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Use of carbon additions to enhance zinc removal from mine drainage in short residence time, flow-through sulfate-reducing bioreactors

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1 **Use of carbon additions to enhance zinc removal from mine drainage in short residence**
2 **time, flow-through sulfate-reducing bioreactors**

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8 **Abstract**

9 The effectiveness of liquid carbon additions to enhance zinc removal in laboratory-scale short
10 hydraulic residence time (19 hours) compost bioreactors receiving synthetic mine water with
11 a high influent zinc concentration (45 mg/L) was investigated. The unique combination of
12 short hydraulic residence time, high strength wastewater and carbon additions was designed
13 to investigate the potential for application of this approach to mine water treatment.
14 Effective removal of zinc could not be sustained by sulfate reduction and / or other
15 attenuation processes, in the presence of such elevated zinc concentrations, without carbon
16 supplementation. Propionic acid addition resulted in improved and sustained performance
17 (mean zinc removal 99%). Comparison of bioreactors receiving continuous propionic acid with
18 those in which carbon addition ceased after a period of time demonstrated distinct
19 differences in the microbial communities. The addition of propionic acid promoted the
20 activities of sulfate reducing bacteria with the compost substrate becoming a net sink for
21 sulfate, which led to efficient zinc removal via bacterial sulfate reduction. Upon cessation of
22 propionic acid addition, carbon limitation resulted in oxidising conditions and the growth of
23 sulfur oxidising bacteria with the compost substrate becoming a net source of sulfate,
24 compromising zinc removal by bacterial sulfate reduction. These research findings show the
25 potential for modest liquid carbon additions to compost-based passive treatment systems to
26 enhance rates of metal attenuation in a short hydraulic residence time, enabling remediation
27 of highly polluting mine drainage at sites with limited land availability.

28 **KEYWORDS:** Zinc, mine drainage, compost bioreactor, carbon addition, sulfate reducing
29 bacteria, residence time

30

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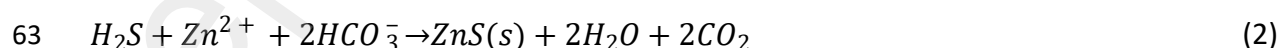
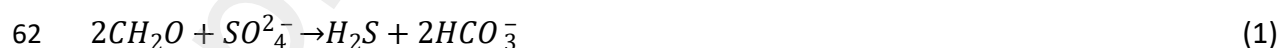
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31 1. Introduction

32 Low pH, high metal concentration mine discharges are among the most ecologically damaging
33 effluent types world-wide. In the UK zinc is particularly prevalent in drainage from abandoned
34 metal mines with over 50% of the total zinc flux to freshwaters of England and Wales
35 attributed to such pollution (Mayes et al. 2013). Although the majority of discharges in the
36 UK are characterised by relatively low zinc concentrations (see Figure S1), a limited number
37 of highly contaminated (up to 45 mg/L zinc) discharges cause severe ecological damage and
38 are therefore a target for remediation. Such heavily polluted mine drainage is also well
39 documented elsewhere in the world (e.g. Castillo et al., 2012; Mosley et al., 2015; Strosnider
40 et al., 2011, 2013).

41 Compost bioreactors utilising bacterial sulfate reduction (BSR) are a favoured approach to
42 metal mine drainage remediation (Gandy et al. 2016; LaBar and Nairn 2018; Neculita et al.
43 2007; Vasquez et al. 2016). However, limitations of such low-energy passive systems, in many
44 locations, include their large footprint and uncertainty regarding their effectiveness in
45 treating high metal concentrations. Many UK discharges occur in remote upland locations,
46 such as in northern England and western Wales, where availability of flat land for treatment
47 systems is limited (see Mayes et al., 2009). Attenuation of zinc in low-cost, low maintenance
48 passive systems with a short hydraulic residence time (HRT) to enable a small footprint is
49 therefore favoured. Whilst many investigations into the potential of compost bioreactors for
50 mine drainage remediation have used systems in which HRT is measurable in days (Biermann
51 et al. 2014; Cruz Viggi et al. 2010; Di Luca et al. 2011; Song et al. 2012; Strosnider et al. 2011,
52 2013), recent research has demonstrated successful removal of zinc in a HRT of less than 14.5
53 hours (Gandy et al. 2016). In the research reported here the limitations of compost
54 bioreactors, particularly for the remediation of highly contaminated UK discharges, were
55 investigated together for the first time. Short residence time passive bioreactors receiving
56 high zinc concentration mine water operated continuously for two years, with controlled
57 testing of the benefits of carbon additions to enhance performance.

58 The principle of BSR is that the reduction of sulfate by sulfate reducing bacteria (SRB) under
59 anaerobic conditions, using a carbon source (represented as CH_2O) as an electron donor,
60 generates sulfide (reaction (1)), which in turn reacts with metals to precipitate metal sulfides
61 (e.g. zinc sulfide, reaction (2)).



64 The choice of carbon source is important to sustain the long-term efficiency of treatment
65 (Costa et al. 2009). Simple organic compounds that are easily degradable, such as carboxylic
66 acids or alcohols, are used by SRB as carbon and energy sources (Gibert et al. 2004; Martins
67 et al. 2009). In laboratory cultures, lactate is the most common carbon source used by SRB

68 but would be prohibitively expensive to employ in full-scale treatment systems (Costa et al.
69 2009). Different types of compost are therefore frequently used to provide a long-term source
70 of carbon (Neculita et al. 2007). These more complex organic sources are far less costly than
71 proprietary carbon sources, are widely available, and often have physical characteristics that
72 make them suitable for use in flow-through water treatment systems.

73 Whilst a number of researchers have explored the benefits of carbon addition to compost-
74 based treatment systems (e.g. Costa et al. 2009; Dvorak et al. 1992; Mayes et al. 2011; Nielsen
75 et al. 2018), these studies are based on either a high HRT (greater than 24 hours) or a
76 comparatively low zinc concentration (less than 20 mg/L). In the research reported here the
77 focus is on the combination of relatively short HRT treatment systems, since their absolute
78 size is a key constraint to wider deployment of the technology in the UK, and waters
79 containing a high zinc concentration. The extent to which the microbial communities key to
80 metal attenuation are influenced by carbon addition under short HRT conditions, and in turn
81 whether they can sustain bacterial sulfate reduction sufficiently to maintain effective zinc
82 removal, is specifically investigated.

83 As compost bioreactors are driven by SRB activity an improved understanding of their
84 microbial community diversity and function is critical for long-term performance (Hiibel et al.
85 2008). Several studies have demonstrated a relationship between system performance and
86 microbial community (e.g. Baldwin et al. 2015, 2016; Drennan et al. 2016, 2017). Engineering
87 design and system operation should thus be configured to ensure optimum activities of the
88 SRB that are responsible for remediation. Enhancement of microbial communities in short
89 HRT bioreactors subjected to high influent zinc concentrations has not previously been
90 investigated.

91 This study, using laboratory scale upflow column experiments, aims to (1) evaluate the
92 effectiveness of liquid carbon additions on zinc immobilisation in short HRT (19 hours)
93 compost bioreactors receiving a high influent zinc concentration (45 mg/L), (2) assess the
94 responses of a microbial community to such metal and carbon additions, (3) determine
95 whether microbial responses favourable to the immobilisation of metals can be engineered
96 in enhanced passive treatment systems receiving carbon additions.

97 **2. Materials and methods**

98 *2.1. Experimental configuration*

99 Two sets of laboratory-scale continuous upflow bioreactors (internal diameter 105 mm,
100 length 500 mm) were operated in triplicate. Limestone gravel (diameter < 10 mm) was placed
101 at the base of each bioreactor (depth 40 mm) and overlain by a reactive substrate (depth 400
102 mm) comprising British Standards Institution (BSI) Publicly Available Specification (PAS) 100
103 compost (45% v/v), wood chips (45% v/v) and activated sludge from a municipal wastewater
104 treatment plant (10%). A 25 mm cover of water ensured that the substrate remained

105 saturated (Figure S2). The substrate was sourced from a decommissioned pilot-scale
106 bioreactor that treated zinc-rich, circumneutral mine water for 2 years (Gandy et al. 2016).
107 This substrate was selected as it was known to have supported BSR previously, but via
108 treatment of a relatively low strength wastewater (mean pH 7.74 and 2.32 mg/L Zn; Gandy et
109 al. 2016) unlikely to invoke any inhibitory effects. Samples from across the entire depth and
110 length of the bioreactor were thoroughly mixed before placement of 3,530 cm³ in each
111 laboratory bioreactor. The substrate was saturated with synthetic mine water to facilitate the
112 calculation of porosity (0.48 – 0.51) and estimate hydraulic residence time. A Watson-Marlow
113 300 series peristaltic pump was set up to give a mean residence time of 19 hours (mean flow-
114 rate 1.6 ml/min).

115 2.2. *Bioreactor operation*

116 Synthetic mine water (mean 45 mg/L Zn, 156 mg/L SO₄, pH 4.1, Table S1), produced by
117 dissolving laboratory-grade salts in deionised water, was passed upwards through the
118 bioreactors for 755 days. The pH was controlled by addition of <10 mL of 1% H₂SO₄ to the
119 mine water. This water quality was representative of an actual mine water discharge in
120 northern England (see Table S1 for details).

121 Propionic acid (13.4M) addition to one set of three bioreactors (1A, B, C) commenced on day
122 234 at a rate of 1 ml per 35 L influent water. The other set of three bioreactors (2A, B, C)
123 operated as a control and continued to receive synthetic mine water only. On day 511
124 propionic acid addition to one bioreactor (1A) ceased.

125 2.3. *Water sampling and analysis*

126 Samples were collected at fortnightly intervals in polypropylene bottles from the influent
127 mine water and the effluent of each bioreactor with more intense (weekly) sampling
128 immediately after propionic acid addition commenced. Flow rate was measured on each
129 sampling occasion by measuring the volume of effluent water collected over a specified time.
130 Measurements of water temperature, pH, oxidation-reduction potential (ORP) and electrical
131 conductivity in the influent and effluent waters were recorded using a pre-calibrated Myron
132 L 6P Ultrameter. Total alkalinity was determined using a Hach digital titrator with 0.16 N
133 sulfuric acid and bromcresol-green methyl-red indicator. Two 30 ml aliquots were acidified
134 with 1% v/v concentrated nitric acid, one following filtration (0.45 µm cellulose nitrate filters)
135 for total and filtered cation analysis. A 30 ml aliquot was filtered and left unacidified for anion
136 analysis. Samples were stored at 4 °C prior to analysis. Cation analysis was performed using a
137 Varian Vista-MPX Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES).
138 Anion concentrations were determined using a Dionex DX320 Ion Chromatograph (IC).

139

140 2.4. Substrate sampling and geochemical analysis

141 Substrate samples were collected from all bioreactors at the end of the trial. In the bioreactors
142 that received propionic acid (1A, B, C), two samples were collected, at approximate depths of
143 220 mm (middle of reactors) and 310 mm (bottom of reactors), in pre-washed (analytical
144 grade nitric acid, 10% v/v) polypropylene bottles which were filled with water from within the
145 bioreactors. One sample was stored at minus 80°C, prior to microbial analysis, and the other
146 at minus 20°C, prior to geochemical analysis. An additional sample was collected at an
147 approximate depth of 90 mm (top of reactors) for microbial analysis only. In the control
148 bioreactors (2A, B, C), two samples were collected at an approximate depth of 220 mm
149 (middle of reactors) and stored as above prior to geochemical and microbial analysis. Samples
150 were allowed to defrost in an anaerobic cabinet before analysis. Geochemical analysis
151 followed the Acid Volatile Sulfide – Simultaneously Extracted Metals (AVS-SEM) method of
152 Allen et al. (1991) with the exception that H₂S was purged from the sample for 3 hours to
153 ensure that all AVS was recovered, as recommended by Standard Method 4500-S²⁻ J (APHA,
154 2005). Metals analysis was undertaken as for water samples. A control sample of the original
155 mixed substrate was subjected to the same analysis.

156 2.5. Microbial analysis

157 Twelve 16S rRNA PCR amplicon libraries were sequenced comprising three (top, middle and
158 bottom) depths for each of Set 1 bioreactors (A, B, C) and an additional three samples from
159 the middle of each one of the three control bioreactors. All bioreactor substrate samples were
160 collected at the end of the trial (see Supporting Information (SI) for a more detailed methods
161 description). Briefly, amplicons of 16S rRNA gene fragments (V4/V5 region) were PCR
162 amplified with barcode-ligated amplification primers from DNA extracts. Amplicons were
163 then pooled and sequenced using the Ion PGM™ sequencing platform. Sequence libraries for
164 each sample were assembled and analysed using the QIIME2 analysis pipeline (Caporaso et
165 al., 2010). A principal components analysis (PCA) of sample diversities was generated using
166 the STAMP v2 software package (Parks et al., 2014). Phylogenetic trees of key representative
167 sequences and their BLAST derived close relatives were generated in MEGA7 (Kumar et al.,
168 2016).

169 3. Results and discussion

170 3.1. Zinc and sulfate removal

171 There was no significant difference between the concentrations of total zinc and filtered zinc
172 in the effluent throughout the trial (Mann-Whitney U test; $p > 0.05$ for replicates 1A, 1B and
173 1C). Therefore, all values reported here are total zinc concentrations.

174 Effective removal of zinc (removal efficiency consistently > 90%) occurred in all bioreactors
175 during the first 90 days of the trial, but effluent zinc concentrations increased in all three

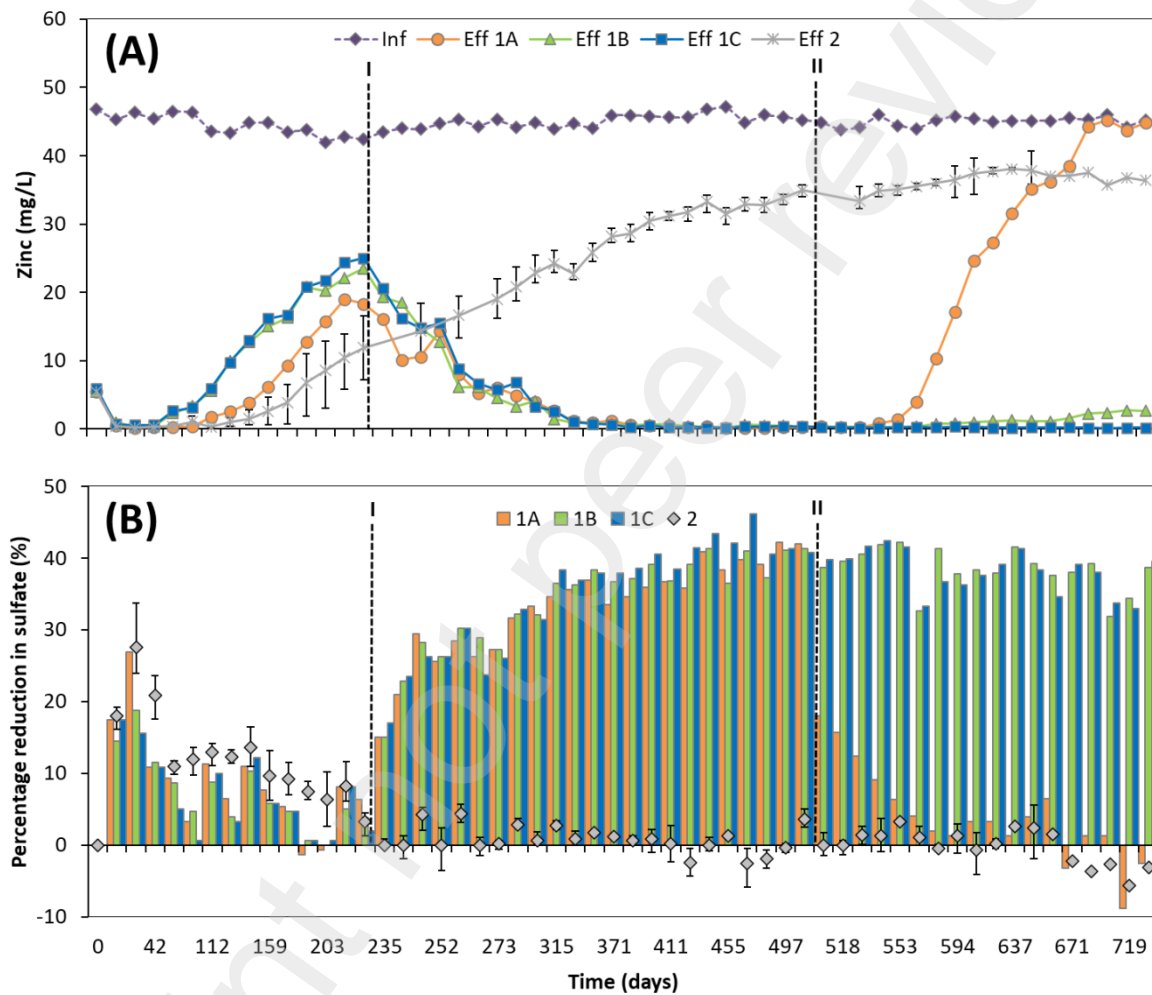
176 bioreactors between 90 and 230 days (Figure 1(A)). Initially there was evidence of a decrease
177 in sulfate concentration between influent and effluent in all bioreactors (Figure 1(B)). Based
178 on a molar ratio of sulfate to zinc of 1:1 (Reactions (1) and (2)), calculation of the predicted
179 effluent zinc concentration, assuming zinc removal only as a sulfide precipitate via BSR (using
180 the difference in influent and effluent sulfate concentration), indicates that actual effluent
181 zinc concentrations during the first 230 days of operation were much less than predicted in
182 all bioreactors (see Figure S3). Processes other than zinc sulfide precipitation (e.g. sorption)
183 were therefore contributing to zinc attenuation during this period.

184 As effluent zinc concentrations increased during the first 230 days of the trial there was a
185 corresponding decrease in mean percentage sulfate reduction (defined as the difference
186 between influent and effluent sulfate concentrations), from 20% to 3.6% (Figure 1(B)). This
187 indicated that effective removal of the high influent zinc concentration (mean 45 mg/L) could
188 not be sustained by sulfate reduction and / or other attenuation processes. Other studies
189 have reported zinc to be toxic or inhibitory to SRB at such concentrations (Poulson et al. 1997;
190 Utgikar et al. 2002, 2003), although Castillo et al. (2012) and Falk et al. (2018) found that
191 bacterial communities later recovered due to the proliferation of more metal-resistant
192 species. Whilst toxicity was not studied specifically in these trials, there is no direct evidence
193 that the elevated zinc concentration was toxic or inhibitory to sulfate reduction.

194 Upon commencement of propionic acid addition on day 234 effluent zinc concentrations
195 decreased substantially in all three replicates, from a mean of 22.3 mg/L to < 0.5 mg/L (mean
196 removal efficiency 99.1%) by day 427 (Figure 1(A)). There was no significant difference in zinc
197 concentration between replicates during the period of propionic acid addition to all
198 bioreactors, between days 235 and 511 (Mann-Whitney U test; $p > 0.05$ for all replicates). A
199 corresponding increase in percentage sulfate reduction, which was sustained at a mean of 41%
200 (Figure 1(B)), indicates that the SRB responded to the supplementary carbon with the result
201 that the rate of attenuation of zinc as its sulfide increased. Like zinc, there was no significant
202 difference in sulfate concentration between the three replicates (Mann-Whitney U test; $p >$
203 0.05 for all replicates). Between days 235 and 511 predicted effluent zinc concentration,
204 assuming only precipitation as its sulfide via BSR, was very close to actual effluent zinc
205 concentration (Figure S3), suggesting that BSR was the key zinc attenuation process during
206 this phase of the trials.

207 After propionic acid addition to bioreactor 1A ceased on day 513, effluent zinc concentration
208 immediately increased (Figure 1(A)), with removal efficiency < 1% by the end of the trial. A
209 substantial decrease in percentage sulfate removal also occurred with effluent sulfate
210 concentrations higher than influent sulfate concentration at times (as shown by negative
211 values in Figure 1B)). These findings suggest that the presence of an easily available electron
212 donor is the limiting factor for sulfate reduction in such systems. Similar observations have
213 been made by others following cessation of methanol addition (Bilek 2006; Mayes et al. 2011)
214 and depletion of lactate (Zhang and Wang 2014). Zinc removal efficiency in bioreactors 1B

215 and 1C, which continued to receive propionic acid, remained > 95% until the end of the trial
 216 and percentage sulfate removal was sustained at 30 - 40%. This suggests that the
 217 deteriorating performance of the bioreactors up to Day 230 of the trial was due to insufficient
 218 labile carbon to maintain high rates of BSR. In the control bioreactor set, which did not receive
 219 propionic acid, effluent zinc concentrations steadily increased until stabilising at around 37
 220 mg/L (mean removal efficiency 17.1%) (Figure 1(A) and Figure S4). Likewise, percentage
 221 sulfate removal progressively decreased throughout the trial indicating that SRB activity was
 222 limited in these control bioreactors (Figure 1(A) and Figure S5).



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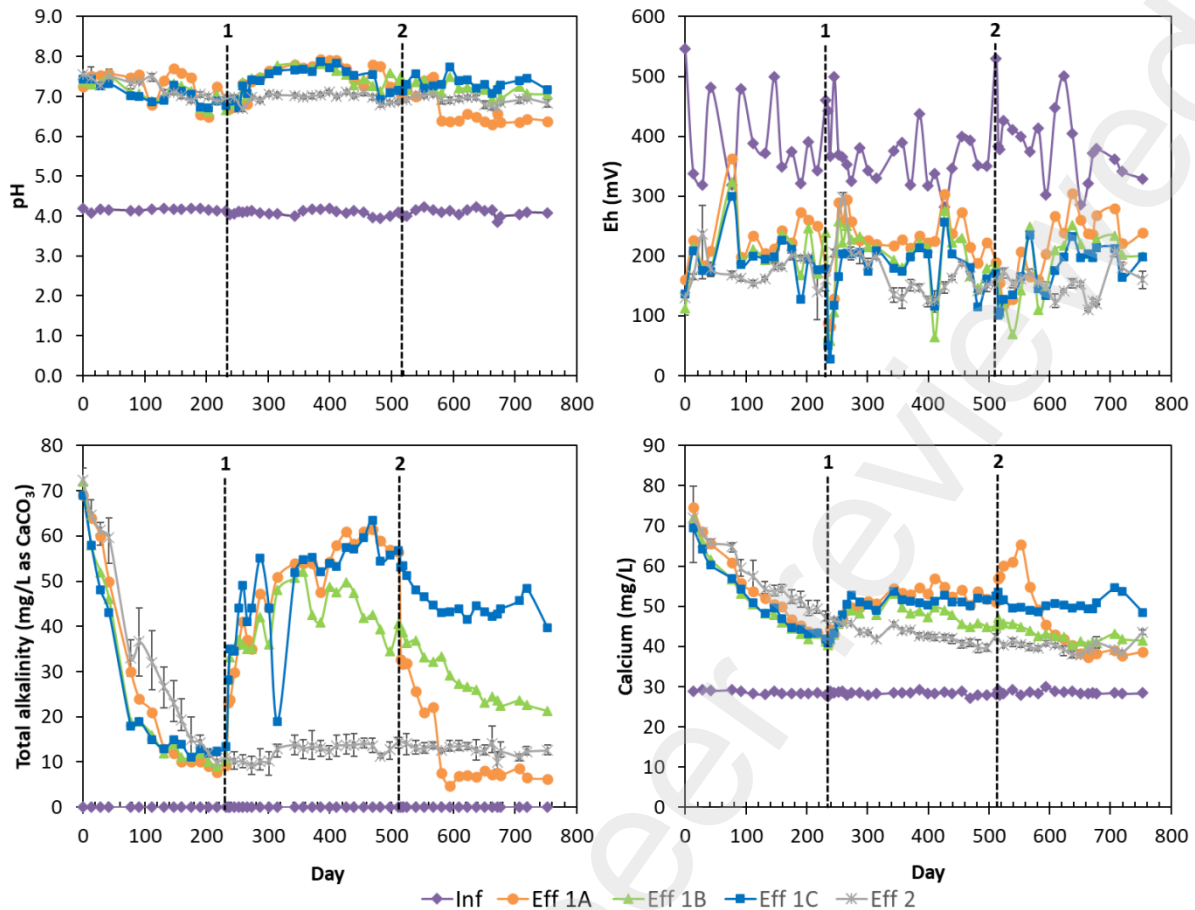
224 **Figure 1.** Effect of propionic acid addition on total zinc removal and sulfate reduction in
 225 laboratory-scale bioreactors. (A) Influent and effluent total zinc concentrations in bioreactors
 226 receiving propionic acid (Eff 1A, Eff 1B, Eff 1C) and mean effluent total zinc concentration in
 227 bioreactors receiving no propionic acid (Eff 2). (B) Percentage reduction in sulfate
 228 concentration in bioreactors receiving propionic acid (1A, 1B, 1C) and mean percentage
 229 reduction in sulfate concentration in bioreactors receiving no propionic acid (2). Error bars
 230 represent the range of results from triplicate samples. Vertical dashed lines refer to: (I)
 231 commencement of propionic acid addition; (II) cessation of propionic acid addition to reactor
 232 1A.

233 3.2. Alkalinity, pH and Eh

234 Changes in pH, Eh and alkalinity concentration between influent and effluent (Figure 2) were
235 consistent with variations in zinc and sulfate removal. Effective buffering of the acidic influent
236 water occurred throughout the trial with an influent mean pH of 4.1 consistently elevated to
237 an effluent pH of 6.29 – 7.92, which is optimal for SRB activity (Neculita et al. 2007). The only
238 notable deviation was in bioreactor 1A, 68 days after propionic acid addition had ceased,
239 when effluent pH decreased from a mean of 7.34 to a mean of 6.41 for the remainder of the
240 trial (Figure 2).

241 Influent and effluent Eh values were also consistent with conditions that favoured BSR and
242 zinc removal as its sulfide. Eh decreased between influent (mean 382 mV) and effluent (mean
243 196 mV) in all bioreactors, with a marked decrease in effluent Eh at commencement of
244 propionic acid addition (Figure 2). Although strongly anaerobic conditions did not appear to
245 become established within the bioreactors, the effluent Eh measurements reported here
246 likely overestimate the actual Eh values within the pore waters. The low flow rates of the
247 bioreactors necessitated an extended period of sample collection and it is possible that
248 oxidising conditions became re-established within the samples before Eh was measured.
249 Furthermore, Eh measurements made on effluent waters are likely not reflective of those in
250 the bulk compost.

251 Effluent alkalinity concentration initially decreased in all bioreactors before increasing upon
252 commencement of propionic acid addition, indicating enhanced alkalinity generation due to
253 BSR (reaction (1)) together with continued calcite dissolution from the limestone gravel
254 (Figure 2). Upon cessation of propionic acid addition effluent alkalinity concentration
255 decreased sharply in bioreactor 1A, compared to reactors continuing to receive propionic acid
256 (1B and 1C), albeit effluent alkalinity was beginning to decrease in all bioreactors (Figure 2).



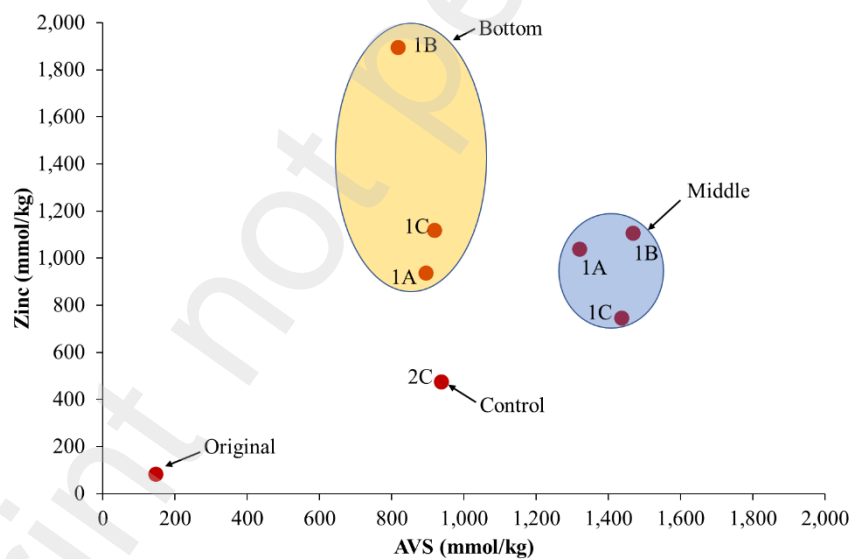
257

258 **Figure 2.** Influent and effluent pH, Eh, total alkalinity and total calcium concentration in
 259 bioreactors receiving propionic acid (Eff 1A, Eff 1B, Eff 1C) and mean effluent pH, Eh, total
 260 alkalinity and total calcium concentration in bioreactors receiving no propionic acid (Eff 2).
 261 Error bars represent the range of results from triplicate samples. Vertical dashed lines refer
 262 to: [1] commencement of propionic acid addition; [2] cessation of propionic acid addition to
 263 reactor 1A.

264 **3.3. Substrate geochemical analysis**

265 Sampling and analysis of the substrates was undertaken at the end of the trial to investigate
 266 metal attenuation processes. The determination of acid volatile sulfides (AVS) and
 267 simultaneously extracted metals (SEM) has previously been used effectively to assess the role
 268 of BSR as a zinc removal mechanism (Gandy et al. 2016; Jong and Parry 2004; LaBar and Nairn
 269 2018). Figure 3 shows that substantial accumulation of both AVS and zinc occurred in the
 270 bioreactors receiving propionic acid. This is consistent with the observed decreases in zinc
 271 and sulfate between the influent and effluent waters (Figure 1) and implies that ZnS was the
 272 main sink for zinc within these bioreactors. Despite having already accumulated some AVS
 273 and zinc during its emplacement in a pilot-scale flow through bioreactor treating zinc-rich
 274 water (Gandy et al. 2016), the original compost substrate contained much lower
 275 concentrations of zinc (81 mmol/kg) and AVS (148 mmol/kg) (Figure 3). Solid phase zinc

276 concentrations in two of the bioreactors receiving propionic acid were higher in the bottom
 277 layer (1B 1,895 mmol/kg; 1C 1,117 mmol/kg) than in the middle layer (1B 1,106 mmol/kg; 1C
 278 745 mmol/kg); in bioreactor 1A, concentrations in the bottom and middle layers were similar
 279 (Figure 3). Conversely, the AVS concentrations were higher in the middle layer (mean of the
 280 three bioreactors 1,410 mmol/kg) than in the bottom layer (mean 878 mmol/kg) (Figure 3).
 281 They also showed little variation between the three bioreactors at equivalent depths (SD = ±
 282 78 mmol/kg in middle layer; SD = ± 53 mmol/kg in bottom layer) compared to zinc
 283 concentrations (SD = ± 191 mmol/kg in middle layer; SD = ± 510 mmol/kg in bottom layer).
 284 Higher zinc concentrations in the bottom layer can be attributed to vigorous BSR close to
 285 where the influent water entered the bioreactors, due to relatively high zinc and sulfate
 286 concentrations. Gandy et al. (2016) and LaBar and Nairn (2018) also noted vertical variations
 287 in metal removal with the highest concentrations found closest to the influent ends of the
 288 systems. No notable difference in either zinc or AVS concentration was observed between
 289 bioreactor 1A, in which propionic acid addition ceased on day 511, and the other bioreactors
 290 receiving propionic acid, albeit the zinc concentration in the middle layer of this bioreactor
 291 was slightly higher than that in the bottom layer. Concentrations of both AVS (939 mmol/kg)
 292 and zinc (473 mmol/kg) were substantially lower in the control bioreactor that did not receive
 293 propionic acid. Nevertheless, the accumulation of some ZnS, particularly in the early stages
 294 of the trial, has resulted in higher concentrations than in the original substrate.



295

296 **Figure 3.** Concentrations of Acid Volatile Sulfide (AVS) and zinc in substrate from laboratory-
 297 scale bioreactors receiving propionic acid (1A, 1B, 1C), from a control bioreactor receiving no
 298 propionic acid (2C) and in the original substrate.

299 The molar ratio of AVS:Zinc in the BSR process is 1:1 (Reactions (1) and (2)) and can be used
 300 to indicate the predominant metal removal mechanism. A molar ratio > 1 demonstrates an
 301 excess of sulfide present within the substrate and implies that metals mainly exist in the form
 302 of sulfide minerals (Vasquez et al. 2016). If the molar ratio is < 1 other attenuation

303 mechanisms, such as adsorption and binding to organic matter, must play an important role
304 in metal attenuation. The AVS:Zinc ratio is > 1 (mean 1.51) in the middle layer of all bioreactors,
305 including the control which received no propionic acid (1.98), which suggests that sufficient
306 sulfide was available to immobilize all of the zinc present as a sulfide. In the bottom layer,
307 however, the AVS:Zinc ratio is < 1 (mean 0.74), albeit close to unity in bioreactors 1A (0.96)
308 and 1C (0.82). Therefore, other attenuation mechanisms must also have taken place in this
309 area of the bioreactors, which is consistent with previous findings (Gandy et al. 2016; Neculita
310 et al. 2008).

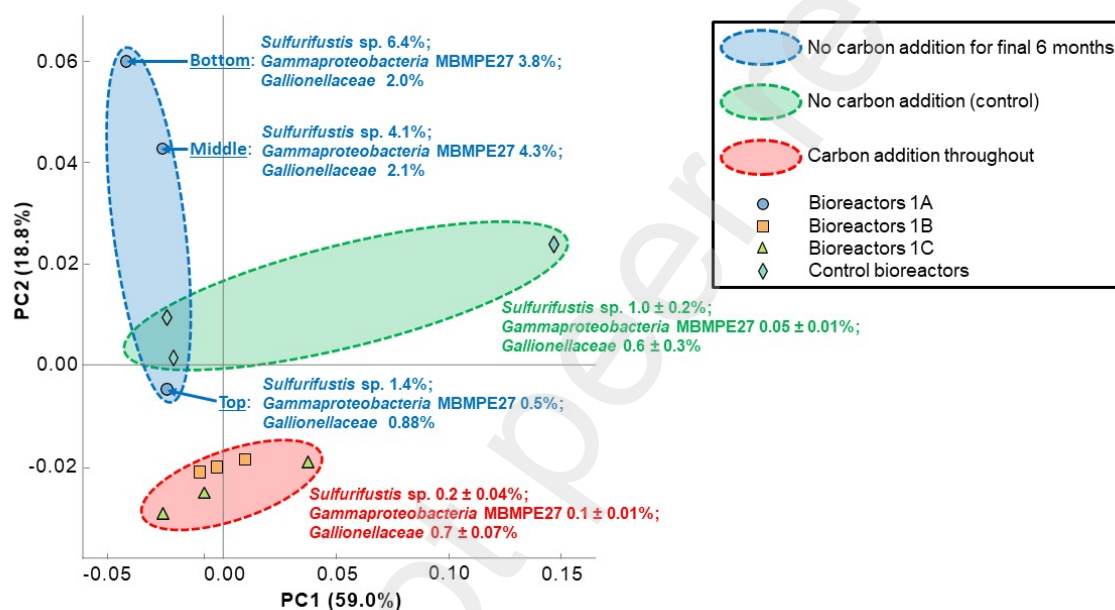
311 3.4. Substrate microbial analysis

312 Community analysis revealed some common features in the libraries consistent with the
313 compost bioreactor origin of the substrate (Gandy et al. 2016). Specifically, putatively sulfate
314 reducing bacteria (SRB) accounted for $7.9\% \pm 0.4$ (average \pm SE) of sequences. These SRB had
315 100% sequence homology with those recovered from natural or engineered anaerobic sulfate
316 reducing systems (Figure S6) and taxa identified indicated a dominance of H_2 utilising SRB
317 autotrophs (see SI for a more detailed discussion). Likewise, syntrophic bacterial partners
318 putatively responsible for fermentative degradation of compost material to supply SRB
319 substrates were also common features. Close relatives of these dominant taxonomic groups
320 (*Candidatus Caldatribacterium*, the family *Anaerolineaceae* and the family *Spirochaetaceae*)
321 were also identified previously in natural or engineered anaerobic environments (Figure S7).

322 Despite these common features, a spatial analysis (PCA) of the compost bioreactor
323 communities (Figure 4) provided useful mechanistic insights into differences in processes and
324 conditional changes. For instance, regardless of depth, all communities from the two
325 bioreactors continuously receiving propionic acid from Day 231 to Day 753 (1B and 1C)
326 clustered together. Contrastingly, depth resolved communities from reactor 1A, in which
327 propionic acid addition ceased on Day 511, were separated not just from the 1B and 1C
328 communities but also from each other. This spatial separation most likely reflected selection
329 by development of a redox gradient within 1A through the absence of propionic acid-driven
330 oxygen consumption, increase in compost Eh and consequent re-oxidation of sulfides
331 accumulated during propionic acid feeding. This redox gradient was evidenced by a
332 substantial enrichment of putatively oxidative chemolithotrophic bacteria (Figure S8), namely,
333 *Sulfurifustis*, Gammaproteobacterial MBMPE27 group, and *Gallionellaceae* spp. in the bottom
334 (i.e. closest to the inlet) and middle sections of the column (see SI for a more detailed
335 discussion). Growth of putative sulfur oxidizers was consistent with effluent compositions
336 after cessation of propionic acid addition (Day 511), from which point bioreactor 1A
337 transitioned from a net sulfate sink to a net source towards the end of the trial (Figure 1(B)).
338 Control reactor communities did not substantially enrich for oxidative chemolithoautotrophs
339 as in reactor 1A, or cluster with reactors 1B and 1C, because without any propionic acid
340 feeding they did not either develop permanently low Eh conditions (as in 1B and 1C) or
341 accumulate reduced sulfur sufficient to sustain oxidative chemolithoautotrophic growth (AVS

342 levels in all the controls were considerably lower than the middle sections of the 1A, B and C
343 bioreactors).

344 A further inference made from these community composition patterns was that toxicity due
345 to elevated Zn concentrations in the influent was not a key constraint on bacterial activity
346 compared to carbon limitation (previously noted above) and changing redox. High influent Zn
347 concentrations, which did not change throughout operation, clearly had no effect on the
348 growth of other functional groups present in the bioreactor compost i.e. the putative sulfur
349 oxidising bacteria *Sulfurifustis*, which responded with growth on cessation of propionic acid
350 addition.



351

352 **Figure 4.** A Principal Component Analysis (PCA) based on amplicon sequence variant (ASV)
353 frequencies within 16S rRNA gene sequencing libraries constructed from the compost
354 bioreactors. Samples from the top, middle and bottom of the 1A (blue circles), 1B (orange
355 squares) and 1C (green triangles) column bioreactors are shown, plus samples from the
356 middle of the three control reactor columns (cyan diamonds). Ellipses are drawn around three
357 data groups: the 1A samples which stopped receiving propionic acid for the last six months of
358 reactor operation; a group comprising the 1B and 1C samples which received propionic acid
359 throughout; and the control reactors which did not receive carbon additions. Mean % ± SE
360 contribution of specific taxonomic groups related to sulfide and iron oxidation are provided
361 for two of the circled groups (1B + 1C and control). Individual sample values presented for the
362 1A group data to illustrate bottom to top progression of changes observed in this bioreactor.

363

364 4. Conclusions and implications

365 Liquid carbon additions to compost-based passive systems, harnessing bacterial sulfate
366 reduction (BSR), offer the potential to enhance rates of attenuation of zinc (mean of 99% zinc
367 removal during carbon addition) in short HRT treatment units receiving high strength
368 wastewater. For the passive units investigated here, deterioration in treatment performance
369 with respect to zinc was due to available carbon limitation, which was overcome by the
370 addition of propionic acid as a carbon source. Other divalent contaminant metals (e.g. lead,
371 cadmium, copper) could potentially be removed too, given the lower solubility products of
372 their sulfides, thus broadening scope for deployment of such low carbon technologies at sites
373 with high strength wastewaters but restricted land availability.

374 Laboratory-scale systems receiving continuous propionic acid and those in which propionic
375 acid addition ceased after a period of time induced distinct differences in the microbial
376 communities in the composts of the respective systems, which are indicative of the dominant
377 processes occurring in relation to metal removal. Addition of propionic acid favoured the
378 activities of the SRB and their syntrophic partners present in high proportions in the compost
379 substrate, inducing a net sink for sulfate via BSR and hence efficient Zn removal. Upon
380 cessation of propionic acid addition, the resulting carbon limitation increased the substrate
381 oxidation potential (as evidenced by the growth of sulfur oxidising bacteria), which
382 compromised zinc removal (as ZnS) via BSR and resulted in a system that was a net source of
383 sulfate. Hence, by such an intervention it is possible to engineer the microbial community and
384 its overall function to enhance treatment with respect to metal removal from the
385 contaminated mine water.

386 The laboratory-scale research described here used a compost commonly available in the UK,
387 a laboratory-grade liquid carbon addition (propionic acid), and a synthetic mine water
388 representing an actual low pH mine water discharge in the UK. The experiments ran for
389 approximately two years. Shortened tests of this type, using different composts, liquid carbon
390 sources and mine waters, would be a useful precursor to design and installation of any pilot-
391 or full-scale system at which liquid carbon addition might be anticipated as a requirement,
392 especially given the large investment overall to construct a full-scale treatment system. Such
393 tests would also provide better understanding of the range of liquid carbon sources
394 deployable for this purpose, and also contribute to better design guidance for enhanced
395 passive treatment.

396 Passive treatment has been defined as using only naturally-available energy sources in
397 systems that require infrequent but regular maintenance (Younger et al. 2002). Use of liquid
398 carbon additions in full-scale treatment systems would be a departure from this definition.
399 However, scaling from the experiments described here to the volume of liquid carbon
400 required for actual mine water discharges reveals that energy requirements could be very
401 modest. In our experiments propionic acid was dosed at a rate of 1 mL per 35 L of synthetic

402 mine water. Scaling to treatment of a mine water discharge with a flow-rate of 10 L/s, as an
403 example, the same dose rate would equate to using 24.7 L of liquid carbon per day, or
404 approximately 9 m³/year. This is a relatively small amount in terms of a full-scale wastewater
405 treatment system, and at a dose rate of approximately 1 L/hour the use of small-scale
406 renewable energy systems to control dosing should be feasible. A key research priority is the
407 identification and reliability testing of waste liquid carbon sources as an alternative to
408 proprietary laboratory chemicals, to strengthen the sustainability case for enhanced passive
409 systems for treatment of metal-contaminated wastewaters in short HRT systems.

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419

420 **Supplementary materials**

421 Supplementary material associated with this article can be found in the online version.

422 **References**

423 Allen, H.E., Fu, G., Boothman, W., DiToro, D.M., Mahoney, J.D., 1991. Determination of acid
424 volatile sulfide and selected simultaneously extractable metals in sediment. USEPA EPA-821-
425 R-91-100, United States Environmental Protection Agency, Office of Science and Technology,
426 Washington, DC.

427 APHA 2005. Standard Methods for the Examination of Water and Wastewater (21st Edition).
428 American Public Health Association, American Water Works Association and the Water
429 Environment Federation, Washington, DC.

430 Baldwin, S.A., Khoshnoodi, M., Rezadehbashi, M., Taupp, M., Hallam, S., Mattes, A., Sanei, H.,
431 2015. The microbial community of a passive biochemical reactor treating arsenic, zinc, and
432 sulfate-rich seepage. *Frontiers in Bioengineering and Biotechnology* 3, 1-13.

433 Baldwin, S.A., Mattes, A., Rezadehbashi, M., Taylor, J., 2016. Seasonal microbial population
434 shifts in a bioremediation system treating metal and sulfate-rich seepage. *Minerals* 6, 17pp.

435 Biermann, V., Lillicrap, A.M., Magana, C., Price, B., Bell, R.W., Oldham, C.E., 2014. Applicability
436 of passive compost bioreactors for treatment of extremely acidic and saline waters in semi-
437 arid climates. *Water Research* 55, 83-94.

438 Bilek, F., 2006. Column tests to enhance sulphide precipitation with liquid organic electron
439 donators to remediate AMD-influenced groundwater. *Environmental Geology* 49, 674-683.

440 Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer,
441 N., Peñna, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E.,
442 Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R.,
443 Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010.
444 QIIME allows analysis of highthroughput community sequencing data. *Nat. Methods* 7, 335-
445 336.

446 Castillo, J., Pérez-López, R., Caraballo, M.A., Nieto, J.M., Martins, M., Costa, M.C., Olías, M.,
447 Cerón, J.C., Tucoulou, R., 2012. Biologically-induced precipitation of sphalerite-wurtzite
448 nanoparticles by sulfate-reducing bacteria: Implications for acid mine drainage treatment.
449 *Science of the Total Environment* 423, 176-184.

450 Costa, M.C., Santos, E.S., Barros, R.J., Pires, C., Martins, M., 2009. Wine wastes as a carbon
451 source for biological treatment of acid mine drainage. *Chemosphere* 75, 831-836.

452 Cruz Viggi, C., Pagnanelli, F., Cibati, A., Uccelletti, D., Palleschi, C., Toro, L., 2010. Biotreatment
453 and bioassessment of heavy metal removal by sulphate reducing bacteria in fixed bed
454 reactors. *Water Research* 44 (1), 151-158.

455 Di Luca, G.A., Maine, M.A., Mufarrege, M.M., Hadad, H.R., Sánchez, G.G., Bonetto, C.A., 2011.
456 Metal retention and distribution in the sediment of a constructed wetland for industrial
457 wastewater treatment. *Ecological Engineering* 37, 1267-1275.

458 Drennan, D.M., Almstrand, R., Lee, I., Landkamer, L., Figueroa, L., Sharp, J.O., 2016.
459 Organoheterotrophic bacterial abundance associates with zinc removal in lignocellulose-
460 based sulfate-reducing systems. *Environmental Science & Technology* 50, 378-387.

461 Drennan, D.M., Almstrand, R., Ladderud, J., Lee, I., Landkamer, L., Figueroa, L., Sharp, J.O.,
462 2017. Spatial impacts of inorganic ligand availability and localized microbial community
463 structure on mitigation of zinc laden mine water in sulfate-reducing bioreactors. *Water*
464 *Research* 115, 50-59.

465 Dvorak, D.H., Hedin, R.S., Edenborn, H.M., McIntire, P.E., 1992. Treatment of metal-
466 contaminated water using bacterial sulfate reduction: results from pilot-scale reactors.
467 *Biotechnology and Bioengineering* 40, 609-616.

- 468 Falk, N., Chaganti, S.R., Weisener, C.G., 2018. Evaluating the microbial community and gene
469 regulation involved in crystallization kinetics of ZnS formation in reduced environments.
470 *Geochimica et Cosmochimica Acta* 220, 201-216.
- 471 Gandy, C.J., Davis, J.E., Orme, P.H.A., Potter, H.A.B, Jarvis, A.P., 2016. Metal removal
472 mechanisms in a short hydraulic residence time subsurface flow compost wetland for mine
473 drainage treatment. *Ecological Engineering* 97, 179-185.
- 474 Gibert, O., de Pablo, J., Cortina, J.L., Ayora, C., 2004. Chemical characterisation of natural
475 organic substrates for biological mitigation of acid mine drainage. *Water Research* 38, 4186-
476 4196.
- 477 Hiibel, S.R., Pereyra, L.P., Inman, L.Y., Tischer, A., Reisman, D.J., Reardon, K.F. Pruden, A., 2008.
478 Microbial community analysis of two field-scale sulfate-reducing bioreactors treating mine
479 drainage. *Environmental Microbiology* 10 (8), 2087-2097.
- 480 Jong, T., Parry, D.L., 2004. Heavy metal speciation in solid-phase materials from a bacterial
481 sulfate reducing bioreactor using sequential extraction procedure combined with acid volatile
482 sulfide analysis. *Journal of Environmental Monitoring* 6, 278-285.
- 483 Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis
484 version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870-1874.
- 485 LaBar, J.A, Nairn, R.W., 2018. Characterization of trace metal removal products in vertical flow
486 bioreactor substrates at the Mayer Ranch Passive Treatment System in the Tar Creek
487 Superfund Site. *Chemosphere* 199, 107-113.
- 488 Martins, M., Faleiro, M.L., Barros, R.J., Veríssimo, A.R., Costa, M.C., 2009. Biological sulphate
489 reduction using food industry wastes as carbon sources. *Biodegradation* 20, 559-567.
- 490 Mayes, W.M., Johnston, D., Potter, H.A.B., Jarvis, A.P., 2009. A national strategy for
491 identification, prioritisation and management of pollution from abandoned non-coal mine
492 sites in England and Wales. I. Methodology development and initial results. *Science of the*
493 *Total Environment* 407, 5435 – 5447.
494
- 495 Mayes, W.M., Davis, J., Silva, V., Jarvis, A.P., 2011. Treatment of zinc-rich acid mine water in
496 low residence time bioreactors incorporating waste shells and methanol dosing. *Journal of*
497 *Hazardous Materials* 19, 279-287.
- 498 Mayes, W.M., Potter, H.A.B., Jarvis, A.P., 2013. Riverine flux of metals from historically mined
499 orefields in England and Wales. *Water, Air and Soil Pollution* 224, 1425.
- 500 Mosley LM, Daly R, Palmer D, Yeates P, Dallimore C, Biswas T, Simpson SL., 2015. Predictive
501 modelling of pH and dissolved metal concentrations and speciation following mixing of acid
502 drainage with river water. *Applied Geochemistry* 59, 1-10.

503 Neculita, C.-M., Zagury, G.J., Bussière, B., 2007. Passive treatment of acid mine drainage in
504 bioreactors using sulfate-reducing bacteria: Critical review and research needs. *Journal of*
505 *Environmental Quality* 36, 1-16.

506 Neculita, C.-M., Zagury, G.J., Bussière, B., 2008. Effectiveness of sulfate-reducing passive
507 bioreactors for treating highly contaminated acid mine drainage: II Metal removal
508 mechanisms and potential mobility. *Applied Geochemistry* 23, 3545-3560.

509 Nielsen, G., Hatam, I., Abuan, K.A., Janin, A., Coudert, L., Blais, J.F., Mercier, G., Baldwin, S.A.,
510 2018. Semi-passive *in-situ* pilot scale bioreactor successfully removed sulfate and metals from
511 mine impacted water under subarctic climatic conditions. *Water Research* 140, 268-279.

512 Parks, D.H., Tyson, G.W., Hugenholtz, P., Beiko, R.G., 2014. STAMP: statistical analysis of
513 taxonomic and functional profiles. *Bioinformatics* 30, 3123-3124.

514 Poulson, S.R., Colberg, P.J.S., Drever, J.I., 1997. Toxicity of heavy metals (Ni, Zn) to
515 *Desulfovibrio desulfuricans*. *Geomicrobiology Journal* 14 (1), 41-49.

516 Song, H., Yim, G.-J., Ji, S.-W., Neculita, C.M., Hwang, T., 2012. Pilot-scale passive bioreactors
517 for the treatment of acid mine drainage: efficiency of mushroom compost vs mixed substrates
518 for metal removal. *Journal of Environmental Management* 111, 150-158.

519 Strosnider, W.J.J., Winfrey, B.K., Nairn, R.W., 2011. Biochemical oxygen demand and nutrient
520 processing in a novel multi-stage raw municipal wastewater and acid mine drainage passive
521 co-treatment system. *Water Research* 45 (3), 1079-1086.

522 Strosnider, W.J.J., Nairn, R.W., Peer, R.A.M., Winfrey, B.K., 2013. Passive co-treatment of Zn-
523 rich acid mine drainage and raw municipal wastewater. *Journal of Geochemical Exploration*
524 125, 110-116.

525 Utgikar, V.P., Harmon, S.M., Chaudhary, N., Tabak, H.H., Govind, R., Haines, J.R., 2002.
526 Inhibition of sulfate-reducing bacteria by metal sulfide formation in bioremediation of acid
527 mine drainage. *Environmental Toxicology* 17, 40-48.

528 Utgikar, V.P., Tabak, H.H., Haines, J.R., Govind, R., 2003. Quantification of toxic and inhibitory
529 impact of copper and zinc on mixed cultures of sulfate-reducing bacteria. *Biotechnology and*
530 *Bioengineering* 82 (3), 306-312.

531 Vasquez, Y., Escobar, M.C., Neculita, C.M., Arbeli, Z., Roldan, F., 2016. Biochemical passive
532 reactors for treatment of acid mine drainage: Effect of hydraulic retention time on changes in
533 efficiency, composition of reactive mixture, and microbial activity. *Chemosphere* 153, 244-
534 253.

535 Younger, P.L., Banwart, S.A., Hedin, R.S., 2002. Mine water: Hydrology, Pollution, Remediation.
536 Kluwer Academic Publishers, The Netherlands.

537 Zhang, M., Wang, H., 2014. Organic wastes as carbon sources to promote sulfate reducing
538 bacterial activity for biological remediation of acid mine drainage. Minerals Engineering 69,
539 81-90.