SPECIALIST STUDIES – SECTION C

C2.3 Thiobacteria

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SUMMARY

Sulphur bacteria distribution in the MLA 170 mine licence area were investigated by Bronwyn Kirby, Next Generation Sequencing Facility Department of Biotechnology, University of the Western Cape

Bacterial genera involved in both sulphur-oxidisation and sulphur-reduction were found to be present in the samples obtained from the proposed mining area. The numbers of sulphur-oxidising bacteria were lower than the numbers of sulphur-reducing bacteria. Sulphur-oxidising bacteria utilise H_2S as fuel, oxidising it into sulphate. This keeps the H_2S within the sediment, and prevents it from being released into the water column. In contrast to this process, sulphur-reducing bacteria reduce sulphate, through respiratory processes, into H_2S, and so are characteristic of sediments where higher concentrations of H_2S are present (Jorgensen 1977). The presence of the sulphur-utilising groups of bacteria corroborates the presence of hypoxic sediments within the mining licence block, as both groups are associated with low oxygen conditions (Jorgensen 1977).

The bacterial genera involved in sulphur reduction identified in the samples obtained included *Desulfobacteralis*, *Desulfovibrionales*, *Syntropobacterialis*, *Desulfoto-maculum*, *Desulfosporomusa* and *Desulfosporosinus*. The bacterial species involved in sulphur oxidisation included *Thiobacillus thiooxidans*, *Thiobacillus ferrooxidans*, *Thiobacillus denitrificans* and *Acidothiobacillus* spp. In general, *Thiobacillus* species have a low growth yield (Jorgensen and Nelson 2004), which explains the lower concentrations of sulphur-oxidising bacteria in comparison to sulphur-reducing bacteria within the study region. Because of these low growth yields and less efficient oxidising capabilities, *Thiobacillus* spp. are of less significance in the oxidisation process of H_2S, compared with the large sulphur bacteria, that have been found in the Namibian continental shelf region (Jorgensen and Nelson 2004, Brüchert *et al*. 2003). None of the large sulphate bacteria, namely from the genera *Thiomargarita*, *Beggiatoa* and *Thioploca*, which have developed more specialised modes of sulphur oxidisation and that play a more significant role in the oxidisation of H_2S, was found in the samples from the mining licence area (MLA 170).

The sediment properties analysed in this verification survey indicate that there are low acid volatile sulphide (AVS) concentrations in the sediment. AVS is used as a proxy for H_2S production and sulphate reduction. The apparent low levels of H_2S production and sulphate reduction occurring within sediments in the mining area corroborate the absence of large sulphur bacteria within the samples and would suggest that the levels of H_2S flux within the area are too low to support large populations of these bacteria. Instead less conspicuous sulphur-oxidising bacteria, such as *Thiobacillus* spp. are active.

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1 SAMPLE INFORMATION

Samples were delivered by Lwandle Technologies in March 2014 in a -20°C freezer (Appendix 1.3 and 1.4). 1 g aliquots were immediately prepared which were stored at -80°C until analysis. Samples were collected in sterile Nalgene bottles. Four separate samples were collected at each sampling time; two samples were used for DNA extraction while two samples were treated with RNAlater (Sigma) for RNA extraction.

Table 1: Samples used for qPCR analysis

Identification on collection bottle	Sample ID	Sample description
To:01 Non1 12:13 26/02/2014	Sample LB1	Sediment/shell mix, course sand. Extraction no metallic layer
To:02 Non1 02:28 26/02/2014	Sample LB2	Sediment/shell mix, course sand. Extraction no metallic layer
To:03 Non1 03:05 26/02/2014	Sample LB3	Sediment/shell mix, course sand. Extraction no metallic layer
To:04 Non1 04:02 26/02/2014	Sample LB4	Soil/seawater Extraction had slight metallic layer
To:05 Non1 05:05 26/02/2014	Sample LB5	Sediment/shell mix, course sand. Extraction had slight metallic layer
To:06 Non1 07:00 26/02/2014	Sample LB6	Medium-Dark brown fine sediment Extraction had slight metallic layer
To:07 Non1 07:15 26/02/2014	Sample LB7	Medium-Dark brown fine sediment Extraction had slight metallic layer
To:08 Non1 07:50 26/02/2014	Sample LB8	Medium-Dark brown fine sediment Extraction had thick metallic layer
To:09 Non1 08:50 26/02/2014	Sample LB9	Dark brown, very fine sediment Extraction had thick metallic layer
To:10 Non1 09:40 26/02/2014	Sample LB10	Dark brown, very fine sediment Extraction had thick metallic layer

*During DNA extraction after the first centrifugation step several samples had a gold/yellow metallic layer floating on the top of the sample. As metals can inhibit the enzymes used for DNA/RNA amplification this layer was removed.

2 METHODOLOGY

Different nucleic acid extraction methods preferentially extract nucleic acids from particular microbial taxa. Therefore, in order to overcome this bias two different extraction methods were employed for both RNA and DNA extraction. The two separated nucleic acid extractions for each sample were pooled in equimolar ratios prior to analysis [DNA analysis – MoBio kit sample and Wang method sample; RNA analysis – RNeasy and $LiCl_2$ method sample].

2.1.1 Extraction of metagenomic DNA

Genomic DNA was extracted using a modified version of the method described by Wang *et al.* (1996). Sediment samples were thawed on ice and approximately 1 g of sediment was used per extraction. Samples were resuspended in 2-3 ml lysis buffer (25 mM Tris-HCl pH 8; 50 mM glucose; 10 mM EDTA; 25 mg lysozyme per ml) and 0.5 g quartz sand (Sigma S-9887) was added to each tube. Microbial cells were lysed by both mechanical and chemical methods. Mechanical shearing involved three cycles of vortexing at maximum speed for 1 min followed by rapid cooling on ice for 2 min. After vortexing the samples were incubated in lysis buffer overnight at 37°C. SDS was added to a final concentration of 1% and the samples were incubated at 65°C for 30 min. Nucleic acids were extracted twice with 1 vol equilibrated phenol (pH 7.6), followed by extraction with 1 vol chloroform:iso-amyl alcohol (24:1, vol/vol). Nucleic acid was precipitated with 1 vol ice-cold isopropanol at room temperature for 30 min and harvested by centrifugation at 14 000 X g for 5 min. The resulting pellet was resuspended in 50 µl 10 mM Tris-HCl; 1 mM EDTA (TE) buffer pH 7.8.

DNA was also extracted using the Ultra PowerSoil kit (MoBio) according to the manufacturer's instructions. Two separate extractions using 500 mg of sediment were performed per sample and the nucleic acid was pooled in the final steps.

2.1.2 Extraction of metagenomic RNA

Total cellular RNA was extracted using the CTAB lysis buffer method adapted from Griffiths *et al*., 2000. Sediment samples stored in RNAlater were thawed at room temperature. In order to remove the RNAlater, samples were vortexed briefly and centrifuged at 4000 x g for 3 min. The RNAlater was removed and the sediment was resuspended in 3 ml DEPC-treated phosphate buffered saline (PBS) and centrifuged as above. After the removal of the PBS, 2 ml CTAB buffer (100 mM Tris-HCl pH 8, 1.4 M NaCl, 20 mM EDTA pH 8, 2% CTAB (wt/vol), 1% polyvinylpyrollidone, 0.4% β-mercaptoethanol (vol/vol), prepared in DEPC-treated H_2O) and 0.5 g each chilled 0.1 mm glass and 0.5 mm silica beads. Lysis was achieved by three cycles of vortexing the samples for 30 s followed by rapid chilling on ice for 1 min. After the addition of 0.8 ml chloroform:isoamylalcohol (24:1, vol/vol), samples were shaken at room temperature for 15 min. Samples were centrifuged for 15 min at 4°C at 15000 x g. Total nucleic acids were precipitated by the addition of 5 M NaCl and 1 vol ice-cold isopropanol. Samples were incubated at -80°C for 30 min. Nucleic acids were harvested by centrifugation at 15000 x g for 15 min at 4°C. The pellet was washed in ice cold 70% ethanol, dried for 15 min and resuspended in 20 µl DEPC-treated water.

In addition, RNA was extracted using the RNeasy kit (Qiagen) according to the manufacturer's instructions.

2.1.3 cDNA conversion

In order to remove genomic DNA, 0.4U shrimp nuclease (ThermoScientific) was added to a 5 µl aliquot of the RNA extraction and incubated at 25°C for 30 min. RNA was extracted with 8 mM $LiCl_2$, harvested by centrifugation at 14000 x g for 30 min at 4°C and the resulting pellet was resuspended in 15 µl DEPC-treated water. The concentration and the purity of the RNA was determined using a NanoDrop ND-100 spectrophotometer and samples were stored at -80°C. 1 µg of total RNA was used as the template in the synthesis of cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions and stored at -20°C. cDNA was quantified using the Nanodrop. To determine RNA integrity the ratio of 23S to 16S rRNA gene was assessed by gel electrophoresis.

2.1.4 Primer design

Primers for quantitative PCR (qPCR) analysis were adapted from published primers used for detection of bacteria in environmental samples. Primers for detecting bacterial genera involved in sulphur reduction detected both the phylum Delta Proteobacteria (the genera *Desulfobacterales, Desulfovibrionales* and *Syntropobacterales*) and members of the phylum Firmicutes (the genera *Desulfotomaculum, Desulfosporomusa* and *Desulfosporosinus*). Primers for the detection of sulphur oxidising bacteria, detected several bacterial species including *Thiobacillus thioooxidans, Thiobacillus ferrooxidans, Thiobacillus denitrificans* cluster and several *Acidothiobacillus* species.

Table 2: Primers designed for qPCR analysis for detection of sulphur utilizing bacteria.

Detection	Primer name	Primer sequence (5' to 3')	Reference
Sulphur reducing bacteria	Delta-13200F	AGGAATWTTGCGCAATGG	Scheid & Stubner, 2001
	Delta-00432R	AGTTAGCCGGTGCTTCCT	Lűcker *et al.*, 2007
Sulphur oxidising bacteria	Thio3-615F	TGGGAATGGCGGTGGAAAC	Kyselkova*et al.,* 2009
	Thio820-810R	CACCAAACATCTAGTATTCATCG	Pecci*et al.,* 2000

2.1.5 Quantitative PCR (qPCR)

qPCR was performed using SYBR Green Master Mix (Roche) according to the manufacturer's instructions. cDNA was diluted 1/5 in sterile water and 5µl was used per PCR reaction. qPCR analysis was performed on a Roche LC480 and Cycling conditions for qRCR were as follows: Detla-13200F/Delta-00432R primers – denaturation 95°C for 2 min, followed by 45 amplification cycles of 95°C for 10 sec, annealing at 51°C for 10 sec and elongation at 72°C for 10 sec. Thio3-615F/Thio820-810R primers denaturation 95°C for 2 min, followed by 40 amplification cycles of 95°C for 10 sec, annealing at 56°C for 20 sec and elongation at 72°C for 25 sec. Absolute quantification was performed using a dilution series of the resulting amplicons (range 100 copies to 10^6 copies per reaction). In order to determine the number of bacteria per gram of soil, the number of copies of the 16S rRNA gene detected per reaction (5µl cDNA used) was multiplied by 15.

3 DATA ANALYSIS

3.1.1 QC of nucleic acid extractions

For metagenomic DNA extracted from sediment an A260/280 ratio of greater than 1.60 is acceptable due to the presence of contaminating humic and/or organic compounds (Table 3). The DNA extracted from all samples was considered to be of reasonably good quality (ratio great than 1.6 and non-degraded). Samples 1, 2, 3, 5 and 10 contained a large amount of PCR inhibitors and required additional clean up steps. DNA and RNA extraction was repeated on sample LB10 three times, but this sample consistently failed to amplify.

Table 3: Concentration of nucleic acid extracted for each sample.

Sample	Extraction method	DNA concentration (ng/µl)	A260/A280	Extraction method	RNA concentration (µg/µl)	A260/A280
LB1	Wang method MoBio Kit	30.9 24.6	1.75 1.72	$LiCl_2$ method RNeasy kit	2.1 1.8	1.92 1.94
LB2	Wang method MoBio Kit	87.1 91.4	1.69 1.71	$LiCl_2$ method RNeasy kit	3.2 4.4	1.88 1.92
LB3	Wang method MoBio Kit	32.2 39.0	1.86 1.82	$LiCl_2$ method RNeasy kit	2.5 2.8	2.10 2.08
LB4	Wang method MoBio Kit	105.5 74.2	1.62 1.65	$LiCl_2$ method RNeasy kit	7.9 6.4	1.94 1.98
LB5	Wang method MoBio Kit	37.5 42.7	1.68 1.72	$LiCl_2$ method RNeasy kit	2.2 1.9	1.97 1.91
LB6	Wang method MoBio Kit	59.7 53.8	1.77 1.61	$LiCl_2$ method RNeasy kit	3.1 3.6	2.03 2.07
LB7	Wang method MoBio Kit	76.4 136.1	1.83 1.67	$LiCl_2$ method RNeasy kit	4.2 5.9	1.93 1.95
LB8	Wang method MoBio Kit	187.4 173.0	1.71 1.71	$LiCl_2$ method RNeasy kit	7.8 6.3	2.04 1.99
LB9	Wang method MoBio Kit	41.1 25.3	1.75 1.80	$LiCl_2$ method RNeasy kit	2.8 3.1	1.94 2.03
LB10	Wang method MoBio Kit	146.6 103.2	2.70 2.43	$LiCl_2$ method RNeasy kit	1.4 1.6	1.85 1.88

The total RNA extracted was of high quality, particularly the RNA extracted with lithium chloride ($LiCl_2$). In addition, the integrity of the RNA was further assessed by gel electrophoresis to ensure ratio of the 23S rRNA to 16S rRNA gene was great than 2. All the sediment samples passed the required QC standards required for qPCR analysis.

3.1.2 Quantitative Polymerase Chain Reaction (qPCR) analysis

The total number of bacteria detected was within the expected range for marine sediments, which are reported to range from 10^4 bacteria per gram to 10^8 bacteria per gram.

It is estimated that the number of sulphur reducing bacteria per gram of soil ranges from less than 3000 (sample LB1) to 7.2x10^6 (sample LB3). In general, samples with a higher total bacteria number also appeared to have a higher number of sulphur reducing bacteria.

It is estimated that the number of sulphur oxidising bacteria per gram of soil ranges from less than 3800 (samples LB1 and LB6) to 8.9x10^5 (sample LB6). The numbers of sulphur oxidising bacteria present appear to be lower than the number of sulphur reducing bacteria. To ensure that this difference is not due to the primers used in the analysis, three different primer combinations were tested for detection of sulphur utilizing bacteria. All primer combinations gave comparable results (data not shown), and it is therefore probable that the difference in bacterial number are not due to primer efficiency. If additional studies are conducted it is proposed that specific genes involved in sulphur oxidation such as the *soxB* gene (Friedrich *et al*., 2001) also be included in the analysis.

Table 4: Estimated number of sulphur utilizing bacteria based on qPCR analysis. Each experiment was performed in triplicate and the average is reported.

Sample	Number of copies of the 16S rRNA gene detected with universal bacterial primers	Estimated total bacterial counts per gram sediment	Number of copies 16S rRNA gene specific for sulphur oxidising genera	Estimated number of sulphur oxidising bacteria per gram	Number of copies 16S rRNA gene specific for sulphur reducing genera	Estimated number of sulphur reducing bacteria per gram
LB1	2522	3.7x10^4	<250*	3.8x10^3 (<10%	<200	<3000**
LB2	13496	2.0x10^5	1080	1.6x10^4 (8%)	3172	4.8x10^4 (24%)
LB3	3816959	5.7x10^7	24098	3.6x10^5 (0.63%)	477167	7.2x10^6 (13%)
LB4	311225	4.6x10^6	59272	8.9x10^5 (19%)	5284	8.0x10^4 (1.8%)
LB5	301456	4.5x10^6	28829	4.3x10^5 (9.6%)	50536	7.6x10^5 (17%)
LB6	2522	3.7x10^4	<250*	3.8x10^3(<10%)	104	1.6x10^3 (4.3%)
LB7	810190	1.2x10^7	18447	2.8x10^5 (2.3%)	450546	6.8x10^6 (57%)
LB8	130720	2.0x10^6	30728	4.6x10^5 (23%)	16238	2.4x10^5 (12%)
LB9	229145	3.4x10^6	30532	4.6x10^5 (13.5%)	5455	8.2x10^4 (2.4%)
LB10	NA		NA		NA	

* Sample had detectible amplification but it was below the range to accurately determine the copy number for this primer set (range would be between 50 and 250 copies)
** Sample had detectible amplification but it was below the range to accurately determine the copy number for this primer set (range would be between 50 and 200 copies)

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