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The enrichment of an alkaliphilic biofilm consortia capable of the anaerobic degradation of isosaccharinic acid from cellulosic materials incubated within an anthropogenic, hyperalkaline environment.

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Abstract

Anthropogenic hyper-alkaline sites provide an environment that is analogous to proposed cementitious geological disposal facilities (GDF) for radioactive waste. Under anoxic, alkaline conditions cellulosic wastes will hydrolyse to a range of cellulose degradation products (CDP) dominated by isosaccharinic acids (ISA). In order to investigate the potential for microbial activity in a cementitious GDF, cellulose samples were incubated in the alkaline (~pH 12), anaerobic zone of a lime kiln waste site. Following retrieval, these samples had undergone partial alkaline hydrolysis and were colonised by a Clostridia dominated biofilm community, where hydrogenotrophic, alkaliphilic methanogens were also present. When these samples were used to establish an alkaline CDP fed microcosm, the community shifted away from Clostridia, methanogens became undetectable and a flocculate

community dominated by *Alishewanella sp.* established. These flocs were composed of bacteria embedded in polysaccharides and protein stabilised by extracellular DNA. This community was able to degrade all forms of ISA with >60% of the carbon flow being channelled into extracellular polymeric substance (EPS) production. This study demonstrated that alkaliphilic microbial communities can degrade the CDP associated with some radioactive waste disposal concepts at pH 11. These communities divert significant amounts of degradable carbon to EPS formation, suggesting that EPS has a central role in the protection of these communities from hyper-alkaline conditions.

Introduction

The UK's national nuclear waste legacy contains approximately 290 000 m^3 (N.D.A., 2013) of intermediate level radioactive wastes (ILW) which includes an estimated (~2000 tonnes) (N.D.A., 2010a) of cellulosic materials (wood, paper and cloth) (Humphreys et al., 2010a). One of the proposed strategies for the disposal of this ILW is a deep geological disposal facility (GDF) (N.D.A., 2010a) employing a multi-barrier system which is likely to include a cement based backfill (Chapman and Hooper, 2012). Upon the closure of such a facility, groundwater ingress combined with corrosion processes will result in the development of a chemically reducing high pH (pH 12.5) environment (Libert et al., 2011, N.D.A., 2010b). Under these conditions the cellulose portion of ILW is expected to undergo chemical, alkaline hydrolysis to form a variety of cellulose degradation products (CDP) (Knill and Kennedy, 2003, Humphreys et al., 2010a).

CDP are comprised of the alpha and beta diastereomers of isosaccharinic acid (ISA), alongside other small chain organic compounds including acetic acid (Van Loon and Glaus, 1997, Motellier et al., 1998, Knill and Kennedy, 2003). The diastereomers of ISA are of significance when considering the performance of a GDF as they possess the ability to enhance the mobility of a range of radionuclides, including nickel, thorium, plutonium and uranium through complexation (Greenfield et al., 1991, Allard and Ekberg, 2006, Warwick et al., 2003). In addition, the hemicellulose fraction of cellulosic waste components will also undergo anoxic, alkaline hydrolysis to form an additional 5-carbon form of ISA, known as xyloisosaccharinic acid (X-ISA) (Almond et al., 2012). Recent work by Randall et al (2013) suggests that X-ISA does not have the same complexation properties as the alpha and beta

forms of ISA but could, however, represent a source of organic carbon available for microbial metabolism.

Although the harsh geochemical conditions of an ILW-GDF place limitations upon microbial life it may not prevent microbes from colonising a facility. An investigation of an anthropogenic analogue of an ILW-GDF at a hyperalkaline contaminated site in Buxton, UK where ISA is generated in-situ (Rout et al., 2015) has revealed a microbially active site despite porewaters of up to pH 13 (Burke et al., 2012). The range of microbes present within the background sediments is diverse, with organisms within the Phyla Bacteroidetes, Proteobacteria and Firmicutes consistently making up large proportions of the sediment taxonomic profiles (Burke et al., 2012, Williamson et al., 2013, Bassil et al., 2014). The subsequent culturing of these sediments has shown that these communities are able to utilise the alpha form of ISA as a substrate under aerobic, nitrate, iron reducing (Bassil et al., 2014) and methanogenic conditions (Rout et al., 2015). Sulphate reduction appears to be inhibited at pH >10 (Bassil et al., 2014), however, the utilisation of ISA under sulphate reducing conditions has been observed at neutral pH indicating that this limitation is thermodynamic (Rizoulis et al., 2012, Rout et al., 2014). The heterogeneity of ILW and its compaction in grout may limit the availability of higher energy terminal electron acceptors such as nitrate and ferric iron, with the inundating ground water also depleted in these electron acceptors due to its passage through the microbial thermodynamic ladder (Bethke et al., 2011). Fermentation processes and subsequent methanogenesis therefore represent the most likely conditions to dominate an ILW-GDF

Microbes in nature can be found in biofilms of mixed syntrophic communities, with microbial biofilms found in a diverse range of environments (Summons et al., 2015, Urbieta et al., 2015). The secretion of extracellular polymeric substance (EPS) such as polysaccharides, proteins, lipids and nucleic acids during biofilm formation assist in bacterial

survival and propagation (Flemming and Wingender, 2010) and confer an increased resistance to environmental stresses such as pH and temperature fluctuations, desiccation and UV radiation (Ordoñez et al., 2009, Gorlenko et al., 2004, Rodrigues et al., 2006, Jones et al., 1994, Conrad et al., 2014). When considering the colonisation of an ILW-GDF, the ability of microbes to migrate and adhere to niche areas such as ungrouted surfaces may allow for both microbial survival and growth under extreme alkaline conditions (Humphreys et al., 2010b). The aim of this work was to culture, *in situ*, a biofilm forming consortium capable of colonising cellulosic materials under anoxic, hyper-alkaline conditions and to determine its ability to degrade CDP, which represent the primary organic carbon source within an ILW-GDF.

Methods

Cellulose cotton preparation

In order to prepare the cellulose cotton for incubation, raw woven cotton fabric (Greige) was treated with NaOH to saponify the natural waxes along with an alkali stable phosphate ester detergent to emulsify the suspended impurities. Further treatment with NaOH and phosphonate stabilised H_2O_2 was carried out to bleach the fabric. The cotton was then rinsed, neutralised under acetic acid before finally being rinsed, dried and autoclaved at 121ºC prior to use.

Analogue site investigation

During May 2014 a 2.2cm Ø borehole was hand drilled to an approximate depth of 0.5m into an area inundated with alkaline leachate at Brook Bottom, Harpur Hill, Buxton, UK (Figure 1). An inert plastic liner with a perforated lower section was placed into the borehole. Approximately 5g of sterile treated cellulose cotton was loaded into a nylon mesh bag and

placed at the bottom of the borehole. After a period of 3 months the cotton was recovered along with sediment and porewater samples from the immediate vicinity of the sample. *In situ* pH and Eh values were determined prior to sample recovery using a handheld portable pH meter with calibrated electrodes and an InLab Redox Micro probe (Mettler Toledo, UK) tested in accordance with BS ISO 11271:2002 (B.S.I, 2002). All recovered materials were sealed in airtight containers along with anaerobic gas packs (Anaerogen, Oxoid, UK) for transport. Sediment and porewater samples were stored at -20˚C until analysis and cotton not used for immediate studies was stored at -20˚C in a solution of 140mL of ultrapure water, 10mL of 1M TRIS-HCl (pH7.5) and 250ml of 96% ethanol after an overnight fixation step in 4% paraformaldehyde in phosphate buffered saline.

Porewater, cotton and sediment ISA content was determined as previously described by Rout et al (2015, 2014) against ISA standards in the alpha, beta and xylo conformations (Almond et al., 2012, Shaw et al., 2012). C1-8 volatile fatty acid (VFA) content of both the sediment and cotton was determined using a standard extraction method outlined in Eaton et al (2005) and analysed via GC-FID as described by Rout et al (2014).

Microscopy

Scanning electron microscopy was undertaken using a JEOL JSM-6060LV microscope (JEOL, USA). Samples were dehydrated using a serial ethanol dilution of 25 %, 50 %, 75% and 100 % for 2 minutes per step then sputter coated via a gold palladium plasma (CA7625 Polaron, Quorum Technologies Ltd, UK). Fluorescence microscopy was carried out using an Olympus BX41 laboratory microscope (Olympus, USA). Live dead staining was carried out using the BAC light Live/dead kit (Life technologies, UK), fluorescein isothiocyanate (FITC) (Sigma-Aldrich, UK) staining was used for protein and visualisation of individual bacteria cells and the polysaccharide components was achieved using ethidium

bromide and Calcofluor White (Sigma-Aldrich, UK) staining, respectively. For DNase digestion, microcosm fluid (1mL) was centrifuged at 10,000 x*g* for 1 minute and re-suspended in ultrapure water (1mL). A 10 fold dilution of this was then subjected to digestion by DNase using a DNase 1 kit (Sigma-Aldrich, UK).

Microcosm

In order to investigate ISA degradation, approximately 1g of colonised cotton was washed with 10mL N_2 purged sterile PBS under an inert environment to remove any transient microorganisms. The washed cotton was then added to a continuously stirred microcosm containing 175mL of pre-reduced 10% CDP and 90% mineral media (B.S.I, 2005) at pH 11 and 20˚C that had been purged with nitrogen and maintained with a nitrogen headspace to ensure anoxic conditions. CDP was produced as previously described by Rout et al (2014). The microcosm was brought up to a final volume of 250mL by feeding 25mL of CDP every 2 weeks with the pH adjusted using 4M NaOH every 7 days. After this period the cotton was removed and the microcosm was switched to a 10% waste/feed cycle with CDP every 2 weeks. The microcosm was maintained with a nitrogen atmosphere and all reagents were reduced prior to use with disodium sulfide nonahydrate (Sigma-Aldrich, UK) and sodium dithionite (Fisher, UK) as per BS ISO 14853:2005 (B.S.I, 2005) and stored under nitrogen. Resazurin redox indicator (Fisher, UK) present within the mineral media provided an indication of anaerobic conditions within the microcosm and all manipulations of the microcosm were carried out under a stream of nitrogen to maintain anoxic conditions. Sufficient time (50 weeks) was allowed for the microcosm chemistry to stabilise and also to allow for the washout of any transient microorganisms. The microcosm was sampled every 2 days over 2 feed/waste cycles to determine the ISA and VFA content. For each sample period microcosm fluid (1mL) was taken, centrifuged at 10,000 x*g* for 1 minute and the supernatant filter sterilised using a 0.45µm syringe filter (Sartorius, UK) and stored at -20˚C prior to

analysis. The gas headspace (75ml starting volume) was sampled every 2 days with the composition determined via gas chromatography using Agilent 6850 gas chromatograph (Hewlett Packard, UK) fitted with a HP-Plot/Q+ PT column and thermal conductivity detection (TCD). Headspace gas (100µL) was removed using a lockable gas syringe from the microcosm and passed through the column under the following conditions: initial temperature of 60°C for 2 minutes, followed by an increase to 120°C at a ramp rate of 30°C min^{-1} with a detector temperature of 250°C. Gas headspace pressure was measured using a digital manometer (TPI, UK) before gas sample periods.

Microcosm fluid (1mL) containing the suspended flocs was taken on days 0, 7 and 14 and spun at 10,000 x*g* for 1 minute for ATP/biomass detection using a 3M™ Clean-Trace™ Biomass Detection Kit and Luminometer employing a modified method (3M, UK). The pellet was washed once with pH 4 PBS and reconstituted in pH 7 PBS to remove interference from excess alkalinity and salts. Following analysis, CFU mL^{-1} and dry weight biomass (DW) were calculated against a standard curve of *E.coli* K12 concentrations. In addition, a set of control microcosms amended with 50µg mL^{-1} chloramphenicol were prepared and were sampled as per the test microcosms. The controls served as an abiotic comparison for the elimination of sorption and precipitation events. All data was processed in Microsoft Excel with calculated means and associated standard error shown in all relevant results. Carbon flow calculations were undertaken using balanced equations 1 and 2 for the fermentation of ISA to acetate and hydrogen.

$$ISA + 4\,H_20 \rightarrow 2\,CH_3COO^- + 2\,HCO_3^- + 4H_2 + 4H^+ \quad \textbf{(1)}$$

$$XISA + 3.33\,H_20 \rightarrow 1.67\,CH_3COO^- + 1.67\,HCO_3^- + 3.33\,H_2 + 3.33\,H^+ \quad \textbf{(2)}$$

Preparation of 16S rDNA clone libraries

Total genomic DNA was extracted from the cotton and microcosm using a Powersoil DNA extraction kit (Mo-BIO, Carlsbad, US) with the following modifications. For the cotton approximately 0.25g was washed with pH 7 PBS and loaded into a glass bead tube with 100µL β- marcaptoethanol and the bead beating step extended to 1 hour in order to overcome dampening effects introduced by the material. For genomic DNA extraction from the microcosm, 25mL of fluid was centrifuged at 5000 xg for 15min and the pellet re-suspended in 25mL pH 4 PBS. The sample was then centrifuged again at 5000 x*g* for 15 minutes and re-suspended in 2mL of pH 7 PBS. 1mL of the concentrated sample was transferred to a 1.5mL tube and centrifuged again at 10,000 x*g* for 1 minute, after which the supernatant was removed and the cell pellet re-suspended in the reaction fluid provided in the glass bead tubes of the Powersoil kit. The resulting mixture was then transferred back to a glass bead tube and bead beaten with 100µL β-marcaptoethanol for an increased time of 20 minutes to overcome clogging due to the EPS and then run as per the supplier's instruction. These modifications were found to increase the yield and purity of DNA obtained from both samples by removing excess salts, inhibiting nucleases and neutralising the samples.

Purified genomic DNA was quantified and quality checked by spectroscopic methods and used as a template to amplify the 16s rRNA gene. A ~1500bp fragment of the Eubacterial 16S rRNA gene was amplified using broad specificity primers pA and pH (Edwards et al., 1989) and a ~750bp fragment of the archaeal 16S rRNA gene was amplified using primers Ar and Af (Gantner et al., 2011). PCR reactions were carried out using BIOMIX red master mix (BIOLINE, UK) with PCR fragments purified via a Qiaquick PCR purification kit (Qiagen, UK) and visualised using a 1.0% agarose TAE gel with SYBR® Safe staining (Life technologies, UK). PCR products were ligated into the standard cloning vector PGEM-T easy (Promega, US) and transformed into *E.coli* JM109 competent cells (Promega, US). Transformed cells were grown on Luria Bertani (LB) agar containing 100µg mL^{-1} ampicillin

overlaid with 40µL of 100mM IPTG and 40µL of 40mg mL^{-1} X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in N'N dimethylformamide for blue-white colour screening. Insert containing colonies were transferred to 96 well plates containing LB agar with 150mg mL^{-1} ampicillin and sequenced using Sanger sequencing technology (GATC Biotech, Germany). Inserts were amplified using a T7 forward primer and the resulting 16S rRNA gene sequences aligned using the multiple sequence alignment package MUSCLE (www.ebi.ac.uk/Tools/services/web/toolform.ebi?tool=muscle) and chimera checked using the UCHIME component of the Mothur suite, where chimeric sequences were omitted from the analysis (Schloss et al., 2009). Sequences were analysed against the NCBI database using Basic Local Alignment Search Tool (MegaBLAST) utilising the 16S ribosomal RNA sequences for Bacteria and Archaea (Altschul et al., 1997). Phylogenetic families were then determined at a 95% confidence level by comparison with the Ribosomal Database Project (Cole et al., 2009).

Nucleotide accession numbers

The 16S rRNA sequence data from the colonised cotton has been submitted to GenBank under accession numbers KP263977 - KP264111 and the microcosm sequences under the numbers KP728118 - KP728176.

Results

Chemical and physiological cotton analysis

The pH in the vicinity of the cotton samples was between pH 11.5 and 12 and redox measurements were found to be negative in both the associated sediment (-77mV) and porewaters (-66mV). Both the alpha and beta forms of ISA were extracted from the cotton (>0.5mg (g dry wt) $^{-1}$), the sediment (>0.5mg (g dry wt) $^{-1}$)) and porewater (7.64mg L^{-1} alpha,

222 6.82mg L^{-1} beta) (Table 1) indicating in-situ alkaline cellulose hydrolysis (Knill and
223 Kennedy, 2003).

224 The surfaces of the colonised cotton showed areas of EPS indicative of biofilm
225 formation and surface associated mineral precipitates (Figure 2B and Figure S1) with
226 individual viable bacterial cells being visible on some fibres (Figure 2C and D).

227 16S rDNA profile of colonised cotton

228 The cotton's Eubacterial clone library (Table S1) was dominated by the Order
229 Clostridiales which represented 58% of the clones obtained (n=67, Figure 3A). Of these
230 Clostridia, 33 sequences most closely matched organisms from the family Clostridiaceae 2,
231 where 13 sequences most closely matched *Clostridium formicaceticum* strain DSM 92 (95%
232 sequence similarity) and a further 10 to *Anaerovirgula multivorans* strain SCA (97%
233 sequence similarity). The remaining 9 clones most closely matched sequences belonging to
234 the genus *Alkaliphilus*, of which 8 were closely related to *Alkaliphilus oremlandii* strain
235 OhILAs (91-93% sequence similarity) and 1 related to *Alkaliphilus transvaalensis* strain
236 SAGM1 (98% sequence similarity). The remaining clones of the Clostridia were represented
237 by sequences most closely related to organisms from the families *Clostridium insertae sedis*
238 *XI* (3 sequences) and *Clostridium insertae sedis XIV* (2 sequences).

239 The remainder of the clone library was made up of a diverse range of taxonomic
240 families (Table S1) including: representatives from Brucellaceae, primarily related to
241 *Ochrobactrum anthropi* strain ATCC 49188 (96-99% sequence similarity);
242 Corynebacteriaceae, dominated by *Corynebacterium marinum* strain D7015 (98-99%
243 sequence match similarity); and the Bacillaceae 1, dominated by *Bacillus pseudofirmus* strain
244 OF4 (89-99% sequence similarity).

The Archaeal clone library (Table S2) was dominated (93%) by sequences most closely matching *Methanobacterium alcaliphilum* strain NBRC 105226 (99% sequence similarity) (n=68, Figure 3B). The remaining sequences were most closely related to *Methanomassiliicoccus luminyensis* strain B10 (4 sequences 89% sequence match) and *Methanosarcina mazei* Go1 (99% sequence similarity).

CDP driven microcosms

The microcosm demonstrated significant degradation of ISA at pH 11.0 over 2 waste/feed cycles (Figure 4) with first order rate constants of 3.33×10^{-2} day^{-1} (SE± 2.0×10^{-2}) for alpha, 9.36×10^{-2} day^{-1} (SD± 2.2×10^{-2}) for beta and 6.78×10^{-2} day^{-1} (SE± 2.85×10^{-2}) for X-ISA. Acetate was the only VFA detected and gradually accumulated in the system reaching a peak of 2.06 mmoles (SE± 0.2), similarly hydrogen gas accumulated in the headspace over the course of the feed cycle reaching 1.00 mmoles (SE± 0.04). Neither carbon dioxide nor methane was detected in the headspace of the microcosm, however, soluble inorganic carbon increased within the system (data not shown) with the pH after each cycle having an average pH of 10.80 (SE ± 0.4). The CDP fed microcosm inoculated with the colonised cotton was dominated by polymicrobial flocs with fluorescence microscopy showing microbial cells embedded in an EPS composed of protein, polysaccharide and extracellular DNA (eDNA) (Figure 5).

Measurement of the ATP concentration of the microcosm showed that cell density increased over the feed/waste cycles (Table S4) indicating that a portion of the organic carbon was used for the generation of both cell biomass and EPS. Carbon flow calculations (Rittmann and Mccarty, 2001) based on the degradation of ISA showed 23.7% of the carbon was converted to acetate and 12.1% converted to carbonate from energy generating processes, 0.5% was converted to cell biomass and a further 63.7% was theorised to be

involved in processes relating to EPS production. The yield of dry cell biomass was 0.012 mg (mg ISA) $^{-1}$ degraded, the system could not be stoichiometrically balanced due to the unknown composition of the flocculate EPS material. Comparison of the samples amended with chloramphenicol showed no ISA degradation and the production of acetate and hydrogen was not detected (Figure S2) indicating that ISA degradation was via microbial activity rather than chemical processes or sorption.

Microcosm clone library

The microcosm microbial populations demonstrated a significant shift away from that associated with the emplaced cotton samples, with Archaeal taxa no longer being detectable and the Eubacterial population no longer dominated by the Clostridiales. The environmental and physiological constraints imposed within the microcosm resulted in a population dominated by clones of *Alishewanella jeotgali* strain MS1 (99% sequence similarity) from the family Alteromonadaceae (Table S3, Figure 6). The remaining clones included representatives of the family Bacillaceae, most closely matching *Bacillus pseudofirmus* strain OF4 (98% sequence similarity) and *Alkaliphilus crotonatoxidans* strain B11-2 (98% sequence similarity) of the family Clostridiaceae 2.

Discussion

Previous authors noted the presence of an organic electron donor within the soils at Harpur Hill that allowed for electron flow into nitrate and iron reducing processes at depth (Burke et al., 2012). The generation of CDPs from the site's soil organic matter has been demonstrated (Rout et al., 2015) and in this study the addition of cotton cellulose resulted in its partial alkaline hydrolysis to CDPs with the concentration of alpha and beta ISA in the porewater and sediments being higher than those measured by Rout et al (2015). This supports the concept that the hyper-alkaline conditions created at this site are capable of

293 generating CDP. The presence of acetate, a common end product of ISA fermentation (Rout
294 et al., 2015, Bassil et al., 2014, Rout et al., 2014), in the porewater, sediment and cotton
295 indicated an active anaerobic microbial community in the immediate proximity of the cotton
296 even though the ambient pH was between pH 11.5 and 12.

297 Cotton fibres were covered with large areas of EPS indicative of biofilm formation
298 (Figure 2A and B) with individual cells being only rarely visible (Figure 2C). This is a
299 marked contrast to the colonisation of cotton incubated in a landfill site under neutral
300 anaerobic conditions reported by McDonald et al (2012) where fibres were heavily colonised
301 with cells and exhibited the characteristic pits and grooves associated with microbial
302 cellulose hydrolysis. The reduced colonisation of the cotton under the hyperalkaline
303 conditions present at the site are further illustrated by the live/dead staining of the cotton
304 (Figure 2D) which revealed a low density of live cells on the individual cotton fibres and
305 within the surrounding biofilm material. Previous work by Grant et al (2002) demonstrated
306 the ability of alkaliphilic microorganisms to form a biofilm upon the surface of the
307 cementitious materials presumably to provide a degree of protection from the alkaline
308 stresses imposed by the local environment. This formation of EPS as a response to
309 hyperalkaline conditions is replicated in these microcosm studies where a polymicrobial,
310 eDNA stabilised floc based population developed (Figure 5A). The importance of EPS
311 generation in this system is illustrated by the fact that >60% of the available carbon is
312 diverted to EPS formation, a finding similar to the carbon distribution in biofilm systems
313 reported by Jahn and Nielsen (1998).

314 The microbial flocs were composed of an EPS containing protein, polysaccharides
315 and eDNA. Polysaccharides are a common component of EPS and moderate a range of
316 bacterial biofilm properties including adhesion, cell aggregation, cohesive nature, protection
317 as well as the sorption of organic compounds and inorganic ions (Flemming and Wingender,

2010). Imaging of the polysaccharide component revealed its distribution throughout the flocculate with large globular like structures (Figure 5A). DNAse treatment caused the loss of these structures resulting a less compact structure of cells associated with polysaccharide, indicating a relationship between the eDNA and the distribution of the polysaccharide components (Figure 5B). The role of eDNA within biofilms appears to serve a number of functions (Dominiak et al., 2011), in this case it is likely to aid the structure and function of the flocculate community (Gloag et al., 2013). The presence of eDNA within the flocculate structure is also likely to act as a phosphate store for the constituent microbial consortia (Dell'anno and Danovaro, 2005). Calcium ions are abundant at the site and as such the interaction between eDNA and these ions is likely to promote cell aggregation and biofilm formation within these alkaliphilic cultures (Das et al., 2014). This is illustrated by the fact that treatment of the flocs with DNase resulted in the loss of flocculate stability (Figure 5A and B). Imaging of the protein component of the flocs showed large concentrated areas of protein within the flocculate (Figure 5C). Protein serves a wide range of functions within biofilm including the permitting of redox activity, protection from environmental conditions, enzymatic reactions and sorption of organic compounds sorption and inorganic ions (Flemming and Wingender, 2010).

The presence of the cotton cellulose within the sediments selected for organisms of the Order Clostridia which contrasts with previous investigations of the background sediments where a larger degree of taxonomic diversity was observed (Bassil et al., 2014, Williamson et al., 2013), presumably due to greater diversity of energy sources and colonisation from surrounding pasture land. Of the Clostridiaceae 2 species identified, *Clostridium formicaceticum* has broad spectrum carbohydrate fermentation capabilities (Andreesen et al., 1970), but was not previously associated with alkaline conditions. This contrasts with species from the genera *Anaerovirgula and Alkaliphilus* which have all been

343 previously associated with alkaline sites (Fisher et al., 2008, Takai et al., 2001, Pikuta et al.,
344 2006).

345 The Archaeal population associated with the cotton was dominated by
346 hydrogenotrophic, alkaliphilic *Methanobacterium* sp. showing sequence similarity to
347 *Methanobacterium alcaliphilum* (Worakit et al., 1986). These findings are in agreement with
348 clone libraries generated from microcosms previously developed from sediment samples
349 from the same site (Rout et al., 2015). Although these organisms are able to utilise acetate as
350 a growth factor (Kotelnikova et al., 1998, Wu et al., 1992), they are incapable of acetoclastic
351 methanogenesis which accounts for the accumulation of acetic acid in extracts from the
352 cotton and surrounding sediment and porewaters. In addition a small number of sequences
353 showing similarity to *Methanomassiliicoccus luminyensis* (Dridi et al., 2012) and
354 *Methanosarcina* sp. (Maestrojuan et al., 1992) were also detected.

355 The microbial population established in the microcosm was much less diverse than
356 that present on the cotton samples with the almost complete removal of Clostridia and the
357 total loss of methanogens from the system. This resulting fermentative system was dominated
358 (95% of clones) by organisms most closely related to *Alishewanella* sp., which was a minor
359 component (3% of clones) of the population present on the colonised cotton. This facultative
360 anaerobic genus is most commonly associated with fermented seafood, but has also been
361 isolated from landfill soils (Jung et al., 2012, Kim et al., 2009, Kim et al., 2010, Kolekar et
362 al., 2013). Its ability to grow in alkaline conditions up to pH 12 has also been reported (Kim
363 et al., 2009, Tarhriz et al., 2012, Kim et al., 2010), and its ability to degrade a range of
364 substrates appears to have enhanced its ability to thrive within the CDP driven microcosm.
365 The ability to form biofilms and pellicles has been reported in *Alishewanella jeotgali* which
366 may indicate a pivotal role for the *Alishewanella* sp. in the formation and maintenance of the
367 bacterial aggregates within the microcosm (Jung et al., 2012) (Figure 5A).

A range of degradation rate constants for the various forms of ISA (alpha, beta and xylo) were observed in the derived microcosms. The rate constant of beta ISA degradation was similar to that reported by Rout et al (2015) at pH 11, whilst the rate constant of alpha ISA degradation was greatly reduced, potentially due to the reduced role of key genera such as *Alkaliphilus* (Rout et al., 2015). This is the first time that a microbial degradation rate constant for xylo ISA has been published.

The loss of methanogens from the microcosm cannot be entirely attributed to the pH, since a pH 11.0 methanogenic microcosm has been successfully established using sediments from the Buxton site (Rout et al., 2015). In that case a similar range of methanogens were observed to that identified here associated with the cotton but with a Eubacterial population dominated by *Alkaliphilus*. The lack of Clostridia species specifically *Alkaliphilus* sp. within the microcosm formed from the colonised cotton appears to have retarded the ability of the associated methanogenic population to become established. Tight adherence to the cotton fibres and a possible differences in redox potential between the internal biofilm environment and the enrichment media may have also contributed to the poor transition of the methanogens and Clostridia species leading to an *Alishewanella* dominated system (Sridhar and Eiteman, 1999, Stuart et al., 1999).

The presence of cotton fibres with the hyper alkaline analogue site at Harpur Hill provided both a source of CDP to drive anoxic metabolism and a surface for microbial colonisation. Subsequent sub culturing indicated that the cotton provided a surface for the adherence of a narrow range of Clostridiaceae 2 species and promoted the development of a floc based alkaliphilic population dominated by *Alishewanella* sp. able to degrade CDP up to a pH of 11.0. Although methanogenic populations were detected on the cotton fibres, they were unable to make the transition to floc based suspended growth.

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592

593 Table 1: Analysis of porewater, sediment and cotton retrieved from sample borehole.

594

Source	pH	eH	Acetate	α-ISA	β-ISA
Porewater (mg L^{-1})	11.92	-66.00	208.90	7.64	6.82
Sediment (mg (g dry wt) $^{-1}$)	11.50	-77.00	127.24	1.01	0.54
Cotton (mg (g dry wt) $^{-1}$)	N/S	N/S	141.16	2.34	0.85

595 *N/S-Not sampled

596

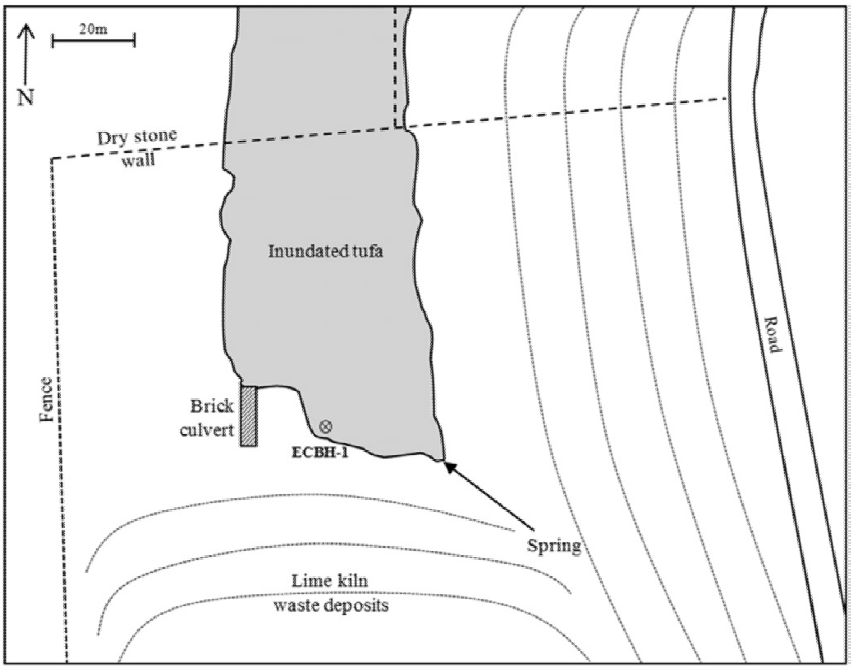
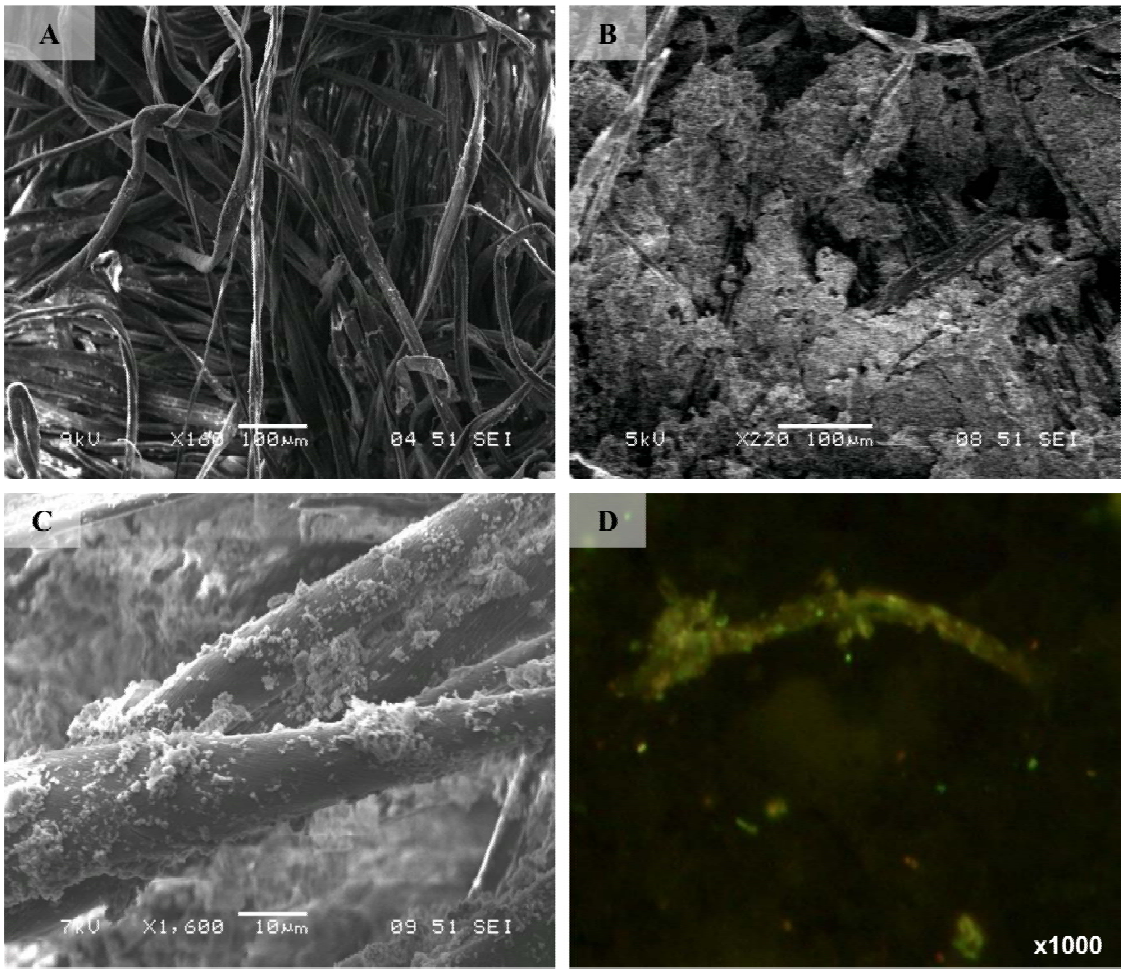


Figure 1: Overview of hyperalkaline contaminated site and position of emplaced cotton within bore hole 1 (ECBH-1)

601



602

603 Figure 2: Microscopy investigation of the cellulose cotton. [A] Sterile cotton. [B]

604 Cellulose cotton from the borehole showing biofilm formation. [C] Close up of

605 individual fibre showing individual cells, EPS aggregates and mineral precipitate. [D]

606 Live/dead image of individual cotton fibre.

607

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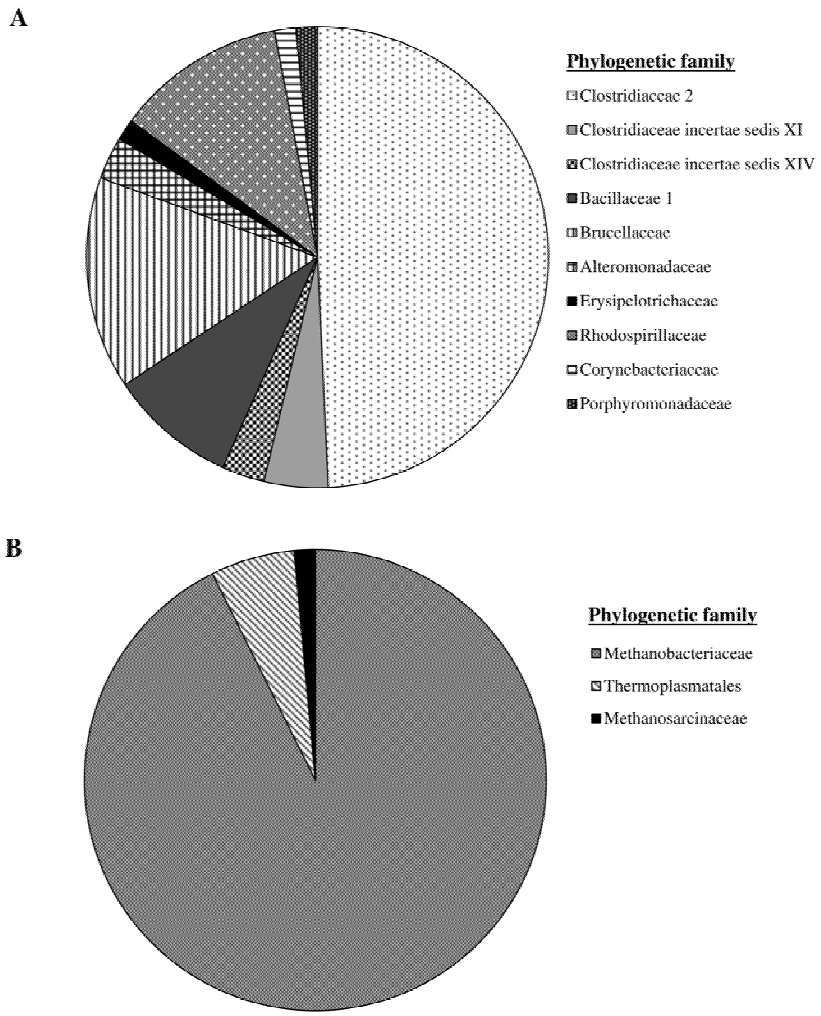

608

609

610 Figure 3: 16S rRNA gene clone libraries of the colonised cotton. [A] Eubacterial

611 (n=67). [B] Archaeal (n=68). Phylogenetic families were assigned to clones through a

612 MegaBLAST database search.

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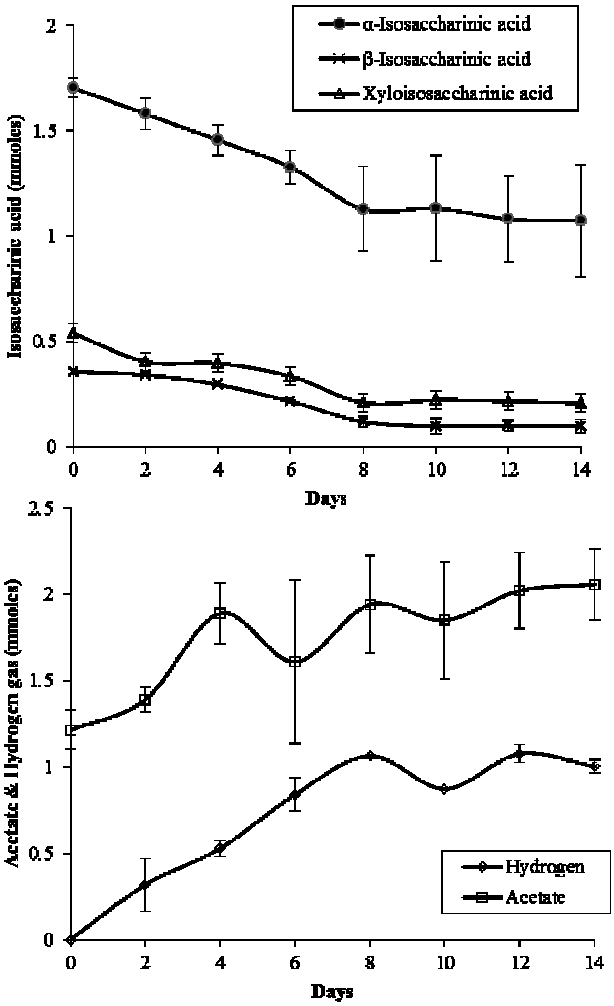

614

615 Figure 4: Chemistry of the CDP driven pH 11 microcosm over two waste/feed cycles
616 using colonised cellulose cotton as an inoculation source. [A] Alpha, beta and xylo
617 isosaccharinic acid degradation profile. [B] Hydrogen and acetate production profile.

618

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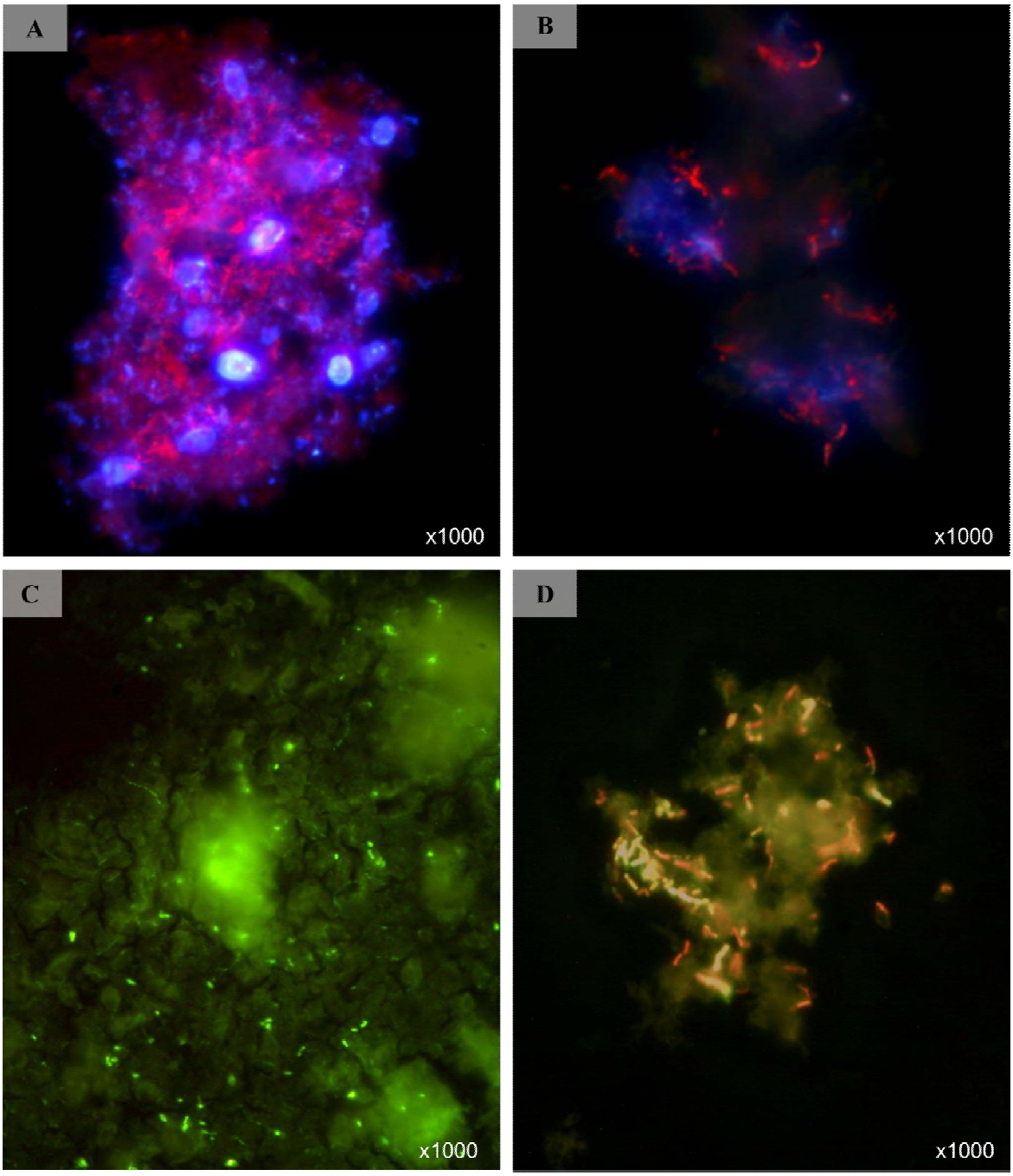
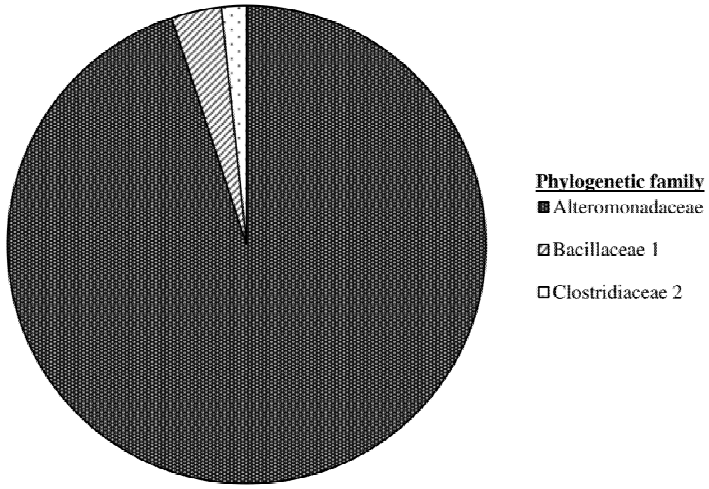


619

620 Figure 5: Microscopy investigation into the morphology of the pH 11 microcosm. [A]
621 Ethidium bromide and Calcofluor white stain of bacterial flocculate showing individual
622 cells and eDNA (red) and extracellular polysaccharides (blue). [B] DNase digest of
623 flocculates stained with ethidium bromide and Calcofluor white. [C] FITC stain of

bacterial flocculate showing areas containing protein (green). [D] Live/dead image of bacterial flocculate.

626



627

628 Figure 6: Eubacterial (n=59) 16s rRNA gene clone library of pH 11 CDP driven

629 microcosm.

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