# Determination of the bacterial diversity of a natural freshwater wetland impacted by acid mine drainage.

by
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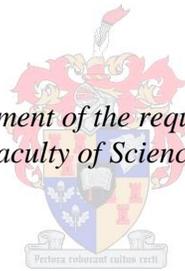
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Declaration

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Abstract

Freshwater resources in semi-arid countries, such as South Africa, are under constant threat from pollution. One of the major pollutants is acid mine drainage, which not only lowers the pH of the water, but also increases sulphate and metal concentrations. Primary producers, such as bacteria and algae, are the first organisms to respond to stressors such as reduced pH and elevated sulphate and metal concentrations. A natural freshwater wetland, the Zaalklapspruit wetland in Mpumalanga, impacted by acid mine drainage and industrial effluent was studied to determine the change in algal and bacterial populations. Five study sites were identified including a reference site and four sites displaying various degrees of degradation. Physical and chemical parameters were measured at each site. Algae were identified microscopically and chlorophyll-*a* concentrations were measured. The algal species present at the five study sites were species previously associated with the conditions present at the various sites. *Gyrosigma rautenbachiae* proved to be an ideal bioindicator for industrial pollution. The diatom species *Synedra ulna*, *Nitzschia* spp. and *Cymbella* spp. were found at the acidic sites. The filamentous green algae *Microspora quadrata* and *Klebsormidium acidophilum* were abundant at the sites the most impacted by AMD. Metal tolerant *K. rivulare* were also identified in this study. The cyanobacteria *Oscillatoria tenius* and *Glaucospira* sp. were associated with enriched conditions.

The bacterial populations were sampled from both the water column and sediment and subjected to next generation sequencing for identification. The phyla that were highly represented throughout all the samples were the *alpha-*, *beta-* and *gamma-Proteobacteria*, *Bacteriodetes* and unclassified species. The *Bacteriodetes* phylum was observed at significantly higher numbers at sites 1, 2, 3 & 5 in the March 2013 water samples and sites 1 & 4 in the March 2013 sediment samples. *Firmicutes* had significantly higher numbers at sites 2 (January 2013), 3 (March 2013) & 4 (January 2013) in the water samples. Both water and sediment samples of sites 2 (March 2013) & 4 (January 2013) had significantly higher numbers of *Actinobacteria*. The *Chloroflexi* phylum had significantly higher numbers in the site 4 & 5 (January 2013) water samples and site 5 (January 2013) of the sediment samples. *Acidobacteria* were only detected in significantly higher numbers in the January 2013 sediment samples of sites 1 & 5. This study was the first to assess the total bacterial diversity in a natural, acid mine drainage impacted wetland in South Africa and also the first to identify sequences from the genus *Marinobacterium*.

The wetland ecosystem health was also determined using a rapid bioassessment tool and a proposed bacterial bioindicator. The bioassessment tool scored the reference site as mostly natural, two sites as severely modified and the last two as modified. The proposed bacterial bioindicator was simplistic in use and reflected the stability of the populations at the five sites accordingly. Lastly, the bacterial bioindicator was incorporated into the established bioassessment tool and was found to correspond with the latter's results.

Opsomming

Varswater bronne in semi-droë lande soos Suid-Afrika is konstant onder druk van besoedeling. Een van die groot besoedeling bronne is suur myn-water, wat beide die pH van die water verlaag en die sulfaat en metal konsentrasies verhoog. Primêre produseerders soos bakterie en alge is die eerste organismes geaffekteer deur stresfaktore soos die bogenoemde. 'n Natuurlike varswater vlei, die Zaalklapspruit vlei in Mpumalanga, besoedeld deur suur myn-waater en industriële uitvloei was bestudeer om die veranderinge in die alge en bakteriese populasies waar te neem. Vyf studie areas was geïdentifiseer, wat 'n verwysings area en vier degradeerde areas insluit. Fisiese en chemiese parameters was gemeet by elke area. Alge was geïdentifiseer deur mikroskopie en chlorofil-*a* konsentrasies was gemeet. Die alge spesies teenwoordig by die vyf studie areas was voorheen gekoppel aan kondisies gemeet by elke area. *Gyrosigma rautenbachiae* was n ideale bioindikator vir industriële uitvloei. Die diatom spesies *Sunedra ulna*, *Nitzchia* spp. en *Cymbella* spp. was geïdentifiseer by studie areas met 'n lae pH. Die filamentige, groen alge *Microspora quadrata* en *Klebsormidium acidophilum* was ook oorvloedig by areas geaffekteer deur die suur myn-water. Metaal-tolerante *K. rivulare* was ook gevind in hierdie studie. Die cyanobakterie *Oscillatoria tenius* en *Glaucospira* sp. was geassosieer met verrykde kondisies.

Die bakteriese populasies was gemonster van beide die water kollom en die sediment en geanaliseer deur middle van volgende generasie volgordebepaling vir identifikasie. Die phyla wat hoogs verteenwoordig was in al die monsters was die *alpha-, beta-* en *gamma-Proteobakterie*, *Bacteriodetes* en ongeklassifiseerde spesies. Die Bacteriodetes phylum was teëgekom teen beduidende hoër getalle by areas 1, 2, 3 & 5 in die Maart 2013 water monsters en areas 1 & 4 in die Maart 2013 sediment monsters. *Firmicutes* het beduidende hoër getalle gehad by areas 2 (Januarie 2013), 3 (Maart 2013) & 4 (Januarie 2013) in die water monsters.

Beide die water en sediment monsters van area 2 (Maart 2013) & 4 (Januarie 2013) het beduidende hoër getalle *Actinobacteria* gehad. Die *Chloroflexi* phylum het beduidende hoër getalle in die area 4 & 5 (Januarie 2013) water monsters en area 5 (Januarie 2013) sediment monster gehad. *Acidobacteria* was slegs verteenwoordig deur beduidende hoër getalle in die Januarie 2013 sediment monsters van areas 1 & 5. Hierdie studie was die eerste van sy soort om die totale bakteriese populasie in 'n natuurlike, suur myn-water geïmpakteerde vleiland in Suid-Afrika te bestudeer asook die eerste studie om 'n organisme van die genus *Marinobacterium* te identifiseer in 'n vlei.

Die vlei se ekosisteem gesondheid was bepaal deur middle van 'n spoedige bioassesseeerings hulpmiddel en 'n voorgestelde bioindikator. Die bioassesserings hulpmiddel het die verwysings area geklassifiseer as meestal natuurlik, twee studie areas as gemodifiseer en twee as ernstig gemodifiseer. Die voorgestelde bakteriese bioindikator was eenvoudig om te gebruik en het die stabiliteit van die populasies verteenwoordig by die verskillende studie areas. Die bakteriese bioindikator was geïnkorporeer in die bioassesseerings hulpmiddel en dit was gevind dat die resultate ooreen stem.

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"We never know the worth of water until the well is dry." – Thomas Fuller

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Figure 2:   Profile bar plot indicating the relative number of sequences assigned to the various bacterial phyla from the 16S rRNA gene sequences of the sediment samples. Comparison is drawn between the two sampling events. Black bars indicate the 95% confidence interval for each and the * indicates significant differences. A: site 1; B: site 2; C: site 3; D: site 4; E: site 5.

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Al: aluminium; Zn: zinc; Ni: nickel; Mn: manganese; Pb: lead; SO4: sulphate; TDS: total dissolved solids; EC: electrical conductivity; Chl(s): suspended chlorophyll-*a*.

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Introduction

South Africa is a water stressed country with an annual average rainfall of 450 mm, below the international average of 860 mm (DWAF, 2004a; Chetty & Luiz, 2014). The importance of conserving our remaining freshwater resources is emphasised by the fact that the quality of the water determines the quantity available (Strydom *et al*., 2010). South Africa is a mineral rich country and the Mpumalanga Province is home to large coal reserves, namely the Springs-Witbank and Highveld coalfields (Vermeulen & Usher, 2006). Mining activities, past and present, have not only put pressure on freshwater resources, but have also contributed to large scale contamination. Decant from closed mines have been estimated at 62 Mℓ/d, with large quantities decanted into the Upper Olifants River Catchment area (DWAF, 2004b; Maree *et al*., 2004). This leads to the production of acid mine drainage (AMD) which is microbially mediated (Johnson & Hallberg, 2005) and characterised by a low pH, a high concentration of dissolved metals such as aluminium, iron and manganese and high concentrations of dissolved sulphates (García *et al*., 2001).

Various methods have been employed to remediate AMD (Fiset *et al*., 2003; Hong *et al*., 2014) including chemical treatment and the use of constructed wetlands. These water bodies not only provide invaluable ecosystem services such as providing habitats for aquatic organisms, but wetlands are also beneficial to humans as they improve water quality and may be used for recreational uses (Kent, 2000). The services that wetlands provide may carry financial value. Wetland services within South Africa, to the community, have been valued at R 382 million for a total area of 72 182 ha in the Upper Olifants River (DWAF, 2010).
Natural wetlands are not only at risk from AMD pollutants, but may also be employed for remediation. Loss and degradation may occur through anthropogenic activities within the catchment (Ehrenfeld, 2000; review: Zedler & Kercher, 2005). As a result, rehabilitation of wetlands has become increasingly important as we begin to understand the importance of these water bodies in South Africa. The aim of rehabilitation of wetlands is to reverse the effects of pollutants on the biota within wetland ecosystems (Zedler, 2000).

Wetlands are unique habitats, where changing hydrology may result in varying aerobic and anaerobic zones that select for specialised species. The microbial populations within a wetland play an important role in nutrient cycling and form the basis of the food web thus are vulnerable to environmental change (Yergeau *et al.,* 2012; Sims *et al.,* 2013). Bacteria and

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algae are potential bioindicators for ecosystem health assessment due to their cosmopolitan nature to inhabit various habitats, their short life-cycle and role as primary producers. Algae have been widely used as bioindicators in the past, yet bacterial assemblages are yet to be used for this purpose (Sims et al., 2013). This is due to the poor understanding of bacterial assemblages in impacted freshwater environments.

There is a need for multidisciplinary studies on complex ecosystems such as wetlands. The organisms that inhabit wetlands respond to varying environmental conditions and stressors such as pollution at each trophic level. Bacteria and algae are known to rapidly respond to change due to their shorter life cycles than higher organisms such as fish and other invertebrates. The aim of this study was to monitor the biological response of algae and bacteria to acid mine drainage in a natural freshwater wetland, the Zaalklapspruit wetland, Mpumalanga, and establish these organisms as bioindicators for ecosystem health.

The objectives of this study were:

- To evaluate the water quality of the studied wetland.
- To determine the algal assemblage composition within an AMD impacted wetland and to link the algal species and environmental conditions present at the study sites.
- To determine the bacterial population diversity within the wetland impacted by AMD and to link the bacterial OTUs to environmental factors present within the study wetland.
- To assess the ecosystem health using the Ecotoxicological Screening Tool (EST) (Oberholster *et al*., 2013), and incorporating the use of bacterial assemblages for the use as a bioindicator.

References

Chetty, S. & Luiz, J.M., 2014, The experience of private investment in the South African water sector: the Mbombela concession, *Development Southern Africa*, ERSA working paper 429: 1-20.

DWAF, 2004a, 'South Africa's Water Situation and Strategies to balance supply and demand', Proposed first edition national water resource strategy, 1st edition, Department of Water Affairs and Forestry, Pretoria.

DWAF, 2004b, *Olifants Water Management Area: Internal Strategic Perspective*, Report P WMA 04/0000/00/0304, Department of Water Affairs and Forestry, Pretoria.

DWAF, 2010, *The nature, distribution and value of aquatic ecosystem services of the Olifants, Inkomati and Ustutu to Mhlatuze Water Management Areas*, RDM project number WP9677, Department of Water Affairs and Forestry, Pretoria.

Ehrenfeld, J.G., 2000, Evaluating wetlands within an urban context, *Ecological Engineering*, 15: 253-265.

Fiset, J.F., Zinck, J.M., Nkinamubanzi, P.C., 2003, Chemical stabilization of metal hydroxide sludge. In: Proceedings of the X International Conference on Tailings and Mine Waste, October 2003, Vail, CO, USA, AA Balkema, pp. 329–332.

García, C., Moreno, D.A., Ballester, A., Blázquez, M.L. & González, F., 2001, Bioremediation of an industrial acid mine drainage water by metal-tolerant sulphate-reducing bacteria, *Minerals Engineering*, 14: 997-1008.

Hong, S., Cannon, F.S., Hou, P., Byrne, T. & Nieto-Delgado, C., 2014, Sulfate removal from acid mine drainage using polypyrrole-grafted granular activated carbon, *Carbon*, 73: 51-60.

Johnson, D.B. & Hallberg, K.B., 2005, Acid mine drainage remediation options: a review, *Science of the Total Environment*, 338: 3-14.

Kent, D.M., 2000, 'Evaluating wetland functions and values', in D.M. Kent (ed.), *Applied wetlands science and technology*, 2nd ed., p.55-80, CRC Press LLC, U.S.A.

Maree, J.P., Hlabela, P., Nengovhela, A.J., Geldenhuys, A.J., Mbhele, N., Nevhullaudiz, T. & Waanders, F.B., 2004, Treatment of mine water for sulphate and metal removal using barium sulphide, *Mine Water and the Environment*, 23: 195-203.

Oberholster, P.J., Genthe, B., Hobbs, P., Cheng, P.H., de Klerk, A.R. & Botha-Oberholster, A.-M., 2013, An Ecotoxicological screening tool to prioritise acid mine drainage impacted streams for future restoration, *Environmental Pollution*, 176: 244-253.

Sims, A., Zhang, Y., Gajaraj, S., Brown, P.B. & Hu, Z., 2013, Toward the development of microbial indicators for wetland assessment, *Water Research*, 47: 1711-1725.

Strydom, W., Hill, L. & Hobbs, P., 2010, 'Water. State of the environment – issue summary', Viewed 9 April 2013, from http://researchspace.csir.co.za/dspace/handle/10204/4220.

Vermeulen, P.D. & Usher, B.H., 2006, Sulphate generation in South African underground and opencast collieries, *Environmental Geology*, 49: 552-569.

Yergeau, E., Lawrence, J.R., Sanschagrin, S., Waiser, M.J., Korber, D.R. & Greer, C.W., 2012, Next-generation sequencing of microbial communities in the Athabasca River and its tributaries in relation to oil sands mining activities, *Applied and Environmental Microbiology*, 78: 7626-7637.

Zedler, J.B., & Kercher, S., 2005, Wetland resources: status, trends, ecosystem services, and restorability, *Annual Review of Environment and Resources*, 30: 39-74.

Zedler, J.B., 2000, Progress in wetland restoration ecology, *Trends in Ecology and Evolution*, 15: 402-407.

Chapter 1: Literature review

1.1 Introduction

South Africa is a water stressed country with limited freshwater supplies. The annual average rainfall is 450 mm which is below the international average of 860 mm (DWAF, 2004a; Chetty & Luiz, 2014; South African Government Online, n.d.).

Dividing water usage into sectors, irrigation dominates followed by domestic use, after which industrial use and forestry follows. Failure in providing potable water to all South Africans can be attributed to poor governance and inadequate technical and management skills (Chetty & Luiz, 2014). Bearing in mind that the quality of water influences the quantity of usable water directly, as well as the cost of converting it to drinkable standards, the importance of conservation of our water bodies is ever increasing (Strydom *et al.*, 2010). Due to the tempo of rapid urbanization and current lack in water supply/infrastructure in some parts, the International Water Management Institute (IWMI) predicts that South Africa will experience water scarcity by 2025 (Seckler *et al.*, 1996).

Mpumalanga Province, where this study was conducted, is home to large coal reserves, namely the Springs-Witbank and Highveld coalfields (Vermeulen & Usher, 2006). The mining of these coalfields started in 1870 and are still continuing to this day. In the 1970s large opencast mines covered the Mpumalanga coalfields. These large scale mining activities have not only brought job opportunities and economic growth to the province, but also a string of water related challenges. Shallow mining operations enter the weathered zone, allowing water to enter the mining shafts. The influx of water leads to oxidation of minerals such as pyrite which can produce acid mine drainage (AMD) (Vermeulen & Usher, 2006). The Olifants River in Mpumalanga (Figure 1) is "one of the most threatened river systems in South Africa" (de Villiers & Mkwelo, 2009) due to the pollution from industry, mining and agriculture activities.
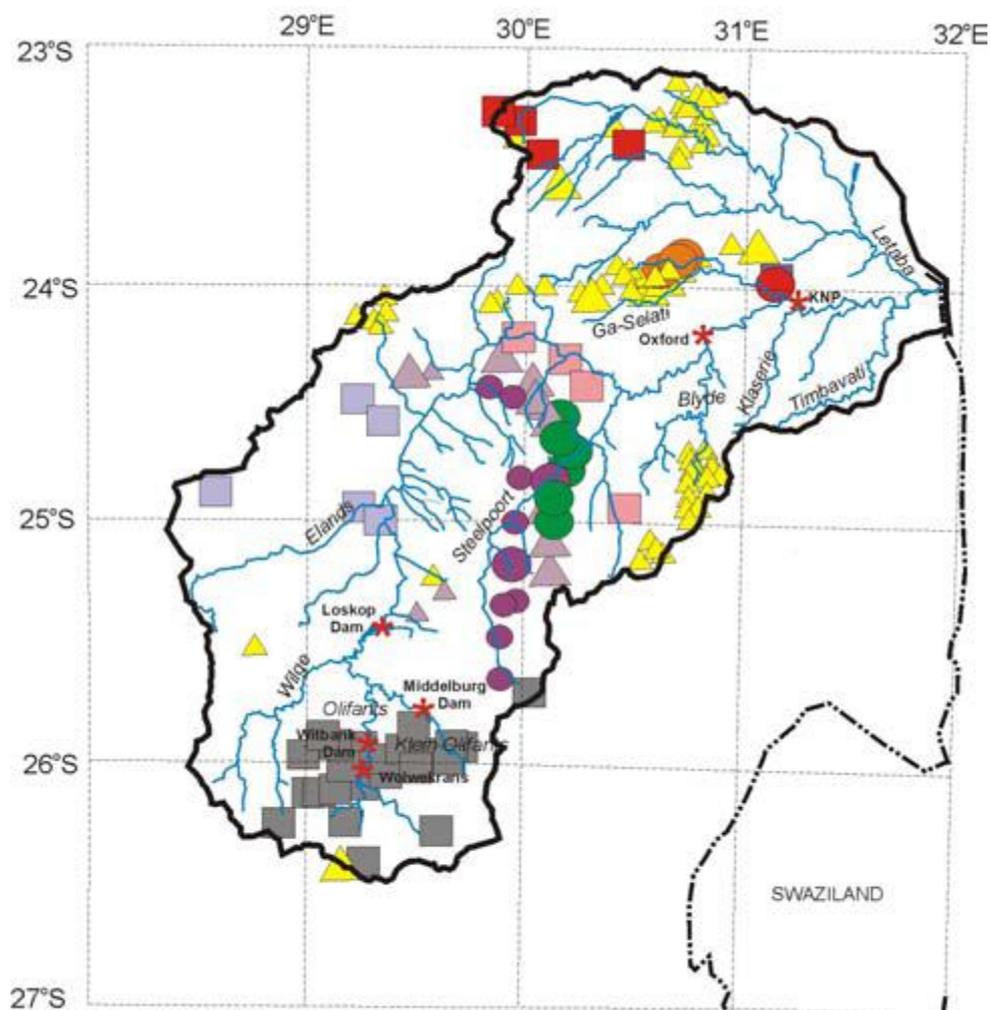
Figure 1. The Olifants River Catchment in the Mpumalanga province, South Africa. The red symbols indicate sites identified for long term monitoring by the Department of Water Affairs and Forestry (DWAF). The coal fields are indicated by grey blocks (de Villiers & Mkwelo, 2009).

Department of Water Affairs and Forestry (DWAF, 2004b) and Maree *et al.* (2004) estimated that water decant after closure from coal mines was around 62 Mℓ/d along with 50 Mℓ/d AMD discharged into the Upper Olifants River Catchment area from 150 closed mines.

In order to avoid the adverse effects of AMD a variety of treatment methods have been developed. Although chemical treatment is widely used for AMD remediation, it is a very costly process. In most cases chemical treatment is only used to neutralise the pH of mine effluent, while metals and sulphates may not be fully remediated (Fiset *et al*., 2003; Hong *et al*., 2014). Constructed wetlands, which are considered a passive treatment system is a more

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cost effective treatment process. In some cases natural wetlands, affected by AMD, may also be used to treat AMD water. However, natural wetlands are among the most endangered ecosystems in the world. It has been estimated that between 35 % and 50 % of South African wetlands and their services have been lost (Dini, 2004). South Africa's wetlands cover only about 7 % of the county's surface area of which 19 are listed as Ramsar sites (Strydom *et al*., 2010).

1.2 Wetlands

Wetlands can be described in many ways. A simplified definition for wetlands is that they are transitional water bodies, between aquatic and terrestrial habitats, with varying shape, size and hydrology. Section 404 of the Clean Water Act (1977) of the United States of America Environmental Protection Agency (EPA) defines a wetland as:

> "The term *"wetlands"* means those areas that are inundated or saturated by surface or ground water at a frequency and duration sufficient to support, and that under normal circumstances do support, a prevalence of vegetation typically adapted for life in saturated soil conditions. Wetlands generally include swamps, marshes, bogs, and similar areas".

South African Water Act defines a wetland as:

> "A wetland is defined as land which is transitional between terrestrial and aquatic systems where the water table is usually at or near the surface, or the land is periodically covered with shallow water and which under normal circumstances supports or would support vegetation typically adapted to life in saturated soil (Water Act 36 of 1998)".

According to the Convention on Wetlands (Ramsar, Iran, 1971):

> "Wetlands include a wide variety of habitats such as marshes, peatlands, floodplains, rivers and lakes, and coastal areas such as saltmarshes, mangroves, and seagrass beds, but also coral reefs and other marine areas no deeper than

six metres at low tide, as well as human-made wetlands such as waste-water treatment ponds and reservoirs."

Wetlands are diverse and differ from one another with respect to shape, size, hydrology and habitat. Apart from these distinguishing characteristics, wetlands function within the ecosystem as a kidney (Oberholster *et al*., 2014), filtering the passing water and increasing the quality. Wetlands also provide an aquatic and wildlife habitat; act as a system for the cycling of elements and flood attenuation, enabling recharge of groundwater, simultaneously stabilising soil and particle retention. Apart from the environmental role wetlands play, they are also beneficial to humans as they are often used for recreational purposes and agricultural needs (Kent, 2000). Human activities threaten the "health" of these water bodies due to the damage done through draining and the disturbance of the biota. Agriculture and mining activities are the main cause of wetland degradation due to increased salinity, increased acidity, increased heavy metals, increased suspended solids and potential eutrophication. Other sources of pollution are industry urban runoff, including sewage plant effluent (Coetzee, 1995).

In South Africa, the Departments of Environmental Affairs (DEA), Water Affairs (DWA) and Agriculture, Forestry and Fishery has been working together on all wetland-related issues by establishing the Working for Wetlands programme, which forms part of the South African National Biodiversity Institute (SANBI). Through this joint-initiative, they collaborate to rehabilitate, protect and ensure the sustainable use of wetlands. Their programme also focuses on job creation within communities through skills development (Working for Wetlands, n.d.). South Africa has more than 120 000 wetlands covering around 7 % of the country, approximately 544 000 hectares. Nineteen of these wetlands have been declared Ramsar sites (Strydom *et al*., 2010). Ramsar sites are defined as a wetland identified as being of international importance upon joining the Ramsar Convention by a contracting party (country) (Ramsar, 2009).

1.2.1 The economic value of wetland services

Previously wetlands were seen as a waste of potential agricultural soil and thus drained to make way for more agricultural land. The largest global example of a developed wetland is the Mississippi River Basin in the USA which had to make way for canals and levees. Fragmentation of this system has led to nutrient starvation of downstream wetland areas as

well as the increased risk of flooding in the downstream New Orleans (Keddy *et al.*, 2009). Presently their value as both water sources and service provider is much more recognised.

In order to emphasise the value of wetlands to society, the services provided by the wetland need to be expressed in terms of aquatic ecosystem services. Thus by allocating an economic value, it has necessitated legal protection of these fragile systems (Mitsch & Gosselink, 2000). Calculating wetland value can become difficult as most of the services provided by the wetland has no market-value, thus a non-market valuation has to be applied. This approach has been widely applied and the results were condensed to comparable measures by Woodward & Wui (2001) to determine what the value determinants are. Key determinants included water quality control, habitat provision and nutrient cycling (Figure 2).
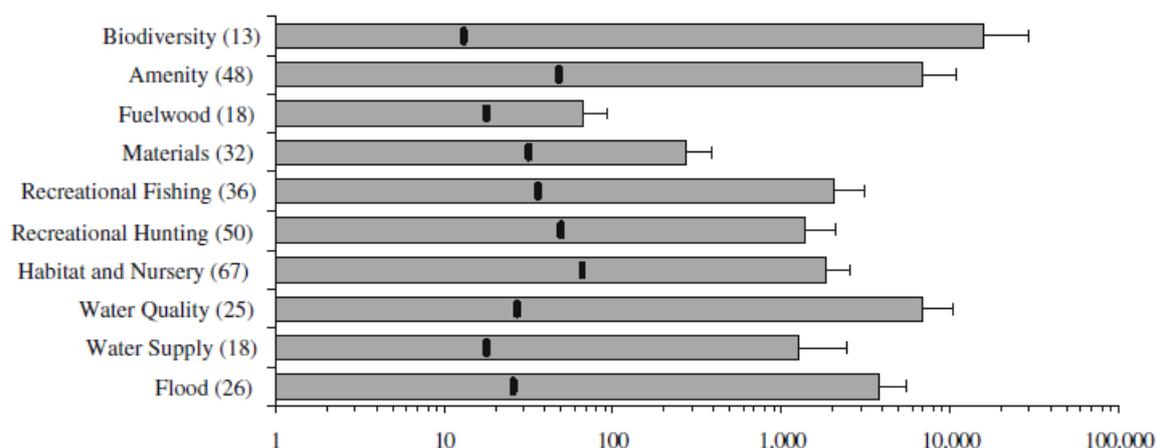
Figure 2. Wetland value (1995 US $ ha^{-1} yr^{-1}; log scale) according to service provided (adapted from Brander *et al.*, 2005).

A study by Carlsson *et al.* (2003) determined the willingness of a community to pay for a wetland and its services in Staffanstorp, southern Sweden. This was done by conducting choice experiments where hypothetical market scenarios were sketched and individuals had to choose between alternatives. Their results indicated that communities in densely populated areas would pay for the aesthetic properties accompanying wetlands such as walkways and biodiversity. The same might not be true for communities in less densely populated areas with more recreational spaces.

Using estimates of the perceived value of wetlands, priorities can be set in accordance to the maintenance, protection and rehabilitation (Woodward & Wui, 2001). Wetlands have an

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estimated overall value of between $ 100/ha/year to $ 10 000/ha/year for the services that it provides (Prime Africa Consultants, 2011) (Table 1). The most valued wetland service is the provision of habitat. Within Southern Africa, wetlands in the Zambezi basin provide between $ 6.57/ha/year to $ 81.70/ha/year. These values may seem small, but the contribution to the poorer communities is much greater in value. When estimating wetland services within South Africa the value to the community in 2011 was around R 1.2 billion for the Olifants water management area (Prime Africa Consultants, 2011). However, DWAF (2010) valued wetlands in the Upper Olifants River at R 382 million for a total wetland area of 72 182 ha.

Table 1. Wetland service values as proposed by Prime Africa (2011), provided by wetlands in the Olifants Water Management Area.

Ecosystem Service	Value (millions Rand)
Livestock watering	725,58
Harvested products	318,23
Flood attenuation	35,01
Groundwater recharge	29,28
Water purification	59,51
Carbon sequestering	10,82
Angling	12,09
Tourism	27,05
Total	**1217,57**

The increase in value can be attributed to a more thorough valuation done or the inclusion of services not valued by DWAF in 2010. Under-valuation can also be attributed to poor scientific evidence, scale issues, wetland delineation, vulnerable communities and lastly the incorrect valuation of regulatory systems (Prime Africa Consultants, 2011).

1.2.2 Rehabilitation of damaged or severely impacted wetlands

Wetland degradation and loss can occur through various anthropogenic activities within the catchment (Ehrenfeld, 2000; review: Zedler & Kercher, 2005). Thus, rehabilitation of wetlands has become increasingly important as we begin to understand the importance of these water bodies in South Africa. Rehabilitation is not only important for restoration and conservation of wetlands, but also to reverse effects on the biota within these ecosystems

(Zedler, 2000). Bacterial communities in tidal wetlands have been reported to be impacted by land-use changes (Bannert *et al*., 2011). These microbial population shifts may either be a result of stress or the adaptation to newly created niches. The latter seems more probable, as was shown by Bossio *et al*. (2006). The authors reported different microbial habitat niches within soil horizons due to aeration and carbon availability. Wetland phospholipid fatty acid (PFLA) profiles shown greater variety than that of agricultural soil samples (Bossio *et al*., 2006). The main goals of rehabilitation are to conserve the biodiversity, extend the water retention time and alleviate floods (Sieben *et al*., 2011), thus in essence the main purpose is to return the wetland to its conditions before it was damaged (Selala *et al*., 2013a).

Wetland rehabilitation strategies used in South Africa include: 1) elimination of ridge and furrow cultivation in the cases where agriculture is practiced in the wetland areas while vegetation is replanted in more suitable areas. 2) Removal of alien vegetation from the area. 3) Preventing or reducing channel formation that alters water flow by deflecting water flow over a larger surface area, weir and beam structures are built (Macfarlane, 2013). These interventions are done to redirecting water flow over areas which was once flooded (Lee *et al*., 2013). Another common practice to counter AMD impacts on wetlands, is liming, where the pH of the water is increased whilst small quantities of metals and sulphides are removed. However this practice was detrimental to microbial communities, decreasing diversity and increasing metabolic stress, negating restoration efforts (Hartman *et al*., 2008; Pound *et al*., 2013). Successful wetland restoration is often done by using biomonitoring methods. There are however some short comings to this approach i.e. 1) various monitoring programs are restricted by the indicator organisms used leading to methological bias, 2) erroneous data reporting, due to the biota recovery lagging behind improved water recovery and 3) full recovery of the wetland limited by broader watershed stressors such as agricultural activities and draining (Walter *et al*., 2012).

1.3 Coal Mining Impact on Wetlands

South Africa is one of the world's largest coal producers. It is also heavily dependent on coal for energy production, as coal provides more than 75 % of the country's energy. There are more than 50 billion tonnes in coal reserves in the Witbank-Middelburg, Ermelo and Standerton-Secunda areas of Mpumalanga (Prime Africa Consultants, 2011) (Figure 3).

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Mining in the Witbank coal fields started in 1895 and open cast mining is the predominant practice (Hobbs *et al*., 2008). The latter has led to enormous land disturbances in the area.
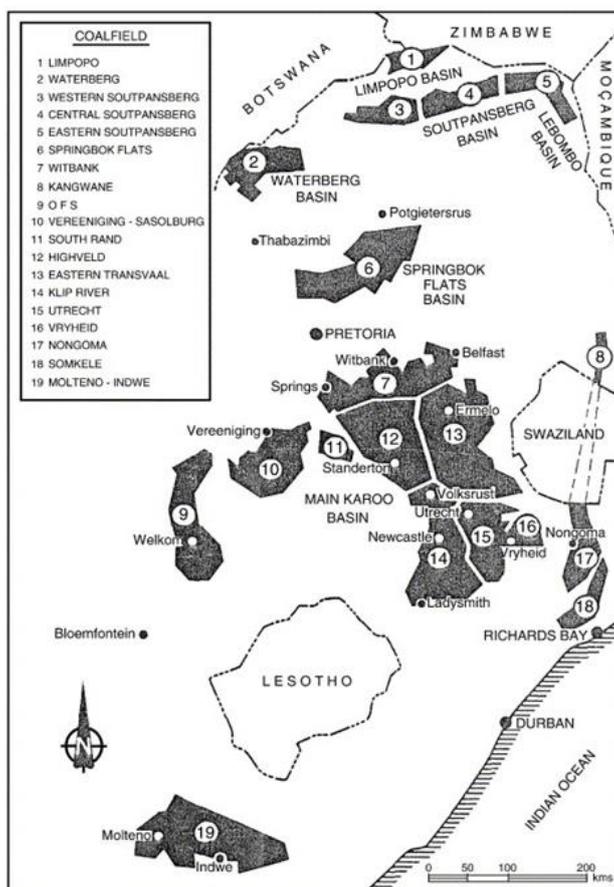
Figure 3. The coalfields found in South Africa (Pinheiro, 2000).

Large volumes of AMD discharge from abandoned, closed coal mines into the Olifants River catchment have reached critical levels. DWAF has spent over R 120 million over the past ten years on clean-up efforts, but much more will be needed. After 1994, the new Constitution made the Government the custodian of all of South Africa's natural resources. There in, the National Water Act (Act 36 of 1998), along with its regulations was instated to protect the country's water resources. This act instated the "Polluter Pays Principle" that required those who produced pollution to be held liable for the cost of the clean-up. Along with the National Environmental Management Act, which addressed AMD and other mining impacts, a strong enforcement was brought about. This was the first step in conserving aquatic resources after the years of lack in environmental governance (Hobbs *et al*., 2008). The impact of AMD in the Mpumalanga region is exacerbated by the combination of climate and geography found in the Witbank coal fields (McCarthy, 2011).

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1.3.1 Acid mine drainage

Acid mine drainage is a pollutant associated mainly with mining and industrial activity (Johnson & Hallberg, 2005). Acid mine drainage is characterised by a low pH, a high concentration of dissolved metals such as aluminium, iron, manganese and high concentrations of dissolved sulphates (García *et al*., 2001). Acid mine drainage is generated when sulphide containing ore (pyrite) is exposed to oxygen and water in an environment without any buffering capacity. When the pH value decreases to below 4, metals become solubilised (Tsukamoto *et al*., 2004). The summarized process of pyrite oxidation (Eq 1.) highlights the multistep reaction of ferric iron attack on minerals present after which ferrous iron is regenerated and reduced sulphur compounds to sulphate (Johnson & Hallberg, 2005).

$$4FeS_2 + 15O_2 + 14H_2O \rightarrow 4Fe(OH)_3 + 8SO_4^{2-} + 16H^+ \qquad (1)$$

The reaction above is microbial-mediated, especially in the case of iron sulphides. The sulphide mineral oxidation produces hydrogen ions responsible for acid production characteristic of AMD. From the equation it is evident that one mole pyrite produces 4 moles hydrogen ions, making this a robust passive acid producing reaction. On the other hand, the amount of acid produced depends on the amount of buffering minerals present such as carbonates. Mining heaps and tailings (Table 2) are primary locations for acid production as they are constantly exposed to the atmosphere through weathering (Johnson, 1995).

Table 2. Sources of acid mine drainage (Akcil & Koldas, 2006).

Primary Source	Secondary Source
Mine rock dumps	Treatment sludge ponds
Tailings impoundment	Rock cuts
Underground and open pit mine workings	Concentrated load-out
Pumped/nature discharged underground water	Stockpiles
Diffuse seeps from replaced overburden in rehabilitated areas	Concentrated spills along roads
Construction rock used in roads, dams, etc.	Emergency ponds

There are many factors that will determine the rate at which AMD is produced, namely: pH; temperature; oxygen content; oxygen concentration; degree of saturation with water; chemical activity of Fe^{3+}; exposed surface area of metal sulphide and chemical activation energy required for the initiation and bacterial activity (Akcil & Koldas, 2006).

One of the financial more attractive treatments of AMD is wetlands, both natural and artificial. Artificial wetlands are constructed mainly for the treatment of mine polluted water, or acid mine drainage. This is done as a cheaper alternative to chemical treatment of water. Wetlands are low maintenance continuous systems that also provide a habitat for introduced fish and birds (Fennessy & Mitsch, 1989). There are two main designs based on the characteristics of natural wetlands, namely surface flow and subsurface flow. Surface flow wetlands are aerobic systems. Oxidation and hydrolysis reactions favour precipitation of metals and do not raise the pH sufficiently. On the other hand, subsurface flow wetlands are anaerobic and also have oxidation and hydrolysis reactions at the surface where oxygen levels are higher and microbial reduction at the lower levels. The microbial sulphate reduction will raise the pH to adequate levels (Barton & Karathanasis, 1999). The level of flow or hydrological conditions will determine the physico-chemical component as well as the biotic component of a wetland. Thus both the water and sediment chemistry will be determined by the level and flow speed of the water but also the macrophytes and other organisms which the system will be able to support (Coetzee, 1995). The success of such a constructed wetland lies in its ability to enhance water quality from that of the effluent. This is monitored through chemical testing of both influent and effluent water as well as metal absorbing macrophytes. Although attractive, constructed wetlands may not be a long term solution as artificial ecosystems, thus natural wetlands should always be considered where appropriate.

1.3.2 Ecosystem health and indicator species

Previously water quality was assessed only by chemical analysis leaving a void in information on the effects of pollutants on the ecosystem within the water body being surveyed. Thus ecosystem health was not assessed. Healthy ecosystems are stable and sustainable, maintaining its organization and automoty over time and while remaining resilient to stress (Costanza, 1992; Rapport *et al*., 1998). In more recent years ecosystem health assessment has been incorporated in monitoring programmes along with physical and chemical analysis to provide more complete information on the state of aquatic ecosystems. Biomonitoring was implemented successfully in numerous studies and many different organisms were used,

including macroinvertebrates and fish (Oberholster *et al*., 2008), *Daphnia magna* and various freshwater algae, including diatoms (Oberholster *et al*., 2013a). It has also been proposed to incorporate microorganisms such as bacteria into biomonitoring studies as they are far more sensitive to abiotic factors such as shifts in nutrient levels (Sims *et al*., 2013) because they are the most abundant organisms in any ecosystem and play major role in the food chain (Atkinson *et al*., 2011). Different organisms can be monitored in parallel to develop a battery of bioassays of different sensitivity to improve biomonitoring results (Oberholster *et al*., 2008). The following factors affect the choice of the suite of organisms used in biomonitoring of ecosystems: ease of sampling, ease of identification, distribution and life cycle (Table 3). These characteristics need to be carefully considered during planning phases in order to select the correct group of organisms for the survey.

1.4 Microbiology in acid mine drainage affected environments

1.4.1 Bacterial assemblages

Wetlands create unique habitats for the organisms that inhabit them, as the changing hydrology of wetlands results in interplay in aerobic and anaerobic zones.

The microbial consortium within a wetland plays an important role in the nutrient cycling as well as catalysing chemical transformations under changing oxic and anoxic conditions within both the soil and water. They form the basis of any food web and are first to respond to changing conditions within a habitat. Thus changing chemical and physical parameters within a wetland will not only impact the microbial populations, but also other organisms which rely on their presence (Yergeau *et al*., 2012; Sims *et al*., 2013).

Wetlands may host both autochthonous and allochthonous microorganisms, the latter will most likely not survive nor function within the habitat (Truu *et al*., 2009). Thus these organisms will have little impact on the resident wetland population dynamics. The changing oxygen availability will in turn dictate the nutrient cycling processes (Gutknecht *et al*., 2006). Biogeochemical processes in wetlands include denitrification, nitrification, methanogenesis and methanotrophy and in wetlands contaminated by AMD, sulphate and iron cycling will also come into play.

Table 3. The advantages and limitations of different bioindicators used in the aquatic environment (Oberholster & de Klerk, 2014b).

Criteria	Benthic filamentous green algae	Diatoms	Macroinvertebrates	Bacteria
Cosmopolitan distribution	Seasonally and periodically available during autumn and spring. Their applicability is thus influenced by their availability.	Not seasonally bound and occur throughout the year.	Seasonally available. Their applicability is thus influenced by their availability.	Not seasonally bound and occur throughout the year.
Low mobility	Stationary and their community characteristics developed entirely around the environmental conditions of a specific site (e.g., nutrient enrichment).	Stationary and their community characteristics developed entirely around the environmental conditions of a specific site (e.g., nutrient enrichment).	Stationary, but can easily drift or move away from pollution impacted areas.	Stationary, but can easily drift or move away from pollution impacted areas.
Life cycle	Short lifecycle and therefore can be expected to reflect short-term impacts.	Short lifecycle and therefore can be expected to reflect short-term impacts.	Medium lifecycle and therefore can be expected to reflect medium-term impacts.	Short lifecycle and therefore can be expected to reflect short-term impacts.
Location and habitat requirements	Require specific habitat characteristics such as velocity, flow, turbulence, sunlight and	Require specific habitat characteristics.	Required specific habitat characteristics such as velocity, flow, turbulence and	Required specific habitat characteristics such as flow. Some species are

	substrate.		substrate.	adaptable.
Sampling procedure and taxonomic identification keys	Easy to sample and visible to the naked eye. Identification is time consuming and labour intensive.	Easy to sample, but not visible to the naked eye. Identification is time consuming and labour intensive.	Easy to sample and visible to the naked eye. Identification is quick.	Easy to sample, but not visible to the naked eye. Identification is time consuming and labour intensive.

Acid mine drainage will lead to an increase in heavy metal contamination of sediment and water leading to a decrease in diversity among microorganisms in the affected area, selecting for organisms that will thrive under these conditions. This was shown to be the case in the study by Guo *et al*. (2009), who evaluated the microbial diversity in the presence of copper pollutants. The authors found in their study that there was a decrease in diversity due to stress yet little variation in the dominant species present. Some degree of impact was noticed in the dominant populations, but was deleterious due to the numbers of dominant bacteria.

The bacteria responsible for the generation of AMD are acid and metal tolerant autotrophic bacteria for example *Sulfobacillus, Acidothiobacillus ferrooxidans* (previously: *Thiobacillus ferrooxidans*), *Leptospirillium ferrooxidans, Acidiphilum* spp. and the numerous yet to be identified uncultured acidophiles that reduce ferrous iron and reduced sulphur for energy production while fixing carbon dioxide for biomass production (Brofft *et al*., 2002; Tyson *et al*., 2004; Ñancucheo & Johnson, 2012). A relative newly identified species of archaeal iron-oxidizing extremophile found to be the dominating prokaryote in pyrite tailings at Iron Mountain, California and have been linked to the production of AMD. This organism, *Ferroplasma acidarmanus*, can grow in the lowest natural occurring pH (pH 0) in acid streamers (Edwards *et al*., 2000). Other unusual sulphur-oxidizing bacteria were identified to be part of the *Burkholdria* species. This group of *β-Proteobacteria* are known to be metabolically versatile (Bhowal & Chakraborty, 2011). Many environmental factors determine which acidophiles are present at a certain site. The main determinants were pH, temperature, nutrient concentration and oxygen concentration. Edwards *et al*. (1999) found that *T. ferrooxidans* occurred at a pH value below 1 and that seasonal changes in pH, temperature and conductivity had an impact on the overall microbial community. Acid mine drainage affected environments host a variety of life forms and are much more complex than what was expected and not as was once thought to be a sterile environment (Xie *et al*., 2011).

Macrophytes growing within the wetland may also affect the rhizosphere bacterial population (Ravit *et al*., 2003). Drying-rewetting frequencies influenced sediment microbial communities, selecting for species that readily adapt, such as Gram-positive bacteria and fungi (Fierer *et al*., 2003). Some studies have also found no or little response to rewetting (Steenwerth *et al*., 2005). Sediment properties such as saturation will also determine the microbial community composition (Adrados *et al*., 2014).

1.4.1.1 Diversity of microbial metabolism

Any environmental system hosts various microbial niches. The diversity in bacteria in an ecosystem is determined by factors such as: temperature (Volant *et al*., 2014), conductivity (Edwards *et al*., 1999), pH (Lear *et al*., 2009; Kuang *et al*., 2012; Chen *et al*., 2013), oxygen gradient (González-Toril *et al*., 2011), metals (Zaidi *et al*., 2012; Volant *et al*., 2014) and nutrients (Faulwetter *et al*., 2009) available to the organisms. Thus the two most important drivers to consider when investigating microbial assemblages are the biotic and abiotic factors. The latter influences microbial community structure the most.

Nutrient availability is one of the key determinants in the microbial community structure in ecosystems (Benlloch *et al*., 1995; Broughton & Gross, 2000; Fisher *et al*., 2000; Lindstrom *et al*., 2000; Øvreas *et al*., 2003). Bacteria are able to utilize a vast amount of electron donors and acceptors due to their respiratory diversity. The respiratory diversity can be found at any temperature, enabling bacteria to colonize many of the most hostile environments. Even oxygen availability can be overcome; *Paracoccus denitrificans* adapt to survive at different concentrations by utilizing different oxido-reductases (Richardson, 2000). Nutrients readily available for bacterial respiration will determine which group is dominant.

Salination, due to land-use changes of a wetland have adverse effects on the microbial communities. Changes in the ionic composition will cause a shift in nutrient availability (i.e. increased iron leading to a decrease in phosphates) and impact microbial respiration. Thus it can be hypothesised that changes in an ecosystem will lead to changes in microbial communities (Baldwin *et al*., 2006). Sediment in wetlands is known to be organic-rich sinks, almost completely anoxic below the first few millimetres, where anaerobic organisms will dominate (Baldwin *et al*., 2006). Another influence on the microbial community composition is the availability of dissolved organic carbon (DOC). In AMD affected systems metal oxides will absorb DOC, making it unavailable for bacterial growth. The latter is due to the sorption of dissolved organic matter by hydrous metal oxides, influencing transport throughout the system and chemical characteristics (McKnight *et al*., 1992).
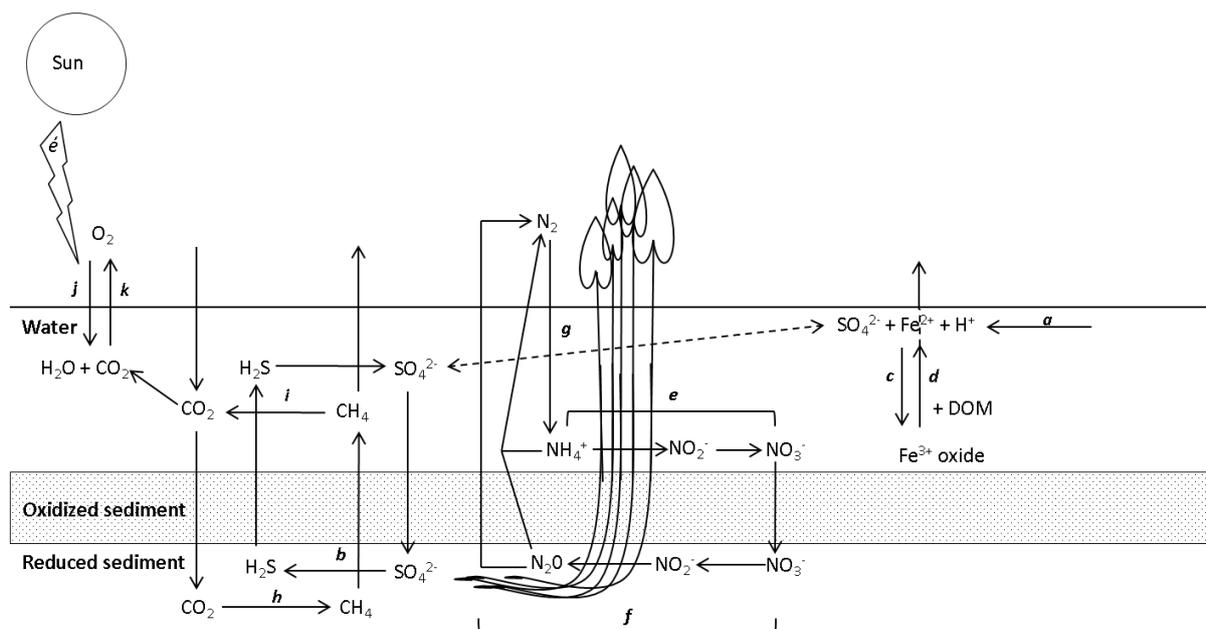
Figure 4. Conceptual bacterial respiration model for an AMD impacted natural wetland. The aquatic environment can be divided into oxic water phase, oxidised sediment transition phase and the reduced (anaerobic) sediment phase. Simplified microbial biogeochemical cycles are indicated as follow: *a* – sulphur oxidation, *b* – sulphate reduction, *c* – iron-oxidation, *d* – iron-reduction, *e* – ammonia-oxidation (nitrification), *f* – ammonia-reduction (denitrification), *g* – nitrogen-fixation, *h* – methanogenesis, *i* – methane-oxidation, *j* – photosynthesis, *k* – aerobic respiration. DOM – dissolved organic matter. Adapted from: Nichols, 1983; Prescott *et al*., 2002; Baker & Banfield, 2003; Krauter *et al*., 2005; Yin *et al*., 2009.

Sulphur

The oxidation of sulphide minerals such a pyrite by bacteria is one of the largest causes of water pollution. Bacteria like *Acidithiobacillus* spp. and *Leptospirillium* spp. have been identified to be the forerunners in utilizing reduced chemicals as energy sources (Ñancucheo & Johnson, 2012). These chemoautotrophic organisms oxidize sulphur minerals like pyrite, in the presence of oxygen or oxygen coupled to nitrate reduction such as the case with *Thiobacillus denitrificans* (Haaijer *et al*., 2012). The oxidation of sulphur minerals also produce hydrogen ions, which contributes to the acid generation (Figure 4 *a*). This type of oxidation is known as direct enzymatic oxidation where cells in close proximity or attached to the pyrite (Baker & Banfield, 2003). However, the amount of acid discharged depends on the amount of carbonates and other neutralizing compounds present. These reactions predominantly occur in abandoned mines and on mine heaps (Johnson, 1995).

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The typical oxidants in the reaction are oxygen and ferric iron, where the large requirement for oxygen is provided by atmospheric air that reaches pyrite surfaces (Baker & Banfield, 2003):

$$FeS_2 + 3.5O_2 + H_2O \rightarrow Fe^{2+} + 2SO_4^{2-} + 2H^+ \qquad (2)$$

Oxygen is a weaker oxidant than that of ferric iron, thus the dominant pathway is through the oxidation of ferrous iron by oxygen, as follow (Baker & Banfield, 2003):

$$14Fe^{2+} + 3.5O_2 + 14H^+ \rightarrow 14Fe^{3+} + 7H_2O \qquad (3)$$
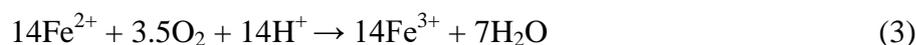
The rate of the reaction above is limiting to the rate of AMD generation due to the slow oxidation by oxygen at low pH. Microbes can catalyse this reaction, thus increasing the rate. The latter is followed by ferric iron regeneration (Baker & Banfield, 2003):

$$14Fe^{3+} + FeS_2 + 8H_2O \rightarrow 15Fe^{2+} + 2SO_4^{2-} + 16H^+ \qquad (4)$$
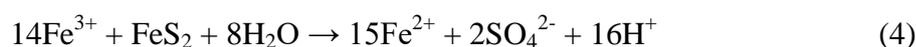
An interesting group of bacteria, closely related to *Burkholderia* sp., were recently identified from an AMD environment that displayed oligotrophic growth alongside with *Acidithiobacillus ferrooxidans.* The five strains identified were shown to be heterotrophic acidophiles that lived off the organic materials produced by autotrophs in their environment (Bhowal & Charkraborty, 2011). Chemolithotrophic bacteria and archaea are less sensitive to increased metal concentrations than other primary producers such as algae, cyanobacteria and higher plant species (Ñancucheo & Johnson, 2010).

Mining ores are rich in sulphate and it is widely known to easily enter aquatic systems such as ground and surface water. Water bodies create an anoxic zone in which sulphate reduction readily takes place through bacterial metabolism. There are a number of sulphur reduction reactions carried out by microorganisms namely reduction of sulphates, the desulphurylation of sulfhydryl groups of proteins as well as disproportionation and reduction elemental sulphur will produce hydrogen sulphide (Prescott *et al*., 2002). The sulphide reduction takes place in the presence of a carbon source and anaerobic conditions (Figure 4 *b*). The precipitation of metals as sulphides on the bacteria's cellular surface can also shield the organisms from the toxic effects of heavy metals at a cost of inhibited metabolism (Castillo *et al*., 2012). The

following two reactions have been proposed by Castillo *et al*. (2012) to be of main importance during sulphate reduction:

$$SO_4^{2-} + 2CH_2O \text{ (simple organic compounds)} \rightarrow H_2S + 2HCO_3^- \hspace{2cm} (5)$$
$$H_2S + Me^{2+} \leftrightarrow MeS_{(S)} + 2H^+ \hspace{3cm} (6)$$

Not only does this remove sulphates from the system, but the pH is also neutralized along with the precipitation of metal sulphides (Boshoff *et al*., 2004). One of the major limiting factors to utilizing this biological reaction for the remediation of AMD is the requirement of a cost efficient carbon source. Sulphate reducing bacteria cannot utilize carbon sources such as polysaccharides, proteins, nor lipids and they depend heavily on the fermentation products of other organisms. Some of the cheaper carbon sources that have been considered include mushroom compost, straw, hay and sewage effluent (Rose *et al*., 1998; Boshoff *et al*., 2004; Sheoran & Bhandari, 2005).

However, the speciation and concentration of sulphide may inhibit sulphate reduction as demonstrated by Moosa & Harrison (2006). This inhibitory activity was observed even though sulphate reducing bacteria (SRB) are more tolerant to sulphide than other anaerobic bacteria. The inhibition could be due to depravation of the cell from vital trace elements required for enzymatic reactions. On the other hand sulphide could be absorbed and denature intercellular proteins. This inhibition is however reversible. Their finding was that as the pH of the environment increased, an increase in growth rate was noted. This phenomenon can be due to a lower concentration of undissociated H_2S present. Sulphate reducing bacteria are also extremely sensitive to low pH, thus when they are to be used for remediation, pre-treatment to increase the pH of the affected water is necessary (Ñancucheo & Johnson, 2012). These organisms are also able to reduce the acidity to an extent through their carbon metabolism. Thus their ideal environment is one where the pH is above 5.5, redox potential below -100 mV and anaerobic (García *et al*., 2001).

The sulphides produced by these organisms are also toxic to other species within the bacterial population. It was found that elevated sulphide concentrations will inhibit aerobic bacteria, especially nitrifiers (Sears *et al*., 2004).

The ability of this bacterial group, the SRB, to remove polluting sulphates from the environment is utilized in constructed wetlands and other bioreactors. The latter is built to provide a more cost effective and less labour intensive alternative bioremediation option instead of chemical treatments currently used (Rose *et al*., 1998). Another approach to bioremediation is the use of microbial mats consisting of predominantly cyanobacteria. This was done on bench-scale by Sheoran & Bhandari (2005). The authors showed that blue-green algal/bacteria mats not only removed AMD pollutants effectively (i.e. sulphates, iron etc.), they also raised the pH of the system within 24 h.

Iron

The oxidation of iron is a major contributor to AMD and microorganisms who aid in this reaction is seen as problem organisms. These organisms' metabolism is also important in the cycling of sulphur in the environment. These aerobic organisms oxidize ferric iron for energy production (Figure 4 *c*). In the rhizosphere of wetland plants this oxidation has been shown to be at a near neutral pH (Weiss *et al*., 2003). This low energy yielding process occurs in the oxic/anoxic interface in aquatic environments, is microbially accelerated and is proposed to be one of the most environmentally important processes (Bacelar-Nicolau & Johnson, 1999; Hauck *et al*., 2001). Thus the roots of wetland macrophytes are the ideal environment for this process as oxygen is slowly released by the roots, creating a microaerophillic zone (Neubauer *et al*., 2002). Previously *Thiobacillus ferrooxidans* were thought to be the most dominant organisms in this reaction, but a study by Edwards *et al.* (2000) found that Archaea are the more dominant prokaryotes found in acidic environments with *Ferroplasma* as the predominant species in their study of the Iron Mountain in California. These organisms, even though they lack cell walls, seem to thrive in the most acidic natural environment. Ferrous iron can also be oxidized anaerobically by either phototrophic bacteria or denitrifiers (Hauck *et al*., 2001). This ability seems not be unique and can occur in various sediment environments (Figure 5). Through direct coupling of reduction of organic carbon and H_2 has been identified to be key factors in enabling the anaerobic oxidation by these organisms (Weber *et al*., 2006).
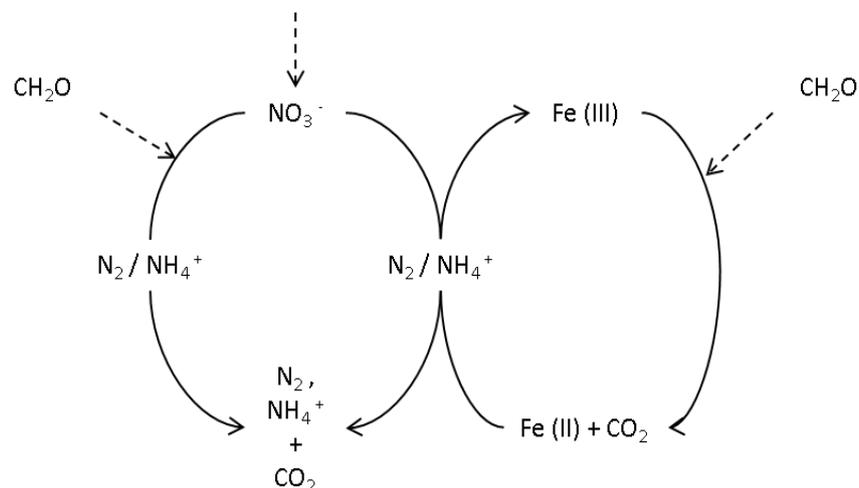
Figure 5. The proposed Fe-N redox pathways in anaerobic sediment environments (Weber *et al*., 2006). Dashed lines depict external loading. Deviations in external load will result in temporal and spatial variation.

Ferric iron respiration has also been reported in mesophillic Gram-negative bacteria, generally in hydrothermal environments. These species have been reported to impact nitrogen and sulphur cycling in environments where they grow. They have overcome the problem of utilizing insoluble ferric iron at circumneutral pH by using tetra-heam and deca-heam *c*-type cytochromes, as reported for the *Shewanella* genus (Richardson, 2000). *Shewanella putrifaciens* can not only utilize iron oxides, but also fumarate, nitrate, tetrathylamine-*N*-oxide (TMAO) and manganese oxides. *S. putrifaciens* does so by coupling anaerobic respiration with these compounds (Myers & Myers, 1997). This respiratory flexibility hints at an intricate electron transport chain in this specie. Species capable of both iron-reduction and nitrate-dependent iron-oxidation have also been reported by Weber *et al.* (2006). Iron reduction by bacteria can be separated into two groups: 1) complete oxidation of multi-carbon compounds to CO_2 (*Geobacter*, *Desulphuromonas*, *Desulphuromusa* & *Geovibrio*) and 2) incomplete oxidation of these carbon compounds (*Shewanella* & *Pelobacter*) (Coates *et al*., 1996). Some species will substitute humic substances for iron as terminal electron acceptor, such as *Geobacter metallireducens* and *Shewanella alga*. This ability enables these microorganisms to thrive in a range of sediment environments, *G. metallireducens* mainly in freshwater habitats and *Desulphuromonas* in marine (Coates *et al*., 1998).

Nitrogen

Almost all of the total nitrogen on earth is stored within soil as organic nitrogen. Nitrogen fertilizers used for agricultural application pose as one of the largest sources of non-point surface water pollutants (Carpenter *et al*., 1998).

Chemolithotrophic ammonia-oxidizing bacteria and archaea (AOB & AOA) play a significant role in the cycling of this nitrogen, where they slowly convert nitrite to nitrate (Figure 4 *e*) (Yin *et al*., 2009; Enowashu *et al*., 2012). Ammonia-oxidizing bacteria showed to be more abundant in some soils at low pH. An investigation into the transcriptional responses of nitrifying organisms showed that their activity increases within one hour of stimulation through wetting of soil. This can lead to the conclusion that AOA and AOB will be more active in permanently waterlogged wetland soil, making more nitrogen available to the root-systems of plants (Placella & Firestone, 2013). These organisms have been used as bioindicators to certain environmental conditions, as they are extremely sensitive to water availability (Bustamante *et al*., 2012). Ammonia-oxidizing bacteria are also extremely sensitive to soluble sulphides in aerobic environments (Sears *et al*., 2004).

Anaerobic microbial respiration along with oxidation of organic material aid in denitrification (ammonia reduction), where by nitrates are removed from a system by the conversion to nitrogen, nitrous oxide or ammonia (Figure 4 *f*). The latter has implications for eutrophication (Morrissey *et al*., 2013). Denitrification along with the remediation of perchlorite was effective in constructed wetlands. Wetland vegetation supported the growth of the microorganisms as well as providing attachment sites, oxygen and carbon. However, this process was dependent on the carbon source (Krauter *et al*., 2005). It has been observed that nitrification decreases in environments with lowered pH, which in turn leads to increased amounts of NH_4^+. The latter is taken up more readily than NO_3^-, which can serve as a terminal electron acceptor during denitrification and thus can decrease denitrification rates and limit the amount of nitrogen transported downstream (Niyogi *et al*., 2003). Denitrification also contributes to the release of N_2O, especially from soils, leading to ozone degradation and global warming (Dandie *et al*., 2007). Denitrification is not isolated from rate influences by other metabolic cycles. Whitmire & Hamilton (2005) reported that denitrification is somewhat linked to the production of sulphates. The authors proposed two explanations for their observation: 1) denitrifiers inhibit SRB by competition for H_2 or 2)

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sulphur-oxidizing bacteria utilize NO_3^- in the place of O_2 for oxidation, the latter was reported to be more likely.

The effect of nitrogen fixation (Figure 4 *g*) counter acts that of denitrification and can reduce a wetland's nitrogen removal capabilities (Nichols, 1983).   During nitrogen-fixation, atmospheric gaseous nitrogen is reduced to ammonia and nitrate to alleviate low levels in the environment that may be limiting to plant growth. The bacteria involved are both free-living species and rhizobium species. It has been reported that nitrogen-fixation directly impacts ecosystem productivity (Karl *et al*., 1997). The balance between nitrogen retention and removal by microorganisms play an important role in the persistence of other organisms such as macrophytes (Findlay *et al*., 2002)

Methane

Wetlands are one of the four largest methane sources, which contribute to about 70 % of total methane production. Bacteria are viewed as one of the major contributors to methane production (Richie *et al*., 1997). Bacteria produce methane through anaerobic metabolism of organic material such as formate, acetate, carbon dioxide and molecular hydrogen (Figure 4 *h*). Bacteria capable of methane oxidation, methanotrophs, are unique in their ability to use it as their main carbon source and for energy production during fermentation (Dedysh *et al*., 1998). All known methanogens are *Archaeae* and most are mesophiles. They cannot utilize complex organic compounds, thus rely symbiotically on other anaerobes to degrade these compounds to products accessible to methanogens (Nazaries *et al*., 2013). The hydrogen consuming species will often occur alongside proton producers such as SRB. There are also a group of bacteria that can oxidize methane, known as methanotrophs that use methane as their sole carbon and energy source. These organisms may also be present in soil and freshwater environments as obligate aerobic bacteria. Methanogens and methanotrophs thus can co-inhabit the same environments (Ritchie *et al*., 1997).

1.4.1.2 Bacteria as bioindicators

Bacteria are promising bioindicators due to their size, versatility and responsiveness to anthropogenic stressors. Of all living organisms, bacteria have the largest surface area to volume ratio. Their thin cell membranes also mean that there is only a small barrier between them and their environment and stressors. These characteristics makes them highly sensitive

to any environmental changes such as pH, nutrients, salts and even water availability (Merkley *et al*., 2004).

When considering microbial bioindicators for AMD, the environment in question and the nutrients available should promote the growth of species that would dominate such a niche. Changes in nutrient availability will shift the bacterial population in order to most effectively utilize available electrons in their environment, thus for stoichiometric reasons. The latter will have an ecosystem effect which will not be limited to the primary producers (Barlett & Leff, 2010). Acid mine drainage environments are extreme and do not support great diversity in microbial species (Edwards *et al*., 1999). The lower diversity reveals the limited amount of electrons available in the environment and also possibly the presence of toxic metals and low pH. The latter environments will hamper the growth of non-extremophiles (Bruneel *et al*., 2006).

Molecular identification techniques have always been more reliable in surveying the community composition of microbes in the environment, as only a hand full of these organisms can be cultured in the lab using current techniques. By combining 16S rRNA techniques with comprehensive genomic sequencing, a snapshot of the population can be obtained. However, investigation into the stability of this community structure is needed to determine whether the population is adapting to changes in the environment or have stabilized at more favourable conditions (Tyson *et al*., 2004).

1.4.2 Phytoplankton assemblages in AMD environments

Algae are abundant in freshwater environments, mostly present as microorganisms. Thus anywhere where sufficient light penetrates a water body, algae will grow. Algae are part the primary producers in most ecosystems and form the basis to any food web in aquatic environments. This is due to them being primary carbon fixers, producing biomass for larger organisms to graze on (Bellinger & Sigee, 2010). These organisms are extremely important in freshwater ecosystems in their role as primary producer. They will occur as either free-floating as phytoplankton or attached to surfaces as benthic communities. Not only does algal biomass serve as food source to invertebrates and small fish, they also form part of wetland metabolism through energy and nutrient cycling. Algal mats can also serve as habitat to smaller organisms and provide refuge against predators. The metabolic activities of these organisms are of great interest in assessment of ecosystem health as they serve as

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bioindicators for various conditions (U.S. EPA, 2002). This is not their only beneficial roles, as summarized in a review by Das *et al*. (2009), they can absorb metals into their cell walls, form complexes with metals within their extracellular polysaccharides (EPS), produce alkalinity and precipitate metal hydroxides. Their most significant role in streams impacted by AMD is that their biomass and EPS serves as nutrient source for SRB.

Algae have remarkable biosorption capabilities, which also can be used as cost effective and safe alternatives for removal of heavy metals. A study by Sheng *et al*. (2004) illustrated the biosorption capabilities of brown marine algae, *Sargassum* sp. and *Padina* sp., where they found that the removal of cations chromium and cadmium was favoured as pH decreased in aqueous solutions. On the other hand, they showed that freshwater green algae exhibits potential in remediation processes, however optimization was needed along with pre-treatment of the water to be treated as some pollutants was inhibitory (Saunders *et al*., 2012)

1.4.2.1 Algal classification

Algae can be defined as the simplest photosynthesizing organisms (both prokaryotes and eukaryotes), that do not have multicellular sexual organs (except for charophytes). Freshwater algae can be divided into the following phyla: *Cyanophyta*, *Chrysophyta*, *Bacillariophyta*, *Cryptophyta*, *Dinophyta*, *Euglenophyta* and *Chlorophyta* (Jansen van Vuuren *et al*., 2006).

The first phylum, the *Cyanophyta* or cyanobacteria, occur mainly in freshwater habitats. These organisms' main photosynthetic pigment is chlorophyll-*a*, they carry out oxygenic photosynthesis and is considered to be the simplest phototrophs. They can occur as either single cells, in colonies or in filaments. Under anaerobic conditions in the presence of high concentrations of sulphide, these organisms can use hydrogen sulphide as electron donor, producing sulphur or thiosulphate. The cells of cyanobacteria contain gas vacuoles which controls buoyancy. The buoyancy mechanism is prevalent when these organisms from blooms on the surface of a water body. These blooms may be toxic due to the production of neuro- and hepato-toxins. Microcystins are part of the latter and are more readily observed in natural environments (Zurawell *et al*., 2005) and are produced by *Anabaena*, *Aphanocapsa*, *Hapalosiphon*, *Microcystis*, *Nostoc* and *Oscillatoria* (Paerl *et al*., 2001). The filamentous species grow their cells in chains that are occasionally interrupted by heterocysts. These heterocysts are responsible for atmospheric nitrogen fixation. They occur mainly in both soil

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and water, but rarely at a pH below 4.0 (Bell & Hemsley, 2000). Cyanobacteria phytoplankton is unique in their ability to utilize atmospheric N_2 gas through biological fixation and can also take up phosphorous in excess of cellular requirements, storing it for periods of deficiency (Paerl *et al*., 2001).

Chrysophyta are more commonly known as golden-brown algae. These organisms are both photosynthetic and heterotrophic. These algae contain chlorophyll *a* or *c*, fucoxanthin, neoxanthin and echinenone (Dawsin, 2007). Their photosynthetic pigment fucoxanthin will mask any green colour of chlorophyll-*a* and *c*. These organisms store their food as oils or leucosin in a large vesicle. The cells are mainly motile with flagella. The cell surface of some species can be covered in scales, which will persist, much like the siliceous remains of diatoms, long after cell death. This group is also known to be good bio-indicators (Prescott, 1984; Jansen van Vuuren *et al*., 2006) due to their wide ecological tolerance (Nixdorf *et al*., 2001). Chrysophytes can also form blooms, most of which are not considered toxic, though they can cause water quality to decline. The genus *Prymnesium* has been reported to produce a toxin that affects gill-bearing organisms (Paerl *et al*., 2001). Algins derived from *Ascophyllum nodosum* and *Laminaria hyperborean* are used in the U.K. as stabilisers and binding agents in food and medicine industries (Dawsin, 2007).

Bacillariophyta is the phylum under which diatoms falls. This group along with green algae are the most abundant algae and are able to thrive in diverse habitats. There are over 1600 diatom species and they occur both as planktonic or benthic organisms. The diatoms are often categorized along with the golden brown algae (*Chrysophyta*). These organisms can occur both unicellular or in filamentous colonies. They possess some motile capabilities through gliding on substratum and only their reproductive cells have flagella. The unique feature of these organisms is that their cells are covered by an opaline silica frustule. The silica frustule makes cells highly refractive under a light microscope, easing the study of morphology. Surface markings can also be seen on the frustule, which has become the basis of classification and identification of diatoms. Morphologically diatoms are divided into two groups, centric and pennate, according to their shape. The silica frustule also aids in the preservation of these organisms, evident from their presence in fossil records. Diatoms have also been reported to form blooms. The key attraction to study these organisms is the fact that they are good bioindicators (Bellinger & Sigee, 2010) due to their diverse physical characteristics, durability and ubiquity. Dokulil (2003) highlighted the different diatom

biocoenosis indices currently in use, including saprobic, biotic, specific diversity and comparative indices.

Cryptophyta is a phylum of Protoctista, under which algae with nanoplanktonic flagellates cryptomonads fall. These organisms are eukaryotic, unicellular and can be asymmetrical, flattened dorsventrally and leaf-shaped. *Cryptophyta* contain genera that are photoautotrophic and these algae are ubiquitous in both marine and freshwater (Marin *et al*., 1998; Baretta-Bekker *et al*., 1999; Jansen van Vuuren *et al*., 2006). All cells are mobile with flagella (Butcher, 1967) and complex plastids which harbour a remnant eukaryotic nucleus (nucleomorph) (Marin *et al*., 1998). They possess chlorophyll *c*, phycocyanin, alloxanthin and α-carotene. Storage of carbohydrates is outside the plastid, which can range in colour from blue to red. They also have a gullet at the flagella end of the cell, which produces ejectisomes (trichocysts) which are explosive. This phylum also contains heterotrophic taxa that either lack the plasmid compartment, such as *Goniomonas*, or contain a non-pigmented plastid (leucoplast), such as in the case of *Chilomonas* (Mignot, 1965; Sepzenwol, 1973; Roberts, 1981; McFadden, 1994a).

Dinophyta is a phylum with a few interesting exceptions. They are mostly unicellular flagellates and only a few are coccoid or filamentous in shape. This phylum also possesses highly specialized heterotrophs. They have two flagella and their chloroplasts are enveloped in three membranes. A few have abnormal chloroplasts which indicate a eukaryotic endosymbiont (Gould *et al*., 2008). Many others are heterotrophic without chloroplasts. They mainly have chlorophyll-*a* and *c*, but in the case where they have endosymbionts, there is no chlorophyll. *Dinophytes* can also have one of many types of eye spots, ranging from the small spherical globules, rows of small globules, layers of globules and a complex 'eye'. There are among 2000 living species and as many fossil species within this phylum (van den Hoek *et al*., 1995).

Euglenoids, *Chlorophyta* and *Charophyta*, contain chlorophylls-*a* and *b*. These organisms store their food as paramylon, a polysaccharide. They occur in both soil and water and have been reported to form blooms. Their cells have contractile vacuoles which are used to control osmotic pressure within the organism. They are also motile organisms with flagella (Prescott, *et al*., 2002). The phylum *Chlorophyta* have both unicellular and multicellular algae. These algae are unique as they have carotenoid pigments as well as chlorophyll-*a* and *b*. This has

led to the belief that plants could have evolved from these algae. They synthesise starch and store it in their chloroplasts like plants as well. There are over 350 species of *Chlorophyta* that are known as snow algae, which thrive in near-freezing temperatures of melting snowfields. Another member of these algae grows in colonies and is known as *Volvox*. The colonies comprise of independent cells but there seems to be some kind of benefit from this adaptation. The latter is said to be how multicellularity could have evolved (Weeks & Alcamo, 2008). *Chlorophyta* species are often associated with low pH waters (Bray *et al*., 2008). Filamentous green algae *Microspira tumidula* (Verb & Vis, 2001) and *M. quadrata* (Das & Ramanujam, 2011) have been reported to remove heavy metals from AMD impacted waters. *Klebsormidium* sp. can be used as iron indicator species (Stevens *et al*., 2001) and along with *Euglena mutabilis* have also been reported to be useful bioindicators of AMD (Valente & Gomes, 2007).

1.4.2.2 Algae as bioindicators

Algae also play an important role in the nutrient cycling within wetlands, as their metabolic contributions are as important as that of microorganisms. Not only do algae photosynthesise, respire and act as primary producers, these organisms can also take nutrients up both actively and passively. As in the case of bacteria, these organisms are also highly impacted by fluctuating phosphorous and nitrogen levels in their environment (U.S. EPA, 2002).
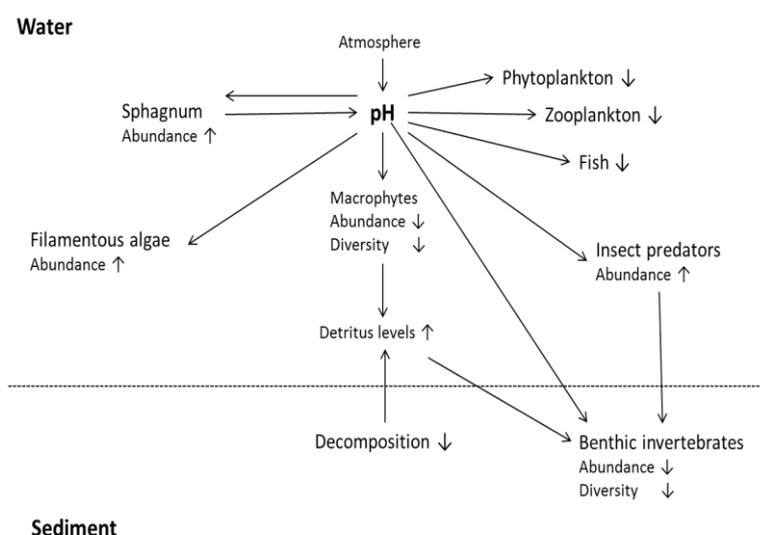
Figure 6. The relationships between organisms at different trophic levels in a lake affected by AMD. The arrows indicate either the increase or decrease of certain components or processed in this particular ecosystem (Lake *et al*., 2000).

Algae are not entirely immune to the devastating effects of AMD. Acid mine drainage has a significant impact on especially benthic algal assemblages, decreasing both their abundance, diversity and the communities as a whole (Figure 6). Other effects on benthic algae are geology, physiographic history and land use. The decrease in species is a result of lethal levels of metallic salts and pH (Verb & Vis, 2005). The biggest effects noted for these assemblages are linked to burial by the iron precipitate, also known as "yellow boy". This could be explained by the hypothesis that "an adhesive metal precipitate coat has less of an effect on periphyton than burial by the substrata in iron hydroxide floc" (DeNicola & Stapleton, 2002). Metal toxicity only occurs where metals are present in free ion form, where uptake exceeds regulatory mechanisms (DeNicola & Stapleton, 2002). In contrast to the decrease in species richness, an increase in biomass will occur. This has been said to be due to a lowered grazing by predatory organisms, decrease in algal competition and shifts in nutrient cycles. In contrast to these notions, alkaline mine drainage has little effect on biomass production and algal diversity (Verb & Vis, 2005).

1.5 Rapid Bioassessment Tools

Rapid assessment methods are needed to establish the condition of a wetland and its ecosystem. The above mentioned include the evaluation of chemical pollutants, physical disturbances as well as habitat changes within a system and summarize the wetlands score in reference to a developed tool or index (Stapanian *et al*., 2013). These indexes are developed to evaluate a specific biotic condition, such as vegetation. The development of such a tool is complicated and in most cases specific to the type of water body and area. By doing this, the assessment is put into ecosystem context which will identify the stressors and the responses (Lemly, 1997). The use of such tools elucidates the direction in which an environmental change can go and can also lead the strategy taken to restore to pre-change conditions (Lee *et al*., 2013). Currently there are a wide variety of conditions used in wetland tools which describe the overall hydrology, soil or substrate condition, vegetation and land use (Department of Sustainability and Environment, 2007). Ecosystem tools use bioindicators, which can be described as particular species which by being present in a particular environment provides information on the surrounding physical and chemical conditions, to assess ecosystem damage. Some organisms are only indicative of one pollutant or change where others are multifunctional.

These tools or indexes form part of environmental risk assessments carried out by government bodies and can become part of legislation if proven effective as an early indicator. Research and scientific monitoring of a wetland does unfortunately not ensure conservation. This is due to the fact that natural resource managers often do not have the resources available to conduct monitoring at such a detailed level and most of wetlands are in fact on privately owned land which further hampers such efforts (Spencer *et al*., 1998). The first step in development is to identify the baseline conditions of the wetland being studied along with the climate, geohydrology, soil, biological processes and ecosystem functioning. After which a problem can be formulated and identified which will then be assessed and measured as end points. The routes of exposure must also be assessed to identify the extent, frequency as well as the magnitude of the stressor that enters the wetland. In parallel biological assessment is carried out to determine the effects of the stressors and the response of the organisms affected. Lastly the ecology is assessed at a population or community level, which is more timely than the previously mentioned as it requires monitoring over years and decades to provide adequate information. This type of ecosystem based approach links the biotic with the abiotic (Lemly, 1997).

In order to choose the correct bioindicators for rapid assessment tools, the following should be considered according to Spencer *et al*. (1998): bioindicators should "show slow natural temporal and spatial variability, be highly responsive to condition change, not be ambiguous in their interpretation, be cost effective and simple to apply, have regional applicability, be biologically relevant, be non-destructive and be able to have results summarized so as to be understood by non-experts." It is also important to consider that the patterns of accumulation of pollutants differ between organisms, depending on the metabolism of the organism (Livingstone, 1993). In order to develop a successful assessment index, a sound knowledge of how assemblages will react to different ecosystem changes is needed (Lee *et al*., 2013). Van Dam *et al.* (1998) summarized a list of attributes a bioindicator should have including anticipatory, sensitive, correlated to actual environmental effects, timely and cost effective, regionally relevant, socially relevant, easy to measure and interpret, diagnostic, broadly applicable and most importantly non-destructive. Along with the list they highlight that an index is only as strong as the indicator chosen.

Algae in particular have been used successfully as bioindicators for years. These organisms are ideal to assess certain changes brought about by anthropogenic activities. Mainly they are

used for water quality assessment, as they can be influenced by inorganic and organic nutrient fluxes, acidity and heavy metals. Planktonic algae are used as indicators of the trophic status of a water body. Wetlands contain a broad range of habitats, but they are also extremely sensitive and can be disrupted by flooding, desiccation, eutrophication as well as an increase in salinity (Bellinger & Sigee, 2010).

Algae are commonly used as bioindicator organisms, indices have mostly been developed for periphyton assemblages. When analysing periphyton assemblages, the focus can be either taxonomic or not. Taxonomic descriptions are mostly used. When using non-taxonomic assessments, biomass and chlorophyll content is measured. The advantage of such an assessment is that it will detect any effects on the population not portrayed in the taxonomic data. There are three ways in which periphyton are used in assessments. The 1st is based on autecology, and is also the oldest system in use. The 2nd is based on the assumption that ecosystem health is revealed in the community structures and that a healthy system will show larger diversity. The 3rd approach use biotic assemblages to assess health and is usually correlated with the physiochemical parameters of the water (Hill *et al*., 2000).

Taxonomic indicators have been used with great success because species composition is determined by the environmental conditions. This is due to the algal assemblages' preferences acting as diagnostic tools to aid the identification of the environmental change or stressor present. Although robust, this type of assessment can be difficult for the non-expert, especially at species level. Similarity between algae communities from the reference and the damaged site should be determined in two ways. First, similarity in biomass of the functional groups and relative abundance of diatoms should be determined. Secondly, the relative abundance of organisms and the number of different taxa should be compared to the reference wetland or site. Lastly, periphyton nutrients can also be related to the soil conditions, providing a glimpse of the past conditions that presided in the wetland (U.S. EPA, 2002).

Diatoms have many characteristics that make them good bioindicators. They occur widely in aquatic environments as primary producers. They also respond rapidly to environmental changes such as pH and metal load of water. Their response is not only through the community structure, but shifts in dominant taxa and diversity can also be observed. Other changes such as size and frustule deformities can also be related to anthropogenic stressors (da Silva *et al*., 2009).

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Most researchers use single bioindicator species for ecological assessment, depending on the variable measured, organisms used include ants (Parr & Chown, 2001), crab (Manuela *et al*., 2001) and fish (Wepener *et al*., 1992). Currently there are a few tools/indexes developed for freshwater systems impacted by an array of pollutants in South Africa: the Wetland Classification and Risk Assessment Index (WCRAI) (Oberholster *et al*., 2014); Phosphorous Sensitivity Index (Oberholster *et al*., 2013b), Ecotoxicological Screening Tool (EST) (Oberholster *et al*., 2013a), South African Scoring System version 5 (SASS 5) (Dickens & Graham, 2002), Index of Habitat Integrity (IHI), Riparian Vegetation Index (RVI) and Fish Assemblage Integrity Index (FAII) (Wepener, 2008).

1.6 Conclusion

Wetlands' services are important ecologically, improving water quality through the displacement of anthropogenic pollutants as well as environmental biotic and abiotic pollutants (Knox *et al*., 2008). Due to the nature of wetlands and the services they provide both for ecosystems and human benefit, rehabilitation and conservation is of utmost importance in water limited areas. However, as highlighted in this review, wetlands and AMD environments are more complex than often thought, with the interactions between the abiotic changes and primary producers easily overlooked. Because of versatile bacterial respiration, a complex food web exists, where the metabolism of one compound may severely affect the other, possibly inhibiting an otherwise beneficial cycle. Thus greater understanding is needed of the relationships between these organisms in order to fully understand possible effects on higher organisms.

1.7 References

Adrados, B., Sánchez, O., Arias, C.A., Becares, E., Garrido, L., Mas, J., Brix, H. & Morató, J., 2014, Microbial communities from different types of natural waste water treatment systems: vertical and horizontal flow constructed wetlands and biofilters, *Water Research*, 55: 304-312.

Akcil, A. & Koldas, S., 2006, Acid mine drainage (AMD): causes, treatment and case studies, *Journal of Cleaner Production*, 14: 1139-1145.

Atkinson, C.L., Golladay, S.W. & First, M.R., 2011, Water quality and planktonic microbial assemblages of isolated wetlands in an agricultural landscape, *Wetlands,* 31: 885-894.

Bacelar-Nicolau, P. & Johnson, D.B., 1999, Leaching of pyrite by acidophilic heterotrophic iron-oxidizing bacteria in pure and mixed cultures, *Applied and Environmental Microbiology*, 65: 585-590.

Baker, B.J. & Banfield, J.F., 2003, Microbial communities in acid mine drainage, *FEMS Microbiology Ecology*, 44: 139-152.

Baldwin, D.S., Rees, G.N., Mitchell, A.M., Watson, G. & Williams, J., 2006, The short-term effects of salination on anaerobic nutrient cycling and microbial community structure in sediment from a freshwater wetland, *Wetlands*, 26: 455-464.

Bannert, A., Kleineidam, K., Wissing, L., Mueller-Niggemann, C., Vogelsang, V., Welz, G.,Cao, Z. & Schloter, M., 2011, Changes in diversity and functional gene abundances of microbial communities involved in nitrogen fixation, nitrification, and denitrification in a tidal wetland versus paddy soils cultivated for different time periods, *Applied and Environmental Microbiology*, 77: 6109-6116.

Baretta-Bekker, H.J.K., Duursma, E.K. & Kuipers, B.R., 1995, *Encyclopedia of Marine Sciences*, 2nd ed., Springer-Verlag Berlin Heidelberg, New York.

Barlett, M.A. & Leff, L.G., 2010, Planktonic bacterial responses to nutrient amendments in wetland mesocosms, *Wetlands*, 30: 1161-1170.

Barton, C.D. & Karathanasis, A.D., 1999, Renovation of a failed constructed wetland treating acid mine drainage, *Environmental Geology*, 39; 39-50.

Bell, P.R. & Hemsley, A.R., 2000, *Green plants: their origin and diversity*, 2nd ed., Cambridge University Press, Cambridge.

Bellinger, E.G. & Sigee, D.C., 2010, *Freshwater algae: identification and use as bioindicators*, Wiley-Blackwell, West Sussex.

Benlloch, S., Rodriguez-Valera, F. & Martinez-Murcia, A.J., 1995, Bacterial diversity in two coastal lagoons deduced from 16S rDNA PCR amplification and partial sequencing, *FEMS Microbiology Ecology*, 18: 267–279.

Bhowal, S. & Chakraborty, R., 2011, Five novel acid-tolerant oligotrophic thiosulphate-metabolizing chemolithotrophic acid mine drainage strains affiliated with the genus *Burkholdria* of *Betaproteobacteria* and identification of two novel *sox*B gene homologs, *Research in Microbiology*, 162: 436-445.

Boshoff, G., Duncan, J. & Rose, P.D., 2004, The use of micro-algal biomass as a carbon source for the biological sulphate reducing systems, *Water Research*, 38: 2659-2666.

Bossio, D.A., Fleck, J.A., Scow, K.M. & Fujii, R., 2006, Alteration of soil microbial communities and water quality in restored wetlands, *Soil Biology & Biochemistry*, 38: 1223-1233.

Brander, L.M., Florax, R.J.G.M. & Vermaat, J.E., 2005, The empirics of wetland valuation: A comprehensive summary and a meta-analysis of the literature, *Environmental and Resource Economics*, 33: 223-250.

Bray, J.P., Broady, P.A., Niyogi, D.K. & Harding, J.S., 2008, Periphyton communities in New Zealand streams impacted by acid mine drainage, *Marine and Freshwater Research*, 59: 1084-1091.

Brofft, J.E., McArthur, J.V. & Shimkets, L.J., 2002, Recovery of novel bacterial diversity from a forested wetland impacted by reject coal, *Environmental Microbiology*, 4: 764-769.

Broughton, L.C. & Gross, K.L., 2000, Patterns of diversity in plant and soil microbial communities along a productivity gradient in a Michigan old-field, *Oecologia*, 125: 420–427.

Bruneel, O., Duran, R., Casiot, C., Elbaz-Poulichet, F. & Personné, J.-C., 2006, Diversity of microorganisms in Fe-As-rich acid mine drainage waters of Carnoulès, France, *Applied and Environmental Microbiology*, 72: 551-556.

Bustamante, M., Verdejo, V., Zúñiga, C., Espinosa, F., Orlando, J. & Carú, M., 2012, Comparison of water availability effect on ammonia-oxidizing bacteria and archaeal in microcosms of a Chilean semiarid soil, *Frontiers in Microbiology*, 3: 1-10.

Butcher, R.W., 1967, An introductory account of the smaller algae of British coastal waters, Part IV: Cryptophyceae. *Fishery Investigations, Ministry of Agriculture, Fisheries and Food,* London, Series IV: 1-52.

Carlsson, F., Frykblom, P., Liljenstolpe, C., 2003, Valuing wetland attributes: an application of choice experiments, *Ecological Economics*, 47: 95-103.

Carpenter, S.R., Caraco, N.F., Correll, D.F., Howarth, R.W., Sharpley, A.N. & Smith, V.H., 1998, Nonpoint pollution of surface waters with phosphorous and nitrogen, *Ecological Applications*, 8: 559-568.

Castillo, J., Pérez-López, R., Caraballo, M.A., Nieto, J.M., Martins, M., Costa, M.C., Olías, M., Cerón, J. & Tuccoulou, R., 2012, Biologically-induced precipitation of sphalerite-wurtzite nanoparticles by sulphate-reducing bacteria: Implications for acid mine drainage treatment, *Science of the Total Environment*, 423: 176-184.

Chen, L.X., Li, J.T., Chen, Y.T., Huang, L.N., Hua, Z.S., Hu, M., & Shu, W.S., 2013, Shifts in microbial community composition and function in the acidification of a lead/zinc mine tailings, *Environmental Microbiology*, 15: 2431-2444.

Chetty, S. & Luiz, J.M., 2014, The experience of private investment in the South African water sector: the Mbombela concession, *Development Southern Africa*, ERSA working paper 429: 1-20.

Coates, J.D., Phillips, E.J., Lonergan, D.J., Jenter, H. & Lovley, D.R., 1996, Isolation of *Geobacter* species from diverse sedimentary environments, *Applied and Environmental Microbiology*, 62:1531-1536.

Coetzee, M.A.S., 1995, 'Water pollution in South Africa: its impact on wetland biota', in G.I. Cowan (ed)., *Wetlands of South Africa.*, Department of Environmental Affairs and Tourism, Pretoria.

Costanza, R., 1992, 'Toward an operational definition of ecosystems health', in, Costanza, R., Norton, B.G. & Haskell, B.D. (eds.), *Ecosystem health: New goals for environmental management*, pp. 239-256, Island Press, Washington D.C.

da Silva, E.F., Almeida, S.F.P., Nunes, M.L., Luís, A.T., Borg, F., Hedlund, M., de Sá, C.M., Patinha, C. & Teixeira, P., 2009, Heavy metal pollution downstream the abandoned Coval da Mó mine (Portugal) and associated effects on epilithic diatom communities, *Science of the Total Environment*, 407: 5620-5636.

Dandie, C.E., Miller, M.N., Burton, D.L., Zebarth, B.J., Trevors, J.T. & Goyer, C., 2007, Nitric oxide reductase-targeted Real-Time PCR Quantification of denitrifier populations in soil, *Applied and Environmental Microbiology*, 73: 4250-4258.

Das, B.K., Roy, A., Koschorreck, K., Mandal, S.M., Wendt-Potthoff, K. & Bhattacharya, J. 2009, Occurrence and role of algae and fungi in acid mine drainage environment with special reference to metals and sulphate immobilization, *Water Research*, 43: 883-894.

Das, M. & Ramanujam, P., 2011, Metal content in water and green filamentous algae *Microspora quadrata* Hazen from coal mine streams of Jaintia Hills District, Meghalaya, India, *International Journal of Botany*, 7: 170-176.

Dawsin, T.L., 2007, Light-harvesting and light-protecting pigments in simple life forms, *Society of Dyers and Colourists*: *Coloration Technology*, 123: 129-142.

de Villiers, S. & Mkwelo, S.T., 2009, Has monitoring failed the Olifants River, Mpumalanga?, *Water SA*, 35: 671-676.

Dedysh, S.N., Panikov, N.S., Liesack, W., Groβkopf, R., Zhou, J., Tiedje, J.M., 1998, Isolation of acidophilic methane-oxidizing bacteria from northern peat wetlands, *American Association for the Advancement of Science*, 282: 281-284.

DeNicola, D.M. & Stapleton, M.G., 2002, Impact of acid mine drainage on benthic communities in streams: the relative roles of substratum vs. aqueous effects, *Environmental Pollution*, 119: 303-315.

Department of Sustainability and Environment, 2007, 'Index of wetland condition: review of wetland assessment methods', Victorian Government, Melbourne.

Dickens, C.W.S & Graham, P.M., 2002, The South African Scoring System (SASS) version 5 rapid bioassessment method for rivers, *African Journal of Aquatic Science* 27: 1-10.

Dini, J., 2004, 'Restoring wetlands and healing a nation: South Africa's Working for Wetlands programme', South African National Biodiversity Institute, South Africa.

Dokulil, M.T., 2003, 'Algae as ecological bio-indicators', in: Markert, B.A., Bruere, A.M. & Zechmeister, H.G. (eds.), *Bioindicators and Biomonitors*, Elsevier Science Ltd., p. 285-327.

DWAF, 2004a, 'South Africa's Water Situation and Strategies to balance supply and demand', Proposed first edition national water resource strategy, 1st edition, Department of Water Affairs and Forestry, Pretoria.

DWAF, 2004b, *Olifants Water Management Area: Internal Strategic Perspective*, Report P WMA 04/0000/00/0304, Department of Water Affairs and Forestry, Pretoria.

DWAF, 2010, *The nature, distribution and value of aquatic ecosystem services of the Olifants, Inkomati and Ustutu to Mhlatuze Water Management Areas*, RDM project number WP9677, Department of Water Affairs and Forestry, Pretoria.

Edwards, K.J., Bond, P.L., Gihring, T.M. & Banfield, J.F., 2000, An archaeal iron-oxidizing extreme acidophile important in acid mine drainage, *Science*, 287: 1796-1799.

Edwards, K.J., Gihring, T. M., & Banfield, J.F., 1999, Seasonal variations in microbial populations and environmental conditions in an extreme acid mine drainage environment, *Applied and Environmental Microbiology*, 65: 3627-3632.

Edwards, K.J., Gihring, T.M. & Banfield, J.F., 1999, Seasonal variations in microbial populations and environmental conditions in an extreme acid mine drainage environment, *Applied and Environmental Microbiology*, 65: 3627-3632.

Ehrenfeld, J.G., 2000, Evaluating wetlands within an urban context, *Ecological Engineering*, 15: 253-265.

Enowashu, E., Kandeler, E., Schloter, M., Rasche, F. & Engel, M., 2012, Development of a primer system to study abundance and diversity of the gene coding for alanine aminopeptidase *pepN* gene in Gram-negative soil bacteria, *Journal of Microbiological Methods*, 91: 14-21.

Faulwetter, J.L., Gagnon, V., Sundberg, C., Chazarenc, F., Burr, M.D., Brisson, J.,. Camper, A.K, & Stein, O.R., 2009, Microbial processes influencing performance of treatment wetlands: a review, *Ecological Engineering*, 35: 987-1004.

Fennessy, M.S. & Mitsch, W.J., 1989, Treating coal mine drainage with an artificial wetland, *Research Journal of the Water Pollution Control Federation*, 61: 1691-1701.

Fierer, N., Schimel, N.J.P. & Holden, P.A., 2003, Influence of drying-rewetting frequency on soil bacterial community structure, *Microbial Ecology* 45:63-71.

Findlay, S.E.G., Dye, S. & Kuehn, K.A., 2002, Microbial growth and nitrogen retention in litter of *Phragmites australis* compared to *Typha angustifolia*, *Wetlands*, 22: 616-625.

Fiset, J.F., Zinck, J.M., Nkinamubanzi, P.C., 2003, Chemical stabilization of metal hydroxide sludge. In: Proceedings of the X International Conference on Tailings and Mine Waste, October 2003, Vail, CO, USA, AA Balkema, pp. 329–332.

Fisher, M.M., Klug, J.L., Lauster, G., Newton, M. & Triplett, E.W., 2000, Effects of resources and trophic interactions on freshwater bacterioplankton diversity, *Microbial Ecology*, 40: 125–138.

García, C., Moreno, D.A., Ballester, A., Blázquez, M.L. & González, F., 2001, Bioremediation of an industrial acid mine drainage water by metal-tolerant sulphate-reducing bacteria, *Minerals Engineering*, 14: 997-1008.

González-Toril, E., Aguilera, Á., Souza-Egipsy, V., Pamo, E.L., España, J. S., & Amils, R., 2011, Geomicrobiology of La Zarza-Perrunal acid mine effluent (Iberian Pyritic Belt, Spain), *Applied and Environmental Microbiology*, 77: 2685-2694.

Gould, S.B., Waller, R.F. & McFadden, G.I., 2008, Plastid evolution, *Annual Review in Plant Biology*, 59: 491-517.

Guo, Z., Megharaj, M., Beer, M., Ming, H., Rahman, M.M., Wu, W. & Naidu, R., 2009, Heavy metal impact on bacterial biomass based on DNA analyses and uptake by wild plants in the abandoned copper mine soils, *Bioresource Technology*, 100: 3831-3836.

Gutknecht, J.L.M., Goodman, R.M. & Balser, T.C., 2006, Linking soil process and microbial ecology in freshwater wetland ecosystems, *Plant Soil*, 289: 17-34.

Haaijer, S.C., Crienen, G., Jetten, M.S.M. & Op den Camp, H.J.M., 2012, Anoxic iron cycling bacteria from an iron sulphide- and nitrate-rich freshwater environment, *Frontiers in Microbiology*, 3: 1-8.

Hartman, W.H., Richardson, C.J., Vilgalys, R. & Bruland, G.L., 2008, Environmental and anthropogenic controls over bacterial communities in wetland soils, *PNAS*, 105: 17842-17847.

Hauck, S., Benz, M., Brune, A. & Schink, B., 2001, Ferrous iron oxidation by denitrifying bacteria in profundal sediments of a deep lake (Lake Constance), *FEMS Microbiology*, 37: 127-134.

Hill, B.H., Herlihy, A.T., Kaufmann, P.R., Stevenson, R.J., McCormick, F.H. & Burch, J.C., 2000, Use of periphyton assemblage data as an index of biotic integrity, *Journal of the North American Benthological Society*, 19:50-67.

Hobbs, P., Oelofse, S.H.H. & Rascher, J., 2008, Management of environmental impacts from coal mining in the Upper Olifants river catchment as a function of age and scale, *Water Resources Development*, 24: 417-431.

Hong, S., Cannon, F.S., Hou, P., Byrne, T. & Nieto-Delgado, C., 2014, Sulfate removal from acid mine drainage using polypyrrole-grafted granular activated carbon, *Carbon*, 73: 51-60.

Jansen van Vuuren, S., Taylor, J., van Grinkel, C. & Gerber, A., 2006, *Easy identification of the most common freshwater algae: A guide for the identification of microscopic algae in South African freshwaters*, Department of Water Affairs and Forestry, Potchefstroom.

Johnson, D.B. & Hallberg, K.B., 2005, Acid mine drainage remediation options: a review, *Science of the Total Environment*, 338: 3-14.

Johnson, D.B., 1995, Acidophilic microbial communities: candidates for bioremediation of acidic mine drainage, *International Biodeterioration and Biodegradation*, 35: 41-58.

Karl, D., Letelier, R., Tupas, L., Dore, J., Christian, J. & Hebel, D., 1997, The role of nitrogen fixation in geobiochemical cycling in the subtropical North Pacific Ocean, *Nature*, 388: 533-538.

Keddy, P.A., Fraser, L.H., Solomeshch, A.I., Junk, W.J., Campbell, D.R., Arroyo, M.T.K. & Alho, C.J.R., 2009, Wet and wonderful: the world's largest wetlands are conservation priorities, *BioScience*, 59: 39-51.

Kent, D.M., 2000, 'Evaluating wetland functions and values', in D.M. Kent (ed.), *Applied wetlands science and technology*, 2nd ed., p.55-80, CRC Press LLC, U.S.A.

Knox, A.K., Dahlgren, R.A., Tate, K.W. & Atwill, E.R., 2008, Efficacy of natural wetlands to retain nutrient, sediment and microbial pollutants, *Journal of Environmental Quality*, 37: 1837-1846.

Krauter, P., Daily, B., Dibley, V., Pinkart, H. & Legler, T., 2005. Perchlorite and nitrate remediation efficiency and microbial diversity in a containerized wetland bioreactor, *International Journal of Phytoremediation*, 7: 113-128.

Kuang, J.-L., Huang, L.-N., Chen, L.-X., Hua, Z.-S., Li, S.-J., Hu, M., Li, J.-T., & Shu, W.-S., 2013, Contemporary environmental variation determines microbial diversity patterns in acid mine drainage, *The ISME Journal*, 7: 1038-1050.

Lake, P.S., Palmer, M.A., Biro, P., Cole, J., Covich, A.P., Dahm, C., Gibert, J., Goedkoop, W., Martens, K. & Verhoeven, J., 2000, Global change and the biodiversity of freshwater ecosystems: Impacts on linkages between above-sediment and sediment biota, *BioScience*, 50: 1099-1107.

Lear, G., Niyogi, D., Harding, J., Dong, Y., & Lewis, G., 2009, Biofilm bacterial community structure in streams affected by acid mine drainage, *Applied and Environmental Microbiology*, 75: 3455-3460.

Lee, S.S., Gaiser, E.E. & Trexler, J.C., 2013, Diatom-based models for inferring hydrology and periphyton abundance in a subtropical karstic wetland: implications for ecosystem-scale bioassessment, *Wetlands*, 33: 157-173.

Lemly, A.D. & King, R.S., 2000, An insect-bacteria bioindicator for assessing detrimental nutrient enrichment in wetlands, *Wetlands* 20: 91-100.

Lemly, A.D., 1997, Risk assessment as an environmental management tool: considerations for freshwater wetlands, *Environmental Management*, 21: 343-358.

Lindstrom, E.S., 2000, Bacterioplankton community composition in five lakes differing in trophic status and humic content, *Microbial Ecology*, 40: 104–113.

Livingstone, D.R., 1993, Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment, *Journal of Chemical Technology & Biotechnology*, 57: 195-211.

Macfarlane, D.M., 2013, 'Phase 1 – Planning report: Zaalklapspruit Wetland' (B20G-01)', Working for Wetlands Rehabilitation Programme, SANBI, Pretoria.

Manuela, P., Schuwerack, M., Lewis, J.W. & Jones, P., 2001, The potential use of the South African river crab, *Potamonautes warreni,* as a bioindicator species for heavy metal contamination, *Ecotoxicology*, 10: 159-166.

Maree, J.P., Hlabela, P., Nengovhela, A.J., Geldenhuys, A.J., Mbhele, N., Nevhullaudiz, T. & Waanders, F.B., 2004, Treatment of mine water for sulphate and metal removal using barium sulphide, *Mine Water and the Environment*, 23: 195-203.

Marin, B., Klingberg, M. & Melkonian, M., 1998, Phylogenetic relationships among Cryptophyta: analysis of nuclear-encoded SSU rRNA sequences support the morphology of extant plastid-containing lineages, *Protists*, 149: 265-276.

McCarthy, T.S., 2011, The impact of acid mine drainage in South Africa, *South African Journal of Science*, 107: 1-7.

McFadden, G.I., Gilson, P.R. & Hill, D.R.A., 1994, *Goniomonas*: rRNA sequences indicate that this phagotrophic flagellate is a close relative of the host component of cryptomonads, *European Journal of Phycology*, 29: 29-32.

McKnight, D.M., Bencala, K.E., Zellweger, G.W., Alken, G.R., Feder, G.L. & Thorn, K.A., 1992, Sorption of dissolved organic carbon by hydrous aluminium and iron oxides occurring at the confluence of Deer Creek with the Snake River, Summit County, Colorado, *Environmental Science & Technology*, 26: 1388-1396.

Merkley, M., Rader, R.B., McArthur, J.V. & Eggett, D., 2004, Bacteria as bioindicators in wetlands: bioassessment in the Bonneville basin of Utah, USA, *Wetlands*, 24: 600-607.

Mignot, J.-P., 1965, Etude ultrastructurale de Cyathomonas truncata From (Flagelle Cryptomonadine), *Journal Microscopie* (Paris) 4: 239-252.

Mitsch, W.J. & Gosselink, J.G., 2000, The value of wetlands: importance of scale and landscape setting, *Ecological Economics*, 35: 25-33.

Moosa, S. & Harrison, S.T.L., 2006, Product inhibition by sulphide species on biological sulphate reduction for the treatment of acid mine drainage, *Hydrometallurgy*, 83: 214-222.

Morrissey, E.M., Jenkins, A.S., Brown, B.L. & Franklin, R.B., 2013, Resource availability effects on nitrate-reducing microbial communities in a freshwater wetland, *Wetlands*, 33: 301-310.

Myers, C.R. & Myers, J.M., 1997, Cloning and sequencing of *cymA*, a gene encoding a tetraheme cytochrome *c* required for reduction of iron (III), fumarate, and nitrate by *Shewanella putrifaciens* MR-1, *Journal of Bacteriology*, 179; 1143-1152.

Ñancucheo, I. & Johnson, D.B., 2010, Production of glycolic acid by chemolithotrophic iron- and sulphur-oxidizing bacteria and its role in delineating and sustaining acidophilic sulphide mineral-oxidation consortia, *Applied and Environmental Microbiology*, 76: 461-467.

Ñancucheo, I. & Johnson, D.B., 2012, Selective removal of transition metals from acidic mine waters by novel consortia of acidophilic sulfidogenic bacteria, *Microbial Biotechnology*, 5: 34-44.

Nazaries, L., Murrell, J.C., Millards, P., Baggs, L. & Singh, B.K., 2013, Methane, microbes and models: fundamental understanding of the soil methane cycle for future predictions, *Environmental Microbiology*, 15: 2395-2417.

Neubauer, S.C., Emerson, D. & Megonigal, J.P., 2002, Life at the energetic edge: kinetics of circumneutral iron oxidation by lithotrophic iron-oxidizing bacteria isolated from the wetland-plant rhizosphere, *Applied and Environmental Microbiology*, 68: 3988-3995.

Nichols, D.S., 1983, Capacity of natural wetlands to remove nutrients from waste waters, *Journal WPCF*, 55: 495-505.

Nixdorf, B., Fyson, A. & Krumbeck, H., 2001, Review: plant life in extremely acidic waters, *Environmental and Experimental Botany*, 64: 203-211.

Niyogi, D.K., Lewis, W.M. & McKnight, D.M., 2003, Direct and indirect effects of mine drainage on bacterial processes in mountain streams, *Journal of the North American Benthological Scociety*, 22: 276-291.

Niyogi, D.K., McKnight, D.M. & Lewis, W.M., 1999, Influences of water and substrate quality for periphyton in a montane stream affected by acid mine drainage, *Limnology and Oceanography*, 44: 804-809.

Oberholster, P.J. & de Klerk, A., 2014, 'Aquatic ecosystems in the coal mining landscape of the upper Olifants River and the way forward', paper presented at the 21st Century challenges to the southern African coal sector Symposium, Emperors Palace Convention Resort, Johannesburg , 4–5 March.

Oberholster, P.J., Botha, A.-M. & Cloete, T.E., 2008, Biological and chemical evaluation of sewage water pollution in the Rietvlei nature reserve wetland area, South Africa, *Environmental Pollution*, 156: 184-192.

Oberholster, P.J., Dabrowski, J. & Botha, A.-M., 2013b, Using modified multiple phosphorous sensitivity indices for mitigation and management of phosphorous loads on a catchment level, *Fundamental and Applied Limnology*, 182: 1-16.

Oberholster, P.J., Genthe, B., Hobbs, P., Cheng, P.H., de Klerk, A.R. & Botha-Oberholster, A.-M., 2013a, An Ecotoxicological screening tool to prioritise acid mine drainage impacted streams for future restoration, *Environmental Pollution*, 176: 244-253.

Oberholster, P.J., McMillan, P., Durapersad, K., de Klerk, A.R. & Botha, A.-M., 2014, Development of a wetland classification and risk assessment index (WCRAI) for natural wetland ecosystems in three ecoregions of South Africa, *Water Air Soil Pollution*, 225: 1-15.

Ochieng, G.M., Seanego, E.S. & Nkwonta, O.I., 2010, Impacts of mining on water resources in South Africa: a review, *Scientific Research and Essays*, 5: 3351-3357.

Øvreas, L., Bourne, D., Sandaa, R., Casamayor, E.O., Benlloch, S., Goddard, V., Smerdon, G., Heldal, M. & Thingstad, T.F., 2003, Response of bacterial and viral communities to nutrient manipulations in seawater mesocosms, *Aquatic Microbial Ecology*, 31: 109-121.

Paerl, H.W., Fulton, R.S., Moisander, P.H. & Dyble, J., 2001, Harmful freshwater algal blooms, with an emphasis on cyanobacteria, *The Scientific World*, 1: 76-113.

Parr, C.L. & Chown, S.L., 2001, Inventory and bioindicator sampling: testing pitfall and Winkler methods with ants in a South African savannah, *Journal of Insect Conservation*, 5: 27-36.

Peralta, A.L., Matthews, J.W., Flanagan, D.N. & Kent, A.D., 2012, Environmental factors at dissimilar spatial scales influence plant and microbial communities in restored wetlands, *Wetlands*, 32: 1125-1134.

Pinheiro, H.J., 2000, *A techno-economic and historical review of the South African Coal Industry in the 19th and 20th Centuries, and analysis of coal product samples of South African Coilleries 1998-1999* (Bulletin 113), South African Bureau of Standards.

Placella, S.A. & Firestone, M.K., 2013, Transcriptional response of nitrifying communities to wetting of dry soil, *Applied and Environmental Microbiology*, 79: 3294-3302.

Pound, K.L., Lawrence, G.B. & Passy, S.I., 2013, Wetlands serve as natural sources for improvement of stream ecosystem health in regions affected by acid deposition, *Global Change Biology*, 19: 2720-2728.

Prescott, G.W., 1984, *How to know the freshwater algae*. Wm. C. Brown Company Publishers, Dubuque.

Prescott, L.M., Harley, J.P. & Klein, D.A., 2002, *Microbiology*, 5th ed., McGraw Hill, Boston.

Prime African Consultants, 2011, *Limiting and mitigating the impact of coal mines on wetlands: resource economic analysis component*, Coaltech, Pretoria.

Ramsar Convention Secretariat, 2013, *The Ramsar Convention Manual: a guide to the Convention on Wetlands* (Ramsar, Iran, 1971), 6th ed., Gland, Switzerland.

Ramsar, 2009, About Ramsar: The Ramsar List of Wetlands of International Importance, from www.ramsar.org/cda/en/ramsar-about-sites/main/ramsar/1-36-55_4000_0__.

Rapport, D.J., Costanza, R. & McMicheal, A.J., 1998, Assessing ecosystem health, *TREE*, 13: 397-402.

Ravit, B., Ehrenfeld, J.G. & Haggblom, M.M., 2003, A comparison of sediment microbial communities associated with *Phragmitese australis* and *Spartina alterniflora* in two brackish wetlands in New Jersey, *Estuaries*, 26: 465-474.

Richardson, D.J., 2000, Bacterial respiration: a flexible process for a changing environment, *Microbiology*, 146: 551-571.

Ritchie, D.A., Edwards, C., McDonald, I.R. & Murrell, J.C., 1997, Detection of methanogens and methanotrophs in natural environments, *Global Change Biology*, 3: 339-350.

Roberts, K.R., Stewart, K.D. & Mattox, K.R., 1981, The flagellar apparatus of *Chilomonas paramecium* (Cryptophyceae) and its comparison with certain zooflagellates, *Journal of Phycology*, 17: 159-167.

Rose, P.D., Boshoff, G.A., van Hille, R.P., Wallace, L.C.M., Dunn, K.M. & Duncan, J.R., 1998, An integrated algal sulphate reducing high rate ponding process for the treatment of acid mine drainage wastewaters, *Biodegradation*, 9: 247-257.

Saunders, R.J., Paul, N.A., Hu, Y. & de Nys, R., 2012, Sustainable source of biomass for bioremediation of heavy metals in waste water derived from coal-fired power plants, *PLoS One*, 7: 1-8.

Sears, K., Alleman, J.E., Barnard, J.L. & Oleszkiewicz, J.A., 2004, Impacts of reduced sulphur components on active and resting ammonia oxidizers, *Journal of Industrial Microbiology and Biotechnology*, 31: 369-378.

Seckler, D., Aurasinghe, U., Molden, D., de Silva, R. & Barker, R., 1996, *World water demand and supply, 1990 to 2025: Scenarios and Issues*, Research Report 19, International Water Management Institute, Sri Lanka.

Selala, M.C., Botha, A-M., de Klerk, A.R., de Klerk, L.P., Myburgh, J.G., Bletter, M.C.M. & Oberholster, P.J., 2013a, Effects of vegetable oil pollution on aquatic macroinvertebrate assemblage in a freshwater wetland and its use as a remediation tool, *Water Air Soil Pollution*, 224: 1650.

Selala, M.C., Oberholster, P.J., Surridge, K.A.K., de Klerk, A.R. & Botha, A.-M., 2013b, Responses of a selected biota after biostimilation of a vegetable oil spill in the Con Joubert Bird Sanctuary wetland, *African Journal of Biotechnology*, 12: 385-399.

Sepsenwol, S., 1973, Leucoplast of the cryptomonad *Chilomonas paramecium*: evidence for presence of a true plastid in a colourless flagellate, *Experimental Cell Research*, 76: 395-409.

Sheng, P.X., Tan, L.H., Chen, J.P. & Ting, Y.P., 2004, Biosorption performance of two brown marine algae for removal of chromium and cadmium, *Journal of Dispersion Science and Technology*, 25: 679-686.

Sheoran, A.S. & Bhandari, S., 2005, Treatment of mine water by a microbial mat: bench-scale experiments, *Mine Water and the Environment*, 24: 38-42.

Sieben, E.J.J., Ellery, W.N., Kotze, D.C. & Rountree, M., 2011, Hierarchical spatial organization and prioritization of wetlands: a conceptual model for wetland rehabilitation in South Africa, *Wetlands Ecological Management*, 19: 209-222.

Sims, A., Zhang, Y., Gajaraj, S., Brown, P.B. & Hu, Z., 2013, Toward the development of microbial indicators for wetland assessment, *Water Research*, 47: 1711-1725.

South African Government Online, About SA: Geography and climate, viewed 17 July 2014, from www.gov.za/aboutsa/geography.htm.

Spencer, C., Robertson, A.I. & Curtis, A., 1998, Development and testing of a rapid appraisal wetland condition index in south-eastern Australia, *Journal of Environmental Management* 54: 143-159.

Stapanian, M.A., Mack, J., Adams, J.V., Gara, B. & Micacchion, M., 2013, Disturbance metrics predict a wetland Vegetation Index of Biotic Integrity, *Ecological Indicators*, 24: 120-126.

Steenwerth, K.L.L.E., Jackson, F.J., Caldreron, F.J., Scow, K.M. & Rolston, D.E., 2005, Response of microbial community composition and activity in agricultural and grassland soils after a simulated rainfall, *Soil Biology and Biochemistry* 37: 2249-2262.

Stevens, A.E., McCarthy, B.C. & Vis, M.L., 2001, Metal content of *Klebsormidium*-dominated (Chlorophyta) algal mats from acid mine drainage waters in southeastern Ohio, *Journal of the Torrey Botanical Society*, 128: 226-233.

Streten-Joyce, C., Manning, J., Gibb, K.S., Neilan, B.A., & Parry, D.L., 2013, The chemical composition and bacteria communities in acid and metalliferous drainage from the wet–dry tropics are dependent on season, *Science of the Total Environment*, 443: 65-79.

Strydom, W., Hill, L. & Hobbs, P., 2010, 'Water. State of the environment – issue summary', Viewed 9 April 2013, from http://researchspace.csir.co.za/dspace/handle/10204/4220.

Torsvik, V., Ovreas, L. & Thingstad, T.F., 2002, Prokaryotic diversity – magnitude, dynamics, and controlling factors, *Science*, 296: 1064–1065.

Truu, M., Juhanson, J. & Truu, J., 2009, Microbial biomass, acitivity and community composition in constructed wetlands, *Science of the Total Environment*, 407: 3958-3971.

Tsukamoto, T.K., Killion, H.A., Miller, G.C., 2004, Column experiments for microbial treatment of acid mine drainage: low temperature, low pH and matrix investigations, *Water Research*, 38: 1405-1418.

Tyson, G.W., Chapman, J., Hugenholtz, P., Allen, E.E., Ram, R.J., Richardson, P.M., Solovyev, V.V., Rubin, E.M., Rokhsar, D.S. & Banfield, J.F., 2004, Community structure and metabolism through reconstruction of microbial genomes from the environment, *Nature* 428: 37-43.

U.S. E.P.A., 2002, 'Methods for evaluating wetland condition: Using algae to assess environmental conditions in wetlands', Office of Water, U.S. Environmental Protection Agency, Washington, DC.

van Dam, R.A., Camilleri, C. & Finlayson, C.M., 1998, The potential of rapid assessment techniques as early warning indicators of wetland degradation: a review, *Environmental Toxicology and Water Quality*, 13: 297-312.

van den Hoek, C., Mann, D.G. & Jahns, H.M., 1995, *Algae: an introduction to phycology*, Cambridge University Press, Cambridge.

Verb, R.G. & Vis, M.L., 2001, Macroalgal communities from an acid mine drainage impacted watershed, *Aquatic Botany*, 71: 93-107.

Verb, R.G. & Vis, M.L., 2005, Periphyton assemblages as bioindicators of mine-drainage in Unglaciated Western Allegheny Plateau lotic systems, *Water Air and Soil Pollution*, 161: 227-265.

Vermeulen, P.D. & Usher, B.H., 2006, Sulphate generation in South African underground and opencast collieries, *Environmental Geology*, 49: 552-569.

Volant, A., Bruneel, O., Héry, M., Casiot, C., Bru, N., Delpoux, F.A., Javerliat, F., Bouchez, O., Duran, R., Bertin, P.N., Elbaz-Poulichet, F. & Lauga, B., 2014, Diversity and spatiotemporal dynamics of bacterial communities: physicochemical and other drivers along an acid mine drainage, *FEMS Microbiology Ecology*, 90: 247-263.

Walter, C.A., Nelson, D. & Earle, J.I., 2012, Assessment of stream restoration: sources of variation in microinvertebrate recovery throughout an 11-year study of coal mine drainage treatment, *Restoration Ecology*, 20: 431-440.

Weber, K.A., Urrutia, M.M., Churchill, P.F., Kukkadapu, R.K. & Roden, E.E., 2006, Anaerobic redox cycling of iron by freshwater sediment organisms, *Environmental Microbiology*, 8: 100-113.

Weeks, B.S. & Alcamo, I.D., 2008, *Microbes and society*, 2nd ed., Jones and Barlett Publishers. Sudbury, MA.

Weiss, J.V., Emerson, D., Backer, S.M. & Megonigal, J.P., 2003, Enumeration of Fe(II)-oxidizing bacteria in the root zone of wetland plants: implications for rhizosphere iron cycling, *Biogeochemistry*, 64: 77-96.

Wepener, V., van Vuuren, J.H.J. & du Preez, H.H., 1992, Effect of manganese and iron at a neutral and acidic pH on the hematology of the banded tilapia (*Tilapia sparrmanii*), *Bulletin of Environmental Contamination and Toxicology*, 49: 613-619.

Whitmire, S.L. & Hamilton, S.K., 2005, Rapid removal of nitrate and sulphate in freshwater wetland sediment, *Journal of Environmental Quality*, 34: 2062-2071.

Woodward, R.T. & Wui, Y.S., 2001, The economic value of wetland services: a meta-analysis, *Ecological Economics*, 37: 257-270.

Working for Wetlands, n.d. Home, Availible from: wetlands.sanbi.org. [30 January 2014].

Xie, J., He, Z., Liu, X., van Nostrand, J.D., Deng, Y., Wu, L., Zhou, J. & Qiu, G., 2011, GeoChip-Based analysis of the functional gene diversity and metabolic potential of microbial communities in acid mine drainage, *Applied and Environmental Microbiology*, 77: 991-999.

Yergeau, E., Lawrence, J.R., Sanschagrin, S., Waiser, M.J., Korber, D.R. & Greer, C.W., 2012, Next-generation sequencing of microbial communities in the Athabasca River and its tributaries in relation to oil sands mining activities, *Applied and Environmental Microbiology*, 78: 7626-7637.

Yin, J., Jiang, L., Wen, Y., Yao, Z. & Zhou, Q., 2009, Treatment of polluted landscape lake water and community analysis of ammonia-oxidizing bacteria in constructed wetland, *Journal of Environmental Science and Health Part A*, 44: 722-731.

Zaidi, A., Wani, P.A., & Khan, M.S. (Eds.), 2012, *Toxicity of heavy metals to legumes and bioremediation*, Springer Science & Business Media.

Zedler, J.B., & Kercher, S., 2005, Wetland resources: status, trends, ecosystem services, and restorability, *Annual Review of Environment and Resources*, 30: 39-74.

Zedler, J.B., 2000, Progress in wetland restoration ecology, *Trends in Ecology and Evolution*, 15: 402-407.

Zurawell, R.W., Chen, H., Burke, J.M. & Prepas, E.E., 2005, Hepatoxic cyanobacteria: a review of the biological importance of microcystins in freshwater environments, *Journal of Toxicology and Environmental Health, Part B*, 8: 1-37.

Chapter 2: Phytoplankton diversity

2.1 Introduction

Algae are cosmopolitan freshwater organisms, serving as primary producers in their environments. Most species are autotrophic with photosynthetic metabolism and a few being secondary heterotrophs (Bellinger & Sigee, 2010). Planktonic and benthic algae assemblages are primary producers and form the basis of the food chain in the habitats where they occur. Algae depend on sunlight for photosynthesis and can live as planktonic organisms in the water column or as sessile organisms attached to substratum in shallow water habitats. They also occur intertwined among macrophytes. Algae are sensitive to disturbances in their environment which can affect their community composition. Environmental factors that influence algal growth include e.g. nutrient concentration, rainfall patterns, water flow regimes, location of the habitat and lastly the water quality (Fonge *et al*., 2012). Water quality is mostly affected by anthropogenic activities such as land use activity resulting in chemical pollution. Anthropogenic disturbances such as phosphorus runoff or from untreated sewerage can result in an increase in algal biomass, as in the case of cyanobacterial blooms in eutrophic waters (Verb & Vis, 2005). Due to the sensitivity of algae to changes in their environment that they are well suited as biological indicators (Stevenson & Smol, 2003).

Biological indicators are increasingly being used in monitoring programs as physical and chemical parameters alone cannot always provide insight into the impact of environmental changes caused by anthropogenic pollution on the ecosystem (Oberholster *et al*., 2008). The latter provides information on only the sampled water at a specific point in space and time. Algae are useful indicators of wetland ecosystem health and assessing changes in environmental conditions (Oberholster & de Klerk, 2014). Algae are a practical approach in biological assessment along with physical and chemical methods. The diversity among algal species, with regard to metabolism and habitat, enable the use of algal bioindicators in various environments (Table 1), depending on the physical characteristics of the study site (Oberholster & de Klerk, 2014). The more commonly assessed parameters include photosynthesis, respiration, net primary production, nutrient uptake and phosphatase activity (U.S. EPA, 2002).

Table 1. Criteria for the use of algal indicators for the assessment of freshwater ecosystem health in the Upper Olifants River Catchment (adapted from Oberholster & de Klerk, 2014).

Criteria	Benthic filamentous green algae	Diatoms
Practicality in non wadable streams.	High	High
Utility in seasonal waterlogged wetlands that are saturated with water, but where the water does not inundate or cover the sediment surface.	Low	High
Indicators of functional and structural process in aquatic ecosystems.	Low	Low
Specific indicators for land use activities.	High: *Microspora* sp. extremely low pH values < 2.5 *Stigeoclonium* sp. high phosphorus values. Wastewater treatment plants	High: *Gyrosigma rautenbachiae*: pollutants from the steel industry and smelters *Gomphonema aff. Gracile*: mining effluent
Practicality in streams impacted by deposits of metal hydroxides, caused by acid mine drainage.	Medium	Low
Biomass suitability for metal bioaccumulation.	High: large benthic mats occur in the upper Olifants River. Their high biomass makes them ideal specimens for pollutant bioaccumulation analyses.	Low: Their small biomass in the upper Olifants River makes them not ideal for pollutant bioaccumulation analyses.

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Freshwater algae can provide either short-term or long-term information on a water body, depending on the type of algae. Long-term information is indicative of the existing state of the habitat and short-term information is usually indicative of environmental change or a shift from the norm. The latter can act as early warning signs to severe changes in the aquatic ecosystem. The time it takes to observe changes in the algal community due to a disturbance can vary between seconds to days and days to weeks (Figure 1).
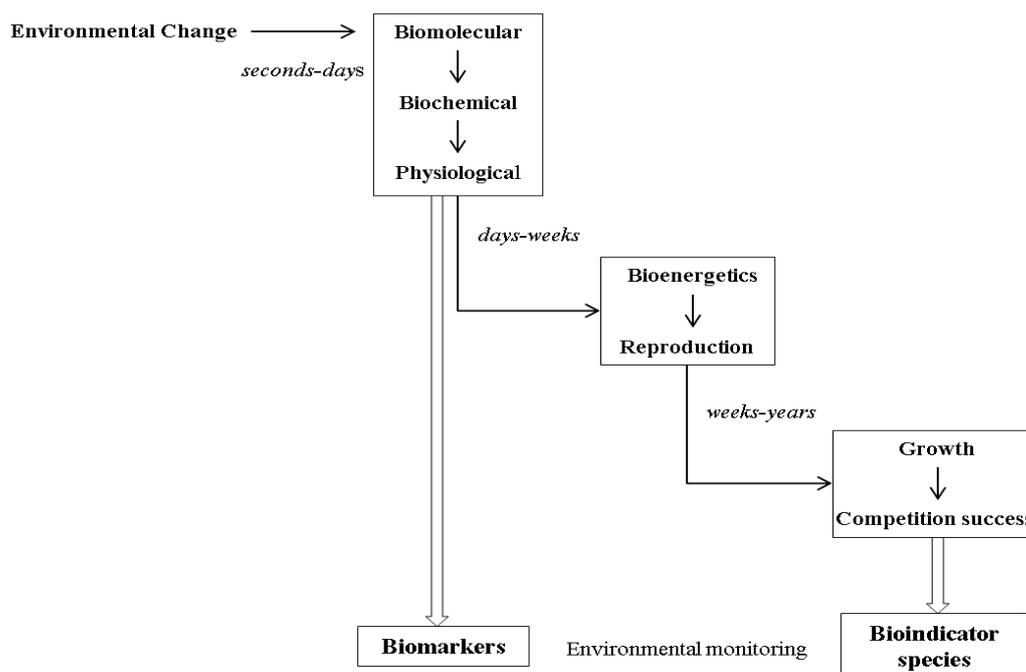
Figure 1. Algal response to environmental change in water quality. Time for response is indicated in italics. The responses follow a hierarchical trend from sub-organismal response (left) to individual response to population response (right). Adapted from Bellinger & Sigee (2010).

A good bioindicator should have a narrow ecological range, respond rapidly to change, be well defined taxonomically, easily identifiable and have a wide geographical distribution. (Bellinger & Sigee, 2010). There are three ways in which algae can be used for water quality assessment; the first is as indicator species using autecology, the second is measuring the community structure and the last is as part of a biotic index, i.e. diatom indices. The latter is considered to be more reliable as it is developed through the establishment of undisturbed conditions of the system being evaluated (Hill *et al*., 2000). However, Oberholster (2011) found that epilithic filamentous green algae to be more reliable indicators of nutrient enrichment and acid mine drainage than planktonic species, as they required a shorter period

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to recover after a flooding event. Thus the use of sessile algae would be more reliable than planktonic algae for monitoring programs (Oberholster, 2011). When considering the use of algal bioindicators, several trade-offs should be considered in order to select an appropriate method/index, i.e. laboratory labour, skill required and cost (Fetscher *et al*., 2014). In most cases, it is best that a combination of indicator organisms is used, offering a multifaceted approach to ecosystem health assessment (Fetscher *et al*., 2014). In the case of algal indicators, different populations may be studied. Epilithic filamentous green algae and diatoms are stationary, making these organisms ideal for physicochemical evaluation of a water body (Oberholster *et al*., 2013a).

Algal communities have been used as bioindicator organisms successfully (Dokulil, 2003; Bérard *et al*., 2004; Lavoie *et al*., 2004; Poulíčková *et al*., 2004; Verb & Vis, 2005; Cohen & Fong, 2006; Schneider *et al*., 2009). Their importance as biological indicators have been proven in AMD affected freshwater environments by various previous studies (Lopes *et al.,* 1999; Meyer & Galatowitsch, 1999; Valente & Gomes, 2007; Oberholster *et al*., 2013a; DeNicola & Stapleton, 2014). The latter is due to the rare occurrence of higher organisms in impacted streams, e.g. invertebrates and macrophytes (Sabater *et al*., 2003). Investigation into the environmental effects of AMD on algal communities has provided a better understanding into the response of the affected communities (Das *et al*., 2009; Oberholster & de Klerk, 2014). The identification of these responses forms the basis of biological indicator studies. Benthic communities are of great importance as they are abundant in all aquatic environments, have great species diversity as well as tolerance to habitat changes such as lowered pH and increased metal concentrations (DeNicola & Stapleton, 2014). Periphyton, i.e. diatoms has also been used in paleolimnological studies as bioindicator organisms to determine the pH history of lakes (Battarbee, 1984; Cameron *et al*., 1999; Kovács *et al*., 2006; Oberholster *et al*., 2013b). A review by DeNicola (2000) found that pH is the most significant environmental variable that affects freshwater diatoms, as these organisms have narrow optimal ranges; which can cause lower species richness. Planktonic algae respond more quickly to environmental changes in the water column whereas benthic algae are mostly influenced by changes in the underlying substrate (U.S. EPA, 2002). Acidophilic algae have been previously reported at a pH as low as 0.05 (Lee, 1999; Gross, 2000). Acidophilic algal species may make up the majority of biomass in highly acidic environments, as previously reported for the Rio Tinto River (Zettler *et al*., 2002). Increased algal biomass has been reported in acidic conditions (where there were low concentrations of metals) (Mulholland *et*

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al., 1986; Niyogi *et al*., 1999). On the other hand it has also been reported that even low metal concentrations may impact biomass, diversity and function of algal populations (Niyogi *et al*., 2002). In a previous, Norwegian study by Schneider & Lindstrøm (2009) it was found that river benthic algae are suitable for the detection of river acidification between a pH of 5.5 and 7. The overall effect of AMD seepage cause an increase in benthic biomass, due to adapted species dominating, depending on metal oxide precipitates (de la Peña & Barreiro, 2009). This could be due to the higher toxicity of especially precipitated iron than its dissolved form (DeNicola & Stapelton, 2002).

Filamentous green algae are known to sequester metals from their water environment, with a clear relationship between water metal concentration and metal uptake (Das & Ramanujam, 2011) as well as optimal growth in acidic conditions, thus making them ideal bioindicators of metal concentrations within a system (Stokes, 1979; Whitton *et al*., 1981). Taxa involved in metal uptake include *Klebsormidium*, *Microspora*, *Mougetia*, *Ulothrix*, *Spirogyra aequinoctialis* and *Stigeoclonium* species (Verb & Vis, 2001; Kaonga *et al*., 2008).   Various studies have reported on the metal uptake of *Klebsormidium*-dominated algal mats (Hargreaves & Whitton, 1976; Stevens *et al*., 2001; Verb & Vis, 2001). *Klebsormidium rivulare (Kützing)* has also been said to be genetically adapted to high Zn concentration in the environment (Say *et al*., 1977). *Euglenia* species were previously reported to have differential metal tolerance, leading to distinct distribution patterns between *E. gracilis* and *E. mutabilis* (Olaveson & Nalewajko, 2000). Green filamentous epilithic algae can also be used to monitor ecosystem health during physical disturbances like flooding, as they are attached to the substrata and do not take as long as water column planktonic algae to recover after such disturbances (Oberholster, 2011).

This study aimed to investigate different phytobenthos communities in the Grootspruit valley bottom wetland as indicators of anthropogenic pollution during the pre-restoration phase before the South African National Biodiversity Institute (SANBI) Working for Wetland programme started to restore the wetland. The objective was to identify the dominant algae species at each site, quantification of primary production along with taking the physical and chemical parameters into consideration in relationship with algal autecology.

2.2 Materials and Methods

2.2.1 Study site

The wetland studied in this study was the Zaalklapspruit wetland. This wetland is a ~139 hectares naturally channelled valley bottom system comprising (Macfarlane, 2013) and is situated in the Upper Olifants River catchment in the province of Mpumalanga and lies along the tributary of the Zaalklapspruit River, known as the Grootspruit. The latter then flows into the Wilge River, one of the main tributaries of the Olifants River. The catchment area of the wetland include cultivated lands, a coal mine and Evraz Highveld Steel and Vanadium foundry (Highveld Steel) which impacts water quality severely. The wetland was not functioning optimally as permanent channel incision from agricultural activities caused concentrated channelized water flow. The study area surrounding the wetland also suffers reduced vegetation growth as a result of previous ridge and furrow cultivation and artificial drainage. A selection of four study sites (sites 2-5) and a reference site (site 1), that acted as a control within this system, was chosen throughout the wetland (Figure 2). The selection of the five study sites were done based on water chemistry and habitat characteristics as basis for determining the land-use impact, i.e. AMD.
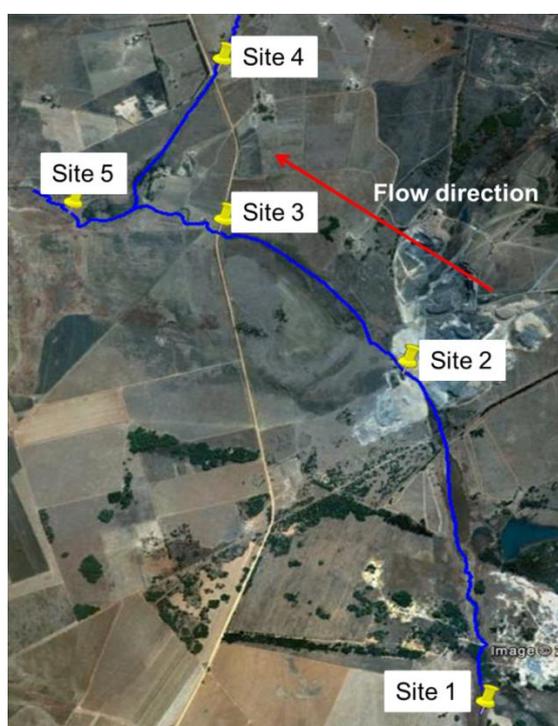
Figure 2. An aerial map of the study area, the Zaalklap wetland in Mpumalanga, South Africa (Google Earth).

The substrate type of each study site (i.e. percentage of cobbles, pebbles, gravel, sand and silt) and in-stream substrate cover (i.e. macrophytes) as well as the riparian canopy cover were determined visually according to the method of Stevenson and Bahls (1999). An assessment of the degree of wetland canal bank erosion was made to distinguish between AMD adverse effects and other land activities according to Spencer *et al.* (1998). Scores were allocated to each site using the following categories: 5 = stable (where the banks or edges of the stream are stable and are protected by good vegetation cover); 4 = good (evidence of minor localised erosion without damage to bank structure or vegetation); 3 = moderate (some erosion evident, with minor damage to bank structure and vegetation); 2 = poor (significant areas of erosion evident with little vegetation present); 1 = unstable (extensive erosion evident, where bare, steep and sometimes undercut banks are present). These physical characteristics were recorded for each site on the Wetland Index Field Sheet (Oberholster *et al.*, 2014).

2.2.2 Physical and chemical analysis

In situ measurements were taken for pH, temperature, redox potential (redox), electrical conductivity, dissolved oxygen (D.O$_2$), total dissolved solids (TDS) using the Thermo five star handheld water quality meter and turbidity using the Hach 2100P Turbidimeter (Loveland, USA) at each site. Surface water sampled at each sampling site was collected in duplicate using prewashed 2 ℓ bottles, according to the sampling method of Shelton (1994). These 2 ℓ samples were divided in a) 1 ℓ for chemical analysis and b) 1 ℓ for suspended chlorophyll-*a* analysis. Water samples were chemically analysed using Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) and Inductively Coupled Plasma-Mass Spectrometer (ICP-MS) using the APHA, AWWA and WPCF methods (APHA, AWWA and WPCF, 1992) for the presence of metals, sulphate, nitrate etc. The substrate type of each study site and in-stream substrate cover etc. was determined visually according to the method of Stevenson & Bahls (1999). Stream flows within the canal of the wetland was done according to Gore (2006).

Benthic chlorophyll-*a* concentrations of the algal mats were used as surrogate for benthic algal biomass according to Biggs (1996). Suspended and benthic chlorophyll-*a* was extracted from lyophilized Whatman GF filters using N,N-dimethylformamide at room temperature for two hours. Chlorophyll-*a* (chl-*a*) was then measured spectrophotometrically at 647 nm and 664 nm using the methodology of Porra *et al.* (1989). The algal biomass (expressed as

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chlorophyll-*a* concentration) may give an indication of the primary production within each of the sites. This in turn could be used to infer the trophic class of each site. This was done following the classification as proposed by Dokulil (2003) (Table 5).

2.2.3 Sample collection of benthic algae

Sampling of benthic algae was conducted from January 2013 to May 2013 at each selected study site (1 m^2). The presence of epilithic filamentous algae was first defined with the naked eye, since these types of algae have a distinct structure (Sheath & Cole, 1992). The percentage cover of filamentous algae was estimated using the method of Sheath and Burkholder (1985). If present, an area of substrate surface (5 cm in diameter) was isolated for epilithic filamentous algae sampling using a syringe extended with a tygon tube (Douglas, 1958; Steinman *et al*., 2006). Epilithic filamentous green algae samples were collected randomly at each site on three sampling occasions and combined in a composite sample of 100 mℓ for each study site; 50 mℓ was fixed in formaldehyde, for identification while the other 50 mℓ was used to determine benthic chlorophyll-*a*. Epilithic algae abundance in the samples was evaluated by counting the presence of each species (as cells in a filament or equal number of individual cells). In the case of diatom sampling, stones were collected from the submerged part (10-50 cm depth) of the Zaalklapspruit wetland at each sampling site. The attached diatoms were removed by brushing an area of 5 cm^2 of each stone and the material was resuspended in 200 mℓ deionised water. An aliquot of 50 mℓ was fixed with formaldehyde at a final concentration of 4 % (v/v) for microscopic examination to identify algal species. In the case of sand and silt samples containing benthic diatoms, the sediment was cleared of organic matter in a potassium dichromate and sulphuric acid solution and the cleared material was rinsed, diluted, and mounted in Pleurax medium for microscopic examination (Taylor *et al*., 2007a). All algae were identified using a compound microscope at 1250 x magnification (Wehr & Sheath, 2001; Van Vuuren *et al*., 2006; Taylor *et al*., 2007b). The samples were sedimented in an algae cell count chamber and were analysed using the strip-count method (APHA, 1992).
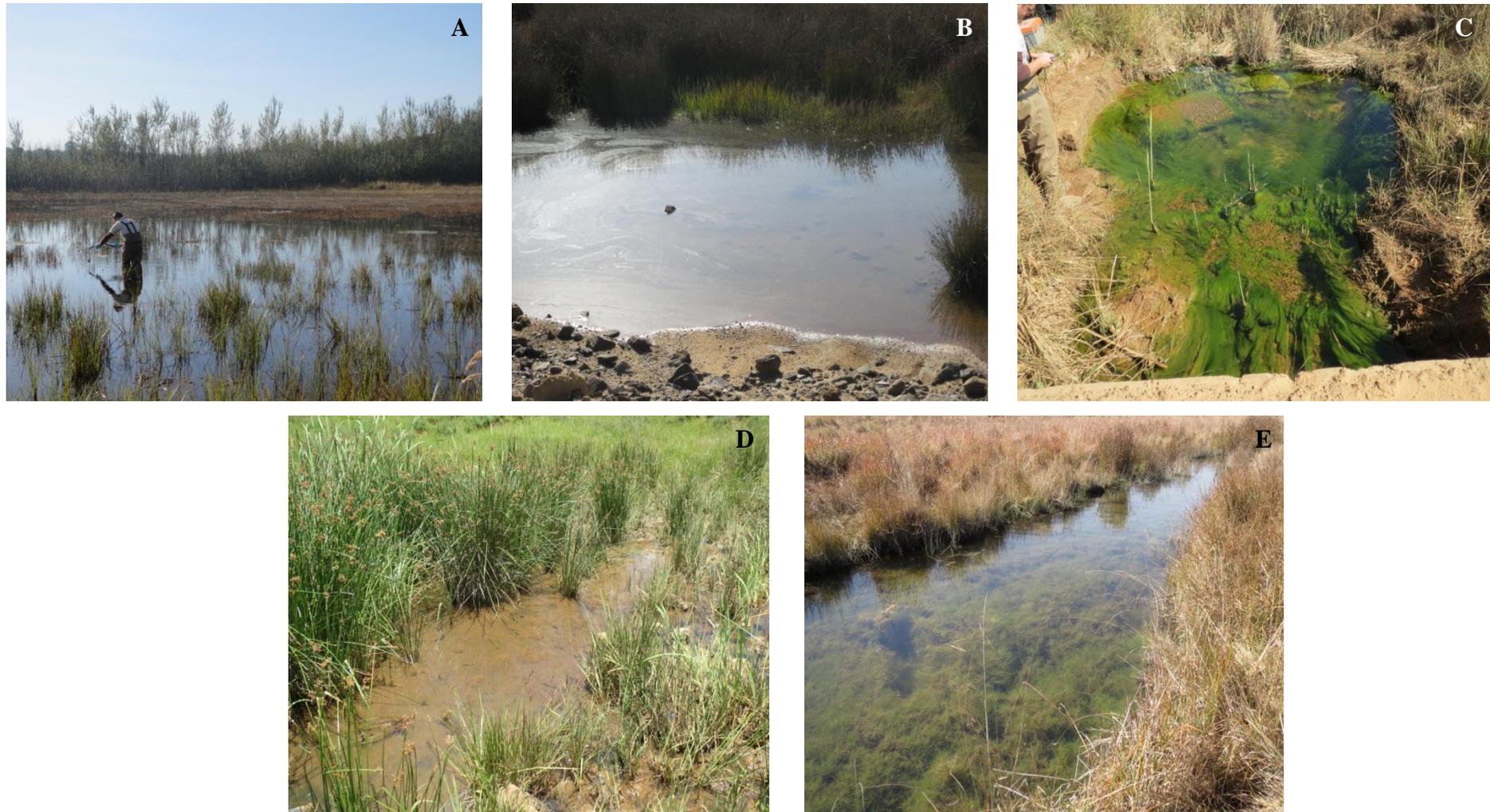
Figure 3. Study sites selected for the study. The least impacted site was chosen as the reference site (A), followed downstream by the most impacted site, namely site 2 (B) and after which site 3 (C), was located 1.2 km downstream from site 2. An adjoining branch into the wetland, receiving effluent from the Highveld Steel was sampled as site 4 (D) which flowed into the wetland just before site 5 (E).

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Table 2. Description of visual characteristics of the five study sites and the anthropogenic impacts at each of the individual sites. Adapted from Oberholster *et al*. (2013b).

Study Station	Site 1	Site 2	Site 3	Site 4	Site 5
Coordinates	25°57'6.13"S 29° 4'57.25"E	25°56'26.50"S 29° 5'0.10"E	25°55'9.37"S 29° 4'41.51"E	25°54'50.47"S 29° 4'26.15"E	25°54'31.41"S 29° 3'55.22"E
Bottom substrate type	Clay, silt	Clay, cobbles	Sand, bedrock	Sand, clay	Sand, cobbles
Canopy cover (%)	0	0	0	0	0
In-stream macrophytes at study site	*Typha capensis*	*T. capensis*	*T. capensis, Phragmites australis*	*T. capensis*	*T. capensis*
Submerged aquatic vegetation					*Isolepis fluitans*
Average flow regime (cm/S)	> 10	> 10	> 10	> 10	> 10
Geology	Dolomite	Dolomite	Dolomite	Dolomite	Dolomite
Stream channel width (m)	5	3	2	2.2	1.6
Average stream channel depth (cm)	72	22	23	14	61
Average bottom depth (cm)	>20	>20	~10	>20	>20
Bank	Good	Poor	Poor	Poor	Stable

stability					
Land use impacts	Agriculture	Abandoned coal mine	Downstream of abandoned coal mine	Highveld Steel effluent	Agriculture, downstream Highveld Steel effluent

2.2.4 Statistical analysis

All data were recorded on standard Excel spreadsheets for subsequent processing and statistical analysis. The Berger-Parker dominance index (Berger & Parker, 1970) was used to measure proportion of the most dominant species in each sample:

$N=N_{max}/N$

Where N_{max} is the number of individuals of the most abundant species in each sample and N is the total number of individuals at each site.

Multivariate analysis was used, namely principle component analysis (PCA) plots, to summarize the variation in species composition and interpret it using the physical and chemical parameters measured during this study. The analysis was done using the CANOCO v.5.04 (trial) software (Ter Braak & Šmilauer, 2002)

2.3 Results

2.3.1 Physical and chemical parameters

The longitudinal effects of *in situ* water quality parameters showed varying impacts throughout the wetland system over the three sampling events. Site 1 was the least impacted site, where the highest average suspended chl-*a* content (11.10±6.24 µg/ℓ) was measured (Table 3), compared to the other sites. The benthic chl-*a* was measured at a concentration of 26±5.5 mg/m^2. A near neutral pH (6.45±0.34) and low concentrations for Al and Fe (under detection limit and 29.22±17.49 µg/ℓ) was measured (Table 3). The DOC load and COD was measured as 7±2.65 mg/ℓ C and 21±5.57 mg/ℓ (Table 3) respectively at this site. In contrast to the reference site, site 2 appeared to be heavily impacted, since this site had the lowest benthic chl-*a* concentrations (11±7.2 mg/m^2) as well as suspended chl-*a* (0.23±0.16 µg/ℓ) in comparison to site 1 (Table 3). The highest concentrations for dissolved Al and Fe, compared to the other study sites, were measured in the water column (8000±7365.46 µg/ℓ

and 8696.67±11653.93 µg/ℓ respectively) of site 2 (Table 3). The lowest pH throughout the study was measured at site 2 (3.23±0.62) (Table 3).

At site 3, pH values did start to increase. The algal biomass increased at site 3 in comparison to site 2, concentrations of 67±13.1 mg/m^2 and 6.78±7.92 µg/ℓ were measured for benthic and suspended chl-*a*. The highest EC was measured at this site (1891.50±159.96 µS/cm) in comparison to the other sites sampled during the study. Metal concentrations for Al, Zn and Mn (3700±953.94, 430±229.13 and 9033.33±3496.19 µg/ℓ respectively) were considerably above the standards for aquatic ecosystems (Table 3) (DWAF, 1996).   The highest concentrations of dissolved sulphates were also measured at site 3 (1001.33±210.04 mg/ℓ) (Table 3) compared to the other sites. At site 4, where effluent from the Highveld Steel is entering the system, the highest turbidity was measured (9.22±6.19 NTU) (Table 3), possibly due to increased sedimentation, very shallow water column present and possible altercations within the water column at this site. The second highest DOC and COD (5.9±1.15 mg/ℓ C and 17±0 mg/ℓ respectively) were measured at this site compared to the other sites (Table 3). At site 5, the highest concentration of Mn (9233.33±4130.78 µg/ℓ) and EC (1925.50±374.44 µS/cm) was measured compared to the other sites (Table 3). High concentrations of Zn (353.33±141.89 µg/ℓ), sulphates (976±247.23 mg/ℓ) and TDS (943.50±183.54 mg/ℓ) were also recorded at this site (Table 3). The overall pH of the wetland varied downstream, with some improvement downstream of the severely impacted site 2 (Table 3).   The D.O$_2$ concentrations increased gradually downstream throughout the wetland (Table 3).

2.3.2 Planktonic and benthic algal assemblages

Site 1: Algal species that was dominant was the diatom *Navicula cryptotenella* (Lange-Bertalot) (Berger-Parker dominance index (BPDI): 0.352) and filamentous green algae *Spirogyra reinhardi* (Chmielevsky) (BPDI: 2.71). The blue-green algae *Oscillatoria tenuis* (Agardh) and *Glaucospira* sp. were also abundant at this site (Table 4). The largest number of species were identified at this site (Table 4), thus indicating a higher diversity and a stable population. Site 2: The dominating diatom species and filamentous green algae were *Frustulia saxonica* (Rabenhorst) (BPDI: 0.179) and *Microspira quadrata* (Hazen) (BPDI: 0.182) respectively.   The diatom *Nitzschia reversa* (Smith) were found to not only be common at this site, but at all sites sampled (Table 4) throughout the study. Site 3: The species identified at this site showed improvement in the water quality, as indicated by the increased diversity. The dominant algal species were *N. reversa* (Smith) (BPDI: 0.278),

Klebsormidium acidophilum (Novis) (BPDI: 0.491) and *Mougeotia cf. leavis* (Kützing) (BPDI: 0.271). Other species also identified at this site were *G. scalproides* (Rabenhorst) and *Synedra ulna* (Nitzsch) (Table 4). <u>Site 4:</u> Species found to dominate at this site were the diatom *Gyrosigma rautenbachiae* (Cholnoky) (BPDI: 0.289) and charophyte *S. reinhardi* (Chmielevsky) (BPDI: 2.81). Species identified to be scarce at this site were *S. ulna* (Nitzsch), *N. cryptotenella* (Lange-Bertalot) and *Merismopedia punctata* (Meyen) whereas *Craticula cuspidate* (Kützing) was identified to be common at this site (Table 4). <u>Site 5:</u> The dominant algae at the last site (site 5) were *Klebsormidium rivulare* (Kützing) (BPDI: 0.274), and was also found to be common at this site (Table 4). The diatoms *G. rautenbachiae* (Cholnoky), *Frustulia vulgaris* (Bory), *G. scalproides* (Rabenhorst) and *S. ulna* (Nitzsch) were detected at low numbers at this site (Table 4).

The Principal Component Analysis (PCA) biplot (Figure 4) summarized the variation in the species composition in relation to the environmental variables. According to the PCA biplot, TDS, EC and $D.O_2$ correlated positively together along with the diatom *N. reversa* (Smith) and *F. vulgaris* (Thwaites). Environmental parameters characteristic of AMD, namely sulphate; Mn; Ni; Zn; Fe; Pb; Al and Cu correlated positively with *F. saxonica* (Rabenhorst), *K. acidophilum* (Novis), *M. leavis* (Kützing), *Zygnema cf. cylindrospermum* (West & G.S. West) and *M. quadrata* (Hazen). Turbidity and Boron correlated positively with the diatoms *Gomphonema parvulum* (Kützing) and *C. cuspidata* (Kützing). pH, temperature and COD correlated positively with one another including the diatoms *S. ulna* (Nitzsch), *Fragilaria ulna* (Nitzsch), *N. cryptotenella* (Lange-Bertalot) and *Diatoma vulgaris* (Bory) and the cyanobacterium *M. punctata* (Meyen).

Table 3. Physical characteristics and chemical concentrations as measured at each of the study sites throughout the study period (n=3).

Parameters		Site 1	Site 2	Site 3	Site 4	Site 5
EC	μS/cm	102.7 ± 45.4	799.7 ± 442.0	1891.5 ± 160.0	1317.3 ± 40.4	1925.5 ± 374.4
pH		6.5 ± 0.3	3.2 ± 0.6	4.4 ± 0.4	6.4 ± 0.3	5.3 ± 0.4
$D.O_2$	mg/ℓ	5.0 ± 2.1	6.9 ± 1.2	7.1 ± 1.3	7.2 ± 1.2	7.8 ± 0.2
	%	62.9 ± 22.9	85.2 ± 9.4	80.5 ± 4.5	92.6 ± 15.6	97.2 ± 7.6
TDS	mg/ℓ	50.3 ± 22.4	391.8 ± 216.3	926.7 ± 78.2	645.5 ± 18.7	943.5 ± 183.5
Temp		20.4 ± 2.4	17.5 ± 6.2	13.4 ± 5.7	18.8 ± 7.5	17.5 ± 4.9
Turbidity	NTU[*]	3.9 ± 0.9	2.5 ± 1.8	1.0 ± 0.5	9.2 ± 6.2	1.9 ± 1.0
Redox	mV	-151.6 ± 14.0	470.9 ± 4.5	284.5 ± 139.9	48.7 ± 263.7	240.0 ± 11.2
Chl-a[w]	μg/ℓ	11.1 ± 6.2	0.2 ± 0.2	6.8 ± 7.9	2.4 ± 0.3	1.6 ± 1.6
Chl-a[b]	mg/m^2	26.0 ± 5.5	11.0 ± 7.2	67.0 ± 13.1	44.0 ± 8.1	27.0 ± 9.3
SO_4^{2-}	mg/ℓ	3.8 ± 0.96	211.7 ± 137.2	1001.3 ± 210.0	371.0 ± 49.1	976.0 ± 247.2
COD	mg/ℓ	21.0 ± 5.6	7.0 ± 2.0	ND	17.0 ± 3.0	9.7 ± 3.5
DOC	mg/ℓ	7.0 ± 2.7	1.0 ± 0.5	1.83 ± 0.3	5.9 ± 1.2	2.2 ± 0.3
Al	μg/ℓ	ND	8000.0 ± 7365.5	3700.0 ± 953.9	20.6 ± 15.7	870.0 ± 240.6
Iron	μg/ℓ	29.2 ± 17.5	8696.7 ± 11653.9	206.7 ± 110.6	17.2 ± 4.2	26.3 ± 11.0
Pb	μg/ℓ	ND	0.7 ± 0.07	ND	ND	ND
Mn	μg/ℓ	18.3 ± 23.2	1846.7 ± 1544.8	9033.3 ± 3496.2	107.0 ± 67.5	9233.3 ± 4130.8
Zn	μg/ℓ	ND	212.3 ± 203.8	430 ± 229.1	ND	353.3 ± 141.9
B	μg/ℓ	13.8 ± 6.9	12.7 ± 2.04	36.3 ± 4.4	169.0 ± 56.0	57.7 ± 7.1

| Copper | µg/ℓ | ND | 13.33 ± 12.74 | 4 ± 1.73 | ND | ND |
| Nickel | µg/ ℓ | 0.97 ± 0.85 | 92.33 ± 88.64 | 246.67 ± 105.04 | 2.2 ± 2.17 | 203.33 ± 30.55 |

[*] NTU: nephleometric turbidity units.

[w] Suspended water-column chlorophyll-*a*.

[b] Benthic chlorophyll-*a*.

ND – not determined/under detection limit of method.
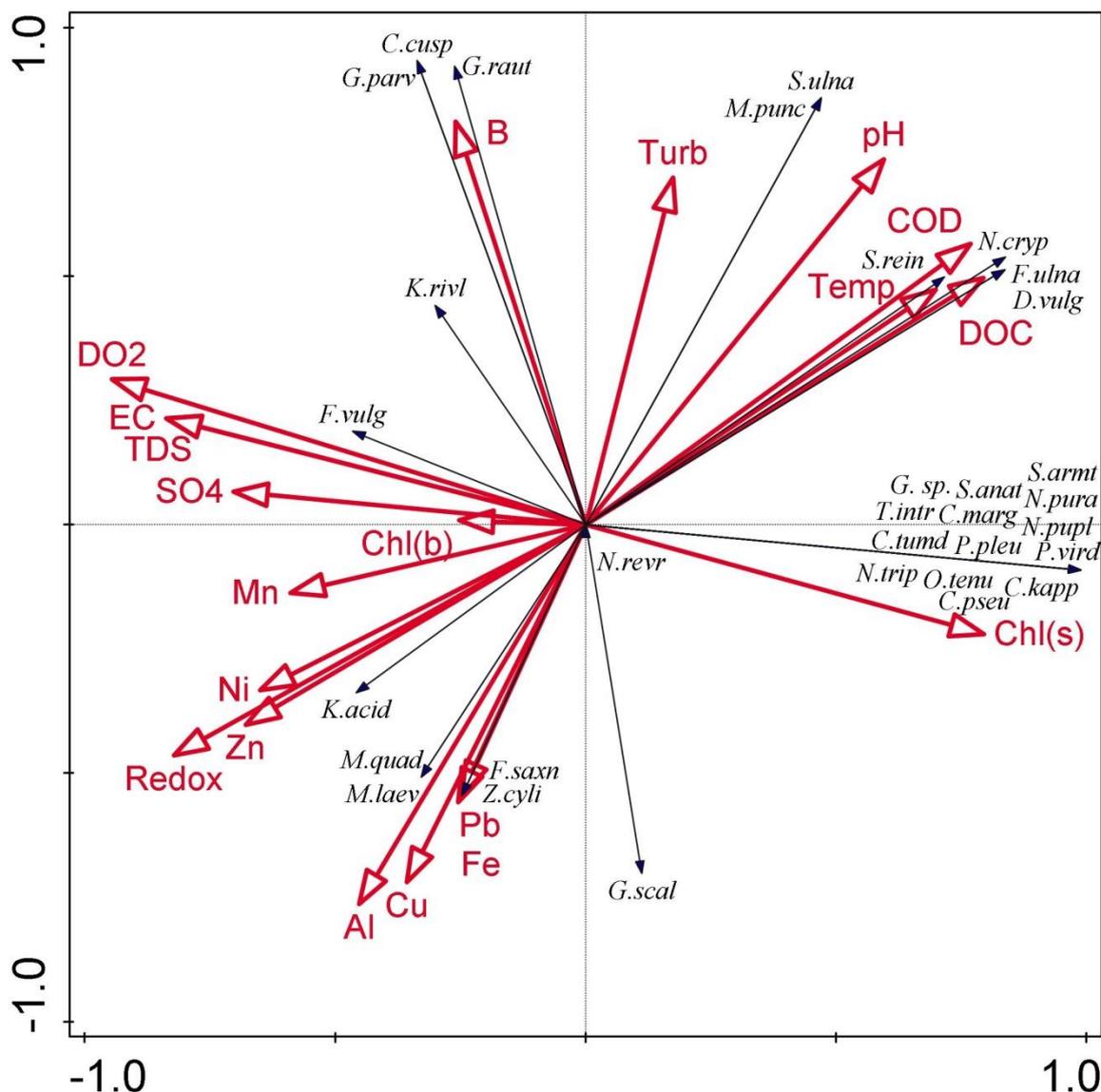
Figure 4. PCA biplot of the algae sampled at the 5 different study sites during January 2013 to May 2013 with the physico-chemical parameters depicted by the red arrows. Al: aluminum; Cu: copper; Zn: zinc; Ni: nickel; Mn: manganese; Fe: iron; Pb: lead; TDS: total dissolved solids; EC: electrical conductivity; DO2: dissolved oxygen; Chl(s): suspended chlorophyll-*a*; Chl(b): benthic chlorophyll-*a*; DOC: dissolved organic carbon; Redox: redox potential; SO4: sulphates; COD: chemical oxygen demand; temp: temperature; turb: turbidity; B: boron.

Tabel 4. Composition of the algal community in the Grootspruit wetland sampled from January 2013 to May 2013. The relative abundance of each algal taxa was grouped into: 1 = ≤ 50 (rare) 2 = 51- 250 (scarce), 3 = 251-1000 (common), 4 = 1001-5000 (abundant), 5 = 5001-25 000 (predominant) cells l^{-1}(n=3), according to Oberholster & Botha (2011).

Division	Major species	Site 1	Site 2	Site 3	Site 4	Site 5
Bacillariophyta	*Cymbella kappii* (Cholnoky)	+ +				
	Craticula cuspidata (Kützing)				+ + +	+ + +
	Diatoma vulgaris (Bory)	+ +			+	+
	Fragilaria ulna (Nitzsch)	+ +			+	+
	Gomphonema parvulum (Kützing)				+	+
	Nitzschia reversa (Smith)	+ + +	+ + +	+ + +	+ + +	+ + +
	Nitzschia pura (Hustedt)	+				
	Gyrosigma rautenbachiae (Cholnoky)				+ + + +	+ +
	Pinnularia viridiformis (Krammer)	+ + +				
	Frustulia saxonica (Rabenhorst)		+ +			
	Frustulia vulgaris (Thwaites)			+		+ +
	Gyrosigma scalproides (Rabenhorst)	+ +	+ +	+ +	+	+ +
	Synedra ulna (Nitzsch)	+ +	+	+	+ +	+ +
	Navicula cryptotenella (Lange-Bertalot)	+ + +			+ +	+
	Navicula tripunctata (OF Müller)	+ + + +				
	Navicula pupula (Kützing)	+ +				
	Cymbella tumida (Brébisson)	+ +				

		C1	C2	C3	C4	C5
Chlorophyta	*Cosmarium pseudopraemorsium* (Corda)	+ +				
	Closterium margaritiferum (Eichwald)	+ + +				
	Scenedesmus armatus (Chodat)	+ +				
	Staurastrum anatinum (Cooke & Wills)	+ +				
	Microspora quadrata (Hazen)		+ + + +			
Charophyta	*Spirogyra reinhardi* (Chmielevsky)	+ + +			+ + +	
	Klebsormidium acidophilum (Novis)			+ + + + +		+ +
	Klebsormidium rivulare (Kützing)					+ + + +
Streptophyta	*Mougeotia cf. laevis* (Kützing)			+ +		
	Zygnema cf. cylindrospermum (West & G.S.West)		+			
Euglenophyta	*Trachelomonas intermedia* (Dangeard)	+ +				
	Phacus pleuronectes (OF Müller)	+ +				
Cyanophyta	*Oscillatoria tenuis* (Agardh)	+ + + +				
	Merismopedia punctata (Meyen)	+ +	+	+	+ +	+ +
	Glaucospira sp.	+ + + +				

+ = rare

+ + = scarce

+ + + = common

+ + + + = abundant

+ + + + + = predominant

2.4 Discussion

In the current study of the Zaalklapspruit wetland it was evident that severe impacts on the aquatic biota occurred, due to AMD discharge and industrial effluent from Highveld Steel into the wetland. Changes in the water chemistry had a significant impact as was observed at site 2, most noticeably the formation of 'yellow boy' on the bottom substrate of the wetland. The latter was owing to the high concentrations of dissolved metals at site 2, due to the low pH, however as the pH was slightly above 3, metal oxide precipitates were expected. The lower pH at this site compared to the other 4 sites, did however increase downstream through the wetland. This was due to the wetlands' natural ability to improve water quality (Verhoeven *et al*., 2006). The conditions at the reference site also appeared tosupport a more diverse algal community. The reference site was chosen due to it being the least impacted site in the study wetland. Circumneutral pH and very low metal concentrations at this site was favourable to more stable algal communities with high diversity (Table 3).

The chl-*a* content was indicative of algal primary production (algal biomass), which decreased downstream in the wetland (Hellawell, 1986).   Site 1 was classified as eutrophic with moderate primary production possibly due to agriculture activities in the form of maize farming surrounding the wetland. Site 2 was oligotrophic with very low primary activity which was related to the low pH values. The high DOC and COD at site 1 may have attributed to the high algal biomass (Table 3). Chemical oxygen demand is a good indicator of the organic pollution within an environment (Emongor *et al*., 2005).   Site 3 was mesotrophic with low to moderate primary productivity (Table 5) and sites 4 and 5 oligotrophic. According to de la Peña & Barreiro (2009), benthic algal biomass will increase and the algal accumulation would decrease in the presence of AMD conditions. Accordingly, benthic algae can be used as a reliable indicator of AMD, as diatoms and green filamentous algae would dominate severely impacted areas, although with lowered richness, and metal hydroxide precipitation on the bottom substrate would lower the accumulation of photosynthetic algal biomass (Oberholster *et al*., 2013a). The same observation was made by de Nicola & Stapleton (2002), where they found that the aqueous AMD chemical environment had a greater effect than the metal hydroxide precipitate on organisms, due to limited recovery of the benthic populations. Thus dissolved metals have less an influence on benthic algae than the acidic pH level (de la Peña & Barreiro, 2009). This study supports the

latter, where lower benthic chl-*a* concentrations were measured at site 2 (low pH) than at site 3 (increased metal concentrations) compared to the other sites.

(mesotrophic and oligotrophic highlighted) Compare to the COD and DOC values in Fig 3. Need to show N and P values too for clarity when referring to trophic status.

Table 5. Classification of trophic levels according to chlorophyll-*a* concentration as a surrogate for algal biomass (adapted from Dokulil, 2003).

Trophic class	Primary production	Chl-*a* (µg/ℓ)
Oligotrophic I	Very low	<1-4
Mesotrophic I-II	Low to moderate	3-8
Eutrophic II	Moderate	7-30
Eu- to Polytrophic II-III	Moderate to high	25-50
Polytrophic III	High	50-100
Poly- to Saprotrophic III-IV	Very high	>100
Saprotrophic IV	Extremely high	>400

Algal biomass was not the only consideration in the evaluation of the wetland, but the autecology of the species revealed much more information in conjunction with the physical and the chemical parameters measured at each site. Diatoms have been previously reported to be important biological indicators of wetland health (Matlala *et al*., 2011). The abundance of *Bacillariophyceae* species at the reference site corresponds with previous studies that found such species to thrive under enriched conditions (Wahlby & El-Moneim, 1979; Selala *et al*., 2013b). Species that have been reported to be indicative of acidic environments in previous studies were *S. ulna* (Lange-Bertalot) (Lampkin & Sommerfield, 1982), *Nitzschia* and *Cymbella* species along with *Gomphonema* species (Matlala *et al*., 2011). A study investigating the impact across an AMD gradient found that severely impacted streams were dominated by chlorophytes, (*M. quadrata* (Hazen) and *M. laevis* (Kützing) and the diatom *F. vulgaris* (Thwaites) (Bray, 2007). Other chlorophytes with an AMD preference were found to include *K. acidophilum* (Novis) and *Euglena mutabilis* (Schmitz) and *M. quadrata* (Hazen) (Bray, 2007). Thus it was not unexpected that *M. quadrata* (Hazen) was the dominant algal species at site 2 which was severely impacted by AMD inflow from nearby abandoned open cast mine. *Microspora* spp. have previously been linked to high metal accumulation,

increased biomass in AMD environments as well as decreased sensitivity to toxic conditions to a certain extent (Das & Ramanujam, 2011). At site 3, with a relatively low pH value throughout the study period was dominated by *K. acidophilum* (Novis) and *M. laevis* (Kützing), both commonly found in highly acidic environments. Mats of *Klebsormidium* species were found to contain higher concentrations of certain metals than measured in the water column (Stevens *et al*., 2002). Consequently, *Klebsormidium rivulare* (Kützing) were found to be tolerant to metals due to their ability to accumulate Zn, Al, Fe and Mn through the production of a slime that forms complexes with these metals (Stevens *et al*., 2002). Species previously reported at near-neutral conditions included *G. parvulum* (Kützing) (Kovács *et al*., 2006; de al Peña & Barreiro, 2009; DeNicola & Stapleton, 2014) and *D. vulgaris* (Bory) (Bray *et al*., 2008). It has also been found that *K. rivulare* (Kützing) is commonly found in acidic waters (Whitton & Diaz, 1981; Morison & Sheath, 1985; Douglas *et al*., 1998; Stevens *et al*., 2001).

A summary of the reported ecological conditions where some South African diatom species may be found is given in Table 6. According to Taylor *et al*. (2007b), the diatom *G. rautenbachiae* (Cholnoky) is an ideal bioindicator for the industrial pollution conditions that were observed at site 4, where the water is slow flowing and industrial effluent enters the system while the diatom *N. cryptotenella* (Lange-Bertalot) is ideal for conditions at site 1. This was also evident from the multivariate analysis (Figure 4) in the case of *N. cryptotenella* (Lange-Bertalot). *Closterium magaritiferum* (Eichwald) could also be considered as an indicator of low impacted (reference) sites. According to Figure 4, *D. vulgaris* (Bory) and *F. ulna* (Nitzsch) corresponded with less impacted environments. The former have been reported to not occur in waters with Zn concentrations above 100 µg/ℓ (Say & Whitton, 1981). In contrast, *D. vulgaris* (Bory) and *F. ulna* (Nitzsch) have been reported to be moderately to highly tolerant, to organic pollution (Rott *et al*., 1998). Thus the occurrence of these organisms at site 1 could be attributed to higher carbon loading at this site compared to the other sites in the study (Table 3). *Klebsormidium acidophilum* (Novis) is expected to correlate with pH rather than AMD metals, however some studies suggest that these organisms correlate more positively with AMD conditions, preferring a pH of 2.7-6 and moderate to high metal precipitates (Bray, 2007). Certain green filamentous algae species are impacted more by the metals present in AMD waters than the low pH (Aguilera, 2013), which may explain why *K. acidophilum* (Novis) correlated positively with AMD metals and not pH (Figure 4). Other species associated with metals in AMD waters were *M. laevis*

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(Kützing), *Z. cylindrospermum* (West & G.S. West), *F. saxonica* (Rabenhorst) and *M. quadrata* (Hazen) (Figure 4). These species have previously been found in acidic or mining impacted waters (Table 6) (Bray *et al*., 2008). *Mougeotia laevis* (Kützing) have also previously been found in waters with high Al and Zn concentrations (Whitton, 1970; Say & Whitton, 1980; Graham *et al*., 1996). Temperature, pH and COD were also correlated along with *F. ulna* (Nitzsch), *D. vulgaris* (Bory) and *N. cryptotenella* (Lange-Bertalot) (Figure 4), all of these species are found in waters with intermediate to high levels of primary productivity (Table 6) and low pollution levels (Say & Whitton, 1981). However, *G. parvulum* (Kützing) and *C. cuspidata* (Kützing) were grouped with boron, these organisms are usually associated with high levels of pollution. Increased boron concentrations observed at the study sites could be due to leaching from coal fly ash, boron is also water-soluble and may be easily transported throughout the wetland (Jankowski *et al*., 2006). The recommended concentration for boron in aquatic ecosystems is 1.2 mg/ℓ (Moss & Nagpal, 2003), however different trophic levels have different tolerance levels and toxicity is also dependent on the form of boron present. The algae *G. parvulum* (Kützing) have previously been associated with polluted and extremely polluted rivers (Ivorra *et al*., 1999).

Gomphonema parvulum (Kützing) is also tolerant to moderate pollution and can be found under high nutrient concentrations, turbidity and temperature (King *et al*., 2006; Matlala *et al*., 2011) as observed at site 4. This site was impacted by industrial pollution from Evraz Highveld Steel upstream and possibly to a smaller extent agricultural impact in the form of cattle farming. Species reported to thrive under increased electrical conductivity (EC) includes *Gomphonema* spp. with both species observed at site 5 with the highest EC, whereas the diatom *Pinnularia* sp. were found to prefer lower EC and turbidity, conditions present at site 1 where this diatom was found (Matlala *et al*., 2011). Environmentally sensitive species affected by AMD, *Cymbella kappii* (Cholnoky), were only found at the reference site (Bray *et al*., 2008). Diatoms that are known to prefer higher pH values than AMD environments were identified as *F. ulna* (Nitzsch) and *Navicula tripunctata* (Müller) (Kovács *et al*., 2006). *N. reversa (*Smith) has previously been reported in both increased sedimentation (Bahls, 2001; Smucker & Vis, 2011) and more saline environments (Medvedeva, 2002; Smucker & Vis, 2011). Two diatom species, *D. vulgaris* (Bory) at sites 1, 4 & 5 and *F. vulgaris* (Thwaites) observed at sites 3 & 5, have previously been reported at high D.O$_2$ and nitrate concentrations (Matlala *et al*., 2011). The diatom *G. rautenbachiae* (Cholnoky) have also been associated with saline environments (Oberholster *et al*., 2009). The cyanobacterium *O. tenuis* (Agardh)

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that was observed at site 1 has been known to occur in eutrophic conditions and can cause an off taste and odour in drinking water (Hoson, 1992). These organisms have been specially adapted to survive low concentrations of Fe by producing two siderophores (Brown & Trick, 1992).

Table 6. Ecological conditions in relationship with the dominant diatoms sampled in the Zaalklap wetland (Taylor *et al*., 2007b).

Diatom species	Ecological conditions
Craticula cuspidata (Kützing)	Cosmopolitan
	Moderate to high EC content
	May tolerate critical to very heavy pollution
D. vulgaris (Bory)	Meso- to eutrophic
	Average EC content
F. ulna (Nitzsch)	Cosmopolitan
	Alkaline waters
	Meso- to eutrophic
G. parvulum (Kützing)	Cosmopolitan
	Tolerant to extremely polluted conditions
N. reversa (Smith)	Cosmopolitan
	Saline inland waters
G. rautenbachiae (Cholnoky)	Standing or slow flowing waters
	Brackish water
	Industrial pollution
P. viridiformis (Krammer)	Cosmopolitan
	Oligo- to eutrophic
	Low to moderate EC content
F. saxonica (Rabenhorst)	Cosmopolitan
	Dystrophic
	Acidic water
	EC content poor
F. vulgaris (Thwaites)	Cosmopolitan
	Fresh to slightly brackish water
	Oligotrophic to highly polluted

N. cryptotenella (Lange-Bertalot)	Cosmopolitan
	Oligo- to eutrophic
	Tolerant to only moderate pollution
N. tripunctata (OF Müller)	Cosmopolitan
	Eutrophic
	High EC content
	Tolerant to critical levels of pollution

Very electrolyte poor – <50 µS/cm

Electrolyte poor (low EC content) – 50-100 µS/cm

Moderate electrolyte content – 100-500 µS/cm

Electrolyte-rich (high EC content) – >500 µS/cm

Brackish (very high EC content) – >1000 µS/cm

Saline – 6000 µS/cm

2.5 Conclusion

From the data generated in this study it was evident that the interaction between environmental conditions, anthropogenic pollution and algal populations were clearly interlinked. The observed results could be broadly attributed to the threefold impact of AMD on the wetland aquatic ecosystem, namely reduced diversity due to lethal pH levels and metal concentrations, dominance of tolerant species and lastly the shift in nutrient availability (Oberholster & de Klerk, 2014). Even though AMD will decrease diversity due to the creation of selective conditions, algal biomass may increase. The latter can be attributed to various changes within the ecosystem, i.e. reduced grazers, decreased competition and altered nutrient cycles (Verb & Vis, 2005; Bott *et al*., 2012). However, algae form an integral part of the trophic food-web within AMD impacted waters (Sabater *et al*., 2003). Their importance have been noted when it was proposed that a component of the water DOC may be produced by them when no allochthonous sources are readily available. If true, algae may support heterotrophic bacteria and some grazers of invertebrates indirectly (Sabater *et al*., 2003). A recent study by Pound *et al*. (2013) has highlighted 3 effects that environmental variables may have on wetland diatoms, namely: 1) the origin and severity of acidity within a stream will control diatom diversity in acid impacted streams; 2) the relationship between wetlands, organic content of the water and diatom exist for acid impacted waters as was suggested for hard streams by Passy (2010). And lastly, 3) acidification and its origin, influences diatom

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population structure in addition to richness (Pound *et al*., 2013). Planktonic algae may be used for general water quality assessment, whereas benthic algae may be used to assess the ecological status and localized water quality assessment (Bellinger & Sigee, 2010). The differences between upstream and downstream assemblages and water chemistry may also reflect reduction of the wetland's remediation ability (Valente & Gomes, 2007).

This study demonstrated that algal autecology could be used to assess valley bottom wetlands, aquatic conditions impacted by AMD and industrial effluent. It has also been shown that many algal features, structural and functional can be used as a diagnostic tool for the evaluation of wetland water quality (U.S. EPA, 2002). This study demonstrated that combining the identification of known indicator species, species diversity and chl-*a* content gave a reliable insight to the conditions present within each site (Hill *et al*., 2000). The use of algae as biological indicators would be advantageous in the future for wetland restoration under South African environmental conditions, as there is vast knowledge on most species in literature, which might not exist for other indigenous bioindicator organisms.

2.6 References

Aguilera, A., 2013, Eukaryotic organisms in extreme acidic environments, the Río Tinto case, *Life*, 3: 363-374.

American Public Health Association (APHA), American Water Works Association (AWWA), and Water Pollution Control Federation (WPCF), 1992, in APHA, AWWA, and WPCF, *Standard Methods for the Examination of Water and Wastewater*, 19th ed., Washington, D.C., USA.

Bahls, L.L., 2001, 'Support of aquatic life uses in Mizpah Creek based on the composition and structure of the benthic algae community', Department of Environmental Quality, Montana, U.S.A.

Battarbee, R.W., 1984, Diatom analysis and acidification of lakes, *Philosophical Transactions of the Royal Society of London, Series B*, 305: 451-477.

Bellinger, E.G. & Sigee, D.C., 2010, *Freshwater algae: identification and use as bioindicators*, Wiley-Blackwell, West Sussex.

Bérard, A., Rimet, F., Capowiez, Y. & Leboulanger, C., 2004, Procedures for determining the pesticide sensitivity of indigenous soil algae: a possible bioindicator of soil contamination?, Archives of environmental contamination and toxicology, 46: 24-31.

Berger, W.F. & Parker, F.L., 1970, Diversity of planktonic foraminifera in deep-sea sediments, *Science*, 168: 1345-1347.

Biggs, B.J.F., 1996, Patterns in benthic algae of streams In: Stevenson R.J., Bothwell M.L., Lowe, R.L. (eds), Algal ecology: freshwater benthic ecosystems, pp. 31-56, Academic Press, San Diego.

Bott, T.L., Jackson, J.K., McTammany, M.E., Newbold, J.D., Rier, S.T., Sweeney, B.W. & Battle, J.M., 2012, Abandoned coal mine drainage and its remediation: impacts on stream ecosystem structure and function, *Ecological Applications*, 22: 2144-2163.

Bray, J.P., 2007, 'The ecology of algal assemblages across a gradient of acid mine drainage stress on the West Coast, South Island, New Zealand', M.Sc. Thesis, University of Canterbury.

Bray, J.P., Broady, P.A., Niyogi, D.K. & Harding, J.S., 2008, Periphyton communities in New Zealand streams impacted by acid mine drainage, *Marine and Freshwater Research*, 59: 1084-1091.

Brown, C.M. & Trick, C.G., 1992, Response of the cyanobacterium, *Oscillatoria tenuis*, to low iron environments: the effects on growth rate and evidence of siderophore production, *Archives of Microbiology*, 157: 349-354.

Cameron, N.G., Birks, H.J.B., Jones, V.J., Berge, F., Catalan, J., Flower, R.J., Garcia, J., Kaeweeka, B., Koining, K.A., Marchetto, A., Sanchez-Castillo, P., Schmidt, R., Sisko, M., Soloviena, N., Stefkova, E. & Torro, M., 1999, Surface-sediment and epilithic diatom pH calibration sets for remote European mountain lakes with Surface Waters Acidification Programme (SWAP) calibration set, *Journal of Paleolimnology*, 22: 291-317.

Cohen, R.A. & Fong, P., 2006, Using opportunistic green macroalgae as indicators of nitrogen supply and sources to estuaries, Ecological Applications, 16: 1405-1420.

Das, B.K., Roy, A., Koschorreck, M., Mandal, S.M., Wendt-Potthoff, K. & Bhattacharya, J., 2009, Occurrence and role of algae and fungi in acid mine drainage environment with special reference to metals and sulfate immobilization, *Water Research*, 43: 883-894.

Das, M. & Ramanujam, P., 2011, Metal content in water and green filamentous algae *Microspora quadrata* Hazen from coal mine streams of Jaintia Hills District, Meghalaya, India, *International Journal of Botany*, 7: 170-176.

de la Peña, S. & Barreiro, R., 2009, Biomonitoring acidic drainages impact in a complex setting using periphyton, *Environmental Monitoring and Assessment*, 150: 351-363.

DeNicola, D.M. & Stapelton, M.G., 2002, Impact of acid mine drainage on benthic communities in streams: the relative roles of substratum vs. aqueous effects, *Environmental Pollution*, 119: 303-315.

DeNicola, D.M. & Stapleton, M.G., 2014, Benthic diatoms as indicators of long-term changes in a watershed receiving passive treatment for acid mine drainage, *Hydrobiologia*, DOI 10.1007/s10750-014-142-4.

DeNicola, D.M., 2000, A review of diatoms in highly acidic environments, *Hydrobiologia*, 433: 111-122.

Department of Water Affairs and Forestry, 1996, South African Water Quality Guidelines, Volume 7: Aquatic Ecosystems, Pretoria.

Dokulil, M.T., 2003, 'Algae as ecological bio-indicators', Markert, B.A., Breure, A.M. & Zechmeister, H.G. (eds.), pp. 285-327, Elsevier Science Ltd.

Douglas, B., 1958. The ecology of the attached diatoms and other algae in a small stony stream. Journal of. Ecology. 46, 295-322.

Douglas, G.E., John, D.M., Williamson, D.B. & Reid, G., 1998, The aquatic algae associated with mining areas in peninsula Malaysia and Sarawak: their composition, diversity, and distribution, *Nova Hedwigia*, 67: 198-211.

Emongor, V., Nkegbe, E., Kealotswe, B., Koorapetse, I., Sankwasa, S. & Keikanetswe, S., 2005, Pollution Indicators in Gaborone Industrial Effluent, *Journal of Applied Science*, 5: 147-150.

Ferreira, L., 2008, Determining the influences of land use patterns on the diatom, macroinvertebrates and riparian vegetation integrity of the lower Harts/Vaal River system, M.Sc. thesis, University of Johannesburg.

Fetscher, A.E., Stancheva, R., Kocielek, J.P., Sheath, R.G., Stein, E.D., Mazor, R.D., Ode, P.R. & Busse, L.B., 2014, Development and comparison of stream indices of biotic integrity using diatoms vs. non-diatom algae vs. a combination, *Journal of Applied Phycology*, 26: 433-450.

Fonge, B.A., Tening, A.S., Egbe, E.A., Yinda, G.S., Fongod, A.N. & Achu R.M., 2012, Phytoplankton diversity and abundance in Ndop wetland plain, Cameroon, *African Journal of Environmental Science and Technology*, 6: 247-257.

Gore, J.A., 2006, 'Discharge Measurements and Streamflow Analysis', in F.R. Hauer and G.A. Lamberti (eds.), *Methods in Stream Ecology*, pp. 51-81, Academic Press. San Diego. California, USA.

Graham, J.M., Arancibia-Avila, P. & Graham, L.E., 1996, Effects of pH and selected metals on growth of the filamentous alga *Mougeotia* under acidic conditions, *Limnology and Oceanography*, 41: 263-270.

Gross, W., 2000, Ecophysiology of algae living in highly acidic environments, *Hydrobiologia*, 433: 31-37.

Hargreaves, J.W. & Whitton, B.A., 1976, Effect of pH on growth of acid stream algae, *British Phycology Journal*, 11: 215-223.

Hellawell, J.M., 1986, *Biological indicators of freshwater pollution and environmental management*, Elsevier Applied Science Publishers, London & New York.

Hill, B.H., Herlihy, A.T., Kaufmann, P.R., Stevenson, R.J., McCormick, F.H. & Burch, J.C., 2000, Use of periphyton assemblage data as an index of biotic integrity, *Journal of the North American Benthological Society*, 19:50-67.

Hoson, T., 1992, Growth characteristics of the musty odor producing alga, *Oscillatoria tenuis*, *Water Science and Technology*, 25: 177-184.

Ivorra, N., Hettelaar, J., Tubbing, G.M.J., Kraak, M.H.S., Sabater, S. & Admiraal, W., 1999, Translocation of microbenthic algal assemblages use for *in situ* analysis of metal pollution in rivers, *Archives of Environmental Pollution and Toxicology*, 37: 19-28.

Jankowski, J., Ward, C.R., French, D. & Groves, S., 2006, Mobility of trace elements from selected Australian fly ashes and its potential impact on aquatic systems, Fuel, 85: 243-256.

Kaonga, C.C., Chiotha, S., Monjerezi, M., Fabiano, E. & Henry, E.M., 2008, Levels of cadmium, manganese and lead in water and algae, *International Journal of Science and Technology*, 5: 471-478.

Kelly, M.G., 1988, *Mining, and the freshwater environment*, Elsevier Applied Science, London.

King, L., Clarke, G., Bennion, H., Kelly, M. & Yallop, M., 2006, Recommendations for sampling littoral diatoms in lakes for ecological status assessment, *Journal of Applied Phycology*, 18: 15-25.

Kovács, C., Kahlert, M. & Padisák, J., 2006, Benthic diatom communities along pH and TP gradients in Hungarian and Swedish streams, *Journal of Applied Phycology*, 18: 105-117.

Lampkin, A.J. & Sommerfield, M.R., 1982, Algal distribution in a small, intermitted stream receiving acid mine drainage, *Journal of Phycology*, 18: 196-199.

Lavoie, I., Vincent, W.F., Pienitz, R. & Painchaud, J., 2004, Benthic algae as bioindicators of agricultural pollution in the streams and rivers of southern Québec (Canada), Aquatic ecosystem health & Management, 7: 43-58.

Lee, R.E., 1999, *Phycology* 3rd ed., pp. 3-59, Cambridge University Press, UK.

Lopez, I., Gonçalves, F., Soares, A.M.V.M. & Ribeiro, R., 1999, Ecotoxicological tools in the remediation of acid mine drainage, *Toxicological and Environmental Chemistry*, 70: 441-460.

Macfarlane, D.M., 2013, 'Phase 1 – Planning report: Zaalklapspruit Wetland' (B20G-01)', Working for Wetlands Rehabilitation Programme, SANBI, Pretoria.

Matala, M.D., Taylor, J.C. & Harding, W.R., 2011, 'Development of a diatom index for wetland health', Water Research Commission, WRC Report No. KV 270/11, p. 65-67.

Medvedeva, L.A., 2002, 'Diversity of diatoms in Sikhote-Aline biosphere reserve, far East Russia', In *Proceedings of the Fifteenth International Diatom Symposium*, ARG Gantner KG, *Ruggell*, p. 193-200.

Meyer, P.M. & Galatowitsch, S.M., 1999, Diatom communities as ecological indicators of recovery in restored prairie wetlands, *Wetlands*, 19: 765-774.

Morison, M.O. & Sheath, R.G., 1985, Responses to desiccation stress by *Klebsormidium rivulare* (Ulotrichales, Chlorophyta) from a Rhode Island stream, *Phycologia*, 24: 192-145.

Moss, S.A. & Nagpal, N.K., 2003, Ambient water guidelines for boron, Water Protection Branch, Ministry of Water, Land and Air Protection, British Columbia, Canada.

Mulholland, P.J., Elwood, J.W., Palumbo, A.V. & Stevenson, R.J., 1986, Effect of stream acidification on periphyton composition, chlorophyll, and productivity, *Canadian Journal of Fisheries and Aquatic Sciences*, 43: 1846-1858.

Naselli-Flores, L., Padisak, J., Dokulil, M.T. & Chorus, I., 2003, Equilibrium/steady-state concept in phytoplankton ecology, *Hydrobiology* 502: 395-403.

Niyogi, D.K., Lewis, W.M. & McKnight, D.M., 2002, Effects of stress from mine drainage on diversity, biomass, and function of primary producers in mountain streams, *Ecosystems*, 5: 554-567.

Niyogi, D.K., McKnight, D.M. & Lewis, W.M., 1999, Influences of water and substrate quality for periphyton in a montane stream affected by acid mine drainage, *Limnology and Oceanography*, 44: 804-809.

Oberholster, P., de Klerk, A.R., Botha, A.-M., Babalola, O.O., Ndlela, L., Staebe, K., van Wyk, J.H., 2013b, 'The adverse effects of anthropogenic pollution on *Xenopsus leavis* with special reference to acid mine drainage (AMD) in a freshwater wetland', in Lombardi, M.P. (ed.), Amphibians: Anatomy, ecological significance and conservation strategies, Nova Publishers.

Oberholster, P.J. & Botha, A.M., 2011, Dynamics of phytoplankton and phytobenthos in Lake Loskop (South Africa) and downstream irrigation canals, *Fundamental and Applied Limnology/Archiv für Hydrobiologie*, 179: 169-178.

Oberholster, P.J. & de Klerk, A., 2014, Aquatic ecosystems in the coal mining landscape of the upper Olifants River, and the way forward, The Southern African Institute of Mining and Metallurgy: 21st Century Challenges to the southern African coal sector symposium proceedings, Emperors Palace Convention Resort, Johannesburg, 4–5 March 2014, pp. 1-14.

Oberholster, P.J., 2011, 'Using epilithic filamentous green algae communities as indicators of water quality in the headwaters of three South African river systems during high and

medium flow periods', in *Zooplankton and Phytoplankton*, pp. 107-122 , Nova Science Publishers, Inc., USA.

Oberholster, P.J., Botha, A.-M. & Ashton, P.J., 2009, Appearance of new taxa: invertebrates, phytoplankton and bacteria in an alkaline, saline, meteoric crater lake, South Africa, *Fundamental and Applied Limnology*, 173: 1-12.

Oberholster, P.J., Genthe, B., Hobbs, P., Cheng, P.H., de Klerk, A.R. & Botha-Oberholster, A.-M., 2013a, An Ecotoxicological screening tool to prioritise acid mine drainage impacted streams for future restoration, *Environmental Pollution*, 176: 244-253.

Oberholster, P.J., Myburg, J.G., Ashton, P.J. & Botha, A.-M., 2010, Responses of phytoplankton upon exposure to a mixture of acid mine drainage and high levels of nutrient pollution, *Ecotoxicology and Environmental Safety*, 73: 326-335.

Olaveson, M.M. & Nalewajko, C., 2000, Effects of acidity on the growth of two *Euglenia* sp., *Hydrobiologia*, 233: 39-56.

Passy, S.I., 2010, A distinct latitudinal gradient of diatom diversity is linked to resource supply, *Ecology*, 91:36-41.

Porra, R.J., Thompson, W.A. & Kriedemann, P.E., 1989, Determination of accurate extinction coefficient and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectrometry, *Biochimica et Biophysica Acta*, 975: 384-394.

Poulíčková, A., Duchoslav, M. & Dokulil, M., 2004, Littoral diatom assemblages as bioindicators of lake trophic status: A case study from perialpine lakes in Austria. European Journal of Phycology, 39: 143-152.

Pound, K.L., Lawrence, G.B. & Passy, S.I., 2013, Wetlands serve as natural sources for improvement of stream ecosystem health in regions affected by acid deposition, *Global Change Biology*, 19: 2720-2728.

Rott, E., Duthie, H.C. & Pipp, E., 1998, Monitoring organic pollution and eutrophication in the Grand River, Ontario, by means of diatoms, Canadian Journal of Fisheries and Aquatic Sciences, 55: 1443-1453.

Sabater, S., Buchaca, T., Cambra, J., Catalan, J., Guasch, H., Ivorra, N., Muñoz, I., Navarro, E., Real, M. & Romaní, A., 2003, Structure and function of benthic algal communities in an extremely acid river, *Journal of Phycology*, 39: 481-489.

Say, P.J. & Whitton, B.A., 1980, Changes in flora down a stream showing a zinc gradient, *Hydrobiologia*, 76: 255-262.

Say, P.J. & Whitton, B.A., 1981, 'Chemistry and plant ecology of zinc-rich streams in Northern Penninies', in P.J. Say & B.A. Whitton (eds.), *Heavy metals in northern England: environmental and biological aspects*, p. 55-63, University of Durham, Durham.

Say, P.J., Diaz, B.M. & Whitton, B.A., 1977, Influence of zinc on lotic plants, I: Tolerance of *Hormidium* species to zinc, *Freshwater Biology*, 7: 377-384.

Schneider, S & Lindstrøm, E.-A., 2009, Bioindication in Norwegian rivers using non-diatomaceous benthic algae: The acidification index periphyton (AIP), *Ecological Indicators*, 9: 1206-1211.

Schneider, S. & Lindstrøm, E.A., 2009, Bioindication in Norwegian rivers using non-diatomaceous benthic algae: the acidification index periphyton (AIP), Ecological indicators, 9: 1206-1211.

Selala, M.C., Oberholster, P.J., Surridge, K.A.K., de Klerk, A.R. & Botha, A.-M., 2013b, Responses of a selected biota after biostimilation of a vegetable oil spill in the Con Joubert Bird Sanctuary wetland, *African Journal of Biotechnology*, 12: 385-399.

Sheath, R.G. & Cole, K.M., 1992, Biogeography of stream macroalgae in North America, *Journal of Phycology*, 28: 448-460.

Shelton, L. R., 1994, Field guide for collecting and processing stream-water samples for the National Water-Quality Assessment Program, US Geological Study Open-File Rep, pp. 94-455.

Smucker, N.J. & Vis, M.L., 2011, Spatial factors contribute to benthic diatom structure in streams across spatial scales: considerations for biomonitoring, *Ecological Indicators*, 11: 1191-1203.

Spencer, C., Robertson, A.I. & Curtis, A., 1998, Development and testing of a rapid appraisal wetland condition index in south-eastern Australia, *Journal of Environmental Management* 54: 143-159.

Steinman, A.D., Lamberti, G.A. & Leavitt, P.R., 2006, 'Biomass and pigmentation of benthic algae', in F.R. Hauer & G.A. Lamberti (eds.), *Methods in stream ecology*, pp.357-377, Elsevier, Academic Press, Amsterdam.

Stevens, A.E., McCarthy, B.C. & Vis, M.L., 2001, Metal content of *Klebsormidium*-dominated (Chlorophyta) algal mats from acid mine drainage waters in southeastern Ohio, *Journal of the Torrey Botanical Society*, 128: 226-233.

Stevens, A.E., McCarthy, B.C. & Vis, M.L., 2001, Metal content of *Klebsormidium*-dominated algal mats from acid mine drainage waters in south-eastern Ohio, *Journal of the Torrey Botanical Society*, 128: 226-233.

Stevenson, R.J. & Smol, J.P., 2003, 'Use of algae in environmental assessments', Freshwater Algae in North America: Classification and Ecology, p. 775-804, Elsevier Science.

Stevenson, R.J.& Bahls, L.L., 1999, 'Periphyton protocols', in M.T. Barbour, J. Gerritren, B.D. Snyder & J.B. Stribling (eds.), *Rapid bioassessment protocols for use in streams and wadeable rivers: Periphyton, benthic macroinvertebrates and fish*, 2nd ed., pp. 6-22, Washington DC: USA EPA 841-B-99-002.

Stokes, P.M., 1979, 'Copper accumulation in freshwater biota', in: Nriagu, J.O. (ed.), *Copper in the Environment, Part 1*, pp. 360-381, Wiley/Interscience, New York.

Taylor, J.C., Harding, W.R. & Archibald, C.G.M., 2007b, 'An illustrated guide to some common diatom species from South Africa', WRC Report TT 282/07, Pretoria, South Africa.

Taylor, J.C., Harding, W.R. & Archibold, C.G.M., 2007a, A methods manual for the collection, preparation and analysis of diatom samples, Water Research Commission, TT 281/07.

Ter Braak, C.J.F. & Šmilauer, P. (2002). CANOCO Reference Manual and Canodraw for Windows User's Guide: Software for Canonical Community Ordination (Version 4.5). Microcomputer Power. New York, USA.

U.S. E.P.A., 2002a, 'Methods for evaluating wetland condition: Using algae to assess environmental conditions in wetlands', Office of Water, U.S. Environmental Protection Agency, Washington, DC.

U.S. E.P.A., 2002b, 'Methods for evaluating wetland condition: Using algae to assess environmental conditions in wetlands', Office of Water, U.S. Environmental Protection Agency, Washington, D.C.

Valente, T.M. & Gomes, C.L., 2007, The role of two acidophilic algae as ecological indicators of acid mine drainage sites, *Journal of Iberian Geology*, 33: 283-294.

Van Vuuren, S., Taylor, J.C., Gerber, A. & Van Ginkel, C., 2006, *Easy identification of the most common freshwater algae*, North-West University and Department of Water Affairs and Forestry, Pretoria.

Verb, R.G. & Vis, M.L., 2000, Comparison of benthic diatom assemblages from streams draining abandoned and reclaimed coal mines and nonimpacted sites, *Journal of the North American Benthological Society*, 19: 274-288.

Verb, R.G. & Vis, M.L., 2001, Macroalgal communities from an acid mine drainage impacted watershed, *Aquatic Botany*, 71: 93-107.

Verb, R.G. & Vis, M.L., 2005, Periphyton assemblages as bioindicators of mine-drainage in Unglaciated Western Allegheny Plateau lotic systems, *Water Air and Soil Pollution*, 161: 227-265.

Verb, R.G. & Vis, M.L., 2005, Periphyton assemblages as bioindicators of mine-drainage in unglaciated Western Allegheny Plateau lotic systems, Water, air, and soil pollution, 161: 227-265.

Verhoeven, J.T.A., Arheimer, B., Yin, C. & Hefting, M.M., 2006, Regional and global concerns over wetlands and water quality, *TRENDS in Ecology and Evolution*, 21: 96-103.

Wahlby, S.D. & El-Moneim, A.M., 1979, The problem of phosphorous in the eutrophic lake Maryût, *Estuarine and Coastal Marine Science*, 9: 615-622.

Wehr, J.D. & Sheath, R.D., 2001, Freshwater algae of North America, ecology and classification, pp. 775-804, Academic Press, San Diego, USA.

Whitton, B.A. & Diaz, 1981, Influence of environmental factors on photosynthetic species composition in highly acidic waters, *Verhandlungen – Internationale Vereinigung für Theoretische und Angewandte Limnologie*, 21: 1459-1465.

Whitton, B.A., 1970, Toxicity of zinc, copper and lead to Chlorophyta from flowing waters, *Archives of Mircobiology*, 72: 353-360.

Whitton, B.A., Say, P.J. & Wehr, J.D., 1981, 'Use of plants to monitor heavy metals in riviers', in: Say, P.J. & Whitton, B.A. (eds.), *Heavy Metals in Northern England: Environmental and Biological Aspects*, pp. 135-146, University of Durham, UK.

Zettler, L.A.A., Gómez, F., Zettler, E., Keenan, B.G., Amils, R. & Sogin, M.L., 2002, Eukaryotic diversity in Spain's river of fire, *Nature*, 417: 137.

Chapter 3: Bacterial population dynamics

3.1 Introduction

Within South Africa, water has become an important resource, as South Africa is a "chronically water stressed country with between 500 m^3 and 1000 m^3 of water available per person per year" (Ashton, 2002; Turpie *et al*., 2008). One of the greatest threats to potable water supply is mining and according to the Environmental Mining Council of British Columbia (2001), water has been described as "mining's most common casualty". Mining is a great consumer, diverter of and seriously polluter of water resources (Miller, 1999). Irresponsible mining practices can result in reduced quantity of water of acceptable quality for future generations (Ochieng *et al*., 2010). One of the long term consequences of mining activity is acid mine drainage (AMD). There is over 10 000 km^2 of hydraulically interlinked coal mines and over 300 km of interlinked gold mines within South Africa (Vureen, 2009). In 1997, South Africa was dependent on coal for 68% of its energy needs (Energy Information Administration, 2000), this number has increased in recent years. Decant from disused and closed coal mines has been estimated to be at 62 mℓ/d (DWAF, 2004). The impact of AMD in the Highveld coalfield in Mpumalanga has had severe implications for Loskop Dam and the Olifants River catchment (Naiker *et al*., 2003). The four major consequences of AMD were identified by Ochieng *et al*. (2010) to be 1) depletion of aquatic life; 2) contamination of the food chain; 3) contamination of drinking water and 4) deterioration of ecosystems. The impact of AMD on South African water resources has been said to be more severe due to the combination of unique climate, geography and population distribution (McCarthy, 2011). These impacts may be further exacerbated by a historic lack of policy addressing mining impact on the environment (Hobbs *et al*., 2008).

AMD is a result of the oxidation of sulphide-bearing rocks due to exposure to oxygen and leads to acid generation and metal dissolution. Most commonly AMD is produced by the weathering of pyrite, during which oxidation occurs. Ferrous iron is oxidized to ferric iron and pyrite is oxidized to produce sulphate and acid (Vermeulen & Usher, 2006). The latter is also responsible for the precipitation of iron oxyhydroxides (Druschel *et al*., 2004). Although AMD is perceived as a passive process, it is highly microbially mediated (Edwards *et al*., 1999). Iron oxidizing chemolithotrophs increase the rate of oxidation (Singer & Stumm, 1970) during the rate limiting step of oxidation of ferrous to ferric iron. Bacterial species implicated in mediating this step include *Acidothiobacillus ferrooxidans* and *Leptospirillium*

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ferrooxidans (Edwards *et al*., 1999; Kelly & Wood, 2000; Brofft *et al*., 2002; Baker & Banfield, 2003; Bruneel *et al*., 2006).   Aerobic, neutrophillic iron oxidation has been documented in various environments, including stream sediments, groundwater iron seeps, wetland surface sediments and plant rhizospheres (Emerson & Weiss, 2004). Phototrophic iron oxidizers have also been identified in anoxic environments (Widdel *et al*., 1993). Bacterial species capable of phototrophic iron oxidation are *Chlorobium ferrooxidans*, *Rhodovulum robiginosum*, *Rhodomicrobium vannielii*, *Thiodictyon* sp., *Rhodopseudomonas palustris* and *Rhodovulum* spp. (Weber *et al*., 2006a). Circumneutral, light-independent iron oxidation coupled to nitrate reduction have also been identified (Straub *et al*., 1996; Straub *et al*., 1998; Weber *et al*., 2001). The latter is an important process within wetlands, supporting abundant microbial communities within the sediment (Kluber & Conrad, 1998; Ratering & Schnell, 2001). Organisms identified to be capable of nitrate dependent iron oxidation are *Thiobacillus denitrificans* (Beller, 2005), *Geobacter* sp. (Weber *et al*., 2006b) and *Geobacter metallireducens* (Finneran *et al*., 2002). Bacteria are also capable of reducing iron. Iron oxides (i.e. ferrihydrite) occur predominantly in crystalline phase or as a component of clays. Iron reducing bacteria have been described as being "on the energetic edge" (Weber *et al*., 2006a) by utilizing these iron resources as an electron acceptor (Kostka & Nealson, 1995; Kostka *et al*., 1996; Fredrickson *et al*., 1998).

Wetlands are sensitive ecosystems (Janssen *et al*., 2005) where water is the primary determinant influencing both the aquatic life and riparian plant growth it supports (Zedler & Kercher, 2005). These unique ecosystems are vulnerable to the detrimental effects of AMD, which will lead to lowered primary producer diversity (Bond *et al*., 2000; Niyogi *et al*., 2002; Baker & Banfield, 2003; Druschel *et al*., 2004; Baker *et al*., 2009). Wetlands are one of four major natural methane sinks, which account to 70% of global emissions (Ritchie *et al*., 1997). Wetlands are unique ecosystems which can support diverse microbial metabolic activities. Methane has also been regarded as one of the most significant greenhouse gasses, accounting for up to 30% of the warming effect (IPCC, 2007). Bacteria within these natural sinks form an integral part of global methane cycling. Methanogenesis (methane production) by microorganisms occurs through anaerobic respiration by *Archaea*.   These *archaeal* methanogens is responsible for the last step in organic matter degradation and therefore relies on the presence of a microbial consortium capable of hydrolytic, fermentative and acetogenic metabolic capabilities (Cicerone & Oremland, 1988). The syntrophic bacterial populations are responsible for the degradation of simple sugars and fatty acids, followed by fermentation

to acetate, formate, methanol, methylamines, H_2 and CO_2 (Ritchie *et al*., 1997; Nazaries *et al*., 2013). Microbial reduction of iron-oxide may suppress methanogenesis in both freshwater surface sediments (Achtnich *et al*., 1995; Roden & Wetzel, 1996) and the rhizosphere of riparian plants (Roden & Wetzel, 1996; van der Nat & Middelburg, 1998; Frenzel *et al*., 1999). Methanotrophic (methane oxidizing) bacteria grow on methane or methanol as sole carbon source as well as energy source (Hanson, *et al*., 1992). These organisms are found at the oxic-anoxic interface in environments such as wetlands and paddy soils (King, 1990; Reim *et al*., 2012) and may use either sulphate (Knittel & Boetius, 2009), manganese/iron (Beal *et al*., 2009) or nitrite/nitrate (Raghoebarsing *et al*., 2006; Haroon *et al*., 2013) as electron acceptors. They are responsible for lowering environmental methane emissions (Conrad & Rothfuss, 1991; Conrad, 1996; Holmes *et al*., 1999; Henckel *et al*., 2000).

Nitrogen is considered to be the most limiting nutrient in ecosystems (Whitford, 1992). Bacteria play a central part in the cycling of nitrogen. Bacterial nitrification cycles nitrogen through the oxidation of ammonia to nitrate by ammonia- and nitrate oxidizers (Nicol & Schleper, 2006). These organisms have previously been used as bioindicators (Kowalchuk & Stephen, 2001). Freshwater wetlands are also important habitats for denitrification, through which downstream nitrogen is mitigated, as well as dissimilatory nitrate reduction (Fisher & Acreman, 2004; Ma & Aelion, 2005; Erler *et al*., 2008). During these processes, nitrate is used as terminal electron acceptor for anaerobic respiration to N_2 and N_2O (denitrification) or ammonia (dissimilatory nitrate reduction). Denitrification is highly dependent on the type and the quantity of carbon available (Krauter *et al*., 2005). Studies have also demonstrated that anaerobic ammonium oxidation (anammox), at some sites, may convert nitrate and ammonium to N_2 gas (Dalsgaard *et al*., 2003; Kuypers *et al*., 2003; Trimmer *et al*., 2005). Dinitrogen fixation is responsible for the conversion of the most abundant and inert form of nitrogen, into a biologically available form (Karl *et al*., 1997). During nitrogen-fixation, atmospheric gaseous nitrogen is reduced to ammonia and nitrate to alleviate low levels in the environment that may be limiting to plant growth. The bacteria involved are both free-living species and rhizobium species. A balance in the nitrogen cycle is important for the survival of macrophytes (Findlay *et al*., 2002).

Oxidation of inorganic sulphur compounds by chemolithotrophic bacteria have been widely studied, from as early as 1887 (Winogradsky, 1887). These microorganisms play an

important role in sulphur cycling, where most candidates fall within the genus *Thiobacillus*, deriving energy from oxidizing reduced sulphur compounds (Kuenen *et al*., 1992). Metabolites utilized by these bacteria are thiosulphate, tetrathionate and sulphite (Visser *et al*., 1997). Through the oxidation of sulphide minerals, sulphide oxidizers contribute to AMD production (Tang *et al*., 2009). Sulphate reducing bacteria (SRB) utilize inorganic sulphate as electron acceptor producing hydrogen sulphide (Rabus *et al*., 2006; Muyzer & Stams, 2008). Sulphate reducing bacteria aid the complete degradation of organic matter as well as sulphide production and/or metal reduction (Barton & Fauque, 2009). These bacteria may grow either heterotrophic, autotrophic or lithoautotrophic (Cypionka, 2000; Fauque & Ollivier, 2004). Sulphate reducing bacteria are able to reduce the highest number of electron acceptors in addition to inorganic sulphur compounds (LeGall & Fauque, 1988; Fauque *et al*., 1991; Fauque, 1995; Fauque & Ollivier, 2004; Rabus *et al*., 2006; Muyzer & Stams, 2008).

The cycling of carbon, nitrogen and sulphur in wetlands are closely tied to bacterial activity and remediation within these ecosystems (Faulwetter *et al*., 2009). By growing our understanding of bacterial communities within complex environments such as wetlands, efforts can be made to use these natural populations for bioremediation. Bacterial population studies have broaden the knowledge base on natural wetland soil communities (Sims *et al*., 2012; Yu *et al*., 2012; Peralta *et al*., 2013) as well as how communities react to environmental change (Hartman *et al*., 2008; Peralta *et al*., 2013; Ansola *et al*., 2014). Bartman *et al*. (2014) found that soil pH is one of the main predictors of community composition. When considering nutrient cycling by bacterial metabolism, substrate competition is a common phenomenon within communities, such as the case with methanogens and SRB. Hydrogen and acetate are vital to methane production however these precursors also serve as electron donors for sulphate reduction (McCarthy & Oleszkiewicz, 1993). Ammonia oxidizing bacteria have also been reported to be "extremely sensitive" to soluble sulphide under aerobic conditions (Sears *et al*., 2004). Heterotrophic nitrate reducing bacteria will also out-compete SRB as nitrate reduction is more thermodynamically favoured (Eckford & Fedorack, 2002). Additionally, the presence of oxidized forms of Fe and Mn may also limit sulphate reduction (Stein *et al*., 2007). Symbiotic metabolic cycling has also been reported. Anaerobic methane oxidation coupled to nitrite/nitrate reduction have been detected in freshwater wetlands (Raghoebarsing *et al*., 2006; Ettwig *et al*., 2010; Zhu *et al.,* 2010; Shen *et al*., 2015), while studies of bacterial population dynamics have mainly concentrated on sole carbon-source profiling (Osam *et al*., 2007; Deng *et al*., 2011),

extracellular enzyme activities (Wobus *et al*., 2003) and potential respiration (Truu *et al*., 2005). Only a few studies have assessed potential metabolic activities from 16S rRNA taxonomic profiling (Arroyo *et al*., 2015).

This study aimed at providing insight into microbial population dynamics within an AMD impacted wetland before rehabilitation. This was done to shed light on the complex interactions within a wetland ecosystem, even at primary producer level. The knowledge gained was then applied to develop a microbial bioindicator for ecosystem health assessment along with indices already used to improve insight provided. Firstly, a culture-independent identification was carried out on the populations at each site for both the water and sediment columns. These results indicated population size and diversity as well as metabolic capabilities. A possible microbial bioindicator was then described according to the results from this study and further adapted to be used with an already existing index for ecosystem health evaluation.

3.2 Materials and methods

3.2.1 Study site

The study sites were selected and described as discussed in Chapter 2. The physical and chemical parameters were determined discussed in Chapter 2.

3.2.2 Sample collection and processing

Sampling was conducted once a month for three months, between January 2013 and May 2013. Water samples were taken in 2 L sterile polyethylene bottles using the scoop-method. Sediment core samples for microbial analysis were taken with a modified Shelby tube sampler from the submerged banks. A sediment core of ~20 cm was taken and the top 10 cm was halved (Selala *et al*., 2013a). This was done to separate the oxic and the anoxic levels. The core sampler was sterilized between sampling.

Samples for microbial DNA extraction were kept on ice and transported to the laboratory within 24 h. Two litres of the water samples were filtered through a 0.45 μm cellulose nitrate membrane (Sartorius Stedium). The debris on the filter was aseptically scraped off using a sterile glass hockey stick and resuspended in 1X PBS solution (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 KH_2PO_4) at pH 7.4. The suspension was then centrifuged at 13 000

rpm to pellet the bacterial cells. DNA was then extracted from the pellet using the DNAeasy Blood and Tissue kit (Qiagen) according the manufacturer's protocol. Sediment microbial genomic DNA was extracted from the top 5 cm of the core sample using the ZR Soil Microbe DNA MiniPrep Kit (Zymo Research, California, USA). The protocol was adapted by using an initial sediment weight of 1.2 g to increase DNA yield.

Genomic DNA samples were sent to Inqaba Biotech™ (Pretoria, South Africa) for Roche 454 pyrosequencing using primer sets 27F and 518R for 16S rRNA universal primers (V1, V2 and V3 regions) (Weisberg *et al*., 1991) prepared using the Titanium emPCR library preparation kit (Lib-A) (Roche Applied Science) on the GS Junior platform according to the manufacturers guidelines. Sequencing data was analysed using Mothur version 1.33.3 (Schloss *et al*., 2009) using the 454 pyrosequencing standard operating procedure (454 SOP) pipeline as derived from Schloss *et al*. (2011). Some adjustments were made to accommodate the data files received, setting the minimum for the flowgrams to 300 and maximum to 720 in order to maximise flowgram length. The sequencing files were merged for each analysis run, as to unpack, denoise and trim the sequences and produce a single file output for downstream analysis. Subsequent alpha and beta diversity measurements were done as recommended by the 454 SOP. Profile bar plots were drawn to illustrate phylum differences between the two sampling events using the STAMP software (http://kiwi.cs.dal.ca/Software/STAMP) (Parks & Beiko, 2010). Default statistical parameters were chosen: the G-test (with and without Yate's continuity correction) plus Fisher's exact test and Storey FDR multiple test correction. The confidence interval method used was the DP (difference between proportions) asymptotic with CC (continuity correction) (0.95) as recommended as default selection (Parks & Beiko, 2010). Multivariate analysis was performed to summarize the variation in species composition and interpret it using physical and chemical parameters measured during this study, using the XLSTAT (trial) software add-in for Microsoft Excel. The first 50 OTUs were used for the analysis.

3.3 Results

3.3.1 Physical and chemical properties of the water column and sediment samples.

Physiochemical properties of the water samples were as discussed in Chapter 2. The sediment chemistry differed from that of the water column samples. At all five sites, the total Al was high, up to 36380.00 ± 25746.78 mg/kg at site 4 (Table 1). The Fe content of sites 2

(11325.67 ± 5524.30 mg/kg) to 5 (10353.33 ± 1769.67 mg/kg) was high (Table 1). Manganese was the highest at sites 3 (639.00 ± 375.66 mg/kg) and 4 (947.00 ± 72.55 mg/kg) (table 4). The total carbon and organic carbon was the lowest at site 5 (2.11 ± 2.09 & 2.02 ± 2.02 %) (Table 1).

3.3.2 Bacterial population dynamics

A total of 10 363 sequences were obtained from the analysis of the 20 samples. The number of reads obtained per sample ranged from 92 to 2010, with an average of 518 reads per sample. Alpha-diversity was calculated using the Mothur software (Table 2). The highest number of sequences in January was obtained in the water sample from site 4 and the sediment samples of site 4. For March the highest number of sequences was for the water sample from site 1 and sediment sample from site 3. The observed species diversity were the highest at sites 4 (January) and 1 (March) for the water samples and sites 5 (January) and site 3 (March) for the sediment samples. The highest species diversity was calculated for the water samples at sites 1 (January) and 3 (March). The highest species diversity for the sediment samples were at sites 5 (January) and 4 (March).

In all of the samples, the dominant phylum was *Proteobacteria* as well as unclassified sequences (Figure 1 & 2). The other phyla identified had a low overall representation (Figure 1 & 2). Profile bar plots revealed how the populations compared over the two sampling events for each site (Appendix A, Figure 1 & 2). For the January water samples, these were unclassified bacteria, *Acidobacteria*, *Actinobacteria*, *Planctomycetes*, *Verrucomicrobia* and *Chloroflexi* at site 1; *Proteobacteria*, *Firmicute*s and unclassified bacteria at site 2; unclassified bacteria, *Actinobacteria* and *Chloroflexi* at site 3; unclassified bacteria, *Proteobacteria*, *Chloroflexi*, *Actinobacteria*, *Firmicutes* and *Acidobacteria* at site 4; and unclassified bacteria, *Chloroflexi*, *Acidobacteria*, *Actinobacteria*, *Firmicute*s and Nitrospira at site 5.

Table 1. Chemical analysis of sediment samples from the different sites within the wetland measured from January to May 2013 (n=3). Site 1 acted as the reference site.

Criteria	Site 1	Site 2	Site 3	Site 4	Site 5
Al (mg/kg)	18872.3 ± 10587.3	32783.3 ± 9530.7	25957.3 ± 22105.1	36380.0 ± 25746.8	14993.3 ± 7011.92
Ni (mg/kg)	11.2 ± 6.8	10.7 ± 4.1	21.3 ± 10.5	26.7 ± 18.6	10.2 ± 2.66
Si (µg/kg)	915.0 ± 721.0	1558.3 ± 1339.9	1449.0 ± 1163.4	2083.0 ± 1945. 6	3232.7 ± 3814.65
% C	4.5 ± 5.8	3.2 ± 0.4	3.5 ± 1.7	4.6 ± 2.1	2.1 ± 2.09
% Organic C	2.9 ± 3.7	2.6 ± 0.3	2.7 ± 1.5	3.3 ± 1.4	2.0 ± 2.02
B (mg/kg)	ND	4.0 ± 2.7	3.0 ± 2.8	3.7 ± 1.5	2.0 ± 0.58
Cu (mg/kg)	11.1 ± 8.4	15.2 ± 5.7	30.0 ± 23.8	36.0 ± 26.9	7.7 ± 0.89
Fe (mg/kg)	4710.0 ± 3549.9	11325.7 ± 5524.3	34913.0 ± 15242.2	41888.0 ± 5983.3	10353.3 ± 1769.67
Pb (mg/kg)	7.0 ± 2.8	8.2 ± 1.9	8.1 ± 1.4	9.7 ± 1.4	5.9 ± 0.64
Mn (mg/kg)	56.7 ± 68.1	30.3 ± 15.6	639.0 ± 375.7	947.0 ± 72.6	73.7 ± 25.66
V (mg/kg)	42.3 ± 40.5	40.0 ± 12.3	106.7 ± 56.1	154.0 ± 25.9	29.3 ± 3.21
Zn (mg/kg)	17.0 ± 13.1	14.8 ± 5.4	35.7 ± 19.9	48.7 ± 8.5	12.7 ± 2.89

ND -   Concentrations measured were under detection limit.

The phyla in the March water samples at significant numbers were *Proteobacteria* and *Bacteriodetes* at site 1; *Actinobacteria* and *Bacteriodetes* at site 2; *Proteobacteria*, *Firmicute*s, *Bacteriodetes* and *Fusobacteria* at site 3; *Bacteriodetes* at site 4; and *Proteobacteria*, *Bacteriodetes* and *Fusobacteria* at site 5. In the sediment samples for January at site 1, *Proteobacteria*, unclassified bacteria and *Acidobacteria* were at significantly higher numbers; at site 2 *Actinobacteria*; at site 4 unclassified bacteria, *Verrucomicrobia*, *Firmicute*s and *Acidobacteria*; and at site 5 unclassified bacteria, *Acidobacteria*, *Chloroflexi* and Nitrospira. Lastly in the March sediment samples, at site 1 it was *Actinobacteria* and *Bacteriodetes*; site 2 unclassified bacteria; site 3 *Proteobacteria* and unclassified; site 4 *Proteobacteria*, *Bacteriodetes* and *Actinobacteria*; and at site 5 *Proteobacteria*, *Firmicute*s and candidate phylum TM7.

Table 2. Population size and diversity (n=2) for the water and sediment bacterial samples[*]. The number of sequences (Nseqs), number of observed species (Sobs), the Chao 1 estimator as well as the inverse Simpson index (InvS) was calculated using the Mothur software.

Water		Nseqs[a]	Sobs[b]	Chao	InvS	**Sediment**	Nseqs[a]	Sobs[b]	Chao	InvS
	1	464	419	2954.7	1820.6		388	291	1096.8	342.82
	2	583	147	261.2	11.02		126	34	51.14	15.2
January	**3**	475	164	689.2	7.84		149	33	57	8.85
	4	2010	1309	4033.5	185.9		594	425	1853	119.7
	5	778	507	1687.3	61.82		477	434	4368.3	1555.2
	1	552	112	230.33	3.99		204	140	765.3	31.37
	2	249	69	184	7.16		92	46	120.4	8.07
March	**3**	549	184	485	10.71		907	621	2730.9	316.3
	4	418	81	417	3.68		369	307	1559.2	556.5
	5	522	120	343.1	4.66		457	382	3689.1	321.6

a Number of individual sequences

b Number of observed species

* Data was only available for two sampling events, due to limited funds the third sample set could not be processed.

3.3.3 Multivariate analyses

Multivariate analyses of community structure and diversity patterns summarized the variation in the species composition in relation to the environmental variables. The multivariate analysis of the bacterial OTUs was used to elucidate the relationship between the environmental conditions and the community composition. The PCA plot of the January 2013 water samples (Appendix A, Figure 3A) showed a positive correlation of the metals Al, Cu and Fe with the bacterial OTUs: 4;

18; 21; 28; 29; 31 & 43. The metals Pb, Zn, Ni and Mn correlated positively with electrical conductivity (EC), total dissolved solids (TDS) and sulphate. The bacterial OUT 32 correlated with these environmental conditions. Suspended chlorophyll-*a* correlated positively with OTUs: 5; 6; 9; 12; 16; 23; 25 & 50. Turbidity and B correlated positively with OTUs: 7; 8; 10; 11; 17; 19; 20; 24; 27; 33; 34; 39; 41; 42 & 44-49. The PCA plot for the January 2013 sediment (Appendix A, Figure 3B) showed a positive correlation between total carbon and total organic carbon. Al, Pb, Ni, Cu and Zn were positively correlated as well as V, Mn, Fe and B with bacterial OTUs: 3; 5; 15; 19; 23; 26; 28; 34-36; 43 & 45-47. Redox potential and Si were positively correlated and Si was in turn with the bacterial OTUs: 1; 2; 4; 6; 7; 11; 13; 14; 16; 17; 25 & 37-41.

The March 2013 water PCA plot (Appendix A, Figure 3C) revealed a positive correlation between sulphates, Mn, Ni, EC, TDS, dissolved oxygen (D.O$_2$) and Zn. The latter and the bacterial OTUs: 2; 12; 14; 16; 23; 24; 31; 37; 39; 45 & 46 correlated positively. Fe and Pb correlated with the OTUs: 7; 11; 25 & 30. Dissolved organic carbon and suspended chlorophyll-*a* with the OTUs: 18; 27; 29; 33; 38 & 47. The COD correlated with OTU 1 & 6 and pH with OUT 3. Lastly, the multivariate analysis for March 2013 sediment samples (Appendix A, Figure3D) showed that Cu and Ni positively correlated with each other and a vast amount of bacterial OTUs: 3-5; 8-11; 13; 15; 17-22; 24; 27; 28; 32; 34; 36-38; 40; 43-45 & 47-50. The metals Cu, Ni, Al, Fe, Zn; Mn and V correlated positively and Si correlated with OTUs: 7; 12; 16 & 29.

3.4 Discussion

3.4.1 Phylum-level taxonomic distribution and bacterial diversity.

Bacteria are ubiquitous organisms that also thrive in the harshest environments. There are many parameters apart from standard physiochemical, which influence their growth and proliferation. Clean water environments are thought to have low concentrations of organisms. On the other hand sediment environments are rich in large bacterial populations with high species diversity (Atlas & Bartha, 1998). Bacterial populations play vital roles in the decomposition of organic matter, remineralisation of nutrients and the biogeochemical cycling within the environments they occur (Zhang *et al*., 2014). Bacterial diversity was at its highest in the water samples of site 1 (January 2013) and site 5 (March 2013) (Table 2). This was attributed to the lower concentrations of metals and sulphate (Chapter 2, Table 3) at these two sites as well as

circumneutral pH (Chapter 2, Table 3) measured at the sites. The improvement in water quality downstream in the wetland could also be attributed to the wetland's natural remediation capabilities (Stern *et al*., 2001). Decreased bacterial diversity in the water samples were found at the sites impacted by the AMD (sites 2 and 4) (Table 2). The low diversity for the water bacterial population at site 3 (Table 2) may be attributed to high water flow rate at this site as well as high sulphate concentration and low DOC concentration (Chapter 2, Table 3). The sediment bacterial populations had overall, low diversity (table 2). However, there was an increase in diversity for the March 2013 populations for sites 3-5 (Table 2). This increase could be caused by increased carbon concentrations at site 4, as well as increased metal concentrations (Table 1), thus supporting the growth of ubiquitous species as well as species adapted to high metal concentrations.

The *Chloroflexi* phylum, which represents most of the green non-sulfur anoxygenic photosynthetic bacteria, were identified in the water samples of site 4 & 5 (January 2013) (Figure 1) at high numbers compared to the other sites. These bacteria mainly grow photoheterotrophically, but are also capable of photoautotrophy, using H_2S and H_2 as electron donors (Prescott *et al*., 2002). These bacteria were also detected in the sediment samples of   site 5 (January 2013) and site 3 (March 2013) (Figure 2) in increased numbers compared to the other sites investigated. This phylum has previously been identified in constructed wetlands (Arroya *et al*., 2013), wetland soil (Ligi *et al*., 2014), reservoir sediment (Röske *et al*., 2012) and in the oxic-anoxic zone in an acidic peat bog (Dedysh *et al*., 2006). The *Firmicutes* phylum, detected at various levels in both the water column and sediment has been reported to occur during phytoplankton blooms. Two studies obtained different results, Riemann & Winding (2001) reported that *Firmicutes* occurred during the first 5-8 days (peak) of a bloom and were free-living. Whereas van Hannen *et al*. (1999) reported these organisms during the post-bloom phase of their study. *Firmicutes*, during the current study, were only associated during the March 2013 sampling at site 3, at increased numbers in the water samples (Figure 1) compared to the other sites sampled, where high numbers of filamentous algae were present (Chapter 2). *Planctomycetes* were not detected in high numbers although elevated numbers were present in the site 3 (March 2013) sediment samples (Figure 2). This phylum contains species capable of performing anammox (anaerobic ammonium oxidation) metabolism (Strous *et al*., 1999; Prescott *et al*., 2002) and have been identified in various freshwater aquatic (Schubert *et al*., 2006; Newton *et al*., 2011), sediment (Zhang *et al*., 2007) environments as well as an acidic bog lake

(Dedysh *et al*., 2006). However *Planctomycetes* are underrepresented in 16S rRNA gene clone libraries and thus may be more abundant than currently reported (Vergin *et al*., 1998).

Anammox species play a vital role in global nitrogen cycling (Hu *et al*., 2011). *Verrucomicrobia* were only identified in low numbers throughout the study, with the exception of the sediment samples of site 4 & 5 (January 2013) and site 3 (March 2013) (Figure 2). Species from this phylum are ubiquitous in both aquatic and terrestrial environments, yet understudied in soil environments (Arnds *et al*., 2010; Bergmann *et al*., 2011; Freitas *et al*., 2012; Liao *et al*., 2013ab; Shange *et al*., 2013). *Verrucomicrobia* have been detected in lakes, soils, oceans and human faeces as well as eukaryotic endo- and ectosymbionts (Wagner *et al*., 2006; Newton *et al*., 2011). Ligi *et al*. (2014) also detected *Verrucomicrobia* in low numbers in their wetland soil study. *Verrucomicrobia* was reported by de Figueiredo *et al*. (2007) in oligotrophic and low pH waters. The latter agrees with this study's findings. This phylum also occurs in extremely low pH, thermophillic environments (Semrau *et al*., 2008) and degrades phytoplankton-derived carbohydrates (Rabus *et al*., 2002). Lastly, *Bacteriodetes*' numbers increased during March 2013 for all the samples compared to the January 2013 results (Figure 1 & 2). The phylum *Bacteriodetes* comprise of chemoorganotrophs (Gómez-Consarnau *et al*., 2007; González *et al*., 2008), who may dominate in freshwater lakes (Pernthaler *et al*., 2004). *Bacteriodetes* species occur as particle-associated (Nold & Zwart, 1998; Lemarchand *et al*., 2006) bacteria, preferring increased DOC concentrations or algal-derived DOC (Eiler & Bertilsson, 2004; Kolmonen *et al*., 2004; Eiler & Bertilsson, 2007; Zeder *et al*., 2009).
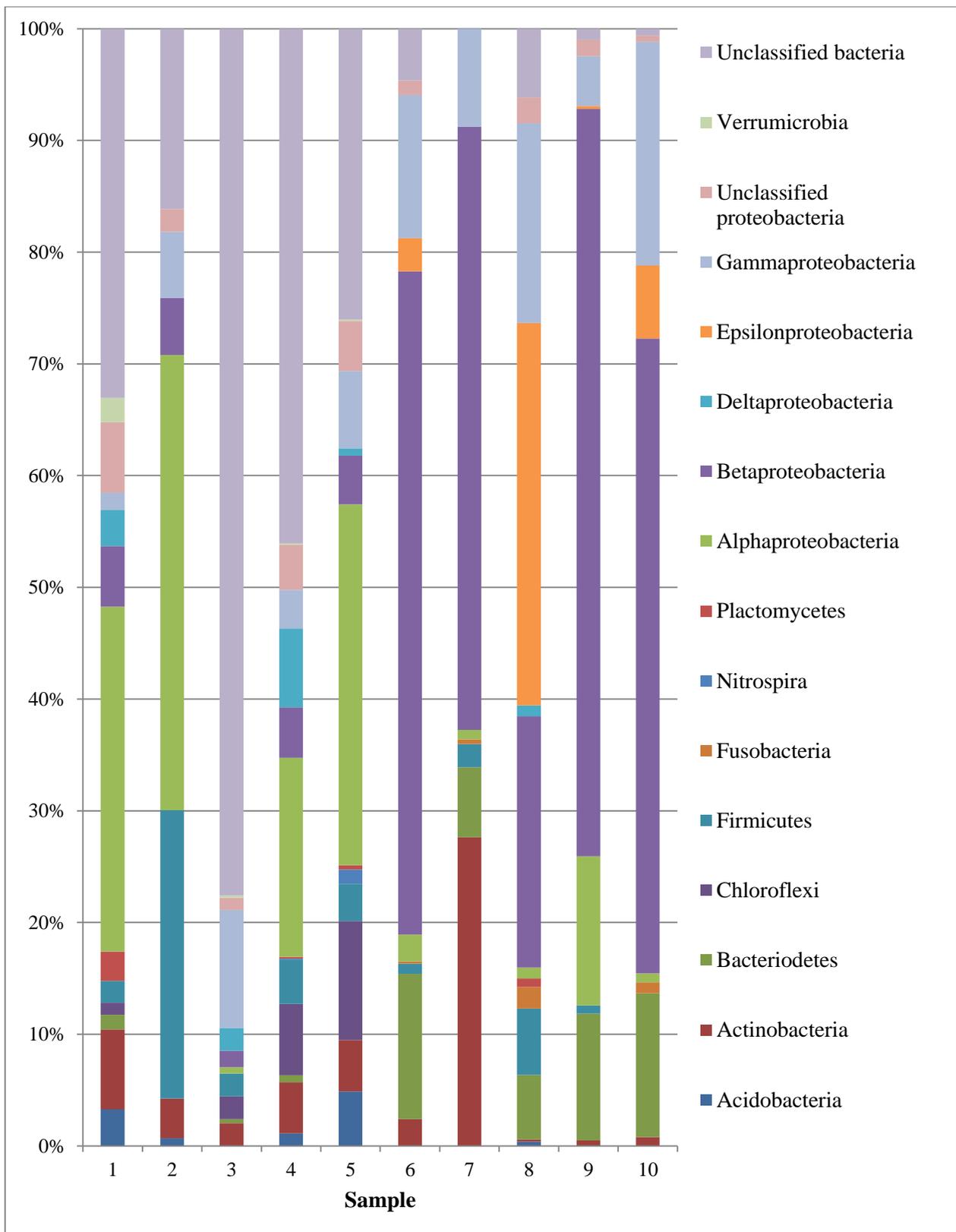
Figure 1. Relative abundance of the most abundant bacterial phyla detected, from 16S rRNA sequencing of the water samples from site 1 -5, for the samplings of January to March 2013 (n=2).
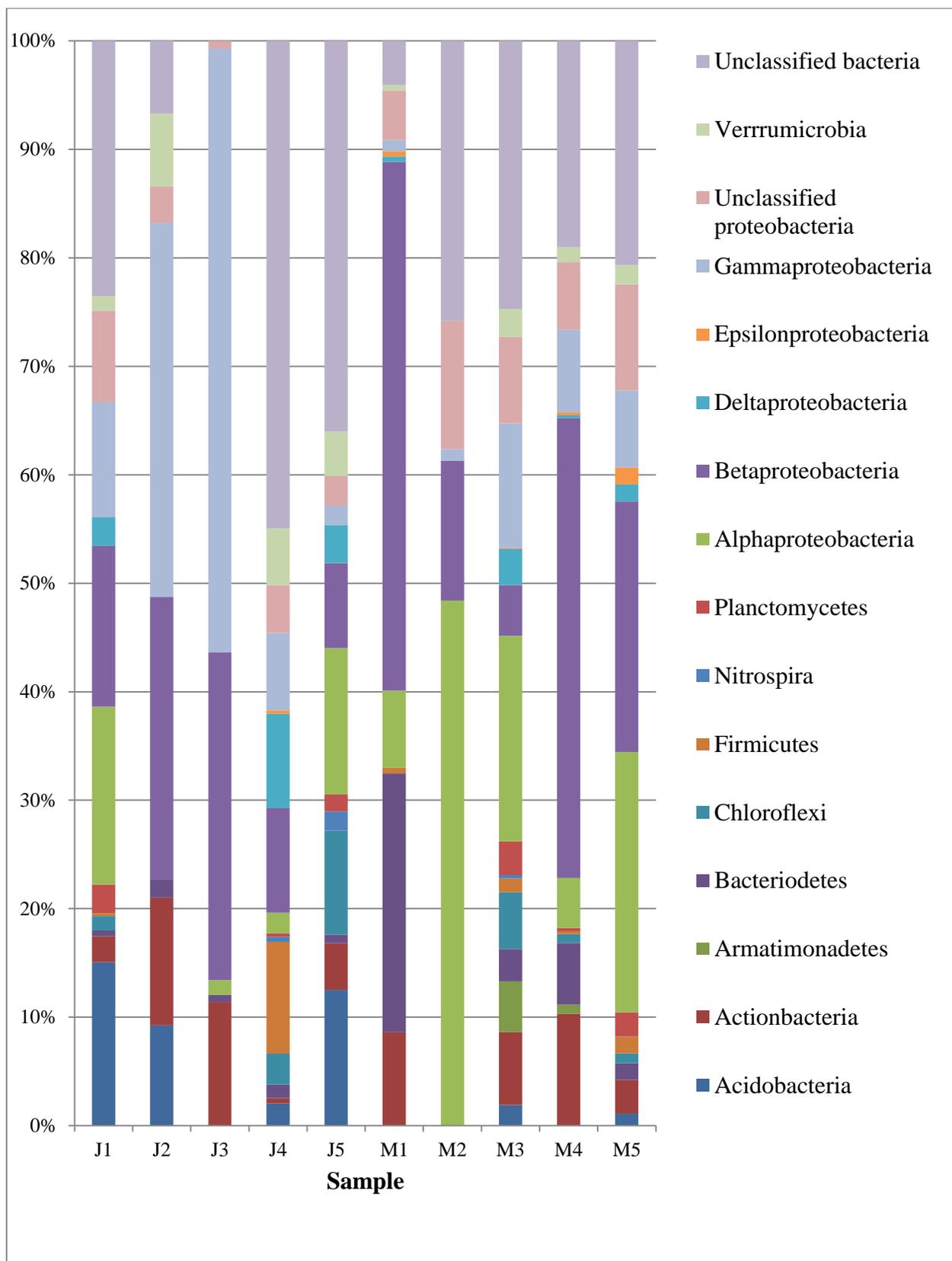
Figure 2. Relative abundance of the most abundant bacterial phyla detected, from 16S rRNA sequencing of the sediment samples from site 1 -5, for the samplings of January to March 2013 (n=2).

Acidobacteria, *Actinobacteria* and *Proteobacteria* are the more dominant phyla in aquatic and sediment environments compared to other phyla (Dedysh *et al*., 2006; Baik *et al*., 2008; Newton *et al*., 2011; Röske *et al*., 2012; Arroyo *et al*., 2013; Peralta *et al*., 2013; Shange *et al*., 2013; Ligi *et al*., 2014). These phyla have also been found in aerobic soils (Lauber *et al*., 2009; Nacke *et al*., 2011) and lower proportions of *Acidobacteria* and *Actinobacteria* in waterlogged soil (Ligi *et al*., 2014). *Acidobacteria* have historically been associated mainly with soil environments (Goodfellow & Williams, 1983; Janssen, 2006; Empadinhas *et al*., 2011; Mendes *et al*., 2011; Shange *et al*., 2013), yet through advancement of detection methods, *Acidobacteria* were more common in a variety of freshwater environments than previously reported (Hiorns *et al*., 1997; Denisova *et al*., 1999; Glöckner *et al*., 2000; Warnecke *et al*., 2005; Buck *et al*., 2009; Perez *et al*., 2010). Bacteria from the phylum *Acidobacteria* were identified in various samples from this study, at site 1, 4 & 5 (January 2013) and site 2 (March 2013) (Figure 1) in the water samples at higher numbers compared to the other samples. For the sediment samples, the highest numbers were at site 5 (January 2013) and site 3 & 4 (March 2013) (Figure 2), compared to the other sites. The bacteria from this phylum are responsible for carbon cycling and the breakdown of organic matter (Gardner *et al*., 2011; Nacke *et al*., 2011). The phylum *Acidobacteria* harbour bacterial species such as *Geothrix fermentans*, which are anaerobic chemoorganotrophs, utilizing Fe(III) as an electron acceptor (Coates *et al*., 1999). This phylum was dominant in wetland soil due to a preference for reduced oxygen concentrations (Jones *et al*., 2009; Dedysh, 2011; Shange *et al*., 2013), where they significantly correlated with pH, increasing in abundance where the pH decreases below 5.5 (Wang *et al*., 2012). *Acidobacteria* was reported by Dedysh *et al*. (2006) to be dominant in the oxic-anoxic zone in an acidic peat bog. Species from the phylum *Acidobacteria* were enumerated at higher numbers in the water samples at site 4 & 5 (January 2013) (Figure 1) and in the sediment samples at site 1 & 5 (January 2013) compared to the other sites.

The *Proteobacteria* phylum is widely dominant in both water and sediment environments (Dedysh *et al*., 2006; Gorra *et al*., 2007; Röske *et al*., 2012; Arroyo *et al*., 2013; Peralta *et al*., 2013; Shange *et al*., 2013; Ligi *et al*., 2014). *Proteobacteria* play an important role in the biodegradation and biogeochemical processes in aquatic ecosystems (Cheng *et al*., 2013). This phylum can be subdivided into classes. One of the subclasses is the *Alphaproteobacteria*, oligotrophic species, some of which are capable of unusual metabolism, such as methylotrophy, chemolithotrophy, nitrite oxidation and nitrogen

fixation. This class also includes the purple non-sulphur bacteria that perform anaerobic photoorganoheterotrophy or facultative photolithotrophy, using organic, reduced sulphur or H_2 as electron donors (Prescott *et al*., 2002). This class of *Proteobacteria* have been called "the hub of global nitrogen cycle" and are ubiquitous in many environments (Newton *et al*., 2011). *Alphaproteobacteria* are known to inhabit mud, water with high concentrations of organic material and low sulphide (Prescott *et al*., 2002). Within soil environments, species from this class have been reported to respond similarly to *Acidobacteria* to oligotrophic environments (Jangid *et al*., 2008; Nacke *et al*., 2011; Shange *et al*., 2012). Environmental pH and nutrient availability is major determinants for the dominance of this class, along with that of *Betaproteobacteria* (Newton *et al*., 2011). Two studies reported that this class of *Proteobacteria* are competitive under nutrient restricted conditions (Eiler *et al*., 2003; Pinhassi *et al*., 2003) and their numbers (as a class) increase as microeukaryotic grazing increases (Šimek *et al*., 1999; Jürgens & Jeppese, 2000; Langenheder & Jürgens, 2001; Salcher *et al*., 2005; Comte *et al*., 2006). The water samples contained very high numbers of *Alphaproteobacteria* at sites 1, 2, 4 & 5 (January 2013) and very high at site 4 (March 2013) (figure 2) compared to the other sites. This class was represented in high numbers at sites 1 & 5 (January 2013) and sites 3 & 5 (March 2013) in the sediment samples compared to the other sites investigated. These results agree with the findings by Kwon *et al*. (2011) that this class, along with the *Beta-* and *Gammaproteobacteria* occur predominately in freshwater habitats and have also been previously reported in a wetland (Dorador *et al*., 2013).

The class *Betaproteobacteria* are morphologically and physiologically diverse; species within this class may grow through chemoheterotrophy, photolithotrophy, methylotrophy or chemolithotrophy. This class of bacteria use substrates which have diffused from organic decomposition within anoxic zones (Prescott *et al*., 2002). *Betaproteobacteria* consist of ammonia oxidizers (Gorra *et al*., 2007), including the *Nitrosospira* sp. from soil environments (Koops & Harms, 1985), are dominant in both water and sediment environments (Hiorns *et al*., 1997; Glöckner *et al*., 2000; Wobus *et al*., 2003; Zwisler *et al*., 2003; Briee *et al*., 2007; Buck *et al*., 2009), and is best studied in freshwater environments. The latter agrees with the results obtained in this study for both the water (Figure 1) and sediment (Figure 1) bacterial populations. Species in this class have been co-cultured with algae (Pernthaler *et al*., 2001), associated with cyanobacteria (Eiler *et al*., 2006) or associated with particles (Weiss *et al*., 1996; Šimek *et al*., 1999; Lemarchand *et al*., 2006).

Deltaproteobacteria are the chemoorganotrophic, anaerobes found in mud, sediment and waterlogged soils. Well known species of this class include the sulphate reducing *Desulfovibrio* sp. and *Desulfotalea* sp. (Rabus *et al*., 2006) which occur in aquatic and waterlogged sediment environments (Madigan *et al*., 2000). However, this class also include metal reducing species such as *Geobacter* sp. and *Desulfuromonas* sp. that oxidises organic compounds, hydrogen or sulfur along with the reduction of Fe(III) oxides (Rodionov *et al*., 2004). These bacteria require sulphur as an electron acceptor in the oxidation of acetate (Brugna *et al*., 1999). Species from this class have been isolated from freshwater sediment previously (Spring *et al*., 2000; Briee *et al*., 2007). High numbers of the *Deltaproteobacteria* were detected in the water sample of site 4 (January 2013) (Figure 1) and sediment samples of site 4 (January 2013) and site 3 (March 2013) (Figure 2) compared to the other sites. Magnetotactic bacterial species are also part of this class, which have recently been isolated from the surface sediment of a freshwater moat (Wang *et al*., 2013). Previously these species were associated with marine sediments (Wenter *et al*., 2009), river estuaries (Bazylinski *et al*., 1993), alkaline environments (Lefèvre *et al*., 2011a) and seldom in freshwater lakes (Kawaguchi *et al*., 1995; Lefèvre *et al*., 2011b). These *Deltaproteobacteria* are active in the biogeochemical cycling of iron and sulphur (Lefèvre *et al*., 2011b). Some aerobic species of *Deltaproteobacteria* are able to digest other bacteria (Kuever *et al*., 2005).

The last class of the *Proteobacteria* to be discussed is the *Gammaproteobacteria*, a class containing the most studied bacterium, *Escherichia coli* (Newton *et al*., 2011). This group of bacteria are more abundant in saltwater lakes and marine environments than freshwater (Wu *et al*., 2006) and have been found dominate filamentous microbial mats in anoxic, sulphide rich cave springs (Prescott *et al*., 2002). In the water bacterial community, these organisms were more abundant at sites 3, 4 & 5 (January 2013) and sites 1, 3 & 5 (March 2013) (Figure 1) and in the sediment communities at sites 1-4 (January 2013) and sites 3-5 (March 2013) (Figure 2). These results were in accordance with previous studies where bacteria from this class was identified in various freshwater and sediment environments (Newton *et al*., 2011; Kwon *et al*., 2011; Röske *et al*., 2012; Dorador *et al*., 2013; Ligi *et al*., 2014). There were also several unclassified *Proteobacteria* as well as unclassified sequences from this study. The latter highlights that there is still little known about wetland environments (Zhang *et al*., 2014) and the bacterial populations which they support. The phylum-level taxonomic analysis revealed similar diversity patterns to that of previous studies on freshwater environments, sediments and wetlands (Wobus *et al*., 2003; Dedysh *et al*., 2006; Briee *et al*.,

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2007; Baik *et al*., 2008; Ye *et al*., 2009; Lauber *et al*., 2009; Nacke *et al*., 2011; Wang *et al*., 2012; Röske *et al*., 2012; Peralta *et al*., 2013; Shange *et al*., 2013; Arroyo *et al*., 2013; Ligi *et al*., 2014).

3.4.2 Linking wetland properties and bacterial community composition.

The multivariate analysis of the bacterial OTUs was used to evaluate the relationships between wetland physical-chemical conditions over the two sampling events and the bacterial community composition. Only the first 50 OTUs were used in the analysis as there were large numbers of OTUs with only a single representative in a sample. The patterns of the bacterial community distribution showed the shifts in the population composition as a function of environmental factors.

The population dynamics of the January 2013 water samples (Appendix A, Figure 3A) revealed that Al, Fe and Cu grouped together as metals commonly associated with AMD (Johnson & Thornton, 1987; Evangelou & Zhang, 1995; Olías *et al*., 2004; Johnson & Hallberg, 2005; Akcil & Koldas, 2006). Bacterial OTUs from this study most influenced by these metals included *Halomonas* sp., *Herbaspirillum* sp., *Paenibacillus* sp., *Delftia* sp. and unclassified organisms (Appendix B). These bacterial species are not extremophiles and thus are considered to be negatively impacted by these metals. The *Halomonas* sp. may be either halophillic or non-halophillic (Mata *et al*., 2002). Some moderately halophillic species have been described, which includes *H. campisalis* and *H. cerina* that are capable of denitrification (Mormile *et al*., 1999; Gonźalez-Domenech *et al*., 2008). *Herbaspirillum* spp. are nitrogen fixing endophytic diazotrophs (Baldani *et al*., 1986; Olivares *et al*., 1996; Elbeltagy *et al*., 2001). *Paenibacillus* spp. may be found in the rhizosphere where these organisms are involved in nitrogen cycling (Berge *et al*., 2002; Behrendt *et al*., 2010) and may promote plant growth (McSpadden Gardener, 2004). Adhesion onto pyrite has been reported by Sharma & Rao (2003) for *P. polymyxa* and *P. guangzhouensis* has been identified to be capable of Fe(III) reduction (Li *et al*., 2014). *Paenibacillus* spp. have also been isolated in wetlands (Baik *et al*., 2011ab). The trace metals Pb, Zn, Ni and Fe-associated Mn correlated with EC, TDS and sulphate (Appendix A, Figure 3A). Only Mn and Zn had elevated concentrations at sites 3 and 5 (Chapter 2, Table 3). The suspended chlorophyll-*a* concentrations correlated with unclassified bacteria (Appendix A, Figure 3A; Chapter 2, Table 3). Turbidity and B were also correlated with a majority of unclassified bacteria (Appendix A, Figure 3B; Appendix B).

The sediment populations had different patterns due to the vast difference in their habitat. Carbon and O.C. of the sediment samples (Appendix A, Figure 3B) were strongly associated according to the PCA analysis. The metals Al, Pb, Ni, Cu and Zn were also weakly correlated, as what is expected of an AMD environment. Vanadium, Mn, Fe and B were largely correlated with unclassified bacteria (Appendix A, Figure 3B; Appendix B), including those identified to be part of the candidate phylum OP11. There are no cultured isolates from this phylum. It was first identified in hot springs of the Yellowstone National Park and has subsequently been identified in both marine and freshwater environments (Ueda *et al*., 1995; Wise *et al*., 1997; Hugenholtz *et al*., 1998; Li *et al*., 1999) as well as in soil (Borneman & Triplett, 1997; Kuske *et al*., 1997; Harris *et al*., 2004). The OP11 phylum was identified in sediment of a mining impacted tropical stream by Reis *et al*. (2013).   Redox potential and Si correlated with OTUs from various genera (Appendix A, Figure 3B). Those identified in higher numbers were *Halomonas* sp., *Herbaspirillum* sp., *Shewanella* sp. and *Propionibacterium* sp. (Appendix B). *Shewanella* spp. are known as metal reducing bacteria that may inhabit both sediment and water environments (Arnold *et al*., 1990; Myers & Nealson, 1990; Lower *et al*., 2001; Heidelberg *et al*., 2002) and minerotrophic wetlands (Todorva & Costello, 2006), are also capable of reducing metal oxides (Bretschger *et al*., 2007). The dissimilatory metal reduction by *Shewanella* spp. is coupled to the oxidation of carbon compounds through reductively dissolving Fe(III) containing minerals (Lower *et al*., 2001). Thus these sediment bacteria identified in the study during January 2013 were in accordance to previous studies.

The multivariate analysis of the March 2013 water populations (Appendix A, Figure 3C) revealed some similar results compared to that of the January 2013 bacterial populations. Similarly, sulphates, Mn, Ni and Zn correlated with EC, TDS and dissolved O_2 and in turn with a large number of bacterial OTUs. High numbers of unclassified *Campylobacteriales* were identified. In lower numbers *Vibrio* spp., *Marinobacterium* sp. and *Legionella* sp. were identified (Appendix B). *Marinobacterium* spp. are sulphate reducing bacteria commonly found in marine waters (Fuse *et al*., 2000; Kim *et al*., 2008). The latter were represented by 8 sequences at site 3, where the EC and TDS were high, thus giving a clear indication of the salinity of the water at this site and may have enabled bacteria from this genus to survive. No report of *Marinobacterium* spp. in a high salinity, freshwater environment could be found. Iron and Pb correlated strongly together and weaker with Al and Cu. Both Fe and Pb correlated with bacterial OTUs identified as *Propionibacterium* spp. and *Legionella* spp. in

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relatively abundant numbers. *Propionibacterium* spp. have previously been identified in wetland sediment (Hallberg & Johnson, 2005) and rice paddy soil (Akasaka *et al*., 2003) and have been linked to methanogenesis in acidic wetland soil (Drake *et al*., 2009). Chlorophyll-*a* and DOC were correlated together (Appendix A, Figure 3C), which is expected as algal biomass may serve as a carbon source to bacteria (Cole *et al*., 1982). The latter correlated with 13 sequences for an unclassified bacterium at site 1 as well as low numbers of *Burkholderia* sp., *Legionella* sp., *Shewanella* sp. and *Pandorea* sp. (Appendix A, Figure 3C; Appendix B). Lastly, COD was correlated with *Pandorea* sp. and *Halomonas* sp. (Appendix A, Figure 3C).

The final analysis of the sediment populations for March 2013 (Appendix A, Figure 3D) showed a relationship between Cu, Ni and a vast diversity of OTUs. *Thermosporothrix* sp. was represented by 29 sequences at site 3. This genus was first described in 2010 by Yabe *et al*. (2010), isolated from hot compost. It was described by the authors to be an aerobic, thermophillic heterotrophy with an optimal pH range of 5.4 to 8.7. (Yabe *et al*., 2010). This new genus also produces secondary metabolites (Park *et al*., 2014). These bacteria form part of the *Chloroflexi* phylum, which indicates that they are capable of using H_2S and H_2 as electron donors (Prescott *et al*., 2002). Unfortunately, there is very little information available on this new genus. Other organisms represented by the most numerable for the sediment samples were *Armatimonadetes* Gp4, Gp5, unclassified bacteria, *Cytophaga* sp. and *Telmatospirillium* sp. (Appendix B). However, most of the top represented sequences were only represented in higher numbers in one site. Lastly, Si correlated with sequences of candidate phylum TM7, unclassified bacteria, *Polynucleobacter* sp. and *Rhodobacter* sp. (Appendix A, Figure 3D; Appendix B).

3.5 Conclusion

Acid mine drainage affects microbial biochemical cycling as stressor through acidity, increased metal concentrations and disruption of hydrous metal oxides. These effects are usually first observed in the nitrification pathway of nitrogen cycling as it is easily disrupted by low pH and increased dissolved metals (Niyogi *et al.,* 2003). Hence AMD environments are not the harsh, simple environments it was once thought to be. Even populations in extreme habitats will contain lithoautotrophs, organoheterotrophs, anaerobes etc. although highly specialised species are expected to dominate through adaptations to these conditions

(Xie *et al.,* 2011). Chemoautotrophs are the abundant primary producers in extremely acidic environments due to the availability of inorganic electron donors and their reduced sensitivity to increased concentrations of soluble transition metals and other solutes (Ñancucheo & Johnson, 2010). Although unusual for AMD environments, diverse acidophilic heterotrophs do inhabit these environments (Kimura *et al.,* 2011). It is the indigenous species of wetland environments that are regularly overlooked (Brantner & Senko, 2014) when these sensitive ecosystems are impacted, thus shifting the focus on AMD-related species. The understanding of the mechanisms shaping microbial populations in AMD impacted environments remain constrained (Miller *et al.,* 2009). Elaborate

This study found higher bacterial diversity than expected although the diversity varied at each site due to individual environmental stressors present (Kuang *et al.,* 2012). Environmental factors shaping the population were conductivity (Edwards *et al.,* 1999) and pH (Nicol *et al.,* 2008; Wang *et al.,* 2009; Lear *et al.,* 2009; Lauber *et al.,* 2009; Kuang *et al.,* 2013; Bartram *et al.,* 2014). The presence of metal concentrations also influenced the populations to a lesser degree. Microorganisms are the first to be impacted by these metals, reducing numbers, metabolic activity, diversity and thus the overall population structure (Giller *et al.,* 1998; Kozdroj & van Elsas, 2001). The relatively high number of unclassified bacteria obtained in this study has been reported before (Volant *et al.,* 2014) and reinforces the need for further study of environmental populations. This was the first study investigating the total bacterial populations in a wetland, impacted by AMD, in South Africa using culture-independent methods. It was also the first study to report the identification of a *Marinobacterium* sp. in a freshwater environment. Conclude your findings do not simply continue literature review/discussion here.

3.6 References

Achtnich, C., Bak, F. & Conrad, R., 1995, Competition for electron donors among nitrate reducers, ferric iron reducers, sulphate reducers, and methanogens in anoxic paddy soils, *Biology and Fertility of Soils*, 19: 65-72.

Akasaka, H., Ueki, A., Hanada, S., Kamagata, Y., & Ueki, K., 2003, *Propionicimonas paludicola* gen. nov., sp. nov., a novel facultatively anaerobic, Gram-positive, propionate-producing bacterium isolated from plant residue in irrigated rice-field soil, *International Journal of Systematic and Evolutionary Microbiology*, 53: 1991-1998.

Akcil, A. & Koldas, S., 2006, Acid Mine Drainage (AMD): causes, treatment and case studies, *Journal of Cleaner Production*, 14: 1139-1145.

American Public Health Association (APHA), American Water Works Association (AWWA), and Water Pollution Control Federation (WPCF), 1992, in APHA, AWWA, and WPCF, *Standard Methods for the Examination of Water and Wastewater*, 19th ed., Washington, D.C., USA.

Ansola, G., Arroyo, P. & de Miera, L.E.S., 2014, Characterisation of the soil bacterial community structure and composition of natural and constructed wetlands, *Science of the Total Environment*, 473: 63-71.

Arnds, J., Knittel, K., Buck, U.,Winkel, M. & Amann, R., 2010, Development of a16S rRNA-targeted probe set for *Verrucomicrobia* and its application for fluorescence *in situ* hybridization in a humic lake, *Systematic and Applied Microbiology*, 33: 139–148.

Arnold, R.G., Hoffmann, M.R., DiChristina, T.J. & Picardal, F.W., 1990, Regulation of dissimilatory Fe(III) reduction activity in *Shewanella putrefaciens*, *Applied and Environmental Microbiology*, 56: 2811-2817.

Arroyo, P., Ansola, G. & de Miera, L.E.S., 2013, Effects of substrate, vegetation and flow on arsenic and zinc removal efficiency and microbial diversity in constructed wetlands. *Ecological Engineering*, 51: 95-103.

Arroyo, P., Sáenz de Miera, L.E. & Ansola, G., 2015, Influence of environmental variables on the structure and composition of soil bacterial communities in natural and constructed wetlands, *Science of the Total Environment*, 506: 380-390.

Ashton, P.J., 2002, Avoiding conflicts over Africa's water resources, *Ambio*, 31: 236-242.

Atlas, R.M. & Bartha, R., 1998, *Microbial ecology: fundamentals and applications*, 4th ed., Benjamin/Cummings, California.

Baik, K.S., Choe, H.N., Park, S.C., Kim, E.M. & Seong, C.N., 2011b, *Paenibacillus wooponensis* sp. nov., isolated from wetland freshwater, *International Journal of Systematic and Evolutionary Microbiology*, 61: 2763-2768.

Baik, K.S., Lim, C.H., Choe, H.N., Kim, E.M. & Seong, C.N., 2011a, *Paenibacillus rigui* sp. nov., isolated from a freshwater wetland, *International Journal of Systematic and Evolutionary Microbiology*, 61: 529-534.

Baik, K.S., Park, S.C., Kim, E.M., Bae, K.S., Ahn, J.-H., Ka, J.-O., Chun, J. & Seong, C.N., 2008, Diversity of bacterial community in freshwater of Woopo wetland, *The Journal of Microbiology*, 46: 647-655.

Baker, B.J. & Banfield, J.F., 2003, Microbial communities in acid mine drainage, *FEMS Microbiology Ecology*, 44: 139-152.

Baker, B.J., Tyson, G.W., Goosherst, L. & Banfield, J.F., 2009, Insights into the diversity of eukaryotes in acid mine drainage biofilm communities, *Applied and Environmental Microbiology*, 75: 2192-2199.

Baldani, J.I., Baldani, V., Seldin, L. & Döbereiner, J., 1986, Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a root-associated nitrogen-fixing bacterium, *International Journal of Systematic Bacteriology*, 36: 86-93.

Barton, L.L. & Fauque, G.D., 2009, 'Biochemistry, physiology and biotechnology of sulfate-reducing bacteria', in A.I. Laskin, S. Sariaslani & G.M. Gadd, *Advances in Applied Microbiology*, Vol. 68, pp.41-98, Academic Press.

Bartram, A.K., Jiang, X., Lynch, M.D., Masella, A.P., Nicol, G.W., Dushoff, J. & Neufeld, J.D., 2014, Exploring links between pH and bacterial community composition in soils from the Crabstone Experimental Farm, *FEMS Microbiology Ecology*, 87: 403-415.

Bazylinski, D.A., Heywood, B.R., Mann, S. & Frankel, R.B., 1993, Fe_3O_4 and Fe_3S_4 in a bacterium, *Nature*, 366: 218.

Beal, E.J., House, C.H. & Orphan, V.J., 2009, Manganese-and iron-dependent marine methane oxidation, *Science*, 325: 184-187.

Behrendt, U., Schumann, P., Stieglmeier, M., Pukall, R., Augustin, J., Spröer, C., Schwendner, P., Moissl-Eichinger, C. & Ulrich, A., 2010, Characterization of heterotrophic nitrifying bacteria with respiratory ammonification and denitrification activity–Description of *Paenibacillus uliginis* sp. nov., an inhabitant of fen peat soil and *Paenibacillus purispatii* sp. nov., isolated from a spacecraft assembly clean room, *Systematic and Applied Microbiology*, 33: 328-336.

Beller, H.R., 2005, Anaerobic, nitrate-dependent oxidation of U(IV) oxide minerals by the chemolithoautotrophic bacterium *Thiobacillus denitrificans*, *Applied and Environmental Microbiology*, 71: 2170-2174.

Berge, O., Guinebretière, M.H., Achouak, W., Normand, P. & Heulin, T., 2002, *Paenibacillus graminis* sp. nov. and *Paenibacillus odorifer* sp. nov., isolated from plant roots, soil and food, *International Journal of Systematic and Evolutionary Microbiology*, 52: 607-616.

Bergmann, G., Bates, S., Eilers, K., Lauber, C., Caporaso, J., Walters, W., Knight, R. & Fierer, N., 2011, The under-recognized dominance of Verrucomicrobia in soil bacterial communities, *Soil Biology and Biochemistry*, 43: 1450–1455.

Bond, P.L., Smriga, S.P. & Banfield, J.F., 2000, Phylogeny of microorganisms populating a thick subaerial, predominantly lithotrophic biofilm at an extreme acid mine drainage site, *Applied and Environmental Microbiology*, 66: 3842-3849.

Borneman, J. & Triplett, E.W., 1997, Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation, *Applied and Environmental Microbiology*, 63: 2647-2653.

Bretschger, O., Obraztsova, A., Sturm, C.A., Chang, I.S., Gorby, Y.A., Reed, S.B., Culley, D.E., Reardon, C.L., Barua, S., Romine, M.F., Zhou, J., Beliaev, A.S., Bouhenni, R., Saffarini, D., Mansfeld, F., Kim, B., Fredrickson, J.K. & Nealson, K.H., 2007, Current production and metal oxide reduction by *Shewanella oneidensis* MR-1 wild type and mutants, *Applied and Environmental Microbiology*, 73: 7003-7012.

Briee, C., Moreira, D. & Lopez-Garcia, P., 2007, Archaeal and bacterial community composition of sediment and plankton from a suboxic freshwater pond, *Research in Microbiology*, 158: 213–227.

Brofft, J.E., McArthur, J.V. & Shimkets, L.J., 2002, Recovery of novel bacterial diversity from a forested wetland impacted by reject coal, *Environmental Microbiology*, 4: 764-769.

Brugna, M., Nitschke, W., Toci, R., Bruschi, M. & Giudici-Orticoni, M.T., 1999, First evidence for the presence of a hydrogenase in the sulfurreducing bacterium *Desulfuromonas acetoxidans, Journal of Bacteriology*, 181: 5505-5508.

Buck, U., Grossart, H.P., Amann, R. & Pernthaler, J., 2009, Substrate incorporation patterns of bacterioplankton populations in stratified and mixed waters of a humic lake, *Environmental Microbiology*, 11: 1854–1865.

Cheng, W., Zhang, J.X., Wang, Z., Wang, M. & Xie, S.G., 2013, Bacterial communities in sediments of a drinking water reservoir, *Annals Microbiology*, 64: 875-878.

Cicerone, R.J. & Oremland, R.S., 1988, Biogeochemical aspects of atmospheric methane, *Global Biogeochemistry Cycles*, 2: 299-327.

Coates, J.D., Ellis, D.J., Gaw, C.V. & Lovley, D.R., 1999, *Geothrix fermentans* gen. nov., sp. nov., a novel Fe(III)-reducing bacterium from a hydrocarbon-contaminated aquifer, *International Journal of Systematic Bacteriology*, 49: 1615-1622.

Cole, J.J., Likens, G.E. & Strayer, D.L., 1982, Photosynthetically produced dissolved organic carbon: An important carbon source for planktonic bacteria1, *Limnology and Oceanography*, 27: 1080-1090.

Comte, J., Jacquet, S., Viboud, S., Fontvieille, D., Millery, A., Paolini, G. & Domaizon, I., 2006, Microbial community structure and dynamics in the largest natural French lake (Lake Bourget), *Microbial Ecology*, 52: 72-89.

Conrad, R. & Rothfuss, F., 1991, Methane oxidation in the soil surface layer of a flooded rice field and the effect of ammonium, *Biology and Fertility of Soils*, 12: 28-32.

Conrad, R., 1996, Soil microorganisms as controllers of atmospheric trace gases (H_2, CO, CH_4, OCS, N_2O, and NO), *Microbiology Review*, 60: 609-640.

Cypionka, H., 2000, Oxygen respiration in *Desulfovibrio* species, *Annual Reviews in Microbiology*, 54: 827-848.

Dalsgaard, T.D.E., Canfield, J., Peterson, B., Thamdrup, B. & Acuna-Gonzalez, J., 2003, N_2 production by the annamox reaction in the anoxic water column of Golfo Dulce, Costa Rica, *Nature*, 422: 606-608.

de Figueiredo, D.R., Pereira, M.J., Moura, A., Silva, L., Barrios, S., Fonseca, F., Henriques, I. & Correia, A., 2007, Bacterial community composition over a dry winter in meso- and eutrophic Portuguese water bodies, *FEMS Microbioly Ecology*, 59: 638–650.

Dedysh, S.N., 2011, Cultivating uncultured bacteria from northern wetlands: Knowledge gained and remaining gaps, *Frontiers in Microbiology*, 2: 184.

Dedysh, S.N., Pankratov, T.A., Belova, S.E., Kulichevskaya, I.S. & Liesack, W., 2006, Phylogenetic analysis and in situ identification of Bacteria community composition in an acidic Sphagnum peat bog, *Applied and Environmental Microbiology*, 72: 2110–2117.

Deng, H., Ge, L., Xu, T., Zhang, M., Wang, X., Zhang, Y. & Peng, H., 2011, Analysis of the metabolic utilization of carbon sources and potential functional diversity of the

bacterial community in lab-scale horizontal subsurface-flow constructed wetlands, *Journal of Environmental Quality*, 40: 1730-1736.

Denisova, L.Y., Bel'kova, N.L., Tulokhonov, I.I. & Zaichikov, E.F., 1999, Bacterial diversity at various depths in the southern part of Lake Baikal as revealed by 16S rDNA sequencing, *Mikrobiologiia*, 68: 475–483.

Department of Water Affairs and Forestry, 2004, Olifants Water Management Area: Internal Strategic Perspective, Report P MWA 04/0000/00/0304, Department of Water Affairs and Forestry, Pretoria.

Dorador, C., Vila, I., Witzel, K.P. & Imhoff, J.F., 2013, Bacterial and archaeal diversity in high altitude wetlands of the Chilean Altiplano, *Fundamental and Applied Limnology*, 182: 135–159.

Drake, H.L., Horn, M.A. & Wüst, P.K., 2009, Intermediary ecosystem metabolism as a main driver of methanogenesis in acidic wetland soil, *Environmental Microbiology Reports*, 1: 307-318.

Druschel, G.K., Baker, B.J., Gihring, T.M. & Banfield, J.F., 2004, Acid mine drainage biogeochemistry at Iron Mountain, California, *Geochemical Transactions*, 5: 13-32.

DWAF (Department of Water Affairs and Forestry), 1996a, *South African Water Quality Guidelines, Volume 1: Domestic use*, Department of Water Affairs and Forestry, Pretoria.

DWAF (Department of Water Affairs and Forestry), 1996b, *South African Water Quality Guidelines, Volume 4: Agricultural Use: Irrigation*, Department of Water Affairs and Forestry, Pretoria.

DWAF (Department of Water Affairs and Forestry), 1996c, *South African Water Quality*, Department of Water Affairs and Forestry, Pretoria.

Eckford, R.E. & Fedorack, P.M., 2002, Chemical and microbiological changes in laboratory incubations of nitrate amendment "sour" produced waters from three western Canadian oil fields, *Journal of Industrial Microbiology and Biotechnology*, 29: 243-254.

Edwards, K.J., Gihring, T. M. & Banfield, J.F., 1999, Seasonal variations in microbial populations and environmental conditions in an extreme acid mine drainage environment, *Applied and Environmental Microbiology*, 65: 3627-3632.

Edwards, K.J., Gihring, T.M. & Banfield, J.F., 1999, Seasonal variations in microbial populations and environmental conditions in an extreme acid mine drainage environment, *Applied and Environmental Microbiology*, 65: 3627-3632.

Eiler, A. & Bertilsson, S., 2004, Composition of freshwater bacterial communities associated with cyanobacterial blooms in four Swedish lakes, *Environmental Microbiology*, 6: 1228–1243.

Eiler, A. & Bertilsson, S., 2007, Flavobacteria blooms in four eutrophic lakes: linking population dynamics of freshwater bacterioplankton to resource availability, *Applied and Environmental Microbiology*, 73: 3511–3518.

Eiler, A., Langenheder, S., Bertilsson, S. & Tranvik, L.J., 2003, Heterotrophic bacterial growth efficiency and community structure at different natural organic carbon concentrations, *Applied and Environmental Microbiology*, 69: 3701–3709.

Eiler, A., Olsson, J.A. & Bertilsson, S., 2006, Diurnal variations in the auto- and heterotrophic activity of cyanobacterial phycospheres (*Gloeotrichia echinulata*) and the identity of attached bacteria, *Freshwater Biology*, 51: 298–311.

Elbeltagy, A., Nishioka, K., Sato, T., Suzuki, H., Ye, B., Hamada, T., Isawa, T., Mitsui, H. & Minamisawa, K., 2001, Endophytic colonization and in planta nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species, *Applied and Environmental Microbiology*, 67: 5285-5293.

Emerson, D. & Weiss, J.V., 2004, Bacterial iron oxidation in Circumneutral freshwater habitats: findings from the field and the laboratory, *Geomicrobiology Journal*, 21: 405-414.

Empadinhas, N. & da Costa, M.S., 2011, Diversity, biological roles and biosynthetic pathways for sugar-glycerate containing compatible solutes in bacteria and Archaea, *Environmental Microbiology,* 13: 2056–2077.

Energy Information Administration, 2000, National Energy Information Centre, 1000 Independence Avenue, SW Washington, DC, USA, www.eia.doe.gov.

Environmental Mining Council of British Columbia (EMCBC), 2001, Acid mine drainage: mining and water pollution issues in British Columbia, www.miningwatch.org/emcbc/publications/amd-water.html.

Erler, D.V., Eyre, B.D. & Davidson, L., 2008, The contribution of annamox and denitrification to sediment N_2 production in a surface flow constructed wetland, *Environmental Science and Technology*, 42: 9144-9150.

Ettwig, K.F., Butler, M.K., Le Paslier, D., Pelletier, E., Mangenot, S., Kuypers, M.M., Schreiber, F., Dutilh, B.E., Zedelius, J., de Beer, D., Gloerich, J., Wessels, H.J.C.T., van Alen, T., Luesken, F., Wu, M.L., van de Pas-Schoonen, K.T., Op den Camp, H.J.M., Janssen-Megens, E.M., Francoijs, K.-J., Stunnenberg, H., Weissenbach, J.,

Jetten, M.S.M. & Strous, M., 2010, Nitrite-driven anaerobic methane oxidation by oxygenic bacteria, *Nature*, 464: 543-548.

Evangelou, V.P. & Zhang, Y.L., 1995, A review: pyrite oxidation mechanisms and acid mine drainage prevention, *Critical Reviews in Environmental Science and Technology*, 25: 141-199.

Faulwetter, J.L., Gagnon, V., Sundberg, C., Chazarenc, F., Burr, M.D., Brisson, J., Camper, A.K. & Stein, O.R., 2009, Microbial processes influencing performance of treatment wetlands: a review, *Ecological Engineering*, 35: 987-1004.

Fauque, G. & Ollivier, B., 2004, Anaerobes: the sulfate-reducing bacteria as an example of metabolic diversity, in A.T. Bull (ed.), *Microbial diversity and bioprospecting*, pp. 169-176, ASM Press, Washington, DC.

Fauque, G., LeGall, J. & Barton, L.L., 1991, Sulfate-reducing and sulfur-reducing bacteria, in J.M. Shively & L.L. Barton (eds.), *Variations in autotrophic life*, pp. 271-337, Academic Press Limited, London.

Fauque, G.D., 1995, 'Ecology of sulfate-reducing bacteria', in T. Atkinson & R.F. Sherwood (eds.), *Biotechnology Handbooks*, L.L. Barton (ed.), *Sulfate-reducing bacteria*, Vol. 8, pp. 217-241, Plenum Press, New York.

Findlay, S.E.G., Dye, S. & Kuehn, K.A., 2002, Microbial growth and nitrogen retention in litter of *Phragmites australis* compared to *Typha angustifolia*, *Wetlands*, 22: 616-625.

Finneran, K.T., Housewright, M.E. & Lovley, D.R., 2002, Multiple influences of nitrate on uranium solubility during bioremediation of uranium-contaminated subsurface sediments, *Environmental Microbiology*, 4: 510–516.

Fisher, J. & Acreman, M., 2004, Wetland nutrient removal: a review of the evidence, *Hydrology and Earth System Sience*, 8: 673-685.

Fredrickson, J.K., Zachara, J.M., Kennedy, D.W., Dong, H., Onstott, T.C., Hinman, N.W. & Li, S.M., 1998, Biogenic iron mineralization accompanying the dissimilatory reduction of hydrous ferric oxide by a groundwater bacterium, *Geochimica et Cosmochimica Acta*, 62: 3239-3257.

Freitas, S., Hatosy, S., Fuhrman, J.A., Huse, S.M., Welch, D.B.M., Sogin, M.L. & Martiny, A.C., 2012, Global distribution and diversity of marine Verrucomicrobia, *The ISME Journal*, 6: 1499–1505.

Frenzel, P., Bosse, U. & Janssen, P.H., 1999, Rice roots and methanogenesis in a paddy soil: ferric iron as an alternative electron acceptor in the rooted soil, *Soil Biology and Biochemistry*, 31: 421-430.

Fuse, H., Takimura, O., Murakami, K., Yamaoka, Y. & Omori, T., 2000, Utilization of dimethyl sulfide as a sulfur source with the aid of light by *Marinobacterium* sp. strain DMS-S1, *Applied and Environmental Microbiology*, 66: 5527-5532.

Gardner, T.G., Acosta-Martínez, V., Senwo, Z. & Dowd, S.E., 2011, Soil rhizosphere microbial communities and enzyme activities under organic farming in Alabama, *Diversity*, 3: 308–328.

Giller, K.E., Witter, E. & Mcgrath, S.P., 1998, Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review, *Soil Biology and Biochemistry*, 30: 1389-1414.

Glöckner, F.O., Zaichikov, E., Belkova, N., Denissova, L., Pernthaler, J., Pernthaler, A. & Amann, R., 2000, Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of *Acidobacteria*, *Applied and Environmental Microbiology*, 66: 5053-5065.

Gómez-Consarnau, L., González, J.M., Coll-Lladó, M., Gourdon, P., Pascher, T., Neutze, R., Pedrós-Alió, C. & Pinhassi, J., 2007, Light stimulates growth of proteorhodopsin-containing marine Flavobacteria, *Nature*, 445: 210-213.

González, J.M., Fernández-Gómez, B., Fernàndez-Guerra, A., Gómez-Consarnau, L., Sánchez, O., Coll-Lladó, M., del Campo, J., Escudero, L., Rodríguez-Martínez, R., Alonso-Sáez, L., Latasa, M., Paulsen, I., Nedashkovskaya, O., Lekunberri, I., Pinhassi, J. & Pedrós-Alió, C., 2008, Genome analysis of the proteorhodopsin-containing marine bacterium *Polaribacter* sp. MED152 (Flavobacteria), *PNAS*, 105: 8724-8729.

González-Domenech, C.M., Martínez-Checa, F., Quesada, E. & Béjar, V., 2008 *Halomonas cerina* sp. nov., a moderately halophilic, denitrifying, exopolysaccharide-producing bacterium, *International Journal of Systematic and Evolutionary Microbiology*, 58: 803-809.

González-Toril, E., Aguilera, Á., Souza-Egipsy, V., Pamo, E.L., España, J. S. & Amils, R., 2011, Geomicrobiology of La Zarza-Perrunal acid mine effluent (Iberian Pyritic Belt, Spain), *Applied and Environmental Microbiology*, 77: 2685-2694.

Goodfellow, M. & Williams, S.T., 1983, Ecology of Actinomycetes, *Annual Reviews of Microbiology*, 37: 189–216.

Gore, J.A., 2006, 'Discharge Measurements and Streamflow Analysis', in F.R. Hauer and G.A. Lamberti (eds.), *Methods in Stream Ecology*, pp. 51-81, Academic Press. San Diego. California, USA.

Gorra, R., Coci, M., Ambrosoli, R. & Laanbroek, H.J., 2007, Effects of substratum on the diversity and stability of ammonia-oxidizing communities in a constructed wetland used for wastewater treatment, *Journal of Applied Microbiology*, 103: 1442-1452.

Hallberg, K.B. & Johnson, D.B., 2005, Microbiology of a wetland ecosystem constructed to remediate mine drainage from a heavy metal mine, *Science of the Total Environment*, 338: 53-66.

Hanson, R.S., Netrusov, A.I. & Tsuji, K., 1992, 'The obligate methanotrophic bacteria *Methylococcus, Methylomonas*, and *Methylosinus*', in A. Balows, H.G. Trüper, M. Dworkin, W. Harder and K.-H. Schleifer (eds.), *The Prokaryotes: A Handbook of Bacteria: Ecophysiology, Isolation, Identification, Applications*, 2nd Ed., Vol. 3, pp. 2350–2364, Springer-Verlag, New York.

Haroon, M.F., Hu, S., Shi, Y., Imelfort, M., Keller, J., Hugenholtz, P., Yuan, Z. & Tyson, G.W., 2013, Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage, *Nature*, 500: 567-570.

Harris,J.K., Kelley, S.T. & Pace,N.R. , 2004, New perspective on uncultured bacterial phylogenetic division OP11, *Applied and Environmental Microbiology*, 70: 845-849.

Hartman, W.H., Richardson, C.J., Vilgalys, R. & Bruland, G.L., 2008, Environmental and anthropogenic controls over bacterial communities in wetland soils, *Proceedings of the National Academy of Sciences*, 105: 17842-17847.

Heidelberg, J.F., Paulsen, I.T., Nelson, K.E., Gaidos, E.J., Nelson, W.C., Read, T.D., ... & Fraser, C.M., 2002, Genome sequence of the dissimilatory metal ion–reducing bacterium *Shewanella oneidensis*, *Nature Biotechnology*, 20: 1118-1123.

Henckel, T., Jäkel, U., Schnell, S. & Conrad, R., 2000, Molecular analysis of novel methanotrophic communities in forest soil that oxidize atmospheric methane, *Applied and Environmental Microbiology*, 66: 1801-1808.

Hiorns, W.D., Methe´, B.A., Nierzwicki-Bauer, S.A. & Zehr, J.P., 1997, Bacterial diversity in Adirondack Mountain lakes as revealed by 16S rRNA gene sequences, *Applied and Environmental Microbiology*, 63: 2957–2960.

Hobbs, P., Oelofse, S.H.H. & Rascher, J., 2008, Management of environmental impacts from coal mining in the Upper Olifants river catchment as a function of age and scale, *Water Resources Development*, 24: 417-431.

Holmes, A.J., Roslev, P., McDonald, I.R., Iversen, N., Henriksen, K. & Murrell, J.C., 1999, Characterization of methanotrophic bacterial populations in soils showing atmospheric methane uptake, *Applied and Environmental Microbiology*, 65: 3312-3318.

Hu, B., Shen, L., Xu, X. & Zheng, P., 2011, Anaerobic ammonium oxidation (anammox) in different natural ecosystems, *Biochemical Society Transactions*, 39: 1811-1816.

Hugenholtz, P., Goebel, B.M. & Pace, N.R., 1998, Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity, *Journal of Bacteriology*, 180: 4765-4774. (Erratum, 180: 6793.)

Intergovernmental Panel on Climate Change (IPCC), 2007, Climate change 2007: the physical science basis, in S. Solomons, D. Quin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, *et al*. (eds.), *Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*, Cambridge, U.K. & New York, U.S.A., U.S.A.: Cambridge University Press.

Jangid, K., Williams, M.A., Franzluebbers, A.J., Sanderlin, J.S., Reeves, J.H., Jenkins, M.B., Endale, D.M., Coleman, D.C. & Whitman, W.B., 2008, Relative impacts of land-use, management intensity and fertilization upon soil microbial community structure in agricultural systems, *Soil Biology and Biochemistry*, 40: 2843–2853.

Janssen, P.H., 2006, Identifying the dominant soil bacteria taxa in libraries of 16S rRNA and 16S rRNA genes, *Applied and Environmental Microbiology*, 72: 1719–1728.

Janssen, R., Goosen, H., Verhoeven, M.L., Verhoeven, J.T.A., Omtzigt, A.Q.A. & Maltby, E., 2005, Decision support for integrated wetland management, Environmental Modelling and Software, 20: 215-229.

Johnson, C.A. & Thornton, I., 1987, Hydrological and chemical factors controlling the concentrations of Fe, Cu, Zn and As in a river system contaminated by acid mine drainage, *Water Research*, 21: 359-365.

Johnson, D.B. & Hallberg, K.B., 2005, Acid mine drainage remediation options: a review, *Science of the Total Environment*, 338: 3-14.

Jones, R.T., Robeson, M.S., Lauber, C.L., Hamady, M., Knight, R. & Fierer, N., 2009, A comprehensive survey of soil *Acidobacteria*l diversity using pyrosequencing and clone library analyses, *The ISME Journal*, 3: 442-453.

Jürgens, K. & Jeppese, E., 2000, The impact of metazooplankton on the structure of the microbial food web in a shallow, hypertrophic lake, *Journal of Plankton Research*, 22: 1047–1070.

Karl, D., Letelier, R., Tupas, L., Dore, J., Christian, J. & Hebel, D., 1997, The role of nitrogen fixation in geobiochemical cycling in the subtropical North Pacific Ocean, *Nature*, 388: 533-538.

Kawaguchi, R., Burgess, J.G., Sakaguchi, T., Takeyama, H., Thornhill, R.H. & Matsunaga, T., 1995, Phylogenetic analysis of a novel sulfate-reducing magnetic bacterium, RS-1, demonstrates its membership of the δ-Proteobacteria, *FEMS Microbiology Letters*, 126: 277–282.

Kelly, D.P. & Wood, K.P., 2000, Reclassification of some species of *Thiobacillus* to the newly designed genera *Acidithiobacillus* gen. nov., *Halothiobacillus* gen. nov., and *Thermothiobacillus* gen. nov., *International Journal of Systematic Evolutionary Microbiology*, 50: 511-516.

Keunen, J.G., Robertson, L.A. & Tuovinen, O.H., 1992, The genera *Thiobacillus*, *Thiomicrospora* and *Thiosphaera*, in A. Balows, H.G. Trüper, M. Dworkin, H. Harder & K.H. Schleifer (eds.), *The Prokaryotes*, 3: 2636-2657, Springer, Berlin Heidelberg New York.

Kim, Y.G., Jin, Y.A., Hwang, C.Y. & Cho, B.C., 2008, *Marinobacterium rhizophilum* sp. nov., isolated from the rhizosphere of the coastal tidal-flat plant Suaeda japonica, *International Journal of Systematic and Evolutionary Microbiology*, 58: 164-167.

Kimura, S., Bryan, C.G., Hallberg, K.B. & Johnson, D.B., 2011, Biodiversity and geochemistry of an extremely acidic, low-temperature subterranean environment sustained by chemolithotrophy, *Environmental Microbiology*, 13: 2092-2104.

King, G.M., 1990, Dynamics and controls of methane oxidation in a Danish wetland sediment, *FEMS Microbiology Letters,* 74: 309-323.

Kluber, H.D. & Conrad, R., 1998, Effects of nitrate, nitrite, NO and N_2O on methanogenesis and other redox processes in anoxic rice field soils, *FEMS Microbiology Ecology*, 25: 301-318.

Knittel, K. & Boetius, A., 2009, Anaerobic oxidation of methane: progress with an unknown process, *Annual Review of Microbiology*, 63: 311-334.

Kolmonen, E., Sivonen, K., Rapala, J. & Haukka, K., 2004, Diversity of cyanobacteria and heterotrophic bacteria in cyanobacterial blooms in Lake Joutikas, Finland, *Aquatic Microbial Ecology*, 36: 201–211.

Koops, H.P. & Harms, H., 1985, Desoxyribonucleic acid homologies among 96 strains of ammonia-oxidizing bacteria, *Archive of Microbiology*, 141: 214–218.

Kostka, J.E. & Nealson, K.H., 1995, Dissolution and reduction of magnetite by bacteria, *Environmental Science and Technology*, 29: 2535-2540.

Kostka, J.E., Stucki, J.W., Nealson, K.H. & Wu, J., 1996, Reduction of structural Fe(III) in smectite by a pure culture of *Shewanella putrifaciens* strain MR-1, *Clays and Clay Minerals*, 44: 522-529.

Kowalchuk, G.A. & Stephen, J.R., 2001, Ammonia-oxidizing bacteria: a model for molecular microbial ecology, *Annual Reviews in Microbiology*, 55: 485-529.

Kozdroj, J. & van Elsas, J.D., 2001, Structural diversity of microorganisms in chemically perturbed soil assessed by molecular and cytochemical approaches, *Journal of Microbiological Methods*, 43: 197-212.

Krauter, P., Daily, B., Dibley, V., Pinkart, H. & Legler, T., 2005. Perchlorite and nitrate remediation efficiency and microbial diversity in a containerized wetland bioreactor, *International Journal of Phytoremediation*, 7: 113-128.

Kuang, J.-L., Huang, L.-N., Chen, L.-X., Hua, Z.-S., Li, S.-J., Hu, M., Li, J.-T. & Shu, W.-S., 2013, Contemporary environmental variation determines microbial diversity patterns in acid mine drainage, *The ISME Journal*, 7: 1038-1050.

Kuever, J., Rainey, F. A. & Widdel, F., 2005, "Class IV. Deltaproteobacteria class nov.", in D.J. Brenner, N.R. Krieg & J.T. Staley (eds.), *Bergey's Manual® of Systematic Bacteriology*, pp. 922-1144, Springer, USA.

Kuske, C.R., Barns, S.M. & Busch, J.D., 1997, Diverse uncultivated bacterial groups from soils of the arid southwestern United States that are present in many geographic regions, *Applied and Environmental Microbiology*, 63: 3614-3621.

Kuypers, M.M.M., Sliekers, A.O., Lavik, G., Schmid, M., Jørgensen, B.B., Kuenen, J.G., Sinninghe Damsté, J.S., Strous, M. & Jetten, M.S.M., 2003, Anaerobic ammonium oxidation by annamox bacteria in the Black Sea, *Nature*, 422: 608-611.

Kwon, S., Moon, E., Kim, T.S., Hong, S. & Park, H.D., 2011, Pyrosequencing demonstrated complex microbial communities in a membrane filtration system for a drinking water treatment plant, *Microbes and Environments*, 26: 149–155.

Langenheder, S. & Jürgens, K., 2001, Regulation of bacterial biomass and community structure by metazoan and protozoan predation, *Limnology and Oceanography*, 46: 121–134.

Lauber, C.L., Hamady, M., Knight, R. & Fierer, N., 2009, Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale, *Applied and Environmental Microbiology*, 75: 5111–5120.

Lauber, C.L., Hamady, M., Knight, R. & Fierer, N., 2009, Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale, *Applied and Environmental Microbiology*, 75: 5111-5120.

Lear, G., Niyogi, D., Harding, J., Dong, Y. & Lewis, G., 2009, Biofilm bacterial community structure in streams affected by acid mine drainage, *Applied and Environmental Microbiology*, 75: 3455-3460.

Lefèvre, C.T., Menguy, N., Abreu, F., Lins, U., Pósfai, M., Prozorov, T., Pignol, D., Frankel, R.B. & Bazylinski, D.A., 2011b, A cultured greigite-producing magnetotactic bacterium in a novel group of sulfate-reducing bacteria, *Science*, 334: 1720–1723.

Lefèvre, C.T., Frankel, R.B., Pósfai, M., Prozorov, T. & Bazylinski, D.A., 2011a, Isolation of obligately alkaliphilic magnetotactic bacteria from extremely alkaline environments, *Environmental Microbiology*, 13: 2342–2350.

LeGall, J. & Fauque, G., 1988, Dissimilatory reduction of sulfur compounds, in A.J.B. Zehnder (ed.), *Biology of anaerobic microorganisms*, pp. 587-639, Wiley, New York.

Lemarchand, C., Jardillier, L., Carrias, J.F., Richardot, M., Debroas, D., Sime-Ngando, T. & Amblard, C., 2006, Community composition and activity of prokaryotes associated to detrital particles in two contrasting lake ecosystems, *FEMS Microbiology Ecology*, 57: 442-451.

Li, J., Lu, Q., Liu, T., Zhou, S., Yang, G. & Zhao, Y., 2014, *Paenibacillus guangzhouensis* sp. nov., an Fe (III)-and humus-reducing bacterium from a forest soil, *International Journal of Systematic and Evolutionary Microbiology*, 64: 3891-3896.

Li, L., Kato, C. & Horikoshi, K., 1999, Bacterial diversity in deep-sea sediments from different depths, *Biodiversity and Conservation*, 8: 659-677.

Liao, X.B., Chen, C., Wang, Z., Wan, R., Chang, C.H., Zhang, X.J. & Xie, S.G., 2013a, Changes of biomass and bacterial communities in biological activated carbon filters for drinking water treatment, *Process Biochemistry*, 48: 312–316.

Liao, X.B., Chen, C., Wang, Z., Wan, R., Chang, C.H., Zhang, X.J. & Xie, S.G., 2013b, Pyrosequencing analysis of bacterial communities in drinking water biofilters receiving influents of different types, *Process Biochemistry*, 48: 703–707.

Ligi, T., Oopkaup, K., Truu, M., Preem, J.-K., Nõlvak, H., Mitsch, W.J., Mander, Ü. & Truu, J., 2014, Characterization of bacterial communities in soil and sediment of a created riverine wetland complex using high-throughput 16S rRNA amplicon sequencing, *Ecological Engineering*, 72: 56-66.

Lower, S.K., Hochella, M.F. & Beveridge, T.J., 2001, Bacterial recognition of mineral surfaces: nanoscale interactions between *Shewanella* and α-FeOOH, *Science*, 292: 1360-1363.

Ma, H. & Aelion, C., 2005, Ammonium production during microbial nitrate removal in soil microcosms for a developing marsh estuary, *Soil Biology and Biochemistry*, 37: 1869-1878.

Macfarlane, D.M., 2013, 'Phase 1 – Planning report: Zaalklapspruit Wetland' (B20G-01)', Working for Wetlands Rehabilitation Programme, SANBI, Pretoria.

Madigan, M.T., Martinko, J.M. & Parker, J., 2000, *Brock Biology of Microorganisms*, 9th edition, New York, Prentice Hall.

Mata, J.A., Martínez-Cánovas, J., Quesada, E. & Béjar, V., 2002, A detailed phenotypic characterisation of the type strains of *Halomonas* species, *Systematic and Applied Microbiology*, 25: 360–375.

McCarthy, D.M. & Oleszkiewicz, J.A., 1993, Competition between methanogens and sulfate reducers: effect of COD:sulfate ratio and acclimation, *Water Environment Research*, 65: 655-664.

McCarthy, T.S., 2011, The impact of acid mine drainage in South Africa, *South African Journal of Science*, 107: 1-7.

McSpadden Gardener, B.B., 2004, Ecology of *Bacillus* and *Paenibacillus* spp. in agricultural systems, *Phytopathology*, 94: 1252-1258.

Mendes, R., Kruijt, M., de Bruijn, I., Dekkers, E., van der Voort, M., Schneider, J.M., Piceno, Y.M., DeSantis, F.Z., Andersen, G.L., Bakker, P. & Raaijmakers, J.M., 2011, Deciphering the rhizosphere microbiome for disease-suppressive bacteria, *Science*, 332: 1097–1100.

Miller, G.T., 1999, *Environmental science: working with the earth*, 7th ed., Belmont, Wadsworth.

Miller, S.R., Strong, A.L., Jones, K.L. & Ungerer, M.C., 2009, Bar-coded pyrosequencing reveals shared bacterial community properties along the temperature gradients of two alkaline hot springs in Yellowstone National Park, *Applied and Environmental Microbiology*, 75: 4565-4572.

Mormile, M.R., Romine, M.F., Garcia, M.T., Ventosa, A. & Bailey, T.J., Peyton, B.M., 1999, *Halomonas campisalis* sp.nov., a denitrifying, moderately haloalkaliphilic bacterium, *Systematic and Applied Microbiology*, 22: 551-558.

Muyzer, G. & Stams, A.J.M., 2008, The ecology and biotechnology of sulphate-reducing bacteria, *Nature Reviews Microbiology*, 6: 441-454.

Myers, C.R. & Nealson, K.H., 1990, Respiration-linked proton translocation coupled to anaerobic reduction of manganese(IV) and iron(III) in *Shewanella putrefaciens* MR-1, *Journal of Bacteriology*, 172: 6232–6238.

Nacke, H., Thurmer, A., Wollherr, A., Will, C., Hodac, L., Herold, N., Schöning, I., Schrumpf, M. & Daniel, R., 2011, Pyrosequencing-based assessment of bacterial community structure along different management types in German forest and grassland soils, *PLoS One*, 6: e17000.

Naiker, K., Cukrowska, E. & McCarthy, T.S., 2003, Acid mine drainage from gold mining activities in Johannesburg, South Africa and environs, *Environmental Pollution*, 122: 29-40.

Ñancucheo, I. & Johnson, D.B., 2010, Production of glycolic acid by chemolithotrophic iron- and sulphur-oxidizing bacteria and its role in delineating and sustaining acidophilic sulphide mineral-oxidation consortia, *Applied and Environmental Microbiology*, 76: 461-467.

Nazaries, L., Murrell, J.C., Millards, P., Baggs, L. & Singh, B.K., 2013, Methane, microbes and models: fundamental understanding of the soil methane cycle for future predictions, *Environmental Microbiology*, 15: 2395-2417.

Newton, R.J., Jones, S.E., Eiler, A., McMahon, K.D. & Bertilsson, S., 2011, A Guide to the Natural History of Freshwater Lake Bacteria, *Microbiology and Molecular Biology Reviews*, 75: 14-49.

Nicol, G.W. & Schleper, C., 2006, Ammonia-oxidising Crenarchaeota: important players in the nitrogen cycle?, *TRENDS in Microbiology*, 14: 207-212.

Nicol, G.W., Leininger, S., Schleper, C. & Prosser, J.I., 2008, The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria, *Environmental Microbiology*, 10: 2966-2978.

Niyogi, D.K., Lewis, W.M. & McKnight, D.M., 2002, Effects of stress from mine drainage on diversity, biomass, and function of primary producers in mountain streams, *Ecosystems*, 5: 554-567.

Niyogi, D.K., Lewis, W.M. & McKnight, D.M., 2003, Direct and indirect effects of mine drainage on bacterial processes in mountain streams, *Journal of the North American Benthological Scociety*, 22: 276-291.

Nold, S.C. & Zwart, G., 1998, Patterns and governing forces in aquatic microbial communities, *Aquatic Ecology*, 32:17–35.

Oberholster, P., de Klerk, A.R., Botha, A.-M., Babalola, O.O., Ndlela, L., Staebe, K. & van Wyk, J.H., 2013b, 'The adverse effects of anthropogenic pollution on Xenopsus leavis with special reference to acid mine drainage (AMD) in a freshwater wetland', in Lombardi, M.P. (ed.), Amphibians: Anatomy, ecological significance and conservation strategies, Nova Publishers.

Oberholster, P.J., McMillan, P., Durapersad, K., de Klerk, A.R. & Botha, A.-M., 2014, Development of a wetland classification and risk assessment index (WCRAI) for natural wetland ecosystems in three ecoregions of South Africa, *Water Air Soil Pollution*, 225: 1-15.

Ochieng, G.M., Seanego, E.S. & Nkwonta, O.I., 2010, Impacts of mining on water resources in South Africa: a review, *Scientific Research and Essays*, 5: 3351-3357.

Olías, M., Nieto, J.M., Sarmiento, A.M., Cerón, J.C. & Cánovas, C.R., 2004, Seasonal water quality variations in a river affected by acid mine drainage: the Odiel River (South West Spain), *Science of the Total Environment*, 333: 267-281.

Olivares, F.L., Baldani, V.L., Reis, V.M., Baldani, J.I. & Döbereiner, J., 1996, Occurrence of the endophytic diazotrophs *Herbaspirillum* spp. in roots, stems, and leaves, predominantly of Gramineae, *Biology and Fertility of Soils*, 21: 197-200.

Osem, Y., Chen, Y., Levinson, D. & Hadar, Y., 2007, The effects of plant roots on microbial community structure in aerated wastewater-treatment reactors, *Ecological Engineering*, 29: 133-142.

Park, J., Kagaya, N., Hashimoto, J., Izumikawa, M., Yabe, S., Shin-ya, K., Nishiyama, M. & Kuzuyama, T., 2014, Identification and biosynthesis of new acyloins from the thermophilic bacterium *Thermosporothrix hazakensis* SK20-1T, *ChemBioChem*, 15: 527-532.

Parks, D.H. & Beiko, R.G., 2010, Identifying biologically relevant differences between metagenomic communities, *Bioinformatics*, 26: 715-721.

Peralta, R.M., Ahn, C. & Gillevet, P.M., 2013, Characterization of soil bacterial community structure and physicochemical properties in created and natural wetlands, *Science of the Total Environment*, 443: 725–732.

Pereyra, L.P., Hiibel, S.R., Prieto Riquelme, M.V., Reardon, K.F. & Pruden, A., 2010, Detection and quantification of functional genes of cellulose-degrading, fermentative

and sulfate-reducing bacteria and methanogenic archaea, *Applied and Environmental Microbiology*, 76: 2192-2202.

Perez, M.T., Hörtnagl, P. & Sommaruga, R., 2010, Contrasting ability to take up leucine and thymidine among freshwater bacterial groups: implications for bacterial production measurements, *Environmental Microbiology*, 12: 74–82.

Pernthaler, J., Posch, T., Šimek, K., Vrba, J., Pernthaler, A., Glöckner, F.O., Nübel, U., Psenner, R. & Amann, R., 2001, Predator-specific enrichment of actinobacteria from a cosmopolitan freshwater clade in mixed continuous culture, *Applied and Environmental Microbiology*, 67: 2145-2155.

Pernthaler, J., Zollner, E., Warnecke, F. & Jürgens, K., 2004, Bloom of filamentous bacteria in a mesotrophic lake: identity and potential controlling mechanism, *Applied and Environmental Microbiology*, 70: 6272–6281.

Pinhassi, J. & Berman, T., 2003, Differential growth response of colonyforming alpha- and gamma-proteobacteria in dilution culture and nutrient addition experiments from Lake Kinneret (Israel), the eastern Mediterranean Sea, and the Gulf of Eilat, *Applied and Environmental Microbiology*, 69: 199–211.

Prescott, L.M., Harley, J.P. & Klein, D.A., 2002, *Microbiology*, 5th ed., McGraw Hill, Boston.

Rabus, R., Gade, D., Helbig, R., Bauer, M., Glöckner, F.O., Kube, M., Schlesner, H., Reinhardt, R. & Amann, R., 2002, Analysis of N-acetylglucosamine metabolism in the marine bacterium *Pirellula* sp. strain 1 by a proteomic approach, *Proteomics*, 2: 649-655.

Rabus, R., Hansen, T.A. & Widdel, F., 2006, Dissimilatory sulfate- and sulfur-reducing prokaryotes, in M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, E. Stackebrandt (eds.), *The Prokaryotes*, 2: 659-768, Ecophysiology and Biochemistry, Springer, Berlin.

Raghoebarsing, A.A., Pol, A., Van de Pas-Schoonen, K.T., Smolders, A.J., Ettwig, K.F., Rijpstra, W.I.C., Schouten, S., Sinninghe Damsté, J.S., Op den Camp, H.J.M., Jetten, M.S.M. & Strous, M., 2006, A microbial consortium couples anaerobic methane oxidation to denitrification, *Nature*, 440: 918-921.

Ratering, S. & Schnell, S., 2001, Nitrate-dependent iron(II) oxidation in paddy soil, *Environmental Microbiology*, 3: 100-109.

Reim, A., Lüke, C., Krause, S., Pratscher, J. & Frenzel, P., 2012, One millimetre makes the difference: high-resolution analysis of methane-oxidizing bacteria and their specific

activity at the oxic-anoxic interface in a flooded paddy soil, *The ISME Journal*, 6: 2128-2139.

Reis, M.P., Barbosa, F.A., Chartone-Souza, E. & Nascimento, A.M., 2013, The prokaryotic community of a historically mining-impacted tropical stream sediment is as diverse as that from a pristine stream sediment, *Extremophiles*, 17: 301-309.

Riemann, L. & Winding, A., 2001, Community dynamics of free-living and particle associated bacterial assemblages during a freshwater phytoplankton bloom, *Microbial Ecology*, 42: 274-285.

Ritchie, D.A., Edwards, C., McDonald, I.R. & Murrell, J.C., 1997, Detection of methanogens and methanotrophs in natural environments, *Global Change Biology*, 3: 339-350.

Roden, E.E. & Wetzel, R.G., 1996, Organic carbon oxidation and suppression of methane production by microbial Fe(III) oxide reduction in vegetated and unvegetated freshwater wetland sediments, *Limnology and Oceanography*, 41: 1733-1748.

Rodionov, D.A., Dubchak, I., Arkin, A., Alm, E. & Gelfard, M.S., 2004, Reconstruction of regulatory and metabolic pathways in metal-reducing *δ-proteobacteria*, *Genome Biology*, 5: R90.

Röske,K., Sachse, R., Scheerer, C., Röske, I., 2012, Microbial diversity and composition of the sediment in the drinking water reservoir Saidenbach (Saxonia, Germany), *Systematic and Applied Microbiology*, 35: 35–44.

Salcher, M.M., Pernthaler, J., Psenner, R. & Posch, T., 2005, Succession of bacterial grazing defense mechanisms against protistan predators in an experimental microbial community, *Aquatic Microbial Ecology*, 38: 215–229.

Schloss, P.D., Gevers, D. & Westcott, S.L., 2011, Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies, *PLoS ONE*, 6: 1-14.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., van Horn, D.J. & Weber, C.F., 2009, Introducing mother: open-source, platform-independent, community-supported software for describing and comparing microbial communities, *Applied and Environmental Microbiology*, 75: 7537-7541.

Schubert, C.J., Durisch-Kaiser, E., Wehrli, B., Thamdrup, B., Lam, P. & Kuypers, M.M.M., 2006, Anaerobic ammonium oxidation in a tropical freshwater system (Lake Tanganyika), *Environmental Microbiology*, 8: 1857-1863.

Sears, K., Alleman, J.E., Barnard, J.L. & Oleszkiewicz, J.A., 2004, Impacts of reduced sulphur components on active and resting ammonia oxidizers, *Journal of Industrial Microbiology and Biotechnology*, 31: 369-378.

Selala, M.C., Botha, A-M., de Klerk, A.R., de Klerk, L.P., Myburgh, J.G., Bletter, M.C.M. & Oberholster, P.J., 2013a, Effects of vegetable oil pollution on aquatic macroinvertebrate assemblage in a freshwater wetland and its use as a remediation tool, *Water Air Soil Pollution*, 224: 1650.

Semrau, J.D., DiSpirito, A.A. & Murrell, J.C., 2008. Life in the extreme: thermoacidophilic methanotrophy, *Trends in Microbiology*, 16: 190–193.

Shange, R., Haugabrooks, E., Ankumah, R., Ibekwe, A.M., Smith, R.C. & Dowd, S., 2013, Assessing the diversity and composition of bacterial communities across a wetland, transition, upland gradient in Macon County Alabama, *Diversity*, 5: 461-478.

Shange, R.S., Ankumah, R.O., Ibekwe, A.M., Zabawa, R. & Dowd, S.E., 2012, Distinct soil bacterial communities revealed under a diversely managed agroecosystem, *PLoS One*, 7: e40338.

Sharma, P.K. & Rao, K.H., 2003, Adhesion of *Paenibacillus polymyxa* on chalcopyrite and pyrite: surface thermodynamics and extended DLVO theory, *Colloids and Surfaces B: Biointerfaces*, 29: 21-38.

Shelton, L. R., 1994, Field guide for collecting and processing stream-water samples for the National Water-Quality Assessment Program, US Geological Survey Open-File Rep, pp. 94-455.

Shen, L., Huang, Q. He, Z., Lian, X., Liu, S., He, Y., Lou, L., Xu, X., Zheng, P. & Hu, B., 2015, Vertical distribution of nitrate-dependent anaerobic methane-oxidizing bacteria in natural freshwater wetland soils, *Applied and Environmental Microbiology*, 99: 349-357.

Šimek, K., Kojecká, P., Nedoma, J., Hartman, P., Vrba, J. & Dolan, J.R., 1999, Shifts in bacterial community composition associated with different microzooplankton size fractions in a eutrophic reservoir, *Limnology and Oceanography*, 44: 1634-1644.

Sims, A., Gajaraj, S. & Hu, Z., 2012, Seasonal population changes of ammonia-oxidizing organisms and their relationship to water quality in a constructed wetland, *Ecological Engineering*, 40: 100-107.

Singer. P.C. & Stumm, W., 1970, Acidic mine drainage: the rate-determining step, *Science*, 167: 1121-1123.

Spencer, C., Robertson, A.I. & Curtis, A., 1998, Development and testing of a rapid appraisal wetland condition index in south-eastern Australia, *Journal of Environmental Management* 54: 143-159.

Spring, S., Schulze, R., Overmann, J. & Schleifer, K.H., 2000, Identification and characterization of ecologically significant prokaryotes in the sediment of freshwater lakes: molecular and cultivation studies, *FEMS Microbiology Reviews*, 24: 573–590.

Stein, O.R., Borden-Stewart, D.J., Hook, P.B. & Jones, W.L., 2007, Seasonal influence on sulfate reduction and zinc sequestration in subsurface treatment wetlands, *Water Research*, 41: 3440-3448.

Stern, D.A., Khanbilvardi, R., Alair, J.C. & Richardson, W., 2001, Description of flow through a natural wetland using dye tracer tests, *Ecological Engineering*, 18: 173-184.

Stevenson, R.J.& Bahls, L.L., 1999, 'Periphyton protocols', in M.T. Barbour, J. Gerritren, B.D. Snyder & J.B. Stribling (eds.), *Rapid bioassessment protocols for use in streams and wadeable rivers: Periphyton, benthic macroinvertebrates and fish*, 2nd ed., pp. 6-22, Washington DC: USA EPA 841-B-99-002.

Straub, K.L., Benz, M., Schink, B. & Widdel, F., 1996, Anaerobic, nitrate-dependent microbial oxidation of ferrous iron, *Applied and Environmental Microbiology*, 62: 1458-1460.

Straub, K.L., Hanzlik, M. & Buchholz-Cleven, B.E.E., 1998, The use of biologically produced ferrihydrite for the isolation of novel iron-reducing bacteria, *Systematic and Applied Microbiology*, 21: 442-449.

Strous, M., Fuerst, J.A., Kramer, E.H.M, Logemann, S., Muyzer, G., van de Pas-Schoonen, K.T., Webb, R., Kuenen, J.G. & Jetten, M.S.M., 1999, Missing lithotroph identified as new planctomycete, *Nature*, 400: 446-449.

Tang, K., Baskaran, V. & Nemati, M., 2009, Bacteria of the sulphur cycle: an overview of microbiology, biokinetics and their role in petroleum and mining industries, *Biochemical Engineering Journal*, 44: 73-94.

Todorova, S.G. & Costello, A.M., 2006, Design of *Shewanella*-specific 16S rRNA primers and application to analysis of *Shewanella* in a minerotrophic wetland, *Environmental Microbiology*, 8: 426-432.

Trimmer, M., Nicholls, J.C., Morley, N., Davies, C.A. & Aldridge, J., 2005, Biphasic behaviour of annamox regulated by nitrate in an estuarine sediment, *Applied and Environmental Microbiology*, 71: 1923-1930.

Truu, J., Nurk, K., Juhanson, J. & Mander, Ü., 2005, Variation of microbiological parameters within planted soil filter for domestic wastewater treatment, *Journal of Environmental Science and Health*, 40: 1191-1200.

Turpie, J.K., Marais, C. & Blignaut, J.N., 2008, The working for water programme: evolution of a payments for ecosystem services mechanisms that addresses both poverty and ecosystem service delivery in South Africa, *Ecological Economics*, 788-798.

Ueda, T., Suga, Y. & Matsuguchi, T., 1995, Molecular phylogenetic analysis of a soil microbial community in a soybean field, *European Journal of Soil Science*, 46: 415-421.

van der Nat, F.W.A. & Middelburg, J.J., 1998, Effects of two common macrophytes on methane dynamics in freshwater sediments, *Biogeochemistry*, 41: 1-22.

van Hannen, E.J., Zwart, G., van Agterveld, M.P., Gons, H.J., Ebert, J. & Laanbroek, H.J., 1999, Changes in bacterial and eukaryotic community structure after mass lysis of filamentous cyanobacteria associated with viruses, *Applied and Environmental Microbiology*, 65: 795–801.

Vergin, K.L., Urbach, E., Stein, J.L., DeLong, E.F., Lanoil, B.D. & Giovannoni, S.J., 1998, Screening of a Fosmid Library of Marine Environmental Genomic DNA Fragments Reveals Four Clones Related to Members of the Order *Planctomycetales*, *Applied and Environmental Microbiology*, 64: 3075-3078.

Vermeulen, P.D. & Usher, B.H., 2006, Sulphate generation in South African underground and opencast collieries, *Environmental Geology*, 49: 552-569.

Visser, J.M., de Jong, G.A.H., Robertson, L.A. & Kuenen, J.G., 1997, A novel membrane-bound flavocytochrome *c* sulphide dehydrogenase from the colourless sulfur bacterium *Thiobacillus* sp. W5, *Archives of Microbiology*, 167: 295-301.

Vureen, L., 2009, World mine-water experts to gather in Pretoria, www.wrc.org.za/News/Pages/Worldmine.

Wagner, M. & Horn, M., 2006, The *Planctomycetes*, *Verrucomicrobia*, *Chlamydiae* and sister phyla comprise a superphylum with biotechnological and medical relevance, *Current Opinion in Biotechnology*, 17: 241–249.

Wang, Y., Lin, W., Li, J. & Pan, Y., 2013, High diversity of magnetotactic Deltaproteobacteria in a freshwater niche, *Applied and Environmental Microbiology*, 79: 2813-2817.

Wang, Y., Sheng, H.F., He, Y., Wu, J.Y., Jiang, Y.X., Tam, N.F.Y. & Zhou, H.W., 2012, Comparison of the levels of bacterial diversity in freshwater, intertidal wetland, and

marine sediments by using millions of Illumina tags, *Applied and Environmental Microbiology*, 78: 8264-8271.

Warnecke, F., Sommaruga, R., Sekar, R., Hofer, J.S. & Pernthaler, J., 2005, Abundances, identity, and growth state of *Acidobacteria* in mountain lakes of different UV transparency, *Applied and Environmental Microbiology*, 71: 5551–5559.

Weber, K.A., Achenbach, L.A. & Coates, J.D., 2006a, Microorganisms pumping iron: anaerobic microbial iron oxidation and reduction, *Nature Reviews*, 4:752-764.

Weber, K.A., Picardal, F.W. & Roden, E.E., 2001, Microbially catalysed nitrate-dependent oxidation of biogenic solid-phase Fe(II) compounds, *Environmental Science and Technology*, 35: 1644-1650.

Weber, K.A., Urrutia, M.M.,Churchill, P.F., Kukkadapu, R.K. & Roden, E.E., 2006b, Anaerobic redox cycling of iron by freshwater sediment microorganisms, *Environmental Microbiology*, 8: 100-113.

Weisburg, W.G., Barns, S.M., Pelletier, D.A. & Lane, D.J. 1991, 16S ribosomal DNA amplification for phylogenetic study, *Journal of Bacteriology*, 173: 697–703.

Weiss, P., Schweitzer, B., Amann, R. & Simon, M., 1996, Identification *in situ* and dynamics of bacteria on limnetic organic aggregates (lake snow), *Applied and Environmental Microbiology*, 62: 1998–2005.

Wenter, R., Wanner, G., Schüler, D. & Overmann, J., 2009, Ultrastructure, tactic behaviour and potential for sulfate reduction of a novel multicellular magnetotactic prokaryote from North Sea sediments, *Environmental Microbiology*,11: 1493–1505.

Whitford, W.G., 1992, Biogeochemical consequences of desertification, *ACS Symposium Series*, 483: 352-359.

Widdel, F., Schnell, S., Heising, S., Ehrenreich, A., Assmus, B. & Schink, B., 1993, Ferrous iron oxidation by anoxygenic phototrophic bacteria, *Nature*, 362: 834-836.

Winogradsky, S., 1887, Über Schwefelbakterien, *Botan Ztg*, 45: 489-507, 513-523.

Wise, M.G., McArthur, J.V. & Shimkets, L.J., 1997, Bacterial diversity of a Carolina bay as determined by 16S rRNA gene analysis: confirmation of novel taxa, *Applied and Environmental Microbiology*, 63: 1505-1514.

Wobus, A., Bleul, C., Maassen, S., Scheerer, C., Schuppler, M., Jacobs, E. & Röske, I., 2003, Microbial diversity and functional characterization of sediments from reservoirs of different trophic state, *FEMS Microbiology Ecology*, 46: 331-347.

Wu, Q.L., Zwart, G., Schauer, M., Kamst-van Agterveld, M.P. & Hahn, M.W., 2006, Bacterioplankton community composition along a salinity gradient of sixteen high-

mountain lakes located on the Tibetan Plateau, China, *Applied and Environmental Microbiology*, 72: 5478–5485.

Xie, J., He, Z., Liu, X., van Nostrand, J.D., Deng, Y., Wu, L., Zhou, J. & Qiu, G., 2011, GeoChip-Based analysis of the functional gene diversity and metabolic potential of microbial communities in acid mine drainage, *Applied and Environmental Microbiology*, 77: 991-999.

Yabe, S., Aiba, Y., Sakai, Y., Hazaka, M. & Yokota, A., 2010, *Thermosporothrix hazakensis* gen. nov., sp. nov., isolated from compost, description of *Thermosporotrichaceae* fam. nov. within the class *Ktedonobacteria* Cavaletti et al. 2007 and emended description of the class *Ktedonobacteria*. *International Journal of Systematic and Evolutionary Microbiology*, 60: 1794-1801.

Ye, W.J., Liu, X.L., Lin, S.Q., Tan, J., Pan, J.L., Li, D.T. & Yang, H., 2009, The vertical distribution of bacterial and archaeal communities in the water and sediment of Lake Taihu, *FEMS Microbiology Ecology*, 70: 263–276.

Yu, Y., Wang, H., Liu, J., Wang, Q., Shen, T., Guo, W. & Wang, R., 2012, Shifts in microbial community function and structure along the successional gradient of coastal wetlands in Yellow River Estuary, *European Journal of Soil Biology*, 49: 12-21.

Zeder, M., Peter, S., Shabarova, T. & Pernthaler, J., 2009, A small population of planktonic Flavobacteria with disproportionally high growth during the spring phytoplankton bloom in a prealpine lake, *Environmental Microbiology*, 11: 2676–2686.

Zedler, J.B. & Kercher, S., 2005, Wetland resources: status, trends, ecosystem services, and restorability, *Annual Review of Envirnomental Resources*, 30: 39-74.

Zhang, Y., Ruan, X.H., Op den Camp, H.J.M., Smits, T.J.M., Jetten, M.S.M. & Schmid, M.C., 2007, Diversity and abundance of aerobic and anaerobic ammonium-oxidizing bacteria in freshwater sediments of the Xinyi River (China), *Environmental Microbiology*, 9: 2375-2382.

Zhu, G., Jetten, M.S., Kuschk, P., Ettwig, K.F. & Yin, C., 2010, Potential roles of anaerobic ammonium and methane oxidation in the nitrogen cycle of wetland ecosystems, *Applied Microbiology and Biotechnology*, 86: 1043-1055.

Zwisler, W., Selje, N. & Simon, M., 2003, Seasonal patterns of the bacterioplankton community composition in a large mesotrophic lake, *Aquatic Microbial Ecology*, 31: 211–225.

Chapter 4: Rapid assessment tool for wetland ecosystem health

4.1 Introduction

With a growing population and already stressed natural resources, South Africa is staring water scarcity in the eye. This is due to the limited natural freshwater resources that are not only due to population growth, but also that of the economy. With agriculture (62%) and mining (2.5%) being the major water consumers, this semi-arid country will face shortages in the future (Mathys *et al*., n.d.). Thus it is important to not only preserve the remaining freshwater sources, but also to rehabilitate impacted waters and prevent future degradation. Research has shifted from treating anthropogenic impacts to possible prevention through early detection methods. Biological indicators have been widely used as surrogates for early detection when ecosystem health is being assessed (Rooney & Bayley, 2012).   These early warning tools firstly measure future effects on the ecosystem and secondly monitor the success of rehabilitation efforts (Lemly & King, 2000). Bioindicators also provide the opportunity to link scientific and monitoring data to wetland conservation, enabling scientists and government to use the same monitoring techniques with ease (Spencer *et al*., 1998), placing risk assessment into ecosystem context (Lemly, 1997). Rapid assessment techniques need to be anticipatory, sensitive, fast, cost effective, reproducible and relevant to the anthropogenic impact and area (van Dam *et al*., 1998).

Various bioindicator organisms have successfully been used in research to assess ecosystem health. Macroinvertebrates are a popular choice for monitoring rivers and wetlands for various nutrient inputs and are also used in the South African Scoring System (SASS) (Dickens & Graham, 2002; Rooney & Bayley, 2012; Selala *et al*., 2013a). Periphyton can be used to evaluate both nutrient input and acidification, including chlorophyll-a quantification (Ayeni *et al*., 2010; Lee *et al*., 2013; Oberholster *et al*., 2013b). Other authors have proposed the use of multiple indicators, such as birds, plants and insects (Rooney & Beyley, 2012), insect-bacteria (Lemly & King, 2000) as well as sediment, fringing vegetation, aquatic vegetation and water quality in a rapid appraisal for wetlands (Spencer *et al*., 1998). Thus these organisms can be either indicative of one certain stressor in the ecosystem or a number, revealing the direction in which a habitat is moving in response to the change (Lee *et al*., 2013). Keeler & McLemore (1996) proposed that monitoring organisms at different trophic levels may reveal bioaccumulation and more rapidly remove these contaminants from the system. Biological indicators are formulated on the notion that the loss or dominance of a

specific species would be a result of biochemical or physical changes in the environment (Bellinger & Sigee, 2010). The three basic approaches used for biological monitoring are 1) indicator species, 2) community structure and 3) biotic indices. The latter are developed to encompass species richness, abundance and trophic structure and should be broadly based, multimetric, responsive and a simplification of complex environmental data (Hill *et al*., 2000). The sensitivity of biomonitoring is dependent on the predetermined endpoints (Oberholster *et al*., 2008). These endpoints should include the evaluation of chemical pollutants, physical disturbances as well as habitat changes within a system and should be summarized in reference to a developed tool or index (Stapanian *et al*., 2013). The ultimate goal of biological indicators is to identify stressors and the response by biota into ecosystem context (Lemly, 1997), and a strategy can be formulated to restore the environment to pre-change conditions (Lee *et al*., 2013). When a bioindicator is selected, there are some considerations that will determine the bioindicator's effectiveness. The selected organisms should have spatial and temporal variability, should not be destructive, highly responsive to environmental change and be cost-effective and simple to apply (Spencer *et al*., 1998). The latter is important when considering the use by governmental non-experts. A sound knowledge of trophic level interaction and pollutant accumulation is also needed (Livingstone, 1993; Lee *et al*., 2013).

Microbial populations are complex, even as primary producers they form an important part of the wetland food chain (Daufresne & Loreau, 2001). Their ubiquitous nature is due to their ability to adapt and proliferate in almost any environment. This is explained by the following ecological theories that are applicable to populations of any organism. Firstly, Liebig's law of the minimum state that the total population of any organism will be determined by the nutrient present in limiting concentrations in relation to the requirements of that organism (Liebig, 1840). The second is Shelford's law of tolerance, which details that an organism's proliferation in an environment is based on a complex set of conditions. Each individual/population has a certain range for an environmental factor or combination of factors that determine the survival of a population (Shelford, 1913). Thus both the presence and success of a population in an ecosystem depend on both the nutritional requirements and tolerance to environmental change. The tolerance range for one parameter may also be influenced by another, as with the case of pH and temperature.

Species diversity also plays a role as it maintains population stability (Atlas & Bartha, 1998). It is essential to keep in mind that a single bacterial species cannot be singled out when assessing environmental conditions and establishing ecosystem health. Bacteria are diverse and adapt rapidly to change through the acquisition of beneficial traits through horizontal gene transfer or mutation. Bacterial population interactions with both biotic and abiotic parameters may however give insight into the ecosystem health. Their rapid generation time enable fast assessment of conditions, enable the use of multiple temporal scales and investigation of community-level response to environmental change (Jessup *et al*., 2004).

A population stability model was used in this study and adapted to analyse next-generation sequencing data from mostly natural environments to severely impacted environments within a wetland system. This along with physicochemical data was used to determine the status of different survey sites within the same wetland. A microbial bioindicator for the use in aquatic environments was developed using abovementioned data and its applicability was tested on bacterial environments such as wetland water column and substrate sediment. The application of this biomonitoring tool was then compared to an existing AMD screening tool.

4.2 Materials and Methods

4.2.1 Model description

The model used to describe and assess the stability of the bacterial populations within the wetland is described below. The model for pattern recognition was adapted from Zdyb (1999). This model describes 12 population scenarios that can arise due to environmental impacts on the population size and species diversity (Figure 1). In this model, a stable community is where high population numbers (sequence numbers) and high species diversity occurs. Increased diversity also increases the food-web stability. Thus the removal or addition of a single significant species may impact for example stability. However, it should be noted that diversity is not the force behind stability. Stability within an ecosystem relies on the system's ability to retain species or groups that can counter changes brought into the environment (Pimm, 1984; McCann, 2000).
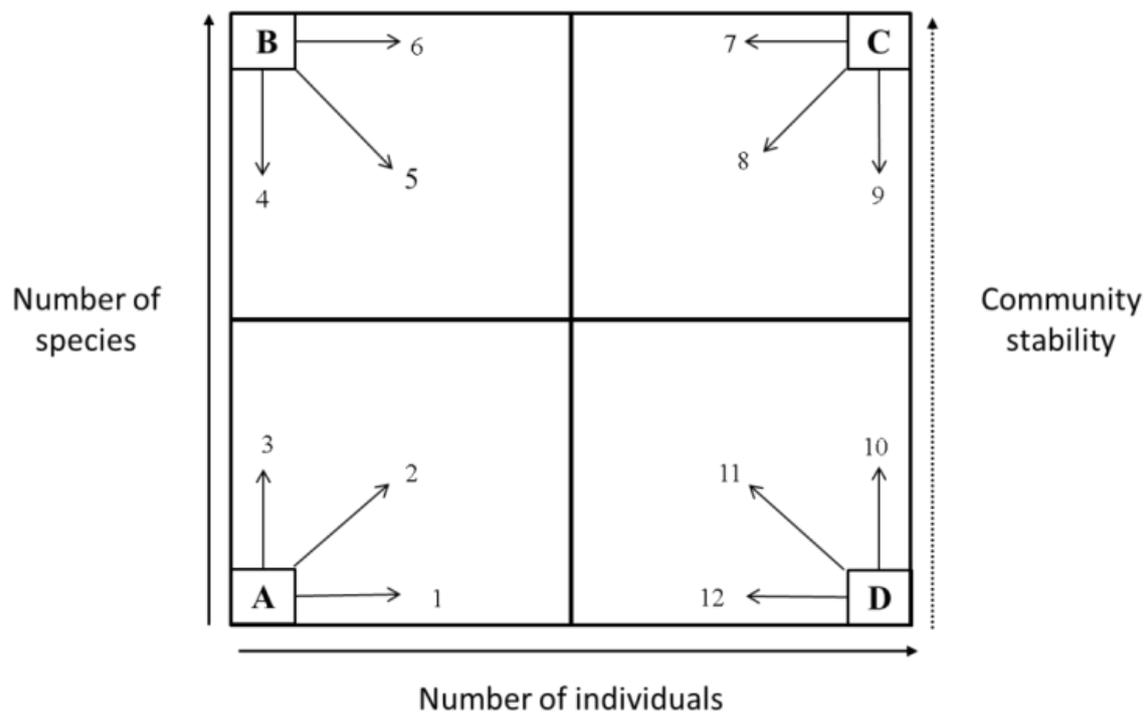
Figure 1. A theoretical model of microbial habitats based on species diversity (adapted from Zdyb, 1999). On the y-axis is the number of different species identified and on the x-axis the total number of individuals in the population. Scenario C represents a stable population, B a stressed population, D an extreme population and A, a sterile or pioneer population.

Description of model environment scenarios based on species richness and population size:

A1 – There is an increase in bacterial cell mass with a low number of species (scenario 'D'). This is due to the dominance of a few species in extreme environments.

A2 – This community is moving towards stability (scenario 'C'). Both the numbers of the individuals and the amount of species are increasing, which will be observed in a nutrient rich environment.

A3 – Increased species diversity while the population numbers is limited by environmental stress. Although there are a low number of individuals, the community is moving towards scenario 'B'.

B4 – A sterile scenario can arise when a high number of species are present at low numbers.

B5 – Increased cell numbers can be observed in the community, but the number of species is decreasing. This indicates a movement to an extreme environment (scenario 'D').

B6 – This community is made up of a high number of species, but very low bacterial cells. This type of community is diverse enough to survive stress, due to the species diversity.

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C7 – This is a stable community with the scenario moving towards scenario 'B'. Any stress/pressure on the population will result in decreasing numbers of individuals, but the species diversity will remain high. This stable community thus contains enough genetic information and will be able to return to its original situation after the stress has been eliminated.

C8 – This is a sterile or pioneer scenario (scenario 'A'), caused by extreme stress placed on the population. This will result in either only one or no species present.

C9 – A decrease in species numbers, but population numbers remain high. The decrease in species is a result of environmental stress, which will drive the change to an extreme environment (scenario 'A').

D10 – There is an increase in the number of species, as the population size remains high. This community is moving towards a stable scenario 'C'. Increased nutrients make the environment more favourable to different species.

D11 – The number of species are increasing but the population is decreasing in size. The population is approaching scenario 'B' as nutrients become more limiting or other stressors are present in the environment.

D12 – A stress situation in the environment is decreasing the population size. This will force the population back to a pioneer state or a sterile environment. The latter is due to the fact that low diversity species cannot persist in the stressed situation due to a lack in genetic diversity.

Table 1. EST framework for AMD impacted aquatic systems (Oberholster *et al.*, 2013a).

Ecological categories of wetland (% score)	Critically modified (0-20%)	EST weighted score	Seriously modified (21-40%)	EST weighted score	Modified (41-70%)	EST weighted score	Largely natural with a few modifications (71-100%)	EST weighted score	EST single cumulative value	References
Length of wetland affected (m)	>0.50	2	0.01-0.50	8	<0.01	12	None	20	**20**	Jarvis & Younger (2000)
Substrate quality/habitat assessment	Substrate cemented, cover 4-5 mm thick.	2	Crust only. Sediment cover 2-3 mm thick.	8	Thin layer. Sediment cover 1 mm thick.	12	Free of colouration	20	**20**	Gray (1997)
Total iron (mg/L)	>3	1	3-2	4	2-1	6	<1	10	**10**	Jarvis & Younger (2000)
pH[a], total Al[b], water column turbidity[c]	3 failures	3	2 failures	12	1 failure	18	No failure	30	**30**	Jarvis & Younger (2000), DWAF (1996)
D. magna survival test (%)	<20% of the negative control	1	<20% of the negative control	1	<20% of the negative control	1	>20% of the negative control	10	**10**	Thursby *et al* (1997), Oberholster (2011)
Benthic	0-1.4	1	1.5-2.4	4	2.5-10	6	>10	10	**10**	Niyogi *et al*

filamentous algae biomass (chl-a mg/m^2)		(1999), Bray (2007), Bray *et al*. (2008), Oberholster *et al* (2010)
Total EST score	**100**	

[a] pH values <7, [b] AL concentrations greater than 1 mg/L, [c] turbidity >20 NTU.

4.2.2 Ecotoxicological Screening Tool (EST)

The EST as developed by Oberholster *et al*. (2013a) was used to assess the extent of damage to the study area. Sample collection and data analysis was done as described by the author, summarized below.

Epilithic filamentous algae were identified with the naked eye first, quantified (percentage cover) and then sampled using an extended syringe at each site. Samples were then evaluated to determine abundance of each species by microscopy using the strip-count method. Diatom sampling was done by removal from submerged stones by brushing, where sand and slit was present followed by acid digestion to clear the material for identification. Samples were also analysed for chlorophyll-a (chl-a) content. Stream bottom substrate, canopy cover and bank stability was determined visually. *Daphnia magna* 48h toxicity tests were conducted in triplicate and data was analysed. Data obtained from the above mentioned analysis was then fitted to the EST framework (Table 1) for each site to establish degree of anthropogenic impact.

4.3 Results

4.3.1 EST data analysis

The EST as formulated by Oberholster *et al*. (2013a) was applied to the monitoring of the Zaalklapspruit wetland. The different criteria was measured and scored according to the EST framework (Table 1). Survey site 1 was determined to be mostly natural with few modifications (71-100%) according to the EST. This was attributed to the largely unmodified channel, low metal concentrations and high chlorophyll-a content compared to the other four sites. This indicated that the site was ideal as a reference site within the studied wetland and other impacted sites can be compared to the mostly undisturbed conditions at site 1. Sites 2 and 3 were deemed seriously modified (21- 40%) and in need for possible future restoration efforts. These sites had 0% survival for the *D. magna* 48h toxicity tests, the highest metal concentrations as well as a layer of iron precipitate visible at site 2. Sites 4 and 5 scored 50%, characterising these sites as modified (41-70%).
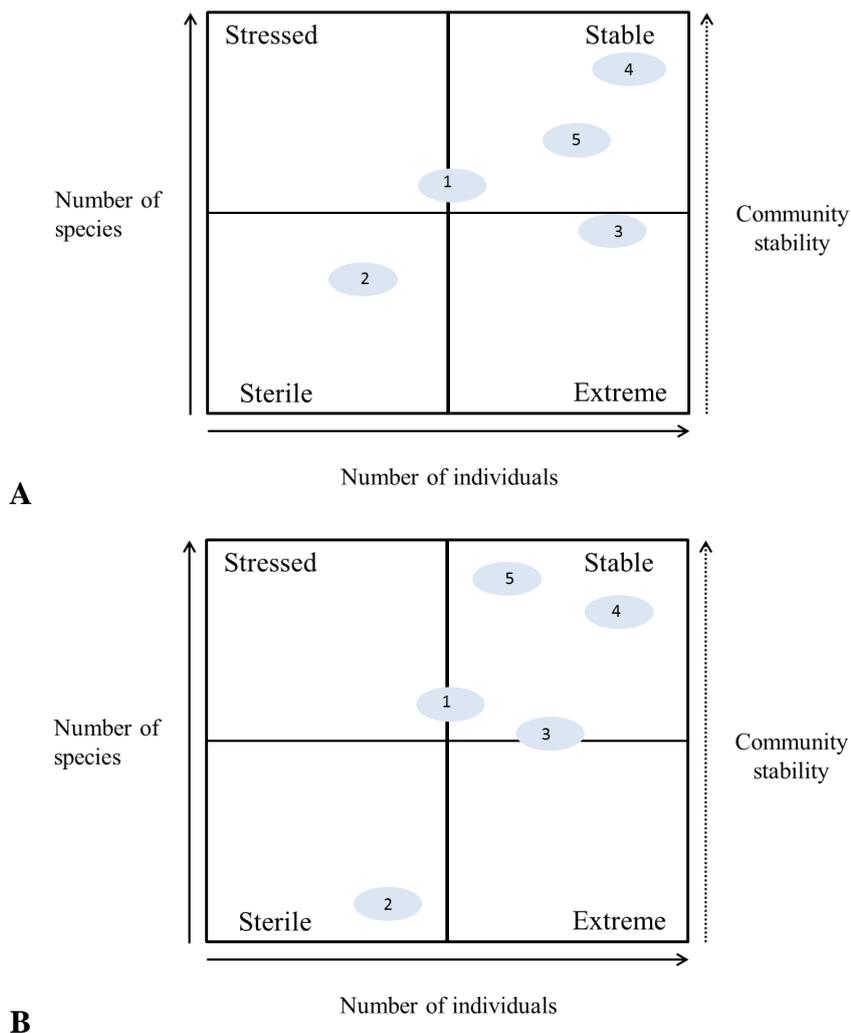
Figure 3. Relative positions of the populations from the surveyed sites according to the environment scenario model. The sites are identified by their site number. A: water bacterial populations and B: sediment bacterial populations.

4.3.2 Bacterial population

The bacterial community structure was defined in terms of the number of individuals, quantified by the number of sequence reads (Nseq) obtained per survey site sample (Chapter 3, Table 2). The number of observed species (Sobs) was also quantified for each instance and served as a measure of diversity. In this study, diversity is defined as the number of different species identified from the 16S rDNA gene sequencing. This definition was adequate for this study (Horner-Devine *et al*., 2003). This made the simplistic approach possible as defined by the Zdyb (1999) model to define population stability. The sites were then spatially compared, assuming site 1 as a stable community as it served as the reference or least impacted site. The bacterial communities of the water column and sediment were assessed separately as

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these two microhabitats differed largely in physicochemical parameters (Figure 3). The bacterial population size (Nseq) and number of species (Sobs) of each site varied both spatially and temporally during the study. These variations may be due to the changing physical environment over the sampling period. The water population at site 4 (January 2013) had both the largest population and diversity (Chapter 3, Table 2), compared to the other sites. In turn the sediment population at site 2 (March 2013) had the lowest compared to the other sites (Chapter 3, Table 2).

The microbial communities were compared spatially and described according to the model proposed (Figure 1). The water bacterial population at site 1, can be described as either C7 (Figure 3A), a stable scenario, yet environmental pressure may push it towards a scenario B population. The sediment population at this site was determined to be C7 (Figure 3B) as well. At site 2, the water population was low in diversity, yet had high numbers of sequences. This represented a C8 (Figure 3A) scenario, a sterile/pioneer population under environmental stress. The scenario for the sediment population of site 2 was A1 (Figure 3B), due to the dominance of only a few species. For site 3, the water population was determined to fit the description of a scenario D10 (Figure 3A), moving towards stability. Sediment populations were best described by a scenario A2 (Figure 3B), moving towards stability. Both populations at site 4 had high numbers of sequences and species, thus the water population was best described by scenario A2 (Figure 3A). Scenario D11, with an increase in species, yet decrease in individuals, best described the sediment populations (Figure 3B). Environmental stress resulted in C9 (Figure 3A) being the best description for the water populations at site 5. Lastly, the sediment population best fit the scenario D11 description (Figure 3B).

4.4 Discussion

Assessing ecosystem health by only monitoring water column chemistry is ineffective in determining ecosystem impact, at most only providing a 'snapshot' of the conditions present. Thus rapid assessment tools have been developed and incorporated in environmental management. Revision of rapid assessment methods would enable validation of suitable techniques and results from assessment studies (Spencer *et al*., 1998).

The microbial populations present in both the water column and sediment provided enough information to determine the ecological state of these habitats. These free-living bacteria make up most of the microbial biomass (Horner-Devine *et al*., 2003). The bacterial populations were classified according to the proposed model's environmental scenarios. The use of next-generation sequencing (NGS) was to include all possible species, both culturable and unculturable. A previous study also proved that using NGS reveals higher diversity than clone libraries or DGGE analysis (Staley *et al*., 2013). The use of the 16S rDNA gene sequence was motivated by the vast database of this gene for identification of bacterial species. Hypervariable regions within this gene have resulted in deep sequencing of microbial populations, sufficient to identify rare species in functional diverse habitats (Kysela *et al*., 2005; Sogin *et al*., 2006). We did not focus on suspected extremophiles, as the significance of heterotrophic bacteria is almost always ignored (Boon *et al*., 1996). Below, the five survey sites are discussed separately along with possible stressors:

Site 1

This site was the most unspoiled and served as a reference site for the wetland system under study. The water quality at this site was the highest than that of all the sites tested, where metal concentrations (Chapter 2, Table 3) were below the DWAF standards for Al, Fe and Mn (DWAF, 1996abc). Because this is the reference site, it was assumed that the microbial population was stable. Thus this population was best described by environment scenario C7 (Figure 3A). The environmental stress that caused this pressure on the population may include the low dissolved oxygen levels (Chapter 2, Table 3) measured at this site that inhibit decomposition capabilities of the population and selecting for facultative organisms within the population to dominate. The high organic carbon levels (Chapter 2, Table 3) may also have contributed to the low metal pollution levels, as it can bind and chelate metals out of solution (Atlas & Bartha, 1998). The sediment microbial population was characteristic of a C7 environment scenario (Figure 3B). The smaller population size was possibly due to environmental stress. This could have been because of the anaerobic conditions within the upper sediment, indicated by the negative redox potential (Chapter 2, Table 3) (Kalff, 2002). The high concentration of Al (Chapter 3, Table 1) could also negatively affect the population, even though it is not as high as at the other sites surveyed in this study. This population was more stable than that of the water community due to the buffering effect of sediment and the availability of carbon at this site (Chapter 3, Table 1).

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Table 3. Ecotoxicological screening tool (EST) actual scores (%) for the five survey sites within the Zaalklapspruit wetland (n=3).

Ecological category of wetland and % score	EST Single cumulative value	Site 1		Site 2		Site 3		Site 4		Site 5	
		Data	Score	Data	Score	Data	Score	Data	Score	Data	Score
Length of wetland affected (m)	**20**	0	20	>0.5	2	>0.5	2	>0.5	2	>0.5	2
Substrate quality	**20**	Free of colour	20	Layer cover 4-5 mm	2	Free of colour	20	Free of colour	20	Free of colour	20
Total Fe (mg/L)	**10**	29.2	2	8696.7	2	206.7	2	17.2	2	26.3	2
pH[a], total Al[b], water column turbidity[c]	**30**	1 failures	18	2 failures	12	2 failures	12	2 failures	12	2 failures	12
D. magna survival (%)	**10**	73%	10	0%	1	0%	1	83%	10	77%	10
Benthic filamentous algae biomass (chl-a mg/m^2)	**10**	11.1	10	6.8	6	0.23	1	2.38	4	1.6	4
Total EST score	**100**	Natural	**82**	Seriously modified	**25**	Seriously modified	**38**	Modified	**50**	Modified	**50**

[a] pH values < 7, [b] Al concentrations greater than 1 mg/L, [c] turbidity > 20 NTU.

Site 2

This site was visually, hydrological sterile with clear water and little apparent aquatic life apart from the macrophytes. Metal hydroxide precipitate (e.g. ferric hydroxide (FeOH3)) (Jarvis & Younger, 2000) was also visual on the bottom substrate (Figure 4). The water conditions also indicated that it would be close to sterile. Extreme conditions were observed including low pH (lowest of 2.8 in January) (Chapter 2, Table 3), high conductivity in the water column, increased redox potential in the sediment and dissolved oxygen levels and overall aerobic conditions were present (Chapter 2, Table 3). Sulphates and dissolved Al, Fe and Mn increased drastically from site 1 and lower levels of organic carbon were also present (Chapter 2, Table 3). These conditions created a hostile environment. The bacterial population in the water column was characteristic of a C8 scenario, due to environmental stress on the bacterial community. These stressors will push the population to a sterile/pioneer state (scenario A), where few species may survive in low numbers, or die off completely. The extreme conditions selected for stenotolerant species, leading to the assumption that if the environmental conditions do not improve, the population will remain in the sterile/pioneer state. Moderate loss of species diversity may impact important functions within the population, hampering survival and recovery (Singh *et al*., 2014). Methane consumption has been reported to take several years to recover after environmental change (Mosier *et al*., 1997). The sterile environment scenario could be compared to the death phase of bacterial growth where either nutrients have become limiting or environmental change decreases the survival rate of microbial cells. The strongest environmental factor to influence populations were pH (Hartman *et al*., 2008; Nicol *et al*., 2008; Wang *et al*., 2009; Lear *et al*., 2009; Lauber *et al*., 2009; Kuang *et al*., 2013; Bartram *et al*., 2014) and increased metal concentrations (Gans *et al*., 2005; Singh *et al*., 2014).

The sediment population was described as an A1 scenario (Figure 3B) due to very low species diversity compared to the other sites. This low diversity could lead to a sterile/pioneer population. The highly selective conditions would allow extremophile organisms to occur here. These organisms would be stimulated by very low pH and increased sulphate, Al and Fe concentrations (Chapter 2, Table 3; Capter 3, Table 1). The dominant influences on soil function are short-term, direct changes in physiology, with long term effects in the population composition (Schimel *et al*., 2007). This could be attributed to the specialized species found in sediment; these organisms tend to be more sensitive to change (Schimel, 1995). However, regular, episodic stress in the environment enhances

tolerance to these types of stressors, a result of selective pressure on the bacterial population (van Gestel *et al*., 1993; Fierer *et al*., 2003; Steenwerth *et al*., 2005).
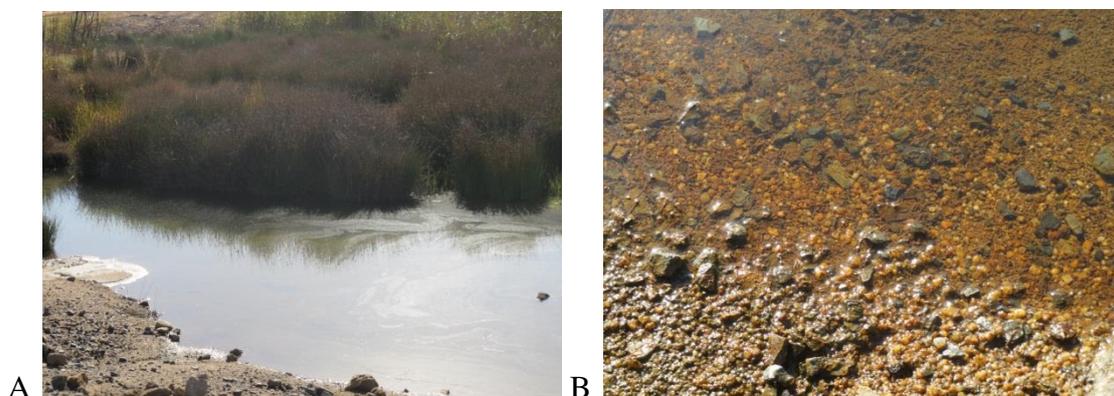

Figure 4. A: Site 2 lacking apparent aquatic life. B: Metal hydroxide precipitation at site 2.

Site 3

Here environmental conditions have improved from site 2, yet are not comparable to the reference site. This was highlighted by increased dissolved solids and dissolved metal concentrations, which remained high despite a decrease from site 2 and decreased carbon concentrations (Chapter 3, Table 3). The situation was aggravated by the location of the sampling site. Due to accessibility, samples were taken at the downstream end of a small bridge. The site was also next to a road. A small in-wetland pond was formed within the wetland stream, reducing flow, which acted as a sink for Fe, indicated by the increased concentrations measured at this site (Chapter 2, Table 3). There was an expected increase in the population size and diversity, as diversity is determined by the energy available in the system (Jessup *et al*., 2004). The water microbial population was classified under scenario D10 (Figure 3A), as the increase in nutrients resulted in an increase in diversity. The microbial population was shifting towards stability, possibly due to adaptation to the environment.

The sediment bacterial population was best represented by the definition for scenario A2 (Figure 3B). As both the population size and diversity increases, the community is approaching stability. It has been proven previously that stability is linked to complexity of the communities, as demonstrated by the shifts mentioned above (MacArthur, 1955). Thus the presence of most if not all major metabolic processes are expected at this site as in any other environment (Xie *et al*., 2011). The latter was not traditionally thought to be the case.

Response by these multifaceted communities to environmental stress may impact diversity and function disproportionately, which could veil the initial response to anthropogenic impact (Yergeau *et al*., 2012). The opposite has also been found in studies of AMD microbial diversity, where the low diversity reported was attributed to limited electron donors and acceptors (Bruneel *et al*., 2006).

Site 4

The conditions at site 4 remained poor as effluent from the Highveld Steel smelters activity upstream was received. Conductivity and TDS remained high even though a decrease was observed, turbidity increased drastically and the redox potential decreased (Chapter 2, Table 3). This could be due to very low water levels (14 cm) at this site. This, along with sulphate concentrations that remained high, placed pressure on the aquatic system (Chapter 2, Table 3). Dissolved metal concentrations were almost at the levels present at site 1 and an increase in available organic carbon was measured (Chapter 2, Table 3). The increased organic carbon could adsorb iron (Chapter 2, Table 3), causing the reduction measured (Limpitlaw, 1996). The water population was determined to fit best the description of scenario A2. The population was stable as both population size and species diversity drastically increased. Both the size and diversity was the highest for this population compared to all the other samples. The sediment at site 4 had the highest concentrations for Al, Fe and Mn (Chapter 2, Table 3), which could limit the population to those that could survive under such conditions, selecting for extremophiles. The sediment population was characterised as a D11 population, with increasing species diversity, yet decreasing population size. The latter could be a result of adaptation to the high metal content. The microbial population differences between and within (water vs. sediment) could be due to small niches forming within the wetland ecosystem, resulting in different community fingerprints (Tyson *et al*., 2004).

Site 5

Water quality conditions improved at this site (compared to the other sites) after flowing downstream through the wetland. However conductivity and TDS was the highest at this site (Chapter 2, Table 3). Sulphate concentrations were almost as high as at site 2 as well as dissolved Mn concentration (Chapter 2, Table 3). Aluminium concentrations were also relatively high compared to site 4 and the available organic carbon concentrations decreased compared to site 4 (Chapter 2, Table 3). These conditions indicated that the microbial populations may be under stress. This stress scenario may only be transient, as it has been

noted that competitors may coexist on the same resource (i.e. carbon), as long as one/all are controlled by another limiting factor (i.e. Al concentrations) (Daufresne & Loreau, 2001). Thus there exists a strict balance within these communities, which may be elucidated through investigation into bacterial function. The water bacterial population decreased in size and diversity, thus best described as a scenario C9 population (Figure 3A). Although there was a marked decrease, the population size remained high. If these decreases continue, the population would be driven to a sterile/pioneer state. The sediment population increased in diversity, yet the population size decreased, thus as a D11 scenario, the population is moving towards a stressed state. Both microbial populations stabilized relatively at this site, as would be expected at a downstream site within a wetland. Water quality is expected to improve as the water flow through the wetland system.

The microbial consortium within a wetland plays an important role in the nutrient cycling as well as catalysing chemical transformations under changing oxic and anoxic conditions within both the soil and water. They form the basis of any food web and are first to respond to changing conditions within a habitat. Thus changing chemical and physical parameters within a wetland will not only impact the microbial populations, but also other organisms which rely on their presence (Yergeau *et al*., 2012; Sims *et al*., 2013). Incorporating these organisms into already existing ecosystem health tools are important to gain a more complete understanding of the impact pollutants have on natural habitats. The influence of wetland modification on bacterial populations has not been investigated to satisfaction (Atkinson *et al*., 2011). Another point where there is a lack of understanding is the link between the wetland bacterial community, biochemical cycling and overall ecosystem function. The latter is due to little interdisciplinary studies being conducted (Gutknecht *et al*., 2006). Dominance of a particular group is facilitated by the competition for common electron donors. Species capable of utilizing electron acceptors with higher redox potential will deplete the common electron donor more rapidly than the unsuccessful species (Achtnich *et al*., 1995). This complex interaction between species capable of different metabolic processes and the potential of genetic exchange within a bacterial population will make it imprudent to select specific species to serve as biological indicators of ecosystem health (Pàl *et al*., 2005). Not ignoring the fact that extreme environments will select for species with unique adaptations to survive. Thus the microbial population as a whole should be evaluated at each selected site.

Thus this study evaluated the population as a whole to identify shifts in its composition rather than focussing on the presence of certain species in response to environmental change. This approach was used because it is simple and easily reproduced, even by non-specialists, which is the aim in the use of bioindicators. The slight adaptation of the model proposed by Zdyb (1999) was adequate to describe both the water and soil microbial populations within this wetland and expected trends were identified. It is important to decide at the start of the survey which populations will be assessed. From the data presented, the author would recommend evaluation of both, as these are two different, yet interlinked habitats. This approach was advantageous as sediment can be used as both short-term and long-term assessment strategies along with water quality monitoring. Metal species retained in wetland soils can be indicative of their time in the system as some form over longer periods in time. Solids formed over short periods of time include ferrihydrite, goethite and amorphous iron sulphides. Those formed over longer periods of time include jarosite and pyrite (Limpitlaw, 1996). Although bacterial biomass in soil only makes up less than 5% of organic content, their impact on ecosystem processes should not be underestimated (Li *et al*., 2009). The most prominent parameter seemed to be pH, influencing both abundance and diversity of communities (Hartman *et al*., 2008).

Comparing the bacterial model to the EST scores revealed some congruity. Both models supported the choice in reference site, with site 1 scoring 82% (mostly natural) according to the EST and both the water and soil microbial populations were deemed stable. However the latter was not definitive as these populations were potentially sensitive to stress in the environment which could cause a shift away from stability. Both sites 2 and 3 scored low according to the EST and were defined as seriously modified. Lastly sites 4 and 5 scored 50%, indicating that these sites are modified and that the water column conditions are improving downstream within the study wetland.

To strengthen the microbial model, the author would suggest including it in the EST framework, for a multifaceted monitoring tool. It would be suggested that a water column population is used for this as a large proportion of the EST framework involves water quality and related tests. Scoring total should be ten, a stable population will score ten, a stressed population six, extreme populations scoring four and a sterile/pioneer population scoring the lowest, one. The total EST score will then be 110, which is reworked to reveal the ecological

category of the wetland. The bacterial model did not change the final classification of the sites according to the EST.

4.5 Conclusion

Sims *et al*. (2013) pressed the importance of a bacterial bioindicator, which was one of the driving forces of this study, as multiple rapid bioassessment tools is needed in order to evaluate ecosystem health. Bacteria are ideal for the use in rapid assessment as they do not only have short generation times, but a thin cell membrane, making them sensitive to environmental change as well as the largest surface-area ratio of all living organisms. Different environmental impacts work on both physiological and community level and these interactions may be interpreted as linkages between the environment and biogeochemical processes (Schimel *et al*., 2007). By engaging in multidisciplinary environmental research, the understanding of wetland functioning can be achieved. This could provide a link between bacterial community composition, biochemical cycling and points of impact (Gutknecht *et al*., 2006). Stressed food webs tend to be short and less complex than that of natural environments, with a decrease in interactions between trophic levels; this in turn may influence nutrient cycling (Hogsden & Harding, 2012). A biological approach is not only more specific than chemical and physical methods, but also more applicable (Kosolapov *et al*., 2004). The notion of basing ecological stability on complexity, whether it is within a food web or population, has been proposed in previous studies. Complexity enables the community to cope with pollutants and recover after the stress has been relieved (Gray, 1997). The physiological variation within a population will determine survival and dominance in changing habitats (Peralta *et al*., 2012). Stressed food webs/communities will be shorten/less diverse due to the loss of sensitive species, the removal of trophic levels and decreased interactions between species. These changes will affect biochemical cycling of nutrients and upper trophic levels (Hogsden & Harding, 2012).

This study demonstrated that bacteria can be used in rapid bioassessment of impacted aquatic ecosystems and may also be used alongside established bioindicators. In future, different bacterial identification techniques could be investigated to lower the cost.

4.6 References

American Public Health Association (APHA), American Water Works Association (AWWA), and Water Pollution Control Federation (WPCF), 1992, in APHA, AWWA, and WPCF, *Standard Methods for the Examination of Water and Wastewater*, 19th ed., Washington, D.C., USA.

Atlas, R.M. & Bartha, R., 1998, *Microbial ecology: fundamentals and applications*, 4th ed., Benjamin/Cummings, California.

Ayeni, O.O., Ndakidemi, P.A., Snyman, R.G. & Odendaal, J.P., 2010, Chemical, biological and physiological indicators of metal pollution in wetlands, *Scientific Research and Essays*, 5: 1938-1949.

Bartram, A.K., Jiang, X., Lynch, M.D., Masella, A.P., Nicol, G.W., Dushoff, J. & Neufeld, J.D., 2014, Exploring links between pH and bacterial community composition in soils from the Crabstone Experimental Farm, *FEMS Microbiology Ecology*, 87: 403-415.

Bellinger, E.G. & Sigee, D.C., 2010, *Freshwater algae: identification and use as bioindicators*, Wiley-Blackwell, West Sussex.

Biggs, B.J.F. (1996). Patterns in benthic algae of streams In: Stevenson R.J., Bothwell M.L., Lowe, R.L. (eds), Algal ecology: freshwater benthic ecosystems. Academic Press, San Diego, pp. 31-56.

Boon, P.I., Virtue, P. & Nochols, P.D., 1996, Microbial consortia in wetland sediments: a biomarker analysis of the effects of hydrological regime, vegetation and season on benthic microbes, *Marine and Freshwater Research* 47: 27-41.

Bruneel, O., Duran, R., Casiot, C., Elbaz-Poulichet, F. & Personné, J.-C., 2006, Diversity of microorganisms in Fe-As-rich acid mine drainage waters of Carnoulès, France, *Applied and Environmental Microbiology*, 72: 551-556.

Daufresne, T. & Loreau, M., 2001, Ecological stoichiometry, primary producer–decomposer interactions ad ecosystem persistence, *Ecology*, 82: 3069-3082.

Department of Water Affairs and Forestry (DWAF), 1996a, *South African Water Quality Guidelines, Volume 1: Domestic use*, Department of Water Affairs and Forestry, Pretoria.

Department of Water Affairs and Forestry (DWAF), 1996b, *South African Water Quality Guidelines, Volume 4: Agricultural Use: Irrigation*, Department of Water Affairs and Forestry, Pretoria.

Department of Water Affairs and Forestry (DWAF), 1996c, *South African Water Quality*, Department of Water Affairs and Forestry, Pretoria.

Dickens, C.W.S & Graham, P.M., 2002, The South African Scoring System (SASS) version 5 rapid bioassessment method for rivers, *African Journal of Aquatic Science* 27: 1-10.

Fierer, N., Schimel, N.J.P. & Holden, P.A., 2003, Influence of drying-rewetting frequency on soil bacterial community structure, *Microbial Ecology* 45:63-71.

Gans, J., Wolinsky, M. & Dunbar, J., 2005, Computational improvements reveal great bacterial diversity and high metal toxicity in soil, *Science*, 309: 1387-1390.

Gore, J.A., 2006, 'Discharge Measurements and Streamflow Analysis', in F.R. Hauer and G.A. Lamberti (eds.), *Methods in Stream Ecology*, pp. 51-81, Academic Press. San Diego. California, USA.

Gray, N.F., 1997, Environmental impact and remediation of acid mine drainage: a management problem, *Environmental Geology*, 30: 62-71.

Gutknecht, J.L.M., Goodman, R.M. & Balser, T.C., 2006, Linking soil process and microbial ecology in freshwater wetland ecosystems, *Plant Soil*, 289: 17-34.

Hartman, W.H., Richardson, C.J., Vilgalys, R., & Bruland, G.L., 2008, Environmental and anthropogenic controls over bacterial communities in wetland soils, *Proceedings of the National Academy of Sciences*, 105: 17842-17847.

Hill, B.H., Herlihy, A.T., Kaufmann, P.R., Stevenson, R.J., McCormick, F.H. & Burch, J.C., 2000, Use of periphyton assemblage data as an index of biotic integrity, *Journal of the North American Benthological Society*, 19:50-67.

Hogsden, K.L. & Harding, J.S., 2012, Anthropogenic and natural sources of acidity and metals and their influence on the structure of stream food webs, *Environmental Pollution*, 162: 466-474.

Horner-Devine, M.C., Carney, K.M. & Bohannan, B.J.M., 2003, An ecological perspective on bacterial biodiversity, interactions, and ecosystem persistence, *The Royal Society*, 271: 113-122.

Jarvis, A.P. & Younger, P.L., 2000, Broadening the scope of mine water environmental impact assessment: a UK perspective, *Environmental Impact Assessment Review*, 20: 85-96.

Jessup, C.M., Kassen, R., Forde, S.E., Kerr, B., Buckling, A., Rainly, P.B. & Bohannan, J.M., 2004, Big questions, small worlds: microbial model systems in ecology, *TRENDS in Ecology and Evolution* 19: 189-197.

Kalff, J., 2002, *Limnology: inland water ecosystems*, Prentice Hall, Upper Saddle River, New Jersey, USA.

Keeler, A.G. & McLemore, D., The value of incorporating bioindicators in economic approaches to water pollution control, *Ecological Economics* 19: 237-245.

Kosolapvo, D.B., Kuschk, P., Vainshtein, A.V., Wieβner, A., Kästner, M. & Müller, R.A., 2004, Microbial processes of heavy metal removal from carbon-deficient effluents in constructed wetlands, *Engineering in Life Sciences* 4: 403-411.

Kuang, J., Huang, L., Chen, L., Hua, Z., Li, S., Hu, M., Li, J. & Shu, W., 2013, Contemporary environmental variation determines microbial diversity patterns in acid mine drainage, *The ISME Journal*, 7: 1038-1050.

Kysela, D.T., Palacios, C. & Sogin, M.L., 2005, Serial analysis of V6 ribosomal sequence tags (SARST-V6): a method for efficient, high throughput analysis of microbial community composition, *Environmental Microbiology* 7: 356-364.

Lauber, C.L., Hamady, M., Knight, R. & Fierer, N., 2009, Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale, *Applied and Environmental Microbiology*, 75: 5111-5120.

Lear, G., Niyogi, D., Harding, J., Dong, Y. & Lewis, G., 2009, Biofilm bacterial community structure in streams affected by acid mine drainage, *Applied and Environmental Microbiology*, 75: 3455-3460.

Lee, S.S., Gaiser, E.E. & Trexler, J.C., 2013, Diatom-based models for inferring hydrology and periphyton abundance in a subtropical karstic wetland: implications for ecosystem-scale bioassessment, *Wetlands*, 33: 157-173.

Lemly, A.D. & King, R.S., 2000, An insect-bacteria bioindicator for assessing detrimental nutrient enrichment in wetlands, *Wetlands* 20: 91-100.

Lemly, A.D., 1997, Risk assessment as an environmental management tool: considerations for freshwater wetlands, *Environmental Management*, 21: 343-358.

Liebig, J., 1840, *Chemistry in its application to agriculture and physiology*, Taylor and Walton, London.

Limpitlaw, D., 1996, Sediments as indicators of wetland efficacy in ameliorating acid mine drainage, *The Journal of the South African Institute of Mining and Metallurgy*, 97: 309-316.

MacArthur, R., 1955, Fluctuations of animal populations, and a measure of community stability, *Ecology* 36: 533-536.

Macfarlane, D.M., 2013, 'Phase 1 – Planning report: Zaalklapspruit Wetland' (B20G-01)', Working for Wetlands Rehabilitation Programme, SANBI, Pretoria.

Mathys, M., Davey, P., Davis, D., Froneman, J., Sternberg, H., Naidoo, S. & Strake, M., n.d., Ripple effect: assessing water risks in the SA resources sector, equity research, Standard Bank Securities.

McCann, K.S., 2000, The diversity-stability debate, *Nature*, 405: 228-233.

Mosier, A.R., Parton, W.J., Valentine, D.S., Ojima, D.S., Schimel, D.S. & Heinemeyer, O., 1997, CH_4 and N_2O fluxes in the Colorado shortgrass steppe: 2 long-term impact of land use change, *Global Biogeochemical Cycles* 11: 29-42.

Nicol, G.W., Leininger, S., Schleper, C. & Prosser, J.I., 2008, The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria, *Environmental Microbiology*, 10: 2966-2978.

Oberholster, P.J., Botha, A.-M. & Cloete, T.E., 2008, Biological and chemical evaluation of sewage water pollution in the Rietvlei nature reserve wetland area, South Africa, *Environmental Pollution*, 156: 184-192.

Oberholster, P.J., Dabrowski, J. & Botha, A.-M., 2013b, Using modified multiple phosphorous sensitivity indices for mitigation and management of phosphorous loads on a catchment level, *Fundamental and Applied Limnology*, 182: 1-16.

Oberholster, P.J., Genthe, B., Hobbs, P., Cheng, P.H., de Klerk, A.R. & Botha-Oberholster, A.-M., 2013a, An Ecotoxicological screening tool to prioritise acid mine drainage impacted streams for future restoration, *Environmental Pollution*, 176: 244-253.

Oberholster, P.J., McMillan, P., Durapersad, K., de Klerk, A.R. & Botha, A.-M., 2014, Development of a wetland classification and risk assessment index (WCRAI) for natural wetland ecosystems in three ecoregions of South Africa, *Water Air Sediment Pollution*, 225: 1-15.

Pimm, S.L., 1984, The complexity and stability of ecosystems, *Nature*, 307: 321-326.

Porra, R.J., Thompson, W.A. & Kriedemann, P.E., 1989, Determination of accurate extinction coefficient and simultaneous equations for assaying chlorophylls *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectrometry, *Biochimica et Biophysica Acta*, 975: 384-394.

Rooney, R.C. & Bayley, S.E., 2012, Community congruence of plants, invertebrates and birds in natural and constructed shallow open-water wetlands: do we need to monitor multiple assemblages?, *Ecological Indicators* 20: 42-50.

Schimel, J., 1995, 'Ecosystem consequences of microbial diversity and community structure', in F. Chapin & C. Korner (eds.), *Artic and alpine biodiversity: patterns, causes, and ecosystem consequences*, pp. 239-254, Springer-Verlag, Berlin, Germany.

Schimel, J., Balser, T.C. & Wallenstein, M., 2007, Microbial stress-response physiology and its implications for ecosystem function, *Ecology* 88: 1386-1394.

Schloss, P.D., Gevers, D. & Westcott, S.L., 2011, Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies, *PLoS ONE*, 6: 1-14.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., van Horn, D.J. & Weber, C.F., 2009, Introducing mother: open-source, platform-independent, community-supported software for describing and comparing microbial communities, *Applied and Environmental Microbiology*, 75: 7537-7541.

Selala, M.C., Botha, A-M., de Klerk, A.R., de Klerk, L.P., Myburgh, J.G., Bletter, M.C.M. & Oberholster, P.J., 2013a, Effects of vegeTable oil pollution on aquatic macroinvertebrate assemblage in a freshwater wetland and its use as a remediation tool, *Water Air Sediment Pollution*, 224: 1650.

Shelford, V.E., 1913, *Animal communities in temperate America*, University of Chicago, Chicago.

Shelton, L. R., 1994, Field guide for collecting and processing stream-water samples for the National Water-Quality Assessment Program, US Geological Survey Open-File Rep, pp. 94-455.

Sims, A., Zhang, Y., Gajaraj, S., Brown, P.B. & Hu, Z., 2013, Toward the development of microbial indicators for wetland assessment, *Water Research*, 47: 1711-1725.

Singh, B.K., Quince, C., Macdonald, C.A>, Khachane, A., Thomas, N., Al-Soud, W.A., Sørensen, S.J., He, Z., White, D., Sinclair, A., Crooks, B., Zhou, J & Campbell, C.D., 2014, Loss of microbial diversity in soils is coincident with reductions in some specialized functions, *Environmental Microbiology* doi: 10.1111/1462-2920.12353.

Singh, B.K., Quince, C., Macdonald, C.A>, Khachane, A., Thomas, N., Al-Soud, W.A., Sørensen, S.J., He, Z., White, D., Sinclair, A., Crooks, B., Zhou, J. & Campbell, C.D., 2014, Loss of microbial diversity in soils is coincident with reductions in some specialized functions, *Environmental Microbiology* doi: 10.1111/1462-2920.12353.

Sogin, M.L., Morrison, H.G., Huber, J.A., Welch, D.M., Huse, S.M., Neal, P.R., Arrieta, J.M. & Herndl, G.J., 2006, Microbial diversity in the deep sea and the unexplored " rare biosphere", *PNAS* 103: 12115-12120.

Spencer, C., Robertson, A.I. & Curtis, A., 1998, Development and testing of a rapid appraisal wetland condition index in south-eastern Australia, *Journal of Environmental Management*, 54: 143-159.

Staley, C., Unno, T., Gould, T.J., Jarvis, B., Phillips, J., Cotner, J.B. & Sadowsky, M.J., 2013, Application of Illumina next-generation sequencing to characterise the bacterial community of the Upper Mississippi River, *Journal of Applied Microbiology* 115: 1147-1158.

Steenwerth, K.L.L.E., Jackson, F.J., Caldreron, F.J., Scow, K.M. & Rolston, D.E., 2005, Response of microbial community composition and activity in agricultural and grassland soils after a simulated rainfall, *Soil Biology and Biochemistry* 37: 2249-2262.

Stevenson, R.J.& Bahls, L.L., 1999, 'Periphyton protocols', in M.T. Barbour, J. Gerritren, B.D. Snyder & J.B. Stribling (eds.), *Rapid bioassessment protocols for use in streams and wadeable rivers: Periphyton, benthic macroinvertebrates and fish*, 2nd ed., pp. 6-22, Washington DC: USA EPA 841-B-99-002.

Tyson, G.W., Chapman, J., Hugenholtz, P., Allen, E.E., Ram, R.J., Richardson, P.M., Solovyev, V.V., Rubin, E.M., Rokhsar, D.S. & Banfield, J.F., 2004, Community structure and metabolism through reconstruction of microbial genomes from the environment, *Nature* 428: 37-43.

van Dam, R.A., Camilleri, C. & Finlayson, C.M., 1998, The potential of rapid assessment techniques as early warning indicators of wetland degradation: a review, *Environmental Toxicology and Water Quality*, 13: 297-312.

Van Gestel, M., Merckx, R. and Vlassak, K., 1993, Microbial biomass response to soil drying and rewetting: the fate of fast- and slow-growing microorganisms in soils of different climates, *Soil Biology and Biochemistry* 25:109–123.

Wang, X.J., Yang, J., Chen, X.P., Sun, G.X. & Zhu, Y.G., 2009, Phylogenetic diversity of dissimilatory ferric iron reducers in paddy soil of Hunan, South China, *Journal of Soils and Sediments*, 9: 568-577.

Weisburg, W.G., Barns, S.M., Pelletier, D.A. & Lane, D.J. 1991, 16S ribosomal DNA amplification for phylogenetic study, *Journal of Bacteriology*, 173: 697–703.

Xie, J., He, Z., Liu, X., van Nostrand, J.D., Deng, Y., Wu, L., Zhou, J. & Qiu, G., 2011, GeoChip-Based analysis of the functional gene diversity and metabolic potential of

microbial communities in acid mine drainage, *Applied and Environmental Microbiology*, 77: 991-999.

Yergeau, E., Lawrence, J.R., Sanschagrin, S., Waiser, M.J., Korber, D.R. & Greer, C.W., 2012, Next-generation sequencing of microbial communities in the Athabasca River and its tributaries in relation to oil sands mining activities, *Applied and Environmental Microbiology*, 78: 7626-7637.

Zdyb, L., 1999, 'Microbial sulphate reduction as a method of passive treatment of acid mine drainage using undefined carbon sources', MSc Thesis, Department of Microbiology and Plant Pathology, University of Pretoria.

Conclusion

The study was undertaken to determine the biological response of algae and bacteria to acid mine drainage in a freshwater wetland and to establish these organisms as bioindicators. Water quality analysis of the five sites of the Zaalklapspruit wetland showed that both acid mine drainage (AMD) and industrial effluent was responsible for the degradation of the wetland. Site 2 was the most impacted with the lowest pH, compared to the other sites studied. Site 4 was impacted by the industrial effluent from Evraz Highveld Steel and Vanadium. Water quality did however improve downstream in the wetland as expected.

The algal species present at the five study sites were species previously associated with the conditions present at the various sites. The diatom *Nitzschia reversa* (Smith) was common at all five of the study site of the wetland. *Gyrosigma rautenbachiae* (Cholnoky) (site 4) and *Navicula tripunctata* (OF Müller) (site 1) were also found to be abundant at these sites. The diatom *G. rautenbachiae* proved to be an ideal bioindicator for industrial pollution. Thus the autecology of the algae proved a valuable assessment of the aquatic conditions. *Bacillariophyceae* species *Synedra ulna* (Nitzsch), *Nitzschia* spp. and *Cymbella* spp. were found at the acidic sites. The filamentous green algae *Microspora quadrata* (Hazen) (site 2) and *Klebsormidium acidophilum* (Novis) (site 3) were abundant at the sites the most impacted by AMD. Metal tolerant *K. rivulare* (Kützing) were abundant at site 5. Lastly the cyanobacteria *Oscillatoria tenius* (Agardh) and *Glaucospira* sp. were abundant at site 1, which indicated enriched conditions. It was clear from the interaction between environmental conditions, anthropogenic pollution and the algae populations were interlinked. There was a clear three-fold impact resulting from the AMD, reduced diversity, dominance of tolerant species and a shift in nutrient availability (Oberholster & de Klerk, 2014). Planktonic algae were shown to be useful for general water quality assessment and benthic algae for localized water quality assessment (Bellinger & Sigee, 2010). The assemblage differences between the five sites may reflect a reduction of the wetland's remediation ability (Valente & Gomes, 2007). It was also demonstrated that the combination of known indicator species, species diversity and chlorophyll-*a* concentration gave a reliable insight to the physical and chemical conditions at each site (Hill *et al*., 2000).

The bacterial population had higher diversity than expected from a polluted environment, challenging the accepted notion that these environments would harbour low diversity and

would be dominated by extremophiles. The two environmental factors found to be the main determinants of the bacterial communities at the various sites were pH and electrical conductivity. The phyla that were highly represented throughout all the samples were the *alpha*-, *beta*- and *gamma-Proteobacteria*, *Bacteriodetes* and unclassified species. The high abundance of unclassified species reiterates the need for taxonomic investigations of open systems such as wetlands and the impact of pollutants on the primary producers present. The effects of AMD are usually first observed in the nitrification pathway of nitrogen cycling due to the effects of low pH and increased metal concentrations (Niyogi *et al*., 2003). However, AMD environments are not as harsh and simple as once thought, as shown by this study by the high diversity in the bacterial population (Xie *et al*., 2011; Kimura *et al*., 2011). Indigenous bacterial species in environments such as wetlands are regulary overlooked (Brantner & Senko, 2014) when environmental impact is investigated, with the focus on specialized species adapted to environmental stressors. Thus there is great potential in the understanding of the mechanisms which shape bacterial populations in AMD impacted environments (Miller *et al*., 2009). This study was also the first to report the identification of sequences from the *Marinobacterium* genus in a freshwater environment. *Marinobacterium* spp. have only been reported in marine samples. This study was also the first, to the author's knowledge, to investigate the total bacterial diversity in the water column and sediment of a wetland, impacted by AMD, in South Africa.

The proposed bacterial bioindicator model was based upon that of Zdyb (1999). This model describes population scenarios based upon the number of individuals measured and the number of species identified. For the purpose of this study, the number of sequences was used as the number of individuals. The proposed model for the bacterial bioindicator indicated that the bacterial populations at site 1 for both the water column and sediment were stable, yet moving towards a stressed scenario and at site 2 both populations were determined to be in a sterile/pioneer scenario. The water column community at site 3 were in an extreme scenario and the sediment was extreme, yet moving towards a stable scenario, thus recovering from environmental stress. Both the water column and sediment bacterial populations at sites 4 & 5 were stable according to the proposed model. The Ecotoxicological Screening Tool (EST) revealed the ecosystem health of the Zaalklapspruit wetland through the use of bioindicator organisms and to select environmental conditions. The scores obtained using the original EST revealed that the reference site (site 1) was largely natural with a few modifications, sites 2 & 3 were seriously modified and sites 4 & 5

were modified. To adjust the EST to include the proposed bacterial bioindicator model, a stable population scenario would score 10 out of ten, a stressed scenario six, extreme scenario 4 and a sterile/pioneer scenario would score a 2. The total EST score of 110 would then be reworked to 100 %. When the proposed bacterial bioindicator was incorporated into the EST, the ecological category score of each site investigated of the Zaalklapspruit wetland did not change. This study demonstrated that a bacterial bioindicator for ecosystem health is possible, as proposed by Sims *et al*. (2013). Environmental stressors impact may be on a physiological and community level, interpreted as linkages between the environment and biogeochemical processed within a bacterial community (Schimel *et al*., 2007). Multidisciplinary environmental research may provide an understanding of wetland functioning (Gutknecht *et al*., 2006). A biological approach was shown to be more specific and applicable than chemical and physical methods for water quality assessment (Kosolapov *et al*., 2004).

This study revealed the complex interactions between biota, at a primary producer level, within a polluted wetland. The importance of algal autecology in determining environmental stressors was also proved. It was evident that the historical approach of identification of certain bacterial species, i.e. extremophiles, within an AMD impacted wetland would not have been sufficient in order to evaluate the bacterial diversity.   The high abundance of unclassified bacterial species indicated that future studies should include taxonomic approaches in order to identify more bacterial species within such environments. The use of various identification techniques, i.e. DGGE and the subsequent application of the bacterial bioindicator would test the model's flexibility towards different techniques.

References

Bellinger, E.G. & Sigee, D.C., 2010, *Freshwater algae: identification and use as bioindicators*, Wiley-Blackwell, West Sussex.

Brantner, J.S. & Senko, J.M., 2014, Response of Soil-Associated Microbial Communities to Intrusion of Coal Mine-Derived Acid Mine Drainage, *Environmental Science and Technology*, 48: 8556-8563.

Hill, B.H., Herlihy, A.T., Kaufmann, P.R., Stevenson, R.J., McCormick, F.H. & Burch, J.C., 2000, Use of periphyton assemblage data as an index of biotic integrity, *Journal of the North American Benthological Society*, 19:50-67.

Kimura, S., Bryan, C.G., Hallberg, K.B. & Johnson, D.B., 2011, Biodiversity and geochemistry of an extremely acidic, low-temperature subterranean environment sustained by chemolithotrophy, *Environmental Microbiology*, 13: 2092-2104.

Kosolapov, D.B., Kuschk, P., Vainshtein, A.V., Wieβner, A., Kästner, M. & Müller, R.A., 2004, Microbial processes of heavy metal removal from carbon-deficient effluents in constructed wetlands, *Engineering in Life Sciences* 4: 403-411.

Miller, S.R., Strong, A.L., Jones, K.L. & Ungerer, M.C., 2009, Bar-coded pyrosequencing reveals shared bacterial community properties along the temperature gradients of two alkaline hot springs in Yellowstone National Park, *Applied and Environmental Microbiology*, 75: 4565-4572.

Oberholster, P.J. & de Klerk, A., 2014, Aquatic ecosystems in the coal mining landscape of the upper Olifants River, and the way forward, The Southern African Institute of Mining and Metallurgy: 21st Century Challenges to the southern African coal sector symposium proceedings, Emperors Palace Convention Resort, Johannesburg, 4–5 March 2014, pp. 1-14.

Schimel, J., Balser, T.C. & Wallenstein, M., 2007, Microbial stress-response physiology and its implications for ecosystem function, *Ecology* 88: 1386-1394.

Sims, A., Zhang, Y., Gajaraj, S., Brown, P.B. & Hu, Z., 2013, Toward the development of microbial indicators for wetland assessment, *Water Research*, 47: 1711-1725.

Valente, T.M. & Gomes, C.L., 2007, The role of two acidophilic algae as ecological indicators of acid mine drainage site, *Journal of Iberian Geology*, 33: 283-294.

Xie, J., He, Z., Liu, X., van Nostrand, J.D., Deng, Y., Wu, L., Zhou, J. & Qiu, G., 2011, GeoChip-Based analysis of the functional gene diversity and metabolic potential of

microbial communities in acid mine drainage, *Applied and Environmental Microbiology*, 77: 991-999.

Zdyb, L., 1999, 'Microbial sulphate reduction as a method of passive treatment of acid mine drainage using undefined carbon sources', MSc Thesis, Department of Microbiology and Plant Pathology, University of Pretoria.

Appendix A

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Figure 1. Profile bar plot indicating the relative number of sequences assigned to the various bacterial phyla from the 16S rRNA gene sequences of the water samples. Comparison is drawn between the two sampling events. Black bars indicate the 95% confidence interval for each and the * indicates significant differences. A: site 1; B: site 2; C: site 3; D: site 4; E: site 5.

A.

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D.

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Figure 2. Profile bar plot indicating the relative number of sequences assigned to the various bacterial phyla from the 16S rRNA gene sequences of the sediment samples. Comparison is drawn between the two sampling events. Black bars indicate the 95% confidence interval for each and the * indicates significant differences. A: site 1; B: site 2; C: site 3; D: site 4; E: site 5.

Figure 3A. Multivariate analysis of the water bacterial populations sampled at the 5 study sites during January with the physical-chemical factors indicated in red. DOC: dissolved organic carbon; B: Boron; Turb: turbidity; COD: chemical oxygen demand; Temp: temperature; DO2: dissolved oxygen; Fe: iron; Cu: copper; Al: aluminium; Zn: zinc; Ni: nickel; Mn: manganese; Pb: lead; SO4: sulphate; TDS: total dissolved solids; EC: electrical conductivity; Chl(s): suspended chlorophyll-*a*.

Figure 3B. Multivariate analysis of the sediment bacterial populations sampled at the 5 study sites during January with the physical-chemical factors indicated in red. Si: silicone; B: Boron; Redox: redox potential; O.C.: organic carbon; C: carbon; Fe: iron; Cu: copper; Al: aluminium; Zn: zinc; Ni: nickel; Mn: manganese; Pb: lead; V: vanadium.

Figure 3C. Multivariate analysis of the water bacterial populations sampled at the 5 study sites during March with the physical-chemical factors indicated in red. DOC: dissolved organic carbon; B: Boron; Turb: turbidity; COD: chemical oxygen demand; Temp: temperature; DO2: dissolved oxygen; Fe: iron; Cu: copper; Al: aluminium; Zn: zinc; Ni: nickel; Mn: manganese; Pb: lead; SO4: sulphate; TDS: total dissolved solids; EC: electrical conductivity; Chl(s): suspended chlorophyll-*a*.

Figure 3D. Multivariate analysis of the sediment bacterial populations sampled at the 5 study sites during March with the physical-chemical factors indicated in red. Si: silicone; B: Boron; O.C.: organic carbon; C: carbon; Fe: iron; Cu: copper; Al: aluminium; Zn: zinc; Ni: nickel; Mn: manganese; Pb: lead; V: vanadium.

Appendix B

Mothur generated database files containing the OUT count and taxonomy of each of the samples sequenced during the study. These spreadsheets are too long to print thus are on the accompanying CD-ROM disk named "Appendix B". The files are named accordingly:

- Jan_water: January 2013 water samples taxonomy from the 5 study sites.
- Jan_sediment: January 2013 sediment samples taxonomy from the 5 study sites.
- March_water: March 2013 water samples taxonomy from the 5 study sites.
- March_sediment: March 2013 sediment samples taxonomy from the 5 study sites.
- Mothur logfiles: Mother generated logfiles of all data used in this study.

Appendix B
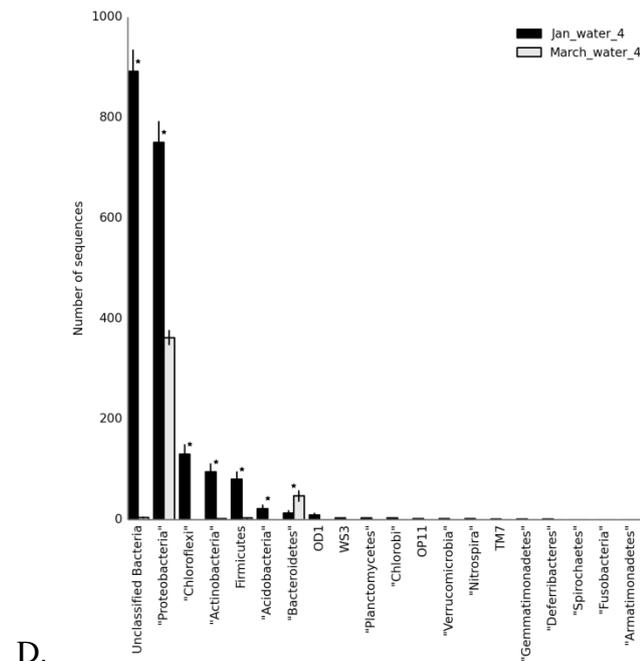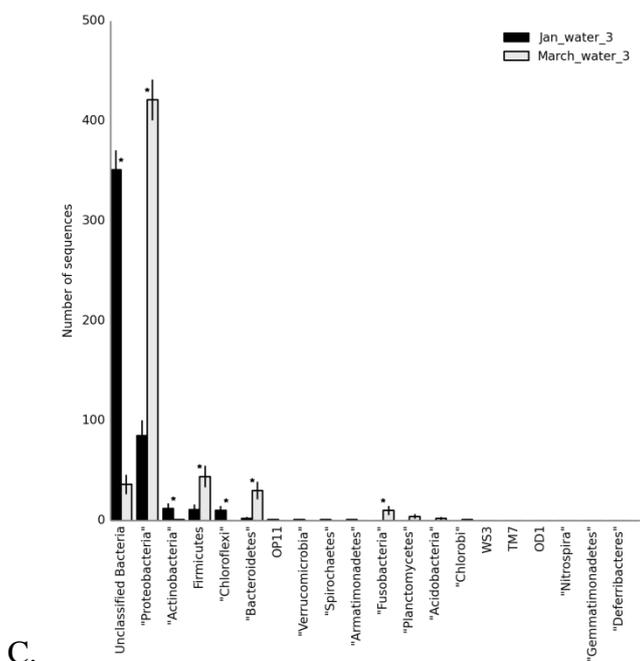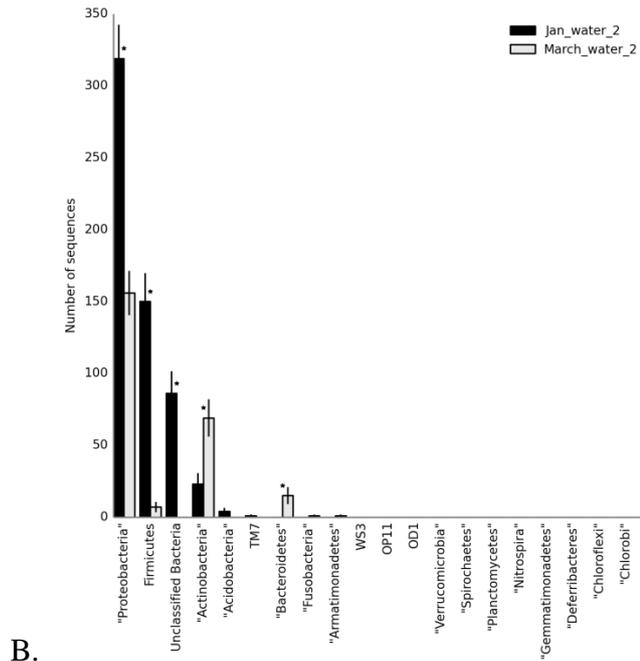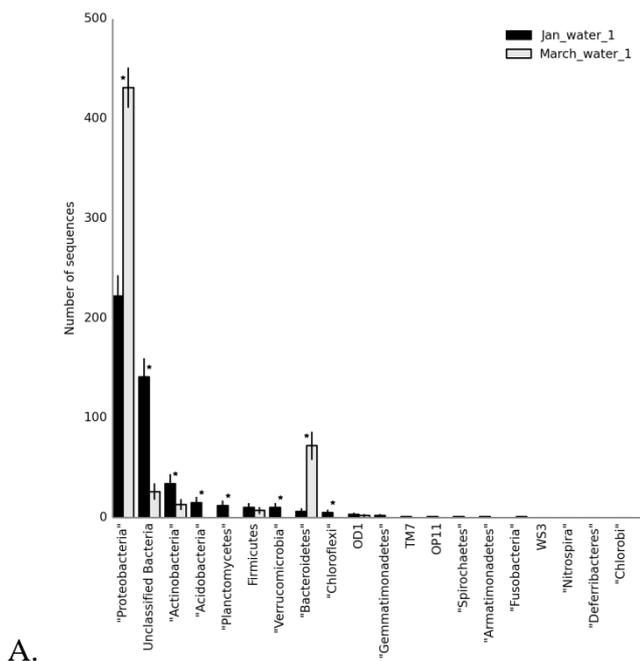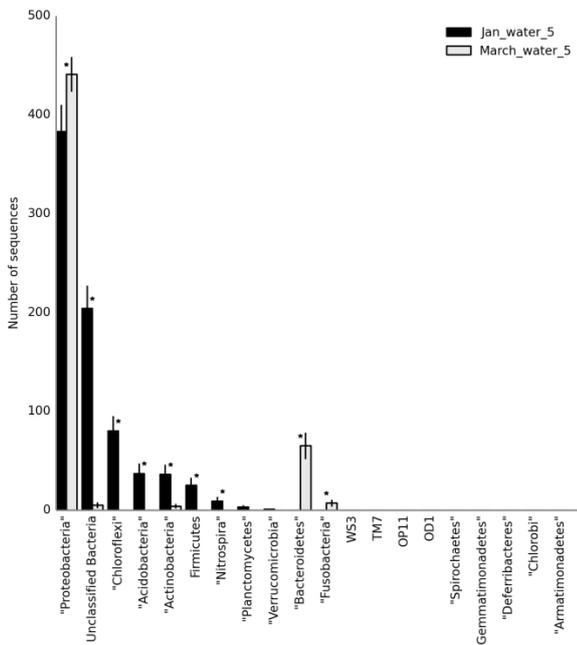
Appendix A

A.

B.

C.

D.

E.

Figure 1. Profile bar plot indicating the relative number of sequences assigned to the various bacterial phyla from the 16S rRNA gene sequences of the water samples. Comparison is drawn between the two sampling events. Black bars indicate the 95% confidence interval for each and the * indicates significant differences. A: site 1; B: site 2; C: site 3; D: site 4; E: site 5.


A.

B.

Figure 2. Profile bar plot indicating the relative number of sequences assigned to the various bacterial phyla from the 16S rRNA gene sequences of the sediment samples. Comparison is drawn between the two sampling events. Black bars indicate the 95% confidence interval for each and the * indicates significant differences. A: site 1; B: site 2; C: site 3; D: site 4; E: site 5.

Figure 3A. Multivariate analysis of the water bacterial populations sampled at the 5 study sites during January with the physical-chemical factors indicated in red. DOC: dissolved organic carbon; B: Boron; Turb: turbidity; COD: chemical oxygen demand; Temp: temperature; DO2: dissolved oxygen; Fe: iron; Cu: copper; Al: aluminium; Zn: zinc; Ni: nickel; Mn: manganese; Pb: lead; SO4: sulphate; TDS: total dissolved solids; EC: electrical conductivity; Chl(s): suspended chlorophyll-*a*.

Figure 3B. Multivariate analysis of the sediment bacterial populations sampled at the 5 study sites during January with the physical-chemical factors indicated in red. Si: silicone; B: Boron; Redox: redox potential; O.C.: organic carbon; C: carbon; Fe: iron; Cu: copper; Al: aluminium; Zn: zinc; Ni: nickel; Mn: manganese; Pb: lead; V: vanadium.

Figure 3C. Multivariate analysis of the water bacterial populations sampled at the 5 study sites during March with the physical-chemical factors indicated in red. DOC: dissolved organic carbon; B: Boron; Turb: turbidity; COD: chemical oxygen demand; Temp: temperature; DO2: dissolved oxygen; Fe: iron; Cu: copper; Al: aluminium; Zn: zinc; Ni: nickel; Mn: manganese; Pb: lead; SO4: sulphate; TDS: total dissolved solids; EC: electrical conductivity; Chl(s): suspended chlorophyll-*a*.

Figure 3D. Multivariate analysis of the sediment bacterial populations sampled at the 5 study sites during March with the physical-chemical factors indicated in red. Si: silicone; B: Boron; O.C.: organic carbon; C: carbon; Fe: iron; Cu: copper; Al: aluminium; Zn: zinc; Ni: nickel; Mn: manganese; Pb: lead; V: vanadium.

Appendix B

Mothur generated database files containing the OUT count and taxonomy of each of the samples sequenced during the study. These spreadsheets are too long to print thus are on the accompanying CD-ROM disk named "Appendix B". The files are named accordingly:

- Jan_water: January 2013 water samples from the 5 study sites.
- Jan_water_PCA: PCA plot of the January 2013 water samples from the 5 study sites.
- Jan_sediment: January 2013 sediment samples from the 5 study sites.
- Jan_sediment_PCA: PCA plot of the January 2013 sediment samples from the 5 study sites.
- March_water: March 2013 water samples from the 5 study sites.
- March_water_PCA: PCA plot of the March 2013 water samples from the 5 study sites.
- March_sediment: March 2013 sediment samples from the 5 study sites.
- March_sediment_PCA: PCA plot of the March 2013 sediment samples from the 5 study sites.
- Mothur logfiles: Mother generated logfiles of all data used in this study.