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

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

Abstract

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

Importance

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

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

Introduction

One of the concepts for the disposal of the UK's intermediate level radioactive waste (ILW) inventory is a geological disposal facility (GDF) employing a cementitious backfill (1, 2). It is anticipated that post closure, groundwater ingress into such a facility will result in a saturated, anaerobic, calcium rich, hyper-alkaline environment that will aid radionuclide retention through sorption and the formation of insoluble complexes (3, 4). These conditions will also result in the alkaline hydrolysis of the cellulose component of the ILW producing a range of small molecular weight organic compounds collectively known as cellulose degradation products (CDP) (5). The biodegradation of CDP has received considerable attention in recent years with a number of authors reporting alkaliphilic degradation under a wide range of growth conditions (6, 7) including alkaliphilic methanogenic consortia operating at pH 11.0 (8). In some cases these alkaliphilic communities have been shown to form flocs where the bacteria are encased in a matrix of extracellular polymeric substances (EPS) (9). The ability of freshwater and marine microorganisms to aggregate into a sustainable microenvironment is documented (10, 11), however the added selective pressure of 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 *Pseudomona*s 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

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

Materials and Methods

Community composition and flocculate concentration

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

111 extension step of 72 ℃ 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^{tm} 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 β-1,4 and

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

EPS extraction, Purification and Characterisation

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

In order to purify the extracted EPS, dialysis was carried out against ultrapure water for 72 hours with the water changed every 24 hours. The protein and carbohydrate fractions were then isolated from the dialysed EPS. Protein was precipitated via treatment with trichloroacetic acid and the carbohydrate fraction precipitated via ethanol treatment as outlined in Marshall, et al. (40). Monomer composition was 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^{2+} (as $CaCl_2$) were prepared to concentrations of 0.1,
168 0.25, 0.5 and 1 g. g^{-1} 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_3.

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

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

Floc survival

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

Accession number

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

Results

Floc Characterisation

Sequencing of the microbial 16S rRNA gene returned 18854 reads via Illumina MiSeq technology. The microcosm community was dominated by sequence reads associated with the Phyla Proteobacteria (39.49 %), Actinobacteria (32.93 %), Firmicutes (24.85 %) and Bacteroidetes (2.34 %) making up 99.6 % of the library (Fig. 1). The Proteobacteria and Actinobacteria were each dominated by sequence reads associated with *Alishewanella* (31.81 %) and *Dietzia* (26.74 %) respectively. Reads associated with *Hydrogenophaga* and *Silanimonas* were also detected within the Proteobacteria group, where *Leucobacter* and *Corynebacterium* represented >1 % of the total reads within the Actinobacteria. The Firmicutes detected were not dominated by a particular species *per se*, where *Anaerobranca* (4.55 %), *GQ356941_g* (4.45 %), *Tissierella* (2.67 %), *Natronobacillus* (2.55 %), *Bacillus* (2.04 %) and *Bacillus_g26* (1.68 %) were all represented within the sequence reads. The Bacteroidetes were almost entirely composed of the sequence reads associated with the genus *Aquiflexum* (2.16 % of the total reads).

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

were composed of concentrated areas of proteins, these regions were surrounded by α-mannopyranosyl and α-glucopyranosyl sugars which were also closely associated with eDNA. These regions of pyranosyl sugars and eDNA surrounded the crystalline precipitates observed under SEM. Bacterial cells were in the centre of the floc, with ~10 µm of EPS material surrounding these cells (Fig. S3). FISH/CLSM probing (Fig. 3) showed that the Actinobacteria (red) and γ-Proteobacteria (blue) were clustered together, with Firmicutes (green) being situated amongst the periphery of these clusters within the centre of the floc. The flocs ranged from 50 – 250 µm in diameter and most featured a central well defined mass with looser less formed sections attached. The denser areas of the flocs showed higher numbers of cells and more concentrated areas of all EPS components.

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

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

Floc profiling and response to alkaline conditions.

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

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

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

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

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

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

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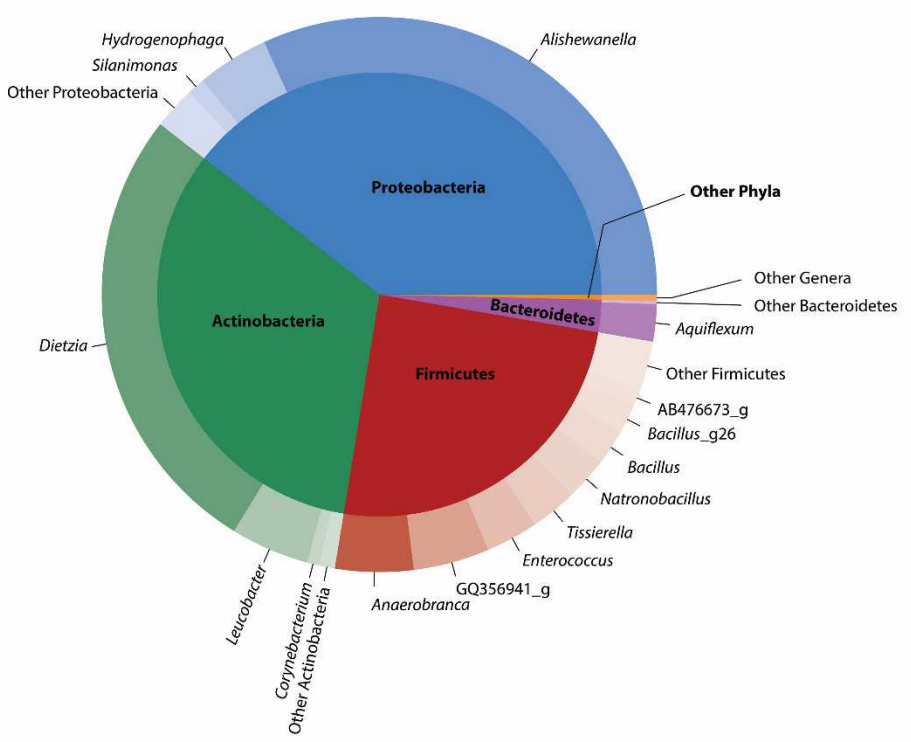
572 **Table and Figure Legends**

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

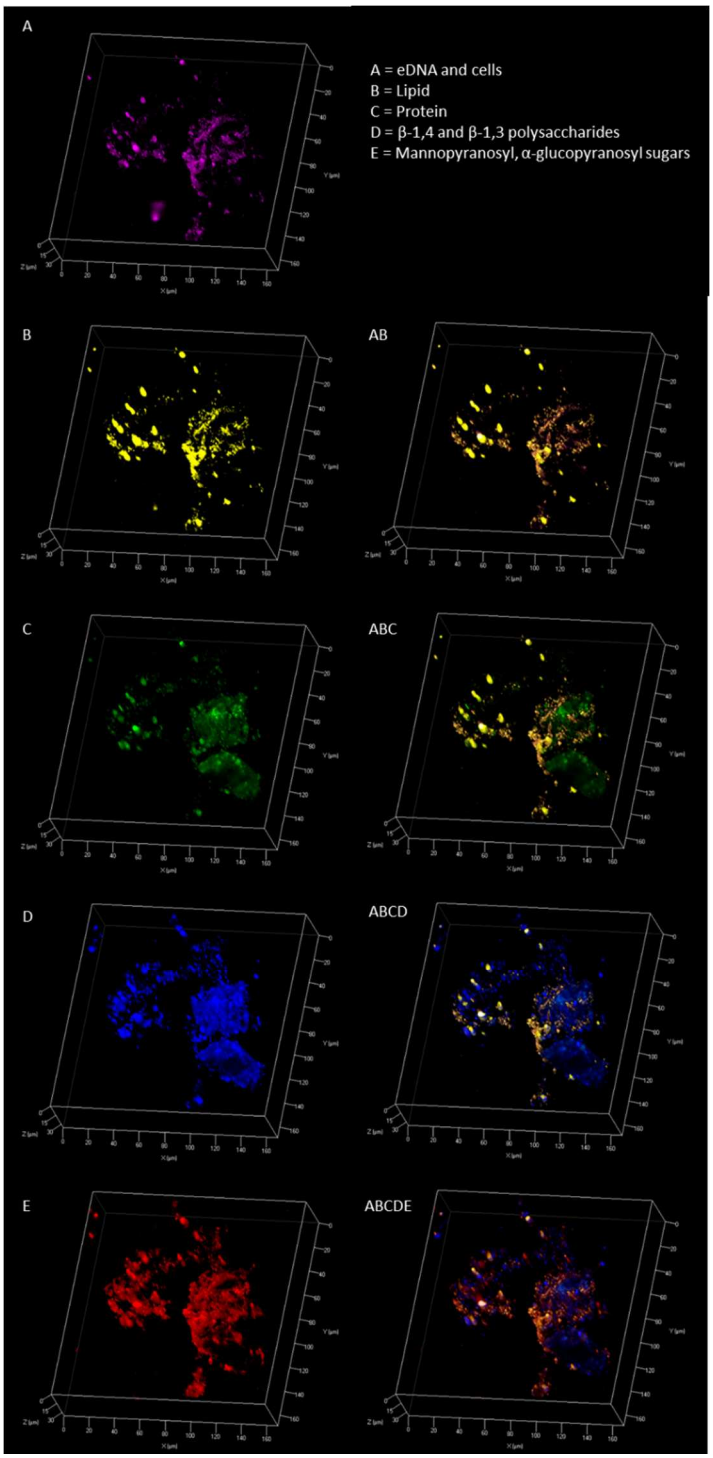
574 **Table 1: Monomer composition of carbohydrates associated with floc EPS.**
575 Mannose accounted for 50 % of the monomers present with ribose and ribitol making
576 up a further 20 %. A varied range of sugars were identified in smaller amounts with
577 uronic acids also present but only making up 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 β-1,4 and β-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 α-mannopyranosyl
593 and α-glucopyranosyl sugars which were also closely associated with eDNA. Images

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

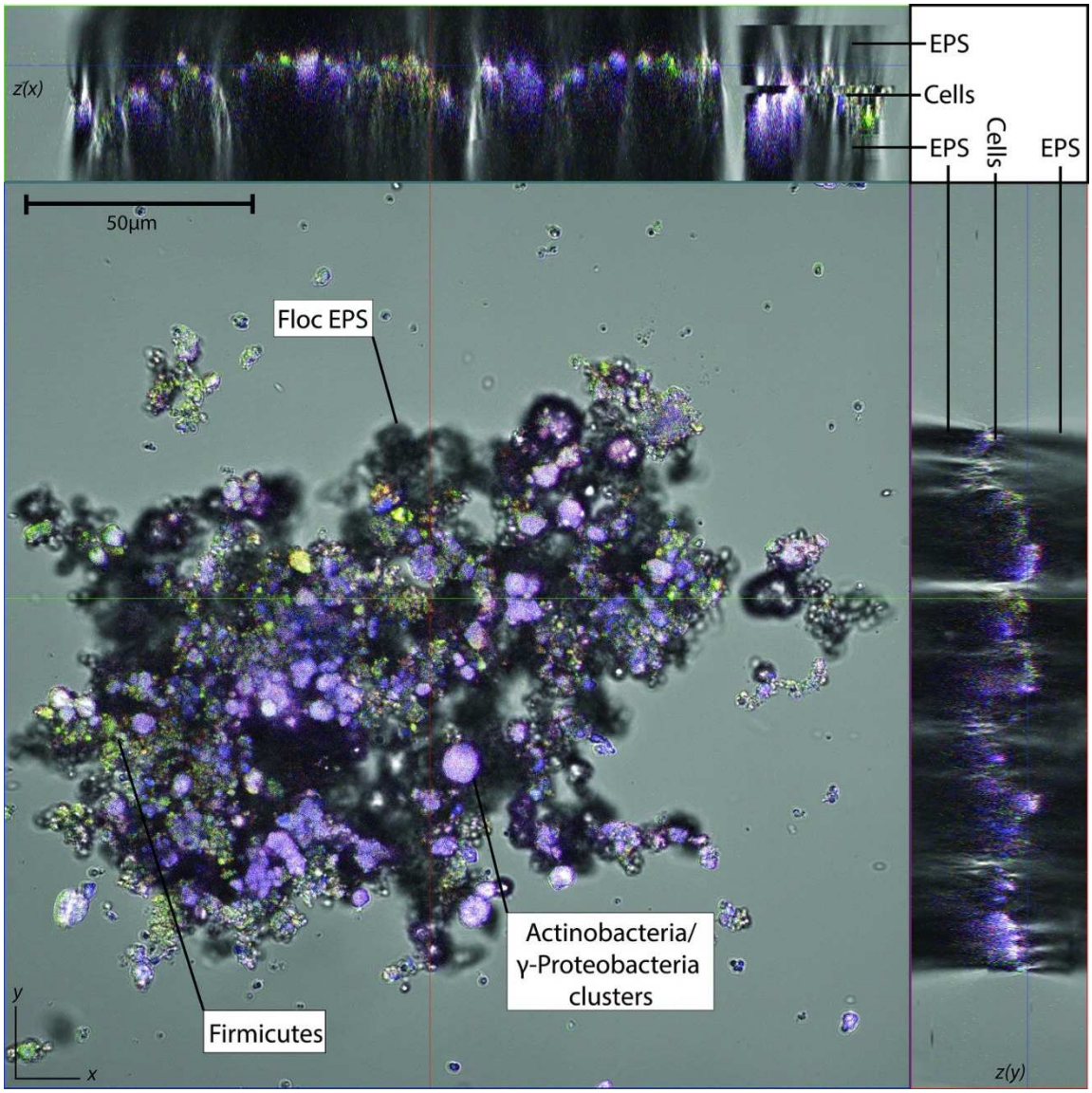
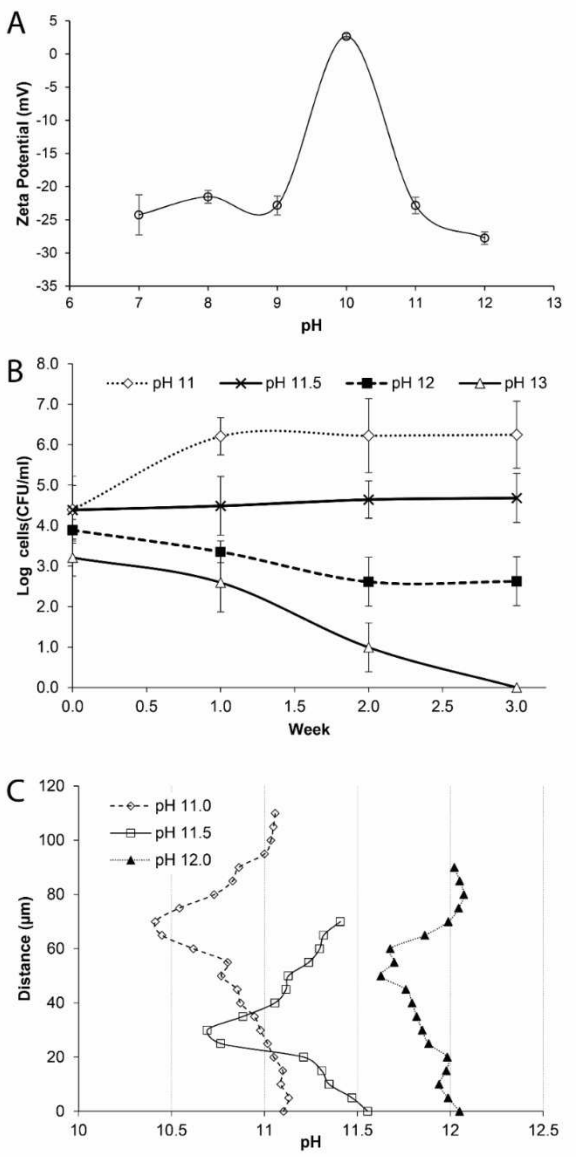


Fig 3. FISH/CLSM imaging of the bacterial floc community. The optical Z stack shows the bacterial cells to be concentrated in the centre of the floc, surrounded by ~10 µm of EPS material (z(y), z (x) images). FISH/CLSM probing showed that the Actinobacteria (red) and γ-Proteobacteria (blue) were clustered together, with Firmicutes (green) being situated to the periphery of these clusters. The flocs ranged 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 35µm (Fig. 4C).

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