Assessing the pollutant removal efficiency of a wetland as a polishing treatment for municipal wastewater

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Declaration

I, **Betty Refilwe Mphuthi**, do hereby declare that this dissertation submitted to the Vaal University of Technology for the fulfilment of Master of Technology (M. Tech) Degree in the Department of Biotechnology, Faculty of Applied and Computer Sciences, is my own independent work. This work has not been submitted before to any institution by me, or any other person in fulfilment of requirements for any qualification

Signature: *brmphuthi* Date:16-02-2021

Dedication

I would like to dedicate this thesis to my wonderful and loving family. I am so grateful for your love and support throughout this journey, thank you all for your inputs. I would have not achieved this if it was not for your love, patience and support. Your unwavering support and company encouraged me every step of the way. To my sons, Tshiamo and Lehlogonolo, thank you so much for your patience and understanding when I was busy and not available to chat and share in your “Aha” moments. To my brother, Promise Skhosana, I pray that God will guide and bless you in everything you do, thank you for your help and support

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Abstract

Pollution of aquatic systems by wastewater containing pathogens, heavy metals and high concentrations of nutrients is of great concern due the ecological risks they impose. The toxic effects of metals may occur even at low concentrations because of potential bio magnification in the food chain. Excessive nutrients cause algal blooms which depletes oxygen and prevents sunlight from penetrating into the water, thereby killing fish and other aquatic organisms. This study investigated the pollutant removal efficiency of a riparian wetland located in Sebokeng, Emfuleni local municipality, South Africa. The study was carried out to assess the water quality of a wetland located downstream of the Sebokeng wastewater treatment plant by monitoring and analysing the physico-chemical parameters which included pH, temperature, electrical conductivity, nutrient levels (nitrates, phosphates, nitrites) and heavy metals. The water samples were collected from the effluent discharge of the treatment plant, upstream and downstream of the wetland. Plant uptake of heavy metals in a riparian wetland, nitrification as well as denitrification processes have been historically recorded as the main processes that contribute to the high removal of pollutants in a wetland. The contaminant concentrations of the influent and the effluent were used to estimate the wetland efficiency in improving the water quality that passes through it and its potential effects on improving the quality of irrigation waters. The heavy metals of interest included Al, Cd, Cr, Cu, Fe, Pb, Mn and Zn. Most heavy metals within the wetland occurred at low concentrations (lower than detectable limits and within the discharge limits for irrigation purposes). The results indicate that the average removal efficiencies for Electrical Conductivity (EC), Total coliforms (TC), *E. coli*, BOD_5, COD, TSS, carbonate hardness, aluminium, iron, manganese, copper, nitrite, nitrate, sulfate and ortho-phosphate were 43 %, 51%, 85%, 60%, 61%, 61%, 21%, 67%, 52%, 51%, 83%, 56%, 89%, 49% and 54% respectively. The study showed that this wetland can provide up to 89% removal efficiency of pollutants. Of particular significance was the high pathogen and nutrient removal efficiency. A t-test was performed in order to determine the statistical significance of the wetland pollutant removal efficiencies. All p-values calculated were well below 0.05 and the removal efficiencies are therefore considered statistically significant. For this particular ecosystem the findings show that there is no great concern about metal pollution since most of the metals tested for were below the minimum limit for irrigation stipulated by the South African water regulation department (DWAF 1996a). Therefore, the wetland effluent water qualifies for both agriculture and landscape irrigation. Future considerations in choosing to use wetlands as a polishing facility for wastewater treatment systems are highlighted in the study.

Key words: Wetlands, Physical parameters, Metal removal efficiency, Nutrients, Eutrophication

Table of contents

List of Figures

List of tables

Abbreviations and Acronyms

APHA	American Public Health Association
BOD	Biological Oxygen Demand
BOD_5	Five-day Biological Oxygen Demand
bp	Base pairs
Cd	Cadmium
$CdCl_2$	Cadmium chloride
COD	Chemical Oxygen Demand
CW	Constructed Wetland
DO	Dissolved Oxygen
EC	Electrical Conductivity
EPA	Environmental Protection Authority
FWS	Free water surface
H_2O_2	Hydrogen peroxide
NCBI	National Centre for Biotechnology Information
NTU	Nephelometric Turbidity Unit
PCR	Polymerase Chain reaction
pH	Power of Hydrogen
PO_4	Phosphate
ppb	Parts Per billion
ppm	Parts Per Million
SO_4	Sulphate
SS	Suspended Solid
TDS	Total Dissolved Solid
TN	Total Nitrogen
TN:TP	Total Nitrogen: Total phosphate

N: P	Nitrogen: Phosphorus
NH_4^+	Ammonium
NO_2^-	Nitrite
NO_3	Nitrate
P	Phosphorus
TSS	Total Suspended Solids
WHO	World Health Organization
WWSP	Wastewater Stabilization Pond
^0C	Degree Celsius
Mg/l	Milligram per Litre
µS/cm	Micro-Siemens per centimetre
m	Meters
M	Molar
min	Minutes
ml	Millilitres
mmol/l	Millimole per litre
mM	Millimolar
mm	Millimetre
nm	Nanometres
OD	Optical density
Pb	Lead

Chapter 1: Introduction

1.1 Background

Water is fundamental for all life, without water no person, plant, animal or any living organism can survive. The water resources of South Africa are vital to the health and prosperity of its people, the preservation of its natural heritage and its economic development. South Africa is a dry country with a low average rainfall. Many areas in the country are facing water shortages, and some people do not have access to portable water. Daily activities by individuals and organisations have an impact on the quality of our rivers and streams, our groundwater and wetlands. The national Water Act provides a framework to protect water resources against over exploitation and to ensure that there is water for social and economic development as well as making sure there is water for the future. National Water Act 36 of 1998 stipulates ways in which water resources (rivers, streams, dams, wetlands and groundwater) must be used, managed, conserved, protected and controlled in an integrated manner**.** Water is a natural resource that belongs to all people in South Africa. The Act aims to protect, use, develop, conserve, manage and control water resources as a whole; and therefore rivers, dams, wetlands, the surrounding land, groundwater, as well as human activities that influence them, will be managed as one cycle. (NWA 1998). Security of water supply has become a key strategic issue as well as a driver for the continued sustainable economic growth and service delivery to the people of South Africa (Manders *et al.* 2009, Sershen *et al.* 2016).

Concerns about water shortage and pollution have received increased attention over the past few years, especially in developing countries. Adequate water needs to be made available for industrial and personal use, and contamination of water sources by the discharge of untreated wastewater from the mining, domestic and industrial sectors should be avoided where possible (Azarch 2011, Afzal *et al.* 2019). Major contaminants found in wastewater include biodegradable, volatile and recalcitrant organic compounds, toxic metals, suspended solids, plant nutrients (nitrogen and phosphorus), microbial pathogens and parasites. Higher concentrations of nutrients, suspended solids, oxygen-demanding compounds, and heavy metals in agricultural waste effluents pose a significant threat to surface and ground water quality (U.S.EPA 2004, You *et al*. 2019). The impact of pharmaceuticals, personal care products and endocrine disrupting compounds in natural systems has become an important issue (Boyd *et al*. 2004, Ebelle *et al.* 2017). These compounds are excreted into sewage systems and enter the aquatic environment with the discharge of untreated municipal wastewater. The discharge of nitrogen as nitrates and phosphorus as phosphates to inland rivers, lakes and dams causes massive growth of algae and plants due to eutrophication (Beaulieu *et. al*. 2019).

The leakage of pollutants may have significant negative impacts on the surrounding environment and threaten the ecosystem and public health. The proper treatment of wastewater before it is discharged into the environment will help to mitigate these damages. Undesirable levels of water quality not only impact negatively on irrigation, crop yields and quality, but also have an adverse impact on industrial water use. For example, should there be a deterioration of the water quality within the Grootdraai dam sub-system, more water must be provided to Eskom through the Vaal River Eastern Subsystem Augmentation Project (VRESAP) pipeline to ensure effective utilization of the cooling systems of their power stations which receive water from this substation. Furthermore, extra costs might be necessitated by bulk water suppliers such as Rand water that provides water to urban centres for domestic and industrial use. The Vaal river system is the most important bulk water supply system in the country supplying water to 60% of the country's economy and 45% of its population. Two of the major impacts dominating the Vaal catchment are water quality impacts and changes in the flow regime. Changes in the flow regime range from too little flow but the most severe impacts are too much flow and changes in the seasonality which mainly relate to transfers, releases, irrigation return flows, mining and urban runoff. The areas of highest water quality impact across the Vaal river catchment include Rietspruit whose quality is category D, largely due to extensive agricultural activities with highly elevated nutrients and salts (DWAF 1997b).

Taking advantage of alternative water sources is one possible response to the challenges of freshwater demand, water shortages and environmental protection. Raw water sources available include rainwater, sea and brackish water, grey water, and domestic/municipal wastewater. Among these, grey water represents the most profitable source in terms of its reliability, availability and raw water quality (Masi *et al.* 2016). Most of the conventional methods in practice for purification of wastewater and removing contaminants are costly and non-ecofriendly. Conventional technologies are also known to consume large amounts of energy and chemicals during the treatment process. Many different technologies for wastewater reclamation have been designed. However, they are generally based on highly optimized physical, chemical and microbial processes (Almuktar *et al*. 2018). In recent years there has been a shift in reclamation strategies for wastewater from high-tech to environmentally sound, sustainable, low-cost and effective technologies based on ecological principles, namely ecological technology. In many cases they offer a more holistic alternative to improve the environmental quality of water (Polprasert and Kittipongvises 2011).

Wastewater treatment works are a major contributing factor to the production of nutrient rich materials and heavy metals which end up being discharged into the aquatic ecosystem if sewage is not effectively treated. Such waste contains phosphorous, nitrates and nitrites. From a South African perspective, nutrient rich materials in wastewater treatment works (WWTW) alone are in higher concentration than in nonpoint sources. In South Africa, only 7.4% of wastewater treatment works (WWTW) were awarded the green drop certification. About 92.6% of the South African WWTW may be said to be noncompliant and their continued operation raises the risks of eutrophication in South African freshwater resources (Chamier *et al*. 2012, Griffin 2017).

Natural wetlands are known to be effective bio-filters and have been utilized for wastewater clarification since ancient times. Both natural wetlands and constructed wetlands clean and filter contaminated water, thus mitigating the effects of contaminants such as excess nutrient input into water bodies that contribute to hypoxic zones. The use of extensive systems, such as constructed wetlands or waste stabilization ponds has attracted considerable interest due to their better landscape integration as well as their low maintenance costs and the greater economic, environmental and social sustainability (Almuktar *et al.* 2018). Wetlands are even applied as a polishing system for the classical wastewater treatment system. Wetland systems are generally inexpensive compared to the high technology treatment systems which are expensive and require extensive maintenance. Wetlands have proven to be a very effective method for the treatment of municipal wastewater and this application of a wetland has led to the study of their use for other kinds of wastewater treatment. Acid mine drainage, agricultural wastewater and industrial wastewater, storm water runoff, landfill leachate as well as airport runoff, are all good candidates for remediation using constructed wetlands (Pang *et al.*2015; Almuktar *et al*. 2018).

A wetland is a natural treatment system in which physical, chemical, and biological processes occur when water, soil, plants, and microorganisms interact. They are considered natural treatment ecosystems designed to take advantage of natural processes to provide wastewater treatment. They treat sewage water using highly effective and ecologically sound principles that use plants, microbes, sunlight and gravity to transform wastewater into garden and reusable water. The water treatment mechanisms include physical filtration and sedimentation, biological uptake, transformation of nutrients by bacteria that are anaerobic (bacteria that flourish in the absence of oxygen) and aerobic (oxygen-needing bacteria), plant roots and metabolism, as well as chemical processes (precipitation, absorption and decomposition). Treating wastewater in semi- natural plant systems is a technique which can in principle be applied in natural wetlands such as marshes, moors and wet fields, in artificial

ponds and lagoons, and in constructed wetlands. The active reaction zone of wetlands is the root zone/rhizosphere (Almuktar *et al*. 2018).

The study area is a natural wetland with minimal current impacts including riverbank erosion, pollution, upstream sewage effluent, as well as wetland disturbances by human settlement (interference) and agricultural holdings. The wetland area falls under the ecological support areas of the SANBI BGIS conservation plan. The wetland is an un-channelled Valley Bottom wetland that provides ecological functions such as biodiversity support due to the presence of the riparian habitat. These wetland types are important for flood attenuation due to their vegetation cover which is of hydrological importance. They are also generally important for their slow release of water during low rainfall periods, which is significant in areas where livestock grazing is a source of livelihood. Direct human benefits associated with this wetland include livestock farming, provision of water for human use; provision of harvestable resources and flood attenuation (Sazi Environmental Consulting 2016).

Vegetation plays a crucial role in the pollutant removal process; hence, the plants must be able to adapt to the conditions of the wetlands and must be suitable for the removal of pollutants. Plant coverage is key in the removal of organic matter, metal pollutants, and pathogens in wetlands. The natural ability of certain plants to bio-accumulate, degrade or render harmless contaminants such as metals, pesticides, organic waste, solvents, crude oil and its derivatives in water is known as phytoremediation. Root, stem, and leaves of vegetation act as a substrate upon which microorganisms can grow and break down organic matter and metal pollutants by one of the five actions: rhizo-filtration, phyto-extraction, phyto-stabilization, rhizosphere bioremediation, or phyto-transformation (Ghimire *et al.* 2019). Generally, the plants used in wetlands are macrophytes such as *Phragmites australis* and species of the *Typha, Scirpus,* and *Cyperus* genera. *Typha capensis* is the dominant species of the study area which is a riparian wetland downstream of a wastewater treatment plant. *T. capensis* is predominant within the permanent zone of the wetland study area. It is commonly seen growing in swampy areas throughout Southern Africa where its rapid growth has been characterized as invasive. *T. capensis* common names include bulrush (English name); papkuil matjiesriet (Afrikaans); ibhuma (Zulu, Swazi); ingcongolo (Xhosa); and motsitla (Sesotho). *T. capensis* stems are erect and terminate in dense, cylindrical flower- spikes. The strap-shaped leaves are long, bluish-grey to light green and have parallel veins. The inflorescence is a dense spike of closely packed yellow flowers that mature to brown. This plant is adapted to muddy and wet conditions and its strong fibrous roots that arise from the rhizomes help anchor the plant so that it can withstand strong winds without being swept

away in the water. Owing to variation in the water content of marsh habitats, the rhizome structure may show both hydric (water) and xeric (dry) adaptations (SANBI 2007).

Bacteria are also an integral part in the phytoremediation process. Bacteria use many of the toxic and complex chemicals as an energy source by converting them into simple and less toxic compounds. This process helps them to sustain their growth under unfavourable conditions e.g., extremes of toxicity. In phytoremediation, organic pollutants are mineralized mostly by plant-associated microbial populations. It has been proposed that the remediation potential of plants is somewhat dependent on the number of bacteria in their surrounding environment. Bacteria can regulate plant growth by N_2-fixation, solubilization of various essential nutrients, production of growth hormones and amelioration of biotic and abiotic stress. In contrast to terrestrial plants that interact with other organisms through certain chemical signals, macrophytes in aquatic systems rely on oxygen, toxic compounds, and organic carbon in water bodies for the proliferation of microbes (Khadeeja *et al.* 2018).

1.2 Problem statement

Phosphates (PO_4^{3-}) and nitrates (NO_3^-) are the main contents of nutrient-rich sediments as well as wastewater material from wastewater treatment works (WWTW). The sources of phosphorus and nitrogen are both point and non-point sources. Point sources may include industrial pollutants through pipelines and non-point sources may include agricultural areas where pollutants are carried away by runoff or infiltration into groundwater. Industrial and agricultural pollutants common in South Africa are: agricultural fertilizers on massive irrigation lands, silt, toxic metals, litter, hot water, and pesticides. However, some of the most common pollutants come from urban wastewater, particularly from informal settlements which lack sewage and water purification facilities. Wetlands are being considered increasingly important for wastewater treatment because of the ability of many wetland plants to absorb large amounts of nutrients and a variety of toxic substances including heavy metals. They have also shown the ability to reduce coliform and *E. coli* concentrations necessary for the effective treatment of urban wastewater.

1.3 Aim of the study

The aim of the study is to evaluate the pollutant removal efficiency of a wetland as a polishing treatment for municipal wastewater, with a focus on the rhizobacteria associated with *Typha capensis.*

1.4 Objectives of the study

1. To evaluate the wetland removal efficiency for the treatment of municipal wastewater with organic load (soluble and non-soluble), nutrient (nitrogen and phosphorus), total suspended solids, inorganics (heavy metals as well as nitrogen and phosphorous compounds), and total coliforms and *E. coli* (an indicator of faecal contamination).

2. To isolate and identify the rhizobacteria associated with *Typha capensis* based on the 16s rDNA sequence data.

3. To determine the heavy metal tolerance of rhizobacteria isolated from *Typha capensis*

Chapter 2: Literature review

2.1 Wetland

In many countries, the term “wetland” is defined more restrictively than in the Ramsar definition, usually with specific reference to the presence of saturated soils and/or hydrophytic vegetation. The South African National Water Act of 1998 defines a wetland as a land that is transitional between terrestrial and aquatic systems where the water table is usually at or near the surface, or land that is periodically covered with shallow water, and which in normal circumstances supports or would support vegetation typically adapted to life in saturated soil. In a South African legal context, the term “watercourse” is often used rather than the terms wetland, or river. The National Water Act, 1998 (Act No. 36 of 1998) includes wetlands and rivers into the definition of the term “watercourse”.

2.2 Wetland Classification

One of the first widely used wetland classification systems devised by Cowardin *et al.* (1979) categorized wetlands into marine (coastal wetlands); estuarine (including deltas, tidal marshes, and mangrove swamps); lacustarine (lakes); riverine (wetlands along rivers and streams); palustrines (marshes, swamps and bogs) based on their hydrological, ecological and geological characteristics. Ramsar convention defined “wetlands” as areas of marsh, fern, peat land or water, whether natural or artificial, permanent or temporary, with water that is static or flowing, fresh, brackish or salty, including areas of marine water the depth of which at low tide does not exceed six meters (Lane *et al.* 2018). A major difference between constructed and natural wetlands is that the hydraulic loadings of constructed wetlands are consistently managed, as a result they can treat wastewater more efficiently than natural wetlands, whereas natural wetlands have variable water flow based on precipitation, climate and seasonality (Almuktar *et al*. 2018).

2.2.1 Natural Wetlands

Natural wetlands are effective as wastewater treatment processes for a number of reasons. Wetlands support a large and diverse population of bacteria which grow on the submerged roots and stems of aquatic plants and are of particular importance in the removal of biological oxygen demand (BOD) from wastewater. In addition to that, the quiescent water conditions of a wetland are conducive to the sedimentation of wastewater solids. Natural wetland systems are typically characterised by emergent aquatic vegetation such as cattails (*Typha*), rushes (*Scirpus*) and reeds (*Phragmites*). They can also contain some of the floating and submerged plant species as well as phreatophytes (plants whose roots extend to the ground-water table or saturated soil area immediately above it). In this particular wetland as shown by figure 1, *Typha capensis* is the main vegetation which was predominant within the permanent zone of the wetland.

Figure 1: Riparian wetland located in Sebokeng, Emfuleni local municipality, South Africa (Picture taken by B.R. Mphuthi)

Wetlands have different characteristics. The most common feature of all wetlands is that the water table (the groundwater level) is very near to the soil surface or shallow water covers the surface for at least part of the year. The main characteristics of a wetland are determined by the combination of the salinity of the water in the wetland, the soil type and the plants and animals living in the wetland. Because of the high variability of the conditions, and because of the different needs for distinguishing among different types of wetlands, so far, there is no single wetland classification system that would account for the manifold aspects of this specific ecosystem type. As transitional environments, wetlands exist under a variety of conditions and as a result a variety of wetland types are produced. There are five main types of natural wetland systems:

1. Marine - coastal and not influenced by river flows (e. g shorelines and coral reefs).
2. Estuarine – where rivers meet the sea and salinity levels are intermediate between salt and freshwater (e. g mangroves and mudflats).
3. Riverine – land periodically inundated with river overtopping (e. g flooded forests and floodplains).
4. Palustrine – where there is more or less permanent water cover (e. g freshwater marshes).
5. Lacustrine – area of permanent water cover with little flow, example is a pond (Tiner 2017).

Another classification system which is dependent mostly on a combination of the above conditions leads to the formation of a particular soil type and distinguishes wetlands into two major types– mineral soil and organic soil wetlands:

a. Mineral Soil Wetlands

The dominant vegetation is the key factor that distinguishes the two major types of mineral soil wetlands: grasses dominate marshes, while trees dominate swamps. Both marshes and swamps may be freshwater or saltwater (Tiner 2017).

- **Marsh** – a type of wetland ecosystem characterized by poorly drained mineral soils and by plant life dominated by grasses. Marshes are common at the mouths of rivers, especially where extensive deltas have been built. The marsh plants slow down the flow of water and allow for the nutrient enriched sediments to be deposited, thus providing conditions for the further development of the marsh (Tiner 2017).

- **Swamp** – a wetland ecosystem characterized by mineral soils with poor drainage and by plant life dominated by trees. Swamps are found throughout the world, most often in low-lying regions (with poor drainage) next to rivers, which supply the swamp with water. Some swamps develop from marshes that slowly fill in, allowing trees and woody shrubs to grow (Tiner 2017).

b. Organic Soil Wetlands

These wetlands are generally referred to as "peatlands" in recognition of their common ability to form peat (organic soil produced by the accumulation of plant material). There are two major types of peatlands – bogs and fens, both of which occur in similar climatic and geographic regions (Tiner 2017).

- **Bog** – a type of wetland ecosystem characterized by wet, spongy, poorly drained peaty soil, dominated by the growth of bog mosses, *Sphagnum*, and heaths (particularly *Chamaedaphne*). Bogs are usually acid areas, frequently surrounding a body of open water. Bogs receive water exclusively from rainfall (Tiner 2017).

- **Fen** – a type of wetland ecosystem characterized by peaty soil, dominated by grasslike plants, grasses, sedges, and reeds. Fens are alkaline rather than acid areas, receiving water mostly from surface and groundwater sources (Tiner 2017).

2.2.2 Constructed Wetlands/Engineered Wetlands

Constructed wetlands (CWs) mimic the simultaneous physical, chemical, and biological processes occurring in natural wetlands for wastewater treatment purposes (Wu *et al.* 2018). Constructed wetlands are engineered systems that have been designed and constructed to utilize the natural processes involving wetland vegetation, soils, and the associated microbial assemblages to assist in

treating wastewaters. They are designed to take advantage of many of the same processes that occur in natural wetlands but do so within a more controlled environment. Constructed wetlands are mainly utilized for secondary or tertiary treatment systems, as primary treatment systems usually involve technical treatment plants or settling tanks. Constructed wetlands are used for further filtration, sedimentation, and biological processes to minimize contaminants entering the receiving water bodies with the effluent. Compared to conventional wastewater treatment plant systems, constructed wetlands require little maintenance and are cost effective (Almuktar *et al.* 2018).

Due to the fact that constructed wetlands emulate natural wetlands, they are robust ecosystems that have the ability to mitigate fluctuations in water flow in a sustainable manner. Constructed wetlands not only treat wastewater, but they can also provide a variety of purposes ranging from aesthetics to the creation of wildlife habitats and flood control (Wang *et al*. 2018). Riggio *et al.* (2018) has demonstrated that constructed wetlands can be a cost-effective option when compared to a traditional treatment process for wastewater reuse. Constructed wetlands are classified according to the water flow regime, and according to the type of macrophytes as well as flow direction (Almuktar *et al*. 2018). Any wetland in which the surface water is exposed to the atmosphere, is classified as a free water surface system, and a system where water is designed to flow through granular media, without coming into contact with the atmosphere is classified as a subsurface wetland. Free water surface (FWS) wetlands are sub-classified according to their dominant type of vegetation: Emergent macrophyte based wetland, free-floating macrophyte, or submerged macrophyte wetland (Vymazal 2018).

2.2.3 Artificial wetlands

Water bodies that mimic wetland characteristics may form in areas where an artificial water source supplies one area of land for an extended period of time. Should that artificial water source be remedied or cut-off; the wetland will also cease to exist. Water sources in these areas may include a burst water pipe, a dripping tap from informal settlements, poorly designed stormwater channels, or illegal draining of water onto the area. These types of artificial wetland systems differ from properly constructed wetlands. This is due to the fact that constructed wetlands are designed for an intended purpose, whereas artificial wetlands described above are often random. There is currently no classification system for artificial wetlands. However, wetlands that are artificially formed can be categorised based on the topography and function they provide in the environment (Sazi Environmental Consulting 2016).

2.3 Sources of water pollution

Various categories of substances are being introduced by anthropogenic activities which give rise to water pollution. Organic and inorganic substances, pathogenic organisms, plant nutrients and oxygen demanding substances are the common types of pollutants (Ali *et. al*. 2017). A pollutant is a substance that enters the environment and elevates the "natural" background concentration of that substance. Point source pollution is pollution originating from a single, identifiable source, such as a discharge pipeline from an industry or a wastewater treatment works. The most common point source surface water pollutants are high temperature discharges, microorganisms (such as bacteria and viruses), and nutrients (such as nitrogen and phosphorus). In contrast, pollution that does not originate from a single point or source is called nonpoint pollution. Nonpoint source pollution is contamination affecting a water resource from diffuse sources, such as polluted runoff from agricultural areas which drain into a river, urban storm water runoff, and runoff from informal un-serviced areas such as the informal settlement. Nonpoint source pollution is usually found to spread over a large area and it is often difficult to trace the exact origin of these pollutants as they result from a wide variety of human activities. The most common nonpoint source pollutants are sediments, nutrients, microorganisms and toxins (U.S.EPA 2004).

The waste disposed of by domestic households and industry is conveyed to wastewater treatment works by means of pipes (sewers). The arrangement of sewers is known as the sewerage system and everything that flows in the sewers is sewage (Scholz 2016). The impact of pharmaceuticals and personal care products and endocrine disrupting compounds in natural systems has become an important issue. These compounds are excreted into sewage systems and enter the aquatic environment with the discharge of untreated municipal wastewater. Impacts from pharmaceutical and personal care products are most pronounced in smaller streams where effluent discharge makes up a large proportion of the flow. Conventional wastewater treatment plants have limited ability to remove pharmaceutical and personal care products due to short retention times while natural and constructed wetlands can promote removal through a number of mechanisms, including photolysis, plant uptake, and microbial degradation (Hunter *et al*. 2018).

With regard to water quality, the primary constituents of interest in treated municipal effluent are nitrogen, phosphorus, and suspended solids, which includes both mineral sediments and particulate organic matter. The basic principle underlying wetland assimilation of these constituents is that the rate of effluent application must balance the rate of removal. The primary mechanisms by which this balance is achieved are physical settling and filtration, chemical precipitation and adsorption, and

biological processes that result in burial, storage in vegetation, and denitrification. The nutrient component of municipal effluent increases wetland vegetation productivity (Rehman *et al*. 2018), which helps offset regional subsidence by increasing organic matter deposition on the wetland surface, thereby decreasing flooding duration and producing a positive feedback loop of increased ecosystem vigour and resilience. Finally, nutrient rich municipal effluent addition promotes increased rates of primary production and soil accretion, an important part of any restoration plan for wetlands (Hunter *et al*. 2018).

2.4 Water quality parameters

A review of the surface water status of selected water quality variables was undertaken in 2010 by the Department of Water Affairs (DWA 2010). Six variables were selected to serve as indicators of the general water quality status, as they provide insight into the salinity and eutrophication status, mining related impacts, and variability of the country's water resources. These variables were selected on the following reasoning:

- Electrical conductivity (EC): to provide an indication of salinization of water resources.
- Orthophosphate (PO_4-P): as an indicator of the nutrient levels in water resources.
- Sulphate (SO_4) (mg/l): as an indicator of mining impacts.
- Chloride (Cl): as an indicator of agricultural impacts, sewage effluent discharges, and industrial impacts.
- Ammonia (NH_3): as an indicator of toxicity.
- pH: as an indicator for mining impacts as well as natural variability.

2.4.1. Temperature and pH

Besides nutrients, the growth rate of microorganisms is also heavily reliant on temperature. Temperature in the surrounding environment directly affects the metabolic rate of organisms within that environment because their enzyme activity depends on the temperature of the environment. Biological activities generally double for every 10 ºC increase in temperature within a given growth range for each organism. Each organism has a minimum temperature, defined as the point below which the organism cannot grow; an optimum temperature range, where enzymatic reactions happen at their greatest possible rates; and a maximum temperature, above which microorganisms can no longer grow due to denaturation of proteins. There are four types of organisms which can be defined depending on their optimal temperature range, namely: psychrophilic (optimal temperature at 15 ºC or lower), mesophilic (optimal temperature between 20-45ºC), thermophilic (optimal temperature between 45- 80 ºC) and hyper-thermophilic which means optimal temperature at 80 ºC or greater (Vymazal 2018).

All life processes leading to growth are catalysed by the enzymes present in the cell. The activity of enzymes is influenced by temperature and therefore temperature is able to influence growth. Temperature impacts most biochemical processes, including those involved in the treatment of pollutants in surface-flow constructed wetlands. Temperature has a major impact on microbiological process rates and plant growth. Nitrogen removal is almost completely inhibited at temperatures below 4 ºC. Temperature factors for biochemical oxygen demand, suspended solids, total phosphorus and faecal coliforms are given as 1.0, meaning removal of these variables is not temperature dependent. This can be explained by the fact that most related processes are physical or chemical in nature and not (micro) biological. Total Nitrogen on the contrary has a temperature factor of 1.05, meaning that the removal efficiency is lowered by 39% when the temperature decreases from 20 ºC to 10 ºC (Vymazal 2018).

pH is a logarithmic expression of the hydrogen ion concentration in water and reflects the degree of acidity (pH less than 7) or alkalinity (pH greater than 7) of the water. pH is the measurement of the activity of hydrogen-ions (H^+) in a liquid solution. In layman's language, pH indicates whether the water is sour (pH <7), or soapy (pH >7) to the taste. The pH value is crucial for all chemical reactions. Some reactions occur only in acidic environments, while others proceed at basic or neutral pH values. The pH of most unpolluted water sources is approximately 6,5 – 8,5. Most fresh waters, in South Africa, are relatively well buffered and more or less neutral, with pH ranging between 6 and 8. pH values below 6,5 may be found wherever acidification processes occur, the most dramatic being that found in acid mine drainage where pH values as low as 3,0 may be found. Alkalanisation processes, such as for example the exposure of water to lime or other alkalis may raise the pH above 8,5. Effects caused by pH may either be direct or indirect. The direct effects includes irritation or burning of mucous membranes by extremes of pH. The indirect effects are a consequence of the health effects of corrosion products formed during cooking or from distribution pipes most commonly occurring at acidic pH values. The pH is affected by factors such as temperature, the concentrations of inorganic and organic ions, and biological activity. The pH may also affect the availability and toxicity of constituents such as trace metals, non-metallic ions such as ammonium, and essential elements such as selenium.Gradual reductions in pH may result in a change in community structure, with acid-tolerant organisms replacing less tolerant organisms. Streams with acidic pH values have different periphyton (micro flora and fauna living on solid surfaces) communities and lower overall production compared with less acidic streams. The discharge of acid wastes into water containing bicarbonate alkalinity results in the formation of free carbon dioxide. If the water is alkaline, free CO may be liberated and be toxic to fish even though the pH does not drop to a level normally considered toxic (DWAF 1996).

2.4.2 Electrical Conductivity and Total dissolved solids/salts

Water quality is usually measured by the total dissolved solids (TDS) or the electrical conductivity of the water (EC). A close relationship exists between the TDS of the water and the EC. Since Electrical conductivity is much easier to measure than total dissolved solids, it is used as an estimate of the TDS concentration. The following relationship can be used: 6.5 x EC (mS/m) = TDS (mg/l) (DWAF, 1996). Electrical Conductivity refers to a substance's ability to hold an electrical current. Units for conductivity are measured in microsiemens per centimeter (uS/cm) or millisiemens per meter (mS/m). This ability to conduct electricity is as a result of the presence of ions such as carbonate, bicarbonate, chloride, sulphate, nitrate, sodium, potassium, calcium and magnesium, in water. All of which carry an electrical charge. Many organic compounds dissolved in water do not dissociate into ions (ionize), and consequently they do not affect the electrical conductivity. Pure water cannot hold an electric charge but water that contains minerals and salts can. Therefore, electrical conductivity is related to the amount of salts and minerals in the water. The salt amount in water is known as total dissolved solids. This is measured in parts per million which can also be converted to mg/l. TDS is made up of inorganic salts, as well as a small amount of organic matter. Common inorganic salts that can be found in water include calcium, magnesium, potassium and sodium, which are all cations, and carbonates, nitrates, bicarbonates, chlorides and sulphates, which are all anions. The total dissolved solids concentration Is a measure of the quantity of all the compounds dissolved in water that carry an electrical charge (WHO 1996).

Total Dissolved Solids (TDS) is a measure of the material in water smaller than 2 microns. It is basically the sum of all minerals, metals, and salts dissolved in the water and is a good indicator of the water quality. While TDS is not considered a primary pollutant, increased concentrations of dissolved solids can also have technical effects as dissolved solids can produce hard water, which reduces efficiency of water filters, leaves deposits and films on fixtures, and on the insides of hot water pipes and boilers, and can also cause aesthetic problems such as a bitter or salty taste. Soaps and detergents do not produce as much lather with hard water as with soft water. Water with a high TDS concentration may indicate elevated levels of ions that pose a health concern, such as aluminium, arsenic, copper, lead, nitrate, and others. These minerals can originate from several sources, both natural and as a result of human activities. Mineral springs contain water with high levels of dissolved solids because the water has flowed through a region where the rocks have a high salt content. These minerals can also come from human activities. Agricultural and urban runoff can carry excess minerals into water sources, as can wastewater discharges, industrial wastewater and salt that is used to de-ice roads (WHO 1996).

2.4.3 Phosphorus and Nitrogen

The two most important nutrients in wastewater are nitrogen and phosphorus as they are both needed for cell growth. Nitrogen and phosphorus are nutrients that are natural parts of the aquatic ecosystems. Nitrogen and phosphorus support the growth of algae and aquatic plants, which provide food and habitat for fish, shellfish and smaller organisms that live in water. Nitrogen is the most abundant element in the air we breathe. Nitrogen is a growth limiting nutrient obtained from organic matter (proteins, amino acids, DNA) and inorganic metabolic waste. Nitrogen is used in protein synthesis while phosphorus is used for cell energy storage. Phosphorus can exist in the form of orthophosphate in water while nitrogen can exist in the form of ammonia, nitrate and nitrite. Nitrogen and phosphorus cycle in a wetland is shown in figure 2 below. Organic nitrogen is associated with cell detritus and volatile suspended solids. Free ammoniacal nitrogen (NH_3-N) results from decay of organic nitrogen, while nitrite- nitrogen (NO_2-N) is formed in the first step of the nitrification process (Scholz 2016; Almuktar *et al*. 2018).

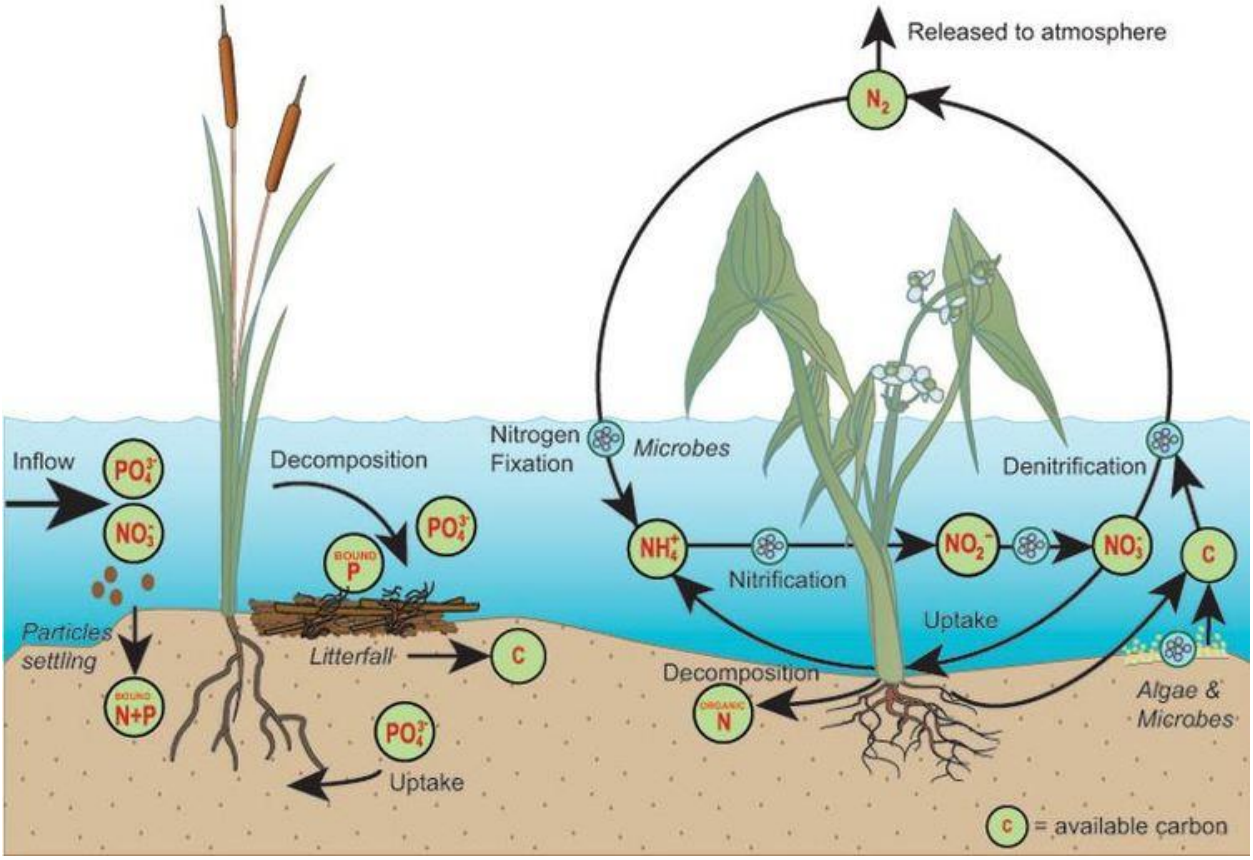

Figure 2: Nitrogen and phosphorus cycle in a wetland (Kadlec and Knight 1996)

Generally, in drinking water, nitrate is not toxic to human health, unless high concentrations in the body may be transformed from nitrate to nitrite. Nitrate, the principal nitrogen-bearing constituent of groundwater, is found mainly in agricultural regions because of the widespread application of synthetic fertilizers and animal manure to agricultural land. Nitrate sources include fertilizers and manure; decayed vegetable matter; animal feedlots; municipal wastewater and sludge disposal to land; industrial discharges; leachates from refuse dumps; septic systems and N-fixation from the atmosphere by bacteria and lightning. Excess nitrogen promotes nitrate leaching to groundwater that, when entering surface water, affects the aquatic environment, through eutrophication. Moreover, nitrate is a danger to human health, especially for pregnant women and children, posing a serious threat to drinking water supplies. Within the wastewater treatment the elimination of nitrogen is an essential element, because of its high impact on the environment. The elimination is done through the microbiological process of nitrification and denitrification. Nitrification is the oxidation of ammonium (nitrogen valency of -3) through nitrate to nitrite (nitrogen valency of +5). Nitrate- nitrogen (NO-N) results from the second and final stage in the nitrification process. The denitrification is a reduction process and can be seen as the inversive process of nitrification. The significant difference is that the reduction process does not take place at the stage of ammonium but at the stage of elemental nitrogen. This way, the unwanted nutrient nitrogen in the treated wastewater is eliminated by being released into the atmosphere as gas (Scholz 2016).

Nitrite is a toxic salt which disrupts the transport of oxygen in the blood by disrupting the haemoglobin to methaemoglobin transformation. This causes stomach pains and nausea for adults and causes blue baby syndrome and blood oxygen deficiency for infants. The dangers from nitrates arise from the fact that they are partially converted to nitrites by bacteria in the body. Tertiary conversion products of nitrate in the human body (from amines and nitrite) can be N-nitroso compounds, which are classified as carcinogenic. Some bacteria can reduce nitrogen gas into ammonia via a process called nitrogen fixation. Nitrogen is measured by organic nitrogen and ammoniacal nitrogen. Nitrate nitrogen (NO_3-N) is difficult to be assimilated by microorganisms for their growth (Scholz 2016).

Inorganic and organic phosphorus compounds are now found in almost all municipal and industrial wastewaters. They are predominantly present in the form of ortho-, poly or organo-phosphates. Together with nitrogen and potassium compounds, phosphates are among the most important fertilizers for adequate plant growth. In addition, phosphates are added as detergent additives for water softening and as preservatives in foodstuffs, since they inhibit growth of fungi and bacteria. However, their use as a water softener is declining since high phosphate levels in waters lead to over-

fertilization. The phosphate content of surface water determines its trophic status. Very high phosphate levels lead to eutrophication (over-fertilization: increased growth of algae and aquatic plants) of rivers and lakes and can ultimately lead to the death of plants and fish. Phosphates play an important role for humans in bone formation in the form of calcium phosphate and are also important in energy metabolism. However, as little phosphate as possible should be present in drinking water, since excessive amounts can lead to digestive upset and are suspected of triggering kidney problems (Scholz 2016).

2.4.4 Sulphate

A high concentration of sulphate has a laxative effect, and this effect increases when sulphate is consumed with magnesium. Gaseous and dissolved sulphides cause physical, chemical, and biological constraints. Sulphides cause scaling and corrosion in industrial water supplies which may lead to process failure and increased chemical oxygen demand in wastewater effluent. Until recently, biological treatment of sulphate rich wastewater was rather unpopular because of the production of H_2S. Sulphate can be reduced by bacteria competing with syntropic acetogens for benzoate as a substrate, converting benzoate either into acetate or directly into bicarbonate. Sulphate can also be reduced by homoacetogenic group of bacteria competing with methanogens for acetate and hydrogen (Scholz 2016).

2.4.5 Biochemical Oxygen Demand, Chemical Oxygen Demand, and Dissolved Oxygen

All living organisms depend upon oxygen to maintain the metabolic processes that produce energy for growth and reproduction. Dissolved oxygen is important in precipitation and dissolution of inorganic substances in water. Oxygen in wetland systems is important for heterotrophic bacterial oxidation and growth. It is an essential component for many wetland pollutant removal processes, especially nitrification, decomposition of organic matter, and other biological mediated processes. It enters wetlands via water inflows or by diffusion on the water surface when the surface is turbulent. Oxygen is also produced photo-synthetically by algae. Plants release oxygen into the water by root exudation into the root zone of sediments. Many emergent plants have hollow stems to allow for the passage of oxygen to their root tissues. The oxygen demand processes in wetlands include sediment-litter oxygen demand (decomposition of detritus), respiration (plants/animals), dissolved carbonaceous BOD, and dissolved nitrogen that utilizes oxygen through nitrification processes (Vymazal 2018).

Gaseous oxygen (O) from the atmosphere dissolves in water and is also generated during photosynthesis by aquatic plants and phytoplankton. Oxygen is moderately soluble in water. Equilibrium solubility, termed the saturation solubility, varies non-linearly with temperature, salinity and atmospheric pressure, and with other site-specific chemical and physical factors. The maintenance of

adequate dissolved oxygen (DO) concentrations is critical for the survival and functioning of the aquatic biota because it is required for the respiration of all aerobic organisms. Therefore, the DO concentration provides a useful measure of the health of an aquatic ecosystem. Measurement of the biochemical oxygen demand (BOD) or the chemical oxygen demand (COD) are inappropriate for aquatic ecosystems but are useful for determining water quality requirements of effluents discharged into aquatic systems, in order to limit their impact. In unpolluted surface waters, dissolved oxygen concentrations are usually close to saturation (Scholz 2016).

There is a natural variation in dissolved oxygen associated with the 24 h cycle of photosynthesis and respiration by aquatic biota. Concentrations decline through the night to a minimum near dawn, then rise to a maximum by mid-afternoon. Seasonal variations arise from changes in temperature and biological productivity. Reduction in the concentration of dissolved oxygen can be caused by several factors:

1. Re-suspension of anoxic sediments, as a result of river floods or dredging activities.
2. Turnover or release of anoxic bottom water from a deep lake or reservoir.
3. The presence of oxidizable organic matter, either of natural origin (detritus) or originating in waste discharges, can lead to reduction in the concentration of dissolved oxygen in surface waters.

The potential for organic wastes to deplete oxygen is commonly measured as biochemical oxygen demand (BOD) and chemical oxygen demand (COD). The COD is used as a routine measurement for effluents and is a measure of the amount of oxygen likely to be used in the degradation of organic waste. However, in aquatic ecosystems it is unlikely that all organic matter will be fully oxidized. The amount of suspended material in the water affects the saturation concentration of dissolved oxygen, either chemically, through the oxygen-scavenging attributes of the suspended particles, or physically through reduction of the volume of water available for solution (Scholz 2016).

The oxygen concentration decreases with depth and distance from the water inflow into the wetland. It is typically high at the surface, grading to very low in the sediment –water interface. Because wetlands are associated with waterlogged soils, the concentration of oxygen within sediments and the overlying water is of critical importance. The rate of oxygen diffusion into water and sediment is slow, and this leads to near-anaerobic sediments within most wetlands. The lack of oxygen in such conditions affects the aerobic respiration of plant roots and influences plant nutrient availability. However, wetland plants have consequently evolved to be able to exist in anaerobic soils. While deeper sediments are generally anoxic, a thin layer of oxidized soil usually exists at the soil-water interface. The oxidized layer is important because it permits the oxidized forms of prevailing ions to

exist. Organic matter discharged to rivers exerts a demand on the dissolved oxygen concentration available in the water as it decomposes. This can have a detrimental effect on aquatic biota if the dissolved oxygen drops to low concentrations. Dissolved oxygen and indicators of organic matter, such as BOD and COD should be routinely monitored (Scholz 2016).

The chemical oxygen demand (COD) is one of the most important parameters for the assessment of industrial and municipal wastewater. As a cumulative parameter, COD determines all chemically oxidizable components present in water. Hence, this includes not only biodegradable substances (as in the BOD_5), but also chemical compounds that cannot be determined by biological oxidation (e.g., nitrogen compounds such as nitrites). The COD indicates the contamination of a water sample. It is therefore also used as an evaluation parameter to determine pollution units in wastewater effluent/discharges. It has also the advantage of a faster availability of results as compared to BOD. On the other hand, BOD is a bioassay test, involving measurement of oxygen consumed by micro-organisms while stabilizing biologically decomposable organic matter under aerobic conditions. The COD test oxidizes material that microorganisms cannot metabolize in 5 days or that are toxic to microorganisms. If COD is much greater than BOD in raw wastewater, then the waste is not readily biodegradable, and it may be toxic to the microorganisms. If COD is similar to BOD, then the waste is readily biodegradable (Scholz 2016).

2.4.6 Pathogens

Wastewater of different kinds, especially municipal wastewater, contains high levels of pathogens, which when mixed with lake or river water poses a threat to human health. Pathogens such as bacteria, protozoa, helminths, and viruses are efficiently removed in wetlands (Arden and Ma 2018), but the performance is affected by the configuration of the wetland. Plant coverage and hydraulic retention time also play a key role in coliform reduction efficiency in wetlands. Macrophytes have shown to remove more than 95% of *E. coli*, *Enterococcus faecalis*, and *Enterococcus faesium* in a winter tolerant macrophyte-dominated artificial wetland system (Donde *et al.*2018). Adrados *et al.* (2018) investigated the removal efficiency of *E. coli,* total coliforms (TC) and intestinal enterococci in a vertical flow constructed wetland and a horizontal flow constructed wetland and reported that the pathogen removal rates were higher (Latrach *et al*. 2018).

Coliform bacteria include genera that originate in faeces (e.g., *Escherichia*) as well as genera that are not of faecal origin (*Enterobacter, Klebsiella, Citrobacter*). The presence of faecal coliforms in water may not be directly harmful and does not necessarily indicate the presence of faeces. In general, increased levels of faecal coliforms provide a warning of failure in water treatment, a break in the integrity of the distribution system, and possible contamination with pathogens. When levels are high

there may be an elevated risk of waterborne gastroenteritis. The presence of faecal coliforms in aquatic environments may indicate that the water has been contaminated with the faecal material of humans or other animals. Faecal coliform bacteria can enter rivers through direct discharge of waste from mammals and birds, from agricultural and storm runoff, and from human sewage. Failing home septic systems can allow coliforms in the effluent to flow into the water table, aquifers, drainage ditches and nearby surface waters. Sewage connections that are connected to storm drain pipes can also allow human sewage into surface waters. Some older industrial cities use a combined sewer system to handle waste. A combined sewer carries both domestic sewage and storm water. During high rainfall periods, a combined sewer can become overloaded and overflow to a nearby stream or river, bypassing treatment (Latrach *et al.* 2018).

2.4.7 Heavy metals

Heavy metals occur naturally in soil, usually at relatively low concentrations, as a result of weathering and other pedogenic processes acting on the rock fragments on which soils develop. The heavy metal concentrations inherited from soil parent material are modified by pedogenic and biochemical processes, and by natural inputs such as dust particles derived from soil, rocks and volcanic ash, and most importantly, by anthropogenic inputs, meaning pollution (Ali *et al*. 2017). Mining and mineral processing activities often generate acidic wastewaters, such as drainage water from mine sites and seepage water from tailings and waste rock piles. Metals in these acidic wastewaters tend to be in soluble forms, capable of penetrating to the groundwater or travelling long distances in surface waters (Bavandpour *et al.* 2018). Heavy metal contamination of water and soil is a growing concern globally because of their potentially toxic effects and long-term persistence. The inhibition of soil microorganism activity by various heavy metals has received increased research attention. Heavy metals are defined as a group of metals whose atomic density is greater than 5 g/cm^3. In nature, there are about 50 heavy metals of special concern because of their toxicological effect to human beings and other living organisms. Many of them, like Zn, Cu, Co, Ni, Mn, and Fe are known as essential “trace elements” and are necessary for living organisms (Ali *et al*.2017).

Heavy metals can accumulate in sediment, thereby affecting aquatic biota due to their toxicity and non-biodegradable nature (Bonanno *et al.* 2017). The toxic effects of metals may occur even at low concentrations because of potential bio-accumulation in the food chains. Metal pollution in lakes and rivers is widespread mainly due to mining, industrial and agriculture activities. Phyto-extraction is considered as an environmentally and economically attractive method for removing heavy metals from contaminated soils. Metal bioavailability is often a limiting factor for the phyto-extraction process (Ali *et al*. 2017). Chemical approaches are available for metal remediation but are often expensive to apply

and lack the specificity required to treat target metals against a background of competing ions. Active treatment systems include sulfidogenic bioreactors, precipitation tanks, adsorption columns/beds, membrane filtration and electrochemical systems. Recent developments in nanotechnology and biodegradable materials have improved the competitiveness of some active systems, but they still have the disadvantages of relatively higher energy input and operating costs, and environmental incompatibility (Bavandpour *et al.* 2018).

In addition, such approaches are not applicable to cost-effective remediation of large-scale subsurface contamination *in situ*, and the chemical techniques may also result in secondary contamination of soils, which often alters the soil properties and makes reestablishment of vegetation in the treated soils difficult. Biological approaches, on the other hand, offer the potential for the highly selective removal of toxic metals coupled with considerable operational flexibility. Moreover, as an *in-situ* plant-based remediation method, phyto-extraction causes no destructive effects on soil properties and often adds aesthetic value to the landscape of contaminated sites. Many such processes utilize microorganisms that have key roles in the biogeochemical cycling of toxic metals and radionuclides. Conventional remediation techniques including excavation, soil washing, and electro-kinetics are prohibitively expensive and often require intensive labour. Advances in understanding the roles of microorganisms in such processes, together with the ability to fine-tune their activities using the tools of molecular biology, has led to the development of novel or improved metal bioremediation processes. On the other hand, phytoremediation can be very time-consuming because most hyper-accumulators have small biomass for effective phyto-extraction (Li *et al.* 2018).

The term metal tolerance refers to the ability of a bacterial strains to grow in the presence of high concentrations of a metal, many bacteria have specific genetic mechanisms of resistance to toxic metals. The toxic effects of heavy metals on microorganisms are influenced by a multitude of factors such as pH, concentration of chelating agents, speciation, and organic matter. There is a vast population of bacteria that reside within the wetland soils and wetland plants that have the potential of accumulating and transforming heavy metals as well as playing a key role in the bioremediation of our polluted environments. In a stressed environment, microorganisms develop tolerance mechanisms to survive. The microorganisms also play an important role in the cycling of toxic metals in the biosphere. Microorganisms can remove heavy metal pollutants through biosorption via van der Waals forces on the cell surface, covalent binding, redox interactions, extracellular precipitation, or a combination of these processes (Cai *et al*. 2019).

(a) Aluminium

Aluminium is the third most abundant element in the earth's crust. It occurs primarily as alumino-silicate minerals which are too insoluble to participate readily in bio-geochemical reactions. Aluminium is a strongly hydrolysing metal and is relatively insoluble in the neutral pH range. Under acidic (pH < 6.0) or alkaline (pH > 8.0) conditions, or in the presence of complexing ligands, elevated concentrations may be mobilized to the aquatic environment. The solubility of aluminium in water is strongly pH dependent. Under acid conditions, it occurs as soluble, available, and toxic hexahydrate species. At intermediate pH values, it is partially soluble and probably occurs as hydroxy- and polyhydroxo- complexes. At alkaline pH values, aluminium is present as soluble but biologically unavailable hydroxide complexes or as colloids and flocculants. Aluminium is described as a *non-critical* element, though there is growing concern over the effects of elevated concentrations of aluminium in the environment, primarily that mobilized because of acid mine drainage and acid precipitation (WHO 2017).

(b) Cadmium

Cadmium is a metal element which is highly toxic to marine and freshwater aquatic life. Elemental cadmium is insoluble in water though many of its organic and inorganic salts are highly soluble. Cadmium occurs primarily in fresh waters as divalent forms including free cadmium (II) ion, cadmium chloride and cadmium carbonate, as well as a variety of other inorganic and organic compounds. Cadmium is defined by the United States Environmental Protection Agency as potentially hazardous to most forms of life and is toxic and relatively accessible to aquatic organisms. Cadmium is present in the earth's crust at an average concentration of 0.2 mg/kg, usually in association with zinc, lead, and copper sulphide ore bodies. Due to its abundance, large quantities of cadmium enter the global environment annually as a result of natural weathering processes. Cadmium is found at trace concentrations in fresh waters mostly as a result of industrial activity. The main sources of cadmium in the environment are due to: emissions to air and water from mining, metal (zinc, lead and copper) smelters, and industries involved in manufacturing alloys, paints, batteries and plastics; agricultural use of sludges, fertilizers and pesticides containing cadmium; burning of fossil fuels (very limited effect); and the deterioration of galvanized materials and cadmium-plated containers (WHO 2017).

(c) Chromium

Chromium (Cr) is a common heavy metal contaminant widely derived from smelting, electroplating, leather tanning, and chemical manufacturing, and is highly toxic for organisms. Most elevated levels of chromium in aquatic ecosystems are a consequence of industrial activity. Cr (VI) is the most toxic among chromium species and can easily penetrate cells and react with intracellular substances to affect the activity of microorganisms (Wang e*t al*. 2017). In the environment, chromium is present only

in the form of compounds; the most important natural source is chromite ($FeCr_2O_4$). In industrial wastewaters, trivalent (chromium (III) ions) and hexavalent (chromate and dichromate ions) are the most common chromium compounds that are found. Chromium (VI) is a highly oxidized state and occurs as the yellow dichromate salt in neutral or alkaline media, and as the orange chromate salt in acid medium. Both Chromium (VI) salts are highly soluble at all pH values. The reduced forms, chromium (II) and chromium (III) are reported as being less toxic and therefore less hazardous than chromium (VI). The most common ore of the metal chromium is chromite, in which chromium occurs in the trivalent state (WHO 2017).

(d) Copper

Copper occurs naturally in the earth's crust, either in mineral deposits or, less frequently, as a metal. Copper can enter water sources from natural processes, such as soil weathering, and human activities such as agriculture, mining, and manufacturing. Copper is one of the world's most widely used metals, copper occurs in four oxidation states, namely, 0, I, II and III. Copper is an acceptable material for service lines and plumbing systems. Copper has been, and continues to be, broadly used in drinking water hardware applications, such as household pipes and in fittings. In natural waters and municipal wastewaters, copper is normally found only at very low concentrations. In industrial effluents, however, it may be present at significantly higher concentrations, e.g., in metal processing plants, in the electroplating industry and in seepages from waste dumps. Copper present in tap water is principally as a result of leaching from copper-containing components of distribution and plumbing systems, consequently, copper in drinking water can also result from the corrosion of copper-containing pipes and fittings, depending on the chemistry of the water. The presence of copper can affect the taste of the water and cause the staining of laundry and plumbing fixtures at levels below the proposed maximum acceptable concentration. Although copper occurs naturally in most waters, it is regarded as potentially hazardous by the USEPA. Based on a review by Canada Health (2018), the proposed guidelines for copper in drinking water are a maximum acceptable concentration of 2 mg/l (2000 µg/l).

Copper is an essential element in humans. Copper deficiency may cause several health effects, The U.S. National Academy of Medicine has established recommended daily allowances of 900 µg/day for adults and 340–890 µg/day for children, as well as tolerable upper intake levels of 10,000 µg/day for adults, and between 1,000 and 8,000 µg/day for children. Short-term exposure to copper may result in effects in the gastrointestinal tract (nausea, pain and vomiting, diarrhea). Long-term effects are less well documented; current evidence indicates that, in the general population, chronic exposure to very high levels of copper may lead to effects in the liver and kidney (Canada Health 2018).

(e) Iron

Iron is the fourth most abundant element in the earth's crust and may be present in natural waters in varying quantities depending on the geology of the area and other chemical properties of the water body. The two common states of iron in water are the reduced (ferrous, Fe^{2+}) and the oxidized (ferric, Fe^{3+}) states. Most iron in oxygenated waters occurs as ferric hydroxide in particulate and colloidal form and as complexes with organic, especially humic, compounds. Ferric salts are insoluble in oxygenated waters, and hence iron concentrations are usually low in the water column. In reducing waters, the ferrous form, which is more soluble, may persist and, in the absence of sulphide and carbonate anions, high concentrations of ferrous iron may be found. The toxicity of iron depends on whether it is in the ferrous or ferric state, and in suspension or solution. Although iron has toxic properties at high concentrations, inhibiting various enzymes, it is not easily absorbed through the gastro-intestinal tract of vertebrates. On the basis of iron's limited toxicity and bio-availability, it is classified as a non-critical element. Iron is an essential micronutrient for all organisms and is required in the enzymatic pathways of chlorophyll and protein synthesis, and in the respiratory enzymes of all organisms (WHO 2017).

Iron is naturally released into the environment from weathering of sulphide ores (pyrite, FeS) and igneous, sedimentary, and metamorphic rocks. Leaching from sandstones releases 2 iron oxides and iron hydroxides to the environment. Iron is also released into the environment by human activities, mainly from the burning of coke and coal, acid mine drainage, mineral processing, sewage, landfill leachates and the corrosion of iron and steel. Various industries that also use iron in their processes, or in their products, include: the chlor-alkali industry, the household chemical industry, the fungicide industry, and the petro-chemical industry. South Africa has extensive gold, uranium and coal mines, drainage from which potentially affects many of our water bodies. Streams may be negatively impacted by high levels of iron in acid mine drainage. Pyrite, iron sulphide, is often found in close association with coal deposits. Upon exposure to moisture and atmospheric oxygen, the ferrous iron is oxidized to the ferric state, a reaction which is frequently accelerated by bacteria of the *Thiobacillus-Ferrobacillus* group. A layer of ferric hydroxide precipitate, so called "yellowboy", on stream bottoms and structures is a common sight in areas affected by acid mine drainage. The receiving water is often also oxygen deficient. The chemical behaviour of iron in the aquatic environment is determined by oxidation -reduction reactions, pH and the presence of coexisting inorganic and organic complexing agents. It has been predicted that, at a low pH, ferrous iron will predominate in the absence of oxygen, whilst ferric iron will predominate in oxygenated water (WHO 2017).

(f) Lead

Lead exists in several oxidation states, that is, 0, I, II and IV, all of which are of environmental importance. Lead occurs as metallic lead, inorganic compounds, and organometallic compounds. The divalent form, lead (II), is the stable ionic species present in the environment and is thought to be the form in which most lead is bio-accumulated by aquatic organisms. In fresh waters, lead is generally present as PbCO and as lead-organic complexes, with a small proportion in the form of free lead ions. Lead is defined by the U.S.EPA as potentially hazardous to most forms of life and is considered toxic and relatively accessible to aquatic organisms. Lead is principally released into the aquatic environment through the weathering of sulphide ores, especially galena. Since metallic lead and common lead minerals such as sulphides, sulphates, oxides, carbonates, and hydroxides are almost insoluble, levels of dissolved lead (acetate and chloride salts) in aquatic ecosystems are generally low. Most of the lead entering aquatic ecosystems is associated with suspended sediments, while lead in the dissolved phase is usually complexed by organic ligands. The photolysis of lead compounds is an important process in the removal of lead from the atmosphere. The products of this photo-degradation are lead oxides and halides, which enter the aquatic ecosystems via direct deposition or surface runoff. The major sources of lead in the aquatic environment are anthropogenic, these include: precipitation, fallout of lead dust and street runoff (associated with lead emissions from gasoline-powered motor vehicles); industrial and municipal wastewater discharge; mining, milling and smelting of lead and metals associated with lead, e.g. zinc, copper, silver, arsenic and antimony; and combustion of fossil fuels (Ali *et al.*2017).Decreasing pH increases the bioavailability of divalent lead, which is accumulated by aquatic biota. At a constant temperature, solubility decreases with increasing alkalinity. Soluble lead is removed from solution by association with sediments and suspended particulates of inorganic and organic material, such as hydrous oxides, clays and humic acids, respectively. Adsorption is the primary factor responsible for low lead concentrations in the aquatic environment (WHO 2017).

(g) Manganese

Globally, the World Health Organization (WHO) has recognized Manganese (Mn) as a potential risk agent since 1958. Manganese is a natural contaminant of water sources. Manganese is the eighth most abundant metal in nature and occurs in a number of ores. Acid mine drainage also releases a large amount of manganese. Iron and steel foundries release manganese into the atmosphere, where it is then redistributed through atmospheric deposition. Manganese is similar to iron in its chemical behaviour and is frequently found in association with iron. The concentration of dissolved manganese is influenced by changes in redox potential, dissolved oxygen, pH and organic matter. In natural waters, a large proportion of manganese is present in suspended and adsorbed forms. In surface waters, divalent manganese is rapidly oxidized to insoluble manganese dioxide, which settles out of

the water column. In aquatic ecosystems, manganese does not occur naturally as a metal but is found in various salts and minerals, frequently in association with iron compounds. It is an essential oligo-element, which may exert toxicity at high doses, particularly via inhalation. Its toxicity by the oral route is less known, but epidemiological and experimental studies tend to support its neurodevelopmental toxicity in infants and children (WHO 2017). Manganese (Mn) is an essential metal that plays a fundamental role for brain development and functioning. Environmental exposure to Mn may lead to accumulation in the basal ganglia and development of Parkinson-like disorders (Lucchini *et al*. 2017).

Symptoms associated with overexposure to manganese may include damage to the central nervous system and pneumonia. Manganese is an essential micronutrient for plants and animals. It is a functional component of nitrate assimilation and an essential catalyst of numerous enzyme systems in animals, plants and bacteria. When manganese is not present in sufficient quantities, photosynthetic productivity may be limited, and plants may exhibit chlorosis (a yellowing of the leaves) or failure of leaves to develop properly. A deficiency in manganese in vertebrates leads to skeletal deformities and reduced reproductive capabilities. High Concentrations of manganese are toxic, and may lead to disturbances in various metabolic pathways, in particular disturbances of the central nervous system caused by the inhibition of the formation of dopamine (a neurotransmitter). Nitrate, sulphate and chloride salts of manganese are fairly soluble in water, whereas oxides, carbonates, phosphates, sulphides and hydroxides are less soluble. Soils, sediments and metamorphic and sedimentary rocks are significant natural sources of manganese. Industrial discharges also account for elevated concentrations of manganese in receiving waters. Various industries use manganese, its alloys and manganese compounds in their processes, such as: the steel industry, in the manufacture of dry cell batteries; the fertilizer industry (manganese is used as a micro-nutrient fertilizer additive); and the chemical industry in paints, dyes, glass, ceramics, matches and fireworks (Lucchini *et al*. 2017).

(h) Zinc

Zinc, a metallic element, is an essential micronutrient for all organisms as it forms the active site in various metallo-enzymes. Zinc occurs in two oxidation states in aquatic ecosystems, namely as the metal, and as zinc (II). In aquatic ecosystems the zinc (II) ion is toxic to fish and aquatic organisms at relatively low concentrations. Zinc occurs in rocks and ores and is readily refined into a pure stable metal. It can enter aquatic ecosystems through both natural processes such as weathering and erosion, and through industrial activity. In aqueous solutions zinc is amphoteric, that is, it dissolves in acids to form the hydrated cations Zn^{2+} and in strong bases it forms zincate anions (probably of the form $Zn(OH)_2^{-4}$). The greatest dissolved zinc concentrations will occur in water with low pH, low alkalinity and high ionic strength. Chemical speciation of zinc is affected primarily by pH and alkalinity.

Soluble zinc salts (for example, zinc chloride and zinc sulphate) or insoluble precipitates of zinc salts (for example, zinc carbonate, zinc oxide and zinc sulphide) occur readily in industrial wastes. The carbonate, hydroxide and oxide forms of zinc are relatively resistant to corrosion and are used extensively in the following industries: metal galvanizing; dye manufacture and processing; pigments (paints and cosmetics); pharmaceuticals; and fertilizer and insecticide. A variety of interactions affect the toxicity of zinc in aquatic ecosystems. The toxicity of zinc is reduced in hard waters. In animals, zinc is a metabolic antagonist of cadmium and its toxicity is reduced in the presence of cadmium. Copper increases the zinc toxicity in soft water, but not in hard water, and zinc toxicity increases at lower oxygen concentrations. Zinc is synergistically toxic with cyanide. Zinc cause stomach cramps, skin irritations, vomiting, nausea, anaemia, damaged pancreas, disturbed protein metabolism, arteriosclerosis, respiratory disorders, danger to infants and unborn and increase in water acidity (Rajan *et al.,* 2019).

2.5 Treatment Processes in Wetlands: Role of microorganisms and wetland plants

Treatment processes within a wetland involve wetland plants and microorganisms. Wetland plants are often central to wastewater treatment via wetlands. The larger aquatic plants growing in wetlands are usually called macrophytes. The macrophyte genera include the *Phragmite spp*, *Typha spp* and *Scirpus spp*. Macrophytes play a fundamental role in wetland geochemistry because they are the principal living accumulators of heavy metals through active and passive absorption (Bonnano *et al*. 2017). Regarding the metals, it has been proposed that metal removal mechanisms in plants are not necessarily the same for different macrophyte species and for different metals (Maine *et al.* 2016). Different species of aquatic plants have different rates of heavy metal uptake, a consideration for plant selection in a constructed wetland used for water treatment**.** Regarding plant tolerance, several species tolerate high metal concentrations in sediment because they limit the absorption and translocation to the leaves maintaining constant and relatively low concentrations in the aerial biomass, independently of the metal concentration in sediment (Hadad *et al*. 2018). These include aquatic vascular plants, aquatic mosses and some larger algae. The presence or absence of aquatic macrophytes is one of the characteristics used to define wetlands, and as such macrophytes are an indispensable component of these ecosystems (Riggio *et al*. 2018). Vegetation in a wetland provides a substrate (roots, stems, and leaves) upon which microorganisms can grow as they break down organic materials. The other roles that plants in wetlands play include radial oxygen loss (oxygen diffusion from roots to the rhizosphere), nutrient uptake and insulation of the bed surface in cold and temperate regions The plants remove about seven to ten percent of pollutants, and when they decay act as a carbon source for the microbes (Almuskar *et al.* 2018).

There are numerous biological, chemical, and physical processes that occur among contaminants and aquatic plants but currently these processes and their relationships are not well understood (Maine *et al*. 2016). The community of microorganisms that grow on the wetland plants is known as the periphyton. The periphyton and natural chemical processes are responsible for approximately 90 percent of pollutant removal and waste breakdown (Almuskar *et al*. 2018). Microbial activities are very important for the renewal of our environment and maintenance of the global carbon cycle. These activities are included in the term biodegradation. Amid the substances that can be degraded or transformed by microorganisms are a huge number of synthetic compounds and other chemicals having ecotoxicological effects like hydrocarbons and heavy metals (Phillips 2019).

Biodegradation is the process by which organic substances are broken down into smaller compounds by living microbial organisms. Biodegradation is associated with environmental bioremediation as it is nature's way of recycling waste or breaking down organic matter into nutrients that can be used by other organisms. In the microbiological sense, "biodegradation" means that the decaying of all organic materials is carried out by a huge assortment of life forms comprised mainly of bacteria, yeast and fungi, and possibly other organisms. When biodegradation is complete, the process is called "mineralisation". However, in most cases the term biodegradation is generally used to describe almost any biologically mediated change in a substrate. Understanding the process of biodegradation requires an understanding of the microorganisms that make the process work. The microorganisms transform the substance through metabolic or enzymatic processes. The process of bioremediation might involve the introduction of new organisms to a site or the adjustment of environmental conditions to enhance degradation rates of indigenous fauna. Different approaches to bioremediation take advantage of the metabolic processes of different organisms for degradation or sequestering and concentration of different contaminants (Phillips 2019).

Biodegradation is based on two processes: growth and co-metabolism. Bioremediation and biotransformation methods harness the astonishing, naturally occurring, microbial catabolic diversity to degrade, transform or accumulate a huge range of compounds including hydrocarbons (e.g., oil), polychlorinated biphenyls, polyaromatic hydrocarbons, radionuclides and metals. There are many reports on the degradation of environmental pollutants by different bacteria. Several bacteria are even known to feed exclusively on hydrocarbons. Microbial activities are very important for the renewal of our environment and maintenance of the global carbon cycle. Due to a wide range of factors: competition with microorganisms, insufficient supply with essential substrates, unfavourable external conditions (aeration, moisture, pH, temperature), and low bioavailability of the pollutant,

biodegradation in natural conditions is limited. It is difficult to study the biodegradation in natural environments because of several factors determining the degradation rate and extent of bacterial metabolism (Almuktar *et al*. 2019).

Phytoremediation is a form of bioremediation and applies to all chemical or physical processes that involve plants for degrading or immobilizing contaminants in soil and groundwater. Phytoremediation refers to technologies that use living plants to clean up soil, air, and water contaminated with hazardous chemicals (Phillips 2019). Phytoremediation is a vital natural process of pollutant removal from aquatic ecosystems. The immersed rhizomes and roots of macrophytes provide a large surface area to develop a biofilm, which plays a key role in the removal of suspended contaminants from the water column. Moreover, roots of some plants supply dissolved oxygen into the waterbody benefiting the growth of aerobic microorganisms, which break down organic substances (Almuktar *et al*. 2019).

Concept of Phytoremediation

Phytoremediation is a cost-effective, plant-based approach to remediation that takes advantage

of the plant's ability to concentrate elements and compounds from the environment and metabolize various molecules in their tissues. It refers to the natural ability of certain plants called hyper-accumulators to bio-accumulate, degrade, or render harmless contaminants in soil, water, or air. Toxic heavy metals and organic pollutants are the major targets for phytoremediation. Since the late 20th century, knowledge of the physiological and molecular mechanisms of phytoremediation have begun to emerge together with biological and engineering strategies designed to optimize and improve phytoremediation. In addition, several field trials confirmed the feasibility of using plants for environmental clean-up. While the technology is not new, current trends suggest its popularity is growing (Phillips 2019).

6 Types of Phytoremediation

1. **Phytosequestration**

Also referred to as phytostabilisation, there are many different processes that fall under this category. They can involve absorption by roots, adsorption to the surface of roots, or the production of biochemicals by the plant that are released into the soil or groundwater in the immediate vicinity of the roots and can sequester, precipitate, or otherwise, immobilize nearby contaminants.

2. **Rhizodegradation**

This process takes place in the soil or groundwater immediately surrounding the plant roots. Exudates from plants stimulate rhizosphere bacteria to enhance biodegradation of soil contaminants.

3. Phytohydraulics

Use of deep-rooted plants to contain, sequester, or degrade groundwater contaminants that come into contact with their roots. For example, poplar trees were used to contain a groundwater plume of methyl-tert-butyl-ether.

4. Phytoextraction

This term is also known as phytoaccumulation. Plants take up or hyper-accumulate contaminants through their roots and store them in the tissues of the stem or leaves. The contaminants are not necessarily degraded but are removed from the environment when the plants are harvested. This is particularly useful for removing metals from soil. In some cases, the metals can be recovered for reuse by incinerating the plants in a process called Phyto mining.

5. Phytovolatilization

Plants take up volatile compounds through their roots, and transpire the same compounds, or their metabolites, through the leaves, thereby releasing them into the atmosphere.

6. Phytodegradation

Phytodegradation is the breakdown of contaminants taken up by plants through metabolic processes within the plant, or the breakdown of contaminants surrounding the plant through the effect of enzymes produced by the plants. Contaminants taken up into the plant tissues are metabolized, or bio transformed. Where the transformation takes place depends on the type of plant and can occur in roots, stems, or leaves (Phillips 2019).

2.6 Wastewater as a reliable alternative source of water in agriculture

The use of wastewater in irrigation is reality that every nation is faced with. Globally, water demand is predicted to increase significantly over the coming decades, especially because of the agricultural sector which is responsible for 70% of water abstractions worldwide. In the face of an ever-growing demand, wastewater is gaining momentum as a reliable alternative source of water, shifting the paradigm of wastewater management from ‘treatment and disposal’ to ‘reuse, recycle and resource recovery’. Wastewater is not merely a water management issue, it affects the environment and all living beings, and can have direct impacts on economies, both the mature and emerging economies. Wastewater constitutes a valuable resource that, if sustainably managed, is set to become a central pillar of the circular economy. It contains several useful materials, such as nutrients, metals and organic material, which can be extracted and used for productive purposes. Improved wastewater management is about reducing pollution at the source, removing contaminants from wastewater flows, recovering useful by-products and reusing it as reclaimed water. Wastewater can also be a cost-efficient and sustainable source of energy. The potential benefits of extracting such resources from

wastewater go well beyond human and environmental health, with implications on food and energy security as well as climate change mitigation. Aquatic ecosystems such as ponds, wetlands and lakes offer additional, low-cost solutions for enhancing wastewater management, provided they are managed sustainably. Although planned use of wastewater for ecosystem services is a relatively recent phenomenon, the valuation of treated wastewater use for ecosystem services reveals favourable environmental and economic benefits (WWAP 2017).

Agricultural activities include crop production, livestock and aquaculture. Wastewater may be used either directly or indirectly in agriculture for crop production, livestock and aquaculture. Direct use refers to planned and deliberate use of treated or untreated wastewater for some beneficial purpose, including irrigation, aquaculture and livestock. Municipal wastewater accounts for the majority of wastewater directly used in agriculture. Indirect use occurs when treated, partially treated or untreated wastewater is discharged into reservoirs, rivers and other water bodies, including groundwater, that supply water for agriculture. Indirect use poses the same health risks as planned wastewater use but may have a greater potential for health problems because the water user is unaware of the pollutants present in the wastewater. The use of wastewater for irrigation has been most successful in urban and peri-urban areas, where wastewater is easily available and reliable, generally free of charge, and where there is a market for agricultural produce. The usage of wastewater in the livestock sector, whether from municipal/industrial production or from the same livestock facility, is primarily dictated by the quality of the wastewater. A minimum of secondary treatment and disinfection is generally recommended. In addition, reclaimed water intended for use with cattle must have been treated to remove helminth parasites. By using treated wastewater for irrigating arable land, organic micro-contaminants are introduced into soils and are potentially transferred to the groundwater. Wastewater-borne micro-contaminants can also be taken up by plants. Food safety is a major health concern If wastewater is used in agriculture without the necessary safety precautions, microbiological and chemical pollutants can accumulate in crops, livestock products, soil or water resources, and lead to severe health impacts for exposed food consumers and farm workers. However, if adequately treated and safely applied, wastewater is a valuable source of both water and nutrients, contributing to food security and the improvement of livelihoods (WWAP 2017).

Chapter 3: Methodology

3.1 Location of study area

The study area is a natural wetland classified as an unchanneled valley-bottom wetland that lies within the Emfuleni Local Municipality, which is part of the Sedibeng District Municipality of the Gauteng Province, South Africa. It is situated about 40 km northeast of Sebokeng and is adjacent to the Sebokeng Wastewater Treatment Works. The wetland is situated along a permanently flowing river which is a tributary to the Rietspruit river. The wetland is situated within the C22H Quaternary Catchment, which lies within the Vaal Water Management area and Highveld Ecoregion. A regional soil classification for the site highlights the presence of clay-rich soils potentially associated with wetland conditions in approximately the same areas as are currently occupied by the Rietspruit and its tributaries (Sazi Environmental consulting 2016). The wetland has typically dark, base-rich and chemically fertile clay soils found in low-lying positions in landscapes with basic parent material. Topsoil is usually dark in the permanent wetness zone due to the accumulation of organic matter (DWAF 2005). *Typha capensis* is the main vegetation which was predominant within the permanent zone of the wetland. The wetland inflow comes from the treatment works plant effluent and stormwater run-off from stormwater channels of the adjacent upstream areas. The direction of flow is towards the west as it mimics the topography. The wetland receives an average inflow of 200 mega litres per day. The study area climate is characterised as a summer rainfall area with a Mean Annual Precipitation (MAP) of 662mm. Much of the rainfall is predominantly in December and January with occasional storms in other wet season months. The winters can be cold with frost being frequent in the area (Mucina and Rutherford 2006).

3.2 Water analysis

3.2.1 Sampling Procedures: Wastewater collection and transportation

Water samples were collected in 1 L Schott glass bottles between 8 and 11 am at the same points over a period of 12 months using manual grab sampling method. Water samples were obtained in such a way that only surface water was collected avoiding debris. A total of 6 bottles were used to collect water samples, 3 bottles for wetland influent and the other three for wetland effluent to enable tests to be done in triplicates. The bottles were clearly labelled and stored in a cool box on ice during transport to the laboratory for same-day testing. Samples were collected from two points, these points were described relative to their location in the wetland, which is upstream (influent/inflow) and downstream (effluent/outflow) of the wetland. The water samples were collected on a monthly basis for a period of twelve months starting from May 2016 until April 2017. Same day testing was done

most of the time but where it was not possible samples were stored in the refrigerator at 4°C and processed within 24 hours after collection. The samples were allowed to reach room temperature prior to any analysis.

3.2.2 Analysis of water quality parameters

The initial wastewater quality of the influent and the effluent from the wetland were critically monitored. The physicochemical parameters were analysed according to the standard methods in the laboratory. Wastewater samples were analysed for indicators as per established protocols. These indicators included total coliforms, *E. coli*, Total Suspended Solids, electrical conductivity, pH, 5-day Biological Oxygen Demand (BOD_5), Chemical Oxygen Demand (COD), nitrates, nitrites, sulphates, orthophosphate, aluminium, cadmium, copper, chromium, iron, lead, manganese and zinc. The wetland effluent values were then compared with the South African irrigation standards to check if the values fall within the target water quality range as outlined by DWAF (1996).

Physico-chemical parameters

3.2.2a Colour

The colour of water samples was determined through visualisation with the human eye. Using the knowledge of general classification of colour, the water samples were observed against both a dark and light background using black and white A4 papers. A human eye was used to assess colour standards and to discriminate colour of the different water samples. The colour of the samples was recorded through visual assessment.

3.2.2b Odour

The odour of the water samples was assessed using the human sense of smell. The exercise was done to pick up any foul odours such as sewage smell (faecal matter), ammoniacal, rotten egg, or rotten cabbage-like smell. The water samples were also assessed for earthy and musty smell.

3.2.2c pH, Electrical Conductivity, Temperature and Total Dissolved Solids

Measurements of pH, Electrical Conductivity, Temperature and Total Dissolved Solids were performed in triplicate and the average was recorded. Crison $MM40^+$ Multimeter was used to record the levels of pH, electrical conductivity (EC) and temperature. Before measurements were recorded, the Multimeter was calibrated according to the manufacturer's instructions. The Multimeter probes were rinsed with sterile distilled water after each and every reading. The probe was inserted in the water and given time to stabilize before the readings were recorded.

Chemical parameters

The following chemical parameters were analysed; BOD_5, COD, nitrate, nitrite, ortho-phosphate, sulphate, $CaCO_3$ hardness, as well as trace elements (heavy metals). A total of 6 bottles were used to collect water samples, 3 bottles for wetland influent and the other three for wetland effluent to enable tests to be done in triplicates. Concentration measurements of the chemical parameters were done using the colorimetric methods that entailed the use of NANOCOLOR® analytical system which includes the Nanocolor test kits and a photometer Nanocolor 500 D. Absorbance (optical density) of the sample is recorded. Absorbance is a measure of the "quenching" or attenuation of radiation through the sample. Cold samples were warmed to room temperature (20–25°C) as per instructions. The concentration of a certain compound is determined by increase or decrease of the absorbance of the solution, caused by a specific colour reaction of the compound to be determined.

3.2.2d Carbonate Hardness

Photometric determination of the carbonate hardness in water with bromophenol blue was performed using carbonate hardness NANOCOLOR test 0-15 (REF 985 015). Water sample to the volume of 4 ml was added to the carbonate hardness 15 test tube and mixed well. NANOFIX reagent R2 was then added to the same test tube and the contents of the tube mixed by shaking. The outside of the tube was wiped with soft paper towel and carbonate hardness was measured after 2 minutes at a wavelength of 585 nm using a Nanocolor 500 D photometer.

3.2.2e Chemical Oxygen Demand (COD)

COD NANOCOLOR test 0-26 (REF 985 026) was performed as per manufacturer's instructions. The COD determinations were performed as soon as possible after sampling. However, where it was not possible, the pH was reduced to 2 or less by the addition of 2 mL of concentrated sulphuric acid (H_2SO_4) per 1 L of sample. COD determination was employed using a Nanocolor kit, a Thermo Scientific Orion COD 165 thermo-reactor and a 500 D photometer. The water sample (200 µl) was pipetted into the COD reagent test tube and the tube was closed and placed into the heating block (Orion COD 165 thermo reactor). The heating block temperature was set to 148°C and the time for digestion was set to 2 hours. After 2-hour digestion the tube was allowed to cool on a test tube rack. Photometer (500 D) was turned on and the outside of the test tube was cleaned using a soft paper towel and COD measured by selecting a built-in photometric test method 028.

3.2.2f Five-day Biological Oxygen Demand (BOD_5)

BOD NANOCOLOR test 8-25 (REF 985 825) was conducted according to the manufacturer's instructions. Determination of the biochemical oxygen demand after 5 days (BOD_5) was done. The storage vessel was filled with tap water and aerated for 1 hour. Twenty millilitres of the aerated water

were transferred into a reaction vessel, the vessel was closed and shaken vigorously for 30 s. One test tube BOD_5-TT was opened and filled to the brim with the control solution and labelled control. Another reaction vessel was filled with undiluted sample and shaken vigorously for 30 s. BOD_5-TT test tube was opened and filled to the brim with sample solution. The prepared test tubes (Undiluted water sample and the control) were incubated for five days at 20°C away from air and light. After 5 days of incubation, the test tubes were opened and 2 drops of BOD_5- TT reagent R1 and 2 drops of BOD_5- TT reagent R2 were added and the test tube and shaken briefly. After 2 minutes the test tube was opened and 5 drops of reagent R3 were added, the tube was closed again and shaken until the precipitate dissolved. The outside of test tube was cleaned by wiping with soft paper towel and BOD_5 was determined at a wavelength of 436 nm using a NANOCOLOR 500 D photometer (with pre-programmed method 8251). The determination of the dissolved oxygen after 5 days was achieved.

3.2.2g Nitrate (NO_3^-)

Nitrate 50 NANOCOLOR test 0-64 (REF 985 064) kit was used to analyse nitrate in the water samples. The method is the photometric determination of 4-nitro-2,6-dimethylphenol, in analogy to the norms ISO 7890-1 and DIN 38405-D9-2. The reaction uses 2,6-dimethylphenol in a mixture of sulfuric acid and phosphoric acid. Direct nitration of dimethylphenol results in the formation of 4-nitro-2,6-dimethylphenol, depending on the nitrate content of the sample. A volume of 0.5 ml water sample and 0.5 ml of reagent R2 were added to the nitrate 50 test tube and the tube closed and mixed gently. The outside of the tube was cleaned and after 10 min nitrate concentration was measured at a wavelength of 385 nm.

3.2.2h Nitrite (NO_2^-)

Photometric determination of nitrite with sulphanilamide and *N*-(1-naphthyl) ethylenediamine. Nitrite 4 NANOCOLOR test 0 – 69 (REF 985-069) was used. The reagents for sample preparation by clarification precipitation (REF 918 937) were used for removal of emulsions, turbidity and colour prior to the nitrite determination. 200 µl of water sample and 1 NANOFIX reagent R2 were added to the nitrite 4 test tube and the tube closed. The contents of the tube were mixed, the outside of the tube cleaned and nitrite concentration measured after 15 minutes at 540 nm.

3.2.2i Orthophosphate

The samples were filtered to remove any existing unwanted suspended solids and turbidity. To remove turbidity, membrane filters with a small pore size (0.45 µm) were used as well as MN 615 qualitative paper filters to filter through the water sample. Water sample (1 ml), one NANOFIX reagent R3 and 200 µl reagent R4 were added into the test tube total phosphate 5. The test tube was closed and

shaken, and the outside of the tube cleaned. After 10 minutes ortho-phosphate concentration was measured at a wavelength of 690 nm using Nanocolor 500 D photometer.

3.2.2j Sulphate

sulphate 200 NANOCOLOR Test 0-86 (REF 985 086) for the determination of barium sulphate was done following the manufacturer's instruction**.** The test sample was filtered before the determination of sulphate if it showed turbidity. sulphate 200 NANOCOLOR TEST 0-86 (REF 985 086) was performed in duplicate where the mean of the two values was recorded. 4 ml water sample was added into sulphate 200 test tube and mixed. The test tube was then placed into the photometer as blank value, after which it was opened and 1 level spoon of R2 was added and the content of the test tube shaken vigorously for 10 s. The outside of the test tube was cleaned and sulphate measured after 2 minutes using Nanocolor 500 D photometer at a wavelength of 436 nm.

3.2.2k Total Suspended Solids (TSS)

For determining TSS in water the analysis was done according to the TSS standard method APHA 2540D and EPA Method 160.2. A total of 6 bottles were used to collect water samples, 3 bottles for wetland influent and the other three for wetland effluent. The bottles were closed with screw caps and placed on ice in a cooler and transported to back VUT laboratory. Analysis was done upon arrival at the laboratory. For TSS analysis the sample maximum holding time is seven days at 4°C. Filtration apparatus consisting of filter pump, Vacuum tubing, 2 L waste receiving flask, 3 position manifold, and filter funnel was set up. Hach TSS pre-weighed 1.5 µm nominal pore size glass microfiber filters (47 mm diameter) were used to filter through the water samples. The 1.5 µm pore size glass fibre filters were inserted onto the filter support using sterile forceps and vacuum applied by turning on the vacuum pump. The water samples were shaken vigorously before filtration. A volume of 1 L sample was measured using a 1 L sterile graduated cylinder. A total sample volume of 1 litre was filtered through each glass fibre filter and the suction continued for five minutes after all the water in the filtration funnels had been completely drained. 20 ml of sterile deionised water was used to rinse each graduated cylinder and the inside of the filter unit, and then filtered until completely drained. The filters were carefully transferred to barcoded aluminium weighing dishes using forceps, and the aluminium dishes each with a filter were placed in an oven set to 104 °C. The filters were dried for an hour before been removed from the oven and transferred to a desiccator to cool to room temperature in a moisture free environment. The dry filters were then weighed on an analytical balance. The tare weight of each filter was recorded, these measurements together with initial weight of the glass fibre filters were used to calculate TSS. Determination of TSS was done using the formula below.

$$TSS\ (mg/l) = \frac{(A - B) \times 1000}{V}$$

Where: A = final mass of filter, meaning mass of filter + dried residue (g),

B = initial mass of filter (g), and

V = volume of sample filtered (l)

Multiplying by 1000 converts grams to milligrams.

Metal Analysis

Metals were analysed by MN Nanocolor photometric methods which entails the use of a test kit and 500 D photometer with built in test method to measure the concentration.

3.2.2l Aluminium

Aluminium 07 NANOCOLOR test 0-98 (REF 985 098**)** was performed to determine the aluminium concentration in water samples. Photometric determination of aluminium with eriochrome cyanine R (NANOCOLOR®) in analogy to APHA 3500-Al D was done. Aluminium 7 test tube containing NANOFIX was opened and 500 µl of R2, 500 µl of R3 and 4 ml water sample were added to the tube and mixed. A volume of 500 µl reagent R4 was added lastly to the tube. The tube was closed and its content mixed by tilting the tube. After 5 minutes, the outside of the tube was cleaned by wiping with a soft paper towel and the concentration of aluminium was measured at a wavelength of 540 nm using a 500 D photometer.

3.2.2m Cadmium

Cadmium 2 NANOCOLOR test 0-14 (REF 985014) was carried as per the manufacturer's instructions. A volume of 4 ml water sample and 200 µl of R2 reagent were added to the cadmium 2 test tube. The tube was then closed and its content mixed. After 5 minutes the outside of the test tube was cleaned with a soft paper towel and cadmium concentration measured at a wavelength of 520 nm using 500 D photometer.

3.2.2n Chromate

Chromate NANOCOLOR test 1-25 (REF 918 25) was done following the manufacturer's instructions. Any samples that were turbid were filtered prior to the determination of dissolved chromate using 1.5 µm glass microfiber membrane filters.

3.2.2o Copper

Copper NANOCOLOR test 1-53 (REF 915 53) was performed to determine the amount of copper. Tube tests were done in triplicates and a mean value recorded. A volume of 4 ml water sample was added into the test tube copper 7, then 200 µl of reagent 2 (R2) was added thereafter and the tube was closed and the content mixed well. The outside of the test tube was cleaned and after 10 minutes the copper measurements were taken using 500 D photometer at 585 nm.

3.2.2p Iron

Iron 3 NANOCOLOR test 0-37 (REF 985 037) was carried out according to the manufacturer's instructions. A volume of 4 ml water samples was added into the iron 3 test tubes containing diphenylpyridyl triazine. The 1 NANOFIX reagent 2 (R2) was then added and the test tube was closed and the content of the tube mixed. After 5 minutes the content of the test tube was poured into 50 mm semi cuvettes and measured at 540 nm wavelength.

3.2.2q Manganese

Mangan 10 NANOCOLOR test 0-58 (REF 985 058) was used for the determination of manganese in the water samples. Water sample (4 ml) was added into test tube manganese 10, the tube was closed and mixed well. R2 reagent (500 µl) was added into the test tube and the content of the tube mixed. A level measuring spoonful of R3 reagent was added after 1 minute and the tube was closed and the content of the tube mixed by shaking. The outside of the tube was cleaned and manganese measured after 5 minutes at 470 nm wavelength using 500 D photometer.

3.2.2r Lead

Lead 5 NANOCOLOR test 0.09 (REF 985 009) was used to analyse the amount of lead in water samples. Test tube of Lead 5 was opened and 0.2 ml of reagent R2 added to the tube and mixed. After which 4 ml of sample was added and mixed well. The outside of the test tube was cleaned and after 3 minutes the test tube solution was used to zero the photometer. The test tube was opened again and 1 NANOFIX reagent R3 added and the contents of the tube were mixed by shaking. The measurement was taken after 3 minutes using a NANOCOLOR 500 D photometer at 520 nm wavelength.

3.2.2s Zinc

Zinc 4 NANOCOLOR test 0-96 (R EF 985 096) was carried and the concentration of zinc was determined at 620 nm wavelength. Water sample to the volume of 4 ml was added into test tube zinc 4 and mixed until the reagent dissolved completely. Reagent R2 (200 µl) was added and the content

of the test tube mixed well by shaking. The outside of the tube was cleaned and Zinc concentration measured after 1 minute at a wavelength of 620 nm.

Analysis of microbiological parameters

Microbiological parameters analysed were Total coliforms and *E. coli*. The tests were done in duplicate and a mean was recorded.

Colilert-18 and Quanti-tray/2000

Colilert-18 and Quanti-Tray/2000 is the worldwide ISO standard 9308-2:2012 method for detecting total coliforms and *E. coli* in water. Colilert-18/Quanti-tray method was used to quantify the total coliforms in wastewater and the most probable number (MPN) was recorded using the MPN table. Chemical substrate containing 4-methyl-umbelliferyl β-D-glucuronide (MUG) was added to a 100 ml water sample, mixed, poured into a multi-well tray. The tray was sealed in a quanti-tray sealer and incubated at 35°C for 18 hours. Positive wells were counted and an MPN table used to enumerate coliforms.

3.2.3 Data Analysis

Data Analysis was performed using a battery of tests which included Microsoft Excel 2016 data analysis tool, Shapiro-wilk test (for normality distribution), After minus before paired sample t-test (left-tailed t-test) and Wilcoxon signed rank test for data that do not follow a normal distribution. Outliers were checked using the Tukey's fences method, K=1.5. These different statistical tests were performed to analyse the pollutant removal efficiency of the wetland system. Standard statistics, which included the mean, median, standard deviation, minimum and maximum were determined. Percentage reduction rate calculations were done using equation 3.3 by Kadlec and Wallace (2008).

Wetland removal efficiency (R).

$$R = \frac{C_i - C_e}{C_i} \times 100$$

Where R is the removal efficiency (percentage reduction rate) (%),

C_e is the effluent concentration value (mg/l) and

C_i is the influent concentration value (mg/l)

3.3 Isolation of rhizobacteria from *T. capensis*

The roots of *Typha capensis* were sampled from approximately 30 cm below the water surface within the wetland. Three samples of fibrous roots were taken from three different locations with about 10 m distance between each point of extraction. A shovel was used to pierce soil to a depth of approximately

30 cm to cut any of the lateral roots holding the plant in the soil. The plant roots were excavated by leveraging the shovel and the entire plant was uprooted. The aboveground plant biomass was cut and discarded on an area opposite the wetland and the root balls were then put in the labelled bucket and transported back to the laboratory at Vaal University of Technology. Upon arrival at the laboratory the roots were cut using sterile forceps and a pair of pruning scissors to approximately 4 cm in length. The roots were washed three times with 500 ml sterile distilled water to remove attached soil and the rinsing water containing soil was then used as an inoculum. The water used to rinse the roots was then plated directly onto nutrient agar and tryptic soy agar plates and incubated at 25°C and 28°C for up to 7 days. The cut roots were then placed into 100 ml flasks containing sterile normal saline solution (0.85 g/100 ml of sodium chloride) and some flasks were incubated overnight at 25°C while others were incubated at 28°C. A volume 0.1 ml of the overnight normal saline with roots was plated onto nutrient agar plates as well as Tryptic soy agar using spread plate method and incubated at 28°C as well. Growth of different colonies were monitored and different colonies were picked according to their distinct colony characteristics (colour and texture). Colonies were streaked onto Tryptic soy agar and nutrient agar and incubated. For maintenance and preservation purposes all cultures were inoculated into the microbank vials and stored at -80°C in an ultralow freezer. The isolates were also grown in trypticase soy broth and the broth culture was mixed with 40% glycerol in a 1:1 ratio and preserved at -80°C ultralow-freezer.

3.4 Gram staining of all isolates

The Gram stain method was performed on all bacterial isolates to classify bacteria into either Gram positive or Gram negative, and to check their purity. A heat fixed smear was prepared, then cells were stained with crystal violet dye for 1 minute. After which cells were rinsed with tap water before addition of Gram's iodine for 1 minute. The next step was the decolorization with 95% Ethyl Alcohol. The cells were counterstained with safranin for 30 seconds, rinsed with tap water, air- dried and viewed under a microscope.

3.5 Metal growth tolerance studies

For metal studies nutrient agar (Oxoid) which is a basal/minimal medium was used to maintain a high free metal concentration in medium as well as Tryptic soy agar (Oxoid). All glassware was acid washed before use to avoid binding of metals. All media was prepared according to the manufacturer's instructions, dissolved in distilled water and sterilized by autoclaving. The metal stock solutions of 1000 mg/l were prepared by dissolving their respective metal salts separately in sterile deionised water and filter sterilizing the solutions through 0.45 µm pore size membrane filters. The amount of metal used to prepare the stock solutions was determined by dividing the molecular weight of the metal salt with the atomic mass of the metal. The amounts of 17.58 g Aluminium potassium sulphate

dodecahydrate ($AlK(SO_4)_2$) x $12H_2O$, 1.85 g Cadmium sulphate ($CdSO_4$), 2.5 g Copper sulphate ($CuSO_4$), 4.98 g Ferrous sulphate heptahydrate ($FeSO_4.7H_2O$), 1.60 g Lead nitrate ($PbNO_3$), 3.08 g Manganous sulphate monohydrate ($MnSO_4.H_2O$), 3.73 g Potassium chromate (K_2CrO_4) and 2.47 g Zinc sulphate ($ZnSO_4$) were prepared separately to a final volume of one litre. Working test metal solutions were prepared through the addition of the metal solution into molten nutrient agar and tryptic soy agar (TSA) before pouring plates to make the concentration of 25mg/l, 30mg/l, 50 mg/l, 75 mg/l, 100 mg/l, 125mg/l, 150mg/l, 200mg/l, 300mg/l. Determination of the final metal concentrations in the media was calculated by using the formula $C_1V_1 = C_2V_2$, where V_1 is the initial volume of the stock solution that needs to be added to the media to achieve the desired final metal concentration (it is the unknown variable that is been calculated), C_1 is the initial concentration of metal stock solution, C_2 is the final concentration of the metal within the culture media, and V_2 is the final volume of the metal supplemented media. Isolates were then tested for heavy metal tolerance by sub-culturing them onto heavy metal supplemented nutrient agar and Tryptic soy agar in the order of the increasing metal concentrations. The isolates that grew on the lower metal concentrations were subsequently cultured onto higher metal concentration supplemented media plates in an increasing order by gradually increasing the concentration of each metal on NA and TSA plates until the strains failed to grow. After the incubation period (24-72 h) the plates were observed for growth. Growth was then quantitatively described as follows: - no growth; + poor growth; ++ good growth; and +++ exuberant growth.

3.6 Biochemical tests for 15 isolates with multiple metal tolerance

The pure cultures were differentiated based on morphology and biochemical characteristics.

3.6.1 Motility Study: Tube method

A semi-solid Sulphide Indole Motility (SIM) medium was used to check for the ability of bacteria to migrate away from a line of inoculation. The Cragie's tube method was used to determine bacterial motility. SIM medium was inoculated with a bacterium inoculum by stabbing the middle of the tube with an inoculation needle and the tubes were incubated at 37 ºC for 18-24 h. Growth along the line of inoculation indicated lack of motility while diffuse growth from the line of inoculation into the rest of the media indicates motility.

3.6.2 Slide method (wet mount)

An overnight actively growing colony was transferred into 5 ml Tryptic soy broth and incubated at 28 °C, a drop of this culture was deposited over a microscope slide, covered with a coverslip and observed under a microscope.

3.6.3 Endospore stain

The endospore stain was performed as per standard protocol to determine the ability of bacteria to produce endospores. A bacterial colony was mixed with a drop of sterile distilled water and allowed to air dry and then the bacterial smear was heat fixed to the slide by passing it through the flame of a Bunsen burner 3-4 times. The slide was placed on top of a beaker of boiling water and a piece of blot/filter paper put on the smear. The endospores were stained with 5 percent Malachite green by flooding the slide with the stain. The warmed Malachite green solution was left on the cells for 5 minutes with continuous steaming. The slide was then rinsed with tap water and the cells were counterstained for 30 seconds with 0.25 percent Safranin O. The slide was air dried and observed under a microscope.

3.6.4 Catalase test: slide method

Catalase is a haemoprotein produced by most aerobic bacteria but not by obligate anaerobes. The catalase test facilitates the detection of the enzyme catalase in bacteria. The enzyme breaks down hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2). A small amount of organism from a well-isolated colony was picked from a 24 h culture and placed onto a microscope slide. Using a sterile Pasteur pipette, 1 drop of 3% H_2O_2 was placed onto the organism and observations were made for formation of bubbles.

3.6.5 Oxidase test

Oxidase test was performed using Remel BactiDrop oxidase reagent (N, N, N, N-tetramethyl-1, 4-phenylenediamine). 1-2 drops of reagent were dispensed onto a strip of Whatman (No.1) filter paper. A colony from a 24-hour isolate was smeared onto the saturated filter paper. Colour development was observed within 10-30 seconds. A positive test was identified by the development of a violet to purple colour on the filter paper.

3.6.6 The indole test: tube method

The formulation of SIM Medium is designed to allow the detection of sulphide production, indole formation and motility. Bacteria which produce the enzyme tryptophanase can metabolize the amino acid tryptophan with the production of Indole (a benzyl pyrole). This test demonstrates the ability of certain bacteria to decompose the amino acid tryptophan to indole. When indole is combined with Kovac's reagent the solution turns from yellow to cherry red/brick red. SIM medium was prepared according to the manufacturer's instructions and dispensed into test tubes. A colony of the bacterial isolate was inoculated by means of a stab inoculation (using a needle to stab into the SIM tube). The tube was then incubated at 37°C for 48 hours. A few drops (5 drops) of kovac's reagent were added

into the SIM medium tube, a positive indole test was indicated by the formation of a red colour in the reagent layer on top of the agar deep within seconds of adding the reagent.

3.6.7 H_2S production

To determine whether the isolates reduce sulphur-containing compounds to sulphides, with hydrogen sulphide gas as a by-product. The formation of a black precipitate within a SIM medium tube indicates hydrogen sulphide gas production.

3.7 Identification of bacterial isolates using molecular techniques (DNA extraction, 16S rDNA PCR amplification and sequencing)

3.7.1 Deoxyribonucleic acid (DNA) extraction

DNA from the 15 isolates was extracted using a Zymo research quick-DNA fecal/soil microbe Micro-prep kit D6012 supplied by Inqaba Biotech. Bacterial isolates were scraped from the culture plate and resuspended in 200 µl of sterile distilled water. The bacterial suspension was then added to a ZR BashingBead™ Lysis Tube (0.1 & 0.5 mm), followed by 750 µl of BashingBead™ Buffer. The lysis tube was then vortexed for 8 minutes on a Disruptor Genie vortex to disrupt the cells. The ZR BashingBead™ Lysis Tube was centrifuged in a microcentrifuge at ≥10,000 x *g* for 1 minute. 400 µl supernatant was transferred to a Zymo-Spin™ III-F Filter in a Collection Tube and centrifuged at 8,000 x *g* for 1 minute. The Zymo-Spin™ III-F Filter was discarded. 1200 µl of Genomic Lysis Buffer was added to the filtrate in the Collection Tube from Step 4 and mixed well by pipetting. A total volume of 800 µl of the mixture from Step 5 was transferred to a Zymo-Spin™ IC Column3 in a Collection Tube and centrifuged at 10 000 x *g* for 1 minute. The flow through from the Collection Tube was discarded and Step 6 repeated. 200 µl DNA Pre-Wash Buffer was added to the Zymo-Spin™ IC Column in a new Collection Tube and centrifuged at 10 000 x *g* for 1 minute. 500 µl g-DNA Wash Buffer was added to the Zymo-Spin™ IC Column and centrifuged at 10 000 x *g* for 1 minute. The Zymo-Spin™ IC Column was transferred to a clean 1.5 ml microcentrifuge tube and ≥ 20 µl DNA Elution Buffer was added directly to the column matrix. The tube was centrifuge at 10 000 x *g* for 30 seconds to elute the DNA. The eluted DNA was transferred to a prepared Zymo-Spin™ II-µHRC Filter in a clean 1.5 ml microcentrifuge tube and centrifuged at exactly 16 000 x *g* for 3 minutes. The filtered DNA was ready for use in PCR.

DNA visualization

8 µl of the isolated genomic DNA was mixed with 2 µl of dye and loaded on 1% agarose gel and separated by agarose electrophoresis in 1 X TBE buffer for 45 minutes at 400 mA. The gel was stained with Ethidium bromide to check the quality of DNA. The gel was photographed under UV in a Gel Documentation System.

3.7.2 16S rDNA Polymerase chain reaction

PCR amplification of the genomic DNA from the bacterial isolates performed using the primers 27F 'GAGTTTGATCCTGGCTCAG' and 907R 'CCCCGTCAATTCATTTGAGTTT'. PCR amplifications were carried out routinely using the following PCR reaction mixture; 4 µl DNA, 1.5 µl forward primer [27F], 1.5 µl reverse primer [907R], 12.5 µl master mix and 5.5 µl water. The PCR was carried out in a BIO RAD T100 thermal cycler and the thermal cycling conditions were as follows; initial denaturation step for 3 min at 95 ºC, followed by 30 seconds at 95, ºC then 30 seconds at 53.9 ºC, 1 minute at 72 ºC, 34 cycles each of 30 seconds at 95 ºC (denaturation), followed by 1 minute 72 ºC and the amplification products were held at 4 ºC.

PCR reaction mix	
DNA	4.0 µl
Forward primer	1.5 µl
Reverse primer	1.5 µl
Master mix	12.5 µl
Sterile DNAse free water	5.5 µl
Total volume	25 µl

Sequencing

The PCR product (17 µl) for each bacterial isolate was sent to Inqaba Biotech for sequencing. PCR products were further amplified with the 907 R primer.

3.8 DNA Sequence analysis and isolate identification through Basic Local Alignment Search Tool (BLAST).

A nucleotide BLAST search with the nucleotide sequences obtained from Inqaba biotech was carried out on the NCBI database to identify the isolated microorganisms. The search was performed on http://blast.ncbi.nlm.nih.gov/.

Chapter 4: Results and Discussion

4.1 Wetland removal efficiency (%) of pathogens, inorganic and organic matter

Target constituent removal percentages were used as a metric to determine the wetland performance. The wetland showed average removal efficiencies of 43%, 51%, 85%, 60%, 61 %, 61 %, 21 %, 67%, 52%, 51%, 83%, 56%, 89%, 49 % and 54% for electrical conductivity, total coliforms, *E. coli*, BOD_5, COD, TSS, carbonate hardness, aluminium, iron, manganese, copper, nitrite, nitrate, sulphate and ortho-phosphate respectively. This study showed that the wetland can provide up to 89% pollutant reduction. The wetland scored highest for reduction of nitrate, *E. coli* and copper with values 89%, 85% and 83% respectively. The wetland has shown significant removal efficiency for various pollutants in a period of one year. BOD_5 and COD percentage removal found in this case study were not as high as previously reported by other researchers who did work on the wetlands in the Mediterranean area who recorded BOD_5 removal efficiency of 96.4 % while COD was 84.6 % (Andreo-Martínez *et al*. 2016).

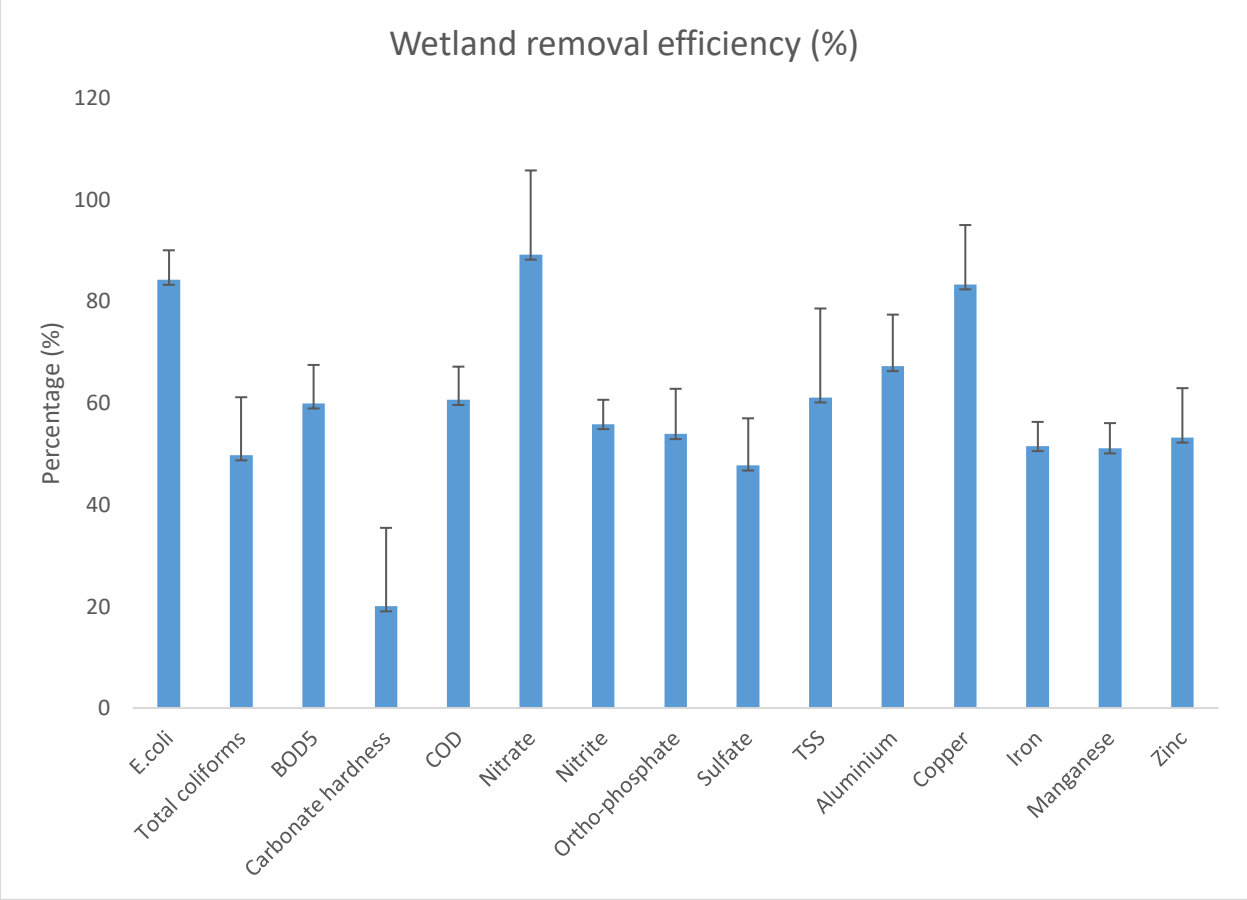

Figure 3: Average wetland pollutant removal efficiency over a 12 months period from May 2016 to April 2017 with standard deviation error bars.

4.1.1 Temperature (°C)

During the study period from May 2016 to April 2017, temperatures ranged from 18 to 28 °C with June and July being the coolest months of the year. As shown in figure 4, temperature remained cooler, around 18 °C, during winter and increased during the summer season to 28°C. Most organisms involved in the wetland processes have an optimal range of 20 to 35 °C. Temperature is known to affect biological processes such as microbially-mediated reactions within the wetland. However, this is only the case at the lower and upper end of the temperature scale (temperatures < 15 °C and > 35 °C). Processes regulating organic matter decomposition are affected by temperature as well as all nitrogen cycling reactions (mineralization, nitrification, and denitrification). Air temperature is usually the single most important factor in determining mean daily water temperature. The insolation factors of shading due to plants within the wetland and width-to-depth ratio have some influence on water temperature as well. (Kadlec and Reddy 2001; Ghasemi-Zaniani *et al*. 2017). Physical processes such as sedimentation and decantation, as well as particulate organic matter removal, are not heavily affected by cooler temperatures. In this study there was no visible pattern to show how different temperatures affected the pollutant removal efficiencies since the temperature range was between 18 to 28 °C and not below 15°C. The temperature range recorded is suitable for the growth of endophytes and other bacterial strains that play a key role in the biodegradation of pollutants.

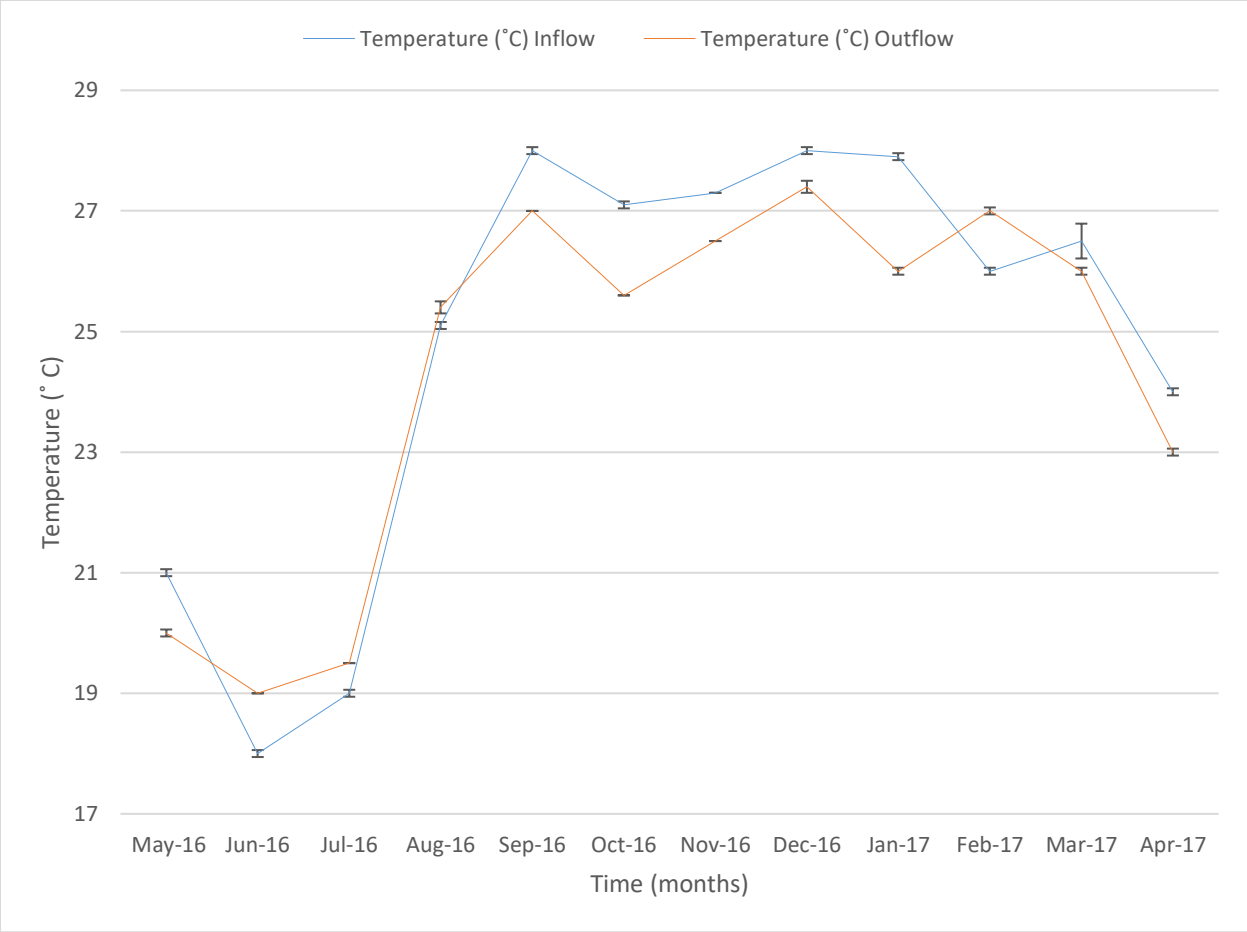

Figure 4. Temperature of wetland inflow and outflow over 12 months with standard deviation error bars.

4.1.2 pH

The wetland upstream and downstream pH values were in the range of 6.8 – 7.4 and 6.8 -7.3 respectfully. The pH was within limits set by the Department of Water and Sanitation national guidelines for wastewater discharge, which states that the effluent pH range for inland surface water, public sewer secondary treatment plant and irrigated land should be between 5.5 and 9.5 (DWAF 1984). Direct contact with crop foliage by either high or low pH waters causes foliar damage, which can, depending on the severity and timing of the damage, result in a decreased yield or damage to fruit or other marketable products. Soil microbial populations are also markedly affected by soil pH levels (DWAF 1996). Hence the pH measurement is very important because pH affects the growth of microbial populations that are involved in the degradation of pollutants and have a direct impact on other water quality parameters such as nutrients, COD and TSS within a wetland (Pang *et al*. 2015**)**.

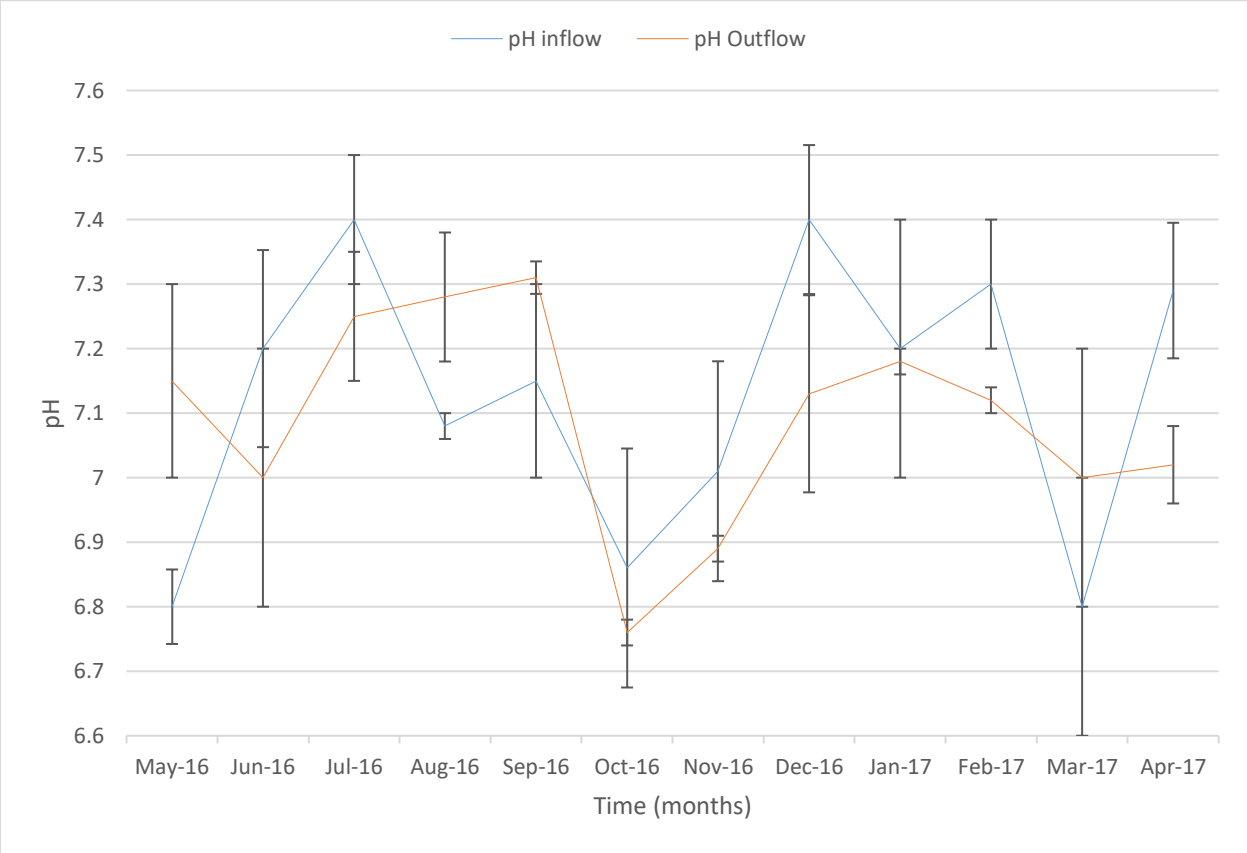

Figure 5: pH of the wetland inflow and outflow over 12 months with standard deviation error bars.

4.1.3 Aesthetic quality of the water: Colour and Odour

Colour and odour are subjective properties of wastewater which are difficult to measure. The observed colour of the water was in most instances light brown during the rainy season, and almost clear during the dry season. The colour of the samples varied from clear to slightly brownish due the humic organic matter in the wetland and also due to the rain. The odour of water within the wetland was no different to the smell of tap water. No fowl odour was recorded throughout the monitoring period as there was no offensive or foul smell. The government gazette 18 May 1984 No. 9225; Regulation No.991 states that wastewater effluent shall not contain any substance in a concentration capable of producing any colour, odour or taste.

4.1.4 Electrical conductivity (µS/cm)

Electrical conductivity (EC) is the most convenient way of measuring water salinity. Average wetland removal efficiency for conductivity was recorded as 43%. The influent electrical conductivity values

ranged from 142 to 507 µS/cm while the effluent values ranged from 58.50 to 334 µS/cm as shown in figure 6. These values were compliant with the irrigation standard that states that EC should not exceed 200 mS/cm. The wetland electrical conductivity removal efficiency of 43% is statistically significant since p <0.05 (p = 8.68996e-8).

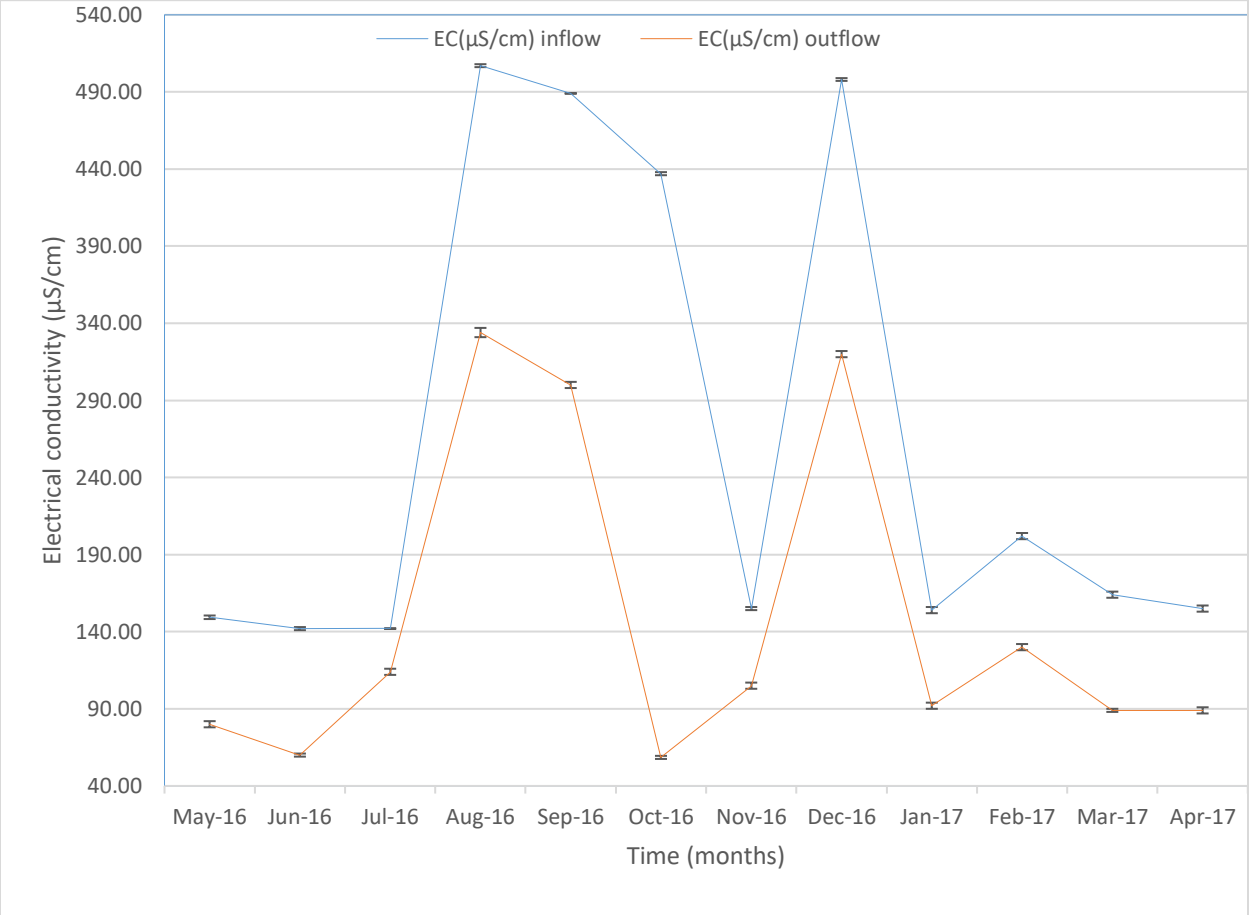

Figure 6**:** Average monthly electrical conductivity (µS/cm) in the wetland inflow and outflow with standard deviation error bars.

4.1.5 Most Probable Number of total coliforms in the wetland inflow and outflow.

The total coliform number in the effluent was outside the approved limits for irrigation water, which states that total coliforms should not exceed 1000 count per 100 ml. As shown in figure 7 the total coliform in the influent ranged from 601.5 to 1011.2 while the effluent total coliforms ranged from 235.9 to 593.8 per 100 ml. The removal efficiency ranged from 36 to 87 %, with an average reduction of total coliforms at 51%. The wetland removal efficiency was lower than the wetland reported by Martin *et al*. (2012). According to Martin *et al*. (2012) vegetated wetlands have higher coliform removal efficiencies (88–95%). A limit of 1000 faecal coliforms per 100 ml of irrigation water was recommended for use in the United States on all crops, including those eaten raw. Guidelines of 100 faecal coliforms per 100

ml and 1000 total coliforms per 100 ml are recommended. Faecal coliforms have been shown to represent 93 % - 99 % of coliform bacteria in faeces from humans, poultry, cats, dogs and rodents. Some faecal coliform tests also enumerate *Klebsiella spp*., which can originate from non-faecal sources, and a few other bacterial strains also of non-faecal origin. *Escherichia coli* may comprises up to 97 % of coliform bacteria in human faeces. The remainder include other *Escherichia spp*., *Klebsiella spp*., *Enterobacter spp.*, *Enterobacter spp.* and *Citrobacter spp.* Total coliform bacteria are primarily used as a practical indicator of the general hygienic quality of water; mainly used in routine monitoring of drinking water. Several natural processes in aquatic systems such as sedimentation, adsorption, coagulation and flocculation may remove microorganisms from water without inactivation and may even protect the organisms against inactivation (DWAF 1996; Canadian Council of Ministers of the Environment 2008). The wetland total coliforms removal efficiency of 51% is statistically significant since p <0.05 (p = 2.7643e-7).

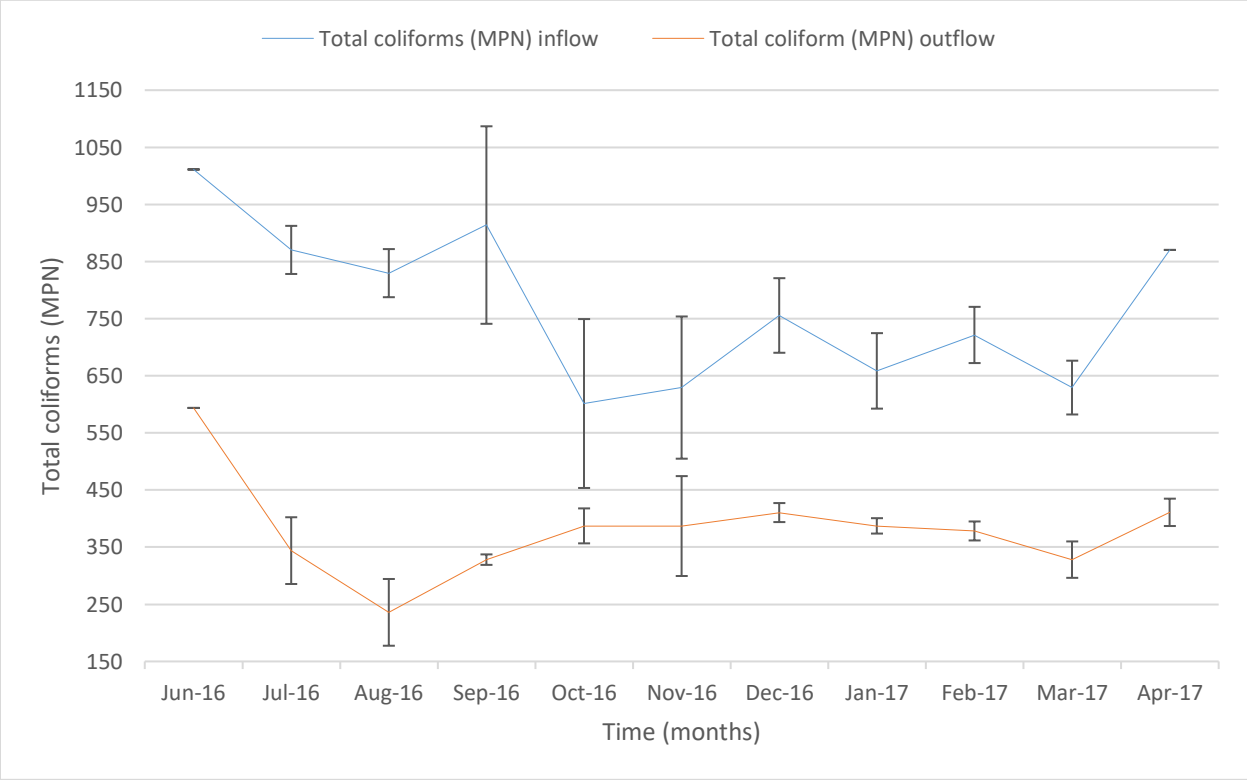

Figure 7: Average monthly total coliform count in the wetland inflow and outflow with standard deviation error bars.

4.1.6 Most Probable Number of *E. coli* in the wetland inflow and outflow

E. coli is a common faecal contaminant present in wastewater and its consumption can cause serious illness. Faecal pollution and high concentration of nutrients in the wastewater favours the development

of *E. coli*. The most probable number of *E. coli* per 100 ml sample in the wetland influent ranged from 219.8 to 517, while in the effluent samples the *E. coli* count ranged from 28.4 to 65.1 as depicted in figure 8. The wetland did show a significant reduction of *E. coli,* the average wetland removal efficiency for *E. coli* was 85%, even though the count was still outside the approved limit of 0 counts per 100 ml for irrigation water as set by the Department of Water Affairs and Forestry, as well as standard limit for purification of wastewater and effluent discharge into a natural water bodies. The wastewater or effluent shall contain no typical faecal coliforms or *E. coli* per 100 ml. The results obtained in this study are consistent with those reported by Vymazal (2018). *E. coli* count (MPN/100 ml) in the wetland influent was a bit higher because of the unsanitary conditions of the surrounding area, the discharge or seepage of sewage and domestic wastewater from the nearby informal settlement and occasional discharges of untreated sewage from the wastewater treatment works. *E. coli* is the best available indicator of faecal contamination from warm-blooded animals, including humans. Higher levels of *E. coli* in the water indicate a higher risk of contracting waterborne diseases, even if small amounts of produce are consumed. The removal of *E. coli* could be attributed to the combination of sedimentation and natural die offs supplemented by the effects of UV radiation and higher temperatures in the open water throughout the wetland. The faecal coliform, specifically *E. coli*, is used as an indication of the microbial quality of irrigation water. The wetland's removal efficiency of *E. coli,* which is 85%, is statistically significant since $p < 0.05$ ($p = 8.584e-8$). Zero standard deviation for each average data set, hence no error bars.

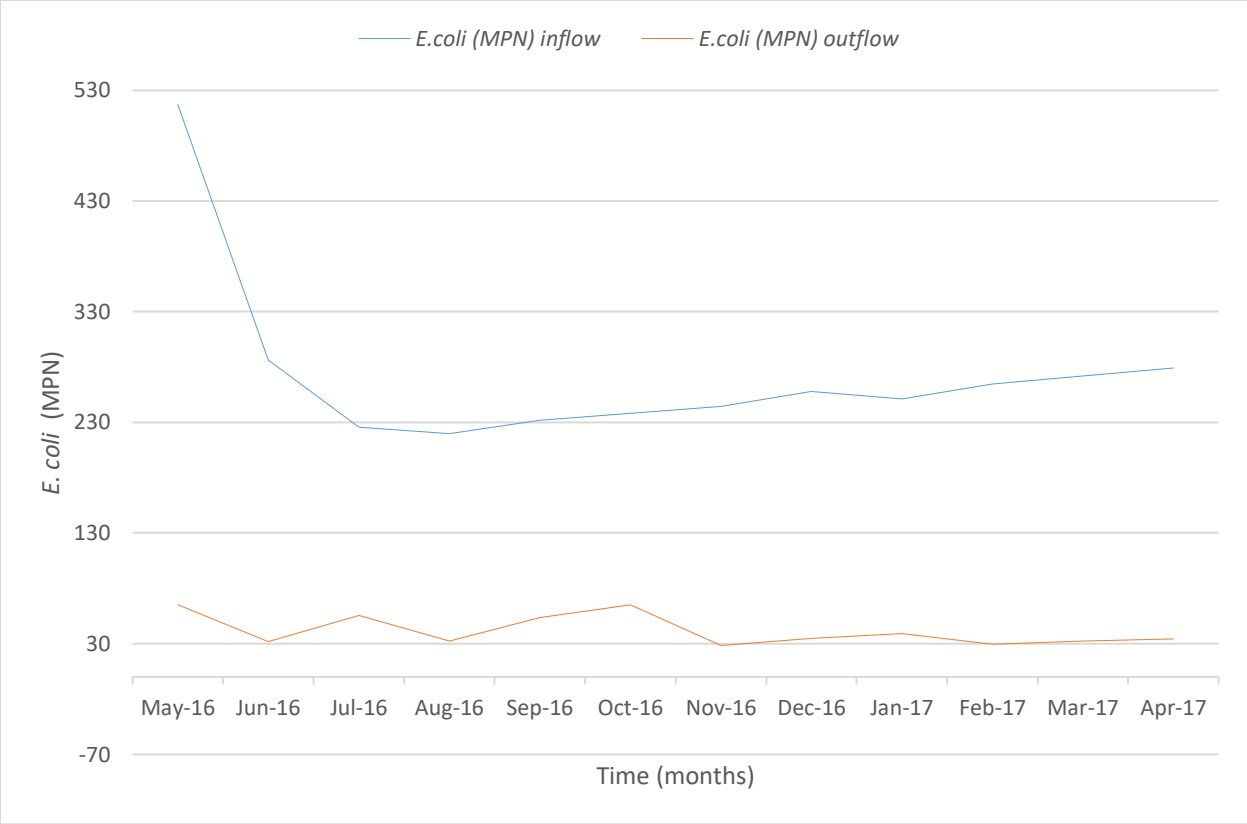

Figure 8: Average monthly *E. coli* count (MPN/100 ml) in the wetland inflow and outflow with no standard deviation error bars

4.1.7 Sulphate concentrations (mg/l)

Figure 9 shows that there was only moderate sulphate reduction. The overall wetland performance in removing sulphate was 49%. The sulphate influent values ranged from 88 to 126 mg/l while those of the wetland effluent ranged between 42 and 81 mg/l. The DWAF (1996b) guidelines did not provide a water quality range for sulphate. The study revealed that there are high levels of nutrients in the wetland thereby bringing forth risks of eutrophication. Chen *et al*. (2016) also reported that the presence of the wetland plant *Typha latifolia* had little effect on sulphate removal. The study showed that the different sulphate removal behaviours were mainly due to the litter decomposition rates and carbon supply in the constructed wetlands. Sulphate is a common contaminant of wastewater and is not usually considered a health concern but it can, under some circumstances, cause diarrhoea. However, sulphate reduction may produce hydrogen sulphide (H_2S) and organic sulphur (S) compounds, which normally cause aesthetic problems (taste, colour and/or odour) in the wastewater and the effluent-dominated river. The wetland sulphate removal efficiency of 49% is statistically significant since p <0.05 (p =3.4034e-16).

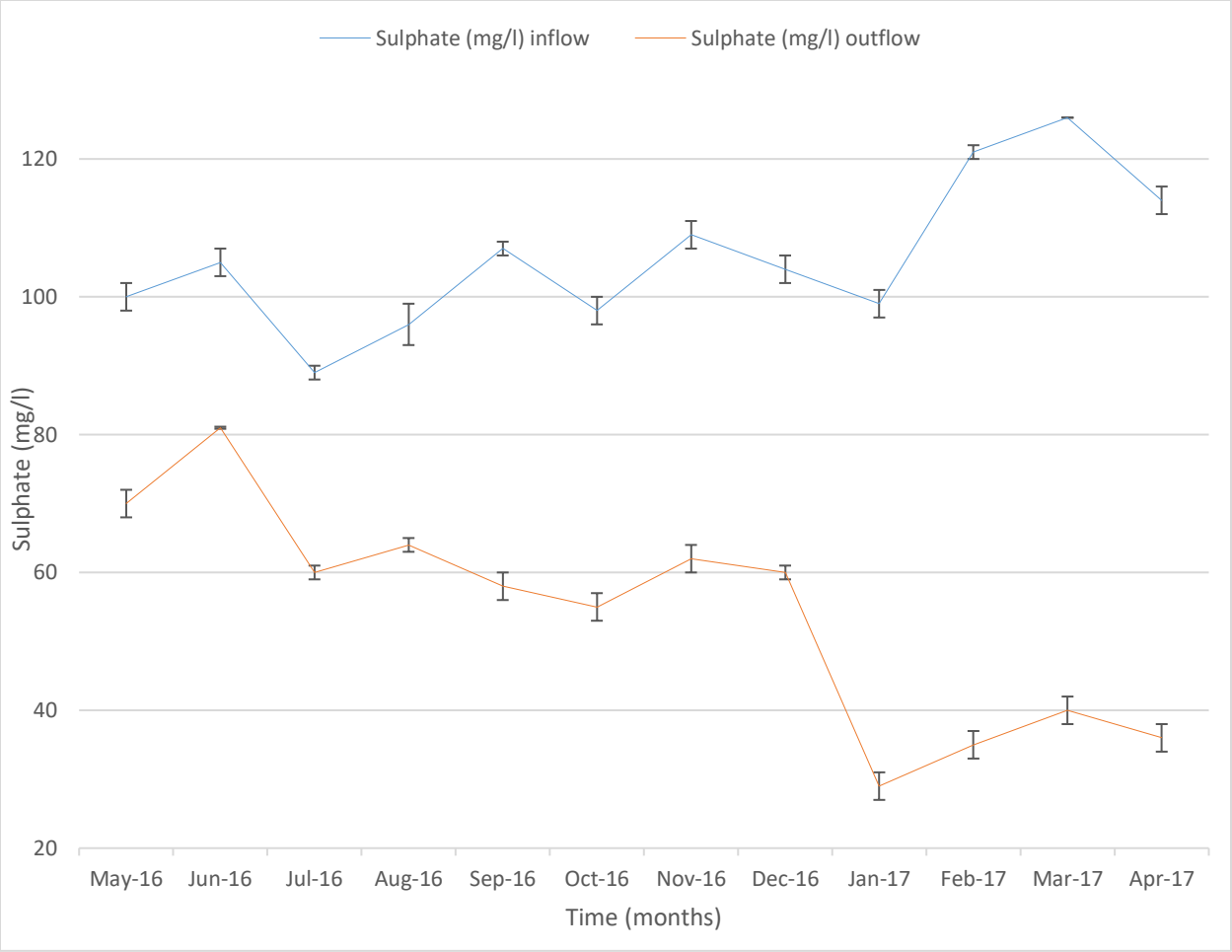

Figure 9: Average monthly sulphate (mg/l) in the wetland inflow and outflow with standard deviation error bars.

4.1.8 Nitrite concentrations (mg/l)

Data obtained during this study shows that NO_3^- concentrations in the influent and wetland effluent measured throughout the 12 months study period were within the agriculture and irrigation standard limits (0 – 15 mg/l) set by the department of Water Affairs. Nitrite values in the influent ranged from 0.63 to 0.99 mg/l while the effluent values ranged from 0.25 to 0.49 mg/l as shown in figure 10. The average removal efficiency for nitrite was 56%. According to Han *et al*. (2019) the contribution of the possible nitrogen removal pathways in the constructed wetlands are as follows; nitrification-denitrification (86.55%)>substrate adsorption (11.70%)>plant uptake (1.15%) >microbial assimilation (0.60%). The wetland nitrite removal efficiency of 56% is statistically significant since p <0.05 (p = 6.1981e-24).

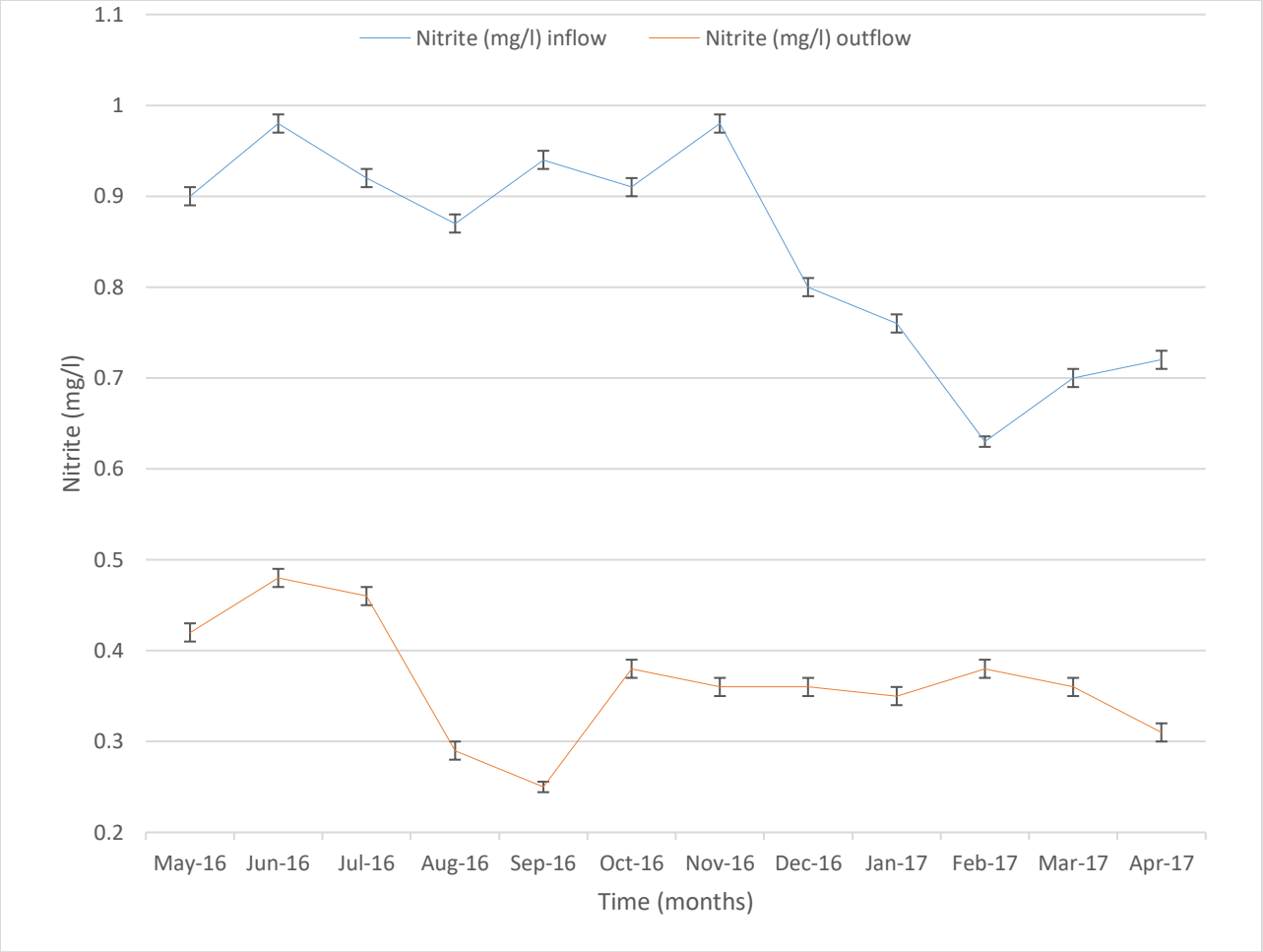

Figure 10: Average monthly nitrite (mg/l) in the wetland inflow and outflow with standard deviation error bars.

4.1.9 Nitrate concentrations (mg/l)

Nitrate concentration values in the influent ranged from 4.50-5.24 mg/l while the effluent values ranged from 0.30 – 0.99 mg/l. The amount of nitrate both in the wetland influent and effluent were within the irrigation limits of mg/l as per DWAF guidelines (0 – 10 mg/l). The wetland exhibited excellent removal efficiency of nitrate throughout the study period as shown in figure 11. The average removal efficiency for nitrate was 89%. Wetlands have proven to be effective in removing nitrate under widely varying weather conditions across seasons and years. Within the wetland there are various groups of microorganisms like algae, fungi and bacteria, which are capable of converting nitrate ions into organic matter through an assimilatory nitrate reduction process. This involves several enzymes including nitrate and nitrite reductases that reduces nitrate to form ammonia. Nitrate is an oxidised form of organic nitrogen and can sometimes occur in high concentrations under natural conditions (e.g., mineral salts derived from rock and soil) or as a result of seepage of sewage systems and leaching of

fertilizers from soil (DWAF 1996). The wetland nitrate removal efficiency of 89 is statistically significant since p <0.05 (p = 5.8042e-35).

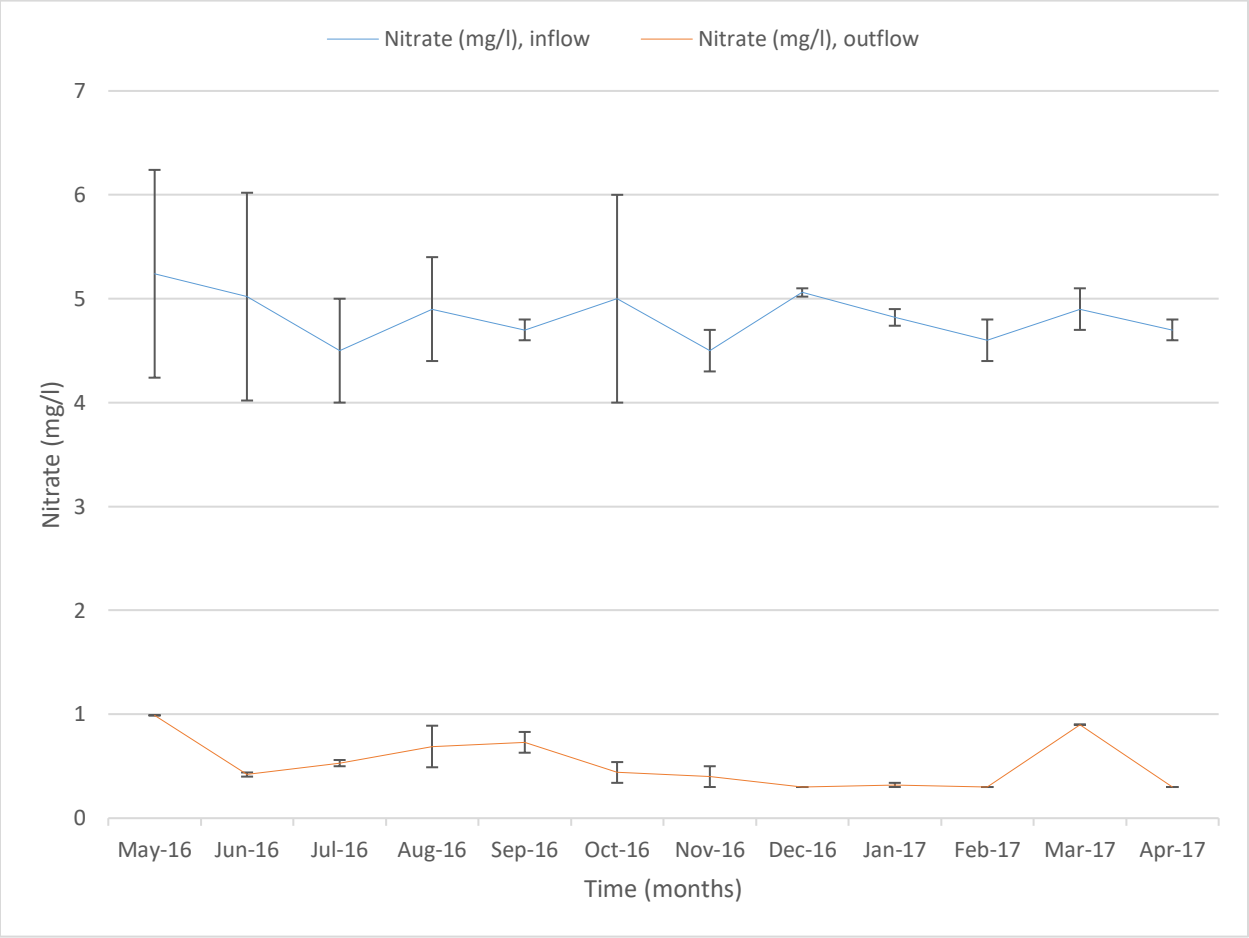

Figure 11: Average monthly nitrate (mg/l) in wetland inflow and outflow with standard deviation error bars.

4.1.10 Carbonate hardness (mmol/l H^+)

Wetland inflow Carbonate hardness values ranged from 3.0 – 4.8 mmol/l H^+ while the values in the effluent were between 1.9 – 3.6 mmol/l H^+. Average removal efficiency for carbonate hardness was 21%, which is by far the lowest removal compared to other pollutants. Calcium and magnesium can be a source of highly alkaline effluent water in constructed wetlands. Hardness is determined by the calcium and magnesium content of water. Since calcium and magnesium are essential plant nutrients, moderate levels of hardness of 1.64 – 2.46 mmol/l H^+ are considered ideal for plant growth. Water that contains high levels of dissolved calcium or magnesium salts, or both, is described as 'hard'. A bicarbonate hazard to soil fertility occurs when irrigation waters containing high concentrations of bicarbonate are used for several decades. As the bicarbonate becomes concentrated in the soil water

because of evapotranspiration, there is an increased tendency for calcium and magnesium to be precipitated as insoluble carbonates. The result over time is an increased Sodium Adsorption Ratio and the manifestation of detrimental effects in the presence of excess sodium. The wetland carbonate hardness removal efficiency of 21% is statistically significant p <0.05 (p = 8.13745e-8).

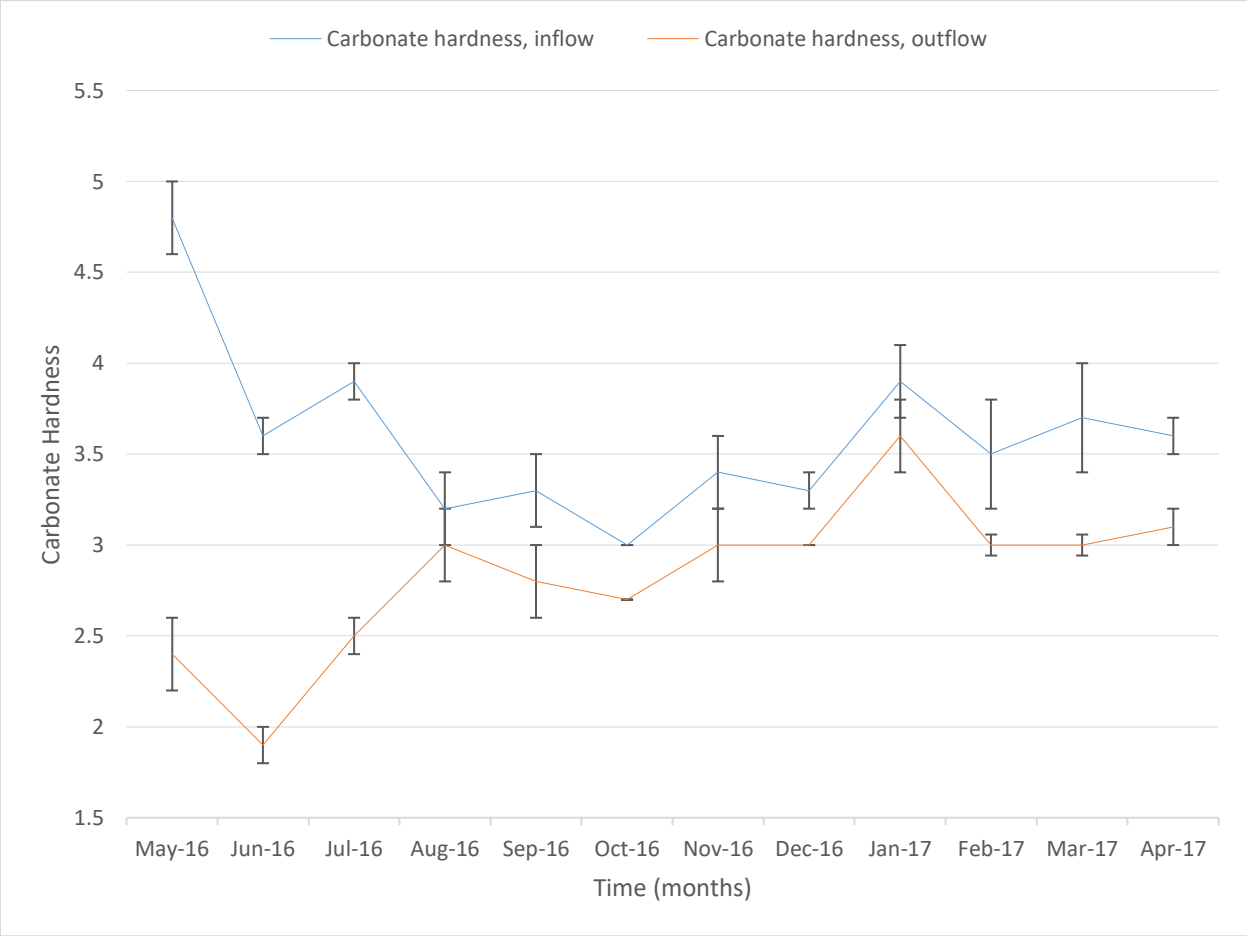

Figure 12: Average monthly carbonate hardness (mmol/l H^+) in the wetland inflow and outflow with standard deviation error bars.

4.1.11 Chemical Oxygen Demand (COD).

The influent Chemical Oxygen Demand levels ranged from 39 to 49 mg/l while the effluent Chemical Oxygen Demand levels ranged between 14 to 20 mg/l and the COD percentage reduction ranged from 51 to 70%. Average removal efficiency for COD = 61%. The COD elimination efficiencies of this field scale wetland are similar to those of the pilot wetlands that achieved COD elimination efficiency of 63% as reported by He *et al.* (2017). It was reported in another study that the real wetland's efficiency range of COD elimination ranged from 84–92%, while in the pilot wetland COD elimination efficiency ranged from 63–69% (Lehl *et al*. 2016). Measurement of COD is very important to evaluate organic

matter concentration in wetlands. Its reduction mechanisms include filtration, adsorption, and aerobic and anaerobic microbial metabolism. The COD reduction in wetlands has been confirmed by many researchers. COD associated with settleable solids in wastewater is removed by sedimentation while colloidal and soluble forms are removed by metabolic activity of microorganism as well as physical and chemical interactions within the root zone and substrate. The rate of biodegradation of various organic substances depends on the temperature, oxygen concentration, pH and presence of microorganisms. The wetland COD removal efficiency of 61% is statistically significant since p <0.05 (p = 2.344e-29).

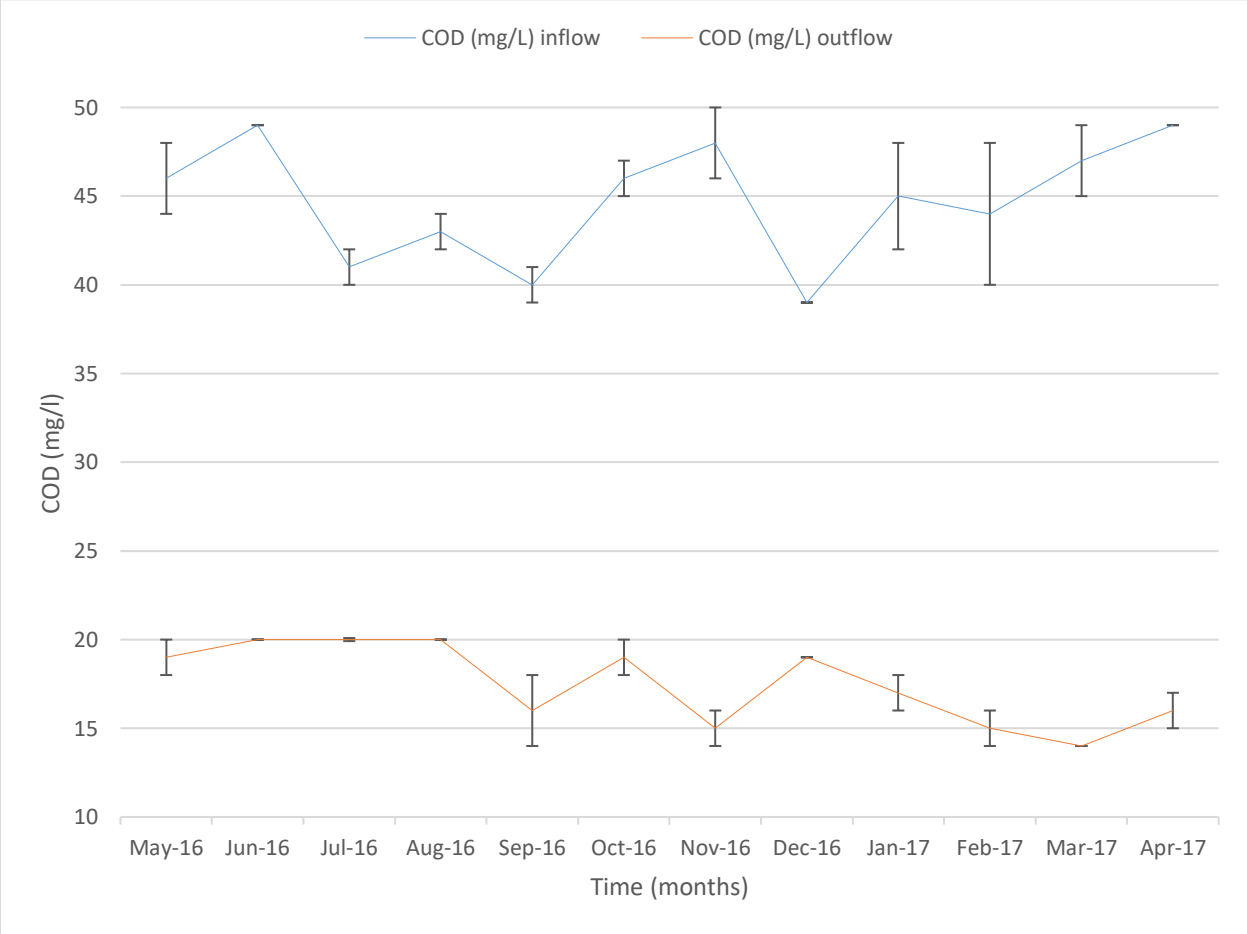

Figure 13: Average monthly COD (mg/l) in the wetland inflow and outflow with standard deviation error bars.

4.1.12 Five-day Biological Oxygen Demand (BOD_5).

Figure 14 shows that BOD_5 concentrations were significantly reduced as water flowed through the unchanneled valley bottom wetland, suggesting efficient treatment of BOD_5 in the wetland. Average BOD_5 Wetland Removal efficiency was 60%. The BOD_5 in the influent water ranged from 18.0 mg/l to

23.0 mg/l while the BOD_5 in the effluent water ranged from 5.5 mg/l to 10.5 mg/l. Singh and Chakraborty (2020) reported the BOD_5 wetland removal efficiency of 65.6%. The removal can be attributed to bacterial community and their enzymes together with the physical processes of sedimentation and filtration. The wetland BOD_5 removal efficiency of 61% is statistically significant since p <0.05 (p = 8.459e-28).

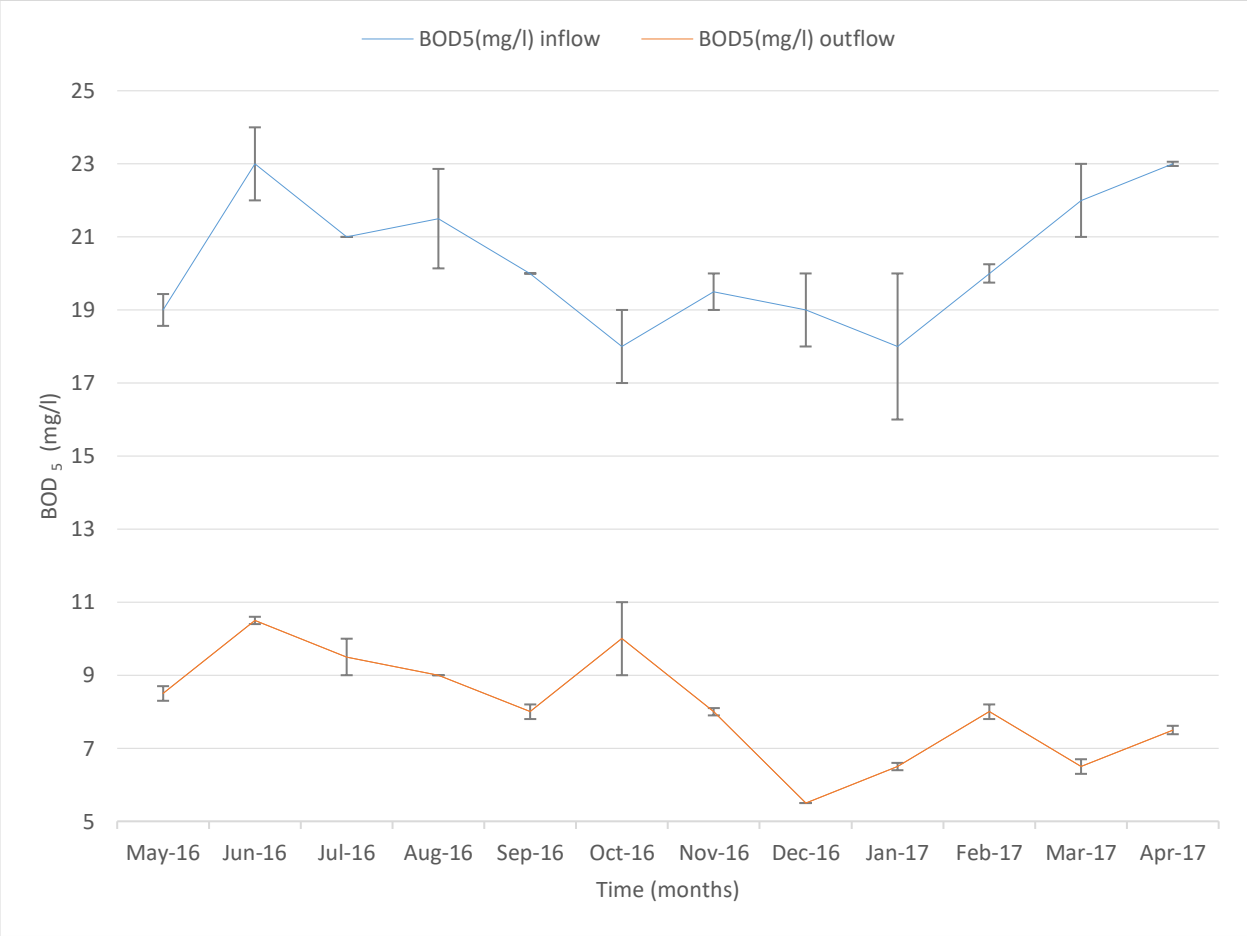

Figure 14: Average monthly BOD_5 (mg/l) in the wetland inflow and outflow with standard deviation error bars.

4.1.13. Total Suspended Solids (TSS).

Total Suspended Solids (TSS) are filterable particles that are larger than 2 microns found in the water column. The wetland influent TSS values varied from 44 mg/l to 200 mg/l, while there was a significant reduction in the effluent with values ranging from 10 mg/l to 80 mg/l as depicted in figure 15 below. The performance efficiency of the wetland with respect to TSS showed a consistent increase. TSS reduction efficiencies ranged from 50% to 77%, with the average removal efficiency of 61%. Even though the average wetland removal efficiency of 61% was achieved, TSS values were still outside

the target water quality range for irrigation water. In a study by Birch *et al.* (2004) the wetland removal efficiency for TSS in storm water from wetland was between 9% and 46%. It was recorded by Birch *et al*. (2004) that substantially higher TSS concentrations were observed in the effluent than in the influent during high water flow in the wetland. These removal efficiencies were a bit lower than 89% removal efficiency of constructed treatment wetlands reported by Vymazal (2018). The removal of total suspended solids takes place through a set of internal processes including production of transportable solids by wetland biota, low water velocity, wetland vegetation, substrate and filtration. The wetland influent samples had more solids still suspended giving them an opaque appearance (showing high turbidity) while the effluent samples were a bit clearer. TSS measurements for the effluent water should not exceed 10 mg/l according to the requirements for purification of wastewater or effluent. The wetland TSS removal efficiency of 61% is statistically significant since p <0.05 (p = 8.59569e-8).

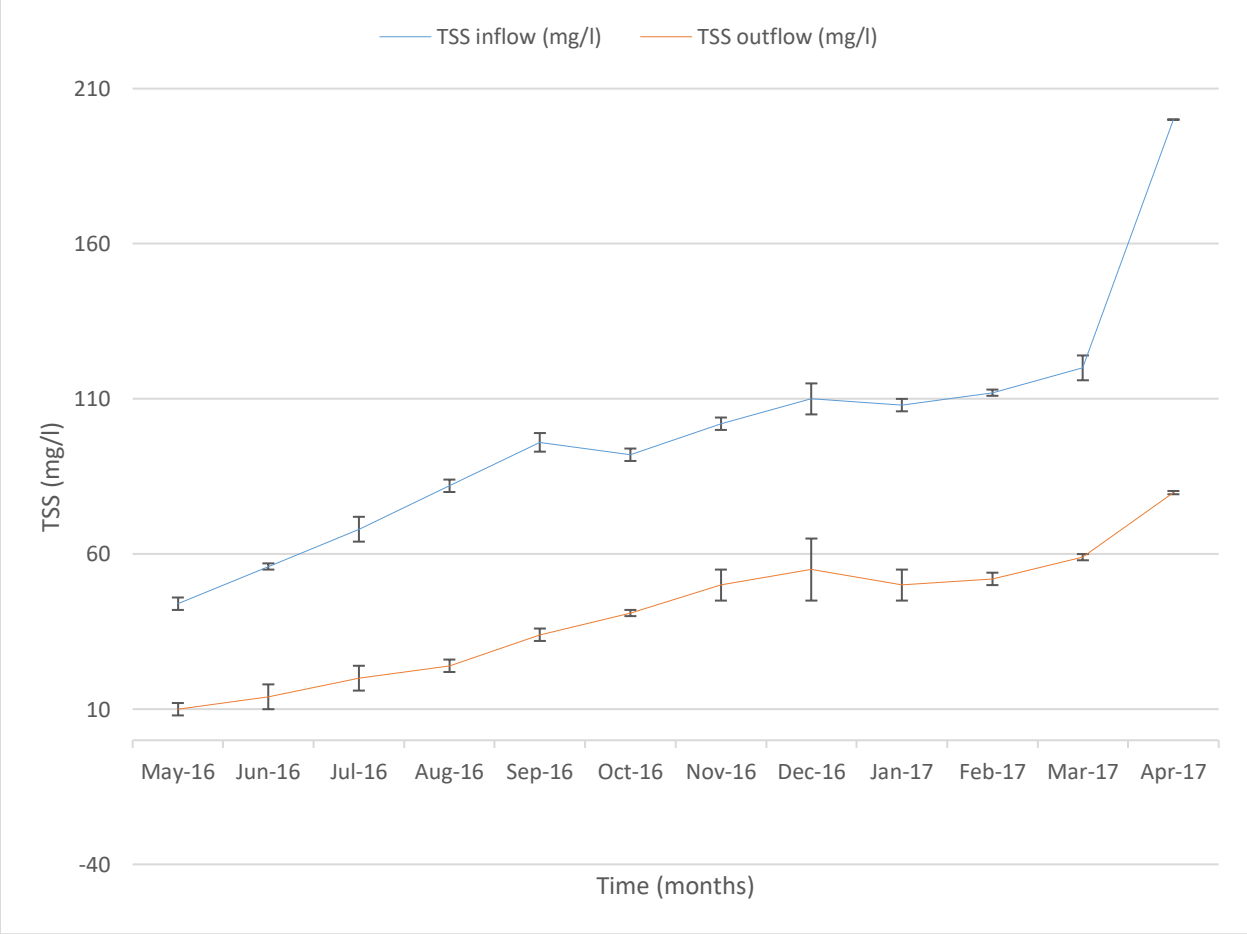

Figure 15: Average monthly TSS (mg/l) in the wetland inflow and outflow with standard deviation error bars.

4.1. 14 Aluminium concentrations (mg/l)

The aluminium values fall within the target water quality range as outlined by DWAF (1996) which is 0 – 5.0 mg/l. Average wetland removal efficiency for aluminium was 67%. Aluminium concentrations in the influent and effluent were very low, and the removal efficiency of the wetland was good. Aluminium is one of the macro soil constituents. Upon being applied to the soil, the aluminium in the irrigation water mixes with the aluminium in the dissolved phase in the soil, which in turn is in dynamic equilibrium with aluminium in other phases. The concentration of aluminium in solution is largely determined by soil pH. Toxicity of aluminium to field crops is an important cause of reduced crop productivity on acid soils, because the solubility of aluminium increases with increasing concentrations of hydrogen ions. The concentration of total aluminium in irrigation water should not exceed 5.0 mg/l for continuous use on all soils, or 20 mg/l for use up to 20 years on neutral to alkaline fine textured soils. Several plants, including wheat and barley, show signs of aluminium toxicity when grown in nutrient solutions that contain between 0.1 and 1.0 mg/l aluminium. These values cannot be applied directly to irrigation waters because of the capacity of soil to bind and hence reduce the solubility of aluminium ions. These values indicate that aluminium can be toxic to plants at relatively low concentrations. Several crops show aluminium toxicity at concentrations as low as0.1 - 0.5 mg/l in soil solution, however soils have the capacity to adsorb complex aluminium ions, thereby reducing their toxicity to plants (Canadian Council of Ministers of the Environment 2008). The wetland aluminium removal efficiency of 67% is statistically significant since $p < 0.05$ ($p = 2.38683e-18$).

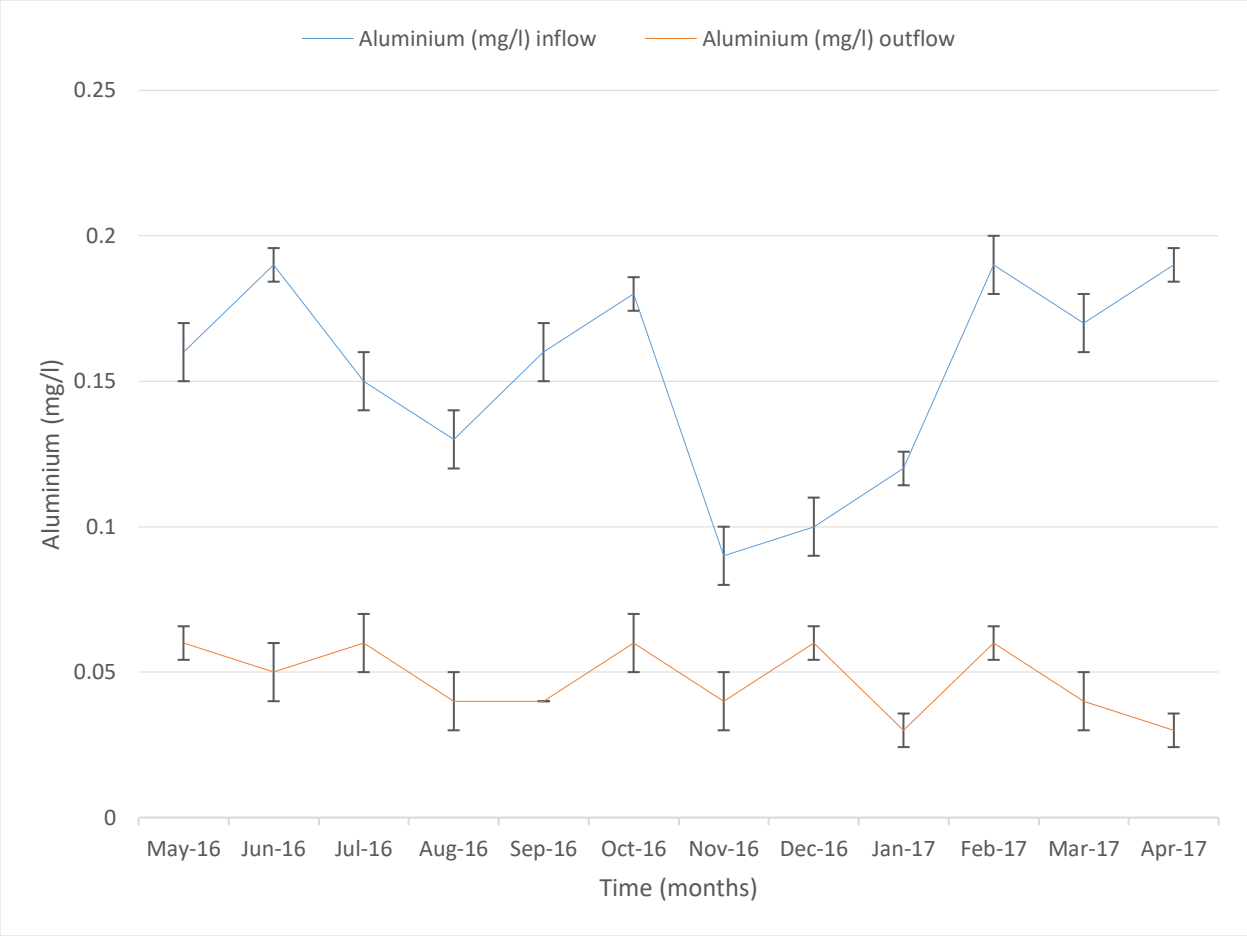

Figure 16: Average monthly aluminium (mg/l) concentrations in the wetland inflow and outflow with standard deviation error bars.

4.1.15 Iron (III) concentrations (mg/l)

Average wetland removal efficiency for iron was 52%. Total iron concentrations for all the twelve samples ranged from 0.40 to 0.54 mg/l in the influent while the values in the effluent ranged from 0.19 to 0.51 mg/l as depicted in figure 17 below. The wetland showed a moderate removal capability of just above 50%. The iron values were within the regulatory limits for irrigation water, the target water quality range for irrigation water is 0 – 5.0 mg/l as set by DWAF (1996)**.** The concentration of total iron in irrigation water should not exceed 5.0 mg/l for continuous use on all soils, or 20.0 mg/l for use up to 20 years on neutral and alkaline soils. Iron is an essential element in human nutrition, but food sources generally provide the minimum requirements (Canadian Council of Ministers of the Environment 2008). The significant iron removal of 98% occurred within a wetland at Whittle Colliery, UK. According to Lesley *et al*. (2008) iron can be removed from wastewater treated by wetland systems mainly through oxidative processes and iron hydroxide formation. The wetland iron removal efficiency of 52% is statistically significant since p <0.05 (p = 1.44834e-33).

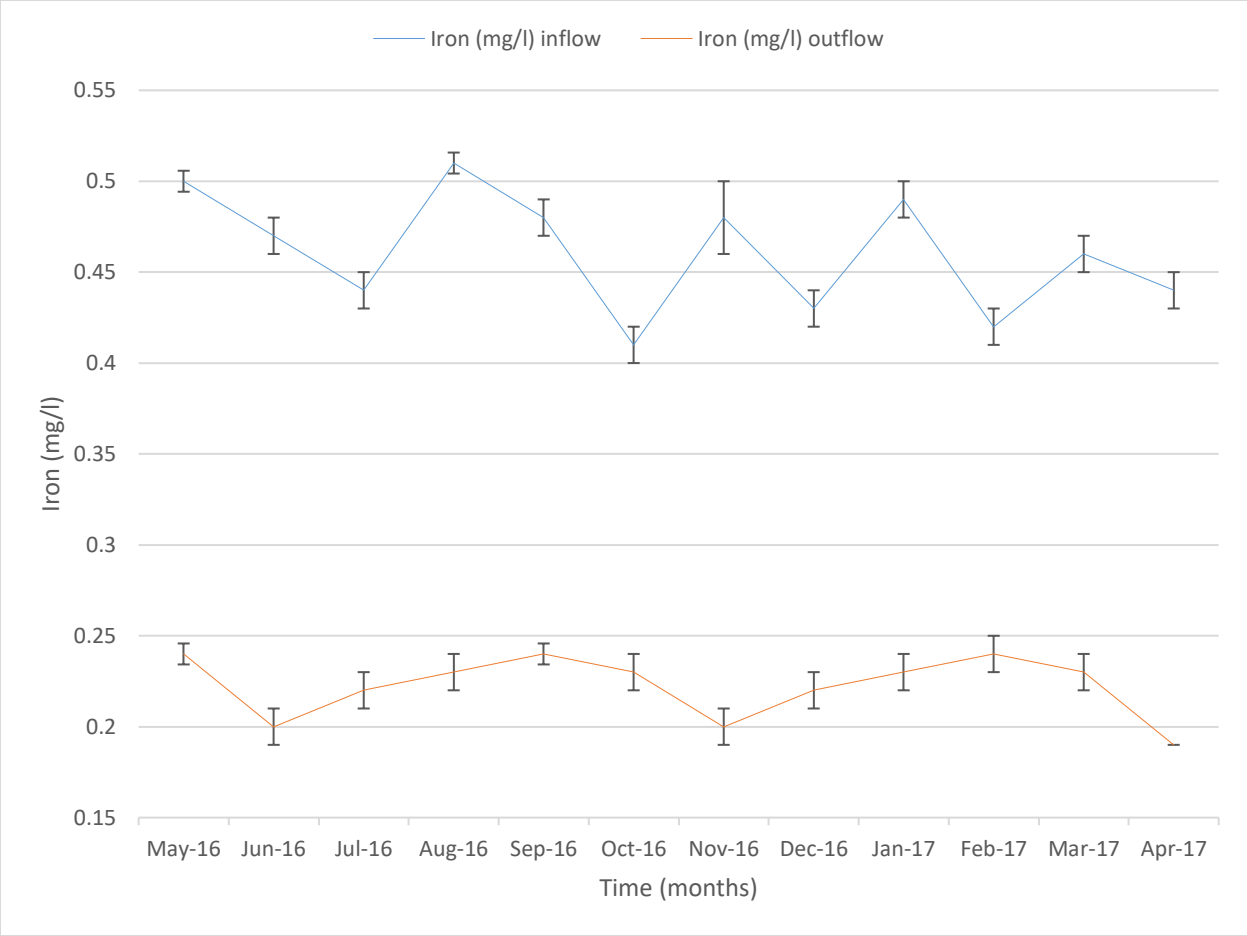

Figure 17: Average monthly Iron (mg/l) concentrations in the wetland inflow and outflow with standard deviation error bars.

4.1.16 Lead concentrations (mg/l)

Lead concentrations in the wetland were lower than the detection limit of 0.10 mg/l throughout the study period. Almost 99% of the samples had less than 0.10 mg/l Lead concentrations in the wetland effluent. During rainy seasons it is expected that the runoff erodes matters of chemical composition and may contribute in increasing levels of lead. The low levels of lead may just be testament that since the use of unleaded fuel in South Africa pollution caused by lead had really dropped. Hence even after the rain when it is expected that the water runoffs will increase concentrations of elements such as lead in the catchment areas, lead concentrations were still low. As a result, no percentage reduction could be calculated and removal wetland efficiency could not be determined. The concentration of total lead in irrigation water should not exceed 0.20 mg/l for continuous use on all soils, and 2.0 mg/l for use on neutral and alkaline fine-textured soils for up to 20 years (Canadian Council of Ministers of the Environment 2008).

4.1.17 Manganese concentrations (mg/l)

Manganese concentration values in the influent ranged from 1.69 – 2.14 mg/l while the effluent values ranged from 0.71 – 1.56 mg/l as depicted in figure 18 below. Average wetland removal efficiency for manganese was 51%. Manganese concentration decreased as it passed through the wetland and the concentration value 0.21 mg/l was a little bit higher than the target water quality range for irrigation set at less or equal to 0.20 mg/l for unrestricted irrigation on all soils. The irrigation standard permits manganese concentrations of 0.2-10.0 mg/l when water is used on fine textured, neutral to alkaline soils. Levels of manganese above 0.05 mg/l can cause clogging of irrigation equipment. Above 2.0 mg/l manganese may be toxic to some sensitive plants and may also be poisonous or injurious to trout, other fish and forms of aquatic life. Other researchers, Kadlec and Knight (2008) recorded 54% as average manganese reduction rate in free water surface flow wetlands. The main mechanism responsible for Manganese removal is precipitation. The concentration of total manganese in irrigation water should not exceed 0.2 mg/l for continuous use on all soils, and 10.0 mg/l for use up to 20 years on neutral and alkaline fine-textured soils (Canadian Council of Ministers of the Environment 2008). The wetland manganese removal efficiency of 51% is statistically significant since p <0.05 (p =8.2958e-28).

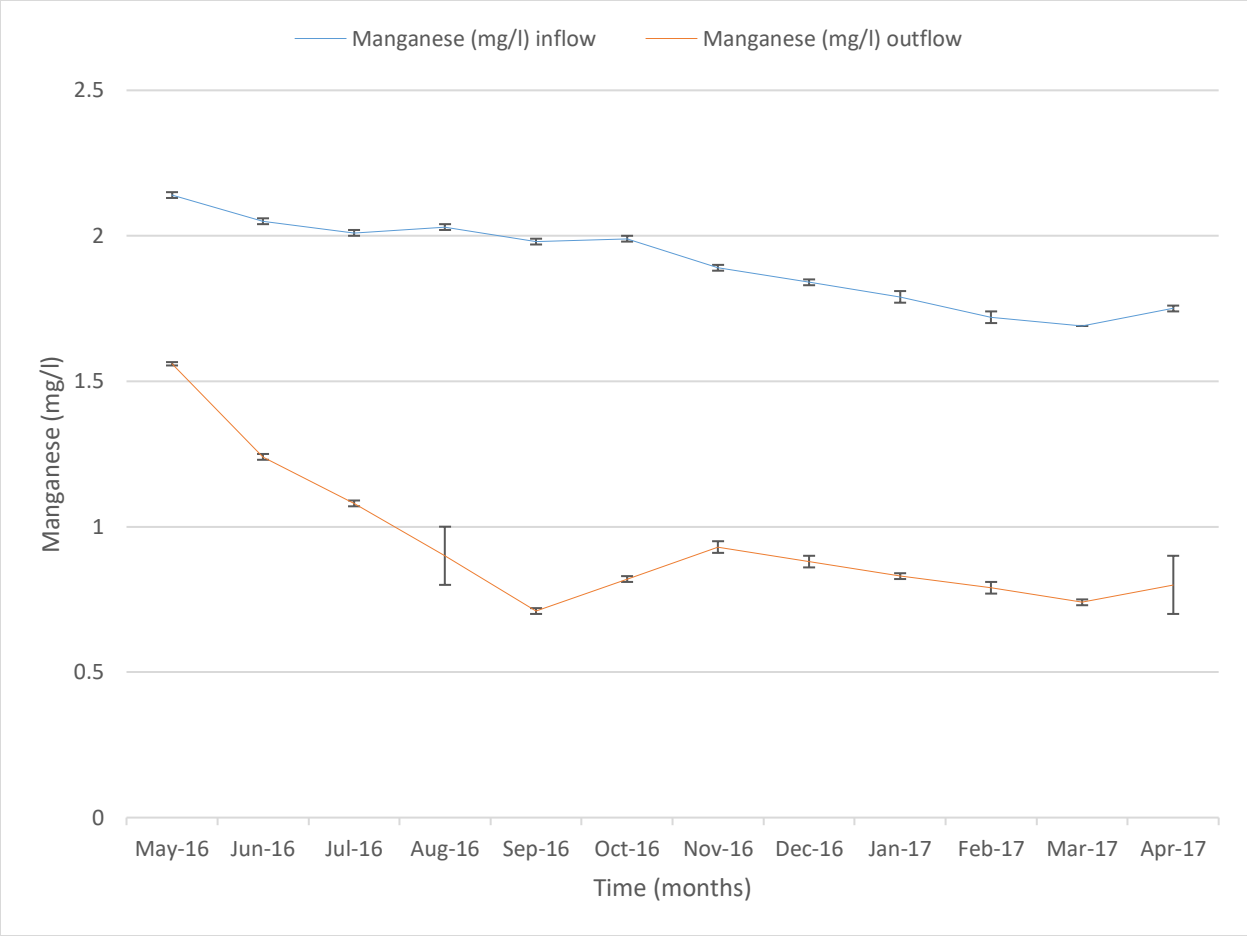

Figure 18: Average monthly manganese (mg/l) concentrations in the wetland inflow and outflow with standard deviation error bars.

4.1.18 Zinc concentrations (mg/l)

Zinc values in wetland influent ranged from 0.20- 0.31 mg/l while in the effluent the values ranged from 0.10 – 0.15 mg/l. Zinc values remained low throughout the twelve months monitoring period while average wetland removal efficiency for zinc was recorded at 53%. Birch *et al.* (2004) reported the average removal efficiency for zinc to be 52%, which is very close to the removal efficiency of the current study. In another study by Xu and Mills (2018) the wetland removal efficiency for zinc was 71%. Sima *et al*. (2016) reported the highest removal efficiency for zinc to be 90%. A study by Song *et al*. (2001) reported the average removal efficiencies for zinc in eight wetlands to be 72%. The concentration of total zinc in irrigation waters should not exceed 1.0 mg/l for continuous use on all soils below pH 6.5; while at higher pH, a limit of 5.0 mg/l is recommended. The zinc levels in the wetland were lower than the Department of Water Affairs and Forestry target range (0-1.0 mg/l) for irrigation as well as discharge of effluent into water bodies. Although zinc is essential to the growth of plants, it causes a toxic response by inducing iron deficiency when minimal requirements are exceeded

(Canadian Council of Ministers of the Environment 2008). The wetland zinc removal efficiency of 53% is statistically significant since p <0.05 (p = 4.16852e-25).

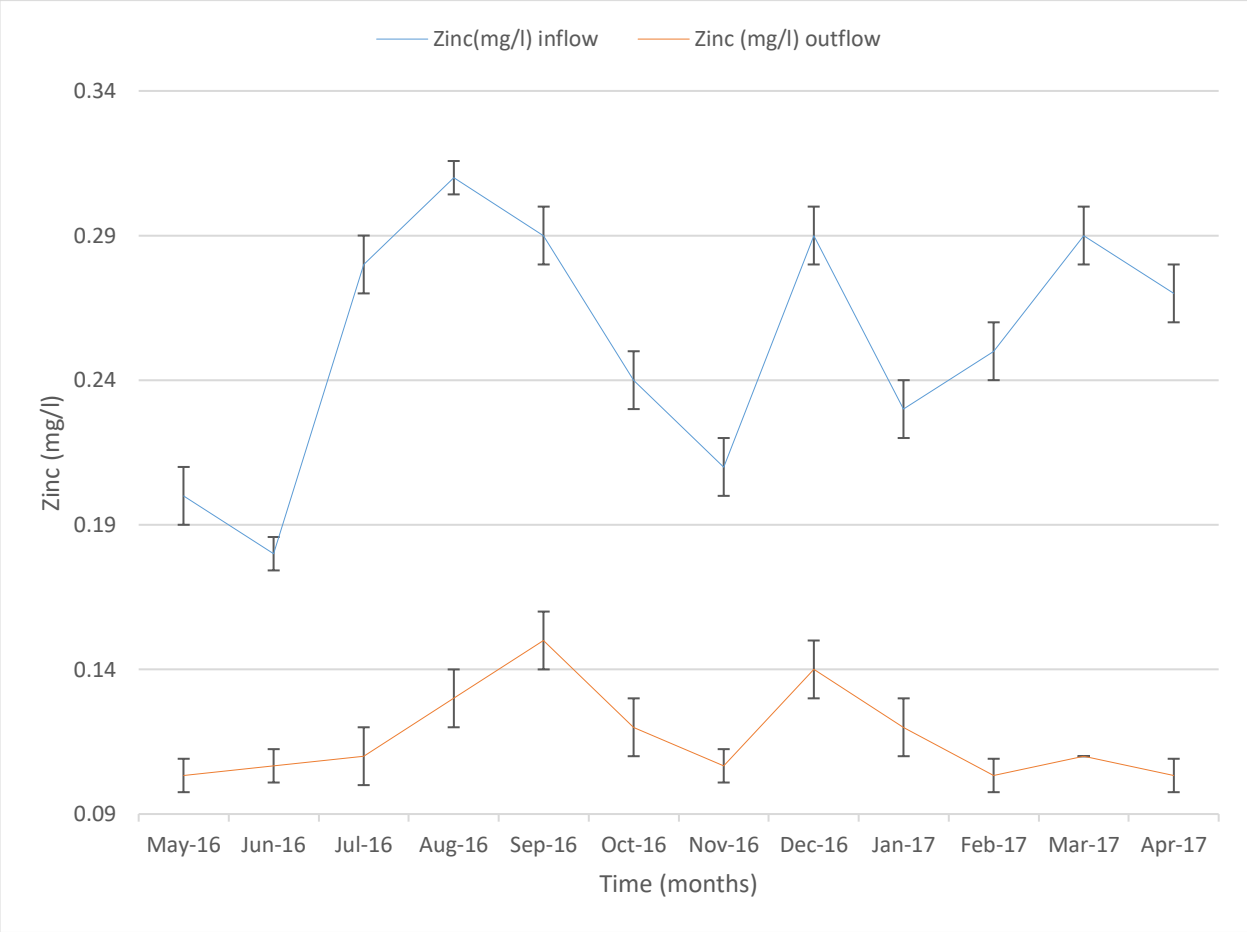

Figure 19: Average monthly zinc (mg/l) concentrations in the wetland inflow and outflow with standard deviation error bars.

4.1.19 Cadmium concentrations (mg/l)

Cadmium concentration in the wetland was very low throughout the sampling period, it always measured less than 0.05 mg/l. The concentration of cadmium was lower than the minimum detectable value, and thus its levels conformed to the water quality for irrigation water which is 0 – 0.01 mg/l (DWAF 1996). The presence of cadmium in the aquatic environment is of concern because it bioaccumulates, and it is highly toxic to living organisms. Cadmium is readily taken up by vegetation, although it is not required by plants for metabolism. The rate of cadmium uptake by plants depends on soil type and on levels of other elements, such as copper and zinc. Cadmium usually occurs at relatively low concentrations in soils. Because cadmium is similar to zinc, an essential element for

plant growth, it can readily interfere with metabolic processes, and is therefore toxic to many plants (Canadian Council of Ministers of the Environment 2008). Cadmium is not usually found in water at concentrations greater than 1 mg/l, unless the water has been subject to pollution from industrial effluents containing cadmium (DWAF 1996).

4.1.20 Chromate concentrations (mg/l)

Chromate levels were very low and the measured concentrations in the influent and effluent were below the detection limit throughout the study period. The concentration was less than 0.01 mg/l throughout the duration of the study. Chromium values for irrigation water should be between 0 – 0.1 mg/l according to DWAF (1996). The concentration of total chromium in irrigation water should not exceed 0.1 mg/l for continuous use on all soils. Chromium at concentrations of 1-10 mg/l in nutrient solution reduces plant yield, depending on the variety and species of plant. When chromium solution is added to soil, however, Cr (VI) remains mobile and available to plants, whereas Cr (III) is adsorbed or complexed. The availability of chromium to Plants also depends on the soil and its moisture content (Canadian Council of Ministers of the Environment 2008). Chromium is not essential for plant growth, but is toxic at high concentrations. Similar to most trace elements, chromium is strongly adsorbed by soil (DWAF 1996).

4.1.21 Copper concentrations (mg/l)

Copper concentrations in the influent ranged from 0.46 – 0.99 mg/l and the effluent values ranged from 0.05 to 0.21 mg/l as depicted in figure 20 below. Average wetland removal efficiency of copper was 83%, which is quite good. In a study by Sima *et al*. (2016) the removal efficiency for copper was 81.5% while a wetland study by Sheoran (2017) attained the removal efficiency of 92%. The removal efficiency of a wetland studied by Xu and Mills (2018) achieved a 65.9%. The concentrations of copper did conform to the target water quality range for irrigation of 0 - 0.2 mg/l (DWAF 2016). The concentration of total copper in irrigation water should not exceed 0.2 mg/l for continuous use on all soils or 0.2-10.0 mg/l on fine textured neutral to alkaline soils. For irrigation of crops that have a low sensitivity to copper, such as cereals, a maximum copper concentration in irrigation water of 1 mg/l is recommended. The concentration of copper may be increased to 5 mg/l for use on neutral to alkaline soils for up to 20 years (Canadian Council of Ministers of the Environment 2008). The wetland copper removal efficiency of 83% is statistically significant since $p < 0.05$ ($p = 8.63706e-8$).

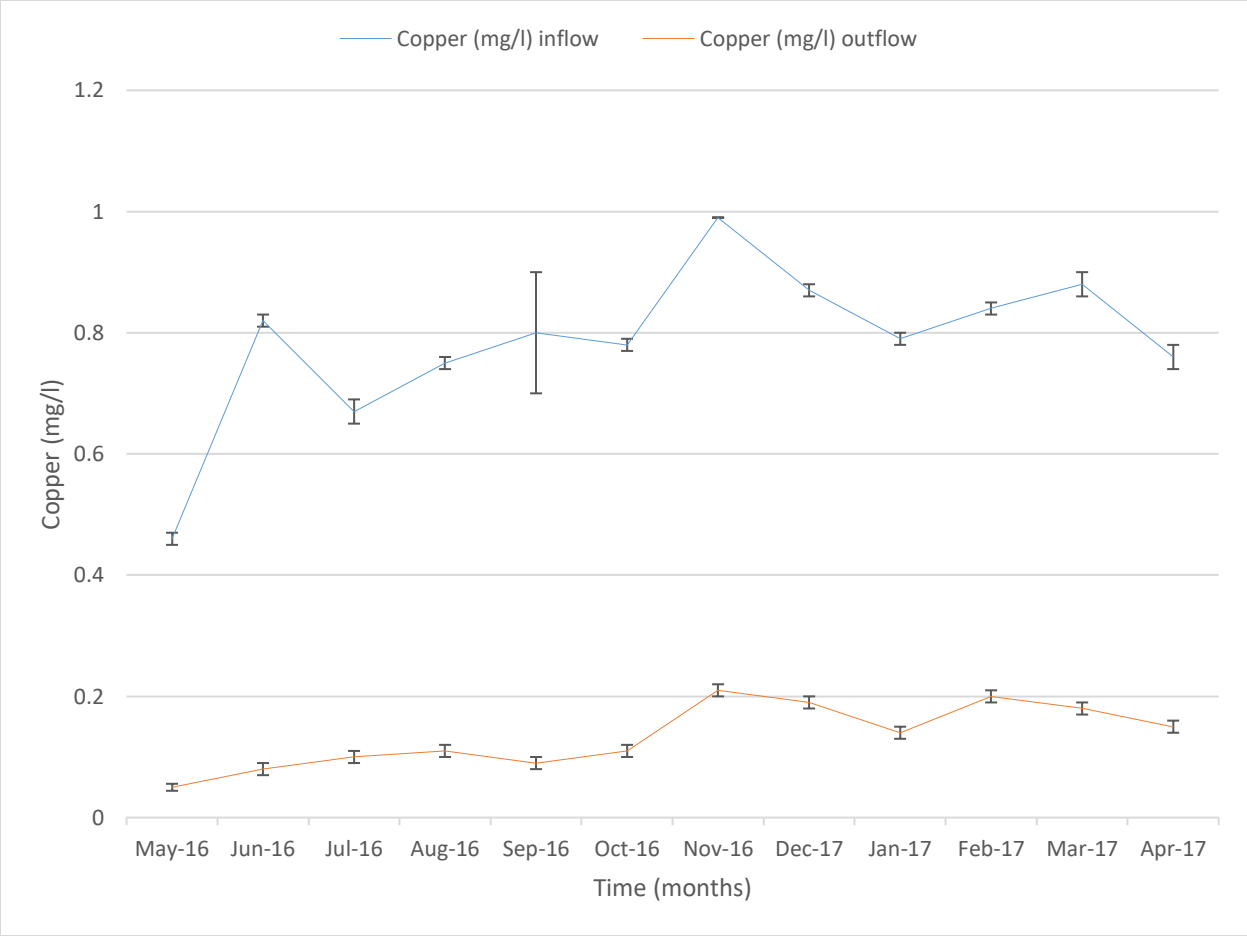

Figure 20: Average monthly copper (mg/l) concentrations in the wetland inflow and outflow with standard deviation error bars.

4.1.22 Orthophosphate concentrations (mg/l)

The ortho-phosphate values in the wetland inflow ranged between 2.0 to 3.02 mg/l while the values in the outflow ranged from 1.01 to 1.30 mg/l as shown on figure 21. The average removal efficiency for ortho-phosphate was 54%. The effluent ortho-phosphate values were within the water quality limits/standards for irrigation of 0-2.0 mg/l as set by DWAF (1996). Phosphates are chemicals that contain the element phosphorus, and when discharged into water bodies such as rivers, lakes and oceans, it affects water quality by causing excessive growth of algae, leading to eutrophication. Eutrophication, dystrophication or hypertrophication is when a body of water becomes overly enriched with minerals and nutrients which induce excessive growth of algae. Algal blooms lead to oxygen depletion, or hypoxia, a common effect of eutrophication in water. The direct effects of hypoxia include fish kills, especially the death of fish that need high levels of dissolved oxygen. Changes in fish

communities may have an impact on the whole aquatic ecosystem and may deplete fish stocks. Phosphorus as soluble ortho-phosphate is a critical nutrient in all biological processes, it is utilized by bacteria in making the energy molecules (ATP molecules) and in creating phospholipids bilayers in their cell structure. Determining orthophosphate content from different points (inflow and outflow) in a wetland is very important because the build-up or excessive phosphates or phosphorus can cause plant to grow poorly and also cause plants to die. This is because excessive phosphorus in the soil reduces the plant's ability to take up other micronutrients, particularly iron and zinc, even when the soil tests show that there is adequate amount of these two nutrients. Phosphorus build-up is caused by excessive use of inorganic fertilizers or the use of composts and manure. The study showed that the wetland is efficient in removing orthophosphate. The wetland removal efficiency of 54% is statistically significant since p <0.05 (p = 8.74313e-8).

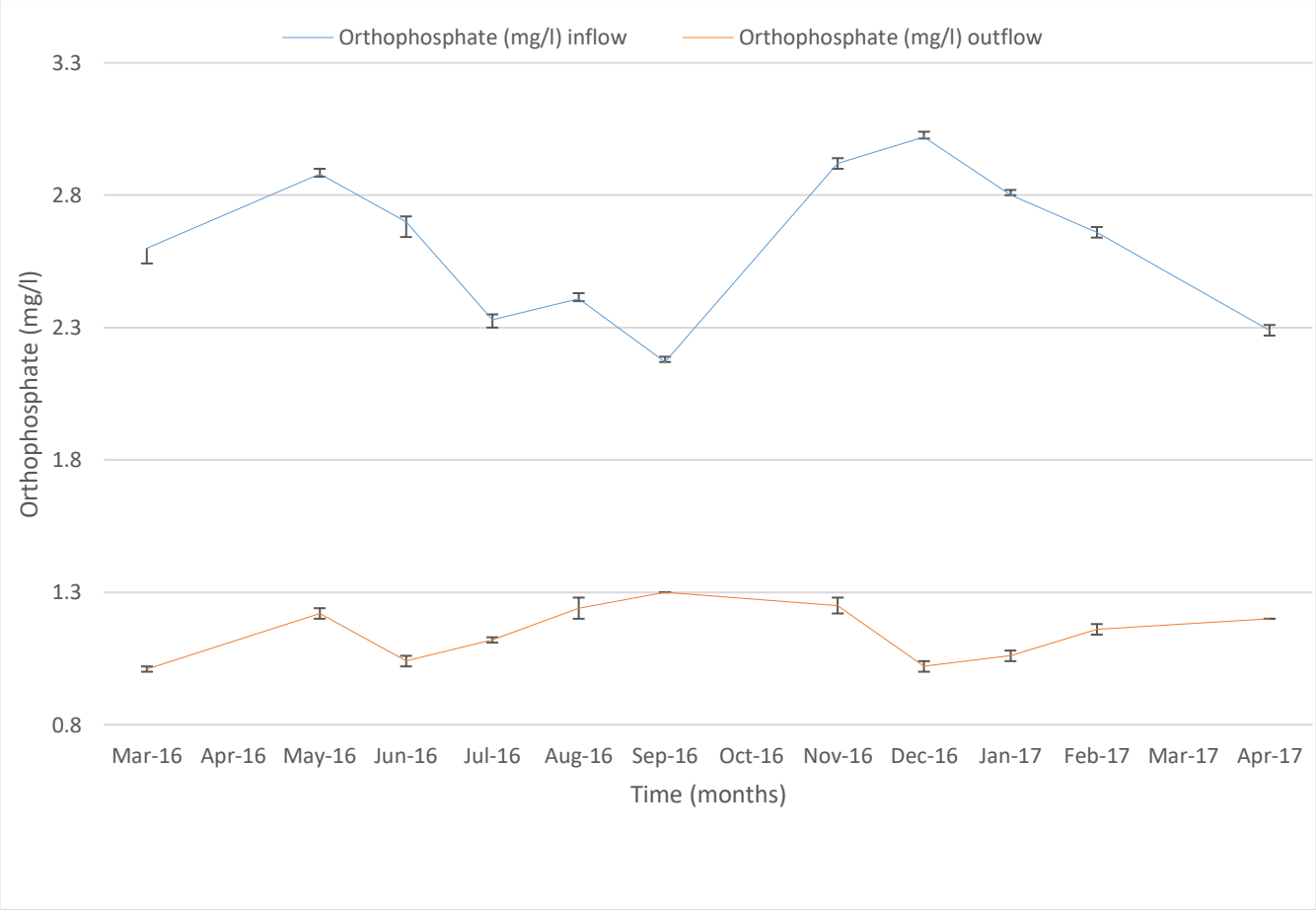

Figure 21: Average monthly orthophosphate (mg/l) concentrations in the wetland inflow and outflow with standard deviation error bars.

Table 4.2a. Maximum and minimum values for wetland influent and effluent compared to South African irrigation water standards

Parameter	Influent		Effluent		Legal requirement for irrigation: South African Water Quality Guidelines for Irrigation water. Target Water Quality Range (TWQR)
	Min	Max	Min	Max	
Total Coliforms	601.5	1011.2	235.9	593.8	1000 counts per 100 ml
E. coli	219.8	517	28.4	65	0 count per 100 ml
Sulfate	88 mg/l	126 mg/l	27 mg/l	81 mg/l	0-20 mg/l
Nitrate	4.50 mg/l	5.24 mg/l	0.30 mg/l	0.99 mg/l	0-10 mg/l
Nitrite	0.70 mg/l	0.98 mg/l	0.25 mg/l	0.48 mg/l	0-15 mg/l
BOD_5	18 mg/l	23 mg/l	5.5 mg/l	10.5 mg/l	-
Carbonate hardness	3.0 mmol/l H^+	5.0 mmol/l H^+	1.7 mmol/l H^+	3.80 mmol/l H^+	-
COD	39 mg/l	50 mg/l	14 mg/l	20 mg/l	Does not exceed 400 mg/l for irrigating 500 m^3 of land, and not exceed 75 mg/l for 2000 m^3
EC	142 µS/cm	507 µS/cm	58.5 µS/cm	334 µS/cm	<200 mS/cm
TSS	44 mg/l	200 mg/l	10 mg/l	80 mg/l	50 mg/l
Orthophosphate	2.17 mg/l	3.02m g/l	1.02 mg/l	1.30 mg/l	0-2.0 mg/l
pH	6.8	7.4	6.8	7.3	pH between 6.5 and 8.4; should not be below 6 and above 9
Aluminium	0.16 mg/l	0.20 mg/l	0.03 mg/l	0. 06 mg/l	5 mg/l unrestricted use on all soils. 5-20 mg/l for fine textured, neutral to alkaline soils
Copper	0.46 mg/l	0.99 mg/l	0.05 mg/l.	0.21 mg/l	0.2 mg/l unrestricted on all soils. 0.2 -5.0 mg/l maximum acceptable on fine textured neutral to alkaline soil.
Iron	0.44 mg/l	0.51 mg/l	0.19 mg/l	0.24 mg/l	5.0 mg/l unrestricted use on all soils;5.0-20 mg/l on fine textured neutral to alkaline soil
Manganese	1.69 mg/l	2.15 mg/l	0.71 mg/l	1.56 mg/l	0.2 mg/l unrestricted use on all soils;0.2-10.0 mg/l on fine textured neutral to alkaline soils
Zinc	0.20 mg/l	0.31 mg/l	0.10mg/l	0.15 mg/l	1.0 mg/l unrestricted use on all soils; 1.0 -5.0 mg/l on fine textured neutral to alkaline soils

Electrical conductivity, ortho-phosphate, aluminium, copper, iron, manganese and zinc were all removed satisfactorily to meet the South African irrigation water standards. The wetland performance

was good in improving the water quality, even though pollutants such as total coliforms, *E. coli*, TSS and sulphate were still higher than the prescribed irrigation water quality limits.

4.3 Biochemical tests

The isolates identified as metal tolerant were then further characterised and the results of biochemical characterisation are shown in table 16. Cellular morphology such as cell shape, arrangement and gram reaction were recorded using the gram stain technique and microscopy. Motility test revealed the ability of bacteria to move by means of a flagellum or multiple flagella. Endospore stain showed the presence or absence of spores. Biochemical tests revealed the ability of bacterial isolates to produce the enzymes catalase and oxidase as well as indole production, mannitol fermentation, lactose fermentation and production of hydrogen sulphide.

Table 1: Biochemical tests

Isolate	Gram reaction	Morphology	Arrangement	Colony colour	Motility	Endospores	Catalase	Oxidase	Indole	H_2S	MSA
1SRA	+	Rods	pairs, chains	Intense yellow	+	-	+	+	-	-	+
4SRD	+	Rods	single	Cream white	+	+	+	+	+	-	+
5SRE	+	Rods	chains	yellow	+	-	+	+	-	-	+
6SRF	+	Rods	single	white	+	+	+	+	-	-	+
8SRH	-	Rods	pairs	white	+	-	+	-	-	-	-
9SRI	+	Cocci	clusters	Cream white	-	-	+	-	-	-	+
10SRJ	+	Cocci	Clusters	Light yellow	-	-	-	-	+	-	+
11SRK	-	Rods	Clusters, chains	white	+	-	+	+	-	-	-
16SRP	+	Cocci	Clusters	Pinkish orange	-	-	+	+	-	-	+
18SRR	+	Rods	Single, pairs	Lemon yellow	+	-	+	-	-	-	+
21SRU	+	Rods	pairs	yellow	-	-	+	-	-	-	+
23SRW	+	Cocci	single	Light yellow	-	-	+	-	-	-	+
24SRX	+	Variable, Cocci-rods	chains	yellow	-	-	+	-	-	-	+
25SRY	+	Cocci	single	Pinkish	-	-	+	+	-	-	+
14TWN	+	Rods	single	Orange yellow	+	-	+	-	-	-	+

4.4 Identification of bacterial isolates using molecular techniques (16 S rDNA PCR and sequencing)

4.4.1 PCR

Genomic DNA was isolated from the metal tolerant rhizobacterial isolates from *Typha capensis* and the 16S rDNA gene was amplified for preliminary identification of the isolates. PCR results are shown in figure 13. The purified PCR product (8 µl) was separated by electrophoresis in 1 % agarose gel. The expected size for PCR products was 1000 bp. Primers used for polymerase chain reactions were 27F (GAGTTTGATCCTGGCTCAG) and 907R (CCCCGTCAATTCATTTGAGTTT). The first lane (M) is a 10 kb KAPA DNA ladder, lane 2 up to lane 16 are PCR amplicons of isolates 1SRA, 4SRD, 5SRE, 6SRF, 8SRH, 9SRI, 10SRJ, 11SRK, 14TWN, 16SRP, 18SRR, 21SRU, 23SRW, 24SRX, and 25SRY respectively.

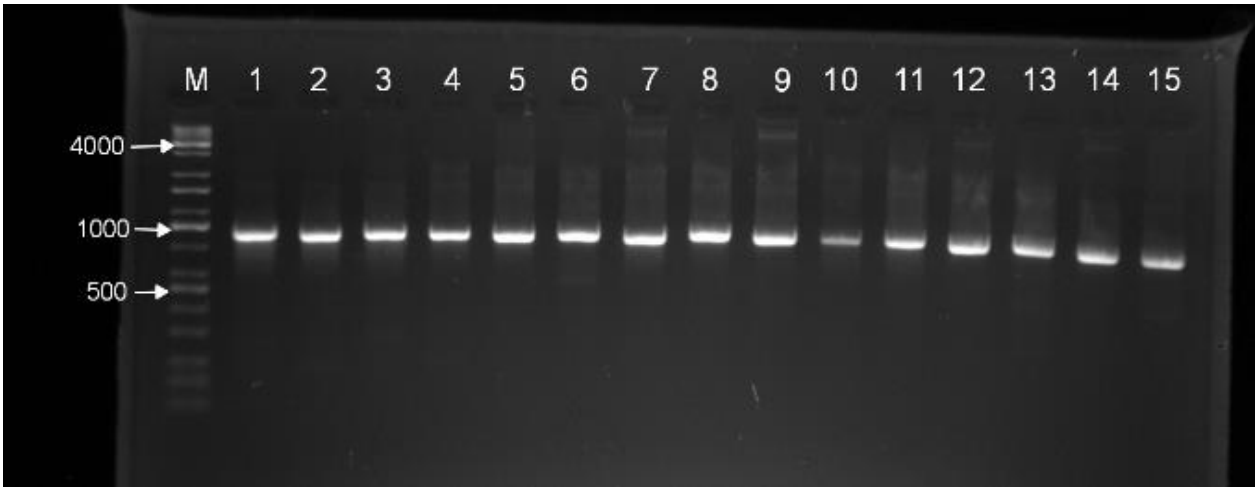

Figure 22: PCR products of the 15 bacterial isolates separated on 1% agarose gel

4.4.2 BLAST analysis

Bacteria isolated from the *Typha capensis* rhizosphere were identified by means of BLAST analysis. As seen in Table 17, the 16 S rDNA sequences were compared with GenBank entries using the Advanced Basic-Local- Alignment-Search-Tool on NCBI (http://www.ncbi.nlm.nih.gov/). Heavy metal tolerant strains isolated and identified in this study included *Acinetobacter pittii, Alcaligenes feacalis, Arthrobacter luteolus, Bacillus safensis, Exigubacterium aretemiae, Exiguobacterium sibiricum, Exiguobacterium indicum, Microbacterium oxydans, Microbacterium maripitycum, Micrococcus luteus, Kocuria oceani,* and *Staphylococcus haemolyticus.*

Table 2: BLAST analysis

Isolate	Accession no.	Database strain name
1SRA	MK229057	*Exiguobacterium sibiricum* B45
4SRD	MK606067.1	*Bacillus safensis* strain LS-111
5SRE	MK2290947.1	*Exiguobacterium indicum* B30
6SRF	MK606067.1	*Bacillus safensis* strain LS-111
8SRH	MH144269.1	*Acinetobacter pittii* PgBE46
9SRI	CP033814.1	*Staphylococcus heamolyticus* FDAAR905 517
10SRJ	CP026366.1	*Micrococcus luteus* SB1254
11SRK	CP033861.1	*Alcaligenes faecalis*
14TWN	MK342507.1	*Kocuria oceani* strain C
16SRP	CP031338 MK424289.1	*Microbacterium oxydans* VIU2A *Microbacterium maritypicum* DSM 12512 (T)
18SRR	MH929796.1	*Arthrobacter luteolus* 3d105
21SRU	CP026366.1	*Micrococcus luteus* SB1254
23SRW	MH299428.1 MH929796.1	*Arthrobacter sp* SN26.1 *Arthrobacter luteolus* 3d105
24SRX	MK342507 KY194315.1	*Kocuria oceani* strain C *Kocuria rosea* JZ87
25SRY	MK229057 MG371989.1	*Exiguobacterium sibiricum* B45 *Exiguobacterium artemiae* LE-13

Fifteen heavy metal tolerant bacterial strains were isolated and identified through molecular techniques. The bacterial isolates displayed good tolerance to multiple heavy metals, which makes them good candidates for bioremediation of heavy metal contaminated environments. Data from literature shows that not only are these isolates tolerant to multiple heavy metals, but some are also antioxidant agents and plant growth promoters. The isolates can be further explored for biotechnological and industrial purposes, including enzyme production, bioremediation, phytoremediation and degradation of toxic substances released into the environment.

4.4.3 Identified bacterial strains

Alcaligenes faecalis (Isolate 11SRK) is a species of Gram-negative, rod-shaped, motile bacteria commonly found in the environment. It was originally named for its first discovery in faeces, but was later found to be common in soil, water, and the environment in association with humans. It is positive for oxidase test and catalase test, but negative for nitrate reductase test. It is alpha-haemolytic and requires oxygen to grow. *A. faecalis* forms colonies that lack pigmentation. While opportunistic infections do occur, the bacterium is generally considered non-pathogenic. When an opportunistic infection does occur, it is usually observed in the form of a urinary tract infection. *A. faecalis* has been used to produce non-standard amino acids. In a study by Abou-Aly *et al*. (2019) another strain, *A. faecalis* MG966440 was found to be an antioxidant agent as well as plant growth promoter under laboratory conditions. It was found to produce all plant growth promoters; phytohormones (indole acetic acid and gibberellins), salicylic acid, prolines, siderophores, exopolysaccharide and biosurfactants. It is considered as heavy metal tolerant-plant growth promoting bacteria. *A. faecalis* is a plant growth promoter and a heavy metal bio-removal agent, that is, it promotes plant growth in heavy metal contaminant environment and reduces the bioavailability of toxic metals displays great potential for bioremediation within the wetland and on crops cultivated on contaminated soils (Abou-Aly *et al*. 2019).

Arthrobacter sp SN26.1 (Isolate 23SRW) is a genus of bacteria that is commonly found in soil. *Arthrobacter* are coryneform bacteria. Most species of *Arthrobacter* are obligate aerobes, but all exhibit a pure respiratory, never fermentative metabolism. Species in this genus are Gram-positive obligate aerobes that are rods during exponential growth and cocci in their stationary phase. Under the microscope, *Arthrobacter* appear as rods when rapidly dividing, and cocci when in stationary phase. Dividing cells may also appear as chevrons ("V" shapes). Arthrobacter have a distinctive method of cell division called "snapping division" or reversion in which the outer bacterial cell wall ruptures at a joint. *Arthrobacter spp* are commonly found in soils, aerial surface of plants, and wastewater sediments. *Arthrobacter spp*. have been isolated from archaeological mural paintings as well as human and veterinary clinical sources. *Arthrobacter spp*. populate foods, such as ready-to-use vegetables, eggs, and raw milk, and contribute to the ripening of smear surface–ripened cheeses. *Arthrobacter* are nutritionally versatile, using a variety of substrates in their oxidative metabolism including nicotine, nucleic acids, and various herbicides and pesticides. They can also be grown on mineral salts pyridone broth, where colonies have a greenish metallic centre, when incubated at 20 °C. Other notable characteristics are that it can use pyridone as its sole carbon source, and that its cocci are resistant to desiccation and starvation. They do not form endospores and are highly

proteolytic. *Arthrobacter* can degrade unusual and polymeric compounds and play an important role in biodegrading agrochemicals and pollutants (Eschbach *et al*. 2003).

Arthrobacter luteolus 3d105 (Isolate 23SRW and 18SRR) is a Gram positive coryneform obligate aerobic bacteria which are motile by virtue of peritrichous flagella and do not produce spores. *Arthrobacter luteolus* grows at temperature range of 20 – 40 °C, with optimum temperature of 30-37°C. Colonies are yellow on nutrient agar and TSA. On blood agar colonies are slightly yellow, smooth, 1.5 mm diameter, after 24 hr incubation at 37 °C. In a study by Emmanuel *et al*. (2012). *A. luteolus* isolated has the ability to sequester metals in their cell walls.

Acinetobacter pittii PgBE46 (Isolate 8SRH) is a Gram-negative diplococcoid rods, oxidase-negative, catalase-positive, strictly aerobic, non-motile bacterium from the genus *Acinetobacter.* A. pittii belongs to the *Acinetobacter calcoaceticus-baumannii* complex and is named after the British microbiologist Tyrone Pitt a gram-negative rod-shaped bacterium that usually occurs in pairs (diplococcoid rods or coccobacilli). Bacteria of the genus *Acinetobacter* are ubiquitously distributed in nature. They are found in various types of soils and waters and are occasionally found in food stocks. They are normal inhabitants of human skin and are capable of transitory colonization of the upper respiratory tract. There have been findings in a study by Chettri *et al*. (2019) about another *A. pittii* strain, *Acinetobacter pittii* strain ABC, which was found to utilize and degrade hydrocarbons. The strain also showed excellent tolerance to 1 mM concentration of the following metal salts; $CuSO_4 \cdot 5H_2O$; $MnCl_2 \cdot 4H_2O$; $FeSO_4 \cdot 7H_2O$; $Al_2(SO_4)_3$; $SrCl_2 \cdot 6H_2O$; $CrCl_3$, $(CH_3COO)_2Pb \cdot 3H_2O$; $ZnSO_4 \cdot 7H_2O$ and $NiCl_2 \cdot 6H_2O$). This indicates *A. pittii* strain ABC as a potential agent for bioremediation of crude oil-polluted environment as well as heavy metal polluted ecosystems. The potential for *A. pittii* strains in bioremediation has been documented to date, including *A. pittii* strain PgBE46 been discovered in this current as a potential candidate for heavy metal bioremediation.

Bacillus safensis strain LS-111 (Isolate 4SRD and 6SRF) is a Gram-positive, motile with polar flagella, spore-forming rod shaped bacterium, originally isolated from a spacecraft in Florida and California. *Bacillus safensis* could have possibly been transported to the planet Mars on spacecraft Opportunity and Spirit in 2004. It produces spores that are resistance to peroxide and radiation. There are several known strains of this bacterium, all of which belong to the Firmicutes phylum and to the large, pervasive genus Bacillus. *Bacillus safensis* strain LS-111 is a salt tolerating bacterium that grows on mannitol salt agar and ferments mannitol. It's growth temperature ranges from 10-50 ºC. It is an aerobic chemoheterotroph that is highly tolerant to UV and gamma radiation, heavy metals as well as

polyaromatic hydrocarbons. *Bacillus safensis* is a powerful plant hormone producer and it is also a plant growth-promoting rhizobacteria, which enhances plant growth after root colonization. Strain *B. safensis* JPL-MERTA-8-2 is the only bacterial strain so far known to grow noticeably faster in micro-gravity environments than on the Earth surface (Satomi *et al.* 2006).

Exiguobacterium indicum B30 (Isolate 5SRE) is a Gram-positive, motile, rod-shaped, non-spore-forming, alkaliphilic bacterium, which was isolated from the melt water of a glacier, the Hamta glacier located at a height of 4270 m above sea level in the Himalayan Mountain ranges of India. *Exiguobacterium indicum* sp. nov., a psychrophilic bacterium from the Hamta glacier of the Himalayan Mountain ranges of India. Stationary-phase cells are coccobacilli and colonies on nutrient agar are yellowish orange or orange, round, shiny, irregular and elevated. It is positive for oxidase and catalase, and also for the reduction of nitrate to nitrite, while testing negative for urease, lipase, gelatinase, methyl red test, tryptophan deamination, indole production, H2S production and starch hydrolysis. The genus Exiguobacterium was created by Collins *et al*. (1983) to accommodate Gram-positive, non-spore-forming, facultative anaerobic, alkaliphilic bacteria isolated from potato-processing effluent (Chaturvedi and Shivaji 2006).

Exiguobacterium sibiricum-B45 (Isolate 25SRY and 1SRA) belongs to the genus Exiguobacterium which are predominantly free living, saprophytic anaerobic bacilli. This genus is repeatedly isolated from the Siberian permafrost but has also been found in hot springs in the Yellowstone National Park, and in the rhizosphere of plants. *Exiguobacterium sibiricum* is a psychrophilic non-spore forming Gram-positive bacterium. The cells are rod-shaped, motile with peritrichous flagella and their growth ranges are from -2.5 to 40 ºC. The cells can vary in shape and size depending on growth temperature. They occur singly, in pairs or infrequently in chains. Colonies appear orange yellow (on TSA media). They are facultative anaerobes but grow more profusely aerobically. Its extremophile nature has made it a target of study for potentially useful industrial genes and enzymes. Some strains, in addition to dynamic thermal adaption, are also halotolerant (up to 13% added NaCl), can grow within a wide range of pH values (5-11), tolerate high levels of UV radiation, and heavy metal stress, including arsenic (Ordoneza *et al*., 2013).

Staphylococcus heamolyticus FDAAR905 517 (Isolate 9SRI) is a member of the coagulase-negative staphylococci. *Staphylococcus haemolyticus* is an opportunistic bacterial pathogen that colonizes human skin and is remarkable for its highly antibiotic-resistant phenotype. It is part of the skin flora of humans and its largest populations are usually found at the axillae, perineum, and inguinal areas of

humans. *S. haemolyticus* also colonizes primates and domestic animals. It is a well-known opportunistic pathogen and is the second-most frequently isolated coagulase negative staphylococci. Infections can be localized or systemic and are often associated with the insertion of medical devices. The highly antibiotic-resistant phenotype and ability to form biofilms make *S. haemolyticus* a difficult pathogen to treat (Fumihiko *et al.* 2005).

The genus *Kocuria c*omprises of gram-positive cocci arranged in pairs, short chains, tetrads, cubical packets of eight and irregular clusters. The genus *Kocuria* has many species that were isolated from various environments such as saline and alkaline desert, rhizoplane of narrow leaved cattail and seafood. *Kocuria* belongs to the phylum Actinobacteria, class Actinobacteria, order Actinomycetales, sub order *Micrococcinae* and family *Micrococcaceae.* The family *Micrococcaceae* includes *Staphylococcus* species and *Micrococcus* species. *Kocuria* can be grown on sheep blood agar and other general-purpose media such as nutrient agar and tryptone soy agar. They are shown to lack haemolytic ability on a blood agar plate. They grow best in neutral pH environments. Depending on the species, they appear in a range of colour such as: orange, pink, red, yellow or cream.

Kocuria oceani strain C (Isolate 24SRX) is Gram-stain-positive, non-spore-forming, irregular spherical-shaped and non-motile. Grows in temperature range from 4-40 degrees celsius with optimal growth occurring at 25–28 degrees celsius. Colonies of *Kocuria oceani* isolate were circular (0.5- 1.5 mm diameter), smooth, convex, and show pinkish orange pigmentation after 3 days of incubation (Zhang *et al*., 2017). *Kocuria rosea* JZ87 is a Gram positive cocci that are arranged in tetrads. It is a Biosafety Level 1 mesophilic bacterium that grows best at 25-37 ºC. *Kocuria rosea* is a Gram-positive coccus found in the environment from soil or water and within normal human skin microbiota, and more recently, it has been potentially implicated as an opportunistic pathogen. It is non-spore-forming, non-motile, catalase positive and oxidase negative. It forms circular, smooth, pink or rose coloured colonies on TSA and nutrient agar. *Kocuria rosea* is known as a saprophyte and also as a commensal of human skin and oropharynx. It is a potential heavy metal bio-removal agent, in a study by Karnwal *et al* (2018) *Kocuria rosea* VB1 and *Arthrobacter luteolus* VB2 reduced the concentration of nickel and chromium by 89 and 78 % respectively.

Micrococcus luteus SB1254 (Isolate 21SRU) is one of six species of genus Micrococcus. *Micrococcus* is a genus of bacteria in the Micrococcaceae family. *Micrococci* have Gram-positive spherical cells ranging from about 0.5 to 3 micrometres in diameter and typically appear in tetrads. *Micrococcus luteus* is a Gram-positive, to Gram-variable, non-motile cocci, tetrad-arranging, yellow pigmented,

saprotrophic bacterium that belongs to the family *Micrococcaceae. M. luteus* is coagulase negative, urease and catalase positive, bacitracin susceptible, and it can grow on diverse concentrations of NaCl (0 to 10%) and at pH range of 7 to 10. It forms bright yellow colonies on nutrient agar. *Micrococcus luteus* occurs in a wide range of environments, including water, dust, and soil and as part of the normal microbiota of the mammalian skin. It can also be isolated from plant roots of Polyspora Axillaris., *M. luteus* is an obligate aerobic bacterium that colonizes the human mouth, mucosae, oropharynx and upper respiratory tract. It was discovered by Sir Alexander Fleming before he discovered penicillin in 1928. *Micrococcus luteus* SB1254 strain was isolated from mussel at Geoje island in Korea. It is an alkaline protease producing extremophile. Alkaline protease enzyme is one of the most important industrial enzymes used in the detergent industry (Madigan and Martinco 2005).

Microbacterium oxydans VIU2A (Isolate 16SRP) belongs to the phylum Actinobacteria, a family of Microbacteriaceae and genus of *Microbacterium. Microbacterium oxydans* is very motile in the vegetative state, and is Gram-positive rods measuring 0.4–0.8 μm in width/diameter and 1.0–2.0 μm in length. *Microbacterium oxydans* is an aerobe, mesophilic bacterium that was isolated from air and from marsh. It occurred both singly or in random groups, and it was catalase-positive and did not form endospores. *Microbacterium oxydans* is a Gram-positive bacterium from the genus of *Microbacterium* which occurs in human clinical specimens. *Microbacterium oxydans* has the ability to degrade alginate and laminarin (Marlene *et al*. 2015)

Microbacterium maritypicum DSM 12512 (Isolate 16SRY) belongs to the phylum Actinobacteria, family Microbacteriaceae and the genus. *Microbacterium maritypicum* DSM 12512 is a mesophilic bacterium that was isolated from sea water and marine mud. It possesses a melanoid pigment and grows optimally at 28 ºC (Learman *et al*. 2019).

4.5 Heavy metal tolerance studies

The isolation and complete characterization of fifteen bacterial strains, which showed tolerance to 8 heavy metals (Al, Cd, Cu, Cr, Fe, Mn, Pb and Zn) was carried out. The results presented in this work revealed that all the 15 isolates, characterized with remarkable tolerance to heavy metals could be potential agents for the development of inoculants applicable in bio-augmentation of heavy metals. Some isolates were able to grow on media supplemented with heavy metal concentrations as high as 300 mg/l, and these isolates can grow across multiple heavy metals. Further studies can be conducted to investigate the genetic capacity of the isolates to gain information about the genes responsible for inferring metal tolerance for prospects of their application in the remediation of heavy metal polluted sites. Microorganisms are ubiquitously present in nature and play a crucial role in elemental biogeochemical cycles of metal transformations between soluble and insoluble species.

Various studies to date have analysed the quality of industrial wastewater and groundwater supplies in terms of heavy metals compared with standards for discharge of environmental pollutants. Various reports have demonstrated that industrial wastewater contains heavy metals beyond permissible limits for drinking water or surface/irrigation water. Applicable permissible limits are listed in Appendix D. Application of heavy metal-contaminated water in agricultural fields has led to their bioaccumulation in crops and associated food chains. Indirect heavy metal pollution results from contaminated surface or groundwater and rainwater. Rivers are one of the most important resources for fresh water and are severely affected by heavy metal pollution. Bioremediation is a technique for removing or converting harmful contaminants like heavy metals into less harmful substances; and/or removing toxic elements from the contaminated environment; or degrading organic substances and ultimate mineralization of organic substances into carbon dioxide, water, nitrogen gas, etc., employing dead or alive biomass. The process of bioremediation can be applied to soil and water media through in- situ and ex-situ techniques.

4.5.1 Isolate 1SRA (*Exiguobacterium sibiricum* B45)

Isolate 1SRA showed the same level of tolerance by growing at 200 mg/l on the seven metals (chromium, aluminium, iron, manganese, copper, lead and zinc) while showing sensitivity on 125 mg/l cadmium. The growth patterns were the same on both nutrient agar and Tryptic soy agar. The bacterial growth (colonies) decreased with increase in concentration of heavy metals indicating the toxic effect of heavy metals.

Table 3: Heavy metal tolerance for isolate1SRA

Concentration	Cr	Cd	Al	Fe	Mn	Cu	Pb	Zn
25 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
30 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
50 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
75 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
100 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
125 mg/l	+++	_	+++	+++	+++	+++	+++	+++
150 mg/l	+++	_	+++	+++	+++	+++	+++	+++
200 mg/l	++	_	++	++	++	++	++	++
250 mg/l	_	_	_	_	_	_	_	_

- no growth; + minimal growth; ++ moderate growth; +++ confluent growth

4.5.2 Isolate 4SRD (*Bacillus safensis* strain LS-111)

Isolate 4SRD showed sensitivity at 300 mg/l of chromium, manganese, copper, lead and zinc as no growth occurred at this concentration for these metals on both media. While for cadmium there was no growth at 100 mg/l, showing cadmium to be the most toxic metal among the eight heavy metals. Isolate 4SRD showed growth on both aluminium and iron at concentrations of 300 mg/l each.

Table 4: Heavy metal tolerance for isolate 4SRD (*Bacillus safensis* strain LS-111)

Concentration	Cr	Cd	Al	Fe	Mn	Cu	Pb	Zn
30 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
50 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
75 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
100 mg/l	+++	_	+++	+++	+++	+++	+++	+++

125 mg/l	+++	_	+++	+++	+++	+++	+++	+++
150 mg/l	+++	_	+++	+++	+++	+++	+++	+++
200 mg/l	++	_	+++	+++	++	++	++	++
250 mg/l	_	_	++	++	_	_	_	_
300 mg/l	_	_	++	++	_	_	_	_

- no growth; + minimal growth; ++ moderate growth; +++ confluent growth

4.5.3 Isolate 5SRE (*Exiguobacterium indicum* B30)

There was no growth on 100 mg/l cadmium, while for chromium, aluminium, iron, manganese, copper, lead and zinc growth still occurred at 200 mg/l. At 250 mg/l all the seven metals proved to be toxic to the bacterium as no growth occurred.

Table 5: Heavy metal tolerance for isolate 5SRE (*Exiguobacterium indicum* B30)

Concentration	**Cr**	**Cd**	**Al**	**Fe**	**Mn**	**Cu**	**Pb**	**Zn**
25 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
30 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
50 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
75 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
100 mg/l	+++	_	+++	+++	+++	+++	+++	+++
125 mg/l	+++	_	+++	+++	+++	+++	+++	+++
150 mg/l	+++	_	+++	+++	+++	+++	+++	+++
200 mg/l	++	_	++	++	++	++	++	++
250 mg/l	_	_	_	_	_	_	_	_

- no growth; + minimal growth; ++ moderate growth; +++ confluent growth

4.5.4 Isolate 6SRF (*Bacillus safensis* strain LS-111)

No growth was observed on both chromium and cadmium at 100 mg/l, while for aluminium, iron, manganese, copper, lead and zinc growth inhibition occurred at 300 mg/l.

Table 6: Heavy metal tolerance studies for isolate 6SRF(*Bacillus safensis* strain LS-111)

Concentration	Cr	Cd	Al	Fe	Mn	Cu	Pb	Zn
25 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
30 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
50 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
75 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
100 mg/l	_	_	+++	+++	+++	+++	+++	+++
125 mg/l	_	_	+++	+++	+++	+++	+++	+++
150 mg/l	_	_	+++	+++	+++	+++	+++	+++
200 mg/l	_	_	+++	+++	+++	+++	+++	+++
250 mg/l	_	_	++	++	++	++	++	++
300 mg/l	_	_	_	_	_	_	_	_

- no growth; + minimal growth; ++ moderate growth; +++ confluent growth

4.5.5 Isolate 8SRH (*Acinetobacter pittii* PgBE46)

No growth at 75 mg/l cadmium, and 150 mg/l of Fe, Mn, Cu, pb and Zn. Growth on media supplemented with 150 mg/l aluminium was very scanty.

Table 7: Heavy metal tolerance studies for isolate 8SRH (*Acinetobacter pittii* PgBE46)

Concentration	Cr	Cd	Al	Fe	Mn	Cu	Pb	Zn
25 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
30 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
50 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
75 mg/l	+++	_	+++	+++	+++	+++	+++	+++
100 mg/l	+	_	+++	+++	+++	+++	+++	+++

125 mg/l	_	_	+++	+++	+++	+++	+++	+++
150 mg/l	_	_	+	_	_	_	_	_

- no growth; + minimal growth; ++ moderate growth; +++ confluent growth

4.5.6 Isolate 9SRI (*Staphylococcus haemolyticus* FDAAR905 517)

Isolate 9SRI showed greater tolerance by growing at 200 mg/l chromium while showing high sensitivity to 75 mg/l of cadmium. Colonies are brownish and very tiny on 100mg/l FeSO4 and there was completely no growth at 125 mg/l of iron. For aluminium and manganese bacterial growth inhibition occurred at 150 mg/l.

Table 8: Heavy metal tolerance studies for isolate 9SRI (*Staphylococcus haemolyticus* FDAAR905 517)

Concentration	**Cr**	**Cd**	**Al**	**Fe**	**Mn**	**Cu**	**Pb**	**Zn**
25 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
30 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
50 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
75 mg/l	+++	_	+++	+++	+++	+++	+++	+++
100 mg/l	+++	_	+++	+++	+++	+++	_	+++
125 mg/l	+++	_	+++	_	+++	_	_	_
150 mg/l	+++	_	_	_	_	_	_	_
200 mg/l	++	_	_	_	_	_	_	_

- no growth; + minimal growth; ++ moderate growth; +++ confluent growth

4.5.7 Isolate 10SRJ (*Micrococcus luteus* SB1254)

Isolate 10SRJ showed the strongest heavy metal tolerance at concentrations of 300 mg/l of chromium, aluminium, iron and lead. There was growth at 100 mg/l cadmium but the organism was inhibited at 125 mg/l cadmium

Table 9: Heavy metal tolerance studies for isolate 10SRJ (*Micrococcus luteus* SB1254)

Concentration	Cr	Cd	Al	Fe	Mn	Cu	Pb	Zn
25 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
30 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
50 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
75 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
100 mg/l	+++	++	+++	+++	+++	+++	+++	+++
125 mg/l	+++	_	+++	+++	+++	+++	+++	_
150 mg/l	+++	_	+++	+++	+++	+++	+++	_
200 mg/l	++	_	++	++	_	_	++	_
250 mg/l	++	_	++	++	_	_	++	_
300 mg/l	++	_	++	++	_	_	++	_

- no growth; + minimal growth; ++ moderate growth; +++ confluent growth

4.5.8 Isolate 11SRK (*Alcaligenes faecalis)*

Isolate 11SRK showed greater tolerance towards cadmium by growing on 150 mg/l cadmium unlike most isolates whose growth were inhibited at 75 mg/l cadmium. No growth occurred at 150 mg/l chromium, and no growth at 200 mg/l cadmium, aluminium, iron, manganese, copper, lead and zinc.

Table 10: Heavy metal tolerance studies for isolate 11SRK (*Alcaligenes faecalis)*

Concentration	Cr	Cd	Al	Fe	Mn	Cu	Pb	Zn
25 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
30 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
50 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
100 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
125 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
150 mg/l	_	++	+++	+++	+++	+++	+++	+++
200 mg/l	_	_	++	++	++	++	+	++

250 mg/l	_	_	_	_	_	_	_	_

- no growth; + minimal growth; ++ moderate growth; +++ confluent growth

4.5.9 Isolate 16SRP *(Kocuria oceani* strain C)

Cadmium inhibited bacterial growth at 75 mg/l. A confluent growth, or growth showing only a few colonies, was considered as a positive. At 125 mg/l of aluminium there was total growth inhibition, while for chromium, manganese, copper, lead and zinc the bacterial growth inhibition occurred at 150 mg/l. Aluminium and iron were inhibitory to the isolate at 125 mg/l.

Table 11: Heavy metal tolerance studies for isolate 16SRP (*Kocuria oceani* strain C)

Concentration	Cr	Cd	Al	Fe	Mn	Cu	Pb	Zn
25 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
30 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
50 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
75 mg/l	+++	_	+++	+++	+++	+++	+++	+++
100 mg/l	+++	_	+++	+++	+++	+++	+++	+++
125 mg/l	++	_	_	_	++	++	++	++
150 mg/l	_	_	_	_	_	_	_	_

- no growth; + minimal growth; ++ moderate growth; +++ confluent growth

4.5.10 Isolate 18SRR (*Microbacterium oxydans* VIU2A)

No growth was recorded at a concentration of 75 mg/l cadmium, 125 mg/l manganese, copper, lead and zinc, and no growth at 150 mg/l chromium, aluminium and iron.

Table 12: Heavy metal tolerance studies for isolate 18SRR (*Microbacterium oxydans* VIU2A)

Concentration	Cr	Cd	Al	Fe	Mn	Cu	Pb	Zn
25 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
30 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
50 mg/l	+++	+++	+++	+++	+++	+++	+++	+++

75 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
100 mg/l	+++	_	+++	+++	+++	+++	+++	+++
125 mg/l	++	_	++	++	_	_	_	_
150 mg/l	_	_	_	_	_	_	_	_

- no growth; + minimal growth; ++ moderate growth; +++ confluent growth

4.5.11 Isolate 21SRU (*Arthrobacter luteolus* 3d105)

Cadmium inhibited bacterial growth at 75 mg/l while aluminium showed total growth inhibition at 125 mg/l. Chromium, manganese, copper, lead and zinc all had their bacterial growth inhibition occurring at 200 mg/l.

Table 13: Heavy metal tolerance studies for isolate 21SRU (*Arthrobacter luteolus* 3d105)

Concentration	**Cr**	**Cd**	**Al**	**Fe**	**Mn**	**Cu**	**Pb**	**Zn**
25 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
30 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
50 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
75 mg/l	+++	_	+++	+++	+++	+++	+++	+++
100 mg/l	+++	_	+++	+++	+++	+++	+++	+++
125 mg/l	+++	_	_	+++	+++	+++	+++	+++
150 mg/l	+++	_	_	+++	+++	+++	+++	+++
200 mg/l	_	_	_	_	_	_	_	_

- no growth; + minimal growth; ++ moderate growth; +++ confluent growth

4.5.12 Isolate 23SRW (*Micrococcus luteus* SB1254)

Cadmium proved to be the most toxic metal that inhibited bacterial growth at 75 mg/l. At 125 mg/l of aluminium there was total growth inhibition, while for manganese, copper, lead and zinc the bacterial growth inhibition occurred at 250 mg/l. There was no growth on 200 mg/l chromium.

Table 14: Heavy metal tolerance studies for isolate 23SRW (*Micrococcus luteus* SB1254)

Concentration	Cr	Cd	Al	Fe	Mn	Cu	Pb	Zn
25 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
30 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
50 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
75 mg/l	+++	_	+++	+++	+++	+++	+++	+++
100 mg/l	+++	_	+++	+++	+++	+++	+++	+++
125 mg/l	+++	_	_	+++	+++	+++	+++	+++
150 mg/l	+++	_	_	+++	+++	+++	+++	+++
200 mg/l	_	_	_	++	++	++	++	++
250 mg/l	_	_	_	_	_	_	_	_

- no growth; + minimal growth; ++ moderate growth; +++ confluent growth

4.5.13 Isolate 24SRX (*Arthrobacter sp* SN26.1; *Arthrobacter luteolus* 3d105)

Bacterial growth inhibition on Cadmium occurred at 75 mg/l. aluminium showed total growth inhibition at a concentration of 125 mg/l, while for chromium, manganese, copper, lead and zinc the bacterial growth inhibition occurred at 150 mg/l.

Table 15: Heavy metal tolerance studies for isolate 24SRX (*Arthrobacter sp* SN26.1; *Arthrobacter luteolus 3d105)*

Concentration	Cr	Cd	Al	Fe	Mn	Cu	Pb	Zn
25 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
30 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
50 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
75 mg/l	++ +	_	+++	+++	+++	+++	+++	+++
100 mg/l	+++	_	+++	+++	+++	+++	+++	+++
125 mg/l	+++	_	_	++	+++	+++	+++	+++
150 mg/l	_	_	_	_	_	_	_	_

- no growth; + minimal growth; ++ moderate growth; +++ confluent growth

4.5.14 Isolate 25SRY (*Kocuria oceani* strain C; *Kocuria rosea* JZ87)

Cadmium inhibited bacterial growth at 75 mg/l. At 125 mg/l of aluminium there was total growth inhibition, while for chromium, manganese, copper, lead and zinc the bacterial growth inhibition occurred at 150 mg/l.

Table 16: Heavy metal tolerance studies for 25SRY **(***Kocuria oceani strain C; Kocuria rosea JZ87)*

Concentration	Cr	Cd	Al	Fe	Mn	Cu	Pb	Zn
25 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
30 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
50 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
75 mg/l	+++	_	+++	+++	+++	+++	+++	+++
100 mg/l	+++	_	+++	+++	+++	+++	+++	+++
125 mg/l	+++	_	_	_	+++	+++	+++	+++
150 mg/l	_	_	_	_	_	_	_	_

- no growth; + minimal growth; ++ moderate growth; +++ confluent growth

4.5.15 Isolate 14TWN (*Exiguobacterium sibiricum* B45;*Exiguobacterium artemiae* LE13)

Cadmium proved to be the most toxic metal that inhibited bacterial growth at even concentrations of 75 mg/l. At 125 mg/l of aluminium there was total growth inhibition, while for chromium, manganese, copper, lead and zinc the bacterial growth inhibition occurred at 150 mg/l.

Table 17: Heavy metal tolerance studies for 14TWN

Concentration	Cr	Cd	Al	Fe	Mn	Cu	Pb	Zn
25 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
30 mg/l	+++	+++	+++	+++	+++	+++	+++	+++
50 mg/l	+++	+++	+++	+++	+++	+++	+++	+++

75 mg/l	+++	_	+++	+++	+++	+++	+++	+++
100 mg/l	+++	_	+++	+++	+++	+++	+++	+++
125 mg/l	+++	_	_	_	+++	+++	+++	+++
150 mg/l	_	_	_	_	_	_	_	_

- no growth; + minimal growth; ++ moderate growth; +++ confluent growth

Chapter 5: Conclusions and Future Studies

Fifteen rhizobacteria associated with *Typha capensis* were successfully isolated and identified based on the 16s rDNA sequence data. All of the fifteen bacterial strains showed multiple tolerance to heavy metals in this study. Some bacterial isolates such as 1SRA, 4SRD, 5SRE,10SRJ,11SRK and 23SRW were able to grow on metal concentrations as high as 200 – 300 mg/l. Throughout the heavy metal tolerance study, cadmium proved to be the most toxic heavy metal among all the eight, managing to inhibit growth of most isolates at concentrations as low as 75 mg/l.

Natural wetlands cannot function with such high removal efficiencies for all kinds of pollutants due to the varied engineering and design, different background concentrations, internal profiles, contact time and mass loading rates. On the other hand, constructed wetlands are engineered wetlands that are precisely and completely optimized in all parameters and there is more control over the bioremediation processes, hence their performance is generally higher than that of natural wetlands. Hydraulic retention time and flow rate play the most important part in ensuring optimum removal efficiency, and these are not controlled in natural wetlands as opposed to constructed wetlands. The most common and practical treatment options for irrigation water includes disinfection of the water with chlorine. Disinfection requires careful process control of dosage and contact time. Filtration which physically removes the pathogens and parasites require further treatment or optimization. Wetlands are a sustainable solution for tertiary treatment of domestic wastewater effluents because wetlands are eco-friendly, cost-effective systems with low maintenance. The wetland in this study has shown significant pollutant removal efficiency in a period of one year even though the data has shown that sulphate, TSS, total coliform, and *E. coli* counts exceeded permissible levels for agricultural use (irrigation) as set by the Department of Water Affair and Forestry. The wetland still performed well in removing other pollutants which includes COD, nitrate, nitrite, EC, ortho-phosphate, pH, aluminium, copper, iron, manganese and zinc.

Future studies could investigate means of improving wetland removal efficiency through the use of carefully selected plant species and directed microbial inoculation of plant rhizospheres. The influent into the wetland area determines the requirements of that wetland. Where industrial and mining run-off increases the heavy metal contamination, inoculation with metal tolerant microorganisms may increase the efficiency of the wetland in removing the metal pollutants.

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Appendix A Statistical tests and parameters.

Table A1. Average characteristic values of the analyzed water quality parameters

Parameter	Wetland Inlet Mean	Wetland Outlet Mean	Wetland inlet Median	Wetland outlet Median	Average removal %
Electrical conductivity	266.2	147.6	159.5	98.5	43
Temperature	24.8	24.4	26.1	25.8	-
pH	7.12	7.09	7.18	7.11	-
Carbonate hardness	3.60	2.83	3.5	2.9	21
BOD5	20.06	8.122	20	8.05	60
COD	44.63	17.51	45	18	61
Ortho-phosphate	2.543	1.158	2.62	1.19	54
Nitrate	4.83	0.53	4.80	0.44	89
Nitrite	0.84	0.37	0.89	0.37	56
Sulfate	105.7	54.17	104.5	59	49
Total Suspended Solids	99.17	40.75	99.5	43.5	61
E. coli	273.9	41.83	254.4	34.35	85
Total coliforms	771.96	381.1	755.6	387.3	51
Aluminium	0.151 ± 0.033	0.05 ± 0.013	0.16	0.05	67
Copper	0.784 ± 0.128	0.135 ± 0.051	0.795	0.13	83
Iron (III)	0.463 ± 0.028	0.221 ± 0.017	0.46	0.23	52
Manganese	1.907 ± 0.143	0.932 ± 0.243	1.935	0.84	51
Zinc	0.256 ± 0.041	0.117 ± 0.017	0.26	0.11	54

Statistical test: Paired sample T-test, using T distribution (df=35) (left-tailed)

The normality assumption based on the Shapiro-Wilk Test. (α=0.05)

Table A2 5-day Biological Oxygen Demand

5-day Biological Oxygen Demand effluent – influent differences scores	
Mean	-11.94166667
Standard Error	0.373409325
Median	-12
Mode	-11
Standard Deviation	2.240455948
Sample Variance	5.019642857
Kurtosis	0.517861243
Skewness	0.449687957
Range	9.4
Minimum	-15.7
Maximum	-6.3
Sum	-429.9
Count	36
Confidence Interval (95%)	[-12.67; -11.21]
Cohen's d	5.93
Effect size	large
t- stats	-31.9801
p-value	8.459e-28
The test priori power is strong	0.9026

Table A3 Chemical Oxygen Demand differences scores

Chemical Oxygen Demand effluent – influent differences scores	
Mean	-27.25
Standard Error	0.766752066
Median	-27
Mode	-27
Standard Deviation	4.600512394
Sample Variance	21.16471429
Kurtosis	-1.152080511
Skewness	0.07509715
Range	15.05
Minimum	-35
Maximum	-19.95
Sum	-981
Count	36
Confidence Interval (95%)	[-28.75; -25.75]
Cohen's d	5.92
Effect size	large
t- stats	-35.53951952
p-value	2.344e-29
The test priori power is strong	0.9026

Table A4 Carbonate hardness differences scores

Carbonate Hardness effluent- influent Differences scores	
Mean	-0.775
Standard Error	0.114113213
Median	-0.5
Mode	-0.5
Standard Deviation	0.684679278
Sample Variance	0.468785714
Kurtosis	0.677727756
Skewness	-1.406828186
Range	2.2
Minimum	-2.4
Maximum	-0.2
Sum	-27.9
Count	36
Confidence Interval (95%)	[-0.993; -0.540]
Wilcoxon signed rank test	
Standardized effect size is large	0.87
z- stats	5.234436
p-value	8.27448e-8
The test priori power is strong	0.9026

Table A5 Electrical conductivity differences scores

Electrical conductivity effluent - influent differences scores	
Mean	-118.5861111
Standard Error	15.86648017
Median	-73
Mode	-82
Standard Deviation	95.198881
Sample Variance	9062.826944
Kurtosis	2.58854987

Skewness	-1.739474173
Range	352.4
Minimum	-378.5
Maximum	-26.1
Sum	-4269.1
Count	36
Confidence Interval (95%)	[-149.7; -87.49]
Wilcoxon signed rank test	
Standardized effect size is large	0.87
z- stats	5.225379
p-value	8.68996e-8
The test priori power is strong	0.9026

Shapiro-Wilk normality test showed that the “After minus Before” data is not normally distributed (p-value = 0.000001068). The data showed a long left tail, a negative skew and three outliers based on the Tukey's fences method, k=1.5. The distribution was skewed to the left with a skewness value of - 1.739474. Null hypothesis claims that there is no difference between the influent electrical conductivity and effluent electrical conductivity medians. Null hypothesis claims that there is no significant median difference in the electrical conductivity concentrations in the water entering the wetland and the electrical conductivity concentrations of the water leaving the wetland. H_0 claims that the influent EC concentration is equal to the effluent EC concentration. The alternative hypothesis H_1 assumes the true median difference between the influent EC concentration and effluent EC concentration is not equal to zero. The Wilcoxon signed test at 0.05 alpha level showed that z-stats = 5.2254 and p-value = 8.68996e-8. Since p <0 .05, the null hypothesis of no difference between the influent and effluent EC medians was rejected. This proves that the wetland removal efficiency for EC (43%) is statistically significant.

Table A6 Nitrate differences scores

Nitrate effluent - influent Differences scores	
Mean	-4.301666667
Standard Error	0.083122113
Median	-4.275
Mode	-3.97
Standard Deviation	0.49873268
Sample Variance	0.248734286
Kurtosis	0.900672757
Skewness	-0.542138859
Range	2.33
Minimum	-5.58
Maximum	-3.25
Sum	-154.86
Count	36
Confidence Interval (95%)	[-4.465; - 4.139]
Cohen's d	8.63
Effect size	large
t- stats	- 51.7511
p-value	5.80424E-35
The test priori power is strong	0.9026

Table A7 Nitrite differences scores

Nitrite effluent - influent differences scores	
Mean	-0.476111111
Standard Error	0.019413127
Median	-0.48
Mode	-0.48
Standard Deviation	0.11647876
Sample Variance	0.013567302
Kurtosis	-0.328276484
Skewness	0.010526888
Range	0.44
Minimum	-0.69
Maximum	-0.25
Sum	-17.14
Count	36
Confidence Interval (95%)	[-0.514; - 0.438]
Cohen's d	4.09
Effect size	large
t- stats	-24.5252
p-value	6.19811e-24
The test priori power is strong	0.902

Table A8 Ortho phosphate differences scores

Ortho phosphate effluent – influent difference scores	
Mean	1.384444444
Standard Error	0.061119769
Median	-1.475
Mode	-0.87
Standard Deviation	0.366718611
Sample Variance	0.13448254
Kurtosis	-0.806005663
Skewness	0.290791947
Range	1.29
Minimum	-2.01
Maximum	-0.72
Sum	-49.84
Count	36
Confidence Interval (95%)	[-1.504 – -1.265]
Standardized effect size is large	0.87
t stat	-22.6513
p-value	8.525e-8
The test priori power is strong	0.9026

Table A9 Sulfate differences scores

Sulfate effluent - influent differences scores	
Mean	-51.51111111
Standard Error	3.683499841
Median	-46.5
Mode	-43
Standard Deviation	22.10099904
Sample Variance	488.4541587
Kurtosis	-1.274164121
Skewness	-0.469336149
Range	65.7
Minimum	-88
Maximum	-22.3
Sum	-1854.4
Count	36
Confidence Interval (95%)	[-58.731; - 44.292]
Cohen's d	2.33
Effect size	large
t- stats	-13.9843
p-value	3.4034e-16
The test priori power is strong	0.9026

Table A10 Total Suspended Solids differences scores

Total Suspended solids effluent - influent differences	
Mean	-58.43333333
Standard Error	3.438036538
Median	-56.5
Mode	-58
Standard Deviation	20.62821923
Sample Variance	425.5234286
Kurtosis	5.165067843
Skewness	-2.215931162
Range	86.8
Minimum	-120.8
Maximum	-34
Sum	-2103.6
Count	36
Confidence Interval (95%)	[-65.172; -51.695]
Cohen's d	2.83
Effect size	large
t- stats	-16.9961
p-value	8.68827e-19
The test priori power is strong	0.9026

Wilcoxon signed rank

p-value	8.59569e-8
Z	5.227396
standardized effect size is large	0.87
common language effect size	1.00

Table A11 *E. coli* differences scores

***E. coli* outflow - inflow differences scores**	
Mean	-232.1166667
Standard Error	12.15363327
Median	-219.6
Mode	-452
Standard Deviation	72.9217996
Sample Variance	5317.588857
Kurtosis	5.2797431
Skewness	-2.326991206
Range	281.6
Minimum	-452
Maximum	-170.4
Sum	-8356.2
Count	36
	24.67318725
Confidence Interval (95%)	[-255.94 – -208.30]

Wilcoxon signed rank test.

p-value	8.58444e-8
Z	5.227638
Standardized effect size is large	0.87
common language effect size	1.00

Standardized effect size of 0.87 is large. That indicates that the magnitude of the difference between the value from Before and the value from After is large.

Table A12Total coliforms differences scores

Total Coliforms effluent - influent differences	
Mean	-390.8606061
Standard Error	22.82139477
Median	-345
Mode	-417.4
Standard Deviation	131.098932
Sample Variance	17186.92996
Kurtosis	-1.293175632
Skewness	-0.319649053
Range	379.6
Minimum	-593.8
Maximum	-214.2
Sum	-12898.4
Count	33
Confidence Interval	[-435.589 –;-346.131]
Test priori power is strong	0.8782
Wilcoxon signed rank.	
p-value	2.76439e-7
Z	5.006990
standardized effect size is large	0.87
common language effect size	1.00

Table A13 Aluminium differences scores

Aluminium effluent - influent differences	
Mean	-0.101944444
Standard Error	0.006195485
Median	-0.1
Mode	-0.09
Standard Deviation	0.037172912
Sample Variance	0.001381825
Kurtosis	-0.417300621
Skewness	0.522623597
Range	0.14
Minimum	-0.16
Maximum	-0.02
Sum	-3.67
Count	36
Confidence Interval (95%)	[- 0.114; - 0.090]
Cohen's d	2.74
Effect size	large
t- stats	-16.4546
p-value	2.38683e-18

Table A14 Copper differences scores

Copper inflow and outflow Differences	
Mean	-0.648888889
Standard Error	0.015925311
Median	-0.645
Mode	-0.64
Standard Deviation	0.095551864
Sample Variance	0.009130159
Kurtosis	1.732881667
Skewness	1.054684392
Range	0.41
Minimum	-0.81
Maximum	-0.4
Sum	-23.36
Count	36
Confidence Level(95.0%)	0.032330099
Confidence Interval (95%)	[-0.680; - 0.618]

Wilcoxon signed test

p-value	8.63706e-8
Z stat	5.226508
standardized effect size is large	0.87
common language effect size	1.00

Table A15 Iron III differences scores

Iron III effluent - influent difference scores	
Mean	-0.241388889
Standard Error	0.005120032
Median	-0.25
Mode	-0.26
Standard Deviation	0.030720191
Sample Variance	0.00094373
Kurtosis	-0.447570358
Skewness	0.188581487
Range	0.13
Minimum	-0.31
Maximum	-0.18
Sum	-8.69
Count	36
Confidence Interval (95%)	[-0.251 – -0.231]
Cohen's d	7.86
Effect size	large
t-stat	-47.146
p-value	1.44834E-33
The test priori power is strong	0.9026

Table A16 Manganese differences scores

Manganese effluent - influent differences scores	
Mean	-0.974444444
Standard Error	0.030452785
Median	-0.955
Mode	-0.96
Standard Deviation	0.182716712
Sample Variance	0.033385397
Kurtosis	0.364445916
Skewness	0.164475376
Range	0.75
Minimum	-1.32
Maximum	-0.57
Sum	-35.08
Count	36
Confidence Level (95.0%)	0.061822441
Confidence Interval (95%)	[- 1.034; -0.915]
Cohen's d	5.33
Effect size	large
t- stats	-31.9985
p-value	8.2958e-28
The test priori power is strong	0.9026

Table A17 Zinc differences scores

Zinc effluent - influent differences scores	
Mean	- 0.138333333
Standard Error	0.005337796
Median	-0.145
Mode	-0.1
Standard Deviation	0.032026775
Sample Variance	0.001025714
Kurtosis	- 1.364260618
Skewness	0.0500360
Range	0.12
Minimum	-0.19
Maximum	-0.07
Sum	-4.91
Count	36
Confidence Interval (95%)	[-0.149;– -0.129]
Cohen's d	4.43
Effect size	large
t- stats	-26.5983
p-value	4.168e-25
The test priori power is strong	0.9026

Influent and effluent descriptive statistics

(a) Aluminum	Mean	Median	Std error	p-value	t - or z-stats	CI (95%)
Influent	0.151	0.16				
Effluent	0.05	0.05				
Effluent- influent	-0.102	-0.1	0.006	2.38683e-18	t=-16.45	[-0.114; -0.090]

(b) BOD_5	Mean	Median	Std error	p-value	t - or z-stats	CI (95%)
Influent	20.06	20				
Effluent	8.122	8.05				
Effluent- influent	-11.94	-12	0.3734	8.4593e-28	t=-31.98	[-12.67; -11.21]

(c) Carbonate Hardness	Mean	Median	Std error	p-value	t - or z-stats	CI (95%)
Influent	3.6	3.5				
Effluent	2.825	2.9				
Effluent- influent	0.775	-0.5	0.114	8.1374e-8	z= 5.2375	[-0.993;-0.540]

(d) COD	Mean	Median	Std error	p-value	t - or z-stats	CI (95%)
Influent	44.63	45				
Effluent	17.51	18				
Effluent- influent	-27.25	-27	0.767	2.3438e-29	t= -35.54	[-28.75;-25.75]

(e)Copper	Mean	Median	Std error	p-value	t - or z-stats	CI (95%)
Influent	0.784	0.795				
Effluent	0.135	0.13				
Effluent- influent	-0.648	-0.645	0.016	8.63706e-8	z=5.2265	[-0.680; -0.618]

(f)*E. coli*	Mean	Median	Std error	p-value	t - or z-stats	CI (95%)
Influent	273.9	254.4				

Effluent	41.83	34.35				
Effluent- influent	-232.12	-219.6	12.15	8.584e-8	z=5.228	[-435.589;-346.131]

(g)Electrical conductivity	**Mean**	**Median**	**Std error**	**p-value**	**t - or z-stats**	**CI (95%)**
Influent	266.2	159.5				
Effluent	147.6	98.5				
Effluent- influent	- 118.6	-73	15.87	8.6899e-18	z=5.2254	[-149.7;-87.49]

(h)Iron III	**Mean**	**Median**	**Std error**	**p-value**	**t - or z-stats**	**CI (95%)**
Influent	0 .463	0.46				
Effluent	0.221	0.225				
Effluent- influent	- 0.241	-0.25	0.005	1.44834e-33	t=-47.15	[-0.251; -0.231]

(i) Manganese	**Mean**	**Median**	**Std error**	**p-value**	**t - or z-stats**	**CI (95%)**
Influent	1.907	1.935				
Effluent	0.932	0.835				
Effluent- influent	-0.974	-0.955	0.030	8.2958e-28	t=-31.999	[-1.034; -0.915]

(j)Nitrate	**Mean**	**Median**	**Std error**	**p-value**	**t - or z-stats**	**CI (95%)**
Influent	4.828	4.80				
Effluent	0.527	0.44				
Effluent- influent	4.302	-4.275	0.083	5.8042e-35	t= -51.75	[-4.465; -4.139]

(k)Nitrite	**Mean**	**Median**	**Std error**	**p-value**	**t - or z-stats**	**CI (95%)**
Influent	0.843	0.885				
Effluent	0.367	0.365				
Effluent- influent	0.476	-0.48	0.019	6.1981e-24	t= -24.52	[-0.514;-0.438]

(l)pH	Mean	Median	Min- Max
Influent	7.12	7.18	6.8 – 7.4
Effluent	7.09	7.11	6.76 – 7.31

(m)Ortho-phosphate	Mean	Median	Std error	p-value	t - or z-stats	CI (95%)
Influent	2.543	2.62				
Effluent	1.159	1.19				
Effluent- influent	-1.384	1.475	0.061	8.74313e-8	z= 5.224250	[-1.504; -1.265]

(n)Sulfate	Mean	Median	Std error	p-value	t - or z-stats	CI (95%)
Influent	105.7	104.5				
Effluent	54.16	59				
Effluent- influent	51.51	-46.5	3.68	3.4034e-16	t = -13.98	[-58.73; -44.29]

(o)Temperature	Mean	Median	Min- Max
Influent	24.8	26.1	18 - 28.1
Effluent	24.4	25.8	19 - 27.5

(p)Total coliforms	Mean	Median	Std error	p-value	t - or z-stats	CI (95%)
Influent	771.9	755.6				
Effluent	381.1	387.3				
Effluent- influent	-390.8	-345	22.82	2.7643e-7	z=5.007	[-435.589;-346.131]

(q)TSS	Mean	Median	Std error	p-value	t - or z-stats	CI (95%)
Influent	99.17	99.5				
Effluent	40.75	43.5				
Effluent- influent	58.43	56.5	3.43	8.59569e-8	z=5.227	[-65.17; -51.70]

®Zinc	Mean	Median	Std error	p-value	t - or z-stats	CI (95%)
Influent	0.256	0.26				
Effluent	0.117	0.11				
Effluent- influent	-0.139	-0.14	0.006	4.16852e-25	t=-26.598	[-0.149; -0.129]

Confidence interval calculations.

5-day BOD

95% Confidence level

Confidence Interval: **-**11.94166667 ±0.732

CI: [-12.674; -11.210]

COD

95% Confidence level

Confidence Interval: -27.25 ±1.503 (±-5.5%)

CI: [-28.753 – -25.747]

Carbonate hardness

95% Confidence level

Confidence Interval: -0.775 ±0.224 (±-28.9%)

CI: [-0.999 – -0.551]

Electrical conductivity

95% Confidence level

Confidence Interval: -118.5861111 ±31.098 (±-26.2%)

CI: [-149.684; -87.488]

Nitrate

95% Confidence level

Confidence Interval: -4.301666667 ±0.163 (±-3.8%)

CI: [-4.465 – -4.139]

Nitrite

95% Confidence level

Confidence Interval: -0.476111111 ±0.0380 (±-8.0%)

CI: [-0.514; -0.438]

Sulfate

95% Confidence level

Confidence Interval: -51.51111111 ±7.220 (±-14.0%)

CI: [-58.731; -44.292]

Ortho phosphate

95% Confidence Interval: -1.407222222 ±0.124 (±-8.8%)

CI: [-1.532; -1.283]

TSS

95% Confidence level

Confidence Interval: -58.43333333 ±6.738 (±-11.5%)

CI: [-65.172; -51.695]

E. coli

95% Confidence Interval:

-199.1555556 ±17.088 (±-8.6%)

[-216.243; -182.068]

Total coliforms

95% Confidence Interval

Confidence Interval: -390.8606061 ±44.729 (±-11.4%)

CI: [-435.590; -346.131]

Aluminium

Confidence Interval: -0.101944444 ±0.0121 (±-11.9%)

CI: [-0.114; -0.090]

Copper

95% Confidence Interval: -0.648888889 ±0.0312 (±-4.8%)

CI: [-0.680; -0.618]

Iron III

95% Confidence Interval: -0.241388889 ±0.0100 (±-4.2%)

CI: [-0.251; -0.231]

Manganese

95% Confidence Interval: -0.974444444 ±0.0597)

CI: [-1.034; -0.915]

Zinc

95% Confidence level

Mean confidence interval: -0.136388889 ± 0.0116972

CI: [-0.148086; -0.124692]

Appendix B: Statistical terms and definitions.

The main goal in many research studies is to check whether the data collected support certain statements or predictions.

Major Methods for making Statistical Inferences about a Population.

- The traditional Method
- The p-value Method
- Confidence Interval

What type of t-test to use?

- If the groups come from a single population (e.g. measuring before and after an experimental treatment), perform a paired t-test.
- If the groups come from two different populations (e.g. two different species, or people from two separate cities), perform a two-sample t-test (a.k.a. independent t-test).
- If there is one group being compared against a standard value (e.g. comparing the acidity of a liquid to a neutral pH of 7), perform a **one-sample t-test**.

One-tailed or two-tailed t-test?

- If you only care whether the two populations are different from one another, perform a two-tailed t-test.
- if you want to know whether one population mean is greater than or less than the other, perform a **one-tailed t-test.**

The t-test estimates the true difference between two group means using the ratio of the difference in group means over the pooled standard error of both groups. It can be calculated manually using a formula, or using statistical analysis software.

T-test formula

The formula for the two-sample t-test (a.k.a. the Student's t-test) is shown below.

$$t = \frac{\overline{x}_1 - \overline{x}_2}{\sqrt{(s^2(\frac{1}{n_1} + \frac{1}{n_2}))}}$$

In this formula, t is the t-value, x_1 and x_2 are the means of the two groups being compared, s_2 is the pooled standard error of the two groups, and n_1 and n_2 are the number of observations in each of the groups.

A larger *t*-value shows that the difference between group means is greater than the pooled standard error, indicating a more significant difference between the groups.

You can compare your calculated *t*-value against the values in a critical value chart to determine whether your *t*-value is greater than what would be expected by chance. If so, you can reject the null hypothesis and conclude that the two groups are in fact different.

When you perform the test on a software, you get an output

The output provides:

1. An explanation of what is being compared, called **data** in the output table.
2. The ***t*-value**: -33.719. Note that you can a negative or a positive t-value; this is fine either way. In most cases, we only care about the absolute value of the difference, or the distance from 0. It doesn't matter which direction.
3. The **degrees of freedom**: 30.196. Degrees of freedom is related to your sample size, and shows how many 'free' data points are available in your test for making comparisons. The greater the degrees of freedom, the better your statistical test will work.
4. The ***p*-value**: 2.2e-16 (i.e. 2.2 with 15 zeros in front). This describes the probability that you would see a *t*-value as large as this one by chance.
5. A statement of the **alternate hypothesis** (H_a). In this test, the H_a is that the difference is not 0.
6. The 95% **confidence interval**. This is the range of numbers within which the true difference in means will be 95% of the time. This can be changed from 95% if you want a larger or smaller interval, but 95% is very commonly used.

Presenting the results of a t-test

When reporting your t-test results, the most important values to include are the t-value, the p-value, and the degrees of freedom for the test. These will communicate to your audience whether the difference between the two groups is statistically significant or if just happened by chance.

You can also include the summary statistics for the groups being compared, namely **the mean** and **standard deviation.**

Paired sample t-test: the samples are paired or matched. Every observation in one sample is linked with an observation in the other sample.

After minus before paired sample t-test: one of the most common cases where dependent samples occur is when both samples have the same subjects and they are "paired by subject." In other words, each subject is measured twice on the response variable, typically before and then after some kind of treatment/intervention in order to assess its effectiveness.

The arithmetic mean: the average of a sum of numbers, which reflects the central tendency of the position of the numbers**.**

The arithmetic mean is calculated by dividing the sum of a collection of numbers by the count of the numbers, which reflects the central tendency of that collection.

A **confidence interval (C.I.):** is a range of values that is likely to include a population parameter with a certain degree of confidence.

The confidence interval: the range of values that you expect your estimate to fall between a certain percentage of the time if you run your experiment again or re-sample the population in the same way.

A confidence interval is the mean of your estimate plus and minus the variation in that estimate. This is the range of values you expect your estimate to fall between if you redo your test, within a certain level of confidence.

Plausible data: data is plausible when you don't see any outrageous values or patterns

Related samples: the samples are related, it means that the numbers in the data set were taken from the same individual or population.

Confidence Intervals for Matched Samples, Continuous Outcome

There is an alternative study design in which two comparison groups are dependent, matched or paired. Consider the following scenarios:

- A single sample of participants and each participant is measured twice, once before and then after an intervention.
- A single sample of participants and each participant is measured twice under two different experimental conditions (e.g., in a **crossover trial**).

A goal of these studies might be to compare the mean scores measured before and after the intervention, or to compare the mean scores obtained with the two conditions in a crossover study.

Yet another scenario is one in which matched samples are used. For example, we might be interested in the difference in an outcome between twins or between siblings.

There are two samples, and the goal is to compare the two means. However, the samples are related or dependent. In the first scenario, before and after measurements are taken in the same individual. In the last scenario, measures are taken in pairs of individuals from the same family. When the samples are dependent, we cannot use the techniques in the previous section to compare means. Because the samples are dependent, statistical techniques that account for the dependency must be used. These techniques focus on difference scores (i.e., each individual's difference in measures before and after the intervention, or the difference in measures between twins or sibling pairs).

The Unit of Analysis

This distinction between independent and dependent samples emphasizes the importance of appropriately identifying the unit of analysis, i.e., the independent entities in a study.

- In the **one sample** and **two independent samples** applications ***participants*** are the units of analysis.
- However, with **two dependent samples** application,***the pair is the unit*** (and not the number of measurements which is twice the number of units).

The parameter of interest is the mean difference, μ_d. Again, the first step is to compute descriptive statistics. We compute the sample size (which in this case is the number of distinct participants or distinct pairs), the mean and standard deviation of the ***difference scores***, and we denote these summary statistics as n, $\bar{X}_d$ and s_d, respectively. The appropriate formula for the confidence interval for the mean difference depends on the sample size. The formulas are shown in Table 6.5 and are identical to those we presented for estimating the mean of a single sample, except here we focus on difference scores.

Computing the Confidence Intervals for μ_d

- If n > 30

$$\bar{X}_d = z\frac{s_d}{\sqrt{n}}$$

Use Z table for standard normal distribution

- f n < 30

$$\bar{X}_d = \pm t \frac{s_d}{\sqrt{n}}$$

Use t-table with df=n-1

When samples are matched or paired, difference scores are computed for each participant or between members of a matched pair, and "n" is the number of participants or pairs, $\bar{x}$ is the mean of the difference scores, and S_d is the standard deviation of the difference scores

Formula for the coefficient of variation is:

Coefficient of Variation: (Standard Deviation / Mean) * 100.

Confidence: another way to describe probability. For example, if you construct a confidence interval with a 95% confidence level, you are confident that 95 out of 100 times the estimate will fall between the upper and lower values specified by the confidence interval.

The confidence level: the percentage of times you expect to reproduce an estimate between the upper and lower bounds of the confidence interval, and is set by the alpha value.

Your desired confidence level is usually one minus the alpha (*a*) value you used in your statistical test:

So if you use an alpha value of $p < 0.05$ for statistical significance, then your confidence level would be 1 − 0.05 = 0.95, or 95%.

When do you use confidence intervals?

You can calculate confidence intervals for many kinds of statistical estimates, including:

- Proportions
- Population means
- Differences between population means or proportions
- Estimates of variation among groups

Median: a statistical measure that determines the middle value of a dataset listed in ascending order (i.e., from smallest to largest value). The measure divides the lower half from the higher **half.**

Statistical Test: uses the data obtained from a sample to make a decision about whether the null hypothesis should be rejected.

Test Value (test statistic): the numerical value obtained from a statistical test.

When we make a conclusion from a statistical test there are two types of errors that we could make. They are called: Type I and Type II Errors

Type I error: reject H0 when H0 is true.

Type II error: do not reject H0 when H0 is false.

Standard Deviation: a statistical term used to measure the amount of variability or dispersion around an average.

It is a measure of variation in data. It allows comparison between two or more sets of data to determine if their averages are truly different

Basically the standard deviation measures how concentrated the data are around the mean; the more concentrated, the smaller the standard deviation.

A small standard deviation means that the values in a statistical data set are close to the mean of the data set, on average,

A large standard deviation means that the values in the data set are farther away from the mean, on average**.**

A large standard deviation indicates that the data points can spread far from the mean and a small standard deviation indicates that they are clustered closely around the mean.

Properties of a standard deviation:

- The standard deviation can never be a negative number, due to the way it's calculated and the fact that it measures a distance (distances are never negative numbers).
- The smallest possible value for the standard deviation is 0, and that happens only in contrived situations where every single number in the data set is exactly the same (no deviation).
- The standard deviation is affected by outliers (extremely low or extremely high numbers in the data set). That's because the standard deviation is based on the distance from the *mean.* And remember, the mean is also affected by outliers**.**
- The standard deviation has the same units as the original data.

Standard deviation and standard error of the mean are both statistical measures of variability. While the standard deviation of a sample depicts the spread of observations within the given

sample regardless of the population mean, the standard error of the mean measures the degree of dispersion of sample means around the population mean.

Standard deviation and standard error of the mean are both statistical measures of variability. While the standard deviation of a sample depicts the spread of observations within the given sample regardless of the population mean, the standard error of the mean measures the degree of dispersion of sample means around the population mean of the dataset. Along with mean and mode, median is a measure of central tendency**.**

Standard error of the mean, or simply **standard error:** indicates how different the population mean is likely to be from a sample mean. It tells you how much the sample mean would vary if you were to repeat a study using new samples from within a single population.

Standard error: a measure of how precisely a sampling distribution represents a population. an important statistical measure and it is related to the standard deviation. It tells the way sample means determine the true population means. A large standard error indicates that there are various changes in the population. A small standard error implies that the population is in a uniform shape.

Null hypothesis: for most tests, the null hypothesis is that there is no relationship between your variables of interest or that there is no difference among groups**.**

Null Hypothesis (H_0): a statistical hypothesis that states that there is no difference between a parameter and a specific value, or that there is no difference between two parameters.

Alternative Hypothesis (H_1): a statistical hypothesis that states the existence of a difference between a parameter and a specific value, or states that there is a difference between two parameters.

p-value: the probability that, if the null hypothesis were true, we would observe a statistic at least as extreme as the one observed.

P-value in null hypothesis significance testing: the p-value is the probability of obtaining test results at least as extreme as the results actually observed, under the assumption that the null hypothesis is correct. A very small p-value means that such an extreme observed outcome would be very unlikely under the null hypothesis**.**

***P*-values and statistical significance**

Significance level (alpha): boundary for specifying a statistically significant finding when interpreting the p-value.

Significance level: the maximum probability of committing a Type I error. This probability is symbolized by α.

P(Type I error|H0 is true) $=\alpha$

Alpha Levels, or p-levels, allow the research to determine how often a Statistically Significant result occurs by chance. The lower the Alpha Level, the more likely you are to draw an erroneous conclusion about whether something is actually affecting the behavior. Most people typically use an Alpha Level of 0.05, so that is what we will use as well.

A Type I Error: a false positive; it means that you found a Statistically Significant result when in fact there is none.

A Type II Error: a false negative; it means that you did not find a Statistically Significant result when in fact there was one

The P-Value (probability value): the probability of getting a sample statistic (such as the mean) or a more extreme sample statistic in the direction of the alternative hypothesis when the null hypothesis is true

Statistical significance: another way of saying that the *p*-value of a statistical test is small enough to reject the null hypothesis of the test.

How small is small enough? The most common threshold is $p < 0.05$; that is, when you would expect to find a test statistic as extreme as the one calculated by your test only 5% of the time. But the threshold depends on your field of study – some fields prefer thresholds of 0.01, or even 0.001.

The threshold value for determining statistical significance is also known as **the alpha value**

Statistical significance is a term used by researchers to state that it is unlikely their observations could have occurred under the null hypothesis of a statistical test. Significance is usually denoted by a *p*-value, or probability value.

Statistical significance is arbitrary – it depends on the threshold, or alpha value, chosen by the researcher. The most common threshold is $p < 0.05$, which means that the data is likely to occur less than 5% of the time under the null hypothesis.

The p-value: the actual area under the standard normal distribution curve of the test value or a more extreme value (further in the tail).

When the *p*-value falls below the chosen alpha value, then we say the result of the test is statistically significant.

The p-value only tells you how likely the data you have observed is to have occurred under the null hypothesis.

If the *p*-value is below your threshold of significance (typically $p < 0.05$), then you can reject the null hypothesis, but this does not necessarily mean that your alternative hypothesis is true.

P-value is a probability. This means that it is a real number from 0 and 1.

P-values are most often used by researchers to say whether a certain pattern they have measured is statistically significant.

How small of a p-value do we need in order to reject the null hypothesis? The answer to this is, a common rule of thumb is that the p-value must be less than or equal to 0.05, but there is nothing universal about this value.

The p-value gets smaller as the test statistic calculated from your data gets further away from the range of test statistics predicted by the null hypothesis.

P-value ≤ α (Critical **value**): Reject the null hypothesis of the statistical test. The critical **value** that most statisticians choose is $\alpha = 0.05$. This 0.05 **means** that, if we run the experiment 100 times, 5% of the times we will be able to reject the null hypothesis and 95% we will not

The p-value is a proportion: if your *p*-value is 0.05, that means that 5% of the time you would see a test statistic at least as extreme as the one you found if the null hypothesis was true.

The p-value is a number, calculated from a statistical test, that describes how likely you are to have found a particular set of observations if the null hypothesis were true.

Critical or Rejection region: the range of values for the test value that indicate a significant difference and that the null hypothesis should be rejected.

Non-critical or non-rejection region: the range of values for the test value that indicates that the difference was probably due to chance and that the null hypothesis should not be rejected.

Critical Value (CV): separates the critical region from the non-critical region, i.e., when we should reject H0 from when we should not reject H0

A Critical Value is simply a 'cut-off' point between the area in a distribution representing the Null Hypothesis and the area in the distribution representing the Alternative Hypothesis."

One-tailed test: a test that indicates that the null hypothesis should be rejected when the test value is in the critical region on one side.

Left-tailed test: when the critical region is on the left side of the distribution of the test value.

Right-tailed test: when the critical region is on the right side of the distribution of the test value.

Two-tailed test: the null hypothesis should be rejected when the test value is in either of two critical regions on either side of the distribution of the test value.

The power of a binary hypothesis test is the probability that the test rejects the null hypothesis when a specific alternative hypothesis is true — i.e., it indicates the probability of avoiding a type II error. The statistical power ranges from 0 to 1, and as statistical power increases, the probability of making a type II error (wrongly failing to reject the null hypothesis) decreases.

Statistical Power: the probability of accepting the alternative hypothesis if it is true.

More intuitively, the statistical power can be thought of as the probability of accepting an alternative hypothesis, when the alternative hypothesis is true.

It is common to design experiments with a statistical power of 80% (0.80) or more, leaving a 20% probability of encountering a Type II error.

A formal way to test for normality is to use the Shapiro-Wilk Test.

The Shapiro–Wilk test: a test of normality in frequentist statistics. It was published in 1965 by Samuel Sanford Shapiro and Martin Wilk.

Shapiro-Wilk normality test and histogram of the differences in endurance performance time between caffeine dosage levels both seem to conclude that our assumptions regarding normality are violated.

This test of a parametric hypothesis relates to nonparametrics in that a lot of statistical methods (such as t-tests and analysis of variance) assume that variables are normally distributed. If they are not, then some nonparametric methods may be needed.

It is classical diagnostics for non-normality: skewness and kurtosis.

Since any normal distribution is symmetric around its mean μ, its skewness is 0

Kurtosis: a measure of the "tailedness" of the probability distribution of a real -valued random variable

Kurtosis: the measure of the thickness or heaviness of the tails of a distribution.

Effect size in statistics

Effect size tells you how meaningful the relationship between variables or the difference between groups is. It indicates the practical significance of a research outcome.

A large effect size means that a research finding has practical significance, while a small effect size indicates limited practical applications.

z * is the test statistic of a z test and t * is the test statistic of a t test

The z test for means: the z test is a statistical test for the mean of a population. It can be used when $n \geq 30$, or when the population is normally distributed and σ is known.

For Non-normal and Symmetrical Data use the Wilcoxon Signed-Rank Test or the Quantile Test for hypothesis testing

What is the Wilcoxon Sign Test?

The Wilcoxon Sign test is a statistical comparison of the average of two dependent samples. The Wilcoxon sign test is a sibling of the t-tests. It is, in fact, a non-paracontinuous level alternative to the dependent samples t-test. Thus the Wilcoxon signed rank test is used in similar situations as the Mann-Whitney U-test. The main difference is that the Mann-Whitney U-test tests two independent samples, whereas the Wilcoxon sign test tests two dependent samples**.**

The tests analyze whether there is a significant difference between the before and after treatment.

Unlike the t-test and F-test the Wilcoxon sign test is a non-paracontinuous-level test. That means that the test does not assume any properties regarding the distribution of the underlying variables in the analysis. This makes the Wilcoxon sign test the analysis to conduct when analyzing variables of ordinal scale or variables that are not multivariate normal**.**

The Wilcoxon signed rank test pools all differences, ranks them and applies a negative sign to all the ranks where the difference between the two observations is negative. This is called the signed rank. The Wilcoxon signed rank test is a non-paracontinuous-level test, in contrast to the dependent samples t-tests. Whereas the dependent samples t-test tests whether the average difference between two observations is 0, the Wilcoxon test tests whether the difference between two observations has a mean signed rank of 0. Thus it is much more robust against outliers and heavy tail distributions. Because the Wilcoxon sign test is a non-paracontinuous-level test it does not require a special distribution of the dependent variable in the analysis. Therefore it is the best

test to compare mean scores when the dependent variable is not normally distributed and at least of ordinal scale.

The paired samples Wilcoxon test (also known as **Wilcoxon signed-rank test)**: a non-parametric alternative to paired t-test used to compare paired data. It's used when your data are not normally distributed.

Statistically at least 20 observations are necessary to evaluate normality properly. On the other hand, skewness can have unpleasant effects on t-tests with small samples, particularly for one-tailed tests, larger sample sizes (30 to 50) may be necessary.

Outliers: extreme scores within the Data Set which have an impact on the mean. If the Outliers are pulling the Mean higher than the majority of scores in the Data Set, then you have Positive Skew, making the Tail on the right side of the graph longer than the Tail on the left side. If the Outliers are pulling the Mean lower than bulk of the scores in the Data Set, then you have Negative Skew. Negative Skew means that the Tail on the left of the graph is longer than the Tail on the right

Outliers: outliers cause distortion in statistical tests. You must scan your data for outliers (the box plot is an excellent tool for doing this). If you have outliers, you have to decide if they are one-time occurrences or if they would occur in another sample. If they are one-time occurrences, you can remove them and proceed. If you know they represent a certain segment of the population, you have to decide between biasing your results (by removing them) or using a nonparametric test that can deal with them. Most would choose the nonparametric test

- Make sure there are no values missing and
- the use of the dollar value makes the data continuous.

Why does effect size matter?

Sample Sizes in statistics are usually a very sensitive and important thing.

However, with the utilization of nonparametric tests, sample size can be as small of 2 participants for some of the tests. Sample sizes can be thought of as small (1–15 participants), medium (16–39 participants), and large (40+ participants),

One example is the Sign Test where a small sample is considered to be less than 35 and a large sample is more than 35 participants. For nonparametric statistics, a small sample size is alright. In contrast, parametric tests all need large sample sizes of 40 or more Data Points. This means

that if a researcher has fewer than 40 Data Points, nonparametric tests are the most appropriate for the research

While **statistical significance** shows that an effect exists in a study, **practical significance** shows that the effect is large enough to be meaningful in the real world. Statistical significance is denoted by *p*-values, whereas practical significance is represented by effect sizes.

Statistical significance alone can be misleading because it's influenced by the sample size. Increasing the sample size always makes it more likely to find a statistically significant effect, no matter how small the effect truly is in the real world.

In contrast, effect sizes are independent of the sample size. Only the data is used to calculate effect sizes.

That's why it's necessary to report effect sizes in research papers to indicate the practical significance of a finding. The APA guidelines require reporting of effect sizes and confidence intervals wherever possible.

How do you calculate effect size?

There are dozens of measures for effect sizes. The most common effect sizes are Cohen's *d* and Pearson's *r*. Cohen's *d* measures the size of the difference between two groups while Pearson's *r* measures the strength of the relationship between two variables.

Cohen's *d*

Cohen's *d* is designed for comparing two groups. It takes the difference between two means and expresses it in standard deviation units. It tells you how many standard deviations lie between the two means.

Cohen's *d* formula	**Explanation**
$d = \frac{\bar{x}_1 - \bar{x}_2}{s}$	• $\bar{x}_1$= mean of Group 1 • $\bar{x}_2$= mean of Group 2 • s = standard deviation

How do you know if an effect size is small or large?

Effect sizes can be categorized into small, medium, or large according to Cohen's criteria.

Cohen's criteria for small, medium, and large effects differ based on the effect size measurement used.

Effect size	Cohen's *d*	Pearson's *r*
Small	0.2	.1 to .3 or -.1 to -.3
Medium	0.5	.3 to .5 or -.3 to -.5
Large	0.8 or greater	.5 or greater or -.5 or less

Cohen's *d* can take on any number between 0 and infinity, while Pearson's *r* ranges between -1 and 1.

In general, the greater the Cohen's *d*, the larger the effect size.

For Pearson's *r*, the closer the value is to 0, the smaller the effect size. A value closer to -1 or 1 indicates a higher effect size.

The criteria for a small or large effect size may also depend on what's commonly found research in your particular field, so be sure to check other papers when interpreting effect size.

Once you've collected your data, you can calculate and report actual effect sizes in the abstract and the results sections of your paper.

Effect sizes are the raw data in meta-analysis studies because they are standardized and easy to compare. A meta-analysis can combine the effect sizes of many related studies to get an idea of the average effect size of a specific finding.

But meta-analysis studies can also go one step further and also suggest why effect sizes may vary across studies on a single topic. This can generate new lines of research.

How to run a paired t-test.

Step 1 – Data Preparation

These data are paired measurements. The sample size is smaller than you would like, but it is 10% of the current

population. There are no missing values, and the use of the dollar value makes the data continuous.

Step 2 – Setup and Run the Paired T-Test Panel

The selection and running of the Paired T-Test from the Analysis menu on the pairs of assessments, Value1 and Value 2, would produce the output that follows. The alpha value has been set at 0.05. Interpretation of the results will come in the steps to follow**.**

Step 3 – Check Assumptions

The major assumption to check for is normality. We begin with the graphic perspectives: normal probability plots, histograms, density traces, and box plots. Since this is paired data, we look at the normality of the differences.

In evaluating normality by numerical measures, look at the Probability (p-value) and the Decision for the given alpha of 0.05. Investigation of the Tests of Assumptions Section confirms that the differences in assessment are normal by all three normality tests since the p-values are greater than 0.05. In fact, the p-values are much greater than 0.05. The "Cannot reject normality" under Decision ($\alpha = 0.05$) is the formal conclusion of the normality tests.

Step 4 – Choose the Appropriate Statistical Test

In Step 3, the conclusions from checking the assumptions were three-fold:

(1) the data are continuous,

(2) the differences are normally distributed, and

(3) there is a strong positive relationship between the two assessments.

As a result of these findings, the appropriate statistical test is the paired t-test, which is shown next.

Step 5 – Interpret Findings

In the Descriptive Statistics Section, the mean difference is -$1.687 thousand with the standard deviation of differences being $6.140 thousand. The 95% interval estimate for the mean difference ranges from -$5.087 thousand to $1.714 thousand. The formal two-tail hypothesis test for this example is shown under the T-Test Section. The p-value for this twotail test is 0.30540, which is much greater than 0.05. Thus, the conclusion of this hypothesis test is acceptance, i.e., there is no difference in the assessments. Remember when checking the assumption of normality, we noted that there was one possible outlier in the normal probability plot in the output. If we had run the Wilcoxon Signed-Rank test instead of the paired t-test, the p-value would be 0.30280. Hence, the conclusion is the same--- there is no difference between assessments. This kind of decision confirmation does not always happen, but it is a simple option on questionable assumption

situations. However, since the data are normally distributed, the paired t-test was the correct statistical test to choose

Step 6 – Record Your Results

The conclusions for this example are that there is no difference between assessors for residential properties evaluated in this area, according to the paired t-test. The Wilcoxon Signed-Rank gave the same conclusion. If you were troubled by the one outlier, you could use a transformation on the differences plus a constant and rerun the paired t-test. Or, further examination of the one outlier might reveal extenuating circumstances that confirm that this is a one-time anomaly. If that were the case, the observation could be omitted, and the analysis redone.

Friedman's test is a non-parametric **test** for finding differences in treatments across multiple attempts. Nonparametric means the **test** doesn't assume your data comes from a particular distribution (like the normal distribution).

Arithmetic mean

$$\text{Arithmetic Mean} = \frac{a_1 + a_2 + a_3 + \ldots + a_n}{n} = \sum_{i=1}^{n} \frac{a_i}{n}$$

Where:

- $\mathbf{a_i}$ – The value of the i^{th} observation
- **n** – The number of observations

Calculating Standard Error of the Mean (SEM)

The SEM is calculated using the following formula:

$$\sigma_{\bar{x}} = \frac{\sigma}{\sqrt{n}}$$

Where:

- **σ** – Population standard deviation
- **n** – Sample size, i.e., the number of observations in the sample

The standard error of the mean can be calculated from a single sample itself. It is calculated by dividing the standard deviation of the observations in the sample by the square root of the sample size.

The standard error of the mean will approach zero with the increasing number of observations in the sample, as the sample becomes more and more representative of the population, and the sample mean approaches the actual population mean.

Calculating a confidence interval: what you need to know

Most statistical programs will include the confidence interval of the estimate when you run a statistical test.

If you want to calculate a confidence interval on your own, you need to know:

1. The point estimate you are constructing the confidence interval for
2. The critical values for the test statistic
3. The standard deviation of the sample
4. The sample size

Once you know each of these components, you can calculate the confidence interval for your estimate by plugging them into the confidence interval formula that corresponds to your data.

Point estimate

The point estimate of your confidence interval will be whatever statistical estimate you are making (e.g. population mean, the difference between population means, proportions, variation among groups).

Example 2: C.I. for a population mean; σ unknown

Find a 95% confidence interval for a population mean, given the following information:

- sample mean x = 12
- sample size n = 19
- sample standard deviation = 6.3

$CI = \bar{X} \pm Z \times s/\sqrt{n}$

$=-0.1368889 \pm 1.9600 \times (0.034571068/\sqrt{36})$

$=-0.1368889 \pm 0.0113$

Finding the critical value

Critical values tell you how many standard deviations away from the mean you need to go in order to reach the desired confidence level for your confidence interval.

There are three steps to find the critical value.

1. **Choose your alpha (*a*) value.**

The alpha value is the probability threshold for statistical significance. The most common alpha value is $p = 0.05$, but 0.1, 0.01, and even 0.001 are sometimes used. It's best to look at the papers published in your field to decide which alpha value to use.

2. **Decide if you need a one-tailed interval or a two-tailed interval.**

You will most likely use a two-tailed interval unless you are doing a one-tailed t-test.

For a two-tailed interval, divide your alpha by two to get the alpha value for the upper and lower tails.

3. **Look up the critical value that corresponds with the alpha value.**

If your data follows a normal distribution, or if you have a large sample size ($n > 30$) that is approximately normally distributed, you can use the ***z*-distribution** to find your critical values.

or a *z*-statistic, some of the most common values are shown in this table:

Confidence level	**90%**	**95%**	**99%**
alpha for one-tailed CI	0.1	0.05	0.01
alpha for two-tailed CI	0.05	0.025	0.005
***z*-statistic**	1.64	1.96	2.57

If you are using a small dataset ($n \leq 30$) that is approximately normally distributed, use the ***t*-distribution** instead.

The *t*-distribution follows the same shape as the *z*-distribution, but corrects for small sample sizes. For the *t*-distribution, you need to know your degrees of freedom (sample size minus 1).

Check out this set of *t* tables to find your *t*-statistic. The author has included the confidence level and *p*-values for both one-tailed and two-tailed tests to help you find the *t*-value you need.

For normal distributions, like the *t*-distribution and *z*-distribution, the critical value is the same on either side of the mean.

The z-score, also referred to as standard score, z-value, and normal score, among other things, is a dimensionless quantity that is used to indicate the signed, fractional, number of standard deviations by which an event is above the mean value being measured. Values above the mean have positive z-scores, while values below the mean have negative z-scores.

The z-score can be calculated by subtracting the population mean from the raw score, or data point in question (a test score, height, age, etc.), then dividing the difference by the population standard deviation.

A z-score describes the position of a raw score in terms of its distance from the mean, when measured in standard deviation units. The z-score is positive if the value lies above the mean, and negative if it lies below the mean. It is also known as a standard score

Standard normal distribution (SND) is a normally shaped distribution with a mean of 0 and a standard deviation (SD) of 1

The formula for calculating a z-score is is $z = (x-\mu)/\sigma$, where x is the raw score, μ is the population mean, and σ is the population standard deviation.

When the population mean and the population standard deviation are unknown, the standard score may be calculated using the sample mean ($\bar{x}$) and sample standard deviation (s) as estimates of the population values.

Performing a Z-test

A z-score describes the position of a raw score in terms of its distance from the mean, when measured in standard deviation units. The z-score is positive if the value lies above the mean, and negative if it lies below the mean.

When the population mean and the population standard deviation are unknown, the standard score may be calculated using the sample mean ($\bar{x}$) and sample standard deviation (s) as estimates of the population values.

Now, a test we would normally perform is the *Z-test*. The formula is: *Z* equals the **sample mean**, minus the **hypothesized mean**, divided by the **standard error**.

The value of the z-score tells you how many standard deviations you are away from the mean. If a z-score is equal to 0, it is on the mean.

Z is close to 0, then we cannot reject the null. If it is far away from 0, then we reject the **null hypothesis**.

A positive z-score indicates the raw score is higher than the mean average. For example, if a z-score is equal to +1, it is 1 standard deviation above the mean.

A negative z-score reveals the raw score is below the mean average. For example, if a z-score is equal to -2, it is 2 standard deviations below the mean.

The procedure for hypothesis testing is based on the ideas described above. Specifically, we set up competing hypotheses, select a random sample from the population of interest and compute summary statistics. We then determine whether the sample data supports the null or alternative hypotheses. The procedure can be broken down into the following five steps.

- **Step 1.** Set up hypotheses and select the level of significance α.

H_0: Null hypothesis (no change, no difference);

H_1: Research hypothesis (investigator's belief); α =0.05

Finding the standard deviation

Most statistical software will have a built-in function to calculate your standard deviation, but to find it by hand you can first find your sample variance, then take the square root to get the standard deviation.

1. **Find the sample variance**

Sample variance is defined as the sum of squared differences from the mean, also known as the mean-squared-error (MSE):

$$S^2 = \sum_{i=1}^{n} \frac{(Xi - \overline{X})^2}{n-1}$$

To find the MSE, subtract your sample mean from each value in the dataset, square the resulting number, and divide that number by $n - 1$ (sample size minus 1).

Then add up all of these numbers to get your total sample variance (s^2). For larger sample sets, it's easiest to do this in Excel.

2. **Find the standard deviation.**

The standard deviation of your estimate (*s*) is equal to the square root of the sample variance/sample error (s^2):

$$S = \sqrt{(S^2)}$$

Confidence interval for the mean of normally-distributed data

In statistics, a confidence interval is a range of values that is determined through use of observed data, calculated at a desired confidence level, that may contain the true value of the parameter being studied. The confidence level, for example, a 95% confidence level, relates to how reliable the estimation procedure is, not the degree of certainty that the computed confidence interval contains the true value of the parameter being studied. The desired confidence level is chosen prior to the computation of the confidence interval and indicates the proportion of confidence intervals, that when constructed given the chosen confidence level over an infinite number of independent trials, will contain the true value of the parameter.

Confidence intervals are typically written as (some value) ± (a range). The range can be written as an actual value or a percentage. It can also be written as simply the range of values. For example, the following are all equivalent confidence intervals:

20.6 ±0.887 or 20.6 ±4.3% or [19.713 – 21.487]

Normally-distributed data forms a bell shape when plotted on a graph, with the sample mean in the middle and the rest of the data distributed fairly evenly on either side of the mean.

The confidence interval for data which follows a standard normal distribution is:

$$CI = \overline{X} \pm Z^* \frac{\sigma}{\sqrt{n}}$$

Where:

- CI = the confidence interval
- $\bar{X}$ = the population mean

- Z* = the critical value of the *z*-distribution
- σ = the population standard deviation
- √n = the square root of the population size

The confidence interval for the t-distribution follows the same formula, but replaces the *Z** with the *t**.

In real life, you never know the true values for the population (unless you can do a complete census). Instead, we replace the population values with the values from our sample data, so the formula becomes:

becomes:

$$CI = \hat{x} \pm Z^* \frac{s}{\sqrt{n}}$$

Where:

- ˆx = the sample mean
- s = the sample standard deviation

Example: Calculating the confidence intervalIn the survey of Americans' and Brits' television watching habits, we can use the sample mean, sample standard deviation, and sample size in place of the population mean, population standard deviation, and population size.

To calculate the 95% confidence interval, we can simply plug the values into the formula.

For example: if we have a sample mean of 35 and a standard deviation of 5

For a two-tailed 95% confidence interval, the alpha value is 0.025, and the corresponding critical value is 1.96.

Sample size: In our survey of Americans and Brits, the sample size is 100 for each group.

Standard deviation: In the television-watching survey, the variance in the GB estimate is 100, while the variance in the USA estimate is 25. Taking the square root of the variance gives us a sample standard deviation (*s*) of:

- 10 for the GB estimate.
- 5 for the USA estimate.

This means that to calculate the upper and lower bounds of the confidence interval, we can take the mean ±1.96 standard deviations from the mean.

$$CI = 35 \pm 1.96 \frac{5}{\sqrt{100}}$$
$$= 35 \pm 1.96(0.5)$$
$$= 35 \pm 0.98$$

The confidence interval only tells you what range of values you can expect to find if you re-do your sampling or run your experiment again in the exact same way.

The more accurate your sampling plan, or the more realistic your experiment, the greater the chance that your confidence interval includes the true value of your estimate. But this accuracy is determined by your research methods, not by the statistics you do after you have collected the data

Critical value

A critical value is the value of the test statistic which defines the upper and lower bounds of a confidence interval, or which defines the threshold of statistical significance in a statistical test. It describes how far from the mean of the distribution you have to go to cover a certain amount of the total variation in the data (i.e. 90%, 95%, 99%). After simplification the confidence interval can be written as a statistic ± margin of error

The Wilcoxon signed-rank test: a non-parametric statistical hypothesis test used to compare two related samples, matched samples, or repeated measurements on a single sample to assess whether their population mean ranks differ (i.e. it is a paired difference test).

The wilcoxon signed-rank test: test that is performed when an analyst would like to test for differences between two related treatments or conditions, but the assumptions of a paired samples t-test are violated. This can occur when when difference between repeated **measurements are not normally distributed, or if outliers exist**. A Wilcoxon signed-rank is considered a “within -subject” or “repeated measures” analysis.

Like a paired samples t-test, a Wilcoxon signed-rank is performed when each experimental unit (study subject), receives both available treatment conditions. Thus, the treatment groups have overlapping membership and are considered dependent.

The Wilcoxon Signed-Rank test is typically used as a last resort. This is because it is a lower power test when compared to the paired t-test.

The two-sided null hypothesis is that mean treatment differences are equal to zero. The alternative hypothesis is that the mean treatment difference is not equal to zero.

H0: Paired rank differences are symmetrically distributed around zero

Ha: Paired rank differences are not symmetrically distributed around zero

Appendix C: Safety considerations

Proper safety precautions must be observed when collecting wastewater samples. Wastewater can contain microbiological disease agents (pathogens), chemical poisons (toxins), and other biological, chemical, and physical components that may cause harm and human health problems or disturb natural aquatic ecosystems. The wetland may contain some pathogens and toxins, analysists and researchers can be exposed to these hazards, hence adherence to the safety procedures and protocols is of utmost importance. Analysts and researchers handling water can be exposed to pathogens and toxins through several pathways:

• respiratory exposure -face shield and masks protect from droplets and

Aerosols.

• dermal exposure -gloves and hand hygiene protect from direct contact

• surface (fomite) exposure - barriers between skin and surfaces protect from wastewater and plant equipment contact.

Special Precautions for Wastewater Sampling

1. A clean pair of new, non-powdered, disposable gloves will be worn each time a different location is sampled and the gloves should be donned immediately prior to sampling. The gloves should not come in contact with the media being sampled and should be changed any time during sample collection when their cleanliness is compromised.
2. Sample containers for samples suspected of containing high concentrations of contaminants shall be stored separately.
3. Sample collection activities shall proceed progressively from the least suspected contaminated area to the most suspected contaminated area. Samples of waste or

highly contaminated media must not be placed in the same ice cooler as environmental (i.e., containing low contaminant levels) or background/control samples.

4. If possible, one member of the field sampling team should take all the notes and photographs, fill out tags, etc., while the other members collect the samples.

Safety considerations when handling biological hazards

Bacteria: The faeces of a healthy person contains large numbers of bacteria, most of which are not pathogenic. Pathogenic or potentially pathogenic bacteria are normally absent from a healthy intestine unless infection occurs. When infection occurs, large numbers of pathogenic bacteria will be passed in the faeces thus allowing the spread of infection to others. Diarrhoea is the most prevalent type of infection, with cholera the worst form. Typhoid, paratyphoid and other *Salmonella* type diseases are also caused by bacterial pathogens.

Protozoa: Many species of protozoa can infect humans and cause diarrhoea and dysentery. Infective forms of these protozoa are often passed as cysts in the faeces and humans are infected when they ingest them. Only three species are considered to be pathogenic: *Giardia lamblia, Balantidium coli* and *Entamoeba histolytica*. An asymptomatic carrier state is common in all three and may be responsible for continued transmission.

Protozoal cysts are poor survivors in any environment. A likely maximum in sewage or polluted water would not exceed that shown in Table 1 for *Entamoeba histolytica*. Helminth eggs vary from the very fragile to the very persistent. One of the most persistent is the *Ascaris* egg which may survive for a year or more. The major concern for this helminth is that the soil is its intermediate host prior to reinfecting humans.

Helminths: There are many species of parasitic worms or helminths that have human hosts. Some can cause serious illnesses and the ones that pass eggs or larval forms in the excreta are of importance in considering wastewater use. Most helminths do not multiply within the human host, a factor of great importance in understanding their transmission, the ways they cause disease and the effects that environmental change will have on their control. Often the developmental stages (life cycles) through which they pass before reinfecting humans are very complex. Those that have soil, water or plant life as one of their intermediate hosts are extremely important in any scheme where wastewater is used directly or indirectly.

The helminths are classified in two main groups: the roundworms (nematodes) and worms that are flat in cross section. The flatworm, in turn, may be divided into two groups: the tapeworms which form chains of helminths "segments" and the flukes which have a single, flat, unsegmented body. Most of the roundworms that infect humans and also the schistosome flukes have separate

sexes. The result is that transmission depends upon infection with both male and female worms and upon meeting, mating and egg production within the human body.

Appendix C. Safety considerations when handling Chemical hazards

Metals

A metal is an electropositive element, metals are crystalline in structure and the individual crystals contain positive metal ions. Copper, gold, iron, platinum, silver, zinc, cadmium, chromium and tungsten. Chemically, in solution, a metal atom releases an electron to become a positive ion. In bulk metals are solids and tend to have high melting and boiling points (an exception is mercury). They are lustrous, relatively dense, malleable, ductile, cohesive and highly conductive to both electricity and heat.

Reactive metals

Reactive metal is a group of metal elements that can form a reaction with acids, water, mineral acids and powerful oxidizing acids. This group can be identified with the activity or reactivity series, which determines the most reactive metals from highest to lowest.

Group I metals are good conductors of **heat and electricity**, are so soft that they can be cut with a knife.

As a result of their low specific gravities, Li, Na, and K float on water. They react vigorously with electronegative elements such as O, S and Cl.

Their highly-reactive nature makes them a hazard to look out for.

Group IIA metals include Be, Mg, Ca, Sr, Ba and Ra. They are grey, moderately-hard, high melting-point substances. Like the alkali metals **they attack water to liberate hydrogen** but with less vigour. The salts of the alkaline earths are generally less stable towards heat and water than those of alkali metals, and less water soluble.

Group IIB metals includes Zn, Cd and Hg. Zinc has some resemblance to magnesium but the other metals in the group have little in common. At room temperature mercury is unaffected by air, water or non-oxidizing agents whereas zinc is more reactive,

Metals form complex ions and their oxides are only weakly basic. Mercury forms no hydride. **Aluminium** is an extremely light, white metal and whilst hard is malleable and ductile. On exposure to air the metal forms a protective oxide film which reduces its reactivity. Its compounds tend to be covalent in nature: the sulphate is hydrolysed in solution and the trichloride is volatile.

Group IV metals includes tin and lead. Both tin and lead can form valency two and four compounds.

Two of the four outer electrons can behave as inert when the atoms are bivalent. Bivalent tin (stannous) derivatives are covalent whereas the nitrate and sulphate of bivalent lead are ionic. Some tetravalent compounds such as the hydrides and chloride are unstable,

e.g.: $PbCl_4 + 2H_2O = PbO_2 + 4HCl$

The transition metals include Cr, Mn, Fe, Co and Ni possess bi- and trivalent states. **Chromium** is a hard, malleable, white metal capable of high polish and does not tarnish in air. It is used for plating steel. Together with nickel it is also used in grades of stainless steel.

Manganese is a grey metal which decomposes water and dissolves in dilute acids. Its chief use is in steel to remove trace quantities of oxygen and sulphur and to produce tough steel. Iron is a white, soft, malleable, ductile magnetic metal when pure and is used mainly in steel production. It is attacked by oxygen or steam to produce an oxide, Fe3O4. When exposed to ordinary atmospheric conditions it becomes covered with rust, i.e. hydrated ferric oxide, 2Fe2O3.3H2O

Group IX metals -the metals copper, silver and gold from Group IX are sometimes termed coinage metals. They possess characteristic metallic lustre, take high polish and resist attack by air. They are extremely malleable and ductile and excellent conductors of heat and electricity. All are attacked by chlorine; copper alone is attacked by oxygen. None of the metals displace hydrogen from acids.

Copper has a characteristic red colour. It is used for cooking utensils and wires in telegraphs, telephones, power lines, and electrical machinery.

Silver is a lustrous, white metal capable of high polish. It is tough, malleable, ductile and an efficient conductor of heat and electricity. Whilst resistant to attack by oxygen, on exposure to air it is slowly covered with a black film of silver sulphide.

Gold is a yellow, malleable, ductile metal which does not tarnish in air and is inert to any mineral acid. It reacts with halogens and aqua-regia.

Sodium

Small pieces of sodium react with water hence sodium must always be submerged and stored in mineral oil. Sodium is soft you can cut it with a scalpel

Metal salts

Zinc sulphate - Zinc sulfate is the inorganic compound with the formula $ZnSO_4$ and historically known as "white vitriol". $ZnSO_4$ is a colorless, orthorhombic crystals

Anhydrous zinc sulfate is a colorless crystalline solid. It is soluble in water and is non-combustible. Zinc sulfate is a metal sulfate compound having zinc(2+) as the counterion. It has a role as a fertilizer. It is a metal sulfate and a zinc molecular entity. It contains a zinc(2+).

Solubility in water, g/100ml at 20 °C is good.

Zinc sulfate is also obtained as a hexahydrate, $ZnSO_4.6H_2O$, and as a heptahydrate $ZnSO_4.7H_2O$. All forms are soluble in water. All are noncombustible

Hazard Statement

- It is harmful if swallowed [Warning Acute toxicity, oral]
- Causes serious eye damage [Danger Serious eye damage/eye irritation]
- Very toxic to aquatic life [Warning Hazardous to the aquatic environment, acute hazard]
- Very toxic to aquatic life with long lasting effects

PPE when handling metals and their respective salts

- Emergency eye wash fountain/station should be available in the immediate vicinity. It should be in a good condition for use
- Wear a lab coat
- Wear closed shoes
- Use a mask when preparing higher concentrations
- Wear gloves that are impermeable to the metals

Acids

Examples of acids used includes hydrochloric acid, nitric acid, and sulphuric acid. These are strong acids which are almost completely dissociated in water. Acids tend to be corrosive. Acids dissolve metals such as copper and liberate hydrogen gas. They also react with carbonates to liberate carbon dioxide.

Acids and alkalis react with each other to produce salts and water,

Thus salts are compounds formed by replacement of hydrogen in an acid by a metal. Clearly nonmetals can also be involved, e.g.: $NH_4OH + HCl = NH_4Cl + H_2O$

Salts are non-volatile and in the fused state or in solution conduct an electric current.

Many salts are hydrated in the solid state with water of crystallization. **These reactions are exothermic** and must be carefully controlled if the reactants are concentrated, since ***the rates can be very rapid!!!!***

Concentrated acids are very corrosive and dangerous chemicals that are commonly encountered in the laboratory. Some acids are also shock sensitive and when subjected to improper handling they can explode. Concentrated acids may be fatal if inhaled; and can cause severe eye and skin burns, severe respiratory and digestive tract burns. Contact with other materials may cause a fire. All operations involving concentrated acids MUST be conducted in the fume hood and the investigator MUST wear appropriate PPE

a. When working with acids,

- **Always consult the MSDS** the Material Safety Data Sheet (MSDS) before using any hazardous material in the laboratory**.**
- Have an acid spill kit in the lab and know the spill clean-up protocol (**what to do in the event of a spill**) and spillage accidents where the acid splash on you
- Verify that emergency eyewash/shower is accessible and in good working order and tested within last month
- Use acid-compatible containers such as PVC, LDPE, PP, NEVER **USE A METAL CONTAINER**
- Handle the acids in a working fume hood, conduct all operations involving acid underneath a fume hood
- Always close the container tightly
- Wear a labcoat that is buttoned all the way up.
- Then wear a tough vinyl lab aprons which are resistant to acids, alkalis, and lab reagents (these aprons provide the protection required against dangerous chemicals, acids and caustics). Or use the Waterproof Rubber Apron, 45°Acid resistant Oil and Stain Proof Protection Work Apron
- chemical resistant Boots, preferably the ones with a steel toe and shank.
- Chemical resistant gloves (such as the ones made with nitrile, or butyl)

- Faceshield
- Add acid to water. When preparing solutions, you should always add acid to water, never the other way around. You should never add water to concentrated acid! This can generate acidic steam, and as a result, can very easily cause spills, accidents, and/or injuries.
- Store acids in a dedicated “corrosion- proof cabinet” acid cabinet. **AND NEVER STORE ACIDS AND BASES TOGETHER**
- In the event of a huge acid spill, a severe case of spillage that cannot be contained with the use of a spill kit, you will have to evacuate the area, and everyone in the laboratory will need to be evacuated. In some cases, you may need to evacuate the whole building. After the most acute injuries have been addressed, make sure that everyone is removed from the area**.**

Appendix D. Standard water quality permissible limits values.

Table D1. Wastewater limit values applicable to the irrigation of any landy up to 2000 m^3 stipulated by the National Water Act.

Variables	Limits
pH	pH not less than 5.5 or more than 9.5 pH units
Electrical Conductivity	does not exceed 70 milliSiemens above intake to a maximum of 150 milliSiemens per metre (mS/m)
Suspended Solids	does not exceed 25 mg/l
Chloride as Free Chlorine	does not exceed 0,25 mg/l
Fluoride	does not exceed 1 mg/l
Soap, Oil and Grease	does not exceed 2,5 mg/l
Chemical Oxygen Demand	does not exceed 75 mg/l
Faecal coliforms	do not exceed 1000 per 100 ml
Ammonia as Nitrogen	does not exceed 3mg/l
Nitrate/Nitrite as Nitrogen	does not exceed 15 mg/l
Ortho-Phosphate as phosphorous	does not exceed 10 mg/l

Table D2. Wastewater limit values applicable to the irrigation of any land or property up to 500 cubic meters stipulated by the National Water Act.

Variables	Limits
pH	pH not less than 6 or more than 9 pH units
Electrical conductivity	not exceed 200 milliSiemens per metre (mS/m);
Chemical Oxygen Demand (COD)	does not exceed 400 mg/l after removal of algae;
Fecal coliforms	do not exceed 100 000 per 100 ml
Sodium Adsorption Ratio (SAR)	does not exceed 5 for biodegradable industrial wastewater

Table D3. Wastewater limit values applicable to discharge of wastewater into a water resource

Substances/parameter	General limit	Special limit
Faecal Coliforms (per 100 ml)	1000	0
Chemical Oxygen Demand (mg/l)	75	30
pH	5.5-9.5	5.5-7.5
Ammonia (ionised and un-ionised) as nitrogen (mg/l)	6	2
Nitrate/Nitrite as Nitrogen (mg/l)	15	1.5
Chlorine as Free Chlorine (mg/l)	0.25	0
Suspended Solids (mg/l)	25	10
Electrical Conductivity (mS/m)	70 mS/m above intake to a maximum of 150 mS/m	50 mS/m above background receiving water, to a maximum of 100 mS/m
Ortho-Phosphate as phosphorous (mg/l)	10	1 (median) and 2,5 (maximum)
Fluoride (mg/l)	1	1
Soap, oil or grease (mg/l) 2,5 0	2.5	0
Dissolved Arsenic (mg/l)	0.02	0.01
Dissolved Cadmium (mg/l)	0.005	0.001
Dissolved Chromium (VI) (mg/l)	0.05	0.02
Dissolved Copper (mg/l)	0.01	0.002
Dissolved Cyanide (mg/l)	0.02	0.01
Dissolved Iron (mg/l)	0.3	0.3
Dissolved Lead (mg/l)	0.01	0.006
Dissolved Manganese (mg/l)	0.1	0.1
Mercury and its compounds (mg/l)	0.005	0.001
Dissolved Selenium (mg/l) 0,02	0.02	0.02

Dissolved Zinc (mg/l)	0.1	0.04
Boron (mg/l)	1	0.5

Table D4. Reagent Grade Water Tests

Parameter	Frequency	Acceptable
Free Residual Chlorine	Monthly	None acceptable
Standard Plate Count	Monthly	<500 colonies/ml
Heavy metals (Pb, Cd, Cu, Cr, Ni, Zn)	Yearly	<0.05 mg/l per metal
Suitability Test	Yearly	Ratio between 0.8-3.0

Table D5. Drinking water quality standards established in South Africa (SANS 241 :2015)

Contaminant	Unit	Risk	standard limit
Total Coliforms count	cfu/100ml	Operational	10
E. Coli (<1 taken as 0)	cfu/100ml	Acute Health Micro	0
Conductivity at 25 ˚C	mS/m	Aesthetic	170
pH at 25 ˚C	pH unit	Operational	≥ 5.0 - ≤9.7
Sulphate as SO4 2-	mg/l	Acute Health Chemical Aesthetic	500 250
Total Dissolved Solids	mg/l	Aesthetic	1500
Nitrate and Nitrite Nitrogen as N	mg/l	Acute Health Chemical	12
Nitrate as N	mg/l	Acute Health Chemical	11
Nitrite as N	mg/l	Acute Health Chemical	0.9
Aluminium as Al	µg/l	Operational	300
Cadmium as Cd	µg/l	Chronic Health	3
Chromium as Cr	µg/l	Chronic Health	50

Copper as Cu	µg/l	Chronic Health	2 000
Iron as Fe	µg/l	Chronic Health Aesthetic	2 000 300
Manganese as Mn	µg/l	Chronic Health Aesthetic	400 100
Lead as Pb	µg/l	Chronic Health	10
Zinc as Zn	mg/l	Aesthetic	5

Heavy metals limits in groundwater

Contaminant	**desirable limit(mg/l)** (BIS 2012)	**permissible limit(mg/l)** (BIS 2012)
Al	0.03	0.2
Cd	0.003	no relaxation
Cr	0.005	no relaxation
Cu	0.05	15
Fe	0.3	No relaxation
Mn	0.1	0.3
Pb	0.01	no relaxation
Zn	5	15

D.6. Relevant wetland legislation and policy

Locally the South African Constitution, seven (7) Acts and two (2) international treaties allow for the protection of wetlands and rivers within South Africa. These systems are protected from destruction or pollution by the following:

- Section 24 of The Constitution of the Republic of South Africa;
- Agenda 21 – Action plan for sustainable development of the Department of Environmental Affairs and Tourism (DEAT) 1998;
- The Ramsar Convention, 1971 including the Wetland Conservation Programme (DEAT) and the National Wetland Rehabilitation Initiative (DEAT, 2000);
- National Environmental Management Act (NEMA), 1998 (Act No. 107 of 1998) inclusive of all amendments, as well as the NEM: Biodiversity Act;
- National Water Act, 1998 (Act No. 36 of 1998);
- Conservation of Agricultural Resources Act, 1983 (Act No. 43 of 1983); and
- Minerals and Petroleum Resources Development Act, 2002 (Act No. 28 of 2002).
- Nature and Environmental Conservation Ordinance (No. 19 of 1974)
- National Forest Act (No. 84 of 1998)
- National Heritage Resources Act (No. 25 of 1999

Appendix E: Glossary of terms.

a) a river or spring;

Active channel bank: the bank of the channel(s) that has been inundated at sufficiently regular intervals to maintain channel form and to keep the channel free of established terrestrial vegetation.

Aeolian: wind-blown.

Agricultural run-off: flows Irrigation tail-water, other field drainage, animal yard, feedlot, or dairy run-off, etc.

Alluvial soil: a deposit of sand, mud, etc. formed by flowing water, or the sedimentary matter deposited thus within recent times, especially in the valleys of large rivers.

Anaerobic: not having molecular oxygen (O2) present.

Anthropogenic: having to do with man, or caused by humans. Anthropogenic means of human creation

Assimilative capacity: the ability of an ecosystem to absorb substances such as human waste and pollutants.

b) a natural channel in which water flows regularly or intermittently;

Bar: accumulations of sediment associated with the channel margins or bars forming in meandering rivers where erosion is occurring on the opposite bank to the bar.

Base flow: long-term flow in a river that continues after storm flow has passed.

Biochemical oxygen demand (BOD_5): a measurement of the amount of oxygen taken up by micro-organisms in oxidizing reducing material in the water sample. Normally measured over a 5day period at 37 degrees celsius.

Biodiversity: the number and variety of living organisms on earth, the millions of plants, animals, and micro-organisms, the genes they contain, the evolutionary history and potential they encompass, and the ecosystems, ecological processes, and landscapes of which they are integral parts.

Buffer: a strip of land surrounding a wetland or riparian area in which activities are controlled or restricted, in order to reduce the impact of adjacent land uses on the wetland or riparian area.

c) a wetland, lake or dam into which, or from which, water flows; and

Catchment: in relation to a watercourse or watercourses or part of a watercourse, this term refers to the area from which any rainfall will drain into the watercourse or watercourses or part of a watercourse, through surface flow to a common point or common points.

Catchment: the area contributing to runoff at a particular point in a river system.

Channel section: a length of river bounded by the banks and the bed.

Chroma: the relative purity of the spectral colour, which decreases with increasing greyness.

Coastal aquifer: groundwater systems found adjacent to the sea.

Community: assemblage of organisms characterized by a distinctive combination of species that occupy a common environment and interact with one another

Compliance monitoring: conducting surveys, inspections and examinations to determine the effectiveness of management strategies and actions to ensure compliance with permit conditions

d) any collection of water which the Minister may, by notice in the Gazette, declare to be a watercourse, and a reference to a watercourse includes where relevant, its bed and banks.

Deflational (hollow): a depression in the ground resulting from loss of material due to wind action.

Delineation (of a wetland or riparian zone): to determine the boundary of a water resource (wetland or riparian area) based on soil and vegetation (wetland) or geomorphological and vegetation (riparian zone) indicators.

Delineation (of a wetland): to determine the boundary of a wetland based on soil, vegetation, and/or

Dilution: the reduction in concentration of a substance due to mixing with water

Domestic effluent: wastewater/effluent arising from domestic and commercial activities and premises, which may contain sewage (as per General Authorizations - GG 20526 GN 1191 of 8 October 1999)

Ecosystem: a community of plants, animals and organisms interacting with each other and with the non-living (physical and chemical) components of their environment

Effluent: any liquid discharged into the coastal environment as waste, and includes any substance dissolved or suspended in the liquid; or liquid which is a different temperature from the body of water into which it is being discharged

EIA: Environmental Impact Assessment

Environmental impact: a positive or negative environmental change (biophysical, social and/or economic) caused by human action.

Environmental monitoring: surveys, inspections and examinations to determine the trends and status of changes in the receiving coastal waters, in terms of the health of important ecosystems and designated beneficial uses.

Environmental quality objective: a statement of the quality requirement for a body of water to be suitable for a particular use (also referred to as Resource Quality Objective)

Ephemeral stream: a stream that has transitory or short-lived flow.

Estuary: a body of surface water- (a) That is part of a water course that is permanently or periodically open to the sea; (b) In which a rise and fall of the water level as a result of the tides is measurable at spring tides when the water course is open to the sea; or (c) In respect of which the salinity is measurably higher as a result of the influence of the sea

Facultative species: species usually found in wetlands (67% – 99% of occurrences) but occasionally found in non-wetland areas.

Fault line: a geological fault resulting from differential movement in the earth"s crust

Flood bench: area between active and macro-channel, usually vegetated (inundated by annual flood). 9 9

Floodplain: a relatively level alluvial (sand or gravel) area lying adjacent to the river channel, which has been constructed by the present river in its existing regime.

Floodplain: wetland inundated when a river overtops its banks during flood events resulting in the wetland soils being saturated for extended periods of time.

Fluvial: resulting from water movement.

Footslope: the lowest portion of a hill-slope.

Geological control: the control over fluvial processes that results from the character of the geological structures in the area.

Gleying: a soil process resulting from prolonged soil saturation, which is manifested by the presence of neutral grey, bluish or greenish colours in the soil matrix.

Groundwater: subsurface water in the saturated zone below the water table.

Groundwater: subsurface water in the zone in which permeable rocks, and often the overlying soil, are saturated under pressure equal to or greater than atmospheric.

Habitat: the natural home of species of plants or animals.

Heavy metals: a group of metals which are sometimes toxic and can be dangerous in high concentrations. The main heavy metals covered by legislation are cadmium, lead, and mercury. Industrial activities such as smelting, rubbish burning, waste disposal and adding lead to petrol increase the amount of toxic heavy metals in the environment.

High terrace: relict floodplains which have been raised above the level regularly inundated by flooding due to lowering of the river channel (rarely inundated).

Hue (of colour): the dominant spectral colour (e.g. red).

Hydrology: the study of the occurrence, distribution and movement of water over, on and under the land surface.

Hydrology: the study of water, particularly the factors affecting its movement on land

Hydromorphic soil: a soil that, in its undrained condition, is saturated or flooded long enough to develop anaerobic conditions favouring the growth and regeneration of hydrophytic vegetation (vegetation adapted to living in anaerobic soils).

Hydromorphy: a process of gleying and mottling resulting from the intermittent or permanent presence of excess water in the soil profile.

Hydrophyte: any plant that grows in water or on a substratum that is at least periodically deficient in oxygen as a result of soil saturation or flooding; plants typically found in wet habitats.

Industrial effluent: wastewater/effluent arising from industrial activities and premises. Contaminated storm water drainage from industrial premises is included in this definition

Intermittent flow: flows only for short periods.

Ion: an isolated electron or positron, or an atom or molecule, which by loss or gain of one or more electrons has acquired a net electric charge.

Land-based treatment: the treatment of effluent at an inland site. Inland treatment, for example includes preliminary, primary, secondary or tertiary treatment of the effluent prior to discharge

Land-derived: means originating from a source on land, also referred to as land-based

Leachate: a leachate is any liquid that, in the course of passing through matter, extracts soluble or suspended solids, or any other component of the material through which it has passed

Macro channel bank: the outer bank of a compound channel.

Measurement parameter within the context of this document: any parameter or variable that is measured to determine specific information about an ecosystem

Mid-channel bar: single bar(s) formed within the middle of the channel; flow on both sides.

Midslope: that portion of a terrain unit, which occurs below a crest and/or scarp and above a footslope and/ or valley bottom.

Mire: peat-containing wetlands also referred to as peatlands.

Mottles: soils with variegated colour patterns are described as being mottled, with the "background colour" referred to as the matrix and the spots or blotches of colour referred to as mottles.

Municipal effluent: domestic effluent or the mixture of domestic effluent with industrial effluent and/or urban storm-water run-off

Munsell colour chart: a standardized colour chart, which can be used to describe hue (i.e. its relation to red, yellow, green, blue and purple), value (i.e. its lightness) and chroma (i.e. its 10 10

NEMA: National Environmental Management Act, Act 107 of 1998.

Non-point source pollution: pollution originating from a number of diffuse sources often associated with run-off from agricultural and urban areas

Numerous viruses may infect humans and are passed in the faeces. Five groups of pathogenic excreted viruses are particularly important: adenoviruses, enteroviruses (including polioviruses), hepatitis A virus, reoviruses and diarrhoea-causing viruses (especially rotavirus).

Obligate species: species almost always found in wetlands (> 99% of occurrences).

Offshore Within the context of ocean outfalls, this is the area of the sea in which wave action has an insignificant effect on water circulation and shoreline processes (erosion and accretion). Also means beyond the surf zone

On site treatment: processes used in reducing or eliminating the contaminants in nondomestic effluent or in altering its nature, before discharging it into any waste treatment system

Organic carbon: carbon derived from or associated with the breakdown of vegetative material.

Peat: a dark brown or black organic soil layer, composed of partly decomposed plant matter, and formed under permanently saturated conditions.

Pedology: a branch of soil science dealing with soils as a natural phenomenon, including their morphological, physical, chemical, mineralogical and biological constitution, genesis, classification and geographical distribution.

Perched water table: the upper limit of a zone of saturation that is perched on an unsaturated zone by an impermeable layer, hence separating it from the main body of ground water (the saturated zone).

Permanent zone of wetness: the inner zone of a wetland that is permanently saturated.

Podzolization: the mobilization in and removal from an A and/or E soil horizon of organic matter and/or sesquioxides.

Point-source pollution: pollution discharged from a specific fixed location, such as a pipe or outfall structure

Pollution: any change in the environment caused by (i) Substances; (ii) Radioactive or other waves; or (iii) Noise, odors, dust, or heat emanating from any activity, including the storage or treatment of waste or substances, construction and the provision of services, whether engaged in by any person or an organ of state, where that change has an adverse effect on human health or well-being or on the composition, resilience and productivity of natural or managed ecosystems, or on materials useful to people, or will have such an effect in the future (NEMA, 1998 and ICMA, 2008)

Precautionary principle avoiding risk through a cautious approach to development and environmental management in that negative impacts on the environment and on people's environmental rights be anticipated and prevented, and when they cannot be altogether prevented, are minimized and remedied

Preferential recharge: area in which a substantial proportion of recharge to groundwater takes place.

Preliminary treatment: treatment that Involves the removal from effluent of 'litter' and solids by coarse and/or fine screens as well as the removal of 'grit' (particles sizes > 0.2 mm and with a specific gravity) or by settling or separation. The effect on the suspended solid concentrations and BOD in the sewage is insignificant

Pretreatment: processes used in reducing or eliminating the contaminants in non-domestic effluent or in altering its nature, before discharging it into a wastewater treatment system

Primary treatment: involves the removal from effluent of settleable organic and inorganic solids by sedimentation tanks. The solids, which settle as sludge, have to be disposed of or treated. Fats (oil and grease) are also skimmed from the top of the settling tank.

purity). Munsell colour charts are available which show that portion commonly associated with soils, which is about one fifth of the entire range.

Redoxymorphic soil features: physic-chemical changes in the soil due to (1) in the case of gleying, a change from an oxidizing (aerated) to reducing (saturated, anaerobic) environment; or (2) in the case of mottling, due to switching between reducing and oxidizing conditions (especially in seasonally waterlogged wetland soils).

Riparian habitat (as defined by the National Water Act): includes the physical structure and associated vegetation of the areas associated with a watercourse which are commonly characterised by alluvial soils (deposited by the current river system), and which are inundated or flooded to an extent and with a frequency sufficient to support vegetation of species with a composition and physical structure distinct from those of adjacent land areas.

Riparian wetlands may be lost to water level increases upstream and flow alterations downstream of the dam. Riparian wetlands are wetlands associated with running water systems found along rivers, streams, and drainageways.

Riparian wetlands" means Class WL wetlands as defined in 15A NCAC 2B .0101(c)(8) whose major primary source of water is ground water or surface water.

Risk: the likelihood of identified hazards causing harm in a specified timeframe, including the severity of the consequences.

Runoff: stream channel flow.

Saturation zone: the zone in which the soils and rock structure are saturated with water.

Scree Pan: a collection of rocks and coarse debris that accumulates at the foot of a steep slope.

Seasonal zone of wetness: the zone of a wetland that lies between the Temporary and Permanent zones and is characterized by saturation for three to ten months of the year, within 50cm of the surface.

Secondary treatment: the separation of liquid and solids contained in primary treated effluent by a stabilizing process, utilizing micro-organisms and oxygen (aerobic biological treatment by bio-filters and/or aeration tanks). The liquid and solids are separated through settling and the

Sedges: grass-like plants belonging to the family Cyperaceae, sometimes referred to as nutgrasses. Papyrus is a member of this family.

Sesquioxides: a general term to describe free iron, aluminium and manganese oxides in the soil.

sludge is disposed of or treated. Normally secondary treatment removes > 70% of suspended solids and BOD

Soil family: a hierarchical level within the S.A. Soil Classification System, below soil form. 11 11

Soil form: a hierarchical level within the S.A. Soil Classification System, above soil family.

Soil horizons: layers of soil that have fairly uniform characteristics and have developed through pedogenic processes; they are bounded by air, hard rock or other horizons (i.e. soil material that has different characteristics).

Soil matrix: the soil framework consisting of the spatially arranged solid particles, which enclose soil air, soil water and biological components.

Soil morphology: pertaining to the form and structure of the soil.

Soil profile: the vertically sectioned sample through the soil mantle, usually consisting of two or three horizons.

Soil saturation: the soil is considered saturated if the water table or capillary fringe reaches the soil surface

Soil survey: the systematic examination, description, clarification and mapping of soils in an area for a specific purpose.

Soil wetness factor: an index indicating the period of wetness of a soil horizon; W1, W2 and W3 being short, long and all year round wetness respectively (correlated to the Forestry Soils Database).

Temporary zone of wetness: the outer zone of a wetland characterized by saturation within 50cm of the soil surface for less than three months of the year.

Terrace: area raised above the level regularly inundated by flooding (infrequently inundated).

Terrain unit morphological classes: areas of the land surface with homogenous form and slope. Terrain may be seen as being made up of all or some of the following units: crest (1), scarp (2), midslope (3) footslope (4), and valley bottom (5).

Tertiary treatment: involves the further treatment of secondary treated effluent to remove nitrogen, phosphorus, ammonia, remaining suspended solids, organic compounds, heavy metals and dissolved solids by special treatment processes

Urban storm water run-off: storm water run-off from paved areas, including parking lots, streets, residential subdivisions, of buildings, roofs, highways, etc.

Value (of colour): the lightness of colour of a soil.

Waste: refers to any substance, whether or not that substance can be re-used, recycled or recovered – (i) That is surplus, unwanted, rejected, discarded, abandoned or disposed of; (ii) That the generator has no further use of, for the purposes of production, reprocessing or consumption; and (iii) That is discharged or deposited in a manner that may detrimentally impact on the environment.

Water table: The upper surface of groundwater or that level below which the soil is saturated with water. The water table feeds base flow to the river channel network when the channel bed is in contact with the water table.

Watercourse (as defined by the National Water Act): means

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

Wetland catchment: the area up-slope of the wetland from which water flows into the wetland and including the wetland itself. Wetland delineation: the determination and marking of the boundary of a wetland on a map

Wetland delineation: the determination and marking of the boundary of a wetland. In terms of the delineation procedure described in this document, delineation means marking the outer edge of the temporary zone of wetness. **Aerobic:** having molecular oxygen (O2) present.