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

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

Information regarding the ability of flocs to attenuate pH within alkaline environments is currently absent from the literature. Consequently, the aim of this study was to determine the ability of alkaliphilic flocs (9) to attenuate environmental pH values relevant to a cementitious GDF. The isolated flocs were also characterised in terms of 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 subsampled 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 °C 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 25 mL 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’TACNVGGGATCTAATCC’3) for both bacteria and archaea (26, 27) with the following overhangs 5’ TCGTCGGCAGCGTGTGTAAGAGACAG’3 and 5’ GTCTCGTGGGCTCGAAGAGACAG’3, respectively. PCR reaction mixture contained 20 ng of purified DNA solution, 0.5 µL 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 °C for 1 minute, and then cycled 35 times through three steps: denaturing (94 °C, 15 s), annealing (60 °C, 15 s), primer extension (72 °C, 10 s). This was followed by a final
extension step of 72 °C for 5 minutes. 16S rRNA gene microbial community analysis was carried out via a MiSeq platform (Illumina, USA) at 250 bp paired ends with chimera detection and removal performed via the UNCHIME algorithm in the Mothur suite (28) (Chunlab, South Korea). Assignment of OTU’s was performed using a CD-HIT clustering method with a 95 % cut-off value with taxonomic assignment performed against the EZtaxon database (29).

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

**Morphology**

The overall morphology of the flocs was investigated using a Quanta FEG 250 scanning electron microscope (SEM) and energy-dispersive X-ray spectroscopy (EDX) used for analysis of sites of interest. Samples were fixed overnight in 4 % paraformaldehyde (Fisher, UK) then dehydrated using a serial ethanol dilution of 25, 50, 75 and 100 % for 2 min per step. Samples were then dried onto a metal disc and sputter coated via a gold palladium plasma (CA7625 Polaron, Quorum Technologies Ltd, UK). The structural morphology of the flocculates was investigated via confocal laser scanning microscopy (CLSM) at the Bio imaging centre of Leeds University using a Zeiss LSM880 inverted confocal microscope with image analysis performed using Zen 2.1 (Zeiss Microscopy). Flocs were fixed in 4% Paraformaldehyde (Fisher, UK) overnight and then stained using the following compounds in accordance with 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
described previously (41) with the exception that sample was eluted with aqueous 0.01 M NaOH for 20 minutes followed by 83 % 10 mM NaOH: 17 % 150 mM NaOH:1 M sodium acetate for 25 minutes for simultaneous determination of monomers and uronic acids. Dry weight and inorganic content of the flocs were determined by methods outlined in BS ISO 6496:1999 (42).

**EPS calcium binding assay**

Aqueous suspensions of EPS:Ca$^{2+}$ (as CaCl$_2$) were prepared to concentrations of 0.1, 0.25, 0.5 and 1 g. g$^{-1}$ dry weight and incubated at room temperature for 15 minutes. The calcium content of the samples was then quantified using a Metrohm 850 Professional IC (Metrohm, Cheshire, UK) with pulsed amperometric detection, employing a Metrohm C4 Column (250 mm x 4.6 mm) with a mobile phase of 4.6 mM phosphoric acid. All reactions were carried out under nitrogen to avoid interference from atmospheric carbon dioxide and EPS bound calcium determined via comparison with EPS-free incubations. Total bound calcium was determined using atomic absorbance spectroscopy (Agilent 200 series AA, Agilent, UK) following digestion of EPS (1 mg) in 10 ml of 0.1 % KCl: 1 % HNO$_3$.

**Floc profiling**

pH profiles of the flocs were undertaken with a micromanipulator and stand (Unisense, Denmark) using a 10 µm diameter pH electrode with an external reference (Unisense, Denmark) connected to a single channel pH/redox meter supplied by the probe manufacturer (Unisense, Denmark). The probe was calibrated against pH 4.0, pH 7.0 and pH 10.0 standards (Fisher, UK) and tested against pH 11.0 and pH 12.0 solutions made using NAOH. Profiles were taken through the flocs at pH 11.0, 11.5 and 12.0. In order to generate the pH profile of the flocs, microcosm fluid was incubated for 1 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 gene sequence 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 $2.0 \times 10^5$ 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. S3) 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 $2.65 \pm 0.36$ 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 $2.5 \times 10^4$ CFU/ml to $1.8 \times 10^6$ CFU/ml (Fig. 4B). When subjected to a pH of 11.5, the flocs showed only a small increase in cell concentration from $2.5 \times 10^4$ CFU/ml to $4.8 \times 10^4$ 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 $7.7 \times 10^3$ CFU/ml to $4.1 \times 10^2$ 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
occurred over a short distances within the flocs with the largest pH shifts occurring over a distance of 35µm within the flocs at each pH value tested. Control profiles showed no change in pH values along profiles of similar lengths (data not shown).

Discussion

The work presented here provides the first comprehensive description of an alkaliphilic floc based microbial community isolated from a calcium dominated, anthropogenic hyperalkaline environment and demonstrates how adopting a floc based life style protects the microbial community from the ambient pH. This further emphasises the fact that it is the microenvironments which microorganisms create rather than the bulk environmental chemistry that determine microbial success in the environment (10, 11).

The flocs isolated from this community were dominated by bacteria from the genera *Alishewanella* and *Dietzia*. *Alishewanella* has been previously reported to form and maintain flocs due to their ability to form biofilm and pellicles (45). Bacteria from the genus *Dietzia* have been reported in a range of hyperalkaline areas and possess the ability to degrade a range of carbohydrates and pollutants in planktonic or biofilm form (46, 47). The large proportion of both *Alishewanella* and *Dietzia* within the community suggests they play a key role in the maintenance of the floc structure (48). Within the EPS environment, these taxa were closely associated with each other, suggesting a synergistic relationship which enhanced survival at these extremes of pH. A number of the Firmicutes detected have been previously associated with alkaline conditions, the diverse metabolic capabilities of these organism would contribute to the overall metabolism capabilities of these flocs (49-51).

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

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

In their cultured state, the flocs had an internal pH of 10.4 in an external environment of pH11.0. Following subculture to a fresh media at this pH, increase in biomass and further floc production was observed. Zeta potential measurements indicated that floc formation was favoured at pH values close to the internal pH of the flocs (pH 10-11). 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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Table 1: Monomer composition of carbohydrates associated with floc EPS.

Mannose accounted for 50% of the monomers present 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 4.3% of the total monomer composition.
**Fig. 1: 16S rRNA gene profile of the microcosm community.** 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 (Inner pie chart). The Proteobacteria and Actinobacteria were each dominated by sequence reads associated with *Alishewanella* (31.81 %) and *Dietzia* (26.74 %) respectively (Outer chart ring).
**Fig. 2: CLSM imaging of EPS components.** CLSM revealed the flocs to be composed of a complex mixture of proteins, carbohydrates, lipids, eDNA and cells. The most basal layer of the floc was composed primarily of lipids and $\beta$-1,4 and $\beta$-1,3 polysaccharides, where the most outer layers of the floc were composed of concentrated areas of proteins, these regions were surrounded by $\alpha$-mannopyranosyl and $\alpha$-glucopyranosyl sugars which were also closely associated with eDNA. 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...
looser less formed sections attached. The denser areas of the flocs showed higher numbers of cells and more concentrated areas of all EPS components.

**Figure 4: Floc response to pH.** Analysis of the zeta potential of the flocs under a range of pH values showed consistent stable potentials of -20 to -30 mV between pH 7-9 and 10-12 (Fig. 4A). Following exposure to elevated pH values flocs demonstrated the ability to grow at pH 11.0, survive for >3 weeks at pH 12.0 and 2 weeks at pH 13.0 (Fig. 4B). Profiling of the pH indicated that the interior of the flocs had a lower pH value than the exterior pH when exposed to pH 11.0, 11.5 and 12.0 indicating pH shifts of
0.6, 0.8 and 0.4 pH units. These pH shifts occurred over short distances with the largest pH shifts occurring over a distance of 35µm (Fig. 4C).