Acid Sulfate Soils

Field report on microbial communities

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1 Overview

1.1 Reminder of the project goals

The project goal was to conduct a pilot assessment of soil microbial communities in a limited number of soil samples (ranging from neutral pH → incubated). The intention is to provide information that will inform potential future work of this nature on a larger scale.

1.2 Summary

Two key goals of the pilot assessment were to ascertain whether or not:

(i) DNA could be successfully extracted from acid sulfate soils (ASS) within River Murray wetlands, and

(ii) Next Generation DNA sequencing could provide a preliminary assessment on the microbial communities present within different wetlands containing a variety of ASS materials.

A total of 12 soil samples were selected from two wetlands: four samples from Spectacle Lakes (Beldora South) and eight samples from Murtho Park (refer to Table 1 below). These wetlands were selected due to the lagoons at Beldora South having fewer wetting and drying cycles than the sites selected at Murtho Park. Soil profiles were located on transects that crossed a waterbody from the top of the bank to the invert of the wetland (lowest position) in the local landscape. At Spectacle Lakes (SPL) two soil profiles were sampled along one transect (named SPL-EF) that crossed a wet lagoon (Beldora South). At Murtho Park (MP) two transects were sampled that crossed wet lagoons, with four soil profiles being sampled. Transect MP-IJ crossed a shallow, saline lagoon in the upper reaches of the wetland complex, while transect MP-EF crossed a fresh water lagoon located nearer pool level. At each profile a topsoil and a subsoil sample were collected, representing (i) the top of the bank at floodplain level and (ii) subaqueous soil (bottom sediment) located at the base of the bank. In addition, two separate (grab) samples were collected from a dry flood-runner creek bed at Mutho Park as this profile contained soil with the most acidic field pH (pHf 5.05) identified at either of the two wetland sites.

To assess how or whether changes in microbial communities could be measured following a period of wetland drying, DNA sequencing was performed on duplicates of all 12 samples, following 8 weeks of progressive oxidation in the laboratory (i.e. the methodology undertake for incubation experiments is detailed in Fitzpatrick et al. 2018). In this report samples that were incubated are demarcated with the suffix “inc” (e.g. MP-IJ 2 2.1.inc).

Incubation experiments supported field observations and analytical testing results which indicated sulfidic material (i.e. soils containing reduced inorganic sulfur (as pyrite) and/or monosulfide) was present at the majority of sites surveyed. Of the sulfidic samples identified, the vast majority maintained a near neutral pH when oxidised (during incubation experiments) and therefore classified as ‘hyposulfidic’ material. Soils that contained no measurable sulfide and did not contain field characteristics indicating post-active ASS processes (such as iron mottles) were classified as ‘non ASS material’. Only one hypersulfidic soil was identified (where soil pH dropped from near neutral to below pH 4 during incubation), which occurred at Murtho Park (sample MP-GS-2.2). No sulfuric material (actual acid sulfate soil) with a pH < 4 was identified at either of these wetlands (Table 1). The sampling methodology used and the classification of ASS materials used herein is provided in Fitzpatrick et al (2018).

Table 1: The sample selection criteria was designed to enable comparisons between similar and contrasting wetland environments and acid sulfate soil materials

Selection criteria		Murtho Park				Spectacle Lakes (Beldora South)			
Stable, wet and dry environments representative of each wetland	Profile position	Transect ID = MP-EF				Transect ID = SPL-EF			
		Top of bank		Bottom of bank (subaqueous)	Top of bank	Top of bank		Bottom of bank (subaqueous)	
	Soil Sample ID	MP-EF-1 1.1	MP-EF-1 1.2	MP-EF-3 3.1	MP-EF-4 4.1	SPL-EF-1 1.1	SPL-EF-1 1.2	SPL-EF-2 2.1	SPL-EF-2 2.2
	Sample depth	0-5 cm	5-50 cm	0-10 cm	0-10 cm	0-20 cm	20-55 cm	0-15 cm	15-45 cm
	ASS Classification	Non-ASS material, sand	Moderately acidic (hyposulfidic), sand	Moderately acidic (hyposulfidic), clay	Moderately acidic (hyposulfidic), clay loam	Hyposulfidic, sandy clay loam	Non ASS Material, clay	Hyposulfidic, clay loam	Non ASS Material, clay
Extremes in ASS environments	Profile position	Transect ID = MP-IJ		Grab sample location = GS					
		Bottom of bank (subaqueous)		Bottom of bank (dry creek)					
	Soil Sample ID	MP-IJ-2 2.1	MP-IJ-2 2.2	MP-GS-2 2.1	MP-GS-2 2.2				
	Sample depth	0-5 cm	2-25 cm	0-5 cm	5-20 cm				
	ASS Classification	Hyposulfidic, monosulfidic, (MBO)	Hyposulfidic, monosulfidic rich clay	Moderately acidic (hypersulfidic) sandy loam	Hypersulfidic sand				

From this data, we also explored how the microbial communities within ASS samples are distinct from non-ASS communities, and generally surveyed factors that may contribute to the microbial communities within ASS soils. From that, we completed a very preliminary analysis to identify microbial biomarkers in ASS soils and to examine how knowledge of microbial communities can inform wetland management strategies.

We successfully examined microbial (bacterial and archaeal) species and diversity present within all 24 soil samples. We identified factors known to drive microbial diversity within soil samples, such as landform, depth or pH, demonstrating the approach selected for this project was sensible and appropriate for future studies. We are able to identify differences in microbial diversity associated with acid sulfate soils (ASS), defined by an alteration of the species that are present and an overall decrease in species diversity. We identified significant differences in ASS soils compared to non-ASS soils, even when non-ASS soils were slightly acidic. Differences in microbial communities (alpha- and beta-diversity) unique to ASS soils were tightly associated with soil pH and electric conductivity, as well as iron oxide mottles (indicative of dynamic redox conditions), not associated/weakly associated the presence of nitrogen, carbon, monosulfide, *etc*. Four unique taxa were identified in ASS soils, although known reference genome sequences for these species do not exist, indicating little is known about these microbial species. Importantly, this preliminary study demonstrates the potential to use Next Generation DNA sequencing to better understand ASS affected soils, and the impacts of management factors on them. Additional microbial DNA analysis on a wider range of sample is required to confirm these pilot study findings. We suggested that more detailed analysis using such approaches is likely to provide novel and useful insights (e.g. role of salinity in wetlands, wetting and drying, acid sulfate soil status) that cannot be gained using conventional methods of soil analysis/assessment.

1.3 Key Findings

Four key questions were raised when selecting soil samples for analysis. These questions were created to guide sample selection, but may also be useful in identifying how this data can be applied to future management decisions. Below are the four key questions, an overall assessment, and a more detailed response.

Questions 1: Is the microbial community at Beldora South different to Murtho which have different management regimes?

Overall assessment: Microbial community composition, and not the numbers of microbial species, is likely related to the soil type (e.g. lagoon, stream bed, etc.), and further research is needed to determine how different management regimes impact unique soil types at different locations.

We examined and compared the microbial community composition at both sites and did not find significant differences between the sites when controlling for soil type. The Spectacle Lakes-Beldora South (SPL) samples collectively contained microbial communities that were distinct from the Murtho Park (MP) site (Kruskal Wallis pairwise comparison of unweighted UniFrac values; pseudo-F = 3.43; q value = 0.001). While this could be due to the management strategies at each site, there were also different soil types present at the sites. For example, the MP site included samples collected from a stream bed, whereas the SPL site did not. Therefore, we controlled for the type of soil examined. When we control for soil type and only compare the lagoon samples collected at each site in the flat areas, the sites were not significantly different from one another in their composition, albeit this could be due to low sample size (Kruskal Wallis pairwise comparison of unweighted UniFrac values; pseudo-F = 2.114; q value = 0.057). The number of species identified at each site (alpha-diversity) was also not significantly different (Kruskal Wallis comparison of Shannon's Diversity; H=0.833; q value = 0.7723). This suggests that we cannot detect significantly differences in the microbial communities between the two sites, given the number of samples that were examined thus far. This is examined in more detail in Section 4

Question 2: Is there a difference in the microbial community between the top of the bank and the bottom, which have had different wetting and drying histories?

Overall assessment: Different wetting and drying histories may result in unique microbial communities, but this may be influenced by the vegetation present.

At SPL, we examined the toe slope and the flat samples (bank and bottom, respectively) to see if the microbial communities differed. Their microbial composition was significantly different between the slope and flat samples at this site (Kruskal Wallis pairwise comparison of unweighted UniFrac values; pseudo-F = 2.586; q value = 0.021), while the number of species identified in the two areas at this site was not (Kruskal Wallis comparison of Shannon's Diversity; H=1.333; q value = 0.248). While both sites were lagoon soil descriptions, the surface vegetation was different in the bank and bottom samples. This result may suggest that different wetting and drying conditions do impact the soil microbial community composition, although this would be better examined experimentally with similar vegetation types. Again, these findings are to be treated as preliminary giving the limited sample size in this pilot study. We also compared differences between samples collected on the slope and the bottom at the MP site (Figure 1). However, different soil types (textures) were present in both the 'top of bank' and the 'bottom of bank' sites, which will influence the data (Table 1). These differences are examined in more detail in Section 4.

Question 3: Is there a difference in the community at time zero (T0) compared to oxidised samples (simulated drying event in laboratory conditions)?

Overall Assessment: Incubation does not alter the microbial community composition, as examined by DNA. This may not be the case for living cells and therefore needs to be examined further using non-DNA sequencing techniques, such as flow cytometry or quantitative PCR.

Overall, incubation did not significantly change the microbial community composition present within these samples (Kruskal Wallis pairwise comparison of unweighted UniFrac and Bray Curtis values; q = <0.734; pseudo F: >0.763). This was true for both acidic soils and monosulfidic soils (q = >0.993; pseudo F: >0.636). However, the method applied here to examine microbial community composition examines DNA from both living and dead microorganisms present in the soil and is therefore unlikely to change through incubation, as microbial species killed during incubation would still have DNA present in the samples. This information is presented in greater detail in Section 6.1.

Question 4: Is there a different microbial community in different ASS types within a wetland?

Overall Assessment: Different microbial communities are present in different types of ASS soils.

The different ASS types do appear to contain distinct microbial communities. While the overall microbial composition in hypersulfidic soils was not unique compared to those that were less acidic (Kruskal Wallis pairwise comparison of unweighted UniFrac values; q = >0.08; pseudo F: <3.39), hyposulfidic soils were significantly different in their microbial composition compared to non-ASS soils (Kruskal Wallis pairwise comparison of unweighted UniFrac values; q = 0.04; pseudo F: 2.669). In addition, several poorly described bacterial species were distinct to hypersulfidic soils, suggesting that this trait may select for unique microbial species that can likely tolerate significant changes in pH. More hypersulfidic soil samples would need to be examined to understand this phenomenon further. This is described in more detail in Section 5.

1.4 Overall Recommendations

In the future, a better understanding the presence/absence of microorganisms that alter pH or respond to changes in pH will likely provide insights into the management options of acidified ASS materials. Further exploration into the microbes that are present in ASS soils will likely reveal more about the process that underpin ASS formation and eventual treatment. This also suggests that unique microbial species may potentially be used as biomarkers to identify hypersulfidic soils and, if they can be tracked during ASS treatment, predict how ASS soils will respond to management strategies. We do, however, caution that given the very limited sample size and preliminary nature of this study, the recommendations herein require more comprehensive assessment and validation before being translated into action in the field. Measuring microbial community changes during managed wetting and drying cycles over the course of time would be beneficial. The preliminary findings in this report suggest this approach would work.

1.5 Limitations

A total of 24 samples were sequenced across two study sites. In doing so, we have a broad sweeping survey of the microbial diversity present within all of these samples. However, we still have limited abilities to identify specific species that are different between different sample types (*e.g.* in ASS soils vs non-ASS soils), as we were limited by the number of samples included in this pilot study. In addition, many more samples (>1400 field and incubated samples are available) would be required to fully identify how treatment and mitigation strategies influence the microbial profile of ASS soils. All of the conclusions made here must be further examined with larger sample sizes. The >500 soil samples from all sites sampled in the main study have been frozen for analysis should additional funding become available.

1.6 Acknowledgements

This project was funded via the Department for Environment and Water's Riverine Recovery Program (RRP). We would also like to acknowledge the Australian Genome Research Facility (AGRF) for technical support and the DNA sequencing discussed in this study. We would also like to acknowledge Sally Maxwell and Andrew Rettig for their generous feedback on the report.

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2 Approach for assessing microbial communities (microbiota)

Soil characteristics (39 in total) were examined according to landscape, soil physical and chemical properties. The soil field and analytical characteristics interrogated are provided in Appendix 1 and are included in the RRP wetlands acid sulfate soils technical report (Thomas et al. 2019). Specific details on the soil sampling and analytical methods are provided in Fitzpatrick et al. (2018).

To assess soil microbial communities, the general strategy was to 1.) extract DNA from the soil samples; 2.) sequence a marker gene sequence (16S ribosomal RNA sequences) that serves as a fingerprint for different types of microorganisms; and 3) use statistical methods to compare or contrast the types and communities of microorganisms that we detected. 1.) First, DNA was extracted by the Australian Genome Research Facility (AGRF) using a standard in-house kit approach, and DNA was successful obtained from all samples, regardless of their pH, mottles type, etc. 2.) 16S ribosomal RNA (rRNA) gene encoding regions (e.g. the marker gene sequence for different types of microbes) were amplified using a polymerase chain reaction (PCR) by AGRF, and all DNA sequences were then sequenced, or recorded, using Next Generation DNA sequencing (Caporaso et al. 2012). Each 16S rRNA sequence serves as a unique 'barcode' or fingerprint for each of the unique bacterial and archaeal species present within a sample. 3.) DNA sequences were then examined using high-throughput computing resources. We assessed how many different species were present in each sample (alpha-diversity; Shannon's and Faith's PD) and how many species were shared or unique across the different samples (beta-diversity; unweighted UniFrac and Bray Curtis). Unweighted UniFrac metrics examines the phylogenetic distance between all of the species present within a sample (*e.g.* examines how related the species are within given sample), while Bray Curtis examines the abundance of species within a sample, without a concern for how related the species are to one another. In both analyses, we performed Kruskal Wallis comparisons with 999 permutations, or subsamplings, to identify if differences between samples were statistically significant, and we report a q-value (a p-value with a false discovery rate (FDR) included; <0.05 is considered significant) and a score to determine how well the statistical test fit the data set (a *pseudo F score* (beta-diversity) or an *H score* (alpha-diversity); the higher the number the better the fit). We then examined if there were individual species that were present in significantly different abundances in different samples using a PERMANOVA test (ANCOM) with 999 permutations. All analyses were conducted using QIIME2 (v2017.11) (Caporaso et al. 2010).

2.1 DNA sequencing quality and success

One of the primary objectives of this work was to ascertain whether or not DNA could be successfully extracted from ASS collected in River Murray wetlands as part of the main project. We were successfully able to extract DNA for each of the samples, and the sequencing data that was returned was of high quality. Excellent quality (>20) was observed for all of the base pairs (300 bp) in each DNA sequence (Figure 1), indicating successful DNA sequencing. This clearly demonstrates the potential to use these approaches to successfully obtain microbial DNA from ASS and non-ASS soils.

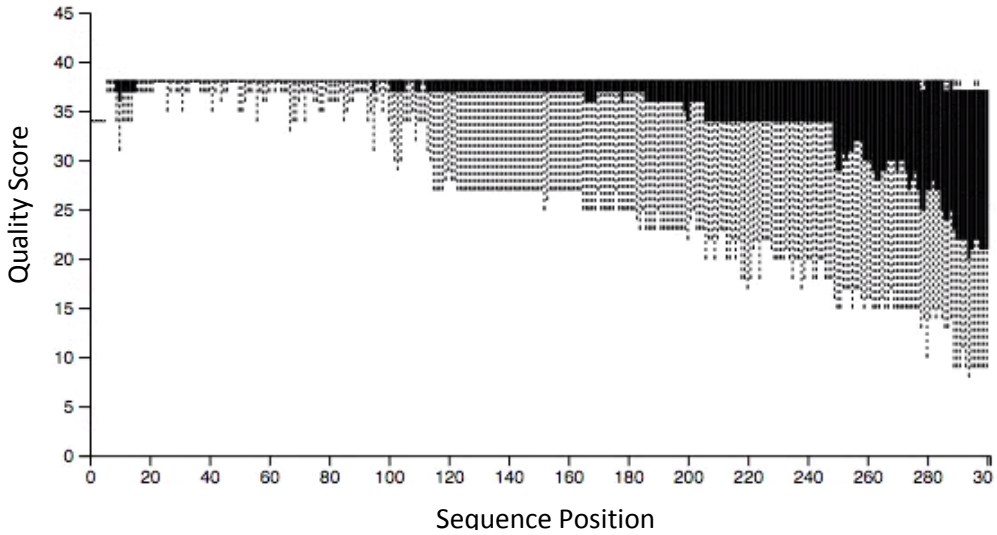

Figure 1: The quality of sequencing for each sequence is averaged at each position.

All forward DNA sequences were then screened for robustness (denoised and fake, chimeric sequences were removed). Robust DNA sequences were then identified (*e.g.* which species originate from) by comparing the 16S rRNA sequences to known, previously described sequences in the SILVA database. Each identified sequence is then named a 'feature' or operational taxonomic unit (OTU). Features or OTUs are analogous to species and are otherwise described as taxa or species in this report. The lowest number of sequences obtained for a single sample was 41,586 (Sample 22.SPL.EF.2.2.1inc), while the most was 193,923 (1.MP.EF.2.2.1), again indicating excellent efficiency in DNA amplification and sequencing. The total number of species identified in this study was 1,487 (Table 2), which was expected for a diverse range of soil sample textures. Sample names are linked to sample characteristics in Table 1.

Table 2: The number features (*i.e.* sequences) is displayed across these samples.

Metric	Sample
Number of samples	24
Number of features	1,487

3 General Taxonomic Summary

We first characterized the species present within all of the samples. These are summarised by classifying the species into their respective phyla (Figure 2). When examining all of the phyla present in each sample, >20% of the microorganisms identified by 16S ribosomal RNA could not be identified (Bacteria; Figure 2), suggesting that many of the species present Murray Darling Basin (MDB) wetland soils are not well described. In addition, >70% of the remaining microbes in these soils were Proteobacteria, suggesting that this is the dominant phyla in MDB wetland soils; Proteobacteria dominance is expected for environmental microbial communities.

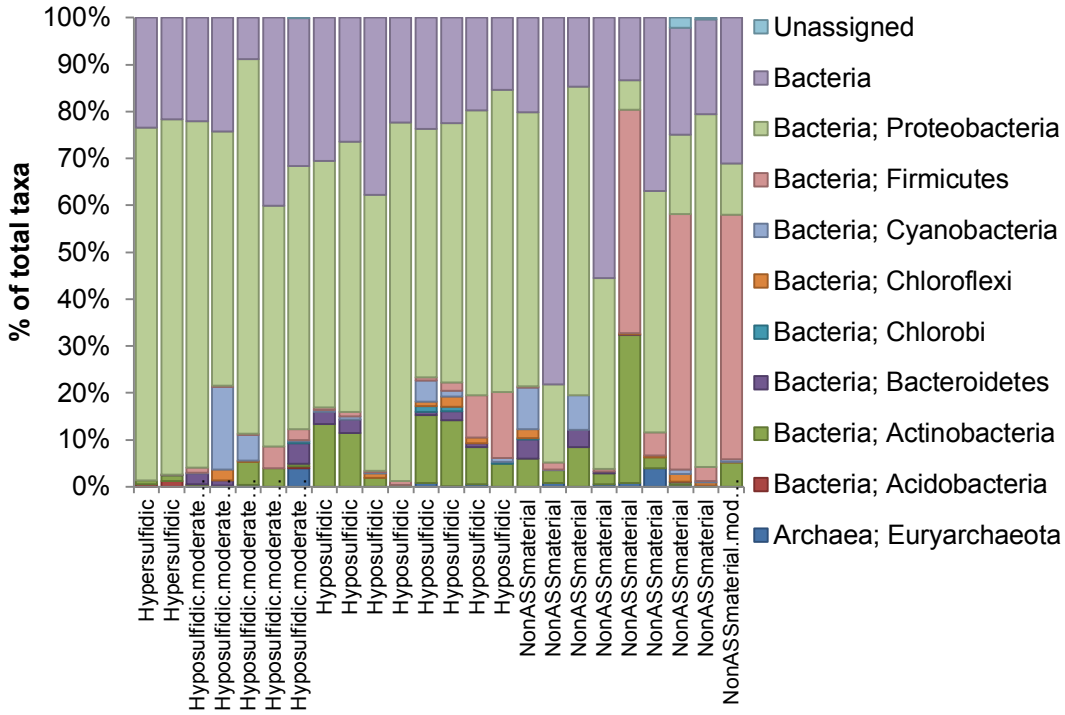

Figure 2: Species identified in each soil sample were summarized by classifying each into their respective phyla. Moderate = moderately acidic (pH 4-5.5).

We next examined all of the features, or species, present in all samples (Figure 3). We observe species that are routinely identified from soils, including *Pseudomonas*, *Streptomyces*, and Acidobacteriaceae species, along with a large diversity of uncharacterized Proteobacterial species and methanogenic archaea. Of note, there were salt-loving archeae, Halobacteriaceae, present throughout all of the soils, indicating a saline environment.

Figure 3: The dominant species identified in this study are shown according to their abundance in each sample. A detailed list of these species was emailed to DEW and that the data is, as of December 2018, internally stored, due to file size.

4 Factors known to alter soil microbial communities

First, we assessed factors that are known to drive microbial diversity within soil microbiotas (Thompson et al. 2017). Identifying the major factors that contribute to soil microbial diversity in MDB wetland soils will help ensure similar microbial communities are compared (Wong et al. 2016). We first identified the top five major characteristics of these soils that drove microbial community structure and diversity (beta-diversity) (Table 3). Each of the top five categories is described in greater detail below.

Table 3: Soil characteristics (metadata factors) that significantly drove microbial community structure.

Statistically Significant Factors that contribute to Soil Microbial Communities			
Soil Characteristic	**p-value**	**Pseudo-F**	**Number of Groups**
Acid Neutralizing Capacity (Categorical)	0.011	1.8066	3
ASS Classification	0.001	2.15855	5
Monosulfidic Soil	0.004	2.41646	2
Consistency Rupture Resistance	0.003	1.79406	5
Landform	**0.001**	**4.34163**	**3**
Site Position	**0.001**	**3.70203**	**2**
Slope Profile	0.041	1.81225	2
Surface Condition	**0.001**	**3.2176**	**4**
Electric Conductivity (pH)	0.001	2.51977	4
Location Code	**0.002**	**3.4373**	**2**
Depth (Categorical)	0.007	1.89519	3
Potential Sulfidic Acidity (Categorical)	0.002	1.88035	4
Primary Mottles Type	0.001	2.12987	3
Sampling Date	0.001	3.68109	3
Site ID	**0.001**	**3.78825**	**7**
Soil pH	0.001	2.06531	5
Soil Matrix Moisture	0.001	2.39358	4
Soil Texture	0.006	1.62336	6
Total Nitrogen	0.01	1.59432	4
Total Carbon	0.001	2.04551	5

The soil characteristics (i.e. metadata factors) (Table 3) are displayed if that characteristic significantly contributed to the differences observed in soil microbial community composition (beta-diversity; unweighted UniFrac values compared using a PERMANOVA based test with an FDR corrected p-value of <0.05). The p-value and the test fit (pseudo-F) are provided, along with the number of groups that were compared in each soil characteristic (see Appendix 1 for more detail on soil characteristics). The five most significant characteristics are highlighted in bold.

4.1 Landform

Landform accounted for a significant level of diversity across samples. Samples collected from the lagoon, wetland, and swamp were all distinct according to the type of landform present at each collection site (q = <0.02; pseudo F = >4.05 for any pairwise comparison between landform) (Figure 4). The type of landform significantly contributed to the microbial diversity present in each sample, although many factors contribute to the description of landform (*e.g.* Site ID, Location Code, Sampling Date (which is associated with sampling location), Surface Condition, and Site Position – all factors that significantly contribute to soil diversity within these samples). For example, the Site

Position (q = 0.001; pseudo F = 3.7), Surface Condition (mud, vegetated, etc.; q = <0.001; pseudo F = 3.2), Location Code (q = 0.002; pseudo F = 3.43), Site ID (q = 0.001; pseudo F = 3.79), Slope Profile (toe slope or flat; q = <0.04; pseudo F = >1.81), Sampling Date (q = <0.001; pseudo F = 3.68), etc. were all additionally significant and are all likely confounded by the landform that underpins this data. For example, all samples taken within a streambed were also the only samples collected in a 'flat' area. Landform, including the key characteristics that are associated with it (site, slope, presence of vegetation, etc.) would need to be controlled experimentally for a more robust analysis. Lastly, alpha-diversity had little effect, suggesting that all samples carry a similar number of species (the only information collected that significantly associated with alpha-diversity in this data set was the Slope Profile of the collection site (Shannon's p = 0.023; H = 5.07; Faith's PD p = 0.003; H = 4.32)); samples collected on a toe slope contained more species than those collected in flat areas, likely due to limited sampling of toe slopes in a single landform type.

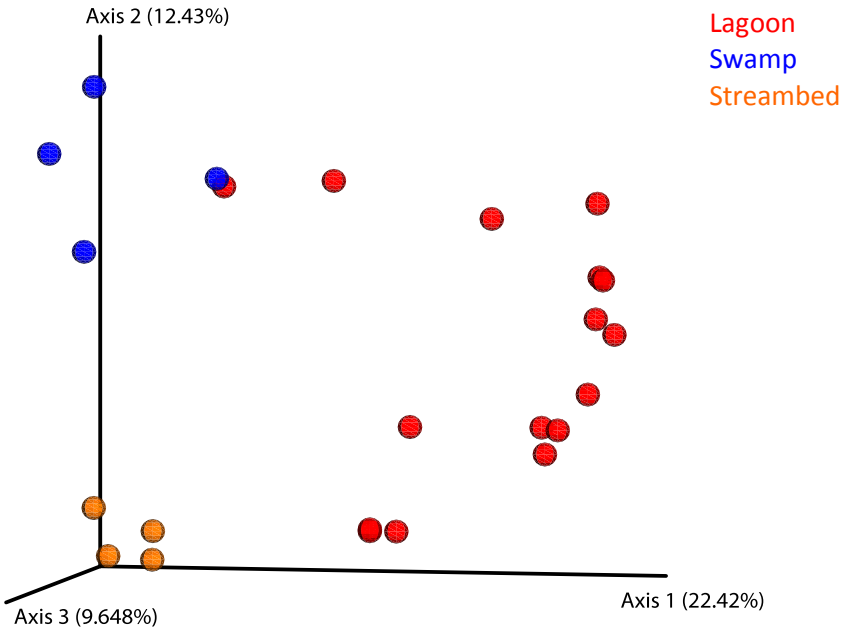

Figure 4: UniFrac distances (beta-diversity) for each sample are visualized on a Principal Coordinates Analysis (PCoA) plot. Dots that are closer together contain species that are more similar, while dots more distant from one another represent microbial communities that share fewer species. Samples are coloured according to their landform type.

4.2 Depth of Sample Collection

We analysed the effect of the depth of soil sample collection in four layers: 0 to 10 cm; 10 to 20 cm; 20 to 30 cm; and 30 to 40 cm. While the same cores are included in this analysis, each individual sample originates from a separate depth. The depth of the sample significantly impacted the microbial community (q = <0.001; pseudo F: >1.89), although this did not explain as much variation as factors such as landform. Specifically, 0 to 10 cm were not significantly different from 10 to 20 cm (q = 0.282; pseudo F = 1.125), but 30 to 40 cm samples were significantly different from all other depths (q = <0.003; pseudo F= >2.17). This suggests that samples should be collected at a consistent depth (e.g. 0 – 20 cm) to avoid depth-related microbial community differences.

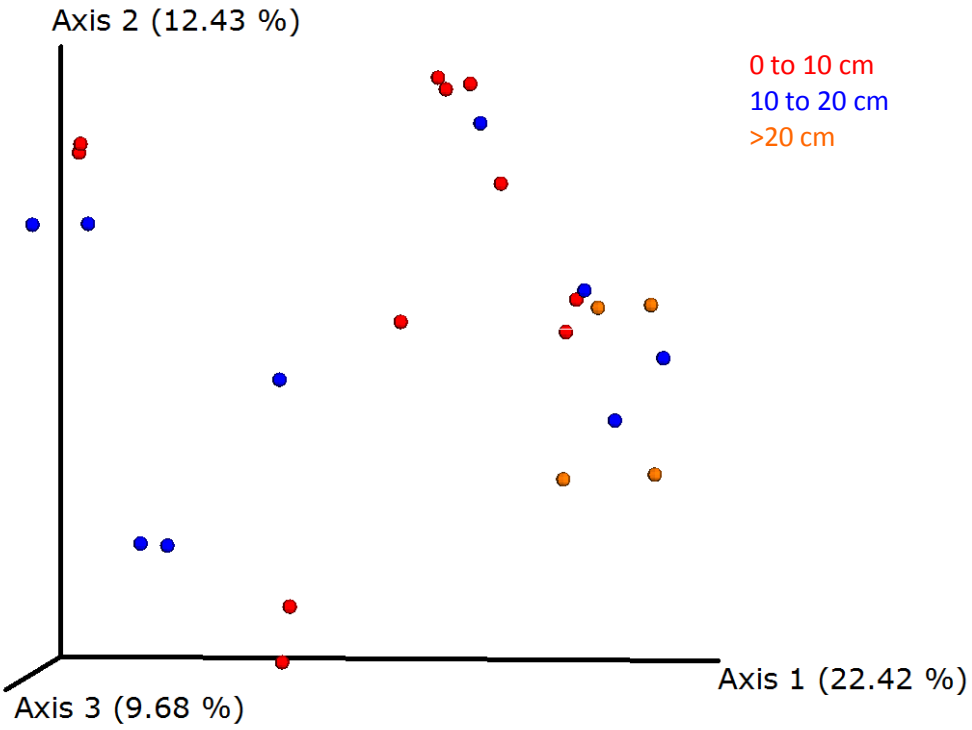

Figure 5: UniFrac distances were calculated for samples obtained from three different depths: 0-10 cm; 10-20 cm; and >20 cm.

5 Acid Sulfate Soil (ASS) specific factors

A key goal of this proposal was to examine how microbial communities may be beneficial in guiding ASS soil remediation and identification.

5.1 Acid Sulfate Soil (ASS) classification

Different ASS soils were identified using standard classification methods (Fitzpatrick et al. 2018). One sample from each site was classified as hypersulfidic (pH<4), while a range of other hyposulfidic (pH>4) and non-ASS soils were also collected. The microbial communities in hypersulfidic soils were not significantly different from hyposulfidic ones (q = 0.08; pseudo F: 3.39) or non-ASS soils (q = 0.08; pseudo F: 3.41) (Figure 6). However, hypersulfuric soils could also not be significantly distinguished from moderately acidic soils of either type (q = >0.14; pseudo F: >1.77).Please note though that very few hypersulfidic soils were examined as part of this study as only one sample was deemed hypersulfidic. In contrast, the microbial composition of hyposulfidic soils were significantly different from non-ASS soils (q = 0.04; pseudo F: 2.669) but were not different from hypersulfidic or moderately acidic soils. Overall, this result suggests that the presence of an overall decrease in acidity may contribute to changes within the microbial communities. This was also true in samples from both a lagoon and swamp, indicating this is independent of landform and depth. We identified four taxa that were unique to hypersulfuric soils (2 unknown bacterial species; 1 Gammaproteobacteria, and 1 Bacillacea; Table 2). All four taxa were specific to hypersulfidic soils, suggesting that they have the ability to thrive in highly acid soil conditions. The lack of better taxonomic descriptions (in the recently updated Silva 119 database) also suggests that they are unique taxa that have not yet been described elsewhere (Quatrini and Johnson 2018).

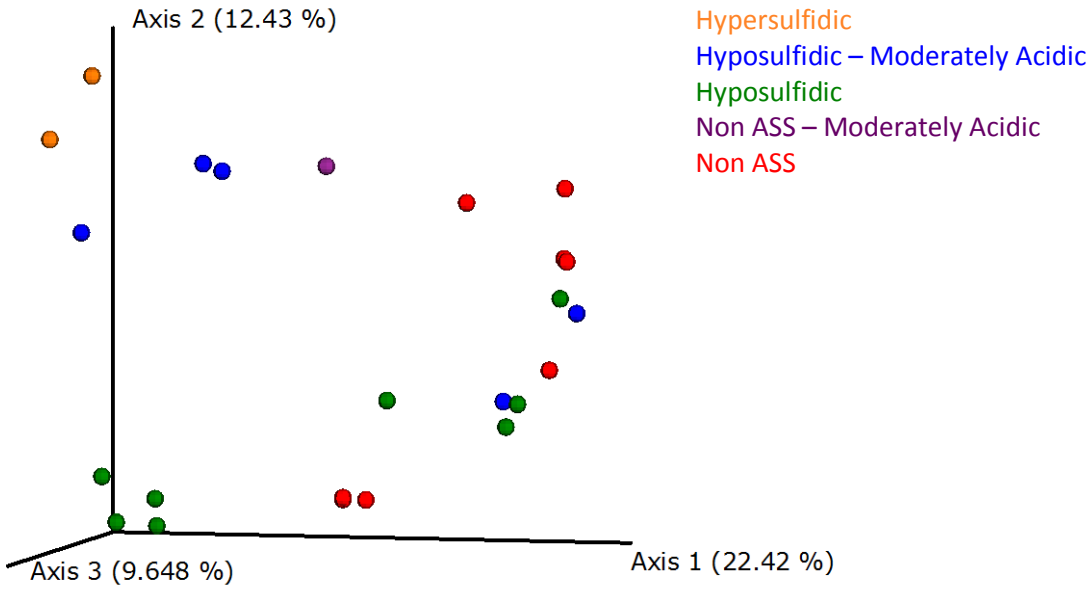

Figure 6: A PCoA plot displaying unweighted UniFrac distances per sample is displayed. Samples are coloured according to their ASS classification. Note that no sulfuric materials were identified at either of the wetlands.

Table 4: Significantly different species between the different ASS Classifications is displayed. Values presented for each significantly different species are the total abundance of that taxa in 100% of the samples.

Taxonomy	ASS Classification: ASS Hypersulfidic	ASS Hyposulfidic	ASS Hyposulfidic moderately acidic	Non ASS material	Non ASS material moderately acidic.
Bacteria	37	1	1	1	1
Bacteria	54	1	1	1	1
Bacteria; Proteobacteria; Gammaproteobacteria	63	1	1	1	1
Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae	1	1	1	1	60

The differences linked to ASS soils cannot be explained by changes in alpha diversity using phylogenetic differences (Faith's PD; q = >0.40; H: >0.2) or Shannon's diversity, although there was a trend for higher Shannon's diversity in hypersulfidic soils (Figure 7), suggesting that some species may be selectively increased in abundance in ASS soils (q = >0.64; H: >2.48).

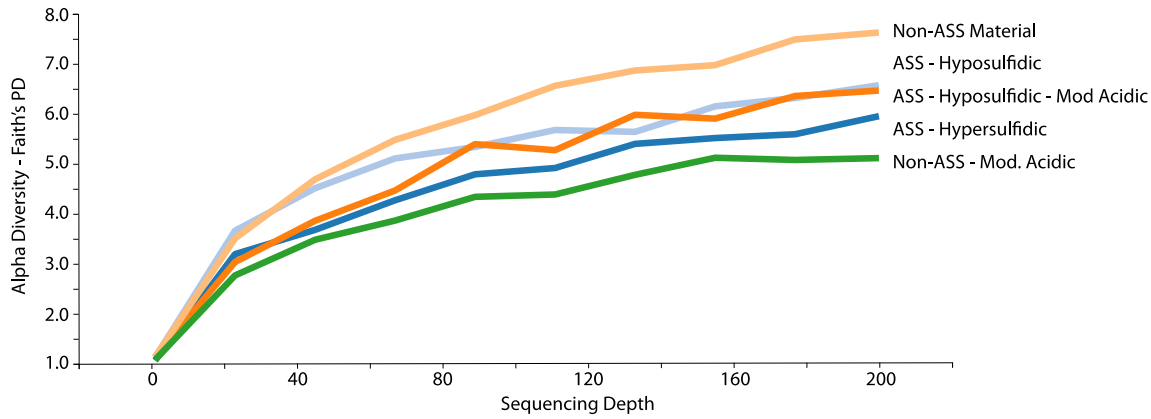

Figure 7: An alpha rarefaction plot displays the phylogenetic diversity (Faith's PD) detected for varying sequences in each sample in each ASS classification.

5.2 pH

Acid sulfate soils that have oxidized and contain sulfuric materials are characteristically low in pH, and the ASS Classifications suggests the pH may be driving differences in microbial diversity. We examined how pH in these samples contributed to microbial diversity, independent of ASS Classification type, and found that pH had significant effects. Specifically, any sample with a pH below 5 was moderately different from soils with a pH between 5 and 7 (q=<0.07; pseudo F = >2.6) and were significantly different from all samples with a pH >7 (q=<0.02; pseudo F = >0.26) (Figure 8), indicating that a drop in pH significantly impacts the microbial communities within the soil. This change in diversity across samples was not due to an introduction or loss of species, as all alpha-diversity values were non-significant but is likely due to a change in abundance of existing species within the soil type. Two species were found significantly linked to low pH soils (<5), a Betaproteobacteria and a Gammaproteobacteria taxa.

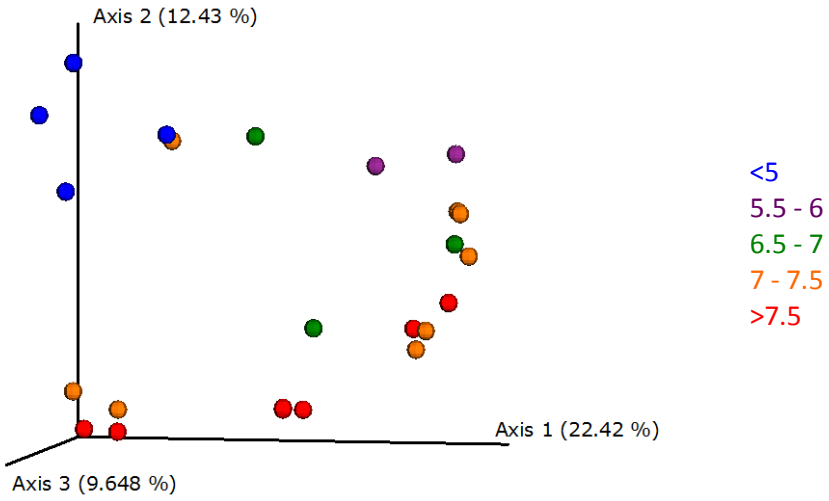

Figure 8: UniFrac beta-diversity visualized on a PCoA plot. Samples are colored according to their pH bin (field pH). The sample pH was used to qualitatively bin samples, as collected in the field: <5, 5-5.5, 5.5-6, 6-6.5, 6.5-7, 7-7.5, >7.5. Samples fell within five bins, displayed in the legend.

5.3 Potential (sulfidic) acidity (PotentialSulfidicAcidity_molH_t_Largebin)

We next examined the microbial communities compared to the potential (sulfidic) acidity in each soil. Sulfidic material forms under highly reducing conditions in soil. Soils with a potential sulfidic acidity of <100 mol H/t were significantly different from all soils with >200 mol H/t (q = <0.03; pseudo F: >2.14). This again suggests that build-up of sulfides significantly changes soil microbial communities.

5.4 Electric Conductivity (EC; salinity)

We next analysed the microbial community compared to the soil electric conductivity (EC; a measure of salinity) of each sample, as salinity has been shown to impact microbial community composition (Lozupone and Knight 2005). Samples were broken down into bins (0 to 1; 1 to 5; 5 to 10; and >10 dS m^{-1}). Samples with an EC of <1 dS m^{-1} were all significantly different to any soil with an EC of >1 (i.e. 1 to 25; q = <0.03; pseudo F: >2.48), while the microbial communities in soils with an EC of >1 dS m^{-1} were not significantly different from one another (q = >0.14; pseudo F: >1.24). Electrical conductivity did not directly correspond to pH. In general, low EC soils (<1 dS m^{-1}) were also likely to have more microbial species diversity (alpha-diversity, Faith's PD) than samples with higher EC, although this was not significant (q = >0.055; H: >7.57). Only one species of

Betaproteobacteria was identified as significantly associated with high EC soils (>10 dS m^{-1}). This suggests that EC changes in the soils contributed to microbial community composition with salinisation (>1 dS m^{-1} EC in a 1:5 soil extract, equivalent to 1000 µS cm^{-1} EC) being sufficient to induce significant changes. The potential impacts of this finding, e.g. following inundation with water of varying EC, requires more detailed investigation.

5.5 Mottles Type

The oxidation of sulfides releases sulfuric acid in ASS soils is known to release other minerals from the soil matrix, such as iron, aluminum, and other metals (Karimian, Johnston, and Burton 2018). The oxidation and reduction processes that are occurring or have occurred in a soil are recorded in the profile as different mottle types. We found that the iron oxide coatings were identified in many of the ASS soil materials, but were absent in non-ASS materials. This distinction was a significant contributor to the microbial diversity within the soils. Samples with iron oxide coatings (*i.e. active and or post active* ASS soils and soils) were significantly different from samples from reducing environments with iron manganese concretions or sulfidic mottles ($q = <0.02$; pseudo F: >2.36). However, we did not find any significantly associated taxa with either mottles type. This suggests that microbial communities present differ between reducing and oxidising micro-environments in acid sulfate soils.

6 Assessing the microbial differences in ASS treatment

Identifying the impacts of ASS soil treatments on microbial communities was a key aim of this proposal. We explored how microbial communities changed and responded to specific laboratory treatments, such as drying through incubation, to explore how microbial ecology may be related to ASS soil treatments.

6.1 Incubation

A laboratory test to examine the response of the ASS soils to oxidation (through wetting and drying cycles) was examined by incubating all soils over 8 weeks and examining the change in pH (Creeper, Fitzpatrick, and Shand 2013). This method is summarised in Fitzpatrick et al. (2018). We first needed to determine if incubation altered the soil microbial profile (i.e. the DNA present in the sample). Incubation did not significantly change the microbial DNA present within each sample ($q = <0.734$; pseudo F: >0.763). However, we used the UniFrac metric that examines phylogenetic differences and not abundance differences, and therefore does not confirm that the microbial community did not change during incubation, as DNA of dead microorganisms would still be present within the soil. To examine if abundances had changed between incubated and non-incubated soils, we examined the Bray Curtis metric that accounts for major abundance changes. We again see that incubation did not have a significant impact on microbial community abundances across all samples ($q = <0.895$; pseudo F: >0.829). We also examined only acidic soils to determine if incubation altered their microbial DNA signatures; incubation again had no significant effect on the microbial communities in acidic soils ($q = 1$; pseudo F: >0.802). This was also the case for the monosulfidic soils ($q = 0.993$; pseudo F: >0.636). However, caution needs to be applied to these interpretations, as the incubation was done in the laboratory on a limited number of samples over a limited time period. In addition, this (and other) DNA-based method may not be the most appropriate for gauging short term changes within soil microbial communities; alternative methods, such as flow cytometry or quantitative PCR of key species identified by 16S rRNA approach would likely provide more informative data. Further testing of acidic field soil samples is strongly recommended.

6.2 Acid neutralizing capacity

Finally, we looked at the inherent acid neutralizing capacity of each soil sample, as this can be a key indicator of ASS soil recovery and response (Mosley et al. 2017) and is a key factor in ASS classification (i.e. incubation experiment results). Samples were group into bins of low (0-100 molH/t), medium (100 – 1000 molH/t), and high (>1000 molH/t). We did see a significant change in microbial communities between low and medium acid neutralizing capacity ($q = 0.009$; pseudo F: >2.29). However, the same effects were not observed between medium and high acid neutralizing capacity, or between low and high acid neutralizing capacity (although this may be due to a low sample size in samples with high neutralizing capacity (n=2)). There were no specific species linked with the change in acid neutralizing capacity, suggesting this capacity may be spread across numerous different types of bacteria. While clear responses to acid neutralizing capacity were difficult to discern (due to low sample numbers), the results are encouraging and warrant further investigation.

6.3 Conclusion

In this collaborative project, we successfully described the bacterial and archeael profile of both ASS and non-ASS soils present at two sites in the River Murray Darling Basin wetlands. We were able to successfully extract DNA from each of the soils types and generate 16S ribosomal RNA profiles using Next Generation Sequencing approaches. Within the analysis of the microbial communities, we identified factors (such as texture, land type, and pH) that significantly contributed to soil microbial community profiles. When examining ASS soils (both hypo- and hypersulfidic soils), we

identified additional factors that contribute to the presence of microbial species, including pH, mottles type, and salinity. Further, incubation of ASS soils did not significantly change the microbial community, as assessed by the presence of 16S rRNA sequences in the soil. Together, these data can be used to guide further study of ASS soils in this region, as this study demonstrates the utility of this approach to assess future management procedures of these wetlands. Again, we caution that while promising, these findings are to be treated as preliminary giving the limited sample size in this pilot study.

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8 Appendix 1

8.1 Classification of soil materials

Acid sulfate soils (ASS) are those soils in which sulfuric acid may be produced, is being produced, or has been produced in amounts that have a lasting effect on main soil characteristics (Pons 1973). This general definition includes:

(i) potential

(ii) actual (or active)

(iii) post-active ASS

which are the three broad generic soil types that continue to be recognised (for example, Fanning 2002). However, definitions of these broad generic types of ASS can be confusing and the Acid Sulfate Soil Working Group of the International Union of Soil Sciences agreed to adopt changes to the classification of ASS materials (Sullivan et al. 2010). This was also adopted;

(i) by the Scientific Reference Panel of the Murray-Darling Basin Acid Sulfate Soils Risk Assessment Project for use in detailed assessment of acid sulfate soil in the Murray-Darling Basin

(ii) in the Second edition of the Australian Soil Classification (Isbell and National Committee on Soils & Terrain 2016).

This report follows these recommendations. Acid sulfate soils are essentially soils containing detectable sulfide minerals, principally pyrite (FeS_2) or monosulfides (FeS).

The definitions used in this report are:

Sulfuric material

Sulfuric material is soil material that has a pH less than 4 (1:1 by weight in water, or in a minimum of water to permit measurement), as currently defined in the Second edition of the Australian Soil Classification (Isbell and National Committee on Soils & Terrain 2016).

Sulfidic materials

Sulfidic materials are soil materials containing detectable sulfide minerals. The intent is for this term to be used in a descriptive context (for example, sulfidic soil material or sulfidic sediment) and to align with general definitions applied by other scientific disciplines such as geology and environment science (for example, sulfidic sediment). The method with the lowest detection limit is the Cr-reducible sulfide method, which currently has a detection limit of 0.005%; other methods (for example, X-ray diffraction, visual identification, Raman spectroscopy or infra-red spectroscopy) can also be used to identify sulfidic materials.

Note that this term differs from previously published definitions in various soil classifications (for example, Isbell 1996).

Hypersulfidic material (Isbell and National Committee on Soils & Terrain 2016).

Hypersulfidic material is a sulfidic material that has a field pH of 4 or more and is identified by experiencing a substantial* drop in pH to <4 (1:1 by weight in water, or in a minimum of water to permit measurement) when a 2-10 mm thick layer is incubated aerobically at field capacity. The duration of the incubation is either:

i) until the soil pH changes by at least 0.5 pH unit to below 4; or

ii) until a stable** pH is reached after at least 8 weeks incubation.

*A substantial drop in pH arising from incubation is regarded as an overall decrease of at least 0.5 pH unit.

**A stable pH is assumed to have been reached after at least 8 weeks of incubation when either the decrease in pH is <0.1 pH unit over at least a 14-day period, or the pH begins to increase.

Hyposulfidic material (Isbell and National Committee on Soils & Terrain 2016):

Hyposulfidic material is a sulfidic material that

(i) has a field pH of 4 or more

(ii) does not experience a substantial drop in pH to <4 (1:1 by weight in water, or in a minimum of water to permit measurement) when a 2-10 mm thick layer is incubated aerobically at field capacity. The duration of the incubation is until a stable pH is reached after at least 8 weeks of incubation.

Monosulfidic materials

These are soil materials with an acid volatile sulfide content of 0.01%S or more (Isbell and National Committee on Soils & Terrain 2016). Monosulfidic materials are subaqueous or waterlogged organic-rich materials that contain appreciable concentrations of monosulfides. Monosulfidic black oozes are specific materials characterised by their gel-like consistence. Monosulfidic materials have a **high index of squishiness or n-Value** as estimated in the field, which is a field estimate of mechanical properties that describes the ability of a saturated soil to support a load. (See field method to estimate **n-Values** in Fitzpatrick et al. 2018**.)**

Non-acid sulfate soil materials

In addition, the Scientific Reference Panel of the Murray-Darling Basin Acid Sulfate Soils Risk Assessment Project agreed to identify 'other acidic soil materials' arising from the detailed assessment of wetland soils in the Murray-Darling Basin even though these materials may not be the result of acid sulfate soil processes (for example, the acidity developed during ageing may be the result of Fe^{2+} hydrolysis (refer to equation 4 above), which may or may not be associated with acid sulfate soil processes). The acidity present in field soils may also be due to the accumulation of acidic organic matter and/or the leaching of bases. These acidic soil materials may also pose a risk to the environment.

The definition of these 'other acidic soil materials' for the detailed assessment of acid sulfate soils in the Murray-Darling Basin is as follows:

1. **Other acidic soil materials** — either
   i) non-sulfidic soil materials that acidify by at least a 0.5 pHw unit to a pH_w of <5.5 during moist aerobic incubation; or
   ii) soil materials with a $pH_w \geq 4$ but <5.5 in the field.
2. **Other soil materials** — soils that do not have acid sulfate soil (or other acidic) characteristics.

8.2 Assessment of Hazards

The following hazard assessment criteria used in this report is related to the classification of acid sulfate soil materials and was developed for the Murray-Darling Basin Authority (MDBA 2010), as described in the Riverine Recovery Project (RRP) Milestone 1 Assessment Methodology Report (Fitzpatrick, Mosley and Thomas. 2018):

Acidification

High level of concern

1. All sulfuric materials
2. All hypersulfidic materials (as recognised by either i) incubation of sulfidic materials or ii) a positive net acidity result with a Fineness Factor of 1.5 being used)

Medium level of concern

1. All hyposulfidic materials with S_{CR} contents ≥ 0.10%S
2. Other acidic soil materials, with pHw<5.5 (as recognised by either i) incubation or ii) field measurement

Low level of concern

1. All hyposulfidic materials with S_{CR} contents < 0.10% S
2. All other soil materials.

Metal Mobilisation

High level of concern

1. All sulfuric materials
2. All hypersulfidic materials (as recognised by either i) incubation of sulfidic materials or ii) a positive net acidity result with a Fineness Factor of 1.5 being used)
3. All monosulfidic materials.

Medium level of concern

1. All hyposulfidic materials with S_{CR} contents ≥ 0.10%S (i.e. within 0-20 cm)
2. Other acidic soil materials, with pHw<5.5 (as recognised by either i) incubation or ii) field measurement

Low level of concern

1. All hyposulfidic materials with S_{CR} contents < 0.10% S
2. All other soil materials.

De-oxygenation

High level of concern

1. All monosulfidic materials.
2. All surface soil materials (i.e. within 0-20 cm) with water soluble sulfate (1:5 soil:water) contents >100 mg SO_4 l^{-1}.

Medium level of concern

1. All hypersulfidic or hyposulfidic materials with S_{CR} contents ≥ 0.10%S (i.e. within 0-20 cm)

Low level of concern

1. All hyposulfidic materials with S_{CR} contents < 0.10% S
2. Other acidic soil materials, with pHw<5.5 (as recognised by either i) incubation or ii) field measurement
3. All other soil materials.

The assessment of acid sulfate soil hazards requires the level of concern to be placed in context with:

- the position of the sample in the soil profile, that is, if it is a surface sample it is more likely to be at the soil water interface and, therefore, to have an impact on surface water in the wetland than a sample deeper in the profile.
- the extent and distribution of the sample, that is, based on information available, e.g. whether the sample is widespread and more likely to have an impact on the wetland water than an isolated local occurrence.

9 Appendix 2 - Soil physical and chemical characteristics

Sample ID	MP-EF-1 1.1	MP-EF-1 1.2	MP-EF-3 3.1	MP-EF-4 4.1	MP-IJ-2 2.1	MP-IJ-2 2.2	MP-GS-2 2.1	MP-GS-2 2.2	SPL-EF-1 1.1	SPL-EF-1 1.2	SPL-EF-1 1.3	SPL-EF-2 2.1	SPL-EF-2 2.2	SPL-EF-2 2.3
Sampling date	14/06/2018	14/06/2018	14/06/2018	14/06/2018	13/06/2018	13/06/2018	14/06/2018	14/06/2018	13/07/2018	13/07/2018	13/07/2018	13/07/2018	13/07/2018	13/07/2018
E	-34.0364328	-34.0364328	-34.0376091	-34.0373532	-34.0223625	-34.0223625	-34.0337360	-34.0337360	-34.3506040	-34.3506040	-34.3506040	-34.3507190	-34.3507190	-34.3507190
N	140.849633 0	140.849633 0	140.846908 2	140.845907 4	140.858745 8	140.858745 8	140.858675 0	140.858675 0	140.399690 0	140.399690 0	140.399690 0	140.399245 0	140.399245 0	140.399245 0
Site position	Mid point	Mid point	Mid point	Low point	Low point	Low point	Low point	Low point	Mid point	Mid point	Mid point	Low point	Low point	Low point
Slope Profile	toeslope	toeslope	flat	flat	flat	flat	toeslope	toeslope	toeslope	toeslope	toeslope	flat	flat	flat
Landform	stream bar	stream bar	lagoon	lagoon	stream bed	stream bed	swamp	swamp	lagoon	lagoon	lagoon	lagoon	lagoon	lagoon
Surface condition	Bare Mud Fl	Bare Mud Fl	Bare Mud Fl	Bare Mud Fl	SubAq MBO	SubAq MBO	Bare Mud Fl	Bare Mud Fl	Veg	Veg	Veg	SubAq Sed	SubAq Sed	SubAq Sed
water pH	7.55	7.66	7.72		7.75	7.75	5.96	5.96	8.16	8.16	8.16	8.16	8.16	8.16
water EC (uS)	759.9	536	504.9		27400	27400	46100	46100	849.5	849.5	849.5	849.5	849.5	849.5
Soil pHf	7.75	7.74	7.44	6.93	7.58	7.07	5.26	5.05	7.36	7.59	8.06	6.94	5.79	5.68
pHinc (min)	6.03	4.04	5.03	4.85	7.24	6.8	4.08	3.41	7.12	7.59	8.06	5.71	5.79	5.68
Upper depth	0	5	0	0	0	5	0	5	0	20	55	0	15	45
Lower depth	5	50	10	10	5	25	5	20	20	55	100	15	45	100
Depth to water	5				-15				-10			-140		
Moist. Status	Wet	Wet	Very moist	Slightly moist	Wet	Wet	Moist	Moist	Wet	Wet	Wet	Wet	Wet	Wet
Class	Sand	Sand	Clay	Clay loam	muck	Clay	Fine sandy loam	Clay	Sandy clay loam	Clay	Sand	Clay loam	Clay	Clay
Matrix Munsell	2.5YR 6/1	10YR 6/1	2.5YR 6/1	2.5YR 6/1	2.5YR 7/3	2.5Y 7/3	2.5YR 6/1	2.5Y 6/1	2.5Y 5/1	5B 5/1	10YR 6/1	2.5Y 4/2	5B 5/1	5B 5/1
Matrix colour	Light grayish reddish brown	Light yellowish brown	Light grayish reddish brown	Light grayish reddish brown	Moderate yellowish pink	Grayish yellow	Light grayish reddish brown	Medium gray	Light olive brown	Bluish gray	Light yellowish brown	Moderate olive brown	Bluish gray	Bluish gray
Structure Type	Single grain	Single grain	Subangular blocky	Subangular blocky	Massive	Subangular blocky	Single grain	Subangular blocky	Massive	Subangular blocky	Single grain	Subangular blocky	Subangular blocky	Massive
Structure Size	Medium	Coarse	Medium	Medium		Medium	Very fine	Medium		Medium	Medium	Medium	Fine	
Rupture Resistance	Loose	Loose	Friable	Friable	Soft	Friable	Very friable	Firm	Soft	Firm	Loose	Soft	Firm	Very firm
Boundary Distinctness	Clear	Gradual	Abrupt	Clear	Clear	Gradual	Abrupt	Abrupt	Clear	Clear		Clear	Gradual	
Boundary Shape	Smooth	Smooth	Smooth	Wavy	Irregular	Irregular	Smooth	Smooth	Wavy	Smooth		Wavy	Wavy	
Mottles Quantity	2-5%	<1%	15-20%	10-15%	2-5%	40-50%	1-2%	5-10%	5-10%	5-10%	2-5%	5-10%	2-5%	10-15%
Mottles Quantity (%)	5.0	0.5	20.5	15.0	5.0	50.5	2.0	10.5	10.5	10.5	5.0	10.5	5.0	15.0
Redoximorphic features					mono S	mono S			mono S	mono S	mono S	mono S	mono S	mono S

Mottles Location	In the matrix (not associated with peds/ pores)		infused into the matrix along faces of peds	In the matrix (not associated with peds/ pores)	In the matrix (not associated with peds/ pores)	In the matrix (not associated with peds/ pores)	infused into the matrix along faces of peds	on faces of peds (all orientations)	infused into the matrix adjacent to pores	In the matrix (not associated with peds/ pores)	In the matrix (not associated with peds/ pores)	infused into the matrix adjacent to pores	In the matrix (not associated with peds/ pores)	In the matrix (not associated with peds/ pores)
Mottle Munsel	7.5YR 5/8	/	7.5YR 5/8	7.5YR 5/8	N 4/4	N 4/4	7.5YR 5/8	7.5YR 5/8	N 4/4	5GY 5/1	5GY 5/1	N 4/4	5GY 5/1	5GY 5/1
Mottle Colour (ISCC-NBS Colour Name)	Strong yellowish brown	/	Strong yellowish brown	Strong yellowish brown	Gray	Gray	Strong yellowish brown	Strong yellowish brown	Gray	Greenish gray green	Greenish gray green	Gray	Greenish gray green	Greenish gray green
Dominant cover type (Aerial mappable)	Bare Mud Flat		Bare Mud Flat	Bare Mud Flat	SubAq MBO		Bare Mud Flat		Veg - Phrag			SubAq Sed		
Representativeness (of WL position)	Somewhat consistent		Moderately consistent	Moderately consistent	Moderately consistent		Somewhat consistent		Moderately consistent			Moderately consistent		
Obs Method (Pit, Auger, etc.)	Small Pit		Shovel Slice	Small Pit	Spear Auger		Large Pit		Spear Auger			Spear Auger		
MBO (Observed Gel)					MBO	MBO								
MBO (Observed mottles)					mono S	mono S			mono S	mono S	mono S	mono S	mono S	mono S
ASS material classifications	Non ASS Material	Moderately Acidic (Hypo sulfidic)	Moderately Acidic (Hypo sulfidic)	Moderately Acidic (Hypo sulfidic)	Hypo sulfidic	Hypo sulfidic	Moderately Acidic (Hypo sulfidic)	Hyper sulfidic	Hypo sulfidic	Non ASS Material	Hypo sulfidic	Hypo sulfidic	Non ASS Material	Hypo sulfidic
Potential Sulfidic Acidity (mol H+/t)	5	10	17	6	202	300	7	7	18	11		164	5	
Titratable Actual Acidity (mol H+/t)	2	3	7	9	0	0	16	11	0	0		4	9	
RA (mol H+/t)	0	0	0	0	0	0	0	0	0	0		0	0	
Net acidity (mol H+/t) (2018 GLs)	7	13	25	14	202	300	23	18	18	11		168	14	
ANCe (mol H+/t)	0	0	0	0	623	312	0	0	1564	384		0	0	
AVS (% Sav DW)			< 0.005		0.15	0.06						0.03		