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Biodegradation of thiocyanate in groundwater by a native aquifer microbial consortium

Liam P Spurr [1], **Mathew P Watts** [1], **Han Ming Gan** [2], **John W Moreau** Corresp. [1]

[1] School of Earth Sciences, University of Melbourne

[2] School of Life and Environmental Sciences, Deakin University, Australia

Corresponding Author: John W Moreau
Email address: jmoreau@unimelb.edu.au

Gold ore processing typically generates large amounts of thiocyanate (SCN^-)-contaminated effluent. When this effluent is stored in unlined tailings dams, contamination of the underlying aquifer can occur. The potential for bioremediation of SCN^--contaminated groundwater, either *in situ* or through *ex situ*, remains largely unexplored. This study aimed to enrich and characterise SCN^--degrading microorganisms from mining-contaminated groundwater under a range of culturing conditions. Mildly acidic and suboxic groundwater, containing ~135 mgL^{-1} SCN^-, was collected from an aquifer below an unlined tailings dam. An SCN^--degrading consortium was enriched from contaminated groundwater using combinatory amendments of air, glucose and phosphate. Biodegradation occurred in all oxic cultures, except with the sole addition of glucose, but was inhibited by NH_4^+ and did not occur under anoxic conditions. The SCN^--degrading consortium was characterised using 16S and 18S rRNA gene sequencing, identifying a variety of heterotrophic taxa in addition to sulfur-oxidising bacteria. Interestingly, few recognised SCN^--degrading taxa were identified in significant abundance. These results provide both proof-of-concept and the required conditions for biostimulation of SCN^- degradation in groundwater by native aquifer microorganisms.

Biodegradation of thiocyanate in groundwater by a native aquifer microbial consortium

Liam P. Spurr[1], Mathew P. Watts[1], Han M. Gan[2] and John W. Moreau[1, *]

[1]*School of Earth Sciences, The University of Melbourne, Parkville, VIC, Australia 3010*

[2]*School of Life and Environmental Sciences, Deakin University, Waurn Ponds, VIC, Australia 3216*

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*Corresponding author: jmoreau@unimelb.edu.au

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ABSTRACT

Gold ore processing typically generates large amounts of thiocyanate (SCN^-)-contaminated effluent. When this effluent is stored in unlined tailings dams, contamination of the underlying aquifer can occur. The potential for bioremediation of SCN^--contaminated groundwater, either *in situ* or through *ex situ*, remains largely unexplored. This study aimed to enrich and characterise SCN^--degrading microorganisms from mining-contaminated groundwater under a range of culturing conditions. Mildly acidic and suboxic groundwater, containing ~135 mgL^{-1} SCN^-, was collected from an aquifer below an unlined tailings dam. An SCN^--degrading consortium was enriched from contaminated groundwater using combinatory amendments of air, glucose and phosphate. Biodegradation occurred in all oxic cultures, except with the sole addition of glucose, but was inhibited by NH_4^+ and did not occur under anoxic conditions. The SCN^--degrading consortium was characterised using 16S and 18S rRNA gene sequencing, identifying a variety of heterotrophic taxa in addition to sulfur-oxidising bacteria. Interestingly, few recognised SCN^--degrading taxa were identified in significant abundance. These results provide both proof-of-concept and the required conditions for biostimulation of SCN^- degradation in groundwater by native aquifer microorganisms.

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Introduction

Thiocyanate (SCN^-) is a toxic contaminant in industrial wastewater streams associated with gold mining (Stott et al. 2001; Akcil 2003; Kenova et al. 2010), steel production (Lay-Son and Drakides 2008), photofinishing (Shukla et al. 2004), electroplating (Aguirre et al. 2010), herbicide and insecticide production (Hughes 1975) and coal coking (Dash et al. 2009; Gould et al. 2012). In gold ore processing, SCN^- is generated through reaction of cyanide (CN^-) with sulfide minerals and other intermediate valence sulfur species (Akcil 2003). Most mine operators promote this reaction, as CN^- is even more toxic than SCN^- (Ingles and Scott 1987). However, SCN^- remains an undesirable end-product that must be removed for safe storage or disposal of waste water.

The waste products of gold ore processing are typically stored for indefinite time periods within large tailings storage facilities (TSFs). Many TSFs were historically unlined, such that tailings-derived SCN^--leachate flows directly into the underlying water table. Although hydrologic recirculation of SCN^--contaminated groundwater to the TSF has been used to retard SCN^- migration, this strategy is unsustainable both as an environmental or an economical long-term solution for aquifer remediation.

SCN^- remediation technologies have drawn recent interest, due to the environmental stability (Gould et al. 2012) and potential toxicity of SCN^- to aquatic life, with an LC_{50} for *Daphnia magna* of 0.55 to 33.47 mg L^{-1} (Watson and Maly 1987). Abiotic SCN^- remediation techniques typically involve chemical oxidation (e.g. SO_2/air, peroxide and Caro's acid) (Wilson and Harris 1960; Breuer and Jeffrey 2011) or adsorption/separation methods (Aguirre et al. 2010). These approaches are often

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62 expensive to implement, and may produce more hazardous waste (Akcil 2003; Dash et
63 al. 2009). Bioremediation of SCN^- would present a more cost-effective alternative
64 (Akcil 2003), either via 1) stimulation of SCN^--degrading microorganisms within
65 extracted groundwater, prior to re-injection into the contaminated aquifer; or 2)
66 promotion of *in situ* biodegradation within the aquifer under ambient conditions. The
67 former approach, likely implemented in the form of a bioreactor has gained much
68 attention for treating SCN^- containing effluent streams (Whitlock, 1990; van Zyl et al.
69 2011; Villemuir et al. 2015), while *in situ* approaches remain largely unexplored.
70
71 SCN^--degrading microorganisms occupy a diverse range of environments, including
72 activated sludge (van Zyl et al. 2011), soils (Vu et al. 2013) and soda lakes (Sorokin et
73 al. 2004, 2014). SCN^--degraders can use SCN^- as a source of sulfur, carbon, nitrogen
74 and energy (Sorokin et al. 2001; Ebbs 2004; Grigor'eva et al. 2006). These
75 microorganisms exhibit both heterotrophic (Vu et al. 2013) and autotrophic
76 metabolisms (Sorokin et al. 2001, 2004; Bezsudnova et al. 2007; Huddy et al. 2015,
77 Watts et al. 2017), with the former typically using SCN^- as a nitrogen source and the
78 latter oxidizing sulfur as an energy source. Much work has been done to understand the
79 complex community interactions in engineered systems treating wastewater (Kantor et
80 al. 2015; Kantor et al. 2017), while no previous studies have focussed on groundwater
81 as the initial enrichment community.
82
83 In this study, we determined experimentally the potential for SCN^- biodegradation by
84 a native microbial consortium in mining-contaminated groundwater, and characterized
85 the diversity and phylogeny of this consortium. Our approach involved 1) enrichment
86 of SCN^--degrading microorganisms from contaminated groundwater, 2) culturing

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experiments involving amendments of DOC (glucose), PO_4^{3-} and NH_4^+; and 3) Illumina MiSeq sequencing of 16S and 18S rRNA genes from the SCN^--degrading microbial consortium. As SCN^- potentially provides carbon and nitrogen to microorganisms, we quantified the extent to which external amendments of NH_4^+ or dissolved organic carbon (DOC) to groundwater impacted SCN^- biodegradation rates (e.g., Paruchuri et al. 1990). We also measured the impact of bioavailable PO_4^{3-} on SCN^--biodegradation rates. Our results present new information and insights into strategies for *in situ* SCN^- bioremediation in mining-impacted groundwater.

Materials and Methods

Groundwater sampling and storage

Mining-contaminated groundwater was extracted from a monitoring well located adjacent to a TSF at an operational gold mine in central Victoria, using a low flow pump. The well was screened in weathered granodiorite and schist, at a depth of 55m below the surface. A sample of approximately 15L was extracted, sealed and stored on ice until it was returned to the lab the next day, where it was refrigerated at 4°C until use. The groundwater was used for enrichments within three days of sampling, while the remaining groundwater was used as a filter-sterilised medium for further culturing transfers, having been stored for up to 10 weeks in the dark at 4°C by the end of the experiments. The chemistry of the groundwater is monitored frequently at the site and is typically moderately saline, has a pH of 6.6-6.8, SCN^- concentrations of 500-1000 mg L^{-1} and free CN^- concentrations of <0.03 mg L^{-1} (courtesy of Kirkland Lake Gold Inc.).

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110

111 *Groundwater geochemical analyses*

112 At the time of sampling, a flow cell was used to determine the pH, E_H and DO
113 measurements taken with a YSI Professional Plus™ multi-parameter meter with
114 calibrated probes. The groundwater was also analysed by colorimetry for SCN^- and
115 NH_4^+ upon return to the laboratory, using the ferric-nitrate method (Eaton and Franson
116 2005) and the salicylate-nitroprusside method (Baethgen and Alley 1989), respectively.
117 During laboratory-based experiments, pH was measured using a Thermo Orion 5 Star
118 Plus™ Electrolyte Analyser and calibrated probes. Growth of the culture was
119 monitored by tracking optical density at 600nm (OD_{600}). All colorimetric analyses were
120 conducted using a Hach DR2800™ Portable Spectrophotometer with standard
121 solutions.

122

123 *Aerobic and anaerobic enrichment culturing experiments*

124 Groundwater was incubated under oxic and anoxic conditions, with various nutrient
125 amendments, to enrich an SCN^--degrading culture. The oxic replicates were made by
126 decanting groundwater (100mL) under sterile conditions into triplicate autoclaved
127 250mL conical flasks, sealed with a cotton wool bung and foil. Anoxic cultures were
128 prepared by adding 30mL of filter-sterilised groundwater to 50mL serum bottles sealed
129 with rubber stoppers and aluminium crimps, and degassed using pressurised nitrogen
130 gas. Both the oxic and anoxic cultures were further amended with additions of either 5
131 gL^{-1} DOC (as glucose), 50 mgL^{-1} PO_4^{3-} (as NaH_2PO_4), or both, alongside no-addition
132 controls. All incubations were maintained in the dark on a rotary shaker at 30°C and
133 120rpm. The SCN^- concentration was monitored to determine if degradation was

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134 occurring; in cases where significant removal was noted, this enrichment provided
135 inoculum for further culturing using filter sterilised (0.22µm filter) groundwater as the
136 medium. The amendments that produced a stable SCN^- degrading culture upon further
137 culturing were selected for further study.
138
139 Oxic cultures were sampled by extracting 2mL with a sterile syringe in a biosafety hood
140 to ensure sterility. Anoxic cultures were sampled by extracting 2mL of the culture with
141 a N_2-degassed sterile syringe and needle, sampled through the rubber stopper. Half of
142 the sample was passed through a 0.22µm filter, while the other half was used for OD_{600}
143 measurement prior to freezing at -20°C. Samples for DNA sequencing were removed
144 at late-log phase growth and immediately frozen at -80°C until thawing for DNA
145 extraction.
146
147 *Culturing of a SCN^--degrading microbial consortium from groundwater*
148 Amendments that resulted in a groundwater culture capable of SCN^- degradation after
149 repeated culturing were further tested. All culturing after the initial enrichment phase
150 was performed in sterilised 250mL conical flasks, containing 100mL of filter-sterilised
151 ground water, stoppered using a cotton wool bung and foil, and with previously used
152 nutrient amendments. For re-culturing, 10% v/v of the inoculum culture was sampled
153 in late log phase of growth and incubated on a rotary shaker at 30°C and 120rpm in the
154 dark. Before subsequent testing, the culture was routinely re-cultured a minimum of
155 five times to ensure a stable microbial community had developed. To determine the
156 behaviour of the end-product, NH_4^+, an identical culturing experiment to those

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previously described was set-up and samples removed to monitor SCN^-, OD_{600} and NH_4^+.

Biodegradation of SCN^- in the presence of ammonium

A further experiment was set up to determine the impact of NH_4^+ on SCN^- biodegradation. This experiment used the re-cultured SCN^--degrading microbial community and was again performed using filter-sterilised (0.22µm filter) groundwater from the same well. As with previous experiments, this was performed in triplicate 250mL conical flasks, containing 100mL of filter-sterilised groundwater, stoppered using a cotton wool bung and foil. The flasks were amended to low (no addition), moderate (10 mgL^{-1}) and high (40 mgL^{-1}) concentrations of NH_4^+ using a filter-sterilised (0.22µm) concentrated $(NH_4)_2SO_4$ solution, in addition to 5 gL^{-1} glucose and 50 mgL^{-1} PO_4^{3-}. An inoculum of the late log-phase culture was then added at 10% v/v concentration and incubated on a rotary shaker at 30°C and 120rpm in the dark.

Whole community microbial DNA extraction and Illumina MiSeq 16S and 18S rRNA gene sequencing

The triplicate samples for microbial ecology analysis were firstly removed from the -80^{0}C freezer and thawed. The genomic DNA was then extracted with the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc. Carlsbad, CA). The primers used for 16S and 18S rRNA gene sequencing consist of partial Illumina adapter at the 5' end. The incorporation of the second-half of the Illumina adapter and dual-index barcode was performed in another round of PCR reaction (Illumina 16S Sequencing Protocol). The

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16S and 18S rRNA gene amplicon sequencing was performed using on Illumina MiSeq platform (Illumina, San Diego, CA) located at the Monash University Malaysia Genomics Facility (2 x 250 bp run configuration).

The 16S rRNA gene was amplified by PCR using primers targeting the V3-V4 region of the 16S gene: Forward 5'-CCTACGGGNGGCWGCAG-3' and Reverse 5'-GACTACHVGGGTATCTAATCC-3' (Klindworth et al. 2013). High-fidelity PCR was performed on 1µL of each DNA sample using 0.5µL of Illumina-compatible universal primers, under the following thermal cycler conditions: initial denaturation step (98°C for 30 seconds), followed by 25 cycles of denaturation (98°C for 10 seconds), annealing (60°C for 30 seconds) and extension (65°C for 60 seconds), followed by a final extension step (65°C for 120 seconds). The product was further purified with 20µL of Ampure 0.8X, and washed with 200µL of 80% ethanol and eluted in 50µL for subsequent index ligation using Nextera XT Index primers i7 forward and i5 reverse Illumina adapters. The subsequent product was purified with 12µL of Ampure 0.8X, washed with 200µL of 80% ethanol, and eluted in 30µL for sequencing.

The 18S rRNA gene from the genomic DNA samples was amplified using the forward primer 1391f 5'-GTACACACCGCCCGTC-3', and the reverse primer EukBr 5'-AGACAGTGATCCTTCTGCAGGTTCACCTAC-3' (Amaral-Zettler et al. 2009). PCR was performed with 1µL of the DNA extract in the presence of 10µM Illumina-compatible primer, under the following thermal cycler conditions; initial denaturation (98°C for 30 seconds), followed by 25 repetitions of denaturation (98°C for 10 seconds), annealing (65°C for 60 seconds) and extension (65°C for 120 seconds) and a final extension (65°C for 120 seconds). This PCR product was purified with 25µL of

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Ampure 1X and washed with 200µL of 80% ethanol, then eluted in 40µL in preparation for index ligation, using Nextera XT Index i7 forward primer Nextera XT Index i5 reverse primer. The product was purified with 10µL of Ampure 1, washed with 200µL of 80% ethanol and eluted in 30µL for 18S sequencing.

16S and 18S rRNA gene sequence analysis

Prior to any bioinformatic processing, the raw 18S and 16S rRNA gene sequences were uploaded to the National Centre for Biotechnology Information's (NCBI's) Sequence Read Archive (SRA), with the BioProject ascension number PRJNA356784. Analysis of the 18S and 16S rRNA gene sequencing data was performed using the QIIME software package in order to determine the phylogenetic structure of the microbial community (Caporaso et al. 2010a). Forward and reverse reads of the 16S and 18S rRNA genes were joined at paired ends and aligned. The sequences were demultiplexed, filtered and processed through the QIIME software package (Caporaso et al. 2010a).

The 16S rRNA gene sequences were compared to those in the GreenGenes Bacterial and Archaeal 16S rRNA gene database (DeSantis et al. 2006) using BLAST, picking operational taxonomic units (OTUs) at a 97% similarity cut-off . Representative sequences from each OTU were aligned using the PyNAST tool (Caporaso et al. 2010b), and chimeric sequences were identified and removed using ChimeraSlayer (Haas et al. 2011). The 18S rRNA gene sequences were assigned to OTUs via a *de novo* approach using USEARCH v5.2.236 (Edgar 2010). Representative sequences from each OTU were checked for chimeric sequences, and these were removed using

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UCHIME v6.1.544 (Edgar et al. 2011). The resulting OTUs were assigned taxonomy by comparison to the SILVA 16S/18S rRNA gene database (SILVA 119, Quast et al. 2013) using Blastall v2.2.22 . All OTUs that were assigned to prokaryotic taxa were then removed from the 18S rRNA gene dataset.

OTUs representing >1% abundance were processed through the NCBI BLAST program and assigned taxonomies according to highest sequence similarity. The resulting BLAST assigned identities were compared to the taxonomic identities assigned by the GreenGenes and SILVA databases for the 16S rRNA (Supplemental Information Table 1) and 18S rRNA sequencing (Supplemental Information Table 2) respectively.

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240 **Results**

241 *Groundwater chemistry*

242 The geochemical conditions of the groundwater at the time of sampling are presented
243 in Table 1. The groundwater pH was slightly acidic and contained SCN^- in addition to
244 a small concentration of NH_4^+. The prevailing redox conditions in the groundwater
245 were reducing, with low oxygen levels.

246

247 *Groundwater enrichment culturing experiments*

248 During the initial enrichment experiment, no SCN^- removal was noted in the absence
249 of oxygen, regardless of nutrient amendment (see Table 2 for initial and final SCN^-
250 concentrations). In the oxic enrichment experiments, SCN^- removal was recorded in the
251 absence of any nutrient amendment; however, upon inoculation of this culture into
252 filter-sterilised groundwater, no SCN^- removal was observed. The sole addition of DOC
253 or PO_4^{3-} also resulted in SCN^- biodegradation in the initial enrichment, but when re-
254 cultured, SCN^- degradation did not occur. The only condition to result in a SCN^-
255 degrading culture, which was culturable in filter-sterilised groundwater, was the
256 addition of DOC and PO_4^{3-} in the presence of air. Initially, complete removal of SCN^-
257 (from approximately 130 mgL^{-1}) was achieved within 4 days, through combined
258 addition of DOC and PO_4^{3-}. This culture was used for subsequent experiments.

259

260 *SCN^- and NH_4^+ biodegradation by a consortium of groundwater microorganisms*

261 The enriched microbial consortium, amended with DOC and PO_4^{3-}, was further
262 investigated to determine the fate of the NH_4^+ released by SCN^- degradation. The

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consortium completely degraded SCN^- in the filter-sterilised groundwater within a period of 50 hours (Fig. 1). The initial NH_4^+ present in the groundwater was consumed prior to any SCN^- removal. After this, an increase in optical density (OD_{600}) was noted, in tandem with the consumption of SCN^- and formation of NH_4^+. The concentration of NH_4^+ decreased to below detection after all SCN^- had been consumed.

Inhibition of SCN^- biodegradation by NH_4^+ addition

Further experimentation was conducted to determine the effect NH_4^+ had upon SCN^- biodegradation. This work revealed that low to moderate concentrations of NH_4^+ did not inhibit biodegradation of SCN^- (Fig. 2). The highest NH_4^+ concentration however, completely inhibited SCN^- biodegradation. SCN^- degradation occurring at lower NH_4^+ concentrations only proceeded after complete NH_4^+ removal (Appendix A, Fig. A1).

Microbial community characterisation by 16S rRNA gene sequencing

The taxonomic assignments for the 16S rRNA gene sequences, from the enriched groundwater community are given in Fig. 3a. At the phylum level, the microbial community enriched through DOC and PO_4^{3-} addition and exposure to air in the groundwater was dominated by *Proteobacteria* (72.8%) and *Bacteroidetes* (25.8%), with a minor proportion of *Actinobacteria* (1.3%). The dominant families within the *Proteobacteria* were *Phyllobacteriaceae* (27.8%), *Rhodobacteriaceae* (12.8%) and *Sphingomonadaceae* (12.5%). The latter was entirely assigned to the *Novosphingobium* genus, and the dominant OTU found to be most closely related to *Novosphingobium panipatense* strain UMTKB-4 (99%), by comparison to the NCBI database (Appendix A, Table A1). The *Phyllobacteriaceae* family was dominated by a single unclassified

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OTU (27.8%), most closely related to an uncultured *Mesorhizobium* sp. clone S3_F08 (99% similarity). The two dominant OTUs for the *Rhodobacteraceae* family were most closely related to an uncultured bacterium clone MAL_E01 (12.8% abundance, 99% similarity), the higher abundance of the two OTUs had equal sequence similarity to an environmental sample, *Thioclava pacifica* (98% similarity) known to be capable of sulfur oxidation and consumption of simple organics (Sorokin et al., 2005). In addition to these high abundance members, sequences assigned to the genera *Martelella* sp. (4.0%) and *Xanthobacter* (5.4%) made up significant minority taxa.

The *Bacteroidetes* phylum was largely dominated by a single OTU unassigned using the Greengenes database below family level, but most closely related to an uncultured *Owenweeksia* sp. Clone (99% similarity). Other *Bacteroidetes* sequences assigned to the *Flammeovirgaceae* family belong to the *Roseivirga* genus.

Microbial community characterisation by 18S rRNA gene sequencing

The 18S rRNA gene analysis showed a simple eukaryotic distribution (Fig. 3b), with only 2 unique OTUs identified: *Tremella indecorata* (98.5%), a fungus of the *Basidiomycota* phylum and *Jakoba libera* (1.5%), a trophic flagellate from the *Loukozoa* phylum (Fig. 3b). The SILVA assigned taxonomies for these OTUs were compared to the identities assigned by the NCBI database by BLAST (Appendix A, Table A2). The dominant OTU, *Tremella indecorata* was most closely related as *Tremellales* sp. LM630 (99% similarity), while the less abundant OTU was most closely related to *Jakoba libera* at 99% gene sequence similarity, which is consistent with the SILVA identity.

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311 **Discussion**

312 The microbial consortium enriched from SCN^- contaminated groundwater was able to
313 consume SCN^- aerobically in the presence of all nutrient amendments; however, under
314 anoxic conditions, no SCN^- degradation was observed. This dependence on oxygen
315 (present as air) supports the interpretation that SCN^- degradation progressed via aerobic
316 respiration. In fact, anoxic pathways have not been observed, with a notable exception
317 through coupling to nitrate or nitrite reduction (Sorokin et al. 2004). Significantly, only
318 the culture amended with both DOC and PO_4^{3-} resulted in a microbial consortium
319 capable of repeatedly performing SCN^- degradation. This suggests should an attempt
320 be made to promote *in situ* SCN^- degradation in the groundwater by the extant microbial
321 community, addition of both these nutrient sources would be required for sustained
322 contaminant removal.

323

324 The observed preferential consumption of NH_4^+ suggests that SCN^- was also being
325 utilised primarily as a nitrogen source by the consortium. Both autotrophic and
326 heterotrophic SCN^--degrading organisms are known to assimilate the NH_4^+ released
327 from SCN^- degradation as their sole source of nitrogen (Stafford and Callely 1969). The
328 presence of NH_4^+ likely represented a preferential source of nitrogen, in comparison to
329 SCN^-, thereby potentially inhibiting degradation (Stafford and Callely 1969). In the
330 absence of added NH_4^+, SCN^- degradation released NH_4^+ as an end product. The NH_4^+
331 was then subsequently removed, potentially due to its consumption as a growth nutrient,
332 as a driver for heterotrophic bacteria to degrade SCN^-, although other microbial
333 community members not capable of this trait may also have assimilated the NH_4^+.
334 Significantly, the consumption of NH_4^+ indicated that the consortium was capable of
335 preventing inhibition of SCN^- biodegradation by higher NH_4^+ concentrations. Although

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NH_4^+ removal through oxidation is also a possibility, no known autotrophic bacteria or archaea widely responsible for this metabolic trait were identified in this microbial community.

As the consortium may have utilised SCN^- as a source of energy, sulfur, nitrogen or carbon (Gould et al. 2012), a number of metabolic niches might be associated with its degradation and the cycling of the released nutrients. 16S rRNA gene sequencing identified few taxa known to contain SCN^--degrading bacteria, the most abundant being an OTU (representing 4.0% abundance) assigned to the *Sphingomonadaceae* family, known to contain SCN^--degrading species within the *Sphingopyxis* and *Sphingomonas* genera (du Plessis et al. 2001; Felföldi et al. 2010).

The consortium was found to include dominant genera that share significant sequence similarity to a known sulfur-oxidising genus, *Thioclava* sp., which has demonstrated chemoautotrophic growth on intermediate sulfur compounds including thiosulphate, and heterotrophic growth on simple organics including glucose (Sorokin et al. 2005). We note that certain *Thioclava* species may consume NH_4^+ as a source of nitrogen (Sorokin et al. 2005). Furthermore, nitrogen-fixing bacteria were also represented in the community: *Novosphingobium* (Kaneko et al. 2000), potentially having a role in nitrogen supply when NH_4^+ was absent.

The cultured microbial consortium was dominated by heterotrophs that likely played an important role in the cycling of carbon and possibly nitrogen. *Martelella* sp., *Thioclava* sp., *Novosphingobium* sp., *Roseivirga* sp. and *Basidiomycota* are all known to consume various forms of organic carbon, including glucose (Chung et al. 2016;

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Sorokin et al. 2005; Chen et al. 2015; Nedashkovskaya et al. 2008; Prillinger and Lopandic 2015). The dominance of heterotrophs in this consortium suggests that the SCN^- may mostly have been degraded by heterotrophs as a source of nitrogen, rather than by autotrophs utilizing sulfur oxidation as an energy source. This interpretation can be compared against previously documented communities dominated by autotrophic SCN^--degraders often belonging to the *Thiobacillus* genus (Felföldi et al. 2010; Huddy et al. 2015; Kantor et al. 2015; Kantor et al. 2017; Watts et al. 2017). The importance of heterotrophs in the SCN^--degrading consortium is not well understood, with only *Sphingomonadaceae* family containing known SCN^--degrading strains. Their ability to prevent the accumulation of the inhibitor NH_4^+, likely through assimilation, may be significant when considering the implementation of a bioremediation strategy. Previous SCN^--degrading communities have also been shown to be incapable of preventing NH_4^+ accumulation from SCN^- degradation (Shoji et al. 2014), while other approaches have coupled SCN^--biodegradation to nitrification/denitrification (Villemur et al. 2015) or assimilation to biomass by algae (Ryu et al. 2014). The coupling of SCN^--biodegradation with microbial NH_4^+-removal is an important requirement to perform the complete bioremediation of SCN^- and its potential intermediate degradation products.

Conclusion

The results presented here demonstrate that naturally occurring SCN^--degrading microbial consortia could be enriched and directly stimulated from SCN^- contaminated groundwater. The promotion of this extant microbial community, already adapted to the presence of SCN^- and the prevailing groundwater chemistry, would preclude the need to bio-engineer externally a mixed community or pure culture, which may be ill-

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suited to these conditions. Interestingly, unlike other reported SCN^--degrading bioreactor communities (Huddy et al. 2015, Ryu et al. 2015, Kantor et al. 2014; Watts et al. 2017), our consortium did not contain significant populations of *Thiobacilli*, previously implicated as the principle SCN^--degraders.

When considering *in situ* SCN^- bioremediation solutions, the lack of oxygen in the groundwater appears to be the most important inhibitor of SCN^- biodegradation. Exposing contaminated groundwater to air may therefore stimulate SCN^- biodegradation, a significant finding considering that most TSFs are unlined and therefore result in seepage of SCN^- through to poorly-oxygenated groundwater. This fact suggests *in situ* natural attenuation may be an oxygen-limited process, with implications for the design of a bioremediation strategy involving both nutrient and air amendments in a controlled bioreactor containing SCN^--degrading microorganisms sourced from locally contaminated groundwater.

Acknowledgements

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585 **TABLE AND FIGURE CAPTIONS**

586

587 **Table 1:** Groundwater chemistry at the time of sampling. Errors are equal to 1
588 standard deviation within triplicate samples of the groundwater.

589

590 **Table 2:** Enrichment experiment results showing changes in SCN^- concentration.
591 Errors are shown as 1 standard deviation within triplicate samples of each culture.

592

593 **Fig. 1.** Concentration profiles of SCN^- and NH_4^+. The profiles are shown alongside
594 OD_{600} measurement during SCN^- removal from filter sterilised groundwater, inoculated
595 with the groundwater culture enriched by addition of DOC and PO_4^{3-}. Error bars are
596 equal to 1 standard deviation within each triplicate.

597

598 **Fig. 2.** Concentration profile of SCN^- and NH_4^+ in groundwater. Profiles represent
599 the inoculated (with the DOC and PO_4^{3-} enriched community) filter sterilised
600 groundwater, in the presence of increasing NH_4^+ concentrations. Error bars are equal
601 to 1 standard deviation within triplicates of each experiment.

602

603 **Fig. 3:** The relative abundance of 16S rRNA gene sequence assignments from the
604 Greengenes database **(a)** and 18S rRNA gene sequence assignments from the SILVA
605 database **(b)**. Relative abundances are shown for the re-cultured groundwater
606 community amended with DOC and PO_4^{3-}. 16S rRNA gene Taxonomic assignments
607 are defined at the phylum (inner circle), family (middle) and genus (outer) levels,
608 while 18S rRNA gene taxonomic assignments are classified at the phylum (inner),

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609 class (middle) and species (outer) levels. Classified taxa comprising ≥ 1% total
610 abundance are labelled, and grey areas represent unclassified taxa.
611
612 **Table A1.** OTU identity comparison for 16S rRNA sequences between BLAST and
613 GreenGenes classification.
614
615 **Table A2.** OTU identity comparison for 16S rRNA sequences between BLAST and
616 GreenGenes classification.
617
618 **Fig. A1.** SCN^- degradation profiles with increasing NH_4^+ concentrations by the
619 enriched microbial consortium. **(a)** With unamended NH_4^+ levels, **(b)** with moderate
620 NH_4^+ levels, and **(c)** with high NH_4^+ levels.
621

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Table 1(on next page)

Groundwater chemistry at the time of sampling.

Errors are equal to 1 standard deviation within triplicate samples of the groundwater.

1

Groundwater Chemistry	
SCN^- (mg L^{-1})	135 ± 1.73
NH_4^+ (mg L^{-1})	8.9 ± 1.9
pH	6.5
Dissolved O_2 (%)	2.0
E_H (mV)	-259.6

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Table 2 (on next page)

Enrichment experiment results showing changes in SCN^- concentration

Errors are shown as 1 standard deviation within triplicate samples of each culture

1

Oxygen Amendment	Nutrient Amendment	SCN^- initial (mgL^{-1})	SCN^- final (18 days) (mgL^{-1})
Anoxic	None	131 ± 2.3	132 ± 6.0
	DOC	125 ± 8.8	132 ± 2.0
	PO_4^{3-}	129 ± 3.6	130 ± 9.3
	DOC, PO_4^{3-}	129 ± 5.3	135 ± 2.4
Oxic	None	128 ± 3.1	0.67 ± 1.2
	DOC	126 ± 2.5	5.00 ± 7.8
	PO_4^{3-}	131 ± 3.0	0.00 ± 0.0
	DOC, PO_4^{3-}	123 ± 3.1	0.33 ± 0.6

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Figure 1 (on next page)

Concentration profiles of SCN^- and NH_4^+

The profiles are shown alongside OD_{600} measurement during SCN^- removal from filter sterilised groundwater, inoculated with the groundwater culture enriched by addition of DOC and PO_4^{3-}. Error bars are equal to 1 standard deviation within each triplicate.

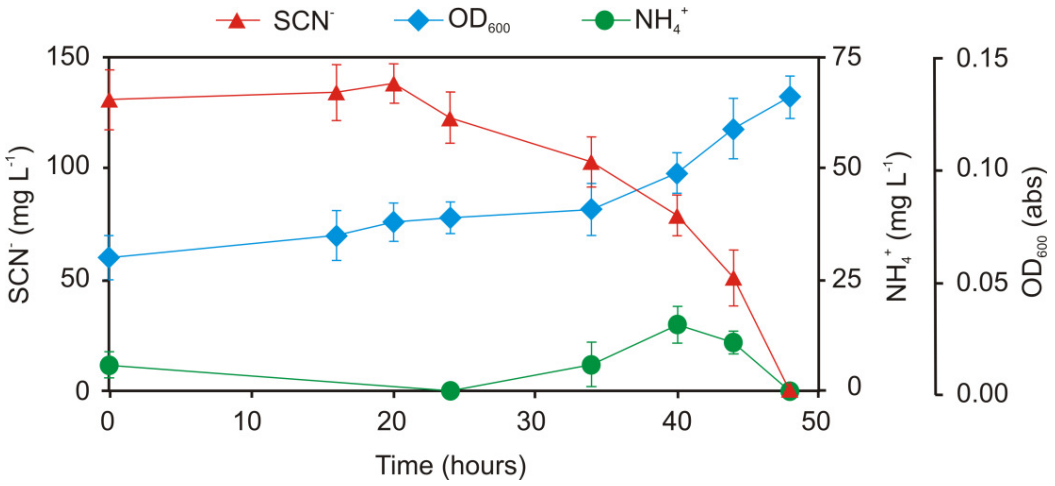

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Figure 2 (on next page)

Concentration profile of SCN^- and NH_4^+ in groundwater

Profiles represent the inoculated (with the DOC and PO_4^{3-} enriched community) filter sterilised groundwater, in the presence of increasing NH_4^+ concentrations. Error bars are equal to 1 standard deviation within triplicates of each experiment

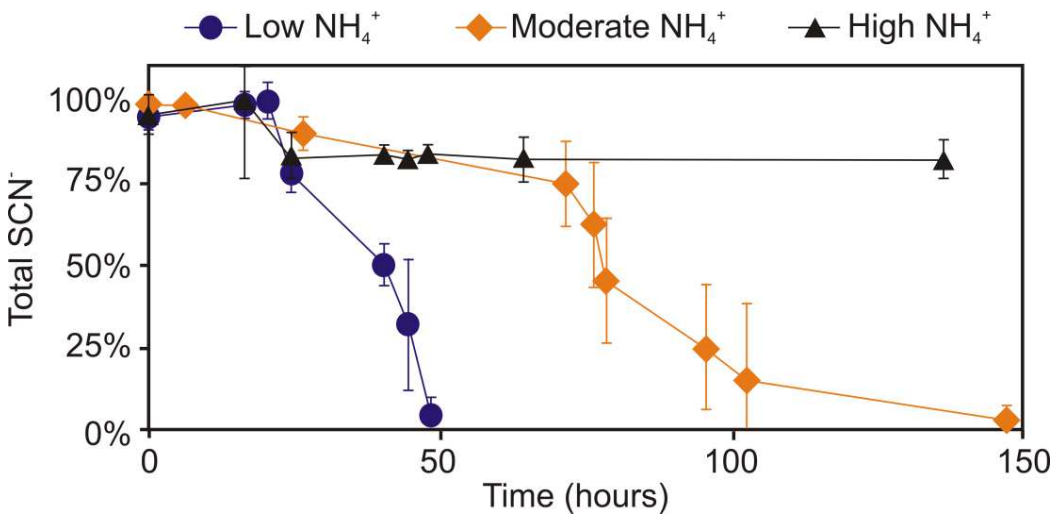

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Figure 3 (on next page)

The relative abundance of 16S rRNA gene sequence assignments from the Greengenes database (a) and 18S rRNA gene sequence assignments from the SILVA database (b).

Relative abundances are shown for the re-cultured groundwater community amended with DOC and PO_4^{3-}. 16S rRNA gene Taxonomic assignments are defined at the phylum (inner circle), family (middle) and genus (outer) levels, while 18S rRNA gene taxonomic assignments are classified at the phylum (inner), class (middle) and species (outer) levels. Classified taxa comprising ≥ 1% total abundance are labelled, and grey areas represent unclassified taxa.

1

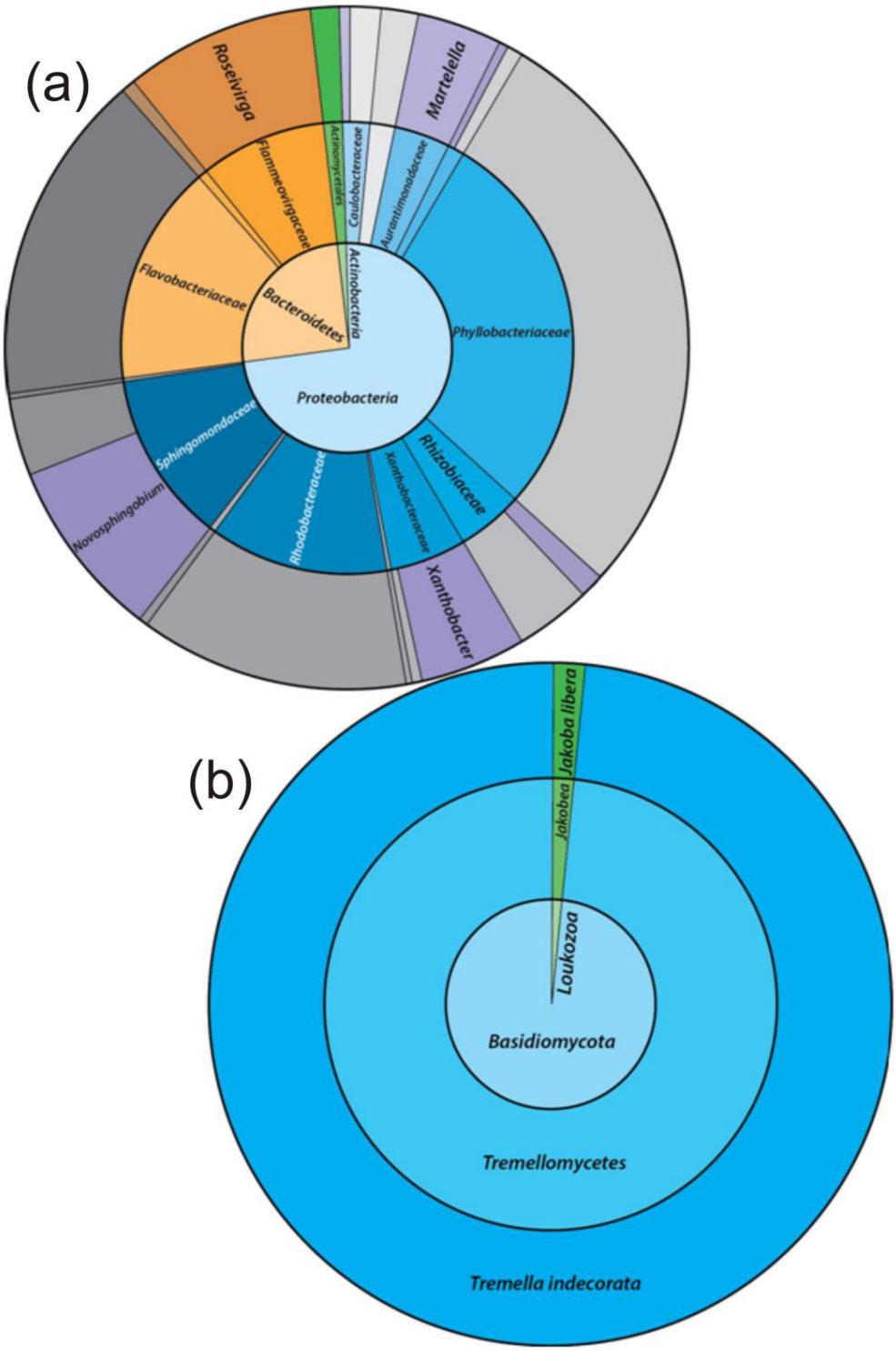

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Table 3(on next page)

OTU identity comparison (1st)

OTU identity comparison for 16S rRNA sequences between BLAST and GreenGenes classification.

Sequence similarity	BLAST Identity	GenBank Sequence ID	GreenGenes Identity
99%	Uncultured *Mesorhizobium* sp. clone S3_F08	KP182007.1	*Phyllobacteriaceae* (family)
99%	Uncultured *Owenweeksia* sp. clone	JX530590.1	*Flavobacteriaceae* (family)
99%	Uncultured bacterium clone MAL_E01 (#2 on list: 98% Thioclava sp.)	KR921275.1	*Rhodobacteriaceae* (family)
99%	*Roseivirga* sp. D-25	KM587636.1	*Roseivirga* sp.
99%	*Novosphingobium panipatense* strain UMTKB-4	KT025847.1	*Novosphingbium* sp.
99%	Uncultured bacterium clone nbw390g07c1	GQ096648.1	Sphingomonadaceae (family)
99%	*Martelella* sp. YC7034	KR233160.1	*Martelella* sp.
99%	*Martelella mediterranea* strain NJES-108	KR140271.1	*Rhizobiaceae* (family)
99%	Uncultured bacterium clone 0010Ak1_E2	KF558803.1	*Martelella* sp.
99%	Uncultured *rhodospirillales* bacterium clone KF130_10F01	EU361456.1	*Kiloniellales* (order)
99%	*Xanthobacter* sp. LAA-2009-i49 strain i49	FN298500.1	*Xanthobacter autotrophicus (sp.)*
99%	Uncultured bacterium clone HK34-1-10-1	KX163485.1	*Caulobacteriaceae* (family)
99%	*Xanthobacter Flavus* strain LEM28	KU180350.1	*Hyphomicrobaceae* (family)

Table 4(on next page)

OTU identity comparison (2nd)

OTU identity comparison for 16S rRNA sequences between BLAST and GreenGenes classification.

Sequence similarity	**BLAST Identity**	**GenBank Sequence ID**	**SILVA Identity**
99%	*Jakoba libera*	AY117418.1	*Jakoba libera* (sp.)
99%	*Tremellales* sp. (LM630 strain)	EF060914.1	*Tremella indecorata* (sp.)

Figure 4(on next page)

SCN^- degradation profiles with increasing NH_4^+ concentrations by the enriched microbial consortium.

(a) With unamended NH_4^+ levels, **(b)** with moderate NH_4^+ levels, and **(c)** with high NH_4^+ levels.