Quantification of in situ heterogeneity of contaminants in soil: a fundamental prerequisite to understanding factors controlling plant uptake.

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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.....(Jacqueline Yvette Thomas)

UNIVERSITY OF SUSSEX

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ABSTRACT

Heterogeneity of contaminants in soils can vary spatially over a range of scales, causing uncertainty in environmental measurements of contaminant concentrations. Sampling designs may aim to reduce the impact of on-site heterogeneity, by using composite sampling, increased sample mass and off-site homogenisation, yet they could overlook the small scale heterogeneity that can have significant implications for plant uptake of contaminants.

Moreover, composite sampling and homogenisation may not be relevant to target receptor behaviour, e.g. plants, and studies, using simplistic models of heterogeneity have shown that it can significantly impact plant uptake of contaminants. The alternative approach, to accept and quantify heterogeneity, requires further exploration as contaminant heterogeneity is inevitable within soils and its quantification should enable improved reliability in risk assessment and understanding variability in plant contaminant uptake.

This thesis reports the development of a new sampling design, to characterise and quantify contaminant heterogeneity at scales, from 0.02m to 20m, using *in situ* measurement techniques, and 0.005m to 0.0005m, using *ex situ* techniques. The design was implemented at two contaminated land sites, with contrasting heterogeneity based upon historic anthropogenic activity and showed heterogeneity varying between contaminants and at different spatial scales, for Pb, Cu and Zn.

Secondly, this research demonstrates how contaminant heterogeneity measured *in situ* can be recreated in a pot experiment, at a scale specific to the plant under study. Results, from 4 different plant species, demonstrated that existing simplistic models of heterogeneity are an inadequate proxy for plant performance and contaminant uptake under field conditions, and significant differences were found in plant contaminant concentrations between simplistic models and those based upon actual site measurements of heterogeneity. Implications of heterogeneity on plant roots were explored in the final experiment showing significant differences in root biomass between patches of differing contaminant concentrations.

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Abbreviations

Contaminants:

As	arsenic
Cd	cadmium
Cr	chromium
Cu	copper
EDTA	ethylenediaminetetraacetic acid
Hg	mercury
Ni	nickel
Pb	lead
Zn	zinc

Related to data quality and analysis:

ANOVA	Analysis of variance
CRM	Certified reference material
HRM	House reference material
NIST	National Institute of Standards and Technology
RSD	Relative standard deviations
s^2_{anal}	Analytical variance
s^2_{meas}	Measurement variance
s^2_{samp}	Sampling variance

Other abbreviations:

AAS	Atomic absorption spectroscopy
EA	Environment Agency
ICP – MS	Inductively coupled plasma – Mass Spectroscopy
P XRF	Portable x-ray fluorescence
XMP	X-ray microprobe

GLOSSARY

analyte

"the compound measured"
(Horwitz, 1990)

bias

"The difference between the expectation of the test result and an accepted reference value. Note: Bias is a measure of the total systemic error as contrasted to random error. There may be one or more systematic error components contributing to this bias. A larger systematic difference from the accepted value is reflected by a larger bias value."
(ISO, 1993b)

bioavailability

"is the fraction of the chemical that can be absorbed by the body through the gastrointestinal system, the pulmonary system and the skin."
(Great Britain. Dept. for Environment and Rural, 2002)

However more relevant to this thesis and for organisms that inhabit soils and sediments:

"as that which is freely available to cross an organism's cellular membrane from the medium the organism inhabits at a given time. Once transfer across the membrane has occurred, storage, transformation, assimilation, or degradation can take place within the organism; however, these processes are obviously distinct from the transfer between the medium (e.g., soil) and the organism." (Semple *et al.*, 2004)

Certified Reference Material. (CRM)

"reference material, accompanied by a certificate, one or more of whose property values are certified by a procedure which establishes traceability to an accurate realisation of the unit in which the property values are expressed and for which each certified value is accompanied by an uncertainty at a stated level of confidence."
(ISO, 1993a)

chlorosis.

A loss of normal green colour of the plant. Colours may be uniform pale green, brown spotted, yellowish over entire leaf, or yellowish only between leaf veins.

contaminant

"A substance which is in, on or under the land and which has the potential to cause harm or to cause pollution of controlled water."
(DETR, 2000)

contaminated land

"...any land which appears to the local authority in whose area it is situated to be in a condition, by

reasons of substances in, on or under the land, that :

(a) significant harm is being caused or there is a possibility of such harm being caused;

or

(b) pollution of controlled waters is being, or is likely to be caused"

(Part IIA of the Environmental Protection Act 1990) (DETR, 2000)

dry weight. The equilibrium weight of the solid particles (plant or soil) after water has been vaporised and no further change is mass is recorded.

duplicate sample "One of the two (or more*) samples or sub-samples obtained separately at the same time by the same sampling procedure or sub-sampling procedure."

(Ramsey and Ellison, 2007)

excluder A plant that is able to regulate the flow of potentially harmful metals into sensitive areas of the plant. Baker (1981)

fit for purpose "The degree to which data produced by a measurement process enables a user to make technically correct decisions for a stated purpose." (Thompson and Ramsey, 1995)

growing medium A prepared replacement for soil containing nutrients, water and air necessary in the environment for plant and root growth.

heavy metals. Those metals of high atomic weight having densities greater than 5 mg/m^3. Many heavy metals are toxic when accumulated into animal bodies. The more common ones of concern are arsenic, cadmium, chromium, copper, beryllium, lead, manganese, mercury, nickel, and zinc among many others.

Homogeneity, heterogeneity "The degree to which a property or a constituent is uniformly distributed throughout a quantity of material.

Notes:

(1) A material may be homogeneous with respect to one analyte or property but heterogeneous with respect to another.

(2) The degree of heterogeneity is the determining factor of sampling error." (IUPAC, 1990)

Hyper-accumulator Plants which take up metals into plant shoots at concentrations that are substantially higher than

	other plants, for which there is usually a specific concentration for each element. Baker (1981)
Indicator	A plant that will tolerate a range metals at elevated concentrations, until a threshold level is reached resulting in chlorosis. Baker (1981)
In situ	On site or in its original location, i.e. an *in situ* analytical technique, analyses the concentration of a contaminant in its original location
Intake dose	"is the amount of a chemical entering or contacting the human body at the point of entry (that is, mouth, nose, or skin) by ingestion, inhalation or skin contact. Actual intake will be a function of the chemical characteristics and the nature of the target population and their behaviour patterns. Intake dose is expressed in terms of mass of substance per kg body weight over a period of time (for example, mg kg-1 bw day-1)." (Great Britain. Dept. for Environment and Rural, 2002)
morphological plasticity (in plant roots)	Changes in root biomass, root length, and/or number of lateral roots in response to patches of differing quality, either contaminant or nutrient concentrations.
nutrient patch	area within growing medium where nutrient concentrations are greater than background.
patch contrast	the degree to which contaminant or nutrient concentrations differ between adjoining patches within growing media.
phyto-management	"describes the manipulation of soil-plant systems to affect the fluxes of trace elements in the environment with the goal of remediating contaminated soils, recovering valuable metals, or increasing micronutrient concentrations in crops." (Robinson *et al.*, 2009)
phytomining	use of plants to extract trace elements from low grade ore. (Robinson *et al.*, 2009)
phytoremediation	The use of plants to decontaminate polluted land, water, or air. (Hine and Martin, 2004)
phytotoxic	Harmful or poisonous to plants. (Park, 2007)

precision	"The closeness of agreement between independent test results obtained under stipulated conditions. *Note:* *(1) Precision depends only on the distribution of random errors and does not relate to the true value or the specified value.* *(2) The measure of precision usually is expressed in terms of imprecision and computed as a standard deviation of the test results. More precision is reflected by a lower standard deviation. (3) 'Independent test results' means results obtained in a manner not influenced by any previous result on the same or similar test object. Quantitative measures of precision depend critically on the stipulated conditions. Repeatability and reproducibility conditions are particular sets of extreme stipulated conditions.*" (ISO, 1993b)
sampling location	"The place where sampling occurs within the sampling target. Perhaps used for location within which duplicate (or replicate) samples are taken at sampling points." (Eurochem)
sampling point	"The place where sampling occurs within the sampling location, perhaps used for point where duplicate (or replicate) sample taken within a sampling location" (Eurochem)
sampling scale	Distance between two duplicate sampling points within the same sampling location.
sampling target	"Portion of material, at a particular time, that the sample is intended to represent. (AMC, 2005)
taproot	"a straight tapering root growing vertically downwards and forming the centre from which subsidiary rootlets spring" (Oxford Dictionary online, 2005)
translocate	"transport (a dissolved substance) within an organism, especially in the phloem of a plant, or actively across a cell membrane." (Oxford Dictionary online, 2005)

Chapter 1 Introduction

1.1 Background to research

With increased global industrialisation and urbanisation the number of **contaminated land** sites, with potentially harmful trace metals, continues to rise. It is estimated that 3 million sites are contaminated in Europe alone and of those, forty percent of the **contaminants** fall within the **heavy metal** category (EEA, 2007). The harmful effects to human health from exposure to heavy metals are well documented, and in the UK, a series of SGV (Soil Guideline Values) reports, from the Environment Agency, provide comprehensive reviews of sources, behaviour in the environment and toxicological data for a range of trace metals and other harmful contaminants. One of the exposure routes, for potentially harmful trace metals, to enter the food chain, is from the consumption of plants growing on contaminated soils. Heavy metals can enter plant cells through both passive and non-passive uptake mechanisms, and depending upon species, be stored in below ground tubers or trans-located to aerial fractions available for consumption by both animals and humans. The same plant uptake mechanisms, that pose a potential risk from trace metals, may also provide a possible solution to remediation. There is considerable research surrounding the key soil based factors affecting plant uptake of trace metals, e.g. pH, cation exchange capacity and organic matter content, and a comprehensive review can be found in Kabata Pendias & Pendias (2000). A key factor that has potentially been underestimated is the spatial **heterogeneity** of contaminants and the scale of the heterogeneity in relation to the target receptor.

To assess the potential risks, or to develop strategies for remediation of contaminated land sites, it is essential to understand the spatial distribution of the contaminants within the soil. Concentrations and spatial distribution of contaminants can only ever be estimated through sampling and substantial literature exists on various sampling strategies and methodologies aimed at producing the most reliable measurements (some examples are given in Ferguson, 1992, DoE, 1994, USEPA, 1996, Lyn *et al.*, 2007). Using statistical

models and sophisticated computer programmes, a range of techniques have been developed to build complex maps of the spatial distribution of target **analytes** from measured concentrations (Webster and Oliver, 1990 for a review). Predominantly employed within geochemical surveys, these techniques have historically been applied at the local or regional scale (10 m -1000 km), more recently these techniques have been used at intermediate scales (1 cm – 10 m) (Jackson and Caldwell, 1993 (nutrient study), Franklin and Mills, 2003, Becker *et al.*, 2006) and small scales (µm - cm) (Nunan *et al.*, 2002) to map a range of soil properties.

There is a stark contrast between the sophisticated models used to map spatial distributions of trace metals in contaminated land investigations and the distributions of trace elements, used in controlled studies, to estimate plant uptake. Much of the research aimed at estimating plant uptake has used either pot experiments or hydroponics with homogeneously distributed trace elements (Kumar *et al.*, 1995, Ebbs *et al.*, 1997, Hooda *et al.*, 1997, Quartacci *et al.*, 2006, Turan and Bringu, 2007) or field experiments where the plant-soil system is unique to that site only (Clemente *et al.*, 2005), and contaminant heterogeneity is overlooked.

Spatial heterogeneity of nutrient distributions at a scale smaller than that of individual roots has been found to have a significant effect on the performance of some plant species, (Robinson, 1994, Einsmann *et al.*, 1999, Hutchings *et al.*, 2003, Hutchings and John, 2004 for comprehensive reviews). Moreover, a smaller number of studies have recently shown that trace metal heterogeneity can also significantly impact on plant performance and uptake (Millis *et al.*, 2004, Manciulea and Ramsey, 2006, Menon *et al.*, 2007, Moradi *et al.*, 2009). Whilst these studies have shown significant differences between their simplistic models of heterogeneity and more traditional homogeneous testing mediums, models used still do not resemble the spatial patterns of analyte heterogeneity actually experienced by the plant under field conditions. It is therefore unsurprising that pot trial results cannot be replicated in field experiments (Banuelos *et al.*, 2005, Grispen *et al.*, 2006).

Spatial scale is also of importance in ecological studies of plants. Nutrient and trace element heterogeneity can have a significant impact on plant performance and trace element uptake, as demonstrated by Wijesinge and Hutchings (1997) and Manciulea and Ramsey (2006). A small plant growing in a heterogeneous environment consisting of patch sizes greater than the plant root system will perceive the environment to be homogeneous.

Traditional methods of soil sampling for geochemical surveys, predominantly involve the removal of one or more (in the case of a composite sampling strategy) cores of approximately 200 grams of the top 15 cm of soil at each **sampling location**. These are then ground and homogenised before chemical analysis. This sample preparation removes nearly all of the small scale heterogeneity that is relevant to many plant species. Fortunately, the relatively recent development of new ***in situ*** analytical techniques, has enabled soil sampling, without disturbing the structural heterogeneity. In a recent study by Taylor *et al*., (2005) using a Portable – X Ray Fluorescence, heterogeneity of Pb and Zn was quantified at scales across five orders of magnitude, using a nested sampling design. The technique analyses a small sample mass, typically less than 1 g, and can therefore quantify small scale *in situ* heterogeneity at the centimetre scale. The pilot study by Taylor *et al*., (2005) characterised heterogeneity at two contaminated land sites and found the degree of spatial heterogeneity (Section 1.4.3 for a review of measurement and quantification) to vary by a factor of two for Zn at the same spatial scale. There is clearly a range of intermediate heterogeneities that exist between the simplistic homogeneous and binary models used in plant uptake studies. The work in this thesis addresses the gap between simplistic models of heterogeneity and realistic *in situ* contaminant heterogeneity by developing a sampling plan aimed at quantifying heterogeneity for a range of contaminants and scales and modelling intermediate levels of heterogeneity based upon actual contaminated land investigations for use in pot experiments.

1.2. Research Objectives.

The broad aim of this research is to establish a methodology for the quantification of *in situ* spatial heterogeneity in soils, and use it to develop more realistic models in pot trials to assess plant performance and uptake of heavy metals from contaminated soils. The more specific aims are:-

1. Assess the existing methodologies for expressing heterogeneity, and its change as a function of scale of measurement.

2. The development of a generic experimental design for quantifying heterogeneity over a range of scales.

   From a review of literature, develop a sampling design that enables quantification of heterogeneity across an entire site at scales ranging from 10 m to 0.001 m.

3. Determine whether heterogeneity significantly differs between different contaminants, and between different sites for the same contaminant.

   The new design will be applied to sites with contrasting contaminants and source characteristics using *in situ* measuring devices, and used to calculate the measurement uncertainty of the resultant measurements (including that from sampling). The sites will be selected to have different levels of heterogeneity, caused by different sources of contamination (e.g. mine wastes, land fill, firing ranges – high expected heterogeneity, and land amended with sewage sludge or from aerial deposition from nearby smelter – low expected heterogeneity. The range of contaminants will be extended beyond those considered by Taylor *et al.,* (2005), (i.e. Pb and Zn) to include other elements (e.g. As, Cd, Cr and Ni) for which *in situ* measurement techniques are now well developed.

4. Develop a method to model *in situ* contaminant heterogeneity, based upon actual site investigations, for use in greenhouse pot trials at scales relevant to receptor plant.

5. Investigate the effect of heavy metal uptake (e.g. Zn) by plants grown in pot experiments with intermediate levels of heterogeneity derived from new sampling design employed at contrasting contaminated land sites.

   Assess whether uptake of heavy metals by plants in intermediate levels of heterogeneity are comparable to estimates from simplistic models to determine whether heterogeneity should be considered a significant factor when estimating plant uptake.

1.3. Thesis outline.

This thesis is formed of six chapters. Chapter 1, Section 1.4 presents a critical review of current literature on the quantification and characterisation of spatial heterogeneity of target analytes in soils. (Brief reviews of current methods for determining plant uptake, heterogeneity models for pot experiments and root responses to heterogeneous soils are given at the beginning of chapters 3, 4 and 5 respectively).

Chapter 2 introduces a new sampling design, to be used in conjunction with *in situ* measurement techniques, to characterise and quantify contaminant heterogeneity over a range of scales from 20 m to 0.0005 m. The design has a systematic approach to sampling that can be easily be adapted to different scales. Heterogeneity is characterised at different scales across the entire site under investigation and differs from the nested sampling design used by Taylor *et al.*,(2005) which focused estimation in a localised sub area. Results are

presented from two contrasting contaminated land site investigations, using percent relative standard deviations to express heterogeneity at each scale for Pb, Cu and Zn.

Chapter 3 presents results from a preliminary experimental study of plant uptake of a range of heavy metals for four plant species grown in sewage amended soils. Four species; *Plantago lanceolata, Taraxacum officinale, Brassica napus* and *Brassica juncea are* assessed for suitability in the main pot experiment which will use five models of differing heterogeneity. Explained within this chapter are; the computer model used to construct pot designs of contaminant heterogeneity measured *in situ*, choice of contaminant (Zn) concentrations used, methods for conducting pot experiment and analytical techniques. The five models contain a homogeneous treatment used in the majority of heavy metal uptake studies, three intermediate levels of heterogeneity based upon the two site investigations in Chapter 2 and the firing range site investigation by Taylor *et al.,* (2005) and a simplistic binary model used by Podar *et al*.,(2004) and Mancuilea *et al*.,(2006)

Chapter 4 analyses plant root and shoot biomass and total measured zinc concentrations in plant dry biomass for the four species, grown in multi-level heterogeneity treatments. Research using simplistic heterogeneity models has previously found significant differences in plant growth and plant uptake of heavy metals (Chapter 4, 4.1. Introduction for a review) and the main aim of this experiment is to determine whether simplistic binary models of heterogeneity provide an adequate estimate of plant uptake in heterogeneous environments. Moreover to consider whether site specific heterogeneity is an important factor controlling plant uptake of heavy metals and should therefore be a fundamental requirement of contaminated land assessment for both risk and remediation.

Chapter 5 analyses a supplementary pot experiment based upon findings in the main experiment. A significant difference of plant response to differing spatial heterogeneity of Zn was observed between the two *Brassica* species, however, both species showed significantly reduced Zn concentrations in shoots in binary

treatments compared to all other treatments. Similar findings have been observed by and Podar *et al*., (2004), Millis *et al*., (2004) and Manciulea *et al*., (2006). The aim of this experiment is to explore root response to treatments of different **patch contrast**, but with the same total concentration throughout. Studies of hyperaccumulating plants e.g. *Thlaspi caerulescens* have demonstrated root foraging into patches of high metal concentration (Schwartz *et al.*, 1999, Whiting *et al.*, 2000, Haines, 2002), whereas non-accumulating plants have been shown to avoid metal rich patches (Menon *et al.*, 2007, Moradi *et al.*, 2009). Using the same method in Chapter 3 two treatments, one binary, the other a simplified high heterogeneity treatment were used to assess root biomass in cells of different Zn concentrations and determine whether root placement is a key mechanism determining heavy metal tolerance in heterogeneous environments.

Chapter 6 summarizes key findings from the thesis in relation to stated aims. Also discussed are the implications of this research to the studies of contaminated land assessment, estimating risk to human health from plant uptake of heavy metals and potential for improvements in strategies to remediate polluted soils using **phytoremediation**.

1.4. Critical review of literature on the characterisation and quantification of contaminant heterogeneity in soils across a range of scales.

1.4.1. Introduction.

One of the consequences of soil heterogeneity is the generation of large uncertainty in environmental investigations. Measurements of analyte concentrations, taken from the same nominal location, within a **sampling target**, can vary substantially (Taylor *et al.*, 2005). Whilst some of the variability may be due to sampling and analytical errors, heterogeneity is most often the main contributor (Ramsey and Argyraki, 1997).

Pitard (1993) proposed that there are only two approaches to coping with the impact of heterogeneity on environmental measurements, either accept with the consequence of quantification and rigorous quality control or to eliminate or minimise through taking larger samples and homogenisation. The former requires isolating the variability due to heterogeneity from that which arises from analytical and sampling errors, the latter requires the removal of the soils, irreversible destruction of the original structure, loss of heterogeneity at finer scales than scale of sample and potential change in the original chemical composition and subsequent increased uncertainty in the final analysis.

Many of the techniques used to characterise spatial distribution patterns have been applied at a specific scale. For example, geostatistics was pioneered by Matheron and Krige, two engineers working in the mining industry, to predict spatial patterns of minerals, hence typically applied at geological scales of (10 – 1000 km). Conversely at finer scales, cellular automata have been used to quantify soil pore spaces, using a cell lattice and transition rules at the molecular scale (less than 1 μm)(Young *et al.*, 2001 for a review). However, processes interacting within soils and between soils and the surrounding environment, occur at a range of scales. For example; heterogeneity of trace

elements in minerals may occur at the micrometer scale, whilst heterogeneity in the distribution of a particular tree species may occur at the hundred meter scale.

The aim of this review is to consider some of the current methods employed to characterize heterogeneity in soils, how different approaches are undertaken and, in particular, to compare the established techniques of geostatistics and the relatively new methods using fractal dimensions. The review will focus on techniques that specifically measure heterogeneity over the range of scales that may occur for plant interactions within soils, with a goal to developing a sampling strategy to characterise heterogeneity of trace metals in soils using relatively new *in situ* analytical techniques.

The effects of contaminant heterogeneity on plant uptake and root response are reviewed in the introductions to Chapter 4 and 5 respectively.

1.4.2. What is heterogeneity

From an ecological perspective, heterogeneity is often referred to as patchiness and can incorporate factors of scale, i.e. size of patch and contrast i.e. the degree to which one patch differs from an adjoining or surrounding patches. Adapting an analogy from Myers, (1997), in relation to soils, the physical concept of scale and contrast of heterogeneity can be illustrated by an inspection of a pile of soil. From a distance the pile appears homogenous, with uniform colour and individual particles indiscernible. As the pile of soil is inspected at a higher resolution (finer scale), individual particles become visible revealing a range of colours, sizes, shapes, opacities and composition etc (contrast). Whilst the analogy relates to the *ex situ* study of soils it is equally applicable to the study of soils, *in situ* and undisturbed.

There are many objective theories that incorporate a clear description of heterogeneity, its sources, quantification and reduction. One such example is that of Pierre Gy's Classical Sampling Theory (CST) that defines total

heterogeneity as arising from two kinds of heterogeneity; constitution heterogeneity and distribution heterogeneity (Gy, 1992).

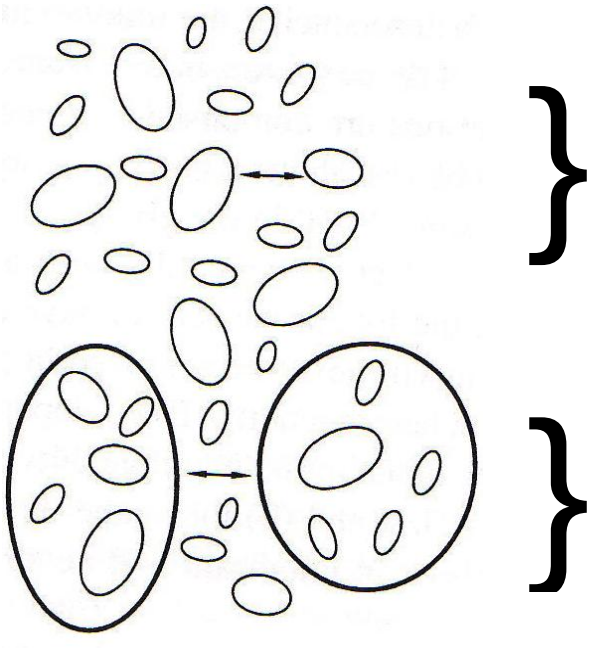
(i) **Constitution heterogeneity** represents the variability between individual fragments and is defined as:

> "The constitution heterogeneity (CH_L) of a lot (L) is the heterogeneity that is inherent to the composition of each fragment or particle making up the lot. The greater the difference in composition between each fragment, the greater the constitution heterogeneity. The constitution heterogeneity could also be called the composition heterogeneity." (Pitard, 1993)

(ii) **Distribution heterogeneity** represents the variability in the arrangement of fragments in groups and is defined as:

> "The distribution heterogeneity DH_L of a lot L is the heterogeneity that is inherent to the manner in which separate and distinct particles or units are scattered or spread out within a lot L. The greater the difference in composition between each fragment, the greater the possible distribution heterogeneity; likewise , the greater the difference in density between each fragment, the greater the possible distribution heterogeneity" (Pitard, 1993)

Figure 1.4.1 illustrates these concepts.



Constitution Heterogeneity: describes variability amongst individual fragments within a sampling target.

Distribution Heterogeneity: describes the manner in which individual fragments separate themselves into groups.

Figure 1.4.1. Demonstrates the difference between constitution and distribution heterogeneity of a zero -dimensional lot. (adapted from Pitard, 1993)

Heterogeneity can be contrasted against **homogeneity**. A batch or sample can be considered constitutionally homogeneous if all the elements making up the batch or sample are strictly identical, in every respect, to each other and distributionally homogenous if all samples or batches contain the same average composition of fragment types. As such homogeneity can be said to have zero heterogeneity and to be the limit case, but it is unlikely to occur in the natural world.

Studies which follow the example of Classical Sampling Theory, from Gy (1992) express heterogeneity mathematically based on the assumption that individual fragments can be quantified, thus, the constitution heterogeneity of a Lot L can be expressed as the variance of the heterogeneities of the number of fragments (N_F) for fragment types F_i making up the lot (Pitard, 1993). Thus, this approach is only applicable to sampling targets where individual fragments can be isolated and categorised.

Distribution heterogeneity is said to be dependent on three factors (Myers, 1997), constitution heterogeneity, the spatial distribution of the constituent parts and the shape of the lot. As constitution heterogeneity quantification is dependant on the ability to identify and count discrete fragments, it makes this approach (outlined in Gy's and in other sampling theories) difficult to apply to field studies of soils *in situ*.

1.4.3. Characterisation of heterogeneity.

Cellular Automata

A review of new methods for characterising structural heterogeneity of soils was undertaken by Young et al., (2001). The review discusses various techniques, for example, cellular automata (CA). The CA technique uses a cell lattice and transition rules based on nearby neighbouring cells to describe how a cell might change in state, e.g. from a pore containing a gas molecule or not. This is not so much a measure of variability, but more a measure of probability of change and relies on defining a number of discrete properties. Additionally, these studies of soil heterogeneity are concerned with the microscopic scale (i.e < 1 μm) rather than the macroscopic scale considered above.

Analysis of variance and nested sampling designs.

At a more intermediate scale (1 m – 100 m), as in for example, a contaminated land site investigation survey, Clark *et al*., (1996) suggest a simple model for quantification of local environmental heterogeneity. If two sites with similar matrices, i.e. soil type, are sampled and analysed by identical means (i.e. personnel, laboratories) at similar times and prevailing climatic conditions, then the sampling and analytical variances can be assumed to be the same for both sites. Based on an original equation in a study by Ramsey *et al*., (1992), (Equation 1.4.1), calculated for each site, Clark *et al.*, (1996) defined environmental heterogeneity as the difference between the observed variance for the two sites (Equation 1.4.2).

Equation 1.4.1 $s^2_{total(observed)} = s^2_{geochem(environment)} + s^2_{samp} + s^2_{anal}$

Where $s^2_{geochem}$ represents the variability across all samples at a site, s^2_{samp} is the variance arising from sampling methods and s^2_{anal} is the variance that arises in measurements from the analytical methods.

Equation 1.4.2 $s^2_{observed,1} - s^2_{observed,2} = s^2_{environment,1} - s^2_{environment,2}$

However, this is impractical for most situations, where only one site is to be investigated. Moreover, this technique only quantifies the difference in heterogeneity between two particular sites, therefore lacking a more descriptive characterisation and assumes that sampling and analytical variance between the two sites to be the same, which is rarely the case.

Measurement uncertainty (Ramsey, 2010b) is a term used to group together all the variance that arises from both random and systematic errors from all methods, both sampling and analytical, in geochemical soil surveys, but excludes geochemical variance, and can be estimated using Equation 1.4.3. The dominant factor in the estimation of measurement uncertainty, of soil at a site investigation, is most often found to be the variance that arises from sampling (s_{samp}) and is primarily caused by heterogeneity (Argyraki, 1997, Taylor *et al.*, 2005).

Equation 1.4.3. $\mathbf{U = s_{meas} = \sqrt{(s^2_{samp} + s^2_{anal})}}$

Estimates of variance (s^2) may also be used to assess the difference between individual samples and the mean ($\bar{x}$) for a particular site investigation. Taylor *et al*., (2005), used analysis of variance, in a study of heavy metals in soils in contaminated land investigations. Incorporating a nested sampling design (Figure 1.4.10, discussed further in section 1.4.4), heterogeneity was characterised at a range of scales, expressed in relative standard deviations, prior to a main investigation.

The variance of sampling, e.g. s^2_{samp} in Equation 1.4.3 can be estimated by taking a **duplicate sample** from the same nominal sampling location. Rearrangement of Equation 1.4.3 to Equation 1.4.4, enables the variance that arises from heterogeneity to be isolated.

Equation 1.4.4. $U = s_{samp} = \sqrt{(s^2_{meas} - s^2_{anal})}$

In geochemical surveys, where sampling distance is typically 10 – 100 m apart, a duplicate sample would be taken approximately 1m distance from the original. In the study by Taylor *et al*. (2005), 8 duplicates were taken at each level (separation distance) to estimate heterogeneity across a range of scales. Using the standard deviations of the 8 measurements, Equation 1.4.4, heterogeneity can then be expressed numerically, as a percentage relative to the mean (%RSD) for each sampling distance. This method of expression minimises the effect of any outlying values or exceptionally high measurements.

Variograms and kriging

Variograms and kriging are two approaches that are widely used for geochemical mapping and are explained in detail in Myers, (1997). Variograms and kriging are geostatistical techniques that follow a statistical method, originally developed by Matheron and Krige, for prediction of gold reserves in South Africa (Swan and Sandilands, 1995). These techniques are based on the assumption that close spatially or temporally related samples exhibit similar values in concentrations (Myers, 1997).

Essentially, the variogram is a graphical plot of variance as a function of distance and is used to characterize the spatial variability of target analytes over a geographical area or region. Initially, the variance is calculated between concentrations for all sample pairs with the smallest spacing (the lag, *h*) in a particular direction. The distance (d) between pairs is then increased to every other sample, therefore the second sample distance is termed lag 2*h*, every third sample is equal to lag 3*h* etc.

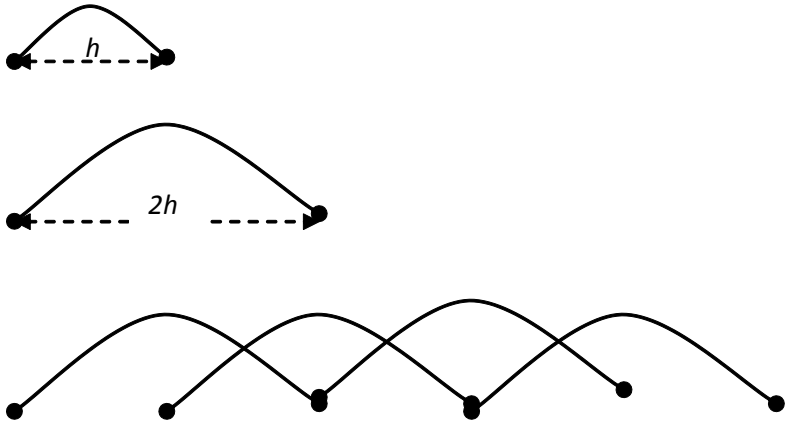


Figure 1.4.2 Pairings of samples (*h*) at initial distance, and subsequent pairing for a distance lag of 2h. (From Myers, 1997).

The calculations to construct a variogram can be expressed mathematically as:

Equation 1.4.5. $$2\gamma(h) = \frac{1}{n}\sum_{i}^{n}\left[g(x_i) - g(x_i + h)\right]^2$$

Where *h* is the distance between sample pairs, *n* is the number of possible sample pairs, $g(x_i)$ is the element concentration at point *x* and $g(x_i+h)$ is the element concentration at distance *h* from point x_i (Bolviken *et al.*, 1992).

The ideal variogram rises from the axis origin, reducing in rate of increase until levelling off. The distance at which the graph flattens is termed the ‘range’. The height at which the plateau is reached is termed the ‘sill’ and represents the variance of the population. When the variogram intercepts the y-axis, this is termed the ‘nugget effect’ (Figure 1.4.3). Nugget effects arise from short-range heterogeneity and are a common feature of environmental surveys where target analytes tend to cluster (Myers, 1997).

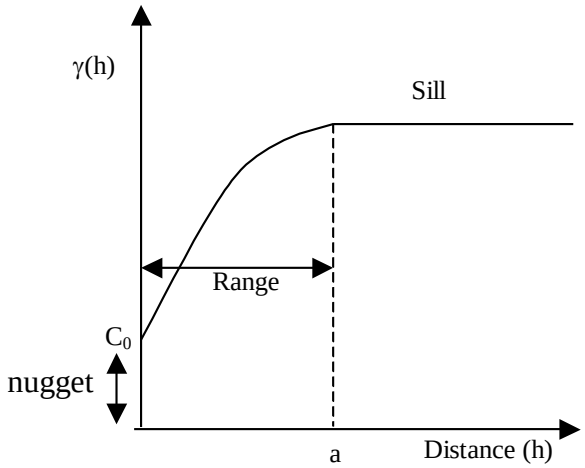


Figure 1.4.3 Illustration of an idealised variogram showing the range, sill and nugget effect (Adapted from Myers, 1997).

According to Myers (1997) the variogram provides three qualitative concepts;

(i) **continuity**, measuring the smoothness of transition between closely spaced samples.

(ii) a **zone of influence**, defined by the range (a), this provides a distance within which the similarity between sampling locations can be predicted. For a specific analyte, for example, if the distance between two points is 20 m, and the γ(*h*) for that distance is equal to 44 µg/g, assuming a normal distribution, the concentration at the second point can be said to be within ± 13.3 µg/g if using 2 standard deviations for 95% confidence.

(iii) **anisotrophy**, derived from calculation of variograms in different directions, quantifies rate of change in variability in spatial structure with direction. It often supports what is intuitive from local factors e.g. prevailing wind from a smelter, down dips and strikes etc.

Kriging is a method used to construct contour maps of estimated concentrations across an area of study (Figure 1.4.4). There are a number of kriging methods, e.g. universal, co-kriging and point kriging (Myers, 1997). Broadly they provide

estimates of concentrations and uncertainty attached to those values, for unsampled locations.

Construction of a kriged contour map usually requires variograms for four cardinal directions calculated from a minimum of 100 samples (Myers, 1997) and is therefore time consuming and expensive.

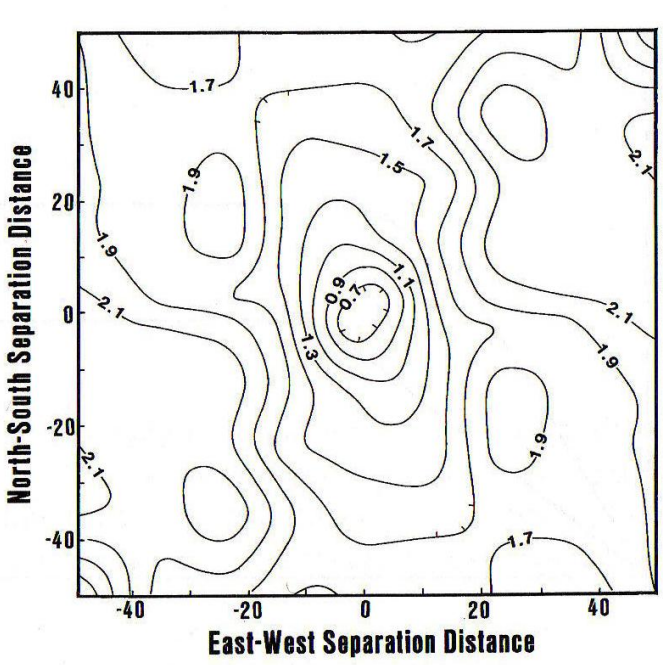


Figure 1.4.4 Illustration of a contour map, for element concentration, constructed using a kriging method (From Myers, 1997)

Using a simple 2-dimensional model, the area to be mapped is overlain with a grid, then values are estimated for grid points. Point values are calculated from surrounding control points within a 'neighbourhood'. The neighbourhood is defined by a circle, surrounding the point to be estimated, with a radius equal to the range of the variogram. Weightings are attached to selected control points based on the semivariance for the distance, *h*, between point and control point. An error estimate is also calculated for each point, usually, the further the point from sampled location the greater the estimate of error. Kriging uses least squares algorithm to produce minimum local error variance (σ_E^2) at each grid point. The result is a smoothing effect of variance for estimated values, with

small values being overestimated and larger values, underestimated (Goovaerts, 1999), consequently this method will underestimate heterogeneity.

A prerequisite for the application of geostatistical models, is that the analyte of interest is distributed spatially in a non-random pattern, i.e a trend in concentration values exists. Yet heterogeneity usually changes across a study area and heteroscedasticity (local variability in data values) (Isaaks and Srivastava, 1989) is often misrepresented by the variogram (Goovaerts, 1999). The proposed solution is to subdivide data points into areas that are relatively homogenous, but this requires sufficient knowledge about the area, to be able to delineate discrete statistical populations.

With the large number of samples and resultant calculations, the process is usually undertaken with complex, computer software. The user requires detailed background knowledge to make informed decisions regarding the correct selection and application of the many possible models, and the consequences of any parameters that may be assumed by default within the software (Taylor *et al*., 2005). Factors, such as nugget effects can increase the uncertainty attached to any estimated values (Myers, 1997) and the resultant map should be interpreted with due care.

Typically variograms are constructed using lag distances of 10 m to 1000 km for geological surveys, however it has more recently been applied at the 1 m to 1 cm ranges for assessing the spatial variability of microorganisms within soils (Franklin and Mills, 2003). Yet, variograms are restricted by the particular sampling interval (Bellehumeur and Legendre, 1998), too fine scale and factors acting on a larger scale may be overlooked and the converse is also true (Levin, 1992). Whilst factorial kriging has been suggested as a method of characterising heterogeneity over a range of scales, it still requires construction of variograms at each scale, which if required to produce a reliable estimate, each variogram will need greater than 150 data values or samples (Webster and Oliver, 1993, cited in Goovaerts, 1999).

Fractal models

Fractal dimensions, as a method of describing geological properties, is a relatively new technique, which has been used in recent studies to describe spatial distributions.

Following a study by Bölviken, *et al*., (1992) that utilized fractals to describe distributions of each of 21 elements over a 250 000 km^2 area, several studies (Cheng *et al.*, 1994, Kravchenko *et al.*, 1999, Li *et al.*, 2004) have used variations of the method to characterise spatial distributions of elements and soil properties.

Fractals is the name that was given by Mandelbrot, B.B. (1983) to a family of shapes that consist of irregular and fragmented patterns, that look similar at a range of scales. Mandelbrot states that the “best fractals are those exhibit maximum invariance”, i.e. something that does not change shape with changes of scale, a good analogy is that of a cauliflower, were individual florets are a miniature of the whole vegetable. For a fractal distribution of heavy metals this may equate to similar variance in measured concentrations at each separation distance across a range of scales. Fractals that are invariant with changes in scale, are termed scaling, or for geometric similarity, ‘self-similar’. The concept is best explained by an example from Mandelbrot, (1983) in a study to determine the length of the British Coastline. Previously coastlines had been described using topology, which defines the coastline of an island as a circle. Mandlebrot argued that this failed to discriminate between different coastlines. He found that coastlines have no definitive length, and that estimates are based upon the unit of measure, with smaller unit measures resulting in larger estimates of length as it traces the degree in variance from the straight line more intricately. A coastline has a fractal dimension between the values of 1 D=1 (dimension of a straight line) and D=2 (dimension of an area).

Fractal dimensions can be calculated by overlaying the feature to be characterized with grids of varying cell sizes. For each grid size, the number of cells intercepted by the feature is counted, and the natural log of the cell count

versus cell size is plotted. The gradient of the resulting best-fit line gives the fractal dimension. The goodness of fit (r^2 value) gives an indication of whether the spatial distribution of the variable is fractal in nature, i.e. is similar over a range of scales (Swan and Sandilands, 1995).

If the geochemical distribution is fractal, then the variance should increase perpetually with increased distance between sample pairs. Bolviken suggests that one implication of fractal dispersion patterns is the identification of geochemical provinces using low-density sampling strategies.

However, due to the smoothing effects of kriged estimates, explained earlier, fractal dimensions derived from kriged contour maps, tend to be underestimated. Plotnick *et al*., (1996) argue that fractal methods are limited, in that they do not describe the full range of patterns that may exist in the environment. For example, some patterns may have the same fractal dimension, but may look very dissimilar due to different textures. Fractal models have been applied at a variety of individual scales, from regional geochemical distributions (10 km – 1000 km) (Bolviken *et al.*, 1992, Cheng *et al.*, 1994, Li *et al.*, 2004), to variability of pore spaces and fractures in rocks (1 mm-1 μm), (Pape *et al.*, 2000, Wagner *et al.*, 2000). Fractals have usually been used to describe a spatial distribution at a particular scale, to date there appears little research that describes the fractal dimensions of a target analyte over a large range of scales e.g. > 2 orders of magnitude. At one scale Cheng *et al.,* (1994) found elements distributions with different types of self similarity, i.e. had more than one fractal dimension, and this may also be the case at different scales. Li *et al*., (2004) go on to suggest that there may be heterogeneous fractal dimensions!

Lacunarity

Lacunarity is a concept that was originally used by Mandelbrot (1983) to describe gaps in fractals. Lacunarity is a measure of the deviation of a fractal from translational invariance and can thus be used to describe the

heterogeneity or texture of an object, regardless of whether the object is fractal or not (Plotnick *et al.*, 1996).

Plotnick *et al*., (1996), used a range of hypothetical distributions for a particular tree species along transects of equal length (Figure 1.4.5), to depict the range impact on lacunarity curves due to different spatial distribution patterns.

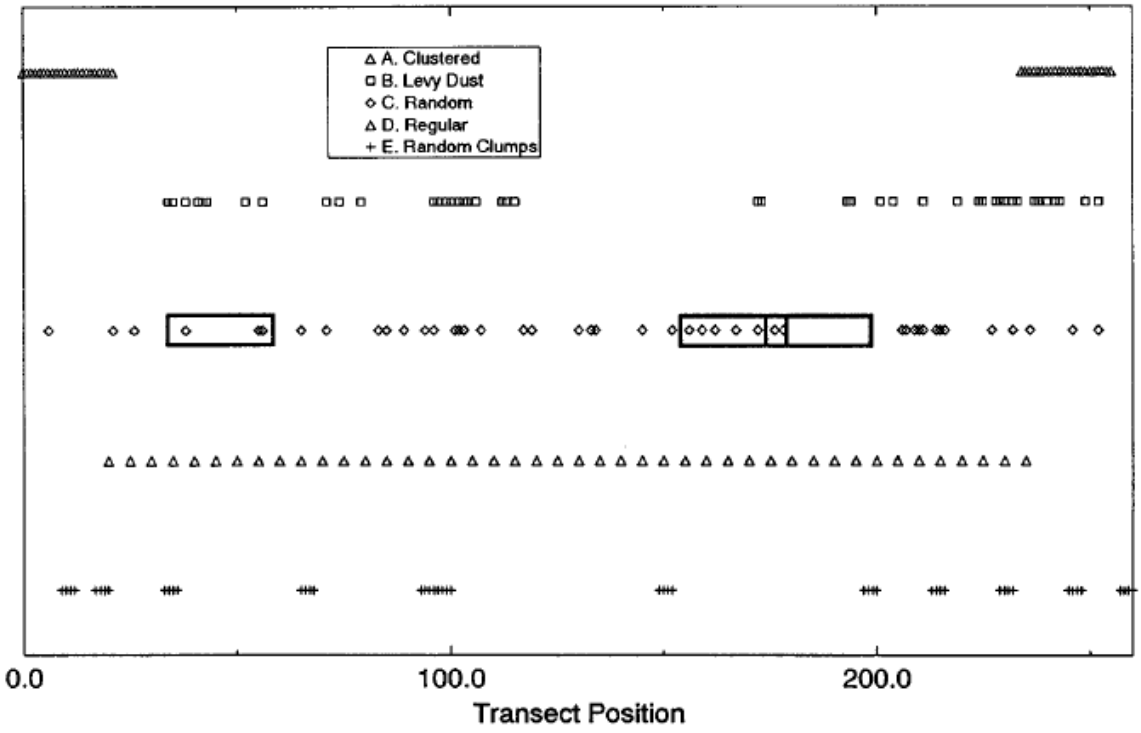


Figure 1.4.5. Five one-dimensional hypothetical sets of the distribution of a tree species. Boxes on transect C, represent three positions of the gliding box. (From Plotnick *et al.*, 1996)

Using the “gliding box” method (Allain and Cloitre, 1991), the box is placed at the origin of each transect and the number of occupied sites within the box is counted. The box is then moved one space along, and the set is counted again. This is repeated along the transect to produce a frequency distribution $n(S,r)$, where S is the number of samples in the box and r is the box size. This is then converted to a probability distribution, $Q(S,r)$, by dividing by the number of boxes. The first and second moments are determined using Equation 1.4.6 and Equation 1.4.7., respectively, and the lacunarity of the box size is calculated

from Equation 1.4.8. This is then repeated for a variety of box sizes and plotted at a log-log plot of lacunarity versus gliding box size (Plotnick *et al.*, 1996).

Equation 1.4.6 $$Z^{(1)} = \sum SQ(S,r)$$

Equation 1.4.7 $$Z^{(2)} = \sum S^2Q(S,r)$$

Equation 1.4.8 $$\Lambda(r) = Z^{(2)}/[Z^{(1)}]^2$$

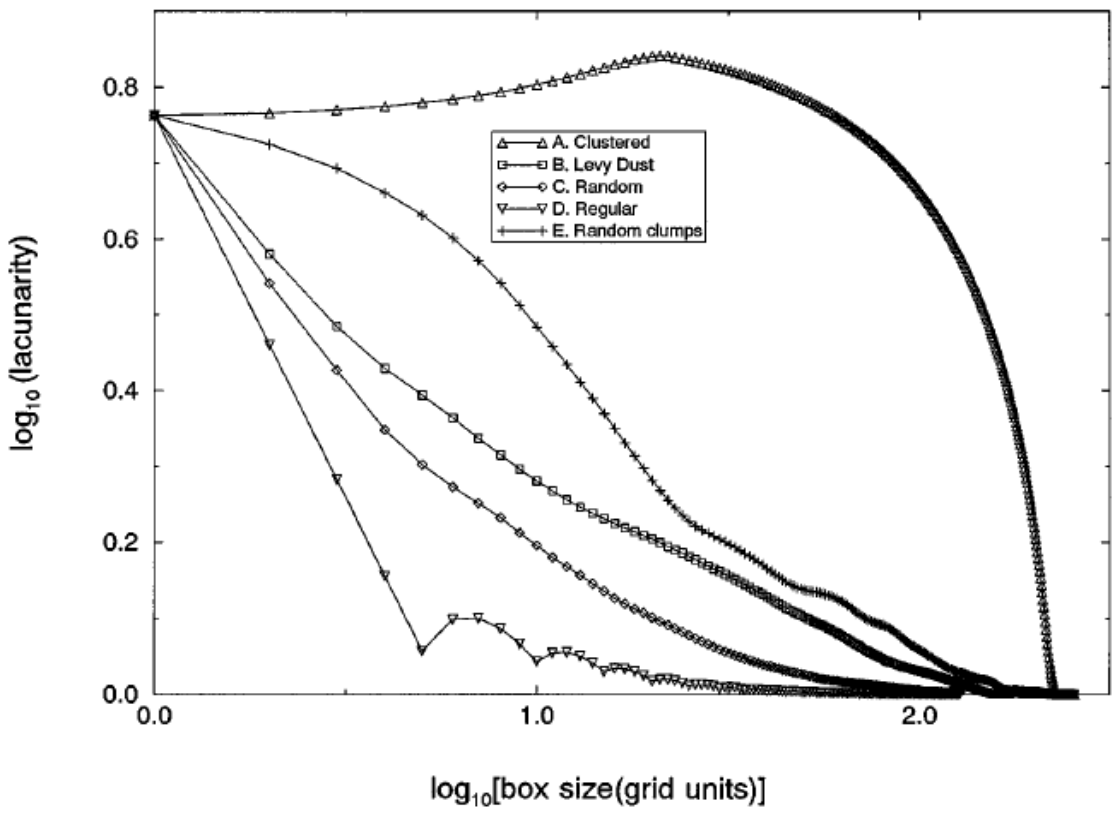


Figure 1.4.6 Log-log plots of data sets depicted in Figure 1.4.5. (From Plotnick et al, 1996)

For characterisation of spatial distribution of analyte concentrations in soils, the method can be adapted by calculating the sum of the distribution within a box size r.

Where a self-similar fractal pattern exists, the lacunarity curve tends towards a straight line. Where distinct breaks in the slope occur, these correspond to scales that exist within particular sets and can therefore be used to detect scale-dependant changes in spatial behaviour. As stated by Levin, (1992), “There is no single natural scale at which ecological phenomena should be studied: systems generally show characteristic variability on a range of spatial, temporal and organisational scales”.

Nearest neighbour

In ecology and geography, a widely used technique for describing spatial relationships is that of ‘nearest neighbour’. Usually the distance relationship between a data point and its nearest neighbours is analysed to determine the departure, or conformity, to a random distribution. Clark and Evans, (1954) define a random distribution as:

“in a set of points on a given area, it is assumed that any point has had the same chance of occurring on any sub-area as any other point, that any sub-area of specified size has had the same chance of receiving a point as any other sub-area of that size, and that the placement of each point has not been influenced by that of any other point.”

The measured mean distance to the nearest neighbour of the population being studied, is compared, as a ratio, to the expected mean distance in a randomly distributed population. This ratio provides a measure of departure from the random. This technique looks at the relationship between individuals within a known population, in characterisation of soils, a sample is taken to be representative of an area for which the true ‘population’ is not known.

A significant limitation of both nearest neighbour and lacunarity techniques is that they only work for binary distributions consisting of discrete individuals. In soils, trace metals can occur over a range of concentrations, with variable effects on plants at different concentrations (Kabata-Pendias and Pendias, 2000). Moreover, based upon studies of **nutrient patch** quality in pot trials

(Gersani and Sachs, 1992, Gleeson and Fry, 1997, Wijesinghe and Hutchings, 1999) patch contrast can be a significant factor in plant responses (Chapter 5 for a review).

Moving window statistics

Moving window statistics is a relatively simple statistical method that can be used to describe variability across a site investigation.

An area can be divided into subunits, within which data values are used to calculate the mean, ($\bar{x}$) and standard deviation, (*s*).

81	77	103	112	123	19	40	111
82	61	110	121	119	77	52	111
82	74	97	105	112	91	73	115
88	70	103	111	122	64	84	105
89	88	94	110	116	108	73	107
77	82	86	101	109	113	79	102
74	80	85	90	97	101	96	72

Figure 1.4.7 Example of overlapping window for calculation of moving average statistics. (From Isaaks and Srivastava, 1989)

The size of the window, defining a subunit, is usually dependant on the average spacing distance between sample points and the total area of the investigation. However there is always a danger that the window may be too large or small or there is insufficient data within to calculate reliable statistics (Isaaks and Srivastava, 1989). One approach, to improve reliability is to overlap windows with adjacent subunits (Figure 1.4.7.) One method of expressing the variability using this technique is correlation coefficients of a plot for the standard deviations versus the means. Whilst the approach may be applied to each data set at a specific range, it is limited by the number of data values required to produce reliable statistics.

1.4.4 Sampling designs.

Before heterogeneity can be quantified, it needs to be measured and this can only be achieved in soils through sampling. Much of the sampling literature for soil and rock surveying is focused towards adopting a sampling strategy that will successfully 'hit' a target, e.g. a contamination 'hot spot,' or a rich vein of a valuable mineral. Other strategies may focus on estimating the mean concentration ($\bar{x}$) of an analyte. In contaminated land investigations, it is important that samples aim to be representative of the whole area, and often sampling designs are based upon historic knowledge of a site. Key requirements of any sampling design are the sampling pattern (e.g. the position at which each sample is taken) and sampling density, i.e. the number of samples. Other factors which are also of importance in sampling strategies and protocols are sample mass, depth, methods of collection and storage. More detailed and comprehensive reviews of considerations can be found in the following papers and government guidelines (Ferguson, 1993, DoE, 1994, Thompson and Ramsey, 1995, BSI, 2001, BSI, 2002).

Generally, the greater the number of samples taken and the smaller the distance between each, the more representative the results will be. However, where prior knowledge exists on the degree of spatial heterogeneity of target analyte, sampling densities can be amended to suit requirements. For example, the BSI (2001) code recommends 50 m to 100 m spacing for exploratory investigations, but at former gas works, where spatial distribution of contaminants is known to be highly heterogeneous a sample spacing of every 10 m is advised. Understanding the spatial heterogeneity of a site can enable a more effective sampling for the main investigation.

There are two approaches to sampling, judgemental, where detailed information about the spatial distribution of a target contaminant exists, and sampling is targeted to confirm what is already known, and non judgemental sampling, where no detailed knowledge exists. For the purposes of characterising heterogeneity across a site the latter is preferred, as targeting within an area

would not provide a representative picture of spatial variability across the entire site.

Numerous, non judgemental, sampling designs exist in the literature and a few are illustrated in Figure 1.4.8. A detailed review of sampling designs by the Department of the Environment and Rural Affairs (DEFRA, formerly Department of the Environment) (1994), concludes that for a sampling design to be efficient it needs to fulfil 4 conditions:

(i) It should be stratified (that is the area to be sampled should be partitioned into regular sub areas);
(ii) Each stratum (sub area) should carry one sampling point;
(iii) It should be systematic;
(iv) **Sampling point**s should not be aligned.

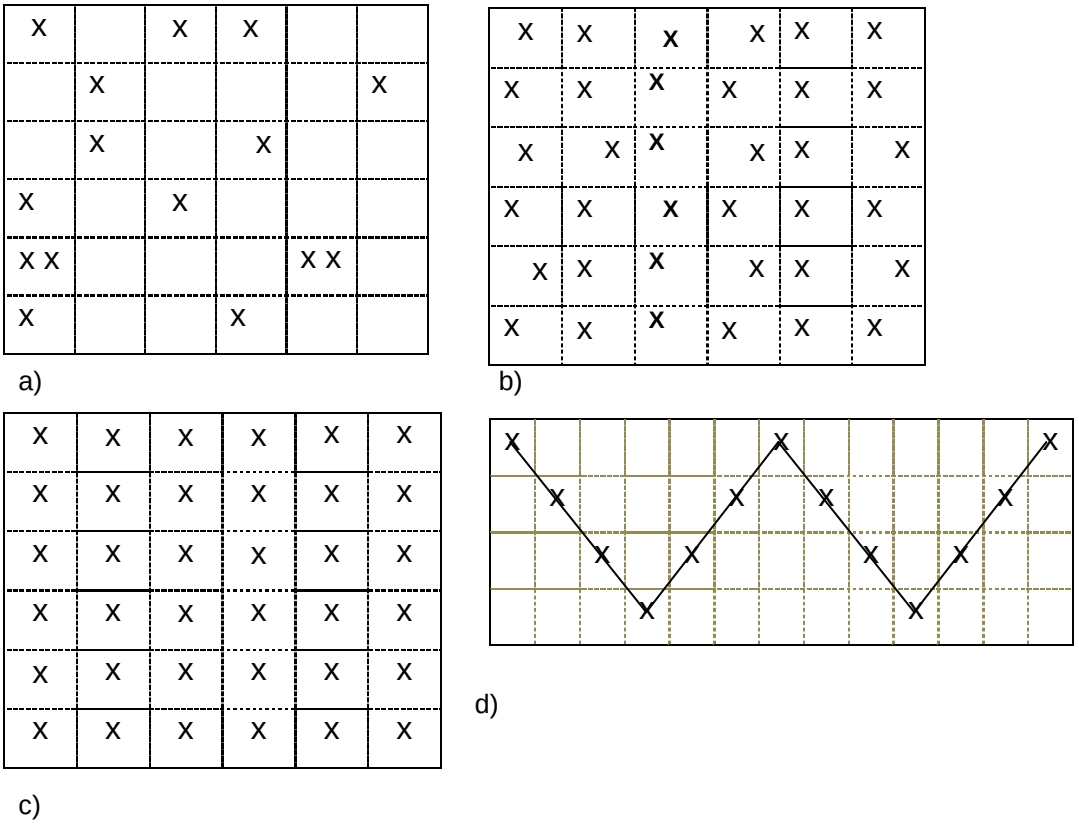


Figure 1.4.8. Diagrams of non judgemental sampling plans, a) represents a simple random sampling pattern, b) stratified random sampling pattern, c) regular sampling grid with equal distance between sampling locations, both from Garret R.G. (in Howarth, 1983) and d) 'W' pattern adapted from Ramsey et al., (1995).

A purely random design (Figure 1.4.8, a) may be appropriate where there are constraints on the total number of samples, however it is often criticised for leaving potentially large gaps (Garret, R. G. in Howarth, 1983, Webster and Oliver, 1990) and fails to fulfil the conditions stated above. Perhaps most commonly used is the regular grid (Figure 1.4.8, c) as it is simple to lay out and suitable for most (Ferguson, 1993) mathematical models and interpolation, but this fails to fulfil criteria (iv). According to the (Figure 1.4.9) herringbone sampling pattern meets all four criteria. Based upon a regular grid, each sampling point is offset by a quarter distance from the original sampling location.

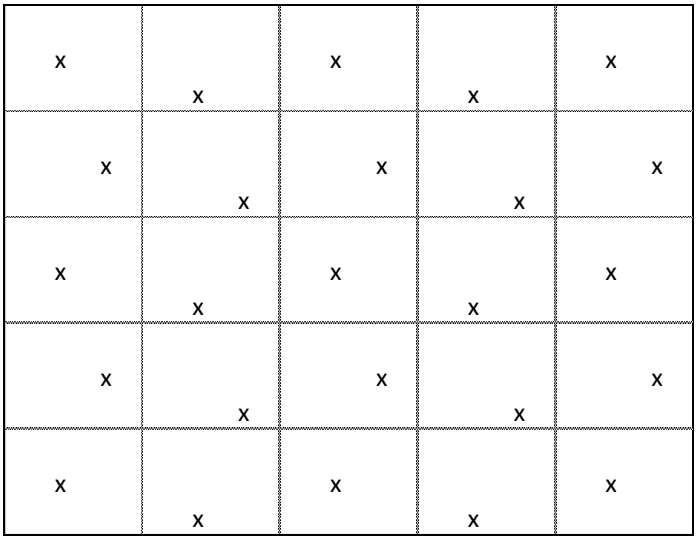


Figure 1.4.9. Diagram of herringbone sampling pattern. Each sampling location is offset from regular grid by a 1/4 of the grid spacing.

These sampling patterns, whilst they all sample across an entire area, are designed to sample at a single separation distance, therefore characterisation of heterogeneity is limited to the scale of the smallest separation distance between two sampling points. When attempting to characterise heterogeneity, the scale of the heterogeneity may vary throughout the site, on a regional basis, both with distance and direction. The occurrence of hot spots may vary in number and size, or distributions may be uniformly homogeneous.

A couple of studies have attempted to address the issue of sampling strategies aimed at quantifying heterogeneity over a range of scales > two orders of magnitude. Firstly, Taylor et al., (2005) employed an eight point nested design, and quantified heterogeneity using %RSD (Section 1.4.3, Analysis of variance and nested sampling designs, pp 14.)

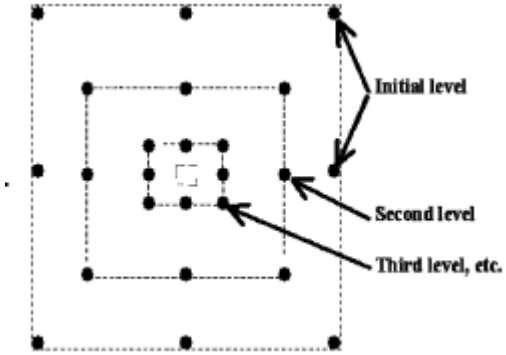


Figure 1.4.10. Example of nested sampling design. Where changes in scale may vary from 100m to 0.001m. (From Taylor, P. *et al*., 2005)

This approach, characterising heterogeneity over a range of scales, showed that heterogeneity did not vary systematically over these different scales at the two sites studied, but did vary significantly between the two sites for both of the two elements measured, Pb and Zn.

A criticism of the nested sampling design by Taylor *et al*.,(2005) is that characterisation of heterogeneity across the range of scales was limited to a small sub area within the site under investigation. To enable characterisation across the entire site would require a more general approach.

The second sampling pattern is another nested design used by Webster *et al*., (2006) aimed at addressing some of the shortcomings in variograms, i.e, not usually covering distances greater than 2 orders of magnitude, and large number of samples usually required at each scale. The technique is based upon dividing the total area to be studied into subclasses. For example, a field (level 1) may be divided into quadrants (level 2), which in turn are sub divided in half (level 3), and so on until the unit size reaches the smallest level of interest.

Points are then randomly selected within the smallest subdivision for sampling, giving a final sampling pattern similar to that in Figure 1.4.11., a. Using hierarchical analysis of variance, the components of variance are then estimated for each separation distance (Figure 1.4.11, b).

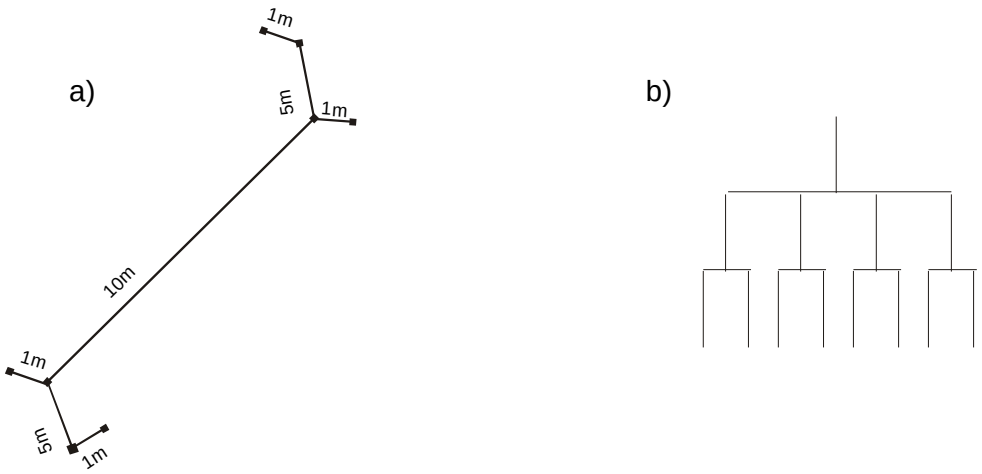


Figure 1.4.11. Illustration of a nested sampling scheme (a) used for hierarchical analysis (b). Samples are taken from each of the points at the smallest sampling distance, e.g 1m. (Adapted from Webster & Oliver, 1990)

The number of samples at each scale reduces by half as distance between sampling points increases and it could be argued that, at larger scales there may not be enough to reliably estimate heterogeneity, based on a study by Lyn et al., (2007), which concluded that a minimum of eight are required. Similarly to the study by Taylor *et al.,* (2005) characterisation is restricted to a smaller subareas within the whole site under investigation.

1.4.5. Summary of review

The methods reviewed cover a range of techniques, which assess the spatial variance, i.e. heterogeneity, of a target analyte. Whilst the work by Gy is detailed in its approach, it is probably impractical to implement in the study of soils, where discrete particles are difficult to isolate and characterise, moreover

it does not address the issue of spatial variance across a range of scales. The complexity and range of models used to produce both variograms and kriged contour maps, make this a thorough and rigorous technique, and it is perhaps the most widely used in studies of spatial heterogeneity. However, it has rarely been used to quantify heterogeneity at scales of > 2 orders of magnitude and construction of the resulting variogram is highly subjective. The shape of the variogram is dependent on the model used to fit, number of samples and distance between each sample. Kriged contour maps interpolate concentrations between sampling locations based upon the assumption that variance increases with distance, however where sample distance is too great and sample mass is too large, small scale heterogeneity, may be over looked. Moreover, complex spatial maps created by kriging would be very difficult to replicate in a plant pot experiment.

The new methods using fractal dimensions, have been employed at a variety of scales, from the microscopic analysis of pore spaces in soils to the geochemical distribution of elements across continents. This would be an interesting approach to use for characterization of soils, however, as highlighted by the study of Plotnick *et al*., (1996) it fails to describe distributions that are not fractal in nature, and has been based on contour maps from analysis of variograms, with their inherent complexity. The lacunarity approach provides a simplistic technique, which can easily be applied at a range of scales and to assess a number of properties, but like nearest neighbour approach is only relevant to discrete populations.

Sampling design patterns range from a simple transect employed by Plotnick *et al*., (1996) to a nested design used by Taylor *et al*., (2005). Designs used in both fractal and variogram approaches to quantify heterogeneity range from a regular spaced grid to a more random sampling design usually due to the geological restrictions of the area to be investigated. Nested sampling designs appear to be the most suitable for assessing heterogeneity over a range of scales, those used to date provide data for localised sub areas within the total site under investigation.

From a review of literature, and to the knowledge of the author, no generic sampling method, that characterises spatial heterogeneity over a range of scales, across an entire site has been developed. Moreover, there is an enormous gap between the complex geochemical spatial mapping techniques and the simplistic heterogeneity models used to estimate the impact of contaminant heterogeneity on plant uptake of trace metals. This thesis aims to bridge the gap between the two academic disciplines by testing a new sampling design to characterise and quantify heterogeneity, using relative standard deviations, at contaminated sites with contrasting spatial distributions of a range of trace metals. Results from actual site investigations will be used to model designs for use in pot trials with %RSD within similar ranges to those found at contaminated sites and used to assess whether heterogeneity is a significant factor in plant uptake of contaminants.

Chapter 2 New Sampling design to quantify in-situ contaminant heterogeneity with results from two contrasting heterogeneous site investigations.

2.1. Introduction.

Soil is an excellent example of the concept of spatial heterogeneity that varies over a range of scales, particularly in terms of contaminant concentrations. Although a single field may appear uniform in colour and composition, from a closer distance, when inspected at a finer scale, a complex range of shapes, colours, pore spaces, biota and other is revealed. One consequence of this is that spatial heterogeneity of contaminants in soil causes uncertainty in environmental measurements of concentration during contaminated land investigations. Where contaminants are more heterogeneously distributed throughout a site there is a greater risk of misclassifying a site as either “contaminated” or “uncontaminated”. There is, therefore, either a potential risk to human health or unnecessary expense in remediation or, in the case of missed hot spots, possible litigation following subsequent discovery. Many sampling designs aim to reduce the impact of on-site heterogeneity, through the use of composite sampling, increased sample mass and off-site homogenisation (Gy, 1992), yet the end result is to potentially overlook the small scale heterogeneity that can have significant implications for exposure assessment and sampling strategies. Moreover, composite sampling and homogenisation may not be comparable to the behaviour of the target receptor, e.g. a child or plant, whose area of exposure maybe on a scale that differs from that of the original averaging area specified in the sampling design. The alternative approach, to accept and quantify heterogeneity, requires further exploration as contaminant heterogeneity is endemic within soils and its quantification should enable improved reliability in risk assessment.

Variability in contaminant uptake by plants is a further consequence of spatial heterogeneity in soils. Concentration factors for contaminant uptake into food plants are used in generic risk assessments to estimate exposure to humans. However, these are based upon pot trials where the contaminant of interest is

distributed homogeneously throughout the soil. Recent studies (Haines, 2002, Millis *et al.*, 2004, Podar *et al.*, 2004, Manciulea and Ramsey, 2006), using simple chequerboard style distributions, have shown that the spatial heterogeneity of heavy metals within soils has a substantial impact on the amount of uptake by plants. Both the degree and scale of heterogeneity are factors for some plant species, particularly in respect of root ball size and distribution. Therefore a model that can characterise heterogeneity across a range of scales can provide greater insight into the interpretation of a site under investigation.

Geostatistical methods of variography and kriging (Chapter 1, section 1.4.3, developed by Matheron and Krige for the mining industry, are the main statistical technique for environmental spatial assessment of contaminant concentrations in contaminated land investigations. Using typical sampling strategies e.g. regular grid or herringbone designs, the technique ideally requires a minimum of 150 (250 if anisotropic (Webster and Oliver, 2001)) samples (Webster and Oliver, 1993). Geostatistical methods assume a trend in concentrations with distance, as such, local variability can be misrepresented without prior understanding of the site (Goovaerts, 1999). Restricted by the typical sampling intervals, variograms often fail to assess heterogeneity over the full range of scales, e.g. 0.001 m – 100 m (5 orders of magnitude).

A nested nine point sampling design (Taylor, 2005) (Chapter 1, section1.4.3) and a balanced hierarchical design (Webster *et al.*, 2006) using analysis of variance, are two techniques that have been employed to tackle characterisation over a range of scales. Both require a large number of samples if applied to an entire site investigation, or only provide localised data within a sub-area, not necessarily representative of the total area, and therefore possibly misleading regarding the potential hazard.

The duplicate method (Ramsey *et al.*, 1992, AMC, 1995) is perhaps the simplest method that can used to estimate heterogeneity as variance (Ramsey *et al.*, 1992). Taking duplicate field samples, with two analytical duplicates on

both, allows the two key components of the variance to be estimated. Analytical variance arises from the random error that can occur during chemical analysis. Sampling variance represents the difference between two samples taken from the same nominal sampling location due to small-scale heterogeneity (Ramsey and Argyraki, 1997). The duplicate method can be easily applied to a site investigation using a balanced sampling design, with duplicate field samples at 10% (minimum of 8, (Lyn *et al.*, 2007)) of all sampling locations.

There are considerable benefits in understanding the degree and scale of heterogeneity of contaminants at a site under investigation. A site, demonstrating low heterogeneity at a range of scales, will require fewer samples in a subsequent secondary site investigation. Conversely, more heterogeneous sites may require greater sampling density to ensure risks are reliably identified.

2.1.1. Objectives.

This chapter introduces a new sampling design that can be used, in conjunction with (relatively new) *in situ* measurements techniques, to characterise the spatial heterogeneity of any contaminant, over a wide range of scales across an entire site of investigation, and addresses the following thesis objectives:

1. The development of a generic experimental design for quantifying heterogeneity over a range of scales.
   From a review of literature, develop a sampling design that enables quantification of heterogeneity across an entire site at scales ranging from 10 m to 0.001 m.

2. Determine whether heterogeneity significantly differs between different contaminants, and between different sites for the same contaminant.
   A new design will be applied to sites with contrasting contaminants and source characteristics using *in situ* measuring devices, and calculate the measurement uncertainty of the resultant measurements (including that

from sampling). The sites will be selected to have different levels of heterogeneity, caused by different sources of contamination (e.g. mine wastes, land fill, firing ranges – high expected heterogeneity, and land amended with sewage sludge or from aerial deposition from nearby smelter – low expected heterogeneity. The range of contaminants will be extended beyond those considered by Taylor *et al.,* (2005), (i.e. Pb and Zn) to include other elements (e.g. As, Cd, Cr and Ni) for which *in situ* measurement techniques are well developed.

2.2. New sampling design.

The proposed sampling strategy has been developed with no **bias** towards finding 'hot spots' of any particular shape, or any prior knowledge of the site for investigation, (i.e. non-judgemental). The foundation of the sampling design is a regular square grid, with samples to be taken at each node. The use of a regular grid for initial sampling is relatively simple to implement, enabling a large number of samples to be undertaken over a 2 day period and at a range of scales. (total number of samples using design in Figure 2.2.1, including analytical/instrumental duplicates, is 170) The number of samples taken at each sample distance is greater than in an eight point nested design and distributed randomly over the entire sampling area. Moreover the design is consistent with most recommendations in current literature (DoE, 1994), in that: (i) the area is partitioned into regular sub-areas; (ii) Each area carries at least one sampling location; (iii) it is systematic; it does not however meet the fourth criteria in that samples should not be aligned (except for the duplicate sample which is offset from the grid).

The new experimental sampling design (Figure 2.2.1), incorporates both the duplicate method and a balanced sampling design. Based on a 50 m by 50 m regular grid with a 5m spaced sampling density, duplicate measurements are taken at 2.0 m, 0.5 m, 0.2 m, 0.05 m and 0.02 m distance in a random direction from randomly selected 5m sampling locations. Sampling points and direction from origin, where duplicate field samples were taken, were randomly selected

using Microsoft Excel® random number generator, where each sampling point has an equal probability of selection. Separation distances on two logarithmic scales were selected to provide increased resolution of any trends in heterogeneity that occur with distance, and to provide data on scales that may be more relevant to plant species growing on contaminated land sites. The analytical variance (or instrumental precision) was estimated by taking two measurements at each of the sample points from one separation distance (0.20 m arbitrarily chosen) e.g. sampling point at origin and sampling point 20 cm from origin.

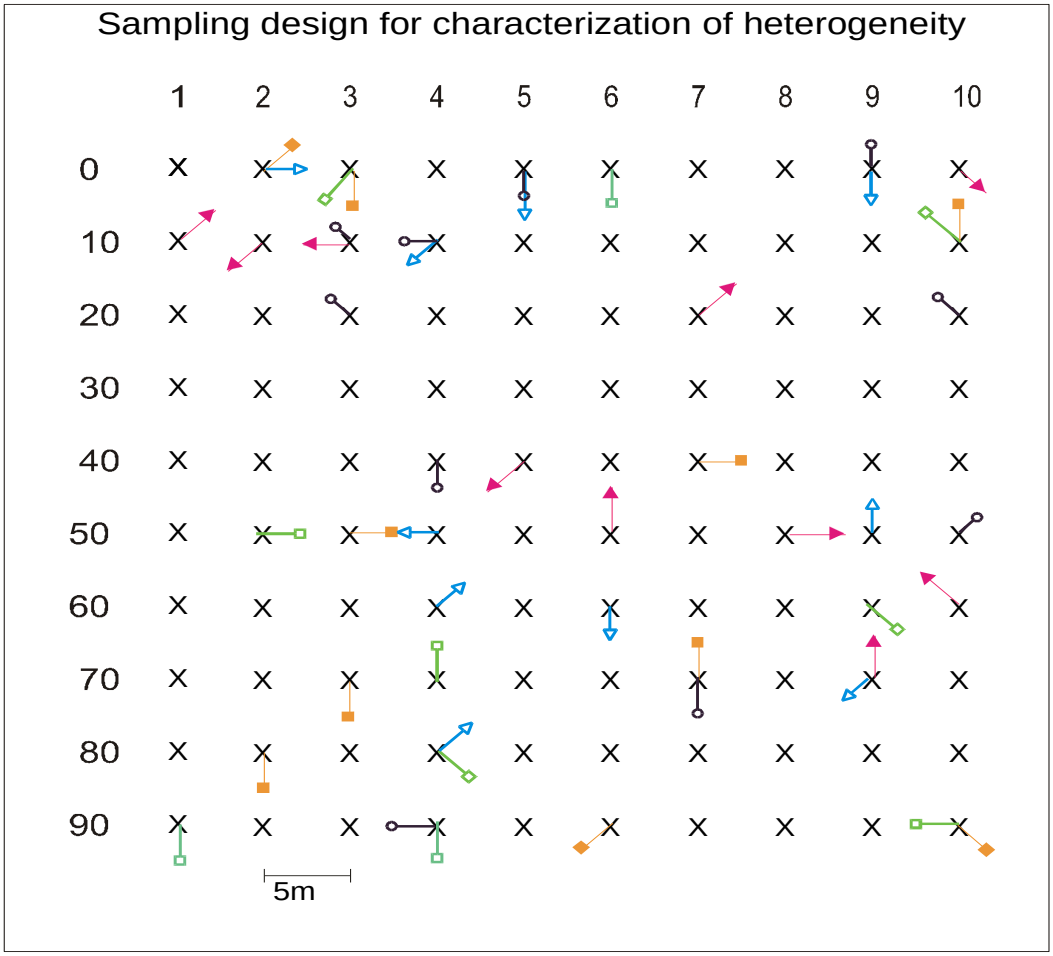


Figure 2.2.1. New sampling design for the characterisation of contaminant heterogeneity over a range of scales, where X represents each sampling point at 5 m spacing and arrows show 10 locations, chosen at random, for duplicate sampling points at each sampling scale.

2.3. Site histories.

This new design was evaluated at two sites, based upon previous research (Dyer, 2007 - Site 1, Coseley and Datta & Young, 2005 - Site 2, Nottingham), and selected to show contrasting degrees of spatial heterogeneity and concentrations of a range of heavy metals at levels that can reliably be detected using *in situ* measurement techniques (Appendix A, A.1 provides a list of detection limits for a range of heavy metals, published by the manufacturer of instrument used, together with a selection of background and regulatory thresholds).

Site 1, (Figure 2.3.1.) was chosen as it was expected to be moderately heterogeneous. Located in Coseley, central Wolverhampton, UK (Grid ref 394492, 295046), the site was once a colliery which has been subsequently in-filled with domestic and industrial waste and dredging from the surrounding canals (a major receptor of effluent from historical metal industries). Today, the site is an urban green space. The small sampling area within the site is mostly grass covered, and bordered by scrub and willow trees.

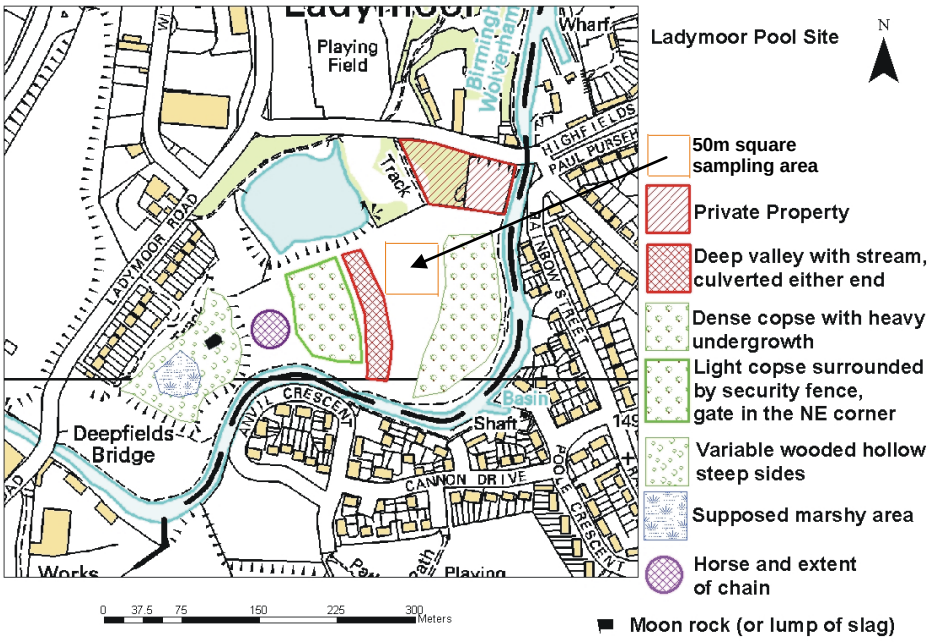


Figure 2.3.1. Map of Lady Pool urban green space in Coseley, Wolverhampton used for Site 1 investigation. Sampling area is highlighted in yellow square. (Source of basic map, Environment Agency)

Site 2 (Figure 2.3.2.) on the outskirts of Nottingham (Grid ref 464380, 340505) was historically used as drying pans for sewage sludge from the neighbouring industrialised area. The site is still used for sewage sludge disposal and agriculture, and at the time of sampling, the small subarea used for sampling was planted with oil seed rape. Sewage sludge is applied by spraying and subsequently ploughed into the top-soil, therefore low heterogeneity is expected at this site.

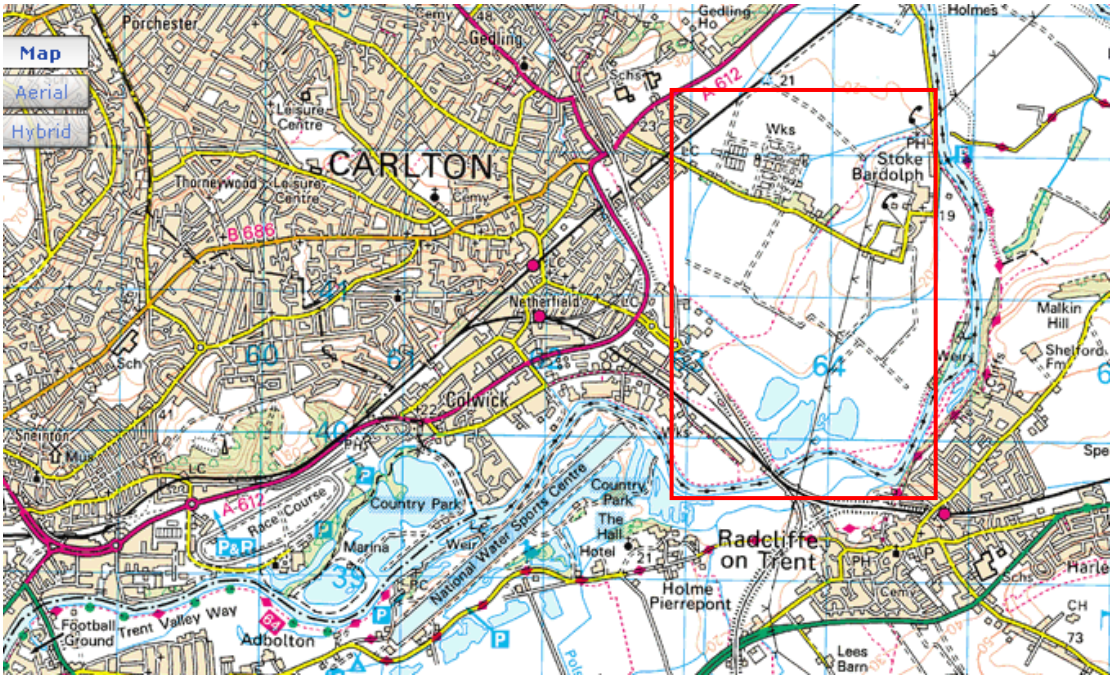


Figure 2.3.2. Aerial view of (Site 2) sampling area located in the south of Stoke Bardolph Farm (Google Maps, satellite image), on the outskirts of Nottingham (*from OS map © Crown Copyright Ordnance Survey. An EDINA Digimap/JISC supplied service*).

2.4. Measurement and analytical methods.

2.4.1. *In situ* methods – sampling scales 0.02 m – 20 m

The new sampling design was laid out at each site using a 5 m spaced regular grid (Figure 2.2.1), with sample duplicates located using a meter rule and compass. Measurements of 17 heavy metals in the topsoil were taken *in situ* using a Portable-X-ray fluorescence spectrometer (model used here was a NITON Xlt 700 with a battery powered x-ray tube as an excitation source). This relatively new technology enables a large number of sample measurements to be made in a short time frame and can be operated without disturbing the spatial heterogeneity of the test material. Soils were analysed, to a depth of approximately 1 mm, without removal or preparation (except to remove surface vegetation) to ensure spatial heterogeneity remained undisturbed (Figure 2.4.2). A Mylar® film disc was placed over the sampling location to protect the analyser window. Measurements at each location were taken for a count time of 60 seconds. A 60 second count time was determined to reliably quantify main target elements; Pb, Cu and Zn at expected concentrations and to ensure all measurement could be completed within a two day period.

Figure 2.4.1. P-XRF taking in situ measurements of undisturbed soil samples (adapted from NITON, 2008).

Figure 2.4.2. Photograph shows sampling area after removal of turf, Mylar discs are placed at sampling distance of 20 cm prior to measurement with P-XRF, labelled containers alongside are for core extraction.

After measurement readings sample cores, of approximately 65 mm diameter and 50 mm depth, were removed at specified sampling points, using a bulb planting device, and stored at 4 ºC to maintain structural integrity, in screw top 500 ml polypropylene straight-sided pots. In the laboratory, smaller cores, 26 mm diameter and approximately 9 mm depth, were extracted for further spatially resolved analysis with X-Ray microprobe to assess spatial heterogeneity of contaminants at scales less than 0.02 m. The estimated water content of soils was determined gravimetrically.

After correction for moisture content at each sampling point, data was analysed using a windows based software package, ROBAN version 1.01 (Water Resource Systems Research Laboratory, 2001) developed from a FORTRAN programme (Ramsey, 1998), based on earlier work (AMC, 1989). The package uses robust analysis of variance which is preferred to classical ANOVA as it accommodates a proportion of outlying values (≤10%), by down weighting outliers.

The use of a balanced sampling design enabled the two main components of random error from sampling (s_{samp}), a measure of heterogeneity, and analysis (s_{anal}) to be estimated (Chapter 1, Equation 1.4.3). A full balanced design (Figure 2.4.3) was used at sampling points where a sampling duplicate, in this instance, was taken at the 0.20 m **sampling scale**.

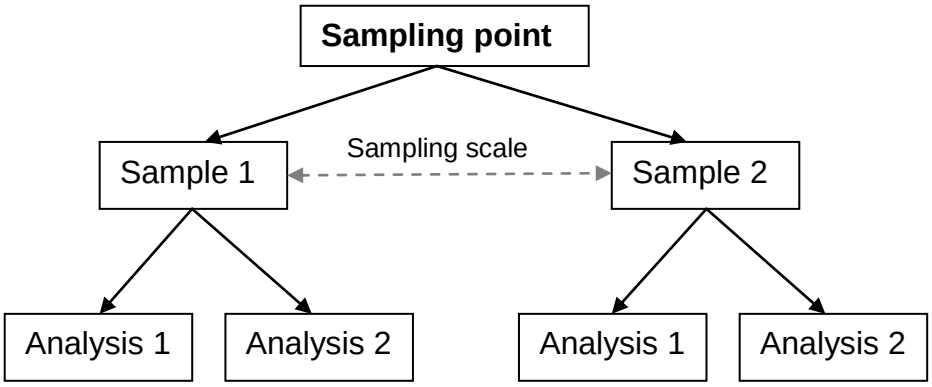


Figure 2.4.3. Full balanced sampling design employed at all sampling points where sampling separation scale is equal to 0.20 m (*ex situ* 0.002 m).

For all other sampling scales a simplified balanced design (Figure 2.4.4) was employed. By rearranging Equation 1.4.3 (Equation 2.4.1), and using the estimate for S_{anal} from the 10 duplicate readings of all sampling points taken for 0.20 m scale, S_{samp} was estimated for all other sampling scales.

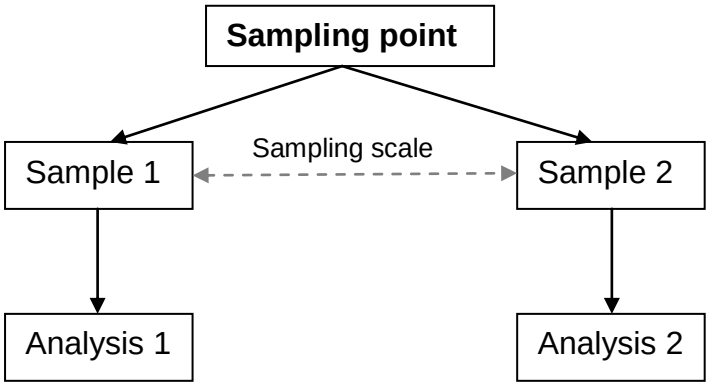


Figure 2.4.4. Simplified balanced sampling design employed at sampling scales 20 m, 5 m, 2.0 m 0.5 m 0.05 m 0.02 m (*ex situ* 0.005 m, 0.0005 m)

Equation 2.4.1. $s_{samp} = \sqrt{(s^2_{meas} - s^2_{anal})}$

2.4.2. *Ex situ* methods – sampling scales 0.0005 m 0.005 m

Sample cores were analysed by an Eagle II Edax energy dispersive spectrometer X-ray microprobe (XMP) to determine the spatial heterogeneity of contaminants (Pb, Zn and Cu) at the finer scale (less than 10 mm).

The XMP emits primary x-rays through a glass capillary, using a 40W rhodium x-ray tube. The glass capillary focuses the x-rays to enable a spot size of approximately 0.3 mm in diameter. The sample is mounted onto an adjustable stage beneath a high magnification camera. The stage is adjusted to obtain a horizontal surface prior to analysis (Figure 2.4.5). X-rays are detected using a nitrogen cooled liquid silicon crystal detector and processed using an EDAX data acquisition model. The XMP produces a characteristic fluorescent

wavelength for each element, with the photon count per second being proportional to the concentration.

Ten duplicate sample readings were taken for each separation distances of 5 mm, 2 mm and 0.5 mm from the original sampling point. Seventy eight sample cores from 39 sampling locations were removed from each site, retaining *in situ* heterogeneity. From the cores taken at the original sampling points, 30 were selected at random, using Excel random number generator, with each core having an equal probability of selection. Small sections of these cores were extracted using a cork boring device (26 mm diameter) to coincide with the area analysed with Portable X-Ray fluorescence (P-XRF). These smaller cores were placed in small petri dishes and sealed using cling film, to retain moisture content, structural integrity and *in situ* heterogeneity. Samples were transported, in cooler bags, to the science laboratory at English Heritage, Fort Cumberland in Fratton, Portsmouth for analysis with XMP. After correcting for moisture content, data were analysed using RANOVA.

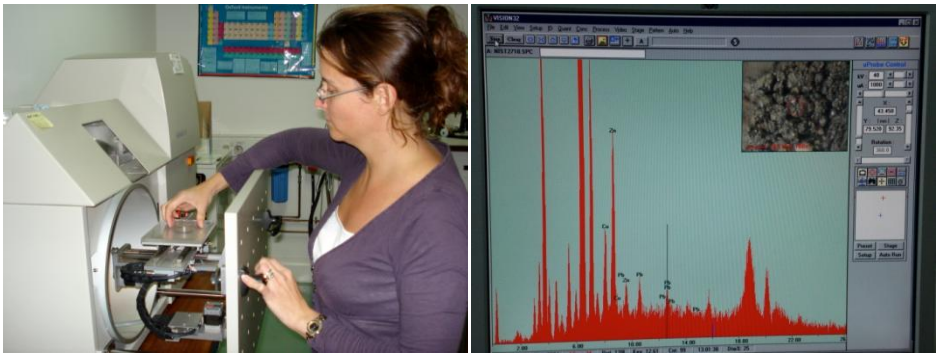


Figure 2.4.5. Photo of sample core being placed into vacuum chamber for analysis with XMP (left) and screen shot showing area of soil for analysis magnified and spectrographic results (right).

Additional details relating to instrument calibrations and settings can be found in Appendix A, A.2.

2.5. DATA QUALITY

2.5.1. Portable X-ray Fluorescence.

Detection limit.

There are a number of approaches to the calculation of detection limits for P-XRF in the literature. For Example: The Niton P-XRF determines detection limits as 2 standard deviations of the counts per second, for each reading, which may differ from published values that are based upon analysis of certified reference materials. (Niton, 2004).

However, Vanhoof et al., (2004) suggest using 3 times the standard deviation of measured concentrations of soil samples with low/background concentrations measured five times in succession. Whilst, Kalnicky and Singhvi (2001) suggest 3 times the standard deviation of twelve non-consecutive measurements of **certified reference materials** (CRM), e.g. National Institute of Science and Technology (NIST) 2709 for low concentrations and NIST 2711 and 2710 for mid – high concentrations, respectively. The P-XRF records a standard deviation value of the counts per second for each 60 second sample reading, and for each element. Detection limits for this research have been estimated by using the median value of 3 times the standard deviation value for counts per second, converted to concentration, of all sample readings (Appendix A A.3., Data Table A.2). Of the 16 elements measured, only 5 (Sb, Zn, Fe, Pb and Cu) were found to be above detection limits at all sampling locations across both sites.

Analytical precision and bias.

At present, there are no established guidelines as to the recommended level of precision specifically required for the validation of *in situ* analytical techniques. For this study instrumental precision was estimated by making two consecutive readings of the same sampling point to form the analytical duplicate required as part of a balanced design (Figure 2.4.3) using the duplicate method. The standard deviation of the analytical variance was calculated using robust

analysis of variance (ANOVA) and expressed relative to the mean for 95% confidence. The analytical precision estimates (Table 2.5.1) for both site investigations were below 8% (at 95% confidence) for Pb, Zn and Cu. This compares favourably to the published guidelines by the Environment Agency, Monitoring Certification Scheme (EA, 2006) which requires an analytical precision of less than 15%, at 95% confidence, for *ex situ* laboratory analytical methods.

Table 2.5.1. Summary estimates of data quality (instrumental precision (95% confidence) and bias) for measurements of Pb, Cu and Zn using P-XRF at Coseley and Nottingham site investigations.

	Instrumental Precision %		**Instrumental Bias %**	
Element	Coseley	Nottingham	Coseley	Nottingham
Pb	± 8.01	± 5.34	-6.57	-7.77
Zn	± 7.46	± 5.20	-6.32	-6.86
Cu	± 6.00	± 6.21	0	-3.8

The bias of the *in situ* method was estimated from repeated analysis, with P-XRF, of three certified National Institute of Standards and Technology (NIST) soil reference materials (2709, 2710 and 2711) at both sites. A regression analysis of P-XRF measurements, at Coseley, found a statistically significant (95% confidence) rotational (i.e. proportional) bias of -6.57% for Pb and –6.32% for Zn, with no significant bias for Cu. At Nottingham, the regression analysis of measurements found rotational bias of -7.77% Pb, -6.86% Zn and –3.80% for Cu. (Detailed regression analysis contained in Appendix A, Data Table A.4 and Data Table A.5, all other raw data on CD attached to rear cover).

2.5.2. X-Ray microprobe.

Detection limit

A silica blank was introduced to try and determine a detection limit, however this generated large abnormal defraction problems. As an alternative, for Coseley samples, several reference materials were analysed more than once to provide

a linear regression model of standard deviation versus measured concentration in parts per million (ppm). From this the standard deviation for a zero concentration can be extrapolated. Detection limits for Nottingham samples were estimated from a regression of the standard error for each measurement, and multiplying by 3, the value extrapolated at zero concentration. Taking the highest value, as a conservative estimate, detection limits for Pb, Zn and Cu are 262, 242 and 217 µg g^{-1} respectively.

Analytical precision and bias.

The precision of the XMP was estimated using the same method as for P-XRF and the instrument demonstrates good repeatability of measurements, with estimated values for precision (Table 2.5.2) below 8% for all elements analysed.

	Instrumental Precision	
Element	Coseley	Nottingham
Pb	0.82	2.80
Zn	2.01	0.75
Cu	2.62	7.42

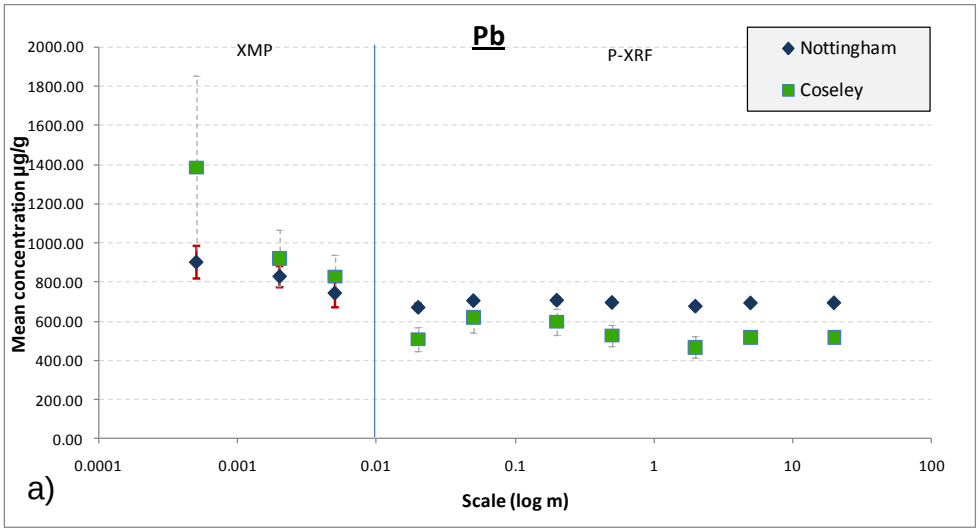
Table 2.5.2. Precision estimates for XMP analysis

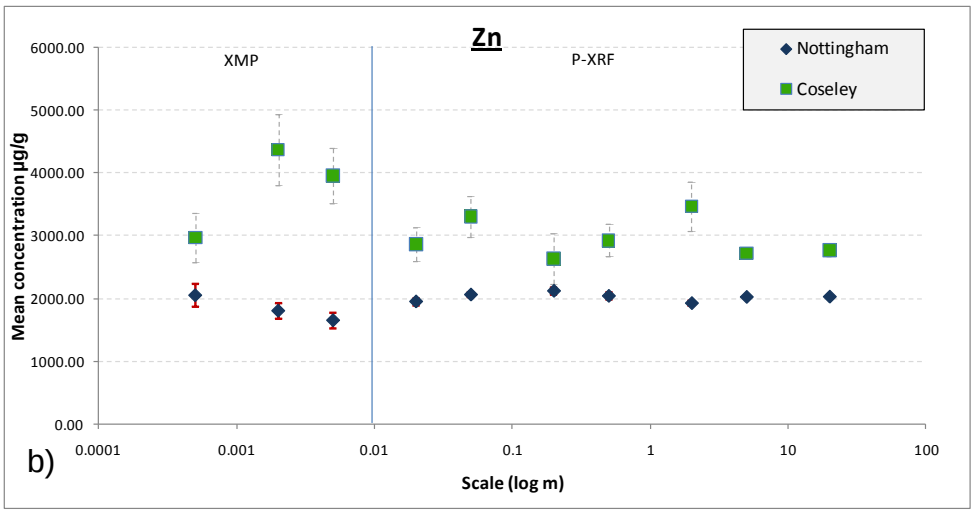
Calibration of the XMP was made using certified reference materials, for estimation of bias, alternative reference materials should be analysed, to preserve an independent estimate of bias. A lack of available reference materials meant that those not used in calibration were of low concentrations and generally below XMP detection limits. Regression analysis found analytical bias was not significant for Pb, and Cu for both sites, and no adjustment made. A positive rotational (19.39%) and a negative translational bias (-38.90 µg g^{-1}) were detected for Zn on samples taken from Coseley site, and measured concentrations were adjusted before analysis. (Data and detailed spread sheet analysis in CD attached to rear cover.)

2.6. Results and discussion.

Measured concentrations taken *in situ* with P-XRF were corrected for the moisture content in the soils (approximately 30-40 %m/m) at each sampling point individually. The robust mean and standard deviation for each sampling scale were estimated for each element and tables of summary statistics can be found in Appendix A, Data Table A.6 (Pb), Data Table A.7 (Zn), and Data Table A.8 (Cu).

The results for Pb, Zn and Cu, (Figure 2.6.1) show that the mean concentration does not vary substantially between each sampling scale at both sites. However, measured concentrations made using XMP are higher than those using P-XRF and this is most likely due to bias that could not be detected with the reference materials available. Larger error bars on mean values for Coseley indicate a greater range in concentration measured at this site than for Nottingham and this arises from the greater heterogeneity of contaminant distribution expected at this site.





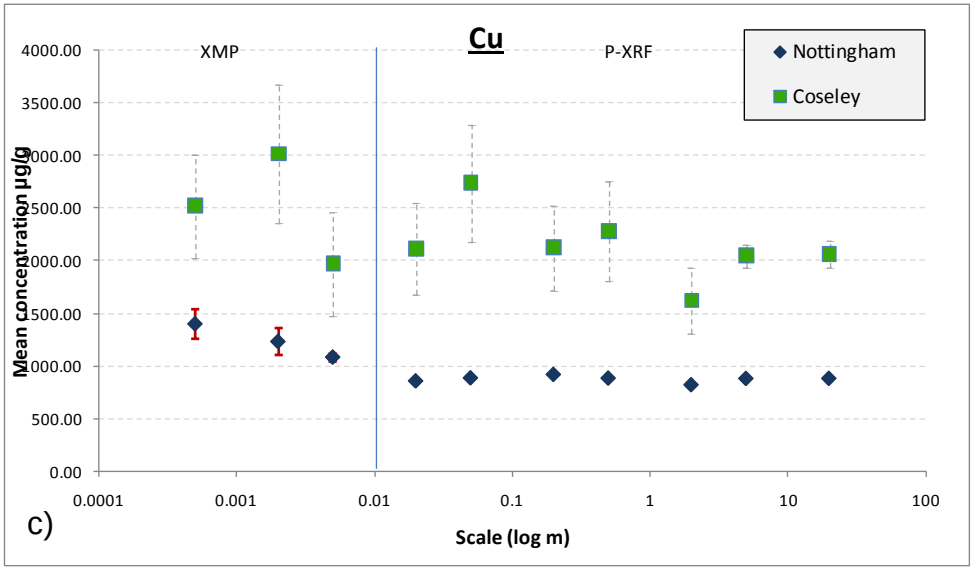
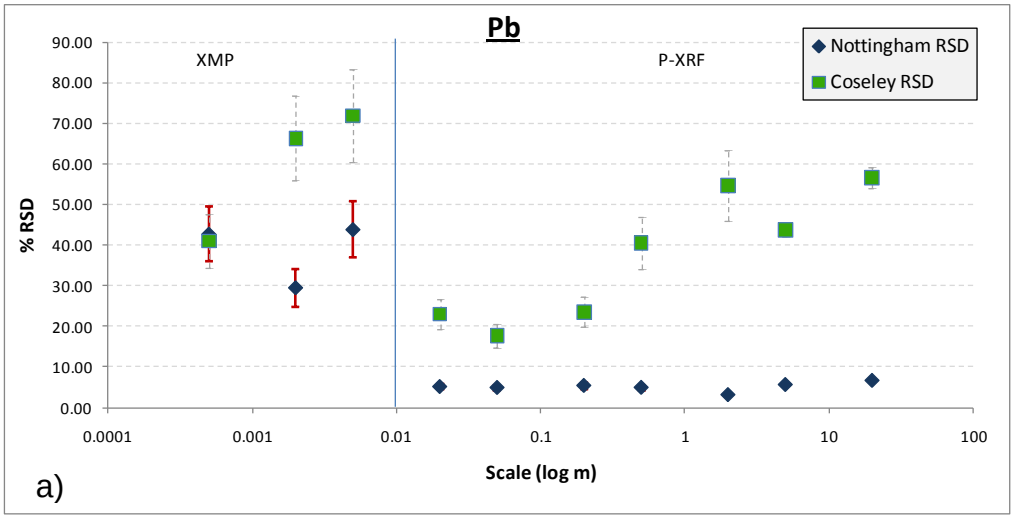
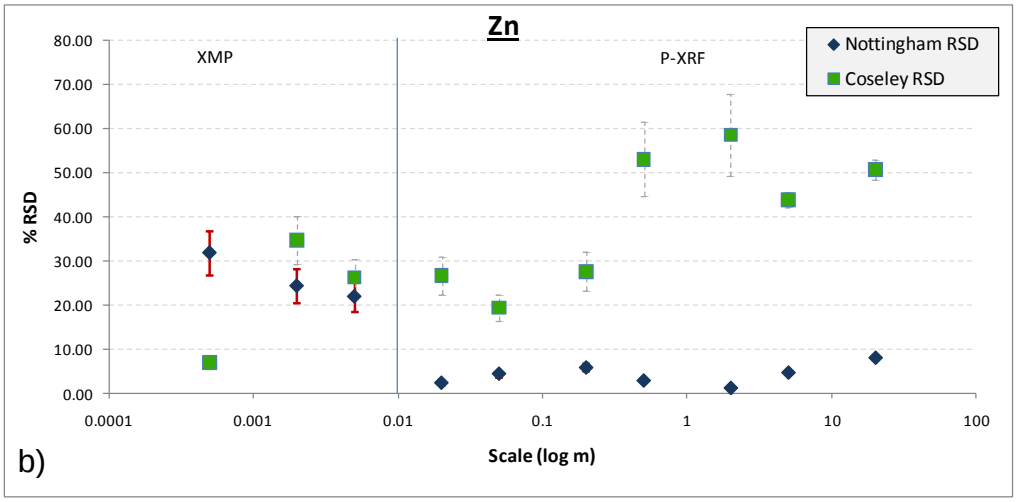


Figure 2.6.1. Mean measured concentration, at logarithm of each sampling scale for a) Pb, b) Zn and c) Cu at each site. Error bars represent the standard error on the mean.





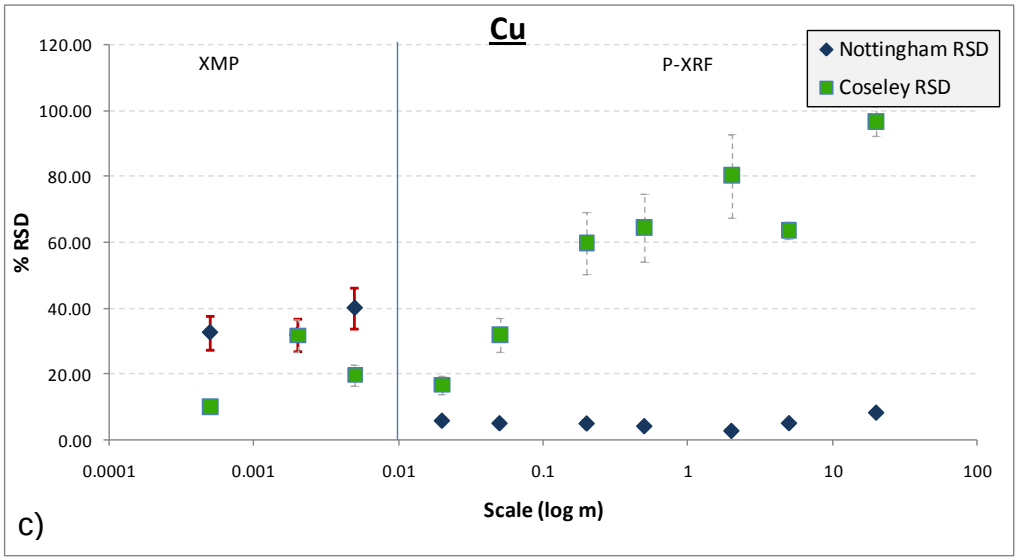


Figure 2.6.2. Heterogeneity, expressed in %RSD at each sampling scale (logarithmically adjusted) for a) Pb, b) Zn and c) Cu at both sites. Error bars represent the relative standard error on the standard deviation, calculated using $((s/\sqrt{2n})/\bar{x}*100)$ **(Baten, 1942).**

Using the duplicate method the degree of heterogeneity can be expressed using the standard deviation (estimated from sampling variance) expressed as a percentage, relative to the mean (%RSD).

Relative standard deviations for all 3 metals, measured *in situ* with P-XRF for cm to m scale, at Nottingham (Site 2), confirm expectations of low heterogeneity. All three elements have a RSD around 5% for each sampling scale, with average values of 5.06% (Pb), 4.17% (Zn) and 5.22% (Cu) (Figure 2.6.2.a), b), and c) respectively). These findings would confirm those of Taylor *et al*.,(2005), who suggested that heterogeneity does not change systematically as a function of scale, at some sites.

Heterogeneity (%RSD), at the finer scales (< 10mm), calculated from measurements made *ex situ* with XMP increases significantly with average values of 38.67% (Pb), 26% (Zn) and 34.78% (Cu). This apparent increase is in some part explained by the difference in aperture size between the two instruments. Aperture of the XMP measures a circular surface area of 0.38 mm^2, more than 500 fold smaller than the surface area measured using P-XRF of 200 mm^2. According to sampling theory by Gy (1992), increasing sample mass has the effect of reducing the sampling error by the square root of the factor of mass increase, equating to a greater than 20 fold increase heterogeneity for XMP measurements. The larger error bars on measurements using XMP, compared to P_XRF suggest again, that heterogeneity at Nottingham does not change as a function of scale.

For Coseley (Site 1), the story is more complex, but it is evident that the contaminants are more heterogeneously distributed overall than at Site 2. %RSD for Cu (Figure 2.6.2, c), measured using P-XRF, ranges from just below 20% at the 0.02 m scale up to 100% at the 20 m scale. For Pb and Zn at Coseley there is a similar distribution, both have RSD ranges from 20% up to maximum of 60%. The difference between Cu and Pb and Zn is possibly due to source of contamination. Metallic Cu shavings have been reportedly found (Ramsey, 2010a) in canal dredging deposited at the site, a legacy of the historic

local copper panning industry, whereas Pb and Zn will arise from general landfill waste. There is a general trend of increased heterogeneity with distance between sampling locations. This change of heterogeneity as a function of scale would fit the conventional variogram model, which assumes a relationship between change in variance with distance (Chapter 1, Section 1.4.3.).

Finer scale heterogeneity for Zn and Cu at Coseley continues to reduce to RSD 7.09% and 10.05% respectively.

Concentrations of Pb are close to the detection limit for XMP, with 9 samples falling below, therefore this instrument is not suitable for analysis of Pb at this site.

2.7. Conclusions and further work.

The results of this study show that the new sampling design, used in conjunction with the duplicate method, can characterise spatial heterogeneity across a range of scales for Pb, Zn and Cu at two contrasting sites. The degree of heterogeneity can be expressed numerically in terms of relative standard deviations for each sampling distance. Heterogeneity was shown to vary significantly between sites, as a consequence of differing historical uses. Moreover spatial heterogeneity was found to vary between sampling scales at the more heterogeneous site and between contaminants at the same scale and site.

The analysis was only completed for 3 elements, but could easily be expanded to include a variety of contaminants. Cr, Ni, Mn and Sr are four further heavy metals found at elevated levels at both sites, whilst some sampling points were below levels of detection, increasing the analysis time for a further 60 seconds, may yield a complete set for these contaminants. The ability to quantify heterogeneity could be used in the development of improved sampling strategies for secondary site investigations. However for the purpose of this research the results, from quantification of spatial heterogeneity of Pb, Zn and

Cu, are **fit for purpose** of assessing the impact of heterogeneity on plant uptake.

Further work is required to compare the %RSD method of quantification to alternative methods, such as variograms using the same sampling design and the number of samples required at any given scale. Also to establish a robust method to characterise heterogeneity at the finer scale and reconcile differences that arise from the differing sizes of instrumental aperture and volume of material.

Chapter 3 Pot experimental methods to assess the impact of variable contaminant heterogeneity, at the 0.02m scale, on root and shoot accumulation and plant biomass for B. napus, B. juncea, P. lanceolata and T.officinale.

3.1 Introduction

This chapter covers the design of pot experiments to simulate realistic field contaminant heterogeneity, at a scale that is relevant to plants selected for experimentation. It will discuss the background to the experiment, and justify choice of contaminant, concentration levels and species selected for testing. Also covered are details of experimental methods in pot preparation, subsequent analysis and data quality control used in Chapter 4 and Chapter 5.

3.1.1. Objectives of experiment

1. To develop an experimental pot trial to mimic field *in situ* heterogeneity of contaminants, in 2 dimensions.
2. To assess whether intermediate variation in *in situ* spatial heterogeneity of soil contaminants has a significant effect on plant uptake of contaminants and whether realistic heterogeneity generates results that are statistically different from predictions made using simplistic binary models of heterogeneity.
3. To select suitable plant species, contaminant of interest and respective concentration.
4. Determine number of replicates.
5. To determine the data quality of measurement results obtained.

3.2. Background to experimental design.

3.2.1. Quantification of *in situ* heterogeneity.

Following two site investigations using a new experimental sampling design (Thomas *et al.*, 2008) and *in situ* measurement techniques, heterogeneity was estimated for several heavy metals, in soil, over a range of spatial scales (Chapter 2). The degree of heterogeneity can be expressed in terms of relative standard deviations (%RSD) (See Analysis of variance and nested sampling designs.Chapter 1, Section 1.4), where a homogeneous distribution would result in a %RSD of zero. Field sites were chosen to provide contrasting heterogeneities and concentration levels of heavy metals present that could be readily detected using the *in situ* measurement technique (i.e. P-XRF).

Concentrations of Pb, Zn and Cu were quantified at all sampling locations, providing a complete dataset, and the results in Table 3.2.1 demonstrate how the spatial heterogeneity, quantified using %RSD (See Chapter 2 for methodology), of contaminants varies; between sites, with an average of 42 %RSD at site A and 4.5 %RSD at site B; between contaminants within the same site, 24 %RSD for Pb and 60 %RSD for Cu at the 0.20 m scale for Site A; and between sampling distances, 16 %RSD at 0.02 m distance and 80 %RSD at 2 m distance, for Cu at Site A.

Table 3.2.1. (% RSD) for Pb, Zn and Cu measured in situ using Portable X-Ray Florescence (scales 0.02 m – 20 m).

	Site A (Coseley) – Moderate heterogeneity - %RSD			Site B (Nottingham) – Low heterogeneity - %RSD		
Scale (m)	**Lead (Pb)**	**Zinc (Zn)**	**Copper (Cu)**	**Lead (Pb)**	**Zinc (Zn)**	**Copper (Cu)**
0.02	**23.0**	**26.8**	**16.7**	**4.3**	**2.3**	**5.9**
0.05	17.7	19.5	32.0	4.1	4.4	5.1
0.2	23.5	27.7	59.8	4.7	5.8	5.1
0.5	40.5	53.1	64.4	4.1	2.8	4.2
2.0	54.6	58.6	80.3	1.4	1.2	2.8
5.0	43.7	43.8	63.7	4.9	4.7	5.1
20.0	56.6	50.7	96.8	6.0	8.0	8.3

3.2.2. Spatial scale of heterogeneity for use in pot experiment.

The values of heterogeneity to be used in the pot experiment reflect the small volume of soil contained within the pot and the heterogeneity that is potentially seen by the roots of a chosen plant species. Therefore, for the purposes of this experiment, *in situ* heterogeneity found at the 0.02 m scale has been chosen, as this can be replicated within 0.11 m sq. pots. Moreover, earlier studies (Manciulea and Ramsey, 2006) of heterogeneity at a similar scale (0.03 m) using simplistic chequer board models (Table 3.2.1, C) have shown changes in heterogeneity can significantly impact plant uptake by as much as 76%.

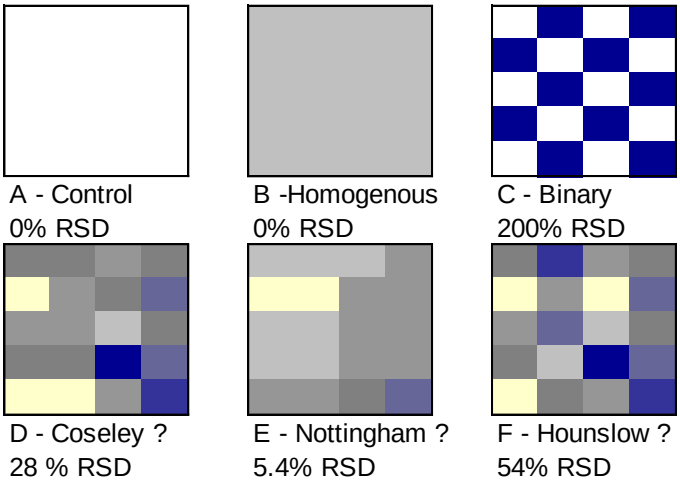


Figure 3.2.1. Heterogeneity models, A – C, simplistic models used in plant uptake and early heterogeneity studies, D – E illustrate how heterogeneity may look based upon actual field *in situ* measurements, based on findings in Chapter 2. F is an illustration of expected heterogeneity of a firing range based upon a site investigation by Taylor et al (2005). Depth of colour is indicative of concentration level.

Using estimated total element concentrations recorded *in situ*, rounded to the nearest 100 µg/g, for 0.02 m sampling distances, experimental pot models can be simulated using simple excel models that generate similar heterogeneity, in %RSD, to that found *in situ* (Section 3.3.5).

3.3.Contaminant and plant species for use in pot experiment (objective 3).

3.3.1. Contaminant of interest.

Selection of a suitable soil contaminant for use in pot trials is based upon the following criteria:

(i) Concentrations found *in situ,* in the field, at levels that can be reliably detected using P-XRF (Chapter 2, Section 2.4) at all sampling locations.

(ii) Bio-availability of particular form of contaminant for plant uptake.

(iii) Contaminant is trans-located to plant shoots at concentration levels that can be reliably measured with low uncertainty to determine statistically

significant effects of heterogeneity, typically at concentrations above 1 part per million for AAS and ICP-MS.

In situ measurements were made for 15 inorganic metals, of these 3 heavy metals were found to occur at all sampling locations at levels above the P-XRF detection limits for; Pb, Cu and Zn.

3.3.2. Plant species and growth conditions.

A preliminary pot trial, using soil containing a range of bioavailable trace elements that were randomly collected from the site at Nottingham, was undertaken to assess plant species suitability for a greenhouse experiment in contaminated soils. Three species, found common to the sites used to estimate *in situ* heterogeneity in Chapter 2; *Plantago lanceolata* (common name ribwort plantain) , *Taraxacum officinale* (dandelion) and *Brassica napus* (oil seed rape) (all tap root species) were chosen to trial in a pilot experiment. A further species with different root morphology (ball root), which has also been shown in earlier research (Blaylock *et al.*, 1997, Ebbs and Kochian, 1998, Podar *et al.*, 2004, Manciulea and Ramsey, 2006, Turan and Bringu, 2007) to take up a range of heavy metals, *Brassica juncea* (Indian Mustard), was chosen for comparison in the pilot experiment.

Seeds were sown in Sinclair® 2.0 - 5.00 mm lightweight density vermiculite, with neutral pH (in the range of pH6 – pH7) and left to germinate in a glasshouse with temperature at 20ºC ± 5ºC and simulated sunlight for 16 hours. After the appearance of the first true leaves (e.g. 10 days for *B. juncea,* 22 days *T. officinale*) 5 seedlings of equal height and appearance were transferred into individual circular, litre pots (13 cm deep and 10 cm wide) containing soil from Site B (sewage sludge amended soil) and watered daily, from below, with tap water. Pots were placed into 5 groups containing one plant from each species. Pot groups and pots within groups were rotated clockwise 90º on a weekly basis to reduce the effects of uneven environmental conditions within the

glasshouse. Plants were harvested when the above ground biomass was sufficient to provide enough material for analysis, or when the plant died, whichever was the earlier. Plant stems were cut 0.01 m above soil surface for harvesting of shoots. Roots were removed from soil, using a sieve. Both plant sections were repeatedly washed with reverse osmosis water and gentle abrasion to remove surface soils. Plant material was dried at 60 ºC for 48 hours and then ground using a zirconium oxide ball mill. Metals were extracted into solution using a nitric and perchloric digest method (Appendix D D.1),(Thompson and Walsh, 1983) and analysed, in batches, using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Reagent blanks and CRM's were included in each batch to estimate analytical precision and bias (Appendix D, D.3).

3.3.3. Preliminary pot trial – Results and discussion

From the analysis of the plant growth, biomass and survival rates (Table 3.3.1), only *B. juncea* had a 100% survival rate until harvest, and yielded the most plants with sufficient biomass for analysis of roots and shoot separately. *B. napus* had very poor root growth in the pot trial, yet yielded similar shoot mass to *B. juncea*. Both *Brassica* species were seen to show signs of **chlorosis** in the form of leaf curling, loss of green pigmentation, changing to white and in some cases purple. Whilst the *P. lanceolata* and *T. officinale* took some time to establish, surviving plants went on to produce good root and shoot biomass. Plant failure was not thought to be caused by high soil contamination, but more likely due to over watering, as all species used in the trial are known to be tolerant of soils with elevated heavy metal content (Wu and Antonovics, 1976, Pollard, 1980, Kabatapendias and Dudka, 1991, Keane *et al.*, 2001, Turan and Bringu, 2007).

Table 3.3.1. Plant growth, time to harvest and available biomass.

Plant species	**Time to harvest (days)**	**Adequate root biomass (no. of plants)**	**Adequate shoot biomass (no. of plants)**	**Total no. of plants surviving**
T. officinale	104	1	1	1
P. lanceolata	105	3	3	4
B. juncea	56	3	3	5
B. napus	56	1	1	4

Currently there are no methods that can determine successful removal of all soil particles from roots prior to analysis, and even a small particle of soil containing high metal concentrations can introduce bias, variability and uncertainty into the measurements of metal concentrations in herbage (Ramsey *et al.*, 1991). However, the results of root and shoot concentrations in the preliminary pot trial provide a useful indication of plant uptake, translocation and accumulation of the four species at a given time in the plant growth cycle.

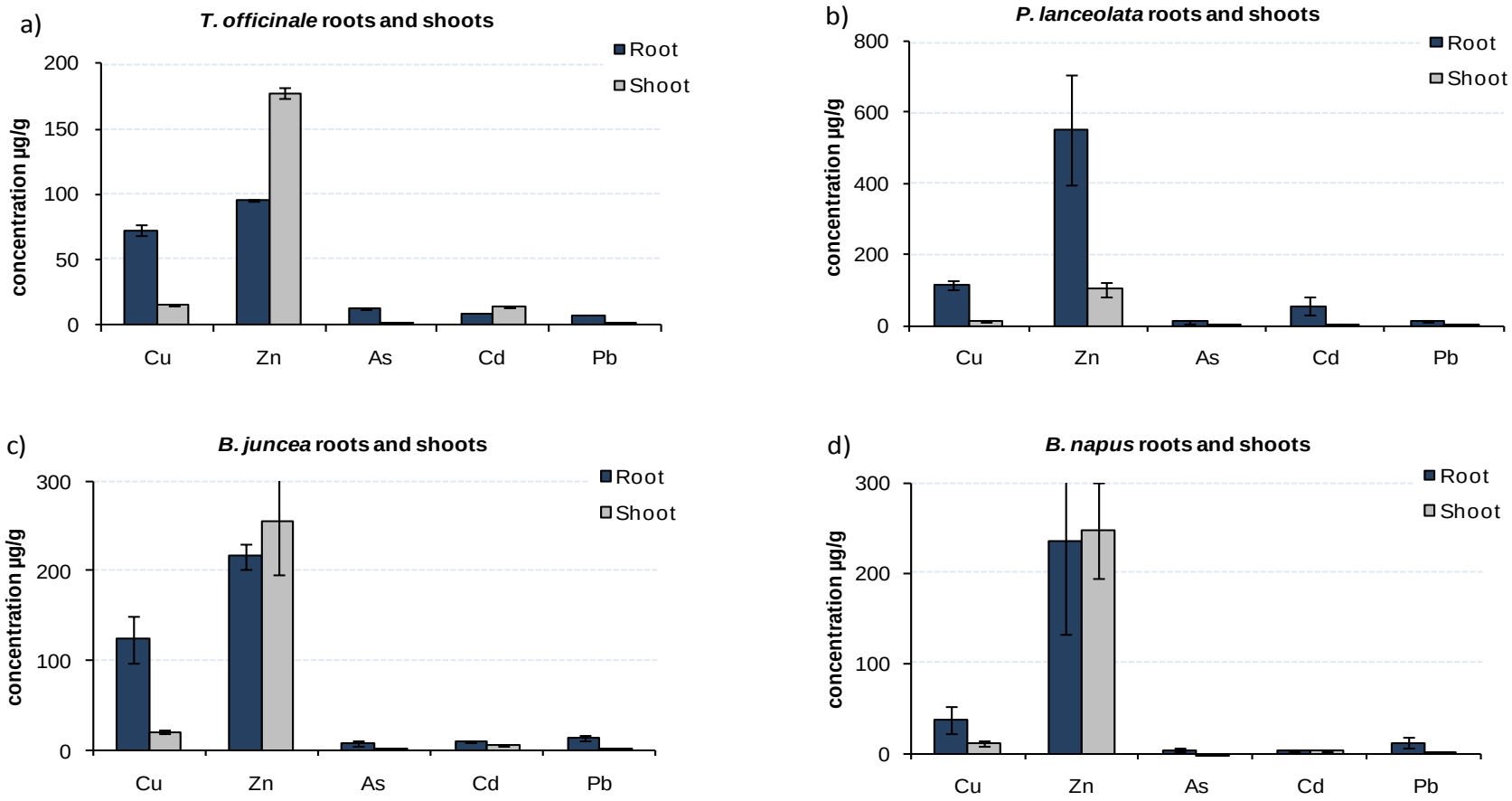


Figure 3.3.1. Measured concentrations (µg/g) of heavy metals in dry weight of roots and shoots for a) *T. officinale*, n = 1 b) *P. lanceolata* n = 3 and c) *B. juncea*. n = 3 In pot trials and d.) *B. napus* taken from field study n = 40 (NB differences on scale of y axis). Error bars represent 1 s.d. (calculated from analytical duplicates in the case of *T. officinale*).

Of the contaminants analysed, zinc is present in the soils at the highest concentrations, averaging 2031 μg g^{-1}, and this is reflected in the concentrations in plant **dry weights**. The highest concentrations of zinc were found in the roots of *P. lanceolata* (Figure 3.3.1.b), averaging 552 μg g^{-1} ± 156 μg g^{-1} (1 s.d.), greater than *T. offinale* (Figure 3.3.1.a) by a factor of 5. *P. lanceolata* roots also contained the highest concentrations of Cd, averaging 55 μg g^{-1} ± 26 μg g^{-1}, which is higher than the maximum measured Cd concentration in the soils of 35.11 μg g^{-1}. This suggests accumulation of Cd in roots by *P. lanceolata*, as opposed to elevated results from inadequate soil removal during washing. Further research is needed to confirm this interesting finding, but is outside the scope of this experiment as Cd cannot easily be measured *in situ* using P-XRF at concentration levels below 20 μg g^{-1}.

Zinc was found at the highest measured concentrations in shoots, rather than roots, of all species, with *B. juncea*, (Figure 3.3.1.c) showing highest uptake into shoots of 254 μg g^{-1} ± 58 μg g^{-1}; *B. napus* (Figure 3.3.1.d) 247 μg g^{-1} ± 53 μg g^{-1}; *T.officinale*, 178 μg g^{-1} (no replicates due to unsuccessful growth) and *P. lanceolata* 103 μg g^{-1} ± 20 μg g^{-1}. Actual levels in plant shoots are likely to be up to twice this value as significant negative analytical bias for zinc of 49% was detected in the analytical method. The cause of the bias was identified and an adjustment made as a result of the use of an inappropriate internal standard used in routine analysis with ICP-MS. No correction has been made to the results shown in Figure 3.3.1, as these are fit for purpose, however the internal standard, causing the error was not used in the main experiment.

T. offinale was the only species to show significant translocation and accumulation of contaminants from roots into shoots, with almost a ratio of 2:1 for zinc in shoots versus roots. Similarly a ratio of 3:2 for Cd was found. Concentrations of Cd in shoots of *T.officinale* were greater than all other species by at least a factor of 2. Whilst Cd cannot easily be detected using P-XRF, Cd is a contaminant that can be harmful to human health even at very low levels (Alloway and Ayres, 1997) and makes this an interesting plant for further study (Chapter 6).

Measured copper concentrations in shoots ranged between 10-20 μg g^{-1}, less than zinc concentration by a factor of 10. Lead and arsenic concentrations in plant shoots were not detected above 1 μg g^{-1}, despite being present in soils at 697 μg g^{-1} and 34 μg g^{-1} respectively. Two key factors may account for this; (i) metals may be bound in the soil in a form that is not readily bioavailable, and/or plant species successfully exclude Pb and As (Kabata-Pendias and Pendias, 2000).

In conclusion, the most suitable contaminant for use in the main pot trial would appear to be zinc, as this is found in the highest concentrations in above ground biomass, and therefore easily detectable with AAS and ICP-MS. *B. juncea* appears to be the most suitable species, as zinc is trans-located to shoots in concentrations that can reliably be detected using various analytical techniques (e.g. AAS, ICP-MS). It is also one of the fastest growing, and most successful plants, with the highest survival success rate, in the preliminary pot trial. Though this domesticated plant species was not found growing wild at any of the sites used for quantifying *in situ* heterogeneity its different root morphology to the other 3 plant species, fast growth rate and success in poor soils make this a useful plant for comparison. Off the 3 species found at heterogeneity study sites, the much less studied *T. officinale* is perhaps a more interesting species as it appears to be translocating a higher percentage of contaminants into above ground biomass, it is also common throughout the UK on waste ground. Also *B.napus*, with similar Zn concentrations in shoots may provide more insight into the effect of root morphologhy in heterogeneous distributions and plant uptake. As each species yielded sufficient Zn concentrations in shoot biomass, all 3 species were included in the main pot experiment.

There are many factors that will influence plant uptake of contaminants, some of which are specific to individual plants, especially where a plant has many genotypes. To isolate the impact of contaminant heterogeneity in soils, it is useful to determine variability between individual plants of the same species, with all other conditions being constant. One approach may be to grow a number of replicates hydroponically, with a spiked solution of zinc to isolate

between plant variance (Flowers, pers comm). An alternative is to obtain seeds grown from a specific accession number, and grow replicates in homogenous soils, for an estimate of between plant variability. Previous studies, e.g. Podar *et al*.,(2004), *Ebbs et al*., (1997) and Hamlin and Barker (2006) have used *Brassica juncea* (L.). czern., accession number 426308. There is no equivalent for *Taraxacum officinale*, however seeds can be collected from a single flower head to reduce genetic variability, with each flower head producing between 50-200 seeds. Seeds for *P. lanceolata* and *B. napus* came from a specific meadow site in Yorkshire (Map red SE 007 823) and an agricultural supplier (Oil seed rape, variety ES Astrid) respectively and the same seeds were used in the main experiment. (For detailed description and suppliers of seeds refer to Appendix B, B.1)

3.3.4. Concentration in spiked soils.

Podar *et al*.,(2004) and Manciulea and Ramsey (2006b) both used powdered Zn oxide (ZnO), diluted in a carrier medium of sand, for spiking of soils, in pot experiments. Powdered Zn oxide is preferred over nitrate solutions for heterogeneity studies as it is not as easily leached from soils, thereby retaining the spatial heterogeneity of the chosen contaminant. ZnO is also more typical of the form of Zn found in contaminated soils (Maskall *et al.*, 1998, Manahan, 2000) that is bioavailable to plants (Kabata-Pendias and Pendias, 2000). The total concentrations for each simulated heterogeneity design should be, nominally, the same, with the only changing factor being the distribution of the contaminant throughout the soil. The aim of this section is to determine the total concentration in each treatment and the distribution.

Measurements of soils using P-XRF give an estimate of the total element concentrations. However, total element concentrations do not provide information regarding how the element is chemically bound to other soil constituents, or more importantly, what percentage of the element is bioavailable for plant uptake (See Semple *et al.*, 2004 for a definition and review of the terms bioavailable and bioaccessible). The total measured concentrations

of Zn, at Site A, ranges from 385 μg g^{-1} up to 8476 μg g^{-1}, and at Site B from 1578 μg g^{-1} to 2650 μg g^{-1}. Soils spiked with concentrations in the upper ranges found at these sites may be **phytotoxic** to a range of plants (Alloway and Ayres, 1997), yet both sites were covered with vegetation, possibly due to the limited **bioavailability** of Zn. Availability of these contaminants to plants will vary depending upon plant species, genotype (Haines, 2002), source of contamination, i.e. how it is chemically bound within the soil (Manahan, 2000), and a wide range of soil properties, including pH, organic matter content and the presence/absence of other elements (Kabata-Pendias and Pendias, 2000, Podar *et al.*, 2004). In the literature there are numerous techniques used to determine the exchangeable/extractable bio-available fraction of a wide range of contaminants in soil and water (See Rao *et al.*, 2008). Two widely used and accepted methods for extractable Zn are those developed by Tessier *et al*., (1979) using magnesium chloride ($MgCl_2$), and the standardized extraction method adopted by the European Commission and developed by Quevauviller (1998) using 0.05 M ethylenediaminetetraacetic acid (EDTA).

The study area at Nottingham was located in a small-sub area of a much larger site that has been the subject of several studies. A study by Datta and Yound (2005), extracted Zn and Cu, using the EDTA method from 17 soils collected across the entire site. The results of this study (Table 3.3.2) show, that on average, 45% of total Zn is available for uptake. In particular, findings for soil sample ID numbers 10 and 11 have similar total concentration values for Zn (2030 μg g^{-1}), Cu (884 μg g^{-1}) and Cd (35 μg g^{-1}) to those measured using P-XRF in the small sub area defined as field numbers 10 and 11 (highlighted values in Table 3.2.1). Taking 45% of the average measured total concentration for Zn of 2030 μg g^{-1} provides a probable bio-available concentration of 913 μg g^{-1}.

Table 3.3.2. *Source: Datta & Young (2005), table 1. p125. Extract from total Zn concentrations (aqua regia) and extractable (0.05M EDTA) and % extractable from analysis of 17 soils collected from site at Nottingham.

Soil sample ID	Total Metal ug/ g	Extractable ug/ g	Extractable fraction %
6	443	182	41.08
7	1211	617	50.95
8	214	96.7	45.19
9	1184	458	38.68
10	**2174**	**961**	**44.20**
11	**2165**	**1100**	**50.81**
12	1768	828	46.83
13	1798	796	44.27
14	202	87.1	43.12
15	186	69.2	37.20

3.3.5. Constructing models for use in pot experiment.

A Excel® based computer model (Appendix D,D.4 shows a worked example for Nottingham site investigation) in conjunction with software package Roban version 1.1. (Water Resource Systems Research Laboratory, 2001) developed from a FORTRAN programme (Ramsey, 1998), based on earlier work (AMC, 1989), was used to model heterogeneity designs for use in pot experiment. Models were produced with similar heterogeneity expressed in %RSD terms to that measured *in situ* at the 0.02 m sampling scale (Figure 3.3.2, a. and b.). Heterogeneity at Site A, Coseley, is estimated at 26.81 %RSD, and Site B, Nottingham, at 2.34 %RSD for Zn at the 0.02m scale (Table 3.2.1). A further pot design, based upon a study of heterogeneity at a firing range by Taylor et al., (2005) was generated at an increased level of heterogeneity (Figure 3.3.2, c.) of 50.94 %RSD, to see if a relationship exists between degree of heterogeneity and plant uptake. Included in the experiment are pot designs previously used in

plant uptake studies by (Manciulea and Ramsey, 2006) and (Podar *et al.*, 2004), a simplistic binary heterogeneity model and a homogenous model.
Where possible, the average nominal concentration to be contained within each pot has been kept within 2% of 900µg g^{-1} of zinc.

900	500	900	1100	750
400	500	1100	1200	900
400	400	800	1100	800
500	900	1400	1600	1200
750	800	800	1400	1400

a) RSD 27.86% Avg. ug/g 900

750	750	800	900	900
750	750	800	900	900
800	800	800	1100	1100
800	800	900	1100	1200
900	900	900	1100	1100

b) RSD 5.63% Avg. ug/g 900

900	1100	750	1100	750
400	400	1200	400	900
400	1600	400	1100	500
900	900	1400	1600	1200
1100	500	900	1400	750

c) RSD 50.94% Avg. ug/g 902

1750	0	1750	0	1750
0	1750	0	1750	0
1750	0	1750	0	1750
0	1750	0	1750	0
1750	0	1750	0	1750

d) RSD 200% Avg. ug/g 910

900	900	900	900	900
900	900	900	900	900
900	900	900	900	900
900	900	900	900	900
900	900	900	900	900

e) RSD 0% Avg. ug/g 900

Figure 3.3.2. Pot trial designs constructed to simulate realistic in situ heterogeneity; a) moderate heterogeneity, Site A, b) low heterogeneity, Site B, c) high heterogeneity; and d) simplistic binary, and e) homogenous designs. Concentrations for each cell are in µg/g, and the average is for the whole pot design.

3.3.6. Number of replicates. – Power analysis

A power analysis was used to estimate the minimum number of replicates required to test for a statistically significant difference between two means of two different treatments (assuming samples taken from normal distribution) (Zar, 1999), based upon data from an earlier study by Millis *et al.,* (2004).
Using an average pooled variance of 113.8 from the study by Millis *et al*., (2004) and a population difference (δ) of 20 μg g^{-1} Zn (equivalent to 10% of average shoot concentration), the estimated minimum number of replicates, at the 95% confidence level and a 90% probability of detecting a difference in population means, is 6.2. Allowing for a 25% failure rate, the main pot experiment used 8 replicates per each treatment for each species, resulting in a total number of 160 pots.

3.4. Main pot trial experimental method.

3.4.1. Treatment preparation.

Dried, analytical grade, Zinc oxide (ZnO) was first added to dry sand, which acts as a carrier medium, compost (John Innes No.2) was then mixed with the spiked sand to provide the necessary nutrients for plant growth. In total 11 **growing mediums** (7 parts sand and 3 parts compost), containing a range of Zn concentrations from 0 µg g^{-1} to 1750 µg g^{-1}, were required to construct pot simulations of the experimental design outlined in Figure 3.3.2. To create the growing medium, firstly the moisture content of the compost was estimated to determine the mass of Zinc Oxide required to yield the desired concentration in growing medium dry weight (DW). Two samples, of approximately 10 g, were randomly taken from each compost bag. Bags were cut with scissors in random locations and samples extracted from the incision point at a depth of 2-3cm. Bags were resealed with sticky tape. Samples were placed in evaporation

dishes, weighed and then left to dry in an oven at 55°C before reweighing and determination of moisture content at 25% (Appendix C, Data Table C.1)
The mass of ZnO to be added to dry sand (c_2) was calculated using Equation 3.4.1

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$$c_2 = c_1 \times z_2/z_1 \qquad \text{Equation 3.4.1.}$$

Where: z_1 and z_2 are equal to the relative atomic mass of Zn and ZnO respectively, c_2 is equal to the mass of ZnO in 1 g of growing media, c_1 is equal to the mass of Zn required in growing media, dry mass to yield desired concentration, which is equal to desired concentration multiplied by dry weight of 1 g. A detailed breakdown of values can be found in Appendix C, Data Table C.2.

The dry, ZnO powder was weighed into pots using an analytical balance and then applied to 5kg batches of horticultural grade silver sand with a nominal particle size <1.0mm. The sand had previously been air dried in a greenhouse at approx. 25°C (sand no longer aggregates and can be poured as if liquid) and then sieved to removed lumps > 2 mm. Removal of larger particles and drying, reduces heterogeneity and facilitates a more even distribution of ZnO throughout the sand. The prepared sand was placed in a cement mixer to which fresh compost was added. The growing media was processed in the mixer until it appeared to be evenly mixed (approximately 30 minutes). Contents were emptied into clean containers and labelled with the relevant concentration. Three 1 kg batches of different concentrations (Zn 0 µg g^{-1}, 400 µg g^{-1} and 900 µg g^{-1}) were prepared to verify concentrations and determine variability. Three replicate samples from each were analysed using the laboratory method outlined in Appendix D, (D.1) and the results, using a students' t-test, were found not to be statistically different from intended nominal concentration (Appendix C, Data Table C.3).

It should be noted that small amounts of trace elements are contained within John Innes No. 2 compost and therefore Zn concentrations will always be

greater than zero. Concentrations in the 3 samples of growing media tested ranged from 3.59 µg g^{-1} to 8.36 µg g^{-1}. Whilst not ideal, against the lowest concentration of spiked growing media at 400 µg g^{-1}, it provides a significant contrast to be suitable for the purposes of this experiment.

The 160 square rigid plastic pots (18 *18 cm and 25 cm deep) were thoroughly washed with detergent then labelled with plant, treatment, block and position ID's, according to randomised block design (Appendix C, Data Table C.4). To recreate the heterogeneity models, a customised cell divider constructed from 0.75 mm clear polyethylene terephthalate glycol (PETG) sheet, was inserted into the pot to yield a 5 by 5 3-dimensional grid, with each cell measuring 25 mm square and 240 mm deep. The divider was constructed using relatively thin PETG to reduce collapse of each column and hence maintain the heterogeneity design as the divider is removed. To maintain the structural integrity of the divider whilst filling with growing media, minimise spillage into adjacent cells and provide a template for filling, labelled paper cuboid inserts were placed in each cell (Figure 3.4.1).

Figure 3.4.1. Construction of growing media to simulate *in situ* 2-dimensional heterogeneity, showing filling of cells, and view from above of 25 mm by 25 mm square cells that go to a depth of 240 mm.

As the pots do not have straight sides, the gap between the insert and the outer edge was packed with an inert material, Sinclair Perlite 2.0 mm – 5.0 mm. Cells were then filled, according to the designs in Figure 3.3.2. To ensure each cell contained an equal volume and growing media compaction was evenly distributed throughout the pot, filling was undertaken as a two stage process. A customised container was used to measure out volumes of 100 ml, of gently compacted growing media, into each cell according to the design. Growing media was then tapped down, before adding a further measured volume of 50 ml and tapping down again.

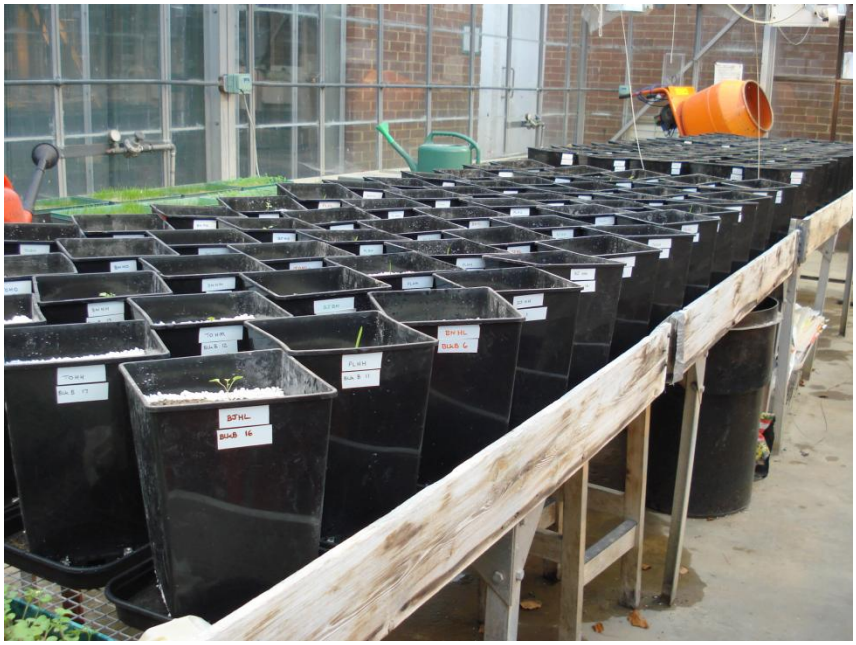


Figure 3.4.2. Completed pots containing seedlings arranged in a randomised block design.

The completed pots were placed upon individual drip trays and arranged on raised benches according to randomised block design in Appendix C, Data Table C.4. Each adjoining block was laid out in 4 rows of 5 columns, with numbers running successively along each column (Figure 3.4.2). Before transplanting seedlings, growing media was moistened from below, by capillary action, from tap water applied to the drip trays and above by hose set on fine spray, to ensure minimal disturbance of the contaminant heterogeneity.

3.4.2. Seed germination and transplantation.

Seeds of the four chosen species (See Appendix B, B.1, for full details of seed suppliers and relevant accessions) were sown in trays (containing Sinclair 2.00 - 5.00 mm lightweight density vermiculite, with neutral pH (in range pH 6.0 – 7.0) and left to germinate in a glasshouse with simulated sunlight for 16 hours and temperature maintained at 20 °C ± 5 °C. Upon the appearance of true leaves, plants of equal size were selected and transplanted into the centre of each treatment (Figure 3.4.3). Tap water was used to water daily, and applied using a fine spray from above, plants were left to grow under the same greenhouse conditions as before.

Figure 3.4.3. a) Germination of seeds, b) template to locate centre and c) transplanting seedling.

3.4.3. Biomass data collection

After 30 days, growth data was recorded for each plant to assess any variation between treatments. Number of true leaves, that could be clearly determined, were counted, and the longest leaf length, to the nearest 5 mm, measured for all species. The stem height from the growing medium interface to the highest leaf stem connection was measured for *Brassica spp.* whilst for *P. lanceolata* the leaf width was also recorded.

Data was collected again after 37 days, 44 days and date of harvest.

3.4.4. Harvest and washing method

Once each plant for a given species (except *B. juncea,* see below) had yielded sufficient shoot biomass for determination of total Zn by acid digest, usually 1-2 grams of dry mass, that species was determined ready for harvest.
After 44 days, *Brassica juncea* plants in the binary treatment began to bolt and flower heads were observed, whilst those in the homogenous treatment were exhibiting considerable chlorosis. A decision was made to harvest this species at this point, to minimise the variance introduced from different growth stages and to yield all plants before necrosis occurs.

Individual plant biomass data was recorded prior to harvesting shoot material, cutting stems approximately 5 mm above shoot-growing media interface. Cutting slightly above this interface minimises the possibility of including growing media particles in the shoot material. This is an important consideration when analysing plant material for metal content, as the smallest soil particle containing target contaminants can significantly distort measured total concentrations and generate excessively large error bars (Ramsey *et al.*, 1991) or bias. The fresh weight of the aboveground biomass for each plant was recorded prior to washing with reverse osmosis water. Shoots were placed in individually labelled bags before drying in an oven for 48 hours at 60 °C.

Roots for each plant were extracted by first sieving then washing. Whilst it is not possible to ensure all growing media particles are successfully removed from roots, each plant root underwent the same cleaning process, in the hope that any variance in Zn concentrations due to treatment is distinguishable from the large error bars associated with root analysis arising from lack of suitable washing methods. Roots were also oven dried for 48 hours.

After drying, dry weight of plant material was recorded prior to grinding using a zirconium oxide ball mill. Grinding reduces heterogeneity of contaminant in the sample and ensures sub-samples extracted for analysis are representative of

total shoot concentrations. Total Zn was extracted using nitric and perchloric acid digestion detailed earlier.

Samples were held in a solution of 1M HCL, and serial dilutions were made using a calibrated pipette to bring estimated concentrations within range of analytical instrument. Samples were analysed using Inductively Coupled Plasma with Mass Spectrometer (ICP-MS), calibrated using a range of prepared solutions from a certified stock solution. (See Appendix D,D.2 for instrument details and calibrations).

3.5. Data Quality Analysis for measured Zn concentrations in Shoots and Roots using ICP-MS.

3.5.1. Detection Limits

Detection limits for Zn in each batch were estimated from 11 replicate analyses of a blank sample and found to be below 3µg Kg^{-1} in each instance (Appendix D3.,Data Table D.1). Samples contain measured concentrations greater than detection limit by a factor of 1000, therefore we can be confident that the precision of the method is sufficiently good to reliably detect Zn concentrations in plant and soil material above background noise.

3.5.2. Reagent blank adjustment.

To estimate background Zn concentration arising from laboratory preparation, a minimum of 6 (10%) reagent blank samples were randomly incorporated within each batch. After analysis, a Student's t-test was performed to determine whether results for blank samples differed significantly (95% confidence) from zero (See Appendix D3., Data table D2). Results show reagent blank to be significant in each batch and measured concentrations were adjusted accordingly.

3.5.3. Analytical Precision

The precision or random error of the analytical method can be estimated by taking and preparing in duplicate two weighing's from the same analytical sample. An optimum number of 8 duplicates (Lyn *et al.*, 2007) were prepared for each plant species. Where adequate sample weight was available 8 duplicates were prepared for both root and shoot, as was the case for *P. lanceolata*. The precision estimate was calculated using the percent absolute difference method outlined in Gill (1997), where 2 times the median value of percent absolute difference of each analytical pair provides an estimate of the analytical precision at 95% confidence. Analytical precision should ideally be below 10%, as is the case for *B.juncea* (8.2%), *P. lanceolata* root (2.3%) and shoot (8.8%). For *B. napus* the precision estimate is not ideal at 21.03%. This

increased random error is likely due to the fibrous nature of the central stem, which did not break down easily in the ball mill. Increasing grinding time may help to reduce the impact of this error.

3.5.4. Analytical bias

For all batches, with the exception of *B. juncea* batch number 2, no significant **bias** was detected. Bias, or systematic error of the analytical technique was estimated from the inclusion of a NIST spinach certified reference material (CRM's), from in each batch and two house plant reference materials. A regression analysis of measured values (Appendix D, Data Table D.3 to Data Table D.9) against accepted (Thompson, 1982) was performed using data analysis toolpak in Microsoft Excel®.

For *B. juncea* in batch number 2, a *p* value of 0.0635 indicated some positive **rotational bias** is present, however no adjustment for bias has been made. Adjusting for rotational bias would only widen the gap between the highest and lowest concentrations of Zn in plant dry biomass and not made any difference to the statistical outcomes for this species.

A key requirement for certified reference materials used to estimate analytical bias is that they should have a similar matrix and target analyte concentrations to the samples produced in the investigation. Currently, plant reference materials do not contain highly elevated Zn concentrations, and those available for this experiment have concentrations ranging from 35 to 82 µg g^{-1} (Appendix D. Data Table D.3). In contrast, sample measured concentrations ranged from 107 to 7489 µg g^{-1}, all above the range of the reference materials and in some cases greater by almost a factor of 100. If further studies are to be carried out on plant accumulation of metals, further work may be required to produce a range of house reference materials, produced by growing a variety of plant species in growing media with a range of concentrations. These can then be used to adequately test the robustness of the analytical method.

Chapter 4 The impact of variable Zn heterogeneity at the 2cm scale on root and shoot accumulations and plant biomass for B. napus, B.juncea, and P. lanceolata.

4.1 Introduction

The chemical, physical and ecological properties within soils affecting plant growth are rarely, if ever, homogeneously distributed. There are many studies aimed at characterising the spatial distribution of soil properties *in situ* using geostatistical techniques (Ettema and Wardle, 2002, Becker *et al.*, 2006, Yavitt *et al.*, 2009), most notably, (Jackson and Caldwell, 1993), who found significant variation in nutrient resources at different scales around a single plant. This in turn has led to a number of studies aimed at quantifying the effects of nutrient heterogeneity on plant growth using controlled pot experiments, with mixed results. Some plants have been found to produce greater biomass in homogeneous treatments (Fransen *et al.*, 2001), whilst others perform significantly better in heterogeneous environments (Cahill and Casper, 1999, Einsmann *et al.*, 1999). Almost all published studies find a strong effect of heterogeneity, even when the nutrient supply is held constant (Einsmann *et al.*, 1999, Wijesinghe and Hutchings, 1999, Fransen *et al.*, 2001, Wijesinghe *et al.*, 2001).

More recently, soil heterogeneity of trace elements, in particular heavy metals has received much attention. Factors controlling plant uptake of heavy and trace metals have significant implications for choice of species used in a range of **phytomanagement** applications (Robinson *et al.*, 2009). In terms of phytoremediation, Haines (2002), Whiting *et al.*,(2000) and Schwartz *et al.*,(1999) have shown the **hyper-accumulator**, *Thlaspi caerulescens,* responding positively to spatial heterogeneity of contaminants, and actively foraging in response to patchily distributed Zn and Cd. Conversely Grey *et al*., (2005) found that the arsenic hyper accumulator *Pteris vittata* does not forage and would need spatial alignment with contamination to be effective. Effects of

heterogeneity are also thought to explain substantial differences in plant uptake between pot experiments in controlled environments and subsequent field studies (Banuelos *et al.*, 1998). Millis *et al* (2004), Manciulea and Ramsey (2006), Podar *et al* (2004) and Moradi *et al.*(2009) have all found significant differences in potentially harmful heavy metals **translocated** to the edible fraction of the plant between plants grown in homogenised growing media and those grown in simulated heterogeneity. This could have serious implications for estimates of risk to human health from the consumption of plants grown in contaminated soils. Predominately, estimates from pot experiments use homogenised contaminants (Quartacci *et al.*, 2006, Turan and Bringu, 2007) or in the case of field studies (Baker *et al.*, 1994) spatial heterogeneity of contaminants is uncharacterised or assumed to be homogeneous. So should the heterogeneous model, in pot trials, now be adopted as a better proxy for metal uptake in field situations?

A limitation of these early, simplistic heterogeneity studies is there is very little resemblance between the heterogeneity that an individual plant will experience in a controlled pot experiment (illustrated in Figure 4.1.1) and the complex spatial maps of target analytes produced by geostatistics and kriging, (Figure 4.1.2). Moreover in Chapter 2, *in situ* surveys of two contrasting sites, contaminated with a range of heavy metals, showed the spatial variation in contaminants varied as a function of both **scale** (distance between two sampling points) and **contrast** (difference in concentration between two adjacent sampling points at the same scale). This clearly demonstrates that it is highly unlikely that plants will experience the simplistic “hit and miss” heterogeneity, used in studies mentioned earlier, when grown in the field.

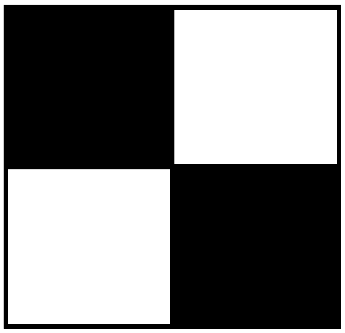


Figure 4.1.1. Model of heterogeneity typically used in pot trials. Shaded quarters contain target analyte, un-shaded quadrants are free from target analyte. Adapted from model of heterogeneity used to estimate plant uptake of cadmium by Millis *et al*,. (2004).

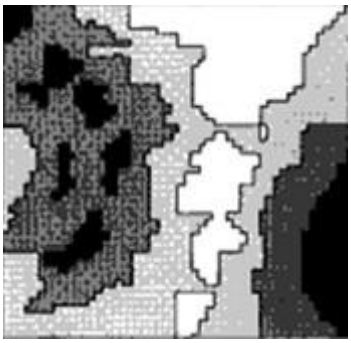


Figure 4.1.2. A kriged contour plot of variable potassium concentrations in a 0.5 m^2 area around a single plant. Taken from (Jackson and Caldwell, 1993) . Darker shading represents higher concentrations of potassium

Actual spatial heterogeneity for a specific contaminant within a field site can only be estimated from sampling, and is therefore impossible to recreate in pot trials exactly. However, Chapter 3 illustrated how site specific heterogeneity, for a given contaminant, at a specified scale may be crudely recreated. Where this current research differs, is that it aims to increase our understanding of heterogeneity, by creating more complex models of contaminants' spatial distribution and consider whether site specific contaminant heterogeneity is a significant factor on plant heavy metal uptake and performance.

In this chapter, data collected from four plant species, grown in 5 different treatments with varying zinc spatial heterogeneity, will be examined against the following hypothesis:

i. Simplistic binary "hit and miss" models of contaminant heterogeneity are unsuitable indicators of plant performance in contaminated land sites for estimation of :-
    a. Plant biomass both roots and shoots
    b. Metal uptake

ii. Site-specific spatial heterogeneity of contaminants has a significant impact on plant uptake.

4.2 Materials and methods.

4.2.1 Study species

Taraxicum officinalis (dandelion), a perennial wild herb, is a member of the *Asteraceae* and native to the British Isles, commonly found in disturbed ground and wasteland (Streeter and Hart-Davies, 2009). It has a basal rosette and a leafless stem growing vertically in height ranging from 5 to 40 cm culminating in a bright yellow flower and generally a clearly defined un-branching tap root.

The second species, *Plantago lancelolata* (ribwort plantain), is also a perennial herb found throughout the British Isles and a member of the *Plantaginacea*. Its narrow ovate leaves grow to 15cm, forming a basal rosette, from which a leafless flower stalk rises vertically, sometimes up to 45 cm, culminating in a brownish flower (Streeter and Hart-Davies, 2009). Both these species were common at the disused landfill site used in the *in situ* sampling investigation to characterise and quantify contaminant heterogeneity (Chapter 2 and (Thomas *et al.*, 2008)). Moreover, results from a preliminary experiment (Chapter 3), show both species can be grown in soils with elevated Zn and accumulate detectable concentrations in aboveground biomass.

Brassica napus (oil seed rape, winter rape, canola) is a cultivated member of the Brassicaceae which has naturalised throughout the British Isles. A biennial that grows up to 100 cm in height, it has long stalked basal leaves and dense bright yellow flowers and a fibrous **taproot**. It was chosen due to its prevalence at the (sewage disposal) contaminated land site used for Chapter 2, and as a useful comparison to the final species *Brassica juncea. Brassica juncea (L.) Czernj. Accession 426308* (Indian mustard) is another member of the *Brassicaceae* with similar growth and size characteristics to *B. napus*. It has

been used in earlier studies of metal uptake in simplistic heterogeneous pot experiments by Podar *et al.(2004)* and Manciulea and Ramsey (2006) and described as having a ball root.

4.2.2. Experimental design and substrate preparation.

The five treatments, of differing spatial Zn heterogeneity, are illustrated and described in Figure 4.2.1. Treatments b and c are based upon actual estimates of heterogeneity at the 2 cm scale from the two *in situ* site investigations at Nottingham (HL) and Coseley (HM), respectively. Model d is a theoretical model of further site investigation at Hounslow Heath firing range (HH) undertaken by Taylor *et al*., in (2005). Finally, models a and e are simplistic homogenous (HO) and the binary heterogeneous (BI) models used in studies to estimate plant uptake of heavy metals described earlier (A full description of model construction and heterogeneity values is given in Chapter 3).

Eight replicates of each treatment per species, were prepared in 18 cm square and 25 cm deep pots. Zinc oxide was applied to a homogenised growing medium comprised of sand and a loam based compost (John Innes no. 2, pH 6.5) in a ratio of 7:3, to produce a range of Zn concentrations from 0 to 1750 µg g^{-1}. Each pot was divided into a 5 x 5 grid with individual cells measuring 2.5 cm square and 15 cm deep and filled with differing concentrations according to the models in Figure 4.2.1. To isolate spatial heterogeneity as the factor affecting plant growth and metal uptake, the total contaminant concentration was held constant in each treatment and modelled to an average Zn concentration of 900 µg g^{-1}.

Seeds were sown in trays (containing Sinclair 2.00 - 5.00 mm lightweight density vermiculite, with neutral pH (in range pH 6.0 – 7.0) and left to germinate in a glasshouse with 16 light/8 hours dark and temperature maintained at 20 °C ± 5 °C (see p 131 for discussion). Upon the appearance of true leaves, plants of equal size were selected and transplanted, singly, into the central cell of each

treatment. Plants were arranged in a randomised block design, watered daily with tap water, applied using a fine spray, and left to grow under the same greenhouse conditions as before.

Use of randomised block enables detection of significant, within greenhouse, variation which may obscure any variation that arises from treatment.

Species were harvested once individual plants had produced sufficient above ground biomass for analysis, ideally 1g dry biomass (Thompson and Walsh, 1983b). Roots and shoots were harvested separately and washed with reverse osmosis water. After drying, Zn content was extracted using a nitric and perchloric digest method (Thompson and Walsh, 1983b), into 1 mol. hydrochloric acid (1 M HCL) matrix solution before analysis with an Inductively coupled plasma mass spectrometer (ICP-MS). Reagent blanks, house and certified reference materials were incorporated into analytical batches to detect any analytical bias in laboratory methods. A detailed description of background to experimental design and methods, and results of data quality analysis can be found in Chapter 3.

900	900	900	900	900
900	900	900	900	900
900	900	900	900	900
900	900	900	900	900
900	900	900	900	900

a) Homogenous (HO) Avg. Zn 900 µg g^{-1}

750	750	800	900	900
750	750	800	900	900
800	800	800	1100	1100
800	800	900	1100	1200
900	900	900	1100	1100

b) Low heterogeneity (HL) Avg. Zn 900 µg g-1

900	500	900	1100	750
400	500	1100	1200	900
400	400	800	1100	800
500	900	1400	1600	1200
750	800	800	1400	1400

c) Medium heterogeneity (HM) Avg. Zn 902 µg g-1

900	1100	750	1100	750
400	400	1200	400	900
400	1600	400	1100	500
900	900	1400	1600	1200
1100	500	900	1400	750

d) High heterogeneity (HH) Avg. Zn 900 µg g-1

1750	0	1750	0	1750
0	1750	0	1750	0
1750	0	1750	0	1750
0	1750	0	1750	0
1750	0	1750	0	1750

e) Binary (BI) Avg. Zn 910 µg g-1

Figure 4.2.1. Illustrates the 5 treatments of differing Zn spatial heterogenity used in a pot based experiment for the four plant species; a and e represent early models of homogenous (HO) and chequerboard binary (BI), respectively. Designs b–d are based upon actual *in situ* geochemical investigations, b) represents (Nottingham) a low heterogeneity (HL) site, c) (Coseley) medium heterogeneity (HM) and d) (Hounslow Heath) High heterogeneity (HH) site (Taylor *et al.*, 2005).

4.2.3 Data analysis.

At harvest, stem height, longest leaf, number of leaves and fresh shoot fresh biomass were recorded. Roots and shoots were washed with reverse osmosis water before drying in an oven at 60°c for 48 hrs. Shoot and root dry biomass were recorded before measured zinc concentrations in both plant parts were obtained.

Statistical tests were performed using SPSS Statistics 17.0 software. One sample Kolmogorov-Smirnov (K-S) tests for normal distribution and Levene's test for equal variance (Dytham, 2003) were performed on each measured variable, per species (See Appendix E, Data Table E.4 for *B. napus*, Data Table E.12 for *B. juncea,* and Data table E.18 for *P. lanceolata*) to determine if parametric statistical tests were appropriate. Where these conditions were not satisfied, data were natural log-transformed and retested for normality and equal variance. A mixed model ANOVA was used to test the hypothesis that different levels of zinc heterogeneity in the growing medium have an effect on plant shoot dry biomass, root dry biomass and concentrations of zinc in each. With treatment as a fixed and block as a random factor, significance of between treatment and within treatment (block) were tested. Block was not found to be significant for any variables. Where significant differences between treatments were detected, Tukey HSD (honestly significant difference) multiple pairwise comparison of means test were performed (Zar, 1999).

4.3 Results – Brassica napus

4.3.1 Biomass results for *Brassica napus*

During the growing period, no visible differences (See Figure 4.3.1 below) between treatments were detected. At 54 days growth, adequate above ground biomass had been produced, with 100 % survival rate, and plants were harvested.

Figure 4.3.1 *Brassica napus*, 54 days after planting seedlings. Plants have been organized in rows (running from front of photograph to rear) according to treatment, decreasing in degree of heterogeneity from left to right, for purposes of photograph only. Plants were arranged in a randomised block design during growth.

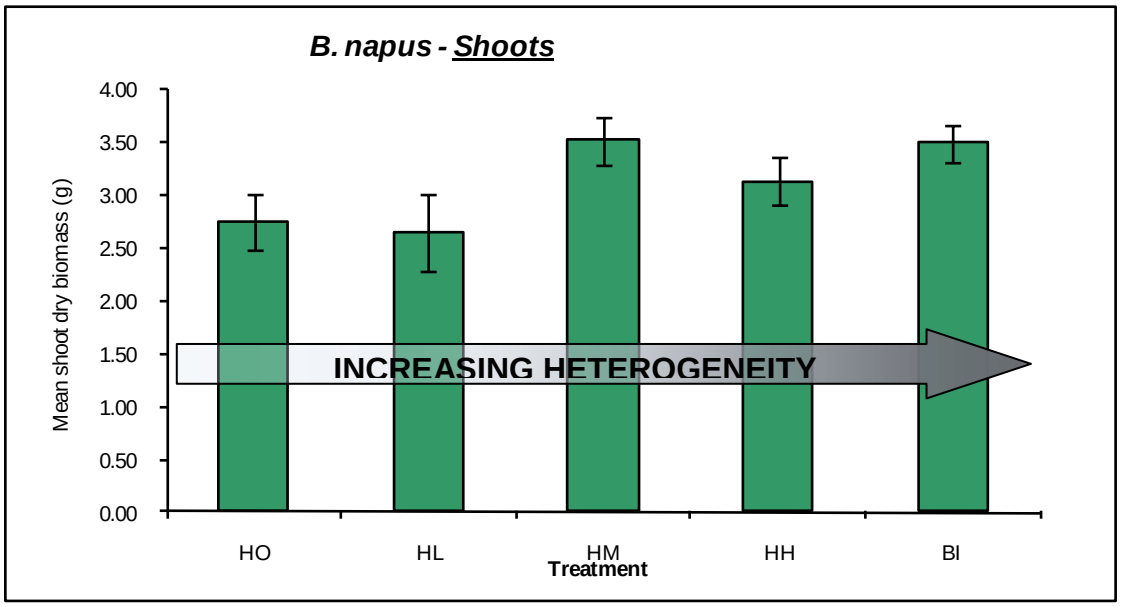


Figure 4.3.2. Mean dry biomass of shoots (g) for *B. napus* in each treatment (No significant differences detected, Tukey c.o.m.test).

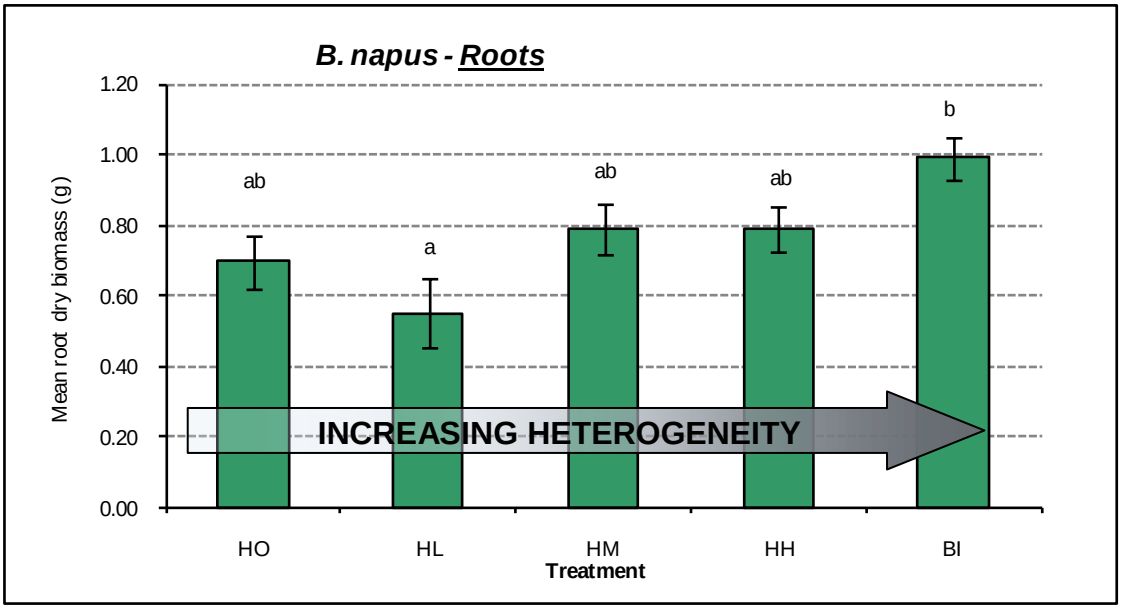


Figure 4.3.3 Mean dry biomass of roots (g) for *B. napus* in each treatment. Means sharing the same data labels do not differ significantly (Tukey c.o.m. test).

Error bars represent the standard error on the mean where n = 8. (HO – homogeneous, HL – low heterogeneity, HM – medium heterogeneity, HH – high heterogeneity, BI – binary)

Mean shoot dry biomass for *B. napus* was reduced in the lower heterogeneity treatments, HO and HL (Figure 4.3.2), compared to higher heterogeneity treatments of HM, HH and BI, though this difference was not statistically significant, (see Table 4.3.1 for ANOVA results). Mean root dry biomass was also reduced in the lower heterogeneity treatments and a significant difference (*P value* 0.002) found between low heterogeneity (HL) and binary (BI) treatments.

Table 4.3.1. Analysis of variance for *B. napus* shoot and root dry biomass and measured zinc concentrations of individual plants grown in 5 treatments of varying spatial Zn heteogeneity. The experiment was conducted using a randomised block design, and tested using a mixed model ANOVA with treatment as fixed factor and block as a random factor. Factor is significant for P values < 0.05 (in bold).

Dependant variable	Factor	d.f.	ss	ms	*F*	*P*
B. napus						
Shoot dry biomass	Treatment	4	5.324	1.331	2.668	0.053
	Block	7	5.517	0.788	1.58	0.183
	Error	28				
Root dry biomass	Treatment	4	0.818	0.204	5.704	**0.002**
	Block	7	0.566	0.081	2.258	0.059
	Error	28				
Shoot Zn conc.	Treatment	4	973381.2	243345.3	17.193	**<0.001**
	Block	7	97481.81	13925.97	0.984	0.463
	Error	28				
[1]Ln root Zn conc.	Treatment	4	2.333	0.583	8.391	**<0.001**
	Block	7	0.356	0.051	0.732	0.647
	Error	28				

[1] Data for variables with Ln prefix have been natural log transformed.

4.3.2 Zinc uptake results in shoots and roots for *Brassica napus*.

A highly significant difference (*P* value < 0.0001) was found between the mean Zn concentrations in shoot dry biomass of plants grown in binary treatments and all other treatments. Mean Zn concentrations (Figure 4.3.4) in intermediate heterogeneity and homogenous treatments were all greater than 600 µg g^{-1}, more than double those measured for binary treatments of 284 µg g^{-1}, and this confirms similar findings of reduced metal concentrations in simplistic heterogeneous studies by Manciulea and Ramsey (2006), Millis *et al.*(2004), Podar *et al.*(2004) and Moradi *et al* (2009).

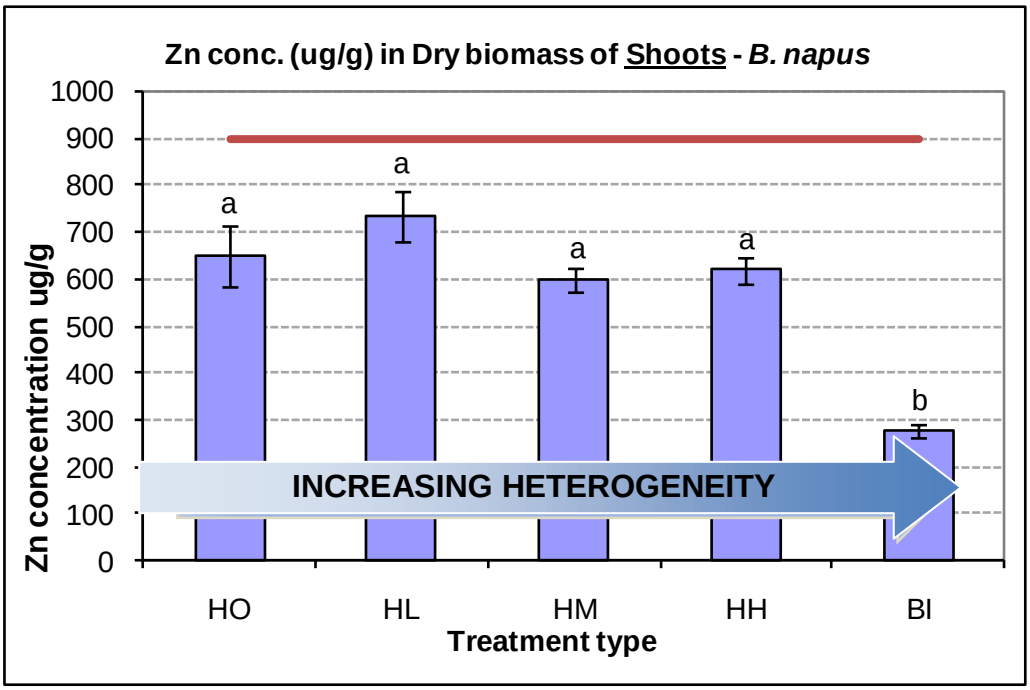


Figure 4.3.4. Measured Zinc concentrations (µg g^{-1}) in shoot dry biomass for *Brassica napus*.

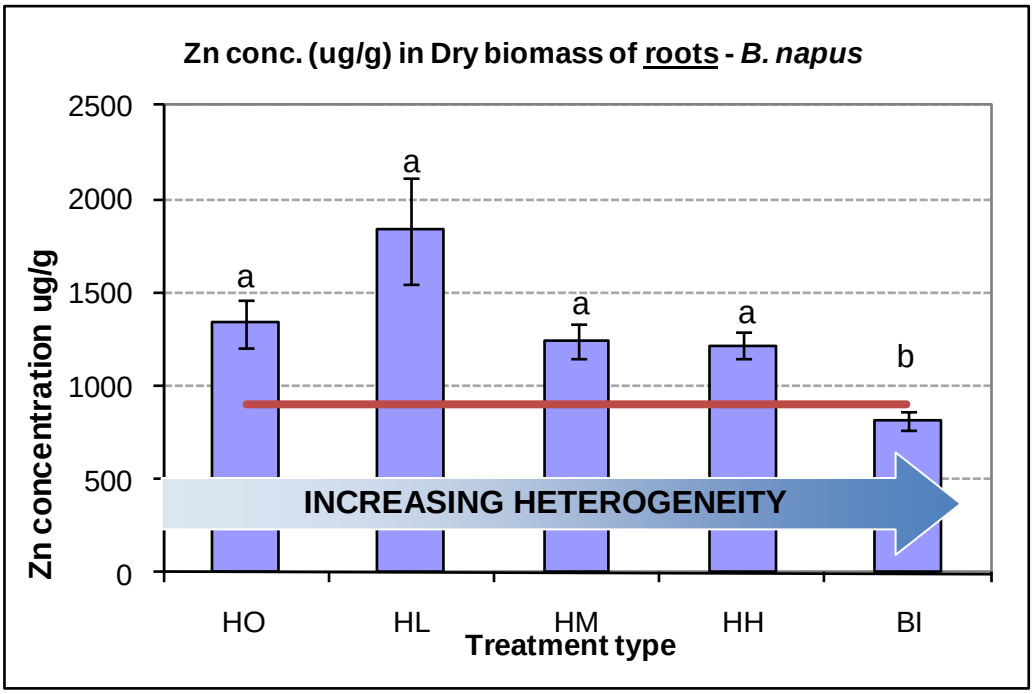


Figure 4.3.5 Measured Zinc concentrations (µg g^{-1}) in root dry biomass for *Brassica napus* grown in 5 treatments with differing heterogeneity.

Error bars represent 1 standard error on the mean. Bars not sharing a letter differ significantly p <0.05 (Tukey H.S.D test). Mean concentration in growing media indicated by, (–) red line on graphs.

No significant differences were detected between the HO treatment and intermediate heterogeneity levels for Zn concentrations in shoots. Similar findings were found for results of measured zinc concentrations in root dry biomass. Mean Zn concentration in roots were 30% lower in BI treatments and significantly different (*P* value <0.05) to all other treatments, a highly significant difference (P value <0.001) was identified between BI and HL treatments. Interestingly, concentrations in roots were found to be higher than the average in the substrate (900 µg g^{-1}, indicated by red line in Figure 4.3.4 and Figure 4.3.5), indicating for homogenous and intermediate levels of heterogeneity, some Zn accumulation within roots.

4.4 Results for Brassica juncea.

4.4.1 Biomass results for *Brassica juncea.*

For *Brassica juncea* there was a visible response to treatments after 30 days growth. Plants in the binary treatment looked healthy and were generating substantial biomass, whilst plants in homogenous treatments were stunted with visible signs of chlorosis, leaf edges discoloured yellow and purple. There was a visible difference between plants in intermediate treatments too, with a general trend towards greater biomass with increased heterogeneity. At 49 days, some plants in the binary treatments began to bolt, whilst plants in homogeneous treatments were at risk of dying (see Figure 4.4.1.for image of plants prior to harvest). A decision was made to harvest at this point to reduce the effect of chemical variation that occurs at different development stages within a plants growth cycle (Miller and Donahue, 1990). A 100% survival rate was recorded and all roots and shoots were harvested..

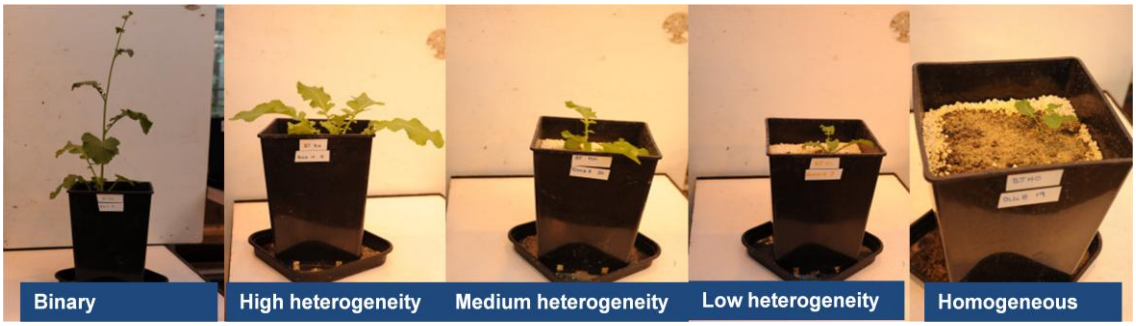


Figure 4.4.1. Images of *Brassica juncea* in different treatments illustrating differences in growth at harvest, heterogeneity is decreasing from left to right.

Figure 4.4.2 and Figure 4.4.3 indicate a trend of increased biomass in response to increasing heterogeneity, both above and below ground. A highly significant difference (P <0.001, Table 4.4.1) in mean dry shoot biomass was detected between simplistic HO and BI treatments with a 20 fold increase from 0.09g in HO to 1.67 g in BI treatments. Similar results were found below ground with a highly significant difference (P <0.001) in mean root dry biomass for simplistic

models, with HO at 0.008 g and BI at 0.48 g this represent a 60 fold increase. Significant differences (P value 0.005) were also found between HH and HM intermediate levels of heterogeneity for both roots and shoots, and results of Tukey comparison of means test (Figure 4.4.2 and Figure 4.4.3) show two distinct groups, with HO, HL and HM treatments not differing significantly, and HH and BI forming the other statistically similar group for both variables. A full set of recorded biomass data can be found in Appendix E, Data Table E.9.

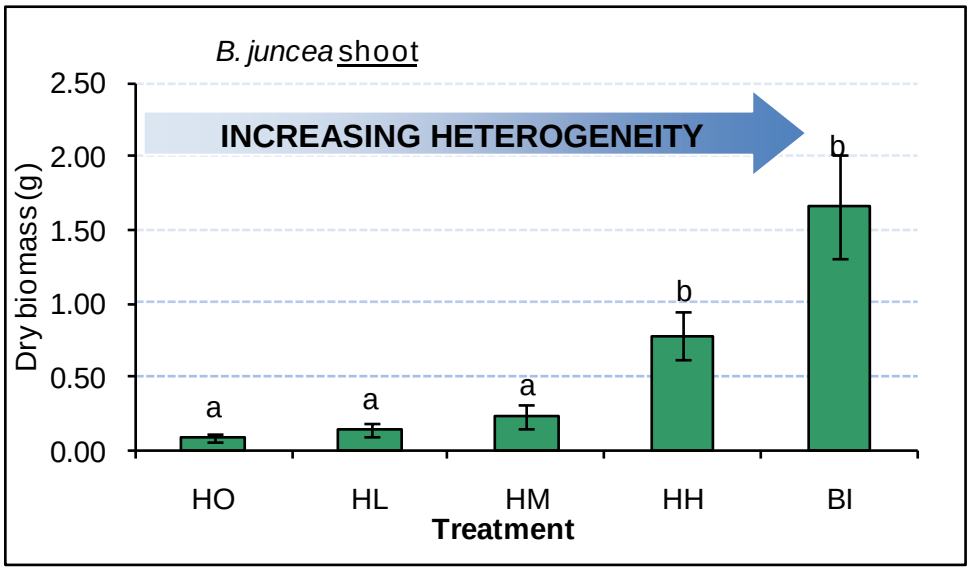


Figure 4.4.2. Mean dry biomass (g) for shoots of B. juncea for each treatment.

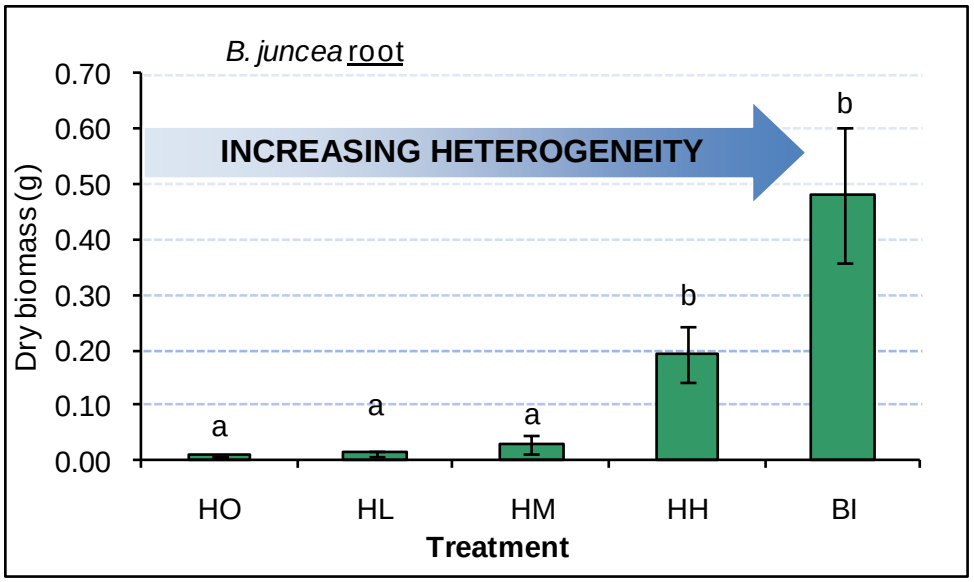


Figure 4.4.3. Mean dry biomass (g) of roots for *B. juncea* for each treatment.

(HO – homogeneous, HL – low heterogeneity, HM – medium heterogeneity, HH – high heterogeneity, BI – binary) Error bars represent the standard error on the mean where n = 8 for shoots and n = 7 for roots. Bars not sharing a letter differ significantly *p* <0.05 (Tukey H.S.D test).

Table 4.4.1. Analysis of variance for *B. juncea* shoot and root dry biomass and measured zinc concentrations of individual plants grown in 5 treatments of varying spatial Zn heterogeneity. The experiment was conducted using a randomised block design, and tested using a mixed model ANOVA with treatment as fixed factor and block as a random factor. Factor is significant for P values < 0.05.

Dependant variable	Factor	d.f.	ss	ms	*F*	*P*
B. Juncea						
Ln Shoot dry biomass	Treatment	4	52.613	13.153	20.024	**<0.001**
	Block	7	4.674	0.668	1.016	0.441
	Error	28				
Ln Root dry biomass	Treatment	4	91.013	22.753	25.071	**<0.001**
	Block	7	2.38	0.397	0.437	0.847
	Error	28				
Ln Shoot Zn conc.	Treatment	4	25.338	6.335	143.533	**<0.001**
	Block	7	0.424	0.061	1.372	0.256
	Error	28				
Ln root Zn conc.	Treatment	4	5.585	1.396	15.791	**<0.001**
	Block	7	0.287	0.048	0.541	0.772
	Error	28				

4.4.2 Zinc uptake results for shoot and roots of *B. juncea*.

Whilst shoot biomass increased with higher heterogeneity, mean measured zinc concentrations in shoot dry biomass was found to significantly decrease (See Figure 4.4.4.). A highly significant difference (*p* value <0.001) was detected between mean zinc in BI and all other treatments, at 250 µg g^{-1}, this is more

than 5 times lower than the mean for HH (1313 µg g^{-1}) and 9 times lower than the mean for HL (2181 µg g^{-1}). Results do show a highly significant difference (p <0.001) between intermediate treatments and simplistic heterogeneity (BI), and homogeneous (HO) models, additionally a highly significant difference (p <0.001) was found between intermediate treatments for HH and HL. Tukey c.o.m. tests (Figure 4.4.2 based upon detailed analysis in Appendix E, Data Table E.14) reveal 3 statistically different groups, with BI forming one, HH and HM the second and HO and HL the third. With the exception of BI treatments, individual plants show higher concentrations of Zn in shoots than average concentration in the growing media (900 µg g^{-1}), more than double in low heterogeneity treatments and greater by a factor of 1.5 in medium to high heterogeneity, indicating some accumulation.

Roots show similar results, again a highly significant difference between BI (p <0.01) and all other treatments was found. Moreover differences between intermediate treatments were also found to be significant, with HH significantly different to HL (p <0.05), though HO, HL and HM treatments were all found to be statistically similar. Accumulation of Zn in dry biomass of roots was also detected, with low heterogeneity treatments (HO, HL and HM), greater than treatment average of 900 µg g^{-1} by a factor of four. Concentrations were significantly reduced in high heterogeneity treatments by a factor of 1.5 and 3.0 for HH and BI respectively.

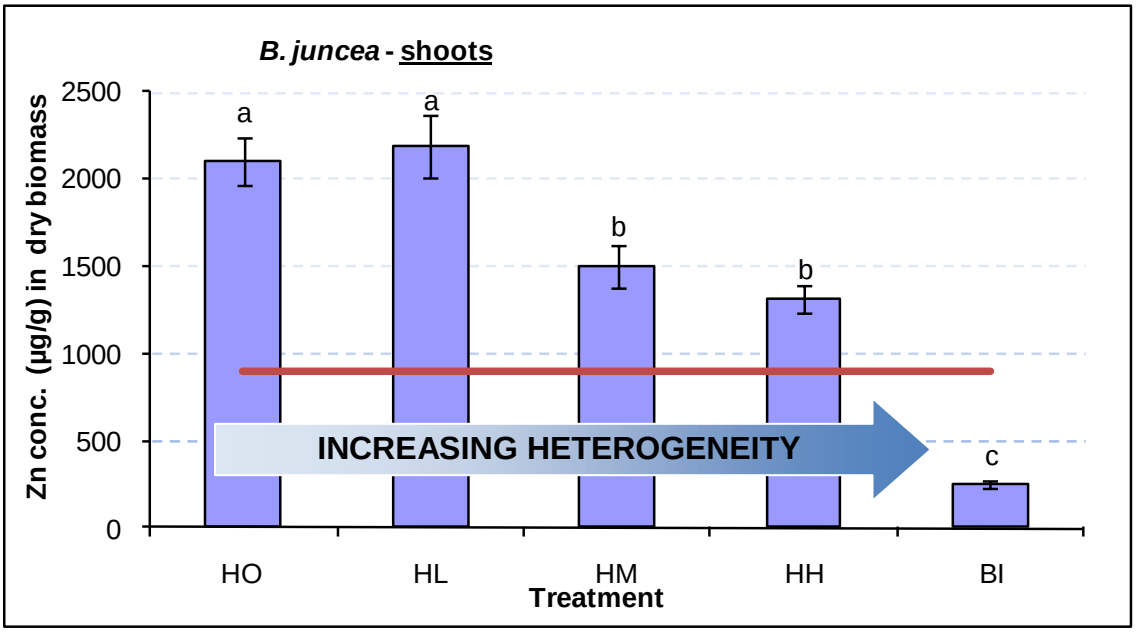


Figure 4.4.4. Mean measured concentration of zinc (µg g^{-1}) in shoot biomass for *B. juncea*.

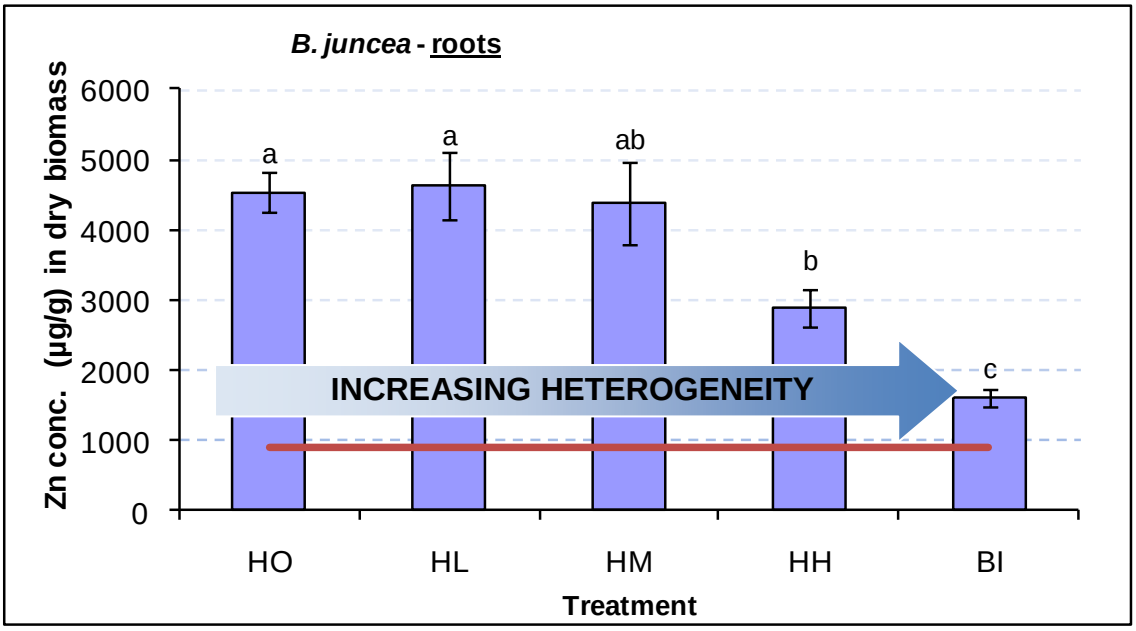


Figure 4.4.5. Mean measured concentration of zinc (µg g^{-1}) in root biomass for *B. juncea*.

Red line represents mean Zn concentration in growing media. (HO – homogeneous, HL – low heterogeneity, HM – medium heterogeneity, HH – high heterogeneity, BI – binary) Error bars represent the standard error on the mean where n = 8 for shoots and n = 7 for roots. Bars not sharing a letter differ significantly *p* <0.05 (Tukey H.S.D test).

4.5 Results for Plantago lanceolata.

Individual plants of *P. lanceolata* were harvested 73 days after transplantation of seedlings, with 1 plant, in a homogenous treatment, dying before harvest. Considerable variability within a single treatment was visible in above ground biomass prior to harvest (Figure 4.5.1).

4.5.1. Biomass for *P. lanceolata*

Figure 4.5.1. Images of two plants from each of the 5 treatments, illustrating variability in above ground biomass, prior to harvest. Treatment heterogeneity is increasing from left to right.

Despite large variability within treatments, some statistically significant differences between treatments were detected in shoot dry biomass, though these did not display any clearly defined trends as had been observed for the two *Brassica* species. Mean dry biomass for plants grown in HO treatments was reduced by more than 50% when compared to BI, HL and HH treatments (Figure 4.5.2), and found to be significantly different (p <0.05, Table 4.5.1) from the two intermediate HL and HH treatments. A significant difference may well be present between HO and BI treatments (p = 0.092, Appendix E, E.20) but is obscured by within treatment variability.

Root dry biomass for *P. lanceolata*, produced similar results (see Figure 4.5.3), with significantly lower biomass in HO treatments compared to HH (p = 0.025), and possibly HL (p = 0.051).

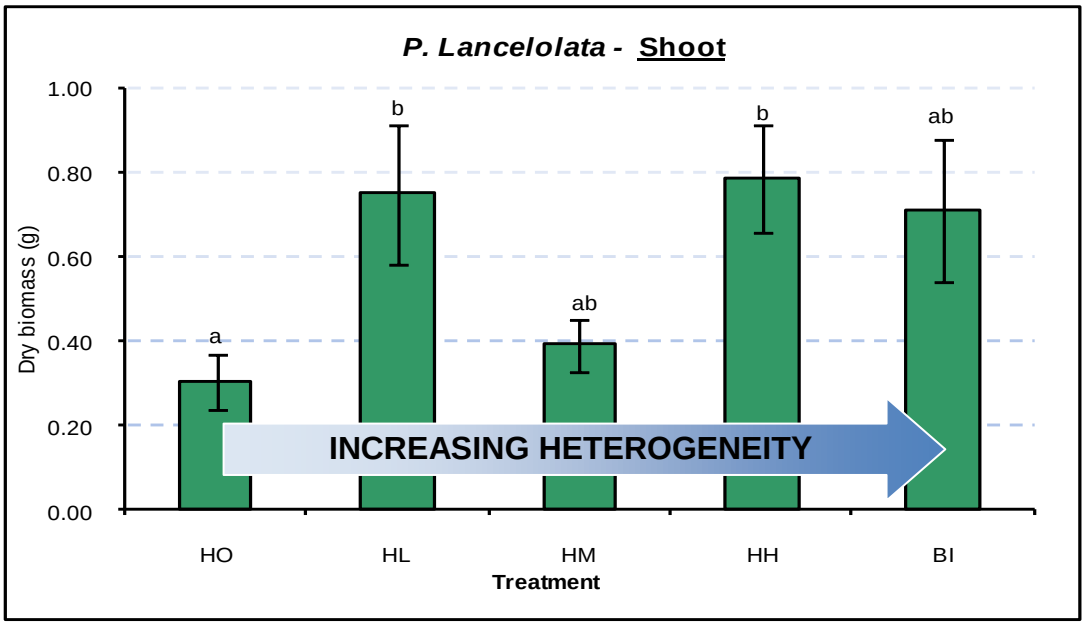


Figure 4.5.2. Mean dry biomass (g) for shoots of *P. lanceolata* for each treatment

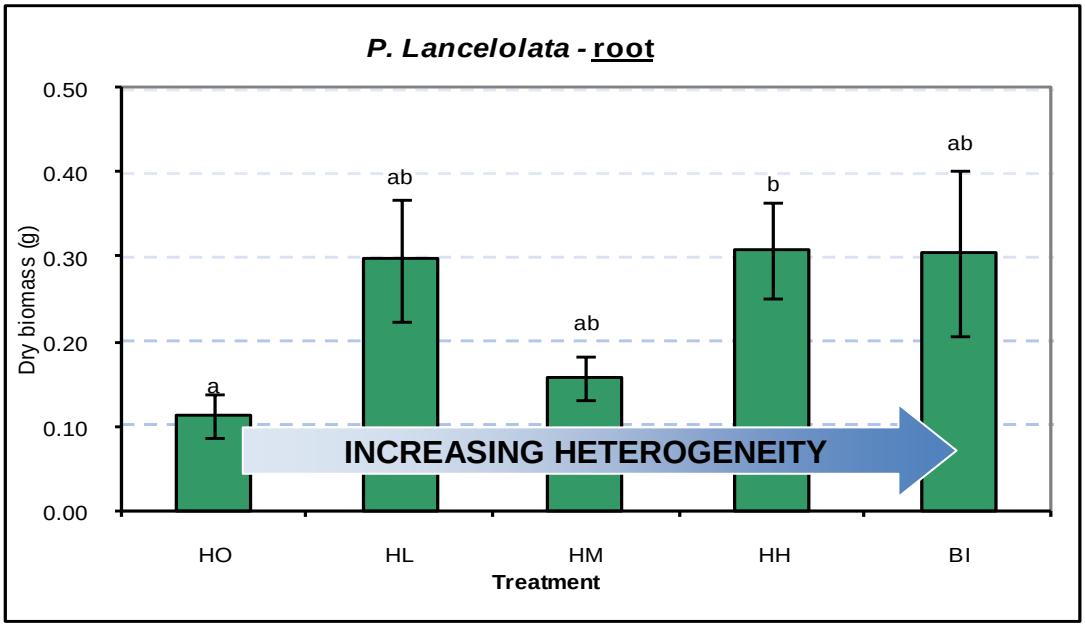


Figure 4.5.3. Mean dry biomass (g) for roots of *P. lanceolata* for each treatment.

(HO – homogeneous, HL – low heterogeneity, HM – medium heterogeneity, HH – high heterogeneity, BI – binary) Error bars represent the standard error on the mean where n = 8, except HO, where n = 7. Bars not sharing a letter differ significantly p <0.05 (Tukey H.S.D test on natural log transformed data).

Table 4.5.1. Analysis of variance for *P. lanceolata* shoot and root dry biomass and measured zinc concentrations of individual plants grown in 5 treatments of varying spatial Zn heterogeneity. The experiment was conducted using a randomised block design, and tested using a mixed model ANOVA with treatment as fixed factor and block as a random factor. Factor is significant for *p* values < 0.05.

Dependant variable	Factor	d.f.	ss	ms	*F*	*P*
Ln Shoot dry biomass	Treatment	4	5.184	1.296	3.783	**0.014**
	Block	7	4.314	0.616	1.799	0.129
	Error	28				
Ln Root dry biomass	Treatment	4	5.326	1.332	3.493	**0.02**
	Block	7	5.35	0.764	2.005	0.092
	Error	28				
Shoot Zn conc.	Treatment	4	768218	192054	11.732	**<0.001**
	Block	7	139153	19879	1.214	0.328
	Error	28				
Root Zn conc.	Treatment	4	10552511	2638128	4.384	**0.007**
	Block	7	5352049	764578	1.271	0.3
	Error	28				

4.5.2 Zinc uptake results for *P. lanceolata.*

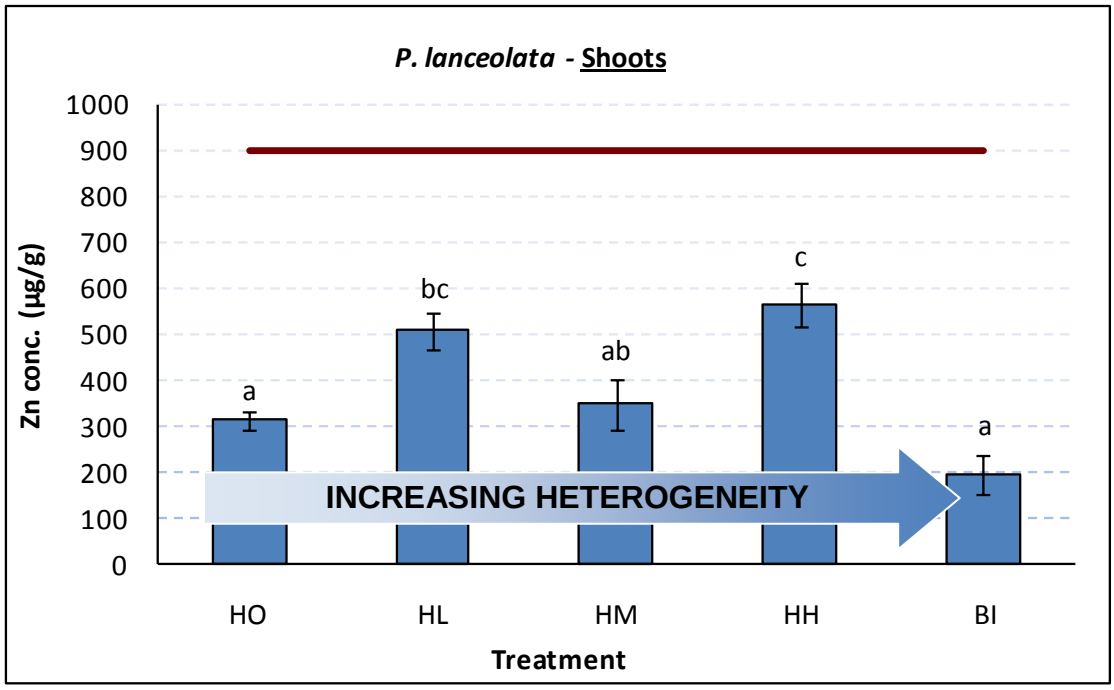


Figure 4.5.4. Mean measured concentration of zinc (µg g^{-1}) in shoot biomass for *P. Lanceolata*.

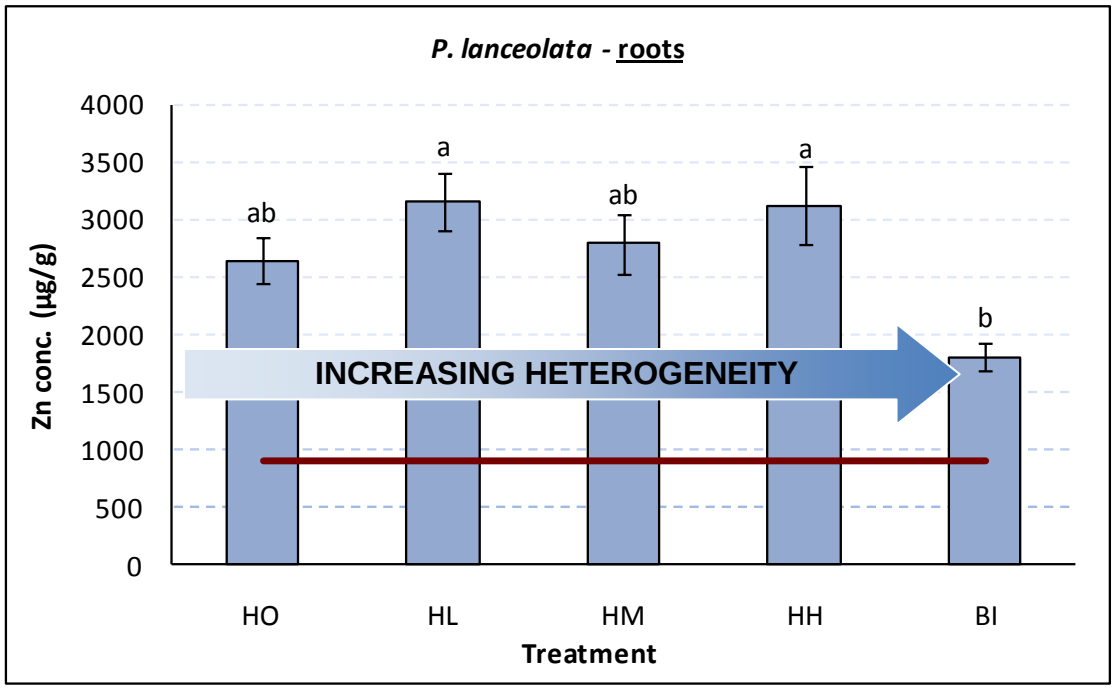


Figure 4.5.5. Mean measured concentrations of zinc (µg g-1) in root biomass for *P. lanceolata*.

Error bars represent 1 standard error on the mean where n = 8, except HO where n = 7. Means with same data label do not differ significantly (Tukey c.o.m test). Red line represents mean Zn concentration in growing media.

Zinc concentrations in shoots between the two simplistic HO and BI models do not differ significantly, with mean concentrations of 196µg g^{-1} and 311µg g^{-1}, respectively (Figure 4.5.4). Across all treatments, concentrations in shoots are lower than mean concentration in substrate. However, there are significant differences between simplistic models and intermediate levels of heterogeneity; a highly significant difference (p <0.01) exists between HO and both the HH and HL treatments, a further highly significant difference (p <0.001) was observed between BI and both HL and HH.

Mean concentrations in root dry biomass (Figure 4.5.3) show zinc is accumulating, with a 2 fold increase in BI treatments compared to mean substrate concentration of 900 µg g-*1*, rising to 3 fold increase for all other treatments (See Figure 4.5.5). A significant difference in zinc concentrations (p < 0.05) was found between simplistic binary heterogeneity treatment and the intermediate HL and HH heterogeneity treatments.

4.6. Results – Taraxacum officinale.

4.6.1. Biomass results for *Taraxacum officinale*

Transplanting seedlings into the centre square was unsuccessful with greater than 50% dying within a few days. An alternative approach was tried, by planting 3 seeds in each centre square and thinning to one seedling after germination. This too, proved to be unsuccessful with only 14 out of 40 plants reaching maturity. More plants reached maturity in treatments with higher levels of heterogeneity, but replicates were too few to draw any significant conclusions. For interest only the number of mature plants in each treatment are; 2 homogeneous (HO), 1 low heterogeneity (HL), 5 medium heterogeneity (HM), 3 high heterogeneity (HH) and 5 binary (BI). No further analysis was undertaken for this species, however results from preliminary study indicate, with perfecting of growth, this species may yield interesting results.

4.7. Discussion

4.7.1. Interpretation of results against stated hypothesis.

A comparison of results for the two extreme spatial heterogeneity treatments, of homogeneous and binary (i.e. simplistic models), to earlier studies, confirms findings by; Podar *et al*., (2004), Manciulea and Ramsey (2006), Menon *et al*., (2007) and Moradi *et al*., (2009), with higher biomass and lower contaminant uptake in binary treatments. However, as demonstrated in the introduction, contaminant spatial heterogeneity is unlikely to have such a simplistic distribution in actual contaminated land sites. Results from intermediate levels of heterogeneity, based upon *in situ* field measurements, provides an insight to plant response to site specific contaminant heterogeneity. Moreover these results challenge whether simplistic binary models are an adequate proxy for estimates of plant uptake of potentially harmful contaminants. Considering results against stated hypothesis:

i. Simplistic binary "hit and miss" models of contaminant heterogeneity in pot trials are unsuitable indicators of plant performance in contaminated land sites for
    a. Plant biomass (both roots and shoots)
    b. Zinc uptake

Where a statistically significant difference is found ($p<0.05$), for measured plant variables, between simplistic binary models of heterogeneity and one of the more realistic field model of heterogeneity, then we accept hypothesis (i) that binary models of heterogeneity in pot trials are unsuitable indicators of plant performance in actual contaminated land sites. For plant growth measurements, (Table 4.7.1, (i)a), results are mixed within the *Brassicacea* species studied, showing significant differences between simplistic binary and field based models of heterogeneity, with the exception of *B. napus* shoots. Though the *p* value, for this variable, is close to significance at 0.053, and increasing the power of the test with more replicates might yield a significant response. However, for *P. lanceolata* we reject hypothesis (i) a, as no significant

differences in plant biomass were detected between binary and field based models of zinc spatial heterogeneity.

For zinc uptake into roots and shoots, results are less ambiguous and we accept the hypothesis for all three species. There is a clear significant difference in Zn uptake (Table 4.7.1. (i) b) between those plants grown in simplistic binary and field based heterogeneity models for all species studied. However, whilst these results show that binary models are an unrealistic proxy for plant uptake of contaminants in the field, should heterogeneity be ignored? The second hypothesis asks the question; when modelling plant uptake of contaminants, does site specific heterogeneity have a significant impact on contaminant concentrations in above and below ground biomass:

ii. The degree of contaminant spatial heterogeneity has a significant impact on plant uptake of contaminants, where total concentration is held constant. (Site specific spatial heterogeneity of contaminants has a significant impact on plant uptake.)

Where a significant difference in zinc uptake into plants exists between different field based models of heterogeneity and also homogenous treatments, then we accept hypothesis (ii). The results summary in Table 4.7.1, shows that for *B. napus*, site specific heterogeneity is not a significant factor for plant zinc uptake, moreover, for this species, there was no significant difference between field models and the simplistic homogenous model, suggesting that the existing approach of modelling plant uptake of contaminants, in homogenous treatments, is adequate.
However, results for *B. juncea* and *P. lanceolata* suggest otherwise, with both species showing significant differences in shoot zinc concentrations in response to different spatial patterns of zinc at the same scale. Though interestingly, roots of *P. lanceolata* show no response to different degrees of heterogeneity.

Table 4.7.1. Summary hypothesis tested for each species based upon Tukey H.S.D. comparison of means statistical tests where p<0.05.

Hypothesis		*B.napus*	*B. juncea*	*P. lanceolata*
		Roots/Shoots	Roots/Shoots	Roots/Shoots
(i)	a. (biomass)	Reject/Accept	Accept/Accept	Reject/Reject
	b. (zinc)	Accept/Accept	Accept/Accept	Accept/Accept
(ii)	Zinc	Reject/Reject	Accept/Accept	Accept/Reject

4.7.2. Implications for risk to human health from consumption of plants grown in contaminated soils.

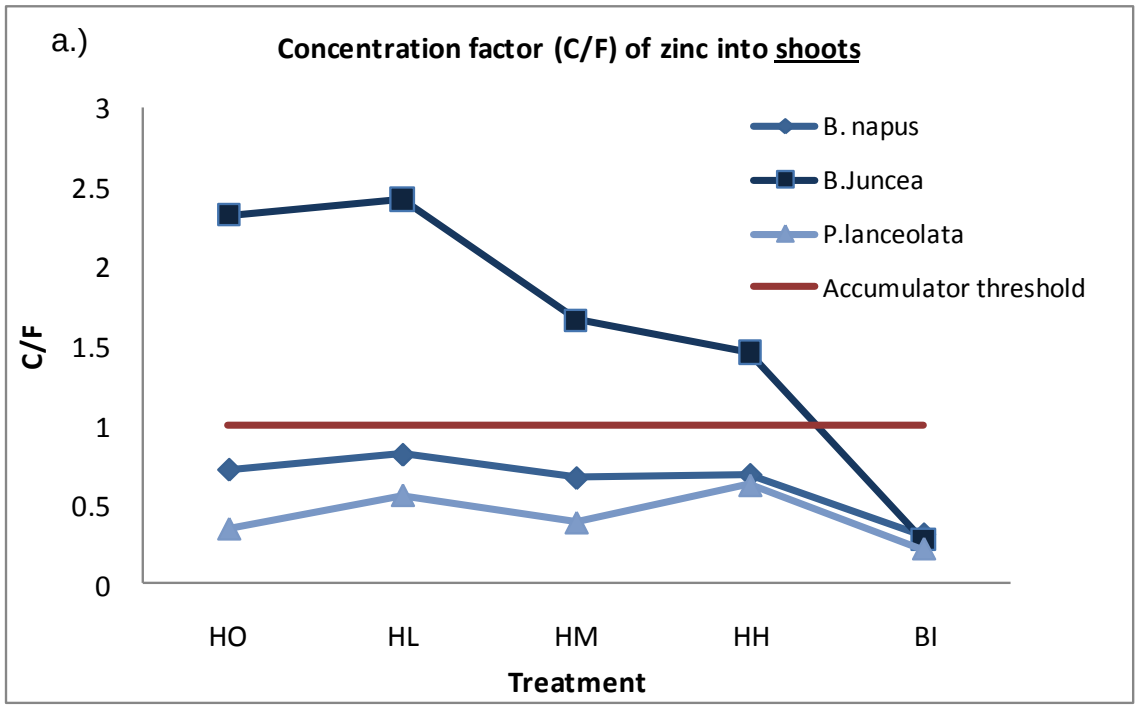
Whilst zinc is essential to human health, the current Provisional Maximum Tolerable Daily Intake (PMTDI) for a UK adult, weighing 60Kg, is 300-100 µg/Kg bodyweight per day as recommended by the Food Standards Agency, (FSA, 2009). Moreover, it is not the only trace element that can enter the human food chain via consumption of plants. Arsenic, Hg and especially Cd are all non essential, harmful trace elements (Alloway and Ayres, 1997) that can be absorbed into plants from the soil. One reason for this is that nutrient uptake pathways lack sensitivity to elements of a similar size, therefore Cd^{2+} which is geochemically similar to Zn^{2+} is readily taken up by plants and found to accumulate in leafy vegetables, carrots, mushrooms and potatoes (Kabata-Pendias and Pendias, 2000).

Currently, in the UK, the risk to human health from the consumption of vegetables grown on contaminated soils, in the absence of a detailed field study, is estimated using generic regression equations. (Great Britain. Dept. for Environment and Rural, 2002). Of which, the concentration factor (CF), “an estimated ratio of the concentration of the contaminant in chosen vegetable to the contaminant concentration in the soil”, is a key parameter of the model.

Concentration factors increase where mechanisms that exclude heavy metals are weaker and contaminant concentrations accumulate in plant tissues.

Currently these concentration factors are based upon a range of values, from studies of 6 home-grown vegetables that are common to the UK diet.

A wide range of concentrations have been reported for each vegetable creating a great deal of uncertainty around this parameter, and these results show that some of this uncertainty can be attributed to the heterogeneity of the contaminant within the soils for some species. The highest variability in concentration factors can be seen in *B. juncea*, with a 90% reduction from CF of 2.4 for shoots (Figure 4.7.1, a), in a low heterogeneous environment (HL) to a CF of 0.3, in binary treatments.



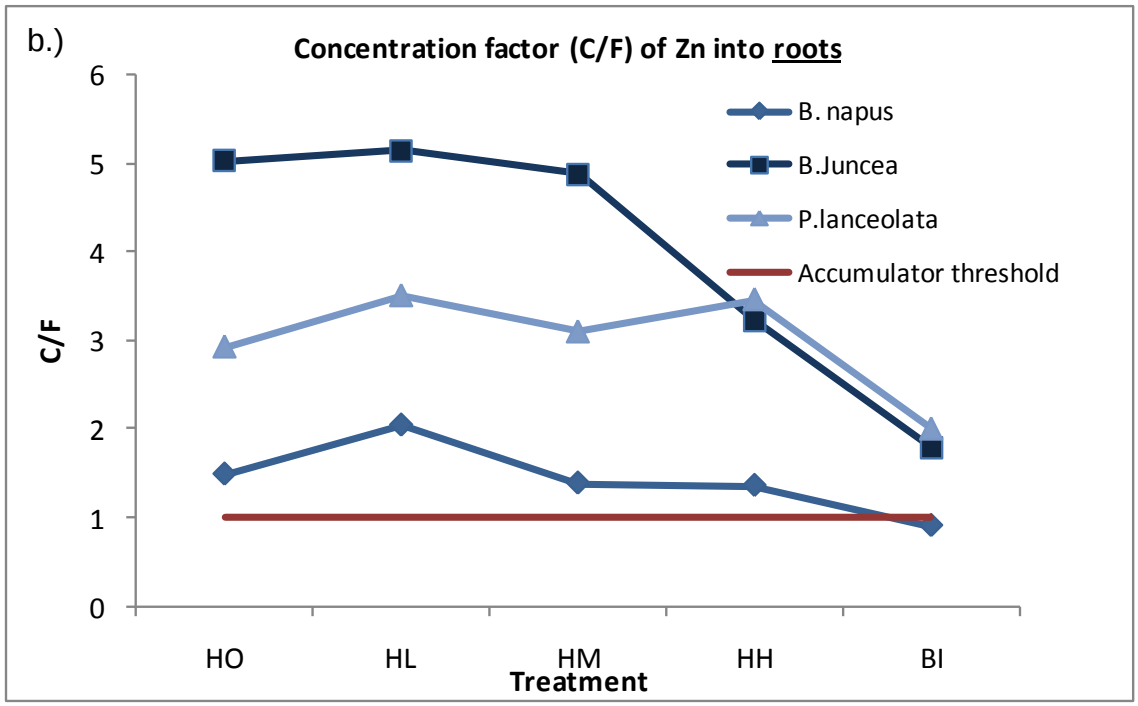


Figure 4.7.1. Concentration Factors (concentration in plant (dry weight)/ mean concentration in soil (dry weight)) of zinc into plant a.) shoots and b.) roots for B. napus, B.juncea and P. lanceolata, grown in 5 treatments of differing Zn spatial heterogeneity but the same total concentration. Where C/F is greater than 1 (accumulator threshold) Zn is accumulated into plants.

The estimated CF values, based upon dry weight, for Cd in leafy vegetables grown in a homogenous medium substrate with pH 7 is 0.793 (Great Britain. Dept. for Environment and Rural, 2002). For *B. juncea*, Podar *et al*., (2004), reported a 73% reduction in CF of Cd when grown in a binary heterogeneous treatment. Zn, being geochemically similar to Cd in its behaviour can be a useful indicator of plant uptake, and results for shoots of the same accession of *B. juncea* grown in binary treatment here, show a similar response, with a 65% reduction in CF compared to published values for Cd. However, for this species, as heterogeneity decreases, then the concentration factor increases above 1, resulting in accumulation of Zn in all other treatments, with HO and HL treatments showing a 3 fold increase in CF.

CFs for *B. napus* and *P. lanceolata* remain below 1 (Figure 4.7.1, a), in all treatments, indicating that these species have stronger control mechanisms, restricting the translocation of heavy metals to shoot tissues. A 50% reduction in CF for *B. napus* in binary treatment also supports findings by Podar *et al*., (2004), however all other treatments have CF's in line with published value for Cd in leafy salads.

Results for both shoots (Figure 4.7.1, a) and roots (Figure 4.7.1, b) indicate that current estimates of CFs based upon studies using a homogeneous spatial distribution provide a more conservative estimate of the risk to human health from the consumption of vegetables grown on contaminated soils, they also show a reduction of CFs in binary treatments compared to more realistic models of heterogeneity. However, *B. juncea* shows CF increasing beyond published values, and potentially hazardous accumulation of trace metals in response to variable spatial heterogeneity, suggesting that further research is required to establish a range of concentration factors to be used in conjunction with site specific geochemical surveys.

4.7.3. Implications for phyto-managment.

B. napus and *B. juncea* have been used in numerous studies (Blaylock *et al.*, 1997, Ebbs *et al.*, 1997, Clemente *et al.*, 2005, Quartacci *et al.*, 2006, Van Ginneken *et al.*, 2007) to assess their potential for phytoremediation of a range of heavy metals. These crop species are preferred over slow growing, element specific, low biomass hyper-accumulators as they meet many of the fundamental requirements for successful phyto-remediating plants, i.e.; high biomass, fast growing and taking up a range of heavy metals (Blaylock *et al.*, 1997, Mench *et al.*, 2010). Many studies using hydroponics, or application of chelating agents such as EDTA have found increased shoot concentrations in response to increased concentration of metal in substrate and/or chelating agent, but equally, significant reductions in biomass and plant success (Blaylock *et al.*, 1997, Ebbs and Kochian, 1997, Turan and Bringu, 2007). However, results for shoot concentrations of target contaminants obtained in hydroponic and homogenized pot experiments have not been realised in field studies (Banuelos *et al.*, 1998, Grispen *et al.*, 2006). Moreover, studies that have compared homogeneous pot experiments to simplistic heterogeneous treatments (Podar *et al.*, 2004, Manciulea and Ramsey, 2006, Menon *et al.*, 2007, Moradi *et al.*, 2009), have found that biomass significantly decreases in homogeneous treatments and metal uptake decreases in heterogeneous treatments. The results of this research support earlier findings for effects of heterogeneity based upon simplistic models for all three species studied, but it is the response to site-specific heterogeneity which provides compelling evidence that the spatial pattern of heavy metals, in contaminated land sites, will be a significant factor in its successful remediation using plants.

B. juncea has the highest concentration in shoots across all treatments compared to other species (Figure 4.7.2., a). However its poor growth performance (Figure 4.7.2 c and d) in low heterogeneity soils, make it unsuitable for use in remediation of sites that have contaminants distributed homogeneously, e.g. aerial deposition from smelting, or (as replicated from Nottingham in this study to form HL treatment) sites with long term application

of sewage sludge. Interestingly, when grown in a treatment representative of a patchy landfill or firing range (Coseley, HM and Hounslow, HH), but with the same average concentration found to be toxic when homogeneously distributed, *B. juncea's* growth is significantly improved, and whilst uptake is reduced, concentrations of Zn are still > 2 fold higher than other species.

B. napus, has been suggested by Turan and Bringu (2007) to be more effective, in the removal of a range of metals, than *B. juncea. B. napus* yielded the highest biomass (Figure 4.7.2. c and d) but shoot concentrations were at least 50% lower than those of *B. juncea* in treatments based upon actual site investigations. Zn concentrations in roots were the lowest of the three species. However, unlike *B. juncea*, *B. napus* appears to be indifferent to contaminant spatial heterogeneity, with the lowest variation between treatments for biomass and shoot Zn, making this a suitable species for use in site remediation where the distribution is unknown or homogeneous. Moreover, the higher biomass of this plant ensures greater total uptake of Zn per plant (Figure 4.7.3,a) being 6 fold higher in homogeneous to medium heterogeneity treatments, however only twice as high in higher heterogeneity treatments, than other species.

P. lanceolata showed no defined patterns in response to increasing contaminant heterogeneity yet significant differences were observed between HM and HH treatments. Plants in the HM treatment, based upon the Coseley site investigation, had lower shoot Zn concentrations and biomass, which is in contrast to earlier studies (Banuelos *et al.*, 1998, Podar *et al.*, 2004, Moradi *et al.*, 2009), covering a range of species and metals, showing that where contaminant concentration decreases in shoots, biomass increases. Of interest with this species is that roots accumulated Zn (Figure 4.7.2), with C/F greater than *B. napus,* by a factor of 2*,* and *B. juncea* (in HH and BI treatments) (Figure 4.7.1). This species demonstrates a strong internal mechanism, restricting the flow of zinc to shoots, making it an ideal species for **phyto-stabilisation**.

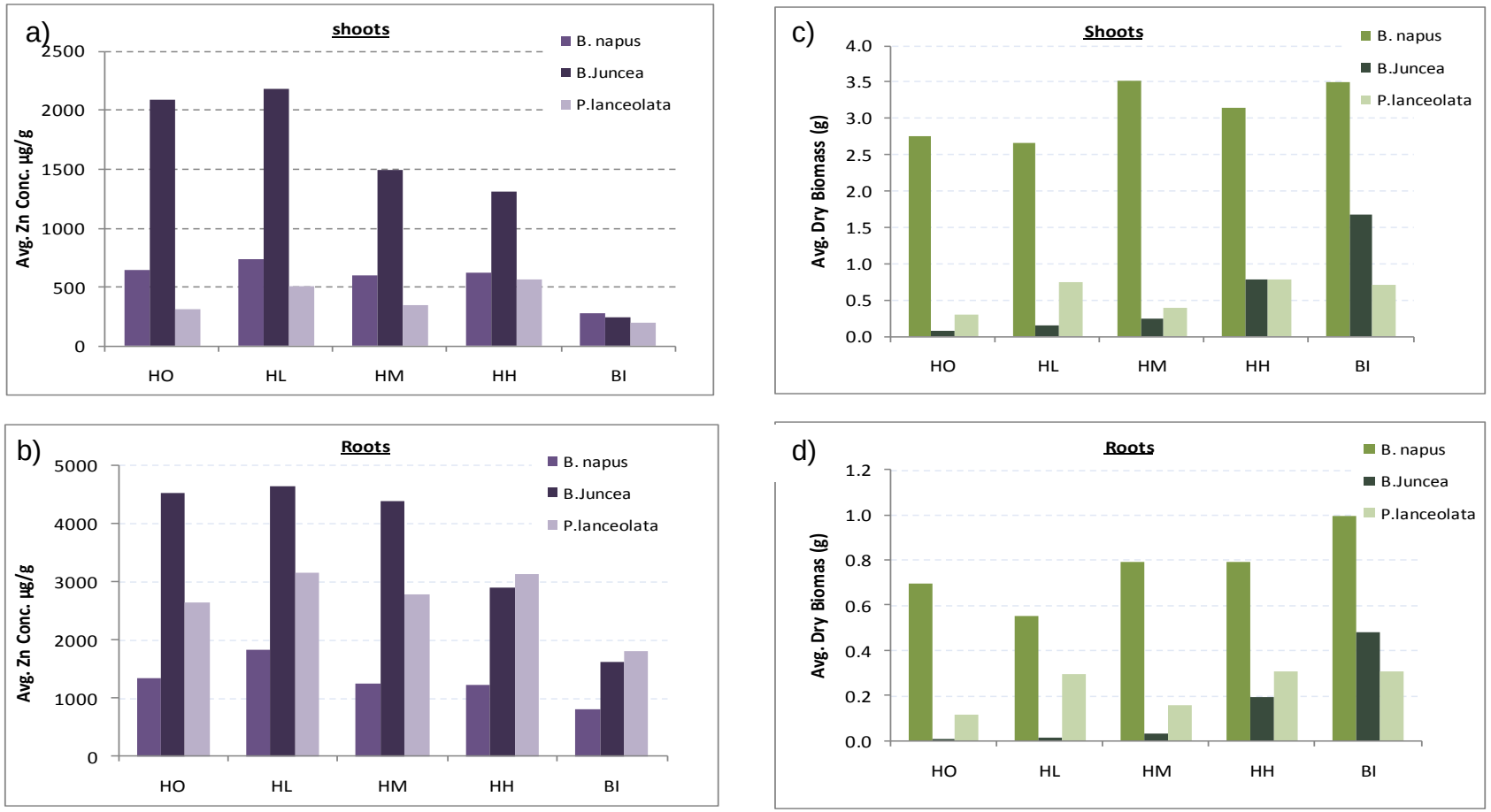
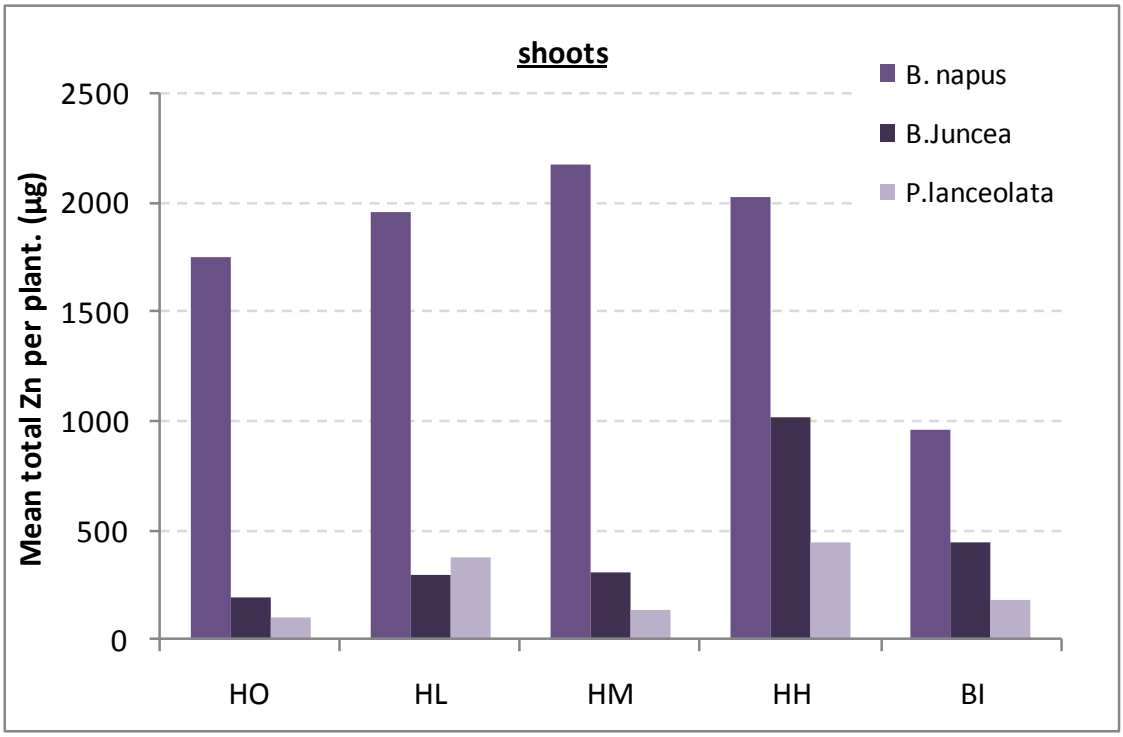


Figure 4.7.2. Average Zinc concentrations (µg g^{-1}) in shoots (a) and roots (b) and average dry weight (in grams) of shoots (c) and roots (d) for *B. napus*, *B. juncea* and *P. lanceolata*, grown in 5 treatments of equal Zn (900 µg g^{-1}) but differing spatial distribution.



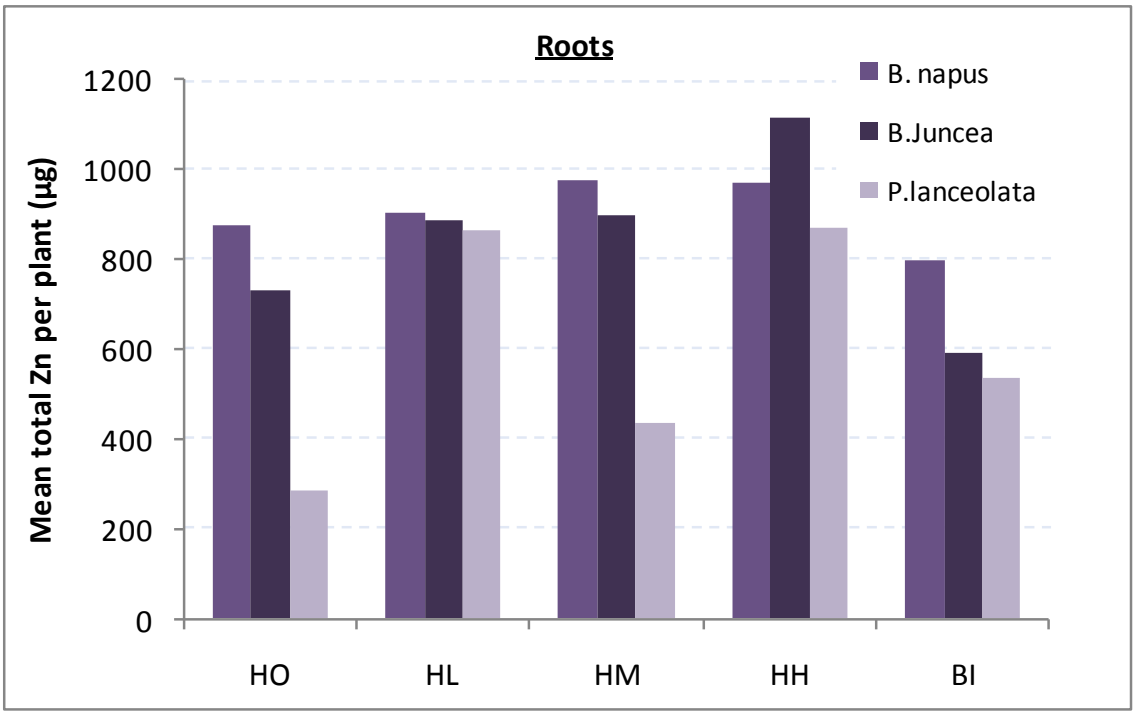


Figure 4.7.3. Mean total Zn per plant (µg) (Zn concentration in µg g^{-1}/total dry biomass) in a.) shoots and b.) roots for *B. napus*, *B. juncea* and *P. lanceolata.*

4.8. Conclusions and further research

This research has demonstrated that the current simplistic binary models, using a “hit and miss” approach are an inadequate proxy for contaminant plant uptake in heterogeneous substrates. Equally, for some species, the existing pot experimental methods, using a homogenised substrate can be misleading, with plants showing symptoms of toxicity at concentrations that may easily be tolerated in a heterogeneous site. The different responses by plants, of a similar size, to contamination at the 2cm scale, has demonstrated that a site specific approach is need for phytoremediation and risk assessment of plant uptake of potential harmful heavy metals. This research has looked at heavy metal contamination, but the models of heterogeneity could equally be applied to other contaminants, nutrient heterogeneity also essential trace elements which may be deficient with heterogeneity occurring at average concentrations which are lower than typical background.

Plants have evolved a complex set of mechanisms to adapt and cope with environmental heterogeneity and the scientific community has only scratched the surface in unravelling these complex mechanisms and interactions. Processes beneath the soil have a significant impact on aboveground performance, and a potential criticism of this study is that no analysis of any mycorrhizae, that may be present in the growing media, was undertaken. Whilst not associated with the two *Brassicas*, there are strong associations for *P. lanceolata* (Harley and Harley, 1987), and a study by Orlowska *et al*.,(2007) has shown that presence of certain strains can have a significant effect on metal uptake for this species.

The results for all three species showed a significant drop in Zn concentrations when patch contrast was highest in binary treatments. It is possible, that when patch contrast is significantly high, plant roots are able to selectively avoid areas of high contamination, and this will be explored in Chapter 5.

Through interdisciplinary research we may better understand how individual plant species react to a complex suite of environmental stimuli that will enable more effective management of the increasing number of global contaminated land sites.

Chapter 5 Second pot trial to investigate root placement as a response to simplistic and field based models of zinc spatial heterogeneity.

5.1. Introduction.

As outlined in the earlier chapter (See 4.1 Introduction) all components of soil are spatially heterogeneous (Ramsey and Argyraki, 1997) and there have been numerous studies determining the effects of spatial heterogeneity, for a wide range of soil properties, on plant growth. Effects of spatial heterogeneity in soils can be clearly visible in above ground shoots, as demonstrated by *B. juncea* in earlier chapter, but perhaps more interesting is the diverse range of responses that can occur below ground, at the coal face, so to speak.

Morphological plasticity of root systems in heterogeneous soils can vary considerably between species and even within individual plants. As roots grow throughout the soil, they can adapt to changes in its composition, for example, where soil is more compact, root architecture will change to produce thinner roots (Pierret *et al.*, 2007). Other studies have shown that some species can discriminate between variable nutrient concentrations by increasing density of fine roots throughout the nutrient patch compared to the less fertile, surrounding soil (Robinson, 1994, Hutchings *et al.*, 2003, Hutchings and John, 2004, Hodge *et al.*, 2009 for comprehensive reviews).

Heterogeneity can vary in time, spatially by scale, i.e. both the patch size in relation to root system and the contrast (the degree to which a resource or contaminant varies between adjoining patches). Research, to date, has predominately focused on the scale of nutrient distributions and its effect on individual root systems, (Campbell *et al.*, 1991, Gross *et al.*, 1993, Wijesinghe and Hutchings, 1997, Cahill and Casper, 1999, Einsmann *et al.*, 1999, Wijesinghe *et al.*, 2001). These studies have looked at a range of plants that have evolved different strategies for exploiting soil resources, with most finding increased root density in nutrient patches when scale is equal to or less than size of root system. There are some exceptions, for example Fransen *et al*.(1998) did not find increased root proliferation (increased root biomass) within

nutrient patches, however plants grown in heterogeneous treatments had increased uptake of nitrogen. Relatively few studies have considered the effects of contrast (Gersani and Sachs, 1992, Gleeson and Fry, 1997, Wijesinghe and Hutchings, 1999), i.e. the variability in nutrient quality or concentrations between patches, those that have done so found that individual plant roots, given an equal chance of proliferation into patches of varying nutrients, will proliferate in patches of highest quality.

However, nutrients are just one of the heterogeneous components within soils affecting plant growth and root systems. Trace or heavy metals, some essential for growth, (e.g. Fe, Ni, Cu, Zn) and some which are not, (e.g. Cd, Pb and As) are also heterogeneously distributed. Often these heavy metals can occur at concentrations that may be phytotoxic to many higher plant species, especially in metalliferous soils occurring both naturally or as a result of anthropogenic activity.

For centuries it has been known that certain plants have adapted to these soils, and in some cases species have a specific tolerance to a just one or two trace metals, making them useful indicators for the mining industry. Baker (1981) grouped these specialist plants into 3 main categories based upon their strategy in response to patches of high metal concentrations; **excluders**, which are able to regulate the flow of potentially harmful metals into sensitive areas of the plant; **indicators**, which will tolerate a range metals at elevated concentrations, until a threshold level is reached resulting in chlorosis; and **hyper-accumulators**, which will trans-locate metals into plant shoots at concentrations which are substantially higher than other plants, for which there is usually a specific concentration for each element. Robinson (2009) went on to ascribe specific root responses of metal tolerant species to a trace metal patch of; avoidance where roots will avoid growth in patches where soil concentrations are elevated compared to surrounding soil; indifference where roots will proliferate equally in patches of differing concentrations and proliferation/foraging, where plants will preferentially place roots in patches with higher concentrations of trace metals.

The phyto-management potential of hyper-accumulating foraging species has led to substantial research in this field, with the focus on the Zn and Cd accumulator, *Thlaspi caerulescens* (McGrath *et al.*, 1993, Baker *et al.*, 1994), as a potential species for remediation of soils containing heavy metals at concentrations toxic to other plants. Moreover recent studies (Schwartz *et al.*, 1999, Whiting *et al.*, 2000, Dechamps *et al.*, 2008) have demonstrated that *T. caerulescens* and *Sedum alfredii* (Liu *et al.*, 2010) respond positively to trace metal heterogeneity, with greater root mass in metal rich patches, though Haines (2002) found the response varied between different ecotypes of *T. caerulescens*. Root avoidance has also been observed for *Lupinus albus* (Menon *et al.*, 2007) in response to boron, and *Acer arietinum* (Moradi *et al.*, 2009) in response to nickel. However these studies have used a very simplified "hit and miss" substrate, with the pot divided into two halves, one with and one without the contaminant of interest.

Millis *et. al*, (2004), Podar *et. al*, (2004), and Manciulea *et. al*, (2006) have all expanded upon the simplistic heterogeneity "hit/miss" designs, of dividing a pot into two halves, to include scale and timing of heavy metal heterogeneity for non hyper-accumulating species. These models also show that for some species, the scale and timing of metal spatial distribution compared to the more traditional homogeneous tests of heavy metal uptake into plant shoots, have a significant impact on the amount of potentially harmful concentrations of metals that are trans-located into edible, above ground, fraction. All three studies found reduced heavy metal uptake and increased growth in some heterogeneous treatments compared to homogeneous controls, where total concentrations in each treatment are equal. However these studies did not look at root response, in terms of differing root density, to patches. Moreover heterogeneous treatments were still based upon simplistic "hit/miss" (similar to binary model, Chapter 4, Figure 4.2.1., e) design, and have not considered how changing patch contrast, to include variable concentrations throughout treatment (see Chapter 4, Figure 4.2.1 models b, c and d), will affect root placement and plant uptake of trace metals.

To date, there is little, if any, research assessing the impact of patch contrast for nutrient or trace metal heterogeneity on root morphology, and of those that have, models have been simplistic with little similarity to field conditions of heterogeneity. Chapter 4 demonstrated that changing the contrast of zinc spatial heterogeneity, whilst keeping scale and total concentrations constant, had significant impacts on total root and shoot growth, and plant uptake of Zn for *B. juncea* between simplistic and field based models. *B. napus*, with a similar root size and morphology, was grown in the same treatments, however, no significant difference was detected between the homogeneous treatment and the intermediate field models of heterogeneity, but a significant difference was detected between these treatments and the simplistic binary model of heterogeneity. This indicates that contrast may be as important to root response as the effect of scale where trace metals are heterogeneously distributed. This chapter will look at how roots of the two *Brassica* species are distributed throughout cells in two different treatments of zinc spatial heterogeneity where scale and total concentrations are held constant, and test the following hypotheses.

Hypotheses.

i.

a. Roots of non-hyperaccumulating plants preferentially proliferate in patches of lower zinc to avoid uptake of this potentially harmful metal.
b. The ability of plants to detect low zinc patches will depend on the degree of contrast between zinc rich and zinc poor patches.

ii. The provision of contamination with greater levels of contrast, but the same overall concentration, will allow roots to proliferate in contaminated patches and improve overall plant performance.

5.2. Materials and methods.

5.2.1. Study species

B. napus and *B. juncea* are two species of the *Brassicacea* family which have a similar root morphology and spatial scale when grown in a medium with elevated levels of zinc, reported in Chapter 4. Both species produce a system of diffuse fibrous roots, with no prominent central tap root, and in the case of *B. juncea*, grown in binary treatments (Chapter 4, Figure 4.2.1), clearly defined lateral roots extending parallel to the growing medium surface. *Brassica napus* (oil seed rape) variety ES Astrid, grade CS seeds, were supplied by Severn Trent Water Authority from Frontier, certification F1621NB30006E1. Seeds had been treated with a fungicide coating of Chinook and Royal Liquid FS, Thiraflo and Seedlife. (Jackson, 2008). *Brassica juncea* seeds, accession PI 426308, origin Pakistan were supplied by the North Central Regional Plant Introduction Station (NCRPIS), forming part of the U.S. National Plant Germplasm System (NPGS). Iowa State University, Regional Plant Introduction Station, Ames, Iowa, United States.

5.2.2 Experimental design and substrate preparation.

Two treatments of spatial zinc heterogeneity were prepared, (See Figure 5.2.1) a high heterogeneity (HH) model based upon *in situ* geochemical studies and binary (BI) model, with the same mean concentration in each pot. The HH model was simplified to a symmetrical design, enabling some pseudo-replication of patches of equal quality and controlled alignment for extraction. The starting central cell was kept at the same concentration for both treatments.

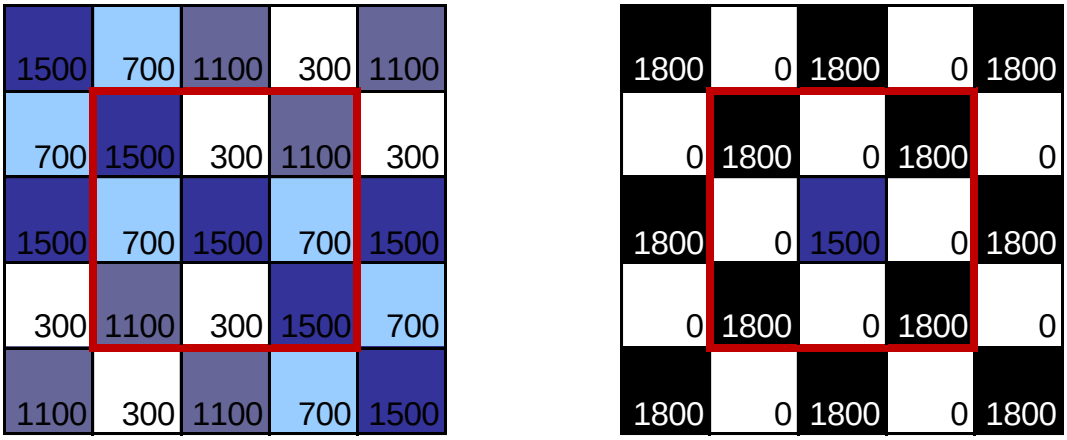


Figure 5.2.1. Heterogeneity designs for root biomass experiment. a) a simplified version of *in situ* (HH) heterogeneity and b) (BI) binary treatment. Level 1 (L1) relates to cells contained within red square, excluding centre, level 2 (L2) cells are outside red square. RSD relates to calculated value for heterogeneity (based upon method in Chapter 3), average value relates to mean concentration throughout pot.

Treatments were prepared in a similar way to Chapter 4 with the following changes.

i. Reduced growing media volume. Each cell within a treatment was reduced in depth to 125mm, yielding a cell volume of 25 x25 x 125mm. The reasons were two fold, a) the growing period was shorter requiring less growing media volume, and b) a smaller volume in the pot enabled easier extraction of each cell.

ii. The models consisted of 25 cells forming a cube, which were contained within custom built barriers, constructed from water-proofed cardboard sleeves. The cube was held in position within the pot by packing the surround void with an inert perlite. The barrier ensured roots would not penetrate the perlite and helped to maintain structure during removal.

iii. The base of the pots were lined with a plastic mesh to prevent growing media escaping and collapse of structure.

iv. Pot heights were reduced from 18 to 15 cm for ease of handling, prevention of shadowing and removing the need for packing the base with sand.

v. Pots were marked to show four corner cell concentrations, to control pot orientation and correct cell extraction at harvest.

20 replicates of each treatment were prepared for each species, giving a total of 80 pots. Seeds were germinated as before (Chapter 4,4.2), in vermiculite, with seedlings of equal size being transferred to central cell of treatment upon the appearance of true leaves. Plants were placed in a randomised block design consisting of 5 blocks, each containing 4 replicates of each species and treatment. Plants were grown in a controlled greenhouse environment (16 hours light at 20°C ±5°C, see p. 131 for discussion) and watered daily with tap water applied with fine mist to avoid displacing growing medium heterogeneity.

5.2.3. Shoot harvest and root extraction.

After 30 days growth, above ground biomass was harvested, washed with tap water and placed in a drying oven for 48 hours at 60°C until dry. Dry biomass was recorded prior to determining zinc concentrations. Total zinc was extracted using a nitric and perchloric digest method (Thompson and Walsh, 1983), into 1 Mol Hydrochloric acid matrix solution before analysis with AAS (Atomic Absorption Spectrometer). Reagent blanks, house and certified reference materials were incorporated into analytical batches to detect any analytical bias in laboratory methods.

To extract roots the growing medium cube in each pot was placed into a wooden holding box. The customised sleeve was removed and cube was held securely in position with a holding block (see Figure 5.2.2 pictures a, b and c). A customised blade was then used to divide growing medium cube into individual cells, using measured grooves on top of the holding box. Cells were grouped according to concentration and level (see Figure 5.2.1 for definition of level) for

each pot, into individually labelled trays before root extraction (See Figure 5.2.3 pictures d and e). Roots were washed and placed in a drying oven before recording dry biomass.

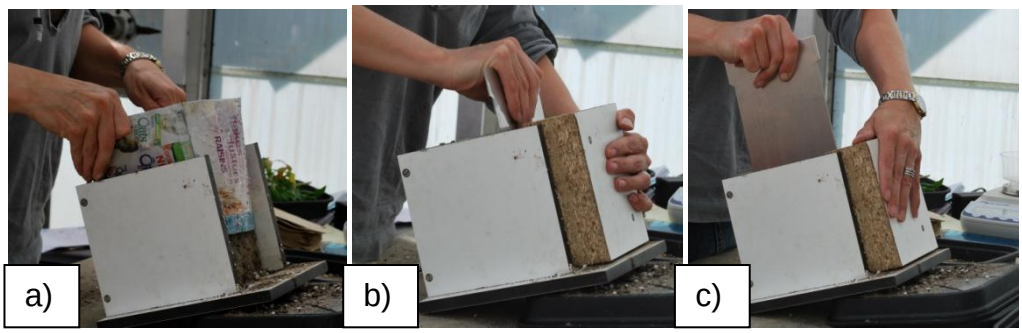


Figure 5.2.2. Dividing growing medium into individual cells of heterogeneity model, prior to root extraction. a) placement of growing medium in holding block and removal of sleeve, b) and c) insertion of blade along vertical and horizontal grid lines.

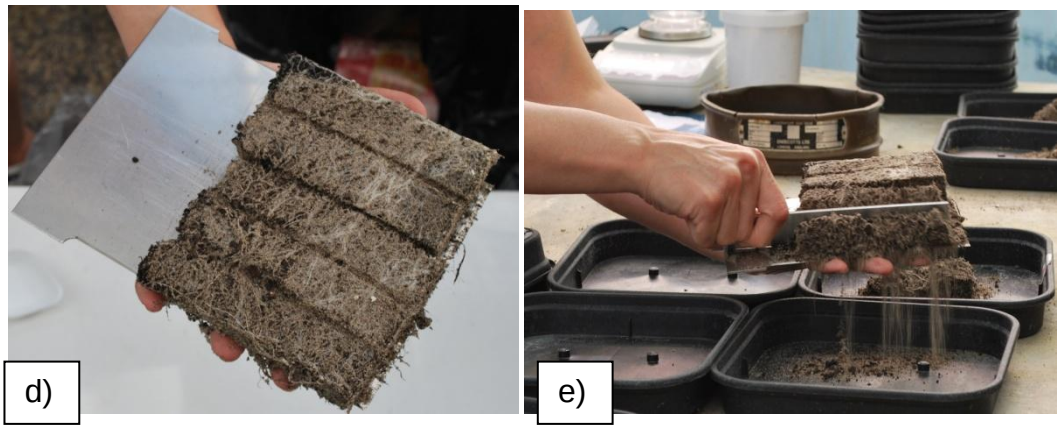


Figure 5.2.3. Picture d) shows a cross section of growing medium, containing a row of 5 individual cells, picture e) shows cells being separated into labelled trays prior to root extraction.

5.2.4 Data analysis.

After washing and drying, shoot biomass was weighed before zinc concentrations were measured. Roots extracted from individual cells were grouped by concentration and level, washed and dried before weighing. A full

dataset of recorded values for each species can be found in Appendix F, Data Table F1.

Statistical tests were performed using SPSS Statistics 17.0 software. One sample Kolmogorov-Smirnov (K-S) tests for normal distribution and Levene's test for equal variance (Dytham, 2003) were performed on variables to determine whether parametric statistical tests were appropriate (See Appendix F, Data Table F.2). Where non-normality and heteroscedasticity of variance were detected, data were logarithmically or square root transformed, or in the case of *B. juncea* in HH treatments, where transformation was not possible due to very low number (3) of complete sets of roots, the non parametric Kruskal-Wallis test was used. For root analysis, equal variance tests were only applied to data from the same level (see Figure 5.2.1 for definition). A mixed model ANOVA was used to test for significance of differences in root density in contrasting patch concentrations at the same level, taking into account any effects of block. Patch concentration was used as the fixed factor and block as random factor.

5.3 Results.

5.3.1 Dry weight Shoot and total root.

For *B. juncea*, highly significant differences ($p<0.001$, see Table 5.3.1) in root and shoot biomass were found between the two contrasting treatments, with plants yielding up to 30 times greater biomass for both root and shoots in binary (BI) treatment compared to the *in situ* high heterogeneity model (HH) (Figure 5.3.1. and Figure 5.3.2.). Analysis of root/shoot (R/S) ratios (Figure 5.3.2) shows *B. juncea* allocates a higher biomass to roots in binary models with the difference in R/S between the two models of heterogeneity to be highly significant ($P < 0.001$).

For *B. napus* no significant difference was found between treatments for roots and shoots, though higher biomass was observed in binary treatments and results from ANOVA for root analysis are close to significance values (p =

0.075). Further analysis of R/S ratios (Figure 5.3.2) shows a significant difference (p = 0.025, see Table 5.3.1) due to model of heterogeneity.

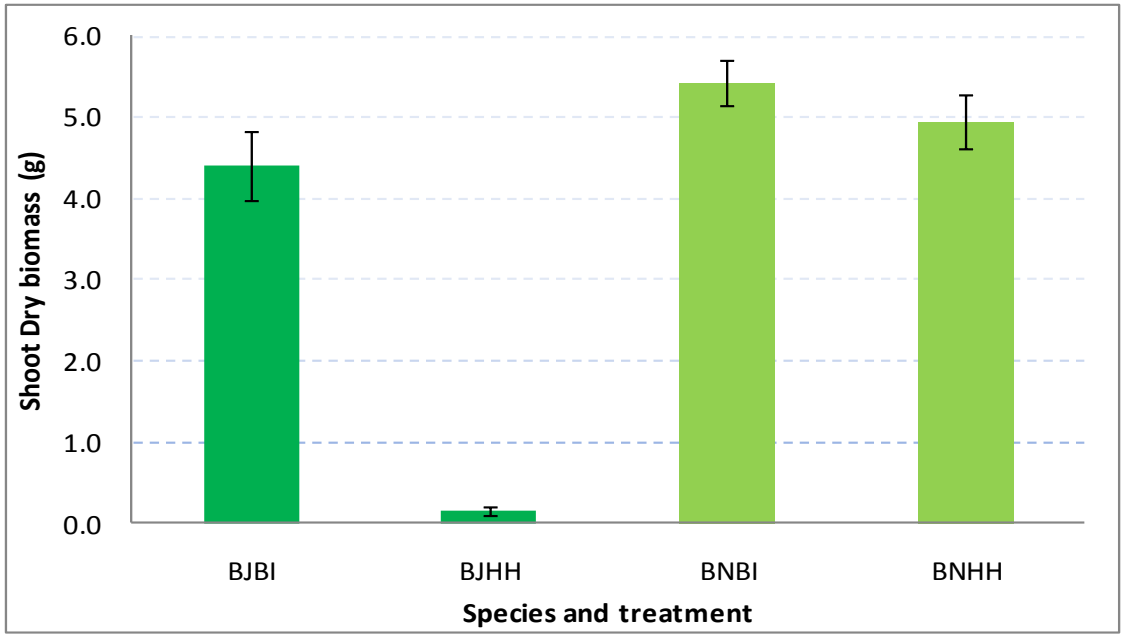


Figure 5.3.1. Mean shoot dry biomass (g) for *B. juncea* (BJ) and *B.napus* (BN) grown in two contrasting Zn heterogeneity treatments; high heterogeneity (HH) and binary (BI). Error bars represent the standard error on the mean where n = 20 for *B. napus* in both treatments, n = 18 for BJBI and n = 15 for BJHH.

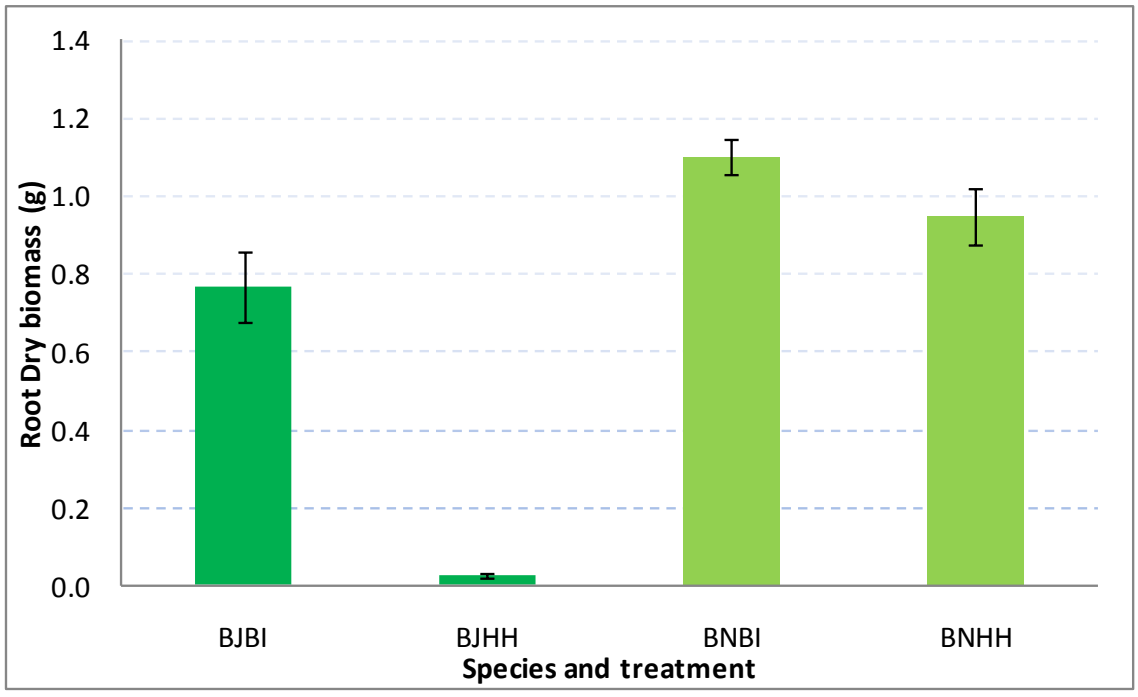


Figure 5.3.2. Mean total root dry biomass (g) for individual for *B. juncea* (BJ) and *B. napus* (BN) grown in High heterogeneity (HH) and Binary (BI). Error bars represent the standard error on the mean where n = 20 for *B. napus*, n = 17 for BJBI and n = 7 for BJHH.

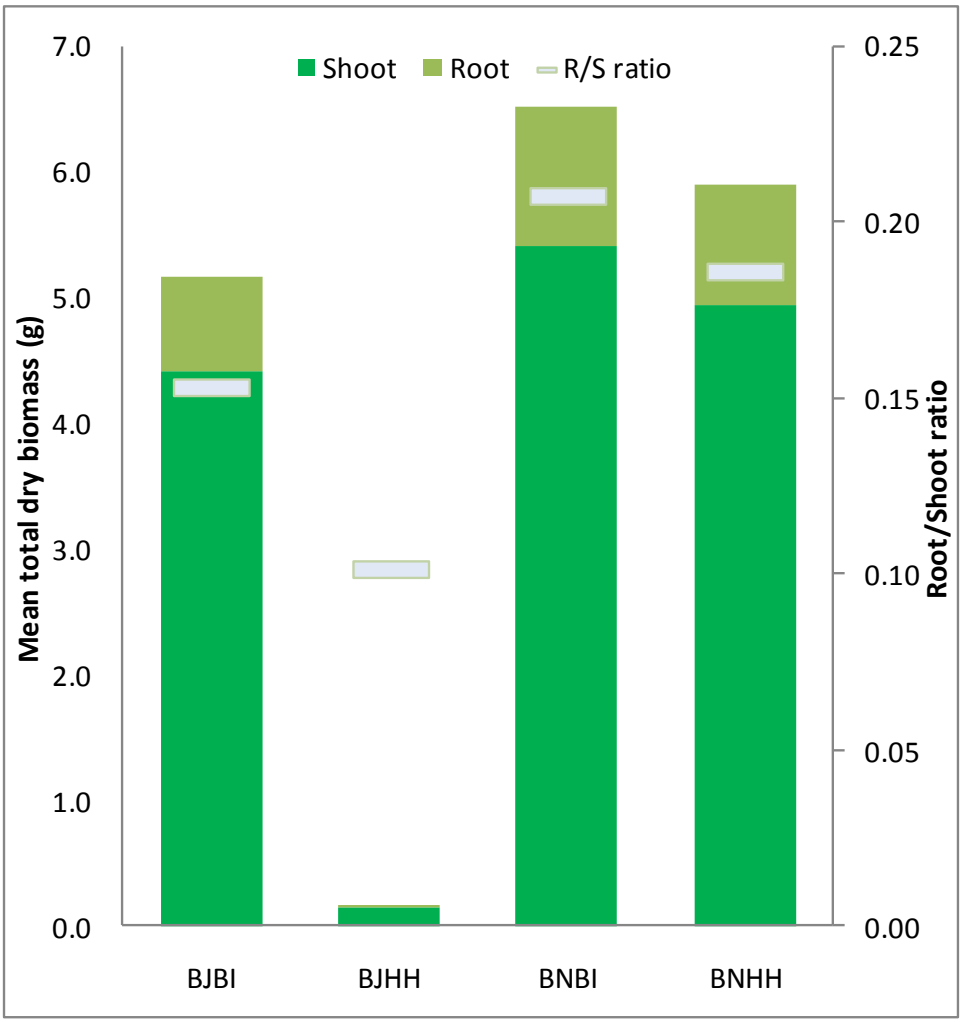


Figure 5.3.3. Total mean dry biomass (g), showing allocation between root and shoot for *B. juncea* and *B. napus* grown in two contrasting treatments of Zn heterogeneity. Respective mean root/shoot ratios are plotted on secondary vertical axis.

Table 5.3.1. Analysis of variance for *B. juncea* and *B. napus* shoot and root dry biomass and measured zinc concentrations of plants grown in 2 treatments of contrasting spatial Zn heterogeneity. The experiment was conducted using a randomised block design, and tested using a mixed model ANOVA with treatment as fixed factor and block as a random factor. Factor is significant for P values < 0.05 and highlighted in bold.

Dependant variable	Factor	d.f.	ss	ms	*F*	*P*
B. juncea						
Shoot dry biomass (Square root transformed)	Treatment	1	21.691	21.691	80.828	**<0.001**
	Block	4	0.621	0.155	4.270	0.681
	Error	27				
Total Root dry biomass	Treatment	1	2.431	2.431	15.850	**0.001**
	Block	4	0.089	0.022	0.145	0.963
	Error	21				
Shoot Zn conc.	Treatment	1	22516825	22516825	80.873	**<0.001**
	Block	4	1024708	256177	0.920	0.467
	Error	27				
Root/shoot ratio	Treatment	1	0.069	0.069	18.931	**<0.001**
	Block	4	0.011	0.003	0.746	0.569
	Error	27				
B. napus						
Shoot dry biomass	Treatment	1	2.196	2.196	1.493	0.230
	Block	4	25.117	6.279	4.270	0.007
	Error	34				
Total Root dry biomass	Treatment	1	0.240	0.240	3.381	0.075
	Block	4	0.511	0.128	1.802	0.151
	Error	34				
Shoot Zn conc..	Treatment	1	1961886	1961886	22.217	**<0.001**
	Block	4	268255	67064	0.759	0.559
	Error	34				
Root/shoot ratio	Treatment	1	0.005	0.005	5.503	**<0.025**
	Block	4	0.009	0.002	2.803	0.041
	Error	34				

5.3.2 Shoot Zinc concentration.

Results for mean measured zinc concentrations (Figure 5.3.4) in above ground biomass were found to be consistent with earlier experiments (Chapter 4), showing reduced uptake into shoot when grown in binary treatments for both species. Concentrations in shoot dry biomass, were significantly reduced, 2.5 times lower in *B.juncea* and 1.5 times lower in *B. napus*, ($p < 0.001$, see Table 5.3.1) when plants were grown in binary treatments compared to *in situ* models.

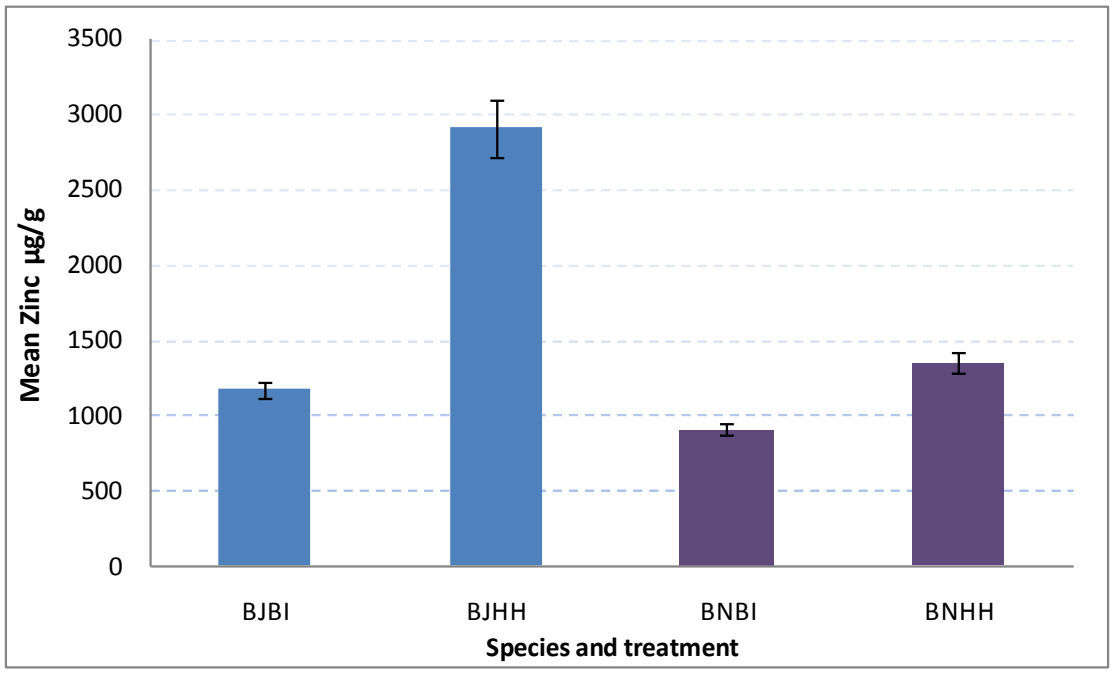


Figure 5.3.4. Mean Zn concentrations (µg/g) in shoot dry biomass for *B. juncea* and *B. napus* grown in two treatments of equal zinc concentration but differing spatial heterogeneity. BI relates to binary model and HH is a high heterogeneity model (based upon field *in situ* measurements). Error bars represent the standard error on the mean where n = 20 for *B. napus*, n = 18 for BJBI and n = 15 for BJHH

5.3.3 Dry weight roots per cell.

Plants of *B. juncea,* grown in simplistic binary treatments (Figure 5.3.5. a), showed no difference in root biomass between cells of contrasting zinc, that were equi-distant from the central cell. As roots penetrated throughout the

growing media away from central cell, dry root mass, measured in the cells with Zn concentration of 1800 µg g^{-1}, was 25 % higher than low zinc cells. No significant difference was detected (Table 5.3.2) using ANOVA but a paired t-test, of dry root mass between L2 0 and L2 1800 cells, reveals a significant difference ($t_{-2.416,\ 16}$ p= 0.028).

B. juncea grown in the more complex, high heterogeneity distribution (Figure 5.3.5, b) of Zn exhibited signs of stress and only 3 complete sets of roots were obtained from individual cells. However a relatively higher root mass was found in cells with the lowest Zn concentration of 300 compared to all other cells with higher concentrations, though due to the low number of replicates, distribution of data did not meet requirements for parametric statistical testing, even after transforming. The less powerful non parametric Kruskal Wallis test for comparison of means found no significant differences (Table 5.3.3).

B. napus plants grown in binary treatments (see Figure 5.3.5, c) showed no difference in root biomass in cells of different Zn concentrations at level 1 (L1) adjacent to central cell. However as roots proliferated to the outer cells a highly significant difference ($P < 0.001$) was found between the two Zn concentrations of 0 and 1800 µg g^{-1} in the outer cells of level 2. Root mass was, on average, 30% higher in the 1800 µg g^{-1} cells than the cells containing no Zn.

In high heterogeneity (Figure 5.3.5, d) treatments there were no significant differences for root biomass between cells of contrasting Zn concentrations at either level 1 or 2 (Table 5.3.2.), however at level 1, results from ANOVA (Table 5.3.2) are close to significance ($P = 0.077$). Further analysis using a paired t test, for cells that have an equal probability of root penetration from central cell, e.g. L1-300 and L1-700 (both are adjacent to central cell) and L1-1100 and L1-1500 (both at corners of central cell, reveals significant differences ($t_{-2.130,19}$ $P = 0.046$ and $t_{3.109,19}$ $P = 0.006$ respectively), with higher root biomass in the cell with lower Zn in both cases.

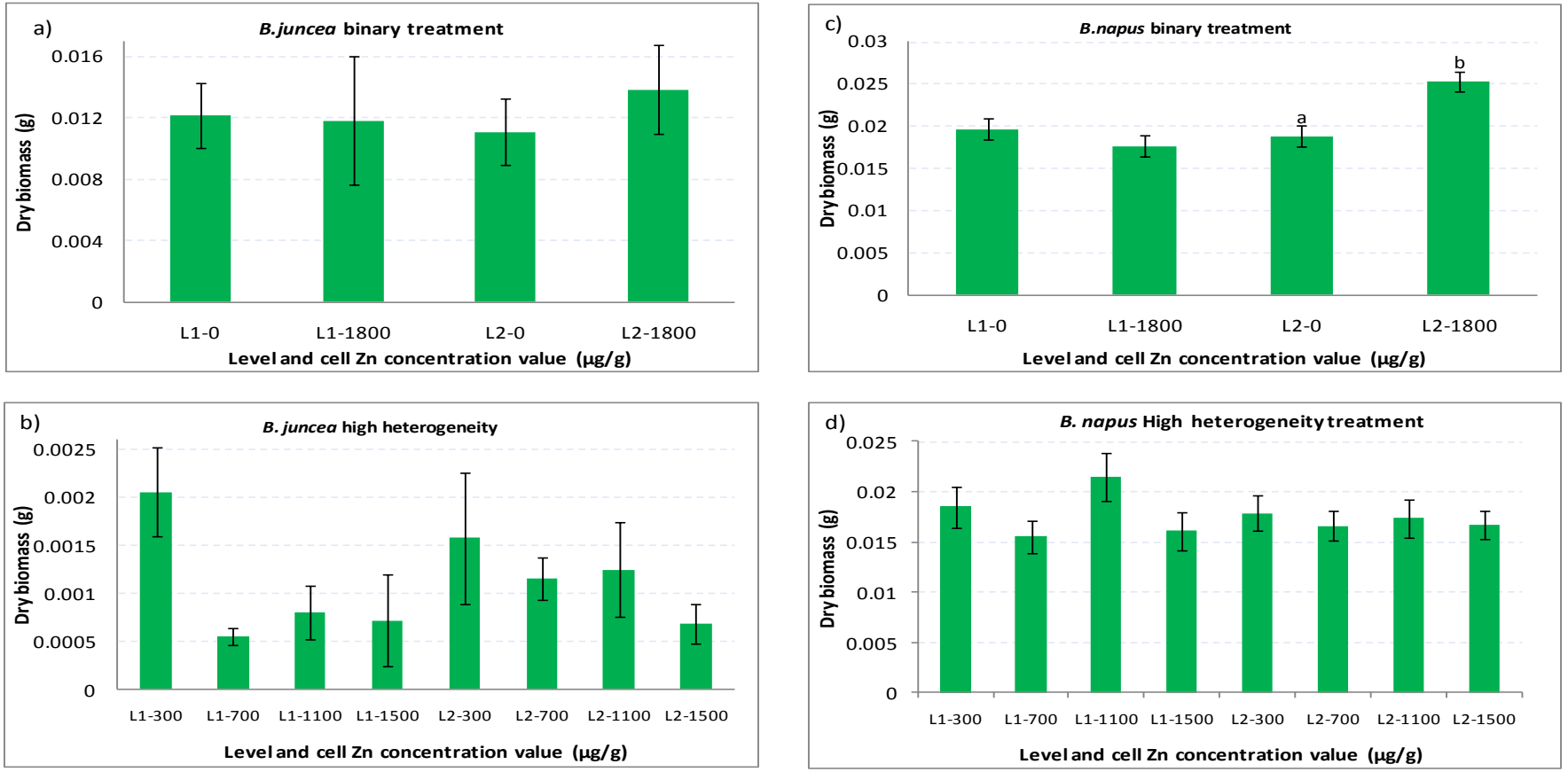


Figure 5.3.5. Indicative root allocation to individual cells of equal zinc concentration and distance (L 1 and L2), for *B. juncea* (a and b) and *B. napus* (c and d), grown in two treatments of equal total zinc but differing patch contrast (see Figure 5.2.1 for design). Numeric values relate to Zn concentration (µg/g) on each cell.

Error bars represent the standard error on the mean where n = 20 for *B. napus* in both treatments and n =17 for BJBN and n = 3 for BJHH, where n is equal to number of surviving plants with extractable roots. Means with different data label differ significantly (based on ANOVA.

Table 5.3.2. Analysis of variance for *B. juncea* and *B. napus* root dry biomass in cells of equal distance and zinc concentration from central cell. The experiment was conducted using a randomised block design at whole plant level, and tested using a mixed model ANOVA with treatment as fixed factor and block as a random factor. Factor is significant for P values < 0.05.

Dependant variable	Factor	d.f.	ss	ms	*F*	*P*
B. juncea						
Binary Level 1 Ln root dry biomass	Cell conc.	1	1.021	1.021	0.870	0.359
	Block	4	4.456	1.114	0.949	0.451
	Error	28				
Binary Level 2 Ln root dry biomass	Cell conc.	1	0.303	0.303	0.319	0.577
	Block	4	8.811	2.203	2.315	0.082
	Error	28				
B. napus						
Binary Level 1 root dry biomass	Cell conc.	1	0.001	0.001	1.274	0.267
	Block	4	0.002	0.000	0.80	0.534
	Error	34				
Binary level 2 root dry biomass	Cell conc.	1	0.027	0.027	14.394	**0.001**
	Block	4	0.003	0.001	0.427	0.788
	Error	34				
HH Level 1 root dry biomass	Cell conc.	3	0.002	0.001	2.377	0.077
	Block	4	0.006	0.002	6.449	**<0.001**
	Error	72				
HH Level 2 root dry biomass	Cell conc.	3	0.000	0.000	0.145	0.932
	Block	4	0.008	0.002	2.565	**0.045**
	Error	72				

Table 5.3.3. Results of non parametric Kruskal Wallis comparison of means test between cells of differing Zn concentrations (µg g^{-1}), but equal distance from central cell, for plants of B. juncea grown in high heterogeneity (HH) model. Factor is significant for *P* < 0.05.

Dependant variable	Factor	Chi Sq.	d.f.	*P*
B. juncea				
HH Level 1 root dry biomass	Cell conc.	4.128	3	0.248
HH Level 2 root dry biomass	Cell conc.	1.256	3	0.741

5.4. Discussion.

5.4.1. Stated hypothesis

Hypothesis.

i.

a. Roots of non-hyperaccumulating plants preferentially proliferate in patches of lower zinc to avoid uptake of this potentially harmful metal.
b. The ability of plants to detect low zinc patches will depend on the degree of contrast between zinc rich and zinc poor patches.

The analysis of root biomass in individual cells in the simplistic binary model of heterogeneity does not support the hypothesis that roots of non-hyperaccumulating plants preferentially proliferate in patches of lower zinc to avoid uptake of this potentially harmful metal. Significant differences in root biomass between cells of different concentrations were detected for both *B. napus* and *B. juncea*, in binary treatments as roots proliferate into outer zone of growing media (L2). Surprisingly, a higher root biomass was found for both *B. juncea* (25 % higher) and *B. napus* (34 % higher), in cells containing the highest concentration of Zn at 1800 µg g^{-1}.

Reduced Zn concentrations were observed in shoots similar to findings by Podar *et. al*, (2004) who found reduced shoot concentrations of Zn and Cd for *B. juncea* grown in simplistic heterogeneity models. Podar suggested that this was due to roots preferentially growing in uncontaminated patches, but results show this not to be the case. These results also contradict the recent findings, using simplistic models, by Moradi *et al*, (2009) for the non hyper accumulating *Acer arietinum* in response to nickel, and Menon *et,al.(2007)* study of *Lupinus albus* in response to boron, who found greater root proliferation, based upon radiography images, in the non contaminated half of the growing media.

Whilst greater root biomass was found in cells of high Zn in the high contrast binary treatments, roots extracted from the field based models of high

heterogeneity, where patch contrast between zinc rich and zinc poor cell is reduced, found roots preferentially proliferating into cells with lower zinc concentrations. Root biomass measured in the lowest zinc concentration cells (300 µg g^{-1}) was 30% greater than in cells, with equal probability of proliferation, but a higher concentration (700 µg g^{-1}), for *B. napus*.

A similar response was observed for *B. juncea,* roots extracted from cells with 300 µg g^{-1} of Zn were found to be nearly 4 times higher than cells containing Zn of 700 µg g^{-1}. Unfortunately, very few plants of *B. juncea* in this treatment survived, resulting in too few replications to yield results of statistical significance. Repeating this experiment with *B. juncea* during the winter months, with lower total concentrations may provide some interesting results for this species which is highly responsive to contaminant heterogeneity.

In conclusion to first stated hypothesis, it is not always true that plant roots will proliferate into patches with the lowest concentration of potentially harmful contaminants (See table Table 5.4.1.) Secondly, both plants were able to detect differences in zinc concentrations at different degrees of contrast, therefore, for the concentrations used in this experiment, we would also reject the second part of hypothesis (i) b. However results show that the response of plant roots will alter where the degree of contrast is altered but the overall concentration remains constant.

ii. The provision of contamination with greater levels of contrast, but the same overall concentration, will allow roots to proliferate in contaminated patches and improve overall plant performance.

The response of *B. juncea* to contrasting, contaminant heterogeneity, provides strong support for the second hypothesis. Where contrast between patches is higher, plant biomass in both roots and shoots is significantly increased. Plant survival increases from 35% in high heterogeneity treatments to 85%, and Zn in shoot of *B. juncea* grown in high contrast binary treatments is 60% lower than plants in lower contrast treatment. For *B. napus*, whilst there are no significant

differences in plant growth performance for shoots and total roots, between treatments of varying contrast, root biomass is 16% greater in binary treatments This difference is close to significance (P = 0.075), and increasing the power of the test, with more replicates, might have produced a significant result. A highly significant difference in shoot Zn concentrations, shows a 33% reduction in Zn uptake between high contrast binary, and lower contrast high heterogeneity treatments, consistent with finding for *B. juncea*. For both species, where contrast is increased, higher root mass is found in patches of high Zn, compared to lower contrast Zn treatments, where root biomass is greatest in patches with lower Zn.

Table 5.4.1. Summary of hypothesis tested for each species and treatments.

Hypothesis	***B. napus***		***B. juncea***	
	Binary	**High heterogeneity**	**Binary**	**High Heterogeneity**
(i) a.	Reject	Accept	Reject	Reject*
(i) b.	Reject		Reject	
(ii)	Accept		Accept	

Conclusions as to whether hypothesis is accepted or rejected are for probability p = 0.05

* Inconclusive, test had poor statistical power, increasing replicates is likely to change outcome.

5.4.2. Comparison to earlier experiment.

This experiment was conducted throughout the summer growing season, June to July 2009, whilst in the earlier experiment (Chapter 4) plants were grown during the winter months of December to February. Variation between treatments was consistent with the earlier study, with no difference in shoot biomass between treatments for *B. napus* and a significant difference between binary and field based heterogeneity treatments for *B. juncea*. Moreover, similar differences in plant uptake of Zn into shoots were found, with significantly lower concentrations in binary treatments for both species. However, mean concentrations for plants grown in summer months were considerably higher

(See Table 5.4.2 below), and this is probably due to higher transpiration rates, as temperatures within the greenhouse were found to be in excess of 30°C on several occasions. These higher levels of Zn, in high heterogeneity field models, may also help to explain the higher mortality of *B. juncea* grown during summer months compared to winter, as a toxic threshold for this species is reached more rapidly.

Table 5.4.2. Average concentration of Zn µg g^{-1}, in dry shoot biomass for *B. napus* and *B. juncea* for binary and field modelled heterogeneity for two experiments conducted during different seasons. Winter values for field modelled heterogeneity show range of concentrations across all treatments.

	***B. juncea* shoot Zn**		***B. napus***	
	Binary	Field modelled heterogeneity	Binary	Field modelled heterogeneity
Summer	1171	2914	908	1351
Winter	250	1313 - 2181	278	600 - 734

5.4.3. Interpretation of results.

Both species of *Brassica*, when grown in the simplistic binary model of heterogeneity, increased root biomass in Zn patches and had shoot Zn concentrations higher than growing media average and at levels phytotoxic to many other species. Based upon the classifications of metal tolerant plant species by Baker (1981) and Robinson *et, al.*(2009), the response observed would indicate that these plants are foraging accumulators. However, Hodge (2004) finds that increased biomass is not always an indication of foraging when considering plant response to nutrient patches, and this may also be true for metal tolerant species.

A possible cause of increased root biomass in Zn patches may be an increase in root birth and death rates. Hamlin and Barker (2006) demonstrated that the

growth of *B. juncea* is characteristic of an indicator plant (Baker, 1981), in the presence of Zn. It will grow in a wide range of concentrations, decreasing biomass as Zn concentrations increase, until a threshold is reached and internal control mechanisms are breached resulting in chlorosis and death. Ebbs and Kochian (1997) undertook research specifically relating to root morphology of *B. juncea* and found lateral root diameter decreased in response to Zn, which would support possible increased death of roots.

Why both these species are able to generate more above and below ground biomass in binary as opposed to high heterogeneity treatments, where total concentration remains constant, may be due more to the cellular mechanisms within the plant, as described by Hall (1999). Plants have developed a range of mechanisms to cope with heavy metal stress and those which may play an important role in the response of the two species to changing spatial heterogeneity are; the ability to avoid concentration build up in sensitive parts of the plant and reduced influx across the plasma membrane and repair mechanisms. For example longer lived roots in low concentration patches may help to rebalance the Zn flowing across the root cell membranes in the high zinc patches, provide the resources for repair mechanisms or sustain continued birth of new roots in high Zn patches after death of existing roots.

Zn concentrations in shoots of *B. juncea* between the two treatments suggest that, in a lower contrast treatment, this species suffers a breakdown in the control of Zn influx, and Zn floods into the plant. Where, once in the plant, this species is less able to control mobility to sensitive parts. *B. napus* is more successful and in coping Zn patches of variable contrast and this species may have evolved several mechanisms.

5.5. Conclusions and further work.

In conclusion, this research shows that, varying the degree of Zn patch contrast in heterogeneity models, produces different responses in root growth, both

within the same species and between different species. Whilst this research has only focused on Zn, it has implications for the numerous studies which have drawn conclusions regarding plant root behaviour, based upon simplistic pot models of heterogeneity, both for trace metals and other components of soil, including nutrients. Both experiments in this and the previous chapter have demonstrated that there are significant differences, in plant response, to high contrast simplistic binary models compared to the more realistic field models of heterogeneity.

The phytoremediation and **phytomining** potential of plants, based upon laboratory studies has not always been realised in field trials (Banuelos *et al.*, 1998), and the results here demonstrate that the *in situ* heterogeneity, replicating that in a field site, is a significant factor. If we are to maximise the phyto-management potential of plants, further work is required to understand the mechanisms within plants, particularly the roots, which control plant uptake of trace metals and its impact on plant growth. Additionally, we need to understand which plants are better adapted to homogeneous substrates and which will be more effective in heterogeneous environments. Results described in Chapter 2 demonstrate that contaminated land sites often have site specific heterogeneity and the ability to match plant response to spatial distribution, both in terms of scale and patch contrast, will enable more effective remediation. This research has focused on high levels of trace metals, but there is also a need to understand the spatial distribution of a range of trace metals that may be deficient in soils. This is currently an important topic for agriculture and human health (Alloway, 2009), where deficiencies in diet, in particular Zn, correlate to a range of life threatening diseases and poor crop performance. If we can understand root response to patchily deficient Zn, then we may be able to select for crops that are more efficient in root foraging and uptake to improve diet.

6.1. Introduction.

This thesis introduces a new sampling design, aimed specifically at quantifying spatial heterogeneity of contaminants in soils across a range of scales. The design was successfully applied at two contrasting contaminated land sites, using relatively new *in situ* measurement techniques, enabling quantification of contaminants in soil, without disturbing the spatial heterogeneity. The new sampling design incorporated a balanced format, which can be used in conjunction with the established statistical technique of robust ANOVA (analysis of variance), to quantify contaminant heterogeneity at each scale.

Four plant species were chosen, based upon site surveys, and historical studies using simplistic models of contaminant heterogeneity. From results of actual site surveys, heterogeneity values, for the scale relevant to chosen species, were selected to create more realistic models of heterogeneity for use in pot experiments. Using the four plant species, a pot experiment was conducted, which for the first time assessed the impact of a range of contaminant heterogeneities on plant contaminant uptake and growth.

Finally, based upon the results from the first pot experiment, a further pot experiment was undertaken, which for the first time assessed root growth in response to varying contaminant contrast, whilst keeping total concentrations equal in each treatment.

The main conclusions from the research are presented here, together with suggestions for further research.

6.2. Quantification of contaminant spatial heterogeneity, across a range of scales, using a new sampling design in conjunction with in situ measurement techniques.

The first objective of this thesis is addressed in Chapter 1, with a review of existing methodologies aimed at the quantification of heterogeneity, and sampling designs used to estimate the spatial variability of contaminants within soils, across a range of scales. Several methods (lacunarity, nearest neighbour and classical sampling theory) were found to only be applicable to discrete variables, or where the variable to be measured is clearly visible, which is not the case for contaminated land investigations of soil.

Two methods, routinely used in contaminated land investigations, are variography and the duplicate method used in conjunction with a balanced sampling design. These methods estimate spatial distributions of contaminants based upon samples taken from the site to be characterised, and are often limited by the distance between each sample, sampling pattern and sampling density.

In Chapter 2, a new sampling design, adapted from a balanced design and incorporating the duplicate method was applied to two sites to address the second objective of this thesis. This new generic sampling design enabled heterogeneity to be quantified across scales of more than two orders of magnitude, (from 20m to 0.0005m). Being generic in design, it makes no assumptions regarding the spatial distribution of contaminants, can be used to quantify a range of contaminants found in soils and can be adapted to different scales. The design is easy to set out in the field, and when used in conjunction with *in situ* measurement techniques, can characterise the heterogeneity of a site within a couple of days.

A standard approach for quantification of spatial heterogeneity, using %RSD (the standard deviation of the measurements for each separation distance expressed relative to the mean of the population) enables comparison between

sites, contaminants and spatial scales. Results showed that heterogeneity can differ significantly between sites for the same contaminant. For example, *in situ* spatial heterogeneity of Pb at the sewage sludge disposal site at Nottingham, ranges between 3% and 7%, indicating very low heterogeneity, compared to Coseley (a landfill topped with dredging from the adjacent industrial canal), where *in situ* spatial heterogeneity ranges from 18% to 57%. At Nottingham, a very similar spatial distribution to that of Pb, was observed for both Zn and Cu, with no change in heterogeneity as a function of scale or between contaminants, which is in stark contrast to Coseley.

At Coseley, results show that spatial heterogeneity can change as a function of scale and differ between contaminants. Generally, heterogeneity decreases with scale for all three contaminants. Lead and Zn demonstrate similar heterogeneity, with maximum heterogeneity at scales greater than 2m of between 50 – 60%RSD, falling to 18% for Pb, and 19% for Zn at the 0.05m scale, before rising again, at the smallest *in situ* scale of 0.02m scale. The spatial heterogeneity of Cu, differs from Pb and Zn, heterogeneity is considerably higher at greater spatial scales, ranging from 80% at 2m scale to 97% at 20m scale, decreasing more rapidly with a continuous downward trend to 17% at 0.02m.

The heterogeneity profile of Cu at Coseley, where heterogeneity decreases with sampling distance is probably more characteristic of a single large hot spot, with concentrations falling with distance from the centre. Lead and Zn, with flatter spatial heterogeneity profiles, possibly operating at two scales (meter and centimetre), is probably more indicative of a number of smaller contamination hot spots, which when viewed on a finer scale are held within coarser particles, which may explain the small increase at the 0.02m scale. In contrast, the continued decline in heterogeneity for Cu may indicate a finer particle size for this contaminant.

Ex situ, finer scale, measurements using XMP (X-Ray Microprobe) were not as successful as those made *in situ* using P-XRF (Portable X-Ray Fluorescence),

partly due to higher detection limits and calibration methods (Section 6.6.further work), but were not a fundamental requirement for subsequent research in this thesis.

Spatial heterogeneity plays a significant part in the correct assessment and sampling strategies of contaminated land investigations. Where the contaminant is more heterogeneously distributed, there is a greater chance of misclassification, resulting in perhaps unnecessary remediation, or in the case of missed hot spots, harmful exposure and possible litigation following subsequent discovery. The use of this new sampling design, with *in situ* measurement techniques, could easily be incorporated into preliminary site surveys, avoiding the need to base main sampling strategy on potentially misguided assumptions. Using the case studies in this thesis, Nottingham with low heterogeneity, has a low risk of misclassification arising from sampling error, where sampling distances are within those used for the preliminary study. The subsequent main investigation would need only a few samples to confirm concentrations that would be applicable to the whole area. Conversely, the more spatially heterogeneous contaminants at Coseley, suggest several contamination hot spots, therefore to minimise risk of missing a hot spot in a routine site investigation, a higher sampling density with smaller spacing to define may be more appropriate (Boon *et al.*, 2010 for further discussion).

6.3. Reconstructing in situ contaminant spatial heterogeneity for use in pot experiments.

To date, as far as the author is aware, no other research has attempted to recreate *in situ* contaminant spatial heterogeneity for use in plant growth experiments, based upon actual site investigations. The fourth objective of this research aimed to address this gap, and in Chapter 3, a new method for recreating a range of contaminant heterogeneities is presented. Two models of Zn spatial heterogeneity at the 0.02m scale were based upon actual *in situ*

measurements obtained from the contrasting site investigations in Chapter 2. Additionally, a further model, based upon the site investigation by Taylor *et al*.,(2005) at a disused firing range, was created, together with the much used homogenous model and a more recent simplistic model of heterogeneity used in studies assessing plant uptake of contaminants.

The five models provided a range of heterogeneities from nominally 0% RSD (effectively homogeneous) through to 200% RSD (simplistic heterogeneity), with intermediate heterogeneities of 5.63% (Nottingham – low), 27.86% (Coseley – medium) and 50.93%RSD (firing range – high). Easy to use, the computer model could also generate heterogeneities for other contaminants measured *in situ* and at different scales.

The method to construct the pot experiments described in Chapter 3, homogenises the other components in the growing medium, and only contaminants are heterogeneously distributed. Total contaminant concentration in each pot remains constant, thereby isolating heterogeneity as the factor.

6.4. Does the degree of contaminant spatial heterogeneity affect plant growth and contaminant uptake?

Many studies, discussed in Chapters 4 and 5 have compared simplistic binary distributions of contaminants to homogeneous distributions and found significant differences in plant growth and contaminant uptake. But which distribution should be used to estimate plant uptake from contaminated land? Neither model is representative of actual heterogeneities experienced by plants in field conditions. Chapter 4 addresses the gap between these two extreme models, and the final objective of this thesis, using intermediate levels of heterogeneity modelled and methods described in Chapter 3. Four different plant species, with root size of a similar scale, were tested in 5 treatments with a range of Zn spatial heterogeneity.

Results varied with each species demonstrating a very different set of responses to the five treatments, and a summary of findings for each species follows

Brassica napus

There was no significant difference in shoot dry biomass between all five treatments, however root dry biomass in the binary (simplistic heterogeneity) model was significantly higher (2 fold) than root biomass of plants grown in low heterogeneity treatments (modelled from Site 2 investigation). Zn concentrations (µg g^{-1}), in both roots and shoots, showed no significant differences between homogeneous and intermediate levels of heterogeneity. However a significant reduction in Zn concentrations (µg g^{-1}) was found in plants in binary treatments compared to all others. These results suggest that realistic spatial heterogeneity of Zn is not a significant factor for this species. Moreover homogeneous models used in contaminant uptake studies are more likely to provide a better estimate of results under field conditions than simplistic binary models.

Brassica juncea

Significant differences were found both within intermediate levels of heterogeneity and between intermediate and simplistic homogeneous and binary treatments for all variables. The degree of Zn spatial heterogeneity is a significant factor for both plant growth and Zn uptake for this species. As heterogeneity increases, plant growth increases and Zn uptake decreases. For this species, site specific heterogeneity is a significant factor to produce reliable estimates of plant growth and Zn uptake. Moreover, the results for *B. juncea* provide strong evidence that simplistic binary and homogeneous models are inadequate for predictions of plant growth and metal uptake for some species.

Plantago lanceolata

Significant differences were found between intermediate levels of heterogeneity and the homogeneous model for plant biomass, both root and shoot. Significant differences were also observed both within intermediate levels of heterogeneity and between intermediate and simplistic binary and homogeneous models for shoot Zn concentrations (µg g^{-1}). A significant reduction in root Zn concentrations was found between binary treatments and two intermediate levels of heterogeneity, suggesting that the level of Zn spatial heterogeneity is a significant factor in estimating plant growth and Zn uptake for this species.

For the 3 species successfully grown in the 5 treatments, 2 showed that the level of Zn spatial heterogeneity is a significant factor for both plant growth and Zn uptake. For both *B. juncea* and *P. lanceolata*, neither the homogeneous, or simplistic binary models of heterogeneity would provide reliable estimates of plant uptake grown under field conditions.

For research concerned with plant uptake of contaminants, either for assessment of risk to human health, or the potential of plants for phyto-management, this new technique assessing different levels of heterogeneity would be extremely useful in providing a range, within which, plant contaminant concentrations will fall when planted in a site where the spatial distribution of the contaminant is not known. Moreover, it is a more robust method to compare the effectiveness of different species for phytoremediation. Based upon results in this research, studies assessing plants in homogeneous pot trials are unlikely to yield similar results under field conditions, with a few exceptions. Results for *B. juncea* provide strong justification for the use of pot trials containing a range of heterogeneities. If conclusions were to be drawn from using only the homogeneous model, where plants are severely stunted, with significant chlorosis, results would suggest that Zn concentrations in soil at 900 ug g^{-1} are phytotoxic to this species. Yet with the same total concentration, but with a heterogeneous distribution, healthy looking plants are produced and total concentration of Zn per plant (Chapter 4, Figure 4.7.3) is 4 times greater than those grown in homogeneous treatments.

6.5. Root proliferation in low concentration patches as a plant root response to patchily distribution contaminants at concentrations above toxicity threshold.

The final experiment in Chapter 5 does not specifically meet any of the original objectives of this research, but arose from an observation of root formation when roots were extracted from growing medium for both *B napus* and *B. juncea* grown in the binary treatment. Roots of larger diameter were observed to radiate horizontally at right angles to each other, suggesting a change in root morphology in response to the pattern of cells in the growing medium.

Despite the low statistical power of this 'look and see' experiment, some significant differences in root biomass were observed between cells of differing concentrations. In binary treatments, where contrast between cells is greatest, biomass for the roots of *B. napus* and *B. juncea* were 34% and 25% higher, respectively, in high Zn concentrations cells compared to cells with no Zn. Conversely, in the treatments where contrast between cell concentrations is reduced, root biomass was 30% higher in low concentration cells (300 µg g^{-1}), compared to slightly higher concentration cells (700 µg g^{-1}). Whilst there is no clear pattern in the response of roots to concentrations, results suggest that heterogeneity and degree of patch contrast have a significant effect on root morphology and biomass.

6.6. Further work.

Quantification of heterogeneity at a range of scales could provide more useful information about spatial patterns of contaminants within soils at any site. Either through empirical studies of actual site investigations, or theoretical studies of heterogeneity using computer modelling, a database of spatial patterns that are characteristic of heterogeneity profiles would greatly assist in the assessment and remediation of contaminated land investigations.

The technique to quantify heterogeneity is relatively simple to apply, compared to the methods required in the field of geostatistics and variography, making it more accessible in interdisciplinary research. However, it would be interesting to compare results quantified using robust ANOVA and expressed using %RSD to a variogram constructed using the same sample measurements. A criticism that often arises in variograms is that a substantial nugget effect, (where distance between two sampling points is zero, variance might also be expected to be zero, however the variogram y-intercept can be greater than zero, Figure 1.4.3) arises from variance at sampling distances less than the smallest sampling interval. The new sampling design, incorporating a range of sampling intervals could provide better resolution at the finer scale, thereby reducing the error on the y-intercept.

Problems exist in reconciling measurements taken across scales of several orders of magnitude, in part due to differences in sample size, but also due to differences in the detection limits of the two analytical techniques used in this study. Whilst fine scale, non destructive instruments allow us to analyse material not visible to the naked eye, it is often at the expense of making highly quantitative measurements with low uncertainty. The author made some progress with the more sensitive, but destructive technique of laser ablation with inductively coupled mass spectrometry, but to maintain structural heterogeneity of the sample, requires impregnation with an epoxy resin. As small scale analytical techniques improve, more robust quantitative estimates of heterogeneity may be possible.

The pot experiment assessed the effect of Zn heterogeneity on plant uptake, but this contaminant is just one of many heavy metals that can be liberated from soils through plant uptake. Further research is needed to assess heterogeneity as a significant factor for plant uptake on a range of contaminants, initially individually but expanding to contaminant combinations, where synergistic and antagonistic effects are known to exist. The models of heterogeneity introduced in this thesis are not only new to contaminant spatial distributions, and further work could expand intermediate levels of heterogeneity to nutrient spatial

distributions, to see how these affect plant growth, with possible implications for precision farming in poor soils.

Four plant species were assessed in this experiment, but only three were successful. Results from the preliminary experiment, for *T. officinale*, in Chapter 3 (Figure 3.3.1) show this species translocates significantly more Cd and Zn into shoots than in roots, differing from the other three species studied. Moreover Cd concentrations in shoots were twice as high as those for other species tested. Cd is a particularly harmful heavy metal and is toxic at relatively low levels (Alloway and Ayres, 1997), making this an interesting plant for further study. There is also scope to extend the experiment to a much wider variety of plant species, including those those which operate at different spatial scale, e.g. small shrubs and trees at the 1m scale.

A key element of the CLEA (Contaminated Land Exposure Assessment) model, in the UK (DEFRA and EA, 2002), to estimate the risk to human health from the consumption of vegetables and food crops, is the calculation of a concentration factor, for either the root or shoot of the plant in question. The equation contains a number of values that specifically relate to soil properties, and assumes that concentrations are homogeneous. This research has shown that the spatial distribution of contaminants can have a significant effect on plant uptake. After further study, the inclusion of a heterogeneity parameter may provide a better estimate of the potential risk.

To conclude, this research has clearly demonstrated that spatial heterogeneity of contaminants is an important factor controlling plant growth and metal uptake. Simplistic chequerboard models should no longer be used in this context and research in this field should adopt realistic models of heterogeneity to examine plant uptake.

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APPENDICES

Appendix A **. Data relating to two site investigations.**

A.1. Detection Limits for Niton Xlt 700 series and respective background concentrations.

El	*PXRF* Detection Limit for Niton XLt 700 series		Canada Residential[1]	SGV-UK[2] Residential with (or without) plants[5]	SGV-UK[2] Commercial/ Industrial	Dutch[3] Target	Dutch[3] Intervention	Inter-national High Threshold	Soil World Median[4]
	60 sec	*120 sec*							
As	*20*	*15*	12	20	500	29.0	55	500[2]	8
Ba			500			160.0	625	2000[1]	300
Ca	750	525	*			*	*	*	200000
Cd	75	65	10	1,2,8[6]	1400	0.8	12	1400[2]	1
Co	200	150	50			9.0	240	300[1]	10
Cr	60	45	0.4(CrVI)	130(200)	5000	100.0	380	5000[2]	43
Cu	100	60	63			36.0	190	190[3]	15
Fe	250	175	*			*	*	*	21000
Hg	20	12	6.6	8 [15[5]]	480	0.3	10	480[2]	0
K	750	525	*			*	*	*	11000
La			*			*	*	*	33
Mn	250	175	*			*	*	*	320
Mo			5			3.0	200	200[3]	3
Ni	120	90	50	50(75)	5000	35.0	210	5000[2]	17
Pb	25	20	140	450	750	85.0	530	750[2]	17
Sb	250	175	*			*	*	*	2
Se	20	15	1	35(260)	8000	*	*	8000[2]	0
Sn	200	150	50			*	*	300[1]	10
Sr	50	30	*			*	*	*	67
U			*			*	*	*	1
V	250	175	130			*	*	*	57
Zn	55	40	200			140.0	750	360[1]	36

[1] Canadian soil quality guidelines for the protection of the environment and human health (1996) http://www.ec.gc.ca/ceqg-rcqe/English/Pdf/soil_protocol.pdf Updated (2002) http://www.ccme.ca.assets/pdf/el_061.pdf

[2] Soil Guideline Values from DEFRA/EA (2002a), (2002b etc,),

[3] Netherlands Ministry of Housing, Spatial Planning and Environment (2000),

[4] Rose Hawkes and Webb (1979)

[5] All values also apply to land use as allotments, except a different value for Mercury [15 mg/kg]

[6] Cd values depend on pH of the soil (1,2 & 8 mg/kg, for pH values 6,7,& 8 respectively)

[7] Detection limit values are for a NIST Standard Reference Material (SRM) matrix, representative of a 'real-world' soil sample. http://www.cysense.com/images/upload/docum/NITON_XLt_792Y_LOD-7-209NEW.pdf

A.2. Instrument settings and calibration for Portable X-Ray Fluorescence and XMP.

P-XRF

There are two approaches to calibrating hand held P-XRF for in situ analysis; (i) Empirical and (ii) Fundamental parameters (FP). The former produces a site-specific calibration, where samples must be taken, prior to analysis with P-XRF, and analysed using traditional laboratory methods to create site-specific reference materials. This method is impractical for analysis of multiple elements at a number of sites and where P-XRF used for primary surveying. The latter uses a theoretical approach using inter element coefficients (Kalnicky and Singhvi, 2001). For the fundamental parameters approach to provide reliable results, the composition of the sample should be known. However, the P-XRF can only detect a limited range of metals, therefore, the average balance of the sample is estimated using the inverse relationship between peak intensities of Rayleigh and Compton scatter to atomic number. The Niton XLt 700 series is calibrated using the FP approach, by analysing a reference material situated on the reverse of the safety shutter.

XMP

Instrument was set to following:
40 kv (maximum)
1000μA (amps)
Time constant 10μs (amount of time electronics will spend estimating the energy of the incoming photon)

The electronics are initially calibrated using an aluminium copper standard. This is for the purpose of signal amplification and definition of peaks.
A range of reference materials, in pelletized form, were analysed to create a linear regression model. Each reference material was analysed as an unknown sample, at a single spot for a period of 2minutes.

Data Table A.1.Summary of certified values in reference materials used for calibration and linear regression models (bold values only).

Reference material	Pb (mg kg^{-1})	Ni (mg kg^{-1})	Sn (mg kg^{-1})	As (mg kg^{-1})	Zn (mg kg^{-1})	Cu (mg kg^{-1})	Cr (mg kg^{-1})
[a] NIST 1834	**0**	**0**	**0**	**0**	**0**	**0**	**0**
[b]NIST 2711	**1162**	**20.6**	**0**	**105**	**350.4**	**114**	**47**
[c]IAEA-SL-1	**37.7**	**44.9**	**0**	**27.6**	**223**	**30**	**104**
[b]NIST 2709	**18.9**	**88**	**0**	**17.7**	**106**	**34.6**	**130**
[b]NIST 2710	**55.3**	**14.3**	**0**	**626**	**6952**	**2950**	**39**
[d]HRM2	**510**	-	**0**	-	**400**	**590**	-
[e]Corning B	**3713**	**778**	**315**	**0**	**1607**	21566	**34.4**
[e]Corning D	**2506**	**471**	**866**	**0**	**803**	9345	**6.9**
[e]Corning C	342641	-	**1575**	**0**	402	3195	**103.2**

[a] National institute of standards and Technology (NIST) Fused simulated Ore for X-Ray fluorescence Spectrometry.

[b]NIST standard soil reference materials

[c]International Atomic Energy Agency (IAEA), trace and minor elements in lake sediment.

[d]House reference material

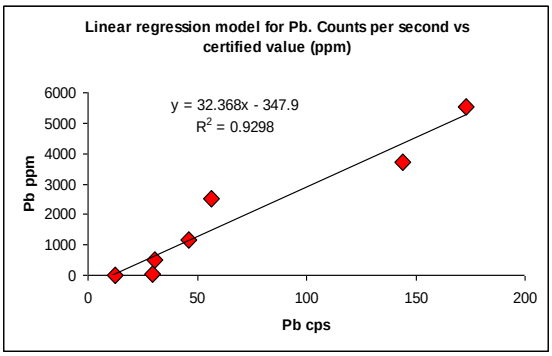
[e]NIST Corning Glass

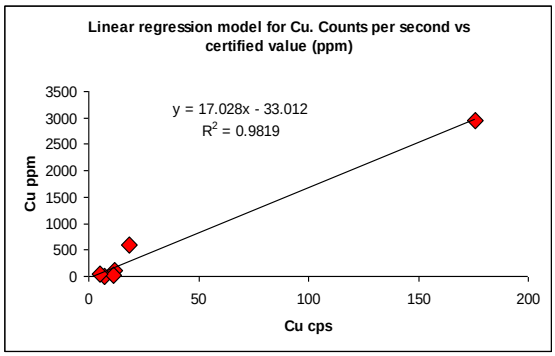
Due to time constraints, samples were only analysed for Pb, Cu and Zn. The respective linear regression model for each is given below.

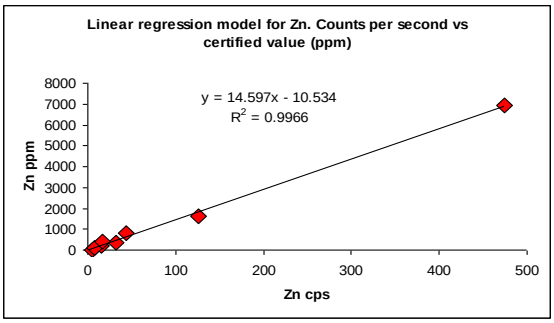
Where possible, reference materials were chosen based on concentration in range of interest, and composition, e.g. if two reference materials had similar concentrations, then the reference material with a matrix more closely related to the samples was used.

N.B. Alpha peaks for As and Pb overlapped, so for purposes of calibration and analysis, the beta peak for Pb was used.

Examples of linear regression models for Pb, Cu and Zn using reference materials listed in Data Table A.1.Summary of certified values in reference materials used for calibration and linear regression models (bold values only).. Data analysed over 3 days, new calibration curve used for each batch.







A.3. Detection Limits

Data Table A.2. Summary statistics of *in situ* measurements using P-XRF at two sites, with contrasting contaminant heterogeneity. Detection limits have been estimated using the median value of 3 x s.d. for each sample reading. (Each sample reading records a counts per second for each element, this is used to estimated a s.d. value over 60 seconds analysis period and reported together with estimated concentration value.)

	Coseley						Nottingham				
Element	Average	Min	Max	Median 3s	% of samples above 3s		Average	Min	Max	Median 3s	% of samples above 3s
Sn (ppm)	217.88	51.01	1465.86	62.87	5.63		73.89	41.35	137.58	42.65	86.39
Cd (ppm)	36.27	20.96	50.91	26.22	1.25		22.02	16.91	35.11	25.26	3.55
Ag (ppm)	<LOD	0.00	0.00	16.48	0.00		18.05	12.86	27.95	16.19	0.59
Sr (ppm)	**81.15**	**20.57**	**294.20**	**7.61**	**100.00**		**74.16**	**62.76**	**89.44**	**6.80**	**100.00**
Rb (ppm)	23.51	3.85	48.58	5.00	99.38		33.50	26.06	41.21	5.21	100.00
Pb (ppm)	**412.36**	**45.37**	**2716.34**	**32.27**	**100.00**		**474.42**	**385.97**	**577.93**	**33.71**	**100.00**
Se (ppm)	6.30	5.17	7.52	7.47	0.63		6.62	4.54	20.68	6.95	10.65
As (ppm)	59.49	12.96	228.99	25.15	76.25		34.15	24.08	52.26	25.62	66.86
Hg (ppm)	9.98	7.19	12.07	10.95	1.25		9.24	6.54	20.66	7.47	47.93
Zn (ppm)	**2125.30**	**270.90**	**6054.56**	**90.02**	**100.00**		**1381.69**	**1121.73**	**1795.75**	**67.56**	**100.00**
Cu (ppm)	**1619.11**	**97.70**	**6221.90**	**84.66**	**100.00**		**601.35**	**468.75**	**785.31**	**64.58**	**100.00**
Ni (ppm)	324.95	40.32	1008.48	82.03	80.00		397.14	310.23	503.33	76.04	100.00
Co (ppm)	260.38	41.64	651.52	214.86	44.38		147.95	106.63	202.39	176.46	4.73
Fe (ppm)	**33379.94**	**2061.21**	**143694.70**	**614.27**	**100.00**		**13629.99**	**11089.02**	**16489.76**	**382.22**	**100.00**
Mn (ppm)	886.06	85.03	4808.43	165.01	95.00		334.34	225.16	475.36	115.47	100.00
Cr (ppm)	558.70	71.94	1848.18	124.24	66.88		651.23	491.90	852.66	123.86	100.00

Data Table A.3. Models used to estimate detection limits for XMP, from repeated analysis of a range of CRMs to generate standard deviations.

	Pb	Zn	Cu
[1]Coseley	188	61	22
[2]Nottingham (Day 1)	180	106	208
[2]Nottingham (Day) 2	262	242	217

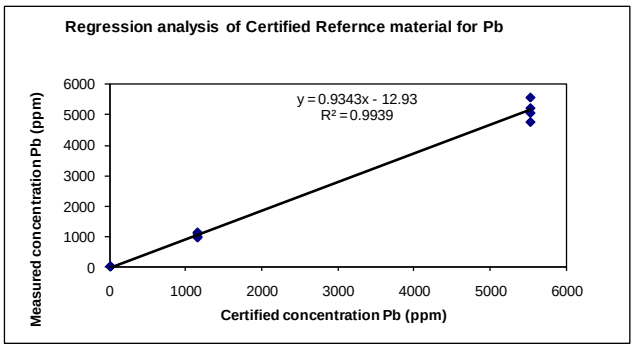
Detection limit calculations

[1]Coseley – regression analysis of standard deviation on repeated analysis of certified reference materials, 3 times the standard deviation extrapolated at zero concentration

[2]Nottingham – regression analysis of standard deviation against measured concentration for each sample. 3* standard deviation extrapolated at zero concentration.

Data Table A.4. Regression analysis of CRMs (NIST 2709, 2710 and 2711) for estimation of instrumental bias for P-XRF and Site 1 (Coseley)

Lead



Measured	Certified
17.67	18.9
17.93	18.9
24.53	18.9
21.21	18.9
5067.34	5532
5575.49	5532
5225.26	5532
4769.87	5532
1141.14	1162
1100.34	1162
969.61	1162
1000.88	1162

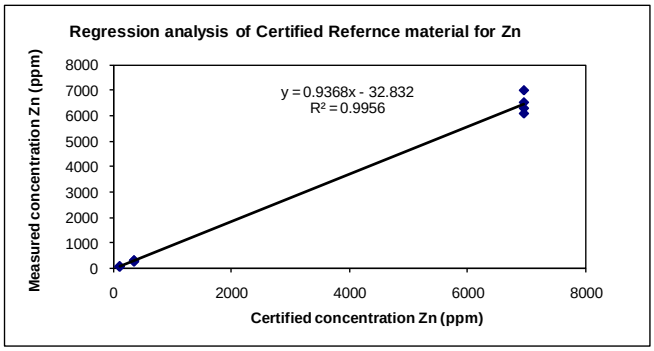
SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.996969
R Square	0.993948
Adjusted R Sq	0.993342
Standard Error	189.7315
Observations	12

R2 Critical value for 11 d.f. = 0.602

	Coefficients	*standard Erro*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-12.92996	75.23965	-0.17185	0.866983	-180.574	154.714445
X Variable 1	0.934262	0.023054	40.52485	2E-12	0.882894	0.98562964
	-0.065738					

Zinc



Measured	Certified
74.48	106
73.93	106
69.52	106
68.15	106
6297.58	6952
7003.92	6952
6528.7	6952
6090.82	6952
315.12	350.4
296.32	350.4
289.72	350.4
259.65	350.4

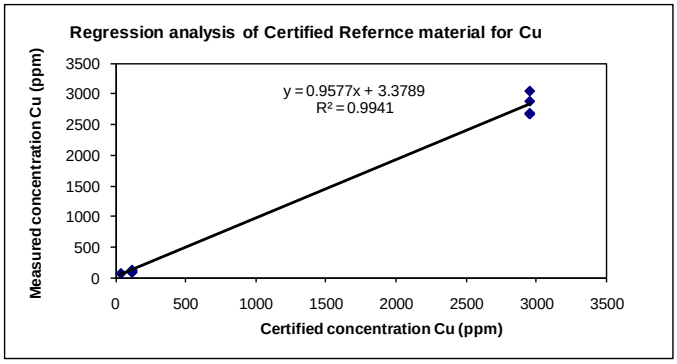
SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.997819
R Square	0.995644
Adjusted R Sq	0.995208
Standard Error	215.2755
Observations	12

R2 Critical value for 11 d.f. = 0.602

	Coefficients	*standard Erro*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-32.83183	78.76454	-0.41684	0.685606	-208.33	142.666532
X Variable 1	0.936838	0.019597	47.80621	3.87E-13	0.893174	0.98050223
	-0.063162					

Copper



Measured	Certified
69.26	34.6
2693.17	2950
3056.23	2950
2888.77	2950
2677.47	2950
84.89	114
108.61	114
98.23	114
124.85	114

SUMMARY OUTPUT

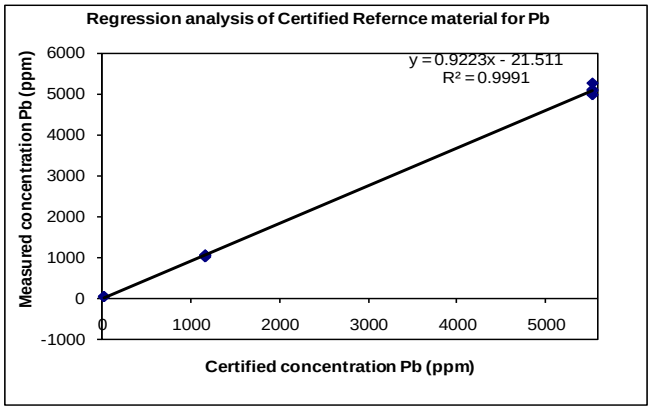
Regression Statistics	
Multiple R	0.997033
R Square	0.994076
Adjusted R Sqı	0.993229
Standard Error	118.8198
Observations	9

R2 Critical value for 8 d.f. = 0.707

	Coefficients	*standard Erro*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	3.378932	55.00035	0.061435	0.95273	-126.676	133.434008
X Variable 1	0.957729	0.027945	34.27201	4.67E-09	0.89165	1.0238087
	-0.042271					

Data Table A.5. Regression analysis of CRM's (NIST 2709, 2710, 2711) for estimation of instrumental bias for P-XRF and Site 2 (Nottingham).

Lead



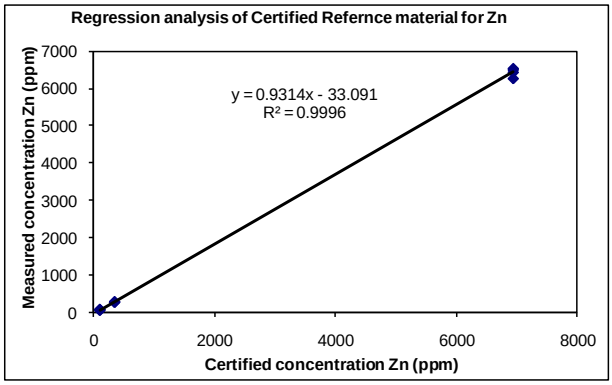
Measured	Certified
23.03	18.9
24.96	18.9
24.96	18.9
4957.51	5532
5244.3	5532
5058.08	5532
5085.31	5532
1042.14	1162
1036.71	1162
991.86	1162
1022.69	1162

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999536
R Square	0.999071
Adjusted R Square	0.998968
Standard Error	74.0157
Observations	11

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-21.51094	31.94944839	-0.673281	0.517682804	-93.78567	50.76379
X Variable 1	0.922314	0.009372819	98.40303	5.86333E-15	0.901111	0.943517
	0.077686					

Zinc



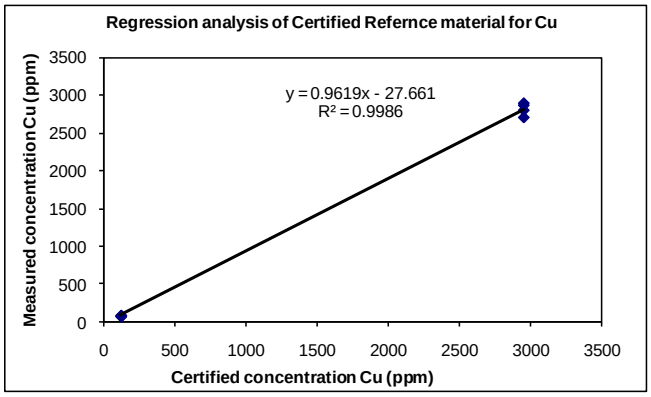
Measured	Certified
73.19	106
81.52	106
58.22	106
86.56	106
6273.71	6952
6544.7	6952
6441.68	6952
6508.88	6952
286.33	350.4
289.64	350.4
275.74	350.4
283.04	350.4

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999787
R Square	0.999574
Adjusted R Square	0.999531
Standard Error	66.79618
Observations	12

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-33.09118	24.4392469	-1.354018	0.20553711	-87.54523	21.36286
X Variable 1	0.931385	0.006080473	153.1765	3.45407E-18	0.917837	0.944934
	0.068615					

Copper



Measured	Certified
2793.95	2950
2859.78	2950
2699.29	2950
2886.96	2950
73.93	114
87	114
88.44	114
78.62	114

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999293
R Square	0.998587
Adjusted R Square	0.998352
Standard Error	59.24275
Observations	8

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-27.66107	30.83507644	-0.897065	0.404239374	-103.1118	47.7897
X Variable 1	0.961917	0.014771139	65.12141	8.81758E-10	0.925774	0.998061
	0.038083					

A.4. Summary statistics and estimates for RSD of measurements made using P-XRF and XMP.

Data Table A.6. Summary statistics for measurement of Pb *in situ* and *ex situ* at each sampling scale.

Pb - Moisture corrected values inclusive of XMP results - Coseley.

	Sanal	Mean	Relative precision
XMP	42.91	1044.99	4.11
PXRF	21.70	535.66	4.05

Scale	Smeas	Robust mean	Ssamp	Ssamp U%	RSD	n	standard error	Mean of Pop.	2 sd of pop	SEM
0.0005	569.51	1384.34	567.89	82.04	41.02	20	6.49	1881.46	4195.93	469.12
0.002	613.31	922.46	611.81	132.65	66.32	20	10.49	1077.53	1278.11	142.90
0.005	596.58	828.16	595.03	143.70	71.85	20	11.36	914.04	974.73	108.98
0.02	118.56	506.40	116.55	46.03	23.02	20	3.64	506.40	556.44	62.21
0.05	111.51	619.72	109.38	35.30	17.65	20	2.79	619.72	686.08	76.71
0.2	142.16	596.80	140.49	47.08	23.54	20	3.72	596.06	616.80	68.96
0.5	213.98	526.30	212.87	80.89	40.45	20	6.40	548.77	477.90	53.43
2	256.14	467.43	255.21	109.20	54.60	20	8.63	489.42	495.80	55.43
5	227.50	517.75	226.46	87.48	43.74	360	1.63	558.41	886.12	23.35
20	292.36	515.25	291.55	113.17	56.58	240	2.58	558.58	898.76	29.01

Pb - Moisture corrected values inclusive of XMP results - Nottingham

	Sanal	Mean (ppm	Relative precision
XMP	61.10	823.93	7.42
PXRF	18.51	693.30	2.67

Scale	Smeas	Robust mean	Ssamp	Ssamp U%	RSD	n	standard error	Mean of Pop.	2 sd of po	SEM
0.0005	384.08	899.35	384.08	85.41	42.71	20	6.75	898.96	748.21	83.65
0.002	244.07	828.02	244.07	58.95	29.48	20	4.66	837.87	466.60	52.17
0.005	326.35	744.42	326.35	87.68	43.84	20	6.93	779.22	655.47	73.28
0.02	34.19	672.60	34.19	10.17	5.08	20	0.68	678.97	157.04	17.56
0.05	34.28	706.30	34.28	9.71	4.85	20	0.65	709.68	98.73	11.04
0.2	37.94	708.37	37.94	10.71	5.36	20	0.74	712.88	141.01	15.77
0.5	34.01	696.99	34.01	9.76	4.88	20	0.65	696.99	123.56	13.81
2	20.93	678.53	20.93	6.17	3.08	20	0.23	678.53	87.28	9.76
5	38.78	694.85	38.78	11.16	5.58	360	0.18	693.61	106.87	2.82
20	45.91	695.49	45.91	13.20	6.60	240	0.28	693.92	102.62	3.31

Data Table A.7. Summary statistics for measurements of Zn *in situ* and *ex situ* at each sampling scale.

Zn - Moisture corrected values inclusive of XMP results (bias corrected) - Coseley.

	Sanal	mean	relative precision
XMP	26.79282152	3768.785	0.710914054
PXRF	105.31496	2959.495	3.558544405

Scale	Smeas	Robust mean	Ssamp	Ssamp U%	RSD	n	S. E.	Mean	2 sd of pop	SEM
0.0005	212.42	2972.40	210.73	14.18	7.09	20	1.12	2972.40	3455.59	386.35
0.002	1517.16	4372.75	1516.92	69.38	34.69	20	5.49	4397.50	5133.13	573.90
0.005	1041.54	3961.20	1041.20	52.57	26.28	20	4.16	3961.17	3963.55	443.14
0.02	776.79	2870.63	769.62	53.62	26.81	20	4.24	2875.86	2415.79	270.09
0.05	652.10	3309.38	643.54	38.89	19.45	20	3.07	3309.38	2868.12	320.67
0.2	737.85	2638.91	730.30	55.35	27.67	20	4.38	2671.99	3586.06	400.93
0.5	1557.96	2929.11	1554.40	106.13	53.07	20	8.39	2939.46	2324.97	259.94
2	2036.38	3470.38	2033.66	117.20	58.60	20	9.27	3523.14	3605.46	403.10
5	1197.86	2723.30	1193.22	87.63	43.82	360	1.63	2793.28	2887.68	76.10
20	1411.05	2774.75	1407.12	101.42	50.71	240	2.31	2832.38	2928.50	94.52

Zn - Moisture corrected values inclusive of XMP results - Nottingham

	Sanal	Mean	Relative precision
XMP	14.102779	1830.764	0.770322062
PXRF	55.272163	2018.156	2.738746145

Scale	Smeas	Robust mean	Ssamp	Ssamp U%	RSD	n	S. E.	Mean	2 sd of pop	SEM
0.0005	523.68	2047.77	523.49	63.62	31.81	20	5.03	2097.79	1565.37	175.01
0.002	437.51	1798.78	437.29	48.62	24.31	20	3.84	1803.61	1099.84	122.97
0.005	448.51	1645.74	448.29	43.78	21.89	20	3.46	1651.85	1037.12	115.95
0.02	71.69	1947.69	45.65	4.69	2.34	20	0.37	1974.24	507.43	56.73
0.05	106.10	2058.24	90.56	8.80	4.40	20	0.70	2065.53	405.49	45.34
0.2	134.56	2118.03	122.68	11.58	5.79	20	0.92	2114.91	569.39	63.66
0.5	80.05	2037.28	57.90	5.68	2.84	20	0.45	2024.66	468.33	52.36
2	50.49	1921.10	22.50	2.34	1.17	20	0.19	1918.73	314.44	35.16
5	108.92	2020.31	93.85	9.29	4.65	360	0.17	2020.76	380.79	10.03
20	171.21	2024.45	162.04	16.01	8.00	240	0.37	2023.34	372.54	12.02

Data Table A.8. Summary statistics for measurements of Cu in situ and ex situ at each sampling scale.

Cu - Moisture corrected values inclusive of XMP results - Coseley.

	Sanal	Mean	relative precision
XMP	9.022977	2504.452	0.360277
PXRF	66.3649	2141.8	3.098557

Scale	Smeas	Robust mean	Ssamp	Ssamp U%	RSD	n	S.E.	Mean	2 sd of po	SEM
0.0005	253.65	2521.97	253.49	20.10	10.05	20	1.59	2709.37	4424.11	494.63
0.002	957.64	3018.86	957.60	63.44	31.72	20	5.02	4026.81	5946.07	664.79
0.005	389.74	1972.53	389.63	39.51	19.75	20	3.12	2137.51	3739.57	494.63
0.02	358.65	2113.13	352.46	33.36	16.68	20	2.64	2191.73	3903.34	436.41
0.05	880.17	2741.23	877.67	64.03	32.02	20	5.06	2803.15	4987.37	557.61
0.2	1272.28	2124.30	1270.55	119.62	59.81	20	9.46	2124.30	3599.32	402.42
0.5	1473.81	2285.12	1472.32	128.86	64.43	20	10.19	2471.11	4241.80	474.25
2	1303.34	1621.57	1301.65	160.54	80.27	20	12.69	1673.73	2847.60	318.37
5	1304.75	2046.86	1303.06	127.32	63.66	360	2.37	2178.46	4068.80	107.22
20	1995.34	2060.40	1994.24	193.58	96.79	240	4.42	2134.03	3920.49	126.53

Cu - Moisture corrected values inclusive of XMP results - Nottingham

	Sanal	mean	realtive precision
XMP	34.59995	1235.086	2.80142
PXRF	27.495159	874.756	3.14318

Scale	Smeas	Robust mean	Ssamp	Ssamp U%	RSD	n	S.E.	Mean	2 sd of po	SEM
0.0005	455.75	1395.28	454.43	65.14	32.57	20	5.15	1426.49	1248.25	139.56
0.002	392.23	1228.59	390.70	63.60	31.80	20	5.03	1258.68	1126.50	125.95
0.005	433.47	1081.39	432.08	79.91	39.96	20	6.32	1079.47	359.48	40.19
0.02	57.47	856.63	50.47	11.78	5.89	20	0.93	863.80	223.68	7.38
0.05	52.99	885.38	45.29	10.23	5.12	20	0.81	889.11	230.91	5.89
0.2	53.88	917.83	46.34	10.10	5.05	20	0.80	917.28	280.98	4.54
0.5	46.48	883.02	37.47	8.49	4.24	20	0.67	883.02	197.75	5.03
2	14.86	820.20	23.13	5.64	2.82	20	0.45	823.67	136.64	1.18
5	52.91	879.72	45.20	10.28	5.14	360	0.19	881.01	180.54	5.86
20	78.24	880.51	73.25	16.64	8.32	240	0.38	881.05	174.97	8.52

Appendix B **List of Suppliers**

B.1. Suppliers of seeds for pot experiments.

Brassica napus (oil seed rape) variety ES Astrid, grade CS, supplied to Severn Trent Water Authority by Frontier, certification F1621NB30006E1. Seeds had been treated with Chinook and Royal Liquid FS, Thiraflo and Seedlife. (Jackson, 2008).

Taraxacum officinale, individual seed heads were collected from grass verge adjacent to slip road leading from the east bound lane of the A27 to the University of Sussex campus. Each head was grown in individual rows. The seed head yielding sufficient plants of similar growth was selected for transplanting to experimental treatments. (Seeds supplied by Herbiseed failed to germinate)

Plantago lanceolata was supplied by Emorsgate Seeds. Seeds were collected from Walden Meadows, Yorkshire (Map ref. SE 007 823) Harvest ID: 640. (individual seed heads were collected from sites on campus but did not yield sufficient plants for transplanting).

Brassica juncea seeds, accession PI 426308, origin Pakistan were supplied by the North Central Regional Plant Introduction Station (NCRPIS), forming part of the U.S. National Plant Germplasm System (NPGS). Iowa State University, Regional Plant Introduction Station, Ames, Iowa, United States.

Appendix C**. Data relating to pot experiment treatment preparation.**

Data Table C.1. Estimated water content of John Innes No. 2 compost.

Dish No	Compost Bag no.	Dish wgt. (g)	Dish + FW (g)	Dish + DW (g)	% moisture
1	1	42.62	53.85	51.34	22.35
2	1	40.32	52.66	49.37	26.66
3	2	46.25	58.94	56.04	22.85
4	2	44.81	54.26	51.37	30.58
5	3	43.31	53.45	50.71	27.02
6	3	39.87	51.71	48.45	27.53
7	4	42.14	53.58	51.06	22.03
8	4	46.33	55.58	53.65	20.86
9	5	40.58	53.23	49.82	26.96
10	5	43.39	55.24	51.56	31.05
11	6	41.84	53.12	50.57	22.61
12	6	40.46	50.04	47.47	26.83
13	7	41.08	54.64	51.97	19.69
14	7	52.18	65.69	62.03	27.09
15	8	41.59	54.47	50.97	27.17
16	8	44.1	57.14	53.98	24.23
17	9	43.88	53.19	51.16	21.80
18	9	47.38	58.78	56.29	21.84
19	10	44.17	54.69	51.91	26.43
20	10	41.97	56.38	52.52	26.79
				Average	25
				Std Dev	3
				%RSD	13

Data Table C.2. Calculation of ZnO mass to achieve desired Zn concentration in growing media dry weight.

Sand	**700**	**Compost**	**300**	**DW Compost**	**225**	**Sand**	Breakdown of concentrations required for each batch				
Moisture content	25.00%	ZnO ATM	81.39	Zn ATM		65.39					
Desired Conc. (mg/kg)	**Number of 150ml reps**	**DW compost (kg)**	**DW Growing medium (kg)**	**Zn in DW (g)**	**Mass of ZnO(g) to 1 Kg of GM (FW)**	**Desired Conc. (mg/kg)**	**Total ZnO**	**Total sand**	**ZnO per 5kg**	**No. 5 Kg reps**	**Residual sand**
0	384	0.225	0.925	0	0.0000	0.00	52.59	0.000	10		
400	256	0.225	0.925	0.37	0.4605	23.07	35.06	2.303	7	0.06	0.028
500	160	0.225	0.925	0.4625	0.5757	18.02	21.91	2.878	4	1.91	1.101
750	288	0.225	0.925	0.69375	0.8635	48.66	39.44	4.318	7	4.44	3.837
800	352	0.225	0.925	0.74	0.9211	63.43	48.21	4.605	9	3.21	2.955
900	1344	0.225	0.925	0.8325	1.0362	272.48	184.07	5.181	36	4.07	4.217
1100	384	0.225	0.925	1.0175	1.2665	95.15	52.59	6.332	10	2.59	3.282
1200	160	0.225	0.925	1.11	1.3816	43.25	21.91	6.908	4	1.91	2.643
1400	160	0.225	0.925	1.295	1.6119	50.46	21.91	8.059	4	1.91	3.084
1600	96	0.225	0.925	1.48	1.8421	34.60	13.15	9.211	2	3.15	5.799
1750	416	0.225	0.925	1.61875	2.0148	163.99	56.97	10.074	11	1.97	3.977

Data Table C.3.Preliminary check of Zn concentrations in 1Kg of growing medium and respective t-test.

Test tube no	Sample ref Conc.	wgt (g)	Dilution factor	measured ug/ml	Conc. Ug/g
1	0	0.2513	100	0.021	8.36
2	blk	0.25	100	-0.044	-17.60
3	2711	0.2508	100	1.159	462.12
4	400	0.2491	100	0.969	389.00
5	0	0.2506	100	0.009	3.59
6	900	0.2509	100	2.025	807.09
7	blk	0.25	100	-0.031	-12.40
8	bcr143	0.2486	100	2.545	1023.73
9	400	0.2496	100	0.901	360.98
10	2711	0.2506	100	0.82	327.21
11	blk	0.25	100	-0.02	-8.00
12	900	0.251	100	2.375	946.22
13	0	0.2503	100	0.01	4.00
14	bcr143	0.2492	100	2.563	1028.49
15	900	0.2498	100	2.317	927.54
16	400	0.2504	100	0.982	392.17

Desired Conc.	0	400	900	blk
Replicate analyses	8.36	389	807.09	-17.6
	3.59	360.98	946.22	-12.4
	4	392.17	927.54	-8
Average	5.316667	380.7167	893.6167	-12.6667
Std dev. (1s)	2.643565	17.16579	75.51413	4.805552
T-calc	1.161152	-0.64857	-0.0488	-1.5218
T-crit	4.302656	4.302656	4.302656	4.302656

Data Table C.4. Randomised block design.

Position Number	Block A	Block B	Block C	Block D	Block E	Block F	Block G	Block H
1	PLHO	BJHH	TOHL	PLHL	TOHM	BJBI	TOHM	BNBI
2	TOHL	PLHM	TOHM	BNHH	PLBI	BJHO	TOHH	BJBI
3	PLHM	TOHL	PLHO	PLHO	TOHH	PLHO	BJHL	PLHO
4	BNHM	BNBI	TOBN	BJHM	PLHH	PLHL	BJHO	BNHH
5	TOHM	PLHL	BNHM	TOHH	TOBI	TOBI	TOHL	PLBI
6	PLBI	BNHL	TOHH	BNHO	BJHM	PLBI	PLBI	BNHM
7	BJHH	BJBI	BJBN	BJHH	BJHH	TOHL	BNBI	TOHM
8	BJHO	BNHM	BNHL	BNBI	BNBI	BJHM	TOBI	BJHL
9	TOBI	BNHO	PLHM	TOHO	BNHM	BJHL	TOHO	PLHH
10	BJHL	TOBI	PLHL	PLBI	PLHM	PLHM	PLHO	PLHL
11	BNHL	PLHH	TOHO	PLHM	PLHL	BNHM	PLHM	BNHO
12	PLHH	TOHM	BJHL	BJHL	BJHL	BNHO	BNHH	BJHM
13	BNHH	BNHH	BNBN	BNHM	BJBI	BNHH	BNHO	TOHO
14	BJBI	TOHO	BNHH	BJBI	BJHO	TOHM	BNHM	TOHL
15	PLHL	PLBI	BJHH	TOHL	BNHL	TOHH	BJBI	BJHH
16	TOHO	BJHL	BJHM	PLHH	BNHH	BNHL	BJHM	BJHO
17	BNBI	TOHH	PLHH	TOHM	PLHO	BJHH	BJHH	PLHM
18	TOHH	PLHO	PLBN	BNHL	BNHO	TOHO	PLHL	TOHH
19	BNHO	BJHO	BJHO	BJHO	TOHO	BNBI	PLHH	BNHL
20	BJHM	BJHM	BNHO	TOBI	TOHL	PLHH	BNHL	TOBI

Plant references: **PL** – *Plantago lanceolata*, **TO** – *Taraxacum officinale*, **BJ** – *Brassica juncea*, **BN** – *Brassica napus*

Treatment references: BI – binary, HH – heterogeneity high, HM – heterogeneity medium, **HL** – heterogeneity low, **HO** – homogenous.

Appendix D**. Laboratory Methods.**

D.1. Nitric and perchloric acid digestion for extraction of heavy metals from herbage and sewage sludge (Thompson and Walsh, 1983a)

DESCRIPTION: Nitric and Perchloric Acid attack

Sample types: Herbage, silage, animal faeces
Sample Weight: 0.100g
Final Volume: 10.0 m
Dilution Factor: 100 ml g^{-1}

COSHH Assessment
Hydrochloric Acid A.R. 36% w/w
Nitric Acid A.R. 70% w/w
Perchloric Acid A.R. 60% w/w

SAFETY POINTS:

1. Do not add Perchloric acid to samples in the absence of Nitric acid.
2. Samples with high organic content may react vigorously with Nitric and Perchloric acids. Watch for frothing when adding Nitric acid. If frothing occurs increase step 1 dwell time to 12 hours.
3. This method must not be attempted on samples containing oil or bitumen.

BATCH ORGANISATION

Maximum Batch Size: 214 samples (252 solutions)
Block Time: 36 hours (or 5 pm day 1 to 9 am day 3)
Total Prep Time: 3 days

Solution Storage Limit: Preferably less than 3 months (if capped)

QUALITY CONTROL

Reagent Blanks: 5% (of total number of samples) □2

Duplicated Samples: 10% (of total number of samples)

Reference Materials: 4% (all RM's should be duplicated)

Possible Reference Materials: HRM11, HRM14 + Certified RM's 1570a

EQUIPMENT

Test tubes 18mm o.d. x 180 mm (PYREX)

Wire test tube racks (plastic coated)

Stainless steel test tube racks

Aluminium heating block (deep, 252 holes)

Shallow aluminium heating block (315 holes)

Centrifuge tubes 18mm x 110mm (polystyrene)

Vortex tube mixer

Balance, top pan

Centrifuge GF8

REAGENTS

Water – Reverse Osmosis (RO)

Nitric Acid A.R. 70% w/w

Perchloric Acid A.R. 60% w/w

Hydrochloric Acid A.R. 36% w/w

5M HCl (Dilute 430ml of Hydrochloric Acid A.R. 36% w/w to 1 litre with DIW).

PROCEDURE

1. Prepare a weighing list.
2. Number a set of test tubes using a waterproof marker pen.
3. Weigh 0.100g (± .001) of sample (oven dried and milled) onto a clean piece of weighing paper using top pan balance. Transfer carefully into clean, dry, numbered test tubes (in wire test tube racks).
4. Add 4.0ml Nitric Acid into each tube from an Oxford dispenser.
5. Place tubes in the aluminium heating block and leave overnight at 50°C.

6. Remove tubes from heating block and add 1.0ml Perchloric Acid from an Oxford dispenser.
7. Place tubes in the aluminium heating block. Switch programmer to 'Manual' mode, then set up as follows:

Rise Rate sec/deg	Dwell Time hrs	Dwell Temp °C
001	0.1	50
001	3.0	150
001	18.0	190
001	0.1	195

8. Check the fume cupboard is on and switch programmer to 'Auto' and press 'Reset' button.
9. When attack cycle complete, check each tube to ensure that residue is dry. If any liquid remains continue heating at 195°C until dry. Transfer tubes to stainless steel racks.
10. When tubes are cool add 2.0ml of 5M HCl to each tube from an Oxford dispenser (calibrated gravimetrically).
11. Place tubes in shallow heating block and leave to leach for one hour at 60°C.
12. Transfer tubes to wire racks and allow to cool.
13. Add 8.0ml DIW from an Oxford dispenser (calibrated gravimetrically) and mix each tube, using a vortex mixer.
14. Decant into polystyrene tubes and cap.
15. Centrifuge at 2000 rpm for 2 minutes.
16. Deliver the tubes (with Analytical Request Form) to room 4.59 at least 12 hours before analysis, to allow solutions to equilibrate at 21°C.

D.2 Instrument settings and of ICP-MS, calibrated using prepared stock solutions

Instrument details: Agilent 7500ce ICP-MS

RF Power: 1500W

Argon Carrier Gas: 0.8 L/min

Argon Makeup Gas: 0.21 L/min

Spray Chamber Temp: 2 degrees C

Using a helium collision mode with helium flow set to 5.0 mls/min

D.3 Data quality Analysis

Data Table D.1.Estimated detection limits for Zn using ICP-MS.

Batch reference	**Standard deviation**	**Detection Limit Zn (ppb)**
Brassica juncea 1	0.26928	0.81
Brassica juncea 2	0.333799	1.01
Brassica napus 1	0.465511	1.40
Brassica napus 2	0.396288	1.19
Plantago lanceolata 1	0.74255	2.23
Plantago lanceolata 2	0.92576	2.78

Data Table D.2. Test for significance of reagent blanks.

	B. juncea		*B.napus*		*P.lanceolata*	
Replicate Number	**Batch 1**	**Batch 2**	**Batch 1**	**Batch 2**	**Batch1**	**Batch 2**
1	10.07	20.25	2.86	6.24	1.67	17.46
2	7.63	13.57	8.69	5.22	-4.239	-10.86
3	10.90	10.82	7.38	6.18	-3.161	-8.549
4	12.79	10.23	9.02	1.28	-4.302	-12.58
5	13.85	8.94	2.85	1.32	1.818	-12.2
6	14.03	8.68	9.51	1.30	0.1733	-13.35
7	20.16				-3.617	-13.25
Mean	12.78	12.08	6.72	3.59	-1.66539	-7.61843
S.d	3.9697227	4.368647022	3.074585	2.535652494	2.776701	11.18388
T.calc	16.96539	14.15810183	9.385674	5.524827479	-2.64423	-6.02724
t- critical	2.446912	2.570581835	2.570582	2.570581835	2.446912	2.446912
Significant Make adjustment	Yes	Yes	Yes	Yes	Yes	Yes

	B. juncea		**B. napus**		**P. lanceolata**		**Certified /accepted Value**
	Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2	
HRM 14	28.69	37.83	37.36	32.49	36.29	30.64	35
HRM 11	34.58	48.14	42.93	36.96	44.43	35.98	45
NIST 1570a	62.34	71.55	70.47	62.02	80.94	65.35	82

Data Table D.3. Table of measured and accepted values for range of certified references materials included in each analytical batch, used for regression analysis.

Data Table D.4. Regression analysis of CRM's - *B. juncea* batch 1

SUMMARY OUTPUT B. Juncea batch 1						
Regression Statistics						
Multiple R	0.999256721					
R Square	0.998513995					
Adjusted R Squ	0.99702799					
Standard Error	0.979599825					
Observations	3					
ANOVA						
	df	*SS*	*MS*	*F*	*Significance F*	
Regression	1	644.8094	644.8094	671.9453	0.02454697	
Residual	1	0.959616	0.959616			
Total	2	645.769				
	Coefficients	*tandard Err*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	2.706459214	1.61316	1.677737	0.342185	-17.790685	23.203604
X Variable 1	0.725221108	0.027977	25.92191	0.024547	0.36973772	1.0807045

Data Table D.5. Regression analysis of CRM's *B. juncea* batch 2.

SUMMARY OUTPUT B.juncea batch 2						
Regression Statistics						
Multiple R	0.995027					
R Square	0.990078					
Adjusted F	0.980156					
Standard E	2.434303					
Observatio	3					
ANOVA						
	df	*SS*	*MS*	*F*	*Significance F*	
Regressior	1	591.3195	591.3195	99.78675	0.0635184	
Residual	1	5.925832	5.925832			
Total	2	597.2454				
	Coefficients	*tandard Err*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	15.00409	4.0087	3.742882	0.166206	-35.93127	65.939445
X Variable	0.69449	0.069523	9.989332	0.063518	-0.188886	1.5778652

Data Table D.6. Regression analysis of CRM's - *B. napus* batch 1.

SUMMARY OUTPUT B. Napus batch 1						
Regression Statistics						
Multiple R	0.998963508					
R Square	0.997928091					
Adjusted R Squ	0.995856182					
Standard Error	1.140899173					
Observations	3					
ANOVA						
	df	*SS*	*MS*	*F*	*Significance F*	
Regression	1	626.9358	626.9358	481.6467	0.02898782	
Residual	1	1.301651	1.301651			
Total	2	628.2375				
	Coefficients	*tandard Err*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	11.63893753	1.878781	6.194942	0.101886	-12.2332344	35.51111
X Variable 1	0.715099217	0.032584	21.94645	0.028988	0.3010825	1.129116

Data Table D.7. Regression analysis of CRM's *B. napus* - Batch 2.

SUMMARY OUTPUT B. Napus batch 2						
Regression Statistics						
Multiple R	0.998052					
R Square	0.996108					
Adjusted F	0.992216					
Standard E	1.404085					
Observatio	3					
ANOVA						
	df	*SS*	*MS*	*F*	*Significance F*	
Regressior	1	504.5557	504.5557	255.9308	0.039742409	
Residual	1	1.971454	1.971454			
Total	2	506.5272				
	Coefficients	*tandard Err*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	9.179638	2.312183	3.970118	0.157085	-20.1994282	38.5587
X Variable	0.641519	0.0401	15.99784	0.039742	0.131995553	1.151042

Data Table D.8. Regression analysis of CRM's - *P. lanceolata* Batch 1

SUMMARY OUTPUT - P.lanceolata batch 1						
Regression Statistics						
Multiple R	0.999511					
R Square	0.999023					
Adjusted F	0.998045					
Standard E	1.051502					
Observatio	3					
ANOVA						
	df	*SS*	*MS*	*F*	*Significance F*	
Regressior	1	1130.021	1130.021	1022.037	0.019906975	
Residual	1	1.105656	1.105656			
Total	2	1131.126				
	Coefficients	*tandard Err*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	2.046429	1.731565	1.181838	0.447065	-19.9551877	24.048046
X Variable	0.960059	0.030031	31.96931	0.019907	0.578483526	1.3416346

Data Table D.9. Regression analysis of CRM's - *P. lanceolata* Batch 2.

SUMMARY OUTPUT - P. Lanceolata batch 2						
Regression Statistics						
Multiple R	0.998214					
R Square	0.996431					
Adjusted F	0.992861					
Standard E	1.579026					
Observatio	3					
ANOVA						
	df	*SS*	*MS*	*F*	*Significance F*	
Regressior	1	696.0428	696.0428	279.1626	0.038056927	
Residual	1	2.493324	2.493324			
Total	2	698.5362				
%	*Coefficients*	*tandard Err*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	3.300341	2.600268	1.269231	0.42482	-29.7391998	36.339883
X Variable	0.753482	0.045097	16.70816	0.038057	0.18047473	1.3264888

D.4. Excel spreadsheet illustrating modelling of Nottingham site heterogeneity (low heterogeneity site) for use in pot experiment.

Measured conc used to estimate 2cm RSD - Step 1		**Bioavailable fraction - 45% adjustment**	
1888	1799	850	810
1666	1619	750	728
1834	1851	825	833
1813	1743	816	785
2095	1972	943	887
1714	1886	771	849
2218	2191	998	986
2064	1996	929	898
2012	2055	905	925
2465	2603	1109	1171
Mean	1974		888

Step 4		Smeas	Robust mean	Ssamp	Ssamp U%	RSD	
Field results Site E	0.02	71.69	1947.69	45.65	4.69		**2.34**
Pot Model B	0.02	50.14	890.74	50.14	11.26		**5.63**

% abs diff in spreadsheet	n	standard er	Mean of Pop. Std dev^2	Average % abs diff
Field results Site E	20	0.37	1974.24	507.43	**3.64**
Pot Model B	80	0.44	897.50	250.52	**5.85**

Procedure

Step 1	Enter measured concentrations from in situ field investigation, and bioavailable fraction is auto matically calculated.
Step 2	Input rounded valued from step one into grid in step 2. Cells below will automatically update for adjoining cells, horizontally and
Step 3	Toggle values in step 2, till desired average concentration and percent absolute difference between paired cells is achieved.
Step 4	Enter paired values into ROBAN software to caculated Robust ANOVA, input results above.

Step 2. Pot model grid		1	2	3	4	5
	a	750	750	800	900	900
	b	750	750	800	900	900
	c	800	800	800	1100	1100
	d	800	800	900	1100	1200
	e	900	900	900	1100	1100

Step 3 **Average** **900**

Step 4 Cell 1	Value	Value	Cell 2	**Mean**	**[S1-S2]**	**[S1-S2]/X**
1a	750	750	2a	750.00	0.00	0.00
2a	750	800	3a	775.00	50.00	6.45
3a	800	900	4a	850.00	100.00	11.76
4a	900	900	5a	900.00	0.00	0.00
1b	750	750	2b	750.00	0.00	0.00
2b	750	800	3b	775.00	50.00	6.45
3b	800	900	4b	850.00	100.00	11.76
4b	900	900	5b	900.00	0.00	0.00
1c	800	800	2c	800.00	0.00	0.00
2c	800	800	3c	800.00	0.00	0.00
3c	800	1100	4c	950.00	300.00	31.58
4c	1100	1100	5c	1100.00	0.00	0.00
1d	800	800	2d	800.00	0.00	0.00
2d	800	900	3d	850.00	100.00	11.76
3d	900	1100	4d	1000.00	200.00	20.00
4d	1100	1200	5d	1150.00	100.00	8.70
1e	900	900	2e	900.00	0.00	0.00
2e	900	900	3e	900.00	0.00	0.00
3e	900	1100	4e	1000.00	200.00	20.00
4e	1100	1100	5e	1100.00	0.00	0.00
1a	750	750	1b	750.00	0.00	0.00
1b	750	800	1c	775.00	50.00	6.45
1c	800	800	1d	800.00	0.00	0.00
1d	800	900	1e	850.00	100.00	11.76
2a	750	750	2b	750.00	0.00	0.00
2b	750	800	2c	775.00	50.00	6.45
2c	800	800	2d	800.00	0.00	0.00
2d	800	900	2e	850.00	100.00	11.76
3a	800	800	3b	800.00	0.00	0.00
3b	800	800	3c	800.00	0.00	0.00
3c	800	900	3d	850.00	100.00	11.76
3d	900	900	3e	900.00	0.00	0.00
4a	900	900	4b	900.00	0.00	0.00
4b	900	1100	4c	1000.00	200.00	20.00
4c	1100	1100	4d	1100.00	0.00	0.00
4d	1100	1100	4e	1100.00	0.00	0.00
5a	900	900	5b	900.00	0.00	0.00
5b	900	1100	5c	1000.00	200.00	20.00
5c	1100	1200	5d	1150.00	100.00	8.70
5d	1200	1100	5e	1150.00	100.00	8.70
		Step 3	**Average**	**897.50**		**5.85**

Appendix E **Tables of results main pot experiment.**

E.1 Brassica Napus biomass and zinc data

Data Table E.1 Biomass data recorded for *B. napus* at harvest (54 days after transplanting seedlings)

Pot No.	Species & Treatment [1]	Height (mm)[2]	No. of true leaves	Longest leaf length	No. Dead Leaves	Shoot FW (g)	Shoot DW (g)	Root DW (g)
A17	BNBI	45	10	225	0	29.6	3.0189	0.8619
B4	BNBI	45	9	240	1	35.26	3.8062	1.1545
C13	BNBI	70	10	250	0	39.16	4.0037	1.0188
D8	BNBI	60	10	250	0	34.88	3.6873	1.1352
E8	BNBI	70	9	230	1	29.53	3.0459	0.8676
F19	BNBI	55	10	240	0	34.05	4.1601	1.2352
G7	BNBI	60	10	250	0	31.09	2.8706	0.7341
H1	BNBI	60	8	255	0	31.9	3.4347	0.9287
A13	BNHH	55	10	210	0	28.39	2.7216	0.7472
B13	BNHH	60	11	265	1	42.6	4.3582	1.0451
C14	BNHH	70	11	250	1	39.8	3.7826	0.8312
D2	BNHH	45	10	220	0	29.59	3.0205	0.8238
E16	BNHH	50	9	230	0	27.58	2.6246	0.6428
F13	BNHH	55	9	220	0	31.24	3.4481	1.0318
G12	BNHH	50	10	250	0	28.5	2.4809	0.5762
H4	BNHH	50	10	240	0	28.91	2.6896	0.6105
A11	BNHL	45	9	240	1	27.85	2.4191	0.4601
B6	BNHL	50	9	250	1	29.92	2.39	0.4715
C8	BNHL	45	10	235	0	21.03	1.5706	0.2315
D18	BNHL	50	9	280	0	39.82	4.0562	0.8149
E15	BNHL	25	10	230	1	29.97	3.0951	0.6925
F16	BNHL	55	9	274	1	35.86	3.4992	0.8826
G20	BNHL	60	10	240	1	32.34	3.3288	0.7426
H19	BNHL	30	8	190	2	11.11	0.8985	0.1253
A4	BNHM	65	10	265	1	40.91	4.1371	0.9267
B8	BNHM	70	10	330	1	52.73	4.1239	0.7011
C5	BNHM	40	10	250	0	33.97	2.7966	0.5503
D13	BNHM	60	11	250	0	37.81	4.2394	1.1627
E9	BNHM	60	9	240	1	30.99	3.1488	0.71
F11	BNHM	60	9	240	0	29.42	3.0149	0.7411

Pot No.	Species & Treatment [1]	Height (mm) [2]	No. of true leaves	Longest leaf length	No. Dead Leaves	Shoot FW (g)	Shoot DW (g)	Root DW (g)
G14	BNHM	45	10	275	1	41.6	4.0289	0.9092
H6	BNHM	55	9	270	1	30.44	2.7237	0.6094
A19	BNHO	50	9	230	0	19.43	1.4491	0.316
B9	BNHO	60	10	250	0	34.96	3.335	0.7899
C20	BNHO	55	10	260	0	36.19	3.5007	0.9975
D6	BNHO	45	9	230	0	25.58	2.4269	0.6883
E18	BNHO	60	9	230	1	20.48	1.8791	0.4941
F12	BNHO	60	11	260	0	33.52	3.3373	0.8794
G13	BNHO	65	9	270	1	34.56	3.068	0.6955

[1] The preceding two letters relate to the species e.g BN – *Brassica napus*, the last two letters refer to the treatment; HO – homogeneous, HL - low heterogeneity, HM – medium heterogeneity, HH – high heterogeneity, BI – binary.

[2] Height is the height of the plant stem

Data Table E.2 *B. napus* measured concentrations of zinc in dry weight of shoots (µg/g)

BLOCK REF	TREATMENT				
	HO	HL	HM	HH	BI
A	591.91	725.16	533.15	636.95	216.53
B	530.38	642.91	576.06	597.68	313.08
C	697.01	831.73	766.61	568.75	266.39
D	578.51	567.20	565.72	706.36	298.12
E	1089.67	760.59	628.80	641.60	262.34
F	607.51	659.61	578.57	775.38	305.60
G	591.13	667.60	618.61	543.20	261.04
H	536.59	1060.52	582.07	529.60	351.47
Average	**652.84**	**739.42**	**606.20**	**624.94**	**284.32**
Std err	64.97	53.91	25.22	29.69	14.59
min	530.38	567.20	533.15	529.60	216.53
max	1089.67	1060.52	766.61	775.38	351.47

Data Table E.3 *B. napus* measured concentrations of zinc in dry weight of roots (µg/g).

BLOCK REF	TREATMENT				
	HO	HL	HM	HH	BI
A	1688.10	1461.60	1179.75	969.65	965.20
B	1458.18	1425.71	1258.12	1515.01	732.38
C	926.46	1587.30	1740.80	1012.40	1047.58
D	1313.07	1479.58	1315.18	1222.18	669.06
E	2021.19	2572.51	999.60	1049.30	853.94
F	1002.80	1010.80	910.33	1332.80	733.97
G	1310.37	1639.31	1303.79	1495.40	914.30
H	1007.20	3542.60	1298.52	1214.89	632.00
Average	**1340.92**	**1839.93**	**1250.76**	**1226.45**	**818.56**
Std err	133.38	288.84	87.98	74.43	52.80
min	926.46	1010.80	910.33	969.65	632.00
max	2021.19	3542.60	1740.80	1515.01	1047.58

Data Table E.4 Results of Kolmogorov - Smirnov test for normality and Levenes test for equal variance, calculated using data collected from *B. napus* grown in 5 different treatments of Zn heterogeneity.

	Treatment K-S statistics[1]					Levenes' statistic (test of equal variance)[2]
Test variable	HO	HL	HM	HH	BI	
Shoot DW	0.703	0.985	0.577	0.764	0.901	0.190
Root DW	0.786	0.925	0.835	0.981	0.973	0.486
Shoot Zn	0.289	0.929	0.665	0.965	0.994	0.145
Root Zn	0.940	0.289	0.606	0.969	0.862	0.004
Ln Root Zn						0.253

[1] Where K-S statistic is <0.05 then data distribution is significantly different from normal and should be transformed or tested using non-parametric statistical techniques.

[2]Where Levenes P value is < 0.05 then data do not have equal variance and should be transformed or tested using non-parametric statistics.

Data Table E.5 Results from mixed model ANOVA test (SPSS) to determine significance of between and within treatments variance for *B. napus* dry biomass (g) and measured zinc concentrations (µg g^{-1}). With Treatment as fixed variable and block as random.

B.napus shoot dry biomass		Type III Sum of Squares	df	Mean Square	F	Sig.
Treatment	Hypothesis	5.324	4	1.331	2.668	.053
	Error	13.969	28	.499[b]		
Block	Hypothesis	5.517	7	.788	1.580	.183
	Error	13.969	28	.499[b]		

B. napus root dry biomass		Type III Sum of Squares	df	Mean Square	F	Sig.
Treatment	Hypothesis	.818	4	.204	5.704	.002
	Error	1.003	28	.036[b]		
Block	Hypothesis	.566	7	.081	2.258	.059
	Error	1.003	28	.036[b]		

B. napus shoot Zn		Type III Sum of Squares	df	Mean Square	F	Sig.
Treatment	Hypothesis	973381.188	4	243345.297	17.193	.000
	Error	396302.675	28	14153.667[b]		
Block	Hypothesis	97481.810	7	13925.973	.984	.463
	Error	396302.675	28	14153.667[b]		

B. napus Ln root Zn		Type III Sum of Squares	df	Mean Square	F	Sig.
Treatment	Hypothesis	2.333	4	.583	8.391	.000
	Error	1.946	28	.070[b]		
Block	Hypothesis	.356	7	.051	.732	.647
	Error	1.946	28	.070[b]		

Data Table E.6 Results of Tukey (HSD) mulitple comparison of means test (SPSS) for *B. napus* root dry weights (g)

Root DW (g)

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HO	HL	.145700	.105887	.647	-.15873	.45013
	HM	-.090487	.105887	.911	-.39492	.21394
	HH	-.090250	.105887	.912	-.39468	.21418
	BI	-.293675	.105887	.063	-.59811	.01076
HL	HO	-.145700	.105887	.647	-.45013	.15873
	HM	-.236188	.105887	.192	-.54062	.06824
	HH	-.235950	.105887	.193	-.54038	.06848
	BI	-.439375*	.105887	.002	-.74381	-.13494
HM	HO	.090487	.105887	.911	-.21394	.39492
	HL	.236188	.105887	.192	-.06824	.54062
	HH	.000238	.105887	1.000	-.30419	.30467
	BI	-.203187	.105887	.327	-.50762	.10124
HH	HO	.090250	.105887	.912	-.21418	.39468
	HL	.235950	.105887	.193	-.06848	.54038
	HM	-.000238	.105887	1.000	-.30467	.30419
	BI	-.203425	.105887	.325	-.50786	.10101
BI	HO	.293675	.105887	.063	-.01076	.59811
	HL	.439375*	.105887	.002	.13494	.74381
	HM	.203187	.105887	.327	-.10124	.50762
	HH	.203425	.105887	.325	-.10101	.50786

*. The mean difference is significant at the 0.05 level.

Data Table E.7 Results of Tukey HSD multiple comparison of means test for measured zinc concentrations (µg g^{-1}) in *B. napus* shoots.

Shoot Zn

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HO	HL	-85.60207	59.38882	.60596	-256.34851	85.14438
	HM	48.39798	59.38882	.92427	-122.34846	219.14443
	HH	29.85468	59.38882	.98656	-140.89176	200.60113
	BI	370.86355*	59.38882	.00000	200.11711	541.61000
HL	HO	85.60207	59.38882	.60596	-85.14438	256.34851
	HM	134.00005	59.38882	.18350	-36.74639	304.74650
	HH	115.45675	59.38882	.31399	-55.28969	286.20319
	BI	456.46562*	59.38882	.00000	285.71918	627.21207
HM	HO	-48.39798	59.38882	.92427	-219.14443	122.34846
	HL	-134.00005	59.38882	.18350	-304.74650	36.74639
	HH	-18.54330	59.38882	.99784	-189.28975	152.20314
	BI	322.46557*	59.38882	.00004	151.71912	493.21201
HH	HO	-29.85468	59.38882	.98656	-200.60113	140.89176
	HL	-115.45675	59.38882	.31399	-286.20319	55.28969
	HM	18.54330	59.38882	.99784	-152.20314	189.28975
	BI	341.00887*	59.38882	.00002	170.26243	511.75532
BI	HO	-370.86355*	59.38882	.00000	-541.61000	-200.11711
	HL	-456.46562*	59.38882	.00000	-627.21207	-285.71918
	HM	-322.46557*	59.38882	.00004	-493.21201	-151.71912
	HH	-341.00887*	59.38882	.00002	-511.75532	-170.26243

*. The mean difference is significant at the 0.05 level.

Data Table E.8 Results of Tukey HSD multiple comparison of means test for measured zinc concentrations (µg g^{-1}) in *B. napus* roots.

Lnrootzn

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HO	HL	-.27795	.12823	.216	-.6466	.0907
	HM	.05411	.12823	.993	-.3146	.4228
	HH	.06946	.12823	.982	-.2992	.4381
	BI	.47683*	.12823	.006	.1082	.8455
HL	HO	.27795	.12823	.216	-.0907	.6466
	HM	.33206	.12823	.094	-.0366	.7007
	HH	.34741	.12823	.073	-.0213	.7161
	BI	.75477*	.12823	.000	.3861	1.1234
HM	HO	-.05411	.12823	.993	-.4228	.3146
	HL	-.33206	.12823	.094	-.7007	.0366
	HH	.01535	.12823	1.000	-.3533	.3840
	BI	.42271*	.12823	.018	.0540	.7914
HH	HO	-.06946	.12823	.982	-.4381	.2992
	HL	-.34741	.12823	.073	-.7161	.0213
	HM	-.01535	.12823	1.000	-.3840	.3533
	BI	.40737*	.12823	.024	.0387	.7760
BI	HO	-.47683*	.12823	.006	-.8455	-.1082
	HL	-.75477*	.12823	.000	-1.1234	-.3861
	HM	-.42271*	.12823	.018	-.7914	-.0540
	HH	-.40737*	.12823	.024	-.7760	-.0387

*. The mean difference is significant at the 0.05 level.

E.2 *Brassica juncea* biomass and zinc data

Data Table E.9. Biomass data recorded at harvest for individual plants of *Brassica juncea* at harvest (49 days after transplanting seedlings).

Pot Number	Treatment	Stem Height	No.True leaves	No. dead leaves	Longest leaf	Shoot FW (g)	Shoot DW (g)	Root DW (g)
A8	HO	40	5	1	20	0.2354	0.0404	cold storage
B19	HO	35	4	2	45	0.4289	0.066	0.0083
C19	HO	40	5	0	30	0.3651	0.0632	0.0055
D19	HO	40	5	1	50	0.5207	0.0764	0.0072
E14	HO	35	5	1	45	0.5843	0.0991	0.0058
F2	HO	40	3	2	25	0.188	0.0341	0.0034
G4	HO	20	6	1	60	0.6419	0.0794	0.0083
H16	HO	50	9	1	120	1.8371	0.232	0.0177
A10	HL	40	4	2	35	0.424	0.0716	cold storage
B16	HL	40	10	1	130	3.7459	0.3876	0.0397
C12	HL	30	6	1	40	0.4725	0.0788	0.0107
D12	HL	25	6	1	45	0.512	0.0772	0.0056
E12	HL	45	2	4	10	0.1653	0.0375	0.0032
F9	HL	35	8	1	80	1.8201	0.2442	0.0192
G3	HL	20	7	1	80	1.5089	0.2157	0.0144
H8	HL	20	4	2	15	0.2	0.0425	0.0048
A20	HM	60	10	1	145	4.7746	0.4576	cold storage
B20	HM	40	4	2	35	0.5242	0.0817	0.0047
C16	HM	30	4	2	45	0.5024	0.0967	0.0073
D4	HM	45	11	0	145	4.5065	0.419	0.0043
E6	HM	25	6	1	25	0.1081	0.0192	0.0667
F8	HM	45	9	0	145	6.1051	0.652	0.1153
G16	HM	40	5	0	25	0.2943	0.0615	0.007
H12	HM	25	6	1	45	0.6344	0.1258	0.0083
A7	HH	40	9	1	140	3.0597	0.3062	cold storage
B1	HH	40	10	1	95	2.6433	0.3431	0.0336
C15	HH	60	11	0	170	9.4667	0.9797	0.1811
D7	HH	55	8	1	150	5.2082	0.432	0.105
E7	HH	55	8	1	140	4.2466	0.3919	0.0703
F17	HH	60	11	0	205	11.8432	1.3241	0.3151
G17	HH	90	12	0	205	13.6333	1.3282	0.3914
H15	HH	90	11	0	205	11.4426	1.1373	0.2508
A14	BI	95	10	1	160	6.6567	0.6382	cold storage
B7	BI	610	17	0	235	29.5466	3.468	0.9252
C7	BI	530	14	0	205	18.4093	1.8964	0.355
D14	BI	150	14	1	235	24.248	2.7573	0.9436
E13	BI	210	10	0	175	9.5088	0.9264	0.2389
F1	BI	175	11	0	190	13.3176	1.5082	0.4732
G15	BI	60	10	0	200	8.3063	0.7243	0.1647
H2	BI	480	17	0	170	12.9636	1.4418	0.2751

Data Table E.10. *B. juncea* **measured zinc concentrations (µg g^{-1}) in shoot dry weights.**

BLOCK REF	TREATMENT				
	HO	**HL**	**HM**	**HH**	**BI**
A	1483.80	2341.07	1196.63	1564.66	192.83
B	2320.66	2418.06	1996.57	1300.90	263.20
C	2379.29	1987.63	1848.63	1483.29	217.65
D	1789.27	1969.00	1530.22	943.11	347.12
E	2140.97	2627.60	1691.38	1387.84	258.35
F	2025.80	1125.60	1053.22	1469.45	288.32
G	1916.10	2260.50	1530.94	1197.22	242.28
H	2707.66	2722.52	1140.52	1158.91	192.99
Average	**2095.44**	**2181.50**	**1498.52**	**1313.17**	**250.34**
Std err	134.50	178.14	121.56	72.77	18.31
min	1483.80	1125.60	1053.22	943.11	192.83
max	2707.66	2722.52	1996.57	1564.66	347.12

Data Table E.11. ***B. juncea*** **measured zinc concentrations (µg g^{-1}) in root dry weights.**

BLOCK REF	TREATMENT				
	HO	**HL**	**HM**	**HH**	**BI**
A					
B	3494.99	3400.29	5436.38	4116.72	1142.27
C	4860.35	4168.54	6596.10	2489.47	1268.94
D	4759.99	5075.34	2871.87	2008.00	1468.02
E	4958.95	3868.21	3763.12	3314.70	1805.50
F	4279.36	4411.95	1944.64	2715.34	2193.70
G	5758.90	7488.94	5654.01	2205.58	1603.94
H	3579.78	4033.04	4477.61	3467.98	1828.78
Average	**4527.47**	**4635.19**	**4391.96**	**2902.54**	**1615.88**
Std err	284.79	480.47	581.75	268.31	127.63
min	3494.99	3400.29	1944.64	2008.00	1142.27
max	5758.90	7488.94	6596.10	4116.72	2193.70

Data Table E.12. Results of Kolmogorov - Smirnov test for normality and Levenes test for equal variance, calculated using data collected from *B. juncea* grown in 5 different treatments of Zn heterogeneity. Where normality and equal variance tests are not satisfied, data has been natural log transformed.

	Treatment K-S statistics[1]					Levenes' statistic (test of equal variance)[2]
Test variable	**HO**	**HL**	**HM**	**HH**	**BI**	
Shoot DW	0.493	0.369	0.426	0.567	0.937	<0.001
Root DW	0.573	0.048	0.119	0.969	0.900	<0.001
Shoot Zn	1.000	0.866	0.947	0.993	0.993	0.019
Root Zn	0.600	0.697	0.997	0.874	0.850	0.04
Ln Shoot DW						0.073
Ln Root DW		0.652				0.053
Ln Shoot Zn						0.785
Ln Root Zn						0.184

[1] Where K-S statistic is <0.05 then data distribution is significantly different from normal and should be transformed or tested using non-parametric statistical techniques.

[2]Where Levene's P value is < 0.05 then data do not have equal variance and should be transformed or tested using non-parametric statistics.

Data Table E.13. Results from mixed model ANOVA test (SPSS) to determine significance of within and between treatment variance for *B. juncea* dry biomass (g) and measured zinc concentrations (µg g-1). With Treatment as fixed variable and block as random. Variable prefixed 'Ln' indicated data has been natural log transformed.

B, juncea Ln Shoot DW		Type III Sum of Squares	df	Mean Square	F	P.
Treatment	Hypothesis	52.613	4	13.153	20.024	.000
	Error	18.393	28	.657[b]		
Block	Hypothesis	4.674	7	.668	1.016	.441
	Error	18.393	28	.657[b]		

B. juncea Ln Root DW		Type III Sum of Squares	df	Mean Square	F	P.
Treatment	Hypothesis	91.013	4	22.753	25.071	.000
	Error	21.782	24	.908[b]		
Block	Hypothesis	2.380	6	.397	.437	.847
	Error	21.782	24	.908[b]		

B. juncea Ln shoot Zn		Type III Sum of Squares	df	Mean Square	F	P.
Treatment	Hypothesis	25.338	4	6.335	143.533	.000
	Error	1.236	28	.044[b]		
Block	Hypothesis	.424	7	.061	1.372	.256
	Error	1.236	28	.044[b]		

B. juncea Ln root Zn		Type III Sum of Squares	df	Mean Square	F	P.
Treatment	Hypothesis	5.585	4	1.396	15.791	.000
	Error	2.122	24	.088[b]		
Block	Hypothesis	.287	6	.048	.541	.772
	Error	2.122	24	.088[b]		

Data Table E.14. Results of Tukey HSD multiple comparison of means test for a) shoot dry biomass (g), b.) root dry biomass (g), c.) zinc in shoot dry biomass and d.) zinc in root dry biomass for *B. Juncea* grown in 5 treatments; HO – homogeneous, HL – low heterogeneity, HM – high heterogeneity, HH – High heterogeneity, BI – binary.

a.) LnShootDW

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HO	HL	-.35908	.40591	.901	-1.5261	.8079
	HM	-.65122	.40591	.505	-1.8182	.5158
	HH	-2.19850*	.40591	.000	-3.3655	-1.0315
	BI	-2.97139*	.40591	.000	-4.1384	-1.8044
HL	HO	.35908	.40591	.901	-.8079	1.5261
	HM	-.29214	.40591	.951	-1.4591	.8749
	HH	-1.83941*	.40591	.001	-3.0064	-.6724
	BI	-2.61230*	.40591	.000	-3.7793	-1.4453
HM	HO	.65122	.40591	.505	-.5158	1.8182
	HL	.29214	.40591	.951	-.8749	1.4591
	HH	-1.54728*	.40591	.005	-2.7143	-.3803
	BI	-2.32016*	.40591	.000	-3.4872	-1.1532
HH	HO	2.19850*	.40591	.000	1.0315	3.3655
	HL	1.83941*	.40591	.001	.6724	3.0064
	HM	1.54728*	.40591	.005	.3803	2.7143
	BI	-.77289	.40591	.334	-1.9399	.3941
BI	HO	2.97139*	.40591	.000	1.8044	4.1384
	HL	2.61230*	.40591	.000	1.4453	3.7793
	HM	2.32016*	.40591	.000	1.1532	3.4872
	HH	.77289	.40591	.334	-.3941	1.9399

*. The mean difference is significant at the 0.05 level.

b.) LnRootDW

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HO	HL	-.33739	.47970	.954	-1.7288	1.0540
	HM	-.60535	.47970	.716	-1.9968	.7861
	HH	-3.01444*	.47970	.000	-4.4059	-1.6230
	BI	-4.02030*	.47970	.000	-5.4117	-2.6289
HL	HO	.33739	.47970	.954	-1.0540	1.7288
	HM	-.26796	.47970	.980	-1.6594	1.1235
	HH	-2.67705*	.47970	.000	-4.0685	-1.2856
	BI	-3.68291*	.47970	.000	-5.0743	-2.2915
HM	HO	.60535	.47970	.716	-.7861	1.9968
	HL	.26796	.47970	.980	-1.1235	1.6594
	HH	-2.40909*	.47970	.000	-3.8005	-1.0177
	BI	-3.41496*	.47970	.000	-4.8064	-2.0235
HH	HO	3.01444*	.47970	.000	1.6230	4.4059
	HL	2.67705*	.47970	.000	1.2856	4.0685
	HM	2.40909*	.47970	.000	1.0177	3.8005
	BI	-1.00587	.47970	.248	-2.3973	.3856
BI	HO	4.02030*	.47970	.000	2.6289	5.4117
	HL	3.68291*	.47970	.000	2.2915	5.0743
	HM	3.41496*	.47970	.000	2.0235	4.8064
	HH	1.00587	.47970	.248	-.3856	2.3973

*. The mean difference is significant at the 0.05 level.

c.) LnShootZn

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HO	HL	-.02511	.10888	.999	-.3381	.2879
	HM	.34414*	.10888	.025	.0311	.6572
	HH	.46402*	.10888	.001	.1510	.7770
	BI	2.12778*	.10888	.000	1.8148	2.4408
HL	HO	.02511	.10888	.999	-.2879	.3381
	HM	.36925*	.10888	.014	.0562	.6823
	HH	.48913*	.10888	.001	.1761	.8022
	BI	2.15290*	.10888	.000	1.8399	2.4659
HM	HO	-.34414*	.10888	.025	-.6572	-.0311
	HL	-.36925*	.10888	.014	-.6823	-.0562
	HH	.11988	.10888	.805	-.1931	.4329
	BI	1.78365*	.10888	.000	1.4706	2.0967
HH	HO	-.46402*	.10888	.001	-.7770	-.1510
	HL	-.48913*	.10888	.001	-.8022	-.1761
	HM	-.11988	.10888	.805	-.4329	.1931
	BI	1.66377*	.10888	.000	1.3507	1.9768
BI	HO	-2.12778*	.10888	.000	-2.4408	-1.8148
	HL	-2.15290*	.10888	.000	-2.4659	-1.8399
	HM	-1.78365*	.10888	.000	-2.0967	-1.4706
	HH	-1.66377*	.10888	.000	-1.9768	-1.3507

*. The mean difference is significant at the 0.05 level.

d.) LnRootZn

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HO	HL	-.00674	.15147	1.000	-.4461	.4326
	HM	.08820	.15147	.977	-.3511	.5275
	HH	.45966*	.15147	.037	.0203	.8990
	BI	1.03801*	.15147	.000	.5987	1.4774
HL	HO	.00674	.15147	1.000	-.4326	.4461
	HM	.09493	.15147	.970	-.3444	.5343
	HH	.46639*	.15147	.033	.0270	.9057
	BI	1.04475*	.15147	.000	.6054	1.4841
HM	HO	-.08820	.15147	.977	-.5275	.3511
	HL	-.09493	.15147	.970	-.5343	.3444
	HH	.37146	.15147	.129	-.0679	.8108
	BI	.94981*	.15147	.000	.5105	1.3892
HH	HO	-.45966*	.15147	.037	-.8990	-.0203
	HL	-.46639*	.15147	.033	-.9057	-.0270
	HM	-.37146	.15147	.129	-.8108	.0679
	BI	.57835*	.15147	.005	.1390	1.0177
BI	HO	-1.03801*	.15147	.000	-1.4774	-.5987
	HL	-1.04475*	.15147	.000	-1.4841	-.6054
	HM	-.94981*	.15147	.000	-1.3892	-.5105
	HH	-.57835*	.15147	.005	-1.0177	-.1390

*. The mean difference is significant at the 0.05 level.

E.3 *Plantago lanceolata* biomass and zinc data

Data Table E.15. Biomass data recorded for *Plantago lanceolata* at harvest (73 days after transplanting seedlings)

Pot Number	Treatment	No.True leaves	No. dead leaves	Widest leaf	Longest leaf	Shoot FW (g)	Shoot DW (g)	Root DW (g)
A1	HO	8	2	10	65	0.595	0.094	0.046
B18	HO	13	2	21	175	4.877	0.527	0.196
C3	HO	DEAD						
D3	HO	12	2	22	150	3.789	0.494	0.189
E17	HO	10	2	18	165	2.631	0.264	0.069
F3	HO	11	2	19	160	2.987	0.345	0.146
G10	HO	9	2	15	160	2.403	0.301	0.092
H3	HO	9	3	10	95	0.762	0.103	0.047
A15	HL	14	1	18	225	4.953	0.564	0.175
B5	HL	17	2	26	145	6.156	0.710	0.258
C10	HL	16	4	26	170	10.013	1.078	0.452
D1	HL	11	0	21	160	2.741	0.314	0.167
E11	HL	13	2	15	120	1.567	0.205	0.101
F4	HL	19	2	40	185	11.467	1.476	0.674
G18	HL	12	1	32	270	10.334	1.226	0.429
H10	HL	11	1	16	200	3.705	0.411	0.121
A3	HM	12	2	13	125	2.381	0.267	0.100
B2	HM	9	2	19	125	1.935	0.258	0.083
C9	HM	14	2	15	150	3.553	0.435	0.172
D11	HM	15	2	19	175	4.675	0.553	0.239
E10	HM	9	2	16	15	2.192	0.277	0.130
F10	HM	15	3	24	155	6.055	0.739	0.296
G11	HM	12	0	13	130	1.876	0.248	0.090
H17	HM	11	2	19	180	3.140	0.349	0.151
A12	HH	13	0	18	190	4.766	0.545	0.253
B11	HH	14	2	25	205	7.401	0.974	0.393
C17	HH	12	1	22	195	4.483	0.608	0.204
D16	HH	13	2	26	190	7.099	1.045	0.541
E4	HH	9	2	15	130	1.561	0.247	0.093
F20	HH	18	2	30	180	11.478	1.377	0.499
G19	HH	14	0	18	170	4.547	0.545	0.168
H9	HH	15	3	25	175	7.295	0.919	0.310
A6	BI	12	2	21	200	5.004	0.563	0.178
B15	BI	12	3	18	165	3.951	0.459	0.160
C18	BI	10	2	20	180	3.567	0.354	0.110
D10	BI	17	0	28	200	10.713	1.489	0.859
E2	BI	17	2	29	185	8.819	1.021	0.448
F6	BI	11	1	21	175	3.268	0.420	0.134
G6	BI	23	2	28	145	9.209	1.221	0.503
H5	BI	9	2	12	160	1.219	0.146	0.050

Data Table E.16. *P. lanceolata* measured concentrations of Zn (µg g^{-1}) in dry biomass of shoots.

BLOCK REF	TREATMENT				
	HO	**HL**	**HM**	**HH**	**BI**
A	265.06	480.30	176.89	514.95	183.02
B	319.41	360.17	209.61	573.29	144.02
C	DEAD	658.42	343.41	403.10	109.71
D	355.14	486.20	342.84	440.75	453.21
E	248.24	674.48	603.23	769.16	163.41
F	383.19	460.02	371.41	754.77	113.73
G	328.76	566.77	197.05	524.40	296.25
H	283.12	363.95	532.62	530.27	106.71
Average	**311.84**	**506.29**	**347.13**	**563.84**	**196.26**
Std err	**18.50**	**42.24**	**55.26**	**47.20**	**42.75**
min	248.24	360.17	176.89	403.10	106.71
max	383.19	674.48	603.23	769.16	453.21

Data Table E.17. *P. lanceolata* measured concentrations of Zn (µg g^{-1}) in dry biomass of roots.

BLOCK REF	TREATMENT				
	HO	**HL**	**HM**	**HH**	**BI**
A	1947.59	4090.49	2288.24	2581.92	1708.79
B	2664.00	2774.39	2369.39	3378.84	1591.89
C	DEAD	3472.67	2659.38	2835.50	2373.15
D	2260.89	3755.91	2289.09	2092.28	2104.26
E	3307.66	3822.12	3624.29	5259.33	1323.34
F	2168.16	2358.31	2471.73	2850.27	1855.81
G	2913.87	2345.31	2408.55	3287.95	1445.55
H	3197.01	2625.16	4207.05	2640.03	2052.62
Average	**2637.03**	**3155.54**	**2789.71**	**3115.76**	**1806.93**
Std err	**199.84**	**249.93**	**255.21**	**338.10**	**126.21**
min	1947.59	2345.31	2288.24	2092.28	1323.34
max	3307.66	4090.49	4207.05	5259.33	2373.15

Data Table E.18. Results of Kolmogorov - Smirnov test for normality and Levenes test for equal variance, calculated using data collected from *P. lanceolata* grown in 5 different treatments of Zn heterogeneity. Where normality and/or equal variance tests are not satisfied, data has been natural log transformed.

	Treatment K-S statistics[1]					Levenes' statistic (test of equal variance)[2]
Test variable	**HO**	**HL**	**HM**	**HH**	**BI**	
Shoot DW	0.960	0.988	0.747	0.944	0.718	0.005
Root DW	0.986	0.803	0.966	0.999	0.459	0.002
Shoot Zn	0.497	0.930	0.939	0.816	0.496	0.892
Root Zn	0.740	0.889	0.379	0.620	0.999	0.474
Ln Shoot DW						0.816
Ln Root DW						1.474

[1]Where K-S statistic is <0.05 then data distribution is significantly different from normal and should be transformed or tested using non-parametric statistical techniques.

[2]Where Levene's P value is < 0.05 then data do not have equal variance and should be transformed or tested using non-parametric statistics.

E.19. Results from mixed model ANOVA test (SPSS) to determine significance of between and within treatment variance for *P. Lanceolata* dry biomass dry (g) and measured zinc concentrations (µg g-1). With Treatment as fixed variable and block as random. Variable prefixed 'Ln' indicated data has been natural log transformed.

P. lanceolata Ln Shoot DW		Type III Sum of Squares	df	Mean Square	F	Sig.
Treatment	Hypothesis	5.184	4	1.296	3.783	.014
	Error	9.250	27	.343[a]		
Block	Hypothesis	4.314	7	.616	1.799	.129
	Error	9.250	27	.343[a]		

P. lanceolata Ln root DW		Type III Sum of Squares	df	Mean Square	*F*	*P*
Treatment	Hypothesis	5.326	4	1.332	3.493	.020
	Error	10.293	27	.381[a]		
Block	Hypothesis	5.350	7	.764	2.005	.092
	Error	10.293	27	.381[a]		

P. lanceolata Shoot Zn		Type III Sum of Squares	df	Mean Square	F	Sig.
Treatment	Hypothesis	768218.149	4	192054.537	11.732	.000
	Error	458346.679	28	16369.524[a]		
Block	Hypothesis	139153.477	7	19879.068	1.214	.328
	Error	458346.679	28	16369.524[a]		

P. lanceolata Rroot Zn		Type III Sum of Squares	df	Mean Square	F	Sig.
Treatment	Hypothesis	10552511	4	2638128	4.384	.007
	Error	16849272	28	601760[a]		
Block	Hypothesis	5352049	7	764578	1.271	.300
	Error	16849272	28	601760[a]		

E.20. Results of Tukey HSD multiple comparison of means test for a) shoot dry biomass (g), b.) root dry biomass (g), c.) zinc in shoot dry biomass and d.) zinc in root dry biomass for *P. lanceolata* grown in 5 treatments; HO – homogeneous, HL – low heterogeneity, HM – medium heterogeneity, HH – High heterogeneity and BI - Binary

a.) LnShootDW

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HO	HL	-.8847	.30292	.050	-1.7695	.0000
	HM	-.3523	.30292	.772	-1.2371	.5324
	HH	-1.0122*	.30292	.019	-1.8970	-.1275
	BI	-.7984	.30292	.092	-1.6831	.0864
HL	HO	.8847	.30292	.050	.0000	1.7695
	HM	.5324	.29265	.384	-.3224	1.3871
	HH	-.1275	.29265	.992	-.9823	.7272
	BI	.0863	.29265	.998	-.7684	.9411
HM	HO	.3523	.30292	.772	-.5324	1.2371
	HL	-.5324	.29265	.384	-1.3871	.3224
	HH	-.6599	.29265	.191	-1.5146	.1948
	BI	-.4460	.29265	.556	-1.3008	.4087
HH	HO	1.0122*	.30292	.019	.1275	1.8970
	HL	.1275	.29265	.992	-.7272	.9823
	HM	.6599	.29265	.191	-.1948	1.5146
	BI	.2139	.29265	.947	-.6409	1.0686
BI	HO	.7984	.30292	.092	-.0864	1.6831
	HL	-.0863	.29265	.998	-.9411	.7684
	HM	.4460	.29265	.556	-.4087	1.3008
	HH	-.2139	.29265	.947	-1.0686	.6409

*. The mean difference is significant at the 0.05 level.

b.) LnRootDW

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HO	HL	-.9302	.31955	.051	-1.8634	.0031
	HM	-.4040	.31955	.715	-1.3372	.5293
	HH	-1.0280*	.31955	.025	-1.9613	-.0947
	BI	-.7928	.31955	.125	-1.7261	.1405
HL	HO	.9302	.31955	.051	-.0031	1.8634
	HM	.5262	.30871	.448	-.3755	1.4278
	HH	-.0979	.30871	.998	-.9995	.8038
	BI	.1374	.30871	.991	-.7643	1.0390
HM	HO	.4040	.31955	.715	-.5293	1.3372
	HL	-.5262	.30871	.448	-1.4278	.3755
	HH	-.6241	.30871	.283	-1.5257	.2776
	BI	-.3888	.30871	.717	-1.2905	.5128
HH	HO	1.0280*	.31955	.025	.0947	1.9613
	HL	.0979	.30871	.998	-.8038	.9995
	HM	.6241	.30871	.283	-.2776	1.5257
	BI	.2352	.30871	.939	-.6664	1.1369
BI	HO	.7928	.31955	.125	-.1405	1.7261
	HL	-.1374	.30871	.991	-1.0390	.7643
	HM	.3888	.30871	.717	-.5128	1.2905
	HH	-.2352	.30871	.939	-1.1369	.6664

c.) Shoot Zn

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HO	HL	-233.4261*	63.9717	.0087	-419.8072	-47.0450
	HM	-74.2688	63.9717	.7729	-260.6499	112.1123
	HH	-290.9714*	63.9717	.0008	-477.3525	-104.5903
	BI	76.6066	63.9717	.7527	-109.7745	262.9877
HL	HO	233.4261*	63.9717	.0087	47.0450	419.8072
	HM	159.1573	63.9717	.1223	-27.2238	345.5384
	HH	-57.5453	63.9717	.8946	-243.9264	128.8358
	BI	310.0327*	63.9717	.0004	123.6516	496.4138
HM	HO	74.2688	63.9717	.7729	-112.1123	260.6499
	HL	-159.1573	63.9717	.1223	-345.5384	27.2238
	HH	-216.7026*	63.9717	.0166	-403.0837	-30.3215
	BI	150.8754	63.9717	.1568	-35.5057	337.2566
HH	HO	290.9714*	63.9717	.0008	104.5903	477.3525
	HL	57.5453	63.9717	.8946	-128.8358	243.9264
	HM	216.7026*	63.9717	.0166	30.3215	403.0837
	BI	367.5780*	63.9717	.0000	181.1969	553.9591
BI	HO	-76.6066	63.9717	.7527	-262.9877	109.7745
	HL	-310.0327*	63.9717	.0004	-496.4138	-123.6516
	HM	-150.8754	63.9717	.1568	-337.2566	35.5057
	HH	-367.5780*	63.9717	.0000	-553.9591	-181.1969

d.) Root Zn

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HO	HL	-848.1466	387.8659	.2140	-1978.1908	281.8975
	HM	-482.3154	387.8659	.7265	-1612.3596	647.7288
	HH	-808.3670	387.8659	.2548	-1938.4112	321.6772
	BI	500.4698	387.8659	.6991	-629.5744	1630.5140
HL	HO	848.1466	387.8659	.2140	-281.8975	1978.1908
	HM	365.8313	387.8659	.8775	-764.2129	1495.8754
	HH	39.7796	387.8659	1.0000	-1090.2645	1169.8238
	BI	1348.6164*	387.8659	.0133	218.5723	2478.6606
HM	HO	482.3154	387.8659	.7265	-647.7288	1612.3596
	HL	-365.8313	387.8659	.8775	-1495.8754	764.2129
	HH	-326.0516	387.8659	.9155	-1456.0958	803.9926
	BI	982.7852	387.8659	.1116	-147.2590	2112.8293
HH	HO	808.3670	387.8659	.2548	-321.6772	1938.4112
	HL	-39.7796	387.8659	1.0000	-1169.8238	1090.2645
	HM	326.0516	387.8659	.9155	-803.9926	1456.0958
	BI	1308.8368*	387.8659	.0171	178.7926	2438.8809
BI	HO	-500.4698	387.8659	.6991	-1630.5140	629.5744
	HL	-1348.6164*	387.8659	.0133	-2478.6606	-218.5723
	HM	-982.7852	387.8659	.1116	-2112.8293	147.2590
	HH	-1308.8368*	387.8659	.0171	-2438.8809	-178.7926

Appendix F**. Tables of results for root placement pot experiment.**

Data Table F.1. Shoot dry biomass (g) for *B. juncea* (BJ) and *B. napus* (BN) grown in two treatments, high heterogeneity (HH) and binary (BI)

Variable	Shoot dry biomass				Total root dry biomass				Measured Zn in shoot DW			
BLOCK	BJBI	BJHH	BNBI	BNHH	BJBI	BJHH	BNBI	BNHH	BJBI	BJHH	BNBI	BNHH
A	4.677	D	4.652	1.670	0.728		0.866	0.251	939.90	2106.91	750.00	2249.71
	4.644	D	4.432	3.576	1.002		1.223	0.722	788.41		751.24	1192.80
	6.207	D	3.934	4.451	1.287		0.783	0.824	1305.02		648.71	1081.64
	3.683	0.047	3.759	5.373	0.210	0.006	0.993	1.078	1042.84		955.93	1262.00
B	D	0.044	5.415	5.077		0.007	1.224	1.201	1432.44	3760.98	908.17	1061.72
	2.462	0.069	4.630	4.560	0.221		1.021	0.961	1287.30	2897.35	871.12	1028.96
	3.996	0.428	4.501	5.107	0.494	0.039	0.757	0.987	1098.78	2237.51	826.82	1293.09
	6.268	0.094	4.581	5.306	1.175	0.006	0.975	0.910		2345.62	880.22	1006.94
C	4.455	0.063	4.416	4.914	0.473	0.007	0.871	1.022	946.56	3053.30	1303.44	1127.00
	4.508	0.069	5.536	6.000	0.598		1.289	1.160	1294.09	3230.76	897.00	1124.87
	6.376	0.333	5.392	3.289	0.915	0.023	0.902	0.442	1058.73	2895.10	1133.36	1346.93
	5.612	0.065	5.192	5.578	1.329		1.150	1.053	1178.06	1888.44	1252.70	1594.21
D	6.631	0.021	5.473	5.640	1.313		1.222	1.129	1487.52	2743.14	1111.64	1211.90
	D	0.906	4.037	6.017		0.082	0.962	1.081	1513.39	1808.18	1058.13	1239.00
	0.433	0.038	5.563	5.121	0.040		1.164	0.988	1555.26	3608.65	978.54	1273.87
	3.643	D	6.780	6.268	0.403		1.405	1.384			919.10	1358.74
E	0.029	0.021	7.365	6.777			1.121	1.297	1103.50		683.92	1379.00
	6.667	D	7.949	7.530	1.111		1.293	1.314	1020.88	3388.16	687.91	1393.81
	5.505	0.031	6.905	1.412	0.971		1.330	0.144	1007.93	4408.04	693.30	2435.89
	3.545	0.034	7.773	5.245	0.721		1.464	0.971	1017.95	3346.81	859.66	1367.49
mean	4.408	0.151	5.414	4.946	0.764	0.024	1.101	0.946	1171.03	2914.60	908.55	1351.48
s.e	0.422	0.052	0.281	0.330	0.090	0.006	0.045	0.072	52.44	191.49	42.62	63.18

Data Table F.2 Results of tests for normality and equal variance for variable in pot experiment no. 3.

[1]Using Kolmogorov-Smirnov test, normal distribution satisfied where value >0.05.

[2]Using levenes test, equal variance assumed where p value >0.05.

[3]Normal distribution was achieved following log transformation, but did not alter the results of ANOVA.

Test	**Normal distribution[1]**		**Equal variance[2]**	**Normal distribution[1]**		**Equal variance[2]**
Treatment / **Variable**	BNBI	BNHH		BJBI	BJHH	
Shoot dry biomass	0.376	0.455	0.145	0.733	0.019*	
Total root dry biomass	0.933	0.298	0.495	0.982	0.540	0.097
Measured Zn conc. in shoot DW	0.901	0.049[3]	0.770	0.641	0.992	0.257
Root/shoot ratio	0.998	0.486	0.282			
***SQR transformed Shoot dry biomass**				0.076	0.176	0.158

Data Table F.3. Results of paired t-test of *B. napus* root dry biomass (g) in cells adjacent to central cell (Level 1) Difference is significant where tcalc is <0.05 (at 95% confidence)

Paired cells Zn concentration values.		95% Confidence Interval of the Difference		t	df	Sig. (2-tailed)
		Lower	Upper			
Pair 1	700 - 1100	-.01746355	-.00627645	-4.442	19	.000
Pair 2	1100 - 1500	.00351406	.01799594	3.109	19	.006
Pair 3	700 - 300	-.01186509	-.00010491	-2.130	19	.046
Pair 4	1100 - 300	-.00166255	.01343255	1.632	19	.119

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	700 & 1100	20	.837	.000
Pair 2	1100 & 1500	20	.688	.001
Pair 3	700 & 300	20	.727	.000
Pair 4	1100& 300	20	.671	.001

List of Publications.

Thomas, J.Y., Ramsey, M.H., John, E.A., Barnes, B. & Helmholtz Centre Environmental, R.-U. (2008) *CASE STUDY USING A NEW EXPERIMENTAL DESIGN FOR THE QUANTIFICATION OF CONTAMINANT HETEROGENEITY IN SOILS.* Leipzig: Helmholtz Centre Environmental Research-Ufz.

Whilst not directly related to the research in this thesis, the measurements made at the first site investigation (Coseley) provide the data for the following discussion paper..

Barnes, B., Glennie, E., Davey, Andrew and Thomas, J., (2010) *Cheby or not Cheby? Is that the question?*, Land Contamination &Reclamation, Vol 18., pp121-133.

The impact of variable Zn heterogeneity at the 2 cm scale on root and shoot accumulations and plant biomass for B. napus, B.juncea, and P. Lanceolata. – In preparation.