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Floc formation reduces the pH stress experienced by microorganisms living in alkaline environments

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1 **Floc formation reduces the pH stress experienced by microorganisms living in**  
2 **alkaline environments.**

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14 **Running title:** Microbial floc formation is a key alkaline survival strategy

15 **Abstract**

16 The survival of microorganisms within a cementitious geological disposal facility for  
17 radioactive wastes is heavily dependent on their ability to survive the calcium  
18 dominated, hyper-alkaline conditions resulting from the dissolution of the cementitious  
19 materials. The present study shows that the formation of flocs, composed of a complex  
20 mixture of extracellular polymeric substances (EPS), provides protection against  
21 alkaline pH values up to pH 13.0. The flocs were dominated by *Alishewanella* and  
22 *Dietzia sp*, producing a mannose rich carbohydrate fraction incorporating extracellular  
23 DNA, resulting in Ca<sup>2+</sup> sequestration. EPS provided a ~10 µm thick layer around the  
24 cells within the centre of the flocs, which were capable of growth at pH 11.0 and 11.5,  
25 maintaining internal pH values of pH 10.4 and 10.7 respectively. Survival was  
26 observed at pH 12.0, where an internal floc pH of 11.6 was observed alongside a  
27 reduced associated biomass. Limited floc survival (<2 weeks) was observed at pH  
28 13.0. This study demonstrates that flocs are able to maintain a lower internal pH in  
29 response to the hyperalkaline conditions expected to occur within a cementitious,  
30 geological disposal facility for radioactive wastes and indicates that floc communities  
31 within such a facility would be capable of survival up to a pH of 12.0.

32 **Importance**

33 The role of extracellular polymeric substances (EPS) in the survival of microorganisms  
34 within hyperalkaline conditions is poorly understood. Here we present data for the  
35 taxonomy, morphology and chemical characteristics of an EPS based microbial floc,  
36 formed by a consortia isolated from an anthropogenic hyperalkaline site. Short term  
37 (<2 weeks) survival of the flocs at pH 13 was observed with indefinite survival  
38 observed at pH 12.0. Micro pH electrodes (ø10 µm) were utilised to demonstrate that

39 floccs were able to maintain a lower internal pH in response to hyperalkaline conditions  
40 (pH 11.0, 11.5 and 12.0), demonstrating for the first time that floc formation and EPS  
41 production is a survival strategy under hyperalkaline conditions. The results indicate  
42 how microbial communities may survive and propagate within the hyperalkaline  
43 environment expected to prevail in a cementitious geological disposal facility for  
44 radioactive wastes, they are also relevant to the wider extremophile community.

## 45 **Introduction**

46 One of the concepts for the disposal of the UK's intermediate level radioactive waste  
47 (ILW) inventory is a geological disposal facility (GDF) employing a cementitious backfill  
48 (1, 2). It is anticipated that post closure, groundwater ingress into such a facility will  
49 result in a saturated, anaerobic, calcium rich, hyper-alkaline environment that will aid  
50 radionuclide retention through sorption and the formation of insoluble complexes (3,  
51 4). These conditions will also result in the alkaline hydrolysis of the cellulose  
52 component of the ILW producing a range of small molecular weight organic  
53 compounds collectively known as cellulose degradation products (CDP) (5). The  
54 biodegradation of CDP has received considerable attention in recent years with a  
55 number of authors reporting alkaliphilic degradation under a wide range of growth  
56 conditions (6, 7) including alkaliphilic methanogenic consortia operating at pH 11.0 (8).  
57 In some cases these alkaliphilic communities have been shown to form floccs where  
58 the bacteria are encased in a matrix of extracellular polymeric substances (EPS) (9).  
59 The ability of freshwater and marine microorganisms to aggregate into a sustainable  
60 microenvironment is documented (10, 11), however the added selective pressure of  
61 alkaline pH is not commonly observed in the natural world.

62 EPS such as carbohydrates, proteins, lipids and extracellular DNA (eDNA) are  
63 ubiquitous components of biofilm matrices (12). In addition to EPS; inorganic materials  
64 (minerals) may also be incorporated to provide structural support where physical  
65 stresses may impact on survival (13). Biofilm formation is known to enhance survival  
66 against a range of environmental stresses such as pH shifts (14), with some biofilm  
67 communities creating more favourable growth conditions through the secretion of  
68 specific EPS components (15, 16). In these cases biofilm formation allows microbial  
69 propagation in extreme environments (16, 17).

70 In the case of hyper-alkaline environments the primary environmental stress is the  
71 extreme pH and there are examples in the literature of flocs and biofilms attenuating  
72 the ambient pH. Aggregates of *Bacillus laevolacticus* modulated their internal pH by  
73 between 0.4 – 2.0 pH units (18). A difference of 1 pH unit in methanogenic aggregates  
74 from an up flow anaerobic sludge blanket have also been observed (19). Large pH  
75 differences have been recorded in *Pseudomonas* biofilms; where a difference of 1.4  
76 pH units was measured across distances of 50µm (20). Further pH shifts have been  
77 seen across a variety of distances in a range of biofilms grown under different  
78 conditions (21-23). However, in all these cases, the pH shifts are associated with near  
79 neutral conditions and as such, the logarithmic nature of the pH scale means that the  
80 modulation of internal pH in response to alkaline conditions represents a significantly  
81 more difficult challenge to the microorganisms concerned.

82 Information regarding the ability of flocs to attenuate pH within alkaline environments  
83 is currently absent from the literature. Consequently, the aim of this study was to  
84 determine the ability of alkaliphilic flocs (9) to attenuate environmental pH values  
85 relevant to a cementitious GDF. The isolated flocs were also characterised in terms of  
86 microbial community structure, morphology and compositional aspects of the EPS

87 produced. The study sought to determine what degree floc formation is a survival  
88 strategy for alkaliphilic microorganisms; and if flocs provide a potential dispersion  
89 vehicle for alkaliphilic microorganisms within hyper-alkaline environments such as a  
90 cementitious GDF.

## 91 **Materials and Methods**

### 92 **Community composition and flocculate concentration**

93 A previously described flocculate producing microcosm operating at pH 11.0 was sub-  
94 sampled and maintained as described previously (9). Briefly, the microcosm was  
95 inoculated with material retrieved from an area inundated with alkaline leachate at the  
96 lime kiln waste site Harpur Hill, Derbyshire, UK. The microcosm was completely mixed,  
97 incubated at 25 °C and fed alkaline cellulose degradation products every two weeks  
98 on a 10% waste/feed cycle. Microcosm fluid (25 mL) was centrifuged at 5,000 x *g* for  
99 10 minutes with the resulting pellet re-suspended in 25mL of phosphate buffer as  
100 described by Hurt et al. (24) Genomic DNA was extracted for analysis using the  
101 methods of Griffiths et al (25). The V4 region of the 16S rRNA gene was amplified  
102 using primers 519F (5'CAGCMGCCGCGGTAA'3) and 785R  
103 (5'TACNVGGGTATCTAATCC'3) for both bacteria and archaea (26, 27) with the  
104 following overhangs 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG'3 and 5'  
105 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG'3, respectively. PCR reaction  
106 mixture contained 20 ng of purified DNA solution, 0.5µL of each primer (20 pmol µL<sup>-1</sup>  
107 concentration), and 25 µL of MyTaq HS red mix (BIOLINE, UK) made up to 50 µL  
108 volume with PCR grade water. The reaction mixture was then incubated at 94 °C for  
109 1 minute, and then cycled 35 times through three steps: denaturing (94 °C, 15 s),  
110 annealing (60 °C, 15 s), primer extension (72 °C, 10 s). This was followed by a final

111 extension step of 72 °C for 5 minutes. 16S rRNA gene microbial community analysis  
112 was carried out via a MiSeq platform (Illumina, USA) at 250 bp paired ends with  
113 chimera detection and removal performed via the UNCHIME algorithm in the Mothur  
114 suite (28) (Chunlab, South Korea). Assignment of OTU's was performed using a CD-  
115 HIT clustering method with a 95 % cut-off value with taxonomic assignment performed  
116 against the EZtaxon database (29) .

117 Floc concentration was determined using a Guava easyCyte™ flow cytometer  
118 (Millipore, US) with flocs stained using FITc (Fisher, UK) in accordance with methods  
119 outlined in Chen, et al. (30). Floc size distribution was analysed using a Mastersizer  
120 2000 (Malvern, UK) with a dispersant refractive index of 1.330 and a particle refractive  
121 index of 1.572, with flocs isolated by centrifugation and re-suspended in ultrapure  
122 water before analysis.

### 123 **Morphology**

124 The overall morphology of the flocs was investigated using a Quanta FEG 250  
125 scanning electron microscope (SEM) and energy-dispersive X-ray spectroscopy  
126 (EDX) used for analysis of sites of interest. Samples were fixed overnight in 4 %  
127 paraformaldehyde (Fisher, UK) then dehydrated using a serial ethanol dilution of 25,  
128 50, 75 and 100 % for 2 min per step. Samples were then dried onto a metal disc and  
129 sputter coated via a gold palladium plasma (CA7625 Polaron, Quorum Technologies  
130 Ltd, UK). The structural morphology of the flocculates was investigated via confocal  
131 laser scanning microscopy (CLSM) at the Bio imaging centre of Leeds University using  
132 a Zeiss LSM880 inverted confocal microscope with image analysis performed using  
133 Zen 2.1 (Zeiss Microscopy). Flocs were fixed in 4% Paraformaldehyde (Fisher, UK)  
134 overnight and then stained using the following compounds in accordance with  
135 methods outlined in Chen et al. (30): Calcofluor white for the visualisation of  $\beta$ -1,4 and

136  $\beta$ -1,3 polysaccharides (Sigma, UK), Nile red (Fisher, UK) for lipids and hydrophobic  
137 sites, concanavalin A, tetramethylrhodamine conjugate (Fisher, UK) for  $\alpha$ -  
138 mannopyranosyl,  $\alpha$ -glucopyranosyl sugars, FITc (Fisher, UK) for protein and Syto 63  
139 (Fisher, UK) for total cells and extracellular DNA. Fluorescence in situ hybridisation  
140 was carried out as per the methods of Ainsworth et al (31), using previously described  
141 probes for the identification of total bacteria (32), Firmicutes (33),  $\gamma$ -Proteobacteria  
142 (34) and Actinobacteria (35).

### 143 **EPS extraction, Purification and Characterisation**

144 Crude EPS was extracted from the flocs using a multiple extraction method outlined  
145 by Ras et al. (36), which extracts different components of EPS based upon the  
146 disruption of different chemical bonds. ATP was measured at each step to ensure the  
147 EPS extraction was not causing the lysis of cells and thus contaminating the extracted  
148 EPS. This was performed using a 3M Clean-Trace Biomass Detection Kit and  
149 Luminometer (3M, UK) employing a modified method described previously by Charles  
150 et al. (9); with CFU mL<sup>-1</sup> calculated using a standard curve of Escherichia coli K12  
151 concentrations (data not shown). The crude EPS extracts were then measured for  
152 carbohydrate content via the phenol sulphuric acid method (37), protein content via  
153 the Bradford assay (38) lipid content via the methods of Bligh and Dyer (39) and the  
154 DNA content via a Genova-nano spectrophotometer (Jenway, Bibby Scientific, UK).

155 In order to purify the extracted EPS, dialysis was carried out against ultrapure water  
156 for 72 hours with the water changed every 24 hours. The protein and carbohydrate  
157 fractions were then isolated from the dialysed EPS. Protein was precipitated via  
158 treatment with trichloroacetic acid and the carbohydrate fraction precipitated via  
159 ethanol treatment as outlined in Marshall, et al. (40). Monomer composition was  
160 determined by TFA hydrolysis followed by anion exchange chromatography as



161 described previously (41) with the exception that sample was eluted with aqueous 0.01  
162 M NaOH for 20 minutes followed by 83 % 10 mM NaOH: 17 % 150 mM NaOH:1 M  
163 sodium acetate for 25 minutes for simultaneous determination of monomers and  
164 uronic acids. Dry weight and inorganic content of the flocs were determined by  
165 methods outlined in BS ISO 6496:1999 (42).

### 166 **EPS calcium binding assay**

167 Aqueous suspensions of EPS:Ca<sup>2+</sup> (as CaCl<sub>2</sub>) were prepared to concentrations of 0.1,  
168 0.25, 0.5 and 1 g. g<sup>-1</sup> dry weight and incubated at room temperature for 15 minutes.  
169 The calcium content of the samples was then quantified using a Metrohm 850  
170 Professional IC (Metrohm, Cheshire, UK) with pulsed amperometric detection,  
171 employing a Metrohm C4 Column (250 mm x 4.6 mm) with a mobile phase of 4.6 mM  
172 phosphoric acid. All reactions were carried out under nitrogen to avoid interference  
173 from atmospheric carbon dioxide and EPS bound calcium determined via comparison  
174 with EPS-free incubations. Total bound calcium was determined using atomic  
175 absorbance spectroscopy (Agilent 200 series AA, Agilent, UK) following digestion of  
176 EPS (1 mg) in 10 ml of 0.1 % KCl: 1 % HNO<sub>3</sub>.

### 177 **Floc profiling**

178 pH profiles of the flocs were undertaken with a micromanipulator and stand (Unisense,  
179 Denmark) using a 10 µm diameter pH electrode with an external reference (Unisense,  
180 Denmark) connected to a single channel pH/redox meter supplied by the probe  
181 manufacturer (Unisense, Denmark). The probe was calibrated against pH 4.0, pH 7.0  
182 and pH 10.0 standards (Fisher, UK) and tested against pH 11.0 and pH 12.0 solutions  
183 made using NAOH. Profiles were taken through the flocs at pH 11.0, 11.5 and 12.0.  
184 In order to generate the pH profile of the flocs, microcosm fluid was incubated for 1  
185 hour at the desired pH and then injected into agar cubes of the same pH to provide

186 support for the profiling. Control profiles were conducted through agarose at pH 11.0,  
187 11.5 and 12.0. In order to investigate floc stability under a range of pH values zeta  
188 potential of the flocs was measured using a Zetasizer nano (Malvern, UK) with the  
189 zeta potential calculated using Smoluchowski's equation. Flocs were isolated by  
190 centrifugation and re-suspended in ultrapure water of the desired pH (values between  
191 pH 7.0 and pH 12.0) before analysis.

## 192 **Floc survival**

193 In order to investigate the ability of the floc communities to survive prolonged exposure  
194 to hyperalkaline conditions small scale microcosms of approximately  $10^4$  cells per ml  
195 were formed using 100 ml Wheaton bottles at pH values 11.0, 11.5, 12.0 and 13.0 in  
196 duplicate. The microcosms were composed of mineral media (43) and CDP to match  
197 the composition of the original microcosm reported in Charles et al. (9). CDP was  
198 produced by methods outlined previously by Rout et al.(44) and pH was measured on  
199 a weekly basis using a portable handheld probe and meter (Mettler Toledo, UK), with  
200 pH adjusted accordingly using sodium hydroxide or hydrochloric acid. The head space  
201 of the microcosms was kept under nitrogen to ensure anaerobic conditions. The  
202 microcosms were sampled for ATP concentration on a weekly basis for three weeks  
203 using a 3M Clean-Trace Biomass Detection Kit and Luminometer (3M, UK) as  
204 previously described. Abiotic controls were established for each pH value within the  
205 pH range and were sampled as per the above.

## 206 **Accession number**

207 16S rRNA gene sequence data was uploaded to the NCBI sequence read archive  
208 under the accession number SRP082489

## 209 **Results**

### 210 **Floc Characterisation**

211 Sequencing of the microbial 16S rRNA gene returned 18854 reads via Illumina MiSeq  
212 technology. The microcosm community was dominated by sequence reads associated  
213 with the Phyla Proteobacteria (39.49 %), Actinobacteria (32.93 %), Firmicutes (24.85  
214 %) and Bacteroidetes (2.34 %) making up 99.6 % of the library (Fig. 1). The  
215 Proteobacteria and Actinobacteria were each dominated by sequence reads  
216 associated with *Alishewanella* (31.81 %) and *Dietzia* (26.74 %) respectively. Reads  
217 associated with *Hydrogenophaga* and *Silanimonas* were also detected within the  
218 Proteobacteria group, where *Leucobacter* and *Corynebacterium* represented >1 % of  
219 the total reads within the Actinobacteria. The Firmicutes detected were not dominated  
220 by a particular species *per se*, where *Anaerobranca* (4.55 %), *GQ356941\_g* (4.45 %),  
221 *Tissierella* (2.67 %), *Natronobacillus* (2.55 %), *Bacillus* (2.04 %) and *Bacillus\_g26*  
222 (1.68 %) were all represented within the sequence reads. The Bacteroidetes were  
223 almost entirely composed of the sequence reads associated with the genus  
224 *Aquiflexum* (2.16 % of the total reads).

225 Flow cytometry indicated that the floc concentration was  $2.0 \times 10^5$  flocs/ml in the pH  
226 11.0 microcosm with 54.4 % of the flocs being >10  $\mu\text{m}$  in diameter reaching a  
227 maximum size of 240  $\mu\text{m}$  (Fig. S1). SEM investigations (Fig. S2) revealed individual  
228 flocs to be clusters of cells, polymeric substance and crystalline precipitates. These  
229 precipitates were composed elementally of calcium, carbon and oxygen indicating  
230 calcium carbonate precipitation (Fig. S2). Confocal laser scanning microscopy (CLSM)  
231 revealed the flocs to be composed of a complex mixture of proteins, carbohydrates,  
232 lipids, eDNA and cells (Fig. 2). The most basal layer of the floc was composed primarily  
233 of lipids and  $\beta$ -1,4 and  $\beta$ -1,3 polysaccharides, where the most outer layers of the floc

234 were composed of concentrated areas of proteins, these regions were surrounded by  
235  $\alpha$ -mannopyranosyl and  $\alpha$ -glucopyranosyl sugars which were also closely associated  
236 with eDNA. These regions of pyranosyl sugars and eDNA surrounded the crystalline  
237 precipitates observed under SEM. Bacterial cells were in the centre of the floc, with  
238  $\sim 10 \mu\text{m}$  of EPS material surrounding these cells (Fig. S3). FISH/CLSM probing (Fig.  
239 3) showed that the Actinobacteria (red) and  $\gamma$ -Proteobacteria (blue) were clustered  
240 together, with Firmicutes (green) being situated amongst the periphery of these  
241 clusters within the centre of the floc. The flocs ranged from 50 – 250  $\mu\text{m}$  in diameter  
242 and most featured a central well defined mass with looser less formed sections  
243 attached. The denser areas of the flocs showed higher numbers of cells and more  
244 concentrated areas of all EPS components.

245 The initial extraction of the floc associated EPS using sonication and ethanol  
246 precipitation removed a primary, lipid rich EPS, consisting of 8.4 mg/g VS of lipids,  
247 carbohydrates (3.2 mg/g VS) and low levels ( $<1$  mg/g VS) of protein and eDNA (Fig.  
248 S4). EDTA extraction yielded a significantly greater mass of volatile solids, with the  
249 EPS extracted being carbohydrate rich (38.2 mg/g VS); again, eDNA and proteins  
250 were also part of the EPS structure, with lipids being the least dominant component  
251 following EDTA extraction. The extraction process was able to extract a combined  
252  $41.4 \pm 3.7$  mg/g VS carbohydrate,  $5.0 \pm 0.7$  mg/g VS of eDNA,  $9.7 \pm 1.0$  mg/g VS and  
253  $4.7 \pm 0.2$  mg/g VS protein with a dry weight content of the microcosm of 15.5 g/L of  
254 which 44.4 % was volatile solids and 55.5 % was inorganic ash. Monomer analysis of  
255 the carbohydrate fraction of the extracted EPS (Table 1) showed half the monomers  
256 to be composed of mannose, with ribose and ribitol making up a further 20 %. A varied  
257 range of sugars were identified in smaller amounts with uronic acids also present but  
258 only making up a small proportion (4.3 %) of the total monomer composition.

259 The calcium content of the liquid component of the floc microcosm was 1.6 g/L which  
260 was lower than that of the abiotic comparison which measured 4.3 g/L. Analysis of the  
261 dialysed EPS via AA spectroscopy revealed 0.203 mg of calcium per mg of EPS.  
262 Investigation into the binding capacity of dialysed EPS indicated that the EPS was able  
263 to bind a further 0.173 mg/mg EPS (Fig. S5) giving a total binding capacity of 0.376  
264 mg/mg EPS.

### 265 **Floc profiling and response to alkaline conditions.**

266 Analysis of the zeta potential of the flocs under a range of pH values showed  
267 consistent stable potentials of -20 to -30 mV when transferred to solutions of pH 7-9  
268 and 10-12 (Fig. 4A). When subjected to a pH of 10 the zeta potential shifted to  $2.65 \pm$   
269  $0.36$  mV. Following exposure of the flocs to elevated pH values representative of a  
270 GDF, flocs demonstrated the ability to grow at pH 11.0 with cell numbers increasing  
271 from a concentration of  $2.5 \times 10^4$  CFU/ml to  $1.8 \times 10^6$  CFU/ml (Fig. 4B). When subjected  
272 to a pH of 11.5, the flocs showed only a small increase in cell concentration from  $2.5$   
273  $\times 10^4$  CFU/ml to  $4.8 \times 10^4$  CFU/ml. Cells within the floc were capable of survival when  
274 sub cultured to pH 12.0, however the concentration of cells fell sharply from  $7.7 \times 10^3$   
275 CFU/ml to  $4.1 \times 10^2$  CFU/ml within the first two weeks and then remained stable up to  
276 the end of week 3. At pH 13 the flocs were able to maintain detectable cell  
277 concentrations for two weeks, after which cells could not be detected. No increase in  
278 ATP values were reported from control microcosms (data not shown).

279 The pH profiling was carried out on a sub sample of each of the surviving flocs (Fig.  
280 4C) exposed to pH 11.0, 11.5 and 12.0. The interior of the flocs in all cases had a  
281 lower pH value than the exterior pH with minimum pH values of 10.4, 10.7 and 11.6 at  
282 pH 11.0, 11.5 and 12.0 indicating pH shifts found within the flocs were 0.6, 0.8 and 0.4  
283 pH points at external pH values of 11.0, 11.5 and 12.0, respectively. These pH shifts

284 occurred over a short distances within the flocs with the largest pH shifts occurring  
285 over a distance of 35µm within the flocs at each pH value tested. Control profiles  
286 showed no change in pH values along profiles of similar lengths (data not shown).

## 287 **Discussion**

288 The work presented here provides the first comprehensive description of an alkaliphilic  
289 floc based microbial community isolated from a calcium dominated, anthropogenic  
290 hyperalkaline environment and demonstrates how adopting a floc based life style  
291 protects the microbial community from the ambient pH. This further emphasises the  
292 fact that it is the microenvironments which microorganisms create rather than the bulk  
293 environmental chemistry that determine microbial success in the environment (10, 11).  
294 The flocs isolated from this community were dominated by bacteria from the genera  
295 *Alishewanella* and *Dietzia*. *Alishewanella* has been previously reported to form and  
296 maintain flocs due to their ability to form biofilm and pellicles (45). Bacteria from the  
297 genus *Dietzia* have been reported in a range of hyperalkaline areas and possess the  
298 ability to degrade a range of carbohydrates and pollutants in planktonic or biofilm form  
299 (46, 47). The large proportion of both *Alishewanella* and *Dietzia* within the community  
300 suggests they play a key role in the maintenance of the floc structure (48). Within the  
301 EPS environment, these taxa were closely associated with each other, suggesting a  
302 synergistic relationship which enhanced survival at these extremes of pH. A number  
303 of the Firmicutes detected have been previously associated with alkaline conditions,  
304 the diverse metabolic capabilities of these organism would contribute to the overall  
305 metabolism capabilities of these flocs (49-51).

306 The carbohydrate fraction of the EPS was mannose rich, previous studies suggest  
307 that mannose rich biopolymers are directly involved in the sequestration of calcium

308 species (52), the results obtained within the calcium binding assay supports this  
309 conclusion. The eDNA component of the EPS although acidic in nature due to the  
310 linkage of nucleotides by the 3' - 5' phosphodiester bonds, most likely plays a role in  
311 calcium sequestering due to its thermodynamically favourable interaction with calcium  
312 ions (53). Calcium has been shown to promote bioflocculation (54), as evidenced by  
313 the production of flocs up to 240µm. Here, the sequestration of calcium appears to  
314 play two key roles; structural support and the buffering of pH. The interactions with  
315 eDNA and formation of carbonates provides structural support to the floc where SEM  
316 investigations clearly showed that EPS was bound to calcium carbonate precipitates  
317 and previous research has shown that eDNA maintains *Alishewanella* associated  
318 biofilm structure (9).

319 CLSM imaging of the flocs suggested that the basal layer of the aggregate provides a  
320 hydrophobic core to the aggregate. This increase in hydrophobicity would reduce the  
321 transport of hydroxide ions into the centre of the flocs resulting in a lower core pH.  
322 Acidic phospholipids are also associated with alkaliphilic bacterial membranes and  
323 may also buffer against the external pH (55). Proteins coated the carbohydrate  
324 fractions of the EPS, where the production of extracellular proteins has been  
325 implicated with the promotion of flocculation (56), however these proteins may also  
326 buffer the local environment through their acidic nature as previously observed in  
327 alkaliphilic bacteria (57).

328 In their cultured state, the flocs had an internal pH of 10.4 in an external environment  
329 of pH11.0. Following subculture to a fresh media at this pH, increase in biomass and  
330 further floc production was observed. Zeta potential measurements indicated that floc  
331 formation was favoured at pH values close to the internal pH of the flocs (pH 10-11).  
332 Sub culturing at pH values >pH 10.0 resulted in decreased viability of the floc

333 community, with a complete cessation of growth at pH 13.0. Survival of the flocs was  
334 observed at pH 11.5, where energy associated with the generation of biomass is likely  
335 to have been diverted into the maintenance of the internal floc pH to  $\approx$  pH 10.7. The  
336 subculturing of flocs at pH 12.0 resulted in the loss of biomass, whilst the flocs  
337 remained stable. The floc internal pH was now pH 11.6, suggesting the survival of only  
338 the most alkaliphilic microorganisms, with much of the energetic process used to  
339 maintain the internal floc pH. Although the shift in pH may only have been 0.4 – 0.8  
340 units, at these extremes of pH the differential in hydroxyl ion concentration between  
341 the internal and external floc surfaces was substantial (between 0.25 to 4.0 mM).

342 The floc community described within this study was grown using the major carbon  
343 source expected within a cementitious GDF for ILW and is the first to be evaluated for  
344 its survival and propagation within the calcium dominated hyperalkaline conditions  
345 expected within such a disposal concept. The ability of flocs to survive at pH 12.0 and  
346 persist short term at pH 13.0 suggest that microbial communities will require regions  
347 of lower pH (<pH 12.0) than that anticipated to dominate a cementitious GDF (pH 12.5  
348 for several tens of thousands of years (2)) if they are to become established. However,  
349 once established the survival of these flocs and the range of sizes observed suggest  
350 that some flocs could migrate from these regions to further colonise the facility. There  
351 is recent evidence (58) that microbial communities can reduce the ambient pH under  
352 ILW conditions suggesting that the creation of low pH regions within an cementitious  
353 GDF by microbial activity is possible. This would then provide initiation points from  
354 which floc forming communities such as those described in this paper could propagate.

355 Overall our study demonstrates that in order to survive hyper-alkaline conditions  
356 microorganisms are able to form multi species flocs composed of a complex mixture  
357 of EPS which provides protection from alkaline pH values up to pH 13.0. The formation



358 of these flocs provides the microbial communities concerned with a means of  
359 dispersion and propagation within hyper-alkaline environments such as a cementitious  
360 GDF for ILW.

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- 570
- 571

572 **Table and Figure Legends**

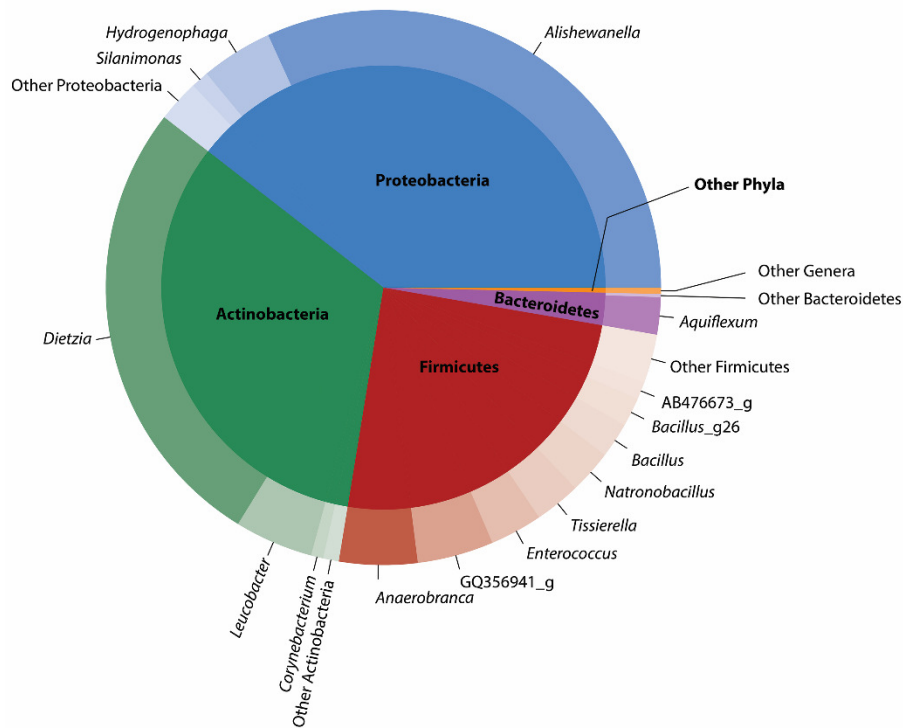
Sugar	Composition (%)
Mannose	50.53
Ribose	9.89
Ribitol	9.73
Arabinose	5.71
Rhamnose	5.07
Galactose	4.45
Trehalose	4.45
Glucuronic	3.51
Xylose	2.91
Fucose	2.00
Glucose	0.99
Galacturonic	0.76

573

574 **Table 1: Monomer composition of carbohydrates associated with floc EPS.**

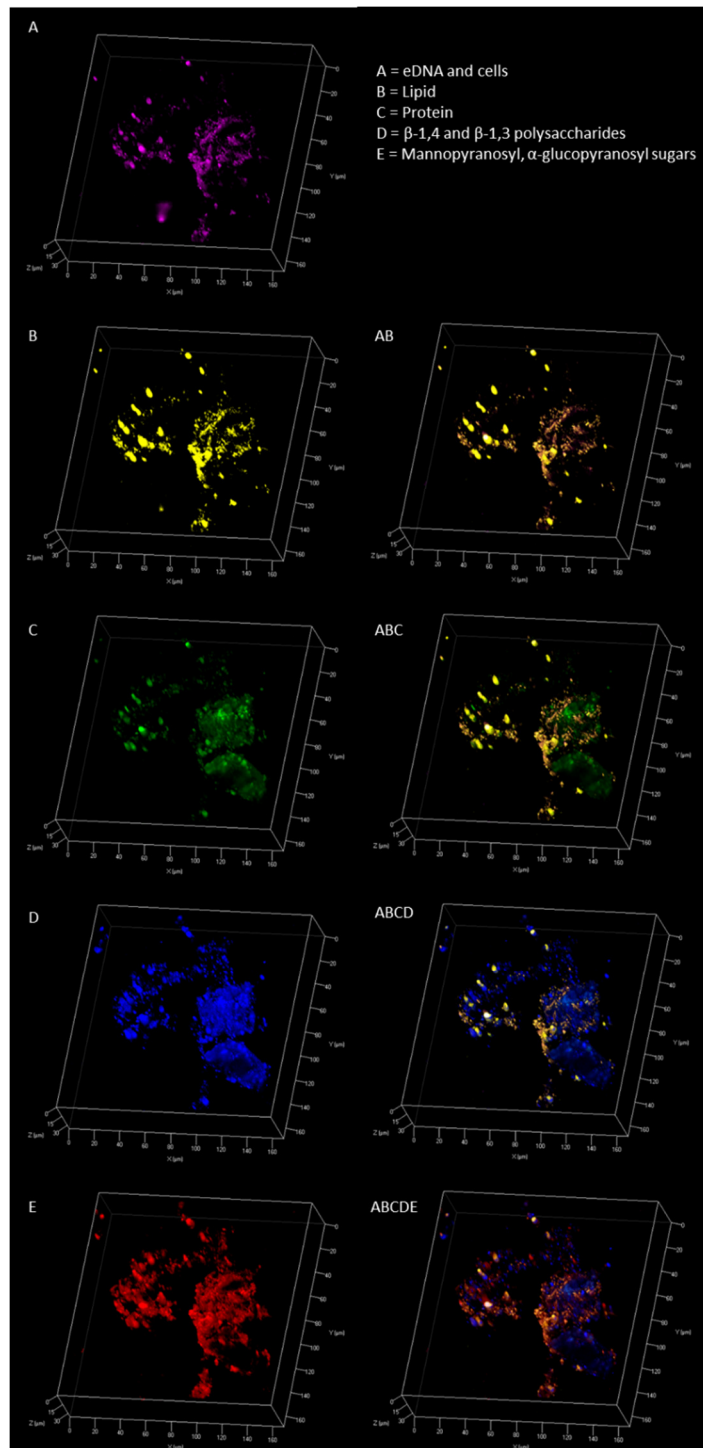
575 Mannose accounted for 50 % of the monomers present with ribose and ribitol making  
 576 up a further 20 %. A varied range of sugars were identified in smaller amounts with  
 577 uronic acids also present but only making up 4.3 % of the total monomer composition.

578



579

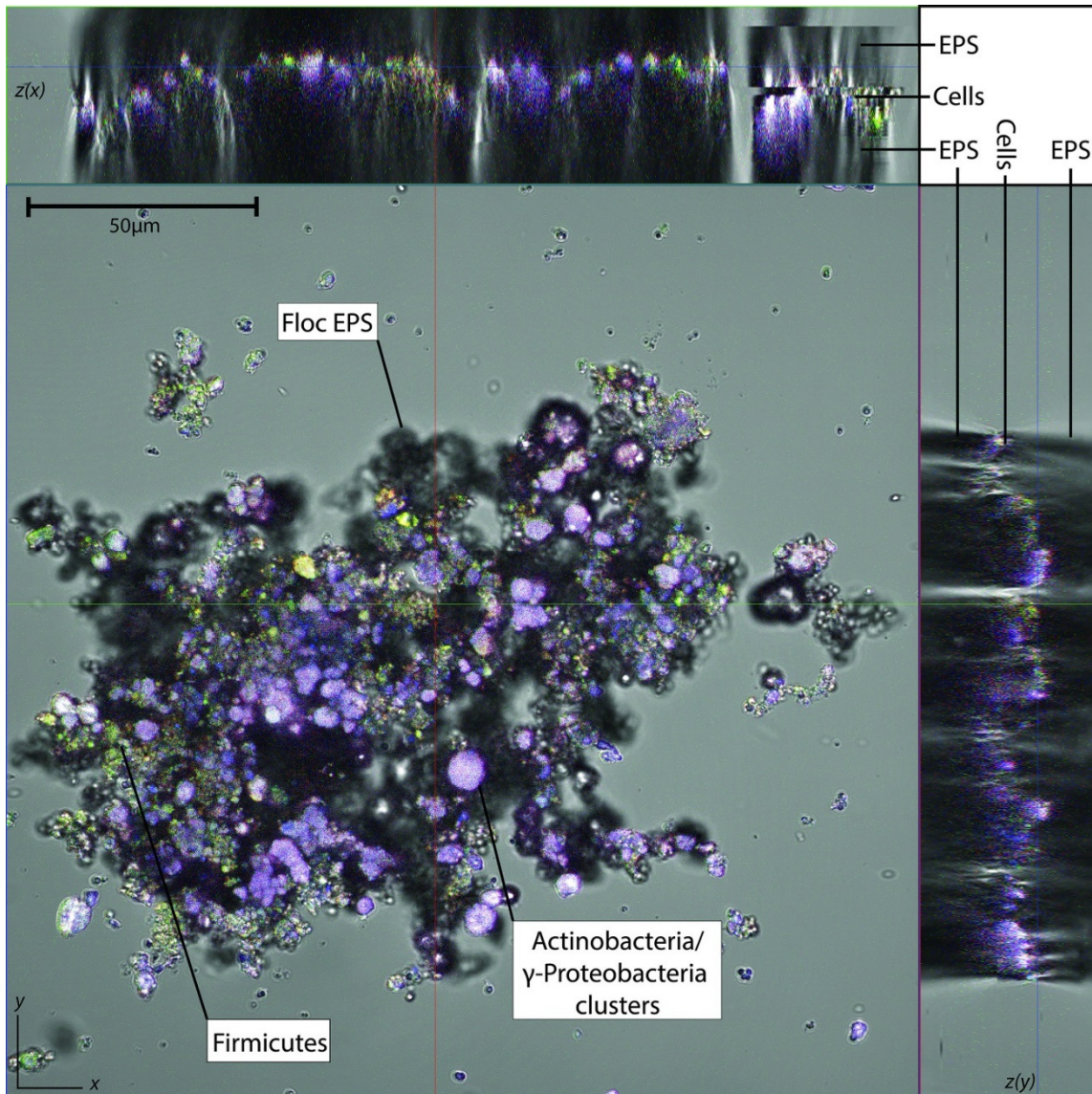
580 **Fig. 1: 16S rRNA gene profile of the microcosm community.** The microcosm  
 581 community was dominated by sequence reads associated with the Phyla  
 582 Proteobacteria (39.49 %), Actinobacteria (32.93 %), Firmicutes (24.85 %) and  
 583 Bacteroidetes (2.34 %) making up 99.6 % of the library (Inner pie chart). The  
 584 Proteobacteria and Actinobacteria were each dominated by sequence reads  
 585 associated with *Alishewanella* (31.81 %) and *Dietzia* (26.74 %) respectively (Outer  
 586 chart ring).



587

588 **Fig. 2: CLSM imaging of EPS components.** CLSM revealed the flocs to be  
 589 composed of a complex mixture of proteins, carbohydrates, lipids, eDNA and cells.  
 590 The most basal layer of the floc was composed primarily of lipids and  $\beta$ -1,4 and  $\beta$ -1,3  
 591 polysaccharides, where the most outer layers of the floc were composed of  
 592 concentrated areas of proteins, these regions were surrounded by  $\alpha$ -mannopyranosyl  
 593 and  $\alpha$ -glucopyranosyl sugars which were also closely associated with eDNA. Images

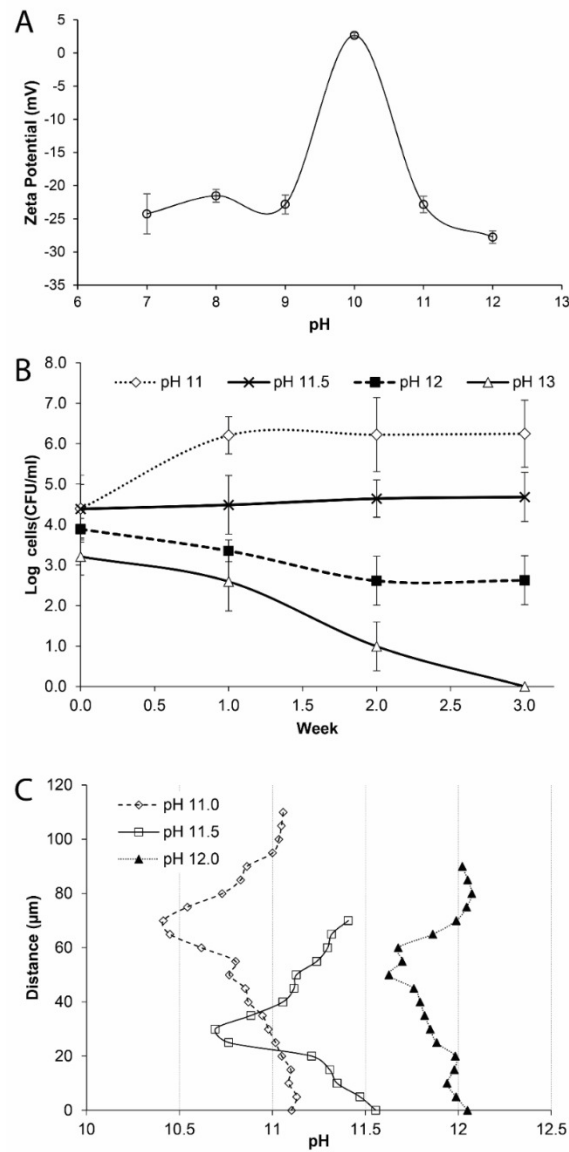
594 in the left column show the individual components of the EPS, images in the right hand  
595 column show the composite images.



596

597 **Fig 3. FISH/CLSM imaging of the bacterial floc community.** The optical Z stack  
598 shows the bacterial cells to be concentrated in the centre of the floc, surrounded by  
599 ~10 µm of EPS material (z(y), z (x) images). FISH/CLSM probing showed that the  
600 Actinobacteria (red) and γ-Proteobacteria (blue) were clustered together, with  
601 Firmicutes (green) being situated to the periphery of these clusters. The flocs ranged  
602 from 50 – 250 µm in diameter and most featured a central well defined mass with

603 looser less formed sections attached. The denser areas of the flocs showed higher  
604 numbers of cells and more concentrated areas of all EPS components.



605

606 **Figure 4: Floc response to pH.** Analysis of the zeta potential of the flocs under a  
607 range of pH values showed consistent stable potentials of -20 to -30 mV between pH  
608 7-9 and 10-12 (Fig. 4A). Following exposure to elevated pH values flocs demonstrated  
609 the ability to grow at pH 11.0, survive for >3 weeks at pH 12.0 and 2 weeks at pH 13.0  
610 (Fig. 4B). Profiling of the pH indicated that the interior of the flocs had a lower pH value  
611 than the exterior pH when exposed to pH 11.0, 11.5 and 12.0 indicating pH shifts of

612 0.6, 0.8 and 0.4 pH units. These pH shifts occurred over short distances with the  
613 largest pH shifts occurring over a distance of 35 $\mu$ m (Fig. 4C).

614

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617