

Uncertainties about analytical methods and removal processes of some drugs of abuse in the biological wastewater treatment

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Abstract. The Wastewater Treatment Plants (WWTPs) can represent a source of release of Emerging Organic Micropollutants (EOMs) to the environment since the removal taking place within their process units is usually very limited; therefore, the final effluent and the excess sludge may still contain a high load of EOMs (Petrie *et al.* 2015). However, the real capability of the WWTPs of removing EOMs is often unknown: the physical and biological processes might be able to increase their efficiency provided that the operating parameters and conditions are suited to achieve the required removal of EOMs (Naidu *et al.* 2016). Along with this uncertainty, the analytical methods commonly used for determining concentrations of EOMs often do not ensure the required reliability and reproducibility, due to the complexity of the matrix represented by either the wastewater or the sludge (Funke *et al.* 2016). Among the wide class of EOMs, the present study focused on some drugs of abuse, specifically Benzoyllecgonine (BE) and 11-nor-9carboxy- Δ^9 -THC (THC-COOH). The double purpose of this study, carried out through laboratory scale investigations, was to evaluate the uncertainty factors of the analytical method used to detect these drugs in the liquid and solid phases of a full-scale WWTP (i.e. wastewater and sludge, respectively) and the contribution of abiotic and biotic processes to the removal of drugs in the biological reactor of the WWTP. The results obtained allowed to assess the optimal conditions of the method used to measure the selected drugs, with the aim to provide a relatively rapid and reproducible analytical tool and to minimize the interferences due to the matrix. Furthermore, the batch tests carried out at laboratory scale highlighted contribution of the biological processes to the overall removal observed in the oxidation tank of the WWTP.

Keywords: analytical method; biodegradation; drugs of abuse; emerging organic micropollutants; wastewater treatment plant

1. Introduction

Illicit drugs are excreted as parent compounds and metabolites through human urine and faeces, and then discharged into the sewage network (Zuccato *et al.* 2005; Castiglioni *et al.* 2007). Drug concentrations in wastewater usually range from a few units to hundreds of ng/l; the type

and concentration of drugs can vary considerably by region (Thomas *et al.* 2012). Measurement of illicit drugs in wastewater, and in general of many emerging contaminants, became possible only in the last 20 years, thanks to the improvement of sensitivity and accuracy of the analytical methods. The medium-high polarity and low volatility of these compounds make liquid chromatography coupled to mass spectrometry a suitable analytical technique. The most important difficulty associated with quantitative and qualitative analysis of illicit drugs in wastewater is related to their low concentration and to the complexity of liquid and solid matrices: the compounds either dissolved or suspended in the sample may interfere and compete with the target residues during the ionization process (Castiglioni *et al.* 2013; Castiglioni *et al.* 2016).

Illicit drugs are only partially removed by wastewater treatment plants (WWTPs) for domestic sewage, because these are not specifically designed to this aim (Zuccato and Castiglioni, 2009). Removal efficiency depends mainly on the type of technology used in the plant and the operating parameters; furthermore, chemical-physical characteristics of wastewater and concentration and properties of drugs may also have some influence. Main removal takes place in the secondary treatment processes, through adsorption, volatilization, and/or biodegradation (Helbling *et al.* 2010). Therefore, by improving efficiency of these processes might be possible to enhance the removal of drugs. According to the European Drug Report (EMCDDA 2015), cannabis is the most used drug in both Europe and worldwide (about five times more than other substances), followed by cocaine, amphetamine-group and opiates. Some studies indicate benzoyllecgonine, ecgonine methyl ester, MDMA, methamphetamine, amphetamine and morphine, like most abundant residues in WWTPs effluents (Pal *et al.* 2013). Moreover, the general consideration of European Directive 495/2015 suggests a future increase of attention about this class of contaminants into the environment and specifically in surface water. In light of these concerns, the aim of the present study was to investigate two selected metabolites of the most used illicit drugs in Europe, i.e. 11-nor-9carboxy- Δ^9 -THC (THC-COOH) and benzoyllecgonine (BE), in term of (1) matrix effect and uncertainties of the analytical methods and (2) contribution of biodegradation and other processes (e.g.,

adsorption and volatilization) to the removal occurring in the biological reactor of a WWTP.

2. Materials and methods

a. Matrix effect

A series of test was performed with the aim to investigate how the wastewater composition may affect the ionization process used to analytically determine drug concentration in the liquid phase. Since main components of a domestic wastewater are represented by carbon (COD), phosphorous (P) and nitrogen (N), therefore 4 standard solutions were prepared having the following contents, with the aim to simulate a typical domestic sewage composition:

Solution 1: 10 ng/L of drug (either BE or THC-COOH); 25 mg/L P solution.

Solution 2: 10 ng/L of drug (either BE or THC-COOH); 60 mg/L NH₃-N solution.

Solution 3: 10 ng/L of drug (either BE or THC-COOH); micro-nutrient solution.

Solution 4: 10 ng/L of drug (either BE or THC-COOH); 900 mg COD/L.

The value of 10 ng/L of the selected drug contained in each solution was chosen since near the LOQ value of the analytical method.

COD, N, P and micro-nutrient solutions were prepared according to the indications provided in Section c.

b. Removal processes

Removal processes were investigated through a series of batch tests performed in a 600 mL volume glass flask (operating volume of 400 mL). Each flask was placed on a jar tester to provide a mechanical stirring in order to maintain the content under completely mixed and aerated conditions; the flasks were covered with aluminium foils to avoid photo-degradation phenomena and the temperature maintained within the range 22±2 °C. The initial concentration of each drug was chosen to be 1000 ng/L, which corresponds approximately to the average concentration of both drugs found in the influent of WWTPs in Italy (Castiglioni *et al.* 2006; Pal *et al.* 2013; Boni *et al.* 2016).

To investigate the removal rate with time, 6 batch tests of different duration were carried out under the same operating conditions: 1 h, 4 h, 8 h, 24 h, 48 h and 52 h. During the tests, dissolved oxygen concentration and temperature value were always monitored and recorded. At the end of each duration, concentrations of the drug and of the following parameters were measured in the liquid phase: COD, NH₃-N, NO₂⁻-N, NO₃⁻-N. Furthermore, concentrations of MLSS and MLVSS were determined at the beginning and at the end of each contact time.

Taking into account that biodegradation usually takes place following adsorption of the compound onto sludge flocs, two series of batch tests (namely Series 1 and Series 2) were carried out for each of the selected drugs. One of the series (Series 2) was made by mixing activated sludge with

the drug-contaminated solution and micro- and macro-nutrients, with the aim to simulate the content of the biological reactor of a WWTP. The other series (Series 1) was used as a control to investigate contribution due to processes other than adsorption+biodegradation (e.g. ionization, hydrolysis, volatilization).

Specific composition of the mixed solution used for each series is reported below:

Series 1-Control test

The flasks were filled with drug solution only, along with carbon, nitrogen, phosphorous and micronutrients to simulate the same conditions as in Series 2.

Series 2-Activated sludge

Each flask was filled with a sample of activated sludge collected at the WWTP (having 3000 mg MLSS/L as in the biological reactor), drug solution (at 1000 ng/L), and carbon, nitrogen, phosphorous (300 mg COD/L, 60 mg NH₃-N/L, 25 mg P/L, respectively) and micronutrients to sustain the microbial metabolism.

Drug removal percentage in each series of batch tests was calculated based on the following equation:

$$R(\%) = \frac{C_{in} - C_e}{C_{in}} 100 \quad [\%]$$

where C_{in} and C_e represent drug concentration at the beginning and the end of the batch test, respectively.

In Series 2, the Average Specific Removal Rate (U) due to adsorption+biodegradation was calculated as follows:

$$U = \frac{C_{in} - C_e}{\Delta t} \cdot \frac{1}{X} \quad [\text{ng mgMLSS}^{-1}\text{h}^{-1}]$$

with X standing for the average concentration of MLSS during the test (mgMLSS/L).

c. Chemicals

Standard solutions of 11-nor-carboxy-Δ⁹-tetrahydrocannabinol (THC-COOH) and benzoylecgonine (BE) were purchased from Cerilliant (Round Rock, TX, USA) at concentration of 100 µg/ml in methanol. Activated sludge was collected at the full-scale WWTP and stored at 4°C until the use for batch tests. All drug solutions were prepared in 4 mg/L methanol solution at 99%. Nitrogen and phosphorous solutions were made by dissolving ammonium chloride, (NH₄Cl) or sodium dihydrogenphosphate (NaH₂PO₄) into deionized water (MilliQ water), respectively. Micro-nutrient solution was made according to OECD n. 209 (OECD 209 2010), i.e. by dissolving into 1 L deionized water the following components: 0.7 g NaCl, 0.4 g CaCl₂·2H₂O, 0.2 g MgSO₄·7H₂O. Organic carbon substrate was supplied by methanol solution at 99% (CH₃OH). All solutions were always stored at 4°C.

d. Analytical methods

APHA methods were used to determine concentrations of the following parameters: COD, NH₃-N, NO₂⁻-N, NO₃⁻-N, MLSS and MLVSS. pH, temperature and DO were measured using standard probes. The analytical technique chosen for the quantitative analysis of the drug metabolites was Ultra-Performance Liquid Chromatography coupled to tandem Mass Spectrometry (UPLC–MS/MS). The analytical method is based on WARC, TZV, NIAES, OCWD, 2008 (Boni *et al.* 2016). Samples were firstly filtered using 0.2 µm membrane filter of regenerated cellulose, and then injected as it is in the following systems:

1) UPLC (Ultra-Performance Liquid Chromatography); Ultimate 3000 RS Thermo, with two pumps, degasser,

chosen for the quantitative determination of concentrations due to the best results of calibrations curves. The repeatability of the analytical method was tested by injecting 5 times a sample at concentration near to LOQ (10 ng/L); the detected repeatability had to be below 5%. Accuracy values were calculated through Multiquant software: it was found to be ±14% and ±3% for BE and THC-COOH, respectively. Overall uncertainty about analytical method was 14%. To analyse concentration of the drugs in the solid phase, two different extraction procedures were used: Accelerated Solvent Extraction (ASE) for BE and Ultrasound assisted extraction (USE) for THC-COOH (since this molecule could be unstable at high temperature and pressure). Method conditions are described in

Compound	UPLC method	Q1	Q3	RT	DP	EP	CE	CXP	LOD	LOQ
		Dalton		min			Volts		ng/L	ng/L
BE-1	Chromatography column Phenomenex Kinetex 2.6µm Biphenyl 100A, 50x2.1 mm with security-guard column at 30°C. Mobile phase A: Milli-Q Reference A+ water with a chromatography column acidified with 0.1% formic acid; mobile phase B: LC-MS methanol acidified with 0.1% formic acid. The gradient elution condition were from 95% A and 5% B to 0% A and 100% B in 10min. Flow 0,4 ml/min.	290.1	168.2	6.9	30	10	25	12	3.7	12.1
BE-2		290.1	105.0	6.9	30	10	41	12	4.5	14.8
THC-COOH -1		343.2	299.2	12.1	-30	-10	-29	-12	7.0	23.2
THC-COOH-2		343.2	245.1	12.1	-30	-10	-37	-12	4.2	13.8

column oven compartment and auto sampler;

2) Mass spectrometer 5500 AB Sciex Q-Trap with Atlas Copco FS2 compressor, FX1 dryer, 270 litres tank and nitrogen generator Zephyr Zero 16 LC-MS.

The analyser and instrumental condition used and qualification limits are reported in

Table 2.

Table 1. Each drug was quantified by MRM (Multiple Reaction Monitoring ratio) using the two most abundant precursor/product ion transitions. The first and second transition for BE and THC-COOH, respectively, were

Table 1 Analyser and instrumental conditions for quantification analysis of BE and THC-COOH in liquid phase (RT= retention time; DP= declustering potential; EP= entrance potential; CE= collision energy; CXP= collision cell exit potential, LOD= limit of detection (S/N>3)); LOQ= limit of quantification (S/N>10))

Compound	UPLC method	Q1	Q3	RT	DP	EP	CE	CXP	LOD	LOQ
		Dalton		min			Volts		ng/L	ng/L
BE-1	Chromatography column Phenomenex Kinetex 2.6µm Biphenyl 100A, 50x2.1 mm with security-guard column at 30°C. Mobile phase A: Milli-Q Reference A+ water with a chromatography column acidified with 0.1% formic acid; mobile phase B: LC-MS methanol acidified with 0.1% formic acid. The gradient elution condition were from 95% A and 5% B to 0% A and 100% B in 10min. Flow 0,4 ml/min.	290.1	168.2	6.9	30	10	25	12	3.7	12.1
BE-2		290.1	105.0	6.9	30	10	41	12	4.5	14.8
THC-COOH -1		343.2	299.2	12.1	-30	-10	-29	-12	7.0	23.2
THC-COOH-2		343.2	245.1	12.1	-30	-10	-37	-12	4.2	13.8

Table 2 Methodology for quantification analysis of BE and THC-COOH in sewage sludge (solid phase)

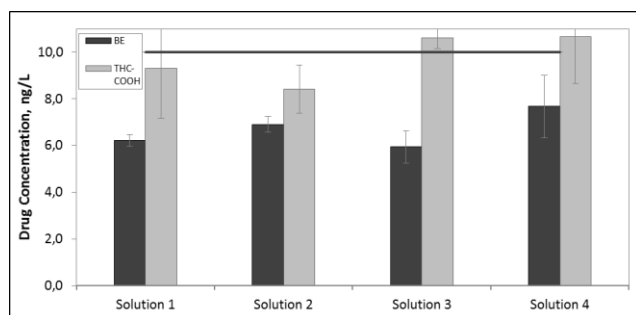
Compound	Sample preparation		Separation and detection	LOQ	
				ng/mgTSS	
BE-1	ASE 350: dichloromethane, 90% methanol	Solvent 10% methanol	Temperature 120 °C Pressure 1500 psi Static time 5 min Cycles 2	HPLC-MS ²	0,01
THC-COOH -2	USE: 10 mL Methanol, 15 min, room temperature. Filtered using 0.2 µm membrane filter.			HPLC-MS ²	0,02

3. Results and discussion

a. Matrix effect

Figure 1 shows the residual concentration of BE and THC-COOH in matrix effect test samples, as average concentration of two replicates; error bar indicates RSD%. For BE, the recovery was about 67%, compared to the expected concentration (10 ng/L). The detected concentration was always underestimated in all samples, with an negligible variability between the different solutions tested. This result indicates homogeneous ionization suppression of BE molecules and this effect might be so relevant because of the tested low concentration.

THC-COOH recovery was about 98%, but with different effect depending on the type of solution: in presence of methanol (Solution 3) and micro-nutrients (Solution 4), the detected concentration was higher than the expected one (10 ng/L), due to the enhancement of the ionization effect.

**Figure 1** Matrix effect

b. Removal processes

BE removal with time is shown in Figure 2; error bar indicates RSD%. In Series 1, removal was about 25% during all tests. This result, according to matrix effect test, shows the homogenous behaviour of BE in the tested solutions; furthermore, it is necessary considering 67% recovery for this type of matrix. Therefore, removal in control tests due to ionization, hydrolysis, volatilization and other processes, can be considered to be less than 25%.

In sludge activated test (Series 2), removal increased with time; for instance, total removal of BE was achieved between 4 and 8 h of contact time, with a Specific Removal Rate at $t=4\text{h}$ equal to $U_{\text{BE}}=0.06 \text{ ng mgMLSS}^{-1} \text{ h}^{-1}$.

At the end of each contact time, drug concentration detected in sludge flocs (solid phase), was always <LOQ;

CEST20

Figure 3 Removal efficiency of BE with time

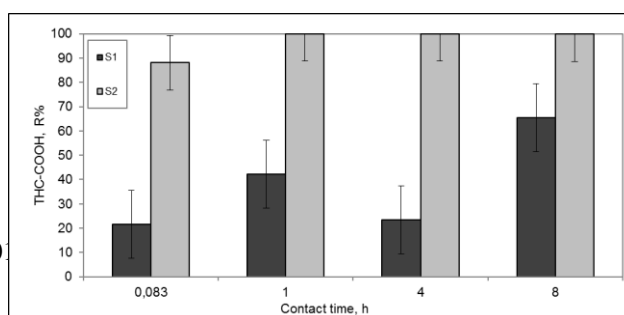
this result suggests that BE was firstly adsorbed onto the sludge flocs and then rapidly biodegraded. COD removal and nitrification proceeded continuously throughout Series 2; furthermore, pH decreased from 7.0 to 5.9 thus confirming the presence of nitrification. These results indicate that BE can be efficiently and rapidly removed in the oxidation reactor of a WWTP, and this process does not affect the biological degradation of carbon and nitrogen compounds.

THC-COOH removal with time is shown in Figure 3; error bar indicates RSD%. Control test (Series 1) shows a removal percentage from 20% to 66%. These high values indicate a relevant instability of THC-COOH, due to its physico-chemical properties like low polarity, and the occurrence of transformation processes. Further studies on this point are required for a better understanding.

In activated sludge tests (Series 2), the removal process was very fast, with total removal occurring after only one hour of contact time and $U_{\text{THC-COOH}}=0.24 \text{ ng mgMLSS}^{-1} \text{ h}^{-1}$. The high values of $\log K_{\text{OC}}$ and $\log K_{\text{OW}}$ (5.51 and 7.60, respectively) (USEPA, 2011) are in agreement with these results. THC-COOH was not found in the solid phase samples, analysed at the end of each contact time (drug concentration always <LOQ). Biological activity took place without being affected by the presence of THC-COOH: COD removal and nitrification proceed continuously and pH decreased accordingly to the oxidation of ammonia into nitrate.

4. Conclusions

The present study represents a start point for a wider study on the removal processes of drugs in WWTPs for domestic sewage. These topics assume a strong interest in light of the recent development of european directives about surface water quality. The results obtained in the present experimental activity show the high potential (rapid and useful method) of the Ultra-Performance Liquid Chromatography coupled to tandem Mass Spectrometry to detect drug concentration in wastewater without pretreatments and of ASE and USE extraction methods to measure drug residue in the sludge flocs. Homogeneous underestimation of BE concentrations was always found



due to nutrient presence; by contrast, the analytical method showed a good recovery of THC-COOH in all solutions.

Removal of both drugs in batch tests was mainly due to biological and adsorption combined processes. Among the drugs, THC-COOH was removed much faster than BE ($U_{\text{THC-COOH}} > U_{\text{BE}}$), whereas was more subjected to different transformation processes than BE.

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