Characterization of an autotrophic bioreactor microbial consortium degrading thiocyanate

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Abstract

Thiocyanate (SCN^-) forms as a by-product of cyanidation during gold ore processing, and can be degraded by a variety of microorganisms utilizing it as an energy, nitrogen, sulfur and/or carbon source. In complex consortia inhabiting bioreactor systems, a range of metabolisms are sustained by SCN^- degradation; however, despite the addition or presence of labile carbon sources in most bioreactor designs to date, autotrophic bacteria have been found to dominate key metabolic functions. In this study, we cultured an autotrophic SCN^--degrading consortium directly from gold mine tailings. In a batch-mode bioreactor experiment, this consortium degraded 22 mM SCN^-, accumulating ammonium (NH_4^+) and sulfate (SO_4^{2-}) as the major end products. The consortium consisted of a diverse microbial community comprised of chemolithoautotrophic members, and despite the absence of an added organic carbon substrate, a significant population of heterotrophic bacteria. The role of eukaryotes in bioreactor systems is often poorly understood; however, we found their 18S rRNA genes to be most closely related to sequences from bacterivorous *Amoebozoa*. Through combined chemical and phylogenetic analyses, we were able to infer roles for key microbial consortium members during SCN^- biodegradation. This study provides a basis for understanding the behaviour of a SCN^- degrading bioreactor under autotropic conditions, an anticipated approach to remediating SCN^- at contemporary gold mines.

Keywords: bioremediation, thiocyanate, autotrophic, bioreactor, environmental biotechnology

Introduction

Thiocyanate (SCN^-) is present at high concentrations in the waste-streams of gold mining and coal gasification (Dash et al. 2009), through the reaction between its precursor compounds cyanide (CN^-) and reduced sulfur (Mudder et al. 2001). Disposal and storage of SCN^--containing waste often results in contamination of ground- and surface-waters, due to the use of unlined storage facilities (Kossoff et al. 2014). Although less toxic than its precursor CN^-, SCN^- can accumulate to concentrations toxic to plant and animal life (Bhunia et al. 2000; Gould et al. 2012). In order to remove SCN^- from waste-streams and contaminated waters, a number of abiotic treatments have been trialled, typically involving chemical oxidation, requiring high inputs of reagents, and resulting in their own waste by-products (Akcil 2003). The ability of some microorganisms to degrade SCN^- offers a cost-effective and scalable approach to SCN^- remediation.

A number of microorganisms are capable of SCN^- degradation, utilizing it as an energy, nitrogen, sulfur and/or carbon source (Gould et al. 2012). The end-products of this degradation are ammonium (NH_4^+), carbon dioxide (CO_2) and sulfide (S^{2-}), subsequent to the formation of carbonyl sulfide (COS) or cyanate (CNO^-) intermediates (Ebbs 2004). The former pathway is well-characterized, proceeding by two stages mediated by the action of SCN^--hydrolase and COS-hydrolase enzymes (Katayama et al. 1992; Ogawa et al. 2013). The latter pathway is proposed to proceed via a thiocyanate:cytochrome *c* oxidoreductase to CNO^- and elemental sulfur (S^0), identified in species of the *Thioalkalivibrio* genus (Berben et al. 2017). In addition, the cyanase enzymes converting CNO^- to NH_4^+ and CO_2 are widely expressed (Anderson 1980). Significantly, CNO^- formed abiotically from urea or cyanide, has recently been recognized as a potentially important source of reduced nitrogen for nitrifying microorganisms (Palatinszky et al. 2015). However, evidence for its production in mixed SCN^- degrading consortia is lacking, leaving its role in SCN^- degradation in environmental systems unresolved.

Microorganisms that carry out SCN^- degradation belong to a variety of metabolic niches (Gould et al. 2012; Watts and Moreau 2016). The most thoroughly studied of these belong to the chemolithoautotrophic sulfur-oxidizing bacteria, which utilize the released reduced sulfur

as an energy source; additionally, some can utilize the nitrogen released as NH_4^+ as a growth nutrient (Boden et al. 2012; Sorokin et al. 2014; Sorokin et al. 2001; Youatt 1954). A phylogenetically diverse group of heterotrophic bacteria are also capable of SCN^- degradation, primarily as a nitrogen source for growth (du Plessis et al. 2001; Stratford et al. 1994). In addition, SCN^- degradation is not restricted to the domain Bacteria, with a eukaryotic fungus, *Acremonium strictum*, also reported to degrade SCN^- (Kwon et al. 2002).

To efficiently harness SCN^- biodegradation, several bioreactor systems have been designed and trialled (Lee et al. 2008; Stott et al. 2001; van Zyl et al. 2014), with some notable examples reaching field deployment (van Buuren et al. 2011; Whitlock 1990). Although isolated SCN^- degraders are easily obtainable, in bioreactor systems microbial consortia are often favoured for their greater resilience to environmental stressors, higher metabolic diversity, and the improved efficiency provided by the division of functions (Brenner et al. 2008; Lindemann et al. 2016). These factors are particularly significant when considering not only SCN^- degradation but also the cycling of its breakdown products, nitrogen and sulfur (Watts and Moreau 2016).

Only recently has a culture-independent approach achieved a consortium level understanding of SCN^--degrading consortia inhabiting bioreactors, through utilization of targeted gene (Huddy et al. 2015; Ryu et al. 2015; Villemur et al. 2015) and metagenomic sequencing (Kantor et al. 2015). These studies, although often performed in the presence of a carbon source, either added or as a co-contaminant, found that autotrophic bacteria are often key community members in SCN^- degrading bioreactor consortia (Kantor et al. 2015). This finding is significant as a fully autotrophic bioreactor system would likely reduce operational costs, biomass fouling, and carbon effluent levels. The role of the inoculum on consortium structure and function also remains unresolved, with the majority of studies employing activated sludge as the inoculum. In addition, although potentially influential as SCN^- degraders, predators, and nutrient cyclers, the role of eukaryotes in SCN^- degrading bioreactor consortia has received limited attention (Huddy et al. 2015; Kantor et al. 2015). In light of these uncertainties regarding the role and function of key consortia members and their impact on bioreactor performance, further work is required.

Here we report the successful selective cultivation and subsequent experimental characterization of an autotrophic SCN^- degrading microbial consortium. We enriched this consortium from SCN^- contaminated gold mine tailings to select for bacteria adapted to high SCN^- concentrations and low labile carbon availability, and used it as inoculum for a lab-scale (5L) bioreactor. During batch operation, we performed chemical analysis of key metabolites in conjunction with high-throughput sequencing of the 16S and 18S rRNA genes. Our work allows for investigation of the community structure, and evaluation of the metabolic potential, of a fully autotrophic SCN^- degrading microbial bioreactor consortium.

Methods

Sample collection and cultivation

An inoculum sample was taken from the surface tailings material of a tailings storage facility at the Stawell Gold Mine, Western Victoria, Australia. These samples were chilled on ice and returned to the lab on the day of collection, where they were stored at 4°C until use. Within 5 days of sampling the tailings samples were sub-sampled, adding approximately 2 g of material to a base media reflective of the chemistry of the SCN^- contaminated water at the storage facility. This media contained 2.3 g L^{-1} Na_2SO_4, 1.8 g L^{-1} NaCl, 1.4 g L^{-1} $CaCl_2$, 0.5 g L^{-1} $MgSO_4$, 0.13 g L^{-1} $NaHCO_3$, 0.1 g L^{-1} KCl and 0.02 g L^{-1} NaH_2PO_4, adjusted to pH 8 using NaOH. This base media was then autoclaved to ensure sterility, after which a volume of filter (0.22 µm)-sterilized NaSCN solution was added to make the desired SCN^- concentration. Initial enrichment of an SCN^- degrading culture was performed in autoclaved 250 mL shake flasks plugged with cotton wool and aluminium foil, containing 50 mL of base media and 17.2 mM SCN^-, maintained on a shaking incubator set to 180 rpm and 30°C. Samples were periodically removed, using aseptic technique, for SCN^- analysis. Upon complete removal of SCN^-, the culture was subcultured into fresh SCN^- containing base media at a transfer volume of 10% v/v of inoculum to fresh media. This process was repeated a minimum of 5 times prior to using the stock culture in any experiments.

Shake flask tests

The control of SCN^- concentration and the potential for inhibition by NH_4^+ was tested in shake flask experiments, prior to deployment of the culture in the lab-scale bioreactor. A culture was grown to late log phase growth, as described above, and used for inoculating, at 10% v/v, triplicate shake flasks containing sterile base media and either variable SCN^- (18.9, 29.3 and 39.6 mM SCN^-) or variable NH_4^+ (0, 1.1, 2.8, 5.5 and 27.7 mM NH_4^+), added as a 0.22 µm syringe-filtered NaSCN and $(NH_4)_2SO_4$ solutions. Once inoculated, the shake flasks were immediately returned to the shaking incubator set to 180 rpm and 30°C. Periodically, samples (1-2 mL) were aseptically removed and passed through a 0.22 µm syringe filter and immediately analyzed for their SCN^- concentration.

Bioreactor - Culture conditions

A 5 L double-jacketed glass bioreactor vessel (Sartorius Stedim UniVessel), equipped with a dissolved oxygen probe, temperature probe, pH probe, impeller, sparger and exhaust cooler, was filled with 4 L of base media, sealed and autoclaved. After sterilization the vessels probes, air supply and impeller were connected to the control unit (Sartorius Stedim Biostat B), while the exhaust cooler and vessel jacket were connected to a chiller unit (Sartorius Stedim Frigomix R), allowing temperature control. A volume of 0.22 µm syringe filtered solution of NaSCN was added, via a needle and syringe and a rubber septum in the vessel lid, giving a final concentration of 22 mM SCN^-. During the batch experiment the bioreactor was operated at 30°C, supplied with air (0.5 lpm) passed through a 0.22 µm filter, the impeller was set to 150 rpm and the bioreactor vessel maintained in the dark. To inoculate the bioreactor vessel, a stock culture was used to inoculate 400 mL of base media, containing 17.2 mM SCN^-, in a sterile 1 L shake flask with a cotton wool bung. This seed culture was again maintained in a shaking incubator at 30°C and 180 rpm, in the dark, until it reached late log phase of growth, determined by OD_{600} measurement. The culture was then harvested by centrifuging at 5000g for 20 minutes, discarding the supernatant and re-suspending in chilled (4°C), autoclaved base media, in the absence of SCN^-. This process was repeated 3 times before concentrating the biomass in to 50 mL of base media, this cell slurry was then added through the rubber septum in to the bioreactor vessel. Initially the pH was prevented from falling below 7.8 using titration of NaOH, however, during the extended lag period this was altered (at 50 hours) to 7.2. Additionally, during the lag phase a further addition of PO_4^{3-} was

added at 66 hours, in a bid to stimulate SCN^- degradation, bringing the total concentration to 0.2 mg L^{-1} in the 4 L media.

Bioreactor – Sampling

At regular intervals, homogenized samples were removed from the bioreactor vessel, using an aseptic sampling apparatus attached to a dip tube, sampling from close to the impeller at the base of the bioreactor vessel. Typically 5 mL of sample was removed, 2.5 mL of which was passed through a 0.22 µm syringe filter, and the remaining unfiltered sample placed in a -80°C freezer. The filtered sample was immediately used for SCN^- determination, prior to being frozen (-80°C freezer) for further analysis later. At strategic timepoints, analysis of the microbial community structure was desired, therefore, in addition to the above 5 mL, a further 10 mL of solution was removed and immediately placed in a -80°C freezer for DNA extraction later.

DNA extraction and high-throughput 16S and 18S rRNA gene amplicon sequencing

When ready for DNA extraction, the unfiltered samples were thawed, centrifuged at 5000g for 20 minutes to form a pellet, the supernatant discarded and the pellet re-suspended in 1 mL of sterile base media. This concentrated slurry was then extracted for its genomic DNA, using the PowerMax Soil DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), alongside a negative (base media only) control. The concentration of the genomic DNA in the extracts were quantified using a Qubit fluorometer, with a broad range assay kit, confirming sufficient DNA in the samples and no detectable DNA in the negative control. The microbial consortium structure of the bioreactor over the course of the experiment was assessed using high-throughput 16S and 18S rRNA gene amplicon sequencing.

The 16S rRNA gene was amplified by PCR using universal primers (S-D-Bact-0341-b-S-17/S-D-Bact-0785-a-A-21) targeting the hypervariable V3-V4 region (Klindworth et al. 2012); Forward 5'-CTACGGGNGGCWGCAG-3', Reverse 5'-ACTACHVGGGTATCTAA-3', containing partial Illumina adapters at the 5' end, in a reaction with NEBNext Q5 Hot Start HiFi PCR Master Mix (NEB, Ipswich, MA, USA). This reaction was performed in a

thermo cycler set to an initial denaturing step (98°C for 30 seconds), followed by 25 cycles of denaturing (10 seconds at 98°C), annealing (30 seconds at 60°C) and extension (60 seconds at 65°C), followed by a final extension (120 seconds at 65°C). Resulting amplicons were purified, using 0.8× volume ratio of Ampure XP bead, prior to indexing with Illumina-compatable adapters; Nextera XT Index i7 and i5, and again purified using 0.8× volume ratio of Ampure XP bead (Beckman Coulter, Brea, CA), prior to sequencing on an Illumina Miseq platform (Illumina, San Diego, CA, USA) at the Monash University Malaysia Genomics Facility using a run configuration of 2 x 250 bp.

The 18S rRNA gene was also amplified from the genomic DNA extract by PCR, using NEBNext Q5 Hot Start HiFi PCR Master Mix (NEB, Ipswich, MA, USA). We used a primer pair (1391f-EukBr) targeting the hypervariable V9 region (Amaral-Zettler et al. 2009), originally developed for the Earth Microbiome Project (http://www.earthmicrobiome.org/emp-standard-protocols/18s/); Forward 5'-GTACACACCGCCCGTC-3', Reverse 5'-TGATCCTTCTGCAGGTTCACCTAC-3'. The thermo cycler conditions for PCR are as follows; initial denaturing at 98°C for 30 seconds, followed by 25 cycles of denaturing for 10 seconds at 98°C, annealing for 30 seconds at 60°C and extension for 60 seconds at 65°C, followed by a final extension for 120 seconds at 65°C. The resulting 18S rRNA gene amplicons were purified using 1× volume ratio of Ampure XP bead (Beckman Coulter, Brea, CA) prior to index litigation using the Nextera XT Index kit, purified again using similar volume ratio ofAmpure XP bead (Beckman Coulter, Brea, CA) and sequenced on the Illumina MiSeq platform.

Bioinformatic data analysis of 16S and 18S rRNA gene sequences

All resulting raw 18S and 16S rRNA gene sequences were uploaded to the National Centre for Biotechnology Information's (NCBI's) Sequence Read Archive (SRA) under the BioProject ascension number PRJNA356781. The 18S or 16S rRNA gene sequences were then analyzed using Qiime (version 1.9.0) (Caporaso et al. 2010). Sequences were first quality filtered, discarding bases with a Phred quality score of <20 and removing reads which contain less than 70% than their original sequences.

The 16S rRNA gene sequences had operational taxonomic units (OTUs) picked by clustering the sequences at 97% sequence similarity. BLASTn (Altschul et al. 1990) was then used to assign taxonomy from the Greengenes database (version 13_8) (DeSantis et al. 2006) and a representative set of sequences were picked and aligned using the PyNAST tool (Caporaso et al. 2010). Chimeric sequences were then identified using the ChimeraSlayer tool and removed (Haas et al. 2011). For alpha and beta-diversity measurements, the sequences were randomly re-sampled to 1000 reads, to remove the bias of sampling depth. The Shannon index and observed OTUs (at 1000 reads) were calculated to give a sense of diversity of each sample (alpha-diversity). To report the dissimilarity between the microbial consortium in the samples, Bray-Curtis dissimilarity was calculated and its dissimilarity matrix used to construct a dendrogram and its principal co-ordinates used to construct a Principal Co-ordinate Analysis (PCoA) plot.

The quality filtered 18S rRNA gene sequences firstly had chimeric sequences identified and removed using USEARCH UCHIME (version 6.1.544) (Edgar et al. 2011). OTUs were then picked at 97% sequence similarity, using a *de novo* approach; UCLUST algorithm (Edgar 2010). Representative sequences were assigned taxonomy using the SILVA (version 119) database and the BLAST method; following this, prokaryotic sequences were removed from the data.

All prokaryotic and eukaryotic OTUs that comprised ≥1% of total sequence abundance in any of the samples were compared to the NCBI database, using the BLASTn search. Uncultured and environmental sequences were excluded and preference was given to the most similar sequence, the maximum sequence coverage and those assigned to species level. In cases where multiple species had the same similarity and coverage, they are all reported; when multiple strains of the same species showed equal matches, only one is reported here.

Aqueous chemistry analyses

The concentration of SCN^- was determined by a colorimetric technique, forming a color complex with a $Fe(NO_3)_3 \cdot 9H_2O$ reagent and its absorbance measured at a wavelength of 420 nm (Eaton and Franson 2005). Ammonia was also analyzed using a colorimetric reaction with salicylate and sodium nitroferricyanide, in the presence of sodium hypochlorite, resulting in a color complex with maximum absorbance at 650 nm (O'Dell 1993). All

colorimetric samples were analysed using a Hach DR2800 spectrophotometer. The concentration of SO_4^{2-} was determined gravimetrically through precipitation of $BaSO_4$. Eppendorf tubes were dried at 80°C overnight and allowed to cool before being weighed, a 1 mL of sample was then added to the tube followed by 10 µL of 1 M HCl, prior to addition of 0.5 mL of $BaCl_2$ solution. The tubes were then placed on a vortex machine for 1 minute and allowed to precipitate $BaSO_4$ at room temperature for 2 hours. The tubes were centrifuged at 10,000g for 5 minutes, all the supernatant completely removed and the tubes and precipitate allowed to dry at 80°C overnight, weighing once cooled to room temperature, allowing calculation of the SO_4^{2-} content of the solution. The concentrations of nitrate, nitrite and cyanate were determined by ion chromatography, performed on a Metrohm 850 Professional IC AnCat, fitted with a Metrosep A Supp 16 150/4.0 column, using a 7.5 mM Na_2CO_3 and 0.75 mM NaOH eluent solution

Results

SCN^- removal during shake flask experiments

The SCN^- concentrations during the shake flask experiments, conducted with increasing SCN^- or NH_4^+ concentrations, are presented in Fig 1 (a) and (b) respectively. No inhibition of SCN^- removal was noted over the SCN^- concentration range tested in the batch experiments, encompassing typical values encountered in gold mine tailings waters (Akcil 2003). All replicates showed similar characteristics; with a lag phase of typically 30-40 hours, followed by rapid removal of all of the SCN^- in solution. The microbial consortium tested here did exhibit limited inhibition of SCN^- removal in the presence of ≥2.8 mM NH_4^+, with the slowest rate of SCN^- degradation in the presence of 27.7 mM NH_4^+. However, under all tested conditions complete SCN^- removal occurred within 200 hours of incubation.

Batch bioreactor chemistry

The changes in concentration of SCN^- and its degradation products, alongside pH and O_2 %, over the batch bioreactor experiment, are presented in Fig 2. The concentration of SCN^- exhibited a lag phase of no removal, over the initial ~135 hours of the experiment, longer than that of previous batch flask experiments (typically 40-50 hours), see Fig 1. During this

lag phase the pH of the system was initially buffered to stay above 7.8, using NaOH, however, as no SCN^- removal was initially noted this was adjusted to 7.2 after 50 hours. Following this the pH decreased slightly, with a sharp decrease and rebound at 66 hours upon addition of PO_4^{2-}.

The lag phase was followed by a period of SCN^- removal, completely removing the starting concentration of ~22 mM SCN^- in 110 hours. SCN^- removal was quickly followed by the accumulation of SO_4^{2-} from initial values of ~24 mM (note this high initial value is due to the presence of significant quantities of SO_4^{2-} in the base medium used). This accumulation started 3.5 hours after SCN^- removal is initiated, reaching a maximum of ~40 mM SO_4^{2-}, ceasing after the complete consumption of SCN^-. This steady state value represents an accumulation of the equivalent of 16 mM S as SO_4^{2-}, accounting for 73% of the initial S added to the system as SCN^-. NH_4^+ also accumulated in solution, concurrent to SCN^- removal, reaching a steady state value of ~9.3 mM NH_4^+ after 223 hours. Other nitrogen containing compounds analysed were not detected at significant concentrations, with no detectable NO_2^- and minimal (<0.5 mM) accumulation of NO_3^-. Although not detected at high concentrations, CNO^- was found to accumulate to concentration of ~0.9 mM during active SCN^- removal, before decreasing to below detection upon complete removal of SCN^-. The sum of the concentrations of nitrogen-containing compounds at the end of the experiment, primarily as NH_4^+, was equivalent to 9.3 mM nitrogen, accounting for 42% of the initial nitrogen in the system, as SCN^-.

Microbial consortium structure

Prokaryotic consortium structure

At the order-genus level, the 16S rRNA gene sequences the initial inoculum was dominated by sequences attributed to *Thiobacillus* and *Moraxellaceae*, the former consisting of three OTUs (OTU# 794020, 850808 and 274500) at ≥1% of all sequences and all most closely related to *Thiobacillus thioparus* strain THI 111, and the latter of a single OTU (OTU# 811253) ≥1%, most closely related to *Pseudomonas stutzeri*, *Pseudomonas xanthomarina* and *Pseudomonas putida* strains (Supporting Information, Table S1). Other taxa make up significant minority sequence abundances; *Bacteroidales*, *Hoeflea*, *Chromatiales* and *Sphingomonadales*, see Supporting Information, Table S1 for details of the NCBI GenBank

assignments to their most abundant OTUs. During the lag phase (52 hours sample) the sequences were predominantly attributed to *Agrobacterium* (Table S1), which went on to dominate during SCN^- degradation and only decreased in abundance after complete SCN^- degradation. The sequences assigned to *Moraxellaceae* also increased during the lag phase, going on to decrease during SCN^- removal and again increase after SCN^- is removed. Several notable taxa in the inoculum, assigned to *Thiobacillus*, *Bacteroidales*, *Chromatiales*, *Hoeflea* and *Sphingomonadales* all significantly decreased during the lag phase, while the *Thiobacillus*, *Bacteroidales* and *Chromatiales* recovered in abundance during and after SCN^- removal.

Interestingly a number of genera, which made up a significant minority of OTU members in the initial inoculum, decreased and did not recover throughout the batch bioreactor experiment. *Sphingomonadales* (OTU# 674655), *Mycoplana* (OTU# 1108959) and *Arenimonas* (OTU# 815996), for example, are all >2% in the inoculum but rarely represented >1% of sequences in later samples from the bioreactor experiment. Other OTUs exhibited the opposite behaviour, representing small minority sequences in the inoculum (<1%) but rose to comprise significant minority consortium members, typically accounting for 1-5% of sequences, these include OTUs assigned to *Thalassospira xiamenensis* (OTU# 348517), *Thiobacillus* (OTU# 274500), *Alcanivorax dieselolei* (OTU# 793461) and *Alcanivorax* (OTU# 275378). Other minority OTUs remained at a stable abundance throughout the batch bioreactor experiment, including *Flavobacteriales* (OTU# 1112867) and *Phaeobacter* (OTU# 580223).

Eukaryotic consortium structure

The eukaryotic population of the bioreactor was far less diverse than that of the prokaryotes, with most sequences (>98%) belonging to three genera; two *Amoebozoa* and one fungi (Fig 2(b)). The most abundant sequences were assigned to the *Acanthamoeba* genus, which dominated the inoculum and all but one sample, where its relative sequence abundance decreased to 2.5%; the late SCN^- degradation stage (209 hours). This genus accounted for multiple OTUs, 6 of which made up ≥1% of total sequence abundance, all equally related to 3 *Acanthamoeba* species (Supporting Information, Table S2). The sequences which dominated

(97%) the late SCN^- degradation stage (209 hours) and form a sizeable minority (11%) of the inoculum, were assigned to the *Reticulamoeba* genus. Using the NCBI GenBank database, the 4 most abundant OTUs (≥1%) all had highest sequence similarity (96-99%) to *Amoebozoa* belonging to the *Vannella* genus (Supporting Information, Table S2). During the lag phase (52 hours) a significant abundance of sequences (2.7%) were also assigned to the fungal species *Capronia pilosella*. This taxa is dominated by a single OTU (OTU# denovo503) which, when compared to the NCBI GenBank database, has equal sequence similarity (98%) to a number of ascomycetous fungi belonging primarily to the *Herpotrichiellaceae* family (Supporting Information, Table S2).

Prokaryotic diversity measurements

The alpha-diversity measurements at 1000 sequences sampling depth, number of OTUs and Shannon Index, for the inoculum and bioreactor samples are presented in Table 1 and their respective rarefaction curves in the Supporting Information, Fig S2(a) and (b). Using both values as indicators of microbial diversity, the initial inoculum is the most diverse sample, in regards its 16S sequence data. A marked decrease in diversity is noted in the lag phase, followed by an increase during early SCN^- degradation and a decrease to a minimum at the end of SCN^- degradation. The equilibrium phase (after complete SCN^- removal) is noted by a marked increase in both diversity measures. These trends in differences in alpha-diversity measurements between samples are well supported by the Bray-Curtis dissimilarity values presented in the PCoA plot (Fig 4) and the dendrogram in Supporting Information Fig S1. The biggest change between samples was recorded between the inoculum and the lag phase (52 hour) sample (Fig 4), this is then followed by smaller differences during SCN^- degradation and a larger change in consortium structure upon complete SCN^- removal. In the dendrogram (Supporting Information Fig S1) this is manifest by the greater similarity between the samples from the lag phase and SCN^- degradation phases, in comparison to the inoculum and the equilibrium samples.

Discussion

Inhibition of SCN^- biodegradation

The results of the batch shake flask experiments corroborate previous observations of the robustness of SCN^- biodegradation (Chaudhari and Kodam 2010; Lay-Son and Drakides 2008). Significantly, the range of high SCN^- concentrations used here is comparable to those reported previously in industrial effluents; 7-1500 mg L^{-1} in coal and coking effluents (Luthy and Bruce Jr 1979) and 500-1000 mg L^{-1} in gold mine effluent (Akcil 2003). However, we did note inhibition of SCN^- degradation in the presence of high concentrations of NH_4^+ (27.7 mM), as has also been previously reported (Chaudhari and Kodam 2010; Lay-Son and Drakides 2008; Stafford and Callely 1969). Inhibition by NH_4^+ is more widely reported by studies of heterotrophic bacteria, thought to degrade SCN^- as a source of nitrogen for growth; the presence of readily available NH_4^+ would, therefore, preclude the need to express enzymes capable of SCN^- degradation. Another potential mechanism for inhibition is the toxicity of NH_3. NH_4^+ itself is only slightly toxic to most bacteria (Müller et al. 2006); however, at the pH of our shake flask experiments (pH 8), the potential exists for formation of NH_3 , as the pK_a of NH_4^+ under standard conditions is ~9.25 (Lay-Son and Drakides 2008). It is worth noting that the maximum NH_4^+ concentration encountered in the bioreactor (~9.3 mM) remained below that of the inhibited batch flask experiment (27.7 mM), suggesting a limited impact on SCN^- degradation.

The cause of the increased lag phase encountered when scaling up the volume of the SCN^- degradation culture, from small flask (50 mL) batch cultures to the bioreactor vessel (4 L), is unclear. However, this inhibition of SCN^- degradation is potentially as a result of the differing maintenance conditions in the bioreactor, where pH was maintained through NaOH addition and O_2 was supplied though sparging air, compared to shaking in the batch flask experiment. Although the causes are not resolved in this study it serves to highlight potential issues when scaling up this method further to treat wastewater.

SCN^- biodegradation

Removal of SCN^- in the batch bioreactor experiment indicates that the microbial consortium contains members capable of SCN^- degradation. Concordantly, a number of bacterial genera previously implicated in SCN^- degradation, such as *Thiobacillus*, *Pseudomonas* and *Sphingopyxis* (Felföldi et al. 2010; Happold et al. 1958; Kelly and Wood 2000; Stafford and Callely 1969), were identified in both the inoculum and bioreactor microbial consortium. The *thiobacilli*, which made up a significant proportion of sequences in the inoculum and increased in abundance during SCN^- degradation, are commonly the dominant SCN^-

degraders in bioreactor communities (Huddy et al. 2015; Kantor et al. 2015; Lee et al. 2008; Villemur et al. 2015). The three most abundant *Thiobacillus* OTUs were all most closely related to *T. thioparus* strain THI 111, chemolithoautotrophic sulfur-oxidizing species known to contain SCN^- degrading strains (Boden et al. 2012). This species is known to utilize the COS pathway, through production of the SCN^--hydrolase (SCNase) enzyme (Katayama et al. 1992).

The most closely related sequences to the *Sphingomonadales* OTU, belong to a *Sphingopyxis* spp., a genus previously implicated in SCN^- biodegradation (Felföldi et al. 2010). *Sphingopyxis* spp. have also previously been identified as consortium members in SCN^- degrading consortia (Joshi et al. 2016; Kantor et al. 2015; Ryu et al. 2015). Little is known about their SCN^- degrading capability, and their role in this system is unclear. Their abundance actually decreased upon inoculation of the bioreactor and through active SCN^- degradation, and only marginally increased after its degradation. Two of the *Pseudomonas* spp. (*P. stutzeri*, *P. putida*), most closely related to the sequences from the bioreactor, are known to contain SCN^- degrading strains (Grigor'eva et al. 2006; Karavaiko et al. 2000; Stafford and Callely 1969). These studies found that these heterotrophic bacteria degraded SCN^- as a source of nitrogen and sulfur for growth, noting inhibition by the presence of NH_4^+ (Stafford and Callely 1969) and oxidation of HS^- to tetrathionate or thiosulfate (Grigor'eva et al. 2006). Although decreasing in abundance during active SCN^- degradation, they again increased after its degradation, potentially benefitting from the subsequent release of nutrients.

In addition to the above-known SCN^- degrading microorganisms, possibly a number of prokaryotic and eukaryotic taxa identified in the bioreactor consortium are capable of SCN^- degradation, but have yet to be identified as possessing this trait. Indeed several OTUs increased in abundance during SCN^- degradation or following it, for example those assigned to *Bacteroidales* or *Chromatiales*. This observation suggests some ecological advantage as a result. However, whether the microorganisms were directly involved in SCN^- degradation, or merely benefitting from the nutrients subsequently released, is not clear.

Sulfur cycling

The direct sulfur containing end product of SCN^- degradation, via the CNO^- or COS pathway, is widely reported to be H_2S (Sorokin et al. 2014). The accumulation of SO_4^{2-}during SCN^- degradation, therefore, indicated that the majority of the sulfur (73%), released as S^{2-}, was fully oxidized by the microbial consortium, with potentially some contribution from abiotic oxidation by O_2 (Moses et al. 1987). The concurrent steady decrease in pH during active SCN^- removal can therefore, at least in part, be attributed to acidogenesis through the release of H_2SO_4 associated with sulfur oxidation (Friedrich et al. 2005; Little et al. 2000). The high oxygen demand of most sulfur oxidation pathways likely contributed towards the rapid decrease of % O_2 in the bioreactor during SCN^- degradation (Toran and Harris 1989). Abiotic sulfur oxidation through a two-electron transfer between O_2 and S^{2-}, resulting in S^0, is thermodynamically favourable, albeit with a significant kinetic barrier in the absence of trace metals capable of H_2S abiotic oxidation (Luther et al. 2011). Biologically mediated oxidation of H_2S, via a chemolithotrophic metabolism, however, has been found to be far more kinetically favourable, with rates 3-4 orders of magnitude higher than in trace metal free reactions, and still 2-3 orders of magnitude faster than in the presence of trace metal oxidants (Luther et al. 2011; Millero et al. 1987; Vazquez et al. 1989). Although trace metals were present in the bioreactor medium, likely increasing the rate of abiotic sulfur oxidation, the kinetics of biological sulfur oxidation, the presence of known sulfur oxidizing taxa, and the conversion of most of the sulfur to SO_4^{2-}, indicate a primarily biologically mediated sulfur oxidation pathway in the bioreactor.

A number of OTUs most closely related to known sulfur-oxidizing chemolithoautotrophs were identified in the bioreactor consortium, potentially utilizing the rich energy source that reduced sulfur provides (Kelly 1999; Kelly et al. 1997). Several of these OTUs belong to the *Thiobacillus* genus, including *T. thioparus*, which is known to be capable of complete oxidation of a wide range of reduced sulfur species (Nordstrom and Southam 1997), often through the intermediate S^0 (Kelly 1999). This genus has been found to be particularly important in H_2S-removing biotechnological applications (Oh et al. 1998; Park et al. 2002), and to play a significant role in environmental sulfur cycling in the absence of light (Chen et al. 2009). The abundant *Chromatiales* OTU was most related to the *Thioprofundium* species, *Thioprofundium hispidum* and *Thioprofundium lithotrophicum*, capable of oxidation of S^0,

$S_2O_3^{2-}$, and $S_4O_6^{2-}$ (Mori et al. 2011). Unlike the *Thiobacilli*, *Thioprofundium* is not known to contain species capable of SCN^- degradation; instead it potentially scavenged reduced sulfur released by SCN^- degradation mediated by other consortium members. The proliferation of both of these taxa during active SCN^- degradation indicated that they gained an ecological advantage from its degradation, likely through their sulfur-oxidizing metabolism.

In addition to the above chemolithotrophic sulfur oxidizers, a number of obligatory heterotrophic bacteria which are capable of sulfur oxidation were identified (Sorokin 2002; Trudinger 1967). These included bacteria belonging to the *Pseudomonas* genus (Chung et al. 1996), which typically oxidize sulfur via an anaerobic pathway associated with denitrification resulting in tetrathionate (Sorokin et al. 1999). The extent and pathways of sulfur oxidation exhibited by heterotrophic bacteria are diverse and often not fully understood and their role in this system remains unresolved (Sorokin 2002).

The incomplete sulfur mass balance in the bioreactor, i.e. not quantified as SCN^- or SO_4^{2-}, could be due to the formation of stable intermediate compounds not analysed in this study, or due to S assimilation into microbial biomass. A number of potential intermediates exist, such as the solid intermediate, S^0 (Sorokin et al. 2001), or volatile intermediates, such as COS (Katayama et al. 1992) and H_2S, not measured in this study. Sulfur is also an essential trace nutrient for life, where it is estimated to comprise 0.81% of the C:N:P:S stoichiometric ratio of microbes (Xu et al. 2015). The assimilation of sulfur was, therefore, required for biomass growth in the bioreactor, and incorporation in to biomass might represent an un-quantified pool of sulfur in the system.

Nitrogen cycling

In addition to H_2S, the other primary end product of SCN^- biodegradation is NH_4^+, either directly from SCN^- degradation, by the enzyme SCN^--hydrolase or via CNO^- and the cyanase enzyme (Sorokin et al. 2014). The formation of CNO^-, evident in the bioreactor chemical data, during SCN^- degradation, has previously been cited as evidence for utilization of this pathway in pure-cultures (Sorokin et al. 2002). However, as previously discussed CNO^- can

also be generated abiotically (Palatinszky et al. 2015) and its presence here does not conclusively provide evidence for the utilization of this metabolic pathway. The subsequent removal of CNO^- after consumption of SCN^- suggests its active degradation to NH_4^+, either by the action of a cyanase enzyme (Anderson et al. 1990) or abiotic chemical decomposition (Palatinszky et al. 2015). Significantly, a number of bacteria in the bioreactor consortium have previously been identified as expressing the cyanase enzyme or containing genes for the enzyme, including; *P. stutzeri* and *P. putida* (Grigor'eva et al. 2006) and *Thiobacillus* spp. (Kantor et al. 2015).

The evident nitrogen mass imbalance in the bioreactor could be due to a variety of factors, such as the accumulation of intermediates not analyzed, loss of volatile intermediates/products, or through assimilation of nitrogen into biomass. A key process for aqueous nitrogen removal in the environment involves nitrification followed by denitrification, producing N_2 gas. This would be firstly initiated as microbial NH_4^+ oxidation to NO_2^- as part of nitrification, principally undertaken by a group of autotrophic bacteria and archaea (Francis et al. 2007; Martens-Habbena et al. 2009). This process did not appear to be occurring significantly in this microbial consortium, due to the lack of accumulation of nitrite or nitrate, in addition to the absence of identification of nitrifying taxa. The bioreactor consortium had the potential for denitrification, with a number of the OTU sequences closely related to known denitrifiying bacteria (Matějů et al. 1992). However, given denitrification primarily occurs under low oxygen conditions, and the apparent absence of nitrate supply, it is unlikely to have been a significant process in the bioreactor under these conditions. As initially SCN^- was the sole nitrogen source in the bioreactor, besides N_2 gas, NH_4^+ was likely the primary nitrogen supply used for growth by the microbial consortium (Magasanik 1982). Indeed SCN^- degrading bacteria are known to assimilate the NH_4^+ released from SCN^- degradation, as their sole nitrogen source (Grigor'eva et al. 2006). This route has the potential therefore to account for the evident loss in nitrogen noted in the batch bioreactor experiment. In addition, nitrogen fixation may also have occurred in the bioreactor, especially prior to SCN^- degradation, where nitrogen is likely a limiting nutrient to non-SCN^- degrading bacteria. Indeed, the most dominant OTU in the bioreactor had highest sequence similarity to two heterotrophic *Rhizobium* spp. capable of nitrogen fixation (Amarger et al. 1997; Diange and Lee 2013).

Carbon cycling

As anticipated, in the absence of the addition of a labile carbon source a number of autotrophic taxa were identified as major consortium members in the batch bioreactor consortium. The previously discussed chemolithotrophic *Thioprofundum* and *Thiobacillus* genera, for example, are able to fix CO_2 from the air supplied to the bioreactor (Mori et al. 2011; Vishniac and Santer 1957). These autotrophs, in the absence of an added organic carbon source, likely provided the primary productivity of the system. This biomass organic carbon was then able to support the large heterotrophic population in the bioreactor. Bacterial predators, in the form of the eukaryotes, *Acanthamoeba* and *Reticulamoeba*, potentially play a role in the cycling of this carbon in the bioreactor, through cell lysis and excretion (Rogerson et al. 1996; Weekers et al. 1993).

Implications for SCN^- degrading biotechnology

This study provides a comprehensive characterization of a SCN^- degrading bioreactor, through analysis of the aqueous chemistry and the phylogeny of the microbial consortium. Unlike the majority of preceding SCN^- bioreactor studies, this culture was enriched from the contaminated gold mine tailings itself, under autotrophy-selective conditions. This consortium was able to degrade high concentrations of SCN^- effectively, requiring minimal nutrient requirements, including the complete absence of added organic carbon. Autotrophs of the *Thiobacillus* genus were implicated in SCN^- degradation, confirming their dominance under a wide variety of culture conditions, while other prokaryotes and eukaryotes likely had roles in carbon, sulfur and nitrogen cycling in the bioreactor. The generation of the potential intermediate cyanate, previously only identified in pure-culture incubation experiments, suggests the hypothetical cyanate pathway may also play a significant role in SCN^- degradation in mixed microbial communities. However, in the absence of abiotic controls it is uncertain if the detected CNO^- is formed abiotically or through biotic SCN^- degradation. Collectively, the results offer an in depth understanding of the SCN^- degradation potential of an autotrophic bioreactor consortium, and help to form the basis for further testing in lab and field scale bioreactors under minimal nutrient input conditions.

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Fig. 1 SCN^- concentration over time during batch shake flask experiments inoculated with the SCN^- degrading microbial consortium. Cultures performed in presence of increasing SCN^- concentrations (a) or increasing NH_4^+ concentrations (b). Error bars represent standard deviation of triplicate flasks

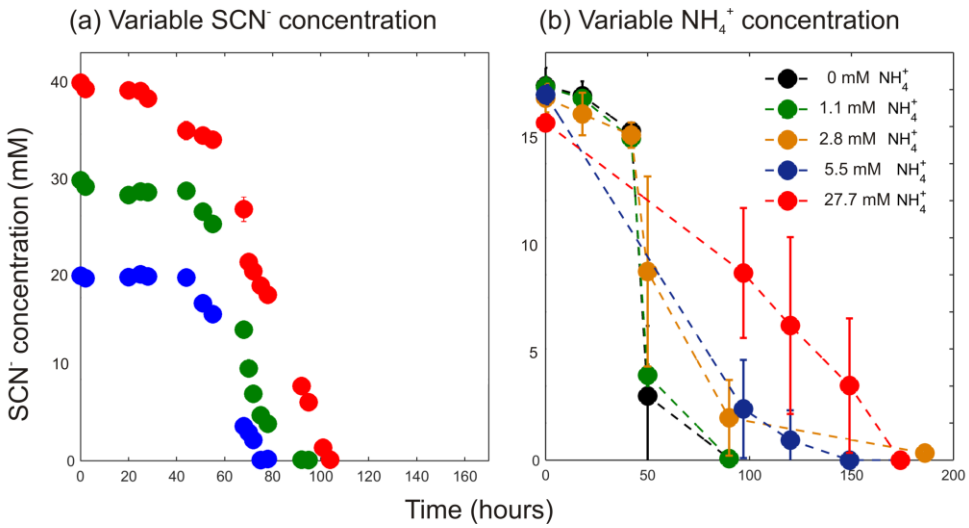
Fig. 2 The concentration of SCN^- and its degradation products, alongside pH and % O_2, in the bioreactor over the batch experiment

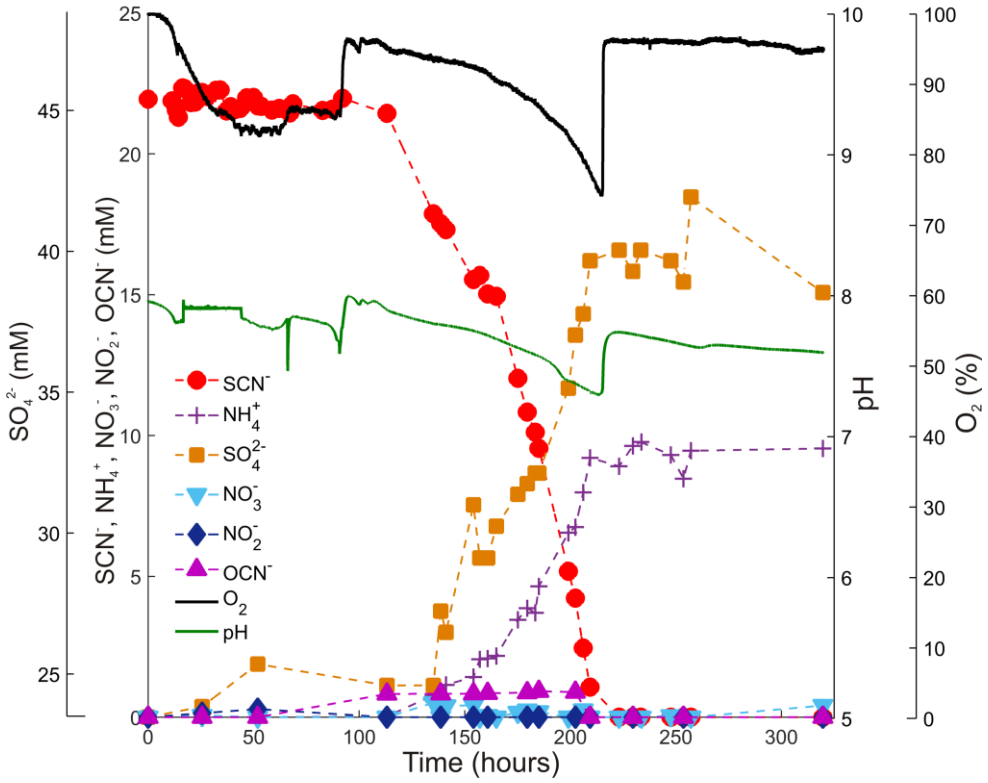
Fig. 3 Relative sequence abundance of 16S rRNA gene sequences assigned at the order, family or genus level (a) and 18S rRNA gene sequences assigned at the genus level (b). Only taxa that make up ≥2% of all sequences at any point in the experiment are presented. The initial inoculum is included to the left of the dotted line, while the subsequent time series has been categorized in stages based on the chemical data, including an initial lag phase (sample at 52 hours), SCN^- degradation phase (samples at 135, 165 and 209 hours) and equilibrium phase (sample at 320 hours), detailed above the top panel

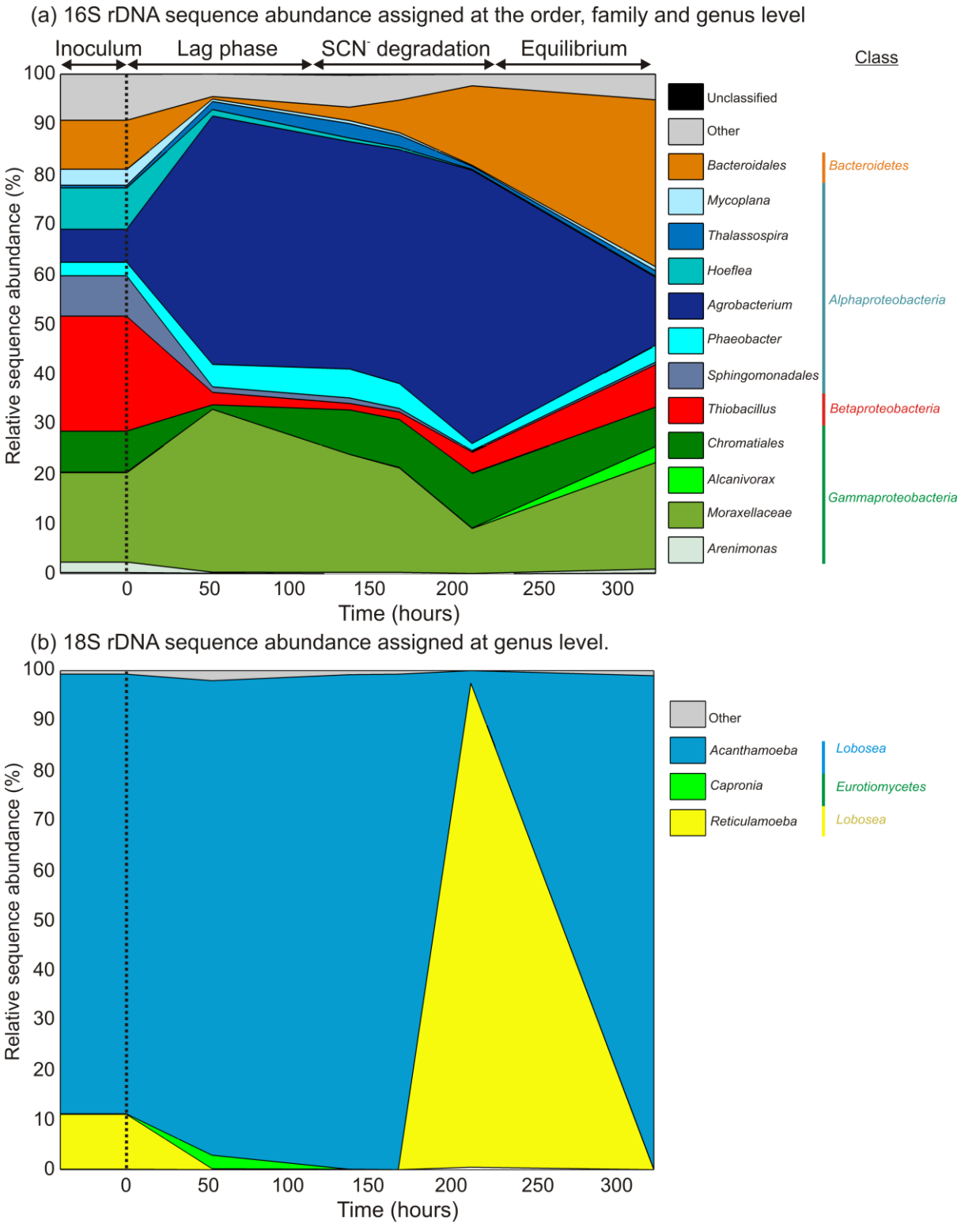
Fig. 4 Principle co-ordinate analysis (PCoA) plot of the Bray-Curtis dissimilarity matrices from the inoculum and bioreactor samples; calculated at 1000 sequences sampling depth of the 16S rRNA gene sequence data

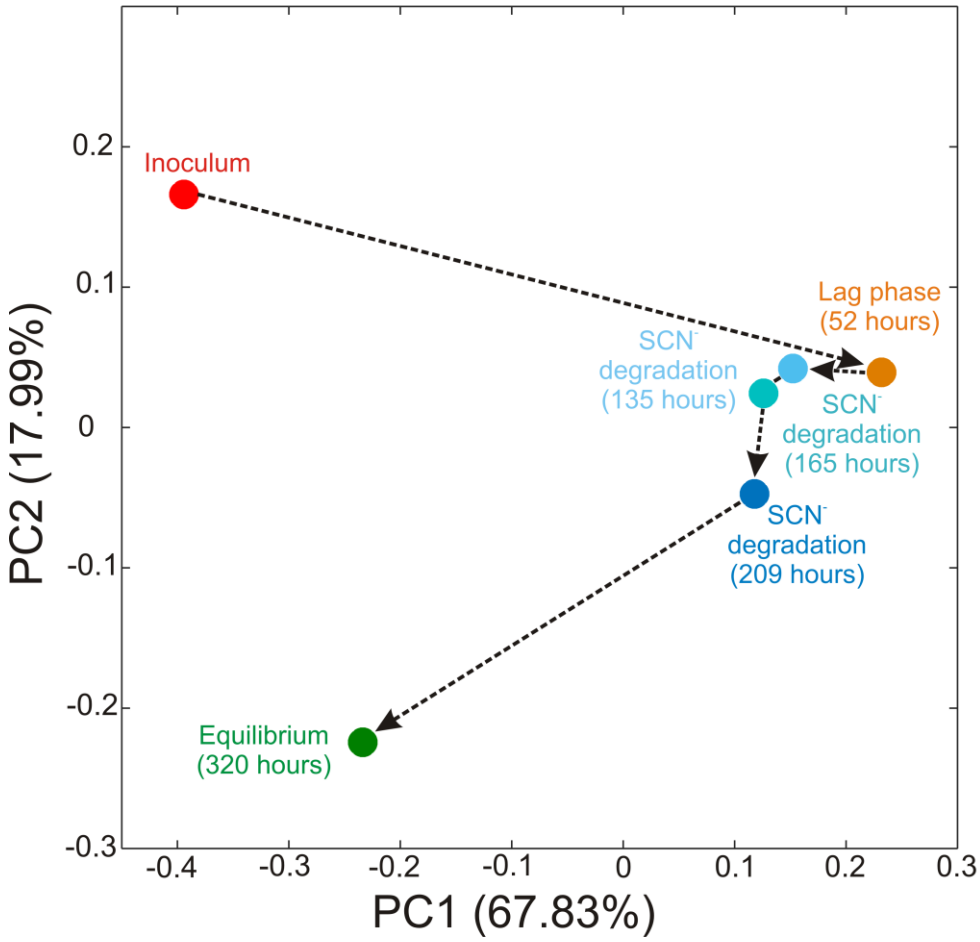
Table 1 Chemical and microbial consortium results of samples removed for 16S rRNA gene sequencing from the batch bioreactor test

Timepoint (hours)	**Bioreactor phase**	**Shannon index (rarefied)**	**OTUs (rarefied)**	**Number of sequences**	**SCN^- (mM)**	**NH_4^+**	**SO_4^{2-}**
0	Inoculum	3.89	52.5	90907	21.9	0	23.5
52	Lag phase	2.27	41.3	51932	21.7	0.0	25.4
135	SCN^-	2.75	44.6	49233	17.9	0.8	24.6
165	degradation	2.65	42.1	51059	14.9	2.2	30.2
209		2.26	31.9	31544	1.1	9.2	39.7
320	Equilibrium	3.18	45.9	37408	0.0	9.5	38.6


(a) Variable SCN⁻ concentration
(b) Variable NH4+ concentration
SCN⁻ concentration (mM)
Time (hours)
0 mM NH4+
1.1 mM NH4+
2.8 mM NH4+
5.5 mM NH4+
27.7 mM NH4+


SCN⁻
NH₄⁺
SO₄²⁻
NO₃⁻
NO₂⁻
OCN⁻
O₂
pH
SCN⁻, NH₄⁺, NO₃⁻, NO₂⁻, OCN⁻ (mM)
SO₄²⁻ (mM)
pH
O₂ (%)
Time (hours)


(a) 16S rDNA sequence abundance assigned at the order, family and genus level
Inoculum
Lag phase
SCN- degradation
Equilibrium
Relative sequence abundance (%)
Time (hours)
Class
Unclassified
Other
Bacteroidales
Mycoplana
Thalassospira
Hoeflea
Agrobacterium
Phaeobacter
Sphingomonadales
Thiobacillus
Chromatiales
Alcanivorax
Moraxellaceae
Arenimonas
Bacteroidetes
Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria
(b) 18S rDNA sequence abundance assigned at genus level.
Relative sequence abundance (%)
Time (hours)
Other
Acanthamoeba
Capronia
Reticulamoeba
Lobosea
Eurotiomycetes
Lobosea


Inoculum
Lag phase
(52 hours)
SCN-
degradation
(135 hours)
SCN-
degradation
(165 hours)
SCN-
degradation
(209 hours)
Equilibrium
(320 hours)
PC2 (17.99%)
PC1 (67.83%)
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.3
-0.2
-0.1
0
0.1
0.2
0.3

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