Supplementary Material

Divergent impacts of antibiotics on ferrihydrite biotransformation by *Shewanella oneidensis*: electron transfer regulation and iron redox cycling

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Content summary

Supplementary materials and methods

Text S1

Sources of chemicals. LB broth was purchased from Sangon Biotech. MOPs and $FeCl_3 \cdot 6H_2O$ were purchased from Aladdin at 99.0% purity and analytical grade, respectively. Penicillin G Sodium (PCG) was purchased from Solarbio at 95% purity. Lincomycin (LCM), acetonitrile, and methanol were all purchased from Sigma-Aldrich at 99.5%, 99.9%, and 99.9% purity, respectively. Other reagents used (e.g. NaCl, L-sodium lactate, sodium fumarate, sulfamethoxazole (SMX), ciprofloxacin (CIP), erythromycin (ERY), tetracycline (TC), and chloramphenicol (CAP), etc.) were all purchased from Macklin at analytical grade. All solutions were prepared using Milli-Q ultrapure water. Furthermore, to facilitate the dissolution process, hydrochloric acid and acetic acid were added to dissolve SMX and CIP, respectively. Ethanol served as the solubilizing agent for ERY, TC, and CAP.

Text S2

Synthesis of ferrihydrite (Fh). Fh was synthesized by following a previous study.[1] In brief, a solution of 0.4 M $FeCl_3 \cdot 6H_2O$ was mixed with a 1 M NaOH solution on a magnetic stirrer for a duration of 3 h, until the pH reached a range of 7.0 to 8.0. Subsequently, the suspension was allowed to react for an additional 6 h. To purify the suspension, ultrapure water was added to remove the supernatant, and this process was repeated at least 5 times until the residual chloride concentration was reduced to below 1 mM. The resultant purified Fh slurry was resuspended in ultrapure water and stored in a refrigerator at 4°C until needed. The concentrations of Fe(III) and Fe(II) ions were then determined using a spectrophotometric method, as described in a later section. The scanning electron microscopy (SEM, MIRA3 LMH, Czech Republic) and particle size analyzer (Brookhaven Instruments Co, NanoBrook Omni, USA) were used to characterize the particle size distribution of synthesized Fh.

Text S3

Measurement of Fe(II) concentration. To characterize the reduction degree of Fh, a modified *o*-phenanthroline spectrophotometric method was adopted to quantify Fe(II) concentration.[2] Initially, 2 mL of the suspension was collected and completely dissolved in 6 M hydrochloric acid (HCl). A specific volume of the aforementioned acidized solution was mixed into 0.5 mL of acetate buffer (prepared by dissolving 400 g of ammonium acetate and 500 mL of ice acetic acid per liter, adjust to pH 4.5). Subsequently, 0.2 mL of 0.5 wt% *o*-phenanthroline solution was added, and the mixture solution was diluted with ultrapure water to a final volume of 5 mL. The absorbance was measured at 510 nm by UV-vis UV-1780 spectrophotometers, after chromogenic reaction for 15 min. To determine the total iron (Fe_{total}) concentration, aliquots of the aforementioned acidized solution were mixed with 1 mL of 10 wt% hydroxylamine hydrochloride solution to fully reduce Fe(III) ions to Fe(II) ions. The total Fe(II) concentration (equal to Fe_{total}) was then determined using the *o*-phenanthroline spectrophotometric method. Subsequently, the Fe(III) concentration was calculated by subtracting the Fe(II) concentration from the Fe_{total} concentration.

Text S4

Determination of concentrations and byproducts for SMX and CAP. At each sampling time, 2 mL of the suspensions were collected from the incubations and filtered through a 0.22 μm nylon filter. Subsequently, the filtrate samples were filtered using a sodium type cation exchange resin filter (IC-Na 1cc) to remove iron, after which the concentrations of SMX and CAP, along with product determination, were conducted. Concentrations of SMX and CAP were measured using ultra performance liquid chromatography with photo-diode array detection (UPLC-PDA; ACQUITY H-class).[3, 4] The stationary phase was a ACQUITY UPLC® BEH C18 (1.7 μm，2.1 mm × 50 mm). The mobile phase flowed at a rate of 0.1 mL min^{-1}, with an injection volume of 10 μL. SMX and CAP were detected at wavelengths of 268 nm and 278 nm, respectively. For SMX concentration analysis, an isocratic mobile phase composed of 80% aqueous buffer (20 mM phosphate buffer adjusted to pH 3.0) and 20% acetonitrile (v/v) was used. In addition, CAP concentration analysis utilized a mobile phase containing 40% water (0.1% formic acid) and 60% methyl alcohol (v/v).

Furthermore, the degradation products of SMX and CAP were determined using a liquid chromatography system (Agilent 1290uplc) coupled with a quadrupole time-of-flight mass spectrometer (Agilent qtof6550), equipped with an electrospray ion (ESI) source.[3, 4] The stationary phase for this analysis was ACQUITY UPLC® BEH C18 (1.7 μm，2.1 mm × 100 mm). The mobile phase flowed at a rate of 0.3 mL min^{-1}, with a sample size of 5 μL. To test the byproducts of SMX, mobile phases A (0.1% formic acid) and B (acetonitrile) were used. The elution gradient began with 100% phase A for 4 min, followed by a transition to 90% phase A and 10% phase B within 2 min. Then, it was converted to 60% of phase A and 35% of phase B within 4 min, kept for 3 min, and finally switched back to 100% phase A within 1 min, which was maintained for 1 min. For CAP byproducts, mobile phases A (0.1% formic acid) and B (methanol) were used, with a 50% elution ratio of phase A and 50% phase B, retention time of 15 min. The mass charge ratio (m/z) of MS analysis was 50~1000, with a sheath temperature of 350℃, a sheath gas flow of 12 L min^{-1}, a voltage of 4000 V in ESI^+ mode, and 3200 V in ESI^-mode.

Text S5

Extraction of extracellular polymeric substance (EPS). The heating method was used according to a previously reported procedure.[5] Initially, 20 mL of the reaction suspension (containing cells) was collected and washed with PBS (0.1 M, pH=7.0). Subsequently, the cells were dispersed in 10 mL of 0.05% (wt%) NaCl solution. The suspension was then heated in a water bath for 30 min at 70℃, centrifuged at 8000 g for 15 min, and the supernatant was regarded as the extracted EPS.

Text S6

Electrochemical analyses. Differential pulse voltammetry (DPV) was performed by an electrochemical workstation (Multi Autolab M204, Metrohm, Switzerland).[6] A conventional quartz three-electrode electrochemical cell was used for all electrochemical experiments with glassy

carbon electrode (GCE), platinum wire, and Ag/AgCl (sat. KCl) as working, counter, and reference electrodes, respectively. For the determination of the extracellular electron transfer type of MR-1, 5 μL of bacterial droplets, washed twice with PBS (100 mM, pH=7.0), were absorbed onto the surface of the GCE. After drying, 2 μL 5% Nafion dispersion was applied to form a film on the surface. The electrolyte, containing 30 mM MOPS (pH=7.0) and 25 mM KCl, was deaerated by bubbling with N_2 for more than 45 min. The DPV parameters were set as follows: E_i = -0.6 V; E_f = 0.4 V; amplitude, 60 mV; pulse width, 200 ms; and potential increment, 6 mV.

Tables

Table S1. The concentrations of antibiotics used in this study.

Antibiotic species	Working concentration (μg/mL)	Antibiotic concentration per unit of bacteria in this system (μg/cells)	Environmental concentration (μg/kg)[7, 8]		Location	Antibiotic concentration per unit of bacteria in environment (μg/cells)
			min	max		
SMX	100.00	2×10^{-6}	1.80	6207.60	Animal farm manure	2.00×10^{-12} - 8.87×10^{-3}
CIP	100.00	2×10^{-6}	1.50	10111.10	Animal farm manure	1.67×10^{-12} - 1.44×10^{-2}
ERY	100.00	2×10^{-6}	4.20	91.00	Animal farm manure	4.67×10^{-12} - 1.30×10^{-4}
PCG	100.00	2×10^{-6}	0.01	974.00	sediment	1.11×10^{-14} - 1.39×10^{-3}
TC	50.00	1×10^{-6}	4.50	9260.00	Animal farm manure	5.00×10^{-12} - 1.32×10^{-2}
LCM	50.00	1×10^{-6}	164.00	17000.00	Animal farm manure	1.82×10^{-10} - 2.43×10^{-2}
CAP	25.00	5×10^{-7}	42.20	42.20	Animal farm manure	4.69×10^{-11} - 6.03×10^{-5}

Given that in natural environments, there are 10^3~10^7 microorganisms in 1 g of soil,[9] and soil bacteria account for 70%~90% of the total number of soil microorganisms, the bacteria count in the environment can be estimated to be the range of 7×10^2 to 9×10^8 cells g^{-1}.

Table S2. Specific primer sequences for quantitative real-time PCR analysis of Mtr pathway-associated genes in MR-1.

Gene ID	Description	Primers
	16sDNA	Forward sequence (5'-3'): CCCTGGACAAAGACTGACGC Reverse sequence (5'-3'): AGACACCAAACTCCGAGTAGACA
SO_RS08155	OmcA	Forward sequence (5'-3'): AAGGTGGCGAGCGTCATAC Reverse sequence (5'-3'): CACAATCTGCGGCTGGTTT
SO_RS21330	CymA	Forward sequence (5'-3'): GATTGGTGTTGTGGGCTATTTT Reverse sequence (5'-3'): GATGCCAGCACTTCATTCTTC
SO_RS08145	Mtr A	Forward sequence (5'-3'): CGTATGTCTTGGAATGGCG Reverse sequence (5'-3'): TGAGTGGGTGACTTGAGCGT
SO_RS08140	Mtr B	Forward sequence (5'-3'): TTACTGGCAACCGCCTTCT Reverse sequence (5'-3'): CTGCACCGACCACATCTACC
SO_RS08150	Mtr C	Forward sequence (5'-3'): CGATTTTGCTGCGGGTAA Reverse sequence (5'-3'): TGAACACTAATTGTGGCTGCTT
SO_1782	Mtr D	Forward sequence (5'-3'): ATCCCAACATCAAAGATTCCC Reverse sequence (5'-3'): GTTGCCTTTCCAAGCGATAC
SO_1781	Mtr E	Forward sequence (5'-3'): CGGACTTACCCACGTTTCTTA Reverse sequence (5'-3'): TACTGCCCGCTTGATCTGA
SO_1780	Mtr F	Forward sequence (5'-3'): AAGCATCAGCAGTGCCAGTAT Reverse sequence (5'-3'): GTTCCCCAGTTAGCATAAATCC

Table S3. Mössbauer hyperfine parameters of spectra from Figure 2 measured at room temperature.

Sample	Attribution	δ (mm/s)	Δ or ε (mm/s)	HF (T)	RA (%)
Initial Fh substrates					
Fh	Fh	0.27	0.75	—	100
Biotic transformation products in 5 h					
CK	Goethite	0.27	0.51	—	10.5
	Fh	0.26	0.79	—	89.5
SMX added	Goethite	0.24	0.52	—	24.7
	Fh	0.24	0.84	—	75.3
CAP added	Goethite	0.27	0.53	—	24.0
	Fh	0.26	0.85	—	76.0
Biotic transformation products in 1 month					
CK	Magnetite (tetra)	0.11	-0.19	48.38	38.4
	Magnetite (octa)	0.63	0.25	47.68	33.7
	Goethite	0.34	-0.07	31.40	21.2
	Fh	0.34	2.28	—	6.7
SMX added	Magnetite (tetra)	0.04	-0.26	48.33	38.6
	Magnetite (octa)	0.51	0.23	47.74	36.3
	Goethite	0.02	0.25	29.68	18.3
	Fh	0.39	2.09	—	6.7
CAP added	Magnetite (tetra)	0.19	-0.21	49.46	2.8
	Magnetite (octa)	0.43	0.21	46.76	16.2
	Goethite	0.24	-0.08	30.38	54.6
	Fh	0.23	0.76	—	26.4

δ, isomer shift taking α-iron as reference at room temperature; Δ or ε, quadrupole splitting; HF, hyperfine magnetic field; RA, relative abundance; tetra, tetrahedral site; octa, octahedral site.

Table S4. Fe(II) concentrations at 5 h for each treatment group in Figure 4a and significance analysis.

Treatment group	Fe(II) concentration (mM)	Significance vs. CK	Significance vs. Corresponding Direct Treatment Group
CK	0.81 ± 0.07	-	-
MR-1 + SMX + Fh	1.70 ± 0.04	$P < 0.01$ (**)	-
pre-equilibrated MR-1 + SMX	1.00 ± 0.13	$P > 0.05$ (ns)	$P < 0.01$ (**) (vs. MR-1 + SMX + Fh)
MR-1 + CAP + Fh	0.36 ± 0.06	$P < 0.01$ (**)	-
pre-equilibrated MR-1 + CAP	0.31 ± 0.02	$P < 0.01$ (**)	$P > 0.05$ (ns) (vs. MR-1 + CAP + Fh)

All data are expressed as mean ± standard deviation. Statistical analysis was performed using one-way ANOVA followed by a pairwise test. Significance levels are marked as follows: **$P < 0.01$ (extremely significant difference), *$P < 0.05$ (significant difference), ns (no significant difference, $P > 0.05$).

158 **Table S5.** The fluorescence intensities of peaks in 3D-EEM of EPS fractions excreted by MR-1
159 incubated with SMX and CAP at 5 h of incubation.

	Peak A		Peak B		Peak C	
	Ex/Em (nm)	Intensity (AU)	Ex/Em (nm)	Intensity (AU)	Ex/Em (nm)	Intensity (AU)
CK	285/335	46.38	365/445	48.13	445/520	90.79
SMX	290/340	77.87	360/450	47.17	445/520	87.95
CAP	290/340	28.19	-	-	445/520	75.78

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Table S6. The pseudo-first-order kinetic consumption rates of SMX and CAP.

	Pseudo-first-order kinetic consumption rates of antibiotics (h^{-1})		
	antibiotic + MR-1	antibiotic + Fh	antibiotic + MR-1 + Fh
SMX	0.001	0.001	1.41
CAP	0.063	0.0008	0.91

Figures

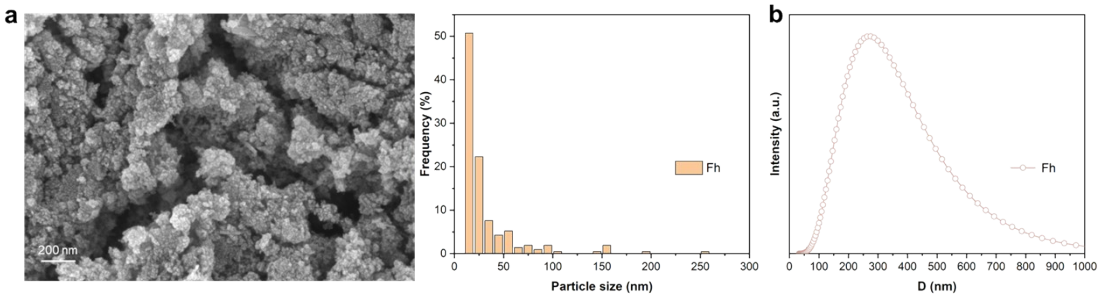


Figure S1. Morphology and particle size distribution of synthesized Fh. (a) SEM image and particle size histogram derived from SEM; (b) particle size distribution determined by dynamic light scattering.

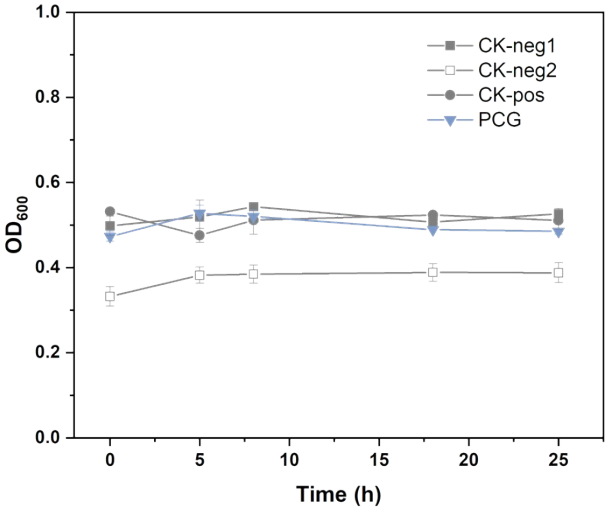


Figure S2. Biomass of MR-1 incubated with PCG. CK-neg1: MR-1 cultured without sodium lactate; CK-neg2: heat-killed MR-1 cells cultured with sodium lactate. The treatment with PCG did not affect the biomass of MR-1.

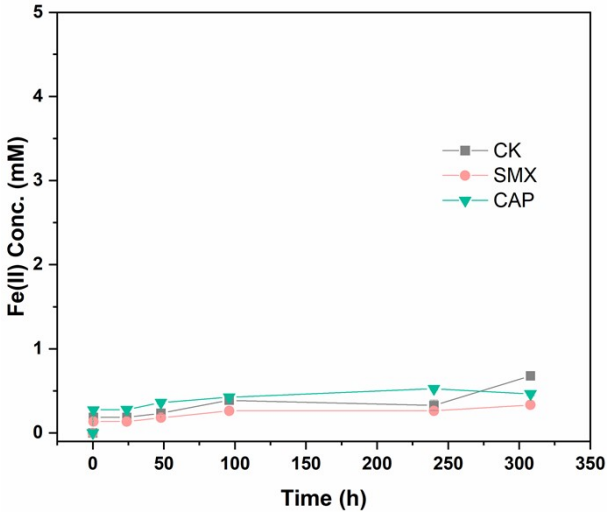
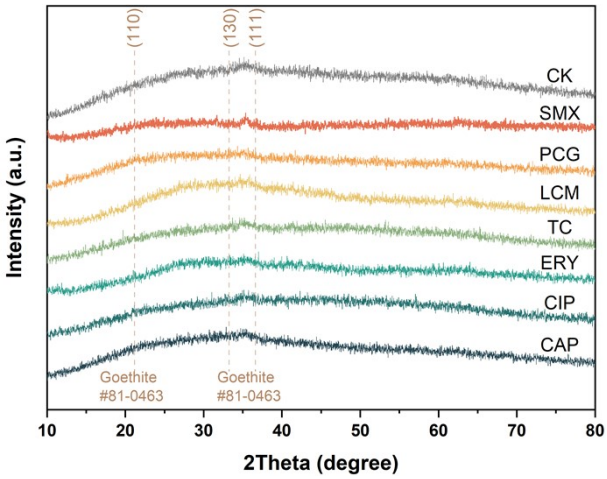


Figure S3. Total Fe(II) production of Fh incubated with SMX or CAP. Fe(II) production of Fh induced by SMX or CAP alone was negligible.



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176 **Figure S4.** XRD spectra of the solids from cultures incubated for 5 h with the addition of various
177 antibiotics. The dashed lines correspond to the positions of the characteristic peaks of goethite.

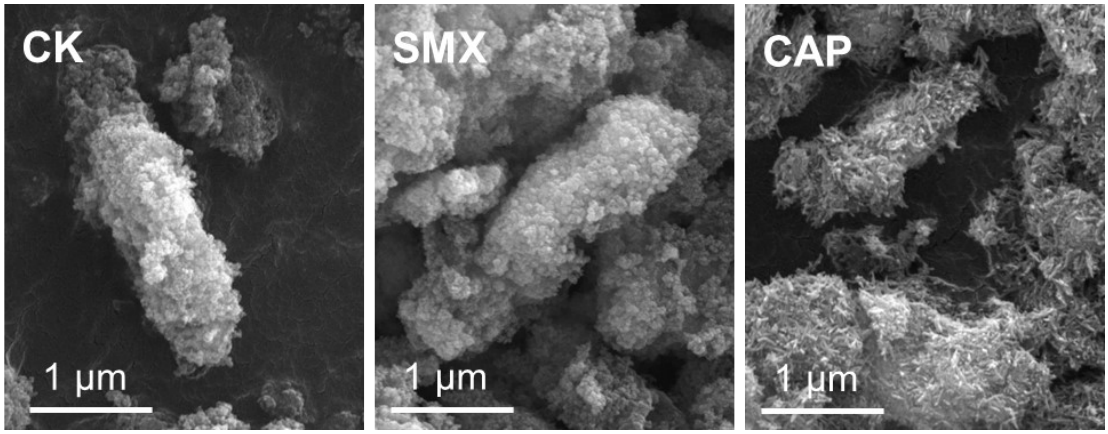


Figure S5. SEM images of solids in cultures incubated for one month with SMX and CAP added.

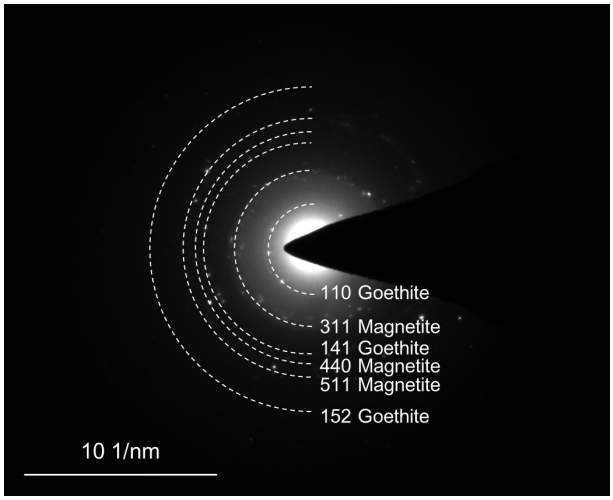
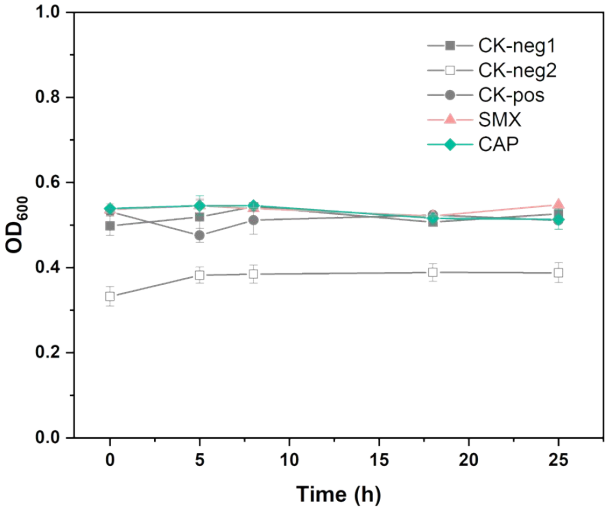


Figure S6. SAED pattern of solids from the biotransformation of Fh with CAP after one month of incubation. The dashed lines correspond to the positions of the characteristic crystal planes of goethite and magnetite, respectively.



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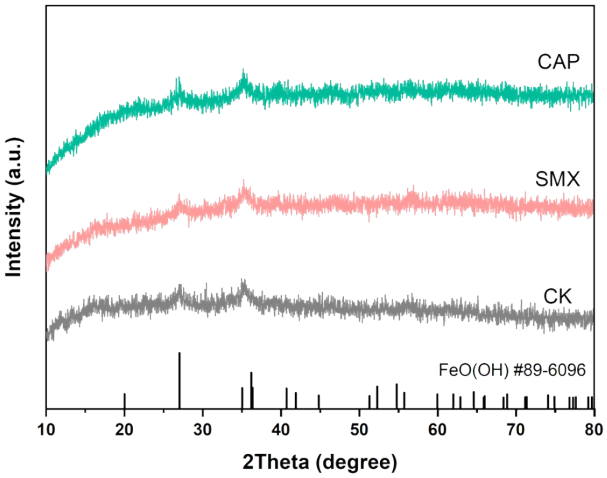
185 **Figure S7.** Biomass of MR-1 incubated with SMX or CAP. CK-neg1: MR-1 cultured without
186 sodium lactate; CK-neg2: heat-killed MR-1 cells cultured with sodium lactate. Treatments with
187 SMX or CAP did not affect the biomass of MR-1.



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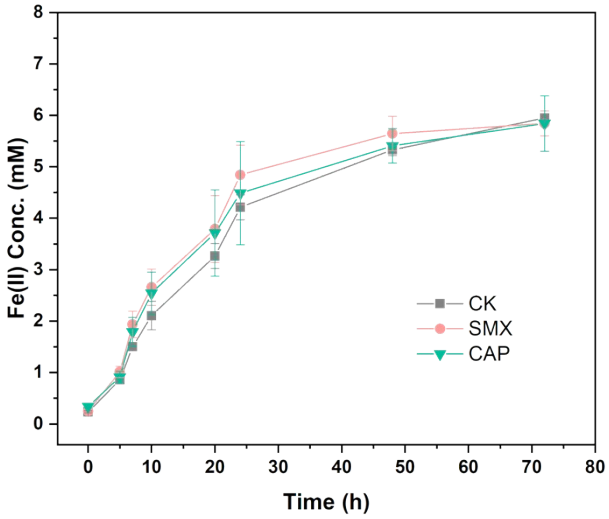
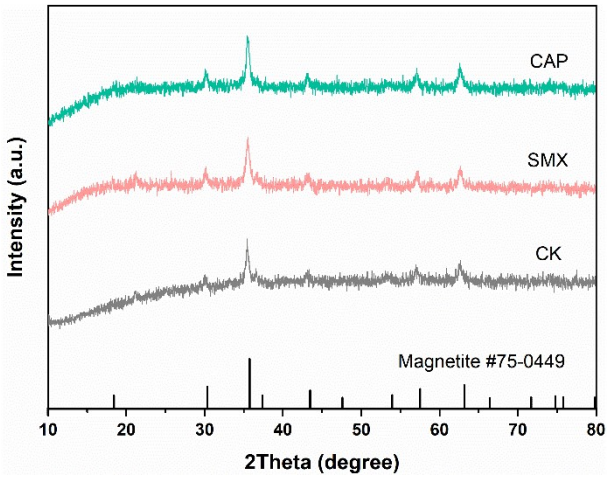
189 **Figure S8.** XRD patterns of solids from Fh incubated with SMX or CAP for one month. Neither
190 SMX nor CAP is capable of catalyzing the conversion of Fh to magnetite or goethite.



191

192 **Figure S9.** Total Fe(II) production from Fh pre-equilibrated with antibiotics and subsequently
193 reduced by MR-1.



194

195 **Figure S10.** XRD spectra of solids from the one-month biotransformation of Fh pre-equilibrated
196 with antibiotics.

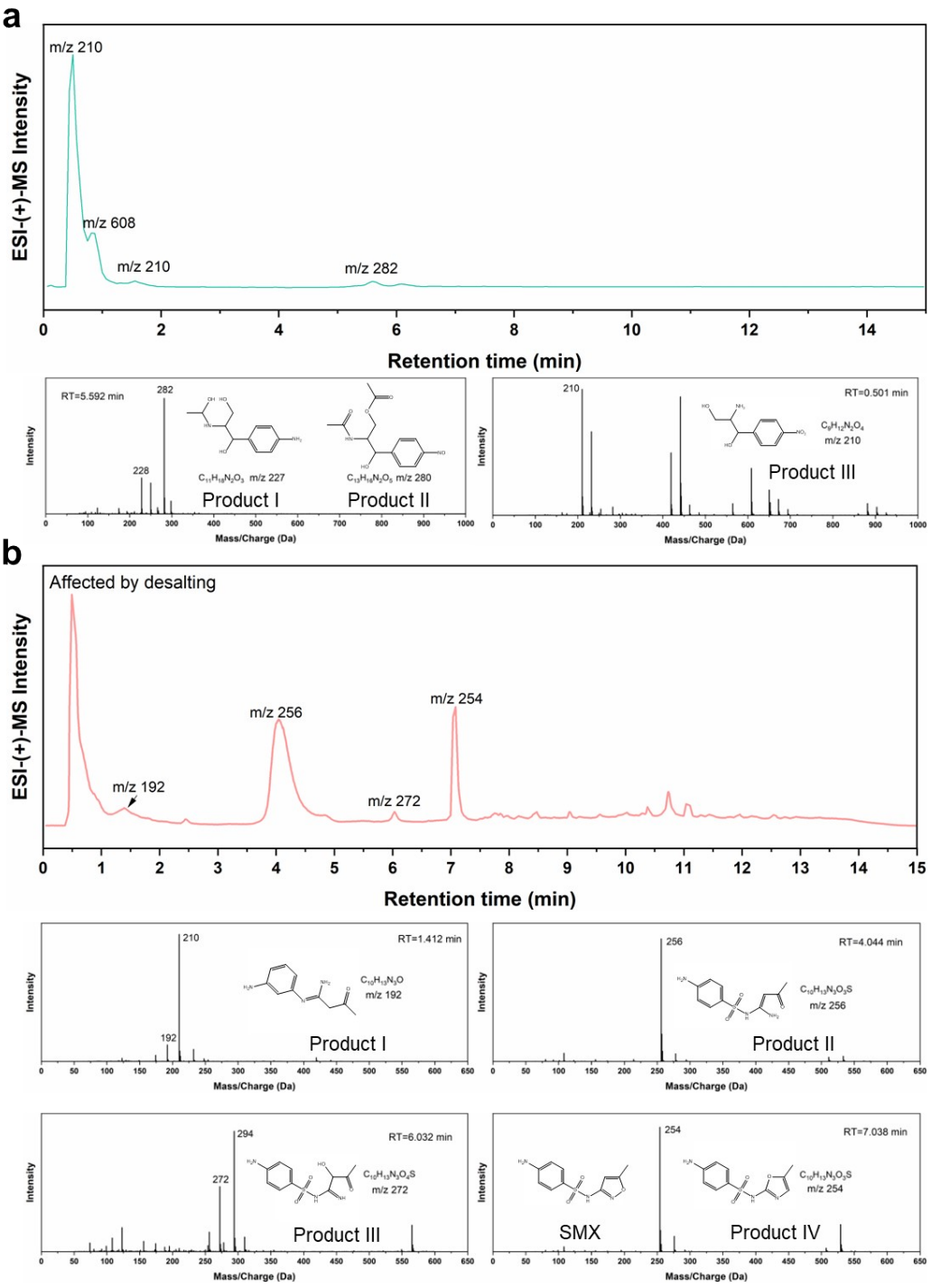


Figure S11. Transformation products of CAP (a) and SMX (b) after 10 h of incubation with Fh and MR-1.

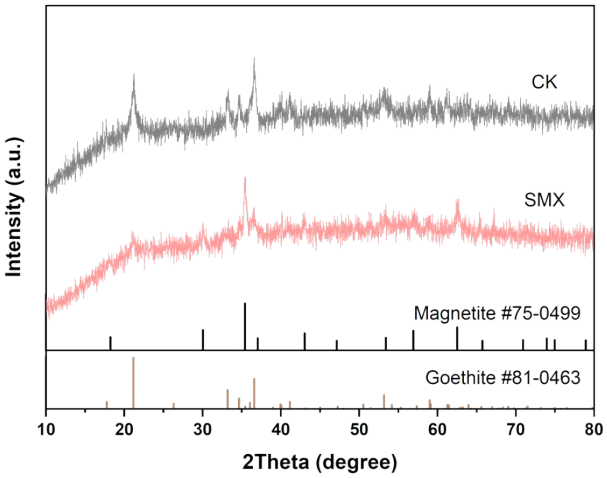


Figure S12. XRD spectra of solids from Fh transformation catalyzed by exogenous aqueous Fe(II), with and without the addition of SMX, following one-month incubation.

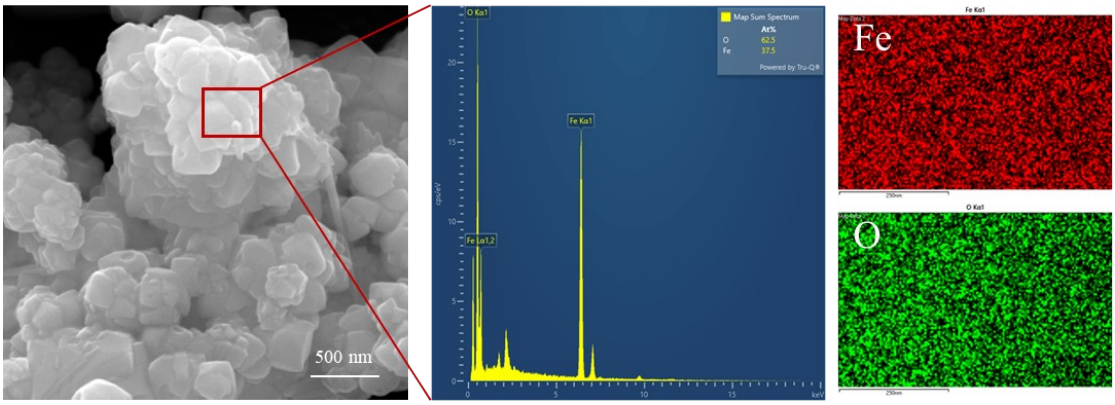


Figure S13. SEM image and EDS mapping of the solids formed from Fe(II) incubated with SMX after 5 h of incubation.

References

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