Hyperspectral Remote Sensing to Detect Biotic and Abiotic Stress in Water Hyacinth, (*Eichhornia crassipes*) (Pontederiaceae)

By

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Declaration

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or any other examination in any other University.

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20th day of May, 2014 in Johannesburg

Abstract

Water hyacinth (*Eichhornia crassipes*) is one of the most notorious aquatic weeds in the world. Its management, despite the release of seven biocontrol agents since 1974, remains a problem in South Africa. This is often attributed to the high level of eutrophication. However, information on the effect of heavy metals or AMD on *Neochetina eichhorniae* and *N. bruchi*, which are the common and most widely established biocontrol agents in the country, is limited. In addition integrated management, which combines herbicides with biological control methods, is the current water hyacinth control method, and requires regular monitoring of the weed's health status. This can be assessed via the canopy chlorophyll and water content, and can facilitate the decision when to intervene and what intervention measures are appropriate and timely. Hyperspectral Remote sensing (HRS) has the potential to be that monitoring tool. This thesis investigates the physiological status of water hyacinth grown with eight different heavy metals in a single-metal tub trial, three different simulated acid mine drainage (AMD) treatments in a pool trial under the influence of biocontrol agent from *Neochetina* spp., and in the Vaal River at the inlets of its tributaries, the Koekemoerspruit and the Schoonspruit. A hand-held spectrometer, the analytic spectral device (ASD), was used to measure reflectance. The hypothesis that HRS can detect the response of the plant to both the heavy metals and the biocontrol-induced stresses and their interactions was tested.

Different spectral indices associated with the canopy chlorophyll and water content of water hyacinth were evaluated. Among these the modified normalized difference vegetation index (mNDVI) and those associated with the red edge position (the linear extrapolation and the maximum first derivative indices) were able to detect the metal, or AMD or weevil-induced plant health stresses and showed a strong positive correlation with the actual leaf chlorophyll content, measured by a SPAD-502 chlorophyll meter. Among the contaminants Cu, Hg, and Zn treatments from the single-metal tub trial and sulphate concentrations exceeding 700 mg/L in the AMD pool trial were detected by the RS as stressful to the plants. The RS also indicated that the water contamination level was greater downstream at the inlet of the Schoonspruit into the Vaal River, compared to the

other sites after rainfall. These results were also consistent with actual measurements of the different plant growth parameters in all the trials and the weevils' feeding and reproductive activities in the tub and pool trials. Thus, the results of this study indicated that the HRS has potential as a tool to assess the physiological status of water hyacinth from a remote position, which could be helpful in management of a serious national problem. The acquisition of spectral reflectance data at a larger scale, from aerial platforms, involves a complex data set with additional atmospheric interference that can mask the reflectance and which demands more complicated image analysis and interpretation. Thus, further such studies in future are recommended.

Dedication

I dedicate this work to my wife Azmera Debesay Mebrahtu, and my brother Yowhannes Wakshum Newete for their unwavering moral support and help during the course of my study.

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Acronyms and their discriptions

Acronyms	Meaning
AMD	Acid mine drainage
BCF	Bioconcentration factor
CF	Chlorophyll florescence
EC	Electrical conductivity
HRS	Hyperspectral remote sensing
HSS	Hyperspectral systems
ICP-MS	Inductively coupled plasma mass spectroscopy
ICP-OES	Inductively coupled plasma optical emission spectroscopy
IPM	Integrated pest management
MSS	Multispectral systems
NIR	Near infrared
PAR	Photosynthetically active radiation
PET	Polyethylene terephthalate
REP	Red edge position
RS	Remote sensing
VIs	Vegetation Indices
WfW	Working for Water

Problem statement

Water has no substitute. South Africa is a water-stressed country with average annual rainfall about half (450mm) of the world average rainfall (860mm); therefore our water resources must be carefully managed (www.dwa.gov.za). Invasions by non-indigenous species result in the destruction of water ecosystems in terms of their function, diversity and economic value (Hulme, 2003). The South African government, through Working for Water (WfW) spends up to R600 million annually and the programme has recently secured a three-year budget of R7.8 billion in invasive alien plant control (van Wilgen *et al*., 2012). Water hyacinth, *Eichhornia crassipes* (Martius) Solms-Laubach (Pontederiaceae), is the most notorious of such invasive aquatic alien weeds (Malik, 2007) and has become unmanageable in some South African water systems despite the enormous resources and efforts allocated to it (Byrne *et al*., 2010).

The country has released seven biocontrol agents since 1974 and it includes: the weevils *Neochetina eichhorniae* Warner (Coleoptera: Curculionidae) and *N. bruchi* Hustache, the moth *Niphograpta albiguttalis* (= Sameodes albiguttalis) Warren (Lepidoptera: Pyralidae), the mirid *Eccritotarsus Catarinensis* Carvalho (Hemiptera: Miridae), the pathogen *Cercospora piaropi* Tharp (Mycosphaerellales: Mycosphaerellaceae), and the mite *Orthogalumna terebrantis* Wallwork (Acarina: Sarcoptiformes: Galumnidae) (Coetzee *et al*., 2011) and the grass hopper *Cornops aquaticum* Brüner (Orthoptera: Acrididae) (Bownes *et al*., 2011). However, none of them have achieved satisfactory results (below a threshold of 10% surface cover of the water body concerned) compared to other parts of the world such as in Uganda (Lake Victoria), Australia, and Papua New Guinea (Coetzee *et al*., 2011). There are several factors that affect the efficacy of water hyacinth biocontrol in South Africa, among which is the high level of water eutrophication (Coetzee and Hill, 2012). Continuous nutrient enrichment of the water system by runoff from agricultural lands and domestic and industrial effluents boosts the growth of water hyacinth and increases its population size exponentially, through rapid regeneration of plant biomass and density that allow the plant to overcome damage by biocontrol agents (Coetzee and Hill, 2012). Equally, the variability of temperature, especially the occurrence of frost during winter affects biocontrol agents, usually giving an advantage to the plant in the following warm season (Byrne *et al*., 2010). For instance the Schoonspruit, which is largely eutrophied by runoff from the nearby gold mining sites, agricultural lands and effluents from the local settlement of Kennan near Orkney, is one of the tributaries, which is a source of pollution and eutrophication of the Vaal River (DWAF, 2009).

Acid mine drainage (AMD) is also a serious problem that compromises the water quality in South Africa (Cukrowsky *et al*., 2010). It is formed from sulfur bearing minerals (e.g. iron sulphide) exposed to water and oxygen, which through their reaction produces sulfuric acid which dissolves heavy metals such as Fe (the most common one), Cu, Pb and Hg into ground and surface waters (Akcil and Koldas, 2006). Such process increases the bioavailability of heavy metals contaminants in water through the release of elements that were previously bound to mineral rocks or other chemical compounds. The Vaal River near Orkney in the North West Province carries waterborne pollution from the closely located slimes dams (solid-water-mixture ('slurry')) of the Buffelsfontein gold mine (Winde and van der Walt, 2004). The effect of AMD on the biological control agents of water hyacinth, particularly the water hyacinth weevils, has not been studied before. The effect of heavy metals on these weevils is limited to the studies conducted by Kay and Haller (1986) and Hussain and Jamil (1992). Other research has demonstrated that metal concentrations in plant shoots affect the efficacy of insect herbivory (Davis *et al*., 2001; Coleman *et al*., 2005; Boyd, 2010).

An integrated pest management (IPM) system, in which a sub-lethal dose of herbicide is used in combination with biocontrol agents, has shown potential to control the water hyacinth weed (Byrne *et al*., 2010). This method has been implemented as a strip-spraying technique, creating refuges for the biocontrol agents where spray drift suppresses plant growth with a low herbicide dose but does not kill the plants nor the insects associated with them. The advantage of such combined method is to reduce the amount of chemical sprayed and cut the cost, while reducing the chemical impacts on the water ecosystem. However, it requires an appropriate method of monitoring the extent of infestation, plant phenology and associated plant physiological status such as canopy chlorophyll and water content, which could be affected by the level of water contaminants (heavy metals or AMD), herbicides and weevil herbivory, to facilitate the correct intervention decisions, which include the release of biological control or spraying with herbicides.

Remote sensors can acquire data from inaccessible sites at a regional and international level (such as from satellite platform). Hyperspectral remote sensing has been used for monitoring plant health status and measuring the encroachment of various alien invasive plants in different habitats (Huang and Asner, 2009). However, studies of water hyacinth using hyperspectral remote sensing have been limited to mapping of infestations (Cavilli *et al*., 2009; Hestir *et al*., 2008; Underwood *et al*., 2006; Everitt *et al*., 1999). In this study hyperspectral remote sensing was used for the first time to evaluate physiological stresses (e.g. reduction in canopy chlorophyll and water contents.) of water hyacinth from heavy metals, AMD and herbivory by biological control agents.

This project is novel because it links the impact of water contamination on the relationship between a weed and a biocontrol agent, while evaluating new monitoring tools to aid in the management of a serious national problem. Ultimately, this approach may improve the management of the weed. This method can be tested at a landscape level either by flying the hyperspectral sensor mounted on a plane or from a satellite platform which will expand its usage across the country as a monitoring tool. Such a tool may eventually be useful against other invasive weeds under normal or polluted conditions.

Chapter 1

Introduction

The impacts and management of water hyacinth and hyperspectral remote sensing

1.1 The success of invasive plants

The fact that most invasive alien plants escape from their co-evolved natural enemies such as pathogens and herbivores, gives them an advantage over their competing local or native plant species (Blumenthal *et al*., 2009). As such these plants grow robustly and extensively, excluding many indigenous plant species in the process, and eventually taking over most of the natural habitat and ecosystem by altering different disturbance regimes such as fire frequencies and other natural processes of the ecosystem (e.g. nutrient cycling, erosion and water availability) (Mack *et al*., 2000; Vitousek, 1990). The European cheatgrass, *Bromus tectorum* L. (Cyperales: Poaceae) successfully spread over five million ha in the great valley of Idaho and Utah, and subsequently exposed the existing natural habitat to destructive fires (Pimentel *et al*, 2005). The invasion increased fire frequency from once every 60-110 years to 3-5 times every year making it virtually impossible for the local woody plants or shrubs to re-establish after such disturbance (Pimentel *et al*, 2005). Water loss through evapotranspiration is another major envoironmental problem of invasive alien plants. The increased evapotranspiration rate from woody invasive alien plants alone accounts for 30% of water loss for many downstream users in South Africa (Pejchar and Mooney, 2009). Similarly the rate of water loss through evapotranspiration by the aquatic invasive species the *Pistia stratiotes* and *Eichhornia crassipes* exceed the open-water evaporation rate by 10- and 3-6 times, respectively (Schmitz *et al*., 1993). Because of such effects, biotic invasions generally have come to be recognized as the leading factor accompanying climatic change as the main causes of global change (Huang and Asner, 2009).

Invasive alien aquatic weeds lead to the destruction of aquatic biodiversity and can degrade the quality of water resources (Hestir *et al*., 2008). Control of aquatic weeds in the United States costs about USD $100 million dollars annually (Pimentel *et al*., 2005). The invasive weed, the purple loosestrife, *Lythrum* *salicaria* L. (Myrtales: Lythraceae) which is known as the “Purple Plague” is identified as “Public Enemy #1 on Federal Lands” by the United States Fish and Wildlife Service (Liu *et al*., 2005). The control costs and forage losses from this weed are estimated at over $45 million dollars every year (Liu *et al*., 2005).

Water hyacinth, *Eichhornia crassipes* (Mart.) Solms-Laubach (Pontederiaceae) is an alien invasive weed from South America (native to Amazonia) (Harley, 1990), and its introduction into South Africa dates back to 1900 (Hill and Cilliers, 1999). It grows best in tropical and subtropical environmental conditions with optimum temperatures between 25-30°C, pH of 6-8 and eutrophied, dry conditions (Gopal, 1987; Wilson *et al*., 2001; Malik, 2007).Currently it is the fastest spreading weed in the world, where it survives in a wide climatic range, tolerates temperatures ranging from 1-40°C and extremes of water nutrient levels (Malik, 2007). In favourable conditions water hyacinth grows vegetatively from stolons and the new daughter plants can double in number within 6-18 days (Malik, 2007). Water hyacinth also reproduces by seeds with a single rosette capable of producing over 3000 seeds annually (Center *et al*., 2002), which can then stay dormant and viable for the next 15-20 years (Gopal, 1987; Lu *et al*., 2007). Albano Pérez *et al*. (2011) found an average density of 1177 seeds/m^2 in seed banks of water hyacinth at 15 sites in South Africa, with a maximum density of up to 4228 seeds/m^2 found at one site. Germination rate was as high as 80% and only 3-4 days are required to germinate under optimal conditions. This potential of the plant, to swap between methods of reproduction under different environmental conditions is the main factor that accounts for its highly dynamic and invasive nature, making it one of the most successful and productive plants on the planet (Malik, 2007). Ogutu *et al*. (1997) calculated that a single plant can expand to cover an estimated area of 1.40 km^2 every year by producing about 140 million daughter plants with a wet weight of 28, 000 tons.

1.2 Environmental problems

Water hyacinth’s enormous capacity to absorb nutrients and its resilience to harsh conditions (wide temperature and nutrient extremes) makes it an aggressive invader which can convert surface water rapidly into a monoculture (Tiwari *et al*., 2007). In ideal conditions water hyacinth grows up to 1.5 m in height creating extensive intertwined mats in the water (Howard and Harley, 1998). Such mats can consist of over two million plants, weighing from 270 to 400 tons per ha (Malik, 2007). Water hyacinth can dominate an entire water system within a short period, propelled by its extremely efficient reproduction and resilience to adverse conditions. Under highly eutrophic and warm conditions a water hyacinth increase in biomass of up to eightfold is possible, compared to the plant in oligotrophic water with low nutrient availability (Reddy *et al*., 1990). Ashton *et al*. (1979) found that water hyacinth shows a vegetative growth rate of up to 6% daily. The weed destroys aquatic biodiversity through its outstanding ability to compete with native plant species and in due course it has the ability to convert an entire water system into a "biological desert" (a one-plant-system). In Lake Caohai in China (in the province of Yunnan), where water hyacinth covered two-thirds of the lake, the number of plant species declined from 16 in 1960 to 3 in 1990 as result of the water hyacinth infestation (Lu *et al*., 2007). The Nile crocodile (*Crocodylus niloticus*) and many birds including Pel's fishing owl (*Scotopelia peli)*, the African Fish eagle (*Haliaeetus vocifer*) and the African Finfoot (*Podica senegalensis*), which once attracted tourists, disappeared from Nseleni River (KwaZulu Natal, South Africa) after the river became infested with extensive mats of water hyacinth between the 1970s to 1990s (Jones, 2009).

Extensive water hyacinth infestations cause other environmental problems including reducing oxygen levels (Malik, 2007). The massive growth of water hyacinth biomass increases water loss by transpiration, reduces water flow, and increases accretion which may lead to catastrophic negative changes to stream and river systems (Tiwari *et al*., 2007).

1.3 Nutrient requirements

Generally the growth rate of water hyacinth is positively correlated with an increase in water nutrient levels (primarily nitrogen and phosphorus) (Reddy *et al*., 1990). The plant responds positively to increases in the phosphorus concentration in water from 0.1-1.06 mg/L, beyond which the growth will stop and in extreme cases the plants will die (such as below 0.06 mg/L of P) (Haller and Sutton, 1973). Similarly water hyacinth growth increases with a rise in nitrogen concentration in the range of 1-25 mg/L (Chadwick and Obeid, 1966), but usually reaches maximum when the concentration is above 21 mg/L (Reddy *et al*., 1989).

The major sources of surface water eutrophication in South Africa are runoff from agricultural and industrial activities, and sewage disposal from highly populated settlements into rivers giving South Africa some of the most eutrophied water systems in the world (Walmsley, 2000). Byrne *et al.* (2010) reported that concentrations of nitrogen and phosphorus in the fresh waters of South Africa ranged from 0.01 mg/L to 7 mg/L and 0.001 mg/L to 2.5mg/L, respectively, allowing water hyacinth to persist and thrive, and requiring continued management interventions.

1.4 Water hyacinth management

Every year enormous amounts of money and effort are expended to reduce the impact of water hyacinth and involve mechanical (manual) removal, herbicides and biological control measures. Manual removal is often costly and labour intensive in addition to being inconvenient and ineffective except for small water bodies or small scale infestations (Sharp, 2009). For instance, even though daily manual removal of water hyacinth in Zhu River of Guangdong Province in China progressively increased over the years from 0.5 tons in 1975, 5 tons in 1985, 50 tons in 1995 and 500 tons in 2000, water hyacinth is still uncontrolled and removal has remained an endless activity (Lu *et al*., 2007). Such tedious control efforts have led to massive use of herbicides as the best alternative measure because of the rapid results they achieve. However, this is offset by the high cost of chemicals and the need to continually apply the chemicals and growing concerns associated with environmental and health hazards. On the other hand, biological control is relatively safe and cost effective, underpinned by extensive research and wide public acceptance. For instance water hyacinth was successfully controlled (usually referring to an infestation level of < 10%) with biocontrol in Australia and the USA (Julien, 2001), Papua New Guinea (Julien and Orapa, 1999), and on Lake Victoria in Uganda (Cock *et al*., 2000), although recent reports in these country are not available in the literature. The extensive water hyacinth mats that once covered large parts of Lake Victoria have been controlled and stabilized by the addition of the biological control agents

(*Neochetina* spp.) in conjuction with other factors such as the El Ninõ incidence of 1997/1998 which may have contributed to sinking of the already weakened plants, thereby facilitating its control (Wilson *et al*., 2007).

1.4.1 The efficacy of *Neochetina* spp.

The first biocontrol agent released against water hyacinth in South Africa in 1974 was the weevil, *Neochetina eichhorniae* Warner (Coleoptera: Curculionidae) and later followed by the release of the *N. bruchi* in 1990 (Coetzee *et al*., 2011). Both weevil species *N. eichhorniae* and *N. bruchi* Hustache have most widely established in South Africa compared to the other five biocontrol agents of water hyacinth (*Niphograpta albiguttalis, Eccritotarsus Catarinensis, Cercospora piaropi, Orthogalumna terebrantis and Cornops aquaticum*), and therefore the country and the continent largely depends on these two weevils in biocontrol programmes (Cilliers and Neser, 1991). However, despite the 'percieved success' (Julien, 2001) of biocontrol of water hyacinth in other parts of the world using these weevils, it has remained unsatisfactory in South Africa (Hill and Olckers, 2001). This is assumed to be due to South African surface waters being exceedingly and consistently enriched with nutrients (Walmsley, 2000), allowing water hyacinth to undergo explosive growth. We now know that several of the biological control agent species will fail to control the plant under high nutrient regimes (Coetzee *et al*., 2007). In addition, parts of South Africa that experience low temperatures, below 10°C in winter and peak around 30°C in summer, often experience a boom-bust growth trend of water hyacinth, while populations of the biocontrol agents take longer, after the cold weather, to reach damaging numbers before the end of summer (Byrne *et al*., 2010). This is because the plants grow at a faster rate than the weevils can reproduce. The lower oviposition and developmental temperature thresholds for the water hyacinth weevils are 10 and 15 °C respectively (King, 2011), as opposed to the host plants which could reproduce in temperatures even lower than that. The resurgence of water hyacinth enables it to prevail over the damage inflicted by the recovering population of biocontrol agents in summer (Hill and Olckers, 2001). The other constraint on biocontrol agents comes from injudicious application of herbicides. Nevertheless this herbicide interference now seems to be resolved since Working for Water (WfW) shifted to an Integrated Pest Management (IPM) system whereby several water hyacinth control measures are optimized (mechanical, herbicide and biological control) and implemented in combination (Sharp, 2009; Cilliers *et al*., 1996). Byrne *et al*. (2010) also showed that a sub-lethal dose of herbicide, resulting from strip spraying the weed, created refuges for the biocontrol insects and improved their efficiency while the sub-lethal dose of herbicide suppressed the water hyacinths' vigour. However, high level of eutrophication enhances the growth of water hyacinth, while acid mine drainage (AMD) could reduce the growth. Thus their interaction with the biocontrol agents (weevils) is a subject that needs an investigation. This is due to the fact that eutrophication and AMD in South African waters are serious problems and the control of the water hyacinth weevils using the biocontrol agents remained ineffective.

1.4.2 Metal accumulation by plants and their response to insect herbivory

Plants that grow under heavily polluted conditions and particularly those which are accumulators or hyperaccumulators (plants capable of accumulating extreme concentrations of heavy metals) are proposed to be resistant to some natural enemies (Boyd, 2010). The toxicity and the deterrent effects of different heavy metal contaminants to insect herbivores is variable and acts either by reducing feeding, retarding larval development or in extreme cases by intoxicating insects, causing death (Davis *et al*., 2001). For instance, when the diamondback moth (DBM), *Plutella xylostella* L. (Lepidoptera: Plutellidae) was fed on an artificial diet (consisting mainly of wheat germ and cabbage leaf powder with varying additions of heavy metals), copper was toxic to the moth at concentrations of 195mg Cu/g of diet and chromium (Cr) at 106mg Cr/g. The threshold for manganese and zinc concentrations at which the survival of DBM was affected and started to decrease were at 1370mg Mn/g and at 275mg Zn/g, respectively (Coleman *et al*., 2005). Boyd (2010) also discussed the advantages of elemental defenses that some plants obtain from the accumulation of high levels of heavy metals such as As, Cd, Ni, Se, and Zn. Such defense against insect herbivores may also occur at lower concentrations of a single element when combined with other heavy metals (Coleman *et al*., 2005). For instance the pairing of Zn with Cd, Ni, and Pd, was found to effectively defend plants at lower concentrations than the concentration level of a single heavy metal element accumulated in the plant tissues (Coleman *et al*., 2005). Similarly Straker *et al.* (2007) found a lower survival rate and density of spores of arbuscular mycrorrhiza in host plants (*Asclepias fruticosa* L., *Cynodon dactylon* (L.) Pers., *Atriplex semibaccata* R. Br., *Phytolacca octandra* L. and *Asparagus laricinus* Burch.), which were planted in never-re-vegetated zones of the slimes dam of gold-mines with the lowest pH, P, organic matter and high potential acidity compared to those in re-vegetated and re-ameliorated zones.

However, some natural enemies have developed strategies to avoid toxicity of hyperaccumulated elements in plant tissues. Boyd *et al*. (2009) indicated that *Berkheya coddii* Rosseler, a plant species known to hyperaccumulate Ni, is a host for *Chrysolina clathrata* Clark. They found Ni concentrations of only 260 µg/g dry weight in *C. clathrata* even though the leaf material this insect species consumed contained 15 100 µg of Ni/g.

Water hyacinth is known to accumulate heavy metals such as Cd, Zn, Ag, Pb (Lu *et al*., 2004), Ni, Se, Cu, and Cr (Malik, 2007), and Hg (Skinner *et al*. 2007). The fact that acid mine drainage from gold mining and effluents from industrial wastes cause a major water pollution problems in South Africa (Manders *et al*., 2009), the growth of water hyacinth under such contaminated waters would accumulate an enormous amount of heavy metals (Mishra *et al*., 2008a; Ismail and Beddri, 2009; Hussain *et al*., 2010; Rahman and Hasegawa, 2011; Chattopadhyay *et al*., 2012). However, heavy metals in plant tissues are known to affect insect herbivory (Boyd, 2010). The efficacy of biocontrol agents (weevils) of water hyacinth could partly be compromised by the level of AMD and the amount of heavy metal becoming bioavailable in water during acidification and therefore, requires more investigation.

1.4.3 Integrated management of water hyacinth

Management of water hyacinth in South Africa for several years was a mismatch of biological and chemical control (Hill and Olckers, 2001), although this has recently changed to an integrated management approach, which combines biocontrol with a sub-lethal herbicides. Currently the control of water hyacinth in China costs over $12.35 million anually (Lu *et al*., 2007). The cost of water hyacinth management in the USA is estimated to be between USD $500,000 (in California) and $3 million (in Florida) annually; while in South Africa the control of water hyacinth with herbicides alone, varies from USD $114 – 687/ha (ZAR800 – 4 800/ha) depending on the spraying method used (Debbie Sharp, 2010, Working for Water, pers. comm.) with annual total estimates being over USD $ 1 714 286 (ZAR12 million) (Byrne *et al*., 2010).

Biological control (potentially integrated with herbicidal interventions) is available and is less expensive than the use of chemical technologies (van Wyk and van Wilgen, 2002). Therefore, an integrated control strategy for water hyacinth, which integrates biocontrol agents with applications of a sublethal dose of herbicide at key points in the annual cycle of the weed, has been developed (Byrne *et al*., 2010; Jadhav *et al*., 2008). This method can work to control water hyacinth depending on the local circumstances of climate, nutrients and pollutants. However, infested sites must be monitored so that the growth trajectory of the weed population is understood, to predict what intervention (biocontrol or herbicides or both) will be required and when. Thus a tool is needed to rapidly assess the status of the plant and the control agents at the plant and landscape level. This information can then be used to guide management interventions.

1.5 Remote sensing reflectance of plants using a spectrometer

The acquisition of information about an object or the surface of earth at larger scale without a physical contact is known as remote sensing and it involves sensing of light reflected or energy emitted from the surface of an object with a sensor (Campbell, 2002). The measurement of reflected light from the earth's surface, such as vegetation cover, as a function of wave length is called spectral reflectance.

Different biochemical reactions, anatomy and physiological processes that occur in plant leaves determine the response curve of the spectral reflectance of vegetation. Among these influential leaf features are the anatomical structure, pigments, proteins, lignin, leaf-water-content, rates of photosynthesis and chlorophyll fluorescence (Cenedese *et al*., 2006). Coloured pigments such as chlorophyll, anthocyanins and carotenoids are the major determinants of leaf spectral features in the visible light range (400-700 nm) of the electromagnetic spectrum (also called Photosynthetically Active Radiation – or PAR), while the effects of intercellular leaf structure and foliar water content on the vegetation spectral curve are primarily observed in the range of 700-1300 nm and 1300-2000 nm respectively (Liew *et al*., 2008). Most plants with healthy green leaves have an increased level of absorption both in the blue (400-500 nm) and red (600-700 nm) ranges, and high reflectance in the green ranges (500-600 nm) and beyond the visible range between (700-1300 nm) of the light spectrum **(**Mirik *et al*., 2007). Leaf chlorophyll includes two prominent pigments known as chlorophyll-a and chlorophyll-b, but chlorophyll-a largely accounts for the red leaf fluorescence in the 600-700 nm range (Liew *et al*., 2008).

Chlorophyll fluorescence is the light re-emitted by chlorophyll molecules of plant leaves after absorption, as opposed to light reflectance which is the amount of incident light directly reflected back from the surface. Both the internal leaf structures and the leaf pigments are directly influenced by the physiological status of the plant, hence any alteration as a result of stressors will change the spectral signature of the vegetation (Blackburn, 1998) and this provides information on the plant's health status (such as photosynthesis, transpiration, metabolism) (Peñuelas and Filella, 1998; Mirik *et al*., 2007). Water deficiency, pests, pathogens, and frost are among some of the environmental factors that depress plant chlorophyll content, which in turn determines the spectral signature of vegetation in remote sensing. Marlin *et al*. (2013) showed that maximal fluorescence (F_m) of water hyacinth leaves decreased as the damage caused by the mite *Orthogalumna terebrantis*, increased and that herbivory was generally correlated negatively with the leaf chlorophyll content (chlorophyll level decreased as mite damage increased). When plants are stressed, the optical properties of the healthy leaf decline (Fig. 1.1). For instance, reflectance will tend to decrease in the NIR (700-1300 nm) and the amount of the red band absorption in the chlorophyll concentrated region (680 nm) will decrease (Yang *et al*., 2009) (Fig. 1.1).

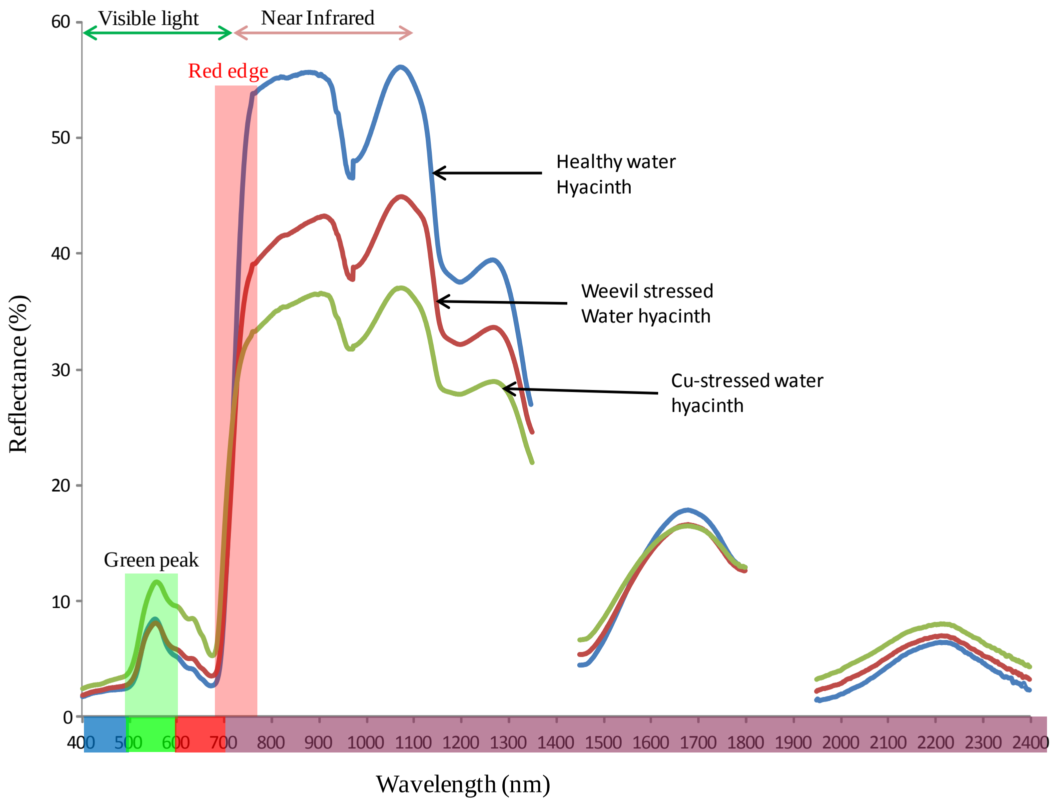

Figure 1.1: Spectral signatures of water hyacinth in a pilot test showing a decrease in both spectral absorption and reflectance at the blue (400-500 nm) and red bands (600-700 nm), because of chlorophyll pigments and in the NIR (700-1300 nm) due to anatomical and intercellular structures, respectively when grown under stress of biotic and abiotic factors (unpublished data).

1.5.1 Vegetation Indices (VIs) used in estimation of plant stresses

Several vegetative indices in the red edge region are used as indicators of plant physiological stress. One such parameter is the ratio of chlorophyll fluorescence (CF) emissions (red and far red light produced in photosynthetic tissue) between 690-740 nm (F690/F740) which is inversely related to the amount of photosynthesis (Liew *et al.*, 2008). Plants growing under stressful conditions exhibit leaf chlorosis – which is a result of chlorophyll pigment disintegration and declines in total chlorophyll concentration. However, the changes in chlorophyll function usually precede changes in chlorophyll concentration, and consequently changes in CF can be detected long before leaf chlorosis (Zarco-Tejada *et al.*, 2002). Thus, the evaluation of CF assists in early detection of stress before the consequences (visual symptoms) appear in plants (Zarco-Tejada *et al.*, 2002). For example, the CF intensity ratio of F690/F730 increased in a sunflower plant stressed by N, P and K deficiency (Subhash and Mohanan, 1997), and in poplars and conifers under water stress (Valentini *et al.*, 1994).

Light reflectance from a vegetation surface depends on several factors among which are the amount and composition of the light that strikes the leaf surface, since solar irradiation varies with time and atmospheric conditions (moisture, clouds, dust particles and gases), which gives inconsistent results in repeated spectral data acquisition (Jackson and Huete, 1991). In addition to this light, reflectance from the leaf surface is also a function of the leaf surface reflectance property. Hence, the absolute value of light reflectance from a surface of vegetation is not a sufficient measure on its own. To overcome such problems vegetation indices (VIs) are used for a more consistent interpretation of leaf properties using spectral data. Vegetation indices are combinations of surface reflectance at two or more wavelengths or bands usually determined as ratios, differences or sums, at different wavelengths, or by using a linear combination of spectral data (Jackson and Huete, 1991). The first vegetation index was the Normalized difference of vegetation index (NDVI), which was attributed to Kriegler *et al*. (1969), although it was later endorsed and used in the Great Plains study by Rouse *et al*. (1973). Over the years many VIs have been developed and published in research papers, but only very few of them are commonly used. Some of these VIs used to detect plant stress are red-edge normalized difference vegetation index NDVI (RE_NDVI) (Gitelson and Merzlyk, 1994), modified red edge NDVI ($mNDVI_{705}$) and modified simple ratio (mSR) (Datt, 1999), photochemical reflectance index (PRI), red-edge position (REP) calculated using first derivative (Dawson and Curran, 1998) and linear extrapolation (Cho and Skidmore, 2006) methods and water band index (WBI), plant senescence reflectance index (PSRI), and other dimensionless spectral indices such as yellowness index (YI) which estimates chlorosis intensity at 550 and 670 nm (maximum and minimum reflectance, respectively) (Adams *et al*., 1999). Similarly the difference in the physiological status of a healthy plant and a stressed plant is also detectable using the soil-adjusted vegetation index (SAVI) (Yang *et al*., 2009). These indices are generally capable of identifying different plant physiological status and plant stress levels. However some are more robust than others depending on the spectral bands selected to identify a specific problem.

NDVI refers to the ratio of the difference between the NIR and the red reflectance bands, to their sum (NDVI = (NIR - RED)/NIR + Red). The NDVI is positively correlated to plant health with concentrated green pigments or active photosynthetic rates due to a high level of reflectance in the NIR bands of the light spectrum (Defries and Townshend, 1999). This is due to an increased absorption of red light in the presence of concentrated leaf chlorophyll pigments of healthy plants; while a high leaf water content results in higher absorption of NIR (Lillesand *et al*., 2004). On the contrary, senescent, dead, dried or highly insect damaged plants will support little or no photosynthesis as a result of chlorophyll pigment degradation, and hence the red light reflectance increases while NIR reflectance decreases (Woldai, 2004). Fisher *et al*. (2007) found a strong negative correlation of NDVI with the insect damage intensity (number of scars per leaf area) on water hyacinth. Mirik *et al*. (2007) also showed that the canopy of wheat plants infested with Russian wheat aphids showed a decrease in the NIR reflectance and an increase in the visible range of the electromagnetic spectrum.

1.5.2 Hyperspectral versus Multispectral Sensors

The major difference between hyperspectral systems (HSSs) and Multispectral scanners (MSSs) is that HSSs record a larger number of narrow-bands (usually at the scale of <1 to 3 nm; Liu *et al*., 2005). The greater the number of narrow spectral bands collected by remote sensors the more explicit information about the surface of a target object can be obtained (Turner *et al*., 2003). Multispectral scanners are relatively inexpensive and can successfully be used in mapping the distribution of land-cover, and general ecosystem types and vegetation systems. However, they are unable to discriminate vegetation by species, due to their low spectral resolution power that results from their collection of only a limited number of broad spectral bands (Everitt *et al*., 2002; Lamb and Brown, 2001), usually greater than 50 nm (Hestir *et al*., 2008). For instance it is difficult to distinguish invasive alien plants (which may have high vigour) from others using multispectral imagery, since healthy vegetation generally looks similar in the visible and near infrared (NIR) ranges of the light spectrum, due to similarity in their cellular chemical properties (Woolley, 1971). However, hyperspectral imagery with narrow (<10 nm) continuous spectral bands provides data more sensitive to specific crop variables with much more spectral information, and is effective in mapping infestation cover and spatial distribution of invasive aquatic weeds even in water systems with high biodiversities of invasive weeds (Hestir *et al*., 2008). Glenn *et al*. (2005) used high resolution hyperspectral imagery to differentiate the infestations of leafy spurge as low as 10% cover in 3.5 m pixel.

The application of hyperspectral imagery has relatively a short history (only ~ 30 years when compared to > 100 years for aerial photography and about 50 years for multispectral satellite platform imaging). The first space-borne hyperspectral sensor on board Earth Observing-1 (EO-1) was the Hyperion sensor (Thenkabail *et al*., 2004a) launched for the first time on November, 2000. This hyperspectral, device with 30 m x 30 m pixel spatial resolution (Thenkabail, 2001), collects data in near-continuous discrete narrow bands in the spectral range of 400-2500 nm (Thenkabail *et al*., 2004b). However, due to the coarse spatial resolution and low signal to noise ratio, the Hyperion imagery is not widely used to map and discriminate alien plant species (Huang and Asner, 2009). Instead AVIRIS, CASI (Compact Airborne Spectrographic Imager), HyMap and PROBE-1 are among some of the airborne hyperspectral sensors which have been successfully used in mapping vegetation at the species level (Pengra *et al*., 2008).

Hyperspectral remote sensing has already been widely used in identifying and mapping encroaching alien invasive vegetation (Huang and Asner, 2009). For instance, the woody vegetation encroaching into grasslands in the Niobrara Valley (Wylie *et al*., 2000), flowering leafy spurge in north eastern Wyoming (Parker and Hunt, 2004), flowering leafy spurge in Idaho (Glenn *et al*., 2005), and hoary cress, *Cardaria draba* an invasive noxious weed in the state of Idaho (Mundt *et al*., 2005) were all mapped and identified using hyperspectral imagery (Lawrence *et al*., 2006). However, studies of water hyacinth using hyperspectral remote sensing have been limited to mapping of infestations (Cavilli *et al*., 2009; Hestir *et al*., 2008; Underwood *et al*., 2006; Everitt *et al*., 1999).

Thus, this study intends to evaluate remote sensing (RS) as a tool of water hyacinth management and will test whether hyperspectral RS can detect the response of water hyacinth to abiotic and biotic stressors, in which case measurements will be instantaneous and easier than laboratory analysis of the plants. Hyperspectral remote sensing will be used to monitor the plant quality (vigour or health status) in relation to water contaminants such as salinity, acidity and selected heavy metal (As, Au, Cu, Fe, Hg, Mn, U, and Zn) induced stresses, insect damage, and the effect of biocontrol agents on water hyacinth plants which have elevated metal concentrations in their tissues (Figure 1.2).

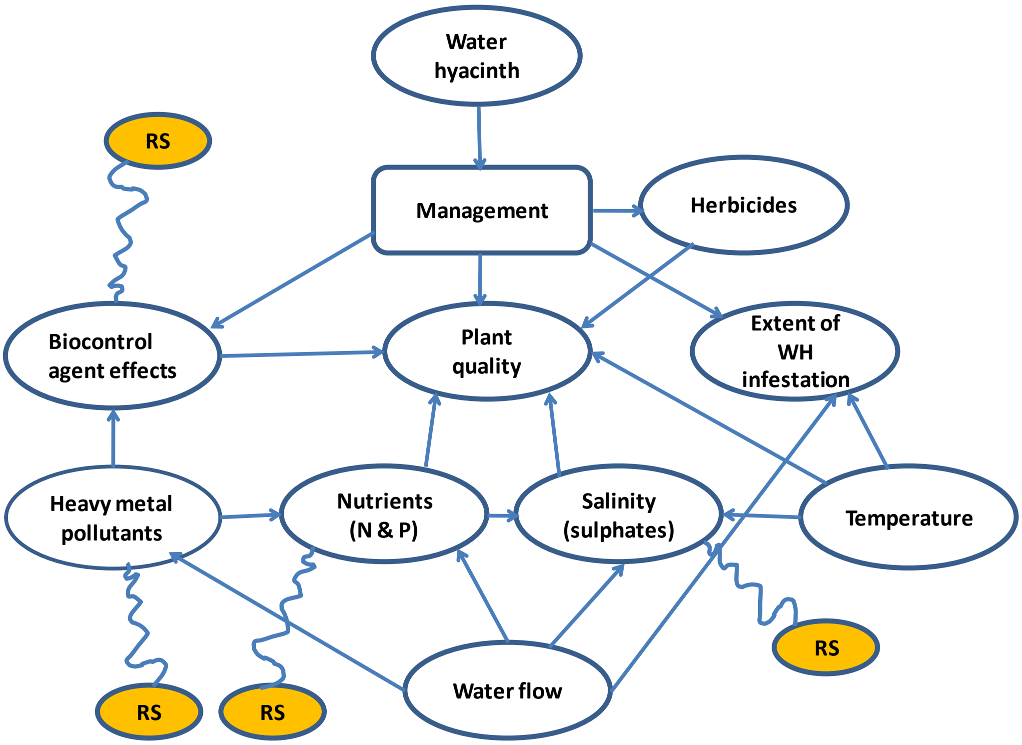

Figure 1.2: Conceptual diagram of water hyacinth management and the potential use of remote sensing (RS) to provide management with necessary information for decision making on the control method.

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1.6 Aims and thesis outline

In summary the following broad aims were addressed:

Aim 1: To investigate if hyperspectral remote sensing can detect the physiological and health status of water hyacinth.

Aim 2: To investigate the capacity of water hyacinth for heavy metal uptake; determine which of the plant parts (root or shoot) accumulate most of the heavy metals and evaluate the amount of heavy metals either adsorbed (binding of the metals outside the negatively charged surface of the roots) or absorbed (metal elements taken into the plant tissues) by the plant's tissues.

Aim 3: To evaluate the interaction of water hyacinth weevils, with the heavy metals in the plant tissues of water hyacinth.

Aim 4: To investigate the plant's growth response to specific heavy metals, acid mine drainage and the biological control agent (the water hyacinth weevils).

South Africa has one of the most eutrophied water systems in the world (Walmsley, 2000) and this has been the main factor behind the success of water hyacinth growth and spread across the country resulting in expensive management measures with variable success in reducing the invasion. In light of this the management system has currently shifted into integrated pest management (IPM) by combining biological control with a sub-lethal dose of herbicides (Byrne *et al*., 2010). However, this requires an efficient tool of data acquisition to facilitate decisions on the appropriate intervention and its timing. Therefore, Chapter 2 investigates the potential of hyperspectral remote sensing as a tool to detect and provide data on the physiological status of water hyacinth, using a hand held spectrometer (Aim 1).

Most aquatic macrophytes are known for their enormous capacity to accumulate heavy metals in their tissues (Misbahuddin and Fariduddin, 2002; Roldán, 2002; Vaillant *et al*., 2004; Bennicelli *et al*., 2004; Kamal *et al*., 2004; Snyder, 2006). A distinctive characteristic that qualifies them for cleaning-up water and wetland systems, contaminated from anthropogenic activities such as runoffs carrying pesticide and fertilizer residues from agricultural activities, acid mine drainage from industrial and mining sites and municipal effluents from local settlements. Such potential of water hyacinth as a tool of phytoremediation is explored both in the lab and field in Chapter 3 (Aim 2).

High levels of heavy metals in plant tissues reduce insect herbivory (Boyd, 2010). Despite the fact that acid mine drainage and water eutrophication are major problems in South Africa, heavy metal interaction with water hyacinth weevils has not been investigated previously, and little information exists in the literature. Therefore, Chapter 4 investigates the interaction of the water hyacinth weevil with eight different heavy metals in a single-element system tub trial and four metals and three different sulphate concentrations in a simulated acid mine drainage pool trial (Aim 3).

Although increased water eutrophication enhances the growth of water hyacinth plants, the impacts of acid mine drainage (AMD) on the plant growth is not well established. AMD from mining wastes such as tailing dams and slimes dams are largely the sources of sulphides, heavy metals and a variety of other salts. Although water hyacinth is capable of removing an enormous amount of heavy metals and localizing them in its roots to avoid their phytotoxicity, some are transported to the shoots where the metal sensitive photosynthetic process occurs. The growth and tolerance of water hyacinth in the presence of selected heavy metals, and simulated acid mine drainage and water hyacinth weevils was investigated in Chapter 5 (Aim 4).

Finally, Chapter 6 is a general discussion that consolidates the findings and discussions of the four preceding chapters.

Chapter 2

Hyperspectral remote sensing to evaluate water hyacinth physiological status

2.1 Introduction

Water hyacinth responds strongly to increased nutrients by increasing biomass and expanding the extent of its infestation, but the effects of other pollutants such as metals, on either the plant or its biocontrol agents are unknown. In this project hyperspectral remote sensing using a hand-held spectrometer was used to assess the health status of water hyacinth, growing under different biotic and abiotic stresses under both “greenhouse” and field conditions. The field trial represents a complex environment, containing different anthropogenic water pollutants in which the water hyacinth grew. Results from this trial allowed comparison with those of the “greenhouse” trials, which include artificial solutions of metal or acid mine drainage pollutants. Being able to assess the plant health status will provide valuable information for the integrated pest management control of water hyacinth by highlighting the appropriate timing of herbicide and biocontrol applications, or indicate when other control methods such as mechanical removal should be used.

2.1.1 Measurement of aquatic weeds with hyperspectral imagery

Measurement of spectral reflectance from water surfaces is influenced by a variety of factors that affect the water quality. Some of these include sediments (turbidity), algae (chlorophylls as well as carotenoid pigments), dissolved organic matter, oils which float on the surface, and aquatic vascular plants, each of which has distinct reflectance properties (Ritchie *et al*., 2003). Water hyacinth can be remotely distinguished from submerged aquatic plants such as hydrilla, *Hydrilla verticillata* (L.F.) Royle (Hydrocharitaceae), since it shows greater spectral reflecantce in the near infrared (NIR) light spectrum compared to the hydrilla (Everitt *et al*., 1999), and from water due to the fact that water absorbs light in the NIR light spectrum as opposed to water hyacinth Woldai (2004). Everitt *et al*. (1999) showed that deep water had lower NIR reflectance than shallow water and the four plant species monitored, among which were water hyacinth and hydrilla; while shallow water had a lower NIR reflectance than the plant species. Such characteristics make it possible to separate water hyacinth from water and submerged aquatic weeds using remote sensors (Lillesand *et al*., 2004). In addition water hyacinth is a succulent floating plant characterized by higher foliar water content than most co-occurring aquatic weeds, and such features enable the acquisition of a distinct spectral signature helpful for identification of water hyacinth (Hestir *et al*., 2008). For instance Cavali *et al*. (2009) was able to separate water hyacinth clearly from Typha sp and the reed, *Phragmites australis* Cav. Trin. Ex Stued., on Lake Victoria based on their distinct reflectance features such as leaf succulence and canopy chlorophyll content.

In this project a hand-held spectrometer (ASD), with a narrow band of 1 nm sampling interval that acquires spectral data between 350-2500 nm and with a 25° Field of View (FOV) through a permanent fibre optic cable was used to evaluate the plants' health status. The spectral reflectance from the plants of water hyacinth was used to assess the growth status, insect damage, and nutrient status and the effect of heavy metals or acid mine drainage on biocontrol agents of the plants.

2.1.2 Use of the "red edge position" to determine plant stress

The red band absorption of vegetation decreases when photosynthetic activities are impaired due to a reduction in the total chlorophyll concentration; a decrease in the chlorophyll to carotenoid ratio and a build-up of extra pigments from tannins when plants are under stress (Rock *et al*., 1988). Such stress-induced variation in chlorophyll and other colour pigments increases chlorophyll fluorescence in the red band as a result of the dissipated excess light energy accumulated by the chlorophyll molecule, which in turn exceeds the limit of the declining photosynthetic activity, to protect the chloroplast from potential damage (Liew *et al*., 2008). This leads to a special spectral feature around the boundary of the red and the infrared range of the light spectrum known as the 'red edge" which is the point at which the maximum spectral reflectance slope occurs in vegetation (Curran *et al*., 1990). This slope occurs between the maximum point of chlorophyll absorption in the red band just below 690 nm and around 750 nm (Fig. 2.1), where the highest spectral reflectance in plants is observed due to increased multiple scattering of radiation in the intercellular spaces of the leaf mesophyll (Smith *et al*., 2004). The red edge varies with the concentration of chlorophyll (Smith *et al*., 2004) and a slight shift in the position of the spectral reflectance curve in the red edge of green plants under stress conditions, such as those induced by heavy metal concentrations, towards the shorter wave length is known as a “blue shift” (Rock *et al.*, 1988; Carter *et. al.*, 1993) (Fig. 2.1). Normally the red edge position of healthy plants shifts towards the longer wave length as they approach maturity until it eventually reaches the wave length of about 712-715 nm where it stabilizes, but in the presence of a stress this shift reverses towards the shorter wave length (Liew *et al.*, 2008) as indicated in the first derivatie curve of Fig. 2.1.

Many researchers use the red edge position (REP) in the region of 680-780 nm as a significant indicator of plants growing under stress. This is because the red edge is not influenced by factors such as trichome density, variation in leaf structure, or leaf chlorophyll heterogeneity. In addition it is robust under some environmental conditions that might result in changes caused by leaf anatomy (Liew *et al.*, 2008). The slope of the red edge changes as a healthy and actively photosynthesizing plant faces different stress levels. For instance Rock *et al.* (1988) indicated that a 5 nm blue shift of the red edge position was detected in spruce specimens collected from spruce forests found at sites with high air pollution (such as acid deposition, ozone, trace metals) damage.

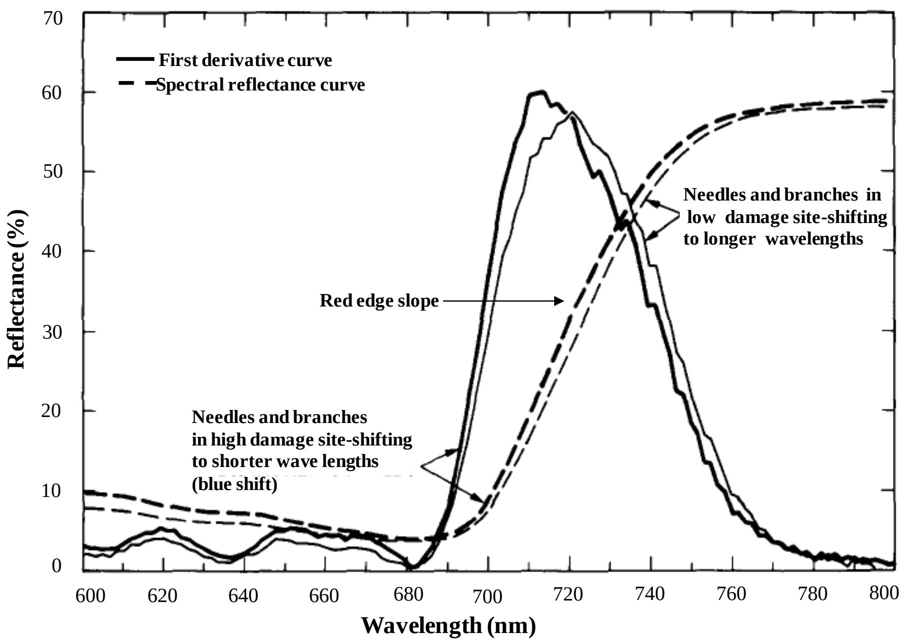


Figure 2.1: Spectral reflectance and first derivative curves of averaged spectral data acqiured using a high-spectral resolution spectrometer known as Visible Infrared Intelligent Spectrometer (VIRIS) in June 1985, to detect air pollution-induced stress on needles and branches of spruce trees (Adapted from Rock *et al.*, 1988).

The slightest decrease in chlorophyll concentration is capable of producing an increased leaf reflectance on the visible to NIR light spectrum and this is an important warning sign (indicator) of plant stress. Zarco-Tejada *et al*. (2002) found two prominent peaks in the first derivative curve of leaf relectance which were associated with chlorophyll concentration of both chlorphyl a+b pigments, from which they developed a derivative chlorphyll index (D_{705}/D_{722}) to track the changes in the double peak and detect vegetation stress. Similarly Smith *et al*. (2004) using the same principle of the derivative ratios between the two important peaks which are related to chlorophyll fluorescence and their concentrations (i.e. the ratio of the derivative values at 725 to that of 702 nm) identified the stress of grasses exposed to gas contamination (Fig. 2.2). Horler also showed that the first peak at 702 nm was an indication of plant stress and the second spectral peak observed at about 725 was due to discontinuous internal leaf structure such as cell-wall and intercellular air spaces (cellular of light scattering in the leaf). Other related studies also showed the association of the first and second peaks to detect plant stress (Jago and Curran, 1996; Lamb *et al.*, 2002). For instance, Llewellyn and Curran (1999) found the stress response of grass, growing on natural gas contaminated soil, with first and second peaks of the first derivative of reflectance at 700 nm and 729 nm respectively. They interpreted the dominance of the first peak with the shift towards the shorter wave length (first derivative spectra at 700 nm) as sites of grass with high levels of soil contamination, while the dominance of the second peak observed in the longer wave length as indication of sites with lower level of contamination.

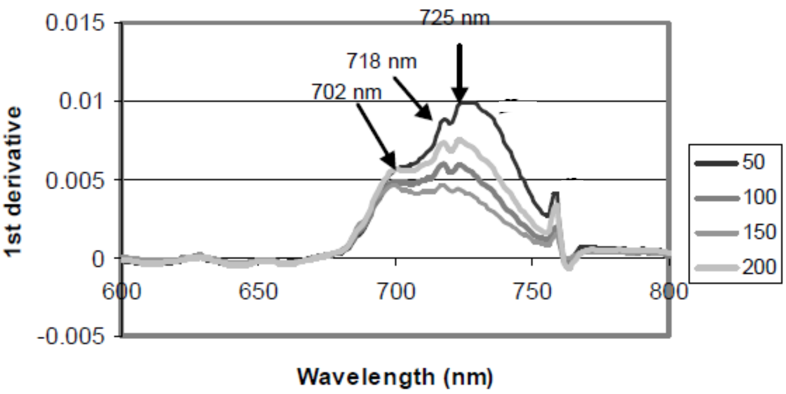


Figure 2.2: The first derivative curve of reflectance of gas contaminated grasses in plots. The different lines are representations of the first derivative of reflectance from grass at 50 cm, 100 cm … etc., along the transect (adapted from Smith *et al*., 2004). NB: 50 cm and 200 cm represent the edges of the plots (with less contamination).

The important association of the REP with foliar chlorophyll content has enabled researchers to evaluate plant health status and has been underpinned by a number of studies in search of a robust technique to determine the REP. Among such methods used to extract the REP are: the maximum first derivative (MAX-FD) (Dawson and Curran, 1998) and the linear extrapolation (REP_LE) (Cho and Skidmore, 2006). Plant health status can also be determined using other spectral indicators such as RE_NDVI (Gitelson and Merzlyk, 1994), $mNDVI_{705}$ and mSR (Datt, 1999) and PRI (Gamon *et al*., 1992) which also evaluate the concentration of leaf chlorophyll pigments or by using water sensitive bands such as water band index, WBI (Peñuelas *et al*., 1995a) to detect the plant water status. Several studies have also used different spectral indices for canopy water content to survey vegetation stress (Peñuelas *et al*., 1995a; Hunt and Rock, 1989; Gao, 1995) and have shown positive correlations of such water indices with canopy chlorophyll content (Claudio *et al*., 2006; Tian *et al*., 2011).

The red edge parameters (mSR, RE_NDVI, and $mNDVI_{705}$) enable the evaluation of a wide range of green canopy structures, since they are not affected by variation in leaf surface reflectance (Sims and Gamon, 2002). Moreover, the adjusted indices of the normalized differences (mSR and $mNDVI_{705}$) which incorporate the reflectance at 445nm produce more reliable results of total chlorophyll concentration of plant canopies compared to the RE-NDVI, since they are not affected by light scattering at 800nm (Sims and Gamon, 2002). In contrast, the blue band index, the photochemical reflectance index (PRI), is used to estimate the photosynthetic light use efficiency by evaluating the spectral features of the carotenoid pigments in the blue band (400-500 nm) as a proportion of the chlorophyll reflectance in the region of the red band (Peñuelas *et al*., 1995b). The PRI reduces leaf surface and mesophyll structural effects that affect plant reflectance and is an important index which enables identification of the physiological and phenological plant status in realtion to plant stressors.

Spectral indicators of canopy water content also have a positive correlation with the concentration of chlorophyll pigments. Claudio *et al*. (2006) found a strong correlation between canopy water content and green canopy structure (between

WBI and NDVI, respectively) for tree species in a semi-arid shrubland ecosystem in southern California. Estimation of the plant water status could therefore, be used to evaluate the plant health status and intensity of both biotic and abiotic plant stressors. In addition to WBI, moisture stress index (MSI) (Hunt and Rock, 1989) and the normalized difference of water index (NDWI) are among some of the common spectral indicators used to estimate plant water stress. However, the WBI (P900/P970) is indicated as a relatively robust spectral indicator of water stress compared to MSI (P1599/P819) and NDWI (P857-P1241)/(P857+P1241) due to the insufficient energy of solar radiation and increased level of spectral impurities caused by the interference of atmospheric water vapour in the longer wavelengths of the latter two water sensitive spectral bands (Sims and Gamon, 2003).

In this chapter the hypothesis that hyperspectral remote sensising can detect both abiotic (heavy metal or acid mine drainage) and biotic (weevil feeding) induced stresses of water hyacinth plants was tested.

2.2 Materials and Methods

2.2.1 General background

Spectral signatures of water hyacinth were collected under different biotic and abiotic conditions, both from trials in a “greenhouse” at the University of Witwatersrand and in the field at the Vaal River near Orkney. The field sites include the Schoonspruit between Klerksdorp and Orkney, and the Vaal River abutting the properties of the AngloGold Ashanti Vaal River Operations, the Simmer and Jack gold mine, and the Harmony / Pamodzi gold mine shafts near Orkney (Fig. 2.3).

Laboratory experiments were conducted in large tubs, as a single-element system trial where plants of water hyacinth were grown with a single heavy metal treatment in each tub. Whereas plants in pools were grown in a multi-component system, where a suite of elements in combination were added to the water to create a simulated acid mine drainage (AMD), similar to conditions in the Vaal River, near the AngloGold Ashanti mining operations. Both tub and pool experiments were covered with a clear, non-UV screening, greenhouse plastic tent

(UVA-clear 200MIC, supplied by Vegtech 2000, Cape Town, South Africa). Plants in the field trial, above and below inlets of both the Schoonspruit and Koekemoerspruit to the Vaal River, were contained in floating cages (rafts) under open environmental conditions, designed to be compared with results from the tub and pool experiments. Both field and lab trials were conducted for a period of 40-55 days between late spring of 2011 and early summer of 2012, during the active growing season of water hyacinth.

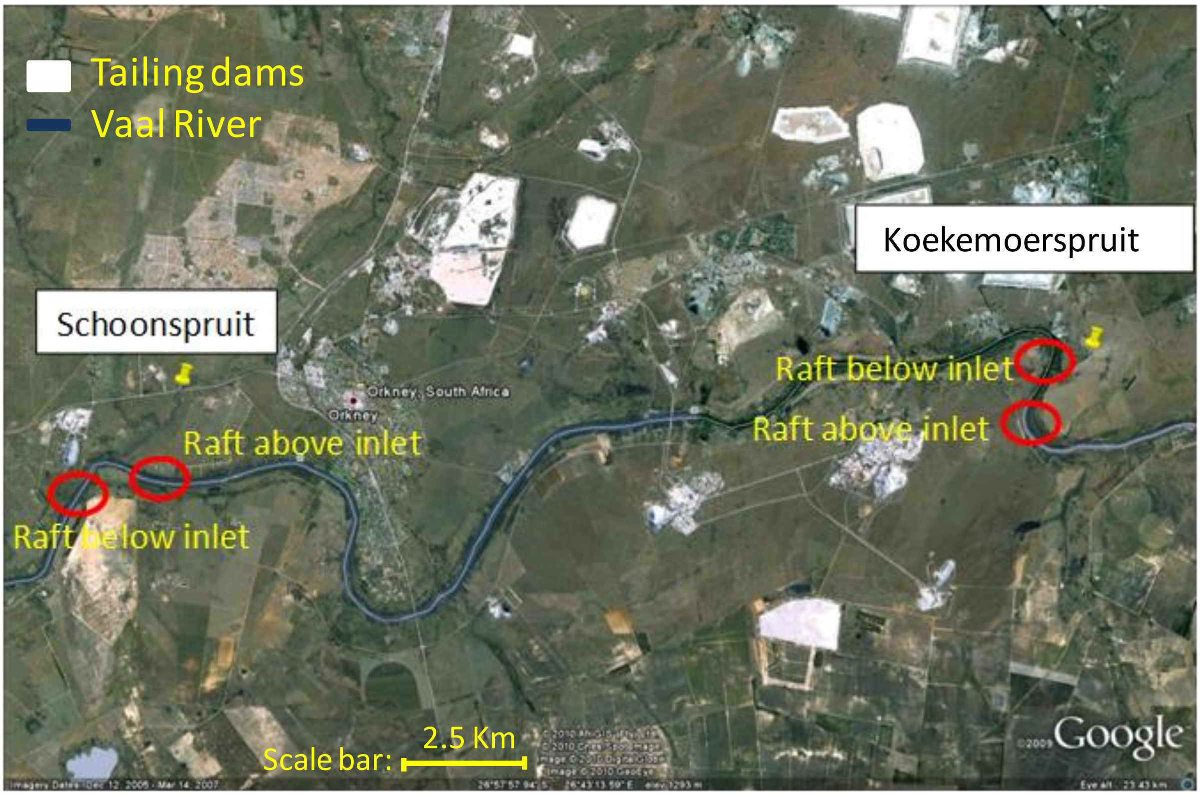


Figure 2.3: Field site map illustrating inlets above and below the Schoonspruit and the Koekemoerspruit on the Vaal River, and the position of four floating rafts of water hyacinth used to evaluate the response of water hyacinth growth to different levels of water contaminants (nutrients, heavy metals) brought to the Vaal River by the two tributaries which are suggested as a source of pollution for the river (Source Google Earth).

The water hyacinth used in the tubs and pools was transplanted from a pond at the University of the Witwatersrand and was originally obtained from Delta Park, Johannesburg two years prior to the experiment. The water hyacinth used in floating cages in the field was transported from one spot at the lower bridge near the Township of Kennan, on the Schoonspruit tributary near Orkney (about 5 km from the Vaal River). At the time of the field trial there were limited number of plants at the river site due to the previous (2009 and 2010) floods on the Vaal River, which had swept all the water hyacinth mats downstream.

Spectral measurements of water hyacinth in all treatments were taken from the continuous plant canopy at a height of 80 cm above the top of the plants, using a hand-held spectrometer (Analytical Spectral Devices (ASD) Boulder, Colorado, USA), with a 25° Field of View (FOV) through a permanent fibre optic cable which covered a ground area of 0.24 m^2. This device has a narrow band of 1 nm sampling interval and acquires spectral data between 350-2500 nm. All spectral measurements were taken on warm days with clear skies between 11:00 in the morning to 14:00 in the afternoon. The reflectance of water hyacinth was taken as a ratio to the reflectance from the 'white reference panel' (a smooth white board made of barium substance) to perform real-time reflectance measurements and to optimize the response of the spectrometer.

Leaf chlorophyll measurements were also quantified with a leaf chlorophyll meter (SPAD-502 Minolta, Japan) after every spectral measurement, for comparison and interpretation of the spectral signature from the ASD. SPAD readings were taken randomly on ten leaf samples from each replicate of each treatment (10 leaves/tub) in the tub totaling 30 leaf SPAD readings per treatment and on 15 leaves per pool or cage from the pool and field experiments respectively. Spectral measurements with the ASD and the SPAD measurements were also repeated in the tub and pool trials after the release of water hyacinth weevils on to the plants.

2.2.2 Single-element system tub trial

A single-element system trial of water hyacinth was conducted in 65 L tubs in a "greenhouse tent" at the University of Witwatersrand, Johannesburg (South Africa). Tubs were first conditioned with sulphuric acid (pH 1.5) for a week. The acidic water was neutralized with sodium hydroxide (NaOH) and disposed of. The tubs were thoroughly washed with tap water, rinsed and dried. Water hyacinth plants were grown with a single heavy metal treatment in each tub. Trials were conducted for a period of 55 days starting in late spring of 2011 and ending in early summer of 2012, with minimum, maximum, and average air temperatures inside the plastic tent being 6°C, 42°C and 24°C respectively. Three replicates of a total of 39 tubs in 13 treatments were arranged randomly in four rows (Fig. 2.4).

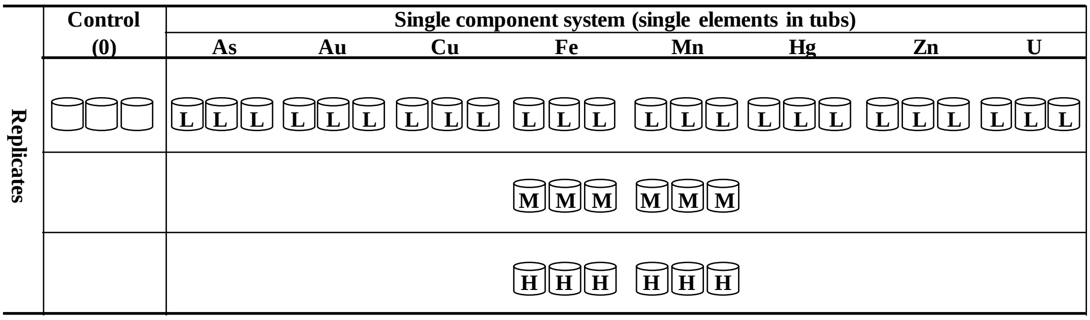


Figure 2.4: Experimental design of the single-component system tub trial with a 1/4th strength Hoagland solution and a concentrations of heavy metals similar to mining and industrial water pollutions. NB: L = low, M = medium and H = high.

Tubs were filled with 45 litres of tap water, and ¼ strength of Hoagland's solution (a hydroponic nutrient solution or recipe) (Hoagland and Arnon, 1950) (Table 2.1) was added to each tub using a plastic syringe and stirred thoroughly with a plastic rod. The use of full strength of Hoagland's solution is more than the actual requirements for ideal plant growth and therefore ¼ of the Hoagland solution was selected based on literature reviews (Zhu *et al*., 1999; Weiss *et al*., 2006; Rajan *et al*., 2008; Hussain *et al*., 2010). Each tub was equipped with a submersible fish tank pump (flow rate 400 litres/hr model PH400; power head pump, Dymax, Singapore) to agitate all treatments.

Ten short, green, healthy water hyacinth plants at the "bulbous" phenostage were washed and rinsed several times with tap water then added to each tub and left to grow for a week. All metal treatments were added to each tub in the same way as the Hoagland's solution, except that the plants were first raised above the water before adding the treatments, to facilitate the stirring process. Metals were added as various compounds as shown in table 2.2 and included As (1 mg/L), Au (1 mg/L), Cu (2 mg/L), Fe (0.5, 2.0, and 4.0 mg/L), Hg (1 mg/L), Mn (0.5, 2.0, and 4.0 mg/L), U (1 mg/L) and Zn (4 mg/L). From this, the Fe and Mn trials were extended to different three concentration treatments (as low, medium and high concentrations) to evaluate the plants' response to dose response treatments

(Table 2.2). The 13th treatment was a control with only the Hoagland's solution (no metals added).

Table 2.1: Composition of Hoagland's solution used in the single-element tub experiment and the final concentration of the solution.

Salt compound	Molecular weight	Conc. of stock solution in Molarity	Conc. of stock solution (g l^{-1})	Final conc. in tubs	
				Elements	(mg l^{-1})
KNO_3	101.11	8.399	849.24	K	234.57
KH_2PO_4	136.09	4.20×10^{-2}	228.631	N	126.34
$CaSO_4.2H_2O$	172.17	8.4004	361.573	P	30.90
$MgN_2O_6.6H_2O$	256.41	3.360	861.538	S	160.62
Fe-EDTA	367.045	1.805×10^{-2}	6.625	Mg	48.64
H_3BO_3	61.83	7.770×10^{-2}	4.804	Ca	200.40
$MnSO_4.H_2O$	169.02	1.529×10^{-2}	2.584	Fe	0.60
$Cu(NO_3)_2.3H_2O$	241.6	5.288×10^{-4}	0.128	B	0.50
$N2O_6Zn.6H_2O$	297.48	1.285×10^{-3}	0.382	Mn	0.50
$(NH4)_6Mo7O_{24}$	1235.86	1.751×10^{-4}	0.216	Cu	0.02
				Zn	0.05
				Mo	0.01

Table 2.2: Composition of heavy metal stock solutions and their final calculated concentrations of each metal treatment in the single-element system tub trial.

Salt compound	Molecular weight	Conc. of stock solution (g l^{-1})	Metal concentration in the stock solution		Volume of the stock solution added per tub (ml)	Final metal conc. in tubs (mg l^{-1})
			Elements	(mg l^{-1})		
AS_2O_3	197.84	1.0	AS	757.4.0	55.45	1
$AuCl_3$	303.33	0.2	Au	129.87	32.00	1
$Cu(NO_3)_2.3H_2O$	241.60	1.0	Cu	263.00	319.40	2
$Fe(NO3)_2.H_2O$	404.00	0.5	Fe	69.11	303.86	0.5
$Fe(NO3)_2.H_2O$	404.00	2.0	Fe	276.46	303.84	2
$Fe(NO3)_2.H_2O$	404.00	4.0	Fe	553.00	303.80	4
$Hg\ (NO_3)_2.H_2O$	342.62	0.5	Hg	297.70	143.50	1
$MnSO_4.H_2O$	169.02	1.0	Mn	325.00	64.60	0.5
$MnSO_4.H_2O$	169.02	1.0	Mn	325.00	258.50	2
$MnSO_4.H_2O$	169.02	2.0	Mn	650.00	258.50	4
$N_2O_6Zn.6H_2O$	297.48	3.0	Zn	659.00	254.93	4
Uranium		1.0	U	1000.00	45.00	1

NB: U is purchased as uranium solution in nitric acid at a concentration of 1000ppm

Water loss from each tub due to evapo-transpiration was compensated for by adding tap water to each tub every four to six days. The experiment was conducted for 55 days in two phases. The first 18 days (metal uptake phase) were used to investigate the spectral signature of water hyacinth as a result of heavy metal impacts, after which 60 water hyacinth weevils (an average of 3.5 weevils per plant) from a mixture of both *N. eichhorniae* and *N. bruchi*) were added to each tub for the second phase (the biocontrol or weevil treatment phase). The weevils were collected and shipped from an insect mass rearing facility at the South African Sugar Cane Research Institute (SASRI) in Kwazulu Natal province. The fact that the mortality per box was negligible showed that the weevils were in good physiological conditions. However, the age of the weevils used in these trials was unknown. Spectral measurements were taken, before (at week 3) and after (week 9) the addition of the weevils, based on the random arrangement of the tubs between 11:30 to 12:30 hrs (around noon to avoid the solar zenith angle effect). Spectral measurements on each replicate were repeated three times, giving a total of nine spectral data for each treatment at each sampling occasion.

2.2.3 Simulated acid mine drainage (AMD) pool trial

The pool experiment was setup outdoors on 18 pools arranged in three rows of six pools each under a “greenhouse” tent. The pools were 1.8 m in diameter and 1 m in height and all six pools in a row were connected in a circuit with pipes to each other and to a water pump (Superflo pump, from Pentair Water Pool and Spa. Inc., Sandford North California, USA) with a flow rate of 2100 L/hr. One pump per row was used. The pools were designed such that water was pumped out from a sump pool in each row, to the bottom of each pool in the row and returned back to the sump pool through gravitational flow from the top surface of each pool in the row. The water circulation between the pools created a gentle water flow and maintained mixing of nutrients and chemicals.

Each row of pools represented one water pollution treatment for water hyacinth. The treatments used in the pools were, sulphates ($MgSO_4$) with Cu, Fe, Mn, and Zn, made from an artificial solution with concentrations spanning those measured in local water-bodies in receipt of acid mine drainage. Water hyacinth weevils were added to every other pool in a row after three weeks. Plant nutrients from a technical grade fertilizer, and the heavy metal treatments were added at the same dose across all pools, whereas the $MgSO_4$ treatment was added to the pools at three different concentrations (Table 2.3), one in each row (low, medium and high) (Fig. 2.5).

	MgSO4	**Biocontrol agents in pools (yes/no)**					
Low conc. of (N, P, Cu, Fe, Mn, and Zn	**Low**	No	yes	No	yes	No	yes
	Medium	yes	No	yes	No	yes	No
	High	No	yes	No	yes	No	yes

Figure 2.5: Experimental design of pools used in the simulated acid mine drainage pool trial to determine spectral reflectance of water hyacinth and the performance of biocontrol agents (weevils) in different concentrations of pollutant mixtures, similar to the acid mine drainage in the Vaal River.

The same pools had previously been used in a pilot trial with Hg, and the same sulphate and heavy metal artificial mixture between April to July, 2011, prior to the start of the experiment. Some water from the pilot test was reused at the "high" concentration treatment row, due to the delay in the disposal of all the water from all the pools for the new trial. This is because of the sizes of the pools each containing 2170 L of contaminated water and associated cost which required time for its safe disposal. However, water and plants samples were taken for further analysis to provide baseline data for the concentration of nutrients and metals in the "high" treatment pools. Therefore, with the exception of the "high" treatment, the existing water from the pilot test was disposed of and the pools were washed and rinsed and filled with fresh tap water. Green, healthy water hyacinth plants (early "bulbous" stage) were washed and rinsed and placed into each pool. About 340 grams of a technical fertilizer ("Lawn and foliage with micronutrients" from Wonder) at a NPK ratio of 7:1:3 with micronutrients such as Zn, Mg and Ca was added in perforated PET (Polyethylene terephthalate) cold drink bottles to each pool. Iron chelate ("Micrel Fe 110D" with 11 % Fe 230 g) was first mixed in five litres of water then added to each pool. The plants were first placed in the pools in early October 2011. The plants were then allowed to grow for two months to completely fill each pool's surface area, after which the metal and sulphate treatments were added. The sulphate concentrations used were: 300 mg/L for the low, 700 mg/L for the medium and 1300 mg/L for the high treatment pools. These concentrations were first mixed and stirred with a plastic rod in tubs of 60 litres of water before being added to each pool of their respective treatments. The metal treatments were added to the pools using a plastic syringes with the correct dose of 2 mg/L Cu, 1 mg/L Fe, 1 mg/L Mn, and 4 mg/L Zn (Table 2.3).

Table 2.3: Composition of the stock solutions of heavy metal treatments, calculated from hydrated metal nitrates and sulphates, and their final concentrations used in the AMD pool trial.

Salt compound	Molecular weight	Conc. of stock solution ($g\ l^{-1}$)	Metal conc. in the stock solution prepared		Volume of the stock solution added per pool (ml)	Final metal conc. in pool ($mg\ l^{-1}$)
			Elements	($g\ l^{-1}$)		
$Cu(NO_3)_2.3H_2O$	241.60	100	Cu	26.302	165.46	2
$Fe(NO3)_2.H_2O$	404.00	95	Fe	13.131	165.70	1
$MnSO_4.H_2O$	169.02	45	Mn	14.627	148.77	1
$N_2O_6Zn.6H_2O$	297.48	240	Zn	52.755	165.00	4

The first phase of the pool experiment (metal uptake phase) ran for 18 days in December, 2011 and two spectral measurements, taken at the start of the experiment before the addition of the AMD treatments and at the end of the metal uptake phase in day 18 (week 3), were acquired from an average height of 80 cm above the plant canopies of each pool, at nadir, in each row. Each spectral measurement was captured three times from each pool during each ASD measurement. In the second phase (biocontrol phase) an average of four weevils per plant was added to every other pool of each row (i.e. on every 2nd, 4th, and 6th pool of each row) while keeping the remaining three pools in the row as control treatments (pools without weevils). A spectal measurement with ASD was taken at the end of the experiment in week 9 (six weeks after the weevils feeding) between 11:30 to 12:30 hrs (consistently taken around noon to avoid the solar zenith angle effect) on clear sunny days. In addition, spectral data was acquired in a regular pattern by shifting from one row to the next after spectral measurement from every two pools per row to randomize the intensity and the angle of sunlight.

Every spectral measurement was also accompanied by a leaf chlorophyll measurement. The water level was topped up every week to compensate for the loss of water due to evapotranspiration.

2.2.4 Acid mine drainage in the field trial

Floating cages (rafts) made of wire mesh with a diameter of 2 m and a height of 75 cm were connected to four buoys (300 mm in diameter and 330 mm long) (Sondor Industries Ltd, Cape Town, South Africa.), set at about 60 m above and below inlets of the Schoonspruit (S27°00'08.4" and E26°37'14.3" and S27°00'10.7" and E26°37'08.5" respectively); and the Koekemoerspruit (S26°56'17.7" and E26°46'46.44.1" and S26°56'14.3" and E26°48'44.8") in the Vaal River (Fig. 2.6). Each of the floating cages was connected to four 50 kg concrete weights anchored on the bottom of the river. In addition to this, the cages were anchored with a 10m steel chain attached to tree trunks in the river bed to prevent the cages from being washed away by water currents or floods.

Figure 2.6: A floating cage of water hyacinth connected to four white buoys positioned below the inlet of the Schoonspruit on the Vaal River. Similar caged water hyacinth plants were also set at three other different positions (in the above inlet of the Schoonspruit and both the above and below inlet of the Koekemoerspruit on the Vaal River).

Green and healthy plants of medium height (approximately 20 cm) were then transported to each of the four cages, from the lower bridge of the nearby local settlement (Kennan) on the Schoonspruit (about 5 km before it reaches the Vaal River). The experiment was run for 44 days in two phases (before and after the start of the season's rain) and spectral measurements were taken twice at a height of about 80 cm from the canopy. Measurements were made from an inflatable boat and repeated four times at each cage. The first ASD measurement was taken before the start of rainfall (two weeks after the start of the experiment) and second one in week 5 after at least three rainfall events had been recorded at the site. For every spectral measurement taken, leaf chlorophyll measurement was also recorded with a SPAD-502 meter.

2.3 Spectral analysis

The repeated spectral measurements from every replicate and the spectral measurements from replicates of each treatment were presented as averages. This also applies to the SPAD- readings in each treatment. Different indices were used to analyse the spectral data from the ASD (Table 2.4). The first derivative spectra were calculated using the first-difference approach, which computes the difference between adjacent wavebands (Dawson and Curran, 1998), while the REP_LE analysis followed the procedures presented in Cho and Skidmore, (2006). The difference in the REP shift (blue shift) before and after the addition of water hyacinth weevils was calculated by subtracting the wavelengths recorded from each heavy metal treatment in week 3 (the metal uptake phase) and week 9 from the respective control treatment.

One-way and Two-way ANOVA followed by Fisher's Least Significant difference (LSD) post hoc test were conducted to evaluate the mean value of the chlorophyll content of the water hyacinth, either measured or calculated under different treatments. The LSD post hoc test was preferred over other post-hoc tests, since it has greater power than the other methods such as Honestly Significant Difference test (HSD) or Tukey test (Abdi and Williams, 2010). Regression analysis was used to assess the relationships between the SPAD-502 reading of leaf chlorophyll content and the spectral stress indicators (spectral indices). The ENVI software (version 4.8), STATISTICA Six Sigma (Statsoft

Release 7, 2006) and Microsoft Office Excel 2007 were the computer packages used for data analysis.

Table 2.4: The spectral indices used to analyse the spectral reflectance of water hyacinth grown with single heavy metal and weevil stressors in the single-element system tub trial, a mixture of heavy metals and sulphates and weevils in the simulated AMD pool trial and in the Vaal River polluted from the nearby mining sites and effluents from the local settlements.

Indices	Name	Formula	Reference
RE_NDVI	Red edge Normalized Difference Vegetation Index	(P750-P705)/(P750+P705)	Gitelson and Merzlyk, 1994
$mNDVI_{705}$	Modified Red Edge Normalized Difference Vegetation Index	$(P_{750}-P_{705})/(P_{750}+P705-2P_{445})$	Datt, 1999
PRI	Photochemical Reflectance Index	(P531-P570)/(P531+570)	Gamon *et al*., 1992
mSR	Modified Red Edge Simple Ratio Index	(P750-P445)/(P705-P445)	Sims and Gamon, 2002
REP_MAX -FDR	Red Edge Position: maximum First Derivative wavelength	$FDR_{(\lambda i)} = (R_{\lambda(j+1)} - R_{\lambda(j)})/\Delta\lambda$	Dawson and Curran, 1998
REP_LE	Red Edge Position: linear extrapolation method		Cho and Skidmore, 2006
WBI	Water Band Index	P900/970	Peñuelas *et al*., 1995b

2.4 Results

The results are divided into the three sections, as tub, pool and field experiments. The hyperspectral data from the ASD and the leaf chlorophyll measurements, as recorded by the SPAD-502, readings are presented in this study. The results of each experiment are described in two phases. In the single-element tub and simulated AMD pool trials the two phases are the metal uptake phase and the biocontrol or weevil phase. The two phases in the field trials were before and after the rainfall. Overall, results from both the single-element tub and simulated AMD pool trials and the field trials showed that the hyperspectral data successfully revealed the different stressors (weevil and heavy metal and nutrients) to which the water hyacinth plants were exposed.

2.4.1 Single-element system tub trial

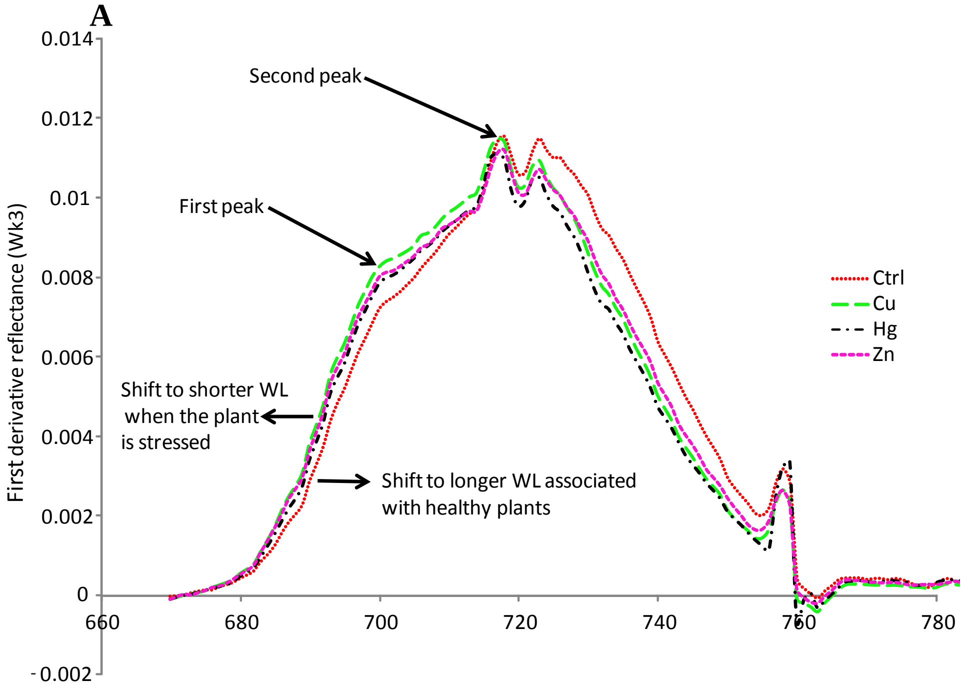
2.4.1.1 Spectral reflectance measures

In the first three weeks after the start of the tub experiment, with a single heavy metal in each treatment, only few treatments indicated symptoms of heavy metal-induced stress. This could be observed from the shift of the REP (blue shift) demonstrated in the first derivative curve of the Cu, Hg and Zn treatments which were significantly different from the control treatment (Fig. 2.7A) based on the linear extrapolation REP (REP_LE) (Fig. 2.8). In the first derivative curve there were two characteristic peaks in the red edge along with the shift of the red edge position to either the shorter (the blue shift) or the longer wavelengths. These peaks were more distinguishable in the treatments after the weevils had fed on the plants (Fig. 2.7B). In the metal uptake phase (week 3), Cu, Zn and Hg showed an increase in the first peak at around 702 nm and decrease in the second at ~ 718 nm, relative to the control treatment (Fig. 2.7A). Similarly, the Cu, Zn treatments followed by Mn-L and Mn-M treatments showed the highest first peak while the control treatment had the highest second peak, after the addition of the weevils (Fig. 2.7B).

The canopy chlorophyll content in the single-element system tub trial calculated using the modified red edge index, $mNDVI_{705}$ indicated that Cu, Hg and Zn treated plants had significantly lower canopy chlorophyll compared to all the other treatments three weeks after the addition of the heavy metal treatments (week 3) ($F_{(12, 101)} = 17.206$, $P < 0.001$) (Fig. 2.8A). Six weeks after the addition of the weevils (week-9), the canopy chlorophyll decreased significantly compared to those before the addition of the weevils in week 3 and Cu was the only treatment showing a significant decrease in $mNDVI_{705}$ compared to the control treatment ($F_{(12, 25)} = 4.4996$, $P < 0.001$) (Appendix 2A). Four more treatments including As, Fe-M, Mn-L, Mn-H showed significantly lower canopy chlorophyll content ($mNDVI_{705}$) compared to those in the control treatment after the weevil's feeding (week 9) ($F_{(12, 101)} = 18.6235$, $P < 0.001$) (Fig. 2.8B). The spectral index, $mNDVI_{705}$ in both trials, before and after the addition of the weevils, of iron and manganese dose response treatments, showed no significant difference between them with the exception of Fe-M which was significantly lower than the Fe-H treatment at week 9 (Figs. 2.8A and B).

The general trend of the REP_LE results followed the same pattern as those in the $mNDVI_{705}$. The Cu, Hg and Zn treatments revealed significant differences from all the other treatments in the first three weeks (Fig. 2.8C). The REP significantly decreased in the second phase (week 9) in which Cu, Hg, Zn, As, Fe-M, Mn-L, and Mn-H treatments were significantly lower than the control treatment ($F_{(12, 25)}$ = 3.9958, $P < 0.001$) and Cu remained significantly different from the rest of the treatments (Fig. 2.8D). The REP showed that Cu, Hg and Zn treatments had the highest blue shift of approximately 5.5nm from the control treatment (Fig. 2.8C). This blue shift increased an additional 14.5, 2.5 and 1.5 nanometres for Cu, Hg and Zn respectively when the weevils were added (Fig. 2.8D).

The canopy water content of the metal and weevil phase trials showed significant differences between treatments (($F_{(12, 101)}$ = 11.3062, $P < 0.001$) and ($F_{(12, 101)}$ = 4.9604, P = 001) respectively) (Fig. 2.8E and F). In the first three weeks of the metal phase Cu and Hg showed significantly the lowest canopy water content (CWC) followed by Zn which was not significantly different from the Fe-L treatment (Fig. 2.8E). The pattern of the canopy water content in the second phase of the trial (week 9) after the addition of the weevils however, was different from those in week 3 results and showed a significant decrease in canopy water content compared to the those in week-3 in all the treatments ($F_{(12, 25)}$ = 2.795, $P < 0.015$). However, the canopy water content in the Cu and Hg treatments did not show any significant decrease compared to those in the control treatment. The WBI in the U treatment went from being the highest to the lowest in the second phase (Fig 2.8F).



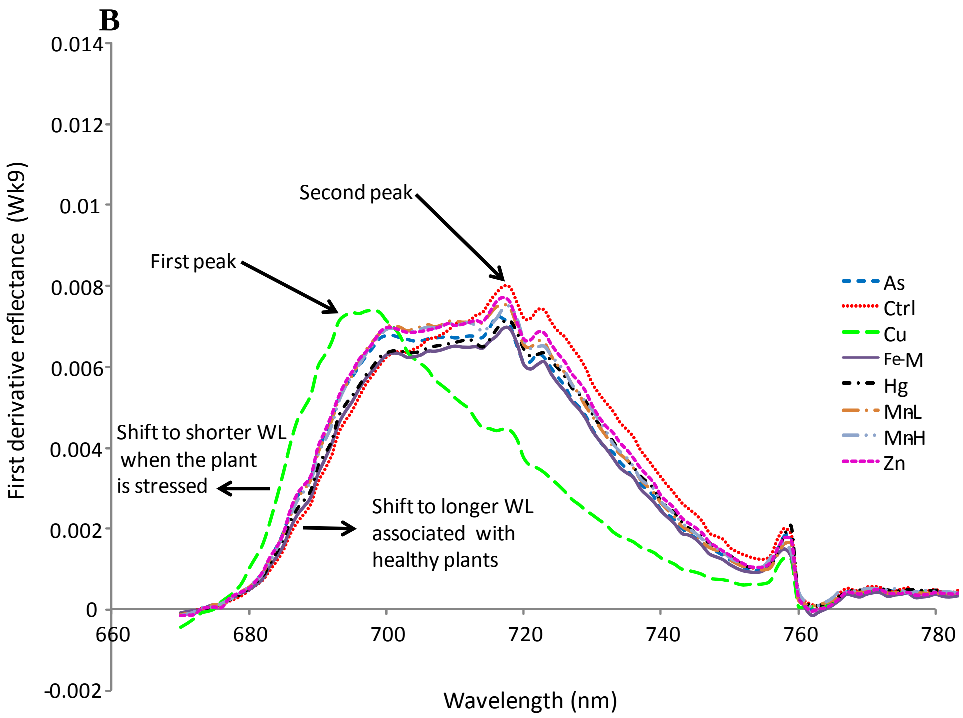


Figure 2.7: Spectral features of water hyacinth growing under different heavy metal and biocontrol treatments in the single-element system tub trial: (**A**) First derivative curve of canopy reflectance three weeks after the addition of heavy metal treatments and before the addition of the weevils (metal uptake phase, week 3), (**B**) First derivative curve of canopy reflectance, in week 9, which is six weeks after the addition of weevils (biocontrol phase) (weevils in the presence of heavy metals).

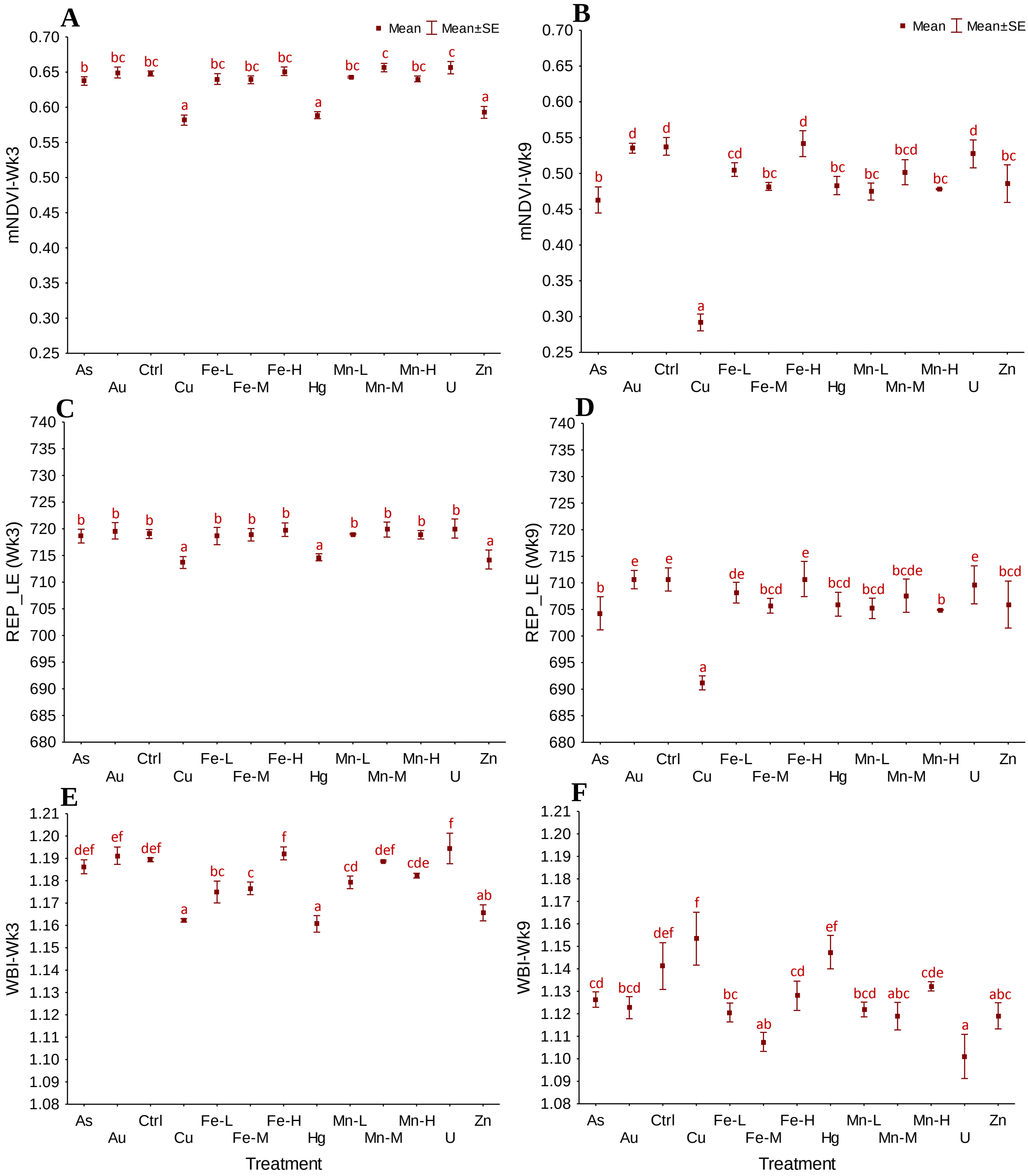


Figure 2.8: The evaluation of canopy chlorophyll and water content of water hyacinth grown under heavy metal and weevil stressors in the single-element system tub trial, using the spectral stress indicators in week 3 (heavy metals only) and week 9 (heavy metals and weevils): (**A**) $mNDVI_{705}$ to detect heavy metal-induced chlorophyll loss in week 3 (**B**) $mNDVI_{705}$ to detect weevil-induced chlorophyll loss in week 9 (**C**) REP_LE to detect heavy metal-induced chlorophyll loss in week 3, (**D**) REP_LE to detect weevil-induced chlorophyll loss in week 9, (**E**) WBI to detect heavy metal-induced canopy water loss in week 3, and (**F**) WBI to detect weevil-induced canopy water loss in week 9. Means were compared by One-way ANOVA and those followed by the same letter(s) are not significantly different ($P>0.05$; Fisher LSD test).

Different spectral indicators of plant stress at week 9 were correlated with SPAD readings, and the number of larval mines and adult feeding scars. All showed positive and significant relationships with the plant stress, although the feeding effects were weak (Table 2.5). Indices based on the red edge bands (mNDVI, REP-LE, RE-NDVI and REP-Max FD) showed stronger correlations compared to the green band index (PRI). The spectral indicator, $mNDVI_{705}$ showed the strongest correlation of all the variables, except the correlation of REP-Max FD with larval feeding which was greater. Of all the spectral indicators the PRI showed the weakest correlations (Table 2.5).

Table 2.5: Correlations of larval mined petioles, adult feeding scars and leaf chlorophyll measured with a SPAD-502 and spectral plant stress indicators of water hyacinth grown in tubs with heavy metal and weevil treatments in week 9; and in the field in week 5 (after the start of the rain). $P < 0.001$.

Spectral Indices	Tub SPAD-reading (R^2)	Tub larval feeding (R^2)	Tub adult feeding (R^2)	Field Wk5 SPAD-reading (R^2)
PRI	0.62	0.15	0.15	0.51
mSR	0.68	0.16	0.36	0.69
REP-Max FD	0.70	0.27	0.33	0.63
RE-NDVI	0.75	0.18	0.36	0.71
REP-LE	0.75	0.18	0.36	0.73
$mNDVI_{705}$	0.79	0.20	0.37	0.71

2.4.2 Simulated acid mine drainage pool trial

The spectral stress indicator, $mNDVI_{705}$ was used to evaluate the canopy chlorophyll content between treatments in the metal uptake and weevil phases. Prior to the addition of the AMD (Week 0), the canopy chlorophyll content in the High AMD concentration treatment was significantly greater than the low and medium AMD concentration treatments ($F_{(5,\ 102)} = 26.8104$, $P < 0.001$) (Fig. 2.9A). However, three weeks after the addition of the AMD, the canopy chlorophyll content in the medium AMD concentration treatment was significantly lower than the other two, which did not show any significant difference between them. The canopy chlorophyll content decreased significantly in all three AMD concentrations three weeks after the addition of the AMD in the metal uptake phase (Week 3) compared to the initial measurements prior to the addition of the AMD (Week 0) (Fig. 2.9A). The canopy chlorophyll content also decreased significantly six weeks after the addition of the weevils (week 9) in all the weevil-treated AMD pools than in the control pools (no-weevils pools) and it was the lowest in the medium AMD, followed by the high AMD treatment ($F_{(5, 48)} = 83.3477$, $P = 0.001$) (Fig. 2.9B). In the control pools the canopy chlorophyll content was significantly greater in the low AMD concentration treatment than in the medium and high AMD treatment, which did not show any significant difference between them.

The pattern of the canopy water content evaluated using the water band index (WBI) was similar to the results shown by the canopy chlorophyll content evaluated with the spectral indicator, $mNDVI_{705}$, except in the metal uptake phase, where the canopy water content did not show any significant difference difference between the three AMD treatments. The canopy water content decreased significantly by 4%, 5% and 7%, for the low, medium and high AMD treatments, respectively six weeks after the addition of the AMD (week 9) and the high AMD concentration showed lower canopy water content compared to the other two ($F_{(5, 102)} = 51.4697$, $P < 0.001$) (Fig. 2.9C). The canopy water content also declined significantly after the weevils' feeding in week 9 in the weevil-treated pools than in the control pools (no-weevil pools) and the medium AMD concentration pool showed significantly the lowest canopy water content of all ($F_{(5, 48)} = 43.9935$, $P < 0.001$) (Fig. 2.9D).

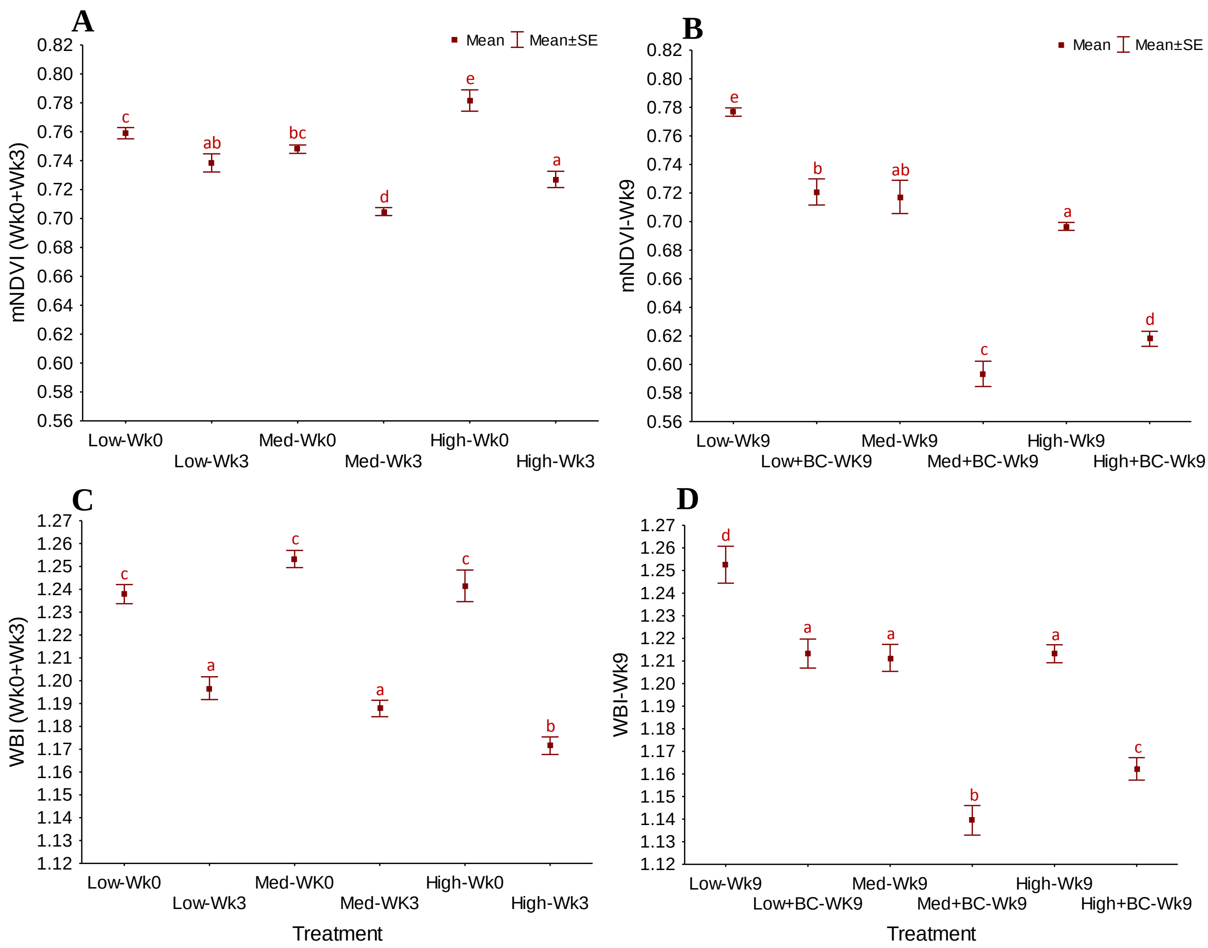


Figure 2.9: Canopy chlorophyll and canopy water contents of water hyacinth grown in simulated acid mine drainage in pool trials at the start of the experiment before the addition of AMD treatment (Wk0), after the addition of AMD treatment (Wk3) and six weeks after the addition of weevils (week 9), calculated using the spectral stress indicators: (**A**) $mNDVI_{705}$ in week 0 and week 3 (**B**) $mNDVI_{705}$ in week 9 in control pools (no-weevil pools) in weevil-treated pools, (**C**) WBI in week0 and week 3, (**D**) WBI in control pools (no-weevil pools) and in weevil-treated pools, in week 9. Means were compared by Two-way ANOVA and those followed by the same letter(s) are not significantly different (P>0.05; Fisher LSD test).

2.4.3 Acid mine drainage trial in the field

The first spectral measurements were taken two weeks after setting the floating cages with water hyacinth, above and below the Koekemoespruit and Schoonspruit on the Vaal River.

Before the start of the rainy season, the canopy chlorophyll content of water hyacinth in the floating cages of Koekemoerspruit was significantly lower than that of the water hyacinth at the inlet of the Schoonspruit into the Vaal River ($F_{(3,}$

$_{13)}$ = 937.7187, $P < 0.001$) (Fig. 2.10A). The mNDVI$_{705}$ of the plants above the inlet of the Koekemoerspruit was significantly lower than those in the cages below the inlet of the Koekemoerspruit. However, the same spectral indicator, (mNDVI$_{705}$) showed that the canopy chlorophyll content in both above and below cages at the inlets of the Schoonspruit into the Vaal River were the same (Fig. 2.10A). The canopy chlorophyll content in the rainy season (week 5) were significantly lower at the sites of the Koekemoerspruit inlet than those at the Schoonspruit inlet on the Vaal River ($F_{(3, 14)}$ = 1263.7005, $P < 0.001$) (Fig. 2.10B). However, there was not any significant difference between the sites within the same tributary of the Vaal River.

The canopy water content before and after the start of the rainy season showed significant differences between the floating cages with similar trends between the sites of the same tributary (($F_{(3, 13)}$ = 323.7679, $P < 0.001$) and ($F_{(3, 14)}$ = 214.7748, $P < 0.001$) respectively) (Fig. 2.10C and D). There was no significant difference between the water hyacinth in the cages above and below the inlet of the Koekemoerspruit, but both cages were significantly different from those cages at the inlet of the Schoonspruit on the Vaal River in both cases, before and after the start of the rain (Fig. 2.10C and D). In contrast the water hyacinth in the above cage of the Schoonspruit showed a significantly lower chlorophyll content than that in the cage below the inlet of the Schoonspruit before and after the start of the rain (Fig. 2.10C and D).

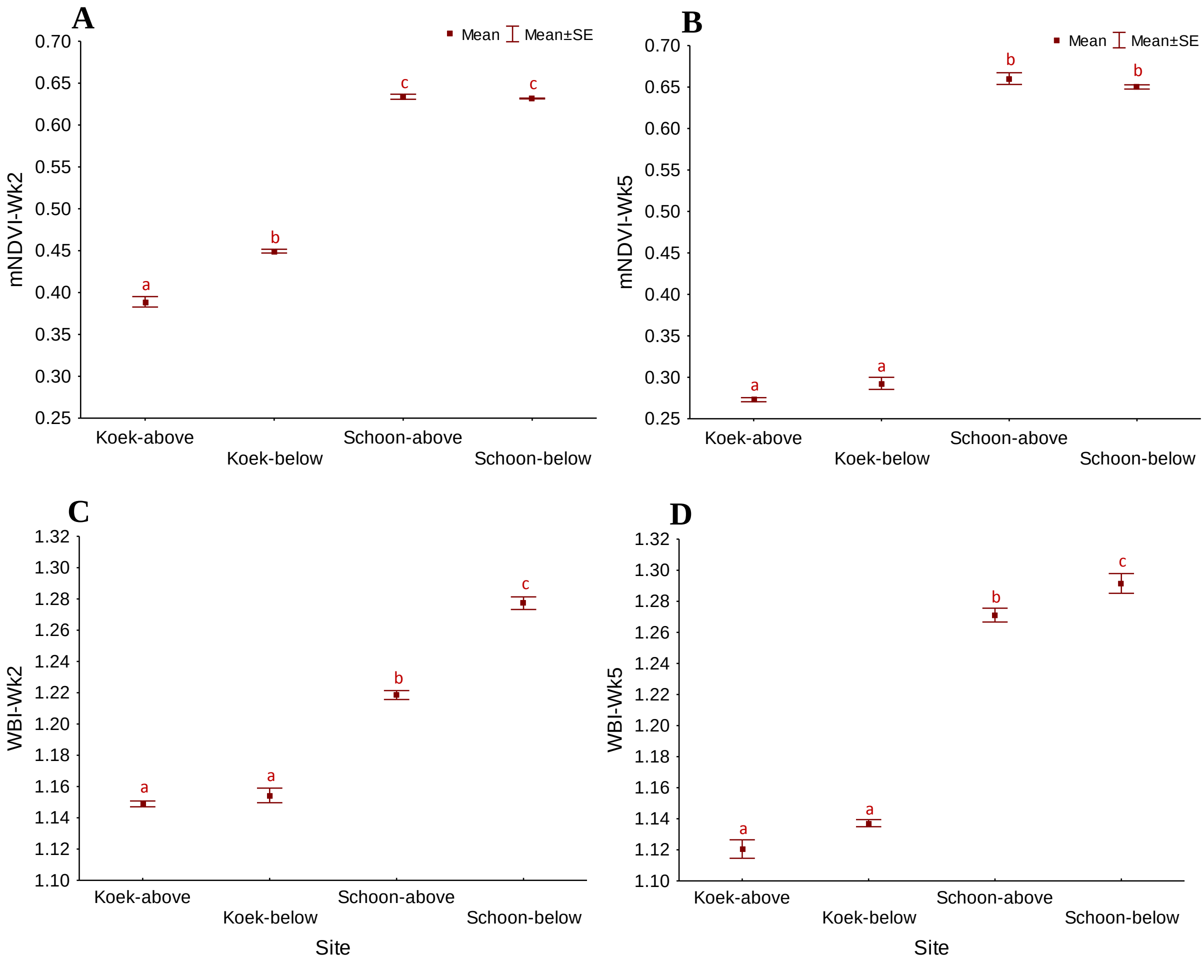


Figure 2.10: The evaluation of canopy chlorophyll and water contents of water hyacinth in acid mine drainage trial, in the field, grown in cages above and below the inlets of the Koekemoerspruit and the Schoonspruit on the Vaal River before (Wk2) and after (Wk7) the start of the rainy season using the spectral stress indicators: $mNDVI_{705}$ for (**A**) and (**B**) and the water band index, WBI for (**C**) and (**D**). Means were compared by One-way ANOVA and those followed by the same letter(s) are not significantly different (P > 0.05; Fisher LSD test). NB: "Koek-above" and "Koek-below" refers to cages above and below the inlet of the Koekemoerspruit on the Vaal River, respectively; whereas the "Schoon-above and "Schoon-below" refers to the cages above and below the inlet of the Schoonspruit on the Vaal River, respectively.

2.5 Discussion

The hand held spectrometer was able to detect plant stress caused by different metals, of which Cu was the most stressful. The simulated AMD pool trial showed that an increased AMD concentration exacerbated the plant stress. The weevil induced plant stress was also visible in the spectrometer results in both the single-element system tub and simulated AMD pool trials.

2.5.1 Spectral features of water hyacinth in the single-element system tub trial

2.5.1.1 Metal uptake phase in the single-element system tub trial

In the first three weeks of the tub trials different spectral indicators showed water hyacinth to be generally tolerant to most heavy metals in which they were grown, with the exception of Cu, Hg, and Zn treatments which consistently caused stress (Fig. 2.8). Several studies have already established the appearance of blue shifts in the red edge of other plant species as an indicator of plant stress associated either with deficiency or excess of organic and inorganic elements, due to their association with plant chlorophyll content (Ayala-Silva *et al*., 2005; Kooistra *et al*., 2004; Horler *et al*. 1980, 1983; Cho and Skidmore 2006). A greater first derivative peak at ~ 702nm (first peak) seen in the Cu, Hg and Zn treatments, when compared to the control treatment and their relative shift towards the shorter wavelength (opposite to the direction seen in the control treatment), indicates a decrease in canopy chlorophyll concentration (Fig. 2.7A). Thus, the blue shift of ~ 5.5 nm in Cu, Hg, and Zn treatments suggests the presence of these heavy metals in the upper (leaf) plant tissues of water hyacinth (Fig. 2.8C). Rock *et al*. (1988) found a blue shift of 5 nm in spruce and fir species as a result of airborne acid deposition causing plant stress. Similarly Ren *et al*. (2008) in a single element trial, using the REP and the blue shift, were able to identify the relative concentrations of lead (Pb) in the canopy leaves of rice during the early tillering stage. Jago and Curran (1996) showed that peaks of 693 nm and 709 nm from stressed grass canopy spectral measurements, growing on oil-contaminated sites, indicated that the first peak decreased (shifting to the shorter wave length) due to decline in canopy chlorophyll content trigerred by plant stress, while the second peak was attributed to the cellular scattering in the leaf.

The modified normalized difference index ($mNDVI_{705}$) also revealed the pattern of plant stress shown by the first derivative reflectance curve (Fig. 2.7A) and the REP calculated by linear extrapolation (Fig. 2.8C), where treatments of Cu, Hg and Zn were indicated to be the most stressful for the water hyacinth plants.

The canopy water stress measured in the tub water hyacinth, using the WBI, matches the results of the spectral indicators associated with leaf chlorophyll concentrations ($mNDVI_{705}$, REP-Max FD and REP-LE). Water hyacinth grown in the same metal elements (Cu, Hg, and Zn) had the lowest WBI, which is an indication of reduced water canopy content due to the heavy metal-induced stress. The fact that this experiment consistently showed that the results of WBI were largely similar to the results of the spectral indicators of canopy chlorophyll content (e.g. $mNDVI_{705}$ and REP-LE), indicates the positive correlation between the water canopy content and the canopy chlorophyll content. Claudio *et al*. (2006) used the WBI to estimate the evapo-transpiration and the canopy water status of vegetation in a semi-arid shrubland ecosystem in Southern California and found a strong link between canopy water content and the green canopy structure.

In the first three weeks (the metal up-take phase), the spectral indicators consistently showed that water hyacinth was more sensitive to Cu, Hg and Zn compared to other heavy metals tested in the tubs.

2.5.1.2 Biocontrol phase in the single-element system tub trial.

Most spectral indicators that detect plant stress are associated with plant chlorophyll. An excess or deficiency of plant nutrients affects plant chlorophyll content. For instance, deficiency of both nitrogen and magnesium results in entire plant chlorisis because they are an essential component of chlorophyll, while a deficiency of Ca, K and P only results in a partial chlorisis (Ayala-Silva *et al*., 2005). Since plant stress, as a result of nutrient deficiency, causes similar symptoms (chlorisis), it is often difficult to distinguish the specific spectral signature of one element from the other. For instance the REP in all nutrient deficiencies is characterized by a shift towards the shorter wavelength (Ayala-Silva *et al*., 2005). The same applies with high levels of heavy metal uptake that reduce leaf chlorophyll by generating higher concentrations of destructive oxyradicals causing “oxidative stress” that eventually impairs photosynthesis (Smolders and Roelofs, 1996). Similarly, pathogenic or insect damage to plants alters the physiological and chemical status of the plant by changing the concentration of chlorophyll pigments, biochemical composition, cell structure and nutrient and water uptake that affect the colour and temperature of the plant canopy (Raikes and Burpee, 1998). Such characteristic changes in the plant canopy as a result of biotic damage also produce spectral features similar to those of excess heavy metal plant uptake or plant nutrient deficiency.

The severity of the plant stress increased after the addition of the weevils to the water hyacinth grown in tubs (week 9) and there were more treatments in week-9 showing stress compared to week 3, and these included Cu, Hg and Zn treatments as the principal plant stressors (Fig. 2.8B and D). The REP of the control treatment decreased by ~ 8 nm by week 9, and the number of stressful treatments increased to seven (adding As, Fe-M, Mn-L and Mn-H) from three, in week 3; indicating that both larval and adult plant feeding increased the intensity of the plant stress (Fig. 2.7B and 2.8B and D). However, considering the fact that plant's water consumption increases with lower nutrient concentrations in water, the relative increment in number of treatments with plant stress in week 9 could also be partly due to the influx of heavy metals into the plants associated with the increased water uptake by plants for more nutrients (Chattopadhyay *et al*., 2012). This also suggests why the canopy chlorophyll content in the control treatment, despite showing the greatest leaf damage by adult weevils (see Chapter-4), still remained significantly greater than most of the metal treatments, which sustained less weevil damage than the control (Fig. 2.8B and D).

Feeding damage by the weevils, in week-9 decreased leaf chlorophyll pigments and changed the canopy structure resulting in increased reflectance in the visible range and decreased reflectance in the near infrared range. Mirik *et al*. (2006), using a hand held spectrometer, also found similar spectral features in a greenbug-damaged wheat canopy compared to undamaged wheat canopies. The distinct appearance of the first derivative curve with an increase in the first peak and decrease in the second peak are linked to the reduction of chlorophyll and change of cellular structure as a result of feeding stress by the weevils. Mirik *et al*. (2007) found that aphid infested wheat had a lower reflectance than non-infested wheat at the red edge (730-750) and up to 900 nm while the reflectance increased in the visible region of the light spectrum. The Cu treatment in this experiment showed the greatest blue shift increase of about 14.5 nm followed by As and Mn-H among others (Fig. 2.8B and D). However, the increased stress of Cu and that of As in week 9, was not solely the consequences of weevil damage, since the adult and larval damage in these two metal treatments were among the lowest (see Chapter-4). Hence, it suggests that even though to a lesser extent the weevil damage might have aggravated the severity of the plant stress, it primarily occurred because of the prevailing metal-induced stress of Cu and As, which could have been translocated into the leaves, after the third week of the experiment.

Some heavy metals are often less bioavailable than others for direct plant uptake, either due to cationic competition or due to their strong binding capacity with ligands. This suggests why Cu and As (among others) showed an increased phytotoxicity after an extended period of plant exposure (week 9). Cu is one of those heavy metals which are commonly less bioavailable for immediate uptake by plants due to its strong affinity to ligands (Fernandes and Henriques, 1991; Daigo, 1997). Hence the distinctive spectral signature of the plants in the Cu treatment throughout this experiment, and more particularly in week 9, is a strong indication of an increase in Cu concentration in the plant tissues and as a result increased stress due to its phytotoxicity. de Almeida *et al*. (2007) showed that extended exposure of plants to Cu led to plant growth and development disorders, with severe chlorotic symptoms, because of inhibition of cellular elongation and interference with a number of enzymatic activities which decreased the photosynthetic processes. Similarly Maksymiec *et al*. (1994) found that increased levels of Cu reaching the plant's leaves resulted in photoinhibitory damage to photosystem-two (PSII).

Considering the As-induced plant stress in week 9, despite the fact that the adult feeding damage in the As treatment was significantly lower than the control treatment, the canopy chlorophyll decreased significantly in week 9 (Fig. 2.8B and D). It is known that plant uptake of phosphates is negatively correlated with As uptake (Mkandawire *et al*., 2004; Rahman *et al*., 2007). The arsenic treatment spectral reflectance was not significantly different from that of the control treatment in week 3, suggesting that phosphates from the Hoagland solution used at the beginning of the experiment could have inhibited the uptake of As by water hyacinth until the complete removal of the phosphates from the water in the first three weeks. Wang *et al*. (2002) found that the uptake of arsenate by the As hyperaccumulator plant, *Pteris vittata*, dropped in the presence of phosphate and increased by 2.5 fold after the depletion of the phosphate after eight days.

Generally the canopy water content of the water hyacinth plants grown in tubs dropped significantly ($F_{(12, 25)} = 2.795$, $P < 0.014$) (Appendix 2B) in the second phase of the weevil trial, indicating the deterioration of the plant's health as a result of additional stress exerted by larval and adult weevil damage compared to the no weevil period in the metal uptake phase, week 3 (Figs. 2.8E and F). Nevertheless, the WBI of Cu, and Hg treatments in week 9 (the weevil phase) was not significantly different from that of the control treatment (with weevils but no heavy metals), (Fig. 2.8F). This could be confounded by greater larval and adult feeding damage in the control treatment which destroyed more leaf tissue and therefore its capacity to hold water compared to Cu and Hg treatments which showed less leaf damage than the control treatment (see Chapter-4).

2.5.2 Spectral features of water hyacinth in the simulated AMD pool trial

2.5.2.1 Pool metal uptake phase in the simulated AMD pool trial

The effect of heavy metals on water hyacinth was further demonstrated in the pool trial, where plants were grown in water which contained more than one element. Initially the high AMD concentration showed significantly greater canopy chlorophyll content than the other two AMD concentration treatments. This could be due to the elevated nutrient levels in the water from the previous pilot test, which was partly reused in the high AMD concentration pools of this trial. The addition of the AMD to the pools decreased the canopy chlorophyll content of water hyacinth plants significantly in all the three AMD concentration treatments after three weeks. The $mNDVI_{705}$ spectral index indicated that the canopy chlorophyll content was significantly lower in the medium and high AMD concentration treatments than in the low AMD concentration treatment (Fig. 2.9A). Nevertheless, the percentage reduction in the canopy chlorophyll content increased with the increase of the AMD concentrations from the low, to the medium and to the high AMD treatments by an average of 3%, 6% and 7%, respectively (Fig. 2.9A). High level of sulphates in water affect plant growth through a variety of effects, among which are severe eutrophication that involves mobilization of P, immobilization of iron and other nutrients, sulphide toxicity or enhancing heavy metal uptake by plants from the water (van der Welle *et al*., 2007). The decline of *Stratiotes aloides* L. (Water Soldier) in the Netherlands was attributed to increased eutrophication levels, which is also known as internal eutrophication, due to increased sulphate contamination from anthropogenic activities (Smolder *et al*., 2003).

Results of the initial WBI taken before the start of the experiment with the addition of the AMD treatments, showed slightly a different pattern between the AMD concentration treatmens compared to that taken usng mNDVI spectral indicator of canopy chlorophyll content. The medium AMD treatment showed significantly greater canopy water content than the low AMD treatment (Fig. 29C). After the addition of the AMD to the pools however, they all decreased significantly in week 3, compared to their initial WBI (week 0) and the high AMD concentration treatment showed significantly lower canopy water content than the other two, indicating the severity of plant stress of water hyacinth grown at concentrations of 1300 mg/L $(SO_4)^{-2}$ in water. Similar to the mNDVI, the WBI revealed a percentage reduction in canopy water content with the increase of the AMD concentration (low, medium and high) by 3%, 5% and 6%, respectively, suggesting that both spectral indicators to some extent could be interchangeably used to detect either the canopy chlorophyll or water content stress in water hyacinth plants.

2.5.2.2 Pool biocontrol phase in the simulated AMD pool trial

Generally the plant health status deteriorated in all the AMD concentration treatments in the biocontrol phase, six weeks after the weevil’s feeding on water hyacinth plants (week 9). Nevertheless, the plant stress was more pronounced in the medium AMD concentration pools followed by the high AMD pools, which showed canopy chlorophyll reduction of 17% and 11% respectively, as opposed to the low AMD (7%), compared to their respective control pools (no-weevil pools) in week 9 (Fig. 2.9B). Similarly, the reduction in the canopy chlorophyll content was greater in the same two AMD treatments than in the low AMD concetration pools (15.8%, 15% and 2.4% respectively) after the addition of the weevils in the biocontrol phase (week 9), compared to those in the metal uptake phase, before the addition of the weevils in week 3 (Figs. 2.9A and B). However, the weevils’ feeding and reproductive activities showed otherwise (see Chapter-4). There was more feeding damage on plants of water hyacinth in the low AMD pools compared than in the other two AMD treatments. Therefore, this suggests that the canopy chlorophyll reduction in the weevil-treated plants in week 9 was not solely due to weevil feeding, but also due to the impact of heavy metals and the high AMD concentrations in the pools. The impact of some heavy metals on the plant could be more substantial with time and extended exposure, due to either the competition between the different heavy metals or nutrients for transport sites on the plants (Prasad *et al*., 2001; Tangahu *et al*., 2011; Chattopadhyay *et al*., 2012). Wang *et al*. (2002) found that the uptake of As by *Pteris vittata*, from water increased over two-fold after a week when all the phosphates in water were first completely taken up by the plant.

The pattern in the canopy water content after the addition of the weevils in the biocontrol phase mirrored that of the $mNDVI_{705}$ results for the canopy chlorophyll content. The reduction in canopy water content of weevil-treated pools compared to the control pools (no-weevil pools) was significantly greater in both the medium and high AMD concentration treatments (6% and 4% respectively) than in the low AMD pools (3%) and the medium AMD concentration treatment showed significantly the most severely water stressed of all the treatments (Fig. 2.9D).

Plant damage by insect herbivory reduces the canopy water content of plants through increased transpiration. Aldea *et al*. (2005) found an increase of 45% water loss in soybean plants damaged by *Popillia japonica* (Japanese beetles) (Coleoptera: Scarabaeidae) and *Helicoverpa zea* (Boddie) (Lepidoptera; Noctuidae) compared to no herbivory. Similarly Marlin *et al*. (2013) found that the damage to water hyacinth by the mite, *Orthogalumna terebrantis* increased the rate of transpiration and water loss. In this trial however, although the weevils' feeding could have contributed to the severity of the plant health status by reducing the canopy water and chlorophyll content, the fact that both the medium and the high AMD concentration treatments sustained significantly lower feeding damage than the low AMD pools, suggests that that plant stress was partly due to the increased AMD concentrations in water. Eaton (1941) found that elevated osmotic pressure in the external medium of plant growth, disrupted the uptake processes of nutrients and other elements by roots, and this could result in plant stress due to nutrient deficiency. The removal of sulphur by roots of water hyacinth in this trial decreased with the increase of the sulphate concentration in water at the end of the metal uptake phase in week 3 (see Chapter-3), suggesting the presence of more sulphates in the water, which could possibly interfere with the nutrient and metal uptake by the roots of water hyacinth. Ayyasamy *et al*. (2009) also found similar reduction in the percentage removal of nitrates in water when the concentration was increased over 300 mg/L.

The fact that the water canopy content before and after the addition of the weevils between the treatments reflected a spectral trend similar to the respective results of canopy chlorophyll content, suggests the positive relationship between canopy water and chlorophyll contents (Fig. 2.9). Claudio *et al*. (2006) found a positive correlation between the spectral indicators, WBI and NDVI when they monitored a drought effect on three tree species in a shrubland ecosystem.

2.5.3 Correlation of spectral reflectance with SPAD meter readings of chlorophyll content

The correlation of the spectral indicators of plant stress to the SPAD-502 chlorphyll readings showed that all indices could reveal the water hyacinth plant stress at a canopy level. Nevertheless, the red-edge normalized difference indices and the spectral indicators for the evaluation of the REP followed by RE-NDVI produced relatively strong correlations compared to the other indices, of which $mNDVI_{705}$ was the best of all (at least in the tub trials) (Table 2.5). Tian *et al*. (2011) found that the $mNDVI_{705}$ was correlated more strongly with chlorophyll content than the RE-NDVI (R^2 of 0.83 and 0.73 respectively). This is due to the fact that addition of the third blue band (reflectance at the wavelength of 445 nm) in the $mNDVI_{705}$ helps to eliminate the effect of surface reflectance and light scattering at 800 nm (Sims and Gamon, 2002). However, the inconsistency between the three red-edge indices ($mNDVI_{705,}$ REP_LE and RE_NDVI) as to which produces the strongest relationship with the SPAD suggests that more than one spectral indicator should be used to get a robust result for plant health status. In the Field trial the REP_LE produced the strongest correlation with the SPAD

(Table 2.5). Mirik *et al*. (2006) found that spectral indicators were strongly correlated with greenbug damaged wheat crops, with correlation coefficients ranging from 0.82 to 0.98, compared to 0.37 on water hyacinth in the current experiment. The fact that the water hyacinth weevils in this study were feeding on heavy metal contaminated plants, suggests their feeding performance was generally reduced.

2.5.4 Spectral features of water hyacinth in the acid mine drainage field-trial

In the field trial, before the start of the first summer rainfall (week 2), water hyacinth grown in cages at the inlet of the Koekemoerspruit on the Vaal River showed that the plants were more stressed than those downstream at the inlet of the Schoonspruit (Fig. 2.10A). However, there were frequent choppy water disturbances to the caged plants at the Koekemoerspruit caused by water skiers from the nearby boating club. This coupled with what looked like a bird feeding, impacted the plants in both cages at the inlet of the Koekemoerspruit to the Vaal, which could be why the canopy chlorophyll content was very low compared to downstream cages (Figs. 2.10A and Appendices 2C, D and E). In addition to this the Schoonspruit, which directly contaminates the cage below its inlet on the Vaal River, carries more nutrients in effluents from the nearby settlements than the Koekemoerspruit (DWAF, 2009). Within the cages at the inlet of the Koekemoerspruit, however, the canopy chlorophyll content in the cage below the inlet of the tributary was significantly greater than those above the inlet. Although, water analysis was only conducted at the end of the experiment after the rain, results indicated that the water around the cage below the inlet of the Koekemoerspruit had greater nutrient concentration (SO_4, Mg, P, Zn) than those at the above-inlet cage (see Chapter-3). DWAF, (2007) also indicated that the Koekemoerspruit is a source of nutrients to the Vaal River and therefore, water hyacinth plants in the cage below the inlet of the tributary could benefit from the nutrients brought in.

After the rainy season (week 5) the water hyacinth canopy chlorophyll content was significantly lower in cages at the inlet of the Koekemoerspruit than downstream in the cages at the Schoonspruit. The caged plants of the

Koekemoerspruit inlet did not show a significant difference between them as previously found in week 2 (before the rain), (Fig. 2.10B). Results from plant tissue analysis also showed that nutrient and heavy metal concentrations in shoots of water hyacinth between the two cages at the inlets of the Koekemoerspruit were not significantly different (see Chapter-3). In addition, the birds' feeding damage on plants in both cages at the Koekemoerspruit was more prevalent and severe compared to the first spectral measurements in week 2, before the rain.

The water canopy content shown by the WBI for most of the single-element system tub and AMD pool trials matched the spectral pattern of the canopy chlorophyll content revealed by different spectral indicators of plant chlorophyll stress. However, the cage trials in the two tributaries of the Vaal River showed a slight mismatch between the spectral patterns of the WBI and the $mNDVI_{705}$ spectral indicators of plant stresses (Figs. 2.10). The mismatch between the two spectral indicators before the start of the rain, in week 2 could probably be attributed to the bird damage to the leaves and petioles, which would reduce light absorption in the NIR spectrum due to water loss from leaf tissues (Appendix 2C and D) (quantitative data not available). The mismatch between the canopy water and canopy chlorophyll contents of plants in the two cages at the inlet of the Schoonspruit after the rain (week 5), however could be attributed to the increased eutrophication levels at the cage below the inlet of the tributary with the rainy season. After the rain, the waters at the cage below the inlet of the Schoonspruit were seen to be silty and highly eutrophied with increased concentration of nutrients such as P, Mn, Mg, Fe, Zn and SO4, caused by runoff from the surrounding mining sites and effluents from the local settlement of Kennan (See Chapter-3). Therefore, the plants in the lower cage of the Schoonspruit were healthier, with thick broad leaves, larger than those in the above inlet cages (see Chapter-5). Such leaf characteristics could also cause the difference in WBI between the two cages in the Schoonspruit.

2.6 Conclusion

A hand held spectrometer (ASD) was used to evaluate the physiological and health status of water hyacinth grown under different abiotic (heavy metals and AMD) and biotic (water hyacinth weevil) stressors. Hyperspectral data was convincingly able to detect the intensity of the stress caused to water hyacinth plants by susch stressors. This indicates that the technique has potential as a tool to determine the health status of water hyacinth from a remote position. However, discrimination between the different plant stressors (between heavy metals or the weevils' feeding) could not be established due to their similarities in their impacts to the plants, which are all associated with degradation of the leaf chlorophyll contents that consequently result in similar spectral plant responses.

Water hyacinth plants were generally tolerant to heavy metals with the exception of Cu, Hg and Zn treatments, which consistently revealed stressful spectral features when analysed using different spectral stress indicators. The plant stress caused by weevil feeding was also detected in the spectral data, extending the total number of treatments with stressed plants to seven at the end of the weevil phase, from three prior to the addition of the weevils. Thus, the success of the hyperspectral remote sensing in gathering different biotic and or abiotic information on the physiological status of water hyacinth could be of importance in management of the plant by facilitating the decision making processes of intervention measures. Such decisions depend on the timely available information such as the phenological stage of the plants (e.g. vegetative or flowering), extent of infestation, plant health status by determining both the canopy chlorophyll and water content which could be stressed due to the effect of previously released biological control agents or sub-lethal herbicides and water contaminants (heavy metals or acid mine drainage).

Chapter 3

Water hyacinth as a tool of phytoremediation

3.1 Introduction

Disposal of untreated sewage and effluents into surface water is still the norm in many countries around the world (Ismail and Beddri, 2009). Both organic and inorganic contaminants of water from such activities put all aquatic life and human health at risk. Contaminants of particular concern are heavy metals, radionuclides, nitrates, phosphates, inorganic acids and organic chemicals (Arthur *et al*., 2005).

Singh *et al*. (2003) reported that an estimated 22,000 t (metric ton), 939,000 t, 783,000 t and 1,350,000 t were released worldwide over the last 50 years for cadmium, copper, lead and zinc, respectively. Since the start of gold mining on the Witwatersrand in 1886, an estimated 6 billion tons of tailings have been generated, and annual uranium (U) disposal on slimes dams from gold mining in South Africa is currently reaching about 6000 tons annually (Winde and van der Walt, 2004). Currently of all wastes generated in the country including U, Zn, Hg, As, Mn, Fe, S, CN … etc., about 70% (318 to 450 million tons per year) comes from the mining industry (particularly the gold/uranium, platinum and coal sectors) (Deat, 2004 cited in Weiersbye, 2007). In the past disposal of mining waste in South Africa was in unlined tailing dams piled on to the surface of the land of which there are over 270 around the Witwatersrand Basin alone (AngloGold Ashanti, 2004). Acid mine drainage (AMD), contamination of both ground and surface water through seepage, runoff and wind erosion from the unvegetated tailing dams are some of the environmental implications of the mining dumps (Oelofse *et al*., 2007). Acid mine drainage is the product of sulphides from the mining waste rock (more often from the iron sulphides in the rock) when they are exposed to oxygen and water (Oelofse *et al*., 2007). This is the worst source of environmental contamination as far as tailings dams are concerned (Ritcey, 2005). Chemical water and sediment analysis has confirmed that gold and uranium slimes dams are sources of contamination of the Vaal River tributary, the Koekemoerspruit, in the North West province of South Africa (about 10 km west of Orkney) through seepage of dissolved U and other metals from tailings dams that eventually drain in to the Vaal River (Winde *et al*., 2004; Winde and Van der Walt, 2004). A recent study also identified mercury (Hg) contamination of the water and sediments of the Schoonspruit in the same region. This is considered to be as a result of the historical use of mercury for the amalgamation of gold, when mining in this region commenced in the late 1800's (Cukrowska *et al*., 2010).

Most heavy metal contaminants reach humans through direct or indirect use of water. For instance, the major route of contaminants such as mercury (Hg) to humans is usually through consumption of fish containing methyl-mercury (Mauro *et al*., 2001). This is due to the fact that Hg is easily transformed into methyl-mercury through microbial activity (Sweet and Zelikoff, 2001) and can be biomagnified up to 106 times through the food web (Fitzgerald *et al*., 1998). Arsenic pollution of drinking ground water is of concern worldwide, whereever arsenic-bearing rocks occur. Well waters of West Bengal and Bangladesh, amongst other countries worldwide, are contaminated by Arsenic as a result of the drilling of drinking water wells into naturally high As rocks (arsenopyrites). Well waters can exceed the WHO recommended levels (10 mg/l) by five fold, threatening the health status of 6 million and 46 million people, respectively (Wang and Zhao, 2009). Mining of arsenopryrite rocks for gold can also exacerbate arsenic pollution of water. South Africa is involved in several mining activities on arsenic-bearing ores, and therefore there is the potential for arsenic contamination of ground waters (van Halem *et al*., 2009).

Tailings from the mining sector and effluents from the non-ferrous metals industry are the main sources of heavy metals and other toxic pollutants in water systems and the environment in general (Ahluwalia and Goyal, 2007). Therefore, intervention by removal or detoxifying these materials in order to provide safe drinking water is an important issue. Phytoremediation, by aquatic plants, is potentially the most strategic approach to "polish" and upgrade such polluted water systems (Ismail and Beddri, 2009).

3.2 Conventional remediation of heavy metals from water

Conventional remediation of heavy metals are very expensive and the removal of chemical sludge generated in the process is even more costly and not eco-friendly (Ahluwalia and Goyal, 2007). The remediation method can be still more costly and or ineffective, when heavy metal contaminants in the aqueous solution are in trace quantities, or between the ranges of 1-100 mg/L (Nourbakhsh *et al*., 1994). The cost of remediation depends on the type of such non-biological technologies implemented and the quantity and the type of contaminant to be removed. A review of global costs over a 10-year period found these to be from US$10-4000 per cubic meter soil or US$100 000 to US$3 million per ha land, and from US$1-300 per kilolitre of groundwater; Whereas, the cost of decontamination per cubic-meter with bio- and/or phyto-technologies over the same period only cost from US$0.02-40 per kilolitre, or US$200 to US$100 000 per ha of land (Weiersbye, 2007). The United States spends up to 2% of its gross national product on remediation and pollution control of the environment (Arthur *et al*., 2005), while in South Africa the Department of Minerals and Energy estimated the cost of rehabilitating all the abandoned mines alone to be a total of about US$14 billion (DME, 2007).

3.3 Phytoremediation

Most aquatic plants have the ability to phytofiltrate heavy metals from water (Misbahuddin and Fariduddin, 2002; Roldán, 2002; Vaillant *et al*., 2004; Bennicelli *et al*., 2004; Kamal *et al*., 2004; Snyder, 2006). Plants that grow vigorously and extensively with high colonization rates can be good candidates as tools of phytoremediation (Sasmaz and Obek, 2009). Even though this is characteristic of most alien invasive aquatic weeds, many have been implemented and redirected to separate heavy metals from water bodies and to improve water quality. Phytoremediation is an emerging technology with a great potential for research and public acceptance as a cost effective and efficient method of remediating environmental contaminants from air, soil and water (Singh *et al*., 2003; Arthur *et al*., 2005).

A plant species' efficiency in phytoremediation is determined by the index of their bioconcentration factor (BCF). This is an index used to evaluate the capacity of a plant to accumulate heavy metals in its tissue and to establish its potential use for phytoremediation (Lu *et al*., 2004). Plants capable of accumulating 5000 mg/kg of heavy metal or those with BCF that exceeds 1000 are considered as good accumulators of heavy metals and they are potentially the best candidate for phytoremediation (Zhu *et al*., 1999). The bioconcentration factor of plants is computed as the final metal concentration in plant tissues divided by the initial metal concentrations in water (Zhu *et al*., 1999).

Several aquatic weeds have shown phytofiltration of different toxic heavy metal contaminants from water. For instance duck weed, *Lemna gibba* L. is one of the aquatic plants largely used in constructed wetlands for wastewater treatment, which efficiently accumulates large amounts of heavy metal pollutants (Vaillant *et al*., 2004). Similarly studies have shown that the small water fern, *Azolla caroliniana* removed about 93% of Hg from polluted water in just 12 days (Bennicelli *et al*., 2004), while nearly all (99.8%) of the Hg was removed after three weeks by parrot's feather (*Myriophyllum aquaticum*), creeping primrose (*Ludwigia palustris*), and water mint (*Mentha aquatica*) (Kamal *et al*., 2004), and most of these metals were accumulated in the root system.

A meshwork of floating roots with porous root caps in aquatic plants provides large surface area with many binding sites for heavy metals in the cell wall of the roots, where absorption takes place by ion exchange and other mechanisms (Elifantz and Tel-or, 2002). Water hyacinth is among the most widely used aquatic plants for the management and monitoring of organic, inorganic and many heavy metals from wastewaters, industrial effluents and polluted waters (Table 3.1). This is largely attributed to its exceptionally high growth rate, and large biomass both below and above water. Wetlands that are invaded by water hyacinth are regarded as nature's kidney, which purifies polluted water (Malik, 2007) and as such, in extreme conditions of heavy metal pollution water hyacinth is even deliberately grown in wetlands for phytoremediation. For instance water hyacinth in a constructed wetland in Taiwan removed large amounts of lead, copper and zinc (Liao and Chang, 2004). Roldán (2002) also reported a removal of over 90% of metals by water hyacinth from effluents from an aluminum factory. The roots of a living water hyacinth plant were found to remove 81% of arsenic from a solution of 400 ppb, while the entire plant removed 100% in less than six hours (Misbahuddin and Fariduddin, 2002). The efficiency of water hyacinth in removing heavy metals from water has even encouraged small scale farmers in Bangladesh to remove arsenic by treating water drawn from wells with water hyacinth overnight before being used (Snyder, 2006).

Most heavy metal contaminants are accumulated in the roots of water hyacinth rather than in the shoot system (Malik, 2007). Linear correlation of metal accumulation was found in the order of roots>stems>leaves of water hyacinth with increasing of Pb, Cu and Cd concentrations in water (Kay *et al*., 1984). Lu *et al*. (2004) also showed that the highest concentration of cadmium (2044 mg/kg) and zinc (9652.1 mg/kg) was in the roots of water hyacinth as compared to the aerial system (113.2 mg/kg and 1926.7 mg/kg, respectively) and this was from Cd and Zn concentrations of 4mg/L Cd and 40 mg/L Zn in water respectively. Liao and Chang (2004) also found that the accumulation of heavy metals in the roots of water hyacinth was 3 to 15 times greater than to the shoots, where lead (Pb) accumulation in water hyacinth was 215.35 and 33.34 mg/kg dry weight in the roots and shoots respectively.

Despite the great potential of water hyacinth for use as phytoremediation plant, and the success already achieved in that regard, it is very important to note its invasive capacity, which makes its use for water management contentious. However, water hyacinth can be exploited as a very efficient plant for water purification, if it is already in the system.

Table 3.1: The phytoremediation capacity of water hyacinth.

Wastewater source	Metal removed	Removal (%)	Exposure (days)	Reference
Coal mine effluent	As	80.00	21	Mishra *et al*., 2008a
Contaminated solution (1.5 mg Cu/L)	Cu	97.00	21	Mokhtar *et al*., 2011
Textile effluents	Cr	94.78	4	Mahmood *et al*., 2005
Textile effluents	Zn	96.88	4	Mahmood *et al*., 2005
Coal mining effluent	Cd	66.4	21	Mishra *et al*., 2008b
Coal mining effluent	Fe	70.5	21	Mishra *et al*., 2008b
Contaminated solution (0.5 mg Hg/L)	Hg	98.79	30	Skinner *et al*., 2007
Contaminated solution (0.8 mg NO_3-N/L)	NO_3-N	62.00	1	Petrucio and Esteves, 2000
Contaminated solution (0.6 mg NO_3-N/L)	PO_4-P	68.20	1	Petrucio and Esteves, 2000

3.3.1 The effect of pH on metal uptake by water hyacinth

Metal uptake in plant tissues is a function of several factors (temperature, Eh, pH, cationic competition or antagonism between elements) but the soil or water pH of the medium where plants grow is particularly important to the fate of metals in the root zone (Saygidegeri *et al.*, 1988). The pH level in water or soil determines metal toxicity in plants and usually at lower pH metal uptake is reduced and so is their phytotoxicity (Huang *et al*., 1988). The roots of many wetland plant species have 'iron-plaques' as a thin-root coating layer of iron (oxyhydro-) oxides, which act as a barrier to some metal uptake by roots, and appear to be a characteristic adaptation of plants used to avoid metal phytotoxcity (Batty *et al*., 2000). Taggart *et al*. (2009) indicated that the iron plaques in macrophyte roots are formed through the oxidation of reduced forms of Fe by the oxygen that diffuses into the water from the roots or from other microbial activities around the root vicinity. For instance such iron-plaques around the root zone were found to adsorb and hinder the uptake of some metals such as Fe, Cu, Zn, Ni, and Cd in rice plants (Greipsson and Crowder, 1992; Greipsson, 1994), in common reed, *Phragmites australis* (Cav.) Trin. ex Steudel (Wang and Peverly, 1996) and As in macrophytes, *Typha dominguensis* (cattail) and *Scirpus maritimus* (alkali bulrush) (Taggart *et al*., 2009) into the plant tissues. Impedance of metal uptake by the iron-plaques occurs by adsorption of the metals onto the plaque surfaces.

Nevertheless, the pH of the root's immediate surrounding also determines the effect of the plaque on the uptake of metals. For instance Batty *et al*. (2000) found that the uptake of both Mn and Cu was reduced at a higher pH when plaques are present as opposed to lower pH, where the presence of the plaques did not significantly affect the uptake of the metals.

Metal movement into the plant tissue can also be inhibited by hydrogen ions around the roots at a low pH, since they compete with the metal ions for pathway sites on the root surface. For instance Mn uptake by *Phragmites australis* was lower at a pH of 3.5 than at 6.0, in the presence, as well as in the absence of the plaques (Batty *et al*., 2000). Mercury uptake was higher in tissues of plants growing under alkaline conditions (Adèle, 1991). However, there is not always a clear cut effect of pH on metal uptake by plants. Plant uptake of Aluminum from water by lake plants and in rice paddies and some forests, is inversely proportional to the pH level (Adèle, 1991). Gambrell *et al.* (1977) however, showed an increased Cd uptake in rice, sorghum, *Spartina alternifolia* and *S. cynosuroides* at a lower pH, while Cd uptake in *Distichlis spicata* was maximum at a higher pH (a range of 5 to 8 pH). Similarly O'Keefe *et al.* (1984) found that the Cd concentration in *E. crassipes* was lower at pH 2. Therefore, the pH of the environment where plants grow affects different metal uptake by different plants, differently, even though the general trend for the uptake of metals decreases in more acidic condition (allowing increased metal availability around the root zone) as opposed to more alkaline or increased pH values.

Water hyacinth grows in fresh water and wetlands and it is widely used for phytoremediation. The uptake removal of heavy metals from water by the plants could therefore be affected by the formation of iron plaques around the root zone and the pH of the water.

3.3.2 The effect of cationic competition in heavy metal uptake

The cationic competition between heavy metals and other nutrients for pathways into the root tissues is an important factor that affects the uptake and removal of heavy metals in water. Competition for sites of uptake is often associated with similarities in chemical properties such as ionic size, and also the microscopic size of the aperture in the root surface through which these elements pass during the process of the uptake (Dun, 2007). Based on the plant's requirement for nutrients, the movement of these elements through the channel in the root surface could either be actively pulled in (by osmosis) or excluded if they are in excess or potentially toxic (Dun, 2007). Some elements can pass freely in and out through the apertures while other can get stuck in the aperture and block the passage of other elements. The uptake of As is negatively related to phosphates in water and as a result its removal from water is inhibited in the presence of phosphates since As uses the same channel of uptake as the phosphates (Wang *et al*., 2002; Rahman and Hasegawa, 2011). In contrast As has a strong affinity with iron although such attraction can still reduce the uptake of As through its adsorption on the iron plaques formed on the surface of the roots (Rahman *et al*., 2008).

Several studies on macrophytes have shown the interaction of heavy metals and their competition for the site of uptake by plants. The uptake of cadmium was inhibited by the presence of Cu, Hg and Pb in a solution with water hyacinth (Wolverton and McDonald, 1978; Tatsuyama *et al.*, 1977). Similarly U was found to enhance the uptake of Ca while inhibiting the uptake of magnesium by the roots of *Azolla filiculoides* exposed to a mixture of 10 ppm of $CuSO_4$, $Cd(NO_3)_2$, or $UO_2(NO_3)_2$ solution (Sela *et al*., 1988). Uranyl ions were also found to compete for binding sites for the uptake of both Ca and Mg by the lichen, *Cladonia rangiferina* (Boileau *et al*., 1985).

3.4 Water pollution in the Koekemoerspruit and the Schoonspruit

The Vaal River Operations is a gold and uranium mining project of AngloGold Ashanti Ltd in the Orkney region (Schatz, 2009). The operation comprises a number of shafts (mines), and neighboring gold mines owned or operated by Harmony and Simmer and Jack. In addition to the current gold mines, historic mining in the region commenced in the late 1800's and the failure of old tailings dams in the early 1900's resulted in large spillages into the Schoonspruit (Isabel Weiersbye 2010, personal comm.). The sediments of the Schoonspruit stream and Vaal River near Orkney are polluted by saline and acid drainage, containing sulphates and some metal contaminants (such as Hg, U, Zn, Mn, and Fe, among others) that have drained from the Black Reef (a surface ore-body), and the historical and current gold mining activities (Isabel Weiersbye 2010, personal comm.). Modern gold mines in South Africa do not use Hg in gold recovery, but it was widely used historically in the whole region, and is still used for the illegal recovery or artisanal mining of gold (Cukrowska *et al*., 2010). The extensive infestation of the river by water hyacinth, despite its economical, social and environmental impacts, helps the phytoremediation of such contaminants by removing them from the water as opposed to killing them with herbicides, since the plants will release most of the contaminants back into the water.

3.5 The fate of water hyacinth removed from water after phytoremediation

Research on aquatic weeds as a tool of phytoremediation started almost three decades ago (Kay *et al*., 1984; Fayed and Abd-EI-Shafy, 1985; Sela *et al*., 1988), and has increased recently (Misbahuddin and Fariduddin, 2002; Kara *et al*., 2003; Liao and Chang, 2004; Ahluwalia and Goyal, 2007; Mishra *et al*., 2008a; Ismail and Beddri, 2009; Hussain *et al*., 2010; Rahman and Hasegawa, 2011; Chattopadhyay *et al*., 2012; Hamilton, 2014). However, despite the fact that most aquatic weeds including water hyacinth, have proved to be effective in removing heavy metals and polishing contaminated waters both in lab and field studies, their practical use in large scale programmes of phytoremediation is limited (Rahman and Hasegawa, 2011). One such example is at Kings Bay in Georgia near St. Mary's, Cadman County (USA), where the conservationist brought back water hyacinth, after being rid of the weed for decades, in order to control the algal population boom and promote denitrification (Hamilton, 2014). One reason for the limited use of the aquatic weeds as phytoremediation tools could be the fact that most of them are invasive and are a threat to the water ecosystem. In addition, the fate of water contaminants locked in the phytoremediating plants is often not addressed. Thus, the safe disposal of such plants remains unresolved.

The use of some aquatic macrophytes such as water hyacinth as biofuel is well established in the literature (Rahman and Hasegaw, 2011; Isarankura-Na-Ayudhya *et al*., 2007; Awasthi *et al*., 2013; Bergier *et al*., 2012; Bhattacharya and Kumar, 2010; Gunnarsson and Petersen, 2007). However, tests for heavy metals in the by-product sludge from biofuel processes (hydrolysis and fermentation) of water hyacinth plants used for phytoremediation require further research. Other disposal methods include carbonization to make charcoal, incineration, and briquetting (Rahman and Hasegawa, 2011). Although, these methods could work for plants contaminated with organic pollutants, their environmental safety still remains a problem for water hyacinth plants containing heavy metals (Rahman and Hasegawa, 2011).

This chapter investigates the efficiency of water hyacinth in removal of eight different metals, each presented to the plants as single water contaminant or acid mine drainage (a mixture of a suit of heavy metals with sulphates). It also investigates the removal capacity of the plant with the increase of the pollutants in water, and the amount of metal removed by root or shoot absorption and adsorption. After the experiment, plants will be safely disposed to the tailing dams (Wanenge, 2012), where originally the heavy metal contaminants are thought to have escaped from and where they will be treated with mining wastes before disposed of again. Harvesting and removal of the contaminated plants are done manually or mechanically. Although the cost of such practice could be expensive, it could be a viable option in a small scale water bodies.

3.6 Materials and Methods

This experiment was conducted in both tubs and pools at the University of the Witwatersrand and in four floating rafts above and below inlets of Schoonspruit and Koekemoerspruit on the Vaal River (refer to sections 2.2.1 to 2.2.3).

3.6.1 Measurement of water pH and electrical conductivity (EC)

Water quality in the single-element system tub trials was monitored using pH (Hanna Instrument Inc, Woonsocket, USA) and electrical conductivity (Hanna Instruments, Italy) measurements at the start of the experiment (Day 1) immediately after the addition of the metal treatments and at the end of the metal uptake phase after three weeks exposure to metals. These sampling occasions were chosen in order to allow comparisons between the pH and EC results and the analytic results of water samples. In the multi-component system pool trials (simulated acid mine drainage trails in pools) measurements of pH and EC were taken one day before the start of the experiment (before the addition of metal solutions to the pools), on the second day after the addition of the metals and finally at the end of the metal uptake phase three weeks after metal addition. Water quality measurements before the start of the experiment were required to determine the water quality before the addition of the metals since water hyacinth had been growing in the pools with technical fertilizers for several weeks. In the field (Vaal River) however, water pH and EC were taken outside the floating cages from four compass directions just adjacent to the cage in the first day after setting the cages above and below the inlets of the Koekemoerspruit and the Schoonspruit with water hyacinth (before the start of the seasonal rains) and after the start of the rain in week 5. The EC after the start of the rain was however, taken in week 7 due to technical problems with equipment.

3.6.2 Sample preparation for water analysis

Water samples were taken at the start of the tub experiments immediately after adding the metal treatments and after three weeks (at the end of the metal uptake phase of the trial). In addition, a sample of water hyacinth plant was also taken before the plants were transferred into the tubs to determine the plants' Fe concentration prior to the start of the experiment. For the pool trials, water samples were collected just before adding and just after adding (the same day) metal treatments at the start of the experiment, and then again after three weeks at the end of the metal uptake phase of the trial. Taking water samples before the addition of the treatments was to provide a baseline data of metal concentrations in water. Water samples in the field (at the Vaal River) were collected at the start and at the end of the experiment (before and after the start of rainfall at the Vaal River), placed in a cool box with ice to transport them to the lab where they were stored in a refrigerator at 4°C. All water samples were collected in 250 ml plastic jars, and were preserved with 1% acetic or hydrochloric acid and stored in a refrigerator in the lab at a temperature of 4°C. Before the start of water analysis all samples were filtered using filter paper (100% cotton fiber, 0.19 mm thickness and with filteration speed of 29 sec/100ml) and finally sent to the chemistry laboratory at the University of the Witwatersrand for metal analysis using the Coupled Plasma-Optical Emission Spectroscopy (ICP-OES), to measure heavy metal content and Flow Injection Atomic Spectrometry (FIAS) to measure Hg concentrations.

3.6.3 Sample preparation for plant tissue analysis

Plant samples were collected from the tub, pool and field trials. Plant samples were collected at the end of the metal uptake phase (three weeks after adding treatments) from each replicate in the tub experiment. The plant samples from the pool trials were collected at the start of the experiment (before adding treatments to the pools) and at the end of the metal uptake phase (after three weeks). The same population of plants had been used in a pilot trial in the previous year and therefore collection of plant samples at the start of the experiment allowed the existing level of contamination in the plants to be assessed before the start of the trial. In the field, plant samples were collected at the start of the experiment from the lower bridge of the Kennan Township on the Schoonspruit. This was the source of all the plants transported to the floating rafts above and below inlets of the Schoonspruit and the Koekemoerspruit on the Vaal River. Plant samples were also taken at the end of the field experiment after five weeks.

The sample plants from each tub were stripped of their leaves (the petiole and lamina) with the exception of the last three leaves at the center of the plant. These three leaves on the plant were split into roots and shoots, and then each of these was bisected with a plastic knife into two halves (resulting in two root samples and two shoots samples). The first half of each root and shoot component was washed three times in deionised water only, while the remaining two samples were first washed in deionised water followed by two washes of acetic acid (pH 3.5) and finally rinsed in deionised water. The four samples prepared from each plant were sealed in individual plastic bags, labelled and stored in a freezer (-20°C) until transferred to a freeze drier. After two weeks in the freeze drier, each sample was ground and placed in a 40 ml plastic jar, sealed and sent for analysis to the chemistry department laboratory, at the University of the Witwatersrand University. The ICP-OES analytical method was used for the analysis of the heavy metals and other elements in the samples, while FIAS was used to analyse Hg only.

3.6.4 Bioconcentration factor (BCF)

The BCF in this study (both in the tub and pool trials) was calculated as the metal concentration in plant tissues divided by the initial metal concentration in the medium (water). BCF data for the field trial was not calculated because the river flow and fluctuating metal concentrations in the water where the plants were growing were unknown.

3.7 Results

Generally the concentration of metals in the water of the single-element system tub trial and the AMD pool trial decreased significantly by the end of the metal uptake phase in the third week. The greatest percentage removal of metal from the single-element tub trial was in the Hg treatment, followed, in order by Mn-H>Mn-M>Mn-L>Zn>Cu>Au>U>Fe-H>As>Fe-M>Fe-L. In the AMD pool trial the percentage metal removal from water was lower compared to the single-element system tub trial, and Fe concentration in the water showed a progressive decrease with the increase of the AMD concentrations in the pools. Percentage removal of Mn was greater in the low AMD treatment than the other two AMD treatments, as opposed to the percentage removal of Cu. In the field heavy metal concentration in the river water increased after the rain (Table 3.10) and was significantly greater in the cages below the inlets of both the Koekemoerspruit and Schoonspruit, compared to the corresponding upstream cages of the two tributaries.

Throughout this experiment greater than 80% of the heavy metals removed by the plants were accumulated in the roots, and the amount of heavy metals taken up by shoot absorption was significantly lower than that taken up by root absorption.

3.7.1 Single-element system tub trial

3.7.1.1 Water pH and electrical conductivity in tubs

The tub water pH in the first day of the experiment (Day 1) after the addition of the metal treatments to the tubs showed significant differences between treatments ($F_{(12, 26)} = 13.659$, $P < 0.001$). However, the only water pH that was significantly lower from all the other treatments was the U treatment (Fig. 3.1A). At the end of the metal uptake phase (week 3) the water pH in all treatments was similar and there were no significant difference between them ($F_{(12, 26)} = 1.084$, $P < 0.411$) (Fig. 3.1A).

The electrical conductivity (EC) on Day 1, immediately after the addition of the metals was not significantly different between the treatments ($F_{(12, 26)} = 1.0237$, $P < 0.457$) (Fig. 3.1B). However, on week 3 of the experiment EC dropped significantly by about 30% compared to the EC at the start of the experiment ($F_{(12, 26)} = 4.7487$, $P < 0.001$) in all tubs (Appendix 3A). There was a significant difference of EC between the treatments ($F_{(12, 26)} = 4.9953$, $P < 0.001$) (Fig. 3.1B). The Hg treatment was the only treatment that showed significantly greater EC than all the other metal treatments including the control (Fig. 3.1B).

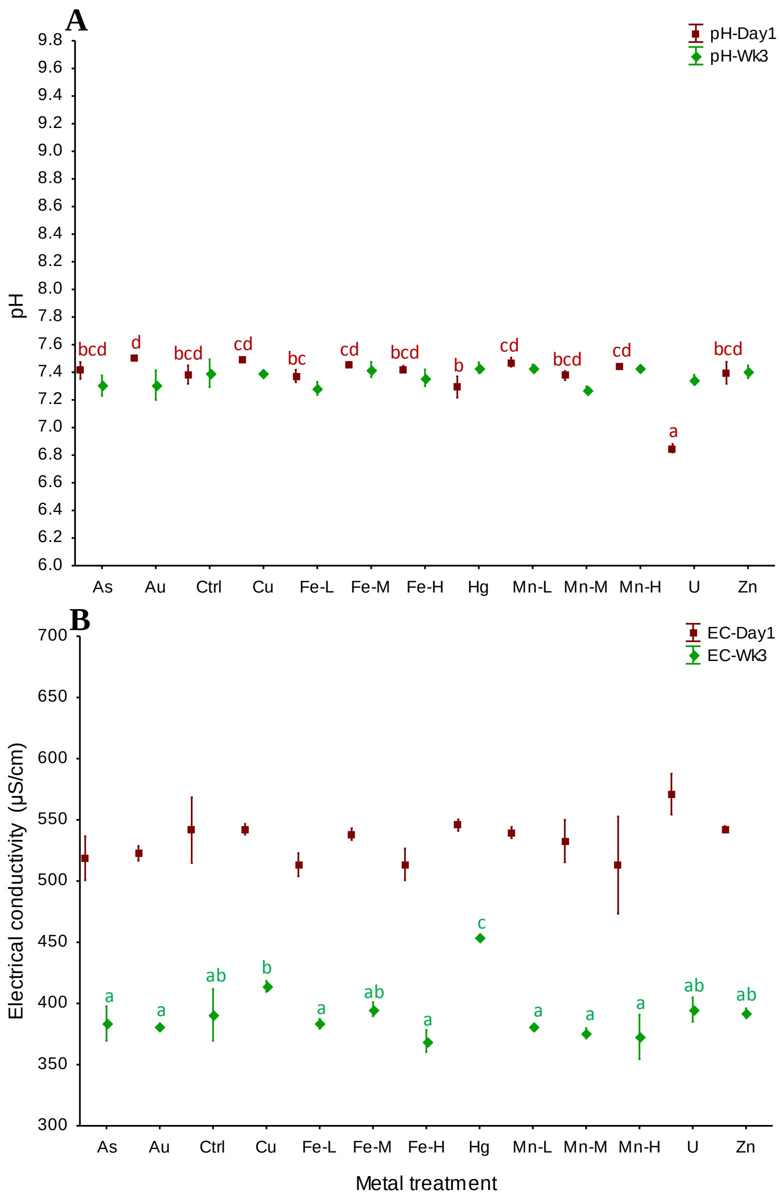


Figure 3.1: Tub water measurements of pH and electrical conductivity: (**A**) pH measurement in Day 1 after the addition of the metal treatments to the tubs and at the end of the metal uptake phase, week 3, (**B**) Electrical conductivity, Day 1 and week 3. Means were compared by One-way ANOVA and those followed by the same letter(s) are not significantly different (P>0.05; Fisher LSD test). NB: n = 3.

3.7.1.2 Concentrations of heavy metals in water and plant tissues in the single-element system tub trial

Analysis of metal concentrations in the tub water samples showed that seven of the total of 12 heavy metal treatments had a significant decrease of over 79% in concentration after three weeks, compared to their initial water concentration at the beginning of the experiment on Day 1. These treatments were Au ($F_{(3,\ 7)}$ = 119.4134, P < 0.001) , Cu ($F_{(1,3)}$ = 126.2531, P < 0.001), Hg ($F_{(3,\ 7)}$ = 164.5977, P < 0.001), Mn-L ($F_{(3,\ 5)}$ = 70.1962, P < 0.001), Mn-M ($F_{(1,\ 3)}$ = 50.5496, P < 0.006), Mn-H ($F_{(1,\ 4)}$ = 68.5902, P < 0.001) and Zn ($F_{(3,\ 7)}$ = 28.9847, P < 0.001) (Table 3.2). The final concentrations of Au, Hg, Zn and Mn-L in the tub water were not significantly different from both the initial and final concentrations of the respective elements in the control treatment (Table 3.2). Most of the heavy metals added to the tubs were dramatically reduced to very low concentration with the exception of the iron dose response treatments (Fe-L, Fe-M and Fe-H) and arsenic treatments. The highest percentage reduction of a metal concentration was shown by Hg (99.90%) followed by Mn-H (98.65%) and Mn-M (94.48) and Mn-L (88%) Zn (83.23%) and Cu (78.72%).

The amount of heavy metal in the shoot and root of the plant samples from each treatment was considered separately. The roots in the metal treatments removed significantly more heavy metals than the shoots (Table 3.3). The same was true for the amount of metals absorbed by the roots compared to those absorbed by the shoots. The absorption of Cu, Fe and Hg by plant roots was between 30 to 50 times greater compared to the absorption by the plant shoots, with Hg showing the greatest difference between the two plant tissues. However the absorption of Mn and Zn by the roots ranged from 3 to 6 times that of the shoot (Table 3.3). The differences between the amounts of heavy metals absorbed and adsorbed by the shoots was not significant with the exception of all the three Mn concentration treatments and Zn. Although there were no metals added to the control treatment other than the Hoagland's solution, of the four elements (Hg, Cu, Mn and Zn) analysed, all showed significantly greater concentration in the roots than in the shoots with the exception of Cu ($F_{(1,\ 4)}$ = 3.3284, P < 0.142). However, these elements did not show any significant differences between their initial and final concentrations in water. Arsenic, Au and U concentrations in the shoots of the metal treatments were below the detectable limit (Table 3.3).

The total amount of metals removed by roots in both the Fe and Mn treatments in the single-element system tub trial was significantly greater compared to that removed by shoots ($F_{(5, 12)} = 3.8431$, $P < 0.026$) and ($F_{(5, 12)} = 4.5577$, $P < 0.014$), respectively), although the total Fe concentration in the plant tissue prior to the start of the experiment was as high as 11856.2 mg/kg d. wt., (before the addition of hoagland’s solution and heavy metals treatments). However, the increase of Fe or Mn concentrations in water did not result in a significant increase in the uptake of Fe or Mn by shoots, nor by roots.

The bioconcentration factor was higher in the iron dose response treatment than all the other heavy metal treatments in the single-element system tub trial. However, the BCF in the iron dose response treatment decreased with increase in Fe concentration in water and the highest BCF was reported in the Fe-L treatment (Table 3.4). In contrast the BCF in the manganese dose response treatment increased with the increase of concentration from Mn-L to Mn-H. In addition to the iron dose response treatments, the BCF in Au, Cu, and Hg treatments was over a 1000. Whereas, U followed by As were at the bottom of the BCF rank (Table 3.4).

Table 3.2: Heavy metal concentrations from water samples in the single-element system tub trial collected immediately after the addition of the metals and three weeks after the addition of metals into the tubs (week 3).

	Metal treatments (mg/L)		Control treatments (mg/L)		% removal of metal by plants
Treatments	**Initial concentration**	**Final concentration**	**Initial concentration**	**Final concentration**	
As	0.294 ± 0.08 a	0.259 ± 0.10 a	nd	nd	11.90
Au	0.047 ± 0.00 b	0.010 ± 0.00 a	0.007 ± 0.00 a	0.008 ± 0.00 a	78.72
Cu	1.61 ± 0.07 b	0.27 ± 0.10 a	_	_	83.23
Fe-L	1.337 ± 0.33 a	2.873 ± 0.72 a	_	_	-114.88
Fe-M	2.787 ± 0.36 a	3.065 ± 0.68 a	_	_	-9.97
Fe-H	3.957 ± 0.04 a	3.31 ± 0.63 a	_	_	16.35
Hg	1.052 ± 0.08 b	0.001 ± 0.00 a	0.0001± 0.00 a	0.0001± 0.00 a	99.90
Mn-L	0.5 ± 0.32 b	0.06 ± 0.00 a	0.024 ± 0.01 a	0.111 ± 0.06a	88.00
Mn-M	1.903 ± 1.90 b	0.105 ± 0.03 a	_	_	94.48
Mn-H	3.7 ± 0.44 b	0.05 ± 0.00 a	_	_	98.65
U	2 ± 0.00 b	0.765 ± 0.10 a	nd	nd	61.75
Zinc	3.387 ± 0.47 b	0.517 ± 0.30 a	0.056 ± 0.03 a	0.026 ± 0.01 a	84.74

Means were compared by One-way ANOVA and means of the same element in a row followed by the same letter(s) are not significantly different (*P* > 0.05; Fisher LSD test). NB: the suffixes L, M and H in the first column stand for Low, Medium and High sulphate concentration treatments respectively. Comparison is between initial and final concentration of the same heavy metal treatment across the row (including the control). NB: the suffix after "±" denotes the standard Error (SE). "%" removal is for the metal treatments only (does not include the control treatment); "-"not tested; "nd" below detectable limit.

Table 3.3: The total concentration of heavy metals (the amount of metals absorbed + adsorbed) by the shoots and roots of water hyacinth grown in single-element system tub trial, and the amount of heavy metals absorbed by the plant parts, three weeks after the addition of the metal treatments (end of the metal uptake phase).

	Metal treatments (mg/kg)				Control treatment (mg/kg)			
Elements	**Metal absorbed by shoots**	**Metal absorbed by roots**	**Total metal uptake by shoots**	**Total metal uptake by roots**	**Metal absorbed by shoots**	**Metal absorbed by roots**	**Total metal uptake by shoots**	**Total metal Uptake by roots**
As	nd	55.1 ± 17.2	nd	80.8 ± 40.2	nd	nd	nd	nd
Au	nd	52.3 ± 22.2	nd	48.9 ± 11.7	nd	nd	nd	nd
Cu	44.9 ± 3.8 a	1360.6 ± 166.6 b	38.1 ± 3.5 a	2837.6 ± 382.5 b	10.1 ± 1.4 a	10.7 ± 1.9 a	9.5 ± 1.4 a	13.8 ± 1.9 a
Fe-L	163.1 ± 50.7 a	6281.7 ± 2249.7 b	139.5 ± 18.8 a	9213.6 ± 4148.0 b	147.2 ± 10.5 a	8442.2 ± 907.1 b	172.9 ± 31.1 a	13691.8 ± 1618.9 b
Fe-M	169.8 ± 20.2 a	7925.2 ± 1034.5 b	151.9 ± 17.8 a	6670.0 ± 3220.5 b	_	_	_	_
Fe-H	199.8 ± 35.2 a	6936.3 ± 1165.6 b	158.1 ± 11.6 a	8414.5 ± 1754.3 b	_	_	_	_
Hg	35.9 ± 6.2 a	1762.3 ± 63.9 b	28.4 ± 2.3 a	1634.2 ± 318.6 b	_	_	_	_
Mn-L	27.7 ± 3.3 a	155.3 ± 21.7 b	29.9 ± 3.5 a	290.9 ± 14.9 b	33.0 ± 10.84 a	74.3 ± 9.4 b	34.1 ± 11.7 a	154.0 ± 31.4 b
Mn-M	258.6 ± 117.1 a	706.68 ± 191.1 b	268.1 ± 116.2 a	1114.5 ± 157.2 b	_	_	_	_
Mn-H	520.1 ± 342.4 a	1837.9 ± 715.4 b	590.5 ± 399.2 a	2900.5 ± 1137.3 b	_	_	_	_
U	nd	927.0 ±131	nd	1339.9 ± 174.6	nd	nd	nd	nd
Zn	373.1 ± 8.7 a	2093.9 ± 205.3 b	401.7 ± 45.2 a	3543.5 ± 696.4 b	66.3 ± 7.0 a	115.7 ± 15.6 b	63.2 ± 7.6 a	231.4 ± 22.1 b

Means were compared using t-test and means of the same element in a row followed by the same letter(s) are not significantly different ($P > 0.05$).
NB: Comparison is in pairs across the rows between the shoot and root of each treatment (the metal or the control treatment); "-" not tested; "nd" below detectable limit. The suffix after "±" denotes the standard Error (SE).

Table 3.4: Bioconcentration factor (BCF) of water hyacinth grown in a single-element system tub trial at the end of the metal uptake phase, three weeks after the addition of metal treatments (week 3).

Treatment	Initial water conc. (mg/L)	Final heavy metal concentration		BCF
		Whole plant (mg/kg)	Root system (%)	
As	0.29	80.78	_	275.074
Au	0.05	48.86	_	1032.25
Cu	1.61	2875.69	98.67	1786.14
Fe-L	1.34	9352.96	98.51	6997.23
Fe-M	2.79	6821.93	97.77	2448.06
Fe-H	3.96	8572.58	98.16	2166.62
Hg	1.05	1662.63	98.29	1579.70
Mn-L	0.50	320.91	90.67	641.82
Mn-M	1.90	1382.6	80.61	726.41
Mn-H	3.70	3490.95	83.09	943.50
U	2.00	1339.87	_	669.93
Zn	3.39	3945.21	89.82	1164.92

3.7.2 Simulated AMD pool trial

3.7.2.1 Water pH and electrrical conductivity in AMD pool trial

All the three pH measurements showed significant differences between the different AMD treatments (low, medium and high sulphate concentrations) (($F_{(2, 15)}$ = 25.3041, $P < 0.001$, ($F_{(2, 15)}$ = 5.4959, $P < 0.01$) and ($F_{(2, 15)}$ = 17.9252, $P <$ 0.001, respectively)) (Fig. 3.2A). The high AMD concentration treatment before the addition of the metals and sulphates (AMD) (Day-1) showed significantly lower water pH than the other two AMD treatments which were not significantly different from each other. After the the addition of AMD treatment (Day 1), the medium and high AMD treatments did not show significant differences between them, but they were both significantly greater than the low AMD treatment. A similar trend was found in the third week (end of the metal uptake phase), where the low AMD treatments showed significantly lower pH than the other two AMD treatments (Fig. 3.2A). The pH decreased by 7.6% and 1.4% from one day before the addition of the AMD treatments to the end of the metal uptake phase in week 3 in the low and medium AMD treatment respectively, while it increased by 30% in the high AMD treatments.

The EC of all the three measurements, before (Day-1) and after (Day 1) the addition of AMD treatments and at the of the metal uptake phase in week 3 also showed significant differences between the AMD treatments (($F_{(2, 15)}$ = 3.3098E5, $P < 0.001$), ($F_{(2, 15)}$ = 165.4186, $P < 0.001$), ($F_{(2, 15)}$ = 284.1163, $P < 0.001$), respectively)) (Fig. 3.2B). The EC of the high AMD treatment on Day- 1, before the addition of the AMD to pools, was significantly greater in the high AMD treatment than the other two treatments, and the medium AMD treatment was significantly the lowest of all. The EC on Day 1, after the start of the experiment, showed that the low AMD treatment was significantly the lowest and the high AMD treatment showed the greatest EC of all the treatments (Fig. 3.2B). The EC trend between the three AMD treatments at the end of the experiment (week 3) did not change compared to those on Day 1, but with slight increases of the EC in the third week. The electrical conductivity generally increased from Day-1 to the end of the metal uptake phase in week 3 with the increase of AMD treatments.

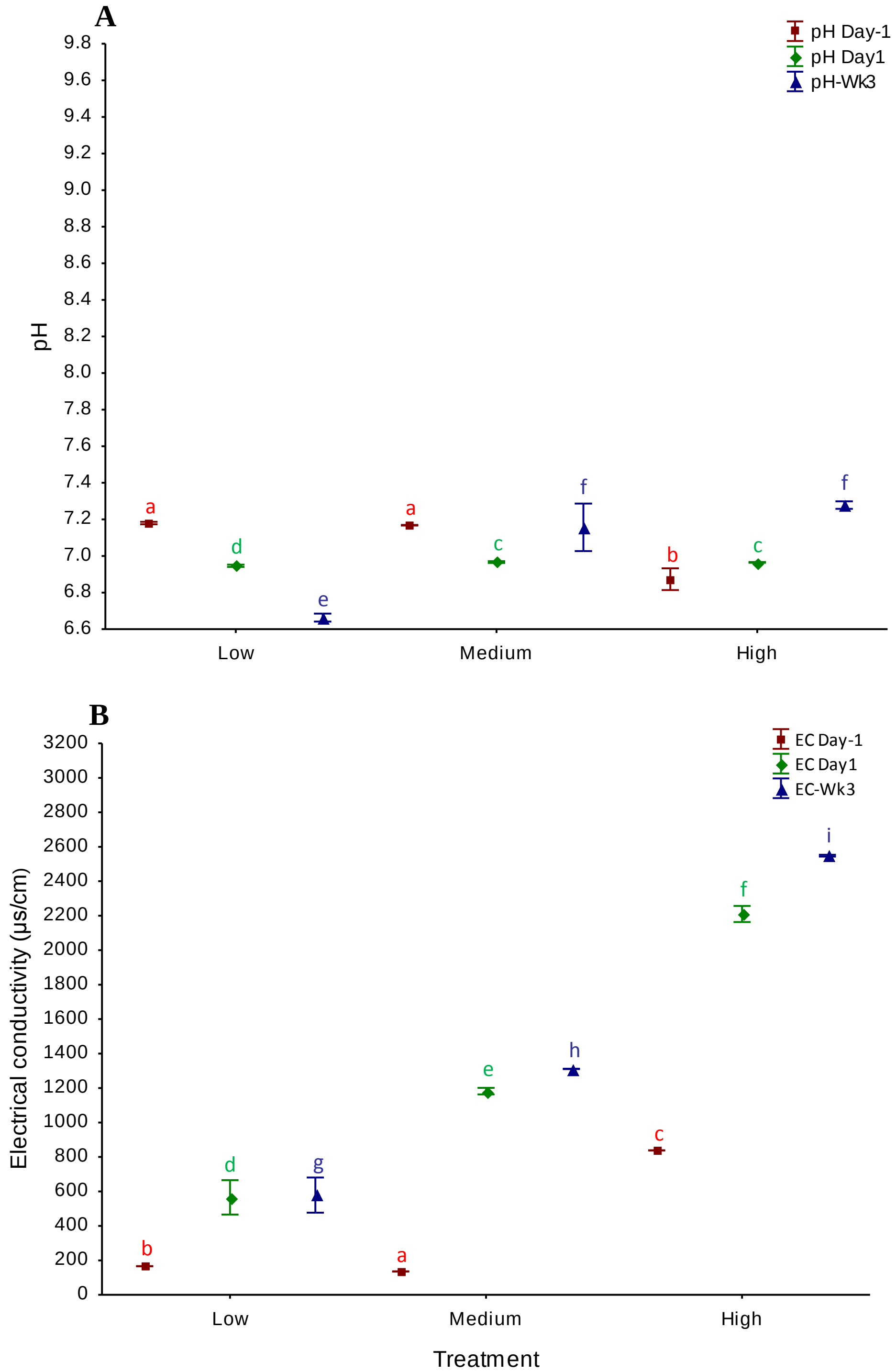


Figure 3.2: Water pH and electrical conductivity measurements in the simulated AMD pool trial: (**A**) pH on Day-1, before the addition of metal and sulphates (Day minus 1), on Day 1, after the addition of metal and sulphates, and three weeks after the addition of metal and sulphates (week 3), (**B**) Electrical conductivity on Day-1, Day 1, and week 3. Means were compared by One-way ANOVA and those followed by the same letter(s) are not significantly different (P>0.05; Fisher LSD test). Low, Medium and H indicates stands for sulphate concentrations of 300, 700 and 1300 mg/L, respectively.

3.7.2.2 Concentrations of heavy metals in water and plant tissues in the AMD pool trial

The analysis of water samples collected on Day-1 showed that the heavy metal concentrations of each of the Cu, Fe, Mn and Zn in the three AMD treatments were similar, with the exception of Fe in the medium AMD treatment, which was significantly lower than the other two AMD treatments ($F_{(8, 9)} = 12.2152$, $P < 0.001$) (Appendix 3B). Nevertheless, results from the initial water samples collected immediately after the addition of heavy metal treatments showed significant differences in the concentration of those metals, between the three sulphate dose response treatments. The four metals showed a significant subsequent reduction in concentration, within the same AMD treatment, by week 3 (($F_{(8, 9)} = 11.3025$, $P < 0.001$), ($F_{(8, 9)} = 12.2152$, $P < 0.001$), ($F_{(8, 9)} = 6.8848$, $P < 0.004$), ($F_{(8, 9)} = 49.2387$, $P < 0.001$), respectively) (Table 3.5). The final concentration of each heavy metal was reduced to a level which was not significantly different between the three AMD treatments, with the exception of Zn in the medium AMD dose response treatment, which was significantly lower than the low and high AMD treatments (Table 3.5). The percentage reduction of Fe in the water declined from 40% in the low AMD, to 32% in the medium, to 29% in the high AMD treatment (Table 3.5). Copper and Mn removal from water in the medium AMD treatment dropped by 4-10% and 16-18% compared to the low and high AMD treatments, respectively. Zinc removal from water was 50% lower than all the other heavy metals in all three AMD concentration treatments.

Plant shoots and roots from the three different AMD treatments were analyzed for metal content. Initially Cu concentration in the shoots and roots of the high AMD treatment was significantly lower than in the other two AMD treatments ($F_{(5, 6)} = 13.1486$, $P < 0.003$), (Appendix 3C). The concentrations of Fe, Mn and Zn in the roots were significantly less at the high AMD treatment compared to the low treatment (Appendix 3C). There was a significant difference in the uptake of Cu, Fe, Mn, S, Zn and Mg by plants between AMD treatments at the end of the metal uptake phase, in week 3 ($F_{(5, 6)} = 678.3707$, $P < 0.001$), ($F_{(5, 6)} = 53.3907$, $P < 0.001$), ($F_{(5, 6)} = 5.0019$, $P < 0.037$), ($F_{(5, 6)} = 84.9371$, $P < 0.001$), ($F_{(5, 6)} = 85.353$, $P < 0.001$), and ($F_{(5, 6)} = 19.2342$, $P < 0.001$), respectively) (Table 3.6). Despite there being a significant difference in the initial concentration of Cu in the shoots between the AMD treatments (Appendix 3C), the final concentration of all elements in the water hyacinth shoots in week 3 showed no significant differences between the AMD treatments (Table 3.6). Similarly, the concentrations of Mg and Mn in the roots showed no significant differences between the treatments. This was however, different for S and Zn in the roots, which progressively declined with the increase of AMD from the low to high treatments (Table 3.6). The Cu concentration in roots showed a significant increase and decrease in the medium and the high AMD treatments, respectively.

The absorption of Cu, Fe, Mn, S, Zn and Mg by either the roots or the shoots of water hyacinth indicated that the two plant parts were significantly different ($F_{(5, 6)} = 795.6036$, $P < 0.001$), ($F_{(5, 6)} = 128.8257$, $P < 0.001$), ($F_{(5, 6)} = 24.3523$, $P < 0.001$), ($F_{(5, 6)} = 3.3619$, p = $P < 0.001$), ($F_{(5, 6)} = 204.8487$, $P < 0.001$), and ($F_{(5, 6)} = 152.8471$, $P < 0.001$) for each element, respectively) (Table 3.7). The absortion of all the heavy metals was significantly greater in the roots than in the shoots. However, there was no significant difference between the two in the absorption of S in the high AMD treatment.

About 65 to 99% of the heavy metals removed were accumulated in the roots with the exception of Mg (Table 3.6). The highest percentage of Zn (88%) taken up by the roots compared to the shoots was in the low sulphate treatment as opposed to Mn where the highest level was in the roots (89%) of the high AMD treatment compared to the shoots (Table 3.8). The highest percentage of shoot uptake of Cu, Zn and Mg were in the high AMD treatment (Table 3.8). Magnesium was the only element that was significantly higher in shoots than in the roots of all the three AMD treatments (Table 3.8).

Table 3.5: Metal concentrations in water in the simulated AMD pool trial, collected just after the addition of metals (Day 1) and sulphates to pools and three weeks after the addition of the same treatments (Week 3).

Treatment	Low sulphate concentration			Medium sulphate concentration			High sulphate concentration		
	Initial (mg/L)	Final (mg/L)	% removal	Initial (mg/L)	Final (mg/L)	% removal	Initial (mg/L)	Final (mg/L)	% removal
Cu	2.16 ± 0.0 b	0.69 ± 0.1 a	68.1	2.16 ± 0.2 b	0.751 ± 0.0 a	65.2	3.63 ± 0.6 c	0.99 ± 0.0 a	72.7
Fe	9.72 ± 0.4 c	5.80 ± 0.9 ab	40.3	6.29 ± 0.3 b	4.260 ± 1.0 a	32.3	7.21 ± 3.2 c	5.08 ± 0.2 ab	29.5
Mn	1.05 ± 0.1 c	0.08 ± 0.0 a	92.4	0.99 ± 0.1 bc	0.243 ± 0.1 ab	75.4	1.89 ± 0.5 d	0.19 ± 0.01 a	89.9
Zn	4.01 ± 0.05 e	2.78 ± 0.3 b	30.7	3.38 ± 0.1 c	2.025 ± 0.0 d	40.1	4.57 ± 0.2 f	2.86 ± 0.0 bc	37.4

Means were compared by One-way ANOVA and means of the same element in a row followed by the same letter(s) are not significantly different (*P* > 0.05; Fisher LSD test). NB: Comparison is across the table in rows for each heavy metal in the three sulphate concentration treatments. The suffix after "±" denotes the standard Error (SE).

Table 3.6: Total concentration of metals (Deionized water washed samples representing the amount of metal absorbed into the tissue plus those adsorbed on the surface of the plant tissue) in the shoots and roots of water hyacinth grown in different simulated AMD treatments (heavy metals plus variable concentration of sulphates) at the end of the metal uptake phase, three weeks after the addition of the AMD treatments (week 3).

Week3	Low sulphate treatment (mg/kg)		Medium sulphate treatment (mg/kg)		High sulphate treatment (mg/kg)	
Treatment	Total metal uptake by shoots	Total metal uptake by roots	Total metal uptake by shoots	Total metal uptake by roots	Total metal uptake by shoots	Total metal uptake by roots
Cu	21.7 ± 0.6 a	100.8 ± 2.7 b	19.6 ± 1.5 a	188.5 ± 5.6 d	25 ± 0.2 a	111.7 ± 0.2 c
Fe	105.8 ± 2.6 a	6453.2 ± 372.7 b	112.4 ± 23.7 a	9476.2 ± 826.2 c	100.1 ± 3.6 a	7819.5 ± 1152.5 bc
Mn	192.1 ± 4.7 a	942.9 ± 252.7 ab	242.1 ± 35.9 a	879.2 ± 123.3 ab	194.1 ± 7.7 a	1694 ± 593.2 b
S	613.8 ± 144.6 a	2408.6 ± 85.7 e	195.2 ± 53.6 b	1849.3 ± 57.6 d	823.8 ± 40.7 a	1318.9 ± 108.8 c
Zn	73.1 ± 17.1 ab	622 ± 27 d	69.5 ± 4.6 ab	465.9 ± 55 c	53.7 ± 6.1 a	152.7 ± 7.9 b
Mg	13540.2 ± 2255.4 b	7671.1 683.4 a	14904.6 ± 341 b	6459.1 ± 96.4 a	15740.7 ± 784.9 b	5763.5 ± 332.5 a

Means were compared by One-way ANOVA and means of the same element in a row followed by the same letter(s) are not significantly different (*P* > 0.05; Fisher LSD test). NB: Comparison is across the table in rows for each heavy metal in the three sulphate concentration treatments. The suffix after "±" denotes the standard Error (SE).

Table 3.7: The amount of metals absorbed (acid washed samples representing the amount of metals absorbed into the tissue) by the shoots and roots of water hyacinth grown in different simulated AMD treatments (heavy metals plus variable concentration of sulphates) at the end of the metal uptake phase, three weeks after the addition of the AMD treatments (week 3).

	Low sulphate treatment (mg/kg)		Medium sulphate treatment (mg/kg)		High sulphate treatment (mg/kg)	
Treatment	**Metal absorbed by shoots**	**Metal absorbed by roots**	**Metal absorbed by shoots**	**Metal absorbed by roots**	**Metal absorbed by shoots**	**Metal absorbed by roots**
Cu	18.8 ± 3.5 a	74.3 ± 1.7 b	20 ± 0.5 a	137.8 ± 0.3 d	22.6 ± 1.3 a	82.4 ± 0.5 c
Fe	118 ± 15.5 a	3673.4 ± 576.0 b	103.6 ± 19.5 a	6207.7 ± 121.6 d	108.1 ± 7 a	4875.7 ± 76.3 c
Mn	180.1 ± 6.9 a	608.7 ± 137.0 b	226.6 ± 37.8 a	498.4 ± 47.6 b	179.3 ± 0 a	939.7 ± 17.2 c
S	773.4 ± 114.5 ab	1557.5 ± 129.8 c	682.3 ± 19.7 a	1440.4 ± 101.7 bc	723.8 ± 79 ab	909 ± 466.2 abc
Zn	87.7 ± 5.9 a	389.1 ± 2.2 d	68.1 ± 1.9 ab	281.1 ± 22.5 c	51.1 ± 0.8 b	98.3 ± 4.1 a
Mg	13307.5 ± 343.7 c	4925.2 ± 202.5 b	14567.7 ± 836.6 cd	4297 ± 251.1 ab	15382.7 ± 303.3 d	3054.1 ± 522.7 a

Means were compared by One-way ANOVA and means of the same element in a row followed by the same letter(s) are not significantly different (P > 0.05; Fisher LSD test). NB: Comparison is across the table in rows for each heavy metal in the three sulphate concentration treatments. The suffix after "±" denotes the standard Error (SE).

Table 3.8: Percentage removal of heavy metals by roots of water hyacinth grown in heavy metal and sulphate treatments in the AMD pool trial, three weeks after the addition of the simulated AMD treatments (week 3).

	Sulphate concentration					
	Low		Medium		High	
Metal	Whole plant (mg/kg)	Root (%)	Whole plant (mg/kg)	Root (%)	Whole plant (mg/kg)	Root (%)
Cu	122.5	82.3	208.1	90.6	136.7	81.7
Fe	6559.0	98.4	9588.6	98.8	7919.6	98.7
Mn	1135.0	83.1	1121.3	78.4	1888.1	89.7
S	3022.4	79.7	2044.5	90.4	2142.7	61.5
Zn	695.1	89.5	535.4	87	206.4	74.0
Mg	21211.3	36.2	21363.7	30.2	21504.2	26.8

The BCF calculated from the whole plant in the AMD pool trial was generally lower compared to the single-element system tub trial. The BCF indices were higher in the medium compared to the low and high AMD treatments, with the exception of Zn which progressively decreased with the increase of the AMD from low to high (Table 3.11). In this trial Fe and Mn were the only two metals with BCF index of greater than a 1000 (Table 3.9).

Table 3.9: Bioconcentration factor (BCF) of water hyacinth grown in a simulated AMD pool trial, three weeks after the addition of the AMD treatments (metals and sulphates) (week 3).

	Bioconcentration factor in sulphate treatments (BCF)		
Metal	Low	Medium	High
Cu	56.71	96.34	37.66
Fe	674.79	1524.42	1098.42
Mn	1080.95	1132.63	1000
Zn	173.34	158.4	45.16

3.7.3 Acid mine drainage trial in the field

3.7.3.1 Water pH and electrical conductivity in the Vaal River

The water pH in the Vaal River before the start (Day 1) and after the start of the seasonal rain (Wk 5) showed significant differences between the sampling occasions (sample dates) at both the Koekemoerspruit and Schoonspruit inlets into the Vaal River (($F_{(3,\ 8)}$ = 4.4628, P < 0.04), ($F_{(3,\ 8)}$ = 188.2143, P < 0.001), respectively) (Fig. 3.3A). Before the start of the rain, the Koekemoerspruit upstream pH was significantly lower by 8% than all the other sites. After rain in week 5 the pH dropped significantly at all sites ($F_{(3,\ 8)}$ = 9.5413, P < 0.005) (Appendix 3D). After the rain, all the sites were significantly different from each other and the sites below the inlets of both the Koekemoerspruit and the Schoonspruit were significantly lower from their respective upstream sites by 7 and 8%, respectively. The pH of the Schoonspruit upstream site was the highest of all the sites, while the pH of the Koekemoerspruit down stream was the lowest of all the sites (Fig. 3.3A).

The EC before the start and after the rain also showed significant differences between sample dates at the four sites of the Koekemoerspruit and Schoonspruit (($F_{(3,\ 8)}$ = 324.6177, P < 0.001), ($F_{(3,8)}$ = 7.1646, P < 0.011), respectively) (Fig. 3.3B). The EC before the start of the rain in all the sites at the Koekemoerspruit was significantly lower compared to the sites at the Schoonspruit. Unlike the sites at the Koekemoerspruit, the EC in Schoonspruit down stream was significantly greater than those in the upstream. A similar trend of EC was also shown after the rain where both sites at the Koekemoerspruit and the upstream site at the Schoonspruit were significantly lower than the downstream site at the the Schoonspruit (Fig. 3.3B).

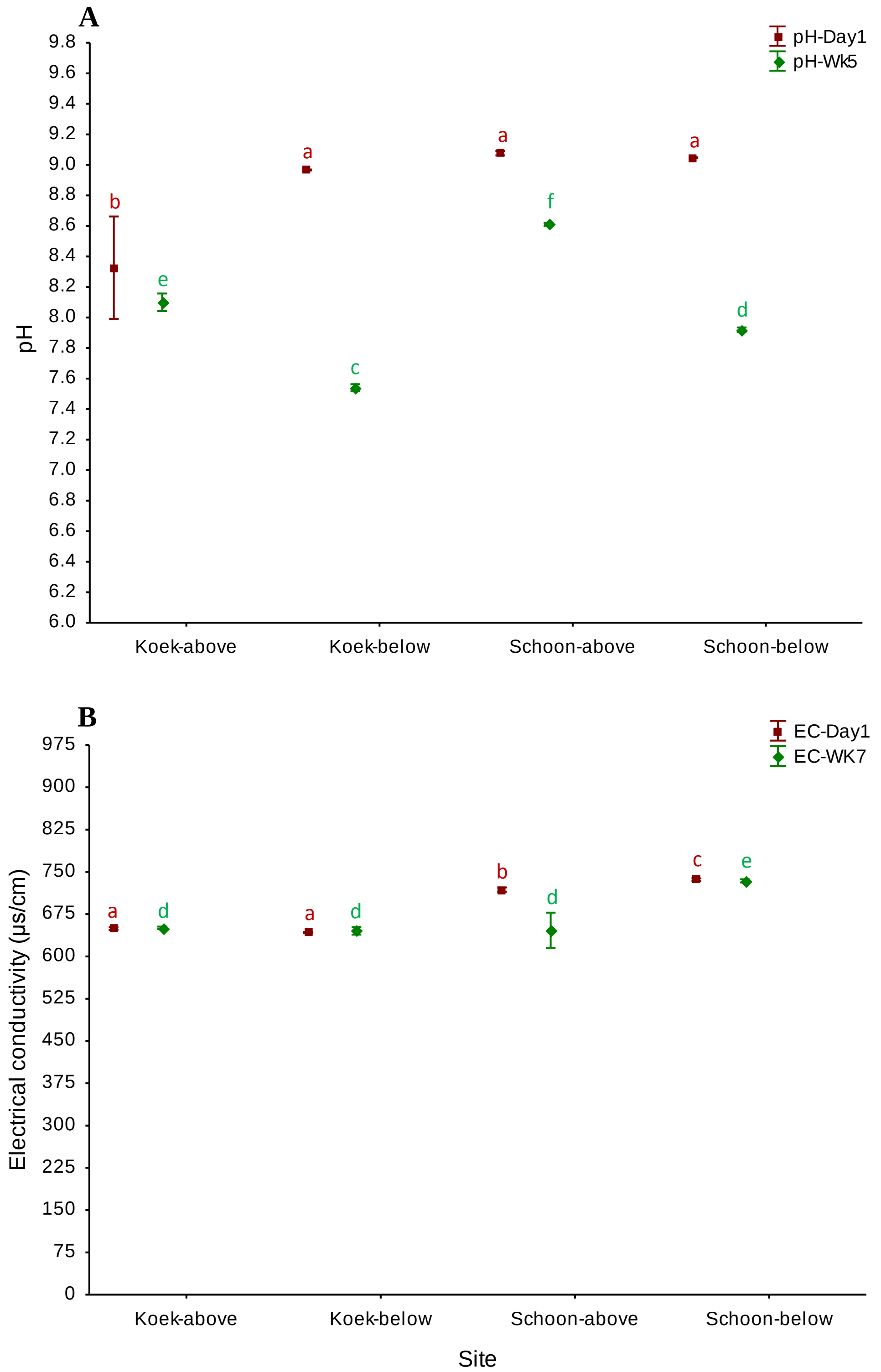


Figure 3.3: Water pH and electrical conductivity in the upstream and downstream sites of the Koekemoerspruit and Schoonspruit inlets on the Vaal River: (A) pH Day 1, before the start of the seasonal rain, and after rain in week 5 (Wk5), and (B) Electrical conductivity on Day 1, and after the rain in week 7. Means were compared by One-way ANOVA and those followed by the same letter(s) are not significantly different ($P>0.05$; Fisher LSD test). NB: Koek-above and below = upstream and downstream sites of the Koekemoerspruit, Schoon-above and below = upstream and downstream sites of the Schoonspruit inlet. n = 3.

3.7.3.2 Metal concentration in water and plant tissues in the Vaal River

Analysis of water samples collected before and after the rainy season in all the four sites at the Koekemoerspruit and Schoonspruit, showed that the As was below the detectable limit for the ICP-OES analytical method. The water concentration of all other metals and nutrients in all the sites however, generally increased after the rain and these concentrations were greater at the site below the inlet of the Schoonspruit compared to all the other sites (Table 3.10). The sulphate concentrations in water were by far the greatest after the rain compared to the other contaminants, with increases ranging from 4 to 66 fold and the site below the Schoonspruit showed the greatest sulphate concentrations in water (729.9 mg/L SO_4^{-2}) of all the other sites (Table 3.10).

Generally there was significantly more Cu, Fe, Hg, K, Mn, P, S, Zn and Mg in the root tissue compared to the shoots of water hyacinth within the same floating cages of both the above and below inlets of the Koekemoerspruit and Schoonspruit tributaries into the Vaal River (($F_{(9, 18)}$ = 12.1285, $P < 0.001$), ($F_{(9, 18)}$ = 26.6256, $P < 0.001$), ($F_{(9, 18)}$ = 3.0743, p = 0.020), ($F_{(9, 18)}$ = 24.5395, $P < 0.001$), ($F_{(9, 18)}$ = 92.0058, $P < 0.001$), (F(9, 18) = 46.3613, $P < 0.001$), ($F_{(9, 18)}$ = 6.7277, $P < 0.001$), ($F_{(9, 18)}$ = 75.081, $P < 0.001$), and ($F_{(9, 18)}$ = 36.4721, $P < 0.001$), respectively) with the exception of K, P and Mg which were greater in the shoots than in the roots (Table 3.11). Iron, Mn and Zn were significantly greater in the roots of plants below the inlet of the Schoonspruit than those in the plants above and below the inlets of the Koekemoerspruit into the Vaal River (Table 3.11). Potassium was also significantly greater in the shoots of the plants below the inlets of the Schoonspruit than those above and below the inlet of the Koekemoerspruit. The water hyacinth roots from the lower bridge of the Schoonspruit near the Township of Kennan (about 5 km before the entry to the Vaal River) showed significantly greater amounts of Hg than the other plant tissues (Table 3.11).

Table 3.10: Metal and sulphate concentration in water samples above and below the inlets of the Koekemoerspruit and Schoonspruit into the Vaal River before (Day 1) and after the rainy season (Week 7).

	Koekemoerspruit (mg/L)				Schoonspruit (mg/L)			
	Above inlet cage		Below inlet cage		Above inlet cage		Below inlet cage	
Elements	**Before**	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**
As	nd	nd	nd	nd	nd	nd	nd	nd
Au	0.016	nd	0.018	0.011	0.016	0.011	0.017	nd
Cu	0.015	0.018	nd	0.016	0.013	nd	nd	0.016
Fe	0.251	0.205	0.213	0.256	0.334	0.329	0.249	0.723
Hg	0.001	0.002	0.001	0.001	0	0.002	0.001	0.002
Mn	0.066	0.103	0.058	0.103	0.116	0.291	0.114	0.549
U	0.004	0.004	0.004	0.004	0.005	0.003	0.005	0.004
Zn	0.285	0.513	0.804	0.805	0.183	0.093	0.16	0.122
P	0.224	0.375	0.215	0.443	0.227	0.662	0.264	0.979
Mg	12.24	17.82	11.61	22.38	14.68	18.5	13.78	25.42
SO_4	6.904	456.6	113.3	440.7	159.9	612.3	147.3	729.9

NB: “nd” not detectable.

Table 3.11: Total concentration of metals (amount of metal absorbed into the tissue plus those adsorbed on the surface of the plant tissue) in shoots and roots of water hyacinth grown in floating cages below and above the Koekemoerspruit and Schoonspruit inlets on the Vaal River, after the start of the seasonal rain (week 7) and in the Schoonspruit near the township of Kennan (5 km from the Vaal River) before the start of the rain (Day 1). All units = mg/kg.

	Kennan		**Koekemoerspruit sites**				**Schoonspruit sites**			
			Above inlet cage		**Below inlet cage**		**Above inlet cage**		**Below inlet cage**	
Elements	Total metal uptake by shoots	Total metal uptake by roots	Total metal uptake by shoots	Total metal uptake by roots	Total metal uptake by shoots	Total metal uptake by roots	Total metal uptake by shoots	Total metal uptake by roots	Total metal uptake by shoots	Total metal uptake by roots
Cu	nd	0.09 ± 0 a	nd	0.1 ± 0 a	0.03 ± 0 a	0.08 ± 0 a	0.05 ± 0 b	0.11 ± 0 b	0.05 ± 0 a	0.13 ± 0 b
Fe	0.67 ± 0.1 a	27.6 ± 2.2 b	1.65 ± 0.1 a	25.49 ± 1.3 b	1.23 ± 0.2 a	12.75 ± 3.3 c	0.42 ± 0 a	17.79 ± 1.6 c	0.47 ± 0 a	28.41 ± 5.3 b
Hg	0.53 ± 0 a	1.26 ± 0.5 b	0.52 ± 0.1 a	0.42 ± 0 a	0.39 ± 0.1 a	0.3 ± 0.1 a	0.4 ± 2 a	0.26 ± 0 a	0.27 ± 0 a	0.33 ± 0 a
K	176.67 ± 0.6 d	100.23 ± 1.8 a	103.53 ± 15 a	44.46 ± 0.5 b	116.8 ± 7.4 a	102.22 ± 6.6 a	279.13 ± 23.2 c	46.62 ± 5.3 b	254.33 ± 37.8 c	76.33 ± 11.9 ab
Mn	1.33 ± 0.1 a	28.33 ± 0.7 d	1.51 ± 0.3 a	9.45 ± 2.2 b	0.9 ± 0 a	3.82 ± 0.3 a	2.27 ± 0.2 a	20.08 ± 3.4 c	2.44 ± 0.1 a	45.37 ± 2.9 e
P	110.48 ± 7.3 e	85.07 ± 2.8 d	54.75 ± 4.8 a	42.96 ± 1.7 ab	46.92 ± 4.4 a	29.32 ± 4.9 b	51.23 ± 2.7 a	26.57 ± 3.0 b	53.13 ±.2.1 a	31.13 ± 0.8 bc
S	2.18 ± 0.8 ab	11.25 ± 0.5 d	1.03 ± 0.2 a	2.35 ± 1.5 ab	2.73 ± 0.3 ab	2.92 ± 0.1 abc	5.93 ± 2.1 c	4.36 ± 0.9 abc	4.9 ± 1.7 bc	3.72 ± 0.6 abc
Zn	0.19 ± 0 ab	0.83 ± 0 f	0.32 ± 0.1 c	0.44 ± 0 d	0.22 ± 0 abc	0.48 ± 0 d	0.13 ± 0 a	0.62 ± 0 e	0.24 ± 0 bc	0.93 ± 0 g
Mg	19.63 ± 0.1 d	23.75 ± 1.4 ad	38.05 ± 2.9 b	26.84 ± 4.5 ae	32.76 ± 3.4 be	44.13 ± 1.9 f	38 ± 0.5 b	11.87 ± 0.9 c	26.57 ± 0.7 a	11.57 ± 0.7 c

NB Means were compared by One-way ANOVA and means of the same element in a row followed by the same letter(s) are not significantly different (*P* > 0.05; Fisher LSD test). Comparisons are within the same heavy metal element across the rows. The suffix after "±" denotes the standard Error (SE).

The amount of Cu, Fe, Hg, K, Mn, P, S, Zn and Mg absorbed by the shoots or the roots also showed significant differences at all the sites (($F_{(9, 18)}$ = 6.0749, P < 0.001), ($F_{(9, 18)}$ = 20.6381, P < 0.001), ($F_{(9, 18)}$ = 51.502, P < 0.001), ($F_{(9, 18)}$ = 58.6933, P < 0.001), ($F_{(9, 18)}$ = 36.467, P < 0.001), ($F_{(9, 18)}$ = 34.6193, P < 0.001), ($F_{(9, 18)}$ = 13.6344, P < 0.001), ($F_{(9, 18)}$ = 8.2006, P < 0.001), and ($F_{(9, 18)}$ = 30.2042, P < 0.001), respectively) (Appendix 3E). However, although absorption of elements at all the sites was generally greater in the roots compared to the shoots, it was the opposite for Mg, P and K (Appendix 3E).

The downstream site at the Schoonspruit generally showed the greatest concentration of heavy metals and nutrient elements such as P and S compared to all other sites, after the rain, with the exception of the site at Kennan (Table 3.10). The percentage concentrations of Cu, Fe, Hg, Mn and Zn in the roots at this site were the highest (Table 3.12).

Table 3.12: The percentage uptake of metals by roots of water hyacinth grown in floating cages above and below the Koekemoerspruit and the Schoonspruit inlets on the Vaal River, after the start of the seasonal rain (week 7).

	Total metal uptake by roots (%)				
		Koekemoerspruit sites		Schoonspruit sites	
Treatment	**Kennan**	**Above inlet**	**Below inlet**	**Above inlet**	**Below inlet**
Cu	nd	nd	71.43	68.75	72.22
Fe	97.63	93.92	91.2	97.69	98.37
Hg	70.4	44.69	43.48	39.39	55
K	36.2	30.04	46.67	14.31	23.08
Mn	95.52	86.22	80.93	89.84	94.9
P	43.5	43.97	38.46	34.15	36.95
S	83.77	69.39	51.73	42.37	43.16
Zn	81.37	58.01	68.57	82.67	79.49
Mg	54.7	41.36	57.4	23.8	30.34

3.8 Discussion

Water hyacinth effectively removed most metals from the water in the single-element system tub trial, and the removal was more pronounced in the tub experiment where plants were exposed to a single metal than in the AMD pool trial with a suite of metal treatments at a variable sulphate concentrations. This is probably because of the complex environment in the AMD trial in pools, compared to the single element trial in the tubs. The uptake of metals is affected by several factors among which are nutrients, exposure time, ion competition for sites of uptake pathway in the root, concentrations of the element, complexing agents and pH (Prasad *et al*., 2001; Tangahu *et al*., 2011; Deng *et al*., 2004). Nevertheless, even under the acid mine drainage (AMD) conditions in the pool trial, the percentage removal of Cu and Mn from the pool water by water hyacinth was over 69%, and this was 52% more than the percentage removal of Fe and Zn.

Among many aquatic plants, water hyacinth is a prominent example of one with a great capacity to accumulate heavy metals in its roots (Malik, 2007; Liao and Chang, 2004). Plants in the controlled tub and pool trials, and the field trials in floating cages accumulated most of the metals removed from water, in their roots. Liao and Chang (2004) and Zhu *et al*. (1999) also showed similar results where the concentrations of heavy metals were between 4 to 16 and 3 to 15 times, respectively in the roots than in the shoots of water hyacinth.

3.8.1 Single-element system tub trial

3.8.1.1 Water pH and Electrical conductivity (EC)

The water pH in the uranium-treated tub water was about 6.8 after the addition of U in water and was the lowest water pH of all the metal treatments. This could be due to the solubility as it gets oxidized resulting in uranyl ion (UO_2^{2+}) that predominantly exist as a monomeric species (monometallic molecule) in water, with a strong potential for anionic binding at pH values close to 7, when it is in contact with anionic substances such as resins, phosphates or carbonates (Bursali *et al*., 2009; DeSilva, 2005). However, the pH for all the other metal treatments was similar and was maintained at an average pH 7.3 (Fig. 3.1A). This is an indication that most of the heavy metals had been removed from the water by water hyacinth since generally greater pH values suggests lower metal concentrations in water. Deval *et al*. (2012) also found pH approaching the neutral value after the exposure of Azolla (*Azolla caroliniana*) to different concentrations of zinc plating effluents for ten days. The pH results in this study also fit the analytic results of water samples from each of the metal treated waters with the exception of the iron and arsenic treated water samples (Table 3.2).

Electrical conductivity of a solution depends on the amount of solutes or anions it contains. In the current study the EC dropped by more than 18% three weeks after the addition of the metals, because of the metal up take by water hyacinch from the solution (Fig. 3.1B). This drop in EC was as a result of metal uptake by the plants. Mahmood *et al*. (2005) and Deval *et al*. (2012) also found similar reduction in heavy metals removed from different concentrations of effluents by *Azolla caroliniana* (after four days) and by water hyacinth (after ten days), respectively.

Water hyacinth removed the heavy metals from water effectively to a level below their initial respective concentrations with the exception of As and Fe (Table 3.2). The percentage removal of the heavy metals from water was in the order of Hg>MnH>MnM>MnL>Zn>Cu. Mishra *et al*. (2008a) found a 71% percentage removal of Hg by water hyacinth plants from an initial concentration of 0.007 mg/L in water in three weeks. Similarly when water hyacinth was exposed to Hg contaminated water in a lab trial for six hours it was able to reduce the initial Hg concentration of 0.875 mg/L in water to less than 0.001 mg/L (i.e. ~ 99.9%) (Wolverton and McDonald, 1975). Skinner *et al*. (2007) showed a percentage removal of 98.79% and 99.54% when water hyacinth was exposed for 30 days to concentrations of 0.5 and 2 mg/L Hg respectively. The root surface of the water hyacinth is negatively charged with strong affinity to cations. Chattopadhyay *et al*. (2012) indicated that Hg is strongly attracted to the negative charges in the water hyacinth roots and the bond formed between them is likened with that of the mercuric chloride bond (strong). Such features of strong ionic attraction make the removal of Hg from water by adsorption much easier than other metals. Similar studies of water hyacinth in contaminated water also showed the affinity of Hg to organic ligands was stronger than those of lead and chromium elements (Nordberg *et al*., 1978).

3.8.1.2 The uptake of Fe by water hyacinth in the single-element tub trial

The percentage removal of Fe from the tub water, besides As, was the lowest of all the treatments (Table 3.2). In fact the Fe concentrations in water, particularly in the Fe-L and Fe-M treatments, were slightly greater than their repective initial concentrations. Iron is a micronutrient and plants require low concentrations of Fe

(0.6 mg/L in Hoagland's solution). Tolerant plants constrain most heavy metals to their roots, where their toxicity is minimal, while others are adapted to reduce the metal toxicity by excretion of cations into the medium (Win *et al*., 2002). Water hyacinth has the ability to leak some excess iron into the medium to avoid iron toxicity (Sutcliffe, 1962). Release of iron into the medium could also be from decaying root and shoot tissues that detached from the mother plant, either due to metal toxicity or senescense. Center and Spencer (1981) showed a water hyacianth plant with 6-7 leaves, grows and sheds a new leaf on average every seven days. Mishra *et al*. (2008a) found a slight increase in Hg and arsenic concentrations in the growth medium at 25 days compared to their concentrations at day 20, as a result of metal discharge from the decaying plant tissues. Thus, the increase of the Fe concentration in water, even after three weeks exposure to the plants, suggests that there was Fe-leakage from the plants to the medium.

The Fe concentration in the roots of the plants before they were transferred to the tubs prior to the start of the experiment was almost as large as those in the control treatment after the addition of the Hoagland's solutions; and the fact that this Fe concentrations in the plants were already greater than those in the iron dose response treatments, suggests the plants were already saturated with Fe and that iron leakage from the iron-treated plants into the medium had occurred in weeks 3 (Table 3.2 and 3.3). Win *et al*. (2002) showed an increased rate of an iron uptake in water hyacinth plants with iron deficiency and a decreased rate as the plant cells saturated with iron, with a possible iron leakage into the medium in the case of iron oversaturation.

3.8.1.3 The uptake of As by water hyacinth in the single-element system tub trial

The arsenic analysis was repeated in three different accredited laboratories, but nevertheless showed that the initial concentration of arsenic in the tub water, collected just after the addition of the metal, could not be matched to the amount of arsenic (1ppm) originally added to the tubs. Both the initial and final concentrations of arsenic in the water did not show a significant difference between them. However, some studies have shown that water hyacinth can effectively remove arsenic from water. Mishra *et al*. (2008a) found the removal of arsenic by fresh plants of water hyacinth exposed to coal mine effluent for 21 days was 80%. The similarity between the initial and final arsenic concentrations in the water in this study could therefore be due to either a technical error, or due to the ICP-OES analytic method being inappropriate instead of ICP-MS, which could be better for lower or trace metal concentrations in water. Nevertheless, arsenic analyses even with ICP-MS, has its own difficulties in establishing accurate result from water samples with arsenic concentrations below 1 ppm (Dunn, 2007).

3.8.1.4 The total uptake of metals by plant roots and shoots in the single-element tub trial

The heavy metal concentrations in the shoots of all the treatments in this trial were significantly lower than the concentrations in the roots, although results for some heavy metals in shoots (e.g. As, Au and U) were below the detectable limit of the analytic method (ICP-OES or FIAS) used (Table 3.3). Most metal accumulations in water hyacinth occur in the plant roots (Kay *et al*., 1984). The translocation of arsenic to the shoot is negatively related to phosphates since they share the same channels of uptake in the roots (Rahman and Hasegawa, 2011). However, they found the largest portion (90%) of the total As removed from water by water hyacinth was retained in roots (Rahman and Hasegawa, 2011), which also agrees with the results in this trial where the As concentration in the shoots was below the detectable limit of the ICP-OES. This could also be due to the strong affinity of arsenic towards the iron plaques, on the surface of the water hyacinth roots which could impede its uptake from the surface of the roots of water hyacinth. The As affinity to the iron plaque depends on its species. The As(V) species is a characteristic feature of oxic conditions, unlike the reduced form of As, the arsenite species As(III), which is more soluble and toxic to plants (Kim *et al*., 2002). The tubs in this trial were equipped with submersible pumps, suggesting that the water was well aerated, enough to oxidize the As(III) added to the tubs, to As(V). This would result in adsorption of As(V) by the iron plaques on the root surface and reduce the As uptake by plants and its transportation into the aerial parts (Rahman and Hasegawa, 2011). Although the metal uptake experiment in this trial was conducted for three weeks, the use of water hyacinth to remove As in overnight by small scale farmers in Bangladish (Snyder, 2006) may not be recommended. This is because of the uptake of As by water hyacinth is affected by P concentration in water due to competition between the two ions, and the removal of As from water takes longer in the presence of P.

The Hg concentration in the roots was 58 times that of the shoot concentration (Table 3.3). This indicates the greatest capacity of water hyacinth to remove and accumulate Hg in their roots compared to other metals. Lenka *et al*. (1990) also found Hg accumulation of four times greater in the roots than in the shoots when a solution of 0.04 mg-Hg/L was exposed to water hyacinth plants for four days The disparity between the results in the literature and the current study could however be due to the different factors that influence the uptake of metals among, which are metal concentration in water, exposure time, nutrients and plant age (Prasad *et al*., 2001; Tangahu *et al*., 2011; Chattopadhyay *et al*., 2012).

The concentrations of the other heavy metals were also greater in the roots than in the shoots. The metal concentration in the roots of the Mn dose response treatment was between 4 to 10 times that of the shoot, while those of the iron dose response treatments was between 44 to 66 times the shoot concentration. Similarly, the concentrations of Cu and Zn in the roots were 75 and 9 times their concentrations in the shoot, respectively. Lu *et al*. (2004) found Zn concentration in roots of water hyacinth was about five times those in shoots, when the plant was exposed to 40 mg Zn /L in water, although their initial Zn concentrations in water were greater than those used here. The plants in the Cu treatment were by far the most detrimentally affected by the heavy metal toxicity and this could be associated to the fact that the Cu concentration in the shoots in this trial was twice that the upper limit of the normal range of Cu in most plants (3-20 mg/kg dwt.) as indicated in several studies (Nriagu, 1979; Clarkson and Hanson, 1980; Howeler, 1983; Stevenson, 1986).

3.8.1.5 Metals absorbed by plant roots and shoots in the single-element tub trial

Generally the amount of metals removed by shoot or root absorption was greater than those removed by adsorption. The removal of heavy metals by absorption in the roots ranged from 3 for the low and medium concentration of manganese treatments to 49 (for Hg) times greater than those absorbed into the shoots (Table

3.3). The root absorption of manganese increased with the increase of its concentration in water, as opposed to Fe dose response treatment, which did not showed any change beween them. Although it is indicated that most of the metals removed from water by macrophytes are accumulated in their roots than in the shoot system (Kay *et al*, 1984; Zhu *et al*., 1999; Liao and Chang, 2004; Malik, 2007, this study), the amount of metals absorbed into plant tissues exceeded the amount adsorbed on the surface of the plant tissues, and the largest portion of the absorption was localized in the roots. The amount of metals absorbed by the roots was generally greater compared to the removal by adsorption. Nevertheless, an adsorption range of 30 to 52% was observed in the roots for most of the metal treatments and the highest was for Cu. This suggests why water hyacinth is tolerant and resilient to most heavy metal phytotoxicity as indicated by Weis and Weis (2004).

3.8.1.6 The bioconcentration factor of water hyacinth (BCF) in the tub trial

The BCF index of half of the metal treatments in tubs was greater than a 1000, which is the lower limit of plants considered as accumulators of heavy metals (Zhu *et al*., 1999) (Table 3.4). This includes Au, Cu, Fe, Hg, and Zn of which Fe from the low concentration treatment of the three different iron dose response treatments showed the highest BCF index of all. Although the BCF of the Fe concentration treatments declined with the increase of Fe concentrations in water, it shows that the water hyacinth plant is a super accumulator of Fe. In contrast the bioconcentration factor of all the manganese dose response treatments was below 1000. However, the Mn BCF increased with increase of Mn concentration in water, suggesting that the plants could be an effective accumulator at concentrations greater than those used in this trial (4 mg/L Mn). This single-element system tub trial indicates that water hyacinth can range from a moderate to good heavy metal accumulator. Thus the plant has an enormous potential in phytoremediation of heavy metal contaminants particularly if the target is the removal of a single element from water.

3.8.2 Simulated AMD pool trial

3.8.2.1 Water pH and EC in the AMD pool trial

On Day-1, before the addition of heavy metals and sulphate treatments, the pH in the high AMD treatment was significantly lower than the other two AMD treatments and it was below 6.9 (Fig. 3.2). This could be due to water from the previous pilot test which was partly reused in the high AMD treatment and the lower pH was an indication of slightly contaminated water condition. Consequently the water quality in the high AMD treatment showed significant decrease in the pH while the EC was greater, on Day-1, than in the other two treatments. Increased concentration of solutes in water decreases the pH and increases the EC, a common characteristic of a contaminated solution (Deval *et al*., 2012). However, on Day 1, after the addition of AMD treatements, the pH was lower in the low AMD treatment (dropping below the pH 6.7) than in the medium and high AMD treatments, while the medium and high AMD treatments increased towards the neutral level, slightly above pH 7.1. The rise of the EC with the increase of the sulphate concentrations from the low to the medium and the high treatments with the passage of time suggests the rapid uptake of sulphates on Day 1 and later in week 3 the plants saturated and started leaking sulphates back to the medium.

The EC before the addition of the metals was lowest in the medium treatment and highest in the high sulphate treatment. Thereafter on Day 1 and at week 3 the EC showed a significant increase with the increase of the AMD concentration (Fig. 3.2B). This was due primarily to the different sulphate concentrations (300, 700 and 1300 mg/L SO_4^{-2}/) respectively. An interaction of the sulphate with the heavy metals in the pool water was possible. Vestena *et al*. (2007) found that the uptake of sulphur by water hyacinth increased with an increase of water sulphate, from 400 to 800 μM in Cd treated water, while in their control treatment, such an increase did not increase the uptake of S, which was suggested to be due to the saturation of the S uptake channels in the plant tissues. They suggested that the Cd-induced plant stress enhanced the uptake of more sulphates by plants for the biosynthesis of peptides known as phytochelatins, used in detoxification of Cd by complexing it with the chelatin. The increase of EC with the increase of sulphate concentrations in this study could therefore be partly due to the saturation of sulphates in the plant cells which consequently led to their greater concentration in water. For instance the total sulphate uptake by plant roots in week 3 was seen to decrease with the increase of the AMD treatments (Table 3.6). Similarly, Ayyasamy *et al*. (2009) found that nitrate removal from water using water hyacinth progressively increased (64, 80 and 83%) with the increase of the nitrate concentrations in water to levels 100, 200 and 300 mg/L, respectively. However, when nitrate concentrations in water were increased to 400 and 500 mg/L the percentage removal decreased, and it was indicated that this was due to increased osmotic pressure in the external medium which impeded the uptake process (Eaton, 1941).

3.8.2.2 The percentage removal of metals from water in the AMD pool trial

The initial concentration of metals, in each of the three sulphate dose response treatments in this experiment dropped significantly lower than the corresponding final metal concentrations in the water in week 3 (Table 3.5). Falbo and Weaks (1990) also found a decline of sulphates, manganese and iron in water hyacinth-treated water compared to their control treatment without plants in 14 days. Similarly Mishra *et al*. (2008b) found removal of Cu and Zn were 76.9%, and 55.4%, respectively by water hyacinth, after an exposure of 21 days, to a coal mining effluent. While Mahmood *et al*. (2005) found removal of Cu and Zn of 94% and 97% respectively, from water after four days of water hyacinth exposure to textile effluents. The highest percentage removals of Cu and Zn in this trial were 73% and 40% respectively. The discrepancy between the pool trial and literature could be due to the differences between the contaminant levels in the different effluents used in the literature and this trial, which also included different sulphate concentration treatments. The uptake of heavy metals by plants is affected by several environmental factors among which are the redox potential of metals, organic chelators, pH, temperature, light intensity, oxygen level, and ionic competition (Prasad *et al*., 2001; Tangahu *et al*., 2011; Deng *et al*., 2004). Copper has a strong affinity to organic matter (ligands) which usually makes it less bioavailable to plants (Fernandes and Henriques, 1991). The Cu percentage removal was greater in the single element trial, suggesting that the pool trial provided more opportunity for binding with organic matter because of the amount of dead plant materials in the pools than in the tubs. Similarly, the percentage removal of Zn in pool water (40%) dropped significantly compared to that in the tub (83%) and this could be attributed partly to its potential to bind with organic substances or with the additives of the technical fertilizer (Lawn and foliage fertilizer from Wonder, with N, P, K, Zn, Mg, Ca and some fillers/additives) applied for plant growth before the trial, and partly due to ionic competition from other heavy metals for uptake channels in the root surfaces. Hardey and Raber (1985) found that the uptake of Zn by water hyacinth was blocked and the removal of Zn from water was reduced by 86% after the addition of a complexing agent (trans-l,2-cyclohexyl.enedinitrilotetraacetic acid (CDTA)) into the solution with water hyacinth. They also found that the uptake of Zn was impeded by the ionic competition from Hg, Cu, and Fe among others for sites of uptake in the root surface.

The initial metal concentrations added to the pools at the beginning of the experiment were the same across all the AMD treatments. Nevertheless, some of the water in the high AMD treatment from the previous pilot test was reused, and also the technical fertilizers contained with N, P and K at a ratio 7:1:3 respectively, with some micronutrients (Fe, Zn, Ca, Mn) and fillers (impurities), the metal concentrations in the water before and after the addition of metal and sulphate treatments showed significant differences between the sulphate dose response treatments (Appendix 3B). As a result the disparity in the percentage removal of heavy metals from water across the different AMD treatments could partly be due to a complex mix of elements in the pools. In addition, several factors influence plant metal uptake and these includes metal concentration in water, complexing substances and cation competition for binding sites on the root surfaces (Prasad *et al*., 2001; Tangahu *et al*., 2011; Deng *et al*., 2004). For instance, Sela *et al*. (1988) showed the uptake of Zn by Azolla (*Azolla filiculoides*) roots was reduced in the presence of uranium because of cation competition for the site of uptake between them, while it enhanced the uptake of calcium. Thus the removal and uptake of the metals in the presence of sulphates in the pool was therefore affected by the concentration of the sulphates and the competition between the metals and different elements from the fertilizer compared to those in the single-element system tub trial.

3.8.2.3 The total uptake of metals by plant roots and shoots in the AMD pool trial

Similar to the single-element system tub trial, the accumulation of metals with the exception of Mg, were also greater in roots than in the shoot in the pool trial (Table 3.8). Magnesium is an essential macro-nutrient in plants and it is the central constituent of chlorophyll molecules involved in absorption of light and fixation and assimilation of CO_2 in the chloroplast (Wilkinson *et al*., 1990). The uptake and transportation of magnesium to the aerial parts of water hyacinth was not affected by the sulphate concentration, which suggests that the magnesium site of uptake in the roots is different from that of the sulphates. Elements with a common uptake route compete for sites. The uptake of selenium (Se) by *Ruppia maritime* (wigeongrass) was reduced with the increase of sulphate concentration in artificial pond water over 21 days of exposure (Bailey *et al*., 1995) due to their similar chemical properties and therefore common pathways for uptake (Germ *et al*., 2007). The metal concentrations in the shoot tissues of each of the heavy metal treatments used in the pool did not show significant differences between the sulphate dose response treatments, which indicates that the metal transportation to the aerial parts was not affected by the sulphate concentrations in water, particularly when plants are not facing a sulphate deficiency (Table 3.6).

The order of the heavy metal concentrations found in the shoots and the roots was largely consistent in both the single-element system tub trial and AMD pool trial. The order of the metal concentrations in shoots and roots of the single-element system tub trial was Fe>Mn>Zn>Cu and Mn>Zn>Fe>Cu respectively, whereas in the AMD pool trial it was the same across the low, medium and high AMD treatments where their concentration was in the order of Mn>Fe>Zn>Cu. Copper concentration in the shoots as well as in the roots in all the trials was the lowest of all, and this could be due to the sensititvity of the plants' photosynthetic system to Cu (Fernandes and Henriques, 1991; Sandman and Boger, 1980) and to some extent to the roots (Lequeux *et al*., 2010). Nevertheless, regardless of the position of Cu in the order of metal accumulation in the shoots, its concentration in water hyacinth from the single-element system tub trial and the high AMD treatment of pool trial exceeded the normal range of 3-20 mg/kg d. wt. of Cu indicated for most plant species (Nriagu, 1979; Clarkson and Hanson, 1980; Howeler, 1983; Stevenson, 1986) and therefore, toxic effects to the plants were unavoidable.

Zinc was the only metal in the pool trial where total uptake by the roots declined significantly with the increase of sulphate (Table 3.6). The amount of absorbed Zn by the roots also showed a similar declining trend from the low to medium to high sulphate treatments (Table 3.7, 3.8). Zinc is primarily soluble and a bioavailable metal ion with relatively weak affinity with complexing agents compared to Cu (Daigo, 1997). The progressive decline in percentage concentration of Zn in the roots could be due to the effect of increased sulphate concentrations which could be blocking the uptake of Zn when sulphates in the root surfaces reach saturation. Increased concentration of sulphates in water also mobilizes phosphates (van Der Welle *et al*., 2007), which enhance the precipitation of Zn as zinc phosphate (Khellaf and Zerdaoui, 2009). When duckweed, *Lemna gibba* L., was exposed to a range of ZnSO4 solutions (6.0, 10.0, 14.0 and 18.0 mg l^{-1} of Zn), the amount of Zn removed from water by precipitation as zinc phosphate was between 49 to 68%, increasing with the increase of sulphates (Khellaf and Zerdaoui, 2009). Nevertheless, the increase of Zn concentration in the shoots, with the increase of sulphate concentrations could be due to Zn transportation to the shoots through the same channels of the sulphates. As the sulphate uptake increased in the high concentration treatment, the Zn transportation into the shoot was also enhanced. Sometimes the uptake of nutrients also enhances the uptake of some heavy metals. At concentrations of 2.5 mg/L PO_4 the removal and translocation of Hg by water hyacinth increased since higher concentrations of phosphate encourage higher influx of water into the plants, which consequently allows the influx and translocation of Hg from the water into the plants (Chattopadhyay *et al*., 2012). Similarly, increase in Cd concentration in water hyacinth plants with increase of sulphur as Na_2SO_4 into the solution was also reported by Vestena *et al*. (2007) and thus, although the sulphate concentration was enormous in this high sulphate treatment compared to their experiment, the sulphate uptake could enhance the uptake of Zn into the aerial parts with the increase of the sulphate concentrations.

3.8.2.4 Metals Absorbed by plant roots and shoots in the pool trial

The patern of metal absorption by the root and shoot tissues in the AMD pool trial was not different from those in the the single-element system tub trial (Table 3.7). Unlike the roots, the absorption of metals by the shoots was not significantly affected by the variation of sulphate concentrations in the different AMD treatments with the exception of Mg. Although the amount of metals absorbed by the roots is generally greater compared to adsorption, an adsorption of up to 52% for Cu in the single-element system tub trial, and 26 – 44% for all the metals in the AMD pool trials was observed. This suggests why water hyacinth is tolerant and resilient to most heavy metal phytotoxicity as indicated by Weis and Weis (2004).

3.8.2.5 The Bioconcentration factor of water hyacinth grown in pools

The BCF index of heavy metals from the simulated AMD trials in pools was relatively low, with the exception of Mn, compared to the tub trial with single elements of heavy metals (Table 3.9). Unlike the single-element system tub trial, the different AMD treatments in the pools could be affected by cationic competition between the different metal and nutrient elements in water for sites of uptake in the roots and by the osmotic pressure in the external medium due to the elevated concentrations of sulphates, which could reduce or inhibit the metal uptake processes by plant roots (Ayyasamy *et al.*, 2009). Concentrations of sulphates exceeding 700 mg/L in water generally cause a decrease in the uptake of most elements (Cu, Fe, S, Mg and Zn) by water hyacinth, although the reduction was not significant for some of these. This also agrees with the results of Bailey *et al*. (1995) who found increased selenate uptake by wigeongrass, *R. maritima* under low sulphate concentrations (0.007 mg/L) compared to high sulphate concentration (1600 mg/L) when exposed to selenium concentrations of 0.01, 0.1 and 1 mg/L for 21 days. Copper and Zn were below the BCF value of 1000. However, this could be associated to the fact that these two elements are relatively less bioavailable for direct uptake by plants due to their strong binding capacity with ligands such as organic matter or sulphidic substances (Fernandes and Henriques, 1991; Hardey and Raber (1985).

The water hyacinth's ability to remove and accumulate metals from the simulated AMD pool trial ranged from poor to good based on the criteria of Zhu *et al*. (1999) for good accumulators of heavy metals. Considering both the single-element system tub trial and the AMD pool trial, water hyacinth is more effective for phytoremediation of a water system with single contaminant and for selective metals in elevated AMD water pollution, such as Fe and Mn.

3.8.3 Acid mine drainage in the field trial

3.8.3.1 Water pH and EC at the Vaal River sites

After the rain in week 5 the pH dropped significantly compared to the pH before the rain in day one, and which is an indication of more effluents and acid mine drainage coming into the water system from the surrounding mining sites and local settlements (Table 3.10). The fact that both the downstream sites at the Koekemoerspruit and the Schoonspruit had a pH significantly lower than those at the upstream sites of the respective tributaries after the rain was an indication of the level of contaminants that flushed into the Vaal River from the two tributaries. EC measurements from the downstream Schoonspruit site before and after the rain were significantly greater from the upstream site (Fig. 3.3B). The water in the Schoonspruit was murky, silty and brownish in colour particularly after the rain. As a result, the downstream EC measures were high because of solutes and or silt sediments in the water.

3.8.3.2 Water contamination at the Vaal River sites before and after the rain

The heavy metal and nutrient analysis samples before and after the rainy season in the two Vaal River tributaries indicated that the Schoonspruit was more enriched with nutrients such as P, S, Fe, Mn, Mg and Zn than the Koekemoerspruit and increased with the rainy season (Table 3.10). Similar increments in concentrations of Cu, Cd, Mn, Pb and Hg were also found in Asia's largest water reservoir (Govind Ballabh Pant Sagar) contaminated by effluents from the coal mining after the rainy season (Mishra *et al*., 2008c). The increase of contaminants at the downstream site of the Schoonspruit could be associated with the increased runoff from the nearby gold mining sites and other contaminants from fertilizers and pesticides in agricultural lands in addition to the effluents from the local settlement of Kennan near Orkney (Table 3.10), which is also reported in DWAF, (2009).

3.8.3.3 The total uptake of metals by plant roots and shoots in the field trial

The fate of the largest concentration of heavy metals removed from water consistently remained the same from the tub and the pool trial to the field trial at the Vaal River. The heavy metal concentrations retained in the roots at each site was significantly greater than those in the shoots, for most of the elements (Table 3.11 and Table 3.12) which is in agreement to results shown by several other studies (Mishra *et al*., 2008c; Chattopadhyay *et al*., 2012; Malik, 2007; Lu *et al*., 2004; Liao and Chang, 2004). Similar to the tub and the pool trials, the concentrations of Fe, Mn, and Zn in roots at the downstream Schoonspruit site was significantly greater than those in the shoots and also than those in the shoots and the roots at both sites of the Koekemoerspruit. This was also true for the amount of absorbed metals in the roots compared to those in the shoots, which was still consistent with results found from the tub and the pool trials, with the exception of the macronutrients Mg, P and K in the field (Appendix 3E). Heavy metals are localized in the roots of aquatic macrophytes and preferably in the root cell wall of such plants as a strategy to enhance tolerance by avoiding their phytotoxic effect when they reach the sensitive photosynthetic system (Mishra *et al*., 2008c; Sela *et al*., 1988). These three heavy metals were also significantly greater at the downstream sites than those at the Koekemoerspruit sites, suggesting that the Schoonspruit is the greater source of contaminants to the Vaal River near Orkney (Table 3.11).

3.9 Conclusion

This study showed the great capacity of water hyacinth for the removal of heavy metals from water. Based on the results of the BCF index, water hyacinth could be rated as a moderate to good accumulator of heavy metals when deployed to remove a single metal contaminant from water. Results from the single metal tub trial showed that most of the metals removed from water were accumulated in the roots compared to those of the shoots and the amount of root removal by absorption was between 3-49 times that of the shoot. Generally, there were not significant differences between the amount of metal absorptions and adsorptions in the roots or shoots except for Mn, U and Zn in the roots and Mn and Zn in the shoots where the absorption significantly exceeded the amount of the adsorption in the single metal tub trial.

The rate and the efficiency at which water hyacinth removes heavy metals from water is often indicated to increase when metal concentrations in water are low or in trace amounts (O'Keeffe *et al*., 1984; Zhu *et al*. 1999; Mishra *et al*., 2008c; Mokhtar *et al*., 2011; Mukhopadhyay, *et al*., 2007). Therefore, considering the fact that the heavy metal concentrations in the Vaal River at the site of the experiment were lower than those concentrations used in the single-element tub trial and simulated AMD pool trial and the fact the sulphate concentration in the river ranged from 6 to 729 mg/L (Table 3.12), slightly over the medium sulphate concentrations in pools (700 mg/L SO_4^{-2}), water hyacinth can be regarded as an important candidate for phytoremediation in the Vaal River, despite its low performance for some metals in the pool trial. However, due to the impact of water hyacinth weed on the integrity of other environmental aspects, its recommendation as a phytoremediation device should be dealt with cautiously and preferably only be used if infestations of the plant pre-exists in the water system targeted for phytoremediation and a safe disposal of the phytoremediating plants has been arranged. One suggestion would be to use the contaminated water hyacinth plants on nearby gold mining tailings dams, where they could be used for mulch, to grow trees that trap dust and other contaminants.

Results of the metal uptake by plant tissues throughout these trials have consistently shown that most of the metals removed from water were accumulated in the roots than in the shoots. This also includes the amount of metals absorbed in the roots which were significantly greater in the roots than in the shoots. Nevertheless, some of these metals were also transported into the aerial parts at concentrations that could result in phytotoxicity, among which was Cu which consistently exceeded the normal range of Cu for most plant species (3-20 mg/kg d. wt.). Heavy metals in plant leaves are known to defend the herbivory of some insects (Boyd, 2010). Despite the fact that water hyacinth accumulated most of the heavy metals taken up in the roots, some metal concentrations in the shoot could potentially be harmfull to biocontrol agents such as the water hyacinth weevils.

This topic will be explored in the next chapter where the effect of these metals on the plant and its biocontrol agents was investigated.

Chapter 4

Heavy metals in water hyacinth plant tissues and their effect on survival and reproduction of *Neochetina* weevils used as biocontrol agents

4.1 Introduction

To date an estimated 450 plant species are reported to have evolved the ability to build up a large amount of trace elements, mainly metals, in their plant tissues (Verbruggen *et al*., 2009). The majority of these plants occur in metalliferous soils. Verbruggen *et al*. (2009), and Brooks *et al*., (1977) define such plants as hyperaccumulators. About 76% of these plants hyperaccumulate Ni while the rest hyperaccumulate As, Cd, Co, Cu, Mn, Pb, Se, and Zn (Reeves and Baker, 2000). The criterion for hypercumulation in plants is determined by the threshold concentration of each element sequestered in the plant tissues (Table 4.1). For instance over 1 000 µg/g dry mass for Co, Cr, Cu, Ni and Pb and over 10 000 µg/g for Mn and Zn are relevant thresholds (Reeves and Baker, 2000).

Table 4.1: The threshold concentration of metals taken-up by plant tissues in the field, above which they are considered as hyperaccumulators (adapted from Coleman *et al*., 2005).

Metal	Normal range	Minimum accumulator level	Minimum hyperaccumulator level
Cd	0.1- 3	20	100
Co	0.03-2	20	1,000
Cr	0.2-5	50	1,000
Cu	5-25	100	1,000
Mn	20-400	2,000	10,000
Ni	1-10	100	1,000
Pb	0.1-5	100	1,000
Zn	20-400	2,000	10,000

All values are expressed in µg/g (dry mass basis).

Several hypotheses have been formulated to explain the uptake of such high concentrations of elemental metals in the tissues of hyperaccumulators. These include metal tolerance, drought resistance, plant allelopathy (a strategy to exclude other competing plants), protection against pathogens and insect damage (Boyd and Martens, 1992). However, most of these hypotheses are either still untested or require further research for clarity. Studies on the elemental metal protection hypothesis against insect herbivory and plant diseases have taken the lead in this regard and there is some evidence to support this (Pollard and Baker, 1997; Jhee *et al*., 1999; Jiang *et al*., 2005; Boyd, 2007, 2010).

On many occasions plants growing in metalliferous sites were noticed to have reduced biotic stresses compared to the same species growing in unpolluted soils. For instance Noret *et al*. (2006) indicated that only one out of the total 63 different types of herbivores that are known to feed on *Silene vulgaris* was actually found to attack this plant when grown on contaminated sites. Both accumulators and hyperaccumulators have different strategies for detoxifying heavy metals that enter into the plant tissues such as: excretion of substances used as binding agents (ligands) to the growth medium to reduce metal bioavailability; selective uptake of elements to exclude toxic metals; metal accumulation in roots; localizing metals in cell walls, vacuoles and inclusions; and development of metal resistant enzymes metal (Fernandes and Henriques, 1991).

4.1.1 Metals and insect interactions

Insects exposed to a heavy metal-contaminated diet accumulate different metals in different body parts before they reach a toxic level. For instance the larvae of *Chironomus yoshimatsui* Martin et Sublette (Diptera: Chironomidae) accumulate cadmium in the digestive tract and fat bodies (Sumi *et al*., I 984). Lead is largely stored in the brain of dragonfly larvae and to a lesser extent in the midgut, fat body, rectum and cuticle (Meyer *et al*., 1986). Some insects accumulate heavy metals in males and females at different concentrations. In adults of the grasshopper, *Aiolopus thalassinus* Fabr., (Saltatoria: Acrididae) cadmium was found largely in the testes, followed by the gut (Schmidt and Ibrahim (1994). Mercury in the same insect was stored in testes, male accessory glands, ovaries and in the midgut. Devkota and Schmidt (2000) found that mercury and Cd concentration in females was greater than that acumumulated in the males of grass hopper species, *Oedipoda caerulesens* L., (Orthoptera: Acrididea) and, *O. germanica* Latr., respectively. At the larval stage, if heavy metals reach a toxic level, they cause morphological deformations such as the development of abnormal wings (Schmidt and Ibrahim, 1994). Heavy metals in the bodies of insects also interfere with proteins, DNA and RNA function (Hussain and Jamil, 1992**)**. For instance (Hussain and Jamil, 1992) showed that the variation in protein and nucleic acid contents in the body of *Neochetina eichhorniae* was due to heavy metal ions suggesting that the metal ions formed complexes with amino acids, and nucleic acids which eventually alter gene transcription and translation activities (Hussain and Jamil, 1992). Insects detoxify accumulated metal ions by binding them with organic acids and forming complex compounds. Nevertheless, the activities of most herbivorous insects are negatively affected by heavy metals accumulated during their feeding.

4.1.2 The trade-off of heavy metals in hyperaccumulating plants

Unlike plant secondary metabolites (defensive organic compounds derived from photosynthesis), elemental metal defences are inorganic metals that are directly removed from the soil or water and moved into the plant tissues (Martens and Boyd, 1994). The metal defence system varies with the type of element taken up by the plants and the minimum threshold concentration needed to impose a negative effect on their natural enemies. This includes growth retardation, reduced reproduction rate, intoxication after foraging and or by acting as an antifeedant against herbivores (Davis *et al*., 2001). Center and Dray (2010) indicated that the performance and fitness of insects from five different orders and 16 families were reduced due to heavy metal toxicity.

4.1.2.1 Toxicity effect of metals on insects' female fecundity

Some organisms have the ability to discriminate between contaminated and uncontaminated host plants. For instance, *Porcellio laevis* (Isopoda: Porcellionidae) is able to discriminate and avoid Cd contaminated food at different concentrations (Odendaal and Reinecke, 1999). Similarly, Weissenburg and Zimmer (2003) found *Porcellio scaber* (Isopoda: Oniscidea) avoiding Cu contaminated leaf litter and feeding on less contaminated litters. From the few similar studies conducted in insects, the ability to discriminate between metal contaminated and uncontaminated hosts for ovipoistion was inconsistent. Trumble and Jensen (2004) found that the female humpbacked fly, *Megaselia scalaris*

(Diptera: Phoridae) did not avoid oviposition on chromium (VI) contaminated artificial food, nor did the beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae) when fed on selenium contaminated host plants (Vickerman *et al*., 2002). Similarly, Konopka *et al*. (2013) found that the cabbage looper, *Trichoplusia ni* (Lepidoptera: Noctuidae) oviposited on both the control and Cd treated *Brassica juncea* (Brassicaceae) host plants without discrimination. However, other female insects such as *Drosophila melanogaster* (Diptera: Drosophilidae) (Bahadorani and Hilliker, 2009) and *Pieris rapae* Linaeus (Lepidoptera: Pieridae) (Freeman *et al*., 2006) were found avoiding contaminated host plants for oviposition. Feeding on heavy metal contaminated host plants generally affects reproduction in most insects. For instance, the egg production of *Culex pipiens* L. (Diptera: Culicidae) exposed to LC_{50} concentrations of 0.11 $CdCl_2$, 5.09 $CuSO_4$, 45.36 $Pb(NO_3)_2$ and 0.44 $Hg(NO_3)$ ppm was significantly reduced by more than 50% compared to the control, as was the hachability of the eggs. Gao *et al*. (2011) found a fecundity decrease of 33 to 47% in the grain aphid, *Sitobion avenae* Fabricius (Hemiptera: Aphididae) fed on Hg, Cd, and Pd contaminated wheat or barley seedlings and oats. Similarly, Görür (2007) found a 30% decrease in fecundity when the cabbage aphid, *Brevicoryne brassicae* L. (Hemiptera: Aphididae) was reared on Cu and Pd contaminated cabbage and radish plants at concentrations of 3.14 mg/L, and 1.39 mg/L), respectively. Heliövaara and Väisänen *et al*. (1990) found a 13% decrease in the European pine sawfly, *Neodiprion sertifer* Geoffroy (Hymenoptera: Diprionidae) grown from larvae collected from Scots pines, *Pinus sylvestris* L. trees near Cu smelter.

4.1.2.2 Toxicity effect of metals on adult insects' feeding and survival

Generally insects do not have chemosensila, which are sensitive to heavy metals. Thus their selection of food quality is suggested to be mediated by tasting of leaves (Augustyniak and Migula, 2000). The amount of biotransfered heavy metals into insect bodies from herbivory of contaminated host plants affects their feeding and survival performance. Zvereva *et al*. (2003) found that the leaf beetle *Chrysomela lapponica* (Coleoptera: Chrysomelidae) collected from polluted sites had accumulated Ni and Cu in their bodies up to 7.7 and 3.6 times greater than those collected from unpolluted sites, respectively. This caused a reduction in adult feeding, survival and reproductive activities through the inhibition of esterase, an enzyme used in insects to degrade allelochemicals or pesticides and in regulation of juvenile hormones. Hanson *et al*., (2004) tested green peach aphids (*Myzus persicae*) on Indian mustard (*Brassica juncea*) growing with and without treatments of Se both in choice and no-choice trials. Their results showed a threshold level of 10 mg Se kg^{-1} dry weight of the plant deterred aphid feeding and as low as 2 mg Se kg^{-1} d. wt. was sufficiently toxic to reduce aphid population growth by 50%. Adult mortality of the Cabbage aphid, *B. brassicae* L. feeding on Cu and Pd contaminated plants was 24 and 64% respectively compared to 17% in the control plant (Görür, 2007). Similarly, adults of *A. thalassinus* Fabr., feeding on wheat seedlings grown at concentrations of 8 mg/L Hg, 10 mg/L Cd and 40 mg/L Pb died early in the experiment before laying eggs (Schmidt *et al*., 1992).

4.1.2.3 Toxic effect of metals on insects' larval feeding and survival

Generally the suitability of the larval host is determined by the female choice for oviposition (Mogren and Trumble, 2010). Thus the larvae are often more susceptible to metal toxicity than their adults due to their limited mobility to choose between contaminated and uncontaminated host plants. Larval mortality of the Cabbage aphid, *Brevicoryne brassicae* L., feeding on Cu and Pb contaminated plant was 54 and 47%, respectively compared to 30% in the control plant (Görür, 2007). Schmidt *et al*. (1992) found a prolonged larval development when *A. thalassinus* was exposed to seedlings and oats contaminated by different concentrations of Hg, Cd and Pb. The mortality of the first instar larvae of mosquitoes, *Culex quinquefasciatus* (Diptera: Culicidae) was greater by 2.5 to 6 times when exposed to lead nitrate concentrations of 0.05, 0.1 and 0.2 mg/L compared to the controls (Kitvatanachai *et al*., 2005). Similarly, Romi *et al.* (2000) found a prolonged larval development and an increased mortality in the first and second instar larvae of *Aedes albopictus* (Diptera: Culicidae) when exposed to Cu concentration of 10 and 20 g/L.

4.1.3 Insect resistance to metal toxicity

Hyperaccumulators are not entirely protected against all types of herbivores, because such elemental metal defences depend on the feeding mode of the herbivores (Boyd, 2004), besides those that are able to circumvent the plant defence system (Gatehouse, 2002; Karban and Agrawal, 2002). For instance even though hyperaccumulated Ni can protect the plant *Streptanthus polygaloides* (Brassicaceae) from caterpillar herbivory (Boyd *et al*., 2002), it gives no protection against aphids. (Boyd and Martens, 1999) found that the pea aphid *Acyrthosiphon pisum* (Homoptera: Aphididae) was not affected by the Ni concentration in *S. polygaloides*. This is associated with insect's preference for different plant parts for feeding. It is often shown that plants transport Ni through the xylem tissues by complexing it with the amino acid histidine and accumulated in the leaf epidermis. This creates an opportunity for insects such as aphids to selectively feed on the carbohydrate rich fluids of phloem tissues of *S. polygaloides* to avoid metal toxicity from the xylem fluid or the leaf epidermis (Boyd and Martens, 1999). Similarly, Jhee *et al*. (2005) showed that hyperaccumulated Ni defended the plant *S. polygaloides* against both leaf chewing ((the grasshopper *Melanoplus femurrubrum* De Geer (Orthoptera: Acrididae) and the (lepidopteran *Evergestis rimosalis* Guenee (Lepidoptera: Pyralidae)) and root-feeding (the cabbage maggot *Delia radicum* L. (Diptera:Anthomyiidae) herbivores, but not against phloem-feeding ((aphid, *Lipaphis erysimi Lipaphis erysimi* Kaltenbach (Homoptera: Aphidae) and whitefly, *Trialeurodes vaporariorum* Westwood (Homoptera: Aleyrodidae)) and xylem-feeding meadow spittlebug, *Philaenus spumarius* (Homoptera: Cercopidae) herbivores. This is due to the fact that most of the heavy metals are either stored in the roots (cell wall, intercellular materials and cell vacuoles) or leaves (epidermis, cuticle, cell cytoplasm with ligands, cell vacuoles) of plants.

However, some herbivores can still feed on hyperaccumulators unharmed. Boyd *et al*. (2006) found the grasshopper, *Stenoscepa* sp accumulated up to 3500 μgNi/g in the body of the insect from feeding on leaves of *Berkheya coddii* Roessler (Asteraceae), with leaf concentrations of up to 19 000 μgNi/g d. wt. without a problem. Such failure of extreme metal concentrations to affect herbivores is suggested to be due to either developed physiological tolerance, or to "diet dilution" (mixing low and high Ni containing diets) (Boyd, 1998) by some polyphagous herbivores. Schwartz and Wall (2001) found that the mirid hemipteran, *Melanotrichus boydi* that feeds only on the hyperaccumulating plant *S. polygaloides* could tolerate a body concentration of 800 mg Ni/g dry mass consumed from Ni-high leaves. Similarly Crawford *et al*., (1995) found that the black aphid *Aphis fabae* (Homoptera: Aphididae) feeding on *Vicia faba* grown in high Cu and Cd concentration treatments was able to accumulate and tolerate Cd in the body with little being excreted, suggesting it bound with metallothionen or removed to the cuticle to reduce its toxic effect, while Cu was largely excreted in the honeydew.

Some insects also transfer excess heavy metals in digestive organs to the lysosomes to reduce their toxicity effect, using metal binding proteins and antioxidant enzymes (Sun *et al*., 2007), while others avoid metal toxicity by directly removing them with their faeces, (Lindqvist, 1994; Kozlov *et al*., 2000); or in larval exuviae and pupal shells (Zhulidov, 1988; Andrzejewska *et al*., 1990), through removal of degraded midgut cells containing metals (Rabitsch, 1995). Heliövaara and Väisänen (1990) also indicated that some insects can avoid metal toxicity by removing them during metamorphosis in their larval skin and other components during moulting of their gut epithelium, and or eliminate them in their pupal remnants, cocoons, gall-walls, or in the droplet excreted by the imago just after hatching. In their study they found the metal concentrations in the adult females of *N. sertifer*, the larval feaces, and empty cocoons containing their last moulted larval skin declined with distance from the Scots pine trees near copper smelter from which they were collected. Therefore, the proposed elemental defense of hyperaccumulated metals is governed by the type of feeding (mode of feeding) and type of herbivores and their adaptations. However, even though it does not provide a complete protection to the plant, it does give some protection against some natural enemies.

4.1.4 Metal accumulation and elemental metal defense in aquatic plants

Most aquatic macrophytes are capable of accumulating large amounts of heavy metals in their tissues, a characteristic feature that has encouraged their wide use in phytoremediation of anthropologically polluted waters. Among these are water hyacinth (Malik, 2007; Liao and Chang, 2004; Misbahuddin and Fariduddin, 2002), duck weed, *Lemna gibba* L. (Vaillant *et al*., 2004), water fern, *Azolla caroliniana* (Bennicelli *et al*., 2004), parrot’s feather (*Myriophyllum aquaticum*), creeping primrose (*Ludwigia palustris*), and water mint (*Mentha aquatica*)

(Kamal *et al*., 2004). *Lemna gibba* L. has been occasionally indicated as a hyperaccumulator of heavy metals by several researchers (Kara *et al*., 2003; Vaillant *et al*., 2004; Mokhtar *et al*., 2011).

Elemental metal influence on herbivores is obviously not just restricted to terrestrial herbivores but can also affect insect performance on aquatic plants. For instance an increased Cd concentration in alligatorweed (*Alternanthera philoxeroides* (Mart.) Griseb) reduced the fecundity of the alligatorweed flea beetle, *Agasicles hygrophyla* Selman and Vogt up to 92% (Quimby *et al*., 1979). Copper concentrations between 0.01 to 0.64 mg/L Cu in water reduced first-instar feeding of *Paratanytarsus parthenogeneticus* Freeman (Diptera: Chironomidae) on green algae (Hatakeyama and Yasuno, 1981). Feeding damage caused by the weevil *Neochetina bruchi* Hustache (Coleoptera: Curculionidae) was significantly reduced when the biocontrol agent was exposed to accumulated concentrations of 232 µg Zn ⁄ 100 g d. wt., and 66.70 µg Cd ⁄ 100 g d. wt. in water hyacinth (Jamil *et al*., 1989a,b).

The research on metal interaction with water hyacinth weevils is limited and largely based on Cd. Even so, results of weevil interaction with such metals are not consistent. For instance, Hussain and Jamil (1992) found no mortality or any other symptoms in adult *N. eichhorniae* feeding on plants grown in Cd, Zn, Hg, and Mn at concentrations up to 100 mg/L. Similarly, Schmidt and Ibrahim (1994) found that *N. eichhorniae* survived a body concentration of 41.45 ppm Pb and 36.67 ppm Cd accumulated by feeding on contaminated leaves of water hyacinth, and suggested either that the weevil was able to detoxify the metals or that body concentration of the metals were still way below the threshold of the toxicity level. Unlike *Neochetina bruchi*, *N. eichhorniae* Warner, was not affected by levels of 8.00 and 17.20 µg of Cd/g in water hyacinth leaves, and did not show a significant difference in feeding from the control when exposed to water hyacinth with concentrations of 21.62 and 44.77 µg Cu/g in leaves and 5.89 and 9.84 µg Pb/g in the leaves (Kay and Haller, 1986). In contrast, Mogren and Trumble (2010) indicated a concentration of 232 µg Zn/100 g d. wt. of water hyacinth was able to reduce feeding in *N. bruchi* significantly compared to those in the control.

Thus, the effects of the heavy metals on the water hyacinth weevils remain unclear and require further studies.

In this study the effect of eight different heavy metals, including some of the heavy metals studied previously (from the literature) in relation to the water hyacinth weevils such as Cu, Hg and Zn and simulated acid mine drainage (AMD) on the water hyacinth weevils were investigated in a single-metal test and a mixture of a suite of metals and sulphates, respectively.

4.1.5 Feeding and reproduction of the *Neochetina* weevils.

Extensive infestation of the Vaal River by water hyacinth, particularly in the upper-middle Vaal, extending up to the Douglas Weir, creates a number of socio-economical and environmental problems. Different individual management techniques have been implemented but none has on its own successefully controlled water hyacinth, and hence the fight against it has shifted to Integrated Pest Management (IPM) (Byrne *et al*., 2010).

There are seven water hyacinth biocontrol agents introduced from Latin America and established successfully in South Africa on water hyacinth (Coetzee *et al*., 2011). Among these agents, the water hyacinth weevils *N. eichhorniae* and *N. bruchi* are the most widely used in the country. These nocturnal weevils are about 4-5mm long and spend the day sheltering in the leaf sheath or inside rolled leaves (DeLoach and Cordo, 1976; Oberholzer, 2001). On average the female produces 350 to 400 eggs in its life span. These are laid either deep in the younger leaf tissue or on the upper surface of older petioles for *N. eichhorniae* or *N. bruchi*, respectively (Oberholzer, 2001). The developmental phase of the *Neochetina* larva includes three instars and a pupal stage before it emerges as an adult weevil (DeLoach and Cordo, 1976). Under optimum conditions the eggs of *N. bruchi* hatch in one week, while the larvae and the pupae take 32 and 30 days, respectively to complete their developmental stages. When the egg of the *Neochetina* species hatches, the larvae start feeding by mining and tunnelling into the petiole towards the crown. The adult weevils feed on the epidermal layer of the leaves, usually leaving behind characteristic feeding scars (Del Fosse *et al*., 1976). DeLoach and Cordo (1976) found 66% of the adult feeding on the upper epidermal layer, 26.7% on the lower surface and the rest on the petioles. Ajuonu *et al*. (2007) measured a maximum of 212 scars per leaf, caused by weevil feeding of weevils and the damage caused by *N. bruchi* was twice that of *N. eichhorniae*. Both weevils can cause considerable damage to water hyacinth but alone have only satisfactorily (brought below surface cover of 10%) controlled the plant at one site in South Africa (New Years Dam in the Eastern Cape) (Byrne *et al*., 2010). The effect of heavy metals in water hyacinth on biocontrol is investigated in this chapter.

The morphological structure of the reproductive system in the *Neochetina* species consists of two ovaries, each of which consists of two ovarioles (Grodowitz *et al*., 1997). The two ovarioles from each ovary are connected by a single duct known as the lateral oviduct, and each of these from the two ovaries lead into the common oviduct, where eggs are fertilized (Fig. 4.1). Each ovariole has two components: the germarium and the vitellarium, where the germ cells and premature follicles and developing follicles are housed, respectively. The follicles are developing eggs with a central ova ensheathed in a follicular epithelium, which sloughs off as the follicle is pushed through the lateral oviduct. The layer of cellular residues (follicular epithelium) deposited at the base of the ovarioles during each ovulation through the lateral oviduct are known as follicular relics and each layer can be used to evaluate the reproductive activity of the weevil. However, since such follicular relics could also be formed as a result of degenerating follicles during lower quality food foraging or starvation of the female weevil, it is not the most reliable method to evaluate the functionality of the ovaries (Grodowitz *et al*., 1997). Nevertheless, the absence of follicular relics in the ovarioles indicates that there has been no any ovulation or reproduction yet (Byrne *et al*., 2010).

Based on the ovary's functionality, they are classified as parous, where the ovaries contains large swollen follicles potentially capable of producing eggs, and nulliparous (non-functional) those with reduced or no follicles (Fig. 4.2). Grodowitz *et al*. (1997) summarized four different stages of the ovarian functional status:

1. Parous, no follicular relics: fully functional ovaries with large matured follicles eggs, but no ovulation has taken place yet.
2. Parous, with follicular relics: fully functional ovaries with large matured follicles, and has reproduced or ovulated eggs before.
3. Nulliparous, no follicular relics: non-functional ovaries with no follicles and has not ovulated before.
4. Nulliparous, with relics: non-functional ovaries with no follicles, but has ovulated eggs before.

The weevil's egg production depends on temperature and the quality of nutrition. Under unfavourable conditions (e.g. poor nutrient quality of host plant), egg production degenerates as they are absorbed allowing the development of flying muscles and a generative phase starts when suitable conditions prevail (Buckingham and Passoa, 1985; Grodowitz *et al*., 1997). In South Africa the weevil reproduction and a surge of their population on water hyacinth starts in spring after September, when the temperature rises above 20°C. However, due to high level of eutrophication in South African water systems, the water hyacinth growth and exponential increase in population size outcompetes the damage caused by the weevils, whose population is building slowly after the cold winter (Coetzee *et al*., 2011). Reproduction and feeding activities of the *Neochetina* weevil could be reduced by heavy metals accumulated in their host plant. This chapter investigates the performance of the *Neochetina* weevils feeding on heavy metal or acid mine drainage contaminated water hyacinth plants, and tests the hypothesis that the weevil's activities such as the fecundity, adult and larval feeding and survival are affected by these water contaminants in the water hyacinth plant tissues.

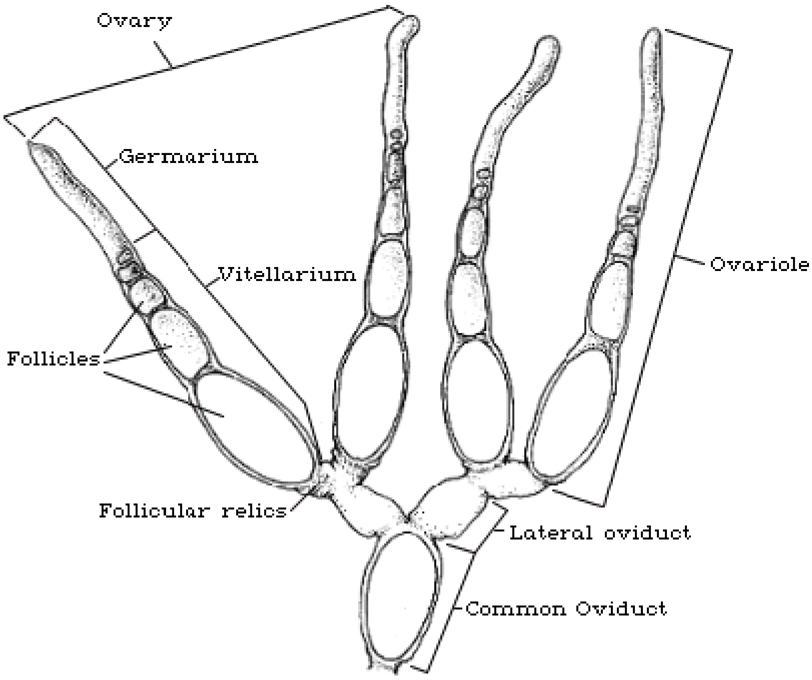


Figure 4.1: The different structures of the reproductive system of *N. eichhorniae* (After Grodowitz *et al*., 1997).

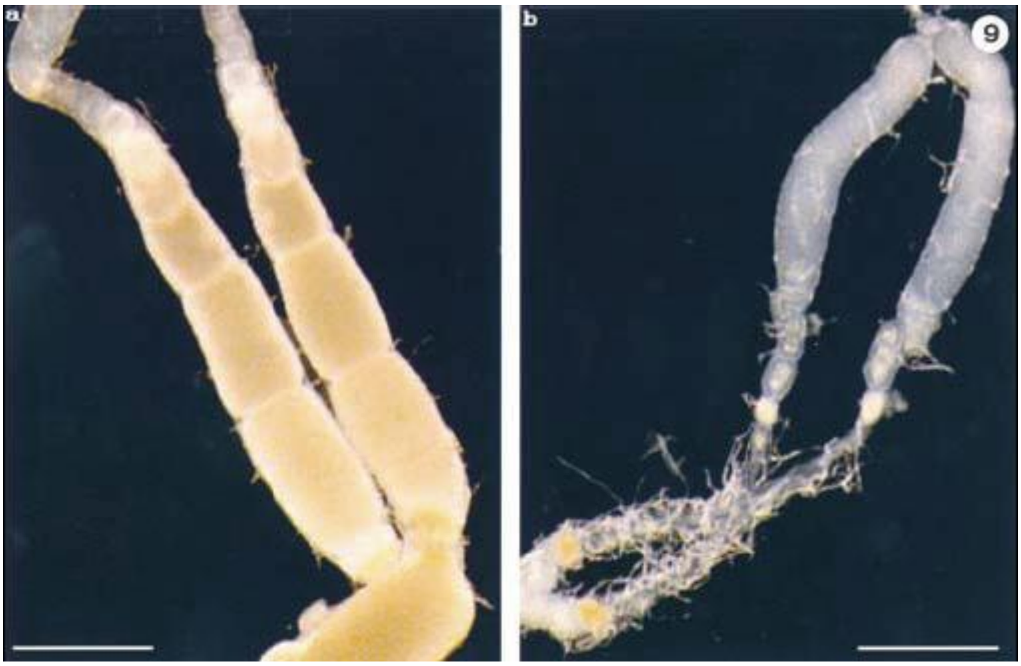


Figure 4.2: Functional status of *Neochetina* female ovaries (**a**) healthy (or parous) and (**b**) degenerate (or nulliparous) ovaries. Follicular relics are also evident at the bases of each ovariole, (Bar = 0.25 mm) (Grodowitz *et al*., 1997).

4.2 Materials and Methods

The effects of heavy metals accumulated in water hyacinth tissues on the feeding and reproduction of the water hyacinth biocontrol agents *N. eichhorniae* and *N. bruchi* were investigated in a single-metal tub and simulated acid mine drainage pool trials as discussed in chapters.two (sections 2.2.2 and 2.2.3) and three. The experiments were conducted in two phases as the metal uptake phase (the first three weeks after the addition of the heavy metals) and the weevil or the biocontrol phase (the following six weeks after the addition of the weevils on the same metal uptake treatments). This allowed the evaluation of metal-weevil interaction on water hyacinth plants. Single heavy metal treatments and a suite of heavy metal treatments were added to the single-element tub trial and AMD pool trial, respectively in different concentrations (for the experimental designs refer to sections 2.2.2 and 2.2.3). The adults released in each of these trials were collected from the South African Sugar Cane Research Institute (SASRI) in Kwazulu Natal province. Thus, the females could have been reproducing before collection. In addition, between the time of their collection and delivery, to the time of their release onto the trials, they were enclosed in perforated boxes with leaves of water hyacinth for one week. Such crowded containment could also affect the female reproductive capacity as the availability and food quality declines (Grodowitz *et al*., 1997). Hence, a sample of insects were dissected to evaluate the number of follicles in the ovaries to determine their functional status (parous or nulliparous) before release into both the single-element metal tub trial and the simulated AMD pool trial. Weevils were not added to the Vaal River trials.

4.2.1 The addition of weevils to the single-element system tub trial

Water hyacinth plants were grown under heavy metal treatments for three weeks in tubs, after which water and plant tissue samples were collected and stored at 4°C for four months for eventual analysis of contamination levels in the plant tissues (refer to sections 3.1.2 for sampling and preparation methods). Three weeks after the addition of heavy metals into each treatment, an average of 3.5 weevils per plant (60 in total) were released on to each tub, including the control treatments. The trial then continued for six more weeks and ended in week 9. At the end of the experiment several indicators of the weevil's efficacy as a biocontrol agent of water hyacinth were measured. These included: the number of weevil larvae found per plant (used as a crude surrogate for egg hatchability or the number of larvae produced by the female) and the number of larval mines; the number of adult survivors per plant and the number of adult feeding scars on leaf-2. The first two weevil parameters were counted from three plants per tub (three tubs per treatment), whereas feeding scars and survival of adult weevils were recorded from five plants per tub and all the plants in the tub, respectively. Two females each of *N. eichhorniae* and *N. bruchi* were dissected from each tub under a stereo microscope using 9X magnification (for details of the dissection technique refer to Byrne *et al*., 2010). The number of follicles from the ovarioles in each ovary was counted. The follicles in this study included the total number of both small and large follicles from the base of germarium to the bottom end of the vitellarium constriction before the lateral oviduct in each ovariole and those follicles present in the lateral and common oviducts. The number of follicles was also recorded from a sample of three female weevils, dissected before the start of the trial, to determine the pre-existing ovarian follicles and the general ovarial functional status. Observation of any follicular relics was also considered during the disection, but they were not clearly visible, which could probably be due to the difficulty of dissecting the ethanol (70%) preserved specimens in a petridish (half-filled beeswax) with tap water to immerse the specimens. Grodowitz *et al*. (1997) used phosphate-buffered saline solution of (about pH 7.0) to soak and maintain the correct osmotic pressure and living specimens to avoid the damage of the delicate reproductive tissues by preservatives and was able to clearly identify all ovarian features including the follicular relics.

4.2.2 The addition of weevils to the AMD pool trial

Water hyacinth was grown in a suite of metal treatments in 2170 L pools with one of the three doses of $MgSO_4$. Water and plant tissue samples were collected after three weeks, before the addition of weevils (refer to section 3.1.3 for sampling and preparation methods). Similar to the tub trial, an average of 3.5 weevils per plant and a total of 800-1000 weevils per pool (depending on the plant density) were released onto three of the six pools in each AMD treatment, while the remaining three pools of each row were kept as a controls without weevils. Weevils were then allowed to feed on the water hyacinth for six weeks before the experiment was terminated in week 9. The weevil survival, feeding and reproduction were recorded. The numbers of adult weevil survivors were counted from a total of ten plant samples per pool, while the number of larvae, mined petioles, and adult feeding leaf scars were counted from a sample of five plants per pool. A total of 12 adult female weevils per treatment (four per pool) were dissected at the end of the experiment in week 9 to quantify total number of follicles in all four ovarioles per female. Twelve female weevils were also dissected before release of the weevils into the pools to determine the pre-existing ovarian follicles of the females prior to their exposure to metal and AMD treated plants. A week in this study is represented by approximately six days.

4.3 Data analysis

One-way ANOVA (the Analysis of Variance) followed by Fisher's Least Significant Difference (LSD) post hoc test was used to compare the number of larvae and adults found per plant, feeding as leaf mines and scars, and the females' fecundity between treatments in the single-element system tub and simulated AMD pool trials. The mean number of follicles in both the single metal tub trail and the simulated AMD pool trial were calculated as a difference, by subtracting the mean number of ovarian follicles found in female weevils before their release from those found in each treatment six weeks after their release in each trial. This allows avoidance of false positives as a result of follicles produced before the start of the experiment. This is because the weevils were not directly used from their pupae and disction prior the start of the experiment had shown that there were some ovarian follicles in the ovarioles. STATISTICA Six Sigma (Statsoft Release 7, 2006) and Microsoft Office Excel 2007 were used for data analysis.

4.4 Results

Flight mucles of the weevils develop only when the food quality deteriorates. Therefore, the issues of emigration and immigration of weevils between the tubs or the pools were negligible, since the weevils managed to feed on all the plants, although feeding was significantly reduced. For instance, no weevils were found in the control (no-weevil treatments) pools. In general Cu, As, Zn, and Hg reduced weevil feeding, survival, and reproduction in both in the tub and the pool trials. The adult feeding in the tub trial was significantly reduced by Cu and As, while survival was only reduced significantly by the Cu treatment compared to all the others treatments. The larvae were more sensitive to heavy metals than the adults. The larval mines in all the metal treatments were significantly fewer than those in the control treatments with the exception of the U treatment. Similarly, all the treatments yielded a significantly lower number of larvae per plant than the control treatment. The number of ovarian follicles per female weevil was significantly reduced in As, Cu, Hg, Mn-H and Zn treatments by over 92% compared to the control treatment and the same heavy metal treatments also showed a similar trend of low numbers of first and second instar larvae per plant, compared to the control treatment. The adult feeding in the pool trial did not show significant differences between the AMD treatments. However, the mean number of larvae and their feeding mines were significantly lower in the medium and high AMD treatments compared to those in the low AMD treatment. The same was true for the mean difference in the number of the ovarian follicles found per female, where the number of follicles in the high AMD concentration treatment was reduced significantly by 64% compared to those found in the low sulphate treatment.

4.4.1 The effect of heavy metal on *Neochetina* weevils in the single-element tub trial

The number of adult feeding scars showed significant differences between treatments and Cu, and As treatments showed the greatest reduction of all ($F_{(12, 104)} = 2.1349$, $P < 0.021$) (Fig. 4.3A). However, only As and Cu had significantly fewer feeding scars than the control treatment. A similar pattern emerged in the number of feeding mines, where all metal treatments except U significantly reduced the number of petioles mined by *Neochetina* weevil larvae ($F_{(12,104)} = 4.259$, $P < 0.001$), and the Cu, As, and Zn treatment had significantly fewer petioles mined than all the other treatments (Fig. 4.3B). Unlike the adult feeding scars, the larval feeding mines on Hg treated plants were significantly fewer by 35%, compared to that of the control. Both the adult and larval feeding showed no significant differences between the different concentrations of iron or manganese treatments (Fig. 4.3A and B).

Adult weevil survival, and the number of ovarian follicles produced per female weevil also showed significant differences between the heavy metal treatments (($F_{(12, 24)} = 3.4108$, $P < 0.005$) and ($F_{(13, 106)} = 4.1777$, $P < 0.001$), respectively) (Fig. 4.3C and D). However, such difference in the adult survival was only shown by the Cu treatment, where the adult weevil adult survival per plant was reduced by 55% compared to the control treatment. The adult survival in the Cu treatment was the lowest of all the treatments.

The number of ovarian follicles per female was significantly lower in the Hg, Cu and Zn treatments compared to the control treatment follicle production (both matured and unmatured follicles in the ovaries). However, all three treatments were not significantly different from the ovarian follicles of the female weevils prior to the start of this experiment ("S" in Fig. 4.3D). The size and the number of ovarian follicles produced by females in each of the As, Cu and Zn treatments are compared to those in the control treatments in Figure 4.4.

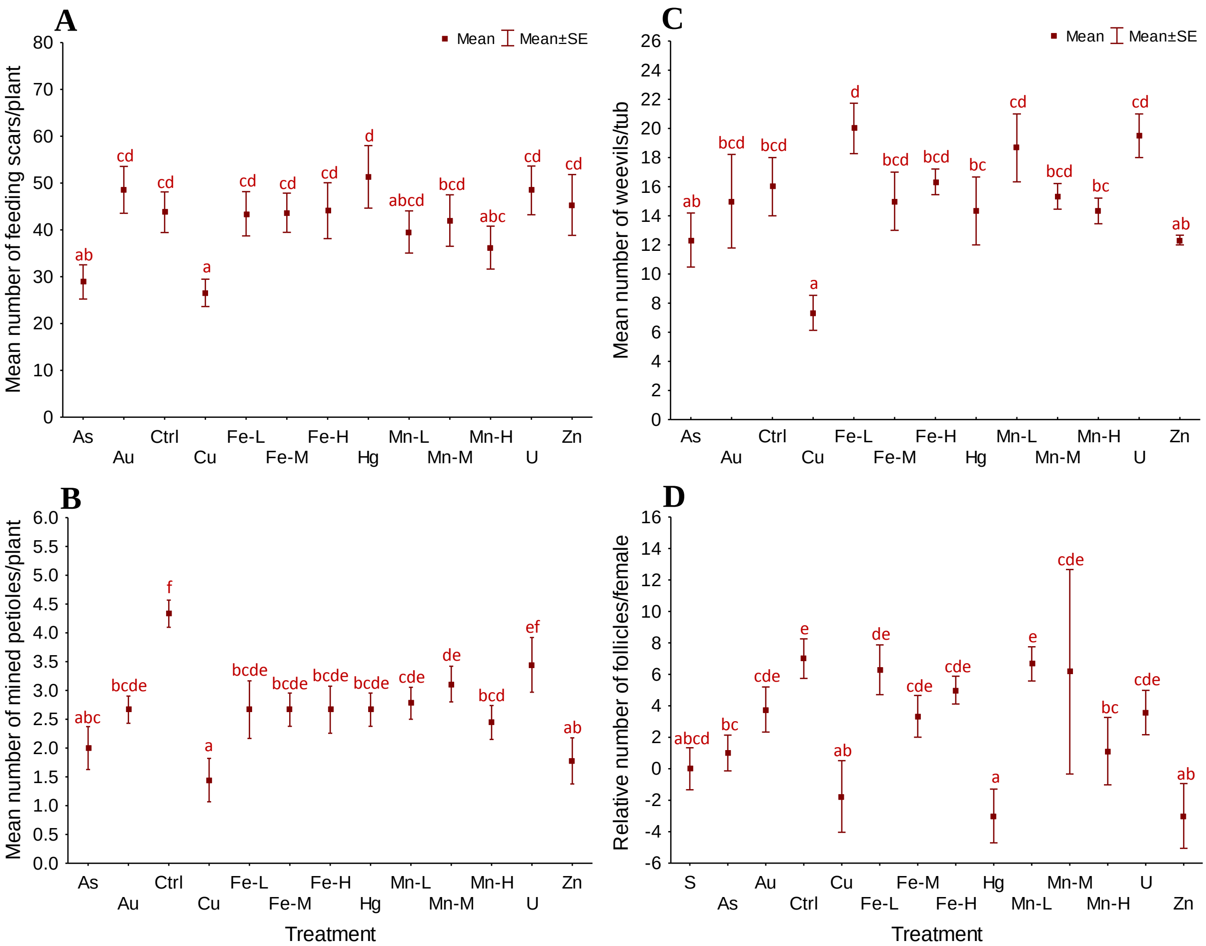


Figure 4.3: The effect of single heavy metal treatments on *Neochetina* weevil activity in the single-element system tub trial in week 9, six weeks after their release: (**A**) Mean number of adult feeding scars per plant, and (**B**) Mean number of larval mined petioles per plant, (C) Mean numbers of adult survivors per tub, and (**D**) Mean number of ovarian follicles per female, related to the number (S) of ovarian follicles in the females at the start of the trial. Means were compared by One-way ANOVA and those followed by the same letter(s) are not significantly different (P>0.05; Fisher LSD test).

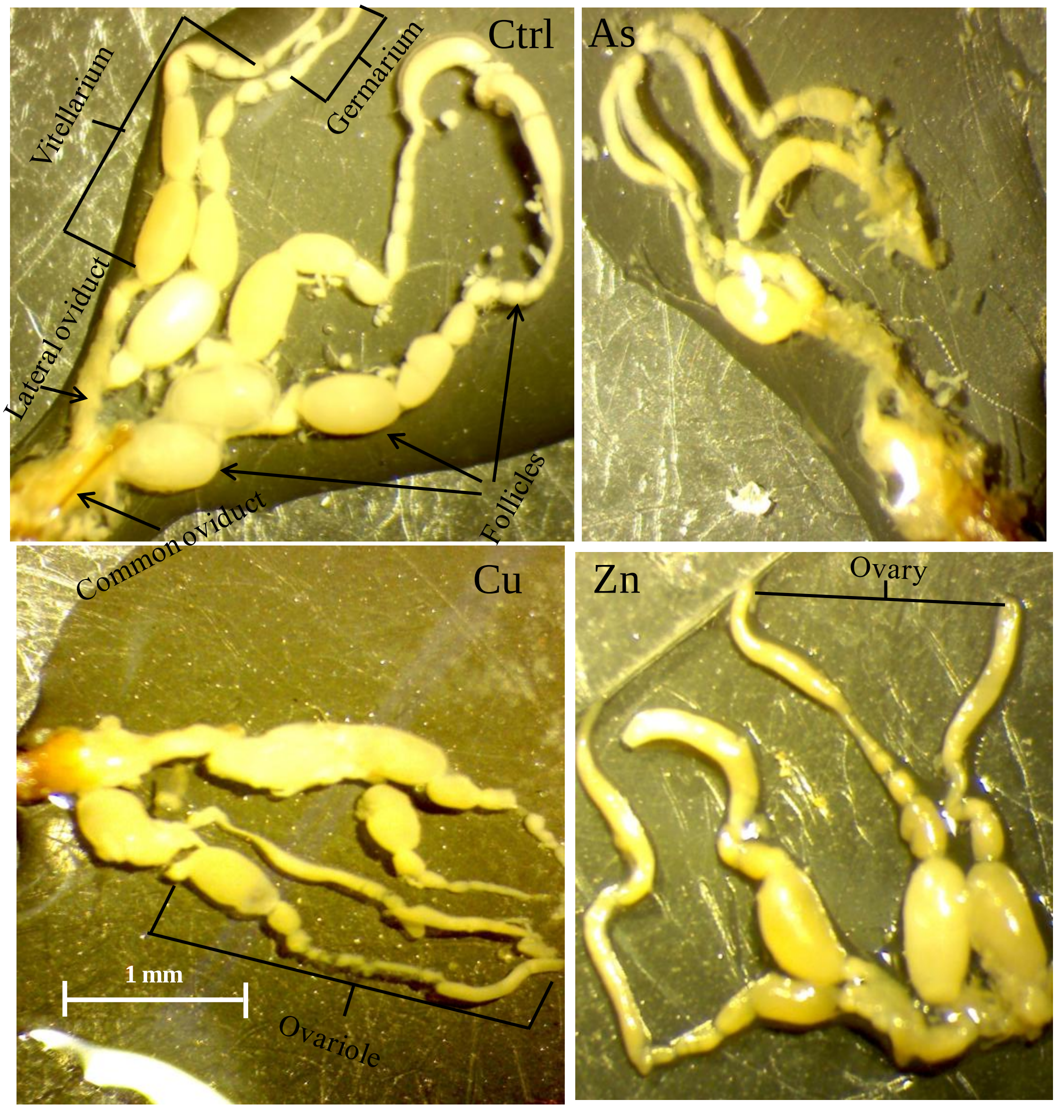


Figure 4.4: Weevil ovaries from female *Neochetina eichhornia* feeding on water hyacinth grown either with or without heavy metal treatments: (Ctrl) ovaries are healthy with many large functional follicles, ovaries from females feeding on metal treated plants (As, Cu and Zn) show reduced numbers of ovarian follicles with degenerating ovaries.

Based on the number of larvae found per plant and their feeding mines, the female weevils in all the treatments had produced eggs. However, the mean numbers of larvae found per plant in all the metal treatments were significantly lower compared to those in the control treatment, and Cu, As, Hg, Mn-H and Zn treatments showed the lowest numbers of all ($F_{(12, 104)} = 3.1264$, $P < 0.001$) (Fig. 4.5A). The mean numbers of the first and second instar larvae and the proportion of the larvae in the second instar were also significantly lower in the same metal treatments compared to the control treatments ($F_{(12, 104)} = 2.7697$, $P < 0.002$), ($F_{(12, 104)} = 2.3803$, $P < 0.009$), and ($F_{(12, 104)} = 1.8588$, $P < 0.048$), respectively) (Fig. 4.5B and C).

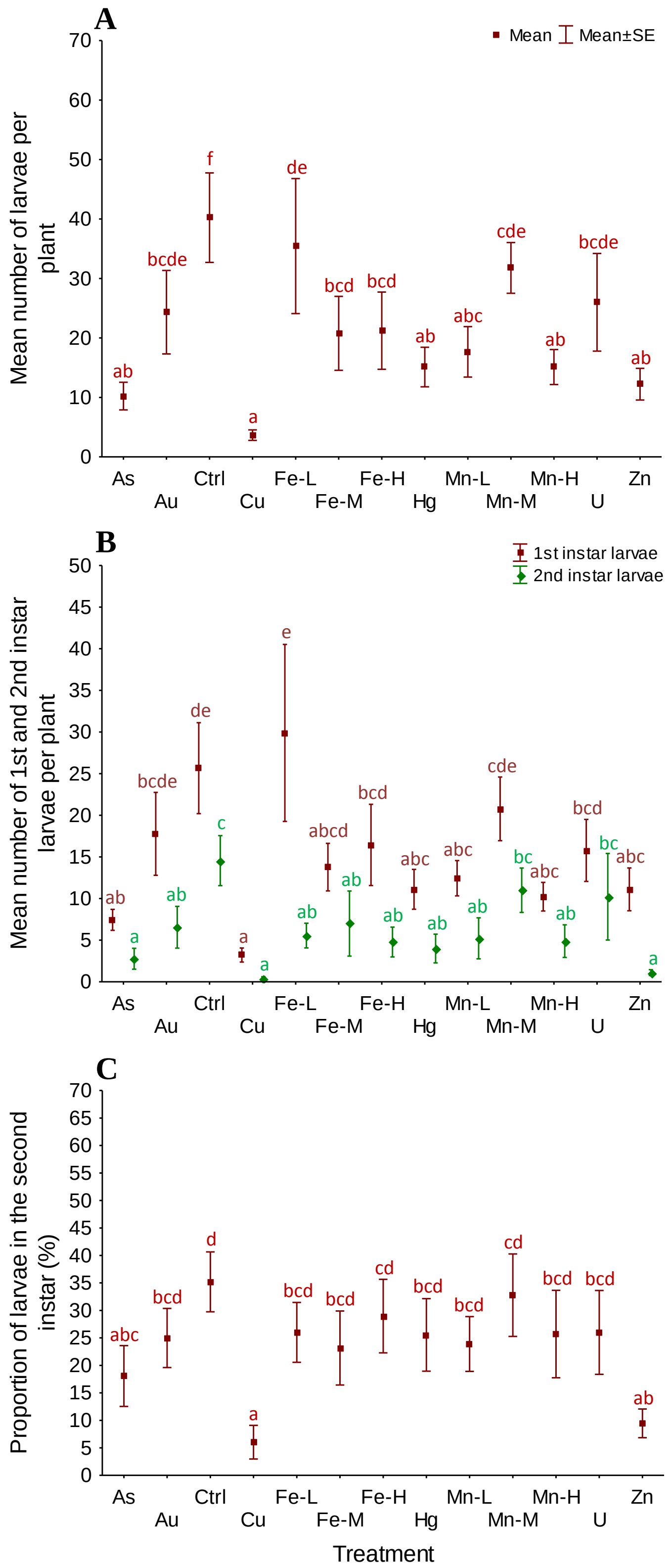


Figure 4.5: The effect of single heavy metal treatments on *Neochetina* weevils in a single-element tub trial in week 9, six weeks after weevil release: (**A**) Mean numbers of larvae produced by the female weevils per plant (**B**) Mean number of first and second instar larvae per plant, and (**C**) The proportion of larvae in the second instar per plant. Means were compared by One-way ANOVA and those followed by the same letter(s) are not significantly different ($P > 0.05$; Fisher LSD test).

4.4.2 The effect of metals and AMD on *Neochetina* weevil in AMD pool trial

The feeding response of the adults and the larvae weevils to the heavy metals and AMD contaminated plants was different. The adult weevil feeding in this trial showed no significant difference between the AMD concentration treatments ($F_{(2, 42)} = 2.2664$, $P < 0.116$) (Fig. 4.6A). However, the larval feeding was significantly lower in the medium and the high AMD concentration treatments than in the low AMD treatment ($F_{(2, 42)} = 12.4444$, $P < 0.001$) (Fig. 4.6B). The relative number of ovarian follicles per female weevil was significantly lower in the high AMD concentration treatment compared to the low AMD treatment (($F_{(3, 23)} = 4.9668$, $P < 0.008$) (Fig. 4.6C). The relative number of ovarian follicles in the high AMD treatment was not significantly different from the number of ovarian follicles found in the female weevils before the start of the trial. The pattern of the number of larvae found per plant mirrored that of the larval feeding mines, where both the medium and high concentration treatments showed significantly lower number of larvae per plant compared to the low AMD treatment ($F_{(2, 42)} = 14.2324$, $P < 0.001$) (Fig. 4.6D). In both cases (the number of larval feeding, and the number of larvae per plant) there were no significant differences between the medium and high AMD concentration treatments.

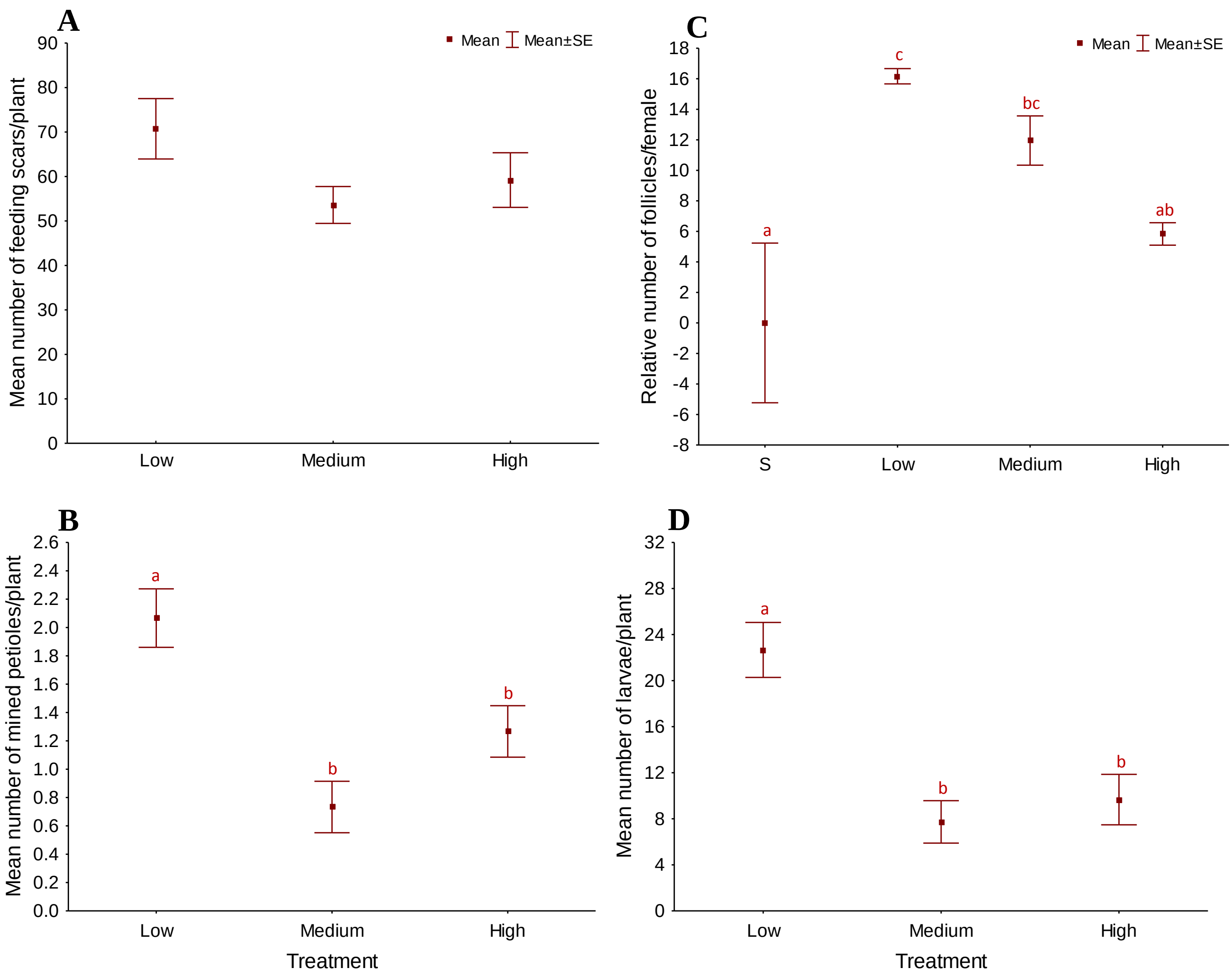


Figure 4.6: The effect of different AMD treatments on *Neochetina* weevils feeding on water hyacinth in a simulated AMD pool trial, in week 9, six weeks after the release of the weevils: (**A**) Mean number of feeding scars per plant, (**B**) Mean number of mined petioles per plant, (**C**) Mean number of ovarian follicles per female weevil related to the number (S) of follicles in the females at the start of the trial, and (**D**) Mean number of larvae found per plant. Means were compared by One-way ANOVA and those followed by the same letter(s) are not significantly different ($P > 0.05$; Fisher LSD test). NB: a graph with no letters indicate the absence of significant difference between treatments.

4.5 Discussion

The performance of the water hyacinth weevil, measured as adult and larval feeding and survival, the relative number of ovarian follicles (both matured and unmatured follicles) per female weevil, and the larval developmental stages, generally decreased in the metal treated-plants compared to the control treatment. Copper and As, followed by Hg and Zn treatments were the most stressful heavy metals to the weevils in the single-element system tub trial. This pattern of weevil response to pollutants in the single-element system was similar to that in simulated AMD pool trial. The high sulphate AMD treatment was the most stressful to the water hyacinth weevils compared to the low and medium AMD treatments. In both the single-element tub and the simulated AMD pool trials, the weevil larvae showed greater sensitivity to the heavy metal and AMD treatments than the adults, and this could be mediated by metal effects on female weevil egg production and larval survival.

4.5.1 Weevil performance in the single-element system tub trial

Plants that grow under heavily polluted conditions and particularly those plants which are metal accumulators or hyperaccumulators may have resistance to some natural enemies conferred on them by the metal (Boyd, 2010). Butler and Trumble (2008), reviewed 16 families of insect herbivores from five different orders and indicated reductions in the insects' feeding and reproductive parameters due to negative effects of heavy metals and metalloids accumulated in plant tissues. The pathways of heavy metals from the environment into insect's body could be through the trachea, cuticle, or the gut (Huang *et al*., 2012). The results in the single-element system tub trial suggests that the larvae of *Neochetina* weevils are more sensitive to Zn and Hg metal accumulation in the plant tissue than the adult weevils, whereas Cu and As reduced both adult and larval feeding (Fig. 4.3A and B). The concentrations of Cu, Hg and Zn in the shoot tissues were 44.9 ± 3.8 mg/kg, 35.9 ± 6.2 mg/kg, and 373.1 ± 8.7 mg/kg d. wt., respectively (see Chapter-3). Mogren and Trumble (2010) showed that the feeding damage of *N. bruchi* decreased significantly on plants with 232 µg Zn/100 g d. wt. Similarly Pollard and Baker (1997) found preferential feeding of two leaf chewing insect herbivores on leaves of *Thlaspi caerulescens* (Brassicaceae) with lower Zn concentrations compared to those with high concentrations, which showed little or no feeding. The low and high Zn concentrations in their studies were 14045 ± 891µg/g and 1474 ± 451 µg/g for the locusts, *Schistocerca gregaria* (Orthoptera: Acrididae), and 528 ± 63 µg/g and 7432 ± 732 µg/g for the caterpillars of *Pieris brassicae* (Lepidoptera: Pieridae), respectively. Similar results were also found in the present study, where the larval feeding, and survival as well as the female fecundity were reduced compared to the control treatment.

On the other hand Kay and Haller (1986) found that the feeding damage caused by adult *N. eichhornia* on water hyacinth grown in a water concentration of 2.5 mg/L Cu, was not significantly different from those of the control treatments, although they found a Cu concentration of 44.77 mg/kg d. wt. of water hyacinth leaves. Furthermore, they found significantly greater mortality in the control plants than in the Cu-treated plants after the weevils fed for 20 days. This contradicts the results of the present study, with similar Cu concentration of 44.9 ± 3.8 mg/kg d. wt., in the leaves of water hyacinth grown at Cu concentrations of 2 mg/L in water, where the weevil feeding damage and the number of weevils found per plant were significantly lower in the Cu-treated plants than in the control. The disparity in the feeding results could however be due to the fact that the plants in the current experiment were exposed to Cu for three weeks, after which the weevils were released and allowed to feed for six weeks, as opposed to that of Kay and Haller (1986), where the weevils were only allowed to feed for 10 days after four weeks of plant exposure to Cu. In addition, although they indicated that the weevil feeding was not affected by Cu contamination, no feeding data was presented in their results.

Generally the weevil activity decreased in the presence of most heavy metals in tubs, and a consistent severe reduction was shown in the As, Cu, Hg and Zn treatments (Figs. 4.3, 4.4 and 4.5). Copper was consistently the most stressful to all activities of the weevil as opposed to the effect of Hg, which was more detrimental to reproduction (female fecundity, larval survival and development) than to the adult weevil feeding and survival. Each of the four ovarioles in the control treatments were full of follicles, three to four times larger than those in the As, Cu, Hg and Zn treated plants. Presumably these were more capable of producing viable egg compared to those in the latter treatments, where the ovarian follicles were degenerate (Fig. 4.4).

Oviposition in the *Neochetina* weevil normally starts within three days after eclosion (adult emergence from pupal case) at a rate of five eggs per day for the first week and thereafter declines to a rate of 1.5 eggs per day (DeLoach and Cordo, 1976). The adult weevils in this trial were not collected directly from their pupae, and the time taken between their shipment from the site of collection to the site of the experiment and to the time of release onto the plants took one week. Thus, from the larval numbers the oviposition rate is calculated to be < 1.5 eggs per day. Although the number of oviposited eggs was not counted, it could be extrapolated from the mean number of larvae found per plant, where the highest number of 40 larvae per plant was found in the control treatment, while the lowest was less than 16 larvae per plant in the order of Hg>Zn>As>Cu (Fig. 4.5A). That is 1.1 eggs/day/female by the weevils in the control treatment and <0.44 eggs/days/female in the latter four metal treatments.

The heavy metal impact on weevils presumably depends on the amount of the element transported to and accumulated in the aerial system of water hyacinth. Hussain and Jamil (1992) showed an increase of heavy metal biotransfer to weevils with the increase of heavy metal concentrations in the leaves of water hyacinth. They found an accumulation of 0.35-0.63 μg Zn/mg and 0.11-0.2 μg Hg/mg in the body of *N. eichhornae* foraging on leaves of water hyacinth with concentrations of 6550-7920 mg/kg d. wt. and 4120-5620 mg/kg d. wt., respectively and unlike Hg (due to its low concentration in the weevil), Zn interfered with the normal protein metabolic processes of the weevils. This included the appearance of new metal binding proteins such as metalothionein, which they suggested to have a role in detoxification of heavy metals; because they also found no symptoms of toxicity in the weevil's feeding or mortality. Their results for Cd and Pb in the same experiment were also not different from that of Zn. Nevertheless, their results were not in agreement with the findings of the current trial, where Hg and Zn, among others, were generally detrimental to most activities of the weevils, at concentrations much lower in the leaves of the water hyacinth compared to those shown by Hussain and Jamil (1992). The disparity between the two results could be due to the fact that their feeding experiment was only conducted for ten days as opposed to six weeks in the current study. Moreover, there is no feeding or mortality data presented in their experiment.

Accumulation of heavy metals such as Hg, Cu, Cd and Zn in some insects induces the synthesis of new proteins, such as metalothionein, a chelatin with a strong affinity for heavy metal ions (Hussain and Jamil, 1992). This is a strategy for detoxification (Maroni *et al.*, 1987), while synthesis of other cellular proteins is inhibited and existing protein molecules may be degraded (Hussain and Jamil, 1992). In the single-element system tub trial the adult feeding in the Hg treated-plants was unaffected (Fig. 4.3A). Schmidt and Fielbrand (1987) found that the acridid, *Acrotylus patruelis* H.-S. (Orthoptera: Acrididae) feeding on wheat germ contaminated by Hg at concentrations between 0.6 to 12 mg/L, avoided toxicity through stimulation of egg production and oviposition process, and suggested that the Hg was decontaminated by the increased oviposition, which further increased at the F1 generation, and suggested that the Hg was removed by the increased oviposition process. Nevertheless, they also found that Hg concentrations of 6 mg/kg in the food reduced the adult lifespan and the hatchability of F1 generation nymph. In the current study however, the ovarial follicles and the mean number of larvae found per plant in the Hg treatment were reduced compared to the control treatments. Hussain and Jamil (1992) found that the adult *N. eichhornae* feeding on water hyacinth plants grown at concentrations of up to 100 mg/L of Hg in water, and accumulated a concentration of 5620 mg/kg d. wt., in leaves, were unaffected, and suggested the adult may have adapted to avoid its toxicity by binding them to protein complexes. This could also explain why the adult feeding on Hg-treated plants in this study was unaffected; in addition to the fact that the Hg concentration of the water hyacinth leaves in the present study was only 35.9 ± 6.2 mg/kg d. wt. (see Chapter-3).

The proportion of second instar larvae dropped by over 49% for Cu, As and Zn treatments compared to the control treatments, and Cu showed the highest reduction (81%) of all the treatments, suggesting increased mortality and delayed larval development as a result of metal toxicity (Fig. 4.5C). Similarly, Schmidt *et al*. (1992) found that the development of the nymphs of *A. thalassinus* fed on Hg and Cd contaminated wheat or barley seedlings at concentrations of 1.5, 3 and 8 mg/L and 2, 5, and 10 mg/L respectively, was prolonged at all the concentrations. Schmidt and Fielbrand (1987) also showed a delay of up to 40% in nymphal development of the Acridide, *Acrotylus patruelis* (H.-S.) (Orthoptera, Acrididae) fed at different concentrations of Hg (0.6, 1.2, 6.1 and 12.1 mg/kg d. wt.) contaminated wheat germs. The reduction in the number of the second instar larvae in the metal treatments in the single-element system tub trial indicates that even if adult weevils manage to feed and lay eggs under polluted circumstances, larval development will be hampered by metal-induced toxicity, which could eventually lead to reduction in the weevil population. Gahukar (1975) found no difference in larval development of *Ostrinia nubilalis* Hbn (Lepidoptera: Crambidae) between a control without $ZnSO_4$ treatments, and $ZnSO_4$ treated artificial diet at concentrations of 0.1-0.4% in the first week. But when extended to three weeks, those larvae fed on the highest a $ZnSO_4$ diets took the longest time to complete development and most died before the prepupal stage.

4.5.2 Weevil performance in the simulated AMD pool trial

The mean numbers of feeding scars inflicted by adult weevils in the simulated AMD pool trial were not significantly different between treatments, suggesting that the adult weevils were tolerant to the different AMD treatments. However, the adult fecundity, and both the mean number of larvae found per plant and their feeding were significantly reduced in the medium and high AMD treatments (Fig. 4.6), suggesting that the simulated AMD levels both in the medium and high AMD treatments adversely affected the weevil performance via oviposition.

Sulphate taken up by plants is sequestered and assimilated as a source of sulphur for plant growth, which is involved in the metabolic process such as in synthesis of proteins, enzymes or their precursors (Koralewska, *et al*., 2009). On the other hand, metals taken up by plants are largely stored in the cell wall, cell vacuoles and intercellular spaces to reduce metal toxicity (Fernandes and Henriques, 1991). For instance the largest portion of metals removed from water by plants of water hyacinth is stored in their roots (Misbahuddin and Fariduddin, 2002; Lu *et al*., 2004; Liao and Chang, 2004; Malik, 2007), followed by the stems. The lowest metal accumulation in water hyacinth is found in the shoot tissue (Kay *et al*., 1984). Adults of *Neochetina* weevil feed on the epidermal layer of leaves, while the larvae feed by tunnelling through the petioles into the crown (DeLoach and Cordo, 1976). Thus, although both stages of the weevils are chewers, the difference in the feeding sites between the weevil adult and the larvae in this trial suggests why the adult feeding was not affected by the AMD in all the different concentration treatments. Konopka *et al*. (2013) found that the green peach aphid, *Myzus persicae* (Hemiptera: Aphididae), which feeds on the phloem tissues, was not affected by Cd which is predominantly stored in the epidermal layer of leaves of the cadmium-tolerant *B. juncea* plants. The reproductive activity of the female weevil however, was reduced in this study. The number of follicles was significantly lower in females from the high AMD concentration treatment than those from the low treatment. The mean number of larvae found per plant was also significantly lower in the high as well as medium AMD treatments than in the low treatment.

4.6 Conclusion

The activities of both species of the *Neochetina* weevils were generally reduced by the metals and more particularly by As, Cu, Hg, and Zn. The larvae were more sensitive to the impacts of the metals or the acid mine drainage pollutants on which the water hyacinth plants were grown, compared to the adult weevils. The weevil experiment was not conducted in the field (the Vaal River) in a natural environment due to low numbers of plants and absence of weevils after the floods of 2009 and 2010. Nevertheless, although the metal concentrations in water were generally lower in the Vaal River at the sites of the plant experiment, compared to the simulated AMD pool trial in the current study, the sulphate concentration at some of the sites, such as the Schoonspruit (729 mg/L SO_4^{-2}) exceeded that of the medium AMD concentration treatment (700 mg/L SO_4^{-2}) in the pool trial (see Chapter 3). Thus, the potential for AMD pollution and heavy metal impacts on the performance of the weevils on water hyacinth in the field could be mirrored by those impacts measured in the simulated AMD pool experiments. The impact of each metal element (in the river water), even at lower concentration than those in the AMD pool trial, could collectively be as harmful to the weevils as a single metal present in the water at high concentration (Coleman *et al*., 2005). Compared to the results of Kay and Haller (1986) and Hussain and Jamil (1992), who found that the activities of the *Neochetina* weevil was generally unaffected by metals such as Hg, Cu and Zn, the current study showed otherwise, and these same metals were among the most stressful elements to the weevils, despite the fact that the concentrations of these metals in the water was less than those used in their studies. Although the general activities of the weevils, particularly in the four worst metals, and the medium (700 mg/L SO_4^{-2}) and high (1300 mg/L SO_4^{-2}) AMD concentration treatments declined significantly compared to the control treatments, the weevils to some extent persisted and managed to damage the plants. Nevertheless, their use as biocontrol agents will be hindered by the pollutants and should be used synergistically with sub lethal dose of herbicides.

The feeding damage of *Neochetina* weevil on growth of water hyacinth plants was therefore investigated in combination with the heavy metals in a single-element system tub trial and different concentration of simulated AMD in pool trial in the next chapter to determine if integrated pest management (IPM) of water hyacinth should include *Neochetina* weevils at AMD and metal contaminated sites.

Chapter 5

Interaction of water hyacinth with heavy metals and weevils

5.1 Introduction

5.1.2 Growth parameters of water hyacinth

Water hyacinth is an invasive aquatic plant that grows best in tropical and subtropical regions of the world (Center and Spencer, 1981). It is a plant that survives in a wide range of environmental conditions and is often referred as the most notorious aquatic weed, characterized by an extremely aggressive and invasive nature in places of its introduction (Malik, 2007). Water hyacinth has a capacity to double its biomass in 7 – 10 days (Malik, 2007; Villamil *et al*., 1979). A single plant of water hyacinth with 6-7 leaves produces a single new leaf per week on average (Center and Spencer, 1981; Byrne *et al*., 2010). The potential of water hyacinth's growth capacity and its ability to accumulate heavy metals has encouraged researchers and stakeholders of water resources and wetlands to utilize the plant as a phytoremediation agent for many water contaminants (Liao and Chang, 2004; Malik, 2007; Misbahuddin and Fariduddin, 2002; Falbo and Weaks, 1990; Mishra *et al*., 2008a).

The largest portion of heavy metals removed from water by water hyacinth is accumulated in the roots (Chapter-3) (Mishra *et al*., 2008c; Lu *et al*., 2004; Liao and Chang, 2004; Rahman and Hasegawa, 2011; Fayed and Abdel-El-Shafy, 1985). Heavy metals are stored predominantly in the root cell walls to avoid their toxic effects (Mishra *et al*., 2008c). Nevertheless, some heavy metals are also translocated into the leaves where they can damage the photosynthetic apparatus and other metabolic processes. Mishra *et al*. (2008c) found that the concentration of Cu, Cd, Mn, Pb and Hg in leaves of water hyacinth was higher compared to other aquatic macrophyte species (*Azolla pinnata*, *Lemna minor*, *Spirodela polyrrhiza*, *Potamogeton pectinatus*, *Marsilea quadrifolia*, *Pistia stratiotes*, *Ipomea aquatica*, *Potamogeton crispus*, *Hydrilla verticillata* and *Aponogeton natans*) sampled from a man made lake in Asia (Govind Ballabh Pant Sagar). This Suggests that some heavy metals are transported to water hyacinth shoots and depending on the kind and concentration of the metal, it could be potentially harmfull to photosynthesis. Some heavy metals are very toxic at lower concentrations than others and therefore water hyacinth responds with different degrees of stress depending on the heavy metal and its quantity in the plant tissues, particularly the aerial parts, by largely localizing most of the metals in the cell walls, cell vacuoles and intercellular spaces in the roots.

5.1.3 Heavy metal induced-stress in water hyacinth

Symptoms of heavy metal phytotoxcity in most aquatic plants are more conspicous in the aerial plant tissues and more specifically the plant leaves. This is because excess heavy metals disrupt photosynthetic and metabolic processes through the inhibition of electron transport at the redox sites in the photosystem I and II (Fernandes and Henriques, 1991). This generates reactive oxyradicals, leading to "oxidative stress", that react and decompose membrane lipid peroxides (Fernandes and Henriques, 1991; Smolders and Roelofs, 1996). Similarly, Prasad *et al*. (2001) showed that excess uptake of Cd and Cu into shoot tissues of *Lemna trisulca* (Araceae) decreased the rate of respiration by altering the gas exchange process. They suggested that mild metal induced stress increases the dark reaction whereas, severe metal induced stress decreases O_2 consumption, which could be due to the fact that excess heavy metals in plant tissues such as Cd, Pb, Hg, Cu, Ni, and Zn can directly influence the cell cytoplasm and cause structural damage to the mitochondria, and that their exclusion requires an increased net respiration. Phytotoxicity of heavy metals also interferes with the function of several enzymes, such as those involved in the dark reaction of photosynthesis (Stiborová *et al*., 1986). Mishra *et al*. (2008a) indicated that the reduction in chlorophyll and cell protein of water hyacinth plants growing in a contaminated man-made lake were due to chlorophyll degradation as a result of increased chlorophyllase and increased protease activities, enhanced by Hg accumulation, respectively. Among several symptoms of heavy metal phytotoxicity, leaf chlorisis and necrosis, stunted growth and water logging of tissues are very common (Kay *et al*., 1984; Shahbaz *et al*., 2010; Mocquot *et al*., 1996; Yruela, 2005; Xiong *et al*., 2006; Han *et al*., 2008; Burkhead *et al*., 2009). These however, depend on the type and concentration of the metal concerned. The natural concentration of Cu in fresh water does not usually exceed 0.002 ppm, and ranges between 0.05 – 0.2 mg/L in waters contaminated with acid mine drainage (Fernandes and Henriques, 1991). While the normal Cu concentration range is 3-20 mg/kg d. wt., for most plant species (Nriagu, 1979; Clarkson and Hanson, 1980; Howeler, 1983; Stevenson, 1986) concentrations exceeding this range in most aquatic plants are toxic. Similarly, Chaney (1989) indicated that the normal range of inorganic arsenic in plants is 0.01–1 mg/kg d. wt., while the phytotoxic concentration ranges between 3 -20 mg/kg d. wt.

Pathogenic or insect damage to plants alters the physiological and chemical status of the plants by changing the concentration of chlorophyll pigments, chemical concentrations, cell structure and nutrient and water uptake that affect the colour and temperature of the plant canopy (Raikes and Burpee, 1998). The hyperspectral results in Chapter-2 showed a decline in the spectral reflectance of water hyacinth grown in some of the heavy metal treatments in the single-element system tub trial and in some of the simulated AMD pool trial. The same treatments, which affected the spectral reflectance of water hyacinth in both trials, were also found to negatively affect the general activities of the biological control agent of water hyacinth (*Neochetina* weevils) (Chapter 4). Therefore, this chapter investigates the effect of different heavy metals and AMD treatments in conmbination with weevil feeding on the growth of water hyacinth plants. This is important to understand as it will influence the integrated pest management (IPM) on how to control water hyacinth at metal contaminated sites.

5.2 Materials and Methods

The effect of heavy metals and acid mine drainage on plants of water hyacinth grown under different heavy metal treatments and water hyacinth weevils was investigated in “greenhouse” experiments, conducted as a single metal tub trial and simulated AMD pool trial; and in the field at the inlets of two tributaries of the Vaal (which are potential sources of contamination). The main objective of this chapter is to evaluate the growth of water hyacinth plants under the influence of heavy metal and AMD and the biological control agent, the *Neochetina* spp. Different plant growth parameters were recorded at the start of the experiment and three weeks after the addition of specific metal treatments in the single-element tub trial and both metal and sulphate treatments in the simulated AMD pool trial. The same measurements were repeated six weeks after the addition of weevils to each of those trials. Measurements of plant parameters at the sites in the Vaal

River were recorded before and after the start of the seasonal rain. The experimental designs of the tub, pool and the Vaal River trials, including the coordinates of the cages at the Vaal River are presented in section 2.2 of Chapter-2. The metal uptake phase was conducted for 18 days which is presented as three weeks in the graphs. The end of the weevil phase in week 9 was 55 days in total. The field trial was conducted over a total of 40 days. The plant and weevil interaction was not included on the Vaal River trial due to the absence of agents at the time of the study, as a result of flooding which had swept away the plants and their agents downstream.

Measurement of the longest petiole, length of petiole of leaf number two (leaf-2 petiole) and the root length were taken from three plant samples per tub in the single element tub trial, resulting in a total of nine plants per treatment. The numbers of ramets, petioles and flowers per plant, were counted from all the plants in each tub. The youngest petioles at the centre (petiole number one) of each of two plants in each tub were tagged at the start of the experiment (week 0) just after the addition of the heavy metals to the tubs and the position of that leaf was recorded at the end of the metal uptake phase in week 3 to evaluate the rate of leaf production per plant. A total of nine leaves per treatment (three leaf-2 from each tub) were traced in outline onto A4 paper and area of each leaf was measured from a cut-out of that outline using a LI-3100 Area Meter (LI-COR, Inc., Lincoln, Nebraska USA 68504).

The same plant parameters were also evaluated in the simulated AMD pool trial. Three plants per pool were randomly selected to count the petioles, ramets and flowers, as well as to measure the longest petiole, length of leaf-2 petiole and root lenth. The rate of leaf production (leaf turnover per plant per week) was determined by tagging two plants per pool as above at the beginning of the experiment (Week 0) and their position was recorded in week 3 at the end of the metal uptake phase. Tagging of plants for leaf turnover was repeated again, just before the addition of weevils and the new leaf position recorded in week 9 (six weeks after the addition of the weevils to the pools). Plant density was also measured from each quadrat (0.25 m^2) per pool from six pools in the metal uptake phase (week 3), and from three quadrats from each of the three pools with weevils and three without weevils (control pools) in week 9, six weeks after the release of the weevil.

Similarly, plant parameters from the water hyacinth grown in cages on the Vaal River, at the sites above and below the inlets of the Koekemoerspruit and the Schoonspruit tributaries, were also taken before the start of the rain two weeks after the plants were placed in the floating cages at the sites (week 2) and seven weeks later (week 7), after the start of the rain. However, plant parameters from the cages at the inlet of the Koekemoerspruit are not presented here due to their damage by what appeared to be birds' feeding and frequent disturbance by water currents from the wake of water skiers from the nearby boating club. The length of the longest petiole, the length of leaf-2 petiole, the root length and the leaf area were recorded from each site on the Vaal River. Using the same sampling method as used in the simulated AMD pool trial, plant density was also determined from cages above and below the inlet of the Schoonspruit.

5.3 Data analysis

Comparisons of the same plant parameters were made between the different phases in the single-element tub trial and the simulated AMD pool trial and between the cages at the above and below the inlets of the Schoonspruit into the Vaal River. These were tested by One-way ANOVA (the Analysis of Variance) followed by Fisher's Least Significant difference (LSD) post hoc test. Comparison of selected metal treatments with the control treatment were also analysed using a Mann–Whitney non-parametric U test, comparing two independent sets of samples. Changes in any plant parameters, between the metal uptake and the weevil phases, in either the single-element system tub trial or simulated AMD pool trial or before and after the rain within and between the cages at the Vaal River were calculated by subtracting a data collected in one occasion from the other.

The relative plant growth in the metal uptake phase of the single element tub trial and simulated AMD pool trial was calculated by dividing the final fresh weight in week 3 (end of metal uptake phase) by the initial fresh weight of plant biomass (at the start of the experiment). The relative plant growth after the addition of the weevils for both trials however, was calculated by dividing the fresh weight of plant biomass at the end of the weevil phase in week 9 (final fresh weight six weeks after the addition of the weevils) by the plant biomass weight taken before the addition of the weevils, in week 3 (initial fresh weight). This allowed comparisons of plant growth to be made between the different trials. STATISTICA and Six Sigma (Statsoft Release 7, 2006) and Microsoft Office Excel 2007 were the computer packages used for data analysis.

5.4 Results

Different plant parameters were considered to evaluate the impact of heavy metals and AMD water pollution and feeding damage of weevils on water hyacinth. Copper and Hg were generally more stressful to the plants than many of the metal treatments in the single-element trial. Their impact during the metal uprtake phase was significant and more detrimental on plant prameteres such as the number of ramets, leaf area and biomass fresh weight than in many of the metal treatments. In the AMD pool trial, the high AMD treatment and to some extent the medium AMD treatment, showed more detrimental negative effects on the growth parameters of water hyacinth in the metal uptake phase (week 3) than the low AMD treatment. The weevils in the same AMD treatmens had also shown more stressful impacts on plant growth parameters than the low AMD treatment, six weeks after their addition to the pools (week 9). The leaf production per plant per week in both the single-element tub and simulated AMD pool trials consistently showed no significant difference between the different treatments. In the Vaal River, only the water hyacinth root length was found to differ between sampling occasions at both the upper and the lower sites on the Schoonspruit inlet on the Vaal River.

5.4.1 Plant growth parameters in the single-element system tub trial

In the metal uptake phase, three weeks after the addition of metals, the length of the longest petiole did not show any significant difference between the metal treatments ($F_{(12, 65)} = 1.0964$, $P > 0.378$) (Fig. 5.1A). After the release of weevils (including in the control treatment), in the weevil phase (week 9) Cu was the only treatment that showed a significantly shorter length of the longest petiole compared to the control ($F_{(12, 65)} = 2.3148$, $P < 0.015$) (Fig. 5.1A), and showed the greatest decrease in length compared to the control treatment, between the two sampling occasions (Table 5.1). The length of leaf-2 also showed significant differences between the metal treatments in week 3, although the Cu treatment did not show a significant difference compared to the control treatment ($F_{(12, 65)}$ = 1.9932, P < 0.039) (Fig. 5.1B).The difference between the initial length just before the addition of metals (Wk0) and three weeks after the addition of Cu (Wk3) was significantly less than that in the control treatment (Table 5.1). The same metal also showed significantly the shortest leaf-2 petiole of all the treatments in week 9 ($F_{(12, 65)}$ = 5.657, P < 0.001) (Fig. 5.1B). There was a significant difference in the root length between treatments on both sampling occasions, although it was only in week 9 that the root length in the Cu treatment was significantly shorter compared to the control and the other metal treatments (($F_{(12, 65)}$ = 2.0096, P < 0.0373), and ($F_{(12, 65)}$ = 8.9712, P < 0.001), respectively) (Fig. 5.1C). However, the differences in the root length of the Cu treatment between the initial (before the addition of metals) and the metal uptake phase in week 3 and between the week 3 and the weevil phase in week 9 were significantly less compared to those in the control treatment (Table 5.1). The root length increased significantly in all the treatments by week 9, after the release of the weevils compared to the metal uptake phase in week 3 ($F_{(12, 65)}$ = 3.9282, P < 0.001). However, the opposite was found in the Cu treatment, where the root length, decreased significantly compared to the control treatment (Table 5.1).

The leaf production recorded per plant per week in the first three weeks, before the addition of the weevils did not show significant difference between treatments ($F_{(12, 65)}$ = 1.0556, P > 0.411) (Fig. 5.1D). The mean number of ramets per plant however, showed a significant difference between treatments in the metal uptake phase in week 3, but not in the weevil phase in week 9 ($F_{(12, 65)}$ = 2.4819, P < 0.009) and ($F_{(12, 65)}$ = 0.9794, P < 0.477), respectively) (Fig. 5.1E). Treatments of Cu and Hg followed by Au, Mn-M and Mn-H treatment revealed significantly lower numbers of ramets than the control treatment in week 3. Unlike in the manganese treatments, the number of ramets did not show significant differences between the Fe-dose response treatments. The number of ramets in the Mn-H treatment was significantly lower than those in the Mn-L treatment (Fig. 5.1E).

The area of leaf-2 of water hyacinth declined significantly by week 9 after the addition of the weevils ($F_{(12,26)} = 2.9877$, $P < 0.009$). The mean area of leaf-2 also showed a significant difference between treatments after the initial three weeks, but not after the feeding of the weevils by week 9 (($F_{(12, 26)} = 3.0384$, $P < 0.008$) and ($F_{(12, 26)} = 1.1919$, $P > 0.338$) respectively) (Fig. 5.1F). The Cu and Hg treatments, along with Mn-H were the only treatments with significantly the smaller leaf areas compared to all the other treatments in the metal uptake phase, in week 3. The differences in leaf area between the initial (at the start of the metal uptake experiment in week 0) and end of the metal uptake phase (week 3) were greater in the Cu and the Hg treatments compared to those in the control treatments. In contrast, the differences in leaf area between week 3 and week 9 were significantly lower in the same two metal treatments and Zn treatment than those in the control treatment (Table 5.1).

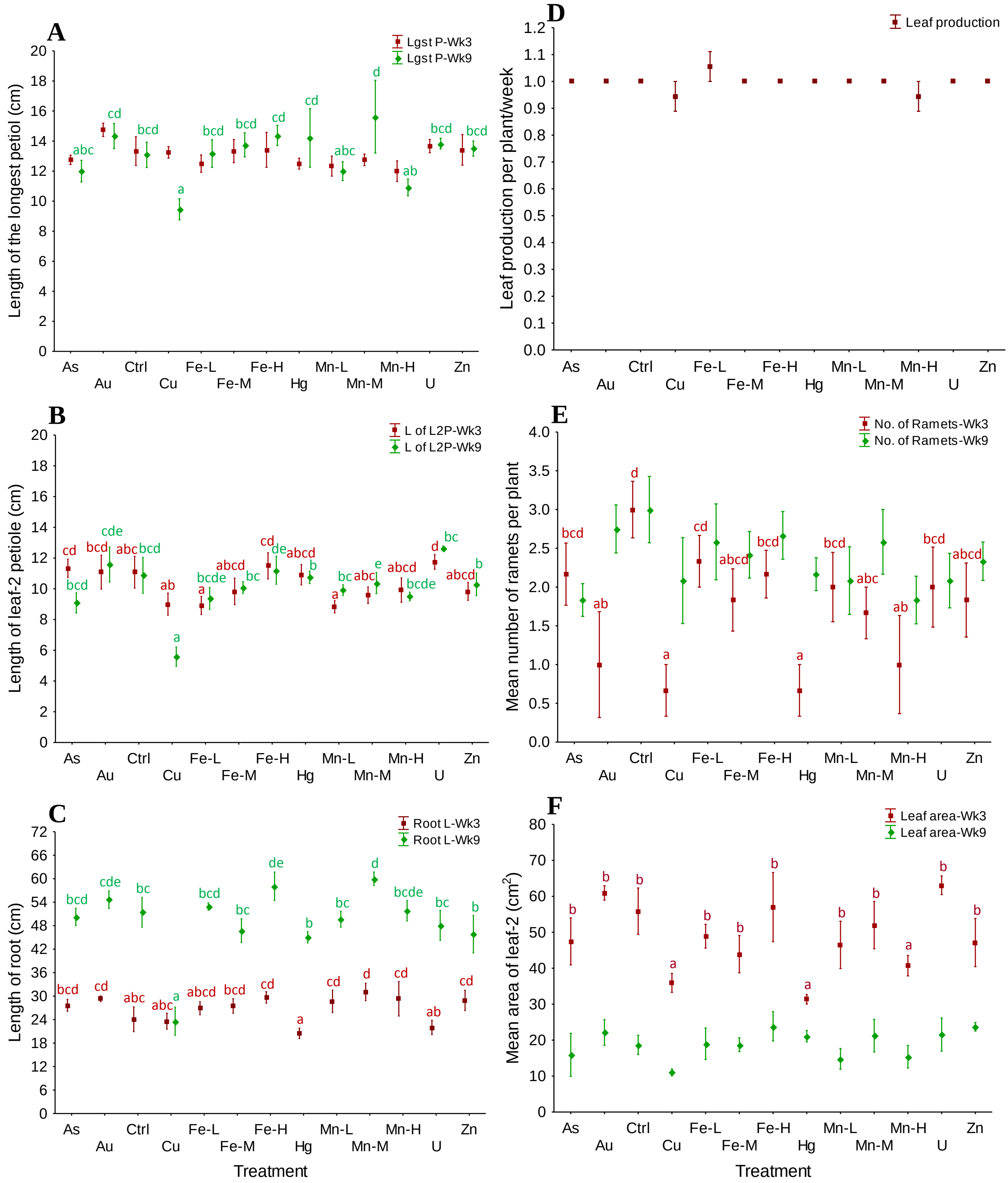


Figure 5.1: The effect of heavy metals on plant growth parameters of water hyacinth in a single-system element tub trial before the addition of the weevils (week 3) or after the addition of weevils (week 9): (**A**), (**B**) and (**C**) Lengths of the longest petiole, leaf-2 petiole and roots in week 3 and week 9, respectively, (**D**) and (**E**) Mean leaf production per plant per week and ramets per plant in week 3 and week 9, respectively, and (**F**) Mean area of leaf-2 in week 3 and week 9. Means compared by One-way ANOVA and those followed by the same letter(s) are not significantly different ($P > 0.05$; Fisher LSD test). Ctrl denotes the control treatment and the suffixes L, M and H denote low, medium and high concentrations, respectively.

Table 5.1: Plant growth parameter differences between different sampling occasions of water hyacinth grown in either Cu, As, Hg or Zn concetration (final metal uptake measurements in week 3 minus the initial measurements in week 0; and final measurements after the weevils in week 9 minus the initial measurements before the addition of the weevils in week 3) compared with water hyacinth plants grown in the control treatment in the single-element system tub trial.

Treatment	**Length of longest petiole**		**Length of leaf-2 petiole**		**Length of Root**		**No. of ramets**	**Leaf area**	
	Wk3-Wk0	**Wk9-Wk3**	**Wk3-Wk0**	**Wk9-Wk3**	**Wk3-Wk0**	**Wk9-Wk3**	**Wk9-Wk3**	**Wk3-Wk0**	**Wk9-Wk3**
Control	1.8 ± 1.4 a	0.3 ± 2.9 a	2.9 ± 1.8 a	- 0.8 ± 4.2 a	15.0 ± 7.7 a	32.6 ± 7.8 a	0.3 ± 1.1 a	- 0.7 ± 4.8 a	- 37.2 ± 5.7 a
Copper	0.8 ± 0.6 a	- 3.8 ± 0.8 b	1.8 ± 0.8 a	- 3.4 ± 1.1 a	7.28 ± 1.85 b	- 0.03 ± 4.1 b	1.4 ± 0.7 a	- 9.8 ± 6.7 b	- 24.9 ± 2.5 b
Arsenic	0.1 ± 0.5a	- 0.8 ± 0.8 a	1.0 ± 1.2 a	- 2.3 ± 0.3 a	13.92 ± 2.8 a	22.6 ± 1.5 a	- 0.33 ± 0.4 a	- 2.1 ± 8.1 a	- 31.6 ± 1.5 a
Mercury	- 0.42 ± 0.7 a	1.7 ± 1.8 a	0.1 ± 0.9	- 0.2 ± 0.9 a	8.17 ± 3.59 a	24.5 ± 1.7 a	1.5 ± 0.5 a	- 26.6 ± 0.6 b	- 10.3 ± 2.1 b
Zinc	0.1 ± 0.6 a	0.1 ± 1.3 a	0.0 ± 0.9 a	0.5 ± 0.7 a	11.3 ± 2.1 a	16.9 ± 6.8 a	0.5 ± 0.4 a	- 2.3 ± 9.4 a	- 23.4 ± 6.1 b

Means compared by non-parametric Mann–Whitney U test; each of the metal treatment was tested against the control treatment and those paired tests in the same column followed by the same letter(s) are not significantly different ($P>0.05$; Mann–Whitney U test). NB: comparisons of the four metals with the control were only selected due to their consistency with results in chapters 2, 3 and 4.

The initial fresh weight of plant biomass taken at the start of the experiment just before the addition of metals (week 0) was about 1.2 kg/tub in all the treatments, and showed no significant difference between treatments ($F_{(12, 26)} = 0.4665$, $P > 0.916$) (Fig. 5.2A). However, the fresh weight generally increased after week 3, but Cu and Hg treatments showed significantly lower plant biomass fresh weight/tub than the control at the end of the metal uptake phase in week 3 ($F_{(12, 26)} = 3.5293$, $P < 0.003$). Six weeks after the addition of weevils to the tubs, Cu was the only treatment that showed significantly lower plant biomass fresh weight/tub compared to al the other treatments in week 9 ($F_{(12, 26)} = 2.2932$, $P < 0.037$) (Fig. 5.2A). Comparison between the initial plant biomass fresh weight taken at the start of the experiment (week 0) and at the end of the metal uptake phase in week 3 revealed that the increase in plant biomass fresh weight/tub was significantly less in the Cu, Hg and Zn treatments compared to the control treatment ($F_{(12, 26)} = 2.4984$, $P < 0.024$) (Fig. 5.2B). Similar comparisons between the sampling occasions of week 3 and week 9 however, did not show any significant difference between the treatments ($F_{(12, 26)} = 0.9632$, $P > 0.505$).

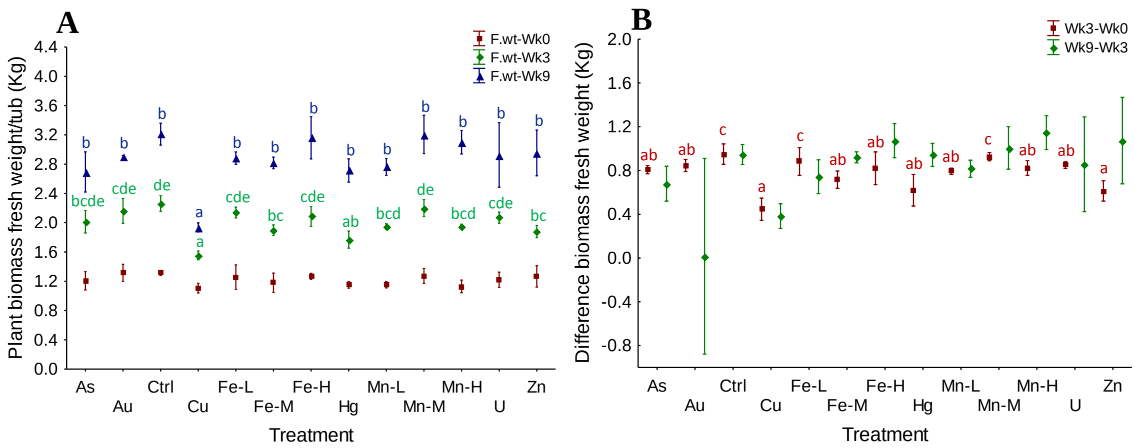


Figure 5.2: The effect of heavy metals on plant growth parameters of water hyacinth grown in the single-element system tub trial with and without weevils: (**A**) Mean fresh weight of plant biomass per quadrat of 0.25m^2, just before the addition of metal treatments (week 0), after the addition of metals (week 3) and after the addition of the weevils (week 9), and (**B**) Difference in plant density per quadrat of 0.25m^2, between week 3 and week 0, and week 9 and week 3. Means compared by One-way ANOVA and those followed by the same letter(s) are not significantly different ($P > 0.05$; Fisher LSD test). NB: Ctrl denotes the control treatment and the suffixes L, M and H denote low, medium and high concentrations, respectively.

Pictures as visual records of plant health, such as leaf chlorisis and necrosis, were also taken during the trial. Both Hg and Cu treated plants showed necrosis (leaves and some petioles dying and turning brown) one week (week 1) after the addition of heavy metal treatments (Fig. 5.3E and F). Leaf chlorisis was more pronounced in the Cu treated plants than in the control and Hg treatments in week seven (week 7) (Fig. 5.3G, H and I). Although leaf chlorisis was observed in all the three treatments at the end of the experiment in week 9 (six weeks after the release of the weevils), it was by far the most pronounced in the Cu treated plants, which turned entirely yellow followed by the Hg treatments (Fig. 5.3J, K and L).

In the metal uptake phase (week 3), results of the relative growth rate (RGR), showed no statistically significant differences between treatments ($F_{(12,\ 25)}$ = 0.6441, $P > 0.785$). However, after the addition of weevils the RGR showed significant differences between treatments in week 9 ($F_{(12,\ 25)}$ = 2.3788, $P < 0.0327$) (Table 5.2) and the Cu treatment showed significantly the lowest RGR of all the treatments with the exception of As, Fe-L and Fe-M.

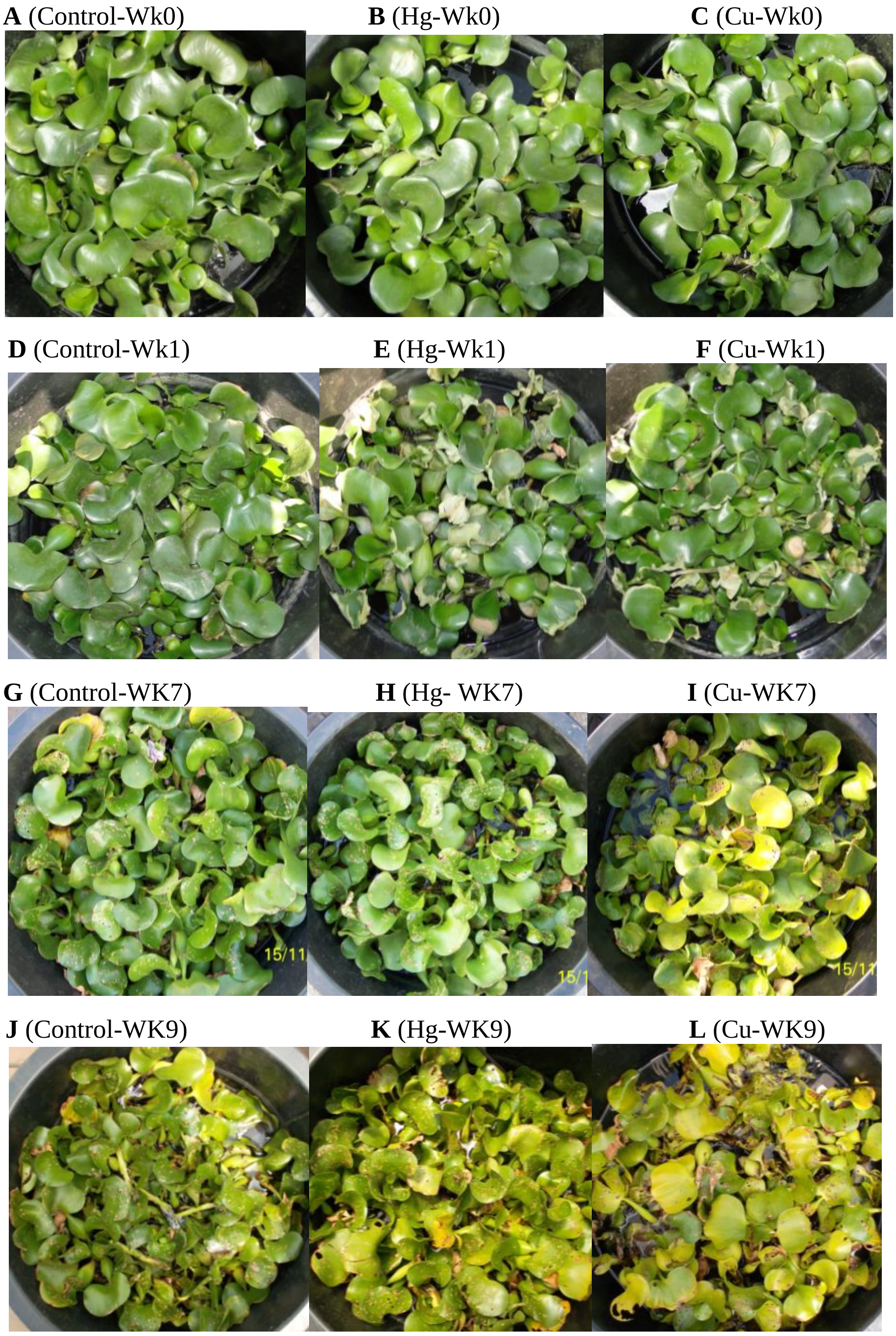


Figure 5.3: Leaf chlorisis and necrosis of water hyacinth plants in the single-element system tub trial: **A**, **B** and **C** control, Hg and Cu treatments respectively, just before the addition of metal treatments in week 0, **D**, **E**, **F**; **G**, **H**, **I, J**, **K**, **L** represent the same treatments in week 1 (before addtion of weevil), week 7 and week 9 (after addition of weevil) respectively.

Table 5.2: Relative growth rate of water hyacinth grown in the single-element system tub trial after the addition of heavy metals (week 3) and after the addition of weevils (week 9).

Treatment	Relative growth (metal phase)	Relative growth (Biocontrol phase)
As	1.68 ± 0.06	1.34 ± 0.07 ab
Au	1.65 ± 0.02	1.46 ± 0.02 bc
Ctrl	1.65 ± 0.02	1.46 ± 0.03 bc
Cu	1.56 ± 0.05	1.19 ± 0.05 a
Fe-L	1.60 ± 0.11	1.47 ± 0.12 ab
Fe-M	1.64 ± 0.03	1.49 ± 0.03 ab
Fe-H	1.65 ± 0.13	1.51 ± 0.05 bc
Hg	1.55 ± 0.14	1.54 ± 0.07 c
Mn-L	1.69 ± 0.04	1.42 ± 0.03 bc
Mn-M	1.74 ± 0.07	1.46 ± 0.09 bc
Mn-H	1.74 ± 0.12	1.59 ± 0.08 c
U	1.71 ± 0.08	1.52 ± 0.14 bc
Zn	1.52 ± 0.04	1.60 ± 0.31 bc

Means compared by One-way ANOVA. Means within the same column followed by the same letter(s) are not significantly different ($P > 0.05$; Fisher LSD test).

5.4.2 Plant growth parameters in the simulated AMD pool trial

The area of leaf-2 in the high AMD treatment at the start of the simulated AMD experiment, just before the addition of the metal and sulphate treatments (week 0), was significantly less than the low and the medium AMD treatments ($F_{(11,60)} = 12.8587$, $P < 0.001$) (Fig. 5.4A). Three weeks after the addition of the AMD treatments (week 3) the leaf area in all the three AMD concentration pools decreased significantly compared to those at the start of the experiment in week 0, but there was not any significant differences between the three treatments. After weevil feeding by week 9 (six weeks after the release of the weevils) the mean area of leaf-2 in both the medium and high AMD treatments was significantly smaller than the control treatments (with no weevils) (Fig. 5.4A).

The pattern of the mean fresh weight of plant biomass per quadrat ($0.25m^2$) in week 0 mirrored that of the area of leaf-2, where the high AMD treatment showed significantly lower plant biomass per quadrat than the low and the medium AMD treatments ($F_{(11, 24)} = 7.3143$, $P < 0.001$) (Fig. 5.4B). However, there was not any significant difference between treatments in the metal uptake phase in week 3. In the weevil phase (week 9), the plant biomass in all the AMD treatments was significantly lower compared to those in the control treatments and both the medium and the high AMD showed significantly lower plant biomass per quadrat than the low AMD treatment (Fig. 5.4B).

The pattern of the mean plant density was opposite to the pattern in the plant biomasss. The high AMD treatment in week 0, at the start of the experiment (just before the addition of treatments) showed significantly greater plant density than the others ($F_{(11, 24)}$ = (17.8886, $P < 0.001$) (Fig. 5.4C). The mean plant density per quadrat in the metal uptake phase (in week 3) dropped significantly compared to those at the start of the experiment in week 0 (before the addition of the AMD treatments) and the density was lower in the low AMD treatment than in the medium and the high AMD treatments. The mean plant density per quadrat also dropped significantly after the addition of the weevils, in week 9 compared to those in the control treatments (no weevil treatments) and the plant density in the low AMD treatment was significantly lower than in the other two AMD treatments (Fig. 5.4C).

The length of the longest petiole in the low AMD treatment increased significantly after the addition of the metal and AMD treatments, in week 3 ($F_{(11, 60)}$ = 8.5369, $P < 0.001$) (Fig. 5.4 D). In the weevil phase (week 9) the length of the longest petiole in the high AMD treatment was significantly shorter compared to the control treatment. The length of the leaf-2 petiole was significantly shorter in the high AMD treatment than the other two, in both week 0 and week 3 ($F_{(11, 60)}$ = 5.4848, $P < 0.001$) (Fig. 5.4E). However, there was not significant difference between the two sampling occasions. In week 9, the length of leaf-2 petiole in both the medium and high AMD treatments was significantly shorter compared to those in the control pools (no weevil pools in week 9), and the leaf-2 petiole in the latter was the shortest of all (Fig. 5.4E). Similarly, the root length at all the three sampling occasions was significantly shorter in the high AMD treatment than in the low and medium AMD treatments ($F_{(11, 60)}$ = 34.2292, $P < 0.001$) (Fig. 5.4F). However, there were not significant differences between the sampling dates (week 0 and week 3; and the control and the weevil treated plants in week 9) for this treatment.

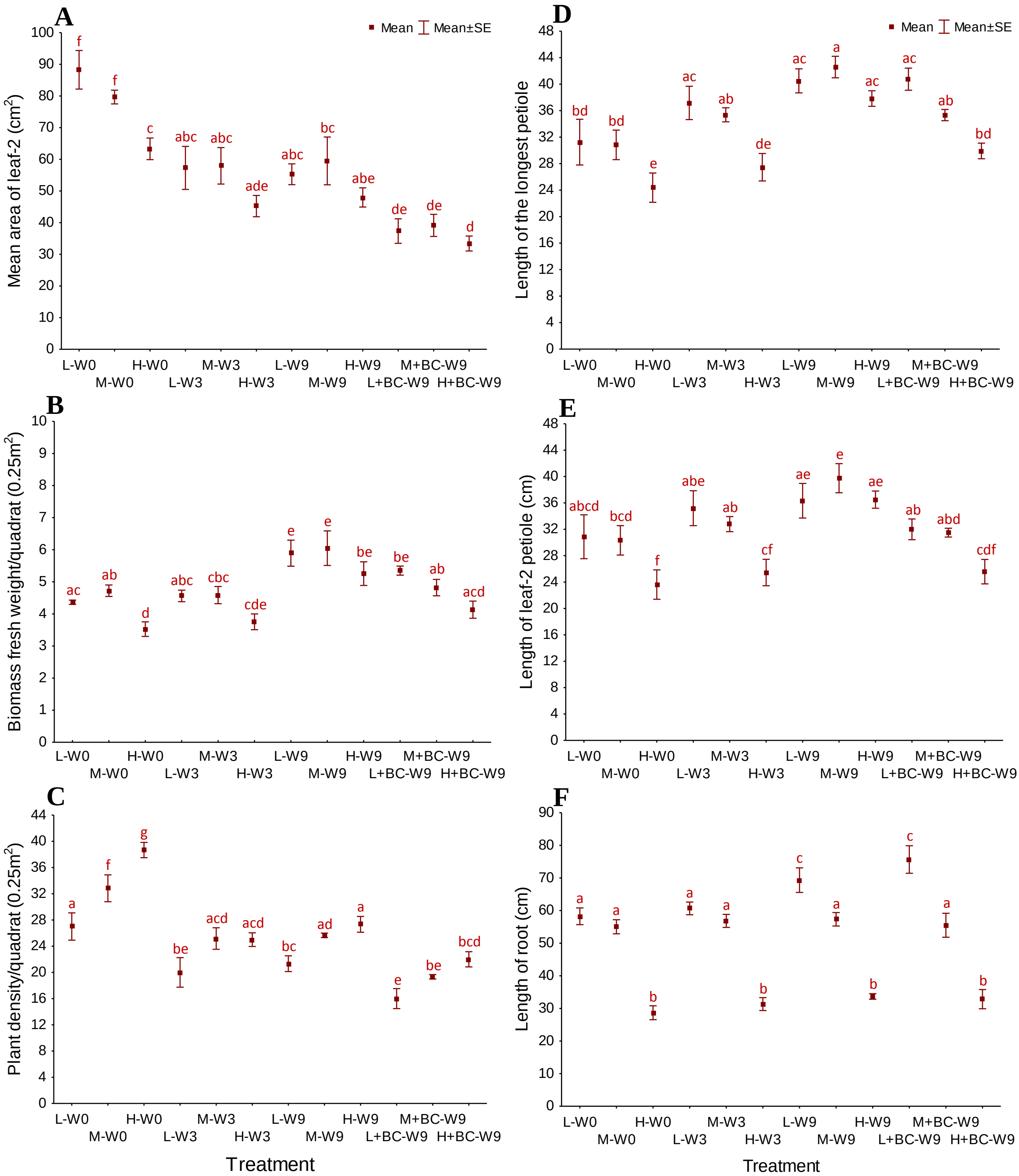


Figure 5.4: Effect of different simulated AMD concentrations on plant growth parameters of water hyacinth in simulated AMD pool trials in different sampling occasions (before the addition of AMD-W0, and before (W3) and after (W9) the addition of weevils (BC): (**A**) Area of leaf-2, (**B**) Plant biomass per quadrat ($0.25m^2$), (**C**) Plant density per quadrat ($0.25m^2$), and **D**, **E**, and **F** are the length of the longest petiole, leaf-2 petiole and root length, respectively. Means compared by One-way ANOVA and those followed by the same letter(s) are not significantly different ($P > 0.05$; Fisher LSD test). NB: the suffixes L, M and H denote low, medium and high concentrations, respectively.

The leaf production per plant did not show significant differences between treatments within and between the two sampling occasions, before and after the addition of the weevils in week 3 and week 9, respectively ($F_{(8, 45)} = 1.0456$, $P > 0.417$) (Fig. 5.5 A). An average of 0.75 leaves was produced per plant per week. The mean number of ramets within treatments on the same sampling occassion did not show any significant difference between the AMD treatments, but the number of ramets in the low and high AMD treatment in week 3, dropped significantly compared to the corresponding treatment at the start of the experiment in week 0 ($F_{(11, 60)} = 5.8586$, $P < 0.001$) (Fig. 5.5B).

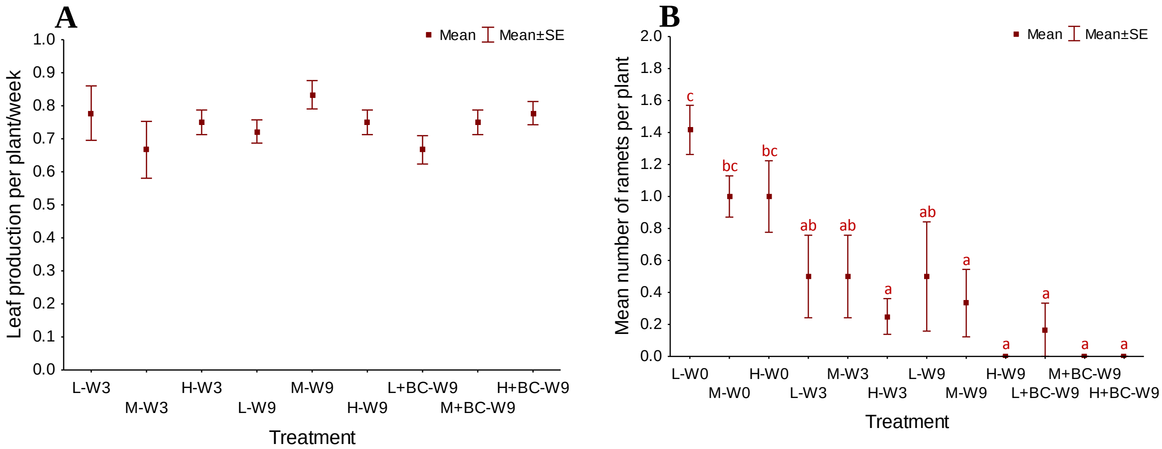


Figure 5.5: Effect of different simulated AMD concentrations on plant growth parameters of water hyacinth in simulated AMD pool trials in different sampling occasions (before the addition of AMD-W0, and before and after the addition of weevils (BC), W3 and W9, respectively: (**A**) Mean number of leaf production per plant per week, and (**B**) Mean number of ramets per plant. Means compared by One-way ANOVA and those followed by the same letter(s) are not significantly different ($P > 0.05$; Fisher LSD test). NB: the suffixes L, M and H denote low, medium and high concentrations, respectively.

The relative growth rate (RGR) of water hyacinth was significantly lower in the high AMD treatment in week 3 (metals) and week 9 (weevils) ($F_{(2, 14)} = 3.8266$, $P < 0.047$) (Table 5.3). The RGR in the medium AMD treatment was not significantly different from either the low or high AMD treatments at week 9 ($F_{(2, 6)} = 9.4426$, $P < 0.014$) (Table 5.3). However, the high AMD treatment was significantly different from the low AMD treatment.

Table 5.3: The relative growth rate (RGR) of water hyacinth grown in a simulated AMD pool trial without (week 3) and with water hyacinth weevils (week 9, six weeks after the release of the weevils).

Treatment	RGR (Week-3)	RGR (Week-9)
Low AMD treatment	1.05 ±0.03 a	0.91 ± 0.05 b
Medium AMD treatment	0.99 ± 0.01 a	0.88 ±0.03 ab
High AMD treatment	0.90 ± 0.02 b	0.79 ± 0.01 a

Means compared by One-way ANOVA. Means within the same column followed by the same letter(s) are not significantly different ($P > 0.05$; Fisher LSD test). NB: '±' is SE

5.4.3 The effect of AMD on the growth of water hyacinth in the Vaal River

Generally water hyacinth plants at the Schoonspruit inlet on the Vaal River showed more growth after the start of the rain than before the start of the rain. The longest petiole before and after the start of the rain did not show any significant differences between the upstream and downstream sites (($F_{(1, 6)} = 0.0004$, $P > 0.985$) and ($F_{(1, 6)} = 3.3282$, $P > 0.117$), respectively) (Fig. 5.6A). However, the longest petiole increased from 18 cm before the rain (week 2) to 41 cm after the rain in week 7. The length of leaf-2 petiole increased by a similar amount and both sites above and below the inlet of the Schoonspruit showed no significant difference between the sampling dates (($F_{(1,6)} = 0.3341$, $P > 0.584$) and ($F_{(1, 6)} = 1.6801$, $P > 0.242$), respectively) (Fig. 5.6B).

Root length was significantly shorter at the downstream site compared to the upstream site before and after rain ($F_{(1,6)} = 48$, $P < 0.001$) and ($F_{(1, 6)} = 35.3744$, $P < 0.001$) respectively (Fig. 5.6C). The root length showed a significant increase after the rain by week 7 at both sites, as did the leaf area ($F_{(1, 5)} = 6.6961$, $P < 0.049$) (Fig. 5.6C). Before the start of the rain (week 2) the mean area of leaf-2 did not show a significant difference between the two sites ($F_{(1,5)} = 0.664$, $P > 0.452$) (Fig. 5.6D). The number of petioles per plant did not show a significant difference between the sites or sampling dates (($F_{(1, 6)} = 1.4421$, $P > 0.275$) and ($F_{(1,6)} = 0.2588$, $P > 0.6291$), respectively) (Fig. 5.6E). The number of ramets per plant showed no significant difference between the sites before or after the start of the rain ($F_{(1, 6)} = 1.875$, $P > 0.219$) and ($F_{(1, 6)} = 0.509$, $P > 0.502$), respectively) (Fig. 5.6F).

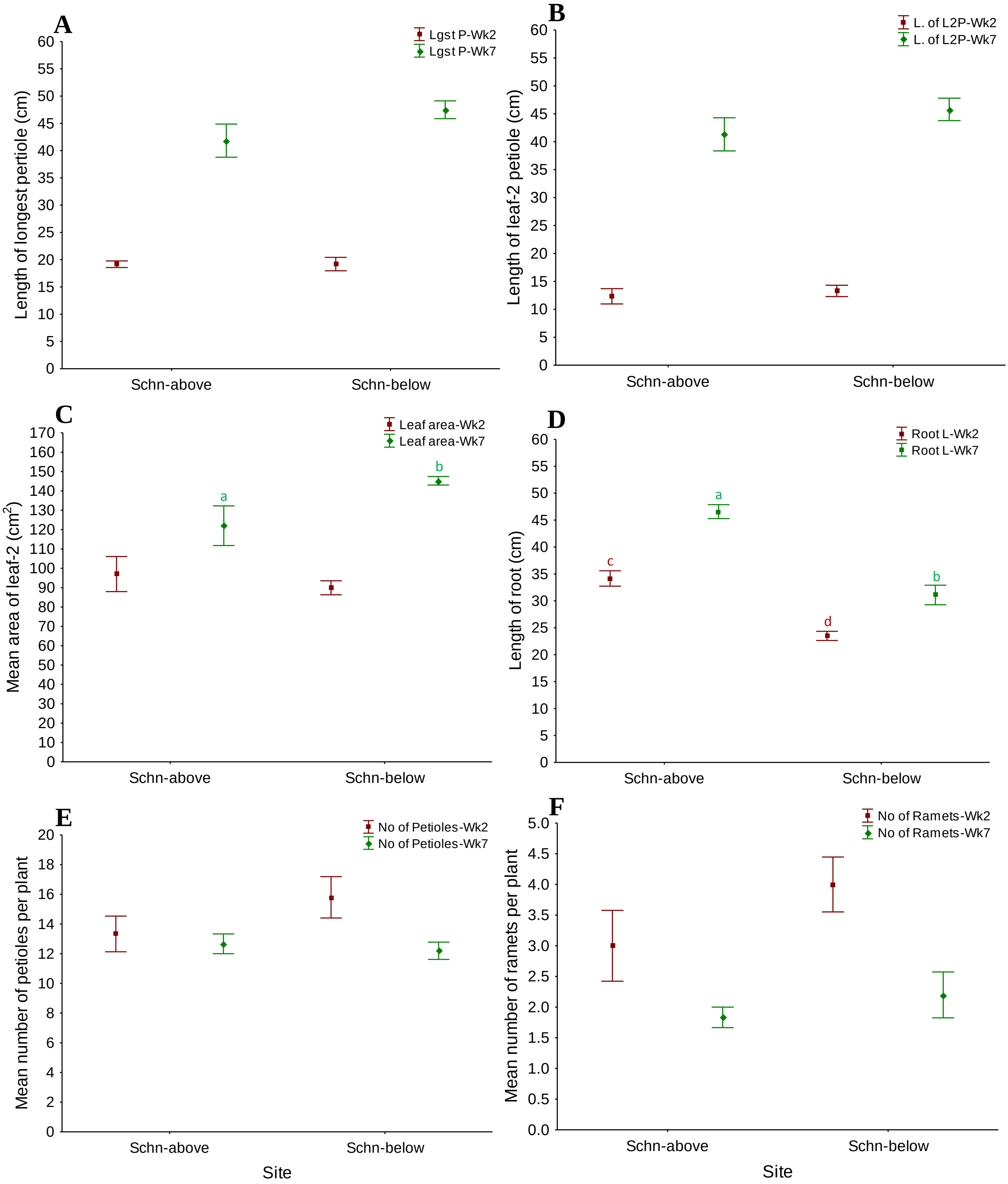


Figure 5.6: The effect of AMD on plant growth parameters of water hyacinth grown in floating cages above and below the inlets of the Schoonspruit (Schn) on the Vaal River at the AngloGold Ashanti mining operations near Orkney before (week 2) and after (week 7) the start of the rainy season: (**A**) Length of the longest petiole (**B**) Length of leaf-2 petiole, (**C**) Mean area of leaf-2, (**D**) Root length (**E**) Mean number of petioles per plant and (**F**) Mean number of ramets per plant. Means compared by One-way ANOVA between sites of the same sampling date and those followed by the same letter(s) are not significantly different ($P > 0.05$; Fisher LSD test). NB: graphs with no letters indicate the absence of significant differences beween the sites.

5.5 Discussion

Generally water hyacinth plants did not stop growing under most heavy metal treatments in the single-element system tub trial and the AMD trials, both in the presence and absence of the weevils, nevertheless with some stress symptoms. The same metal and AMD treatments identified as stressful to both plants and weevils in the preceding two chapters (three and four), were also found to negatively affect the plant growth in this study (Chapter). Copper (and Hg and Zn to some extent) in the single-element system tub trial and the high AMD treatment in the AMD pool trial, frequently appeared as the most stressful to both the plant growth and the weevil's feeding activities. The mean area of leaf-2, the numbers of ramets, fresh weight of plant biomass and plant density (only in the pool trial) were among the plant parameters consistently affected by the metals and AMD trials and their impact was further amplified by the weevils' feeding after their release to both the tubs and the pools. The leaf production however, remained unaffected under all the growth conditions in tubs and pools with an average production of 1 and 0.8 leaf/plant/week, respectively. In the Vaal River, the plant growth relatively increased after the rain than before the rain, and plants at the downstream site were bigger than the plants in the upstream site. These results were also similar to those in the hyperspectral data using the red edge spectral indices, in chapter-2.

5.5.1 The effect of heavy metal and weevil feeding on growth of water hyacinth plants in the single-element system tub trial

Several plant parameters were used to evaluate the influence of heavy metal contamination in water and its combination with weevils on the growth of water hyacinth. This discussion is presented in two sub-sections, one covering the effect of the metals on the plant growth, and the other on the effect of feeding damage by the weevils on water hyacinth.

5.5.1.1 The effect of heavy metals on plant growth of water hyacinth

Generally the water hyacinth plants were tolerant to most metal treatments based on different plant growth parameters evaluated. It is however, worth noting that the root length in some treatments, such as the Hg treatments, was significantly reduced compared to some of the metal treatments, among, which were Zn, Fe-M, Fe-H, Mn-L and Mn-H. Unlike these metals, Hg does not have any vital role in plant metabolism (Dunn, 2007). The Hg concentration in the roots was 58 times greater than the Hg concentration in the shoot system (see Chapter-3). The roots of water hyacinth have an enormous ability to bind and accumulate Hg (Wolverton and McDonald, 1975; Mishra *et al.,* 2008a; Chattopadhyay *et al*., 2012). Although, accumulation of heavy metals in roots of most aquatic plants is a strategy for avoiding phytotoxicity, the effects on root permeability, by altering the uptake process of nutrient elements, is unavoidable. Excess Cu in roots can also damage the cell wall and cell membrane and compromise the root's selective permeability, enhancing passive flows of some metals into the root tissues (Fernandes and Henriques, 1991). After the addition of the metals to tubs, only plants in the Cu treatment showed significantly smaller increase in root length than all the other metals compared to the control treatment (Table 5.1). This suggests that the roots of water hyacinth are sensitive to the toxic effects of Cu. Kay *et al*. (1984) also showed similar results where Cu at concentrations of 2.5 mg/L in water, inhibited the growth of new water hyacinth roots and disrupted the root functions. Although the concentration of Cu in water in this study was 2 mg/L, Cu may have inhibited the root growth at that concentration.

In this trial the mean area of leaf-2 and the plant biomass fresh weight were the only two plant parameters in the single-element tub trail which were significantly reduced due to heavy metal toxicity in the metal uptake phase in week 3, compared to their initial measurements at the start of the experiment. However, such toxicity effects were only revealed in Cu and Hg treatments compared to the control treatments (Table 5.1). This is because toxicity of heavy metals depends on the type of the metals and their concentrations in plant tissues. For instance the Cu concentration in the shoots (44.9 ± 3.8 mg/kg d. wt.) exceeded the normal range of Cu for most plant species (3-20 mg/kg d. wt.) (Nriagu, 1979; Clarkson and Hanson, 1980; Howeler, 1983; Stevenson, 1986). Therefore, at such concentrations of Cu in plant tissues, it was not surprising to see that most of the plant parameters revealed stunted and stressed water hyacinth due to the Cu phytotoxicity and to some extent due to Hg toxicity. Several studies also indicated that an increased ionic Cu concentration in the shoot system resulted in stunted root growth, reduced shoot development and leaf chlorises as well as disruption of plant photosynthesis in different plant species (Yruela, 2005; Xiong *et al*., 2006; Han *et al*., 2008; Burkhead *et al*., 2009; Shahbaz *et al*., 2010).

Despite the negative effect of Cu and Hg on several plant parameters, the leaf production rate was unaffected (Fig. 5.1D). The fact that the water hyacinth plant was able to maintain the normal rate of leaf production, (1 leaf/plant/week (Center and Spencer, 1981; Byrne *et al*., 2010)) across the different heavy metal treatments regardless of the metal toxicity level is evidence of its wide resilence and adaptation to grow under polluted water systems. This plant sheds a leaf (older leaf) with the growth of a new one every week (Center and Spencer, 1981). In addition, metal contaminated leaves show early chlorotic and necrotic symptoms which cause decay and detaching of leaves from the mother plant. This is indicated by the fact that the fresh weight of plant biomass in the Cu and Hg treatments in the current trial was the lowest of all the treatments, which suggests that shedding of more contaminated leaves was as a result of heavy metal toxicity.

5.5.1.2 The effect of weevil feeding on plant growth of water hyacinth

Generally, the six weeks of weevil feeding did not amplify the metal induced plant stresses observed during the metal uptake phase. In contrast, after the addition of the weevils the root length in week 9 in all the tub treatments increased by 45% compared to the lengths before their release in week 3, with the exception of Cu which did not show any increase (Fig. 5.1C). The removal of Cu and Hg by the roots of water hyacinth was among the highest of all the metals treatments, (over 98% in roots) (Chapter-3). This is considered to be an adaptation of the plant to avoid metal toxicity reaching the aerial parts. However, some metals such as Cu are also toxic to the roots and reduce the root growth. Hasan *et al*. (2007) found the growth of new roots was inhibited when water hyacinth was exposed to Cd and Zn at concentrations of 1 mg/L and > 4 mg/L, respectively for 16 days. Similarly, Lequeux *et al*. (2010) found that Cu in the hydroponic plant, *Arabidopsis thaliana* (L.) Heynh (Brassicaceae) exposed to concentrations of 5μM in water, reduced the root biomass more than the shoot biomass.

The decrease of leaf area (area of leaf-2) by 61% after the addition of the weevils compared to those before the addition of the weevils in week 3, suggests that the weevils' feeding amplified the reduction in the leaf area. Such stresses were particularly conspicuous in the same two treatments, the Cu and Hg metals, which also showed the worst leaf chlorosis compared to the control (Fig. 5.3). However, the differences in the leaf area before and after the addition of weevils revealed significantly smaller leaves in Cu, Hg and Zn compared to the control treatements (Table 5.1F). This suggests that the reduction in leaf area, particularly in Cu and Hg treatments and the severe chlorotic appearance of the plant after the addition of the weevils, was largely due to the continued effect of the metal toxicity over extended period of the trial. This could be due to increased transportation of Cu and Hg metals from the roots to the shoots in week 9 compared to those in week 3. Throughout this trial no supplementary nutrients were added to the tubs.

The amount of water uptake by plants is associated with the availability of nutrients, where plants growing in nutrient-poor growth medium take more water than plants growing in a nutrient-rich medium, and such dynamics of water uptake by plants influences the uptake of heavy metals and their transportation from the root into the shoot system (Chattopadhyay *et al*., 2012). For instance, O'Keeffe *et al*. (1984) and Gothberg *et al*. (2004) showed an increase of Cd in the shoots of water hyacinth and water spinach (*Ipomoea aquatica*), respectively with the decrease of nutrient concentration in the growing medium. Thus, the decrease of nutrients in the water after the end of the metal uptake phase (week 3) might have increased the net uptake of water by plants and in the process Cu and Hg were transported to the shoots, where their toxic effect is detrimental. In addition to the leaf area, the length of the longest petiole, leaf-2 petiole and roots were also further reduced in week 9 after the addition of weevils in the Cu treatments (Table 5.1). However, both adult weevil and larval feeding and all other weevil performance parameters, such as number of adults and larvae found per plant, and the count of ovarian follicles in the female weevil, were significantly lower in the Cu and Hg treatments than in the control treatments, except for the adult feeding in Hg treatment (see Chapter-4). The amplified plant stress in these two treatments, after the addition of the weevils, was therefore largely due to the prevalence of the Cu and Hg toxicity beyond the metal uptake phase in week 3. The weevil's feeding worsend the stress, acting synergistically to reduce the plant vigour despite the weevils themselves being under considerable stress from the metals.

Both in the metal uptake and weevil phases, Cu was consistently the most harmful metal to the water hyacinth plants. The only treatment with a significantly lower relative growth rate compared to the control treatment was the Cu treatment in week 9, in the weevil phase (Table 5.2). Kay *et al*. (1984) also found a reduction of 50% in the relative growth rate of water hyacinth exposed to Cu concentrations > 2.5 mg/L for three weeks. The fact that Cu, Hg and Zn treatments in the metal uptake phase showed leaf chlorosis, turning yellow compared to the control treatment, agrees with the spectral data detected using red edge indices (Chapter-2), which showed the lowest canopy chlorophyll in these treatments. The same metals in the single-element tub trial in Chapter-4 also showed the greatest reduction in the weevil's activities, which includes the fecundity, adult and larval feeding and their survivals. Nevertheless, the fact that there was not significant difference in relative growth rate between all the metal treatments in the metal uptake phase generally shows the resilence of water hyacinth plants, despite the symptoms of metal-induced plant stresses in some of the treatments. This suggests the potential of this plant for phytoremediation of contaminated waters.

5.5.2 The effect of AMD and weevil feeding on growth of water hyacinth plants

Unlike the single-element system tub trial, the pool trial was designed with an artificial mixture of heavy metals and different concentrations of sulphates to create a simulated acid mine drainage. The effect of the AMD and its combined effect with the water hyacinth weevils on water hyacinth plant growth is discussed in two sub-sections, one covering the effect of the simulated AMD on the plant growth (week 3), and the other on feeding damage of the weevils on the water hyacinth (week 9).

5.5.2.1 The effect of AMD on growth of water hyacinth plants in the metal uptake phase

Plant growth indicators were used to determine the interaction of plants of water hyacinth with different simulated AMD concentrations in the pool trial, in the metal uptake phase in week 3 by comparing the plant growth difference between the initial measurements at the start of the experiment, just before the addition of the AMD treatment (week 0) and those taken later, three weeks after the addition of the AMD.

The pattern of the change in the area of leaf-2 and the plant density per quadrat ($0.25m^2$) in all the three different AMD treatments was similar, and both were significantly reduced by 31% and 29% respectively, compared to their initial measurements in week 0 (Fig. 5.4A and C). This suggests that the plant density of water hyacinth could be affected by AMD concentrations as low as 300 mg/L SO_4^{-2}. On the contrary the plant biomass before and after the addition of the AMD treatments was unaffected (Fig. 5.4B). The same was true for the plant growth parameters, the length of the longest petiole, leaf-2 petiole and root length. All of them showed a similar pattern, and did not change much as a result of the different AMD treatments, compared to the initial measurements of the same plant parameters at the start of the experiment (Fig. 5.4D, E and F).

Nevertheless, plants in the the high AMD treatment accumulated greater Cu concentrations in their shoots (25 ± 0.2 mg/kg d. wt.), which exceeded the normal range of Cu concentrations of most plant species (3 – 20 mg/kg d. wt.) (Nriagu, 1979; Clarkson and Hanson, 1980; Howeler, 1983; Stevenson, 1986), as opposed to the low and medium AMD treatments, which approached the proposed upper limits (21.7 ± 0.6 and 19.6 ± 1.5 mg/L d. wt., respectively) (Chapter-3). Considering the toxic characteristic of Cu in the aerial parts, Cu is therefore, suggested to be contributing to reduction in some growth parameters, to some extent, in the high AMD treatment compared to the other two AMD treatments. For instance, both the rate of leaf production and the number of ramets per plant decreased significantly in the high AMD treatment, three weeks after the addition of the AMD, by 70% and 30% respectively, compared to those before the addition of the AMD at the start of the experiment (week 0). However, the other two AMD treatments did not show any change in plant parameters between the two sampling occasions, with the exception of the number of ramets in the medium AMD treatment, which was lower in week 3 than in week 0 (Fig. 5.5A and B). Kay *et al*. (1984) also found few ramets with poorly developed roots in water hyacinth stressed by Cu or Cd metals.

Similar to the single-element tub trial, the rate of leaf production did not change after the addition of the AMD, compared to that before the addition of the AMD, nor did it show any significant difference between the different AMD treatments on each of the sampling occasions (week 0 or week 3). However, 0.75 leaves per plant/week, was below the normal rate of one leaf per plant/week as indicated by Center and Spencer, (1981) and Byrne *et al*., (2010). The disparity of the rate of leaf production with the literature is suggested to be due to the sampling dates in this trial, where the metal uptake phase was conducted for 18 days (with a week in this trial was designated by an average of six days) (see Materials and Methods).

Over half of the plant parameters evaluated as plant growth indicators in the metal uptake phase, three weeks after the addition of the AMD, were negatively affected by the AMD, and the high AMD treatment caused the greatest impact on the plant parameters, followed by the medium AMD treatment on some occasions, compared to their initial measurements at the strart of the experiment. This was in agreement with the results found in Chapter-2, where the high AMD treatment was the most stressful to the plant as measured using the red edge spectral indicators and the water band indices.

5.5.2.2 The effect of weevil feeding on the growth of water hyacinth plants grown in AMD

Generally, the same growth plant parameters reduced by the AMD treatments in the metal uptake phase, three weeks after the addition of the AMD, were also affected negatively by the weevil feeding in week 9. The pattern of the mean area of leaf-2 in all the three AMD treatments after the feeding of the weevils in week 9, mirrored those patterns resulting from the effects of the AMD before the addition of the weevils in week 3. The leaf area further decreased by an average of 32% compared to the control, no-weevil treatment (Fig. 5.4A). After the addition of the weevils the plant biomass in both the medium and the high AMD treatments were also significantly lower compared to the control treatment. Although no signifcant decrease in plant biomass weight was observed in the metal uptake phase in week 3, before the addition of the weevils, it suggests that the reduction in weight was partly due to the weevil's feeding but largely due to the AMD effect (Fig. 5.4B). This is because there were no significant differences in the adult weevils' feeding between all the three AMD treatments (see Chapter-4). Similarly, the reduction in plant density per quadrat was further amplified after the addition of the weevils, compared to the control in week 9, and as opposed to the plant biomass, the plant density per quadrat decreased with the decrease of the AMD concentration (Fig. 5.4C). This suggests that healthier plants with broader leaves and greater biomass will have fewer new ramets produced due to the overcrowding (Center and Spencer, 1981), enhancing the growth of longer petioles instead, as in the low AMD treatment (Fig. 5).

The length of the longest petiole and leaf-2 petiole were significantly reduced after the feeding of the weevils in week-9 in the high AMD treatment, and at least the leaf-2 petiole length in the medium AMD treatment, compared to the control treatment. However, the fact that the weevil feeding in these two AMD treatments was lower than in the low AMD treatment, suggests that the stress in the growth plant parameters is a combination of both the high level of AMD and the weevil feeding (see Chapter-4). Ayyasamy *et al*. (2009) found the increase of nitrates from 300 to 500 mg/L, in water reduced the uptake of nutrient elements due to the increase of osmotic pressure in the water. Such effects, particularly in the medium and high AMD treatments at concentrations of 700 and 1300 mg/L SO_4^{-2} could interfere with the nutrient uptake process leading to plant stress. The low AMD treatment sustained greater adult and larval feeding than the other two AMD treatments (Chapter-4). However, the plants in the low AMD treatment continued to grow with relatively less symptoms than the other two AMD treatments and the plants were able to overcome the low rate of the weevil infestation (3.5 weevils/plant; Chapter-4). Hill and Olckers (2001) indicated that the impact of the weevils on water hyacinth growing under eutrophic water condition was overcome by the rapid and massive vegetative growth of the plant and their control efficiency is reduced, and they suggested an inundative release of weevils for greater impact. The weevils showed no significant effect between treatments in the the rate of leaf production, and ramets per plant compared to the control treatments in week 9 (Fig. 5.5). This also suggests that increasing the rate of the weevil infestation could result in detrimental plant damage, despite the metal and/or AMD pollution on which the water hyacinth grows.

Different studies use relative growths rate to determine the stress level of plants grown under heavy metal pollutions (Mokhtar *et al*., 2011; Kay *et al*., 1984; Lu *et al*., 2004). In this trial, the relative growth rate of plants in the metal uptake phase showed that plants in the high AMD treatment were more stressed compared to the other two treatments and the same applied in the weevil phase, although the relative growth was not significantly different from the medium AMD treatment (Table 5.3). Mokhtar *et al*. (2011) and Kay *et al*. (1984) also showed a significant reduction in the relative growth rate of water hyacinth when exposed to high concentrations of Cu applied as $CuSO_4$. The total Cu, Fe and Mg concentrations in the roots of the medium and high AMD treatments were significantly greater than those in the roots of the low treatment (Chapter-3). These metals, apart from their toxicity effects, also interfere with the root uptake and translocation processes of other elements. For instance, the presence of excess Cu in roots of *A. thaliana* reduced K, P, S and Mn concentrations in roots, while the concentrations of K, Ca, P, Fe, Mn in shoots and the translocation of Ca from the roots decreased (Lequeux *et al*., 2010).

The AMD trial showed that both plants and weevils were negatively affected by AMD concentrations greater than 700 mg/L SO_4^{-2} ions. Nevertheless, the weevil feeding amplified the plant stress to a certain degree and their use on water hyacinth plants growing under AMD contaminated water systems is still worthwhile despite the fact that their activity was reduced by elevated AMD concentrations.

5.5.3 The response of water hacycinth to water pollution in the Vaal River

The only plant parameters clearly affected by water pollution in the Vaal River were the root length and the leaf area (Fig. 5.6C and D). The water hyacinth roots at the site below the inlet of Schoonspruit were significantly shorter than those from the upstream site both before and after the rain. The site below the inlet of the Schoonspruit into the Vaal River was in receipt of more nutrients than the upstream particularly after the rain although the nitrogen concentration in water was not measured. This was attributed to the drainage of the tributary into the Vaal River carrying effluents from the local settlement of Kennan and other contaminants as a result of runoff from the surrounding old and new mining wastes (DWAF, 2009). Plants growing under such eutrophic water systems generally grow short roots and a large shoot biomass (Xie *et al*., 2005). Xie *et al*. (2005) found a decrease in root length of submerged macrophytes, *Vallisneria natans*, when nutrient availability was increased in the water column. They also found that the root:leaf mass ratio, and root:leaf length ratio decreased at enhanced nutrient levels in water.

The leaf area before the rain was not significantly different between the two sites. However, after the rain the leaf area from water hyacinth at the downstream site was significantly greater than that from the upstream site. The number of petioles and ramets per plant before and after the rain were not significantly different between the two sites. Nevertheless, the number of ramets per plant in both cages dropped from three and four ramets per plant before the rain to slightly below two after the rain. This could be attributed to the fact that after the rain the water nutrient level in the two cages was greater than before the rain (Chapter-3), leading to a massive plant growth and overcrowding that reduced production of new ramets due to lack of space (Fig. 2.6 in Chapter-2). This is reflected in the increase of the lengths of the longest and leaf-2 petioles in addition to greater leaf area area of leaf-2 (Fig. 5.6A, B and D). Byrne *et al*. (2010) found that plants in the hypertrophic water produced the longest petioles and the greatest length of leaf-2 petiole and the least number of ramets per plant. They also found the number of ramets produced per plant decreased with increasing plant density of water hyacinth grown in confinement in pools at the University of the Witwatersrand. Similarly, Center and Spencer (1981) indicated that in crowded conditions, leaves of water hyacinth became very large and petioles reach up to a meter long while plant density and production of new ramets decreases.

Most of the plant growth parameters evaluated from both sites at the Schoonspruit inlet on the Vaal River showed an increase after the summer rain, and showed no significant difference between the sites, except in the leaf area. The same results were also found using the red edge and the water band indices from the canopy of water hyacinth in Chapter-2, although the red edge did not show significant differences between the upstream and down stream sites except in the water band spectral indices.

5.6 Conclusion

The water hyacinth plants showed a wide range of tolerance to the heavy metals in the single-element system tub trial and the simulated AMD pool trial. However, symptoms of plant stress were revealed in some of the plant parameters, among which were leaf area, plant density and fresh weight of plant biomass in all the trials in addition to leaf chlorisis. Copper in the single-element system tub trial and the medium and the high AMD treatments in the pool trial were consistently the most stressful to the growth of water hyacinth plants. In the single-element tub trial, Cu as well as Hg caused severe and more visible chlorotic effects on leaves than others. The same heavy metals and AMD treatments in both trials were also detected as the more stressful treatments than others treatments in the hyperspectral remote sensing data using the red edge and the water band indices, to detect plant stresses in Chapter-2. The results in this chapter also agree with those found in Chapter-4 where, Cu and Hg in the single-element system tub trial and high AMD treatment in the AMD pool trial were among the most stressful treatments to reproduction and feeding activities of the water hyacinth weevil. The six weeks feeding of the weevils in both the single-element tub and AMD pool trials, amplified the stress levels of those plant parameters negatively affected prior to the addition of the weevils. Thus, despite the decline in the activity of the weevils, their usage as biocontrol agents of water hyacinth growing under contaminated water systems could still be recommended, except under elevated Cu and AMD concentrations in water. The results of this chapter and the preceding three chapters are further discussed and summerized in the next chapter.

Chapter 6

General Discussion

The invasion of water hyacinth in to freshwaters spanning more than 50 countries around the world, mainly in tropical and subtropical regions, could potentially spread further to higher altitudes and latitudes with the rise of temperatures due to climate change (Villamagna and Murphy, 2010). Its management measures include mechanical or manual, herbicide and biological control methods. None of these methods has satisfactorily controlled the weed and reduced its scourge in South Africa. As a result the paradigm of water hyacinth management in the country has shifted to an integrated management, which combines the application of herbicides with the biological control methods (Byrne *et al*., 2010). However, this requires a regular monitoring of the water hyacinth's physiological and health status in relation to the habitat in order to facilitate the decision when to intervene and what intervention measures are appropriate and timely. In line with this, hyperspectral remote sensing was investigated as the main aim of this study to detect both biotic (damage by biocontrol agents) and abiotic (heavy metal and acid mine drainage effects) factors at plant level of water hyacinth (Chapter-2). The hyperspectral remote sensing results were calibrated against, different aspects of water hyacinth growth including the metal uptake potential of the plant (Chapter-3), the interaction of heavy metals in the plant's tissues with its biological control agents and their interaction with heavy metals (Chapter-4), and the effect of heavy metals and biological control agents on the plants' growth (Chapter-5).

6.1 The success of hyperspectral RS in the detection of plant stress

Different spectral indicators of plant stress were evaluated, among which were $mNDVI_{705}$, REP_LE and WBI. Results from all the three spectral indices were similar and of all the eight different heavy metals (As, Au, Cu, Fe, Hg, Mn, U and Zn) used in the single-element tub trial, Cu, Hg and Zn were the only elements detected as stressful to water hyacinth plants in the first three weeks (the metal uptake phase). Spectral indicators in the red-edge are associated to the level of leaf chlorophyll in plants. Generally the correlation between different such spectral indices in the red edge including REP-Max FD, REP-LE, $mNDVI_{705}$ and

RE-NDVI with the leaf chlorophyll content measured using the SPAD chlorophyll meter produced a strong positive relation between them (R^2 = 0.7 to 0.8) . Copper, Hg and Zn were the only elements showing a stressful effect compared to the control treatments, indicating the decline of canopy chlorophyll content in plants treated with those metals in the metal uptake phase (week 3).

Six weeks after the addition of weevils to the single-element tub trial, the plant canopy chlorophyll and water content declined significantly. Seven treatments had significantly lower chlorophyll content ($mNDVI_{705}$) than in the metal uptake phase largely as a result of the water hyacinth weevil-induced stress, which was clearly detected by the spectral indicators $mNDVI_{705,}$ and REP-LE. However, the stress in Cu treated plants was largely attributable to the metal (Chapter-4).

Similarly both the canopy chlorophyll and water contents spectral indicators ($mNDVI_{705}$ and WBI, respectively) were able to detect the plant stress of water hyacinth grown in the simulated AMD pool trial, where stress increased with the increase of sulphate concentration in water from 300 to 1300 mg/L SO_4^{-2}. In the metal uptake phase plant stress was more pronounced in the high AMD treatment (1300 mg/L SO_4^{-2}) than in the the low and medium AMD treatments (300 and 700 mg/L SO_4^{-2} respectively). Six weeks later the degree of stress in the medium and the high AMD treatments was similar. The weevil feeding in both treatments was lower than in the low AMD treatment, suggesting that the feeding activities of the weevils were reduced by the AMD (Chapter-4). However, the fact that the medium and high AMD treatment showed similar stress in the spectral indices, suggests that the weevil feeding had clearly amplified the AMD induced stress in both AMD treatments.

This study showed that hyperspectral remote sensing using spectral indices associated with the red edge bands such as mNDVI, REP-LE, RE-NDVI and REP-Max, successfully detected plant stress of water hyacinth induced either by heavy metals and or acid mine drainage pollution or water hyacinth weevil-induced damage. The heavy metals Cu, Hg, Zn were stressful to plants of water hyacinth and Cu was by far the most stressful. The spectral indicators resulted in a strong positive correlation with chlorophyll meter reading via a SPAD-502. This study also found that the canopy water index, WBI matched most of the results from the spectral indicators of the canopy chlorophyll contents. Due to the metals' similar phytotoxic effect on plants, which all are associated with the degradation of the chlorophyll, specific distinguishing spectral features using the red edge indices could not be established. Neverthless, the fact that the hyperspectral remote sensing was clearly able to detect the water hyacinth physiological status (e.g. the presence and the degree of the plant stressors) could be used in the field to monitor and aquire information on water hyacinth useful for its management.

6.2 Success of water hyacinth in cleaning water

Most aquatic macrophytes avoid heavy metal phytotoxicity by largely localizing them in the roots (Weis and Weis, 2004). Once metal ions enter the root cells, the plant forms complexes of the metal elements with amino acids, organic acids, or metal binding peptides, or impounds the metals in vacuoles to prevent them from being transported to the aerial shoots (Sela *et al*., 1988; Hall, 2002; Mishra *et al*., 2008c). In the current study, all heavy metal results from the plant tissues showed that the water hyacinth roots had significantly greater metal concentrations than the corresponding shoot system. This was in agreement with several other studies on water hyacinth heavy metal uptake (Malik, 2007; Liao and Chang, 2004; Zhu *et al*. 1999). The plants' phytoremediation efficiency was however, greater in the single metal pollution than in the AMD pollution. This could be due to several factors that affect the metal uptake process by plants. Among which are the time of exposure, nutrient levels, plant age, cationic competition for pathway of uptake, complexing agents and bioavailability (Prasad *et al*., 2001; Tangahu *et al*., 2011; Chattopadhyay *et al*., 2012). The accumulation of Mn, Zn, Cu and Fe in the shoots and roots of water hyacinth in the single-element tub trial were in the order of Fe>Mn>Zn>Cu and Mn>Zn>Fe>Cu respectively, while in all the AMD pool trial the accumulation of these four metals was the same in all the plant parts (Mn>Fe>Zn>Cu). Copper was consistently at the bottom of the rank in both trials (Chapter-3). Gupta *et al*. (2012) and Lokeshwari and Chandrappa (2006) also found similar results for Cu.

The trend of the bioconcentration factor (BCF) in the simulated AMD pool trial generally showed a decline at concentrations greater than 700 mg/L SO_4^{-2}in water.

Among the four metal elements (Cu, Fe, Mn and Zn) used to create the simulated AMD treatment in the pool trial, both Cu and Zn had the lowest BCF (38 and 45, respectively) as oppossed to the single-element tub trial (1786 and 1165 respectively). Such decline of metal removal at the high AMD could be due to the elevated osmotic pressure in the growth medium that disrupts the entire metal and nutrient uptake process by plants (Eaton, 1941; Ayyasamy *et al*., 2009).

Results from both sites of the Vaal River at the inlets of the Koekemoerspruit and the Schoonspruit however, showed that water pollution increased after the the rain and it was greater below the inlet of the Schoonspruit, than above the inlet of the Koekemoerspruit. DWAF, (2009) also found increased contamination in these tributaries during the rainy season. The sulphate concentration in water after the rain increased from between 113 - 160 before the rain, to 441 - 730 mg/L after the rain at the sites of the two tributaries. Although the water hyacinth BCF was not calculated in the field trial, the plants' removal of both metal and non-metal elements had generally increased significantly with the increase of the sulphates in water after the rain. This suggests that, water hyacinth can be used in phytoremediation of both heavy metal and AMD pollution, although it is more efficient in sulphate concentrations not exceeding 700 mg/L, which is within the range of the Vaal River.

The information on the fate of most heavy metals removed from water by water hyacinth plant is not well documented, apart from the fact that they are largely accumulated in the roots than in the shoots (Kay *et al*., 1984; Zhu *et al.*, 1999; Liao and Chang, 2004; Malik, 2007; Rahman and Hasegawa, 2011). Generally the absorption of metals by the shoots and the roots in the AMD treatments mirrored results of the total uptake of metals by the respective plant parts. Unlike in the roots, absorption of metals by the shoots was not significantly affected by the variation of sulphate treatments. Metal uptake by root adsorption ranged from 26 to 44% and it was higher for Fe and Mn than for Cu and Zn in the AMD pool trial. This could be due to the formation of iron plaques through the oxidation of reduced forms of Fe at the roots surfaces by oxygen that diffuses from the roots into the water (Taggart *et al*., 2009). The iron plaques adsorb other metals such as Zn, Cu, Mn and Fe on to their surfaces, reducing their absorption by roots, although this reaction is dependant on the pH of the surrounding water (Greipsson and Crowder, 1992; Greipsson, 1994). The adsorbed amount of Cu, Mn and Zn in the single-element tub trial was 52%, 46% and 40% respectivley. Increased adsorption of toxic metals at the surface of roots indicates an additional strategy of adaptation in aquatic plants to reduce metal phytotoxicity as also indicated by Batty *et al*. (2000). The fact that the largest portion of metals removed by water hyacinth is stored in the roots, the plants should be harvested manually or mechanically and removed from the water after their use for phytoremediation. The knowledge of the fate of metals removed by water hyacinth also provide an information on future studies and new introduction of biocontrol agents of the plant, to avoid the toxic effects of the metals based on the insects' feeding choice of the plant tissues (roots or shoot feeders).

The highest proportions of Cu, Hg and Zn were accumulated in the roots of water hyacinth. Nevertheless, at concentrations of 44.9 ± 3.8, 35.9 ± 6.2 and 373.1 ± 8.7 mg/kg d. wt., in the shoots of water hyacinth respectively, showed stressful effects, with symptoms of leaf chlorisis and necrosis (Chapter-5), which was detected by the red edge spectral indices (Chapter-2). However, these metal concentrations in the shoots were not individually correlated with each of the red edge spectral indices used in chapter-2, due to the size of plant samples (two samples per metal) analysed because of the cost of the analysis. It is worth investigating further the correlation of each of the metals used in this study with the hyperspectral data in future studies.

6.3 The effect of heavy metals in plant tissues on water hyacinth weevils

In this chapter the effect of heavy metals on the water hyacinth weevils was investigated in a single-element system tub and simulated AMD pool trials. Generally results from the single element trial showed that the larval feeding and development were more sensitive to metals than the adult weevils were. Copper, As, Hg and Zn were more deterent to the feeding of the weevils than the other metals; and the latter two metals only reduced the larval feeding but not the adult feeding, suggesting either their concentrations were not high enough in the weevil's body to cause a negative effect or the adults were able to detoxify or circumvent these metals. Hussain and Jamil (1992) also found the feeding activity of the adult weevil was unaffected, when exposed to water hyacinth grown at concentrations of 100 mg/L of Zn and Hg in water. Maroni *et al.* (1987) showed *Drosophila melanogaster* was able to circumvent toxicity of Hg, Zn, Cu and Cd through a detoxification process that invloves a synthesis of a new protein (metalothionein) to which the metal ions get chelated. The mean number of follicles, larvae, and the first and the second instars, were significantly reduced in the Cu, As, Hg and Zn treatments, of which Cu was consistently the most stressful metal of all (Chapter-4). Although the larval development was not investigated further than the second instar, the relative decline in the number of second instars compared to the first instars and moreover the decline of the larval development in Cu, As, Zn and Hg by 79% in the second instar compared to those in the control treatment, suggests that it may take longer or fail to complete the life cycle due to metal toxicity, particularly in the worst four heavy metals. The larvae of *Ostrinia nubilalis* insect feeding on an artificial diet contaminated with 0.1-0.4% of $ZnSO_4$ died at the prepupal stage before completing its development (Gahukar, 1975). Similarly the number of ovarian follicles, and larval feeding and survival of water hyacinth weevils were significantly reduced in the simulated AMD pool trial with the increase of the sulphate concentrations. A reduction in egg production of 50% in *C. pipiens* exposed to concentrations of 5 ppm of CuSO4 (El-Sheikh *et al*., 2010) and 33-47% in grain aphids, *S. avenae* fed on Hg, Cd and Pd contaminated wheat seedlings and oats (Gao *et al*., 2011) were also found in other studies. This suggests the large drop in the proportion of the second instar larvae in the current study will eventually result in a dramatic drop in the weevils' population.

The adult feeding did not show any significant reduction between the AMD treatments. However, the fact that the follicles as well as the number of larvae were reduced significantly in both the medium and high AMD treatments, could suggest that both the adult male and female weevils were avoiding the metal toxicity by sequestering them in their reproductive organs. Schmidt and Ibrahim (1994) found some Hg stored in the ovaries of *A. thalassinus*. Thus, concentration of AMD above 700 mg/L SO_4^{-2} in water reduced the general reproductive activities of the water hyacinth weevils, particularly the fecundity and larval feeding and development.

Concentrations of 44.9 ± 3.8, 35.9 ± 6.2 and 373.1 ± 8.7 mg/kg d. wt., in the shoots of Cu, Hg and Zn, respectively as well as arsenic (shoot concentration not detected in the ICP-OES) (see Chapter-4), had detrimental effects on weevil female fecundity and larval feeding and development. Such toxic impacts on the weevils could also occur in lower concentrations in the shoot system of water hyacinth, if weevils fed on plants contaminated by a suite of metals rather than on plants contaminated by a single metal at similar or relatively higher concentration. For instance, the combined impacts of Cu and Zn at concentrations of 19.6 ± 1.5 and 69.5 ± 4.6 mg/kg d. wt., respectively, in the water hyacinth shoots in the medium AMD treatment resulted in the reduction of the number of follicles/female, number of larvae/plant and number of mined petioles as in the single-element system tub trial with the respective concentrations of 44.9 ± 3.8 and 373.1 ± 8.7 mg/kg d. wt. Thus, although the weevil trial was not pursued in the field at the Vaal River due to their absence at the time of the experiment, the trace amount of heavy metals found in the plant tissues and the increased sulphate concentrations in the water (729 mg/L SO_4^{-2}) particularly at the downstream site of the Schoonspruit inlet on the Vaal River, which exceeded the 700 mg/L SO_4^{-2} in the medium AMD pool trial, suggests that water hyacinth weevils used as biocontrol agents on water systems contaminated with heavy metals or AMD will largely be hindered by the pollutants. Furthermore, unlike other similar studies on the interaction of the water hyacinth weevils with heavy metals and AMD, the current study showed that the general activity of the weevils was reduced, and suggests such pollutants could reduce the efficiency of the weevils used as biocontrol agents of water hyacinth.

6.4 The impact of heavy metal and weevil feeding on water hyacinth growth

The uptake of heavy metals can directly or indirectly affect plant growth and therefore the weevils that feed on them. The effect of metals on the photosynthetic apparatus of plants is widely established (Fernandes and Henriques, 1991; Stiborová *et al*., 1986; Smolders and Roelofs, 1996; Rascio and Navari-Izzo, 2011). The uptake of excess heavy metals in macrophytes can also have an indirect effect by modifying the root permeability and altering the metal and nutrient uptake processes, by enhancing passive mass flow of poisonous metals into the roots (Fernandes and Henriques, 1991). The most common symptoms of heavy metal toxicity are leaf chlorisis, necrosis and stunted plant growth (Kay *et al*., 1984; Shahbaz *et al*., 2010; Mocquot *et al*., 1996; Yruela, 2005; Xiong *et al*., 2006; Han *et al*., 2008; Burkhead *et al*., 2009). Insect herbivory on plants also causes similar sysmptoms (Marline *et al*., 2013). They found a reduction in the photosynthetic rate in general and a decrease in the efficiency of the photosystem II with the increase of feeding damage by mites (*Orthogalumna terebrantis*) on water hyacinth plants, and eventually in the reduction of chlorophyll content with prolonged mite feeding.

Although water hyacinth was generally tolerant to most heavy metals, some plant growth parameters in the single-element tub trial and simulated AMD pool trial were reduced by the same metal treatments which were shown to be stressful to plants by the remote sensing (Chapter-2), and to the weevils' feeding (Chapter-4).

In the metal uptake phase of the experiment, most of the plant growth parameters, in both trials, were unaffected by the heavy metals or the AMD treatments (see Chapter-5). However, the leaf area, plant density and the plant biomass declined significantly in the Cu and Hg treatments of the single-element tub trial and in the medium and high AMD treatment of the AMD pool trial. The red edge spectral indices in Chapter-2, also showed similar plant stress in the same treatments, which detected reduced canopy chlorophyll at the spectral bands between 670 and 750 nm. This indicates that even if water hyacinth appears healthy in contaminated waters, plant stresses can still be detected using the hyperspectral remote sensing and this could be used to determine the water quality as a result of pollution.

Generally the same plant growth parameters affected by the heavy metals and AMD in the metal uptake phase in week 3, showed an increased stress after six weeks of weevil impact in week 9, in the Cu, Hg and Zn treatments in the single-element tub trial, and the medium and high AMD treatments in the AMD pool trial (see Chapter-5). Nevertheless, since both adult and larval feeding were significantly reduced particularly in Cu in the single-element tub trial, and the larval feeding in the medium and high AMD treatment in the AMD pool trial, the deterioration of plant stress in week 9, after the addition of the weevils was partly due to the metal or AMD treatment (Chapter-4). Therefore, the use of the weevils as biological control agents at high AMD or elevated Cu concentrations in water will have a reduced effect. Nevertheless, despite the fact that the general activity of the weevils in both trials declined as a result of heavy metals compared to the control treatments, the weevils, had managed to amplify the level of the plant stress in the second phase of both trials, after six weeks of feeding on them. Such deterioration in the physiological health status of the plant after the addition of the weevils was particularly conspicuous in Cu, Hg, As and Zn in the single-metal tub trial and the medium and high AMD treatments in the AMD pool trials, which were also shown in the hyperspectral results, where the same treatments showed significantly greater levels of plant stress, compared to the control treatments (see Chapter-2).

In conclusion, the results of this thesis can be summarized as follows:

1. The hyperspectral remote sensing identified effectively both the heavy metal or AMD and weevil feeding induced plant stresses, and its use as potential tool for monitoring the water hyacinth physiological and plant health status is recommended, although discrimination between the plant stressors using this tool was confounded by the similarities of all the metal toxicity to the plants which are all involved in the distraction of the photosynthetic apparatus (photosystem I and II) and consequent degradation of chlorophyll pigments, as did the weevils' feeding. Although, due to its invasive nature, water hyacinth often exclude other aquatic plants through light and nutrient competition, ground truthing might be required when using hyperspectral remote sensing from aerial platforms. In addition, such data collection from aerial platforms at larger scale involves a complex data set and atmospheric interferences, which further complicate image analysis and interpretation, and therefore such studies in future could be important.
2. Based on a BCF of 1000 (Zhu *et al*., 1999) which qualifies plants as good accumulators of metals, water hyacinth can be categorized from a moderate to good accumulator of heavy metals and AMD. It is however, more effective in phytoremediation of a single water contaminant than a suit of heavy metals or AMD contaminated waters, particularly with sulphate concentrations of >700

mg/L. Nevertheless, the use of water hyacinth for cleaning polluted water systems can only be effective if safe disposal of the phytoremediating plants is pre-arranged. This could include incineration, and briquetting, or could be by disposal to nearby tailings dams on the mining sites which are often the main sources of heavy metal and AMD contaminants. In addition the use of water hyacinth for phytoremediation could be recommended, if a water hyacinth infestation pre-exists on the targeted sites to avoid further infestation and environmental problems. Although manual or mechanical removal of water hyacinth is often expensive, if the purpose of the removal is to clean contaminated water, it might be cost effective compared to the cost of conventional cleaning of such water systems.

3. Despite the high level of pollutants in the current experiments compared to water pollution levels in the natural environment, the weevil persisted and continued to feed and reproduce cuasing a considerable damage to the plants. Nevertheless, these activities were significantly reduced compared to the control treatments, particularly in the Cu, Hg, Zn, As treatments of the single-metal tub trial and in the medium and high AMD concentration treatments in the pool trials. Thus, their use as biocontrol agents in water systems contaminated by increased concentrations in the four metal treatments and AMD with concentrations greater than 700 mg/L SO_4^{-2} such as those in the downstream site of the Vaal River at the inlets of the Schoonspruit tributary will be reduced. Therefore for effective control of water hyacinth, the use of the weevils as biocontrol agents is recommended in combination with a sub-lethal dose of herbicides applied in strip-spraying (leaving the fringes or river banks unsprayed to harbour the weevils) as indicated by Byrne *et al*. (2010).
4. Generally water hyacinth was tolerant and survived the different heavy metal or AMD pollutants to which the plant was exposed. Neverthelss, some symptoms of phytotoxicity were observed in some of the plant growth pramenters evaluated in this experiment, among which were leaf chlorisis, leaf area, plant desity and fresh weight of plant biomass. The metal or AMD treatments with such stressful symptoms were consistent with those found in the hyperspectral and the weevil datas.

Finally, although discrimination between the different metal or AMD induced stresses and/or the weevils plant stresses could not be established using the hyperspectral data with red edge spectral indices, the fact that the hyperspectral remote sensing was able to detect the presence of plant stresses (both abiotic and biotic) and the degree of their severity, can be used to monitor the physiological status of water hyacinth in the field to facilitate its management decision. For future studies I recommend the investigation of physical plant stresses due to insect herbivory (structure such as leaf curling, orientation, … etc.) in experimental set up with and without biocontrol agent (insect) and with metals separately to explore distinctive spectral features that could distinguish the heavy metal or AMD stresses from the insect feeding stresses.

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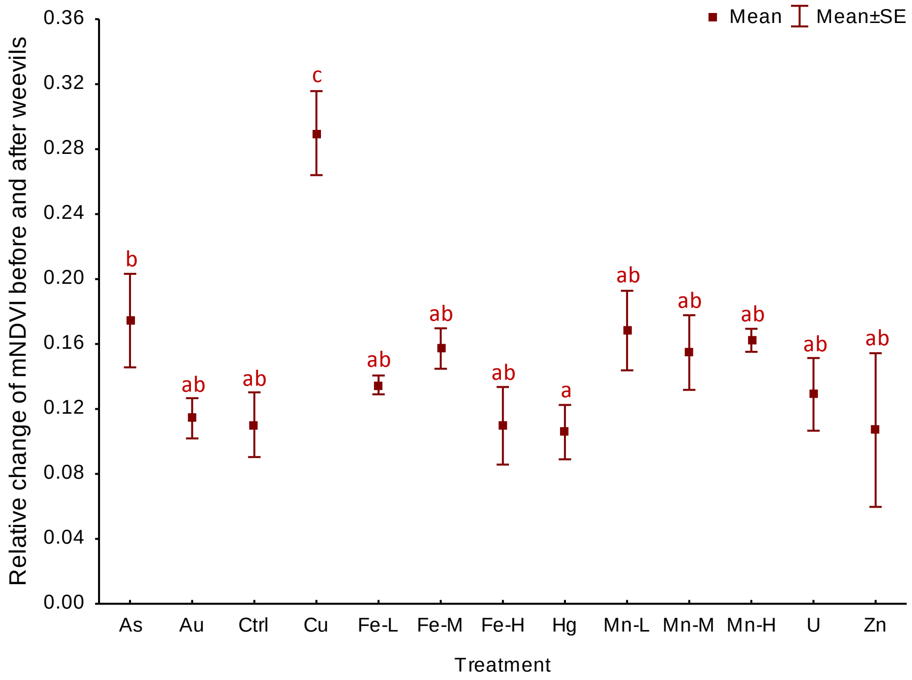
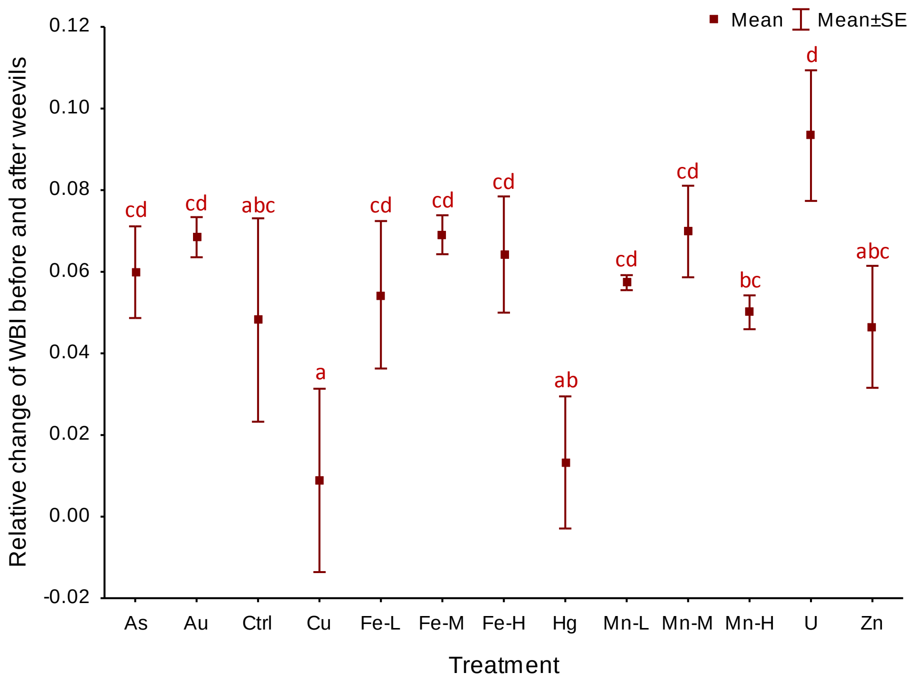
Appendices



Appendix 2A: The relative change in canopy chlorophyll content ($mNDVI_{705}$) between treatments before (week 3) and after the addition of weevils (week-9). Means were compared by One-way ANOVA and those followed by the same letter(s) are not significantly different (P>0.05; Fisher LSD test).

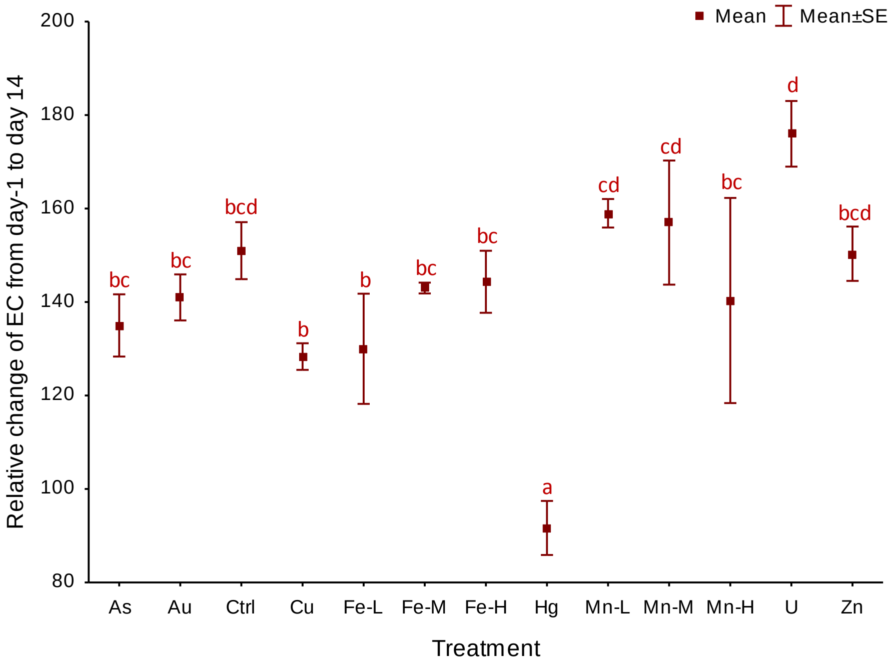


Appendix 2B: The relative change in canopy water content (WBI) between treatments before (week 3) and after the addition of weevils (week 9). Means were compared by One-way ANOVA and those followed by the same letter(s) are not significantly different (P>0.05; Fisher LSD test).

Appendix 2C: Caged-plants above inlet of the Koekemoerspruit into the Vaal River showing the plant damage due to bird feeding.

Appendix 2D: Caged-plants below inlet of the Koekemoerspruit into the Vaal River showing the plant damage due to birds'.

Appendix 2E: Caged-plants below inlet of the schoonspruit into the Vaal River with no physical plant damage from bird feeding.



Appendix 3A: The relative change between measurements of water electrical conductivity (EC) in the first day (just after the addition of the metal treatment into the tubs) and day-14 (at the end of the metal uptake phase) of water hyacinth grown in different heavy metal treatments in a single-element system tub trial.

Appendix 3B: Heavy metal concentrations in pool water samples collected just before the addition (before) and just after the addition (initial) of the different simulated AMD treatments and three weeks (final) after the addition of the treatments (week 3) in the AMD pool trial.

	Pool low sulphate concentration (mg/L)			Pool medium sulphate concentration (mg/L)			Pool high sulphate concentration (mg/L)		
Treatment	**Before**	**Initial**	**Final**	**Before**	**Initial**	**Final**	**Before**	**Initial**	**Final**
Cu	0.11 ± 0.0 a	2.16 ± 0.0 b	0.69 ± 0.1 a	0.06 ± 0.0 a	2.16 ± 0.2 b	0.751 ± 0.0 a	0.10 ± 0.0 a	3.63 ± 0.6 c	0.99 ± 0.0 a
Fe	9.71 ± 0.0 c	9.72 ± 0.4 c	5.80 ± 0.9 ab	6.56 ± 0.0 abd	6.29 ± 0.3 b	4.260 ± 1.0 a	9.07 ± 0.0 cd	7.21 ± 3.2 c	5.08 ± 0.2 ab
Mn	0.01 ± 0.0 ab	1.05 ± 0.1 c	0.08 ± 0.0 a	0.01 ± 0.0 ab	0.99 ± 0.1 bc	0.243 ± 0.1 ab	0.02 ± 0.0 ab	1.89 ± 0.5 d	0.19 ± 0.01 a
Zn	0.90 ± 0.0 a	4.01 ± 0.05 e	2.78 ± 0.3 b	0.29 ± 0.0 a	3.38 ± 0.1 c	2.025 ± 0.0 d	0.74 ± 0.0 a	4.57 ± 0.2 f	2.86 ± 0.0 bc

NB: Means were compared by One-way ANOVA and means of the same element in a row followed by the same letter(s) are not significantly different ($P > 0.05$; Fisher LSD test). NB: The high level of Fe in water compared to the actual amount of Fe applied as treatment is due to the amount of Fe chelate applied (technical fertilizer) during the plants' growing period, before the start of the experiment.

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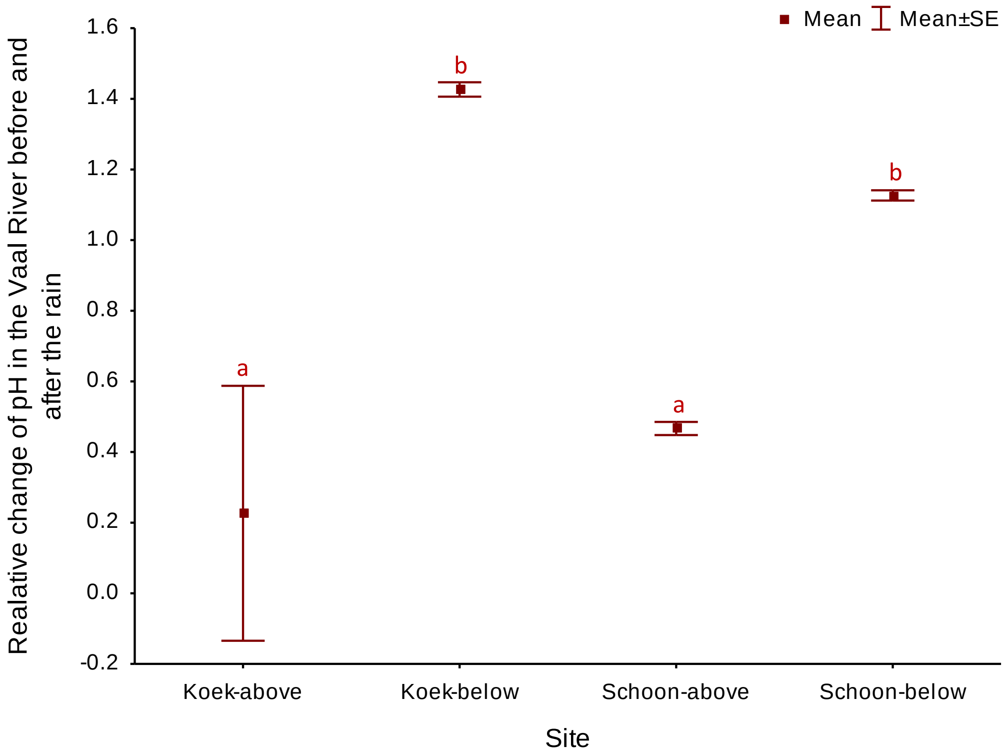
Appendix 3C: The initial total concentrations of metals in water hyacinth shoots and roots, just after the addition of AMD treatments in the simulated AMD pool trial (Week 0).

Week-0	Low sulphate treatment (mg/kg)		Medium sulphate treatment (mg/kg)		High sulphate treatment (mg/kg)	
Treatment	Total metal uptake by shoots	Total metal uptake by roots	Total metal uptake by shoots	Total metal uptake by roots	Total metal uptake by shoots	Total metal uptake by roots
Cu	15.8 ± 0.7 a	20.9 ± 2.6 c	14.9 ± 0.6 a	18.4 ± 1.7 ac	8.5 ± 0.1 b	10.1 ± 0.1 b
Fe	135.9 ± 28.4 a	8143.9 ± 620.3 c	131.5 ± 11.7 a	5139.8 ± 741.3 b	153.1 ± 30.7 a	4436.7 ± 1018.2 b
Mn	67.4 ± 2.0 a	331.3 ± 85.1 b	109.7 ± 9.1 a	116.2 ± 12.4 a	39.4 ± 8.4 a	68.8 ± 30.7 a
S	286.1 ± 132.1 a	2865 ± 775.4 bc	598.6 ± 204.1 a	4097 ± 316.3 c	606.5 ± 271.9 a	2298.8 ± 37.8 b
Zn	51.8 ± 0.4 a	198.4 ± 31.5 b	50.1 ± 2.0 a	158.7 ± 10.3 b	49.3 ± 1.4 a	91.6 ± 2.7 a
Mg	8160.7 ± 1015.1 ab	5908.6 ± 395.6 a	9395.1 ± 392.0 a	9397.4 ± 1043.2 a	11953 ± 355.5 c	7833 ± 288.4 ab

NB: Means were compared by One-way ANOVA, and means in rows under the same element followed by the same letter(s) are not significantly different (*P* > 0.05; Fisher LSD test).



Appendix 3D: The relative change between measurements of water pH before the start of the rain (Wk-2) and after the start of rain (week 5) in cages with water hyacinth above the inlets of the Koekemoerspruit and Schoonspruit into the Vaal River.

Appendix 3E: The concentration of metal absorbed by shoots and roots of water hyacinth grown in floating cages above and below the Koekemoerspruit and Schoonspruit inlets on the Vaal River after the start of the rain (weel 7) and for those at the site of Kennan (5 km before the entry of the Schoonspruit into the Vaal River near the Township of Kennan) at the start of the experiment, before the start of the summer rain. Concentration unit is (mg kg^{-1}).

	Kennan		**Koekemoerspruit sites**				**Schoonspruit sites**			
			Above inlet cage		**Below inlet cage**		**Above inlet cage**		**Below inlet cage**	
Elements	**Metal absorbed by shoots**	**Metal absorbed by roots**	**Metal absorbed by shoots**	**Metal absorbed by roots**	**Metal absorbed by shoots**	**Metal absorbed by roots**	**Metal absorbed by shoots**	**Metal absorbed by roots**	**Metal absorbed by shoots**	**Metal absorbed by roots**
Cu	nd	0.09 ± 0 a	0.02 ± 0 bc	0.06 ± 0 ab	0.028 ± 0 cd	0.07 ± 0 a	0.05 ± 0 a	0.08 ± 0 a	0.05 ± 0 a	0.09 ± 0 a
Fe	0.66 ± 0.4 a	29.13 ± 5.3 e	1.28 ± 0.1 a	18.1 ± 1.0 cd	0.97 ± 0.3 a	11.58 ± 3.5 bc	0.4 ± 0 a	8.83 ± 0.9 b	0.63 ± 0.1 a	20.04 ± 1.7 d
Hg	0.55 ± 0.3 b	1.7 ± 0.1 c	0.43 ± 0.1 ab	0.4 ± 0 ab	0.35 ± 0.1 a	0.3 ± 0.1 a	0.39 ± 0 ab	0.24 ± 0 a	0.32 ± 0.1 a	0.33 ± 0 a
K	143.4 ± 82.8 f	61.8 ± 7.6 ac	71.68 ± 8.9 cd	38.76 ± 8.6 ab	97.08 ± 8.2 d	61.11 ± 3.8 ac	213.9 ± 4.1 e	31.45 ± 4.8 b	190.47 ± 7 e	42.11 ± 5.4 ab
Mn	1.34 ± 0.8 a	26.08 ± 3.0 c	1.23 ± 0.2 a	6.18 ± 1.4 ab	0.78 ± 0 a	2.03 ± 0.2 a	2.21 ± 0.1 a	10.2 ± 1.1 b	2.49 ± 0.3 a	34.12 ± 4.8 d
P	95.07 ± 54.9 e	84.37 ± 3.6 e	42 ± 4.9 ac	34.58 ± 0.9 abc	36.42 ± 0.3 abc	27.64 ± 1.1 bd	40.8 ± 0.5 a	20.42 ± 0.6 d	45.5 ± 2.9 a	28.19 ± 2.0 bcd
S	2.23 ± 1.3 a	9.72 ± 0.3 d	2.43 ± 1.5 ab	1.54 ± 1.0 a	2 ± 0.3 a	2.38 ± 0.8 ab	4.17 ± 0.7 bc	3.48 ± 0.2 abc	3.3 ± 0.7 ab	5.33 ± 0.7 c
Zn	0.21 ± 0.1 ab	0.7 ± 0.1 c	0.18 ± 0 ab	0.33 ± 0 a	0.34 ± 0.2 a	0.32 ± 0 a	0.11 ± 0.0 b	0.36 ± 0 a	0.25 ± 0 ab	0.6 ± 0 c
Mg	14.85 ± 8.6 d	19.07 ± 1.6 bd	28 ± 1.9 a	20.54 ± 5.5 b	29.15 ± 0.6 a	30.78 ± 0.6 a	28.7 ± 0.4 a	6.75 ± 0. c	20.73 ± 0.9 b	8.71 ± 0.4 c

NB: Means were compared by One-way ANOVA and means in rows followed by the same letter(s) are not significantly different ($P > 0.05$; Fisher LSD test). Means are compared across the table in rows. NB: Kennnan represents the Schoonspruit before reaching the Vaal River, (5 km away from the Vaal River), and it was the site from which all the plants used in cages at the inlets of the Koekemoerspruit and the Schoonspruit to the Vaal River, were transported from. The Kennan data is included in this table as a base line data to show the initial metal concentration in plant tissues before the start of the experiment at the Vaal River.