Remediation of New Zealand sheep dip sites using biochar and phytoextraction technologies

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ABSTRACT

The practice of sheep dipping, which subjected livestock to inorganic and organic agricultural pesticides to eradicate pests such as lice and keds, is a historic practice; sheep dipping is no longer practiced in New Zealand today. Animals would be submerged in solid structures known as dips containing chemicals such as arsenicals and organochlorines with the leftover solution pumped onto surrounding soil. The use of pesticides such as these is now banned by law due to their persistence in the environment. Today an estimated 50,000 contaminated sheep dip sites exist in New Zealand representing perhaps the countries' most significant, but understated, environmental challenge.

To determine whether this historic agricultural practice had led to contamination of the environment, an investigation into the extent of contamination resulting from sheep dipping at a known historic dip site in Te Mahia, New Zealand was carried out. Characterisation of the site by arsenic soil concentration mapping revealed that 500 m^2 of agricultural land has been contaminated with this metalloid and that arsenic exists at varying high concentrations through the soil profile. Environmental risk from these historic pesticides was established by analysing plant and water samples below the dip site. Staple Maori food varieties such as watercress were significantly contaminated with arsenic while water samples taken from the stream below the dip returned spiked arsenic concentrations. Based on this, it was justified that arsenic/organochlorine contamination would need to be managed to reduce their effect on these food sources. The design of a coupled remediation strategy using phytoextraction and biochar was utilized to reduce remediation times and is the basis of this thesis.

Contaminated soil from the site was removed and amended with two types of biochar produced from willow feedstock. These biochars, known as 350°C and 550°C biochar were added into the soil at application rates of 30 t ha^{-1} and 60 t ha^{-1}. During a series of 180 d glasshouse trials, the phytoextraction of arsenic into *Lolium perenne* (ryegrass) shoot tissue was analysed along with growth parameters of shoot and root biomass and corresponding response to arsenic at the molecular level. In soil; microbial activity,

soil bacterial community, organochlorine concentration, and element dynamics were analysed as a function of biochar amendment.

Soil microbial activity, analysed using the dehydrogenase assay (DHA), was significantly increased ($P<0.01$) under all biochar treatments compared to the control after 180 d during two glasshouse trials. Metagenomic analysis of the soil bacterial community revealed that biochar amended soils were selecting for bacterial species such as Chryseobacterium, Flavobacterium and Dyadobacter and the family Pseudomonadaceae which are known bioremediators of hydrocarbons. This resulted in isomers of the organochlorine hexachlorocyclohexane (HCH), particularly alpha-HCH and gamma-HCH (lindane), undergoing 10-fold and 4-fold reductions in soil concentrations respectively (2.2 mg kg^{-1} and 0.4mg kg^{-1}) compared to the control (25 mg kg^{-1} and 1.6 mg kg^{-1} respectively). Amendment of soil with both biochars also caused a significant reduction ($P<0.01$) in soil DDT levels.

Biochar promoted a 2-fold increase in shoot dry weight (DW) and a 3-fold increase in root DW after 180 d during one glasshouse trial while during the second trial only ryegrass root biomass was significantly increased as a function of biochar amendment. This increase was attributed, at least in part, to the fertility value of biochar. No negative effect of biochar amendments on ryegrass germination was observed. All biochar amendments resulted in significant increases in arsenic concentrations within ryegrass shoot material. Through extrapolation, 350°C biochar amended soils was estimated to have the potential to increase ryegrass sward DW growth by 0.68 t ha^{-1} compared to ryegrass grown on unamended soils and would correspond to an increase in the extraction of total arsenic by 14,000 mg ha^{-1} compared to unamended soils and in doing so decrease soil remediation times by over 50 %.

Increased arsenic uptake as a function of biochar amendment resulted in increased enzymic activity of components of the antioxidant pathway including SOD and APX in most biochar treatments but across all treatments a reduction in GPX activity was observed. Analysis of specific metabolites utilizing metabolomics also suggest a definitive metabolite profile under biochar amendment compared to contaminated

control ryegrass samples. However, there was no significant difference ($P<0.05$) in chlorophyll content in response to the total arsenic concentration in ryegrass shoot tissue grown on contaminated soil. The observed increases in activity of SOD, APX and steady CAT activity is suggested to be efficiently catalysing the production of harmful ROS in this soil.

A 6-month field investigation into the effect of biochar amendment on the extraction of arsenic into a high biomass crop (*Salix sp*) resulted in significant increases of arsenic in stem biomass as a function of biochar amendment. When data was extrapolated to predict results of a long-term field trial and scale under willow treatment (stem) it was calculated that over 67.7 g of arsenic could be extracted in soils amended with 350°C biochar compared to 5.9 g extracted under control treatment. This could result - assuming a similar rate of extraction with time - in levels of arsenic concentration in soils reaching background concentrations in as little as 6 years, a reduction in remediation times of 92%.

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ABBREVIATIONS

AAS	Atomic Absorption Spectrometry
AOAC	Association of Analytical Communities
APX	Ascorbate Peroxidase
ARML	AgResearch Metabolomic Laboratory
As	Arsenic
ATP	Adenosine-5'-Triphosphate
BET	Brunauer–Emmett–Teller
bp	Base Pair
°C	Degrees Celsius
CaCO3	Calcium Carbonate
CAT	Catalase
CEC	Cation Exchange Capacity
cm	Centimetre
C_{org}	Organic Carbon
d	Day
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DHA	Dehydrogenase Activity
DM	Dry Matter
DNA	Deoxyribonucleic Acid
DW	Dry Weight
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
eV	Electronvolt
FIAS	Flow Injection Atomic Spectroscopy

FW	Fresh Weight
GC-MS	Gas Chromatography – Mass Spectrometry
GFAAS	Graphite Furnace Atomic Absorption Spectrometry
GPX	Guaiacol Peroxidase
H_2O_2	Hydrogen Peroxide
HCH	Hexachlorocyclohexane
H/C_{org}	Hydrogen / Organic Carbon Ratio
HILIC	Hydrophilic Interaction Liquid Chromatography
HNO_3	Nitric Acid
hr	Hour
IAA	Indole-3-acetic Acid
K_2PO_4	Potassium Phosphate
KI	Potassium Iodide
LC-MS	Liquid Chromatography – Mass Spectrometry
M	Molar
Meq	Milliequivalents
min	Minute
mL	Milli Litres
mmol	Millimoles
MQ	Milli Q
mV	Milli Volts
N_2	Nitrogen
NaCl	Sodium Chloride
NBT	Nitro-blue Tetrazolium
ng	Nanogram
O/N	Overnight
PC	Phytochelatin

PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PGPR	Plant Growth Promoting Rhizobacteria
PLS-DA	Partial Least Squares - Discriminant Analysis
ROS	Reactive Oxygen Species
rpm	Revolutions Per Minute
s.e	Standard Error
SOD	Superoxide Dismutase
sp.	Species
TGA	Thermogravimetric Analysis
t ha^{-1}	Tonnes per Hectare
WHC	Water Holding Capacity
WHO	World Health Organisation
w/v	Weight to Volume
w/w	Weight to Weight
XPS	X-ray Photoelectron Spectroscopy

CHAPTER 1 : INTRODUCTION

1.1 Background

The backbone of New Zealand's economy has been the agricultural sector, namely sheep and beef farming, for decades. But with most agricultural activities a certain environmental cost is incurred, particularly for historical farming practices. For much of history, farmers were required by law to treat sheep with a range of chemicals to prevent the spread of a number of pests and to assist in the eradication of a select few that were known to cause a loss in financial return (e.g. damage to fleece) (Ministry for the Environment, 2006). These chemicals, although highly successful, were removed from the markets due to their persistence and ability to accumulate in the wider environment (Edvantoro et al., 2003; van Zwieten et al., 2003; Chopra et al., 2007). Pesticides such as organochlorines and arsenicals were applied to animals by submerging individual sheep in a chemical bath (usually a solid concrete structure built into the ground) that featured on every sheep farm in New Zealand (Ministry of Agriculture and Fisheries, 1976). Because of this, it is estimated that up to 50,000 historic dip sites exist in this country.

Although most of these structures are no longer in use due to modern pour-on techniques, the persistence of these toxic chemicals continues. Moreover, land-use changes from farming into rural sub-division are creating a sudden drive to identify contaminated sites and reduce or remove the contamination that exists in these areas (Juhasz et al., 2003). Also, historically-contaminated soil can negatively affect water quality where arsenic leaches through the soil profile and becomes a diffuse source of pollution to streams and rivers. Arsenic and organochlorines in soil can detrimentally affect soil microbial activity which is an important index of nutrient cycling and soil health (Klose & Ajwa, 2004; Pampulha & Oliveira, 2006; Zhou et al., 2006). Therefore, the remediation of historically contaminated sheep-dip sites has been defined as an important goal for future environmental sustainability in New Zealand (NZ Ministry for the Environment, 2006). Two technologies that have the potential to manage and/or remediate arsenic and organochlorines in soil are biochar and phytoextraction.

Phytoextraction (from the umbrella term phytoremediation), uses the natural ability of plants to take up pollutants from the soil and is used around the world to remediate contaminated soil. The metalloid arsenic, which is prevalent at a number of dip sites around New Zealand, can be targeted using this method. A number of plant species can be used, including arsenic hyperaccumulating ferns to remove this contaminant from the soil. However, in any environmental study, to better understand the nature of removal, plant defence enzyme pathways will be analysed, particularly the antioxidant pathway, which is responsible for protecting the plant against oxidative stress.

A unique approach for this research is the tandem use of biochar along with phytoremediation for the remediation of these sites. Biochar has been signalled as an initiative to mitigate climate change by capturing and storing carbon as a stable form in soil. Due to the co-contamination of arsenic and organochlorines in the soil, soil microbial health is thought to be low. Biochar will be produced and serve a number of purposes, namely the stimulation of microbial activity to breakdown persistent organochlorines but also to study the possible change in arsenic dynamics due to changes in soil pH that biochar will cause. This will eventually create an increase/decrease in arsenic adsorption in soil resulting in an increase/decrease in uptake for phytoextraction or stabilisation particularly around nearby waterways.

1.2 Research Objectives

The aim of the research consisted of two main objectives:

- **To investigate the impact of sheep-dip derived soil contamination on food sources and the wider environment.**
- **To design a culturally acceptable management plan for the control (either immobilisation on removal/degradation) of these contaminants.**

This research was designed to understand the role of biochar in contaminated soil and to define how it could change soil-contaminant dynamics and associated dynamics with plant growth.

1.3 Thesis Outline

This thesis reports the current knowledge of soil contaminants associated with sheep dip sites and the history surrounding these sites. Current research into both biochar and phytoremediation technologies is explained and detail is presented on how these technologies are implemented. The thesis comprises 10 chapters in total. The first two chapters are general introduction and literature review respectively. Chapters 5-7 are research chapters and have been submitted (or are in the process of submission) as journal articles. To reduce duplication and repetition, these chapters have been modified to include a shortened, succinct introduction along with removal of both materials and methods and reference sections, to consolidated parts of the thesis (Chapter 3).

Chapter 1 is a general introduction to the thesis and provides background information relating to its format.

Chapter 2 provides a review of (i) the history of sheep dipping, (ii) the nature of chemicals used, (iii) the association these chemicals have with a number of soil properties and also their main degradative pathways, (iv) the use of biochar and phytoremediation technologies and their effects on soil properties and plant growth and (v) plant defence mechanisms associated with stress and growth. This leads onto the specific objectives of the thesis.

Chapter 3 details the materials and methods used throughout laboratory, glasshouse and field analysis in regards to soil and biochar analysis.

Chapter 4 details the locality of the sheep dip site under investigation and its history. Included in this chapter is the effect of current soil arsenic concentrations on nearby environmental ecosystems and also an investigation into the spread of contamination resulting from arsenic and organochlorine use.

Chapter 5 reports the results of contaminated soil amended with biochar produced from willow at two temperatures of pyrolysis (350 and 550°C) and the effect of amendment on microbial activity. Following on from this a detailed analysis of

bacterial community structure is carried out and the degradation of organochlorines is studied.

Chapter 6 describes the effect of biochar produced at 350°C and 550°C on arsenic uptake and plant growth in *L. perenne* L when amended to contaminated soil. This investigation also details how phytoextraction efficiency can be increased using biochar as a soil amendment.

Chapter 7 describes a field trial carried out at the site described in Chapter 4. This chapter assesses the effectiveness of biochar amendment in the field as compared to the controlled conditions described in Chapter 5 and 6. The field trial was conducted to determine the time needed to mitigate arsenic environmental risk at the site. In this study the objectives were to determine whether the amendment of soil with a willow biochar produced at 350°C and 550°C would affect soil microbial activity, and promote arsenic uptake in *Salix sp*. (willow) a high biomass crop.

Chapter 8 describes an array of molecular analyses carried out on ryegrass tissue and includes enzymatic defence related activity in response to arsenic. Chapter 8 presents metabolomic analysis that were used, in part, to quantify whether arsenic uptake caused metabolite upregulation/downregulation. Included in this chapter is a materials and methods section pertinent soley to this part of the study.

Chapter 9 is a general discussion of the whole thesis with remarks designed to bring site characterisation and environmental risk together with the results from the research described in Chapters 5-7. Also included is an overall summary of the thesis concluding with some future management recommendations for historic sheep dip sites.

CHAPTER 2 : LITERATURE REVIEW

2.1 History and the Practice of Dipping

2.1.1 History of Sheep Dipping

Commercial sheep farming began soon after the signing of the Treaty of Waitangi in 1840 with the focus of sheep welfare paramount to the success to the New Zealand economy (Ministry for the Environment, 2006). Not long after, the colonial government that existed at the time passed the Scab Ordinance bill which was designed to prevent and eventually eradicate the very infectious disease sheep scab (Ministry of Agriculture and Fisheries, 1976). Although dipping was not compulsory during this time, inspectors at sale yards had the power to force dipping of infected flocks before sale or impose fines on owners of infected stock.

The Sheep Act of 1878 soon superceded the Scab Ordinance bill and required all farmers to dip their stock by either complete immersion or plunging (Ministry of Agriculture and Fisheries, 1976). Because of this large sheep farms all across the country soon constructed dip runs built into the ground for the immersion of stock (Kettle, 1985). Smaller farms usually constructed smaller ring type baths or transported stock to a known dip on nearby larger farms. By 1894 New Zealand was declared free of scab with the focus shifting to the control of other sheep parasites namely lice, flies and keds which were common during this time (Kettle, 1985; Ministry for the Environment, 2006). Further legislation was passed in the 1900s such as the 1908 Stock Act which required owners to dip their stock once a year during a specified period for better overall protection against the above named parasites (Oelsner, 1985a). This was in light of a number of farmers complaining to the local authorities of lazy neighbours who did not dip their sheep, creating a new infestation even though the farmers were stringent with their dipping protocols.

Finally, The Animal Remedies Act of 1967 required sheep owners to either dip or dust their stock once a year for the prevention of parasitic infestation (Kettle, 1985). An improvement to this Act in 1982 required that all sheep should be dipped in accordance with the directions on the chemical label and that dipping shall be carried out in a specified time after sheep had been shorn (Charleston, 1985). Presently,

dipping is no longer required by law but sheep should be inspected regularly and infestations monitored if present.

2.1.2 Dipping Practices

Sheep have a natural oily fleece that protects against adverse climate and exposure. However, the protective nature also acts as a habitat for external parasites which, depending on the parasite, feed on the wool or animal flesh itself (Ministry for the Environment, 2006). This natural oily fleece poses a problem in itself as the chemicals needed to remove parasites from the fleece needed to be very potent and would require full immersion of the animal in a chemical bath (Oelsner, 1985b).

Not long after the signing of the Treaty of Waitangi the idea of chemical immersion in trenches or baths was conceptualised. These dipping structures differed according to the size of the farm, the logistics of dipping and also stocking numbers of sheep. Pot dips (**Figures 2.1 and 2.2**) were often used for smaller flocks of sheep on smaller farms but swim dips (**Figure 2.3**) that were very long and trench-like in construction were often built on the larger sheep farming stations to process the dipping of sheep more efficiently (Chopra et al., 2007). Both methods at the time required sheep to swim through a deep chemical bath finally ending with the farmer using a crutch to fully immerse the animal's head under the dip (Ministry for the Environment, 2006). Manual handling of sheep was highly reduced with the invention of power spray machines in the 1940s which allowed for the construction of above-ground holding pens to treat sheep (Figure 4) (Oelsner, 1985b; Chopra et al, 2007). Groups of sheep would pass into a holding pen and be sprayed with jets of chemicals, mitigating the need to be individually handled like earlier methods (Ministry for the Environment, 2006). An enormous environmental benefit of this shower method was the construction of an underground sump which allowed the recycling of unused chemicals. This is a significant factor when identifying contamination at historic sheep farms as swim and pot dips would contain large amounts of left over chemical dip that would eventually be discharged into nearby areas.

Figure 2.1. Ring bath or pot dip in use (Duncan, 1941).

Figure 2.2. Disused and dilapidated ring bath (Duncan, 1941).

Figure 2.3. A swim dip showing immersion of stock (Duncan, 1941).

Figure 2.4. Sheep shower unit in use (Duncan, 1941).

2.1.3 Chemicals Used and Likely Contamination

At present, pour-on techniques use a combination of organophosphates and insect growth regulators (IGRs) to control parasite reproduction. These are highly toxic but do not pose an environmental threat as they readily breakdown in the environment. However, typical historical treatments are often perceived to be persistent and very damaging to a number of ecological indicators (McBride et al., 1998; Ministry for the Environment, 2006). In the context of the environment, organochlorines can be passed up the food chain (i.e. livestock ingesting contaminated soil and passing chemicals to the developing fetus) while arsenic can bioaccumulate in aquatic plant and animal life to toxic levels. A summary of chemicals used since legislation was passed for the treatment of sheep after 1840 is presented in **Table 2.1.**

Table 2.1. Chemicals used to treat sheep for parasite control since 1840

Chemical	Period of Use
Arsenic	1840s – 1980
Nicotine	1840s – mid 1900s
Carbolic Acid and Potash	1880s
Derris	1910 – 1952
Lime Sulphur	1849 – 1891
Zinc	1950s – present
Copper	1950s – present
Organochlorines	
- DDT	1945 – 1961
- Lindane	1947 – 1961
- Dieldrin	1955 – 1961
- Aldrin	1955 – 1961
Organophosphates	1960s – present
Synthetic Pyrethroids	1970s – present
Insect Growth Regulators	present

In this review, an emphasis is placed on arsenicals and organochlorines due to their persistence and hazardous nature and widely reported use at historic dip sites.

2.1.4 Organochlorines and Their Use in Dip Solution

Organochlorines were a common pesticide choice for use in dips due to their ability to diffuse through the oily fleece of sheep. They did not, however, completely replace the use of arsenic and were often applied in tandem for treatment in the longer swim dips (Chopra et al, 2007). Organochlorines often used in dip solution included the chlorinated cyclodienes (aldrin, dieldrin, heptachlor) and the chlorobenzene derivatives (DDT and lindane) (National Rivers Authority, 1994). These were often applied as a suspension in dip but required additions of the chemical at regular intervals as the fleece of passing sheep would remove the chemical from solution (Ministry for the Environment, 2006). All organochlorines were found to be much more effective than arsenic for the control of parasites but in turn, each developed derivative surpassed the former based on its time of use (see **Table 2.1**) (McBride et al., 1998; Edvantoro et al., 2003; van Zwieten et al., 2003; Chopra et al., 2007). By 1961, issues relating to organochlorines were being raised because of their ability to accumulate in body fat and be passed onto higher life forms. This accumulation pattern resulted in the group becoming prohibited under the 1961 Stock Regulations Act.

2.1.5 Arsenic and its Use in Dip Solution

Although dip chemicals evolved from organochlorine to organophosphate treatments, the use of arsenic as a pesticide was standard practice during dip events. Arsenic remained on the market until deregistration in 1978 when farmers and the NZ government started to realise its detrimental potential as a persistent environmental hazard. Its main target was the control of lice and blowfly but its effectiveness as a toxic agent was determined by its efficacy to be ingested by the target host (Ministry for the Environment, 2006). Even during early use, farmers noticed drawbacks from

arsenic, mainly in relation to animal health (Chopra et al., 2007). Dipped sheep would often ingest the chemical and in doing so, would suffer the harmful effects of arsenic poisoning. On hot days, arsenic would cause skin scald due to the arsenic salt reacting with the intense heat (Ministry for the Environment, 2006). Over the next 100 years arsenic use resulted in contaminated soil and waterways surrounding dip sites (Edvantoro et al., 2003; van Zwieten et al., 2003; Chopra et al., 2007) due mainly to unknown effects of these chemicals.

2.1.6 Likely Contamination Zones Surrounding Dip Sites

Before defining contamination, a 'risk' concentration for each of the likely contaminants at the site must be addressed. These are concentrations that have been published by the World Health Organisation (WHO) in response to increasing areas suffering from ill-health in relation to chemicals from industrial and natural sources. New Zealand has its own stringent values (**Table 2.2**).

Table 2.2. Soil guideline values for the protection of ecological receptors. Modified from (Ministry for the Environment, 2006) values in mg kg^{-1}

Value Name	Arsenic	DDT	Dieldrin	Lindane
Minimal Risk Value	12	1.8	0.002	0.006
Serious Risk Value	22	13	0.5	2.1

These values, in most cases, are exceeded at dip sites for arsenic and usually for organochlorines, particularly dieldrin, but the zone of contamination is often ill-defined due to the surrounding topography and design of the sheep dip (van Zwieten et al., 2003). When an investigation begins on a known dipping site it is safe to assume excessive contamination would exist in and directly around the dip structure but in most cases there are other areas in close proximity that exceed guideline values

(Edvantarro et al., 2003). **Figure 2.5** gives a representation of associated structures found surrounding a common dip site. Sheep would enter from the northern end of the dip, swim through the bath, then be held in a drainage pen to remove excess chemical solution remaining on the fleece. From here they would be held in larger sheep yards until the dipping of the flock had been completed. More often than not the surrounding sheep yards would be one area of contamination, while greater contamination would exist in the drainage pen which varied in size depending on the size of the farm (Dewar et al., 2005). In relation to drainage of leftover chemical solution, this was often scooped out and placed next to the dip or pumped down a nearby bank or directly into waterways (Ministry for the Environment, 2006). These past practices give an understanding into the variability of contamination that can exist between sites as if a pump or gravity-fed slope was located at the site, then contamination may be spread over a wider distance.

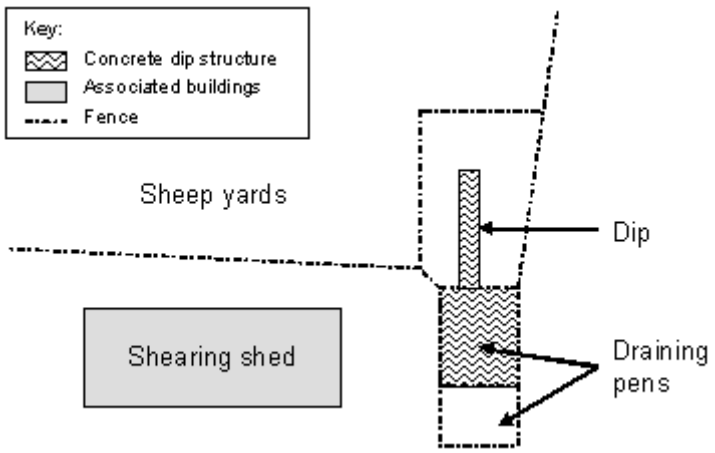

Figure 2.5. A sketch of a typical swim dip site with related structures (Ministry for the Environment, 2006).

2.2 Organochlorines and the Environment

Chemical technology developed specifically for the agricultural industry has produced many organic compounds which are resistant to physical, chemical and biological degradation (Holden, 1975). A number of these compounds have some historic significance to past sheep dipping events and are recognised as chemically stable and persistent in the environment. Environmental persistence of organic compounds such as organochlorines should not be strictly related to what contamination level is found in soil surrounding a dip site. Instead persistence should be defined by form and what organisms are affected. For instance, soil contamination levels of organochlorines could be negligible but these chemicals could accumulate in water or plant biota and thus be persistent in that environment (Edvantarro et al., 2003). Organochlorine pesticides are of significant concern because of their toxicity and bioaccumulation properties (Babu et al., 2003). Even the slightest level of contamination may contribute to sustained long-term effects in the surrounding environment and as such the NZ government has implemented strategies to deal with this problem (Ministry for the Environment, 1998a).

2.2.1 Organochlorine Use and NZ Sheep Dipping

In 1995, the Ministry for the Environment intitiated a national programme to assess the extent of contamination leftover from the use of organochlorine pesticides in New Zealand. Targeted in this programme were pesticides commonly used (during the 1950-1960 period) in sheep dipping such as aldrin, DDT and lindane (Ministry for the Environment, 1998b). Research and assessment carried out during this programme eventually showed that apart from sheep dipping sites and industrial areas New Zealand is moderately clean (Ministry for the Environment, 1998b; Stockholm Convention On Persistent Organic Pollutants, 2006). Background research such as this is vital to understanding contamination levels that may exist at dip sites through definition of a natural benchmark for uncontaminated soil. **Table 2.3** lists each pesticide and its derivatives commonly found in the environment. Such values are

often used to decide what represents organochlorine contamination in a defined environment.

Pesticides were a major success in NZ agriculture when their effect on sheep health and welfare is considered. Although they were banned from developed countries such as New Zealand they are still being widely used in developing countries to control diseases such as malaria (Lal & Saxena, 1982; Loganathan & Kannan, 1994). As with most pesticide formulations available on the market, the organochlorines were designed to be toxic to target organisms only, and thus less toxic to ecological non-target organisms such as aquatic biota. However, this aim was not necessarily achieved with all pesticides (Juhasz et al., 2002). Their persistence in the environment potentially makes them an ecological problem due to their long last effects (Edvantoro et al., 2003; van Zwieten et al., 2003; Chopra et al., 2007). Commonly, the applied pesticide reaching the target organism is about 0.1%, the remaining solution contaminates the environment (Hussain et al., 2009).

One of the most important ecological interactions is with soil microbial communities which are synonymous with nutrient cycling, decomposition and geochemical processes (Lal & Saxena, 1982). In a contaminated environment, soil microbes are an effective means for the natural degradation of organochlorines but the co-contamination of arsenic that exists at a number of dip sites has been shown to cause significant inhibition of degradation (Edvantoro et al, 2003; van Zwieten et al., 2003). Onsite analysis at Australian dip sites compared co-contaminated sites (DDT and arsenic) with sites containing background levels of arsenic (5 mg kg^{-1}) and found that a 50 % reduction in DDD breakdown has occurred. A similar trend yielded the same results when analysing DDE levels (van Zwieten et al., 2003). Edvantoro et al. (2003) made similar conclusions when analysing cattle dip sites. Inhibition of microbial breakdown due to co-contamination seems to be a key component for increased residency of these pesticides.

2.2.2 Breakdown of Organochlorine Pesticides

Pesticides like the organochlorines can be degraded by a number of processes, both chemically (Rodriguez-Garrido et al., 2004) and biotically (Kuiper et al., 2004; Chaudhry et al., 2005) yet it is the biotic process of degradation that is environmentally safer and cheaper. Microorganisms have the ability to accumulate very high levels of organochlorines and thus biomagnify the target pesticide up the trophic chain (Lal & Saxena, 1982) but it is their ability to breakdown pesticides which is a beneficial factor for the remediation of sheep dip sites. Different species and densities of microorganisms are known to degrade organochlorines at different rates and that certain microorganisms like *S.cerevisiae* degrade isomers of lindane more efficiently ($\gamma<\beta<\alpha$) (Rice & Sikka, 1973a; Rice & Sikka, 1973b).

Four main pathways exist as described by Lal & Saxena (1982) for microbial degradation. These are reductive dechlorination, dehydrochlorination, oxidation and isomerisation. All four pathways are used to differing degrees (depending on the pesticide) by soil microbes and as such degradation of each pesticide is discussed as follows.

2.2.3 DDT Breakdown

The primary microbial breakdown pathway for DDT is reductive dechlorination to form DDD (Aislabie et al., 1997). This reaction replaces a chlorine atom on a non-aromatic carbon with a hydrogen atom however, dehydrochlorination is also a degradative pathway for some microorganisms. In this case, DDT is degraded to DDE by the simultaneous removal of hydrogen and chlorine that takes place between saturated chlorinated carbons (Lal & Saxena, 1982). Metabolism studies have shown that reductive dechlorination dominates under anaerobic conditions while dehydrochlorination dominates under aerobic conditions (Guenzi et al., 1967). **Figure 2.6** describes the primary degradation of DDT and its derivatives by microorganisms. Many microbial species contribute to this pathway and as such play an individual role in the breakdown of a specific derivative (Juhasz et al., 2002; Juhasz et al., 2003). **Table**

2.4 lists specific species of microorganisms along with primary metabolites derived from the other main organochlorines when subjected to microbial degradation.

2.2.4 Lindane Breakdown (γ – HCH)

Lindane is less persistent in the environment relative to DDT but as it was commonly used in dipping procedures it would be unwise to discount it entirely from analytical procedures. Similarly to DDT breakdown, co-contamination of arsenic restricts its breakdown under natural conditions but anaerobic conditions are thought to increase metabolism of lindane breakdown (Kohnen et al., 1975; Siddaramappa & Sethunathan, 1975). Dehydrochlorination is a common pathway for metabolism (Lal & Saxena, 1982).

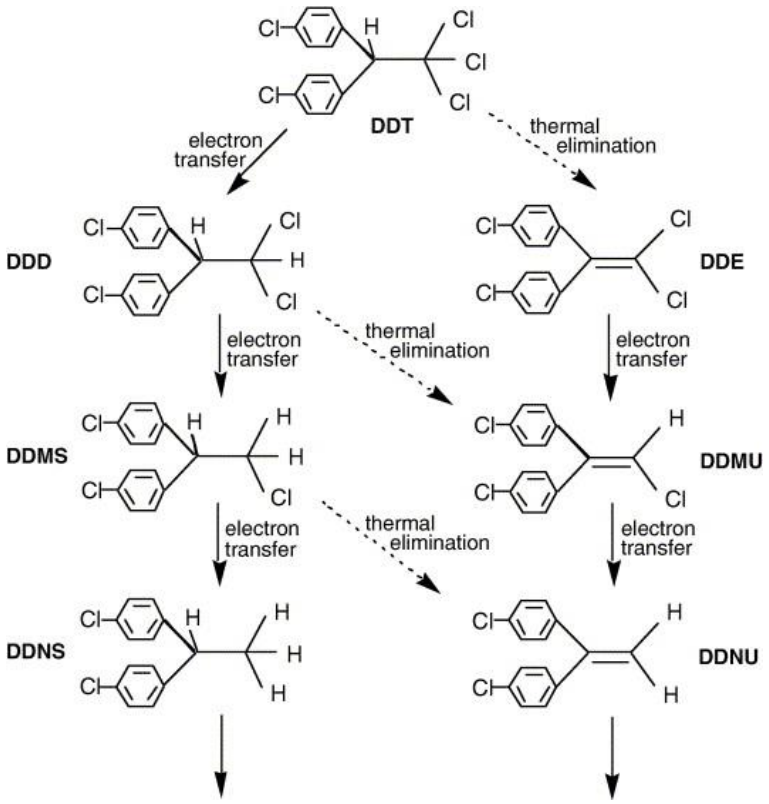

Figure 2.6. The primary breakdown pathway of DDT and its derivatives by soil microorganisms. Main pathways include reductive dechlorination and dehydrochlorination. Degradation can be assisted by thermal means. Figure modified from (Lal & Saxena, 1982).

Table 2.3. Summary of median organochlorine pesticide concentrations in NZ soil. Concentrations are in **µg kg^{-1}** DW soil basis. na = not analysed , nc = not calculated (detected less than 66% of occasions). Table modified from : Ministry for the Environment (1998a).

Pesticide	**Indigenous Forest**	**Indigenous Grassland**	**Hill Country Pasture**	**Flat Land Pasture**	**Provincial Centre**	**Metropolitan Centre (Auckland)**	**Metropolitan Centre (Christchurch)**
	(n = 7)	(n = 5)	(n = 8)[3]	(n = 8)	(n = 8)[3]	(n = 9)	(n = 6)
α - HCH	< 0.02	< 0.01	na	na	< 0.01	< 0.01	< 0.01
β - HCH	< 0.01	< 0.01	na	na	< 0.01	< 0.01	< 0.03
γ - HCH	< 0.02	< 0.02	na	na	< 0.02	< 0.04	< 0.04
Aldrin	< 0.01	< 0.01	na	na	< 0.02	< 0.02	< 0.02
Dieldrin	0.24	0.23	na	na	0.41	0.69	1.44
α - Chlordane	< 0.03	< 0.04	na	na	< 0.03	0.08	< 0.04
γ – Chlordane	< 0.02	< 0.03	na	na	< 0.03	0.05	< 0.03
pp' - DDE	1.20	0.67	na	na	9.32	4.09	190
pp' - TDE	0.087	0.13	na	na	1.52	0.48	4.38
op' - DDT	0.11	0.10	na	na	0.76	0.95	20.6
pp' - DDT	0.83	0.76	na	na	12.0	8.54	131

Table 2.4. Degradation of organochlorines by select soil microorganisms and the metabolite products formed. Table modified from (Lal & Saxena, 1982).

Organochlorine	Microorganism	Metabolite (s)
DDT	*Streptomyces annamoneus*	DDD
	Echerichia coli	DDD, DDMU, DDMS, DDA
	Pseudomonas sp.	DDD
	Isochrysis galbana	DDE
	Saccharomyces cerevisiae	DDD
Lindane (γ-HCH)	*Clostridium sp.*	γ-TCCH
	Pseudomonas putida	γ-PCCH, γ-TCCH, CO_2
	Echerichia coli	γ-PCCH, α-BHC
Aldrin	*Pseudomonas sp.*	*trans*- Aldrindiol
	Dunaliella sp.	Dieldrin
	Aspergillus flavus	Dieldrin
	Trichoderma viride	*trans*- Aldrindiol, photodieldrin
Dieldrin	*Bacillus sp.*	*Trans*- Aldrindiol
	Pseudomonas syringae	photodieldrin, *trans*-Aldrindiol
	Pseudomonas sp.	CO_2
	Bacillus sp.	CO_2
	Mycoccus	CO_2
	Neurospora sp.	*Trans*- Aldrindiol, photodieldrin

2.2.5 Aldrin and Dieldrin Breakdown

Dieldrin is considered to be a very persistent pesticide even when conditions suitable for its degradation exist. In contrast, aldrin is believed to volatilise rapidly from soil but its conversion to dieldrin by soil microbes is of great concern (Harner et al., 1999). Evidence for such a conversion reaction was observed initially by Korte et al. (1962) and later proved by Tu et al. (1968) who showed that a majority of microorganisms carry out this reaction. The oxidation reactions of aldrin to dieldrin is shown in **Figure 2.7**. A high proportion of dieldrin degradation is achieved by soil microbes, yet only 10 isolates of an original 577 of soil microorganisms named in Lal & Saxena (1982) were attributed to this degradation.

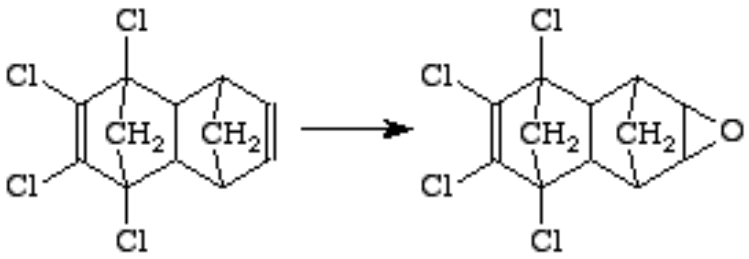

Figure 2.7. Oxidation-reduction conversion reaction between aldrin and dieldrin observed in the environment.

2.2.6 Effects of Pesticides on Microbial Communities

Depite being non-target organisms of pesticide applications, soil microbes are nonetheless affected by dip chemicals once applied to soil. Generally, pesticides have the capability to disturb the ecosystem of the soil and affect the natural community of microorganisms and their associated biochemical activities (Hussain et al., 2009). One major problem with pesticides such as the organochlorines is that they reduce soil enzyme activity which acts as a key component for analysing soil health (Antonious, 2003; Monkiedje et al., 2002). It is well known that applications of organochlorines can

play a detrimental role into the makeup of a microbial community such as that which exists around and in a dip site (Pampulha & Oliveira, 2006; Zhou et al., 2006; Klose & Ajwa, 2004). However, a less explored relationship is one of whether a change in community or change in microbial enzymatic activity significantly alters the breakdown of organochlorines in dip-contaminated soil. **Table 2.4** lists a number of soil microorganisms that are efficient in metabolizing key organochlorines, but the underlying presence of co-contamination (organochlorines and arsenic) at such a site could may be inhibiting microbial activity. Certain pesticides have the ability to influence or inhibit specific organisms which allows another to out-compete for food and thus thrive in a newly created habitat (Chen et al., 2001a). Alternatively, the presence of organochlorines at dip sites may initially inhibit microbial populations with normal activity only being re-instated after a period of time (Niewiadomska, 2004). Organochlorines such as lindane have even been observed to stimulate certain groups of microorganisms yet inhibited other anaerobic bacteria and affect the general morphological characteristics of microbial populations (Kanungo et al., 1995; Das & Mukherjee, 2000; Madhaiyan et al., 2006). Co-contamination studies carried out by Wang et al (2006) and Saez et al (2006) who studied the effect of a mixture of lindane, aldrin and simazine have recorded a differential effect of each chemical on soil microorganisms. Specific focus has been paid to *Xanthobacter autotrophicus*. The denitrifying ability of this micro-organism was negatively affected by simazine while nitric oxide release was strongly inhibited by both aldrin and lindane. Such an effect is possible at sheep dip sites on microbial populations in response to organochlorine-arsenic contamination. Lal and Saxena (1982) agree that if the possibility of co-contamination exists, microbes will behave somewhat differently (reduction in enzymatic activity, possibility of microbial phyla shifts). Studies on a loam soil in the presence of DDT and dieldrin decreased soil bacteria while fungi populations increased. Yet, a sandy loam soil contaminated with the same chemicals showed no decrease or change at all for bacteria and fungi (Tu et al., 1976). Both Edvantarro et al (2003) and Zwieten et al (2003) suggest that co-contamination that exists at cattle dip sites dramatically reduces fungal and bacterial biomass and thus a change and

inhibition of microbial activity must exist that detrimentally affects the ability of this community to breakdown organochlorines in these soils. Although microorganisms are the main source for organochlorine breakdown they have certain limitations to degrade these chemicals (Chaudhry et al., 2005). A synergistic relationship can exist between microorganisms and plant species to remove or breakdown these pesticides. The rhizosphere (a zone around the immediate vicinity of the plant root which holds most of the microbial community) can potentially be of significant importance to remediation. Diversity of microbes in this region is significantly high, which can house multiple strains of microorganisms required for the eventual breakdown of organochlorines such as DDT (Kuiper et al., 2004; Chaudhry et al., 2005). This zone is synergistic as plants provide root exudates, a carbon source for flourishing microbes which in turn eventually remove organochlorines that can be toxic to plants (Kuiper et al., 2004; Robinos et al., 2007). This carbon source from root exudates is a natural stimulator for microbial activity but this can also be achieved with the addition of carbon amendments such as biochar.

2.3 Biochar

Biochar has its origins set deep in history dating right back to when humans first used uncontrolled burn-offs of forestry to clear land for agricultural use. Its concept relates to the partial or complete absence of oxygen during thermal decomposition of plant biomass to form a number of products which all come under the biochar 'product umbrella' (Chan et al., 2007; Liang et al., 2010). In simple terms, biochar is charcoal added to soil to improve soil functions and reduce emissions from organic material that would otherwise naturally degrade to greenhouse gases (Kookana et al., 2011). The process of producing biochar is pyrolysis and this produces end-products such as oils, vapours and a solid carbon residue similar to charcoal. One major characteristic of biochar in terms of its relation to normal charcoal is its stable aromatic form of organic carbon that generally is very stable and persistent when applied to soil (Sohi et al., 2010). In this sense, biochar is a form of organic carbon that can be applied to soil and remain for decades, if not centuries, and resist biotic/abiotic degradative processes

(Glaser et al., 2002). This persistence is dependent on the type of biomass originally used, the processing conditions and also the pedoclimatic conditions of the soils which biochar is applied to (Czimczik & Masiello, 2007). To date biochar technology is seen as an avenue to mitigate climate change due to its ability to store carbon in a stable form without the release of greenhouse gases (Macias & Camps-Arbestain, 2010). Yet depending on design (feedstock, pyrolysis temperature) biochar can potentially be used in a number of other beneficial ways.

2.3.1 Biochar and the Environment

Biochar is strongly positioned as a force for carbon abatement (climate change mitigation) but is also linked intrinsically with food production, land-use change and improving the overall quality of the environment (Joseph et al., 2010; Sohi et al., 2010). In the context of its link with the global carbon cycle, biochar has the potential once produced on a commercial global scale, to negate climate change. Global modelling estimates that through photosynthesis plants draw in 120 Gt of carbon dioxide per year and roughly half of this is invested into new growth for the plant (Schlesinger, 1995). If converted to biochar, the amount of carbon being released from degradative processes would be diverted into a pyrolysed stable form (Yoshida & Antal, 2009) and this would reduce the Earth's carbon dioxide footprint. Remediation projects on contaminated land could use such a concept to create a closed loop system where plants used for phytoremediation can be returned back to the soil as biochar, creating a subsequent carbon-negative cycle (Major & Hopper, 2010).

Biochar amendment to soil also has the ability, under certain processing conditions, to improve yields in key agronomic crops. This unique characteristic has future benefits in nutrient management as inputs could potentially be scaled back, decreasing the possibility of nutrient overloading (Gaunt & Lehmann, 2008). Recent analysis of biochar additions into soil have shown that biochar is a realistic tool to reduce the need for intensive fertiliser use in selected soils (Wang et al., 2012a; Wang et al., 2012b). Asai et al. (2009) observed significant increases in first-year yield in rice when biochar was incorporated in soil with nitrogen-fertiliser compared to a control

(nitrogen-fertiliser only). Yamato et al. (2006) found a similar response in yields of maize and peanuts with the addition of bark-based charcoal and nitrogen-fertiliser. However, other studies have shown no response in increased yield and consider the possibility of nutrient immobilisation by biochar additions (Blackwell et al., 2007; Rondon et al., 2007). Where positive yields have been observed, enhanced nutrient dynamics have been cited as an explanation (Lehmann, 2007a; Lehmann, 2007b; Steiner et al., 2007; Laird, 2008). A long term trial using charcoal and chicken manure compost resulted in higher yields of rice and sorghum. Available nutrient status did not decrease in comparison to a control mineral fertiliser and the higher yields observed significantly increased nutrient export in charcoal amended fields (Steiner et al., 2007).

2.3.2 Biochar Production and its Indirect Effects in Soil

As variability of pyrolysis temperatures and feedstock types exist, it could be assumed that once applied to soil, an array of reactions within the soil may come to the fore. Biochar is usually produced at a temperature between 400 and 500°C (also known as low temperature pyrolysis) but can be produced at temperatures above 700°C (high temperature pyrolysis also known as gasification) (Lehmann, 2007a). Temperature changes during pyrolysis results in heterogeneity to the chemical nature of biochar. Its stability in the environment results from its recalcitrance based on aromatic ring structures but can also contain more easily degradable aliphatic carbon structures (Lehmann, 2007b; Nguyen & Lehmann, 2009). Such degradable structures are more numerous when feedstocks are subjected to low pyrolysing temperatures. A biochar designed in this way provides an available source of carbon that can be used by microbes as an energy source in a poor environment such as that that exists in contaminated soils and also favours the recovery of nutrients that are lost at higher temperatures (Joseph et al., 2010).

Carbon properties within the feedstock type can also be related to the extent of aliphatic carbon properties of biochar (Cheng et al, 2006). Increased pyrolysing temperatures have also been observed to increase the ash content of biochar due to the high degradative temperature (Glaser et al., 2002; Sohi et al., 2010). This feature

can modify the soil environment it is added to, particularly soil pH. Most historic sites in the Amazon that have received historic biochar applications have recorded higher soil pH readings than surrounding soils not containing charcoal (Lehmann, 2007b; Steiner at al., 2007; Major & Hopper, 2010). Soil reactions involving biochar additions have proposed new questions into its potential uses for the future, one being its effect on soil microbe communities.

2.3.3 Biochar and Soil Micro-organisms

Although biochar is deemed inert to degradation it must slowly degrade over time otherwise a mass build-up of charcoal in the soil would occur (Sohi et al., 2010). Clearly, biochar has a long residence time depending on its properties, but oxidation and mineralisation will also be a factor for its degradation (Cheng et al., 2006). High temperature pyrolysis is also likely to lead to an increase in surface area and microporosity which soil microbes may utilise to decrease the likelihood of predation from other microbes (Joseph et al., 2010; Kookana, 2010). Low temperature pyrolysed biochar has a larger fraction of carbon available for soil microbes to be used as a possible energy source for increased microbial stimulation (Warnock et al., 2007; Zimmerman, 2010). Biochar types therefore behave differently in soil depending on properties. Mineralisation of black carbon produced at three temperatures (225, 300, 375°C) in soil, was observed to decrease with increasing temperature providing evidence that microbes use this source of carbon as a possible food source (Brunn et al., 2008).

Zimmerman (2010) carried out an extensive year long trial into microbial oxidation of biochar and observed a general decrease in oxidation with increase in charring temperature. Zimmerman proposed that biochar lability was strongly controlled by the temperature it was pyrolysed at. Biochar produced at 500°C from bull and dairy manure still managed to stimulate microbial biomass and activity in a range of soil types with increased activity observed at higher concentrations (Kolb et al., 2009). Steinbeiss et al. (2009) elaborated that feedstock properties do indeed cause a change in response in the microbial community with additions of biochar. Yeast-derived

biochar and glucose-derived biochar produced at 850°C was observed to promote fungal stimulation and gram-negative bacteria in the soil respectively while mycorrhizal colonisation was increased through biochar amendment in wheat crops (Solaiman et al., 2010). This is potentially interesting as a promotion in fungal biomass has also been observed to degrade organochlorines such as dieldrin and DDT in laboratory incubation studies (Kataoka et al., 2010; Purnomo et al., 2010). Stimulation of soil microbe health as quantified by basal respiration and microbial biomass has been observed with the addition of smoke extract (pyroligneous acid), a by-product of the charring process (Steiner et al., 2008). It is possible that biochar additions will stimulate organochlorine breakdown by way of microbial stimulation in soil.

2.3.4 Biochar and its Interaction with Pesticides

Properties of certain biochars are able to interact with the soil environment once they have been incorporated into the soil profile. One of these properties is cation exchange capacity (CEC) as biochar is reported to have high CEC (Lehmann, 2007a,b). Evidence of high CEC was first observed in Anthrosols from the Amazon where charred material had been added between 600 and 8700 years ago (Liang et al., 2006). High CEC in this case, can be attributed to the surface area of biochars along with heterogeneous surface properties of the char material in the soil (Liang et al., 2006; Yu et al., 2009). This is an important property for designing biochars as high CEC can affect nutrient dynamics and also increase sorption of pesticides onto biochar surfaces (Warnock et al., 2007; Yu et al., 2009; Liang et al., 2010; Chen & Yuan, 2011; Chen et al., 2011). Biochar produced from *Eucalyptus spp*. wood chips at 450 and 850°C caused a loss in degradation of two well used pesticides and decreased their bioavailability for plant uptake with increased concentrations of biochar (Yu et al., 2009).

2.3.5 Biochar and its Interaction with Heavy Metals

The likelihood of arsenic contamination at sheep dip sites is significant. To increase the plant-available fraction of arsenic within the soil with application of biochar at these sites it must be known how biochar will interact with this metalloid over time. The useful surface properties of biochar can lead to contaminant control and release.

Fresh biochars usually have a low cation exchange capacity (CEC). However with time the surface will tend to oxidise as a result of weathering and increase CEC (Cheng et al., 2006; Calvelo-Pereira et al., 2011). High CEC can lead to retention of many heavy metals in soil but not that of metalloids such as arsenic, as it mainly exists as an oxyanion. Biochar can also have high liming equivalence which may raise the soil pH and thus has the potential to increase the mobility of arsenic making it more available for uptake by plants (Hartley et al., 2009; Joseph at al., 2010). This scenario can increase the extraction efficiency for arsenic uptake into plants and reduce remediation times for contaminated soil.

It has also been observed that compost applications resulted in increased arsenic adsorption in soil after application (Cao & Ma, 2004; Perez-de-Mora et al., 2007; Sharma et al., 2011). Biochar produced at 400°C from *Quercus spp*. and *Prunus spp*. did not cause any significant effect on arsenic mobility in soils even though its final pH of 9.4 may have resulted in a liming effect of soils in the UK (Hartley et al., 2009b). Similar results in relation to plant uptake of arsenic into maize shoots identified that biochar additions cause a reduction in uptake (Namgay et al., 2010). However, extractable arsenic in soil was increased with increased biochar applications in a sandy soil. Further, Beesley et al. (2010) carried out studies relating to biochar and bioavailability of arsenic to plants and found that arsenic concentrations in soil pore water increased as much as 30-fold after addition of biochar. Conclusions to this increase were based on the liming effect of the biochar and increases in dissolved organic carbon in soil (Beesley et al., 2010).

2.4 Arsenic

Arsenic is a naturally occurring metalloid found throughout soil profiles around the world. It ranks 20th out of the elements found on the periodic table for abundance in the earth's crust and is deemed a metalloid due to chemical properties (Cullen & Reimer, 1989; Smith et al., 1998). These properties relate arsenic to sub-group V of the

periodic table and its decrease in electronegativity as seen in this sub-group is not sufficient to give arsenic a metallic character (Smith et al., 1998). Ubiquitous in its distribution, arsenic is found in rocks, sediments and air but enters both terrestrial and aquatic environments through natural processes such as weathering, volcanic emissions and biological reactions (Mahimairaja et al., 2005). Although it is found throughout the world its concentration in a number of environments varies and can be explained by both natural and anthropogenic factors.

2.4.1 Arsenic Background Concentrations

The global distribution of arsenic in soil varies with soil type and is highly dependent on the nature of the parent material (Smith et al., 1998) but the mean value of arsenic found in crystal rock is around 2 mg kg^{-1} (Zhang & Selim, 2008). Natural arsenic minerals found in the earth's crust include arsenopyrite, arsenides, colbalite and orpiment which after weathering processes cause dissolved arsenic to enter into the soil environment changing the background concentration in a number of adsorption/precipitation reactions (Mahimairaja et al., 2005; Zhang & Selim, 2008). Due to those reactions, crustal values of arsenic range from 0.1 - several hundred mg kg^{-1} depending on the rock type being considered (Cullen & Reimer, 1989). Sedimentary rocks contain much higher concentrations of arsenic compared to igneous rocks (Bhumbla & Keefer, 1994; Smith et al., 1998). Generally speaking, igneous concentrations range from 1.5 – 3.0 mg kg^{-1} and sedimentary concentrations 1.7 – 400 mg kg^{-1} (Smith et al., 1998; Mahimairaja et al., 2005). After reaction processes have occurred (weathering, biological) it is generally believed that soil concentrations do not exceed 15 mg kg^{-1} although ranges of arsenic concentrations start at 0.2 – 40 mg kg^{-1} (Smith et al., 1998; Mahimairaja et al., 2005). In New Zealand concentrations range from 0.2 – 30 mg kg^{-1} (Zhang & Selim, 2008) therefore any concentration exceeding this is observed to be classed as contaminated.

2.4.2 Anthropogenic Influence on Arsenic Concentration

A range of organic and inorganic arsenic compounds are introduced into the environment both through geogenic and anthropogenic mechanisms (Mahimairaja et

al., 2005). However, human activities have led to arsenic concentrations above natural background levels originally presented. **Figure 2.8** by Mahimairaja et al. (2005) describe a range of anthropogenic sources of arsenic into the environment.

Such activities vary in nature and arsenic compound release, and in doing so varies the chemical nature (speciation) and bioavailability of the arsenic compound (Mahimairaja et al., 2005) but these sources are mainly related to primary and secondary industries (Smith et al., 1998). One activity, mining of metal ores, often releases arsenic as a result of chemical reactions to separate metals such as gold from the rock in the form of arsenopyrite and can reach concentrations of up to 30,000 mg kg^{-1} (O'Neill, 1995). A mode of anthropogenic release of arsenic into the atmosphere is achieved through the combustion of coal (coal ash is naturally high in arsenic) for power consumption which eventually settles in terrestrial and aquatic environments (Qafoku et al., 1999; Ishak et al., 2002). The global average concentrations of arsenic in coal is approximately 84 mg kg^{-1} but concentrations of 9000 mg kg^{-1} have been recorded (Liu et al., 2002). Globally, one of the largest sources of arsenic contamination exists due to the use of arsenic trioxide (As_2O_3) which is commonly used in the manufacture of glass, antifouling agents and electronics. Contemporary contamination is coming from wood preservatives where CCA (copper, chrome, arsenic) is used to treat timber in many countries including NZ (Loebenstein, 1993; Smith et al., 1998).

2.4.3 Use of Arsenic in Agriculture

Until the introduction of organochlorines in the late 1900s arsenic was used extensively in the agricultural industry as a common pesticide (McLaren et al., 1998). These forms of arsenic exist in a number of chemical compounds, namely lead arsenate ($PbAsO_4$), zinc arsenate ($ZnAsO_4$) and Paris green [$Cu(CH_3COO)_2.3Cu(AsO_2)_2$] but organoarsenicals were also used as herbicidal treatments in aquatic and terrestrial environments (Smith et al., 1998; Mahimairaja et al., 2005). With respect to agricultural use of arsenic for the dipping of sheep, it was commonly used to control parasites such as ticks and lice.

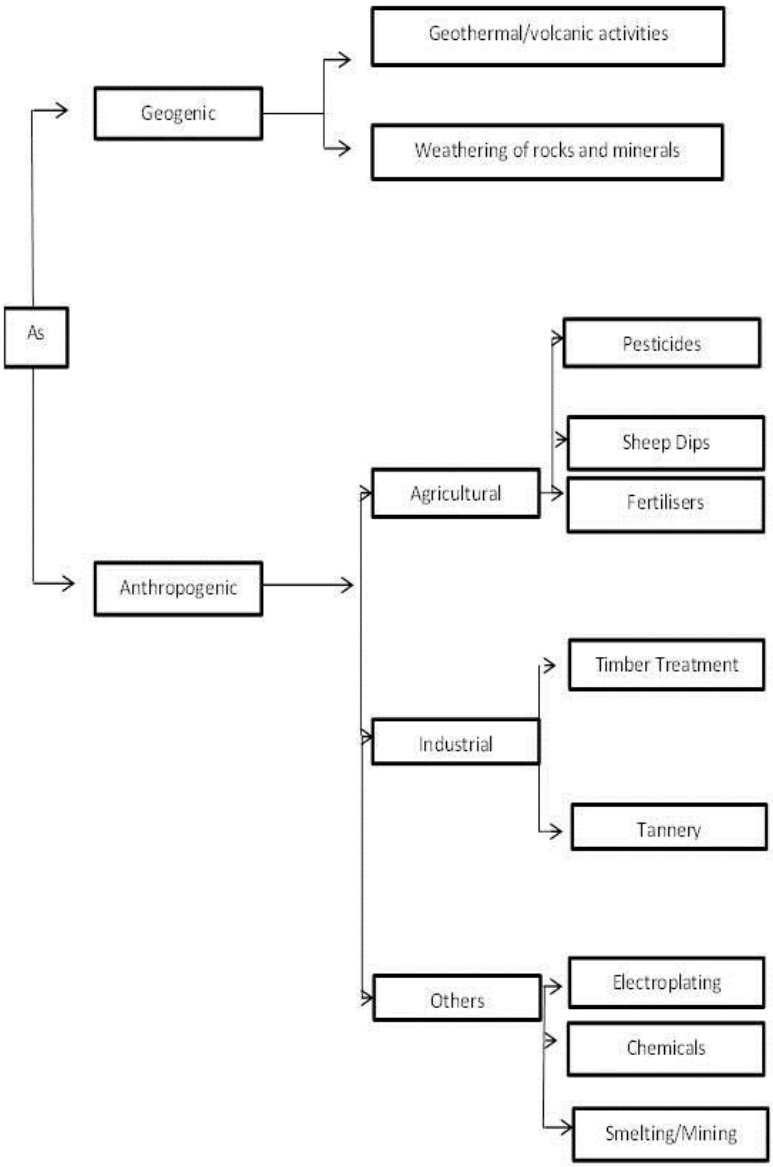

Figure 2.8. Major sources of arsenic by anthropogenic and natural processes. Modified form Mahimairaja et al. (2005)

Contamination at these sites can be excessive as identified by McLaren et al (1998) at eleven dip sites in Australia noting considerable surface contamination (0-10 cm) of arsenic (37-3542 mg kg^{-1}). Zwieten (2003) and Chopra et al (2007) also provide evidence of heterogeneous arsenic at such sites ranging from 1000 – 4500 mg kg^{-1}. Okonkwo (2007) identified significant contamination of surface soils at cattle dipping sites ranging from 20-1369 mg kg^{-1} in South Africa. Movement of arsenic at such sites into water-bodies may create an environmental and ecological disaster in future years (Kimber et al., 2002).

2.4.4 Arsenic in Aquatic Environments

As arsenic occurs naturally, a basic assumption can be made that it is present in water bodies around the world at variable concentrations. Because of the parent material that is located in some countries arsenic exists at substantial higher concentrations particularly in south-east Asia. In Bangladesh contaminated water has often been applied to agricultural crops and affects drinking water supplies. Naidu et al (2009) identified that bioavailability of soil-bound arsenic is relatively high in soils containing over 10 mg kg^{-1} of arsenic. Arsenic desorption was as high as 0.06 mg L^{-1} in samples analysed (Naidu et al., 2009). Globally, the World Health Organisation (WHO) has imposed a water drinking standard of 10 µg L^{-1} of arsenic. This is often exceeded in parts of Bangladesh (Mahimairaja et al., 2005). In New Zealand aquatic areas containing sediment material of greywackes, schists, volcanic lithology and peats have shown elevated levels of arsenic (Davies, 2001). Geothermal areas that contain volcanic lithology such as the Waikato River often exceed WHO drinking standards (Environment Waikato, 2006).

2.4.5 Health Effects and Human Exposure to Arsenic

Ingestion of arsenic is the main exposure pathway for poisoning. Exposure pathways can be contaminated water or ingestion of plants grown on contaminated soil (Smith et al., 1998). The average human intake of arsenic is 0.5 – 1.0 mg/daily for water and food sources combined (Cullen et al., 1995) and exceeding this is very detrimental to human health. **Table 2.5** identifies key concentrations that have been observed to

cause human health problems from drinking contaminated water. Upon ingestion of arsenic, the metalloid binds strongly to plasma proteins and leukocytes, resulting in toxicity symptoms as quickly as 30 min after exposure depending on the source (Cullen et al., 1995). Hindmarsh & McCurdy (1986) noticed that soluble arsenic compounds are rapidly absorbed in the gastrointestinal tract but Buchet et al (1981) observed that 55-80 % of daily arsenic intakes are excreted by the urinary tract. The remaining arsenic compounds in the body will begin to concentrate in various organs including the kidney, lungs and liver (Cullen et al., 1995) and interact at the cellular level by inactivating essential proteins such as those responsible for DNA synthesis and repair (Ratnaike, 2003). Critical exposure or humans that are exposed to sustained long-term treatment with contaminated water may eventually succumb to the effects of arsenic toxicity (Smith et al., 1998; Mahimairaja et al., 2005; Zhang & Selim, 2008).

2.4.6 Arsenic Speciation in the Environment

Arsenic as a metalloid actually exists as a number of inorganic and organic forms depending on the properties of the environment it is situated in (Cullen & Reimer, 1989; Carrillo-Gonzalez et al., 2006). Inorganic forms are commonly known as arsenate (As^{V}) and arsenite (As^{III}) and are the main forms of arsenic present in the environment but these species can undergo various chemical/microbial oxidation-reduction reactions to yield organic forms of arsenic (Smith et al., 1998). These two species are extremely important because of their high solubility in water (Vaughan, 1993). Depending upon the properties of the soil, arsenic speciation can alter and analysis of soil properties can determine which species is likely to be dominant.

Table 2.5. Effects and symptoms linked to various concentrations of arsenic in drinking water. Modified from (Environment Waikato, 2006).

Arsenic Concentration	Type of Poisoning	Symptoms and Effects Linked to Arsenic Drinking Water
> 1200 µg L^{-1}	Acute	Abdominal pain, vomiting, diarrhoea, swelling of eyelids, hands and feet, deterioration of motor skills, shock and DEATH
> 115 µg L^{-1}	Chronic	Non-cancerous effects : skin lesions, diabetes mellitus, adverse effects on cardiovascular, digestive and respiratory systems Cancerous effects : Skin, bladder, kidney, lung colon
> 50 µg L^{-1}	Chronic	Intellectual impairment in children. Cancer risk significant
20 µg L^{-1}	Chronic	Lung and bladder cancer risk = 1 : 140
10 µg L^{-1}	Chronic	Cancer risk = 1 : 300
5 µg L^{-1}	Chronic	Cancer risk = 1 : 500
3 µg L^{-1}	Chronic	Cancer risk = 1 : 900

Masscheleyn et al (1991) concluded that when analysing soil redox conditions 65-98 % of total arsenic in soil existed as arsenate at 200 – 500 mV. If soil pH was raised to alkaline, substantial proportions of arsenic was released into solution (Masscheleyn et al., 1991; Marin et al., 1993). Generally speaking under oxic conditions, those greater than Eh > 200 mV, arsenic will be present as arsenate (pH 5-8) (Marin et al., 1993; Smith et al., 1998).

The two inorganic species do however differ in their toxicity to plants and other higher organisms by the way they react once taken up or ingested. In plants arsenate replaces phosphate in the production of ATP, forming unstable ADP-As complexes which leads to damaging energy flows while arsenite is more toxic and reacts with sulfhydryl groups in enzymes leading to the inhibition of protein and cell synthesis (Meharg & Hartley-Whittaker, 2002; Quaghebeur & Rengel, 2003). Organic forms of arsenic exist in both trivalent and pentavalent states with concern to speciation (Smith et al., 1998). Microbial methylation is a major pathway for the conversion of inorganic arsenic to organic arsenic, forming products such as monomethylarsonic and di and tri – methylarsines (O'Neill, 1990). The relatively higher concentrations of inorganic species makes arsenate and arsenite of greater concern at most contaminated sites (Smith et al., 1998; Zhang & Selim, 2008).

2.4.7 Interacting Factors of Soil Properties

Adsorption of arsenic in soil eventually determines its persistence, mobility and thus toxicity to the surrounding environment. Because of this it is crucial to relate soil properties which change from site to site to this adsorption process (Mahimairaja et al., 2005; Ministry for the Environment, 2006). Carrillo-Gonzalez et al (2006) state that soil pH is the main variable for controlling the mobility of arsenic but properties such as organic matter, clay and metal fractions and competing and non-competing ions all play a part in the adsorption/desorption process (Frost & Griffin, 1977; Masscheleyn et al., 1993; Mahimairaja et al., 2005; Carrillo-Gonzalez et al., 2006).

The effect of soil pH on arsenic sorption has been studied widely and the effect on sorption depends upon the arsenic species present (Smith et al., 1998). In the presence

of silicate minerals arsenate exhibits strong adsorption between pH 4-6 whereas arsenite has maximum adsorption from pH 4-9 (Frost & Griffin, 1977; Smith et al., 1998). Zhang & Selim (2008) state that arsenite is strongly bound to metal oxides when the pH is increased. This is because clay minerals and metal oxides such as iron oxide have a high surface area for arsenic sorption (Blute et al., 2004; Zhang & Selim, 2008). Mahimairaja et al (2005) identified that 90 % of arsenic in soil is associated with the clay fraction. Clay fractions are often correlated with high levels of iron and aluminium oxides which have the potential to modify arsenic interactions yet cation sorption varies depending on the clay properties and arsenic species (Smith et al., 1998; Carrillo-Gonzalez et al., 2006). A number of observation have been made that amorphous aluminium and iron hydroxides adsorb more arsenate than arsenite and in soil containing high oxidic materials adsorption of arsenate decreases with increasing pH (Mahimairaja et al., 2005; Carrillo-Gonzalez et al., 2006). This can be explained by the increasing number of positive charges observed on clay lattices at acid pH resulting in arsenate ions becoming attracted to these surfaces for adsorption (Mahimairaja et al., 2005).

Fertiliser and soil amendments also control the cycling and mobility of arsenic through soil. Phosphate is an essential nutrient for plant growth and is responsible for a number of plant enzymatic reactions. It also belongs to the same chemical group as arsenic and both have comparable disassociation constants for their given acids (Mahimairaja et al., 2005). This similarity also means that they compete strongly for soil colloid binding sites. In doing so, a natural high abundance of phosphate or intensive nutrient additions can cause the leaching or increased mobility of arsenic through the soil profile. Studies have shown that the addition on phosphate-based fertilisers can displace up to 77 % of arsenic in soil however this desorption is highly dependent on soil type (Woolsen et al., 1973; Smith et al., 1998).

Other organic based amendments also cause a significant change in adsorption/desorption reactions of arsenic in soil. Allard & Grimvall (1991) studied the effect of fulvic acid on binding reactions and observed a general decrease in

adsorption of arsenic at a pH range of 5-7. Green waste compost was also observed to increase arsenic desorption at an arsenic contaminated site in the United Kingdom (Hartley et al., 2009). Amendments such as those described by Xu et al (1991) are also naturally carried out by plants which secrete organic acids around the locality of roots to increase the bioavailability of nutrients in the direct vicinity. Such a process is important in the remediation of contaminated sites by using plants to increase the availability of toxic elements and eventually causing that element to be taken up by the plant in a process known as phytoremediation.

2.5 Phytoremediation

Natural remediation using plants to clean contaminated soils is not a new idea to science however the advent of this concept cannot be traced back to a given time (Meers et al., 2008). This process, termed phytoremediation, uses plants and their influence to aid in the accumulation, degradation, sequestration and metabolism of contaminants to restore the soils natural state (Saleh et al., 2004). It is not an efficient, quick method for the remediation of soils, but it is an environmentally friendly way that utilises natural methods. Phytoremediation has a number of beneficial final effects on the environment such as removing carbon dioxide from the atmosphere and creating habitats from biota both above and below ground (Tsao, 2003; Doty, 2008). This method is also popular among indigenous communities whose historical perspective of sustainability governs how they control their land (Harmsworth, 2014).

2.5.1 Division of the Term Phytoremediation

Phytoremediation is a broad concept that can be separated into a number of categories, each with a differing result depending on the remediation goals. Four main concepts are proposed which include phytostabilisation, a containment process which uses plants to immobilise contaminants; phytovolatilisation, which takes advantage of microbial communities occupying the rhizosphere to transform/volatilise contaminants; phytodegradation, which refers to the metabolic capabilities of rhizosphere organisms to degrade contaminants and finally phytoextraction (Fitz & Wenzel, 2002; Leung et al., 2006; Doty, 2008; Wenzel, 2009). Phytoextraction takes

into account the idea that plant species have a natural ability to influence element bioavailability surrounding the root system and by passive or controlled means, accumulate elements such as arsenic from the soil and translocate these elements to aerial regions of the plant (Tsao, 2003; Saleh et al., 2004; Meers et al., 2008; Zhao et al., 2009).

2.5.2 Phytoextraction

Phytoextraction as a remediation technology has advantages and disadvantages just like any other, but its use for the removal of contaminants must be put into context before it can be debated. The vision of Matauranga Maori (science) aims to utilise natural systems put in place and to enhance sustainability during land management practices. Matauranga Maori shies away from scarring processes such as soil filtering or removal which detrimentally affects papatuanuku (the land) and mahinga kai (food gathering areas). Although a slow process, phytoextraction naturally enhances uptake and degradation of metalloids such as arsenic in a sustainable manner. Disadvantages of such a technology on a large scale relate to poor growth due to the toxic environment which also inhibits metalloid translocation in the plant (Vamerali et al., 2010). The biggest factor affecting the success of extraction is the limitations of the rooting zone (Saleh et al., 2004). This variable is paramount for the success of extraction as it is the root system which is responsible for uptake of metalloids (Keller et al., 2003; Tsao, 2003).

Detail on the zone of contamination (in depth) and known information on root systems for a plant species can be used to define the extent to which remediation will result in complete removal of a contaminant over time (Fitz & Wenzel, 2002; Zhao et al., 2009). Pastoral grass species such as the *Lolium* genus (ryegrass) have a rooting system that can reach down to 120 cm (1.2m) but growth and thus translocation of metalloids may be slow. Increased uptake can be achieved by integrating high biomass crops such as *Populus sp*. (poplar) and *Salix sp.* (willow) which may not necessarily accumulate a high concentration of metals but they can increase phytoextraction efficiency due to biomass production (Terry et al., 2003; French et al., 2006; Purdy & Smart, 2008).

Woody biomass crops such as poplar and willow can be integrated into a remediation project by removing metalloids at greater depths as their rooting systems can reach up to 3m (Tsao, 2003; French et al., 2006; Purdy & Smart, 2008).

2.5.3 Arsenic Uptake Processes

Due to arsenic having a high chemical affinity/similarity with phosphate it is observed from physiological and electro physical studies that arsenate shares the same transport pathway in higher plants, yet phosphate transporters will select phosphate for transport if both elements are in abundance (Quaghebeur & Rengel, 2003). Competition for uptake transporters occurs in the root plasma lemma yet uptake of arsenic can be supressed by internal mechanisms via the phosphate/arsenate system (Meharg & Hartley-Whittaker, 2002).

Arsenic uptake usually involves co-transport of phosphate and/or arsenate with proton exchange governing the transport across the root plasma lemma (Zhao et al., 2008). Once inside plant tissues, arsenate is reduced to arsenite and complexed by a number of thiol groups produced by plant reactions (biotransformation to less toxic organic species is also undertaken)(Peralta-Videa et al., 2009; Smith et al., 2009). This reduction step can be a critical process for translocation of metalloids to aerial plants parts and is carried out by internal components, mainly arsenic reductase (Tripathi et al., 2006; Peralta-Videa et al., 2009).

Of biological interest is the ability of rhizosphere based soil micro-organisms to oxidise arsenite to arsenate as a pre-cursor step for selective arsenate uptake (Fitz & Wenzel, 2002; Quaghebeur & Rengel, 2003; Tripathi et al., 2006; Trotta et al., 2006). However arsenite may also be transported across the soil-plant interface as suggested by Zhao et al. (2008). Here transport is achieved by aquaporins, large pores that allow passage of neutral molecules to pass the plant interface. Evidence of such transport has only independently been carried out recently but its complete functioning is only speculative (Bienart et al., 2008; Isayenkov & Maathius, 2008; Ma et al., 2008).

To date, the identity of the arsenic species transported from root to shoot has been debated mainly because differing forms of arsenate/arsenite has been found in aerial plant parts. Recent X-ray analysis provides strong evidence that not only does arsenate reductase reduce arsenate to arsenite, untransformed arsenate is also transported to aerial parts (Pickering et al., 2006; Tripathi et al., 2006). Both species are sequestered in vacuoles of both root and shoot tissue where they are detoxified and removed from the plant by natural shedding/abscission processes (Meharg & Hartley-Whittaker, 2002).

2.5.4 Phytoextraction of Arsenic

Desirable characteristics for phytoextraction include a plant species that exhibits fast growth and high biomass, a large root system for extended coverage of contaminated soil, high metal translocation, and good tolerance to the contaminated area (Vamerali et al., 2010). Finding a suitable species can be time consuming so natural high biomass crops such as willow and poplar are routinely used to remediate arsenic contaminated soil. Although arsenic is adsorbed to soil surfaces, phytoextraction of this metalloid can be altered by soil amendments that can change soil pH and increase bioavailability for phytoextraction (Pilon-Smits & Freeman, 2006; Saleh et al., 2006).

A range of chelating agents such as EDTA have been used in the past to improve rate of uptake but recently these agents have been restricted due to their environmental persistence and the risk of soil leaching (Terry et al., 2003; Meers et al., 2008). These chemicals enhance metal bioavailability by forming a chelating complex with targeted metals causing formerly insoluble compounds to become more readily available to plants (Meers et al., 2008). A more natural method to increase phytoextraction of arsenic can be achieved by the rhizosphere, with interest, particularly toward organic acid production.

Plant roots naturally secrete low molecular weight organic acids to increase nutrient solubility by acidifying the rhizosphere and forming organic-element complexes (Fitz & Wenzel, 2002; Wenzel, 2009). Phosphate fertilisers have been extensively studied for their effect on arsenic bioavailability due to their chemical similarity. Jankong et al.

(2007) observed an increase in phytoextraction ability of a fern *Pityrogramma calomelanos* when phosphate treatments were applied to an arsenic contaminated soil in Thailand. Interestingly, Lambkin & Alloway (2002) carried out adsorption interactions of phosphate and arsenate and discovered that at low arsenate additions phosphate desorption remained consistently low meaning only high phosphate additions would result in increased arsenic phytoextraction.

Elements similar to arsenic have also been studied for their effect on arsenic bioavailability. Additions of silicon to soil as a fertiliser resulted in decreases of internal shoot and root arsenic, identifying an interaction of competition for uptake between the two elements (Guo et al., 2005). Tolerance and uptake is also a varied feature as detailed by Purdy and Smart (2008) who investigated arsenic uptake between clones of shrub willow (*Salix spp*). Stem cuttings were taken and subjected to 100 µM arsenate (7.49 mg L^{-1}) and 250 µM arsenate (18.73 mg L^{-1}) with varying degrees of phytoextraction between clones exhibited. Translocated concentration in the leaves ranged from 2-329 µg g^{-1} and 2-201 µg g^{-1} in stems under 250 µM. Such an effect can alter a phytoextraction project as the maximum uptake of arsenic must be achieved by sourcing the correct clones (French et al., 2006; Purdy & Smart, 2008).

Hydroponic and soil experimentation has also been carried out to determine a given plant species' natural uptake of arsenic in a contaminated environment. An assumption in plant uptake of arsenic and contaminated levels of arsenic can generally be made where increasing soil contamination gives rise to higher internal plant uptake (Keller et al., 2003; Arienzo et al., 2004; Ruiz-Chancho et al., 2008). A site ranging from 51.9 – 66.8 mg kg^{-1} arsenic resulted in internal concentrations of total arsenic reaching 1.38 mg kg^{-1} in a fern *Dryopteris filix-max* and 2.30 mg kg^{-1} in *Buxus sempervirens*. In contrast a similar site with contamination at 21,200 mg kg^{-1} resulted in concentrations in plant samples reaching 98.5 mg kg^{-1} in *Juncus inflexus* and 1750 mg kg^{-1} in *Brachythecium cf*. (Ruiz-Chancho et al., 2008). The discovery of plants that could survive and yet accumulate high levels of contaminants without showing toxic effects

soon changed the face of phytoextraction technology as these plants, known as hyperaccumulators, could be utilised for effective remediation.

A coupled remediation system between biochar and phytoextraction can reduce this remediation time due to the alkaline properties of biochar increasing the elemental solubility of arsenic within soil for plant extraction (Beesley et al., 2011a). Applications of material that is high in organic matter such as composts have in the past yielded a varying degree in response in relation to arsenic mobility in soils. Both Mench et al. (2003) and Hartley et al. (2009a) observed an increase in arsenic mobility when green waste compost was added to soil. This was probably due to competition for sorption sites between arsenic ions and dissolved organic carbon resulting in leaching arsenic through the soil profile.

2.5.5 Arsenic Hyperaccumulators

Hyperaccumulators are defined as plant species that are able to bio-accumulate high metal concentrations within plant parts and usually at higher concentrations than those found in soils they are growing on. Most arsenic hyperaccumulators are members of the *Pteris* genus with the first hyperaccumulator identified being *Pteris vittata* (Fitz et al., 2003). Twelve members of the *Pteris* genus have since been identified as arsenic hyperaccumulators but not all members are able to carry this trait (Zhao et al., 2008). Hyperaccumulation results in ferns being able to accumulate higher concentrations of arsenic in fronds then what is found in the soil. These plants also show the important phytoextraction trait of having a high translocation factor defined as the ability to take up the metalloid and efficiently transport the contaminant from the roots and into aerial parts (Zhao et al., 2002; Xie et al., 2009). Most arsenic translocated is found in the fronds as arsenite with vacuolar sequestration being the key mechanism for arsenic detoxification (Poynton et al., 2004; Verbruggen et al., 2009).

Key studies have presented evidence that *P.vittata* can hyperaccumulate arsenic to internal concentrations of 22,630 mg kg^{-1} (2 % of total weight) (Zhao et al., 2002; Ebbs et al., 2010). Utilising this trait for remediation of sheep dip sites would be very beneficial, yet this species is not naturalised in New Zealand and would require very unique conditions in order to survive this countries temperate climate. Wan et al. (2010) carried out extensive propagation studies on *P.vittata* and found that germination of fern spores was accelerated with high soil pH and calcium concentrations. Sexual propagation from gametophytic to sporophytic stage required a natural high temperature with soil pH below 6 detrimentally affecting fern growth (Gumaelius et al., 2004; Wan et al., 2010).

These constraints on propagation and growth sadly render most contaminated sheep dip sites untreatable with this type of fern. However, the *Pteris* genus has a number of hyperaccumulators and a number are commercially sold by NZ nurseries. One hyperaccumulator currently available in New Zealand is *P.cretica* which has arsenic uptake characteristics similar to *P.vittata*. In an experiment conducted by Fayiga and Ma (2005) *P.cretica* exhibited high translocation of arsenic internally from a cattle dip soil contaminated with 294 mg kg^{-1} arsenic. Fronds removed 297 µg of arsenic while roots removed 158 µg of arsenic per plant with a total uptake up arsenic being 455 µg of arsenic. *P.vittata* significantly removed more arsenic with total arsenic removal being 778 µg of arsenic (Fayiga & Ma, 2005).

Variability between genus species and across genus is also noted in a number of studies in relation to hyperaccumulation. Niazi et al. (2010) conducted field experiments in Australia to determine key concentration uptake characteristics between *P.vittata* and *Pityrogramma calomelanos* var. *austroamericana*. They found a high correlation in biomass weight and uptake factor with *P.vittata* exhibiting a high dry weight (2569 mg kg^{-1}) and uptake (3941 mg kg^{-1}) of arsenic compared to *P.calomelanos* (775 mg kg^{-1} DW and 1262 mg kg^{-1} arsenic respectively). Species within genus also show a marked contrast in arsenic uptake even though in some cases two

ferns are hyperaccumulators (Singh & Ma, 2006; Huang et al., 2008; Singh et al., 2010; Xu et al., 2010).

Genetic research is currently being undertaken to understand the mechanisms of hyperaccumulation in the *Pteridaceae* and how this could be passed on to non-hyperaccumulators. Indriolo et al. (2010) isolated and characterised two genes from *P.vittata* which encode proteins for arsenite efflux. These are naturally missing in flowering plants with knockout of one of the genes resulting in arsenic sensitivity in *P.vittata*. Research has also been carried out in rice and its association with arbuscular mycorrhizal fungi (AMF) in regards to gene regulation of arsenic uptake (Chen et al., 2012). Research similar to this may provide high biomass species to one day hyperaccumulate metalloids as arsenic (Natarajan et al., 2009; Indriolo et al., 2010; Shim et al., 2013).

2.6 Stress Response and the Antioxidant Pathway

A reduction in plant growth is a common response of plants to toxic environments like sheep dip sites. Yet every plant species has differing tolerance levels to such a stress as arsenic toxicity. Naturally, arsenic hyperaccumulators will be very tolerant of high metalloid stress (due to a very efficient antioxidant system) yet others (plants) will be very low. Shaibur et al. (2008) observed a significant repression in sorghum shoot and root dry matter yield in as little as 33.5 µM arsenate present in a hydroponic solution (52 % reduction compared to control). This reduction can be related to the plant response which must regulate a tight control between growth and protection against abiotic stress. Such a stress is controlled by a natural pathway in plants and is related not only to heavy metal stress, but all abiotic/biotic stresses such as drought to insect herbivory (Logan et al., 2006; Krieger-Liszkay et al., 2008).

A process known as oxidative stress is elevated in a detrimental environment and it relates intimately to the production of reactive oxygen species (ROS) which are produced. ROS are created naturally in all life systems by the misplacement of electrons onto oxygen along the electron transport pathway yet are quickly detoxified by the antioxidant pathway (Foyer & Shigeoka, 2011). ROS are normally unreactive but

can form similar species damaging to essential systems such as DNA synthesis and protein synthesis (Bowler et al., 1992; Mates, 2000). Arsenic stress causes this misplacement of electrons to increase resulting in oxidative stress which the plant must respond to by up-regulating the antioxidant pathway.

2.6.1 Components of the Antioxidant Pathway

The major ROS scavenging enzymes involved in the antioxidant pathway in plants include superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), dehydroascorbate reductase, monodehydrateascorbate reductase (MDHAR), glutathione reductase (GR) and glutathione peroxidase (GOPX) (Boscolo et al., 2003; Kuo & Kao, 2003; Polesskaya et al., 2004; Ruley et al., 2004; Simonovicova et al., 2004). These enzymes combine with the non-enzymic components to detoxify ROS to oxygen and water using ascorbate, reduced glutathione and NADPH as electron donors (Gupta et al., 1993; Ezaki et al., 2004). Hyperaccumulators like *P.vittata* commonly exhibit high levels of SOD, CAT and APX than non-hyperaccumulators and therefore do not show toxic symptoms from arsenic uptake (Srivastava et al., 2005). In contrast rice (*Oryza sativa* L.) and mustard (*Brassica juncea* L.) seedlings grown in 500 μM and 25 μM arsenate concentrations respectively exhibited reduced growth and reduction in overall antioxidant activity compared to seedlings under no arsenate treatment (Khan et al., 2009; Shri et al., 2009).

The first antioxidant enzyme to be covered is of great importance. SOD is primarily the first line of defence in detoxifying the superoxide anion (O_2^-), as it is removed by converting it to hydrogen peroxide (H_2O_2) and oxygen in the reaction:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Equation 1 : SOD catalysed reaction showing formation of hydrogen peroxide (H_2O_2) and oxygen (O_2) from superoxide anions (O_2^-)

This enzyme is unique as its activity determines the concentrations of O_2^- and H_2O_2 and is therefore central to the antioxidant pathway defence response (Bowler et al., 1992; Guo et al., 2006).

Hydrogen peroxide, or the product of the SOD reaction, then undergoes a series of successive reactions to produce water and oxygen as end-products. This is mainly achieved by the activity of catalase (CAT) and guaiacol peroxidase (GPX). Hydrogen peroxide is directly eliminated by CAT in peroxisomes and by GPX in the cytosol, vacuole and other extracellular spaces (Boscolo et al., 2003; Sharma & Dubey, 2007; Dominguez-Valdivia et al., 2008).

CAT has an extremely high catalytic rate, but low substrate affinity, and is abundant in peroxisomes. It is also thought to have another important role in protecting SOD against inactivation by high levels of H_2O_2 (Gratao et al., 2006). Depending on the plant species, isoenzymes (as distinct genes) of CAT can differ in number, from two in barley to as many as twelve in mustard (Gratao et al., 2006). Variability in CAT activity has been observed since the antioxidant pathway has been studied in relation to environmental stresses. Kahn et al. (2009) observed that arsenic treatment (25 μM) increased the activities of SOD, APX and GPX significantly. Further, Srivastava et al. (2005) exposed *P.vittata* to increasing concentrations of arsenate (0, 150, 300 μM) and discovered a dose responsive pattern of increased activity of CAT, SOD and APX.

Ascorbate peroxidase (APX) uses ascorbate as a substrate for the removal of H_2O_2 from plant cells. The APX protein family consists of at least five different isoforms which include apoplastic and thylakoid forms (Gratao et al., 2006; Morgan et al., 2008). Guaiacol Peroxidase (GPX) can be distinguished from APX when isolated from plant systems. Differences exist in the amino acid sequence and also physiological function. A number of elements at toxic levels are able to induce the activity of GPX significantly such as cadmium, nitrogen and lead (Polesskaya et al., 2004; Ruley et al., 2004; Guo et al., 2006).

2.7 Specific Research Objectives

At present, gaps exist in the understanding of the potential for biochar to be used in the remediation of co-contaminated soil (that being soil contaminated with inorganic and organic chemicals). The potential use of biochar as a soil amendment to increase arsenic phytoextraction has not been quantified in literature. Furthermore, the effect of biochar on plant enzymic and metabolic processes as a consequence of subsequent arsenic uptake, are poorly understood.

The specific objectives of this research were therefore:

- To study the effect of biochar on soil microbial activity in soil highly contaminated with arsenic and organochlorines (glasshouse and field trial);
- To investigate what effect biochar would have on organochlorine concentrations within the soil and whether associated changes in microbial activity can lead to their degradation (glasshouse trial);
- To understand the role biochar may have on soil dynamics, including soil fertility, change in pH and more importantly, the mobility of arsenic (glasshouse and field trial);
- To investigate the potential influence of soil amended with biochar on plant growth, namely *Lolium perenne* L. (ryegrass) and whether uptake of arsenic is increased in aerial tissues (glasshouse trial);
- To investigate the response of ryegrass at the molecular level to internal arsenic concentrations, and the influence of biochar amendment, by studying plant defence mechanisms and transcriptome-metabolome profiling (glasshouse trial);
- To design and implement a field trial to investigate whether biochar amendment of sheep dip soil is likely to influence soil microbial activity, phytoextraction efficiency and plant growth leading to phytoremediation and contaminated site management.

The overall purpose of this research was to design and implement a new strategy for the remediation of historic sheep dip sites using phytoextraction and biochar technologies that is both effective and efficient for the removal and degradation of contaminants that exists at these sites.

CHAPTER 3 : MATERIALS AND METHODS

3.1 Biochar and Associated Analytical Procedures

All analytical procedures for soil, biochar, and plant samples were carried out at Massey University and analysed in triplicate (unless stated). Methodology reported in this chapter is common for Chapters 5-7.

3.1.1 Feedstock and Pyrolysis Conditions

Biochar used in Chapters 5 and 6 originate from 1 and 5 year old willow (*Salix sp*.), representing young and old tissue respectively. Feedstock was collected from a commercial willow farm and chipped into approximately 0.5 cm size fragments and dried at 30°C until constant weight. The wood was pyrolysed using a 25 L gas-fired rotating drum kiln with an average heating rate of 23.3 and 36.6°C min^{-1} for the 350°C and 550°C biochars respectively. The peak temperatures were achieved by controlled release of pyrolysis gasses in the kiln, and were maintained for 1 to 2 min followed by a short cooling period of 1 hr prior to discharge into a sealed plastic bag.

Biochar used in Chapter 6 also originates from young 1 – 2 year old willow stem (*Salix sp.*) and was commercially chipped into 0.5 cm size. The chipped willow required an initial drying phase (pre-pyrolysis) of less than 200°C with manual mixing for about 12 hr in a 2 m^3 batch pyrolyser. Following this, a second firing to achieve final pyrolysis temperatures of 350°C and 550°C was carried out. The pyrolyser consists of two cylindrical 1 m^3 stainless steel retorts mounted in a lined steel fire box with a central door and flap to allow the addition of flue (wood) and to control air flow. The retorts were loaded with 60 kg willow chips and sealed by closing the retort doors. A k-type thermocouple was positioned in the load area to monitor temperatures. Initially, the pyrolyser was fired by wood until pyrolytic temperatures were reached. Thereafter exhaust gases were burnt in the fire box to provide the correct temperature. Peak temperatures were maintained for 1 to 2 min followed by a cooling period prior to discharge of the produced biochar into a sealed steel barrel.

3.1.2 Elemental and Compositional Analysis

Carbon, hydrogen and nitrogen concentrations in biochars were determined using an Elementar vario MACRO CUBE analyser (Elementar;Germany). The pH of biochar was measured in deionized water at a 1:100 (w/w) ratio according to the method of Ahmedna et al. (1997) after preheating for 20 min at 90°C and cooling to room temperature. Extractable P in biochar was estimated using 2 % formic acid following the method of Rajan et al. (1992) and as modified by Wang et al. (2012b). Extractable K, Mg, Ca and SO_4-S were analysed according to the methods of Blakemore et al. (1987) but with modification. Briefly 0.35 g of finely ground biochar was added to 35 mL 1 M HCl acid solution and placed in 35 mL centrifuge tubes on an over-end shaker overnight. Samples were then filtered and the concentration determined by atomic absorption spectroscopy (AAS) (auto-analyser for SO_4-S).

The ash content of each biochar was determined by thermal analysis using a thermogravimetric analyser (SDT Q600, TA Instruments, Melbourne, Australia) at Massey University. For TGA analysis biochars (10-15 mg) were placed in an Al_2O_3 crucible and heated from room temperature to 900°C (at a rate of 5°C min^{-1}) under a N_2 atmosphere before an air current was provided (Calvelo Pereira et al., 2011). The ash content of each sample was determined when there was no further weight change.

Surface analysis of fresh biochars was conducted by X-ray photoelectron spectroscopy (XPS) with Mg K α (1253 eV) radiation emitted from a double anode at 50 W (Sydney, Australia). Binding energies for the high resolution spectra were calibrated by setting C to 1s at 284.6 eV. The liming equivalence of biochar ($CaCO_3$ equivalence) was determined according to the AOAC standard method (AOAC, 1999) using an auto-titrator (TIM 865 Titration Manager, Radiometer Analytical).

Specific surface area of each biochar was calculated from N_2 physisorption data according to the Brunauer-Emmett-Teller (BET) method using P/Po values in the range 0.05 - 0.2 N_2 physisorption measurements were performed at liquid nitrogen temperature (-195°C) using a Micromeritics Tristar 3000 instrument at the University

of Auckland, New Zealand. Samples were degassed at 300°C in N_2 for 4 hr prior to the N_2 adsorption measurements.

3.2 Soil Analytical Procedures

Soil pH was measured in deionised water at a ratio of 1:2.5. Available phosphate (Olsen P), available sulphate and soil cations were measured according to the methods of Blakemore et al. (1987). Available phosphate (Olsen P) was extracted in 0.5 M sodium bicarbonate at a soil:solution ratio of 1:20 and determined colorimetrically. Available sulphate was extracted with 0.1 M potassium phosphate at a soil:solution ratio of 1:5 and determined by automated colorimetric technique. Cations were extracted by leaching with 1 M ammonium acetate (pH 7) at a soil:solution ratio of 1:50 and determined by atomic absorption spectroscopy (AAS). Cation exchange capacity was determined by the summation of extractable cations and the extractable acidity (Hesse, 1971).

3.2.1 Arsenic Analysis

3.2.1.1 Total Soil Arsenic

In preparation for total arsenic analysis, a sub-sample of air-dried homogenised soil (1 g) was pre-digested in aqua regia (3:1 mixture of concentrated HCl and HNO_3) overnight. Each sample was thereafter digested at 120°C on a heat block for 2 hr and left to cool before filtering through filter paper (Whatman 42C). The digest solution was made to a final volume of 100 mL with deionised water. Arsenic analysis was carried out using a Flow Injection Analysis System 400 (FIAS, Perkin Elmer) coupled to a Graphite Furnace Atomic Absorption Spectrophotometer (GFAAS) (AAnalyst 600, Perkin Elmer). Prior to analysis, aliquots of each digest were pre-reduced by taking 1 mL of sample and adding 1 mL of HCl, and 1 mL of 5 % (w/v) KI + 5 % (w/v) ascorbic acid solution. The treated samples were allowed to stand for 45 min at room temperature and finally diluted to 10 mL with 10 % (w/v) HCl. These samples were analysed for arsenic concentration using FIAS. Working standards were prepared from the As (V) salt $Na_2HAsO_4.7H_2O$ and a minimum correlation coefficient for the standard

curve of 0.995 was required before analysis could proceed. The certified soil reference CRM - GBW 07403 from the National Research Centre for CRMs of China was used to determine the accuracy of the analytical method to determine the concentration of arsenic in soil. Analysed values of the soil reference material differed by less than 10 % from the certified mean concentration.

3.2.1.2 Water-Extractable Arsenic

A modified version of the extraction method reported by Ko et al. (2008) was used for the extraction of water soluble arsenic. Two g of air-dried sieved soil was placed in a 25 mL polycarbonate centrifuge tube, mixed with 20 mL of deionised water and shaken overnight (16 hr) on a rotating over-end platform. Tubes were then removed and centrifuged at 10,000 x g for 3 min. The extractant solution was filtered through filter paper (Whatman 42C) and then pre-reduced and analysed according to the method described for total arsenic.

3.2.2 Organochlorine Analysis

Analysis of soil for organochlorine type and concentration was conducted by an international accredited laboratory (Hill Laboratories, Hamilton). Where soil sampling was conducted for organochlorine determination fresh soil cores were taken using a 10 cm steel corer, and placed in sealed glass jars, and stored at 4°C prior to analysis. Organochlorines were extracted using a hexane-acetone sonication extraction. Phosphoric acid was utilised as a pre-wetting step to expand the soil matrix before full extraction using Gas Chromatography-Mass Spectrometry (GC-MS).

3.2.3 Dehydrogenase Activity (DHA)

Microbial oxidation of organic compounds is directly linked to the electron transport chain (ETC) that utilises oxygen as a final electron acceptor. Dehydrogenases form a main branch of the ETC and are a fundamental enzyme system found in microorganisms (Camina et al., 1998). Such an attribute makes dehydrogenase activity a good indicator of total microbial activity and can be determined in laboratories by using artificial electron acceptors like tetrazolium salt. In this work, microbial activity

was quantified by measuring the dehydrogenase activity in the soil according to the method of Chandler & Brooks (1991) with modification. Briefly, DHA measurement was performed using 5 g of fresh soil with the addition of 0.1 g $CaCO_3$ for activation. Three mL of tetrazolium chloride (TTC) was added in the absence of light and the resulting mixture vortexed to remove any trapped air spaces. The mixture was then incubated at 28°C overnight and the resulting tri-phenyl formazan (TPF) extracted with 20 mL methanol. Colorimetric intensity was measured at 490 nm using a Jenway Spectrophotometer (Jenway 7315) to determine formazan concentration. Standard curves were constructed for each treatment to limit the effect of TPF colour absorption caused by biochar.

3.2.4 Soil Bacterial Community Structure and Diversity

Fresh soil cores for analysis of metagenomic DNA were extracted using a 10 cm steel corer with 1 cm diameter from each pot. Between each core extraction, the corer was thoroughly rinsed in 70 % EtOH (ethanol) and dried to prevent cross-contamination. Each core was placed in a sterile 15 mL falcon tube and stored at 4°C prior to DNA extraction.

3.2.4.1 Sample extraction for metagenomic analysis

Metagenomic DNA was extracted from 3 replicates of each treatment (0.25g) using the Powersoil DNA extraction kit according to the manufacturer's instructions (Mo Bio Laboratories). Following elution, DNA was stored at -80°C until use.

3.2.4.2 PCR amplification

PCR was performed using SRV3 universal primers (SRV3-1/SRV3-2) (Lee et al., 1996) which amplifies the V3 region of the 16S ribosomal subunit (rDNA). Primers had 5' extensions with unique MID tags. PCR reactions were performed using 2 µl of extracted DNA, 1 X high fidelity PCR master mix (Roche Cat. No. 12 140 314 001) and 0.5 mM SRV3 primers. Amplification conditions were: ((1x 95°C, 3min); (35 x (95°C, 30sec; 52°C 30sec; 73°C, 30sec)); (1x 72 °C, 30min); (1x 4°C, ∞)).

3.2.4.3 Sequencing preparation

SRV3 PCR products amplified from the soil sample DNA extractions and their respective negative controls were checked by electrophoresis (1 % agarose 7.5 V/cm) prior to sequencing preparation to ensure no contamination. Amplicons were purified using Agencourt AMPure magnetic beads (Beckman Coulter) according to the manufacturer's instructions, assayed for purity and quantified using a nanodrop. Amplicons were pooled together in equal amounts (119 ng) and sent for sequencing. These pooled amplicons were then distributed amongst six paired-end sequencing runs (of 250 bp) that were performed using an Illumina MiSeq by the Massey Genome Service (part of New Zealand Genomics Limited) with the runs containing PhiX174 as a sequencing control.

3.2.4.4 Sequence analysis

MiSeq reads were initially processed for quality using custom Perl scripts on a run by run basis. The sequences were analysed with SolexaQA (Cox et al., 2010) to obtain an overall quality of the runs. The sequences were analysed to remove any PhiX174 reads by mapping to the PhiX174 genome to create SAM (Sequence Alignment/Map Format) (http://samtools.sourceforge.net/SAMv1.pdf) mapping files by using Bowtie2. The sam2fastq tool within Picard (http://picard.sourceforge.net/) was used to recreate two Fastq files for each read. Length adjustment of the short reads on a per-read basis was performed using FLASh (http://ccb.jhu.ed/software/FLASH) (Magoc & Salzberg, 2011). Reads were then split into nine files (three for each of the three treatments) according to their MID using fastx_barcode_splitter.pl (part of the fastx toolkit; http://hannonlab.cshl.edu/fastx_toolkit/). Reads were subjected to a second round of quality trimming using DynamicTrim (a part of SolexaQA) at and an error probability cutoff of 0.05 to remove any FLASh introduced anomalies. For a given MID the reads from each of the 6 runs were then combined randomly to generate a concatenated file. Two separate sets of analyses were performed on these files. In the first, a random set of 6,000 reads were taken from each of the concatenated files to investigate the sample variation. In the second, files containing 100,000 trimmed reads

were chosen randomly from the pooled sequences for each sample. Both sequence datasets were formatted by custom Perl scripts to provide the correct input fasta format for QIIME (Caporaso et al., 2010). These fasta-formatted reads were analysed in QIIME using default parameters including the classification of operational taxonomic units (OTUs) at 97 %.

3.3 Plant Analytical Procedures

3.3.1 Plant Growth Analysis

Three plant species were investigated as part of this study; *Lolium perenne*, *Pteris cretica (*Frondz, Hamilton) and *Salix moutere (*Matatoa Nursery, Shannon). Germination of ryegrass seeds was scored on the first sign of radicle emergence by observing each seed under magnification. To determine fresh weight (FW) and dry weight (DW) of shoot and root material in respect to ryegrass seedlings, tissue was cut at the remaining seed husk junction (emergence of plumule and radicle). Fern material was harvested by using commercial secateurs to separate the frond leaf (lamina) from the stem (stipe). The stem was cut approximately 1 cm from the base and thoroughly washed with deionised water before drying. Roots were separated from the soil media by passing through a 5 mm sieve and thoroughly washed in distilled water to remove any attached soil particles. Both root and shoot were placed in paper bags and oven-dried at 75°C for 3 d to obtain dry weights. Plant material from willow treatments in the field (stem, leaves) was harvested by manually separating the petiole from the stem.

3.3.2 Arsenic Analysis

A composite sample of plant material was generated and finely ground using a mechanical grinder. In preparation for total arsenic analysis, a sub-sample (1 g) was pre-digested in aqua regia (3:1 mixture of concentrated HCl and HNO_3) overnight. Plant material was thereafter digested at 120°C on a heat block for 2 h and left to cool before filtering through filter paper (Whatman 42C). The digest solution was made to a final volume of 50 mL with deionised water. Arsenic analysis was carried out using

Flow Injection Analysis System 400 (FIAS, Perkin Elmer) coupled with Graphite Furnace Atomic Absorption Spectrophotometer AAnalyst 600 (GFAAS, Perkin Elmer). Prior to analysis, aliquots of each digest were pre-reduced by taking 1 mL of sample and adding 1 mL of HCl, and 1 mL of 5 % (w/v) KI + 5 % (w/v) ascorbic acid solution. The treated samples were allowed to stand for 45 min at room temperature and finally diluted to 10 mL with 10 % (w/v) HCl. These samples were analysed for arsenic concentration using FIAS. Working standards were prepared from the As (V) salt $Na_2HAsO_4.7H_2O$ and a minimum correlation coefficient for the standard curve of 0.995 was required before analysis could proceed. The certified reference plant sample 1573a (tomato) from the National Institute of Standards and Technology was used to determine the accuracy of the analytical method to determine the concentration of arsenic in plant material. Analysed values of the plant reference material differed by less than 10 % from the reported mean concentration.

CHAPTER 4 : SITE CHARACTERISATION

4.1 Site Locality and History

A potentially contaminated sheep dip site was identified through correspondence with landowners on the 16th of August 2010 in Te Mahia on the East Coast of New Zealand **(Figure 4.1)**. The area has a rich history of sheep and beef farming and in the past was associated with the Maori Land Wars. In 1832 the local pa (Maori village or defensive settlement) was besieged by Ngāti Raukawa, Ngāti Tūwharetoa, Waikato, Te Arawa and Tūhoe and after three months the defenders had exhausted all their food and resorted to sucking on uku, a white soapy clay. The place was subsequently given the name Kaiuku, 'to eat clay' (Whaanga, 2012). At present, the Kaiuku Marae is situated approximately 100 m from the dip. The land where the sheep dip site is located has been continuously used for sheep and beef grazing since the late 1800s and the soil has therefore been relatively undisturbed.

With the permission of the current land owners, a survey of the inferred sheep dip run and holding pad was carried out. As the site is no longer in use, a number of associated structures such as shearing sheds, chemical stores and the dip itself have been destroyed or buried **(Figures 4.2 and 4.3).** The current farm manager recollects using the dip site in the 1980s for sheep dipping and believed the site to be active from as long ago as the 1860s (J.Bowen, pers. communication). A sump system existed at the site and left over solution was pumped down a nearby bank (**Figure 4.4)** and into a nearby waterway (Waihakeke Stream) which connects with the ocean 100 m away. At the time of use, local Maori would often gather kaimoana (seafood) and watercress from these two point sources.

To ascertain the current day risk that contaminants from the site may pose to Mahinga Kai (food gathering areas) and the nature of possible arsenic/organochlorine soil contamination, a complete mapping of the area was carried out. This exercise included an initial mapping of arsenic migration through the soil profile.

Figure 4.1. Site location of the studied dip site on the Mahia Peninsula, East Coast, North Island, NZ. The studied dip site has been marked in black.

Figure 4.2. A representation of the historic structures that are no longer on-site as viewed by Google Earth. The dip itself is represented by the upside-down T with associated holding pens and shearing sheds to the right and below of the dip.

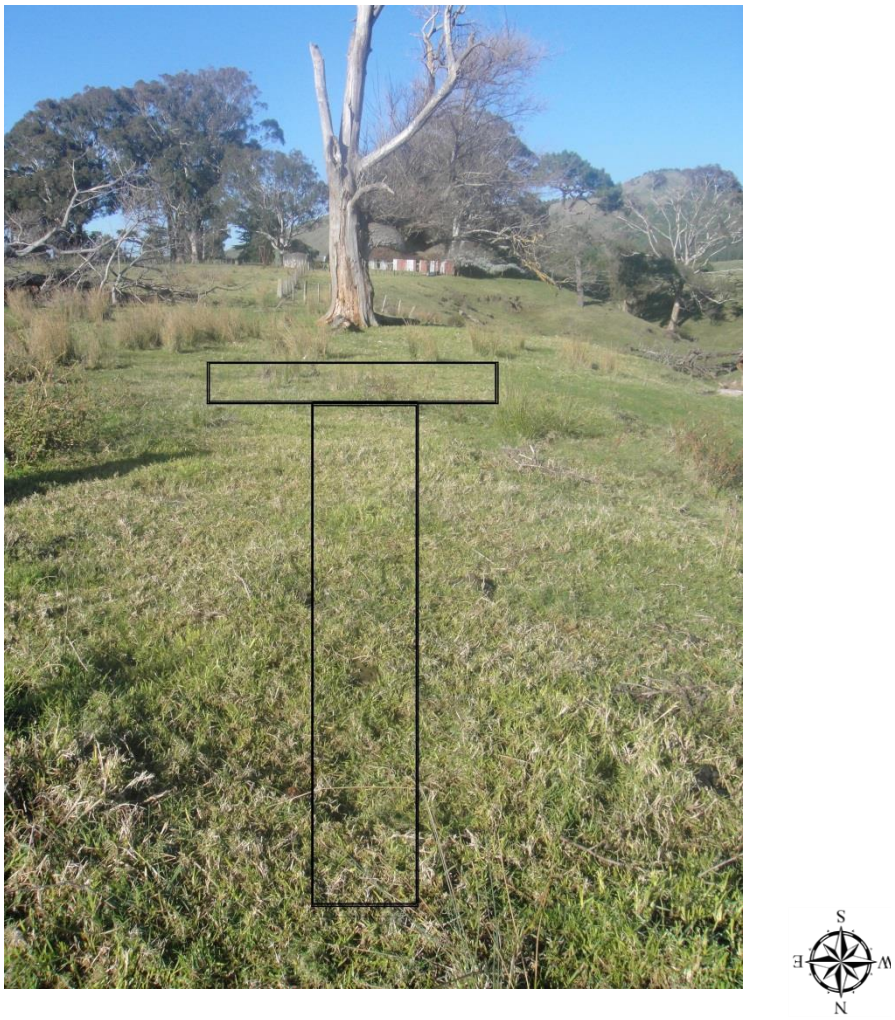

Figure 4.3. Inferred location of the now covered dip site at Te Mahia. Dip features have been completely destroyed.

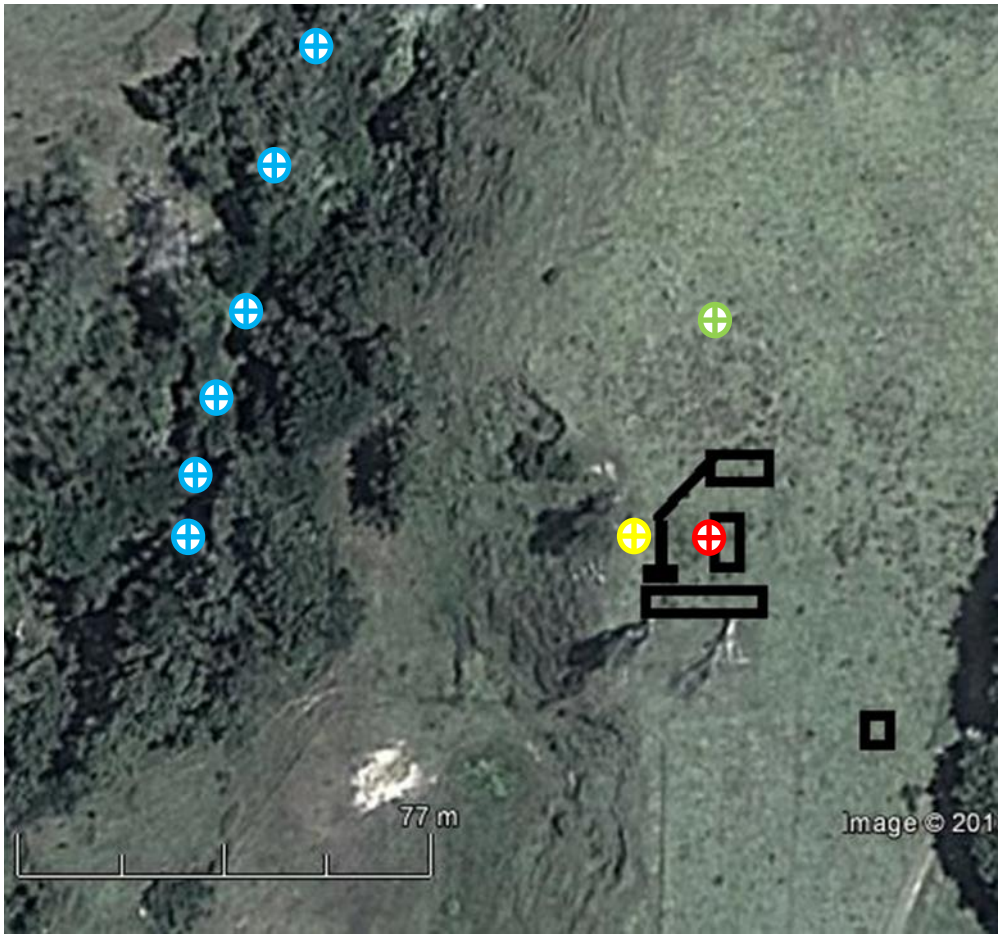

Figure 4.4. Sampling source map at Te Mahia site. Soil used for initial organochlorine and arsenic testing (Table 4.1) and subsequent glasshouse trials (Chapter 5, 6 and 8) was removed from the yellow sampling point. All other sampling points are as noted: Blue cross (water and watercress sampling, Table 4.2); Red cross (field trial site, Chapter 7); Green cross (uncontaminated control soil, Chapter 8).

4.2 Soil Description and Characteristics

The location of the sheep dip site is situated on an old marine terrace with the soil type described as Orthic Pumice. The top 30cm of soil profile is a mixture of Taupo pumice (1850 YBP) and Waimihia pumice (2400 YBP) with a layer of 'pipe clay' directly below it (30-60cm) (Alan Palmer, pers. comm.). This clay layer is pale white in colour and consists of iron oxide mottling throughout.

4.3 Soil Arsenic Mapping On-Site

To determine the extent of arsenic contamination a 50 m x 80 m sampling grid was established, with cores to a depth of 30 cm taken every 10 m. This grid was designed to include possible areas that might be expected to exhibit high arsenic soil concentrations (such as holding pens, shearing sheds). Soil depth analysis was carried out at each point to determine the likelihood of arsenic migration through the soil profile. Core samples were taken at a depth of 0-7.5 cm, 7.5-15 cm, and 15-30 cm and analysed for total soil arsenic (one core was separated into corresponding depth profiles).

4.3.1 Contamination at 0-7.5 cm Depth

Arsenic concentrations in surface soil ranged from 4 mg kg^{-1} – 334 mg kg^{-1} (**Figure 4.5**). Moderate to high contamination exists in direct proximity to the dip site yet elevated levels above 160 mg kg^{-1} are observed around the drainage pen area behind the drip pad. Relative to the run-off zone this area is narrow as soil samples only showed one elevated point of contamination (that being the highest concentration analysed).

4.3.2 Contamination at 7.5-15 cm Depth

Concentrations ranged from 1 – 792 mg kg^{-1} as observed in **Figure 4.6**. In comparison to the 0-7.5 cm depth, soil arsenic concentrations were elevated in direct proximity to the dip. Moderate to high contamination exists either side of the dip and includes the drainage pens. The highest concentration at this depth occurs 10 m from the dip but in line with the proximal flow of left-over dip solution entering the waterway.

4.3.3 Contamination at 15-30 cm Depth

Concentrations ranged from 4 – 1003 mg kg^{-1} **(Figure 4.7)**. Soil surrounding the dip has elevated arsenic soil concentrations compared to 7.5-15 cm. At this depth, approximately 500 m^2 of land exists as that described as arsenic contaminated or above recommended guideline values (that being soil exceeding background arsenic concentrations of 30 mg kg^{-1}). At this depth, two points in the vicinity of the run-off resulted in the highest soil arsenic concentration values of 850-1003 mg kg^{-1}.

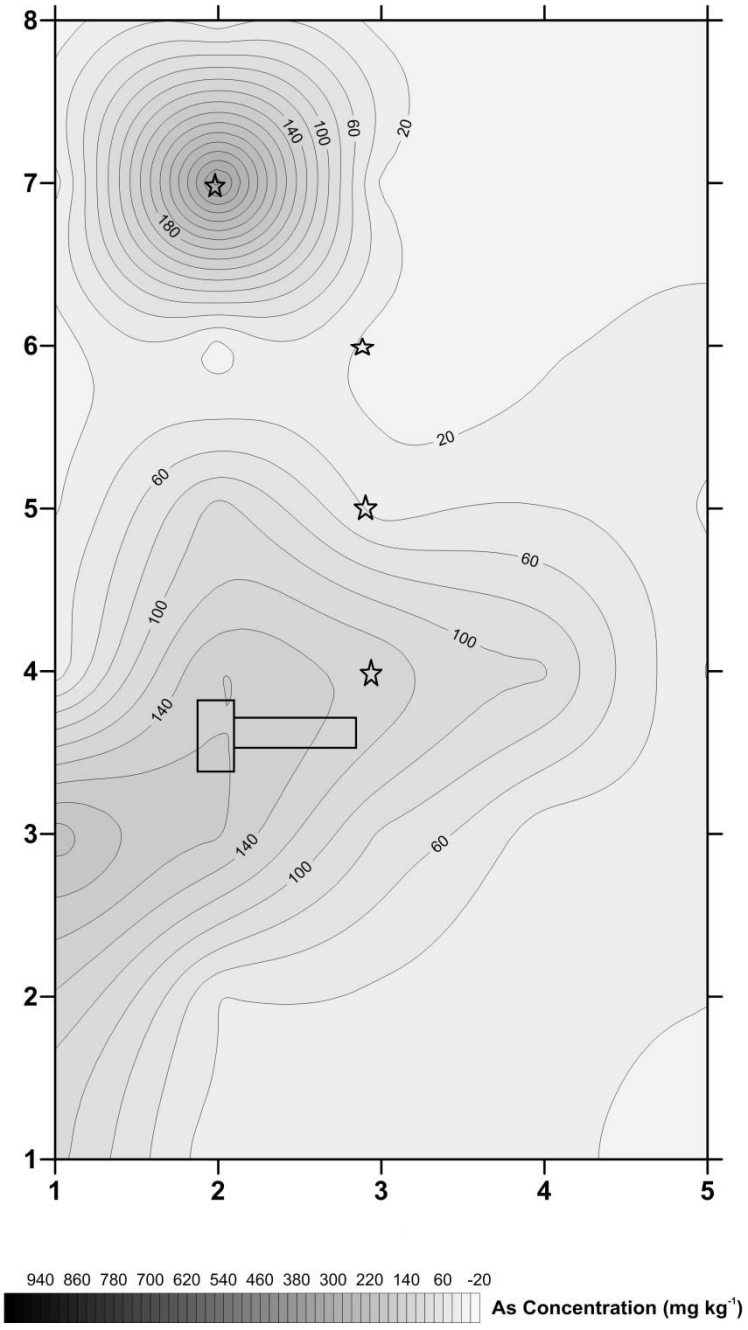

Figure 4.5. Soil total arsenic concentration (mg kg^{-1}) at 0-7.5 cm depth. The outline of the dip and drainage pad has been inlayed with major divisions representing 10 m. Star symbols are a representation of sampling points showing the 10 x 10 m spacing between cores.

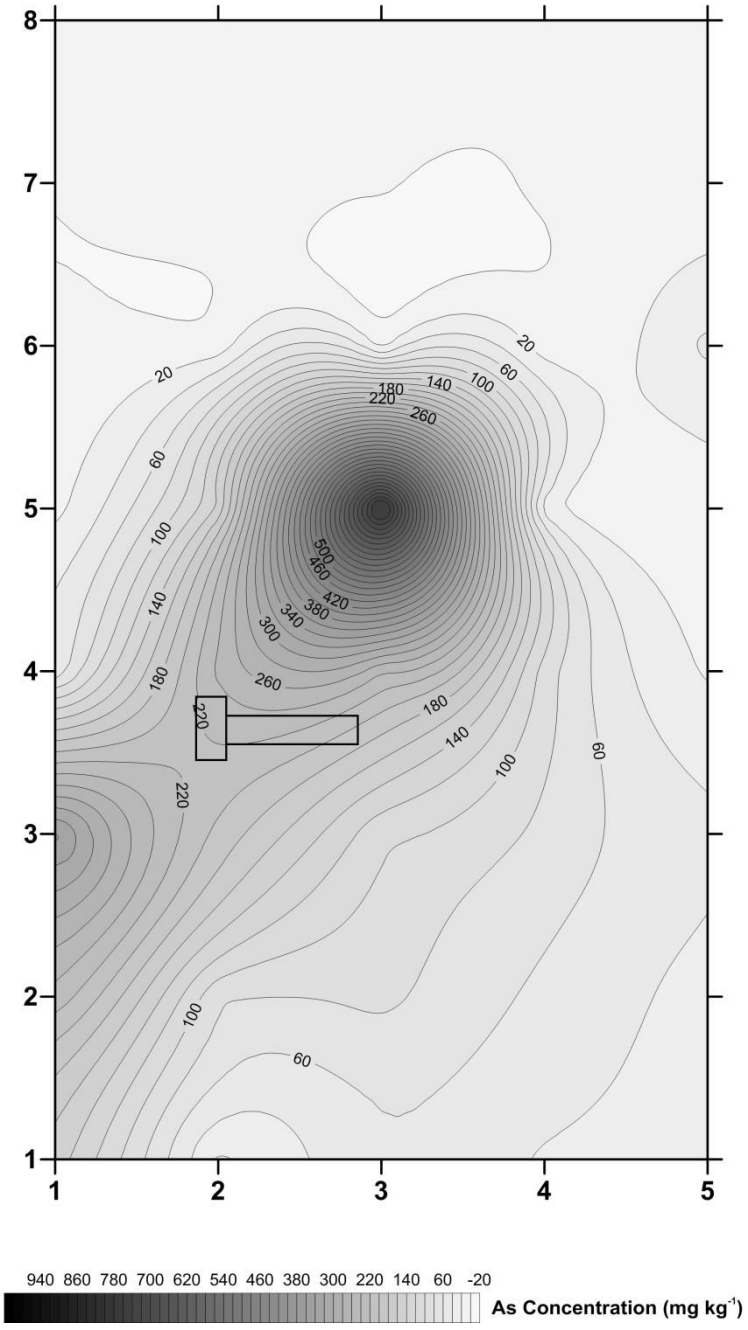

Figure 4.6. Soil total arsenic concentration (mg kg^{-1}) at 7.5-15 cm depth. The outline of the dip and drainage pad has been inlayed with major divisions representing 10 m.

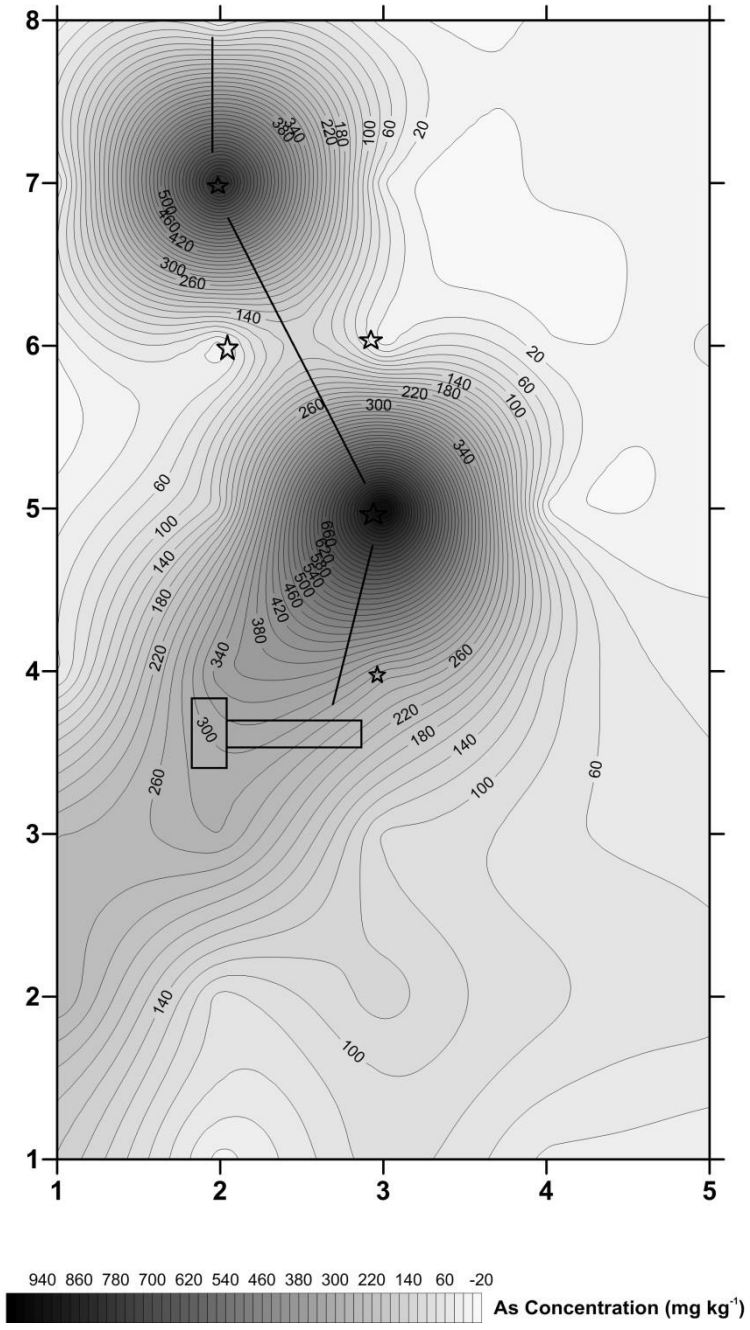

Figure 4.7. Soil total arsenic concentration (mg kg^{-1}) at 15-30 cm depth. The outline of the dip and drainage pad has been inlayed with major divisions representing 10 m. In this figure the proposed flow of left-over dip solution is marked by lines.

4.4 Organochlorine Contamination

Initial soil testing (taken from the position in Figure 4.4) at the site revealed a suite of organochlorines present at high concentrations (**Table 4.1**). Aldrin and dieldrin which are known to interconvert depending on soil redox conditions were found at high concentrations (20 and 163 mg kg^{-1} respectively). On average DDT and its breakdown products (∑DDT) were found at 17 mg kg^{-1}. Isomers of hexachlorocyclohexane were also found at moderate-high concentrations in soil.

Table 4.1 Soil organochlorine concentrations (mg kg^{-1}) from initial testing on-site at Te Mahia dip. (mean *n* = 3 ; ± s.e.)

Organochlorine	Concentration (mg kg^{-1})
Aldrin	20 ± 2.1
Alpha-HCH	52 ± 1.2
Beta-HCH	26 ± 0.8
Delta-HCH	4 ± 0.1
Gamma-HCH (Lindane)	5 ± 0.2
2,4'-DDD	2 ± 0.1
4,4'-DDD	5 ± 0.1
4,4'-DDE	1 ± 0.0
2,4'-DDT	1 ± 0.0
4,4'-DDT	8 ± 0.0
Dieldrin	163 ± 11.8
Endrin	1 ± 0.0
Endrin Ketone	4 ± 0.1

4.5 Effects on Food and Water Sources

To determine present day risk to food and water sources, staple Maori food varieties such as watercress were identified in the stream located directly below the dip site. Analysis of watercress samples (*Nasturtium officinale*) taken 40-50 m directly below the dip exhibited significant levels of arsenic contamination (**Table 4.2**). These plant samples are aquatic in nature and are found in a tributary located approximately 10 m from where the proposed run-off zone intersects with the stream bank. Watercress 1, a sample taken from the presumed entry of run-off solution into the waterway significantly exceeds WHO guidelines for arsenic in foodstuffs. All other watercress samples which were taken downstream (100-150 m) of Watercress 1 were well below WHO guidelines. Water samples taken from the waterway and also pooled surface water near the run-off zone exceeded water drinking guidelines in a number of areas. Surface waters below the dip site ranged from 5.8 – 140 μg L^{-1}. Waterway samples were generally lower yet one sample spiked at 21 μg L^{-1}.

4.6 Site Characterisation Discussion and Conclusions

Characterisation of the site by arsenic soil concentration mapping revealed that 500 m^2 of agricultural land has been contaminated with this metalloid and that arsenic exists at varying high concentrations through the soil profile. Not only are soil arsenic concentrations high around the proximity of the dip but the highest concentrations were from the run off zone which drains into a nearby stream (which subsequently discharges to the ocean approximately 300 m away).

Due to soil coring being carried out at 10 m intervals, the entire run-off zone was not 'captured' by the arsenic concentration map. Soil cores were taken from either side of the run-off zone **(Figure 4.7)** showing the very narrow passage of arsenic contamination from this area. The possibility of both surface and sub-surface flow of arsenic is a real possibility in this zone as a soil core taken at 7-5-15 cm (**Figure 4.6**) showed low soil arsenic concentrations in this run-off zone at this depth.

The potential for environmental contamination from these historic pesticides was established by analysing plant and water samples below the dip site. Staple Maori food varieties such as watercress were significantly contaminated with arsenic while water samples taken from the stream below the dip returned elevated arsenic concentrations.

Present day contamination due to historical application of chemicals associated with sheep dipping practices is occurring on site and must be managed. It was determined that 80% of an 80 x 50m exceeded serious contamination values for arsenic (Table 2.2) yet the true extent of contamination was not captured within in this grid. Limitations of this investigation involve the cost of organochlorine analysis and the nature of the grid used. Because of this, the extent of organochlorine contamination could not be determined. Secondly, by coring every 10m as part of the grid pattern used, failed to establish the existence of true arsenic soil hotspots. It would be beneficial to future researchers to extend the area under investigation but if needed, also carry out coring at smaller intervals.

Table 4.2. Arsenic concentrations in water and plant samples taken from waterway below the dip site which discharges into the ocean. World Health Organisation (WHO) arsenic standards are 2 mg kg^{-1} for plants and 10 µg L^{-1} for water.

Sample	As Concentration	pH
Water 1	139.7 µg L^{-1}	8.4
Water 2	45.4 µg L^{-1}	8.5
Water 3	5.8 µg L^{-1}	8.4
Water 4	1.5 µg L^{-1}	8.3
Water 5	21.4 µg L^{-1}	8.5
Water 6	1.8 µg L^{-1}	8.3
Watercress 1	31 mg kg^{-1}	
Watercress 2	0.4 mg kg^{-1}	
Watercress 3	0.02 mg kg^{-1}	
Watercress 4	0.001 mg kg^{-1}	
Watercress 5	0.001 mg kg^{-1}	
Watercress 6	0.002 mg kg^{-1}	

CHAPTER 5 : BIOCHAR ENHANCES SOIL MICROBIAL ACTIVITY AND DEGRADATION OF ORGANOCHLORINES IN A CO-CONTAMINATED SOIL

(TO BE SUBMITTED)

ENVIRONMENTAL SCIENCE & TECHNOLOGY

5.1 Abstract

This research described in Chapter 5 examined the effect of biochar on soil microbial activity and pollutant degradation in a co-contaminated soil (with arsenic and organochlorines) during a 180 d incubation experiment under glasshouse conditions. Biochar made from willow feedstock (*Salix sp*) was pyrolysed at 350 and 550°C and added to the co-contaminated soil at final rates of 30 t ha^{-1} and 60 t ha^{-1}. Water-extractable arsenic concentrations were significantly reduced ($P<0.05$) under biochar treatments after 30 d (by as much at 40 % compared to the control) but increased to control soil concentration levels from 60 d onwards. Soil microbial activity, analysed using the dehydrogenase assay (DHA), was significantly increased ($P<0.01$) under all biochar treatments with >100% increase in total activity after 60 d of treatment compared to the control. This initial reduction in water-extractable arsenic may reduce selection pressure for soil microbes which in turn, exhibit significant stimulation of microbial activity. Metagenomic analysis of the soil bacterial community revealed that biochar amended soils were selecting for bacterial species such as Chryseobacterium, Flavobacterium and Dyadobacter and the family Pseudomonadaceae which are known bioremediators of hydrocarbons. Isomers of the organochlorine hexachlorocyclohexane (HCH), particularly alpha-HCH and gamma-HCH (lindane), underwent 10-fold and 4-fold reductions in soil concentrations respectively (2.2 mg kg^{-1} and 0.4mg kg^{-1}) compared to the control (25 mg kg^{-1} and 1.6 mg kg^{-1} respectively). Amendment of soil with biochar also caused a significant reduction ($P<0.01$) in soil DDT levels. This investigation provides evidence towards the beneficial attributes of biochar in contaminated soil and its potential to reduce the time needed to remediate these soils.

5.2 Keywords

Biochar; Arsenic; Soil Microbial Activity; Organochlorines, Remediation

5.3 Introduction

From the 1940s organochlorines began to replace arsenicals in dip operations as awareness of the environmental danger of arsenic increased (Chopra et al, 2007). The chlorinated cyclodienes (aldrin, dieldrin, heptachlor) and the chlorobenzene derivatives of these cyclodienes (dichlorodiphenyltrichloroethane, known as DDT; and ϒ-hexachlorocyclohexane, known as lindane) were commonly used and in respect with lindane, was often applied as technical HCH (a mixture of HCH isomers) (National Rivers Authority, 1994; Chaudhry et al., 2005). By 1961, issues relating to organochlorines were being raised because of their ability to accumulate in body fat and to be passed onto consumers.

Organochlorines in soil will break down with time, and can be degraded or volatilised from soil through a number of reactive steps such as dechlorination, dehydrochlorination, isomerisation and oxidation (Lal & Saxena, 1982; Aislabie et al., 1997; van Zwieten et al., 2003). These degradative processes can be facilitated by microbiological activity in soil, and the technology of promoting the degradation of a soil contaminant is known as bioremediation. The effectiveness of bioremediation techniques can be increased through the addition of specific amendments to soil (Quilty & Cattle. 2011) like biochar (Anderson et al., 2011; Beesley et al., 2011a).

Previous studies have shown that biochar amendment can manipulate soil microbial activity and lead to increased adsorption of soil contaminants. However, to our knowledge no studies have analysed the specific response of soil microbes to carbon amendment of a co-contaminated soil. In particular, the biochar-promoted degradation of organochlorines in an arsenic contaminated soil is poorly defined in literature. The objectives of this study were to investigate if biochar produced from willow at two temperatures of pyrolysis (350 and 550°C) (i) has an effect on microbial activity; to further test this hypothesis, perform a bacterial 16S ribosomal DNA metagenomic analysis of the soils at the end of the treatment to characterise the bacterial communities present and (ii) promotes the degradation of organochlorines in soil.

5.4 Glasshouse Setup

A bulk soil sample (300 kg) was collected to 20 cm depth from a 2 m x 2 m area where residual dip solution was identified to have been disposed of at the end each dipping activity (**Figure 4.4**). The soil was then transported back to the Plant Growth Unit, Massey University, Palmerston North in 50 kg plastic bags and stored under a black PVC sheet to prevent drying. The soil was then mixed using a nursery grade mixer with two sets of revolving sleeves that slowly fold the soil to homogenise arsenic and organochlorine contamination. The mixed soil was subsequently sieved through a 5 mm-mesh to remove constituent coarse inorganic and organic material before pot experiments were initiated.

Pot experiments were conducted under greenhouse conditions using square plastic pots measuring 20 x 20 cm and 30 cm deep, with drainage holes drilled at the base of the containers. Two doses of each biochar (0.5 x 0.5 cm particle size) were selected for both glasshouse trials : 10 and 20 g biochar kg^{-1} soil, which when incorporated to 30 cm depth corresponds to a loading of 30 t ha^{-1} and 60 t ha^{-1}, respectively. Biochar was manually mixed with soil prior to filling of the plastic pots with a final weight (soil + biochar) of 4kg. All pots were kept at 70 % water holding capacity (WHC) with the addition of distilled water and left for 1 week to incubate (defined as T = 0 d).

5.4.1 Soil Sampling

Soil samples were collected from every pot each month for chemical and biological analysis starting (T = 30 d) Samples were taken using a stainless steel corer with an internal diameter of 1 cm. These cores were analysed for microbial activity (fresh soil) and water-extractable arsenic (air-dried soil). Cored holes were filled with initial (control) soil and marked with a toothpick during monthly sampling. A new sampling location roughly 3 cm adjacent to the previous was used at each sampling time.

5.5 Results

5.5.1 Properties of Biochars

Elemental analysis and the yield of the two biochars are described in **Table 5.1**. Both biochars had high pH (8.6) with yields of 25% and 27.5% for the 550°C and 350°C biochar, respectively. Organic C in both biochars was >70%, whereas inorganic C was almost negligible (<0.8%). The 350°C biochar had a higher volatile C content than the 550°C biochar (30.8% compared to 27.5%), higher atomic H/C_{org} ratio (0.52 compared to 0.48), and a lower proportion of fixed C (63.6% compared to 68.4%). The liming equivalence was 8.9% and 9.2% for the 350°C and 550°C biochar, respectively. Increased pyrolysation temperature significantly increased the specific surface area ($P<0.01$) from 6.1 $m^2\ g^{-1}$ for the 350°C biochar to 59.8 $m^2\ g^{-1}$ for the 550°C biochar.

High-energy XPS spectra of fresh biochar samples describes the surface functionality of carbon (C1s – **Table 5.2**). The XPS spectra define energy signals corresponding to four functional groups: aliphatic/aromatic (284.6eV assigned to CHx, C-C, C=C), hydroxyl and ether (285.8eV to C-OR), carbonyl (287.2eV to C=O) and carboxyl groups (288-289eV to C-OOR). Biochars had a very high proportion of aliphatic/aromatic carbon (83.56% and 87.08% for 350 and 550°C biochars) and low proportions of carboxylic and carbonyl groups (**Table 5.2**). Both biochars contained high concentrations of 1M HCl-extractable K^+ (10-12 g kg^{-1}) and Ca^{2+} (24 g kg^{-1}) and moderate concentrations of Mg^{2+} (1.5 g kg^{-1}), and SO_4-S (1.8 g kg^{-1}). The Formic acid-extractable P concentration was ~0.7 g kg^{-1} (**Table 5.1**).

5.5.2 Soil Properties at the End of the Experiment

The chemical properties of the control soil and the biochar-amended soils are summarised at T = 180 d (**Table 5.3**). Soil amended with both types of biochar had significantly ($P < 0.05$) higher concentrations of extractable SO_4^{2-}, K^+, Ca^{2+} and Mg^{2+} than the control soil yet Olsen P values were slightly lower. Soil pH was significantly increased at high dose for both biochars (0.2 unit increase from pH 5.6 to pH 5.8, $P < 0.05$).

Table 5.1. Physiochemical characterisation of fresh biochar.

Parameter	350°C Biochar	550°C Biochar
Feedstock	Salix sp. (1y.o)	Salix sp. (5.y.o)
Pyrolysis Temp (°C)	350°C	550°C
pH	8.6	8.6
Yield (%)	27.5	25.0
Organic C Content (C_{org} %)[a]	73.9	77.4
N (%)	0.8	0.7
S (%)	0.3	0.2
O (%)[b]	19.4	14.3
H (%)	3.5	3.3
Volatile C (%)	30.8	27.5
Atomic H/Corg	0.52	0.48
Ash Content (%)	5.6	4.1
Liming Equiv (%)	8.9	9.2
Surface Area (m^2 g^{-1})	6.1	59.8
1M HCl-extractable K (g kg^{-1})	11.9	10.7
1M HCl-extractable Ca (g kg^{-1})	23.6	24.0
1M HCl-extractable Mg (g kg^{-1})	1.5	1.4
1M HCl-extractable SO_4-S (g kg^{-1})	1.9	1.8
2 % Formic acid-extractable P (g kg^{-1})	0.7	0.8

[a]Biochars contained < 0.8 % C_{inorg} thus C_{org} basically represents total C. [b]Estimated by difference O = 100 – (C + N + S + Ash).

Table 5.2. Chemical composition of carbon (C1s) determined through high-energy resolution XPS analysis of biochar particles.

	C1s Composition (%)			
	CHx, C-C, or C=C	**C-OR**	**C=O**	**C-OOR**
350°C Fresh BC	83.6	11.3	3.3	1.8
550°C Fresh BC	87.1	9.1	2.4	1.4

The binding energy of C1s at 284.6eV was assigned to CHx, C-C, C=C; at 285.8eV to C-OR; at 287.2eV to C=O; and at 288-289eV to C-OOR

5.5.3 Dehydrogenase Activity

Activity in the control soil did not change over the six months of the trial, and remained at a constant level of 100 μg g^{-1} DM (**Figure 5.1A**). On addition of 350°C biochar, dehydrogenase activity significantly and immediately increased ($P<0.01$) at both concentrations (30t ha^{-1} and 60t ha^{-1}) at every sampling point relative to the control, although there was a general trend to decreasing activity with time. On addition of 550°C biochar, dehydrogenase activity was significantly increased at both dosage rates at all sampling times from one-month after biochar incorporation into the soil (**Figure 5.1B**). This increase was immediate for the high rate of application, but delayed for the low rate; activity for the low rate on the day of incorporation was the same as the control. Again for the 550°C biochar, there was a trend of decreasing activity with time.

5.5.4 Biochar Amendment of Soil and its Effect on Microbial Composition

Metagenomic soil DNA analysis identified 14 named phyla (and 2 unknown phyla) contributing significantly to the composition of the bacterial community (**Table 5.4**). Across all treatments the bacterial phyla that dominated bacterial community structure were Actinobacteria (31.4%), Proteobacteria (17.3%), Firmicutes (10.6%), Acidobacteria (5.0%) and Bacteroidetes (4.4%).

Amendment of soil with both biochars (60 t ha^{-1}) caused a significant shift ($P<0.05$) in the relative position of a number of bacterial phyla after 180 d of treatment. Therefore it can be proposed that biochar promoted a change in bacterial community structure. For 350°C biochar amendment of soil, the composition shifted away from Actinobacteria and towards Bacteroidetes and Planctomycetes, while a general shift towards Firmicutes and Planctomycetes and away from Proteobacteria was observed for 550°C biochar amendment.

At the genus level a pronounced shift was observed in a number of phyla for the 350°C biochar amendment (**Table 5.5**). Within the Firmicutes a significant ($P<0.05$) 3-fold increase in Chryseobacterium and Flavobacterium was observed along with a 2-fold increase in Dyadobacter. This shift was also represented in the family Pseudomonadaceae (phylum : Proteobacteria) with a 2-fold increase observed, suggesting a shift towards this family within the phyla compared to 550°C biochar amended soil. For 550°C biochar amendment of soil a significant ($P<0.05$)10-fold increase in the order Streptophyta (phylum : Cyanobacteria) was evident along with small increases in the genus Bacillis (phylum : Firmicutes), although this result was not significant ($P<0.05$).

5.5.5 Concentration of Organochlorines and Arsenic in Soil at the end of Experiment

Soil from each pot was analysed for organochlorine concentration at the end of the experiment (**Table 5.6**). The dilution effect of biochar was taken into account when calculating the final concentration of each contaminant.

Table 5.3. Chemical characterisation of biochar-amended and control soil at the end of the experiment (T = 180 d) (mean *n* = 3 ; ± s.e.). Cations have been depicted as extractable cations (meq/100g).

	pH	Olsen P	SO4	K	Ca	Mg	Na	CEC	Total As
		µgP/g	µgS/g	me/100g	me/100g	me/100g	me/100g	me/100g	(mg kg^{-1})
Control Soil (T = 0 d)	5.6	48.7 ± 0.2	13.4 ± 0.2	0.8 ± 0.1	3.3 ± 0.4	1.4 ± 0.1	0.4 ± 0.1	12 ± 0.0	202 ± 3.9
Control Soil	5.6	48.6 ± 0.3	12.8 ± 0.2	0.7 ± 0.5	3.5 ± 0.2	1.0 ± 0.6	0.3 ± 0.1	12.0 ± 0.0	200 ± 2.1
Soil + 350°C BC (30t ha^{-1})	5.6	47.9 ± 2.1	15.9 ± 0.3	1.0 ± 0.0	4.8 ± 0.2	1.3 ± 0.0	0.3 ± 0.0	16.0 ± 0.5	199 ± 4.0
Soil + 350°C BC (60t ha^{-1})	5.8	47.6 ± 2.1	24.8 ± 2.0	1.4 ± 0.1	5.9 ± 0.2	1.7 ± 0.1	0.4 ± 0.0	19.0 ± 0.4	199 ± 0.8
Soil + 550°C BC (30t ha^{-1})	5.6	48.4 ± 0.8	18.6 ± 0.7	1.2 ± 0.1	4.9 ± 0.4	1.2 ± 0.0	0.4 ± 0.0	15.0 ± 0.3	198 ± 3.6
Soil + 550°C BC (60t ha^{-1})	5.8	49.3 ± 1.3	19.5 ± 4.1	1.3 ± 0.1	6.3 ± 0.2	1.5 ± 0.1	0.3 ± 0.1	17.0 ± 0.8	199 ± 3.1

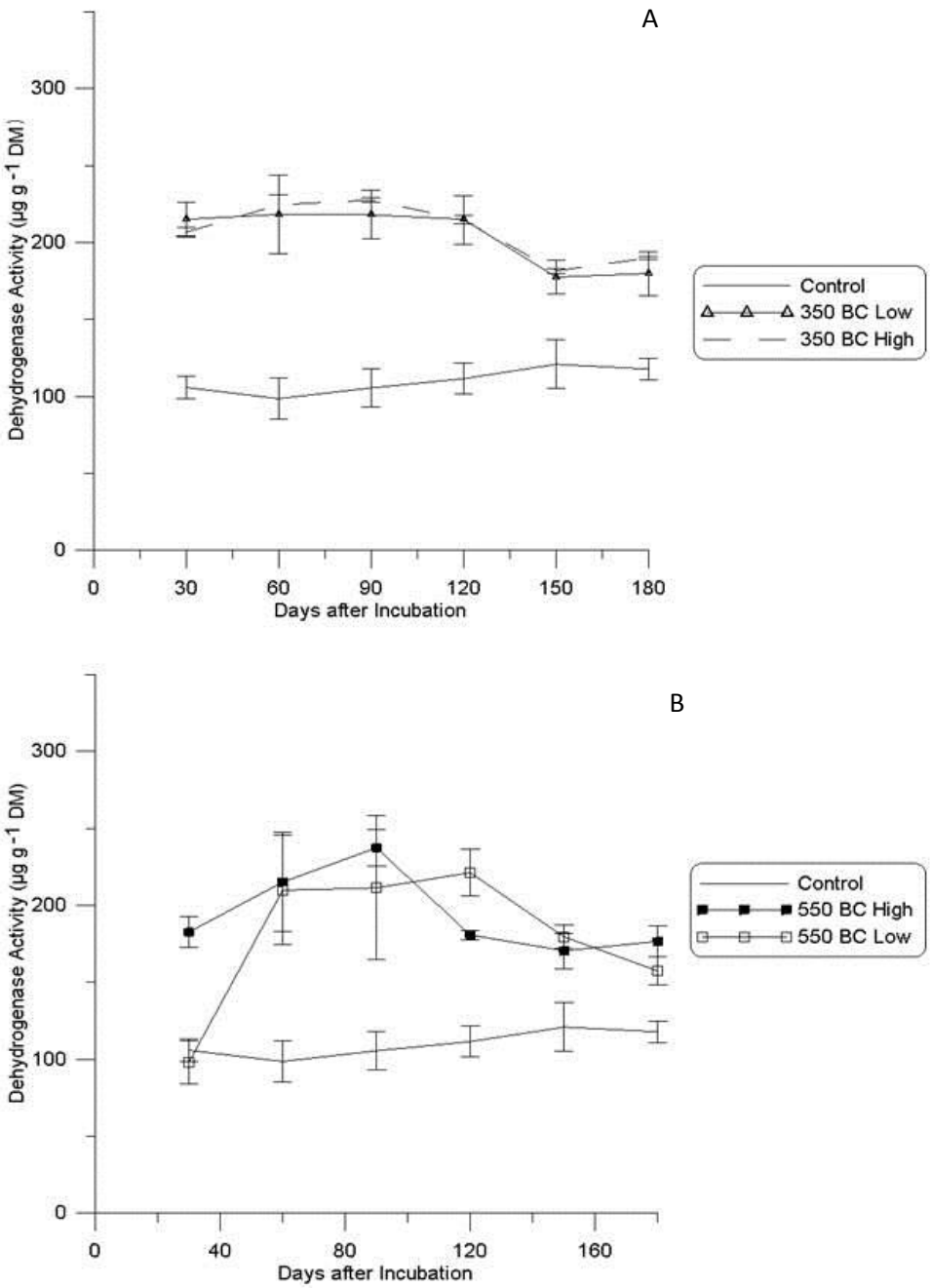

Figure 5.1. Soil dehydrogenase activity measured in µg per g of dry matter (DM) as a function of A) 350 °C biochar treatment and B) 550 °C biochar treatment (mean *n* = 3 ; ± s.e.).

5.5.5.1 Organochlorines

Additions of both types of biochar at both rates caused a significant decrease in the soil concentration of two isomers of HCH, alpha-HCH (**Figure 5.2A**) and gamma-HCH (**Figure 5.2B**) ($P<0.01$). Alpha HCH was observed to undergo nearly a 10-fold reduction under all biochar treatments compared to the control while lindane (gamma HCH) underwent a 4-fold reduction under all biochar treatments. There was no significant difference ($P<0.05$) in the extent of reduction between the four biochar treatments. Delta HCH concentrations also reduced slightly but not significantly under each biochar treatment (**Figure 5.2D**) and there was no difference attributable to the biochar treatment used. The isomer beta HCH was unaffected by biochar amendment and after 6 months of treatment did not significantly differ ($P<0.05$) in concentration compared to the control.

A decrease in the soil concentration of DDT (including its breakdown products: sum DDT) was also significantly affected ($P<0.01$) by three of the four treatments under study (350°C biochar at 30 t ha^{-1} and 60 t ha^{-1} and also 550°C biochar at 60 t ha^{-1}) compared to the control (**Figure 5.2C**). The magnitude of the decrease in DDT concentration was influenced by the biochar treatment used. 350°C biochar at 60 t ha^{-1} resulted in a reduction of 38% of total DDT in soil compared to the control, with 350°C biochar (30 t ha^{-1}) and 550°C biochar (60 t ha^{-1}) reduced total DDT concentrations by 27 and 25% respectively compared to the control. Interestingly dieldrin and aldrin exhibited a significant reduction ($P<0.05$) in the extent of degradation as a function of treatment ($P<0.05$).

5.5.5.2 Arsenic

The total concentration of arsenic in soil did not change during the experiment. However, all biochar treatments promoted a significant reduction in the concentration of water available arsenic in soil collected one month (T = 30 d) into the experiment (**Table 5.7**). By the third month, the concentration of water-soluble arsenic had increased to be the same as that recorded for the control soil.

5.6 Discussion

5.6.1 Effect of Biochar on Soil Properties and H_2O-Extractable Arsenic

Biochar is widely reported to have a liming effect, although this is biochar type dependent, and any change in soil pH also depends on the pH-buffering capacity of the amended soil (Calvelo Pereira et al., 2011). The addition of biochar to soil slightly raised the soil pH over the six months of the incubation, and this is attributed to the liming potential of biochar. Both biochars contained greater than 8.8% liming equivalence.

An increase in pH should favour desorption and increased bioavailability of arsenic in soil (Mahimairaja et al., 2005; Carrillo-Gonzalez et al., 2006; Hartley et al., 2009; Beesley et al., 2011a; Namgay et al., 2010) which would lead to a gradual increase in the water-available fraction of arsenic in each biochar treatment (Sohi et al., 2010; Kookana et al., 2011). Yet this effect was not evident in this study. In this work, an initial reduction in water-extractable arsenic was observed under biochar amendment (T = 30 d) yet after possible weathering of biochar particles arsenic was re-released (T = 60 d). The mechanistics of this initial reduction in water-available arsenic could be the precipitation of arsenic with alkaline salts such as Ca^{2+} found in the initial ash fraction. Once these salts dissolve the arsenic may become available for extraction (Joseph et al., 2010; Beesley et al., 2011a,b).

5.6.2 Stimulation of Microbial Activity Under Biochar Amendment

Dehydrogenase activity is an index of the microbial health of soil and has been used to assess the environmental toxicity of chemicals and heavy metals in contaminated soils (Chandler and Brooks, 1991). Low dehydrogenase activity is common in co-contaminated soils as microbial populations are often supressed by surrounding toxic elements especially arsenic and selected organochlorines (Edvantoro et al., 2003; van Zwieten et al., 2003). A labile carbon source may restore microbial communities to uncontaminated levels. In this work a background level of 250 µg g^{-1} DM was observed for uncontaminated soil adjacent to the dipping site).

Table 5.4. Metagenomic analyses from QIIME based on taxonomic structure of the soil bacterial community (%). Taxonomy is listed based on Phyla (Kingdom : Bacteria) as a function of treatment. Significant differences are noted by different letters (mean *n* = 3 ; ± s.e.) compared to the control.

	Control	550 BC (60t ha^{-1})	350 BC (60t ha^{-1})
Actinobacteria	34.9 ± 2.1	31.3 ± 1.7	**28.0 ± 1.1^{a}**
Other	17.3 ± 0.0	16.9 ± 0.1	18.3 ± 2.9
Proteobacteria	17.7 ± 2.6	**14.2 ± 0.3^{a}**	19.9 ± 2.4
Firmicutes	9.9 ± 0.2	**12.8 ± 0.9^{a}**	9.2 ± 0.4
Bacteroidetes	3.2 ± 0.5	3.1 ± 0.8	**6.9 ± 1.3^{a}**
Acidobacteria	5.0 ± 0.3	5.2 ± 0.3	4.8 ± 0.5
Planctomycetes	2.5 ± 0.1	**3.9 ± 0.4^{a}**	**3.3 ± 0.1^{a}**
Verrucomicrobia	2.4 ± 0.2	3.1 ± 0.4	2.9 ± 0.2
Chloroflexi	1.9 ± 0.3	2.1 ± 0.3	1.7 ± 0.1
Gemmatimonadetes	1.8 ± 0.1	2.0 ± 0.1	1.8 ± 0.2
Cyanobacteria	0.4 ± 0.1	2.3 ± 2.0	0.5 ± 0.2
Nitrospirae	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
Chlamydiae	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Unknown	2.1 ± 0.2	2.0 ± 0.0	1.7 ± 0.2

Table 5.5. Metagenomic analyses from QIIME based on taxonomic structure of the soil bacterial community (%). Selected taxonomy is based on significant changes within phyla as a function of treatment. Significant differences are noted by different letters (mean *n* = 3 ; ± s.e.).

	Control	550 BC (60t ha^{-1})	350 BC (60t ha^{-1})
(genus) Chryseobacterium	0.0 ± 0.0	0.0 ± 0.0	1.5 ± 0.8[a]
(genus) Flavobacterium	0.5 ± 0.1	0.3 ± 0.1	1.2 ± 0.5[a]
(genus) Dyadobacter	0.5 ± 0.3	0.1 ± 0.0	1.0 ± 0.1[a]
(family) Pseudomonadaceae	3.0 ± 1.1[a]	0.8 ± 0.1	4.8 ± 0.8[a]
(order) Streptophyta	0.2 ± 0.0	2.1 ± 0.5[a]	0.3 ± 0.2
(genus) Bacillus	5.5 ± 0.5	7.1 ± 1.8	4.9 ± 0.1

Table 5.6. Organochlorine concentrations (mg kg^{-1}) in soil as a function of treatment. Different letters corresponding to each organochlorine represent significant differences. Significant differences are noted at P<0.05 (aldrin, dieldrin, δ-HCH, endrin ketone) and P<0.01 (α-HCH, Lindane, ∑DDT) and are noted by different letters (mean *n* = 3 ; ± s.e.).

	Aldrin	**Dieldrin**	**α-HCH**	**δ-HCH**	**Lindane**	**Endrin ketone**	**∑DDT**
Control Soil	6.17^{a} ±0.37	111^{a} ± 4.04	25^{a} ± 1.15	1.8^{a} ± 0.06	1.6^{a} ± 0.07	1.8^{a} ± 0.08	6.3^{a}
350°C BC (30t ha^{-1})	7.6^{a} ± 0.25	117^{a} ± 3.84	2.6^{b} ± 0.31	1.6^{b} ± 0.04	0.5^{b} ± 0.06	1.4^{b} ± 0.05	4.6^{b}
350°C BC (60t ha^{-1})	9.9^{b} ± 0.52	152^{b} ± 4.84	2.2^{b} ± 0.06	1.5^{b} ± 0.03	0.4^{b} ± 0.01	1.7^{a} ± 0.03	3.9^{b}
550°C BC (30t ha^{-1})	8.4^{b} ± 0.59	134^{b} ± 8.50	3.8^{b} ± 1.19	1.6^{b} ± 0.05	0.5^{b} ± 0.01	1.5^{b} ± 0.03	6.0^{a}
550°C BC (60t ha^{-1})	6.1^{a} ± 0.70	106^{a} ± 2.52	2.4^{b} ± 0.40	1.6^{b} ± 0.04	0.4^{b} ± 0.02	1.4^{b} ± 0.04	4.7^{b}

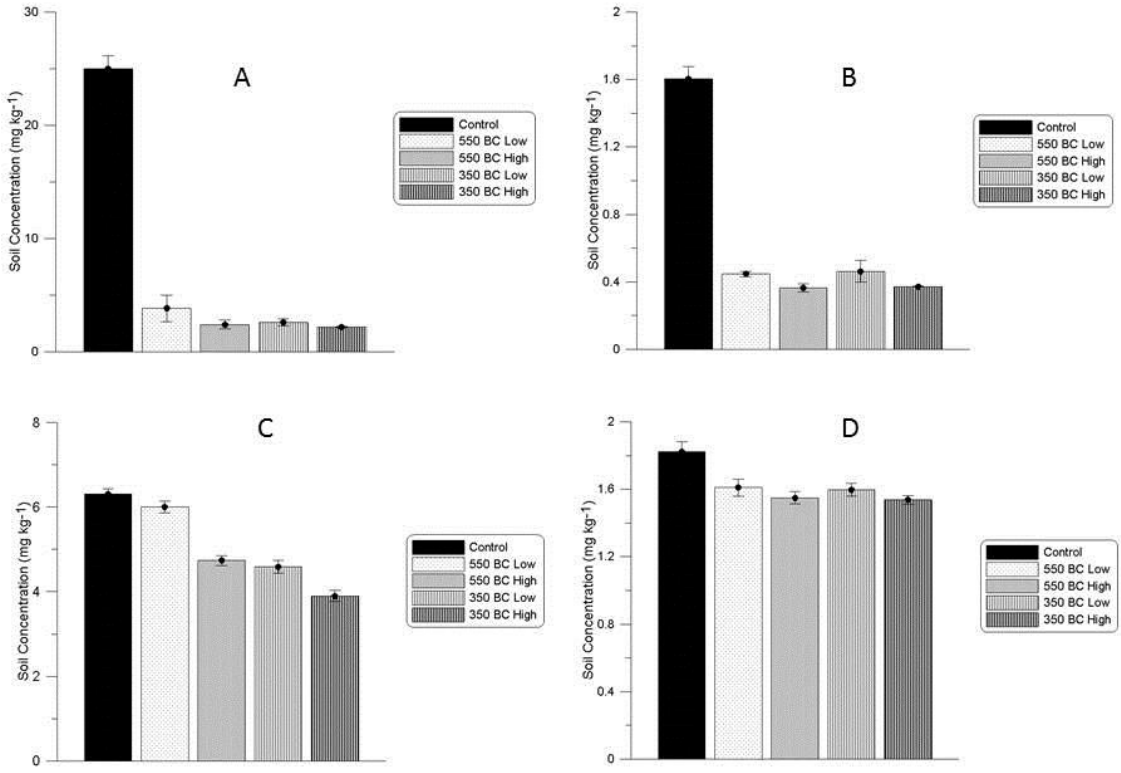

Figure 5.2. Soil concentrations (mg kg^{-1}) for A) Alpha-HCH B) Lindane C) DDT and D) Delta-HCH at the termination of the glasshouse trial as a function of treatment. Significant differences are observed between all biochar types and the control ($P<0.01$) (mean $n = 3$; ± s.e.).

Warnock et al (2007) suggest that biochar with high microporosity may form refuges for microbes against soil parasites when added to soil. This mechanism may explain an increase in soil microbial activity associated with the 550°C biochar (compared to the control) which had a greater surface area relative to the 350°C biochar ($P<0.01$) (**Table 5.1**). However, there was a difference in the labile C content between the two biochars. The 350°C biochar had a higher volatile C content than the 550°C biochar (30.8% compared to 27.5%) and a lower fixed C content (63.6% compared to 68.4%) potentially meaning a greater source of labile C. It is possible that soil microbes may

use the volatile or labile fraction of biochar as a stimulant, and the microporosity of biochar as a refuge with labile C the most important stimulant.

Despite being non-target organisms of pesticide application, soil microbes are affected by dip chemicals once these are added to the soil. Pesticides, such as organochlorines and arsenic, reduce soil enzyme activity which can be used as a key index for soil health (Antonious, 2003; Monkiedje et al., 2002). The initial reduction in water-extractable arsenic due to biochar amendment may have released soil microbes from arsenic related toxicity and combined with a biochar –source of labile carbon, stimulated microbial activity.

Organochlorines have been shown to detrimentally affect the makeup of microbial communities in contaminated soils (Pampulha & Oliveira, 2006; Zhou et al., 2006; Klose & Ajwa, 2004), and along with arsenic compounds will similarly inhibit microbial activity. Co-contamination of organochlorines and arsenic has been observed to reduce the rate of degradation of these chemicals by microbial means; there is a well recorded reduction in soil microbial activity at historic sheep dip sites (Zwieten et al., 2003; Edvantoro et al., 2003). The low stable soil dehydrogenase activity observed for the control soil throughout our incubation study may result from this co-contamination. Wang et al (2006) and Saez et al (2006) studied the effects of co-contamination in a mixture of lindane, aldrin and simazine, and found that each chemical affected soil microorganisms differently. The denitrifying ability of *Xanthobacter autotrophicus,* a ubiquitous bacterium in soil that can degrade halogenated short-chain hydrocarbons, was negatively affected by simazine, while nitric oxide release was strongly inhibited by both aldrin and lindane. A similar effect on bacterial species caused by organic and inorganic chemicals (such as organochlorines and arsenic) could be expected at sheep dip sites.

Table 5.7. Water extractable arsenic concentration (mg L^{-1}) in soil as a function of treatment. Significant differences are denoted by different letters at P<0.05 (mean *n* = 3 ; ± s.e.). Soil pH values are listed in italics and in brackets for each treatment.

	T = 0	**T = 30**	**T = 60**	**T = 90**	**T = 120**
Control Soil	1.3 ± 0.14^{a} (*5.8*)	1.4 ± 0.10^{a} (*5.8*)	1.2 ± 0.08^{a} (*5.7*)	1.3 ± 0.04^{a} (*5.8*)	1.4 ± 0.01^{a} (*5.8*)
350°C BC (30t ha^{-1})	1.4 ± 0.12^{a} (*6.1*)	0.8 ± 0.00^{b} (*6.0*)	1.1 ± 0.04^{a} (*5.8*)	1.2 ± 0.02^{a} (*5.9*)	1.4 ± 0.05^{a} (*5.9*)
350°C BC (60t ha^{-1})	1.2 ± 0.18^{a} (*6.2*)	0.9 ± 0.05^{b} (*6.1*)	1.1 ± 0.03^{a} (*6.0*)	1.2 ± 0.02^{a} (*5.9*)	1.4 ± 0.04^{a} (*6.1*)
550°C BC (30t ha^{-1})	1.3 ± 0.08^{a} *(6.1)*	0.9 ± 0.04^{b} *(5.9)*	1.2 ± 0.00^{a} *(5.9)*	1.1 ± 0.07^{a} *(5.8)*	1.3 ± 0.08^{a} *(5.9)*
550°C BC (60t $ha^{-1)}$	1.4 ± 0.14^{a} *(6.2)*	1.0 ± 0.11^{b} *(6.1)*	1.1 ± 0.07^{a} *(5.9)*	1.3 ± 0.16^{a} *(5.8)*	1.5 ± 0.02^{a} *(6.0)*

Table 5.8. The soil concentration of 4,4'-DDT and its breakdown derivative 4,4'-DDE (mg kg^{-1}) and the DDE : DDT ratio as a function of treatment in co-contaminated soil after T = 180 d. Significant errors are listed after the mean with all samples (mean *n* = 3 ; ± s.e.).

	DDE	**DDT**	**DDE:DDT**
Control Soil	0.50 ± 0.06	3.13 ± 0.26	0.16 ± 0.03[a]
350°C BC Amended (30t ha^{-1})	0.45 ± 0.01	2.10 ± 0.06	0.21 ± 0.00[b]
350°C BC Amended (60t ha^{-1})	0.43 ± 0.01	1.72 ± 0.08	0.25 ± 0.01[b]
550°C BC Amended (30t ha^{-1})	0.49 ± 0.01	2.90 ± 0.32	0.17 ± 0.02[a]
550°C BC Amended (60t ha^{-1})	0.47 ± 0.02	2.17 ± 0.07	0.22 ± 0.01[b]

Lal and Saxena (1982) first commented that if the possibility of co-contamination exists, microbe communities may shift in structure such that previously rare bacterial species may out-compete other bacterial strains and increase in abundance. Chen et al. (2001) suggested that pesticides differentially influence the viability of specific groups of organisms, leading to dominance of some species of microorganism over others in a contaminated ecosystem. The increase in dehydrogenase activity and the decrease in organochlorine levels that were observed as a function of biochar treatment may be a consequence of changing microbial community structure that favours organochlorine degradation, or simply by an activation of the existing microbial community.

5.6.3 Key Organochlorines Degraded as a Function of Biochar Amendment

Biochar significantly decreased the concentration of HCH in soil although the extent of degradation varied as a function of specific isomer. Two formulations of HCH were used during sheep dipping, technical HCH (a commercial mixture of main isomers) and lindane (Calvelo Pereira et al., 2008). It is known from manufacturing formulations that lindane and the alpha HCH isomer are the most readily degraded in aerobic environments, while other HCH isomers are more persistent due to their lipophilic nature and stable equatorial position of chlorine atoms (Doesburg et al., 2005; Rodriguez et al., 2004; Rubinos et al., 2007). This may explain why lindane concentrations decreased significantly compared to the more chemically persistent delta and beta HCH isomer.

In this research, one objective of adding biochar to historically contaminated dip soil was to stimulate microbial activity for the degradation of organochlorines. Lindane and alpha HCH decreased in concentration and DDT and its breakdown derivatives also exhibited reductions. Associated with these decreases was a significant increase in dehydrogenase activity that was used as an index of microbial activity and activation. Biochar may have been selecting for certain species of microorganisms that are known to degrade organochlorines such as the Chloroflexi. This could potentially decrease remediation times through amendment of contaminated soils with biochar as

microorganisms known for degradative potential of other key HCH isomers and organochlorines could be selected for. The bacterial phyla composition that was observed is in agreement with Sutton et al. (2013) who analysed the microbial community composition and diversity at a diesel-contaminated railway site by pyrosequencing bacterial 16S rRNA gene fragments. Sutton et al. (2013) described relatively high abundances of Proteobacteria, Firmicutes, Actinobacteria, Acidobacteria and Chloroflexi bacterial phyla in all samples. In the current study Chlorofexi were observed to be present in the co-contaminated sheep-dip soil (**Table 5.4**).

Microbial degradation of organochlorines occurs via four main pathways (Lal & Saxena, 1982; Lu et al., 2010): reductive dechlorination, dehydrochlorination, oxidation and isomerisation. Each of these pathways occurs through soil microbiological activity, however different species and densities of microorganisms will lead to the degradation of organochlorines at different rates. For example, the yeast (fungus) *Saccharomyces cerevisiae* has been observed to degrade isomers of lindane more efficiently than other species (γ gamma<β beta<α alpha) (Rice & Sikka, 1973a; Rice & Sikka, 1973b). In this study, biochar amendment caused a shift towards a number of soil bacterial genera that have been used around the world as bioremediation agents. In a study carried out by Saimmai et al (2012), the genus Chryseobacterium was used to degrade oil hydrocarbons and resulted in 40.5% oil-degrading activity within 7 days of application. Select species of Flavobacterium have been used in batch reactions to completely degrade the organochlorine PCP (pentachlorophenol) at concentrations of 30 and 50 mg l^{-1} (Lo et al., 1998). In the sheep dip soil the proportion of these bacteria in the microbiological community was significantly increased as a result of biochar amendment, along with Dyadobacter which has the recorded ability to degrade a wide range of organic compounds and hydrocarbons in soil (Krieg, 2010).

With respect to DDT degradation, a DDE:DDT ratio of 20:1 has been proposed by Elliott et al (1994) to define a soil that has not received DDT input for a period of 20 years. A high ratio therefore indicates active degradation of DDT in soil. We recorded a DDE:DDT ratio of 0.16 for the control soil in our study, and this ratio was significantly

increased ($P<0.05$) for three of the four biochar treatments (**Table 5.8**). The generally low ratios for all soils of this study suggest that degradation of DDT has been historically inhibited, and this may be a consequence of low microbial activity due to the presence of the co-contaminant arsenic. This scenario is in agreement with Gaw et al. (2006) who used the DDE:DDT ratio to quantify the magnitude of copper co-contamination and its effect on degradation. They reported that 4,4'-DDT is degraded to 4,4'-DDE by a number of biotic and abiotic reactions within the soil (Zwieten et al., 2003; Gaw et al., 2006) and the presence of copper as a co-contaminant has been reported to inhibit the natural degradation pathway of DDT to DDE, possibly by inhibiting the biological activity that is responsible for degradation. In this study the DDE:DDT ratio increased as a function of biochar amendment to soil and it is proposed that this is direct evidence of both a biochar-promoted increase in organochlorine-degrading microbial activity, as a result of the amelioration of the co-contaminant effect of arsenic.

The presence of co-contamination at historic sheep dips (arsenic) may cause significant selection pressure on soil microbes and prevent the breakdown of organochlorines like DDT. The observed reduction in water soluble arsenic immediately after amending soil with biochar may have decreased toxicity pressure on some soil microorganisms. The onset of a significant increase in dehydrogenase activity was correlated to this reduction in water soluble arsenic.

5.6.4 Possible Mechanisms for Organochlorine Reduction

This data shows that amendment of contaminated soil with biochar caused a significant decrease of lindane and alpha isomers compared to the control. This degradation is proposed to be a result of increased microbial activity in the soil (as quantified by an increase in soil dehydrogenase activity) due in part, to an initial reduction in water-extractable arsenic and a shift at the genus level for a number of bacteria.

While variance was found between individual biochar treatments and the control soil, few systematic differences in the bacterial communities between the treated and

control soils at the phylum level were found (**Table 5.4**). This suggests that biochar treatment had minimal effect on community composition at the phylum level, but caused significant shifts at the genus level within phyla, yet preserved the relative proportions of the different phyla.

Although a shift in a number of genus and orders was observed as a result of biochar amendment and that have bioremediation properties for the degradation of organic contaminants such as Streptophyta (550°C biochar) (Subashchandrabose et al., 2013) and Pseudomonadales (350°C biochar) (Leigh et al., 2006), the co-contaminated soil (including the control) found at this dip site contained other known bioremediation agents. The phyla Chloroflexi, genus Luteibacter and Burkholderia were represented in the microbial community and are known for their efficiency at degrading polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs). All have the natural ability to breakdown organochlorines such as HCH isomers found at high levels within this contaminated soil yet it is their proposed increased microbial activity under biochar amendment that they have likely played a significant part in the degradation of these isomers.

5.7 Conclusion

Co-contamination of soil with organic and inorganic chemicals at sheep dip sites is a long lasting environmental issue. The work in this thesis has shown that after decades of existence, soil extracted from these sites is still highly contaminated with arsenic and a range of organochlorine compounds. Under controlled glasshouse conditions the use of biochar as a soil amendment led to an increase in soil microbial activity and ultimately the degradation of a number or organochlorines. This follows a reduction in water-extractable arsenic which is proposed to decrease selective pressure on soil microbes, allowing for an increase in microbial activity. Once re-solubilisation had occurred, increased soil microbial activity was resilient towards soluble arsenic. The use of biochar made from willow feedstock resulted in a 25% reduction in DDT (and its products), a 75% decrease in lindane and 80% reduction in alpha-HCH levels in the soil 6 months after soil amendment. Other known organochlorines were not significantly

affected by biochar amendment. These results suggest that the use of biochar can stimulate the activity of existing microbial communities and cause small increases at the genus level of known organic contaminant bioremediaters, leading to degradation of certain organochlorines.

CHAPTER 6 : RESPONSE OF PLANT AND SOIL MICROBES TO BIOCHAR AMENDMENT OF AN ARSENIC-CONTAMINATED SOIL

IN REVIEW : JOURNAL OF AGRICULTURE, ECOSYSTEMS AND ENVIRONMENT

- SPECIAL ISSUE ON BIOCHAR

6.1 Abstract

The historical treatment of livestock with arsenical-based pesticides has resulted in large areas of pastoral land being highly contaminated with arsenic. This study investigated the effect of biochar on soil microbial activity and arsenic phytoextraction in an arsenic-contaminated soil during a 180 d glasshouse experiment. Biochar made from willow feedstock (*Salix sp*) was pyrolysed at 350 and 550°C (representing a low- and high-temperature biochar) and amended to soil at rates of 30 t ha^{-1} and 60 t ha^{-1} to 30 cm depth. Ryegrass (*Lolium perenne* L.) was seeded and plant growth was monitored. Soil microbial activity, quantified using the dehydrogenase assay (DHA), was significantly increased ($P<0.01$) under all biochar treatments. This increase was in excess of 100% after 30 d of treatment and was significantly higher than the control (unamended soil) throughout the trial for 350°C amended soils. The increase for the 550°C amended soils relative to the control was greater than 70%. No negative effect of biochar amendments on ryegrass germination was observed. Biochar promoted a 2-fold increase in shoot dry weight (DW) and a 3-fold increase in root DW after 180 d under all biochar amendments and this was attributed, at least in part, to the fertility value of biochar. By increasing dose rates of biochar amendment from 30 t ha^{-1} to 60 t ha^{-1} shoot tissue of ryegrass extracted significantly higher concentrations of arsenic ($P<0.05$). Through extrapolation, 350°C biochar amended soils was estimated to have the potential to increase ryegrass sward DW growth by 0.68 t ha^{-1} compared to ryegrass grown on unamended soils. Due to increased arsenic uptake into ryegrass tissue under biochar amendment, this would correspond to an increase in the extraction of total arsenic by 14,000 mg ha^{-1} compared to unamended soils and in doing so decrease soil remediation times by over 50%. This investigation provides insight into the beneficial attributes of biochar in contaminated soil and its potential to reduce the time needed to remediate these soils. However, more studies are needed to understand the mechanisms through which these benefits are provided.

6.2 Keywords

Biochar; Arsenic; Soil Microbial Activity; Phytoextraction; Soil Remediation; Environmental Risk

6.3 Introduction

Soil contamination is a global problem and occurs when the concentration of an element or compound in soil exceeds a natural background threshold value (Chapman, 2007). Contamination can occur through geogenic or anthropogenic processes (Beesley et al., 2011a). The agricultural development of New Zealand through the 20th Century saw the use of a range of inorganic and organic compounds as pesticides to control production-limiting insects. These pesticides included arsenicals and a range of organochlorines used specifically to control parasites on sheep. Animals would be submerged in baths containing these chemicals with the leftover solution pumped onto surrounding soil. Today an estimated 50,000 contaminated sheep dip sites exist in New Zealand with soil concentrations of arsenic reaching as high as 11,000 mg kg^{-1} to a depth of 30 cm (NZ Ministry for the Environment, 2006).

Plants have a natural ability to take up metals from soil by either passive or active means, but the technological application of this trait to remediate contaminated areas can take hundreds of years depending on the severity of contamination (Tsao, 2003; Saleh et al., 2004; Meers et al., 2008; Zhao et al., 2009). The plant species that have been most commonly used to extract arsenic from soil (phytoextraction) are arsenic hyperaccumulating ferns such as *Pteris vittata* and *Pteris cretica*. These plants have been used for arsenic remediation under both greenhouse and field conditions (Zhao et al., 2002; Xiao et al., 2008). Niazi et al. (2010) reported that hyperaccumulator ferns grown in the field at a historic cattle dip site had an arsenic concentration of more than 3500 mg kg^{-1} after 6 months of growth (Niazi et al., 2010). Ferns, however, have slow growth rates and require low light-intensity conditions to flourish. Their use in field applications may therefore be limited.

The target characteristics for a plant species being used in phytoextraction include adequate rates of growth and biomass production (including the development of root biomass) along with tolerance to the metal (metalloid) being targeted for remediation (Vamerali et al., 2010). Any strategy that can increase the rate of plant growth and that can manipulate soil chemistry and biology to increase arsenic bioavailability for plant uptake may improve the potential and timeframe for phytoremediation. It is in the context of optimised phytoextraction using a non-hyperaccumulator but high biomass and growth rate species that biochar may be a mechanism to reduce the timeframe of remediation.

Previous studies investigating the interactions between biochar and soil contaminants in a contaminated soil have focussed on metal availability and retention (Namgay et al., 2010; Beesley et al., 2011b). Less attention has been paid to the dual effect of biochar on plant growth and soil microbial activity, and the effect that these parameters may have in the stimulation of arsenic phytoextraction. Here, I investigate the influence of biochar on soil chemistry and biology when applied to a highly arsenic-contaminated soil in the presence of plants. Here, *Lolium perenne* L. (perennial ryegrass) is used as a model non-arsenic accumulator plants species. Ryegrass is a common species grown in New Zealand pastoral systems that thrives on soil with a pH between 5.5 and 7.5 (Sartie, 2006). Ryegrass is routinely used to investigate plant growth responses (germination, root and shoot growth) to changing environmental conditions, and has been used in this study to better understand plant-soil dynamics in a biochar amended soil.

The specific objectives of this study were (i) to determine whether biochar produced at 350°C and 550°C would promote arsenic uptake in *L. perenne* L., (ii) to investigate whether biochar additions to a contaminated soil would affect soil microbial activity, and iii) to ascertain the potential that biochar amendment of sheep-dip contaminated soil has to improve the efficacy of phytoextraction for soil remediation and management.

6.4 Glasshouse Setup

The trials that make up this chapter and chapter 5 were run con-currently in the same glasshouse. A bulk soil sample (300 kg) was collected to 20 cm depth from a 2 m x 2 m area where residual dip solution was identified to have been disposed of at the end each dipping activity (**Figure 4.4**). The soil was then transported back to the Plant Growth Unit, Massey University, Palmerston North in 50 kg plastic bags and stored under a black PVC sheet to prevent drying. The soil was then mixed using a nursery grade mixer with two sets of revolving sleeves that slowly fold the soil to homogenise arsenic and organochlorine contamination. The mixed soil was subsequently sieved through a 5 mm-mesh to remove constituent coarse inorganic and organic material before pot experiments were initiated.

Pot experiments were conducted under greenhouse conditions using square plastic pots measuring 20 x 20 cm and 30 cm deep, with drainage holes drilled at the base of the containers. Two doses of each biochar were selected for both glasshouse trials : 10 and 20 g biochar kg^{-1} soil, which when incorporated to 30 cm depth corresponds to a loading of 30 t ha^{-1} and 60 t ha^{-1}, respectively. Biochar was manually mixed with soil prior to filling of the plastic pots with a final weight (soil + biochar) of 4 kg. All pots were kept at 70 % water holding capacity (WHC) with the addition of distilled water and left for 1 week to incubate.

6.4.1 Ryegrass Growth Trial

Fifty (*Lolium perenne* L. cv *Nui*) seeds obtained from AgResearch Grasslands NZ (Accession No : A13509 Nil Endophyte), that were imbibed overnight in distilled water, were placed on top of soil using forceps and lightly sprayed with distilled water daily. A growth experiment was conducted over 6 months with three replicate pots per treatment. Contaminated soil that was not amended with biochar, but planted, was the control treatment in this study.

6.4.2 Soil Sampling

Soil samples were collected from every pot each month for chemical and biological analysis (T = 30 d). Samples were taken using a stainless steel corer with an internal diameter of 1 cm. These cores were analysed for microbial activity and water-extractable arsenic. Cored holes were filled with initial (control) soil and marked with a toothpick during monthly sampling. A new sampling location roughly 3 cm adjacent to the previous was used at each sampling time.

6.5 Results

Biochars used in this study have identical physiochemical properties as previously described in Chapter 5 yet their effect on soil properties and water-extractable arsenic differ due to the germination and growth of *Lolium perenne* seedlings.

6.5.1 Biochar Properties and Characteristics

Elemental analysis and the yield of the two biochars are described in **Table 5.1**. Both biochars had high pH (8.6) with yields of 25% and 27.5% for the 550°C and 350°C biochar, respectively. Organic C in both biochars was >70%, whereas inorganic C was almost negligible (<0.8%). The 350°C biochar had a higher volatile C content than the 550°C biochar (30.8% compared to 27.5%), higher atomic H/C_{org} ratio, and a lower proportion of fixed C (63.6% compared to 68.4%), as expected. The liming equivalence of biochar was 8.9% and 9.2% for the 350°C and 550°C biochars, respectively. Increased pyrolysation temperature significantly increased the specific surface area ($P<0.01$) from 6.1 $m^2\ g^{-1}$ for the 350°C biochar to 59.8 $m^2\ g^{-1}$ for the 550°C biochar. High-energy XPS spectra of fresh biochars describe the surface functionality of carbon (C1s). The XPS spectra define energy signals corresponding to four functional groups: aliphatic/aromatic (284.6eV assigned to CHx, C-C, C=C), hydroxyl and ether (285.8eV to C-OR), carbonyl (287.2eV to C=O) and carboxyl groups (288-289eV to C-OOR). Biochars had a very high proportion of aliphatic/aromatic carbon (83.56% and 87.08% for 350 and 550°C biochars) and low proportions of carboxylic and carbonyl groups (**Table 5.2**). Both biochars contained high concentrations of 1M HCl-extractable K^+ (10-12 g kg^{-1})

and Ca^{2+} (24 g kg^{-1}) and moderate concentrations of Mg^{2+} (1.5 g kg^{-1}), and SO_4-S (1.8 g kg^{-1}) while Formic-P yielded 0.7 g kg^{-1} (**Table 5.1**).

6.5.2 Effect of Biochar Amendment on Soil Nutrient Properties

The chemical properties of the control soil and the biochar-amended soils were analysed at T = 180 d (**Table 6.1**). Soil amended with both types of biochar had significantly ($P < 0.05$) higher concentrations of extractable SO_4^{2-}, K^+, Ca^{2+} and Mg^{2+} than the control soil yet Olsen P values were slightly lower. Olsen P values are consistent with fertiliser use on grazed farming land. Soil pH was significantly increased at high doses for both biochars from pH 5.6 to pH 5.8 ($P < 0.05$). Total arsenic was lower under all biochar treatments compared to the control soil yet at T = 30 d total arsenic was comparable under all treatments.

6.5.3 Effect of Biochar on Water-extractable Arsenic

All biochar treatments promoted a significant reduction in the concentration of water available arsenic in soil collected at T = 30 d(**Table 6.2**). By T = 120 d, the concentration of water-soluble arsenic had increased to be the same as that recorded for the control soil. All treatments initially (T = 0 d) had similar water-extractable arsenic concentrations.

6.5.4 Soil Dehydrogenase Activity

Dehydrogenase activity was significantly increased with the addition of both types of biochar at 30 t ha^{-1} and 60 t ha^{-1} for all time points relative to the control ($P<0.05$) (**Figure 6.1**). Dehydrogenase activity also increased with time for the control treatment. This increase was of 155 µg g^{-1} DM (dry matter) in the control soil over 180 d. Biochar (350°C) added at 30 t ha^{-1} increased dehydrogenase activity by 163 µg g^{-1} DM and 235 µg g^{-1} DM (30 t ha^{-1} and 60 t ha^{-1} respectively). Biochar (550°C) added at 30 t ha^{-1} increased by 169 µg g^{-1} DM and 60 t ha^{-1} by 175 µg g^{-1} DM. Biochar-amended soils had a significantly higher dehydrogenase activity compared to the control yet the relative rate of increase over the trial was the same for all three treatments.

6.5.5 Ryegrass Germination

Total germination of ryegrass seeds was not significantly affected by biochar treatments ($P<0.01$) (**Figure 6.2**). The control soil had a final germination rate of 93% while the biochar treatments had a germination rate ranging from 93-97%. Germination started 3 d after imbibition and reached 60% after 6 d for all treatments. Both biochars at 60 t ha^{-1} caused a decrease in germination percentage over the period 6 – 13 d relative to the control. A similar decrease was apparent for the 350°C biochar at 30 t ha^{-1}. However, by day 15 the cumulative germination rate was the same for all treatments.

Table 6.1. Elemental analysis of soil characterisation after 180 d as a function of biochar treatment (mean *n* = 3 ; ± s.e.). Cations have been depicted as extractable cations (meq/100g).

	pH	Olsen P µgP/g	SO4 µgS/g	K me/100g	Ca me/100g	Mg me/100g	Na me/100g	CEC me/100g	Total As (mg kg^{-1})
Control Soil (T = 0)	5.6	48.8 ± 0.4	13.2 ± 0.5	0.7 ± 0.1	3.5 ± 0.4	1.2 ± 0.1	0.4 ± 0.1	12 ± 0.0	202 ± 3.3
Control Soil	5.6	48.6 ± 0.3	12.5 ± 0.7	0.7 ± 0.0	3.5 ± 0.0	1.0 ± 0.0	0.3 ± 0.0	12.0 ± 0.0	200 ± 2.3
Soil + 350°C BC (30t ha^{-1})	5.6	47.9 ± 2.1	16.0 ± 0.3	1.0 ± 0.0	4.6 ± 0.2	1.1 ± 0.0	0.3 ± 0.0	16.0 ± 0.6	198 ± 4.5
Soil + 350°C BC (60t ha^{-1})	5.8	47.6 ± 2.1	25.7 ± 2.3	1.3 ± 0.0	5.8 ± 0.2	1.3 ± 0.0	0.4 ± 0.0	19.0 ± 0.6	197 ± 0.4
Soil + 550°C BC (30t ha^{-1})	5.6	48.4 ± 0.8	18.5 ± 0.6	1.0 ± 0.0	4.9 ± 0.1	1.1 ± 0.0	0.4 ± 0.0	15.0 ± 0.3	197 ± 3.2
Soil + 550°C BC (60t ha^{-1})	5.8	49.3 ± 1.3	19.0 ± 4.3	1.2 ± 0.1	5.9 ± 0.1	1.2 ± 0.0	0.3 ± 0.1	17.0 ± 1.0	196 ± 3.2

Table 6.2. Water extractable arsenic (mg L^{-1}) in soil as a function of biochar and control treatment. Arsenic concentration is obtained by pre-reducing samples and analysed by FIAS-GFAAS. Significant differences are denoted by different letters at P<0.05 (mean *n* = 3 ; ± s.e.). Soil pH values are listed in italics and in brackets for each treatment.

	T = 0	**T = 30**	**T = 60**	**T = 90**	**T = 120**
Control Soil	1.3 ± 0.16^{a} (*5.7*)	1.4 ± 0.11^{a} (*5.8*)	1.2 ± 0.13^{a} (*5.7*)	1.3 ± 0.08^{a} (*5.8*)	1.4 ± 0.00^{a} (*5.8*)
350°C BC (30t ha^{-1})	1.4 ± 0.10^{a} (*6.0*)	0.8 ± 0.03^{b} (*6.0*)	1.1 ± 0.04^{a} (*5.8*)	1.2 ± 0.04^{a} (*5.9*)	1.4 ± 0.02^{a} (*5.9*)
350°C BC (60t ha^{-1})	1.2 ± 0.22^{a} (*6.2*)	0.9 ± 0.08^{b} (*6.0*)	1.1 ± 0.02^{a} (*6.0*)	1.2 ± 0.04^{a} (*5.9*)	1.4 ± 0.05^{a} (*6.1*)
550°C BC (30t ha^{-1})	1.3 ± 0.05^{a} *(6.1)*	0.9 ± 0.09^{b} *(5.9)*	1.2 ± 0.03^{a} *(5.9)*	1.1 ± 0.07^{a} *(5.8)*	1.3 ± 0.01^{a} *(5.9)*
550°C BC (60t ha$^{-1)}$	1.4 ± 0.15^{a} *(6.1)*	1.0 ± 0.16^{b} *(6.1)*	1.1 ± 0.06^{a} *(5.9)*	1.3 ± 0.14^{a} *(5.8)*	1.5 ± 0.05^{a} *(6.0)*

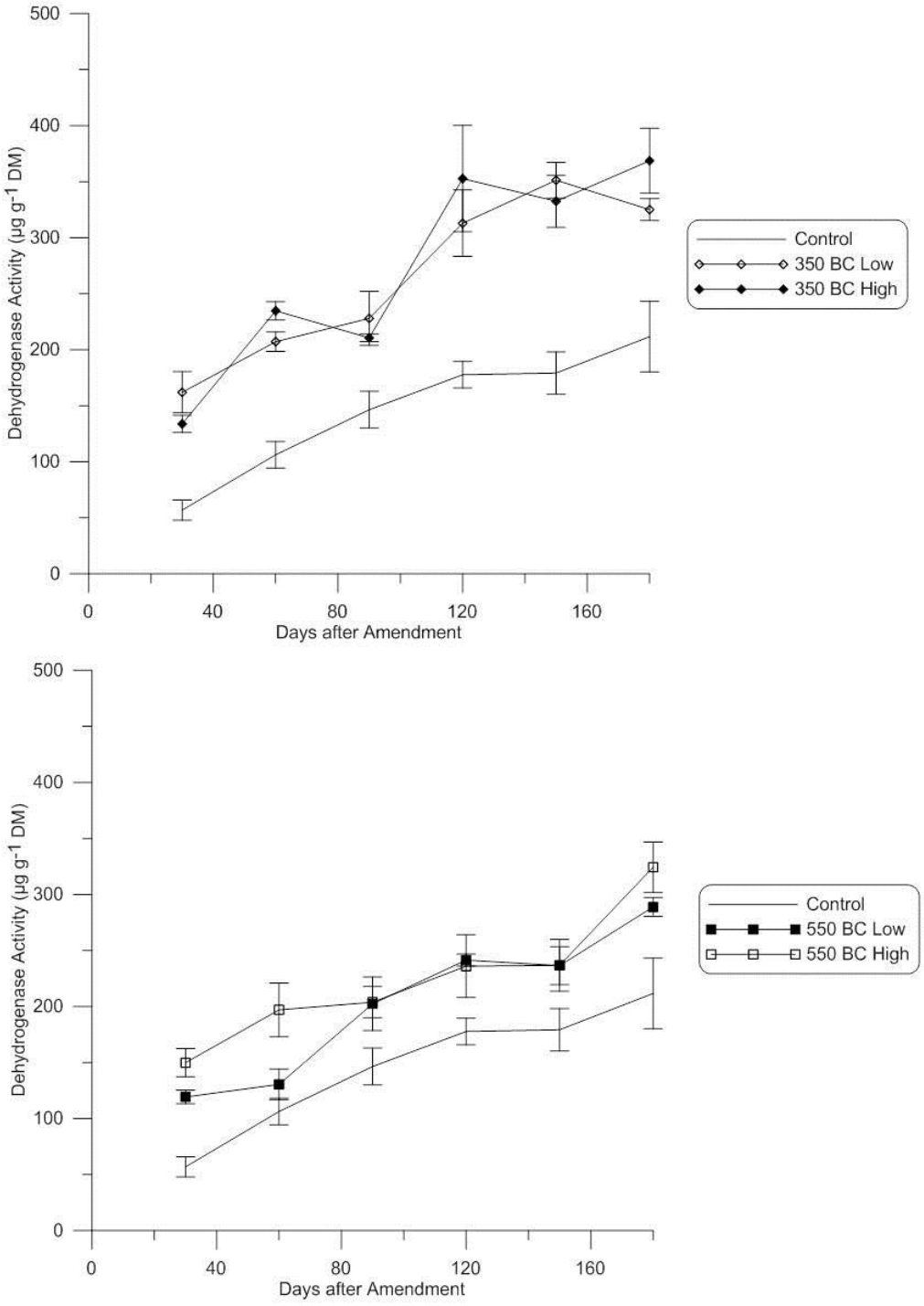

Figure 6.1. Soil dehydrogenase activity measured in µg per g of dry matter (DM) as a function of 350°C and 550°C biochar treatment (mean *n* = 3 ; ± s.e.).

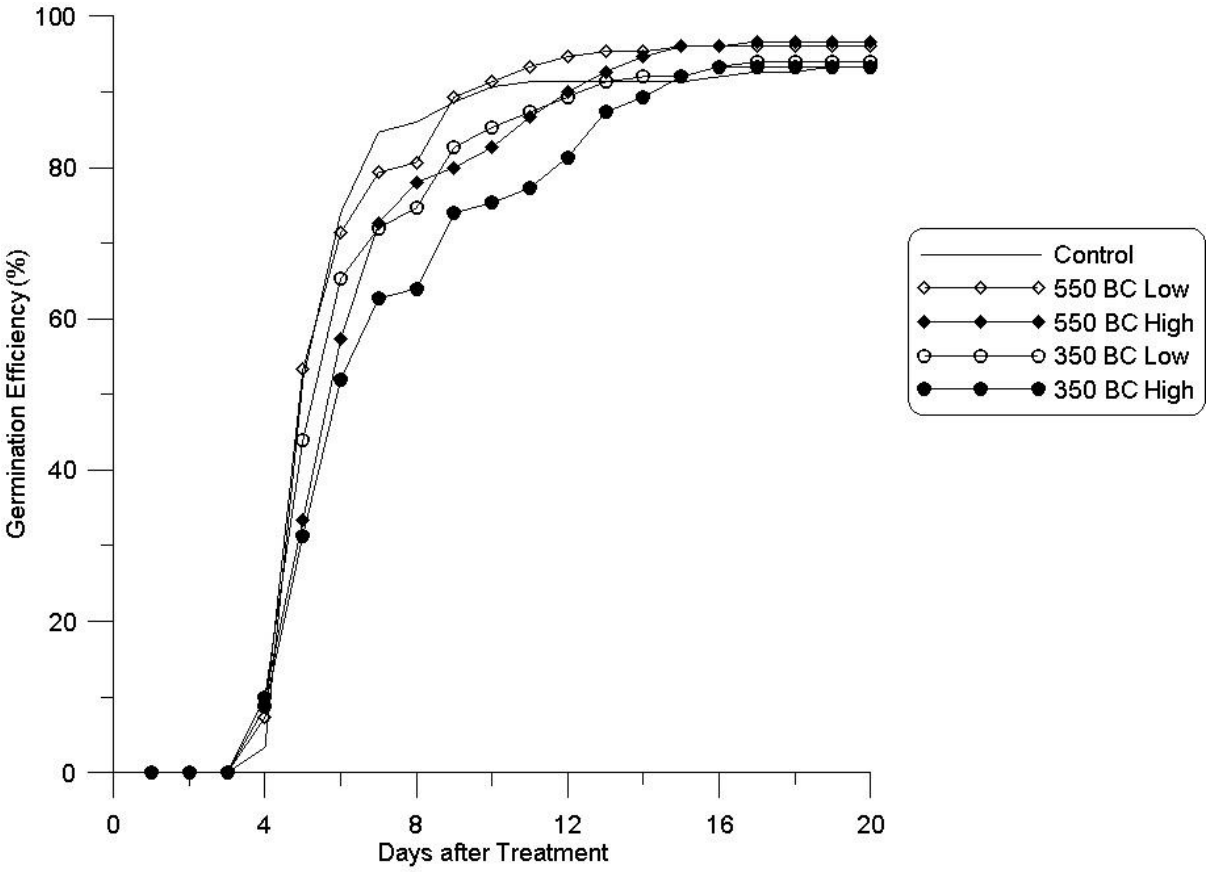

Figure 6.2. Ryegrass seed germination (%) between 0 and 20 d after seeding onto soil as a function of biochar treatment (30t ha^{-1} and 60t ha^{-1}) . The control soil is soil not amended with biochar.

6.5.6 Ryegrass Dry Weight (biomass)

Lolium perenne L. root and shoot dry weight (biomass) was significantly increased for all biochar treatments relative to the control ($P<0.01$ and <0.05 respectively) (**Figure 6.3**), although no significant differences were observed between the four biochar treatments. In general, both biochars nearly doubled total shoot DW regardless of dosage with 350°C biochar providing more evident changes in DW. Biochar (550°C)

resulted in an increase in shoot DW to 6.05 g and 5.48 g per pot for 30 t ha^{-1} and 60 t ha^{-1}, respectively. Shoot biomass for the control treatment was 3.67 g per pot. Shoot DW for the 350°C biochar treatments was slightly higher compared to the 550°C treatments with biomass values of 6.43 g and 6.16 g recorded for 30 t ha^{-1} and 60 t ha^{-1} respectively. However, these results were not significantly different (<0.05) between the biochar treatments. Root DW for the control treatment (4.5 g per pot) was nearly three-fold lower than for the biochar treatments. Root DW biomass for the biochar-amended soil was 10.8 g and 11.2 g for the 350°C biochar (30 t ha^{-1} and 60 t ha^{-1}) and 11.1 and 11.0 g for the 550°C biochar (30 t ha^{-1} and 60 t ha^{-1} respectively).

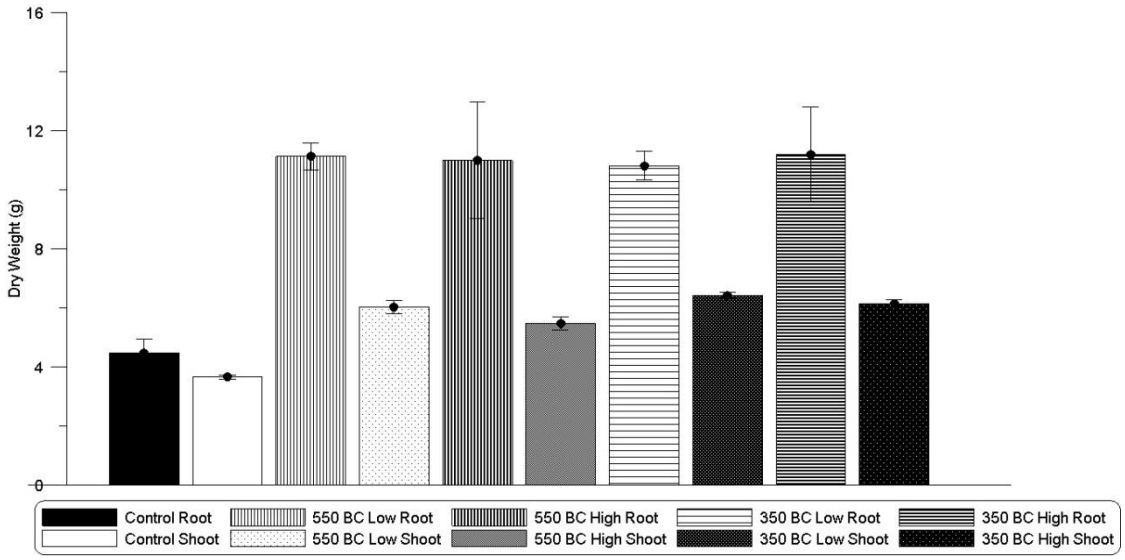

Figure 6.3. Averaged ryegrass DW (g) yield at harvest (T = 180 d) as a function of biochar treatment (mean *n* = 3 ; ± s.e.).

6.5.7 Ryegrass Tillering

Ryegrass tillering was significantly increased by all biochar treatments (P<0.05) (**Figure 6.4**). The control pots yielded an average 89 tillers per pot. At 30 t ha^{-1} for the 550°C

biochar treatment, each pot yielded 113 tillers and 107 tillers for 60 t ha^{-1} although no significant difference was noted between the rates of 550°C biochar. For soil amended with 350°C biochar, pots yielded 120 tillers and 140 tillers for 30 t ha^{-1} and 60 t ha^{-1}, respectively. A significantly greater tillering production was observed for the 350°C biochar treatment at a dose of 60 t ha^{-1} compared to the other three biochar treatments ($P<0.05$).

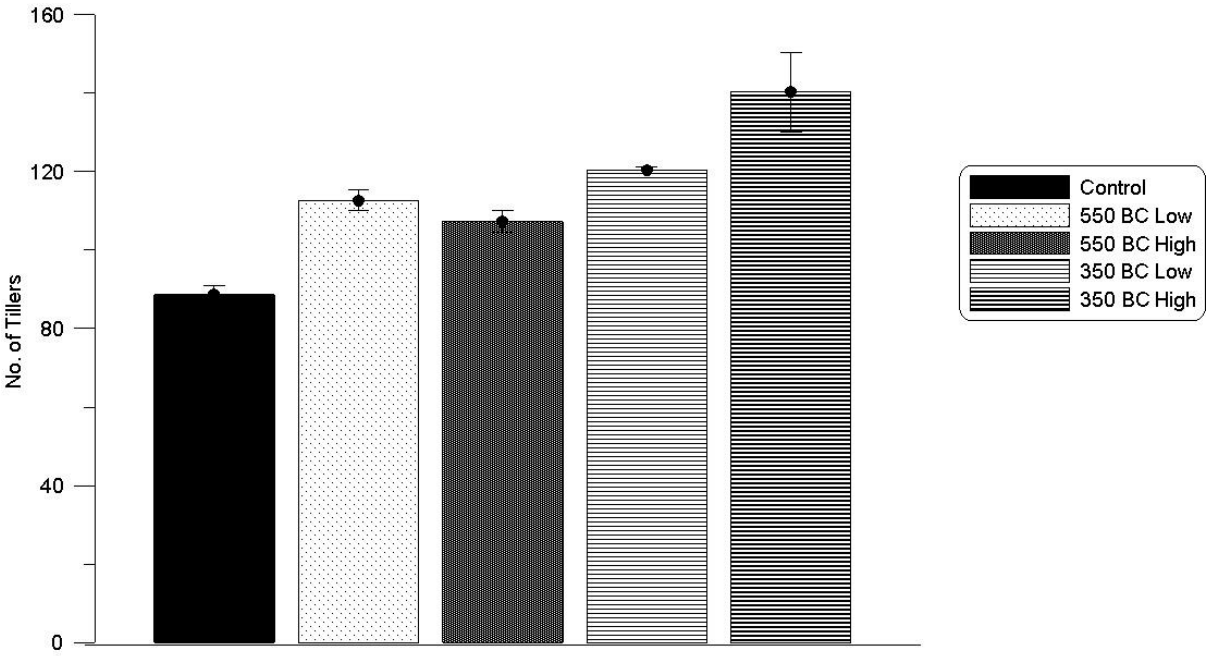

Figure 6.4. Average number of ryegrass tillers per pot as a function of biochar treatment (mean $n = 3$; ± s.e.).

6.5.8 Arsenic Concentration in Ryegrass Shoot Biomass

The arsenic concentration in ryegrass shoot biomass was significantly increased as a function of both biochar treatments at 60 t ha^{-1} relative to the control (**Figure 6.5**).

However no change was apparent for biochar applied at a rate of 30 t ha^{-1} compared to the control ($P<0.05$). The maximum concentration of shoot arsenic was 15.3 mg kg^{-1} for the 60 t ha^{-1} application of 350°C biochar. This was an increase of 33% over the control concentration (11.5 mg kg^{-1}). The element composition was also increased in ryegrass shoot tissue under biochar amendment. Both Na^{+} and Ca^{2+} concentrations were increased in ryegrass tissue but these concentrations were not significant ($P>0.05$) compared to control grown ryegrass (Appendix A).

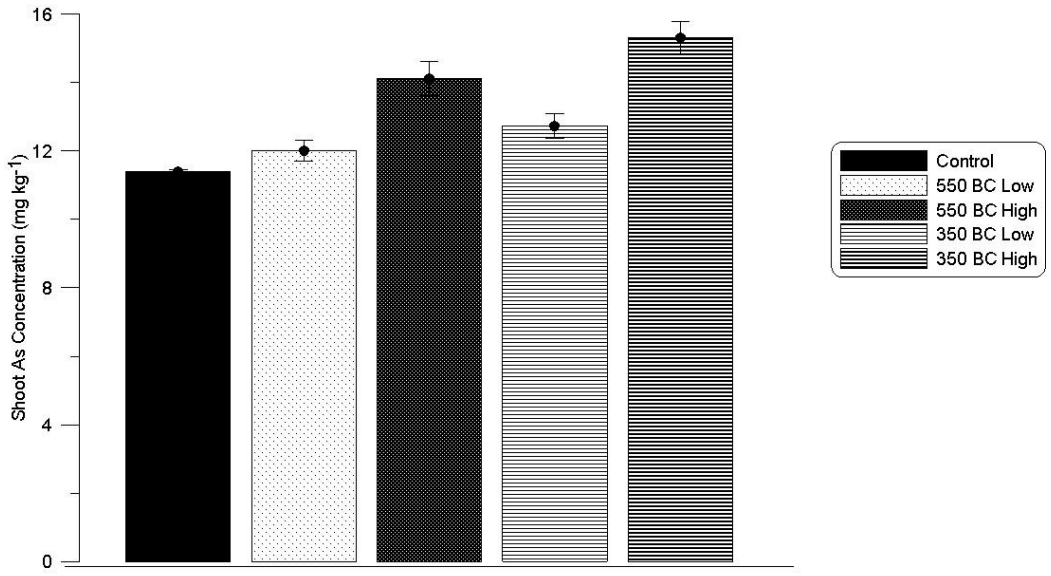

Figure 6.5. Total average arsenic concentration (mg kg^{-1}) in ryegrass shoot biomass as a function of biochar treatment (mean n = 3 ; ± s.e.).

6.6 Discussion

In this study a description on the specific effects of biochar on both soil properties and plant response to arsenic in the soil is presented. Biochar technology was initially developed as a means to sequester carbon in the soil for long periods of time in order to offset the effects of increasing carbon dioxide emissions and to improve soil fertility

(Kookana et al., 2011; Lehmann and Joseph, 2009). However, in recent years many researchers have observed that biochar addition to soil may effect environmental improvement within contaminated soil as a result of changes to plant-soil-microbial interactions (Beesley et al., 2011a; Beesley et al., 2011b; Hartley et al., 2009).

6.6.1 Biochar Stimulates Soil Dehydrogenase Activity

Quantification of total soil microbial activity was estimated by using the dehydrogenase assay to assess whether biochar amendment caused a change in the environmental toxicity of arsenic, as proposed by Chandler and Brooks (1991) when working with lead-contaminated soils. The addition of biochar pyrolysed at 350°C and 550°C caused a significant increase in DH activity relative to the control, but there was no difference between the two dose treatments used (30 t ha^{-1} and 60 t ha^{-1}). Low temperature (350°C) biochar has a greater labile fraction of carbon (C) than the high temperature biochar, as inferred from its volatile fraction, which can be used as an energy source by soil microorganisms. Increased energy supply may stimulate microbial activity leading to a higher soil dehydrogenase activity. However, the lack of differences between the two types of biochar suggests that other factors may be also influencing microbial activity. A greater root development, as quantified by root biomass, in the presence of biochar may have affected microbial activity through a greater input of root exudates into the rhizosphere.

Microbial activity in a contaminated environment can be negatively affected by the chemicals that constitute contamination. Despite being non-target organisms of pesticide applications, soil microbes are affected by dip chemicals once added to the soil. Pesticides such as arsenicals reduce soil enzyme activity which is commonly used as a key index for soil health (Antonious, 2003; Monkiedje et al., 2002) and can have a detrimental impact on the makeup of soil microbial communities that exist in and around a dip site (Pampulha & Oliveira, 2006; Zhou et al., 2006; Klose & Ajwa, 2004). A short term reduction to the water-extractable concentration of arsenic in soil may have decreased toxicity pressure on some soil microorganisms. The onset of a

significant increase in dehydrogenase activity was correlated to this reduction in water soluble arsenic (**Table 6.2, Figure 6.1**).

This reduction could be attributed to the precipitation of arsenic with metal cations, such as Ca^{2+}, from soluble salts found in the initial ash fraction (e.g., chlorine and sulphate salts). Once these arsenic salts dissolved with time, the arsenic becomes then available for plant extraction (Beesley et al., 2011a,b). Furthermore, as the incubation proceeded, the liming effect of the amendment may have become more evident thus increasing arsenic solubilisation along with an increase in soil pH. Specific adsorption of arsenic onto the non-weathered biochar surface cannot be discarded, although the positive charge on biochar surface is generally low (Liang et al., 2006).

6.6.2 Manipulation of Ryegrass Growth with Biochar Additions

In this study, biochar made from willow feedstock did not affect the extent of final germination but did slow the germination rate relative to the control for three doses between 6 - 13 d. Germination was scored at the first sign of radicle emergence and we observed that some seeds were placed directly on top of biochar chips. However, after 18 d the extent of germination for the biochar treatments was the same as that for the control. This research, thus shows that the effect of biochar on plant germination varies in the short term (<15 d) but is not significantly beneficial or detrimental in the long term (>20 d). This effect is in agreement with Free et al. (2010) who studied maize germination in soil amended with biochar. However, variation in germination is likely to be a function of plant species and soil type. Biochars have been suggested to contain phytotoxic compounds that may decrease overall seed germination (Kookana et al., 2011) or contain organic compounds such as butenolide that can trigger germination (Dixon, 1998). Biochars derived from papermill waste (applied at 10 t ha^{-1}) have been shown to improve germination in wheat (Van Zwieten et al., 2010) and lettuce (*Lactuca sativa*) when using a sorghum feedstock (Keller et al., 2010) while biochar made from wheat chaff did not affect wheat seed germination (Solaiman et al., 2012). The results of this study suggest that biochar made from willow feedstock does not negatively affect germination rate of *Lolium perenne*.

The nutrient value of biochar is strongly associated to the type of feedstock and the pyrolysing temperature used (Wang et al, 2012a; Wang et al, 2012b). Soil amended with both types of biochar in our work had significantly ($P<0.05$) higher concentrations of SO_4^{2-}, K^+, Ca^{2+} and Mg^{2+} when compared with the control soil. This is related to the element loading of the soils with biochar which contain a relatively high concentrations of both available K^+ and Ca^{2+} but also moderate concentrations for available Mg^{2+} (**Table 5.1**). In this study, addition of biochar to soil caused an increase in both root and shoot DW after 180 d of growth. This increase can be partly correlated to the nutrient composition of biochar **(Table 5.1).** However, increased soil fertility may also promote increased activity of soil microorganisms that are responsible for element cycling and transformation. Some soil microbes such as the plant growth promoting rhizobacteria (PGPR) are able to fix nitrogen and solubilise unavailable forms of nutrient such as phosphate, making these more available for plant uptake (Ambrosini et al., 2012; Harel et al., 2012). The extent to which such soil bacteria may constitute increased dehydrogenase activity is unknown, but genetic fingerprinting of the abundance of soil microorganisms in the biochar-treated soils relative to the control soils has been completed (Chapter 5) and has determined that the structure of the soil community has shifted (specifically under 350°C biochar amendment) towards microorganisms such as the PGPR.

6.6.3 Biochar Increased Arsenic Uptake in Ryegrass Shoot Tissue

Biochar addition to soil led to an increase in the arsenic concentration of ryegrass shoots relative to the control. This can be attributed to an increase in overall arsenic bioavailability as a function of soil alkalinity due to biochar amendment (**Table 6.1 and 6.2**), despite the initial reduction in water-soluble arsenic concentration detected. The liming effect of biochar and resulting pH increase plays a major part in arsenic mobility within the soil (Frost & Griffin, 1977; Masscheleyn et al., 1991; Mahimairaja et al., 2005; Carrillo-Gonzalez et al., 2006; Hartley et al., 2009; Beesley et al., 2011a; Namgay et al., 2010). The addition of biochars slightly raised soil pH over the six-month experimental period and this was attributed to the liming effect of biochar as both

biochars contained a lime equivalence of greater than 8.8%. This effect was more evident in the 60 t ha^{-1} treatments.

Conflicting results are reported in literature regarding the effect of biochar on arsenic mobility, but there is consensus that biochar will change the chemistry of arsenic in soil. This change may be mediated through change in redox status of the soil or by an effect on soil pH (e.g. liming effect) (Joseph et al., 2010). Microbial activity within the soil can also affect both the redox status of the soil and arsenic speciation. Recent studies have provided insights into a change in arsenic speciation from arsenate to arsenite leading to greater bioavailability (Bolan et al., 2012). In contrast to the results reported in this chapter, Beesley and Marmiroli (2011) showed that adding a hardwood biochar pyrolysed at 400°C to a soil (pH 6.2) increased the concentration of pore water arsenic in a multi-element contaminated soil.

Research into the effect of biochar-arsenic interactions on metal uptake by plants has also provided contrasting views. Both Hartley et al (2009) and Namgay et al (2010) observed no significant increase in arsenic uptake in *Miscanthus* sp. and *Zea mays* respectively when soils were amended with biochar. However, the fact that the soil used by the former was naturally alkaline (pH 7-8) may have masked the effect of biochar amendment.

6.6.4 Application of an Integrated Biochar and Phytoextraction Strategy to Remediate Sheep Dip Sites

Environmentally-friendly technologies that not only reduce the impact of a contaminant on the environment but that also have beneficial additional effects such as increasing soil functions are becoming popular with the general public and researchers (Adriano et al., 2004; Pilon-Smits et al., 2006). The tandem use of biochar and phytoremediation has been shown here to provide benefit for the remediation of soil contaminated with arsenic. Under controlled glasshouse conditions this study has shown that biochar amendment can have a number of interacting beneficial effects in a contaminated soil that ultimately has the potential to decrease soil remediation time using techniques that can be applied *in situ*. Here, we have shown that a coupled

biochar-phytoextraction system can both stimulate plant growth and also the uptake of arsenic across root and into shoot tissue.

The potential benefit of integrating biochar with phytoextraction can be quantified through consideration of the biomass and arsenic uptake data that we have recorded. Extrapolation of the recorded shoot biomass shows that amendment of soil with 350°C biochar (at 60 t ha^{-1}) can increase DW yield from 0.95 t ha^{-1} (control) to 1.63 t ha^{-1}. Total arsenic removal from soil can be determined by multiplying these biomass yields by the arsenic concentration in the above-ground biomass. Using the data recorded in our study, total arsenic removal from the control soil is 10,900 mg ha^{-1}, but this increases to 24,900 mg ha^{-1} for the 350°C biochar amended soil (60 t ha^{-1}). Our calculations show that this increase would lead to a 56% reduction in the timeframe needed to remediate soil to a defined target.

Despite the figures presented in the above paragraph, we do not propose that the phytoextraction of arsenic could be realistically achieved using ryegrass; this is neither a hyperaccumulator nor a high biomass species. We have used ryegrass in this work as a model plant species to better understand plant-soil dynamics in a biochar amended soil. Biochar would, however, lead to real increases in the efficiency of phytoextraction where the same biochar-promoted biomass and arsenic concentration effects were apparent in known hyperaccumulator or high-biomass species. We propose that the costs of biochar would not preclude the usefulness of the described scenario. Arsenic (at high concentrations) at dip sites usually occupies an area of about 2,500 m^2. Assuming a cost for the preparation and incorporation of biochar to soil of $400/t (Jones, 2013) then the additional cost of using biochar at a rate of 30 t ha^{-1} as part of a remediation option would be approximately $3,000 per site.

Nonetheless, the extent to which the same effect as that observed here can be achieved by adding lime and fertilisers instead of biochar needs to be investigated. To fully understand biochar amendment effects, the use of controls that mimic biochar (i.e soil amended with a liming agent with the same liming equivalence as biochar) should be investigated to determine biochars' unique properties (Jeffery et al., 2013).

If the biomass and arsenic concentration can be similarly manipulated without the use of biochar, then this may be a more feasible route to enhance phytoextraction efficiency. The extent to which the effects we have observed are attributable to biochar is an area that needs further validation. Moreover, it has yet to be seen what effect these type of biochars will have on arsenic mobility and phytoextraction under field conditions when using high biomass crops like willow (*Salix sp*.).

The design and utilisation of a remedial plan for arsenic using a biochar – phytoextraction system is aimed at being carbon negative and creating zero waste. Under this scenario, plant biomass is harvested and chipped on site. The chipped contaminated biomass is then transported to an on-site pyrolyer that contains a filter system to capture volatilised arsenic, with the clean biochar returned to the soil (Gregory, 2013). This would create a closed loop system where carbon is being stored in the soil while providing a means for the sustainable remediation of the contaminated site and would provide an end-use for the arsenic-contaminated plant material.

6.7 Conclusion

We have shown that in a highly arsenic-contaminated soil, microbial activity, soil-arsenic dynamics, and plant growth can be both manipulated with the additions of biochar. Using an indicator plant species, *Lolium perenne* L., significant changes in soil-arsenic dynamics in relation to the bioavailability of arsenic for phytoextraction were observed. Extrapolation of our data infers that soils amended with 350°C biochar (60 t ha^{-1}) have the potential to yield increased ryegrass sward DW growth by 0.68 t ha^{-1} compared to ryegrass grown on unamended soils. This represents an increase of 14,000 mg ha^{-1} of arsenic being extracted compared to unamended soils. Biochar amended soils also evidenced noticeably increased soil microbial activity. Increases in both plant growth and soil microbial activity may be due to the inherent fertility of biochar (K^{+}, Ca^{2+}, Mg^{2+}) and to the labile C fraction of biochar that can be used as a microbial food source. More research is needed to develop greater mechanistic understanding of the soil-plant processes that are associated with the amendment of

soil with biochar, as well as to investigate whether the use of alternative soil amendments (e.g., lime, fertilisers, organic wastes) would lead to similar results.

CHAPTER 7 : FIELD AMENDMENT OF ARSENIC CONTAMINATED SOIL WITH BIOCHAR: EFFECT ON MICROBIAL ACTIVITY AND PHYTOEXTRACTION

IN REVIEW : JOURNAL OF AGRICULTURE, ECOSYSTEMS AND ENVIRONMENT

7.1 Abstract

A field trial was setup on a dip site in New Zealand which was operational from 1860-1980. Replicate plots with relatively homogenous arsenic concentration were delineated, and soil was amended with 350°C and 550°C willow-derived biochar at a rate equivalent to 30 t ha^{-1} to a depth of 30 cm. The fern *Pteris cretica* L. and high biomass tree species *Salix sp.* were planted and represented different treatments along with ryegrass a pasture species (representing three individual potential phytoextraction species). Soil microbial activity (analysed as dehydrogenase activity, DHA) and arsenic chemistry in soil was monitored over 180 d, and the extent of arsenic phytoextraction was quantified at the end of the trial. Biochar treatment did not significantly ($P<0.05$) stimulate soil DHA relative to the control. However, a reduction in microbial activity was correlated with decreasing soil moisture content over the duration of the trial. The concentration of water-soluble arsenic did not change at any sampling point during the trial. The arsenic concentration in plants at the end of the trial was increased in biochar-amendment plots but the result was only significant for willow leaf and *P.cretica* L. stem amended with 550°C biochar, and for willow stem amended with 350°C biochar ($P<0.05$). When data was extrapolated to predict results of a long-term field trial and scale under willow treatment (stem) it was calculated that over 67.7 g of arsenic could be extracted in soils amended with 350°C biochar compared to 5.9 g extracted under control treatment. This could result – assuming a similar rate of extraction with time – in levels of arsenic concentration in soils reaching background concentrations in as little as 6 years. This would result in a decrease in remediation time frame compared to no soil-biochar amendment under these field conditions of 92%. These results highlight the potential of this willow biochar for the extraction of metalloids from contaminated soil by manipulating both plant and soil characteristics to improve phytoextraction efficiency. However, more research is needed to fully understand the mechanisms through which biochar enhance arsenic uptake by plants. A system analysis study on the feasibility of this technology would also be needed before it is considered as a commercially viable option for soil remediation.

7.2 Keywords

Biochar; Arsenic; Soil Microbial Activity; Phytoextraction; Timeframe for Soil Remediation

7.3 Introduction

Biochar is generated through the pyrolysis of biomass and the stability of biochar in the environment results from its recalcitrance against decomposition based on condensed aromatic ring structures (Liang et al., 2006). However, biochar can also contain more easily degradable carbon structures (Lehmann, 2007; Nguyen & Lehmann, 2009) referred to as labile C, which are more abundant when feedstock is pyrolysed at low temperatures. These labile forms of C can be utilised as energy and C sources by some soil microorganisms. Therefore, any soil amendment that increases labile C may promote increased microbial activity in soil.

In the context of sheep-dip sites this might be useful as soil microbes have the ability to degrade organic pollutants such as organochlorines which are often present as co-contaminants in historic sheep dip soils (Edvantoro et al., 2003; van Zweiten et al., 2003; Warnock et al., 2007). These co-contaminants where toxic to plants can limit biomass production. Certain soil microbes may also oxidise arsenite to arsenate which utilises phosphate transporters for plant uptake and thus enhance the phytoextractability of arsenic. This mechanism usually involves co-transport of phosphate and/or arsenate with proton exchange governing the transport across the root plasma lemma (Zhao et al., 2008).

High temperature biochars have a higher ash content and therefore a greater liming effect than low-temperature biochars (Glaser et al., 2002; Uchimiya et al., 2011). Once incorporated into soil, high-temp biochars may raise soil pH (Beesley et al., 2010), and this can affect arsenic bioavailability in soil as pH is a key variable controlling arsenic-soil solution concentration (Beesley et al., 2011b).

Biochar amendment of soil therefore has the potential to increase the efficacy of phytoextraction by two mechanisms. Low temperature biochar may increase soil

microbial activity, and subsequently increase arsenic bioavailability through biological pathways as well as enhance the degradation of co-contaminants that may restrict plant growth. Alternatively, high temperature biochar may increase soil pH and subsequently increase arsenic bioavailability through chemical pathways. When coupled at a contaminated field site, biochar and phytoextraction may reduce the length of time necessary to meet remediation goals.

For the current work plots of contaminated soil at a historic sheep dip site were amended with biochar produced at two temperatures. Changes in soil chemistry and biology as function of time and amendment were then investigated. Specifically, the objectives of this study were to investigate whether the amendment of a willow biochar produced at 350°C and 550°C to a contaminated soil would (i) affect soil microbial activity, and (ii) promote arsenic uptake in *Salix sp*. (willow) a high biomass crop and a naturalised arsenic hyperaccumulating fern *Pteris cretica* L.. The purpose of the work described in this chapter was to investigate the extent to which biochar amendment of soil can reduce the time needed to mitigate arsenic environmental risk at the site under study. No attempts were made to investigate changes in organochlorine concentrations, although previous studies on the same soil have shown a significant reduction in organochlorine soil concentration that was attributed to an increase in microbial activity as a function of biochar amendment to soil (Chapter 5).

7.4 Field Trial Setup

To design a statistically relevant trial, detailed soil sampling was first conducted over a 100 m^2 area of soil that had been previously identified as contaminated with arsenic (see Chapter 4 : Site Characterisation) and Chapter 7 for detail.

7.4.1 Soil Sampling

Soil samples were collected from every plot at the establishment of the experiment, and 30, 90 and 180 d thereafter for chemical and biological analysis. Samples were taken using a stainless steel corer with an internal diameter of 1 cm. These cores were analysed for microbial activity and water-extractable arsenic. To ensure sampling

points did not overlap, cored holes were filled with initial (control + no biochar) soil and marked with a toothpick during sampling. A new sampling location was randomly chosen per plot during each sampling period.

7.5 Site Selection and Pre-Treatment

Field research was conducted at a historic sheep dip site in Te Mahia, Hawkes Bay, New Zealand. To design a statistically relevant trial, detailed soil sampling was first conducted over a 100 m^2 area of soil that had been previously identified as contaminated with arsenic (Chapter 3). Soil cores (to a depth of 30 cm) were taken every 1 m inside a 10 x 10 m grid and analysed for total-soil arsenic (**Figure 7.1**). The 100 m^2 area was then cleared manually of vegetation using a mechanical excavator with 1 m wide bucket to a depth of 5 cm (the use of herbicides was unwanted at this stage due to possible negative influences on soil microbial activity). Three grids (3 m x 2.5 m) were constructed inside this 10 x 10 m area in areas selected for arsenic homogeneity (representing 3 blocks and setup to be a replicated block experiment). Each grid was thus designed to contain one replicate of each of 12 treatments (biochar and plant combinations).

Soil in each grid was homogenised to a depth of 30 cm with a mechanical rotary hoe to ensure homogenous arsenic concentrations throughout the soil profile. Each grid was then further divided into 12 squares measuring 0.5 x 0.5 m representing an area for each treatment (a 0.2 m gap between treatments was utilised to prevent crossover of treatments) which in total made 36 sub-plots (**Figures 7.2A-E**). This 0.2m gap between treatments was put in place to separate teatments and developing root zones over a period of 6 months, yet would need to be increased if the field trial was designed to run for longer periods. Each sub-plot was randomly allocated to a treatment using a random number generator. Soil was amended with 350°C biochar and 550°C biochar at 30 t ha^{-1} (representing a low and high temperature biochar respectively), or received no biochar amendment (control soil). For the sub-plots receiving biochar, pits measuring 0.5 x 0.5 m and to a depth of 0.2 m were dug and the soil removed and placed in 60 L plastic bins. Biochar (1 kg representing 30 t ha^{-1}) was added to the soil

and mixed by hand and then placed back into the pits using a spade. Control sub-plots not receiving biochar were treated the same.

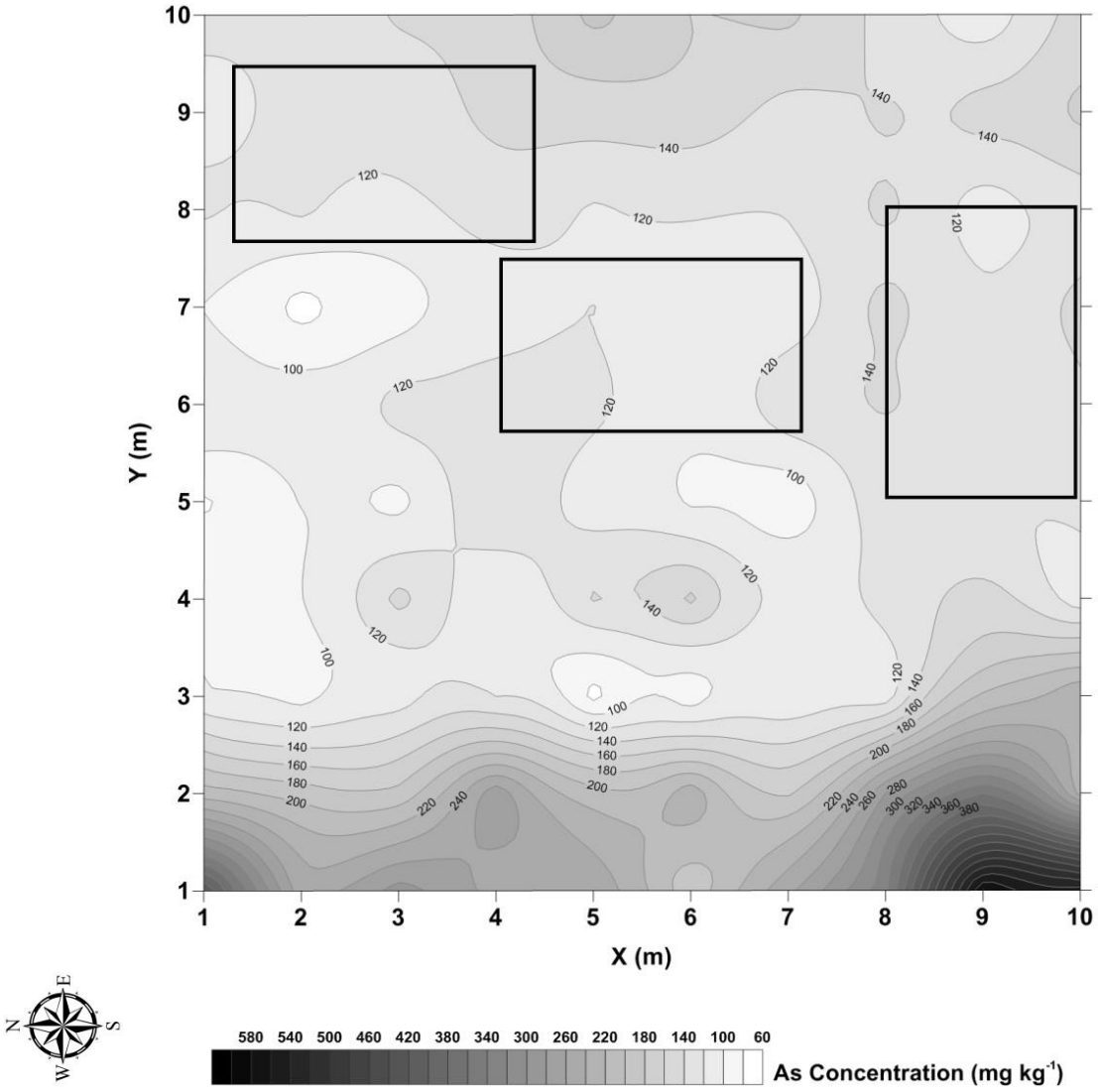

Figure 7.1. 2-D isocontour map depicting the heterogeneity of soil arsenic within the 10 x 10 m grid localised to the left of the actual dip. High arsenic concentrations (mg kg^{-1}) are relative to the proximity from the dip itself which is situated to the west. The three plots used for the trial are situated to the east

Figure 7.2A. Observation of the initial field trial setup **T = 1 d**. The initial three grids can be observed

The field plot assessed the effect of each treatment on arsenic uptake by three plants species; willow (*Salix sp*), fern (*P. cretica* L.) and ryegrass (*Lolium perenne* cv Nui). Plant species were individually planted in their own plots. Two willow poles (*Salix sp*. 1.5 m length) were planted to a depth of 20 cm in relevant plots and were spaced 15 cm apart. Ryegrass seeds (Accession No: A13509 Nil Endophyte) (250 per plot) were placed over the surface of the soil and covered lightly (<2 mm) with soil. Mature ferns were planted in duplicates per plot. *Pteris cretica* is a naturalised fern in New Zealand but is susceptible to high light intensity. Fern material was harvested after 60 d as fern fronds became sunburnt/scorched with the onset of summer conditions. Total willow growth in the field was estimated using key woody biomass indicators for small trees as reported in Ritson & Sochacki (2003). The field trial ran for 180 d (6 mo) from late winter to mid-summer. Due to insect and pest herbivory damaging both ryegrass shoot material and disturbing the rooting zone in soil no further analysis was carried out for ryegrass plots in regards to plant growth and arsenic uptake.

Figure 7.2B. Observation of the initial field trial setup **T = 1 d**. Randomisation can be observed in one of the three grids showing sub-plots.

Figure 7.2C. Observation of the field trial setup **T = 30 d**

Figure 7.2D. Observation of the field trial setup **T = 90 d**

Figure 7.2E. Observation of the field trial setup **T = 180 d**

7.6 Results

7.6.1 Biochar Properties and Characteristics

The biochar used for the field trial differed slightly from that used for the glasshouse trials. The elemental composition and yield of the two biochars is summarised in **Table 7.1**. Each biochar had a high pH; 8.1 and 9.0 for 350°C and 550°C biochar, respectively. As the amount of inorganic C was less than 0.5 % (data not shown), total C was considered organic C (C_{org}). The 350°C biochar had a higher volatile C content than the 550°C biochar (26.9% compared to 25.6%) but the fixed C content was the same for the two biochars (66.6%). The ash content of the 350°C biochar (6.5%) was lower than that for the 550°C biochar (7.9%). The molar H/C_{org} ratio decreased as the temperature of pyrolysis increased (**Table 7.1**). The liming equivalence increased with pyrolysis temperature from 9.0% to 12.0% for the 350°C and 550°C biochar respectively. Increased pyrolysed temperature also significantly increased the surface area of the 550°C biochar compared to the 350°C biochar ($P<0.01$). This increase for the high temperature biochar relative to the low temperature preparation was considerable (37.1 and 0.1 $m^2\ g^{-1}$ respectively). The surface functionality of carbon (C1s) from high energy resolution XPS spectra of fresh biochars showed signals that could be attributed to one of four groups: aliphatic/aromatic groups (284.6eV assigned to CHx, C-C, C=C), hydroxyl and ether groups (285.8eV to C-OR), carbonyl groups (287.2eV to C=O) and carboxyl groups (288-289eV to C-OOR) (**Table 7.2**).

7.6.2 Effect of Biochar on Selected Soil Properties (water-soluble arsenic, soil pH)

Soil pH and the concentration of water-soluble arsenic was determined for the control soil and the biochar-amended soils containing willow at T = 0 d and T = 180 d (**Table 7.4**). Soil amended with both biochars had a higher pH ($P<0.05$) compared to the control at the end of the trial (T = 180 d). Soil pH of the control did not change (pH 5.97) significantly over 180 d yet there was a substantial increase under both biochar amendments. There was no difference in soil pH between the two biochar treatments at the end of the trial (0.28 and 0.29 pH units for the 350°C and 550°C biochar, respectively). The percentage of soil arsenic that was water-soluble did not change

over the duration of the trial as a function of either time or biochar amendment to soil (**Table 7.4**).

Table 7.1. Physio-chemical characterisation of biochars

Parameter	350°C Biochar	550°C Biochar
Feedstock	Salix sp. (1 – 2 y.o)	Salix sp. (1 -2 y.o)
Pyrolysis Temp (°C)	350°C	550°C
pH	8.1	9.0
Organic C Content (C_{org} %)[a]	76.5	73.1
N (%)	1.2	1.3
S (%)	0.39	0.21
O (%)[b]	12.3	14.8
H (%)	3.1	2.7
Volatile C (%)	26.9	25.6
Atomic H/Corg	0.45	0.41
Ash Content (%)	6.5	7.9
Liming Equiv (%)	9.0	12.0
Surface Area (m^2 g^{-1})	0.1	37.1
1M HCl-extractable K (g kg^{-1})	11.0	14.2
1M HCl-extractable Ca (g kg^{-1})	15.2	24.5
1M HCl-extractable Mg (g kg^{-1})	1.3	1.9
1M HCl-extractable SO_4-S (g kg^{-1})	0.3	1.4
2 % Formic acid-extractable P (g kg^{-1})	0.5	0.7

[a]Biochars contained < 0.8 % C_{inorg} thus C_{org} represents total C. [b]Estimated by difference O = 100 – (C + N + S + Ash).

Table 7.2. Chemical composition of carbon (C1s) determined through high-energy resolution XPS analysis of fresh biochar particles (T = 0 d).

	C1s Composition (%)			
	CHx, C-C, or C=C	**C-OR**	**C=O**	**C-OOR**
350°C BC	81.8	13.3	2.8	2.1
550°C BC	88.4	8.2	2.2	1.2

The binding energy of C1s at 284.6eV was assigned to CHx, C-C, C=C; at 285.8eV to C-OR; at 287.2eV to C=O; and at 288-289eV to C-OOR.

Table 7.3. Selected physio-chemical properties of control soil (T = 0 d). (mean *n* = 36 ; ± s.e.)

	Soil Properties
CEC (meq/100g)	20.1 ± 0.1
Olsen P (µg g^{-1})	205.6 ± 3.2
SO_4 (µg g^{-1})	14.1 ± 4.4
Ca (mg kg^{-1})	842.4 ± 10
K (mg kg^{-1})	380.1 ± 12
Mg (mg kg^{-1})	108.7 ± 8
Na (mg kg^{-1})	74.5 ± 6

Table 7.4. Effect of biochar on soil pH and water-extractable arsenic. Significant differences are denoted by different letters at P<0.05 (mean *n* = 12; ± s.e.)

	Soil pH	**Soil pH**	**H_2O Extractable Arsenic (%)**	**H_2O Extractable Arsenic (%)**
	T = 0 d	**T = 180 d**	**T = 0 d**	**T = 180 d**
Control Soil	5.95^{a} ± 0.11	5.97^{a} ± 0.06	1.40^{a} ± 0.01	1.42^{a} ± 0.01
350°C BC Amended	5.96^{a} ± 0.06	6.25^{b} ± 0.03	1.40^{a} ± 0.00	1.39^{a} ± 0.11
550°C BC Amended	5.93^{a} ± 0.12	6.27^{b} ± 0.03	1.40^{a} ± 0.02	1.46^{a} ± 0.16

7.6.3 Soil Dehydrogenase Activity

There was no significant effect of biochar on soil dehydrogenase activity (P<0.05). After 30 d, control soils exhibited a 12 μg g^{-1} dry matter (DM) decrease in DHA compared with T = 0 d and averaged a 24 μg g^{-1} DM reduction in total activity at every sampling point up to 120 d (**Figure 7.3**). After 180 d control soils exhibited an increase of 47 μg g^{-1} DM compared with T = 0 d. In comparison, biochar amended soils exhibited increases of 12 and 14 μg g^{-1} DM for the 350°C and 550°C biochar treatments after 30 d respectively. A similar decrease in total DHA at T = 90 d and 120 d treatment yet 550°C amended soils exhibited a slight increase in DHA of 5 μg g^{-1} DM at 120 d. This trend continued at 180 d with increases of 103 μg g^{-1} DM in the 350°C soil and 63 μg g^{-1} DM in the 550°C soil. Results were not significant (P<0.05) at any time point when comparing different treatments.

7.6.4 Effect of Biochar on the Arsenic Concentration in Plant Tissue

Biochar amendment had a differential effect on the arsenic concentration in leaf and stem biomass at the end of the experiment (T = 60 d for the ferns; T = 180 d for the willow) (**Figures 7.4 and 7.5**). There was no significant difference between the

concentration of arsenic in willow leaf biomass for the control and 350°C biochar treatments (0.28 and 0.28 mg kg^{-1} respectively, **Figure 7.4**). However, the 550°C biochar treatment promoted a significant increase ($P<0.05$) in the arsenic concentration of the willow leaf biomass relative to the control (0.38 mg kg^{-1} and 0.28 mg kg^{-1} respectively).

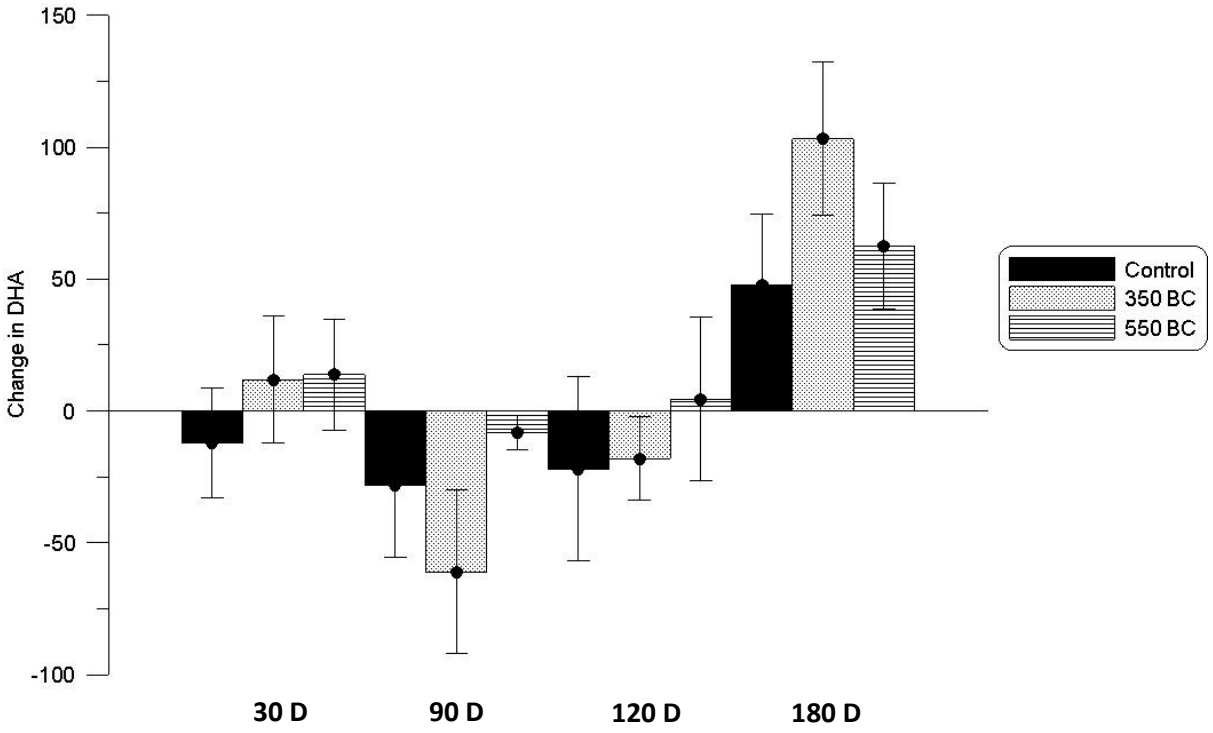

Figure 7.3. Change in soil dehydrogenase activity (DHA) in µg g^{-1} DM (dry matter) between sampling points for the three treatments. Changes are relative to the previous sampling point (mean *n* = 12 ; ± s.e.)

In contrast, 350°C biochar promoted a significant increase ($P<0.05$) in the arsenic concentration of the stem biomass compared to control and 550°C biochar treatments. The arsenic concentration of the control willow stem was 0.11 mg kg^{-1} while that grown on soil amended with 350°C and 550°C biochar was 1.27 and 0.14 mg kg^{-1} respectively (**Figure 7.4**). Total willow biomass did not differ between treatments at

the end of the trial (180 d) and therefore biochar was inferred to have no stimulatory effect on plant growth (total stem DW ranged from 5.5 kg to 6 kg).

Pteris cretica L., in comparison, exhibited a high concentrations of arsenic in both lamina and stem tissue (**Figure 7.5**) relative to willow (**Figure 7.4**) for all treatments. Differences in arsenic concentration of *Pteris cretica* L. were apparent between the two biochar treatments and the control, with concentrations significantly increased for the 550°C biochar treatment relative to the 350°C treatment. Leaf concentrations reached 18.1 mg kg^{-1} for 550°C treatment and were significantly higher ($P<0.05$) than the control (13 mg kg^{-1}) and 350°C amendment (8.1 mg kg^{-1}). Higher concentrations were also noted in stem material (10.5 mg kg^{-1} and 7 mg kg^{-1} in 550°C amended soils

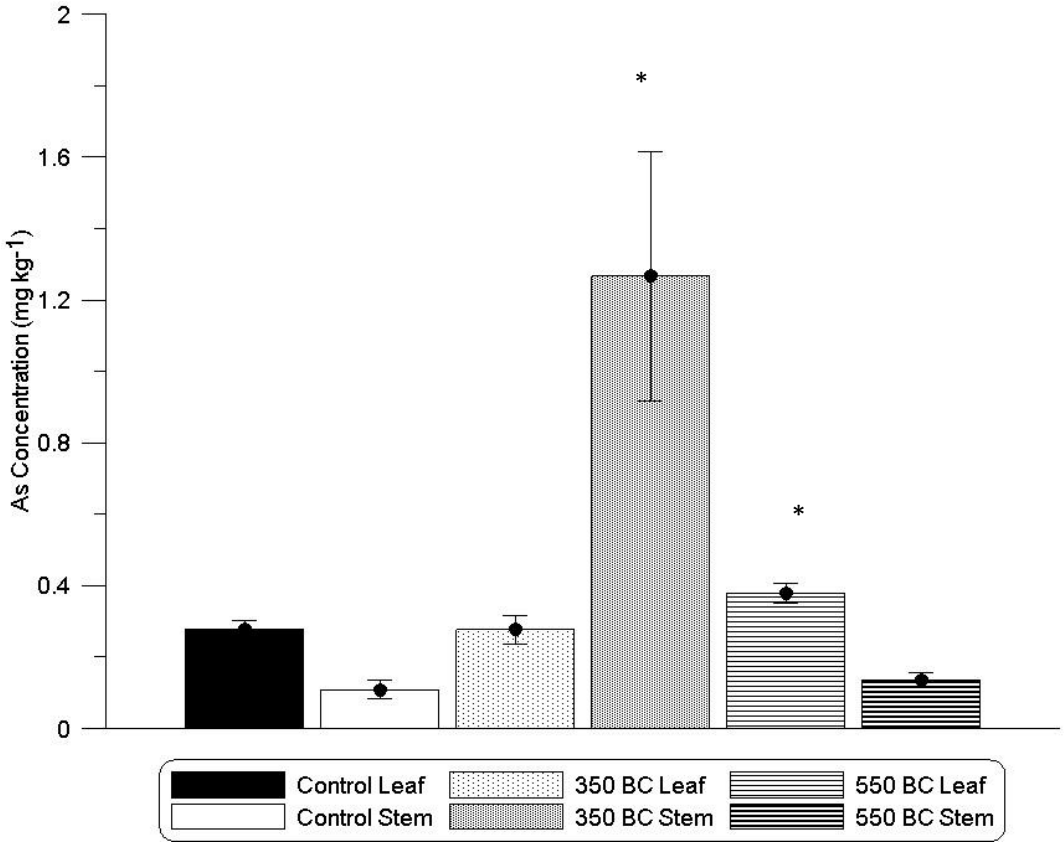

Figure 7.4. Total mean arsenic concentration (mg kg^{-1}) in willow leaf and stem biomass as a function of biochar treatment. * Denotes a significant difference ($P<0.05$) between treatments (mean n = 12 ; ± s.e.)

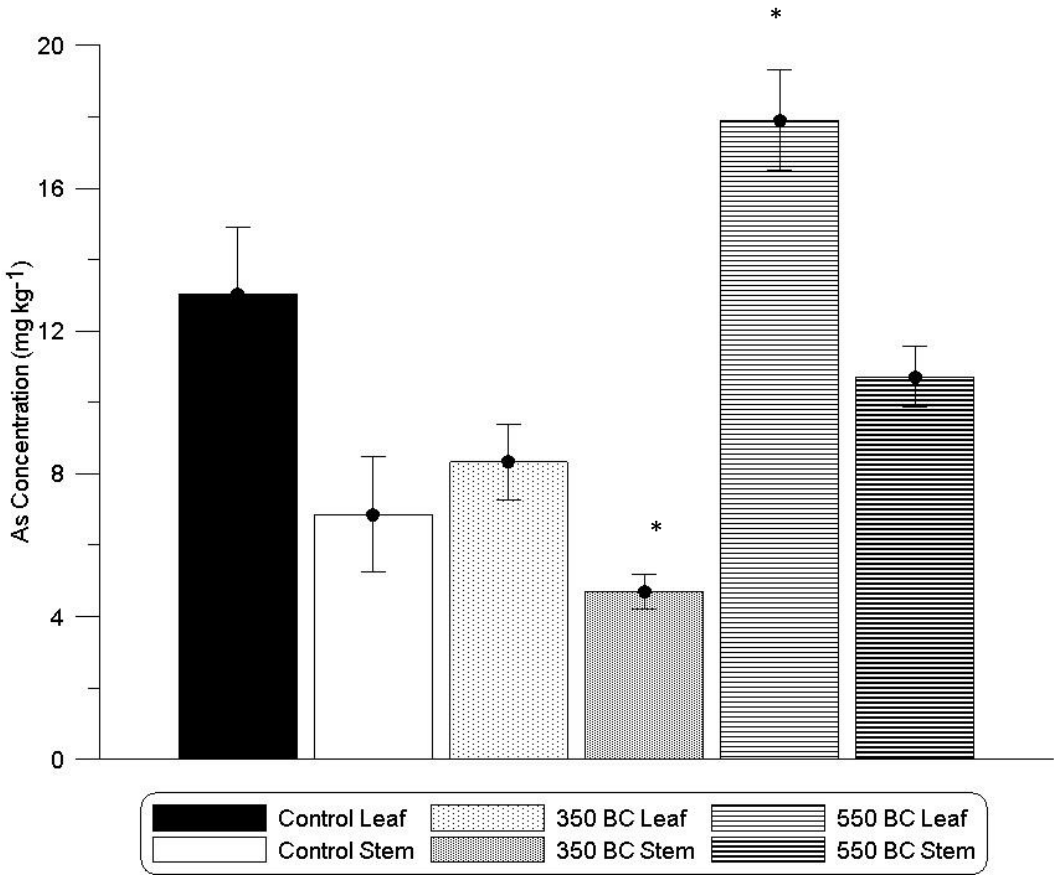

Figure 7.5. Total mean arsenic concentration (mg kg^{-1}) in *Pteris cretica* leaf lamina and stem biomass as a function of biochar treatment. *Denotes a significant difference ($P<0.05$) between treatments (mean $n = 12$; ± s.e.)

and control soils respectively) with a decreased concentration noted in 350°C amendment compared to the control (4.2mg kg^{-1}).

7.7 Discussion

7.7.1 Distribution of Arsenic at the Site under Study

At dip sites the total concentration of soil arsenic can vary greatly due to the heterogeneous distribution of this contaminant in the environment (**Figure 4.5-4.7, 7.1**). Heterogeneous arsenic distribution is primarily related to stock management and

the methods used to dispose of dip solution at the end of a dipping event. Soil arsenic concentrations were elevated at the site where the dip bath and associated scooping mound were positioned (west of **Figure 7.1**) and decreased with increasing distance away from the actual dip. Soil sampling further away from the dip site showed an average background level of arsenic at this site of 30 mg kg^{-1}, and therefore any concentrations above 30 mg kg^{-1} could represent contamination (Chapman, 2007). The soil arsenic concentration within the three blocks ranged from 55 – 91 mg kg^{-1} whereas the soil arsenic concentration ranged from 73 – 582 mg kg^{-1} within the 10 x 10 m grid. A lower concentration for the planted grids relative to the range of concentrations defined for the 10 m x 10 m grid can be explained by soil mixing to a depth of 30 cm. The original values stated in **Figure 7.1** are of cored values to a depth of 20 cm. Mixing effectively diluted the total soil arsenic concentration to below the values reported during the original grid sampling. Contaminated soil from sheep and cattle dipping has shown heterogeneity of arsenic with distance from the actual dip and with soil depth (van Zweiten et al., 2003; NZ Ministry for the Environment; Chopra et al., 2007). Within a 10 m radius, soil arsenic concentrations between sites can range from 200 to 11,000 mg kg^{-1} (NZ Ministry for the Environment).

7.7.2 Changes of Soil Microbial Activity

There was no significant difference in soil microbial activity during our trial that could be attributed to the treatments. However, microbial activity varied over time, and we attribute this variation to be a response to fluctuation in soil moisture content over the 180 d of the trial. Soil dehydrogenase activity decreased as a function of decreasing soil moisture content (**Figure 7.3 and Table 7.5**). Analysis of weather patterns during the field trial indicated that rainfall over this period was lower than usual and correlates with the soil moisture content determined for soil collected at each sampling time (**Table 7.5**).

Table 7.5. Analysis of climate parameters in the area (Mahia, New Zealand) including temperature (max, min °C) and rainfall (mm) during the time period of the field trial (mean *n* = 36 ; ± s.e. for soil moisture)

	July **T = 0 d**	**August** **T = 30 d**	**October** **T = 90 d**	**November** **T = 120 d**	**January** **T = 180 d**
[a]**Temperature °C (min-max)**	1 – 19	2 – 19	3 – 27	6 – 28	8 – 32
[a]**Rainfall (mm)**	234	120	14	0	34
Soil Moisture Content (%)	30 ± 0.01	35 ± 0.02	23 ± 0.02	15 ± 0.01	13 ± 0.01

[a]**Source : www.metservice.com/towns-cities/Gisborne**

Between day 30 and 120, the moisture content of the control soil reduced by 66% and this was correlated (r^2=0.85, P<0.05) with a 25% decrease in microbial activity. However, no significant relationship was apparent for the biochar-amended soils. There was a 58% reduction in soil moisture content but 6% increase in microbial activity for the 350°C biochar treatment. For the 550°C biochar treatment there was a 63% reduction in soil moisture content and 18% reduction in microbial activity. Soil amended with biochar retained more soil moisture content over the trial period than the control soils, and this is in agreement with previous studies (Herath et al., 2013). However, the extent to which this affected microbial activity is unknown, as other factors such as the labile C fraction in the biochar may have also affected soil microbial activity. Controlled incubation studies carried out in a glasshouse, using the same field soil and the same application rate of biochar (30 t ha^{-1}) showed a significant increase (P<0.01) in soil microbial activity over 180 d from 105 to 215 µg g DM for amendment of soil with 350°C biochar (Chapter 5). Stimulation of microbial activity was further increased by growing the pasture species *Lolium perenne* L. (ryegrass) (Chapter 6). This discrepancy between the soil microbial activity in field and greenhouse studies is likely

to be a function of the reducing soil moisture content of the field soil over the spring and summer of 2012.

7.7.3 Changes in Plant-Arsenic Dynamics

The addition of biochar to the field site caused a general increase in the concentration of arsenic in the plants relative to the control treatment (willow and a fern). The increase in pH caused by biochar amendment may have enhanced arsenic mobility in soil, but properties such as metal fractions and competing and non-competing ions (e.g. phosphate) present in the biochar ash fraction may have also influenced arsenic bioavailability (Frost & Griffin, 1977; Masscheleyn et al., 1991; Mahimairaja et al., 2005; Carrillo-Gonzalez et al., 2006; Wang et al., 2012b). In general, arsenic becomes more soluble as the soil pH increases (Beesley & Marmiroli, 2011); this is mainly caused by the increase in stronger ligands (e.g., OH^-) able to displace As oxy-anions from reactive surfaces as pH increases (Cheng et al., 2008; Hartley et al., 2009; Beesley et al., 2011). However, biochar did not increase (or decrease) the net water soluble arsenic concentration in soil (**Table 7.4**). The existence of other mechanisms that may have affected the arsenic concentration in solution, such as the precipitation of soluble arsenic with constituent elements of the ash in biochar (calcium and magnesium) and plant uptake may explain these results (Chapter 6).

An increased arsenic concentration in plant biomass as a function of biochar amendment of soil was only observed for specific combinations of biochar type and plant species. For willow, the arsenic concentration was significantly increased relative to the control ($P<0.05$) in leaf tissue for the 550°C biochar and in stem tissue for the 350°C biochar. It could be presumed that this differential effect of the two biochar types indicates that 550°C biochar amendment may alter soil-arsenic dynamics for plant uptake faster as the transfer of arsenic from stem to leaf tissue has already occurred compared to that seen under 350°C biochar. Zheng et al. (2012) showed that under various biochar additions made from different feedstocks, translocation of arsenic from root to shoot in rice was significantly affected ($P<0.001$) during a contaminated soil trial. Naturally, arsenic is transferred across the soil-plant interface

mostly as inorganic arsenic (arsenite, arsenate). At the plant interface, these arsenic species are actively and passively transported across the plant membrane by a number of transport systems. Once inside the plant arsenic is reduced by arsenate reductase and complexed with phytochelatin and translocated up the stem and finally to the leaf (Fitz & Wenzel, 2002; Meharg & Hartley-Whittaker, 2002; Huang et al., 2008; Zhao et al., 2009; Ebbs et al., 2010; Indriolo et al., 2010).

7.7.4 Management and Remediation of Sheep Dip Sites using an Integrated Phytoextraction-Biochar System

Phytoextraction is considered to be an environmentally friendly and relatively cheap option for the remediation of some areas of contaminated soil (Meers et al., 2008; Zhao et al., 2009). This method is also popular among indigenous communities, such as Maori in New Zealand, whose historical perspective of sustainability governs how they control their land. However, a major drawback is the time required for remediation. As plants will only remove a small percentage of the total arsenic concentration in the soil in any plant crop, many years of cropping may be needed to reduce the concentration of arsenic to an acceptable level. Therefore, mechanisms to increase the concentration of arsenic in plants, and therefore to reduce the time needed for remediation, are needed. Amendment of soil with biochar, as investigated in this work, has potential as one mechanism to meet this objective with incorporation also providing flow-on beneficial effects such as removing carbon dioxide from the atmosphere and creating habitats from biota both above and below ground (Tsao, 2003; Doty, 2008).

Under the natural environmental conditions observed at the site calculations show that 350°C biochar treatment effected removal of 7.6 mg of total arsenic in willow stem biomass compared to 0.66 mg in control treatments (based on stem concentrations of 1.27 and 0.11 mg kg^{-1} per 6 kg DW pole). On an annual basis this extraction would be 15.2 mg and 1.3 mg for the 350°C and control treatments respectively with remediation times, assuming rates of extraction remain constant, of 5.9 years and 68.2 years for each of the treatments (based on soil with 120 mg kg^{-1} arsenic reduced to 30 mg kg^{-1}). This extraction of arsenic was estimated considering

that after each 6-monthly period, willow poles would be removed and replaced with new poles. This, however, is not the most feasible plan in the field as new plantings would need to sourced and planted, which can be time-consuming. In most cases, willow could be coppiced and allowed to regenerate, which may in itself provide a potential means for greater arsenic extraction due to increased growth. Incorporation of biochar into contaminated soil in this scenario, and under willow treatment, resulted in a decrease in remediation time of approximately 60 years.

The increase in arsenic concentration in willow grown in plots amended with 350°C biochar was small, but significant, and when extrapolated to long-term field scale, the timeframe for phytoextraction is considerably reduced. Assuming a bi-annual biomass production figure of 53 t ha^{-1}, total coppicing of a willow crop under 350°C biochar amendment could result in the extraction of 609 g of arsenic compared to 53 g under control treatment. This analysis can also be extrapolated for stem material under 550°C biochar treatment. Although stem material only reached 0.14 mg kg^{-1} under the 550°C biochar treatment, on a large scale this would allow for the total extraction of 67.2 g on a hectare basis, an increase in extraction compared to the control of 14.2 g of arsenic.

Extrapolating arsenic extraction based on nursery grown *P.cretica* L. after 6 mo in the field yielded similar results among treatments yet greater extraction was observed in the 550°C biochar treatments. Based on average DW yield of stem (10 g) and leaf (4 g) it can be assumed that willow, as a high biomass phytoextraction species, would be more efficient and viable for arsenic extraction due in great part, to its higher biomass. If the total yield of an individual fern was 14 g DW with a stocking rate of two ferns per 1.5 m^2 a bi-annual biomass production could reach 0.12 t ha^{-1}. A breakdown of total DW by stem and leaf tonnage would yield 89 kg ha^{-1} and 35 kg ha^{-1} respectively. If stem tissue alone is extrapolated for total arsenic concentration it can be assumed that 550°C biochar-amended ferns could extract 0.9 g compared to 0.6 g for control soils. With concern to leaf tissue the same two treatments would extract 0.6 g and 0.5 g for every hectare treated.

7.8 Conclusion

In this study, two plant species, a fern *P.cretica* L. and a high biomass crop, willow (*Salix sp*.) were planted and grew in contaminated soil with arsenic concentration ~120 mg kg^{-1}. Soils were homogenised and either amended with one of two biochars or left as a control (no biochar). Soil microbial activity was unaffected by biochar additions yet was highly correlated with a decrease in soil moisture content over the duration of the trial. Under field conditions both *P.cretica* L. and willow exhibited increases in arsenic extraction from the soil but this pattern of uptake was differentially expressed. Slight but significant increases in arsenic concentration were observed in willow stem for 350°C biochar amendment and *P.cretica* leaf stem under 550°C treatment compared to control soil grown ferns. These numbers were consequently extrapolated to determine if biochar amendment in a field situation was feasible for the extraction of arsenic and to decrease remediation times. Calculation by tonnage revealed that willow poles, acting as a high biomass crop, has the potential to greatly decrease remediation times by as much as ~60 years and that using a high biomass crop has greater potential than using *P.cretica* L.*,* a hyperaccumulating fern. These results have the potential to make remediation due to phytoextraction of contaminated soil a more appealing option for contaminated soil landowners.

CHAPTER 8 : ANALYSIS OF ANTIOXIDANT AND METABOLITE PROFILES IN LOLIUM PERENNE

8.1 Chapter Background

This chapter describes results from a glasshouse trial carried out in June 2012 that was designed to supplement the findings of a previous glasshouse trial carried out in May 2011 (see Chapter 6). This trial was designed to provide additional information regarding plant response mechanisms and aspects such at metabolite profiling within shoot tissue, in relation to uptake of arsenic and changes to plant growth.

8.2 Introduction to Plant Response Mechanisms to Arsenic

As described in Chapter 2 (**section 2.5 and 2.6**) arsenic is transported from the soil and into the root through a number of mechanisms including the use of plant phosphate transporters. As arsenic in a non-essential element for growth and function within plant tissue its de-toxification relies on an efficient response by the plant to complex it for eventual removal.

Due to arsenic having a similar chemical affinity to phosphate, its uptake results in a number of reactions occurring where arsenic replaces or reacts with metabolites containing phosphate. These reactions include the degradation of molecules such as ATP, DNA and proteins (Gomes et al., 2013) which are responsible for normal functioning within plant tissues.

The accepted model for the arsenic detoxification process is the use of arsenate reductase (AR) to reduce arsenate to arsenite and coupling the metalloid with phytochelatin to form a stable complex which is available for transport. Phytochelatins (PCs) are synthesized within plants from glutathione, a non-enzymatic component of the antioxidant pathway, and the newly formed PC-arsenite complexes are transported to the vacuoles. To sense arsenic toxicity, plants are reported to elicit a number of signaling mechanisms such as an upregulation of the antioxidant pathway in response to increasing ROS levels and changes to metabolite regulation. An ability to respond quickly to arsenic uptake determines whether a plant is susceptible or not to high soil arsenic levels.

8.2.1 ROS and the Antioxidant Pathway

All plants, like other aerobic organisms that have evolved in the presence of oxygen have developed processes that harness the energetic potential of this molecule (Navrot et al., 2007). Oxygen is reduced during respiration and different pathways are involved in the reduction. This reduction of oxygen can occur in a number of ways with one being a stepwise system where oxygen accepts electrons one by one and leads to the formation of a reactive oxygen species (ROS), one example being the superoxide anion (Basu et al., 2001) .

ROS are generated by aerobic metabolism and include active molecules derived from oxygen including peroxides, hydroxyl radicals, superoxide anions and hydrogen peroxide. These are formed and detoxified as a normal process during photosynthesis especially in the chloroplasts and peroxisomes. In most living cells, ROS also play a key role as signaling molecules especially in response to a number of various stresses or threats to plant integrity such as arsenic toxicity. They may act as messengers to induce gene transcription or trigger protein activation but the levels of these oxidants must be tightly controlled as increased levels may lead to cell damage (Bowler et al., 1992; Navrot et al., 2007).

However during arsenic uptake, ROS have been observed to increase to levels which can cause damage to plant integrity in a process known as oxidative stress (Goupil et al., 2009; Gunes et al., 2009; Shri et al., 2009). The accumulation of ROS can include superoxide radicals which are relatively unreactive but can form species damaging to cellular components, such as hydroxyl radicals in the presence of metals (Huang et al., 2008; Rangel et al., 2009). Hydroxyl radicals are able to react indiscriminately, causing lipid peroxidation, protein oxidation and DNA damage.

In general the activation of the antioxidant pathway follows transduction events as a consequence of ROS signaling. This pathway consists of a number of enzymic and non-enzymic components which work in concert to detoxify ROS (**Figure 8.1**).

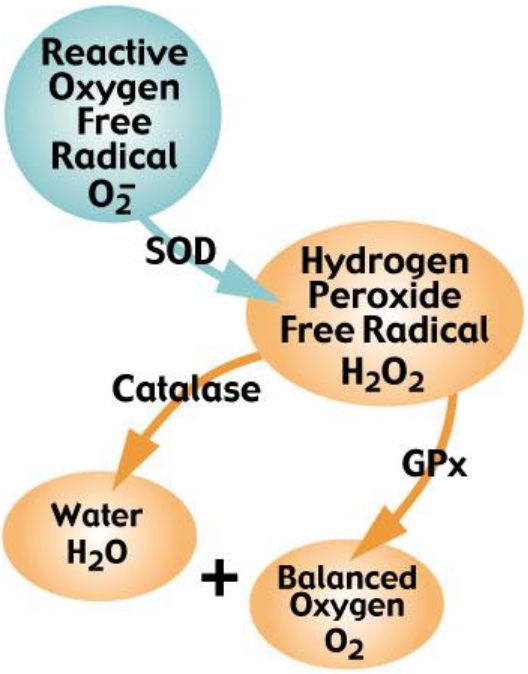

Figure 8.1. ROS and their catalysation to form water, by enzymic components.

8.2.2 Plant Metabolomics

Plant metabolomics is the scientific study of the entire plant metabolome, which represents a collection of all metabolites in a specific sample, in this case, ryegrass shoot tissue (Fraser et al., 2013). Metabolites are end products of a plant cellular process and are commonly referred to in plant based metabolomics as primary and secondary metabolites. Primary metabolites are directly involved with plant growth and development, while secondary metabolites are usually involved in ecological functioning such as pigmentation of leaves.

Currently no technology is available to analyse the entire metabolome at once, but a myriad of extraction techniques are available to detect both primary and secondary metabolites and separate them into 'streams' based on their chemical structure (Rasmussen et al., 2012). Metabolomics was developed to provide an analysis of the chemical composition of a plant and is used as a powerful tool to find desirable traits for plant breeding. It can also be used to obtain a snapshot of the effect of abiotic/biotic plant stress (Fraser et al., 2012). Therefore, the use of metabolomics as a technique to monitor a response to biochar amendment and arsenic uptake could provide insights into changes in regulation of different metabolite streams.

8.3 Materials and Methods

8.3.1 Plant Materials and Growth Conditions

Soil (see section **6.4 Glasshouse Setup**) was collected from an area identified as where remaining dip solution was disposed of and represented a contaminated soil (**Figure 4.4**). For the glasshouse trial underpinning the research in this chapter, soil was also removed from an area located 10 m to the north of the dip entrance **(designated as the uncontaminated soil, Figure 4.4)**. The glasshouse setup and associated analytical procedures are as described in **section 3.3** and **section 6.4** and sections from therein. Pots were filled with soil and seeded with ryegrass as described in Chapter 6. Each treatment consisted of 5 pots.

8.3.2 Ryegrass Harvesting

At T = 90 d and T= 180 d, the 2nd flag leaf of each individual growing seedling was removed using a scalpel and all leaves excised from all plants in each pot were combined in a sterile 15 mL falcon tube and the tissue snap frozen in liquid nitrogen and stored at -80°Cto be used for enzymatic analyses. To limit the effect of ROS fluctuations and changes in enzymatic activity due to circadian rhythm patterning, samples were harvested at midday on both occasions, and the minimum time was allowed to elapse between leaf excision and freezing. Before storage at -80°C, frozen tissue was ground down to a fine powder in a mortar and pestle using more liquid nitrogen. All protein in samples was determined as described by Bradford (1976), using bovine serum albumin (BSA) as a standard.

8.3.3 Chlorophyll Content

Chlorophyll content was analysed as described by Evans et al. (2011) but with modification. Into a 0.2 mL Eppendorf tube, 0.5 mL of 96% (v/v) ethanol (EtOH) was pipetted and 50 mg of ryegrass tissue (FW) added. Briefly, the tube was subjected to 30 sec 'pulse-spin' before being placed in the dark O/N at 4°C. The extract was then spun at 12,000 x g for 2 min and the resulting supernatant pipetted into fresh sterile eppendorf tubes and kept at 4°C.

Immediately, 2 μL of the supernatant was quantified for chlorophyll content on a 'Nanodrop' spectrophotometer (Thermo Fisher Scientific Inc.) (UV-Vis option) at 649 nm and 665 nm. Total chlorophyll was calculated using the following equation:

Equation 2 : chl a + b = (6.1(A665) + 20.04(A649))

Each sample was analysed in triplicate to gain a relative average. Results are expressed as total chlorophyll (μg mL^{-1}).

8.3.4 Enzyme Analysis

Homogenised tissue (0.1 g) was added to 1 mL of extraction buffer solution comprising 50mM K_2HPO_4 (pH 7.0), and 1mM EDTA. Samples were then centrifuged at 4°C for 15 min at 12,000 x g and the supernatant used as a crude extract of cytoplasmic fractions for the determination APX, CAT and SOD activity. The resulting pellet was extracted prior to GPX determination.

As GPX is a cell-wall protein, the pellet was subjected to multiple clean-up steps (5 washes with 1 mM DTT, with centrifugation at 12,500 x g for 5 min at 4°C after each wash, followed by 5 washes with sterile MQ H_2O followed by centrifugation at 12,500 x g). A small volume of 1 M NaCl (containing a protease inhibitor, Sigma Aldrich) was used to cover the pellet and the suspension incubated O/N at 4°C. The following day, the extract was centrifuged at 12,500 x g for 10 min at 4°C, and the supernatant (the ionically-bound cell wall fraction) collected. The remaining pellet was again suspended in enough 1 M NaCl (containing protease inhibitor) to cover the pellet and the slurry incubated at 37°C for 1 hr. After this, the extract was centrifuged at 12,500 x g for 10 min at 4°C and both supernatant fractions pooled together for immediate GPX analysis.

8.3.4.1 SOD Enzymatic Assay

For estimation of SOD activity, samples was assayed by measuring the photoreduction of nitroblue tetrazolium (NBT) at 560 nm (Beauchamp & Fridovich, 1971). Briefly, 25 μL of crude extract was added to an assay reaction mixture comprising 50 mM K_2HPO_4 (pH

7.8), containing final concentrations of 0.1 mM EDTA, 13 mM L-Methionine, 161 μM NBT and 22 μM riboflavin in a final volume of 2.8 mL.

Samples were prepared in standardised glass tubes of equal diameter and length and carried out in triplicate. To begin the reaction, samples were prepared in the dark before initialising the reaction by illuminating the samples (representing T_0) with two 15 W fluorescent lights for 10 min (T_{10}) and terminating the reaction by switching the lights off. A sub-sample of the reduced reaction (100 μL) was pipetted into a microplate and the absorbance of each well read at 560 nm using a plate reader (Biotek) with Gen 5 software at 560 nm. One unit of SOD is considered to be the activity amount required to inhibit the photoreduction of NBT by 50% and is expressed in units (U) per mg^{-1} of protein.

8.3.4.2 CAT Enzymatic Assay

CAT activity was determined according to the method of Aebi (1984) and monitors the disappearance of H_2O_2 as a decrease in absorbance at 240 nm. To determine CAT activity, a sample containing 10 μg of protein was added to a 1mL reaction mixture containing 12.5 mM H_2O_2 and 50 mM K_2HPO_4 (pH7.0) in a UV cuvette.

The absorbance was recorded every 10 secs for 3 min and the difference in absorbance between 80 sec – 140 sec was used to calculate CAT activity, using an extinction co-efficient for H_2O_2 of 43.6 M^{-1} cm^{-1}. Results are expressed as mmol per μg protein. Each sample was assayed in triplicate.

8.3.4.3 APX Enzymatic Assay

The activity of APX was measured through the oxidation of ascorbate to dehydroascorbate as described by Nakano & Asada (1981). Briefly, the reaction assay mixture comprised 50 mM K_2HPO_4 (pH 7.0), containing 0.25 mM ascorbic acid, and 1 mM H_2O_2. To initiate the reaction, 10 μL of undiluted sample was added to 3 mL assay mixture and the decrease in absorbance at 290 nm was measured every 20 sec for 3 min. Results are expressed as mmol per μg protein using an extinction co-efficient of 2.8 mM^{-1} cm^{-1}. Each sample was assayed in triplicate.

8.3.4.4 GPX Enzymatic Assay

GPX activity was measured by the oxidation of guaiacol. The assay (1 mL) comprised 50 mm K_2HPO_4 (pH 6.0), 5 µL of H_2O_2 with 25mM guaiacol, and a 10 µL sample of a 50-fold dilution of cell-wall extract was used. The reaction was started by the addition of guaiacol and followed spectrophotometrically as the increase of absorption at 470 nm. Readings were taken every 10 sec for 3 min. Results are expressed as mmol per µg protein using an extinction co-efficient of 26.6 mM^{-1} cm^{-1}. Each sample was assayed in triplicate.

8.3.5 Metabolomic Analysis

Detection of metabolites within ryegrass tissue in a high throughout and untargeted manner was performed by AgResearch's Metabolomics Laboratory (ARML) and consisted of four streams that comprising:

- C18 +ve - amines, flavonoids, condensed tannins
- C18 –ve - aromatic acids, lignins, short chain fatty acids
- Hilic +ve - amino acids
- Hilic –ve - organic acids, mono/disaccharides, low DP fructans, sugar alcohols

Ryegrass shoot tissue grown on four treatments, that being the uncontaminated control, contaminated control, and both 350 and 550°C biochar amended soils at 60 t ha^{-1} were used for metabolomics analysis. Due to ARML confidentiality regarding methods/protocols for carrying out analysis of these streams, the following methods are described as published by cited references herein.

8.2.5.1 Method of Detection for C18 Streams

The detection of metabolites from C18 streams was carried out as described by Fraser et al. (2013) and analysis performed by ARML. Analysis used an LC–MS system (Thermo Fisher Scientific, Waltham, MA, USA) consisting of an Accela 1250 quaternary UHPLC pump, a PAL auto-sampler fitted with a 15,000 psi injection valve (CTC Analytics AG.,

Zwingen, Switzerland) and 20 µL injection loop, and an Exactive Orbitrap mass spectrometer with electrospray ionisation. Samples were cooled in the auto-sampler at 4°C and a 2 µL aliquot was resolved on an Agilent RRHD SB-C18 column (150 mm × 2.1 mm, 1.8 µm) at 25°C with a gradient elution programme and a flow rate of 400 µL min^{-1}. The mobile phase was a mixture of water–formic acid (solvent A) and acetonitrile–formic acid (solvent B).

The gradient elution programme was as follows: held at 5% B (0–0.5 min), 5–99% B (0.5–13 min), held at 99% B (13–15 min), returned to 5% B (15–16 min) and allowed to equilibrate for a further 4 min prior to the next injection. The first 1.5 min and the last 6 min of the chromatogram were diverted to waste. Mass spectral data were collected in profile mode over a mass range of m/z 60–1200, at a mass resolution setting of 25,000 with a maximum trap fill time of 100 ms using the Xcalibur software package provided by the manufacturer.

Samples were run in both positive and negative ionisation modes separately. Positive ion mode parameters were as follows: spray voltage, 3.5 kV; capillary temperature, 325°C; capillary voltage, 50 V, tube lens 120 V. Negative ion mode parameters were as follows: spray voltage,−3.5 kV; capillary temperature, 325°C; capillary voltage, −90 V, tube lens −80 V. The nitrogen source gas desolvation settings were the same for both modes (arbitrary units): sheath gas, 40; auxiliary gas, 10; sweep gas, 5. Samples were spiked with dichlorofluorescein (DCF) which was used throughout the procedure as an internal standard.

8.3.5.2 Method of Detection for HILIC Streams

The detection of metabolites from HILIC streams was carried out as described by Fraser et al. (2012) and analysis performed by ARML. The Thermo LC–MS system in this case is coupled with a Merck polymeric bead based ZIC-pHILIC column (100 mm x 2.1 mm, 5µm) which was used for the chromatographic separation. The samples were separated at 25°C with a gradient elution program and a flow rate of 250 µl min^{-1}. The mobile phase was a mixture of acetonitrile-formic acid (solvent A) and water–ammonium formate (solvent B).

The gradient elution programme was: held at 97% A (0–1 min), 97–70% A (1–12 min), 70–10% A (12–14.5 min), held at 10% A (14.5–17 min), returned to 97% A (17–18.5 min) and allowed to equilibrate for a further 5.5 min prior to the next injection. The samples were cooled in the auto-sampler at 4°C and the injection volume of each sample was 2 µl. The first 1.5 min and the last 5 min of the chromatogram were diverted to waste.

Data was collected in profile data acquisition mode over a mass range of m/z 55–1100 at a mass resolution setting of 25,000 with a maximum trap fill time of 100 ms using the Xcalibur software package provided by the manufacturer. Samples were run in both positive and negative ionisation modes separately. Positive ion mode parameters were as follows: spray voltage, 3.5 kV; capillary temperature, 325°C; capillary voltage, 90 V, tube lens 120 V. Negative ion mode parameters were as follows: spray voltage, -3.0 kV; capillary temperature, 325°C; capillary voltage, -90 V, tube lens -100 V. The nitrogen source gas desolvation settings were the same for both modes (arbitrary units): sheath gas, 40; auxiliary gas, 10; sweep gas, 5. Samples were spiked with dichlorofluorescein (DCF) which was used throughout the procedure as an internal standard.

8.3.5.3 Data Extraction and Multivariate Analysis

Data processing and normalisation of peak files returned from ARML for each stream were subjected to MetaboAnalyst 2.0 (Xia & Wishart, 2011). This online software package uses raw, annotated peak lists in combination with multivariate statistics to identify statistically different spectral features. Data was first normalised by auto-scaling (mean-centred and divided by the standard deviation of each variable) and then subjected to the following multivariate analysis:

- Principal Component Analysis (PCA)
- Partial Least Squares – Discriminant Analysis (PLS-DA)
- Hierarchical Clustering

Principal component analysis (PCA) is a mathematical technique commonly used for exploratory data analysis to differentiate sample sets. It is an unsupervised method that transforms the possibly correlated variables (component peaks) into a smaller number of uncorrelated variables called principal component scores which are weighted averages of the original variables.

PLS uses multivariate regression techniques to extract linear combinations of variables and often uses VIP (Variable Importance in Projection) to account for the amount of explained variation in a dimension. VIP can be used to calculate the importance of a feature or in this case, the upregulation/downregulation of a metabolite.

Once important features (metabolites) had been calculated, two online metabolite databases were used in conjunction to determine the likelihood of a given named metabolite. The raw peak list was subjected to online databases from:

- The Human Metabolome Database (HMDB) (http://www.hmdb.ca/)
- Scripps Center for Metabolomics (http://metlin.scripps.edu/index.php)

8.4 Results

8.4.1 Biochar Properties and Effect on Soil Elemental Status

The effect of biochar (**Table 5.1**) on soil properties in the current glasshouse trial was similar to that for the glasshouse trial described in Chapter 6 (refer to **Table 6.1**). Soil amended with both types of biochar had significantly ($P < 0.05$) higher concentrations of extractable SO_4^{2-} and Ca^{2+} when compared to the control soil, yet Olsen P values were slightly lower (**Table 8.1**). Soil pH was significantly increased at high doses for both biochars from pH 5.5 to pH ~5.8 ($P < 0.05$). A difference between the two trials is the total soil arsenic, with concentrations significantly lower in this trial. For Glasshouse Trial 2, soil was removed from a position 20 cm to the left of the original

position suggesting arsenic heterogeneity is a major contributor to soil arsenic concentrations.

Uncontaminated soil analysed contained significantly higher Olsen P values than the contaminated soil (202 µgP g^{-1} compared to ~47.1 µgP g^{-1} respectively) yet lower extractable SO_4^{2-} ($P<0.01$). This treatment also exhibited the highest CEC value of 21 which was significantly higher than the control (12) and biochar amended soils (16). Analysis of this soil also identified low contamination with arsenic (26 mg kg^{-1}) which is within soil WHO guidelines for New Zealand. Methods for soil and biochar characterisation can be found in **section 3.1 and 3.2**.

8.4.2 Soil Dehydrogenase Activity

Soil dehydrogenase activity (measured as described in **section 3.3**) was significantly increased with the addition of both types of biochar at both 30 t ha^{-1} and 60 t ha^{-1}, although only after 90 d for 550°C (**Figure 8.2**) and 120 d for 350°C biochar amended soils (**Figure 8.3**). Both high rates (60 t ha^{-1}) significantly increased ($P<0.05$) dehydrogenase activity in as little as 60 d after amendment. After 180 d of treatment, dehydrogenase activity in the 550°C biochar amended soils (60 t ha^{-1}) had 75% higher activity compared to the control and 89% higher in 350°C biochar amended soils (60 t ha^{-1}). This data is in agreement with the observed increase in activity from the glasshouse trial described in Chapter 6 (**Figure 6.1**), although activity in this trial is slightly higher compared to the control. An uncontaminated soil used in this trial remained low compared to the biochar amended soils, and yet its activity was the same as the contaminated control soil at all sampling points except 150 d. In general, biochar- amended soils had higher dehydrogenase activity compared to the controls, yet the relative rate of increase was the same for 550°C amended soils but higher under 350°C amended soils.

8.4.3 Ryegrass Dry Weight (DW) Biomass

Lolium perenne L. grown under glasshouse conditions (**Figure 8.4**) and in contaminated soil (control and biochar amended pots) exhibited significantly increased shoot and

root DW ($P<0.05$) when compared with plants grown in uncontaminated soil. Shoot DW, under the 350°C biochar amendment, increased nearly 2-fold compared to that grown on uncontaminated soil (**Figure 8.5**) and was also significantly increased ($P<0.05$) compared to the control. In comparison, ryegrass grown on the 550°C soils did not significantly differ from the control. Shoot DW for the control treatment was 9.9 g per pot. Shoot DW for the 350°C biochar treatments was higher compared to 550°C treatments with biomass values of 13.0 g and 13.1 g recorded for 30 t ha^{-1} and 60 t ha^{-1}, respectively per pot.

Root DW (**Figure 8.6**) in the uncontaminated treatment (17.5 g total per pot) was nearly 2-fold lower than the 60 t ha^{-1} 350°C amended treatment (33.1 g per pot). All other treatments had significantly increased root DW compared to the uncontaminated treatment ($P<0.01$). When compared with the control ryegrass (25.5 g per pot), all biochar amended pots had higher total root DW but this increase was significant ($P<0.05$) only in 350°C amended pots (60 t ha^{-1}). Root DW growth for the biochar amended soil was 28.2 g and 33.1 g for the 350°C biochar (30 t ha^{-1} and 60 t ha^{-1}, respectively) and 27.2 and 28.1 g for the 550°C biochar (30 t ha^{-1} and 60 t ha^{-1}, respectively).

8.4.4 Ryegrass Tillering

Ryegrass tillering was only significantly increased under 350°C amended soil (60 t ha^{-1}) when compared with the control (**Figure 8.7**). Increased ryegrass tillering was observed in both 350°C amended soils when compared with the control (3.4 tillers per plant) with 3.6 tillers and 4.1 tillers per plant (30 t ha^{-1} and 60 t ha^{-1}, respectively). At 30 t ha^{-1} for the 550°C biochar treatment, each plant yielded 3.2 tillers and 3.3 tillers for 60 t ha^{-1} although no significant difference was noted between the rates of 550°C biochar and the control. Ryegrass grown on all contaminated soil treatments (that representing the control and biochar amended soils) exhibited increased tillering when compared to ryegrass grown on uncontaminated soils (2.8 tillers per plant).

8.4.5 Arsenic Concentration in Ryegrass Shoot Biomass

The arsenic concentration in ryegrass shoots was significantly increased ($P<0.05$) as a function of both biochar treatments at 30 and 60 t ha^{-1}, relative to the control (**Figure 8.8**). The maximum concentration of shoot arsenic was 23.6 mg kg^{-1} for the 30 t ha^{-1} application of 350°C biochar compared to the control of 14.6 mg kg^{-1}. This was an increase of 62% over the control concentration. Ryegrass shoot grown in uncontaminated soils contained 2.5 mg kg^{-1} of arsenic.

8.4.6 Chlorophyll Content of Ryegrass Shoot

Ryegrass shoots in general exhibited a decrease in chlorophyll content with time across all plants grown on contaminated soil (**Figure 8.9**). Shoot material analysed from plants grown on uncontaminated soil treatment exhibited stable total chlorophyll content between 90 and 180 d (380 and 375 μg mL^{-1}, respectively).

At 90 d and 180 d of treatment, all leaves had lower chlorophyll content compared to leaves from plants grown on the uncontaminated soil, yet these results were not significant ($P<0.05$). Within the contaminated soil treatments, a significant decrease ($P<0.05$) in chlorophyll content was observed for the control and 350°C (30 t ha^{-1}) ryegrass between 90 and 180 d samples.

Table 8.1. Elemental analysis of soil and biochar characterisation after 180 d of treatment (values are mean ± s.e. *n* = 5). Cations have been depicted as extractable cations (meq/100g).

	pH	Olsen P µgP/g	SO4 µgS/g	K me/100g	Ca me/100g	Mg me/100g	Na me/100g	CEC me/100g	Total As (mg kg^{-1})
Uncontaminated	5.7	202 ± 1.6	6.9 ± 0.5	1.2 ± 0.1	3.3 ± 0.4	0.7 ± 0.1	0.2 ± 0.1	21 ± 0.0	26 ± 2.5
Control	5.5	47.1 ± 0.2	12.8 ± 0.8	1.0 ± 0.1	3.1 ± 0.2	0.9 ± 0.0	0.2 ± 0.0	12.0 ± 0.4	145 ± 1.6
Soil + 550°C BC (30t ha^{-1})	5.7	50.6 ± 1.1	20.0 ± 0.6	1.1 ± 0.0	4.0 ± 0.1	1.1 ± 0.0	0.2 ± 0.0	16.0 ± 0.1	138 ± 5.4
Soil + 550°C BC (60t ha^{-1})	5.8	43.2 ± 6.1	33.0 ± 1.3	1.2 ± 0.1	4.7 ± 0.3	1.1 ± 0.0	0.2 ± 0.0	16.0 ± 0.0	137 ± 1.4
Soil + 350°C BC (30t ha^{-1})	5.6	45.9 ± 0.7	23.0 ± 0.8	1.1 ± 0.0	4.1 ± 0.3	1.0 ± 0.0	0.2 ± 0.0	16.0 ± 0.1	136 ± 3.3
Soil + 350°C BC (60t ha^{-1})	5.9	46.4 ± 2.3	26.5 ± 2.9	1.3 ± 0.1	5.9 ± 0.1	5.0 ± 0.6	0.2 ± 0.0	16.0 ± 0.0	141 ± 2.1

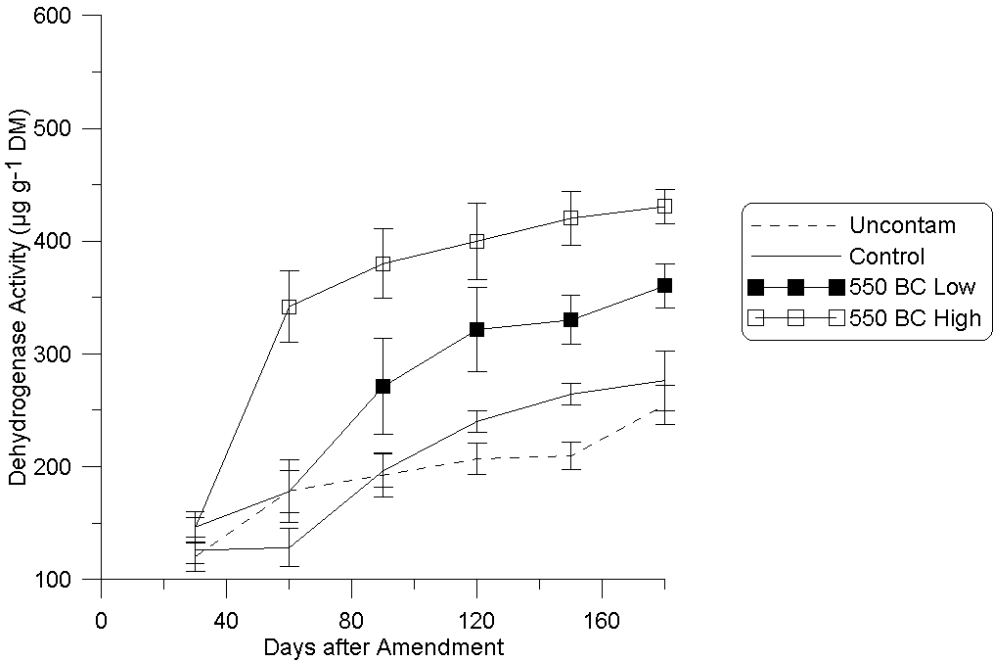

Figure 8.2. Change in soil dehydrogenase activity, measured in µg per g of dry matter (DM), over a 180 d time period as a function of two 550°C biochar treatments and uncontaminated and contaminated controls (values are mean ± s.e. *n* = 5).

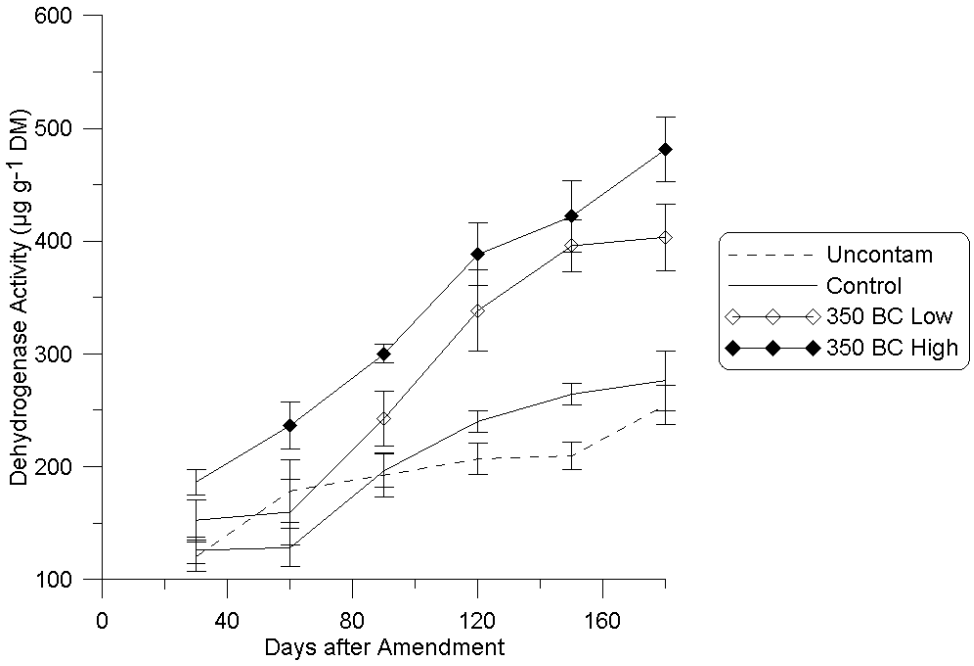

Figure 8.3. Change in soil dehydrogenase activity, measured in µg per g of dry matter (DM), over a 180 d time period as a function of two 350°C biochar treatments and uncontaminated and contaminated controls (values are mean ± s.e. *n* = 5).

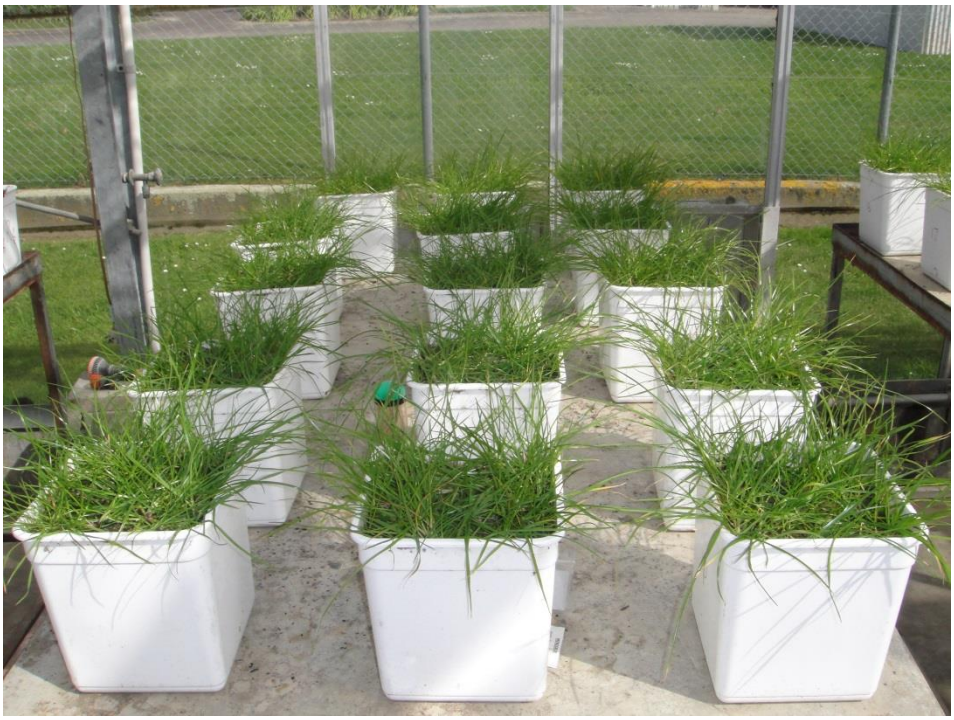

Figure 8.4. Glasshouse setup of amended soil pots containing growing ryegrass plants (T = 90 d)

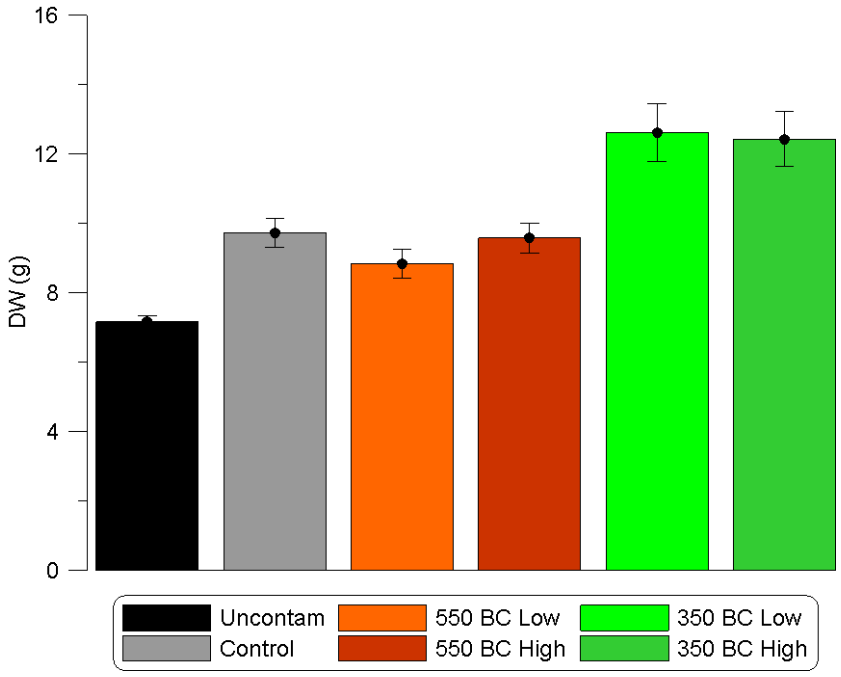

Figure 8.5. Averaged ryegrass shoot DW (g) yield at harvest (T = 180 d) as a function of control and biochar treatment (values are mean ± s.e. *n* = 5).

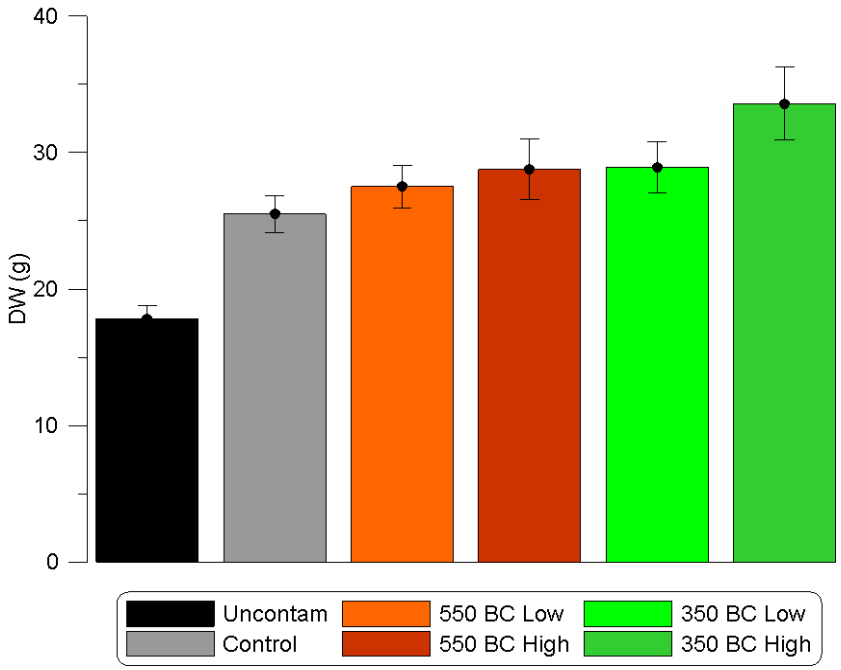

Figure 8.6. Averaged ryegrass root DW (g) yield at harvest (T = 180 d) as a function of control and biochar treatment (values are mean ± s.e. *n* = 5).

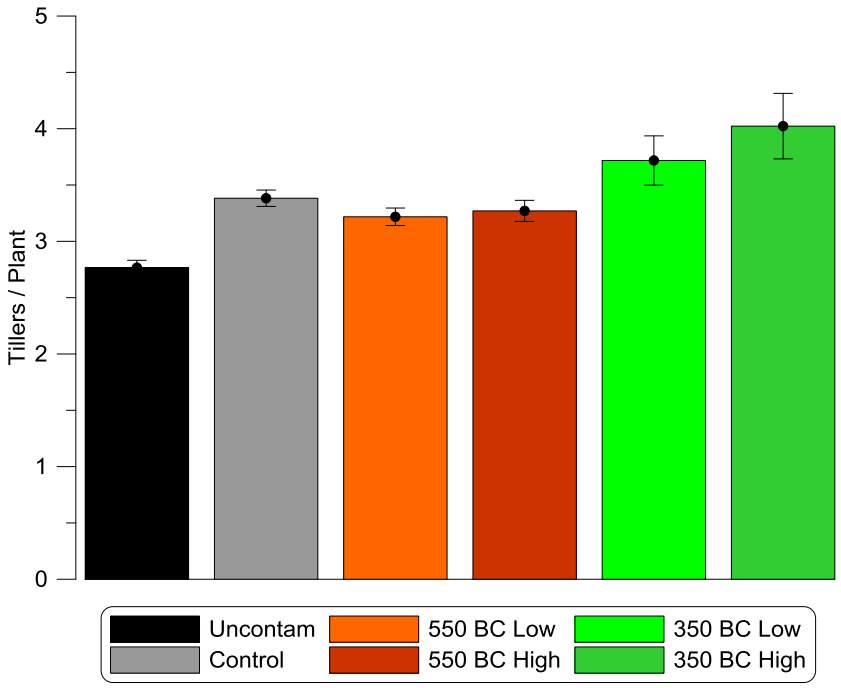

Figure 8.7. Average number of ryegrass tillers per plant as a function of biochar treatment (values are mean ± s.e. *n* = 5).

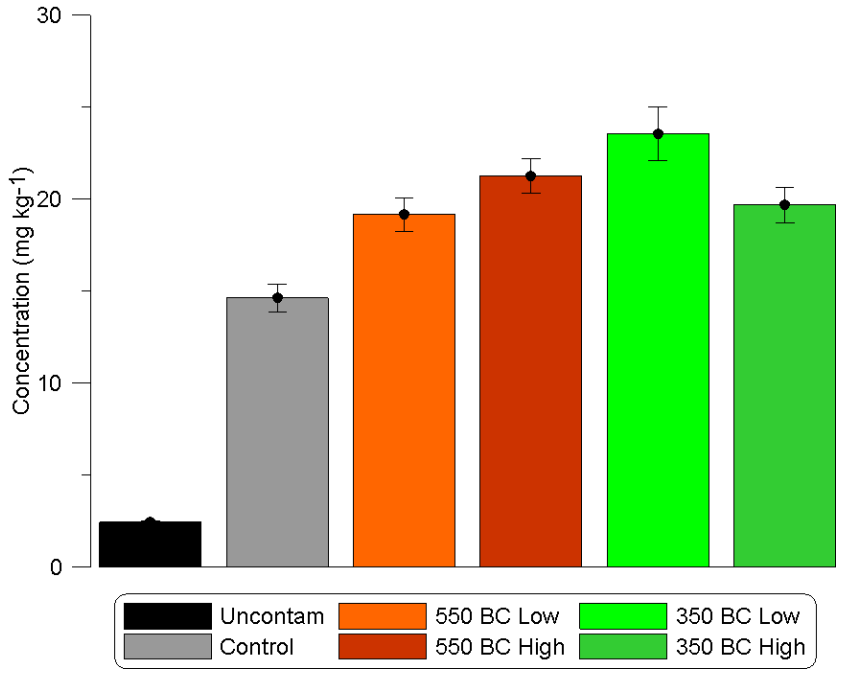

Figure 8.8. Total average arsenic concentration (mg kg^{-1}) in ryegrass shoot biomass as a function of biochar treatment (values are mean ± s.e. *n* = 5).

8.4.7 Analysis of Ryegrass Shoot Enzyme Activity

8.4.7.1 SOD Activity

In general, shoot tissue from ryegrass grown on both biochar amended soils at both rates (30 t ha^{-1} and 60 t ha^{-1}) had increased SOD activity compared to the control at 90 and 180 d of treatment (**Figure 8.10**). At 90 d, shoot tissue from ryegrass grown on biochar amended soil (60 t ha^{-1} 550°C and 30 t ha^{-1} and 60 t ha^{-1} 350°C) had nearly 2-fold increase in SOD activity compared to the control (0.68 U mg $protein^{-1}$). These results at 90 d were significantly different ($P<0.05$) compared to the control. A significant increase in SOD activity was also observed at 180 d for all ryegrass shoots from plants grown on biochar amended soils compared to the control ($P<0.05$). There was no significant difference between the uncontaminated and control treatment, although the mean SOD activity was higher in plants grown on the control soil at both time points.

8.4.7.2 CAT Activity

Catalase activity across all treatments was very low compared to APX and GPX activity due mainly to its high catalytic capacity to de-toxify H_2O_2. There were no significant differences ($P<0.05$) in activity across treatments at both 90 and 180 d time points (**Figure 8.11**). Ryegrass grown on uncontaminated soil generally had a lower CAT activity than other treatments with activity of 0.043 and 0.040 mmol μg $protein^{-1}$ (at 90 and 180 d respectively), but this was not significant.

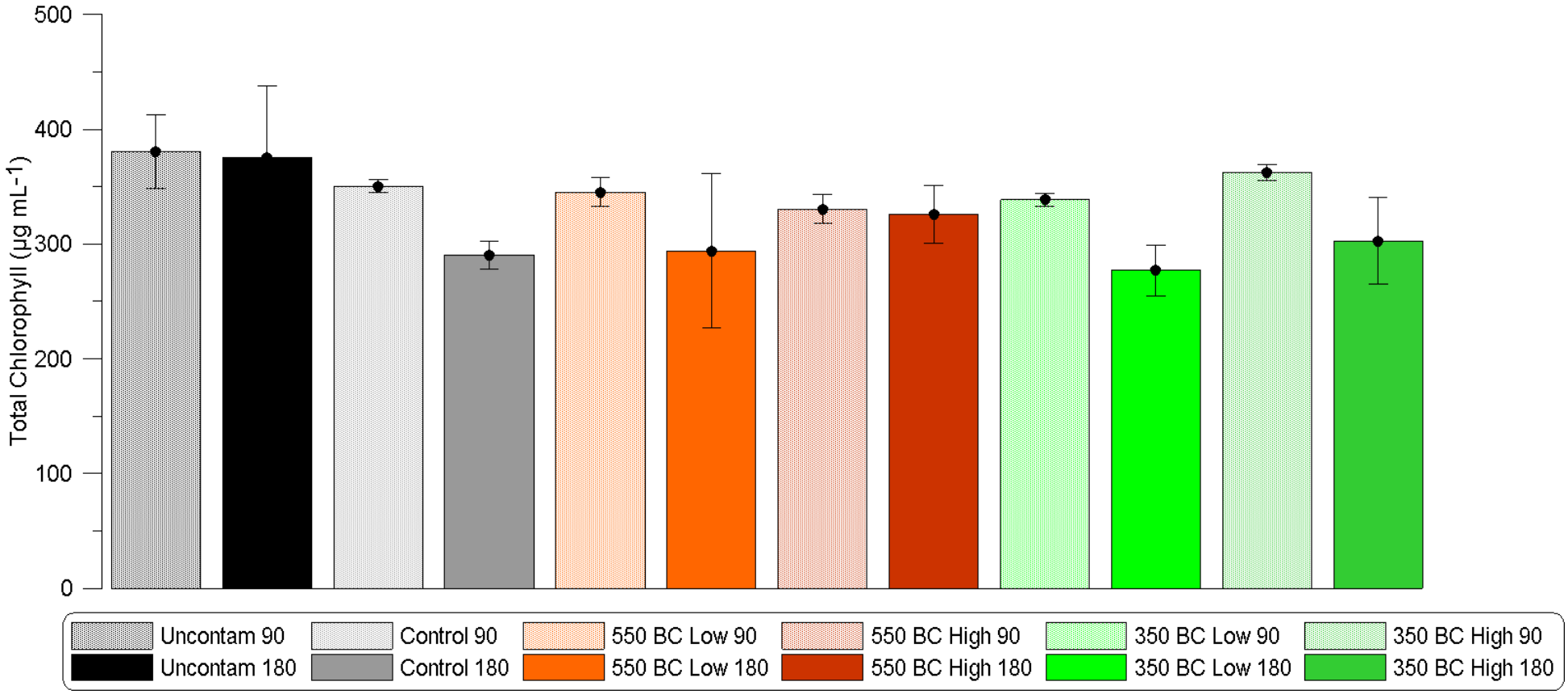

Figure 8.9. Chlorophyll content of the excised 2nd flag leaf of ryegrass samples grown under different treatments at 90 d and 180 d (values are mean ± s.e. *n* = 5).

8.4.7.3 APX Activity

Ryegrass grown on biochar amended soils had significantly higher APX activity after 180 d (**Figure 8.12**) when compared with the control ($P<0.05$), yet APX activity was only significantly higher in ryegrass shoots grown on 350°C biochar amended soils at 90 d ($P<0.05$). At 90 d, ryegrass grown on control soil had a higher APX activity (0.61 mmol µg protein^{-1}) than after 180 d (0.39 mmol µg protein^{-1}). Ryegrass grown on all biochar amended soils (except 350°C at 30 t ha^{-1}) had increased APX activity after 180 d when compared with 90 d, but these results were not significant ($P<0.05$). APX activity in ryegrass samples grown on uncontaminated soil was the lowest across all treatments at each time point and remained relatively stable.

8.4.7.4 GPX Activity

GPX activity was significantly increased ($P<0.05$) in ryegrass grown on 550°C amended soils (30 t ha^{-1} and 60 t ha^{-1}) but also on 350°C soil (at 30 t ha^{-1}) when compared with the control at 90 d (**Figure 8.13**). GPX activity in the control (12.6 mmol µg protein^{-1}) was slightly higher than ryegrass grown on 350°C soil (11.5 mmol µg protein^{-1} at 60 t ha^{-1}) yet this was not significantly different. GPX activity in ryegrass grown on uncontaminated soil was very similar to that on control soil at 90 d. In comparison, all samples at 180 d had decreased activity compared to 90 d within each treatment and this decrease was significant for all treatments except for uncontaminated ($P<0.05$).

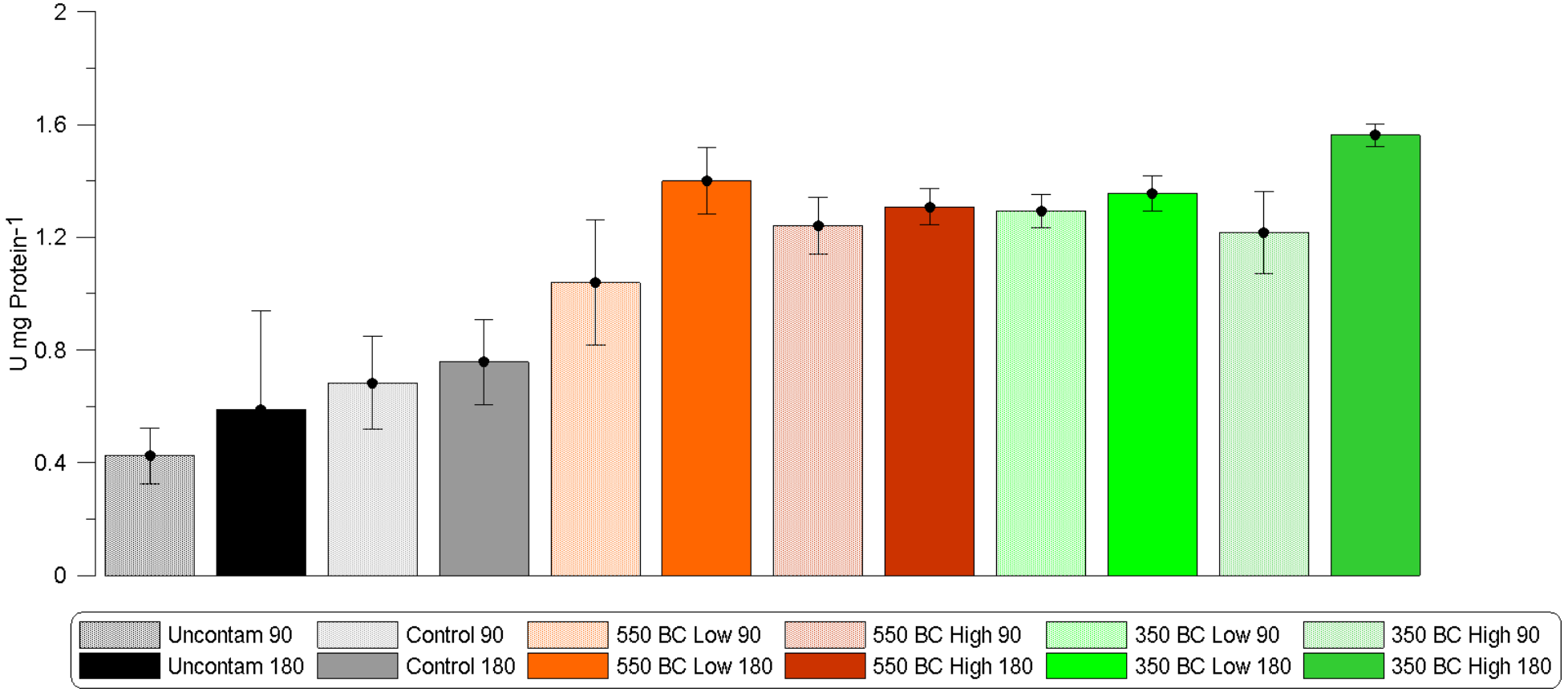

Figure 8.10. SOD activity of the excised 2nd flag leaf of ryegrass samples grown under different treatments at 90 d and 180 d (values are mean ± s.e. *n* = 5).

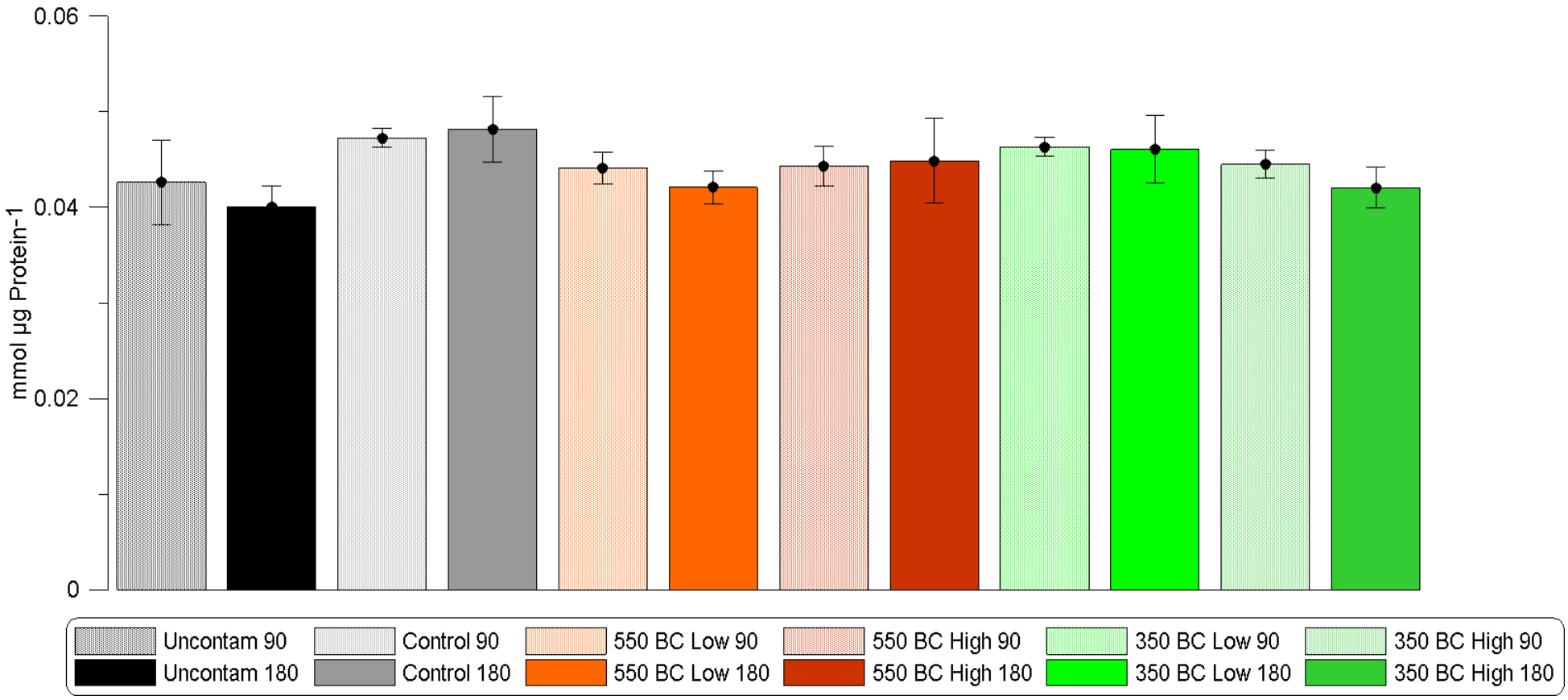

Figure 8.11. CAT activity of the excised 2nd flag leaf of ryegrass samples grown under different treatments at 90 d and 180 d (values are mean ± s.e. *n* = 5).

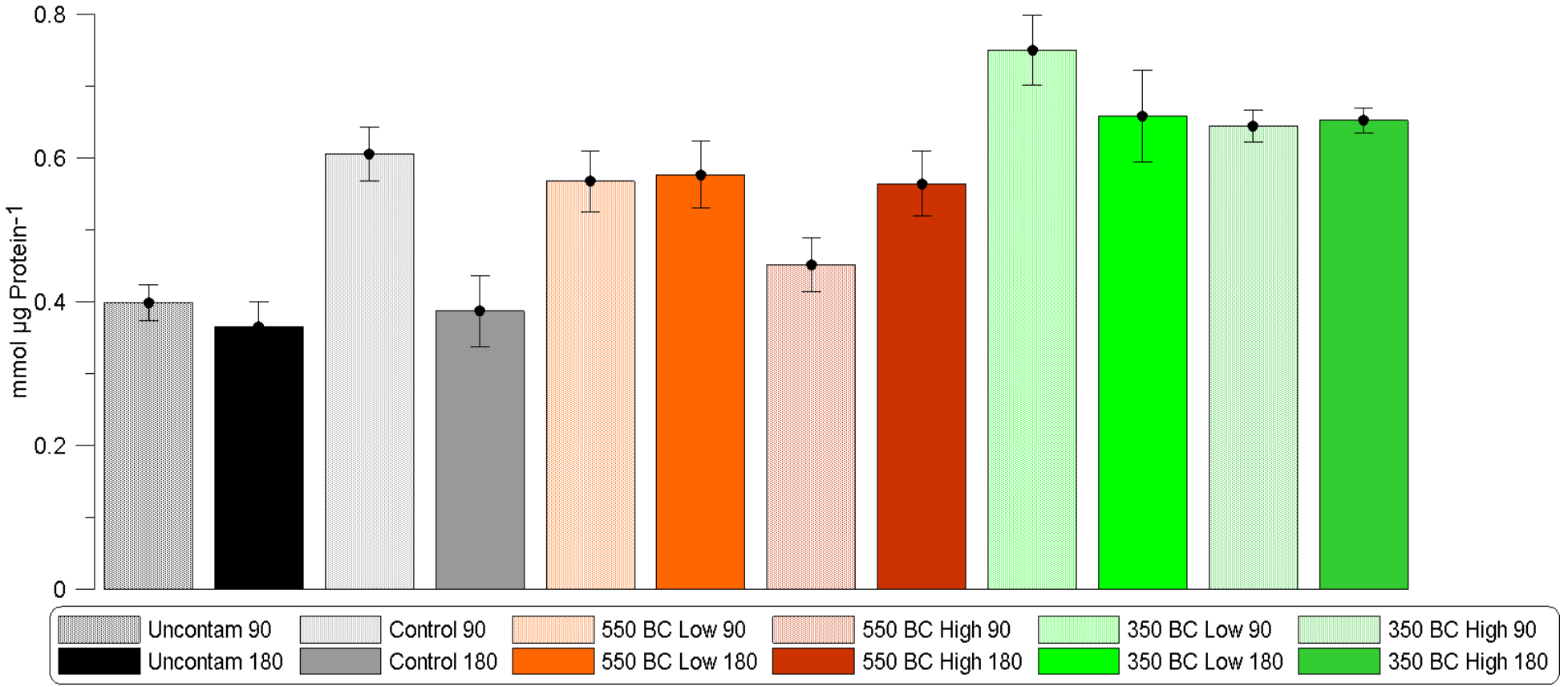

Figure 8.12. APX activity of the excised 2nd flag leaf of ryegrass samples grown under different treatments at 90 d and 180 d (values are mean ± s.e. *n* = 5).

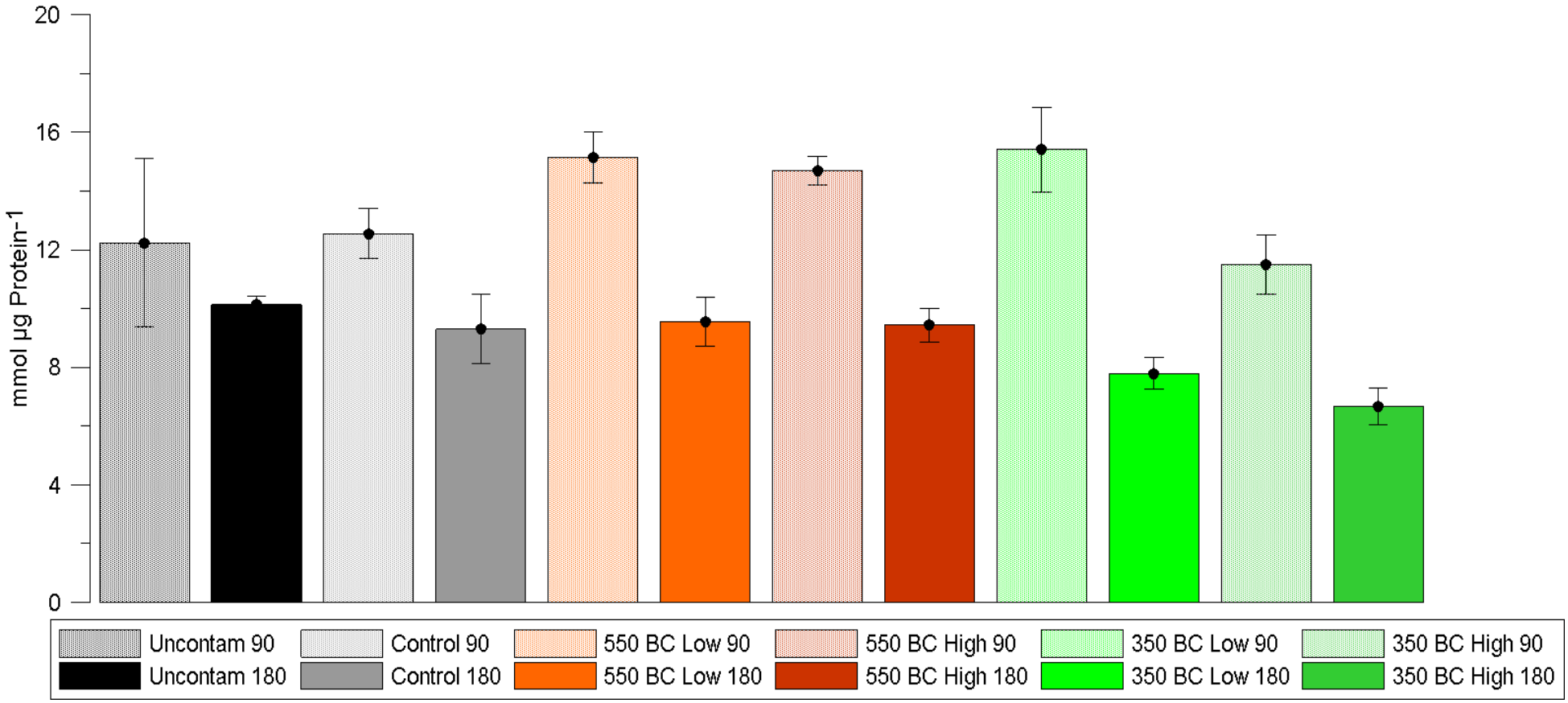

Figure 8.13. GPX activity of the excised 2nd flag leaf of ryegrass samples grown under different treatments at 90 d and 180 d (values are mean ± s.e. *n* = 5).

8.4.8 Metabolomic Analysis of C18 Streams

The PCA score plots for principal components 1 and 2 for the C18 Negative and Positive stream demonstrate clear resolution of the four treatments (**Figure 8.14**). Further analysis showed that the principal components within ryegrass grown on biochar amended soils were different to that of both the control and uncontaminated grown ryegrass when compared against principal components 1, 2 and 3 (**Figure 8.15**). An overlap in this instance exists between ryegrass samples from control and uncontaminated treatments for both C18 Negative and Positive.

Important features as determined by PLS-DA (VIP Scoring) indicated a number of metabolites that were highly upregulated in ryegrass shoot grown on uncontaminated and control soil when compared with the biochar amended soils in the C18 Negative stream (**Figure 8.16**). Analysis using two online search engines revealed that a number of possible metabolites including traumatic acid, jasmolone and methyl jasmonate were upregulated in uncontaminated soils compared to those amended with biochar. Important features identified by PLS-DA for C18 Positive indicated high upregulation in biochar amended soils including IAA, diphenylcarbazide and lysinoalanine (**Figure 8.17**).

Clustering results of all metabolites of each stream exhibited clear differences in upregulation/downregulation for each treatment for C18 Positive (**Figure 8.18**). Clear clustering for metabolite regulation was observed for ryegrass growing on uncontaminated soil for both streams yet control ryegrass did not exhibit clear clustered profiles for C18 Negative. Generally speaking, both C18 streams clustered biochar amended soils together (**Figure 8.18**).

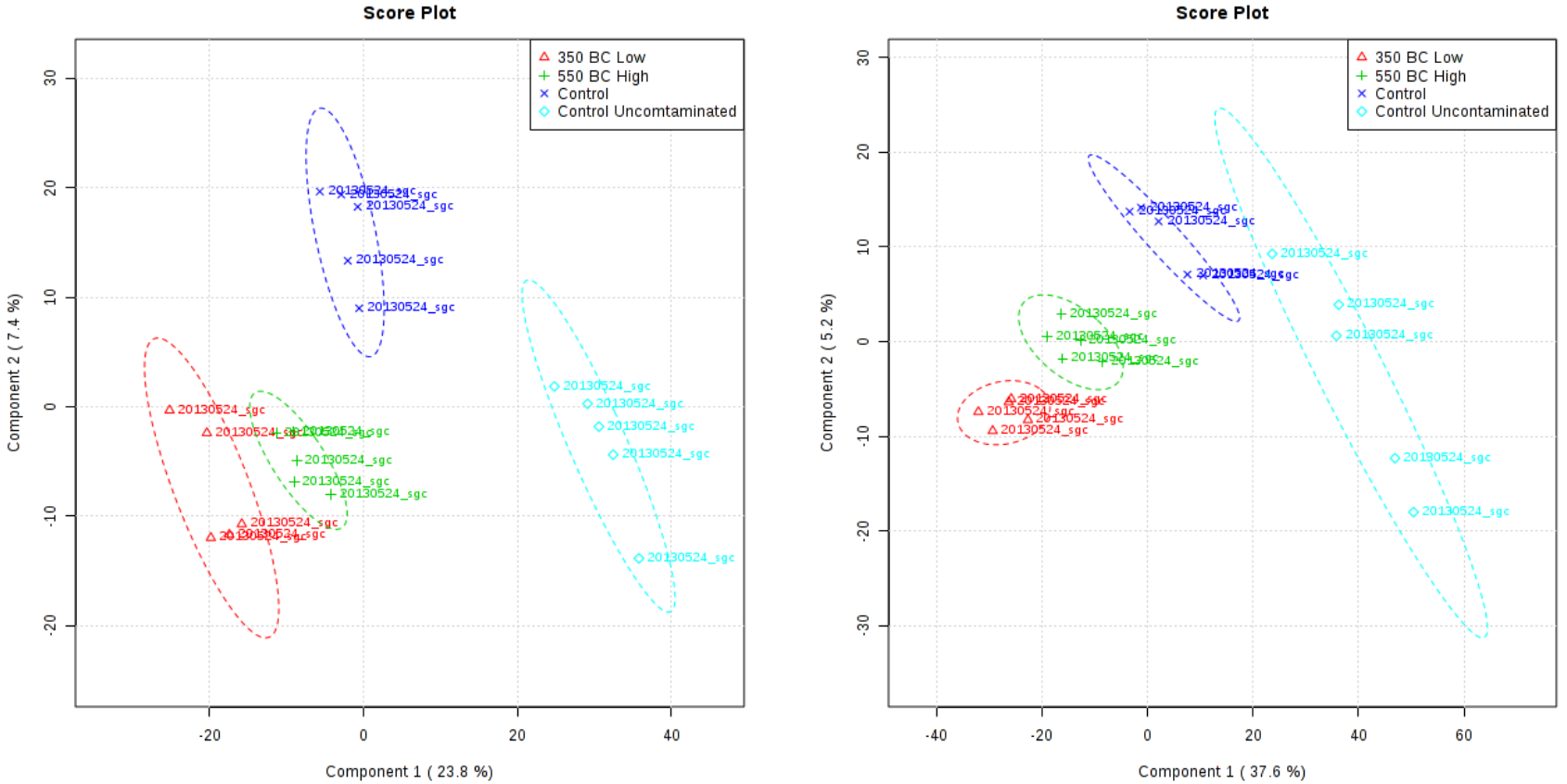

Figure 8.14. Score plot between the selected principal components (PCs) for C18 Negative (Left) and C18 Postive (Right). Variances are shown in brackets on each axis.

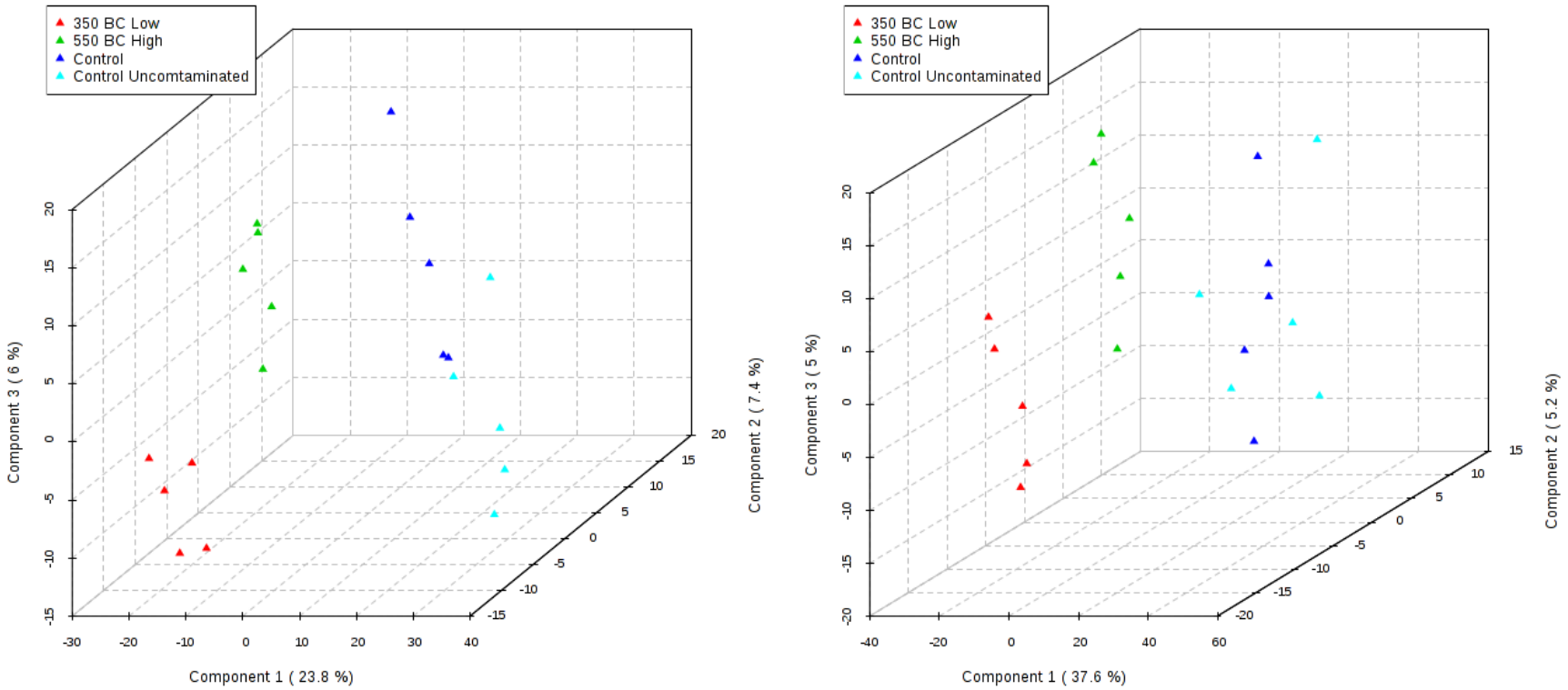

Figure 8.15. 3D score plot between the selected principal components (PCs) for C18 Negative (Left) and C18 Postive (Right). Variances are shown in brackets on each axis.

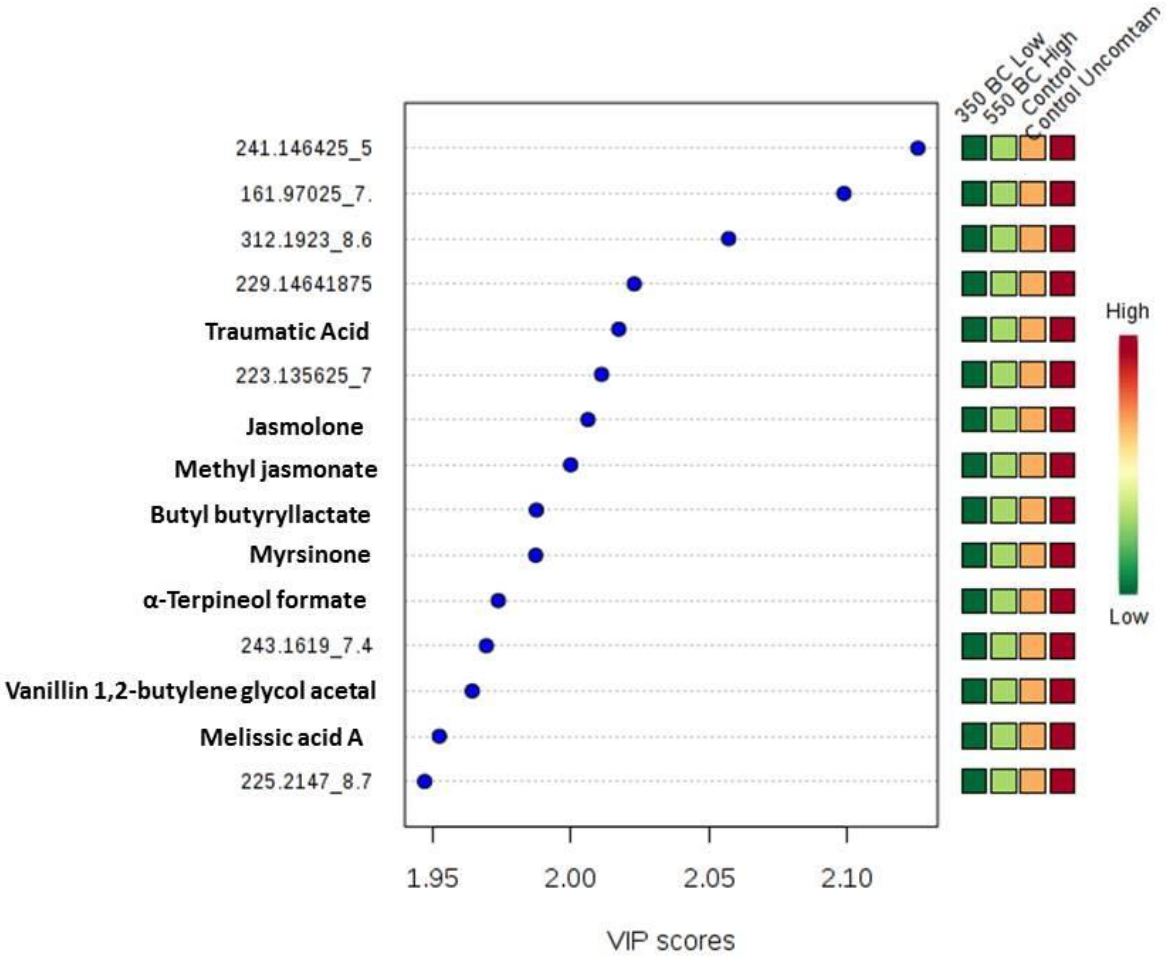

Figure 8.16. Important features (calculated by their difference among treatments) identified by PLS-DA for C18 Negative. The coloured boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.

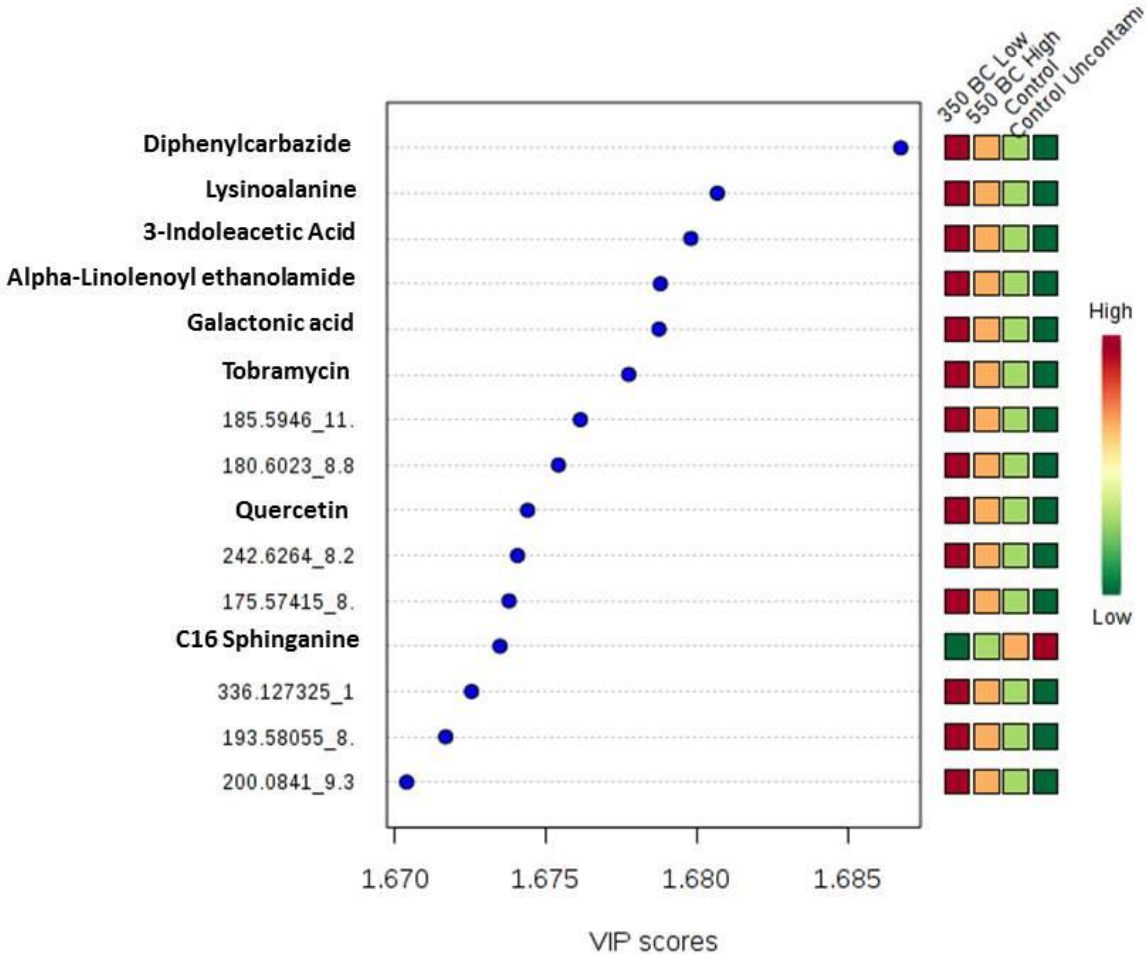

Figure 8.17. Important features (calculated by their difference among treatments) identified by PLS-DA for C18 Positive. The coloured boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.

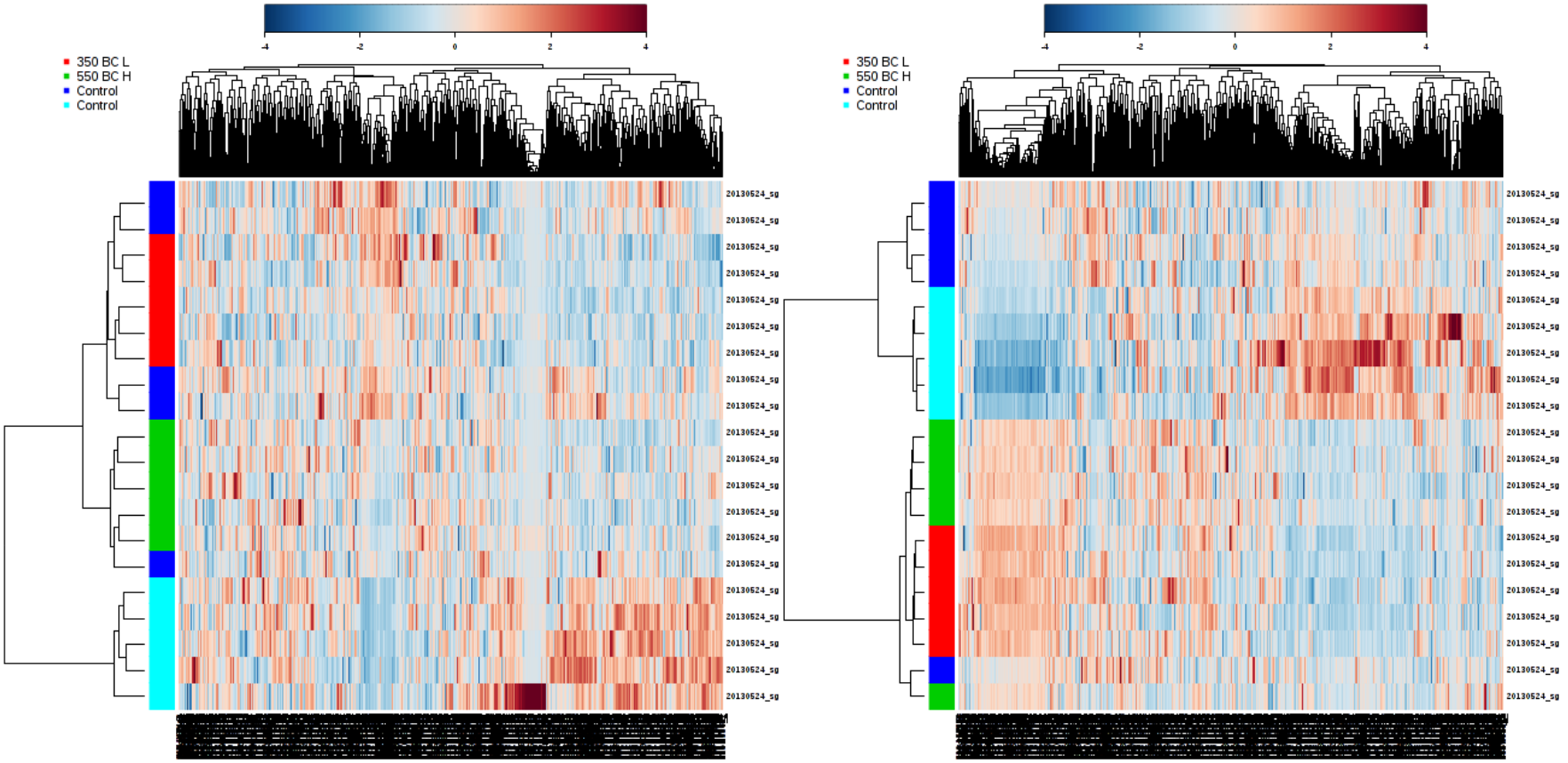

Figure 8.18. Clustering result shown as heatmap for C18 Negative (Left) and C18 Postive (Right). Metabolites (blue) represent downregulation and metabolites (red) are upregulated

8.4.9 Metabolomic Analysis of HILIC Streams

The PCA score plots for principal components 1 and 2 for the HILIC Negative and Positive stream demonstrate clear resolution of the four treatments (**Figure 8.19**). Further analysis showed that metabolites within ryegrass grown on biochar amended soils were different to that of both the control and uncontaminated grown ryegrass when checked against principal components 1, 2 and 3 (**Figure 8.20**). An overlap in this instance exists between ryegrass samples from control and uncontaminated treatments for both C18 Negative and Positive, but also one sample for 350°C biochar amended soils for HILIC Negative.

Important features as determined by PLS-DA (VIP Scoring) indicated a number of metabolites that were highly upregulated in ryegrass growing on uncontaminated and control soil compared to biochar amended soils in the HILIC Negative stream (**Figure 8.21**). Analysis using the two online search engines revealed a number of possible metabolites including osmundalactone, $_{D}$-glutamate and epicatechin upregulated in ryegrass on uncontaminated soils compared to those amended with biochar. Yet $_{L}$-isoleucine was in this instance, highly upregulated compared to the other treatments in 350C biochar amended soils. Important features identified by PLS-DA for HILIC Positive indicated high upregulation in biochar amended soils including Squalmalone and $_{L}$-Glutamine but also upregulation of phenylpyruvic acid and $_{N}$-malonyltryptophan in uncontaminated ryegrass grown soils (**Figure 8.22**).

Clustering results of all metabolites of each stream exhibited clear differences in upregulation/downregulation for each treatment for HILIC Negative (**Figure 8.23**). Heat mapping of metabolite profiles showed ryegrass samples grown on biochar amended soils to be clustering together with clear differences between control and uncontaminated ryegrass treatments. Metabolite clustering for HILIC Positive was in this case, not as clear with ryegrass grown on control soils exhibiting similar metabolite profiles to 350°C amended soils and slighty to 550°C amended soils (**Figure 8.23**).

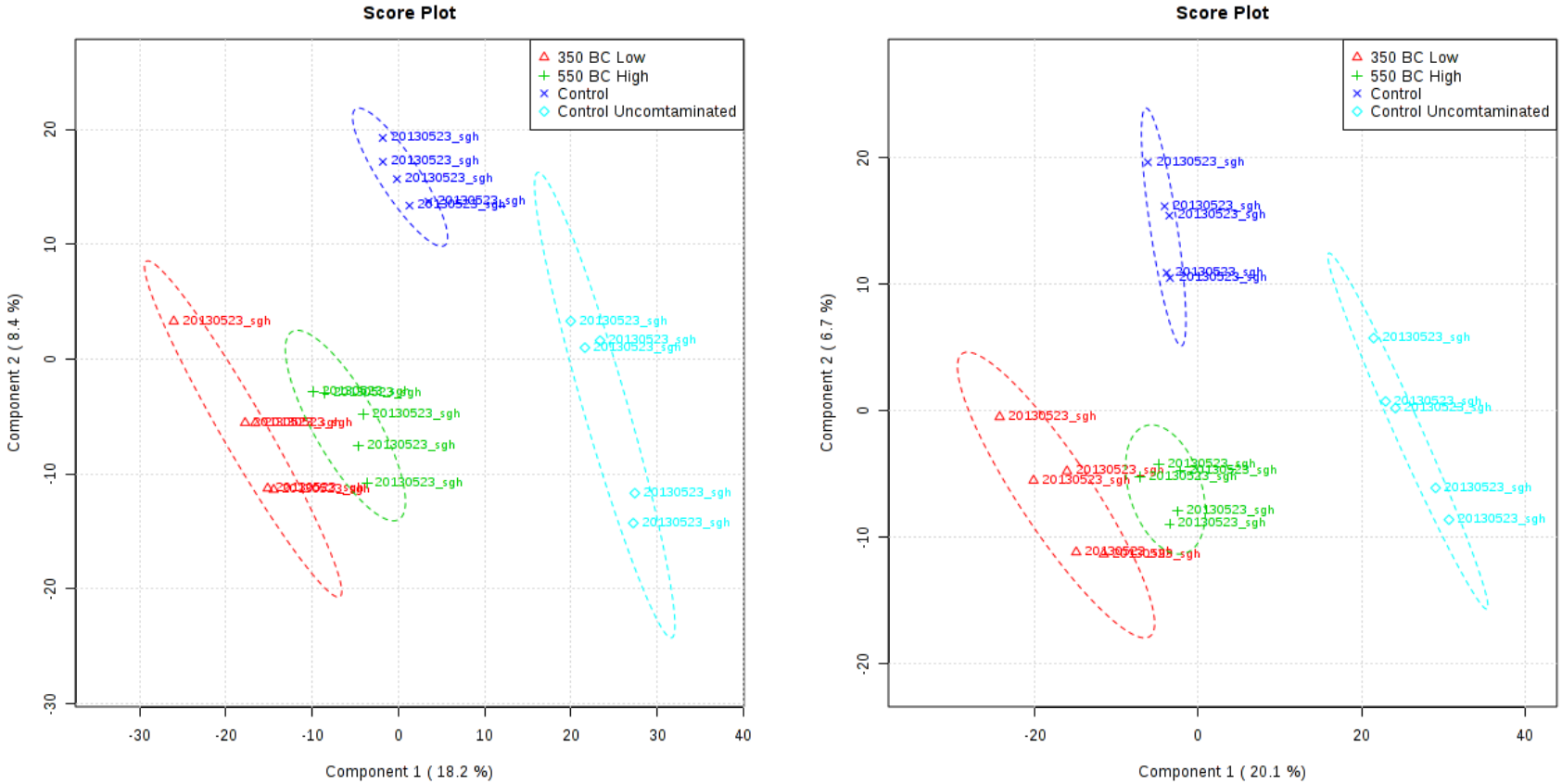

Figure 8.19. Score plot between the selected principal components (PCs) HILIC Negative (Left) and HILIC Positive (Right). Variances are shown in brackets on each axis.

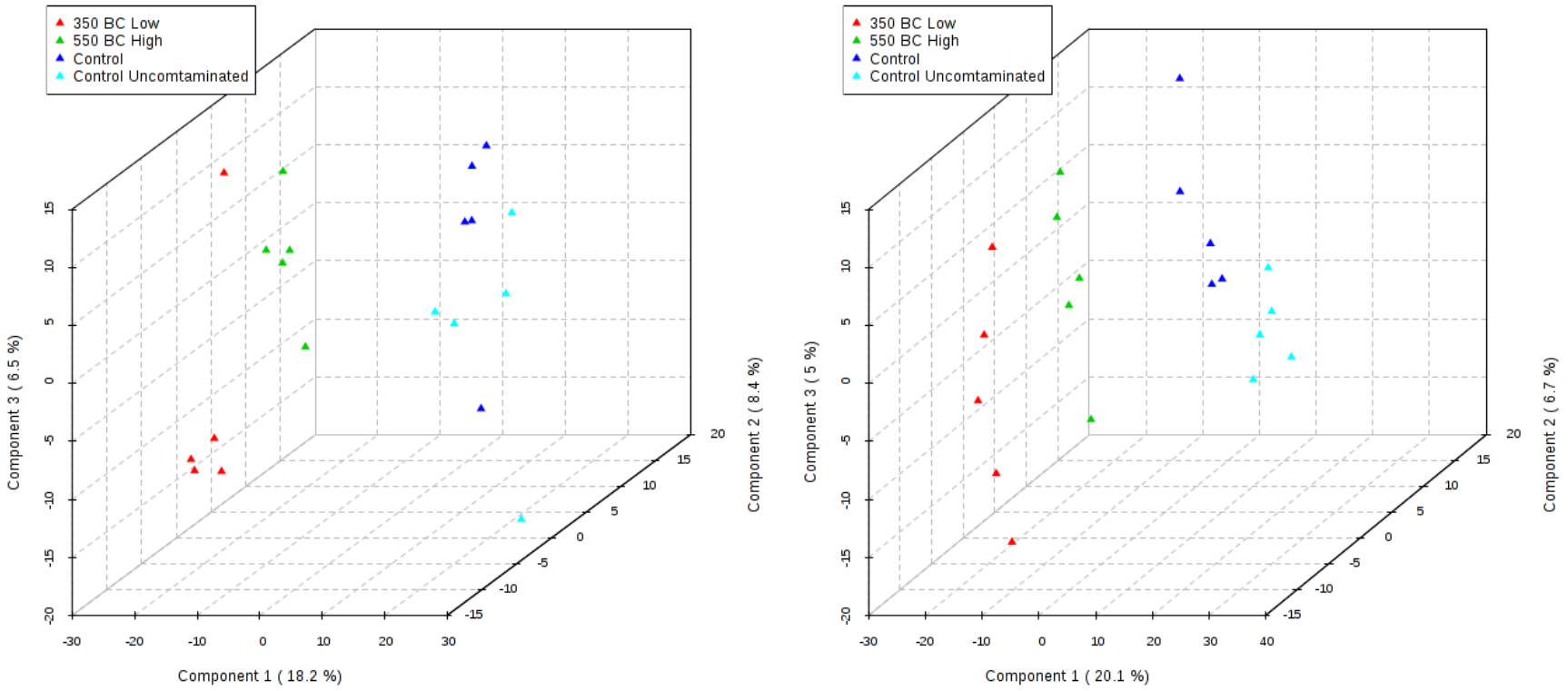

Figure 8.20. 3D plot between the selected principal components (PCs) HILIC Negative (Left) and HILIC Positive (Right). Variances are shown in brackets on each axis.

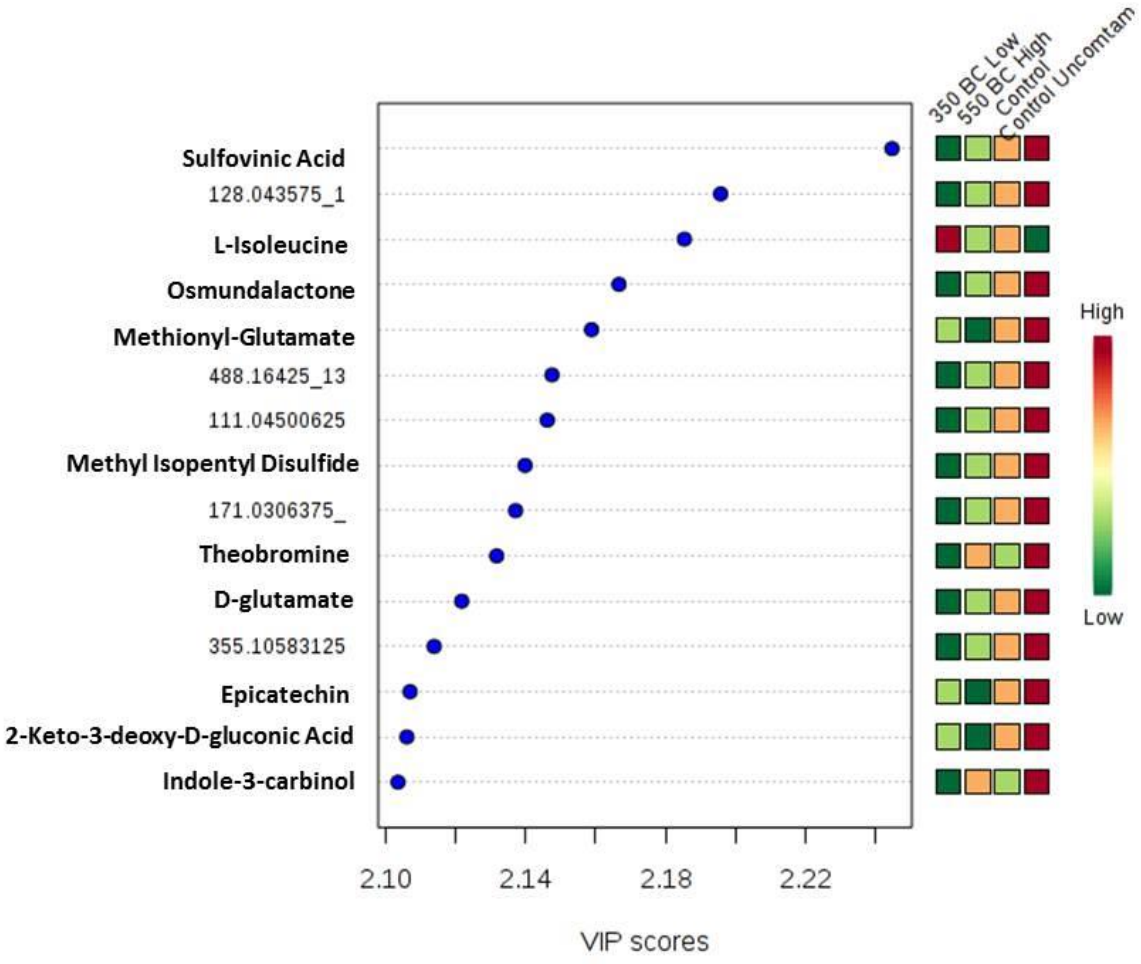

Figure 8.21. Important features (calculated by their difference among treatments) identified by PLS-DA for HILIC Negative. The coloured boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.

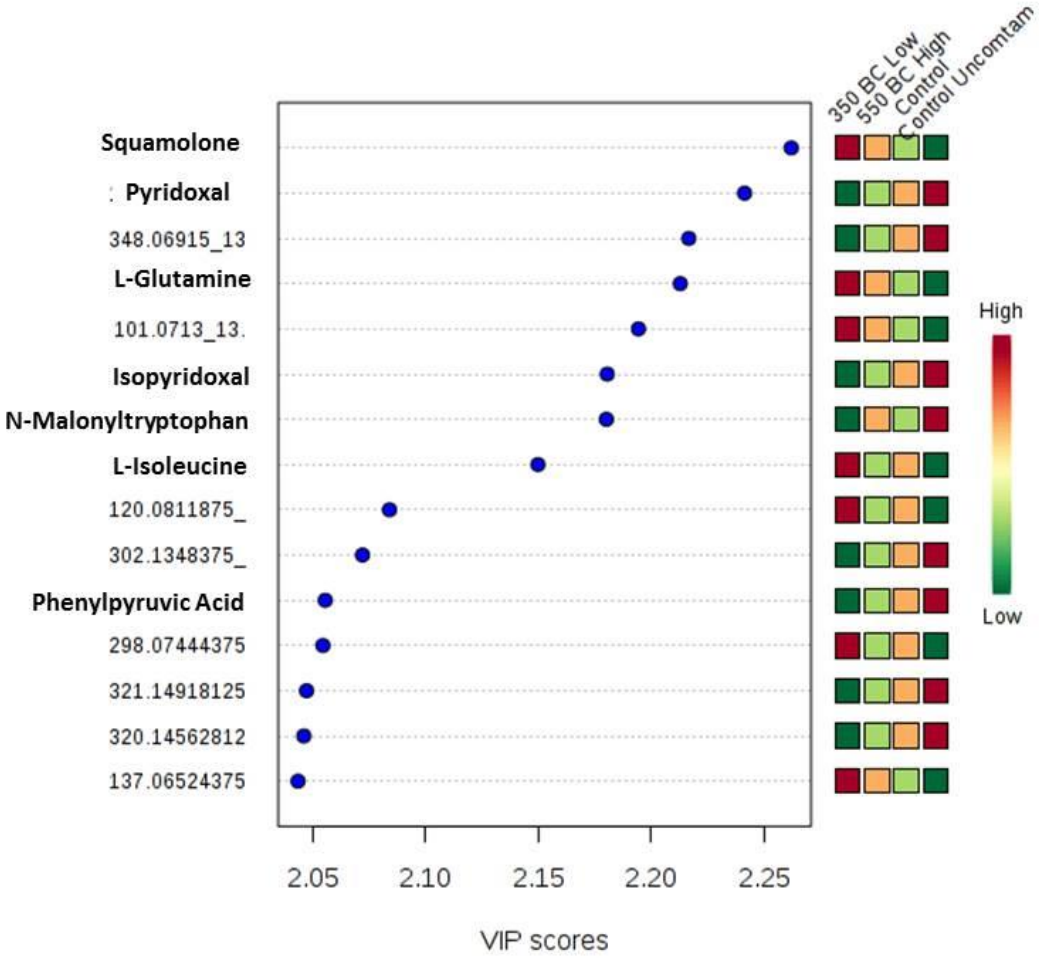

Figure 8.22. Important features (calculated by their difference among treatments) identified by PLS-DA for HILIC Positive. The coloured boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.

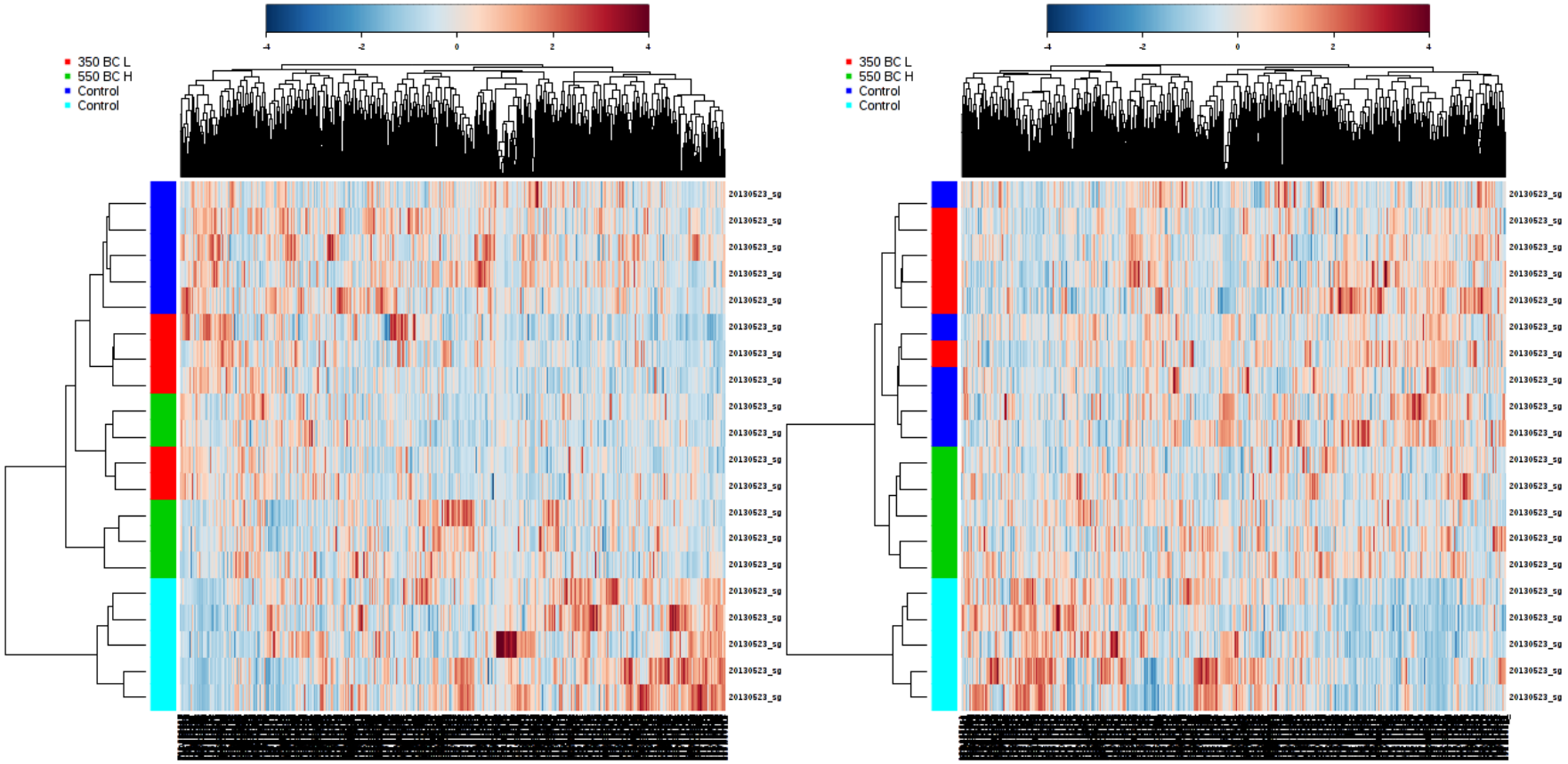

Figure 8.23. Clustering result shown as heatmap for HILIC Negative (Left) and HILIC Positive (Right). Metabolites (blue) represent downregulation and metabolites (red) are upregulated

8.5 Discussion

In this glasshouse trial, ryegrass was grown to test for changes in enzymic activity and metabolite profiles in response to arsenic uptake in ryegrass shoot tissue. The increased uptake of arsenic in soil amended with biochar caused both metabolic and enzymological changes to components of the antioxidant pathway and also to known and unknown metabolites.

8.5.1 Effect on Growth and Enzymic Components of the Antioxidant Pathway

After 180 d of treatment, ryegrass tissue growing in both 350°C and 550°C biochar amended soils contained significantly greater concentrations of arsenic compared to both controls (**Figure 8.8**). While ryegrass growing on an uncontaminated soil contained significantly reduced concentrations of arsenic compared to all other treatments. Analysis of soil properties show that this uncontaminated soil contains very high concentrations of Olsen P (**Table 8.1**) which has the ability to out-compete arsenic for uptake and decrease its concentration within plant tissues (Meharg & Macnair, 1991; Quaghebeur & Rengel, 2003; Zhao et al., 2008). It has also been suggested that high phosphate concentrations within soil may have a protective function against arsenic phytotoxicity by increasing the activity of a number of enzymic components of the antioxidant pathway (Gomes et al., 2013).

Ryegrass growing in the contaminated soil exhibited a reduction in total chlorophyll content under all treatments compared to that growing in uncontaminated soil. This suggests a phytotoxic response to arsenic uptake, although this reduction was only significantly evident under the contaminated control and 30 t ha^{-1} 350°C biochar amended soil. Analysis of ascorbate peroxidase activity (APX) which is responsible for the catalysation of H_2O_2 suggests that the cytosolic fraction is reduced under these two treatments, and possibly resulting in a slightly higher ROS concentration in the photosynthetic apparatus. ROS are produced naturally in plant tissues and are often known for their oxidative signalling and include superoxide anions ($O2^-$) and H_2O_2 (Foyer & Noctor, 2005). Their level is controlled by the antioxidant system but when this system is overcome, plant tissues can undergo oxidative stress (Mates, 2000). In

regard to chloroplasts, the major defence mechanism in detoxifying H_2O_2 is the ascorbate-glutathione cycle which detoxifies H_2O_2 to water and oxygen.

In response to arsenic uptake, an increased activity of superoxide dismutase (SOD) but decreases in activity of guaiacol peroxidase (GPX) was observed in all treatments. Increased uptake of arsenic did not affect catalase activity (CAT) as evident by similar activity between ryegrass grown on contaminated and uncontaminated soil (**Figure 8.11**). This is not suprising as CAT is one of the most efficient enzymes and cannot be saturated with H_2O_2, meaning even moderately elevated levels of H_2O_2 may only cause a slight increase in total CAT activity. This is in agreement with Khan et al (2009) who studied the effect of arsenic uptake in Indian mustard (cv. Pusa Jai Kisan) and found that at increasing concentrations of arsenic resulted in significant increased activity of SOD and APX but activity of CAT remained steady (Khan et al., 2009).

Phytotoxicity or tolerance to arsenic caused by decreased/increased antioxidant responses has also been documented in a number of different plant species including hyperaccumulators (Srivastava et al., 2005; Gunes et al., 2009; Khan et al., 2009; Shri et al., 2009; Srivastava et al., 2010). In a natural arsenic hyperaccumulator *P.vittata*, increased accumulation of arsenic resulted in higher activity levels of of SOD, CAT and APX in comparison to non-hyperaccumulators yet no differences were observed for GPX (Srivastava et al., 2005). In comparison, oxidative stress was effected in chickpea under arsenic stress. Seedlings grown in soil spiked with 60 mg kg^{-1} of arsenic contained reduced SOD compared to 0 mg kg^{-1} arsenic and resulted in reduced shoot growth (Gunes et al., 2009). Interestingly, activity of both CAT and APX were increased in response to a reduction in SOD in the study.

The observed increase in SOD activity under all treatments between 90 and 180 d of treatment suggests that inactivation of SOD and its catalysation of O^{2-} to H_2O_2 has not occurred which would result in oxidative stress. Consequently, the production of H_2O_2 in plant tissues from this reaction has caused a change in antioxidant profiles between treatments for APX and GPX. As mentioned previously the ascorbate-glutathione cycle is responsible for the removal of H_2O_2 from chloroplasts and consists of non-enzymic

and enzymic components to carry this out. When arsenic in taken up by plants, a major non-enzymic component of this cycle, glutathione, is responsible for synthesising phytochelatins, the main mechanism for forming a detoxifying complex around arsenic. If the available pool of gluthione is being reduced by synthesising phytochelatins then it is possible that this cycle is not performing effectively and may lead to oxidative stress (Foyer & Noctor, 2005; Logan et al., 2006; Krieger-Liszkay et al., 2008). This effect can also reduce the available pool for substrates (such as glutaredoxins) that GPX require to function which may explain the decrease in GPX activity with time as seen in this study.

8.5.2 Metabolite Profiling in Response to Soil Amendment

Ryegrass grown on biochar amended soils generally had different metabolite profiles compared to both control soils (**Figures 8.18 and 8.23**) while ryegrass grown on the uncontaminated soil was significantly different to the other three treatments.

Analysis of the HILIC Negative (**Figure 8.21**) stream which detects a number of organic acids, mono/disaccharides, low DP fructans, and sugar alcohols resulted in the identification of $_{D}$-glutamate being upregulated in both contaminated control and uncontaminated control grown ryegrass tissue. This metabolite is a potent inhibitor of glutathione synthesis which is responsible for synthesising phytochelatins. This provides some evidence towards why the control grown ryegrass has low total chlorophyll content due to the possibility of increased arsenic phytotoxic reactions with plant metabolic functions.

Analysis of the C18 Positive stream (**Figure 8.17**) detected very high upregulation of IAA under biochar amendment. IAA is a plant hormone and its functions include regulating cell growth and stimulating increased rooting. Under biochar amendment, ryegrass underwent increased root growth (**Figure 8.6**) compared to the control and was increasingly significant under 350°C biochar amendment (60 t ha^{-1}), suggesting a growth effect mediated by higher concentrations of IAA.

Overall metabolite profiling suggests that ryegrass grown on contaminated soil differs markedly compared to ryegrass grown on uncontaminated soil for aromatic acids, lignins, short chain fatty acids (C18 Negative); organic acids, mono/disaccharides, low DP fructans, sugar alcohols (HILIC Negative) and amino acids (HILIC Positive). But in comparison to ryegrass grown on contaminated control soil, ryegrass grown on biochar amended soil has an unrelated metabolite profile in respect to upregulation/downregulation for organic acids, mono/disaccharides, low DP fructans, sugar alcohols (HILIC Negative) and amines, flavonoids, condensed tannins (C18 Postive) suggesting that either an increase in arsenic uptake under these treatments or as a direct effect of biochar stimulating soil microbial activity and increasing element soil element concentrations is causing this change in metabolite regulation.

8.5.3 Conclusion

Increased arsenic uptake as a function of biochar amendment has resulted in increased enzymic activity of SOD and APX under most biochar treatments but across all treatments a reduction in GPX activity is observed. This could be in response to a reduced gluthione pool causing a decrease in available substrate for the ascorbate-gluthione cycle to be functioning effectively for the detoxification of arsenic. Metabolomics also suggest a definitive metabolite profile under biochar amendment compared to the contaminated control ryegrass samples.

Compared to the control, ryegrass grown on biochar amended soils is able to extract and uptake significantly more arsenic and yet overcome the phytotoxic damage that usually occurs with increased arsenic uptake. This is likely due to a change in metabolite profiling and changes in enzymatic activity of the antioxidant pathway. It is clear that there are limitations to this work in regards to optimal conditions being provided as part of this glasshouse study. Metabolite profiling and enzyme activity are likely to fluctuate under field conditions.

The study of key metabolite upregulation/downregulation and corresponding changes to antioxidant profiles allows for a better understanding of how plant species are able to cope with plant stress. If metalloid toxicity such as arsenic uptake can be tightly controlled then plants being used in a glasshouse or field trial setting can efficiently deal with the stress, without causing negative flow-on effects such as decreased biomass, reduced soil mining by plants roots and breakdown of chlorophyll. Here, it has been observed that ryegrass grown in contaminated soil amended with biochar exhibits increased biomass and increased chlorophyll content compared to the control. These attributes allow for a plant species (in this case ryegrass) to perform more effectively as a phytoextraction species.

CHAPTER 9 : GENERAL DISCUSSION AND RECOMMENDATIONS FROM THIS RESEARCH

9.1 Overview of the Current Research

The practice of sheep dipping, which subjected livestock to inorganic and organic agricultural pesticides to eradicate pests such as lice and keds, is a historic practice; sheep dipping is no longer practiced in New Zealand today. The use of pesticides such as arsenicals and organochlorines is now banned by law due to their persistence in the environment. The starting point for the research described in this thesis was an investigation of the extent of contamination resulting from historical sheep dipping at a known historic dip site in Te Mahia, New Zealand. This investigation, begun in 2010, was conducted to determine if the presence of arsenic and organochlorines in the environment presented any current-day risk to environmental ecosystems and potentially to human health. To provide an overview of contamination at the site, soil mapping of arsenic concentration and organochlorine analysis was carried out.

This overview contamination mapping exercise revealed extensive contamination of land by arsenic and a range of organochlorines (lindane, DDT, dieldrin, aldrin). The study site was therefore co-contaminated with both arsenic and organochlorines. Fresh water and aquatic plants (watercress) downstream of the inferred likely discharge point of drainage from the dip site had arsenic concentrations that exceeded WHO guidelines for water and food for a number of tested samples. To manage the effects of these chemicals, an environmental management intervention was planned and implemented, based on phytoremediation and conjunction with soil amendment with biochar. This intervention was designed to mitigate the risk of soil contaminants through phytoextraction and microbial degradation during a series of glasshouse and experimental field trial studies.

9.2 Discussion of Research Objectives

This research was designed to understand the potential of biochar to affect contaminant properties and processes in contaminated soil and to define how biochar could be used to change soil-contaminant dynamics and parameters associated with plant growth. The main objective was to investigate the environmental impact associated with soil contaminants found at sheep dip sites and to ascertain whether

the use of biochar and phytoextraction technologies could be integrated into a management plan to reduce this impact.

9.2.1 Analysis of Soil Microbial Activity and Microbial Community

Microbial activity as measured by the dehydrogenase assay defines the activity of the entire microbial communtiy within the soil. Amendment of soil with both biochars led to an immediate and significant increase in soil microbial activity (**Figures 5.1, 5.2, 6.1, 6.2**).

This stimulation of activity is suggested to be related to biochar acting as a source of carbon (labile C) that is used as a energy source for increased metabolic activity. The addition of 350°C biochar to contaminated soil (**Figure 5.1A**) with no plant growth resulted in activity of ~180 µg g DM (± 0.9) after 180 d compared to ~170 µg g DM (± 8.9) for 550°C biochar amended soils and ~100 µg g DM (± 6.8) for the control treatment (no amendment). Analysis of these biochars showed that the 350°C biochar had a slightly higher volatile C content (3.3%) compared to the 550°C biochar and that this labile C fraction was likely being used by soil microbes until its exhaustion at 120 d of treatment (**Figures 5.1A, B**). The study reported in Chapter 5 is a non-planted study. The addition of root exudates through the introduction of plants to the contaminated environment in Chapter 6 showed that the addition of an additional source of available carbon (in the form of exudates for all planted soils) is able to further stimulate soil microbial activity (**Figures 6.1, 6.2**). The addition of these exudates resulted in a steady rate of increase in microbial activity under all treatments due to the increased form of carbon (exudates) resulting in higher microbial activity.

Metagenomic analysis of the soil bacterial community in the non-planted pots revealed the presence of 12 named bacterial phlya with a shift (in %) for phyla in biochar amended soils (**Table 5.4**). Of interest, was a shift towards a number of known bacterial family, order, and species in biochar amended soils that have been reported in the literature as beneficial for the bioremediation of hydrocarbons (**Table 5.5**). The soil in the biochar-amended experimental treatments more closely modelled a technological bioremediation environment than the control pots, and this similarity

was achieved without attention to the microbiological status of the co-contaminated soil.

9.2.2 Degradation of Organochlorines as a Function of Biochar Amendment

The increased soil microbial activity promoted in this research by biochar amendment of soil caused a significant increase in the degradation of lindane, DDT (and its products), and the two isomers of hexachlorocyclohexane alpha-HCH and delta-HCH (**Figure 5.2**). This degradation is proposed to have been caused by soil microbes via the process of reductive dechlorination, although it is unknown which microbes were the main degradative members of the bacterial community.

Analysis of metagenomic DNA of the bacterial community revealed that biochar-amended soils were selecting for a number of known bioremediating agents of chlorinated hydrocarbons. However, in some cases (especially for the family Pseudomonadaceae) biochar amended soils (550°C) were shifting away from these bacterial members. This provides a snap shot of the entire, known, bacterial community within the soil but does not give analysis of the dormant or active nature of these microbes. The active microbial community was therefore quantified using the dehydrogenase assay which suggests that even though a number of these bioremediating agents have been selected against in biochar amended soils, they could actually be more active in their degradation of organochlorines.

9.2.3 Effect of Biochar Amendment on Soil Properties and Plant Growth

Biochar amendment of soil resulted in an increase in soil pH with increased rates of application (**Table 5.3, 6.1, 8.1**). Generally, soil elemental status (concentration of Mg^{2+}, SO_4^{2-}, Ca^{2+}) and CEC was increased as a function of biochar amendment, however Olsen P levels were slightly decreased in amended soil due to the dilution effect of biochar on soil volume. Biochar produced from willow feedstock will contain some inherant fertility and beneficial physio-chemical properties (**Table 5.1**), explaining the obseved increase in soil fertility and properties relating to microporosity and surface charge that are proportional to application rate.

Increased soil fertility in biochar amended soils led to an increase in the shoot and root biomass (**Figure 6.3, 8.5, 8.6**) of *Lolium perenne* in two controlled glasshouse studies. Metagenomic analysis also provided evidence that biochar amendment was shifting and selecting for certain bacterial orders and species. Three agents, that being Pseudomonadaceae, Streptophyta and Bacillus are known plant growth promoting rhizobacteria (PGPR) agents and were observed to be selected for in biochar amended soils. These bacteria exhibit traits such as the production of siderophores (which make elements such as iron available for plant uptake), production of phytohormones (such as auxin) and solubilisation of previously bound forms of soil phosphate (Singh et al., 2011). Interestingly, ryegrass grown on biochar amended soils exhibited significantly upregulated levels of auxin (IAA) in shoot tissue, possibly from the production of phytohormonic levels of auxin supplied by PGPR (**Figure 8.17**). An upregulation of auxin also provides evidence towards the observed increase in root growth under biochar amendment. Increased microbial activity observed in biochar amended soils suggests that PGPR could be playing an active role in plant growth increases.

9.2.4 Plant Response to Arsenic as a Function of Biochar Amendment of Soil

Lolium perenne exhibited significantly higher concentrations of arsenic within shoot tissue as a function of biochar amendment relative to a control treatment at the termination of two glasshouse trials (**Figure 6.5, 8.8**). Compared to ryegrass grown on an uncontaminated soil (soil containing 26 mg kg^{-1} total arsenic), ryegrass grown on contaminated soil (soil containing ~150 mg kg^{-1} total arsenic) exhibited the onset of chlorotic symptoms, resulting in a decrease in total chlorophyll (**Figure 8.9**). However, there was no significant difference ($P<0.05$) in chlorophyll content in response to the total arsenic concentration in ryegrass shoot tissue grown on contaminated soil. The observed increases in activity of SOD, APX and steady CAT activity is suggested to be efficiently catalysing the production of harmful ROS in this soil. Increased arsenic concentration in shoot tissue grown on biochar amended soils is suggested, in this case, to be affording no increase in oxidative stress relative to ryegrass tissue grown in the contaminated soil and acting as a control. The increased concentration of arsenic

in ryegrass grown on biochar-amended soil is not detrimentally affecting the health and growth of this pasture species when compared with the contaminated control.

9.3.4.1 Significance of Arsenic-Phosphate Interactions at Study Site

The unusually high plant-available P concentrations in the soil (analysed as Olsen P) could have affected the results recorded in this study. The Olsen P concentration is soil used for the studies in Chapter 7 and 8, collected from the flat area around the dip site reached as high as 200 μg P g^{-1} i(**Table 7.3 and Table 8.1**). However, the soil collected for the study described in Chapter 6 had an Olsen P concentrations between 40-50 μg P g^{-1} (**Table 6.1**). The higher Olsen P concentrations are possibly the result of a nearby phosphate fertiliser storage area. yet its exact proximity to the dip is unknown (J.Bowen, pers. comm), although, a build up of sheep faeces (P fertiliser source) on this land cannot be ruled out (M.Hedley, pers. comm).

Fertiliser and soil amendments control the cycling and mobility of arsenic through soil. Phosphate belongs to the same chemical group as arsenic and both have comparable disassociation constants for their given acids (Mahimairaja et al., 2005). This similarity also means that they compete strongly for soil colloid binding sites. In doing so, a natural high abundance of phosphate or intensive nutrient additions can cause the leaching or increased mobility of arsenic through the soil profile. In this case, it is possible that high Olsen P concentrations in soil may promote the leaching of arsenic through the soil profile. This result thus, could explain the increase of arsenic concentration with depth through the soil profile (**Figures 4.5-4.7**) yet analysis of the water-extractable concentration of arsenic was comparable (<1% of total soil arsenic) across all control treatments (contaminated control, Chapter 5 and 6; uncontaminated control, Chapter 8). Further identification of phosphate-arsenic dynamics with soil properties such as pH, organic matter, redox and clay content may complex the interaction in relation to extractable arsenic and leachability (Carrillo-Gonzalez et al., 2006; Zhang & Selim, 2008).

The most significant interaction of increased Olsen P concentrations in regards to arsenic is likely to be arsenic uptake (phytoextraction) and consequently remediation

times for contaminated soil. Due to arsenic having a high chemical affinity/similarity with phosphate it is observed from physiological and electro physical studies that arsenate shares the same transport pathway in higher plants, yet phosphate transporters will select phosphate for transport if both elements are in abundance (Quaghebeur & Rengel, 2003). This attribute would result in low arsenic uptake if soil Olsen P concentrations are high such as those analysed at the study site. Yet a number of studies have shown that high phosphate concentrations may actually promote arsenic uptake due to increasing plant biomass. Jankong et al. (2007) observed increased plant biomass and arsenic accumulation in *P.calomelanos* in response to increased usage of P-fertilisers while Purdy & Smart (2008) observed that phosphate fertilisers actually alleviated arsenic phytotoxicity and caused an increase in arsenic accumulation within willow tissue.

High soil arsenic and Olsen P concentrations at the trial site thus could be providing ideal conditions for arsenic phytoextraction while alleviating possible phytotoxic symptoms caused by arsenic uptake yet it is difficult to gauge whether this scenario is occuring or not. Ryegrass tissue analysed (Chapter 8) revealed that chlorophyll content decreased in response to increased arsenic uptake in ryegrass grown on soil containing 145 mg kg^{-1} arsenic and 47 µgP g^{-1} compared to stable chlorophyll content in ryegrass grown on an uncontaminated soil, that having 20 mg kg^{-1} arsenic and 202 µgP g^{-1} (**Table 8.1, Figure 8.9**).

9.3 Research Summary

Extensive soil mapping of a historic sheep dip site located on the East Coast of New Zealand revealed significantly high arsenic concentrations in soil to a depth of 30 cm and WHO guideline values exceeded for organochlorines. Further, water and aquatic samples in the proximity of the dip exceeded health guidelines for arsenic.

The addition of biochar as a soil amendment under glasshouse conditions resulted in immediate and significantly higher microbial activity. Analysis of soil bacterial DNA through metagenomics revealed that biochar amendment was causing a shift (%) in a number of bacterial phyla and in some cases increasing the proportion of phlya known

for their bioremediation properties. After 180 d under glasshouse conditions, biochar amended soils exhibited highly reduced soil concentrations of lindane, alpha-HCH, and DDT and its derivatives.

An increase in soil fertility was also noted in biochar amended soils which resulted, in part, to an increase in root and shoot biomass of *Lolium perenne*. By increasing biomass, plant phytoextraction was also be increased. Analysis of arsenic mobility showed that the water-extractable fraction of arsenic in soil was reduced compared to the control over the course of 90 d, but re-solubilised to concentrations similar to the control at 180 d. This increased the phytoextraction of arsenic into an indicator plant species, *Lolium perenne* in biochar amended soil. In response, shoot tissue of ryegrass exhibited an increase in enzymic activity to prevent oxidative stress. Analysis of the metabolic profile for ryegrass grown on biochar amended soil showed that metabolites (untargeted) like $_{L}$-glutamate, a potent inhibitor of glutathione synthesis (the main mechanism for arsenic detoxification) were downregulated but upregulated in control ryegrass tissue. Biochar potentially could be acting as a primer for plants grown in biochar amended soils to upregulate specific defensive pathways / or metabolites to protect against metalloid-induced oxidative stress.

By increasing both arsenic uptake and biomass during two glasshouse trials and arsenic uptake in a high biomass crop in the field (under one amendment) it was calculated that potential remediation times could be reduced by between 53 and 91%, providing an appealing option for regional councils and contaminated landowners for remediation.

9.4 Relevance of This Research to a Remediation Strategy for Co-contaminated Sheep-Dip Sites : Recommendations for Future Work

Historic sheep dip sites represent perhaps New Zealand's most significant, but understated, environmental challenge. New Zealand has an estimated 50, 000 dip sites found throughout the country, mostly found on present-day farmland. Contaminated land that has an origin with dipping has recently been discovered in sub-divided residential properties, and an escalation of such findings can be expected due to urban

encroachment on past farmland throughout New Zealand. Land use change provides a direct exposure pathway for humans towards these chemicals. Justification for the conjoint use of biochar and phytoextraction to remediate such sites soils is therefore strongly grounded in the idea of 'sustainable remediation', that being, the undertaking of soil remediation with an end goal of reducing soil contaminants that are detrimental to ecosystems and food sources where the cost of action is less outweighs the cost of ignoring the problem.

Justification for the design of a coupled biochar-phytoextraction system to be used in the treatment of co-contamination at historic dip sites soil has been developed and advanced for a number of reasons. The technology is based on natural products and processes which align with cultural value, specifically Maori cultural values in New Zealand. The conventional mechanism to remediate contaminated land associated with sheep dip sites is to extract soil using excavators and to transfer this to contaminated landfill sites (or any recycling centre that can deal with contaminated waste). This can often be an expensive process and culturally speaking, unacceptable.

Biochar as used during this research increased soil pH and this resulted in a change in the mobility of arsenic, leading to enhanced phytoextraction. To further justify the biochar strategy or implementation in this case, biochar is designed to have a soil liming effect similar to the addition of calcium carbonate but also, to mitigate a farmers carbon footprint by offsetting and storing carbon away in the soil. Essentially, it is hoped that this system would provide farmers or contaminated land owners a 'win-win' situation where remediation times are reduced while limiting their carbon footprint.

The eventual design of a closed-loop sustainable remediation plan for the management of historic sheep dips is the final step for the implentation of this technology (**Figure 9.1**). Initially, biochar would be added to contaminated soil and planted with high biomass plant species such as willow. After a period of growth, plants are harvested and contain the phytoextracted arsenic. This contaminated material is then dried, coppiced, and returned to a pyrolyser reactor to produce fresh

biochar. When this biochar is produced, arsenic is volatilised from the biochar and trapped within a built-in filter within the pyrolyser, resulting in clean biochar and a filter containing arsenic that can be returned for manufacturial use (arsenic is used in the manufacture of semi-conductors and electronics).

Recommendations for future research before this coupled technology could be implemented nationwide for the remediation of these historic co-contaminated soil include:

- Analysis of biochar interactions in different sheep dip contaminated soils (i.e recently formed, volcanic, sandy) under controlled conditions and utilising a high biomass crop for phytoextraction
- Analysis of leachates if flooded soil conditions are used to determine the likelihood of increased arsenic leaching in the field
- Analysis for the specific fraction of arsenic available for plant uptake (bioavailable As-fraction)
- Detailed investigation into As-P interactions, especially in response to phytotoxicity and As uptake into plant tissues.

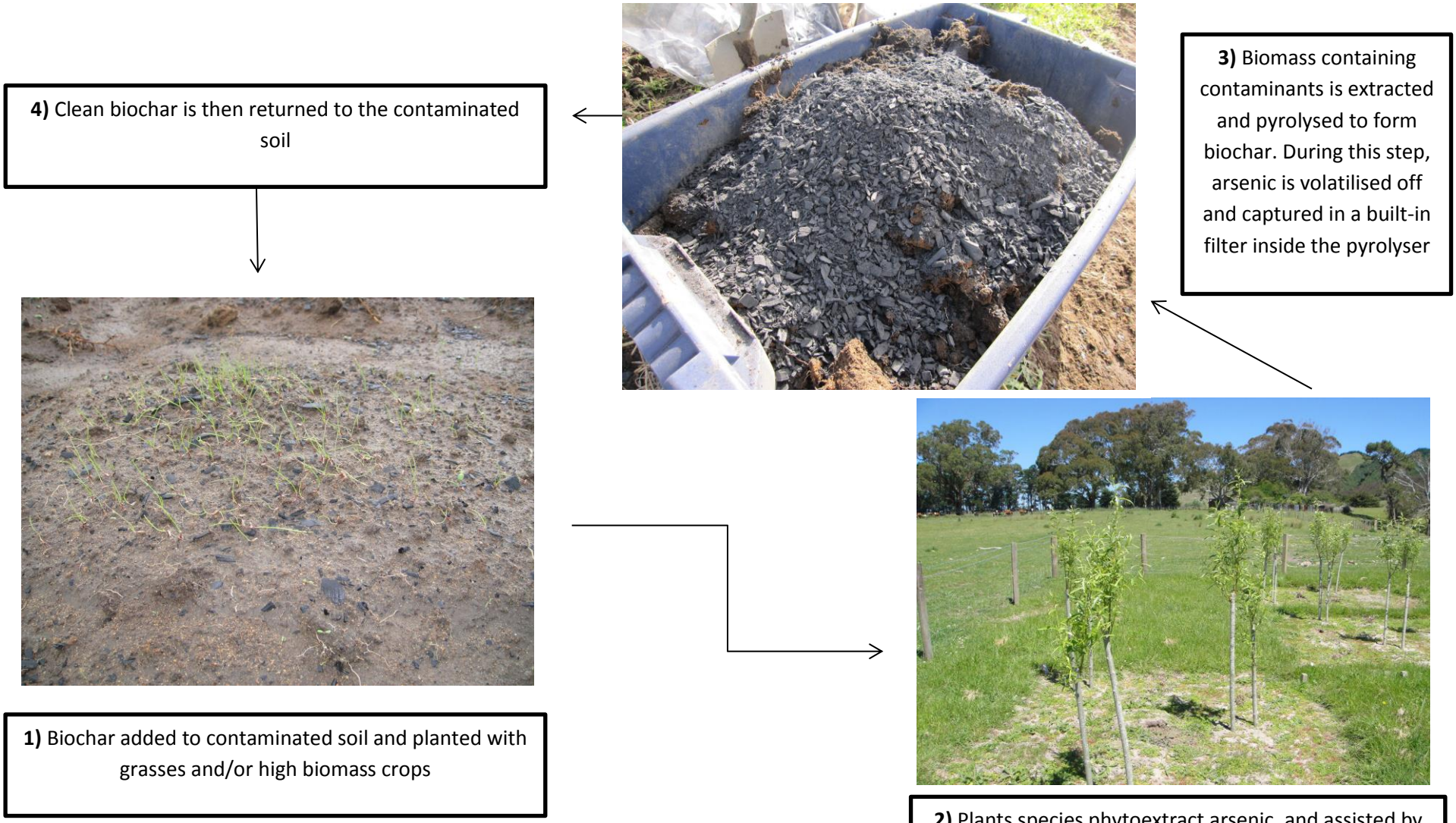

Figure 9.1. Flowchart depicting eventual closed loop remediation system for biochar-phytoextraction treatment of historic sheep dip soil

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APPENDIX A

	Control	550L	550H	350L	350H
Na	7.4 ± 2.3	10.0 ± 1.2	10.4 ± 1.4	10.5 ± 1.8	10.6 ± 1.2
Ca	17.8 ± 2.1	23.1 ± 2.6	22.6 ± 2.5	26.8 ± 3.1	26.7 ± 2.8
Mg	11.4 ± 0.8	11.8 ± 0.2	12.7 ± 0.5	13.1 ± 0.5	12.5 ± 0.9
K	46.9 ± 2.1	46.6 ± 2.2	46.3 ± 1.8	49.9 ± 3.2	46.6 ± 3.0

Selected elemental composition of ryegrass shoot tissue under differing amendments at T = 180 d. Concentrations are in mg kg^{-1} (values are mean ± s.e. *n* = 3).