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1 pH influences the distribution of microbial rock-weathering
2 phenotypes in weathered shale environments

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21 ABSTRACT

22 Microbial rock weathering of shale forms an important part of global biogeochemical cycling
23 and soil formation. Culture-independent analyses have revealed diverse microbial communities
24 in weathered shale environments, yet few studies have attempted to discern the functional
25 ecology of such communities in relation to their rock weathering capabilities. In this study,
26 phenotypic plate assays were used to determine the abundance of microbes with different rock
27 weathering phenotypic traits in weathered shale environments. A physicochemical parameter
28 (pH) is shown to influence the abundance of aerobic rock weathering microbes in weathered
29 shale. Iron and manganese oxidizers were restricted to acidic environments, while siderophore
30 producing and alkaline phosphatase producing microbes were largely confined to pH neutral
31 environments. Furthermore, a clear separation in the spatial distribution of aerobic iron
32 oxidizing and siderophore-producing microbes, as defined by a pH gradient across the sites
33 sampled, was demonstrated. Phylogenetic analysis of isolates revealed that siderophore
34 producing and alkaline phosphatase producing bacteria belonged to commonly identified rock
35 weathering genera including *Arthrobacter*, *Pseudomonas* and *Streptomyces*. These results
36 enhance our understanding how physicochemical parameters can define the composition and
37 rock weathering potential of microbial communities.

38 KEY WORDS

39 Functional ecology, iron oxidizers, siderophore producers, microbial rock weathering, pH

40 INTRODUCTION

41 Shale rock comprises 25 % of the Earth's exposed continental landmass (Amiotte-Suchet et al.
42 2003) and forms two thirds of all sedimentary rock deposits (Ilgen et al. 2017). The weathering
43 of shale in the natural environment therefore makes a significant contribution to global

44 biogeochemical cycles (e.g. C, Fe and S) and soil formation (Uroz et al. 2009; Brantley et al.
45 2012). The role of microbes in the weathering of shale has been extensively studied in natural
46 environments (Petsch et al. 2001; Petsch et al. 2005; Joeckel et al. 2005; Cockell et al. 2011;
47 Yesavage et al. 2012; Zhu and Reinfelder, 2012; Li et al. 2014; Włodarczyk et al. 2018).
48 However, most of these studies have used culture-independent phylogenetic analysis and/or
49 biogeochemical data to infer the impact of microbial activity on rock weathering processes,
50 rather than directly quantify the rock weathering potential, or the phenotypic traits, of microbes
51 present in those environments.

52 The phenotypic capabilities of rock weathering microbes have been of significant interest to
53 those studying shale weathering within industrial contexts. For example, harnessing microbial
54 activity within shale has been proposed to enhance metal recovery from low-grade ores (Anjum
55 et al. 2012; Kutschke et al. 2015). Furthermore, microbial rock weathering activity has been
56 indicated to increase environmental pollution from mining wastes (Wengel et al. 2006;
57 Kalinowski et al. 2006) and to contribute to rock expansion in shale bedrock, causing
58 significant damage to infrastructure (Anderson, 2008; Hoover and Lehmann, 2009). Such
59 application-focused studies often quantify the biologically enhanced rate of elemental leaching
60 from rock as a measure of the weathering potential of individual microbial strains (Tasa et al.
61 1997; Anjum et al. 2010) or microbial communities (Lee et al. 2005; Matlakowska et al. 2012;
62 Włodarczyk et al. 2015). These studies have revealed numerous microbial rock weathering
63 mechanisms including the oxidation of iron (Tasa et al. 1997, Grobelski et al. 2007; Spalore
64 et al. 2011) secretion of siderophores (Kalinowski et al. 2006, Włodarczyk et al. 2015) and
65 organic acid production (Anjum et al. 2010; Włodarczyk et al. 2016) that result in the enhanced
66 dissolution and degradation of shale.

67 Functional ecology is the study of the functional roles species play within their native
68 ecosystem (Prosser et al. 2007). Within the context of geomicrobiology, one approach to

69 explore the functional ecology of weathered rock environments is through the use of agar plate
70 phenotypic assays, which allow microbial isolates with specific capabilities to be identified
71 (Hirsch et al. 1995). Microbial colonies grown on a defined agar type can be recorded as
72 positive or negative for a phenotype based upon a visual test result (Schwyn and Neilands,
73 1987; Pérez et al. 2007). Matlakowska and Sklowdowska (2009) used this approach to
74 characterise eight bacterial strains isolated from black shale, determining their ability to
75 degrade a variety of recalcitrant organic compounds (e.g. phenanthrene), produce degradative
76 enzymes (e.g. dioxygenases), to uptake and degrade metalloporphyrins, and to produce
77 siderophores. As black shale is rich in sedimentary organic matter (2-15 % weight
78 composition), the ability of shale-inhabiting organisms to degrade organic matter contributes
79 to their ability to weather shale. Matlakowska and Sklowdowska (2009) demonstrated that
80 while some capabilities were shared by most of the isolates (e.g. haem degradation), only one
81 isolate was identified that could produce dioxygenases and degrade phenanthrene. From a
82 microbial ecology perspective, it is interesting to consider if the differences in rock weathering
83 potential between isolates can be explained by the environmental conditions in which those
84 isolates were obtained. The rock weathering effect of microbes is known to be constrained by
85 the physicochemical parameters of their surrounding environment (Calvaruso et al. 2006;
86 Calvaruso et al. 2010). For example, as ferric iron is significantly less soluble at pH values
87 above 4, the production of siderophores in pH neutral environments to increase iron
88 bioavailability is advantageous to capable organisms (Kalinowski et al. 2006). In contrast, the
89 bioenergetic favourability of aerobic iron oxidation is known to increase below pH 4 (Hedrich
90 et al. 2011). Therefore, the abundance of siderophore-producing and iron oxidizing microbes
91 across rock weathering environments could be predicted to be negatively correlated, but there
92 is a lack of empirical evidence from culture-based studies to support this hypothesis.

93 Chen et al. (2016) investigated the variability of weathering potential across 150 bacterial
94 isolates obtained from across a weathered rock gradient (less-altered siltstone, more-altered
95 siltstone and soil). To quantify rock weathering potential of isolates, the authors quantified the
96 enhancement of elemental leaching from unaltered siltstone, the secretion of siderophores and
97 the release of organic acids in liquid-rock culture. They identified numerous differences in
98 these capabilities between isolates obtained across the weathered rock gradient, with isolates
99 taken from soils having a greater ability to leach iron, silicon and potassium from the rock
100 compared to isolates from less-altered rock. Interestingly however, the extent of siderophore
101 production (a rock-weathering trait) did not significantly vary between isolates taken from
102 across the weathered rock gradient (Chen et al. 2016).

103 In this study, we attempt to determine if rock surface pH influences the distribution of microbes
104 with rock weathering phenotypic traits, which could constrain the potential for microbial
105 communities to contribute to weathering processes. We chose six phenotypic plate assays that
106 enrich for: 1-2) isolates that grow on nutrient agar (at both neutral and acidic pH), 3)
107 siderophore producers, 4) alkaline phosphatase producers, 5) iron oxidizers and 6) manganese
108 oxidizers. These assays have been used to enumerate the presence of these phenotypic trait
109 groups in samples taken from across eroding shale cliffs and other weathered shale
110 environments on the North Yorkshire coastline, UK.

111 Coastal erosion of the rocky cliffs on the North Yorkshire coastline is a pervasive hazard and
112 poses substantial risk to infrastructure (Guthrie and Lane, 2007; Rosser et al. 2013). Recent
113 studies have obtained accurate rates of erosion (Lim et al. 2005; Rosser et al. 2007) and
114 attempted to determine which geomorphological processes constrain those rates (Lim et al.
115 2010; Van Jones et al. 2015). An important finding of this work is that cliff erosion at studied
116 sites was best described by progressive rock mass deformation, with rock falls of smaller
117 magnitude proceeding larger magnitude events (Lim et al. 2010). The role of rock weathering

118 processes, including microbial activity, which could accelerate the rate of these low magnitude
119 events was explored in our previous work (Cockell et al. 2011; Samuels et al. 2019). This study
120 has continued to use the North Yorkshire coastline to investigate microbial shale weathering
121 activity, based upon the significant environmental and economic consequences that weathering
122 processes potentially impact.

123 METHODS

124 **Field sites and sample collection**

125 Rock and water samples were obtained across nine different sites on the North Yorkshire
126 coastline (Figure 1) in August 2015. The following is a list of these sites with their numeric
127 ID: 1) cliff surface at Hole Wyke, a bay near to Boulby Head, 2) cliff surface revealed by a
128 rock fall at Hole Wyke, 3) walls of jet mines located near Tellgreen, 4) cliff surface at
129 Keldhowe Steel, 5) ochreous water from a stream within Deepgrove quarry, 6) outcrop surface
130 at Sandsend Ness quarry 7) scree slope of Gaytres quarry, 8) walls from one of the levels of
131 Assholm cement stone mines and 9) cliff surface at Saltwick Bay. These sites include a variety
132 of natural weathered cliff outcrops (sites 1-2, 4 and 9) and historic industrial sites including
133 quarries (5-7) and mines (3 and 8). The position of these sites, including that of the whole
134 sampling area on the UK coastline, can be seen in figure 1; representative images of some of
135 these field sites can be seen in figure 2.

136 Samples obtained in this study came from strata within two geological stages within the Early
137 Jurassic period (Hobbs et al. 2012). Samples taken at sites 1 and 2 were from pyritous shale in
138 the Redcar Mudstone Formation (RMF), deposited during the Pleinsbachian stage (182.7-190.8
139 Ma). All other samples were taken from two members (Alum shales and Mulgrave shales) of
140 the Whitbian Mudstone Formation (WMF), deposited during the Toarcian stage (174.1-182.7
141 Ma). Samples taken from sites 3-6 and 7-9 were taken from the Mulgrave shale and Alum shale

142 members respectively. The mineralogy of both the RMF and WMF are similar, being largely
143 comprised of quartz (24-31 %), mica (34-42 %) and kaolinite (16-21 %), with minor amounts
144 of pyrite (2-5 %). All shales contained sedimentary organic matter in the form of type II
145 kerogen (1.5-5.5 %), and are enriched in a number of metallic elements including iron and
146 manganese (Hobbs et al. 2012). Further information on the geology and geochemistry of the
147 sampling sites can be found in Samuels (2018) and selected references within (in particular,
148 see Hobbs et al. 2012).

149 Sites were chosen based upon evidence of rock weathering at that site. Weathered surfaces of
150 shale rock in these environments were heavily coated in red and purple mineral deposits that
151 in appearance could be putatively identified as ferromanganese deposits (Figure 2), which are
152 heavily comprised of iron and manganese oxides and hydroxides (Carmichael et al. 2013).

153 Previous geochemical characterisation of weathered shale on the North Yorkshire coastline
154 (Cockell et al. 2011) demonstrated that weathered rock surfaces were coated in authigenic iron
155 oxyhydroxides and localised acicular, platy and aggregated gypsum. Geochemical analyses of
156 rock on the North Yorkshire coastline established the high abundance of both iron (5.88-11.04
157 %) and manganese (160-550 ppm) within the shale strata (Gad et al. 1968). Phylogenetic
158 analysis of the microbial community inhabiting the rock surface revealed a single iron
159 oxidizing microbial species (*Acidiferrobacter thiooxydans*) within a low-diversity microbial
160 community dominated by Proteobacteria (Cockell et al. 2011).

161 Within each sampling site, multiple locations (i.e. different spots on a cliff surface) were chosen
162 for sampling. The number of locations sampled at each individual site varied (2-5), based upon
163 the availability of surfaces to sample: site 1 (2 locations), site 2 (3), site 3 (4), site 4 (2), site 5
164 (2), site 6 (2), site 7 (2), site 8 (5) and site 9 (3). A flame sterilised rock hammer was used to
165 either break away or dislodge pieces of bulk rock or saprolites (chips of slaked, weathered rock)

166 from rock surfaces into Whirlpak© bags. Water from a stream running through Deepgrove
167 quarry was collected in 50 mL Falcon tubes. A water sample was included in this study due to
168 the abundance of ochre material visually identifiable within the water (Figure 2F), that
169 appeared to be weathered mineral products derived from the surrounding rock. Samples were
170 kept at ambient temperature until return to the University of Edinburgh, whereupon they were
171 stored at 4 °C until use. Due to the large number of samples to be processed, it took one month
172 to finishing processing and plating out all samples. Storing samples at 4 °C for a prolonged
173 period potentially affected microbial viability within the samples. However, the storage
174 conditions were the same for all samples, meaning they should have effected equally.

175 **pH measurements**

176 Within one month of collection, ferromanganese crusts on shale samples were scraped from
177 rock surfaces using a scalpel. Care was taken to only scrape surface coatings until un-weathered
178 rock below was revealed, to obtain the most representative estimate of rock surface pH. Rock
179 chips from all sampling locations within a site were pooled, due to the low mass of scrapings
180 that could be obtained from samples taken at any one sampling location. For each pH
181 determination, 50 µg of scrapings were suspended in 2 mL of Milli-Q filtered water and
182 allowed to equilibrate for 30 minutes. Triplicate measurements were then taken for each
183 equilibrated sample. For water samples collected at Deepgrove quarry, the pH of the sample
184 was measured directly. All pH values were converted into hydrogen ion concentrations for use
185 in the statistical analyses (see results, figure 4).

186 **Phenotypic trait agar plate assays**

187 To determine the abundance of aerobic, culturable microbes with a specific phenotype
188 inhabiting weathered shale, rock sample suspensions were plated onto petri dish plates
189 comprised of one of six different agar types. These agar types enabled the positive

190 identification of colonies with the following phenotypic traits: 1) heterotrophic growth on pH
191 neutral media, 2) heterotrophic growth on acidic media, 3) siderophore production, 4) alkaline
192 phosphatase production, 5) iron oxidation and 6) manganese oxidation. Each agar type allowed
193 positive identification of microbial isolates with a specific phenotype, either through a visually
194 recognisable color change or through the presence alone of microbial colonies on that agar
195 type.

196 We choose to specifically study aerobic rock weathering organisms based upon their relevance
197 to the sites sampled (primarily rock-air interfaces). Phenotypic traits were chosen based upon
198 either their known importance in microbial rock weathering of shale (Kalinowski et al. 2006;
199 Spalore et al. 2011; Włodarczyk et al. 2015) or of rock weathering more generally (Uroz et
200 al. 2009; Gadd, 2010; Carmichael et al. 2013), and based upon the availability of agar plate
201 phenotypic assay methodology.

202 The authors appreciate that the agar types used in this study enrich for microbial isolates within
203 narrow, specific metabolic/phenotypic groupings, rather than being broad, all-encompassing
204 assays. For example, the iron oxidizers enriched in this study are those isolates that can
205 specifically grow on WAYE agar (below and Table 1), meaning they are at least acidotolerant,
206 if not acidophilic. Furthermore, we cannot comment whether these organisms are autotrophic
207 or heterotrophic iron oxidizers, as the medium contains nutrients that would facilitate growth
208 and activity of both groups. Finally, as all incubations carried out in this study were under
209 aerobic conditions, our culturing approach could not identify anaerobic or microaerophilic iron
210 oxidizers.

211 With the exception of nutrient agar based media (both pH neutral and acidic media were used),
212 all phenotypic groups were isolated within a single pH range. For example, the CAS agar plates
213 (used to detect siderophore production) had a media pH of 6.8. This limitation could not be

214 overcome by preparing media at differing pH levels (e.g. pH neutral and acidic CAS agar), as
215 the chemical reactions that provide a visual result in these media types are pH sensitive (e.g.
216 acidified CAS agar will not be functional). Further methodological limitations apply to the
217 media used to enrich for alkaline phosphatase producers, manganese oxidizers and iron
218 oxidizers. Despite these issues, our approach enabled the isolation of geomicrobiologically-
219 relevant microbial groups from our sample sites. Throughout this manuscript, the microbial
220 groups isolated in this study are referred to by their general name for ease (e.g. aerobic,
221 acidotolerant/acidophilic iron oxidizers are called “iron oxidizers”).

222 No attempt to distinguish between bacteria, fungi or archaea was made, however only
223 successfully amplified 16S rDNA sequences from bacterial isolates were submitted for
224 sequencing (see below, Colony PCR and Sanger sequencing). All agar types contained an
225 added source of organic carbon, in addition to organic material within the shale rock
226 supplement, therefore both autotrophic and heterotrophic isolates could be cultivated on all
227 media types and could not be distinguished from each other with the methods used. Brief details
228 of each agar type are included in Table 1, with media recipes detailed in the “Agar media”
229 section below. Further details on each agar type can be found in the provided selected
230 references.

231 One gram aliquots of samples from each location (25 locations across 9 field sites) were
232 suspended in 5 mL of 1x PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄ and 0.24 g KH₂PO₄, 1 L
233 water - adjusted to pH 7 with HCl) in 50 mL Falcon tubes and hand inverted for 5 minutes to
234 ensure thorough mixing. Suspensions were serially diluted in phosphate buffered saline (PBS)
235 and plated out onto each of six agar types (see media below) in triplicate. This created a total
236 of 75 plates (triplicate plates for 25 samples) for each agar type. Plates were wrapped in
237 parafilm and incubated aerobically at room temperature (~23 °C) for one month, and the
238 number of colonies positive for that phenotype recorded. These colony counts were then

239 adjusted to create CFU g⁻¹ values for each phenotype within a sample. Un-inoculated plates
240 and plates inoculated with just PBS were incubated alongside sample plates as sterile controls.

241 **Agar media**

242 All agar media recipes are for 1 L preparations made with Milli-Q UV- purified and filtered
243 (0.22 µm) water, and autoclaved at 121 °C for 20 minutes unless otherwise specified. All agar
244 media was supplemented with 2 g L⁻¹ of black shale rock powder obtained by collection and
245 subsequent crushing of (<0.5 mm size fractionated) slabs of black shale from the Mulgrave
246 shale member at Saltwick Bay.

247 *Nutrient agar*: 28 g nutrient agar powder, 2 g rock powder.

248 *Acidic nutrient agar*: Solution A contains 28 g nutrient broth powder and 2 g rock powder
249 mixed in 750 mL of water, which is then adjusted to pH 3 with H₂SO₄. Solution B is prepared
250 by mixing 7 g agarose in 1 L of water and stirred for 30 minutes to allow agarose hydration.
251 The agarose was then allowed to settle, and 750 mL of the water removed, leaving a remaining
252 250 mL agarose suspension. Both solutions A and B are autoclaved and then mixed while still
253 molten.

254 *Chrome Azurol S (CAS) blue agar*: Solutions A, B and C were prepared as follows, A) 0.324
255 g FeCl₃•6H₂O was mixed in a 100 mL aqueous solution of 0.01 M HCl, B) 60.5 mg of CAS
256 powder in 50 mL of water and C) 72.9 mg of hexadecyltrimethylammonium bromide
257 (HDTMA) in 40 mL of water. Ten millilitres of solution A was mixed with all of solution B,
258 which was then mixed with solution C, creating solution D. Solution E is prepared from 200
259 mL of 5x M9 salts (34 g Na₂HPO₄, 15 g KH₂PO₄, 2.5 g NaCl and 5.0 g NH₄CL in 1 L water -
260 autoclaved), 15 g agar, 30.25 g piperazine-N,N-bis(2-ethanesulfonic acid) (PIPES) and 2 g of
261 rock powder, mixed in 650 mL of water and adjusted to pH 6.8 with NaOH. Both solutions D

262 and E were autoclaved, and then allowed to cool to 50 °C in a water bath before being mixed
263 to create the final media.

264 *Phenolphthalein phosphate agar*: 15 g of Phenolphthalein phosphate agar (PPA, purchased
265 from Sigma-Aldrich) and 2 g of rock powder.

266 *Manganese oxidizer agar*: 2 g Peptone, 0.5 g yeast extract, 1 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 150 mg
267 $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 2.38 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 8 mg
268 LBB, 2 g rock powder and 15 g agar were added to 1 L water and adjusted to pH 7.4 using
269 NaOH/HCl.

270 *Washed Agarose - Yeast Extract (WAYE) agar*: A basal salts solution (1 L - autoclaved) was
271 prepared by the addition of 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5 g $(\text{NH}_4)_2\text{SO}_4$, 1 g KCl and 0.1 g of
272 $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$. Solution A was prepared by mixing 100 mL of basal solution to 650 mL of
273 water, with 0.2 g of yeast extract powder and 2 g of rock powder added. This solution is then
274 adjusted to pH 3 with sulfuric acid before autoclaving. Solution B is prepared by mixing 7 g
275 agarose in 1 L of water and stirred for 30 minutes to allow agarose hydration. The agarose was
276 then allowed to settle, and 750 mL of the water removed. The remaining 250 mL containing
277 the hydrated agarose is then autoclaved. Solution C) 800 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, adjusted to pH 3
278 with sulfuric acid before filter sterilization (0.22 μm filter) was prepared. When hot, solutions
279 A and B are mixed (ensuring agar is fully molten) along with 5 mL of solution C under sterile
280 conditions.

281 **Colony PCR and Sanger sequencing**

282 Forty six colonies were picked from phenotypic assay plates, based upon visual assessment of
283 unique colony morphology, for phylogenetic identification of the microbial isolates. Isolates
284 that were obviously fungal in morphology were not selected, as only 16S rDNA sequencing to
285 identify bacterial species was carried out. Selected individual colonies were picked and

286 suspended in 50 μ L of molecular grade water. One microlitre of this solution was used as the
287 DNA template for the PCR reaction. Each 25 μ L reaction included a 12.5 μ L PCR Mastermix,
288 1 μ L of both the forward and reverse primer solutions and 10.5 μ L of molecular grade water.
289 PCR Mastermix (New England Biolabs) contained Taq DNA Polymerase, deoxynucleoside
290 triphosphates (200 μ M) and buffer ($MgCl_2$ 1.5 mM). Primer concentration was 0.4 μ M in the
291 final PCR reaction volume.

292 The forward primer used was 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse
293 primer used was 1389R (5'-ACGGGCGGTGTGTACAAG-3'). The PCR procedure started
294 with a 10 minute denaturation step at 96° C for 10 minutes, followed by 30 cycles of
295 denaturation at 95° C for 1 minute, annealing at 60° C for 1 minute and extension at 72° C for
296 1 minute, with a final step of extension at 72° C for 10 minutes. A negative control lacking
297 added DNA template was also performed to rule out false positive results from reagent
298 contamination. PCR products were analysed using gel electrophoresis.

299 Successfully amplified products were processed using a DNA Purification Kit (Qiagen)
300 according to the manufacturer's instructions, and analysed by Sanger sequencing at Edinburgh
301 Genomics with the same primer set used for PCR reactions. Processed reads (quality control
302 checked and trimmed) were provided by Edinburgh Genomics, which were then aligned and
303 forward and reverse sequences stitched to produce contigs using the software DNA Sequence
304 Aligner (v4) from DNA Baser. Contigs were then aligned in the alignment, classification and
305 tree (ACT) service of ARB-Silva (Pruesse et al. 2012), allowing neighbour sequences with
306 >97% similarity to be obtained and isolate identity to be determined (Quast et al. 2013; Yilmaz
307 et al., 2014; Glöckner et al. 2017). Isolate identifications were taken from the European
308 Molecular Biology Laboratory (EMBL) database within ACT. ACT was also used to produce
309 a .tree file (Beccati et al. 2017) which was visualized in iTOL (Letunic and Bork, 2016).

310 Cultured isolates are labelled on the phylogenetic tree with the following label structure:
311 phenotype (SP/APP)_ site number (e.g. 1)_ unique identifier.

312 **Data and statistical analysis**

313 Basic data manipulation and statistical analyses were carried out in R (version 3.5.3) (R Core
314 Team, 2019). A Spearman's Rank Correlation analysis was performed on CFU g⁻¹ data and pH
315 values to determine if significant positive and/or negative correlations occurred between
316 phenotypic trait groups and with pH. Figures were produced using the R packages ggplot2
317 (Wickham, 2016) and corrplot (Wei and Simko, 2017).

318 **RESULTS**

319 Isolates cultured on nutrient agar were identified in samples collected from all nine sampling
320 sites, ranging in abundance between 10³ and 10⁷ CFU g⁻¹ (Figure 3). This was the only
321 phenotype tested for that was identified in samples from all sampling sites. In contrast, isolates
322 cultured on acidic nutrient agar were only identified in samples taken from four sites, three of
323 which were identified as highly acidic (< pH 3) from pH measurements taken of rock surface
324 scrapings (Figure 3).

325 All phenotypes screened for were identified in samples from at least one site (Figure 3).
326 Alkaline phosphatase producers were widely distributed among the samples collected, with
327 abundance ranging across two orders of magnitude (5 x 10³ – 3 x 10⁵ CFU g⁻¹) from eight of
328 nine sites sampled (Figure 3). Manganese oxidizing isolates (identified at sites 6, 7 and 9 only)
329 were the least distributed among the sites sampled (Figure 3).

330 Interestingly, manganese oxidizers were isolated from sites (6, 7 and 9) below pH 3 (Figure 3),
331 despite the fact the media used to culture these isolates was circumneutral (Table 1). This
332 indicates that the manganese oxidizers isolated in this study could tolerate a wide pH range,

333 and that media pH did not constrain the enrichment of manganese oxidizers from sample sites
334 with a differing pH. It should be noted that “tolerate” in this context refers to the viability of
335 the microbes within the sample, and does not indicate the extent of their activity (active vs
336 dormant) within the environment.

337 Samples taken from highly acidic (< pH 3) sites yielded positive isolates for the most number
338 of phenotypes tested for, with phenotypically positive isolates cultured from samples 6, 7 and
339 9 being found on five out of six of the agar types tested (all except siderophore producers). In
340 comparison, samples collected from sites 1-4, which had moderately alkaline (~ pH 8) to
341 moderately acidic (pH 5.5-7) rock surfaces yielded phenotypically positive isolates for only 2-
342 3 of the phenotypes tested for (nutrient agar isolates and alkaline phosphatase producers in all
343 cases) (Figure 3).

344 Histograms of CFU g⁻¹ abundance data for each tested phenotype demonstrated that the data
345 collected was not normally distributed, meaning that a non-parametric (Spearman’s rank-order
346 correlation) analysis was required to test for associations between each of the six groups and
347 with the environmental parameter pH (Figure 4). Eight associations were identified as
348 significant at the 0.99 confidence level, with a further 3 significant associations identified at
349 both confidence levels of 0.95 and 0.9 respectively (total associations=14) (Figure 4).

350 pH significantly correlated with all six phenotypic trait groups, with positive associations
351 identified with iron oxidizers ($r_s=0.45$, $p<0.01$), acidic nutrient agar isolates ($r_s=0.55$, $p<0.01$)
352 and manganese oxidizers ($r_s=0.46$, $p<0.01$), and negative associations identified with nutrient
353 agar isolates ($r_s=-0.3$, $p=0.01$), siderophore producers ($r_s=-0.46$, $p<0.01$) and alkaline
354 phosphatase producers ($r_s=-0.22$, $p=0.06$). These two sets of associations with pH are internally
355 supported by further positive correlations between the abundances of the phenotypes within
356 each set (Figure 4). For example, the abundance of iron oxidizers positively correlated with the

357 abundance of both manganese oxidizers ($r_s=0.79$, $p<0.01$) and acidic nutrient agar isolates (r_s
358 $=0.57$, $p<0.01$). Positive correlations between those phenotypes that negatively correlated with
359 pH were weaker but still significant, such as the correlation between siderophore producers
360 with both alkaline phosphatase producers ($r_s=0.27$, $p<0.05$) and nutrient agar isolates ($r_s=0.21$,
361 $p=0.07$). Furthermore, two negative correlations in the abundance of phenotypes between the
362 pH association sets was identified, with siderophore producers negatively correlating with iron
363 oxidizers ($r_s=-0.26$, $p<0.05$) and manganese oxidizers ($r_s=-0.19$, $p<0.1$).

364 Twenty two isolates were successfully sequenced and the genus of the organism identified
365 (Figure 5, Table 2). The result of this analysis was the identification of eight different genera
366 across two phenotypic trait groups. These eight genera were from a diverse range of phyla,
367 including Actinobacteria, Firmicutes, α -Proteobacteria and γ -Proteobacteria (Figure 5, Table
368 2).

369 Roughly equal numbers of isolates from each of the weathering phenotypes processed for
370 sequencing were identified: 10 siderophore producers and 12 alkaline phosphatase producers.
371 Strains of *Aeromonas*, *Rhizobium* and *Pseudomonas* were identified within both phenotypic
372 groups. Of the 10 siderophore producing isolates successfully sequenced, eight belonged to the
373 genus *Pseudomonas* (Figure 5, Table 2).

374 No phylogenetic data for the iron and manganese oxidizing colonies could be obtained in this
375 study. Initial difficulty was encountered in performing colony PCR with iron and manganese
376 oxidizing colonies, however in some cases this was overcome by culturing those isolates on
377 acidic nutrient agar. However, those successfully amplified 16S rDNA amplicons failed in
378 subsequent sequencing reactions. The reason for these technical difficulties is unknown, but
379 metal content bound to the biomass/nucleic acids could have affected reaction success.

380 DISCUSSION

381 The abundance of microbes within six phenotypic trait groups from samples of weathered shale
382 was determined. As microbial isolates were likely cultured on more than one of the six agar
383 types (e.g. siderophore producing microbial species were also likely to grow on nutrient agar),
384 combined with the fact that most microbial species in environmental samples are known to be
385 non-culturable (Kirk et al. 2004), an accurate assessment of the total biomass within the
386 samples collected was not made. However, the abundance of nutrient agar and acidic nutrient
387 agar isolates have been used in this study to provide a baseline for total biomass within the
388 samples of weathered shale (Puente et al. 2009). Nutrient agar isolates were cultured from all
389 nine sites sampled, ranging in abundance between 3×10^3 and 1.5×10^7 CFUg⁻¹, while acidic
390 nutrient agar isolates were identified at four sites and ranged in abundance from 1.3×10^3 and
391 4.4×10^5 CFU g⁻¹ (Figure 3). This range of microbial abundance in samples of weathered shale
392 is consistent with a previous assessment made by Cockell et al. (2011), who used extractable
393 DNA content to estimate a total microorganism abundance of 4.2×10^4 cells cm⁻².

394 The occurrence of the remaining phenotypic trait groups (siderophore producers, alkaline
395 phosphatase producers, iron oxidizers and manganese oxidizers) varied substantially between
396 field sites (Figure 3). For example, at sites 1, 2 and 4 isolates were cultured from only two
397 phenotypic trait groups, nutrient agar isolates and alkaline phosphatase producers. In
398 comparison, sites 6, 7 and 9 yielded isolates from five of the six phenotypic trait groups (Figure
399 3). These differences can be qualitatively explained by differences in rock surface pH values,
400 with sites 1, 2 and 4 being pH neutral to moderately acidic (pH 5.8-7.7) while sites 6, 7 and 9
401 were strongly acidic (pH 2.6-2.8) (Figure 3). The low pH rock surface values obtained in this
402 study are consistent with the analysis of weathered shale crusts previously studied (pH 3.5,
403 Cockell et al. 2011).

404 To quantitatively determine if the abundance of isolates within phenotypic trait groups
405 correlated with rock surface pH, Spearman's rank correlation coefficient analysis was used

406 (Figure 4). Correlation analyses were also run for each pair combination of phenotypic trait
407 groups (e.g. iron oxidizers with siderophore producers) to determine abundance co-associations
408 between groups (Figure 4). pH was found to significantly influence the abundance of all six
409 phenotypic trait groups enumerated in this study, with the set of acidic nutrient agar isolates,
410 iron oxidizers and manganese oxidizers correlating with acidic pH values (r_s value range 0.45
411 to 0.55), and the set of nutrient agar isolates, siderophore producers and alkaline phosphatase
412 producers correlating with neutral to mildly acidic pH values (r_s value range -0.22 to -0.46)
413 (Figure 4). This control of pH over phenotypic trait abundance was supported by further
414 positive correlations between phenotypic trait groups within sets (e.g. iron oxidizers with acidic
415 nutrient agar isolates, $r_s=0.57$, $p<0.01$) and negative correlations between phenotypic trait
416 groups between sets (e.g. iron oxidizers with siderophore producers, $r_s=-0.26$, $p<0.05$) (Figure
417 4).

418 The association of aerobic, acidotolerant/acidophilic iron oxidizers with acidic environments
419 in this study (Figure 4) is unsurprising, as the bioenergetic favourability of aerobic microbial
420 iron oxidation is known to be restricted below pH 4 (Hedrich et al. 2011). Previous studies
421 have shown that microbial iron oxidation in shale is predominated by the oxidation of pyrite
422 (FeS_2), in which both the iron and sulfur become oxidized (Tasa et al. 1997; Joeckel et al. 2005;
423 Li et al. 2014). The oxidation of pyritic sulfur to sulphuric acid lowers environmental pH,
424 which in turn facilitates the growth of iron oxidizing microbes and the continued dissolution
425 of pyrite (Vera et al. 2013, but see also Samuels et al. 2019). As such, the interaction of iron
426 oxidizing and sulfur oxidizing microbial activity generates a positive feedback loop that
427 maintains a suitable habitat ($< \text{pH } 4$) for iron oxidizing microbes.

428 Siderophore production is known to of greater adaptive significance in pH neutral
429 environments where ferric iron bioavailability is low (Kalinowski et al. 2006), which explains
430 why siderophore producers were largely restricted to pH neutral environments in weathered

431 shale samples (Figure 3). These opposing relationships of iron oxidizers and siderophore
432 producers with pH are further supported by the negative correlation between these phenotypic
433 trait groups ($r_s=-0.26$, $p<0.05$), demonstrating that the approach we have used can effectively
434 distinguish between rock weathering niches along a physicochemical gradient (Figures 3 and
435 4). As far as the authors are aware, this is the first empirical evidence of the spatial separation
436 of aerobic iron oxidizers and siderophore producers across a pH gradient in rock weathering
437 environments.

438 Unlike microbial iron oxidation, microbial manganese oxidation is not known to be limited to
439 acidic environments, with the recommended pH value for the cultivation of manganese
440 oxidizers ranging between pH 7-8 (Nealson, 2006). Despite this, the abundance of manganese
441 oxidizers correlated positively with acidic environments ($r_s=0.46$, $p<0.01$), and with both iron
442 oxidizers ($r_s=0.79$, $p<0.01$) and acidic nutrient agar isolates ($r_s=0.57$, $p<0.01$). A potential
443 explanation for our findings is that the manganese oxidizing organisms isolated in this study
444 have the ability to oxidize multiple metallic elements (e.g. iron and manganese) (Corstjens et
445 al. 1992), and therefore are not actively oxidizing manganese in the acidic environments they
446 were isolated from. However, as the rate of abiotic manganese oxidation of manganese is
447 relatively slow compared to microbially-facilitated oxidation, and that the formation of
448 manganese oxide deposits is believed to mainly biologically mediated (Tebo et al. 2004), it
449 seems likely that the manganese oxide deposits identified at our field sites (Figure 2) are at
450 least the partial result of microbial activity. An alternative hypothesis for our results is that the
451 organisms isolated here may not be active oxidizers, i.e., they do not use enzymes such as
452 multicopper oxidases to facilitate manganese oxidation (Geszvain et al. 2012), but that biomass
453 of these organisms passively acts as a nucleation site for manganese oxidation (Tebo et al.
454 2005; Gadd, 2010). Further work, including phylogenetic identification and characterisation of

455 manganese oxidizing activity, would be required to resolve the exact role of the manganese
456 oxidizing microbes isolated in this study in their natural environment.

457 The pH of the individual media types used in this study potentially contributed to the
458 constraints on the distribution of the phenotypic trait groups enriched on those media types.
459 For example, aerobic acidotolerant iron oxidizers were isolated from three sites that ranged in
460 average surface pH from 2.6 to 5.2 (Figure 3). As the media used to enrich for this group
461 (WAYE agar) was at pH 3, aerobic iron oxidizers from pH neutral sites that could not tolerate
462 this pH were unlikely to be enriched. This is an inherent limitation of a culture-based approach
463 to investigate the distribution of microbial groups, however we believe that in the case of our
464 results this limitation likely had a minimal impact. The manganese oxidizers isolated in this
465 study were cultivated on a pH neutral agar, but were entirely isolated from acidic sites, while
466 siderophore producers cultivated on pH neutral agar were isolated from sites ranging in pH
467 from 5.20 to 8.07. Finally, alkaline phosphatase producers were isolated from eight of the nine
468 sites sampled, including from the most acidic and alkaline sites (Figure 3). Therefore, although
469 media pH may have partially constrained our results, it does not explain the pattern and general
470 trends we have observed.

471 The phenotypic trait agar plate assay used in this study to enumerate phosphate solubilizers
472 tested for the production of alkaline phosphatases, which have been previously indicated in
473 inorganic phosphate solubilisation in neutral and alkaline environments (Hughes and Lawley,
474 2003; Sharma et al. 2013). The correlation between the abundance of alkaline phosphatase
475 producing microbes and neutral pH environments in this study can therefore be expected
476 (Figure 4). In future work it would be interesting to compare the abundance of microbial
477 isolates that produce acid and alkaline phosphatases, and determine if pH influences their
478 distribution in rock weathering environments. In addition, determining the distribution of

479 anoxic and anaerobic microbial groups that contribute to microbial rock weathering in shale,
480 such as neutrophilic iron oxidizers, would be a logical progression of this work.

481 Phylogenetic identification of the isolates cultured in this study was attempted for four
482 phenotypic trait groups (not including nutrient agar and acidic nutrient agar isolates), but was
483 only successful for siderophore producers and alkaline phosphatase producers. In total 22
484 isolates were successfully identified, revealing eight genera across four different phyla (Table
485 2, Figure 5). A single alkaline phosphatase producing isolate was identified as *Moraxella* (γ -
486 Proteobacteria), which is the first time this genus has been associated with weathered shale
487 environments. The genera of the remaining isolates have been previously identified in
488 weathered shale, either through isolation from culture-based studies (e.g. Jiang et al. 2015) or
489 in culture-independent analyses (e.g. Włodarczyk et al. 2018). Furthermore, most of these
490 genera are well-known rock weathering organisms such as *Arthrobacter* (Frey et al. 2010),
491 *Bacillus* (Puente et al. 2004), *Pseudomonas* (Matlakowska and Sklodowska, 2009), *Rhizobium*
492 (Zhao et al. 2013) and *Streptomyces* (Cockell et al. 2013). In most cases, these organisms have
493 been previously recognised to have the rock weathering phenotypic traits that they are
494 associated with in this study, such as the siderophore production of *Pseudomonas* (Kalinowski
495 et al. 2006) and *Rhizobium* (Zhao et al. 2013), or the phosphate solubilisation of *Bacillus*
496 (Puente et al. 2004) and *Streptomyces* (Hamdali et al. 2008). It is therefore highly likely that
497 the organisms identified in this study are active rock weathering organisms within weathered
498 shale environments.

499 CONCLUSIONS

500 In this study, the abundances of microbes with differing phenotypic traits were found to be
501 influenced by a physicochemical parameter (pH). Iron and manganese oxidizing microbes were
502 restricted to acidic environments, while siderophore producing and alkaline phosphatase

503 producing microbes were largely constrained to pH neutral environments. Furthermore, the
504 first empirical evidence for a clear separation of the distribution of aerobic iron oxidizers and
505 siderophore producers in rock weathering environments was shown, which can be readily
506 explained by changes in iron geochemistry across pH gradients. Phylogenetic analysis of
507 siderophore producing and alkaline phosphatase producing bacterial isolates revealed several
508 commonly identified rock weathering genera, indicating that these organisms are likely active
509 rock-weathering organisms in their native environments.

510 Our results show that physicochemical parameters can be used to predict the distribution of
511 functional groups. Future research should attempt to discern the effect physicochemical
512 parameters have on the expression and efficacy of rock weathering phenotypic traits within
513 these groups. Combined, this knowledge could be used to better estimate the contribution of
514 microbial communities to rock weathering rates in the field (Yesavage et al. 2012; Ilgen et al.
515 2017). Finally, our study demonstrates the importance of culture-based approaches to
516 investigate the functional ecology of microbial communities.

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521 ICL Boulby operate an active mine within the local area of the field sites sampled in this study.

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