchments, sewage stays between 30 min and 12 h in sewers, with a typical 6-h average, before reaching WWTPs (Heuett et al., 2015). For context, the WWTP personnel at our sampled facility estimated that the mean sewage residence time within the small sewershed is 3–4 h.

In a majority of WBE studies, wastewater samples are collected at the WWTP facilities (European Monitoring Centre for Drugs and Drug Addiction, 2016). More recently, efforts to rapidly track COVID-19 infections via sewage renewed interest in the feasibility of manhole sampling (Harris-Lovett et al., 2021) for WBE. Manhole sampling increases

the granularity of WBE data and improves WBE model estimates by reducing model uncertainties related to analyte stability and population estimation. Drawing wastewater samples from manholes reduces the in-sewer residence time of the sample, and simplifies WBE modeling since accounting for the effect of in-sewer stability on individual drugs can be reduced or eliminated altogether. However, manhole sampling is a tremendously complex and resource-intensive undertaking, and requires close coordination with many personnel (Harris-Lovett et al., 2021). It is likely that the majority of WBE efforts in the foreseeable future will entail sampling at WWTPs rather than sampling at manholes. Therefore, a better understanding of the stability and complex transformation process of drugs in sewers will help reduce uncertainties in WBE modeling.

3.7. Study limitations and considerations for future work

While our findings provide critical insights on the stability of priority drugs in sewage, this study also has several limitations. Firstly, the degradation experiments were conducted over limited environmental settings (25 °C, natural wastewater pH, aerated). Environmental conditions in actual sewer networks vary over a wide range. For instance, in our sampled wastewater facility, influent wastewater temperature and pH range from 10 °C to 25 °C and from 6.9 to 8.2, respectively, over four seasons. Environmental factors play a critical role in the rate kinetics of biochemical processes in wastewater systems (Gao et al., 2017), and should be carefully considered when building robust kinetic models of in-sewer drug degradation. Secondly, this study examined sorption effects for biofilm in suspension similar to McCall et al. (2016b). Other studies (Li et al., 2019, 2018; Thai et al., 2014; Ramin et al., 2017) evaluated biofilm effects using attached growth systems, which are more complex to perform but more closely mimic environmental sewer conditions. While the microbial community composition can be kept intact in suspended and attached growth configurations, the difference in biofilm morphologies can lead to different degradation kinetics (Falås et al., 2013). Furthermore, other studies have also examined biosorption as a combined contribution of suspended particulates and biofilms (i.e., using unfiltered wastewater with added biofilm in degradation tests (McCall et al., 2016b; Ramin et al., 2016)). Suspended particulates can play a significant role in drug biotransformation in sewage (Ramin et al., 2016) alongside other important transformation pathways such as abiotic hydrolysis (McCall et al., 2016b). Thirdly, this study did not monitor microbial growth in the microcosms on the assumption of steady state biomass over the short duration of the degradation tests. Similar assumptions were made and validated (by monitoring oxygen demand) in previous 24-h drug degradation tests (McCall et al., 2016b). In contrast, other studies noted rapid biomass growth in sewage over the same duration (Ramin et al., 2016), though the microcosms were artificially seeded with external carbon sources for biomass growth. Furthermore, we did not monitor wastewater quality parameters during 48-h degradation runs but a separate preliminary test indicated no significant changes in pH and dissolved oxygen throughout 72 h. Lastly, the study microcosms were built from a single-source sewer system thus the derived kinetic models may be specific to the sampled sewershed. The microbial community profiles in wastewater and sewer biofilms can change spatio-temporally and strongly influence drug degradation kinetics. For example, LaMartina et al. (2021) noted seasonal shifts in microbial community profiles in residential sewers, while in the work of McCall et al. (2016a, 2016b), large variabilities in drug stabilities were noted in wastewater-biofilm microcosms that were sourced from different but adjacent locations. Transitions in microbial community composition might be expected to occur over longer periods of time than we tested (48 h) and influence longer-term degradation dynamics, for instance, microbial community shift was observed in a 21-day exposure test for amphetamine (Lee et al., 2016). Overall the present study highlights the importance of biofilms to in-sewer drug degradation. Further studies are needed to examine how environmental factors influence sewer microbiomes, and consequently drug transformation kinetics during sewage transport.

4. Conclusion

In this study, we evaluated the stability of morphine, fentanyl, cocaine, and amphetamine in sewage, and examined the effect of sewer biofilm on the drug degradation kinetics. The experiments were performed using wastewater-biofilm microcosms spiked with drugs at environmentally relevant concentrations. Results indicate that amphetamine is stable in wastewater while the other drugs were not. Abiotic chemical transformation played a major role in the degradation of morphine, fentanyl, and cocaine. Fentanyl removal from wastewater was also influenced strongly by biofilm sorption. This study is the first to report on the effect of sewer biofilm on fentanyl degradation, and highlights the need to account for in-sewer drug stability in wastewater-based drug use estimation, particularly for chemicals with high affinity for organics.

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CRediT authorship contribution statement

JL performed the experiments and data analysis, and contributed in manuscript writing. **SP** and **FC** contributed in experimental design, data analysis, and manuscript writing and review.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.127666.

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