Accumulation of zinc and its role in plant defence

By

Samuel Valbonesi

A thesis submitted for the Degree of Doctor of Philosophy
of the University of Sussex

Department of Biology and Environmental Science
School of Life Sciences
University of Sussex

Submitted September 2009

Declaration

I hereby declare that this thesis has not been and will not be, submitted in whole or in part to another university for the award of any other degree.

Signature:…….(Samuel Valbonesi)

Acknowledgements

My biggest thanks goes first and foremost to my supervisors, Professors Sue Hartley and Mike Ramsey, without their help, advice and encouragement, this thesis would not have happened. Thanks for not giving up on me.

Thanks to the Natural Environment Research Council (NERC) and Rio Tinto for the funding that made it possible for me to do this research. Dr Phil Potts for accompanying us to Derbyshire and the use of his P-XRF. Thanks to the following for all of their technical support over the last three years, Dianne Ellis, Mick Henry, and Chris Dadswell.

A special thank you goes to Dr Boon, Dr Ewald and David Fisher-Barham and all my friends from 5b1 (past and present) for all their help in the lab and proof reading of my work.

Lastly, but not least I would like to thank all of my family, for their love and support during my time as a DPhil student.

University of Sussex

Samuel Valbonesi

A Thesis submitted for the Degree of Doctor of Philosophy

Accumulation of Zinc and its role in plant defence

Abstract

The elemental defence hypothesis was first put forward by Boyd and Martens in 1992. It suggested that plants concentrate high levels of heavy metals into their biomass to act as a defence against herbivory. This thesis focused on testing this theory using zinc (Zn) as the accumulated element.

Several plant species, including *T. caerulescens*, are known as hyperaccumulators of Zn and can contain concentrations greater than 10,000 mg kg^{-1}. The research used a novel technique to assess the levels of Zn in this plant species in the field.

Other plant species, which contain more than 2000 mg Zn kg^{-1} but less than hyperaccumulators, are known as accumulators. They have received increased amounts of attention because research has shown that these concentrations can still have a negative impact on herbivores (Coleman *et al*., 2005). One of these accumulators, *B. juncea*, is a fast growing species, well suited to test the defensive qualities of Zn against herbivores and so was the focus of the rest of the thesis.

B. juncea was tested to see if the uptake of Zn was inducible by assessing if there was an increased uptake of Zn after plants were subjected to attack by the herbivore *H. aspersa*. Previous research (Hodge *et al*, 2000) has shown that many plants are capable of increasing their concentration of defence compounds following herbivore attack. However, damage did not increase Zn uptake in *B. juncea*, so this elemental defence does not appear to be inducible. The preference and performance of herbivores (juvenile and adult *H. aspersa* and larvae of *P. brassicae*) on leaves of *B. juncea* containing high and low concentrations of Zn was tested. It found that the growth rate of both juvenile snails and larvae of *P. brassicae* was reduced by a diet of leaves high in Zn concentration and both species selected leaves low in Zn in preference tests, suggesting that Zn is an effective defence.

In the final experimental chapter, the thesis evaluates the defensive properties of Zn in *B. juncea* plants grown in the field. In contrast to the earlier experiments, this field experiment found that plants containing elevated concentrations of Zn were significantly more damaged than those that had been grown on a control treatment, a result which goes against the elemental defence hypothesis. The thesis concludes by suggesting that although increased concentrations of Zn may have an impact on the growth and behaviour of herbivores in the laboratory, these effects do not necessarily occur under field conditions.

Table of contents:

List of tables:

Chapter 1:

Chapter 2:

Chapter 3:

Chapter 4:

Chapter 5:

Chapter 6:

List of Figures:

Chapter 1:

Chapter 2:

Chapter 3:

Chapter 4:

Chapter 5:

Chapter 6:

Chapter 1: Introduction

1.1 Chapter overview

This chapter highlights the rationale and purpose of undertaking the research for this DPhil. Beginning by outlining the environmental issue of contaminated land, it then puts forward the reasons for investigating the study metal, zinc (Zn). It then discusses the remarkable traits of hyperaccumulator plant species, plants that are capable of concentrating high concentrations of heavy metals in their above ground biomass. Following this, it summarises the current hypotheses as to why plants hyperaccumulate heavy metals. It then focuses its attention on the hypothesis that has received the most amount of research, and is the focal point of the thesis, the elemental defence hypothesis. It follows on by considering the plant species *Thlaspi caerulescens*, a 'model' hyperaccumulator of Zn and summarises the relevant published work to date. *Brassica juncea*, an accumulator of heavy metals including Zn, is then reviewed as a possible alternative to the use of *T. caerulescens* in experiments for assessing the plant defence hypothesis. Having examined possible plant species for experimentation, the chapter then focuses on possible herbivore species to be used in experiments to test the elemental defence, by examining the impact of Zn on herbivores in any published literature. Lastly, the chapter introduces the hypothesis of the thesis and concludes with the outline of the chapters to follow.

1.2 Heavy metal contamination: A global problem

One of the major environmental hazards that must be addressed internationally is contaminated land. This is defined in England and Wales by part IIa of the Environmental Protection Act of 1990 as:-

> *"Land which appears to the local authority in whose area it is situated to be in such a condition, by reason of substances in, on or under the land, that:*
>
> *(a) Significant harm is being caused or there is a significant possibility of such harm being caused; or*

(b) Pollution of controlled waters is being, or is likely to be, caused" (DETR, 2000).

In 2002 the Environment Agency (EA) estimated that there were 100,000 sites across England and Wales that qualified as coming under the title of contaminated land (DEFRA, 2008), with approximately 20,000 sites requiring treatment (EA, 2004). These types of estimates are not isolated to areas of the UK, but are a small proportion of a global problem. For example, in 1990 the US predicted that there were 31,000 sites which required some form of remediation, generated from mining activity alone (Moore and Luoma, 1990); increased mining activity over the past 20 years would have increased this number (Tolcin and McNair, 2007). The number of different pollutants that can contaminate land is broad and extensive, but the group of contaminants that are of particular interest to this thesis are heavy metals. For the purpose of this thesis metals will be defined as elements with an atomic number greater than 20 and that possess typical metallic properties i.e. conductivity and ductility (Raskin *et al*., 1994).

Heavy metal contamination in soils can occur from natural sources, such as those present in sulphide minerals (Podar *et al*, 2004), although the vast majority of contamination is from a range of anthropogenic activities. Table 1.1 describes the most significant of these.

Sector	Pathway to Environment
Mining	Smelting, furnace slag and flute dust, recycling and disposal of waste from mine ore (Han *et al*, 2002 ; Moore and Luoma, 1990)
Agriculture	Application of sewage sludge, livestock manure, compost fertilizers and agrochemicals to arable land (Nicholson *et al*, 2003).
Motor Vehicles	Emissions from vehicles, depositing heavy metal particulates at the road side and further afield that increase in concentration with increased emissions (Bilos *et al*, 2001; Viard *et al* 2004).

Table 1.1: Anthropogenic sources of heavy metal contamination in soils

Han *et al*., (2002) suggests that there are seven heavy metals in particular that present the greatest danger to ecosystems, these are cadmium (Cd), copper (Cu), chromium (Cr), mercury (Hg), lead (Pb), nickel (Ni) and zinc (Zn). These seven heavy metals are often the cause of land being classified as contaminated. At present the UK government uses soil guideline values (SGV's), produced by DEFRA (Department for Environment, Food and Rural Affairs) and the EA, governmental bodies, to determine whether land is contaminated with heavy metals. At present there are 7 generic heavy metal SGV's, available for the following metals: As, Cd, Cr, Hg, Ni, Se and Pb. The SGV's have been introduced to replace the old ICRCL (Interdepartmental Committee on the Redevelopment of Contaminated Land, 1987) which assigned target values that soils had to be below, dependent on the land use, to be considered safe. Even though many scientists and regulators now deem them out of date, as they can no longer be used for regulatory decisions, for the purpose of this thesis the ICRCL trigger value for Zn (300 mg kg^{-1}) will be used (as an indicator for contaminated land) as no SGV has been produced as it is only phytotoxic and not harmful to human health. In addition, although Zn is an essential metal for all plant species (at a concentration between 10 – 80 mg kg^{-1}) for the production of enzymes, while also thought to play a role in protecting plants from drought and disease. At concentrations in excess of >300 mg kg^{-1} (equal to the ICRCL trigger value) it can be phytotoxic to plants, by either stunting growth or killing the plant completely (Emsley, 2001, Kabata-Pendias, 2001).

From the seven metals named by Han *et al*., (2002) above, Zn has been chosen as the target metal to be studied in this thesis. Zn is the 24^{th} most abundant element on the planet. It is an essential component for protein production in all terrestrial life (Barak and Helmke, 1994) and is the second metal only to iron in terms of abundance in living organisms (Broadley *et al*., 2007). Zinc is a commercially valuable material used in the production of brass and other Zn based alloys, it is also used in the galvanization of other metals to reduce corrosion to name but a few uses. At present, it is still in the top ten most demanded heavy metals, with the US alone mining 63,500 metric tonnes in the year 2007 (Tolcin and McNair, 2007). While mining activity and demand for Zn remains high, so will the production of waste contaminated with Zn produced by the mining, smelting, processing,

recycling and disposal of the Zn. These activities are all sources and potential pathways to contaminate land (Han *et al*., 2000, 2001, 2002). So there is still a strong motivation for undertaking research on Zn uptake by plants, particularly those plants that are capable of hyperaccumulating Zn into their above ground biomass. A large amount of land is contaminated and a range of plants are capable of dealing with the contamination in the soil.

1.3 Hyperaccumulator plant species

Land contaminated with heavy metals at very high concentrations may become barren due to the phytotoxic effects they have on plant life. Even essential micro-nutrients, such as Zn and Cu, may prove to be deadly to vegetation if they are present in the soil above a certain concentration. For instance, Zn is essential in the production of thousands of proteins in plants, but beyond a soil concentration of 300 mg kg^{-1} can produce toxic effects such as biomass reduction and fatality of the plant (Broadley *et al.*, 2007, Markert, 1993).

However, a number of plant species have evolved to cope with these potentially fatal concentrations of metals in soil (Cobbett, 2003). There are two methods by which plant species can cope with the stress of phytotoxic concentrations of heavy metals in soil. Firstly by the prevention of the uptake of metal into the plant; these species are known as excluders. Secondly, by the accumulation of the metal(s) in the plant's above or below ground biomass. Plant species that adopt the accumulation method can be further subdivided into three categories (Figure 1.1). Indicators are plants that have concentrations of metals in their above ground biomass that directly reflect concentrations found in the soil (Baker, 1981; McGrath *et al*., 2000). Accumulator species accumulate metal concentrations above that of the soil concentration and that found in surrounding vegetation (Reeves *et al*, 1999; Reeves and Baker, 2000; Coleman *et al*, 2005). Lastly, hyperaccumulators are plants that capable of accumulating extraordinary high concentrations of metals in their above ground biomass even when soil metal concentrations are relatively near background (Baker, 1981; Brooks *et al*., 1977; Morrison *et al*., 1980). The subdivision of plants that are accumulators and hyperaccumulators of metals is relatively arbitrary, but is discussed later.

The concentration factor of metals in plants can be defined as below.

$$\text{Concentration Factor (CF)} = \frac{\text{Metal concentration in the plant (mg kg}^{-1}\text{)}}{\text{Metal concentration in the soil (mg kg}^{-1}\text{)}}$$

The concentration factor of metal tolerant plants differs depending on the plants response to an increasing metal concentration in the soil. Hyperaccumulators will achieve a high concentration factor even when the metal concentration in the soil is relatively low, however, as the metal concentration in the soil increases only a minimal increase in the metal concentration in the plant will occur. While the concentration factor in an indicator species will increase approximately linear with increased metal concentration in the soil. An excluder meanwhile will retain an extremely low concentration factor until the concentration reaches such a high concentration that the plant is unable to prevent the metal entering.

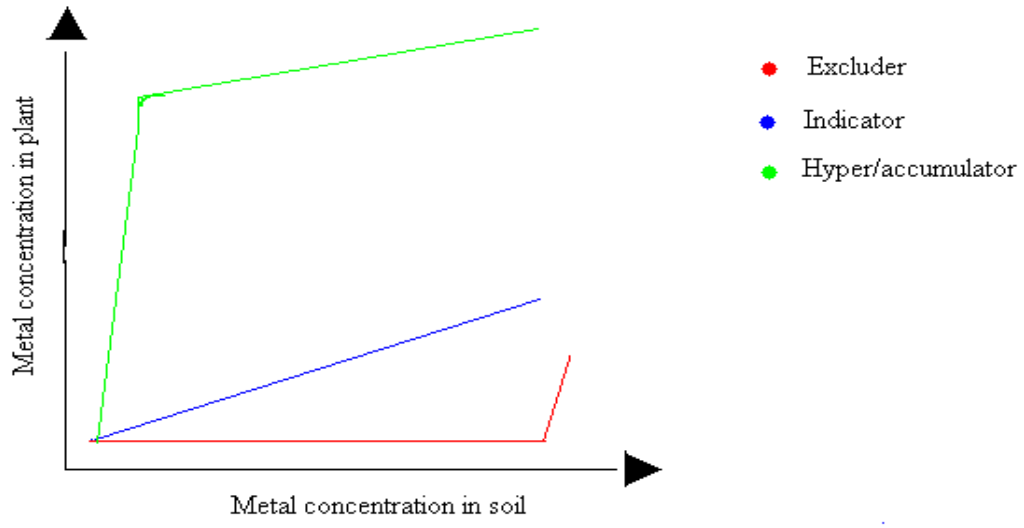

Figure 1.1: Response of plants tolerant to heavy metals with increasing metal concentration in soils. Adapted from work by Baker, 1981; McGrath et al., 2000.

1.3.1 Classification of hyperaccumulator species

Peterson in 1971 was the first author to define the two ways in which metals accumulate in plants, namely (1) the accumulation of an element within a plant to a concentration that exceeds that of the growth medium, (2) the possession of greater concentrations of an element than is usual for that organism. Six years later Brooks *et al.*, (1977) defined the concept of hyperaccumulator thresholds, i.e. values that if a plant is capable of concentrating pollutants equal to or above these thresholds then they may deemed as a hyperaccumulator of that contaminant. These were defined initially for a number of heavy metals including Ni and Zn. Table 1.2 below contains threshold values for different metals, and the plant species that are considered to be hyperaccumulator species of each metal.

Metal	Threshold value to be considered a hyperaccumulator mg kg^{-1}	Threshold values in contaminated land assessment (e.g. SGV's values) mg kg^{-1}	Typical concentrations in non-hyperaccumulator mg kg^{-1} (Brooks, 1998; Anderson et al, 1999)	Known hyperaccumlators
Cadmium	100[abc]	30 residential [d] 500 industrial [d]	1	*Arabidopsis halleri* (Bert *et al*., 2003).
Copper	5000[a]	130 residential [e]	1	*Silene vulgaris* (Freot *et al*., 2003).
Nickel	5000[a]	75 residential [f] 5000 industrial [f]	2	*Berkheya coddii* (Anderson *et al*., 1999; Angle *et al*., 2003).
Selenium	1000[a]	35 residential [g] 8000 industrial [g]	1	*Astragalus pattersoni* (Anderson *et al*., 1999).
Zinc	10,000[abc]	300 residential [h]	100	*Thlaspi caerulescens* (Baker and Brooks, 1989; Brown *et* al., 1995).
Lead	1000[bc]	450 residential [i] 750 industrial [i]	5	*Sedium alfredi* (Sun *et al*., 2005).

Table 1.2: Hyperaccumulator threshold values, soil contamination values and examples of hyperaccumulator plant species n.b. pH dependent.
[a] (Brooks, 1998.) [b] (Baker and Brooks, 1989) [c] (Baker *et al*., 1994) [d] (DEFRA and EA, 2002a) [e] (DEFRA and EA, 2002b) [f] (DEFRA and EA, 2000c) [g] (DEFRA and EA, 2002d) [h] (ICRCL, 1987) [i](DEFRA and EA, 2002e).

Scientific research has identified approximately 400 known species of hyperaccumlators, which are able to extract a range of contaminants (Roosens *et al*., 2003). However, nearly 75% of the plants species that have been identified as hyperaccumulators are solely associated with the accumulation of nickel (Assunção *et al*., 2003).

1.3.2 Why do plants hyperaccumulate heavy metals in their above ground biomass?

At present our understanding of why these plant species accumulate such high concentrations of metals in their above ground biomass is still unclear. However, several researchers (Reeves, 1981; Boyd and Martens, 1992; Hanson *et al*., 2004) have put forward five possible hypotheses as to why they do, most of which are proposed to have some benefit for the plant.

1) Inadvertent uptake – Plants grown on nutrient poor soils tend to have high affinity systems to overcome the low concentrations of nutrients. This in turn increases the inadvertent uptake of heavy metals into the plant (Baker and Walker, 1989; Severne and Brooks, 1972).

2) Metal tolerance – Plants that are tolerant to high concentrations of heavy metals sequester the metal into inert parts of the plant such as the vacuole in leaves. These leaves are then shed from the plant, thus enabling the plant to cope with the high concentrations of heavy metals in the soil (Brooks, 1998; Baker, 1981; Baker, 1987; Ernst, 1972; Farago and Cole, 1988; Kruckeburg and Wu., 1992). A competitive advantage of this is the interference with other plants.

3) Interference with other plants – Similar to hypothesis (2) in that it involves plants that are tolerant to high concentrations of heavy metals sequestering the metal into inert parts of the plant such as the vacuole in leaves. These leaves are then shed from the plant and their decomposition increases the concentration of metals on the surface of the soil, making harder for neighboring plants species with a lower metal

tolerance to compete in the same area (Baker and Brooks, 1989; Gabrielli *et al*, 1991).

4) Drought resistance – Some authors have found that the uptake of Ni by some plants species may help them to cope with drought stress, although to date the exact mechanism underpinning this is still not fully understood (Baker and Walker, 1989; Robertson, 1998; Severne, 1974).

5) Pathogen/herbivore defence – The uptake of high metal concentrations in the plants above ground biomass acts as a secondary defence mechanism for the plant by either directly or indirectly affecting the amount of damage the plant is subjected to by pathogens or herbivores (Ernst, 1987; Ernst *et al*., 1990; Reeves, 1981). Since the initial proposal of this hypothesis it has been further refined by Boyd and Martens (1992) as the elemental defence hypothesis, discussed in more detail in section 1.4.

There have only been a limited number of studies conducted on hypotheses 1-4, partly as a result of the complexity of testing some of these hypotheses (Boyd, 1994). It has been the elemental defence hypothesis that has received most of the academic attention recently, although research in this area is still relatively limited. For example Boyd (2007) states that the number of published papers testing the elemental defence hypothesis with respect to Zn is eight, in a review of work conducted on the elemental defence hypothesis.

1.4 The Elemental defence hypothesis against herbivores

As mentioned above, the elemental defence hypothesis proposes that plants sequester high concentrations of elements (typically heavy metals) into their above ground biomass to act as a secondary defence mechanism against herbivores (Boyd and Martens, 1992).

This hypothesis has two modes of action by which the plant may exhibit the elemental defence to defend against attacking herbivores: (1) direct toxicity of the consumed plant

material on the attacking herbivore, by either acute toxicity of the individual resulting in mortality, or by chronic toxicity reducing the growth and development of herbivores resulting in lower fecundity (Boyd and Moar, 1999; Freeman *et al*., 2006; Freeman *et al*., 2007; Jhee *et al*., 2006); (2) deterrence of herbivores, whereby plant tissue containing elevated concentrations of elements is consumed less as a direct result of the herbivores preferring plant material that does not have such high concentrations (Boyd and Martens, 1992; Boyd and Martens, 1994; Boyd, 2007; Martens and Boyd, 1994).

The action of plants concentrating elements in their tissues against herbivores may be more desirable than the use of other types of secondary chemical defences produced by plants, because there is no synthetic cost to the plant, and the energy to sequester and transport elements around the plant is relatively low (Baker, 1981; Krämer *et al*., 1996; Lee *et al*., 1978 as cited in Brooks, 1998). In addition to this, it may be harder for the herbivores to adapt to metal-based defences as, for example, metals cannot be broken down by enzymes or easily excreted by the herbivore (Baker, 1987; Martens and Boyd, 1994).

However, this is not to say that herbivores can't circumvent defence systems involving metals. Herbivores may overcome the toxic affects of the metal in plants by the avoidance of plant tissue that contains the metal, or by diluting the metal rich plant material with other components in their diet and the possession of a physiological tolerance to the specific metal (Boyd and Martens, 1992; Boyd and Martens, 1994; Boyd, 2007; Martens and Boyd, 1994).

It has been suggested by a number of authors that there is a trade-off in plants using elemental defence between the concentration of metals in the plants tissue and the amount of chemical defences produced by the plant (Davis and Boyd, 2000; Noret, 2005; Tolrà *et al*., 2001). As the availability of the element in the soil increases, and a plant capable of sequestering high concentrations of elements (i.e. a hyperaccumulator species) increases its metal concentration, the concentration of chemical defence compounds decreases (Figure 1.2). Tolrà *et al*., (2001) witnessed a reduction in the levels of glucosinolates, a group of chemical defences found in plants from the *Brassicaceae* family, when they grew a Zn

hyperaccumulator species of *T. caerulescens* on a high Zn media compared with the level of glucosinolates found in a non-hyperaccumulator ecotype of *T. caerulescens*. There have been similar studies to the one above that have demonstrated a decrease of organic defences by hyperaccumulators when supplied with media high in the concentration of element they are capable of hyperaccumulating, (e.g. Freeman (2005), Krämer (2005) and Tolrà *et al*., (1996)). However, these trade offs are not always evident and the possibility that there is a synergistic effect between the two defence systems has also been suggested (Dyer, 2003 and Jhee *et al*., 2006).

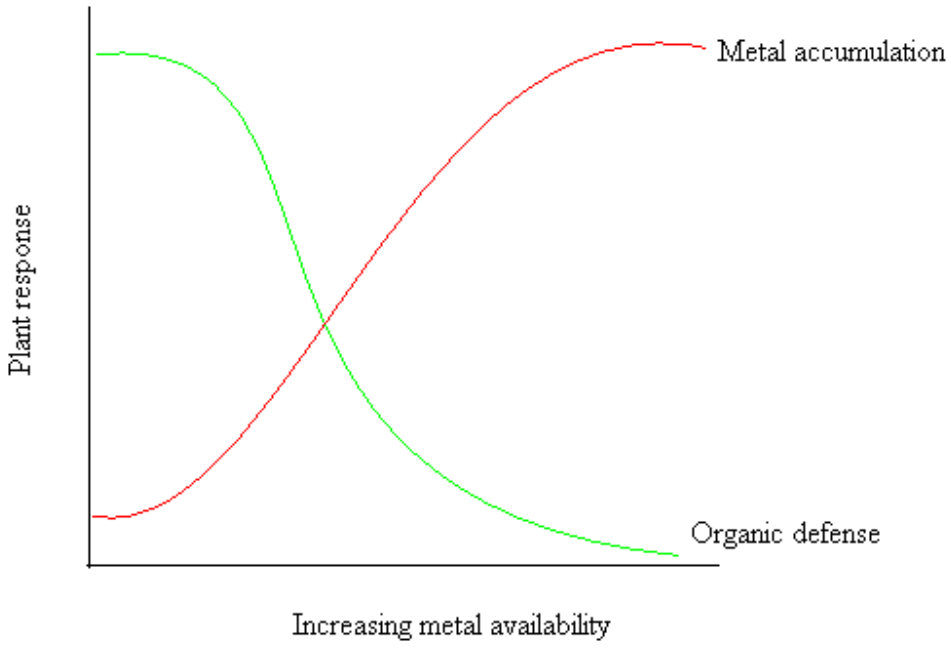

Figure 1.2: Trade-off Hypothesis adapted from Boyd (2007)

It is important to note that elemental defences can be very specific, for example a plant species may only demonstrate a elemental defence for only one or more elements, against only one or two herbivores, i.e. elemental defences are plant species, element and herbivore specific (Boyd and Martens, 1992). This makes testing the defence hypothesis effectively a challenge, particularly in terms of establishing any general patterns.

1.4.1 Features of elemental defence?

Plants have two main ways of defending against herbivores, physical defences, for instance spines or hairs on the plant which provide an obstacle the herbivore must overcome and chemical defences, i.e. the production of secondary compounds, such as phenolics or glucosinolates, which are toxic to herbivores. The use of metals in plant defence is analogous to a chemical defence, due to the pathway in which metals are sequestered, transported and ultimately stored by the plant. Chemical defences, including heavy metals, have two potential modes of action: the elevated concentrations of metals within the plant material may directly result in herbivores being deterred from attacking the plant; the consumption of plant material containing heavy metals can have a negative impact on the growth and survival of herbivores, which may ultimately lead to a reduction in the overall damage to the plant.

Many chemical defences are inducible, i.e. they first appear, or increase in concentration following damage to the plant by herbivores. Having proposed that the use of metals in plants as a type of chemical defence in plants, it is logical to also examine the possibility of this defence being inducible. Many examples of inducible defences are known (Karbon and Baldwin 1997), although it is important to emphasize that many induced chemical changes may not benefit the plant because there is no clear evidence they have an adverse impact on the herbivore. For example, research has found that phenolic compounds accumulate in plants following exposure to herbivory (Coleman and Jones 1991), but how much and which type of phenolic induced was dependent on the type of herbivore inflicting the damage (Hartley & Lawton 1987). Furthermore, many herbivores were unaffected by such changes (Hartley & Jones 1989). However, many induced defences have been shown to be effective (e.g. Massey and Hartley, 2007) and it may be beneficial for plants to sequester an increased amount of metal into their above ground biomass having been exposed to herbivory, hence elemental defences could be inducible.

1.5 *T. caerulescens* – a hyperaccumulator of Zn

T. caerulescens is described by some researchers as a 'model' hyperaccumulator species (Baker *et al*., 1994; Krämer, 2000; Lombi *et al*., 2001), with a high affinity for Zn. It is capable of accumulating Zn concentrations in its above ground biomass greater than 10,000 mg kg^{-1}, 1 % dry weight (DW). Research by Baker and Brooks (1989), Baker *et al*., (1994b) and Brown *et al*., (1995) has shown that this plant is capable of accumulating levels of Zn up to 3% of its biomass DW. The Zn is taken up through the root and deposited in vacuoles in leaf cells, where the Zn is stored in a soluble form (Kupper *et al*., 1999; Frey *et al*., 2000). In addition to being classed as a Zn hyperaccumulator, *T. caerulescens* has been shown to be tolerant to a number of other heavy metals, i.e. when they are present at phytoxic concentrations in anthropogenic contaminated soils (e.g. Ingrouille and Smirnoff, 1986; Meerts and Isacker, 1997). For example, there is mounting experimental evidence to suggest, that *T. caerulescens* should also be classed as a cadmium hyperaccumulator (>100 mg kg^{-1}) (Robinson *et al*., 1998; Bert *et al*., 2000).

This plant species, commonly known as alpine penny-cress, is found in certain areas of central Europe and a number of sites in the north of the UK, usually on serpentine soils that contain a range of heavy metal contaminants at concentrations that would be phytotoxic to most other plant species. The plant is a biennial or short lived perennial, which has no commercial value. Physically it is relatively small in stature, formed of a basal rosette with smooth spoon shaped leaves, and which may produce one or more racemes, which have arrow shaped leaves and white flowers, there is no documentation on which herbivores feed on this plant species (Baker and Procter., 1990; Haines, 2002). Figure 1.3 shows *T. caerulescens* growing in the wild.

Although the reason *T. caerulescens* takes up such high concentrations of Zn is unknown, it has been shown to be a plant that has a high affinity and increased internal requirement for Zn (Chaney *et al*., 1997; Shen *et al*., 1997). Research conducted by Whiting (1998) showed that this plant species is capable of discriminating between areas of different Zn concentrations, and Haines (2002) showed that its roots actively search out areas with high

concentrations of Zn. Table 1.3 summaries some key experiments involving the Zn hyperaccumulator plant species *T. caerulescens*.

Figure 1.3: *T. caerulescens* growing in the wild (Anderberg, 2008).

Type of experiment	Zn Concentration in growth medium (mg kg^{-1})	Range of Zn in plant shoots (mg kg^{-1})	Main experimental outcomes	Reference
Pot trial	0-75	<15 – 650	Even at low Zn concentrations in soil *T. caerulescens* was capable of accumulating Zn into its biomass.	Ozturk *et al.*, (2003).
Pot trial	Sections of a pot contained 0, 200 , 300, 1000 additional Zn.	Not measured	*T. caerulescens* seems to use its roots to actively seek out areas of high Zn.	Haines (2002).
Field study	19326 ± 2476	19071 ± 2485	*T. caerulescens* grown in wild upon highly contaminated soil. Plants had an approximate bioaccumulation factor of 1.	Baker *et al.*, (1994).
Field study	107-365	5259-10,858	The potential for phytoextraction is increased 2 fold if seeds are planted directly into the ground, while chelating agents did not increase Zn uptake.	McGrath *et al.*, (2006).

Table 1.3: Experiments involving uptake of Zn by *T. caerulescens*

Despite the existence of several previous studies (e.g. Table 1.3) there are drawbacks to using *T. caerulescens* for research on the elemental defence hypothesis. Although it has the ability to hyperaccumulate high concentrations of Zn in its above ground biomass while tolerating phytotoxic concentrations of other contaminants, it is slow growing and produces relatively low biomass (Ernst, 2000; Lombi *et al*., 2001; McGrath and Zhao, 2003), typically a maximum of 0.2-1.6 g DW per plant (Haines, 2002). It should be noted that both the genotype and ecotype of the any plant species can have a significant impact on the plants ability to sequester Zn into to biomass.

1.6 *Brassica juncea* – an accumulator of Zn

Since the introduction of the system classifying plant species into accumulators of heavy metals (as opposed to hyperaccumulators) by Reeves and Baker (2000), there has been an increased interest in the use of heavy metal accumulator plant species. This is due to the fact that the potential numbers of plant species falling into the accumulator category are far greater than those of hyperaccumulating status and frequently have greater biomass.

One of these accumulator species is *B. juncea* (Indian mustard, Figure 1.4), a commercial crop plant (contributing to the third largest edible oil production, Sinha *et al*, 2010) that produces high biomass (Salt *et al*., 1995). Found predominately in central Asia and northern Europe, but also in a few locations of Britain, it is a fast growing annual crop that is capable of self-pollination. This plant species has been found to be tolerant to a number of heavy metals, including Pb, Zn, Cd and Se (Clemente *et al*., 2005; Ebbs and Kochian, 1997; Irtelli and Navari-Izzo, 2006; Kumer *et al*., 1995). The majority of the work on this species has focused on its uptake of Se, with concentrations exceeding 1500 mg kg^{-1} in its above ground biomass DW, categorising it as a hyperaccumulator of Se, with experimental results providing evidence to support the elemental defence hypothesis for this metal (Hanson *et al*., 2003; Hanson *et al*., 2004; Freeman *et al*., 2006; Freeman *et al*., 2007).

It has also been found to be an accumulator of Zn, with above ground biomass concentrations greater than 2000 mg kg^{-1} (Clemente *et al*., 2005; Podar *et al*., 2004). The

main scientific focus on this plant has been assessing its suitability for phytoremediation (Section 1.8), with very little attention having been paid to its link with the elemental defence hypothesis, as its Zn concentrations are not as extreme as those found in hyperaccumulators. Clemens (2006) however, was one of the first authors to suggest that even the heavy metal concentrations found in accumulator plant species may have an impact on feeding herbivores. Therefore, this plant species could be useful for testing whether the elemental defence is effective in accumulators as well as hyperaccumulators of Zn. In addition to there being a range of both generalist and specialist *Brassicae* herbivores that feed on it in the wild.

Figure 1.4: *B. juncea* growing in the wild (Redfearn, 2008).

1.7 Herbivores – which species to study

The ecological importance of studying the defence hypothesis and its effects on both plants and herbivores arises from the possibility of the feeding herbivore becoming a pathway for heavy metals entering the food-chain (Nascimento *et al*., 2006). Determining which herbivore species to use when investigating the interactions between heavy metal uptake by plant species and the effect on feeding herbivores, is not easy. The species needed to have been shown to respond to Zn but also readily available and convenient to study. The species should also encounter Zn rich plants in natural systems.

1.7.1 Snails

Snails have been used in a number of studies, looking at the effect of a high Zn diet on the herbivore. They have also been used in a number of preference feeding trial experiments, in which the snails were given a choice between a control plant and one with a high concentration of Zn. Snails are important herbivores to study, as most of the species are generalists and are common across most of Europe, as well as being an important food source for many predator species further up the food chain.

A preference feeding trial by Noret *et al*., (2005), found that snails (*Helix aspersa*) were not deterred from consuming plant matter that had concentrations of Zn present in the range of 2600 and 5000 mg kg^{-1}. These results however do not provide conclusive evidence that snails are not deterred by increasing levels of Zn in plant biomass, but rather that their tolerance to Zn might be higher than that in this experiment. Also the experiment only looked at the preference the snails had for a particular “contaminated” leaf over a control, while not examining the overall performance effects that the consumption of high levels of Zn might be having upon the snails.

Growth performance trials on snails have however been conducted by Gomot-De Vaufleury (2000). This experiment involved the feeding of snails (*H. aspersa)* with a feed that was spiked with Zn, (4000, 6000, 10,000 mg kg^{-1}) as well as a control that contained no additional Zn. The trials were conducted on juvenile snails (five-six weeks old) into

adulthood; snails were fed one of the four different feeds. Results from the experiment were very interesting as it was found that the 4000 mg kg^{-1} feed started to cause toxic effects in the snails. The EC^{50} value (the concentration that has a lethal effect on 50% of the population) was found to be 5500 mg kg^{-1}. This may explain the observations seen by Noret *et al* (2005), in which the snails where not deterred because the high concentration used, was just below that of the EC_{50} value found by Gomot-De Vaufleury (2000). So it may be possible to speculate that although the high concentration of Zn used by Noret *et al*., (2005) has adverse health effects on snails it is not high enough for the snails to sense it. Therefore a choice performance test that uses higher concentrations of Zn in leaves may yield a different result than that found by Noret *et al*., (2005).

Laskowski and Hopkins (1996) conducted work into the accumulation of heavy metals into the body and shell of the snail *H. aspersa*, to see what implications it might have for its predators. Zn, which was one of the metals they used in their experiments, was presented to the snails at concentrations in the range of 39 to 12,200 mg kg^{-1}. Results from the experiment showed that Zn was accumulated in the soft tissue of the snails at the same proportion as that of the feed they where given, with 60% of the Zn being assimilated from the food into the body tissue. Their findings also show that the amount of contaminants sequestered into the shells of snails is not significant. They conclude that snails are not important sources of Zn to higher levels of the food chain. This conclusion seems open to interpretation, because for a snail population in the wild feeding on a plants species that are hyperaccumulators of Zn, the level of Zn in their soft tissue may be of significance to species further up the food chain, especially when predators of snails tend to feed on several snails a day, presenting a possible route for Zn to bioaccumulate in higher trophic species.

B. juncea leaves containing elevated levels of Se were found not to affect the growth of the snail species *Mesodon ferrissi*. The snails were not found to have a preference when presented with *B. juncea* leaves containing elevated concentrations of Se and a control leaf (Hanson *et al*., 2003). But as the elemental defence is plant-metal-herbivore specific, leaves

of *B. juncea* containing a high level of Zn as opposed to Se may prove to give a different outcome.

1.7.2 Slugs

Slugs like snails form an important link in the food chain to higher trophic level species; hence this may also be a route for the transport and accumulation of Zn in species further up the food chain. Pollard and Baker (1997) examined the preference feeding habits of *Deroceras caruanae* (common garden slug) on leafs from the Zn hyperaccumulator *T. caerulescens*, that had been grown in a range of Zn concentrations. The results showed that although the slugs sampled the higher level leaves, a trait observed by many generalist herbivores, the overall consensus showed a choice for leaves with a lower concentration of Zn. Greville and Morgan (1991) also worked with *D. caruanae*, they transplanted slugs from sites where there was no Zn contamination to sites where there was and grew them for twenty days. Results from the experiment showed that transplanted slugs contained, after twenty days, higher levels of Zn in their body tissues than those living on the contaminated site, thus showing that slugs may inherit tolerance to Zn.

1.7.3 *Lepidoptera*

Preference trials by Pollard at *et al*., (1997) on the caterpillar species *Pieris brassicae* using leaves of *T. caerulescens* again showed preference for plant leaves with lower Zn levels.

Pieris rapae was found by Hanson *et al*., (2003) to have a preference for *B. junce*a leaves containing low concentrations of Se. They also ascertained that the high concentration of Se in *B. juncea* leaves had a significant impact in the growth of the caterpillars, when compared to *Pieris rapae* reared on a diet with a control level of Se. This experiment provides strong evidence for the elemental defence hypothesis, an extension of this will be to change the element, i.e. to our target metal of Zn, again to increase our understanding of the elemental defence hypothesis.

1.8 Phytoremediation: the potential use of hyperaccumulator plant species

Whilst this thesis focuses on the potential ecological problems for herbivores feeding on hyperaccumulators and the implications of this for organisms up the foodchain, it is important to remember that hyperaccumulators also have the potential to be used in the clean-up of contaminants of soil by remediation. If this is the case and their use becomes widespread, the potential impacts on natural food chains will need to be considered.

The two main ways in which land can be remediated is by removal of the contaminated soil or by stabilization of the pollutant thus preventing it from being a threat to the environment (Susarla *et al*., 2002). However, current techniques in land remediation are very intrusive and are very expensive, typically between £38 and £215 per ton (Ciccu *et al*., 2003; Cl:aire, 2005; Glass, 1999; Mulligan, 2005).

Phytoremediation however, has been described as an *in-situ*, environmentally friendly process for remediating water systems, atmospheric pollutants and contaminated land (EPA, 2001; Raskin *et al*., 1997; Susarla *et al*., 2002). There are seven known ways in which plants, trees or fungi can be used to remediate the environment (Table 1.4).

Process:	Category:	Mode of action:
Phytodegradation	Degradation	The breakdown and transformation of pollutants within the plant by enzymes [a].
Phytodegradation	Degradation	Similar to the above but takes place in the root zone by fungi and bacteria [a].
Phytovolatisation	Dissipation	Contaminants are metabolized and then volatized into the atmosphere as harmless chemicals [a].
Phytostabilization	Immobilisation	The root zone of the plants binds contaminants to itself thus making them unavailable to other species [a].
Hydraulic control	Immobilisation	Similar to the above but in an aquatic environment [a].
Phytoextraction	Accumulation	The accumulation of pollutants by plants in their above ground biomass, that may then be harvested and disposed of [b].
Rhizofiltration	Accumulation	Similar to the above but for aquatic environments with trace amounts of contamination, where by the pollutants are sequestered in the roots [a].

Table 1.4: Methods of phytoremediation

[a] (EPA, 2001) [b] (Raskin *et al.*, 1997)

It is the phytoextraction method of phytoremediation that is relevant to this research, where contaminants are concentrated into the plants above ground biomass.The range of contaminants suitable for phytoextraction is quite extensive, it includes a number of heavy metals, organic and inorganic pollutants (notably DDT along with chlorinated solvents) and some radionucli (EPA, 2001 and Susarla *et al.*, 2002), it is only limited by the identification of a plant species that accumulates the particular pollutant. There are two main strategies that can be employed when examining the possibility of using phytoextraction for land

remediation; these are (1) to use plants that are considered to be hyperaccumlators which naturally take up high concentrations of pollutants in their biomass, though typically produce small amounts of vegetation (McGrath *et al*., 1993; Baker *et al*., 1994). (2) A second technique is to use plants that produce large amounts of biomass that can tolerate moderate amounts of contaminants in the soil but are not deemed as hyperaccumulator species (McGrath, 2005).

The advantages and disadvantages of phytoextraction as a method for phytoremediation are summarised in Table 1.5.

Advantages	Disadvantages
Environmentally friendly – as it doesn't involve the use of potentially environmentally damaging chemicals or removal of waste to be taken to landfill (Raskin *et al*., 1997).	**Contaminated waste** – the biomass produced by the plant will contain 'high' concentrations of contaminants, therefore could be classed as hazardous waste (McGrath and Zhao, 2003; Rock, 1997).
Phytomining – there is a possibility that heavy metals with a high market price such as copper or gold may be reclaimed by incinerating the biomass produced, thus reclaiming the metal (Baker and Brooks, 1989; Nicks and Chambers, 1995).	**Not economically viable** – at present plant yields have not accumulated sufficient concentrations of metals to make this a viable financial option (Nevel *et al*., 2007)
Cost – current predictions are that phytoextraction would be considerably cheaper that current remediation strategies. Estimates by Cl:aire (2005) are as low as £0.16 per ton of soil.	**Time** – most sites that are considered as being contaminated would require a number of crop cycles to bring the concentration in line with government's legislation of what is acceptable. Also additional cost for biomass treatment, which can include the incineration of plant matter and landfill of residual waste. (Cunningham *et al*., 1995; Cl:aire, 2005)
Insitu – phytoextraction will take place at the source of the contamination and although may not remediate land immediately would provide a more aesthetically pleasing option to denuded brown field sites at present (Cunningham *et al*., 1995; Susarla *et al*., 2002).	**Potential ecological impact** – it has been hypothesised that phytoextraction may have a negative ecological impact by moving contaminants 'locked' in the soil to become available above ground herbivores with possible adverse effects on higher trophic levels (Friesl *et al*., 2006; Nevel *et al*., 2007).

Table 1.5: Advantages and disadvantages of phytoextraction

Phytoextraction is still very much in the developmental stages and has not yet become a widely used strategy. There have been numerous lab based studies but only a handful of field experiments to date (Baker and Whiting, 2002; McGrath and Zhao, 2003).

1.9 Thesis hypotheses

The research contained within this thesis combines the use of Zn hyperaccumulator/accumulator plant species and the interaction of herbivores feeding on them, using both lab and field based studies. The work aims to further our understanding of the elemental defence hypothesis from both the plants' and herbivores' perspective.

From this review of the literature the following hypotheses were established:

1) The concentration factor of Zn in the above ground biomass of *T. caerulescens*, along with other physiological factors, such as biomass above and below ground, total Zn concentration and root: shoot ratio, will alter significantly when grown in media of varying Zn concentration (Chapter 3).

2) Zn concentration in *B. juncea* plants will differ significantly between those grown on media that contain additional concentrations of Zn and those grown on a control medium. The Zn concentration in both leaf and stems of plants grown on all the treatments will change significantly as the plant grows through time (Chapter 4).

3) *B. juncea* plants grown on a high Zn medium will increase their concentration of Zn in their above ground biomass when exposed to herbivory damage from the generalist herbivore *H. aspersa*, to a greater extent than plants grown on the same media that are not exposed to herbivory. This hypothesis addresses whether the elemental defence mechanism of Zn uptake by *B. juncea* is inducible by herbivore attack (Chapter 4).

4) Increased Zn concentration in leaves from plants grown on a medium containing additional Zn consumed by selected herbivores will have an impact on their growth and performance, compared with the performance of herbivores fed on leaves from plants grown on a media not containing any additional Zn (Chapter 5). This hypothesis was tested using juvenile *H. aspersa*, adult *H. aspersa* and *P. brassicae* caterpillars.

5) Selected herbivores will choose to consume leaves of low Zn concentration over those with high Zn concentrations (Chapter 5). This hypothesis was tested using juvenile *H. aspersa*, adult *H. aspersa* and *P. brassicae* caterpillars.

6) *P. brassicae* that have consumed a diet of *B. juncea leaves* from plants grown on soil containing additional Zn, will have a higher concentration of Zn in their biomass, than caterpillars that have consumed *B. juncea* leaves from plants that have been grown without any additional Zn (Chapter 5).

7) Plants grown to have higher Zn concentrations in their above ground biomass will experience less herbivory when grown in the field than plants containing 'normal' concentrations of Zn in their above ground biomass (Chapter 6). This hypothesis tests whether the elemental defence hypothesis is effective under field conditions.

1.10 Outline of chapters

Below is a brief summary of each of the chapters that are to follow:-

Chapter 2: General experimental methods – This Chapter outlines the general experimental techniques which were employed in the majority of the experiments contained in the thesis.

Chapter 3: Evaluation of the Zn hyperaccumulator *T. caerulescens* in lab and in the field – The first of four experimental chapters uses *T. caerulescens* as a 'model' species for the investigation of the uptake of Zn by plants as a defence against herbivores. Plants were

grown in three different Zn treatments and without additional Zn and fed to *H. aspersa* in a small scale preference trial. It also includes a field evaluation of *T. caerulescens* in terms of soil and plant foliage Zn concentrations, grown in the wild at two sites in Derbyshire, UK.

Chapter 4: Accumulation of Zn in *B. juncea* with and without herbivory over time and the possible induction uptake by the herbivore *H. aspersa* – The Zn accumulator species *B. juncea* was assessed in two experiments. Firstly uptake of Zn over time in the stems and leaves of plants in two Zn treatments and a control; in addition it investigates whether the general herbivore *H. aspersa* is capable of inducing increased Zn uptake in plants grown in additional Zn, hence testing whether elemental plant defences are inducible.

Chapter 5: Testing the elemental defence hypothesis of Zn accumulation in *B. juncea* using the generalist herbivore *H. aspersa* and the brassicaceae specialist *P. brassicae* in the lab – Having established that *B. juncea* is a suitable plant species for accumulating Zn in its above ground biomass, the potential impact this could have on *H. aspersa* juvenile growth and adults fed on a diet of high and low Zn, in addition it also examines the preference of adult snails when repeatedly given the feeding choice between high and low Zn leaves. Furthermore it examines the feeding preference and performance of the caterpillar *Pieris brassica* fed on a high and low Zn diet of leaves of *B. juncea.*

Chapter 6: Evaluating the elemental defence hypothesis of *B. juncea* (an accumulator of Zn) under field conditions – The experimental chapters so far have concentrated on lab based experiments with plants grown in glasshouses and herbivores being observed under laboratory conditions, the experiment in this chapter breaks the mould, by being a large scale field experiment. Here plants grown in four different Zn concentrations were placed in three locations on a field, for a time period of three weeks, during this time the plants were assessed for percentage damage caused by herbivores and abundance of herbivores on the plants. At the end of experiment mean Zn concentrations in the plants biomass was determined to and evaluated to see if this was the factor for the trends in herbivory.

Chapter 7: Discussion – This final chapter collates all of the findings from the four experimental chapters and summaries their outcomes, both in terms of their results and their success at fulfilling the aims of the thesis, in addition to evaluating them against research conducted by other scientists. It concludes by suggesting further research questions that have been generated from conducting this work.

Chapter 2: General experimental methods

2.1 Introduction

The purpose of this chapter is to describe the general analytical methods and plant/soil experimental techniques that have been implemented repeatedly in the numerous experiments that follow in subsequent chapters. It incorporates any method that is referred to in more than one chapter. Beginning with the initial growth, transplanting and harvest of plants, it continues to describe the production of growth media, the decomposition of plant and soil samples by acid digestion, the chemical analysis by Flame-Atomic Absorption Spectrometry (F-AAS), the analysis of plant samples for Carbon and nitrogen content (C:N) and concludes by defining plant:metal concentration factor.

2.2 Plants

2.2.1 Germination of *B. juncea* seeds

Germination was carried out by dispersing seeds approximately 1 cm apart, onto wet vermiculite in a seed tray 2.5 cm deep. The seed trays were then placed on a greenhouse work bench with a temperature of 22/16 ^{o}C and a day/night cycle of 16/8 hr. Prior to use, *B. juncea* seeds purchased from Herbiseed (2006) were stored in a cold room (3-5 ^{o}C). The of number of seeds germinated for any particular experiment was calculated as the number of plants required, plus an additional 20 % to allow for any seeds that failed to germinate.

2.2.2 Initial growth of *B. juncea* plants

After 7-10 days, the seeds had germinated and grown to approximately 3 cm in height, with their cotyledons present. The number of plants required for any given experiment, plus an additional 10 %, were then pricked out of the vermiculate and cultivated in plug trays, containing only compost (John Innes no.2) see Figure 2.1. They were grown for a further 10 days by which time they had reached a mean height of 6 cm and the first set of true leaves had emerged. At this stage, similar sized individuals were selected and transplanted into the relevant growth media for the experiment being conducted.

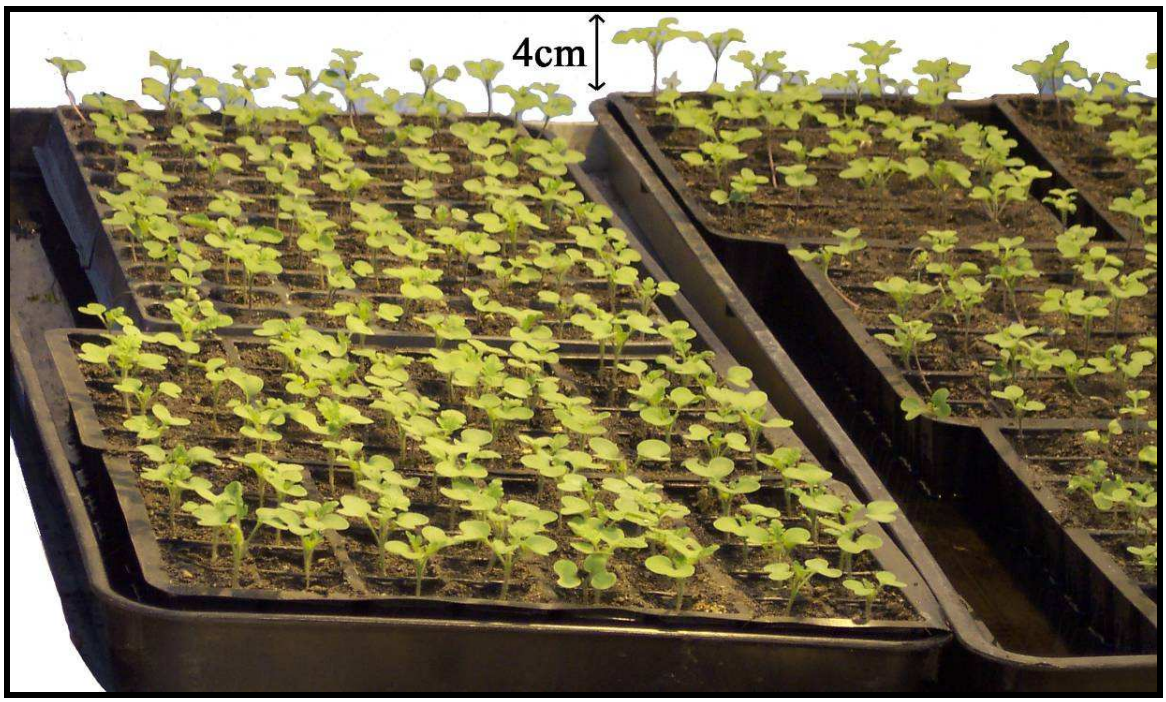

Figure 2.1: Initial growth of *B. juncea* in plug trays

2.2.3 Growth and maintenance of plants

Once the plants were established in the relevant growth medium, they were grown under the same conditions as mentioned in Section 2.2.1 for the remainder of the experiment, unless specified differently in the individual experimental design. All plants were kept well watered with standard tap water.

2.2.4 Harvest of plants

Plants were harvested by severing the stem approximately 0.5 cm above the growth medium, with any remaining particulate material removed from the plant by tap water from a hose. Once collected, the plant material was dried to a constant weight in an oven at 60 ^{o}C for 48 hrs, at which point they reached a constant weight. Samples could then be separated into leaf and stem material. The samples were then homogenised by being ground in a ball-mill (Pulversette 23; Fritsch) and stored in snap lock polythene bags prior to chemical analysis. Roots from plants were not analysed due to the physical difficulties involved in

successfully removing all soil particulate matter from roots. As even a small amount of soil present on root matter would significantly skew the analytical results.

2.3 Preparation of growth media

The growth media used to cultivate the plants wwas prepared using dried potting sand and fresh compost (John Innes no. 2), in a 4:1 mixture respectively. This was weighed into a cement mixer a kilogram at a time, in a mass ratio of 800 g sand to 200 g compost. The mixer was turned on for 2 minutes after each addition to increase homogenisation. The media that required additional zinc were supplemented with zinc oxide (ZnO), in an amount calculated on a dry weight (DW) basis (Table 2.1). ZnO is present in soils contaminated with Zn produced from industrial processes. For example, ZnO represents 10.5-16.0 % of the goethite waste that arises from the procedure for producing (mining and smelting) Zn as a metal (Orru *et al*., 1999). It has also been used in a wide number of previous experiments with zinc accumulating plants (e.g. Haines 2002), because in this form of ZnO the Zn is ready available for uptake into plants (Broadley *et al*., 2007). The baseline concentration of Zn in the prepared medium with no additional Zn was typically 17 mg kg^{-1}.

Target concentration of additional Zn mg kg^{-1} DW	Sand (g) per kg	Moisture content sand %	Compost (g) per kg	Mean moisture content of compost % m/m	Mass of ZnO required to reach target values mg
0	800	0	200	17.6	0.00
400	800	0	200	17.6	480.35
600	800	0	200	17.6	720.53
800	800	0	200	17.6	960.70

Table 2.1: Addition of Zn to growth media

2.4 Acid digest of growth media and plant samples

2.4.1 Preparation of sample tubes

As tubes (made of glass) are reused repeatedly in different batches of acid digest of plant and growth medium samples, it is imperative that they are adequately cleaned between batches. Table 2.2 outlines the procedure for ensuring tubes are correctly cleaned before use.

Step:	Action:	Purpose:
1	Rinsed 3 times with Reverse osmosis (RO) water.	Remove any particulate debris from tubes.
2	Washed on a high temperature cycle in dishwasher.	Removal of any stubborn particulate matter.
3	Set to soak for 4 hrs in 10 % v/v Nitric acid (HNO_3) bath, made from analytical grade acid and RO water.	Dissolve and remove any remaining metal ions on tubes into solution.
4	Rinsed 3 times with RO water.	Remove traces of metal and nitric acid.
5	Placed in oven at 60 ^{o}C until dry.	Dry tubes.

Table 2.2: Procedure for cleaning sample tubes

2.4.2 Nitric/Perchloric digestion of growth media samples

The digestion of both plant and growth medium samples was conducted using two different acid attacks with nitric and perchloric acids, a method adapted from Thompson (1982) and Thompson and Walsh (1983). A risk assessment was completed prior to starting any work.

The acid digest of soil samples was carried out as follows:-

Step1: Weighed 0.1 g (± 0.001) of sample into a clean dry test tube using a 4 point balance.
Step 2: Added 4 ml of HNO_3 (70 % w/w trace analysis grade) using a bottle top dispenser.

Step 3: Added 1 ml of Perchloric acid (60 % w/w AnalaR grade) using a bottle top dispenser.

Step 4: Place in hot block and run for the following heating cycle in Table 2.3.

Rise rate sec/ deg	Dwell time hrs	Temperature ^{o}C
001	3.0	50
001	3.0	150
001	18.0	190
001	0.1	200
Cool down	Hold at	40

Table 2.3: Heating cycle for acid digest of soil

Step 5: Once samples were at 40 ^{o}C they were removed from the hot block and 2 ml of 5M hydrochloric acid (HCl, trace analysis grade) was added from a gravimetrically calibrated bottle top dispenser.

Step 6: Returned test tubes to hot block to leach for 1 hr at 60 ^{o}C.

Step 7: Removed samples from hot block and added 8 ml of high purity water (e.g. RO water) using a gravimetrically calibrated bottle dispenser.

Step 8: Mixed using a vortex mixer for 20 seconds.

Step 9: Decanted sample into polypropylene tubes with caps and labelled.

2.4.3 Acid digest of plant samples

Step1: Weighed 0.1 g (± 0.001) of sample into a clean dry test tube.

Step 2: Added 4 ml of HNO_3 (70 % w/w trace analysis grade) using a bottle top dispenser.

Step 3: Left over night in hot block at 50 ^{o}C.

Step 4: Added 1 ml of Perchloric acid (60 % w/w AnalaR grade) using a bottle top dispenser.

Step 5: Placed in hot block and run for the following heating cycle in Table 2.4.

Rise rate sec/deg	Dwell time hrs	Temperature ^{o}C
001	0.1	50
001	3.0	150
001	18.0	190
001	0.1	195
Cool down	Hold at	40

Table 2.4: Heating cycle for acid digest of herbage

Then continued using steps 5-9 in section 2.4.2

2.5 Quality Control

In addition to running samples, sample duplicates and trip blanks, certified reference materials (CRMs) and house reference materials (HRMs) were analysed at the same time to help provide an estimate of the analytical bias (B), a component of the measurement uncertainty. The bias and relative bias (B%) is calculated according to equations 1 and 2, where the mean measured value of the CRM is $\bar{x}_m$ and $\bar{x}_c$ is the certified accepted value. Table 2.5 gives the accepted concentration of Zn in the CRMs and HRMs. Sample, duplicates, trip blanks and CRM/HRMs were typically represented as 5% of the total number of samples analysed.

Equation 1: Bias

$$B = \bar{x}_m - x_c$$

Equation 2: Relative bias

$$B\% = \frac{\bar{x}_m - x_c}{x_c} \times 100$$

	Zinc		
Matrix	**CRM/HRM**	**Accepted value mg kg^{-1}**	**Std deviation**
Soil	HRM 1	22.0	4.00
Soil	HRM 2	400.0	45.00
Soil	Nist 2709	106.0	0.75
Soil	Nist 2710	6952.0	22.25
Soil	Nist 2711	350.4	1.25
Plant	Nist 1570 A	82.0	0.75
Plant	HRM 11	45.0	-
Plant	HRM 14	35.0	-

Table 2.5: Zn concentration in CRM and HRM.

2.6 Analysis of plant and soil samples by F-AAS

The flame atomic absorption spectrometer (Perkin Elmer Analyst 100) was calibrated using zinc solutions containing 1, 3 and 5 µg ml^{-1} respectively dissolved in 1M HCl (trace analysis grade). Calibration solutions were made by diluting a zinc stock 1000 µg ml^{-1} solution, dissolved in 1M nitric acid (Fisher Scientific). Samples whose absorbance exceeded that of the calibration curve were diluted by either a factor of 10 (1 ml sample + 9 ml 1M HCl) or 33.3 (3 ml sample + 7 ml 1M HCl), using gravimetrically calibrated pipettes and bottle top dispensers.

The sensitivity and detection limit of the F-AAS was calculated according to equations 3 and 4 respectively. The sensitivity was then checked against the manufacturers' value for the instrument to ensure F-AAS was working satisfactorily.

Equation 3: Sensitivity of F-AAS

$$\frac{\text{Concentration of the second calibration solution}}{\text{Absorbance of the second calibration solution}} \times 0.0044$$

Equation 4: Detection limit of F-AAS

3 x standard deviation (N = 11) of the calibration blank

2.7 Carbon and Nitrogen (C:N) analysis

The carbon and nitrogen ratio of samples were determined by the use of an elemental combustion system (Costech instruments, Milan). 2.5 mg of plant sample was weighed into a tin capsule and was combusted. The ratio of carbon and nitrogen was then calculated by comparing the emission of the sample against a calibration line. Calibration of the instrument was conducted by combusting 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg of BBOT (a synthetic standard $C_{26}H_{26}N_2O_2S$ produced by Costech). The standard has known percentages of C = 72.53% and N = 6.51%. During combustion of the standard/sample there are specific time intervals at which the N and C are released which is plotted as a function of time. The area of the peak produced can then be quantified against the calibration line.

2.8 Protocol for the storage and acid digest of insects

Insects that were used in experiments and required for analytical analysis at a later date were placed in a -20 ^{o}C freezer within zip-lock bags. Insects were not defrosted prior to acid digest, but were weighed. The following outline the protocol for the acid digest of insects.

Step1: Placed a weighed sample into a clean dry test tube.

Step 2: Added 4 ml of HNO_3 (70 % w/w trace analysis grade) using a bottle top dispenser.

Step 3: Left over night in hot block at 50 ^{o}C.

Step 4: Added 1 ml of Perchloric acid (60 % w/w AnalaR grade) using a bottle top dispenser.

Step 5: Placed in hot block and run for the following heating cycle in Table 2.4.

Rise rate sec/deg	Dwell time hrs	Temperature ^{o}C
001	0.1	50
001	3.0	150
001	18.0	190
001	0.1	195
Cool down	Hold at	40

Table 2.6: Heating cycle for acid digest of insects.

Step 6: Once samples were at 40 ^{o}C removed from hot block and added 2 ml of 5M nitric acid (HNO_3, trace analysis grade) from a gravimetrically calibrated bottle top dispenser.
Step 7: Returned test tubes to hot block to leach for 1 hr at 60 ^{o}C.
Step 8: Removed samples from hot block and added 8 ml of high purity water (e.g. RO water) using a gravimetrically calibrated bottle dispenser.
Step 9: Mixed using a vortex mixer for 20 seconds.
Step 10: Decant sample into labelled polypropylene tubes with caps.

At the same time as the insect samples being analysed, five blanks were incorporated into the sampling regime, also an effort was made to help assess quality control, in form of a Zn spike recovery. The concentrations of the Zn spikes used were as follows: 2, 5, 10, 15, 20 mg l^{-1}. Zn spikes were made from diluting a Zn stock solution (1000 mg l^{-1}), spiked solutions were taken to dryness using the hot block as per samples, before being brought back up in 2ml HNO_3 and then followed steps 7 – 10 of the above protocol.

2.9 Concentration Factor

Concentration factor (CF) is a measure of metal mobility from soil to plants and can be applied to calculate the CF of any bioavailable metal in a plant (Boruvka *et al*, 1997). It is derived as the ratio between the metal concentration in a plants biomass and that in the soil, as seen in equation 5 and more specifically for the work in this thesis in equation 6. Plants which exhibit a high CF for Zn are typically accumulators or hyperaccumulators (as discussed in Chapter 1) and is key to being a suitable plant for phytoremediation.

Equation 5:

$$\text{Concentration Factor (CF)} = \frac{\text{Plant metal concentration (mg kg}^{-1})}{\text{Soil Concentration (mg kg}^{-1})}$$

Equation 6:

$$\text{Zinc Concentration Factor (CF)} = \frac{\text{Zn concentration in plant material mg kg}^{-1}}{\text{Zn concentration in soil (mg kg}^{-1})}$$

Chapter 3: Evaluation of the Zn hyperaccumulator *T. caerulescens* in the lab and in the field

3.1 Introduction

Thlaspi caerulescens as described in Chapter 1 is a 'model' hyperaccumulator of Zn (Cosio *et al*., 2004; Saison *et al*, 2004). The vast majority of previous experiments on this species have focused on the ability of *T. caerulescens* to sequester Zn (and other heavy metals) into its above ground biomass. These studies have been driven by the commercial desire to produce a working plant-metal system that could be used as an effective tool in the remediation of contaminated land (Alkorta *et al*., 2004; Brooks, 1998; Marchiol *et al*., 2004).

Fewer studies have examined the role of Zn accumulation in above ground biomass as an elemental defence against herbivores (Table 3.1 provides a summary these studies). The elemental defence hypothesis states that accumulation or hyperaccumulation of metals by plants may defend them from damage by herbivores (Boyd, 2007). This may play an important role in determining whether a plant-metal system is a feasible option for remediating contaminated land. Although a plant may be capable of sequestering high concentrations of contaminant into living tissue, if this is then accumulated in the herbivores that feed on them, it is effectively introducing a pathway for the previously 'locked' contaminant to become mobile and enter the food chain. This could possibly have a more detrimental effect on the environment than leaving land contaminated and may be less effective than traditional remediation techniques. Therefore, it is imperative to fully understand the impact of elemental defence on higher trophic levels before moving on to consider whether these plant species are suitable for use in phytoremediation.

Herbivore type (species)	Max concentration mg Zn kg^{-1} DW in leaf	Experimental overview	Author
Snail and slugs	15,000	A field study which showed that herbivores were **not deterred** from consuming leaf tissue containing high concentrations of Zn.	Noret *et al*., 2007.
Snails (*Helix aspersa*)	13,500	The growth of *H. aspersa* was **not affected** when fed a diet of high Zn concentration.	Noret *et al*., 2005.
Slug (*Deroceras caruanae*)	14,000	**No preference** was found when *D. caruanae* was presented with a choice of leaves containing high and low Zn concentrations.	Pollard and Baker, 1997.
Thrips (*Frankliniella occidentalis)*	2,700	The growth of *F. occidentalis* was **not affected** when fed a diet of high Zn concentration.	Jiang *et al*., 2005.
Grasshopper (*Schistocerca gregaria)*	5,770	A choice experiment which found that *S. gregaria* **preferred leaves with low Zn** concentrations. In addition, a diet of the leaves with high Zn concentrations had a **negative impact** on the growth of the grasshoppers.	Behmer *et al*., 2005.
Grasshopper (*Schistocerca gregaria)*	14,000	In contradiction to the work by Behmer *et al* (2005), this study found that *S. gregaria* **did not show a preference** when presented with a choice of leaves containing high and low Zn concentrations.	Pollard and Baker, 1997.
Butterfly (*Pieris napi oleracea)*	34,000	**No preference** was found when *P. napi oleracea* was presented with a choice of leaves containing high and low Zn concentrations.	Jhee *et al*., 1999.
Butterfly (*Pieris brassicae)*	7,400	**No preference** was found when *P. brassicae* was presented with a choice of leaves containing high and low Zn concentrations.	Pollard and Baker, 1997.

Table 3.1: Experiments which have examined the response of herbivores to elemental defence using different concentrations of Zn in the leaves of *T. caerulescens* (adapted from Noret 2007).

Previous experiments to test the elemental defence hypothesis in *T. caerulescens* have been based on different concentrations of Zn in the leaves, from as little as 2,700 mg Zn kg^{-1} (DW) to as much as 34,000 mg Zn kg^{-1} (DW) (Baker and Brooks, 1989). It may be difficult to apply the findings of these experiments to phytoremedation work, as the concentration of Zn in the leaves of *T. caerulescens* growing in contaminated land in the wild is not precisely known. The expected Zn concentration of *T. caerulescens* leaves based on concentrations of Zn in soils from previous experiments, may be expected to be as high as 35,000 mg kg^{-1} (Table 3.2).

Nominal Zn Concentration mg kg^{-1} added to soil (Dry weight)	**Predicted concentration of Zn in plant biomass**
0	2000 mg kg^{-1} (a)
300 (ICRCL,1987 phytotoxicity threshold)	6000 mg kg^{-1} (b)
1000 (typical contaminated site)	35000 mg kg^{-1} (b)

Table 3.2: Composition of initial soils and predicted Zn concentrations in the plants
(a) Based on figures produced by Noret *et al*., (2005).
(b) Based on figures produced by Haines (2002).

The choice of herbivore used in previous studies has also differed markedly. Whether these species are found feeding on *T. caerulescens* in the wild on contaminated land has obvious implications for the validity of experimental results. Most studies in Table 3.1 show that high levels of Zn appear to have no adverse effects on herbivore preference and performance. Snails have been used in a number of the studies (Laskowski and Hopkin, 1996; Noret *et al*., 2005), discussed in more detail in Chapter 1 (Section 1.7.1) and the preference and performance of adult *H. aspersa* seems unaffected by Zn. However, findings by Gomot-De Vaufleury (2000) suggest that Zn did have an impact on the growth of juvenile snails, when reared on an artificial diet containing a high concentration of Zn. It may be that the artificial constraints of laboratory experiments force the herbivores into making unrealistic choices. The apparent discrepancy between the effects of Zn on juvenile snails, as opposed to adult snails needs to be investigated as part of the elemental defence

hypothesis. The effects of plant defences often differ according to the age of the herbivore and younger herbivores maybe more vulnerable to their effects (Massey and Hartley, 2009).

There are few studies which examine the elemental defence hypothesis under field conditions, even though this type of research could determine whether there was a defence mechanism taking place which occurs without the artificial constraints of laboratory based experiments. Justification for this is that field studies can be difficult to execute effectively and challenging to interpret correctly. One limitation is the difficulty of taking accurate measurements in the field. It is common practice for soil and composite plant material samples from contaminated sites to be analysed by lab-based techniques such as F-AAS at a later date, as for the reasons stated above this is thought to ensure greater accuracy. However, there is an alternative procedure using Portable x-Ray Fluorescence (P-XRF) equipment as an *in-situ* measurement device to determine the level of Zn concentrations in soil (Anderson *et al*., 1998; Argyraki *et al*., 1997). In order to determine the accuracy of this equipment it should be compared with the results of F-AAS. In addition, the P-XRF has the potential to be used for *in-situ* measurement of Zn concentrations in the living tissue of the plants at field sites, a technique which has not been documented before.

3.2 Aim and hypotheses

The aim of this chapter was to establish the suitability of *T. caerulescens* for evaluating the elemental defence hypothesis using Zn. This was achieved by testing three hypotheses:

(i) The amount of Zn sequestered by *T. caerulescens* would depend on the concentration of Zn in the soil. This is important to increase our understanding of how this plant species sequesters Zn.

(ii) The herbivore (*H. aspersa*) would not show any preference for *T. caerulescens* containing different levels of Zn. It is important to know whether the herbivore can distinguish between high and low Zn concentrations.

(iii) In regions where land has been contaminated by Zn, the amount of Zn accumulated by *T. caerulescens* depends on the concentration of Zn in the soil. These data were needed to determine realistic concentrations of Zn to be used in future experiments to ensure that the results would be applicable to the concentrations herbivores encounter in a real world situation.

In addition, an assessment was made of the usefulness of using a piece of equipment (P-XRF) for *in-situ* measurements of Zn concentrations in both soils and plants.

3.3 Assessing the suitability of the Zn hyperaccumulator *T. caerulescens* to test the elemental defence hypothesis under greenhouse conditions.

3.3.1 Materials and methods

3.3.1.1 Experimental design

Plants were grown in three different Zn concentration treatments (Treatment A: control; Treatment B: 300 mg kg^{-1}; Treatment C: 1000 mg kg^{-1}) for 11 weeks (23rd November 2005 to 2nd of February 2006). The greenhouse was kept on a 16hr light, 8hr dark cycle and maintained at approximately 20^{o}C ± 5^{o}C. The experiment used a randomised block design, with 20 replicates of each treatment, a total of sixty plants. After the required growing period, plants were harvested for chemical analysis. In addition to above-ground biomass, plants roots were also harvested to be used in the analysis, including root mass and root: shoot ratio. Prior to harvest, leaves from treatment A and C were used in a choice preference trial with the generalist herbivore *H. aspersa* (common garden snail).

3.3.1.2 Seed germination and soil spiking

Seeds of *T. caerulescens* of the Bradford Dale ecotype (which has been identified as a suitable hyperaccumulator of Zn, Haines, 2002) were germinated on uncontaminated wet potting sand, at 25^{o}C, under natural lighting conditions, for a period of 10 days. Upon germination the seedlings were planted directly into soil containing a 60:40 mix by fresh mass of John Innes number two compost and dry potting sand (for detail of preparation see Chapter 2, Section 2.3). Plants were grown in one of three soil treatments, a control (containing no additional Zn) (Treatment A), or a soil spiked with Zn to concentrations of either 300 mg kg^{-1} (Treatment B) or 1000 mg kg^{-1} (Treatment C). All Zn used in the preparation of soils was in the form of Zn oxide (ZnO). In soils containing additional Zn, 2000 mg kg^{-1} of Zn as ZnO was added to 400g of dry sand to act as a carrier. When mixed with the same quantity of John Innes the highest concentration of 1000 mg kg^{-1} (Treatment C) had been achieved. The other treatment was made by serially diluting a single kilogram of the soil mixture spiked at 1000 mg kg^{-1} with soil mixture containing no added Zn. Sub-samples of soil (~15g) were collected during soil preparation. These were dried at 60^{o}C for

48hrs, ground using a pestle and mortar and then analysed to determine the Zn concentration using F-AAS.

3.3.1.3 Plant harvest

The plants were harvested after 12 weeks of growth, as described in Section 2.2.4 of Chapter 2.

3.3.1.4 Preference experiment using the herbivore *H. aspersa*

Nine adult snails (collected from the grounds of the University of Sussex) were placed in individual cages. In each cage, a snail was left equidistant (2.5cm) from two leaves of known area (measured using a leaf area meter, by ADC BioScientific) (Figure 3.1). One leaf had been selected from treatment A (no added Zn) and the other, treatment C (1000 mg kg^{-1}). The experimental cages were left in the dark for eight hours. The leaves were then removed and their area re-measured.

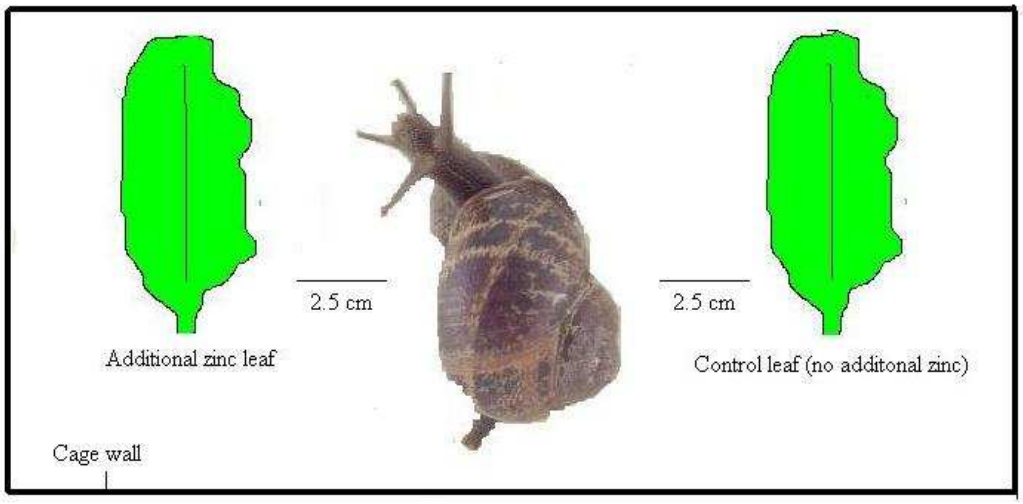

Figure 3.1: Diagram of the preference trial set-up, involving *H. aspersa* (not to scale)

3.3.1.5 Chemical analysis of plant and soil samples

Samples of soil (n = 27) and plant material (n = 42) were analysed by F-AAS after nitric and perchloric acid digestion, as described in Sections 2.4.2 and 2.4.3 in Chapter 2. In

addition, both Certified Reference Materials (CRM's) and House Reference Materials (HRM's) were analysed to estimate analytical bias.

3.3.1.6 Statistical analysis

Data were analysed using Minitab (Version 14) and presented using Microsoft Excel (Microsoft Office, 2003). Binary regression was used to assess the survival of plants grown in different Zn concentrations. One-way ANOVA (Analysis of Variance) was used to determine differences between Zn concentrations in the growth media, leaves and concentration factor (CF) in addition to above and below ground biomass and root: shoot ratio. Data were checked for normality (probability plots) and equal variance (Levene's test) to ensure they conformed to the assumptions of this test. Significant results were subjected to post-hoc Tukey's test to determine how they differed from another at $P < 0.05$. Data from the snail preference test was not normally distributed therefore the non-parametric Wilcoxon signed rank test was used.

3.3.2 Results

Before data were analysed to determine whether the elemental defence hypothesis held true in *T. caerulescens* contaminated with Zn, data were analysed to ensure that the experimental design was robust.

There was no significant difference in the number of plants which survived (Figure 3.2) when grown in the three different Zn treatments. The plant fatalities (5) that occurred were not related to the treatment they were grown on. It was important to establish this, as the concentrations used in treatments B and C were known to be phytotoxic to some plants (Broadley *et al*., 2000) and may have affected plant survival.

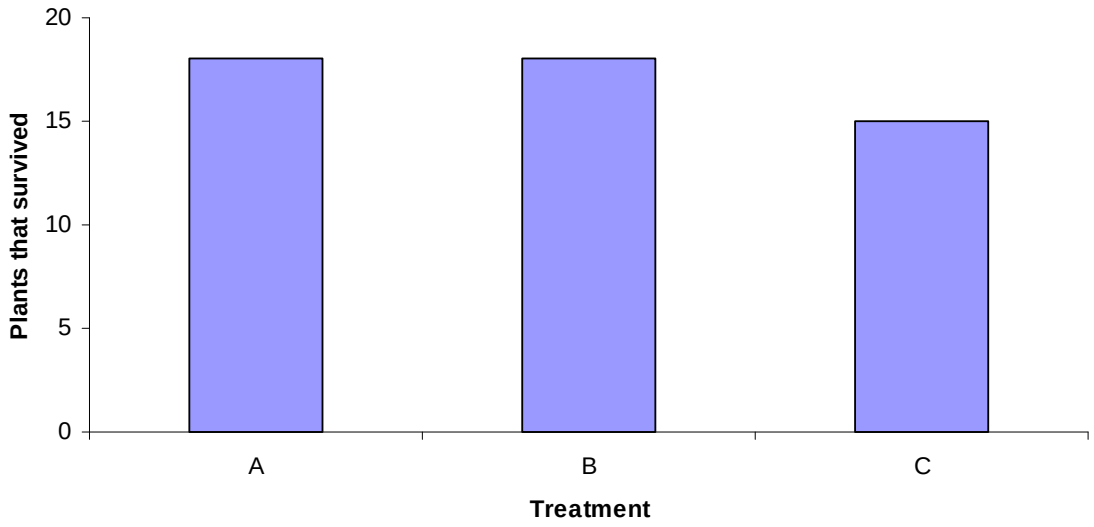

Figure 3.2: *T. caerulescens* (n = 20) plant survival when grown on three different Zn treatments. Binary logistic regression of Zn treatment on plant survival, $G_{19} = 2.225$, $P = 0.329$ (Treatment A: control, Treatment B: 300 mg kg^{-1}, Treatment C: 1000 mg kg^{-1}).

To determine whether the nominal concentrations of Zn had been achieved for each treatment, soil samples from each treatment were analysed. The mean concentrations of Zn present in the three growth media were, 38 mg kg^{-1} (Treatment A), 278 mg kg^{-1} (Treatment B) and 974 mg kg^{-1} (Treatment C) (Figure 3.3). These were very close to the nominal target values of 0 mg kg^{-1} (Treatment A), 300 mg kg^{-1} (Treatment B) and 1000 mg Zn kg^{-1} (Treatment C). Treatment A, the control, was found to contain a Zn concentration of 38 mg kg^{-1}. This resulted from Zn already present in the compost; therefore it was not possible to have a treatment with no Zn. The three Zn treatments were also found to be significantly different from one another.

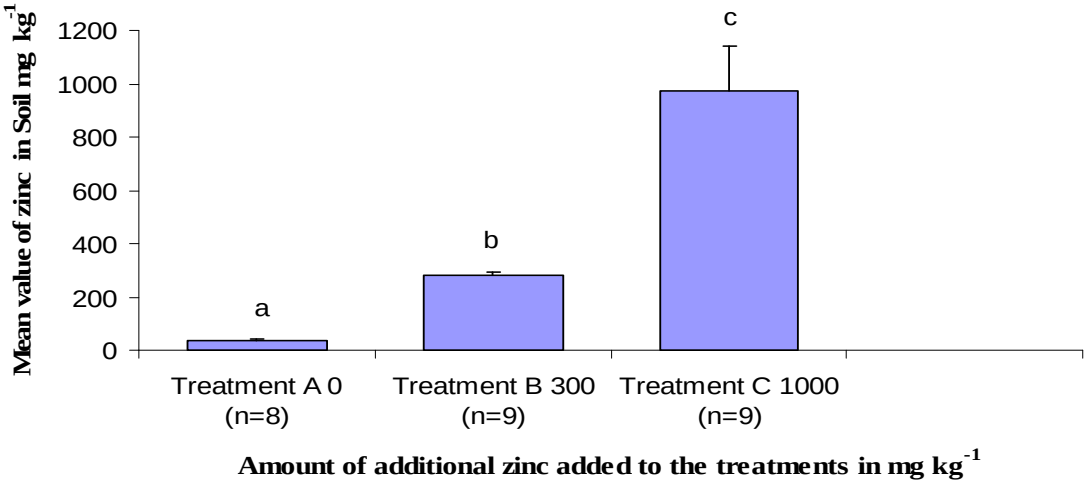

Figure 3.3: Mean ± SE Zn concentration in the growth media treatments (A-C) ANOVA. Different letters above bars indicate significant differences between treatments (Tukey Test).

Source	d.f.	SS	MS	F	P
Soil Zn concentration	2	9.70	4.84	49.97	**<0.001**
Error	23	2.23	0.09		
Total	25				

Table 3.3: Statistical output of the Zn concentration in the growth media treatments (A-C) ANOVA.

These results confirm that the experimental design was robust. Data from the greenhouse experiment were then analysed to determine the affect of different soil Zn concentrations on *T. caerulescens*.

Mean Zn concentrations in the above ground biomass of plants, in treatments B (4582 mg kg^{-1}) and C (3651 mg kg^{-1}) were significantly different from plants grown in treatment A (847 mg kg^{-1}) the control (Figure 3.4). When these results were compared against the predicted concentrations, there was a noticeable discrepancy. The concentrations of Zn were lower than those predicted from the soil concentrations and uptake values of Zn *T. caerulescens* by other researchers as shown in Table 3.2. Treatment A was 2.4 times lower than predicted (expected concentration = 2000 mg kg^{-1}), Treatment B was 1.3 times lower than predicted (expected concentration = 6000 mg kg^{-1}) and Treatment C was 9.6 times lower than predicted (expected concentration = 35000 mg kg^{-1}).

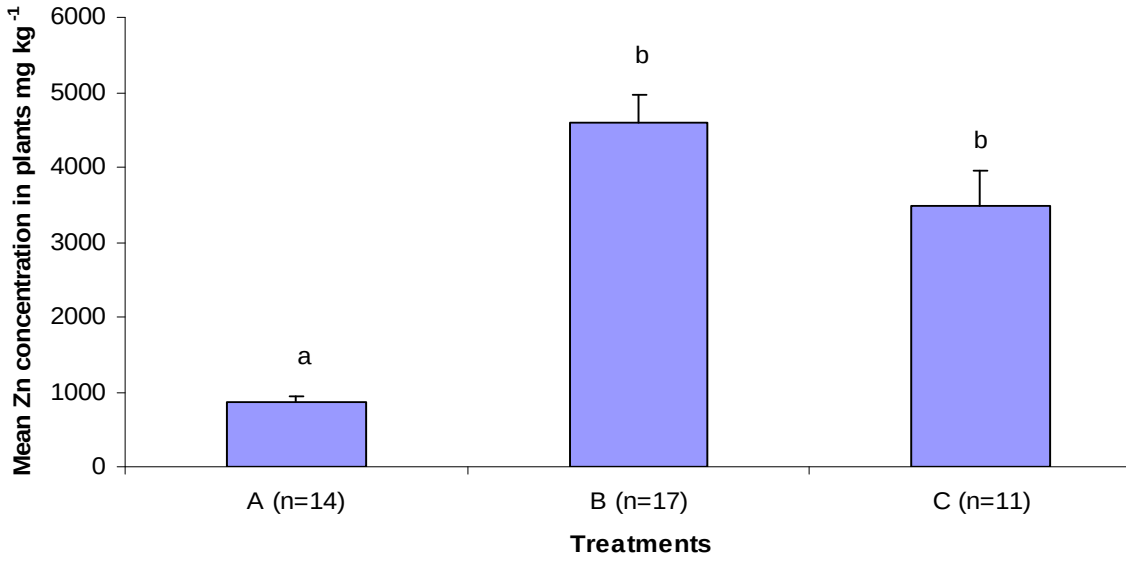

Figure 3.4: Mean ± SE Zn concentration in the above ground biomass of *T. caerulescens* ANOVA. Letters above bars indicate significant differences between treatments (Tukey Test).

Source	d.f.	SS	MS	F	P
Plant Zn concentration	2	1.1×10^8	5.4×10^7	36.92	**<0.001**
Error	39	5.8×10^7	1.5×10^6		
Total	41	1.7×10^8			

Table 3.4: Statistical output of the concentration in the above ground biomass of *T. caerulescens* ANOVA.

The concentration factor (CF) is the ratio of Zn concentration in the plants above ground biomass to soil.

$$\text{Concentration Factor (CF)} = \frac{\text{Plant concentration (mg kg}^{-1}\text{)}}{\text{Growth Media concentration (mg kg}^{-1}\text{)}}$$

The CF was highest in Treatment A and lowest in Treatment C (Figure 3.5). This suggests that *T. caerulescens* is most effective at concentrating Zn in its above ground biomass when the growth media contains a low concentration of Zn.

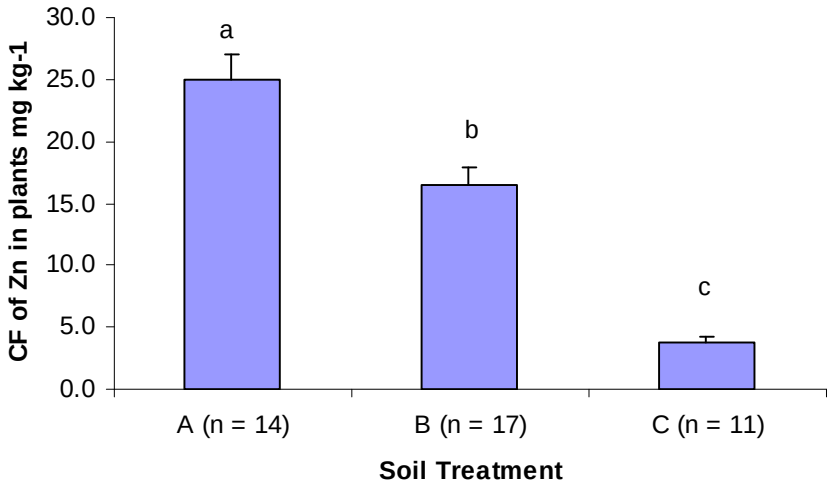

Figure 3.5: Mean ± SE of Zn concentration factor in the above ground biomass of *T. caerulescens*, ANOVA. Letters above bars indicate significant differences between treatments (Tukey Test).

Source	d.f.	SS	MS	F	P
Concentration factor	2	2.3×10^3	1.1×10^3	23.47	**<0.001**
Error	42	2.1×10^3			
Total	44	4.5×10^3			

Table 3.5: Statistical output of the Zn concentration factor in the above ground biomass of *T. caerulescens*, ANOVA.

The mean above ground biomass (DW) of *T. caerulescens* was greatest (1.38g) in Treatment B (278 mg Zn kg^{-1}) (Figures 3.6 and 3.7). Plants grown in treatments A and C produced significantly lower biomass. This implies that Treatment B contained an optimal concentration of Zn for biomass production. Treatment A may contain too little Zn for optimal growth, whilst the high levels of Zn in Treatment C may have stressed the plant and as a result reduced biomass.

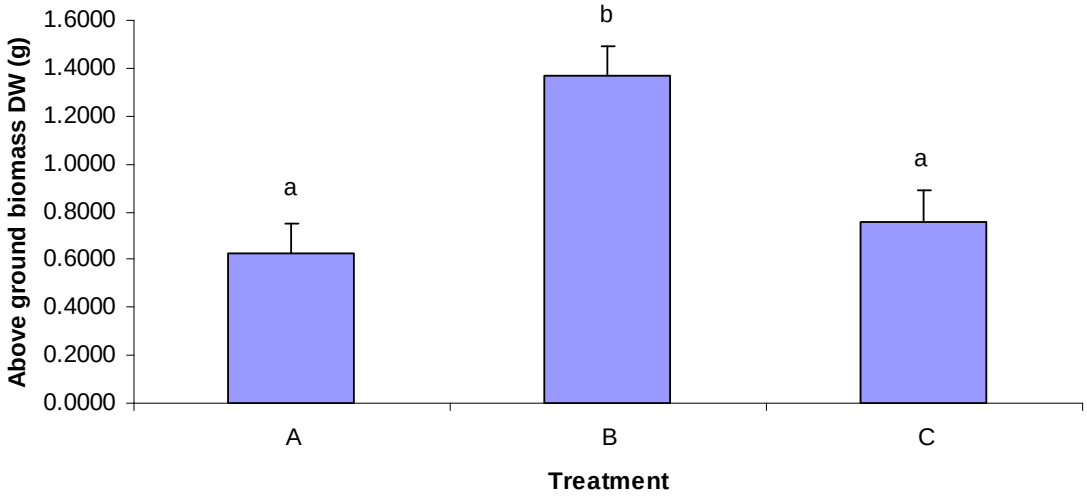

Figure 3.6: Mean ± SE biomass production of *T. caerulescens* grown on different Zn treatments ANOVA. Letters above bars indicate significant differences between treatments (Tukey Test).

Source	d.f.	SS	MS	F	P
Biomass production	2	5.6	2.8	10.39	**<0.001**
Error	48	12.9	0.27		
Total	50	18.6			

Table 3.6: Statistical output of the biomass production of *T. caerulescens* grown on different Zn treatments ANOVA.

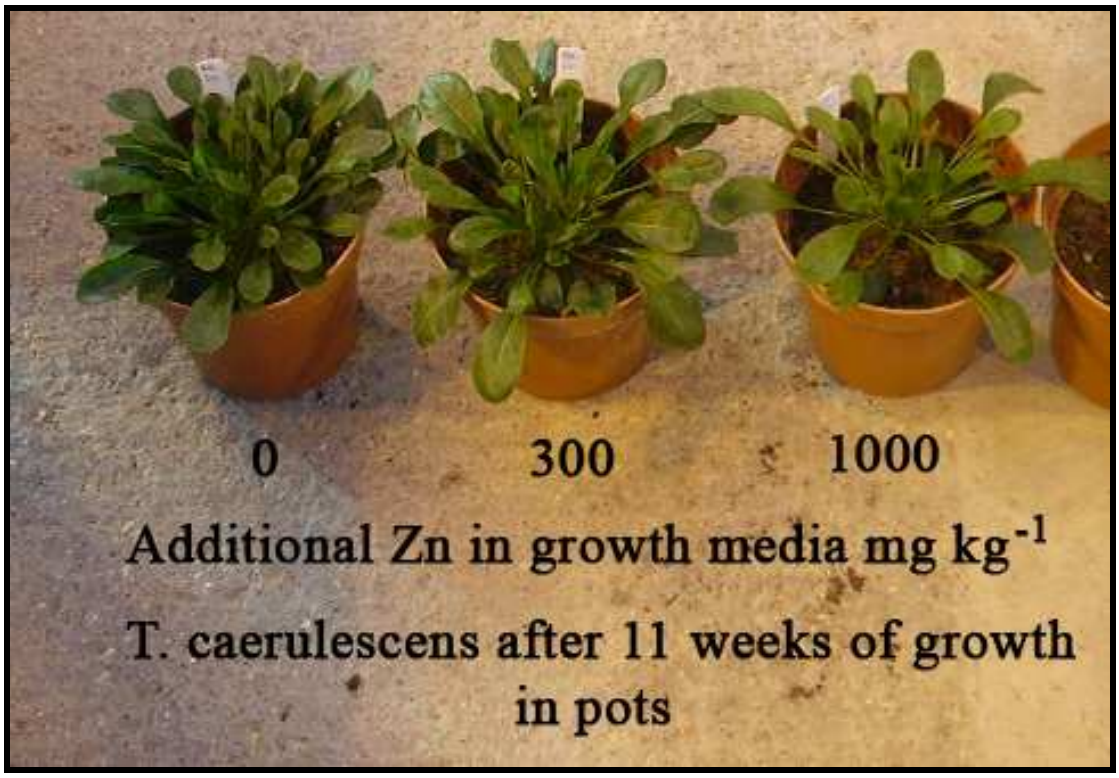

Figure 3.7: Photograph of *T. caerulescens* grown on three different Zn concentrations at the end of the experiment.

The mean root mass DW (Figure 3.8) produced by plants in the different treatments revealed the same trend as for above ground biomass (Figure 3.6). Plants in Treatment B produced significantly more root mass than plants grown in Treatment A or C.

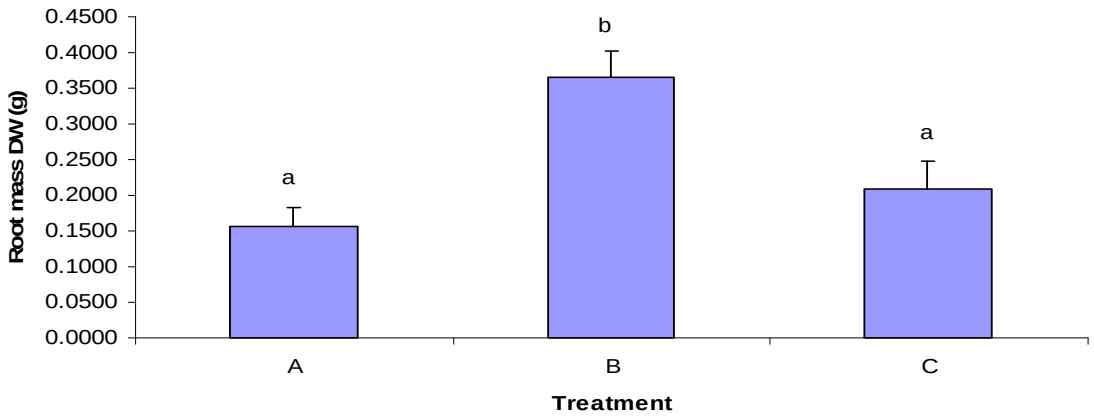

Figure 3.8: Mean ± SE root mass (dry weight) produced by *T. caerulescens* grown on three different Zn treatments ANOVA. Letters above bars indicate significant differences between treatments (Tukey Test).

Source	d.f	SS	MS	F	P
Root mass	2	0.42	0.21	10.08	**<0.001**
Error	48	1.00	0.02		
Total	50	1.42			

Table 3.7: Statistical output of the root mass (dry weight) produced by *T. caerulescens* grown on three different Zn treatments ANOVA.

The root: shoot ratio for *T. caerulescens* was not significantly different for plants grown in the three Zn concentration treatments (Figure 3.9). This suggests that although the plants differed in terms of both above and below ground biomass, the relative allocation to roots and shoots is unaffected by Zn.

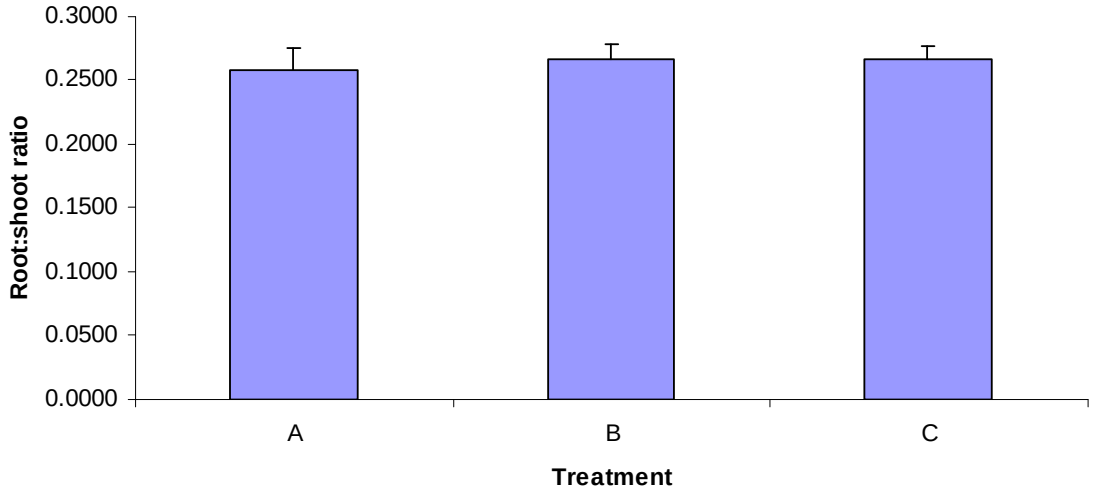

Figure 3.9: Mean ± SE root: shoot ratio (dry weight) produced by *T. caerulescens* grown in three different Zn treatments ANOVA.

Source	d.f	SS	MS	F	P
Root: shoot ratio	2	7.5×10^{-4}	3.7×10^{-4}	0.12	0.887
Error	48	14.8×10^{-2}	3×10^{-3}		
Total	50	14.8×10^{-2}			

Table 3.8: Statistical output of the root: shoot ratio (dry weight) produced by *T. caerulescens* grown in three different Zn treatments ANOVA.

To determine the effect of Zn accumulation on higher trophic levels a choice feeding preference trial (Figure 3.10) using adult *H. aspersa*, presented with a choice of a *T. caerulescens* leaves, grown on control soil (Treatment A) and on high Zn concentration soils (Treatment C) was used. The results showed that *H. aspersa* did not have a significant preference for leaves from either treatment ($W_9 = 8$, $P = 0.097$) Table 3.3, even though the leaves from Treatment C had Zn concentrations approximately four times higher than the control.

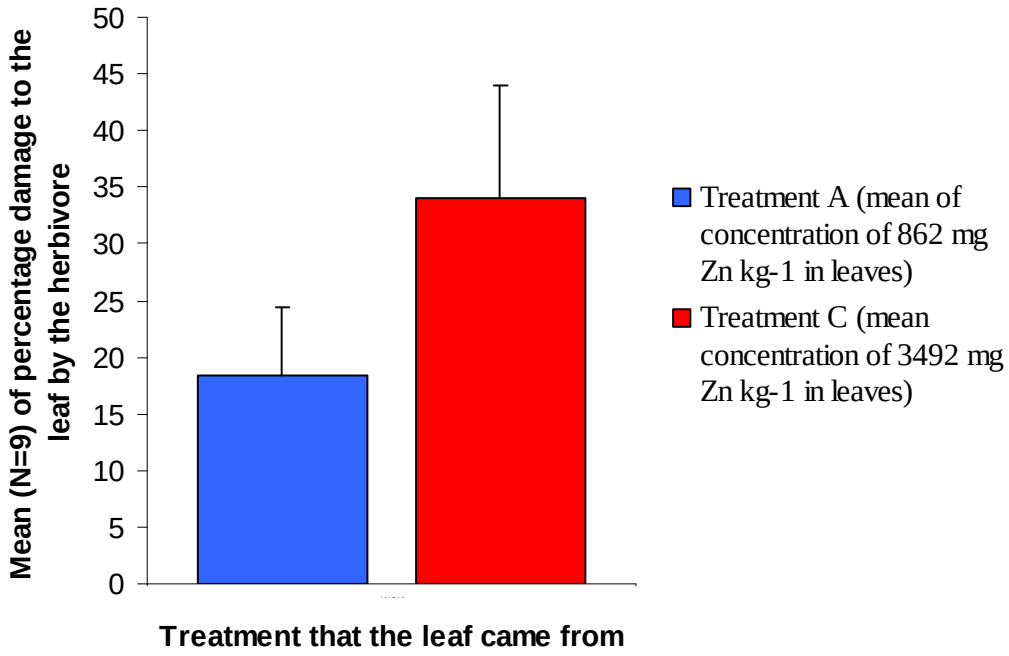

Figure 3.10: Mean ± SE percentage damage to leaves from the different Zn treatments in a preference trial using the herbivore *H. aspersa*. $W_8 = 8$, $P = 0.097$, showing no significant difference between treatments.

The statistical results from chapter 1 have been summarised in Table 3.9 below.

Response	Factor	d.f.	F/G/W statistic	P
Plant survival	Zn Treatment	2	2.25*	0.329
Zn concentration in growth media	Zn Treatment	2	49.98	**<0.001**
Zn concentration in plants	Zn Treatment	2	86.39	**<0.001**
Concentration Factor	Zn Treatment	2	23.47	**<0.001**
Above ground biomass	Zn Treatment	2	10.39	**<0.001**
Root mass	Zn Treatment	2	10.08	**<0.001**
Root: Shoot ratio	Zn Treatment	2	0.12	0.887
Percentage damage in feeding trial	Zn Treatment	8	8.0**	0.097

Table 3.9: Summary of statistical analysis of *T. caerulescens* grown on three different Zn treatments. Data were analysed by ANOVA when the data conformed to the assumptions, unless indicated by * for binary regression or ** for a Wilcoxon ranked sign test. Bold type indicates a significant result.

3.4 Field survey to determine the concentration of Zn in T. caerulescens growing wild on contaminated land.

3.4.1 Materials and methods

3.4.1.1 Experimental design

Derbyshire, England was chosen as a study site for the field survey for three main reasons; (1) it has a large number of areas classed as contaminated land with Zn concentrations exceeding 10,000 mg kg^{-1} (Baker *et al*., 1994); (2) It is one of the locations within the UK where populations of *T. caerulescens* are common (Baker and Brooks, 1989); (3) Field research was carried out by Baker *et al*., (1994) in this area so any results may be comparable with those from previous studies. Three sites were selected for the field survey during May 2006. The first two were at Black Rocks, O/S grid reference SK 293 556 (Figure 3.11).

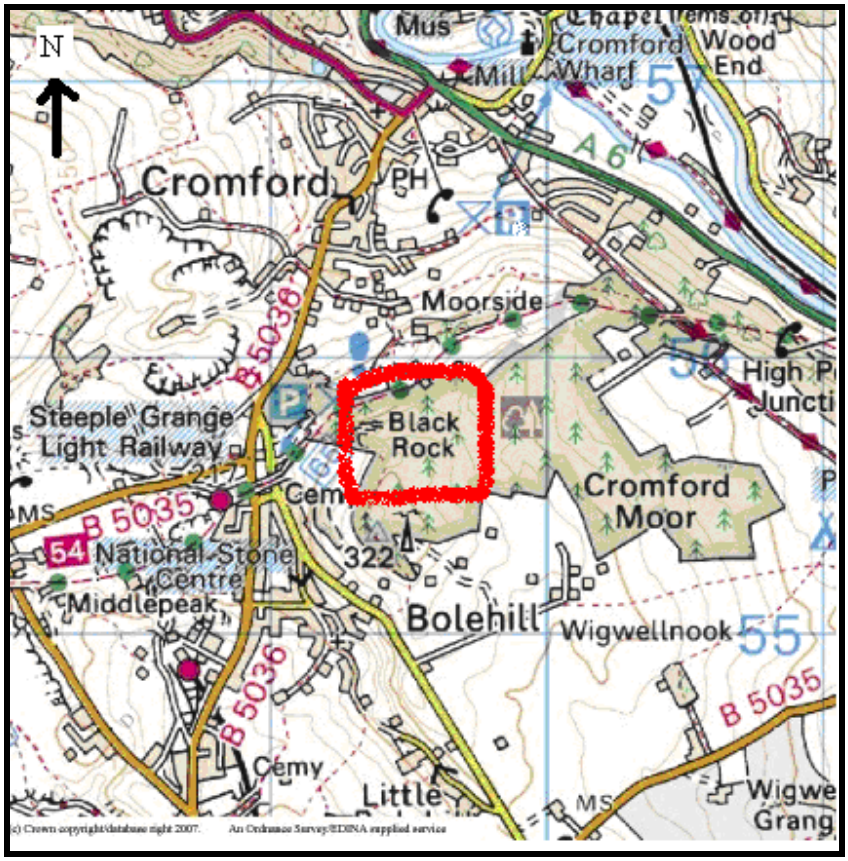

Figure 3.11: O/S Map of the location of Black Rocks sample site in Derbyshire, UK. This map was generated using EDINA Digimap.

Due to the size and nature of the site at Black Rocks it was deemed necessary to split it into two. The first, Black Rocks top (BRT), was at the top of the site and on a steep gradient running north to south (Figure 3.12). The second site at Black Rocks was termed Black Rocks bottom (BRB) and was on a flat gradient running east to west.

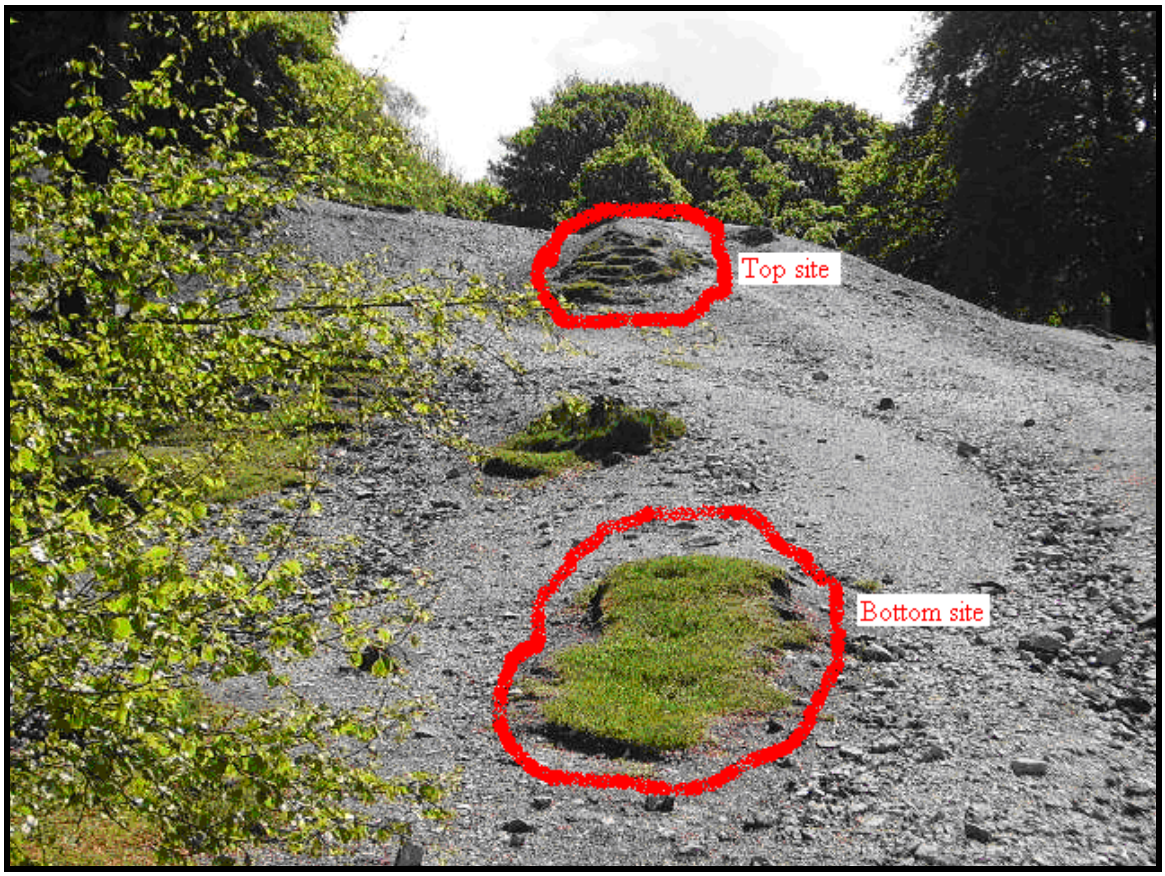

Figure 3.12: Photograph of Black Rocks. The bottom site is in the foreground and the top site in the background, sample site in Derbyshire.

The second location was approximately 7 miles from Black Rocks, in Clough Wood (CW), O/S grid reference SK 254 616 (Figure 3.13). As Figure 3.13 illustrates, the site at Clough Wood was also on a gradient.

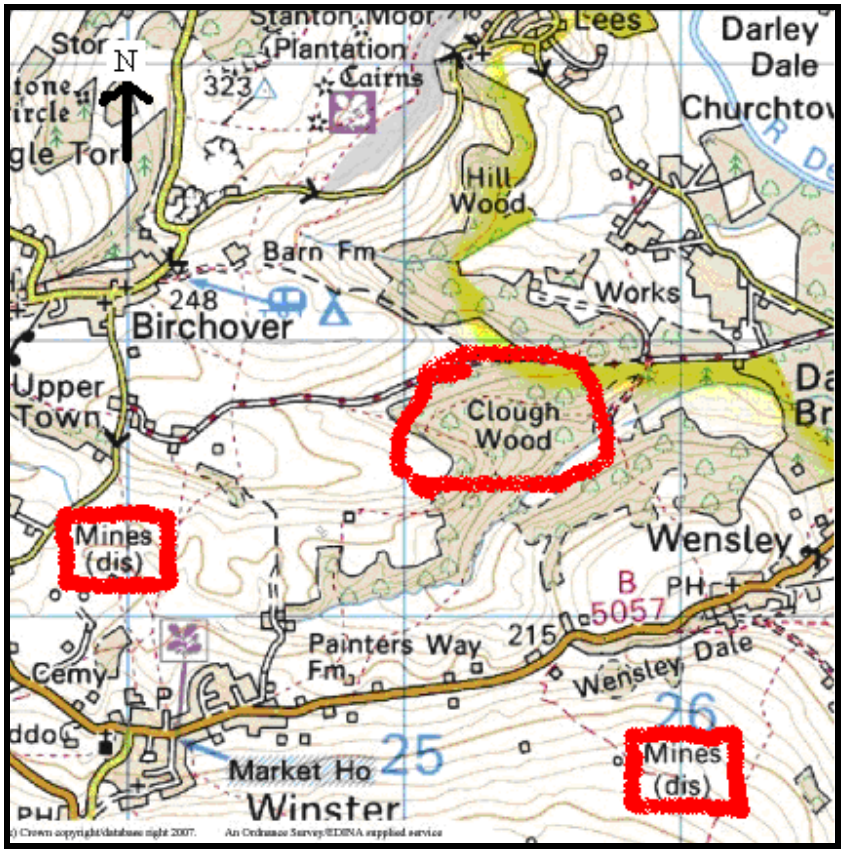

Figure 3.13: O/S Map of the location of Clough Wood sample site in Derbyshire, UK. This map was generated using EDINA Digimap. Note the presence of the two disused mine sites close to the Clough Wood.

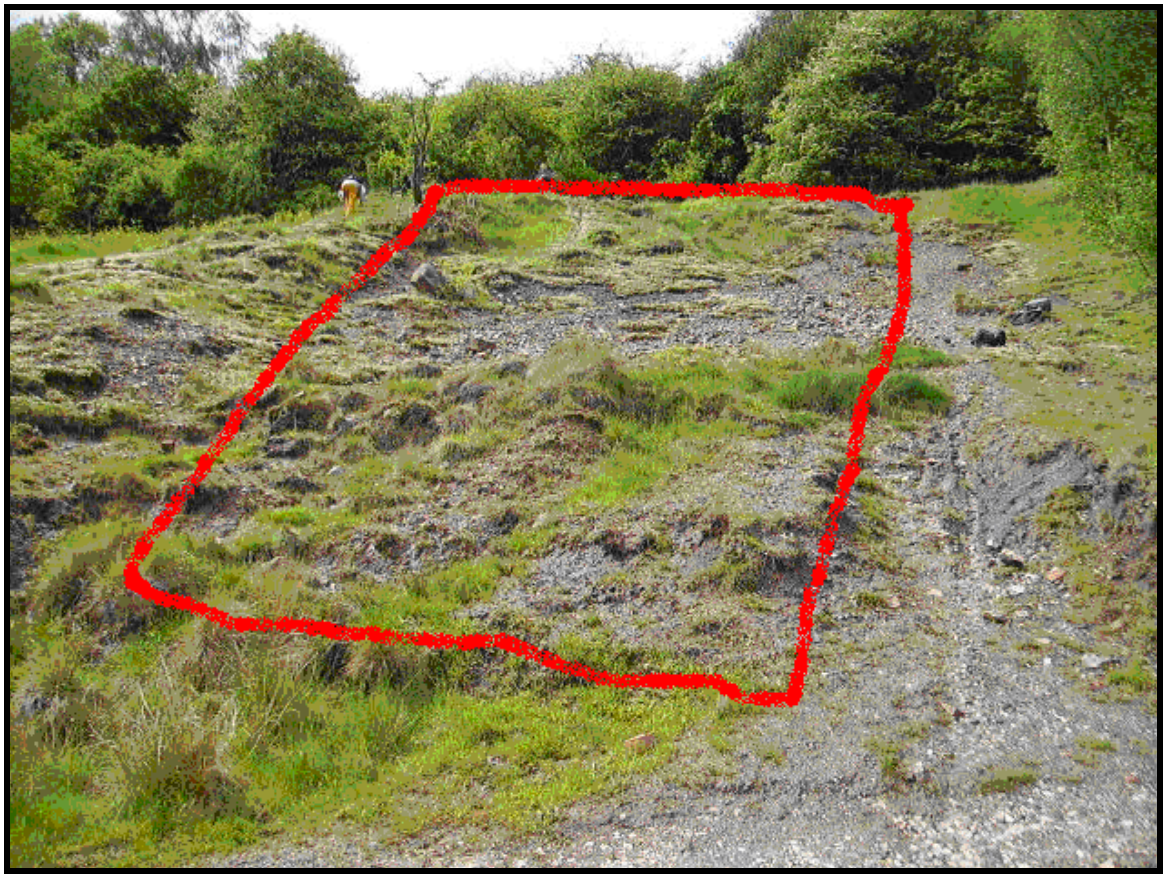

Figure 3.14: Photograph of Clough Wood sample site in Derbyshire.

All three sites were examined to confirm that *T. caerulescens* (Figure 3.15) was present in sufficient quantities for the survey.

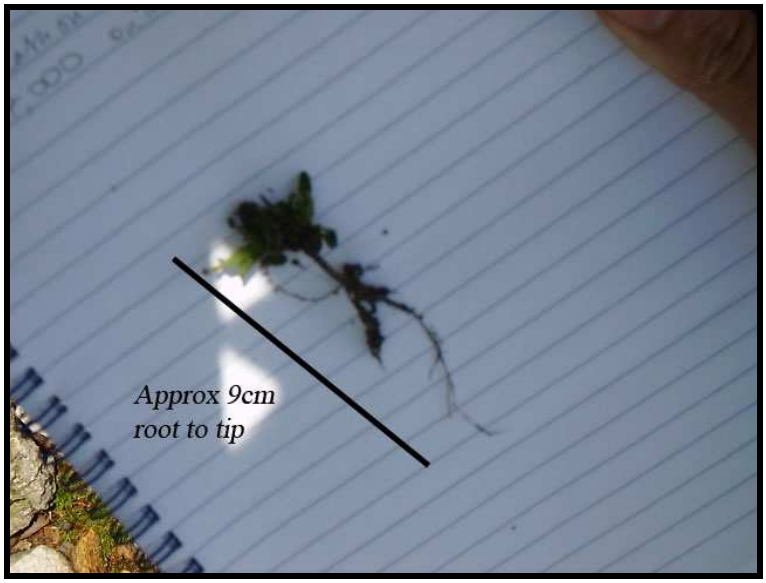

Figure 3.15: *T. caerulescens* in the wild in the middle of May, from Black Rocks in Derbyshire, UK.

At each of the three sites (Clough Wood, Black Rocks top and Black Rocks bottom) a nine point sample grid was arranged as displayed in Figure 3.16. At each of the points the surface vegetation was scraped away using a trowel to reveal bare soil over an area of approximately 5 by 3 cm. The P-XRF (Figure 3.17) was then used to record Zn concentration measurements. P-XRF works by the emission of radiation from an electronically excited X-ray tube. The presence of a particular element fluoresces to a specific wave length and the strength of the return signal to the P-XRF is used to quantify the concentration of the element in the media.

Having analysed the soil by P-XRF, a soil scrape (approximately 0.5 cm in depth) was collected at each of the points for *ex-situ* analysis by F-AAS at a later date. At five of the sample points at Black Rocks top and Black Rocks bottom and all nine of the Clough Wood sample points, *T. caerulescens* were collected, to be analysed by F-AAS at a later date. In addition an attempt was made to measure the Zn concentration of plant leaves directly using the P-XRF window onto the leaf. *T. caerulescens* were also examined for herbivore damage in order to provide a comparison with damage observed in the feeding preference experiment. However, none of the plants surveyed had damage in sufficient quantities to warrant detailed recording.

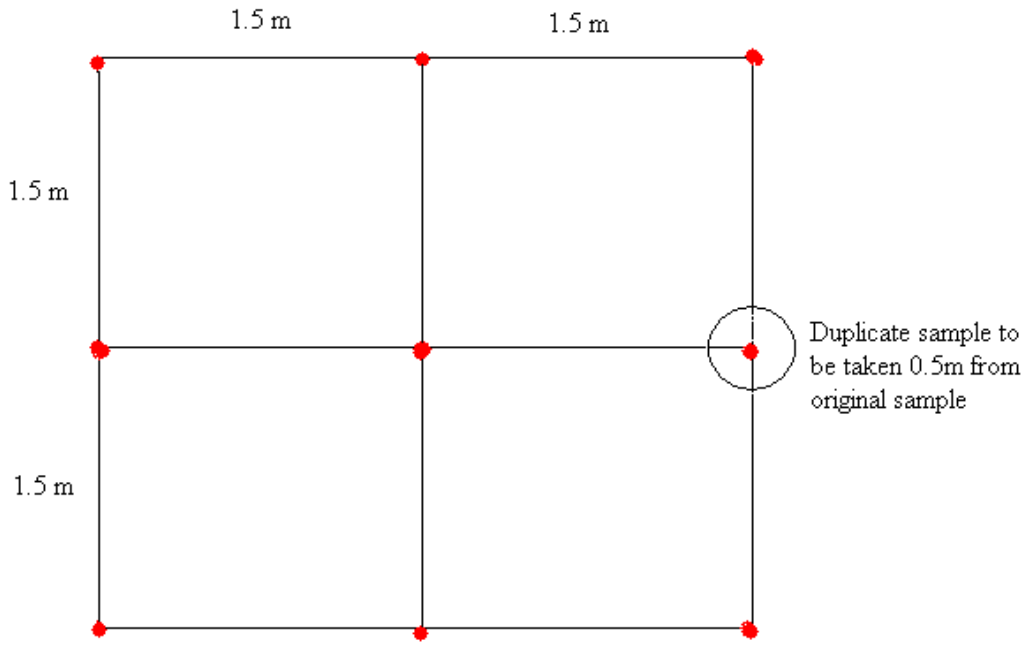

Figure 3.16: Schematic illustration of the nine point sampling system, each red dot represents a sampling point.

Figure 3.17: Photograph of Spectrace TN 9000, P-XRF equipment.

3.4.1.2 Chemical analysis

Composite plant and soil samples collected from the three sites were also analysed in the lab by F-AAS after having undergone acid digests described in Sections 2.4.2 and 2.4.3 of Chapter 2.

3.4.1.3 Statistical analysis

Data were analysed using Minitab (Version 14). One-way ANOVA (Analysis of Variance) was used to assess whether there was any significant differences between the Zn concentration in the soil and leaves at the three sites. Data were assessed for normality (probability plots) and equal variance (Levene's test) to ensure that they conformed to the assumptions of ANOVA. Post-hoc Tukey's test was then used to determine which treatments differed significantly from one another at $P < 0.05$. Regression analysis was used to compare F-AAS and P-XRF data.

3.4.2 Results

The Zn concentration of soils at all three sites, determined by F-AAS, exceeded 20,000 mg kg^{-1}. The mean Zn concentration of soil at Black Rocks bottom was in excess of 35,000 mg kg^{-1}. When the results were compared to ICRCL values it was confirmed that all three sites were heavily contaminated with Zn. Any vegetation growing on these soils must possess some form of adaptation to cope with the high levels of Zn contamination. Statistical analysis of the data revealed that there was a significant difference between the mean soil concentrations found at Black Rocks bottom and the other two sites, Black Rocks top and Clough Wood (Figure 3.18).

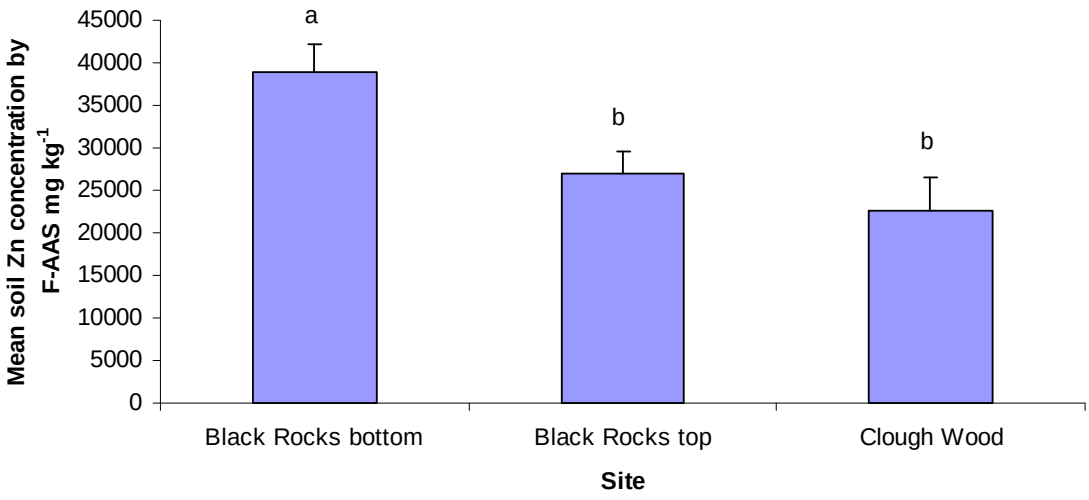

Figure 3.18: Mean ± SE Zn concentration (mg kg^{-1}) of the soil samples (n = 9) from the three sample sites in Derbyshire, analyzed by F-AAS ANOVA. Letters above bars indicate significant differences between treatments (Tukey Test).

Source	d.f.	SS	MS	F	P
Soil Zn concentration	2	1.2×10^9	6.4×10^9	6.26	<0.001
Error	24	2.4×10^9	1.0×10^9		
Total	26	3.7×10^9			

Table 3.10: Statistical output of the Zn concentration (mg kg^{-1}) of the soil samples (n = 9) from the three sample sites in Derbyshire, analyzed by F-AAS ANOVA.

The F-AAS analysis of *T. caerulescens* plant samples collected from the three sites established that all plant material contained Zn concentrations in excess of 6000 mg kg^{-1}. The highest mean concentration was found in the plant matter collected from the Clough Wood site, with a mean value just over 10,000 mg kg^{-1}, which was significantly higher than the results from the Black Rocks top site. However, there was no significant difference between the mean Zn concentrations of plants collected at the two Black Rocks sites (Figure 3.19). The mean Zn concentration found in the above ground biomass of *T. caerulescens* was notably higher than reported in other plant species (Raskin *et al*., 1994), where such a concentration would be phytotoxic (Broadley *et al*., 2000). Of the three sites, only Clough Wood had plant Zn concentrations exceeding 10,000 mg kg^{-1}. *T. caerulescens* at these sites fall within the definition of a Zn hyperaccumulator (Baker and Brooks, 1989).

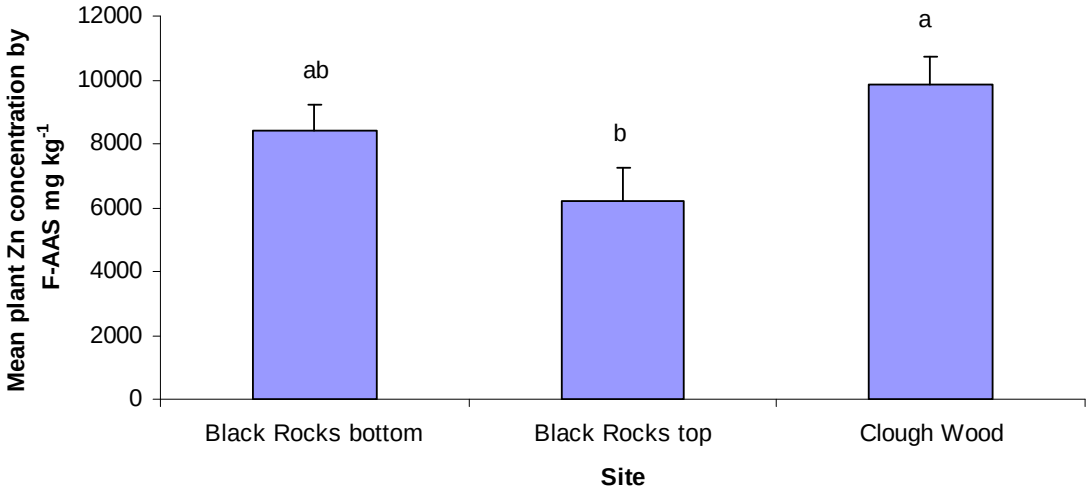

Figure 3.19: Mean ± SE Zn concentrations (mg kg^{-1}) of plant samples from the three sample sites in Derbyshire, analysed by F-AAS ANOVA. Letters above bars indicate significant differences between sites (Tukey Test).

Source	d.f	SS	MS	F	P
Plant Zn concentration	2	4.26×10^7	2.1×10^7	3.96	**0.04**
Error	16	8.6×10^7	5.3×10^6		
Total	18	1.2×10^8			

Table 3.11: Statistical output of the Zn concentrations (mg kg^{-1}) of plant samples from the three sample sites in Derbyshire, analysed by F-AAS ANOVA.

The concentration factor (CF) of Zn in plants, based on F-AAS data, was highest at the Clough Wood site (0.6) (Figure 3.20). There was a significant difference in the CF of this site and the two sites at Black Rocks (0.2 at BRT and 0.24 at BRB). Interestingly Clough Wood had the lowest mean concentration of Zn in the soil. These results were comparable with the results of greenhouse experiments, where plants grown in low Zn concentration soils had the highest CF.

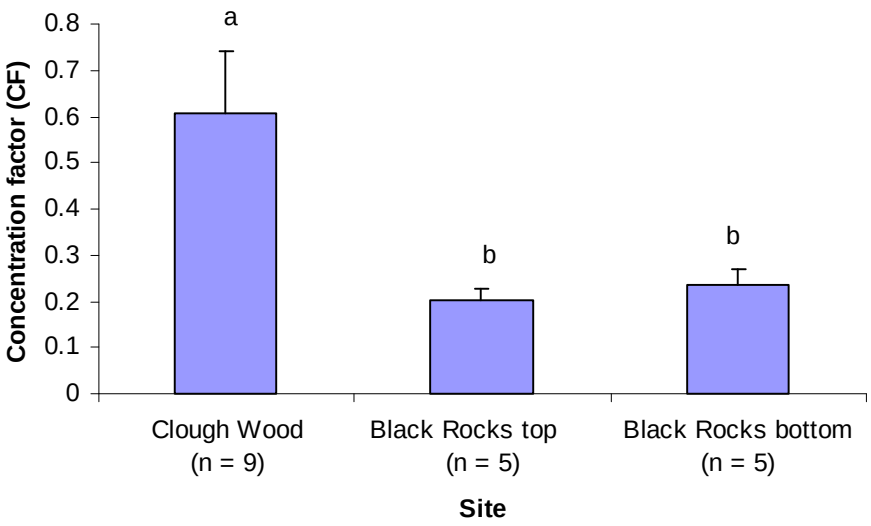

Figure 3.20: Mean ± SE Zn concentration factor (CF) of plants from the three different sites in Derbyshire using the F-AAS data ANOVA. Letters above bars indicate significant differences between treatments (Tukey Test).

Source	d.f.	SS	MS	F	P
Zn concentration factor	2	0.71	0.35	4.01	**0.039**
Error	16	1.41	0.088		
Total	18	2.12			

Table 3.12: Statistical output of the Zn concentration factor (CF) of plants from the three different sites in Derbyshire using the F-AAS data ANOVA.

In order to assess the performance of the *in-situ* measurements on soils by P-XRF, a regression analysis of the P-XRF and F-AAS results was made between soil samples from the three sites (Figure 3.21). It found that there was a positive relationship between the two

techniques. This established that it was possible to use P-XRF measurements as a reliable source of data for the concentration of Zn in soils. The slope of the regression line (0.224) indicates bias of -78%, this may be due field moisture and pore spaces and is not dissimilar to the value of -63% reported by Argyraki (1997). Therefore, any future work on the concentration of Zn in soils could use the P-XRF, after suitable correction for bias, to provide accurate *in-situ* measurements. However, there was no relationship between the F-AAS and P-XRF results for plant leaf Zn concentration. This may be because of a number of reasons including the P-XRF was determining the Zn concentration of fresh biomass, whilst the F-AAS used dry plant matter. Other plausible explanations include the leaves being thinner than x-ray penetration or soil contamination on the leaves.

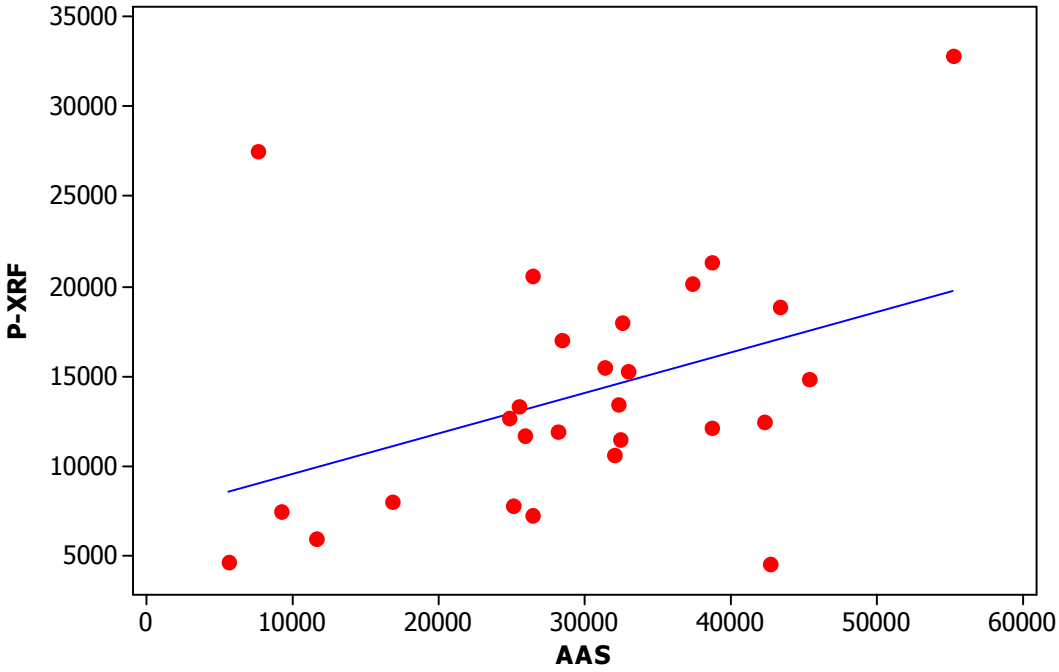

Figure 3.21: Regression analysis of the Zn concentration in soils by P-XRF and F-AAS in mg kg^{-1}. F_{26} = 4.85, P = 0.037. Equation of line P-XRF = 7348 + 0.224 x F-AAS.

Response	Figures	Factor	d.f.	F	P
Zn concentration of soils (F-AAS)	3.18	Site	2	6.26	**<0.001**
Zn concentration in plant material (F-AAS)	3.19	Site	2	3.96	**0.04**
Concentration factor	3.20	Site	2	4.01	**0.039**
Regression analysis of soil Zn concentration by F-AAS and P-XRF	3.21	F-AAS Vs P-XRF	1	4.85	**0.037**

Table 3.13: Summary of the statistical analysis of the field survey at Derbyshire in the UK

3.5 Discussion

3.5.1 Plant Zn concentrations

In greenhouse experiments, the highest mean Zn concentration occurred in plants grown on soils with lower additions of Zn (278 mg kg^{-1}). The mean Zn concentration of these plants was five times higher than the Zn concentration found in plants grown on soil which contained no additional Zn. The Zn concentrations of these plants were also 25% greater than the Zn concentration of plants grown on soils with the highest concentrations on Zn (974 mg kg^{-1}). However, the Zn concentration of plant material (4582 mg kg^{-1}) grown on soils with lower levels of Zn addition were still considerably lower (under half) than the threshold value of 10,000 mg kg^{-1} required for a plant species to be defined as a hyperaccumulator (defined by Baker and Brooks, 1989). These results were also lower than Zn concentrations achieved in some previous experiments. For instance Saison *et al*., (2004) grew *T. caerulescens* in a range of Zn growth treatments. Plants grown on their highest treatment (1280 mg Zn kg^{-1}) produced foliar Zn concentrations in the range of 13,716 - 17,516 mg kg^{-1}, 3 - 4 times higher than those found in this experiment. However, other researchers achieved Zn concentrations in *T. caerulescens* leaves within a similar

range to this experiment (2700 - 5770 mg kg^{-1} (Jiang *et al*., 2005; Behmer *et al*., 2005)). The concentrations achieved by Behmer *et al*., (2005) were high enough to have an effect on their chosen herbivore grasshopper species. This provides support for the concentrations of Zn chosen in this experiment, as the levels of Zn in the leaves may be high enough to have an effect on herbivores, but only if snails respond similarly to grasshoppers.

Zn concentrations found in plants at the three sites in Derbyshire UK, had Zn concentrations far greater than those produced in the laboratory experiment, almost three times the highest mean Zn concentration obtained in the greenhouse study. It is however, not surprising that plants grown in the wild than those cultivated under laboratory conditions as those growing on site are possibly better 'adapted' to cope/accumulate with high concentrations of Zn in the soil. The Zn concentration of these plants was just beyond the threshold value required for a plant species to be classified as a Zn hyperaccumulator (Baker and Brooks, 1989). Data collected by Baker *et al*., (1994) from the Black Rocks site found plant Zn concentrations of 19,071 mg kg^{-1} twice that of the results found in this investigation. The discrepancy between these results may have arisen from a difference in time, i.e. Baker *et al*., (1994) collected samples later in the year. Later in the year plants would be more mature which may increase their ability to accumulate Zn, but would also increase the length of time over which they had been accumulating, thereby increasing the concentration of Zn in their above ground biomass.

The attempt to quantify Zn concentrations in plants by the *in-situ* technique of P-XRF, did not prove overly successful. On comparison of results from the P-XRF with those from that of the *ex-situ* analysis of F-AAS (a more robust method) showed no correlation. However, there are a multitude of reasons for the difference in results, which including that the P-XRF was measuring the Zn concentration in plants wet weight, while analysis by F-AAS measures the Zn concentration in a dry homogenised sample. This was the first document attempt to try such an analysis, further research into this approach would further determine if it is possible.

3.5.2 Zn concentration factor

A metal concentration factor greater than one is typically found in plant species that are metal accumulators. A concentration factor less than one usually indicates that a species is an excluder of metal (Baker, 1989). *T. caerulescens* grown in greenhouse experiments is clearly an accumulator as the plant concentration factor for all treatments was greater than one. It was apparent that the concentration factor decreased with increased Zn concentration in the soil, which agrees with work by Zhao *et al*., (2003).

They analysed the results from nine different researchers that had grown *T. caerulescens* in varying concentrations of Zn and concluded that there was a negative log-linear relationship between Zn concentration in soil and that in the plant, a trend that was also observed by Robinson *et al*., (1998). The reason for the relationship between declining concentration factors with increasing soil concentration has been proposed by Pence *et al*., (2000). Plants are thought to become over saturated with Zn, when concentrations in the soil go beyond a certain level. This is thought to occur even when Zn toxicity is not observed in the plant (Zhao *et al*., 2003). *T. caerulescens* may be able to avoid the toxic effects of Zn at very high concentrations, but their ability to concentrate Zn into above ground biomass is diminished.

The results from the field survey supported those produced in the greenhouse experiment. Plants growing on contaminated sites in Derbyshire were growing in extremely high concentrations of Zn (39,013 – 22681 mg kg^{-1}), Plants found growing on soils with the highest Zn concentration had the lowest concentration factor, whilst plants growing on soils with the lowest Zn concentrations had the highest concentration factor. Baker *et al*., (1993) conducted field work at Black Rocks and concluded that the concentration factor of *T. caerulescens* at the site was on average 0.99, five times higher than those observed in this investigation. As explained above, the difference between these results may be because of the difference in sample date and therefore plant age.

3.5.3 Biomass production

One of the main concerns of using *T. caerulescens* for work involving herbivores species is that this plant produces low amounts of biomass (Zhao *et al*,. 2003; Freot *et al.*, 2003). The results of the greenhouse experiment support these findings, as the mean values for above ground biomass were low for all treatments and within the range found by Haines (2002). The most biomass was produced in the medium Zn treatment which agrees with results obtained by Ozturk *et al*., (2003). They found that the largest amount of biomass was produced by plants grown on a media containing 300 mg kg^{-1} of Zn. The least amount of biomass was found in plants grown on media that contained no additional Zn (38 mg kg^{-1}). This evidence supports the claim by Chaney *et al*., (1997) and Shen *et al*., (1997) that *T. caerulescens* has a high internal requirement for Zn and that stunted growth occurs when Zn concentrations are low (Broadley *et al*., 2007). Other authors who have grown plants over a longer time frame (133 – 200 days) have not been able to increase biomass (Knight *et al*., 1997; Schwartz *et al*., 1999), suggesting that the amount of biomass produced is not that much affected by increase in the age of the plant.

3.5.4 Feeding preference of *H. aspersa*

The result of the feeding trial showed that *H. aspersa* did not significantly prefer to consume either high or low Zn leaves, which does not support the elemental defence hypothesis. This result differed from that found by Noret (2005, 2007), in which *H. aspersa* both in laboratory and field experiments preferred *T. caerulescens* containing elevated concentrations of Zn (2000 – 15,000 mg kg^{-1}). This could be because their low Zn plants contained lower concentrations of Zn than those in this experiment and therefore *H. aspersa* were able to distinguish between the treatments. Noret (2007) suggested that *H. aspersa* would show a preference for *T. caerulescens* with high Zn concentrations because these plants have depleted concentrations of glucosinolates, compared with those containing low concentrations of Zn. This concurs with the finding of both Newman (1992) and Tolrà *et al*., (1998), who found similar trends in the levels of glucosinolates in plants with different levels of Zn. Notten (2006) puts forward the notion that plants which have evolved to cope and prosper in soils high in heavy metal concentrations have lower levels

of glucosinolates due to low herbivory pressure, as a result of metalliferous soils. If these authors are correct, their findings do not support the elemental defence hypothesis, because higher levels of Zn should reduce the herbivory of these plants.

A possible explanation for the results obtained in this experiment may be that *H. aspersa* were unable to distinguish between the two Zn concentrations in the leaves. Leaves from the control (containing no additional Zn) still had a mean Zn concentration of 847 mg kg^{-1} and although this was significantly lower than leaves from the higher treatment (3651 mg kg^{-1}), the Zn concentration in the control was a lot higher than plants containing no Zn. Therefore, *H. aspersa* may have been unable to make a choice, if both options were equally unpalatable to them.

3.5.5 The use of *T. caerulescens* in future experiments

Although the experiments in this chapter have demonstrated that *T. caerulescens* is capable of accumulating extremely high concentrations of Zn into its above ground biomass, this species has some serious drawbacks as an experimental species to test the elemental defence hypothesis. The first of these problems is the limited amount of above ground biomass produced after a growth period of 12 weeks in greenhouse experiments. Samples from the field which had grown in even higher concentrations of Zn appeared to produce even less biomass. This limits the use of *T. caerulescens* when researching the elemental defence of Zn in these plants, because a large quantity of biomass is required for performance trials using herbivores. The second problem arises because *T. caerulescens* is too efficient at accumulating Zn, as it is capable of obtaining high concentrations of Zn in the plants biomass even when grown in a treatment containing no additional Zn (typically only 17 mg kg^{-1}). This is problematic because it means that herbivores faced with the 'low' Zn leaves are actually being offered Zn concentrations far beyond those found in other plant species. Furthermore, in the field *T. caerulescens* remained relatively undamaged. Therefore, a different plant species will be required to test the elemental defence of Zn in plants against herbivores. This species should produce more above ground biomass over a short growth period and only accumulate minimal concentrations of Zn when grown on the

low Zn treatment. This species is a known accumulator of Zn (Kumar *et al*., 1995; Salt *et al*., 1995) and will be used as the study species for the remaining experimental chapters of this thesis.

Chapter 4: Accumulation of Zn in *B. juncea* with and without herbivory over time and the possible induction of Zn uptake by the herbivore *H. aspersa*

4.1 Introduction

Chapter 3 of this thesis evaluated the possibilities of using *Thlaspi caerulescens* in the study of Zn as an elemental defence in plants. However, it was concluded that because of certain physiological properties, slow growing producing small amounts of above ground biomass, *T. caerulescens* may not be the most appropriate candidate to test the elemental defence hypothesis. Ideally the study plant should produce large quantities of above ground biomass over short growth periods and only accumulate minimal concentrations of Zn when grown on low Zn concentration soils. A plant species which seems to fit these criteria is the Zn accumulator *Brassica juncea* (Kumar *et al*., 1995; Salt *et al*., 1995), although further investigation is required to fully understand how *B. juncea* responds to metal uptake.

B. juncea is truly a remarkable plant species, as it has been found to be an accumulator of Zn and Cd (Mobin and Khan, 2006; Speiser *et al*., 1992), as well as a hyperaccumulator of Se (>1000 mg kg^{-1}), (Reeves and Baker, 2000; Hanson *et al*., 2003). As a result *B. juncea* has been used in previous studies on metal hyperaccumulation and elemental defence. However, although a number of authors have researched the uptake of Zn in *B. juncea* (Table 4.1), there has only been published work on experiments involving the uptake of Se, in terms of assessing the elemental defence hypothesis (Banuelos *et al*., 2002; Freeman *et al*., 2007; Hanson *et al*., 2003). The role of Zn as an elemental defence in *B. juncea* is still unknown.

Zn concentration in growth media mg kg^{-1}	**Highest Zn concentration in *B. juncea* above ground biomass mg kg^{-1}**	**Reference**
3326	1600	Quartacci *et al*., 2006
1109	>2000	Clemente *et al*., 2005
704	1500	Podar *et al*., 2004
0-160	624	Cui *et al*., 2004

Table 4.1: Summary table of experiments that have researched the uptake of Zn in *B. juncea.*

As seen (Table 4.1), work by previous authors has obtained Zn concentrations in the above ground biomass of *B. juncea* in excess of 2000 mg kg^{-1} Zn concentration (Clemente *et al*., 2005). This defines *B. juncea* as a Zn accumulator species (Reeves and Baker, 2000), with sometimes an overall concentration factor of approximately 2. The Zn concentrations of these plants were achieved after a set growth period of 30 days (Clemente *et al*., 2005). The study did not examine the uptake of Zn during plant development; even though Boyd (1998) states that there is little understanding of how metals are accumulated over time. This is of extreme importance because of the possible wider ecological implications. In terms of experimental research, it is also important to understand how long it takes for plants to sequester 'high enough' concentrations of Zn (concentrations significantly higher than plants grown with no additional Zn) to have an impact on herbivores. If plants slowly sequester Zn into above ground biomass early on in their development, they may not contain the same concentrations at the beginning and end of an experiment. By furthering our understanding of the levels of Zn accumulation over time, it would be possible to ascertain an optimal time for leaves to be used in experiments with herbivores.

Plants such as *B. juncea* may also increase their uptake of Zn when exposed to herbivores. In essence the uptake of Zn into above ground biomass may be an inducible defence (Karbon and Baldwin, 1997). In this case a stimulus, such as the action of an herbivore, produces a response (Smith, 1996), an increase in the concentration of Zn in the plants

leaves. Although no literature to date has examined if herbivores have an impact on the concentration of metals plants sequester, many authors have examined the induction of plant defences by herbivores. Hodge *et al*., (2000) concluded that Lepidoptera larvae could increase the production of an anti-feed compound in plants, resulting in the herbivore being impelled to move away and stop feeding on the plant. If the concentration of Zn in *B. juncea* were increased by the action of a herbivore feeding on its leaves, this could cause the plant to become unpalatable. This would be advantageous in terms of the phytoremediation potential of *B. juncea*, because it would maximise the amount of above ground biomass. However, it could have serious ecological implications, in terms of affecting herbivores feeding on plants growing on contaminated land. An alternative outcome may be that Zn uptake does not increase as a result of herbivory, but that plants increase their above ground biomass to compensate for the damage (Karbon and Baldwin, 1997).

4.2 Aim and hypotheses

This chapter aims to assess the suitability of *B. juncea* for evaluating the elemental defence hypothesis using Zn. This was established by testing three hypotheses:

i. The concentration of Zn sequestered by *B. juncea* will differ depending on the concentration of Zn in the soil and throughout plant development.

ii. Increased Zn uptake will have a negative impact on plant attributes, such as biomass production at different Zn treatments and plant ages.

iii. Increased Zn uptake by *B. juncea* may be induced when attacked by the herbivore species *H. aspersa*.

4.3 Experiment to determine the physiological and chemical changes of *B. juncea* when grown on different concentrations of Zn over time.

4.3.1 Materials and methods

4.3.1.1 Experimental design

Following seed germination and initial growth (Chapter 2 Sections 2.2.1 and 2.2.2), 108 *B. juncea* plants (Czernj accession 426308, a known accumulator of Zn, Podar *et al*, 2004) were transplanted at random into one of three growth treatments (36 plants per treatment). Treatment A had a target Zn concentration of 0 mg kg^{-1}, Treatment B 300 mg kg^{-1} and Treatment C 1000 mg kg^{-1} Zn concentration, above the baseline (prepared as according to Section 2.3 of Chapter 2). The plants were arranged in the form of a Latin square (9 x 12) on the greenhouse work bench and plants maintained in accordance with Section 2.2.3, Chapter 2.

4.3.1.2 Plant harvest and chemical analysis of growth media and plant samples

Samples of the growth media were collected after production and analysed for Zn concentration, as described in Section 2.4.2 of Chapter 2.

The experiment was conducted over four weeks. At the end of each week nine plants from each of the treatments were harvested (Chapter 2, Section 2.2.4) a total of 27 plants per week. These weekly harvests continued for the duration of the experiment, to determine how *B. juncea* uptakes Zn over time. Once harvested plants were weighed before and after being dried in an oven at 50 ^{o}C, so both wet weight and dry weights could be recorded.

It was important to accurately record the concentration of Zn in the leaves, as this is where herbivore damage was expected. Therefore leaves and stems were separated prior to grinding (Section 2.4.3, Chapter 2), making it possible to analyse the two above ground components of the plant separately.

The quality of the data produced from any research is extremely important. Experimental design should reduce the risk of Type I and Type II errors, whilst the data collected must be accurate and reliable. Obviously it is not always feasible to assess the quality of data, such as those produced by direct counts of insects for example. However, for the majority of analytical techniques it is possible to quantify and qualitatively comment on how close data is to the 'true' value. In this and subsequent chapters the quality of the data produced by F-AAS was assessed by bias and relative bias, a component of the measurement uncertainty of the data (equations of which are in Section 2.5 of Chapter 2). This was done by comparing the measured value of CRM's and HRM's against accepted values (Table 2.5, Chapter 2). Typically a bias/relative bias of below 20% is considered to show that data is reliable and of high quality.

4.3.1.3 Statistical analysis

All data analysis was completed using the statistical package Minitab (Version 14), with Microsoft Excel (Microsoft Office, 2003) used for the production of all graphs. One and two-way ANOVA's (Analysis of Variance) were used to assess whether there was any statistical difference in Zn concentration between different growth media, leaf and stem components of the plants, biomass production, Zn concentration factor and the amount of nitrogen in the leaves. Data was checked to ensure it fulfilled the assumptions of the test i.e. data was normally distributed (using probability plots) and had equal variance (Levene's test for equal variance). If differences were found to be significant ($P < 5\%$), data were subjected to a post-hoc Tukey's test, to determine which treatment means differed significantly from one another at $P < 0.05$.

4.3.2 Results

4.3.2.1 Quality of data

Data from the growth media samples (Table 4.2) and the plant samples (leaf and stem) all had an overall relative bias below 10%. This is half of the recommended value for measurements not to be drastically affected by a systematic analytical error, therefore the results can be considered to be accurate and of good quality.

Samples	CRM/HRM	N	Mean measured Zn concentration mg kg^{-1}	Mean Bias	Mean relative Bias %	Overall relative Bias %
Growth media samples	NIST 2709	3	92.55	-13.45	-12.69	-9.62
	NIST 2710	3	6456.37	-495.63	-7.13	
	NIST 2710	3	318.31	-31.69	-9.05	
Leaf samples	NIST 1570 A	4	83.96	1.96	2.36	7.56
	HRM 11	4	48.48	3.48	7.74	
	HRM 14	4	39.39	4.39	12.54	
Stem samples	NIST 1570 A	4	76.61	-5.39	-6.58	-5.59
	HRM 11	4	40.69	-4.30	-9.56	
	HRM 14	4	34.78	-0.22	-0.63	

Table 4.2: Summary table of the mean bias and relative bias of CRM's and HRM's. Growth media and plant material samples were analysed at the same time to provide an estimate of the quality of the data.

Table 4.3 clearly demonstrates how close the 'actual' measured values of the soil treatments were to the target values in terms of Zn concentration. The Zn concentration in both treatments B and C were well within 10% of the target values. The reason for Zn soil concentration being so far from Zero (14 mg kg^{-1}) is due to Zn contained within the compost which is almost impossible to remove, but for the purposes of the experiment are more than adequate as a control soil containing no 'additional' Zn.

Soil treatment	Zn concentration target value mg kg^{-1}	Mean measured Zn concentration mg kg^{-1}	Nominal difference from target value mg kg^{-1}	% difference from target value
A	0	14	14	n/a
B	300	318	18	6 %
C	1000	918	-82	-8.2 %

Table 4.3: Zn soil target concentrations vs. measured concentrations

4.3.2.2 Zn soil concentration

Addition of Zn to the three growth treatments (Figure 4.1) was successful in producing significantly different concentrations of Zn between treatments (F_3 = 117.79, P = <0.001). The actual mean concentrations of Zn (Treatment A = 14, B = 318 and C = 918 mg kg^{-1}) in the treatments was very close to that of the target Zn concentrations (Treatment A = 0 mg kg^{-1}, B = 300 mg kg^{-1} and C = 1000 mg kg^{-1}).

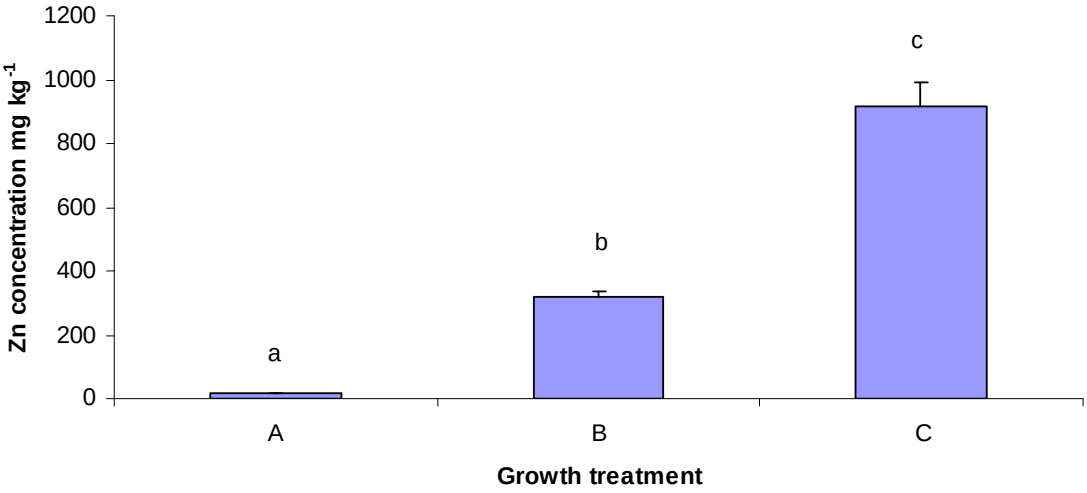

Figure 4.1: Mean (n = 5) ± SE Zn concentration in the three soil treatments ANOVA (Target Zn concentrations: A = 0 mg kg^{-1}, B = 300 mg kg^{-1} and C = 1000 mg kg^{-1}). Different letters above bars in the graph indicate significant differences between treatment means (post-hoc Tukey's analysis).

Source	d.f.	SS	MS	F	P
Soil Zn concentration	2	2.1×10^6	1.0×10^6	117.79	**<0.001**
Error	12	1.0×10^5	8.9×10^3		
Total	14	2.2×10^6			

Figure 4.4: Statistical output of the Zn concentration in the three soil treatments ANOVA.

4.3.2.3 Concentration of Zn in leaves

Figure 4.2 (i-iv) displays the concentration of Zn in plants harvested from weeks one to four of the experiment respectively. It can clearly be seen that the Zn concentration of plants grown on treatment B (318 mg kg^{-1}), were significantly higher than those plants grown on treatments A and C (14 and 918 mg kg^{-1} respectively) during the four week experiment. The peak Zn concentration 1179 mg kg^{-1}) occurred in plants grown on treatment B from week one. The Zn concentration of plants grown on treatments A and C appeared to have an almost constant Zn concentration, 62 and 690 respectively.

It would appear that plants from treatment B had a leaf Zn concentration that oscillated slightly, between an increase and decrease on a week by week basis. This may coincide with the growth of the plant.

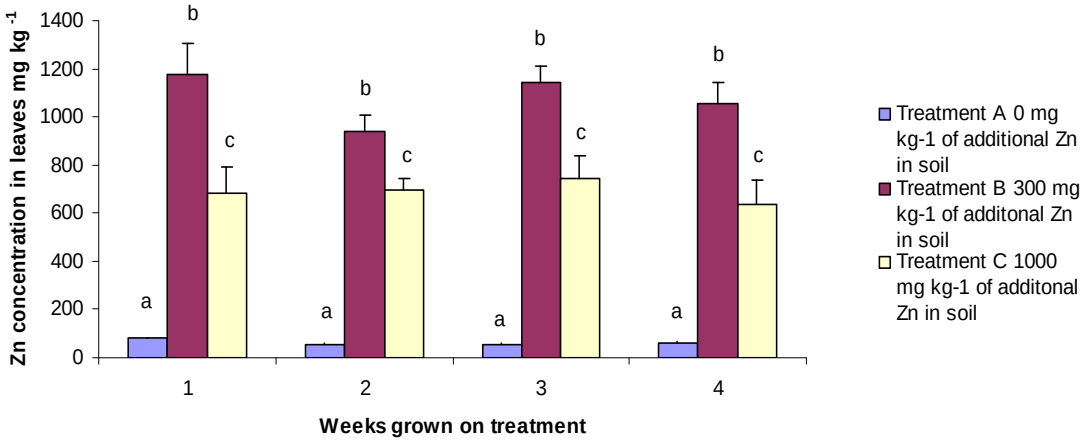

Figure 4.2: Mean ± SE Zn concentration in *B. juncea* leaves grown on the three different soil treatments over the four week experiment. Different letters above in the graph indicate significant differences between treatment means (post-hoc Tukey's analysis).

Experiment week	Source	df	SS	MS	F	P
1	**Treatment**	2	5.4×10^6	1.9×10^6	92.85	**<0.001**
	Error	24	1.9×10^5	8.2×10^4		
	Total	26	7.4×10^6			
2	**Treatment**	2	3.7×10^6	1.9×10^6	92.85	**<0.001**
	Error	24	4.9×10^5	2.0×10^4		
	Total	26	4.2×10^6			
3	**Treatment**	2	5.1×10^6	2.5×10^6	62.76	**<0.001**
	Error	24	9.8×10^5	4.1×10^4		
	Total	26	6.1×10^6			
4	**Treatment**	2	4.5×10^6	2.2×10^6	43.90	**<0.001**
	Error	24	1.2×10^6	5.1×10^4		
	Total	26	5.7×10^6			

Table 4.5: Statistical analysis output of the Zn concentration in *B. juncea* leaves grown on the three different soil treatments over the four week experiment.

4.3.2.4 Concentration of Zn in stems

The concentration of Zn in the stems of the plants from treatment B showed an overall decline over the four weeks (from 531 to 256 mg kg^{-1}), a steep decline was observed between weeks 1 and 2, while a much more subtle decline took place between weeks 2 and 4. This suggests that there was a large uptake of Zn from root to stem in *B. juncea* during plant establishment in the growth treatment, followed by a decline in uptake over time. The Zn concentration in the plants stems grown on treatment C, the highest Zn concentration treatment (918 mg kg^{-1}), followed a similar trend to those grown on treatment B. However, Zn concentration in the leaves was at a lower magnitude (745 – 638 mg kg^{-1}) at each time interval. This may be because the growth medium was oversaturated with Zn and as a result the plant was unable to accumulate the leaf Zn concentrations seen in treatment B. The stem Zn concentration in plants from treatment C declined after the first week from a starting value similar to that found in plants from treatment B (558 – 330 mg kg^{-1}), it then steadily increased over the remaining two weeks to 406 mg kg^{-1} where it was significantly higher than plants grown on treatment B.

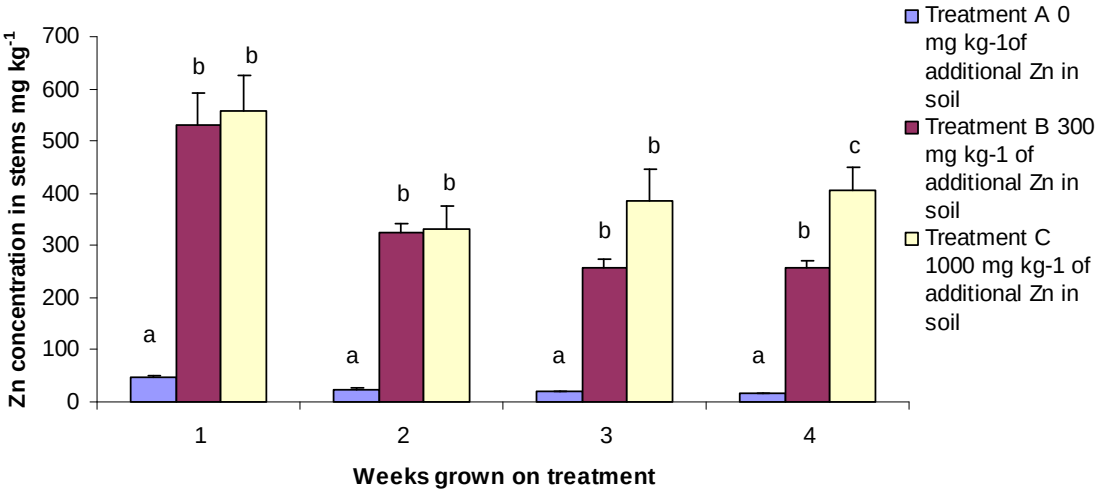

Figure 4.3: Mean ± SE Zn concentration in stems of *B. juncea* grown over the four week experiment. Different letters above in the graph indicate significant differences between treatment means (post-hoc Tukey's analysis).

Experiment week	Source	d.f.	SS	MS	F	P
1	**Treatment**	2	1.5×10^6	7.4×10^6	29.56	**<0.001**
	Error	24	6.0×10^5	2.5×10^5		
	Total	26	2.1×10^6			
2	**Treatment**	2	6.4×10^5	3.2×10^5	47.94	**<0.001**
	Error	24	1.6×10^5	6.6×10^3		
	Total	26	8.0×10^6			
3	**Treatment**	2	6.1×10^5	3.1×10^5	24.35	**<0.001**
	Error	24	3.0×10^5	1.3×10^4		
	Total	26	9.2×10^5			
4	**Treatment**	2	6.9×10^5	3.5×10^5	55.91	**<0.001**
	Error	24	1.5×10^5	6.2×10^3		
	Total	26	8.4×10^6			

Table 4.6: Statistical summary of the analysis Zn concentration in stems of *B. juncea* grown on soil containing three different concentrations of additional Zn over the four week experiment.

4.3.2.5 Zn concentration factor

The Zn concentration factor between plants leaves and the growth media was calculated at the end of the experiment (week 4). The Zn concentration factor (CF) (Figure 4.4) was highest in plants grown on the medium that contained no additional Zn (Treatment A). The Zn concentration in this growth media was very low (14 mg kg^{-1}) and plants must uptake a certain concentration for them to be healthy and not Zn deficient, creating a big difference between the two values. Far more interesting is that the CF in the medium Zn concentration treatment (Treatment B 318 mg kg^{-1}) was greater than 3, and not statistically different from the CF in treatment A. Treatment C which contained the highest Zn concentration had the lowest CF, less than 1. This was lower than that required for a plant to be considered to be an accumulator. This may suggest that the Zn concentration in this growth media was close to saturation and may even have been having a phytotoxic effect on the plants.

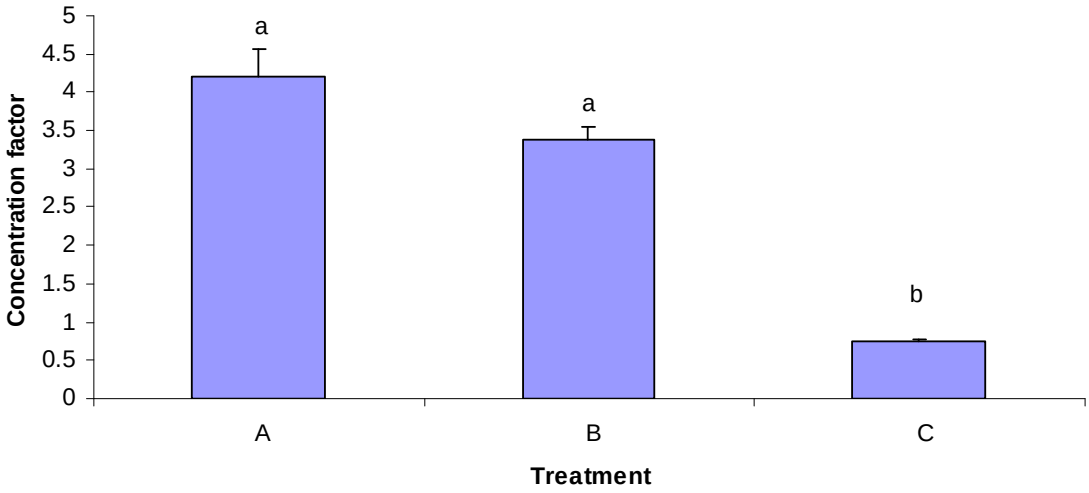

Figure 4.4: Mean ± SE concentration factor for plants grown on three different Zn treatments ANOVA (Treatment A = 14 mg kg^{-1}, Treatment B = 318 mg kg^{-1} and Treatment C = 918 mg kg^{-1}). Different letters above bars in the graph indicate significant differences between treatment means (post-hoc Tukey's analysis).

Source	d.f.	SS	MS	F	P
Concentration factor	2	26.08	13.04	62.39	**<0.001**
Error	9	1.88	0.20		
Total	11	27.96			

Table 4.7: Statistical output of the concentration factor for plants grown on three different Zn treatments ANOVA.

4.3.2.6 Biomass production

The above ground biomass DW (Figure 4.5) of *B. juncea* grown on different Zn concentrations (Treatment A = 14 mg kg^{-1}, B = 318 mg kg^{-1} and C = 918 mg kg^{-1}) did not differ significantly between treatments (F_{107} = 1.77, P = 0.176), but did differ significantly between weeks (F_{107} = 317.87, P <0.001) for the three treatments. This suggested that the growth media Zn concentrations were not phytotoxic to *B. juncea* when grown under laboratory conditions. Between weeks 1 and 4 plants increased their overall biomass approximately five fold, from their biomass after the first week's growth. *B. juncea* therefore fulfilled the criteria for being a fast growing plant species, required to effectively study the elemental defence hypothesis.

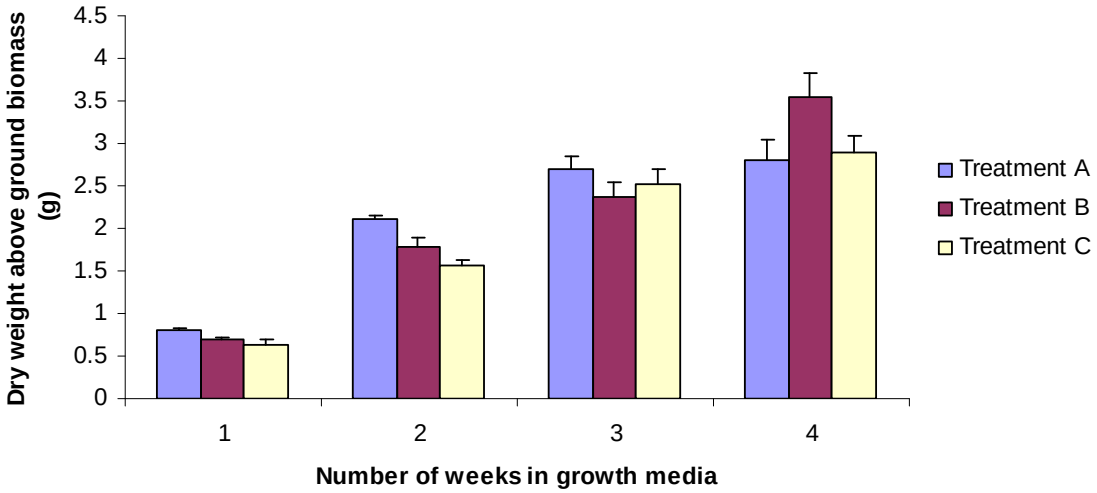

Figure 4.5: Mean (n = 9) ± SE above ground biomass of *B. juncea* (DW) grown on three different Zn treatments (Treatment A = 14 mg kg^{-1}, Treatment B = 318 mg kg^{-1} and Treatment C = 918 mg kg^{-1}) over time.

4.4 Experiment to determine whether damage by the generalist herbivore *H. aspersa* could induce increased uptake of Zn by *B. juncea*.

4.4.1 Materials and methods

4.4.1.1 Experimental design

The aim of this experiment was to determine whether Zn uptake in *B. juncea* can be increased by subjecting plants to a sustained (one week) herbivore attack. Growth media containing a Zn concentration of approximately 400 mg kg^{-1} was prepared as described in Section 2.3 of Chapter 2. After germination and an initial growth period (Chapter 2, Sections 2.2.1 and 2.2.2), 100 plants were transplanted into individual pots containing the growth medium and arranged as in Figure 4.6 (a) (Week 0). The plants were then allowed to grow for 1 week, after which time 20 of the plants were harvested to record a baseline Zn concentration in the plants (Week 1). At the same time snails were caged on to half (40) of the remaining plants. The other 40 plants were also caged (Figure 4.6) so as not to bias the growth of the plants, but without herbivores present (Figure 4.7 (b)). Plants were watered (using tap water) on a daily basis via a saucer underneath the plant pot for a further week.

After this time (Week 2) the snails were removed from all plants. The heights of all plants were recorded. Plants in the snail addition treatments were assessed for damage on a leaf by leaf basis, and percentage damage per leaf recorded. 20 plants on which snails had been present and 20 plants which had had no snails were harvested. The wet weight of these plants was recorded before being placed in a drying oven. The remaining 40 plants (Figure 4.7 (c)) were left to grow for an additional week in the absence of snails but with the cages still in place. After this time (Week 3) they were harvested using the same procedure as the second harvest. This would determine whether plants increased Zn uptake into their above ground biomass in response to herbivore damage immediately, whether there was a delay in Zn uptake, or whether leaf Zn concentration decreased once active herbivore damage had ceased.

Samples of growth media (n = 10) were analysed to determine how close the Zn concentration of the growth media was to the target concentration of 400 mg kg^{-1}. All samples (all leaves from an individual plant constituted one sample) were analysed by F-AAS, following an acid digest (Chapter 2; Section 2.4).

Figure 4.6: Photograph of caged *Brassica juncea* plants.

In order to effectively study the role of Zn uptake as an elemental defence in plants, certain variables need to be controlled for in the experimental design. Nitrogen content is often used to describe the nutritional quality of a plant (Valentine and Heck, 2001). Plants with lower nitrogen content may be less desirable to herbivores; a form of plant defence (Feeny, 1970; Schroeder, 1986; Augner, 1995). Growth rate and total biomass may affect nitrogen content, both of which may be affected by Zn concentration. To determine whether a change in Zn concentration between plants and over time was affecting herbivore preference or whether a difference in nitrogen content between plants was the underlying cause, nitrogen levels were measured throughout the experiment.

(a)

(b)

(c)

Figure 4.7: Experimental setup of (a) pot arrangement at start when *B. juncea* were first transplanted; (b) pot arrangement after 1 week growth with addition of *H. aspersa* and (c) pot arrangement of remaining plants grown for a further week after the removal of *H. aspersa*.

Figure 4.7 key

- Week 0 *B. juncea* transplanted. Week 1 plants harvested.
- Week 0 *B. juncea* transplanted. Week 1 *H. aspersa* added. Week 2 snails removed and plants harvested.
- Week 0 *B. juncea* transplanted. Week 1 no H. aspersa added. Week 2 plants harvested.
- Week 0 *B. juncea* transplanted. Week 1 H. aspersa added. Week 2 snails removed. Week 3 plants harvested.
- Week 0 *B. juncea* transplanted. Week 1 no *H. aspersa* added. Week 3 plants harvested.
- Shows where plants have been harvested.

4.4.1.2 Statistical analysis

All data analysis was undertaken using the statistical package Minitab (version 14), with Microsoft Excel (Microsoft Office, 2003) used in the production of all graphs. Two-way ANOVA (Analysis of Variance) was used to determine whether there were any significant differences in the Zn concentrations or biomass of plants subject to herbivory or harvested at different times. Data was checked to ensure they fulfilled the assumptions of the test i.e. data were normally distributed (using probability plots) and with equal variance (Levene's test for equal variance). If differences were found to be significant, data were subjected to a post-hoc Tukey's analysis to determine which group means differed significantly from one another at $P < 0.05$.

4.4.2 Results

4.4.2.1 Quality of data

The quality of the data (Table 4.4) for the growth media samples was good with an overall relative bias value of 3.73%. Therefore, there was a high degree of certainty that the Zn concentration recorded for these samples was the true value. However, the overall relative bias for the leaf sample CRM's and HRM's was above the desired 20% value at -29.73%. These data should be interpreted with caution, as results may be significantly lower than the true value. However, all three CRM/HRM's had a persistent negative bias of relatively the same magnitude, suggesting that the bias arose from an analytical systematic error, for example an error in the calibration of a pipette or bottle top dispenser. This error would be consistent for all samples; therefore although the data collected may have lower values than expected, the trends in the data would be the same.

Samples	CRM/HRM	n	Mean measured Zn concentration mg kg-1	Mean Bias	Mean relative Bias %	Overall Relative Bias %
Growth media samples	NIST 2709	2	125.01	19.02	17.94	3.73
	NIST 2710	2	6198.95	-753.05	-10.83	
	NIST 2710	2	364.33	14.33	4.09	
Leaf samples	NIST 1570 A	4	64.71	-17.28	-25.9	-29.73
	HRM 11	4	32.43	-12.57	-27.93	
	HRM 14	4	22.62	-12.38	-35.35	

Table 4.8: Summary table of the mean bias and relative bias of CRMs and HRMs of growth media and plant material samples to provide an estimate of the quality of the data. Samples were analysed at the same time.

4.4.2.2 Zn soil concentration

The mean (n = 5) Zn concentration of the growth media was 349 mg kg^{-1} with a standard error of ± 40 mg kg^{-1}. This result was lower than expected (target Zn concentration = 400 mg kg^{-1}), however it was still above concentrations considered phytotoxic to most plant species and high enough for land to be classed as contaminated (ICRCL, 1987).

4.4.2.3 Concentration of Zn in the leaves

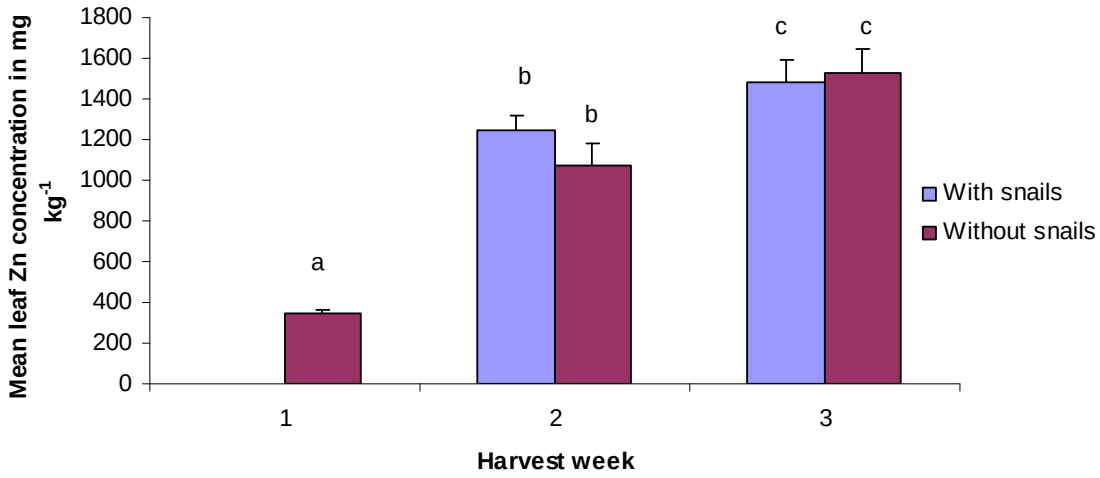

Figure 4.8: Mean (n = 20) ± SE Zn concentration in *B. juncea* leaves harvested in week 1, 2 and 3, with and without snails. Two-way ANOVA presence of snail $F_2 = 0.20$, $P = 0.654$, harvest week $F_2 = 42.60$, $P < 0.001$. Different letters above bars in the graph indicate significant differences between treatment means (post-hoc Tukey's analysis).

In week 1, after one week growing on the high Zn concentration growth media, 20 *B. juncea* were harvested. The mean leaf Zn concentration (Figure 4.8) of these plants was 345 mg kg^{-1}. The plants had sequestered a mean concentration almost exactly the same as the growth media (349 mg kg^{-1}), a concentration factor of 0.99. *H. aspersa* was added to half the remaining 80 plants and they were then allowed to continue growing for a week.

4.4.2.4 Damage to plants by herbivores

The level of herbivore damage was recorded in week 2 once snails had been removed. There was no significant difference in the level of herbivory (Figure 4.9) between plant treatments, i.e. those harvested in week 2 and those harvested in week 3. Therefore, they were comparable in terms the amount of herbivore damage they received. Plants that did not have snails placed in a cage with them were also observed for damage. A visual inspection of these plants confirmed that no significant damage was evident.

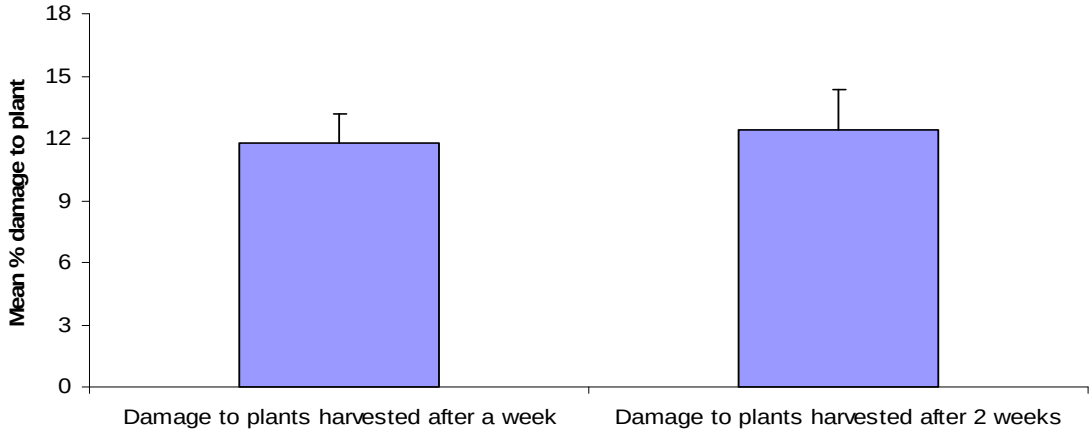

Figure 4.9: Mean (n = 20) ± SE % damage to plants after being subjected to herbivory by *H. aspersa*. One-way ANOVA confirmed that there was no significant difference between plants harvested in week 3 and those harvested in week 4, $F_2 = 0.03$, P = 0.874.

In addition in week 2, a further 40 plants were harvested, half of which had been exposed to one weeks worth of herbivore damage. There was no significant difference in the leaf Zn concentration of plants which had been subjected to *H. aspersa* damage (1246 mg kg^{-1}) and those which had not. The mean Zn concentration of these leaves had increased by approximately 900 mg kg^{-1} since week 1 (Figure 4.8), with a concentration factor of 3.5.

The remaining plants (n = 40) were harvested in week 3, half of which had experienced herbivore damage between week 1 and 2. The mean leaf Zn concentration of these plants was 1490 mg kg^{-1}, a concentration factor of 4.27. The leaf Zn concentration had increased by 350 mg kg^{-1} compared with those harvested the previous week, but the increase was not as high as it had been between week 1 and 2 (Figure 4.8). There was no significant difference between the leaf Zn concentration of plants that had been subject to herbivore damage and those which had not ($F_2 = 0.20$, P = 0.654).

Plant leaf Zn concentration increased significantly over time, but did not differ significantly in the presence or absence of the herbivore *H. aspersa* (Figure 4.8).

If *B. juncea* does not increase Zn uptake in response to herbivory, it may be that the defense strategy of the plants was to increase their biomass to compensate for the lost vegetation. Therefore, the biomass of plants subjected to herbivory and those which were not, were compared after each harvest. The results showed that there was no significant difference in the biomass of plants irrespectively of herbivory (Figure 4.10). There was a significant difference between plants harvested in week 2, 1.25g DW and those harvested in week 3, 3.00g DW above ground biomass. Therefore, *B. juncea* plants do not increase above ground biomass in response to herbivory from *H. aspersa*.

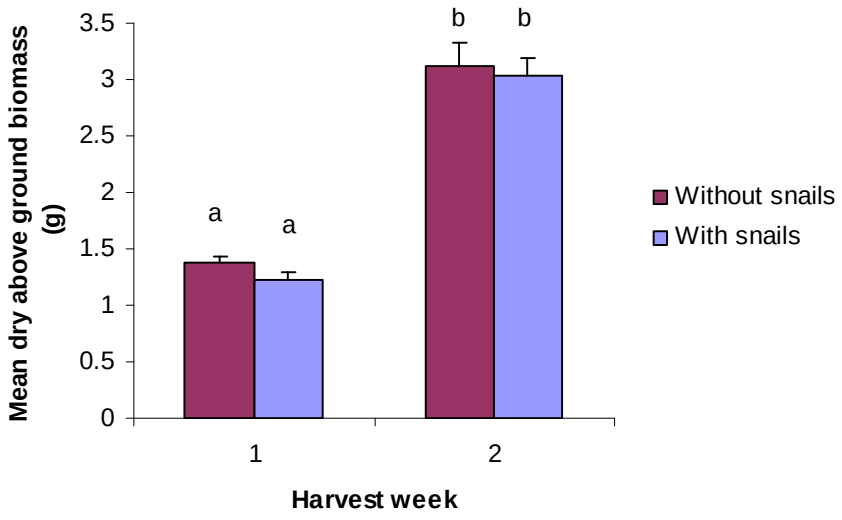

Figure 4.10: Mean (n = 20) ± SE above ground biomass of *B. juncea* harvested in weeks 2 and 3, that had or had not been subjected to herbivory by *H. aspersa*. Two-way ANOVA: Herbivory $F_2 = 0.68$, $P = 0.411$, Time $F_1 = 16, 1.99$, $P < 0.001$. Different letters above bars in the graph indicate significant differences between treatment means (*post-hoc* Tukey's analysis).

A regression analysis for the amount of damage per plant caused by herbivores against the concentration of Zn in the plant leaves for the plants harvested in week 2, at the same time as the removal of snails ($R^2 = 6.4\%$, $P = 0.296$,) and those harvested in week 3 ($R^2 = 1.4\%$, $P = 0.624$,) were analysed separately. There was no significant relationship between the leaf Zn concentration and the level of herbivory in either harvest.

Response	Factor	d.f.	F	P
Mean damage to the plants	Harvested after one and two weeks	1	0.03	0.874
Zn concentration in leaves	Presence of snail	1	0.20	0.654
	Harvest week	2	42.60	**<0.001**
Above ground biomass production	Presence of Snail	1	0.68	0.411
	Harvest week	1	161.99	**<0.001**

Table 4.5: Summary of one-way and two-way ANOVA from the Zn induction experiment. Results in bold indicate significant results.

4.5 Discussion

4.5.1 Zn concentration

Previous experiments (Table 4.1) examining Zn uptake by *B. juncea* had recorded Zn concentrations in above ground biomass of between 624 – 2000 mg kg^{-1} (Quartacci *et al*., 2006; Clemente *et al*., 2005; Podar *et al*., 2004). The highest mean leaf Zn concentrations presented in this chapter were between 1179 and 1490 mg kg^{-1}. The results from both experiments fell short (by approximately 500 mg kg^{-1}) of the 2000 mg kg^{-1} threshold for plants to be considered accumulators of Zn (Reeves and Baker, 2000). However, they were still higher than the Zn concentrations typically required for normal growth, which is in the range of 15 – 20 mg kg^{-1} (Broadley *et al*., 2007). The leaf Zn concentrations of plants grown in the 400 mg kg^{-1} was approximately 100 times greater than this minimum value. *B. juncea* is considered to be an accumulator of Zn rather than a hyperaccumulator species such as *T. caerulescens*. Therefore, it is not surprising that the highest Zn concentration for *B. juncea* in this experiment was under half of that recorded for *T. caerulescens* in Chapter 3.

However, because it is not a hyperaccumulator *B. juncea* has been shown to be a better study species to investigate the elemental defence hypothesis than *T. caerulescens*. *B. juncea* plants grown in the control growth media (containing no additional Zn) did not sequester high concentrations of Zn into their above ground biomass. The high internal requirement of *T. caerulescens* and its ability to hyperaccumulate Zn resulted in plants

grown in the control growth media accumulating concentrations exceeding 800 mg kg^{-1}. The Zn concentrations of *B. juncea* grown in the control media were below 100 mg kg^{-1}. If these were to be presented to herbivores along with high Zn concentration leaves in preference feeding trials, there would be a big difference in Zn concentrations between the leaves presented, making it easier for snails to make a choice. Using *T. caerulescens* the difference between low and high Zn leaf concentrations was by a factor of approximately 4 (Chapter 3). If this experiment were to be repeated using *B. juncea* the difference between the two would be by a factor of over 15.

However, the apparent change in Zn concentration within leaves over the four weeks may arise from a dilution effect, as plants increased in biomass over time. More leaf material was ground per sample as biomass increased and therefore the Zn in the plants may have been diluted by the increasing volume of plant material. Large plants may have contained more Zn per plant because they had sequestered more into their above ground biomass but would have appeared to have the same or lower Zn concentration compared to smaller plants, because smaller plants had less biomass. The differences in Zn concentration between small and large plants could result in changes in herbivore behaviour or impact differently upon herbivore health. Smaller plants with a higher Zn concentration may deter herbivore attack if herbivores are able to detect Zn in the plant. As a result herbivore behaviour may alter if they try to avoid consuming the plant. Herbivores may not be able to detect Zn in plants which are larger, due to the dilution effect. As a result herbivores may consume large volumes of leaf material containing Zn. This may have an impact on the fitness of herbivores, reducing herbivore numbers and herbivory on the plant in the long term.

Even though the majority of work on elemental defence has focussed on plants with very high metal concentrations, there is mounting evidence by authors such as Coleman *et al*., (2005) which suggests that metals can have an impact on herbivores at much lower concentrations. These low concentrations are similar to those found in plant accumulator species such as *B. juncea*, rather than those found in metal hyperaccumulators such as *T. caerulescens*.

The results from the sequential harvest experiment, provides valuable evidence to suggest that *B. juncea* leaves, are capable of reaching a Zn soil/plant equilibrium (albeit non-linear) within one week's growth on any given treatment used in the experiment. It then appears that plant leaves maintain an approximate constant Zn concentration throughout their development over a four week growth period. This is invaluable information for furthering research into the elemental defence characteristics of Zn, as it enables 'us' as researchers to have a reasonable degree of confidence of leaves Zn concentration after one weeks growth on a Zn treatment, which will remain approximately constant for a four week period. This is most important when setting up experiments that contain the additional complexity of using leaf chewing herbivores. However, the Zn concentration within the stems of *B. juncea* is far less variable over a four week growth period, therefore far more care and planning is required when setting up experiments, which involve herbivores that feed from the stems of *B. juncea* plants.

4.4.2 Zn concentration factor

B. juncea like *T. caerulescens* had an optimal concentration of Zn in the growth media to produce the highest concentration of Zn in the leaves. In both plant species the CF was significantly lower in plants that were grown in the highest Zn concentration treatment. When oversaturated with Zn it may be that Zn uptake is inhibited, to prevent the plant sequestering levels which would be toxic to them. The optimum Zn concentration for maximum uptake was at moderate Zn concentrations (300 – 600 mg kg-1), even though this range is considered to be phytotoxic to most other plant species (Broadley *et al*., 2007). The unimodal response of *B. juncea* to Zn concentrations is supported by the CF factors recorded by authors such as Podar *et al*., (2004), who produced a CF of just over 2 (less than the CF of plants grown on treatment B) when grown on a medium containing a Zn concentration of 704 mg kg^{-1} (higher than the growth media Zn concentration of treatment B). This trend continues with a CF of approximately 0.5 in plants grown on a soil containing 3326 mg kg^{-1} Zn (Quartacci *et al*., 2006).

4.4.3 Biomass production

One of the constraints of using *T. caerulescens* as a study species to investigate elemental defence in plants was the low amount of biomass produced (Chapter 1). It has been shown that *B. juncea* is capable of producing considerably more above ground biomass over a very short time scale. This confirms claims by other authors that it is a fast growing, high biomass producing plant, capable of accumulating Zn (Kumar *et al*., 1995; Salt *et al*., 1995). Zn concentration in growth media did not have a significant impact on the amount of biomass produced, a trait consistent with plants that are capable of sequestering high concentrations of Zn (Broadley *et al*., 2007). The biomass produced (4g DW after 28 days) was more than that found by Quartacci *et al*. (2006), a mean total of 0.5 – 0.8 g DW after 43 days growth. However, the Zn concentration of their growth media was in excess of 3000 mg kg^{-1}, three times that of highest Zn treatment in these experiments. A different study (Wu *et al*., 2006) recorded a biomass of over 2.6 g DW in their Zn treatments, which is more comparable with the current study. Therefore, *B. juncea* is a more desirable study species than *T. caerulescens* to assess elemental defence in plants using Zn, as experiments involving herbivores, such as performance trials, can require a lot of biomass.

4.4.4 Hypotheses

i. The concentration of Zn sequestered by *B. juncea* will differ depending on the concentration of Zn in the soil and throughout plant development.

This hypothesis may be accepted as plants did express different leaf and stem concentrations depending in which soil concentration they were grown on. It would seem that *B. juncea* is best suited at accumulating Zn into its leaves when the soil concentration is 'moderately' contaminated 318 mg kg^{-1}, its performance at sequestering Zn decreases to half when the soil concentration reaches 1000 mg kg^{-1}.

ii. Increased Zn uptake will have an impact on plant attributes, such as biomass production at different Zn treatments and plant ages.

The second hypothesis must be rejected as *B. juncea* plants did not reduce the amount of biomass produced irrespective of the Zn treatment they were grown on. This differs from the results from the previous experiment where it was seen *T. caerulescens* biomass reduced drastically with Zn concentration in the soil.

iii. Increased Zn uptake by *B. juncea* may be induced when attacked by the herbivore species *H. aspersa*.

The third hypothesis must be rejected as herbivore attack did not result in plants increasing the amount of Zn sequestered into their above ground biomass.). It may be that the Zn concentration of the growth medium was too high, saturating the plant irrespective of herbivore damage; or too low, limiting the ability of the plant to increase Zn uptake. It may be that the level of herbivory was not severe enough, or over too short a time period to elicit a response. Alternatively, it may take the plant longer to respond to herbivory than the length of this experiment allowed. However, the most likely explanation is that, herbivory does not induce an increase in Zn uptake in *B. juncea*.

To conclude, *B. juncea* a Zn accumulator, was able to grow in similar Zn concentrations as those of the Zn hyperaccumulator *T. caerulescens*, without suffering any significant negative impact in terms of biomass production. The concentration of Zn in *B. juncea* was higher in the leaves than the stems, with leaf concentrations exceeding 1000 mg kg^{-1} after a single week's growth. This suggests that the elemental defence could have more of an impact on leaf chewing herbivores, rather than sap suckers. However, this experiment concluded that herbivory by *H. aspersa* did not result in an increase in Zn uptake by *B. juncea* thus suggesting that the elemental defence of Zn uptake is not inducible.

Chapter 5: Testing the elemental defence hypothesis of Zn accumulation in *B. juncea* using the generalist herbivore *H. aspersa* and the brassicaceae specialist *P. brassicae* in the lab

5.1 Introduction

Chapter 4 analysed the effect of Zn uptake in *B. juncea* as a defence mechanism from the plant's perspective. This chapter aims to determine if Zn acts as defence against herbivory in *B. juncea* plants from the perspective of two herbivores, the generalist herbivore *H. aspersa* (the common garden snail) as well as the *brassicaceae* specialist *P. brassicae* (the cabbage white butterfly).

5.1.1 Measuring the effectiveness of the elemental defence hypothesis using herbivores

Boyd (2007) stipulates that the two main ways to evaluate the effectiveness of metal uptake by plants in terms of a defence characteristic against herbivores, are conducting preference tests and performance trials using herbivores. Therefore, contained within this Chapter is an extensive evaluation of the possible effects that elevated concentrations of Zn in *B. juncea* leaves may have on two herbivore species, in terms of their preference and performance.

Preference tests, described in detail in Chapter 3, provide evidence of the effect of Zn on the feeding behaviour of herbivores. Previous work has established that heavy metals, including Zn, can deter herbivore feeding (Greville and Morgan, 1991; Hanson *et al*., 2003; Pollard *et al*., 2007) and therefore provide support for the elemental defence hypothesis.

However, deterrence does not demonstrate that that the defence has a negative impact on herbivore performance, i.e. their growth development, survival or fecundity. Furthermore, sometimes herbivores are not deterred by a defence because, for example, they cannot detect it, but the defence is still effective one because it impacts negatively on the herbivore's performance.

There is less evidence that Zn and other metals affect herbivore performance as fewer studies have been carried out than for preference.

Hence it is important to demonstrate effects on both preference and performance for a number of herbivores before the elemental defence hypothesis can be supported.

5.1.2 Choice of Herbivore in tests

When conducting research into the effectiveness of metals as a defence for plants, there are two very important factors that must be thoroughly researched before experiments commence. The first is the plant species being used; in this case *B. juncea* which from the results of the previous chapter has demonstrated that it is a plant species capable of accumulating Zn into its biomass. The second factor is the choice of herbivore. After an extensive literature investigation, two species, *H. aspersa* and *P. brassicae*, were deemed to be suitable candidates for experimentation.

H. aspersa – The common garden snail is a generalist herbivore referred to by many researchers as a key ecological species (Menta and Parisi, 2001) due to its wide geological distribution. This species of snail has also been used for such purposes as an indicator of environmental pollution and biological monitoring (Phillips, 1977; Gomot and Phian, 1996). Dallinger *et al*., (1993) even suggested that this species was a micro-concentrator of Zn.

P. brassicae – Is a Lepidoptera species found wild throughout Europe, commonly known as the large white butterfly (Feltwell, 1979). When in larval form it feeds predominately on *Brassica* plant species and are specialists when tackling these species of plant. Pollard and Baker (1997) used this species of caterpillar in one of their preference trials, presenting individuals with a choice of *T. caerulescens* containing either a low Zn concentration of 528 mg kg^{-1} or a high Zn alternative with a Zn concentration of 7432 mg kg^{-1}. They concluded that *P. brassicae* had a significant preference for *T. caerulescens* leaves that contained the lower Zn concentration. Although this herbivore species has been involved in

experiments with the Zn hyperaccumulator species *T. caerulescens*, it has not been used in any published experiments with plants that are classed as Zn accumulators. Thus this chapter aims to see if even a specialist herbivore such as *P. brassicae* can show a preference when presented with low and high Zn leaves from a Zn accumulator species *B. juncea*, hence refining the possibility of less extreme concentrations of Zn affecting herbivore preference and growth performance.

5.1.3 Implications for phytoremediation

This research has wider implications for the potential application of phytoremediation for remediating land contaminated with heavy metals. Although phytoremediation is a highly desirable technique (as discussed in the introduction chapter) it does have a fundamental flaw, as it is effectively mobilising a contaminant from a 'locked' source zone (the soil) into the environment. As plants accumulate the heavy metals into their above ground biomass it presents environmental and ecological problems as it produces pathways by which the contaminant can enter the ecosystem. The most concerning of these problems is the movement of heavy metals entering and bioaccumulating up the food chain. Hence it is important to test to what extent insects feeding on plants containing high levels of Zn accumulate Zn in their tissue. This has been assessed for snails by previous work but using a different plant species (Noret *et al*., 2005, 2007), but not for Lepidoptera.

5.2 Aims

i. To establish if elevated Zn concentrations in *B. juncea* has on an impact on the feeding preference of juvenile *H. aspersa* and caterpillars of *P. brassicae.*

ii. To ascertain if adult *H. aspersa* change their feeding preference when repeatedly presented with leaves of *B. juncea* containing low, medium and high concentrations of Zn.

iii. To determine if a diet of *B. juncea* leaves containing elevated concentrations of Zn have an impact on the growth and performance of juvenile and adult *H. aspersa*, as well as on caterpillars of *P. brassicae*.

iv. To assess if a diet of *B. juncea* leaves containing elevated concentrations of Zn increases the Zn concentrations in *P. brassicae* when compared to caterpillars fed on a diet of *B. juncea* leaves that do not contain elevated concentrations of Zn.

5.3 Preference of juvenile *H. aspersa* on leaves of *B. juncea* containing high and low concentrations of Zn

5.3.1 Materials and methods

5.3.1.1 Rearing of juvenile snails

Juvenile snails used in the experiment were bred in captivity from eggs produced from adult *H. aspersa* that had been collected from the grounds of the University of Sussex. These adult snails had not been used in any other experiments prior to producing eggs and therefore had no known prior exposure to elevated concentrations of Zn. The juvenile snails were reared on a diet of washed (using tap water) lettuce after they hatched from their eggs until they were used in the experiments.

5.3.1.2 Experimental design

Snails were starved for a 24hr period before the start of the experiment. Twenty five juvenile snails were placed in individual cages 24 hours prior to the start of the experiment in the aim to increase herbivory. Snails were then presented with two leaves of *B. juncea* that had been grown on media either containing no additional Zn or additional Zn (800 mg kg^{-1}). The snails were then positioned in the middle of the cage equal distance from the leaves and facing neither. Prior to the leaves being placed in the cage, their area was recorded using a leaf area meter (by ADC BioScientific). The snails were then placed in a dark area of the laboratory and monitored over a 12 hour feeding period to ensure the

herbivores did not completely consume the leaf material. At the end of the elapsed time period the remaining leaf material was removed from the cage and the area recorded.

5.3.1.3 Statistical analysis

Data were analysed using Minitab (Version 14) and presented using Microsoft Excel (Microsoft Office, 2003). A Wilcoxon signed rank test was used to analyse the data to see if there was any significant differences in the amount of leaf material consumed by the snails, with a 95% confidence interval.

5.3.2 Results

When juvenile *H. aspersa* snails were presented with the choice of *B. juncea* leaves containing low and high concentrations of Zn, there was a significant difference ($W_{24} = 57$, $P = 0.005$) between the amounts consumed by the snails (Figure 5.1). The results from the preference trial provides evidence that feeding behaviour of juvenile *H. aspersa* snails is affected when presented with *B. juncea* leaves containing high concentrations of Zn. The mean percentage of leaf consumed containing the high Zn was only 3.5 %, while leaves containing the lower concentration of Zn had a mean nearly three times higher (9.9 %) in terms of leaf material consumed.

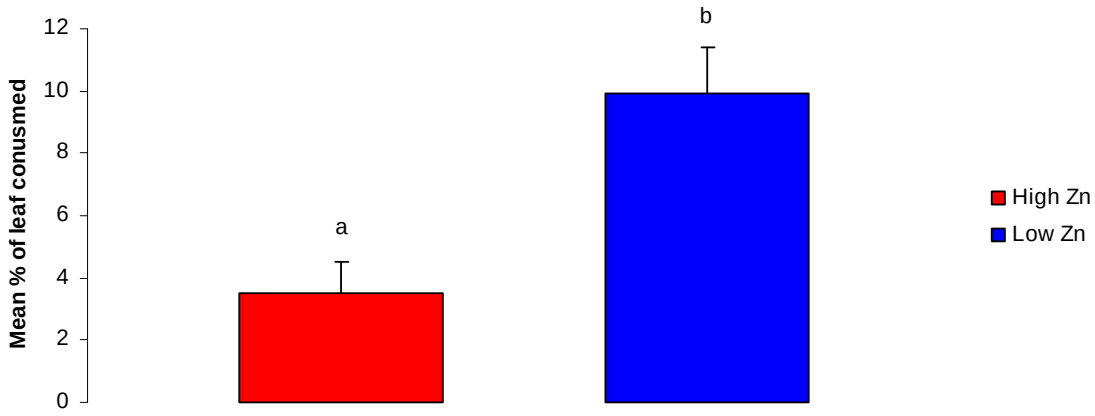

Figure 5.1: Mean (n = 25) ± SE percentage leaf of *B. juncea* consumed by juvenile *H. aspersa* when presented with a choice of leaves containing high and low concentrations of Zn. Means with different letters overhead differed significantly after running a Wilcoxon signed rank test, $W_{24} = 57$, P = 0.005.

5.4 Multiple preference trials of adult *H. aspersa* on leaves of *B. juncea* containing high, medium and low concentrations of Zn

5.4.1 Materials and methods

5.4.1.1 Experimental design

Sixty adult snails were collected from the area around the University of Sussex. Prior to the experiment they were placed in a large glass animal cage containing a compost layer of approximately 4cm in depth. The cage was kept in an experimental room with an approximate temperature of 16^{o}C, the lights in the room set on a 16hr light 8hr dark cycle. High humidity was maintained within the cage by daily monitoring and by the use of a water spraying bottle to ensure the soil/cage was moist but not wet. The snails were fed on a diet solely comprising of cabbage washed thoroughly with tap water before being given to the snails. A week before the experiment began, the snails were transferred to individual cages, to make sure that they were not carrying eggs, which might affect their preference, and fed on the same diet as before. 24 hours prior to the start of each experiment all food was removed from the cages in an effort to promote herbivory. The experimental design consisted of three preference options:-

1) Low Zn leaves – Leaves from *B. juncea* plants which had been grown on soil containing no additional Zn.

2) Medium Zn leaves – Leaves from *B. juncea* plants which had been grown on soil containing 400 mg kg^{-1} of additional Zn.

3) High Zn leaves – leaves from *B. juncea* plants which had been grown on soil containing 800 mg kg^{-1} of Zn.

The sixty snails were randomly allocated to one of three experimental trials (when placed in their individual cages), to test the preference of snails for (i) low vs. medium Zn leaves, (ii) low vs. high Zn leaves, and (iii) medium vs. high Zn leaves. Due to the fact that preference can be altered through previous experience it was concluded that each snail should carry out their preference trial three times, to see if preference alters with experience. In between each of the trials for the snails there was a 48 hr 'cooling off period'; where individuals although remained in their cages were fed the original diet of cabbage, before being starved for 24 hrs and used in another trial.

Each trial was conducted by placing two *B. juncea* leaves, one from each treatment (being tested) into the individual cages. The snail was then positioned equal distance from both leaves and facing nether. Cages were then placed in a dark and quiet area of the lab and monitored over a 12 hr period. Prior to leaves being placed in the cages their area was scanned using an ADC BioScientific area meter; leaves were rescanned at the end of the experiment so that total area consumed could be calculated. To reduce leaves wilting and becoming unappealing to the herbivores their stems were wrapped in damp paper towel. To reduce characteristic favourability (in terms of size or colour) of one leaf over another in any individual trial, leaves were matched paired from the two different experiments as well as possible, in terms of age, colour and size.

5.4.1.2 Statistical analysis

Data were analysed using Minitab (Version 14) and presented using Microsoft Excel (Microsoft Office, 2003). A non-parametric Mann Whitney U test was used to test for any significant differences (at the 95% confidence level) from the results of each trial. Due to the data produced from the trials being in percentage form it was necessary to convert the data using an angular transformation prior to statistical analysis.

5.4.2 Results

Snails did not have a significant preference during the first two trials (Figures 5.2). However, by the end of the third trial the snails did have a significant preference (W_{19} = 486.5, P = 0.037) for the leaves that came from plants grown on a soil that contained no additional Zn.

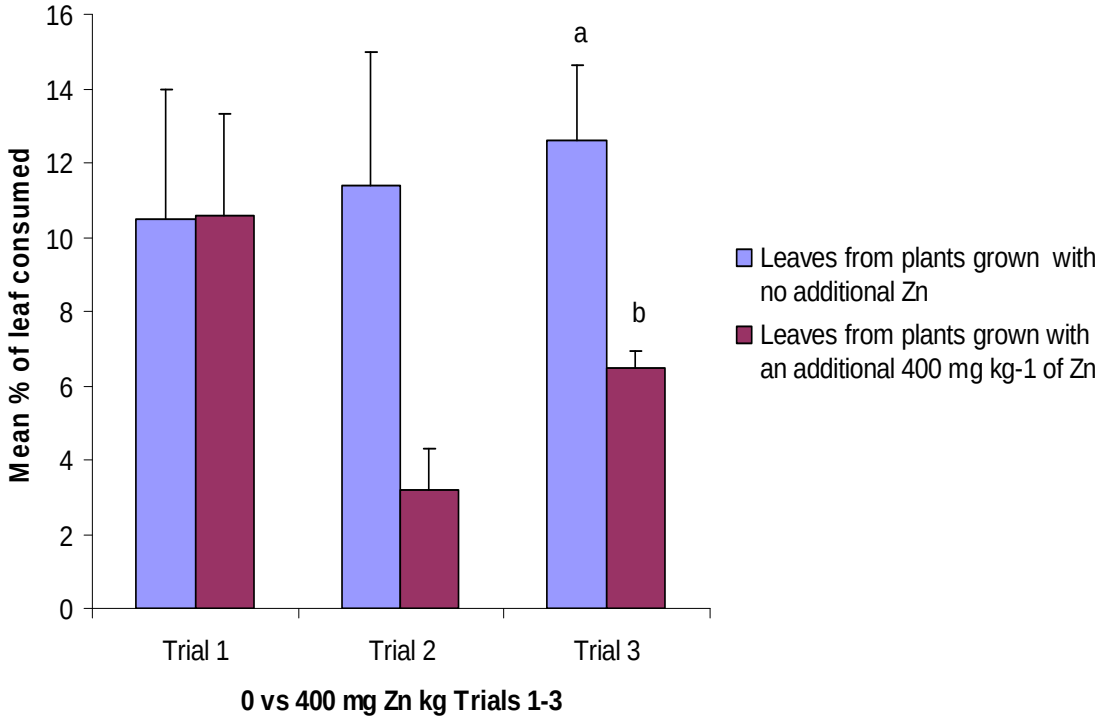

Figure 5.2: Mean (n = 20) ± SE percentage of *B. juncea* leaves consumed by *H. aspersa* when presented with a choice of leaves containing no additional Zn and medium concentrations, trials 1-3.

Figures 5.3 shows the results of the three repeated preference trials of adult snails, when presented with a choice of a low and high Zn containing leaf of *B. juncea*. The first two trials yielded results that were not significantly different, therefore the snails were not displaying a preference for either leaf. However, the result from the third trial produced a significant result (W_{19} = 521, P = 0.001). In this trial the snails had made preference for the low Zn leaves, consuming on average 3.9 times more of the low Zn leaf (12.9 %) than the high Zn leaves (3.3 %).

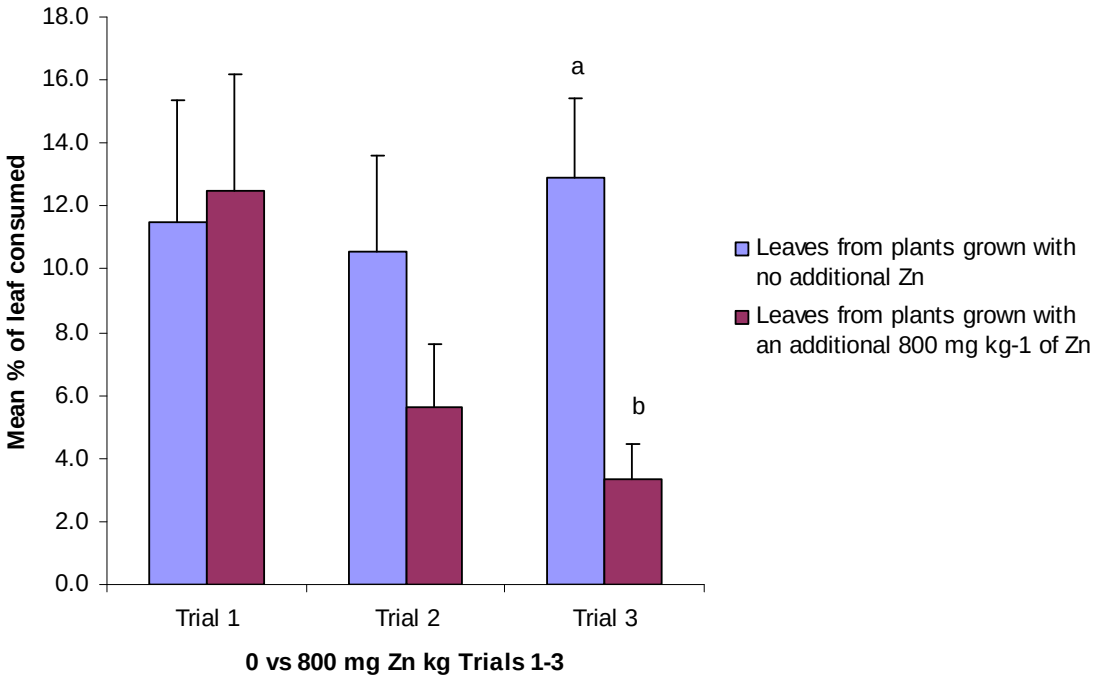

Figure 5.3: Mean (n = 20) ± SE percentage of *B. juncea* leaf consumed by *H. aspersa* when presented with a choice of leaves containing no additional Zn and the addition of high concentrations of Zn (800 mg kg^{-1}), trials 1-3.

Figures 5.4 displays the results from the three preference trials, in which adult snails were provided with a choice of a medium Zn containing leaf or high Zn containing leaf. The first trial produced a result that differed significantly (W_{19} = 495, P = 0.019), with the snails making a preference for leaves from the plants grown on a soil containing 400 mg kg^{-1} of additional Zn. This result was not observed in the second and third trial conducted on the snails, which instead produced results which showed that snails did not have a statistically significant preference for either leaf.

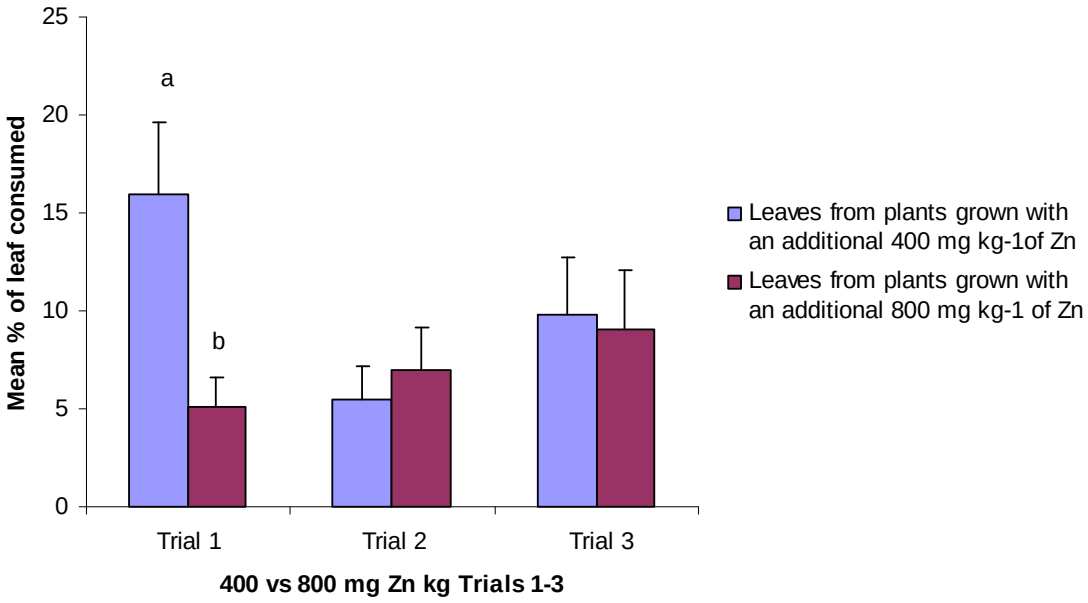

Figure 5.4: Mean (n = 20) ± SE percentage of *B. juncea* leaf consumed by *H. aspersa* when presented with a choice of leaves containing medium and high concentrations of Zn, trials 1-3.

Due to the complexity of the experimental design it is not possible to quantitatively analyse the differences between the results of all the feeding trials. It is possible though, to qualitatively comment on the outcomes as a whole. When snails were presented with a choice between a leaf that had come from a plant that had been grown on a soil containing no additional Zn and leaves from a plant grown on a soil containing additional Zn (either 400 or 800 mg kg^{-1} in the soil) snails did not make a preference until the third trial. When they did make a preference it was always for the leaf from the plant that had been grown on the lowest Zn concentration in the soil of the two.

5.5 Preference of *P. brassicae* on leaves of *B. juncea* containing high and low concentrations of Zn

5.5.1 Materials and methods

5.5.1.1 Experimental design

P. brassicae eggs purchased from Blades Biological (Kent, UK) were allowed to hatch in a Petri dish that contained a damp paper towel and the cabbage leaf to which the eggs were attached. After the eggs had hatched and the caterpillars were feeding independently they were transferred to a larger container, again containing cabbage leaves and damp paper towel, and left to grow until they were in their 3rd instar (approximately 3 cm in length).

Having reached their 3rd instar, 16 caterpillars were transferred to individual cages in a laboratory environment. Each cage contained two *B. juncea* leaves, one from each treatment, either from plants grown on soils containing no additional Zn or 800 mg kg^{-1}. Plants had been growing in their respective soils for approximately three weeks. To prevent leaves from wilting and thus losing their appeal to the caterpillars, damp paper towel was wrapped around the leaves stem for the duration of the trial.

Prior to leaves being placed within the cages, they were first match paired, in terms of age, colour and size. Following this, a leaf area meter (by ADC BioScientific) was used to measure the area of the leaves.

The caterpillars were then carefully placed into individual cages facing neither leaf, and approximately equal distance from each leaf. The herbivores were then closely monitored over an eight hour period to ensure they did not completely consume any of the leaves. After this time had elapsed the caterpillars were returned to the main growth cage and the remainder of the leaves were rescanned using the leaf area meter.

5.5.1.2 Statistical analysis

Data were analysed using Minitab (Version 14) and presented using Microsoft Excel (Microsoft Office, 2003). A non-parametric Mann Whitney U test was used to test for a significant difference (at the 95% confidence level) in the quantity of leaf consumed by *P. brassicae* from the two different treatments.

5.5.2 Results

The results from the choice preference trial of *P. brassicae* presented with a *B. juncea* leaf from a plant grown on a low (no additional Zn) and high (an additional 800 mg kg^{-1}) Zn soil concentration. These findings as displayed in Figure 5.11 show that *P. brassicae* have a significant preference (W_{11} = 108, P = 0.013) for the low Zn leaves, consuming on average three times more of the low Zn leaf (24% mean amount consumed) than the high Zn leaf (7.45% mean amount consumed).

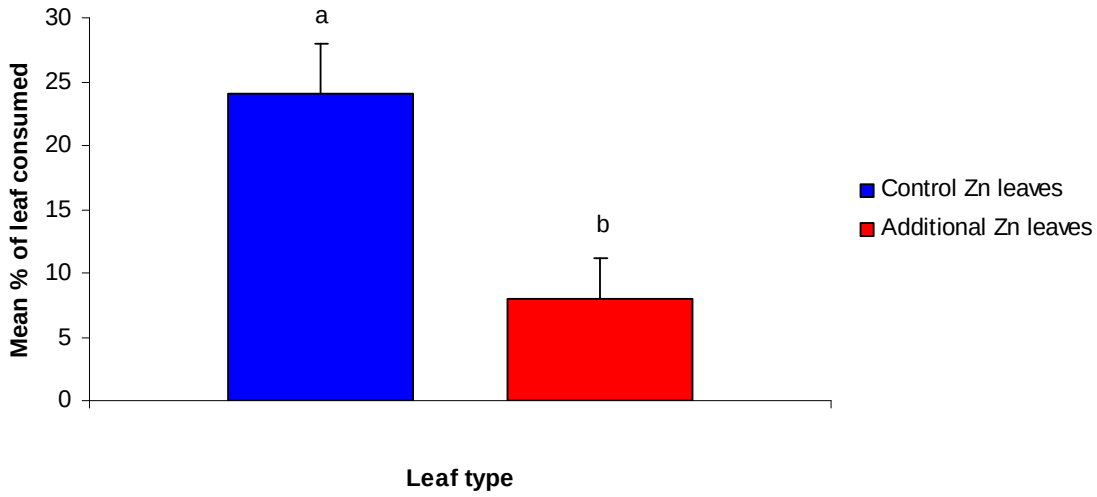

Figure 5.5: Mean (n = 12) ± SE of percentage of leaf consumed by *P. brassicae* from the two treatments containing either high or low concentrations of Zn. Means with different letters overhead differed significantly after running a Mann Whitney U test, $W_{11} = 108$, $P = 0.013$.

5.6 Growth performance of juvenile *H. aspersa* on a high and low Zn diet of leaves of *B. juncea*

5.6.1 Materials and methods

5.6.1.1 Experimental design

Forty juvenile snails (reared as discussed in section 5.3.1.2 of this Chapter), which were approximately four weeks old, were allocated at random to one of two diets (20 replicates on each diet) which comprised *B. juncea* leaves. The difference in the two diets were the growth media the plants were grown on, either containing additional 400 mg kg^{-1} of Zn (high Zn diet) or containing no additional Zn (low Zn diet) which subsequently led to differences in Zn concentration within the leaves.

Snails had their mass recorded on a four point balance at the start of the experiment and then every third day over an eighteen day period. The snails were provided with a fresh *B. juncea* leaf from the allocated diet each morning in their cage (Petri dish), which was also sprayed daily with a small amount of water to increase humidity in the cage and provide an

'optimal' environment for the snails as presented by Madec *et al.*, 2000. The experimental setup can be seen in Figure 5.12.

5.6.1.2 Statistical analysis

Data were analysed using Minitab (Version 14) and presented using Microsoft Excel (Microsoft Office, 2003). The initial and final mass of snails was analysed using a non-parametric Mann Whitney U test for significant differences, at the 95% confidence interval.

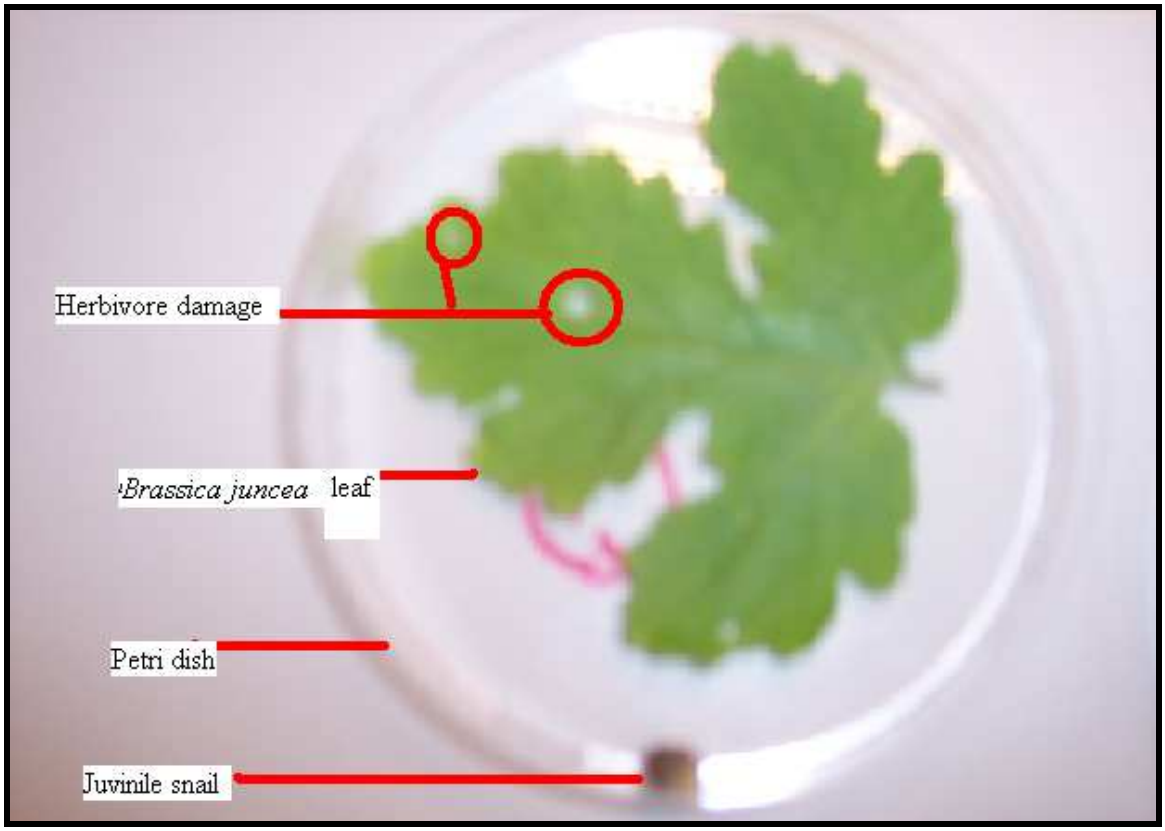

Figure 5.6: Experimental set-up of the growth performance of juvenile snails on a diet of *B. juncea* leaves containing high or low concentrations of Zn.

5.6.2 Results

As demonstrated in Chapter 4 the Zn concentration of *B. juncea* changes over time. Therefore, during this trial the concentration of Zn in the *B. juncea* leaves was sampled at the beginning and the end of the trial. The mean Zn concentration in the leaves of the plants grown on the treatment containing no additional Zn did not differ a great deal at the beginning of trial (35.0 mg kg^{-1}) and at the end (49.4 mg kg^{-1}). While the concentration of Zn in the plants that were grown in the additional Zn treatment did significantly increase from the beginning (439.5 mg kg^{-1}) and eighteen days later at the end of the experiment (994.8 mg kg^{-1}), with the Zn concentration more than doubling over this course of time. But as discussed in more detail in Chapter 4, it was expected that Zn concentration in the leaves would change over time.

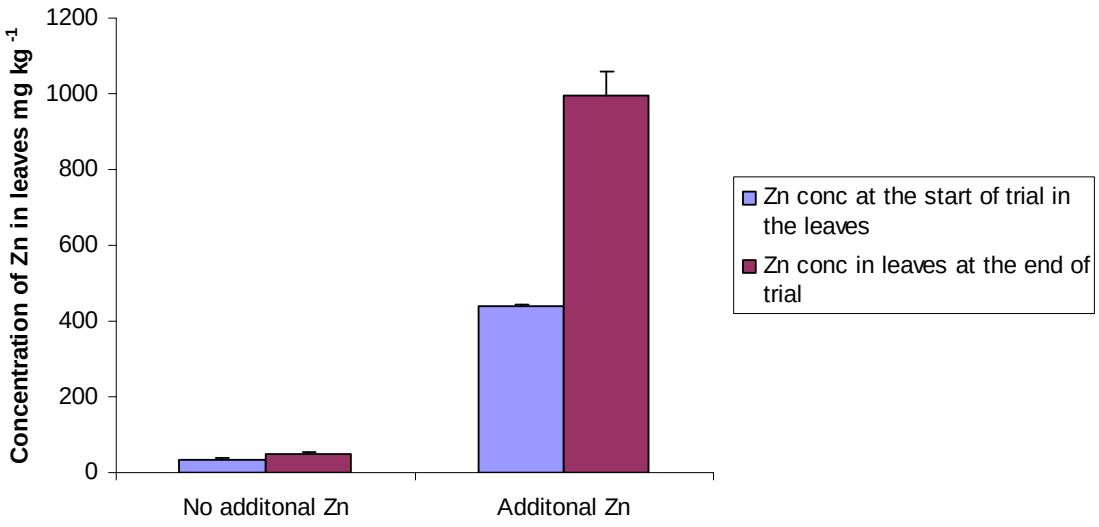

Figure 5.7: Mean ± SE concentration of Zn in the *B. juncea* leaves that formed the two diets for the performance trial of juvenile snails, from samples taken at the beginning and end of the trial.

The masses of both sets of twenty snails that were fed the high and low Zn leaves, did not differ significantly (W_{39} = 361, P = 0.1895) at the start of the experiment. The snails on the high Zn diet had a mean mass of 0.024 g and for the snails on the low Zn diet weighed 0.028 g on average. For the first 6 days of the trial, snails on both diets steadily increased in

mean mass, but by the twelve day snails on the high Zn treatment had started to decrease in mass, while those on the low Zn diet continued to gain mass. This trend continued for the remainder of the trial. On the eighteenth and final day of the trial the snails mean mass from the two diets were significantly different (W_{39} = 235, P = < 0.001), with the mean mass of snails on the diet of *B. juncea* leaves not containing any additional Zn having a mean mass almost double of their starting mass (0.046 g). In contrast the mean mass of the snails on the high Zn diet was actually lower than (0.022 g) than their starting mass, Figure 5.14. These results suggest that the *B. juncea* leaves containing elevated concentrations of Zn have a negative impact on the growth of juvenile snails.

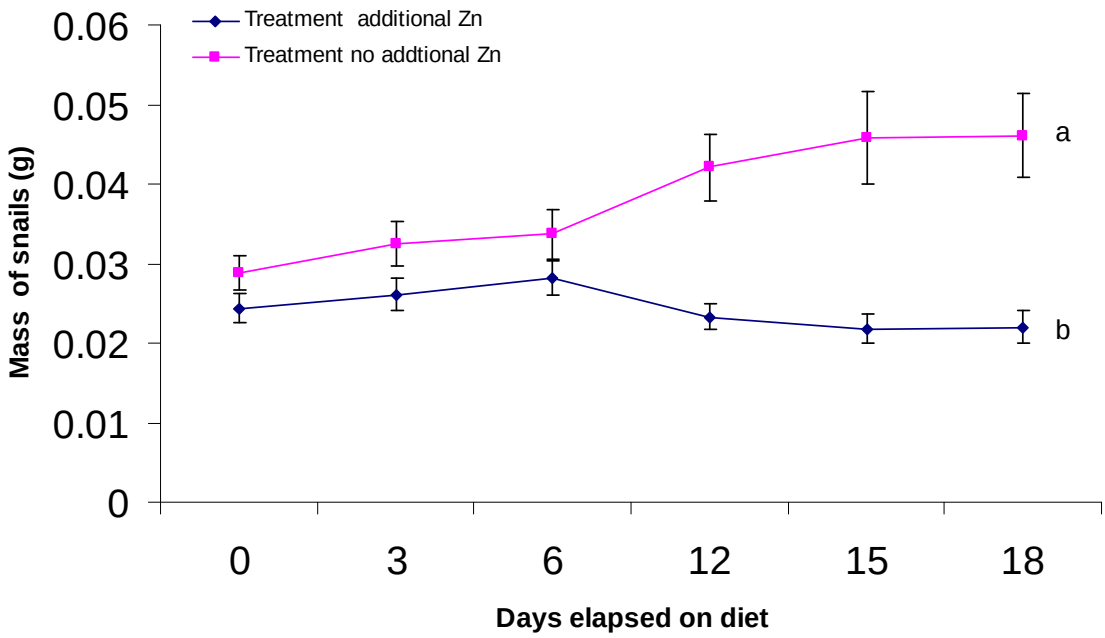

Figure 5.8: Mean (n = 40) ± SE juvenile snail masses fed on the two different diets of *B. juncea* leaves, either containing high or low concentrations of Zn. Means with different letters differed significantly following a non-parametric Mann Whitney U test.

5.7 Performance of adult *H. aspersa* on a high and low diet of leaves of *B. juncea*

5.7.1 Materials and methods

5.7.1.1 Experimental design

Twenty four adult *H. aspersa* snails were collected from the grounds of the University of Sussex. The snails, none of which has been used in any previous experiments, were housed as described in section 5.4.1.1 of this chapter, prior to taking part in the experiment. The initial mass of the snails as recorded before the start of the experiment and then they were randomly allocated to individual cages that contained one of two diets. The diets comprised *B. juncea* leaves that came from plant either grown in soils containing additional concentrations of Zn (400 mg kg^{-1}) or not. The leaves in the snail cages were replaced on a daily basis with leaves picked freshly from plants growing in greenhouses. Snails were then fed their allocated diets for a period of 15 days, during which the mass of individuals were recorded every other day.

5.7.1.2 Statistical analysis

Data were analysed using Minitab (Version 14) and presented using Microsoft Excel (Microsoft Office, 2003). Mann Whitney U test were used to test for any significant (with a 95% confidence interval) differences in snail masses at the start and end of the experiment.

5.7.2 Results

Figure 5.15 displays the mass of the snails over the course of the 15 day trial; it clearly shows that there was no significant difference in the starting weight of both groups of snails at the start of the experiment (W_{11} = 150, P = 0.99). There was very limited change in the snail's masses over the duration of the experiment. The two groups of snails almost had identical final masses at the end of the experiment (W_{11} = 152, P = 0.931).

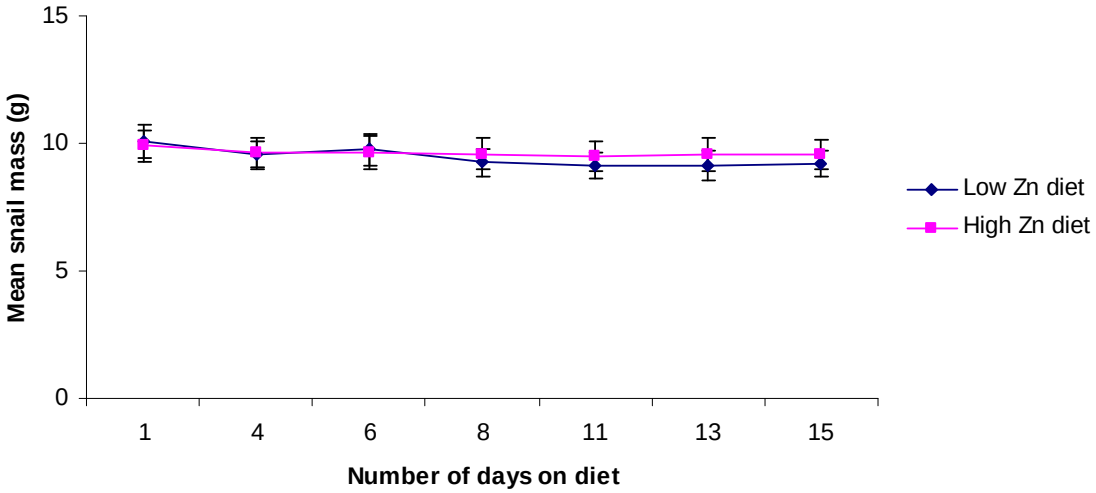

Figure 5.9: Mean (n = 12) ± SE snail masses fed on the two different diets of *B. juncea* leaves either containing high or low concentrations of Zn.

5.8 Growth performance of *P. brassicae* on a high and low Zn diet of *B. juncea* leaves

5.8.1 Materials and methods

5.8.1.1 Experimental design

P. brassicae eggs from Blades Biological were hatched as described in section 5.5.1. Once they were feeding independently, forty individuals were transferred to separate feeding cages after being weighed on a four point balance and randomly assigned one of the two diets.

The diets consisted of *B. juncea* leaves from plants that had either been grown on one of two growth treatments, containing no additional Zn (low Zn diet) or 800 mg kg^{-1} additional Zn (high Zn diet) for 10 days, having undergone seed germination and initial growth as described in Chapter 2.

Caterpillars were provided with a fresh leaf of the diet they were allocated each day, leaves had damp paper towel wrapped around the petiole to keep the leaves as fresh and palatable as possible. The mass of caterpillars was recorded every other day over the course of a week, until just before they pupated. The experiment was carried out in an experimental chamber that had lights set on a 16/8 hr and mean temperature of 20/16 ^{o}C on a day/night cycle. Figure 5.16 shows an example of the experimental set-up.

Five days after the caterpillars had reached their pupa state, they had their mass recorded so that a comparison between the two diets could take place. The pupae were then transferred to labelled self seal bags and placed in a -20 ^{o}C freezer so that may be analysed at a later date to establish if an accumulation of Zn occurred in the caterpillars on the high Zn diet (see section 2.8 of Chapter 2).

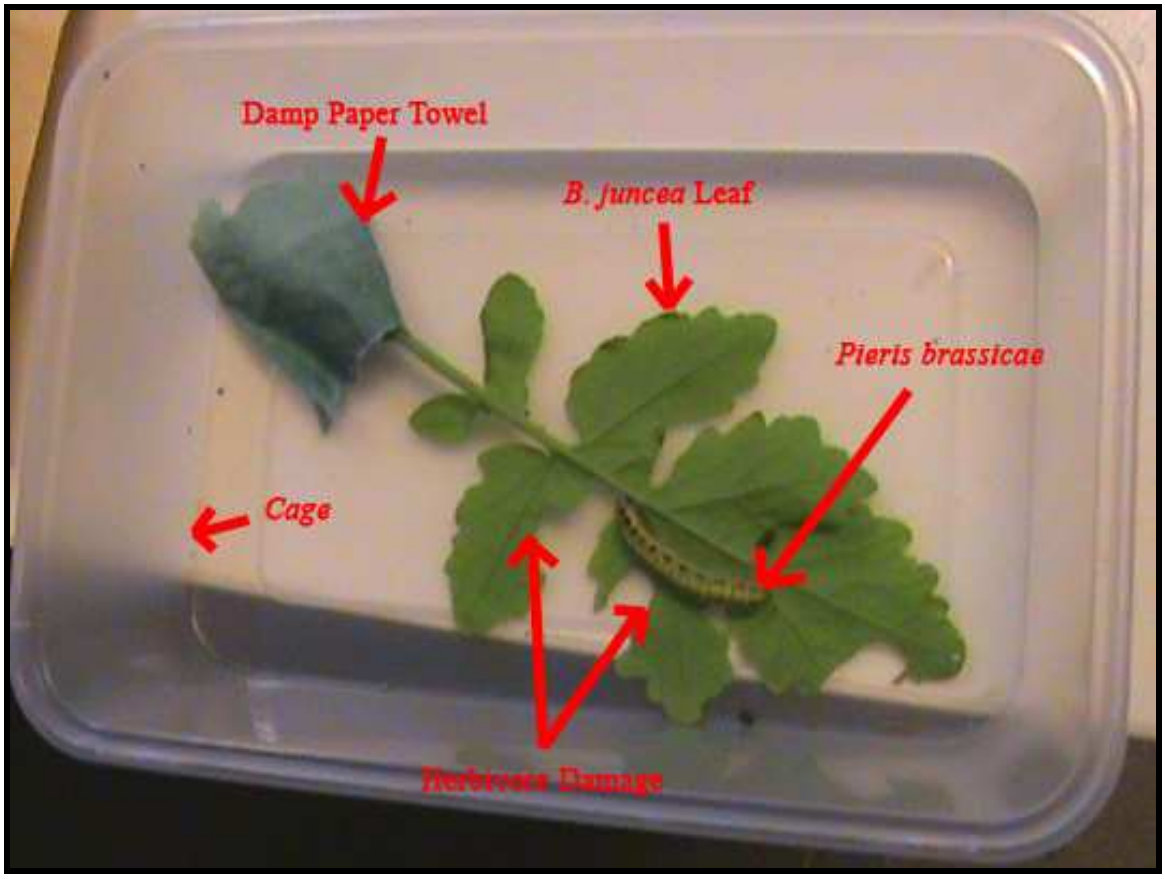

Figure 5.10: Experimental set-up of *P. brassicae* performance on a low and high Zn diet

5.8.1.2 Statistical analysis

Data were analysed using Minitab (Version 14) and presented using Microsoft Excel (Microsoft Office, 2003). The initial and final weights of the Lepidoptera reared on the different Zn diets were analysed as separate one-way ANOVA's.

5.8.2 Results

The starting masses of the two groups of *P. brassicae* were not significantly different (F_{31} = 0.24, P = 0.63) as displayed in Table 5.1. The caterpillars on the low Zn diet increased in mass between day three and seven in a linear fashion, while those on the elevated Zn diet were slower to increase in weight. The final masses of the caterpillars (Table 5.2) reared on the diet of *B. juncea* leaves containing an elevated concentration of Zn had significantly lower masses (F_{31} = 6.54, P = 0.022) at the end of the experiment than those on the diet of *B. juncea* leaves from plants grown without any additional Zn.

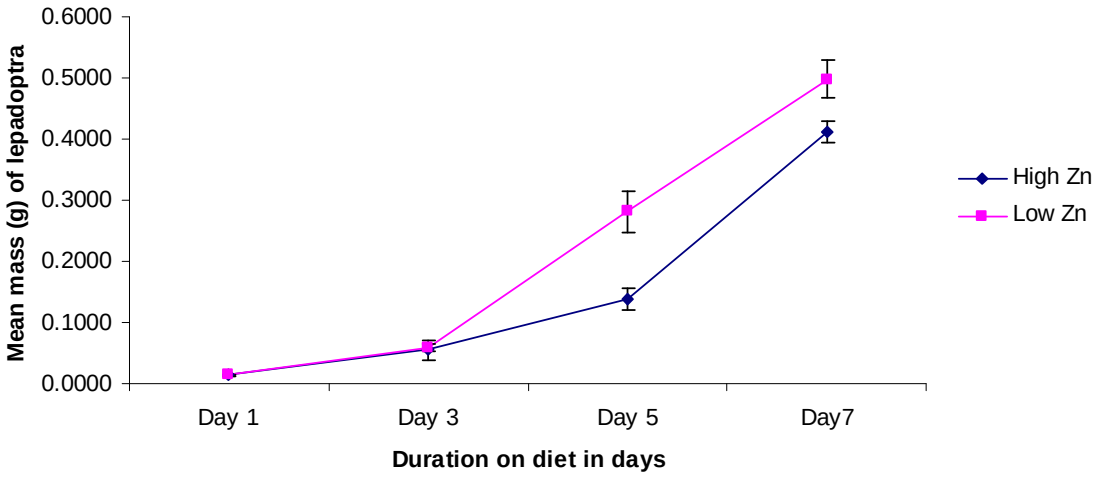

Figure 5.11: Mean (n = 20) ± SE mass of caterpillars on a high and low Zn diet, over the growth period from the second instar to just before pupating.

Initial Weight	d.f.	SS	MS	F	P
Diet	1	4.3×10^{-6}	4.3×10^{-6}	0.24	0.63
Error	38	6.9×10^{-4}	1.8×10^{-5}		
Total	39	6.9×10^{-4}			

Table 5.1: One-way ANOVA output for the comparison of the initial mass of the caterpillars.

Final Weight	d.f.	SS	MS	F	P
Diet	1	2.6×10^{-2}	2.6×10^{-2}	6.54	**0.022**
Error	15	6.0×10^{-2}	4.0×10^{-3}		
Total	16	8.6×10^{-2}			

Table 5.2: One way ANOVA output for the comparison of the final mass of the caterpillars.

Table 5.3 displays the mean results for the recovery of Zn spikes that were run at the same time as the insect samples. The results show that for four of the five spikes ranging between 5 and 100 mg l^{-1} of Zn, the percentage recovery exceeded over 90 %. The percentage recovery only dropped below 90 % on the spikes that had a Zn concentration of 2 mg l^{-1}. This was most likely due to analytical errors arising from incorrectly calibrated pipettes and the like. The concentrations in the insect samples were at least ten times this and within the range where Zn recovery exceeded 90 %, so that results from the insect digest can be viewed with a high degree of certainty in terms of the values produced.

N	Spiked Zn concentration mg l^{-1}	Mean actual Zn concentration mg l^{-1}	Mean % recovery of Zn spikes
3	100	94.34	94.34
3	50	47.97	95.93
3	25	24.03	96.10
3	5	4.84	96.80
3	2	1.60	79.83

Table 5.3: Recovery of Zn spikes, used as a data quality control tool for the Zn concentration in the insects.

Caterpillars reared on a diet of *B. juncea* leaves that contained an elevated Zn concentration contained a mean Zn concentration in their biomass of 42 mg kg^{-1}. This was significantly higher ($F = 12.72$, $P = < 0.001$) than the concentration of Zn (29mg kg^{-1}) in pupae from

larvae reared on low Zn diet. These results suggest that the relationship between Zn concentrations in pupates and Zn concentration consumed from the diets is not linear.

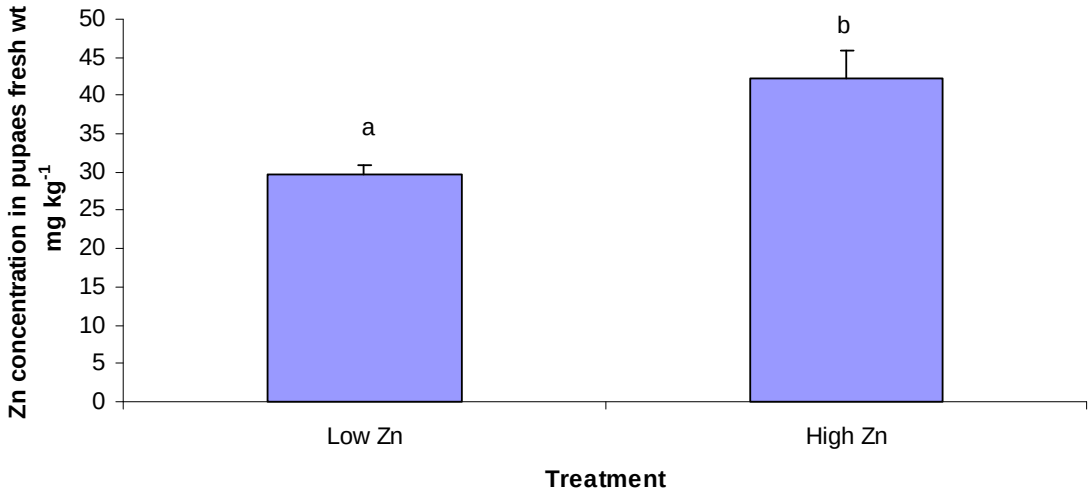

Figure 5.12: Mean ± SE Zn concentration in pupae at the end of the experiment reared on the two different Zn diets. Different letters above bars indicate significant differences in Zn concentration determined by a One-way ANOVA.

Zn Concentration	d.f.	SS	MS	F	P
Diet	1	1260	1260	12.72	**<0.001**
Error	31	3072			
Total	32	4332			

Table 5.4: Statistical output of the Zn concentration in pupae at the end of the experiment reared on the two different Zn diets.

5.9 Discussion

5.9.1 Effects of Zn on juvenile snails

i. To establish if elevated Zn concentrations in *B. juncea* have an impact on the feeding preference of **juvenile *H. aspersa*** and caterpillars of *P. brassicae.*

The preference trial conducted in this experiment has produced conclusive evidence that juvenile snails have a preference for *B. juncea* leaves that contain low concentrations of Zn over those containing elevated concentrations in their leaves.

It is plausible to suggest that the preference of juvenile snails for *B. juncea* leaves that do not contain elevated concentrations of Zn arises due to their possible ability to 'taste' the Zn.

iii. To determine if a diet of *B. juncea* leaves containing elevated concentrations of Zn have an impact on the growth and performance of **juvenile** and adult ***H. aspersa*** as well as caterpillars of *P. brassicae*.

The results from the performance trial show that the juvenile snails on the diet of *B. juncea* leaves containing elevated concentrations of Zn increased their mass significantly less than the snails on the control diet. This suggests that Zn at these elevated concentrations is having a negative impact on the growth of juvenile *H. aspersa*. In the case of juvenile snails performance matched preference.

5.9.2 Effects of Zn on adult snails

ii. To ascertain if adult *H. aspersa* change their preference when repeatedly presented with leaves of *B. juncea* containing low, medium and high concentrations of Zn.

In the two experiments in which adult *H. aspersa* were offered a choice of a low Zn leaf and either a medium or high Zn leaf, the snails showed a significant preference for the low Zn leaves in the third and final trial. This indicates that repeated exposure has an affect on feeding preference, with the results suggesting that increased Zn concentration in leaves is less desirable in a diet for snails than low Zn leaves.

In the experiment where snails were given a choice between medium and high Zn leaves, the first trial resulted in a significant preference for the medium leaves. However, this result can not be claimed to be conclusive as no preference was observed in the following two trials.

These results suggest that snails predominately have a preference for low Zn leaves over leaves containing elevated concentrations of Zn, but that preference is established through repeated exposure and experience. This supports theories put forward by Pliner (1982). Therefore, naïve snails are just as likely to consume *B. juncea* leaves high in Zn as those containing non-elevated concentrations.

iii. To determine if a diet of *B. juncea* leaves containing elevated concentrations of Zn have an impact on the growth and performance of juvenile and **adult *H. aspersa*** as well as caterpillars of *P. brassicae*.

Snails on the two different diets of *B. juncea* leaves from plants grown on soils containing no additional Zn and 800 mg kg^{-1} of additional Zn, did not alter their mass significantly over the 15 day trial. These results are contradictory to those found in the juvenile performance experiment, but this maybe due to a number of reasons. Firstly, the mass of adult snails is large and relatively stable and so less likely to display dramatic changes over a relatively short time period. Secondly, having reached adult status they are possibly in a better position to deal with increased levels of 'toxins' in their diet. Some authors including Dallinger *et al*, 1993 have suggested that adult snails may be capable of coping with elevated concentrations of heavy metals, by depositing them in their shells, thus in effect reducing the energy expenditure that may be required by juveniles to detoxify them.

5.9.3 Effects of Zn on *P. brassicae*

i. To establish if elevated Zn concentrations in *B. juncea* has on an impact on the feeding preference of juvenile *H. aspersa* and **caterpillars of *P. brassicae***.

P. brassicae had a significant preference for *B. juncea* leaves containing no additional Zn than those that contained elevated concentrations of Zn. This mirrors the outcome of that from the juvenile snail preference experiment and again suggests both types of herbivore are capable of 'tasting' the increased amounts of Zn in the plants.

iii. To determine if a diet of *B. juncea* leaves containing elevated concentrations of Zn have an impact on the growth and performance of juvenile and adult *H. aspersa* as well as **caterpillars of *P. brassicae*.**

P. brassicae reared from the second instar until just prior to pupation increased their mass significantly less if on a diet of *B. juncea* leaves with an increased concentration of Zn than leaves that did not. It appears that the increased Zn in the diet has negative impact on the growth rate of *P. brassicae*; this possibly suggests that a high Zn concentration in plants biomass is toxic to these herbivores

iv. To assess if a diet of *B. juncea* leaves containing elevated concentrations of Zn increases the Zn concentrations in **Pieris brassicae** when compared to caterpillars fed a diet of *B. juncea* leaves that do not contain elevated concentrations of Zn.

A diet of *B. juncea* leaves with elevated concentrations of Zn resulted in pupae containing a significantly higher concentration of Zn than pupae from caterpillars individuals raised on a diet that not contain increased Zn levels. However, the relationship between Zn concentration in the plant material and of that in the pupae was non-linear; an explanation for this maybe that caterpillars are trying to detoxify the additional Zn concentrations, at a cost of reduced growth rate.

To conclude, both the generalist (juvenile *H. aspersa*) and the brassica specialist (*P. brassicae*) experienced a reduction in biomass production when fed exclusively on leaves of *B. juncea* containing elevated concentrations of Zn. This provides a very compelling argument to suggest that Zn is toxic and adversely affects the growth rate of these herbivores.

H. aspersa (juvenile and adult) and *P. brassicae* caterpillars all made a significant preference for *B. juncea* leaves that did not contain increased concentrations of Zn. The results of all the experiments are summarised in Table 5.5.

	Significant experimental outcome in the herbivore species		
Experimental type	**Juvenile *H. aspersa***	**Adult *H. aspersa***	***P. brassicae***
Preference for low Zn leaves	**YES**	**YES**	**YES**
Performance adversely affected by high Zn leaves	**YES**	**NO**	**YES**

Table 5.5: Summary Table of results from Chapter 5

This chapter has presented evidence to suggest that increased concentrations of Zn in the plant *B. juncea* have a negative impact on both *H. aspersa* and *P. brassicae*. The next logical step having now investigated Zn as an elemental defence in *B. juncea* in the laboratory is to conduct experiments under field conditions. Results demonstrated in an artificial laboratory environment may not occur in the field, so the next chapter of this thesis contains one of the largest field based experiments testing the elemental defence characteristics of Zn in *B. juncea*.

Chapter 6: Evaluating the elemental defence hypothesis of *B. juncea* (an accumulator of Zn) under field conditions

6.1 Introduction

The experimental chapters so far have focused predominantly on laboratory based experiments. However, results produced in the lab often contradict data collected under field conditions. This is because experiments conducted in greenhouses under laboratory conditions are highly controlled and are more likely to produce clear positive results. These results are not always observed in field based experiments, due to the vast number of uncontrollable natural factors which occur in this type of experiment, such as the amount of light plants receive, changes in temperature and wind exposure etc, which affect plant behaviour and metabolism (for example the amount of Zn plants sequester). But these experiments are imperative in testing theories such as the elemental defence hypothesis, because plant species are exposed to populations of natural herbivores with climatic conditions more typical than the artificial parameters of green house based experiments. There are relatively few tests of the elemental defence hypothesis conducted under field conditions (Galeas *et al*., 2008; Boyd, 2007). Table 6.1 summaries the studies published to date.

Many researchers conducting field trials with plants that accumulate heavy metals are focused on the optimisation of metal concentration uptake in plants and designing crop rotations to produce a commercially viable phytoremediation, rather than assessing the potential effects these plants may have on ecosystems. This is why Boyd (2007) considers the elemental defence hypothesis to be relatively new and in need of further experimental investigation. The elemental defence hypothesis has important implications for phytoremediation, in terms of ensuring that the uptake of metals by plants is not going to have significant impacts of the movement of contaminants (including heavy metals) to be bioaccumulated up the food chain (Laskowski and Hopkin, 1996*;* McGrath, 2006).

The field work that has been conducted to test the elemental defence hypothesis has used plant species that are hyperaccumulators of heavy metals and is summarised in Table 6.1). Two of the experiments used the Zn hyperaccumulator plant species *T. caerulescens* but these have produced mixed results. The possible reasons for these conflicting results are discussed below. A review of these experiments is detailed below.

Noret *et al*., (2007) transplanted *T. caerulescens* plants that had been germinated and grown initially in a greenhouse, in soils with and without the addition of Zn. The plants were transplanted (within countersunk pots) into an area where *T. caerulescens* is found to grow in the wild. The plants were monitored over a number of months and a record of the damage they received recorded. A positive aspect of this experiment is that plants were grown in an area where natural herbivores of *T. caerulescens* reside, thus increasing the realism of the experiment. The amount of damage the plants received was not reduced by increased concentration of Zn in the plants. However, the concentrations of glucosinolates, a secondary plant defence compound, did affect the rate of herbivory.

Dechamps *et al*., (2007) also used *T. caerulescens* in a field experiment, but rather than grow plants in soils containing predetermined heavy metal concentrations, they located two areas where *T. caerulescens* was growing in the wild. The locations differed in terms of heavy metal content, one site had 'normal' concentrations of heavy metals, below soil guideline values (SGV's), while the other had metal concentrations that greatly exceed SGV's and classed as heavily contaminated. Plants were then reciprocally transplanted between the two and the herbivore damage monitored over time. Their conclusion was that plants transferred from contaminated sites to non-contaminated sites suffered more herbivore damage than those plants moved from the clean site to the contaminated site. However, this result is open to alternative interpretations, as several important factors were not addressed by this experimental design. For example, neither the abundance nor the identity of the herbivores at the two sites was recorded, so it is possible that there were more herbivores on the uncontaminated site than the contaminated site, or that the herbivore species at this site were the best adapted to consuming T. *caerulescens*.

Both the above experiments have tried to some extent to evaluate the effectiveness of Zn as an elemental defence in plants, they have used a plant species, that as defined by this thesis is not suitable to test this hypothesis. Due to the naturally high Zn internal requirement of *T. caerulescens* it will hyperaccumulate Zn into its above ground biomass far beyond 'normal' plant species even when the availability in the soil is low. Therefore, herbivores in the wild have not truly been offered the same plant species with high and low concentrations of Zn but rather, high and very high concentrations of Zn.

It is for the reasons stated above that the research for this chapter has been carried out. It is to date the first documented example of a large scale field experiment testing the elemental defence characteristics of Zn in an accumulator (*B. juncea*) plant species. This experiment is an advance on previous work, as it examines the growth nutrient content as well as the conducting an assessment on the abundance and type of herbivore, against increasing Zn concentration in soils and thus plants on a very large scale.

6.2 Aim and Hypotheses

To determine if *B. juncea* plants grown on a high Zn soil and subsequently containing higher Zn concentrations in their foliage are protected against herbivory compared to plants grown on a control soil, not containing any additional Zn.

i. Plants that have sequestered more Zn will be less damaged by herbivores.

ii. Insects will be present in fewer numbers on plants that contain a higher Zn concentration than those with a lower concentration.

iii. Growth of plants on soils containing varying Zn concentrations will not be affected in terms of nutritional quality (C:N content) and plant height.

Plant species	Metal and concentration mg kg^{-1}	Brief description of experiment	Outcome of experiment	Reference
T. caerulescens	With or without an additional 2000 mg kg^{-1} of Zn in growth media.	Greenhouse grown plants were transplanted into field conditions where natural populations exist and the extent of herbivore damage monitored.	Higher Concentrations of Zn did not correspond to lower levels of herbivore damage however, glucosinolate levels did.	Noret *et al*., 2007.
T. caerulescens	Plants were grown on a soil containing a range of metals, which included Zn with concentrations of between 13,145 -30678 mg kg^{-1} at the metalliferous sites and 15- 23 mg kg^{-1} at the non-metalliferous sites.	Reciprocal transplantation experiment in which plants from a metalliferous environment were moved to a non-metalliferous environment and vice-versa.	Plants from metalliferous sites transplanted to non-metalliferous suffered more herbivore damage than the plants transferred to the metalliferous site from the non-metalliferous sites.	Dechamps *et al*., 2007.
Streptanthus polygaloides	Ni 9,100 mg kg^{-1}.	Greenhouse grown plants were transplanted into field conditions where natural populations exist and the extent of herbivore damage monitored.	Unknown invertebrates were deterred from consuming plants grown on the soil containing higher concentrations of Ni.	Martens and Boyd, 2002.

Table 6.1: Summary table of the experiments that have assessed the elemental defence hypothesis under field conditions.

6.3. Materials and methods

6.3.1 Seed germination and initial growth of plants

Eight trays (6” x 4”) containing an inch of damp vermiculite had approximately 100 *B. juncea* seeds sown on each of them. Germination rates exceeded 90% within three days. Seedlings continued to grow on the vermiculate, which was routinely kept damp but not wet for a week. After this time had elapsed 700 seedlings which exceeded 1.5” were transplanted into 2” plug trays, containing John Innes no. 2. The seedlings were then allowed to grow in the plug trays for a further week. At the end of growth period in the plug trays 600 plants were at random allocated and transplanted into pots containing one of the four soil treatments. The plants were then allowed a further five days in the confines of the greenhouse to fully establish, before being moved to the field trials plot. Any plants that did not survive the transplant while in the greenhouse were replaced immediately with plants still growing in the plug trays.

6.3.2 Preparation of soil treatments

Soils were prepared as described in section 2.3 of Chapter 2. To obtain additional Zn concentrations in the soil of 0, 400, 600 and 800 mg kg^{-1}.

6.3.3 Experimental design

Three replicate sites approximately 4 x 5 m in area were mammal proofed using chicken wire around the perimeter. Each site had a synthetic liner covering the ground to prevent weed growth. Although plants remained in pots, a thin layer < 2 cm of John Innes no. 2 compost covered the surface of each of the plots, to help aid herbivore access to the plants.

Each site housed 200 plants (50 replicates of each treatment), these were arranged with a five centimetre spacing between pots in a Latin square styled design (10 x 20).

The plants were watered daily and allowed to grow on the sites for a total of three weeks.

The herbivore damage that plants received was monitored after ten days and at the end of the three week experiment. The damage assessment was conducted by examining every leaf on all of the plants and counting the total number of leaves on each plant. Leaf damage was assessed by eye to the nearest 5 %, on each leaf of the plant. Plant height was recorded at the same time as the damage assessments taking place..

In addition to this, a herbivore count was conducted halfway through the experiment to try to record the number of herbivores both feeding and residing on the plants. Four main species of herbivore were identified and their abundances recorded: weevils, leaf beetles, green and black aphids from a 30 second observation period of each plant.

6.3.4 Site description

The trial was carried out in a field at Sussex University, (OS grid reference: TQ 34 09) upon the grasslands of the Sussex downs. The surrounding area is used as grazing farmland. The total site area is approximately 55m by 185m. Figures 6.1 and 6.2, display this area from an aerial satellite view and an O/S map respectively.

Figure 6.1: Aerial image of the three experimental sites on the field trials plot at the University of Sussex (Adapted from Google maps, 2008).

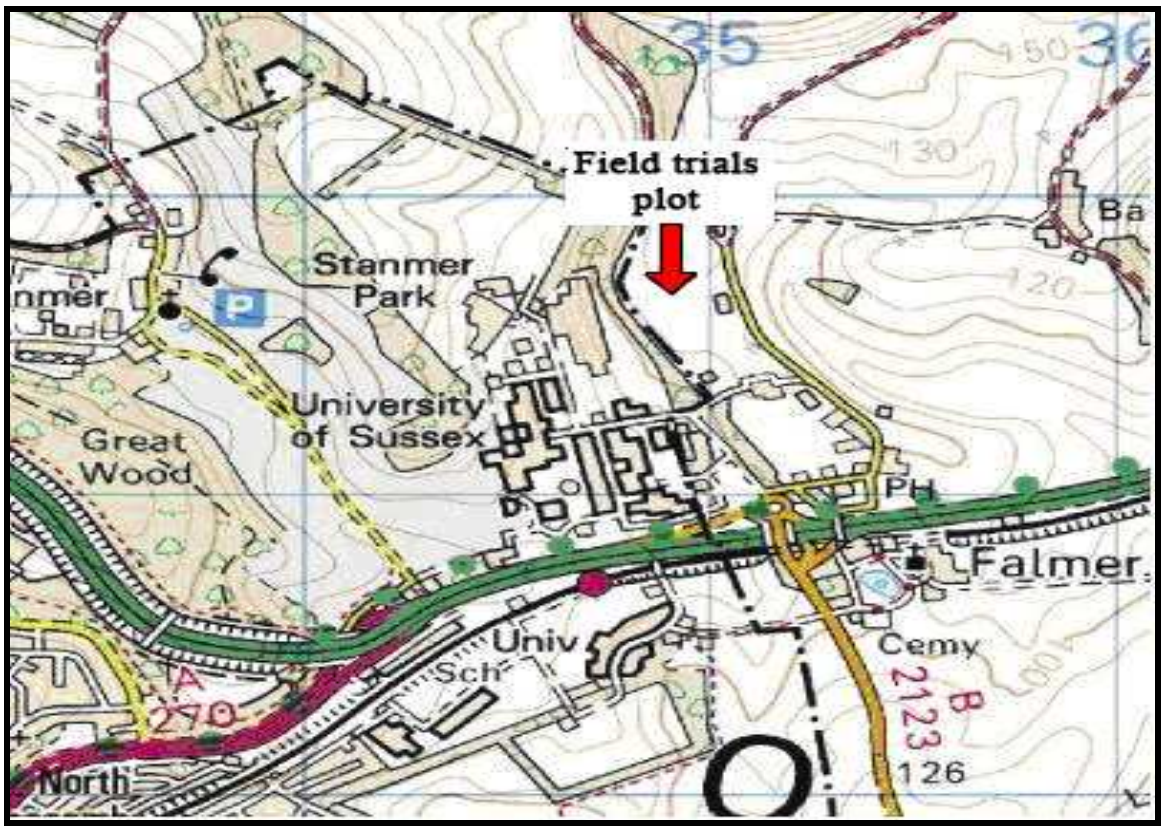

Figure 6.2 Ordinance Survey map of the field trials site in respect to Sussex University.

All plants were harvested after the second round of leaf damage analysis. They were cut 0.5cm above the soil surface using scissors and placed into labelled paper bags and returned to the lab for drying and analysis.

Figure 6.3: Experimental set-up of site one on the field trials plot at the University of Sussex, on the day of the Harvest.

6.3.5 Plant and soil analysis

Upon harvesting the plants they were placed in drying ovens at 40^{o}C for three days by which time all samples were dry. Ten plants from each treatment and plot (120 plants in total) were then randomly selected for Zn analysis by F-AAS. Each plant that was used for analysis was dried in an oven at 50^{o}C for 48 hrs, before being segregated into leaves and stems. The plant material was then ground using a ball-mill and then analysed in accordance with chapter 2.

6.3.6 Statistical analysis

Data were analysed using Minitab (Version 14) and presented using Microsoft Excel (Microsoft Office, 2003). One-way ANOVA (Analysis of Variance) was used to determine differences between Zn concentrations in the growth media. Two-way ANOVAs were used to test for the effect of treatment on the Zn concentration in the leaves of the plants grown

on the four treatments at the three sites, as well as the percentage damage on the plants half-way and at the end of the experiment. Data were checked for normality (probability plots) and equal variance (Levene's test) to ensure they conformed to the assumptions of the tests. Significant results were subjected to *post-hoc* Tukey's test to determine how they differed from another at $P < 0.05$. The insect data were analysed using two nonparametric Friedman's tests, to evaluate both the effect of treatment and site on total herbivore numbers on the plant. The effect of the treatments on C:N ratio and % nitrogen content was tested by separate GLM analysis, with *post-hoc* Tukey tests to determine significant differences at the $P < 0.05$ level.

6.4 Results

6.4.1 Analytical data quality

The CRMs that were analysed at the same time as the soil and plant samples gave estimates of bias that were below 13 % for both media, Table 6.2. This would indicate that the results obtained are not subject to any unacceptable bias.

Samples	**CRM**	**n**	**Mean measured Zn concentration mg kg^{-1}**	**Mean Bias**	**Mean relative Bias %**	**Overall relative Bias %**
Growth media samples	NIST 2709	3	92.55	-13.45	-12.69	-9.62
	NIST 2710	3	6456.37	-495.63	-7.13	
	NIST 2711	3	318.31	-31.69	-9.05	
Plant samples	NIST 1570A	5	87.47	5.47	6.67	

Table 6.2: Summary table of the mean bias of CRM measurements run at the same time as samples of soil and plant material to provide an estimate of the systematic error in the data.

6.4.2 Zn concentration in soil

The measured mean concentration of Zn in the four growth treatments (19, 374, 587 and 764 mg kg^{-1}) was very close to the target added values of 0, 400, 600 and 800 mg kg^{-1} (Table 6.3). A one way ANOVA (F = 71.02, P = <0.001) followed by a *post-hoc* Tukeys analysis of the data, confirmed that the concentrations in the four growth treatments differed significantly from each other (Figure 6.4).

Soil treatment	**Zn concentration target value mg kg^{-1}**	**Mean measured Zn concentration mg kg^{-1}**	**Nominal difference from target value mg kg^{-1}**	**% Difference from target value**
A	0	19	19	n/a
B	400	374	-26	-16.5
C	600	587	-13	-2.2
D	800	764	-36	-4.1

Table 6.3: Zn soil target concentrations vs. measured concentrations.

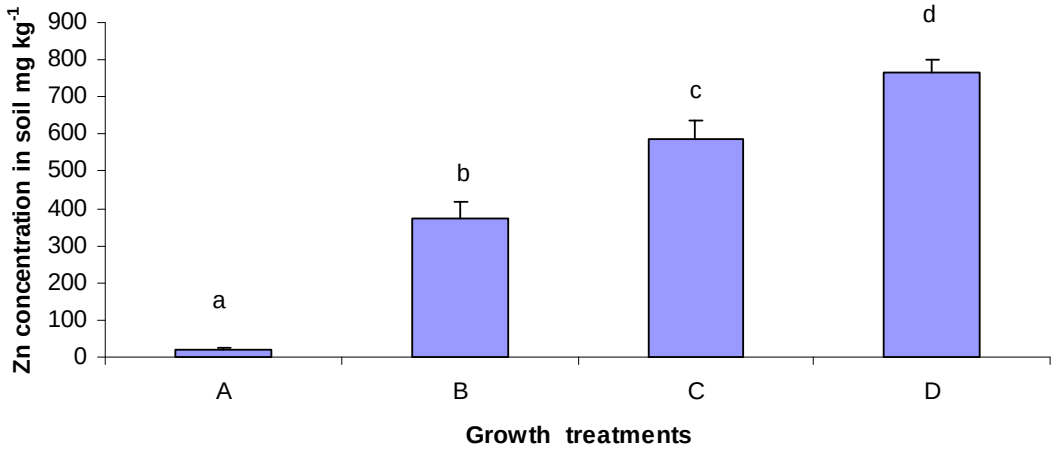

Figure 6.4: Mean (n = 5) ± SE concentration of Zn in the four soil treatments (A-D). Means with different letters overhead differed significantly after running a *post-hoc* Tukeys analysis.

Source	d.f.	SS	MS	F	P
Zn soil concentration	3	1.53 x 106	5.13×10^5	71.02	**<0.001**
Error	16	1.15×10^5	7.22×10^3		
Total	19	1.65×10^6			

Table 6.4: Statistical output of the concentration of Zn in the four soil treatments (A-D).

6.4.3 Zn concentration in plant material

At site 1 the highest Zn concentration (1508 mg kg^{-1}) was found in leaves from plants that had been growing on treatment D (767 mg kg^{-1}) containing the highest additional amount of Zn. However, this was not significantly different from the leaf Zn concentration (1297 mg kg^{-1}) grown on treatment B (soil 374 mg kg^{-1}), while plants from treatment C (soil 587 mg kg^{-1}) had a leaf Zn concentration (899 mg kg^{-1}) significantly lower than found in plants from treatment D but not from plants grown on treatment B. Plants grown on the treatments B-D had Zn leaf concentrations significantly higher by between 15-25 times more, than the plants grown on treatment A that contained no additional Zn.

The leaf Zn concentration in the plants leaves at site 2, followed a different trend to that demonstrated at site 1. At this site there was a more consistent relationship between the concentration of Zn in the growth media and the concentration of metal in the plants leaves i.e. treatment D that contained the most amount of additional Zn (767 mg kg^{-1}) also contained the highest mean Zn concentration in its leaves (1348 mg kg^{-1}). The difference in Zn leaf concentration between those grown on treatment A (19 mg kg^{-1}) and those grown on treatments (B-C) that contained additional Zn ranged between 10-18 times higher, slightly less than that found in plants from site 1.

The trend at site 3 in Zn concentration in the plants leaves grown on the treatments A-D is similar to that found in the plants from site 2 but not identical and is as follows $A < B \leq C \leq D$, not quite the near linear relationship found at site 2 but leaning towards that direction. The difference in Zn leaf concentration between those grown on treatment A (19 mg kg^{-1}) and those grown on treatments (B-C) that contained additional Zn ranged between 11`-18 times higher, again slightly less than that found in plants from site 1, but very similar to that at site 2. Figure 6.5 displays the mean Zn concentration in the leaves of the plants at the end of the experiment.

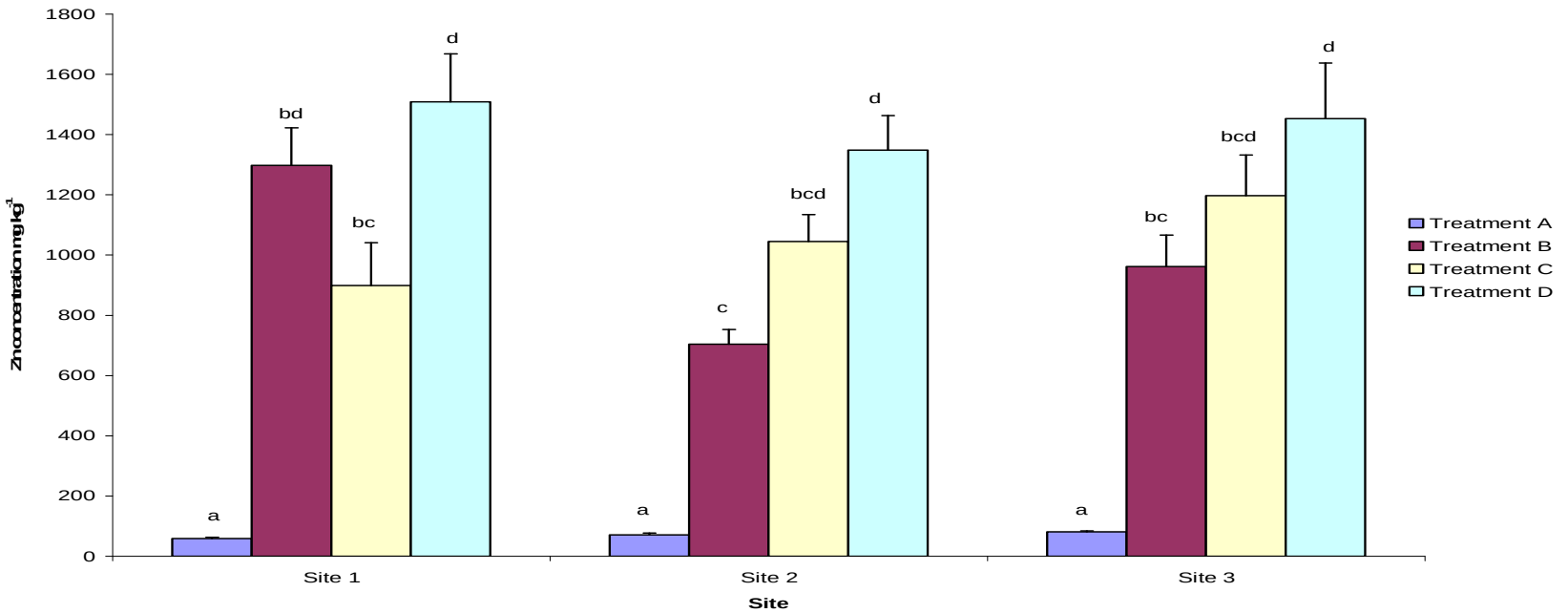

Figure 6.5: Mean (n = 10) ± SE Zn concentration in the plants leaves at the end of the experiment from the plants grown on the four different treatments at the three sites (1-3). Letters above bars indicate significant differences between treatments and site (Tukey Test).

Source	d.f.	Seq SS	Adj SS	Adj ms	F	P
Treatment	3	3.01×10^7	3.01×10^7	1.00×10^7	81.52	**< 0.001**
Site	2	5.28×10^5	5.28×10^5	2.64×10^5	2.15	0.122
Interaction	6	1.82×10^6	1.82×10^6	3.03×10^5	2.46	**0.029**
Error	108	1.33×10^7	1.33×10^7	1.23×10^5		
Total	119	4.58×10^7				

Table 6.5: Statistical output of the Zn concentration in the plants leaves at the end of the experiment from the plants grown on the four different treatments at sites 1-3.

6.4.4 Plant damage

Figure 6.6 displays the mean % herbivore damage that occurred to the plants growing on the four different Zn treatments (A-D) half-way through the experiment (after a week and a half). There were no significant differences in the amount of herbivore damage that occurred to plants between the sites 1-3 (F_{571} = 0.88, P = 0.416) which is why it has not been included in the Figure 6.6 but was involved in the analysis of the data. In this instance there was also, no interaction between treatment and site (F_{571} = 0.53, P = 0.788) on mean plant damage caused by herbivores. There was however, a significant difference (F = 10.18, P = <0.001) between the damage exhibited on the plants containing 'normal' concentrations of Zn i.e. those grown on treatment A, and the damage on the plants that had been cultivated on growth media containing the additional concentrations of Zn and thus having a higher concentration in their above ground biomass. Between the plants from treatments B-D there was no significant difference to the extent of the damage that the plants received. Therefore at this stage of the experiment increased concentrations of Zn in plants results in significantly less damage occurring, a result which supports the elemental defence hypothesis.

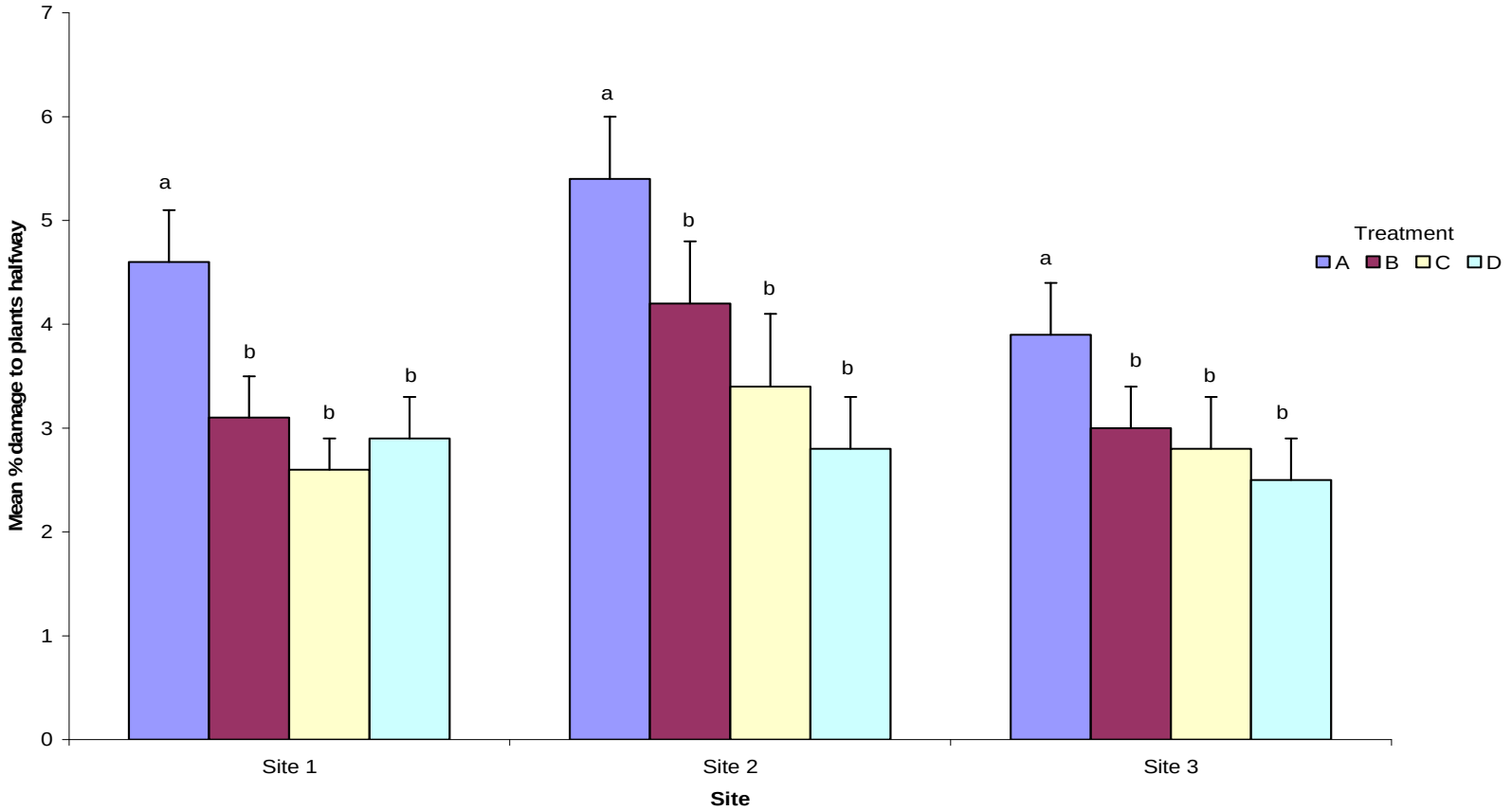

Figure 6.6: Mean ± SE % damage to plants grown on the four different treatments, half-way through the experiment. Letters above bars indicate significant differences (Tukey test).

Source	**d.f.**	**Seq SS**	**Adj SS**	**Adj ms**	**F**	**P**
Treatment	3	1049	1056	352.14	10.18	< **0.001**
Site	2	63.01	60.72	30.36	0.88	0.416
Interaction	6	109.41	109.41	18.23	0.53	0.788
Error	571	1.98×10^4	1.97×10^4	34.59		
Total	582					

Table 6.6: Statistical output of the % damage to plants grown on the four different treatments, half-way through the experiment.

At the end of the experiment, by which time the plants had been on the field trials plot for a total of three weeks, a second assessment of herbivore damage that had occurred to the plants was conducted, at the same time plants were harvested. The results from the mean % damage the plants received is displayed in Figure 6.7. Even a quick glance can confirm that there have been some marked differences in both the trend and the extent of the damage that occurred at the end of the experiment compared to the damage that was seen half way.

The most notable difference is that all of the plants at site 1 regardless of which treatment they were grown on were significantly less damaged than all the plants grown on the different treatments at the two other sites, receiving in some cases less than a quarter of the damage. The most plausible explanation for this difference is that at site 1 the plants spend much of their day in shade and is edged on one side by large trees which are not present at sites 2 and 3. At site two the plants grown on the media containing no additional Zn received significantly less damage (approximately 10 % less) than the plants grown on the treatments B-C a result which goes against the findings of the half-way assessment and those that would support the elemental defence hypothesis. A similar result at site 3 is seen to that at site 2, where by the plants containing the lowest concentration of Zn received significantly less damage than those plants with higher concentrations of Zn, with the exception of plants from treatment C which was not significantly different from any of the plants grown on the other treatments.

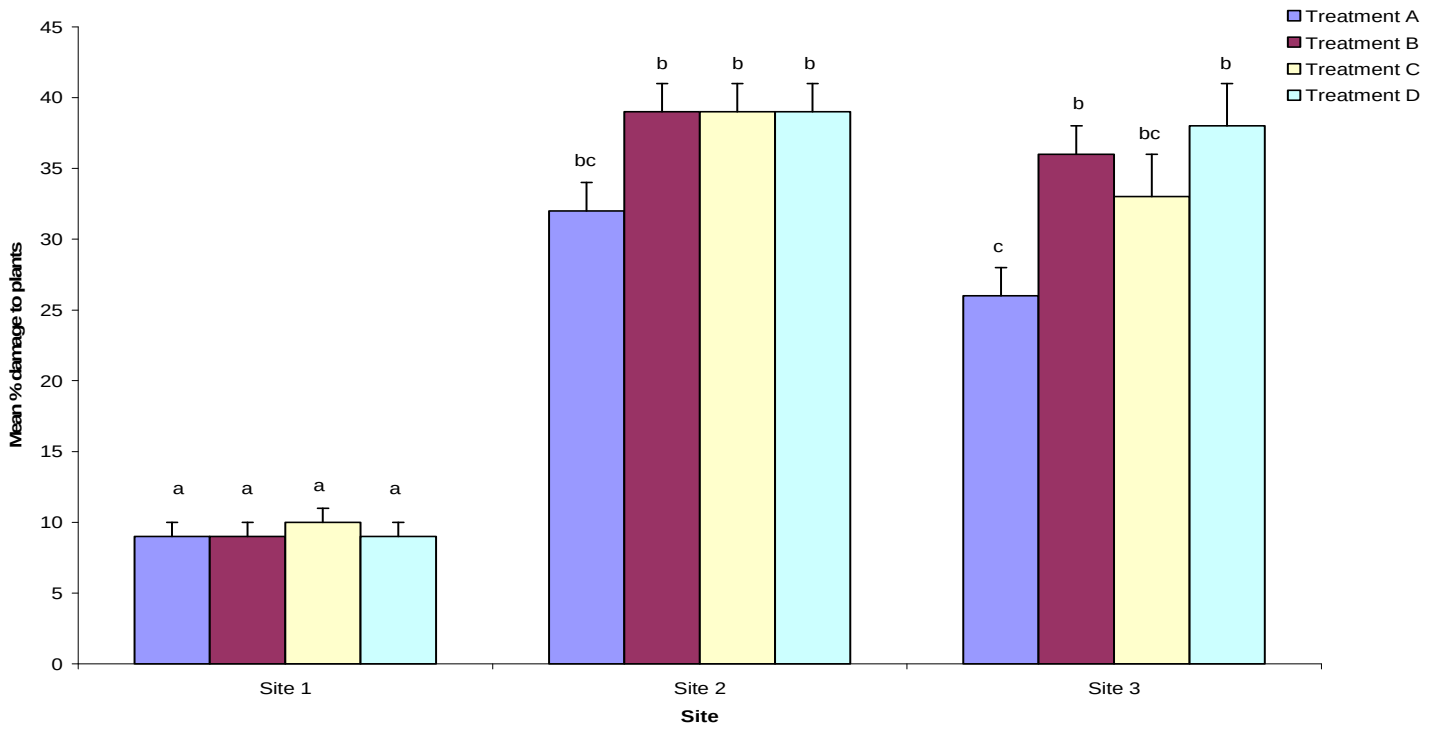

Figure 6.7: Mean ± SE % damage to plants grown on the four different treatments at the three sites at the end of the experiment. Letters above bars indicate significant differences (Tukeys test).

Source	d.f.	Seq SS	Adj SS	Adj ms	F	P
Treatment	3	1410.4	1378	459.3	7.07	**<0.001**
Site	2	4.45×10^4	4.44×10^4	2.22×10^4	342.44	**<0.001**
Interaction	6	1014.7	1014.7	169.1	2.60	**0.017**
Error	551	3.57×10^4	3.57×10^4	65		
Total	562	8.28×10^4				

Table 6.7: Statistical out put of the % damage to plants grown on the four different treatments at the three sites at the end of the experiment.

6.4.5 Plant height

6.4.5.1 Halfway through the experiment

Figure 6.8 displays the mean plant height data of the plants grown on the four treatments at the three sites, halfway through the experiment. The most notable difference is that between site 1 and sites 2 and 3, as all of the plants from site 1 were significantly (F_{582} = 65.61, P = <0.001) taller than from the other two sites.

At all three sites there is a strong trend towards plants height being in the following height order A > B > C $\geq$ D. There was a significant difference (F_{582} = 65.61, P = <0.001) of plant height associated with Zn treatment, clearly indicating that plant height is adversely affected by an increasing concentration of Zn in the soil.

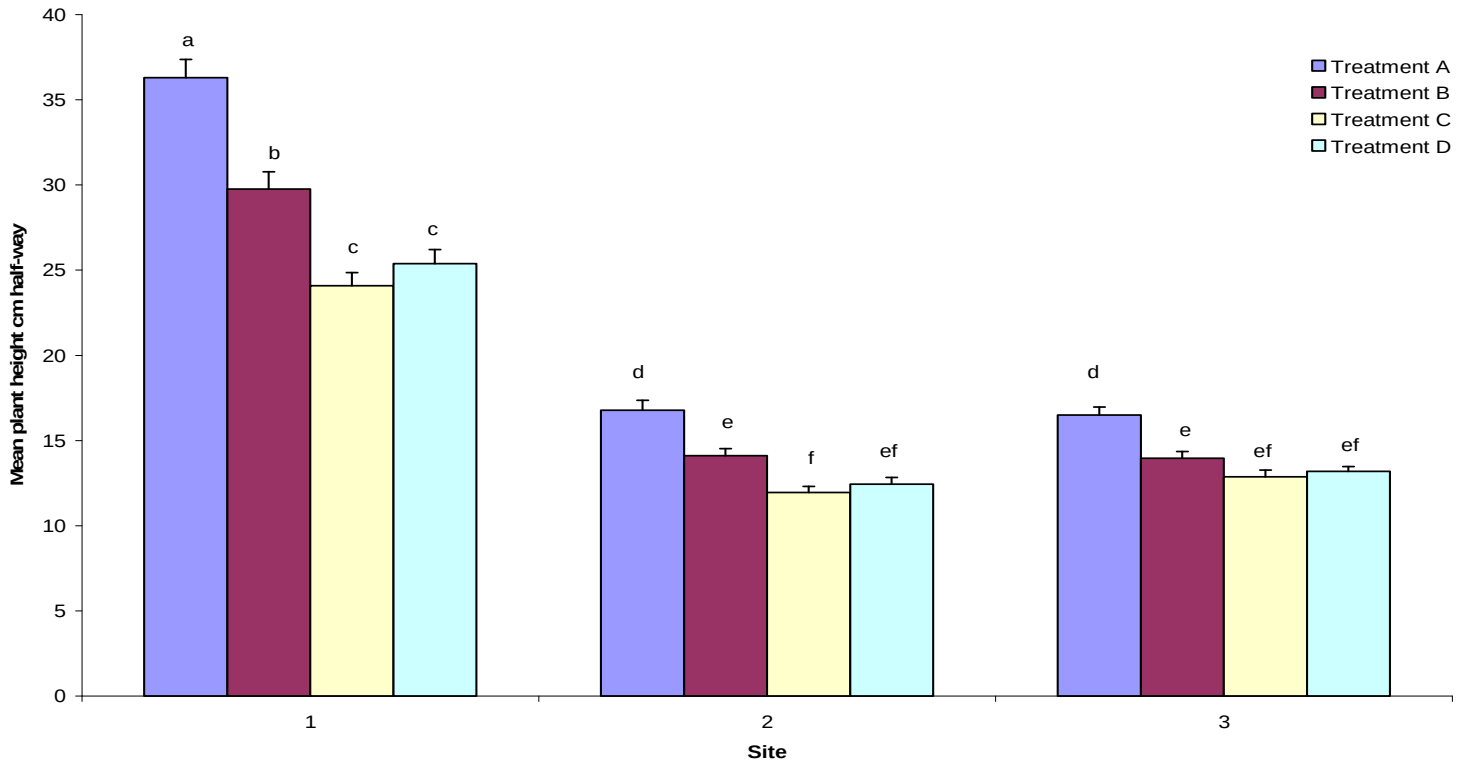

Figure 6.8: Mean ± SE plant height half-way through the experiment from the plants grown on the different treatments from the three sites. Letters above bars indicate significant differences (Tukey test).

Source	d.f.	Seq SS	Adj SS	Adj ms	F	P
Treatment	2	66.97	67.17	33.59	692.91	**<0.001**
Site	3	9.64	9.54	3.18	65.61	**<0.001**
Interaction	6	0.49	0.48	0.081	1.68	0.124
Error	571	27.68	27.68	0.048		
Total	582	104.78				

Table 6.8: Statistical output of the plant height half-way through the experiment from the plants grown on the different treatments from the three sites.

6.4.5.2 Plant height at the end of the experiment

The height of the plants was also recorded at the same time as the harvest of plants, in attempt to quantify 'plant success' the results can be seen in Figure 6.9. There were significant differences between growth treatment (F_{562} = 759.30, P = <0.001), site (F = 40.34, P = < 0.001) and the interaction between them (F_{562} = 3.01, P = 0.007).

The plants grown at site 1 were all significantly taller than those at sites 2 and 3, possibly reflecting the more shady location. At site 1 there was a significant difference between the plants grown on treatments A and B and those from treatments C and D, suggesting that at this site a Zn concentration in the growth media above 400 mg kg^{-1} was having a significant impact on plant height.

The main trends that were identified at the halfway point of the experiment have not changed for sites 2 and 3. However, at site 1 it shows that plants grown on treatment B have now caught up with the plants grown on the control soil. This is possibly suggesting that although plant height is initially decreased in plants grown on soils containing additional Zn, this might have less of an impact as the plant matures.

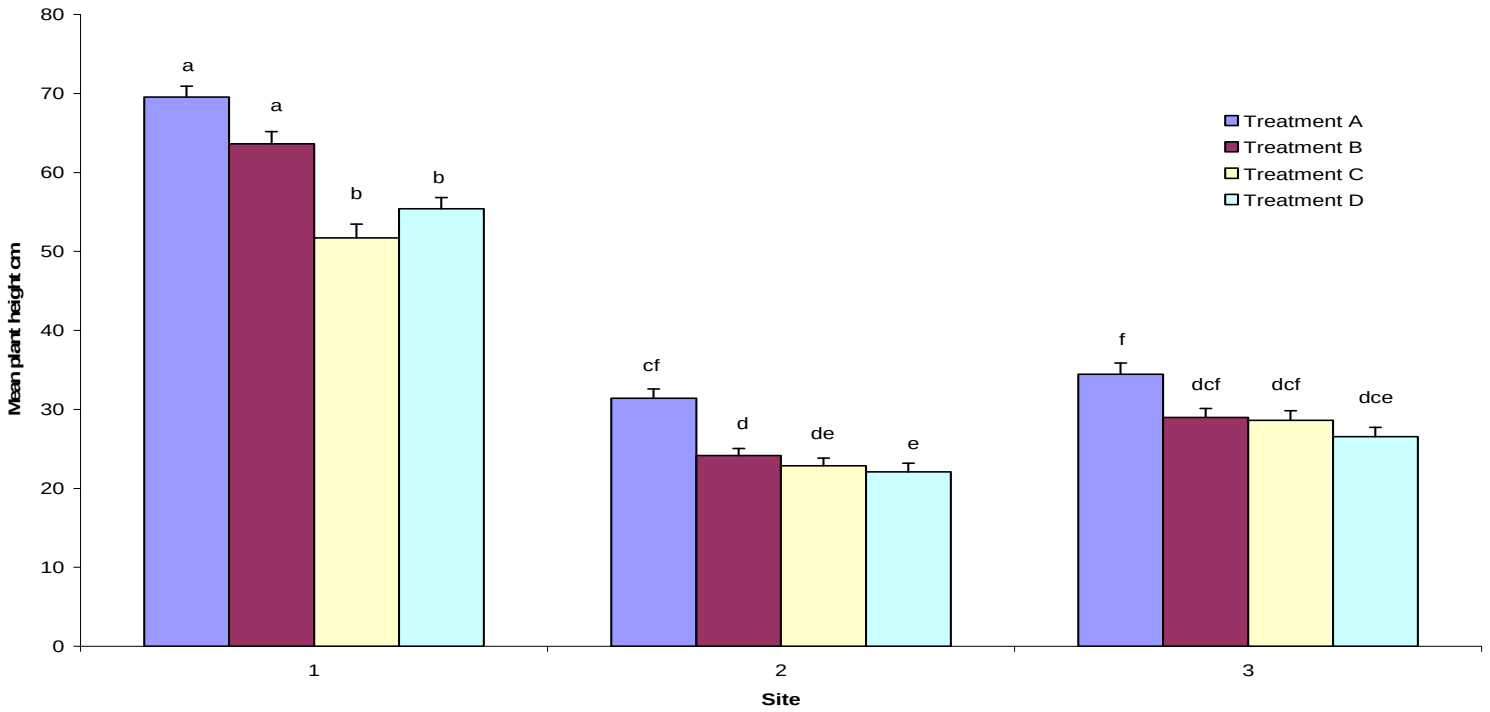

Figure 6.9: Mean ± SE plant height at the end of the experiment from the plants grown on the different treatments from the three sites. Letters above bars indicate significant differences between treatments and sites (Tukeys test).

Source	d.f.	Seq SS	Adj SS	Adj ms	F	P
Treatment	2	843.07	839.68	419.84	759.30	**<0.001**
Site	3	67.83	66.91	22.30	40.34	**<0.001**
Interaction	6	9.972	9.97	1.66	3.01	**0.007**
Error	551	304	304.66	0.553		
Total	562	1225				

Table 6.9: Statistical output of plant height at the end of the experiment from the plants grown on the different treatments from the three sites.

6.4.6 Herbivore count

A day after the half-way damage assessment of the plants, an insect count was conducted of the four main types of insects observed at the site i.e. weevils, leaf beetles, green and black aphids, present on the plants growing on the four different Zn treatments. A total count of all four taxa is shown in Figure 6.10, results for the four different taxa separately is shown in Table 6.6.

There was a significant difference in total insect numbers both between sites (S_2 = 8.00, P = 0.018) and between the treatments (S_3 = 8.20, P = 0.042) that the plants were grown on. The most striking trend occurred at site 1, where plants grown containing the lowest concentration of Zn had the greatest number of insects (118 in total) on the plants, almost twice the number on plants grown on treatment B, and then four times the number on plants from treatment C and D.

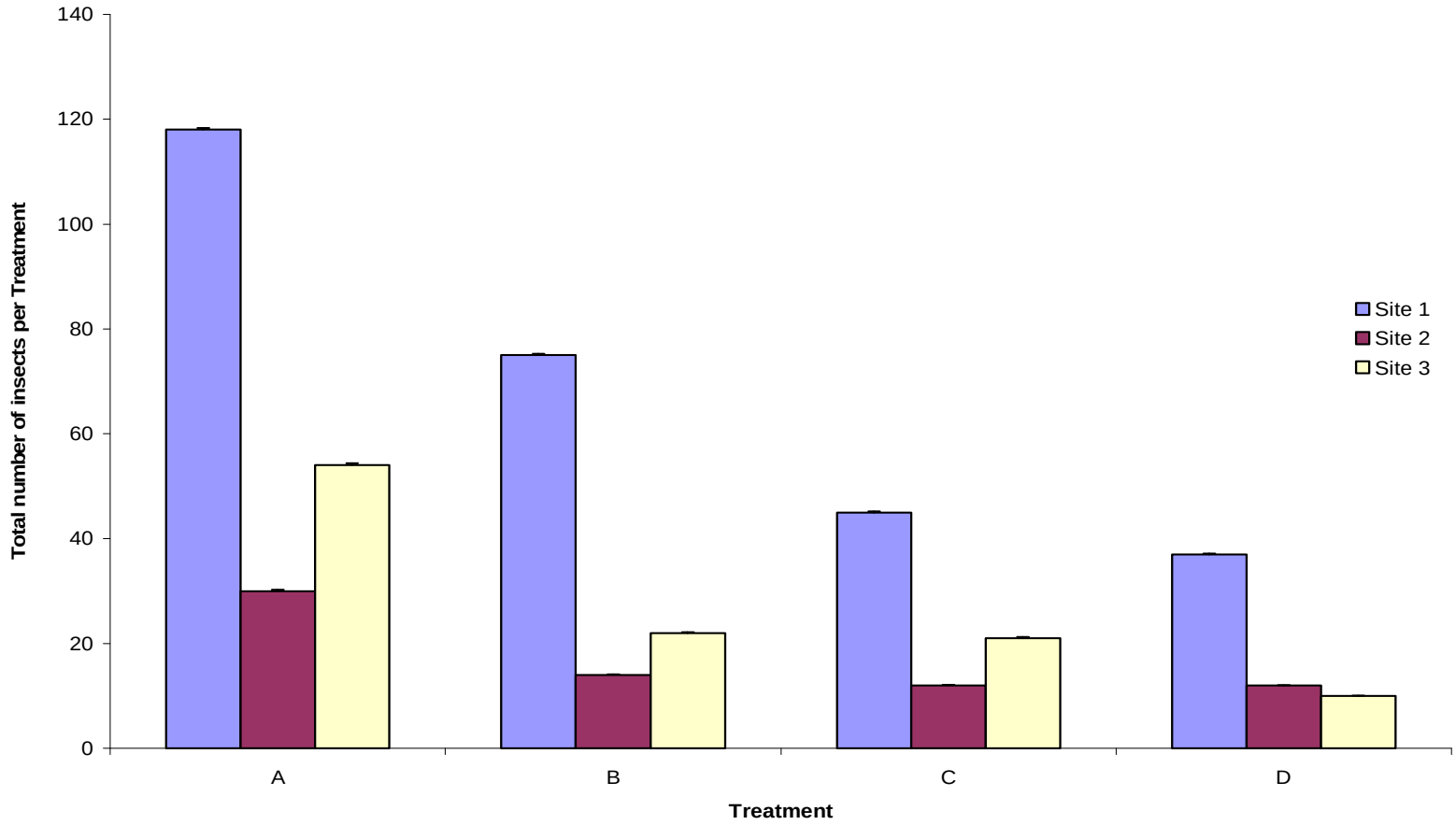

Figure 6.10: Total ± SE number of insects observed on the plants grown on the different Zn treatments at the three sites. Non-parametric Friedman test between sites showed a significant difference $S_2 = 8.00$, $P = 0.018$ as well as between treatment $S_3 = 8.20$, $P = 0.04$.

	Treatment	Treatment A			Treatment B			Treatment C			Treatment D		
	Site	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Herbivore	**Weevil**	15	3	6	8	2	1	2	2	4	4	1	0
	Leaf beetle	54	24	17	48	8	4	30	6	0	22	6	3
	Green aphid	13	0	3	5	0	3	0	0	2	4	1	2
	Black aphid	36	3	28	14	4	14	13	4	15	7	4	5
	Total	118	30	54	75	14	22	45	12	21	37	12	10

Table 6.10: Total number of insects observed on plants grown on the four different treatments (A-D) at the three sites.

6.4.7 Plant nutritional content

6.4.7.1 C:N ratio

The plants grown on treatment A at sites 1 and 3 had a C:N ratio that was significantly (F_2 = 1.41, P = 0.007) lower than plants from the other sites and treatments (Figure 6.11).

6.4.7.2 Nitrogen content

Plants grown on treatment soil A at sites 2 and 3 had significantly (F_2 = 4.83, P = 0.004) lower nitrogen content than all the other plants. This is displayed in Figure 6.12.

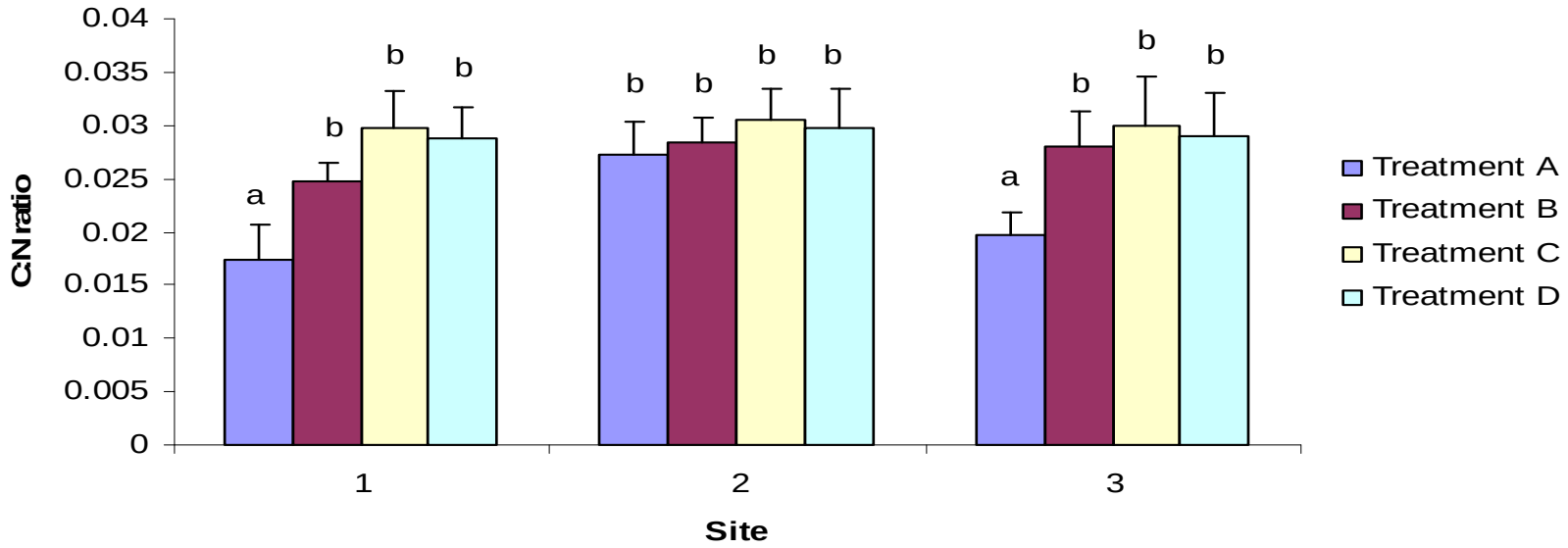

Figure 6.11: Mean ± SE C:N ratio. Different letters above bars indicates significant differences identified by a *post-hoc* Tukeys test.

Source	d.f	Seq SS	Adj SS	Adj MS	F	P
Site	2	2.6×10^{-4}	2.2×10^{-4}	1.1×10^{-4}	1.41	0.250
Soil treatment	3	1.1×10^{-3}	1.0×10^{-3}	3.4×10^{-4}	4.23	**0.007**
Interaction	6	2.3×10^{-4}	2.3×10^{-4}	3.8×10^{-5}	0.48	0.822
Error	96	7.7×10^{-3}	8.0×10^{-3}	8.0×10^{-5}		

Table 6.11: Statistical output of the C:N ratio of the plants grown on the four different treatments at the three sites.

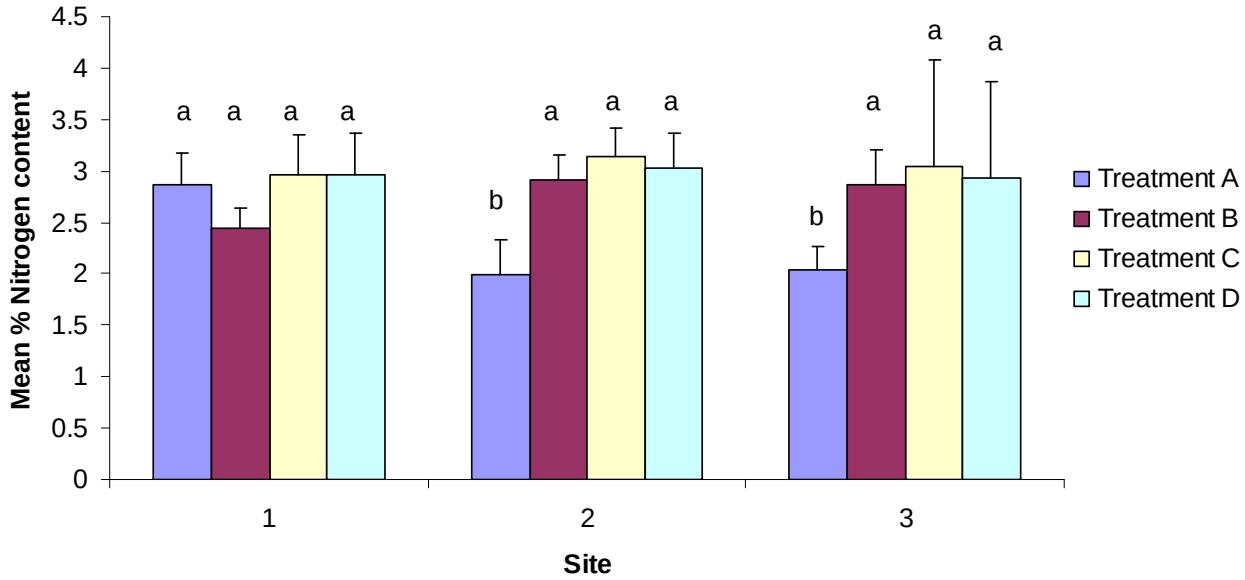

Figure 6.12: Mean (n = 9) ± SE % nitrogen content of leaves. Letters above bars indicate significant differences identified by a *Post-hoc* Tukeys test.

Source	d.f	Seq SS	Adj SS	Adj MS	F	P
Soil treatment	3	1.73	1.58	0.53	4.83	**0.004**
Site	2	0.60	0.56	0.28	2.37	0.081
Interaction	6	0.44	0.44	0.07	0.66	0.679
Error	96	10.50	10.51	0.11		
Total	107	13.27				

Table 6.12: Statistical output of the % nitrogen content of leaves from plants grown on the four treatments at the three sites.

6.5 Discussion

This experiment is the first field based study to compare the effect of Zn on herbivore numbers and damage levels in *B. juncea* with low and high concentrations of Zn. The experiment aimed to test three key predictions which are discussed below.

(i) Plants that have sequestered more Zn will be less damaged by herbivores.

The results from the damage assessment halfway through the experiment were in agreement with the elemental defence hypothesis (Boyd *et al*., 2002; Boyd and Davis, 2008; Butler and Trumble, 2008; Boyd, 2009) as all of the plants grown on the control soil containing no additional Zn were significantly more damaged than plants grown on either of the treatments containing additional amounts of Zn at all of the sites. However, the damage assessment at the end of the experiment saw a reversal in the results obtained, with plants grown on control soils at sites 2 and 3 receiving significantly less damage than those grown on the Zn rich soils. This was not observed in plants growing on site one, reasons for this can be explained in the slight differences between the sites. Sites 2 and 3 have very similar surrounding, in terms of surrounding vegetation and light and wind exposure, but site 1 differed because it was on the edge of the field trials plot, where it received less light, was sheltered by the wind by the surrounding trees and was closer to the vegetation that borders the field. These micro environmental factors commonly affect the outcome of field based experiments (Diamond, 1986;Yates and Peckol, 1993; Calisi and Bentley, 2009). However, as these factors were not measured or recorded throughout the experiment, these factors can not be definitively defined as the cause.

What can be concluded from this damage assessment (at the end of the experiment) is that plants containing elevated concentrations of Zn in the leaf tissue are not less likely to be damaged by herbivores. In fact, it would appear that it may result in the plants becoming more damaged. This result suggests that Zn does not defend plants effectively against herbivore damage in the field, thus not supporting the elemental defence hypothesis. Some of the results suggest that the Zn is affecting *B. juncea* in some way that appears to make it

more appealing to herbivores. Plants that grow on metalliferous soils can have lower concentrations of secondary defence compounds such as glucosinolates (Noret *et al*., 2007), so this may explain why damage levels increase in plants grown on the high Zn treatments. Antonious and Kochlar (2009) screened seven *Brassica* plant species for glucosinolates and found that *B. juncea* contained the highest concentration of the plant species assessed. Therefore, it is plausible that glucosinolate levels (which can be analytically measured) in *B. juncea* plants are affected by an increased uptake of Zn, which in turn affects herbivore preference.

(ii) Insects will be present in fewer numbers on plants that contain a higher Zn concentration than those with a lower concentration.

The insect counts from site 1 do support the elemental defence hypothesis, as those plants with the lowest concentration of Zn in there above ground biomass had the most insects on them. The trend at sites 2 and 3 does seem to mimic that found at site 1 though to a lower magnitude, hence the significant difference found between sites. However, this is very dependent on sap-suckers such as aphids being affected in the same way as leaf chewing herbivores, which is unlikely as different feeding guilds respond differently to different plant defences (White, 2009).

These results support the findings of the first damage assessment whereby plants with enhanced concentrations of Zn in their foliage had been subjected to less herbivory. It should be noted that there was not a second herbivore count when the second damage assessment at the end of the experiment took place. This was because of the logistical reasons associated with conducting a second damage assessment and the harvest of 600 plants within a relative short time frame, so that harvest time was not an additional statistical factor.

(iii) Growth of plants on soils containing varying Zn concentrations should not be affected in terms of nutritional quality (C:N content) and height

It would appear that the growth (height) of *B. juncea* plants grown on four soil treatments containing an increasing amount of Zn were adversely affected by the increasing presence of the metal. It can therefore be proposed that there is a direct cost for *B. juncea* to uptake Zn. Verbrugger *et al* (2009) observed that an increased concentration of Zn in plants above ground material resulted in reduced biomass production, supporting the proposal that there are costs associated with Zn uptake in plants.

By using nitrogen content and the C:N ratio as a proxy for plant nutritional quality it was surprising to obtain results that plants grown on the control soil were the only ones to be significantly lower in nitrogen. However, research conducted by White (1984) found that plants under physical stress such as drought etc actually increase the amount of nitrogen in their above ground biomass. Therefore the increased amount of nitrogen observed in *B. juncea* plants that have been grown on soils containing increased Zn concentrations, could perhaps be a result of the plants coping with the stress of the increased Zn concentrations in the soil.

The amount of nitrogen contained within plant material is of extreme importance to herbivores as it is a key limiting nutrient for them (White, 1976). It is therefore feasible that herbivores consider increased nitrogen content in plant material to be more beneficial than the negative impact of consuming plant material with increased Zn concentrations.

To conclude, it would appear that *B. juncea* may be more susceptible to some types of herbivore attack if grown on a soil containing additional Zn and that these plants will be shorter but nutritionally superior to plants grown on a control soil containing no additional Zn. Although these results go against the fundamentals of the elemental defence hypothesis much more experimentation is required before it can conclusively be said that Zn does not act as defensive property for *B. juncea*. Further experimentation should evaluate the

importance of nitrogen content in plants with the increasing concentration of Zn against the feeding preference and performance of herbivores. In addition to assessing if levels of glucosinolates in *B. juncea* plants is affected with increased concentrations of Zn in their above ground biomass.

Chapter 7: General Discussion

7.1 Introduction

The elemental defence hypothesis was first put forward by Boyd in 1992 and since then a number of researchers (Greville and Morgan, 1991; Hanson *et al*., 2003; Laskowski and Hopkins, 1996; Noret *et al*., 2005; Pollard *et al*., 1994) have attempted to assess its validity. At present there is some evidence for and some against the hypothesis. This is part due to the fact that the elemental defence hypothesis is very plant, metal, herbivore specific with the majority of previous work having been on Ni hyperaccumulating plant species, other metals potentially involved in the elemental defence, such as Zn , have been relatively neglected. Therefore, the principle goal of this thesis was to further our understanding of the possible elemental defensive role of Zn in plants on herbivores, and if possible, to clarify the conflicting results produced by previous researchers on the role of this metal.

The first steps towards conducting this research involved establishing a working plant: metal system. This was first attempted by assessing the potential of *T. caerulescens*, a plant species that had been deemed a model hyperaccumulator of Zn (Baker *et al*., 1994; Brown *et al*., 1995; Krämer, 2000; Lombi *et al*., 2001). However, although *T. caerulescens* displayed remarkable abilities in terms of accumulating Zn into its above ground biomass in both the lab and field, it did have some major drawbacks as a study system. The first of these is the low amount of biomass that was produced over a reasonable growth period, while a second was its ability to sequester such high concentrations of Zn that plants grown on control treatments contained concentrations that far surpassed those of what could be considered a low Zn containing plant. Hence the plant: metal system was re-evaluated and it was decided that the Zn accumulator *B. juncea* (Clemente *et al*., 2005; Ebbs and Kochian, 1998; Irettei and Navari-Izzo, 2006; Kumer *et al*., 1995) was a more suitable experimental system. Results from early experiments confirmed that this plant species could be used to test the elemental defensive properties as it was capable of sequestering Zn concentrations in its foliage beyond those found in typical plant species. Although the concentrations of Zn in *B. juncea* were lower than those found in *T. caerulescens*, *B. juncea* does have several clear advantages over the hyperaccumulating species: plants grown on a control soil have

concentrations similar to 'normal' plant species, and it is a fast growing species that produces large amounts of biomass, which is essential for experiments involving herbivores of moderate size.

Having established a suitable a suitable plant species, the thesis then tested whether an increase in Zn uptake by *B. juncea* was an inducible defence in this plant species by measuring changes in Zn levels following herbivory by the generalist herbivore *H. aspersa*. Following this, the plant species was used to test the impact of Zn levels on the preference and performance of the brassica specialist *P. brassicae*, as well as juvenile and adult *H. aspersa*. The experimental research concludes with a large scale field investigation using *B. juncea*, which appears to be the first such experiment using this plant species and metal.

Chapter 7 first discusses the conclusions of this research in the context of the six hypotheses set out in Chapter 1. It then examines the wider implications of these results in terms of ecological importance and phytoremediation. Finally it suggests further avenues of study that possibly could be employed to continue to advance our understanding of the elemental defence hypothesis.

7.2 Hypothesis 1

The concentration factor of Zn in the above ground biomass of *T. caerulescens*, along with other physiological factors, such as biomass above and below ground, total Zn concentration and root: shoot ratio, will alter significantly when plants are grown in media of varying Zn concentration (Chapter 3).

As predicted by the hypothesis, Zn concentration in the soil did alter the growth of *T. caerulescens*, but surprisingly the maximum biomass obtained was achieved by plants grown on the relatively low Zn concentration of 300 mg kg^{-1}.

An evaluation of the literature to date has found that this is not an isolated result. For example, Ozturk *et al*., (2003), noted that *T. caerulescens* plants grown on soils that did not contain elevated concentrations of Zn produced less biomass, while plants that were grown

with moderate concentrations of Zn produced the most amount of biomass. Therefore, it may be concluded that the plants grown on the soil that contained no additional Zn produced less biomass than plants grown with additional Zn as a result of being Zn deficient. This has been observed by a number of authors (Chaney *et al*., (1997), Shen *et al*., (1997) and Broadley *et al*., (2007)) and presumably reflects the fact that *T. caerulescens* is Zincophilic and only found growing naturally in soils that contain elevated concentrations of Zn (Baker *et al*., 1994).

The highest Zn concentration in *T. caerulescens* plants above ground biomass achieved in the experiment was a moderate 4582 mg kg^{-1}; this is 55% lower than the concentration defined by Baker and Brooks (1989) for a plant species to be classed as a Zn hyperaccumulator (10,000 mg kg^{-1}). However, this disparity has been documented by a number of authors (e.g. Jiang *et al*., (2005), Behmer *et al*., (2005) and Ozturk *et al*., (2003), all of whom found that *T. caerulescens* did not manage to produce Zn concentrations at the hyperaccumulator level in their experiments. This suggests that plants grown under greenhouse conditions are not correctly replicating the natural environment that *T. caerulescens* requires to exhibit its full potential as a Zn hyperaccumulator. There are numerous factors that could be affecting the ability of *T. caerulescens* to bioaccumulate Zn to hyperaccumulator concentrations when grown under artificial conditions. The most prevalent of these factors is soil pH, as any change in this parameter will have both effects directly on the plant and on the availability and mobility of the metal. Another factor that may be limiting Zn accumulation is the speciation of the metal contained within the soil. All of the experiments contained within this thesis artificially contaminated the soil with ZnO (zinc oxide) due to its high mobility. However, in naturally occurring soils metals are rarely found in only one form. It is also possible that due to ZnO being highly mobile, it was physically removed from the soils during routine watering of the plants and hence was not then available to be sequestered by plants.

The bioconcentration factor (BCF) of Zn in plants decreased with soil concentration. Research conducted by both Robinson *et al*., (1998) and Zhao *et al*., (2003) saw a similar decline in BCF, with increasing Zn concentration in the soil. So far two authors have put

forward suggestions as to why the BCF of *T. caerulescens* plants decrease with increase Zn concentrations in the soil. These include body burden, i.e. plants become over saturated with Zn and stop sequestering it from the soil (Pence *et al*., 2000; Liang *et al*., 2009). Liang *et al*., (2009) have gone further to suggest that although body burden plays a role in the phenomenon of decreased BCF in *T. caerulescens* with increasing Zn soil concentration, growth dilution and other elimination processes by plants also play a significant part.

7.3 Hypothesis 2

Zn concentration in *B. juncea* plants will differ significantly between those grown on media that contain additional concentrations of Zn and those grown on a control media. The Zn concentration in both leaf and stems of plants grown on all the treatments will change significantly as the plant grows through time (Chapter 4).

Part of this hypothesis may be accepted, as results from Chapter 4 shows that the Zn concentration in leaves and stems of *B. juncea* significantly differed dependent on the Zn level in the soil the plants were grown on. The concentrations achieved in the plants biomass closely approached the target concentration (2000 mg kg^{-1}) for plant species to be classed as a Zn accumulator, as per the classification set out by Reeves and Baker (2000).

However, part of the hypothesis must be rejected as the Zn concentration did not change significantly over the four week experimental period. It may be concluded that Zn uptake in *B. juncea* takes place early on from plants being transplanted into contaminated soil and then remains relatively constant throughout the plants development. This is an advantage for an experimental plant: metal system with which to test the impact of Zn on herbivores, as the plants reach a relatively stable Zn concentration in a short time period and then maintain this over an additional three weeks of plant growth. This provides confidence that herbivores can be fed leaves from plants will not significantly alter their Zn content over the course of an experiment, at least one that lasts several weeks.

This has been first time that the concentration of Zn uptake in *B. juncea* has been assessed over time to establish how the plant sequesters the metal over time.

7.4 Hypothesis 3

***B. juncea* plants grown on a high Zn media will increase their concentration of Zn in their above ground biomass when exposed to herbivory damage from the generalist herbivore *H. aspersa*, to a greater extent than plants grown on the same media that are not exposed to herbivory. This hypothesis addresses whether the elemental defence mechanism of zinc uptake by *B. juncea* is inducible by herbivore attack (Chapter 4).**

Results from the experiment in Chapter 4 show that herbivory by *H. aspersa* did not induce an increased uptake of Zn in *B. juncea* plants. Therefore, it would appear that the uptake of Zn by *B. juncea* is not an inducible defence and hence this hypothesis must be rejected. It was hypothesised that Zn uptake may be an inducible defence because many other forms of plant defence have been found to be inducible by herbivory and this is thought to make them a more "cost-effective" form of defence (Karbon and Baldwin 1997).

During the 1990's researchers (Coleman and Jones, 1991; Hartley and Lawton, 1991) established that phenolic compounds are inducible in plants by herbivore damage. Arimura *et al*., (2005) states that for a defence to be induced in a plant it is not just the wounding, but also the exposure to elicitors (chemicals) released from herbivores while feeding on plants that induces a defence mechanism. The work by Arimura concludes by saying that *P. brassicae* produces lots of elicitors while feeding, therefore this may have been a more suitable choice of herbivore to induce an increase in Zn uptake than *H. aspersa*. It may then be fairer to conclude that Zn uptake did not exhibit an increased uptake in Zn by the damaging of *B. juncea* by *H. aspersa*. In addition to changing the herbivore species to one that produces more or different elicitors, it may have been more effective to run the experiment over a slightly longer time frame, thus resulting in increased levels of damage to plants.

This is the first time that a researcher has tried to induce an increase in Zn uptake by herbivore damage. Although induction of Zn-based defences was not demonstrated in this

case, if a different herbivore species had been used a very different result may have been achieved.

7.5 Hypothesis 4

Increased Zn concentration in leaves from plants grown on a medium containing additional Zn consumed by selected herbivores will have an impact on their growth and performance, compared with the performance of herbivores fed on leaves from plants grown on a medium not containing any additional Zn (Chapter 5). This hypothesis was tested using juvenile *H. aspersa*, adult *H. aspersa* and *P. brassicae* caterpillars.

The growth of juvenile *H. aspersa* and *P. brassicae* was significantly impeded when reared on a diet of *B. juncea* leaves from plants that had been grown on a soil containing elevated concentrations of Zn. The results show that increased concentrations of Zn in diets of both a generalist (*H. aspersa*) and specialist (*P. brassicae*) herbivores are adversely affected by elevated concentrations of Zn.

The results of the juvenile snails performance is same as that found by Gomot-De Vaulfleury (2000). However, this experiment is an advance over their study as they used an artificial diet, while this experiment used a real plant. Thus, this is first instance of juvenile snails being impacted by Zn in *B. juncea* leaves.

It was also found that the *P. brassicae* caterpillars reared on the high Zn diet contained increased Zn concentrations in their biomass. This emphasizes the potential ecological dangers of using plants to remediate contaminated soils and possible mobilisation and bioaccumulation of contaminants up the food chain (Friesl *et* al., 2006; Nevel *et* al., 2007; Peralta-Videa *et al*., 2009).

Both of the above results reinforce the idea that plant species that are deemed as Zn accumulator species produce metal concentrations that are capable of negatively impacting on herbivores (Coleman, 2005; Iturrade, 2004; Reeves and Baker, 2000).

The performance of adult *H. aspersa* was not affected by Zn containing leaves of *B. juncea*. This may reflect the age of the herbivore; adult herbivores may be less susceptible to the effects of plant defences than juveniles (Hanley *et al*., 2007), possibly because they can sequester the Zn into their shells (Laskowski and Hopkins, 1996).

7.6 Hypothesis 5

Selected herbivores will choose to consume leaves of low Zn concentration over those with high Zn concentrations (Chapter 5). This hypothesis was tested using juvenile *H. aspersa*, adult *H. aspersa* and *P. brassicae* caterpillars.

Adult and juvenile *H. aspersa* along with *P. brassicae* all showed a significant preference for *B. juncea* leaves containing low Zn concentrations, under lab conditions. It raises the question whether herbivores are actually capable of tasting the contaminant and hence able then to choose an alternative.

It is of special interest to note that both adult and juvenile *H. aspersa* had a preference for the low Zn *B. juncea* leaves. As mentioned above, the growth of juvenile *H. aspersa* is adversely effected by an increase in Zn concentration within a diet, so it is not overly surprising that they should opt for the low Zn option. However, adult *H. aspersa* were not affected by a high Zn diet, yet they still had a significant preference for low Zn leaves. This suggests that either snails are capable of 'tasting' the Zn and thus instinctively avoid it, or that it has an adverse impact on an aspect of snail performance other than growth. One possibility is that elevated Zn reduces fecundity, a hypothesis that could be investigated in future research.

These findings contradict those found by Noret *et al*., (2005) who concluded that *H. aspersa* did not show a preference for leaves containing high or low concentrations of Zn. However, Noret conducted the experiment using *T. caerulescens*, which this thesis has shown to not be the most suitable candidate for conducting such experiments due to its ability to sequester high concentrations of Zn in its above ground biomass even at low soil

concentrations. Therefore, it may be concluded that this is the first time to show that *H. aspersa* have a preference for low Zn containing leaves.

Previous work by Pollard *et al*., (1997) found that *P. brassicae* had a preference for low Zn containing leaves of *T. caerulescens*. By combining this finding with the results produced in this thesis it has increased our confidence that Zn uptake by both these plant species has an influence on the feeding preference of *P. brassicae*. This is a particularly interesting result as this particular herbivore is a *Brassicae* specialist, meaning that it has evolved to eat specifically on *Brassicae* species and do its best to circumvent or cope with the defences produced by these plants, therefore a preference for low Zn leaves provides evidence that this defence works against feeding specialists.

7.7 Hypothesis 6

P. brassicae that have consumed a diet of B. juncea leaves from plants grown on soil containing additional Zn, will have a concentration of Zn in their biomass, significantly greater than caterpillars that have consumed *B. juncea* leaves from plants that have been grown without any additional Zn.

The results from chapter five concluded that *P. brassicae* that consumed a diet of *Brassica juncea* leaves that had been grown on a soil that contained additional Zn, had a significantly lower mass after five days, than caterpillars reared on plants that had been grown with no additional zinc in the soil.

This result indicates that potentially there is a ‘cost’ (reduced mass) to the caterpillars that are consuming the diet elevated in Zn. However, due to the fact that caterpillars reared on leaves containing additional Zn, appeared (by visual inspection) to consume approximately the same amount of leaf mass. This could signify that the caterpillars are unaware of the ‘costs’ involved as they did not appear to compensate for the additional Zn in the feed, by consuming more leaf matter. Although, this contradicts the findings from hypothesis 5, were evidence indicated that *P. brassicae* has a preference for low Zn plant material.

7.8 Hypothesis 7

Plants grown to have higher Zn concentrations in their above ground biomass will experience less herbivory when grown in the field than plants containing 'normal' concentrations of Zn in their above ground biomass (Chapter 6). This hypothesis tests whether the elemental defence hypothesis is effective under field conditions.

The results from Chapter six concluded that plants that were grown on soils containing additional concentrations of Zn, subsequently had elevated concentrations of Zn in their above ground biomass actually suffered a significantly higher degree of herbivore damage than those grown on a control soil by end of the experiment. Therefore, this hypothesis must be rejected. It is also a result that goes against the elemental defence hypothesis; however, this is not conclusive evidence against the elemental defence hypothesis, but rather that the results contradict those found from the lab based experiments from this thesis. Discrepancies between herbivore behaviour in lab experiments compared with in the field are not uncommon in ecology (e.g. Valledares & Hartley 1994).

One possible explanation for the results observed in the field trial comes from the Carbon Nitrogen ratio data. The C:N results showed that the plants grown on soils containing elevated concentrations of Zn were actually nutritionally superior to those grown on the control soils. This possibly suggests that herbivores are more concerned with the nutritional quality of plants and the benefits associated with consuming nitrogen rich food, over the negative implications associated that with consuming higher concentrations of Zn. Some researchers (Davis and Boyd, 2000; Noret, 2005; Tolrà *et al*., 2001) have also suggested that secondary plant defences such as glucosinolates have more of an influence on herbivore preference than plant metal concentration. Alstyne *et al*., (2009) demonstrated that herbivores will opt for plants that have higher nitrogen content even if they contain higher concentrations of plant chemical defences. However, this seems the first documented research to demonstrate that plant nutritional quality may be a more important factor than metal concentration in plants in terms of herbivore preference in the field.

Other factors such as site location had a significant effect on the results. In the field, environmental factors such as shade, shelter from the wind and amount of sunlight can influence the outcome of experiments testing the elemental defence hypothesis, making them harder to interpret than lab experiments where conditions are more controlled.

7.9 What impact may these findings have for phytoremediation?

Fully understanding the elemental defence hypothesis is central to our ability to correctly implement phytoremediation as a viable and safe option for land remediation.

One very positive outcome for phytoremediation that has come from this research, has been the further demonstration that *B. juncea* is a high biomass producing, fast growing plant species capable of coping with moderate concentrations of Zn in soil. Furthermore, the results obtained in Chapter 4, showed that Zn uptake in this species occurs shortly after transplantation into Zn impacted soils. Hence, it would be possible to conduct many harvests in a year, thus theoretically removing Zn more rapidly compared to Zn hyperaccumulators which are typically slow growing low biomass producing species.

However, the fact remains that at present our limited knowledge and understanding of the elemental defence hypothesis, means that phytoremedation is still a potential pathway for the movement of contaminants both into the environment and foodchain. Such movement has been observed in experiments carried out in this thesis, which saw a significant increase of the Zn concentration in the pupae of *P. brassicae* reared on an elevated Zn diet. Until this fundamental principle and potential implications are fully understood phytoremedation should remain as a purely experimental technique.

7.10 Suggestions for future work

Although this thesis has answered a number of questions on the elemental defence qualities of Zn in *B. juncea* and *T. caerulescens*, it has also highlighted many additional questions.

Altering the plant nutritional quality:

In the final experimental chapter the research suggested that the nutritional quality of the plants had an impact on herbivore preference. However, the results were not conclusive enough to definitely establish if this factor over shadows the effects of metal concentration in plants above ground biomass in shaping herbivore preference. Therefore, a combination of lab and field based experiments should be conducted in a similar vein to those contained within this thesis, although an additional factor, that of plant nutrient content should be integrated. The nutritional quality of plants could be altered using either a slow release nutrient compound in the soil or a soluble based nutrient supplement, provided to the plants at varying concentrations. By including this additional factor it would be possible to fully establish the combined effects of Zn and nutrient concentration in plants on herbivore preference.

Increasing the range of herbivores used in experiments:

The plant: metal system that has been established in thesis appears to be relatively robust in terms of producing *B. juncea* leaves with varying concentrations of Zn. The lab based research has focused on two herbivore species, the generalist *H. aspersa* and the *Brassicae* specialist *P. brassicae*. Although a marked achievement in terms of the results that were obtained, increasing the number of herbivore species used in preference and performance experiments would strengthen our understanding of the elemental hypothesis and better establish its generality. The type of herbivore species that could be used would be ones that have a different feeding action, as the herbivores experimented on so far are both leaf chewers. Herbivores such as leaf miners and aphids exhibit a different mode of feeding to leaf chewers, therefore Zn may have a different impact on these herbivores in terms of performance and preference.

Using a soil containing a range of metals:

A limited number of researchers have conducted experiments where by plants have been transplanted into contaminated soil containing a range of heavy metals. However, few if any have used this soil in greenhouse based experiments which have then involved herbivores. The advantage of conducting such research as it may overcome the problems

that have so far plagued researchers in terms of replicating metal concentrations that have been found in plant species growing in the field. For example, soils which are high in Zn are often high in other heavy metals such as in lead, cadmium and copper from (mainly historic) industrial activities such as lead smelting (Kwan-Rae and Owens *et al*., 2009). Thus experiments using a combination of metals in the soil would produce results closer to a real world scenario, while at the same time benefiting from the controls that can be introduced through lab based experiments, for example the use or exclusion of a specific herbivore.

7.11 Conclusions

To conclude, *T. caerulescens* is a remarkable plants species capable of growing on soils heavily contaminated with Zn (>35,000 mg kg^{-1}). However, due to its high Zn affinity even in soils that contain nominal concentrations of Zn it hyperaccumulates the metal into its biomass at concentrations above those found in 'normal' plants species, it is an extremely atypical species, perhaps not best suited for testing the generality of the elemental defence hypothesis.

B. juncea is a suitable Zn accumulating plant species for testing whether the elemental defence hypothesis applies to accumulator species with moderate concentrations of Zn. In addition, as a potential candidate species for phytoremediation, the findings may be relevant to applied settings.

The herbivores *H. aspersa* and *P. brassicae* have a feeding preference for plant material that do not contain elevated concentrations of Zn. The growth of young herbivores of both of these species is adversely impacted by increased concentrations of Zn.

However, under field conditions, other conditions such as plant nutrient content, site location and plant size impact on herbivore feeding behaviour, so the effects of Zn on food selection are less clear cut.

This thesis has made significant progress towards understanding the elemental defence characteristics of Zn in plants and its impact on herbivores, although many questions relating to the effectiveness of elemental defence, particularly under field conditions, remain.

References:

Alkorta I., Hernandez-Allica J., Garbisu S. (2004). 'Plants against the global epidemic of arsenic poisoning'. *Environment International*, **30**, 949-951.

Alstyne K.L., Pelletreau K.N. and Kirby A. (2009). 'Nutritional preferences override chemical defenses in determining food choice by a generalist herbivore, Littorina sitkana'. *Journal of Environmental Marine Biology and Ecology*, **379**, 85-91.

Anderberg (2008) *http://linnaeus.nrm.se/flora/di/brassica/thlas/thlacae4.jpg*.

Anderson C.W.N., Brooks R.R., Stewart R.B. and Simcock R. (1998). 'Harvesting a crop of gold in plants'. *Nature*, **395**, 553-554.

Anderson C.W.N., Brooks R.R., Chiarucci A., LaCoste C.J., Leblanc M., Robinson B.H., Simcock R. and Stewart R.B. (1999). 'Phytomining for nickel, thallium and gold'. *Journal of Geochemical Exploration*, **67**

Angle J.S., Baker A.J.M., Whiting S.N. and Chaney R.L. (2003). 'Soil moisture effects on uptake of metals by *T. caerulescens*, *Alyssum* and *Berkheya*. *Plant and Soil*, **256**, 325-332.

Antonious G.F. and Kochlar T.S. (2009). 'Mobility of heavy metals from soil into hot pepper fruits: a field study'. Environmental Contamination Toxicology, **82**, 59–63.

Augner, M. (1995). 'Low nutritive quality as a plant defence: effects of herbivore-mediated interactions'. *Evolution Ecology*, **9**, 1–12.

Argyraki A., Ramsey M. H. and Potts P. J. (1997). 'Evaluation of portable X-ray fluorescence instrumentation for in situ measurements of lead on contaminated land'. *Analyst*, **122,** 743-749.

Arimura G., Kost C. and Bolad W. (2005). 'Herbivore induced, indirect plant defences'. *Biochemical Biophysical Molecular Cell Biology*, **1734**, 91-111.

Assunção A.G.L., Bookum W.M. and Neilssen H.J.M. (2003). 'Differential patterns among *T. caerulescens* populations originating from different soil types'. *New Phytologist*, **159**, 411-419.

Baker A.J.M. (1981). 'Accumulation and excluders – strategies in the response of plants to heavy metals'. *Journal of Plant nutrition*, **3**, 643-654.

Baker A.J.M. (1987). 'Metal tolerance'. *New Phytologist*, **106**, 93-111.

Baker A.J.M. and Brooks R.R. (1989). 'Terrestrial higher plants which hyperaccumulate metallic elements – A review of their distribution, ecology and phytochemistry'. *Biorecovery*, **1**, 81-126.

Baker A.J.M. and Walker P.L. (1989). 'Ecophysiology of metal uptake by tolerant plants.' In *Heavy Metal Tolerance in Plants: Evolutionary Aspects.* Edited by Shaw A.J. Boca Raton: CRC Press, 155-177.

Baker A.J.M and Procter J. (1990). 'The influence of cadmium, copper, lead and zinc on the distribution and evolution of metallophytes in the British Isles'. *Plant Systematics and Evolution*, **173**, 91-108.

Baker A.J.M., Proctor J., van Balgooy M. and Reeves R.D. (1993). 'Hyperaccumulation of nickel by flora of the ultramafics of Palawan, republic of the Phillippines'. In *The Vegetation of Ultramafic (Serpentine) Soils. Proceedings of the First International Conference on Serpentine Ecology*. Andover, Hampshire: Intercept Ltd, UK, 291-304.

Baker A.J.M., Reeves R.D. and Hajar A.S.M. (1994). 'Heavy metal accumulation and tolerance in British populations of the metallophyte *T. caerulescens*. J. & C. Presl (*Brassicaaceeae*). *New Phytologist*, **127**, 61-68.

Baker A.J.M and Whiting S.N. (2002). 'In search of the Holy Grail – A further step in understanding metal hyperaccumulation'. *The New Phytologist*, **155**, 1-4.

Banuelos G.S., Vickerman D.B., Trumble J.T., Shannon M.C., Davis C.D., Finley J.W. and Mayland H.F. (2002). 'Biotransfer possibilites of selenium from plants used in phytoremedation'. *International Journal of Phytoremediation*, **4**, 315-331.

Barak P. and Helmke P.A. (1994). 'Zinc in soils and plants'. *Development in Plant and Soil Science*, **55**, 1-13.

Behmer S.T., Lloyd C.M. and Raubenheimer D. (2005). 'Metal hyperaccumulation in plants : Mechanisms of defence against insect herbivores'. *Functional Ecology*, **19**, 55-66.

Bert V., McNair M.R., Delaguerie P., Saumitou-Laprade P. and Petite D. (2000) 'Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri* (*Brassicaceae*). *New Phytologist*, **146**, 225-233.

Bert V., Bonnin I. and Saumitou-Laprade P. (2002). 'Do *Arabidopsis halleri* from nonmetallicolous populations accumulate zinc and cadmium more effectively than those from metallicolous populations?'. *New Phytologist*, **155**, 47-57.

Bert V., Meerts P., Saumitou-Laprade P., Salis P., Gruber W. and Verbruggen N. (2003). 'Genetic basis of Cd tolerance and hyperaccumulation in *Arabidopsis halleri*'. *Plant and Soil*, **249**, 9-18.

Bilos C., Colombo J.C. and Skorupka C.N. (2001). 'Sources, distribution and variability of airborne trace metals in La Plata City area, Argentina'. *Environmental Pollution*, **111**, 149-158.

Boruvka L., Kozak J. and Kritoufkova S. (1997). 'Heavy metal accumulation in plants grown in heavily polluted soils'. *Journal of Folia Microbiologica*, **24**, 524-526.

Boyd R.S and Martens S.N. (1992). 'The raison d'etre for metal hyperaccumulation by plants'. In Baker A.J.M., Procter J., Reeves R.D., eds. *The vegetation of Ultramafic (Serpentine) soils*. Andover, UK: Intercept, 279-289.

Boyd R.S., Shaw J., Martens S.N. (1994). 'Nickel hyperaccumulation defends *Streptanthus polygaloides* (Brassicaceae) against pathogens'. *American Journal of Botany*, **81**, 294-300.

Boyd R.S. and Martens S.N. (1994). 'Nickel hyperaccumulated by *Thlaspi-montanum* is actively toxic to insect herbivore'. *Olkos*, **70**, 21-25.

Boyd R.S and Martens S.N. (1998). 'Nickel hyperaccumulation of *Thlaspi montanum* a constitutive trait'. *American Journal of Botany*, **85**, 259-265.

Boyd R.S. and Moar W.J. (1999). 'The defensive function of Ni in plants: Response of the polyphagous herbivore *Spondoptera exigui* to hyperaccumulator and accumulator species of *Streptanthus brassicae*'. *Oecologica*, **118**, 218-224.

Boyd R.S. (2004). 'Ecology of metal hyperaccumulation'. *New Phytologist*, **162**, 563-567.

Boyd R.S., Davis M.A., Wall M.A. (2002). 'Nickel defends the South African hyperaccumulator Senecio coronatus (Asteraceae) against Helix aspersa (Mollusca: Pulmonidae)'. *Chemoecology*, **12**, 91-97.

Boyd R.S., Davis M.A. and Wall M.A. (2007). 'Host herbivore studies of *Stenoscepa* (*Orthoptera pygromorphide*), a high Ni herbivore of the south African Ni accumulator *Berkheya coddii*'. *Insect Science*, **14**, 133-143.

Boyd R.S. (2006). 'A test of elemental defence against slugs by Ni in hyperaccumlator and non-hyperaccumulator species'. *Chemoecology*, **15**, 179-185.

Boyd R.S. (2007). 'The defence hypothesis of elemental hyperaccumulation: Status, challenges and new directions'. *Plant and Soil*, **293**, 153-176.

Boyd R.S. and Davis M.A. (2008). 'Elemental patterns in Ni hyperaccumulating and non-hyperaccumulating ultramafic soil populations of *Senecio coronatus*'. *South African Journal of Botany*, **74**, 158-162.

Boyd R.S. (2010). 'Heavy metal pollutants and chemical ecology: Exploring new frontiers'. *Journal of Chemical Ecology*, **36**, 46-58.

Butler C.D. and Trumble J.T. (2008). 'Effects of pollutants on bottom-up and top-down processes in insect–plant interactions'. *Environmental Pollution*, **156**, 1-10.

Broadley M.R., Escobar-Gutierrez A.J. and Burns A. (2000). 'What are the effects of nitrogen deficiency on growth components of lettice?'. *New Phytologist*, **147**, 519-526.

Broadley M.R., White P.J., Hammond J.P., Zelko I. and Lux A. (2007). 'Zinc in plants'. *New Phytologist*, **173**, 677-702.

Brooks R., Lee J., Reeves R.D. and Jaffre T. (1977). 'Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants'. *Journal of Geochemical Exploration*, **7**, 49-77.

Brooks R.R. (1998). '*Plants that hyperaccumulate heavy metals*'. Wallingford, UK: CAB International.

Brown S.L., Chaney R.L., Angle J.S. and Baker A.J.M. (1995). 'Zinc and cadmium uptake by hyperaccumulator *Thlaspi caerulescens* and metal tolerant *Silene vulgaris* grown on sludge amended soils'. *Environmental Science Technology*, **29**, 1581-1585.

Calisi R.M. and Bentley G.E. (2009). 'Lab and field experiments: Are they the same animal'. *Hormones and Behavior*, **56**, 1-10.

Chaney R.L., Malik M., Li Y.M., Brown S.L., Angle J.S. and Baker A.J.M. (1997). 'Phytoremediation of soil metals'. *Current Opinion in Biotechnology*, **8**, 279-284.

Ciccu R., Ghiani M., Serci A., Fadda S., Peretti. R. and Zucca A. (2003). 'Heavy metal immobilization in the mining-contaminated soils using various industrial wastes'. *Minerals Engineering*, **16**, 187-192.

CL:AIRE (2005). 'CL:AIRE Research Project Report: RP6 Phytoextraction of metals: Investigation of hyperaccumulation and field testing'. Contaminated Land: Applications in Real Environments, UK.

Clemens S. (2006). 'Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants'.

Clemente R., Walker D.J. and Bernal P. (2005). 'Uptake of heavy metals and As by *B. juncea* grown in a contaminated soil in Aznalcollar (Spain): The effect of soil amendments'. *Environmental Pollution*, **138**, 46-58.

Cobbett C. (2003). 'Heavy metals and plants – model systems and hyperaccumulators'. *The New Phytologist*, **159**, 289-293.

Coleman, J. S. and Jones C.G. (1991). Plant stress and insect herbivory: toward an integrated perspective. - In: Mooney, H. A., Winner, W. E. and Pell, E. J. (eds), Response of plants to multiple stresses. Academic Press, San Diego, CA, pp. 249-280. Jones, C. G. and Hartley, S. E. 1998. Global.

Coleman C.M., Boyd R.S. and Eubanks M.D. (2005). 'Extending the elemental defence hypothesis: Dietry metal concentrations below hyperaccumulator levels could harm herbivores'. *Journal of Chemical Ecology*, **31**, 1669-1681.

Cosio C., Martinoia E. and Keller C. (2004). 'Hyperaccumulation of cadmium and zinc in *T. caerulescens* and *Arabidopsis halleri* at the leaf cellular level'. *Plant Physiology*, **134**, 716-725.

Cui Y., Wang Q., Dong Y., Li H. and Christe P. (2004). 'Enhanced uptake of soil Pb and Zn by Indian mustard and winter wheat following combined soil application of elemental sulphur and EDTA'. *Plant and Soil*, **261**, 181-188.

Cunningham S.D., Berti W.R. and Huang J.W. (1995). 'Phytoremediation of contaminated soil'. *Tibtech*, **13**, 393-397.

Dallinger R., Berger B. and Gruber A. (1993). 'Quantitative aspects of zinc and cadmium binding in *Helix* pomatia, differences between essential and non-essential and non-essential trace elements'. *Ecotoxicology of Metals Invertebrates*, Book series: Setac special publication series, 315-332.

Davis M.A and Boyd R.S. (2000). 'Dynamics of Ni base defence and organic defences in the Ni hyperaccumulator, *Streptanthus polygaloides* (*Brassicaceae*). *The New Phytologist*, **146**, 211-217.

Dechamps C., Lefebvre C. and Noret N. (2007). 'Reaction norms of the life history traits in response to zinc in *Thlaspi caerulescens* from metalliferious and nonmetal liferous sites'. *New Phytologist*, **173**, 191-198.

Dechamps C., Noret N. and Mozaek R. (2008). 'Cost of adaptation to metalliferious environment for *Thlaspi caerulescens*: A field reciprocal transplantion approach'. *New Phytologist*, **177**, 167-177.

DEFRA (2008) *Guidance on the legal definition of contaminated land*.

DEFRA and EA (2002a). *SGV3 Soil guideline values for cadmium contamination*. Environment Agency, Bristol.

DEFRA and EA (2002b). The contaminated land exposure assessment (CLEA) model: Technical basis and algorithms (CLR10). Environment Agency, Bristol.

DEFRA and EA (2002c). *SGV7 Soil guideline values for nickel contamination*. Environment Agency, Bristol.

DEFRA and EA (2002d). *SGV9 Soil guideline values for selenium contamination*. Environment Agency, Bristol.

DEFRA and EA (2002e). *SGV10 Soil guideline value for lead contamination*. Environment Agency, Bristol.

DETR (Department of the Environment, Transport and the Regions) (2000). *Environmental Protection Act 1990: Part IIA. Contaminated Land Circular 02/2000*. The Stationery Office, PO Box 29, Norwich NR3 1GN.

Diamond J.M. (1986). 'The environmentalist myth'. *Nature*, **324**, 19-20.

Dyer M. (2003). 'A field trial for *in-situ* bioremediation of 1,2 DCA'. *Engineering Geology*, **70**, 315-320.

EA (Environment Agency) (2004). *Integrated Pollution Prevention and Control (IPPC) - Guidance on the Protection of Land Under the PPC Regime: Surrender Site Report (Consultation Draft Version 1.0) (Technical Guidance Note IPPC H 8)*, pp. 29. Environment Agency, Bristol.

Ebbs S.D. and Kochian L.V. (1997). 'Toxicity of Zinc and copper to *Brassica* species: Implications for phytoremediation'. *Journal of Environmental Quality*, **26**, 776-781.

Ebbs S.D. and Kochian L.V. (1998). 'Phytoextraction of Zn by oat (*Avena sativa*), barley (*Holdeum vulgare)*, and Indian mustard (*Brassica juncea*)'. *Environmental Science and Technology*, **32** 802-806.

Emsley J. (2001). 'Natures Building Blocks: An A-Z guide to the elements'.

EPA (Environmental Protection Agency US). (2001). 'Phytoremediation Resource Guide'. U.S. Environmental Protection Agency, Office of Solid Waste and Emergency Response Technology Innovation Office, Washington, DC 20460.

Ernst W.H.O. (1972). 'Zinc and cadmium pollution of soils and plants in vicinity of a zinc smelting plant'. *Berichte Der Deutschen Botanischen Gesellschaft*, **85**, 295-300.

Ernst W.H.O. (1987). 'Perennial herbs as monitor for moderate levels of metal fall-out'. *Chemosphere*, **16**, 233-238.

Ernst W.H.O., Schat H. and Verkleij J.A.C. (1990). 'Evolutionary biology of metal resistance in *Silene vulgaris*'. *Evolutionary Trends in Plants*, **4**, 45-51.

Ernst W.H.O. (2000). 'Evolution of metal hyperaccumulation and phytoremediation'. *New Phytologist*, **146**, 357-358.

Farago M.E. and Cole M.M. (1988). 'Nickel and plants'. *Metal ions and biological systems*, **23**, 47-90.

Feeny, P. (1970). 'Seasonal changes in the oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars'. Ecology, ***51***, *565–581*.

Feltwell J.S.E. (1979). 'Carotenoids of *Pieris Brassicae* and of its food plant'. *Journal of insect Physiology*, **18**, 2203-2215.

Freeman D.C., Brown M.L. and Duda J. (2005). 'Leaf fluctuating asymmetry, soil disturbance and plant stress: a multiple year comparison using two herbs, *Ipomoea pandurata* and *Cnidoscolus stimulosus*'. *Ecological Indicators*, **5**, 85-95.

Freeman J.L., Quinn C.F. and Marcus M.A. (2006). 'Selenium tolerant diamond moth disarms hyperaccumulator plant defence'. *Current Biology*, **16**, 2181-2192.

Freeman J.L. Lindblom S.D. and Quinn C.F. (2007). 'Selenium accumulation protects plants from herbivory by *Orthoperta* via toxicity and deterrence'. *New Phytologist*, **175**, 490-500.

Freot H., Petit C., Lefebvre C., Gruber W., Collin C., and Escarre J. (2003). 'Zinc and cadmium accumulation in controlled crosses between metallicolous and nonmetallicolous populations of *T. caerulescens*'. *New Phytologist*, **157**, 643-648.

Frey B., Keller C., Zierold K. and Schulin R. (2000). 'Distribution of Zn in functionally different leaf epidermal cells of the hyperaccumulator *T. caerulescens*. *Plant Cell and Environment*, **23**, 675-687.

Friesl W., Friedl J., Platzer K., Horak O. and Gerzabek M.H. (2006). 'Remediation of contamination agricultural soils near a former Pb/Zn smelter in Austria: Batch, pot and field experiments'. *Environmental Pollution*, **144**, 40-50.

Gabrielli R., Mattioni C. and Vergnano O. (1991). 'Accumulation mechanisms and heavy metal tolerance of a nickel hyperaccumulator'. *Journal of Plant Nutrition*, **14**, 1067-1080.

Galeas M.L., Klamper E.M. and Benett L.E. (2008). 'Selenium hyperaccumulation reduces plant arthropod loads in the field'. *New Phytologist*, **177**, 715-724.

Glass D.J. (1999). 'Phytoremediation applications – An emerging market for the introduction of specialty crops and transgenic crops'. *Genetic Engineering News*, **18**, 17.

Gomot A. and Pihan F. (1996). 'Comparison of the bioaccumulation capacites of copper and zinc in two snail subspecies (*Helix*)'. *Ecotoxicology and Environmental Safety*, **38**, 85-94.

Gomot-De Vaulfleury A. (2000). 'Standardized Growth Toxicity Testing (Cu, Zn, Pb, and Pentrachlorophenol) with *H. aspersa*'. *Ecotoxicology and Environmental Safety*, **46**, 41-50.

Greville R.W. and Morgan A.J. (1991). 'A comparison of (Pb, Cd and Zn) accumulation in terrestrial slugs maintained in microcosms: Evidence for metal tolerance'. *Environmental Pollution*, **74**, 115-127.

Haines B.J. (2002). 'Zincophilic root foraging in *T. caerulescens*'. *New Phytologist*, **155**, 363-372.

Han F.X., Kingery W.L., Selim H.M. and Gerald P. (2000). 'Accumulation of heavy metals in a long-term poultry waste-amended soil'. *Soil Science*, **165**, 260-268.

Han F.X., Kingery W.L. and Selim H.M. (2001). 'Accumulation, redistribution, transport and bioavailability of heavy metals in waste-amended soils'. *Trace Elements in Soil*, 141-168.

Han F.X., Banin A., Su Y., Monts D.L., Plodinec M.J., Kingery W.L. and Triplett G.E.(2002). 'Industrial age anthropogenic inputs of heavy metals into the pedosphere'. *Naturwissenschaften*, **89**, 497-504.

Hanley M.E., Lamont B.B. and Meredith M. (2007). 'Plant structural traits and their role in anti-herbivore defence'. *Perspectives in Plant Ecology, Evolution and Systematics*, **8**, 157-178.

Hanson B., Graifullina G.F., Lindblom S.D., Wangeline A., Ackley A., Kramer K., Norton A.P., Lawrence C.B. and Pilon-Smits E.A.H. (2003). 'Selenium accumulation protects *B. juncea* from invertebrate herbivory and fungal infection'. *New Phytologist*, **159**, 461-469.

Hanson B., Lindblom S.D., Loeffer M.L., and Pilon-Smits E.A.H.(2004). 'Selenium protects plants from phloem-feeding aphids due to both deterrence and toxicity'. *New Phytologist*, **162**, 453-459.

Hartley S.E. and Lawton. (1987). 'Effects of different types of damage on the chemistry of birch folage and the responses of birch feeding insects'. *Oecologia*, **74**, 432-437.

Hartley S.E. and Jones. (1989). 'Phenolic biosynthesis, leaf damage and insect herbivory in birch (*Betula pendula*)'. *Journal of Chemical Ecology*, **45**, 275-283.

Hartley S.E. and Lawton. (1991). 'Host plant manipulation by gall insects – A test of the nutrition hypothesis'. *Journal of Animal Ecology*, **61**, 113-119.

Hodge A., Robinson D. and Fitter A. (2000). 'Are microorganisms more effective than plants at competing for nitrogen'. *Trends in Plant Science*, **5,** 304-308.

ICRCL (Inter-departmental Committee on the Redevelopment of Contaminated Land). (1987). 'Guidance on the assessment and redevelopment of contaminated land'. ICRCL paper 59/83, Department of the Environment London.

Ingrouille M.J. and Smirnoff N. (1996). '*Thlaspi caerulescens* J. & C . Presl (*T. alpestre* L.) in Britain'. *New Phytologist*, **102**, 219-233.

Irtelli B. and Navari-Izzo F. (2006). 'Influence of sodium nitriotriacetate (NTA) and citric acid on phenolic and organic acids in *B. juncea* grown in excess cadmium'. *Chemosphere*, **65**, 1348-1354.

Jiang R.F., Ma D.Y., Zhao F.J., and McGrath. (2005). 'Cadmium hyperaccumulation protects *Thlaspi caerulescens* from leaf feeding damage by thrips *(Frankliniella occidentalis*)'. *New Phytologist*, **167**, 805- 814.

Jhee E.M., Dandridge K.L., Christy A.M.Jr and Pollard A.J. (1999). 'Selective herbivory on low zinc phenotypes of the hyperaccumulator *T. caerulescens* (*Brassicaceae*)'. *Chemoecology*, **9**, 93-95.

Jhee E.M., Boyd R.S. and Eubanks M.D. (2006). 'Effectiveness of metal-metal and metal organic compound combinations against *Plutella xylostella*: Implications for plant elemental defence'. *Journal of Chemical Ecology*, **32**, 239-259.

Kabata-Pendias, A. (2001). 'Trace Elements in Soils and Plants, 3rd ed. CRC Press LLC, Boca Raton.

Karbon and Baldwin. (1997). 'Induced responses'. *HE*.

Knight B., Zhao F.J., Mcgrath S.P. and Shen Z.G. (1997). 'Zinc and cadmium uptake by the hyperaccumulator *Thalspi caerulescens* in contaminated soils and its effects on the concentration and chemical speciation of metals in soil solution'. *Plant and Soil*, **197**, 71-78.

Krämer U., Cotter-Howelis J.D., Charnock J.M., Baker A.J.M and Smith J.A.C. (1996) 'Free histidine as a metal chelator in plants that accumulate nickel'. *Nature*, **379**, 635-638.

Krämer U., Smith R.D. and Wensel W.W. (1997). 'The rate of metal transport and tolerance in nickel hyperaccumulation by *Thlaspi goesingense hanlacsy*'. *Plant Physiology*, **115**, 1641-1650.

Krämer U. (2000). 'The use of transgenic plants in the bioremediation of soils contaminated with trace elements'. *Applied Microbiology Biotechnology*, **55**, 661-672.

Krämer U. (2003). 'Phytoremediation to phytochelatin – plant trace metal homeostasis'. *New Phytologist*, **158**, 1-9.

Krämer U. (2005). 'Phytoremediation: Novel approaches to cleaning up polluted soils'. *Current Opinion in Biotechnology*, **16**, 133-141.

Kruckeberg A.L. and Wu L. (1992). 'Copper tolerance copper accumulation of herbaceous plants colonizing inactive California copper mines'. *Ecotoxicology and Environmental Safety*, **23**, 307-319.

Kumar P.A.B.N., Dushenkov S., Motto H. and Raskin I. (1995). 'Phytoextraction: The use of plants to remove heavy metals from soils'. *Environmental Science and Technology*, **29**, 1232-1238.

Kupper H., Zhao F.J. and McGrath S.P. (1999). 'Cellular compartmentation of zinc in leaves of the hyperaccumulator *T. caerulescens*. *Plant Physiology*, **119**, 305-311.

Kwan-Rae K. and Owens G. (2009). 'Chemodynamics of heavy metals in long-term contaminated soil: Metal speciation in soil solution'. *Journal of Environmental Sciences*, **11**, 1532-1540

Laskowski R. and Hopkin S.P. (1996). 'Accumulation of Zn, Cu, Pb and Cd in the garden snail (*H. aspersa*): Implications for predators'. *Environmental Pollution*, **91**, 289-297.

Lee J., Reeves R.D. and Brooks R.R. (1977). 'Relation between nickel and citric acid in some nickel accumulating plants'. *Phytochemistry*, **17**, 1033-1035.

Liang H., Lin T., Chiou J. and Yeu J. (2009). 'Model evaluation of the phytoextraction potential of heavy metal hyperaccumulators and non-hyperaccumulators'. *Environmental Pollution*, **157**, 1945-1952.

Lombi E., Zhao F.J., Dunham S.J. and McGrath S.P. (2001). 'Phytoremediation of heavy metal-contaminated soils: Natural hyperaccumulation versus chemically enhanced phytoextraction. *Environmental Quality*, **30**, 1919-1926.

Marchiol L., Sacco P., Assolari S. and Zerbi G. (2004). 'Reclamation of polluted soil: Phytoremediation potential of crop-related *Brassica*, species'. *Water, Air, and Soil Pollution*, **158**, 345-356.

Markert B. (2003). ' Plants as Biomarkers'. Weinheim VCH Press.

Martens S.N. and Boyd R.S. (1994). 'The ecological significance of nickel accumulation: a plant chemical defence'. *Oecologia*¸ 98, 379-384.

Martens S.N. and Boyd R.S. (2002). 'The defensive role of Ni hyperaccumulation by plants : A field experiment'. *American Journal of Botany*, **86**, 998-1003.

Massey F.P. and Hartley S.E. (2007). 'Herbivore specific induction of silica based environmental plant defences'. *Oecologica,* **152**, 677-683.

Massey F.P. and Hartley S.E. (2009). 'Impacts of silica based defences in grasses on the feeding preferences of sheep'. *Basic and Applied Ecology*, **10**, 622-630.

McGrath S.P. (1993). 'The potential for the use of metal accumulating plants for the *in-situ* decontamination of metal polluted soils'. *Intergrated Soil and Sediment Research*, **673**.

McGrath S.P., Dunham S.J. and Correll R.L. (2000). 'Potential for phytoextraction of zinc cadmium from soils using hyperaccumulator plants'. In Norman T. ed. *Phytoextraction of contaminated soil*. Boca Raton, FL, USA, CRC Press, 189-198.

McGrath S.P., Zhao F.J. and Lombi E. (2002). 'Phytoremediation of metals, metalloids, radionuclides'. *Advances in Agronomy*, **75**, 1-56.

McGrath S.P. and Zhao F.J. (2003). 'Phytoextraction of metals and metalloids from contaminated soils'. *Current Opinion in Biotechnology*, **14**, 277-282.

McGrath S.P. (2005). 'An inter-laboratory study to test the availability of amendments to reduce the availability of Cd, Pb and Zn *in-situ*'. *Environmental Pollution*, **138**, 34-45.

McGrath, S.P., Lombi, E., Gray, C.W., Caille, N., Dunham, S.J. and Zhao, F.J. (2006). 'Field evaluation of Cd and Zn phytoextraction potential by the hyperaccumulators *Thlaspi caerulescens* and *Arabidopsis halleri*. *Environmental Pollution*, **141**, 115-125.

Menta C. and Parisi V. (2001). 'Metal concentrations in *Helix pomatia*, *H. aspersa* and *Arion rufus*: A comparative study'. *Environmental Pollution*, **115**, 205-208.

Meerts P. and Van Isacker N. (1997). 'Heavy metal tolerance and accumulation in metallicolous and non-metallicolous populations of *T. caerulescens* from continental Europe'. *Plant Ecology*, **133**, 221-231.

Minitab (1996) Minitab for windows, release 14. Minitab Inc., State College, Pennsylvania.

Mobin M. and Khan N. (2006). 'Phytosynthetic activity, pigment composition and antioxidative response of two mustard (*B. juncea*) cultivars differing in photosynthetic capacity subjected to cadmium stress'. *Journal of Plant Physiology*.

Moore J.N. and Luoma S.N. (1990). 'Hazardous wastes from large scale metal extraction – A case study'. *Environmental Science and Technology*, **24**, 1278.

Morrison R.S., Brooks R.R. and Reeves R.D. (1980). 'Nickel uptake by *Alyssum* species'. *Plant Science Letters*, **17**, 451-457.

Mulligan C.N. (2005). 'Environmental applications for biosurfactants'. *Environmental Pollution*, **133**, 183-198.

Nascimento A. and Xing B. (2006). 'Comparison of natural organic acids and synthetic chelates at enhancing phytoextraction of metals from a multi-metal contaminated soil'. *Environmental Pollution*, **140**, 114-123.

Nevel L., Mertens J., Oorts K. and Verheyen K. (2007). Phytoextraction of metals from soils: How far from practice?'. *Environmental Pollution*, **150**, 34-40.

Newman M.A. (2002). 'Prior exposure to lipopolysaccharide potentiates expression of plant defenses in response to bacteria'. *Plant Journal*, **29**, 487-495.

Nicholson F.A., Smith S.R., Alloway B.J.,Carlton-Smith C. and Chambers B.J.(2003). 'An inventory of heavy metals inputs to agricultural soils in England and Wales'. *The Science of the Total Environment*, **311**, 205-219.

Nicks L.J. and Chambers M.F. (1995). 'Farming for metals'. *Mining Environment.*

Noret N., Meerts P., Tolera R., Poschenrieder C., Barcelo J. and Escarce J. (2005). 'Palatability of *T. caerulescens* for snails: Influence of zinc and glucosinolates'. *New Phytologist*, **165**, 763-772.

Noret N., Josens G. and Escarre J. (2005). 'Development of *Issoria lathonia* on zinc accumulating and non-accumulating *Viola* species'. *Environment Toxicology and Chemistry*, **26**, 565-571.

Noret N., Meerts P. and Vanhaelen M. (2007). 'Do metal rich plants deter herbivores? A field test of the elemental defence hypothesis'. *Oecologica,* **152**, 92-100.

Notten M.J.M., Oosthoek A.J.P., Rozema J. and Aerts R. (2006). 'The landsnail *Cepaea nemoralis* regulates internal Cd levels when fed on Cd enriched stinging nettle (*Urtica dioica*) leaves at low, field relevant concentrations'. *Environmental Pollution*, **139**, 296-305.

Orru R., Sannia M., Cincotti A. and Cas G. (1999). 'Treatment and recycling of zinc hydrometallurgical wastes by self-propagating reactions'. *Chemical Engineering Science*, **54**, 3053-3061.

Ozturk L., Karanlik S., Ozkutlu F., Cakmak I. and Kochian L.V. (2003). 'Shoot biomass and zinc/cadmium uptake for hyperaccumulator and non-accumulator *T. caerulescens*species in response to growth on a zinc deficient calcareous soil'. *Plant Science*, **164**, 1095-1101.

Pence N.S., Larson P.B., Ebbs S.D., Letham D.L.D, Lasat M.M., Garvin D.F., Eide D. and Kochian L.V. (2000). 'The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *T. caerulescens*. *Proceedings of the National Academy of Sciences*, **97**, 4956-4960.

Peralta-Videa J.R., Gardea-Torresdey J.L., Gomez E., Tiemann K.J., Parsons J.G and Carrillo G. (2009) 'Effect of mixed cadmium, copper, nickel and zinc at different pHs upon alfalfa growth and heavy metal uptake'. *Environmental Pollution*, **119**, 291-301.

Peterson P.J. (1971). 'Unusual accumulations of elements by plants and animals'. *Science Progress (Oxford)*, **59**, 505-536.

Philips D.J.H. (1977). 'The use of biological indicator organisms to monitor trace metal pollution in marine and estuarine environments', *Environmental Pollution*, **13**, 281-317.

Pliner P. (1982). 'The effects of mere exposure on liking for ediable substances'. *Appetite*, **3**, 283-290.

Podar D., Ramsey M.H. and Hutchings M.J. (2004). 'Effect of cadmium, zinc and substrate heterogeneity on yield, shoot metal concentration and metal uptake by *B. juncea*: Implications for human health risk assessment and phytoremediation'. *New Phytologist*, **163**, 313-324.

Pollard A.J. and Baker A.J.M. (1997). 'Deterrence of herbivory by zinc hyperaccumulation in *T. caerulescens* (*Brassicaceae*)'. *The New Phytologist*, **135**, 655-658.

Pollard A.J., Dandridge K.L. and Jhee E.M. (2000). 'Ecological genetics and evolution of the trace element hyperaccumulation in plants'. *Phytoremediation of contaminated soil and water*, 251-264.

Pollard A.J., Powell K.D., Harper F.A. and Smith J.A.C. (2002). 'The genetic basis of metal hyperaccumulation in plants'. *Critical Reviews in Plant Sciences*, **21**, 539-566.

Quartacci M.F., Argilla A., Baker A.J.M. and Navari-Izzo F. (2006). 'Phytoextraction of metals from a multiply contaminated soil by Indian mustard'. *Chemosphere*, **63**, 918-925.

Raskin I., Kumar P.B.A., Dushenkov S. and Salt D.E. (1994). 'Bioconcentration of heavy metals by plants'. *Current Opinion in Biiotechnology*, **5**, 285-290.

Raskin I., Kramer U. and Smith R.D. (1997). 'Phytoremediation and mechanism of metal accumulation in plants'. *Plant Physiology*, **114**, 1253.

Raskin I., Smith R.D. and Salt D.E. (1997). 'Phytoremediation of metals: Using plants to remove pollutants from the environment'. *Current Opinion in Biotechnology*, **8**, 221-226.

Redfern. (2008). mgnaturalsonline.com/images/Brassica%20juncea.jpg

Reeves R.D. (1981). 'Nickel uptake by *Calfornia streotanthus* and *caulanthus* with particular reference to the hyperaccumulator *S-polygaloides* grey (*Brassicaceae*)'. *American Journal of Botany*, **68**, 708.

Reeves R.D. and Baker A.J.M. (1984). 'Studies on metal uptake by plants from serpentine and non-serpentine populations of *Thlaspi*'. *New Phytologist*, **98**, 191-204.

Reeves R.D. and Brooks R.R. (1983). 'European species of *T. caerulescens* L. (Cruciferae) as indicators of nickel and zinc'. *Journal of Geochemical Exploration*, **18**, 275-283.

Reeves R.D., Baker A.J.M., Borhidi A., and Berazain R. (1999). 'Nickel hyperaccumulation in the serpentine flora of Cuba'. *Annals of Botany*, **83**, 29-38.

Reeves R.D. and Baker A.J.M. (2000). 'Metal-accumulating plants'. In Raskin I. Ensley B.D, eds. *Phytoremediation of toxic metals: Using plants to clean up the Environment*. New York , NY,USA, John Wiley & Sons, 193-229.

Roberts R.D. and Johnson M.S. (1978). 'Dispersal of heavy metals from abandoned mine workings and their transference though the terrestrial food chains'. *Environmental Pollution*, **16**, 293-310.

Robertson J.M., Hubuck K.T. (1992). 'Developmental responses to drought and abscisic acid in sunflower roots'. *Journal of Experimental Botany*, **41**, 325-337.

Robinson B., Leblanc M., Petit D., Brooks R., Kirkman J. and Gregg P. (1998). 'The potential of *T. caerulescens* for phytoremediation of contaminated soils'. *Plant and Soil*, **203**, 47-56.

Rock S.A. (1997). 'Land remediation and pollution control' In Freeman H (Ed). The standard handbook of hazardous waste treatment and disposal, second ed. McGraw Hill, New York.

Roosens N.H., Verbruggen N., Meerts P., Ximenez-Embun P. and Smith J.A.C. (2003). 'Natural variation in cadmium tolerance and its relationship to metal hyperaccumulation for seven populations of *T. caerulescens* from western Europe. *Plant Cell and Environment*, **26**, 1657-1672.

Saison C., Schwart C and Morel JL. (2004). 'Hyperaccumulation of metals by *T. caerulescens* as affected by root development and Cd-Zn/Ca-Mg interactions'. *International Journal of Phytoremediation*, **6**, 49-61.

Salt D.E., Blaylock M. and Kumar N.P.B.R. (1995). ' Phytoremediation – A novel strategy for the removal of toxic metals from the environment using plants'. *Biotechnology*, **13**, 468-474.

Schroeder L.A. (1986). 'Changes in tree leaf quality and growth performance of Lepidoptera larvae'. *Ecology*, **67**, 1628-1636.

Schwartz C., Morel J.L., Saumier S., Whiting S.N. and Baker A.J.M. (1999). 'Root development of the zinc hyperaccumulator plant *T. caerulescens* as affected by metal origin, content and localization in soil'. *Plant and Soil*, **208**, 103-115.

Severne B.C. and Brooks R.R. (1972). 'Nickel accumulating plant from western Australia'. *Planta*, **103**, 91.

Severne B.C. (1974). 'Nickel accumulation by *Hybanthus floribundus*'. *Nature*, **248**, 807-808.

Shen Z.G., Zhao F.J. and McGrath S.P. (1997). 'Uptake and transport of zinc in the hyperaccumulator *T. caerulescens* and the non-hyperaccumulator *T. caerulescens ochroleucum*'. *Plant Cell and Environment*, **20**, 898-906.

Sinha S., Sinam G., Mishra R.K. and Mallick S. (2010). 'Metal accumulation, growth, antioxidants and oil yield of *Brassica juncea* L. exposed to different metals'. *Ecotoxicology and Environmental Safety*, in Press.

Smith M.L. (1996). 'Examination of methods for evaluating remining a mine waste site'. *Engineering Geology*, **43**, 23-30.

Speiser D.M., Abrahamson S.L., Banuelos G. and Ow D.W. (1992). '*B. juncea* produces a phytochelatin-cadmium-sulfide complex'. *Plant Physiology*, **99**, 817-821.

Sun Q., Ye Z.H., Wang X.R. and Wong M.H. (2005). ' Increase of glutathione in population of *Sedium alfredi*: A Zn hyperaccumulator and Pb accumulator'. *Phytochemistry*, **66**, 2549-2556.

Susarla S., Medina V.F. and McCutchean S.C. (2002). 'Conference information: International symposium on the ecology of post-mining landscapes Mar 1999, Brandeburg Uni Tech, Germany'. *Ecological Engineering*, **18**, 647-658.

Thompson M. (1982). 'Regression Methods in the Comparison of Accuracy'. *Analyst*, **107,** 1169-1180.

Thompson M. and Walsh J.N. (1983). *A handbook of inductively coupled plasma spectrometry*. Blackie and Son Ltd, Glasgow.

Tolcin A.C. and McNair B.J. (2007). Mineral Industries Surverys.

Tolrà R.P., Poschenrieder C., and Barcelo S. (1996). 'Zinc hyperaccumulation in *T. caerulescens*, influence on growth and mineral nutrition'. *Journal of Plant Nutrition*, **19**, 1531-1540.

Tolrà R.P., Gomis M., Barcelo J. and Poschenrieder C. (1998). 'Influence of metal hyperaccumulation on glucosinolate content'. *Plant Mineral Nutrition*, 471-476.

Tolrà R.P., Poschenrieder C., Alonso B., Barcelo D. and Barcelo J. (2001). 'Influence of zinc hyperaccumulation on glucosinolates in *Thlaspi caerulescens*'. *New Phytologist*, **151**, 621-626.

Valladres G.R., and Hartley S.E. (1994). 'Effects of scale on detecting interactions between *Coleophora* and *Eriocrania* leaf-miners', *Ecology*, **19**, 257- 262.

Valentine J.F. and Heck K.L. (2001). 'The role of leaf nitrogen content in determining turtlegrass (*Thalassia testudinum*) grazing by a generalized herbivore in the northern Gulf of Mexico'. *Journal of Experimental Marine Biology and Ecology*, **258**, 65-86.

Vamerali T., Bandiera M. and Mosca G. (2010). 'Filed crops for phytoremediation of metal contaminated land: A review'. *Environmental Chemistry Letters*, **8**, 1-17.

Van Nevel L., Mertens J., Oorts K. and Verheyen K. (2007). 'Phytoextraction of metals from soils: How far from practice?' *Environmental Pollution*, **150**, 34-40.

Verbrugger N., Hermans C. and Schat H. (2009). 'Molecular mechanisms of metal hyperaccumulation in plants'. *New Phytologist*, **181**, 759-776.

Viard B., Pihan F., Promeyrat S. and Pihan J. (2004). 'Intergrated assessment of heavy metal (Pb, Zn, Cd) highway pollution: Bioaccumulation in soils Graminaceae and land snails'. *Chemosphere*, **55**, 1349-1359.

White T.C.R. (1976). 'Weather, food and plagues of locust'. *Oecologica*, **22**, 119-134.

White T.C.R. (1984). 'The abundance of invertebrate herbivores in relation to the availability of nitrogen in stressed food plants'. *Oecologica*, **63**, 90-105.

White T.C.R. (2009). 'Plant vigour versus plant stress: A false dichotomy'. *Olkos*, **118**, 807-808.

Whiting S.N. (1998). 'Metal mobilization and uptake by the zinc hyperaccumulator *T. caerulescens* J. & C. Presl. PhD thesis. Sheffield, UK, University of Sheffield.

Whiting S.N., Leake J.R., McGrath S.P. and Baker A.J.M. (2001). 'Zinc accumulation by *Thalspi caerulescens* from soils with different Zn availability: A pot study'. *Plant and Soil*, **236**, 11-18.

Wu S.C., Cheung K.C., Luo Y.M. and Wong M.H. (2006). 'Effects of inoculation of plant growth-promoting rhizobacteria on metal uptake by *B. juncea*'. *Environmental Pollution*, **140**, 124-135.

Yates J.L. and Peckol P. (1993). 'Effects of nutrient availability and herbivory on polyphenolics in the seaweed *Fucus vesiculosus*'. *Ecology*, **74**, 1757-1766.

Zhao F.J., Lombi E. and McGrath S.P. (2003). 'Assessing the potential for zinc and cadmium phytoremediation with the hyperaccumulator *T. caerulescens*. *Plant and Soil*, **249**, 37-43.

`