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Ciprofloxacin biotransformation during anaerobic digestion: Major transformation products elucidation and environmental proteomics

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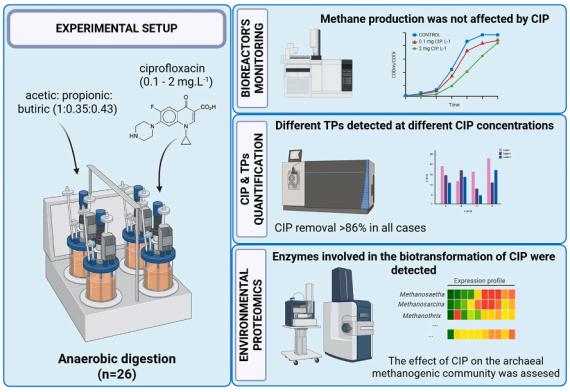
HIGHLIGHTS

- Methanogenic activity was not affected by CIP presence and CIP removal was > 88 %.
- Sorption and biological transformation co-occurred at lower CIP concentrations.
- Six TPs pointed to hydroxylation and loss of the cyclopropyl ring.
- *Methanothrix* and *Methanosaeta* were the main methanogens in all conditions.
- MCRA expression decreased at 2 mg CIP L^{-1} suggesting an inhibitory threshold.

GRAPHICAL ABSTRACT



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ABSTRACT

Anaerobic membrane bioreactors (AnMBR) ar a promising alternative for treating high-load sewage, like black water (BW), due to the energy saving (biogas production and lack of aeration). Antibiotics like ciprofloxacin (CIP) are expected to be more concentrated than in a typical urban sewage matrix. Its fate under these conditions remains poorly understood. To broaden this understanding, we combined analysis of transformation products (TPs) and proteomics on 26 batch experiments inoculated with an AnMBR sludge, fed with volatile fatty acids (VFAs) and spiked with CIP. Methanogenic activity was not affected by CIP and the removal was higher than 88 % in all cases. Abiotic controls indicated the removal was mainly driven by sorption processes. However, at the lower concentrations tested (0.1 and 0.5 mg L^{-1}) biotransformation also occurred. A total of six TPs were identified, some only present in low concentrations while one of them only appeared at the highest concentrations, pointing to hydroxylation and loss of the cyclopropyl ring as the main biological transformations. *Methanothrix* and *Methanosaeta* were the main methanogens and not negatively affected by CIP, except at 2 mg

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CIP. L^{-1} suggesting an inhibitory threshold. Environmental proteomics pointed to the key role of dehydrogenases under methanogenic anaerobic conditions.

1. Introduction

In a study of 258 of the world's rivers, over a quarter of these carried concentrations of active pharmaceutical ingredients including antibiotics that exceeded safe limits [36]. Whilst their exact derived effects are not fully known, the predicted increase in antibiotic resistance poses a serious threat to humankind. Under the Water Framework Directive, macrolide antibiotics as a group were included in the European Union watch list in 2015 [6], while later in 2018, amoxicillin and ciprofloxacin (CIP) were also incorporated [7].

CIP was launched into clinical practice in 1987 and is listed as an essential medicine by the World Health Organization [44], for controlling infections of the lungs, joints, bones, airways, and the urinary tract. As with other fluoroquinolones, CIP enters cells by simple diffusion and selectively inhibits DNA replication in bacteria by forming an irreversible ternary complex with the DNA gyrase, preventing gyrase activity, blocking replication, and rapidly inhibiting DNA synthesis. Cell death derives from the chromosome breakage, partially due to the accumulation of toxic reactive oxygen species (ROS). The targets of fluoroquinolones are widely distributed among bacterial species, which allows them to have broad-spectrum activity [25]. CIP effect on the bacteria is well known, however, to the best of our knowledge, few reports are available on the susceptibility of isolated archaea to CIP. All archaebacteria tested in Sioud et al., [31] were resistant to CIP except *Natronobacterium gregoryi* and *Methanosarcina barkeri* and CIP did not induce cell lysis of archaebacteria at doses up to 200 mg L^{-1}.

Nonetheless, bacteria and archaea rapidly develop and spread resistance mechanisms. Three main types of microbial resistance to fluoroquinolones have been described: i) mutations in the DNA gyrase, topoisomerases IV, or regulatory genes controlling the expression of the outer membrane proteins or efflux pumps, ii) a mutant aminoglycoside-modifying enzyme (AAC(6')-Ib-cr) that modifies certain quinolones, including CIP by adding an acetyl group, which reduces the antibiotic activity, and iii) active pumping of the CIP molecules out of the cells by the efflux pumps QepA and oqxAB [9].

Wastewater treatment is crucial to minimize the impact of organic micropollutants including antibiotics derived from our daily life into the environment and our safety. Among the different technologies developed, the anaerobic treatment of high-loaded sewage is attractive in terms of sustainability, not only because of the energy recovery through biogas production but also because of less sludge production and easy disposal. AnMBRs are becoming a promising alternative thanks to their high sludge retention time (SRT) and the high quality of the derived effluents [5]. Despite of the current concern about the presence of antibiotics in urban sewage, few studies have explored the role of this high SRT mixed cultures in their removal. Considering the widespread presence of CIP in rivers and lakes as well as in most types of wastewaters [34], it is of great importance to understand the potential mechanisms of CIP biotransformation during AnMBR treatment.

Despite CIP being known to be an easily biodegradable compound under anaerobic conditions during sewage treatment [2], literature regarding the elucidation of the degradation pathway of CIP during anaerobic digestion is still limited. Some studies focus on pure cultures, while others in the effluent of anaerobic digestion [12,16,43]. Only few studies focus on the different main stages of this process: hydrolysis, acetogenesis and methanogenesis [34,4] and found that microorganisms carrying out the acetogenesis were main actors in the degradation of CIP. [34] and Do & Stuckey described a dose-effect phenomena within the addition of CIP in an anaerobic digestor and an AnMBR, respectively. However, methanogenic archaea and bacteria are still a black spot for the antibiotic biotransformation.

The elimination of antibiotics present in the wastewater matrix may occur through different mechanisms. The biodegradation of antibiotics typically does not reach complete mineralization due to the chemical stability of their molecular structure. However, biological reactions cause modifications leading to the formation of transformation products (TPs) with different physical-chemical characteristics than the parent compound [11]. These TPs may show similar or higher toxicity, leading to the underestimation of the environmental impact [17]. In the case of CIP, previous literature identified TPs derived from the hydroxylation of the fluoroquinolone group or the piperazine ring [4].

The information obtained by environmental proteomics allows to decipher the different activities of each microbial taxa [15], as well as to correlate them with the operational conditions applied in an experimental design [28]. The study of the set of proteins expressed by complex mixed cultures has become possible thanks to the great technical advances achieved in mass spectrometry in recent decades [45]. The combination of biotransformation profiles and TPs identification with environmental proteomics might allow to elucidate not only the key players involved in the biotransformation of organic micropollutants during biological wastewater treatment [14] but also present the potential for the elucidation of the biotransformation routes.

This study aimed to assess the biological degradation mechanisms of the antibiotic ciprofloxacin (CIP) under anaerobic methanogenic conditions in different concentrations through a combination of methanogenic assays, TPs identification, and environmental proteomics.

2. Material and methods

2.1. Bioreactors

The experimental design consisted of 500 mL pyrex bottles with approximately 275 mL of liquid phase. The bottles were inoculated with 1 g VSS L^{-1} of anaerobic sludge from an AnMBR treating black water (BW) from the toilets of a car industry. This inoculum was not previously exposed to a relevant concentration of CIP. Thus, it was considered as a non-adapted biomass. To avoid microbial activity until the addition of carbon source and nutrients, the biomass was washed with phosphate buffer. A mixture of volatile fatty acids (VFAs) was selected as carbon source, consisting of acetic:propionic:butyric acids in a ratio 1:0.35:0.43 on a COD basis [26], reaching a final concentration of 2 g COD L^{-1}. Due to the high methanogenic activity, a second pulse of carbon source was added at 26 h. The batch tests were spiked with four different initial CIP concentrations: 0.1, 0.5, 1, and 2 mg L-1. The range of concentration selected is higher than the typical values of CIP in domestic sewage [35] although reported in other wastewaters [18] since the scope of this study was to elucidate biodegradation pathways and TPs identification. This is a common strategy used in other studies [14,21,34,4] in order to improve the detection of both the parent compound and TPs with the same analytical methods, to clearly elucidate the degradation pathways and to evaluate the effect of higher concentrations of CIP in the microbial community. Each batch test was spiked with the corresponding volume of a previously prepared CIP stock solution (1000 mg L^{-1} in HPLC grade methanol) and then evaporated at room temperature 2 h before starting the experiment.

Also, three controls were included: sterilized sludge (spiked at 0.5 mg L^{-1} of CIP), blank (no sludge, milliQ water with 0.5 mg L^{-1} of CIP), and a control without CIP. The samples were taken at 6 h and 48 h (corresponding to the hydraulic retention time (HRT) of the inoculum in the AnMBR). Each experiment was conducted in triplicate, while control experiments were conducted in duplicate (total number of bioreactors $n = 26$). Bottles were sealed and placed in a shaker for continuous

shaken at 150 rpm in a thermostatic room at 25ºC. On each sampling point, the biogas produced was released after pressure and composition was monitored and 2 mL of liquid phase were collected for further analyses.

2.2. Bioreactor's monitoring

Conventional parameters of the liquid phase (COD, total nitrogen (TN), CH_4, etc.) were analysed according to Standard Methods [3]. Biogas production was calculated by measuring the gas pressure in the headspace of the bottles at 6, 24, 30, and 48 h of the experiments, and releasing it after every measure. The biogas composition was measured using a gas chromatograph (GC) HP® 5890 Series II with a column SOPELCO® Porapack Q 80/100 2 m x 1/8".

2.3. CIP and TPs analyses

To monitor the CIP removal and TPs formation, samples from all the digesters were directly taken with a syringe and centrifuged. The supernatant was filtered at 0.45 µm (HAWP04700, Millipore). High-resolution mass spectrometry (HRMS) was used to determine CIP's concentration through a calibration curve and by matching the fragmentation spectra with an analytical standard using an Orbitrap Exploris 120 mass spectrometer (MS) (Thermo Fisher Scientific Inc) coupled to an ultra-performance liquid chromatography (UPLC). Moreover, six major transformation products (TPs) were identified using HRMS by a non-target analysis. The TP identification was done by correlating the *m/z* spectra of all the detected compounds with the corresponding fragmentation pattern. The analysis was carried out using a chromatographic column Hypersil GOLDTM (50 ×2.1 mm, particle size 1.9 µ, Thermo Fisher). The mobile phases were acetonitrile (eluent A), and water (eluent B), both with 0.1 % or formic acid. The gradient expressed as the ratio of B was as follows: 0–0.2 min, 2 %; a linear increase from 2 % to 98 %; 0.2–4.75 min, hold at 98 % until 6 min, followed by a linear decrease from 98 % to 2 %; 6–9 min at a constant flow rate of 0.4 mL min^{-1}. Orbitrap Exploris 120 was equipped with an electrospray ionization source (ESI), working in positive ionization mode (3500 V). The samples were analyzed in full scan mode, from 100 to 1000 *m/z*, with Orbitrap MS (resolution of 30,000) to identify suspect *m/z* and further fragmented at a normalized collision energy of 30 %. The fragmentation spectra were acquired by Orbitrap working at a resolution of 15,000 to obtain structural information on the suspected TPs. All the data was acquired and processed with Compound Discoverer™ 3.0 Software.

2.4. Protein extractions

For proteomics analysis, 1 mL samples were collected from the bioreactors at the end of the operation (48 h) and frozen until further proteome extraction, following the protocol described in [14]. Samples were centrifuged at 9000 rpm for 10 min at 4ºC to pellet the bacterial cells. Briefly, the pellet was washed with phosphate buffer solution (PBS) and then incubated at 90 °C for 20 min in an extraction buffer (50 mM Tris buffer, 1 % SDS, pH = 7.5). After this, cell lysis was achieved by mechanical beating of the samples in tubes containing 0.1 mm glass beads and placed in a cell disruptor (Thermo Scientific). Three cycles of 4 min bead beating alternated with 1 min- incubations in ice were performed. Samples were then centrifuged for 20 min at 3300 rpm and 4 °C, and the supernatants were transferred to a fresh tube for protein precipitation. Two steps of −20 °C-cold acetone precipitation were performed to remove the organic contaminants and salts. Next, acetone was carefully removed, and pellets were resuspended in molecular grade water. Protein concentration was quantified with the BCA Protein Assay Kit (Thermo Fisher Scientific) following the instructions provided by the manufacturer. Protein electrophoresis in denaturing conditions was performed with the proteome samples as an integrity check control in NuPAGE Bis-Tris 4–12 % gels (Thermo Fisher Scientific) at 200 V for 30 min. A standard blue Coomassie staining protocol was used for the visualization of the protein bands.

2.5. Proteome analyses

The protein samples were processed in solution by enzymatic digestion and further desalted using ZipTip-µC18 material (Merck Millipore, Burlington, MA). Peptide samples (0.3 µg of protein) were analysed in a timsTOF Pro (Bruker, Bremen, Germany) equipped with a nano-electrospray source (CaptiveSpray) and a tims-QTOF analyzer. Proteomic analyses were performed at the Mass Spectrometry and Proteomics facilities of the University of Santiago de Compostela (RIAIDT-USC).

2.6. Proteomic data analyses

MS/MS spectra were processed with PEAKS Studio Xpro (Bioinformatics Solutions, Waterloo, ON) software for protein identification. The database used contained the sequences available in the UniProtKB protein database uniprot_anaerobic_digester_2023_07_04. The Label-Free module from PEAKS Studio Xpro was used for protein quantification. The data obtained was further processed with GraphPad and Unipept Desktop [37]. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [27] partner repository with the dataset identifier PXD062699.

3. Results and discussion

3.1. Bioreactor's performance

Fig. 1 shows the evolution of the different batch experiments regarding the biomethanization of the VFAs. The methanogenic activity in all cases was high enough to reduce the initial COD for > 88 %, which is in the typical COD removal range for AnMBRs [5]. The obtained results confirm two different scenarios for the TPs and proteomics sampling (6 and 48 h): at 6 h, all experiments (except abiotic) were in the 50 % of COD final removal, suggesting a moment of high metabolic activity whereas at 48 h COD removal was completed, expecting a lower microbial activity.

CIP removal was assessed at different initial concentrations, ranging from 0 to 2 mg L^{-1}. CIP removal efficiencies were ≥ 90 % in all the conditions tested except the blank and the control without CIP addition (as shown in Fig. 2). Varying CIP removal efficiencies under anaerobic conditions have been reported in previous literature. For instance, [4] obtained values between 64 % and 85 % in an anaerobic structured bed reactor (ASBR) and 66–85 % in an anaerobic packed bed reactor (APBR) with a HRT of 12 h, operated at 30 °C and fed with CIP at 400 ng L^{-1}. Do

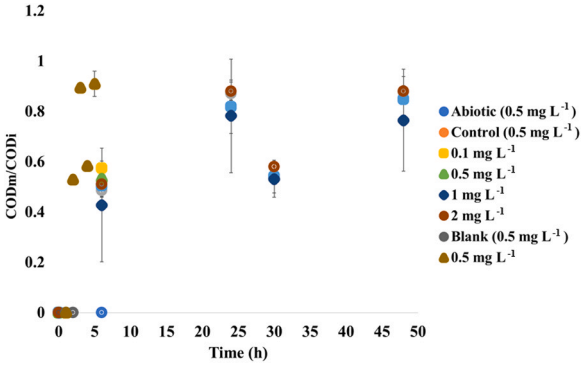

Fig. 1. Methanization of the VFAs mixture in different batch experiments. COD basis: COD_m is the COD of the produced methane in the gas phase, COD_i is the initial concentration of COD in the liquid phase.

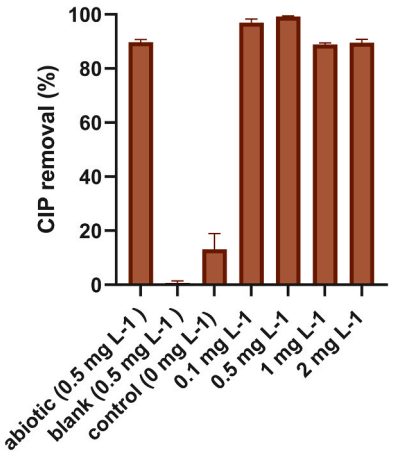

Fig. 2. Removal of CIP (%) in different experimental conditions.

& Stuckey, [5] obtained a removal of 50–76 % when applying 0.5–1.5 mg CIP L^{-1} in the feed, although at 4.7 mg L^{-1} its removal efficiency decreased to < 20 % in an AnMBR over the long-term (120 days), suggesting that an inhibition threshold might exist above the concentrations used in the present study.

The abiotic control (consisting of autoclaved sludge + 0.5 mg CIP L^{-1}) showed a high CIP removal activity (90 % ± 1 %) while in the case of the blank (not inoculated reactor + 0.5 mg L^{-1}) the removal was negligible (i.e., 1 % ± 1 %) (Fig. 2). These results are consistent with the finding of Do & Stuckey, [5], where they found that sorption kinetics did not present significant differences between active or sterilised biomass in AnMBR. Therefore, abiotic transformations due to the elements present in the medium composition, the pH or light can be discarded and this removal in the abiotic control can be attributed to adsorption onto the biomass. Fluoroquinolones are known to adsorb onto solid matrices, including soils, sediment, and sludge [20], with an adsorption coefficient (K_d) of 54,600 L kg^{-1}. Particularly, ciprofloxacin has a log K_{ow} of 0.28 and contains the carboxylic acid, carbonyl, and amide groups, facilitating the adsorption process [10]. Jia et al., [13] determined that the major factor in the removal of fluoroquinolones in a municipal sewage treatment plant was sorption to sludge. In another study [19,46], CIP sorption was a pH-dependent process strongly correlated with the sludge properties via multiple adsorption mechanisms including electrostatic attraction, cation exchange, and bridging, π-π interaction or hydrogen bonds.

On the control without CIP addition, we determined a removal of 13 % ± 6 % due to the presence of CIP on the sludge used as inoculum (0.0013 mg L^{-1}). The CIP removal detected in the experiments spiked with 0.1 mg L^{-1} was 97 % ± 1 % and in the case of 0.5 mg L^{-1} it was 99 % ± 1 % (Fig. 2) and considering the above-mentioned results on the abiotic control (90 % ± 1 %) this is indicative of a certain degree of biotransformation activity. However, at the highest concentrations tested (1 and 2 mg L^{-1}) the values obtained were similar to the abiotic control (89 % ± 1 % and 90 % ±1 %, respectively) (Fig. 2). This might indicate very low biotransformation activity at the highest CIP concentrations tested, and the fact that the removal at these high concentrations in short operational times is mainly due to adsorption.

3.2. *Transformation products elucidation*

The anaerobic biodegradation of CIP resulted in the identification of six TPs through HRMS analysis (see Supplementary Material). The proposed TPs are listed in Table S1, and their fragmentation profiles are detailed in Figures S1-S7. The TPs identified were the following: TP-347 (*m/z* 348.1351), TP-346 (*m/z* 347.1238), TP-332 (*m/z* 333.1430), TP-273 (*m/z* 274.1188), TP-259 (*m/z* 282.1014), and TP-255 (*m/z* 256.1081). The TPs were classified into two groups: hydroxylation of the CIP structure (TP-347, 346, and 332); and loss of the cyclopropyl group (TP-273, 255, and 259). Fig. 3 presents the proposed biodegradation

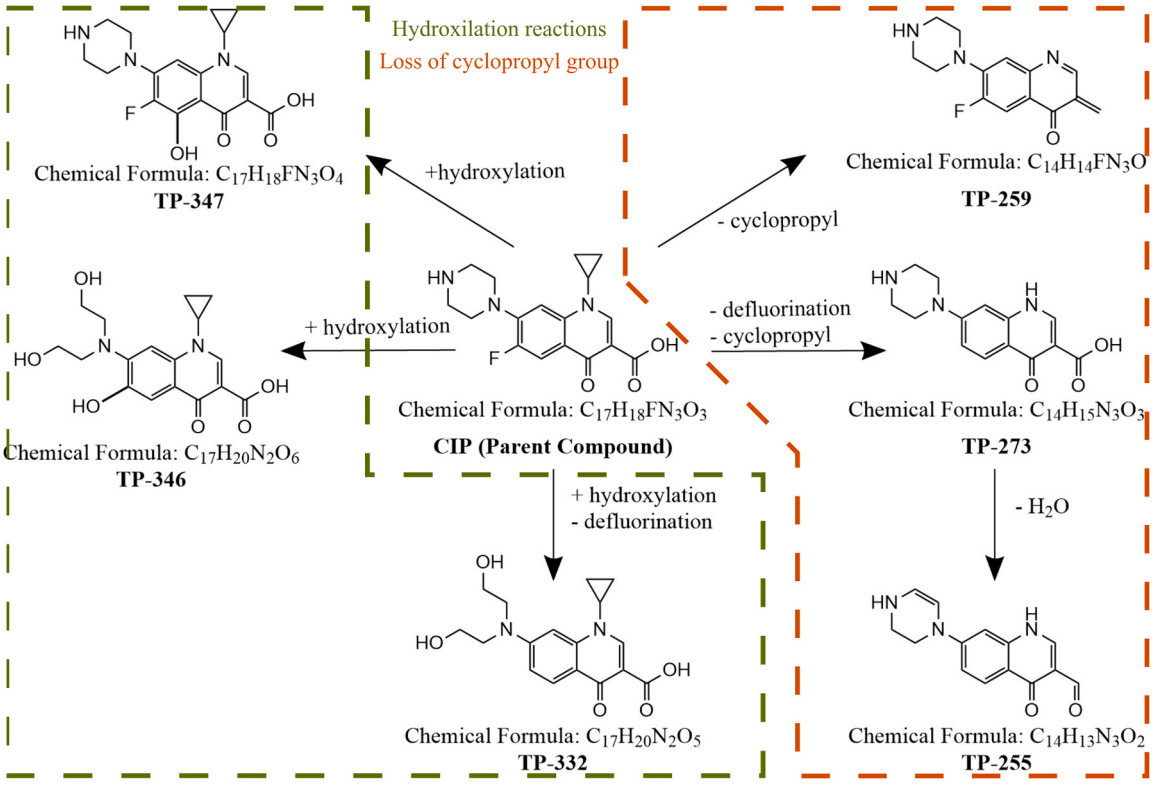

Fig. 3. Proposed degradation pathway for ciprofloxacin (CIP) and the different transformation products (TP) detected.

pathway.

The relative abundance of the TPs derived from CIP biodegradation is presented as their peak areas normalized to the initial CIP concentration signal ($A_{TP}(t)/A_{CIP}(t_o)$) in Fig. 4. An initial CIP concentration ≤ 0.5 mg L^{-1} led to the formation of four TPs (i.e. TP-346, TP 332, TP-259, and TP-255). TP-346 and TP-332 were products of hydroxylation at the piperazine ring, this pathway involves the introduction of hydroxyl groups (-OH) into the six-membered ring and their cleavage. The reaction usually targets carbon or nitrogen atoms, the most common in biological systems being the hydroxylation of carbon atoms in the piperazine ring. This biotransformation of the CIP has been demonstrated in bacterial sludge systems via enzymatic pathways [29]. The position of the fluorine substitution plays a crucial role in catabolism, as it can influence whether the compound undergoes further breakdown [24], in this case, defluorination is observed in both TP-332 and TP-273. The TPs identified in this study are aligned with other studies, such as Tang et al., [34] and [24], which demonstrated similar defluorination processes in microbial metabolism.

On the other hand, the electron-withdrawing nature of the nitrogen in the quinolone nucleus contributes to the reactivity of the system, particularly by facilitating interactions with enzymes [30], leading to the formation of reactive intermediates near the cyclopropyl group as radical species followed by ring opening and loss of this moiety. As mentioned above, the loss of the cyclopropyl group from CIP was a common step in TP-273, TP-259, and TP-255. Carneiro et al. [4] reported similar biotransformation pathways of CIP with an anaerobic packed bed biofilm reactor treating municipal sewage.

The TPs detected in CIP concentrations ≥ 1 mg L^{-1} reactors were TP-347 and TP-273. The relative abundance of TP-347 was higher than TP-273, indicating a predominance of hydroxylation in the quinolone ring. This finding is consistent with previous studies [42,8] that have reported the formation of TP-347 under anaerobic and enzymatic conditions. TP-273 was detected at 6-hour samples, pointing as a TP of secondary reactions. Although this TP (TP-347) was not detected in samples from reactors with CIP concentration < 1 mg L^{-1}, its formation cannot be discarded since the concentration might be below the detection limit. An initial CIP concentration ≤ 0.5 mg L^{-1} led to the formation of three main TP (i.e. TP-346, TP-259, and TP-255). TP-259 was formed by the loss of the cyclopropyl group from CIP, followed by the reduction of hydroxyl groups. Likewise, TP-255 was produced from TP-273, which was detected at 6-hour samples ($A_{TP}(t)/A_{CIP}(t_o) = 0.0007$), whose transformation consisted of the loss of the cyclopropyl group and a defluorination reaction.

The main TP detected in CIP concentrations ≥ 1 mg L^{-1} reactors was TP-347, together with TP-273 which presented a very low signal ($A_{TP}(t)/A_{CIP}(t_o) < 0.0001$). Although this TP (TP-347) was not detected in samples from reactors with CIP concentration < 1 mg L^{-1}, its formation cannot be discarded since the concentration might be below the detection limit. The formation of TP-347 was previously reported to occur under anaerobic conditions [42]. Moreover, a recent study reported that dehydrogenase enzymes can catalyze the hydroxylation of the quinolone ring, resulting in the formation of TP-347 [8]. Nevertheless, the TPs are not necessarily less toxic than the parent compound, which may preserve the bioactive groups [1,40]. Previous studies reported that the TP of fluoroquinolone antibiotics, derived from photodegradation, may double the inhibitory effect caused by the parent compound [32]. However, others suggest that the biological transformation of fluoroquinolone antibiotics reduces the ecotoxicological effect [33]. For instance, in silico analysis regarding the ecotoxicity of fluoroquinolones and their transformation products concluded that hydroxylation, specifically in the piperazine moiety, decreased the ecotoxicity in comparison with the parent compound [38,39]. Therefore, future studies should focus on exploring the mineralization of these emerging contaminants.

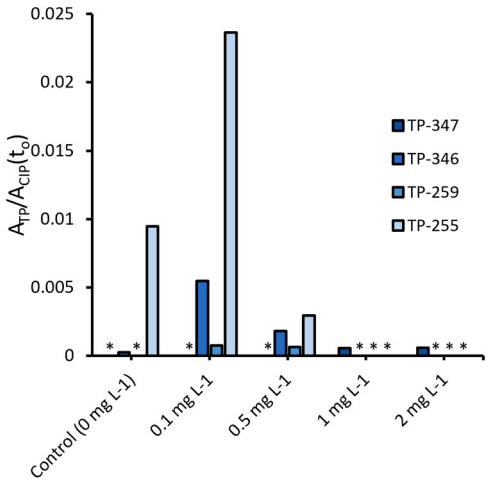

Fig. 4. Relative abundance of each TP after 48 h, normalized to the initial signal of ciprofloxacin (CIP) ($A_{TP}(t)/A_{CIP}(t_o)$). Asterisk (*) indicates TP was not detected.

3.3. Environmental proteomics

Regarding the active microbial community, this was dominated by the archaeal genera *Methanothrix* and *Methanosaeta*, representing 24 and 14 % of the peptide contributions to the total in the control (no CIP) at the end of the experiment and followed by *Methanosarcina* in a significantly lower proportion (3 %) (see Supplementary Material). Our results suggest a positive correlation between the concentration of ciprofloxacin and the activity of *Methanothrix*, which increases its peptide contribution in those bioreactors spiked with CIP, and being this effect remarkably higher in the concentration of 1 mg L^{-1} (36 %). Our results are in contrast with the genomic-based study of [22], which reported a significant negative effect of CIP on the *Methanothrix* population. In the case of *Methanosaeta,* no clear trend was detected, and the relative activity of this genus remains similar in all the concentrations tested (ranging from 14 % to 18 %).Fig. 5

In contrast, *Methanosarcina* strongly decreased its activity in the lower concentrations (<1 % in 0.1 and 0.5 mg L^{-1}) compared to the control (3 %), while the decrease is less accused in the higher concentrations (3 and 2 % in 1 and 2 mg L^{-1}). The same occurred with *Methanoregula* and *Methanoculleus*.

Other genera not predominant in the control archaeal community (<1 %) also increased their activity in the presence of CIP. That was the case of *Methanobrevibacter* and *Methanospirillum*.

The main functional activity of both *Methanothrix* and *Methanosaeta* was methanogenesis, as demonstrated by the high abundance of their proteins Methyl-coenzyme M reductase subunit alfa, and this was true in all the conditions tested, including the control and all the concentrations of CIP applied (Table 1). The number of peptides detected at different CIP concentrations applied suggest no negative impact of the antibiotic, maintaining the expression of mcrA from 0 to 1 mg L^{-1}, while in the presence of 2 mg L^{-1} the abundance decreased, indicating a potential

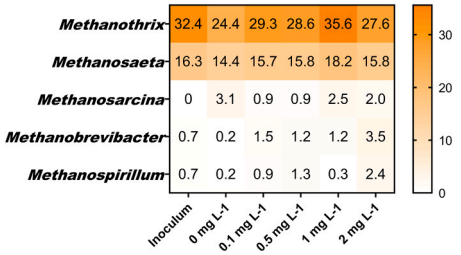

Fig. 5. Peptide contribution to the total of the sample of the active archaeal community at the taxonomic genus level.

Table 1
Number of peptides identified belonging to methyl-coenzyme M reductase (mcrA) protein of potential methanogens present in the bioreactor's microbial community at different concentrations of CIP applied (in mg L^{-1}).

Genera	Inoculum	Control (0 mg L^{-1} CIP)	0.1 mg L^{-1} CIP	0.5 mg L^{-1} CIP	1 mg L^{-1} CIP	2 mg L^{-1} CIP
Methanothrix	140	55	103	125	139	92
Methanosaeta	82	44	71	100	90	69

inhibitory threshold of the methanogenic activity. In longer operational periods (e.g. 26 days in [46]) CIP has been already described to negatively affect the methanogenic yield of anaerobic digestors at the macroscopic level. The presence of CIP in higher concentrations than those used in our study affected the microorganisms using acetic, propionic, and butyric acid utilization pathways ([47]; Tang et a, 2022) leading to an accumulation of VFAs, particularly acetate that has been proved to affect the final steps of methanogenesis. [47] investigated the molecular level determining the expression of mcrA gene in the presence of various antibiotics. Their results showed that mcrA expression is particularly sensitive to CIP, compared to tylosin and sulfaminidine, leading to a mcrA abundance 49 % lower in the presence of CIP than in the control. However, to the best of our knowledge, the specific mechanisms of mcrA inhibition by this antibiotic remain undescribed. Another possible explanation for the mcrA decrease in the presence of the highest CIP dose tested would be the accumulation of derived intermediate TPs, as already mentioned in [34]. Both hypotheses deserve more research effort in the future.

On the contrary, in the case of *Methanosarcina*, *Methanobrevibacter* and *Methanospirillum*, none of its detected proteins corresponded to mcrA (peptides values <1) and therefore these genera cannot be directly related to methanogenesis. So, these data suggest that different main activities for these taxa in the community in all the treatments applied and including the control.

Our results differ from the genomic-based study of [34] where *Methanosarcina* was the main methanogenic archaea in low CIP concentrations, while at a higher concentration (2 mg L^{-1}) this activity shifted to *Methanosaeta*. This was attributed to changes in acetate concentration during the operation of the bioreactors.

The corresponding gene of the catalytic subunit of Methyl-coenzyme M reductase (mcrA) is often used as a biomarker of methanogenic activity when qPCR analyses are applied to track microorganism activities [23]. It is therefore of great interest that a metaproteomic analysis can also offer this information, with the added value of identifying the taxa responsible for the activity.

The list of the identified proteins from *Methanosarcina* are: CO-methylating acetyl-CoA synthase (that works primarily through the Wood–Ljungdahl pathway which converts carbon dioxide to Acetyl-CoA) and Acetyl-CoA decarbonylase/synthase complex subunit beta (activates acetate into acetyl-CoA via the reductive acetyl-CoA pathway). Other proteins involved in the general maintenance of the cell structures, like the S-layer family duplication domain-containing protein, the S-layer PGF-CTERM sorting domain-containing protein, the V-type ATP synthase or movement (methyl-accepting chemotaxis proteins).

The proteins identified for *Methanobrevibacter* and *Methanospirillum* with assigned functions are Glutamate dehydrogenase and Glyceraldehyde-3-phosphate dehydrogenase) and for *Methanospirillum* (i.e., Methyl-coenzyme M reductase subunit beta and 5,10-methylenetetrahydromethanopterin reductase). Other proteins were not functionally assigned.

Regarding bacteria, the most active phylum were Bacteroidetes and Firmicutes. There is a positive correlation between Bacteroidetes and the concentration of erythromycin.

3.4. Enzymes potentially involved in CIP biotransformation

Certain enzymes have been linked to CIP biotransformation in previous literature reports. Specifically, Tang et al. [34] indicated partial adsorption of CIP outside of the bacterial cells, while the majority of its molecules crossed the cell membrane and entered the cell, being then degraded by the intracellular enzymes CYP450 enzymes, acetate kinases and dehydrogenases. In Wang et al. [41], their transcriptomics study highlighted the role of different types of dehydrogenases during CIP degradation in a simultaneous manner to VFA production. In [9], they describe a second quinolone resistance mechanism involving a mutant aminoglycoside-modifying enzyme (AAC(6′)-Ib-cr) capable of modifying certain quinolones, including CIP and norfloxacin, by adding an acetyl group, thereby reducing their antibacterial activity.

In our samples, we did not detect CYP450 enzymes or aminoglycoside modifying enzymes. However, we did detect different types of dehydrogenases, including F420-dependent methylenetetrahydrome thanopterin dehydrogenase (from the archaeal *Methanofollis*), malate dehydrogenase (from *Betaproteobacteria* members), 3-hydroxyacyl-CoA dehydrogenase (from *Deltaproteobacteria* members) and succinate dehydrogenase flavoprotein subunit (from *Bacteroidetes*). This presence of dehydrogenases is particularly interesting, as Wang et al. [41] speculated that the 23.3 % of dehydrogenases detected in their experiments play a role in CIP metabolism while converting starch into VFAs. However, in our study, the initial feeding was a mixture of VFAs, strengthening the hypothesis of their implication in the CIP biotransformation.

Finally, as mentioned above, the detection of TP-347 occurred in bioreactors where CIP concentrations were $\geq$ 1 mg L^{-1}, but its formation in reactors with CIP concentrations $<$ 1 mg L^{-1} cannot be discarded. Moreover, although CIP was effectively removed from the liquid phase, the removal efficiency of $>$ 90 % cannot be exclusively attributed to biotransformation since other processes, such as absorption/adsorption, can be involved. CIP was reported to stimulate the dehydrogenase-enzymes activity significantly [8]. The evidence provided hereafter: i) dehydrogenase enzymes were reported to catalyze hydroxylation reactions in anaerobic processes, ii) these are intracellular enzymes (intracellular degradation, and therefore, absorption of CIP), iii) the CIP removal efficiency is $>$ 90 %, and iv) the low relative abundance of TP, lead to attribute the biotransformation of CIP to dehydrogenase enzymes after the absorption of this emerging contaminant.

4. Conclusions

A high removal (>88 %) of CIP was observed during methanogenic conditions. The most likely removal mechanism was sorption for those experiments with higher initial antibiotic concentrations (>1 mg L^{-1}) and sorption + further biodegradation for the lower concentrations (<1 mg L^{-1}). Six TPs were identified and quantified by their relative abundance compared to the parent compound. The higher relative abundances were found in the lower initial concentrations, which supports the previous statement that biotransformation was likely carried out under these conditions. The biodegradation pathways for each compound were hydroxylation and loss of cyclopropyl group the main processes involved. *Methanothrix* and *Methanosaeta* were the main methanogens in all conditions and interestingly, the expression of the methanogenic activity biomarker methyl-coenzyme M reductase

decreased at 2 mg CIP L^{-1} suggesting an inhibitory threshold. Environmental proteomics analysis pointed to the potential key role of dehydrogenases enzymes during biological degradation under methanogenic anaerobic conditions, and not only under hydrolysis and acetogenesis processes.

Environmental implication

Antibiotic resistances are considered one of the main hazards to the humanity in the near future. Ciprofloxacin is ubiquitously detected in the urban water cycle and its biotransformation has been reported. However, the biological mechanisms involved are poorly understood yet, partially because the identification of transformation products and enzymes involved in these complex phenomena is technically challenging. In this study a combination of mass spectrometry and proteomics allowed us to determine hydroxylation and loss of the cyclopropyl ring and the key role of dehydrogenases enzymes during CIP biological transformations under methanogenic anaerobic conditions.

CRediT authorship contribution statement

Elisabeth Cuervo-Lumbaque: Writing – review & editing, Supervision, Investigation, Formal analysis, Data curation. **Oriol Casabella-Font:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Francisco Omil:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Jelena Radjenovic:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Matías Rivadulla:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Alba Trueba-Santiso:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sonia Suárez:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2025.139159.

Data availability

Data will be made available on request.

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