APPLICATION OF FUNCTIONAL MOLECULAR BIOLOGY TECHNOLOGIES TO THE STUDY OF BIOLOGICAL PROCESSES IN SULFIDIC ENVIRONMENTS.

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SUMMARY
The formation of potential acid sulphate soils is the result of the reduction of SO_4^- and Fe_3^+ under anaerobic reducing conditions. Anaerobic chemoautotrophic bacteria notably the genus *Desulfovibrio* are known to be responsible for the enzymic reduction of Fe and S under these conditions. Biogeochemical transformations of sulfide minerals responsible for the formation of actual Acid Sulfate Soils are carried out by a group of chemolithotrophic bacteria collectively known as sulfur oxidisers. Whilst this group of organisms has been studied in detail, recent advances in molecular genetic analysis have led to the re-classification of many species previously classified as *Thiobacillus*. The ecology of these organisms in sulfidic environments and the relationship between chemolitotrophic bacterial activity and rates of sulfide mineral oxidation are poorly understood. Application of new functional molecular biology techniques is discussed, based on the extraction of nucleic acids from sulfidic sediments and the determination of the presence and expression of the sulfur oxidase gene *sox*B, as a means of determining the mechanistic relationship between biological activity and biogeochemical transformation of sulfide minerals in these environments. Additional molecular techniques that allow the determination of microbial community population dynamics and identification of species based on 16S rRNA analysis are also described.

INTRODUCTION
Formation of floodplain, inland and coastal acid sulfate soils (ASS) is the result of the biological oxidation of pyritic and sulfide minerals. This process is carried out by a group of bacteria known as chemolithoautotrophes. These organisms are highly adapted to low pH environments, and utilise Fe or S as electron donors in their respiratory chain, catalysing Fe and S oxidation through the production of iron or sulfur oxidase enzymes. Bacteria are therefore the key biochemical catalysts of geochemical transformations in these environments. Bacteria responsible for the oxidation of sulfide minerals including As, Cu, Co, Fe, Ni, Mo, Pb, Zn have been isolated from sulfidic environments and have been historically classified as members of the Thiobacillus genus notably (*Thiobacillus* sp., *Acidothibacillus* sp., *Sulfobacillus thermosulfidooxidans* and *Acidanus brierleyi*). Mukhopadhyaya *et al*. (2000) note that chemolithotrophic growth on sulfur was previously considered a conserved genetic trait and taxonomic characteristic of Thiobacillus, i.e., any isolate displaying this biochemical property was automatically classified as Thiobacillus. However, modern molecular analysis (mainly 16S rDNA/rRNA sequence analysis) has discovered a wide range of physiologically and genetically unrelated species inhabiting low pH sulfidic environments that are S chemolithotrophes belonging to the α, β, and γ subgroups of proteobacteria, all these organisms were previously classified as Thiobacillus. For example *Thiobacillus versutus* has been re-classified as *Paracoccus versutus* based on its 16S ribosomal RNA gene sequence (Wodara *et al.* 1997).

The environmental impact and chemistry of ASS are described elsewhere in this proceedings (Hicks *et al.* 2003, Fitzpatrick 2003, Lamontagne *et al.* 2003). Whilst the chemistry of ASS formation is relatively well understood, the role of bacteria in Fe and S oxidation transformations in sulfidic environments, specifically:

- the environmental conditions under which biological S oxidation processes operate such as the role of organic matter fractions (adequate organic C availability is a key requirement for S oxidation);
- the relationship between mineral geochemistry and biological oxidation/reduction (what chemical forms of S are preferentially utilised by chemolithotrophic bacteria). Petri *et al*. (2001) note that chemolithotrophes can utilise a range of S compounds as an energy source including sulfide, elemental S, polythionates and thiosulfate;
- the mechanistic relationship between microbial activity and rates of biogeochemical transformation; and,
- the basic ecology of microbial populations in such environments. This is, in comparison poorly understood, as evidenced by the reclassification of organisms previously grouped as Thiobacillus. This is especially the case under 'field' conditions. Determination of the mechanistic relationship between the activity of S oxidizing chemolithotrophes and the biogeochemical transformation of S compounds in sulfidic environments is essential in the development of tools to predict the likelihood of ASS formation, and rates of geochemical transformation under a range of environmental conditions.

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BIOLOGICAL ACTIVITY AND BIOGEOCHEMICAL TRANSFORMATIONS

Much of the current approach to the study of bacterial S oxidation in sulfidic environments has focussed on the identification of chemolithotropic organisms, species diversity and population dynamics, (Johnson *et al.* 2001, Baker & Banfield 2003) and their relationship to rates of S oxidation. In fact, environmental microbiology research concerning defining the role of biota in driving biogeochemical transformations in general has focussed on establishing the relationship between bacterial diversity and rates of biogeochemical processes (Rogers & Colloff 1999). In general there is a paucity of evidence from available studies linking changes in species diversity or shifts in soil populations to changes in biogeochemical functionality. Wardle & Giller (1997) note that the relationship between the productivity of soil organisms and population diversity is unclear, and most of the data is ambiguous. Bengtsson (1998) went as far as to say that there is no mechanistic relationship between diversity and soil ecosystem function, and that correlations between diversity and function in soils will be non-causal.

FUNCTIONAL MOLECULAR TOOLS

An alternative approach to determining the mechanistic relationship between microbial activity and biogeochemical transformations is to target the 'functional' attributes of microbial populations as opposed to their phylogenic attributes (Rogers *et al.* 2002). This approach is based on targeting the genes that encode for enzymes responsible for key biogeochemical transformations, measuring the presence and expression of the functional gene (core biological driver of geochemical transformations) in environmental samples, and determining its relationship to rates of biogeochemical transformation. We have developed molecular techniques based on the direct extraction of nucleic acids (DNA and RNA) from environmental samples that remove the need to isolate/culture and identify individual organisms. They allow direct comparison of biogeochemical reaction kinetics, gene expression and biotic diversity of organisms, all in the same sample, and represent a major advance over traditional microbiology methods. These techniques are especially applicable to the study of extreme environments such as ASS and specialised groups of organisms such as the chemolithoautotrophes involved in S oxidation. Figure 1 summarises the key stages in our functional molecular technology platform. This technology is currently being successfully applied to the detection, and measurement of expression, of key functional genes involved in C and N biogeochemical transformations such as ammonia monoxygenase (*amo*A), nitrogenase reductase (*nif*H), periplasmic nitrate reductase (*nap*A), nitrite reductase (*nir*S, *nir*K), and chitinase (*chiA*), (Rogers *et al*. 2002)

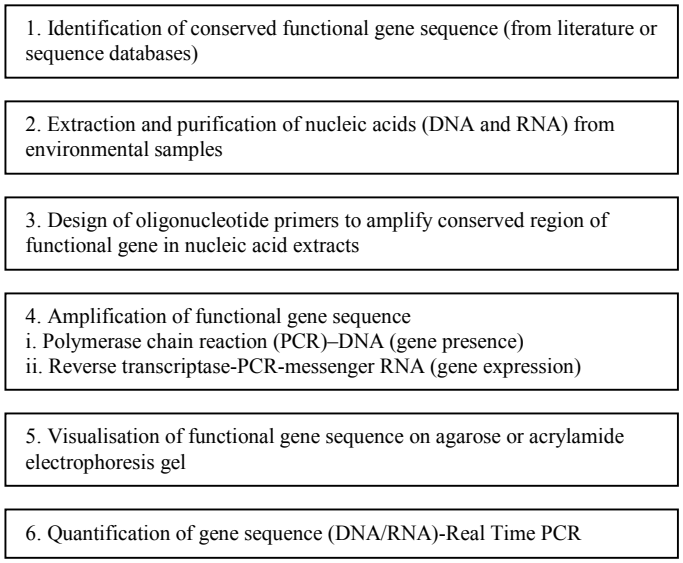

Figure 1: Key stages in functional molecular technology platform for determination and quantification of microbial functional genes in environmental samples

MOLECULAR ANALYSIS OF SULFUR OXIDATION IN SULFIDIC ENVIRONMENTS

Identification of chemolithotropic microbial functional genes involved in S oxidation

The suite of genes encoding for sulfur oxidation in chemolithotrophes has recently been identified (Rother *et al.* 2001). The sulfur oxidase (sox) gene cluster comprises nine genes; soxXYZABCDEF (Friedrich *et al.* 2000). The soxB gene encodes for a diheme cytochrome c enzyme and has been shown to be essential for chemolithotrophic sulfur oxidation (Mukhopadhyaya *et al.* 2000). This functional gene has also been identified in representatives of all known groups of chemolithotrophic sulfur oxidising bacteria, making it an ideal candidate for our functional molecular approach. Degenerate oligonucleotide PCR primers have been designed that amplify a conserved 1000 base pair region of the soxB gene sequence (Petri *et al.* 2001),

allowing us to apply our existing functional molecular techniques to the study of sulfur oxidising chemolithotrophic bacteria activity and presence in sulfidic sediments.

In brief, DNA and RNA will be extracted from environmental samples using established techniques. Oligonucleotide primers will be designed to amplify through PCR soxB gene sequences in sample DNA extracts. The presence of functional genes indicates the presence of the biological potential for S oxidation reactions. Measurement of soxB gene expression indicates active biological S oxidation is taking place, and will in turn be related to rates of S mineral oxidation determined by complementary LEME projects (Hicks *et al.* 2003).

Microbial ecology/population diversity of ASS environments

In addition to the studies of specific bacterial genes involved in chemolithotrophic S oxidation, it is recognised that the general microbial ecology of ASS environments is poorly understood. In order to identify organisms present, numbers of species and population diversity in sulfidic environments 16S rRNA Density Gradient Gel Electrophoresis (DGGE) analysis will be performed (Muyzer 1999). The 16S ribosomal RNA (rRNA) gene is functionally conserved in all prokaryotic organisms (Amann 1995). However, within the 16S gene sequence there are a number of 'variable' regions where the nucleotide sequence is different, each bacterial species has a specific nucleotide sequence within the variable region. In brief the V3 variable region of the 16S gene can be amplified in DNA extracts by PCR using degenerate 16S oligonucleotide primers. PCR products are separated on a DGGE electrophoresis gel, that separates double stranded DNA based on its nucleotide composition, and allows determination of 16S sequence diversity (identification of species numbers and diversity). Individual sequences (bands) from the DGGE gel can be further analysed by sequence analysis in order to identify individual species present. Figure 2 shows a representative DGGE acrylamide gel showing bacterial species 16S gene diversity in DNA extracted for a soil sample. Each band on the gel represents an individual bacterial species.

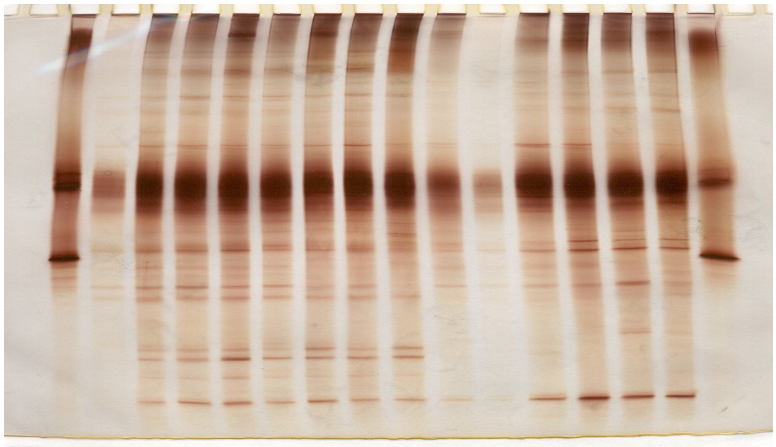

Figure 2: 16S rRNA Density Gradient Gel Electrophoresis profiles of soil bacterial populations. 16S V3 region amplified in soil DNA extracts with degenerate oligonucleotide PCR primers 27FGC and 534R. PCR product visualised on a 35-60% urea/formamide acrylamide gel (8%), 120V, 20hrs.

Extraction of nucleic acids from sulfidic sediments

Work has commenced on extracting nucleic acids from sulfidic sediments collected from sites along the Murray floodplain, for site descriptions (see Hicks *et al.* 2003). Highly reducing or oxidising environments represent challenging conditions from which to extract intact nucleic acids, suitable for functional gene determinations. Exposure of potential ASS to air initiates rapid oxidation of organic material, including nucleic acids, anaerobic sediments also have high levels of DNase enzyme activity, a microbial enzyme responsible for the degradation of DNA (Ruiz *et al.* 2000). In addition sediments are characterised by relatively high organic matter contents, which have the potential to interfere with gene sequence amplification using PCR. Procedures have been developed to prevent degradation of DNA in samples including the addition of EDTA to samples, which halts the activity of the DNase enzyme (Ruiz *et al.* 2000). Figure 3 summarises successful DNA extraction from sulfidic sediments collected from sites along the Murray floodplain. We will be commencing analysis of these samples for the *sox*B sulfur oxidase gene and 16S rRNA bacterial population diversity determination shortly.

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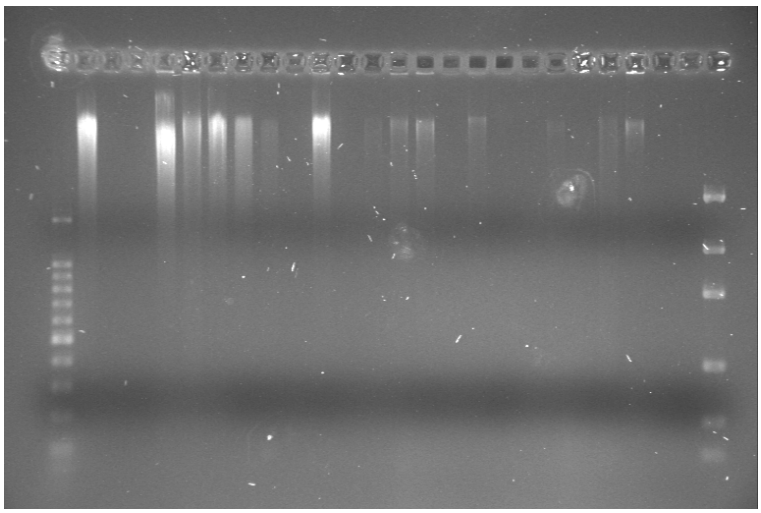

Figure 3: Examples of DNA extracted from Murray floodplain sulfidic sediments visualised on an EtBr stained 2% agarose gel**. Lane 1** 100 bp DNA marker; **Lanes 2-8** Ramco lagoon; **Lanes 9-11** Hearts lagoon; **Lane 12** Ross lagoon; **Lanes 13, 14, 15** Bottle Bend lagoon; **Lane 16** DNA quantification marker.

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