

1 **Use of propionic acid additions to enhance zinc removal from mine drainage in short**
2 **residence 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. Effective removal of such
12 elevated zinc concentrations could not be sustained by sulfate reduction and / or other
13 attenuation processes without carbon supplementation. Propionic acid addition resulted in
14 improved and sustained performance by promoting the activities of sulfate reducing bacteria,
15 leading to efficient zinc removal (mean 99%) via bacterial sulfate reduction. In contrast,
16 cessation of propionic acid addition led to carbon limitation and the growth of sulfur oxidising
17 bacteria, compromising zinc removal by bacterial sulfate reduction. These research findings
18 demonstrate the potential for modest liquid carbon additions to compost-based passive
19 treatment systems to engineer microbial responses which enhance rates of zinc attenuation
20 in a short hydraulic residence time, enabling remediation of highly polluting mine drainage at
21 sites with limited land availability.

22 KEYWORDS: Zinc; Mine drainage; Compost bioreactor; Carbon addition; Sulfate reducing
23 bacteria; Residence time

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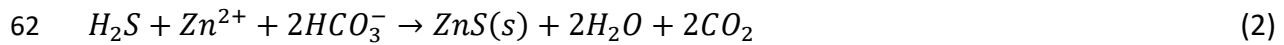
25 1. Introduction

26 Low pH, high metal concentration mine discharges are among the most ecologically damaging
27 effluent types world-wide (Olías et al. 2020). In the UK zinc is particularly prevalent in drainage
28 from abandoned metal mines with over 50% of the total zinc flux to freshwaters of England
29 and Wales attributed to such pollution (Mayes et al. 2013). Although the majority of
30 discharges in the UK are characterised by relatively low zinc concentrations (see Figure S1), a
31 limited number of highly contaminated (up to 45 mg/L zinc) discharges cause severe
32 ecological damage. Whilst zinc is an essential trace element, it can be toxic to humans and
33 ecosystems (Wei et al. 2020) and such discharges are therefore a target for remediation.
34 Heavily polluted mine drainage is also well documented elsewhere in the world (e.g. Castillo
35 et al., 2012; Mosley et al., 2015; Strosnider et al., 2011, 2013).

36 Compost bioreactors utilising bacterial sulfate reduction (BSR) are a favoured approach to
37 metal mine drainage remediation (Gandy et al. 2016; LaBar and Nairn 2018; Neculita et al.
38 2007; Vasquez et al. 2016). However, limitations of such low-energy passive systems, in many
39 locations, include their large footprint and uncertainty regarding their effectiveness in
40 treating high metal concentrations (Mayes et al. 2011). Many UK discharges occur in remote
41 upland locations, such as in northern England and western Wales, where availability of flat
42 land for treatment systems is limited (Mayes et al., 2009). Attenuation of zinc in low-cost, low
43 maintenance passive systems with a short hydraulic residence time (HRT) to enable a small
44 footprint is therefore favoured (Gandy et al. 2016). Whilst many investigations into the
45 potential of compost bioreactors for mine drainage remediation have used systems in which
46 HRT is measurable in days (Biermann et al. 2014; Cruz Viggi et al. 2010; Di Luca et al. 2011;
47 Song et al. 2012; Strosnider et al. 2011, 2013), recent research has demonstrated successful
48 removal of zinc in a HRT of less than 14.5 hours (Gandy et al. 2016). In the research reported
49 here the limitations of compost bioreactors, particularly for the remediation of highly
50 contaminated UK discharges, were investigated together for the first time. Short residence
51 time passive bioreactors receiving high zinc concentration mine water operated continuously
52 for two years, with controlled testing of the benefits of carbon additions to enhance
53 performance. The residence time chosen for these trials (mean 19 hours) was based on the
54 results of preliminary trials (unpublished results, Newcastle University) and was operationally
55 defined as short with the key requirement being that it was less than 24 hours to make it
56 applicable to the remediation of UK discharges.

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





63 The choice of carbon source is important to sustain the long-term efficiency of treatment (Xu
64 and Chen 2020). Simple organic compounds that are easily degradable, such as carboxylic
65 acids or alcohols, are used by SRB as carbon and energy sources (Gibert et al. 2004; Martins
66 et al. 2009). In laboratory cultures, lactate is the most common carbon source used by SRB
67 but would be prohibitively expensive to employ in full-scale treatment systems (Costa et al.
68 2009). Different types of compost are therefore frequently used to provide a long-term source
69 of carbon (Neculita et al. 2007). These more complex organic sources are far less costly than
70 proprietary carbon sources, are widely available, and often have physical characteristics that
71 make them suitable for use in flow-through water treatment systems. However, the long-
72 term efficiency of traditional compost-based treatment systems is limited by the supply of
73 readily available carbon (Tsukamoto et al. 2004). This is particularly pertinent in the UK given
74 limited land availability and system sizing constraints which necessitate a short HRT. To
75 extend system lifetime and to stimulate microbial sulfate reduction the supplementation of
76 the compost substrate with additional carbon sources has been successfully applied (e.g.
77 methanol (Mayes et al. 2011), glycerol (Santos and Johnson 2017), molasses (Nielson et al.
78 2018), ethanol (Costa et al. 2009), acetate (Yildiz et al. 2019) and lactate (Zhang and Wang
79 2014)). Propionic acid was chosen as a carbon additive in the research reported here as,
80 together with propionate, it has been recognised as an effective carbon source for SRB (Qian
81 et al. 2019; Virpiranta et al. 2021; Xu and Chen 2020) and in preliminary trials using a range
82 of carbon sources (unpublished results, Newcastle University) it proved the most effective at
83 zinc removal.

84 Whilst improved treatment efficiency has been demonstrated by carbon supplementation,
85 previous studies were based on either a high HRT (greater than 24 hours) or a comparatively
86 low zinc concentration (less than 20 mg/L). In the research reported here the focus is on the
87 combination of relatively short HRT treatment systems, since their absolute size is a key
88 constraint to wider deployment of the technology in the UK, and waters containing a high zinc
89 concentration. The extent to which the microbial communities key to metal attenuation are
90 influenced by carbon addition under short HRT conditions, and in turn whether they can
91 sustain bacterial sulfate reduction sufficiently to maintain effective zinc removal, is
92 specifically investigated.

93 As compost bioreactors are driven by SRB activity an improved understanding of their
94 microbial community diversity and function is critical for long-term performance (Hiibel et al.
95 2008). Several studies have demonstrated a relationship between system performance and
96 microbial community (e.g. Baldwin et al. 2015, 2016; Drennan et al. 2016, 2017). Engineering
97 design and system operation should thus be configured to ensure optimum activities of the
98 SRB that are responsible for remediation. Enhancement of microbial communities in short
99 HRT bioreactors subjected to high influent zinc concentrations has not previously been
100 investigated.

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

107 **2. Materials and methods**

108 *2.1. Experimental configuration*

109 Two sets of laboratory-scale continuous upflow bioreactors (internal diameter 105 mm,
110 length 500 mm) were operated in triplicate. Limestone gravel (diameter < 10 mm) was placed
111 by hand at the base of each bioreactor (depth 40 mm) and overlain by a reactive substrate
112 (depth 400 mm), sourced from a decommissioned pilot-scale bioreactor that treated zinc-rich,
113 circumneutral mine water for 2 years (Gandy et al. 2016). The substrate comprised British
114 Standards Institution (BSI) Publicly Available Specification (PAS) 100 compost (45% v/v), wood
115 chips (45% v/v) and activated sludge from a municipal wastewater treatment plant (10%).
116 Activated sludge, which contains high concentrations of organic matter (Peng et al. 2017), has
117 previously been shown to be an effective carbon source for SRB (Virpiranta et al. 2021). A 25
118 mm cover of water ensured that the substrate remained saturated (Figure S2). This substrate
119 was selected as it was known to have supported BSR previously, but via treatment of a
120 relatively low strength wastewater (mean pH 7.74 and 2.32 mg/L Zn; Gandy et al. 2016)
121 unlikely to invoke any inhibitory effects. Samples from across the entire depth and length of
122 the bioreactor were thoroughly mixed before placement of 3,530 cm³ in each laboratory
123 bioreactor. The substrate was saturated with a measured volume of synthetic mine water and
124 porosity calculated according to the ratio of mine water volume to substrate volume. A
125 Watson-Marlow 300 series peristaltic pump was set up to give a mean flow-rate of 1.6 ml/min,
126 which, based on a calculated porosity of 0.48 to 0.51, equated to a mean residence time of
127 19 hours.

128 *2.2. Bioreactor operation*

129 Synthetic mine water (mean 45 mg/L Zn, 156 mg/L SO₄, pH 4.1, Table S1), produced by
130 dissolving laboratory-grade salts (Table S2) in deionised water, was passed upwards through
131 the bioreactors for 755 days. The pH was controlled by addition of <10 mL of 1% H₂SO₄ to
132 each 35 L batch of mine water which, at such a low concentration, had an immeasurable
133 impact on the sulfate concentration of the synthetic mine water. This water quality was
134 representative of an actual mine water discharge in northern England (see Table S1 for
135 details).

136 Propionic acid (13.4M) addition to one set of three bioreactors (1A, B, C) commenced on day
137 234 at a rate of 1 ml per 35 L influent water. The other set of three bioreactors (2A, B, C)

138 operated as a control and continued to receive synthetic mine water only. On day 511
139 propionic acid addition to one bioreactor (1A) ceased.

140 2.3. *Water sampling and analysis*

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

154 2.4. *Substrate sampling and geochemical analysis*

155 Substrate samples were collected from all bioreactors at the end of the trial. In the bioreactors
156 that received propionic acid (1A, B, C), two samples were collected, at approximate depths of
157 220 mm (middle of reactors) and 310 mm (bottom of reactors), in pre-washed (analytical
158 grade nitric acid, 10% v/v) polypropylene bottles which were filled with water from within the
159 bioreactors. One sample was stored at minus 80°C, prior to microbial analysis, and the other
160 at minus 20°C, prior to geochemical analysis. An additional sample was collected at an
161 approximate depth of 90 mm (top of reactors) for microbial analysis only. In the control
162 bioreactors (2A, B, C), two samples were collected at an approximate depth of 220 mm
163 (middle of reactors) and stored as above prior to geochemical and microbial analysis. Samples
164 were allowed to defrost in an anaerobic cabinet before analysis. Geochemical analysis
165 followed the Acid Volatile Sulfide – Simultaneously Extracted Metals (AVS-SEM) method of
166 Allen et al. (1991) with the exception that H₂S was purged from the sample for 3 hours to
167 ensure that all AVS was recovered, as recommended by Standard Method 4500-S²⁻ J (APHA,
168 2005). Metals analysis was undertaken as for water samples. A control sample of the original
169 mixed substrate was subjected to the same analysis.

170 2.5. *Microbial analysis*

171 Twelve 16S rRNA PCR amplicon libraries were sequenced comprising three (top, middle and
172 bottom) depths for each of Set 1 bioreactors (A, B, C) and an additional three samples from
173 the middle of each one of the three control bioreactors. All bioreactor substrate samples were
174 collected at the end of the trial (see Supporting Information (SI) for a more detailed methods

175 description). Briefly, amplicons of 16S rRNA gene fragments (V4/V5 region) were PCR
176 amplified with barcode-ligated amplification primers from DNA extracts. Amplicons were
177 then pooled and sequenced using the Ion PGM™ sequencing platform. Sequence libraries for
178 each sample were assembled and analysed using the QIIME2 analysis pipeline (Caporaso et
179 al., 2010). A principal components analysis (PCA) of sample diversities was generated using
180 the STAMP v2 software package (Parks et al., 2014). Phylogenetic trees of key representative
181 sequences and their BLAST derived close relatives were generated in MEGA7 (Kumar et al.,
182 2016).

183 **3. Results and discussion**

184 *3.1. Zinc and sulfate removal*

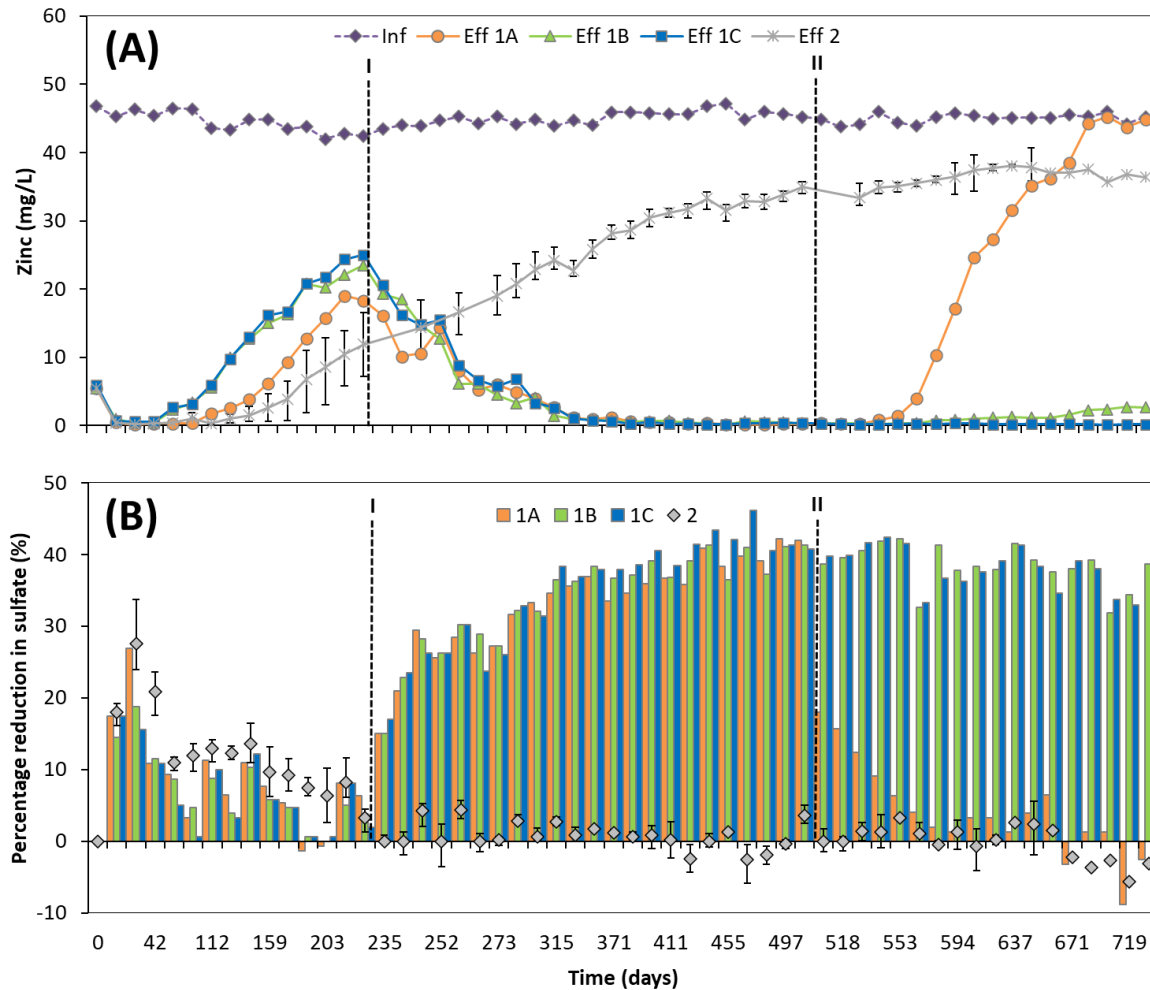
185 There was no significant difference between the concentrations of total zinc and filtered zinc
186 in the effluent throughout the trial (Mann-Whitney U test; $p > 0.05$ for replicates 1A, 1B and
187 1C). Therefore, all values reported here are total zinc concentrations.

188 Effective removal of zinc (removal efficiency consistently $> 90\%$) occurred in all bioreactors
189 during the first 90 days of the trial, but effluent zinc concentrations increased in all three
190 bioreactors between 90 and 230 days (Figure 1(A)). Initially there was evidence of a decrease
191 in sulfate concentration between influent and effluent in all bioreactors (Figure 1(B)). Based
192 on a molar ratio of sulfate to zinc of 1:1 (Reactions (1) and (2)), calculation of the predicted
193 effluent zinc concentration, assuming zinc removal only as a sulfide precipitate via BSR (using
194 the difference in influent and effluent sulfate concentration), indicates that actual effluent
195 zinc concentrations during the first 230 days of operation were much less than predicted in
196 all bioreactors (Figure S3). Processes other than zinc sulfide precipitation (e.g. sorption) were
197 therefore contributing to zinc attenuation during this period. Others have similarly reported
198 additional processes to be taking place (e.g. Gandy et al. 2016) whilst Neculita et al. (2008)
199 attributed metal removal to a combination of metal hydroxide precipitation and sorption to
200 the compost substrate.

201 As effluent zinc concentrations increased during the first 230 days of the trial there was a
202 corresponding decrease in mean percentage sulfate reduction (defined as the difference
203 between influent and effluent sulfate concentrations), from 20% to 3.6% (Figure 1(B)). This
204 indicated that effective removal of the high influent zinc concentration (mean 45 mg/L) could
205 not be sustained by sulfate reduction and / or other attenuation processes. Other studies
206 have reported zinc to be toxic or inhibitory to SRB at such concentrations (Poulson et al. 1997;
207 Utgikar et al. 2002, 2003), although Castillo et al. (2012) and Falk et al. (2018) found that
208 bacterial communities later recovered due to the proliferation of more metal-resistant
209 species. Whilst toxicity was not studied specifically in these trials, there is no direct evidence
210 from the microbial community composition patterns discussed below that the elevated zinc
211 concentration was toxic or inhibitory to sulfate reduction.

212 Upon commencement of propionic acid addition on day 234 effluent zinc concentrations
213 decreased substantially in all three replicates, from a mean of 22.3 mg/L to < 0.5 mg/L (mean
214 removal efficiency 99.1%) by day 427 (Figure 1(A)). There was no significant difference in zinc
215 concentration between replicates during the period of propionic acid addition to all
216 bioreactors, between days 235 and 511 (Mann-Whitney U test; $p > 0.05$ for all replicates).
217 Minor deviations in effluent zinc concentration can be attributed to operational issues. A
218 corresponding increase in percentage sulfate reduction, which was sustained at a mean of 41%
219 (Figure 1(B)), indicates that the SRB responded to the supplementary carbon such that the
220 rate of attenuation of zinc as its sulfide increased. Like zinc, there was no significant difference
221 in sulfate concentration between the three replicates (Mann-Whitney U test; $p > 0.05$ for all
222 replicates). Between days 235 and 511 predicted effluent zinc concentration, assuming only
223 precipitation as its sulfide via BSR, was very close to actual effluent zinc concentration (Figure
224 S3), suggesting that BSR was the key zinc attenuation process during this phase of the trials.
225 Effective attenuation of both zinc and sulfate during periods of carbon addition to sulfate
226 reducing bioreactors has previously been reported by others. Mayes et al. (2011) observed
227 almost complete removal of zinc during a phase of methanol addition whilst Neilsen et al.
228 (2018) reported up to 90% zinc removal when using molasses as a carbon source, albeit the
229 HRT was 2 weeks which is considerably longer than that in the study reported here. Similarly,
230 Costa et al. (2009) achieved over 90% zinc removal with the addition of both ethanol and wine
231 wastes but in a HRT of 8 days.

232 After propionic acid addition to bioreactor 1A ceased on day 513, effluent zinc concentration
233 immediately increased (Figure 1(A)), with removal efficiency < 1% by the end of the trial. A
234 substantial decrease in percentage sulfate removal also occurred with effluent sulfate
235 concentrations higher than influent sulfate concentration at times (as shown by negative
236 values in Figure 1B)). This decline in BSR upon cessation of propionic acid addition suggests
237 that the microbial community adapted rapidly and that the presence of an easily available
238 electron donor is the limiting factor for sulfate reduction in such systems. Similar observations
239 have been made by others following cessation of methanol addition (Bilek 2006; Mayes et al.
240 2011) and depletion of lactate (Zhang and Wang 2014). Zinc removal efficiency in bioreactors
241 1B and 1C, which continued to receive propionic acid, remained > 95% until the end of the
242 trial and percentage sulfate removal was sustained at 30 - 40%. This suggests that the
243 deteriorating performance of the bioreactors up to Day 230 of the trial was due to insufficient
244 labile carbon to maintain high rates of BSR. In the control bioreactor set, which did not receive
245 propionic acid, effluent zinc concentrations steadily increased until stabilising at around 37
246 mg/L (mean removal efficiency 17.1%) (Figure 1(A) and Figure S4). Likewise, percentage
247 sulfate removal progressively decreased throughout the trial indicating that SRB activity was
248 limited in these control bioreactors (Figure 1(A) and Figure S5).



249

250 **Figure 1.** Effect of propionic acid addition on total zinc removal and sulfate reduction in
 251 laboratory-scale bioreactors. (A) Influent and effluent total zinc concentrations in bioreactors
 252 receiving propionic acid (Eff 1A, Eff 1B, Eff 1C) and mean effluent total zinc concentration in
 253 bioreactors receiving no propionic acid (Eff 2). (B) Percentage reduction in sulfate
 254 concentration in bioreactors receiving propionic acid (1A, 1B, 1C) and mean percentage
 255 reduction in sulfate concentration in bioreactors receiving no propionic acid (2). Error bars
 256 represent the range of results from triplicate samples. Vertical dashed lines refer to: (I)
 257 commencement of propionic acid addition; (II) cessation of propionic acid addition to reactor
 258 1A.

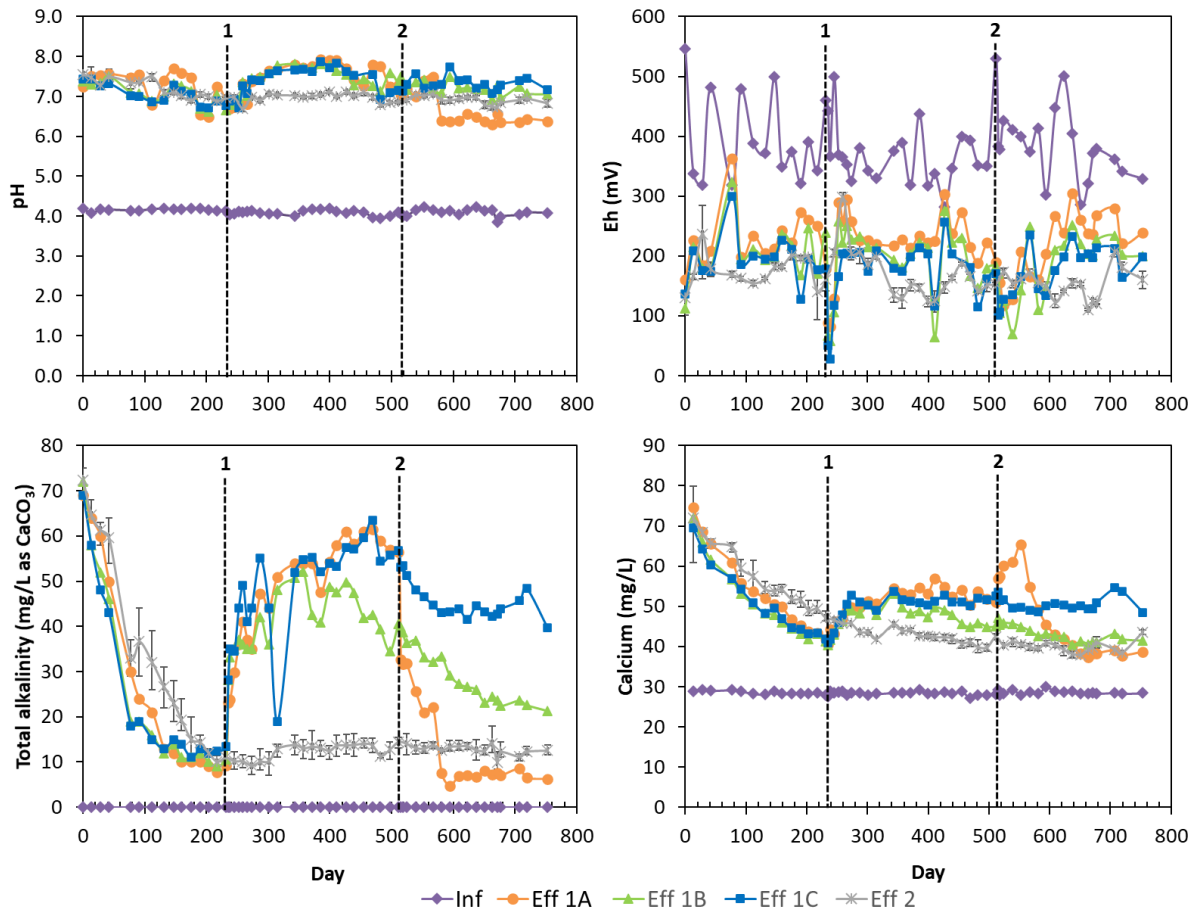
259 **3.2. Alkalinity, pH and Eh**

260 Changes in pH, Eh and alkalinity concentration between influent and effluent (Figure 2) were
 261 consistent with variations in zinc and sulfate removal. Effective buffering of the acidic influent
 262 water occurred throughout the trial with an influent mean pH of 4.1 consistently elevated to
 263 an effluent pH of 6.29 – 7.92, which is optimal for SRB activity (Xu and Chen 2020). The only
 264 notable deviation was in bioreactor 1A, 68 days after propionic acid addition had ceased,
 265 when effluent pH decreased from a mean of 7.34 to a mean of 6.41 for the remainder of the
 266 trial (Figure 2).

267 Influent and effluent Eh values were also consistent with conditions that favoured BSR and
268 zinc removal as its sulfide. Eh decreased between influent (mean 382 mV) and effluent (mean
269 196 mV) in all bioreactors, with a marked decrease in effluent Eh at commencement of
270 propionic acid addition (Figure 2). Although strongly anaerobic conditions, as observed by
271 others (e.g. Mayes et al. 2011) during carbon additions, did not appear to become established
272 within the bioreactors, the effluent Eh measurements reported here likely overestimate the
273 actual Eh values within the pore waters. The low flow rates of the bioreactors necessitated
274 an extended period of sample collection and it is possible that oxidising conditions became
275 re-established within the samples before Eh was measured. Furthermore, Eh measurements
276 made on effluent waters are likely not reflective of those in the bulk compost.

277 Effluent alkalinity concentration initially decreased in all bioreactors before increasing upon
278 commencement of propionic acid addition, indicating enhanced alkalinity generation due to
279 BSR (reaction (1)) together with continued calcite dissolution from the limestone gravel
280 (Figure 2). Mayes et al. (2011) also noted increased alkalinity due to enhanced sulfate
281 reduction during methanol addition. Upon cessation of propionic acid addition effluent
282 alkalinity concentration decreased sharply in bioreactor 1A, compared to reactors continuing
283 to receive propionic acid (1B and 1C), albeit effluent alkalinity was beginning to decrease in
284 all bioreactors (Figure 2).

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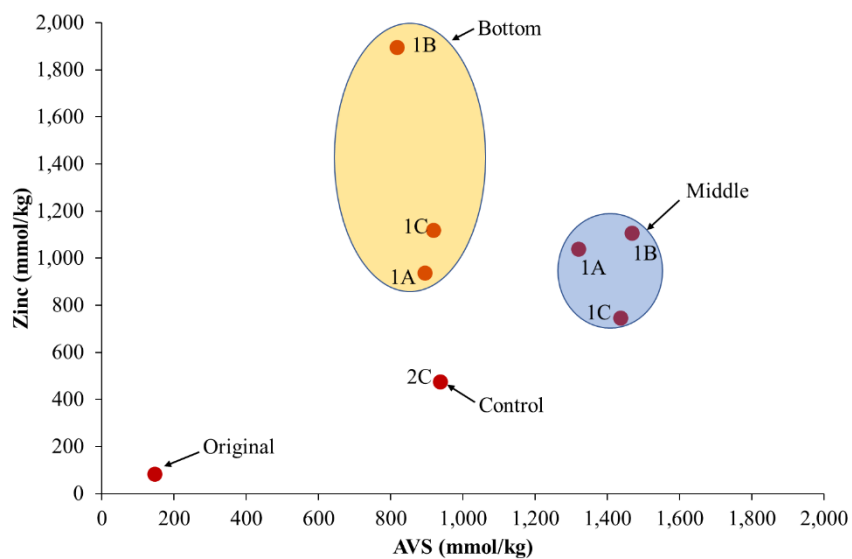
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287 **Figure 2.** Influent and effluent pH, Eh, total alkalinity and total calcium concentration in
 288 bioreactors receiving propionic acid (Eff 1A, Eff 1B, Eff 1C) and mean effluent pH, Eh, total
 289 alkalinity and total calcium concentration in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

293 3.3. Substrate geochemical analysis

294 Sampling and analysis of the substrates was undertaken at the end of the trial to investigate
 295 metal attenuation processes. The determination of acid volatile sulfides (AVS) and
 296 simultaneously extracted metals (SEM) has previously been used effectively to assess the role
 297 of BSR as a zinc removal mechanism (Gandy et al. 2016; Jong and Parry 2004; LaBar and Nairn
 298 2018). Figure 3 shows that substantial accumulation of both AVS and zinc occurred in the
 299 bioreactors receiving propionic acid. This is consistent with the observed decreases in zinc
 300 and sulfate between the influent and effluent waters (Figure 1) and implies that ZnS was the
 301 main sink for zinc within these bioreactors. Despite having already accumulated some AVS
 302 and zinc during its emplacement in a pilot-scale flow through bioreactor treating zinc-rich
 303 water (Gandy et al. 2016), the original compost substrate contained much lower
 304 concentrations of zinc (81 mmol/kg) and AVS (148 mmol/kg) (Figure 3). Solid phase zinc

305 concentrations in two of the bioreactors receiving propionic acid were higher in the bottom
 306 layer (1B 1,895 mmol/kg; 1C 1,117 mmol/kg) than in the middle layer (1B 1,106 mmol/kg; 1C
 307 745 mmol/kg); in bioreactor 1A, concentrations in the bottom and middle layers were similar
 308 (Figure 3). Conversely, the AVS concentrations were higher in the middle layer (mean of the
 309 three bioreactors 1,410 mmol/kg) than in the bottom layer (mean 878 mmol/kg) (Figure 3).
 310 They also showed little variation between the three bioreactors at equivalent depths (SD = ±
 311 78 mmol/kg in middle layer; SD = ± 53 mmol/kg in bottom layer) compared to zinc
 312 concentrations (SD = ± 191 mmol/kg in middle layer; SD = ± 510 mmol/kg in bottom layer).
 313 Higher zinc concentrations in the bottom layer can be attributed to vigorous BSR close to
 314 where the influent water entered the bioreactors, due to relatively high zinc and sulfate
 315 concentrations. Gandy et al. (2016) and LaBar and Nairn (2018) also noted vertical variations
 316 in metal removal with the highest concentrations found closest to the influent ends of the
 317 systems. No notable difference in either zinc or AVS concentration was observed between
 318 bioreactor 1A, in which propionic acid addition ceased on day 511, and the other bioreactors
 319 receiving propionic acid, albeit the zinc concentration in the middle layer of this bioreactor
 320 was slightly higher than that in the bottom layer. Concentrations of both AVS (939 mmol/kg)
 321 and zinc (473 mmol/kg) were substantially lower in the control bioreactor that did not receive
 322 propionic acid. Nevertheless, the accumulation of some ZnS, particularly in the early stages
 323 of the trial, has resulted in higher concentrations than in the original substrate.



324

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

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

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

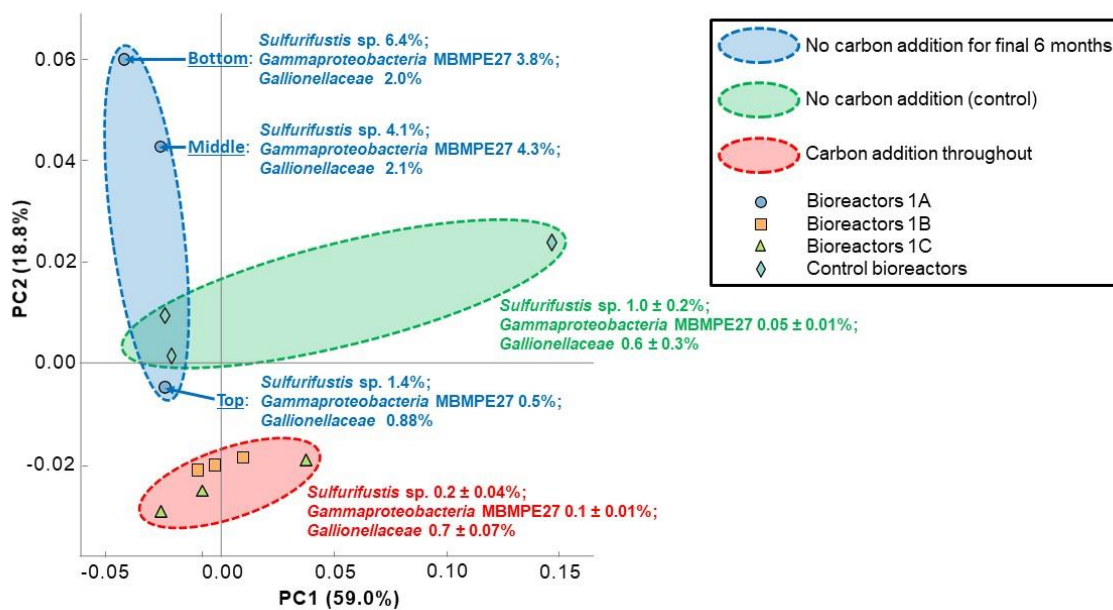
340 3.4. Substrate microbial analysis

341 Community analysis revealed some common features in the libraries consistent with the
342 compost bioreactor origin of the substrate (Gandy et al. 2016). Specifically, putatively sulfate
343 reducing bacteria (SRB) accounted for $7.9\% \pm 0.4$ (average \pm SE) of sequences. These SRB had
344 100% sequence homology with those recovered from natural or engineered anaerobic sulfate
345 reducing systems (Figure S6) and taxa identified indicated a dominance of H_2 utilising SRB
346 autotrophs (see SI for a more detailed discussion). Likewise, syntrophic bacterial partners
347 putatively responsible for fermentative degradation of compost material to supply SRB
348 substrates were also common features. Close relatives of these dominant taxonomic groups
349 (*Candidatus Caldatribacterium*, the family *Anaerolineaceae* and the family *Spirochaetaceae*)
350 were also identified previously in natural or engineered anaerobic environments (Figure S7).

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

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

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



380

381 **Figure 4.** A Principal Component Analysis (PCA) based on amplicon sequence variant (ASV)
 382 frequencies within 16S rRNA gene sequencing libraries constructed from the compost
 383 bioreactors. Samples from the top, middle and bottom of the 1A (blue circles), 1B (orange
 384 squares) and 1C (green triangles) column bioreactors are shown, plus samples from the
 385 middle of the three control reactor columns (cyan diamonds). Ellipses are drawn around three
 386 data groups: the 1A samples which stopped receiving propionic acid for the last six months of
 387 reactor operation; a group comprising the 1B and 1C samples which received propionic acid
 388 throughout; and the control reactors which did not receive carbon additions. Mean % \pm SE
 389 contribution of specific taxonomic groups related to sulfide and iron oxidation are provided
 390 for two of the circled groups (1B + 1C and control). Individual sample values presented for the
 391 1A group data to illustrate bottom to top progression of changes observed in this bioreactor.

392

393 3.5. *Implications*

394 Sustained zinc removal from high strength wastewater in short HRT sulfate-reducing
395 bioreactors, as required in the UK due to limited land availability and associated treatment
396 unit size constraints, necessitates carbon supplementation. Use of liquid carbon additions in
397 full-scale treatment systems would be a departure from the definition of passive treatment
398 as using only naturally-available energy sources in systems that require infrequent but regular
399 maintenance (Younger et al. 2002). However, the volume of liquid carbon required could be
400 very modest. A dosing rate of 1 mL propionic acid per 35 L of synthetic mine water in the
401 laboratory experiments trialled here equates to 24.7 L per day, or approximately 9 m³/year,
402 for treatment of a mine water discharge with a flow-rate of 10 L/s, as an example. This is a
403 relatively small amount in terms of a full-scale wastewater treatment system, and at a dose
404 rate of approximately 1 L/hour the use of small-scale renewable energy systems to control
405 dosing should be feasible. Given such low volumes of propionic acid required, this would have
406 minimal impact on overall treatment costs.

407 The laboratory-scale research described here used a compost commonly available in the UK,
408 a laboratory-grade liquid carbon addition (propionic acid), and a synthetic mine water
409 representing an actual low pH mine water discharge in the UK. The experiments operated for
410 approximately two years. Shortened tests of this type, using different composts, liquid carbon
411 sources and mine waters, would be a useful precursor to design and installation of any pilot-
412 or full-scale system at which liquid carbon addition might be anticipated as a requirement,
413 especially given the large investment overall to construct a full-scale treatment system. Such
414 tests would also provide better understanding of the range of liquid carbon sources
415 deployable for this purpose, and contribute to better design guidance for enhanced passive
416 treatment.

417 **4. Conclusions**

418 An evaluation of the effectiveness of liquid carbon additions on zinc immobilisation in
419 laboratory-scale short HRT (19 hours) compost bioreactors receiving a high influent zinc
420 concentration (45 mg/L) showed that enhanced rates of zinc attenuation (mean of 99% zinc
421 removal) are possible. Without supplementation, available carbon limitation led to a
422 deterioration in treatment performance, with respect to zinc. This was overcome by the
423 addition of propionic acid which acted as an electron donor for the reduction of sulfate by
424 SRB and led to enhanced zinc removal as a sulfide.

425 The different responses of the microbial communities in systems receiving continuous
426 propionic acid addition and in systems in which propionic acid addition ceased after a period
427 of time are indicative of the dominant processes occurring in relation to metal removal.
428 Addition of propionic acid favoured the activities of SRB and their syntrophic partners present
429 in high proportions in the compost substrate, inducing a net sink for sulfate via BSR and hence
430 efficient zinc removal. Upon cessation of propionic acid addition, the resulting carbon

431 limitation increased the substrate oxidation potential (as evidenced by the growth of sulfur
432 oxidising bacteria), which compromised zinc removal (as ZnS) via BSR and resulted in a system
433 that was a net source of sulfate.

434 These research findings demonstrate the potential for microbial responses favourable to the
435 immobilisation of zinc to be engineered in enhanced passive treatment systems receiving
436 carbon additions. Even modest liquid carbon additions to compost-based passive treatment
437 systems can increase rates of metal attenuation in a short HRT, enabling remediation of highly
438 polluting mine drainage at sites with limited land availability, typical of abandoned mine sites
439 in the UK. Given the lower solubility products of the sulfides of other divalent contaminant
440 metals (e.g. lead, cadmium, copper), these metals could potentially be removed too, thus
441 broadening scope for deployment of such low carbon technologies at sites with high strength
442 wastewaters but restricted land availability. A key research priority is the identification and
443 reliability testing of waste liquid carbon sources as an alternative to proprietary laboratory
444 chemicals, to strengthen the sustainability case for enhanced passive systems for treatment
445 of metal-contaminated wastewaters in short HRT systems.

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455

456 **Supplementary materials**

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

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