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Investigation of Pharmaceutical Degradation in Urine via MFC and Assessment of Their Removal Efficiency Using UPLC-MS/MS

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ABSTRACT

Source-separated human urine has become an attractive nutrient resource due to its high levels of nitrogen and phosphorus, which can be repurposed as fertilizer. Nevertheless, it also contains contaminants such as pharmaceuticals and steroid hormones, whose elimination is vital to prevent adverse environmental and health effects. In this study, a double-chamber microbial fuel cell (MFC) was employed to explore the degradation of pharmaceuticals. Urine was intentionally spiked with four compounds—trimethoprim, lamivudine, levofloxacin, and estrone—at 2 µg/mL, and the MFC was run in batch mode for seven months using this feed. A sensitive UPLC-MS/MS method was developed and validated to quantify the pharmaceuticals, achieving a lower quantification limit of 0.39 ng/mL. The system achieved up to 96% ± 2% removal of the target compounds. Degradation was driven by processes including sorption and anoxic biodegradation. Monitoring of voltage indicated that pharmaceuticals initially hindered power generation and raised organic content, but after reactor acclimation, both power output and organic removal improved, reaching peak efficiency at a retention time of 30 h. These findings provide a novel perspective on anoxic pharmaceutical degradation and highlight potential strategies for bioremediation in nutrient-rich waste streams.

Keywords: Liquid chromatography mass spectroscopy, Biodegradation, MFC, Urine, Pharmaceuticals

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Introduction

The global population has now exceeded 8 billion, with each individual producing approximately 1.2 L of urine per day, whose composition can vary. Although commonly regarded as waste, urine contains valuable nutrients such as nitrogen (N) and phosphorus (P) in forms that plants can directly assimilate [1]. Separating urine from the main wastewater stream to recover these nutrients for use as fertilizer not only creates a sustainable resource but also reduces the energy demand associated with synthetic fertilizers and mitigates N and P overload in wastewater treatment facilities [2]. However, urine also contains a range of other compounds, including enzymes, organic matter, heavy metals, pharmaceuticals, and hormones. The presence of pharmaceuticals and hormones is particularly concerning due to their potential environmental impact, as they can leach into groundwater and enter the food chain [3]. Often present at very low concentrations (µg/L to ng/L), these substances are classified as micropollutants.

Pharmaceuticals, widely used to prevent and treat diseases, are largely excreted in unchanged form, entering sewage systems and eventually the environment. The application of urine as fertilizer can therefore facilitate the transfer of these compounds to soils, crops, and ultimately humans. Many pharmaceuticals exhibit endocrine-disrupting properties that can significantly impact wildlife [4]. Pharmaceuticals encompass various classes, including antipyretics, analgesics, antidepressants, antibiotics, antivirals, and hormones, with antibiotics, antivirals, and hormones particularly noted for their environmental relevance [5]. Antivirals inhibit microbial replication, antibiotics can induce bacterial toxicity, and hormones tend to persist in the environment, potentially

exacerbating ecological impacts. This highlights the critical need to monitor and remove pharmaceutical residues from urine.

Conventional wastewater treatment technologies face challenges in degrading these compounds due to their complexity and high concentration [6]. Although techniques such as chlorination, advanced oxidation, ozonation, electrochemical treatment, and biological methods have been investigated for micropollutant removal, they often require significant energy, time, and investment [7-10]. Consequently, alternative treatment approaches are necessary.

Microbial fuel cells (MFCs) have recently emerged as a promising technology in this field [11, 12]. MFCs exploit microorganisms' ability to convert organic matter in wastewater into electricity through microbially catalyzed redox reactions, simultaneously enabling the degradation of pollutants. Previous studies have demonstrated the effectiveness of MFCs in removing pharmaceuticals such as sulphamethoxazole and ciprofloxacin [13, 14]. Traditionally, pharmaceutical analysis in biological matrices such as serum, plasma, and urine has relied on UV and HPLC-based methods [15], which are limited to detecting concentrations in the mg/L range. To address this, ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS/MS) has been employed to accurately quantify pharmaceuticals at much lower concentrations (ng/L) in MFC effluents.

Integrating bioelectrochemical systems with biodegradation offers considerable potential for treating urine containing pharmaceutical residues. In this study, an MFC was operated with urine spiked with four pharmaceuticals from different classes—levofloxacin (LFL, fluoroquinolone antibiotic), trimethoprim (TRI, dihydrofolate reductase inhibitor), lamivudine (3TC, antiviral), and estradiol (EST, hormone)—to evaluate their degradation in the system. These compounds are commonly excreted in urine, detected in wastewater and surface waters [16], and are known for their persistence and environmental recalcitrance, exhibiting varied half-lives.

This work focuses on developing a UPLC-MS/MS method to quantify pharmaceutical degradation in MFCs, while assessing the reactor's performance in treating spiked urine through microbial voltage production and chemical oxygen demand (COD) removal. The study ultimately establishes the relationship between pharmaceutical presence and MFC operational efficiency.

Materials and Methods

Chemicals and reagents

The pharmaceuticals trimethoprim (TRI), lamivudine (3TC), levofloxacin (LFL), estradiol (EST), dexamethasone (DXM), carbamazepine (CBZ), and ciprofloxacin (CIP) were procured from Sigma-Aldrich (Germany). Organic solvents—acetonitrile (ACN), methanol (MeOH), formic acid (FA), and ethyl acetate—of LC-MS grade were obtained from Merck (Germany), while ammonium acetate was also sourced from Sigma-Aldrich. Ultrapure water was prepared using a Millipore system (Merck). Urine was collected from healthy volunteers, filtered through 0.22 µm membranes, and stored at 4 °C prior to experiments. Stock solutions of LFL and CIP (1 mg/mL) were prepared in 0.5% FA in Milli-Q water, whereas 3TC, TRI, CBZ, EST, and DXM stocks were prepared in MeOH. The internal standards selected were CIP for LFL, CBZ for 3TC and TRI, and DXM for EST. Additional salts used for media preparation—KCl, NH_4Cl, NaH_2PO_4, $NaHCO_3$, and CH_3COONa—were purchased from HIMEDIA.

Microbial fuel cell (MFC) reactor

A double-chamber MFC, constructed from Plexiglas with a total working volume of 230 mL, was employed. The anode and cathode compartments were separated using a cation exchange membrane (8 cm × 8 cm, Ultrex CMI7000, Membranes International Inc.), and stainless steel mesh (SS-304) functioned as electrodes. The reactor operated at room temperature under a fixed external load of 100 Ω.

The anode (anaerobic) was initially filled with synthetic media composed of KCl (0.1 g/L), NH_4Cl (1.5 g/L), NaH_2PO_4 (0.6 g/L), $NaHCO_3$ (2.5 g/L), CH_3COONa (0.82 g/L), 10 mL vitamin solution, and 10 mL trace element solution, along with a microbial inoculum obtained from a biogas plant treating food waste (Birla Institute of Technology & Science, Pilani, KK Birla Goa Campus, Zuarinagar, India) mixed with activated sludge. During inoculation, the anolyte was refreshed weekly. The cathode chamber contained a 20 mM phosphate buffer, was aerobic, and was replaced twice per week, with oxygen acting as the electron acceptor. After approximately one month, steady voltage was achieved, and urine replaced the synthetic media as feed. Voltage was recorded manually using a digital multimeter.

Batch experiments with spiked pharmaceuticals

Following two months of MFC operation with urine, pharmaceuticals were introduced at a final concentration of 2 mg/L. Experiments were conducted in five sequential phases: (1) fresh urine without pharmaceuticals, (2) urine with EST, (3) urine with EST and TRI, (4) urine with EST, TRI, and 3TC, and (5) urine containing all four compounds (EST, TRI, 3TC, LFL). Each phase lasted one month in batch mode, with stock solutions added and refreshed twice weekly. After reactor acclimatization, the operation mode shifted from batch to continuous flow. The effects of pharmaceuticals on MFC performance were monitored through voltage output and chemical oxygen demand (COD) analysis using the closed reflux colorimetric method [17]. Additional parameters, including orthophosphate (Ortho-P), ammonium (NH4+), and nitrate (NO3−), were measured according to established methods [18].

Development and validation of UPLC-MS/MS method

A UPLC-MS/MS method was developed and validated to quantify all four pharmaceuticals in urine and to evaluate their degradation in the MFC. Samples were stored at 4 °C until analysis.

Chromatography and mass spectrometry

UPLC-MS/MS analysis was performed using a Series-1290 Infinity-11 HPLC system (Agilent Technologies, Inc.) with a flexible pump, autosampler (vials kept at 4 °C), and a thermostatted column oven.

Levofloxacin was separated on a Zorbex Eclipse C18 column (100 mm × 2.1 mm, 1.8 µm) at 30 °C using a gradient of 0.5% FA in 1 mM ammonium acetate (A) and MeOH (B) [13], for 3TC and TRI, a C8 column (150 mm × 4.6 mm, 5 µm) was used, with mobile phase A (0.1% FA in 90% MeOH) and B (0.1% FA in 10% water). Estradiol was analyzed on the same column in isocratic mode using mobile phase A (10 mM ammonium acetate with 0.01% FA) and B (MeOH with 0.01% FA). The detailed chromatographic conditions are summarized in **Table 1**.

Table 1. Chromatography conditions maintained for UPLC-MS/MS analysis of pharmaceuticals.

Compound	Column used	Mobile phase	Flow rate (mL/min)	Gradient/Isocratic	Retention time (min)	Injection volume (µL)	Linearity range (ng/mL)
LFL	Agilent C_{18} (2.1 mm × 100 mm, 1.8 µm)	0.5% FA in 1 mM ammonium acetate and MeOH (1:1, *V/V*)	0.2–0.3	Binary gradient (0–10 min)	4.3	5	1.56–100
TRI	Agilent C_8 (150 mm × 4.6 mm, 5 µm)	0.1% FA in MeOH (90%) and milliQ water (10%)	0.3	Isocratic	3	5	0.78–50
3TC	Agilent C_8 (150 mm × 4.6 mm, 5 µm)	0.1% FA in MeOH (90%) and milliQ water (10%)	0.3	Isocratic	3.2	5	0.78–50
EST	Agilent C_8 (150 mm × 4.6 mm, 5 µm)	0.01% FA in ammonium acetate (10 mM) and MeOH	0.3	Isocratic	4.2	5	0.39–100

LFL: levofloxacin; TRI: trimethoprim; 3TC: lamivudine; EST: estradiol.

All target analytes were detected and quantified using an Agilent 6460 Triple Quadrupole UPLC-MS/MS (Agilent Technologies, Santa Clara, CA, USA) equipped with a positive-mode electrospray ionization (ESI) interface and an ion spray voltage of 4 kV. Measurements were conducted in multiple reaction monitoring (MRM) mode. The optimized instrumental parameters, elemental compositions, and measured ion masses from standard solutions for each compound are listed in **Table 2**. The triple quadrupole was operated at unit resolution, and peak areas were processed and integrated using MassHunter software, version B.08 (Agilent Technologies, Inc.).

Table 2. MRM parameters and LLOQ concentration for the tested pharmaceuticals.

Compound	Precursor ion, ESI^+ (molar mass)	Product ions (molar mass)	MRM transitions	Fragmented voltage (V)	Collision energy (V)	LLOQ (ng/mL)
LFL	362 ($C_{18}H_{21}FN_3O_4^+$)	261 ($C_{14}H_{14}FN_2O_2^+$) 318 ($C_{17}H_{21}FN_3O_2^+$) 344 ($C_{18}H_{19}FN_3O_3^+$)	362–318	135	20	1.56
TRI	291 ($C_{14}H_{18}N_4O_3$)	110 ($C_4H_6N_4$) 123 ($C_5H_7N_4$) 230 ($C_{12}H_{14}N_4O$) 257 ($C_{13}H_{13}N_4O_2$) 261 ($C_{12}H_{13}N_4O_3$) 275 ($C_{13}H_{15}N_4O_3$)	291–230	100	10	0.78
3TC	230 ($C_8H_{11}N_3O_3S$)	112 ($C_4H_7N_3O$) 202 ($C_7H_{10}N_3O_3S$)	230–112	110	15	0.78
EST	271 ($C_{18}H_{23}O_2$)	157 ($C_{11}H_9O$) 196 ($C_{14}H_{13}O$) 253 ($C_{18}H_{21}O$)	253–271	110	20	0.39

LLOQ: lower limit of quantitation.

Preparation of calibration standards and quality control (QC) samples

The working solution of LFL was prepared by diluting its stock with a mixture of 0.5% FA in 1 mM ammonium acetate buffer (pH 4.7) and MeOH in a 1:1 (v/v) ratio. For 3TC, TRI, and EST, working solutions ranging from 0.039 to 10 μg/mL were prepared in MeOH. Calibration standards and QC samples were generated by spiking blank urine with the appropriate working solutions. Calibration concentrations included 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 ng/mL. QC samples were prepared in triplicate at four levels: the low-low QC for LFL and EST was 0.39 ng/mL (0.78 ng/mL for 3TC and TRI), the low QC for LFL and EST was 0.78 ng/mL (1.56 ng/mL for 3TC and TRI), the medium QC was 6.25 ng/mL, and the high QC was 100 ng/mL for all pharmaceuticals. Twenty microliters of the respective internal standard (5 μg/mL) were added to all spiked urine samples, and all solutions were stored at 4 °C until analysis.

Sample preparation

LFL was extracted from urine using a simple dilute-and-shoot procedure, whereas 3TC, TRI, and EST were isolated via liquid-liquid extraction. For LFL, 500 μL of urine was combined with 20 μL of the internal standard (5 μg/mL) and vortexed for 20 s, followed by the addition of 1 mL of mixed buffer to precipitate proteins. Samples were vortexed for 3 min and centrifuged at 13,000 rpm for 15 min. The resulting supernatant was transferred to autosampler vials for UPLC-MS/MS injection.

For 3TC, TRI, and EST, 100 μL of urine was mixed with 3 mL of ethyl acetate containing 0.1% FA, along with 20 μL of the corresponding internal standard (5 μg/mL). Samples were vortexed for 10 min and centrifuged at 5,000 rpm for 30 min. The organic layer was collected, evaporated under vacuum, and reconstituted in 200 μL of MeOH/H2O (2:1, v/v) before injection. Calibration standards, QC samples, and MFC samples spiked with all four pharmaceuticals were prepared following the same procedure.

Method validation and quantification

The analytical method was validated for selectivity, linearity, accuracy, and precision. Identification of analytes was based on their retention times. Calibration standards were prepared in the 0.39–100 ng/mL range from working stock solutions, with each concentration injected in triplicate daily to generate calibration curves by plotting the analyte-to-internal standard peak area ratios against nominal concentrations. Quadratic regression was applied, with squared coefficients of determination (r^2) exceeding 0.998 for all analytes.

Selectivity was confirmed by comparing chromatograms of blank urine with those spiked with pharmaceuticals, ensuring no interference or carryover. Linearity was evaluated over five consecutive days using a $1/x^2$ weighting factor. Accuracy (% bias) and precision (% RSD) were determined at four QC levels from repeated injections (n = 5) for both intra-day (repeatability) and inter-day (reproducibility) measurements, with acceptable limits set

at ±15%. The lower limit of quantification (LLOQ) was established by analyzing spiked urine (n = 3) with signal-to-noise ratios of 3:1 and 10:1, consistent with RSD calculations from the calibration curve intercept and slope.

Results and Discussion

Reactor startup and operation

The anode chamber was inoculated with a mixture of microbial inoculum and synthetic media. Microorganisms oxidized organic matter, transferring electrons to the electrical circuit and generating current, making anode inoculation a critical step during MFC startup. Microbial growth led to biofilm formation on the anode surface, with bacteria consuming media components for survival and replication, thereby initiating reactor activity. Voltage production was monitored throughout inoculation. On the first day, the open-circuit voltage was measured at (57 ± 4) mV. The system was then operated under a closed-circuit configuration with a 100 Ω external load, reaching (234 ± 17) mV by the end of the first week. After one month, the feed was switched to fresh urine whenever the voltage fell to 50 mV. Within three one-week operating cycles, the maximum voltage reached (480 ± 22) mV, remaining stable for two months. This consistent voltage indicated successful bacterial attachment and formation of an electrochemically active biofilm. **Figure 1** illustrates the voltage profile during reactor startup.

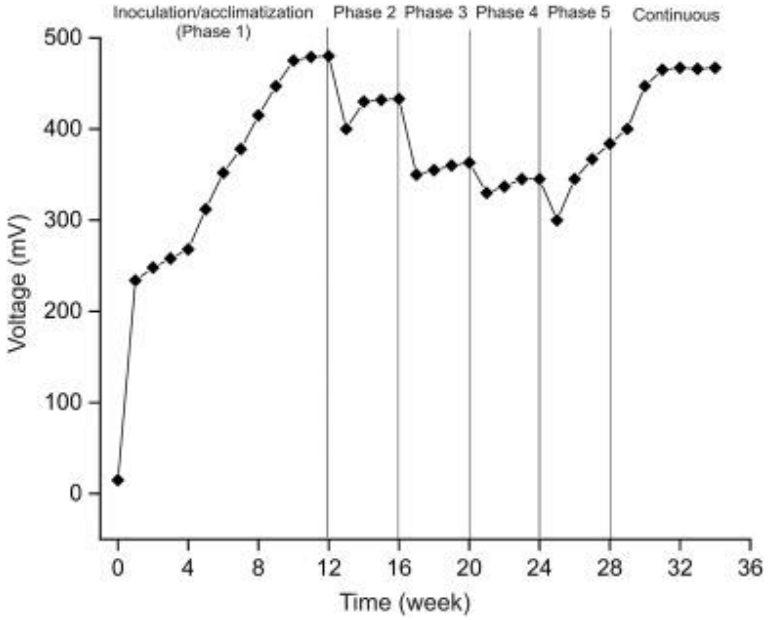


Figure 1. Voltage Profile of the MFC Reactor Across Different Pharmaceutical Addition Phases. Phase 1: fresh urine without pharmaceuticals, Phase 2: urine spiked with EST, Phase 3: urine with TRI and EST, Phase 4: urine containing 3TC, TRI, and EST, and Phase 5: urine spiked with LFL, 3TC, TRI, and EST.

After three months of reactor operation, urine containing 2 μg/mL of EST was introduced, a concentration chosen based on trace pharmaceutical levels reported in environmental samples [19-21]. The addition of EST caused a voltage drop, indicating its inhibitory effect on the biofilm, reducing the voltage to (400 ± 14) mV in Phase 2. When TRI was added a month later, the voltage further decreased to (350 ± 11) mV. Subsequent addition of 3TC lowered the voltage to (330 ± 15) mV, and finally, LFL addition in Phase 5 reduced it to 300 mV.

The voltage profile demonstrates that each pharmaceutical caused an initial decline in MFC performance, with EST exhibiting the strongest inhibitory effect, likely due to its longer half-life and complex structure, while LFL showed the least impact. After seven months, the voltage gradually increased to (467 ± 12) mV and stabilized, indicating that the biofilm adapted to the pharmaceuticals and the microbial community could efficiently utilize the organic matter. The reactor achieved a maximum power output of 2.18 mW at 4.67 mA under a 100 Ω load, corresponding to a power density of 0.88 W/m² and a current density of 1.89 A/m².

MFC performance was further assessed via COD removal. The decrease in COD reflected microbial oxidation of organic matter in urine. Initially, the COD of the urine influent at Phase 1 was (7892 ± 205) μg/mL, which dropped to (1736 ± 113) μg/mL after two months, achieving an average removal efficiency of 78% without pharmaceuticals. EST addition temporarily increased COD to (9345 ± 402) μg/mL, and subsequent

pharmaceutical additions initially caused fluctuations in both COD and voltage. However, after 7–8 months, COD stabilized at (1682 ± 104) μg/mL, with a maximum removal efficiency of 93% observed in Phase 5 once the biofilm acclimatized to all four compounds. This trend indicates that microbial adaptation allowed effective degradation of pharmaceuticals. The effect of each pharmaceutical on COD removal is summarized in **Figure 2**. The reduction in pharmaceutical concentrations was quantified using UPLC-MS/MS analysis.

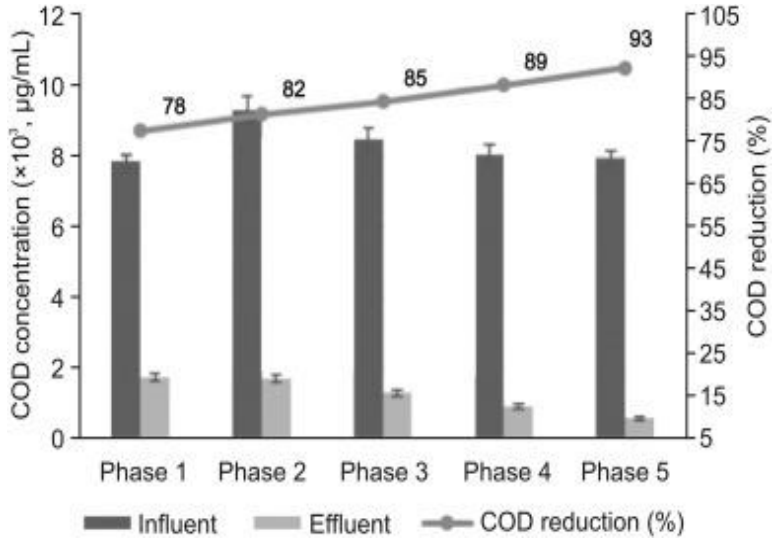


Figure 2. COD Variation in the MFC During Different Pharmaceutical Addition Phases
Phase 1 corresponds to fresh urine with no pharmaceuticals, Phase 2 to urine containing EST, Phase 3 to urine with TRI and EST, Phase 4 to urine spiked with 3TC, TRI, and EST, and Phase 5 to urine containing LFL, 3TC, TRI, and EST.

Development of UPLC-MS/MS analytical method

To evaluate how pharmaceuticals affect MFC performance, a UPLC-MS/MS approach was employed to simultaneously track the concentrations of the added compounds and assess their removal efficiency.

Chromatographic and mass spectrometric optimization

The method was optimized by repeatedly injecting each standard individually into the mass spectrometer. Both positive and negative electrospray ionization (ESI) modes were tested, with the positive mode ultimately selected due to its superior sensitivity.

Mobile phase selection was critical for achieving efficient separation. Various solvents, including methanol (MeOH), formic acid (FA), ammonium acetate, and acetonitrile (ACN), were examined. For LFL, conditions from previous work [13] were applied. Formic acid concentrations of 0.5% (LFL), 0.1% (3TC and TRI), and 0.01% (EST) yielded sharp peaks and improved detection sensitivity. EST was separated under isocratic conditions using ammonium acetate and MeOH, while 3TC and TRI were eluted using MeOH and Milli-Q water. Multiple columns were tested, including Agilent Eclipse Plus C18 (2.1 mm × 150 mm, 3.5 μm), Agilent C18 (2.1 mm × 100 mm, 1.8 μm), Agilent C100 (2.1 mm × 50 mm, 1.0 μm), and Agilent C8 (150 mm × 4.6 mm, 5 μm). The best separation and peak resolution were achieved using the Agilent Zorbex Eclipse Plus C18 (2.1 mm × 100 mm, 1.8 μm) for LFL and the Agilent C8 column (150 mm × 4.6 mm, 5 μm) for 3TC, TRI, and EST.

The MRM mode was employed to enhance sensitivity and reduce peak interference. The precursor ion for each compound was the [M+H]+ species, and product ions were generated via induced fragmentation **(Table 2)**. Representative MRM and positive ion spectra for all analytes are shown in **Figures 3-5**.

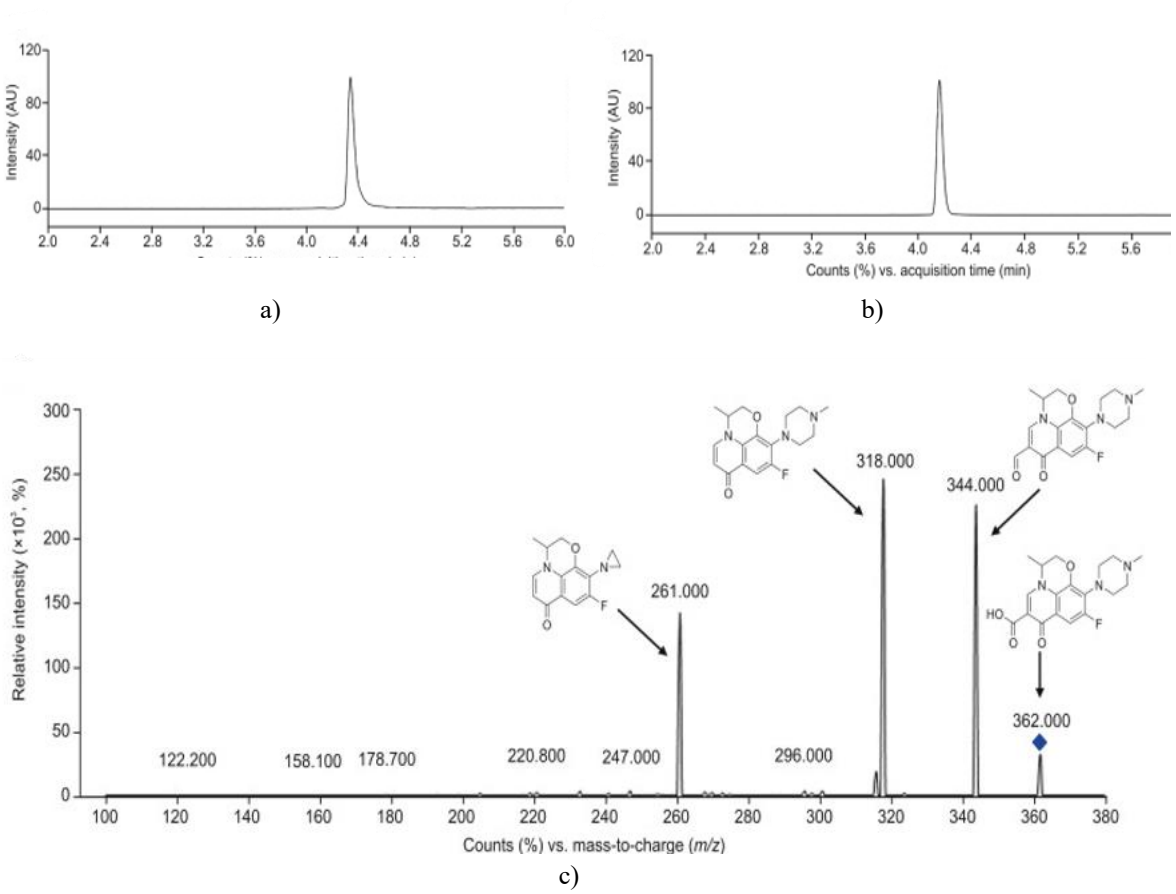
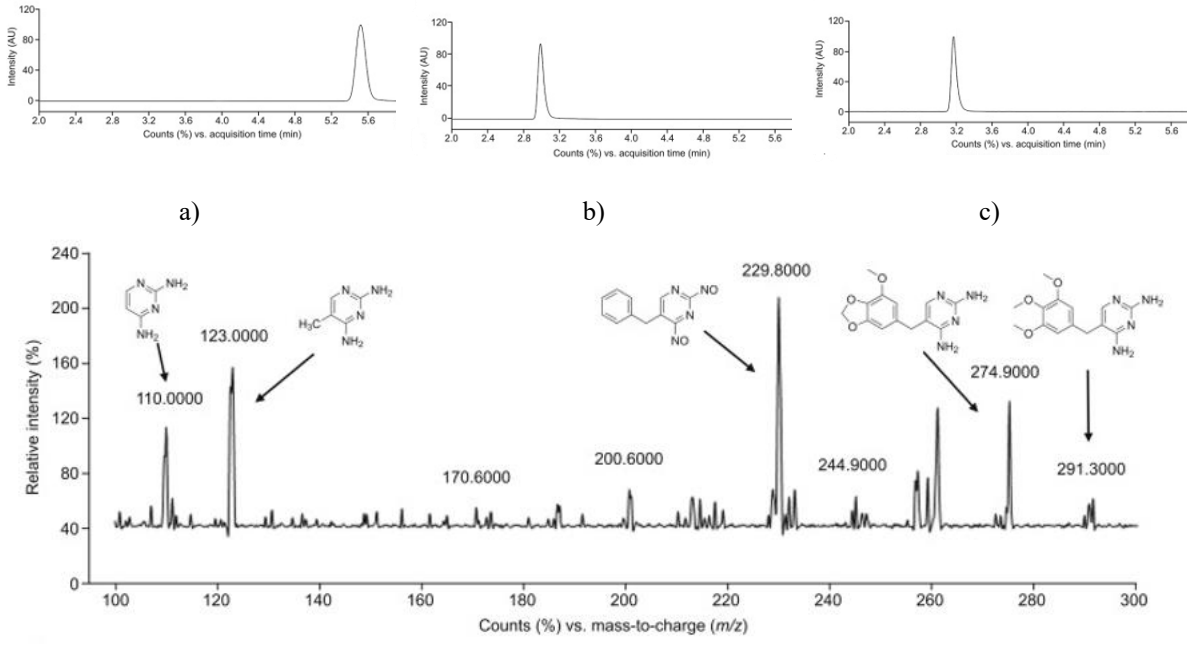
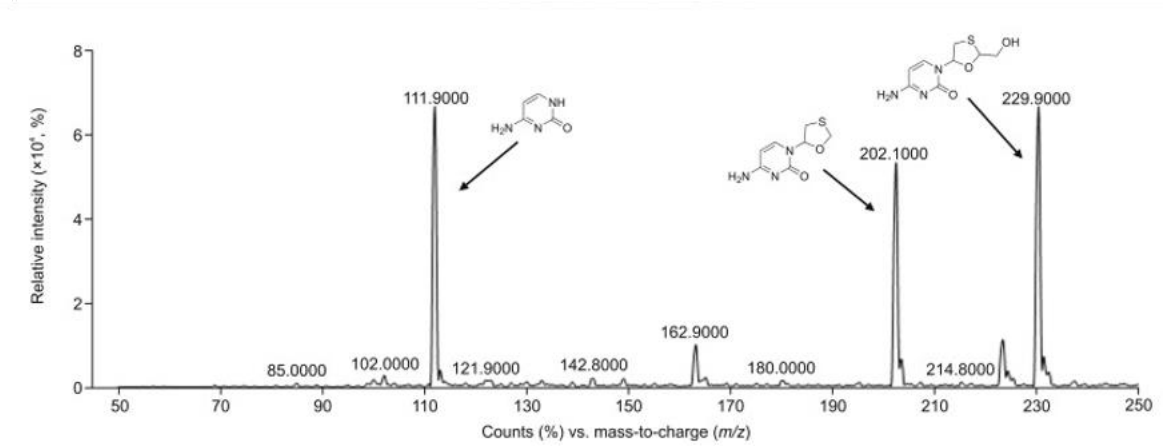


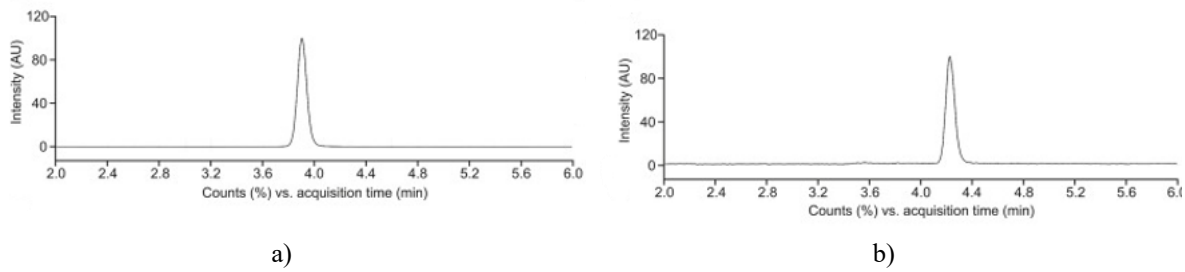
Figure 3. Chromatographic and positive-mode electrospray mass spectra for LFL: (a) MRM trace of CIP (retention time: 4.4 min), (b) MRM trace of LFL (retention time: 4.1 min), (c) product ion spectrum of LFL. Abbreviations: LFL – levofloxacin; MRM – multiple reaction monitoring; CIP – ciprofloxacin.



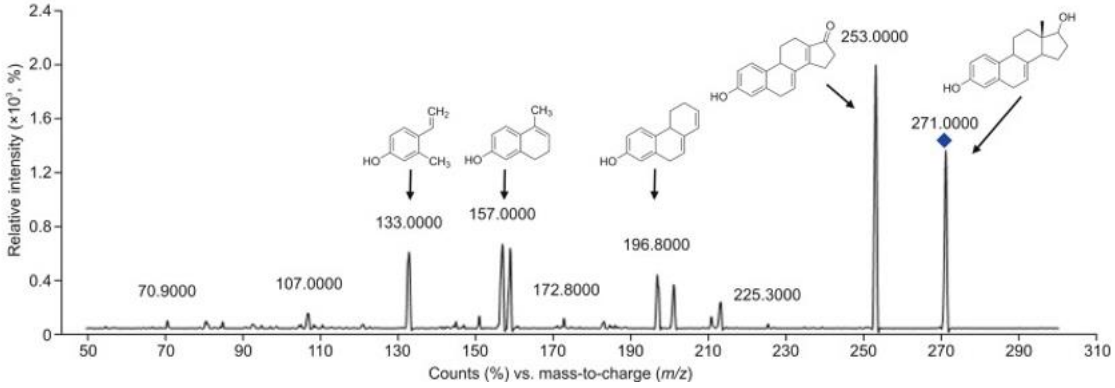


e)

Figure 4. Chromatograms and positive-mode electrospray mass spectra for TRI and 3TC: (a) MRM trace of CBZ (retention time: 5.5 min), (b) MRM trace of TRI (retention time: 2.9 min), (c) MRM trace of 3TC (retention time: 3.1 min), (d) product ion spectrum of TRI, (E) product ion spectrum of 3TC. Abbreviations: TRI – trimethoprim; 3TC – lamivudine; CBZ – carbamazepine.



a) b)



c)

Figure 5. Chromatograms and positive-mode electrospray mass spectra of EST: (a) MRM trace of DXM (retention time = 3.8 min), (b) MRM trace of EST (retention time = 4.25 min), (c) product ion spectrum of EST.

The extraction of pharmaceuticals from biological samples can be challenging due to the presence of complex matrix components. Solid-phase extraction (SPE) is commonly used to minimize matrix effects in serum and plasma, but its high cost makes it less practical. In this study, SPE was not applied because urine has a simpler composition compared to plasma. Instead, straightforward sample preparation techniques, including protein precipitation, liquid-liquid extraction, and dilute-and-shoot approaches, as reported in previous studies [22], were explored for efficient drug extraction from urine. LFL was extracted using the dilute-and-shoot method, while 3TC, TRI, and EST were isolated via liquid-liquid extraction as described earlier [22].

Method validation

The analytical method was validated following FDA Bioanalytical Method Validation guidelines [23]. Linear calibration responses were achieved across the ranges of 0.39–100 ng/mL for EST, 0.78–50 ng/mL for 3TC and TRI, and 1.56–100 ng/mL for LFL, with all analytes exhibiting an r^2 of 0.998. The absence of interfering peaks in blank urine at the retention times of the analytes relative to their internal standards confirmed the method's specificity. Each compound was effectively separated from its corresponding internal standard under the described chromatographic conditions **(Table 2)**, and the MRM spectra demonstrated clear resolution for all analytes.

Matrix effects were evaluated by comparing the QC sample accuracy in fresh urine against the calibration curve. Compounds eluting earlier generally exhibited lower matrix effects. For all analytes, matrix effects were negligible (<10%), indicating minimal interference in quantification.

Accuracy and precision were assessed at four concentration levels using five replicates per level over three consecutive days. Accuracy was expressed as the percentage bias of QC samples, and precision was determined using one-way ANOVA on the RSD data. The intra-day and inter-day accuracy and precision values for all analytes are summarized in **Table 3**.

Table 3. Intra-day and inter-day assay precision and accuracy of used pharmaceuticals.

Compound	Concentration (ng/mL)	Accuracy (%bias)		Precision (%RSD)	
		Intra-day	Inter-day	Intra-day	Inter-day
TRI	0.78	−5.10	−4.48	10.43	10.73
	1.56	−0.67	−1.37	4.72	5.71
	6.25	0.44	1.46	5.06	4.54
	100	2.13	1.53	4.01	4.1
3TC	0.78	−6.56	−6.38	7.33	6.58
	1.56	1.45	−0.86	5.75	6.08
	6.25	0.68	1.44	5.12	4.42
	100	2.11	1.09	4.39	3.79
LFL	0.39	−12.84	−9.39	12.16	13.04
	0.78	−3.49	−2.33	9.17	7.89
	6.25	0.80	2.54	5.45	4.39
	100	−1.20	−1.00	3.77	3.78
EST	0.39	−9.38	−11.13	9.47	8.24
	0.78	−3.18	−0.42	9.06	8.44
	6.25	−0.98	6.63	5.39	3.69
	100	1.47	2.14	3.07	3.55

The results were evaluated using the linear regression equations derived from the calibration curves, which demonstrated excellent linearity with $r^2 = 0.998$. The regression equations obtained were $y = 21.28x$ for TRI, $y = 50.11x$ for 3TC, $y = 41.71x$ for LFL, and $y = 32.46x$ for EST. Method validation was further confirmed by the intercept and slope values, along with their standard deviations. The lower limit of quantification (LLOQ) was determined as 0.39 ng/mL for EST, 0.78 ng/mL for 3TC and TRI, and 1.56 ng/mL for LFL.

Analysis of MFC-treated urine samples

Urine is a rich source of nitrogen (N) and phosphorus (P); however, microbial utilization of these nutrients in the anode is relatively low, around 5 ± 2% [18], indicating significant potential for nutrient recovery. Urease enzymes facilitate the conversion of nitrogen into ammonium ions (NH4+/NH3). In the MFC, NH4+ and other cations migrate from the anode to the cathode through the cation exchange membrane, creating a concentration gradient between the two chambers over time. The cathode pH rises due to hydroxyl ion accumulation, and at alkaline conditions, phosphates and ammonium in the cathode can be precipitated using a magnesium source.

The presence of various pharmaceuticals in urine and their environmental persistence has become an important concern due to potential ecological and human health risks. Therefore, it is critical to screen urine and assess the

recovered products, such as struvite (N and P), to ensure they are free from pharmaceutical residues. The physicochemical properties of untreated urine and the percentage reductions in key parameters following the addition of pharmaceuticals and hormones are summarized in **Table 4**. To monitor the fate and quantify the concentration of all pharmaceuticals in the MFC, a UPLC–MS/MS method was developed and validated, and it was applied to analyze samples collected at specific time intervals from the reactor.

Table 4. Physicochemical parameters analysis of neat urine and reduction percentage on pharmaceuticals addition.

Parameters	Neat urine (mg/L)	Reduction (%)				
		Without any pharmaceutical addition	EST addition	TRI addition	3TC addition	LFL addition
Chemical oxygen demand	7892 ± 22	78 ± 3	82 ± 2	85 ± 4	89 ± 3	93 ± 1
Ortho-P	201 ± 15	94 ± 1	95 ± 1	94 ± 2	93 ± 2	94 ± 2
NH_4^+	674 ± 23	49 ± 2	53 ± 2	57 ± 1	59 ± 3	60 ± 4
NO_3^-	246 ± 42	54 ± 3	57 ± 2	61 ± 4	62 ± 3	64 ± 3

The use of MFCs for the degradation of antibiotics has been widely investigated due to their low operational costs [12]. In this study, MFCs were applied to recover nutrients from urine in the form of struvite (MgNH4PO4·6H2O), a slow-release phosphorus fertilizer. Urine spiked with four different pharmaceuticals was used to evaluate their degradation within the MFC. Voltage measurements during the inoculation phase confirmed the successful colonization of microorganisms on the anode, while changes in COD reflected microbial oxidation of the organic matter present in urine.

The addition of pharmaceuticals initially impaired MFC performance, as the increased organic load disrupted microbial metabolism and slowed the activity of the biofilm. However, continuous operation with spiked urine gradually allowed the microbial community to acclimate to the pharmaceuticals.

Initial effluent samples exhibited higher concentrations of the added drugs, but after several batch cycles, the reactor adapted to their presence. After seven months of operation, with anolyte replaced every two days, samples were collected at specific intervals and analyzed. The results **(Figure 6)** indicated that pharmaceuticals were effectively biodegraded within 25–30 h of retention time. Starting from an initial concentration of 2000 ng/mL (2 µg/mL), the levels decreased to 78.58 ng/mL for EST, 58.39 ng/mL for TRI, 70.15 ng/mL for 3TC, and 37.29 ng/mL for LFL, corresponding to an overall cumulative removal efficiency of 96 ± 2%.

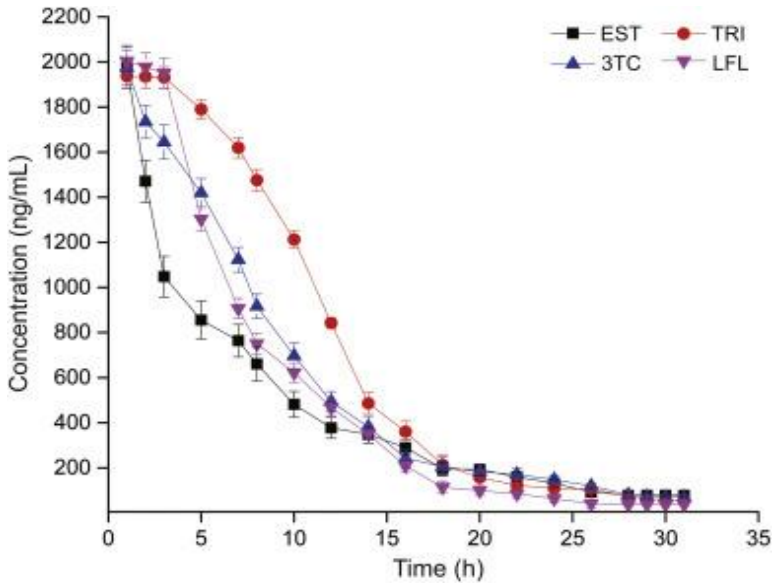


Figure 6. Variation in effluent concentration of spiked pharmaceuticals with time

Previous studies have reported that MFC treatment of tetracycline-containing wastewater achieved a 90% reduction in COD (initial concentration of 850 ± 50 µg/mL) along with 99% degradation of tetracycline [24]. In

contrast, the higher-strength urine used in our study, with an initial COD of 7000 ± 200 μg/mL, reached a maximum COD removal of 93%. This reduction is largely attributed to microbial activity, as microorganisms facilitate direct oxidation of pharmaceuticals through adsorption onto the anode surface [25]. Microbial electron transfer mechanisms, reflected in ATP levels, are sufficient to degrade antibiotics within the MFC, while electrochemical processes combined with osmosis have also been applied for antibiotic removal [24].

The introduction of pharmaceuticals altered the bacterial community, slightly weakening electron transfer, as indicated by the decrease in voltage from 480 mV to 467 mV. Nevertheless, both COD removal and pollutant degradation were enhanced in the presence of pharmaceuticals, highlighting the MFC's capability to reduce organic matter and pharmaceutical compounds through a combination of microbial oxidation and electrochemical transformation.

Biodegradation mechanism

Biodegradation offers an environmentally friendly and cost-effective approach to contaminant removal, as microorganisms mineralize organic pollutants into less harmful intermediates that are subsequently metabolized. However, the pathways for microbial degradation of most pharmaceuticals and hormones remain largely uncharacterized, making it challenging to pinpoint the genes involved in the mineralization process. Organic compound removal generally involves two processes: biosorption and biodegradation, with the latter being more prominent and favorable. Microbial degradation can occur through metabolism-based or co-metabolism-based pathways, with the majority relying on metabolism, where compounds serve as carbon or energy sources to support microbial growth. The initial characterization of the biofilm, inoculum, and urine in this study was detailed previously [18], revealing bacterial communities dominated by Proteobacteria and Firmicutes, with key genera including Pseudomonas, Escherichia, Bacillus, Proteus, Tissierellia, and Alcaligens.

Prior research has demonstrated that human gut and intestinal bacteria contribute to the breakdown of organic pollutants. Specifically, E. coli, Alcaligens, and Pseudomonas have been implicated in estrone (EST) degradation via growth-linked reactions [26]. The EST degradation pathway involves hydroxylation of the aromatic ring (C-4), hydroxylation of the saturated ring, dehydration of the cyclopentane ring (C-17), and dehydrogenation of ring D (C-17). For trimethoprim (TRI), biodegradation has been well-studied in Rhodococcus species, and similar pathways are followed by certain Pseudomonas and Bacillus strains, which produce enzymes such as acrylamine N-acetyltransferase that target aromatic amines [27].

Fluoroquinolone antibiotics like levofloxacin (LFL) undergo either aerobic or anaerobic microbial degradation, where mono- or dioxygenase/reductase enzymes attack the aromatic structure, forming dihydroxy aromatic intermediates. These intermediates are further cleaved by intradiol or estradiol dioxygenases or enter pathways involving dearomatizing reductases, ultimately being metabolized into simple compounds used for microbial growth [28].

The microbial degradation of lamivudine (3TC) has not been previously documented, and it is generally considered non-biodegradable and inhibitory to bacterial consortia in activated sludge [5]. In this study, 3TC removal likely occurred primarily through biosorption on the anode surface, though further research is needed to elucidate its biodegradation. Overall, the study suggests that Pseudomonas, Escherichia, Bacillus, and Alcaligens remain active in the biofilm after pharmaceutical addition, contributing to biodegradation, while the observed reduction in MFC performance may result from disrupted metabolic activity among other co-metabolizing microorganisms in the biofilm.

Conclusion

Chemical precipitation in MFC, induced by elevating pH, provides an effective approach to recover nitrogen and phosphorus from urine in the form of struvite, which can serve as a slow-release phosphorus fertilizer for agricultural use. The formation of struvite was achieved by adding the required amount of magnesium to the MFC effluent. Nevertheless, prior to its application in agriculture, the struvite must be evaluated for the presence of micropollutants such as pharmaceuticals and hormones.

The developed UPLC-MS/MS method proved to be an effective analytical tool for quantifying four pharmaceuticals in urine samples obtained from the MFC, exhibiting good linearity over the range of 0.39–100 ng/mL. The MFC successfully degraded pharmaceuticals in urine spiked at an initial concentration of 2 mg/L (2000 ng/mL) over seven months of operation under anoxic conditions. The reactor was operated in batch mode,

with pharmaceuticals introduced after a three-month inoculation period. During this phase, monitoring COD provided insights into microbial biodegradation of organic matter. Significant changes in pharmaceutical concentrations were observed only after the biofilm had acclimatized to the added compounds, indicating microbial adaptation within the MFC. The biofilm utilized the pharmaceuticals as carbon and nitrogen sources and secreted enzymes that facilitated their breakdown. With a hydraulic retention time of 30 h, the system achieved approximately $96 \pm 2\%$ biodegradation. The removal of pharmaceuticals can be attributed to microbial biodegradation, oxygen diffusion at the cathode, and the electricity generated within the MFC. These findings demonstrate that MFCs can be effectively applied in the bioremediation of environmentally persistent pollutants.

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