INFLUENCE OF ACID MINE WATER ON THE DIVERSITY AND METABOLITE SHIFT OF MICROBIAL POPULATIONS OF THE RHIZOSPHERE OF COMMON REED (*PHRAGMITES AUSTRALIS*)

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Abstract. Common reed (*Phragmites australis* (Cav.) Trin. ex Steud.) grows widely in various acid mine water bodies and remediates these polluted sites. There exists only a paucity of studies on the effect of acid mine water on microbial diversity and secondary metabolite compositions of the rhizosphere of the common reed. The aim of this study was therefore to determine the influence of acid mine drainage on the microbial diversity of the rhizosphere of the common reed as well as on its secondary metabolite composition. Two acid mine water bodies were selected for study and were compared with a normal water body. The acid mine status of the water bodies was confirmed by assessing a set of physicochemical properties, microbial diversity was measured using BIOLOG EcoPlates and secondary metabolite compositions were determined by nuclear magnetic resonance. Results showed that acid mine drainage reduces rhizosphere microbial diversity as well as influences secondary metabolite composition.

Keywords: rhizoremediation, microbes, bioremediation, Shannon index, BIOLOG EcoPlates

Introduction

Acid mine drainage (AMD) also known as acid rock drainage is a process that occurs as a result of the contact between oxygenated water and mineral pyrite which leads to the oxidation of the pyrite and the production of acids (Evangelou, 1995; Blowes et al., 2003). Areas affected by AMD are usually lost but there are various remediation technologies adopted by many countries to mitigate the impact of AMD, among them is the use of reeds such as *Typha* sp. and *Phragmites* sp. which can adsorb heavy metals. *Phragmites australis* (Cav.) Trin. ex Steud.) is a large perennial grass that is predominant in wetlands in the temperate and tropical regions of the world. The microbes associated with the rhizosphere of *P. australis* are believed to contribute to the remediation ability of the plant via the synthesis of metabolites which enhances the growth of plant and hyperaccumulation of the pollutants by both the plant and associated microbes (Bulgarelli et al., 2012; Kawasaki et al., 2016; Robertson-Albertyn et al., 2017; Pétriacq et al., 2017).

Rhizoremediation effectiveness is based on the complementary process between the plants and the microbes where plants pull contaminants into their rhizosphere and the microbes degrade them either in the rhizosphere or in the plant itself or both (Barac et al., 2004; Muratova et al., 2008). *Phragmites australis* employs this method of remediation (Scholes et al., 1999). However, very little is known about the rhizosphere microbial diversity and secondary metabolite profiles of *P. australis* in acid mine sites. This study is aimed at determining the influence of acid mine drainage on the diversity of rhizosphere microbes and the secondary metabolite profiles of *P. australis*. The effect of acid mine drainage on microbial diversity, in general, is understood and therefore the concern of the authors of this manuscript focused on the rhizosphere microbes due to their role in

bioremediation and the uniqueness of their habitat. The understanding of the influence of acid mine water on the diversity of microbial populations in the rhizosphere of the bioremedial *P. australis* and the metabolite shift may prove to be vital knowledge for bioremediation processes and microbes adaptations in acid mine.

Materials and Methods

This study was undertaken to assess the effect of acid mine drainage on the microbial diversity and secondary metabolite shifts of rhizosphere soil of two acid mine sites (Wuinze 17 and Lan 3) and one non-acid mine site (Florida Lake) in the Gauteng province in South Africa which were all within a 17.7 Km radius. Rhizosphere soil sampling was done on the edge of the water body where the plant roots are constantly submerged in water. In three field study regimes undertaken, water was also sampled to determine its physicochemical parameters to assess the extent of acid mine drainage. Acid mine water and contaminated soils are characterised by high electrical conductivity (EC), high total dissolved solutes (TDS), high salinity (Sa), and low pH which is why these parameters were measured to confirm acid mine contamination of the selected sites. The temperature was also measured to evaluate the temperature variation in the acid mine drainage sites and the non-acid mine drainage site. Following the sampling of the water, and soil, microbial diversity of the rhizosphere soil was assessed as well as secondary metabolite profiles of the rhizosphere soil.

Study Area

Lan 3 (S 26°07.820, E 027°46.680' and elevation of 1693 m), a tailing dam of Mintails Mogale Gold Mine in Randfontein and Wuinze 17 (S 26°07.171', E 027°43.305' and elevation of 1670 m), a tailing dam of Sibanye Gold Mine were the acid mine sampling sites in this study. Both sampling sites are located in Randfontein, Gauteng Province, South Africa. Florida Lake is a normal water body which serves as a control in this study. It is also found in the Gauteng Province of South Africa and has a GPS of S 26°10.625', E 027°54.220' and 1673 m elevation. Details of the sampling sites are shown in *Fig. 1*.



Figure 1. Map showing the sampling sites (Mogale Gold Mine, Sibanye Gold Mine and Florida Lake)

Sample Collection

From each of three sampling sites, three sampling points were identified. Water was scooped with a bucket from each sampling point and physicochemical parameters, namely, pH, temperature, salinity, total dissolved solutes and electrical conductivity were measured on-site using a multi-parameter ion-specific meter (Hanna instruments, version HI9828, SN 08334776). Furthermore, three rhizosphere soil samples (one sample per each uprooted reed plant that was 1 m apart) were collected and pooled equally to form a 1 gram sample. The soil samples were transported in ice to the laboratory where a further composite sample of 3 grams was pooled from each of the 1 gram samples which represented a sampling site. Details of the sampling and analysis done are shown in *Fig. 2* and *Table 1* respectively.



Figure 2. Diagrammatic representation of rhizosphere soil sample collection in a water body. Notice that the sampling was done on the edge of muddy waters. Water was scooped with a bucket from the same spots where rhizosphere soil samples were collected. This process was done on each sampling period

Table 1. Summary of the samples collected and analysis done. Lan and Wuinze 17	are acid
mine sites and Florida Lake is a normal water body	

Sampling sites	Samples collected	Analysis done
Wuinze 17	Water	Physicochemical properties
Wuinze 17	Rhizosphere soil	Biodiversity and secondary metabolite profile
Florida Lake	Water	Physicochemical properties
Florida Lake	Rhizosphere soil	Biodiversity and secondary metabolite profile
Lan 3	Water	Physicochemical properties
Lan 3	Rhizosphere soil	Biodiversity and secondary metabolite profile

Assessment of Microbial Community Functional Diversity

Within two hours of sampling three grams of rhizosphere soil (one composite sample for each of the three sites) were mixed with 27 mL of sterile 0.85% sodium chloride solution, vortexed for five minutes and the silt was allowed to settle for an additional five minutes. 180 μ L of the water fraction was dispensed into each of the 96 wells of the BIOLOG Ecoplate. One BIOLOG Ecoplate was used for each of the sampling sites and

therefore a total of three plates were used per sampling regime resulting in a total of 9 plates used for the three regimes. The plates were sealed with parafilm and incubated in the dark at 25° C. Absorbance was measured at 590 nm with a VarioSkan Flash (Thermoscientific) absorbance scanner at 0, 24, 48, 72, 96 and 120 hours after incubation. The purple coloration of the BIOLOG Ecoplate is an indication of the substrate utilization and functional diversity. The measured absorbance is used to calculate the Shannon index of biodiversity which indicates the measure of the microbial community present in the sampling sites.

Physicochemical Data Analysis

Measurements of physicochemical parameters were analyzed by analysis of variance (ANOVA). One way ANOVA was done using SAS version 9.4 with a significant level of p<0.05. Post hoc testing was carried out using the Tukey test to test the differences among all possible pairs of treatments. The mean and standard deviation of all the samples were recorded for each sampling sites. Results of this analysis are presented in *Table 2A-C*.

Table 2A. Rhizosphere soil and water collected from the three sampling sites (Wuinze 17 at Sibanye Gold Mine, Florida Lake at Florida Roodepoort, and Lan 3 at Minitails Mogale Gold Mine) on September 2017

Sample ID	Temp.	EC	TDS	Sa	pН	SI
^{WW1} 1	12±1.23 °	2286±128.3 ^b	1969.50±15.9 ^b	1.59±0.05 ^b	6.12±1.58 ^b	NA
ww22	12±1.23 °	2286±128.3 ^b	1969.50±15.9 ^b	1.59±0.05 ^b	6.12 ± 1.58^{b}	NA
ww33	12±1.23 °	2286±128.3 ^b	1969.50±15.9 ^b	1.59±0.05 ^b	6.12 ± 1.58^{b}	NA
^{WS1} 4	NA	NA	NA	NA	NA	3.24±0.13 ^b
^{WS2} 5	NA	NA	NA	NA	NA	3.24±0.13 ^b
^{WS3} 6	NA	NA	NA	NA	NA	3.24±0.13 ^b
^{FW4} 7	16.0±1.46 ^b	167.9±4.56 °	135.2±19.5 °	$0.10{\pm}0.02^{\circ}$	8.11±2.53 ^a	NA
FW58	16.0±1.46 ^b	167.9±4.56 °	135.2±19.5 °	$0.10{\pm}0.02^{\circ}$	8.11±2.53 ^a	NA
FW69	16.0±1.46 ^b	167.9±4.56 °	135.2±19.5 °	$0.10{\pm}0.02^{\circ}$	8.11±2.53 ^a	NA
FS410	NA	NA	NA	NA	NA	4.55±2.60 ª
FS511	NA	NA	NA	NA	NA	4.55±2.60 a
FS612	NA	NA	NA	NA	NA	4.55±2.60 ª
^{LW7} 13	22.3±2.04 ^a	6142±267.8 ^a	4407±236.9 ^a	3.75±0.89 ^a	5.03±1.56 °	NA
^{LW8} 14	22.3±2.04 ^a	6142±267.8 ^a	4407±236.9 ^a	3.75±0.89 ª	5.03±1.56 °	NA
^{LW9} 15	22.3±2.04 ^a	6142±267.8 ^a	4407±236.9 ^a	3.75±0.89 ª	5.03±1.56 °	NA
^{LS7} 16	NA	NA	NA	NA	NA	$2.89{\pm}0.04^{\circ}$
^{LS8} 17	NA	NA	NA	NA	NA	2.89±0.04 °
LS918	NA	NA	NA	NA	NA	2.89±0.04 °

Mean \pm standard deviation. Mean with the same superscript letters are not significant at p<0.05. On the sample ID, the first superscript letter is for the site (W=Wuinze 17, F=Florida Lake, L=Lan 3), the second superscript letter is for sample type (W=Water, S=Soil), and the superscript number is for sampling points (1-9). Three rhizosphere soil samples were collected from each of the nine points (1-9) shown in *Fig. 2*. From each sampling point, water was also scooped and its temperature (Temp.), electrical conductivity (EC), total dissolved solids (TDS), salinity (Sa), and pH determined. Shannon indices (SI) of soil samples collected from two acid mine sites (W 1-3 for Wuinze 17 sampling points; and L 7-9 for Lan 3 sampling points) and the non-acid mine site (F 4-6 for Florida Lake) are represented

Sample ID	Temp.	EC	TDS	Sa	pН	SI
^{ww1} 1	10.7±1.05 °	1724±104.6 ^b	1546.3±12.7 ^b	1.22±0.03 ^b	6.54±1.08 ^b	NA
^{WW2} 2	10.7±1.05 °	1724 ± 104.6^{b}	1546.3±12.7 ^b	1.22±0.03 ^b	$6.54{\pm}1.08^{b}$	NA
^{WW3} 3	10.7±1.05 °	1724 ± 104.6^{b}	1546.3±12.7 ^b	1.22±0.03 ^b	$6.54{\pm}1.08^{b}$	NA
^{WS1} 4	NA	NA	NA	NA	NA	$2.81{\pm}0.08^{b}$
^{WS2} 5	NA	NA	NA	NA	NA	$2.81{\pm}0.08^{b}$
^{WS3} 6	NA	NA	NA	NA	NA	$2.81{\pm}0.08^{b}$
^{FW4} 7	14.2±1.11 ^b	111.9±2.74 °	102.4±14.3°	0.05 ± 0.01 ^c	7.05 ± 1.12^{a}	NA
FW58	14.2±1.11 ^b	111.9±2.74 °	102.4±14.3°	0.05 ± 0.01 ^c	7.05 ± 1.12^{a}	NA
FW69	14.2±1.11 ^b	111.9±2.74 °	102.4±14.3°	0.05±0.01 °	7.05±1.12 ^a	NA
FS410	NA	NA	NA	NA	NA	$3.34{\pm}0.04^{a}$
FS511	NA	NA	NA	NA	NA	3.34±0.04 ^a
FS612	NA	NA	NA	NA	NA	$3.34{\pm}0.04^{a}$
^{LW7} 13	19.87±1.2 ^a	4236±148.2 ª	3682±156.2 ª	3.10±0.36 ^a	5.87±1.15°	NA
^{LW8} 14	19.87±1.2 ^a	4236±148.2 ª	3682±156.2 ª	3.10±0.36 ^a	5.87 ± 1.15 °	NA
^{LW9} 15	19.87±1.2 ^a	4236±148.2 ª	3682±156.2 ª	3.10±0.36 ^a	5.87 ± 1.15 °	NA
^{LS7} 16	NA	NA	NA	NA	NA	2.7±0.12 °
^{LS8} 17	NA	NA	NA	NA	NA	2.7±0.12 °
^{LS9} 18	NA	NA	NA	NA	NA	2.7±0.12°

Table 2B. Rhizosphere soil and water collected from the three sampling sites (Wuinze 17 at Sibanye Gold Mine, Florida Lake at Florida Roodepoort, and Lan 3 at Minitails Mogale Gold Mine) on November 2017

Mean \pm standard deviation. Mean with the same superscript letters are not significant at p<0.05. On the sample ID, the first superscript letter is for the site (W=Wuinze 17, F=Florida Lake, L=Lan 3), the second superscript letter is for sample type (W=Water, S=Soil), and the superscript number is for sampling points (1-9). Three rhizosphere soil samples were collected from each of the nine points (1-9) shown in *Fig.* 2. From each sampling point, water was also scooped and its temperature (Temp.), electrical conductivity (EC), total dissolved solids (TDS), salinity (Sa), and pH determined. Shannon index (SI) of samples collected from two acid mine sites (W 1-3 for Wuinze 17 sampling points; and L 7-9 for Lan 3 sampling points) and the non-acid mine site (F 4-6 for Florida Lake) are represented

Biodiversity Data Analysis

Absorbance values obtained from the VarioSkan Flash absorbance scanner were adjusted by subtracting the baseline absorbance value (0-hour absorbance). The 96-hour absorbance values differentiated between the three water bodies the most and were, therefore, the only ones considered for further analysis. The Shannon index of diversity (*H*) was calculated following the approach of Gryta et al. (2014). One way analysis of variance (ANOVA) was done on all the biodiversity indicators using SAS version 9.4 at a significant level of p<0.05. Shannon index was correlated with temperature, electrical conductivity, total dissolved solutes, salinity, and pH; and 0.8 and -0.8 were set as the cut-off for Pearson correlation coefficients at a significant level of p<0.05 (*Table 3*). Results of the microbial diversity are presented in *Table 2A-C* and *Fig. 3*.

Secondary Metabolite Shift Assessment

Secondary metabolite profiles of rhizosphere soil from the three sites were done using a 600 MHz Varian NMR spectroscopy. NMR spectra were processed with ACD/NMR

and principal component analysis of intensity data was analysed using MetaboAnalyst. Principal component analysis plots are presented in *Fig. 4*.

Table 2C. Rhizosphere soil and water collected from the three sampling sites (Wuinze 17 at Sibanye Gold Mine, Florida Lake at Florida Roodepoort, and Lan 3 at Minitails Mogale Gold Mine) on June 2018

Sample ID	Temp.	EC	TDS	Sa	pН	SI
^{WW1} 1	7.9±0.31 ^b	1791.3±10.9 ^b	1557.2±1.15 ^b	1.24±0.02 ^b	6.50±0.31 ^b	NA
ww22	7.9±0.31 ^b	1791.3±10.9 ^b	1557.2 ± 1.15^{b}	1.24±0.02 ^b	6.50±0.31 ^b	NA
^{WW3} 3	7.9±0.31 ^b	1791.3±10.9 ^b	1557.2 ± 1.15^{b}	$1.24{\pm}0.02^{b}$	6.50 ± 0.31^{b}	NA
^{WS1} 4	NA	NA	NA	NA	NA	3.33±0.05 ^a
^{WS2} 5	NA	NA	NA	NA	NA	3.33±0.05 ^a
^{WS3} 6	NA	NA	NA	NA	NA	3.33±0.05 ^a
^{FW4} 7	5.4±0.2 °	114.5±0.36 °	119.3±0.42 °	0.09±0.01 °	7.20±0.01 ^a	NA
FW58	5.4±0.2 °	114.5±0.36 °	119.3±0.42 °	0.09±0.01 °	7.20±0.01 ^a	NA
FW69	5.4±0.2 °	114.5±0.36 °	119.3±0.42 °	0.09±0.01 °	7.20±0.01 ^a	NA
FS410	NA	NA	NA	NA	NA	3.34±0.01 ^a
FS511	NA	NA	NA	NA	NA	3.34±0.01 ^a
FS612	NA	NA	NA	NA	NA	3.34±0.01 ^a
^{LW7} 13	12.3±0.15 ^a	4424.3±1.15 ^a	3823±1.0 ª	3.20±0.10 ^a	5.53±0.11 °	NA
^{LW8} 14	12.3±0.15 ^a	4424.3±1.15 ^a	3823±1.0 ª	3.20±0.10 ^a	5.53±0.11 °	NA
^{LW9} 15	12.3±0.15 ^a	4424.3±1.15 ^a	3823±1.0 ª	3.20±0.10 ^a	5.53±0.11 °	NA
^{LS7} 16	NA	NA	NA	NA	NA	$2.80{\pm}0.71^{b}$
^{LS8} 17	NA	NA	NA	NA	NA	2.80±0.71 ^b
^{LS9} 18	NA	NA	NA	NA	NA	2.80±0.71 ^b

Mean \pm standard deviation. Mean with the same superscript letters are not significant at p<0.05. On the sample ID, the first superscript letter is for the site (W=Wuinze 17, F=Florida Lake, L=Lan 3), the second superscript letter is for sample type (W=Water, S=Soil), and the superscript number is for sampling points (1-9). Three rhizosphere soil samples were collected from each of the nine points (1-9) shown in *Fig. 2*. From each sampling point, water was also scooped and its temperature (Temp.), electrical conductivity (EC), total dissolved solids (TDS), salinity (Sa), and pH determined. Shannon index (SI) of samples collected from two acid mine sites (W 1-3 for Wuinze 17 sampling points; and L 7-9 for Lan 3 sampling points) and the non-acid mine site (F 4-6 for Florida Lake) are represented

Table 3. Correlation coefficients of the Shannon index of biodiversity and the physicochemical parameters for rhizosphere soil and water samples collected from the sampling points

Donomotona	Shannon index of biodiversity				
Farameters	September 2017	November 2017	June 2018		
pН	0.48	0.24	0.95		
Temperature	-0.20	-0.88	-0.97		
Salinity	-0.47	-0.29	-0.97		
TDS	-0.46	-0.27	-0.97		
EC	-1.00	-0.88	-0.96		

Values greater or equal to 0.80 or-0.80 are taken as the threshold cut off in this study (n=3). Correlation is significant at the level p<0.05. TDS and EC stand for total dissolved solids and electrical conductivity

FLORIDA LAKE	LAN 3	WUINZE 17
0000000	00000000	
00000000		
00000000	00000000	
00000000	0000000	
0.000000	0 0 0 0 0 0 0 0 0	0000000
	0000000	
	0000000	0000000
	00000000	
00000000	0000000	
	00003000	
0, 0, 0, 0, 0, 0, 0, 0	00000000	

Figure 3. Ninety-six well BIOLOG Ecoplates showing catabolic activities of the microbial community of the rhizosphere of Phragmites australis after 96 hours of incubation. The ecoplates are precoated with 31 carbon substrates and the purple colour of the well indicates utilization of the substrate by the microbes in the water. Notice that no substrate turned purple in the middle 96 well ecoplate showing complete loss of microbial activities as a result of acid mine drainage. All the sets of BIOLOG Ecoplates looked similar and therefore only one set (sampled in September 2017) was shown



Figure 4. Principal component analysis showing the secondary metabolite shifts of soil samples of Phragmites australis. Florida Lake and Wuinze 17 clustered together indicating closeness in their metabolites. The metabolites of Lan 3 were differentiated from the other two sites

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Results

Measurements of physicochemical parameters of the water bodies confirmed that the sites selected as acid mine sites indeed showed characteristics of acid mine pollution and the normal site had characteristics of a regular water body. This was shown by the low pH, and high electrical conductivity (EC) and total dissolved solids (TDS) values in the acid mine sites and relatively normal in the water samples from Florida Lake and also the significant difference at p<0.05 across the three sampling sites for the physicochemical parameters. The applications of BIOLOG EcoPlates in the evaluation of microbial diversity by many researchers have proven to be effective in providing a good indication of microbial diversity (Wang et al., 2008; Nautiyal et al., 2010; Ling et al., 2012; Zhang et al., 2013, 2014; Deng et al., 2014; Xu et al., 2015; Liu et al., 2015; Raghawendra et al., 2019). In this study, BIOLOG EcoPlates were used to measure the microbial diversity of rhizosphere soil of *Phragmites australis*. Microbial diversity in the two acid mine sites was reduced compared to the normal water body. However, the Wuinze 17 site retained most of its microbial diversity despite having a relatively high electrical conduction and low pH characteristic of acid mine water. Although these parameters had values higher than those of Florida they were still far less than those of the very harsh Lan 3 site. Proton NMR spectroscopy has also proved effective in secondary metabolite profiling of different samples (Blow, 2008; De Meyer et al., 2008; Nicholson and Lindon, 2008; Alves et al., 2009; Pontoizeau et al., 2010; Ravanbakhsh et al., 2015; Alonso et al., 2015). The secondary metabolites profiles as observed in this study showed diversity among the sampling sites. However, one of the acid mine sites (Wuinze 17) showed close clustering of the metabolites with the normal water body (Florida Lake). This could be attributed to the ability of the rhizosphere microbes associated with *Phragmites australis* to increase the pH of Wuinze 17 that was 1.03 above the pH of Lan 3 site (an acid mine site) to meet the acceptable limits of DWAF (1996) guidelines as well as the not so high EC in Wuinze 17. This could lead to the production of metabolites similar to the ones produced by the microbes in Florida Lake.

Physicochemical Characteristics

The results of the physicochemical parameters showed that there were significant differences between each of the parameters across the two acid mine sites (Wuinze 17 and Lan 3) and the non-acid mine site (Florida Lake) (Table 2A-C). The acid mine drainage samples from the two acid mine sites (Wuinze 17 and Lan 3) showed higher electrical conductivity, salinity, total dissolved solids and low pH when compared to the non-acid mine drainage (Florida Lake) (Table 2A-C). The findings of many researchers have shown acid mine drainage to be characterized by pH values that are less than five (Dagmar Kock, 2008; Denef et al., 2010; USGS, 2012; Masindi et al., 2015; Chen et al., 2016; Teng et al., 2017). The guidelines stipulated for acid mine treated water according to DWAF (1996) are 6 – 9 for pH, 0 – 1 200 mg/L for total dissolved solutes, and $0-450 \mu$ S/cm for electrical conductivity. The pH values of Wuinze 17 (acid mine site) and Florida Lake (normal water body) were observed to be within the recommended guideline. This could be an indication of more remediation activities of *Phragmites* australis and the associated rhizosphere microbes in Wuinze 17 than in Lan 3. This could be attributed to the difference in the pH between the two acid mine drainage sites that ranges from 0.67 to 1.09. Furthermore, the electrical conductivity and total dissolved solids of Florida Lake was within the required guideline confirming the lake as a regular water body.

Microbial Community Functional Diversity

In this study, the Shannon index of biodiversity was done to assess the microbial diversity of the two acid mine water and the non-acid mine water. High Shannon index indicates more diversity of microbial communities in the sampling sites. The non-acid mine water showed more diversity than the acid mine water.

Shannon Index of Biodiversity

The Shannon indices differ significantly across the three sampling sites in this study (*Table 2A-C* and *Fig. 3*). Florida Lake (non-acid mine drainage site) was observed to be more diversified in the microbial community than the two acid mine sites (Wuinze 17 and Lan 3). The Shannon diversity index of Florida Lake was higher than the two acid mine sites and this was also shown by the higher number of BIOLOG Ecoplate wells which had turned purple indicating the presence of microbes that had the potential to utilize the substrate pre-coated in the well. Wuinze 17 showed higher Shannon index values than Lan 3 presenting Lan 3 as the less diversified site.

Correlation analysis of the Shannon index of Biodiversity and Physicochemical Parameters

The acid mine drainage influenced the microbial diversity of the common reed's rhizosphere. This finding is based on the correlation between the physicochemical parameters and the Shannon index (*Table 3*). Pearson correlation coefficients of 0.8 and -0.8 were set as the cut-off in this study at a significant level of p<0.05. Correlated with salinity and total dissolved solute, the SI had a negative correlation coefficient with the highest negative correlation recorded in the third sampling. The negative correlation means that as salinity and total dissolved solids increase, the rhizosphere microbial diversity decreases. Similarly, electric conductivity showed a strong negative correlation with the Shannon index of biodiversity in all the three sampling periods. However, pH showed a positive correlation with the Shannon index with the strongest correlation recorded in the third sampling negative correlation implied that the increase in pH results in the corresponding increase in microbial diversity. This correlation study provided an insight into the response of the microbial diversity with changes in environmental conditions.

Secondary Metabolites Assessment

The acid mine drainage influenced the secondary metabolite compositions of the rhizosphere soil and roots of *Phragmites australis*. This is evident in the metabolite profiles observed in the principal component analysis (PCA) of secondary metabolites in the soil samples (*Fig. 4*). Surprisingly, the non-acid mine Florida Lake and the acid mine Wuinze17 soil samples showed similar metabolite shift that differs from Lan 3 soil samples. It was expected that the acid mine sites would cluster together instead of the grouping of Florida Lake and Wuinze 17 which resulted from the PCA of NMR data. This could be attributed to the normality of Florida Lake and the fact that Wuinze 17 is not static water and therefore receives fresh supplies of water which may sometimes be fresh. Furthermore, the compliance of the pH values of the two sites to the DWAF (1996)

guidelines could be another contributing factor to the clustering of the sites. This is reaffirm by the closeness of Shannon index of both sites indicating the presence of similar microbes capable of producing similar metabolites.

Discussion

Although there are dynamics in studies investigating aspects of pollution due to mining, conclusions drawn from these studies confirm the loss of biodiversity as a result of pollution. As such the production of secondary metabolites by the affected organisms is likely to change to reflect the new natural imbalances. This phenomenon was further re-established in this work. However, the focus of the authors of this manuscript was on microbes associated with the rhizosphere of *Phragmites australis* because of their role in bioremediation and the uniqueness of their habitat. Efforts in ecosystem restoration in acid mine environments needs to focus on preserving rhizosphere associated microbes to maintain their mutual relationship with their associated bioremediating plant. Results of this study clearly showed that the diversity of the P. australis rhizosphere associated microbes of the two acids mine drainage sites (Wuinze 17 and Lan 3) were reduced when compared to the non-acid mine drainage site (Florida Lake) in all the sampling periods. The correlation between the physicochemical parameters and the Shannon index of biodiversity showed that the acid mine drainage influenced microbial diversity (Table 3). The secondary metabolite analysis showed metabolite shifts among the soil samples in the three sampling sites indicating diversity and difference in their secondary metabolite composition.

The reasons for the loss of diversity in the acid mine sites in this study could have been caused by the pH gradient. A neutral pH of 7 could pose no environmental problems to the existing organisms. Brady and Weil (1996) suggested that extreme pH affects the survival of microorganisms directly or indirectly via the control of auxillary environmental parameters relating to pH like nutrient availability and cationic metal solubility. This suggestion was supported by the work done by Amaral-Zettler et al. (2011), Li et al. (2014), Sun et al. (2014) and Sun et al. (2015) who observed that pH affects the microbial structure and composition of environments. Kuang et al. (2013) stated that the acidic nature of acid mine drainage water tends to pose a major risk factor to existing diversity of microbial community and structure when discharged directly into water bodies. Furthermore, many researchers have reported that the low pH in acid mine drainage water accelerates the solubility of many metals, and in addition with low availability of organic carbon, it creates highly challenging and extreme environmental growth conditions for the microbial community (Dopson et al., 2003; Slonczewski et al., 2009). The work of these researchers was consistent with the results obtained in this study because the acid mine sites with low pH showed less microbial diversity than the non-acid mine site based on the Shannon index.

Sun et al. (2015) observed a strong correlation between temperature and microbial community. The authors further observed a strong and significant link between temperature and microbial community variance. Volant et al. (2014), and Edwards et al. (1999) reported temperature as a crucial factor that shapes the community composition of microbes in acid mine drainage. Similarly, in this present study, the temperature was observed to correlate strongly with the Shannon index of biodiversity. This finding indicated the influence of the environmental temperature on microbial community composition. However, it should be stated that the three sampling sites in this study are

within a 17.7 km radius and therefore fall under the same climatic zone and therefore the difference in the water temperature was not large but had however a significant influence on the Shannon index an indication of the elasticity of the Shannon index. Further studies on water temperature and the Shannon index are recommended to deepen understanding of the dependence of microbial activity and water temperature.

Salinity can influence and possibly change soil environment leading to changes in the soil microbial composition, structure, and biomass. This implied that salinity brought about by the presence of dissolved solids in the acid mine plays a major role in determining the diversity and composition of microbes and the metabolites produced by the microbes in order to adapt to the changing environment. The correlation analysis of the Shannon index of biodiversity with the physicochemical parameters also showed that indeed salinity must have reduced diversity in this study. A plethora of similar studies agree with the results of this study (Batra and Manna, 1997; Pathak and Rao, 1998; Rousk et al., 2011; Setia et al., 2011; Andronov et al., 2012; Yan et al., 2015; Chen et al., 2017). The reduction of microbial biomass in the high saline environment is due to osmotic stress that leads to lysing and drying of cells (Laura, 1974; Sarig and Steinberger, 1994; Sarig et al., 1996; Batra and Manna, 1997; Pathak and Rao, 1998; Rietz and Haynes, 2003; Yuan et al., 2007a).

Yaish et al. (2016) discovered that soil salinity causes a reduction in the bicarbonate and calcium carbonate of the soil. A discovery that was consistent with the work done by Setia et al. (2013) where the effect of salinity in the reduction of the global carbon stocks was observed due to the reduction in the microbial activity. This implies that an increase in the salinity of the environment results in a corresponding decrease in the microbial structure and possibly the diversity. The extreme environmental condition of the acid mine drainage might be the cause of the change in the microbial activity and the diversity of the heterogeneous microbial community in this study. This was evidenced in the work done by Keshri et al. (2015) who discovered that the Vanadium mine water samples were less acidic and saline with higher bacterial diversity. This result concurs with the results obtained in this study where the Florida Lake, a non-acid mine drainage sites and Wuinze 17, an acid mine drainage site, which were less acidic were observed to have higher microbial diversity than Lan 3, an acid mine drainage site that was more acidic.

Previous studies showed a correlation between the physicochemical parameters and the microbial community composition (Dopson et al., 2003; Slonczewski et al., 2009). This present study supported the work done by Dopson et al. (2003) and Slonczewski et al. (2009) because correlation coefficients of the biodiversity indicator, showed both positive and negative correlation with different physicochemical parameters. For example, electrical conductivity showed a strong negative correction throughout the sampling periods in this study. Some studies indicated that increase in soil electrical conductivity leads to decrease in soil respiration and consequently affect the soil microbial community (Adviento-Borbe et al., 2006; Yuan et al., 2007b; Wong et al., 2009). These results were in agreement with the results obtained in this study where the two acid mine sites with high electrical conductivity experienced low microbial diversity in terms of the Shannon index. This reduction in Shannon index could be attributed to the reduction in the cell respiration or the osmotic stress faced by the soil microbes.

There are variations in the sensitivity of enzymatic activities to varying salinity in the soil (Frankenberger and Bingham, 1982; Garcia and Hernandez, 1996; Pan et al., 2013). This could be attributed to variation in the metabolite profiles caused by the varying salinity state in the environment. Work done by many researchers has shown that

variation in salt tolerance among microorganisms' influences the microbial community structure and metabolite profiles in saline soils more than the non-saline soils (Quesada et al., 1982; Del-Moral et al., 1987; Zahran et al., 1992; Killham, 1994; Pankhurst et al., 2001; Oren, 2001; Gros et al., 2003; Sagot et al., 2010). Furthermore, some studies have shown the relationships between enzyme activities and the metabolites produced by both plants and microbes in response to harsh environment factors (Sagot et al., 2010; Sulpice et al., 2010; Méndez-García et al., 2015; Jian et al., 2016). Similarly, in this study, the diverse metabolites produced by the microbes could be a surviving factor for those organisms and possibly be responsible for the metabolite shifts observed between the acid mine sites and non-acid mine sites in this study.

Tian et al. (2014) pointed out that for effective remediation in constructed wetlands using macrophytes like *P. australis*, microbial communities present in the rhizosphere of plant species selected for the bioremediation play major roles in promoting the growth of plants and biodegrading the contaminants. The understanding of the influence of the acid mine water on the rhizospheres' microbes in this present study unveiled the need to preserve these microbes in order to enhance the remediation processes of the macrophytes.

Conclusion

This study evaluated the influence of the acid mine drainage on the microbial diversity and metabolite shift of the common reed's rhizosphere. The evaluation was successful and provided a clear distinction of the metabolite profiles and microbial diversities of the acid mine drainage (AMD) sites and the non-acid mine drainage site. The AMD sites showed a low microbial diversity which is evident in the biodiversity indicator. However, Wuinze 17 and the non-acid mine site showed similar metabolite profile. This could be attributed to remediating ability of the microbes associated with the rhizosphere of *Phragmites australis* leading to the compliance of pH values of Wuinze 17 to DWAF (1996) guidelines and the closeness of the Shannon index of biodiversity values of both sites (Wuinze 17 and Florida Lake). The closeness in their Shannon index could imply the presence of similar microbes capable of producing similar metabolites. The present study suggests further study on the identification of the microbial communities in the three sampling sites and the remediation ability of the identified microorganisms across environmental pollutants.

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