

1 **Selective metal extraction by biologically produced siderophores during**
2 **bioleaching from low-grade primary and secondary mineral resources**

3

4 *Adam J. Williamson^{1,2,‡,*}, Karel Folens^{1,2,‡}, Sandra Matthijs³, Yensy Paz Cortez¹, Jeet Varia¹, Gijs Du*
5 *Laing⁴, Nico Boon^{1*}, Tom Hennebel^{1,5}*

6

7 ¹ Center for Microbial Ecology and Technology (CMET), Faculty of Bioscience Engineering, Ghent
8 University, Coupure Links 653, 9000 Ghent, Belgium.

9 ² SIM vzw, Technologiepark 48, 9052 Zwijnaarde, Belgium.

10 ³ Institut de Recherche LABIRIS, Av. E. Gryzon 1, 1070 Brussels, Belgium.

11 ⁴ Department of Green Chemistry and Technology, Faculty of Bioscience Engineering, Ghent
12 University, Coupure Links 653, 9000 Ghent, Belgium.

13 ⁵ Umicore, Broekstraat 33, Brussels, Belgium.

14

15 * Corresponding authors. *Email addresses:* nico.boon@ugent.be (N. Boon), williams@cenbg.in2p3.fr
16 (A.J. Williamson)

17 ‡ Both authors contributed equally to this work.

18

19 **Abstract**

20 Siderophores are a class of biogenic macromolecules that have high affinities for metals in the
21 environment, thus could be exploited for alternate sustainable metal recovery technologies. Here, we
22 assess the role of siderophores in the extraction and complexation of metals from an iron oxide-rich
23 metallurgical processing residue and a low-grade primary Ni ore. Evaluation of the biological
24 siderophore bioproduction by three pseudomonads, *P. fluorescens*, *P. azotoformans* and *P. putida*
25 identified that *P. putida* could generate the highest siderophore yield, which was characterized as a
26 hydroxamate and catecholate mixed-type pyoverdine PyoPpC-3B. Key physiochemical parameters
27 involved in raw siderophore mediated metal extraction were identified using a fractional factorial
28 design of experiments (DOE) and subsequently employed in purified PyoPpC-3B leaching
29 experiments. Further targeted experiments with hydroxamate and catecholate functional analogues of
30 PyoPpC-3B confirmed their marked ability to competitively or selectively leach and chelate hard
31 metal ions, including $\text{Al}(\text{OH})_4^-$, Mn^{2+} and Zn^{2+} . Interestingly, complexation of Mn and Zn ions
32 exceeded the natural affinity of pyoverdine for Fe^{3+} , thus despite the low metal recoveries from the
33 materials tested in this study, this work provides important new insights in siderophore-metal
34 interactions.

35

36 **Keywords:** Waste processing; Metal complexation; Pyoverdine; *Pseudomonas putida*; Resource
37 recovery

38

39

40 **1. Introduction.** Siderophores are an important group of secondary metabolites produced by
41 microorganisms and plants to facilitate the uptake of iron, which is typically insoluble in most
42 terrestrial environments¹⁻³. Siderophore concentrations in the environment typically lie in the μM -
43 mM range⁴ and are intrinsically involved in weathering soil minerals^{5,6}, thus can significantly
44 contribute to the mobility of metals in the environment. Siderophores also form complexes with a
45 diverse range of other metals including Al, Cd, Cu, Ga, In, Pb, REE, Zr, Hf^{7,8}, essential
46 macronutrients Mo, Mn, Co and Zn⁹, as well as radionuclides U, Np, Th and Pu¹⁰⁻¹². Whilst the
47 evolutionary reasoning behind this remains unclear, it could represent the sequestration of essential
48 macronutrients, or detoxification of metals which would otherwise result in oxidative cellular stress
49^{9,12}.

50 Over 500 siderophores have been characterized to date, and can be classified by their ligand
51 functionalities¹³: (i) catecholates (aryl caps) of which include phenolates, (ii) hydroxamates, (iii)
52 carboxylates or hydroxycarboxylates¹⁴. It is widely documented that *Pseudomonas* sp. can produce
53 pyoverdine-type siderophores, and less complex siderophores ('secondary siderophores') such as
54 pyochelin, pseudomonine, thioquinolobactin and pyridine-2,6-bis(monothiocarboxylic acid), yet there
55 is a paucity of studies towards the characterisation of their metal binding properties in mixed element
56 systems. Pyoverdines have been shown to have high affinities for a range of metals including Zn, Cu
57 and Mn (K_a 10^{17-22}) yet with a clear preference for iron (K_a 10^{32}) under their respective experimental
58 conditions^{9,15,16}.

59 Siderophores have received much attention in recent years because of their potential
60 application in various areas of environmental research, including medicine (e.g. anemia treatments),
61 agriculture (plant-bacteria synergism and bio-pesticides)^{17,18}, bio-sensors, chelating agents and bio-
62 remediation¹⁴ (Table 1). Siderophores can also offer perspectives for recovering raw materials from
63 sustainable metal reserves. The depletion of high-grade mineral resources at a reasonable accessibility
64 (<1 km depth) has forced the mining industry to search for alternative processes that exploit low-
65 grade mineral deposits and avoid a high energy consumption. This is further exacerbated by the
66 predicted exhaustion of Zn, Ga, Ge, As, Rh, Ag, In, Sn, Sb, Hf, Pb, Mn and Au within 50 years, Ni,
67 Cu, Cd, Tl, Fe and U within 100 years and platinum group metals in 150 years, based on current
68 consumption rates¹⁹. Metal recovery from alternate low-grade primary and secondary sources

69 provides a great opportunity to meet the demand of raw materials. Zinc refining operations, for
 70 instance, have been generating large amounts of iron oxide-rich jarosite and goethite wastes, posing
 71 serious environmental, social and economic difficulties²⁰. Primary laterite ores are increasingly being
 72 investigated due to their abundance and significant quantities of important metals Co and Ni²¹. Over
 73 the last century, bioleaching is being increasingly investigated as a more sustainable mode of
 74 hydrometallurgical metal extraction²². Recent work has implicated the involvement of siderophores
 75 in the leaching of metals from fayalite slags^{23,24} and chromite tailing²⁵ by *P. aeruginosa* and *P.*
 76 *putida*, however, no further siderophore characterisation or metal-siderophore interactions were
 77 assessed.

78 Accordingly, this study aimed at evaluating the production of siderophores by strains of
 79 *Pseudomonas* and their potential to extract metals, including Zn, Mn and Al, from two low-grade
 80 mineral resources; the first, an iron oxide-rich residue from zinc processing, and secondly, a Ni-
 81 bearing laterite ore. Whilst improving our mechanistic understanding of siderophore-metal
 82 interactions in complex mineralogical environments, it contributes to the early development of
 83 alternate bio-metallurgical technologies for sustainable metal extraction.

84 **Table 1.** Overview of studies towards the application of siderophores towards non-Fe metals

Siderophore	Microorganism	Metal targeted	References
Deferoxamine	<i>Streptomyces pilosus</i>	Fe, Al, Ga, In	26
		Mn	27
Micacocidin	<i>Pseudomonas</i> sp.	Zn	15
		Cu	28
		Ga, Ni	29
Protochelin	<i>Azotobacter vinelandii</i>	Mo, V	15
		Other non-iron metallophore	30
Pyochelin	<i>Pseudomonas aeruginosa</i>	Cu	15
		Other non-Fe metallophore	31,32
		Actinides/REEs	11
Pyoverdines	<i>Pseudomonas aeruginosa</i> <i>Pseudomonas fluorescens</i>	Ga	33
		Zn/Cu	15
		U/ REEs	11,34,35
		As	36

	<i>Pseudomonas putida</i>		
Pyridine-2,6-	<i>Pseudomonas</i> spp.	Zn	15
dithiocarboxylate		Cu	37
Yersiniabactin	<i>Pseudomonas</i>	Zn	15
	<i>syringae</i> pv.	Cu	38
		Ni, Pd	39
Azobactin	<i>Azobacter vinelandii</i>	Fe, V, Mo	15
		Mo, V	40

85

86

87 2. Materials and Methods

88

89 **2.1 Cultivation of strains.** The bacterial strains used in this study were *Pseudomonas putida* PpF1
90 (LMG 24210), *Pseudomonas fluorescens* (LMG 1794) and *Pseudomonas azotoformans* (DSMZ
91 18862). *P. putida* and *P. fluorescens* strains were obtained from the Belgian Coordinated Collection
92 of Micro-organisms and *P. azotoformans* was purchased from Leibniz Institute DSMZ-German
93 Collection of Microorganisms and Cell Culture. Bacterial strains were first plated on LB agar (Carl
94 Roth, Germany) from glycerol stocks and incubated at 28 °C for 24 h. Single colonies were further
95 sub-cultured in 10 mL of LB broth (Carl Roth, Germany) and incubated at 28 °C for 24 h with
96 constant shaking at 120 rpm until an optical density at 600 nm, OD₆₀₀ (DR Lange ISIS 900 MPA
97 photometer) was approximately 1.5. All microbial cultivation and siderophore production experiments
98 were carried out under strictly sterile and aerobic conditions.

99

100 **2.2 Microbial siderophore purification and characterization.** To stimulate the production of
101 siderophores, strains were first grown on LB growth media, centrifuged and washed twice with 0.9 %
102 NaCl before transferring at a starting OD₆₀₀ of 0.02 into a modified selective medium (MSM)
103 previously applied for siderophore production (6 g L⁻¹ K₂HPO₄, 3 g L⁻¹ KH₂PO₄, 5 g L⁻¹ (NH₄)₂SO₄,
104 0.2 g L⁻¹ MgSO₄, 4 g L⁻¹ Na-succinate and 4 g L⁻¹ casamino acids)⁴¹. The pH was set to 7 prior to
105 autoclaving and to avoid precipitation, the casamino acids solution was filter-sterilised and added
106 after autoclaving the medium. Strictly Fe-free conditions were established and maintained by pre-
107 washing all glassware in 2 (v/v)% HCl, to prepare the medium in order to maximize the siderophore

108 production⁴². Before inoculation from LB sub-cultures to MSM, cells were centrifuged at 5000 rpm
109 for 2 min and washed twice with MSM. The growth of each strain was evaluated in triplicate serum
110 flasks by measuring the OD₆₀₀ for a period of 5 days. The siderophore concentration was
111 approximated with a high throughput chrome azurol sulfonate (CAS) assay and measuring the UV-
112 VIS absorbance at 620 nm⁴³. The resulting pyoverdine siderophore was purified from 4 L of a 72 h
113 old culture by previously described methods⁴⁴. Briefly, the filtered culture supernatant was loaded
114 onto a C-18 column that was activated with methanol and washed with distilled water. Elution was
115 performed with acetonitrile/ H₂O (70/30 %). Preparative-scale purification of the pyoverdine was
116 performed using a Prep 150 LC system (Waters). A SunFire Prep C18 column (C-18, 19 x 250 mm, 5
117 µm particle size) was used with a flow rate of 20 mL min⁻¹ and a gradient from H₂O/CH₃CN 9:1
118 containing 0.1 % CF₃COOH to H₂O/CH₃CN 6:4 containing 0.1 % CF₃COOH in 20 min. CH₃CN was
119 evaporated from the extract in vacuo and the sample was lyophilized. LC/MS analyses were
120 performed to identify the pyoverdine on a Kontron 325 system, coupled to the mass spectrometer and
121 equipped with a UV detector (model 322), an automatic injector (model 465) and LC-6A pumps. The
122 column used was a Vydac 218TP54 RP column (C18, 5 µm, d = 0.46 cm, l = 25 cm) and a flow rate
123 of 1 mL min⁻¹ was maintained. Mass spectral data (MS) were recorded on a VG Quattro II
124 spectrometer (ESP ionization, cone voltage 70 V, capillary voltage 3.5 kV, source temperature 80 °C).
125 Data collection was performed using Masslynx software. Structural information from the LC/MS
126 spectrum was visualized using ChemSketch (ACD Labs).

127

128 **2.3 Metal bioleaching.** The elemental composition of the two materials used in this study, an iron
129 oxide-rich processing residue from Zn production and a Polish laterite ore was determined via a
130 pseudo total acid digest via aqua regia⁴⁵ (Table 2) after sieving through a 1000 µm mesh sieve and
131 have been previously characterised⁴⁶. Briefly, the iron oxide mineral residue primarily consisted of
132 gypsum, quartz, calcite, hematite, willemite, jarosite and franklinite, whilst the laterite comprised of
133 lizardite, forsterite, magnesioferrite, quartz and willemseite. All batch leaching experiments were
134 performed in closed polypropylene tubes (Greiner, Germany) at 28 °C in a vertical shaker.

135 For the fractional factorial design of experiments (DOE), five parameters were evaluated:
136 sonication of the material prior to leaching (yes/no), pulp density (5%/20%), pH (2/9), particle size

137 fractions (0.2 mm/1 mm) and the presence or absence of microbial biomass, i.e. separation prior to
138 leaching via centrifugation (yes/no). All subsequent experiments were performed without sonication,
139 a pulp density of 5%, no pH buffering and on material sieved with a 0.2 mm steel wire mesh. After 24
140 h, the pH was measured and the suspensions were filtered using 0.2 µm syringe filters (Chromafil
141 Xtra, Germany). All experiments were performed in triplicates and control experiments were
142 conducted with demineralized H₂O and uninoculated MSM (pH 7). The negative controls in
143 demineralized H₂O and MSM had a pH value of either 2 or 9.5. Siderophores produced by the three
144 strains of *Pseudomonas* were harvested at the previously determined maximum siderophore unit (SU)
145 production time point of 48 h and verified for siderophore concentration using the CAS assay, prior to
146 bioleaching. The siderophore content was calculated according to Equation 1 where A_r and A_s
147 correspond to the absorbance at 630 nm of the reference (sterile growth media) and sample,
148 respectively ¹⁸.

149

$$(1) \quad \% \text{ siderophore units} = (A_r - A_s)/A_r \times 100$$

150

151 Leaching experiments were also conducted with synthetic siderophore functionalities, using catechol
152 (Sigma Aldrich, Germany), acetohydroxamic acid (Sigma Aldrich, Germany) and a commercial
153 purified siderophore, 1.52 mmol L⁻¹ deferoxamine (DFO, Sigma Aldrich, Germany). The functional
154 analogues catechol and acetohydroxamic acid (AHA) were added at both low (0.1 wt/v %) and higher
155 1 and 10 wt/v % in order to exaggerate differences in leaching and to develop insights into
156 siderophore-metal interactions in these systems.

157

158 **Table 2.** Elemental composition of the investigated materials, expressed as mg metal per g of
159 material. Concentrations are mean values and standard deviations derived from chemical analysis in
160 triplicate (N = 3).

161

Element	Iron oxide mineral residue (mg g ⁻¹)	Laterite (mg g ⁻¹)
Al	6.03 ± 0.09	1.44 ± 0.11

Cd	0.287 ± 0.006	< 0.0003
Co	0.020 ± 0.001	0.164 ± 0.011
Cr	0.337 ± 0.006	0.412 ± 0.058
Cu	2.95 ± 0.10	0.001 ± 0.001
Fe	125.5 ± 4.24	67.9 ± 3.0
Mn	2.45 ± 0.07	1.36 ± 0.08
Ni	0.050 ± 0.001	10.3 ± 0.4
Pb	12.5 ± 0.6	< 0.015
Zn	43 ± 2	0.084 ± 0.002

162

163 **2.4 Chemical analysis.** The metal concentrations in the filtrate were in-line diluted with a $1 \mu\text{g L}^{-1}$
164 Rh internal standard and determined by Inductively Coupled Plasma – Optical Emission Spectroscopy
165 (ICP-OES, Varian Vista MPX, US), after appropriate dilution using 1 (v/v)% HNO_3
166 . Quantification was performed using an external standard series and linearity criteria in the
167 calibration of $R^2 > 0.9990$. All reported concentrations exceeded the method detection limit. The pH
168 was measured using a Consort multiparameter analyzer C3020.

169

170 **3. Results**

171

172 **3.1 Microbial siderophore production and purification.** The siderophore production by *P.*
173 *fluorescens*, *P. azotoformans* and *P. putida* was compared under previously reported optimal
174 siderophore production conditions^{18,41}. Similar average growth rates of $0.092 \pm 0.009 \text{ h}^{-1}$, $0.080 \pm$
175 0.001 h^{-1} , $0.095 \pm 0.008 \text{ h}^{-1}$ were observed for *P. putida*, *P. fluorescens* and *P. azotoformans*,
176 respectively (Figure 1A). Whilst *P. putida* and *P. fluorescens* had similar maximum SU production
177 rates ($3.0 \pm 0.2 \text{ h}^{-1}$ and $2.6 \pm 0.8 \text{ h}^{-1}$, respectively) compared to *P. azotoformans* ($1.6 \pm 0.7 \text{ h}^{-1}$), near
178 maximal SU units were measured at an earlier time point with *P. putida* (21 h) with respect to the
179 other 2 strains (Figure 1B). No significant enhancement of siderophore production was observed by
180 using a 10 fold higher inoculum concentration (starting OD_{600} 0.2 vs 0.02), highlighting siderophore

181 production was an active process during the growth of these strains. The maximum yield of 75 % SU
182 by *P. putida* in this study is slightly lower than the 83 % and 87 % reported by Sayyed and coworkers
183 ¹⁸ and higher than the value of 69 % SU for *P. aeruginosa* reported by Shaikh and coworkers ⁴⁷. With
184 its optimal siderophore production, *P. putida* was therefore chosen for further leaching experiments in
185 this study.

186 To identify the pyoverdine produced by *P. putida* under the experimental conditions of this
187 study, siderophores were harvested from the growth media and analyzed by ESI-MS. Structural
188 analysis of semi-purified siderophores by ESI-MS (Figure S1) showed a predominant molecular mass
189 at m/z 1370 and at its double ionization of m/z 685, corresponding to a previously identified a
190 pyoverdine-type siderophore, PyoPpC-3B ⁴⁸, with the chromophore group linked to a 9 residues long
191 peptide chain consisting out of Asp-OHbutOHOrn-Dab-Thr-Gly-Ser-Ser-OHAsp-Thr. The
192 abbreviations OHbutOHOrn, Dab and OHAsp represent N^δ-hydroxybutyryl-N^δ-hydroxy-Orn,
193 diamino-butanoic acid and threo-β-hydroxy-aspartic acid, respectively. The peptide sequence suggests
194 a metal-binding pocket formed by three moieties; the catecholate of the chromophore, the
195 hydroxamate of N⁵-hydroxy-Orn and β-hydroxybutyric acid, and the α-OH-carboxylate from OHAsp.
196 PyoPpC-3B has been shown to be actively involved in iron acquisition by another closely related
197 strain, *P. putida* C ⁴⁹.

198

199 **3.2 Physiochemical impacts towards metal extraction by biogenic siderophores.** To efficiently
200 identify biological (presence of cells) and physiochemical (pH, particle size, pulp density and
201 sonication of the material) parameters that may influence metal extraction from an iron oxide mineral
202 residue by siderophores, a fractional factorial DOE was employed (Figure 2). No metals were
203 extracted in the growth media control, indicating that metals leached were through a combination of
204 siderophore and/ or physiochemical modifications. Whilst sonochemical leaching has been
205 demonstrated to impact (bio)physiochemical parameters and improve (bio)leaching processes, no
206 significant impact on metal extraction was observed in our experiments ($p = 0.250$) ⁵⁰. Aside from
207 copper, a slightly poorer bioleaching performance in the presence of residual *Pseudomonas* cells was
208 observed. Further production of siderophores may have been suppressed due to the initially extracted

209 metals in the pregnant leachate, and the overall metal extraction performance may have been
210 counteracted by their adverse sorption to cell surfaces. Nevertheless, the difference was not
211 significant ($p = 0.851$). The highest metal extracted over all conditions was Zn, with a marked
212 selectivity (900 fold average response Zn vs Fe) over the highest abundant metal in the starting
213 material, Fe (24 wt %). The most significant factor on metal extraction was lowering the pH to 2 ($p <$
214 0.05 for Zn), highlighting the importance of proton attack towards metal solubilisation from this
215 material. Lowering the pulp density from 200 g L^{-1} to 50 g L^{-1} generally improved the leaching of all
216 metals analyzed, which is typically reported from previous leaching studies with biogenic acids as it
217 improves reactive processes at the surface interface^{51,52}. With the exception of iron, increasing the
218 total particle size fraction from 0.2 mm to 1 mm hindered leaching, which may represent more facile
219 surface reactions on smaller particle sizes for non Fe metals and has also been reported for inorganic
220 acid leaching⁵³.

221

222 **3.3 Bioleaching with pyoverdine produced by *Pseudomonas putida*.** To further explore
223 pyoverdine-metal extraction mechanisms and to support the initial physiochemical screen with raw
224 siderophore solutions, a series of leaching experiments were conducted with the purified PyoPpC-3B
225 in contact with the iron oxide mineral residue. To augment metal extraction, experiments were sieved
226 with a $200 \mu\text{m}$ mesh at a pulp density of 50 g L^{-1} without sonication and after removal the biomass of
227 *P. putida* via centrifugation. Whilst dramatically improving the leaching efficiency of Zn, the pH was
228 not changed in order to dissociate the effect of proton activity from pure siderophore activity.

229 To determine whether pyoverdine purity, concentration or material contact time influenced
230 metal extraction, materials were brought in contact with the lixiviant either directly in the liquid phase
231 after centrifugation of cells, or following a purification step by C18 chromatography and re-dissolving
232 the lyophilized siderophore sample in ultra-pure water at two concentrations over a contact time of
233 seven days (Figure 3A and 3B). The purification step had no effect on the leaching of Mn ($p = 0.830$)
234 or Zn ($p = 0.900$) from the iron oxide mineral residue, thus harvested pyoverdines can be applied
235 directly for bioleaching without the need for additional purification steps. Furthermore, expanding the
236 contact time from 1 to 7 days (Figure 3B) only moderately enhanced the leaching of Mn ($p = 0.206$)
237 and Zn ($p = 0.804$), showing limited kinetic dependence of bioleaching at neutral pH. The pyoverdine

238 concentration has a certain, although not significant, effect towards Mn ($p = 0.173$) and Zn ($p =$
239 0.346) extraction from iron oxide mineral residue (Figure 3C). Lower concentrations of Al (2.1 ± 0.2
240 $\mu\text{mol g}^{-1}$) and Cu ($1.1 \pm 0.1 \mu\text{mol g}^{-1}$) were also observed after leaching with the higher pyoverdine
241 concentration (Figure 3C). The maximal extraction of Mn ($4.6 \pm 2.7 \mu\text{mol g}^{-1}$) and Zn ($6.0 \pm 2.1 \mu\text{mol}$
242 g^{-1}) was obtained at the longest contact time of 7 d and highest pyoverdine concentration of 3.6 mM,
243 whereas Cu and Al concentrations dropped at later time points (data not shown), indicative of a
244 reprecipitation event.

245 To explore pyoverdine interactions with other metals and varying mineral phases, leaching
246 experiments with the semi purified pyoverdine were performed on a Polish Ni laterite ore. Similar to
247 the iron oxide mineral residue, higher pyoverdine concentrations favored metal extraction, with $1.3 \pm$
248 $0.1 \mu\text{mol g}^{-1}$ Al extracted only at 3.6 mmol L^{-1} , coupled to a pH drop from 8.3 to 7.8 (Figure 3D). Ni
249 ($0.42 \pm 0.03 \mu\text{mol g}^{-1}$) and Fe ($5.2 \pm 0.5 \mu\text{mol g}^{-1}$) were also present after leaching with the higher
250 pyoverdine concentration, consistent with the Ni:Fe ratio of the primary Ni mineral in this material,
251 forsterite. One-way ANOVA with Holm-Šídák post-hoc testing showed an enhancement ($p < 0.001$)
252 at a pyoverdine concentration of 3.6 mM compared to the control group with only H₂O. A marked
253 increase of Co extraction was also observed from the laterite using 3.5 mmol L^{-1} pyoverdine ($12.5 \pm$
254 0.1 nmol g^{-1}) compared to 0.4 mmol L^{-1} , where Co was below the detection limit ($<1 \text{ nmol g}^{-1}$).
255 However, these results represent a very low extraction yield and selectivity.

256
257 **3.4 Leaching using synthetic chelating functionalities.** In order to gain a mechanistic understanding
258 of the affinity in metal complexation reactions, metal extraction from the iron oxide mineral residue
259 and the Ni laterite ore was evaluated using synthetic chelating functionalities that resemble the
260 chromophore groups found in siderophores. An initial screening was carried out with catechol and
261 acetohydroxamic acid, as representation of catecholate and hydroxamate functional analogues of
262 PyoPpC-3B at 1 wt/v % ($133 \mu\text{mol L}^{-1}$ and $90.8 \mu\text{mol L}^{-1}$ respectively) ⁵⁴ (Figure 4).

263 In general, as predicted, more pronounced levels of metal extraction were observed using
264 synthetic analogues that were poised at elevated concentrations in comparison to the biogenic
265 pyoverdine. Amongst the different chelating molecules, AHA showed capable of extracting and
266 complexing the largest amount of metals. This was apparent for Zn and Fe in iron oxide mineral

267 residue (Figure 4A) and Fe, Mn and Ni in laterite (Figure 4B). However, the selectivity towards Zn,
268 Mn or Ni over Fe was limited, with relatively high concentrations of Fe being extracted in solution:
269 $170 \mu\text{mol g}^{-1}$ from iron oxide mineral residue and $23 \mu\text{mol g}^{-1}$ from laterite. Preferential extraction via
270 acidolysis and subsequent metal complexation by AHA functionalities would suggest that this
271 functional group may have the most pronounced influence towards quantitative bioleaching.

272 To further explore the impact of hydroxamate and catecholate concentrations on the leaching
273 of metals from the iron oxide mineral residue, leaching experiments were performed at two additional
274 concentrations, 0.1 wt/v % ($13.3 \mu\text{mol L}^{-1}$ and $9.08 \mu\text{mol L}^{-1}$) and 0.01 wt/v % ($1.33 \mu\text{mol L}^{-1}$ and
275 $0.91 \mu\text{mol L}^{-1}$) (Figure 4A). Lower catechol concentrations showed a marked improvement of
276 selectivity and Mn extraction. Furthermore, no impact on Mn extraction was observed between two
277 higher concentrations (9.1 and $91 \mu\text{mol L}^{-1}$), where the biggest difference in the final pH was
278 observed ($\sim\text{pH}5-6.5$) (Figure 4C), highlighting that catechol-Mn interactions could not be explained
279 by the pH and could proceed at low stoichiometric ratios. With respect to the hydroxamate
280 functionality, a significantly improved selectivity of Zn against Fe was observed at lower
281 hydroxamate concentrations, from a 1:2 Zn:Fe ratio at $133 \mu\text{mol L}^{-1}$ to 10:1 and 5:0 for $13.3 \mu\text{mol L}^{-1}$
282 and $1.33 \mu\text{mol L}^{-1}$. In this case, the pH shift from $\sim\text{pH} 5.9-6.8$ between the two higher concentrations
283 had a greater role in Zn and Fe extraction (Figure 4A and 4C).

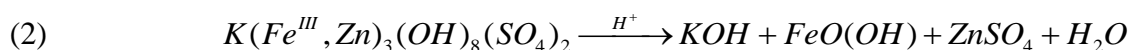
284 To benchmark PyoPpC-3B against other biogenic siderophores, leaching experiments with a
285 commercial hydroxamate-type siderophore DFO produced by *Streptomyces pilosus* at a concentration
286 of 1 g L^{-1} (1.52 mmol L^{-1}) was performed on the iron oxide residue (Figure 4). Whilst Zn leaching
287 was higher for DFO over PyoPpC-3B at $7.65 \pm 2,3 \mu\text{mol g}^{-1}$, a lower selectivity against iron was also
288 observed, with extracted iron at $3.58 \pm 0.1 \mu\text{mol g}^{-1}$. The low mg L^{-1} concentration range of metals
289 leached by PyoPpC-B3 and DFO are comparable with previous reported values from a study using 3
290 mmol L^{-1} DFO, particularly for Fe ($5.2-12.2 \mu\text{mol g}^{-1}$) that was also present in hydroxide form and at
291 an elevated concentration ($7.2-9.2 \text{ wt } \%$)⁵⁵.

292

293 **4. Discussion.** Bioleaching by heterotrophic microorganisms occurs by one or a combination of the
294 following mechanisms: acidolysis, complexolysis or redoxolysis^{56,57}. Upon contact of the 5 g L^{-1} , pH
295 7.7 PyoPpC-3B lixiviant with the iron oxide-rich mineral residue, the pH of the resulting solution

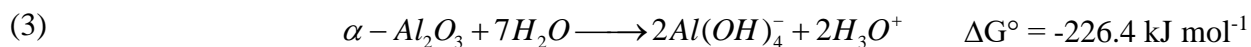
296 raised from 5.72 ± 0.01 increased to 6.88 ± 0.01 after 1 day. The subsequent pH transition from weak
297 acid to neutral conditions is consistent with the dissolution of Zn^{2+} from the Zn bearing mineral
298 jarosite in the iron oxide mineral residue⁵⁸ (Figure 3), given according to reaction Equation 2. A
299 similar pH evolution was observed with demineralized water, yet resulted in a lower leaching yield,
300 clearly highlighting that PyoPpC-3B enhanced the metal extraction process. Indeed, results from DOE
301 screening demonstrated that reducing the initial pH (2) through inorganic acid addition could further
302 enhance metal extraction (Figure 2), however no significant difference was observed between the
303 siderophore solution and the acidified siderophore solution, indicating proton attack overrules the
304 siderophore chelating activity.

305



306 In this study, acidification by acetohydroxamic acid, particularly at higher concentrations
307 highlighted that acidolysis may play a more direct role in metal extraction. Santos and coworkers also
308 showed through pH dependence studies that metal extraction by trihydroxamate is accelerated with
309 increasing acidity of the medium⁵⁹. Mechanistically, they inferred two parallel pathways: one is a
310 bimolecular process involving the direct attack of the acid on the siderophore analogue complex, the
311 other involves initially the protonation of the trihydroxamate group, followed by a rapid attack of the
312 competing ligand. Conversely, pure complexolysis by siderophores that contain hydroxamate groups
313 is expected to have a higher efficiency at neutral to alkaline conditions. Hence, dissolution and
314 complexation of Al from the laterite in this study is only occurring at neutral pH. The small decrease
315 in pH (from 8.3 to 7.8) during bioleaching can be explained by the release of hydroxonium ions in the
316 pregnant leachate solution (Equation 3).

317



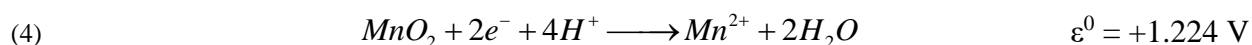
318

319 The balance of pH on the added effect of siderophore action is therefore a key parameter to
320 consider towards the application of this technology. Kraemer and coworkers also found that a low pH

321 (< 2) indeed limits metal chelation with siderophores due to a change in the protonation⁵⁵. Moreover,
322 high H₃O⁺ concentrations can even cause degradation of the pH-sensitive DFO molecule. Conversely,
323 at pH 2-7, the tertiary amine of hydroxamate moieties in DFO have a lone electron pair, allowing for
324 tris-hydroxamate to coordinate with Fe(III)⁶⁰. Slightly basic conditions of pH > 8 have also been
325 shown to be preferable for chelation of Pt and Pd by DFO from PGM-rich ores⁵⁵. In the same way,
326 Neubauer and coworkers showed that DFO could chelate Co³⁺ better than Fe³⁺ at higher pH values⁶¹.
327 Whilst preliminary experiments in this study at pH 2 indicated no sign of pyoverdine degradation at
328 early time points and that functional groups were predominantly protonated (data not shown), further
329 work is needed to monitor siderophore stability under these experimental conditions over longer time
330 frames. Identifying whether siderophore degradation (via e.g. ester/amide hydrolysis) or ion exchange
331 with other metals in the pregnant leachate could help to elucidate observations towards Cu and Al
332 reprecipitation from the iron oxide mineral residue over longer leaching time frames.

333 Redoxolysis can also contribute to the bioleaching process, considering catecholate has a
334 comparable redox potential ($\epsilon^0 = +0.795$ V) for e.g. Mn(IV) reduction ($\epsilon^0 = +1.224$ V), thus can act as
335 a specific mediator and transform to *o*-benzoquinone⁶². The dissolution of metal ions from pyrolusite
336 (Equation 4) and manganite (Equation 5) is hereby accompanied by an electron transfer and reduction
337 of Mn(IV) or Mn(III). The relatively high yields of Mn solubilisation in comparison to the lower
338 catecholate concentrations, non-shifted between 16.9 ± 0.34 $\mu\text{mol g}^{-1}$ and 15.2 ± 0.03 $\mu\text{mol g}^{-1}$ for
339 90.8 and 9.08 $\mu\text{mol L}^{-1}$ respectively (Figure 4), may also give support to an electron shuttling
340 reductive solubilisation mechanism.

341



342

343 Figure 5 illustrates the central hexadentate metal coordination of the pyoverdine characterised in this
344 study that contains both catecholate and hydroxamate functional groups. A comparison of metal
345 extraction with synthetic functional analogues (Figure 4), pointed towards AHA being the main metal

346 chelating group and that catecholate groups may be involved in the reductive dissolution of Mn
347 oxides in the materials tested in this study. Nevertheless, other non-specific functional groups, as part
348 of the peptide chain attached to C1 of the chromophore group may collectively act as metal cation
349 chelators⁶³, but further work is needed to clarify these observations.

350 The selectivity in metal complexation is greatly determined by their affinity with functional
351 groups. In mixed metals solutions, competition for siderophore complexation exists between different
352 metal cations, typically Fe³⁺, Al³⁺, Ca²⁺, Cu²⁺ and Zn²⁺⁶⁴. Selectivity of siderophores towards hard
353 metal ions of coordination number 6 became clear from our results (Figure 3), thus in the absence of
354 significant labile Fe³⁺ concentrations, Mn²⁺ and Zn²⁺ may take over its role in complexation with
355 pyoverdine. Both Mn²⁺ and Zn²⁺ possess a similar charge to ionic radius ratio (2 : 0.07 nm) that it is
356 determinative for cationic metallophore complexation⁷. On the contrary, Al(OH)₄⁻ follows another
357 geometric complexation as a result of its hydroxyanion speciation.

358 Pyoverdines are well documented to be selective towards Fe in aqueous mixed metal systems,
359 but little work has looked at the extraction of metals from solid mineral residues. The selectivity of
360 pyoverdines towards Zn and Mn over Fe in this study likely represents a recalcitrance of the Fe
361 mineral phases and/or more labile Zn and Fe for siderophore extraction. Whilst hematite, a principal
362 iron mineral in the iron oxide-rich mineral residue has been previously demonstrated to be susceptible
363 to Fe extraction by siderophores, these studies were performed either through siderophore expression
364 during in situ contact with the material, using an alternate hydroxamate-type siderophore, DFO and/or
365 with nanoparticle fractions⁴. The larger particle sizes in this study, in combination with its presence
366 within a mixed highly weathered sample may thus impede such processes. This may be compounded
367 by the limited metal binding siderophore substrate. This effect may be less apparent in the laterite
368 system, with lower reported aluminum-hydroxamate formation constants (log K 21.5)⁶⁵. The higher
369 observed Zn to Fe ratios at lower concentrations of acetohydroxamic acid, where a pH shift from 6
370 (133 μmol L⁻¹) to 6.75 (13.3 and 1.33 μmol L⁻¹) occurred may also favor metal complexolysis of Zn
371 over acidolysis, but should be validated with other materials in follow up studies.

372

373 **5. Conclusions.** This study demonstrates the selective extraction of Zn²⁺ and Mn²⁺ metal ions, and
374 competitive extraction of Al(OH)₄⁻ from a primary and secondary mineral residue, by a hydroxamate

375 and catecholate mixed-type pyoverdine PyoPpC-3B, produced by *Pseudomonas putida* PpF1. We
376 propose the following mode of action of this biogenic macromolecule and its functional analogues:
377 (1) (in)direct metal dissolution via acidolysis (e.g. for Zn from jarosite) (2) reductive mineral
378 dissolution via redoxolysis (e.g. for catecholate groups with Mn oxides) (3) subsequent complexolysis
379 via the chromophore that can further drive metal release from the substrate. Whilst relatively low
380 yields are reported for these materials, metal solubilisation could be further enhanced by increasing
381 siderophore or functional analogue concentrations, and to a lesser extent, the contact time. For further
382 follow up studies, combinations of siderophores and more established bio-hydrometallurgical
383 lixivants such as biogenic organic acids under weakly acidic conditions could also be of interest to
384 identify synergy between higher leaching yields and selectivity. The separation of the metal-
385 siderophore complex and subsequent metal recovery from the pregnant leachates should also be
386 investigated and recent work has demonstrated that this could be feasible ⁶⁶.

387 Whilst some work has reported concomitant or alternate metal complexation by siderophores
388 over iron, little work has shown a clear preference for Zn and Mn extraction over Fe. Whilst Al was
389 not selectively leached over Fe in the laterite, the co-leaching of these metals by this particular
390 siderophore has not been reported before and clearly warrants further follow up. Comparison with
391 synthetic functional analogues revealed that AHA has strong metal chelating properties. It is
392 suggested that these hydroxamate moieties in biogenic siderophores also represent the primary route
393 in the metal dissolution and complexation process, although advanced chemical characterization of
394 the metal complexes in the leachate are needed to support this working hypothesis. The stoichiometric
395 excess of Mn extracted against the lower catechol concentrations suggests that reductive dissolution
396 may play an additional role, but further work is also needed to clarify these observations. Finally, the
397 stability of siderophores during the metal extraction process should be evaluated to determine their
398 impact on metal release post extraction and their potential recovery for reuse.

399 This study has, for the first time, implicated the direct role of siderophores in metal extraction
400 from low grade primary and secondary resources. Whilst currently providing low relative metal
401 yields, it supplies important first steps towards sustainable metal extraction and recovery. Moreover, it
402 provides important insights in siderophore-metal interactions in complex and refractory primary and

403 secondary mineral sources, that also have implications for the acquisition of metals by
404 microorganisms in the environment.

405

406 **Acknowledgements.** The research was financed by SBO Project SMART (Sustainable Metal
407 Extraction from Tailings) that fits in the SIM (Strategic Initiative Materials) program of Flanders
408 (*HBC.2016.0456*) and was supported by the EU Horizon 2020 METGROW+ project (Grant
409 Agreement n° 690088) on Metal Recovery from Low Grade Ores and Wastes. We thank Dr. Jasmine
410 Heyse for critically reading the manuscript.

411

412 **Author Contributions.** AJW conceived the project, designed experiments, assisted in data
413 acquisition, interpreted the data and wrote the manuscript. KF assisted in data acquisition, interpreted
414 the data and wrote the manuscript. YPC performed growth experiments and DOE analysis. SM
415 characterised the pyoverdine structure, assisted in data interpretation and reviewed the manuscript. JV
416 assisted in DOE design and analysis, GDL, NB and TH assisted in project conceptualisation, data
417 interpretation and reviewed the manuscript.

418

419 **References**

420

- 421 (1) Ahmed, E.; Holmström, S. J. M. Siderophores in Environmental Research: Roles and Applications. *Microb. Biotechnol.* **2014**,
422 7 (3), 196–208. <https://doi.org/10.1111/1751-7915.12117>.
- 423 (2) Neilands, J.B., *Siderophores: structure and function of microbial iron transport compounds*. J Biol Chem, 1995. **270**(45): p.
424 26723-6.
- 425 (3) Venkat Kumar, S.; Menon, S.; Agarwal, H.; Gopalakrishnan, D. Characterization and Optimization of Bacterium Isolated from
426 Soil Samples for the Production of Siderophores. *Resour. Technol.* **2017**, 3 (4), 434–439.
427 <https://doi.org/10.1016/j.reffit.2017.04.004>.
- 428 (4) Hersman, L.; Huang, A.; Maurice, P.; Forsythe, J. Siderophore Production and Iron Reduction by *Pseudomonas mendocina* in
429 Response to Iron Deprivation. *Geomicrobiol. J.* **2000**, 17 (4), 261–273. <https://doi.org/10.1080/01490450050192965>.
- 430 (5) Barton, L. E.; Quicksall, A. N.; Maurice, P. A. Siderophore-Mediated Dissolution of Hematite (α -Fe₂O₃): Effects of
431 Nanoparticle Size. *Geomicrobiol. J.* **2012**, 29 (4), 314–322. <https://doi.org/10.1080/01490451.2011.558566>.
- 432 (6) Saad, L. B.; Duckworth, O. W. Synergistic Dissolution of Manganese Oxides as Promoted by Siderophores and Small Organic
433 Acids. *Soil Sci. Soc. Am. J.* **2010**, 74 (6), 2021. <https://doi.org/10.2136/sssaj2009.0465>.
- 434 (7) Kraemer, S. M.; Duckworth, O. W.; Harrington, J. M.; Schenkeveld, W. D. C. Metallophores and Trace Metal

- 435 Biogeochemistry. *Aquat. Geochemistry* **2015**, *21* (2–4), 159–195. <https://doi.org/10.1007/s10498-014-9246-7>.
- 436 (8) Christenson, E. A.; Schijf, J. Stability of YREE Complexes with the Trihydroxamate Siderophore Desferrioxamine B at
437 Seawater Ionic Strength. *Geochim. Cosmochim. Acta* **2011**, *75* (22), 7047–7062. <https://doi.org/10.1016/j.gca.2011.09.022>.
- 438 (9) Braud, A.; Geoffroy, V.; Hoegy, F.; Mislin, G. L. A.; Schalk, I. J. Presence of the Siderophores Pyoverdine and Pyochelin in
439 the Extracellular Medium Reduces Toxic Metal Accumulation in *Pseudomonas aeruginosa* and Increases Bacterial Metal
440 Tolerance. *Environ. Microbiol. Rep.* **2010**, *2* (3), 419–425. <https://doi.org/10.1111/j.1758-2229.2009.00126.x>.
- 441 (10) Rajkumar, M.; Ae, N.; Narasimha, M.; Prasad, V.; Freitas, H. Potential of Siderophore-Producing Bacteria for Improving
442 Heavy Metal Phytoextraction. *Trends Biotechnol.* **2010**, *28* (3), 142–149. <https://doi.org/10.1016/j.tibtech.2009.12.002>.
- 443 (11) Desouky, O. A.; El-Mougith, A. A.; Hassanien, W. A.; Awadalla, G. S.; Hussien, S. S. Extraction of Some Strategic Elements
444 from Thorium–Uranium Concentrate Using Bioproducts of *Aspergillus ficuum* and *Pseudomonas aeruginosa*. *Arab. J. Chem.*
445 **2016**, *9*, S795–S805. <https://doi.org/10.1016/j.arabjc.2011.08.010>.
- 446 (12) Schalk, I. J.; Hannauer, M.; Braud, A. New Roles for Bacterial Siderophores in Metal Transport and Tolerance. *Environ.*
447 *Microbiol.* **2011**, *13* (11), 2844–2854. <https://doi.org/10.1111/j.1462-2920.2011.02556.x>.
- 448 (13) Petrik, M.; Zhai, C.; Haas, H.; Decristoforo, C. Siderophores for Molecular Imaging Applications. *Clin. Transl. Imaging* **2017**,
449 *5* (1), 15–27. <https://doi.org/10.1007/s40336-016-0211-x>.
- 450 (14) Hernlem, B. J.; Vane, L. M.; Sayles, G. D. The Application of Siderophores for Metal Recovery and Waste Remediation:
451 Examination of Correlations for Prediction of Metal Affinities. *Water Res.* **1999**, *33* (4), 951–960.
452 [https://doi.org/10.1016/S0043-1354\(98\)00293-0](https://doi.org/10.1016/S0043-1354(98)00293-0).
- 453 (15) Johnstone, T. C.; Nolan, E. M. Beyond Iron: Non-Classical Biological Functions of Bacterial Siderophores. *Dalt. Trans.* **2015**,
454 *44* (14), 6320–6339. <https://doi.org/10.1039/c4dt03559c>.
- 455 (16) Chen, Y.; Jurkevitch, E.; Bar-Ness, E.; Hadar, Y. Stability Constants of Pseudobactin Complexes with Transition Metals. *Soil*
456 *Sci. Soc. Am. J.* **1994**, *58* (2), 390–396. <https://doi.org/10.2136/sssaj1994.03615995005800020021x>.
- 457 (17) Ali, S. S.; Vidhale, N. N. Review Article Bacterial Siderophore and Their Application : A Review.
458 *Int.J.Curr.Microbiol.App.Sci* **2013**, *2* (12), 303–312.
- 459 (18) Sayyed, R. Z.; Badgajar, M. D.; Sonawane, H. M.; Mhaske, M. M.; Chincholkar, S. B. Production of Microbial Iron Chelators
460 (Siderophores) by Fluorescent Pseudomonads. **2005**, *4* (October), 484–490.
- 461 (19) Watling, H. R. Review of Biohydrometallurgical Metals Extraction from Polymetallic Mineral Resources. *Minerals* **2014**, *5*
462 (1), 1–60. <https://doi.org/10.3390/min5010001>.
- 463 (20) Pelino, M.; Cantalini, C.; Abbruzzese, C.; Plescia, P. Treatment and Recycling of Goethite Waste Arising from the
464 Hydrometallurgy of Zinc. *Hydrometallurgy* **1996**, *40* (1–2), 25–35. [https://doi.org/10.1016/0304-386X\(95\)00004-Z](https://doi.org/10.1016/0304-386X(95)00004-Z).
- 465 (21) Newsome, L.; Solano Arguedas, A.; Coker, V. S.; Boothman, C.; Lloyd, J. R. Manganese and Cobalt Redox Cycling in
466 Laterites; Biogeochemical and Bioprocessing Implications. *Chem. Geol.* **2020**, *531* (March 2019), 119330.
467 <https://doi.org/10.1016/j.chemgeo.2019.119330>.
- 468 (22) Nancharaiah, Y. V.; Mohan, S. V.; Lens, P. N. L. Biological and Bioelectrochemical Recovery of Critical and Scarce Metals.
469 *Trends Biotechnol.* **2016**, *34* (2), 137–155. <https://doi.org/10.1016/j.tibtech.2015.11.003>.
- 470 (23) Yin, N. H.; Sivry, Y.; Avril, C.; Borensztajn, S.; Labanowski, J. Ô.; Malavergne, V.; Lens, P. N. L.; Rossano, S.; van
471 Hullebusch, E. D. Bioweathering of Lead Blast Furnace Metallurgical Slags by *Pseudomonas Aeruginosa*. *Int. Biodeterior.*
472 *Biodegrad.* **2014**, *86*, 372–381. <https://doi.org/10.1016/j.ibiod.2013.10.013>.

- 473 (24) van Hullebusch, E. D.; Yin, N. H.; Seignez, N.; Labanowski, J.; Gauthier, A.; Lens, P. N. L.; Avril, C.; Sivry, Y. Bio-
474 Alteration of Metallurgical Wastes by *Pseudomonas aeruginosa* in a Semi Flow-through Reactor. *J. Environ. Manage.* **2015**,
475 *147*, 297–305. <https://doi.org/10.1016/j.jenvman.2014.09.018>.
- 476 (25) Bolaños-Benítez, V.; van Hullebusch, E. D.; Lens, P. N. L.; Quantin, C.; van de Vossenberg, J.; Subramanian, S.; Sivry, Y.
477 (Bio)Leaching Behavior of Chromite Tailings. *Minerals* **2018**, *8* (6). <https://doi.org/10.3390/min8060261>.
- 478 (26) Kraemer, D.; Tepe, N.; Pourret, O.; Bau, M. ScienceDirect Negative Cerium Anomalies in Manganese (Hydr) Oxide
479 Precipitates Due to Cerium Oxidation in the Presence of Dissolved Siderophores. *Geochim. Cosmochim. Acta* **2017**, *196*, 197–
480 208. <https://doi.org/10.1016/j.gca.2016.09.018>.
- 481 (27) Duckworth, O. W.; Sposito, G. Siderophore-Manganese(III) Interactions II. Manganite Dissolution Promoted by
482 Desferrioxamine B. *Environ. Sci. Technol.* **2005**, *39* (16), 6045–6051. <https://doi.org/10.1021/es050276c>.
- 483 (28) Kobayashi, S.; Hidaka, S.; Kawamura, Y.; Ozaki, M.; Hayase, Y. *Micacocidin A, B and C, novel antimycoplasma agents from*
484 *Pseudomonas sp. II. Structure elucidation.* *J. Antibiot (Tokyo)*, 1998. **51**(3): p. 328-32.
- 485 (29) Kreutzer, M. F.; Kage, H.; Gebhardt, P.; Wackler, B.; Saluz, H. P.; Hoffmeister, D.; Nett, M. Biosynthesis of a Complex
486 Yersiniabactin-like Natural Product via the Mic Locus in Phytopathogen *Ralstonia solanacearum*. *Appl. Environ. Microbiol.*
487 **2011**, *77* (17), 6117–6124. <https://doi.org/10.1128/AEM.05198-11>.
- 488 (30) Cornish, A. S.; Page, W. J. Role of Molybdate and Other Transition Metals in the Accumulation of Protochelin by *Azotobacter*
489 *vinelandii*. *Appl. Environ. Microbiol.* **2000**, *66* (4), 1580–1586. <https://doi.org/10.1128/AEM.66.4.1580-1586.2000>.
- 490 (31) Brandel, J.; Humbert, N.; Elhabiri, M.; Schalk, I. J.; Mislin, G. L. A.; Albrecht-Gary, A. M. Pyochelin, a Siderophore of
491 *Pseudomonas aeruginosa*: Physicochemical Characterization of the Iron(III), Copper(II) and Zinc(II) Complexes. *Dalt. Trans.*
492 **2012**, *41* (9), 2820–2834. <https://doi.org/10.1039/c1dt11804h>.
- 493 (32) Ankenbauer, R. G.; Quan, H. N. FptA, the Fe(III)-Pyochelin Receptor of *Pseudomonas aeruginosa*: A Phenolate Siderophore
494 Receptor Homologous to Hydroxamate Siderophore Receptors. *J. Bacteriol.* **1994**, *176* (2), 307–319.
495 <https://doi.org/10.1128/jb.176.2.307-319.1994>.
- 496 (33) Guo, Y.; Li, W.; Li, H.; Xia, W. Identification and Characterization of a Metalloprotein Involved in Gallium Internalization in
497 *Pseudomonas aeruginosa*. *ACS Infect. Dis.* **2019**, *5* (10), 1693–1697. <https://doi.org/10.1021/acsinfecdis.9b00271>.
- 498 (34) Hussien, S. S.; Desouky, O. A.; Abdel-Haliem, M. E. F.; El-Moughith, A. A. Uranium (VI) Complexation with Siderophores-
499 Pyoverdine Produced by *Pseudomonas fluorescens* SHA 281. *Int. J. Nucl. Energy Sci. Eng.* **2013**, *3* (4), 95.
500 <https://doi.org/10.14355/ijnese.2013.0304.03>.
- 501 (35) Mullen, L.; Gong, C.; Czerwinski, K. Complexation of Uranium (VI) with the Siderophore Desferrioxamine B. *J. Radioanal.*
502 *Nucl. Chem.* **2007**, *273* (3), 683–688. <https://doi.org/10.1007/s10967-007-0931-5>.
- 503 (36) Nair, A.; Juwarkar, A. A.; Singh, S. K. Production and Characterization of Siderophores and Its Application in Arsenic
504 Removal from Contaminated Soil. *Water. Air. Soil Pollut.* **2007**, *180* (1–4), 199–212. [https://doi.org/10.1007/s11270-006-](https://doi.org/10.1007/s11270-006-9263-2)
505 [9263-2](https://doi.org/10.1007/s11270-006-9263-2).
- 506 (37) Sebat, J. L.; Paszczynski, A. J.; Cortese, M. S.; Crawford, R. L. Antimicrobial Properties of Pyridine-2,6-Dithiocarboxylic
507 Acid, a Metal Chelator Produced by *Pseudomonas* Spp. *Appl. Environ. Microbiol.* **2001**, *67* (9), 3934–3942.
508 <https://doi.org/10.1128/AEM.67.9.3934-3942.2001>.
- 509 (38) Bultreys, A.; Gheysen, I.; De Hoffmann, E. Yersiniabactin Production by *Pseudomonas syringae* and *Escherichia coli*, and
510 Description of a Second Yersiniabactin Locus Evolutionary Group. *Appl. Environ. Microbiol.* **2006**, *72* (6), 3814–3825.

- 511 <https://doi.org/10.1128/AEM.00119-06>.
- 512 (39) Moscatello, N. J.; Pfeifer, B. A. Yersiniabactin Metal Binding Characterization and Removal of Nickel from Industrial
513 Wastewater. *Biotechnol. Prog.* **2017**, *33* (6), 1548–1554. <https://doi.org/10.1002/btpr.2542>.
- 514 (40) Thomas, W.; Bellenger, J. P.; Morel, F. M. M.; Kraepiel, A. M. L. Role of the Siderophore Azotobactin in the Bacterial
515 Acquisition of Nitrogenase Metal Cofactors. *Environ. Sci. Technol.* **2009**, *43* (19), 7218–7224.
516 <https://doi.org/10.1021/es8037214>.
- 517 (41) Murugappan, R. M.; Aravinth, A.; Rajaroobia, R.; Karthikeyan, M.; Alamelu, M. R. Optimization of MM9 Medium
518 Constituents for Enhancement of Siderophoregenesis in Marine *Pseudomonas putida* Using Response Surface Methodology.
519 **2012**, *52* (3), 433–441. <https://doi.org/10.1007/s12088-012-0258-y>.
- 520 (42) Kalinowski, B. E.; Liermann, L. J.; Givens, S.; Brantley, S. L. Rates of Bacteria-Promoted Solubilization of Fe from Minerals:
521 A Review of Problems and Approaches. *Chem. Geol.* **2000**, *169* (3), 357–370.
- 522 (43) Arora, N. K. Modified Microplate Method for Rapid and Efficient Estimation of Siderophore Produced by Bacteria. *3 Biotech*
523 **2017**, *7* (6), 1–9. <https://doi.org/10.1007/s13205-017-1008-y>.
- 524 (44) Matthijs, S.; Brandt, N.; Ongena, M.; Achouak, W.; Meyer, J. M.; Budzikiewicz, H. Pyoverdine and Histicorrugatin-Mediated
525 Iron Acquisition in *Pseudomonas thivervalensis*. *BioMetals* **2016**, *29* (3), 467–485. <https://doi.org/10.1007/s10534-016-9929-1>.
- 526 (45) Ure, A. M. Methods of Analysis for Heavy Metals in Soils. In *Heavy metals in soils, 2nd ed*; Alloway, B. J., Ed.; Blackie:
527 London, UK, 1990; pp 58–102.
- 528 (46) Williamson, A. J.; Verbruggen, F.; Chavez Rico, V. S.; Bergmans, J.; Spooren, J.; Yurramendi, L.; Laing, G. Du; Boon, N.;
529 Hennebel, T. Selective Leaching of Copper and Zinc from Primary Ores and Secondary Mineral Residues Using Biogenic
530 Ammonia. *J. Hazard. Mater.* **2021**, *403*, 123842. <https://doi.org/10.1016/j.jhazmat.2020.123842>.
- 531 (47) Shaikh, S.; Wani, S. J.; and Sayyed R. Z. Statistical-Based Optimization and Scale-up of Siderophore Production Process on
532 Laboratory Bioreactor. *3 Biotech* **2016**, *6* (1), 1–10. <https://doi.org/10.1007/s13205-016-0365-2>.
- 533 (48) Seinsche, D.; Taraz, K.; Budzikiewicz, K. *Neue Pyoverdin-Siderophore aus Pseudomonas putida C*. *Journal für Praktische*
534 *Chemie/Chemiker-Zeitung*, 1993. **335**(2): p. 157-168.
- 535 (49) Ongena, M.; Jacques, P.; Delfosse, P.; Thonart, P. Unusual Traits of the Pyoverdin-Mediated Iron Acquisition System in
536 *Pseudomonas putida* Strain BTP1. *BioMetals* **2002**, *15* (1), 1–13. <https://doi.org/10.1023/A:1013157824411>.
- 537 (50) Vyas, S.; Ting, Y. P. A Review of the Application of Ultrasound in Bioleaching and Insights from Sonication in (Bio)Chemical
538 Processes. *Resources* **2018**, *7* (1). <https://doi.org/10.3390/resources7010003>.
- 539 (51) Guo, Z.; Zhang, L.; Cheng, Y.; Xiao, X.; Pan, F.; Jiang, K. Effects of PH, Pulp Density and Particle Size on Solubilization of
540 Metals from a Pb/Zn Smelting Slag Using Indigenous Moderate Thermophilic Bacteria. *Hydrometallurgy* **2010**, *104* (1), 25–
541 31. <https://doi.org/10.1016/j.hydromet.2010.04.006>.
- 542 (52) Panda, S.; Mishra, S.; Rao, D. S.; Pradhan, N.; Mohapatra, U.; Angadi, S.; Mishra, B. K. Extraction of Copper from Copper
543 Slag: Mineralogical Insights, Physical Beneficiation and Bioleaching Studies. *Korean J. Chem. Eng.* **2015**, *32* (4), 667–676.
544 <https://doi.org/10.1007/s11814-014-0298-6>.
- 545 (53) Ruan, Z.; Li, M.; Gao, K.; Zhang, D.; Huang, L.; Xu, W.; Liu, X. Effect of Particle Size Refinement on the Leaching Behavior
546 of Mixed Rare-Earth Concentrate Using Hydrochloric Acid. *ACS Omega* **2019**, *4* (6), 9813–9822.
547 <https://doi.org/10.1021/acsomega.9b01141>.
- 548 (54) Jing, Y.; Bremer, P. J.; Lamont, I. L.; McQuillan, A. J. Infrared Spectroscopic Studies of Siderophore-Related Hydroxamic

- 549 Acid Ligands Adsorbed on Titanium Dioxide. *Langmuir* **2006**, 22 (24), 10109–10117. <https://doi.org/10.1021/la061365l>.
- 550 (55) Kraemer, D.; Junge, M.; Oberthür, T.; Bau, M. Improving Recoveries of Platinum and Palladium from Oxidized Platinum-
551 Group Element Ores of the Great Dyke, Zimbabwe, Using the Biogenic Siderophore Desferrioxamine B. *Hydrometallurgy*
552 **2015**, 152, 169–177. <https://doi.org/10.1016/j.hydromet.2015.01.002>.
- 553 (56) Gadd, G. M. *Fungal Production of Citric and Oxalic Acid: Importance in Metal Speciation, Physiology and Biogeochemical*
554 *Processes*; 1999; Vol. 41. [https://doi.org/10.1016/S0065-2911\(08\)60165-4](https://doi.org/10.1016/S0065-2911(08)60165-4).
- 555 (57) Simate, G. S.; Ndlovu, S. The Fungal and Chemolithotrophic Leaching of Nickel Laterites — Challenges and Opportunities.
556 **2010**, 103, 150–157. <https://doi.org/10.1016/j.hydromet.2010.03.012>.
- 557 (58) Williamson, A. J.; Folens, K.; Van Damme, K.; Olaoye, O.; Abo Atia, T.; Mees, B.; Nicomel, N. R.; Verbruggen, F.; Spooren,
558 J.; Boon, N.; Hennebel, T.; Du Laing, G. Conjoint Bioleaching and Zinc Recovery from an Iron Oxide Mineral Residue by a
559 Continuous Electrodialysis System. *Hydrometallurgy* **2020**, 195, 105409. <https://doi.org/10.1016/j.hydromet.2020.105409>.
- 560 (59) Santos, M. A.; Bento, C.; Esteves, M. A.; Farinha, J. P. S.; Martinho, J. M. G. Iron Release Mechanism in a Trihydroxamate
561 Siderophore Analogue. Kinetics and Effect of pH. *Inorganica Chim. Acta* **1997**, 258 (1), 39–46. [https://doi.org/10.1016/S0020-1693\(96\)05510-7](https://doi.org/10.1016/S0020-1693(96)05510-7).
- 562
- 563 (60) Matsumoto, K.; Ozawa, T.; Jitsukawa, K.; Masuda, H. Synthesis, Solution Behavior, Thermal Stability, and Biological
564 Activity of an Fe(III) Complex of an Artificial Siderophore with Intramolecular Hydrogen Bonding Networks. *Inorg. Chem.*
565 **2004**, 43 (26), 8538–8546. <https://doi.org/10.1021/ic048761g>.
- 566 (61) Neubauer, U.; Nowack, B.; Furrer, G.; Schulin, R. Heavy Metal Sorption on Clay Minerals Affected by the Siderophore
567 Desferrioxamine B. *Environ. Sci. Technol.* **2000**, 34 (13), 2749–2755. <https://doi.org/10.1021/es990495w>.
- 568 (62) Lhenry, S.; Leroux, Y. R.; Hapiot, P. Use of Catechol as Selective Redox Mediator in Scanning Electrochemical Microscopy
569 Investigations. *Anal. Chem.* **2012**, 84 (17), 7518–7524. <https://doi.org/10.1021/ac301634s>.
- 570 (63) Lai, B.; Yu, S.; Bernhardt, P. V.; Rabaey, K.; Virdis, B.; Krömer, J. O. Biotechnology for Biofuels Anoxic Metabolism and
571 Biochemical Production in Pseudomonas Putida F1 Driven by a Bioelectrochemical System. *Biotechnol. Biofuels* **2016**, 1–13.
572 <https://doi.org/10.1186/s13068-016-0452-y>.
- 573 (64) Albrecht-Gary, A. M.; Crumbliss, A. L. Coordination Chemistry of Siderophores: Thermodynamics and Kinetics of Iron
574 Chelation and Release. *Met. Ions Biol. Syst.* **1998**, 35, 239–327.
- 575 (65) Del Olmo, A.; Caramelo, C.; SanJose, C. Fluorescent Complex of Pyoverdine with Aluminum. *J. Inorg. Biochem.* **2003**, 97 (4),
576 384–387. [https://doi.org/10.1016/S0162-0134\(03\)00316-7](https://doi.org/10.1016/S0162-0134(03)00316-7).
- 577 (66) Jain, R.; Fan, S.; Kaden, P.; Tsushima, S.; Foerstendorf, H.; Barthen, R.; Lehmann, F.; Pollmann, K. Recovery of Gallium from
578 Wafer Fabrication Industry Wastewaters by Desferrioxamine B and E Using Reversed-Phase Chromatography Approach.
579 *Water Res.* **2019**, 158 (2019), 203–212. <https://doi.org/10.1016/j.watres.2019.04.005>.
- 580

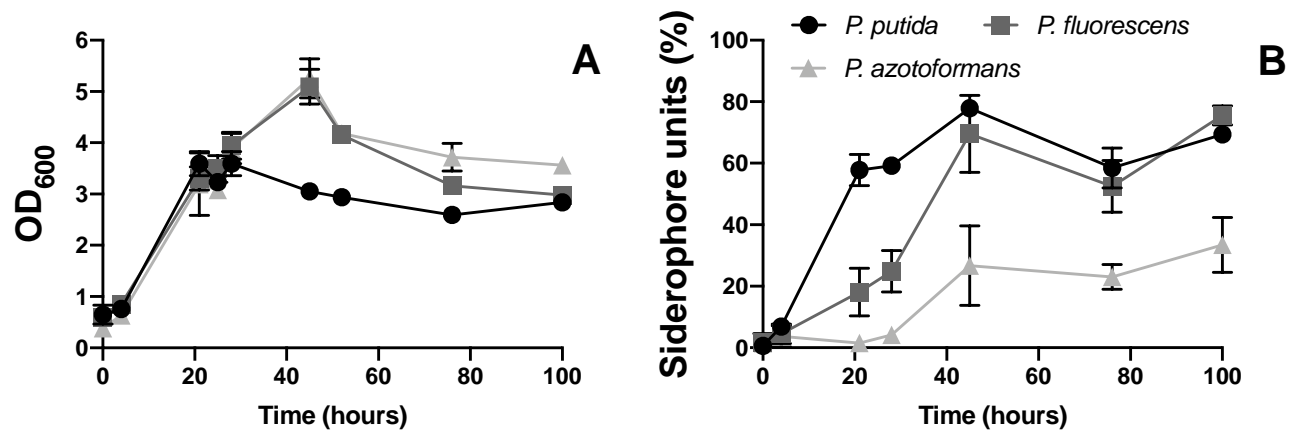


Figure 1. Growth curve (A) and siderophore production (B) by *Pseudomonas putida*, *Pseudomonas fluorescens* and *Pseudomonas azotoformans* as a function of time. Error bars arise from $n = 3$ independent samples.

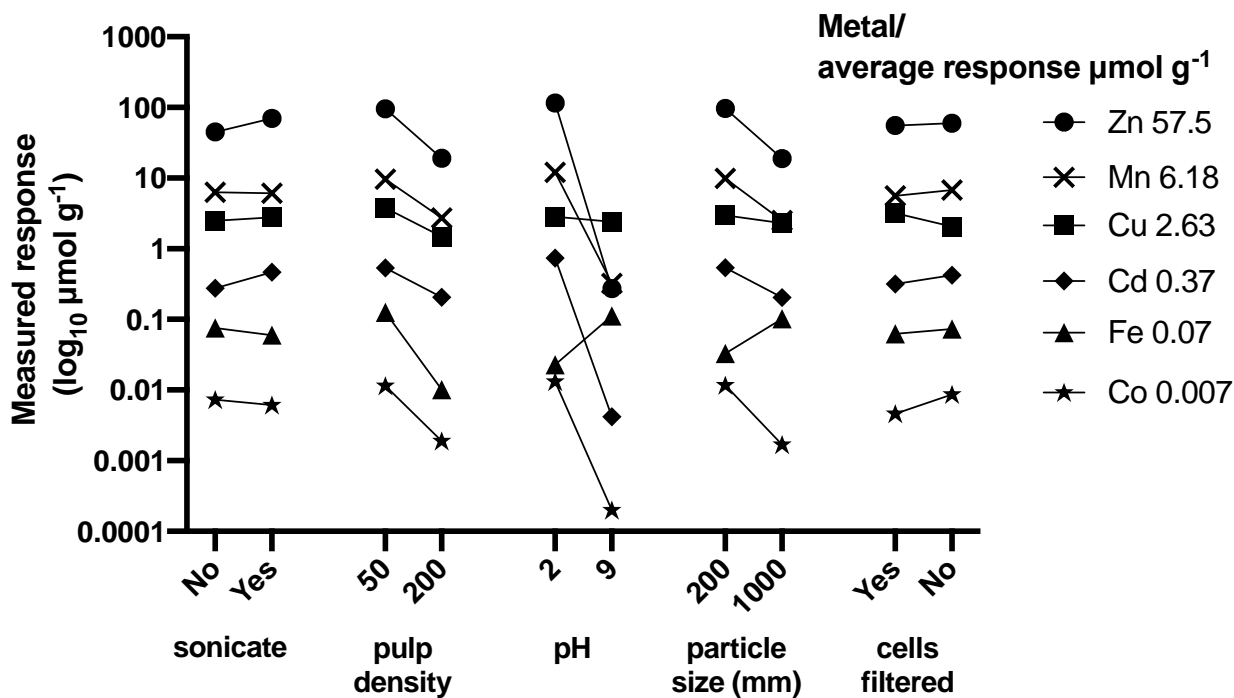


Figure 2. Effect of sonication, solid to liquid (S/L) ratio, pH, sieving mesh size of the substrate and presence of cells on the Zn, Mn, Cu, Cd, Fe and Co extraction from iron oxide mineral residue, presented as its calculated response ($\log_{10} \mu\text{mol g}^{-1}$) to each treatment. The average response for each metal is given in the legend

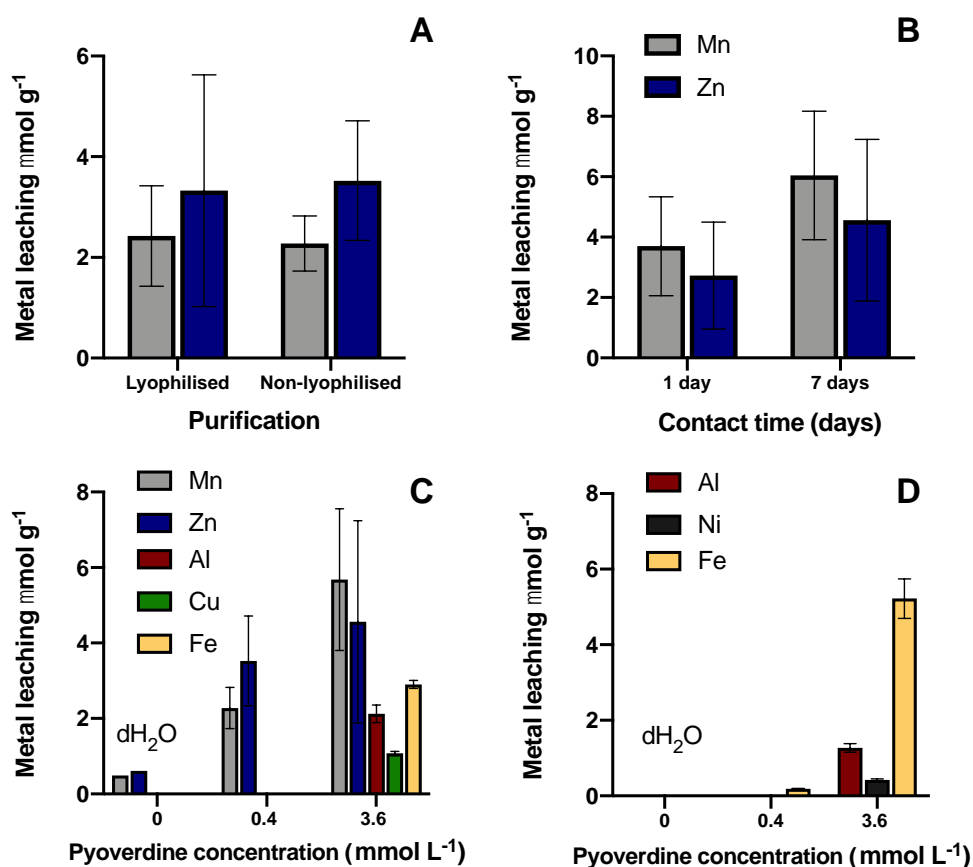


Figure 3. Metal equivalents extracted from low grade mineral residues by PyoPpC-3B. The effect of a purification step to enhance the pyoverdine purity (A), contact time (B) and pyoverdine concentration after a contact time of 1 day with iron oxide mineral residue (C) and laterite (D) on the extracted metal content is shown ($n = 3$).

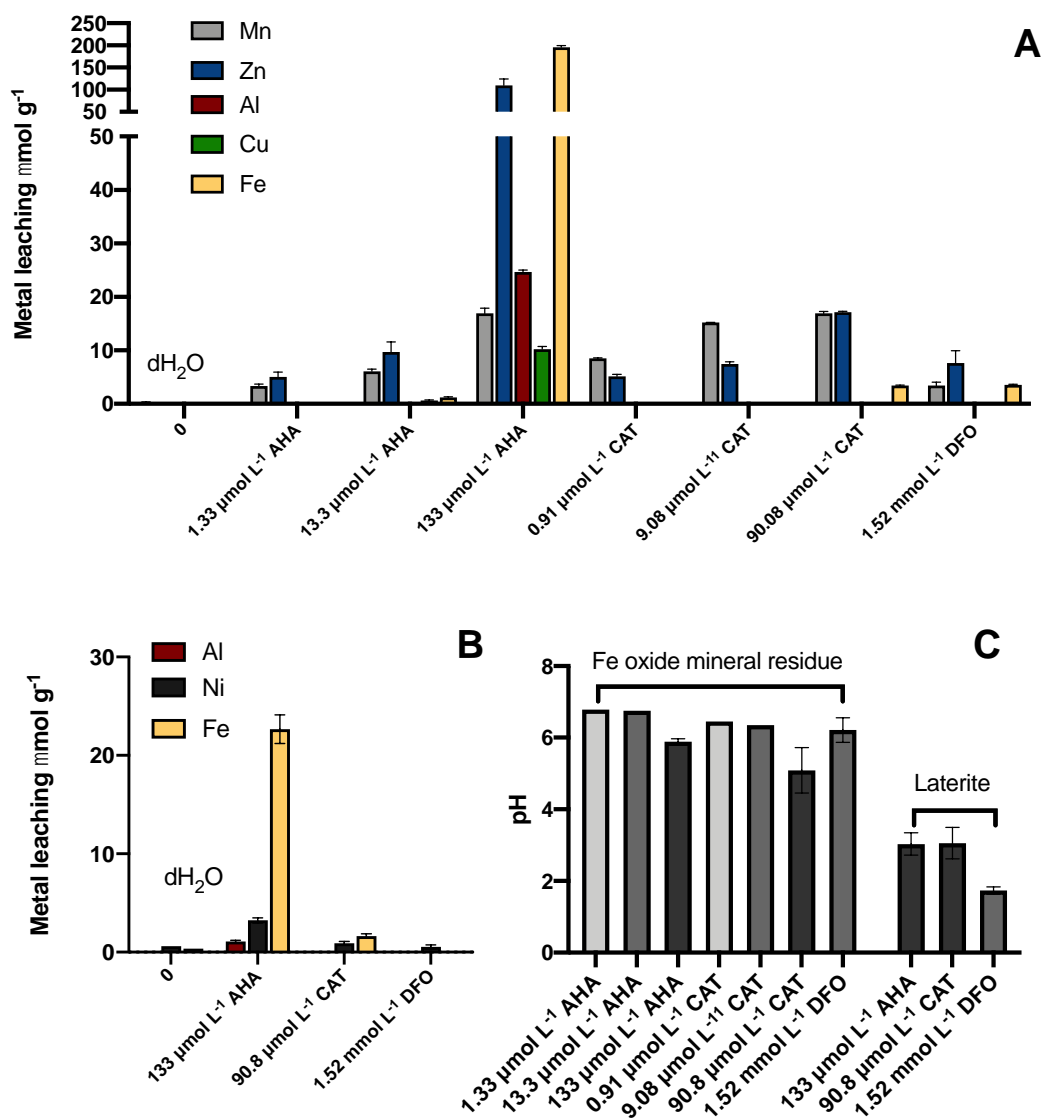


Figure 4. Metal leaching from iron oxide mineral residue (A) and laterite (B) by purified water (negative control), deferoxamine (DFO), acetohydroxamic acid (AHA) and catechol (CAT), with their respective final pH after a contact time of one day. Error bars arise from $n = 3$ independent samples.

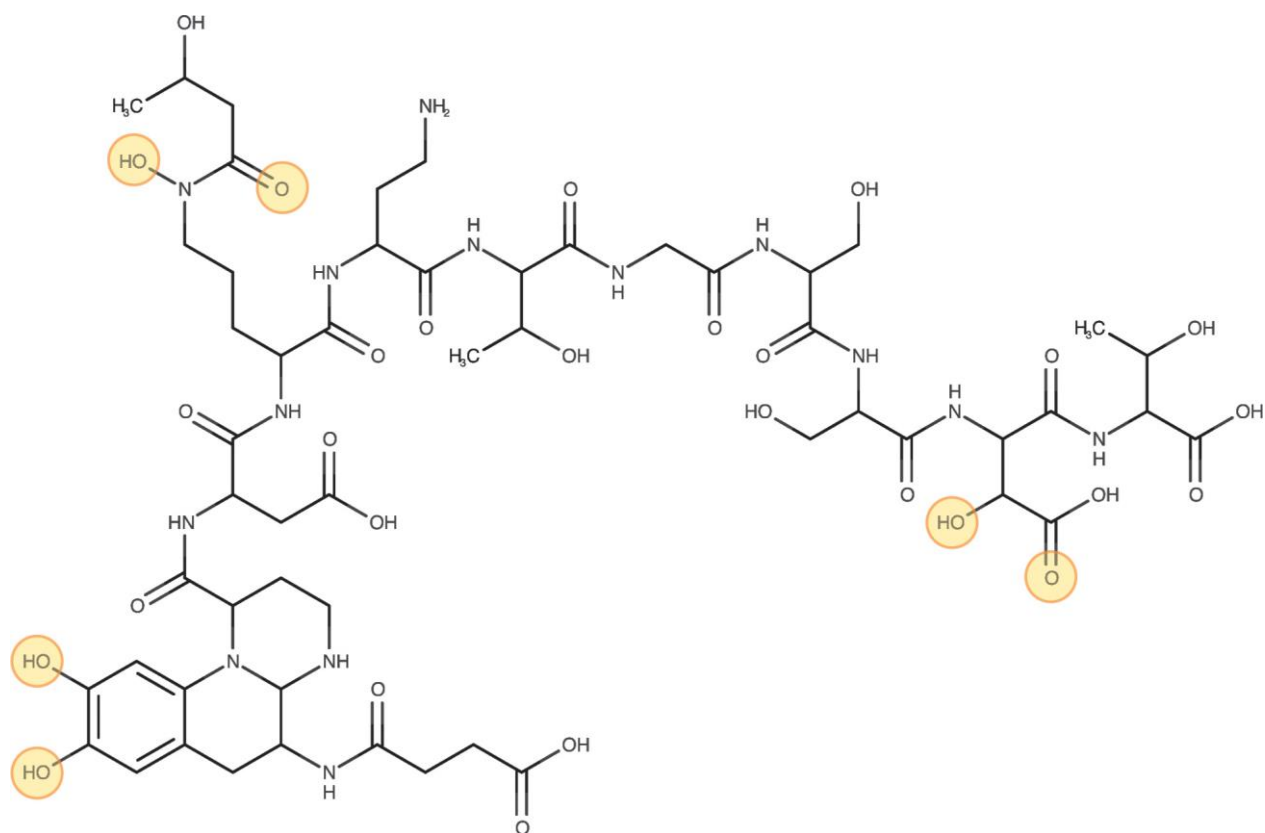


Figure 5. Chemical structure of PyoPpC-3B. The 6 circled functional groups are responsible for metal interaction and together form the metallophore complex