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## How mineral induced antibiotic transformation products 2 impact bacterial growth and denitrification activity

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PRE-PROOF

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## Abstract

18

20 The abiotic transformations of quinolones and tetracyclines facilitated by redox-active  
22 minerals has been studied extensively, however limited information is available regarding the  
24 antimicrobial activity and toxicity of their resultant transformation products. In this study, we  
26 first investigated the mechanisms underlying the transformation of two commonly used  
28 antibiotics, ciprofloxacin (CIP) and tetracycline (TC), by the ubiquitous redox soil mineral,  
30 birnessite (MnO<sub>2</sub>). Subsequently, we evaluated the impact of these transformation products  
32 on both the growth and activity of the environmental denitrifier *Pseudomonas veronii*.  
34 Following the reaction with birnessite, four transformation products for CIP and five for TC  
36 were identified. Remarkably, the antibacterial activity of both CIP and TC was lost upon the  
38 formation of transformation products during their interaction with birnessite. This loss of  
antimicrobial efficacy was associated with specific chemical transformations, such as the  
opening of the piperazine ring for CIP and hydroxylation and demethylation for TC.  
Interestingly, denitrifying activity, quantified in terms of nitrate reduction rates, remained  
unaffected by both CIP and TC at low concentrations that did not impact bacterial growth.  
However, under certain conditions, specifically at low concentrations of CIP, the second step  
of denitrification—nitrite reduction—was hindered, leading to the accumulation of nitrite.  
Our findings highlight that the transformation products induced by the mineral-mediated  
reactions of CIP or TC lose the initial antibacterial activity observed in the parent compounds.  
This research contributes valuable insights into the intricate interplay between antibiotics,  
redox-active minerals, and microbial activity in environmental systems.

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42 **Keywords:** antibiotic; abiotic degradation; minerals; nitrate reduction; nitrite production;  
44 environmental bacteria.

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## 48 1. Introduction

50 The extensive use of antibiotics and their subsequent presence in the environment  
(Kovalakova et al., 2020; Kummerer, 2009) as well as their impact on environmental bacteria  
(Kim and Carlson, 2007) are of increasing concern. Although the abiotic transformation of  
52 antibiotics has been documented (Loffler et al., 2023), little is known about the impact of  
these transformation products on microbial communities and ecosystem functioning. Two  
54 frequently detected antibiotics in the environment are the fluoroquinolone ciprofloxacin (CIP)  
and tetracycline (TC). CIP and TC transformation products have been detected in the  
56 environment, reaching ng- $\mu\text{g L}^{-1}$  concentrations (Loffler et al., 2023), so far the possible  
antimicrobial and ecotoxicological effects of these products remain largely unstudied.

58 Tetracyclines possess connected ring systems and are amphoteric molecules with  
multiple ionizable functional groups, inhibiting bacterial protein synthesis by binding 30S and  
60 50S ribosome subunits. Quinolones interfere in bacterial DNA replication and are electron  
donor-acceptor compounds with the piperazinyl group serving as the electron donor and  
62 carboxylic group as the electron acceptor in the neutral and zwitterion forms. Both CIP and TC  
have high adsorption capacities towards soil and sediment (Sassman and Lee, 2005; Zhou et  
64 al., 2011) and can be transformed upon interactions with environmental mineral surfaces,  
such as hematite ( $\alpha\text{-Fe}_2\text{O}_3$ ) and birnessite ( $\delta\text{-MnO}_2$ ) (Chen and Huang, 2011; Martin et al.,  
66 2015; Wang et al., 2016; Zhou et al., 2019). Generally, binding of redox-active antibiotics to  
Fe(III) or Mn(IV) oxides is followed by an electron transfer process resulting in the concomitant  
68 oxidation of sorbed compound and reduction of surface-bound metal. This electron exchange  
forms a radical, followed by a series of reactions including radical coupling, fragmentation,  
70 substitution and elimination to generate multiple byproducts (Li et al., 2022).

To the best of our knowledge, the impact of mineral induced CIP and TC transformation  
72 products on environmental bacteria, particularly beneficial microbes playing a role in the  
biogeochemical cycling of elements, has not been investigated. The only documented study  
74 examining the effects of manganese oxide-induced CIP transformation products tested the  
acute toxicity of the model organism *Vibrio fischeri*, suggesting a decrease in toxicity (Deng et  
76 al., 2017). Therefore, the goal of the current study was to investigate the effects of mineral  
induced transformation products on both bacterial growth and activity of an environmental  
78 relevant denitrifying bacterial isolate. Manganese oxide (birnessite), which is ubiquitous in  
soils and sediments, and was used as a redox-active mineral (Post, 1999). The Minimum  
80 Inhibiting Concentration (MIC), *i.e.* the concentration that prevents growth, of the  
environmental isolate *Pseudomonas veronii* towards CIP or TC was determined by broth  
82 micro-dilution susceptibility method (Reller et al., 2009; Wiegand et al., 2008). The  
antimicrobial activity of the corresponding transformation products were evaluated applying  
84 the same approach and compared CIP and TC. The toxicity of CIP or TC and their  
transformation products was also determined on the nitrate reducing and intermediate nitrite  
86 producing activity of *P. veronii* in mineral medium at CIP and TC concentrations below the MIC,  
allowing growth. These results are a first attempt to assess the antimicrobial activity of  
88 mineral induced transformation products on the growth and activity of environmental  
bacteria and processes.

90

## 92 2. Material and Methods

92

### 2.1. CIP and TC transformation in the presence of birnessite

94 ~~Manganese oxide (birnessite), which is ubiquitous in soils and sediments was used as a~~

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96 redox-active mineral. Acid birnessite was synthesized by reacting  $\text{KMnO}_4$  and  $\text{HCl}$  as described  
in previous studies (McKenzie, 1971). The purity of synthesized birnessite was confirmed by  
powder X-ray diffraction (XRD). The  $\text{N}_2$  Brunauer-Emmett-Teller (BET) specific surface area of  
98 the synthetic acid birnessite was  $65 \pm 1 \text{ m}^2 \text{ g}^{-1}$ .

100 CIP and TC transformations were carried out in 15 mL polyethylene tubes, wrapped with  
aluminum foil to eliminate the possibility of a photolysis reaction. The pH of the suspensions  
was adjusted to the desired values over the reaction time using an auto pH titrator with  
102 addition of small volumes of  $\text{HCl}$  (0.1 M) or  $\text{NaOH}$  (0.1 M) solutions. Aliquots of solution were  
periodically withdrawn and filtered through a  $0.20 \mu\text{m}$  membrane filter for analysis of  
104 antibiotic concentration and identification of transformation products.

For parent and transformation products analyses, previous published methods were  
106 employed (Martin et al., 2015; Wang et al., 2016; Zhou et al., 2019). Concentrations of CIP, TC  
as well as the transformation products were analyzed with a Waters Ultra LCMS (Acquity  
108 UPLC) system using a Waters BEH C18 column ( $100 \text{ mm} \times 2.1 \text{ mm}$ ,  $1.7 \mu\text{m}$ ) at a flow rate of  
 $0.4 \text{ mL}$  and a mass spectrometer. The mobile phase consisted of acetonitrile containing 0.1%  
110 of formic acid (eluant A) and mixture acetonitrile/water 10%/90% containing 0.1% of formic  
acid (eluant B) with gradient 0 min/0% A – 1 min/0% A – 9 min/100% A – 12 min/0% A, and a  
112 flow rate of  $400 \mu\text{L}/\text{min}$ . Mass spectrometric analysis was conducted by positive electrospray  
ionization with a mass scan range of  $m/z$  50–1000.

## 116 2.2. Antimicrobial activity assessment of CIP and TC transformation products

Growth of *P. veronii* was tested at different concentrations of CIP and TC (SI Figure 1).  
118 Based on these tests, growth was tested at high concentrations under optimal conditions on  
rich medium and oxic conditions in microtiter plates. The effects on denitrifying activities as  
120 nitrate and nitrite reduction rates were tested at concentrations allowing growth. All  
experiments were done in triplicate.

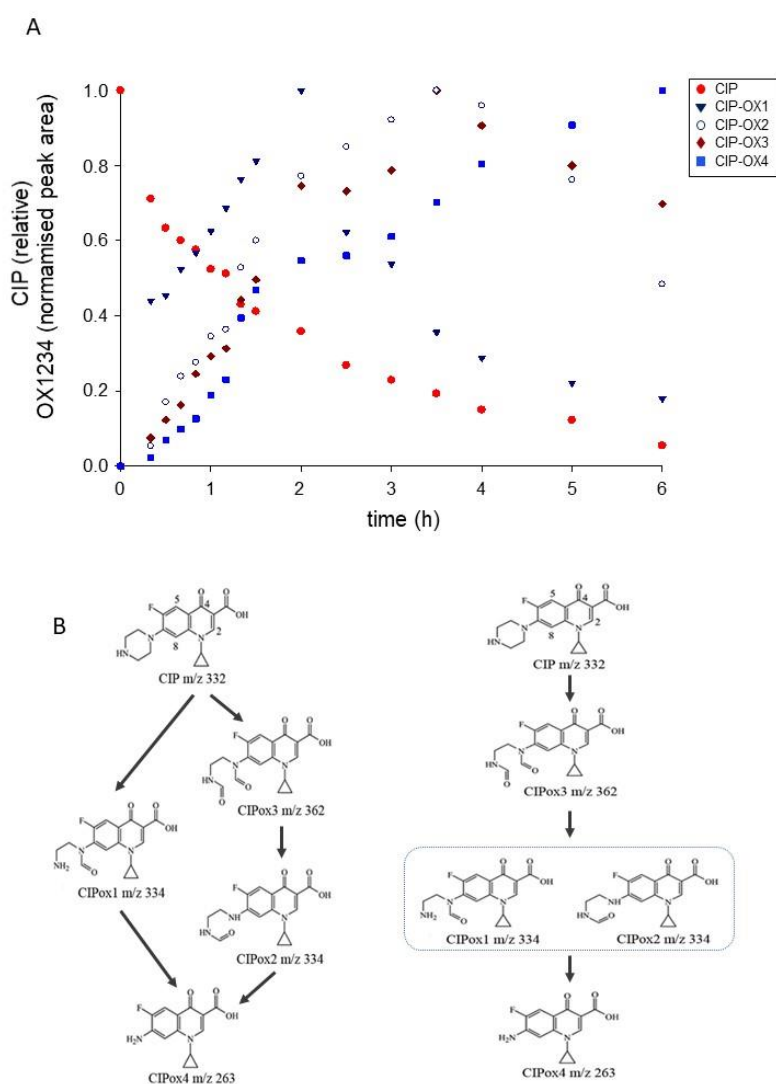
122 Growth curves of *P. veronii* were determined by inoculating  $100 \mu\text{L}$  aliquots of  
exponential growing cells ( $\text{OD}_{660}$  0.1,  $10^8 \text{ cells mL}^{-1}$ ) to a 96 well plate and exposed to CIP or TC  
124 as well as to their different transformation products. CIP concentrations of  $10 \mu\text{M}$  ( $3 \text{ mg L}^{-1}$ )  
and TC at  $100 \mu\text{M}$  ( $44 \text{ mg L}^{-1}$ ) were used, known to inhibit growth of *P. veronii* exceeding the  
126 Minimum Inhibiting Concentrations for CIP and TC, respectively  $0.5$  and  $16 \text{ mg L}^{-1}$  (See SI Figure  
1). Growth was determined in an antibiotic free control, in the presence of CIP or TC and the  
128 different transformation products generated at different times (1h interval, see 2.1). The  
plates were incubated at  $25^\circ\text{C}$  in a microplate reader (SAFAS Xenius XM, Monaco),  
130 continuously stirred (4Hz), measuring the  $\text{OD}_{660}$  every 10 minutes.

The effect of the antibiotics and their different transformation products on nitrate  
132 reducing activity was determined in batch experiments in mineral denitrifying medium in  $120$   
 $\text{mL}$  serum bottles. A mineral medium with  $5 \text{ mM}$   $\text{KNO}_3$  and succinate, lactate and acetate  
134 (each  $5 \text{ mM}$ ) at  $\text{pH } 7.00 \pm 0.05$  (Laverman et al., 2006). The sterile, anoxic medium ( $90 \text{ mL}$ ) was  
inoculated with  $10 \text{ mL}$  bacterial culture ( $\text{OD}_{660}$  0.1) with  $0.25 \mu\text{M}$  CIP ( $0.1 \text{ mg L}^{-1}$ ) or  $15 \mu\text{M}$  TC  
136 ( $6.7 \text{ mg L}^{-1}$ ) and the corresponding transformation products. These concentrations were lower  
than the MICs for CIP and TC, allowing growth and at the same time testing the impact on the  
138 denitrifying activity. The serum bottles were incubated at  $180 \text{ rpm}$  at  $25^\circ\text{C}$  and sampled twice  
a day for 3 days to determine  $\text{NO}_3^-$  and  $\text{NO}_2^-$  concentrations.  $\text{NO}_3^-$  and  $\text{NO}_2^-$  concentrations  
140 were determined on a Gallery Sequential analyser (Thermofisher)

### 3. Results and discussion

#### 3.1. Abiotic transformation of CIP and TC in birnessite suspension

Birnessite-mediated oxidation of CIP (100  $\mu\text{M}$ ) led to four transformation products, identified based on the retention time and mass-to-charge ratio, and named CIP-Ox1, CIP-Ox2, CIP-Ox3 and CIP-Ox4 (SI Table 1). The concentration of CIP decreased rapidly in the presence of birnessite. 50% CIP was transformed within 1 hour and less than 5% of the initial concentration remained after 6 hours of reaction time (Figure 1A). In accordance with previous reports investigating CIP transformation mechanisms by metal oxides (Chen and Huang, 2011; Martin et al., 2015; Zhang and Huang, 2005; Zhou et al., 2019), we have proposed two transformation pathways of CIP by birnessite as shown in Figure 1B.



152 **Figure 1. Relative abundance of CIP (C/C<sub>0</sub>) and the normalized peak areas of the CIP transformation**  
154 **products upon reaction with birnessite (A), peak areas of each compound were normalized towards**  
156 **the maximum peak area. Two proposed ciprofloxacin degradation pathways (B) based on the**  
**identification of transformation products in this study and related literature.**

158

The opening of the piperazine ring was expected due to the angle stress caused by inductive effect of electronegative fluorine atom. A “double iminium ion” species is likely to act as a reactive intermediate as previously reported (Zhang and Huang, 2005). Oxidation of the latter led to formation of a dialdehyde compound CIP-Ox3 (m/z 362), and two monoaldehyde compounds CIP-Ox1 and CIP-Ox2 (m/z 334), with differences in stability (Figure 1A). The concentration of CIP-Ox1 gradually decreased while the CIP-Ox2 and CIP-Ox3 peak intensities increased and accumulation of CIP-Ox4 (m/z 263). The abundance of CIP-Ox4 increased over the reaction time, suggesting this fully dealkylated product as a final product under our experimental conditions.

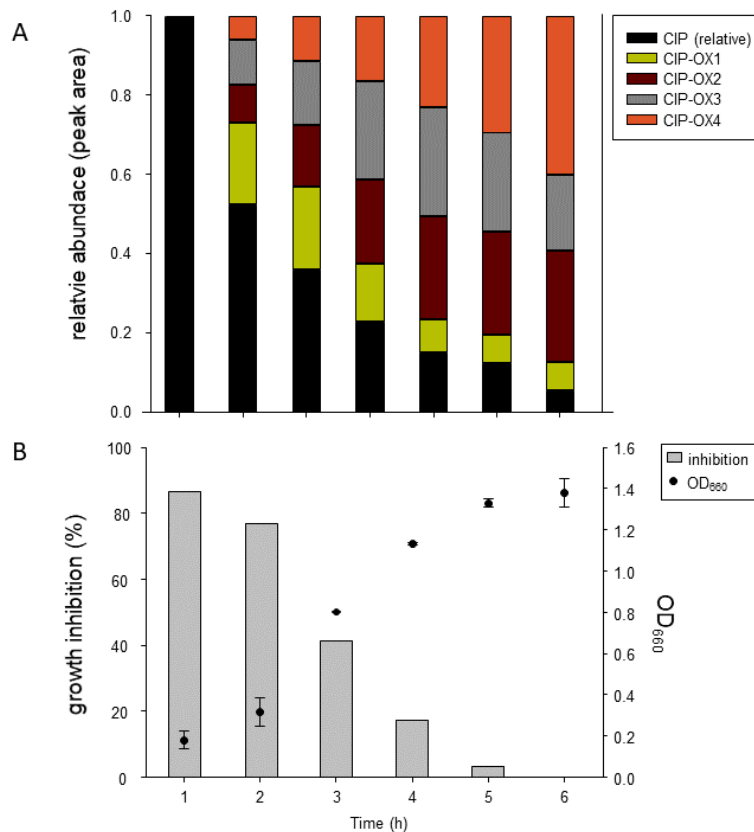
Five transformation products were identified by LC/MS during birnessite-mediated oxidation of TC (SI Table 2). TC showed a higher transformation rate than CIP, as almost 60% of initial TC was transformed by birnessite during the first 10 seconds. A possible reaction pathway of birnessite induced TC oxidation was proposed based on the identification of transformation products via LC/MS (SI Figure 2). Hydroxylation and demethylation have been suggested as the main transformation pathways in the birnessite induced oxidation (Chen and Huang, 2011). The D ring of TC is a phenol moiety which can readily form a phenoxy radical through one-electron transfer with MnO<sub>2</sub> (SI Figure 2). The phenoxy radical can be further oxidized by Mn<sup>IV</sup> to yield a hydroquinone product. The addition of a –OH group can occur at either the ortho or para position relative to the present phenolic –OH group. The demethylation process could be due to the oxidation of the dimethylamino group by Mn<sup>IV</sup>. The oxidation process first produces a methylene radical. This methylene radical can be further oxidized to an iminium cation, which undergoes rapid hydrolysis to yield N-demethyl derivative and formaldehyde.

182

### 3.2. Effect of the mineral induced transformation products on growth of *P. veronii*

The generated transformation products, collected at different time intervals in the presence of birnessite, with different proportions of the transformation products (Figure 2A) showed different antimicrobial activities (Figure 2B). CIP inhibited cell growth (85%), with the reaction time increasing, the concentration of CIP gradually decreased, coinciding with a decreasing antimicrobial activity (Figure 2B), suggesting that the antimicrobial activity is alleviated upon exposure to the transformation products.

190



192 **Figure 2. Relative abundance of CIP and CIP transformation products upon reaction with birnessite**  
 194 **over time (A). Growth at 24h (OD<sub>660</sub>) as well as growth inhibition compared to a positive control,**  
 196 **upon exposure to the CIP and the CIP transformation products (B). The growth exposure tests**  
 196 **(triplicate) were done with 10  $\mu$ M CIP (3 mg L<sup>-1</sup>) and the corresponding transformation products.**

198 The carboxylate and carbonyl groups are considered essential to the antimicrobial activity  
 200 due for binding to cleaved or perturbed DNA (Tillotson, 1996), whereas the fluorogroup on  
 202 fluoroquinolone molecules facilitates cell penetration and gyrase affinity (Peterson, 2001).  
 204 The piperazine ring improves the effect of CIP by facilitating the cell penetration (Domagala,  
 1994). The quinolone core structure with its essential functional groups for gyrase binding  
 206 remained unaffected in the present birnessite system. Therefore, based on the molecular  
 208 structure of the transformation products (fluoro, carboxylate and carbonyl group present), an  
 210 antimicrobial activity would have been expected. Indeed, the piperazine ring plays a crucial  
 206 role in the antimicrobial efficacy of CIP. This structural component facilitates the penetration  
 208 of CIP into bacterial cells. Without the presence of this ring, it is probable that CIP cannot  
 210 efficiently enter the cell, thereby thwarting its antimicrobial effects, which primarily target the  
 210 activity of DNA gyrase. Consequently, the loss of the piperazine ring likely mitigates the  
 210 toxicity associated with the transformation products.

212 Growth of *P. veronii* was completely inhibited in the presence of 100  $\mu$ mol L<sup>-1</sup> (44 mg L<sup>-1</sup>)  
 214 TC, whereas the growth inhibition was overruled in the presence of TC transformation  
 214 products (data not shown). The antimicrobial activity of the tetracycline molecule relies on  
 216 the linearly arranged DCBA naphthacene ring system with an A-ring diketo substructure and  
 216 an exocyclic carbonyl or amide group (Fuoco, 2012). The LC/MS results showed that the A-ring  
 216 dimethylamino group of the transformation products were changed by a demethylation



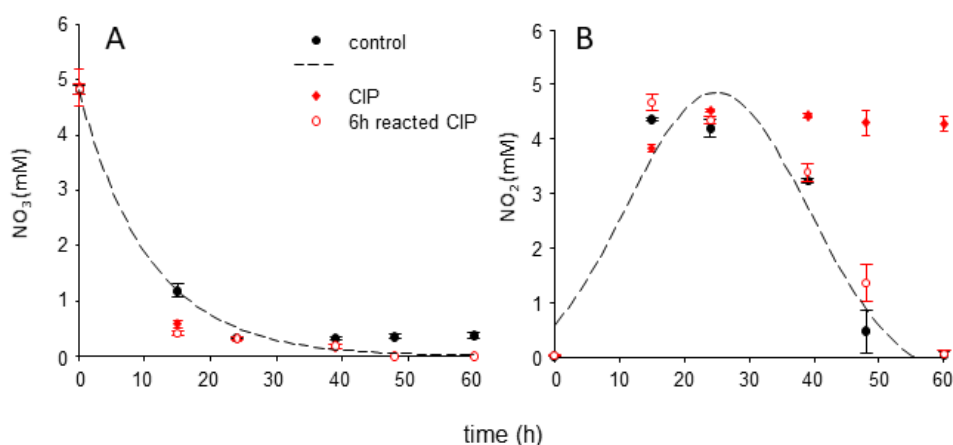
218 process. The A-ring dimethylamino group is pivotal for the mode of action of TC, part of the  
220 lower peripheral non-modifiable region of the TC molecule that interacts with the 30S rRNA.  
The change in the A-ring dimethylamino group is most likely responsible for the loss of the  
antimicrobial activity of the TC transformation products.

222

### 224 3.3 Effect of CIP and TC transformation products on denitrifying activity

The effect of low, environmentally relevant concentrations of CIP and TC as well as the  
226 associated transformation products were tested on the denitrifying activity of *P. veronii*  
(Figure 3). Nitrate concentrations decreased rapidly for all the treatments and consequent  
228 production of nitrite was observed. More than 85% of the nitrate was reduced into nitrite  
within 15 hours for the positive control as well as the CIP and the CIP transformation products  
230 (6h reacted CIP) exposed cultures, without significant differences (student t-test,  $p < .05$ ). The  
produced nitrite was rapidly further reduced in the positive control as well as in the CIP  
232 transformation products treatment. During exposure to CIP, in which no effect was observed  
on the reduction of nitrate (Figure 3A), nitrite accumulated and was not further reduced to  
234 gaseous nitrogen. Interestingly, at these low concentrations of CIP, with neither effect on  
growth nor nitrate reduction, the denitrification process is inhibited at the step of nitrite  
236 reduction.

238



240 **Figure 3. Concentrations of NO<sub>3</sub><sup>-</sup> (A) and NO<sub>2</sub><sup>-</sup> (B) over time in the positive control, exposed to 0.25**  
242 **μM CIP and the 6h reacted CIP (CIP-Ox1234, see figure 2A) transformation products (triplicate). Dotted lines indicate the nitrate reduction rate (A) and the production and reduction of nitrite (B)**  
244 **in the positive control. The dotted lines were fitted employing the "exponential decay" function for nitrate and a "sigmoidal" function for nitrite in Sigmaplot, solely for the purpose of illustrating**  
246 **the trends.**

248 Interestingly, low concentrations of CIP inhibited the reduction of nitrite to gaseous  
nitrogen, without impacting growth. It appears thus nitrate reducing enzymes (NAR) were  
250 unaffected, whereas the further reduction of nitrite into NO, N<sub>2</sub>O and N<sub>2</sub> is inhibited in the  
presence of CIP, possibly due to an effect of CIP on the nitrite reductase (NIR). The stable

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252 embedding of nitrate reductase (NAR) in the membrane, compared to the freely distributed  
253 nitrite reductase in the periplasm (Zumft, 1997) could make the latter more vulnerable to CIP.  
254 This is in line with previous observations that showed a lower expression of the *nirS* gene  
(coding NIR) than *narG* gene (coding NAR) upon exposure to CIP (Li et al., 2021)..

256 The antimicrobial activity of the mineral induced TC oxidation products was also tested  
257 on *P. veronii*. The tested TC concentration, which was below the MIC of 16 mg L<sup>-1</sup> (15 μmol L<sup>-1</sup>,  
258 6.7 mg L<sup>-1</sup>), to allow some growth, had no impact on growth and denitrifying activity of *P*  
259 *veronii* compared to the control (data not shown). Apparently at these low TC concentrations  
260 *P. veronii* resisted towards the antibiotic. Consistent with the lack of antibacterial activity of  
261 these low concentrations (below the MIC), the TC transformation products also lacked  
262 antibacterial activity.

263 The current study shows the importance of abiotic transformation by redox-active  
264 minerals and the loss of antibacterial activity. It is noteworthy that antibiotics, besides  
265 undergoing transformation via mineral surfaces, can adsorb to mineral surfaces found within  
266 sediment-water interfaces. The extent of adsorption determines the availability of antibiotics  
267 for microbial degradation and can affect their persistence in aquatic environments. In addition  
268 to this certain bacteria can metabolize antibiotics. Besides abiotic transformation by minerals,  
269 these minerals may influence the rates of antibiotic degradation by providing surfaces for  
270 microbial attachment and facilitating nutrient exchange and metabolic interactions. Mineral-  
271 mediated adsorption of antibiotics can sequester these compounds, limiting their  
272 bioavailability and potential for microbial degradation. In turn, minerals may serve as  
273 substrates for microbial colonization and biofilm formation at sediment-water interfaces,  
274 these microbial-mineral interactions can influence microbial community composition,  
275 diversity, and metabolic activities, ultimately impacting antibiotic degradation processes.  
276 These interactions among antibiotics, minerals, and microorganisms at sediment-water  
277 interfaces can create feedback mechanisms that modulate the fate of antibiotics.

278

## 4. Conclusion

280

281 Abiotic transformations of pharmaceuticals, particularly antibiotics, can occur in  
282 environments rich in redox-active minerals. These transformations play a crucial role in  
283 reducing the levels of parent compounds while concurrently generating multiple degradation  
284 products. Our study focused on birnessite-induced transformation products of ciprofloxacin  
(CIP) or tetracycline (TC) and their impact on the antibacterial growth of the environmental  
285 denitrifying isolate *Pseudomonas veronii*.

286 Notably, our results revealed that the transformation products induced by birnessite did  
287 not exhibit any antibacterial effect on the growth of *P. veronii*. This suggests that the  
288 antimicrobial activity, observed in the parent compounds, was lost during the abiotic  
289 transformation process. It is essential to note, however, that our investigation is confined to  
290 a limited number of antibiotics and minerals, necessitating a broader exploration  
291 encompassing different antibiotic families and various soil minerals to derive more  
292 comprehensive conclusions.

293 Additionally, our observations indicated that a low concentration of CIP had no  
294 discernible impact on bacterial growth but did affect denitrifying activity, leading to nitrite  
295 accumulation. This underscores the need for a focused examination of the effects of lower  
296 antibiotic concentrations on both the growth and activity of environmental bacteria. In  
297 conclusion, our findings underscore the intricate dynamics between antibiotics, redox-active  
298 minerals, and microbial activity, emphasizing the importance of expanding research to  
299 encompass a wider range of antibiotics and minerals for a more holistic understanding.

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384 *Statement and Declarations*

386 **Compliance with Ethical Standards:**

- 388 • Ethical approval. The authors mentioned in the manuscript have agreed for authorship, read and approved the manuscript, and gave consent for submission and subsequent publication of the manuscript.
- 390 • Consent to participate: All authors have consented the manuscript content for publication.
- 392 • Consent to publish: The paper is submitted with the consent of all listed authors.

394 **Author contributions:**

396 Conceptualization [Khalil Hanna, Anniet Laverman]; Methodology: Formal analysis: [Chen  
396 Chen], Writing - original draft preparation: [Chen Chen, Anniet Laverman]; Writing - review  
and editing: [Khalil Hanna, Celine Roose-Amsaleg, Anniet Laverman]; Funding acquisition:  
398 [Khalil Hanna], Supervision: [Khalil Hanna, Celine Roose-Amsaleg, Anniet Laverman]

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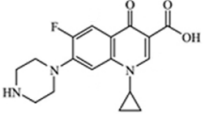
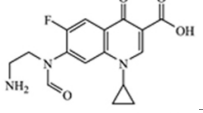
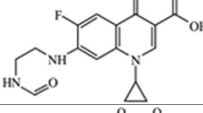
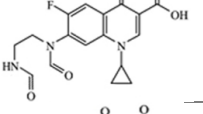
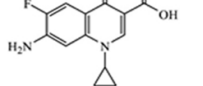
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404 **Competing Interests:** The authors declare that they have no known competing financial  
406 interests or personal relationships that could have appeared to influence the work reported  
in this paper.

408 **Availability of data and materials:** The study’s data are available on request.

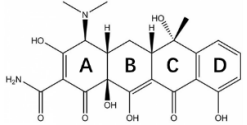
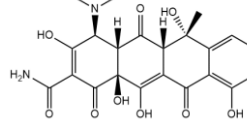
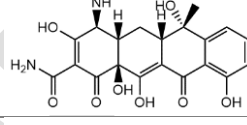
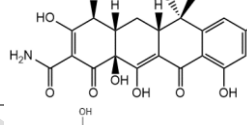
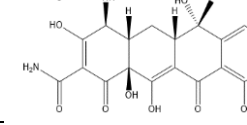
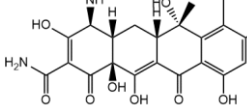
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412 **Supplementary Information**414 **Tables**416 **SI Table 1. LC/MS data for birnessite induced degradation of CIP**

Compound name	Retention (min)	time	m/z value	(Proposed) structure
CIP	3.8		334	
CIP-Ox1	3.0		332	
CIP-Ox2	4.7		362	
CIP-Ox3	5.4		334	
CIP-Ox4	5.8		263	

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SI Table 2. LC/MS data for birnessite induced degradation of TC

Compound name	Retention time (min)	m/z value	(Proposed) structure
TC	4.1	455	
TC ox 1	2.3	459	
TC ox 2	4.2	431	
TC ox 3	2.5/4.0	447	
TC ox 4	5.5	461	
TC ox 5	1.1/1.5/2.5/4.0	463	

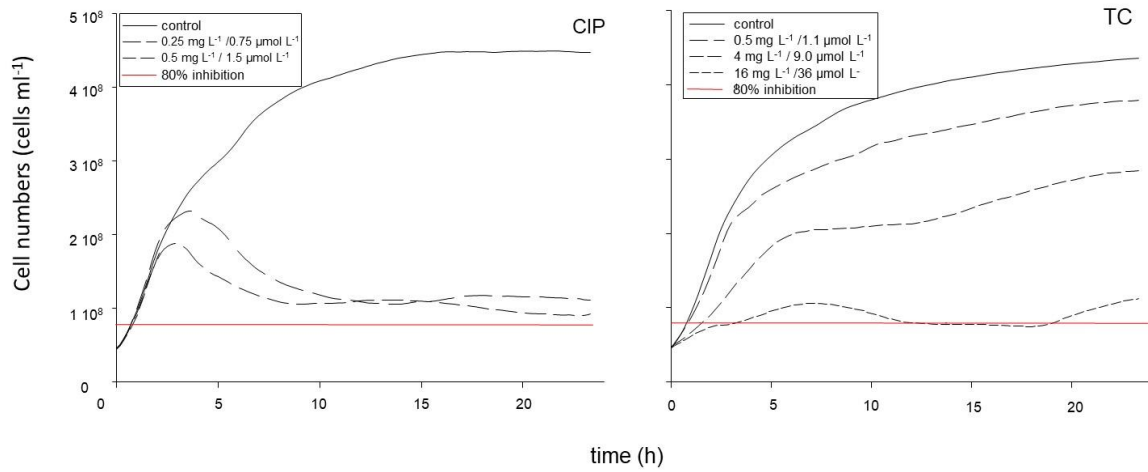


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## Figures

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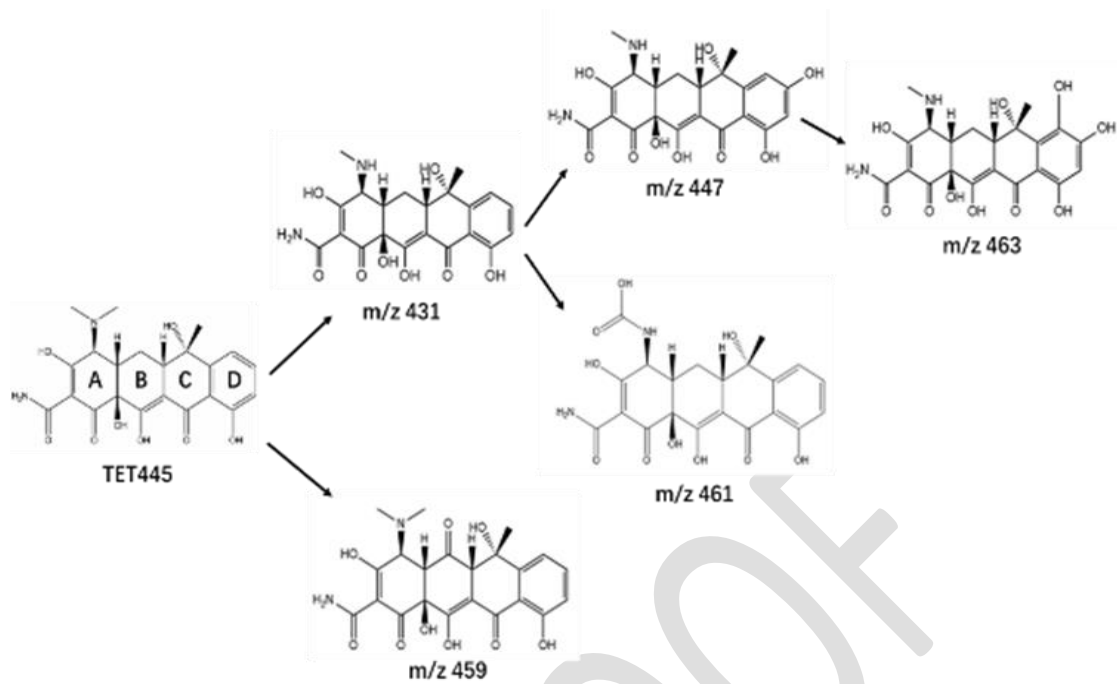
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SI Figure 1. Growth curves of *P. veronii* at different CIP (left panel) and TC (right panel) concentrations indicated in mg L<sup>-1</sup> as well as in μmol L<sup>-1</sup>. The red line indicates the 80% growth inhibition compared to the positive control. The Minimum Inhibiting Concentrations were deduced from the 80% inhibition compared to the control and were 0.5 mg L<sup>-1</sup> for CIP and 16 mg L<sup>-1</sup> for TC



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SI Figure 2. Proposed TC degradation pathway based on the identification of transformation products in this study and related literature.