EFFECTS OF QUICKLIME TREATED ACID MINE DRAINAGE (AMD) IRRIGATION ON THE SOIL MICROBIAL ACTIVITY, PHYSIOLOGICAL PARAMETERS AND BIOCHEMICAL PERFORMANCE OF POTATO (*Solanum tuberosum* L.)

by

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Doctor of Philosophy in Agriculture

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DECLARATION BY CANDIDATE

I, Rabelani Munyai, declare that this thesis entitled,

‘Impacts of quicklime treated Acid Mine drainage (AMD) irrigation on the soil microbial activity, physiological parameters and biochemical performance of potato cultivars (Solanum tuberosum L.)’

is my own work and all sources that I have used or quoted have been indicated and acknowledged by means of complete references. Prior to the commencement of the research project, both the researcher and the Unisa library conducted a literature review and ascertained that no other similar research had been conducted in South Africa/ or globally, prior to the registration of this project.

Signature………………………………………… Date…03/02/2022…………………

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DEDICATION

This dissertation is dedicated to my family for the patience of caring for my two beautiful girls (Roanda ngatshilidzi and Anzanivhudihawe) and siblings (Uhone-Thama and Rikhode-Zwothe) in my absence and the encouragement they gave for the duration of the study. A special thanks to my dad, mom and siblings who were supportive throughout the period and viewed this as a source of encouragement for me to do better.

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Munyai, R. Nemutanzhela M.V. & Modise, D.M. (2022). LC-MS Based Metabolomics Analysis of Potato (*Solanum tubersum* L.) Cultivars Irrigated with Quicklime Treated Acid Mine Drainage Water. Metabolites, 12, 221 (1-16). doi.org/10.3390/metabo12030221

Raletsena, M.V., Munyai, R., Modise, D.M. & Woldesemayat, A.A. (2022). Heavy Metals Risk Assessment and Nutritional composition of Two Potato (Solanum tubersum L.) Cultivars irrigated with Fly Ash Treated Acid Mine Drainage. Author list: *Sustainability*.

ABBREVIATIONS AND UNITS OF MEASUREMENT

AMD	Acid Mine Drainage
ARC	Agricultural Research Council
DNA	Deoxyribonucleic acid
DAP	Days after planting
DWAF	Department of Water and Forestry
EC	Electrical Conductivity
FAO	Food and Agriculture Organization
g	gram
HM	Heavy Metal
HPLC	High-performance liquid chromatography
ICP-EOS	Inductively coupled plasma optical emission spectrometry
LC-MS	Liquid Chromatograph Mass spectrometry
mg/l	Milligram Per Litre
OTU	Operational taxonomic unit
PCR	Polymerase chain reactions
rRNA	Ribosomal ribonucleic acid
ROS	Reactive oxygen species
TDS	Total Dissolved Solids
WHO	World Health Organization
FeS_2	pyrite
H_2SO_4	sulphuric acid
µS cm-1	micro Siemens per cm

ABSTRACT

The increasing need for irrigation water due to water scarcity and decreasing precipitation has led to the use of both treated and untreated acid mine drainage (AMD) as irrigation water. However, there is a paucity of studies on the impact of quicklime treated AMD irrigation on the physiological parameters and biochemical performance on the potato cultivars as well as bacterial diversity of the irrigated soil. The present study investigated the physiological parameters and biochemical performance of *Solanum tuberosum* as well as the soil bacterial diversity abundance and variations when subjected to quicklime treated AMD irrigation. A randomized complete block design experiment was conducted under greenhouse conditions with five treatments levels replicated four times for each of the treatments. The results showed that the quicklime treatment increased the pH of the AMD water, and reduced the concentration of electrical conductivity, NO_3^-, SO_4^{2-} as well as other heavy metals such as Al, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, and Zn. The plant height, phenodays, chlorophyll content, stomatal conductance, number of tubers, fresh tuber weight, and dry tuber weight of the Marykies and Royal potato cultivars were improved when irrigated with quicklime treated AMD water. The elevation in the abundance of metabolites such as glycine, dopa, pyruvic acid, dimethylglycine, aspartic acid, acetylcarnitin, norepinephrine, 4-hydroxyproline, threonine, orotic acid, serine, adenine, creatinine, cartinine, and 4-aminobutyric acid under the irrigation of treated AMD water was observed. Changes in bacterial diversity were also observed in the soil irrigated with treated AMD with Acidobacteria and Chloroflexi as the dominant phyla.

KEYWORDS: Acid mine drainage, irrigation physiology, heavy metals, metabolites, bacterial diversity.

MANWELEDZO

U engedzea ha ṱhoḓea ya maḓi a tsheledzo zwi tshi itiswa nga ṱhahalelo ya maḓi na u fhungudzea ha mvula zwo livhisa kha u shumiswa ha vhuvhili hazwo muelelo wa maḓi a esidi o ḓalaho methala a songo kunakisiwaho na o kunakisiwaho (AMD) sa maḓi a tsheledzo. Naho zwo ralo, hu na ṱhahelelo ya ngudo nga ha masiandaitwa a ḽaimi yo kunakiswaho ya tsheledzo ya AMD nga ha kushumele kwa mivhili ya vhathu na ya zwipuka na kushumele kwa khemikhala dzine dza wanala kha zwi tshilaho kha tshaka dzo fhambanaho dza maḓabula kha zwitshili zwo fhambanaho zwa mavu a tsheledzo. Ngudo ya zwino i khou ṱoḓisisa nga ha kushumele kwa mivhili ya vhathu na ya zwipuka na kushumele kwa khemikhala dzine dza wanala kha zwi tshilaho zwa *Solanum tuberosum* na zwithu zwinzhi zwo fhambanaho zwi tshilaho mavuni na phambano zwi tshi ḓa kha ḽaimi yo kunakiswaho ya tsheledzo ya AMD Nyolo ya tshilinganyo tsho itelwaho zwiedza zwa vhulimi hune yuniti dzine dza fana dza kuvhanganywa nga zwibuḽoko nga fhasi ha nyimele ya nnḓu ine ya fhisa hune zwimela zwa aluwa khayo hu na ḽeveḽe ṱhanu dza kushumisele dzo bveledzwaho hafhu zwiṋa kha kushumisele kuṅwe na kuṅwe. Mvelelo dzo sumbedza u shumiswa ha ḽaimi hu engedza pH ya maḓi a AMD, na u fhungudza u khwaṱha ha kutshimbidzele kwa muḓagasi, NO_3^-, SO_4^{2-} na dziṅwe methala dzine dza lemela dzi ngaho sa Al, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, na Zn. Vhulapfu ha tshimela, maduvha, zwi re ngomu kha kolorofili, sitomatala khondakitentse, tshivhalo tsha khufhi, tshileme tsha khufhi thethe, na tshivhalo tsha khufhi yo omaho ya tshaka dzo fhambanaho dza maḓabula a Marykies na Royal dzo khwinisea musi dzi tshi sheledzwa nga maḓi a AMD ane a shumisa ḽaimi. U gonya ha vhunzhi ha methabolizimu dzi ngaho giḽeserina, dopa, esidi ya pyruviki, giḽeserina ya dimetheyeli, esidi ya asiparatiki, acetylcarnitin, norepinephrine, 4-hydroxyproline, threonine, esidi ya orotic, serine, adenine, creatinine, cartinine, na esidi ya 4-aminobutyric nga fhasi ha tsheledzo ya kushumisele kwa maḓi a AMD zwo sedzwa. Tshanduko kha u fhambana ha zwitshili na zwone zwo sedzwa kha mavu a tsheledzo na tsireledzo ya AMD na Acidobacteria na Chloroflexi sa khethekanyo khulwane ya zwi tshilaho.

MAIPFI A NDEME: Muelelo wa maḓi a esidi o ḓalaho methala, tshivhumbeo tsha tsheledzo, methala dzine dza lemela, methabolizimu, zwitshili zwo fhambanaho.

NKOMISI LOWU NGA NA MONGO WA NDZAVISISO

Ku ngeteleleka ka xilaveko xa ku cheleta hikokwalaho ka ku pfumaleka ka mati na ku ya ehansi ka minyuku ya mati swi endle leswo ku tirhisiwa mati yo huma eka timayini yo basisiwa no ka ya nga basisiwangi ya esidi ku nga acid mine drainage (AMD) tanihi mati yo cheleta. Kambe, a ku na mindzavisiso yo enela eka ku tirhisiwa ka quicklime-treated ADM ku cheleta eka rimba ra swo khomeka (physiological parameteres) na ku tirha kahle ka swa bayokhemiikali ka matapula yo byariwa na le ka ku hambana-hambana eka misava leyi nga cheletiwa. Ndzavisiso lowu wa sweswi wu lavisise tipharamita ta ku khomeka na tirhelo ra bayokhemikali eka Solanum tuberosum xikan'we na ku hambana ka tibhaktheriya ta misava hi xitalo na ku hambana loko swi pimanisiwa na ncheleto wa quicklime-treated AMD. Ku endliwe ekspirimente ya *block design* yo helela ehansi ka xiyimo xa *greenhouse* hi swiyenge swa ntlhanu swa ku ongola eka mikarhi ya mune eka n'ongolo wun'wana na wun'wana. Vuyelo byi kombise leswo quiklime treatment yi ngetele pH ya mati ya ADM, no hunguta ku fambiseka ka swa electrical conductivity, NO3-, SO42- xikan'we na ti-heavy metal to fana na Al, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, and Zn. Vulehi bya ximila, phenodays, na chlorophyll content, stomatala conductance, nhlayo ya ti-tubers, na ntiko wa tuber ya frexe, na ntiko wa tuber yo oma ya matapula ya Marykies and Royal cultivars swi antswiswile loko swi cheletiwa hi mati ya quicklime-treated AMD. Ku tlakusiwa ka vunyingi bya metabolites byo fana na glycine, dopa, pyruvic acid, dimethylglycine, aspartic acid, acetylcarnitin, norepinephrine, 4-hydroxyproline, threonine, orotic acid, serine, adenine, creatinine, cartinine, and 4-aminobutyric acid ehansi ka vucheleti hi mati yo ongoriwa ya AMD swi voniwile. Ku cinca eka ku hambana ka tibhaktheriya, naswona swi voniwile eka misava leyi yi nga cheletiwa hi mati yo ongoriwa ya AMD hi Acidobacteria na Chloroflexi tanihi hi *phyla* yo tala ngopfu.

MARITO YA NKOKA: mati ya esidi yo huma eka timayini, vucheleti byo khomeka, heavy metal, metabolites, ku hambana ka tibhaktheriya.

CHAPTER 1: INTRODUCTION

1.1 Background of the study

Water is a vital and essential resource for all living things on the Earth planet; without it, no person, animal, or plant can exist (Bates *et al*., 2008; Pedro-Monzons *et al*., 2015; Junguo *et al*., 2016). Globally, the ongoing increase in population, climate change and pollution of water supply sources challenges its availability and, as a result, the lack of fresh water already encountered in many parts of the world (Rijsberman, 2006; Kammu *et al.,* 2010; Ochieng *et al.,* 2010; Mishra & Dehuri, 2011; Nawaz *et al.,* 2012). The world is entering an era of increasing water scarcity, with potential severe implications for many countries, especially in the developing world. South Africa (SA) is one of the countries expected to experience serious water scarcity (Mukheibir & Sparks, 2003).

South Africa is water-stressed (Ashton, 2007), of which about two-third is arid or semi-arid (Kamara & Sally, 2004). The annual rainfall across the country averages around 450 mm, which is well below the world average of about 860 mm per year (Scholes & Biggs, 2004; Mukheibir & Sparks, 2005). Water may be everywhere, but its use has always been constrained in terms of availability, quantity, and quality (Biswas, 2004). The country's annual available freshwater is less than 1700 m^3 per capita (the index for water stress) with the present estimation by FAO being 1154 m^3 per capita. This indicates a declining trend in the available freshwater in the country. Congruent to the above statement, International Water Management Institute (IWMI, 1996) pointed out that in 2025 there will be a physical water scarcity in South Africa with an annual freshwater availability of less than 1000 m^3 per capita. Presently, 77 % of surface water, 9 % of groundwater, and 14 % of recycled water are being utilized in South Africa (UN Water, 2006).

Water problems are expected to become more complex in the future, potentially competing or interfering with other development sectors such as energy, agriculture, mining, transportation, and social sectors such as education, environment, health, and rural and regional development (Biswas, 2004; Molebola & Sinha, 2011). The country's available supplies of fresh water are decreasing due to increased population growth, low precipitation, competing demands from industry, agricultural and urban development (Johnson *et al*. 2001; Amer *et al.* 2016). Furthermore, some of the world's water supplies are currently being affected by climate change (Blignaut *et al*. 2009), the occurrence and spread of invasive alien plant species (Blignaut *et al.*, 2007; Cullis *et al.*, 2007) and mining activities (Annandale *et al.*, 2006). Thus, water will increasingly become the single most limiting resource in SA. Water supply will become a major restriction to the future socio-economic development of the country especially in terms of the quantity and quality of available resources. Alternative water sources, such as seawater, storm water, wastewater, and industrial effluent, are costly and do not generate enough water. Therefore, to guarantee equitable and sustainable use of water resources in South Africa, control of both supply and quality is required. There has been an increasing concern that has been raised concerning the limited water supply available for major industries, drinking water and other activities such as irrigation for agriculture. Currently, industrial effluents, home and commercial sewage, acid mine drainage, agricultural run-off, and solid waste damage most water resources (Khatri & Tyagi, 2015).

South Africa has a highly diverse agricultural sector, consisting of commercial and private intensive and vast crop farming systems, including the cultivation of food crops including vegetables, fruits, cereals, and legumes. Agriculture is a major source of income and therefore a significant proportion of the population depends on agricultural activity for their livelihoods (El Chami & El Moujabber, 2016). It accounts for more than 60 % of South Africa's water utilisation for its irrigation practices (Otieno & Ochieng, 2004; Fischer *et al*. 2007; Nkondo *et al*., 2012). Currently, one of the most cultivated food crops is potato (*Solanum tuberosum* L.) and its successful cultivation requires irrigation with very high quantities of water (Fabeiro *et al*. 2001; Pimentel *et al*., 2004). The scarcity of water in SA ranks among major challenges especially in agriculture and some crop farmers have resorted to using alternative water sources such as acid mine water as a source of irrigation. However, crops grown under wastewater irrigation which is associated with contaminants such as heavy metals and pathogenic microorganisms among many harmful substances exhibit poor morphological and physiologically response. Of noteworthy, some crops adopt mechanisms to tolerate and/or withstand the adverse effect of various contaminants reported in wastewater.

Hence, understanding the physiological response of crops to the application of treated and untreated wastewater will provide an insight into the metabolic activities of crop.

1.2 Problem statement

South Africa is faced with declining volume and quality of water. The poor quality of the country's water is partly caused by pollution by AMD. Interestingly, published studies have reported that AMD treated with various agro-inputs can be used to irrigate food crops and as an alternative innovative solution for the SA country's increase water shortage crisis and undoubtedly play an important role in the future of the country's agriculture.. As to be expected, such crops exhibit altered growth, yield and concentration of heavy metals depending on the cultivar or rate or ratio of AMD:agro-input. Currently, there are rarely published studies that considered the effects of AMD treated with quicklime on the physiological and metabolic performance as well as microbial richness and diversity of rhizospheric soil bacteria of potato cultivars. Will the quicklime treated AMD water be an innovative water crisis solution and the water be used for long-term performance within the crop production? This study therefore aims to provide answers to these questions through examining the effects of acid mine treated water mixed with quicklime on microbial community of selected potato cultivars.

1.3 Justification of the study

Crop farmers are interested in using AMD to irrigate food crops. According to published research, AMD treated with lime can be used as an alternate source of irrigation water for food crops. Irrigation of food crops with AMD treated with quicklime can elicit varying physiological and metabolic responses as well as rhizospheric microbial richness and diversity. It is therefore the uniqueness of this combination that merits reporting. This research project's emphasis on evaluating the role of treating AMD with quicklime on potato cultivars is therefore an attempt to identify previously unknown positive effects, thus contributing to existing literature and knowledge. Research on irrigation of potato with quicklime-treated AMD is likely to produce results that will guide on the effects such an alternative has on the quality of potato. For example, understanding how different rates of quicklime-treated AMD alter the growth-related parameters, physiological performance, and soil bacterial richness and diversity could potentially lead to adoption of the alternative and therefore cultivation of irrigated crops with less pressure on the already dwindling water resources of the country. Rivers, streams, lakes, ponds, and springs provide about 70% of the water utilized in South Africa for drinking and agricultural purposes. Most of those water resources are declining in many provinces due to AMD pollution. This study seeks to address the potential of quicklime treated AMD water for irrigation of commercial potato cultivars and other crops at large. It will enable making informed decisions related to the use of treated AMD water in crop growing practices under drastic climate conditions and water deficit seasons of the current times.

1.4 Aims and objective of the study

1.4.1 Aim of the study

The aim of the study was to investigate whether the use of quicklime-treated AMD for irrigation alters the physiological parameters, biochemical performance as well as the diversity and abundance of soil bacterial of two potato cultivars (*Solanum tuberosum* L.).

1.4.2 Objectives of the study

To meet the aim, the specific objectives of the study were to investigate:

- The effects of quicklime-treated AMD on the physiological parameters and heavy metals toxicity on both water, soil, and the potato cultivars.
- The metabolic profile on potato cultivars when subjected to quicklime-treated AMD irrigation.
- Identification of bacterial diversity within quicklime-treated AMD irrigated soil levels.

1.5 Research questions

- Would irrigating potato cultivars with quicklime-treated AMD change their physiological parameters and at what treatment level will heavy metals be considered toxic?
- Which metabolites of the potato cultivars will be affected?
- Will quicklime-treated AMD irrigation affect the soil bacterial diversity?

1.6 Reliability and validity

Consistency and replicability throughout time define reliability. Furthermore, reliability is defined as the degree to which a test is free of measurement errors, with the number of measurement errors increasing the test's reliability (Neuman, 2003). The techniques and equipment used to generate information and analyze data in order to answer the research questions of interest determine the degree of credibility in scientific research. In this study, it was critical to employ reliable, valid, and reasonable methodologies, manage and monitor experiments, and most importantly, accurately record results. Reliability is an important part of assessment, and it is portrayed as a factor that contributes to validity rather than as a factor that is opposed to validity. To ensure that data obtained in the study is valid and reliable, the experimental treatments were replicated three times, and where the data is not normally distributed, it was transformed before analysis. Also, methods used to set-up, manage, sample, analyse data, and for extractions, are trusted and used widely by the scientific community.

1.7 Thesis outline

- Chapter one of this study presented the background, problem statement, and significance of the study, as well as the main and specific objectives, research question, research hypothesis, and thesis outline.
- Chapter two reviewed existing literature on the shortage of water in South Africa, the use of acid mine drainage (AMD) water as an alternative resource for irrigating food crops and its effects, various techniques use dto remediate AMD, effects of heavy metals contained in AMD on crops, physiological and biochemical response of AMD on crops, metabolomics analysis on crops and microbial diversity associated with AMD and the effects of remediation techniques on soil microbial diversity.
- Chapter three presents the effects of quicklime-treated AMD on selected physiological parameters as well as heavy metals toxicity on the water, soil, and the potato cultivars.
- Chapter four presents the metabolic changes on potato cultivars when subjected to quicklime treated AMD irrigation.
- Chapter five presents the soil bacterial diversity after the application of different quicklime treated AMD irrigation.
- Chapter six presents recommendations and conclusion

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CHAPTER 2: LITERATURE REVIEW

2.1. INTRODUCTION

This section reviews published and/or available literature on water scarcity, acid mine drainage (AMD) water as an alternative resource, environmental impacts of AMD, techniques used in remediating AMD, reuse of (un)treated AMD for irrigating food crops, physiological and biochemical response of crops supplied with AMD water, metabolomic profile on crops and microbial diversity associated with (un)treated AMD and effects of remediation techniques on microbial diversity.

2.1.1 Water scarcity: A global crisis

Literature background shows that water scarcity will be among major challenges that humans and animals will face in the next decade (Levy *et al*., 2013; Urbano *et al*., 2017) particularly for food production. The decline in sources of freshwater particularly in arid and semi-arid areas found in Africa, south Asia, southern Europe and the Middle East, is becoming a major concern especially for the agricultural sector due to an expansion in human population and degradation of water quality (Hong *et al*., 2014; Chouchane *et al*., 2018). The scarcity has led to the need to consider the utilisation of alternative water sources including that discharged from industrial, commercial, and domestic activities (Khalid *et al*., 2017). Many countries have been irrigating landscape and in some cases food crops using both treated and untreated wastewater (Khalid *et al*., 2017). Interestingly, the practice has been reported to increase in recent years particularly in countries where access to or availability of freshwater is limited. Intriguingly, irrigation of food crops with treated and untreated wastewater has positive and negative effects. For example, the water reportedly contains large amounts of pollutants (salts, heavy metals), beneficial nutrients, organic matter, viruses, pathogenic bacteria, nematodes, and protozoa (Rusan *et al*., 2007; Murtaza *et al*., 2010; Uyttendaele *et al*., 2015; Alghobar & Suresha, 2017). Some of the contaminants are very disastrous for agricultural production and the environment. In most cases, wastewater is discharged into streams, rivers, and lakes without being treated at all, only partially treated, or without being treated at all (Thapliyal *et al*., 2011). The utilization of wastewater not only conserves freshwater resources for domestic purposes such as drinking water and irrigation, but it also reduces pollution in adjacent bodies of water and the environment (Murtaza *et al*., 2010; Chamorro *et al*., 2013; Jaramillo *et al*., 2017; Libutti *et al*., 2018). Furthermore, using treated waste-water to irrigate crops improves the growth and yield and reducing the need for fertilizers (Libutti *et al*., 2018). Also, it reduces the cost associated with the treatment of wastewater and that of crop production. The reduction in the quantity of mineral fertilisers needed is because wastewater contains essential nutrient elements and therefore plays a crucial role in livelihoods through increasing food production thus, it receives attention due to the scarcity of quality water (Norton-Brandáo *et al.,* 2013; Yao *et al*., 2013; Mojid *et al*., 2016; Khalid *et al*., 2018). There are countries that have had success regarding the use of wastewater in agriculture and these include China where about 70% of wastewater is used for agriculture (Drechsel & Evans, 2010).

The major contributors of acid mine drainage are operational and abandoned mines (Johnson & Hallberg, 2005). Without a doubt, AMD reduces the value of water meant for use in agriculture, recreation, or industry, and renders it unsafe for consumption by either humans or animals (Tripole *et al*., 2006). South Africa ranks among countries that host plenty of mines that are abandoned and operational that drains acid mine drainage water mostly into proximal waterbodies (Pulles *et al.,* 2005). Although mining is a major contributor to country's GDP, its activities can result in the release of by-products that have negative impacts on the fauna and flora of environments that surround mines (McCarthy, 2011). South Africa is ranked among 30 of the driest countries in the world and is expected to experience severe water scarcity in the future (Dabrowski *et al.*, 2009; De Lange *et al.*, 2009; Engelbrecht *et al*., 2009; Grewar, 2019). To increase the sources of water, there are several proposals for alternatives including that the re-use of treated wastewater. As a result, there is a critical need to reduce toxins linked to AMD by implementing appropriate technology, eliminating waste, and implementing reuse and recycling strategies across the country. When treated, AMD water can be used for multiple purposes including the irrigation of crops and serve as an innovative solution for the current and future water crisis.

2.2 Acid Mine Drainage (AMD): An alternative source

2.2.1 Sources, formation, and chemistry of AMD

Mining is associated with the production of enormous volumes of harmful acidic water containing high concentrations of heavy metals (HM) and sulfates which are referred to as acid mine drainage (AMD) or acid rock drainage (Adler *et al*., 2007; Egiebor & Oni, 2007; Ochieng *et al*., 2010; Rambadu *et al*., 2020). Acid Mine Drainage has been reported in various mining regions around the world including Australia (Lei *et al*., 2010), Brazil (Rubio *et al*., 2007), Canada (Sracek *et al*., 2004), England, Wales, Spain, Norway (Hallberg, 2010), Morocco (Boularbah *et al*., 2006), South Africa (Ochieng *et al*., 2010), the United Kingdom (Johnson & Hallberg, 2005) and United States of America (Blowes *et al*., 2014). In South Africa (SA), AMD is widely reported in mines located in the Witwatersrand Gold fields (Western basin, Eastern and Central basin in Gauteng Province), Mpumalanga and KwaZulu Natal Coalfields (Manders *et al*., 2009; McCarthy, 2011; Durand, 2012).

Several researchers (Akcil & Koldas, 2006; Jennings *et al*., 2008; Ochieng *et al*., 2010; Zipper *et al*., 2011; Nieto *et al*., 2013; Simate & Ndlovu, 2014) define acid mine drainage defined as metal-rich water that results from a chemical interaction between rocks and sulphur-bearing minerals in water. Among the heavy metals found in AMD is aluminium, copper, iron, lead, and zinc (Blowes *et al*., 2014) and their concentration is high due to the acid-driven leaching of the rocks (Johnson, 2003). Depending on the intricate interactions of hydrological, chemical, and biological processes in some acid mine drainage systems, the water can be very acidic with a low pH (2-4) (Akcil & Koldas, 2006; Abbassi *et al*., 2009). In general, majority of AMDs exhibit pH values in a range from 2.5 to 6.5, with most mining systems covering the lower half of that range.

Drainage from underground mine shafts, run-off from open mine waste dumps, mine structure, springs, seepage sites, contaminated boreholes, waste rock, ore stockpiles, and wastes from metallurgy processes are all well-known primary causes of AMD (Johnson & Hallberg, 2005; Akcil & Koldas, 2006; Sheoran & Sheoran, 2006; Manders *et al*., 2010; McCarthy, 2011; Durand, 2012; Blowes *et al*., 2014). The three primary chemical reactions that cause acid mine drainage are pyrite oxidation, ferrous oxidation, and iron hydrolysis. Acid mine drainage is mainly formed through chemical and biological reactions where pyrite (FeS_2) reacts with oxygen and water to produce sulphuric acid (H_2SO_4) and ferrichydrite precipitate called 'yellow boy' (Johnson & Hallberg, 2005; Jennings *et al*., 2008; Das *et al*., 2009; McCarthy, 2011; Blowes *et al*., 2014). The oxidation in most AMD sites is primarily catalysed by naturally occurring bacteria called *Acidithiobacillus* and *ferrooxidans* (Tyson *et al*., 2004; Akcil & Koldas, 2006; Egiebor & Oni, 2007) and these bacteria break down sulphides. Poor management strategies, failure to control and treat AMD water within and from abandoned mines causes severe impacts (Akcil & Koldas, 2006; Durand, 2012; García-Gómez *et al*., 2014).

2.2.3. Major known environmental impacts of AMD pollution

The discharge of untreated AMD into the environment causes severe environmental impacts on the soil, aquatic communities, and water resources (Shin *et al*., 2015). These impacts depend on the AMD's chemical properties, composition, and pH which in turn may vary depending on the geology of the mine sites or sources (Grewar, 2019). As previously discussed on section 2.2.2, the oxidation of sulphidic minerals (such as pyrite) promotes the formation of sulphuric acid, which then causes the release of a variety of metals. As a result, AMD has high acid and dissolved metal concentrations. For instance, Hedrich & Johnson, (2014) reported water draining in Sweden at the Maurliden mine as highly acidic (pH 2.3), rich in Zn (~460 mg L^{-1}) and iron (~400 mg L^{-1}), and had smaller concentrations of metals such as Mn, Co, Cd, Mn, Ni and As. In some areas, the low level of pH causes the dissolution of minerals which then create an acidic environment which is not suitable for the cultivation of vegetables, vegetation, survival of aquatic life and negatively affects human health (Ochieng *et al*., 2010; Martinez *et al*., 2013; Choudhury *et al*., 2017). A study conducted in South Africa by Kefeni *et al*. (2015) reported that the concentration of Fe (II) was very high however, the concentration varied between type of mineral that was mined. For example, the concentration of Fe (II) detected in coal and gold mines ranged between 2135 and 835 mg L^{-1} (Kefeni *et al*., 2017).

The negative effects of AMD are largely reported on aquatic flora and fauna, human health, terrestrial plants and the overall terrestrial ecosystem. When aquatic systems are contaminated with HM, it results in the creation of reactive oxygen species (ROS), which can harm fish, aquatic macroinvertebrates, and other creatures (Jenning *et al*., 2008; Jiwal & Kalamdhad, 2011; Simate & Ndlovu, 2014). Oberholster *et al*. (2010) reported elevated levels of heavy metals (Zn, Cu, Mn, Pb, Cr, Ni, Al, and Fe) as well as their bioaccumulation in macrophytes and fish that habited an aquatic system. Elevated levels of HM in aqutic systems can also decrease the richness and diversity of algal species (Luís *et al*., 2009). The bioaccumulation of heavy metals such as cadmium, copper, lead, and zinc has been discovered to be particularly hazardous to aquatic life (Simate & Ndlovu, 2014; Saha *et al*., 2019). Furthermore, the presence of elevated concentration of heavy metals in water bodies has a negative impact on human health (Ying *et al*., 2016). These include causing diseases and disorders such as bronchitis, skin and bladder cancer, liver and kidney failure, and mental impairment in children (Saha *et al*., 2019; Rambabu *et al*., 2020).

The closure of mines or their abandonment has serious consequences for communities or waterbodies near such mines (Akcil & Koldas, 2006; Egiebor & Oni, 2007). AMD affects the ecological integrity of stream ecosystems receiving inflow from surface and underground mines (Hogsden & Harding, 2012). For example, a small-scale mining site in Ghana enhanced the concentration of Pb, Hg, Cd, and K and contributed to the pollution of waterways used for crop irrigation (Nukpezah *et al*., 2017). In the Lousal area of Portugal, AMD polluted irrigation water sources through increasing the levels of SO_4^{2-}, Fe, Al, Cu, Pb, Zn, As and Cd (Luís *et al*., 2009). Studies have also documented the contamination of downstream water sources and agricultural land in China by high concentrations of Pb, Cu, and Zn contained in AMD (Wu *et al*., 2009). Over the years, there are several strategies that have been developed for the remediation of AMD water and AMD-polluted soils and some of them are discussed below.

2.2.4 Remediation techniques for AMD water

Most nations have environmental regulations that regulate and/or enforce environmentally friendly mining operations to protect the environment, land, and water resources. These include guidelines on the proper remediation and rehabilitation of AMD which is necessary for restoration of mining landscapes. In many parts of the world, the quality of AMD water does not meet standards that are approved for drinking or irrigation water. Without a doubt, there is a critical need for the development and/or implementation of strategies that limit AMD water contamination. Where methods that properly treat AMD water are utilized, waste is eliminated, and the water is reused and recycled. Pulles (2005) outlined and summarized technologies that can promote waste reuse and recycling, including 1) pollution prevention at the source, (2) reuse and recycling of polluted water to reduce the volume of polluted water discharged, (3) treatment of AMD effluents if the problem is not resolved through prevention, reuse, and recycling, and (4) discharge of treated effluent, which is considered the last resort. Although some of these management strategies result in the purification of water to acceptable standards for humans, animals and the ecosystem, there are limitations because some management and treatment need complex technologies and are costly (Annandale *et al*., 2006).

Therefore, there are various passive and active treatment methods that have been proposed and used for the remediation of wastewater. To date, some of known methods range from the use of limestone and lime for neutralization of AMD (Gitari *et al*., 2006), wetlands (Sheoran & Sheoran, 2006; RoyChowdhury *et al*., 2015), iron exchange (Buzzi *et al*., 2013), precipitation (Bologo *et al*., 2012), adsorption (Gitari *et al*., 2014), the use of fly ash (Gitari *et al*., 2006; Shaheen *et al*., 2014; Nemutanzhela *et al*., 2017) and sulfidogenic bioreactors (Panda *et al*., 2016). Blowes *et al*. (2014) tested both active and passive treatment technology to reduce the toxicity of AMD. Ramla and Sheridan (2015) tested the effectiveness of indigenous South African grasses, *Hyparrhenia hirta* and *Setaria sphacelatab* in the purification of AMD. Overall, the use of the various methods recommended for the purification of AMD has several limitations. In fact, their adoption has been limited by high cost, generation of excessive secondary sludge, or constant management (Sheoran & Sheoran, 2006; Simate & Ndlovu, 2014). Gericke *et al*. (2001) reported that in some cases, the water produced after those treatments can only be utilised for industrial purposes, provided the HMs have been sufficiently removed. Research has emerged that show that AMD can be treated using lime such as quicklime (CaO: Calcium oxide) and hydrated lime method (Othman *et al.*, 2017). Lime is a versatile chemical that is used in a variety of industrial, environmental, and chemical applications (Dowling *et al*., 2015). For instance, a recent study by Othman *et al.* (2017) reported that the application of quicklime can increase the pH value and decrease the concentration of HMs such as arsenic (As), cadmium (Cd), chromium (Cr) found in AMD. On the other hand, Leopold & Freese (2009) and Tolonen *et al*. (2014) reported that the by-product from quicklime also has potential to treat AMD. Its application removed above 99% of Al), As, Cd, Co, Cu, Fe, Mn, Ni, Zn and approximately 60% of SO_4 from AMD water. In addition, Caires *et al*. (2006) also found that supplying quicklime can adjust pH adjustment, remove HMs (e.g. impurities such as Arsenic) as well as kill bacteria and viruses. Caution should be taken however because treating AMD using some methods does not effectively purify it to recommended standards for agricultural use. Therefore, there is a need to evaluate the effects of using some of these including quicklime on the physiology, biochemical and metabolic activities on plants. Quicklime has been chosen as AMD treatment for this study.

2.3 Reuse of (un)treated AMD for Agricultural production

Several published studies have shown that untreated AMD is used for agricultural purposes (van Zyl *et al*., 2001; 2002; Lin *et al*., 2005; Annandale *et al.,* 2001, 2002, 2009; Oporto *et al*., 2007; van der Laan *et al.,* 2014; Garido *et al*., 2017; Nemutanzhela *et al*., 2017; Nevhulaudzi *et al*., 2020; Shabalala & Ekulo, 2019). Although some of the results have shown that it can have positive effects, in the main, majority of literature revealed negative effects largely caused by the activity of heavy metals.

2.3.1 Effects of heavy metals on crops

Food crops are crucial as they supply the human body with essential mineral nutrients, vitamins, carbohydrates, and fibres among others benefits (Yang *et al*., 2009). However, when cultivated in growth media or soil that is contaminated with heavy metals, the edible parts of food crops do accumulate them at times at levels higher than that recommended even when HM are found in lower concentrations in the growth media or soils. The mechanisms and/or quantity of HM that can be taken up by soil-grown plants is influenced by their concentration in a particular soil, the genetic composition of the plant species, and the physic-chemical or biological properties of the soil (Chen *et al*., 2006; Zhou *et al*., 2016). With regard to the concentration of HM in soil, several research studies have reported that when plants are established in soil that exhibit high concentrations, they show negatively affected morphology, physiology and biochemistry (Ghavri & Singh, 2012; Ebbs *et al*., 2015; Gautam *et al*., 2016; Mathur *et al*., 2016;).

For example, Henry *et al*. (2018) showed that irrigating cabbage and tomatoes with pond water taken from an exhausted mine did not accumulate Chromium and cadmium while the concentration of Mn, Pb and Fe were above that permitted by the WHO permissible and Cr at permissible level in the Cabbage. In another study, potatoes planted in soil cored from a Zn smelting area in the northwest of the Guizhou Province of China exhibited metal(loid) content below that permitted except for Cd (all samples), Pb and Se (some samples) with bioconcentration factors below 0.5, and no health risk index value higher than 0.1 (Peng *et al*. (2018). In South Africa, Nemutanzhela *et al*. (2017) reported that potato tubers (*Solanum tuberosum* L.) of Fianna and Lady Rosetta cultivars accumulated unsafe levels of Ni, Zn, and Sr when irrigated with Fly ash-treated AMD water. A published study by Islam *et al*. (2016) recorded higher concentration of Cr, Ni, Cu, As, Cd, and Pb in potato (*Solanum tuberosum*), red onion (*Allium cepa*), and wild carrot (*Daucus carota*) established in multimetal-contaminated soils relative to that recommended by the FAO (2014) /WHO, an indication that consumption of such crops could pose a risk. The accumulation of increased levels of HMs in vegetables could be due to their enhanced uptake from soil. A multivariate principal component analysis (PCA) showed that humans contribute a significant content of Cr, Ni, Cu, and Pb in samples. THQs showed that the consumption of vegetables cultivated in metal-contaminated soils enhanced the intake of Cu, As, and Pb which were higher than the recommended health standards and could cause non-carcinogenic risk. A study by Liao *et al*. (2016) revealed that irrigating sugarcane, vegetables and paddy rice with untreated AMD polluted the soils with Cd, Cu, and As.

Zhuang *et al*. (2014) showed significantly higher concentrations of Cd and Pb in rice grain, vegetable, and soybean compared to the maximum permissible level in the vicinity of Dabaoshan mine, located in southern China. When irrigating with mine wastewater, Ma *et al*. (2013) revealed that the grain of winter wheat had significantly higher Cr, Pb, Cu and Zn relative to that in their counterparts irrigated with tapwater, thus implying that the irrigation with mine wastewater could result in the accumulation of heavy metals in wheat grain. A study showed that cassava and plantain cultivated in soil surrounding a small-scale gold mining located in the Wassa-Amenfi-West District had higher levels of metals in plant tissue compared to that in the soils while the Pb, Cd, Zn and Cu accumulated in the plantain exceeded that recommended by the FAO/WHO and only values of Pb, Cd and Zn in the cassava exceeded recommended levels (Zango *et al*., 2013). Garrido *et al*. (2009) reported that untreated AMD exhibited higher levels of heavy metals which resulted in the contamination of surface water used for irrigating agricultural soils and potato in the arid desert of Potosí, Bolivia. In fact, the heavy metals recorded in the study exceeded that in guidelines of the United Nations Food and Agriculture Organization (UNFAO), Canada, and Australia. In a review article, Annandale *et al*. (2009) reported that lime-treated AMD supplied to sugar-beans, wheat, maize and potatoes using sprinkler irrigation promoted higher yields and that the impact of gypsiferous mine water was both minimal and manageable. Oporto *et al*. (2007) studied the effects of supplying untreated AMD on potato and their results showed that potato tubers accumulated high levels of Cd.

2.3.2 Physiological and biochemical response on crops

When plants are exposed to stressful environmental conditions, their physiological and biochemical performances and altered (Hasanuzzaman *et al*., 2013). Currently, there are few studies published studies that assessed the use of treated or untreated wastewater (sewage, municipal, and industrial) mixed with AMD for irrigation. For instance, a study by Ma *et al*. (2015) examined the effects of irrigating wheat with mine wastewater (leacheate of coal gangue, coal-washing wastewater, and precipitated coal-washing wastewater) on soil enzymes, physiological properties and potential risks of heavy metal contamination. The results showed that mine wastewater irrigation caused adverse effects on rhizospheric enzymes, physiological properties, and grain yield of the winter wheat. Similarly, when wheat was supplied with mine wastewater, its growth, grain yield, leaf area, dry mass per stem, root activity, and net photosynthetic rate were markedly decreased realtive to that irrigated with tap water (Ma *et al*., 2013). In another study, Kamaruzzaman *et al.* (2013) reported a significant increase on the height, spike length, grains spike and grain yield of wheat grown with the application of quicklime.

There is a need for more research on the feasibility of using quicklime-treated AMD for irrigating food crops especially in South Africa. This is more relevant because currently, the country is faced with water scarcity problems and high population growth rate. Therefore, there is a need to investigate the response and performance of agricultural crops. The findings can be helpful to farmers, resolve water crisis, and add scientific value to the board of science.

2.3.3 Metabolomic profiling on crops

Metabolomics is one of the newest omics technologies (Razzaq *et al*., 2019) that is used to study the abiotic stress tolerance, disease resistance, robust ecotypes, and metabolic-assisted breeding of crops. Plant metabolomes consist of primary and secondary metabolites. The plant kingdom comprises of over 200,000 different metabolites, the majority of which are yet unknown (Aliferis & Jabaji, 2012). According to Obata *et al*. (2015), several metabolites play a major role in enhancing the yield and nutritional quality of crops. For instance, Dawid et al. (2018) stated that each plant requires primary metabolites to produce lipids, carbohydrates, and amino acids. Primary metabolites are known to mediate the tricarboxylic acid and glycolysis cycle during photosynthesis (Daz *et al*., 2004). However, variations in primary metabolite production can cause photosynthesis to malfunction and osmotic adjustment to become unbalanced in plants.

Metabolomics has been extensively studied in crops for years and numerous methods have been devised for the detection and identification of specific metabolites (Sung *et al*., 2015). It is very crucial to understand how plants respond to such stresses, particularly drought, heavy metals, salt stress, temperature, infection, nutrient deficiency. Using a GC−MS technique, Gundaraniya *et al*. (2020) investigated key metabolites that play a role on drought tolerance and revealed that pentitol, phytol, xylonic acid, D-xylopyranose, stearic acid, and D-ribose were the main drought-responsive metabolites. This study contributed knowledge to the metabolic response of peanuts to drought stress and paved the way for more transcriptome and proteome research. Bernardo *et al*. (2019) revealed that subjecting wheat to drought promoted AMF colonization and modulation of a variety of secondary metabolites, the majority of which were connected to sugars and lipids. In another study, Moschen *et al*. (2017) used combined transcriptome and metabolic profile analyses to determine the response of sunflower to drought stress. Their findings revealed that candidate genes and key metabolic pathways prolonged the senescence of sunflower by increasing photosynthesis expression levels.

Lu *et al*. (2013) and other researchers evaluated metabolomics of different plants in response to growth under salt stress. Other studies include that by Cu *et al*. (2018) who established peanut under salt stress and revealed that 92 metabolites were altered in response to the salt stress while 1,742 transcripts in shoots and 3,281 transcripts in roots were altered in response to the stress, and 372 transcripts in shoots and 1,386 transcripts in roots responded particularly to recovery but not to salt stress. In addition, Guo et al. (2015) used GC-MS analysis to profile metabolics of wheat and identified 75 metabolites that were different between the treatments, including organic acids, amino acids, sugars/polyols, and more. Also, salt stress and alkali stress generated various metabolic changes.

Even though re-using wastewater and mine water could contribute to dwindling agricultural water, save water resources and lessen environmental difficulties, there are potential negative consequences to crop production (Libutti *et al*., 2018). Riemenschneider *et al*. (2016) identified 12 micropollutants and six carbamazepine metabolites in field-grown vegetables. Although there are international and national standards that govern the quality of irrigation water in terms of hygienic parameters, salinity, and (heavy) metals, currently, there is no regulation for the prevalence of trace contaminants (WHO, 2006). Abreu *et al*. (2018) showed that zucchini plants irrigated with desalinated saltwater produced more zucchini, had greater glucose, fructose, and vitamin B3 concentrations in their fruits, and had higher antioxidant activity. In the same stuy, plants irrigated with groundwater increased their sugar levels while irrigating zucchinis with groundwater increased the concentrations of trigonelline, histidine, and phenylalanine.

Earlier literature background on potato tuber development, metabolism, and the controlling mechanisms largely focussed on targeted analysis of gene and protein expression and metabolite/flux analysis (Matsuda *et al*., 2003; Morandini, 2009). However, the introduction of the ‘‘omics’’ technologies such as transcriptomics, proteomics and metabolomics provide important and additional information that facilitates a profounder understanding of issues of trait development and trait differentiation between species or genotypes. Studies in the early year 2000’s by Roessner *et al*. (2000); Davies *et al*. (2005); Urbanczyk-Wochniak *et al*. (2005) and

Shepherd *et al*. (2010) reported potato untargeted metabolomic approaches using gas chromatography and liquid chromatography mass spectrometry (LC-MS and GC-MS) to evaluate changes in primary metabolites under different conditions to assess metabolic response to various genetic modifications, abiotic stress and to determine the phytochemical diversity among cultivars. Metabolomic studies in potato (*Solanum tuberosum*) have gradually increased partly as a result of the tubers exhibiting traits (quality of starch, chipping quality, flesh colour, taste, and glycoalkaloid content) that can be linked to a wide range of metabolites (Dobson *et al*., 2008; Carrera-Quintera *et al*., 2012). Thus, tuber quality can be assessed by evaluating a range of metabolites. Some metabolites are strongly affected by growth factors such as light, temperature, type of soil, application of fertilizers, pests, diseases, heavy metals (Hounsome *et al*., 2008).

2.4 Microbial diversity in AMD

2.4.1 Microbial diversity in AMD habitats

Acid mine drainage comprises of components that alter the diversity of microorganisms that inhabit them. As mentioned above, the key factors that shape AMDs associated with microbial diversity are pH, temperature, concentrations of dissolved metals and other solutes, total organic carbon (TOC), and dissolved oxygen (DO) (Mendez-Garcia *et al*., 2015). Several studies have documented the microbial diversity from both exhausted and operational mining sites and a variety of organisms detected in environments affected by AMD. Advances including the use of 16S rRNA gene and meta-omics-based molecular analyses in combination with culture-dependent approaches have contributed to an increase in knowledge on the microbial diversity and functioning of AMD microenvironments over the last three decades. The observation of microbial diversity within most studied AMD sites includes organisms belonging to the domains *Bacteria*, *Archaea* and *Eukarya* (predominantly fungi and algae) by means of both classical microbiological methods and molecular genetic techniques (Kamika & Momba, 2014; Mendez-Garcia *et al*., 2015; Kadnikov et al., 2016).

There are several studies that documented the diversity of soil microbes particularly bacteria in sites treated with AMD (Kamika & Momba, 2014; Sun *et al*., 2015; Chen *et al*., 2016; Mesa *et al*., 2017; Lukhele *et al*., 2020). *Proteobacteria, Nitrospirae,* *Acidobacteria, Chloroflexi,* and *Actinobacteria* are the prominent taxa in varied AMD polluted environments (Kuang *et al*., 2013; Méndez-Garca *et al*., 2015; Clapa *et al*., 2019; Lukhele *et al*., 2019). For example, Clapa *et al*. (2019) reported that the pH of microbial communities that inhabit extreme acid mine drainage (AMD) polymetallic mine ranges from 1.0 to 1.5. Furthermore, bacteria belonging to the Proteobacteria, Acidobacteria, and Actinobacteria groups were found. Similar results were also reported by Kadnikov *et al*. (2016) who showed that the dominant microbial populations in AMD were *Proteobacteria*, *Nitrospira*, *Firmicutes* and *Acidobacteria*. The most common *Proteobacteria* are *Acidithiobacillus* species (*Acidithiobacillia* classis nov.) and is characterized as a mesophilic member of the γ-proteobacteria. He *et al*. (2007) also identified *Nitrospira, a-Proteobacteria, b-Proteobacteria,* and Proteobacteria as main dominating bacterial families as well as *Acidithiobacillus* and *Gallionella* genera in the Yunfu Sulfide Mine in China. Acid mine drainage dams associated with tailings of the deep mines of South Africa revealed *Proteobacteria, Firmicutes,* and *Planctomycetes* including *Marinobacteria* spp. and *Anabaena spp*. respectively (Keshri *et al*., 2015; Lukhele *et al*., 2019; Sibanda *et al*., 2021).

2.4.2 Effects of remediation techniques on microbial diversity

Remediation techniques have been shown to have effects on the microbial diversity within AMD sites (Nayak *et al*., 2015; Narendrula-Kotha & Nkongolo, 2017; Rambabu *et al*., 2020). For instance, Liang *et al*. (2021) reported that the application of quicklime on severely acidic soils used to grow tobacco enhanced the diversity of dominant bacteria and fungi and enriched bacterial genera *Rhodanobacter, Gaiellales, Streptomyces,* and *Terrabacter*. Pang *et al*. (2019) reported improved microbial community richness as well as abundance and functions of *Acidobacteria* and *Chloroflexi*, soil nutrient status, and crop yield in a sugarcane cropping system associated with application of lime. The supply of fly ash in acid/metal-contaminated soils increased the diversity of bacterial and fungal communities (Garcia-Sánchez *et al*. 2015). Nayak *et al*. (2015) showed that application of FA at lower levels in soil enhanced micronutrients content, microbial activities, and yield of soil-grown crops.

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CHAPTER 3: EFFECTS OF QUICKLIME TREATED ACID MINE DRAINAGE IRRIGATION ON PHYSIOLOGICAL PARAMETERS AND AVAILABILITY OF HEAVY METALS ON POTATO CULTIVARS

3.1 INTRODUCTION

The supply of clean and safe water has become a serious concern in many districts across South Africa. As alluded to in chapter 1, South Africa (SA) is one of the driest countries in the world and projections show that the county experiences serious water scarcity. The country's water availability is relatively restricted, unevenly distributed, and badly impacted by climate change (Blignaut *et al*., 2009). Hoffman *et al*. (2009) and Vetter (2009) remarked that the problem is very likely to get worse if climate change trends continued to rise as projected. In 2015, the country's weather forecast was marked by unprecedentedly dry and hot weather (El Chami & El Mojabber, 2016). Water that is available to consumption across the world is at times contaminated by resource depletion, expanding populations, industrialisation, and urbanization which has resulted in a shortage of high-quality water supplies (Johnson *et al.,* 2001; Biswas, 2004; Ochieng *et al.,* 2010; Molebola & Sinha, 2011; Amer *et al.,* 2016). Water shortage seems to be one of the most important commonly faced problems particularly in arid and semi-arid regions. Mancosu *et al*. (2015) projected a high demand for food which would have a direct effect on the usage of water for agricultural purposes due to the increasing world population growth and high volume of wastewater generated every day. Countries can be able to cope with water shortages if they design and/or adopt measures intended to improve the efficiency of water consumption especially in the agricultural sector given that crop production demands huge volumes of water (Mancosu *et al*., 2015). Research conducted in several developing countries has suggested that irrigating with wastewater could be a solution (Hussain *et al*., 2013; Khalid *et al*., 2017; Nzediegwu *et al*., 2019). South Africa's rural and urban populations rely on surface water, and with the country's economy heavily reliant on mining, its water supplies are progressively being polluted, notably by acid mine drainage (Ochieng *et al.,* 2010).

As alluded in chapter 2, Acid mine drainage (AMD) has low pH with high levels of heavy metals. These characteristics make AMD unsuitable for drinking or irrigation.

Also, AMD water comprises diverse microorganisms belonging to the following domains: *Bacteria*, *Archaea* and *Eukarya* (Tyson *et al*., 2004; Akcil & Koldas, 2006; Egiebor & Oni, 2007; Chen *et al*., 2015) that lowering their pH. Research has shown that ecosystems that are polluted with AMD water show soil acidification, high concentration of heavy metals, are polluted with Fe and sulphate (SO_4), and decreased crop health (Nieto *et al*., 2013). Because of these characteristics, AMD is not suitable for drinking or irrigation. Its contamination of irrigation water and agricultural ecosystems has been recorded in several parts of the world, including Bolivia (Oporto *et al*., 2007), South Africa (Oberholster *et al*., 2010), Tunisia (Boussen *et al*., 2013), Vietnam (Bui *et al*., 2016). The Fraser Institute (2012) recorded changes to the types of crops that can be grown as well as decreases in crop productivity and quality among consequences of irrigating with AMD water.

Potatoes (*Solanum tuberosum* L.) along with rice (*Oryza saliva* L.) and wheat (*Triticum aestivum* L.), is a significant staple food in various parts of the world and requires adequate supply of water to achieve a high-quality yield (Levy & Tai, 2013). One of the most critical factors affecting potato yield and quality is the supply or availability of good unpolluted soil water (Yuan *et al*., 2003). Lerna & Mauromicale's (2012) found that the potato crop is highly vulnerable to water stress particularly during the tuber formation and tuber bulking growth stages and these decrease yield. Overall, the foremost factor that negatively influence the production of potato is the type of irrigation (Elzner *et al*., 2018). Some studies have examined the effects of using AMD and/or wastewater to irrigate crops. One of these studies assessed the effects of mixing fly ash (FA)-treated acid mine drainage with fly ash at different rates on the growth, tuber yield, elemental composition, stomatal conductance, and chlorophyll content of two potato cultivars (Nemutanzhela *et al*. 2017). They showed that irrigation with 75 % AMD considerably enhanced growth and tuber output while (Fly ash: FA) FA-treated AMD tubers exhibited substantial hazardous levels of Ni, Zn, and Sr. In addition, FA-treated AMD potatoes had reduced leaf stomatal conductance and chlorophyll content. Due to the abundance of free lime in South African coal, combustion creates FA that is very alkaline. Fly ash has been found to control AMD formation in situ in mine spoils and for acidic soil rehabilitation by several authors (Gitari *et al*., 2008; Madzivire *et al*., 2010; Vadapalli *et al*., 2008, 2012; Kalombe *et al*., 2020). As amorphous hydroxides or oxyhydroxides, FA raises the pH and immobilizes the contaminating elements. The mixing rate influenced the change in pH and water composition. Globally, there is little or no published literature on how irrigating potato with AMD ameliorated with quicklime could alter their physiology and growth parameters. The present study was aimed at determining the effects of quicklime-treated AMD on the physiological parameters of two potato cultivars and heavy metals toxicity on the water, soil, and tubers.

3.2 MATERIALS AND METHODS

3.2.1 Ethical statement

This research was approved by the Ethics Committee of University of South Africa (UNISA) in the Department of Agriculture and Animal Health. Two cultivars of potato seeds were donated by McCain company from Delmas, South Africa. For the described field investigation, the required permits were obtained in accordance with the Ethical Clearance processes. There were no endangered or protected species in the field research.

3.2.2 Study Area

The study was conducted at the University of South Africa (UNISA), Florida Science Campus, Johannesburg, Gauteng Province (S 26° 10′ 30″ S, 27° 55′ 22.8″ E). The greenhouse experiment was conducted between the months of August and November 2018. The temperature in the greenhouse was between 20- 25 ° degrees aligned with potato growth temperature requirement.

3.2.3 Water sampling and pre-treatment

Acid mine drainage water samples were collected from Sibanye Gold Mine (S 26˚07.171', E027˚43.305' at an elevation of 1670 m) using sterile 50 Litres (L) UV-sterilized plastic containers washed with 20% sodium hypochlorite and UV-sterilized for one hour. Prior to analysis, water samples were accurately measured into 2 L containers. A total of five experimental treatments with different solution ratios (amount (g) of quicklime (QL): percentage of fly ash (FA): percentage of AMD) as shown below:

(i) Treatment 1(T1) = 0:0, Tapwater.
(ii) Treatment 2 (T2) = 0:100, AMD water.
(iii) Treatment 3 = 1:100, 1 g Quicklime and AMD water.
(iv) Treatment 4 (T4) = 2:100, 2 g Quicklime and AMD water
(v) Treatment 5 (T5) = 2:75:100, 2 g Quicklime, 75 % FA and AMD water.

Before irrigating with AMD, as alluded above, the water was treated with quicklime based on the Othman *et al*. (2017) protocol. Quicklime (QL) was obtained from Lecco Pty located near Springs in Johannesburg. For the Lab segment of the experiment, each weight of QL was added into a 2 L beaker that contained 1 L of AMD (1g of QL equivalent to 1 L of AMD water). The AMD water that contained QL was stirred using mechanical stirrer. The method to carry out the experiments is known as the Jar test, a well-known active treatment technique (Figure **3.1**). Before adding Ql, AMD water was reddish-brown and after the QL was added, the AMD water colour changed to orange and precipitation was simply observed.

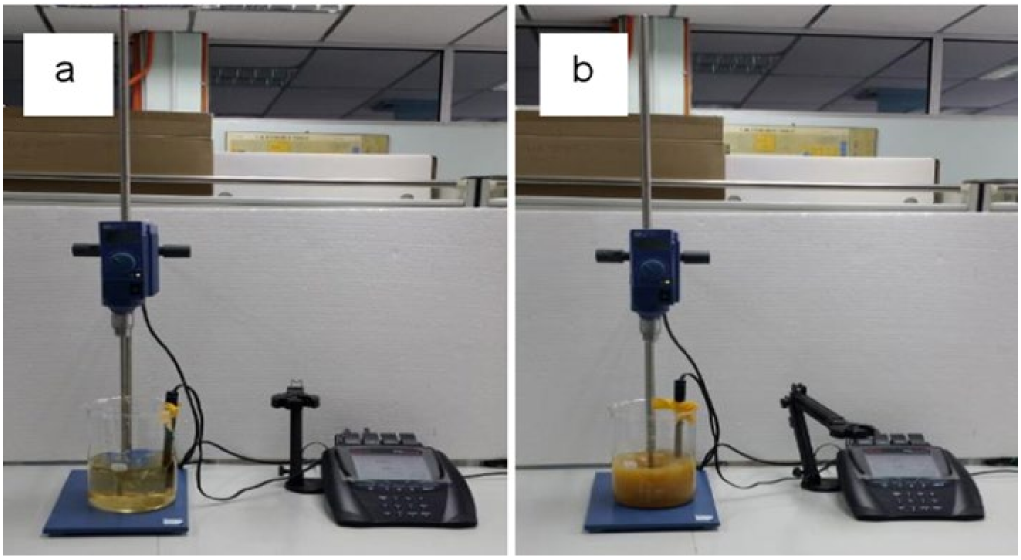


Figure 3.1 Jar test showing AMD water treatment used for irrigation (a) before and (b) after reaction between AMD and quicklime **(Source: adapted from Othman *et al*., 2017).**

3.2.4 Physicochemical analysis

For (un) treated water samples, the pH and heavy metals concentration of the used AMD were recorded before and after treatment using pH meter (A329, Thermo

Scientific, Indonesia) and ICP-EOS (Agilent Technologies 700 series ICP-OES). A multi-probe field meter (YSI TM 6 series, Sonde Marion, Germany) was used to measure physicochemical parameters such as pH, temperature (T), total dissolved solids (TDS), dissolved oxygen (DO), and electrical conductivity (EC) in situ. Triplicate water samples from each experimental treatment level namely T1:Tap water, T2: 100% Acid mine water, T3: 100% AMD + 1g QL, T4: 100% AMD + 2 g QL and T5: 100% AMD + 1 g QL + 75% FA mixture was prefiltered via Porafil 0.45 m, 47 mm cellulose acetate membrane filters under negative pressure. All heavy metal analyses filtered samples were acidified to pH 2 with 70% nitric acid and stored at 4 °C awaiting analysis.

Soil samples were collected in triplicates from each treatment and stored at 4 °C before being dried in a benchtop vacuum freeze dryer in the laboratory (Labconco, USA). Before passing through a 200-mesh sieve, all dry materials were homogenized in a pestle and mortar. To avoid cross-contamination, each sample was ground separately and in ascending sequence of concentration. Heavy metal content was determined in both water and soil samples. The anions (sulphates and nitrates) were measured spectrophotometrically from non-acidified pre-filtered water according to the manufacturer's instructions using a Spectroquant Pharo 300 (Merck, RSA). The denotations T1 to T5 represent treated water samples whereas ST1 to ST5 represent soil samples obtained from soil irrigated with the treated water and TT1 to TT5 represent the tuber of potato harvested from soil irrigated with treated AMD.

3.2.5 Pot experimental design, potato planting schedule and irrigation water treatments

The greenhouse experiment was conducted between the months of August and November 2018. The factorial experiment involved randomized blocks which comprised of six pots (2 × 5) in which Royal and Marykies (Cultivars) potato tubers of almost equal diameters between 30 – 60 mm were planted in a 20 x 20 cm pot (Figure **3.2**). A mixture of 3:1:1 made up of Culterra topsoil, vermiculite and river sand was used as a substrate. After planting, all the pots were immediately irrigated with tap water for 1 week until sprouts developed before application the treatments. As previously stated, the irrigation treatments consisted of acid mine drainage that was improved with different quantities of quicklime. From emergence until crop maturity, irrigation with the various AMD treatments was applied every two days (senescence). An Irrometer Soil Moisture Meter (SN: 946,776) (Model 30 – KTCD – NL) was used to accurately schedule irrigation. When the Irrometer reading was between 60 and 100 centibars, 500 ml irrigation water was applied to all experimental pots every cycle.

Figure 3.2 A randomized block design greenhouse pot experimental setup for Royal and Marykies potato seeds. (**Photo**: Rabelani Munyai, 2018).

3.2.6 Physiological response analysis

Plant growth analysis was done to determine how irrigation with quicklime-treated AMD water affected the growth and yield of the test potato cultivars. The two cultivars were evaluated for following growth parameters such as plant height, chlorophyll content and stomatal conductance at 4-day intervals starting on day 40 after planting (DAP). At the end of the experiment, when the plants had achieved maturity 85 DAP, the number of tubers, as well as fresh and dry weight, were recorded. A 30 cm ruler was used to measure the height of the plants. All tubers were rinsed with tap water after harvesting, dried with paper towels, and weighed. The UW4200H top loading Balance scale was used to determine the tuber fresh weights per plant. The scale can weigh anything from 0.5 grams to 4200 grams. Fresh tubers per individual plant were freeze-dried and weighed to determine tuber dry matter using a freeze dryer (Free

Zone Plus 2.5 L Cascade Benchtop Freeze Dry System Vacutec, USA). All plant parameters were measured following the protocols from Nemutanzhela *et al*. (2017). The chlorophyll content and stomatal conductance were measured on a plant leaf (abaxial and adaxial) using a hand-held Minolta SPAD (Soil Plant Analysis Development)-502 meter (2900 PDL, Spectrum Technologies, Inc.) and Porometer Model SC–1 Leaf Porometer, Pullman, USA respectively. The 4th matured completely expanded leaf from the apex of the plant was used for both measures (chlorophyll and stomatal conductance) at weekly intervals from 40 to 72 days after planting.

3.2.6 Determination of heavy metals for potato tubers and soil

To determine heavy metal content, hydrochloric-nitric acids HNO_3–HCl method was used adopted from Uddin *et al*. (2016). The method was used due to the ability of hydrochloric-nitric acids HNO3–HCl to liberate metal ions from such complex matrices of tuber materials and, as a result, to limit noise levels during the detection technique, has proven to be the optimum acid combination suitable for potato tuber samples. A total of 1 g of potato tubers and soil sample from each of the five treatments were weighed using a UW4200H top loading Balance scale, then placed in microwave vessels and mixed with 9 mL nitric acid (65%) and 3 mL hydrochloric acid (37%). As previously stated by Sekhohola-Dlamini *et al*. (2020), the digestion procedure was performed at 175 °C for 60 min at 6 Watts power. After digestion, the samples were left to cool and thereafter centrifuged at 10 000 Xg for 10 minutes. The supernatant was then collected, diluted with deionized water (1:3), filtered through Whatmann No 1. filter paper, and in each tube, volume was made to 50 ml in a volumetric flask. The suspension settles overnight at room temperature. An Inductively Coupled Optical Emission Spectrometer (Agilent Technologies 700 series ICP-OES) was used to assess the presence of heavy metals

3.2.7 Determination of Enrichment Factor (EF) in soils

The enrichment factor (EF) is a commonly used method for evaluating the anthropogenic influence of heavy metals (Huu *et al*., 2010; Chopra *et al*., 2013). The ratio of the sample metal enrichment over the concentration present in the reference material is used to determine the degree of contamination (Mediolla *et al*., 2008). The Enrichment Factor was determined using expression below: EF = (Metal/RE) soil/(Metal/RE) water. Where, RE is the value of metal, adopted as Reference Element. Al (Chen et al., 2007a) and Fe (Chen et al., 2007b) are two commonly used elements for normalization (Ghrefat & Yusuf 2006). Iron (Fe) was utilized as a conservative tracer in this work to distinguish natural from anthropogenic components and prefer to represent metal contamination in terms of average water to quantify the extent and degree of metal pollution. Fe contents were adjusted to metal concentrations in ordinary water and used as a reference metal using this procedure. There are five contamination categories used on the enrichment factor (Sutherland, 2000). The EF < 2 is deficiency to minimal enrichment, EF = 2–5 is moderate enrichment, EF = 5–20 is significant enrichment, EF = 20–40 is very high enrichment and EF > 40 is extremely high enrichment.

3.3 STATISTICAL ANALYSIS

The measurements' data were subjected to analyses of variance (ANOVA) in the same way that a completely randomized block design would be. The Duncan's Least Significant Difference (LSD) test was used to compare the means of each parameter at $p < 0.05$. The Statistica v. 10, StatSoft (USA) application was used for all statistical studies.

3.4 RESULTS AND DISCUSSION

3.4.1 Physicochemical properties of the water and soil

The physicochemical parameters of different water treatments and soils are summarised in Table **3.1** and **3.2**. Significant difference ($p < 0.05$) was observed across the treatments for all the measured parameters (Table **3.1** and **3.2**). After the application of quicklime in the AMD water, the pH increased from 3.85 (T2) to 6.23-8.63 (T3-T4) and 8.85 (T5), respectively. These values are within the permissible limit of WHO standards (WHO, 2006). Consistent to the present study, quicklime and fly ash treatment has been reported to increase the pH of water to levels that are acceptable for irrigation of crops (Tolonen *et al*., 2014; Othman *et al*., 2017; Chen *et al*., 2020; Liang *et al*., 2021). This is attributed to the ability of the two compounds to neutralise the acid formed in the AMD (Geldenhuys *et al*., 2003).

The EC and TDS values of treated water (T3, T4 and T5) decreased when compared to untreated water (T2); and were also within the recommended limit (WHO, 2006). In general, high EC values might make it difficult for plants to absorb ions from the soil solution (Oladeji *et al*., 2012). On the other hand, TDS has a significant impact on plant growth, yield, and quality. The EC of treated mine water varied from 421.64 (T4), 434.61 (T5) to 917.43 (T3); and the TDS values ranged from 853.14 to 2431.16 mg/L. Oyem *et al*. (2014) observed a strong positive correlation between EC and TDS. This could account for the concurrent decrease of both parameters in this studied under the ameliorating effect of QL and FA (Table **3.1**). However, only T3 (1 g of QL) was above the maximum permissible limit (700 µS/cm) of EC and TDS (1000 mg/L) for irrigation water. In another study, Shabalala & Ekolu (2019) also reported high EC (3100 to 13 000 µS/cm) values of treated mine water that were above the maximum permissible limit for irrigation water. Similar to the findings of study, EC values of the studied samples collected from Eshidiya Mines in South Jordan differed from mine waste water and ranged from 3689 to 3795 S/cm with a mean value of 3724 12 S/cm, while those from another mine waste water site ranged from 3869 to 3960 S/cm with a mean value of 3919 11 S/cm, both of which were above the WHO standard value (Al-Hwaiti *et al*., 2016). This implies that irrigation of crops with water treated with 1 g of QL (T3) would not be recommended. The EC, or specific conductance, of a water sample is used to measure the salinity suitability of irrigation water. Irrigating with excessively salty water elevates soil salt levels, which can be harmful to crops (Mansilha *et al*., 2021). The ability of plants to absorb water and nutrients can be restricted or inhibited when grown in saline environments, leading to stunted growth, and reduced yields. Results of this study showed that the solution obtained from mixing AMD with 2 g of QL (T4) and 2 g of QL and spiking with 75 % of FA (T5) was suitable for irrigating the selected potato cultivars.

The treatment of AMD with quicklime and fly ash precipitates the sulphates associated with AMD, leading to a decrease in its concentration (Tolonen *et al*., 2014; Qureshi *et al*., 2016). In consonance to our findings, the sulphate was decreased by the application of quicklime and fly ash treatments (T4 and T5) compared to irrigating with the raw AMD (T2). However, values were still above the recommended standard for irrigation (WHO, 2006). According to Al-Hwaiti *et al*. (2016), sulfate can interfere with a plant's ability to absorb other nutrients when present in high amounts. However, for this study, the high concentration could be associated with quantity of QL and FA that were used. Shirin *et al.* (2021) alluded in their work that there was a need to increase the quantity of QL in the treatment of AMD to achieve a desirable result. However, the disadvantage of this suggestion is that it could lead to an increase in the amount of unstable secondary waste that can be produced during the process. Hence, application of appropriate amount of QL is required to maximise its ameliorating effect and reduction of the unstable secondary waste production.

Table 3.1 Physiochemical properties of quicklime and fly ash (un)treated AMD water.

	Mean concentration of water treatment levels					
Parameters	**TW1**	**TW2**	**TW3**	**TW4**	**TW5**	**WHO**
pH	8.45±0.11c	3.98±0.01e	6.23±0.06d	8.63±0.06b	8.85±0.08a	**6.5-8.5**
Temp (°C)	25.33±0.58a	19.42±0.24e	22.33±0.58d	24.33±1.15c	24.00±1.00b	**-**
EC (μS/cm)	45.98±0.98e	3641.33±52.05a	917.43±3.75b	421.64±4.93d	434.61±5.29c	**700**
TDS (mg/ L)	128.35±1.89e	4874.00±24.27a	2431.16±71.70b	922.61±1.44c	846.47±4.65d	**1000**
NO_3^- (mg/ L)	2.17±0.13e	6.29±0.19a	2.34±0.06d	2.38±0.33c	2.66±0.10b	**50**
DO (mg/ L)	16.09±0.19a	5.54±0.18e	11.13±0.29d	13.24±0.40c	14.84±0.21b	**-**
SO_4^{2-} (mg/ L)	224.55±3.86e	5255.33±49.08a	1127.55±3.16d	1182.28±14.62c	1195.81±1.72b	**500**

Mean ± SE in same row with dissimilar letter are significantly different at $p < 0.05$.

*DO: Dissolved oxygen, EC: Electrical conductivity, NO: Nitrate, TDS: Total dissolved solids, and SO_4^{2-}: Sulfate.

Comparable to the physiochemical parameters of the treated water, the physicochemical parameters of soil irrigated with treated AMD water were significant ($p < 0.05$) across the treatments for the measured parameters (Table **3.2**). There was a significant ($p < 0.05$) increase in the pH of the soil irrigated with treated AMD (5.67 (ST3), 6.70 (ST4) and 7.23 (ST5) as compared to the untreated AMD (T2) and tapwater (T1) (Table **4.2**). In agreement with our findings, Natsheh *et al*. (2021) recorded an increase value of pH for soil irrigated with treated AMD water. Heavy metal mobility and bioavailability are greatly influenced by soil pH (Nigam *et al*., 2001). The change in pH values appears to be due to the type of water utilized (Nigam *et al*., 2001). Through altering the soil chemistry, pH plays a significant role on plant growth and health especially on increasing the availability of nutrients in the soil (Roohi *et al*., 2017). The electrical conductivity (EC) of soil has a direct effect on the parameters that influence crop yield. Decreased EC values were also recorded for the soil irrigated with treated AMD water (Table **4.2**). Contrary to our study, Singh *et al*. (2012) and

Saleh *et al*. (2013) observed an increase in EC values of soil irrigated with treated wastewater. This could be attributed to the different wastewater treated with QL and fly ash. In summary, a notable decrease in pH and EC; and increase in sulphate were observed in soil irrigated with treated AMD water (Table **3.2**) when compared to the treated AMD water (Table **3.1**). This could be attributed to the interaction of plant-microbes that have the tendency to alter the soil environment making it suitable for the growth of crops (Xin *et al*., 2021). Furthermore, the increase in sulphate in AMD polluted environment is associated with sulphate oxidizing bacteria present in the AMD (Wang *et al*., 2019). This implies that the increase in the sulphate in the soil that was irrigated with treated AMD could be associated with the presence of sulphate oxidizing bacteria that was not removed through the treatment as well as the plant-microbe interaction between the bacteria and potato cultivars.

Table 3.2 Physicochemical properties of soil samples irrigated with quicklime and fly ash (un)treated AMD water.

	Mean concentration of soil treatment levels				
Parameters	**ST1**	**ST2**	**ST3**	**ST4**	**ST5**
pH	7.13±0.12a	3.85±0.14d	5.67±0.11c	7.70±0.05b	7.87±0.06a
EC (mS/m)	0.52±0.09e	163.40±2.77a	50.29±1.49d	72.32±1.47c	85.00±0.95b
NO_3^-	0.74±0.08e	8.78±0.31a	4.21±0.08b	2.80±0.02c	2.17±0.06d
SO_4^{-2}	16.25±0.43e	12706.01±19.60a	848.32±1.86d	1208.90±16.92c	1264.99±9.06b

Mean ± SE in same row with dissimilar letter are significantly different at $p < 0.05$.

*EC: Electrical conductivity, NO: Nitrate, TDS: Total dissolved solids, and SO_4^{2-}: Sulfate.

3.4.2 Heavy metal contents of irrigation water

To assess whether the treated AMD water was suitable for irrigation, the concentration of heavy metal in the treated water samples was analysed. The treated AMD water showed significant difference ($p < 0.05$) for all the measured parameters (Table **3.3**). The treated AMD water (T3, T4, and T5) exhibited reduced concentration of Al, As, Co, Cu, Cd, Cr, Fe, Mg, Mn, Ni, and Zn when compared to the untreated AMD (T2). Congruent to our findings, Tolonen *et al*. (2014) reported that treating AMD with QL removed 99 % of Al, As, Cd, Co, Cu, Fe, Mn, Ni, and Zn. Further, Othman *et al*. (2017)

observed a reduction in the concentration of As, Cd, and Cr under QL treatment of AMD. Comparing the findings of this study to the stipulated standard, T3 did not meet any recommended standard with Al, Cd, Cr, Fe, Ni and Zn whereas most of the heavy metals in T4 and T5 were reduced to levels below that stipulated in standards except for Pb, Mg and Mo (Table **3.3**). The difference in the three treatments could be associated with the quantity of QL applied as well as the spiking of QL treatment with fly ash as indicated by Shirin *et al.* (2021). According to Shabalala and Ekolu (2019), high levels of metals in irrigation water cause pollution of agricultural soils and metal uptake by crops produced on these soils. As a result, the level of heavy metals in irrigation water must be kept within a certain stipulated standard.

Table 3.3 Heavy metal concentrations in irrigation water (Sources: DWAF and FAO. FAO standards: adapted from Jeong *et al*. (2016).

	Heavy metal mean concentration (mg/L)						
Metals						**Permissible limit**	
(mg/L)	**T1**	**T2**	**T3**	**T4**	**T5**	**DWAF**	**FAO**
Al	0.29±0.01e	305.28±1.65a	23.89±0.25b	4.31±0.01c	1.40±0.01d	**5-20**	**5.0**
As	0.02±0.00d	2.06±0.11a	1.78±0.14b	0.02±0.00d	0.09±0.01c	**0.1-2.0**	**1.0**
Cd	0.01±0.00d	1.36±0.02a	0.08±0.00b	0.01±0.00d	0.03±0.00c	**0.01-0.05**	**0.05**
Co	0.03±0.00c	6.57±0.05a	2.40±0.02b	0.03±0.00c	0.02±0.00d	**0.05-5.0**	**0.05**
Cr	0.04±0.00c	3.78±0.00a	0.66±0.01b	0.04±0.00c	0.03±0.00d	**0.1-1.0**	**0.05**
Cu	0.06±0.00d	1.72±0.12a	0.11±0.00c	0.01±0.00e	0.14±0.00b	**0.2-0.5**	**0.2**
Fe	3.73±0.01d	1029.45±13.87a	22.97±0.29b	2.57±0.03e	4.52±0.01c	**5.0-20**	**-**
Mg	27.94±0.99e	294.41±3.39d	453.18±3.07a	377.18±1.66c	409.44±1.67b	**-**	**0-5**
Mn	0.02±0.00d	34.14±0.34a	4.96±0.05b	0.02±0.00d	0.06±0.00c	**0.02-10**	**0.2**
Mo	0.06±0.01d	0.05±0.00e	129.27±1.01c	285.71±1.73b	349.03±1.74a	**0.01-0.05**	**0.01**
Ni	0.02±0.00e	6.50±0.35a	3.33±0.03b	0.15±0.01c	0.08±0.00d	**0.2-2.0**	**0.2**
Pb	0.02±0.00e	0.33±0.01d	8.30±0.30a	6.28±0.01b	6.10±0.01c	**0.2-2.0**	**5.0**
Sr	0.05±0.00e	0.86±0.02d	12.03±0.12a	2.45±0.01c	5.25±0.04b	**-**	**-**
Zn	0.73±0.01e	50.32±0.66a	5.94±0.08b	2.09±0.05c	1.16±0.05d	**1.0-5.0**	**2.0**

Mean ± SE in same row with dissimilar letter are significantly different at $p < 0.05$.

*In the mean concentration names, T indicates treatment level. *T1 = 0:0, Tapwater; T2 = 0:100, AMD water; T3 = 1:100, 1 g QL and AMD water; T4 = 2:100, 2 g QL and AMD water and T5 = 2:75:100, 2 g QL, 75 % FA and AMD water.

3.4.3 Effect of the irrigation water on the physiological parameters of cultivars

Plant height

When the two potato cultivars (Marykies and Royal) were irrigated with the treated AMD, their height and phenodays varied significantly ($p < 0.05$) (Table **3.4**). This implied that the two cultivars responded differently to the treated AMD water. This could be attributed to the difference in the cultivar as well as differential physiological response of the two cultivars to diverse environmental stress (Rahman *et al.*, 2009).

Table 3.4 Effects of acid mine water drainage (AMD) water mixed with QL and FA on the growth of two cultivars of potato grown.

	Season 1		**Season 2**	
Cultivar	**Phenodays**	**Height (cm)**	**Phenodays**	**Height (cm)**
Marykies	56.01±0.63a	49.27±0.4a	56.5±0.9b	51.7±1.39b
Royal	56.06±0.63a	41.22±0.4b	56.6±0.9a	43.8±0.98a

At $p < 0.05$, values (Mean ± S.E.) followed by similar letters in a column are not significantly different.

Figure **3.3** show a summary of the response of the two cultivars to the different treatments of AMD water. Significant difference ($p \leq 0.05$) was observed across the treatments for both cultivars. In addition, the progress growth of the crops was recorded as shown in Figure **3.4**. In general, Marykies cultivar responded better than the Royal across the treatments. This could be attributed to differences in the physiological response of the two cultivars as impacted by their molecular properties under AMD environment (Baebler *et al.*, 2009). Kiiskila *et al.* (2021) also indicated that different plants respond differently in the synthesis of proteins that could play a crucial role in their adaptation and survival under AMD conditions. The differential protein abundance in the Marykies or the plant-microbe interactions could have played a crucial role in their better adaptation under the treated AMD condition. However, there is a need for further studies to validate this assumption.

Among the treatment levels, T4 and T5 enhanced the plant height of the two cultivars better with reference to the AMD sample (T2) and control (T1). This may be due to lime and fly ash's beneficial benefits in reducing soil acidity, as they are well-known for their strong acid neutralizing capabilities, which can effectively eliminate existing acid, increase biological activity, and minimize heavy metal toxicity. (Anetor & Ezekiel, 2007; Ameyu, 2019; Shirin *et al*., 2021). For instance, a study conducted by Achalu *et al*. (2012), applying lime to acid soil increased barley height, fresh biomass, dry biomass, grain yields, harvest index, and P-uptake. Furthermore, the use of lime enhanced maize growth and yield, owing to the reduction in Al toxicity (Beukes *et al*., 2012). Furthermore, a study conducted in a greenhouse showed that the grain and straw yield of rice were significantly increased by applying up to 20% of fly ash (Nayak *et al*., 2015). For this study, T2 and T3 exhibited low plant height as compared to T1, T4 and T5. Similar results were reported by Ma *et al*. (2013) who observed negative effects on the growth and grain yield of winter wheat irrigated with mine wastewater.

Several studies have shown that the presence of heavy metals in growth media largely retards the growth of plants. Of the toxic heavy metals, Cd, Pb and Hg reportedly exert the most detrimental effects on plant growth (Okcu *et al.,* 2009). Yildirim *et al*. (2019) also reported that stress exerted by Cd and Pb itions could negatively affect plant growth of rocket (*Eruca sativa* L.) plants established under greenhouse conditions. Furthermore, the significantly taller plants of the Marykies compared to that of the Royal cultivar could be attributed to genetic differences, differences in their ability to accumulate metals or variation in their ability to withstand or translocate heavy metals that were not completely trapped and removed by the treatment. Long-term irrigation of both cultivars with the different treatments also significantly affected their height in both seasons when compared to the control. For instance, there was a steady increase in plant height from 40 (D1) up to 56 (D5) but at 70 days after planting, there was a decrease in plant height in both cultivars (Figure **3.4**). This could suggest that there could have been an inhibition of growth possibly due to the accumulation of some heavy metals over time. Other studies have also shown that plants exposed to an excess amount of certain nutrients are stressed and their growth is inhibited (Ali *et al*., 2003).

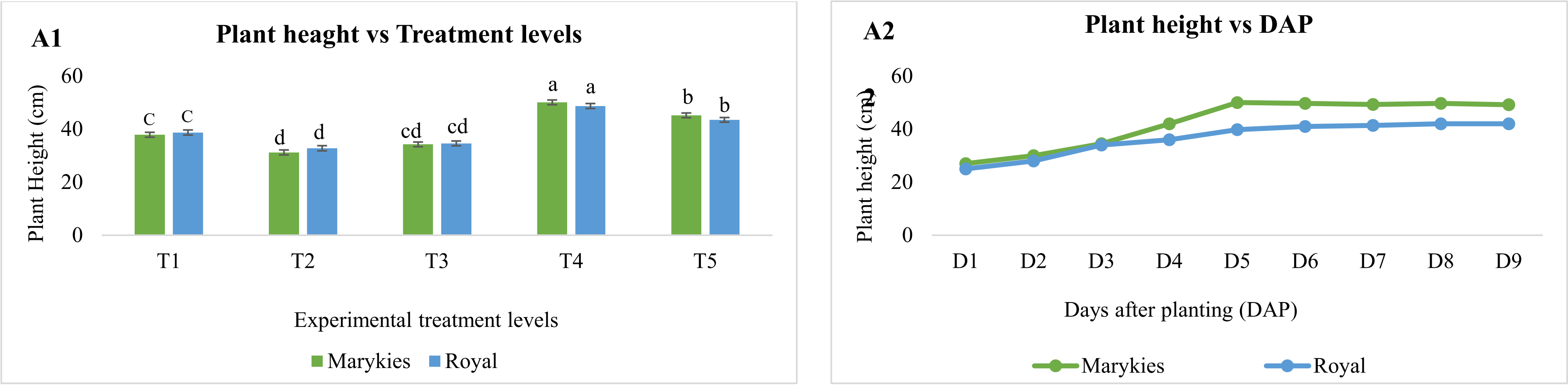


Figure 3.3 Plant height response of two cultivars of potato (Marykies and Royal) irrigated with quicklime and Fly Ash (FA) treated acid mine drainage (AMD) and progressive growth days for a period of 40 to 72 days after planting (Season 1(A1 & 2). Similar letters across the treatments are not significantly different at $p \leq 0.05$.

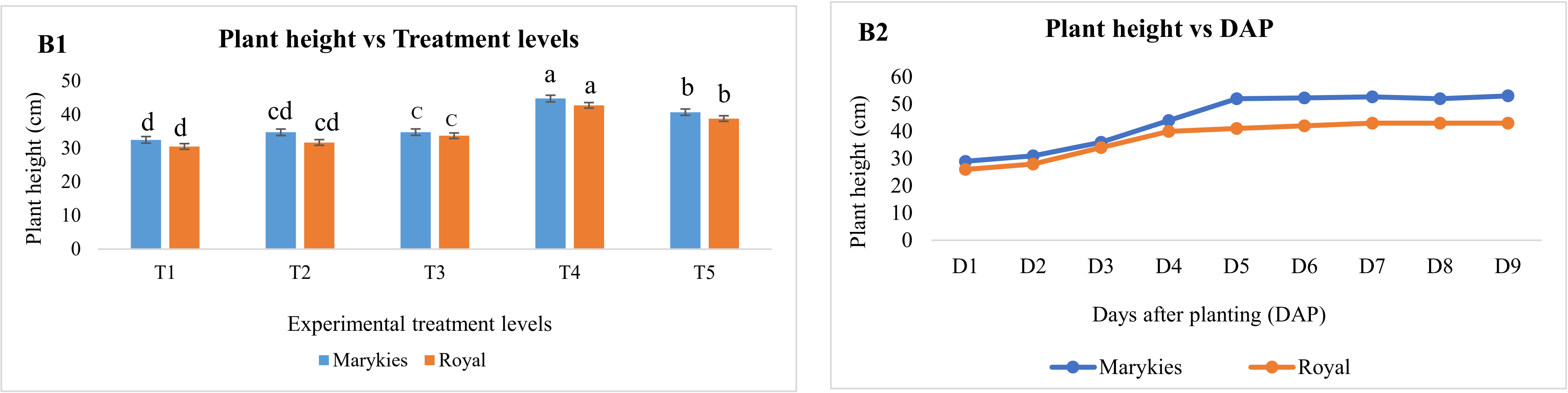


Figure 3.4 Plant height response of two cultivars of potato (Marykies and Royal) irrigated with quicklime and Fly Ash (FA) treated acid mine drainage (AMD) and progressive growth days for a period of 40 to 72 days after planting (Season 2(B1 & 2). Similar letters across the treatments are not significantly different at $p \leq 0.05$.

Chlorophyll content

Plants exhibit dynamism that cuts across physiological, metabolic, and molecular response in their struggle to survive adverse environmental conditions (Kalu *et al*., 2021). Ebrahim (2004) stated that chlorophyll concentration, stomatal conductance, and biomass of roots, stems, leaves, and fruits can all be used to determine a plant's physiological growth. Several studies have suggested that factors such as water stress and soil types might affect the chlorophyll concentration of plant leaves (Shu *et al*., 2013). For this study, the chlorophyll content (CC) and stomatal conductance (mmol m^2 s^1) of two cultivars of potato grown under the irrigation of treated AMD water were measured to ascertain whether their physiological response could promote their survival under (un)treated AMD water stress. The measured physiological parameters showed significant difference ($p < 0.05$) across the two cultivars. The Royal cultivar produced more chlorophyll content than the Marykies (Figure **3.5**). Like in these findings, Nemutanzhela *et al*. (2017) also observed variation in the chlorophyll response when different cultivars of potato were treated with AMD water. This could be linked to crops' physiological responses to stress, which vary depending on the type and level of crops, as well as the type of crops involved (Kalu *et al*., 2021). Clearly, the chlorophyll content (chlorophyll a & b) determined on the leaf of the selected cultivars were significantly affected by long-term irrigation with the different quicklime AMD treatments. For example, the highest chlorophyll content a & b was recorded on T2 and T3 as compared to the controls (T1) and TW4 and TW5 for both seasons (Figure **3.5**). The increase in chlorophyll can be due to possible accumulation of metals (toxic effect) in comparison with the other treatments. A similar trend was observed on season 2 as well.

The chlorophyll content varied on days after planting (DAP) (Table **3.5**) with the highest recorded on D5 which is day 56 after planting or 56 DAP). There was a trend in that the chlorophyll content decreased as the day of planting increased. Congruent to our study, other studies have also observed variation in the chlorophyll content with DAP as impacted the AMD treatment (Sangannavar & Kalshetty, 2011; Ali *et al*., 2013; Nemutanzhela *et al*., 2017). For this study, the implication is that long-exposure of the plants to QL treated AMD could impair the physiological processes of the crops.

Table 3.5 Effects of acid mine drainage (AMD) water treated with quicklime on the chlorophyll content and stomatal conductance of Marykies and Royal.

Chlorophyll content for Marykies and Royal potato cultivars				
	Season 1		**Season 2**	
Cultivar	**Chlorophyll a**	**Chlorophyll b**	**Chlorophyll a**	**Chlorophyll b**
Marykies	16.15±0.44b	27.90±0.57b	26.90±0.57b	33.3±1.0b
Royal	18.91±0.53a	30.39±0.64a	29.39±0.64a	36.9±1.1a
F-Statistics	**342.51 s**	**428.50 s**	**12325 s**	**19903 s**
Treatment levels				
T1	15.96±0.57e	25.63±0.76e	21.80±0.82e	28.50±1.09e
T2	21.50±1.09a	32.37±1.30a	30.62±1.64a	42.18±1.92a
T3	19.89±0.86b	30.42±1.07b	28.89±1.29b	39.23±1.56b
T4	16.04±0.69d	27.47±0.81d	25.02±0.90d	31.37±1.70d
T5	18.29±0.60c	29.83±0.80c	26.30±0.88c	37.92±1.13c
F-Statistics	**34.25 s**	**56.30 s**	**1475 s**	**2200 s**
Days After Planting (DAP)				
D1 (40)	14.14±0.28f	25.05±0.28f	18.00±0.40f	29.22±0.38f
D2 (44)	17.25±0.28e	28.20±0.29e	20.63±0.42e	31.80±0.40e
D3 (48)	22.24±0.52c	33.47±0.53c	26.44±0.77c	35.10±0.78c
D4 (52)	25.24±0.54b	36.63±0.57b	29.54±0.83b	45.12±0.80b
D5 (56)	29.94±0.92a	44.31±0.79a	36.27±1.10a	48.84±1.08a
D6 (60)	20.27±0.40d	31.36±0.42d	24.47±0.57d	33.22±0.55d
D7 (64)	13.23±0.31g	17.44±0.32g	12.18±0.42g	18.86±0.40g
D8 (68)	9.64±0.28h	13.62±0.28h	11.17±0.39h	16.27±0.41h
D9 (72)	5.85±0.24i	8.21±0.21i	6.73±0.33i	10.30±0.37i
F-statistics	**1174.52 s**	**3078.20 s**	**48108 s**	**136353 s**
Treatment x Cultivar				
F-statistics	**1.6 s**	**2.19 s**	**41 s**	**63 s**
Treatment x DAP				
F-statistics	**6.2**	**35.78 s**	**1518 s**	**2877 s**
Cultivar x DAP				
F-statistics	**18.40 s**	**22.97 s**	**440 s**	**823 s**
Treatment x Cultivar x DAP				
F-statistics	**1.38 s**	**1.4 s**	**86 s**	**184 s**

Mean ± SE in same colomn with dissimilar letter are significantly different at $p < 0.05$.

Below, figure 3.5 summarised the information on the effect of the treated AMD on chlorophyll content of both potato cultivars. Significant differences ($p < 0.05$) were observed in the chlorophyll content (abaxial and adaxial) across the treatment for both cultivars of potato.

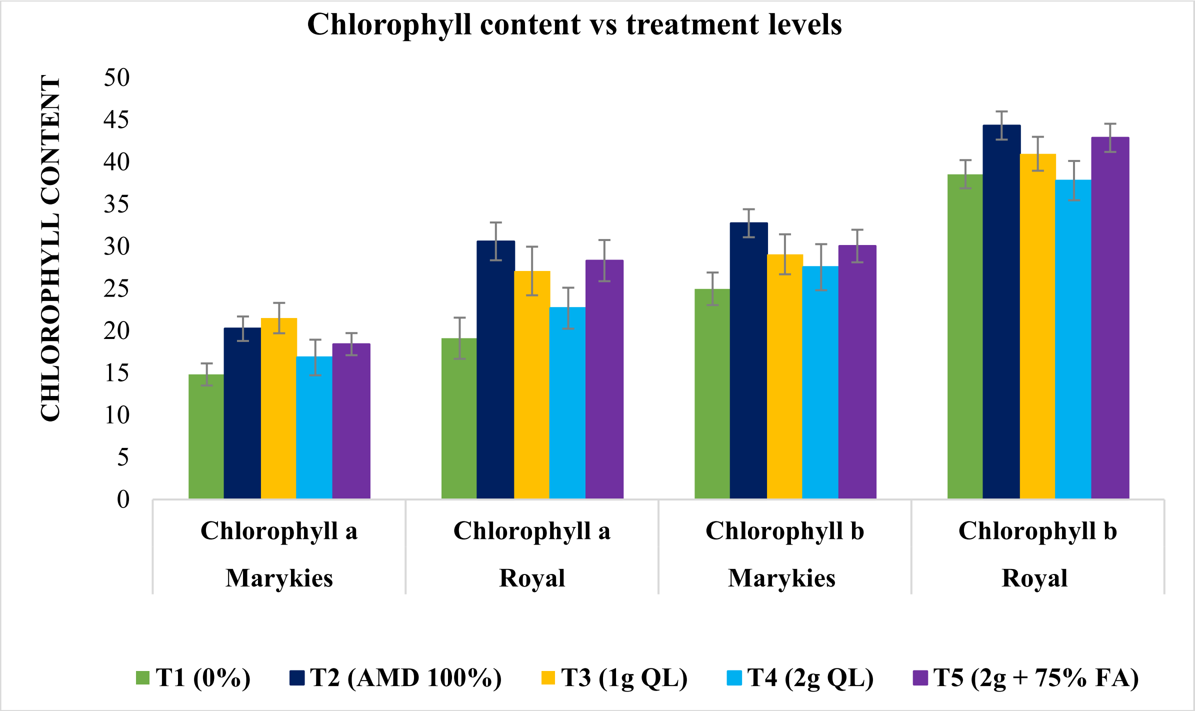


Figure 3.5 Effects of quicklime treated AMD experimental treatments levels on leaf chlorophyll content of Marykies and Royal cultivar.

Stomatal conductance

Stomatal conductance is referred to as a measure of the degree of physical resistance to gas movement between air and leaf interior (Pietragalla *et al*. 2012). Such an exchange supports the exchange in CO_2 intake and water loss (transpiration) through the stomatal aperture. Stomatal adjustments help to maintain plant water status under varying soil moisture and atmospheric conditions. Acid mine drainage is known to constitute an environmental stress to plants, and this culminates into diverse physiological and morphological responses by plants that include reduction in transpiration rate and stomatal conductance (Abegunrin *et al*., 2016; Akhkha *et al*., 2017). The two measured physiological parameters showed significant differences ($p < 0.05$) across the two cultivars. The Royal cultivar produced greater stomatal conductance than the Marykies (Table **3.6**). Nemutanzhela *et al*. (2017) also observed variation in stomatal conductance between Fiana and Lady Rosetta cultivars that were treated with AMD water. In consonant to the findings of this study, other research studies conducted under extreme environmental stress, plants exhibit a plethora of physiological adjustment which reduces stomatal conductance and chlorophyll content as a mechanism to aid their survival (Khaleel *et al*., 2013; Manisha & Angoorbala, 2013; Wen *et al.*, 2017; Sahay *et al.*, 2019; Naz *et al.*, 2020). The treatment of the AMD with QL was promising as the treatments were able to improve the stomatal conductance of the two cultivars. Similarly, there were significant differences (p <0.05) in the stomatal conductance on DAP across the different days of measurement with D5 also showing the highest stomatal conductance (Table **3.6**). Stomatal conductance continued to decrease as the plant grew older.

Table 3.6 Effects of acid mine drainage (AMD) water treated with quicklime on the stomatal conductance of Marykies and Royal.

Stomata conductance of Marykies and Royal potato cultivars				
	Season 1		**Season 2**	
Cultivar	**Abaxial**	**Adaxial**	**Abaxial**	**Adaxial**
Marykies	84.08±2.30b	92.65±2.40b	87.15±3.31b	92.55±3.46b
Royal	102.80±1.85a	111.99±1.85a	106.68±2.74a	116.14±2.81a
F-Statistics	**2059.10 s**	**2255.70 s**	**72761 s**	**114492 s**
Experimental Treatment levels				
Tapwater 0% (T1)	92.17±3.18e	100.67±3.33e	95.47±4.58d	103.86±4.87e
AMD 100% (T2)	95.27±2.99a	104.42±3.04a	99.01±4.40a	108.20±4.47a
1g + AMD (T3)	94.44±4.15b	103.64±4.23b	98.34±6.08b	107.73±6.26b
2g + AMD (T4)	92.31±2.98d	101.13±3.14d	95.30±4.35d	104.08±4.62d
2g + AMD + 75% (T5)	93.00±3.69c	101.74±3.77c	96.46±5.41c	105.36±5.58c
F-Statistics	10 s	11.70 s	562 s	679 s
Days After Planting (DAP)				
D1 (40)	86.82±0.56g	95.97±0.51g	90.18±0.57g	98.44±0.75g
D2 (44)	92.73±0.75f	102.75±0.71f	96.06±0.80f	105.51±0.96f
D3 (48)	103.14±1.08d	112.81±1.10d	108.27±1.30d	118.16±1.22d
D4 (52)	120.74±0.95c	129.39±1.08c	125.30±1.26c	134.59±1.40c
D5 (56)	130.78±0.97a	140.68±1.10a	134.14±1.40a	145.60±1.50a
D6 (60)	127.80±2.26b	137.04±2.22b	133.31±3.39b	142.63±3.58b
D7 (64)	95.06±3.11e	103.78±3.06e	97.98±4.55e	106.84±4.53e
D8 (68)	57.71±3.55h	65.89±3.70h	59.37±5.02h	67.49±5.09h
D9 (72)	26.17±2.00i	32.58±2.45i	27.63±2.95i	33.34±3.36i
F-statistics	**38531.0 s**	**87917.0 s**	**6.0 s**	**1.0 ns**

Mean ± S.E values followed by similar letters in a column are not significantly different at p ≤0.05.

Yield parameters and its components

A two-way ANOVA analysis showed that the quicklime and fly ash treatments of AMD were significant (p <0.05) across the treatments for the tuber yield, fresh tuber weight, and dry tuber weight for both cultivars (Table **3.7** and **3.8**) with subtle variation between them. The treated AMD water samples (T3, T4, and T5) improved all the yield parameters for the two potato cultivars with T2 showing higher potential in the improvement of the yield (Table **3.7**). Maize (*Zea mays*) and sunflower (*Helianthus annuus*) grown in a heavy metal enriched AMD environment showed enhanced growth and copper uptake, according to Li & Ramakrishna (2011). Additionally, using copper-resistant *Pseudomonas* strains improved Zn and Pb bioaccumulation as well as plant growth-promoting indole-3-acetic acid (IAA), iron chelating siderophore, and mineral phosphate and metals solubilization capacity. The increased crop yield observed under T2 could have been because of the presence of growth-promoting bacteria that could have promoted the growth promotion and heavy metal bioaccumulation of the potatoes but could have also enhanced the remediation function through plant-microbes' interactions (Rathi & Yogalakshmi, 2021).

Results on season 2 (Table **3.8**) revealed slight variation between treatments compared to season 1. Marykies had higher number of tubers on T4 and T5 and this enhancement on yield of can be in response to quicklime and fly ash application and other environmental factors in the greenhouse. Several researches including Pandey *et al*. (2010); Singh *et al*. (2012) and Parab *et al*. (2013) reported that fly ash has a potential to improve the yield of wheat (*Triticum aestivum*), rice (*Oryza sativa*), maize (*Zea mays*), mung bean (*Vigna unguiculata*), eggplant (*Solanum melongena*), onion (*Alium cepa*) and chickpea (*Cicer arietinum*) cultivated on different types of soils. Irrigation with AMD water generally causes a shift in the parameters of soils, has the potential to positively alter the microbial diversity and play vital roles in the ecology of the rhizosphere of plants through the maintenance of soil health and therefore increasing the yield of crops (Narendrula-Kotha *et al*., 2019; Wu *et al*., 2019; Li *et al*., 2020; Xin *et al*., 2021). The observed decrease in the yield of crops irrigated with treated AMD water can be explained as a function of the important microbe's reduction during the process of treatment. Hence, there is a need to evaluate a system where AMD treatment can protect the important microbial communities while removing the harmful substance that plants can translocate from the soil.

Table 3.7 Effects of treated quicklime AMD water on the Marykies and Royal yield.

Season 1 (A)	**Number of tubers**	**Fresh tuber weight (g)**	**Dry tuber weight (g)**
Marykies	8.27±0.55a	122.19±2.82b	45.56±2.17b
Royal	6.33±0.34b	287.08±9.91a	91.90±4.80a
F-Statistics	**25.49 s**	**623.24 s**	**45.45 s**
	Treatment levels		
Tapwater 0% (T1)	6.67±0.49d	224.97±45.76a	76.24±13.43b
AMD 100% (T2)	9.33±0.71a	218.29±37.73b	79.75±10.84a
1g QL + AMD (T3)	7.00±0.45c	203.67±38.12c	71.75±13.01c
2g QL + AMD (T4)	6.00±0.26e	182.83±35.14e	56.18±11.20e
2g QL + AMD + 75% FA (T5)	7.50±1.23b	183.40±26.08d	59.90±7.35d
F-statistics treatment	**8.67 s**	**7.26 s**	**6.88 s**
Treatment x Cultivar	**5.94 s**	**4.98 s**	**3.97 s**

Mean ± S.E. values followed by similar letters in a row are not significantly different at $p \leq 0.05$.

Table 3.8 Effects of treated quicklime AMD water on the Marykies and Royal yield.

Season 2 (B)	**Number of tubers**	**Fresh tuber weight (g)**	**Dry tuber weight (g)**
Marykies	9.01±0.30a	123.16±2.13b	45.93±1.17b
Royal	6.47±0.41b	236.39±9.81a	79.16±4.68a
F-Statistics	**25.78 s**	**175.21 s**	**56.60 s**
	Treatment levels		
Tapwater 0% (T1)	8.00±0.58b	215.26±46.15a	68.61±13.05a
AMD 100% (T2)	7.67±0.61c	189.78±38.61b	64.92±10.24b
1g QL + AMD (T3)	7.00±1.03d	145.75±16.93e	59.09±2.72d
2g QL + AMD (T4)	8.00±1.03b	180.46±36.73c	62.19±10.50c
2g QL + AMD + 75% FA (T5)	8.17±1.07a	167.59±20.75d	57.90±5.53e
Treatment	**0.67 s**	**1.89 s**	**0.78 s**
Treatment x Cultivar	**1.30 s**	**2.95 s**	**2.55 s**

Mean ± S.E. values followed by similar letters in a row are not significantly different at $p \leq 0.05$.

Table 3.9 Effects of acid mine drainage (AMD) water treated with quicklime on the yield of two cultivars of potato. Mean ± S.E. values followed by similar letters in a row are not significantly different at $p \leq 0.05$.

Season 1						
	Number of tubers		**Fresh tuber weight (g)**		**Dry tuber weight (g)**	
Treatment	**Marykies**	**Royal**	**Marykies**	**Royal**	**Marykies**	**Royal**
Tapwater 0% (T1)	7.33±0.67d	6.0±0.57d	122.87±1.74c	327.8±6.58a	46.22±0.22c	106.26±0.93a
AMD 100% (T2)	10.67±0.66a	8.08±0.58a	136.32±1.48a	300.27±19.97b	55.76±1.56a	103.75±2.97b
1g QL + AMD (T3)	7.67±0.30c	6.33±0.67c	120.29±0.36d	288.06±17.66c	42.67±1.17d	100.48±3.15c
2g QL + AMD (T4)	5.66±0.33e	6.35±0.34b	105.10±0.41e	260.54±11.57d	33.70±0.42e	78.67±11.03d
2g QL + AMD + 75% FA (T5)	8.22±0.60b	5.01±0.57e	126.35±4.06b	240.45±11.36e	49.47±5.07b	70.33±11.63e

Season 2

	Number of tubers		**Fresh tuber weight (g)**		**Dry tuber weight (g)**	
Treatment	**Marykies**	**Royal**	**Marykies**	**Royal**	**Marykies**	**Royal**
Tapwater 0% (T1)	9.00±0.56b	7.00±0.56b	120.27±0.17d	310.25±40.37a	42.13±0.67d	95.10±12.23a
AMD 100% (T2)	8.00±0.58c	7.33±1.20a	122.22±0.1b	257.33±53.77b	46.34±1.06b	83.51±13.33b
1g + AMD (T3)	9.00±1.00b	5.00±0.57e	137.70±0.89a	153.79±73.21e	53.27±0.57a	64.88±1.80e
2g + AMD (T4)	9.67±0.67a	6.33±1.45d	113.90±1.69e	245.03±48.10c	42.20±1.15d	82.19±12.28c
2g + 75% FA (T5)	9.66±0.33a	6.67±0.33c	121.69±0.32c	213.50±61.64d	45.69±1.87c	70.12±0.29d

3.4.3 Determination of heavy metals for potato tubers

Irrigation of crops with AMD water, be it derived from industrial, municipal, sewage and whether treated or untreated has been reported to be detrimental to crops and agricultural soil because of the possibilities of crops taking up heavy metals (Singh & Agrawal, 2010; Shabalala & Ekolu, 2019). In this study, we irrigated potato cultivars with various concentrations of quicklime (QL) and fly ash (FA) and examined the content of heavy metals in tubers. The results showed the presence of heavy metals in the tubers and their concentrations were significantly different ($p<0.05$) across the treatments (Table **3.10**). There was a reduction in the concentration of some of the heavy metals in the tubers especially under the quicklime and fly ash treatments (TT3, TT4, TT5) when compared to the 100% AMD (TT2). This could be attributed to the effectiveness of the treatment to reduce the concentration of heavy metal in the 100% AMD. Several studies have found that heavy metal stress causes an increase in the production of a variety of metabolites that are crucial in the signaling, sequestration, and transportation of heavy metals like Fe, Cu, Zn, and Cd. (Johnson *et al*., 2011; Zhou *et al*., 2013; Yang *et al*., 2015; Jalmi *et al*., 2018; Safdarian *et al*., 2019).

Apart from the impact of the quicklime and fly ash on the content of heavy metals, the production of diverse metabolites could also account for the reduction in the translocation of heavy metals bythe potato cultivars. Of noteworthy is that not all the reduced heavy metals that were within the acceptable standards recommended by the WHO. For example, Al, Co, Cu, Fe, Mg, Mn, Ni and Zn were within the stipulated standard. By contrast, the concentrations of As, Cd, Cr, Pb and Mo were above the safe limit for WHO standards. The differential response by the selected potato cultivars regarding the translocation and sequestration of heavy metals could have been due to their genetic and metabolite synthesis variations and could have accounted for reduction of the above-mentioned heavy metals within the stipulated standard. Hence, further studies that could unveil the underlying shift in metabolite that could be responsible for the crop not sequestrating many of the heavy metals are recommended. Also, such studies could explain the mechanisms behind the heavy metal hyperaccumulation ability of potato cultivars.

Compatible to this study, Mafayai *et al*. (2019) found unsafe concentration of As, Cd, Cr, Pb and Mn in carrot (*Daucus carota* subsp. *Sativus*), spinach (*Spinacia oleracea*

L.), tomato (*Lycopersicon esculatum* L.), cabbage (*Brassica oleraceae*), red pepper (*Capsicum annum*) and Garden Egg (*Solanum melongena*) vegetables irrigated with Tin mining pond water as prescribe by WHO (2007). In addition, higher levels of heavy metals such as As, Cd, Pb and Zn were recorded in the soils and vegetables irrigated with water from areas adjacent to a mine in Portugal (Avila *et al*., 2017). While Bui *et al*. (2016) reported contamination of irrigation in areas adjacent to mining areas with high concentration of As and Pb from fresh vegetable samples that exceeded maximum levels set by international food standards. Dong *et al*. (2011) measured Cd, Pb, Cu and Zn in 11 edible vegetables including *Solanum tuberosum* (potato) in Xiguadi village around Lechang Pb/Zn mine in Guangdong province, South China and the results showed that local mining activity caused heavy metal contamination with Cd concentration exceeding the required standards for all vegetables. In a different study, paddy fields nearby a Pb/Zn mine at a Karst area in Guangxi Province, South China, were severely polluted by Cd, Zn, Pb and Cu (Li *et al*., 2009). Similarly, irrigation with mining wastewater caused contamination of a paddy field and rice grain with Cd in Lechang, Guangdong Province, South China (Yang *et al*. 2006). A study by Zhang *et al.* (2009) investigated the extent to which heavy metals (Cu, Zn, Pb, and Cd) contaminate soils, vegetables, and rice grown in the vicinity of the Dabaoshan mine, South China and the results showed that concentrations of Pb and Cd in rice grain had exceeded the maximum permissible limits of China. In Potosı (Bolivia), Cd concentrations in potato tubers irrigated with mining impacted streams were observed to be higher than the ones irrigated with spring water (Oporto *et al*., 2007). Similarly, heavy metal content of potato tubers grown under acid mine water discharged from mining companies in Potosı accumulated As, Cd, Pb and Zn above the recommended limits (Garido *et al*. 2017).

Table 3.10 Mean ± standard deviation of the potato tubers (Marykies and Royal) heavy metal concentration irrigated with treated AMD in comparison with the permissible limits of World Health Organization standards **(WHO, 2007).**

Heavy metal concentration (mg/kg)											
Metals	**Marykies tuber**					**Royal tuber**					**WHO**
	TT1	**TT2**	**TT3**	**TT4**	**TT5**	**TT1**	**TT2**	**TT3**	**TT4**	**TT5**	
Al	9.62±0.23e	32.27±0.15a	25.12±0.32b	10.59±0.14d	12.68±0.27c	10.75±0.21e	35.57±0.06a	26.85±0.29b	11.85±0.12d	13.72±0.04c	**-**
As	0.04±0.00e	33.39±0.31a	12.79±0.06b	5.04±0.06d	6.54±0.24c	0.07±0.03e	33.81±0.29a	13.04±0.01b	5.59±0.00d	6.89±0.27c	**0.1-0.2**
Cd	0.21±0.00e	6.80±0.04a	3.08±0.02b	1.96±0.01d	2.05±0.01c	0.36±0.00e	7.20±0.08a	3.64±0.04b	2.13±0.01d	2.14±0.01c	**0.02-0.2**
Co	0.02±0.00e	5.48±0.15a	0.17±0.00b	0.07±0.00d	0.10±0.00c	0.03±0.00e	5.93±0.01a	0.23±0.00b	0.08±0.00d	0.10±0.00c	**0.05-0.1**
Cr	0.70±0.02e	4.96±0.01a	3.47±0.03b	2.83±0.07c	2.57±0.01d	0.80±0.01e	5.03±0.01a	3.59±0.04b	3.01±0.01c	2.95±0.01d	**1.3**
Cu	2.90±0.05e	47.83±0.08a	11.56±0.15b	5.02±0.06c	3.12±0.02d	3.40±0.08e	50.25±0.46a	12.19±0.09b	5.54±0.05c	3.95±0.01d	**10-60**
Fe	2.81±0.01e	46.55±0.12a	14.76±0.23b	4.54±0.01c	3.16±0.00d	3.02±0.05e	49.18±0.07a	16.52±0.09b	4.97±0.01c	3.72±0.05d	**425**
Mg	36.03±0.30b	62.50±0.53a	17.70±0.09e	28.03±0.18c	26.12±0.11d	37.38±0.29b	65.05±0.19a	19.76±0.17e	30.72±0.13c	29.08±0.12d	**-**
Mn	7.80±0.12d	46.11±0.09a	14.80±0.17b	8.04±0.04c	6.26±0.06e	8.28±0.04d	49.40±0.27a	15.09±0.06b	8.41±0.05c	6.89±0.01e	**500**
Mo	0.35±0.03e	13.65±0.00a	2.37±0.09b	1.61±0.03d	1.91±0.05c	0.44±0.02e	14.07±0.02a	2.84±0.03b	1.99±0.05c	1.60±0.02d	**100**
Ni	0.28±0.00e	13.03±0.01a	2.31±0.01b	1.65±0.00c	0.75±0.00d	0.30±0.00e	13.51±0.09a	2.91±0.01b	1.93±0.01c	0.95±0.14d	**10**
Pb	0.07±0.00e	45.92±0.06a	17.82±0.01b	4.95±0.03c	3.40±0.03d	0.09±0.00e	46.07±0.01a	17.96±0.01b	5.08±0.01c	3.50±0.01d	**0.3-2.0**
Sr	3.87±0.06d	0.81±0.01e	7.49±0.01a	4.17±0.02b	4.01±0.01c	3.97±0.01d	0.87±0.01e	7.78±0.03a	4.30±0.05b	4.11±0.01c	**0.30**
Zn	7.05±0.04e	164.82±0.77a	20.63±0.01b	9.37±0.10c	8.25±0.05d	7.53±0.02e	170.85±0.12a	21.08±0.01b	10.30±0.02c	8.89±0.07d	**23**

*In the sample names, T indicates tuber and T indicates treatment level. TT1: Tap water, TT2: 100% Acid mine water, TT3: 100% AMD + 1g quicklime, TT4:100% AMD + 2g quicklime and TT5: 100% AMD + 2g quicklime + 75% Fly Ash mixture.

3.4.4 Determination of heavy metals for soil

The study also evaluated the concentration of selected heavy metals in the soils irrigated with quicklime and fly ash treated AMD. An analysis was done to delineate the impact of heavy metal contamination of the soil on the crops. Studies have shown that when plants are raised in soils that are contaminated with heavy metals, they absorb and accumulate these in their edible parts of plants and these could be beyond the permissible limits, which could be harmful to human it consumed (Ahmad *et al*., 2015). The present results showed significant differences ($p < 0.05$) across the treatments for both cultivars (Table **3.11**). The soil irrigated with treated AMD (ST3, ST4, and ST5) showed variation in the concentrations of heavy metals and were within the permissible limit of the WHO except for As, Cd and Cr. As to be expected, the soil that was irrigated with untreated AMD water (ST2) showed a higher concentration of heavy metals in most of the measured metals that were not within the stipulated standard. This is due to the transfer of heavy metals from the untreated AMD and the inability of the crops to sequester such high concentration (Rai *et al*., 2019; Rambadu *et al*., 2020).In agreement with these findings, some studies have also reported an increase in the concentration of heavy metals of soil polluted with heavy metal-laden waste (Sadiq Butt *et al*., 2005; Roy & McDonald, 2015). A study by Qu *et al*. (2017) investigated the degree of contamination of heavy metals in paddy soil irrigated with acid mine drainage showed that Cu, Zn, and Cd in topsoil exceeded the maximum permissible concentrations for Chinese agricultural soil. Hu *et al*. (2014) observed similar findings on paddy fields that had been extensively polluted by Cu, Zn, and Cd due to long-term irrigation with nearby stream water contaminated by mine wastes. Rimawi *et al*. (2009) reported higher concentrations of heavy metals and soil salinity during the experimental period for plots irrigated with mine wastewater, when compared to plots irrigated with fresh water. Shu *et al*. (2001) also reported Cd contamination in study soils and had been caused using untreated mining wastewater from mine tailings. Paddy soils and rice from Kočani Field (Eastern Macedonia) were contaminated by heavy metals contributed by irrigation with riverine water (Rogan *et al*., 2009). Overall, a significant number of studies on heavy metals in plants have been conducted in Chinese paddy fields, which is likely due to a large amount of mining in that region of the world, which has resulted in AMD accumulation (Chen *et al*., 2007b; Zhao *et al*., 2007; Sun *et al*., 2012; Zhuang *et al*., 2013).

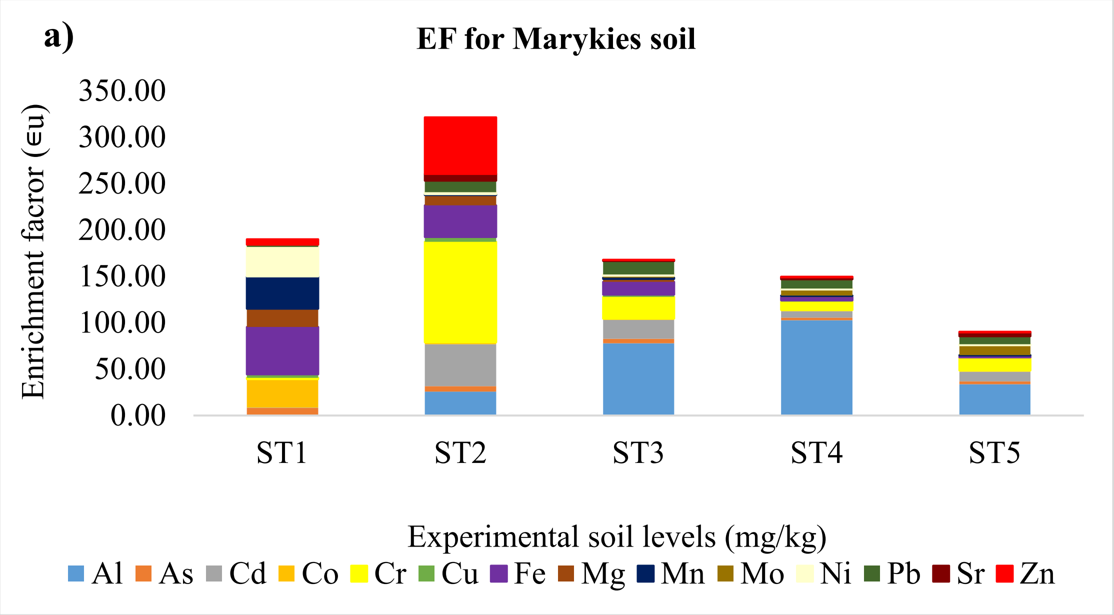
Table 3.11 Mean ± standard deviation of the soil heavy metal concentration irrigated with treated AMD and tapwater (control) in comparison with the acceptable level of World Health Organization (WHO) standards and Department of Environmental Affairs (DEA: Adapted from Nyoni *et al*., 2015) standards.

Heavy metal concentration (mg/kg)												
Metals	**Soil for Marykies cultivar**					**Soil for Royal cultivar**					**mg/kg**	
	ST1	**ST2**	**ST3**	**ST4**	**ST5**	**ST1**	**ST2**	**ST3**	**ST4**	**ST5**	**WHO**	**DEA**
Al	0.31±0.03e	8.05±0.33	24.16±0.16	31.81±0.10	10.43±0.09	0.41±0.20e	8.67±0.87d	23.46±0.41b	33.07±0.40a	9.65±0.33c	**-**	**-**
As	7.88±0.21	45.46±0.84	38.74±0.92	22.85±0.49	24.93±1.01	9.97±0.01e	46.94±0.28a	39.57±0.26b	23.51±0.24c	25.97±1.15d	**20**	**5.5**
Cd	0.51±0.00e	22.99±0.43	10.26±0.40	3.52±0.04	5.35±0.01	0.60±0.00e	23.92±0.38a	10.86±0.64b	3.66±0.01d	5.38±0.02c	**3**	**-**
Co	30.51±0.58	60.57±1.59	47.92±0.94	40.20±0.05	36.92±0.20	31.41±0.14e	62.20±0.57a	50.05±0.22b	42.72±0.06c	38.36±1.07d	**50**	**300**
Cr	1.30±0.01e	141.22±.2.31	30.30±0.04	11.94±0.09	17.88±0.04	1.43±0.03e	143.61±1.05a	30.39±0.03b	12.14±0.03d	18.15±0.01c	**-**	**6,5**
Cu	3.83±0.01d	19.74±0.77	9.30±0.03	4.28±0.11	2.13±0.01e	3.99±0.05d	20.50±0.57a	9.57±0.18b	4.84±0.04c	2.19±0.05e	**100**	**16**
Fe	51.03±0.01e	1747.50±8.42a	705.45±1.06b	177.99±0.57c	79.02±0.32d	51.08±0.01e	1743.60±6.16a	701.13±1.87b	174.64±0.18c	78.30±0.31d	**5000**	**-**
Mg	19.76±0.17d	213.26±0.77a	66.81±0.71b	26.12±0.11c	10.79±0.02e	18.93±0.02d	211.18±0.85a	65.99±0.67b	27.90±0.03c	10.68±0.01e	**-**	**-**
Mn	34.80±0.09c	42.56±0.37a	36.32±0.16b	13.34±0.57d	7.88±0.09e	35.03±0.74c	44.46±0.11a	35.64±0.10b	13.92±0.05d	8.08±0.05e	**740**	**2000**
Mo	ND	ND	2.35±0.17c	6.85±0.11b	10.78±0.18a	ND	ND	2.99±0.05c	7.53±0.05b	10.26±0.24a	**5**	**-**
Ni	31.87±0.13d	63.05±1.43a	42.80±0.53b	32.62±0.11c	30.31±0.26e	31.83±0.25d	62.63±0.09a	45.13±0.41b	33.74±0.20c	31.01±0.12e	**50**	**91**
Pb	2.65±0.04e	32.55±0.04b	38.43±0.02a	26.06±0.27c	22.98±0.13d	2.69±0.04e	33.13±0.02b	40.01±0.18a	26.51±0.07c	23.06±0.15d	**100**	**20**
Sr	ND	7.49±0.09a	0.84±0.01d	1.94±0.01c	4.15±0.08b	ND	7.68±0.07a	0.81±0.01d	1.81±0.06c	4.46±0.01b	**-**	**-**
Zn	5.09±0.00c	345.89±4.34a	3.41±0.05e	5.61±0.07b	4.44±0.32d	5.12±0.00c	352.30±3.46a	3.46±0.0e	5.81±0.05b	4.79±0.13d	**300**	**240**

*In the sample names, S indicates soil and T indicates treatment level. ST1: Tap water, ST2: 100% Acid mine water, ST3: 100% AMD + 1g quicklime, ST4:100% AMD + 2g quicklime and ST5: 100% AMD + 2g quicklime + 75% Fly Ash mixture.

3.4.5 Enrichment Factor Analysis

The EF values for the metals examined in this study are shown in Figure **3.6** below. There were variations that were observed in the EF (Єu) for different heavy metals across the treatments in the soil for Marykies and Royal cultivars (Figure **3.6** a & b). Generally, the quicklime and fly ash treatments (ST3-ST5) were less enriched with heavy metals indicating the impact of the treatments in reducing the uptake of the heavy metals present in AMD. The soil irrigated with 100% AMD and the tubers grown in the soil were highly enriched with most of the heavy metals. This was expected because the 100% AMD (T2) was not treated and therefore had high levels of heavy metals (Roy & McDonald, 2015). As shown in this study, several studies have observed soil that is polluted with heavy metal-laden pollutants (Gupta *et al*., 2010; Kesser, 2013). According to Singh *et al*. (2009), EF is influenced by metal bioavailability, which is influenced by metal content in the soil, chemical form, plant uptake capability, and plant growth rate.This may be the reason that similar trends of reduced EF were observed in the soil under the treated AMD. This implies that the quantity of heavy metals absorbed by the plants may be dependent on the available heavy metals in the soil and the variations in the heavy metals in the tubers could be attributed to the potential of the different cultivars to absorb and sequester the heavy metals.



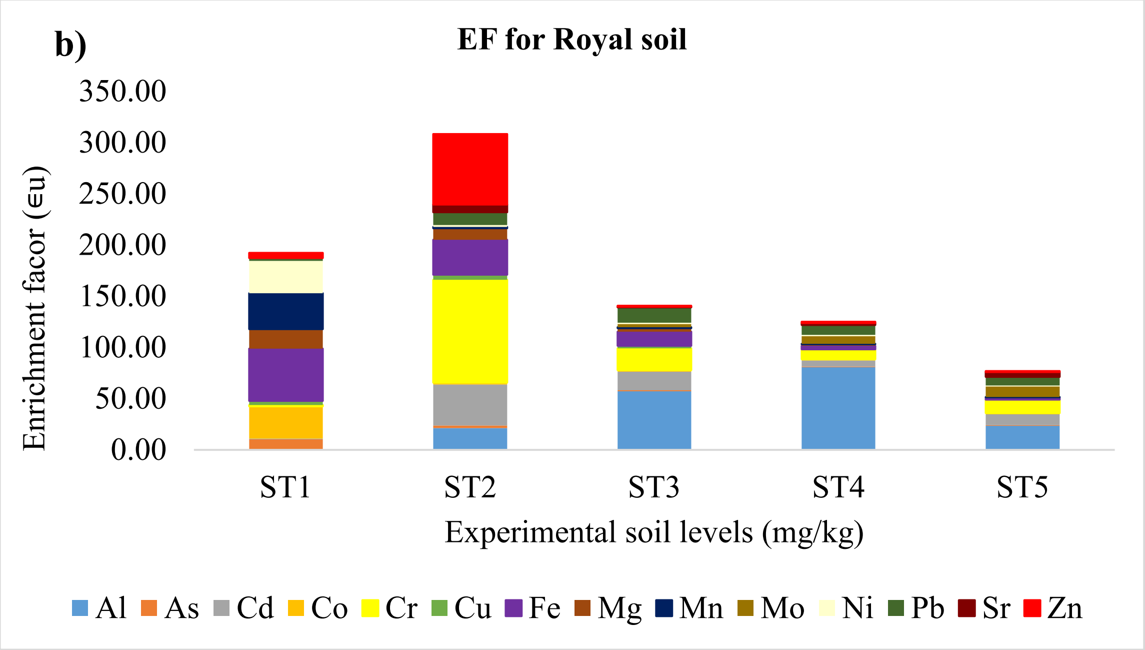


Figure 3.6 Enrichment factor analysis for soil irrigated with treated AMD (A: Marykies and B: Royal cultivar soils).

3.5 CONCLUSION

The influence of quicklime treated AMD irrigation water on the growth, development, and physiology of the potato cultivars as well as the availability and concentration of selected heavy metals on the plants and soil was assessed. The results showed that the quicklime treatments were able to reduce the concentration and detrimental impact of AMD on the physiological and growth parameters of the potatoes. Since water is a scarce resource in South Africa, these findings make it possible to consider the possibility of using treated AMD in agriculture, without negative consequences to plants and by extension, to human life. Since AMD is available abundantly and quicklime is also cheap to obtain, this study presents a great opportunity to ameliorate AMD water for food security. However, to avoid the eventual risks, the use of AMD must be regularly monitored, and the reuse standards should be developed and strictly observed.

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CHAPTER 4: METABOLOMIC ANALYSIS OF POTATO CULTIVARS IN RESPONSE TO QUICKLIME TREATED ACID MINE DRAINAGE IRRIGATION

4.1 INTRODUCTION

When plants are exposed to harzadous levels of heavy metals contained in acid mine drainage water that is used for their irrigation, they reveal a wide range of physiological and metabolic changes (Shulaev *et al*., 2008; Villiers *et al*., 2011). An uptake and accumulation of a high concentration of heavy metals reduces the growth of plants, biomass output, protein content, and chlorophyll pigment synthesis, and potentially resulting in significant crop yield reductions (Nagajyoti *et al*., 2010; Singh *et al*., 2013). For instance, when taken up in high concentration, Cd, Pb and Cr have been reported to affect several metabolic processes in plants (Zemanová *et al*., 2017). Even though the re-use of mine water could be a possible source of agricultural water in that it save water resources and reduce environmental issues associated with effluent discharge into bodies of water (Libutti *et al*., 2018); it has potential detrimental effects on crop production. Therefore, understanding plant responses to such stresses, particularly that caused by exposure to heavy metals is very crucial as it can guide the development of other novel ways for improving crops quantitatively and qualitatively. According to Singh *et al*. (2016), plants respond to the toxicity of heavy metals in a variety of ways: recognizing external stress stimuli, signal transduction and transmission into the cell, and initiating appropriate responses to counteract the detrimental effects of stress stimuli by modifying the cell's physiological, biochemical, and molecular status. Several researches focusing on the effects of using mine water for irrigation have been published, with varied degrees of success. (Annandale *et al*., 2001, 2006; Van Zyl *et al*., 2001; Nemutanzhela *et al*., 2017; Nevhulaudzi *et al*., 2020). However, it is not clear as to whether quicklime-treated AMD would not be lethal to crops despite meeting the prescribed standards and treatments in some cases. Hence, there is a need to treat the AMD before discharge into the waterbodies and/or used as irrigation water. Irrigation with AMD water has a direct impact on anatomical and physiological performances of plants (Shabalala & Ekolu, 2019; Rambadu *et al*., 2020).

In recent years, the metabolomics phenomenon has been widely used to categorize how different cultivation methods, environments and irrigation types affect plants, as well as to assess the quality of agricultural products (Sung *et al*., 2015; Garcia *et al*., 2016). Metabolomics is the study of all low-molecular-weight metabolites required by organisms during developmental stages (Arbona *et al*., 2013), and some of these metabolites have been linked to heavy metal stress tolerance levels. To date, several studies have been performed on plant metabolomics of crops such as Zucchini (Abreau *et al*., 2018), soyabean (Jiao *et al*., 2018), lettuce (Tamura *et al*., 2018), rice (Liu *et al*., 2020), and peanut (Srutiben *et al*., 2020). Plants can produce over 100,000 primary and secondary metabolites, but only around 10% have been identified so far (Aliferis & Jabaji, 2012; Lisec *et al*., 2006; Kusano *et al*., 2014). Metabolites have a variety of functions, including growth and development, respiration and photosynthesis, hormone, and protein synthesis, and are associated with increasing crop survival in stressful situations (Kusano *et al*., 2014; Das *et al*., 2017). Primary metabolites in plants, such as amino acids, enzymes, and carbohydrates, ensure that plant growth processes function optimally and supports the growth and development of plants (Daz *et al*., 2004; Koch, 2004). Plants can respond to various stresses by altering their gene expression, protein abundance, and metabolite accumulation at the molecular (Feng *et al*., 2020).

Because metabolites have such a wide range of chemical properties, new functional genomics technologies have evolved in recent years, including high-throughput transcriptomic, proteomic, metabolomic, and ionomic studies and used to reveal plant biochemical responses (Putri *et al*., 2013; Komatsu *et al*., 2013; Singh *et al*., 2016). Feng *et al*. (2020) recently confirmed that analysis of metabolites using plant metabolomic technologies can certainly provide information on how crop species respond to abiotic stress. A mass spectrometry (MS) based metabolomic methodology has been integrated with a variety of analytical separation techniques, including gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE), since it produces very sensitive results and allows for high-throughput data gathering (Gowda & Djukovic, 2014). For this present study, Liquid Chromatograph (LC) - Mass spectrometry (MS) was used because of its unique properties that allow for direct probing of metabolites in any sample without the necessity for derivatization (Wang *et* *al*., 2017). Furthermore, both targeted and non-targeted approaches for LC-MS-based metabolic profiling are used on LC-MS technique.

The crop of interest in this study, potato (*S. tuberosum*) is a highly sensitive crop that requires adequate supply of water needed to achieve high-quality yield (Levy & Tai, 2013; Joshi *et al*., 2016). However, there is dearth of published work on the effects of using quicklime-treated AMD for irrigation on the metabolomics profile of Marykies and Royal cultivars. Since the pioneering study of Roessner *et al*. (2000) on simultaneous analysis of metabolites in potato tubers by gas chromatography-mass spectrometry, there have been several publications on the potato tuber metabolome (Chaparro *et al*., 2018). The current study thus aimed to compare metabolic changes using LC-MS analytical technique on Marykies and Royal potato cultivars when subjected to quicklime treated AMD irrigation. Results of this study could provide a baseline information on the primary metabolites shift in potato that enhance their survival and growth under AMD condition.

4.2 MATERIALS AND METHODS

4.2.1 Metabolomic analysis for Potato tubers

Potato plants (Marykies and Royal cultivars) were planted at the UNISA in a greenhouse using randomized blocks. The factorial experiment comprised of six pots (2 × 5) planted in a 20 x 20 cm pot as explained in detail in Chapter 3, Section 3.2.3. The potato plants were irrigated with five types of water treatments. A total of five experimental treatments with different solution ratios (amount (g) of QL: percentage of fly ash (FA): percentage of AMD) as shown below: Treatment 1(T1) = 0:0, Tapwater; Treatment 2 (T2) = 0:100, AMD water; Treatment 3 = 1:100, 1g Quicklime and AMD water; Treatment 4 (T4) = 2:100, 2g Quicklime and AMD water and Treatment 5 (T5) = 2:75:100, 2 g Quicklime, 75 FA and AMD water. From emergence to crop senescence, irrigation with the various AMD treatments was applied every two days. Potatoes for both cultivars were harvested, cleaned with distilled water, dried, then frozen in liquid nitrogen immediately. They were later dried using a freeze dryer and then ground to a powder using a benchtop grinder and stored in glass vials below – 50° C for further analysis. For metabolites, the treatment was denoted as Treatment 1(T1): TM1; Treatment 2 (T2): TM2; Treatment 3 = TM3; Treatment 4 (T4) = TM4 and Treatment 5 (T5) = TM5 respectively.

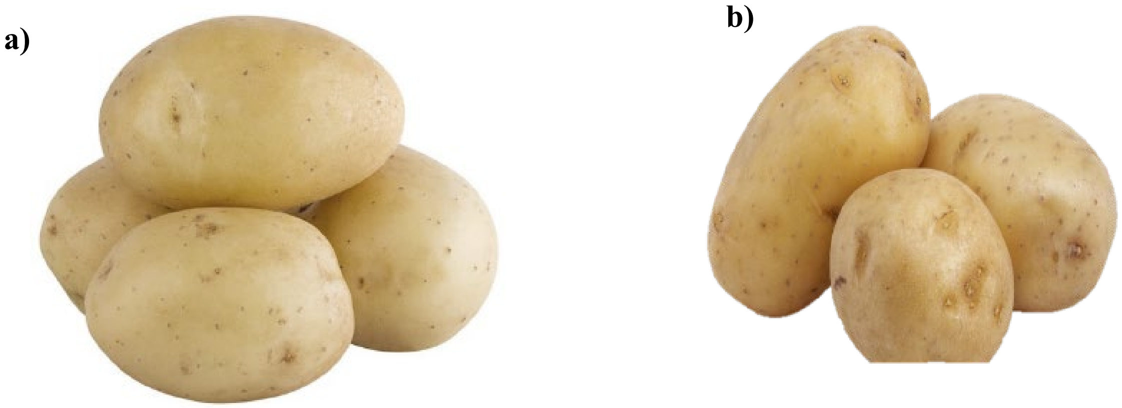

Figure 4.1 Potato tubers for Marykies (A: oval) and Royal (B: round) grown in the greenhouse used for metabolomic analyses.

4.2.2 Metabolite Extraction for LC-MS

Kim & Verpoorte (2010) procedure was used to extract metabolites. In a 2 mL Eppendorf tube, a total of 0.5 g of freeze-dried ground potato tuber was weighed, thereafter, 1.5 mL of MeOH (75 % MEOH/25 % water) was added and mixed with a vortex mixture. Using the BRANDSON 1800, the mixture was sonicated for five minutes (Germany). The sonicated supernatant concentrate was then filtered through 0.2-micron syringe filters (Sartorius Minisart RC 4) with 1 mL pipette. The supernatant filtered concentrate was then centrifuged in an Eppendorf tube (Centrifuge 5424, South Africa) at 10 000 revolutions per minute (rpm). Seven hundred microliters of the supernatant were then pipetted into HPLC vials for LC-QQQ-MS analysis, triple quad MS.

4.2.3 Metabolite Detection

The separation analysis was carried out using a Dionex Ultimate 3000 UHPLC system (Dionex Softron GmbH, Dornierstr. 4, Germany) equipped with an electrospray ion source (ESI). The separation and detection of metabolites was achieved using a reversed-phase C18 analytical column of 100 mm × 2.1 mm and 1.7 µm particle size (Acquity UPLC® BEH, Waters, Ireland), maintained at 35 °C. The injection volume was 2µL. The mobile phase consisted of 0.1% formic acid in water (solvent A) and LC-MS grade methanol (sol-vent B), at a flow rate of 0.3 mL/min. The gradient elution applied was: 85% A: 15% B to 65% A: 35% B in 4 min, changed to 50% A: 50% B for 2 min, then to 20% A: 80% B for 1 min, and back to the initial ratio (85% A: 15% B to 65% A: 35% B) for 0.5 min. Furthermore, the UHPLC system was interfaced with a Xevo G2 QT of water, then applied using the source-ESI positive and negative modes; capillary voltage-3 kV; cone voltage 30 V; cali-bration-sodium formate; lock spray-leucine enkephalin. The data acquisition was per-formed using LabSolutions software LC-MS Ver.5.82 (Shimadzu, Kyoto, Japan) and Lab-Solutions database, at an acquisition rate and range of 30 spectra/s and *m*/*z* 60–1500, re-spectively. To compare the treatments, the quantities of metabolites were plotted in STA-TISTICA (StaSoft Inc., Tulsa, OK, USA, 2011) package. The extracts were analyzed by re-verse phase LC-MS for their metabolomic contents. The MS analysis was carried out in the electron spray (ESI) positive and negative modes used for checking mass accuracy. A method followed by [71] was adopted, whereby peak intensity-showing LCMS-8040 triple quadrupole mass spectrometer intensities represented the quantities of the metabolites, varying with the treatments. The denotations T1 to T5 represent metabolites in the various treated tuber samples.

4.2.4 Data Processing and Statistical Analysis

MetaboAnalyst 5.0 software (https://www.metaboanalyst.ca/) was used to process the data and perform statistical analysis (Chong *et al.*, 2018; Pang *et al.*, 2021). Peak areas were considered for statistical analysis. Pre-processing includes data alignment (Koh *et al.*, 2010), normalization (Sysi-Aho *et al.*, 2007) or internal standard correction, missing value correction, scaling (pareto), and transformation (logarithm transformation) before employing various chemometrics algorithms (van den Berg *et al.*, 2006; Veselkov *et al.*, 2011). The top metabolites for tapwater versus treated AMD water were depicted on heat maps based on the Pearson distance measure and the Ward clustering technique to visualize relative levels. Multivariate tests such as partial least-squares discriminant analysis (PLS-DA) and Principal Component Analysis (PCA) were used to display significant metabolites among the studied groups. The PLS-DA method is a supervised method for analysing huge data sets. With a significance level of $p \leq 0.05$, the variable importance in projection (VIP) score ranks the overall impact of each variable. The links between metabolites were discovered using dendrogram analysis. PLS-DA, VIP scores, and heat maps were used to identify the key metabolites.

4.3 RESULTS

4.3.1 Metabolomes profile variations in two cultivars of potato under irrigation with treated AMD water using LC-MS/MS

A metabolomic evaluation approach involving the application of LC-MS/MS was exploited in delineating the metabolites profile associated with the two potato cultivars, Marykies and Royal as impacted by the quicklime treated AMD irrigation water. As alluded in Chapter 3, irrigation of crops with AMD water has been documented to be harmful to crops because of the possibility that crops could absorb the heavy metals. Several studies have also found that stress caused by heavy metals increases the formation of a variety of metabolites that are crucial in the signaling, sequestration, and transportation of heavy metals like Fe, Cu, Zn, and Cd (Johnson *et al*., 2011; Zhou *et al*., 2013; Yang *et al*., 2015). Despite the impact that quicklime and fly ash treatments could have on the concentration of heavy metals, the development of a variety of metabolites could also account for the reduction in the translocation of heavy metals bypotato cultivars. Results from this study showed that the concentration of heavy metals such as As, Cd, Cr, Pb and Sr in the potato tubers was at unsafe levels as compared to acceptable limit by WHO standards. Previous research has shown that different types of metabolites accumulate in different plant species in response to abiotic stress, depending on the species and severity of stress in different experiments (Du *et al*., 2012).

Analysis done in this study revealed 40 metabolites in Marykies and 36 from Royal (Tabel **4.1**). The two potato cultivars exhibited spatially distinct metabolome under AMD conditions as well as when treated with the quicklime. Metabolites ranging from amino acids, organic acid, and aromatic amines showed differential spatial exudation and accumulation at the tuber level of the two potato cultivars. Overall, the two cultivars shared similarity in the detected metabolites however, Adenosine monophosphate, Cytidine, Xanthine, Lactic acid, and Isocitric acid were only detected in the Marykies potato cultivar. These results were consonant with those of several studies that reported the accumulation of higher quantity of amino acids, organic acids, sugars, and sugar alcohol are vital protective responses of plants in response to abiotic stress (Kovács *et al*., 2012; Benzarti *et al*., 2014; Iqbal *et al*., 2015; Reddy *et al*., 2015; Ahanger *et al*., 2018).

Table 4.1 Metabolites identified from a methanol tissue extract of Marykies and Royal potato tubers

Irrigation Treatments Used	**Metabolites**		
	Amino acids	**Organic acids**	**Aromatic amines**
Marykies			
Treatment 1 (TM1)	Acetylcarnitine, Acetylcholine, Adenosine monophosphate, Adenine, Allantoin, Asparagine, Aspartic acid, Carnitine, Creatinine, Cytidine, Cytosine, Dimethylglycine, Epinephrine, Glutamic acid, Glycine, Guanosine 3',5'-cyclic monophosphate, Histamine, Hypoxanthine, Inosine, Methionine sulfone, Methionine sulfide, Niacinamide, Norepinephrine, Serine, Threonine, Uridine, Xanthine, 4-Aminobutyric acid, 4-Hydroxyproline	Cholic acid, Fumaric acid, Isocitric acid, Lactic acid, Malic acid, Nicotinic acid, Orotic acid, Pyruvic acid, and 2-Morpholinoethanesulfonic acid	Dopa
Treatment 2 (TM2)	Acetylcarnitine, Acetylcholine, Adenine, Alanine, Asparagine, Aspartic acid, Carnitine, Creatinine, Histamine, Histidine, Hypoxanthine, Niacinamide, Norepinephrine, Serine, 4-Aminobutyric acid, 4-Hydroxyproline	Fumaric acid, Nicotinic acid	
Treatment 3 (TM3)	Acetylcarnitine, Adenine, Alanine, Asparagine, Aspartic acid, Carnitine, Creatinine, Dimethylglycine, Dimethylglycine, Histamine, Niacinamide, Norepinephrine, Serine, Xanthine, 4-Aminobutyric acid, 4-Hydroxyproline	Fumaric acid, Nicotinic acid	
Treatment 4 (TM4)	Acetylcarnitine, Adenine, Asparagine, Aspartic acid, Carnitine, Creatinine, Dimethylglycine, Glycine, Histamine, Niacinamide, Norepinephrine, Serine, Threonine, 4-Aminobutyric acid, 4-Hydroxyproline	Fumaric acid, Isocitric acid, Nicotinic acid, Orotic acid, Pyruvic acid	Dopa
Treatment 5 (TM5)	Acetylcarnitine, Adenine, Asparagine, Aspartic acid, Carnitine, Creatinine, Dimethylglycine, Glycine, Histamine, Norepinephrine, Serine, Threonine, Xanthine, 4-Aminobutyric acid, 4-Hydroxyproline	Isocitric acid, Nicotinic acid, Orotic acid, Pyruvic acid	Dopa
Royal			
Treatment 1 (TM1)	Acetylcarnitine, Ace-tylcholine, Adenine, Allantoin, Asparagine, Aspartic acid, Carnitine, Creatinine, Cytosine, Dimethylglycine, Epinephrine, Glutamic acid, Glycine, Guanosine 3',5'-cyclic monophosphate, Histamine, Hypoxanthine, Inosine, Methionine sulfone, Me-thionine sulfide, Niacinamide, Norepinephrine, Serine, Threonine, Uridine, 4-Aminobutyric acid, 4-Hydroxyproline	Cholic acid, Fumaric acid, Malic acid, Nicotinic acid, Orotic acid, Pyruvic acid, Succinic acid and 2-Morpholinoethanesulfonic acid	Dopa

Treatment 2 (TM2)	Acetylcarnitine, Acetylcholine, Adenine, Asparagine, Aspartic acid, Carnitine, Creatinine, Dimethylglycine, Histamine, Histidine, Hypoxanthine, Niacinamide, Norepinephrine, Serine, 4-Aminobutyric acid, 4-Hydroxyproline	Fumaric acid, Nicotinic acid	
Treatment 3 (TM3)	Acetylcarnitine, Adenine, Alanine, Asparagine, Aspartic acid, Carnitine, Creatinine, Dimethylglycine, Histamine, Niacinamide, Norepinephrine, Serine, 4-Aminobutyric acid, 4-Hydroxyproline	Fumaric acid, Nicotinic acid	
Treatment 4 (TM4)	Acetylcarnitine, Adenine, Asparagine, Aspartic acid, Carnitine, Creatinine, Dimethylglycine, Glycine, Histamine, Niacinamide, Norepinephrine, Serine, Threonine, 4-Aminobutyric acid, 4-Hydroxyproline	Fumaric acid, Nicotinic acid, Pyruvic acid, Succinic acid	Dopa
Treatment 5 (TM5)	Acetylcarnitine, Adenine, Carnitine, Creatinine, Dimethylglycine, Glycine, Histamine, Niacinamide, Norepinephrine, Serine, Threonine, 4-Aminobutyric acid, 4-Hydroxyproline	Nicotinic acid, Pyruvic acid	Dopa

*TM (1-5) TM1 represent metabolites on treated tuber samples across (un)treated AMD water used for irrigation.

4.3.2 Effect of the quicklime treatment of AMD on the two cultivars metabolomes using LC-MS/MS

The unsupervised Principal Component Analysis (PCA) approach for pattern recognition analysis was applied to the LC-MS chromatograms of Marykies and Royal potato tubers to give a comparative interpretation and visualization of metabolic differences between them amongst five irrigation treatment used in this study. The PCA revealed that the control sample was clearly separated from the samples with AMD (both treated and untreated) along PC2 axis accounting for 2.8 % and 2.1 % for Marykies and Royal cultivars respectively (Figure **4.2** A & B). Furthermore, along the PC1 accounting for 97.1 % and 97.6 % for Marykies and Royal cultivars respectively, the 2g QL + 100% AMD (T4: Green) and 2g QL + 75 % FA (T5: Dark blue) were well separated indicating the impact of the treatments on the metabolite profiling in the two cultivars (Figure **4.2** A & B).

However, to obtain a higher level of treatments separation and a better understanding of variables responsible for classification, a supervised PLS-DA was applied. In contrast to the PCA, the PLS-DA is a supervised approach that can categorize observations into groups based on the greatest predicted indicator variable (Kelsey *et al.*, 2020; Bi *et al.*, 2021). Barker (2012) employed statistical theory to demonstrate that PLS-DA was capable of accurate classification. The results showed a significant discrimination of the T1 (tapwater: control) and other treatments (T2-5) (Figure **4.3** A & B) below. The treatments differentiated from each other on the first two-components of PLS-DA score plot by the principal component t(1) (51.9%), principal component t(2) (44.7%), and principal component t(3) (3%) for Marykies cultivars and principal component t(1) (55.3%), principal component t(2) (41.8%), and principal component t(3) (2%) for Royal cultivar (Figure **4.3** A & B). For Marykies cultivar, the PLS-DA revealed five distinct groups, tapwater (T1) grouped at the top towards the right side of the PLS-DA score plot while the 100% AMD (T2) and 1g QL+100% AMD (T3) aligned together at the middle towards the right and left respectively of the PLS-DA score plot. The 2g QL + 100% AMD (T4) and 2g QL + 75 % FA (T5) aligned at the bottom towards the right of PLS-DA score plot. A different alignment was observed for Royal cultivar with tapwater (T1) appearing at similar position with the Marykies cultivar in the PLS-DA score plot. The 2g QL + 100% AMD (T4) and 2g QL + 75 % FA

(T5) aligned at the middle towards the left and right respectively of PLS-DA score plot. The 100% AMD (T2) and 1g QL+100% AMD (T3) aligned together at the bottom towards the right and left respectively of the PLS-DA score plot. The Partial Least Squares-Discriminate Analysis (PLS-DA) score plots, a supervised discriminant analysis, clearly delineated the metabolites profile of the different treatments for both cultivars of potato. The discrimination of the metabolites accumulated by the tubers of the two cultivars of potato under 100% AMD (T2) condition indicated the spatial exudation of metabolites by the cultivars possibly at the rhizospheric level to promote the adaptability of the crops to the acidic conditions of the AMD as well as the heavy metal toxicity. The differences in the alignment of the different metabolites under the various treatments could be attributed to the variation in the physiological response of the cultivars under environmental stress (Demirel *et al*., 2020; Toubiana *et al*., 2020). Through metabolic alterations, plants can adjust their physiology to diverse situations (Khan *et al*., 2017). According to Khan *et al*. (2019), plants have a variety of metabolic adaption mechanisms that they use in order to protect themselves from the harmful impacts of stress, and these systems can play an important part in the adaptive mechanisms of plants.

In agreement to our findings, Tan *et al*. (2021) reported that the rhizosphere and tissue of *Brassica juncea* planted under Cd stress exuded diverse metabolites that included amino acids, linoleic acid, arginine, valine, leucine, and isoleucine. In general, when plants are subjected to conditions of metal toxicity, they coordinate metabolic activities involved in plant growth and development, inducing higher levels of amino acids, their derivatives, and organic acids necessary for their adaptation (Feng *et al*., 2021). The close grouping of the 2g QL + 100% AMD (T4) and 2g QL + 75 % FA (T5) treatments PLS-DA score plots, provide an indication of the effect of the two AMD treatments on the metabolite's exudation of the two cultivars of potato. This could be attributed to effect of the treatments in the reduction of the contaminants present in the AMD such as heavy metals as well as the stimulation effect of the quicklime on the potato. Shanmugaraj *et al*. (2013) reported the association of metabolites such as organic acids, amino acids, peptides, glutathione, and phytochelatins in the detoxification of heavy metal toxicity. Acid mine drainage water contains high concentration of heavy metals, therefore, during heavy metal stress, amino acids play important roles in metal binding, antioxidant defense, and signaling in plants (Jezek *et al*., 2011; Xu *et al*., 2012a, b). On the other hand, organic acids are other compounds that are reportedly involved in the defense against heavy metal stress (Haydon & Cobbert, 2007). This could account for the close grouping of the treated AMD water in the PLS-DA used as a source of irrigation of the potato as any trace of heavy metals could trigger the exudation of similar metabolites. Furthermore, one could be tempted to say that the presence of quicklime as well as the fly ash used in the treatment of the AMD might also induce the production of similar metabolites. However, further studies are recommended, and they could elucidate the direct impact of quicklime and fly ash on the metabolite profiles of the two potato cultivars.

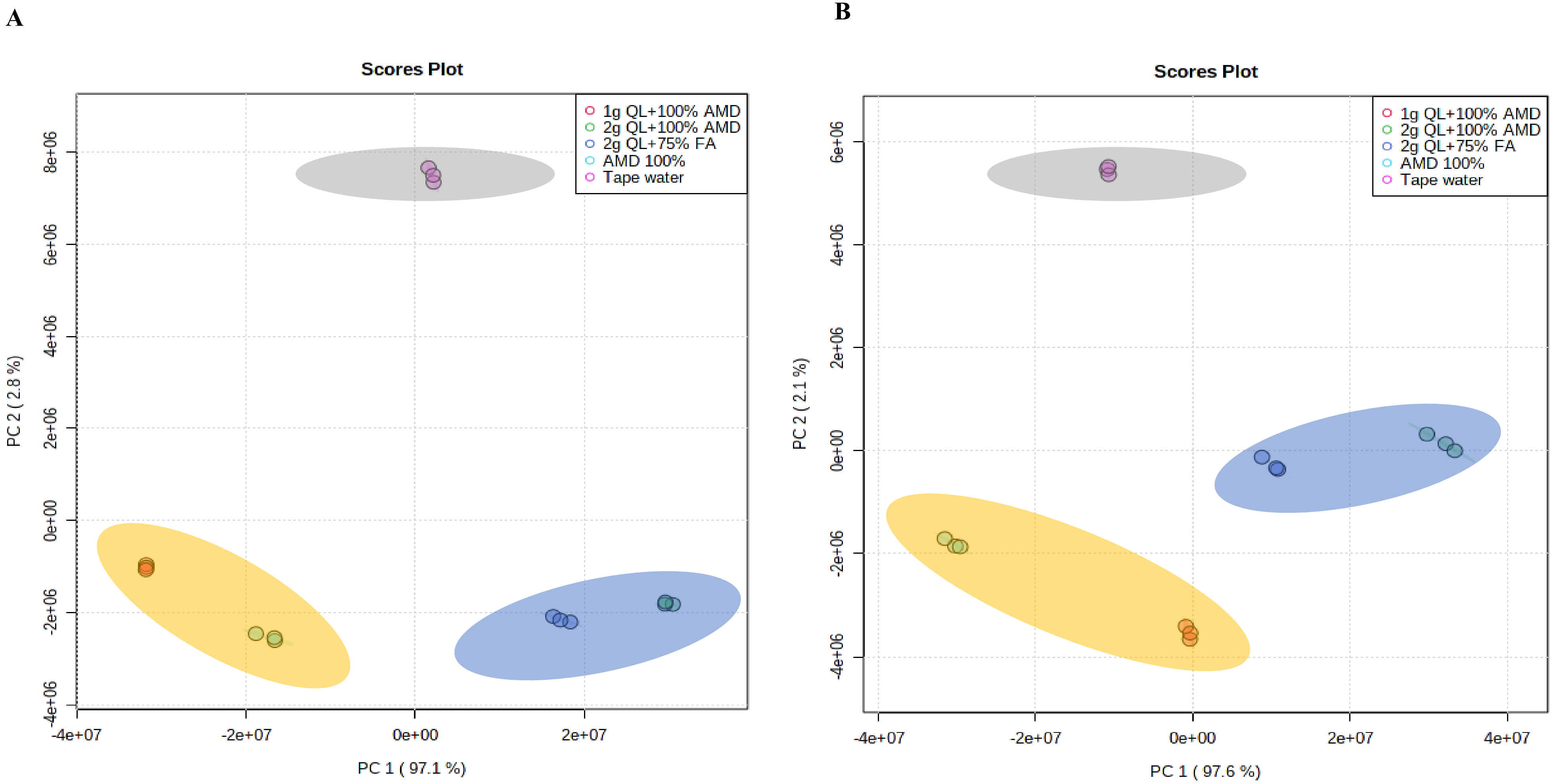

Figure 4.2 2D Principal component (PCA) analysis score plot from LC-MS data of samples from Marykies (A) and Royal (B) potato tuber samples. Different colours denoted different treatments: Tap water (T1): Purple, AMD 100% (T2): Light blue, 1g QL + 100% (T3): Red, 2g QL + 100% AMD (T4): Green and 2g QL + 75 % FA (T5): Dark blue respectively.

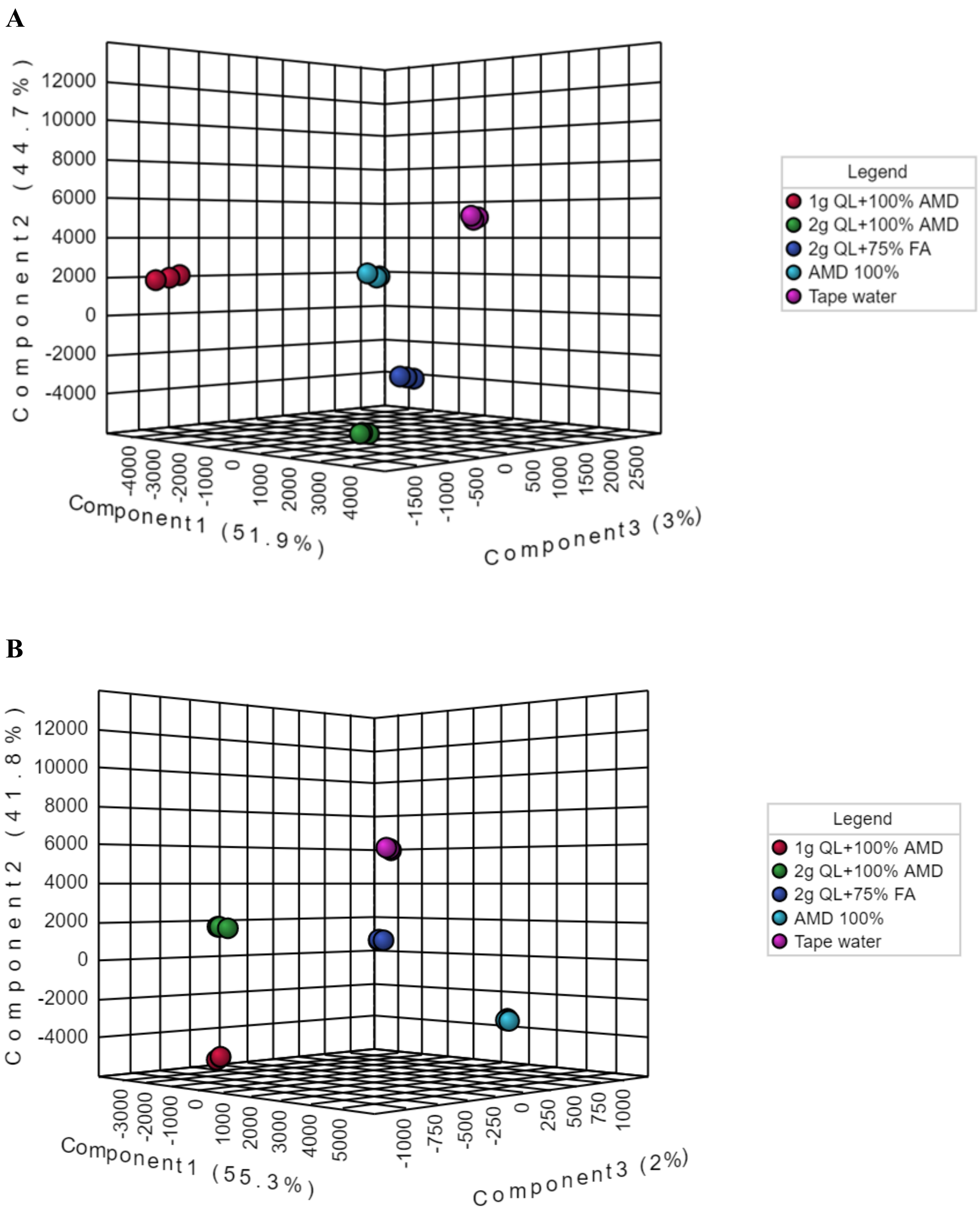

Figure 4.3 Partial Least Squares-Discriminate Analysis (PLS-DA) score plots of metabolic profiles from LC-MS data of samples from Marykies (A) and Royal (B) potato tuber samples. Different colours denoted different treatments: Tap water (T1): Purple, AMD 100% (T2): Light blue, 1g QL + 100% (T3): Red, 2g QL + 100% AMD (T4): Green and 2g QL + 75 % FA (T5): Dark blue respectively.

The most important discriminant metabolites (identified by PLS-DA) ranked by variable importance in projection (VIP) scores in component 1, delineated the metabolites abundance in the two cultivars as impacted by the AMD and the treatments (Figure **4.4**). For both cultivars, the 100% AMD (T2) and 1g QL +100% AMD (T3) resulted in the minimal production of the identified metabolites (glycine, dopa, pyruvic acid, dimethylglycine, aspartic acid, acetylcarnitin, norepinephrine, 4-hydroxyproline, threonine, Orotic acid, serine, adenine, creatinine, Cartinine, and 4-aminobutyric acid) in comparison to the tapwater (T1) (Figure **4.4**).

The VIP scores delineated metabolites in component 1 were higher in the 2g QL + 100% AMD (T4) and 2g QL + 75 % FA (T5) for both cultivars when compared to the control (T1). This could imply that two treatments did not only decontaminate the AMD but had the potency to initiate the production of vital metabolites necessary for the growth of the potato as was mentioned by Mahale *et al*. (2012) on the study of the growth and accumulation of heavy metals in wheat (*Triticum aestivum*), mung bean (*Vigna radiata*), and urad beans (*Vigna mungo*) experiments. In cases where the impact of the metabolites as a distinguishing trait among cultivars is greater, the VIP score becomes higher (Sato *et al*., 2021). Only metabolites having a VIP score of greater than one was considered. These results imply that different metabolites can be stored in different tissues and cells depending on the function of the metabolites and the environmental stress that promoted their secretion (Massalha *et al*., 2017). Amino acids that are present in plants contribute to the detoxification process by regulating ion transport, chelating ions, and nitrogen (N) metabolism under heavy metal stress (Feng *et al*., 2021). The elevated abundance of the amino acids and other metabolites in the selected potato tubers that were irrigated with quicklime and fly ash treated AMD could be attributed to their response to heavy metal stress exerted by the treated AMD water as well as the individual impacts of the quicklime and fly ash on the crops.

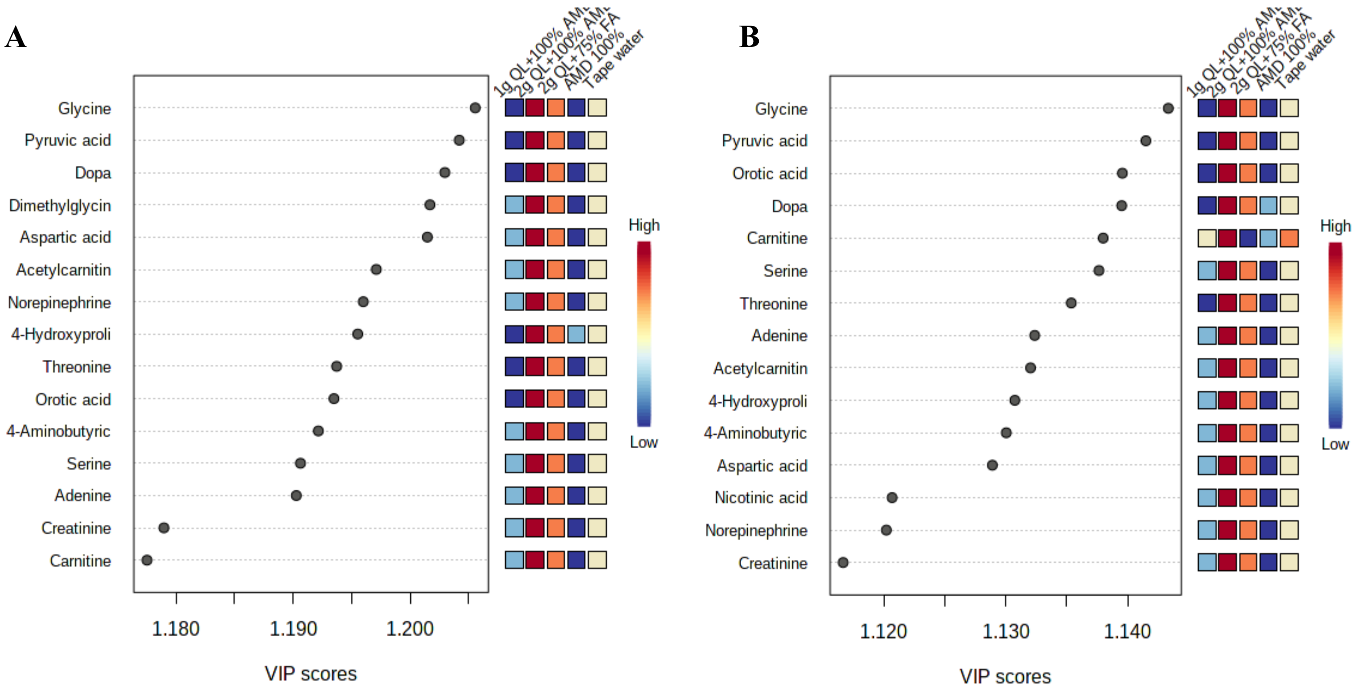

Figure 4.4 Discriminant metabolites identified by PLS-DA ranked by variable importance in projection (VIP) scores in component 1. The relative abundance of each metabolite from Marykies (A) and Royal (B) cultivars are indicated with a colour code scaled from blue (low) to red (high). Tap water (T1); AMD 100% (T2); 1g QL + 100% (T3); 2g QL + 100% AMD (T4) and 2g QL + 75 % FA (T5) respectively.

4.3.3 Hierarchical cluster analysis of metabolomes in two cultivars of potato under irrigation with treated AMD water

Based on the dendrogram analysis (Figure **4.5** A & B) shown below, treatments used for this study for both potato cultivars were scattered into two main clusters comprising of cluster 1 (T1, T4 and T5) and cluster 2 (T2 and T3) respectively. Thus, showing that most of the metabolites accumulated on each cluster as per treatments were similar. Heat map analysis of all the metabolites in the two potato cultivars subjected to quicklime treated AMD irrigation water is presented below (Figure **4.6** A & B). The results showed that primary metabolites were patterned into three different clusters. The analysis revealed variation on the two cultivars. Metabolites such as Adenosine monophosphate, Cytidine, Xanthine, Lactic acid, and Isocitric acid were only detected in Marykies. Some amino acids and organic acids may have accumulated in response to stress.

The separation of metabolites was further revealed on heatmap (Figure **4.6** A & B). The heatmap revealed different groups of primary metabolites of two potato cultivars irrigated with treated AMD indicating that potato metabolome patterns are dependent on treatments levels. The heatmap comprises of five clusters as per the used treatments for this study. It showed treatments 2g QL + 100% AMD (T4) and 2g QL + 75 % FA (T5) clustered together indicating the effect of the two treatments in the spatial distribution of synthesized metabolites for both cultivars. Further, AMD 100% (T2) and 1g QL + 100% (T3) showed clustering indicating similarities in the metabolites produced by the two cultivars as the 1g QL may not have strong impact in the treatment of the AMD leading the plants to respond similarly in the production of metabolites under the two treatments.

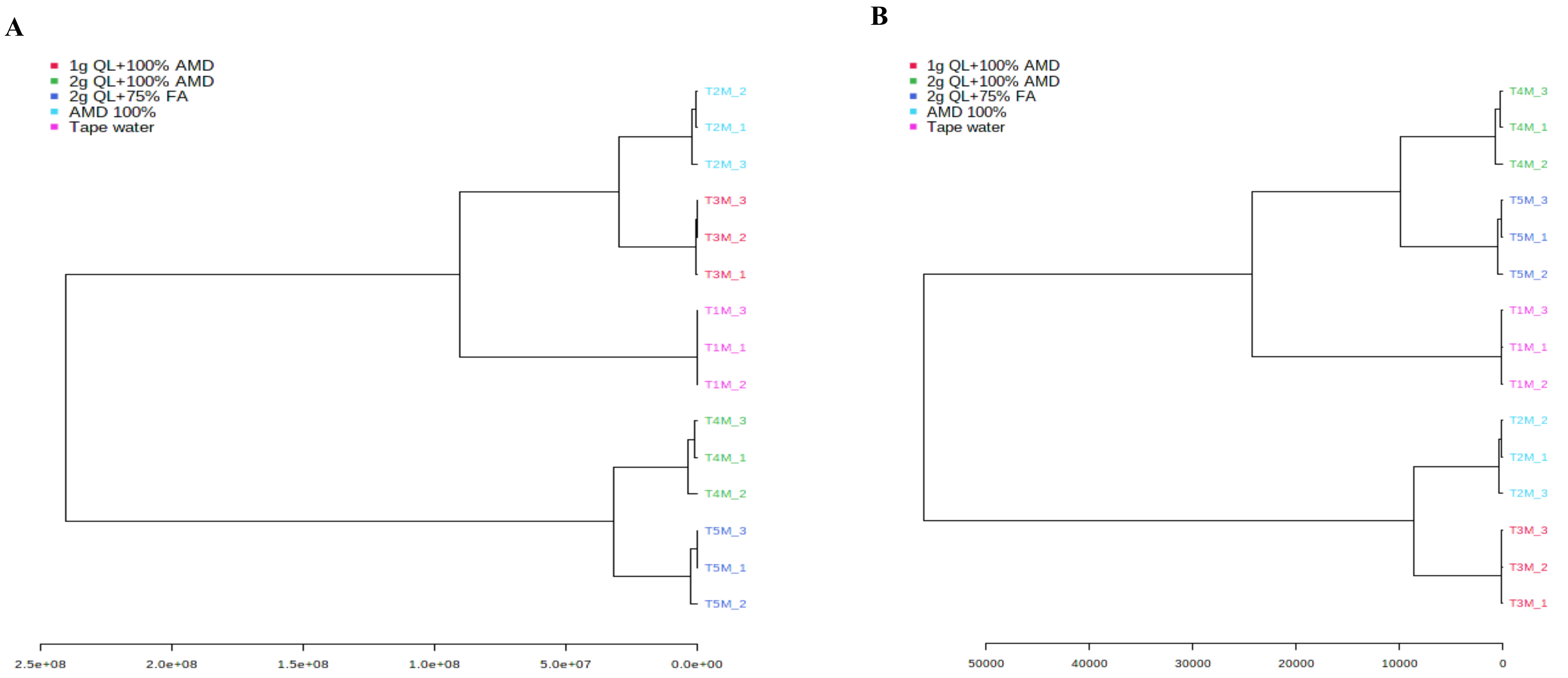

Figure 4.5 Clustering pattern shown as the dendrogram of potato cultivars A (Marykies) and B (Royal). Different colours denoted different treatments: Tap water (T1): Purple, AMD 100% (T2): Light blue, 1g QL + 100% (T3): Red, 2g QL + 100% AMD (T4): Green and 2g QL + 75 % FA (T5): Dark blue respectively. Row represents metabolites and column represents treatments.

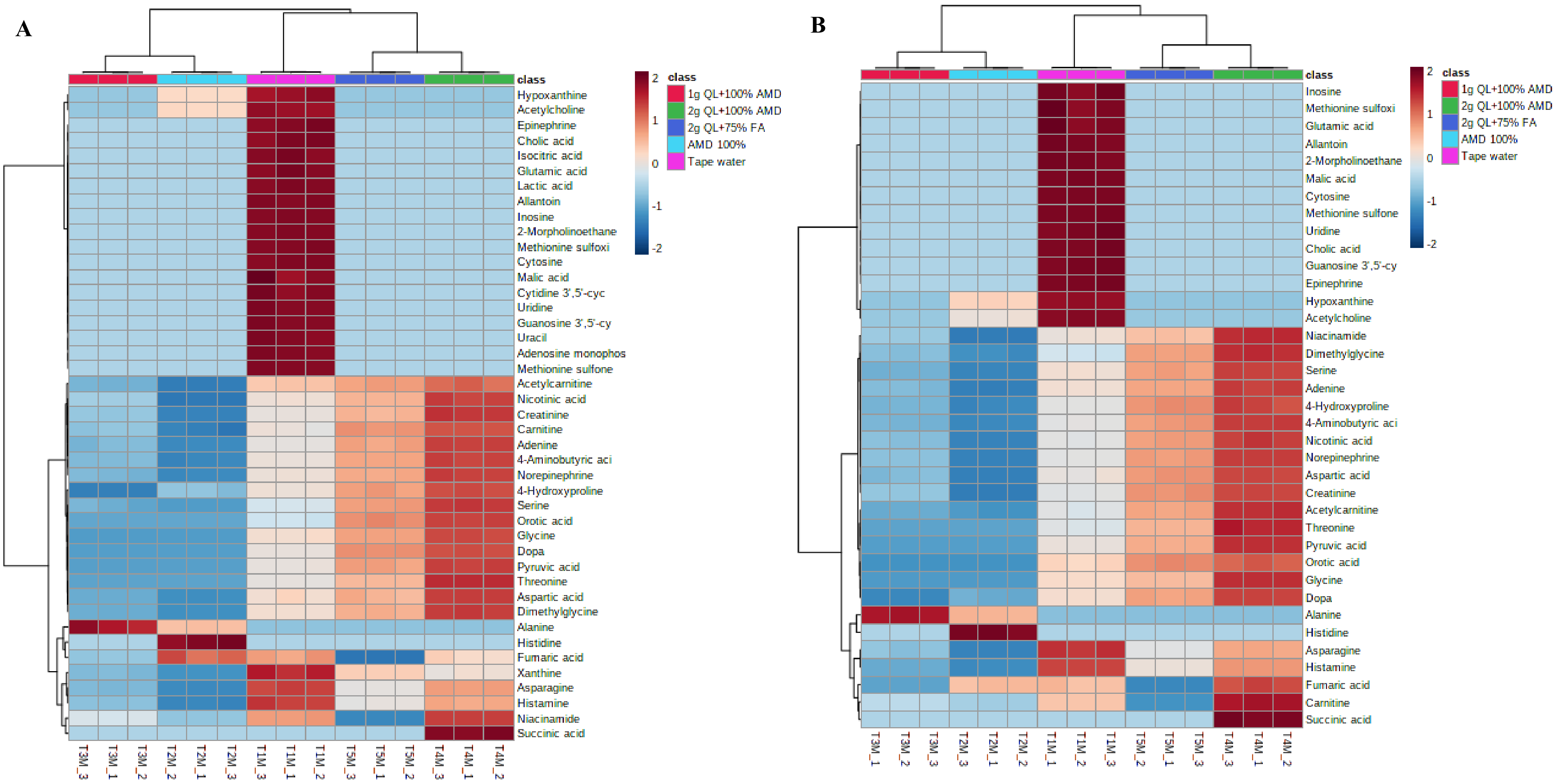

Figure 4.6 The heatmap for metabolites from liquid chromatography linked to mass spectrometry data of samples from Marykies (A) and Royal (B) potato tuber samples. Different colours denoted different treatments: Tap water (T1): Purple, AMD 100% (T2): Light blue, 1g QL + 100% (T3): Red, 2g QL + 100% AMD (T4): Green and 2g QL + 75 % FA (T5): Dark blue respectively. Row represents metabolites and column represents treatments.

4.4 CONCLUSION

This study elucidated the impact of AMD and quicklime/fly ash treated AMD on the spatial exudation and accumulation of metabolites in tubers of two potato cultivars. Overall, the results showed that the AMD and the treatments influenced the exudation and accumulation of metabolites in the tuber of the two cultivars with subtle difference in the exudation within the two cultivars. The elevation in the abundance of glycine, dopa, pyruvic acid, dimethylglycine, aspartic acid, acetylcarnitin, norepinephrine, 4-hydroxyproline, threonine, orotic acid, serine, adenine, creatinine, cartinine, and 4-aminobutyric acid in the tubers of crops irrigated with treated AMD water, imply their role in the maintenance of the health and growth of the two crops; as the metabolites are believed to be protective response of crops to environmental stress. Despite the role of quicklime and fly ash in the removal of heavy metal constituent of AMD water, their presence in the irrigated water have been observed to trigger the exudation of metabolites in the crops as the crops might have recognised the chemicals as stress condition. This is evident in the lower abundance of glycine, dopa, pyruvic acid, dimethylglycine, aspartic acid, acetylcarnitin, norepinephrine, 4-hydroxyproline, threonine, orotic acid, serine, adenine, creatinine, cartinine, and 4-aminobutyric acid in the 100% AMD (T2) when compared to the control (T1). However, further studies are recommended to evaluate the direct impact of quicklime and fly ash on the metabolite's exudation in potato cultivars (whole plant).

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CHAPTER 5: IDENTIFICATION OF BACTERIAL DIVERSITY ABUNDANCE AND VARIATION IN QUICKLIME TREATED ACID MINE DRAINAGE IRRIGATED SOILS

5.1 INTRODUCTION

Acid mine drainage (AMD) is one of major challenges that affect the availability of water used for domestic and agricultural purposes and has been observed in countless mining regions around the world. It is characterised by diverse microorganisms belonging to the following domains: *Bacteria*, *Archaea* and *Eukarya* (Akcil & Koldas, 2006; Egiebor & Oni, 2007; Baker *et al*., 2010; Chen *et al*., 2015); and to various phyla such as *Proteobacteria*, *Nitrospirae*, *Actinobacteria, Acidobacteria, Chloroflexi, Saccharibacteria,* and *Firmicutes* (Tyson *et al*., 2004; Logares *et al*., 2012; Méndez-García *et al*., 2015; Kadnikov *et al*., 2016; Liang *et al*., 2017; Lukhele *et al*., 2019) that aid in their acidity by lowering the pH of the acid mine water.

There are many bioremediation approaches that have been suggested depending on the ability of a plant to absorb and retain heavy metals. Two distinct methods are adopted in AMD treatment. The first is a conventional method which involves biological and chemical treatment of effluent in a centralised wastewater treatment plant. The second is passive and involves natural or constructed wetlands where effluent is treated through the aid of both aerobic and anaerobic microorganisms, algae strains, and other macroorganisms (Bwapwa *et al*., 2017). Active and passive treatment systems are widely used for AMD remediation. Both treatments remove heavy metals and sulfate as well as increasing the pH of AMD (Johnson & Hallberg, 2002). Passive remediation systems comprise of several retention ponds, wetlands, and alkaline materials that enhance heavy metal precipitation especially, in AMD. The addition of active chemicals such as hydrated lime, quicklime, or soda ash contribute to the remediation of polluted sites form part of active treatment systems. The work of Othman *et al*. (2017) showed that the application of quicklime to AMD increased the pH and lowered the amount of arsenic, cadmium, chromium in the mine water and increase microbial community. In addition to heavy metals and sulfate reduction, active treatment systems play important role in altering the microbial structure of the environment. The active treatment has been recorded to play crucial role in soil microbial composition and physicochemical parameters shift necessary for the growth of crops. Application of limestone increases soil biological activity, control soil microbial composition and function and promote the growth of crops (Xun *et al*., 2016; Narendrula-Kotha & Nkongolo, 2017). Qaswar *et al*. (2020) observed that the interaction between liming and long-term application of fertilizer enhanced the growth and yield of wheat and maize and increased the pH of the soil. A study carried out by Liang *et al*. (2021) also reported that application of quicklime positively affected the microbial community in strongly acidic soils of tobacco crops. In addition to the impact of liming on the growth of crop and increasing the pH of soil, it also has an influence on the microbial diversity and structure of the soil. Short-term liming in winter wheat and summer corn rotation systems was reported to enhance the proliferation and abundance of *Bacteroidetes* and *Alphaproteobacteria* and reduced *Actinobacteria* and *Betaproteobacteria*. The work of Ly *et al*. (2019) revealed shifts in microbial community structure from acidophilic bacteria in raw AMD discharge to a more metabolically diverse set of taxa (i.e. *Acidimicrobiales*, *Rhizobiales* and *Chthoniobacteraceae*) in a passive treatment of acidic abandoned coal mine. Several research projects done provided an insight into the impact of passive and active treatment on the bacterial structure of AMD. However, little or no work has been done on the identification of the diversity and functional profile of bacterial community in the treated-AMD irrigated water and soil. The present study aimed to identify the soil bacterial diversity abundance and variation in treated AMD irrigated soil.

5.2 MATERIALS AND METHODS

5.2.1 Study area

As described in chapter 3, section 3.2.2.

5.2.2 Sample collection

As detailed in chapter 3, section 3.2.3, acid mine drainage water samples were collected from a Gold Mine in Mogale city. Water samples were collected using sterile 50 L plastic containers (that had been cleaned with 20 % sodium hypochlorite and UV-sterilized for one hour). Prior to analysis, water samples were accurately measured into 2 litres (L) containers as explained in detail in Chapter 3, Section 3.2.2. A total of five experimental treatments with different solution ratios (amount (g) of QL: percentage of fly ash (FA): percentage of AMD as shown below: Control: Tapwater (T1); AMD: 100 % AMD (T2): 1g QL + 100 % AMD (T3: A1Q); A2Q: 2g QL + 100 % AMD (T4); AFQ: 2g QL + 100 % AMD +75 % FA (T5). For this study, the treatments were denoted as T1: control; T2: AMD; T3: A1Q, T4: A2Q and T5: AFQ respectively. Before irrigating with AMD, as explained in detail in Chapter 3, Section 3.2.2, the water was treated with quicklime based on the Othman *et al*. (2017) protocol. All the treatments were in triplicates. For this study, the quicklime treated water was used for irrigation of two potato cultivars (Marykies and Royal). Soil samples that were randomly collected in triplicates from each treatment level for both Marykies and Royal potato cultivars pots from the greenhouse were stored in into sterile Ziplock bags and thereafter kept under 4°C in the laboratory for metagenomic analysis.

5.2.3 Soil Geochemical Properties

For this study quicklime-treated AMD irrigated soils physiochemical properties and heavy metals were recorded and analysed as explained in detail in Chapter 3, Section 3.2.4 and reported on section 3.4.1 and 3.4.2. The study also focused on environmental variables identified as influencing soil bacterial communities. As mentioned above, pH was recorded using pH meter (A329, Thermo Scientific, Indonesia) and ICP-EOS (Agilent Technologies 700 series ICP-OES). A multi-probe field meter (YSI TM 6 series, Sonde Marion, Germany) was used to measure physicochemical parameters such as pH, temperature (T), total dissolved solids (TDS), dissolved oxygen (DO), and electrical conductivity (EC) in situ. The presence of heavy metals was assessed using an Inductively Coupled Optical Emission Spectrometer (Agilent Technologies 700 series ICP-OES).

5.2.4 DNA extraction and PCR Amplification

For soil bacterial analysis, metagenomic DNA was extracted from 5 g of triplicates soil samples using the PowerSoil ® DNA isolation kit (MoBio Laboratory, CA, USA) according to the manufacturer's instructions. PCR reactions were performed in triplicate in a 50 µL mixture which contains 25 µL Qiagen Top Taq Master Mix (2.5 units Taq DNA polymerase, dNTPs (200 µM each), 1.5 Mm $MgCl_2$), 1 µL each of forward primer and reverse primer, metagenomic DNA template (50–100 ng/µL), and sterile nuclease-free water added to make up the final reaction volume of 50 µL. To detect contamination, each reaction contained a template-free control. The Polymerase chain reactions (PCR) reaction was amplified using universal primers 27F (5’-AGR GTT TGA TCM TGG CTC AG-3’) and 1492R (5’-GGT TAC CTT GTT ACG T-3’). This set of primers produces 1200 bp amplicons from the bacterial variables (V1-3) region of the 16S rRNA gene sequence. The following cycling conditions were used to perform PCR reactions in a BioRad T100 thermal cycler: initial denaturation step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min 30 s, with a final extension at 72 °C for 10 min. The infinite hold was set at 4 °C. Extracted DNA was quantified on a Qubit 3.0 Fluorometer (Life Technologies, RSA) and the purity was determined by measuring the A260/280 and A260/230 ratios in a Biodrop µLite spectrophotometer (Biochrom, USA).

The PCR products were placed onto a horizontal agarose gel in 1 TAE buffer stained with 5% of 10 mg/mL ethidium bromide (Merck, SA). The samples were run in 1 TAE buffer at 80 volts for 90 minutes, then the gel was withdrawn from the buffer solution and visualized with a Biorad UV illuminator. For validation and comparison of band size and authenticity, a CSL-MDNA-100BP DNA ladder was utilized. A Biodrop spectrophotometer was used to quantify the concentrations of DNA to determine the quality and amount of extracted DNA (Nanodrop2000, Thermo Scientific, Japan). Recovered DNA samples were re-amplified with universal primers 27F (5′-TCG TCG TCA GAT GTG TAT AAG AGA CAG AGA GTT TGA TCM TGG CTC AG-3′) and 518R (5′- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GAT TAC CGC GGC TGC TGG-3′) with adapters and barcodes suited for running samples. According to Caporaso *et al*. (2011), this set of primers amplifies the bacterial V1-3 region of the 16S rRNA gene sequence and produces 500-550 bp amplicons. The PCR reaction was re-analyzed at a different annealing temperature of 50 °C, as previously stated, as published by Tekere *et al*. (2011).

5.2.5 Illumina MiSeq sequencing

Ampure XP beads were used to further purify the amplicons. The Agilent DNA 1000 Bioanalyser was used to validate the barcoded libraries and the Qubit DNA BR reagent assay was used to quantify them. The PCR product was sequenced using paired-end sequencing chemistry on the Illumina Miseq. This sequencing method has been widely employed in the research of soil bacterial diversity because it provides more detailed information on microbial diversity with high accuracy.

5.2.6 Data Processing and Statistical Analyses

The raw data files from MiSeq sequencing of the 16 rRNA gene, region V1-3, sets in FASTQ format were trimmed to remove PCR artefacts, Illumina tags, and low-quality reads using QIIME (version 1.7) prior to data analysis. QIIME v. 1.7.0 (Caporaso *et al*., 2010) was used to filter reads and determine Operational Taxonomic Units (OTUs) at the ≥ 97 % similarity level (Kuczynski *et al*., 2011). The quality data was filtered, and all data sets were uploaded onto the Mothur pipeline v.1.40.0 for further analysis as reported by Schloss *at al*. (2009). Sequence reads with fewer than 50 nucleotides, > 2% ambiguity, or 7% homopolymers, as well as those from mitochondria or chloroplasts, were all discarded. Chimeric sequences were deleted using UCHIME settings, as described by Edgar *et al*. (2011). All statistical analysis was performed using R software (v.3.6.0. R core Team, 2019). To assess alpha diversity among the sequencing data sets, researchers employed the number of discovered OTUs per sample, as well as alpha non-parametric indicators such as Chao, Dominance, Shannon H, Simpson D, and Species evenness (Zakrzewski *et al*., 2017). At a genetic distance of 0.03, all non-parametric diversity indices were calculated. The relative abundance of each taxon was computed by dividing the number of sequences associated with that taxon by the total number of sequences recovered for that sample. To identify significant differences in community composition (beta diversity), redundancy analysis (RDA) was employed to measure the effects of environmental variables on bacterial community structures. Venn diagrams for graphical descriptions of unique and shared bacterial genera between different quicklime-treated AMD irrigated soils were calculated using the “Venn Diagram” package in R package.

5.3 RESULTS

5.3.1 Soil Geochemical Properties

The physiological properties of the soil were fully discussed on Chapter 3, section 3.4.1 and 3.4.2 across the treatments for all the measured parameters and reported in Table **3.2**. There was an increase in the pH of the soil irrigated with treated AMD (5.67 (ST3), 6.70 (ST4) and 7.23 (ST5) as compared to the untreated AMD (T2) and tapwater (T1). The EC and TDS values of treated water (T3, T4 and T5) decreased when compared to untreated water (T2) and and increase in sulphate (SO_4^2) were observed in soil irrigated with treated AMD water. Thus, could be attributed to the interaction of plant-microbes that have the tendency to alter the soil environment making it suitable for the growth of crops (Xin *et al*., 2021). As alluded to, on Chapter 3; the study also evaluated the concentration of selected heavy metals in the soils irrigated with quicklime and fly ash treated AMD. The results showed that soil irrigated with treated AMD (ST3, ST4, and ST5) showed variation in the concentrations of heavy metals and were within the permissible limit of the WHO except for As, Cd and Cr (Table **3.11**) respectively.

5.3.2 Soil Bacterial Diversity Derived from 16S rRNA Gene Sequence

Reduction in annual precipitation due to climate change and other environmental factors has become a major challenge in the agricultural industrial, especially in crop propagation (Molden *et al*., 2007; FAO, 2016). This challenge has been the major contributor to the use of diverse sources of water by farmers for irrigation. Among which, is the use of untreated and treated acid mine drainage (AMD) as well as other wastewater by the farmers as irrigation water (Annandale *et al*., 2011). AMD has been reported as a global challenge due to its devastating effect on agricultural land (Rezaie & Anderson, 2020). This imply that the use of AMD water as a source of irrigation could alter the soil microbial composition as well as the health of the soil necessary for an improved crop propagation (Musvoto & de Lange, 2019). The present study examined the impact of quicklime/fly ash treatment on the diversity of bacterial community in AMD and treated-AMD irrigated soil. Overall, both untreated and quicklime/fly ash treated contributed to the change in the soil bacterial community diversity. In consonant to the present study, AMD and treated-AMD irrigation has been reported by several authors to alter the microbial composition of the soil which could improve the health of the soil and promote the growth of crops (Narendrula-Kotha & Nkongolo, 2017; Wu *et al*., 2019; Li *et al*., 2020; Xin *et al*., 2021).

The results through 468 MiSeq sequencing analysis revealed a total of 145,080 quality reads and 2,202 OTU's were obtained from 45 samples across the five soil treatment levels of the two potato cultivars (Table **5.1**). Each library contained 10648 to 53275 reads, with different OTU's ranging from 93 to 689 respectively. The comparative alpha diversity (α-diversity) indices of soil bacterial community as affected by different AMD-treated water are presented below (Figure **5.1**). The rarefaction and rank abundance curves clearly showed an asymptotic approach that formed a visible plateau (Figure **5.2**). This implied that the curves accurately reflected the bacterial community and that the sample deepness was good enough for the estimation of the bacterial diversity covering all major bacterial communities presented in the treated AMD irrigated soil. The rarefaction curve indicated the presence of considerable variance in the total number of OTU's in different samples from quicklime treated AMD irrigated soils. A significant difference was observed across the treatments for the number of OTU's presented with the AMD site (100% AMD water) as the lowest with OTU's of 93, followed by AFQ, AMD water treated with fly ash and 2 g quicklime (394), A2Q, AMD water treated with 2 g quicklime (465), A1Q, AMD water treated with 1 g quicklime (561), and Control, tap water (689). This indicated that the soil irrigated with tap water is more enriched with bacterial communities.

Similarly, Chao-1 index that also estimates the richness of microbes in an environment was significant across the treatment with the control samples showing more enrichment and the AMD less enrichment (Figure **5.1** a, b, c & d) below. The diversity indices (Shannon and Simpson index) were also significant across the treatments and showed a decreasing trend under 100 % AMD water with subtle variation with the treated AMD irrigated soil. Different colours denoted different treatments: Control, tap water (Apple green); AMD 100% water (Red); A1Q, 1 g QL + 100 % AMD water (Green); A2Q, 2 g QL + 100 % AMD water (Blue); and AFQ, 2 g QL + 100 % AMD + 75 % Fly Ash (Purple).

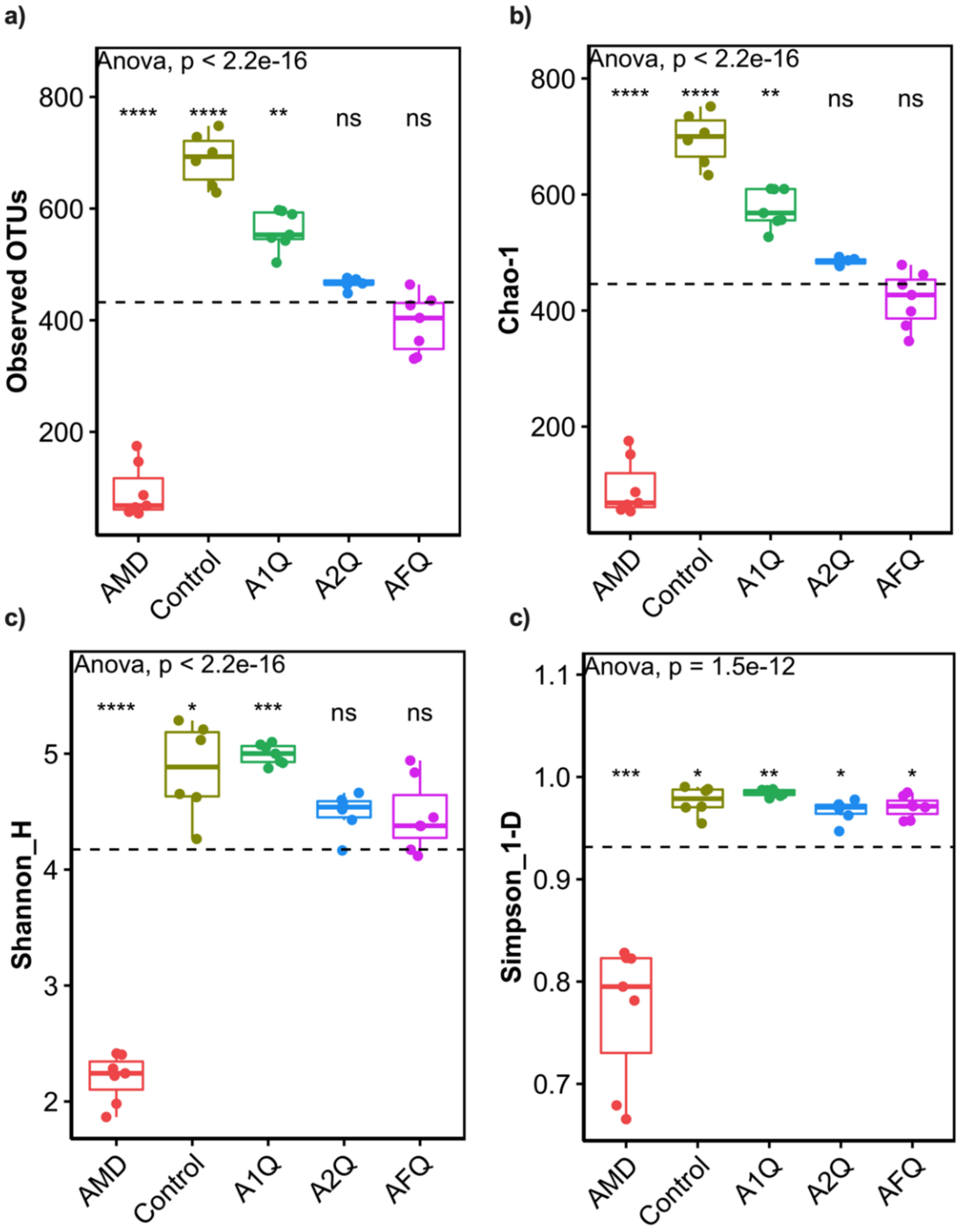


Figure 5.1 Comparative alpha diversity indices of bulk soil bacterial community as affected by quicklime AMD irrigation water. Different colours denoted different treatments levels: Control, tap water (Apple green); AMD 100% water (Red); A1Q, 1 g QL + 100 % AMD water (Green); A2Q, 2 g QL + 100 % AMD water (Blue); and AFQ, 2 g QL + 100 % AMD + 75 % Fly Ash (Purple).

Table 5.1. MiSeq sequencing results and diversity estimates for each quicklime treated AMD irrigated soils level.

Treatment levels	**Sequencing results**		**Diversity estimates**[a]		
	Observed OTUs	**Valid reads**	**Simpson 1_D**	**Shannon_H**	**Chao-1**
AMD (n=7)	93±43*e*	48403±40800*a*	0.771±0.070*b*	2.20±0.21*c*	94±49*e*
A1Q (n=7)	561±35*b*	16686±3210*ab*	0.984±0.003*a*	4.99±0.09*a*	576±33*b*
A2Q (n=6)	465±10*c*	16068±3840*ab*	0.967±0.011*a*	4.49±0.18*a*	485±8*c*
AFQ (n=7)	394±52*d*	10648±4117*b*	0.971±0.011*a*	4.47±0.31*b*	419±48*d*
Control (n=6)	689±47*a*	53275±33301*a*	0.977±0.014*a*	4.86±0.41*b*	696±45*a*
Significance	T: $F_{4,23}$ = 193.5, $p < 0.0001$***	T: $F_{4,23}$ = 5.302, $p = 0.0036$**	T: $F_{4,23}$ = 44.2, $p < 0.0001$***	T: $F_{4,23}$ = 209.8, $p < 0.0001$***	T: $F_{4,23}$ = 221.3, $p < 0.0001$***
	Cultivar: $F_{1,23}$ = 2.25, $p = 0.147$	Cultivar: $F_{1,23}$ = 1.798, $p = 0.193$	Cultivar: $F_{1,23}$ = 0.366, $p = 0.551$	Cultivar: $F_{1,23}$ = 4.355, $p = 0.0482$*	Cultivar: $F_{1,23}$ = 1.623, $p = 0.250$

Control: Tapwater (T1); AMD: 100 % AMD (T2); A1Q: 1g QL + 100 % AMD (T3); A2Q: 2g QL + 100 % AMD (T4); AFQ: 2g QL + 100 % AMD +75 % FA (T5). [†] Diversity indices (observed OTUs, *Chao1*, Shannon and Simpson) were based on rarefied datasets of 10648 Sequences representing the lowest number of reads in a sample.

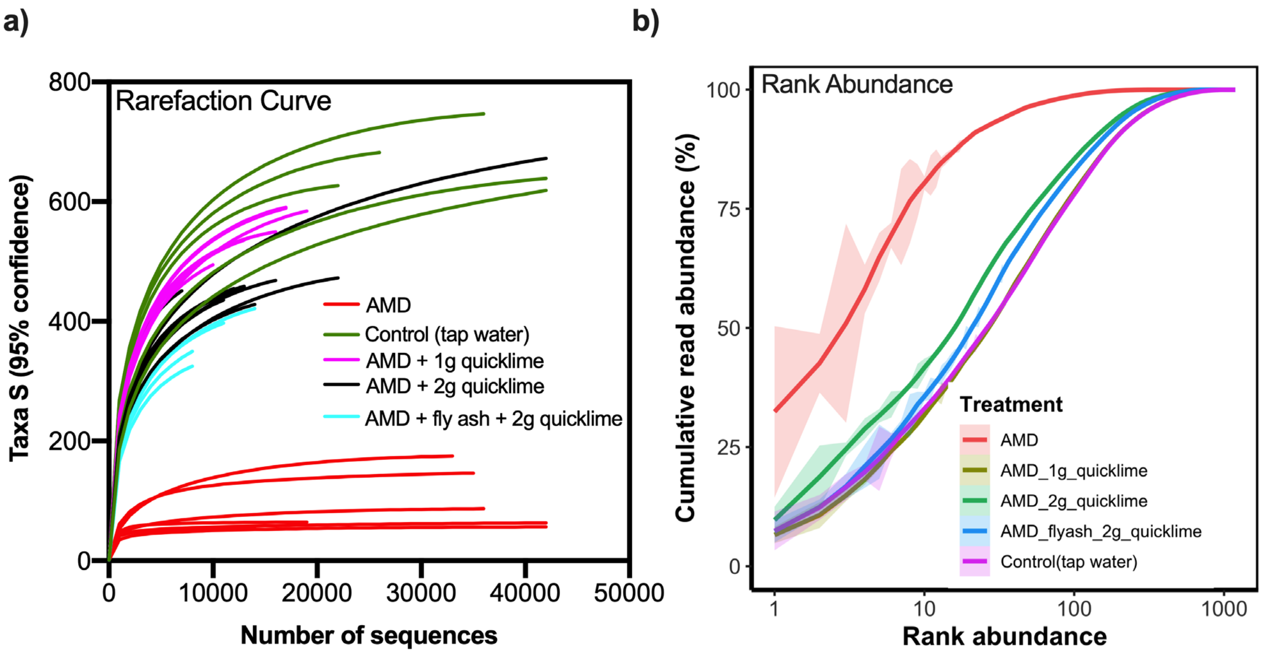


Figure 5.2 Rarefaction curve of the OTU number at 97% boxplot.

5.3.4 Taxonomic Composition of the Soil Bacterial Diversity

The microbial richness and diversity of AMD irrigated soil has been reported to be less due to the inability of diverse microbes to survive (Villegas-Plazas *et al*., 2019). A similar observation was recorded in this study as the bacterial richness and diversity of the 100% AMD were less than the control (tap water) and quicklime/fly ash treated AMD water (Table **5.1** and Figure **5.2**). Lime treatment of soils has also been reported to promote and increase the bacterial richness and diversity of the soil either directly or mixed with irrigated water (Narendrula-Kotha & Nkongolo, 2017; Pang *et al*., 2019; Liang *et al*., 2021). This report agreed with our study as the alpha diversity and species richness of the bacteria community in the quicklime/fly ash treated AMD, were higher than the 100% AMD water. This implied that quicklime and fly ash have the potential to create suitable micro-environment necessary for the proliferation of diverse bacterial communities in AMD impacted environment and possibly promote the growth of plants through plant-microbe’s interactions.

Unclassified sequences were assigned to sequences that could not be classified into any known group. Based on relative abundances, bacterial communities OTU's in quicklime treated irrigated soils for the two potato cultivars were assigned into 10 different phyla, 27 classes, 80 orders, and 242 genera. Six different phyla

(*Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes* and *Proteobacteria*) were the most common. The summary of the bulk soil bacterial composition at phylum and class level as affected by different AMD irrigation water treatment for the two-potato cultivars is presented in Figure **5.3**.

The dendrogram revealed distinct groups of bacterial phyla and classes associated with the soil irrigated with AMD and AMD treated water indicating that the bacterial phyla and classes are dependent on the treatment and the potato cultivars (Figure **5.3** a). The dendrogram comprises of two major clusters with the second cluster further divided into two. The 100 % AMD treatments grouped in the first cluster on the left. The cluster was divided into two groups, and the control samples grouped together in one sub-cluster while the treated samples grouped together with the AMD + 1 g quicklime and AMD + 2 g quicklime dominating the sub-cluster on the right (Figure **5.3** a). In contrast to the present findings, Wang *et al*. (2018) reported that *Acidobacteria* was dominant in paddy rice soil irrigated with AMD water. This could be attributed to the ability of the quicklime/fly ash to create a conducive environment for the proliferation of the members of the phyla. Pang *et al*. (2019) observed increased the dominance of the members of *Acidobacteria* and *Chloroflexi* in the soil treated with lime indicating the impact of lime on the bacterial diversity. Further, the survival of the two phyla in the irrigated soil maybe due the plant-microbe interaction that could exist between the Marykies and Royal potato cultivars used in this study and could promote the growth of the two cultivars. In support of this hypothesis and finding, Wang *et al*. (2021) reported the role of *Acidobacteria* in the generation of an alkaline environment due to increase metabolic activities that enhances the proliferation of diverse sulfate- (SRB) and iron-reducing bacteria.

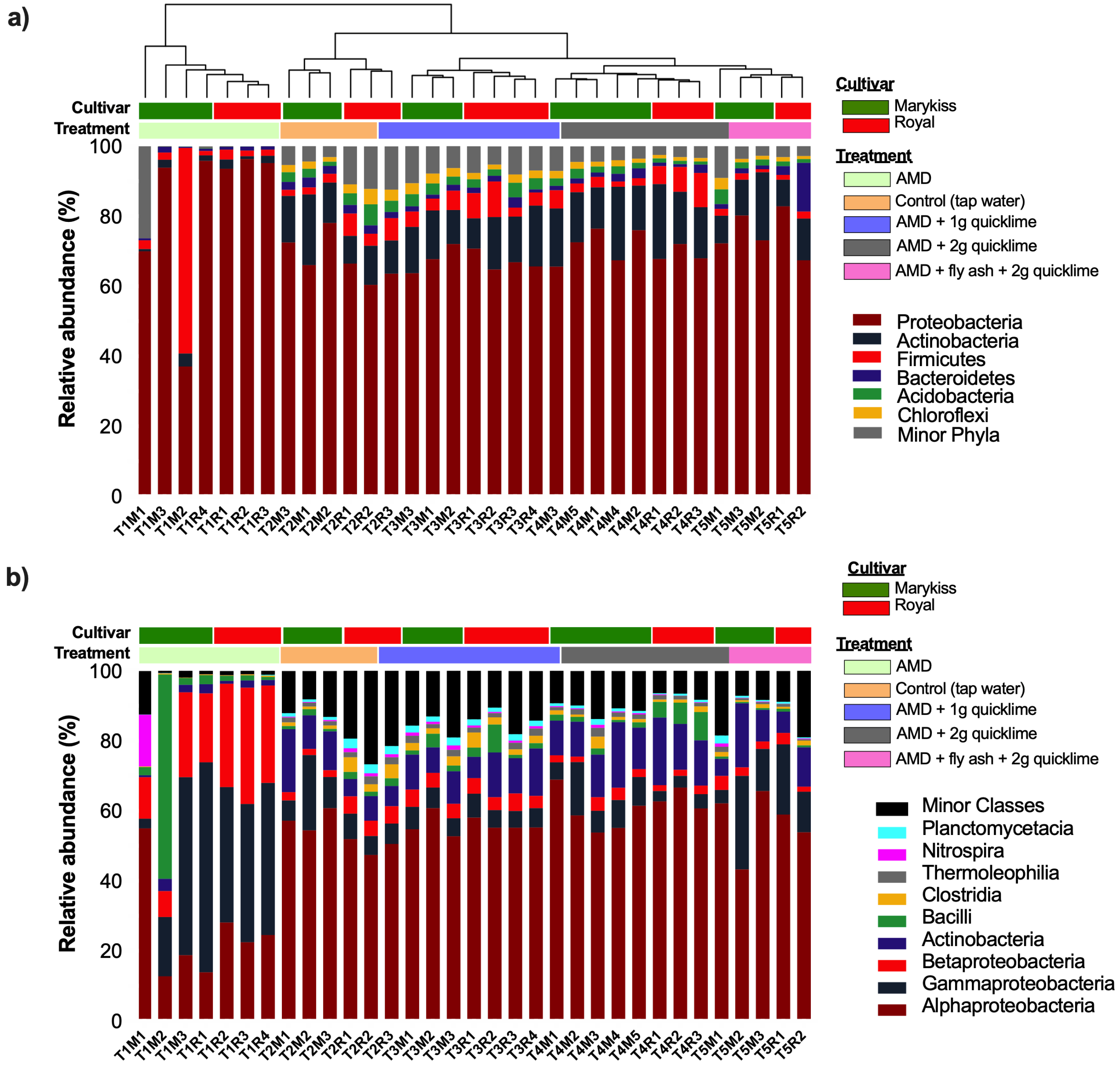


Figure 5.3. Taxonomic composition of soil bacterial diversity at phylum and class level as affected by quicklime treated AMD irrigation within the two-potato cultivars (Marykies and Royal). The dendrogram shows complete-linkage agglomerative clustering based on a Euclidean distance.

To further delineate the impact of the irrigation of the soil with AMD treated water on the soil bacterial diversity of the two cultivars, the relative abundance of the different phyla was done for the AMD treated water before irrigation and after irrigation. The relative abundance of the bacterial community at phylum level for quicklime treated AMD irrigated soils is summarized below (Figure **5.4**). Overall, members of the phylum *Firmicutes, Proteobacteria, Actinobacteria, Planctomycetes, Cyanobacteria, Acidobacteria, Chloroflexi, Nitrospirae,* and *Bacteriodetes* were the most abundant across all the samples accounting for >75% of the observed taxa for quicklime treated AMD irrigated soils (Figure **5.4** a) and the soil irrigated with the quicklime treated AMD water (Figure **5.4** b) with subtle variations especially, in the soil irrigated with treated AMD under two potato cultivars (Figure **5.3**). The Proteobacteria constituted the major phylum in both the 100% AMD water and treated AMD water before and after irrigation with a decreasing trend down the treated AMD water. Similar trends were observed in Firmicutes, Cyanobacteria, and Nitrospirae. This could be attributed to the impact of the treatments on the bacterial phylum diversity through the alteration of the environmental conditions suitable for the proliferation of the phyla.

At the class level, *Clostridia, Betaproteobacteria, Bacilli, Alphaproteobacteria, Actinobacteria, Planctomycetia,* and *Gammaproteobacteria* were the abundant classes with variation in their abundance across the treatments before (Figure **5.5** a) and after irrigation (Figure **5.5** b). A decreasing trend from the 100% AMD down to the treated AMD was also observed for the members belonging to the classes *Betaproteobacteria, Bacilli,* and *Gammaproteobacteria*. Subtle variations in the relative abundance of the bacterial classes were observed across the treatments on the soil irrigated with treated AMD for the two potato cultivars. This provided a clear indication of the influence of the treatments and the two potato cultivars on the bacterial diversity of the soil. Jung *et al*. (2014) observed the dominance of the members of bacteria belonging to the classes *Clostridia* and *Deltaproteobacteria* in an AMD treated water. This observation confirms the findings in this study, as the members of the above-mentioned classes were higher in abundance in the treated AMD water as well as the irrigated soil. The study found that some genera have close relation with heavy metals and may provide a new way to explore natural bioremedial genera. However, several genera which had not been reported for their resistance to turned out to be dominant in quicklime-treated AMD irrigated soils and needed further study to explore their influencing factors.

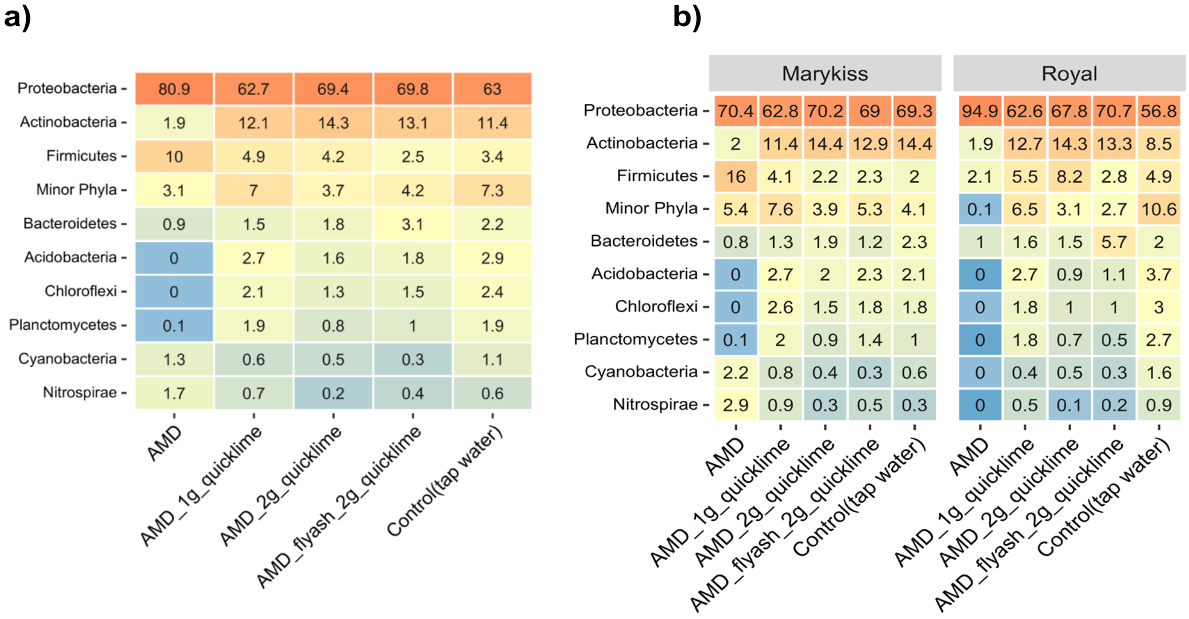


Figure 5.4 The composition and relative abundance of major bacterial phyla as impacted by the quicklime treated AMD water before (**a**) and after (**b**) irrigation on the soils of the two potato cultivars

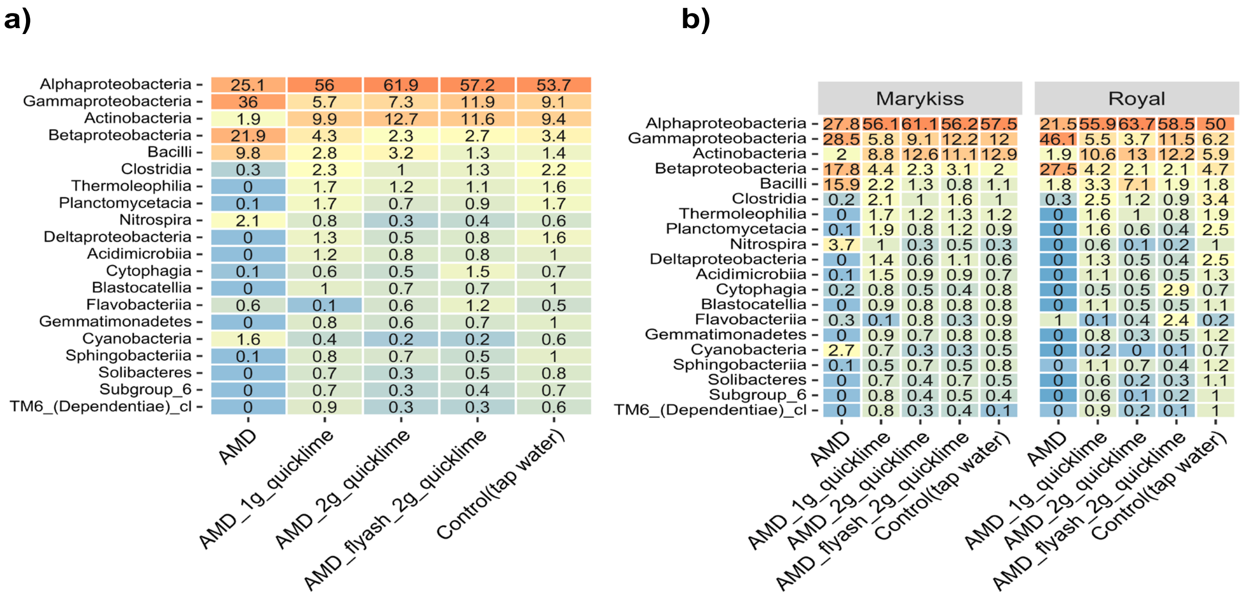


Figure 5.5 The composition and relative abundance of major bacterial classes as impacted by the treatment of AMD water before (a) and after (b) irrigation on the soils of the two potato cultivars.

5.3.5 Correlation Between Soil Bacterial Structure and Environmental Factors

The relative importance of each individual environmental variable on bacterial community composition was measured by redundancy analysis, to further identify the major environmental variables controlling the soil bacterial structure (Figure **5.6**). Results indicated that both heavy metals (Cu and Pb) and soil (pH and EC) were identified to be the most influential factors on soil bacterial dstructure. The first and second axis of the RDA explained 6.2 % and 25.7 % of the variance in the bacterial community, respectively. Moreover, RDA ordination revealed distinct differences in bacterial community composition between quicklime-treated AMD irrigated soils.

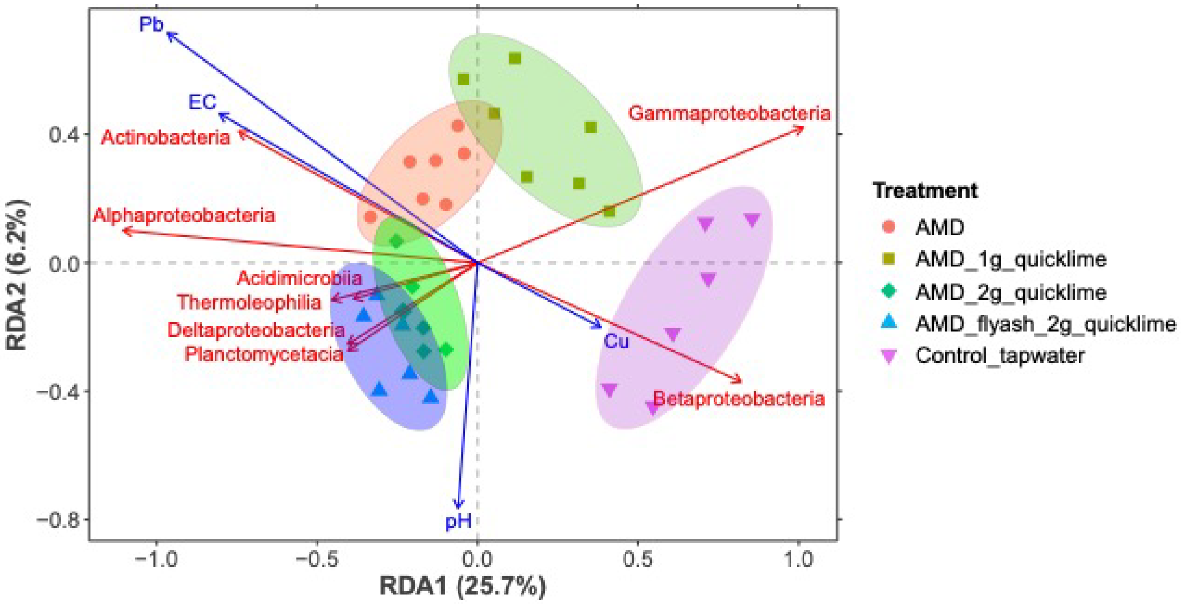
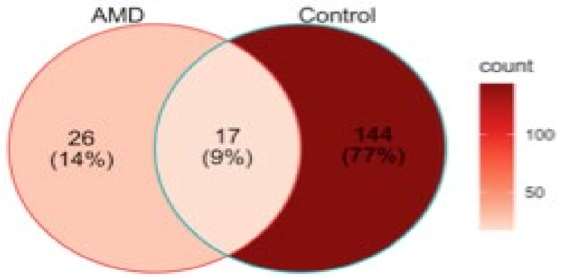


Figure 5.6 Redundancy analysis (RDA) triplot of the bacterial community composition at the class level (relative abundance > 1 %) and environmental variables in the bulk soil samples. Red arrows indicate the members of the bacterial community. The blue arrows represent environmental variables (Cu, Pb, EC and pH) with significant correlation lower than 0.05, based on ordistep forward selection after Holm's correction.

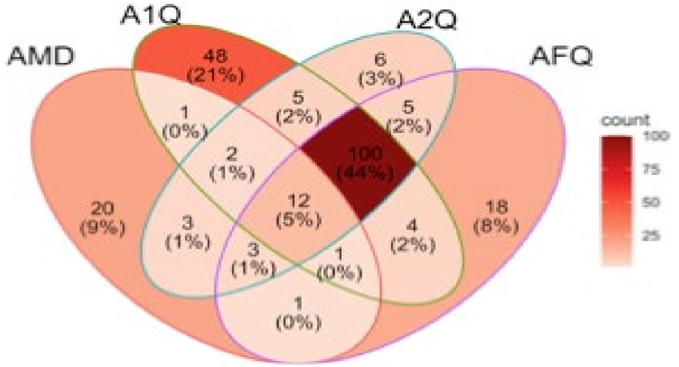
Based on the relative abundance of the genera from figure **5.3** above, the genera with an average abundance of >1 % in at least one group were defined as dominant. Combined with Venn diagram. Most dominant genera belonged to the genera irrigated by tapwater

(Figure **5.7**). However, their relative abundance changed with different quicklime-traeted AMD iirigated soils.

a) Control (tapwater) vs untreated AMD irrigation water



b) Treated vs untreated AMD irrigation water



c) control (tapwater) vs untreated AMD irrigation water

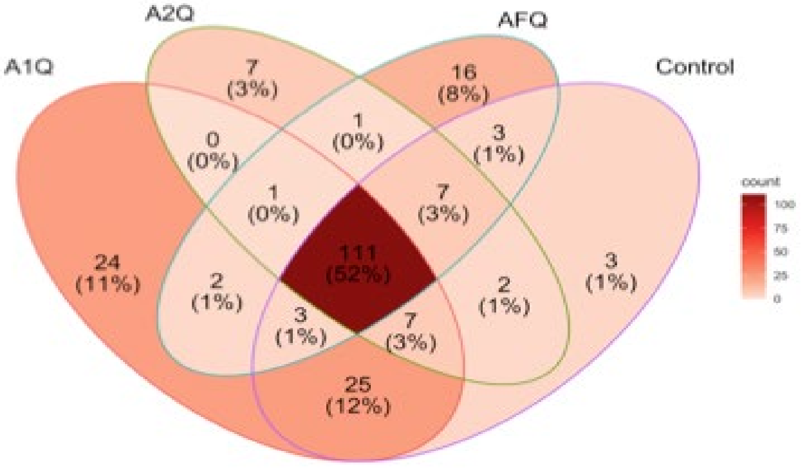


Figure 5.7 Venn diagrams showing core shared and unique OTUs to different bulk soil irrigated with tapwater (control), untreated AMD (AMD) and treated (A1Q, A2Q and AFQ) water. The core OTUs were calculated at athreshold abundance cutoff (cut_a) = 0.1% and frequency cutoff (cut_f) = 80%

5.4 CONCLUSION

The present study evaluated the diversity of bacterial community in the soil used to grow the selected potato cultivars and irrigated with both AMD and quicklime/fly ash treated AMD. In general, the bacterial diversity of the irrigated soil was influenced by the AMD and AMD treated water with variations with respect to the cultivars of the potato. The diversity and species richness of the AMD irrigated water was less than that of the control and AMD treated water. This indicated that there was a shift in the microenvironment that can promote the growth of diverse bacterial communities in AMD treated water as impacted by the quicklime/fly ash. *Firmicutes, Proteobacteria, Actinobacteria, Planctomycetes, Cyanobacteria, Acidobacteria, Chloroflexi, Nitrospirae*, and *Bacteriodetes* were dictated as the dominant bacterial phyla with subtle variation across the treatments and irrigated soil. However, the dominant of the members of the phyla *Acidobacteria* and *Chloroflexi* in the treated AMD indicated that the treatment selects the bacterial community that can proliferate in the environment.

Furthermore, the survival of these phyla in the soil could also be attributed to the plant-microbes interaction that could promote the health of the soil as well as the growth of the crops. In addition, members of the classes *Clostridia* and *Deltaproteobacteria* were abundant in the AMD treated water and irrigated soil. In summary, the quicklime/fly ash may have created an environment that selects the bacterial communities that survive and proliferate in the treated AMD water as well as the irrigated soil. However, further study is required to elucidate the role of the bacterial communities in the soil and plant-microbes interaction between the two cultivars of potato when irrigated with treated AMD.

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CHAPTER 6: SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 INTRODUCTION

The aim of this chapter is to present the conclusions drawn from the results of the analysis from each objective in the study. It includes a brief discussion presented according to each of the objectives then followed by the findings of this study; summarizes the conclusions originating from this study; and covers the lesson learned, as well as its merits, flaws, and unique contributions to the knowledge of science. Lastly, it concludes with a list of recommendations that will enable researchers to work on future research.

6.2 CONCLUSIONS

The main aim of the study was to evaluate the impacts on the physiological parameters and biochemical performance on the *S. tuberosum* and identify soil bacterial diversity abundance and variations when subjected to quicklime-treated AMD irrigation.

To achieve the aim, the following objectives of the study investigate:

1) The effects of quicklime treated AMD irrigation on the physiological parameters and heavy metals toxicity on the water, soil, and the potato cultivars.
2) The metabolic profile on potato cultivars when subjected to quicklime treated AMD irrigation.
3) Identify soil bacterial diversity variation and abundance within different quicklime treated AMD levels.

In accordance with the first objective of study, the results revealed that after the application of quicklime in the AMD water, the physiochemical properties such as pH increased, EC, TSD and SO_4^{2-} decreased. However, SO_4^{2-} was still unsafe as per WHO limit. To validate the suitability of the quicklime treated AMD water for irrigation, quicklime can reduce heavy metal concentrations. The growth, physiological parameters, and yield of the two potato cultivars showed significant differences after irrigation with quicklime-treated AMD water.

Regarding the second aim, even though mine water re-use has positive effects, the metabolite profiles of the two potato cultivars were significantly affected by quicklime-treated AMD irrigation, according to findings of this study. The results revealed a total of 40 metabolites were identified from Marykies and 36 from Royal which included amino acids, organic acids, and aromatic amines. However, Adenosine monophosphate, Cytidine, Xanthine, Lactic acid, and Isocitric acid were only detected in the Marykies potato cultivar. There was a clear separation on PCA analysis between control sample (tapwater) and AMD both treated and untreated.

Regarding the third aim which involved using the Illumina MiSeq sequencing technique to identify soil bacterial community structure in quicklime-treated AMD irrigated soils for the two potato cultivars, the rarefaction results revealed a significant difference across the treatments for the number of OUT's. The relative abundances of bacterial communities in the different soils irrigated with 100% AMD and treated AMD water for the two potato cultivars were grouped into 10 phyla, 27 classes, 80 orders, and 242 genera.

6.3 RECOMMENDATIONS

Understanding how plants respond to such stressors, particularly heavy metals, is critical for developing new crop-improvement strategies.

- Therefore, although, the treatment used in this stdy showed positive results, there is a need to explore it on other potato cultivars.
- This experiment was carried out in a greenhouse environment. It is assumed that when quicklime is used in an open field, the effects may vary due to the influence of numerous field characteristics/ factors.

Metabolomics came after proteomics and genomes in terms of development, and its detection method has several drawbacks.

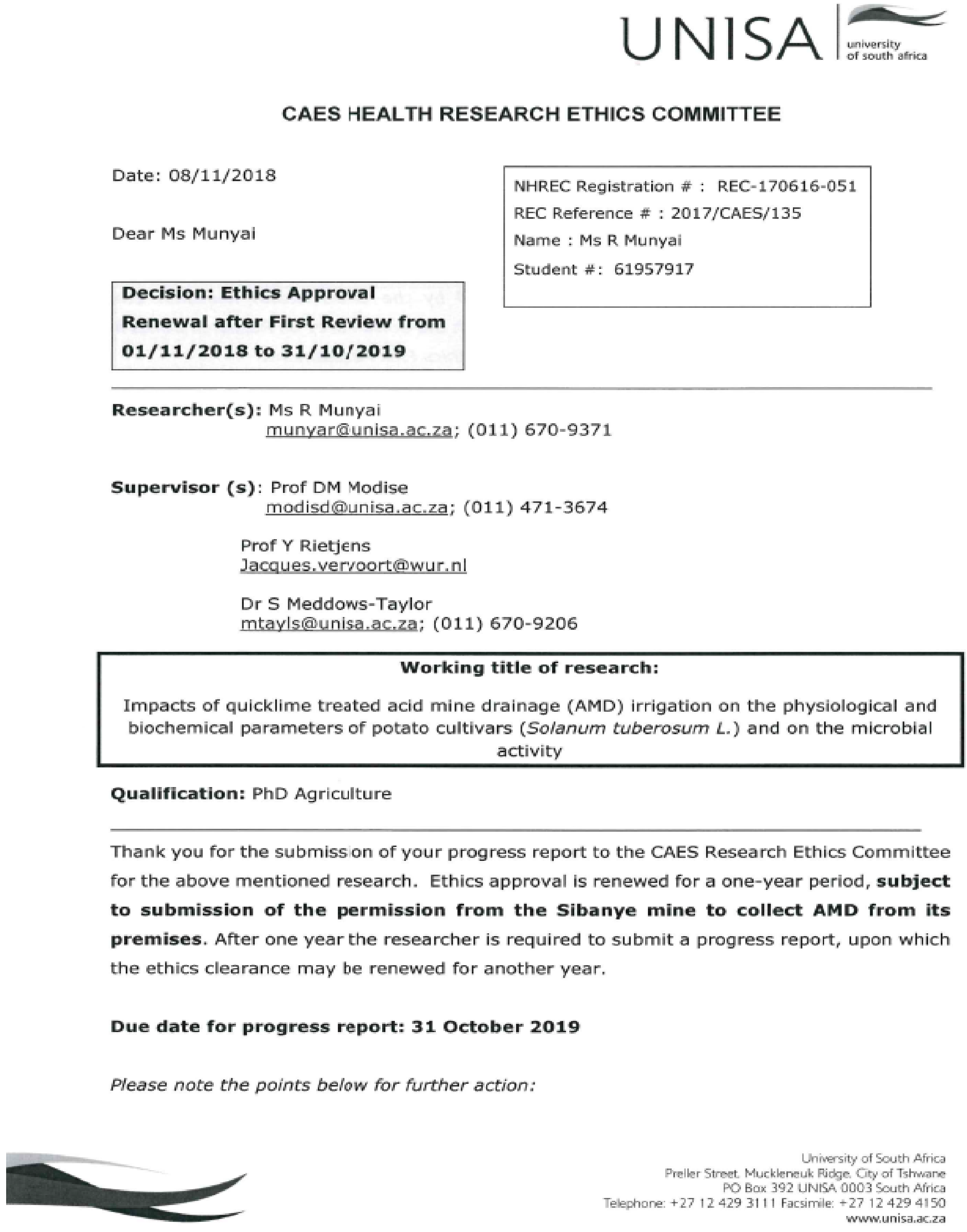
- In the future, metabolomics combined with other omics would help to improve our understanding of the effects of treated acid mine drainage water irrigation on crops in general, as well as establish a comprehensive biological knowledge base.

- There is a need for more studies including metabolomics as an integral part of the systems biology approach to studying plant response to a variety of stress conditions. A holistic view of how plants respond to abiotic and biotic stress would be provided by combining metabolomics, proteomics, transcriptomics, and mathematical modeling, allowing us to develop advanced strategies to improve the tolerance of different plants and crops to biotic and abiotic stress conditions.

The use of next-generation sequencing and metagenomics technologies has considerably improved the ability to identify bacterial taxa and quantify bacterial abundance and diversity in AMD environments.

- There is a need to understand the effects of AMD water on microbial ecology and evolution, identifying microorganisms with bioremediation properties, and understanding the mechanisms of microbial AMD tolerance and remediation all require characterization of microorganisms in polluted environments.

APPENDIX I Ethical Clearance Certificate



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CAES HEALTH RESEARCH ETHICS COMMITTEE

Date: 08/11/2018

Dear Ms Munyai

NHREC Registration # : REC-170616-051
REC Reference # : 2017/CAES/135
Name : Ms R Munyai
Student #: 61957917

Decision: Ethics Approval Renewal after First Review from 01/11/2018 to 31/10/2019

Researcher(s): Ms R Munyai
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Working title of research:

Impacts of quicklime treated acid mine drainage (AMD) irrigation on the physiological and biochemical parameters of potato cultivars (*Solanum tuberosum L.*) and on the microbial activity

Qualification: PhD Agriculture

Thank you for the submission of your progress report to the CAES Research Ethics Committee for the above mentioned research. Ethics approval is renewed for a one-year period, **subject to submission of the permission from the Sibanye mine to collect AMD from its premises**. After one year the researcher is required to submit a progress report, upon which the ethics clearance may be renewed for another year.

Due date for progress report: 31 October 2019

Please note the points below for further action:

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1. The researcher(s) will ensure that the research project adheres to the values and principles expressed in the UNISA Policy on Research Ethics.
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3. The researcher(s) will conduct the study according to the methods and procedures set out in the approved application.
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5. The researcher will ensure that the research project adheres to any applicable national legislation, professional codes of conduct, institutional guidelines and scientific standards relevant to the specific field of study. Adherence to the following South African legislation is important, if applicable: Protection of Personal Information Act, no 4 of 2013; Children's act no 38 of 2005 and the National Health Act, no 61 of 2003.
6. Only de-identified research data may be used for secondary research purposes in future on condition that the research objectives are similar to those of the original research. Secondary use of identifiable human research data require additional ethics clearance.
7. No field work activities may continue after the expiry date. Submission of a completed research ethics progress report will constitute an application for renewal of Ethics Research Committee approval.

Note:
The reference number ***2017/CAES/135*** *should be clearly indicated on all forms of communication with the intended research participants, as well as with the Committee.*

Yours sincerely,

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APPENDIX II Language Editing Certificate

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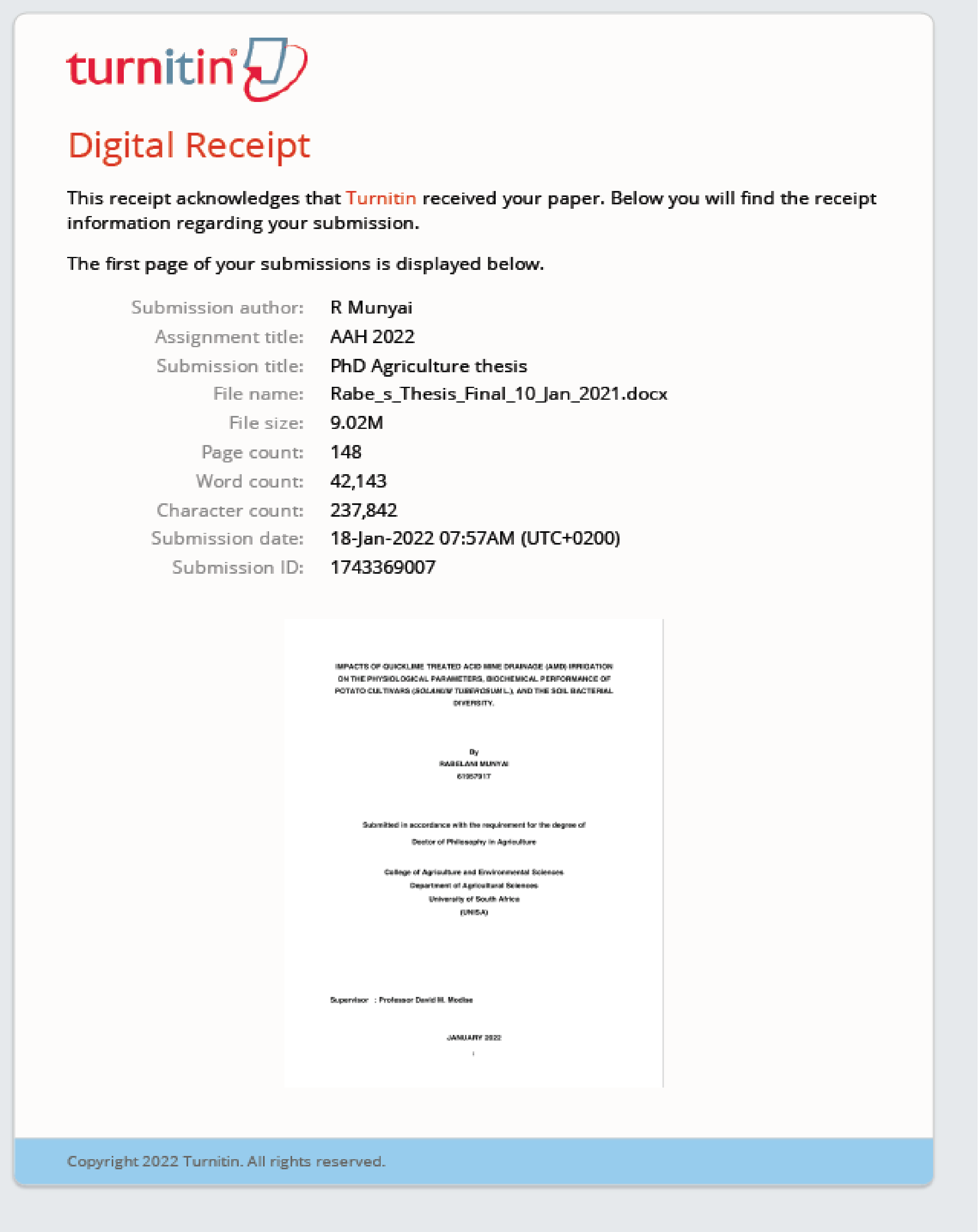

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IMPACTS OF QUICKLIME TREATED ACID MINE DRAINAGE (AMD) IRRIGATION ON THE PHYSIOLOGICAL PARAMETERS, BIOCHEMICAL PERFORMANCE OF POTATO CULTIVARS (SOLANUM TUBEROSUM L.), AND THE SOIL BACTERIAL DIVERSITY.

By
RABELANI MUNYAI
[illegible]

Submitted in accordance with the requirement for the degree of
Doctor of Philosophy in Agriculture

College of Agriculture and Environmental Sciences
Department of Agricultural Sciences
University of South Africa
(UNISA)

Supervisor : Professor David M. Modise

JANUARY 2022

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APPENDIX IV Published paper

frontiers
in Sustainable Food Systems

REVIEW
published: 21 September 2021
doi: 10.3389/fsufs.2021.701870

Microbial Community Diversity Dynamics in Acid Mine Drainage and Acid Mine Drainage-Polluted Soils: Implication on Mining Water Irrigation Agricultural Sustainability

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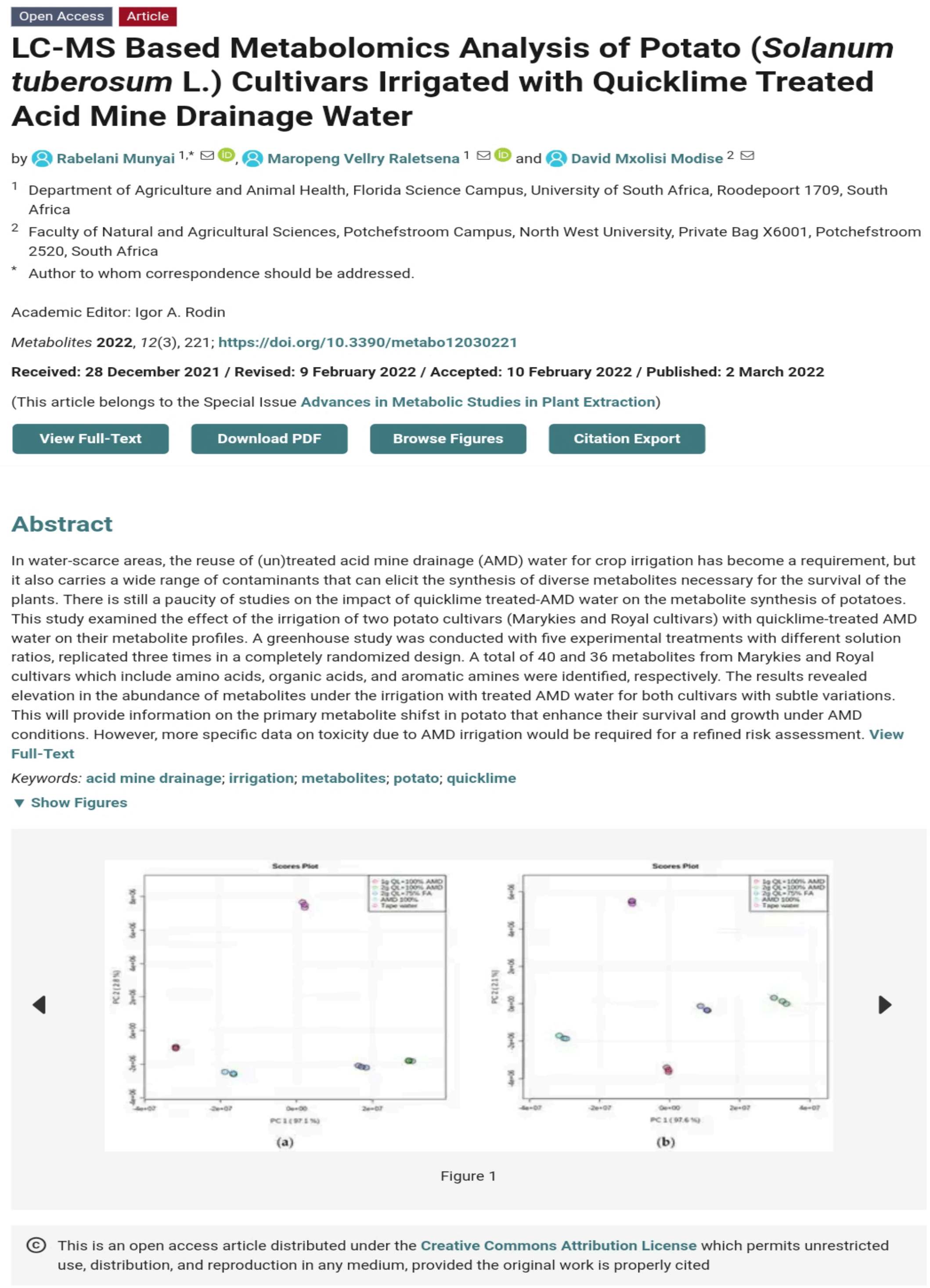
Environmental degradation related to mining-generated acid mine drainage (AMD) is a major global concern, contaminating surface and groundwater sources, including agricultural land. In the last two decades, many developing countries are expanding agricultural productivity in mine-impacted soils to meet food demand for their rapidly growing population. Further, the practice of AMD water (treated or untreated) irrigated agriculture is on the increase, particularly in water-stressed nations around the world. For sustainable agricultural production systems, optimal microbial diversity, and functioning is critical for soil health and plant productivity. Thus, this review presents up-to-date knowledge on the microbial structure and functional dynamics of AMD habitats and AMD-impacted agricultural soils. The long-term effects of AMD water such as soil acidification, heavy metals (HM), iron and sulfate pollution, greatly reduces microbial biomass, richness, and diversity, impairing soil health plant growth and productivity, and impacts food safety negatively. Despite these drawbacks, AMD-impacted habitats are unique ecological niches for novel acidophilic, HM, and sulfate-adapted microbial phylotypes that might be beneficial to optimal plant growth and productivity and bioremediation of polluted agricultural soils. This review has also highlighted the impact active and passive treatment technologies on AMD microbial diversity, further extending the discussion on the interrelated microbial diversity, and beneficial functions such as metal bioremediation, acidity neutralization, symbiotic rhizomicrobiome assembly, and plant growth promotion, sulfates/iron reduction, and biogeochemical N and C recycling under AMD-impacted environment. The significance of sulfur-reducing bacteria (SRB), iron-oxidizing bacteria (FeOB), and plant growth promoting rhizobacteria (PGPRs) as key players in many passive and active systems dedicated to bioremediation and microbe-assisted phytoremediation is also elucidated

LC-MS Based Metabolomics Analysis of Potato (Solanum tuberosum L) Cultivars Irrigated with Quicklime Treated Acid Mine Drainage Water. Authors: Rabelani Munyai *, Maropeng Velry Nemutanzhela, David Mxolisi Modise. *Metabolites*.



Open Access Article

LC-MS Based Metabolomics Analysis of Potato (*Solanum tuberosum* L.) Cultivars Irrigated with Quicklime Treated Acid Mine Drainage Water

by Rabelani Munyai [1,*], Maropeng Vellry Raletsena [1] and David Mxolisi Modise [2]

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(This article belongs to the Special Issue **Advances in Metabolic Studies in Plant Extraction**)

Abstract

In water-scarce areas, the reuse of (un)treated acid mine drainage (AMD) water for crop irrigation has become a requirement, but it also carries a wide range of contaminants that can elicit the synthesis of diverse metabolites necessary for the survival of the plants. There is still a paucity of studies on the impact of quicklime treated-AMD water on the metabolite synthesis of potatoes. This study examined the effect of the irrigation of two potato cultivars (Marykies and Royal cultivars) with quicklime-treated AMD water on their metabolite profiles. A greenhouse study was conducted with five experimental treatments with different solution ratios, replicated three times in a completely randomized design. A total of 40 and 36 metabolites from Marykies and Royal cultivars which include amino acids, organic acids, and aromatic amines were identified, respectively. The results revealed elevation in the abundance of metabolites under the irrigation with treated AMD water for both cultivars with subtle variations. This will provide information on the primary metabolite shifst in potato that enhance their survival and growth under AMD conditions. However, more specific data on toxicity due to AMD irrigation would be required for a refined risk assessment. **View Full-Text**

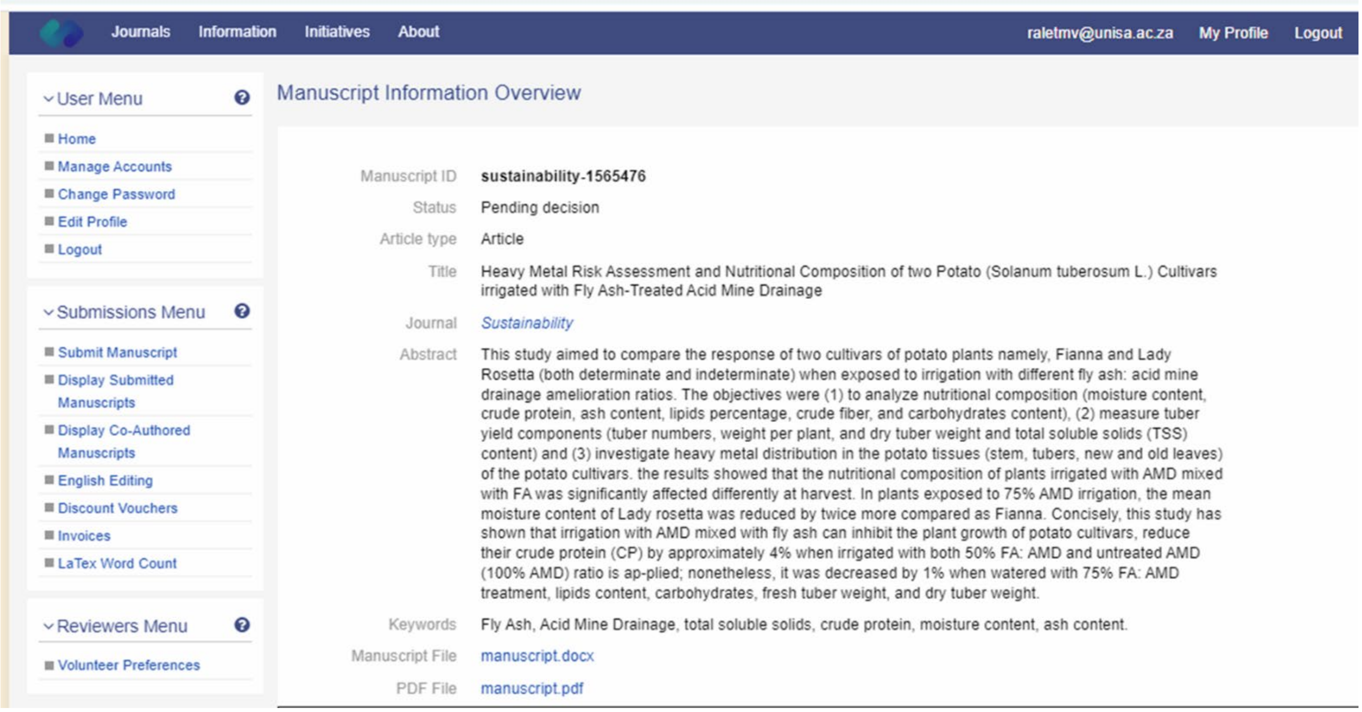
Keywords: **acid mine drainage**; **irrigation**; **metabolites**; **potato**; **quicklime**

▼ **Show Figures**

Figure 1

APPENDIX V: **Awaiting publication**

Heavy Metals Risk Assessment and Nutritional composition of Two Potato (Solanum tubersum L.) Cultivars irrigated with Fly Ash Treated Acid Mine Drainage. Author list: Maropeng Vellry Raletsena*, Rabelani Munyai, David Mxolisi Modise, Adugna Abdi Woldesemayat. *Sustainability*. 2022



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Article type	Article
Title	Heavy Metal Risk Assessment and Nutritional Composition of two Potato (Solanum tuberosum L.) Cultivars irrigated with Fly Ash-Treated Acid Mine Drainage
Journal	*Sustainability*
Abstract	This study aimed to compare the response of two cultivars of potato plants namely, Fianna and Lady Rosetta (both determinate and indeterminate) when exposed to irrigation with different fly ash: acid mine drainage amelioration ratios. The objectives were (1) to analyze nutritional composition (moisture content, crude protein, ash content, lipids percentage, crude fiber, and carbohydrates content), (2) measure tuber yield components (tuber numbers, weight per plant, and dry tuber weight and total soluble solids (TSS) content) and (3) investigate heavy metal distribution in the potato tissues (stem, tubers, new and old leaves) of the potato cultivars. the results showed that the nutritional composition of plants irrigated with AMD mixed with FA was significantly affected differently at harvest. In plants exposed to 75% AMD irrigation, the mean moisture content of Lady rosetta was reduced by twice more compared as Fianna. Concisely, this study has shown that irrigation with AMD mixed with fly ash can inhibit the plant growth of potato cultivars, reduce their crude protein (CP) by approximately 4% when irrigated with both 50% FA: AMD and untreated AMD (100% AMD) ratio is ap-plied; nonetheless, it was decreased by 1% when watered with 75% FA: AMD treatment, lipids content, carbohydrates, fresh tuber weight, and dry tuber weight.
Keywords	Fly Ash, Acid Mine Drainage, total soluble solids, crude protein, moisture content, ash content.
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APPENDIX VI Soil bacterial diversity taxonomic spectrum

Bacterial genera significantly associated with distinct bulk soil irrigated with AMD treated water representing a broad taxonomic spectrum. The generalized indicator value (IndVal.g) and corresponding p-values used to assess the predictive value of a taxon for each treatment category as implemented in *indicspecies* in R *(de Cáceres et al., 2010)* is given.

Genera	**IndVal.g**					**p-value**
	AMD	**A1G**	**A2G**	**AFQ**	**Control**	
Hydrogenophaga	0.530	-	-	-		0.0128*
Unclassified Erythrobacteraceae	0.532	-	-	-		0.0170*
Azoarcus	0.527	-	-	-		0.0179*
Blastococcus	0.522	-	-	-		0.0230*
Microbacterium	0.514	-	-	-		0.0296*
Rhodococcus	0.497	-	-	-		0.0354*
Parasegetibacter	0.487	-	-	-		0.0195*
Flavihumibacter	0.486	-	-	-		0.0062**
Rhizorhapis	0.486	-	-	-		0.0150*
Ornithinimicrobium	0.481	-	-	-		0.0150*
Rhizobium	0.446	-	-	-		0.0307*
Pseudoxanthomonas	0.440	-	-	-		0.0313*
Bosea	0.429	-	-	-		0.0458*
Azospirillum	0.400	-	-	-		0.0449*
Dyella	-	0.637	-	-		0.0035**
Rhodanobacter	-	0.602	-	-		0.0069**
Paenarthrobacter	-	0.516	-	-		0.0317*
Coxiella	-	-	0.522	-		0.0167*
Blastopirellula	-	-	0.504	-		0.0388*
0319-6G20_ge	-	-	0.502	-		0.0373*
Chlorogloeopsis	-	-	0.424	-		0.0500*
Myroides	-	-	-	-	0.614	0.0064**
Methylobacterium	-	-	-	-	0.545	0.0160*
Staphylococcus	-	-	-	-	0.440	0.0335*
Actinomyces	-	-		-	0.410	0.0066**
Lelliottia	-	-	-	-	0.401	0.0426*
Pseudarthrobacter	0.530	0.530				0.0356*
Fluviicola	0.498	0.498				0.0343*
Knoellia	0.489	0.489				0.0336*
Unclassified Micrococcaceae	0.487	0.487				0.0435*
Leifsonia		0.505	0.505			0.0227*
Unclassified Proteobacteria	0.562	0.562	0.562	0.562		0.0102*