CRANFIELD UNIVERSITY

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Long-Term Impacts of Zinc and Copper on Microbial Biomass, Phosphatase Enzyme Activities, and the Mineralisation of Organic Phosphorus in Sludge Amended Soils

School of Environment, Energy, and AgriFood

PhD

Academic Year: 2014 – 2015

Supervisors:

Dr Ruben Sakrabani

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ABSTRACT

The agricultural demand for inorganic phosphate fertilisers needs to be reduced whilst the dependence on more sustainable sources of phosphorus, such as sewage sludge, is increased. However, the presence of heavy metals in sewage sludge pose a threat to soil microorganisms and can inhibit the action of phosphatase enzymes if present in high concentrations. The long-term impact of Zn and Cu on soil microorganisms and phosphatase enzyme activity was investigated at four field sites from the Defra 'Long-Term Sludge Experiment' in order to determine the overall impact on organic phosphorus mineralisation. Following the final applications of sludge in 1997, the total concentrations of Zn and Cu at each site were comparable to the UK statutory limits for sludge amended soils. Almost 20 years later 63-91 % of the applied metal loadings still remained and total concentrations of Zn and Cu were found to be significantly higher in contaminated soils, in comparison to untreated soil, and soils receiving uncontaminated sewage sludge. A significant correlation between exchangeable and total metal concentration could still be seen in soils contaminated with Zn, whereas the solubility of Cu was found to be very low (<1 %); though a significant percent remained bound to soil organic matter. No long-term decrease in microbial biomass carbon (C_{mic}) could be detected in the contaminated soils at any of the sites. However analysis of ergosterol showed an increase in the proportion of microbial biomass carbon derived from fungi. Significant changes were also observed in the PLFA profiles of microbial communities within contaminated soils, indicating the microbial community has adapted and become tolerant to the heavy metal contamination. Combining results using meta-analysis indicated that Zn and Cu caused an increase in fungal biomass carbon of approximately 25-35 % in comparison to soil receiving uncontaminated sludge. Whereas overall decreases of 16 and 8 % were seen for C_{mic} in soils contaminated with Zn and Cu, respectively, indicating a loss of bacterial biomass from the contaminated soils. Hence, the current UK statutory limits may not be sufficient to prevent changes in soil microbial community and a reduction in the limits set for sludge amended soils is recommended in order to protect microbial diversity. No long-term decrease in the activity of phosphomonoesterase was detectable in the contaminated soils. However combining the results using meta-analysis indicated phosphomonoesterase activity per milligram of biomass carbon was greater in soils contaminated with Cu. This may be an indication that Cu is inhibiting extracellular phosphatase enzyme activity within the soil environment, though this remains undetermined. Orthophosphate, phosphomonoesters, and pyrophosphate were the predominant forms of phosphorus within the applied sludge treatments; a broad phosphodiester signal was also present. Overall, the greatest range of organic phosphorus forms was seen in the uncontaminated sewage sludge. However, no difference in the range of organic phosphorus compounds was observed between the untreated and sludge amended soils at the field sites investigated. It was therefore concluded that the organic phosphorus content of sludge amended soils is returning to that of untreated soil, with no long-term interference caused by the presence of Zn and Cu.

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Many thanks,

Alex Charlton

"…"With your intensive agriculture," he went on, "you're simply draining the soil of phosphorus. More than half of one per cent a year. Going clean out of circulation. And then the way you throw away hundreds of thousands of tons of phosphorus pentoxide in your sewage! Pouring it into the sea. And you call that progress. Your modern sewage systems!" His tone was witheringly scornful. "You ought to be putting it back where it came from. On the land."…"

Aldous Huxley [1894 – 1963]

Point Counter Point (Published in 1928)

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NOMENCLATURE

AAS:	Atomic Absorption Spectroscopy
ADAS:	Agricultural Development and Advisory Service
Al:	Aluminium
Al_2O_3:	Aluminium Oxide
ANOVA:	Analysis of Variance
AUC:	Auchincruive
BCR:	Community Bureau of Reference
^oC:	Degrees Celsius
C:	Carbon
Ca:	Calcium
$CaCl_2$:	Calcium Chloride
$CaCO_3$:	Calcium Carbonate (Lime)
$CaSO_4$:	Calcium Sulphate
Cd:	Cadmium
C_{fungi}:	Fungal Biomass Carbon
$CHCl_3$:	Chloroform
$CL_{95\%}$:	Confidence Limits (95 %)
cm:	Centimetre
C_{mic}:	Microbial Biomass Carbon
C=O:	Carboxyl Functional Group
CO_2:	Carbon Dioxide
CRM:	Certified Reference Material
Ctrl1:	Uncontaminated Digested Sludge Treatment
Ctrl2:	Uncontaminated Undigested Sludge Treatment
Cu:	Copper
δ:	Chemical Shift
d:	Soil Cultivation Depth
D:	Dilution Factor
D_2O:	Deuterium Oxide (Heavy Water)
Defra:	Department for Environment, Food and Rural Affairs
DIN:	Duetsche Institut Für Normung
DNA:	Deoxyribonucleic Acid
DoE:	Department of Environment
ds:	Dry Solids
E:	Enzyme
ED:	Ecological Dose
EDTA:	Ethylenediaminetetraacetic Acid
EU:	European Union
F_1:	Principal Component Factor 1
F_2:	Principal Component Factor 2
F_3:	Principal Component Factor 3
FAME:	Fatty Acid Methyl Esters
F_C:	Organic Matter to Organic Carbon Conversion Factor
Fe:	Iron
Fe_2O_3:	Iron Oxide
F_F:	Fungal Biomass to Fungal Biomass Carbon Conversion Factor
g:	Gram
GC:	Gas Chromatography
GLE:	Gleadthorpe

H^+:	Hydrogen Ion
ha:	Hectare
HAR:	Hartwood
HCl:	Hydrochloric Acid
Hg:	Mercury
HNO_3:	Nitric Acid
H_2O:	Water (Hydrogen Oxide)
HPLC:	High Performance Liquid Chromatography
H_3PO_4:	Orthophosphate
H_2SO_4:	Sulphuric Acid
Hz:	Hertz
i:	Independent Study
ID:	Internal Diameter
IFDC:	International Fertiliser Development Centre
°K:	Degrees Kelvin
K:	Potassium
K_{EC}:	Microbial Biomass Correction Factor
K_F:	Ergosterol to Fungal Biomass Conversion Factor
K_M:	Michaelis Constant
K_2SO_4:	Potassium Sulphate
kg:	Kilogram
K_M:	Michaelis Constant
K_M^H:	Michaelis Constant for Enzyme with High Substrate Affinity
K_M^L:	Michaelis Constant for Enzyme with Low Substrate Affinity
L:	Litre
LOI:	Loss on Ignition
LTSE:	Long-Term Sludge Experiment
µg:	Microgram
µL:	Microlitre
µm:	Micrometre
µM:	Micromolar
µmol:	Micromole
µs:	Microseconds
m:	Metre
m:	Mass of Soil
M:	Moles per Litre (Molar)
M^+:	Metal Ion
MAFF:	Ministry for Agriculture, Fisheries, and Food
MeOH:	Methanol
mg:	Milligram
min:	Minute
mL:	Millilitre
mm:	Millimetre
mM:	Millimolar
mol%:	Percent of Total Concentration
Mt:	Million Metric Tonnes
4-MUF:	Methylumbelliferone
4-MUF-P:	Methylumbelliferone Phosphate
bis-4-MUF-P:	bis-Methylumbelliferone Phosphate
v:	Enzyme Activity
n:	Number of Observations
N:	Nitrogen
N_2:	Nitrogen Gas
$NaHCO_3$:	Sodium Hydrogen Carbonate
NaOH:	Sodium Hydroxide

Na_2SO_4:	Sodium Sulphate
N- NH_4^+:	Ammonium Nitrogen
NH_4NO_3:	Ammonium Nitrate
Ni:	Nickel
nm:	Nanometre
nmol:	Nanomole
NMR:	Nuclear Magnetic Resonance Spectroscopy
NS:	Untreated Soil (No Sludge)
OH:	Hydroxyl Functional Group
ρ:	Soil Bulk Density
P (^{31}P):	Phosphorus
$P_{Loading}$:	Estimated Phosphorus Loading
Pb:	Lead
PCA:	Principal Component Analysis
PLFA:	Phospholipid Fatty Acid
P_{mic}:	Microbial Biomass Phosphorus
pmol:	Picomole
P_2O_5:	Phosphate Rock
ppm:	Parts per Million
QF:	Fluorescence Quenching Factor
R:	Organic Moiety
RCF:	Relative Centrifugal Force
RFU:	Relative Fluorescence Units
R_i:	Log Response Ratio
rpm:	Revolutions per Minute
s:	Second
S:	Substrate
SH:	Thiol Functional Group
SOC:	Soil Organic Carbon (%)
SOM:	Soil Organic Matter (%)
SPE:	Solid Phase Extraction
t:	Tonne
UK:	United Kingdom
US:	United States
USEPA:	United States Environmental Protection Agency
USGS:	United States Geological Survey
UV:	Ultra Violet
v:	Volume
V_{MAX}:	Maximum Enzyme Activity
V_{MAX}^H:	Maximum Enzyme Activity for Enzyme with High Substrate Affinity
V_{MAX}^L:	Maximum Enzyme Activity for Enzyme with Low Substrate Affinity
W_{H_2O}:	Soil Moisture Content (%)
WOB:	Woburn
$\overline{x}_C$:	Arithmetic Mean of Control Group
$\overline{x}_E$:	Arithmetic Mean of Experimental Group
yr:	Year
Zn:	Zinc

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. Peak Phosphorus

Over the past 150 years, it is estimated that 10 000 million metric tonnes (Mt) of inorganic phosphate rock (P_2O_5) has been mined and applied to agricultural land as mineral fertiliser in order to meet the growing demands of global food production (Buckingham & Jasinski, 2014; Tiessen et al., 2011). The annual production of phosphate rock has increased steadily throughout the 20th century, reaching an estimated peak of 166 Mt in 1988, prior to the collapse of the Soviet Union (Buckingham & Jasinski, 2014; Van Kauwenbergh, 2010). Global annual production subsequently declined in the following years, reaching a minimum of 119 Mt in 1993, before increasing steadily again to a record 225 Mt in 2013 (Jasinski, 2015). Current estimates of remaining global phosphate rock resources range from 290 000 to 460 000 Mt (Van Kauwenbergh, 2010), however the quantities of utilisable phosphate rock reserves (i.e. that for which extraction is currently economically viable) are continually debated and revised (Edixhoven et al., 2014). Most recently, the 2010 'Mineral Commodity Summaries', published by the U.S. Geological Survey (USGS), estimated global phosphate rock reserves to be 16 000 Mt (Jasinski, 2010), however this figure was drastically increased the following year (**Figure 1.1**) to 65 000 Mt (Jasinski, 2011), and now currently stands at 67 000 Mt (Jasinski, 2015). The reason for such an increase was due to the inclusion of additional 'hypothetical' phosphate rock resources in an IFDC (International Fertiliser Development Centre) report into global phosphate rock reserves and resources (Cooper et al. 2011; Edixhoven et al., 2014; Rosemarin et al., 2010; Van Kauwenbergh, 2010). Nevertheless, despite this increase, the forecasts for phosphate rock production throughout the 21st century still remain unclear (Cooper et al., 2011; Cordell et al., 2009; Walan et al., 2014).

Historically, China and the United States (US) have been the two primary producers of phosphate rock, respectively producing 90.5 and 28.5 Mt yr^{-1}, on average, over the past five years (2010 to 2014). However, if these levels of production are maintained the phosphate rock reserves of both countries will be depleted within the next 40 years (Jasinski, 2012, 2013, 2014, 2015). At present, approximately 88 % of global phosphate rock reserves are located within just five countries (Morocco, China, Algeria, Syria, and South Africa (**Figure 1.1**)), with approximately 74 % located in Morocco alone (Jasinski, 2015). Hence, both Cooper et al. (2011) and Walan et al. (2014) predict that in the future, production and distribution of phosphate rock globally, will be increasingly controlled by a single country. At present, both the European Union (EU) and the United Kingdom (UK) rely heavily on importing mineral fertilisers derived from phosphate rock (EFMA, 2000; Soil Association, 2010), therefore such a geopolitical scenario is becoming an increasingly major cause for concern. Particularly following the dramatic increases (approximately 700-800 %) in the price of phosphate rock observed during the 2008

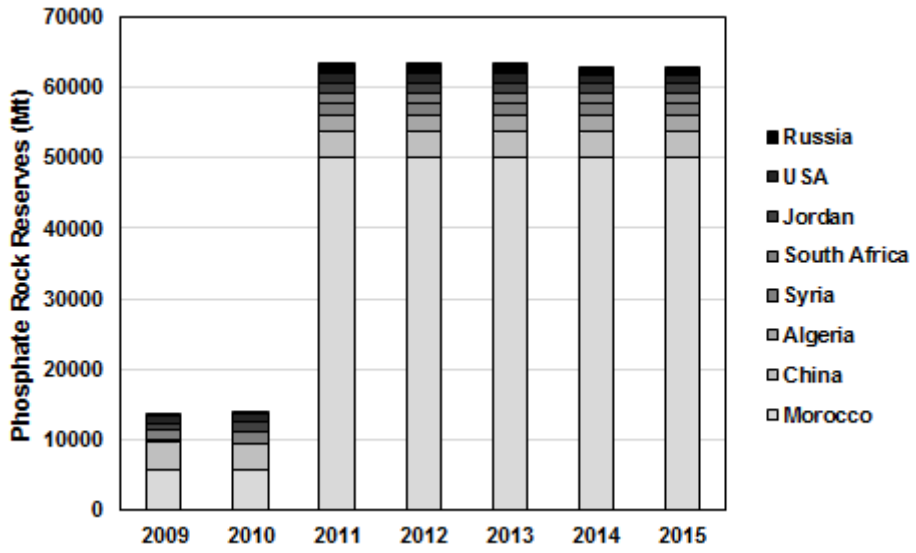


Figure 1.1:- Estimated phosphate rock reserves (Mt) for the top eight global producers reported by the US Geological Survey over the past seven years. Data obtained from Jasinski (2009, 2010, 2011, 2012, 2013, 2014, 2015).

food crisis, which caused China to impose a 135 % export tariff in order to secure a domestic supply, essentially halting all Chinese exports that year (Cordell et al., 2009; Cordell & White, 2011; Elser & White, 2010; Rosemarin et al., 2010; Soil Association, 2010).

Phosphorus (P) is an essential element for all living organisms, having an important structural role in both nucleic acids and phospholipids, as well as a vital metabolic role in adenosine triphosphate; for which there is no biochemical substitute (Childers et al., 2011; EFMA, 2000; Stevenson & Cole, 1999). Hence it is no surprise that around 85-90 % of the phosphate rock produced each year is used to produce the quantities of mineral fertilisers and animal feeds required to meet the demands of global agriculture and food production (Cordell et al., 2009; EFMA, 2000); approximately 200 Mt yr^{-1} at current production levels (Jasinski, 2015). Therefore, in order to maintain sufficient food production to feed a growing global population (9.6 billion by 2050 (United Nations, 2013)), demand for phosphate rock is predicted to increase dramatically throughout the 21st century (Cooper et al., 2011; Cordell et al., 2009; Rosemarin et al., 2010; Walan et al., 2014)

Phosphate rock is a finite resource, and the longevity of global phosphate rock reserves is still widely debated. Cordell et al. (2009) have predicted that 'peak phosphorus', i.e. the point in time where global demand for phosphate rock exceeds the production capacity of current phosphate rock reserves, could potentially occur in 2033. However, this analysis used USGS data (Jasinski, 2007, 2008) published prior to the IFDC report in 2010. Most recently Walan et al. (2014) have estimated 'peak phosphorus' will occur around 2084 using the revised USGS data, however this is reduced to 2030-2041, in agreement with Cordell et al. (2009), if the unrevised data is used. The possibility of 'peak phosphorus' occurring within the 21st century was subsequently challenged in the IFDC report

which stated that phosphate rock reserves are sufficient to last for 300-400 years (Van Kauwenbergh, 2010); though, in this case, it was assumed that annual production of phosphate rock would remain constant at the 2010 rate. Rosemarin et al. (2011) extended the IFDC analysis and calculated the longevity of phosphate rock reserves using the United Nations global population growth forecasts. In this case, a figure of 172 years was calculated assuming phosphate rock production is proportional to the predicted increases in global population. Though, if additional demands for phosphate rock are taken into account, principally the intensive development of African agriculture and then the proliferation of bio-fuel crops, this figure is reduced to 126 and 48 years, respectively (Rosemarin et al. 2011). However, Cordell & White (2011) stress that these 'depletion scenarios' are too simplistic and suggest that the declining quality of phosphate rock reserves, i.e. the concentration of P per kg of phosphate rock, will require the production of greater quantities in order to maintain even a constant level of food production, and eventually there will come a time when it is no longer economically viable to meet the global demand for phosphate rock. Furthermore, Cordell & White (2011) also stress that this debate is focussed too heavily on the longevity of phosphate rock reserves, and emphasise that phosphorus exists in a variety of utilisable forms, hence there is no actual shortage of phosphorus itself.

1.2. Sewage Sludge as a Sustainable Source of Phosphorus

In undisturbed ecosystems, the phosphorus cycle is essentially a closed biogeochemical process as interactions with the atmosphere are negligible (Stevenson & Cole, 1999). In this situation, phosphorus is returned to the soil in organic forms as part of the residual organic matter from plants and animals (**Figure 1.2**). However in agricultural systems, soils will become phosphorus deficient if the phosphorus taken up during plant growth, and subsequently removed during harvest (**Figure 1.2**), is not replenished (Kirkham, 1982; Stevenson & Cole, 1999). For this reason, global agriculture has relied heavily on the application of inorganic mineral fertilisers in order to maintain food production (Cordell et al., 2009). However, this practice is not sustainable and substantial losses occur due to current mining and agricultural processes (Childers et al., 2011; Cordell et al., 2009; Cordell et al., 2011). For instance, Cordell et al. (2009) estimate that only 20 % of the phosphorus applied annually as mineral fertiliser is actually consumed by the population, with only 10 % of that excreted returning to agricultural land in wastewater or sewage sludge. With the global population set to increase, the safe recycling of increasing quantities of sewage sludge also poses a significant management problem for the 21st century (Defra, 2007b; Water UK, 2010). Therefore several authors have suggested that increasing the quantities of sewage sludge applied to agricultural land could help reduce the demand for inorganic mineral fertilisers (Childers et al., 2011; Cordell et al., 2011; Soil Association, 2010).

It has long been known that the application of organic residues, such as sewage sludge, can help improve soil quality and fertility by restoring organic matter, nitrogen, and phosphorus, plus a range of

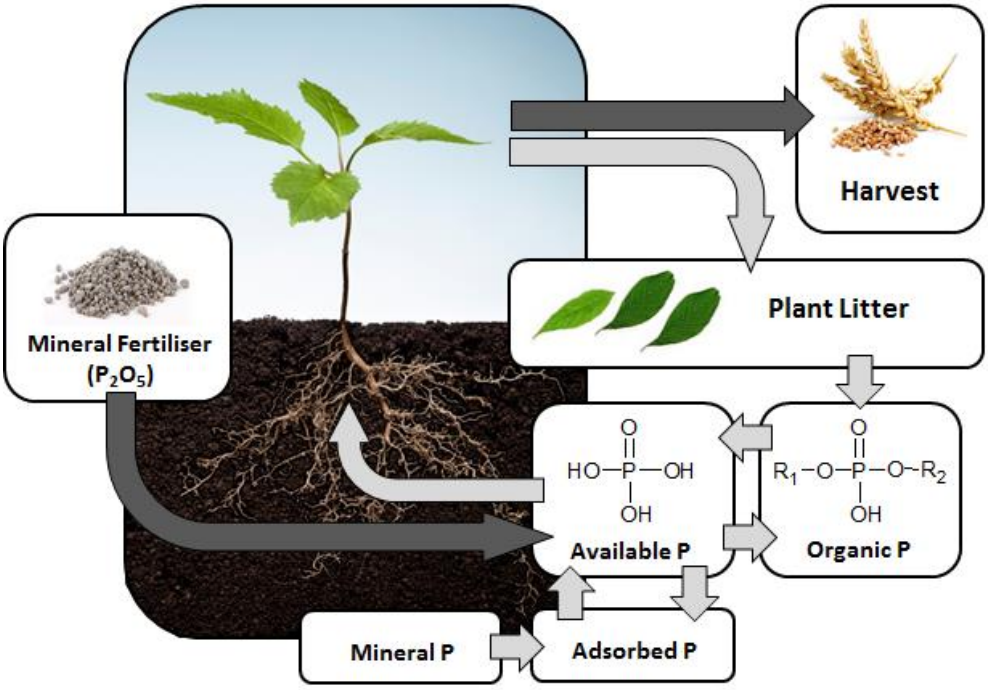


Figure 1.2:- The terrestrial phosphorus cycle. Available phosphorus (P) present in the soil solution is taken up by plants and soil microorganisms during growth. This is returned to the soil as organic matter in plant litter, cell detritus, and the manure of grazing animals (not shown). Phosphorus is then released back into the soil solution following the mineralisation of organic matter by soil microorganisms. In agricultural systems the phosphorus present in plant litter is removed during harvest, therefore the application of mineral fertilisers (P_2O_5) is required to prevent the soil becoming phosphorus deficient. The solubility of phosphorus is pH dependent and in acidic soils phosphorus is readily adsorbed to the surface of clay particles and iron (Fe)/aluminium (Al) oxides. Phosphorus is also present in soil in a range of mineral forms (apatites), however these are extremely insoluble and release phosphorus to the soil solution over geological timescales (Stevenson & Cole, 1999).

micronutrients, to the soil (Kirkham, 1982; Smith, 1996; Sterrit & Lester, 1980); and for these reasons the application of sewage sludge to agricultural land is considered to be the best practical environmental option for sludge recycling within Europe and the UK (Defra, 2007b; Water UK, 2010). Over the past two decades the approach to the treatment and handling of sewage sludge has gone through considerable change within the EU, with the options for disposal becoming increasingly restricted (**See Section 1.3**). Nevertheless, the total quantity of sewage sludge produced per annum in the UK has increased steadily within this period (**Figure 1.3**), reaching an estimated 1.6 Mt (dry matter) in 2010 (Defra, 2007b); at present approximately 77 % of sludge produced in the UK is applied to agricultural land (Water UK, 2010). On average, the total amount of phosphorus present in sewage sludge ranges from approximately 1-5 % of the dry matter content, depending on the treatment processes involved (Kirkham, 1982; Smith, 1996; Stevenson & Cole, 1999). Sludge cake generally has higher concentrations of phosphorus than liquid sludge, which can be increased further, in both cases, by anaerobic digestion (Smith, 1996). Hence, assuming a median phosphorus content of 3 %, an estimated 37 000 tonnes of phosphorus was recycled to agricultural land in the UK in 2010 (**Figure 1.3**). However, Lima et al. (1996) estimate as much as 70 % of the total phosphorus in sewage can be present in organic forms. Although more recent

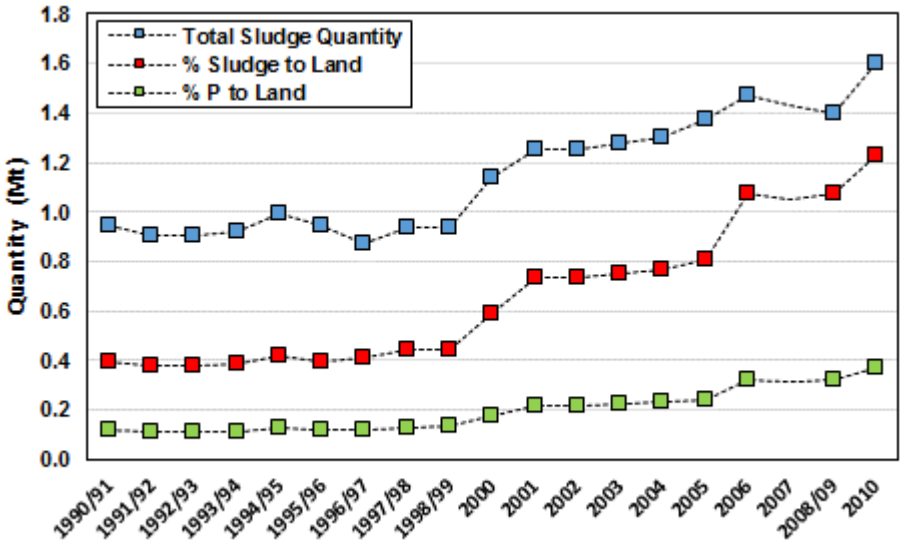


Figure 1.3:- Estimated increases in the total annual quantity of sewage sludge (Mt dry matter) produced in the UK over the past two decades, the percentage applied to agricultural land, and the percentage of phosphorus, assuming a median content of 3 %. Data obtained from Defra (2007b), Gendebien (1999), Smith (1996), and Water UK (2010).

measurements by Peng et al. (2010), using ^{31}P-nuclear magnetic resonance spectroscopy (^{31}P-NMR), suggest the organic phosphorus content of sewage sludge may be lower, ranging from 13-51 % of the total phosphorus content. This is in agreement with the results of Xie et al. (2011) who report the organic phosphorus contents of aerobic and anaerobically digested sludge ranging from 20-44 % and 14-52 % of total phosphorus, respectively. Similarly, Hanotiaux et al. (1981) observed an organic phosphorus content of 15-35 % in sludge produced by four wastewater treatment plants in Belgium. Whereas, Chae and Tabatabai (1981) found that organic phosphorus constituted 41-67 % of total phosphorus in sludge produced in the US. Hence a considerable fraction of the total phosphorus applied in sewage sludge is likely to be present in organic forms. Information on the forms of organic phosphorus in sewage sludge is relatively scarce, though it is assumed that phosphomonoesters, such as inositol phosphates (Cosgrove, 1973; Peng et al., 2010; Annaheim et al., 2015), and phosphodiesters, such as phospholipids and nucleic acids, are the predominant forms (Chae & Tabatabai, 1981; Criquet et al., 2007; Hinedi et al., 1989). However, Hinedi et al., (1989) have observed that the distribution of phosphorus between organic and inorganic forms is influenced by digestion processes, with higher concentrations of phosphomonoesters and phosphodiesters present in aerobically digested sludge in comparison to sludge undergoing anaerobic digestion; for which inorganic phosphorus was predominant. The long-term fate of organic phosphorus in sludge amended soils is also not yet fully understood, and can be influenced by a range of environmental factors (Harrison, 1987; Magid et al., 1996); hence investigations often produce conflicting results. For instance, Otabbong et al. (1997) determined the forms of phosphorus in soils receiving annual applications of sewage sludge at the 'Long-Term Soil Organic Matter Experiment' in Ultuna, Sweden. Over a period of 35 years, an

estimated 6.62 t P ha^{-1} was applied, causing a significant (p <0.05) accumulation of both organic and inorganic phosphorus in soils receiving the sludge treatment, in comparison to untreated soil. Whereas, at the 'Organic Fertilizer Experiment' in Zürich, Switzerland, Annaheim et al. (2015) only observed a significant (p <0.05) increase of orthophosphate in soils receiving annual applications of sewage sludge over a period of 62 years (6.88 t P ha^{-1}). Furthermore, the forms of organic phosphorus characterised by ^{31}P-NMR remained unchanged in the sludge amended soils over the course of the experiment, and were no different to those observed in untreated soil.

1.3. Protecting Soil Quality

Sewage sludge is typically produced in two stages. In the first instance, particulate organic matter present in the wastewater stream is allowed to settle producing raw primary sludge. Wastewater then continues on to a secondary biological treatment process, where microorganisms are used to remove dissolved organic matter from wastewater forming a secondary 'activated' sludge (Smith, 1996; Water UK, 2010). The degradation of organic matter present in sewage sludge is then subsequently stabilised by aerobic or anaerobic digestion, before the sludge is dewatered and dried for storage (Water UK, 2010). However, due to the sources of wastewater, both domestic and industrial, as well as urban run-off, and the nature of wastewater treatment processes themselves, sewage sludge frequently contains concentrations of potentially toxic, and extremely persistent, heavy metals that are significantly greater than the background concentrations found in soils (Berrow & Webber, 1972; Smith, 1996; Thornton, 2001). This is problematic and, understandably, there is concern that increasing the quantities of sewage sludge used in agriculture could lead to dangerous levels of heavy metals accumulating in the environment and a reduction in the quality of agricultural soils (Giller et al., 1998, 2009).

Soil quality has been defined as '*the continued capacity of soil to function as a vital* ***living system****, within ecosystem and land-use boundaries, to sustain biological productivity, promote the quality of air and water environments, and maintain plant, animal, and human health*'; and its preservation has become an environmental priority for many countries worldwide (Doran & Safley, 1997). Therefore, in order to prevent the accumulation of heavy metals in the environment, the EU Sludge Directive 86/278/EEC (CEC, 1986) sets statutory maximum limits, in both sludge and sludge amended soils, for the concentrations of six heavy metals (cadmium (Cd), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni) and zinc (Zn)) considered to pose the greatest risk to soil quality and human health (**Table 1.1**). It has now been 25 years since the EU Sludge Directive was enacted throughout the United Kingdom as the UK Sludge (Use In Agriculture) Regulations (SI, 1989), and within this period additional legislation has been implemented in order to encourage the recycling of sewage sludge where possible. The disposal of sewage sludge at sea was prohibited in 1998 under the EU Urban Wastewater Treatment Directive 91/676/EEC (CEC, 1991a), with further restrictions imposed the following year

Table 1.1:- Maximum concentrations (mg kg^{-1}) for heavy metals in sludge and sludge amended soils permitted by the EU Sludge Directive (CEC, 1986).

	Total Metal Concentration (mg kg^{-1})		
	Soil[1]	Sludge	Annual Mean (kg ha^{-1} yr^{-1})
Cadmium (Cd)	1-3	20-40	0.15
Copper (Cu)	50-140	1000-1750	12
Lead (Pb)	50-300	750-1200	15
Mercury (Hg)	1-1.5	16-25	0.1
Nickel (Ni)	30-75	300-400	3
Zinc (Zn)	150-300	2500-4000	30

[1]Concentration in soils with pH 6-7.

Table 1.2:- Maximum concentrations of heavy metals in sludge amended soils, and average annual rate of application over a 10 year period, permitted by the UK Sludge (Use In Agriculture) Regulations (SI, 1989).

	Total Metal Concentration (mg kg^{-1})				Annual Mean over 10 Years
	pH 5.0<5.5	pH 5.5<6.0	pH 6.0-7.0	pH >7.0	(kg ha^{-1} yr^{-1})
Copper (Cu)	80	100	135	200	7.5
Nickel (Ni)	50	60	75	110	3
Zinc (Zn)	200	250	300	450	15
	For pH 5.0 and Above				
Cadmium (Cd)	3				0.15
Lead (Pb)	300				15
Mercury (Hg)	1				0.1
Chromium (Cr)[1]	400				15
Molybdenum (Mo)[1]	4				0.2
Selenium (Se)[1]	3				0.15
Arsenic (As)[1]	50				0.7
Fluoride (Fl)[1]	500				20

[1]Values are precautionary limits taken from UK 'Code of Practice' (DoE, 1996).

by the EU Landfill Directive 1999/31/EC, to reduce the quantities of sewage sludge sent to landfill (CEC, 1999). In the same year (1999) the EU Nitrate Directive 91/676/EEC (CEC, 1991b) set maximum limits for the application of nitrogen fertilisers (including sewage sludge) to agricultural soils: 210 kg N ha^{-1} yr^{-1} in 1999, reducing to 170 kg N ha^{-1} yr^{-1} in 2003. These directives now form the basis of a regulatory framework in place throughout the EU, however member states are permitted to set their own statutory limits for heavy metals in sludge and sludge amended soils, provided they do not exceed those specified in the EU Sludge Directive (CEC, 1986).

The majority of EU member states permit a certain degree of metal accumulation in sludge amended soils and have generally adopted intermediate values within the ranges specified in **Table 1.1** (McGrath et al., 1994); although some do take a more cautious approach (Witter, 1996). The statutory limits for sludge amended soils specified by the UK Sludge (Use In Agriculture) Regulations (**Table 1.2**) are based on scientific evidence and were set below the lowest concentrations of heavy metals known to impact on soil quality; thus providing some margin of safety (MAFF/DoE, 1993a, 1993b; McGrath et al., 1994). The UK Sludge (Use in Agriculture) Regulations are further supported by an industry 'Code of Practice', drafted by the UK Department of Environment (DoE, 1996), which provides precautionary limits for five additional heavy metals (**Table 1.2**). In addition, a voluntary agreement between ADAS (Agricultural Development and Advisory Service), Water UK (representing water and sludge operators

Table 1.3:- Number annual of applications of sewage sludge required to raise the total concentration of heavy metals (mg kg^{-1}) in sludge amended soils (pH = 6-7) to the statutory limits specified in the UK Sludge (Use In Agriculture) Regulations (MAFF/DoE, 1993b). Estimates are determined using permitted application rates for heavy metals and nitrogen (See Text).

	Background (mg kg^{-1})[1]	Application Rate (kg ha^{-1} yr^{-1})			No. of Applications		
		Metal	250 kg N ha^{-1}	170 kg N ha^{-1}	Metal	250 kg N ha^{-1}	170 kg N ha^{-1}
Cadmium (Cd)	0.8	0.15	0.04	0.03	48 (29)[2]	179 (110)	238 (147)
Copper (Cu)	20	7.5	4.76	3.27	50 (31)	78 (48)	115 (71)
Lead (Pb)	50	15	1.67	1.15	54 (33)	491 (302)	712 (438)
Mercury (Hg)	0.1	0.1	0.03	0.02	29 (18)	98 (60)	146 (90)
Nickel (Ni)	20	3	0.54	0.37	60 (37)	332 (204)	483 (297)
Zinc (Zn)	80	15	7.65	5.26	48 (29)	93 (57)	136 (83)

[1]Values are geometric means taken from the UK National Soil Inventory (MAFF/DoE, 1993a). [2]Values in parenthesis are calculated by Smith (1996).

Table 1.4:- Number of years required to remove 100 % of applied metal loading based on percentage uptake of total metal concentration (mg kg^{-1}) by plants over a 20 year period at the Woburn Market Garden Experiment (McGrath, 1987).

	Cadmium (Cd)	Copper (Cu)	Lead (Pb)	Nickel (Ni)	Zinc (Zn)
Metal Loading (mg kg^{-1})	19.4	239.4	209.1	42.3	635.4
Uptake (% 20 $years^{-1}$)	0.28	0.16	0.06	0.37	0.57
Time (years)	7500	13100	35000	5700	3700

in the UK), and the British Retail Consortium known as the 'Safe Sludge Matrix' (ADAS, 2001), provides guidelines for the safe use of sewage sludge in agriculture. For instance, a time interval of 10 weeks is recommended between the application of sewage sludge and the harvesting of crops, whilst an interval of 3 weeks is recommended before allowing animals to graze on sludge amended soil. Additionally, in order to reduce pathogen risks, the use of raw untreated sludge is now strongly discouraged, as is the application of sewage sludge to soil with pH less than 5; pH being an important environmental factor controlling the bioavailability, and hence the toxicity, of heavy metals in soils (Alloway, 1995; Alloway & Jackson, 1991). Most recently, Water UK, in collaboration with ADAS, have begun compiling the material covering both legislative requirements and non-legislative best practices into a single 'Bio-Solids Assurance Scheme', aimed at improving and promoting the safe recycling of sewage sludge to agricultural land (Water UK, 2013).

Using the UK regulatory framework and agricultural best practices as a guide, the number of sludge applications required to increase the total concentration of heavy metals in sludge amended soils to the proposed statutory limits was determined, as follows, by the UK Ministry of Agriculture, Fisheries and Food and the Department of Environment (MAFF/DoE; now combined as the Department for Environment, Food and Rural Affairs (Defra)):

$$No.\ of\ Applications = \frac{([M^+]_{Limit} - [M^+]_{Background})}{Application\ Rate} \times \left(\frac{d \times \rho}{0.1}\right) \qquad (\mathbf{E.1.1})$$

where $[M^+]_{Limit}$ and $[M^+]_{Background}$ are the statutory (**Table 1.2**) and background (**Table 1.3**) total metal concentrations (mg kg^{-1}) for sludge amended soils in the UK, d is the soil cultivation depth (0.25 m),

ρ is the soil bulk density (1.3 kg m^{-3}), and 0.1 is a conversion factor (1000 mg kg^{-1}/10 000 m^2 ha^{-1} = 0.1) to give metal loadings in kg ha^{-1} (MAFF/DoE, 1993b). Similar calculations were also carried out by Smith (1996) using values of 0.2 m and 1.0 kg m^{-3} for *d* and ρ, respectively (**Table 1.3**); these values are recommended by Water UK and give more precautionary estimates (Smith, 1996). Based on the current maximum application rates specified by the UK Sludge (Use in Agriculture) Regulations, the statutory limits for each heavy metal would be reached within 60 years, or 40 years if the more precautionary values for *d* and ρ are applied (**Table 1.3**); however, it should be noted that sludge applications would cease once the first limit is reached (i.e. approximately 20-30 years for Hg). In contrast, McGrath (1987) calculated the number of years required to remove 100 % of the total metal concentration added to sludge amended soils during the Woburn Market Garden Experiment (**See Section 1.4**), based on the uptake of heavy metals by plants over a 20 year period (**Table 1.4**); the values determined here range from thousands to tens of thousands of years, thus demonstrating the extreme persistence of heavy metals in agricultural soils. However, estimating the longevity of sludge application using application rates based on sludge metal content gives a 'worst-case' scenario, and in practice sewage sludge is generally applied every 2-3 years, depending on crop nitrogen requirements (Smith, 1996). Therefore more reasonable estimates of longevity have been calculated using application rates based on sludge nitrogen content (**Table 1.3**). In comparison to other heavy metals, Zn and Cu were identified as the principal elements limiting the application of sludge to agricultural land (MAFF/DoE, 1993b; Smith, 1996), reaching the current statutory limits after 78 and 93 years, respectively, when sludge is applied at a rate of 250 kg N ha^{-1} yr^{-1} (previously the UK advisory rate for nitrogen in organic residues), or 115 and 136 years when applied at a rate of 170 kg N ha^{-1} yr^{-1} (the current limit specified by the EU Nitrate Directive); with more precautionary estimates of 48 and 57 years, or 71 and 83 years, when applied at the respective rates (**Table 1.3**).

Following the implementation of the UK Sludge (Use In Agriculture) Regulations, two independent scientific reviews, conducted by the Steering Group on Chemical Aspects of Food Surveillance (MAFF/DoE, 1993a) and an Independent Scientific Committee (MAFF/DoE, 1993b), were commissioned on behalf of the UK Ministry for Agriculture, Fisheries and Food (MAFF) and the Department of Environment (DoE; now combined as the Department of Environment, Food, and Rural Affairs (Defra)), in order to determine possible risks to food safety, assess the potential long-term impacts of repeated sludge application to agricultural land, and confirm that the legislation put in place was sufficient to protect soil quality. Due to their known toxicity in humans, Cd, Cu, and Pb were considered to pose the greatest risk to food safety, however the reported concentration factors for these metals (i.e. the concentration ratio between plant and soil ($[M^+]_{Plant}/[M^+]_{Soil}$)) indicated negligible uptake of Pb (0.001) and a moderate uptake of Cu (0.05-0.5) and Cd (0.05-4); however, it was considered unlikely that the concentration of Cu in plants would exceed the recommended food safety limit of 20 mg kg^{-1} without phytotoxic effects being observed (MAFF/DoE, 1993a). Furthermore, based on the

assumption that 10 % of food consumed is grown on sludge amended soil with a Cd content of 3 mg kg^{-1}, it was shown that the dietary intake of Cd for an average consumer would only increase by 6.9 %; from 18.8 to 20.1 µg Cd day^{-1} (MAFF/DoE, 1993a). Although it was recognised that increases of approximately 63 % (30.6 µg Cd day^{-1}) could potentially occur in 'upper-range' consumers (i.e. those who consume greater quantities of bread, cereal, offal, and potatoes), both increases remained below the World Health Organization 'tolerable daily intake' (for a 60 kg person) of 60 µg Cd day^{-1} (MAFF/DoE, 1993a). Therefore it was generally accepted that metal uptake by plants was unlikely to pose significant food safety problems, hence the limits proposed in the UK Sludge (Use In Agriculture) Regulations were deemed sufficient to protect plants, animals, and humans from metal toxicity; however this could not be said for soil microorganisms (MAFF/DoE, 1993b).

1.4. Impact on Soil Microbiology

The definition of soil as a '***living system***' emphasises the role of microorganisms in the mineralisation of soil organic matter and the cycling of soil nutrients (Doran & Safely, 1997), for which the soil microbial community has been described as *'the eye of the needle through which all organic materials must pass'* (Jenkinson, 1977). Evidence for the potential impact of heavy metals on the soil microbial community was only beginning to emerge when the UK Sludge (Use In Agriculture) Regulations were first drafted, hence soil microorganisms were not considered when setting the statutory limits. However in light of the emerging evidence there was growing concern that a decrease in the diversity and activity of soil microorganisms, due to metal toxicity, could disrupt processes essential for crop production (MAFF/DoE, 1993b).

The alarm was raised in 1983 when Brookes and McGrath (1984) began measuring microbial biomass carbon (C_{mic}) in sludge amended soils at the Woburn Market Garden Experiment (Bedfordshire, UK). Started in 1942, during the Second World War, the Woburn Market Garden Experiment was established in order to investigate how various organic residues could be used to improve soil quality and increase the yield of market garden crops (Johnston & Wedderburn, 1974). Sewage sludge was applied annually to duplicate experimental plots at rates of 8.2 and 16.4 t ha^{-1}, however in 1959 it was discovered that the concentrations of Cu, Cr, Pb, Ni, and particularly Zn, in the sludge amended soils had increased dramatically; with concentrations of Zn (360 and 430 mg kg^{-1} in the plots sampled), extractable by 0.5 M acetic acid, above the statutory limits subsequently set by the UK Sludge (Use In Agriculture) Regulations (Le Riche, 1968). As a precaution applications of sewage sludge were ceased in 1961, prior to the end of the experiment in 1967, following which all soils have since received inorganic fertilisers. Using archived samples of soil and sludge, McGrath (1984) calculated metal loadings for the period 1942-1960, as well as estimating the overall heavy metal 'enrichment' (i.e. the concentration ratio between sludge amended and untreated soil ($[M^+]_{Experimental}/[M^+]_{Control}$)) in the sludge amended soils.

Annual loadings for Cd (0.98 kg ha^{-1} yr^{-1}), Cu (12.5 kg ha^{-1} yr^{-1}) and Zn (31.6 kg ha^{-1} yr^{-1}) all exceeded the maximum average application rate over a 10 year period (**Table 1.2**) now specified by the UK Sludge (Use In Agriculture) Regulations, hence these metals were respectively enriched by factors of 44 to 97, 5.5 to 10.5, and 4.6 to 8.5, in soils receiving sludge at the lower and higher application rates. By plotting C_{mic} against the concentration of heavy metals extractable by EDTA, Brookes and McGrath (1984) observed that C_{mic} had decreased, by up to 50 % in some cases, in the sludge amended soils in comparison to 'low-metal' soils receiving farmyard manure or inorganic fertilisers. However, since all metals were present simultaneously it was not possible to determine which metal was responsible for the decrease.

The following year a striking decline in health was observed in white clover (*Trifolium repens*) plants growing on the sludge amended soils, in comparison to those growing on the 'low-metal' soils. Investigations carried out by McGrath et al. (1988) and Giller et al. (1989) later confirmed that the decline in clover health was due to ineffective N-fixation, caused by the toxic effect of heavy metals on *Rhizobium leguminosarum* biovar *trifoli* in the root system of clover plants, and not a phytotoxic effect on the clover itself, as plant health was restored by the addition of inorganic N fertiliser. In an attempt to elucidate which metals were having an effect on *Rhizobium*, Chaudri et al. (1992) added metal salt solutions (Cd, Cu, Ni, and Zn), at six different rates, to soils amended with farmyard manure. After 18 months, no *Rhizobium* cells were found in soils treated with the Zn ($\geq$ 385 mg kg^{-1}) or Cd ($\geq$ 7.1 mg kg^{-1}) salt solutions, with reductions of 82 and 99 % also observed in soils containing Cu concentrations of 191 and 225 mg kg^{-1}, respectively; whereas Ni appeared to have no effect on *Rhizobium* over the concentration range of 26-54 mg kg^{-1}.

In order to verify these results, Chaudri et al. (1993) carried out an additional investigation in 1991, at the long-term sludge experiment in Brunswick (Lower Saxony, Germany). Established in 1980, applications of inorganic fertiliser, 'low metal', and 'high metal', sludge treatments were applied annually to an ex-woodland soil (pH = 5.1-6) and an arable soil (pH = 6-7), at rates of 100 m^3 ha^{-1} yr^{-1} and 300 m^3 ha^{-1} yr^{-1}, for a period of 10 years. Sludge treatments were prepared by combining Cd, Cu, Pb, Ni, and Zn metal salts with anaerobically digested sludge and storing under anaerobic conditions for six weeks to allow the metals to incorporate into the sludge (Fließbach et al., 1994). By 1991, the total concentrations of Zn in soils receiving 300 m^3 ha^{-1} yr^{-1} of the 'high metal' sludge treatment, at both sites (ex-woodland soil = 265-427 mg kg^{-1}; arable soil = 383-441 mg kg^{-1}), were above the UK statutory limits for the respective soil pH values. Multiple regression analysis was used to determine the total concentrations of Cd, Cu, and Ni, corresponding to a given total concentration of Zn, in each of the sludge amended soils, before plotting the number of *Rhizobium* cells against the total concentration of Zn (Chaudri et al., 1993). The number of cells (cells g $soil^{-1}$) found in soils receiving inorganic fertilisers ranged from 4.2-9.3$\times10^3$ cells g $soil^{-1}$ and 9.3-40$\times10^3$ cells g $soil^{-1}$ in the arable and ex-woodland sites, respectively (Chaudri et al., 1993); with similar ranges seen in soils receiving the 'low-metal' sludge

treatment at 100 m^3 ha^{-1} yr^{-1}. However, cell numbers decreased dramatically at both sites as the quantity of applied sludge increased to 300 m^3 ha^{-1} yr^{-1}. The number of *Rhizobium* cells decreased steadily in the arable soil receiving the 'low metal' sludge treatment, eventually reaching zero cells as the total concentration of Zn increased from 218 to 254 mg kg^{-1}. This decline occurred at concentrations below the UK statutory limit for Zn, however it was noted that the corresponding limit set by Germany (200 mg Zn kg^{-1} for soils with pH > 6) would have prevented such an occurrence. Zero cells were observed in three of the four plots receiving the 'high metal' sludge treatment where total Zn concentration ranged from 441 to 495 mg kg^{-1}. This was also observed in the more acidic ex-woodland soil where again significant reductions in the number of *Rhizobium* cells were observed in soils receiving the 'low metal' and 'high metal' sludge treatments at 300 m^3 ha^{-1} yr^{-1} over a concentration range of 185-427 mg Zn kg^{-1}. These results were taken into account by the Independent Scientific Committee when reviewing the UK Sludge (Use In Agriculture) Regulations, prompting the recommendation that, as a precaution, advisory limits of 200 and 300 mg Zn kg^{-1} should be applied respectively to sludge amended soils with pH 5-7, and pH >7 (MAFF/DoE, 1993b).

Expanding their initial investigation, Brookes and McGrath (1984) also sampled sludge amended soils from the Luddington Experimental Horticulture Station (Warwickshire, UK), where contaminated sludge was either applied annually at a rate of 31 t ha^{-1} yr^{-1} over a four year period (1968-72), or as a single amendment (125 t ha^{-1}) in 1968, in order to investigate the accumulation and long-term 'availability' of heavy metals in sludge amended soils. Eight sludge treatments were prepared in total: four 'high-metal' treatments (8000 mg Cu kg^{-1}; 8800 mg Cr kg^{-1}; 4000 mg Ni kg^{-1}; and 16000 mg Zn kg^{-1}) and four 'low-metal' treatments (4000 mg Cu kg^{-1}; 4400 mg Cr kg^{-1}; 2000 mg Ni kg^{-1}; and 8000 mg Zn kg^{-1}). Uncontaminated sludge treatments, applied at both rates, were also included as experimental controls along with two plots of untreated soil (Berrow & Burridge, 1980). The quantities of C_{mic} in soils receiving the Zn (265 µg C_{mic} g $soil^{-1}$) and Cr (287 µg C_{mic} g $soil^{-1}$) sludge treatments were of a similar magnitude to that measured in untreated (249 µg C_{mic} g $soil^{-1}$) soil and those receiving the uncontaminated (269 µg C_{mic} g $soil^{-1}$) sludge treatments. Whereas a reduction in C_{mic} was observed in soils receiving the Cu (156 µg C_{mic} g $soil^{-1}$) and Ni (127 µg C_{mic} g $soil^{-1}$) sludge treatments, now over 10 years since the last sludge applications were made (Brookes & McGrath, 1984); however the rate at which the sampled soils had received the sludge treatments was not specified. In addition, the total concentration of Zn had also increased in soils receiving the Cu and Ni sludge treatments, therefore the actual cause of the decrease was not entirely clear.

Chander and Brookes (1991) continued this investigation in 1989, looking at C_{mic} in sludge amended soils at both the Luddington and Lee Valley (Hertfordshire, UK) Experimental stations; with the same experimental design at both sites (see above). Samples were collected from untreated soil, plus soils receiving uncontaminated sludge, 'high metal' Zn, Cu, and Ni sludge treatments, and the 'low metal' Cu sludge treatment, each as a single application of sludge (125 t ha^{-1}) in 1968; samples from the 'low

metal' Zn sludge treatment were also collected at Lee Valley (Chander & Brookes, 1991). In all cases soil pH was between 5.5 and 6 with the total concentrations of Zn, Cu, and Ni in soils receiving the respective sludge treatments (both 'high metal' and 'low metal treatments) all above the UK statutory limits for this pH range (**Table 1.2**). Concentrations of Cd were also above the UK statutory limit in each of the sludge amended soils (both experimental and control) at Lee Valley (Chander & Brookes, 1991). At both sites, C_{mic} in soils receiving the Ni sludge treatment were comparable to that in soils receiving uncontaminated sludge, despite the total concentrations of Ni being twice the UK statutory limit; this was also the case in soil receiving the 'low Zn' sludge treatment at Lee Valley. In soils receiving the 'high Zn' sludge treatment, C_{mic} had decreased by approximately 9 % and 28 %, at Luddington and Lee Valley, respectively; possibly due to a higher total concentration of Zn at Lee Valley (857 mg kg^{-1}). In contrast, C_{mic} in soils receiving the 'low Cu' sludge treatment appeared to be unaffected at Lee Valley, again despite the total concentration of Cu (212 mg kg^{-1}) being more than twice the UK statutory limit; however a decrease of 18 % was observed at the Luddington site. This apparent discrepancy was thought to be due to differences in soil texture between sites. Soils at the Lee Valley site had both a higher clay and organic matter content, in comparison to Luddington, therefore Chander and Brookes (1991) have suggested that the bioavailability of Cu may be reduced in these soils. However, at both sites, C_{mic} had decreased by approximately 40 % in soils receiving the 'high Cu' sludge treatment. Overall it was concluded that Zn and Cu posed the greatest risk to C_{mic}, with no apparent effects caused by Ni or by the presence of Cd at either site (Chander & Brookes, 1991).

Chander and Brookes (1993) also investigated the impact of heavy metals on C_{mic} in sludge amended soils at the Gleadthorpe Experimental Husbandry Farm (Nottinghamshire, UK), where single applications of contaminated sewage sludge were made in 1982. Five sludge treatments were prepared using metal salts and applied at four different rates in order to produce dose response curves for each treatment, these were: Cu (1200-3000 kg ha^{-1}), Ni (50-200 kg ha^{-1}), Zn (600-2800 kg ha^{-1}), Zn + Cu (300:250-2300:1600 kg ha^{-1}), and Zn + Ni (150:25-600:100 kg ha^{-1}). Additional applications of sludge cake, contaminated with either Zn or Cu, were made to soils receiving the respective sludge treatments (Zn, Cu, and Zn + Cu) in 1986. Untreated soil, and two uncontaminated sludge treatments ('low Zn and Cu', and 'low Ni') were also included in the experimental design as controls (Chander & Brookes, 1993); soil pH was maintained at 6.5 by adding lime ($CaCO_3$). In comparison to untreated soil, C_{mic} had increased by approximately 8 % in soils receiving both uncontaminated sludge treatments, plus the Ni and Zn + Ni sludge treatments, though the total concentrations of Zn (89-200 mg kg^{-1}) and Ni (20-53 mg kg^{-1}) in these soils were below the UK statutory limits (**Table 1.2**). In soils receiving the Zn and Cu sludge treatments, C_{mic} decreased by approximately 6-40 % and 18-55 %, respectively, in comparison to soil receiving the uncontaminated control, as the total concentration of these metals (Zn = 375-705 mg kg^{-1}; Cu = 197-690 mg kg^{-1}) increased above the UK statutory limits. Most striking, however, was that greater decreases in C_{mic} were observed when Zn and Cu were applied in combination. For example,

Table 1.5:- Minimum total metal concentrations (mg kg^{-1}) at which decreases in soil microbial biomass have been observed in sludge amended soils. Data obtained from McGrath et al. (1995).

			Total Metal Concentration (mg kg^{-1})					
Field Site	**Location**	**pH**	**Cd**	**Cu**	**Cr**	**Pb**	**Ni**	**Zn**
Woburn	UK	6.5	6.0	**70**	105	**100**	**22**	**180**
Luddington	UK	6.5	ND[1]	150	ND	ND	ND	**281**
Lee Valley	UK	5.7	ND	384	ND	ND	ND	857
Ultuna	Sweden	5.3	**0.7**[2]	125	85	**40**	**35**	**230**
Brunswick 1	Germany	6-7	**2.8**	**102**	95	**101**	**23**	360
Brunswick 2	Germany	5.5	**2.9**	111	105	**114**	**24**	386

[1]Not Determined. [2]Values in bold are below UK Statutory limits for respective soil pH.

decreases of 6 % and 18 % were observed in soils where the total concentrations of Zn and Cu were 375 and 197 mg kg^{-1}, respectively, whereas, a decrease of 23 % was observed when Zn and Cu were both present at respective concentrations of 322 and 176 mg kg^{-1}.This effect was even more pronounced when Zn and Cu were present at concentrations of 427 and 262 mg kg^{-1}, respectively, just over half the maximum concentrations used for the metals individually. In this case a 57 % decrease in C_{mic} was observed. It was therefore suggested that the toxic effects of Zn and Cu might be additive, and more harmful to microorganisms when present simultaneously (Chander & Brookes, 1993).

A follow up investigation into the status of soil microorganisms in sludge amended soils at the Woburn Market Garden Experiment was carried out by Abaye et al. (2005) in 1998; almost 40 years since the final sludge applications were made. Although, in general, total metal concentrations in the sludge amended soils remained more than double that observed in the 'low-metal' soils, it was possible to take samples from two plots where only the concentration of Cd (6.3 and 6.0 mg kg^{-1}) exceeded the UK statutory limits; thus allowing a more accurate discussion of the protection offered by the UK Sludge (Use In Agriculture) Regulations. In agreement with Brookes and McGrath (1984), C_{mic} remained approximately 20 % lower in the sludge amended soils in comparison to the 'low-metal' soils, while analysis of phospholipid fatty acids (**See Section 5.4**) suggested that the microbial communities in the sludge amended soils were now dominated by Gram-negative bacteria, while those in 'low-metal' soils were dominated by Gram-positive species. Although the decreases were not as severe as those observed by Brookes and McGrath (1984), Abaye et al. (2005) expressed concern that an adverse effect on soil microorganisms could still be observed, decades after sludge applications have ceased, and therefore asserted that, at the very least, the current UK statutory limits should not be increased.

McGrath et al. (1995) reviewed data from a number of investigations into the long-term impacts of heavy metals on soil microorganisms and report the minimum concentrations of Cd, Cu, Cr, Pb, Ni, and Zn at which an adverse effect on soil microbial biomass was observed (**Table 1.5**). A number of these fell below the statutory limits set by both the UK Sludge (Use In Agriculture) Regulations (**Table 1.2**) and the EU Sludge Directive (**Table 1.1**), though because all of these metals were present in the soil simultaneously, again it could not be said which metal, or combination thereof, was responsible for the observed decreases in C_{mic}; therefore McGrath et al. (1995) exercised caution when making these

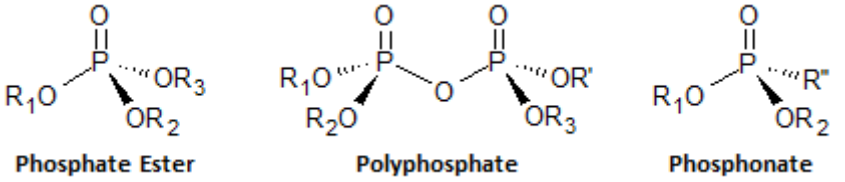


Figure 1.4:- Forms of organic phosphorus compounds found in soils: R_1, R_2, and R_3, can be either hydrogen or an organic compound; R' can be an organic compound or another phosphate group; R'' is an organic compound bound by a P-C bond.

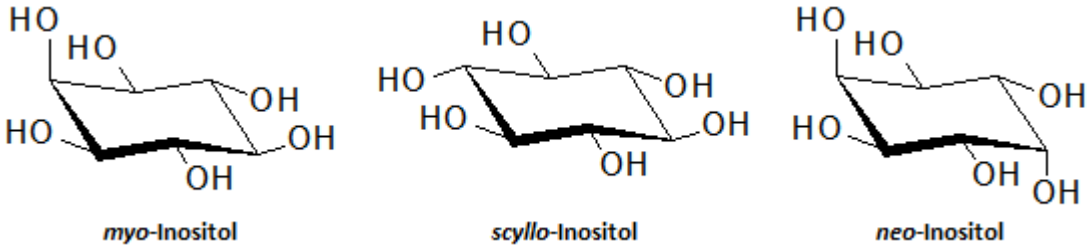


Figure 1.5:- Stereoisomers of inositol. Each OH group can be substituted by phosphate to give mono-, bis-, tris-, tetrakis-, pentakis-, and hexakis- phosphates. The most common form found in soil is *myo*-inositol hexakisphosphate (Turner et al. 2002).

comparisons. However, as mentioned previously, the current statutory limits enforced in the UK are considered sufficient to prevent exposure to heavy metals via the food chain (MAFF/DoE, 1993a), therefore two questions have been posed when considering the potential impact of sewage sludge applications on soil microorganisms; these are, 'do microbes matter?' (Giller et al., 1999) and, if so, 'where's the limit?' (Dahlin et al., 1997).

1.5. Mineralisation of Organic Phosphorus

Phosphorus exists in a variety of organic forms within the soil environment which, in general, account for approximately 30 % of the total phosphorus content; although in some cases this can be as high as 80 % (Dalal, 1977). The predominant forms of organic phosphorus in soils are phosphate esters (phosphomonoesters and phosphodiesters), polyphosphates, and phosphonates (**Figure 1.4**). Inositol phosphates are the most common form of phosphomonoesters, accounting for up to 80 % of soil organic phosphorus (Dalal, 1977), however only the *myo*-, *scyllo*-, and *neo*-inositol stereoisomers (**Figure 1.5**) are regularly found in soil (Cosgrove & Irving, 1980; Turner et al., 2002). Phosphodiesters, such as phospholipids and nucleic acids derived from plant, animal, and microbial cells, make up a significant fraction of the organic phosphorus returned to soils, however in general they only account for approximately 0.5-7 % and <3 % of soil organic phosphorus, respectively (Dalal, 1977; Stevenson & Cole, 1999). The stability of organic phosphorus compounds in soil is largely determined by the organic carbon moieties, which can adsorb onto the surfaces of soil particles or become incorporated into soil humus (Magid et al., 1996). However, in compounds with low molecular weight, particularly phosphomonoesters, the phosphate group can interact with the soil environment in a similar manner to

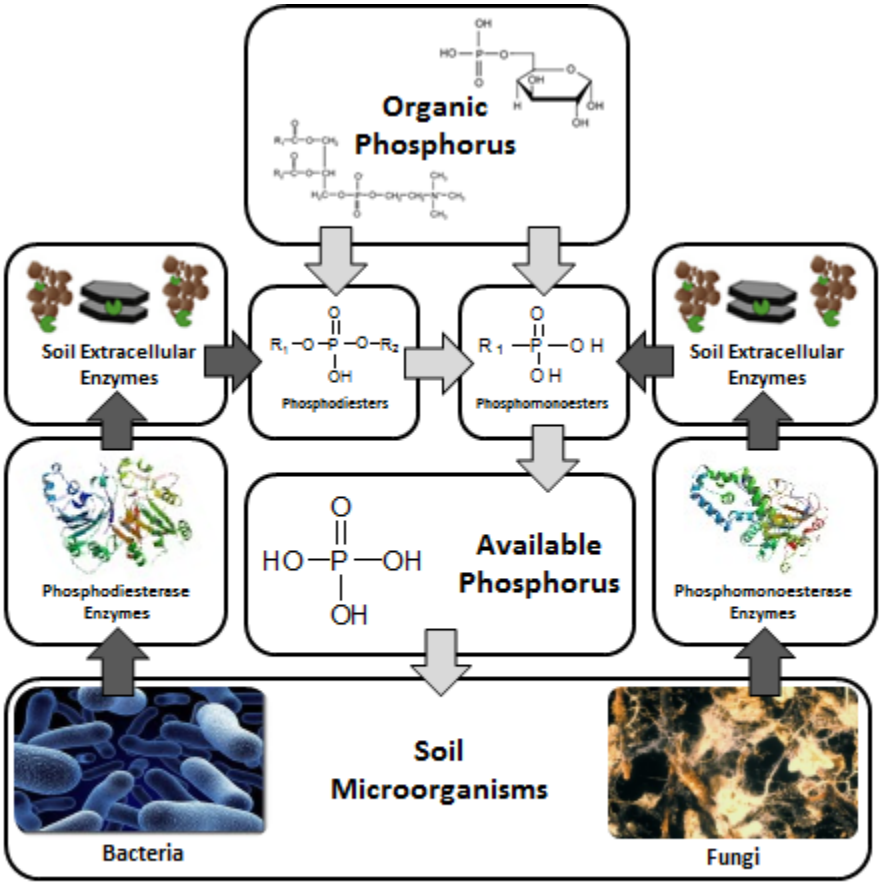


Figure 1.6:- The mineralisation of organic phosphorus in soils. Extracellular enzymes, stabilised in the soil environment by organic matter and clay particles, act on fresh sources of organic phosphorus, releasing bioavailable orthophosphate (H_3PO_4). Soil microorganisms then respond to the increase in available nutrients by releasing additional enzymes into the soil environment (Burns, 1982). A range of enzymes is often required to mineralise organic phosphorus compounds. For instance, phosphodiesters, such as nucleic acids and phospholipids make up a significant fraction of soil organic phosphorus, however two enzymes (phosphodiesterase and phosphomonoesterase) are required to hydrolyse the ester bonds and release orthophosphate (Turner & Haygarth, 2005).

orthophosphate (**See Section 4.2**). The adsorption affinity of phosphomonoesters has been shown to increase with the number of phosphate groups present, hence compounds such as inositol hexakisphosphate can be adsorbed more strongly within the soil environment in comparison to orthophosphate (Magid et al., 1996). In contrast, the adsorption of phosphodiesters occurs primarily through the organic carbon moieties. This regularly leaves the phosphate diester bond exposed to the hydrolytic activity of phosphatase enzymes (Magid et al., 1996). Hence phosphodiesters are generally less stable within the soil environment and readily undergo mineralisation.

The mineralisation of organic matter in soil, and therefore the chemical cycling of essential plant nutrients such as carbon, nitrogen, and phosphorus, is largely facilitated by a diverse range of enzymes, released into the soil environment by plants and microorganisms (Ladd, 1978). Extracellular enzymes can become stabilised in the soil environment by adsorption, or binding, to the surfaces of clay particles and soil organic matter where they remain active for extended periods of time (Burns et al. 2013; Skujins, 1976; Theng, 2012). Burns (1982) describes the relationship between soil microorganisms and

extracellular enzymes in the soil environment as a feedback loop, whereby the activity of extracellular enzymes initiates the mineralisation of organic matter substrates which may be inaccessible to soil microorganisms due to size and/or location (**Figure 1.6**). The nutrients released are subsequently absorbed by soil microorganisms, which then respond by taking an active role in mineralisation and release additional enzymes into the soil environment (**Figure 1.6**). As a result of this process Burns (1982), and Burns et al. (2013), identify a number of possible locations for enzymes within the soil environment, these are predominantly: intracellular enzymes (present within the cytoplasm and membrane of living, dormant, or dead cells), enzymes associated with cell detritus, unbound extracellular enzymes present in the soil solution, and extracellular enzymes bound to soil organic matter and clay particles. However, isolating and determining the enzyme activity in each location, and hence the relative contribution to the overall soil enzyme activity, remains a significant problem for soil enzymology.

Organic phosphorus compounds in particular are mineralised by a range of hydrolytic enzymes known collectively as phosphatases (Quiquampoix & Mousain, 2005). Five main groups of phosphatase enzymes have been identified based on the type of organic phosphorus compounds they utilise as a substrate, these are: phosphomonoesterases, phosphodiesterases, phosphotriesterases, enzymes acting on phosphoryl-containing anhydrides (e.g. polyphosphates (**Figure 1.4**)), and enzymes that hydrolyse P-N bonds (Tabatabai, 1994). The activity of phosphomonoesterases in soil has been studied extensively over the past 50 years, whereas the other groups have received relatively little attention (Tabatabai, 1994). In general, phosphomonoesterases are grouped according to the pH at which maximum enzyme activity is observed; hence acid phosphomonoesterases are predominant in acidic soils, whereas alkaline phosphatases are predominant in alkaline soils (Eivazi and Tabatabai, 1977). For instance, Eivazi and Tabatabai (1977) reported pH optima of 6.5 and 11 when investigating the effect of buffer pH on the activity of phosphomonoesterase in a range of acidic and alkaline soils, respectively, from the US. Subsequently, Juma and Tabatabai (1978) observed a negative correlation ($R = -0.80$; $p < 0.01$) between acid phosphomonoesterase activity and soil pH (pH 4.5-8), whereas a positive correlation was seen for alkaline phosphatase ($R = 0.9$; $p < 0.01$); in addition, both enzymes were found to be positively correlated to soil organic matter ($R = 0.80$ and 0.66, respectively; $p < 0.01$). However it could not be determined if soil pH caused direct inhibition due to a change in the ionisation state of the enzyme, of if pH influenced the rate of enzyme synthesis by soil microorganisms (Juma & Tabatabai, 1978). Similar results were reported the same year by Browman and Tabatabai (1978) investigating the activity of phosphodiesterase in the same set of US soils. A positive correlation ($R = 0.80$; $p < 0.01$) between phosphodiesterase activity and soil organic matter was also observed, however, although maximum enzyme activity was observed using buffer solution at pH 8, the influence of soil pH on phosphodiesterase activity was not investigated.

In order for plants and soil microorganisms to utilise phosphodiesters present in the soil as a source of nutrients, both phosphodiesterase and phosphomonoesterase enzymes are required to mineralise the organic phosphorus compound (**Figure 1.6**). Therefore, Turner and Haygarth (2005) compared the relative activities of acid phosphomonoesterase and phosphodiesterase in a range of acidic soils (pH 4.4-6.8) and again found that the activity of acid phosphomonoesterase was negatively correlated ($R = -0.60$; $p < 0.01$) to soil pH, in agreement with Juma and Tabatabai (1978), whereas phosphodiesterase activity was positively correlated ($R = 0.69$; $p < 0.01$). Higher concentrations of organic phosphorus were seen in the more acidic soils where the activity of phosphodiesterase was significantly lower than that of acid phosphomonoesterase, therefore Turner and Haygarth (2005) suggested that at lower soil pH phosphodiester hydrolysis becomes a rate limiting step in the mineralisation of organic phosphorus.

The maximum rate of enzyme activity in soils, and hence the rate of organic phosphorus mineralisation, is largely determined using the Michaelis-Menten model of enzyme kinetics (Michaelis & Menten, 1913). This model provides a basis from which the rate and mechanism of organic phosphorus mineralisation can be determined. However due to the heterogeneous nature of the soil environment (McLaren & Packer, 1970), adsorption of reaction products (Irving & Cosgrove, 1976), the presence of isozymes, i.e. different enzyme species catalysing the same reaction (Nannipieri et al., 1982), and potential enzyme inhibitors in the soil, several modifications often need to be applied in order to account for deviations in the predicted behaviour (**See Section 6.4.1**).

1.6. Impact on Phosphatase Enzymes

Both soil microbial biomass and enzyme activities are sensitive to changes in soil properties, environmental factors, and land management practices, and have therefore been suggested as biological indicators of soil quality (Dick, 1997; Sparling, 1997), and proxies for monitoring the impact of heavy metal contamination in soil (Brookes, 1995). Frankenberger, Jr. and Dick (1983) have reported significant correlations ($R = 0.69$; $p < 0.05$) between microbial biomass and the activities of acid phosphomonoesterase and phosphodiesterase in soils; although Lima et al. (1996) report a slightly weaker correlation for acid phosphomonoesterase ($R = 0.57$; $p < 0.01$). Nevertheless this implies that a decrease in soil microbial biomass, due to heavy metal toxicity, could cause corresponding decreases in phosphatase enzyme activity, the rate of organic phosphorus mineralisation, and an overall decline in soil quality. Therefore if sewage sludge is to be used as an alternative and sustainable source of phosphorus, it is important to understand what lasting effects the co-application of organic matter and heavy metals has on soil microbiology and the capacity for sludge amended soils to mineralise organic phosphorus.

The first account of enzyme inhibition in soil was reported by Tyler (1974, 1976), who found significant

Table 1.6:- Total metal concentrations (mg kg^{-1}) and ecological dose (ED) at which 10 % (ED_{10}), 50 % (ED_{50}), and 90 % (ED_{90}) inhibition of acid phosphomonoesterase activity is estimated to occur in different soil types after 18 months exposure to metal contamination. Comparisons to UK statutory limits for respective soil pH have been made. Adapted from Doleman & Haanstra (1989).

			Total Metal Concentration (mg kg^{-1})			
Soil	**pH**	**Metal**	**ED_{10}**	**ED_{50}**	**ED_{90}**	**UK Limit**
Sand	7.0	Cd	15.7	330.5	7000	3
		Cu	**8.3**[1]	170.3	3600	135
		Pb	201	78943	100100	300
		Ni	**40.5**	757.2	14500	75
		Zn	**5.2**	**170**	6000	300
Sandy Loam	6.0	Cd	8071	9869.6	12100	3
		Cu	438.5	1893.7	8100	135
		Ni	6878	7918	9100	75
		Zn	570.1	2968.3	15.4	300
Silty Loam	7.7	Cd	13.5	230.4	3800	3
		Cu	**170.3**	743.5	3200	200
		Pb	**16.6**	7521.4	63600	300
		Ni	246.2	150.3	5300	110
		Zn	**300.1**	4870.8	65400	450
Clay	7.5	Cd	829.6	5305.8	33700	3
		Cu	959.5	2770.6	7.9	200
		Zn	**36.0**	2844.0	65.4	450
Sandy Peat	4.4	Cu	**57.8**	2440.2	63.5	80

[1]Values in bold are below UK Statutory limits for respective soil pH.

decreases in the activities of urease and acid phosphomonoesterase in soils contaminated with Zn and Cu from a Swedish brass foundry. Subsequent investigation by Juma and Tabatabai (1977) found that acid phosphomonoesterase activity was reduced by a range of metal ions, including Cd, Cu, Pb, Ni, and Zn, with the extent of inhibition proportional to the total concentration of each metal. During the investigation, 2.5 µmol of each metal was added to 1 g of soil, giving concentrations of 281, 158, 518, 146, and 163 mg kg^{-1} for each metal, respectively. With the exception of Zn, the total metal concentration of each was higher than the statutory limits imposed by the EU Sludge Directive (**Table 1.1**) and the UK Sludge (Use in Agriculture) Regulations (**Table 1.2**), for soils with pH 6-7. Of these metals, the greatest inhibition observed was that of Cu (-11-18 %), with comparable inhibition also observed for Cd (-14 %) and Ni (-11 %), however it should be noted that the concentration of Cd used was almost 95 times greater than the current UK statutory limit, with Ni at almost twice the limit. However, perhaps most concerning is that inhibition of acid phosphomonoesterase by Zn (-5 %) was observed at a concentration just over half the current UK statutory limit. Expanding on this idea, Doleman and Haanstra (1989) modelled dose response curves for the inhibition of acid phosphomonoesterase by each of the metals mentioned above in order to help establish suitable statutory limits for these metals in soils. Approximately, 55, 150, 400, 1000, 3000, and 8000 mg kg^{-1} of each metal was added to a range of soil types and incubated for 18 months, before measuring the activity of phosphomonoesterase. Their results suggested that Zn and Cu had the greatest impact on enzyme activity however the extent of inhibition varied considerably between different soil types (**Table 1.6**). This variation was attributed to differences in soil organic matter and clay content, though the influence of these properties was not investigated, nor was the possible influence of pH and metal speciation.

However, when exposed to increasing concentrations of Cu (0.1-0.4 mM Cu) at pH 5.5, Huang and Shindo (2000a), observed little difference in the inhibition of acid phosphomonoesterase immobilized on the clay fractions (-50-55 % (0.1 mM) and -75-80 % (0.4 mM)) of two Chinese soils, in comparison to the free enzyme (-48.4 (0.1 mM) and 72.8 % (0.4 mM)) in solution. Similarly for Zn, little difference was observed between the inhibition of free (-44.1 % (1 mM) and -71.6 % (4 mM)) and immobilised enzyme (-47.6 % (1 mM) and -70.4 % (4 mM)) over a concentration range of 1 to 4 mM at pH 5.5 (Huang & Shindo, 2000b); although the inhibition of the immobilised enzyme was greater at pH 6. Therefore, in summary, it was suggested that clay minerals may not prevent the inhibition of acid phosphomonoesterase by Zn and Cu. Subsequent investigation, showed that Cu had a greater impact on the activity of acid phosphomonoesterase in comparison to Zn, causing approximately 50 % and 70-75 % inhibition, as before, at concentrations of 0.1 and 0.4 mM respectively, whereas Zn only caused 20-27 % and 45-50 % inhibition at the same concentrations (Huang & Shindo, 2001). In addition, the effect of Cd was also investigated, however at each of the concentrations tested inhibition was less than 2 %.

In general, investigations regarding the inhibition of soil enzymes by heavy metals are based on total metal concentrations, however Wang et al. (2007) have stressed the importance of determining metal speciation, and hence bioavailability, when investigating the impact of heavy metal contamination on soil microorganisms and enzyme activity. In an investigation similar to that of Tyler (1974, 1976), Wang et al. (2009) observed a decrease in C_{mic} and the activity of acid phosphomonoesterase in soils contaminated with Zn and Cu from a nearby metal smelter. In this case, a greater correlation was seen between enzyme activity and the concentrations of Zn ($R = -0.76$; $p < 0.01$) and Cu ($R = -0.85$; $p < 0.01$) extractable by NH_4NO_3 than was seen for their respective total concentrations (Zn, $R = -0.61$; Cu, $R = -0.82$); the correlation with soluble Zn ($R = -0.68$; $p < 0.01$) was also greater. However, it was not established whether the reduction in enzyme activity was due to direct inhibition by each metal, or indirectly, as a result of decreased microbial biomass.

1.7. Project Rationale and Knowledge Gaps

It has become apparent that the agricultural demand for inorganic phosphate fertilisers needs to be reduced, and their use managed efficiently, whilst attempts are made to increase the dependence on more sustainable sources of phosphorus, such as sewage sludge. The application of sewage sludge to agricultural land is currently considered to be the best practical environmental option for the management of sewage sludge within the EU and UK. However, the presence of potentially toxic heavy metals in sewage sludge poses a threat to soil microorganisms and the mineralisation of applied organic matter, thereby disrupting the cycling of essential nutrients. Of the six heavy metals listed in the UK Sludge (Use In Agriculture) Regulations, Zn and Cu have been identified as the most problematic with

regards to the application of sewage sludge to agricultural soil, potentially accumulating to the maximum statutory limits within a few decades (**Table 1.3**). Significant decreases in C_{mic} have been observed in soils where Zn and Cu are present at concentrations close to the current UK statutory limits, with greater decreases observed when both metals are present simultaneously (**See Section 1.4**). In addition, Zn and Cu have been found to significantly reduce the activity of phosphatase enzymes, and therefore inhibit the release of phosphorus from organic compounds, at concentrations below the UK statutory limits, with Cu causing greater inhibition in comparison to Zn (**See Section 1.6**). Both Ni and Pb have been seen to inhibit phosphatase enzyme activity (**Table 1.6**), however Ni appears to have no overall impact on soil microorganisms (**See Section 1.4**), whereas the solubility, and hence the bioavailability, of Pb is very low in soils thereby reducing the risk to microorganisms (McGrath, 1987). Significant decreases in C_{mic} have been observed in soils heavily contaminated with Cd, however concentrations greatly exceed the UK statutory limits and often the observed effect is confounded by the presence of other metals (**See Section 1.4**). Furthermore Cd appears to have little impact on the activity of phosphatase enzymes within the soil environment (**See Section 1.6**). Hence Zn and Cu are most likely to cause significant disruptions to the mineralisation of organic phosphorus within sludge amended soils, which could limit the use of sewage sludge as an alternative to inorganic phosphate fertilisers. It is therefore important to understand what lasting effects the co-application of organic matter and heavy metals, particularly Zn and Cu, has on soil microbiology and the capacity for sludge amended soils to mineralise organic phosphorus; particularly if adverse effects can occur when the total concentration of these metals are below the limits set by the UK Sludge (Use In Agriculture) Regulations.

Not only is it possible for heavy metals to directly inhibit the activity of phosphatase enzymes, there is evidence to suggest that a decrease in soil microbial biomass, due to heavy metal toxicity, can also cause a corresponding decrease in enzyme activity. However, these conclusions are derived from short-term incubations, whereas questions regarding the long-term impact of heavy metal contamination (i.e. adverse effects observable after a period of 10 years or more) on soil microorganisms and phosphatase enzymes remain unanswered. For instance, are the observed decreases in microbial biomass, due to metal contamination at or below the UK limits, permanent or can the microbial population become tolerant and proliferate? Also, how are extracellular enzymes affected by metal contamination in the long-term? Is enzyme activity restored as new enzymes are subsequently released into the soil environment over time, and what effect does this have on the organic phosphorus content of sludge amended soils? Furthermore, investigations into the impacts of metals on soil microbial biomass and enzyme activities are frequently based on total metal concentrations, often overlooking metal speciation and bioavailability, which could lead to an overestimation of the metal concentrations causing adverse effects. Therefore it may be that stricter regulatory limits are required if sewage sludge is to be used effectively as a sustainable source of phosphorus and a safe alternative to inorganic chemical fertilisers.

1.8. Project Aims and Objectives

The overall aim of this project is to determine the long-term impacts of Zn and Cu contamination, as a result of historical sewage sludge application, on soil microorganisms and their capacity to mineralise organic phosphorus by phosphatase enzyme activity. Four field sites are to be investigated which have received applications of sewage sludge, containing elevated concentrations of Zn and Cu, more than 15 years prior to the current investigation. The presence of Zn and Cu within sludge amended soils has been seen to cause significant reductions in soil microbial biomass and enzyme activity, therefore a number of research questions have been devised which will help to evaluate the use of sewage sludge as a sustainable source of phosphorus and a safe alternative to inorganic phosphate fertilisers. Achieving this project aim will contribute towards a greater understanding of the mineralisation of organic phosphorus compounds in the environment and help to elucidate the potential limitations of using sewage sludge as an agricultural fertiliser.

1.8.1. Research Question 1

- Does the presence of residual heavy metal contamination (Zn and Cu) cause a lasting decrease in soil microbial biomass and phosphatase enzyme activity, or can soil microbial communities become tolerant and recover?
- ***Hypothesis 1:*** *After a period of 15 years soil microbial biomass and extracellular phosphatase enzyme activities will still be reduced in soils contaminated with Zn and Cu at concentrations approaching the UK statutory limit. An observed decrease in microbial biomass will indicate that microbial communities have not become tolerant to the presence of Zn and Cu. A corresponding decrease in phosphatase enzyme activity will also indicate that the rate of organic phosphorus mineralisation is reduced in contaminated soils.*

1.8.2. Research Question 2

- What role does metal speciation play in the inhibition of phosphatase enzymes and toxicity to soil microorganisms?
- ***Hypothesis 2:*** *Greater inhibition of phosphatase enzyme activity will be observed in soils contaminated with Cu, in comparison to soils contaminated with Zn, due to the greater binding affinity of this metal for soil organic matter and proteins.*

1.8.3. Research Question 3

- What is the long-term fate of organic phosphorus compounds derived from sewage sludge in sludge amended soils?
- ***Hypothesis 3:*** *After a period of 15 years organic phosphorus will be greater in contaminated soils, in comparison to uncontaminated soils, due to a reduced rate of organic phosphorus mineralisation caused by decreases in soil microbial biomass and enzyme activity.*

1.8.4. Research Question 4

- Are the current limits specified for Zn and Cu in the UK Sludge (Use in Agriculture) Regulations sufficient to protect microorganisms and phosphatase enzyme activity in sludge amended soils?
- ***Hypothesis 4:*** *The UK statutory limits for Zn and Cu are currently set too high to prevent disruption to the mineralisation of organic phosphorus and may need to be reduced if the agricultural use of sewage sludge, as a sustainable alternative to inorganic phosphate fertilisers, is expected to increase.*

Several objectives have been established in order to achieve the project aim, these are:

1.8.5. Objective 1

Compile historical field survey data from untreated and sludge amended soils at four UK field sites and determine the current soil properties (pH and organic matter), phosphorus content (total P, available P, and organic P), and the extent and speciation of Zn and Cu contamination.

1.8.6. Objective 2

Assess the current status of soil microbial communities by analysing soil microbial biomass C, as well as phospholipid fatty acid (PLFA) and ergosterol biomarkers in both sludge amended and untreated soils.

1.8.7. Objective 3

Quantify the activity of phosphatase (acid phosphomonoesterase and phosphodiesterase) enzymes in untreated and sludge amended soils and determine the extent of enzyme inhibition.

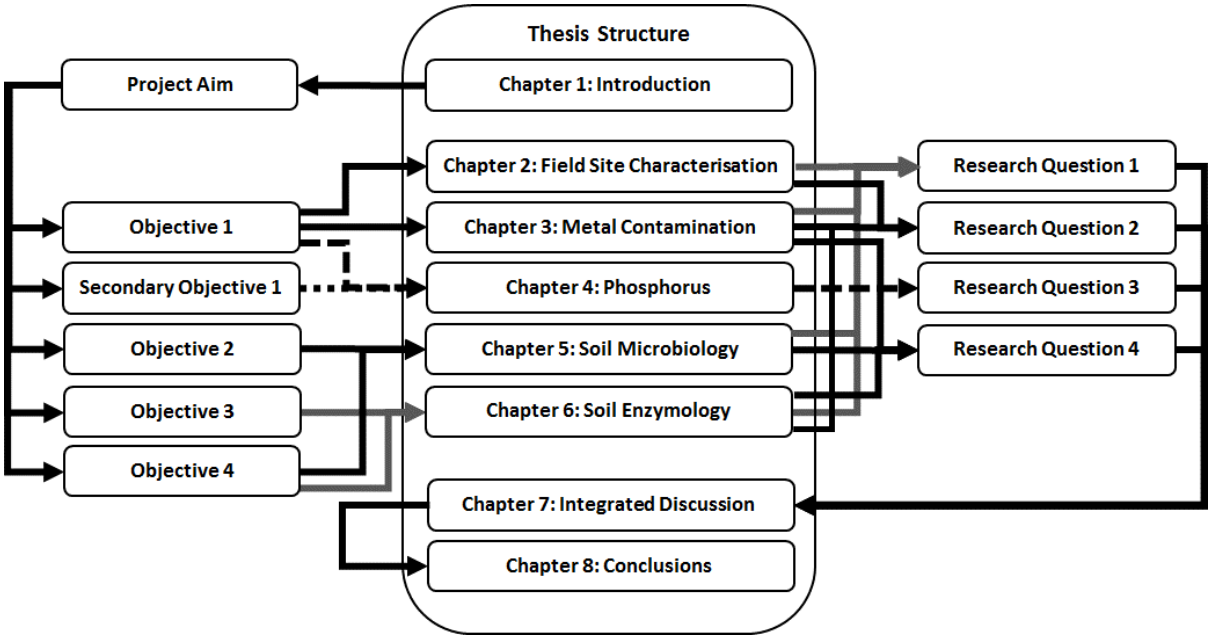


Figure 1.7:- Overview of research project and thesis structure.

1.8.8. Objective 4

Evaluate the impact of heavy metal contamination on the proliferation of microorganisms, by making a fresh application of sewage sludge to both contaminated and uncontaminated soils and monitoring the overall microbial response (i.e. changes in C_{mic} and phosphatase enzyme activity) over time.

1.8.9. Secondary Objective 1

A secondary objective was also established in collaboration with the University of Sheffield: Using ^{31}P-NMR, characterise the form of organic phosphorus compounds present within the applied sewage sludge, and determine their fate in sludge amended soils in comparison to untreated soil.

1.9. Thesis Structure and Overview

Figure 1.7 gives an overview of the thesis structure and outlines how each objective contributes toward answering the proposed research questions. Following the literature review and project description presented here in **Chapter 1**, an overview of the Defra 'Long-Term Sludge Experiment' and historical details of the four field sites chosen for the current investigation are presented in **Chapter 2**. The sampling protocol employed at each site, the storage and treatment of samples prior to analysis, and an overview of the statistical methods used in the analysis of experimental data are included. The current status of soil pH and organic C are also discussed. **Chapter 3** presents both historical and contemporary

data for the Zn and Cu contamination present at each of the field sites investigated. The long term behaviour of each metal, and the current metal speciation, are also discussed. **Chapter 4** focuses on phosphorus, discussing the current status of the total, available, and organic fractions of phosphorus in sludge amended soils. Additional ^{31}P-NMR data is also presented showing the forms of organic phosphorus in each of the applied sludge treatments, as well as sludge amended and untreated soils. **Chapter 5** presents historical and contemporary data for soil microbial biomass carbon in sludge amended soils at each of the field sites. Additional data from the analysis of ergosterol and PLFA biomarkers are also presented to give an overview of how the soil microbial community has adapted to the presence of heavy metal contamination. **Chapter 6** describes the extent of phosphatase enzyme inhibition (phosphomonoesterase and phosphodiesterase) by Zn and Cu and explores the kinetics of organic phosphorus hydrolysis in sludge amended soils. The results presented in **Chapters 2-6** are followed by a discussion of the experimental data (both historical and contemporary), examining the trends and relationships between variables across all four of the field sites, whilst placing them in a wider context by comparison to results reported in the scientific literature. The conclusions drawn from **Chapters 2**, **3**, **5**, and **6** will contribute in answering **Research Question 1**; discussion of the soil properties, metal contamination, and phosphatase enzyme activities in **Chapters 2**, **3**, and **6** will also contribute in answering **Research Question 2**. The conclusions drawn from **Chapter 4**, focussing specifically on phosphorus, will contribute in answering **Research Question 3**. Finally, **Chapter 7** gives an integrated discussion of the data presented in each of the preceding chapters within the context of current EU and UK sludge recycling policy. From the conclusions of **Chapters 3**, **5**, and **6**, a discussion of the current UK statutory limits for Zn and Cu is given in order to answer **Research Question 4**. The overall conclusions of the current investigation are then summarised in **Chapter 8**.

CHAPTER 2

FIELD SITE CHARACTERISATION

2. FIELD SITE CHARACTERISATION

2.1. Introduction

This chapter provides historical information regarding the Defra 'Long-Term Sludge Experiment' and the four field sites chosen for the current investigation, thus allowing discussion of the current data set in a wider context. The sampling and storage of soil samples collected during the current investigation are also described, along with an overview of the experimental and statistical methods used in the acquisition and analysis of data. The current physical and chemical characteristics (soil moisture content, pH, and organic carbon) of the soils are also discussed.

2.2. Site Selection

Following the implementation of the UK Sludge (Use in Agriculture) Regulations in 1989, a review of the proposed statutory limits was conducted by an Independent Scientific Committee (**See Section 1.3**), on behalf of the UK Ministry for Agriculture, Fisheries and Food and the UK Department of Environment, now Defra, which concluded that the proposed limits were sufficient to protect plants, animals, and humans from metal toxicity, but could pose a risk to soil microorganisms (MAFF/DoE, 1993a, 1993b). However, one of the major obstacles encountered by policy makers in deciding statutory limits remains a lack of data available to establish dose-response curves, thus giving a more accurate estimation of the minimum total metal concentrations likely to have adverse effects on soil microorganisms. The need for more long-term experimental field sites has been recognised by several authors (McBride, 2003; McGrath et al., 1994, 1995), and in particular, the Independent Scientific Committee described a number of ideal experimental criteria aimed at improving the reliability and interpretation of experimental data (MAFF/DoE, 1993b).

Principally, a long-term field experiment should aim to determine the correlation between individual metal concentrations and a range of chemical and biological soil properties over a range of different soil textures and climatic conditions. The rate at which any adverse effects occur following the application of sewage sludge, along with the rate of attenuation, persistence, or intensification of such effects should be monitored for the duration of the experiment (MAFF/DoE, 1993b). Therefore, as part of continuing research into the effects of heavy metals on soil fertility, an investigation into the long term impact of sewage sludge applications on soil microorganisms was established following the MAFF/DoE review (MAFF/DoE, 1993b).

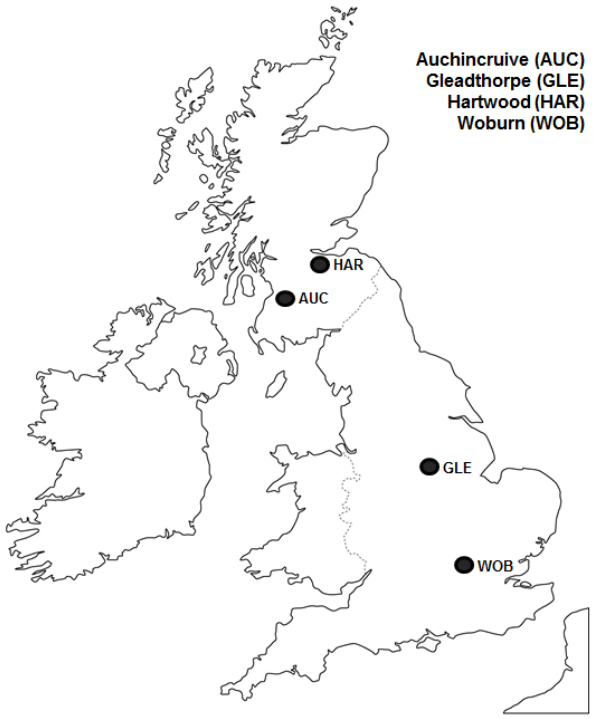


Figure 2.1:- Location of Long-Term Sludge Experiment field sites.

The 'Long Term Sludge Experiment' (LTSE) began in 1994, at nine UK field sites, chosen to provide a range of soil properties from varying climatic regions (Defra, 2002, 2007a; Gibbs et al., 2006). In order to determine the long-term impacts of Zn and Cu (after a period of 15 years), on soil microorganisms and the mineralisation of organic phosphorus (**See Section 1.8**), four of the remaining LTSE field sites (three of the original nine sites have since been decommissioned) were selected to provide soils with concentrations of the two heavy metals comparable to the UK statutory limits (**Table 1.2**). The four sites chosen for the current investigation are shown in **Figure 2.1**, these are: Auchincruive (AUC; Ayrshire; currently managed by Scotland's Rural College), Gleadthorpe (GLE; Nottinghamshire; currently managed by ADAS), Hartwood (HAR; Lanarkshire; currently managed by the James Hutton Institute), and Woburn (WOB; Bedfordshire; currently managed by Rothamsted Research). A preliminary investigation of the remaining six LTSE field sites, in 2012, revealed that the site at Hartwood had the highest soil organic carbon content, whereas, conversely, the site at Woburn had the lowest. Hence, these two sites were chosen to provide contrasting locations with regards to soil organic matter. Similarly, the conditions under which the field sites were maintained were also contrasting, with Hartwood managed as a grassland for the duration of the LTSE, whereas a ley/arable cropping regime was implemented at Woburn (**See Section 2.3**). Two additional sites were selected to investigate the potential differences in the effects of Zn and Cu between field sites. Auchincruive was chosen to provide an additional grassland site, with a soil organic carbon approximately half of that seen at Hartwood, whereas the site at Gleadthorpe was chosen to provide an additional arable site. At Gleadthorpe soil organic matter was approximately 1.5 times that of the Woburn site (**See Section 2.7**).

Table 2.1:- Physical, chemical, and biological properties of soils (0-25 cm) at the Long-Term Sludge Experiment field sites prior to initial sludge applications in 1994. Data obtained from Gibbs et al. (2006).

	Field Site			
	Auchincruive (AUC)	**Gleadthorpe (GLE)**	**Hartwood (HAR)**	**Woburn (WOB)**
Sand (> 63 µm) (%)	51 (1.33)[1]	71 (1.73)	59 (2.56)	80 (1.20)
Silt (2-63 µm) (%)	29 (0.29)	22 (1.45)	20 (1.20)	12 (0.88)
Clay (< 2 µm) (%)	20 (1.04)	7 (0.33)	21 (3.38)	8 (0.33)
Soil Texture	Sandy Clay Loam	Sandy Loam	Sandy Clay Loam	Loamy Sand
pH	6.0 (0.09)	7.1 (0.06)	5.8 (0.04)	7.2 (< 0.01)
Organic C (%)	2.5 (0.09)	1.2 (0.06)	4.7 (0.19)	1.3 (0.07)
Fe_2O_3 (%)	4.24 (0.10)	1.66 (0.11)	3.32 (0.06)	2.90 (0.09)
Al_2O_3 (%)	3.54 (0.09)	1.51 (0.09)	7.78 (0.22)	1.13 (0.02)
Microbial Biomass C (µg g^{-1})	428 (6.8)	66 (3.5)	663 (24.8)	108 (4.5)

[1] Values in parenthesis are standard error (n = 3).

Table 2.2:- Physical and chemical properties of sludge treatments applied at the Long-Term Sludge Experiment field sites from 1994-1997. Data obtained from Gibbs et al. (2006).

	Sludge Treatment			
	Zinc (Zn)	**Copper (Cu)**	**Digested Control (Ctrl1)**	**Undigested Control (Ctrl 2)**
Dry Matter (%)	23.5	18.0	18.3	36.7
Organic C (%)	31.6	37.6	38.1	42.9
N (% ds)	3.95	4.35	5.25	3.50
NH_4^+-N (% ds)	0.82	0.77	0.55	0.87
P (% ds)	2.87	0.76	1.98	1.61
K (% ds)	0.18	0.17	0.16	0.13
Fe (mg kg^{-1})	22 750	8500	9800	4800
Al (mg kg^{-1})	24 350	8450	15 900	11 200
pH	7.5	5.2	7.3	7.3

2.3. Site Histories

Experimental Phase I (1994-1997)

Prior to the start of the long-term sludge experiment in 1994, a range of chemical, physical, and biological properties were determined for each of the soils at the LTSE field sites (Gibbs et al., 2006); these results are summarised in **Table 2.1**. Two contaminated sludge treatments, containing significantly higher concentrations of Zn and Cu, in relation to each of the other metals present, were identified from two different sources (Defra 2002, Defra 2007a). Digested sludge cake, contaminated with zinc (Zn), and raw undigested sludge cake, contaminated with copper (Cu), was applied annually over the course of four years (Phase I: 1994-1997), in order to increase the total concentration of these metals in each soil to specified targets and establish dose response curves for each metal (**See Section 3.2**). The total quantity of organic matter applied, at each level of the dose-response curve, was maintained at a constant level by additional applications of 'low-metal' uncontaminated sludge cake which contained concentrations of heavy metals typical of sewage sludge produced in the UK (Gibbs et al., 2006). Separate applications of the 'low-metal' uncontaminated sludge cakes were also included in the experimental design as control treatments, with the Zn sludge treatment matched by an uncontaminated digested sludge cake (Ctrl1) and the Cu sludge treatment matched by an uncontaminated undigested sludge cake (Ctrl2); untreated soil, receiving no sludge (NS), was also included as an experimental control. Each of the four sludge treatments were replicated three times

Table 2.3:- Total carbon loading and percentage remaining in the topsoil (0-25 cm) of the Long-Term Sludge Experiment field sites following final sludge applications in 1997. Data obtained from Gibbs et al. (2006).

Sludge Treatment	Total Sludge C Loading (t ha^{-1})	Soil Org. C in 1997 (t ha^{-1})	Increase in Org. C (%)[1]	Remaining C Load in 1997 (t ha^{-1})
Auchincruive (AUC/NS)	N/A[2]	66	N/A	N/A
Digested Sludge (AUC/Zn and AUC/Ctrl1)	69	115	74.2	51.1 (74)[3]
Undigested Sludge (AUC/Cu and AUC/Ctrl2)	83	104	57.6	36.5 (44)
Gleadthorpe (GLE/NS)	N/A	36	N/A	N/A
Digested Sludge (GLE/Zn and GLE/Ctrl1)	69	59	63.8	22.8 (33)
Undigested Sludge (GLE/Cu and GLE/Ctrl2)	68	49	36.1	12.9 (19)
Hartwood (HAR/NS)	N/A	107	N/A	N/A
Digested Sludge (HAR/Zn and HAR/Ctrl1)	74	137	28.0	33.3 (45)
Undigested Sludge (HAR/Cu and HAR/Ctrl2)	81	127	18.7	17.8 (22)
Woburn (WOB/NS)	N/A	32	N/A	N/A
Digested Sludge (WOB/Zn and WOB/Ctrl1)	81	51	59.4	20.3 (25)
Undigested Sludge (WOB/Cu and WOB/Ctrl2)	81	47	46.9	15.4 (19)

[1]Percentage increase based on the organic C content of untreated soil (Gibbs et al., 1996). [2]Not Applicable. [3]Values in parenthesis are percentage of Total C loading remaining (Gibbs et al., 1996).

in fully randomised blocks consisting of 6 m × 8 m plots (**Figure 2.2**), and annually cultivated by spading machine to a depth of 25 cm (Gibbs et al., 2006). Physical and chemical properties of the applied sludge treatments are summarised in **Table 2.2**.

Total C loadings, applied during experimental Phase I, ranged from 69 (AUC and GLE) to 81 (WOB) t ha^{-1} in soils receiving digested sludge treatments, and 63 (GLE) to 83 (AUC) t ha^{-1} in soils receiving undigested sludge treatments (**Table 2.3**). By 1997, organic C was reported to be 0.9-2 % higher in the sludge amended soils, in comparison to untreated soil (Defra, 2002). The greatest increases were seen at Auchincruive, where the sludge amended soils now contained additional organic carbon equivalent to 60-75 % of that seen in untreated soil (**Table 2.3**); whereas in contrast the lowest increases were seen at Hartwood, presumably due to the higher organic C content already present at that site (**Table 2.1**). With the exception of the Auchincruive field site, less than half of the applied C remained in the top 25 cm of soils receiving digested sludge treatments, whereas less than 25 % of applied C remained in soils receiving undigested sludge treatments (**Table 2.3**). These losses were attributed to the rapid mineralisation of applied organic matter by soil microorganisms, with the differences between sludge treatments attributed to the anaerobic digestion pre-treatment of digested sludge cake, which would have previously removed large quantities of labile organic matter (Gibbs et al., 2006).

Experimental Phase II (1998-2001)

An Italian ryegrass (Cotswold Grass Seeds, 'Special Mix A') was sown (40 kg ha^{-1}) at all sites in autumn 1997, following the final sludge applications. Each site was then harvested in summer 1998, and

Figure 2.2:- Site layout and sample locations for the Long-Term Sludge Experiment field sites at Auchincruive (Ayrshire, Scotland, UK), Gleadthorpe (Nottinghamshire, England, UK), Hartwood (Lanarkshire, Scotland, UK), and Woburn (Bedfordshire, England, UK).

cultivated an additional three times that year to ensure the organic matter applied during Phase I was evenly distributed throughout each plot (Defra, 2002). A ley/arable cropping regime was subsequently implemented at the two English field sites, with spring wheat (Chablis) sown in February/March 1999 and 2001, whereas both Scottish field sites were managed as grassland (Italian ryegrass) for the duration of Phase II (Defra, 2002). Soil nutrient status, metal content, organic C, and microbial biomass C (C_{mic}), were measured following sampling events in spring 1999 and 2001.

Experimental Phase III (2002-2005)

Cropping regimes remained unchanged for both English and Scottish field sites throughout Phase III (Defra, 2007a). Soil nutrient status, metal content, organic C, and C_{mic} also continued to be monitored during this period following sampling events in 2003 and 2005. Over the course of the experiment, soil pH was maintained at all sites by applications of lime ($CaCO_3$). The English sites were maintained at a target pH of 6.5, considered to be optimal for arable soils in the UK, whereas soil pH at the Scottish sites, Hartwood and Auchincruive, were kept at pH 5.8 and pH 6.0, respectively, typical of Scottish grasslands (Gibbs et al., 2006).

Experimental Phase IV (2006-2011)

Official funding for the 'Long-Term Sludge Experiment' ceased in 2005. Therefore, although soil pH and cropping regimes are still maintained at each site by their respective institutions, the chemical, physical, and biological properties of the sludge amended soils only continue to be monitored at the Scottish field sites.

2.4. Sampling and Analysis

Soil samples were collected from the four LTSE field sites described in **Section 2.3** over the course of three years (2012-2014); dates for each sampling event are given in **Table 2.4**. In order to investigate whether the current UK statutory limits, for Zn and Cu, are sufficient to protect C_{mic} and phosphatase enzyme activity, samples were collected from soils receiving contaminated sludge (Zn and Cu) at dose response 'Level 3' (**See Section 3.2**); the target concentrations of Zn (350 mg kg^{-1}) and Cu (150 mg kg^{-1}) at 'Level 3' of the established dose response curve were closest to the statutory limits currently enforced in the UK (**See Section 1.3**). Additional samples were collected from soils receiving uncontaminated sludge treatments (digested (Ctrl1) and undigested (Ctrl2) controls), and from untreated soil (no sludge (NS)). Each sludge treatment was replicated three times giving a total of n = 15 samples per field site. Samples were collected to a depth of 20 cm from each of the plots shown in **Figure 2.2**. Each sample was a composite of five soil cores (approximately 1000 g) collected using a gouge auger (except at HAR where screw augers were used) and arranged in a herringbone ('W') pattern (**Figure 2.3**); augers were cleaned with ethanol then rinsed with deionised water between sampling each plot.

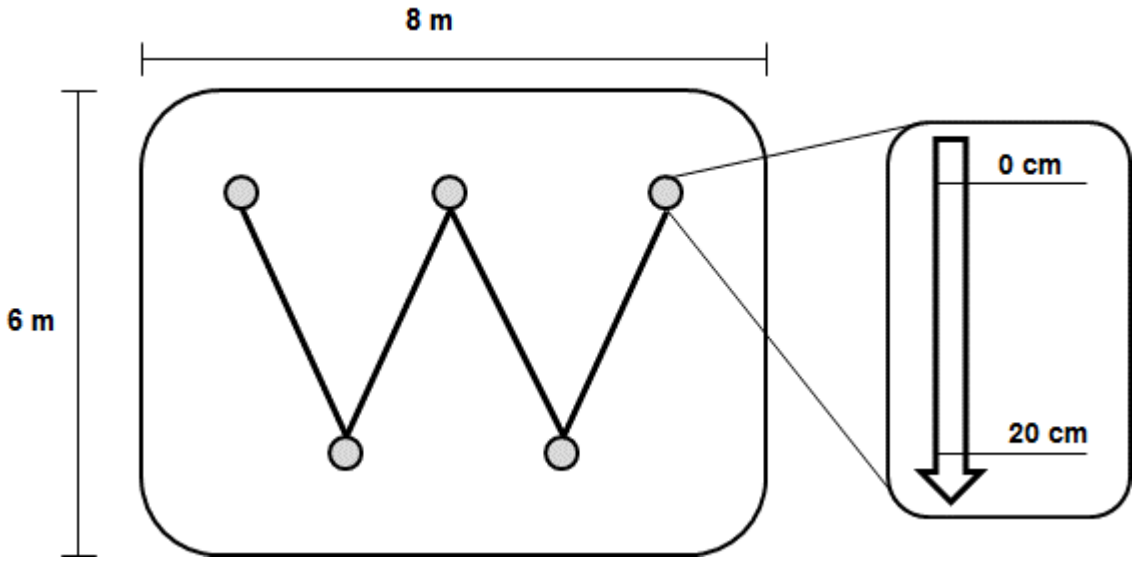


Figure 2.3:- Schematic of sampling protocol employed at each of the Long-Term Sludge Experiment field sites.

The field conditions encountered during each sampling event are given in **Table 2.4**. Approximately 150 g of soil from each sample was air-dried (~30^{o}C) immediately and sieved (<2 mm). A further 50 g was then frozen (-85^{o}C) prior to freeze drying. Air- and freeze-dried samples were stored at room temperature (**Figure 2.4**) and analysed for a range of soil properties as shown in **Table 2.4**. The remainder of each sample (approximately 800 g) was sieved (<4 mm) and stored in sealed plastic bags at 4^{o}C pending the analysis of C_{mic} and phosphatase enzyme activity (**Figure 2.4**). Analysis of soil microbial biomass (**See Section 5.2**) was carried out immediately (<24 hours) following the 2013 and 2014 sampling events (**Table 2.4**), whereas phosphatase enzyme activities (**See Section 6.2**), determined for samples collected in 2014 (**Table 2.4**), were analysed within one month of sampling. An incubation experiment was established following the 2014 sampling event in order to investigate the changes in C_{mic} and phosphatase enzyme activity observed in contaminated soils following a fresh application of organic matter (**See Section 5.3**). In this case 300 g of field moist soil was divided into five replicate samples of 60 g (**Figure 2.4**) and incubated at 20^{o}C for a period of three months.

2.4.1. Statistical Analysis

All statistical analysis of experimental data was carried out using STATISTICA (64-bit) Version 12 (StatSoft Inc., 2014) with graphics produced using Microsoft Excel® 2013. Statistical significance ($\alpha = 0.05$) was determined by one-way analysis of variance (ANOVA) using Fisher's least significant difference (LSD) to compare differences *between* means; *p* values are given throughout the text as <0.05 (5 %), <0.01 (1 %), or <0.001 (0.1 %) depending on the level of significance. For each of the analyses listed in **Table 2.4** comparisons were made between soils receiving corresponding sludge treatments and untreated soil, with the Zn sludge treatment compared to the digested control, and Cu sludge

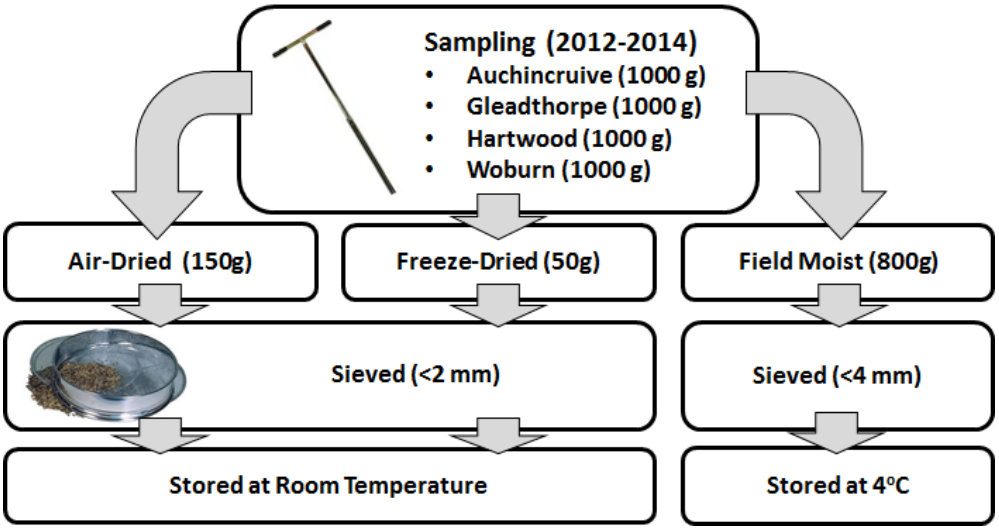


Figure 2.4:- Schematic of sample pre-treatment, preparation, and storage conditions prior to analysis. Soil quantities used are given in parenthesis.

Table 2.4:- Dates of sampling events, field conditions encountered, and methods of analysis, for each of the samples collected from the Long-Term Sludge Experiments field sites during 2012-2014.

Field Site	Sampling Date	Field Conditions[1]	Methods of Analysis[2]
		2012	
Auchincruive (AUC)	12th July	Grassland	• Soil pH (10 mL) • Organic Carbon (1 mg) • Organic Matter (5g) • Total Zn, Cu, and P (0.5 g) • Available and Organic P (3 x 5 g) • PLFA (10 g – WOB only) • Ergosterol (2 x 5 g – WOB only)
Gleadthorpe (GLE)	09th July	Ley (Grassland)	
Hartwood (HAR)	12th July	Grassland (Waterlogged)	
Woburn (WOB)	23rd May	Arable (Under Wheat)	
		2013	
Auchincruive (AUC)	24th October	Grassland	• Soil Moisture Content (30 g) • Soil pH (10 mL) • Organic Matter (5g) • Total Zn, Cu, and P (0.5 g) • Available and Organic P (3 x 5 g) • Microbial Biomass C (2 x 12.5 g) • PLFA (10 g – AUC,GLE, and HAR) • Ergosterol (2 x 5 g – AUC, GLE, and HAR)
Gleadthorpe (GLE)	11th October	Ley (Ploughed Soil)	
Hartwood (HAR)	24th October	Grassland	
Woburn (WOB)	06th June	Ley (Grassland)	
		2014	
Auchincruive (AUC)	11th August	Grassland	• Soil Moisture Content (30 g) • Soil pH (10 mL) • Organic Matter (5g) • Total Zn, Cu, and P (0.5 g) • Zn and Cu Speciation (5 g) • Available and Organic P (3 x 5 g) • Microbial Biomass Carbon (2 x 12.5 g) • Enzyme Activity (3 x 1 g) • PLFA (10 g) • Ergosterol (2 x 5 g) • Incubation Experiment (5 x 60 g)
Gleadthorpe (GLE)	10th June	Arable (Under Wheat)	
Hartwood (HAR)	17th July	Grassland	
Woburn (WOB)	1st April	Arable (Under Wheat)	

[1]Notable field conditions encountered during sampling are given in parenthesis. [2]Methods of Analysis were applied to all of the samples collected that year, unless specified otherwise.

treatment compared to the undigested control (**Table 2.2**). For each of the soils analysed, comparisons were also made between different sampling events to determine if any changes in the analysed properties occurred over the course of the current investigation. Residual changes ($\Delta\bar{x}$) in soil organic carbon, metal contamination, phosphorus content, and microbial biomass were then determined for sludge amended soils at each of the field site as follows:

$$\Delta\bar{x} = \overline{x_{SA}} - \overline{x_{NS}} \qquad (\mathbf{E.2.1})$$

where $\bar{x}_{SA}$ and $\bar{x}_{NS}$ are, respectively, the mean values for a given property in sludge amended and untreated soils over the course of the current investigation (2012 to 2014). Comparisons between the residual changes were then made to determine the differences between field sites and give an overview of the long-term impact of historic sludge application; a comparison between untreated soils was also made to determine the differences in soil properties between field sites.

Regression analysis was used to investigate the relationship between continuous variables, such as total metal concentration and C_{mic}, across field sites. Samples collected from the sludge amended and untreated soils at each field site were considered as a separate case giving n = 12 cases for each of the variables investigated. Regression and correlation coefficients were then determined by fitting a linear model to the data. For regression models with more than one independent variable, an F-test was carried out to determine whether the combined effect on the dependent variable was statistically significant. The proportion of variance explained was then determined by calculating R^2; in each case R^2 is reported as the adjusted value to allow comparison between regression models. Residual plots were examined in order to determine the accuracy of the regression model. A histogram of residual values, along with a plot of residual against predicted values, was used to ensure a normal and random distribution of residual values and identify possible outliers or heteroscedasticity within the data set.

2.4.2. Meta-Analysis

A common tendency of primary studies is to focus predominantly on statistical significance rather than effect size. It is often assumed that the absence of statistical significance provides evidence for the null hypothesis, however non-significant results can simply be due to low statistical power where a small sample size has been used during an investigation (Borenstein, 2000). In the case of the LTSE, the sludge treatments were applied in triplicate which may not be sufficient to overcome the natural variation in chemical, physical, and biological properties observed within the soil environment. Therefore it could be that the effect of applying contaminated sewage sludge was consistently negative across all field sites (e.g. C_{mic} was lower in contaminated soils), but produced non-significant results due to a low sample size, hence the overall effect may have been overlooked. Therefore experimental data for soil organic C (**See Section 2.7.**), C_{mic} (**See Section 5.2.**), ergosterol (**See Section 5.3**), and

phosphatase enzyme activity (**See Section 6.4**) were subsequently combined using meta-analysis in order to increase statistical power (**See Section 7.2**) and provide an overview of the treatment effects observed in the data set.

Meta-analysis also aims to test the null hypothesis of no effect, however, this is achieved by establishing a common framework in which direct comparisons can be made between independent studies (*i*). The observed effects (R_i), between experimental (E) and control groups (C), are then combined to give an overall summary effect (M) across a number of studies, thus improving statistical power and allowing more accurate estimations of statistical significance (Hedges & Pigott, 2001). All calculations were carried out according to Borenstein et al. (2009). Effects were calculated as the log response ratio (Hedges, et al. 1999), as follows:

$$R_i = \frac{\overline{x_E}}{\overline{x_C}} \qquad (\mathbf{E.2.2})$$

$$\ln(R_i) = \ln\left(\frac{\overline{x_E}}{\overline{x_C}}\right) = \ln \overline{x_E} - \ln \overline{x_C} \qquad (\mathbf{E.2.3})$$

where $\overline{x}_E$ and $\overline{x}_C$ are the respective means of the experimental and control groups. The *within* study variance (V_i) and standard error (SE_i) of each effect were then calculated:

$$V_i = \left(\frac{SD_E^2}{n_E \overline{x_E}^2}\right) + \left(\frac{SD_C^2}{n_C \overline{x_C}^2}\right) \qquad (\mathbf{E.2.4})$$

$$SE_i = \sqrt{V_i} \qquad (\mathbf{E.2.5})$$

where SD_E and n_E, and, SD_C and n_C are the standard deviation and sample size of the experimental and control groups, respectively. Upper (UL_i) and lower (LL_i) confidence limits were determined as:

$$UL_i = R_i + (1.96 \times SE_i) \qquad (\mathbf{E.2.6})$$

$$LL_i = R_i - (1.96 \times SE_i) \qquad (\mathbf{E.2.7})$$

where 1.96 is the Z-value corresponding to a confidence interval of 95 % ($CL_{95\%}$). Each effect was assigned a weight (W_i), as the reciprocal of **E.2.4** ($1/V_i$), and combined to give an overall summary effect as follows:

$$M = \frac{\sum W_i R_i}{\sum W_i} \qquad (\mathbf{E.2.8})$$

The overall variance (V_M) was calculated as:

$$V_M = \frac{1}{\sum W_i} \qquad (\mathbf{E.2.9})$$

with standard error (SE_M) and confidence intervals determined, as above, by substituting M and V_M into

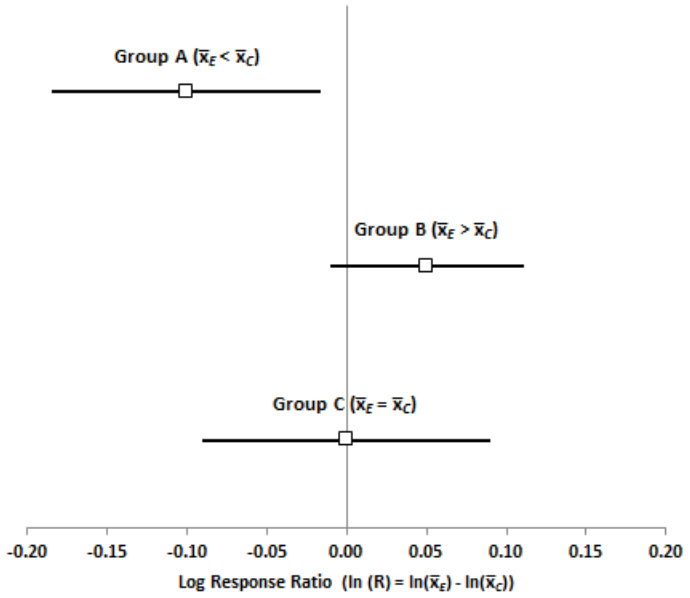


Figure 2.5:- Forest plot showing negative (Group A), positive (Group B), and zero (Group C) summary effects as log response ratio. Horizontal lines represent 95 % confidence interval. Effects are not statistically significant (p <0.05) if the 95 % confidence interval crosses the centre line.

equations **E.2.5**, **E.2.6**, and **E.2.7**, where applicable. The summary effect and 95 % confidence limits (LL_M and UL_M) were then converted back into the original metric and expressed as the percentage of overall increase or decrease:

$$M_{\%} = 100 - \left(e^{(M)} \times 100\right) = 100 - \left(\frac{M_E}{M_C} \times 100\right) \qquad (\mathbf{E.2.10})$$

The results of each meta-analysis are presented as 'forest plots', showing overall summary effect sizes for each analysis with 95 % confidence intervals represented as horizontal lines (**Figure 2.5**); the observed effect is not statistically significant (p <0.05) if the confidence limit spans the centre line, at which point the effect is zero (i.e. $\bar{x}_E = \bar{x}_C$).

2.5. Soil Moisture Content

As mentioned in **Section 1.5**, the mineralisation of soil organic matter is carried out by a range of hydrolytic enzymes, therefore the presence of water within the soil environment is a required for the cycling of nutrients. The transport of nutrients, as well as heavy metals, and their uptake by microorganisms is also facilitated by the soil solution (Marschner & Kalbitz, 2003; Metting, 1993), hence the soil moisture content can play a significant role in determining bioavailability (Marschner & Kalbitz, 1993). The overall moisture content of a soil, and its ability to retain water, is largely determined by soil texture, organic matter content, and the local climatic conditions experienced by the soil (Rawls et al., 1982; Rawls et al., 2003; Saxton et al. 1986).

Table 2.5:- Soil moisture content (%) determined for samples collected during years 2013 and 2014.

Sludge Treatment	Soil Moisture Content (%)	
	2013	2014
AUC/Zn	32.23 (1.00)[a][1][2]	33.93 (1.01)[a]
AUC/Ctrl1	35.01 (1.45)[a]	34.79 (1.08)[a]
AUC/Cu	31.25 (2.35)[a]	31.77 (1.54)[a]
AUC/Ctrl2	32.36 (0.24)[a]	32.90 (1.66)[a]
AUC/NS	30.39 (1.44)[a]	31.40 (0.93)[a]
GLE/Zn	19.52 (1.28)[a]	21.70 (1.07)[a]
GLE/Ctrl1	19.67 (0.95)[a]	21.15 (1.54)[a]
GLE/Cu	19.76 (0.95)[a]	22.23 (1.77)[ab]
GLE/Ctrl2	18.14 (1.02)[a]	19.73 (0.95)[ab]
GLE/NS	16.39 (0.48)[a]	17.47 (0.40)[b]
HAR/Zn	57.68 (4.83)[a]	46.79 (2.99)[a]
HAR/Ctrl1	53.90 (6.67)[a]	46.43 (4.13)[a]
HAR/Cu	53.85 (3.11)[a]	44.98 (0.57)[a]
HAR/Ctrl2	58.08 (9.07)[a]	47.35 (3.99)[a]
HAR/NS	47.21 (4.29)[a]	41.78 (3.58)[a]
WOB/Zn	15.37 (1.09)[a]	19.73 (0.53)[a]
WOB/Ctrl1	14.78 (0.62)[ab]	18.05 (0.53)[ab]
WOB/Cu	14.67 (0.13)[ab]	18.16 (0.56)[ab]
WOB/Ctrl2	12.78 (0.64)[b]	16.78 (0.68)[bc]
WOB/NS	10.46 (0.59)[c]	14.96 (0.68)[c]

[1]Values in parenthesis are standard errors (n = 3). [2] Values without corresponding letters denotes statistical significance at $\alpha = 0.05$ for individual sampling events.

2.5.1. Soil Moisture Content Method

The moisture content (W_{H_2O}) of soils sampled in 2013 and 2014 was determined as a percentage of total mass by measuring the change in mass due to the evaporation of water. Approximately 30 g of fresh field moist soil was weighed out and heated at 105°C ± 5°C for ≥ 24 hours, following which the mass of dried soil was recorded immediately. Soil moisture content was then calculated as follows:

$$W_{H_2O} = \frac{m_1}{m_2} \times 100 \qquad (\mathbf{E.2.11})$$

where m_1 is the total mass of soil prior to heating, and m_2 is the mass of dried soil. The average moisture contents determined for the sludge amended and untreated soils at each of the LTSE field sites are shown in **Table 2.5**. For analytical methods requiring fresh field moist soil (i.e. the determination of C_{mic} and phosphatase enzyme activities), the quantities of soil used were adjusted in order to take W_{H_2O} into account, thereby allowing results to be expressed per gram of dry mass:

$$m_{H_2O} = \frac{m_{dm}\left(100 + W_{H_2O}\right)}{100} \qquad (\mathbf{E.2.12})$$

where m_{H_2O} is the required mass of field moist soil, m_{dm} is the required mass of dried soil, and W_{H_2O} is the soil moisture content (**E.2.11**).

2.5.2. Soil Moisture Content Results (2013-2014)

The only significant differences in soil moisture content between sludge amended and untreated soils were observed at the English sites, Woburn and Gleadthorpe. In 2014 the moisture content of the untreated soil at Gleadthorpe was significantly (p <0.05) lower in comparison to soils receiving the Zn and Cu sludge treatments (**Table 2.5**). Whereas at Woburn, the moisture content of the untreated soil was significantly (p <0.05) lower in comparison to each of the sludge amended soils during both 2013 and 2014 (**Table 2.5**); with the exception of soil receiving the undigested control in 2014. In addition, the moisture content for each of the soils sampled at Woburn in 2014 was significantly (p <0.05) higher in comparison to the previous year. Conversely, in 2014 the moisture content for soil receiving the Cu sludge treatment at Hartwood was significantly (p <0.05) lower in comparison to samples collected in 2013.

2.5.3. Soil Moisture Content Overview (2013-2014)

For both sampling events the mean soil moisture content, for all of the soils sampled at each field site (n = 15), was significantly (p <0.001) higher at the Scottish field sites in comparison to the English sites, with the moisture content at Hartwood also significantly (p <0.001) higher in comparison to Auchincruive. The moisture content at the Gleadthorpe was also significantly higher in comparison to Woburn during both 2013 (p <0.01) and 2014 (p <0.05). The observed differences in moisture content are most likely due to the differences in soil texture, with the clay content of the Scottish field sites more than twice that of the English field sites (**Table 2.2**), and organic matter content (**See Section 2.7**). In addition, the field site at Hartwood was waterlogged during the 2012 sampling event (**Table 2.4**), hence, although no rainfall was encountered for the reminder of the investigation, the differences in climatic conditions and the amount of rainfall received at each of the sites will also influence the moisture content of the collected samples.

2.6. Soil pH

Soil pH is an important environmental factor affecting the solubility, and therefore bioavailability, of both heavy metals (Alloway, 1995) and orthophosphate anions (Stevenson & Cole, 1999). In general the solubility of heavy metal cations is higher in acidic soils, and increases as the soil pH decreases. This is because positively charged H^+ ions are attracted to negatively charged surfaces within the soil environment and are capable of replacing most metal cations at these sites (Alloway, 1995). Analysis of pH is therefore necessary in determining the speciation of heavy metals in soil and the potential impact on microorganisms. In contrast the solubility of orthophosphate anions decreases as soil pH decreases, due to surface adsorption on either clay minerals or iron (Fe)/aluminium (Al) oxides, and

precipitation as insoluble Fe/Al-phosphates. Furthermore, phosphorus solubility also decreases with increasing soil pH due to precipitation as calcium (Ca)-phosphates, therefore the optimum pH for phosphorus availability is generally considered to be pH 6.5 (Stevenson & Cole, 1999). The activities of phosphatase enzymes are also influenced by pH (**See Section 1.5**), hence pH is also an important factor in determining the rate of organic phosphorus mineralisation within the soil environment.

As mentioned previously, soil pH at each of the LTSE field sites was controlled, for the duration of the experiment, by applications of $CaCO_3$. Soils at Woburn and Gleadthorpe were kept at a target pH of 6.5, while the Auchincruive and Hartwood sites were maintained at pH of 6.0 and 5.8, respectively. Soil pH was determined over the course of experimental Phases II, III, and IV by measuring the pH of soil suspended in water (Gibbs et al., 2006); deviations from the target pH values are shown in **Figure 2.6**. By 2011, the pH values for each of the soils sampled from Auchincruive were within 0.2 pH units below the target value, whereas soil pH at Hartwood was within 0.3 pH units above the target value (**Figure 2.6**). Soil pH at the Gleadthorpe site appeared to be persistently lower than the target pH of 6.5 for the duration of experimental Phases II and III. Whereas soil pH at Woburn increased steadily during experimental Phases II and III and exceeded the target pH, in all cases, by approximately 0.2-0.4 pH units in 2005 (**Figure 2.6**).

2.6.1. Soil pH Method

Soil pH was measured during the current investigation as the pH of a 1:5 (v/v) soil suspension in deionised water. Approximately 10 mL of air dried soil was suspended in 50 mL deionised water, mechanically shaken for 1 hour, then allowed to stand for at least 1 hour. Measurements were taken using an MA235 Advanced pH/Ion/^{o}C meter (Mettler Toledo) with a glass electrode. Results are summarised in **Table 2.6**.

2.6.2. Soil pH Results (2012-2014)

Auchincruive

In comparison to the previous two years, a significant decrease (p <0.01) in pH, was observed in all of the soils sampled at Auchincruive in 2014 (**Figure 2.7**). However soil pH still remained within 0.5 pH units of the target value. Significant differences (p <0.05) in pH were observed between soils receiving the Zn sludge treatment and the uncontaminated controls (Ctrl1 and Ctrl2) in 2013, as well as soils receiving the contaminated sludge treatments (Zn and Cu) in 2014 (**Table 2.6**). However, grouping the data according to the type of sludge applied showed no overall significant difference between untreated

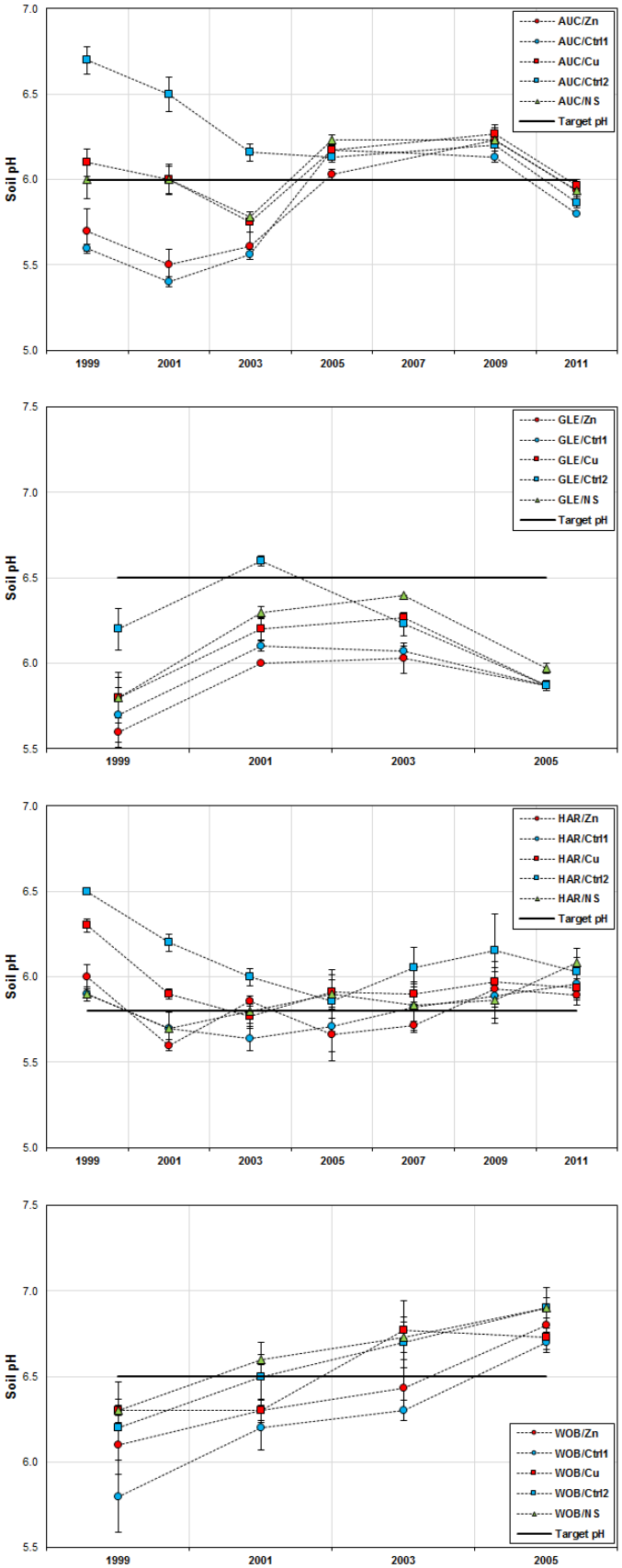


Figure 2.6:- Changes in soil pH during experimental Phase II (1999 – 2001), Phase III (2003 – 2005), and Phase IV (2006-2011), at the Long-Term Sludge Experiment field sites. Applications of lime ($CaCO_3$) were made throughout the experiment to maintain soil pH at speficfied target values (pH 6.5 for GLE and WOB, pH 6 for AUC, and pH 5.8 for HAR). Data obtained from Defra (2002, 2007a), Cooper (Personal Communication), and Crooks (Personal Communication). Error bars represent standard error (n = 3).

Table 2.6:- Soil pH measured over the course of three years (2012-2014) at the Long-Term Sludge Experiment field sites.

Sludge Treatment	Soil pH			
	2012	2013	2014	Mean
AUC/Zn	6.31 (0.05)[a][1][2]	6.19 (0.02)[a]	5.79 (0.05)[a]	6.10 (0.16)
AUC/Ctrl1	6.17 (0.07)[a]	6.04 (0.03)[b]	5.66 (0.05)[ab]	5.96 (0.20)
AUC/Cu	6.25 (0.04)[a]	6.11 (0.02)[abc]	5.58 (0.08)[b]	5.98 (0.20)
AUC/Ctrl2	6.16 (0.06)[a]	6.08 (0.03)[bc]	5.64 (0.04)[ab]	5.96 (0.16)
AUC/NS	6.28 (0.06)[a]	6.15 (0.02)[ac]	5.73 (0.03)[ab]	6.05 (0.16)
GLE/Zn	6.67 (0.03)[a]	6.54 (0.01)[a]	6.68 (0.07)[ab]	6.63 (0.05)
GLE/Ctrl1	6.85 (0.04)[b]	6.60 (0.06)[ab]	6.67 (0.02)[ab]	6.71 (0.07)
GLE/Cu	6.73 (0.10)[ab]	6.70 (0.05)[bc]	6.66 (0.07)[ab]	6.70 (0.02)
GLE/Ctrl2	6.82 (0.02)[ab]	6.74 (0.05)[bc]	6.58 (0.06)[b]	6.71 (0.07)
GLE/NS	6.87 (0.02)[b]	6.83 (0.04)[c]	6.79 (0.04)[a]	6.83 (0.02)
HAR/Zn	6.07 (0.06)[ab]	6.12 (0.10)[a]	5.82 (0.09)[ab]	6.00 (0.09)
HAR/Ctrl1	5.95 (0.14)[b]	5.87 (0.07)[a]	5.63 (0.06)[a]	5.82 (0.10)
HAR/Cu	6.05 (0.05)[b]	5.95 (0.08)[a]	5.67 (0.05)[a]	5.89 (0.11)
HAR/Ctrl2	6.30 (0.05)[a]	6.10 (0.10)[a]	6.00 (0.16)[b]	6.13 (0.09)
HAR/NS	6.12 (0.01)[ab]	5.96 (0.12)[a]	5.89 (0.10)[ab]	5.99 (0.07)
WOB/Zn	6.53 (0.09)[a]	6.43 (0.16)[a]	6.43 (0.06)[a]	6.46 (0.04)
WOB/Ctrl1	6.37 (0.17)[a]	6.27 (0.06)[a]	6.47 (0.05)[a]	6.37 (0.06)
WOB/Cu	6.37 (0.23)[a]	6.58 (0.25)[a]	6.94 (0.03)[b]	6.63 (0.17)
WOB/Ctrl2	6.43 (0.15)[a]	6.76 (0.39)[a]	6.50 (0.07)[a]	6.56 (0.10)
WOB/NS	6.23 (0.13)[a]	6.38 (0.05)[a]	6.22 (0.09)[c]	6.28 (0.05)

[1] Values in parenthesis are standard error (n = 3). [2] Values without corresponding letters denotes statistical significance at $\alpha = 0.05$ for individual sampling events.

soil (NS), soils receiving digested sludge (Zn and Ctrl1), or soils receiving the undigested sludge treatments (Cu and Ctrl2), for any of the sampling events.

Gleadthorpe

At Gleadthorpe soil pH remained, on average, 0.2 pH units above the target pH value for the duration of the current investigation (**Figure 2.8**); with the only significant ($p < 0.05$) changes observed over time in soils receiving the two uncontaminated sludge treatments (Ctrl1 and Ctrl2). Significant differences ($p < 0.01$) in pH were observed between soils receiving the digested sludge treatments in 2012, with pH of soil receiving the Zn sludge treatment, and soils receiving both of the digested sludge treatments (Zn and Ctrl1) also significantly ($p < 0.01$) lower than the untreated soil in 2012, and 2013, respectively (**Table 2.6**). As a result of this, an overall significant ($p < 0.01$) difference in pH was observed between soils receiving the different sludge types in 2013. However, by 2014, the only significant ($p < 0.05$) difference in pH was between untreated soil (pH 6.79) and soil receiving the undigested control (pH 6.58).

Hartwood

Soil pH at Hartwood declined steadily over the course of the current investigation, although the only significant ($p < 0.05$) changes over time were observed in soils receiving the Zn and Cu sludge treatments (**Figure 2.9**). The only significant differences between treatments were observed in 2012 (**Table 2.6**), where pH of soil receiving the undigested control was higher in comparison to both untreated soil ($p < 0.05$) and soil receiving the Cu sludge treatment ($p < 0.01$); no overall significant

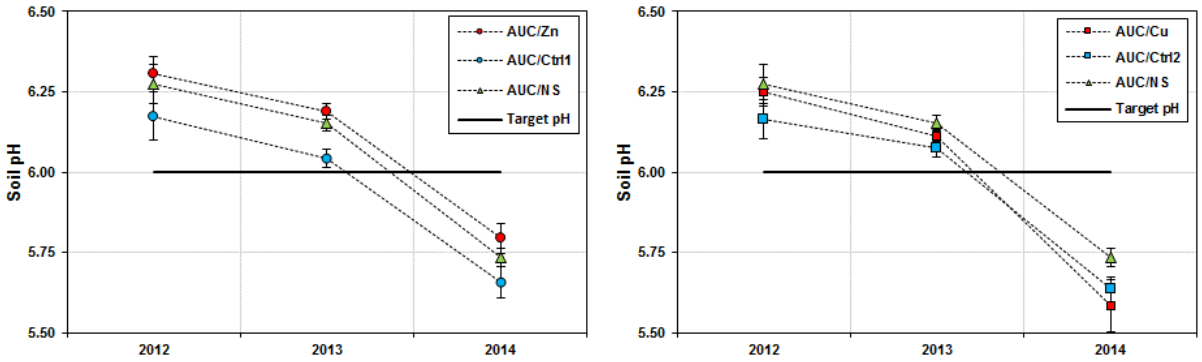


Figure 2.7:- Change in soil pH over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Auchincruive (AUC) field site. Error bars represent standard error (n = 3).

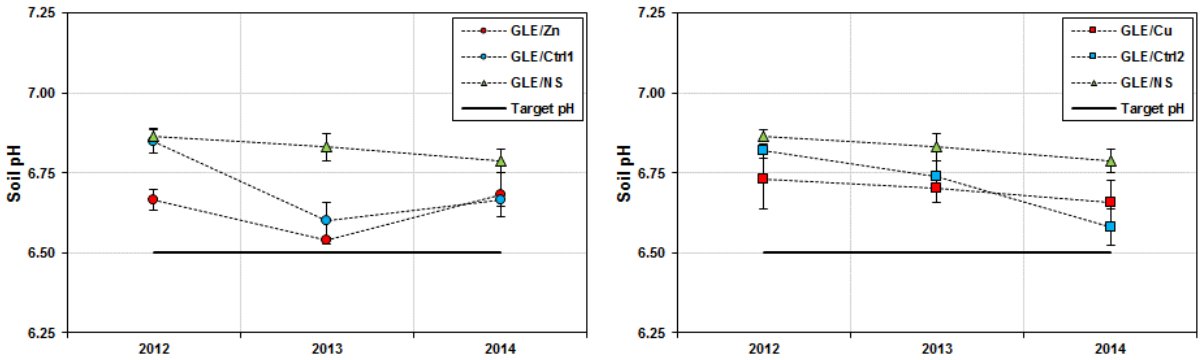


Figure 2.8:- Change in soil pH over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Gleadthorpe (GLE) field site. Error bars represent standard error (n = 3).

difference in pH was observed at any time between soils receiving either digested or undigested sludge types.

Woburn

With the exception of soil receiving the Cu sludge treatment, which increased (p <0.05) steadily to pH 6.94, soil pH at Woburn remained within 0.3 pH units of the target pH for the duration of the current investigation (**Figure 2.10**). Only in 2014 were any significant differences observed between soil treatments, with pH in soils receiving the digested control (p <0.05), undigested control (p <0.05), and Cu sludge treatment (p <0.001), all higher than the untreated soil (**Table 2.6**). In addition, due to the high pH observed in soil receiving the Cu sludge treatment, the difference in pH between soils receiving digested and undigested sludge was also significant (p <0.01) in 2014.

2.6.3. Soil pH Overview (2012-2014)

Overall, the mean soil pH values over the course of the current investigation (**Table 2.6**), were generally in agreement with the target pH values for each of the field sites and increased as follows:

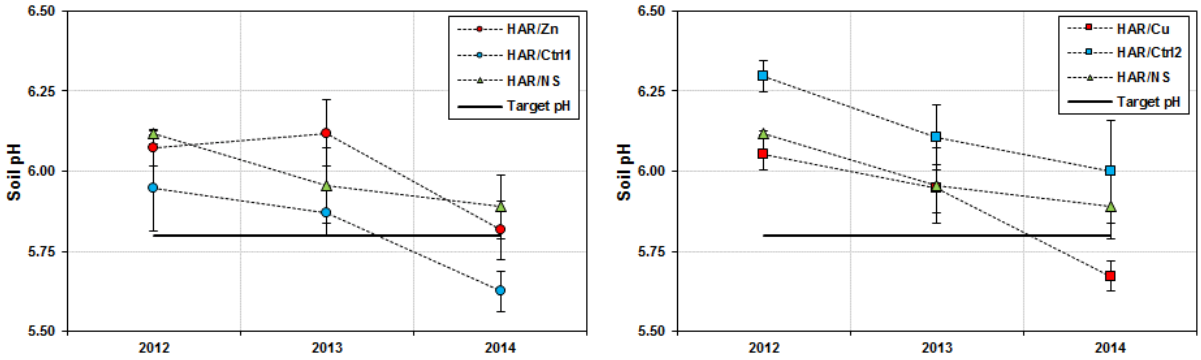


Figure 2.9:- Change in soil pH over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Hartwood (HAR) field site. Error bars represent standard error (n = 3).

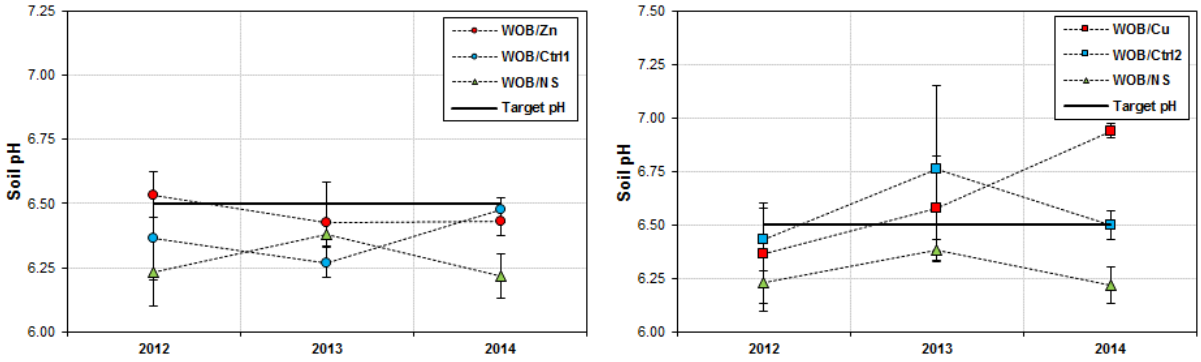


Figure 2.10:- Change in soil pH over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Woburn (WOB) field site. Error bars represent standard error (n = 3).

HAR (pH 5.82-6.13) ≤ AUC (pH 5.96-6.10) < WOB (pH 6.28-6.63) < GLE (pH 6.63-6.83)

Therefore it can be expected that available phosphorus (**See Section 4.2**) will be greatest at the two arable field sites (GLE and WOB), whereas the solubility of heavy metals (**See Section 3.5**) is likely to be greatest at Hartwood.

2.7. Soil Organic Carbon

Soil organic carbon (SOC), and organic matter as a whole, has an important structural role within soils influencing water holding capacity by facilitating the formation of soil aggregates. In addition it provides a feed-stock for both plants and soil microorganisms, whereby nutrients are released through the process of mineralisation (**See Section 1.5**), and, in general, the size of a soil microbial community is proportional to the amount of organic carbon present within the soil (Jenkinson & Ladd, 1981). Soil organic matter contains a number of chemical functional groups, such as hydroxyl (OH), and carboxyl (C=O), which can act as ligands forming coordination complexes with a range of metal cations present in the soil environment (Alloway, 1995); this has implications for the long-term use of sewage sludge as the bioavailability of heavy metals in soil is also dependent on soil organic matter.

McBride (1995) has previously described two opposing hypotheses, known as the '*sludge protection*' and '*sludge time-bomb*' hypotheses, which predict changes in the bioavailability and long-term fate of metal contamination according to the capacity of organic and inorganic components in soil and sludge to immobilise heavy metals. The '*sludge protection*' hypothesis predicts that the additional inorganic components (carbonates, phosphates, silicates, Fe/Al oxides) present in sewage sludge will maintain the adsorption capacity of the soil when applied, therefore the overall bioavailability of heavy metals should decrease over time. However, in contrast, the '*sludge time-bomb*' hypothesis places more emphasis on the immobilisation of metals by soil organic matter and acknowledges that soils have a finite capacity to adsorb metals. Therefore an increase in metal availability is predicted to occur over time as soil organic matter is mineralised and previously complexed metals are released into the soil solution (McBride, 1995).

Changes in SOC during experimental Phases II, III, and IV were determined using the method of potassium dichromate oxidation as described by MAFF (1986); changes over the course of the LTSE are shown in **Figure 2.11**. At the end of experimental Phase I, approximately 74 and 44 % (**Table 2.3**) of the organic C applied in the digested and undigested sludge treatments, remained in the respective soils at Auchincruive, hence SOC was approximately 10 % higher in soils receiving digested sludge in 1997. However, although this difference could still be seen at the start of experimental Phase II (**Figure 2.11**), no significant differences in SOC were reported for soils receiving the different sludge types, and by 2001, even the difference in SOC between sludge amended and untreated soils was no longer reported as significant (Defra, 2002). Similarly, for experimental Phase III, although SOC was reported to be higher in sludge amended soils, no significant differences in SOC were reported between soils receiving different sludge treatments (Defra, 2007a). A similar increase was also seen at Gleadthorpe, with SOC in soils receiving the digested sludge treatments approximately 20 % higher, at the end of experimental Phase I, in comparison to soils receiving undigested sludge (**Table 2.3**). However, by 1999, although SOC was highest (2.86 %) in soil receiving the Zn sludge treatment (**Figure 2.11**), this difference was not reported to be significant (Defra, 2002). No other significant differences in SOC were reported at Gleadthorpe for the remainder of the experiment (Defra, 2007a). Soil organic carbon appeared to be most variable at the Hartwood field site (**Figure 2.11**), with SOC at the end of Phase I only 8 % higher in soils receiving digested sludge than in those treated with undigested sludge (**Table 2.3**). Even the differences in SOC between sludge amended soils and untreated soils were not reported as significant during 1999 (Defra, 2002), and subsequently no significant changes in SOC were reported for the remainder of the experiment (Defra, 2007a). At the Woburn field site, SOC in soils receiving digested sludge was 9 % higher, at the end of experimental Phase I, than in soils receiving undigested sludge (**Table 2.3**), however no significant differences in SOC were reported for the duration of the experiment (Defra, 2002, 2007a).

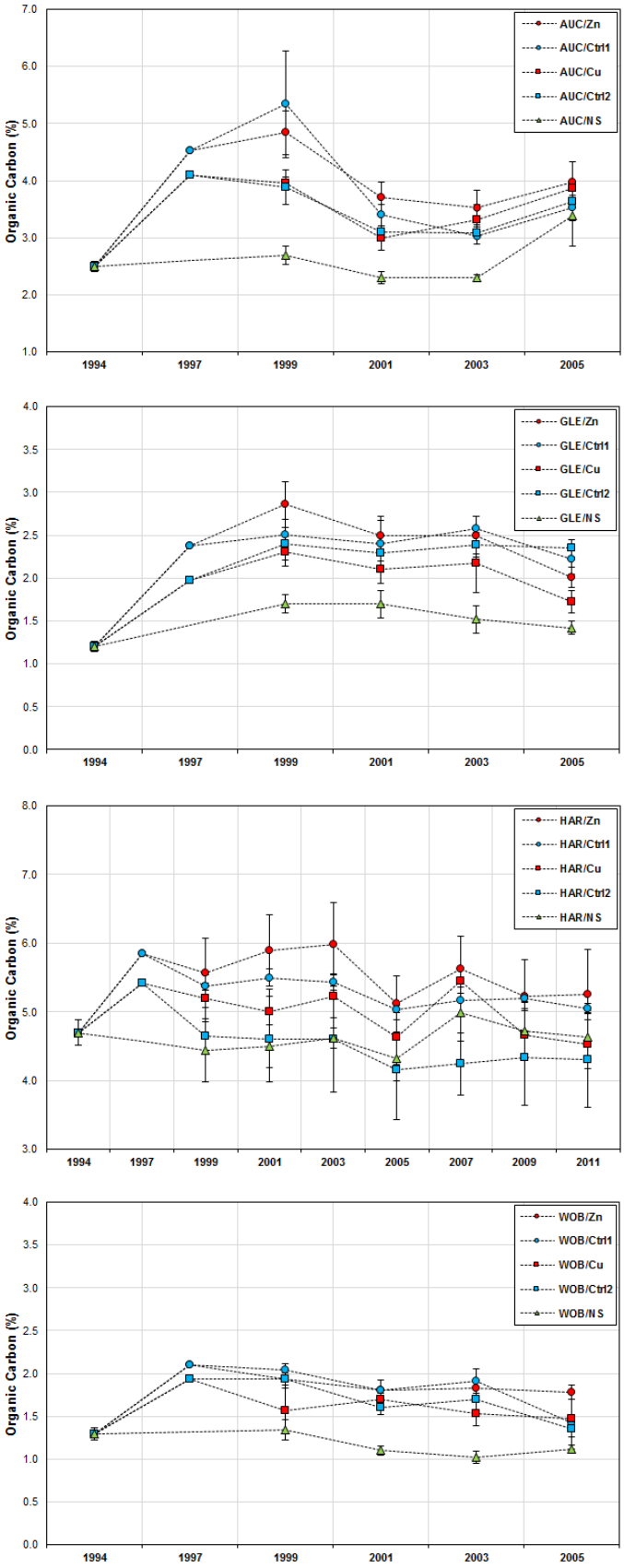


Figure 2.11:- Changes in soil organic carbon (%) during experimental Phase I (1994-1997), Phase II (1999 – 2001), Phase III (2003 – 2005), and Phase IV (2006-2011), at the Long-Term Sludge Experiment field sites. Initial values of soil organic carbon prior to sludge application in 1994 are also shown. Data obtained from Gibbs et al. (2006), Defra (2002, 2007a), and Cooper (Personal Communication). Error bars represent standard error (n = 3).

Table 2.7:- Soil organic carbon (%) and soil organic matter (%) measured over the course of three years (2012-2014) at the Long-Term Sludge Experiment field sites. Values of SOC were obtained for each sample by conversion of SOM using a scaling factor (F_C).

Sludge Treatment	Soil Organic Matter (%)			Soil Organic Carbon (%)			
	2012	2013	2014	2012	2013	2014	F_C
AUC/Zn	7.37 (0.21)$^{a[1][2]}$	7.26 (0.35)^{a}	6.73 (0.44)^{ab}	3.30 (0.19)^{a}	3.25 (0.16)^{a}	3.01 (0.24)^{ab}	0.45 (0.01)
AUC/Ctrl1	6.72 (0.35)^{b}	7.00 (0.36)^{ab}	6.93 (0.45)^{a}	3.00 (0.23)^{ab}	3.13 (0.24)^{a}	3.09 (0.24)^{b}	0.45 (0.01)
AUC/Cu	6.63 (0.52)^{b}	6.14 (0.52)^{bc}	6.50 (0.68)^{ab}	2.97 (0.27)^{ab}	2.75 (0.27)^{ab}	2.91 (0.34)^{a}	0.45 (0.01)
AUC/Ctrl2	6.67 (0.32)^{b}	6.55 (0.29)^{abc}	6.25 (0.25)^{ab}	2.98 (0.20)^{ab}	2.93 (0.18)^{ab}	2.79 (0.13)^{a}	0.45 (0.01)
AUC/NS	5.77 (0.12)^{b}	5.61 (0.13)^{c}	5.53 (0.05)^{b}	2.41 (0.18)^{b}	2.34 (0.17)^{b}	2.31 (0.13)^{a}	0.42 (0.02)
GLE/Zn	4.18 (0.32)^{ab}	4.57 (0.33)^{a}	3.95 (0.44)^{a}	2.11 (0.28)^{ab}	2.31 (0.29)^{ab}	2.01 (0.38)^{ab}	0.50 (0.04)
GLE/Ctrl1	4.50 (0.25)^{a}	4.24 (0.37)^{a}	3.93 (0.37)^{a}	2.35 (0.08)^{b}	2.23 (0.21)^{ab}	2.07 (0.20)^{ab}	0.52 (0.01)
GLE/Cu	3.24 (0.67)^{b}	3.94 (0.31)^{a}	3.81 (0.58)^{a}	2.04 (0.27)^{ab}	2.59 (0.33)^{b}	2.48 (0.36)^{b}	0.66 (0.09)
GLE/Ctrl2	3.58 (0.05)^{ab}	3.85 (0.16)^{ab}	3.59 (0.28)^{a}	2.10 (0.05)^{ab}	2.25 (0.08)^{ab}	2.10 (0.15)^{ab}	0.59 (0.01)
GLE/NS	3.13 (0.16)^{b}	3.05 (0.16)^{b}	3.00 (0.17)^{a}	1.67 (0.13)^{a}	1.63 (0.13)a	1.60 (0.14)^{a}	0.53 (0.01)
HAR/Zn	10.31 (0.69)^{a}	10.95 (1.06)^{a}	9.34 (0.74)^{a}	5.40 (0.39)^{a}	5.75 (0.62)^{a}	4.90 (0.48)^{a}	0.52 (0.01)
HAR/Ctrl1	9.12 (0.59)^{a}	10.12 (1.13)^{a}	7.90 (0.76)^{a}	4.53 (0.59)^{a}	5.05 (0.89)^{a}	3.94 (0.63)^{a}	0.49 (0.03)
HAR/Cu	9.22 (0.51)^{a}	9.96 (0.93)^{a}	8.44 (0.41)^{a}	4.64 (0.48)^{a}	5.03 (0.69)^{a}	4.24 (0.39)^{a}	0.50 (0.03)
HAR/Ctrl2	8.76 (1.14)^{a}	9.21 (0.69)^{a}	7.88 (1.09)^{a}	4.29 (0.80)^{a}	4.48 (0.55)^{a}	3.86 (0.72)^{a}	0.48 (0.03)
HAR/NS	8.31 (0.36)^{a}	9.43 (0.31)^{a}	7.56 (0.20)^{a}	4.21 (0.06)^{a}	4.81 (0.07)^{a}	3.83 (0.05)^{a}	0.51 (0.01)
WOB/Zn	3.36 (0.13)^{ab}	3.44 (0.29)^{ab}	3.53 (0.08)^{a}	1.66 (0.09)^{ab}	1.69 (0.11)^{ab}	1.74 (0.07)^{a}	0.49 (0.01)
WOB/Ctrl1	3.76 (0.06)^{b}	3.91 (0.08)^{b}	3.40 (0.21)^{a}	1.83 (0.02)^{a}	1.91 (0.02)^{a}	1.66 (0.10)^{a}	0.49 (0.01)
WOB/Cu	3.39 (0.12)^{ab}	3.53 (0.14)^{ab}	3.29 (0.16)^{a}	1.81 (0.11)^{a}	1.88 (0.10)^{a}	1.76 (0.14)^{a}	0.53 (0.02)
WOB/Ctrl2	3.09 (0.10)^{a}	3.21 (0.12)^{ac}	2.98 (0.21)^{ab}	1.47 (0.12)^{b}	1.52 (0.12)^{bc}	1.42 (0.18)^{ab}	0.47 (0.03)
WOB/NS	2.47 (0.20)^{c}	2.73 (0.23)^{c}	2.58 (0.18)^{b}	1.14 (0.08)^{c}	1.26 (0.09)^{c}	1.20 (0.07)^{b}	0.46 (0.01)

[1] Values in parenthesis are standard error (n = 3). [2] Values without corresponding letters denotes statistical significance at $\alpha = 0.05$ for individual sampling events.

By the end of Phase II (2001), soil organic carbon at both of the English field sites was reported to be approximately 0.9 % higher in soils receiving digested sludge treatments (Zn and Ctrl1) and 0.7 % higher in soils receiving undigested sludge treatments (Cu and Ctrl2) in comparison to untreated soil; whereas increases of 1.2 % and 0.6 % were seen for the two sludge types, respectively, at the Scottish sites (Defra, 2002). A general decrease in SOC was subsequently observed in 2005 at the end of Phase III, with SOC across all sites now reported to be 0.8 % higher in soils receiving digested sludge, and 0.6 % higher in soils receiving undigested sludge, in comparison to untreated soil (Defra, 2007a).

2.7.1. Soil Organic Carbon Method

Soil organic carbon was initially determined for samples taken in 2012, by heating approximately 1 mg of finely ground oven dried soil (105°C ± 5°C for ≥ 2 hours) to 900°C and calculating the percentage of total mass oxidised to carbon dioxide (CO_2) using a thermal conductivity detector (Vario EL III CHNOS Elemental Analyser, Elementar). Samples were pre-treated with a few drops of 4 M HCl, then oven dried (90°C; 4 hours), in order to completely remove inorganic carbonates. Soil organic carbon was then subsequently determined (for samples collected in 2013 and 2014 (**Table 2.4**)) by measuring the percentage of mass loss on ignition (LOI), and applying a scaling factor (F_C) in order to convert soil organic matter (SOM) to SOC:

$$F_C = \left(\frac{SOC}{SOM}\right) \qquad (\mathbf{E.2.13})$$

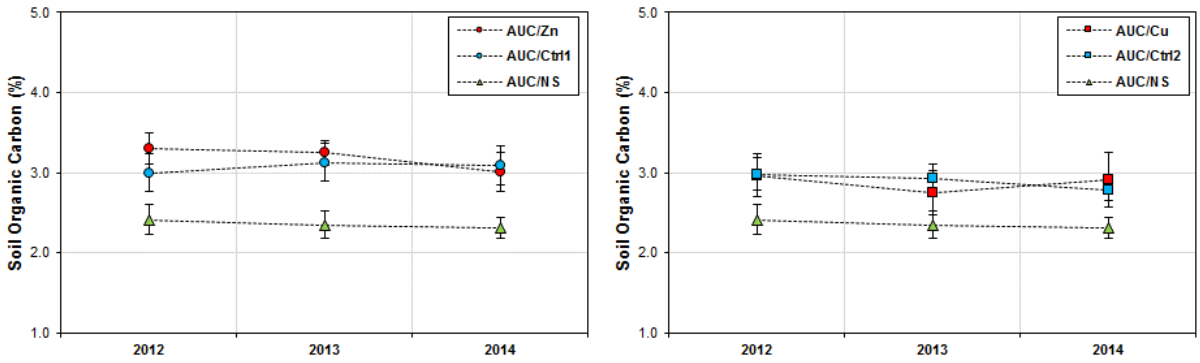


Figure 2.12:- Change in soil organic carbon (%) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Auchincruive (AUC) field site. Error bars represent standard error (n = 3).

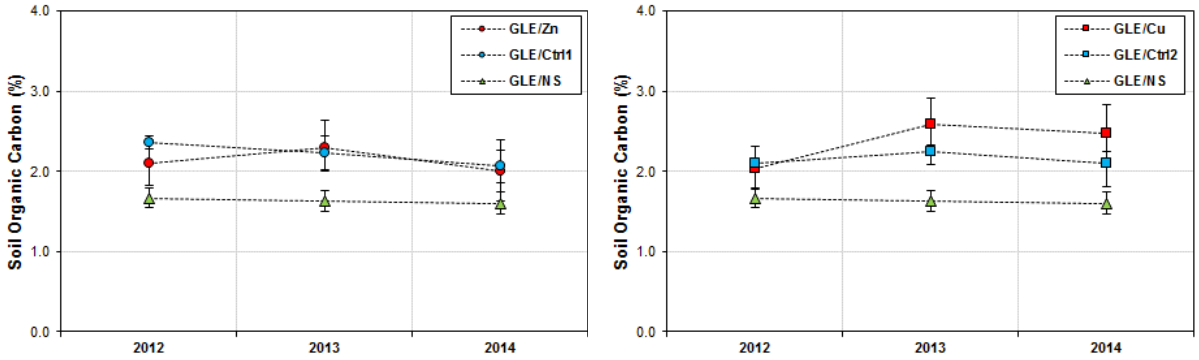


Figure 2.13:- Change in soil organic carbon (%) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Gleadthorpe (GLE) field site. Error bars represent standard error (n = 3).

Approximately 5 g of air-dried soil was weighed out and pre-heated at 105°C ± 5°C for ≥ 17 hours prior to recording the total mass of soil. Soil samples were then transferred to a muffle furnace (Carbolite AAF 1100) and heated at 450°C ± 10°C for 4 hours ± 15 minutes. The mass of ignited soil was recorded immediately after ignition. Soil organic carbon was then determined as follows:

$$\text{SOC}\ (\%) = \left(\frac{m_1}{m_2} \times 100\right) \times F_C \qquad \textbf{(E. 2. 14)}$$

where m_1 is the total mass of soil prior to ignition, and m_2 is the mass of ignited soil; results are summarised in **Table 2.7**.

2.7.2. Soil Organic Carbon Results (2012-2014)

Auchincruive

By 2012, SOC at Auchincruive had decreased to a mean value of 3.15 % in soils receiving digested sludge, and 2.97 % in soils receiving undigested sludge, with no significant difference between sludge types nor any significant change over the course of three years (**Figure 2.12**). However, in comparison to untreated soil, SOC was found to be significantly (p <0.05) higher in soil receiving the Zn sludge

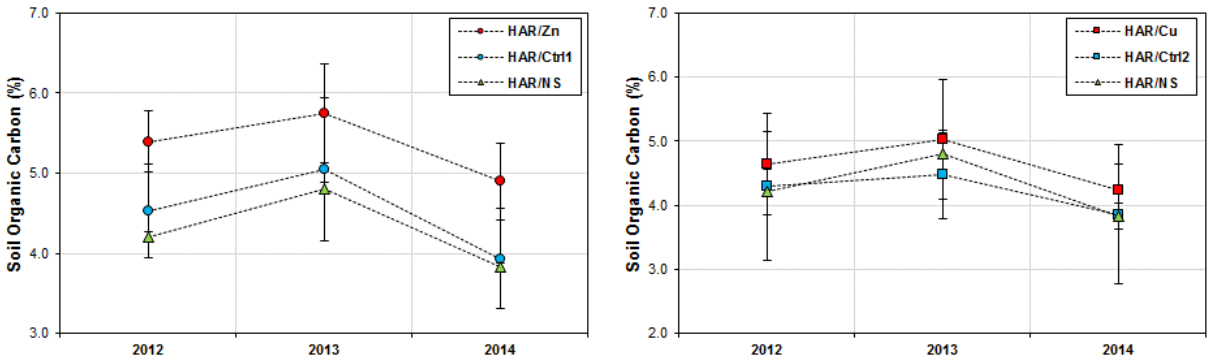


Figure 2.14:- Change in soil organic carbon (%) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Hartwood (HAR) field site. Error bars represent standard error (n = 3).

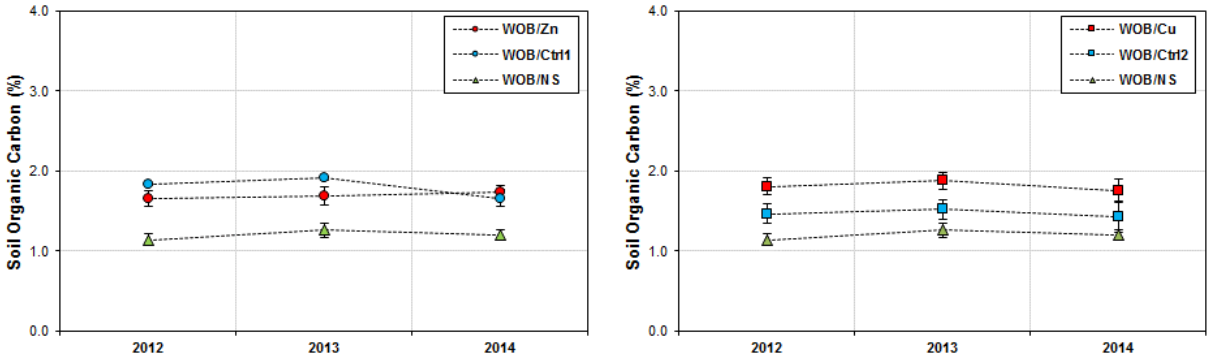


Figure 2.15:- Change in soil organic carbon (%) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Woburn (WOB) field site. Error bars represent standard error (n = 3).

treatment in years 2012 and 2013, and in soil receiving the digested control in years 2013 and 2014 (**Table 2.7**).

Gleadthorpe

In comparison to untreated soil, SOC at Gleadthorpe was found to be significantly (p <0.05) higher in soil receiving the digested control in 2012, and in soil receiving the Cu sludge treatment in years 2013 and 2014 (**Table 2.7; Figure 2.13**); however no significant differences were observed between soils receiving the different sludge types or over time.

Hartwood

No significant differences in SOC were observed at Hartwood between sludge amended and untreated soils or between soils receiving different sludge types over the course of the current investigation (**Table 2.7**). However, SOC in the untreated soil appeared to fluctuate significantly (p <0.01) over time reaching a maximum value of 4.8 % in 2013, before decreasing to 3.8 % in 2014 (**Figure 2.14**).

Woburn

In comparison to untreated soil, SOC at Woburn was found to be significantly higher in soils receiving the digested sludge treatments (Zn (p <0.05) and Ctrl1 (p <0.01)) for the duration of

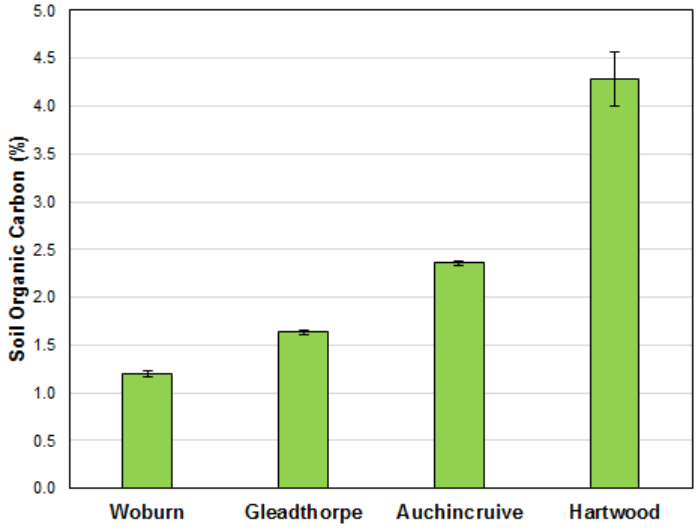


Figure 2.16:- Mean soil organic carbon (%) in untreated soil (NS) at each of the Long-Term Sludge Experiment field sites over the course of three years (2012-2014). Error bars represent standard error (n = 3).

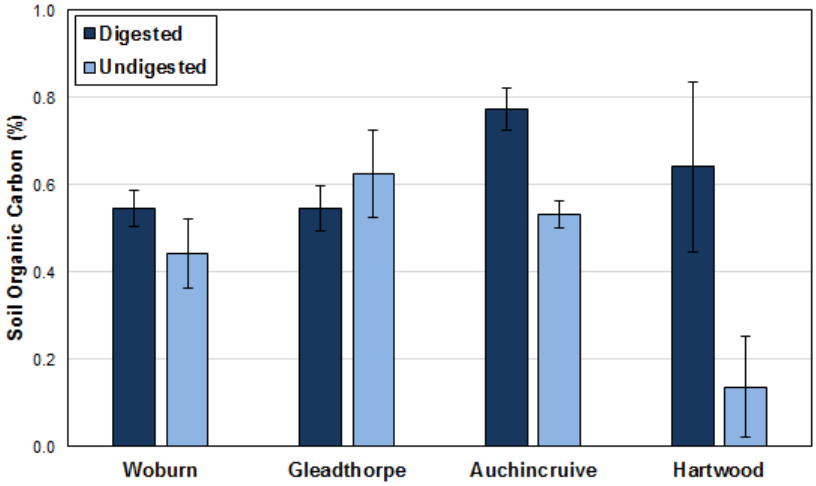


Figure 2.17:- Residual increase in soil organic carbon (%) at each of the Long-Term Sludge Experiment field sites in soils receiving either digested (Zn and Ctrl1) or undigested (Cu and Ctrl2) sludge types. Values are mean soil organic carbon over the course of three years (2012-2014). Error bars represent standard error (n = 3).

the current investigation (**Table 2.7**), despite a significant (p <0.05) decrease, in comparison to the previous year, observed in soil receiving the digested control in 2014 (**Figure 2.15**). Soil organic carbon also remained significantly higher (p <0.05) in soil receiving the Cu sludge treatment over the same time period (**Figure 2.15**). No other significant changes in SOC were observed over time, and no overall significant difference was observed between soils receiving different sludge types for any of the sampling events.

2.7.3. Soil Organic Carbon Overview (2012-2014)

Figure 2.16 shows the overall mean value of background SOC at each field site, over the course of three years (2012-2014), increasing in the following order:

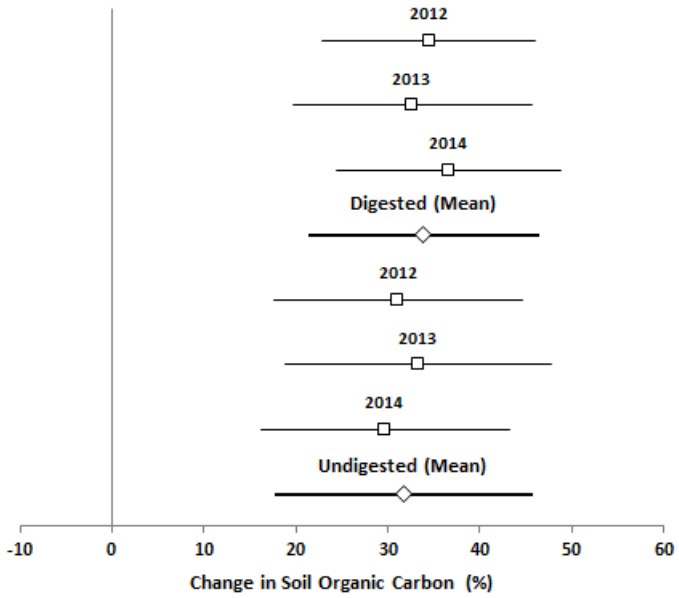


Figure 2.18:- Forest plot showing the overall change in soil organic carbon in soils receiving digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments, in comparison to untreated soil (NS), across all four LTSE fields sites for years 2012 to 2014. Horizontal lines represent 95 % confidence intervals. Effects are not statistically significant (p <0.05) if the 95 % confidence interval crosses the centre line.

WOB (1.20 %) < GLE (1.63 %) < AUC (2.36 %) < HAR (4.29 %)

With the exception of Woburn and Gleadthorpe, all differences in mean background SOC between sites were statistically (p <0.01) significant. Hence both sites managed as grassland contained higher levels of SOC in comparison to those where a ley/arable cropping rotation was implemented. These values also correspond to the differences in moisture content observed between soil samples collected from each of the field sites in 2013 and 2014 (**See Section 2.5**), hence due to higher levels of SOC the Scottish sites also appear to have a higher water holding capacity in comparison to the English sites.

In order to compare residual organic carbon from the applied sludge treatments between sites, mean values for background SOC were subtracted from that measured in sludge amended soils for each respective year. These values were then averaged to give the overall differences in SOC due to the application of different sludge types (**Figure 2.17**). In this instance, SOC in soils receiving digested sludge was significantly (p <0.05) higher in comparison to soils treated with undigested sludge, at both the grassland sites (AUC and HAR), reflecting the differences in SOC observed at the end of experimental Phase I; again this is likely due to the nature of the applied organic matter as more labile, and therefore readily degradable, organic matter would have been applied with the undigested sludge (Gibbs et al., 2006). No significant differences in SOC were observed when comparing between field sites, with the exception of soils receiving the undigested sludge treatments (Cu and Ctrl2) at Hartwood where SOC was significantly (p <0.05) lower than at the other three sites (**Figure 2.17**). This may be

due to the higher moisture content seen in soils at the Hartwood site (**Section 2.5**), which can increase the rate of organic C mineralisation (Rodrigo et al., 1997; Thomsen et al., 1999).

Combining the results for SOC (**Table 2.7**) using meta-analysis (**See Section 2.4.2**) showed that, over the course of the current investigation, soils receiving the digested sludge treatments (Zn and Ctrl1) still contained additional organic C equivalent to 34 % ($CL_{95\%}$ = 21 to 48 %) of that in the untreated soil (i.e. SOC was approximately 0.6 % higher). Whereas an additional 32 % ($CL_{95\%}$ = 18 to 47 %) organic C remained in soils receiving undigested sludge, in which SOC was approximately 0.4 % higher in comparison to untreated soil (**Figure 2.18**).

2.8. Chapter Discussion

2.8.1. Previous Findings (1997-2011)

Of the four field sites chosen, Woburn received the greatest C loadings (81 t ha^{-1}) for soils receiving both digested and undigested sludge treatments. However the least amount of the applied C loading remaining in sludge amended soils at the end of experimental Phase I was also observed at Woburn (**Table 2.3**); which would indicate a faster rate of organic C mineralisation in comparison to the other sites. Soils receiving the digested and undigested sludge treatments at Gleadthorpe also contained comparable quantities of the applied C loading at the end of experimental Phase I (**Table 2.3**). Though in this case, the quantities applied were the lowest of the four sites (68-69 t ha^{-1}). Therefore of the English sites, organic C mineralisation over the course of Phase I appears to have been faster at Woburn (digested = 15.2 t ha yr^{-1}; undigested = 16.4 t ha yr^{-1}), in relation to Gleadthorpe (digested = 11.5 t ha yr^{-1}; undigested = 13.7 t ha yr^{-1}), having reduced greater quantities of applied organic C over the same period of time.

In each case, the rate of organic C mineralisation at the Scottish sites, for soils receiving comparable sludge treatments, was lower in comparison to the English sites. The English sites were cultivated several times over the course of experimental Phases I-III (**See Section 2.3**), and continued to be cultivated during the current investigation; for instance the soil at Gleadthorpe had recently been ploughed prior to sample collection in 2013 (**Table 2.4**). This would have remobilised fresh organic C, increasing the accessibility to soil microorganisms, and therefore increasing the rate of organic C mineralisation. In contrast, the Scottish sites were maintained as grasslands for the duration of experimental Phases I-III. Hence the mobilisation of organic C, and therefore the bioavailability to soil microorganisms (Marschner & Kalbitz, 1993), would largely be due to the soil moisture content (Ladd et al., 1993). The moisture content of the Scottish sites, Hartwood in particular, was found to be greater in comparison to the English sites (**Section 2.5**), which would normally increase the rate of organic C mineralisation, however this may have been mitigated by the colder climatic conditions experienced by

the Scottish sites (Rodrigo et al., 1997). Hence, the differences in C loss between the Scottish grassland and English arable sites is most likely due to differences in the cropping regimes, and land management practices implemented at each site.

A greater rate of organic C mineralisation was seen at Hartwood (digested = 10 t ha yr^{-1}; undigested 15.8 t ha yr^{-1}) in comparison to Auchincruive (digested = 4.5 t ha yr^{-1}; 11.63 t ha yr^{-1}), where of the four sites the least amount of organic C was mineralised over the course of experimental Phase I; particularly in soils receiving the digested sludge treatments (**Table 2.3**). Since the organic C content at Hartwood was almost double that seen at Auchincruive, prior to the application of sludge treatments (**Table 2.1**), the rate of C reduction would be expected to be greater at Hartwood according to the relationship described by Bellamy et al. (2005), i.e. the rate of C loss is proportional to the original C content. This could also be a possible explanation for why the residual organic C in soils receiving the undigested sludge treatments at Hartwood was significantly lower in comparison to the other sites (**Figure 2.15**). However, since the climate, cropping regimes, and original C content at the English sites have been approximately equal for the duration of experimental Phases I-III (Defra, 2002, 2007a; Gibbs et al., 2006), it could be that the greater rate of C mineralisation seen at Woburn has been encouraged, in accordance with the relationship mentioned above (Bellamy et al., 2005), by the greater C loading applied.

2.8.2. Current Investigation (2012-2014)

Excepting the few significant differences in SOC observed between contaminated and uncontaminated soils described above (**Table 2.7**), the presence of Zn and Cu in soils receiving the respective sludge treatments appears to have had no effect on the mineralisation of organic C over the course of the LTSE. This observation is in agreement with the results of Cornfield et al. (1976) who found no significant effect on the rate of C mineralisation, measured as CO_2 production, in soils amended with sludge treatments containing 2652-5650 mg kg^{-1} and 758-999 mg kg^{-1} of Zn and Cu, respectively. Though in this case the concentrations of heavy metals applied in the sludge treatments were lower than in the treatments applied at the LTSE sites (**See Section 3.2**). However, as mentioned previously, if C mineralisation remains uninhibited in sludge amended soils, the loss of organic C from sludge amended soils still has implications for the bioavailability of heavy metal contamination. McGrath et al. (2000) investigated the decreases in soil organic matter in sludge amended soils at the Woburn Market Garden Experiment in order to determine the changes in Zn and Cd availability over time (**See Section 3.6**). Again, the presence of heavy metals appeared to have no effect on the rate of C loss from sludge amended soils, with 83-85 % of the applied C loading removed over a period of 23 years (1961-1984). However, it was concluded that although rapid mineralisation of applied organic C is observed in the short-term following sludge applications, there remains a recalcitrant fraction of organic matter that

could potentially remain in the soil for hundreds of years thereby reducing the overall bioavailability of heavy metals (McGrath et al., 2000). Presumably this is now the situation at the LTSE field sites. As described above, rapid mineralisation of organic C was observed over experimental Phase I, whereas little change was observed for the remainder of the experiment (**Figure 2.11**). Now, SOC generally remains higher in sludge amended soils (**Figure 2.18**) with few significant changes in SOC observed over time (**See Section 2.7.2**), therefore it may take decades before SOC in sludge amended soils returns to that of that of untreated soil. McGrath et al. (2000) also suggest that the presence of a recalcitrant fraction of organic matter could prevent accurate testing of the '*sludge time-bomb*' hypothesis as this may still provide some degree of protection and bind metals more strongly than more labile organic matter. Only when the entire C loading has been lost from the soil can the influence of organic matter on metal bioavailability be accurately determined. In addition, the soil pH at the Woburn Market Garden Experiment was also maintained at approximately pH 6.5 using $CaCO_3$ (McGrath et al. 2000). This may need to be a common practice for sludge amended soils as significant acidification has been observed in soils receiving regular applications of sewage sludge at the 'Long-Term Soil Organic Matter Experiment' in Ultuna, Sweden. Kirchmann et al. (1994) report decreases of approximately 0.7-1.2 pH units over the course of 35 years in soils receiving biennial applications of sewage sludge, where no applications of lime were made. Hence the protection offered by a recalcitrant fraction of organic matter may be offset by an increase in metal solubility due to decreasing soil pH, in this case a '*sludge time-bomb*' scenario would be due to acidification rather than the loss of organic C.

It should also be noted here that the dry combustion and ignition methods used to determine SOC during the current investigation (**See Section 2.7.1.**) differ from the chemical oxidation method used previously. The comparability of these methods has been investigated by Da Silva Dias et al. (2013) who found that values for SOC determined by the dry combustion method were comparable to those determined by chemical oxidation whereas values determined by the loss on ignition method were approximately 3 times greater in comparison. This discrepancy is possibly due to incomplete oxidation by potassium dichromate, the additional combustion of inorganic carbon materials during ignition, or both. Different conversion factors are also applied during the two methods in order to determine a final value for organic C (Bisutti et al., 2004; Da Silva Dias et al., 2013). For chemical oxidation this takes into account the incomplete oxidation of organic C, whereas for the ignition method the value for total organic matter is converted to total organic C (**See Section 2.7.1.**). However the accuracy of using a single conversion factor for a range of different soil samples is often debated (**See Section 7.2**). The values for SOC determined by chemical oxidation at the Hartwood site in 2011 (4.31-5.26 %; **Figure 2.11**) are in good agreement with those determined by dry combustion in 2012 (4.21-5.40 %; **Table 2.7**) which gives some indication that the conversion factors subsequently used to determine are accurate.

2.9. Conclusions

2.9.1. Previous Findings (1997-2011)

Following the final applications of sewage sludge at the end of experimental Phase I, rapid mineralisation of the applied organic matter appears to have occurred at each site. The fastest rate of C loss was observed at Woburn, with the slowest rates seen at Auchincruive; C loss at Hartwood and Gleadthorpe were approximately equal. Despite some fluctuations over the course of experimental Phases II, III, and IV, SOC now appears to have stabilised and in general has remained constant for the duration of the current investigation.

2.9.2. Current Investigation (2012-2014)

With the exception of the Gleadthorpe site, the target pH for each of the LTSE field sites fell within the range of pH values measured for both sludge amended and untreated soils during the current investigation; soil pH at Gleadthorpe was persistently higher than the target pH of 6.5. Background quantities of SOC differed significantly across field sites, with the highest values observed at Hartwood and lowest at Woburn. The differences in SOC also corresponded to the differences in soil moisture content observed between each site. Combining the results across all four field sites using meta-analysis indicated that the remaining SOC in sludge amended soils was approximately 0.4-0.6 % higher than in untreated soil.

No significant differences in residual organic C, derived from the applied sludge treatments, were observed between soils receiving the digested sludge treatments (Zn and Ctrl1) at any of the LTSE field sites. Hence, with regards to **Research Question 2**, differences in the speciation of Zn is likely to be most influenced by the inherent SOC content present in the untreated soil at each field site and the target pH maintained at each site. Soil pH was lowest at Hartwood, therefore it can be expected that the solubility of Zn will be greatest at this site, however, Hartwood also had the greatest SOC content hence this may offer some protection. For soils receiving the undigested sludge treatments (Cu and Ctrl2), residual organic C was significantly lower at the Hartwood site in in comparison to the other sites, which could mean a potential increase in the bioavailability of Cu in comparison to the other field sites. Though again the potential release of Cu into the soil solution may be mitigated by the high SOC content present at the Hartwood site.

CHAPTER 3

METAL CONTAMINATION

3. METAL CONTAMINATION

3.1. Introduction

The aim of this chapter is to describe the current state of metal contamination at each of the Long-Term Sludge Experiment field sites. Background information regarding the long-term behaviour of Zn and Cu contamination in sludge amended soils at each site is also provided and the total concentrations of each metal are compared to the current UK statutory limits. The chemical speciation of Zn and Cu is also investigated in order to estimate the potential bioavailability of metals to soil microorganisms.

3.2. Dose Response Curves

Prior to the application of sludge in 1994, mean background concentrations of Zn and Cu at the Long-Term Sludge Experiment field sites ranged from 34.4 to 82.4 mg kg^{-1} and 7.4 to 22.6 mg kg^{-1}, respectively (**Table 3.1**; Gibbs et al., 2006); with the lowest concentrations of both metals seen at Gleadthorpe and the highest at Auchincruive. As mentioned in **Section 2.2**, digested sludge cake, contaminated with Zn, and raw undigested sludge cake, contaminated with Cu were applied annually during experimental Phase I (1994-1997) in order to produce dose response curves whereby the total concentrations of Zn and Cu in the sludge amended soils increased from background levels in untreated soil, to above the current UK regulatory limits. The heavy metal content of the applied sludge treatments are shown in **Table 3.2**, with the target concentrations for each metal, at each level of the dose response curve, shown in **Table 3.3**. At the end of experimental Phase I, concentrations of Zn and Cu at dose response 'Level 3' were, respectively, within ± 20 and ± 30 % of the target concentration at each of the field sites (**Table 3.3**; Gibbs et al., 2006), and were closest to the current maximum concentrations for each metal permitted by the UK Sludge (Use In Agriculture) Regulations (**Table 1.2**).

3.3. Experimental Confounds

A statistical review of data from the Long-Term Sludge Experiment was subsequently carried out in 2008, following the end of experimental Phase III (Defra, 2008). During this investigation, a number of experimental confounds were identified which affect both the analysis and interpretation of data obtained from the LTSE field sites. In the first instance, as mentioned previously, not all of the target metal concentrations were achieved (**Table 3.3**). Therefore multiple regression analysis

Table 3.1:- Background total metal concentrations (mg kg^{-1}) of Zinc (Zn) and Copper (Cu) in untreated soil (0-25 cm) at the Long-Term Sludge Experiment field sites prior to initial sludge applications in 1994. Data obtained from Gibbs et al. (2006).

	Total Metal Concentration (mg kg^{-1})	
Field Site	**Zinc (Zn)**	**Copper (Cu)**
Auchincruive (AUC)	82.4 (0.42)[1]	22.6 (0.14)
Gleadthorpe (GLE)	34.4 (0.92)	7.4 (0.28)
Hartwood (HAR)	72.3 (1.87)	19.8 (0.33)
Woburn (WOB)	44.8 (1.06)	13.9 (0.36)

[1] Values in parenthesis are standard error (n = 3).

Table 3.2:- Mean total metal concentration (mg kg^{-1}) of sludge treatments applied at the Long-Term Sludge Experiment field sites over the course of four years (1994-1997). Data obtained from Defra (2002, 2007a).

		Total Metal Concentration (mg kg^{-1})	
Sludge Treatment	**Source**	**Zinc (Zn)**	**Copper (Cu)**
Zinc (Zn)	Coleshill, Warwickshire	6002	1423
Digested Control (Ctrl1)	Banbury, Oxfordshire	559	589
Copper (Cu)	Selkirk, Scottish Borders	553	5049
Undigested Control (Ctrl2)	Carterton, Oxfordshire	491	453

Table 3.3:- Target and achieved total metal concentrations (mg kg^{-1}) for Zinc and Copper dose response curves measured at each of the Long-Term Sludge Experiment field sites following the final sludge application in 1997. Data obtained from Gibbs et al. (2006).

		Concentration Achieved (mg kg^{-1})			
Dose Response Level	**Target Concentration (mg kg^{-1})**	**AUC**	**GLE**	**HAR**	**WOB**
Zinc					
Level 1 (Zn)	150	147 (1.8)[1]	134 (6.6)	171 (16.8)	125 (14.1)
Level 2 (Zn)	250	236 (10.2)	232 (49.0)	240 (11.6)	211 (8.1)
Level 3 (Zn)[2]	**350**	**314 (8.1)**	**334 (44.4)**	**406 (30.9)**	**304 (24.9)**
Level 4 (Zn)	450	342 (30.3)	291 (29.4)	443 (4.2)	224 (16.4)
Digested Control (Ctrl1)	-	111 (3.3)	85 (2.3)	108 (0.4)	76 (14.7)
Copper					
Level 1 (Cu)	50	66 (4.2)	69 (6.8)	74 (8.4)	56 (3.3)
Level 2 (Cu)	100	103 (9.1)	120 (18.3)	129 (6.6)	92 (10.3)
Level 3 (Cu)	**150**	**140 (17.0)**	**188 (39.4)**	**195 (15.7)**	**161 (15.0)**
Level 4 (Cu)	200	206 (31.6)	166 (23.6)	239 (17.1)	188 (37.6)
Undigested Control (Ctrl2)	-	53 (2.3)	37 (2.2)	56 (8.6)	36 (1.1)

[1] Values in parenthesis are standard error (n = 3). [2]Values in bold are the target concentrations for dose-response Level 3, closest to the UK statutory limits.

was used, instead of ANOVA, to directly relate variables, such as soil microbial biomass, to the actual concentrations of heavy metals at each site (Defra, 2008). More importantly, however, it was noted that the concentration of Cu in the sludge used to prepare the Zn sludge treatment, was more than twice of that in the sludge used as the digested control (**Table 3.2**). As a result of this, the total concentration of Cu in soils receiving the Zn sludge treatment was also significantly higher than in soils receiving the digested control (Defra, 2008). However, this was not the case for soils receiving the Cu sludge treatment, in which only the total concentration of Cu had been significantly increased. Therefore, in order to account for the effect of Cu, it was possible to adjust the data obtained from soils receiving the Zn sludge treatment using the coefficients obtained from the regression analysis of a dependent variable on total Cu concentration (Defra, 2008). For instance the effect of Cu on C_{mic} was accounted for by

adding $-m \times \ln([\mathrm{Cu}])$ to the value of C_{mic} determined in soils receiving the Zn sludge treatment, where *m* is the slope of regression between ln (Cu) and C_{mic}, for soils receiving the Cu sludge treatment, and [Cu] is the total concentration of confounding Cu contamination (Defra, 2008).However this approach assumes individual effects for Zn and Cu and does not take into account the possibility of an additive effect as suggested by Chander and Brookes (1993; **See Section 1.4**). Furthermore, the error determined for the effect of Cu was not taken into account when adjusting the data for soils receiving the Zn sludge treatment, hence the effect determined for Zn is an approximation and must be considered with caution (Defra, 2008).

3.4. Total Metal Concentration

Figure 3.1 and **Figure 3.2** show, respectively, the changes in the total concentrations of Zn and Cu, determined by *aqua-regia* ($HCl:HNO_3$) acid digestion (Defra 2002, Defra 2007a), in both untreated and sludge amended soils, at each of LTSE field sites, over the course of experimental Phases I-IV (1994-2011). In all cases, following the final applications of sludge in 1997, the total concentration of Zn in soils receiving the Zn sludge treatment (at Level 3) had increased above the current UK statutory limit of 300 mg Zn kg^{-1} in sludge amended soil (**Table 3.3**; Gibbs et al., 2006), with the greatest increase seen at Hartwood (**Figure 3.1**). Slight decreases in total Zn concentration were reported during experimental Phase II at the English field sites, however, these were attributed to the continuing cultivation of the plots and further homogenisation of the applied sludge within the soil matrix (Defra, 2002). By the end of experimental Phase III, total Zn concentration in soils receiving the Zn sludge treatment, at all of the field sites, was still within ± 10 % of the values reported for 1997 (**Table 3.3**). However, it should be noted that at this point the total Zn concentration at Woburn had fallen to 273.28 mg Zn kg^{-1}; below the UK statutory limit (**Figure 3.1**). Additional data obtained for the Scottish field sites during experimental Phase IV shows little change in the total concentration of Zn, with values at both sites still within ± 10 % of that measured in 1997 (**Figure 3.1**). Although concentrations of total Zn in soils receiving the remaining sludge treatments (Cu, Ctrl1, and Ctrl2) were generally higher than in the untreated soil, no statistical analysis has been reported which would indicate whether the increases were significant.

By the end of experimental Phase I, the total concentration of Cu in soils receiving the Cu sludge treatment had also increased above the current UK statutory limit (135 mg Cu kg^{-1}) for Cu in sludge amended soil at each of the LTSE field sites, again with the greatest increase seen at the Hartwood field site (**Table 3.3**; Gibbs et al., 2006). Subsequent changes in total Cu concentration in both sludge amended and untreated soils during experimental Phases II-IV are shown in **Figure 3.2**. A slight decrease in the total Cu concentration of soils receiving the Cu sludge treatment appears to have occurred at Auchincruive, and both of the English field sites, during experimental Phases II and III,

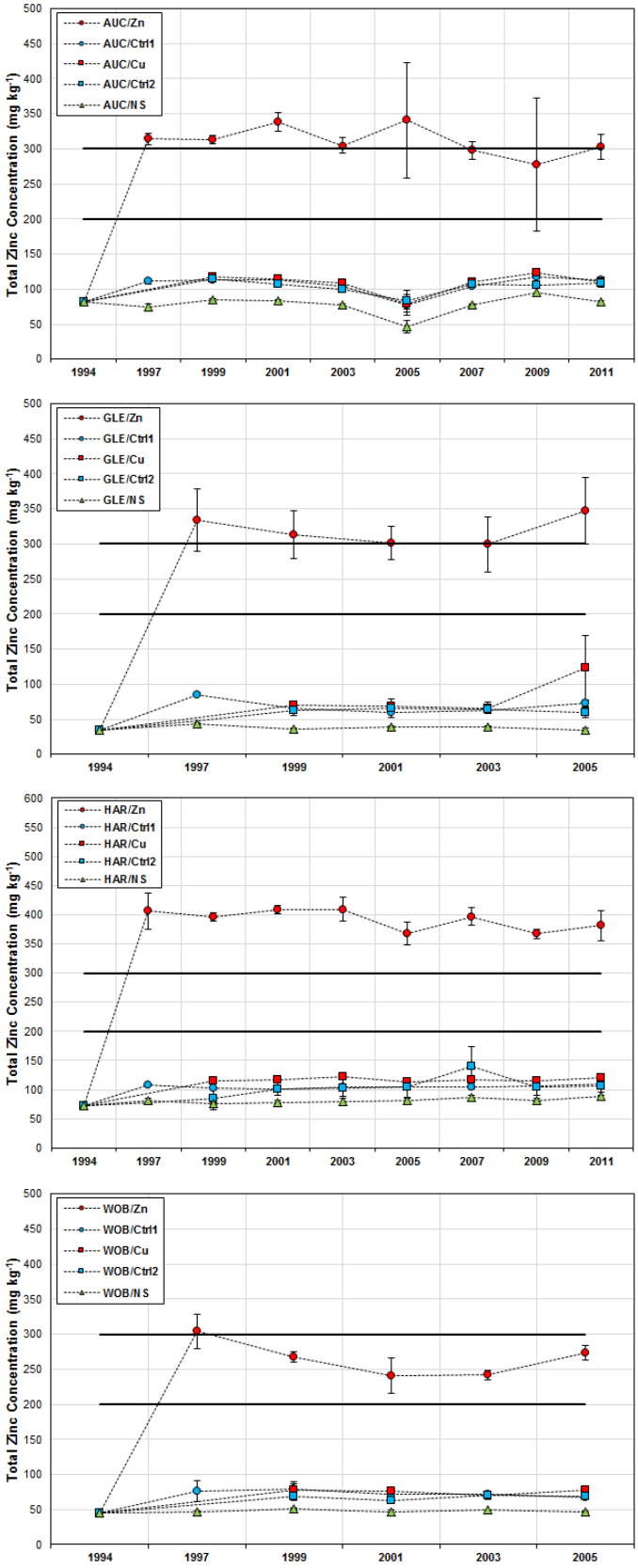


Figure 3.1:- Changes in total Zinc concentration (mg Zn kg^{-1}) during experimental phases I-IV (1994 - 2011) in soils receiving digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments, plus untreated soil (NS), at the Long-Term Sludge Experiment field sites. Current UK statutory (300 mg Zn kg^{-1}) and Advisory (200 mg Zn kg^{-1}) limits for Zn in sludge amended soil are also shown. Data obtained from Gibbs et al. (2006), Defra (2002, 2007a), Cooper (Personal Communication), and Crooks (Personal Communication). Error bars represent standard error (n = 3).

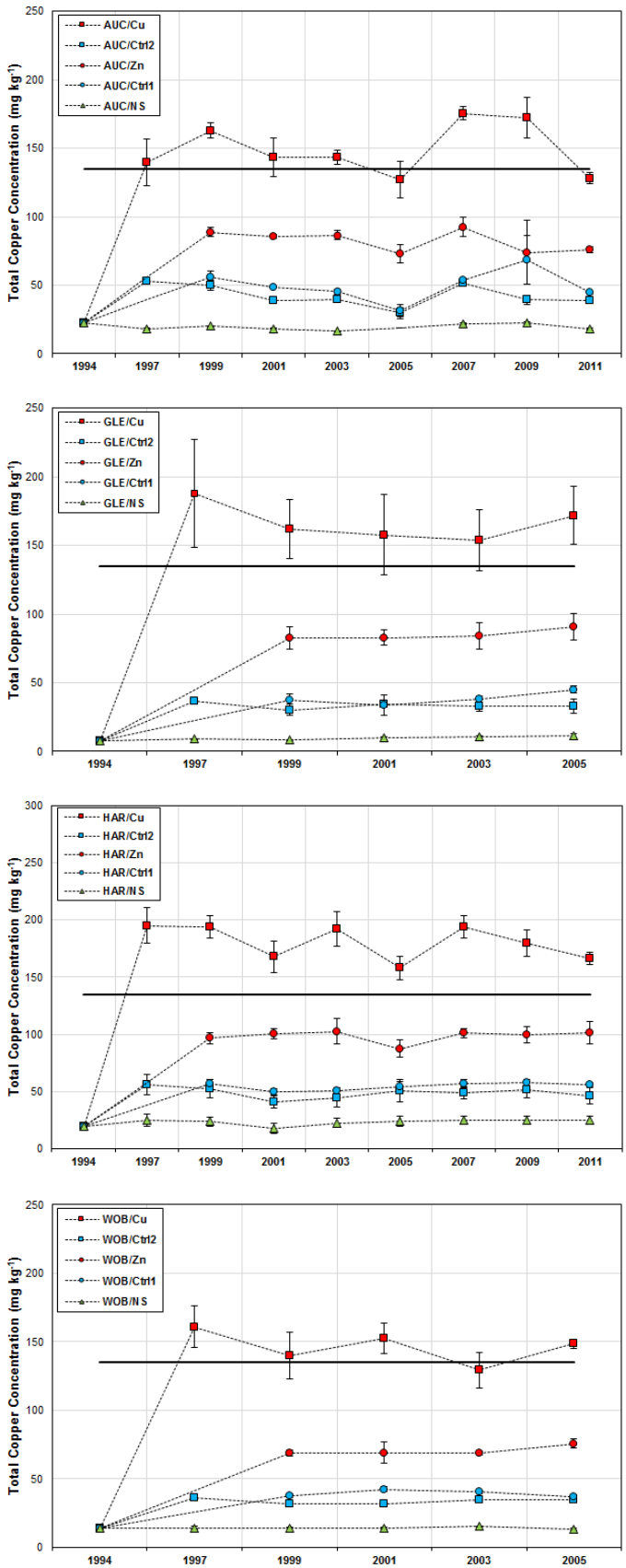


Figure 3.2:- Changes in total Copper concentration (mg Cu kg^{-1}) during experimental phases I-IV (1994 - 2011) in soils receiving digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments, plus untreated soil (NS), at the Long-Term Sludge Experiment field sites. Current UK statutory (135 mg Cu kg^{-1}) limit for Cu in sludge amended soil is also shown. Data obtained from Gibbs et al. (2006), Defra (2002, 2007a), Cooper (Personal Communication), and Crooks (Personal Communication). Error bars represent standard error (n = 3).

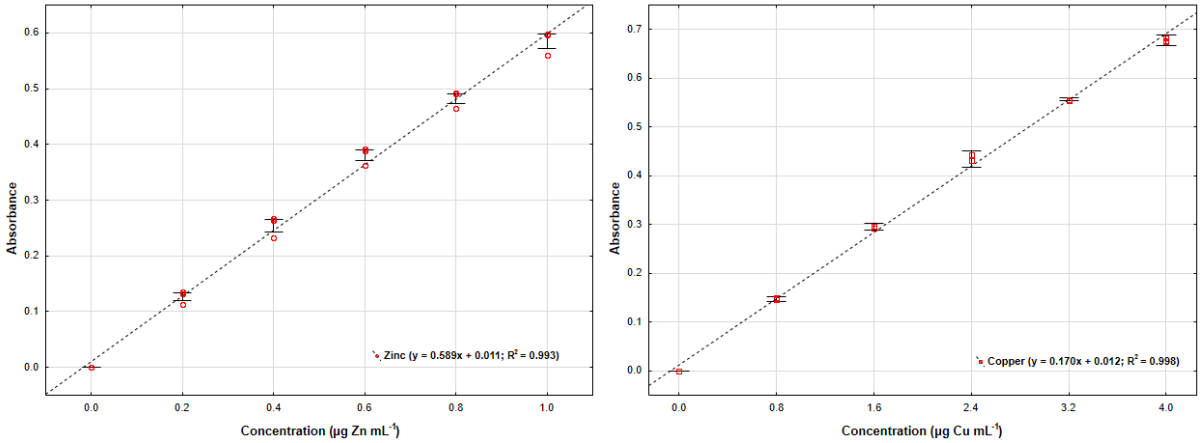


Figure 3.3:- Calibration graphs used to determine concentrations (µg mL^{-1}) of Zinc (left) and Copper (right) by atomic absorption spectroscopy. Error bars represent standard error (n = 3).

with values measured in 2005 approximately 10 % lower than in 1999, whereas the value at Hartwood had decreased by 20 % (**Figure 3.2**). Additional data for the Scottish field sites during experimental Phase IV indicates no further change in total Cu concentration has occurred, with values in 2011 still within 10 and 20 % of the initial values at Auchincruive and Hartwood, respectively. As mentioned previously, the total concentration of Cu in soil receiving the Zn sludge treatments also increased in comparison to the digested and undigested controls, which in turn, contained higher concentrations of Cu in comparison to the untreated soil (**Figure 3.2**). These increases occur at all of the field sites and are present for the duration of the experiment, however, only the increases in soil receiving the Zn sludge treatment have been reported as statistically significant (Defra, 2008).

3.4.1. Total Metal Concentration Method

For the current investigation, total metal concentration (mg kg^{-1}) was determined by flame atomic absorption spectroscopy (AAS) following microwave digestion of soil samples in *aqua-regia* (HCl: HNO_3). Approximately 0.5 g of air-dried soil was weighed out into a microwave digestion sample tube and suspended in 6 mL HCl (1.18 specific gravity), plus 2 mL HNO_3 (1.42 specific gravity), before leaving to dissolve for ≥ 24 hours. Samples were then heated (170°C) under pressure (20 bar) using a Multiwave 3000 Microwave Reaction System (Anton Paar). Following digestion, samples were filtered (Whatman No. 542) and diluted to 100 mL final volume in deionised water.

Calibration graphs for AAS analysis were prepared as follows:

- **Zinc:** 1 mL of 1000 µg mL^{-1} Zn working standard solution (Pure Grade AS Calibration Standard, PerkinElmer) was diluted to 200 mL final volume in deionised water to give 5 µg mL^{-1} stock solution. Aliquots of 0, 1, 2, 3, 4, and 5 mL were diluted to 25 mL final volume in deionised water to give 0.0, 0.2, 0.4, 0.6, 0.8, 1.0 µg Zn mL^{-1} calibration standards.

Table 3.4:- Concentrations of Zinc and Copper measured in Community Bureau of Reference (BCR) certified reference material (CRM) No. 143 – Trace Elements in a Sewage Sludge Amended Soil.

	Copper (mg kg^{-1})	Zinc (mg kg^{-1})
Specified Concentration	236	1301
CRM #1[1]	223.20	1174.18
CRM #2	215.78	1198.10
CRM #3	210.10	1219.76
CRM #4	207.18	1236.82
CRM #5	204.66	1235.31
CRM #6	209.29	1241.99
Mean	211.70	1217.69
Standard Deviation	6.74	26.64
Standard Error	2.75	10.87
Accuracy %	90	94

[1] Samples were diluted x10 for analysis by AAS following *aqua-regia* acid digest.

- **Copper:** 1 mL of 1000 µg mL^{-1} Cu working standard solution (Pure Grade AS Calibration Standard, PerkinElmer) was diluted to 50 mL final volume in deionised water to give 20 µg mL^{-1} stock solution. Aliquots of 0, 1, 2, 3, 4, and 5 mL were then diluted to 25 mL final volume in deionised water to give 0.0, 0.8, 1.6, 2.4, 3.2, 4.0 µg Cu mL^{-1} calibration standards.

Total metal concentration was measured using an AAnalyst 800 Atomic Absorption Spectrometer (PerkinElmer) running WinLab 32 for AA (v 6.4.0.0191) software. Samples were aspirated into an air-acetylene flame and the absorbance of light at wavelengths 213.9 nm (electric discharge lamp) and 324.8 nm (hollow cathode lamp) was used to determine the total concentrations of Zn and Cu, respectively, by reference to a calibration graph (**Figure 3.3**).

The total metal concentration in the original sample of digested soil was then calculated as follows:

$$[\mathrm{M}^+] = \frac{([M_S^+] - [M_B^+]) \times v \times D}{m} \qquad (\mathbf{E.3.1})$$

where [M^+_S] is the total metal concentration in the diluted sample (µg mL^{-1}), [M^+_B] is the total metal concentration in a method blank (µg mL^{-1}), v is the final volume of solution (mL), m is the original mass of soil sample (g), and D is a dilution factor. Analysis of a certified reference material (BCR No. 143 – Sewage Sludge Amended Soil) was also carried out to determine the accuracy of the method. For both metals the mean total concentration determined was ≥ 90 % of the specified concentration (**Table 3.4**).

3.4.2. Total Zinc Concentration Results (2012-2014)

Auchincruive

Total Zn concentration at Auchincruive remained significantly (p <0.001) higher in soil receiving the Zn sludge treatment than in each of the other soils for the duration of the current investigation (**Figure 3.4**). In addition, the total concentration of Zn was also found to be significantly

Table 3.5:- Total concentration of Zinc (mg kg^{-1}) measured over the course of three years (2012-2014) at the Long-Term Sludge Experiment field sites.

Sludge Treatment	Total Zinc (mg kg^{-1})		
	2012	2013	2014
AUC/Zn	**312.12 (6.00)^{a}**[1][2][3]	**307.71 (23.22)^{a}**	**277.46 (5.41)^{a}**
AUC/Ctrl1	113.35 (1.94)^{b}	94.39 (3.28)^{b}	97.00 (1.39)^{b}
AUC/Cu	118.30 (4.18)^{b}	95.12 (6.16)^{b}	101.30 (2.33)^{b}
AUC/Ctrl2	116.16 (1.16)^{b}	90.53 (3.90)^{b}	96.31 (3.73)^{b}
AUC/NS	91.51 (1.52)^{c}	75.40 (1.62)^{b}	78.76 (0.57)^{c}
GLE/Zn	**260.21 (36.62)^{a}**	**236.05 (54.17)^{a}**	**215.18 (77.75)^{a}**
GLE/Ctrl1	70.36 (1.20)^{b}	53.68 (1.49)^{b}	53.17 (8.49)^{b}
GLE/Cu	78.09 (9.55)^{b}	59.22 (5.55)^{b}	57.21 (8.49)^{b}
GLE/Ctrl2	68.83 (5.85)^{b}	52.71 (4.39)^{b}	50.45 (5.39)^{b}
GLE/NS	48.75 (2.63)^{b}	34.17 (1.97)^{b}	36.60 (1.74)^{b}
HAR/Zn	**371.39 (15.18)^{a}**	**357.53 (12.26)^{a}**	**368.12 (7.49)^{a}**
HAR/Ctrl1	100.33 (7.01)^{bc}	78.36 (5.44)^{b}	85.22 (8.18)^{bc}
HAR/Cu	124.28 (3.84)^{c}	100.55 (4.59)^{b}	102.65 (5.51)^{b}
HAR/Ctrl2	109.63 (14.40)^{bc}	86.39 (9.57)^{b}	99.32 (9.06)^{b}
HAR/NS	90.15 (5.90)^{b}	75.94 (5.90)^{b}	74.31 (5.71)^{c}
WOB/Zn	181.91 (1.58)^{a}	**172.58 (40.18)^{a}**	**219.45 (17.27)^{a}**
WOB/Ctrl1	69.64 (2.18)^{b}	70.50 (4.86)^{b}	54.64 (4.11)^{b}
WOB/Cu	73.89 (6.96)^{b}	67.50 (3.85)^{b}	57.14 (3.56)^{b}
WOB/Ctrl2	62.38 (3.53)^{b}	61.79 (4.08)^{b}	51.59 (3.22)^{b}
WOB/NS	48.40 (1.56)^{c}	52.34 (6.13)^{b}	39.54 (4.72)^{b}

[1]Values in parenthesis are standard errors (n = 3). [2] Values without corresponding letters denotes statistical significance at $\alpha = 0.05$ for individual sampling events. [3]Values in bold are for soils receiving the Zn sludge treatment. Samples were diluted x2 for analysis by AAS.

($p < 0.01$) higher in soils receiving the remaining sludge treatments, in comparison to the untreated soil, during years 2012 and 2014 (**Table 3.5**).

Although the total concentration of Zn in soil receiving the Zn sludge treatment dropped below the UK statutory limit to 277.46 ± 5.41 mg kg^{-1} in 2014 (**Figure 3.4**), the overall change for the duration of the current investigation was not statistically significant. However, a significant decrease ($p < 0.05$) in the total concentration of Zn did occur in each of remaining soils from 2012 to 2013 (**Figure 3.4**).

Gleadthorpe

Similarly at Gleadthorpe, despite decreasing to 215.18 ± 77.75 mg kg^{-1}, just above the UK advisory limit for Zn in sludge amended soils, no significant change in total Zn was observed in soil receiving the Zn sludge treatment over the course of the current investigation (**Figure 3.5**). Significant decreases in total Zn occurred from 2012 to 2013 in soil receiving the digested control ($p < 0.001$) and untreated soil ($p < 0.01$), whereas in soil receiving the undigested control, total Zn declined steadily over the course of the current investigation, with a significant ($p < 0.05$) difference in concentration between years 2012 and 2014 (**Figure 3.5**); no significant change in total Zn concentration was observed in soil receiving the Cu sludge treatment.

Again, for the duration of the current investigation the total concentration of Zn in soil receiving the Zn sludge treatment was significantly ($p < 0.01$) higher in comparison to the remaining soils (**Figure 3.5**), between which no significant differences in total Zn were observed (**Table 3.5**).

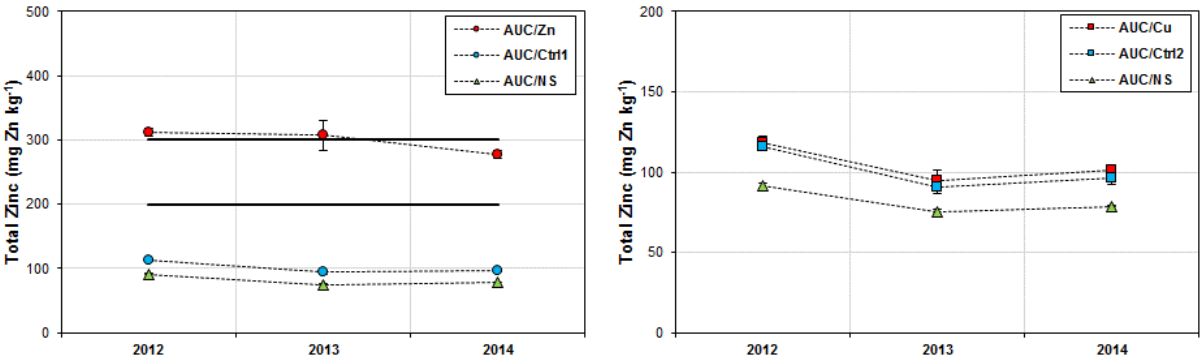


Figure 3.4:- Change in total Zinc concentration (mg Zn kg^{-1}) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Auchincruive (AUC) field site. Current UK statutory (300 mg Zn kg^{-1}) and Advisory (200 mg Zn kg^{-1}) limits for Zn in sludge amended soil are also shown. Error bars represent standard error (n = 3).

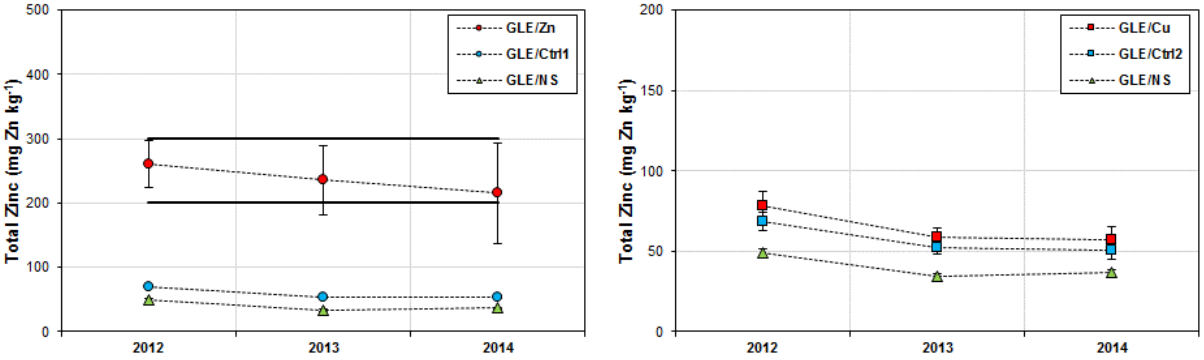


Figure 3.5:- Change in total Zinc concentration (mg Zn kg^{-1}) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Gleadthorpe (GLE) field site. Current UK statutory (300 mg Zn kg^{-1}) and Advisory (200 mg Zn kg^{-1}) limits for Zn in sludge amended soil are also shown. Error bars represent standard error (n = 3).

Hartwood

At Hartwood the concentration of total Zn was significantly (p <0.001) higher in soil receiving the Zn sludge treatment in comparison to all other soils, and remained above the UK regulatory limit for the duration of the current investigation (**Figure 3.6**). Additionally, in comparison to untreated soil, total Zn was significantly higher in soil receiving the Cu sludge treatment (p <0.05) during years 2012 and 2014, and in soil receiving the undigested control (p <0.05) in 2014 (**Table 3.5**).

With the exception of soil receiving the Cu sludge treatment, where total Zn decreased significantly (p <0.05) between 2012 and 2013, no significant changes in total Zn were seen over time at the Hartwood field site.

Woburn

Excepting a significant decrease (p <0.05) in total Zn from year 2013 to 2014 in soil receiving the digested control, no significant change in total Zn concentration was observed in any of the soils at the Woburn field site.

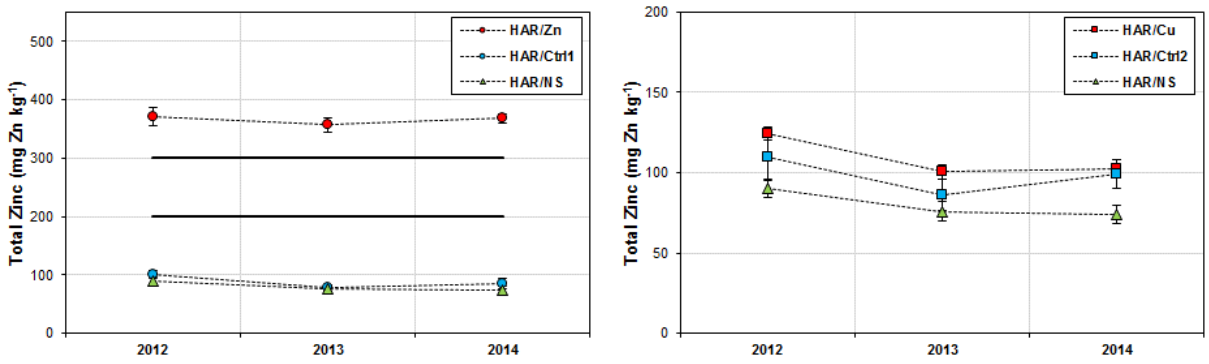


Figure 3.6:- Change in total Zinc concentration (mg Zn kg^{-1}) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Hartwood (HAR) field site. Current UK statutory (300 mg Zn kg^{-1}) and Advisory (200 mg Zn kg^{-1}) limits for Zn in sludge amended soil are also shown. Error bars represent standard error (n = 3).

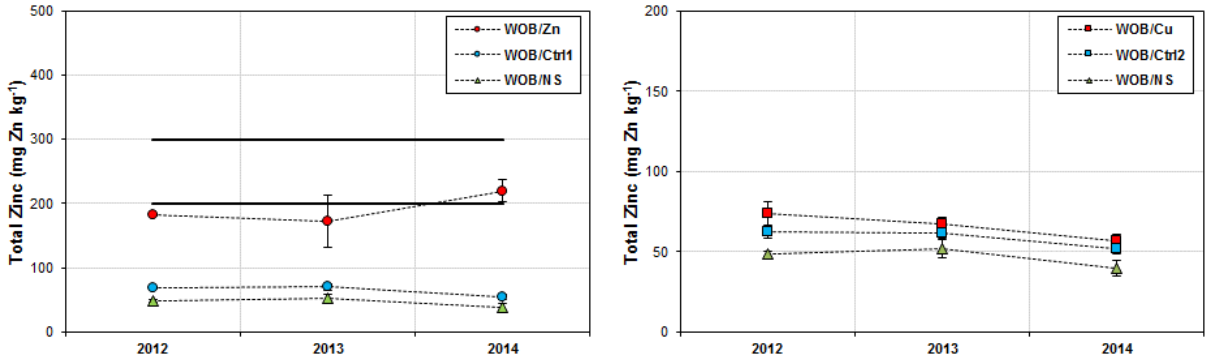


Figure 3.7:- Change in total Zinc concentration (mg Zn kg^{-1}) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Woburn (WOB) field site. Current UK statutory (300 mg Zn kg^{-1}) and Advisory (200 mg Zn kg^{-1}) limits for Zn in sludge amended soil are also shown. Error bars represent standard error (n = 3).

However, although the change over time was not seen to be significant, the mean concentration of total Zn in soil receiving the Zn sludge treatment increased above the UK advisory limit for Zn in sludge amended soils during 2014 (**Figure 3.7**), and was significantly higher (p <0.01) in comparison to the remaining soils for the duration of the current investigation (**Table 3.5**). During 2012 total Zn was also significantly higher in soils receiving the digested (p <0.05) and undigested (p <0.05) controls, as well as the soil receiving the Cu sludge treatment (p <0.001), in comparison to the untreated soil. However no significant differences in total Zn were seen between these soils for the remainder of the current investigation (**Table 3.5**).

3.4.3. Total Zinc Concentration Overview (2012-2014)

Values for the overall background concentration of Zn at the four field sites were in agreement with those determined in 1994 prior to experimental Phase I (**Table 3.1**), indicating little change has occurred in the total concentration of Zn in the untreated soil over the past 20 years. Background concentrations of Zn were significantly (p <0.001) higher at the Scottish field sites, in comparison to

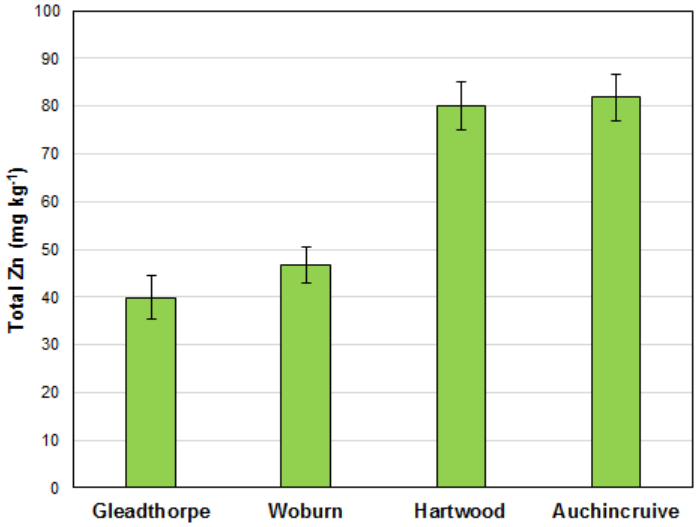


Figure 3.8:- Mean values for total Zinc (mg kg^{-1}) in untreated soil (NS) at each of the Long-Term Sludge Experiment field sites, over the course of three years (2012-2014). Error bars represent standard error (n = 3).

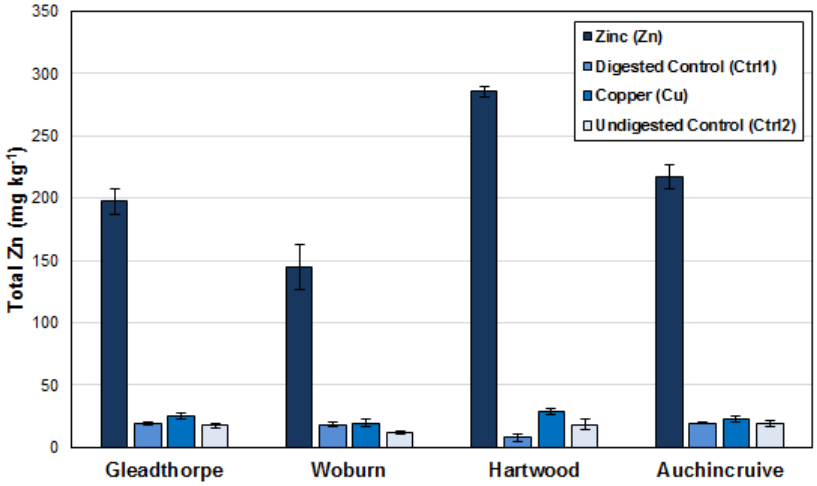


Figure 3.9:- Residual increase in total Zinc (mg kg^{-1}) at each of the Long-Term Sludge Experiment field sites in soils receiving digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments. Values are mean the total metal concentration over the course of three years (2012-2014). Error bars represent standard error (n = 3).

the English sites, with no significant differences observed between the sites of each region (**Figure 3.8**).

The total concentration of Zn (mg kg^{-1}) remaining in soils receiving the Zn sludge treatment at each of the four sites increased as follows:

WOB (144.55 ± 18.09) < GLE (197.31 ± 9.76) < AUC (217.21 ± 9.85) < HAR (285.55 ± 4.13)

Residual Zn at the Hartwood field site was significantly (p <0.01) higher than each of the other sites, whereas residual Zn at Woburn was significantly (p <0.05) lower (**Figure 3.9**); no significant difference was observed between Auchincruive and Gleadthorpe. Again, this is in agreement with the achieved concentrations reported for dose response 'Level 3' (**Table 3.3**), indicating no change in the relative total concentration of Zn has occurred between sites since the end of experimental Phase I.

Table 3.6:- Total concentration of Copper (mg kg^{-1}) measured over the course of three years (2012-2014) at the Long-Term Sludge Experiment field sites.

	Total Copper (mg kg^{-1})		
Sludge Treatment	**2012**	**2013**	**2014**
AUC/Zn	83.38 (1.85)$^{a[1][2]}$	74.92 (2.27)^{a}	73.90 (3.59)^{a}
AUC/Ctrl1	44.47 (2.85)^{b}	45.74 (2.88)^{b}	49.50 (2.64)^{b}
AUC/Cu	**130.30 (10.16)$^{c[3]}$**	**113.22 (15.33)^{c}**	**141.27 (2.77)^{c}**
AUC/Ctrl2	43.42 (48.85)^{b}	40.14 (3.36)^{bd}	43.23 (2.55)^{b}
AUC/NS	25.51 (0.39)^{d}	21.59 (0.14)^{d}	26.14 (0.74)^{d}
GLE/Zn	61.29 (9.08)^{a}	56.24 (10.14)^{a}	55.38 (17.83)^{a}
GLE/Ctrl1	31.99 (1.55)^{ab}	27.13 (2.37)^{b}	27.19 (1.76)^{ab}
GLE/Cu	**121.58 (18.67)^{c}**	**126.56 (13.46)^{c}**	**118.20 (23.72)^{c}**
GLE/Ctrl2	27.43 (3.35)^{b}	25.85 (3.33)^{b}	22.82 (4.76)^{ab}
GLE/NS	10.15 (0.81)^{b}	8.17 (0.39)^{b}	10.26 (0.85)^{b}
HAR/Zn	102.59 (3.00)^{a}	98.97 (4.44)^{a}	101.00 (4.08)^{a}
HAR/Ctrl1	50.29 (3.56)^{b}	45.82 (3.14)^{b}	50.56 (6.36)^{b}
HAR/Cu	**180.94 (6.57)^{c}**	**163.84 (9.14)^{c}**	**166.79 (10.19)^{c}**
HAR/Ctrl2	48.20 (6.79)^{b}	48.18 (5.08)^{b}	51.32 (6.51)^{b}
HAR/NS	22.84 (2.97)^{d}	24.04 (3.60)^{d}	24.16 (4.28)^{d}
WOB/Zn	54.27 (1.01)^{a}	52.09 (4.01)^{a}	57.09 (1.61)^{a}
WOB/Ctrl1	30.31 (1.68)^{b}	40.20 (3.57)^{ab}	27.77 (0.92)^{b}
WOB/Cu	**109.51 (12.99)^{c}**	**127.01 (6.50)^{c}**	**97.59 (6.62)^{c}**
WOB/Ctrl2	22.64 (0.80)^{bd}	30.70 (0.96)^{b}	21.82 (1.02)^{b}
WOB/NS	11.37 (0.30)^{d}	17.83 (1.73)^{d}	10.81 (0.51)^{c}

[1]Values in parenthesis are standard errors (n = 3). [2] Values without corresponding letters denotes statistical significance at $\alpha = 0.05$ for individual sampling events. [3]Values in bold are for soils receiving the Cu sludge treatment.

3.4.4. Total Copper Concentration Results (2012-2014)

Auchincruive

For the duration of the current investigation, the total concentration of Cu at Auchincruive was significantly higher in soils receiving the Zn ($p < 0.05$) and Cu ($p < 0.001$) sludge treatments, in comparison to the other soils (**Figure 3.10**; **Table 3.6**). In general, concentrations of Cu in soils receiving the digested and undigested controls were also significantly higher ($p < 0.05$) than in the untreated soil, with no significant differences observed between the two soils themselves (**Table 3.6**).

No significant change in total Cu concentration was observed in soil receiving the Cu sludge treatment over the course of the current investigation, despite increasing above the UK statutory limit to 141.27 ± 2.77 mg kg^{-1} in 2014 (**Figure 3.10**). In contrast, a significant decrease ($p < 0.05$) in total Cu occurred in soil receiving the Zn sludge treatment which declined steadily from 83.38 ± 1.85 mg kg^{-1} in 2012, to 73.90 ± 3.59 mg kg^{-1} in 2014 (**Table 3.6**). Total Cu also decreased significantly ($p < 0.01$) in the untreated soil during 2013, however no difference was observed between values measured in 2012 and 2014.

Gleadthorpe

Total Cu was significantly ($p < 0.01$) higher at Gleadthorpe in soil receiving the Cu sludge treatment, in comparison to the other soils, but remained below the UK statutory limit for the duration of the current investigation (**Figure 3.11**). Again, total Cu had increased significantly ($p < 0.05$) in soil receiving the

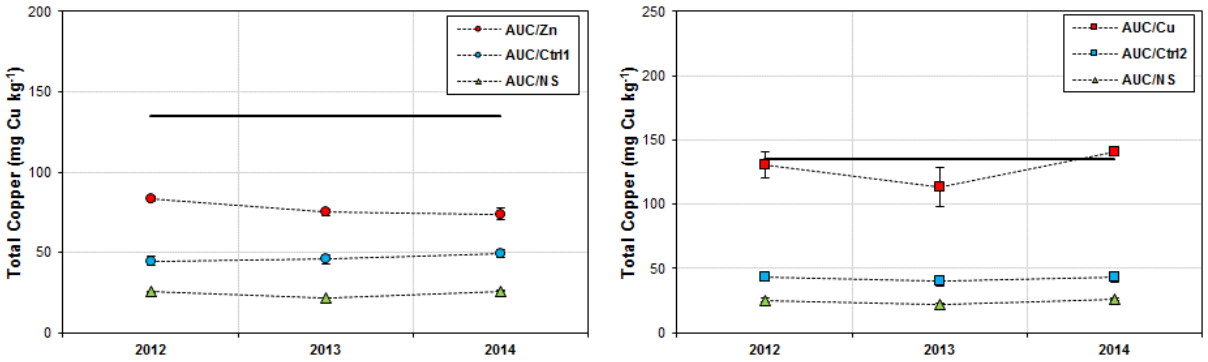


Figure 3.10:- Change in total Copper concentration (mg Cu kg^{-1}) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Auchincruive (AUC) field site. Current UK statutory (135 mg Cu kg^{-1}) limit for Cu in sludge amended soil is also shown. Error bars represent standard error (n = 3).

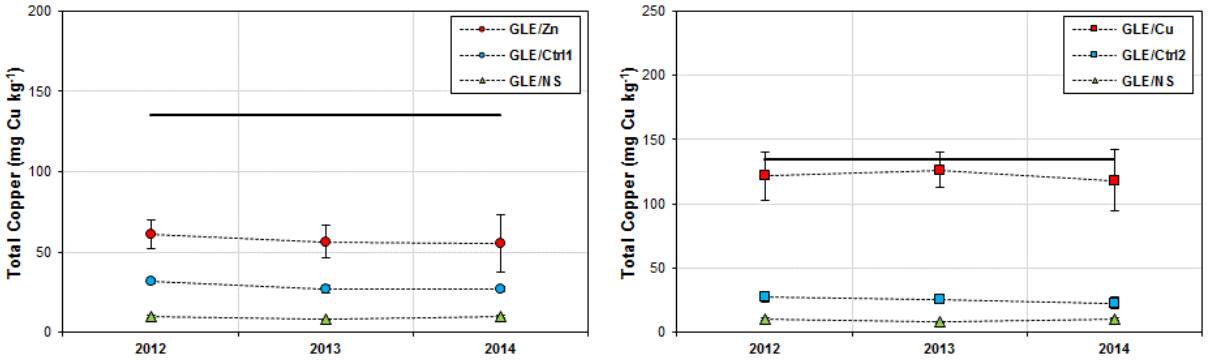


Figure 3.11:- Change in total Copper concentration (mg Cu kg^{-1}) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Gleadthorpe (GLE) field site. Current UK statutory (135 mg Cu kg^{-1}) limit for Cu in sludge amended soil is also shown. Error bars represent standard error (n = 3).

Zn sludge treatment, in comparison to the untreated soil, and was also significantly (p <0.05) higher than soils receiving the digested and undigested controls in 2013 (**Table 3.6**). No significant differences in total Cu were observed between the untreated soil and soils receiving the digested and undigested controls at any point during the current investigation.

No significant changes in the total concentration of Cu occurred in any of the soils sampled at the Gleadthorpe site over the course of the current investigation.

Hartwood

In comparison to the other soils, total Cu concentration was significantly higher (p <0.001) in soil receiving the Cu sludge treatment at Hartwood, and remained above the UK statutory limit for the duration of the current investigation (**Figure 3.12**). In comparison to the other field sites, soil receiving the Zn sludge treatment showed the greatest increase in total Cu concentration, which also remained significantly (p <0.001) greater than the untreated soil and soils receiving the digested and undigested controls over the course of the current investigation; concentrations of Cu in these soils were also significantly (p <0.05) greater than in untreated soil (**Table 3.6**).

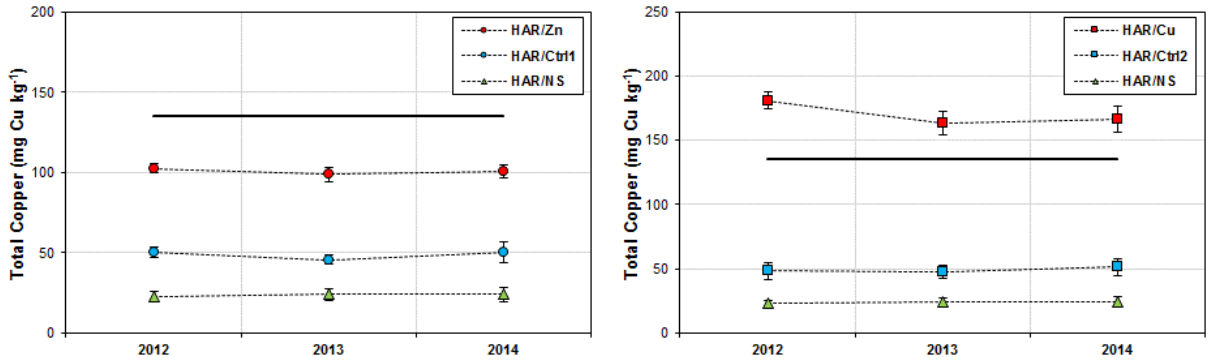


Figure 3.12:- Change in total Copper concentration (mg Cu kg^{-1}) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Hartwood (HAR) field site. Current UK statutory (135 mg Cu kg^{-1}) limit for Cu in sludge amended soil is also shown. Error bars represent standard error (n = 3).

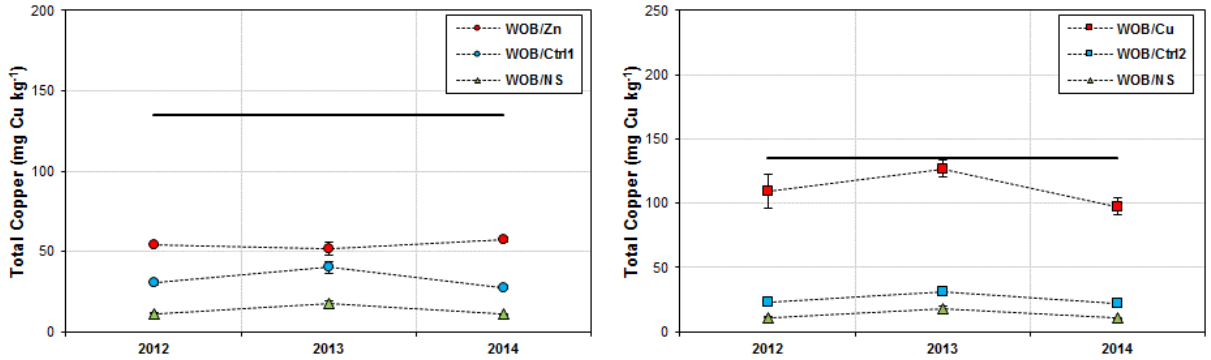


Figure 3.13:- Change in total Copper concentration (mg Cu kg^{-1}) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Woburn (WOB) field site. Current UK statutory (135 mg Cu kg^{-1}) limit for Cu in sludge amended soil is also shown. Error bars represent standard error (n = 3).

No significant changes in total Cu concentration were observed over time in any of the soils sampled at the Hartwood field site.

Woburn

At the Woburn field site, total Cu increased significantly in the untreated soil (p <0.01), and soils receiving the digested (p <0.05), and undigested (p <0.001) controls during 2013, however, no overall significant change was seen over the course of the current investigation (**Figure 3.13**). No significant change in total Cu was observed over time in soils receiving either the Zn or Cu sludge treatment.

Total Cu in soil receiving the Cu sludge treatment was significantly (p <0.001) higher than each of the other soils for the duration of the current investigation, though, like Gleadthorpe, had now decreased below the UK statutory limit (**Figure 3.13**). Again, total Cu in soil receiving the Zn sludge treatment was significantly higher than in untreated soil (p <0.001) and, in general, soils receiving digested (p <0.05), and undigested (p <0.01) controls (**Table 3.6**); with no significant difference in total Cu concentration observed between these two soils. Total Cu in soil receiving the digested control was also significantly (p <0.05) higher than the untreated soil over the course of the current investigation,

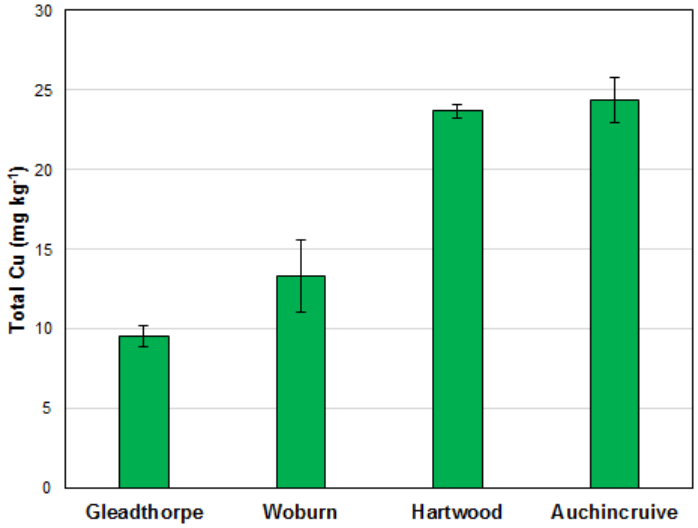


Figure 3.14:- Mean values for total Copper (mg kg^{-1}) in untreated soil (NS) at each of the Long-Term Sludge Experiment field sites, over the course of three years (2012-2014). Error bars represent standard error (n = 3).

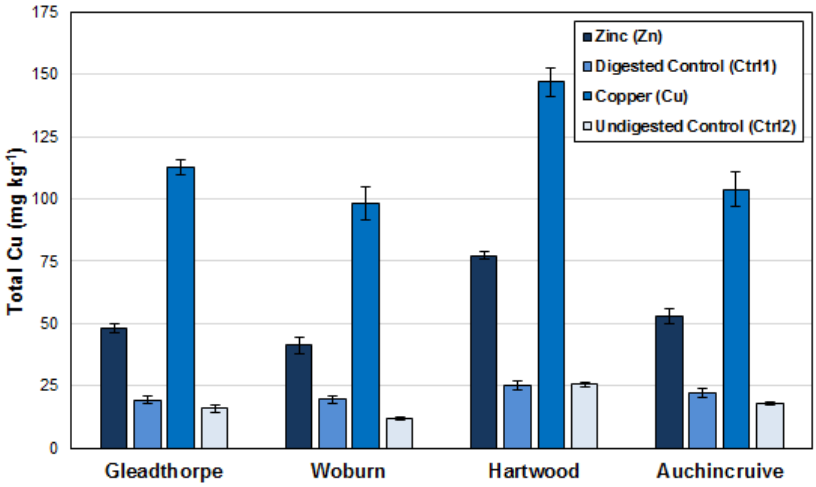


Figure 3.15:- Residual increase in total Copper (mg kg^{-1}) at each of the Long-Term Sludge Experiment field sites in soils receiving digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments. Values are mean the total metal concentration over the course of three years (2012-2014). Error bars represent standard error (n = 3).

whereas total Cu in soil receiving the undigested control was only significantly (p <0.05) higher in 2013 and 2014 (**Table 3.6**).

3.4.5. Total Copper Concentration Overview (2012-2014)

Values for the overall mean background concentration of Cu in the untreated soil at each site were also in agreement with those determined prior to the start of experimental Phase I (**Table 3.1**); again indicating that little change has occurred over the past 20 years. As seen for Zn, the background concentrations of Cu were also significantly (p <0.001) higher at the Scottish sites in comparison to the English sites, again with no significant differences observed between sites of the same region (**Figure 3.14**).

Similarly, in agreement with the concentrations reported for dose response 'Level 3' (**Table 3.3**), the greatest concentration of Cu remaining in soils receiving the Cu sludge treatment was seen at the Harwood field site which was significantly (p <0.01) greater than at the other three sites (**Figure 3.15**). However now the lowest concentration of Cu (mg kg^{-1}) was observed at Woburn, as follows:

WOB (98.03 ± 6.47) < AUC (103.85 ± 6.80) < GLE (112.58 ± 3.07) < HAR (146.85 ± 5.68)

Though no significant differences were observed between Auchincruive, Woburn and Gleadthorpe. Furthermore, due to the confounding Cu contamination in the Zn sludge treatment (**See Section 3.3**), soil receiving the Zn sludge treatment at Hartwood also contained significantly (p <0.001) higher concentrations of Cu in comparison to the other sites (**Figure 3.15**); residual Cu at the Auchincruive field site was also significantly (p <0.05) higher in comparison to that at Woburn (**Figure 3.15**).

3.5. Metal Speciation

The bioavailability of a heavy metal in soil is largely determined by the chemical nature of the metal cation itself combined with its current chemical speciation within the soil environment (Alloway, 1995). Hence despite a total metal concentration above recommended statutory limits, only a small fraction of the metal contamination may actually be bioavailable and present in the soil solution at any given time. Viets, Jr. (1962) describes five principal fractions between which the total concentration of a metal can be distributed, these are: soluble cations (i.e. free (M^+) or complexed ions present in the soil solution), exchangeable cations, adsorbed, chelated, or complexed cations (including organically bound cations), occluded cations (in secondary clay minerals and precipitates such as metal oxides), and residual cations (present in primary minerals). However, the distribution of a metal between each fraction is not easily determined and a range of chemical extractants are currently employed in order to determine the relative concentrations of a heavy metal within each fraction (Lake et al., 1984).

The bioavailability of Zn was monitored over the course of experimental Phases I-III by extraction of exchangeable Zn with ammonium nitrate (NH_4NO_3) as described by DIN (1997). Following the final sludge applications in 1997, the concentration of Zn extractable by NH_4NO_3, in soils receiving the Zn sludge treatment, were higher at the Scottish field sites in comparison to the English sites (**Figure 3.16**), with the highest concentration (36.7 ± 4.31 mg kg^{-1}) seen at Hartwood (Gibbs et al., 2006). However, over the course of experimental Phases II and III, the availability of Zn at the Hartwood field site appeared to fluctuate, whereas a steady increase in the amount of Zn extractable by NH_4NO_3 was observed at Auchincruive (**Figure 3.16**), reaching a maximum of 47.9 ± 6.36 mg kg^{-1} in 2001. Similar trends were also observed at the English field sites over the same period. The availability of Zn at the Gleadthorpe site also increased to a maximum of 20.4 ± 2.46 mg kg^{-1} in 2001 before declining (Defra,

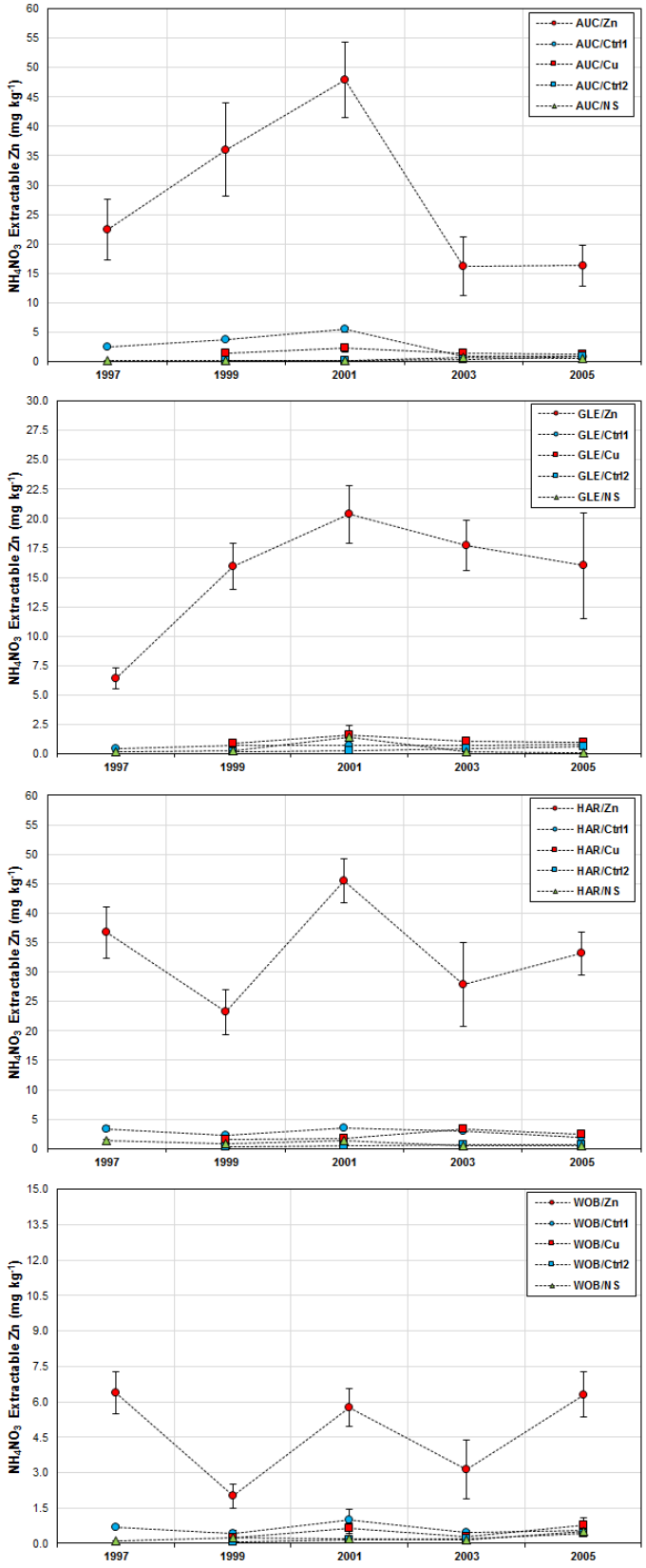


Figure 3.16:- Changes in the concentration of Zn (mg Zn kg^{-1}) extractable by NH_4NO_3 during experimental phases I-III (1997 - 2005) in soils receiving digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments, plus untreated soil (NS), at the Long-Term Sludge Experiment field sites. Data obtained from Gibbs et al. (2006), Defra (2002, 2007a). Error bars represent standard error (n = 3).

2002, 2007a), whereas, like Hartwood, the concentration of Zn extractable by NH_4NO_3 at Woburn also appeared to fluctuate.

Additional measurements on the speciation of Zn in the soil solution were also made at the two English sites during years 2001 and 2004 using the method of cation exchange described by Holm et al. (1995) and Knight et al. (1998). At the end of experimental Phase II the concentration of soluble Zn (GLE = 1.6 ± 0.26 mg L^{-1}; WOB = 0.8 ± 0.21 mg L^{-1}) at both sites was significantly (p <0.05) higher in soils receiving the Zn sludge treatment in comparison to the remaining soils, with approximately 65 % of Zn present as free Zn^{2+} ions (Defra, 2002). In both cases, the total concentration of Zn present in the soil solution (GLE = 0.94 mg L^{-1}; WOB = 0.46 mg L^{-1}) decreased in 2004, though remained significantly (p <0.05) higher in comparison to the other soils (Defra, 2007a). However, despite this decrease the proportion of Zn present as free Zn^{2+} ions appeared to increase at Gleadthorpe (73 %), whereas a slight decrease was observed at Woburn (59 %).

The concentrations of NH_4NO_3 extractable Cu were markedly lower than those observed for Zn, and following the final applications of sludge in 1997, ranged from 0.62 ± 0.07 mg kg^{-1} at Auchincruive to 2.33 ± 0.73 mg kg^{-1} at Gleadthorpe in soils receiving the Cu sludge treatment (Gibbs et al., 2006). With the exception of Auchincruive, where the available Cu concentration remained relatively constant at approximately 0.56 ± 0.05 mg kg^{-1} (**Figure 3.17**), a decrease in the amount of Cu extractable by NH_4NO_3 was seen in soils receiving the Cu sludge treatment at each of the LTSE sites during experimental Phase II (**Figure 3.17**). For the remainder of the experiment, concentrations of NH_4NO_3 extractable Cu were highest at Gleadthorpe, ranging from 1.07 ± 0.15 mg kg^{-1} in 2001 to a maximum of 1.31 ± 0.23 mg kg^{-1} in 2003 (Defra, 2002, 2007a). Similar increases were also observed at the Woburn and Hartwood sites, where NH_4NO_3 extractable Cu respectively increased from 0.62 ± 0.04 mg kg^{-1} and 0.42 ± 0.06 mg kg^{-1} in 2001, to 1.03 ± 0.04 mg kg^{-1} and 0.51 ± 0.05 mg kg^{-1} in 2003 (**Figure 3.17**).

In addition, as a result of the confounding Cu contamination in the Zn sludge treatment (**See Section 3.3**), concentrations of NH_4NO_3 extractable Cu also appear to have increased in the receiving soils (**Figure 3.17**); though the statistical significance of these increases has not been reported (Gibbs et al., 2006; Defra, 2002, 2007a). However, in 2001 it was observed that the concentration of Cu present in the solution of soil receiving the Zn sludge treatment at Woburn (0.149 ± 0.008 mg L^{-1}) was not significantly different to that in soil receiving the Cu sludge treatment (0.195 ± 0.012 mg L^{-1}), which in turn, was significantly (p <0.05) higher in comparison to untreated soil, and soil receiving the undigested control, at both of the English sites (Defra, 2002). Subsequently in 2004, the total solution concentration of Cu in soils receiving the Cu sludge treatment had decreased at Gleadthorpe (0.504 mg L^{-1} to 0.306 mg L^{-1}) and increased at Woburn (0.235 mg L^{-1}). Both were significantly (p <0.001) higher in comparison to untreated soil and those receiving the remaining sludge treatments, with less than

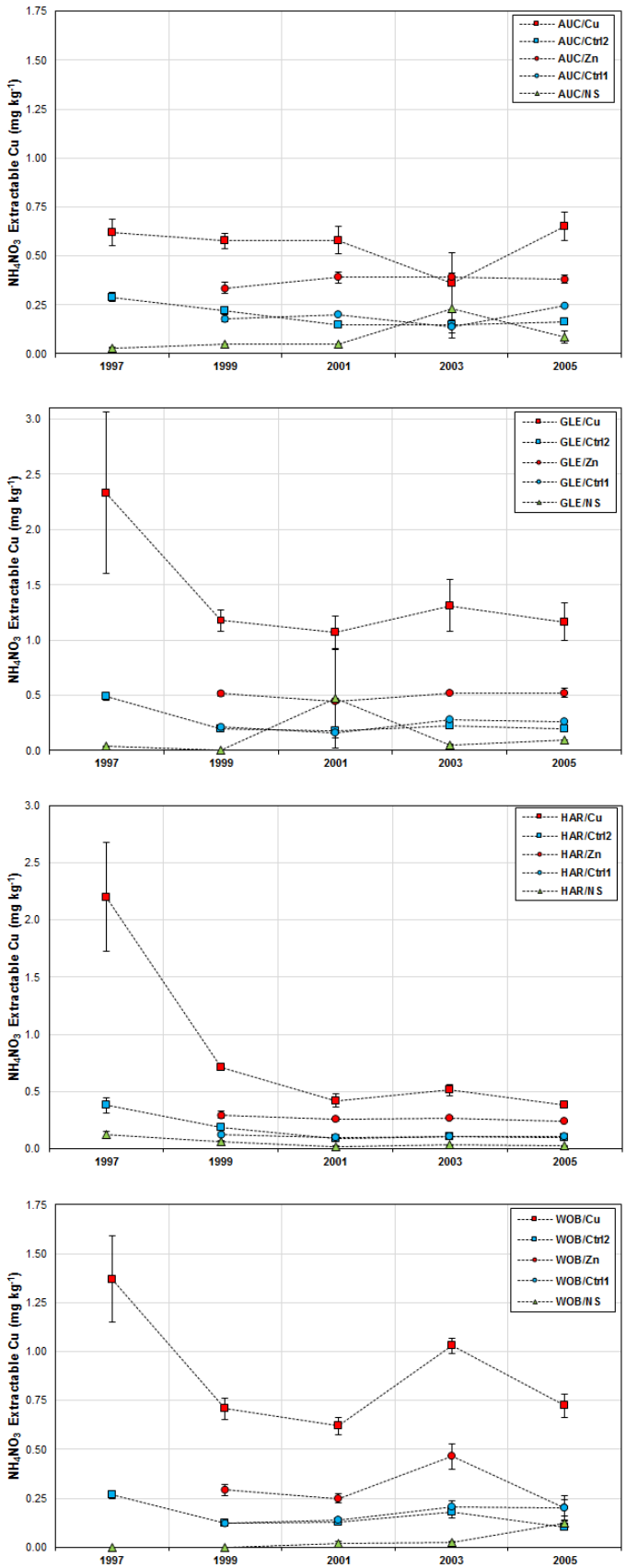


Figure 3.17:- Changes in the concentration of Copper (mg Cu kg^{-1}) extractable by NH_4NO_3 during experimental Phases I-III (1997 - 2005) in soils receiving digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments, plus untreated soil (NS), at the Long-Term Sludge Experiment field sites. Data obtained from Gibbs et al. (2006), Defra (2002, 2007a). Error bars represent standard error (n = 3).

0.001 % present as free $Cu2^{+}$ ions at each site; in both cases the total solution concentration of Cu had decreased in soils receiving the Zn sludge treatment (Defra, 2007a).

3.5.1. Metal Speciation Method

The chemical speciation of Zn and Cu was determined for the current investigation by sequential extraction of heavy metals using extractants of increasing strength. The method of extraction used was based on that described by McGrath and Cegarra (1992), which in turn was an adaptation of the methods described by Lund et al. (1980) and Stover et al. (1976). Solutions of calcium chloride ($CaCl_2$), NaOH, and EDTA were used to respectively extract, water-soluble and exchangeable metal species, metals bound to soil organic matter, and precipitated metals associated with carbonates (McGrath and Cegarra, 1992). Approximately 3 g of air-dried soil, sampled during year 2014, was weighed into a 50 mL centrifuge tube and extracted with 30 mL of 0.1 M $CaCl_2$ solution by mechanically shaking for ≥ 16 hours. Samples were then centrifuged (4800 RCF) for 20 minutes before decanting the supernatant solution. Samples were subsequently extracted with 30 mL of 0.5 M NaOH solution, followed by 30 mL of 0.05 M EDTA solution. In each case, the weight of the sample plus residual solution was recorded before addition of the next extractant. Approximately 30 mL of 5 M HCl was added to both NaOH and EDTA solutions in order to remove soil organic matter from the extracts prior to analysis by AAS. Solutions were allowed to stand for ≥ 16 hours, before filtering (Whatman No. 542) and diluting to 100 mL final volume with deionised water. Each sample was then air-dried and analysed for the remaining, residual, metal content by acid digestion in *aqua-regia* (**See Section 3.4.1**).

The concentrations (mg kg^{-1}) of Zn and Cu in each of the extraction solutions were measured by AAS as previously described (**See Section 3.4.1**). Concentrations of exchangeable Zn and Cu were calculated using **E. 3.1**, whereas concentrations of the subsequent metal fractions were adjusted as follows, in order to account for the extracted metals still present in the residual solution following each extraction stage (n = 1 to 4):

$$[M^{+}_{Organic}] = \frac{\left(([M^{+}_{S2}] - [M^{+}_{B2}]) \times (v_2 + r_1) \times D\right) - ([M^{+}_{S1}] \times r_1 \times D)}{m} \quad (\mathbf{E.3.2})$$

$$[M^{+}_{Carbonate}] = \frac{\left(([M^{+}_{S3}] - [M^{+}_{B3}]) \times (v_3 + r_2) \times D\right) - ([M^{+}_{S2}] \times r_2 \times D)}{m} \quad (\mathbf{E.3.3})$$

$$[M^{+}_{Residual}] = \frac{\left(([M^{+}_{S4}] - [M^{+}_{B4}]) \times v_4 \times D\right) - ([M^{+}_{S3}] \times r_3 \times D)}{m} \quad (\mathbf{E.3.4})$$

where $[M^{+}_{S(n)}]$ is the total metal concentration in each extraction solution (μg mL^{-1}), $[M^{+}_{B(n)}]$ is the total metal concentration in the corresponding method blank (μg mL^{-1}), $v_{(n)}$ is the final volume of extraction

Table 3.7:- Concentrations (mg kg^{-1}) of Zinc species measured by sequential extraction in soil samples taken from the LTSE field sites in 2014. Accuracy is expressed as a percentage of total metal concentration determined by *aqua-regia* acid digestion (See Section 3.4.1).

Sludge Treatment	Zinc (mg Zn kg^{-1})					Accuracy (%)
	Exchangeable	Organic	Carbonate	Residual	Σ Species	
AUC/Zn	**23.12 (1.97)[a][1][2][3]**	8.92 (2.07)	**21.51 (4.02)[a]**	188.73 (5.26)[a]	242.28 (8.66)[a]	87 (3)
AUC/Ctrl1	1.69 (0.38)[bc]	ND[4]	3.45 (0.37)[b]	74.89 (4.53)[b]	80.03 (3.97)[b]	83 (5)
AUC/Cu	3.48 (1.02)[b]	ND	31.8 (1.15)[b]	92.14 (1.72)[c]	98.80 (2.39)[c]	98 (4)
AUC/Ctrl2	2.55 (0.07)[bc]	ND	1.78 (0.81)[b]	98.68 (6.34)[c]	103.01 (6.89)[c]	108 (11)
AUC/NS	0.23 (0.03)[c]	ND	ND	61.76 (4.40)[b]	61.98 (4.42)[d]	79 (6)
GLE/Zn	**11.62 (5.85)[a]**	**19.87 (4.20)[a]**	**74.64 (23.90)[a]**	71.63 (26.94)[a]	177.76 (55.76)[a]	86 (8)
GLE/Ctrl1	0.49 (0.15)[b]	0.15 (0.15)[b]	6.75 (1.42)[b]	30.94 (5.65)[ab]	38.33 (4.48)[b]	73 (10)
GLE/Cu	1.21 (0.18)[b]	0.70 (0.70)[b]	11.79 (5.64)[b]	42.07 (7.70)[ab]	55.77 (1.32)[b]	102 (15)
GLE/Ctrl2	0.62 (0.20)[b]	ND	2.43 (1.57)[b]	28.18 (4.51)[b]	31.23 (3.03)[b]	64 (12)
GLE/NS	ND	ND	ND	33.67 (2.88)[ab]	33.67 (2.88)[b]	92 (4)
HAR/Zn	**39.73 (13.01)[a]**	**14.92 (3.72)[a]**	**26.96 (3.12)a**	**249.38 (32.06)[a]**	331.00 (30.76)[a]	90 (7)
HAR/Ctrl1	3.21 (1.13)[b]	1.67 (1.67)[b]	2.17 (1.01)[b]	63.74 (9.70)[b]	70.78 (9.32)[b]	83 (7)
HAR/Cu	**6.09 (2.20)[b]**	ND	2.94 (0.26)[b]	91.68 (2.70)[b]	100.72 (2.39)[b]	99 (5)
HAR/Ctrl2	1.80 (0.29)[b]	0.12 (0.12)[b]	14.76 (7.17)[c]	85.29 (3.87)[b]	101.97 (10.83)[b]	103 (8)
HAR/NS	2.16 (0.44)[b]	ND	0.65 (0.33)[b]	75.65 (6.84)[b]	78.47 (6.81)[b]	106 (5)
WOB/Zn	**22.49 (2.04)[a]**	**18.73 (4.85)[a]**	**48.49 (3.79)[a]**	48.47 (4.19)[a]	138.18 (9.20)[a]	64 (9)
WOB/Ctrl1	3.05 (0.10)[b]	ND	9.84 (0.83)[b]	30.99 (3.32)[b]	43.88 (4.04)[b]	80 (4)
WOB/Cu	3.20 (0.46)[b]	0.06 (0.06)[b]	10.84 (1.22)[b]	34.46 (1.36)[b]	48.56 (1.73)[b]	85 (3)
WOB/Ctrl2	2.03 (0.05)[b]	ND	9.51 (0.99)[b]	30.19 (1.91)[b]	41.73 (2.85)[b]	81 (5)
WOB/NS	1.53 (0.16)[b]	ND	1.67 (0.49)[c]	32.89 (3.37)[b]	36.09 (3.30)[b]	94 (13)

[1]Values in parenthesis are standard errors (n = 3). [2] Values without corresponding letters denotes statistical significance at α = 0.05. [3]Values in bold indicates samples were diluted x5 for analysis by AAS. [4] ND = Not Detected.

solution (mL), $r_{(n)}$ is the residual volume of extraction solution, m is the mass of extracted soil sample (g), and D is a dilution factor.

The accuracy of the sequential extraction method was estimated by comparing the sum of the concentrations for each chemical species ($\sum[M^+]$) to the total metal concentrations determined by microwave digestion described in **Section 3.4.1**. The concentrations of Zn determined by the sequential extraction method were, in general, within ± 20 % of those determined by microwave acid digestion indicating reasonable accuracy (**Table 3.7**). However, the sequential extraction method appears to have overestimated the total concentration of Cu species, with accuracy ranging from + 10 % to + 218 % (**Table 3.9**); hence, these results should be considered with caution, particularly when comparing the observed effects on soil microorganisms (**See Section 5.5**) to current UK statutory limits.

3.5.2. Zinc Speciation Results (2014)

Auchincruive

The total sum of Zn species at the Auchincruive field site, in soil receiving the Zn sludge treatment, was significantly (p <0.001) higher than in each of the other soils (**Table 3.7**). In addition, soils receiving the digested (p <0.05) and undigested (p <0.001) controls, and the Cu sludge treatment (p <0.01), also contained total concentrations of Zn significantly higher than in the untreated soil.

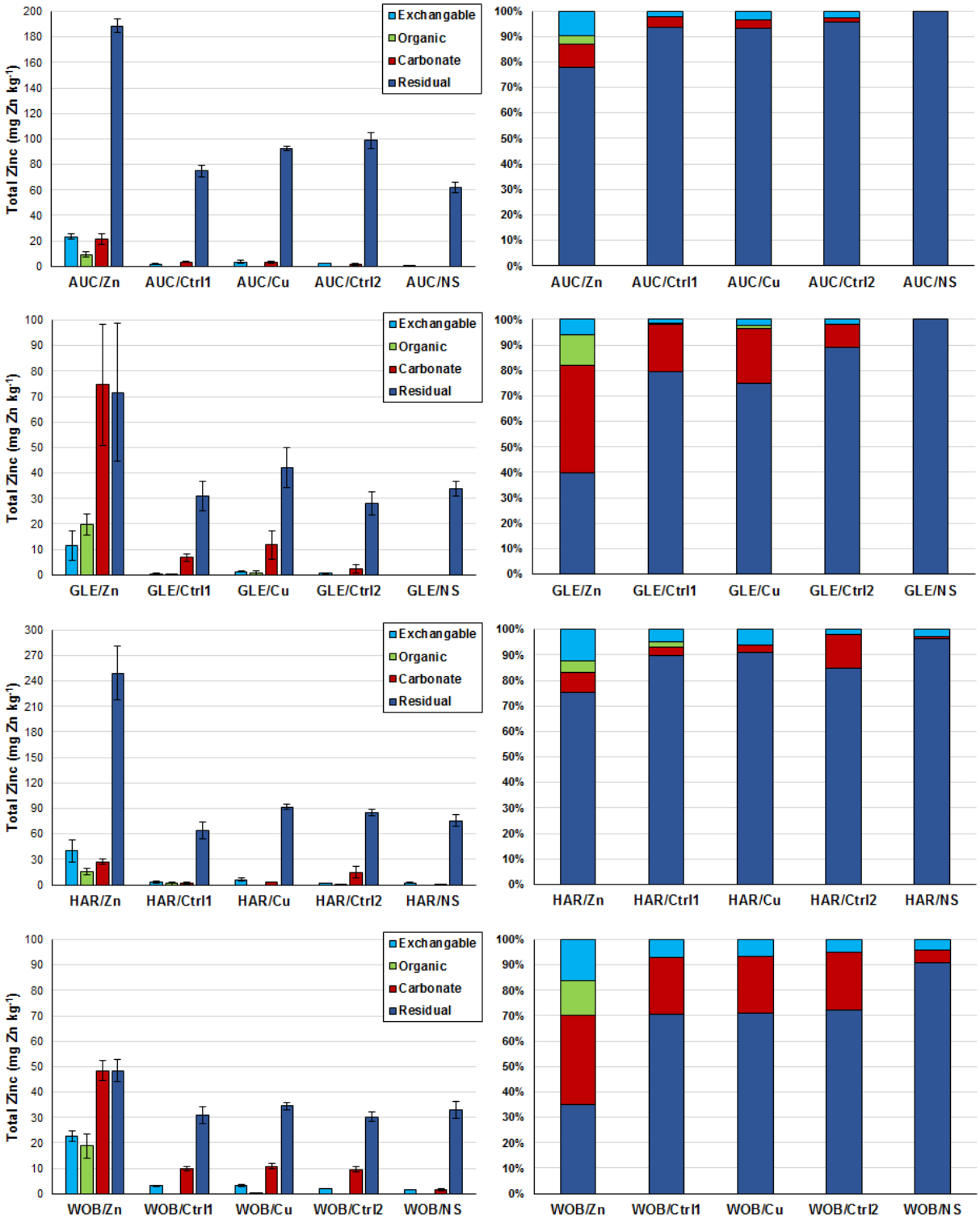


Figure 3.18:- Concentrations (mg kg^{-1}) of Zinc species determeind by sequential extraction at the LTSE field sites in 2014 (left). Results are expressed as a percentage of the total Zinc concentration (right). Error bars represent standard error (n = 3).

However, the total concentration of Zn in soil receiving the digested control was found to be significantly (p <0.05) lower than in soils receiving the undigested sludge treatments (**Table 3.7**).

Concentrations of all Zn species were significantly (p <0.001) higher in soil receiving the Zn sludge treatment in comparison to the other soils (**Table 3.7** and **Figure 3.18**). As a percentage of total

concentration (i.e. the sum of chemical species), Zn was predominantly found in the residual fraction (78 %), with approximately equal amounts of exchangeable (10 %) and carbonate (8 %) forms present (**Figure 3.18**). Organically bound Zn (4 %) was only observed in soil receiving the Zn sludge treatment.

In each of the remaining soils, over 90 % of Zn (99 % in untreated soil) was present in the residual fraction (**Figure 3.18**), with significantly higher concentrations in soils receiving the Cu sludge treatment (p <0.05) and undigested control (p <0.01) in comparison to untreated soil. Exchangeable Zn was also significantly (p <0.05) higher in soil receiving the Cu sludge treatment than in the untreated soil, but was not significantly different to soils receiving the digested and undigested controls (**Table 3.7**). The remaining Zn in the sludge amended soils was present as carbonates (approx. 2-4 %) however no significant differences were found between soils receiving different sludge treatments (**Table 3.7**).

Gleadthorpe

In agreement with the results described in **Section 3.4.2**, the total sum of Zn species in soil receiving the Zn sludge treatment at Gleadthorpe was significantly (p <0.01) higher than in each of the remaining soils, with no significant differences observed between them (**Table 3.7**). Seemingly due to the large carbonate fraction (42 %), the amount of Zn present in the residual fraction (40 %) of soil receiving the Zn sludge treatment was only significantly (p <0.05) higher in comparison to soil receiving the undigested control. However, concentrations of Zn present in exchangeable (6 %; p <0.05), organic (12 %; p <0.001), and carbonate (42 %; p <0.01) forms were significantly higher in comparison to each of the other soils (**Figure 3.18**); again, with no significant differences observed between them (**Table 3.7**).

All of the Zn present in untreated soil at Gleadthorpe was found in the residual fraction, hence only in sludge amended soils was Zn found to be associated with carbonates (**Figure 3.18**). Exchangeable Zn ranged from approximately 1-2 % in the remaining soils, with trace amounts of organically bound Zn also observed in soils receiving the Cu sludge treatment (1.5 %) and digested control (0.5 %).

Hartwood

At Hartwood, the total sum of Zn species in soil receiving the Zn sludge treatment was significantly (p <0.001) higher than in each of the remaining soils; with no significant differences observed between them (**Table 3.7**). Zinc was predominantly (75 %) present in the residual fraction of soil receiving the Zn sludge treatment (**Figure 3.18**), with approximately 12 %, 4.5 %, and 8 % present in the exchangeable, organic, and carbonate fractions, respectively; concentrations of each species were significantly (p <0.01) higher than in each of the other soils (**Table 3.7**). A greater percentage of Zn (13 %) was present in the carbonate fraction of soil receiving the undigested control (**Figure 3.18**), which was also significantly (p <0.05) higher than in the remaining soils (**Table 3.7**).

Approximately 3 % of Zn was present in the exchangeable fraction of the untreated soil at Hartwood, with only trace amounts associated with carbonates (1 %). Speciation of Zn in soils receiving the Cu sludge treatment and digested control were similar with approximately 5-6 % and 3 % present in exchangeable and carbonate fractions, respectively (**Figure 3.18**). Trace amounts of organically bound Zn were also observed in soil receiving the digested (2 %) and undigested (0.1 %) controls, however no significant differences were observed between the concentrations of Zn species in these soils (**Table 3.7**).

Woburn

Again, in agreement with the results described in **Section 3.4.2**, the total sum of Zn species in soil receiving the Zn sludge treatment at Woburn was significantly (p <0.001) higher than in each of the remaining soils, with no significant differences observed between them (**Table 3.7**).

Soil receiving the Zn sludge treatment had the lowest residual Zn fraction (35 %) of all of the field sites which, similarly to Gleadthorpe, was equal to the amount of Zn associated with carbonates (35 %); with approximately 18 % and 22 % present in organically bound and exchangeable forms, respectively (**Figure 3.18**). In each instance the concentration of Zn in these fractions was significantly (p <0.01) greater in comparison to the other soils (**Table 3.7**).

Concentrations of Zn associated with carbonates were approximately 22-23 % in each of the other sludge amended soils and were significantly (p <0.05) higher in comparison to the untreated soil (**Table 3.7**). Exchangeable forms of Zn were approximately 5-7 % of the total Zn concentration in these soils (**Figure 3.18**), but were not significantly higher than in untreated soil (4 %).

3.5.3. Exchangeable Zinc as a Function of Total Zinc

Regression of exchangeable Zn (extractable by $CaCl_2$) on total Zn, for samples collected at each of the LTSE field sites during 2014 (**Table 3.5**), showed a significant (R = 0.627; $p < 0.05$) positive correlation between the amount of exchangeable Zn present in soils receiving the Zn sludge treatment and the total concentration of Zn (**Table 3.8**); accounting for approximately 33 % of the observed variance. Examination of residual plots indicated possible heteroscedasticity within the data set, with residual values, predominantly for data obtained from the Hartwood site, increasing in proportion to the predicted values. For soil receiving the Cu sludge treatment, a significant (R = 0.608; p <0.05) correlation between exchangeable and total Zn was also observed, accounting for 30 % of the observed variance. However examination of residual plots showed this was due to an outlier present at the Hartwood field site, for which exchangeable Zn was greatest (**Table 3.7**); the correlation was no longer significant once the data point was removed (R = 0.504; p = 0.114). No significant correlation was

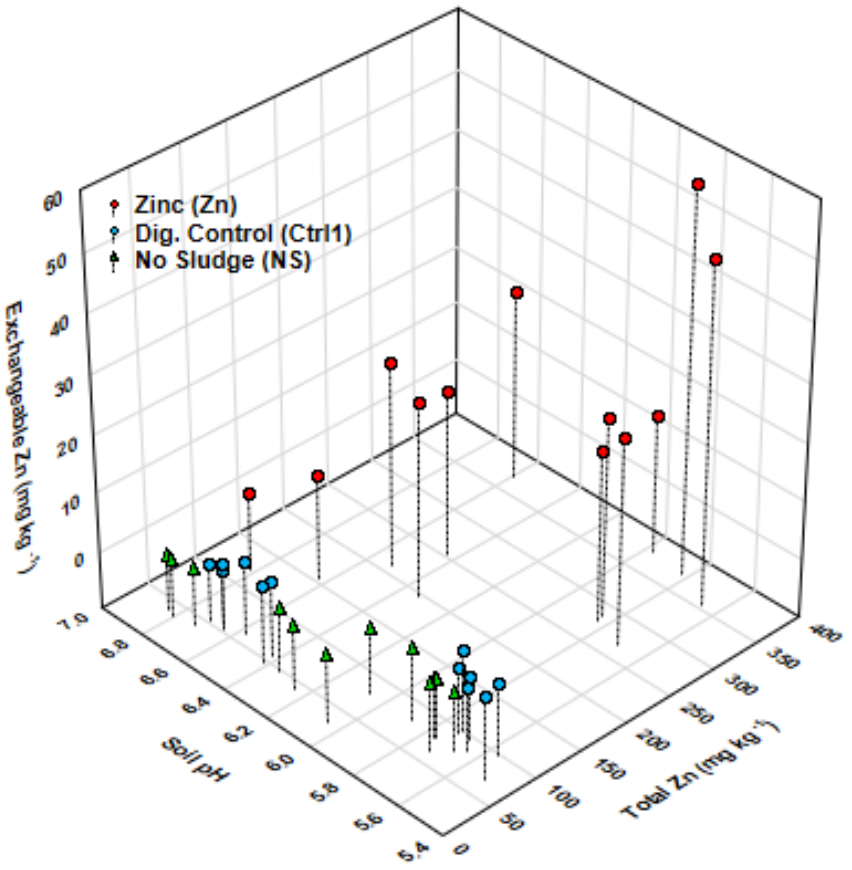


Figure 3.19:- Plot of exchangeable Zinc (mg kg^{-1}), extractable by $CaCl_2$, as a function of soil pH and total Zinc concentration (mg kg^{-1}), in untreated soil (NS), and soils receiving digested sludge treatments (Zn and Ctrl1), taken from each of the LTSE fields sites in 2014.

Table 3.8:- Regression and correlation coefficients from regression analysis of exchangeable Zinc on total Zinc concentration and soil pH.

	Regression of Exchangeable Zn on Total Zn						
Sludge Treatment	**Slope (Total Zn)**	***p***	**R**	**Intercept**	***p***	**R^2[2]**
Zinc (Zn)	0.108 (0.04)[1]	<0.05	0.627	-4.82 (11.94)	0.695	**0.333**
Dig. Control (Ctrl1)	0.012 (0.02)	0.604	0.167	1.27 (1.62)	0.449	**-0.07**
Copper (Cu)	0.063 (0.03)	<0.05	0.608	-1.54 (2.17)	0.494	**0.307**
Un. Dig Control (Ctrl2)	0.013 (0.01)	0.135	0.457	0.73 (0.65)	0.288	**0.130**
No Sludge (NS)	0.008 (0.02)	0.585	0.175	0.50 (0.90)	0.592	**-0.07**
	Regression of Exchangeable Zn on Soil pH					
	Slope (Soil pH)	***p***	**R**	**Intercept**	***p***	**R^2**
Zinc (Zn)	-23.01 (8.83)	<0.05	0.636	166.45 (54.71)	<0.05	**0.345**
Dig. Control (Ctrl1)	-1.07 (0.86)	0.243	0.365	8.66 (5.30)	0.133	**0.047**
Copper (Cu)	-1.83 (1.16)	0.147	0.445	14.85 (7.26)	0.068	**0.118**
Un. Dig Control (Ctrl2)	-1.30 (0.39)	<0.01	0.722	9.75 (2.44)	<0.01	**0.473**
No Sludge (NS)	-0.94 (0.67)	0.187	0.408	6.78 (4.11)	0.130	**0.083**
	Regression of Exchangeable Zn on Soil pH and Total Zn								
	Slope (pH)	***p***	**R**	**Slope (Tot. Zn)**	***p***	**R**	**Intercept**	***p***	**R^2**
Zinc (Zn)	-14.41 (11.1)	0.228	0.398	0.06 (0.05)	0.253	0.375	95.96 (78.69)	0.254	**0.375**
Dig. Control (Ctrl1)	-2.65 (1.75)	0.163	0.903	-0.04 (0.04)	0.325	0.618	21.39 (13.32)	0.143	**0.054**
Copper (Cu)	2.56 (2.47)	0.326	0.624	0.12 (0.06)	0.082	1.176	-22.15 (20.0)	0.296	**0.313**
Un. Dig Control (Ctrl2)	-1.65 (0.64)	<0.05	0.917	0.01 (0.01)	0.495	0.252	12.47 (4.56)	<0.05	**0.446**
No Sludge (NS)	-1.61 (1.09)	0.173	0.697	-0.02 (0.02)	0.454	0.369	11.89 (7.76)	0.160	**0.046**

[1] Values in parenthesis are standard error (n = 12). [2]Values are adjusted R^2.

observed between exchangeable Zn and total Zn in either the untreated soil or soils receiving the uncontaminated controls (**Table 3.8**).

A significant (R = 0.636; p <0.05) negative correlation was observed between exchangeable Zn and soil pH (**Table 3.8**), indicating, as mentioned previously (**See Section 2.5**), that the solubility of Zn increases as soil pH decreases. Similarly for the remaining soils, the regression coefficients for the relationship between total Zn and soil pH were all negative (**Table 3.8**), however only in the case of soil receiving the undigested control was the correlation statistically significant (R = 0.772; p <0.01). This is possibly due to the relatively small pH range (pH 5.8-6.5) covered by the data set, and the similar concentrations of total Zn observed in these soils (**See Section 3.4.2**). Hence the data set may be too limited to detect the influence of pH on the solubility of Zn in these soils using regression analysis.

With the exception of soil receiving the undigested control, addition of soil pH to the original regression model increased the proportion of variance explained; now accounting for 38 % of the variance observed in soil receiving the Zn sludge treatment (**Table 3.8**). However, only in soil receiving the undigested control was the correlation to total Zn statistically significant (R = 0.917; p <0.05), with no significant correlation now observed between exchangeable Zn and soil pH (**Table 3.8**). However, the F-test result was statistically significant (p <0.05) for soil receiving the Zn sludge treatment, indicating that both total Zn and soil pH have a combined influence on the amount of exchangeable Zn, extractable by $CaCl_2$ (**Figure 3.19**); this was also the case for soil receiving the undigested control (p <0.05).

Therefore it follows that Zn will be most soluble, and therefore most bioavailable, at the Hartwood field site, having both the highest concentration of total Zn remaining in soil receiving the Zn sludge treatment (**Figure 3.8**), and the lowest soil pH (**See Section 2.5**). This is shown in **Figure 3.19**, as the two points corresponding to the highest concentrations of exchangeable Zn (56.16 and 49.0 mg kg^{-1}) were obtained from the Hartwood site. Hence, with regards to **Research Question 1**, it can be predicted that the greatest impact on soil microorganisms due to Zn toxicity is likely to be observed at the Hartwood site, with a potentially similar impact also observed at Auchincruive; again due to relatively high concentrations of Zn and low soil pH in comparison to the English sites.

3.5.4. Copper Speciation Results (2014)

Auchincruive

The total sum of Cu species at Auchincruive was significantly (p <0.001) higher in soils receiving the Zn and Cu sludge treatments in comparison to the other soils. However, due to the apparent overestimation of Cu (+80 %) in the untreated soil, the difference in the total concentrations of Cu species between untreated soil and soils receiving the digested and undigested controls was no longer found to be statistically significant (**Table 3.9**).

Table 3.9:- Concentrations (mg kg^{-1}) of Copper species measured by sequential extraction in soil samples taken from the LTSE field sites in 2014. Accuracy is expressed as a percentage of total metal concentration determined by *aqua-regia* acid digestion (See Section 3.4.1).

Sludge Treatment	Copper (mg Cu kg^{-1})					Accuracy (%)
	Exchangeable	Organic	Carbonate	Residual	Σ Species	
AUC/Zn	0.81 (0.11)[a][1][2]	32.17(1.86)[a]	17.08 (1.12)[a]	66.55 (4.34)[a]	116.61 (6.81)[a]	158 (7)
AUC/Ctrl1	0.14 (0.03)[b]	16.33 (1.63)[b]	6.96 (0.13)[b]	42.88 (1.17)[b]	66.30 (0.82)[b]	135 (6)
AUC/Cu	0.98 (0.24)[a]	55.27 (5.97)[c]	31.29 (5.36)[c]	71.05 (3.45)[a]	158.59 (13.20)[c]	113 (11)
AUC/Ctrl2	0.06 (0.03)[b]	15.64 (0.39)[b]	7.83 (1.89)[b]	44.08 (1.27)[b]	67.61 (2.00)[b]	158 (14)
AUC/NS	ND[3]	4.75 (0.73)[d]	5.85 (0.20)[b]	36.33 (1.25)[b]	46.93 (1.63)[b]	180 (8)
GLE/Zn	ND	20.17 (5.18)[a]	28.56 (5.46)[a]	37.34 (5.04)[ab]	86.07 (15.08)[a]	169 (23)
GLE/Ctrl1	ND	8.30 (0.56)[ab]	12.26 (0.63)[a]	30.53(1.90)[b]	51.08 (1.15)[ab]	189 (12)
GLE/Cu	0.01 (0.01)	63.40 (8.05)[c]	71.43 (20.84)[b]	41.35 (4.39)[a]	176.19 (24.31)[c]	153 (10)
GLE/Ctrl2	ND	7.06 (1.89)[ab]	10.55 (2.85)[a]	27.80 (1.04)[b]	45.41 (3.67)[ab]	211 (30)
GLE/NS	ND	2.08 (0.33)[b]	2.82 (0.50)[a]	27.74 (0.68)[b]	32.64 (0.73)[b]	321 (19)
HAR/Zn	ND	37.90 (3.35)[a]	19.34 (1.61)[a]	64.75 (6.32)[a]	121.99 (8.61)[a]	120 (4)
HAR/Ctrl1	ND	12.31 (0.23)[b]	7.21 (0.92)[bd]	42.65 (3.50)[b]	62.17 (4.60)[b]	125 (6)
HAR/Cu	0.13 (0.13)	63.61 (5.08)[c]	40.51 (4.57)[c]	78.58 (2.68)[a]	182.83 (7.53)[c]	110 (2)
HAR/Ctrl2	ND	12.33 (1.01)[b]	10.50 (1.84)[b]	46.02 (6.05)[b]	68.85 (8.66)[b]	134 (1)
HAR/NS	ND	4.55 (1.48)[b]	2.69 (0.45)[d]	32.30 (2.64)[b]	39.54 (4.15)[d]	167 (11)
WOB/Zn	ND	23.83 (0.19)[ac]	26.42 (1.60)[a]	24.38 (1.59)[a]	74.64 (2.12)[a]	131 (6)
WOB/Ctrl1	ND	31.57 (3.53)[ab]	10.84 (0.62)[b]	17.12 (0.53)[bc]	59.53 (4.61)[ab]	215 (18)
WOB/Cu	ND	38.46 (7.36)[b]	57.21 (6.37)[c]	23.43 (1.42)[ab]	119.09 (15.02)[c]	121 (8)
WOB/Ctrl2	ND	15.99 (0.25)[cd]	10.40 (0.37)[b]	14.50 (0.55)[c]	40.89 (1.17)[bd]	188 (3)
WOB/NS	ND	6.65 (0.80)[d]	2.40 (0.41)[b]	15.42 (4.50)[c]	24.47 (3.39)[d]	230 (43)

[1]Values in parenthesis are standard errors (n = 3). [2] Values without corresponding letters denotes statistical significance at $\alpha = 0.05$. [3] ND = Not Detected.

Concentrations of organically bound Cu were significantly higher in soils receiving the Zn ($p < 0.01$) and Cu ($p < 0.001$) sludge treatments in comparison to the other soils, and were approximately 28 and 35 % of total Cu, respectively (**Figure 3.20**). Organically bound Cu ranged from 23-25 % in soils receiving the digested and undigested controls and was also found to be significantly ($p < 0.05$) higher than in untreated soil (10 %). Similarly, the concentrations of Cu associated with carbonates, were also significantly higher in soils receiving the Zn (15 %; $p < 0.05$) and Cu (20 %; $p < 0.001$) sludge treatments in comparison to the other soils (11-13 %), with no significant differences observed between them (**Table 3.9**). Exchangeable forms of Cu were only detected in the sludge amended soils at Auchincruive and were less than 1 % of total Cu in each case (**Figure 3.20)**, however the concentration in soils receiving the Zn and Cu sludge treatments were significantly ($p < 0.05$) higher than in soils receiving the uncontaminated controls (**Table 3.9**).

Gleadthorpe

In all cases, the total concentrations of Cu species were overestimated by ≥ 50 % at the Gleadthorpe field site (**Table 3.9**); particularly in the untreated soil where the sum of Cu species was approximately 3 times greater than the value determined by microwave acid digestion (**Table 3.6**). Nevertheless, the relative differences in total Cu concentrations determined by sequential extraction were still in agreement with the results described in **Section 3.4.3**. The sum of Cu species in soil receiving the Cu sludge treatment was significantly ($p < 0.001$) higher in comparison to the other soils (**Table 3.9**). The total concentration of Cu species in soil receiving the Zn sludge treatment was also significantly

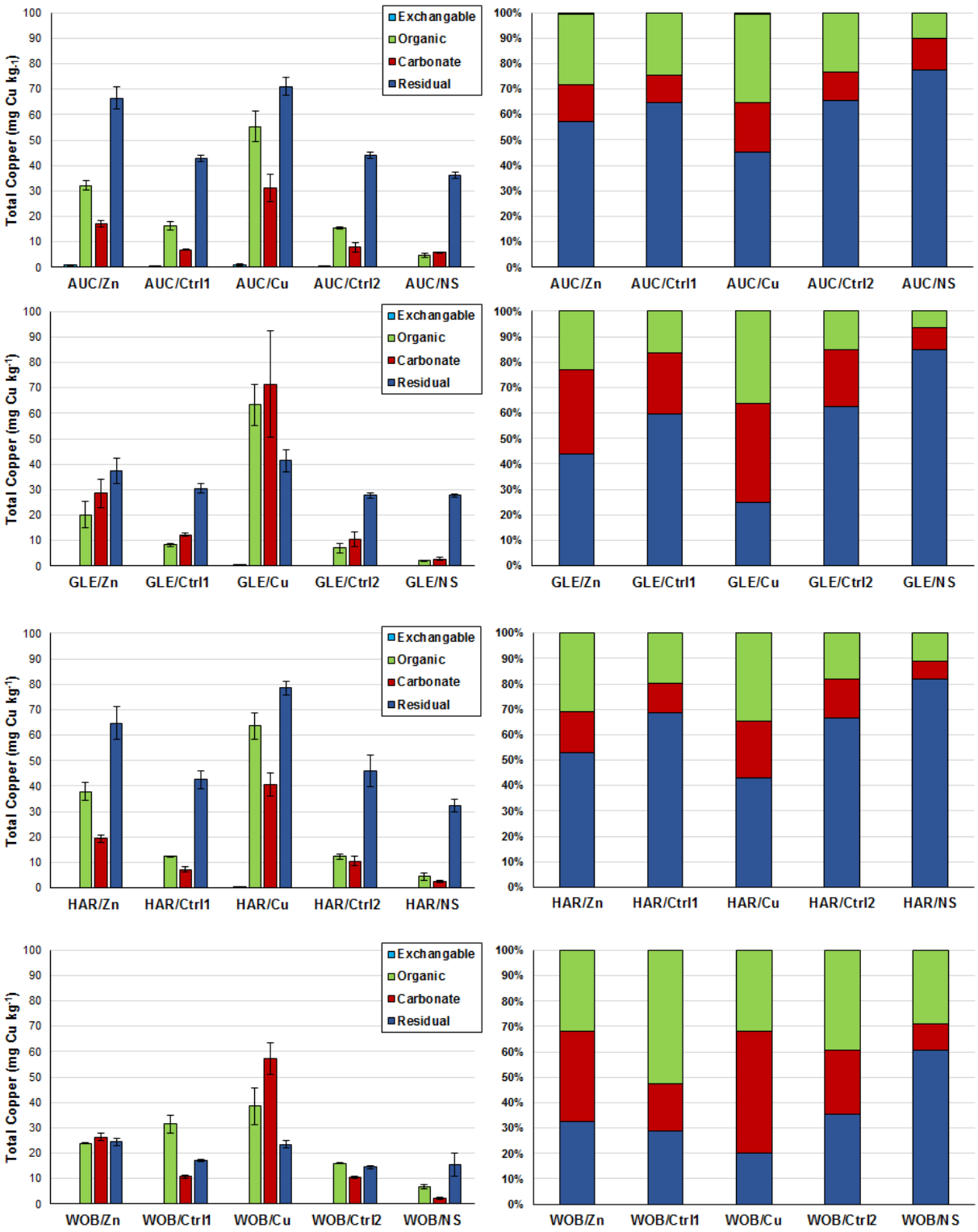


Figure 3.20:- Concentrations (mg kg^{-1}) of Copper species determeind by sequential extraction at the LTSE field sites in 2014 (left). Results are expressed as a percentage of the total Copper concentration (right). Error bars represent standard error (n = 3).

(p <0.05) higher in comparison to untreated soil, but not those receiving the digested and undigested controls (**Table 3.9**).

The concentration of organically bound Cu in soil receiving the Cu sludge treatment (36 %) was significantly (p <0.001) higher in comparison to all other soils (**Table 3.9**); with the only other

significant (p <0.05) difference observed between soil receiving the Zn sludge treatment (23 %) and untreated soil (6 %). However, at Gleadthorpe, Cu was predominantly associated with carbonates (39 %) in soil receiving the Cu sludge treatment (**Figure 3.20**) and, again, was significantly (p <0.01) higher in comparison to the other soils, with no significant differences observed between them (**Table 3.9**). Only trace amounts (<0.5 %) of exchangeable Cu were observed in soil receiving the Cu sludge treatment (**Table 3.9**).

Hartwood

Values for total Cu concentration at the Hartwood field site, determined by sequential extraction, appear to be the most accurate, with values within 10-70 % of the values reported in **Section 3.4.3** (**Table 3.9**). The total sum of Cu species in soil receiving the Cu sludge treatment was significantly (p <0.001) higher in comparison to the other soils; with the sum of Cu species in soil receiving the Zn sludge treatment also significantly (p <0.001) higher than in the remaining soils (**Table 3.9**). No significant difference was observed between soils receiving the digested and undigested control, however, the sum of Cu species in both soils was significantly (<0.5 %) higher in comparison to the untreated soil (**Table 3.9**).

In all cases, Cu was predominantly in the residual fraction at Hartwood, ranging from 43 % in soil receiving the Cu sludge treatment to 82 % in untreated soil (**Figure 3.20**), and was significantly higher in soils receiving the Cu (p <0.001) and Zn (p <0.05) sludge treatments than in the other soils (**Table 3.9**). Organically bound Cu was the secondary fraction in all cases (**Figure 3.20**), with concentrations in soils receiving the Zn (31 %) and Cu (35 %) sludge treatments significantly (p <0.001) higher than the other soils; no significant differences were observed between them. Concentrations of Cu associated with carbonates were also significantly higher in soils receiving the contaminated sludge treatments (Zn (p <0.05) and Cu (p <0.001)) in comparison to the other soils, with concentrations in soil receiving the uncontaminated control (15 %) also significantly (p <0.05) higher than in untreated soil (7 %). Exchangeable Cu was only present in soil receiving the Cu sludge treatment (<0.5 %).

Woburn

The accuracy of results obtained by the sequential extraction of metals varied considerably at Woburn, with sum of Cu species ranging from approximately +20 % to +130 % of the values reported for total Cu in **Section 3.4.3** (**Table 3.9**). The sum of Cu species in soil receiving the Cu sludge treatment remained significantly higher (p <0.01) in comparison to the other soils, however total Cu in soil receiving the Zn sludge treatment was no longer significantly higher in comparison to soil receiving the digested control, as described above (**See Section 3.4.3**).

As seen at Gleadthorpe, Cu was predominantly associated with carbonates (48 %) in soil receiving the Cu sludge treatment and was significantly higher (p <0.001) than in the other soils (**Figure 3.20**). This was also the case for soil receiving the Zn sludge treatment, which also contained concentrations

Table 3.10:- Regression and correlation coefficients from regression analysis of organic Copper on total Copper concentration and soil organic carbon.

	Regression of Organic Cu on Total Cu					
Sludge Treatment	**Slope (Total Cu)**	***p***	**R**	**Intercept**	***p***	**$R^{2[2]}$**
Zinc (Zn)	0.34 (0.04)[1]	<0.001	0.933	3.86 (3.14)	0.248	**0.859**
Dig. Control (Ctrl1)	-0.16 (0.06)	<0.05	0.656	26.88 (4.18)	<0.001	**0.373**
Copper (Cu)	0.30 (0.10)	<0.05	0.683	16.23 (13.58)	0.260	**0.413**
Un. Dig. Control (Ctrl2)	0.09 (0.08)	0.296	0.329	9.59 (3.10)	<0.05	**0.019**
No Sludge (NS)	0.07 (0.08)	0.382	0.278	3.21 (1.55)	0.065	**-0.02**
	Regression of Organic Cu on Organic C					
	Slope (Organic C)	***p***	**R**	**Intercept**	***p***	**R^2**
Zinc (Zn)	5.59 (0.90)	<0.001	0.892	12.20 (2.88)	<0.01	**0.775**
Dig. Control (Ctrl1)	-3.62 (2.62)	0.198	0.400	26.85 (7.53)	<0.01	**0.076**
Copper (Cu)	7.88 (3.57)	0.052	0.573	32.75 (10.77)	<0.05	**0.261**
Un. Dig. Control (Ctrl2)	0.16 (1.17)	0.892	0.044	12.34 (3.23)	<0.01	**-0.10**
No Sludge (NS)	-0.09 (0.65)	0.889	0.045	4.72 (1.60)	<0.05	**-0.10**

	Regression of Organic Cu on Organic C and Total Cu								
	Slope (Org. C)	***p***	**R**	**Slope (Tot. Cu)**	***p***	**R**	**Intercept**	***p***	**R^2**
Zinc (Zn)	1.59 (1.70)	0.373	0.254	0.26 (0.10)	<0.05	0.704	5.29 (3.51)	0.166	**0.857**
Dig. Control (Ctrl1)	-3.92 (1.87)	0.065	0.434	-0.16 (0.05)	<0.01	0.677	37.74 (6.30)	<0.001	**0.533**
Copper (Cu)	-0.66 (6.52)	0.921	0.048	0.32 (0.21)	0.161	0.724	15.75 (15.0)	0.322	**0.348**
Un. Dig. Control (Ctrl2)	-5.09 (2.12)	<0.05	1.362	0.43 (0.16)	<0.05	1.559	10.64 (2.59)	<0.01	**0.332**
No Sludge (NS)	-0.95 (0.86)	0.298	0.462	0.16 (0.11)	0.186	0.598	3.84 (1.64)	<0.05	**0.007**

[1] Values in parenthesis are standard error (n = 12). [2]Values are adjusted R^2.

of carbonate Cu (35 %) significantly (p <0.01) higher than the remaining soils (**Table 3.9**). In contrast, organically bound Cu was the predominant fraction in soils receiving the digested (53 %) and undigested (39 %) controls (**Figure 3.20**), with no significant difference observed between soil receiving the digested control and those receiving the contaminated sludge treatments (**Table 3.9**); the difference in organic Cu between soil receiving the undigested control and Zn sludge treatment was also not statistically significant. Exchangeable Cu was not observed at Woburn.

3.5.5. Organic Copper as a Function of Total Copper and Organic Carbon

Regression of organic Cu (extractable by NaOH) on total Cu, for samples collected at each of the LTSE field sites during 2014 (**Table 3.6**), showed significant positive correlations for both soils receiving the Zn (R = 0.933; p <0.001) and Cu (R = 0.683; p <0.05) sludge treatments (**Table 3.10**); respectively accounting for approximately 86 and 41 % of the observed variance in these soils. However in contrast, a significant negative correlation was seen in soils receiving the digested control (R = 0.656; p <0.05), due to the large fraction of organically bound Cu (53 %) observed in this soil at Woburn (**Figure 3.20**). In this case, due to the apparent overestimation of Cu by the sequential extraction method (**Table 3.9**), the value for organically bound Cu determined in soil receiving the digested control at Woburn (31.57 ± 3.53 mg kg^{-1}) was approximately equal to that of total Cu (27.77 ± 0.92 mg kg^{-1}) determined by microwave acid digestion.

A significant positive correlation was also found between the concentration of organically bound Cu and SOC in soils receiving the Zn sludge treatment (R = 0.892; p <0.001), though the proportion of

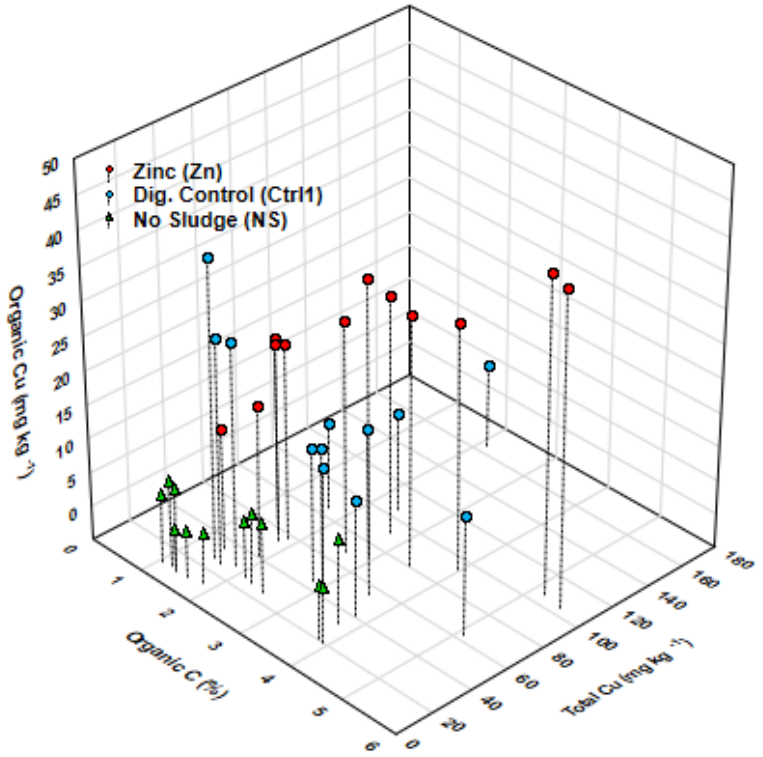


Figure 3.21:- Plot of organic Copper (mg kg^{-1}), extractable by NaOH, as a function of soil organic carbon (%) and total Copper concentration (mg kg^{-1}), in untreated soil (NS), and soils receiving digested sludge treatments (Zn and Ctrl1), taken from each of the LTSE fields sites in 2014.

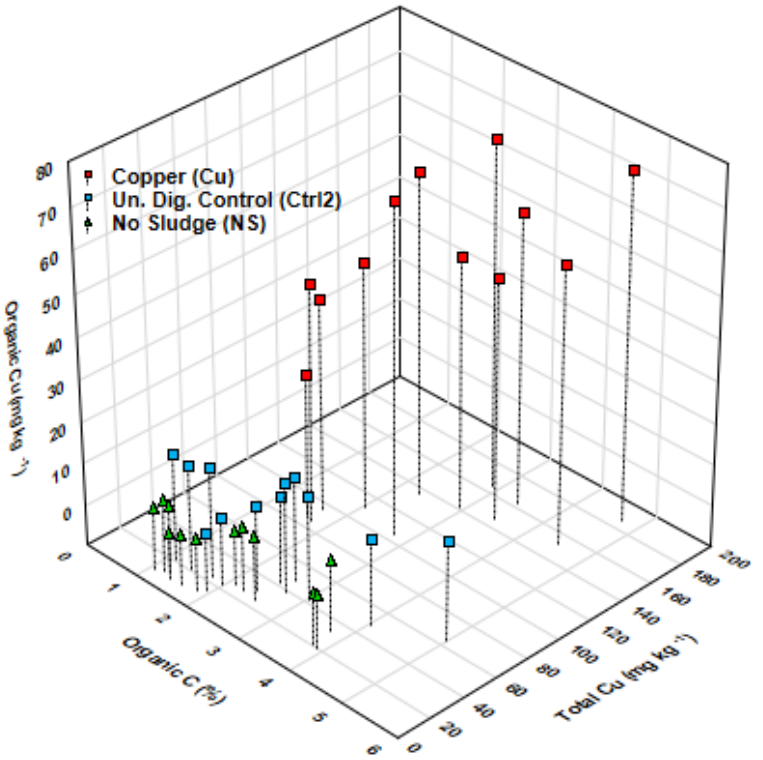


Figure 3.22:- Plot of organic Copper (mg kg^{-1}), extractable by NaOH, as a function of soil organic carbon (%) and total Copper concentration (mg kg^{-1}), in untreated soil (NS), and soils receiving undigested sludge treatments (Cu and Ctrl2), taken from each of the LTSE fields sites in 2014.

Table 3.11:- Regression and correlation coefficients showing multicollinearity between the independent variables total Copper and soil organic carbon.

	Regression of Organic C on Total Cu					
Sludge Treatment	**Slope (Total Cu)**	***p***	**Intercept**	***p***	**R**	**$R^{2[2]}$**
Zinc (Zn)	0.05 (0.01)	<0.001	-0.90 (0.59)	0.156	0.907	**0.805**
Digested Control (Ctrl1)	-0.001 (0.01)	0.878	2.77 (0.61)	<0.01	0.050	**-0.10**
Copper (Cu)	0.04 (0.01)	<0.001	-1.89 (0.81)	<0.05	0.888	**0.767**
Undigested Control (Ctrl2)	0.06 (0.01)	<0.01	0.64 (0.50)	0.223	0.816	**0.632**
No Sludge (NS)	0.09 (0.03)	<0.05	0.66 (0.56)	0.270	0.695	**0.432**

[1] Values in parenthesis are standard error (n = 12). [2]Values are adjusted R^2.

Table 3.12:- Regression and correlation coefficients from regression analysis of organic Copper on Σ Copper species and soil organic carbon.

	Regression of Organic Cu on Σ Cu Species								
Sludge Treatment	**Slope (Σ Cu)**			***p***	**R**	**Intercept**		***p***	**$R^{2[2]}$**
Zinc (Zn)	0.32 (0.04)[1]			<0.001	0.921	-3.32 (4.39)		0.467	**0.832**
Dig. Control (Ctrl1)	0.43 (0.38)			0.277	0.342	-8.76 (22.68)		0.708	**0.028**
Copper (Cu)	0.40 (0.03)			<0.001	0.969	-8.67 (5.29)		0.132	**0.932**
Un. Dig. Control (Ctrl2)	0.09 (0.08)			0.314	0.318	7.95 (4.68)		0.120	**0.011**
No Sludge (NS)	-0.01 (0.07)			0.864	0.055	4.96 (2.65)		0.091	**-0.10**
	Regression of Organic Cu on Organic C and Σ Cu Species								
	Slope (Org. C)	***p***	**R**	**Slope (Σ Cu)**	***p***	**R**	**Intercept**	***p***	**R^2**
Zinc (Zn)	2.42 (1.36)	0.111	0.386	0.20 (0.08)	<0.05	0.589	1.07 (4.70)	0.825	**0.862**
Dig. Control (Ctrl1)	-6.78 (2.34)	<0.05	0.749	0.90 (0.33)	<0.05	0.712	-18.5 (17.5)	0.319	**0.441**
Copper (Cu)	-0.45 (1.43)	0.760	0.033	0.41 (0.04)	<0.001	0.989	-8.71 (5.55)	0.151	**0.925**
Un. Dig. Control (Ctrl2)	-4.69 (2.18)	0.060	1.264	0.39 (0.16)	<0.05	1.455	2.69 (4.70)	0.581	**0.274**
No Sludge (NS)	-0.05 (0.81)	0.957	0.022	-0.01	0.914	0.044	4.96 (2.80)	0.110	**-0.22**

[1] Values in parenthesis are standard error (n = 12). [2]Values are adjusted R^2.

variance explained was reduced slightly to 78 % (**Table 3.10**). The relationship between the two variables in soil receiving the Cu sludge treatment was also reasonably strong (R = 0.573; p = 0.052), accounting for 26 % of the observed variance, however the correlation was not found to be statistically significant (**Table 3.10**).

Figure 3.21 and **Figure 3.22** show the relationship between organically bound Cu, total Cu, and organic C. In each case, combining total Cu and SOC in a single regression model increased the proportion of variance explained for untreated soil and soils receiving the digested and undigested controls (**Table 3.10**). Whereas the proportion of variance explained for soil receiving the Cu sludge treatment (35 %) decreased in comparison to the original regression model (**Table 3.10**); no change was seen in the proportion of variance explained for soil receiving the Zn sludge treatment. Strong positive correlations between total Cu and organic Cu were still observed in soils receiving the Zn (R = 0.704; p <0.05) and Cu (R = 0.724; p = 0.161) sludge treatments, however the relationship was no longer significant in soil receiving the Cu sludge treatment. In addition, the correlation between organic Cu and SOC was no longer significant in soil receiving the Zn sludge treatment (R = 0.254; p = 0.373) and was now negative in soil receiving the Cu sludge treatment (R = 0.048; p =0.921). For soil receiving the Zn sludge treatment, the result of an F-test was statistically significant (p <0.001) suggesting SOC and total Cu do have a combined influence on the concentration of organically bound Cu, though this was not the case for soil receiving the Cu sludge treatment (p = 0.059).

In contrast, the relationship between organic Cu and the two variables, SOC and total Cu, in soil receiving the digested control were both negative (**Table 3.10**) with a significant correlation to total Cu ($R = 0.677$; $p < 0.01$); in this case the F-test result was also significant ($p < 0.05$). However again, it should be noted that the method of sequential extraction appears to have overestimated the concentration of Cu species, with accuracy ranging from +20-69 % and +10-53 % for soils receiving the Zn and Cu sludge treatments, respectively. Whereas, in the case of soil receiving the digested control at Woburn, the total concentration of Cu determined by sequential extraction is overestimated by approximately +115 %, again, with the concentration of organically bound Cu approximately equal to the value of total Cu determined by microwave acid digestion. Furthermore, there appears to be high multicollinearity between the variables total Cu and SOC within the current data set (**Table 3.11**), particularly in soils receiving the contaminated sludge treatments. As described previously, the lowest values for both total Cu (**Table 3.6**) and SOC (**Table 2.7**) were observed at Woburn, whereas the highest values for each were observed at Hartwood. Therefore, given the current data set, it is not possible to accurately determine the effect of each variable. Nevertheless, it seems that organically bound Cu does appear to increase in proportion to both the total concentration of Cu and SOC. However, although in some cases the relationship between organic Cu and total Cu appears to be statistically significant, these results should still be considered with caution due to the discrepancies in Cu concentration determined by the two methods.

For soil receiving the Cu sludge treatment, the correlation between organic Cu and the sum concentration of Cu species, determined by sequential extraction, was greater in comparison to the original regression model (**Table 3.12**); now accounting for approximately 93 % of the observed variance. Whereas little change was seen for soil receiving the Zn sludge treatment. Again including organic C in the regression model caused little change in the proportion of variance explained for soils receiving the Zn and Cu sludge treatments (**Table 3.12**). However, in both cases the F-test result remained statistically significant (Zn ($p < 0.001$); Cu ($p < 0.001$)), further indicating both total Cu concentration and SOC content influence the amount of organically bound Cu present in a soil.

3.6. Chapter Discussion

3.6.1. Previous Findings (1997-2011)

Application of both the Zn and Cu sludge treatments has undoubtedly caused a significant and persistent increase in the total concentration of each metal found in soils receiving the respective sludge treatments (**See Section 3.4.2** and **Section 3.4.3**). Over the past 20 years (1994-2014), the total concentrations of each metal have respectively decreased, on average, by approximately 10-37 % and 9-35 %. Greater decreases in total metal concentration were seen at the English sites, in comparison to the Scottish sites,

which is presumably due to the ley/arable cropping regime implemented at the English sites, and the uptake of Zn and Cu by wheat crops (**See Section 2.1**). However, in most cases the total concentration of Zn still remained above the UK advisory limit of 200 mg kg^{-1}, or in the case of Hartwood above the UK statutory limit (300 mg kg^{-1}), whereas the total concentration of Cu remained close to the UK statutory limit (135 mg kg^{-1}); total Cu at Hartwood was also above the statutory limit. Hence, with regards to **Research Question 4**, any adverse effect observed on soil microorganisms, or phosphatase enzyme activity, particularly at the two English sites, will indicate that the current statutory limits for Zn and Cu may be set too high.

With the exception of Woburn, significant (p <0.05) correlations between available Zn, extractable by NH_4NO_3, and total Zn concentration (measured across dose response Level 1-4 at each site (**Table 3.3**)) were observed at each of the LTSE field sites during the course of experimental Phase II (Defra, 2002). Extractable Cu was only significantly (p <0.05) correlated to total Cu at Woburn in 1999, and Auchincruive in 2001; and in the case of Woburn, the relationship was negative (Defra, 2002). In addition, NH_4NO_3 extractable Zn was found to be consistently higher at the Scottish sites in comparison to the English sites, presumably due to lower values of pH that the soils were kept at (**See Section 2.5**). A strong significant (R^2 = 0.960; p <0.001) correlation between the total concentration of Zn in the soil solution and free ion concentration was seen at both of the English sites during Phase II (2001), with approximately 80 % of Zn in the soil solution present as Zn^{2+}.

Throughout experimental Phase III, concentrations of NH_4NO_3 extractable Zn were consistently higher at Hartwood (% 7 of the total concentration, in comparison to the other sites (2.7 % at AUC and GLE, and 1.7 % at WOB)), again this is presumably due to the lower soil pH (Defra, 2007a). In contrast, NH_4NO_3 extractable Cu was consistently lower at Hartwood, possibly due to the higher SOC content (**See Section 2.6**). Gibbs et al. (2006) also found that NH_4NO_3 extractable Cu was inversely proportional (p <0.001) to the soil Fe content, which was lower at the two English sites in comparison to the Scottish sites (**Table 2.1**). For both metals, the relationship between total metal concentration and that extractable by NH_4NO_3 was significantly (p <0.01) positive at each of the sites during experimental Phase III (Defra, 2007a). Again at the English sites, significant correlations were observed between the total concentration of Zn (R^2 = 0.850; p <0.001) and Cu (R^2 = 0.580; p <0.001) present in soil solution and the concentration of free ions. Though in each case less than 0.1 % of Cu in the soil solution was present as Cu^{2+}, whereas the proportion of Zn present as Zn^{2+} ranged from 60-80 % (Defra, 2007a). Significant (p <0.001) correlations were also found between the total solution concentrations of Zn and Cu, and the concentration of each metal extractable by NH_4NO_3 over the course of experimental Phases II and III (Defra, 2002; 2007a); hence, since the soluble metal fraction is the most bioavailable, the concentration of each metal determined by NH_4NO_3 extraction was considered to be a good indicator of metal bioavailability.

3.6.2. Current Investigation (2012-2014)

Exchangeable Zn (**See Section 3.5.2**) and organically bound Cu (**See Section 3.5.4**) metal species still remain significantly higher in soils receiving the respective contaminated sludge treatments 20 years following the final sludge applications. In most cases, these metal species have also increased in soils receiving the uncontaminated controls compared to untreated soil. Hence, a consistent trend seems to be evident over the course of the Long-Term Sludge Experiment, whereby increasing the total concentrations of Zn and Cu in a soil, has caused proportional increases in the concentration of exchangeable Zn and organically bound Cu. Therefore with regards to **Research Question 1** it can be expected that microorganisms in soils receiving the Zn sludge treatment have been exposed to more bioavailable metal contamination over the past 20 years, which could still potentially cause an observed decrease in microbial biomass. Above pH 5, Cu has been shown to have a high binding affinity to soil organic matter (Alloway, 1995; McClaren & Crawford, 1973b), and in general is considered to be immobile in soils (Alloway, 1995); this is consistent with the results presented here. Hence, with regards to **Research Question 2**, although the risk to soil microorganisms may be reduced due to the apparently low solubility of Cu, soil organic matter includes extracellular enzymes such as phosphatases. Therefore even in this situation Cu may still directly interfere with the mineralisation of organic P due to the inhibition of phosphatase enzymes within the soil environment.

In agreement with the results from experimental Phases I-III, the availability of Zn was also found to increase in proportion to the total concentration of the metal during the current investigation; though, with regards to Cu, the results are not as consistent. It should be noted that a different extractant ($CaCl_2$) was used to determine the 'available' metal fraction during the current investigation, hence the results are not directly comparable, to those obtained during experimental Phases I-III. However, Pueyo et al. (2004) have compared the amounts of Zn and Cu extractable by both $CaCl_2$ and NH_4NO_3 and found both methods to be in agreement. Nevertheless, the accuracy of the sequential extraction method used for the current investigation needs to be questioned as the concentration of Cu species appear to be overestimated (**Table 3.9**). It is possible that the $CaCl_2$ solution used in the current investigation was too weak an extractant to displace adsorbed Cu within the soil environment, as only in soils receiving the Cu sludge treatment was exchangeable Cu detected, hence the concentrations of Cu determined in the subsequent fractions would be overestimated. A weaker solution of $CaCl_2$ (0.05 M) was used by McLaren and Crawford (1973a) to determine exchangeable Cu in a range (n = 24) of UK soils. However despite analysing a greater quantity of soil (20 g) Cu extractable by $CaCl_2$ still only accounted for <1 % of the total concentration. A quantity of 5 g was also extracted, but produced insufficient Cu for reliable analysis (McLaren & Crawford, 1973a). Therefore it is possible that the quantity of soil used in the current investigation was also too small to produce sufficient exchangeable Cu, though it is

more likely that, over the soil pH range of the LTSE sites, the availability of Cu has now reduced and the metal is now predominantly bound by organic matter (Alloway, 1995; McClaren & Crawford, 1973b). Another possibility for the overestimation of Cu species is that the acid solution used to remove organic matter from the organic and carbonate fractions (**See Section 3.5.1**) was too weak (McGrath and Cegarra (1992) originally used *aqua-regia* to remove organic matter). The presence of residual organic matter, such as humic and fulvic acids, within the extracts could therefore, potentially, cause interference when determining Cu by AAS (Todolí & Mermet, 1999).

As an alternative to the '*sludge time-bomb*' and '*sludge protection*' hypotheses (**See Section 2.7**), McBride (1995) also describes a '*constant partitioning*' model which suggests that the adsorptive capacity of a sludge amended soil increases in proportion to the quantity of sludge applied. Therefore metal availability will simply be a linear function of the total metal loading due to the partitioning of heavy metal contamination between soluble and immobilised forms. This appears to be the case for the LTSE sites, but has also been observed at a number of long-term sludge experiments. For instance, McGrath and Cegarra (1992) investigated changes in the speciation of heavy metal contamination, in sludge amended soils, at the Woburn Market Garden Experiment over a period of 42 years (1942-1984). Prior to the application of sludge in 1942, over 80 % of Zn, Cd, Cr, and Ni, were found in the residual fraction, whereas Cu appeared to be distributed between residual (40 %), organic (35 %), and carbonate (25 %) fractions; Pb was also distributed between residual (65 %) and carbonate (30 %) fractions. Significant changes in metal speciation occurred during the period of sludge application (1942-1961), following which very little change in metal speciation was observed (1961-1984); despite losing approximately 88 % of the total C loading over the same period (McGrath et al., 2000). In general a shift from the residual fraction to the carbonate fraction was seen for all metals, though for Cr this change was slight due to the low solubility of the metal. The proportion of Pb present as carbonates increased to about 80 %, with the remaining 20 % in the residual fraction, again indicating low metal solubility. Concentrations of Zn and Ni, present in exchangeable and organically bound forms increased to approximately 5 % and 10 % respectively. Whereas a marked increase in exchangeable Cd was observed reaching 20 % of the total metal concentration. Approximately 30 % of Cu remained organically bound for the duration of the experiment, with carbonates accounting for approximately 60 % of the total concentration by 1984. A slight increase in exchangeable Cu was observed following the final applications of sludge in 1961, however this eventually decreased by 1984, suggesting the metal had become immobilised. McGrath et al. (2000) re-examined the availability of Zn and Cd over the same period and confirmed that the proportion of total metal concentration present in exchangeable forms (extractable by $CaCl_2$) remained relatively constant at approximately 1.5-3.5 % and 15 %, respectively, for a period of almost 25 years; with no significant change observed in the ratio of SOC to exchangeable metal concentration.

Table 3.13:- Change in total metal concentration (mg kg^{-1}) and percentage in exchangeable form (%), over the course of nine years (1972-1981) at the Luddington and Lee Valley long-term sludge experiments. Data obtained from Berrow & Burridge (1990).

	Luddington				Lee Valley[1]			
	Zn	**Cu**	**Ni**	**Cr**	**Zn**	**Cu**	**Ni**	**Cr**
Total Conc. (mg kg^{-1})								
1968[2]	50	20	15	30				
1972	650	420	285	350				
1981	420	320	110	255				
Exchangeable (%)								
1972	54	25	34	1.5	54	22	35	1.5
1981	54	22	30	1.3	57	17	31	1.1

[1]Total metal concentrations for Lee Valley are not reported (Berrow & Burridge, 1990). [2]Values are background concentrations in untreated soil prior to sludge application in 1968.

Similarly, Berrow and Burridge (1990) monitored the availability of heavy metal contamination over a period of 9 years (1972-1981), in sludge amended soils at the Luddington and Lee Valley sludge experiments. In agreement with the results of McGrath and Cegarra (1992), changes in the speciation of heavy metals occurred during the period of sludge application (1968-1972), following which little change was observed. For instance, at the Luddington field site, the total concentrations of Zn, Cu, Ni, and Cr increased from 50, 20, 15, and 30 mg kg^{-1}, respectively, in the untreated soil, to 650, 420, 285, and 350 mg kg^{-1} following four applications of 'high-metal' sludge treatments (125 t ha yr^{-1}). Total metal concentrations subsequently declined over the next 9 years (**Table 3.13**), however the average percentage (n = 14) of the total metal concentration present in exchangeable forms, in this case extractable by acetic acid ($C_2H_4O_2$), remained essentially constant over the same period, again indicating the partitioning of heavy metal contamination between soluble and immobilised forms (**Table 3.13**); this trend was also observed at the Lee Valley site (Berrow & Burridge, 1990).

3.7. Conclusions

3.7.1. Previous Findings (1997-2011)

Application of the Zn and Cu sludge treatments during experimental Phase I, at 'Level 3' of the dose response curve, increased the total concentration of each metal, in the receiving soils at each field site, above the respective UK statutory limits. With the exception of the Woburn site, the total concentration of Zn remained above the UK statutory limit of 300 mg Zn kg^{-1} for the duration of experimental Phases II, III, and IV. Similarly, the total concentration of Cu, in soil receiving the Cu sludge treatment, remained above the UK statutory limit of 135 mg Cu kg^{-1} at each of the LTSE field sites during experimental Phases II, III, and IV; though in some instances dropped below the limit at Woburn and Auchincruive. At each site, application of the Zn sludge treatment also caused increases in the total concentration of Cu in the receiving soils. These were significantly greater in comparison to soils

receiving the digested control, hence, with regards to **Research Question 1**, any adverse effect on soil microorganisms observed in these soils could potentially be due to a combination of Zn and Cu toxicity.

At each of the LTSE field sites the concentration of Zn extractable by NH_4NO_3, and hence more bioavailable, had increased in proportion to the total concentration of Zn, and was therefore greatest at the two Scottish sites. A steady increase in NH_4NO_3 extractable Zn was observed at Auchincruive and Gleadthorpe, reaching a maximum in 2001, at the end of experimental Phase II, whereas values at Woburn and Hartwood fluctuated over the course of experimental Phases II and III. With the exception of Auchincruive, where values remained fairly steady over the course of experimental Phases I-III, NH_4NO_3 extractable Cu decreased by approximately 50 % in 1999, at the start of experimental Phase II, at each of the LTSE field sites before stabilising for the remainder of the experiment.

3.7.2. Current Investigation (2012-2014)

For the duration of the current investigation, the total concentration of Zn at the Scottish sites, Hartwood in particular, remained above the UK limit, whereas values at Gleadthorpe had now decreased below; and in general total Zn at Woburn was below the current advisory limit of 200 mg Zn kg^{-1}. In each case, the total concentration of Zn remained significantly higher in soil receiving the Zn sludge treatment in comparison to both untreated soil and soil receiving the digested control. Similarly, concentrations of exchangeable Zn, extractable by $CaCl_2$ also increased in proportion to total Zn concentration, with a negative correlation observed between the concentration of exchangeable Zn and soil pH; the solubility of Zn was therefore found to be greatest at the Hartwood field site. Hence, with regards to **Research Question 1**, it can be predicted that the greatest impact on soil microorganisms, due to application of the Zn sludge treatment, will be seen at the Scottish field sites due to the higher concentrations of Zn, and lower soil pH, in comparison to the English sites.

For the duration of the current investigation, the total concentration of Cu at the English field sites was below the UK limits, whereas concentrations at Hartwood remained above and were significantly higher in comparison to the other sites; total Cu at Auchincruive was approximately equal to the UK limit. Hence, with regards to **Research Question 4**, any adverse effect observed on soil microorganisms, or phosphatase enzyme activity, particularly at the two English sites, will give some indication that the current statutory limits for Zn and Cu may be set too high. In agreement with previous findings, the solubility of Cu appeared to be very low during the current investigation, with less than 1 % of the total concentration observed present in exchangeable form, extractable by $CaCl_2$. For soils receiving the Zn and Cu sludge treatments, the concentration of Cu bound to soil organic matter was significantly correlated to the total concentration of Cu in the soils, in each case. However, due to an apparent overestimation of Cu by the method of sequential extraction the relationships determined by multiple

regression analysis should be considered with caution. Regression analysis, using the sum of concentrations for each Cu species determined by sequential extraction, also showed a strong relationship between organically bound Cu and the total concentration of Cu present in the soil. However, due to multicollinearity within the data set, it was not possible to accurately determine the effect of soil organic matter on Cu speciation. Nevertheless, with regards to **Research Question 2**, the apparent increase in the concentration of organically bound Cu, in proportion to the quantity of Cu applied, indicates Cu could still interfere with the mineralisation of organic phosphorus by binding directly to phosphatase enzymes within the soil environment.

CHAPTER 4

PHOSPHORUS

4. PHOSPHORUS

4.1. Introduction

This chapter discusses the current state of total, available, and organic phosphorus fractions in the untreated and sludge amended soils at each of the Defra 'Long-Term Sludge Experiment' field sites. Possible effects of various soil properties on the availability of inorganic orthophosphate are also discussed. The long-term fate of the organic phosphorus compounds, applied as part of the sludge treatments, is also investigated using ^{31}P-NMR.

4.2. Inorganic Phosphorus

Like heavy metals, the total concentration of phosphorus (P) in soil is also distributed across a number of different fractions. Over geological timescales, phosphorus is solubilised from primary phosphate minerals and is released into the soil solution (Stevenson & Cole, 1999). As described in **Section 1.2**, phosphorus is only available as a nutrient to plants and microorganisms when present in the soil solution as inorganic orthophosphate (H_3PO_4). However, only a small percentage of the total phosphorus concentration is present in the soil solution as orthophosphate at a given time. Within the pH range of most soils (pH = 5-8) orthophosphate exists predominantly as $H_2PO_4^-$ and HPO_4^{2-} orthophosphate anions (**Figure 4.1**), with approximately 94 % and 60 % present as $H_2PO_4^-$ at pH 6 and pH 7, respectively (Stevenson & Cole, 1999). Orthophosphate anions are readily adsorbed to the surfaces of metal oxides and clay minerals, where they constitute a labile fraction in equilibrium with soluble phosphorus in the soil solution (Stevenson & Cole, 1999). However other factors within the soil environment can also influence the availability of phosphorus (Stevenson & Cole, 1999). In acidic soils, with pH < 6, orthophosphate can precipitate as Fe/Al-phosphates, whereas in calcareous alkaline soils, orthophosphate can precipitate as Ca-phosphates, or hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$); each of these precipitated compounds are highly insoluble. Hence, the availability of orthophosphate is largely determined by soil pH, with pH 6.5 considered to be the optimum value for phosphorus availability (Stevenson & Cole, 1999). Phosphorus is also incorporated into plant and microbial biomasses, where it becomes immobilised in organic forms. Organic phosphorus compounds therefore constitute a significant percentage of the total phosphorus content in soils (**See Section 4.3**).

The percentages of Fe (Fe_2O_3) and Al (Al_2O_3) oxides present in the untreated soils at each of the 'Long-Term Sludge Experiment' field sites, prior to the beginning of experimental Phase I in 1994, are given

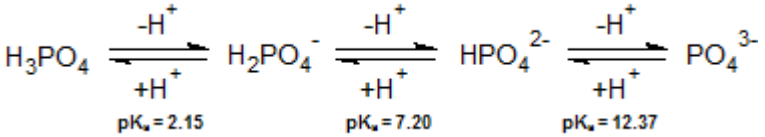


Figure 4.1:- Forms of inorganic orthophosphate. Within the pH range of most soils orthophosphate exists as $H_2PO_4^-$ and HPO_4^{2-}. Forms are present in equal amounts when pH = pK_a (Stevenson & Cole, 1999).

Table 4.1:- Estimated total phosphorus loadings (t ha^{-1}), applied during experimental Phase I (1994-1997), to soils receiving digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments at each of the Long-Term Sludge Experiment field sites. Data obtained from Gibbs et al. (2006).

Sludge Treatment	Sludge Properties[1]			Estimated Phosphorus Loading (t ha^{-1})[2]			
	C (%)	P (%)	C:P	AUC	GLE	HAR	WOB
Zinc (Zn)	31.6	2.87	11.01	6.27	6.27	6.72	7.36
Digested Control (Ctrl1)	38.1	1.98	19.24	3.59	3.59	3.84	4.21
Copper (Cu)	37.6	0.76	49.47	1.68	1.37	1.64	1.68
Undigested Control (Ctrl2)	42.9	1.61	26.65	3.11	2.55	3.04	3.04

[1]Values for C (%) and P (%) are from **Table 2.2**. [2] Values were calculated using total C loadings (t ha^{-1}) from **Table 2.3** (P (t ha^{-1}) = C (t ha^{-1})/(C:P)).

in **Table 2.1**. As mentioned in **Section 2.6**, adsorption of orthophosphate to Fe/Al oxide surfaces and the formation of insoluble Fe/Al-phosphates increases as pH decreases. Therefore, given that the Hartwood field site has the highest concentration of Fe/Al oxides (Fe_2O_3 = 3.32 %; Al_2O_3 = 7.78 %), and has been kept at a pH of approximately 5.8 for the duration of the experiment, it can be assumed that phosphorus availability will be the least at this site. In contrast, phosphorus availability is likely to be highest at the Gleadthorpe field site which has both the lowest concentration of Fe/Al oxides (Fe_2O_3 = 1.66 %; Al_2O_3 = 1.51 %) and the highest range of soil pH (**See Section 2.6**).

The sludge treatments applied during the 'Long-Term Sludge Experiment' were comprised of four different sludge types of varying phosphorus content (**Table 2.2**), with the digested sludge treatments (Zn and Ctrl1) containing more phosphorus as a percent of dried mass in comparison to the undigested sludge treatments (Cu and Ctrl2). Hence the soils amended with the digested sludge treatments received a greater phosphorus loading during experimental Phase I, proportionally increasing the total phosphorus concentration in these soils. An estimate of the applied phosphorus loadings have been calculated, as follows:

$$\mathrm{P}_{Loading}\ (\mathrm{t\ ha^{-1}}) = \frac{C_{Loading}\ (\mathrm{t\ ha^{-1}})}{C{:}P} \qquad (\mathbf{E.4.1})$$

where $C_{Loading}$ is the total C loading (t ha^{-1}) applied during experimental Phase I (**Table 2.3**), and C:P is the ratio of the C and P contents (%) for each of the sludge treatments (**Table 2.2**); estimated phosphorus loadings are given in **Table 4.1**. However, in order to ensure uniform quantities of organic carbon were applied across all levels of the dose-response curves (**See Section 3.2**), applications of the Zn and Cu sludge treatments were supplemented with corresponding sludge material from the digested and undigested controls (Gibbs et al., 2006). Hence for soils receiving the Zn and Cu sludge treatments the

total phosphorus loading will be the sum of the phosphorus loadings derived from the individual quantities of contaminated and uncontaminated sludge that were applied:

$$P_{Loading}\ (\mathrm{t\ ha^{-1}}) = \left(\frac{C_{Zn/Cu}\ (\mathrm{t\ ha^{-1}})}{C{:}\,P_{Zn/Cu}}\right) + \left(\frac{C_{Ctrl1/Ctrl2}\ (\mathrm{t\ ha^{-1}})}{C{:}\,P_{Ctrl1/Ctrl2}}\right) \qquad (\mathbf{E.4.2})$$

where $C_{Zn/Cu}$ and $C_{Ctrl1/Ctrl2}$ are the assumed individual C loadings, and $C{:}P_{Zn/Cu}$ and $C{:}P_{Ctrl1/Ctrl2}$ are the C:P ratios (**Table 4.1**) for contaminated and uncontaminated sludge treatments, respectively. These values have not been reported, therefore the estimated phosphorus loadings determined here assume the total C loadings are derived entirely from the contaminated sludge used for the Zn and Cu sludge treatments (**Table 2.2**). For this reason the values given in **Table 4.1** are likely to be overestimated for soils receiving the Zn sludge treatment and underestimated for soils receiving the Cu sludge treatment. For instance, during experimental Phase I, the total C loading applied to soils receiving the Zn and Cu sludge treatments at the Woburn field site was 81 t ha^{-1} in both cases (**Table 2.3**). Therefore, if it is assumed that, in this case, 11 t ha^{-1} of the total value was derived from the corresponding uncontaminated controls, digested and undigested, then the respective phosphorus loadings would be 6.93 and 1.82 t ha^{-1} using **E. 4.2**.

Plant 'available' phosphorus, extractable by $NaHCO_3$ (MAFF, 1986), was measured at all of the LTSE field sites during experimental Phase II and Phase III to determine soil nutrient status (Defra, 2002, 2007a). As predicted, available phosphorus in untreated soil was lowest at Hartwood (2.38-4.30 µg P g soil^{-1}) for the duration of the experiment, with the highest values seen at Woburn (13.6-9.53 µg P g soil^{-1}). The relative concentrations of 'available' phosphorus determined in 2001, at the end of Phase II, corresponded to the estimated phosphorus loadings given in **Table 4.1**, with concentrations in soils receiving digested and undigested sludge treatments ranging from 19.8-41.7 µg P g soil^{-1} and 17.3-31.8 µg P g soil^{-1}, respectively.

An overall decrease in 'available' phosphorus was seen at both of the English sites from 2001 to 2005, presumably due to the uptake, and harvest, of phosphorus within wheat crops planted during 2001 and 2003. The greatest decreases were seen at Woburn where 'available' phosphorus decreased by approximately 26-30 % in both sludge amended (21.5-26.6 µg P g soil^{-1} reduced to 15.5-19.3 µg P g soil^{-1}) and untreated soils (13.6 µg P g soil^{-1} reduced to 9.53 µg P g soil^{-1}). Similar decreases were also seen in the untreated soil at Gleadthorpe, and in soil receiving the Cu sludge treatment, where 'available' phosphorus in 2005 decreased by approximately 28 % (8.7 µg P g soil^{-1} to 6.3 µg P g soil^{-1}) and 30 % (17.3 µg P g soil^{-1} to 11.9 µg P g soil^{-1}), respectively (Defra 2002, 2007a). Whereas, the lowest decreases at Gleadthorpe were seen in soils receiving the digested (19.80 to 18.80 µg P g soil^{-1}; 5 %) and undigested (17.40 to 15.73 µg P g soil^{-1}; 10 %) controls.

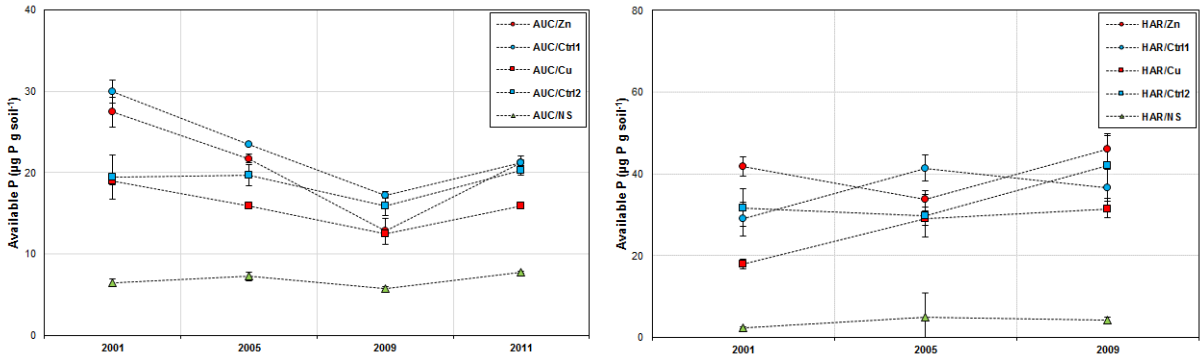


Figure 4.2:- Changes in 'available' phosphorus (µg P g soil^{-1}), extractable by $NaHCO_3$, during experimental Phase II (1999 – 2001), Phase III (2003 – 2005), and Phase IV (2006-2011), at the Long-Term Sludge Experiment field sites situated in Scotland. Data obtained from Defra (2002, 2007), Cooper (Personal Communication), and Crooks (Personal Communication). Error bars represent standard error (n = 3).

Despite being managed as a grassland, a steady decline in 'available' phosphorus was also observed at the Auchincruive field site during experimental Phases II and III (**Figure 4.2**). Over the same period of time, an average decrease of 22 % was seen in soils receiving the digested sludge treatments (Zn = 27.46 to 21.73 µg P g soil^{-1}; Ctrl1 = 30.00 to 23.47 µg P g soil^{-1}) while 'available' phosphorus in soil receiving the Cu sludge treatment decreased by 16 % (18.98 to 15.87 µg P g soil^{-1}). Phosphorus availability continued to be monitored at both of the Scottish sites during experimental Phase IV. However, no general trend in phosphorus availability could be seen at the Hartwood site, 'available' phosphorus either reached a maximum (NS = 5.01 µg P g soil^{-1}; Ctrl1 = 41.46 µg P g soil^{-1}) or minimum (Zn = 33.84 µg P g soil^{-1}; Ctrl2 = 29.80 µg P g soil^{-1}) in 2005, or increased steadily (Cu = 17.9 µg P g soil^{-1} to 31.3 µg P g soil^{-1}) over an eight year period from 2001 to 2009 (**Figure 4.2**). Little change in 'available' phosphorus was seen at Auchincruive in the untreated soil or soil receiving the undigested control during this time, although 'available' phosphorus continued to decline in the remaining soils before increasing, in all cases, in 2011 (**Figure 4.2**).

4.2.1. Inorganic Phosphorus Method

Phosphorus (i.e. orthophosphate) concentrations were determined by colourimetric analysis using the molybdenum blue method described by Murphy and Riley (1962) and Watanabe and Olsen (1965). A 5 mL aliquot of phosphorus extract was mixed with 20 mL of 7.65 mM (0.15 % m/v) ammonium molybdate ($(NH_4)_2MoO_4$) solution and reduced to molybdate blue by adding 5 mL of 85 mM (1.5 % m/v) ascorbic acid solution. Colour was allowed to develop for 2 hours before measuring absorbance at 880 nm using a spectrophotometer (Nicolet Evolution^{TM} 100 UV-Visible Spectrophotometer, Thermo Electron Corporation). Total phosphorus was determined using a 5 mL aliquot of trace element solution obtained following *aqua-regia* acid digest of air-dried soil sample (**See Section 3.4.1**). Plant 'available' phosphorus was extracted using a 0.5 M solution of sodium hydrogen carbonate ($NaHCO_3$) as described

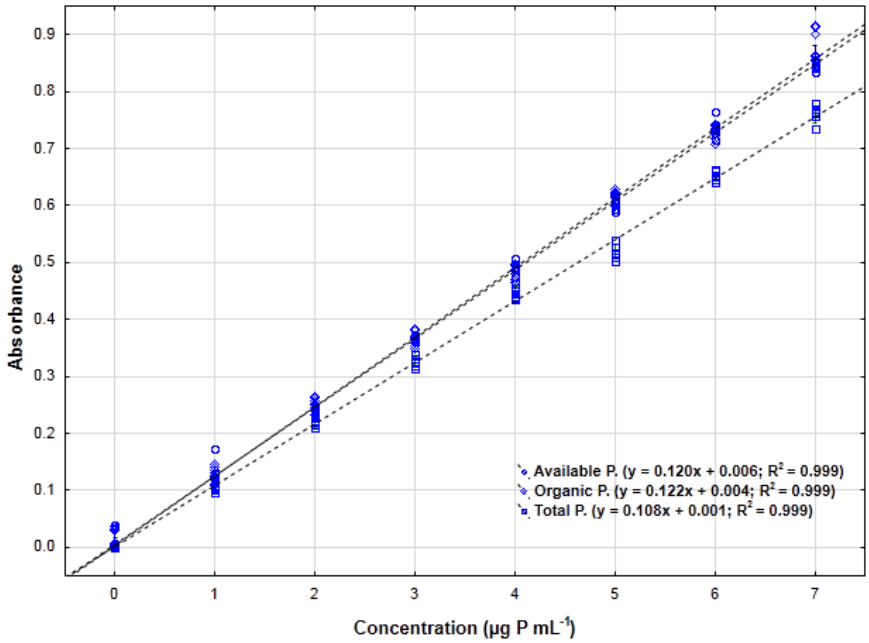


Figure 4.3:- Calibration graphs used to determine phosphorus concentrations (µg P g soil^{-1}) by colorimetric analysis. Error bars represent standard error (n = 9).

by MAFF (1986; after Olsen et al. (1954)). Approximately 5 g of air-dried soil was suspended in 100 mL $NaHCO_3$, and mechanically shaken for ≥ 30 minutes before filtering (Whatman No. 2); 5 mL aliquots were then acidified with 1 mL of 1.5 M sulphuric acid (H_2SO_4) prior to colorimetric analysis.

A 100 µg P mL^{-1} stock solution was prepared by diluting 1 mL of 10 000 µg P mL^{-1} working standard solution (Pure Grade AS Calibration Standard, PerkinElmer) to 100 mL final volume in deionised water. Calibration graphs (**Figure 4.3**) were prepared for colourimetric analysis by diluting 0, 0.5, 1, 1.5, 2, 2.5, 3, and 3.5 mL aliquots of 100 µg P mL^{-1} stock solution to 50 mL final volume in the respective extractants (i.e. deionised water for 'total P' analysis (**See Section 3.4.1**), $NaHCO_3$ for 'available' P analysis, 0.1 M H_2SO_4 for organic P (**See Section 4.3.1**)) to give 0, 1, 2, 3, 4, 5, 6, and 7 µg P mL^{-1} calibration standards; standards were analysed using the same molybdate blue method described above.

The concentration of phosphorus in the original soil sample was then calculated as follows:

$$[\mathrm{P}] = \frac{([P_S] - [P_B]) \times v \times D}{m} \qquad (\mathbf{E.4.3})$$

where $[P_S]$ is the phosphorus concentration in the diluted extract (µg mL^{-1}), $[P_B]$ is the phosphorus concentration in the method blank (µg mL^{-1}), v is the final volume of the extraction solution (mL), m is the original mass of extracted soil sample (g), and D is a dilution factor.

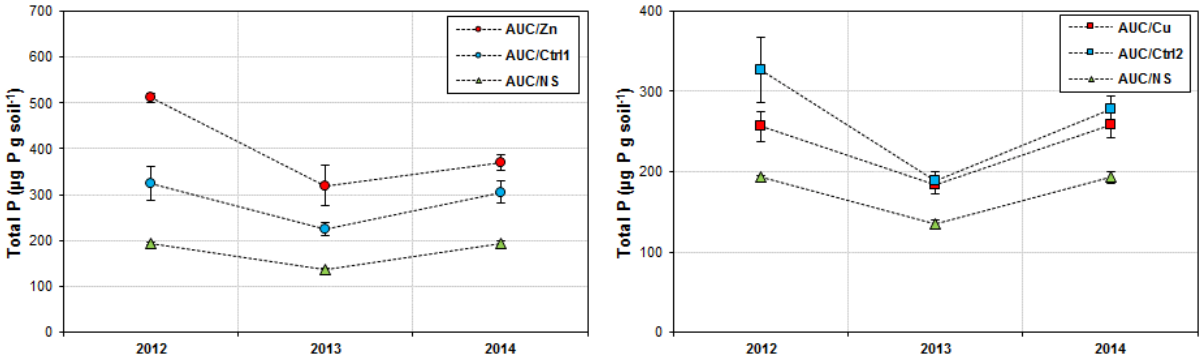


Figure 4.4:- Change in total phosphorus (µg P g soil^{-1}) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Auchincruive (AUC) field site. Error bars represent standard error (n = 3).

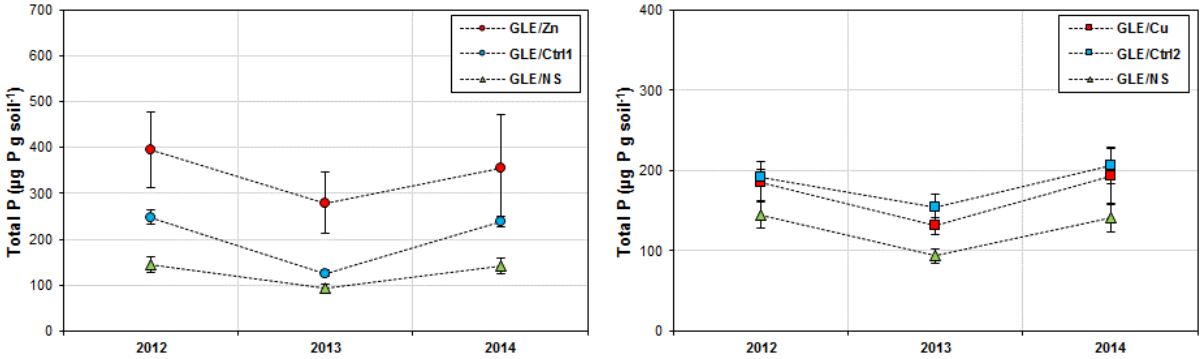


Figure 4.5:- Change in total phosphorus (µg P g soil^{-1}) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Gleadthorpe (GLE) field site. Error bars represent standard error (n = 3).

4.2.2. Total Phosphorus Results (2012-2014)

Auchincruive

Total phosphorus in soil receiving the Zn sludge treatment remained significantly higher than both untreated soil (p <0.01) and soil receiving the digested control (p <0.05) for the duration of the current investigation (**Table 4.2**), despite a significant (p <0.01) decrease in total phosphorus from 2012 to 2013 (**Figure 4.4**). In addition, total phosphorus in soil receiving the digested control was also significantly (p <0.01) greater than in the untreated soil for years 2012 and 2014. No significant difference in total phosphorus was observed between soils receiving either of the undigested sludge types (**Table 4.2**), although (with the exception of soil receiving the Cu sludge treatment in 2012) both had significantly (p <0.05) greater total phosphorus concentrations in comparison to the untreated soil (**Figure 4.4**). Some significant (p <0.05) decreases in total phosphorus were observed in 2013, however in all cases (excepting soil receiving the Zn sludge treatment) total phosphorus in 2012 was not statistically different from total phosphorus in 2014 (**Figure 4.4**).

Gleadthorpe

Total phosphorus in soil receiving the Zn sludge treatment varied considerably at the Gleadthorpe site (**Figure 4.5**), and was found to be significantly (p <0.05) higher than in untreated soil during years 2012

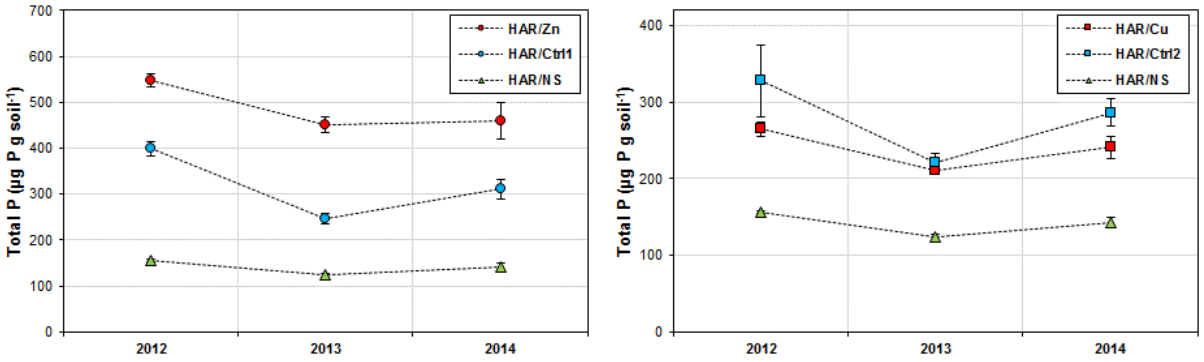


Figure 4.6:- Change in total phosphorus (µg P g soil^{-1}) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Hartwood (HAR) field site. Error bars represent standard error (n = 3).

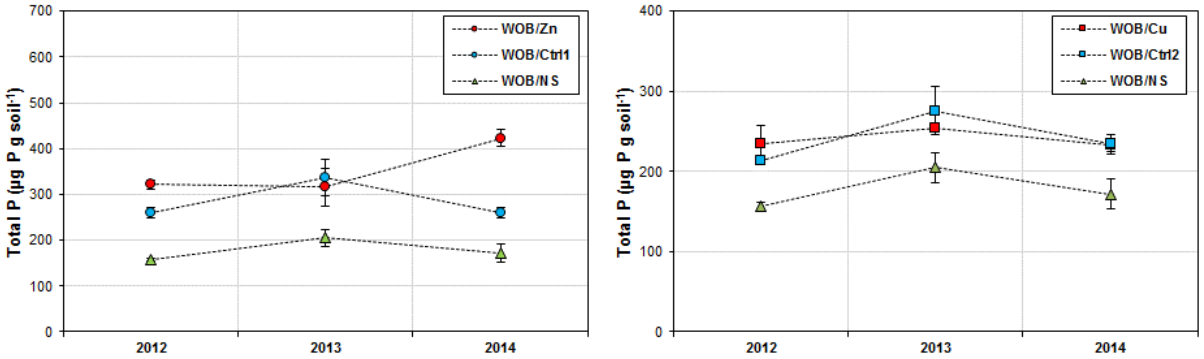


Figure 4.7:- Change in total phosphorus (µg P g soil^{-1}) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Woburn (WOB) field site. Error bars represent standard error (n = 3).

and 2013; but was only significantly (p <0.05) higher than soil receiving the digested control in 2013 (**Table 4.2**). Similarly to the Auchincruive site, no significant difference in total phosphorus was observed between soils receiving the undigested sludge treatments for the duration of the current investigation (**Table 4.2**), nor was any significant difference in total phosphorus observed (with the exception of soil receiving the undigested control in 2013 (p <0.05)) when compared to the untreated soil. The only significant (p <0.001) change in total phosphorus over time was seen in soil receiving the digested control which decreased from 247.97 ± 15.78 µg P g soil^{-1} in 2012 to 126.03 ± 6.62 µg P g soil^{-1} in 2013 (**Figure 4.5**).

Hartwood

Marked differences in total phosphorus were observed at the Hartwood field site, between soils receiving both digested sludge types and untreated soil, which were all significantly (p <0.01) different from each other for the duration of the current investigation (**Table 4.2**); the highest concentrations of total phosphorus were seen in soil receiving the Zn sludge treatment (**Figure 4.6**). No significant difference was observed in the total phosphorus content of soils receiving undigested sludge treatments, however total phosphorus in both soils was significantly higher (Cu (p <0.05); Ctrl2 (p <0.01)) than in

Table 4.2:- Concentrations of 'total' and 'available' phosphorus (μg P g soil^{-1}) fractions measured over the course of three years (2012-2014) at the Long-Term Sludge Experiment field sites.

Sludge Treatment	Total Phosphorus (μg P g soil^{-1})			Available Phosphorus (μg P g soil^{-1})		
	2012	2013	2014	2012	2013	2014
AUC/Zn	511.57 (9.91)$^{a[1][2]}$	320.19 (44.06)^{a}	369.90 (16.63)^{a}	20.53 (2.13)^{a}	23.34 (1.42)^{a}	17.27 (0.69)^{a}
AUC/Ctrl1	323.98 (36.26)^{b}	225.06 (13.04)^{b}	305.42 (23.85)^{b}	15.15 (1.81)^{a}	23.80 (2.43)^{a}	18.33 (1.58)^{a}
AUC/Cu	256.34 (18.22)^{cd}	183.17 (11.05)^{c}	259.26 (17.37)^{d}	10.61 (0.95)^{c}	15.34 (1.53)^{c}	14.07 (0.87)^{c}
AUC/Ctrl2	326.77 (40.90)^{d}	188.98 (10.44)^{c}	278.26 (16.44)^{d}	15.18 (0.32)^{d}	19.09 (2.00)^{c}	15.40 (1.78)^{c}
AUC/NS	193.06 (2.82)^{c}	135.74 (4.83)^{b}	192.88 (6.60)^{c}	6.46 (1.08)^{b}	7.96 (0.18)^{b}	7.50 (0.60)^{b}
GLE/Zn	395.21 (83.31)^{a}	279.49 (66.87)^{a}	356.30 (115.23)^{a}	16.88 (1.67)^{a}	20.05 (1.47)^{a}	16.82 (2.58)^{a}
GLE/Ctrl1	247.97 (15.78)^{ab}	126.03 (6.62)^{b}	238.16 (10.87)^{a}	15.82 (0.92)^{a}	16.62 (0.97)^{a}	16.90 (0.24)^{a}
GLE/Cu	185.89 (24.56)^{c}	130.73 (10.38)^{cd}	192.97 (34.34)^{b}	10.70 (1.61)^{c}	12.22 (0.86)^{c}	11.68 (1.80)^{bc}
GLE/Ctrl2	190.81 (10.37)^{c}	154.38 (15.67)^{d}	206.46 (23.22)^{b}	12.53 (0.35)^{c}	13.81 (0.95)^{c}	14.10 (1.67)^{c}
GLE/NS	144.84 (17.15)^{bc}	93.74 (9.34)^{bc}	140.98 (17.23)^{ab}	5.55 (0.77)^{b}	6.52 (1.00)^{b}	7.26 (1.01)^{b}
HAR/Zn	547.42 (14.06)^{a}	452.12 (16.98)^{a}	458.50 (40.06)^{a}	22.34 (1.22)^{a}	25.15 (2.32)^{a}	25.09 (2.22)^{a}
HAR/Ctrl1	399.01 (15.09)^{b}	246.46 (12.66)^{b}	310.86 (17.76)^{b}	19.78 (1.28)^{a}	20.92 (0.48)^{a}	21.41 (1.12)^{a}
HAR/Cu	264.88 (9.94)^{d}	211.60 (5.92)^{d}	240.90 (14.00)^{d}	11.13 (0.46)^{c}	12.98 (1.14)^{c}	11.92 (1.12)^{c}
HAR/Ctrl2	327.93 (46.84)^{d}	221.66 (12.28)^{d}	286.66 (17.76)^{d}	12.76 (0.54)^{d}	14.89 (0.54)^{c}	15.46 (0.46)^{d}
HAR/NS	156.42 (2.42)^{c}	123.83 (4.26)^{c}	142.31 (6.73)^{c}	3.09 (0.26)^{b}	3.88 (0.83)^{b}	3.50 (0.09)^{b}
WOB/Zn	321.81 (10.13)^{a}	315.54 (41.51)^{ab}	422.28(18.39)^{a}	17.32 (0.19)^{a}	14.03 (0.84)^{a}	21.84 (0.66)^{a}
WOB/Ctrl1	259.55 (11.56)^{b}	336.30 (39.13)^{b}	260.08 (10.87)^{b}	19.41 (0.26)^{b}	16.83 (0.56)^{b}	18.75 (0.73)^{b}
WOB/Cu	234.04 (22.92)^{d}	254.21 (5.68)^{cd}	232.92 (8.42)^{d}	14.64 (0.43)^{d}	12.55 (0.14)^{d}	17.83 (2.04)^{d}
WOB/Ctrl2	214.01 (1.66)^{d}	275.88 (29.75)^{d}	233.92 (11.87)^{d}	16.05 (0.61)^{d}	14.34 (0.26)^{e}	16.07 (0.61)^{d}
WOB/NS	157.43 (3.55)^{c}	204.80 (18.65)^{ac}	172.02 (19.26)^{c}	9.69 (0.99)^{c}	8.09 (0.59)^{c}	10.35 (0.63)^{c}

[1]Values in parenthesis are standard error (n = 3). [2]Values without corresponding letters denotes statistical significance at α = 0.05 for individual sampling events, note comparisons between different sludge types (i.e. digested (Zn and Ctrl1) and undigested (Cu and Ctrl2)) have not been made.

untreated soil for the duration of the current investigation (**Figure 4.6**). Again, total phosphorus appeared to fluctuate significantly (p <0.05) over time in soil receiving the digested control, decreasing to 246.46 ± 12.66 μg P g soil^{-1} in 2013, whereas no significant difference over time (i.e. between 2012 and 2014) was observed in any of the other soils (**Figure 4.6**).

Woburn

At the Woburn field site, total phosphorus in soil receiving the Zn sludge treatments was only significantly higher than soil receiving the digested control (p <0.01) and untreated soil (p <0.001) in years 2012 and 2014 (**Table 4.2**); with a significant (p <0.05) increase from 315.5 ± 41.51 μg P g soil^{-1} in 2013 to 422.3 ± 18.39 μg P g soil^{-1} in 2014 (**Figure 4.7**). No significant changes in total phosphorus were observed over time in soils receiving either of the undigested sludge treatments (**Figure 4.7**), which, with the exception of soil receiving the Cu sludge treatment in 2013, were significantly (p <0.05) higher than in untreated soil for the duration of the current investigation. No significant differences in total phosphorus were found between soils receiving either of the undigested sludge treatments (**Table 4.2**).

4.2.3. Available Phosphorus Results (2012-2014)

Auchincruive

Plant 'available' phosphorus appeared to fluctuate in soils receiving the digested sludge treatments (Zn

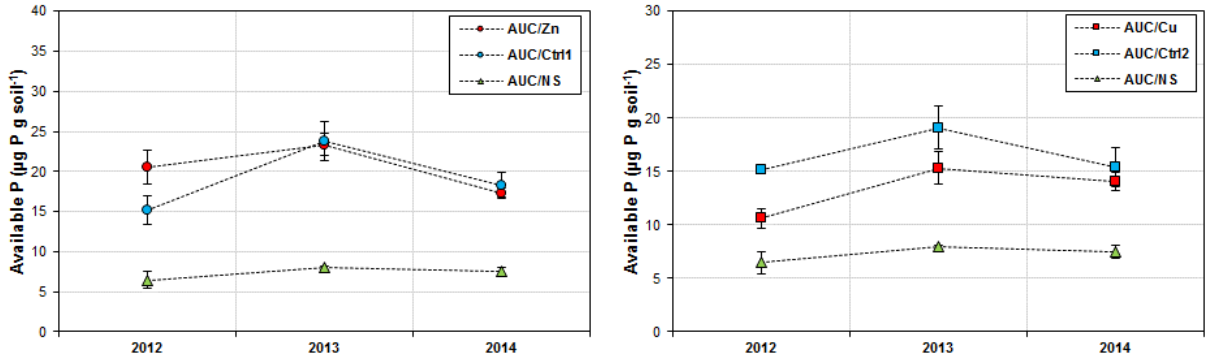


Figure 4.8:- Change in 'available' phosphorus (µg P g soil^{-1}) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Auchincruive (AUC) field site. Error bars represent standard error (n = 3).

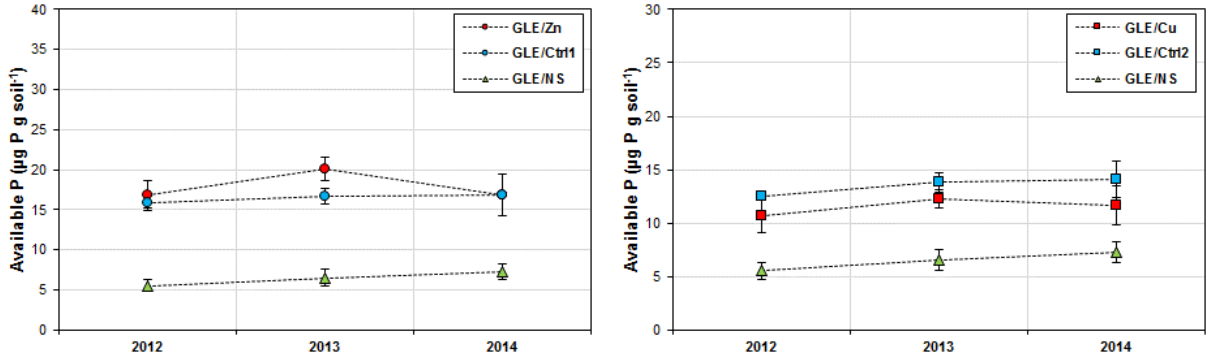


Figure 4.9:- Change in 'available' phosphorus (µg P g soil^{-1}) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Gleadthorpe (GLE) field site. Error bars represent standard error (n = 3).

and Ctrl1) and the Cu sludge treatment, reaching maximum values in 2013 which were significantly (p <0.05) higher than the previous year (**Figure 4.8**). However, in no instance was there any significant difference observed between 'available' phosphorus in 2012 and 2014; nor was 'available' phosphorus in 2014 significantly different to 2013. No significant change in 'available' phosphorus was seen over time in the untreated soil or soil receiving the undigested control (**Figure 4.8**).

In comparison to untreated soil, 'available' phosphorus was significantly higher, for the duration of the current investigation, in all of the sludge amended soils at Auchincruive (**Table 4.2**): Zn (p <0.01), Ctrl1 (p <0.05), Cu (p <0.05), and Ctrl2 (p <0.01). However, with the exception of soils receiving the undigested sludge treatments (Cu and Ctrl2; p <0.01) in 2012, no significant difference in 'available' phosphorus was observed between comparative sludge treatments (**Table 4.2**), i.e. the Zn sludge treatment and digested control, and the Cu sludge treatment and undigested control.

Gleadthorpe

In all cases, values for 'available' phosphorus remained similar for the duration of the current investigation, with no significant changes observed over time (**Figure 4.9**). In addition, all of the sludge amended soils contained significantly higher concentrations of 'available' phosphorus in comparison

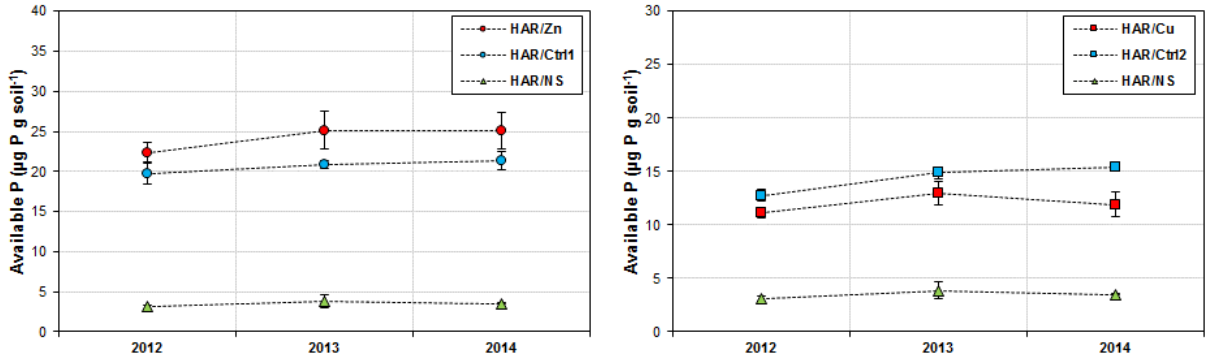


Figure 4.10:- Change in 'available' phosphorus (µg P g soil^{-1}) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Hartwood (HAR) field site. Error bars represent standard error (n = 3).

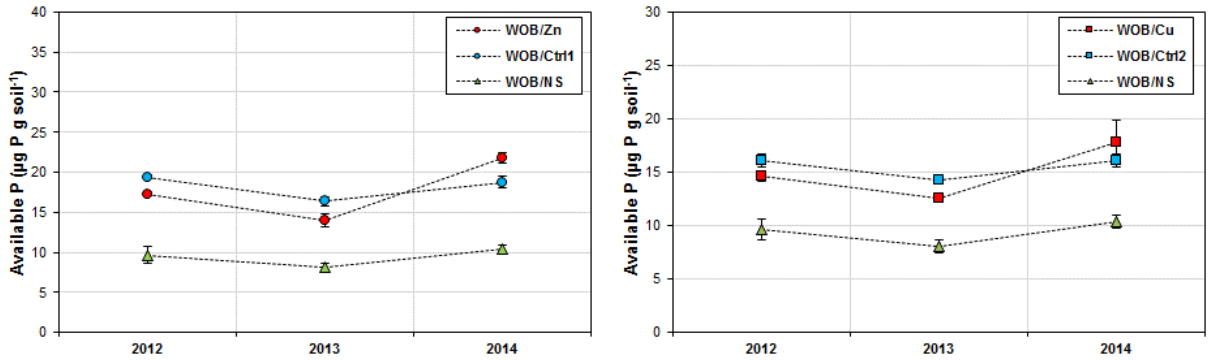


Figure 4.11:- Change in 'available' phosphorus (µg P g soil^{-1}) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Woburn (WOB) field site. Error bars represent standard error (n = 3).

to untreated soil (Zn (p <0.01), Ctrl1 (p <0.01), Cu (p <0.05), and Ctrl2 (p <0.05)), although, 'available' phosphorus in soil receiving the Cu sludge treatment did decrease to 11.68 ± 1.80 µg P g soil^{-1} in 2014, which was not significantly different to the value observed in untreated soil (7.26 ± 1.01 µg P g soil^{-1}). No significant differences were found between comparative sludge treatments (**Table 4.2**).

Hartwood

Similarly, no significant changes in 'available' phosphorus were observed over time at Hartwood, although 'available' phosphorus in soil receiving the undigested control did increase significantly (p <0.05) from 12.76 ± 0.54 µg P g soil^{-1} in 2012 to 14.89 ± 0.54 µg P g soil^{-1} in 2013 (**Figure 4.10**). Again, 'available' phosphorus in sludge amended soils was significantly (p <0.001) higher than in untreated soil for the duration of the current investigation (**Table 4.2**), and in some instances, during 2012 and 2014, was significantly (p <0.05) higher in soil receiving the undigested control in comparison to soil receiving the Cu sludge treatment (**Table 4.2**). No significant difference was observed between digested sludge treatments at any time.

Woburn

In contrast, 'available' phosphorus at Woburn, appeared to increase over time in soil receiving the Zn

sludge treatments (**Figure 4.11**) reaching a maximum of 21.84 ± 0.66 μg P g soil^{-1} in 2014, significantly (p <0.01) higher than the previous two years. A similar increase was also observed in soil receiving the Cu sludge treatment, which increased significantly (p <0.05) from 12.55 ± 0.14 μg P g soil^{-1} in 2013 to 17.83 ± 2.04 μg P g soil^{-1} in 2014; however the value in 2014 was not significantly different to that observed in 2012 (**Figure 4.11**). A significant (p <0.01) decrease in 'available' phosphorus was also observed in soil receiving the digested control in 2013, whereas no significant changes over time were observed in the untreated soil or soil receiving the undigested control.

Again, in all cases, 'available' phosphorus was significantly higher in sludge amended soil than in untreated soil (Zn (p <0.001), Ctrl1 (p <0.001), Cu (p <0.01), and Ctrl2 (p <0.05)). However, in contrast to the other field sites, 'available' phosphorus was also significantly (p <0.05) different in soils receiving the digested sludge treatments, with higher concentrations of 'available' phosphorus seen in soils receiving the digested control in 2012 and 2013, whereas higher concentrations were seen in soil receiving the Zn sludge treatment in 2014 (**Table 4.2**); a significant (p <0.05) difference in 'available' phosphorus was also observed between soils receiving the undigested sludge treatments in 2013.

4.2.4. Total and Available Phosphorus Overview (2012-2014)

The mean values for both total and 'available' phosphorus, over the course of three years (2012-2014), are shown in **Figure 4.12** for the untreated soils at each of the LTSE field sites. Total phosphorus (μg P g soil^{-1}) increased as follows:

GLE (126.52 ± 16.43) < HAR (140.85 ± 9.44) < AUC (173.89 ± 19.08) < WOB (178.08 ± 14.01)

however, the only statistically significant (p <0.05) difference observed was between Woburn and Gleadthorpe. With the exception of Hartwood, for which 'available' phosphorus was significantly (p <0.01) lower in comparison to each of the other sites, 'available' phosphorus (μg P g soil^{-1}) appeared to increase in proportion to total phosphorus in the untreated soils (**Figure 4.12**):

HAR (3.49 ± 0.23) < GLE (6.44 ± 0.50) < AUC (7.31 ± 0.44) < WOB (9.38 ± 0.67)

As mentioned previously, the apparent reduction in phosphorus availability in the untreated soil at Hartwood is likely due to a combination of low pH (**See Section 2.6**) and the presence of Fe/Al oxides within the soil (**Table 2.1**). The value for Woburn was significantly (p <0.05) higher in comparison to the other sites, with no significant difference in 'available' phosphorus observed between Gleadthorpe and Auchincruive.

The relative differences between the total concentrations of phosphorus remaining in the sludge amended soils at each site during the current investigation (**Figure 4.13**) were still in agreement with

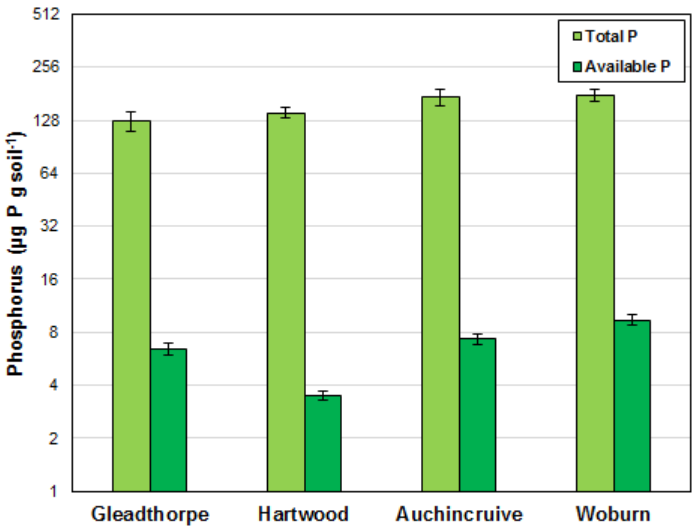


Figure 4.12:- Mean values for total and 'available' phosphorus (µg P g soil^{-1}) in untreated soil (NS) at each of the Long-Term Sludge Experiment field sites, over the course of three years (2012-2014). Error bars represent standard error (n = 3).

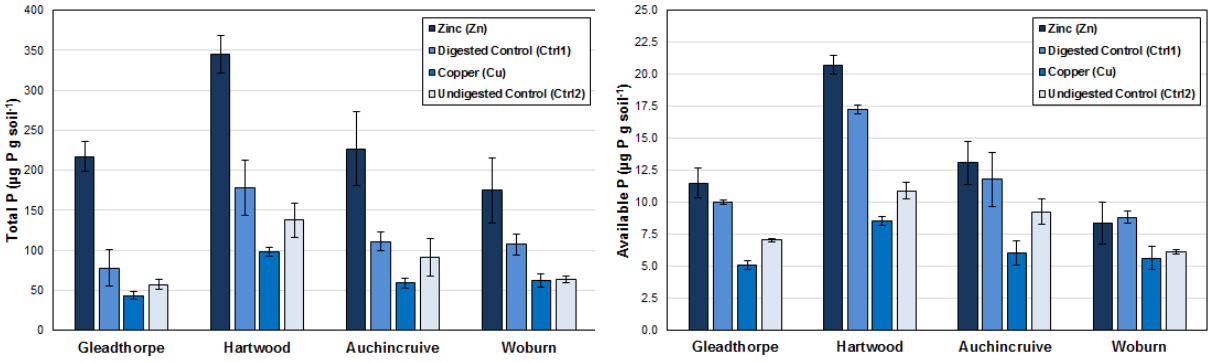


Figure 4.13:- Residual increase in total (left) and 'available' (right) phosphorus (µg P g soil^{-1}) at each of the Long-Term Sludge Experiment field sites in soils receiving digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments. Values are mean phosphorus over the course of three years (2012-2014). Error bars represent standard error (n = 3).

the phosphorus contents of the applied sludge treatments (**Table 4.1**). Hence the greatest increases were observed in soils receiving the Zn sludge treatment, particularly at Hartwood, where the concentration of total phosphorus remaining was significantly (p <0.05) higher in comparison to the other sites (**Figure 4.13**). Similarly, the concentrations of total phosphorus remaining in soils receiving the digested control and undigested sludge treatments (Cu and Ctrl2) were also greatest at the Hartwood site (**Figure 4.13**). With the exception of soil receiving the undigested control at Auchincruive, residual total phosphorus in soils receiving the undigested sludge treatments at Hartwood were also significantly (Cu (p <0.01) and Ctrl2 (p <0.05)) higher than at each of the other sites. Whereas the concentration of total phosphorus remaining in soil receiving the digested control was only significantly (p <0.05) higher in comparison to Gleadthorpe (**Figure 4.13**). In general, the concentrations of total phosphorus remaining in the sludge amended soils at Auchincruive were also higher in comparison to the English sites, however no significant differences in residual total phosphorus were observed. However, for the

current investigation, comparing the residual increases in total phosphorus across each of the field sites did not correspond to the estimated phosphorus loadings applied (**Table 4.1**). For instance, despite having the highest phosphorus loading for soils receiving the Zn sludge treatment (7.36 t P ha^{-1}), the value for total phosphorus remaining in this soil at the Woburn field site was now the lowest of the four sites (**Figure 4.13**).

The concentrations of 'available' phosphorus remaining in each of the sludge amended soils increased in proportion to the concentration of total phosphorus remaining, and were therefore also greatest at the Hartwood site (**Figure 4.13**). In soils receiving the digested sludge treatments (Zn and Ctrl1) the increase was significantly (p <0.01) greater in comparison to each of the other sites, with the concentration of 'available' phosphorus at Auchincruive also significantly (p <0.05) higher in comparison to the Woburn site. The concentration of 'available' phosphorus in soil receiving the Cu sludge treatment was significantly (p <0.05) higher at Hartwood in comparison to the other sites, whereas for soil receiving the undigested control, the increase in 'available' phosphorus was significantly (p <0.05) higher at both of the Scottish sites in comparison to the English sites.

4.2.5. Available Phosphorus as a Function of Total Phosphorus

As mentioned in **Section 1.5**, the availability of phosphorus can affect the release of phosphatase enzymes into the soil environment (Burns, 1982). Hence it is important to understand the environmental factors that may influence the availability of phosphorus at each field site, particularly when discussing possible differences in enzyme activity between sites. Regression of 'available' phosphorus on total phosphorus, using the mean values determined over the course of the current investigation (2012-2014), gave positive correlations for both sludge amended and untreated soils showing that 'available' phosphorus generally increased as a function of total phosphorus across the LTSE field sites (**Table 4.3**). However, although total and 'available' phosphorus were highly correlated in both soils receiving digested sludge treatments (Zn (R = 0.908; p < 0.001) and Ctrl1 (R = 0.791; p <0.01)), the higher concentrations of total phosphorus seen in soils receiving the Zn sludge treatment did not appear to produce corresponding increases in 'available' phosphorus, greater in comparison to soil receiving the digested control (**Figure 4.12**). This is possibly due to the higher Fe and Al content within the Zn sludge treatment (**Table 2.2**), in comparison to the digested control, which could have subsequently mitigated the increase in available phosphorus, despite the greater phosphorus loading (**Table 4.1**). For instance, the percentage of total phosphorus in 'available' form was greater in soils receiving the digested control (8.2-12.9 %) in comparison to soil receiving the Zn sludge treatment (4.7-6.0 %). Hence, with the exception of Woburn, the differences in 'available' phosphorus between the soils receiving the digested sludge treatments, at each site, were not statistically significant as described above (**Table 4.2**). In contrast, total and 'available' phosphorus in soils receiving the undigested sludge

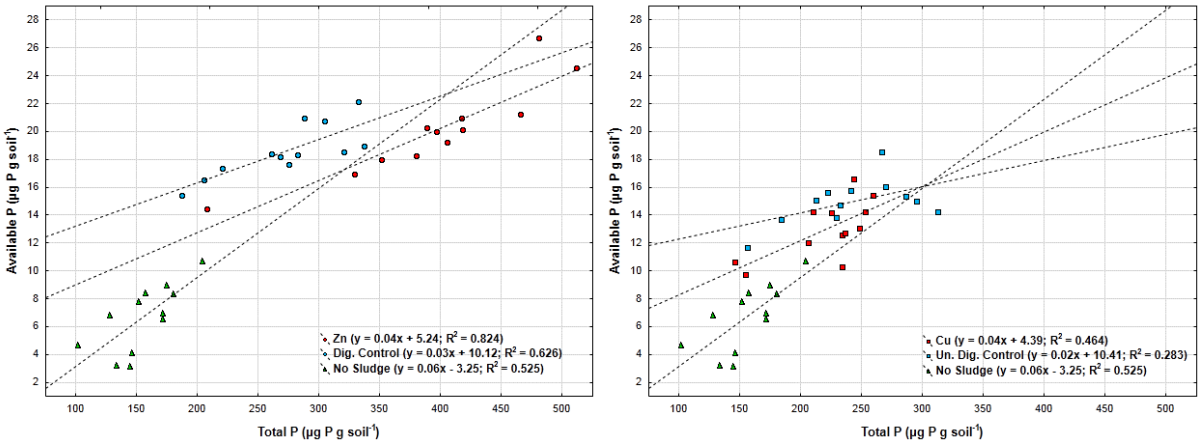


Figure 4.14:- Plot of 'available' phosphorus (µg P g soil^{-1}) as a function of total phosphorus (µg P g soil^{-1}). Data represents mean values of total and 'available' phosphorus measured over the course of three years (2012-2014) in each of the samples taken from the LTSE field sites.

Table 4.3:- Regression and correlation coefficients from regression analysis of 'available' phosphorus on total phosphorus.

	Regression of Available P on Total P					
Sludge Treatment	**Slope (Total P)**	***p***	**Intercept**	***p***	**R**	**R²[2]**
Zinc (Zn)	0.037 (0.005)[1]	<0.001	5.24 (2.21)	<0.05	0.908	0.806
Digested Control (Ctrl1)	0.031 (0.006)	<0.01	10.12 (2.10)	<0.001	0.791	0.589
Copper (Cu)	0.039 (2.941)	<0.05	4.39 (2.95)	0.168	0.681	0.410
Undigested Control (Ctrl2)	0.019 (0.009)	0.075	10.41 (2.33)	<0.01	0.532	0.212
No Sludge (NS)	0.064 (0.019)	<0.01	-3.25 (3.02)	0.307	0.724	0.477

[1] Values in parenthesis are standard error (n = 12). [2]Values are adjusted R^2.

treatments showed the weakest correlations (Cu (R = 0.681; p <0.05) and Ctrl2 (R = 0.532; p = 0.074), particularly in soils receiving the undigested control for which the regression coefficient was not significant (**Table 4.3**).

Therefore if it is assumed that 'available' phosphorus is proportional to total phosphorus, this could be an explanation for the greater decreases in total phosphorus observed at the English field sites. As mentioned in **Section 4.2**, a steady decline in 'available' phosphorus was observed at both of the English field sites during experimental Phases II and III. Hence, this could be an indication that total phosphorus has also been depleted as orthophosphate is released into the soil environment to replace that which is removed during harvest (**Figure 1.2**). This would be in agreement with the findings of the current investigation, as soil pH at the English sites continued to be held at pH 6.5 (**See Section 2.6**), the optimum value for orthophosphate solubility (**See Section 4.2**), therefore the applied phosphorus loading would continue to remain bioavailable to the wheat crops, and therefore continue to be removed over the course of the current investigation; wheat crops were encountered at the Woburn and Gleadthorpe sites during the 2012 and 2014 sampling events (**Table 2.4**). However, in the case of the Scottish grassland sites, particularly at Hartwood, both low soil pH and the presence of Fe/Al-oxides appear to have immobilised phosphorus within the soil, hence the total phosphorus remaining in sludge amended soils is greatest at this site. Furthermore, since the phosphorus cycle is closed at these sites, i.e. phosphorus is returned to the soil in organic forms within plant matter, the only mechanisms

Table 4.4:- Concentrations of organic phosphorus (µg P g soil^{-1}) measured over the course of three years (2012-2014) at the Long-Term Sludge Experiment field sites.

	Organic Phosphorus (µg P g soil^{-1})		
Sludge Treatment	**2012**	**2013**	**2014**
AUC/Zn	160.63 (10.06)$^{a[1][2]}$	155.54 (24.97)^{a}	161.11 (26.98)^{a}
AUC/Ctrl1	118.76 (12.67)^{b}	149.18 (9.54)^{a}	172.21 (16.59)^{a}
AUC/Cu	112.56 (5.96)^{c}	116.55 (8.47)^{b}	137.72 (31.24)^{b}
AUC/Ctrl2	125.83 (17.18)^{c}	151.17 (9.40)^{c}	184.13 (5.42)^{b}
AUC/NS	97.65 (1.95)^{bc}	107.88 (8.00)^{ab}	134.82 (4.17)^{ab}
GLE/Zn	109.30 (19.91)^{a}	108.66 (24.74)^{a}	128.82 (10.60)^{a}
GLE/Ctrl1	89.23 (7.62)^{ab}	91.35 (12.01)^{a}	83.11 (5.31)^{b}
GLE/Cu	86.59 (8.38)^{c}	71.37 (1.28)^{b}	86.60 (5.50)^{c}
GLE/Ctrl2	82.76 (1.12)^{cd}	86.75 (11.76)^{b}	89.56 (5.21)^{c}
GLE/NS	61.99 (6.11)^{bd}	63.66 (6.38)^{ab}	66.28 (5.51)^{bd}
HAR/Zn	143.88 (8.99)^{a}	117.25 (6.90)^{a}	184.73 (20.52)^{a}
HAR/Ctrl1	127.48 (10.96)^{a}	114.23 (11.52)^{ab}	150.11 (12.30)^{a}
HAR/Cu	110.62 (6.82)^{cd}	119.47 (9.23)^{c}	140.81 (18.75)^{c}
HAR/Ctrl2	121.59 (11.76)^{d}	112.07 (14.87)^{c}	161.98 (30.36)^{c}
HAR/NS	68.86 (16.28)^{bc}	66.56 (19.98)^{bc}	95.26 (2.77)^{bc}
WOB/Zn	59.02 (13.24)^{a}	77.92 (11.47)^{ab}	90.01 (11.78)^{a}
WOB/Ctrl1	54.87 (6.80)^{a}	99.34 (1.52)^{a}	73.65 (4.55)^{ab}
WOB/Cu	58.89 (1.63)^{b}	75.67 (1.72)^{c}	68.77 (1.53)^{c}
WOB/Ctrl2	62.96 (8.48)^{b}	81.70 (1.01)^{c}	57.99 (4.67)^{cd}
WOB/NS	47.61 (1.45)^{ab}	56.25 (2.67)^{bd}	51.64 (3.25)^{bd}

[1]Values in parenthesis are standard error (n = 3). [2]Values without corresponding letters denotes statistical significance at $\alpha = 0.05$ for individual sampling events, note comparisons between different sludge types (i.e. digested (Zn and Ctrl1) and undigested (Cu and Ctrl2)) have not been made.

for phosphorus removal would be soil erosion and leaching of phosphorus (Stevenson & Cole, 1999); hence the rate of phosphorus removal at these sites would be considerably slower.

4.3. Organic Phosphorus

As described in **Section 1.5**, organic phosphorus constitutes a significant fraction of the total phosphorus content in soil (Dalal, 1977), and represents an important reservoir of potentially 'available' phosphorus as orthophosphate is continually cycled through the soil biomass (plant and microbial) via microbial activity and the process of mineralisation (Magid et al., 1996; Stevenson & Cole, 1999). However, the long-term fate of organic phosphorus compounds in sludge amended soils is not fully understood, and the potential for sludge derived organic phosphorus as a sustainable source of phosphate fertiliser is yet to be determined. To date, the organic phosphorus content of the sludge amended and untreated soils at the LTSE field sites have not been investigated.

4.3.1. Organic Phosphorus Method

Organic phosphorus was determined as the difference in acid extractable orthophosphate between ignited and unignited soil samples, using the ignition method described by Saunders and Williams (1955) and Legg and Black (1955). Approximately 1 g of air-dried soil was weighed out in duplicate. One of the duplicates was then transferred to a muffle furnace (Carbolite AAF 1100) and heated at

450°C ± 10°C for 4 hours ± 15 minutes to convert organic phosphorus to inorganic orthophosphate. Both ignited and unignited samples were extracted with 100 mL of 0.1 M H_2SO_4 solution by mechanically shaking for ≥ 16 hours and filtering (Whatman No. 2). A 2.5 mL aliquot of each sample was then made up to 5 mL final volume (×2 dilution) with deionised water prior to colourimetric analysis (**See Section 4.2.1**); however in some cases ×5 dilution was required (1 mL in 4 mL deionised water). Results are summarised in **Table 4.4**. It should be noted, however, that the ignition temperature used was higher than the 240°C recommended by Legg and Black (1955), therefore it is possible that organic phosphorus has been overestimated due to increases in the solubility of inorganic orthophosphate at the higher temperature. Though, Legg and Black (1955) have found the overestimation to be within 10 % of the alternative HCl/NaOH extraction method described by Mehta et al. (1954).

4.3.2. Organic Phosphorus Results (2012-2014)

Auchincruive

No significant change in organic phosphorus was observed over time in soils receiving the Zn and Cu sludge treatments at Auchincruive. In both soils receiving the uncontaminated controls, organic phosphorus appeared to increase over time and was significantly (p <0.05) higher in 2014 than at the start of the current investigation (**Figure 4.15**). Organic phosphorus also increased in the untreated soil and was significantly (p <0.05) higher in 2014 than in the previous two years (**Figure 4.15**).

However only in 2012 and 2013 were any statistically significant differences observed between untreated and sludge amended soils. In comparison to the untreated soil (p <0.01), and soil receiving the digested control (p <0.05), organic phosphorus was significantly higher in soil receiving the Zn sludge treatment in 2012 (**Table 4.4**). Whereas in 2013, organic phosphorus was significantly (p <0.05) higher in soil receiving the undigested control than in the untreated soil and soil receiving the Cu sludge treatment (**Table 4.4**).

Gleadthorpe

Despite an apparent increase in soil receiving the Zn sludge treatment to 128.82 ± 10.60 µg P g $soil^{-1}$ in 2014 (**Figure 4.16**), no statistically significant changes in organic phosphorus were observed over time in any of the sludge amended soils or the untreated soil at the Gleadthorpe field site.

In comparison to untreated soil, organic phosphorus was significantly (p <0.05) higher in 2012, in soils receiving the Zn and Cu sludge treatments (**Table 4.4**). Organic phosphorus in soil receiving the Zn sludge treatment was also significantly higher in comparison to both the untreated soil (p <0.01) and soil receiving the digested control (p < 0.01) in 2014, whereas in the same year organic phosphorus was

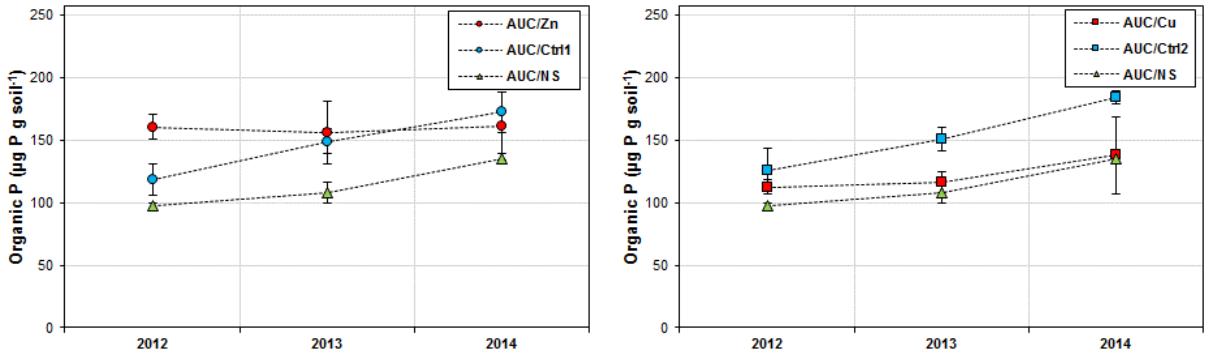


Figure 4.15:- Change in organic ohosphorus (µg P g soil^{-1}) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Auchincruive (AUC) field site. Error bars represent standard error (n = 3).

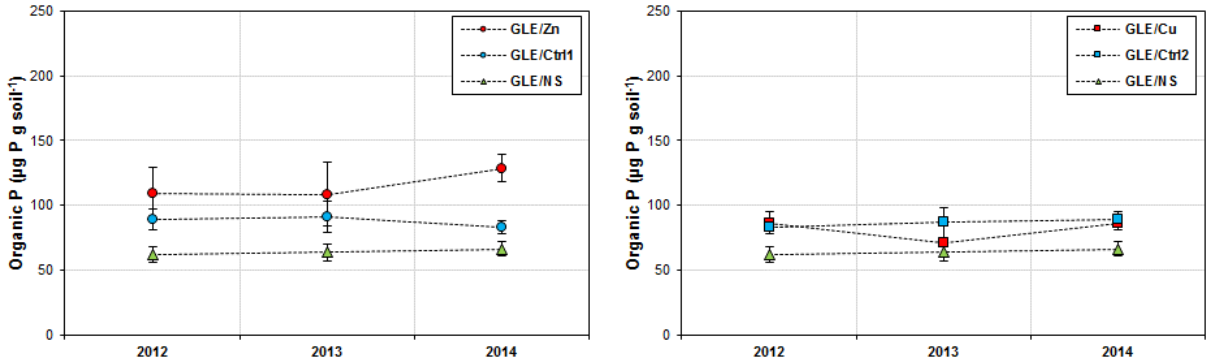


Figure 4.16:- Change in organic phosphorus (µg P g soil^{-1}) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Gleadthorpe (GLE) field site. Error bars represent standard error (n = 3).

significantly ($p < 0.05$) lower in the untreated soil in comparison to soils receiving the undigested sludge treatments (**Table 4.4**).

Hartwood

At Hartwood, organic phosphorus in soil receiving the Zn sludge treatment decreased to 117.25 ± 6.90 µg P g soil^{-1} in 2013 before increasing significantly (p <0.05) to a maximum of 184.73 ± 20.52 µg P g soil^{-1} in 2014; however this was not significantly different to the value observed in 2012 (**Figure 4.17**). No other significant changes in organic phosphorus were observed over time for any of the other soils at the Hartwood field site.

Organic phosphorus in soils receiving the digested sludge treatments (Zn and Ctrl1) was significantly (p <0.05) higher in comparison to the untreated soil for the duration of the current investigation, with the exception of soil receiving the digested control in 2013 (**Table 4.4**). In contrast, the only significant (p <0.05) difference observed for soils receiving the undigested sludge treatments (Cu and Ctrl2) was in 2012, where organic phosphorus in soil receiving the undigested control was significantly higher than the untreated soil (**Table 4.4**).

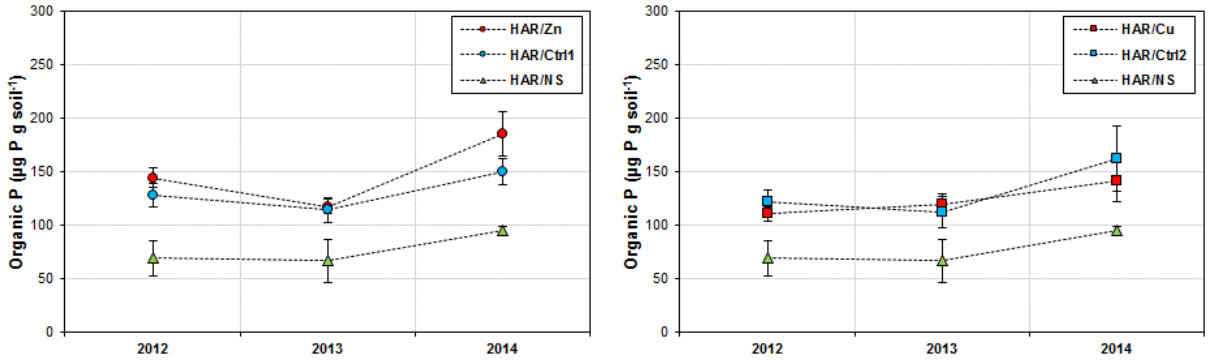


Figure 4.17:- Change in organic phosphorus (µg P g soil^{-1}) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Hartwood (HAR) field site. Error bars represent standard error (n = 3).

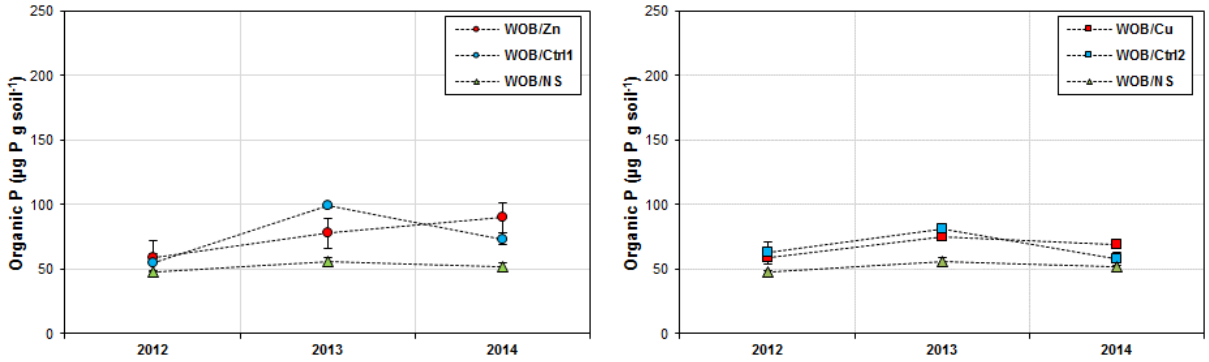


Figure 4.18:- Change in organic phosphorus (µg P g soil^{-1}) over the course of 3 years (2012-2014) in soils receiving digested (left) and undigested (right) sludge treatments at the Woburn (WOB) field site. Error bars represent standard error (n = 3).

Woburn

In soils receiving the Cu sludge treatment and the digested control, organic phosphorus increased significantly (p <0.001) at Woburn to maximum values of 75.67 ± 1.72, and 99.34 ± 1.52 µg P g soil^{-1}, respectively, in 2013 (**Figure 4.18**). In both cases organic phosphorus decreased significantly (p <0.05) the following year, but remained significantly higher than the initial values observed in 2012 (Cu (p <0.01), Ctrl1 (p <0.05)). In addition, organic phosphorus was significantly (p <0.05) lower in soil receiving the undigested control in 2014, in comparison to the previous year, but was not significantly different to that observed at the beginning of the current investigation. No significant change over time was observed in the untreated soil or the soil receiving the Zn sludge treatment (**Figure 4.18**).

No significant differences in organic phosphorus were observed between soils receiving the digested sludge treatments (Zn and Ctrl1), or those receiving the undigested sludge treatments (Cu and Ctrl2) for the duration of the current investigation (**Table 4.4**). However, in 2013 organic phosphorus in soil receiving the digested control was significantly (p <0.01) higher than the untreated soil, whereas the following year organic phosphorus in soil receiving the Zn sludge treatment was significantly (p <0.01) higher (**Table 4.4**). Organic phosphorus in both of the soils receiving the undigested sludge treatments

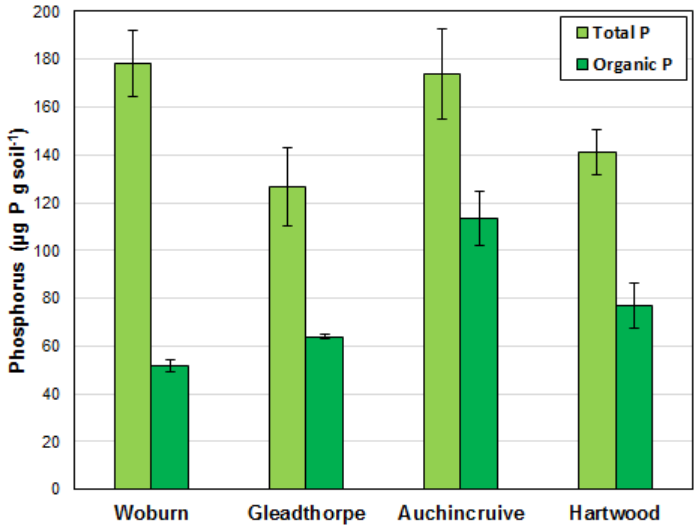


Figure 4.19:- Mean values for organic phosphorus (µg P g soil^{-1}) in untreated soil (NS) at each of the Long-Term Sludge Experiment field sites, over the course of three years (2012-2014). Error bars represent standard error (n = 3).

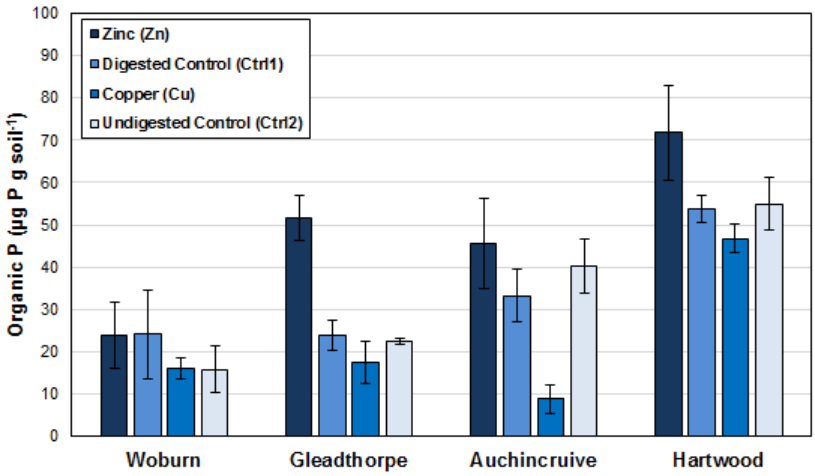


Figure 4.20:- Residual increase in organic phosphorus (µg P g soil^{-1}) at each of the Long-Term Sludge Experiment field sites in soils receiving digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments. Values are mean organic phosphorus over the course of three years (2012-2014). Error bars represent standard error (n = 3).

were significantly (p <0.01) higher than the untreated soil in 2013, however only soil receiving the Cu sludge treatment remained significantly (p <0.05) higher the following year.

4.3.3. Organic Phosphorus Overview (2012-2014)

Figure 4.19 shows the mean values for organic phosphorus (µg P g soil^{-1}) over the course of three years (2012-2014), arranged in order of increasing soil organic C (**See Section 2.6**). In general, the mean background organic phosphorus increased in proportion to the amount of organic C measured at each site, however the Auchincruive field site was anomalous to this trend. The untreated soil at Auchincruive has both the highest organic phosphorus content (113.45 ± 11.08 µg P g soil^{-1}),

significantly (p <0.01) higher than the other three sites, and the highest percentage of total phosphorus (65.24 %) present in organic form (**Figure 4.19**). This could possibly be due to a higher total phosphorus content of the soil parent material at the Auchincruive site, subsequently enriching the soil organic matter with organic phosphorus compounds during soil formation (Walker & Adams, 1958), or due to historical land management practices (Harrison, 1987), however there is no data available to draw a firm conclusion. The mean value for organic phosphorus at Hartwood was also significantly (p <0.01) higher in comparison to the Woburn site.

The residual increases in organic phosphorus, observed in the sludge amended soils, were greatest at the Hartwood field site (**Figure 4.20**), in agreement with those described previously for total phosphorus (**See Section 4.2.4**). In addition, the percentage of residual total phosphorus present in organic forms was also greatest in each of the sludge amended soils at the Hartwood site (Zn = 41 %; Ctrl1 = 50 %; Cu = 75 %; Ctrl2 = 87 %) indicating a possible accumulation of organic P in comparison to the other sites (**Figure 4.20**). Residual organic phosphorus was significantly (p <0.01) higher at Hartwood in soil receiving the Zn sludge treatment in comparison Woburn, and significantly (p <0.001) higher in soil receiving the Cu sludge treatment in comparison to each of the remaining sites. Since the total concentrations of both Zn and Cu were found to be significantly higher at Hartwood in comparison to the other sites, this could be an indication that the presence of Zn and Cu have reduced the rate of organic phosphorus mineralisation at Hartwood in comparison to the other sites. Furthermore, with regards to **Research Question** 2, it appears that Cu may have had a greater effect in comparison to Zn. However, residual organic phosphorus at Hartwood was also significantly (p <0.05) higher in soil receiving the digested control in comparison to both of the English sites, whereas residual organic phosphorus in soil receiving the undigested control was significantly (p <0.05) higher at both of the Scottish sites in comparison to the English sites. Furthermore, no significant differences in organic phosphorus were observed at Hartwood between soils receiving the contaminated and uncontaminated sludge treatments over the course of the current investigation (**Table 4.4**); hence Zn and Cu appear to have had no effect on organic phosphorus mineralisation at the site itself. Therefore the differences in residual organic phosphorus are more likely due to the differences in climatic conditions and land management practices, and the rates of organic C mineralisation (**See Section 2.7**), at each site.

4.3.4. Organic Phosphorus as a Function of Organic Carbon

Harrison (1987) provides a number of regression models that describe the relationships between organic phosphorus and a range of soil properties, principally: total phosphorus, organic C, soil pH, soil texture, and sample depth. The relationships with total phosphorus and organic C are both described as significant positive correlations, respectively accounting for approximately 58.5 % and 44 % of the observed variation in soil organic phosphorus globally. However, when organic phosphorus is

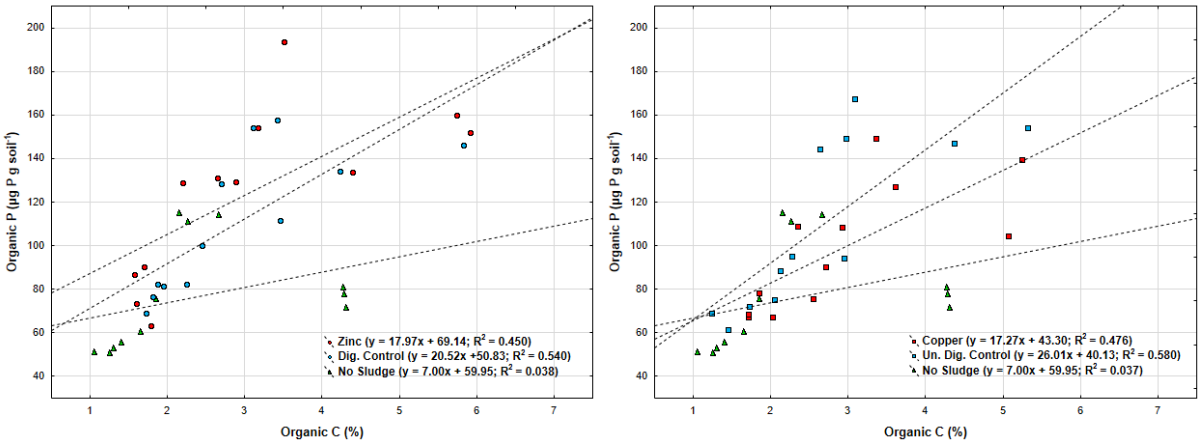


Figure 4.21:- Plot of organic phosphorus (µg P g soil^{-1}) as a function of organic carbon (%). Data represents mean values of orgnaic phosphorus and organic carbon measured over the course of three years (2012-2014) in each of the samples taken from the LTSE field sites.

Table 4.5:- Regression and correlation coefficients from regression analysis of organic phosphorus on organic carbon and total phosphorus.

	Regression of Organic P on Organic C					
Sludge Treatment	**Slope (Org. C)**	***p***	**R**	**Intercept**	***p***	**$R^{2[2]}$**
Zinc (Zn)	17.97 (5.68)[1]	<0.05	0.707	69.14 (19.47)	<0.01	**0.450**
Dig. Control (Ctrl1)	20.52 (5.50)	<0.01	0.763	50.83(17.18)	<0.05	**0.540**
Copper (Cu)	17.27 (5.21)	<0.01	0.723	43.30 (16.39)	<0.05	**0.476**
Un. Dig Control (Ctrl2)	26.01 (6.47)	<0.01	0.786	40.13 (18.83)	0.059	**0.580**
No Sludge (NS)	7.00 (5.85)	0.259	0.354	59.95 (15.51)	<0.01	**0.038**
	Regression of Organic P on Total P					
	Slope (Total P)	***p***	**R**	**Intercept**	***p***	**R^2**
Zinc (Zn)	0.29 (0.13)	<0.05	0.585	10.10 (51.17)	0.847	**0.276**
Dig. Control (Ctrl1)	0.32 (0.19)	0.116	0.478	22.81 (51.57)	0.668	**0.151**
Copper (Cu)	0.26 (0.24)	0.302	0.325	42.33 (52.55)	0.439	**0.016**
Un. Dig Control (Ctrl2)	0.57 (0.20)	<0.05	0.674	-28.85 (48.87)	0.568	**0.400**
No Sludge (NS)	0.25 (0.27)	0.392	0.272	38.58 (43.05)	0.391	**-0.02**

	Regression of Organic P on Organic C and Total P								
	Slope (Org. C)	***p***	**R**	**Slope (Total P)**	***p***	**R**	**Intercept**	***p***	**R^2**
Zinc (Zn)	16.92 (9.99)	0.124	0.666	0.03 (0.19)	0.898	0.052	62.27 (56.18)	0.296	**0.390**
Dig. Control (Ctrl1)	19.92 (6.91)	<0.05	0.717	0.06 (0.17)	0.754	0.083	39.21 (40.23)	0.355	**0.494**
Copper (Cu)	16.49 (5.76)	<0.05	0.691	0.08 (0.19)	0.690	0.099	33.33 (40.19)	0.428	**0.428**
Un. Dig Control (Ctrl2)	19.87 (8.25)	<0.05	0.600	0.25 (0.21)	0.273	0.290	-3.27 (41.56)	0.939	**0.594**
No Sludge (NS)	8.66 (5.84)	0.172	0.438	0.33 (0.27)	0.241	0.371	4.29 (46.78)	0.929	**0.091**

[1] Values in parenthesis are standard error (n = 12). [2]Values are adjusted R^2.

expressed as a percentage of the total phosphorus content, only 1.7 % of the observed variation is accounted for by total phosphorus, whereas 27.4 % is still accounted for by organic C. Hence it was concluded that soils with a greater SOC content are, in general, more likely to contain greater quantities of organic phosphorus, in comparison to those with a lower organic C content, but with similar concentrations of total phosphorus (Harrison, 1987).

With the exception of untreated soil, regression of organic phosphorus on SOC gave significant positive correlations (**Figure 4.21**), accounting for approximately 45-60 % of the variation in organic phosphorus observed in the sludge amended soils at each of the LTSE field sites (**Table 4.5**). However

Table 4.6:- Regression and correlation coefficients showing multicollinearity between the independent variables organic carbon and total phosphorus.

	Regression of Organic C on Total P					
Sludge Treatment	**Slope (Total P)**	***p***	**Intercept**	***p***	**R**	**$R^{2[2]}$**
Zinc (Zn)	0.02 (0.003)	<0.01	-3.08 (1.49)	0.065	0.801	**0.606**
Digested Control (Ctrl1)	0.01 (0.01)	0.063	-0.85 (1.82)	0.065	0.551	**0.234**
Copper (Cu)	0.01 (0.01)	0.299	0.55 (2.20)	0.809	0.327	**0.018**
Undigested Control (Ctrl2)	0.02 (0.01)	<0.05	-1.29 (1.54)	0.423	0.638	**0.348**
No Sludge (NS)	-0.01 (0.01)	0.481	3.96 (2.20)	0.102	0.226	**-0.044**

[1] Values in parenthesis are standard error (n = 12). [2]Values are adjusted R^2.

examination of residual plots showed the values of organic phosphorus determined at the Auchincruive site to be outliers in most cases, particularly in untreated soil (**See Section 4.3.3**), and soils receiving the uncontaminated controls. Removing data for the Auchincruive site dramatically increased the correlation between organic phosphorus and SOC in all cases. A significant (R = 0.851; p <0.01) correlation was now observed for the untreated soil, with stronger correlations seen in soils receiving the contaminated (Zn (R = 0.855; p <0.01) and Cu (R = 0.871; p <0.01)) and uncontaminated (Ctrl1 (R = 0.969; p <0.001) and Ctrl2 (R = 0.976; p <0.001)) sludge treatments. In this case, SOC now accounted for 68 % of the variation in organic phosphorus observed in the untreated soils and approximately 69-72 % and 93-95 % of the variation in soils receiving the contaminated and uncontaminated sludge treatments, respectively. Regression of organic phosphorus on total phosphorus only gave significant correlations in soils receiving the Zn sludge treatment (R = 0.585; p <0.05) and undigested control (R = 0.674; p <0.05), respectively, accounting for approximately 30 and 40 % of the observed variation of organic phosphorus in each soil.

Including total phosphorus in the original regression model more than doubled the proportion of variance accounted for in the untreated soils (R^2 = 0.038 to R^2 = 0.091), but only caused a slight increase for the soils receiving the undigested control (R^2 = 0.580 to R^2 = 0.594). For the remaining soils the proportion of variance explained by the regression model decreased (**Table 4.5**), however this may be due to multicollinearity between the variables SOC and total phosphorus, particularly in soil receiving the Zn sludge treatment, as values for SOC (**See Section 2.7**) and total phosphorus (**See Section 4.2.4**) in these soils were generally greater at the Scottish sites in comparison to the English sites. However, it is interesting to note that the proportion of variance explained in untreated soil, although small, doubled when total phosphorus was added to the regression model as no multicollinearity was observed between SOC and total phosphorus in this case. In no instance was the correlation between organic phosphorus and total phosphorus statistically significant for the multi-variable model (**Table 4.5**), however, in each case an F-test was carried out which showed that the values for R^2 determined for each of the sludge amended soils were statistically significant (Zn (p <0.05); Cu (p <0.05); Ctrl1 (p <0.05); Ctrl2 (p <0.01)), hence organic C and total phosphorus do appear to have a combined effect on organic phosphorus.

Removing the data for Auchincruive markedly improved the correlation between organic phosphorus and organic C, with 63 %, 66-67 %, and 91-93 % of the variation in organic phosphorus observed in the untreated soil, and soils receiving the contaminated (Zn and Cu) and uncontaminated (Ctrl1 and Ctrl2) sludge treatments, respectively, now explained by the regression model. In each case the correlations to total phosphorus remained non-significant, and were now negative for untreated soil (R = -0.071) and soil receiving the digested control (R = -0.023), however values for R^2 were now statistically significant for both sludge amended and untreated soils (Zn (p <0.05); Cu (p <0.05); Ctrl1 (p <0.001); Ctrl2 (p <0.001); NS (p <0.05)). Therefore, in agreement with the regression models given by Harrison (1987), the amount of organic C appears to be the principal factor in determining the amount of organic phosphorus present in the sludge amended soils at each of the LTSE field sites, with a minor contribution from total phosphorus. This also appears to be the case for the untreated soils at both of the English field sites and at Hartwood. Whereas, the high values of organic phosphorus observed in the untreated soil at Auchincruive are potentially due to both high SOC content and a high total phosphorus concentration present within the soil parent material (Walker & Adams, 1985). However, the processes responsible for enriching the soil organic matter with organic phosphorus compounds, in comparison to the other LTSE field sites, cannot be determined.

4.4. ^{31}P-NMR Spectroscopy Analysis

In collaboration with the University of Sheffield, an investigation into the long-term fate of organic phosphorus compounds in sludge amended soils was carried out using ^{31}P-Nuclear Magnetic Resonance (NMR) Spectroscopy. Samples of untreated and sludge amended soils, collected from the Woburn and Hartwood field sites in 2014, were sent for analysis along with samples of both digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments originally applied during experimental Phase I (archived samples of sludge treatments applied in 1996 were obtained from Rothamsted Research).

The Woburn and Hartwood field sites were chosen to provide samples with contrasting levels of SOC (**See Section 2.7**), C_{mic} (**See Section 5.2**), and metal contamination (**See Section 3.4**), as well as contrasting climatic conditions and land management practices (**See Section 2.3**). Therefore, with regards to **Research Question 3**, these two sites provide two contrasting scenarios for the discussion of the long-term fate of organic phosphorus compounds in sludge amended soils. However, it should be noted that only one replicate was analysed for each of the applied sludge treatments, plus the sludge amended and untreated soils at each site. Furthermore, ^{31}P-NMR signals were not quantified against an internal standard, hence, although signal peak areas do correspond to the amount of organic phosphorus determined in each sample, the results presented here can only be discussed qualitatively.

4.4.1. ^{31}P-NMR Method

Approximately 1 g of air dried sample was extracted with 20 mL of a 0.25 M NaOH and 0.05 M EDTA mixture (Bowman & Moir, 1993; Cade-Menun & Preston, 1996) by mechanically shaking for ≥ 16 hours. Extracts were then centrifuged (4800 RCF) for 30 minutes. The supernatant was immediately decanted into a clean sample vial and frozen (-80°C) prior to freeze-drying. Freeze dried sample extracts were then subsequently ground with a mortar and pestle to create a powder.

Approximately 100 mg (50 mg was used for sludge samples) of the ground freeze dried material was dissolved in 0.9 mL of 1 M NaOH and 0.1 M EDTA and 0.1 mL of D_2O (1 mL total volume), mixed briefly with a vortex mixer and left to stand for 10 minutes. Sample extracts were then centrifuged (12,000 rpm) for 5 minutes before transferring a 0.5 mL aliquot to a 5 mm NMR tube ready for NMR spectroscopy. Samples were dissolved immediately (<1 hour) before being run in the spectrometer to minimise degradation (Robertson, Personal Communication).

All spectra were obtained on a 500 MHz Bruker Avance DRX Spectrometer (Bruker) operating at 202.5 MHz with a 5 mm broadband probe for ^{31}P; D_2O was included in all samples as the signal lock. Samples were left to stand inside the spectrometer for 10 minutes prior to analysis to allow sample and spectrometer temperatures to equilibrate. Spectra were obtained using an acquisition time of 0.403 seconds, a 90° pulse angle (pulse length of 10 µs), 1 second delay time, 20,000 scans and a probe temperature of 298 °K (~25°C). Chemical shifts (δ) were determined relative to an external standard of 85% H_3PO_4 which was used to set 0 ppm. Spectra were plotted using a line broadening of 2 Hz (Robertson, Personal Communication). Individual ^{31}P-NMR spectra for each of the applied sludge treatments, plus sludge amended and untreated soils at the Woburn and Hartwood field sites, are shown in **Figure 4.22**, and **Figure 4.23**, respectively. Spectral assignment of organic phosphorus compounds was based on the chemical shifts reported by Turner et al. (2003) and Cade-Menun (2005); these results are summarised in **Table 4.7**.

4.4.2. Forms of Organic Phosphorus in Applied Sludge Treatments

Of the four sludge treatments, the greatest range of organic phosphorus compounds was seen in the undigested control (**Figure 4.22** and **Table 4.7**). Whereas, inorganic species, such as orthophosphate and pyrophosphate, were predominant in the Zn sludge treatment; though phosphonate and phosphodiester signals were visible at δ = 17.40 ppm and δ = -0.43 ppm, respectively, however, they were the weakest of the four sludge treatments (**Figure 4.22**). In agreement with the data presented in Section 4.2.4, and the values for $P_{Loading}$ given in **Table 4.1**, the signal for orthophosphate was greatest in the spectra obtained from the Zn sludge treatment, and weakest in that of the Cu sludge treatment (**Figure 4.24**); similarly the orthophosphate signal was greater for the digested control than the

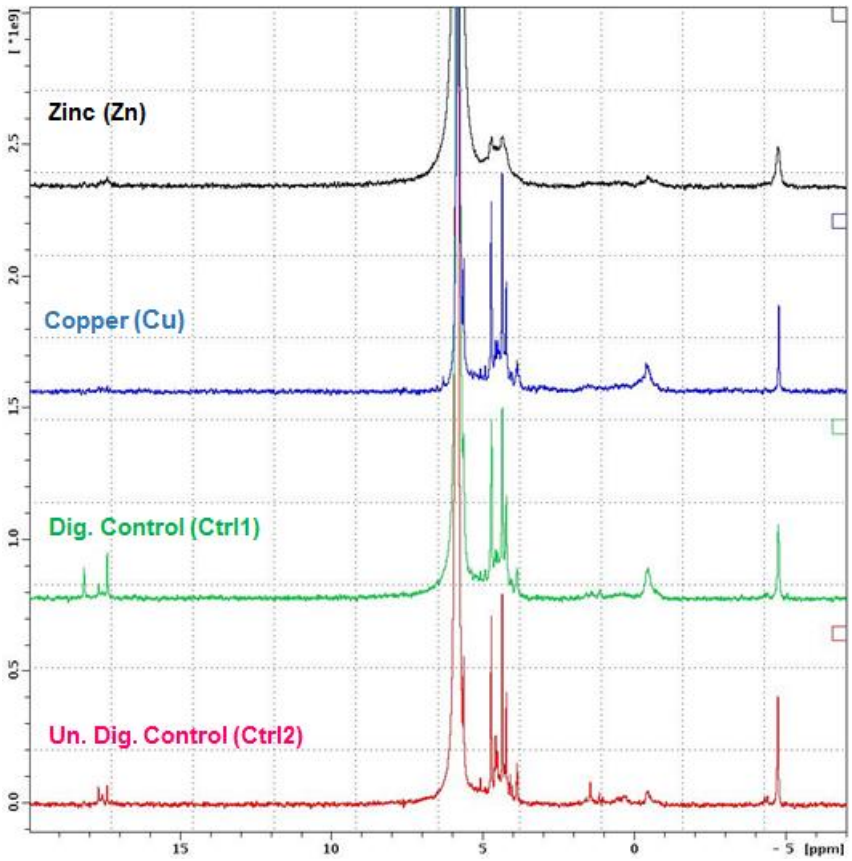


Figure 4.22:- ^{31}P-NMR specrta of digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments applied at the LTSE field sites during experimental Phase I (1996). Phosphonate (δ = 19 to 18 ppm), Phosphomonoester (δ = 6.5 to 4 ppm), Orthophosphate (δ = 6 to 5 ppm), Phosphodiester (δ = 2 to -1.5 ppm), and Pyrophosphate (δ = -4 to -5 ppm) regions are shown. Concentration of compounds are proportional to peak area.

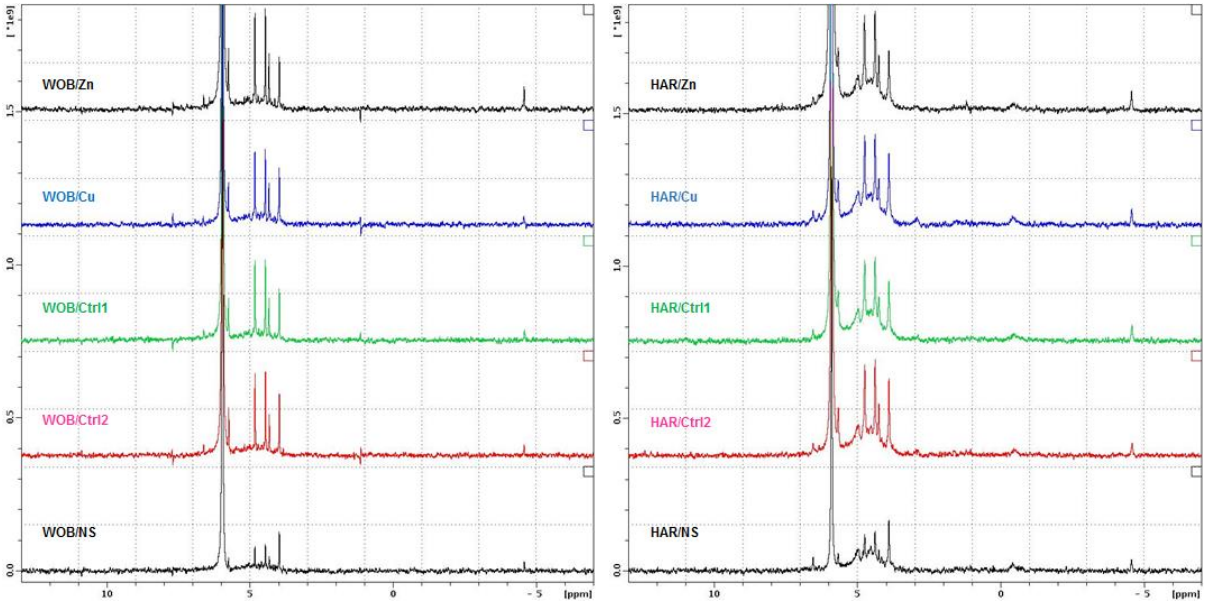


Figure 4.23:- ^{31}P-NMR specrta of soils receiving digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatents, plus untreated soil (NS), at the Woburn (WOB; left) and Hartwood (HAR; right) field sites. Samples were collected in 2014, approximately 18 years after the final sludge applications. Phosphonate (δ = 19 to 18 ppm), Phosphomonoester (δ = 6.5 to 4 ppm), Orthophosphate (δ = 6 to 5 ppm), Phosphodiester (δ = 2 to -1.5 ppm), and Pyrophosphate (δ = -4 to -5 ppm) regions are shown. Concentration of compounds are proportional to peak area.

Table 4.7:- Spectral assignments for peaks present in ^{31}P-NMR spectra of digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments applied at the LTSE field sites. Assignments are based on chemcial shifts reported by Turner et al. (2003), Turner & Richardson (2004) and Cade-Menun (2005).

	Chemical Shift (δ ppm)				
Spectral Region	**Zinc (Zn)**	**Copper (Cu)**	**Dig. Control (Ctrl1)**	**Un. Dig. Control (Ctrl2)**	**Assignment**
Orthophosphate	5.0-7.0	5.5-6.5	5.25-6.5	5.25-6.5	Orthophosphate
Phosphomonoester		5.65	5.65	5.65	
δ = 3 to 6 ppm	Unresolved	4.75	4.75	4.75	*myo*-Inositol
		4.40	4.40	4.40	Hexakisphosphate
		4.25	4.25	4.25	
		4.53	4.53	4.53	
				4.09	*scyllo*-Inositol
		4.08	4.08		Pentakisphosphate
		3.87	3.87	3.87	
		3.90	3.90	3.90	*scyllo*-Inositol Hexakisphosphate
		4.60	4.60	4.60	Adenosine Monophosphate
		4.04		4.04	Choline Phosphate
Phosphodiester				1.45	Phospholipids
δ = -1 to 2 ppm				1.15	or
				1.05	Teichoic Acids
		-0.40			
	-0.43			-0.43	DNA
			-0.50		
Phosphonate			18.18		
δ = 12 to 24 ppm			17.70	17.70	
				17.58	Phosphonolipids
			17.55		
	17.40		17.40	17.40	
Polyphosphate	-4.75			-4.75	
δ = -20 to -3			-4.76		Pyrophosphate
		-4.78			

undigested control. It should be noted that due to the high concentration of Zn present in the Zn sludge treatment, the concentration of EDTA used to chelate metal ions during extraction (Bowman & Moir, 1993) does not appear to have been sufficient. As a result peak broadening has occurred in the spectra for the Zn sludge treatment, particularly in the phosphomonoester region (δ = -6.5-4 ppm) which is largely unresolved (**Figure 4.22** and **Figure 4.25**). This may be due to the aggregation of phosphate groups facilitated by the binding of Zn^{2+} ions, however this has not been determined (Craven, Personal Communication).

For the remaining sludge treatments, the phosphomonoester region is dominated by signals from *myo*-inositol hexakisphosphate (**Figure 4.22** and **Figure 4.25**). The four signals present at δ = 5.65 ppm, δ = 4.75 ppm, δ = 4.40 ppm, and δ = 4.25 ppm (**Table 4.7**), are due to the 1-equitorial/5-axial conformation of the phosphate groups around the inositol ring (**Figure 1.5**), with the signal at δ = 5.65 ppm produced by the equitorial phosphate group (Turner et al., 2003). A signal from *scyllo*-inositol hexakisphosphate (**Figure 1.5**) is also present at δ = 3.90 ppm, however in each case this could not be fully resolved due to an overlapping signal at δ = 3.87 ppm (**Figure 4.26**), possibly due to the presence of *scyllo*-inositol pentakisphosphate. Additional peaks in the spectra for the the undigested control at δ = 4.09 ppm and δ = 4.53 ppm give further indication to the presence of *scyllo*-inositol pentakisphosphate

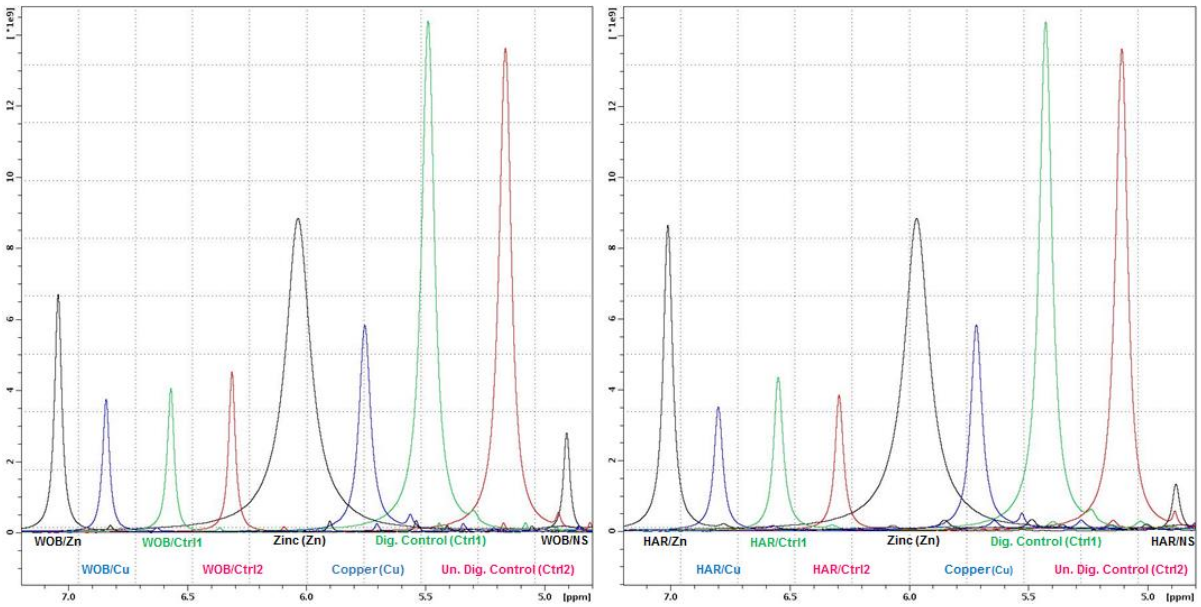


Figure 4.24:- Enlargement of Orthophosphate region (δ = 6 to 5 ppm) in the ^{31}P-NMR specrta of digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments, plus sludge amended and untreated soils at the Woburn (left) and Hartwood (right) field sites. Spectra of sludge and sludge amended soils are colour coordinated. Concentration of orthophosphate is proportional to peak area.

(Turner & Richardson, 2004); these signals are also present in the Cu sludge treatment and digested control spectra, but are slightly weaker (**Figure 4.26**). In each case, the inositol phosphate signals were strongest in the undigested sludge treatments, with the strongest *myo*-inositol signals in the Cu sludge treatment spectra, and the strongest *scyllo*-inositol signals in the undigested control spectra. An additional signal was present in the phosphomonoester region at approximately δ = 4.60 ppm for each of the sludge treatments (**Figure 4.25**), with a further signal at δ = 4.04 ppm also in the spectra of the undigested sludge treatments (**Figure 4.26**). These could potentially be due to adenosine monophosphate and choline phosphate, respectively, which are degradation products from the hydrolysis of nucleic acids and phospholipid diesters (Turner et al. 2003). However it is not possible to determine whether these compounds were originally present in the sludge treatments when applied, or are due to the hydrolysis of phosphodiester compounds during the extraction process (Turner et al. 2003). For each of the sludge treatments, the phosphodiester region is dominated by a broad signal for DNA centred at approximately δ = -0.43 ppm, with the strongest signals seen in the spectra for the Cu sludge treatment and digested control (**Figure 4.27**). Additional signals are also present in the undigested control spectra at δ = 1.45, 1.15, and 1.05 ppm which could either be due to phospholipid diesters (Turner et al. 2003) or teichoic acids (Makarov et al., 2002). Phosphonate signals appeared predominantly in the spectra for the digested and undigested controls, at approximately δ = 17.70, 17.57, and 17.40 ppm, with an additional signal at δ = 18.18 ppm present in the digested control spectra (**Figure 4.28**). A weak signal at δ = 17.40 ppm was also observed for the Zn sludge treatment, whereas no phosphonates were present in the Cu sludge treatment. Possible assignment for these signals is to phosphonolipids (Cade-Menun et al., 2002). A strong signal for pyrophosphate (**Figure 1.4**) also

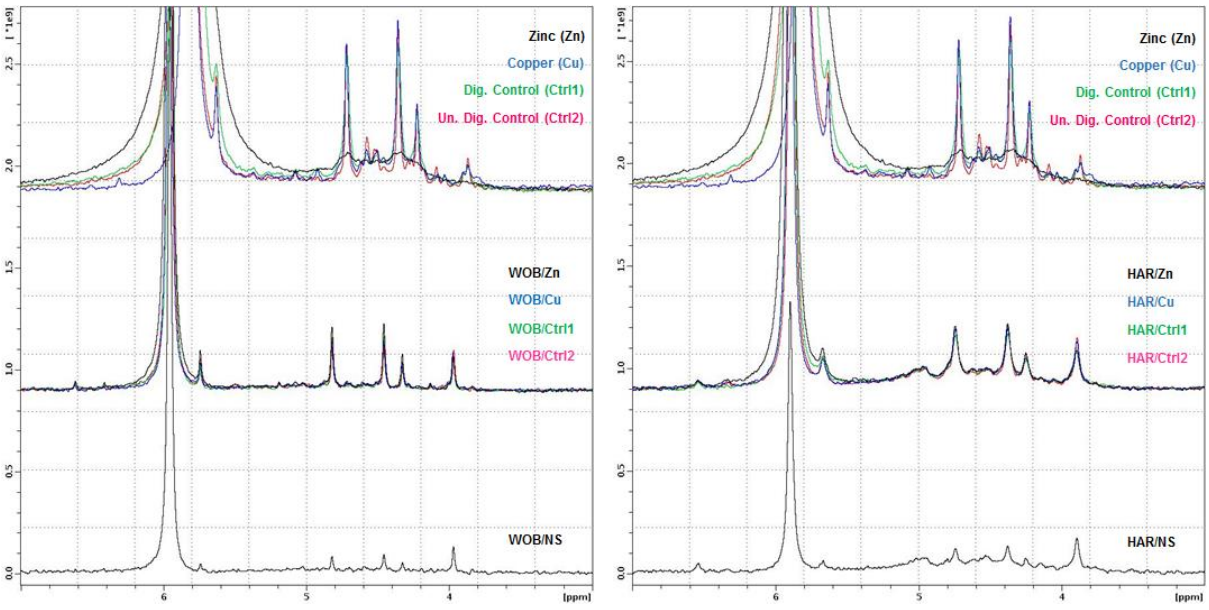


Figure 4.25:- Enlargement of Phosphomonoester region (δ = 3 to 6 ppm) in the ^{31}P-NMR spectra of digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments, plus sludge amended and untreated soils at the Woburn (left) and Hartwood (right) field sites. Spectra of sludge and sludge amended soils are colour coordinated. Concentration of phosphomonoester compounds is proportional to peak area.

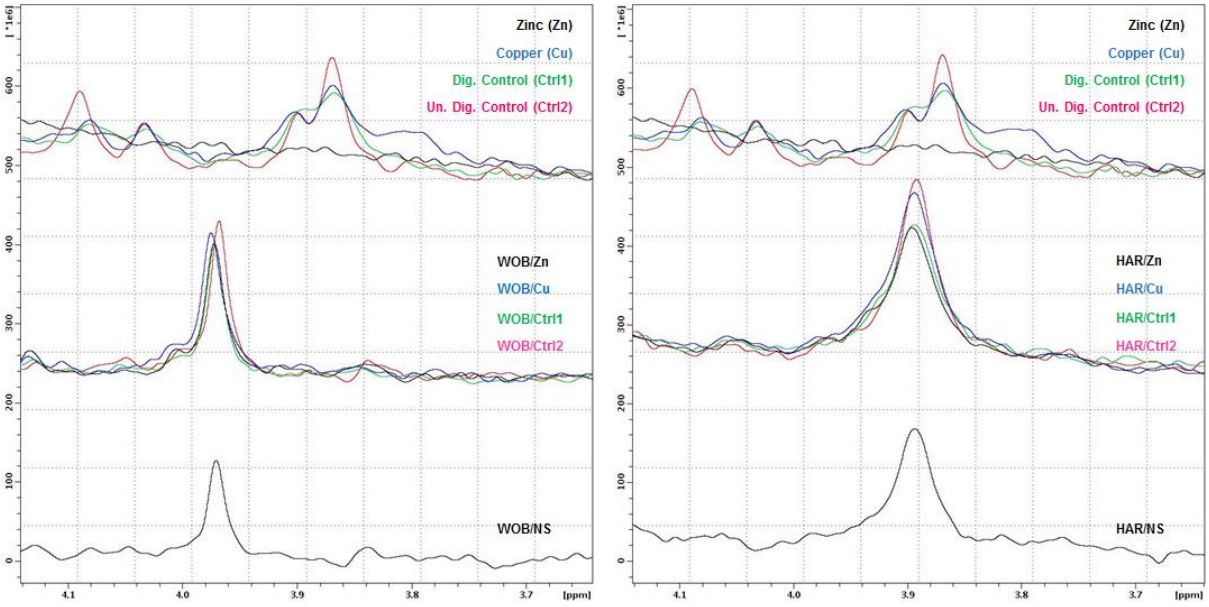


Figure 4.26:- Enlargement of *scyllo*-Inositol Phosphate signals in the ^{31}P-NMR spectra of digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments, plus sludge amended and untreated soils at the Woburn (left) and Hartwood (right) field sites. Spectra of sludge and sludge amended soils are colour coordinated. Concentration of phosphomonoester compounds is proportional to peak area.

occurred in the polyphosphate region for each of the sludge treatments at approximately at δ = -4.76 ppm (**Figure 4.29**). Due to the absense of signals between δ = -19 to -21 ppm, caused by mid-chain phosphate groups, organic polyphosphates, such as adenosine triphosphate, or inorganic polyphosphates with chain length n > 2 did not appear to be present in any of the sludge treatments (Turner et al. 2003).

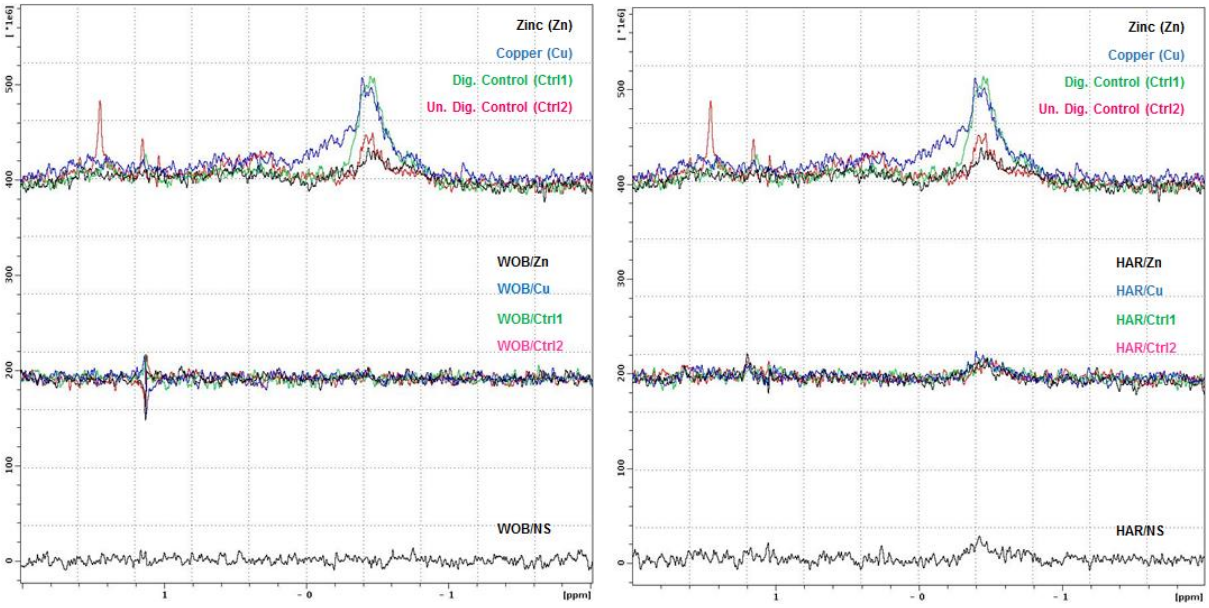


Figure 4.27:- Enlargement of Phosphodiester region (δ = -1 to 2 ppm) in the ^{31}P-NMR spectra of digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments, plus sludge amended and untreated soils at the Woburn (left) and Hartwood (right) field sites. Spectra of sludge and sludge amended soils are colour coordinated. Concentration of phosphodiester compounds is proportional to peak area.

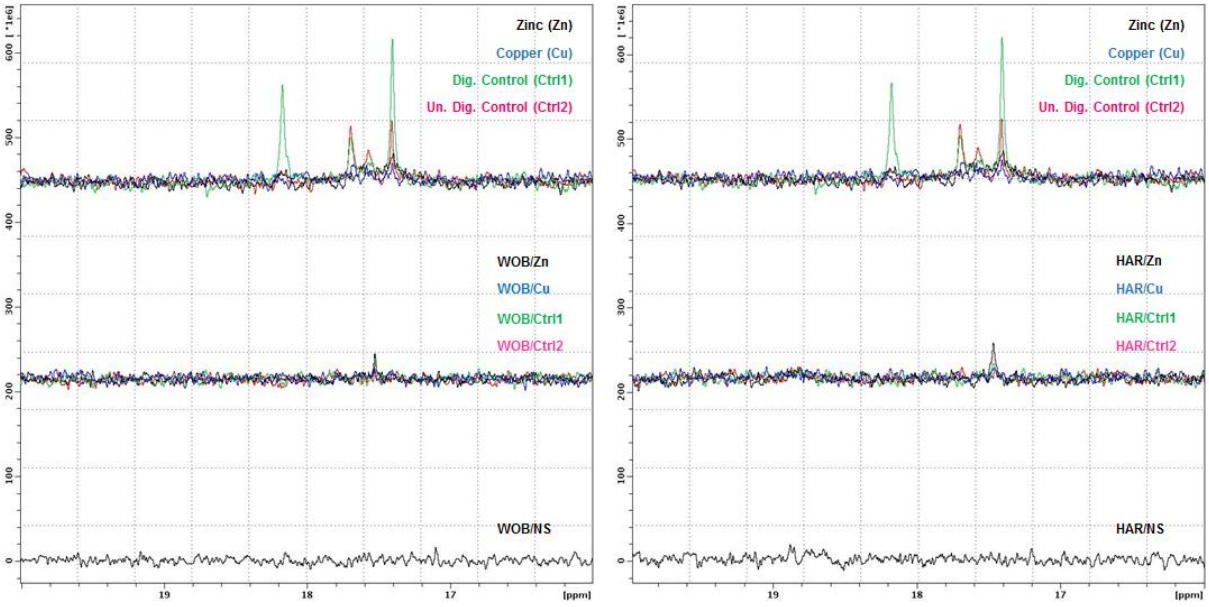


Figure 4.28:- Enlargement of Phosphonate region (δ = 12 to 24 ppm) in the ^{31}P-NMR spectra of digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments, plus sludge amended soils at the Woburn (left) and Hartwood (right) field sites. Spectra of sludge and sludge amended soils are colour coordinated. Concentration of phosphonate compounds is proportional to peak area.

4.4.3. Long-Term Fate of Organic Phosphorus Compounds

In agreement with the data presented for total p in **Section 4.2.2**, the orthophosphate signal in the untreated soil at Woburn was greater in comparison to that at Hartwood (**Figure 4.24**). Similarly, the orthophosphate signals in sludge amended soils at Hartwood also reflected the relative differences

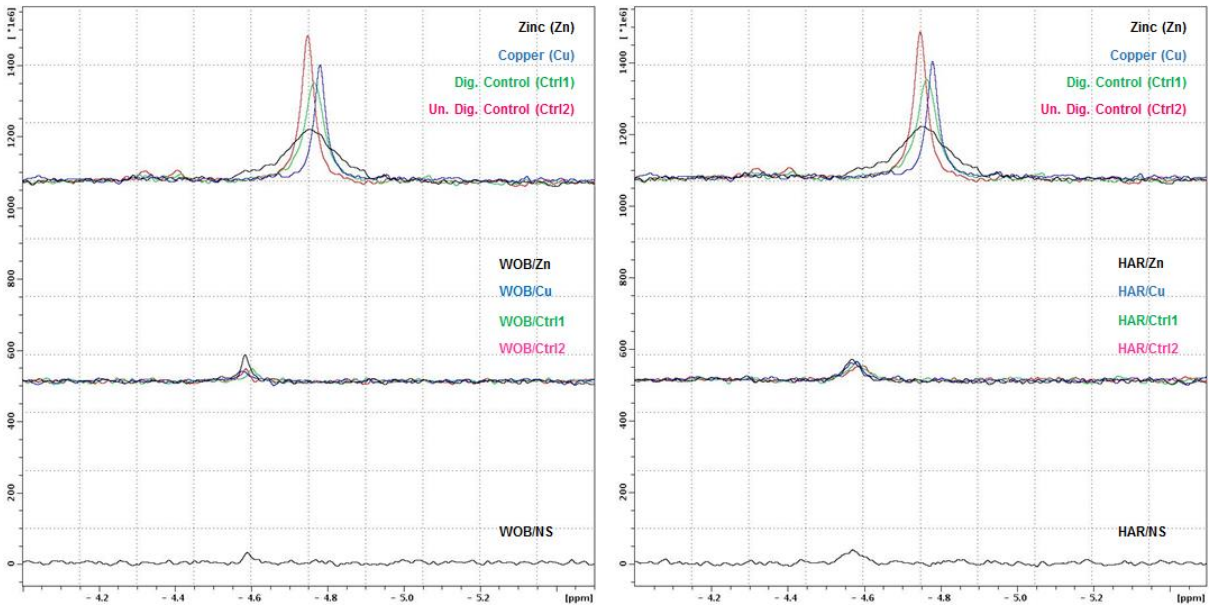


Figure 4.29:- Enlargement of Polyphosphate region (δ = -20 to -3 ppm) in the ^{31}P-NMR spectra of digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments, plus sludge amended soils at the Woburn (left) and Hartwood (right) field sites. Spectra of sludge and sludge amended soils are colour coordinated. Concentration of phosphonate compounds is proportional to peak area.

observed at this site during 2014 (**Table 4.2**), with the greatest increase in orthophosphate observed in soil receiving the Zn sludge treatment (**Figure 4.24**). Similarly at Woburn, the greatest increase in orthophosphate signal was also seen in soil receiving the Zn sludge treatment, which was lower than that seen at Hartwood (**Figure 4.24**). Again this was in general agreement with the total phosphorus data given in **Section 4.2.2.** Although in this case the orthophosphate signal for soil receiving the undigested control at Woburn was greater in comparison to soils receiving the digested control and the Cu sludge treatment, and was also greater in comparison to the respective soil at Hartwood (**Figure 4.24**). However, it should be noted here that the orthophosphate signal is not directly comparable to the total phosphorus concentrations determined by *aqua-regia* acid digestion (**See Section 4.2.2**), as these also include phosphorus derived from organic matter. Furthermore, it was demonstrated that the NaOH-EDTA extraction method used to produce the NMR spectra, was stronger than the $NaHCO_3$ method used to determine the concentration of 'available' phosphorus (**Figure 4.30**; Craven & Robertson, Personal Communication). Therefore although the relative differences in the orthophosphate signals of the sludge amended soils are in agreement with the data presented in **Section 4.2**, comparisons between the two data sets should be made with caution.

The phosphomonoester regions of both untreated and sludge amended soils at the Hartwood field site show a broad signal from approximately δ = 5.2 to 4.0 ppm (**Figure 4.23** and **4.25**) due to phosphomonoesters present within the humic fraction of soil organic matter (Doolette et al., 2011). The signal is also present in the spectra for Woburn, but is not as pronounced due to the lower SOC content at this site (**See Section 2.6**). Hence in comparison to Hartwood, the signals for *myo*-inositol hexakisphosphate at Woburn are more defined, however, in both cases the concentration of

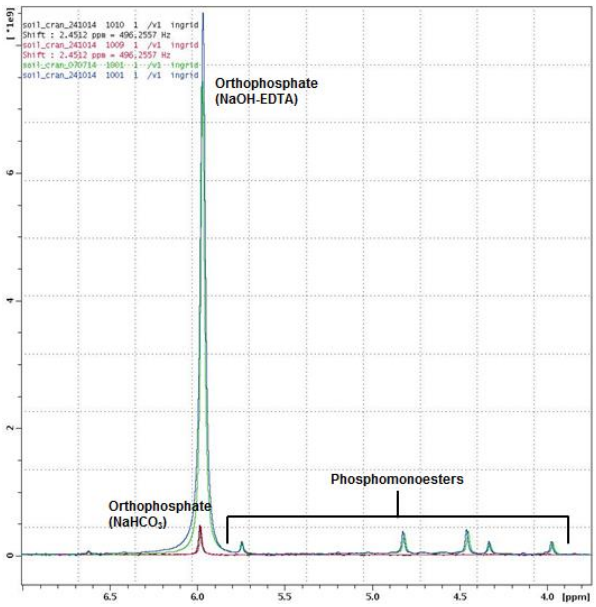


Figure 4.30:- Comparison of Orthophosphate signals (δ = 6.0) obtained using NaOH-EDTA and $NaHCO_3$ extraction methods on soil receiving the Zn sludge treatment at the Woburn Field Site.

myo-inositol hexakisphosphate has increased due to the application of the sludge treatments (**Figure 4.25**). Similarly, the signal for *scyllo*-inositol hexakisphosphate is greater in the spectra for sludge amended soils at both sites, in comparison to that of the untreated soils, with a higher content of *scyllo*-inositol hexakisphosphate seen in soils receiving the undigested sludge treatments in both cases (**Figure 4.26**). In addition, the signals for *scyllo*-inositol pentakisphosphate are no longer present in the spectra for soils receiving the undigested sludge treatments at either Woburn or Hartwood indicating this compound may have been utilised as a source of phosphorus following sludge application.

A weak DNA signal can be seen in the phosphodiester region of the spectra for untreated soil at Hartwood; whereas no signal is present at Woburn (**Figure 4.27**). In both cases, the spectra for each of the sludge amended soils reflects that of the untreated soil, suggesting that the DNA applied in each of the sludge treatments has now been mineralised, and the DNA content of these soils has returned to background levels. Similarly, the phospholipid signals present in the spectra for the undigested control are also absent in the spectra for the respective soils at each site (**Figure 4.27**).

For both sites, a weak signal is present at approximately δ = 17.5 ppm in the phosphonate region of the spectra for soils receiving the digested sludge treatments which is not present in the spectra for untreated soil (**Figure 4.28**). This may correspond to the signal at δ = 17.4 ppm seen in the spectra for the digested sludge treatments (**Table 4.7**). Nevertheless, with this exception, the remaining phosphonate signals present in the sludge treatment spectra were no longer present in those of the receiving soils (**Figure 4.28**). The signals for pyrophosphate, with the exception of soil receiving the Zn sludge treatment at Woburn, also appear to have returned to that of the untreated soil at both sites (**Figure 4.29**).

In summary, there appears to be no apparent difference in the range of organic phosphorus compounds present in the sludge amended and untreated soils at either of the field sites. This suggests that following the final sludge applications made in 1997, in contrast to the differences oberved for inorganic orthophosphate (**See Section 4.2.4**), the extraneous organic phosphorus compounds present in the sludge treatments have been mineralized, or physically removed from the receiving soils, and the organic phosphorus content is now returning to that of untreated soil, with no aparrent interference caused by the presence of heavy metals.

4.5. Chapter Discussion (2012-2014)

As seen for heavy metals (**See Section 3.4**), application of the sludge treatments has increased the total phosphorus content of the receiving soils, which, in most cases, particularly at the Scottish sites, still remain significantly higher in comparison to the untreated soil almost 20 years since the final sludge applications were made (**Table 4.2**). In addition, the concentration of 'available' orthophosphate in each of the sludge amended soils increased in proportion to the quantities of phosphorus applied (**Table 4.1**) during experimental Phase I (1994-1997). In this case, in comparison to untreated soil, 'available' phosphorus still remained significantly higher in each of the sludge amended soils at each of the LTSE field sites (**Table 4.2**).

With the exception of the Cu sludge treatment, for which the phosphorus content was slightly low (0.76 %), the phosphorus content of the applied sludge treatments (**Table 4.1**) were in agreement with the range of values given by Kirkham (1982); with higher phosphorus contents seen in the digested sludge treatments (Zn and Ctrl1) due to the concentrating effect of anaerobic digestion (Smith, 1996). Again after almost 20 years, the relative differences observed for total and 'available' phosphorus in the sludge amended soils at each of the LTSE field sites still reflected the phosphorus content of the applied sludge treatments (**Figure 4.13**). Although, in general, no significant differences in 'available' phosphorus could now be detected between soils receiving comparable sludge treatments (**Table 4.2**). Regression analysis also showed a significant relationship between total and 'available' phosphorus concentrations across the LTSE field sites (**Table 4.3**), however, the higher concentrations of total phosphorus in soils receiving the Zn sludge treatment did not produce greater concentrations of 'available' phosphorus in comparison to soils receiving the digested sludge treatments (**Figure 4.14**). However, this may be due to the greater Fe/Al content of the Zn sludge treatment in comparison to the digested control (**See Section 4.2.5**). In contrast to the differences at each site, the relative differences in the total and 'available' phosphorus content of sludge amended soils between field sites, no longer reflected the initial $P_{Loading}$ applied during Phase I (**Table 4.1**), due to differences in soil properties and the land management practices implemented at each site. In general, concentrations of total and 'available' phosphorus remaining in the sludge amended soils at the Scottish grassland sites during the

current investigation, particularly at Hartwood, were greater in comparison to the English arable sites (**Figure 4.13**).

In contrast to the findings of Hawkes et al. (1984), that a wider range of organic phosphorus compounds are generally found at lower pH values, the greatest number of organic phosphorus forms were seen in the undigested control (Ctrl2; **Figure 4.22**), which had a pH of 7.3 (**Table 2.2**); second to this was the digested control (Ctrl1) which also had a pH of 7.3. Though in agreement with Hawkes et al. (1984), the Zn sludge treatment, with the highest pH of the four sludge treatments (pH 7.5; **Table 2.2**), did contain the smallest range of organic P compounds (**Figure 4.22**); though it is possible some of the signals present were not resolved (**See Section 4.4.2**). Nevertheless, the ^{31}P-NMR spectra determined for each of the sludge treatments were broadly in agreement with those presented by Peng et al. (2010), in that orthophosphate, phosphomonoesters, and pyrophosphate were the predominant forms of phosphorus within the sludge treatments (**See Section 1.3**). However, a broad signal, assigned to DNA (**Table 4.7**), was present in each of the ^{31}P-NMR spectra for the LTSE sludge treatments (**Figure 4.22**), whereas only 15 % of the sludge samples (n = 13) analysed by Peng et al. (2010) contained phosphodiesters.

Comparing the sludge treatment spectra with those for the respective receiving soils, plus untreated soil, at the Woburn and Hartwood field sites, showed no overall change in the phosphodiester, pyrophosphate, or phosphonate (with the exception of soil receiving the Zn sludge treatment) content of the sludge amended soils (**Figure 4.27** to **4.29**). In each case, the signals for the respective organic phosphorus species were comparable to that seen in the spectra for untreated soil. However, at both sites, the inositol phosphate signals were greater in the spectra for sludge amended soils, in comparison to untreated soil, showing an increase in these compounds (**Figure 4.25** and **Figure 4.26**). This is not too surprising as inositol phosphates are considered to fairly recalcitrant (Celi et al., 1999; Cosgrove & Irving, 1980; Magid et al., 1996) and are often reported to accumulate in a wide range of soils (Turner et al., 2002). Therefore contrary to the hypothesis posed for **Research Question 3** (**See Section 1.8.3**) the presence of Zn and Cu in soils receiving the contaminated sludge treatments appears to have no long-term impact on the mineralisation of organic phosphorus. These findings are in agreement with the results of Annaheim et al., (2015) who also observed no change in either the number, or quantity, of organic phosphorus compounds present in soils receiving annual applications of sewage sludge (2.5 t ha^{-1} yr^{-1}) over the course of 62 years. As described by Magid et al. (1996), the possible fates for the organic phosphorus compounds derived from the applied sludge treatments are: contribution to the increase in orthophosphate observed in sludge amended soils at the LTSE sites (**Figure 4.24**), incorporation into soil humus (Doolette et al., 2011), or incorporation into plant and microbial biomass. Therfore it can be predicted that the organic phosphorus in sludge amended soils, measured using the ignition method (**See Section 4.3.1**), is now predominantly comprised of humic phosphorus, microbial biomass phosphorus, and sludge derived inositol phosphates.

Brookes et al. (1984) investigated the phosphorus content of the soil microbial biomass at a range of arable (n = 5) and grassland (n = 6) sites around the UK. Values for organic phosphorus ranged from 180-270 µg P g soil^{-1} (an extreme case of 810 µg P g soil^{-1} was also observed) and 190-500 µg P g soil^{-1} in arable and grassland soils, respectively. In each case the lowest values for organic phosphorus reported by Brookes et al. (1984), were higher than those observed in the LTSE English arable, and Scottish grassland sites, by factors of approximately 2-4. This was probably due to differences in the methods used, as Brookes et al. (1984) used both ignition (Saunders & Williams, 1955) and acid-base extraction (Mehta et al. 1954) methods to determine organic phosphorus, then reported the mean value. Nevertheless, the percentage of organic phosphorus derived from the soil microbial biomass was found to be lower at the arable sites, ranging from 2.5-3.4 % (mean = 3.0 ± 0.2 %; and outlier of 7.8 % was also observed), in comparison to the grassland sites where microbially derived organic phosphorus varied considerably, ranging from 5.0-24.3 % (mean = 13.7 ± 3.5). As described above, organic phosphorus was significantly correlated to SOC (**See Section 4.3.3**), therefore given the relationship between SOC and the size of soil microbial biomass (**See Section 5.2.3**) described by Jenkinson and Ladd (1981), it follows that organic phosphorus will also be significantly correlated to the size of the soil microbial biomass, hence it can be assumed that a greater proportion of the organic phosphorus at the Scottish grassland sites is compried of microbial phosphorus; Brookes et al. (1984) estimate that soil microbial biomass contains approximately 3.25 ± 0.25 % phosphorus (range = 1.9-4.7 %).

Kahn et al. (2007) investigated the long-term effects of heavy metals on soil microbial biomass P (P_{mic}) and the ratio of biomass C to biomass P (C_{mic}:P_{mic}) in sludge amended soils at five field sites in Germany (two of which came from the Brunswick field experiment mentioned in **Section 1.4**). Concentrations of Zn, Cu, and Pb ranged from 119-565, mg kg^{-1}, 26-126 mg kg^{-1}, and 34-115 mg kg^{-1}, respectively. Therefore only Zn was present above the current UK statutory limits (**Table 1.2**), though in some soils Zn, Cu, and Pb exceeded the statutory limits set by Germany (**See Section 7.3**). In each case, no significant correlation was observed between P_{mic} and total metal concentration, nor were any significant changes in the C_{mic}:P_{mic} ratio observed. An attempt to determine P_{mic} was carried out as part of the current investigation using the method of Brookes et al. (1982), though the results were inconsistent and often produced negative values for P_{mic}; this is probably because the % recovery of phosphorus was not determined for fumigated and non-fumigated samples. However, in contrast to the results of Kahn et al. (2007), data obtained from the Woburn site in 2013, showed a decrease in the C_{mic}:P_{mic} ratio (hence an increase in P_{mic}) of soils receiving the Zn and Cu sludge treatments (**Figure 4.31**) in comparison to soils receiving the uncontaminated controls; although in comparison to the values reported by Brookes et al. (1984) P_{mic} appears to be underestimated. This could be an indication that the presence of heavy metals has reduced phosphorus availability thereby prompting soil microorganisms to increase the concentration of phosphorus stored within their biomass. However, further investigation would be required in order to test this hypothesis.

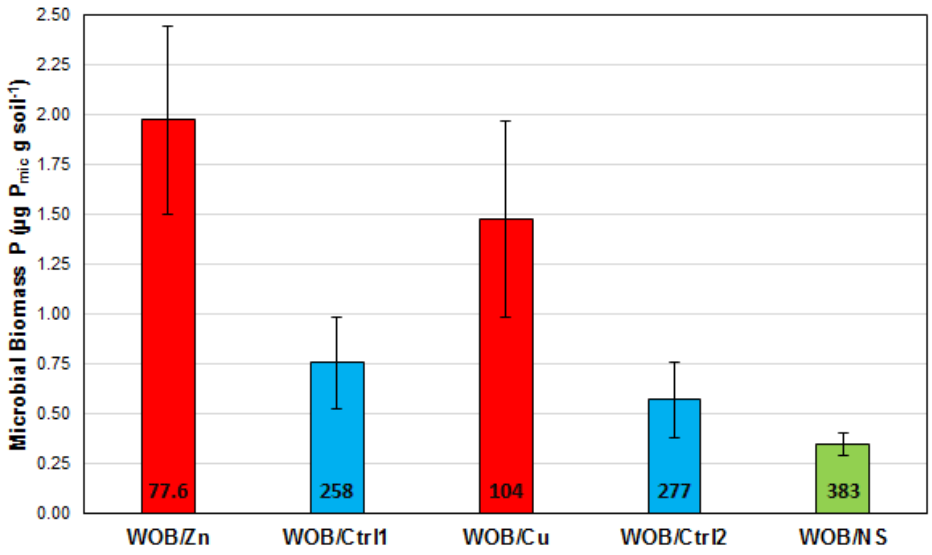


Figure 4.31:- Change in soil microbial phosphorus (µg P_{mic} g $soil^{-1}$) in untreated soil (NS), and soils receiving contaminated (Zn and Cu) and uncontaminated (Ctrl1 and Ctrl2) sludge treatments at the Woburn (WOB) field site in 2013. Ratios of C_{mic}/P_{mic} are shown for each soil. Error bars represent standard error (n = 3).

4.6. Conclusions (2012-2014)

For each of the LTSE field sites, the total phosphorus concentrations found in sludge amended soils have increased in proportion to the phosphorus content of the sludge treatments applied during experimental Phase I. For almost 20 years the relative differences in total phosphorus observed between the sludge amended soils at each site remain unchanged, with the greatest concentrations of total phosphorus still found in soils receiving the sludge treatment with the highest phosphorus content, i.e. the Zn sludge treatment. However, the decrease of total phosphorus in the sludge amended soils appears to have occurred at different rates at each site, and the relative differences in total phosphorus for respective soils at each site are no longer in agreement with the initial $P_{Loading}$ applied. This is likely to be due to the different land management practices implemented at the English arable and Scottish grassland sites.

In each of the sludge amended soils the concentration of 'available' phosphorus remained significantly higher in comparison to the untreated soil at each site and was found to increase in proportion to total phosphorus concentration, particularly in soils receiving the digested sludge treatments (Zn and Ctrl1). However, although a positive correlation was observed between total and 'available' phosphorus in soils receiving the digested sludge treatments, the percentage of total phosphorus present as available orthophosphate differed, hence no significant difference in 'available' phosphorus was observed between the two soils, despite a greater total phosphorus concentration in soils receiving the Zn sludge treatment.

Application of the sludge treatments also increased the organic phosphorus content of the receiving soils, however in most cases the increase was no longer significantly different to the organic phosphorus content of untreated soil at each site, nor were the differences between sludge amended soils significantly different; although some significant differences between the English and Scottish field sites were observed. Analysis of the sludge treatments by ^{31}P-NMR showed that a range of organic phosphorus compounds were applied to each of the receiving soils. However, subsequent analysis of the sludge amended soils at Woburn and Hartwood indicated that, with the exception of inositol phosphates, the organic phosphorus compounds applied to the soil had now been mineralised and the organic phosphorus content now resembled that of untreated soil. These findings are contrary to the hypothesis posed for **Research Question 3**, therefore, based on the current investigation, it can be concluded that the presence of Zn and Cu contamination in soil has no long-term impact on organic phosphorus mineralisation. In addition, organic phosphorus was also strongly correlated to organic C, and by inference soil microbial biomass. Hence the quantities of organic phosphorus, determined by the ignition method, that remain in the sludge amended soils at the LTSE field sites are likely to be comprised of microbial biomass phosphorus, phosphorus incorporated into the soil humus fraction, and inositol phosphates derived from the applied sludge treatments.

Cranfield UNIVERSITY

CHAPTER 5

SOIL MICROBIOLOGY

5. SOIL MICROBIOLOGY

5.1. Introduction

The aim of this chapter is to discuss the current state of soil microbiology in the sludge amended and untreated soils at each of the Long-Term Sludge Experiment field sites. Measurements of soil microbial biomass carbon, plus ergosterol and phospholipid fatty acid biomarkers, are used to determine the effect of sludge application on soil microorganisms by comparison to untreated soil. The long-term effects of Zn and Cu contamination are determined by comparing soil microbial status in contaminated soils to soils receiving uncontaminated sludge treatments. The design of an incubation experiment, established to determine the effect of heavy metal contamination on the short-term response of microbial biomass proliferation and phosphatase enzyme synthesis, is presented. The effects of Zn and Cu on the short-term proliferation of microbial biomass C are also discussed.

5.2. Microbial Biomass Carbon

Soil microbial biomass comprises the total mass of fungi, bacteria, protozoa, and algae per unit weight of soil and is regarded as an undifferentiated single compartment for the purpose of studying biochemical processes within the soil system and the cycling of nutrients. Taken as a gross measure of the microbial community size, soil microbial biomass carbon (C_{mic}) accounts for approximately 1-3 % of soil organic carbon (Jenkinson & Ladd, 1981) and, as described in **Section 1.4**, has frequently been used to investigate the impact of applying heavy metal contaminated sewage sludge on soil microorganisms (Brookes, 1995).

Throughout experimental Phases I-III, C_{mic} was monitored at each of the LTSE field sites (**Figure 5.1**) using the method of chloroform fumigation described by Vance et al., (1987); with additional measurements made at Hartwood during experimental Phase IV (2006-2011). In general, following the final sludge applications in 1997, C_{mic} had increased in each of the sludge amended soils in comparison to untreated soil, with greater increases seen in soils receiving the undigested sludge treatments (Cu and Ctrl2); presumably due to the application of more labile forms of organic matter (**See Section 2.6**). At Gleadthorpe C_{mic} was observed to be significantly (p <0.05) lower in soils receiving the Zn (-30 %) and Cu (-45 %) sludge treatments, in comparison to their respective controls (**Figure 5.1**). However, the overall effect observed across sites was not consistent enough to be attributed to metal toxicity, hence it was concluded that the application of contaminated sludge had little short term impact on C_{mic} (Gibbs

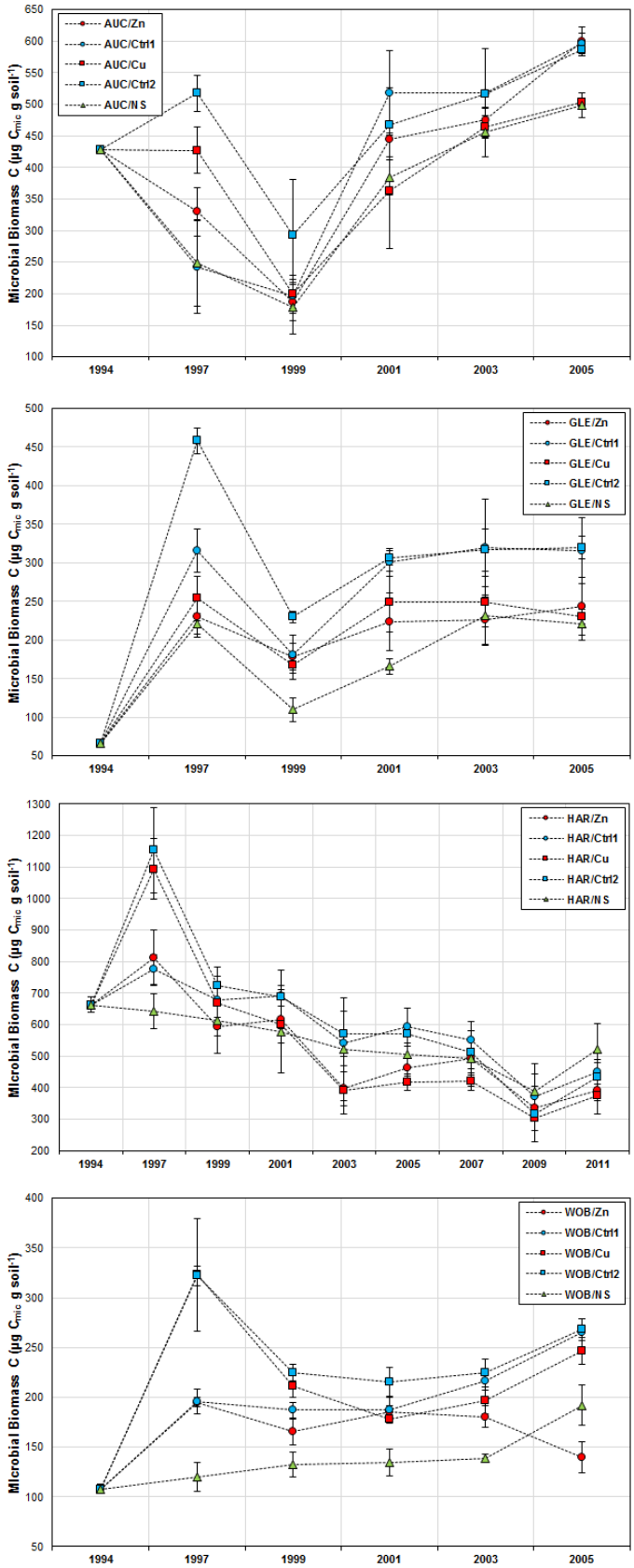


Figure 5.1:- Changes in soil microbial biomass carbon (µg C_{mic} g $soil^{-1}$) during experimental Phases I-III (1994 - 2005) in soils receiving undigested (Cu and Ctrl2) and digested (Zn and Ctrl1) sludge treatments, plus untreated soil (NS), at the Long-Term Sludge Experiment field sites. Additional data for experimentl Phase IV (2006-2011) is shown for Hartwood. Data obtained from Gibbs et al. (2006), Defra (2002, 2007), Cooper (Personal Communication). Error bars represent standard error (n = 3).

et al., 2006). In comparison to untreated soil, C_{mic} was reported to be generally higher in sludge amended soils during experimental Phase II (1998-2001), though no significant differences in C_{mic} were reported between soils receiving the digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments (Defra, 2002). During 2003, C_{mic} at the Woburn field site remained significantly higher (p <0.05) in soils receiving the digested sludge treatments, plus the Cu sludge treatment, in comparison to the untreated soil; whereas no significant differences were observed at the remaining sites. Additionally, only at Woburn was C_{mic} in soil receiving the Zn sludge treatment reported to be significantly (p <0.05) lower in comparison to the digested control (Defra, 2007a); whereas no significant differences were observed between soils receiving the undigested sludge treatments. By 2005, C_{mic} in soil receiving the digested control at Auchincruive and Gleadthorpe, plus soil receiving the undigested control at Woburn and Gleadthorpe, remained significantly (p <0.05) higher in comparison to the untreated soil at each site. In comparison to soil receiving the digested control, C_{mic} remained significantly (p <0.05) lower in soil receiving the Zn sludge treatment at the Woburn field site (Defra, 2007a). Whereas C_{mic} in soil receiving the Cu sludge treatment at Gleadthorpe was now reported to be significantly (p <0.05) lower in comparison to soil receiving the undigested control (Defra, 2007a). Overall, subsequent statistical review of the data from the LTSE field sites (**See Section 3.3**), indicated that, in general, C_{mic} decreased by 15-20 % in soils receiving the Cu sludge treatment, whereas a decrease of approximately 6 % was observed in soils receiving the Zn sludge treatment, once the confounding effect of Cu had been taken into account (Defra, 2008).

5.2.1. Microbial Biomass Carbon Method

For the current investigation (2013-2014), C_{mic} was also determined using the method of chloroform fumigation described by Vance et al. (1987). Samples of field moist soil, equivalent to 12.5 g of dry matter (**E.2.2**), were weighed into 100 mL glass containers and placed in a desiccator lined with moist filter paper. Approximately 25 g of soda lime was placed in each desiccator, followed by 25 mL of chloroform ($CHCl_3$). Desiccators were evacuated, allowing the $CHCl_3$ to boil for approximately 2 minutes, sealed, and left to stand for ≥ 24 hours. Following fumigation (*f*), residual $CHCl_3$ was removed from each desiccator using a vacuum pump before extracting each sample. Organic C was extracted using 50 mL of 0.5 M potassium sulphate (K_2SO_4) solution by mechanically shaking for 30 minutes then filtering (Whatman No. 42). Duplicate, non-fumigated (*nf*), samples were also weighed out and extracted immediately with K_2SO_4.

The concentration of dissolved organic C was determined by colourimetric analysis. A 5 mL aliquot of K_2SO_4 extract was mixed with 5 mL (× 2 dilution) of sodium polyphosphate ($(NaPO_3)_n$) reagent (1:20 m/v) in order to dissolve any $CaSO_4$ which may have precipitated. Samples were then loaded onto a segmented flow analyser (SFA 2000, Burkard Scientific), acidified with 0.5 M H_2SO_4, and mixed

with CO_2 free air in order to remove inorganic C as CO_2. The remaining organic C was mixed with 0.15 M potassium persulphate ($K_2S_2O_8$) solution and oxidised to CO_2 by irradiation with UV light. The evolved CO_2 was then passed through a gas diffusion membrane and mixed with a buffered solution of 50 µM phenolphthalein solution causing a loss of colour. The concentration of dissolved organic C in each extract was determined colourimetrically (CFX, Burkard Scientific) by measuring the absorbance of phenolphthalein at 550 nm and comparing the results to a calibration graph produced using C working standard solutions (0, 10, 20, 30, 40, and 50 µg C mL^{-1}). The concentrations of organic C in both fumigated and non-fumigated samples were then calculated as follows:

$$Org.C = \frac{(([C_S] \times D) - ([C_B] \times D)) \times (v + m_{H_2O} + m_{dm})}{m_{dm}} \quad (\mathbf{E.5.1})$$

where $[C_S]$ and $[C_B]$ are the measured concentrations of organic C in each sample and a method blank, respectively, D is a dilution factor, v is the volume of K_2SO_4 used for extraction, and m_{H_2O} and m_{dm} are the field moist and dry matter mass of extracted sample, respectively. Microbial biomass C (µg C_{mic} g $soil^{-1}$) was then calculated as the difference in organic carbon extracted from fumigated (C_f) and non-fumigated (C_{nf}) samples as follows:

$$C_{mic} = \frac{C_f - C_{nf}}{K_{EC}} \quad (\mathbf{E.5.2})$$

where K_{EC} is a correction factor equal to 0.45, assuming only 45 % of C_{mic} is extractable by K_2SO_4 following fumigation with $CHCl_3$ (Vance et al., 1987).

5.2.2. Microbial Biomass Carbon Results (2013-2014)

Auchincruive

Values for C_{mic} measured at the Auchincruive field site during 2013 were higher in comparison to those obtained for 2014 (**Table 5.1**), however the only significant (p <0.001) change observed over time occurred in soil receiving the undigested control which decreased from 387.28 ± 4.45 µg C_{mic} g $soil^{-1}$ in 2013 to 309.16 ± 6.73 µg C_{mic} g $soil^{-1}$ in 2014. This may be due to seasonal differences between the two sampling events, as samples for 2013 were collected in autumn, whereas samples for 2014 were collected in summer (**Table 2.4**). No significant differences in C_{mic} were observed between any of the sludge amended or untreated soils at any point during the current investigation (**Table 5.1**).

Gleadthorpe

With the exception of soil receiving the Zn sludge treatment, values for C_{mic} at the Gleadthorpe site were also higher in 2013 in comparison to that of 2014 (**Table 5.1**), with significant decreases in C_{mic}

Table 5.1:- Soil microbial biomass carbon (μg C_{mic} g $soil^{-1}$) concentrations measured over two consecutive years (2013 and 2014) at the Long-Term Sludge Experiment field sites.

Sludge Treatment	Microbial Biomass Carbon (μg C_{mic} g $soil^{-1}$)	
	2013	2014
AUC/Zn	345.20 (14.89)[a][1][2]	293.52 (41.05)[a]
AUC/Ctrl1	435.28 (67.43)[a]	317.11 (21.29)[a]
AUC/Cu	383.33 (33.43)[b]	271.67 (29.53)[b]
AUC/Ctrl2	387.28 (4.45)[b]	309.16 (6.73)[b]
AUC/NS	340.77 (21.42)[ab]	284.50 (7.71)[ab]
GLE/Zn	209.53 (8.37)[a]	220.51 (18.25)[a]
GLE/Ctrl1	277.30 (34.35)[ab]	266.64 (47.66)[a]
GLE/Cu	248.04 (9.78)[c]	213.90 (30.84)[b]
GLE/Ctrl2	345.08 (18.96)[d]	261.81 (8.65)[b]
GLE/NS	325.10 (3.24)[bd]	202.33 (25.62)[ab]
HAR/Zn	560.51 (51.60)[a]	526.26 (55.11)[a]
HAR/Ctrl1	651.81 (73.15)[a]	593.82 (83.69)[a]
HAR/Cu	478.67 (13.96)[b]	524.96 (79.70)[b]
HAR/Ctrl2	466.21 (112.63)[b]	559.60 (89.17)[b]
HAR/NS	615.95 (78.22)[ab]	634.66 (49.39)[ab]
WOB/Zn	153.16 (34.47)[a]	144.75 (8.15)[a]
WOB/Ctrl1	194.85 (8.33)[a]	158.77 (11.06)[a]
WOB/Cu	152.99 (5.60)[bc]	105.14 (34.39)[b]
WOB/Ctrl2	157.80 (6.62)[b]	162.90 (11.24)[b]
WOB/NS	132.53 (5.68)[ac]	124.01 (14.73)[ab]

[1]Values in parenthesis are standard errors (n = 3). [2] Values without corresponding letters denotes statistical significance at α = 0.05 for individual sampling events, note comparisons between different sludge types (i.e. digested (Zn and Ctrl1) and undigested (Cu and Ctrl2)) have not been made.

observed in the untreated soil (p <0.01) and soil receiving the undigested control (p <0.05). Again, this may be due to seasonal differences between the two sampling events (**Table 2.4**). In 2013, C_{mic} was significantly (p <0.01) lower in soil receiving the Zn sludge treatment in comparison to the untreated soil, whereas C_{mic} in soil receiving the Cu sludge treatment was significantly (p <0.01) lower in comparison to both untreated soil plus soil receiving the undigested control (**Table 5.1**). However, no significant differences in C_{mic} were observed between any of the sludge amended and untreated soils the following year.

Hartwood

Only in soils receiving the digested sludge treatments (Zn and Ctrl1) were values for C_{mic} higher in the samples collected during autumn 2013. However no significant changes in C_{mic} were observed over time at the Harwood field site (**Table 5.1**), nor were any significant differences in C_{mic} observed between soils.

Woburn

Similarly, no significant changes in C_{mic} occurred over time at the Woburn field site. However, in 2013, C_{mic} in soil receiving the undigested control was found to be significantly (p <0.05) higher in comparison to the untreated soil (**Table 5.1**).

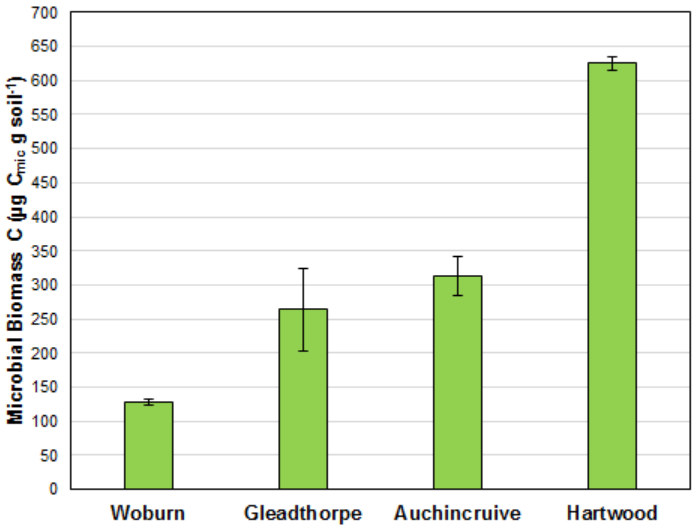


Figure 5.2:- Mean values for microbial biomass carbon (µg C_{mic} g $soil^{-1}$) in untreated soil (NS) at each of the Long-Term Sludge Experiment field sites, over two consecutive years (2013-2014). Error bars represent standard error (n = 2).

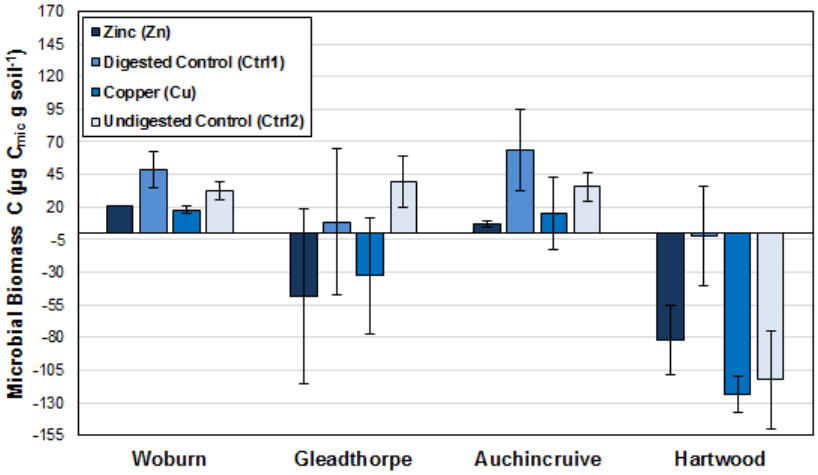


Figure 5.3:- Change in microbial biomass carbon (µg C_{mic} g $soil^{-1}$) in soils receiving digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments relative to the untreated soil at each of the Long-Term Sludge Experiment field sites. Values are mean microbial biomass carbon over two consecutive years (2013-2014). Error bars represent standard error (n = 2).

5.2.3. Microbial Biomass Carbon Overview (2013-2014)

In agreement with the relationship described by Jenkinson and Ladd (1981), the overall mean values for C_{mic} (µg C_{mic} g $soil^{-1}$), for years 2013 and 2014, increased in proportion to the SOC content of untreated soil (**See Section 2.7**) at each of the LTSE field sites, as follows:

WOB (128.27 ± 4.26) < GLE (263.72 ± 61.39) < AUC (312.63 ± 28.13) < HAR (625.31 ± 9.36)

with the value observed at Hartwood, significantly (p <0.01) higher in comparison to the other sites. In contrast, the value of C_{mic} at the Woburn field site was significantly (p <0.05) lower in comparison to the other sites, whereas no significant difference was observed between Auchincruive and Gleadthorpe (**Figure 5.2**).

Relative differences in C_{mic} between sludge amended and untreated soil are shown in **Figure 5.3** for each of the LTSE field sites. Only at Auchincruive and Woburn did C_{mic} in the sludge amended soils remain, on average, greater in comparison to untreated soil. At Gleadthorpe, values for C_{mic} in soils receiving the Zn and Cu sludge treatments were now lower in comparison to untreated soil; in contrast to that in soils receiving the uncontaminated controls. Whereas in each case, C_{mic} in the sludge amended soils at Hartwood had now decreased, on average, below the value seen in the untreated soil. Though, as described above, the only values for C_{mic} determined in sludge amended soils that were found to be significantly different from that in untreated soil were at Gleadthorpe and Woburn (**Table 5.1**); with no significant differences observed between soils receiving the Zn and Cu sludge treatments and their respective uncontaminated controls. However, the lack of statistically significant results may be due to seasonal variations in C_{mic} between sampling events, particularly at Auchincrive, Hartwood, and Gleadthorpe, as samples were collected from these sites in autumn 2013, and then in summer during 2014 (**Table 2.4**). For instance, Bardgett et al. (1999) observed significant seasonal changes in C_{mic} over the course of a year (1994-1995) in four grassland soils at the Institute of Grassland and Environmental Research in North Wyke (Devon, UK). Although, in this case C_{mic} reached a maximum during summer whereas values for C_{mic} determined during the current investigation were generally higher for samples collected in autumn 2013, in comparison to those collected in summer 2014 (**Table 5.1**).

The effect of heavy metal contamination on C_{mic} was investigated further using meta-analysis in order to increase statistical power and determine the overall impact of Zn and Cu on C_{mic}. The differences in C_{mic} between soils receiving the Zn and Cu sludge treatments and their respective controls (Ctrl1 and Ctrl2), for each of the LTSE field sites, were expressed as log response ratios and combined to give a summary effect across sites for each sampling event (**See Section 2.4.2**). These effects were then combined to give an overall mean effect for each of the contaminated sludge treatments (**Figure 5.4**). In this instance, the application of the Zn sludge treatment appears to have an overall significant (p <0.05) effect decreasing C_{mic} by approximately 15.8 % ($CL_{95\%}$ = -28 to -1 %) in comparison to the uncontaminated digested control. A negative effect was also seen for the Cu sludge treatment, decreasing C_{mic} by approximately 7.8 % ($CL_{95\%}$ = -7 to 2 %) in comparison to the uncontaminated undigested control. However in this case the overall effect was not statistically significant (p = 0.128), although the overall effect of the Cu sludge treatment during 2014 (-13.9 %) was found to be statistically significant (p <0.05). This is in agreement with the results presented in **Section 3.5** which suggest that Zn is more bioavailable, and therefore likely to be more toxic to microorganisms in comparison to Cu. Hence, with regards to **Research Question 1** it appears that, Zn may have caused an overall lasting decrease in C_{mic} in soils receiving the Zn sludge treatment that is still observable 15 years since the final sludge applications were made. This could have potential implications for the UK statutory limit (**See Section 1.3**), since the total concentrations of Zn have remained relatively unchanged over the past 15

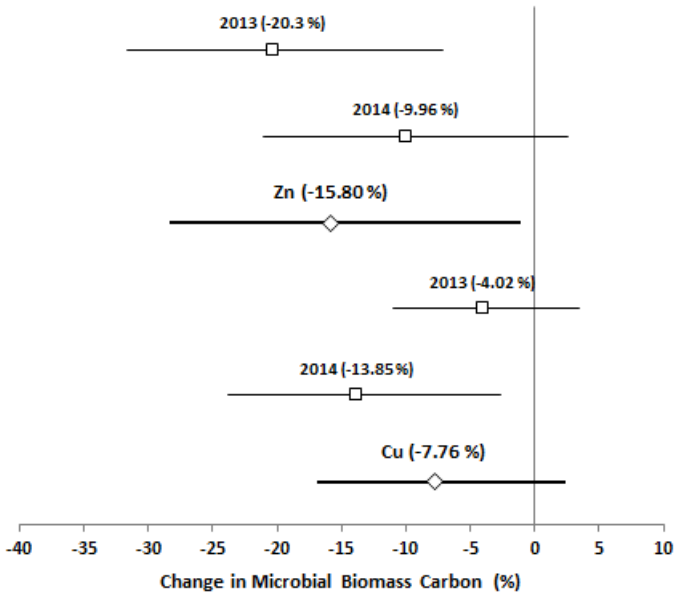


Figure 5.4:- Forest plot showing the change in soil microbial biomass carbon (µg C_{mic} g $soil^{-1}$) in soils receiving the Zn and Cu sludge treatments, in comparison to uncontaminated controls (Ctrl1 and Ctrl2), across all four LTSE fields sites for years 2013 and 2014. Horizontal lines represent 95 % confidence intervals. Effects are not statistically significant (p <0.05) if the 95 % confidence interval crosses the centre line.

years, and remain close to, or even below, the current statutory value in some cases (**Section 3.4**). However, it should be noted that, with the exception of soil receiving the Cu sludge treatment at Gleadthorpe in 2013 (**Table 5.1**), no significant differences in C_{mic} were observed between soils receiving contaminated and uncontaminated sludge treatments when considering each site separately. Furthermore, as described in **Section 3.3** and **Section 3.4**, soils receiving the Zn sludge treatment also contained significantly higher concentrations of Cu in comparison to soils receiving the digested control. Therefore it is possible that the effect observed here (**Figure 5.4**) is due to an interactive effect of Zn and Cu which augments the toxicity to soil microorganisms. It may be that a total concentration of Zn comparable to the UK statutory limit could be tolerated by soil microorganisms if Cu were not also present. Hence, with regards to **Research Question 4**, it may not be possible to assess the suitability of the current UK statutory limit for Zn in protecting C_{mic}.

5.2.4. Microbial Biomass Carbon as a Function of Organic Carbon

Regression of C_{mic} on SOC showed a strong positive relationship between the two variables in both untreated and sludge amended soils (**Figure 5.5**). In each case the correlation was statistically significant (p <0.001) with the proportion of variance explained ranging from 68 % in soil receiving the undigested control, to 90 % in untreated soil (**Table 5.2**). The regression coefficients for sludge amended soil were lower than that observed for untreated soil, indicating a slight decrease in the quantity of C_{mic} produced per unit of SOC (**Table 5.2**); potentially due to the presence of heavy metals

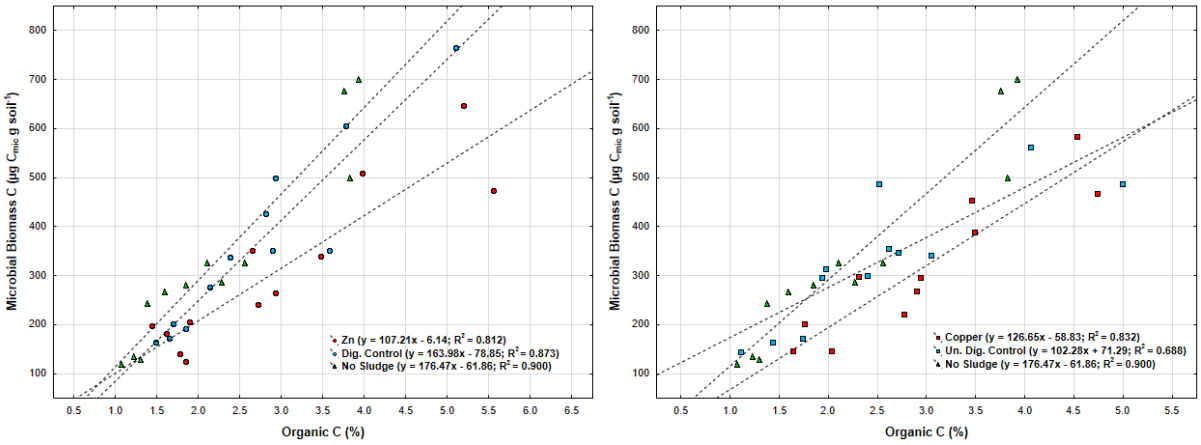


Figure 5.5:- Plot of microbial biomass carbon (µg C_{mic} g $soil^{-1}$) as a function of soil organic carbon (%). Data represents mean values of microbial biomass (2013-2014) and soil organic carbon (2012-2014) measured over the course of the current investigation in each of the samples taken from the LTSE field sites.

Table 5.2:- Regression and correlation coefficients from regression analysis of microbial biomass carbon on soil organic carbon.

	Regression of Biomass C on Organic C					
Sludge Treatment	**Slope (Org. C)**	***p***	**Intercept**	***p***	**R**	**$R^{2[2]}$**
Zinc (Zn)	107.21 (6.97)[1]	<0.001	-6.14 (49.30)	0.903	0.911	**0.812**
Digested Control (Ctrl1)	163.98 (18.71)	<0.001	-78.85 (53.82)	0.174	0.941	**0.873**
Copper (Cu)	126.65 (17.79)	<0.001	-58.83 (55.48)	0.317	0.921	**0.832**
Undigested Control (Ctrl2)	102.28 (20.37)	<0.001	71.29 (56.02)	0.232	0.846	**0.688**
No Sludge (NS)	176.47 (17.65)	<0.001	-61.86 (43.33)	0.184	0.953	**0.900**

[1] Values in parenthesis are standard error (n = 12). [2]Values are adjusted R^2.

within the applied sludge treatments (**Table 3.2**). In addition, the regression coefficient for soil receiving the Zn sludge treatment (slope = 107.21 ± 6.97) was lower than that seen for soil receiving the digested control (slope = 163.98 ± 18.71) which also indicates the Zn sludge treatment has had greater impact on C_{mic}. Whereas, in contrast, a higher regression coefficient seen for soils receiving the Cu sludge treatment (slope = 126.65 ± 17.79) in comparison to soil receiving the undigested control (slope = 102.28 ± 20.37). These results are in agreement with the meta-analysis above (**See Section 5.2.3**), however, it was not possible to include the total concentrations of Zn and Cu in the regression model due to multicollinearity with SOC (**See Section 3.5.4**). In general, total metal concentrations (**See Section 3.4**) and SOC content (**See Section 2.7**) increased in proportion across the four LTSE field sites chosen for the current investigation, with both the highest values for total metal concentration and SOC content seen at Hartwood and the lowest values seen at Woburn. Therefore it was not possible to determine the effect of heavy metal contamination using multiple regression analysis.

5.2.5. Sludge Incubation Experiment Method

In order to investigate the long term impact of heavy metal contamination on the proliferation of soil microbial biomass and phosphatase enzyme synthesis, an incubation experiment was established

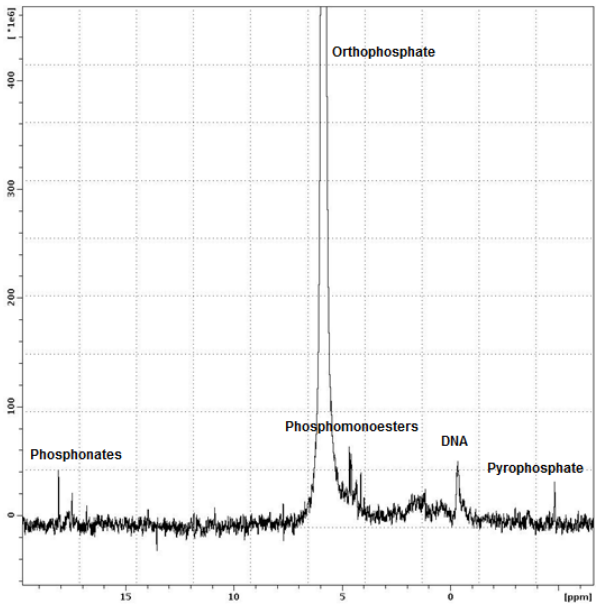


Figure 5.6:- ^{31}P-NMR spectra of liquid sludge amendment added to incubated soil samples from each of the LTSE field sites as part of an investigation into microbial response (See Sections 5.2.6 and 6.5).

whereby the response of soil microorganisms to the application of fresh phosphodiester compounds (DNA) was monitored over the course of three months. Approximately 300 g (dry matter basis) of field moist soil, sampled in 2014, was weighed out, sieved (<4 mm), and thoroughly mixed with 20-25 mL of anaerobically digested liquid sludge. Each sample was divided into five 60 g (dry matter basis) aliquots and stored in glass jars, sealed with perforated Parafilm M® to allow air flow. Samples were then incubated at 20°C, for <24 hours, 2 weeks, 1 month, 2 months, and 3 months. Moisture content was maintained by weighing each sample once a week and replenishing the amount of water lost by evaporation (1 g = 1 mL H_2O). Samples were removed at the specified time points and analysed for microbial biomass carbon (incubated samples were analysed for C_{mic} as previously described (**See Section 5.2.1**)) and the activities of acid phosphomonoesterase and phosphodiesterase (**See Section 6.5**). The organic phosphorus content of the applied sludge was determined by ^{31}P-NMR (**Figure 5.6**). However, it was not possible to detect the DNA signal present in the liquid sludge spectra once applied to each soil as the dilution of DNA compounds in the sludge amended soil was too great (Craven, Personal Communication). Therefore the relative rate of the phosphodiester mineralisation in contaminated and uncontaminated soils could not be determined. The changes in C_{mic} observed over the course of the incubation study are shown in **Figure 5.7** and **Table 5.3**.

5.2.6. Change in Microbial Biomass Carbon Results: Incubation Study (2014)

Auchincruive

A steady decline in C_{mic} was observed in each of the soils sampled from the Auchincruive field site over the course of 1 month following a fresh application of liquid sludge (**Figure 5.7**), and, with the

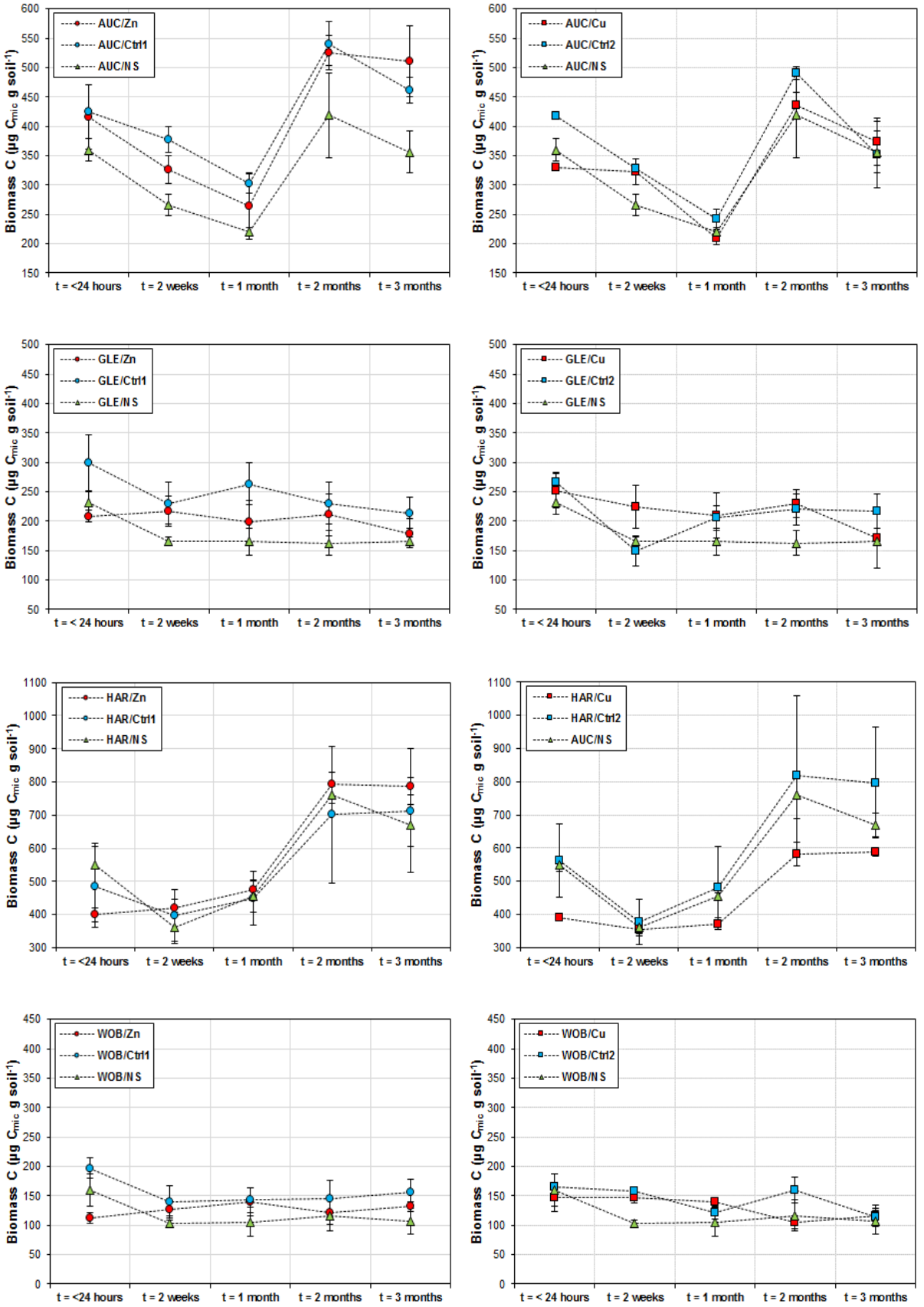


Figure 5.7:- Changes in soil microbial biomass carbon (µg C_{mic} g $soil^{-1}$) following a fresh application of liquid sludge (See Section 5.2.5). Samples of soil receiving digested (Zn and Ctrl1; left) and undigested (Cu and Ctrl2; right) sludge treatments, plus untreated soil (NS), were taken from each of the LTSE field sites and incubated at 20°C for 3 months. Error bars represent standard error (n = 3).

Table 5.3:- Changes in soil microbial biomass carbon (μg C_{mic} g $soil^{-1}$) measured over the course of 3 months following a fresh application of liquid sludge (See Section 5.2.5). Samples were incubated at 20ºC.

	Microbial Biomass Carbon (μg C_{mic} g $soil^{-1}$)				
Sludge Treatment	**<24 Hours**	**2 Weeks**	**1 Month**	**2 Months**	**3 Months**
AUC/Zn	415.75 (55.38)$^{a[1][2]}$	326.65 (24.25)^{ab}	264.16 (57.25)^{a}	524.85 (29.18)^{a}	510.54 (60.59)^{a}
AUC/Ctrl1	424.25 (2.63)^{a}	376.83 (22.18)^{b}	302.20 (16.78)^{a}	540.37 (37.27)^{a}	461.48 (22.63)^{ab}
AUC/Cu	329.75 (4.90)^{b}	322.34 (21.36)^{cd}	209.20 (10.61)^{b}	436.20 (21.26)^{b}	373.84 (40.97)^{c}
AUC/Ctrl2	418.49 (1.36)^{c}	327.76 (3.71)^{c}	241.59 (17.69)^{b}	490.65 (10.76)^{b}	352.52 (56.58)^{c}
AUC/NS	359.47 (19.06)^{ab}	265.60 (18.39)^{ac}	220.79 (7.11)^{ab}	419.07 (72.31)^{ab}	356.35 (35.49)^{bc}
GLE/Zn	208.44 (9.99)^{a}	216.60 (25.73)^{a}	199.33 (35.39)^{a}	210.82 (35.86)^{a}	179.60 (25.09)^{a}
GLE/Ctrl1	298.92 (48.03)^{a}	230.30 (36.00)^{a}	263.70 (35.07)^{a}	230.58 (36.07)^{a}	214.36 (26.68)^{a}
GLE/Cu	251.22 (29.13)^{b}	224.61 (36.72)^{b}	209.88 (38.86)^{b}	229.98 (23.21)^{b}	170.93 (51.09)^{b}
GLE/Ctrl2	266.15 (17.24)^{b}	149.87 (25.09)^{b}	205.35 (20.71)^{b}	220.42 (26.37)^{b}	217.07 (29.02)^{b}
GLE/NS	231.48 (19.94)^{ab}	166.16 (6.66)^{ab}	165.24 (23.04)^{ab}	162.60 (21.21)^{ab}	165.37 (7.44)^{ab}
HAR/Zn	400.40 (21.28)^{a}	421.48 (24.00)^{a}	476.94 (23.90)^{a}	793.24 (37.32)^{a}	787.63 (24.93)^{a}
HAR/Ctrl1	483.47 (120.29)^{a}	396.89 (78.67)^{a}	449.76 (81.35)^{a}	701.25 (206.36)^{a}	714.02 (187.26)^{a}
HAR/Cu	390.25 (5.49)^{b}	355.31 (20.93)^{b}	371.88 (18.85)^{b}	580.55 (35.98)^{b}	586.91 (12.39)^{b}
HAR/Ctrl2	561.41 (110.13)^{b}	377.99 (68.74)^{b}	479.99 (124.13)^{b}	817.48 (241.90)^{b}	797.54 (167.64)^{b}
HAR/NS	550.41 (63.29)^{ab}	360.34 (47.35)^{ab}	456.32 (49.75)^{ab}	760.90 (25.72)^{ab}	668.65 (62.16)^{ab}
WOB/Zn	112.77 (8.92)^{a}	127.45 (11.54)^{a}	139.63 (23.62)^{a}	122.00 (21.50)^{a}	131.74 (8.84)^{a}
WOB/Ctrl1	197.20 (17.56)^{b}	139.60 (26.88)^{a}	142.59 (21.00)^{a}	145.40 (31.40)^{a}	156.16 (21.45)^{a}
WOB/Cu	147.79 (25.18)^{c}	147.49 (10.10)^{b}	139.16 (8.00)^{b}	105.58 (11.09)^{b}	115.95 (18.73)^{b}
WOB/Ctrl2	166.07 (21.18)^{c}	158.63 (5.93)^{b}	121.69 (10.75)^{b}	159.67 (22.39)^{b}	113.90 (10.76)^{b}
WOB/NS	159.23 (27.23)^{abc}	103.33 (5.35)^{ac}	105.74 (25.30)^{ab}	116.57 (26.33)^{ab}	106.90 (21.52)^{ab}

[1]Values in parenthesis are standard error (n = 3). [2]Values without corresponding letters denotes statistical significance at α = 0.05 for individual sampling events, note comparisons between different sludge types (i.e. digested (Zn and Ctrl1) and undigested (Cu and Ctrl2)) have not been made.

exception of soil receiving the Zn sludge treatment, was significantly (p <0.01) lower at 1 month than at the start of the incubation period. At this point no significant differences in C_{mic} were observed between any of the soils (**Table 5.3**). A subsequent increase in C_{mic} was observed in all cases after a period of 2 months (**Figure 5.7**) with the highest concentrations of C_{mic} observed in soils receiving the Zn sludge treatment (524.85 μg C_{mic} g $soil^{-1}$) and digested control (540.37 μg C_{mic} g $soil^{-1}$), however no significant differences in C_{mic} were observed between these soils (**Table 5.3**); nor were any significant differences observed between soils receiving the undigested sludge treatments (Cu and Ctrl2) and untreated soil at this point. Microbial biomass C subsequently declined in all soils (**Figure 5.7**), and was significantly lower at 3 months, in comparison to the previous month, in soils receiving the digested (p <0.05) and undigested (p <0.01) controls. At the end of the incubation period C_{mic} was significantly (p <0.05) higher in soil receiving the Zn sludge treatment (510.54 μg C_{mic} g $soil^{-1}$) in comparison to the untreated soil (356.34 μg C_{mic} g $soil^{-1}$), whereas no significant differences were observed between the remaining soils; nor in any case was C_{mic} at 3 months significantly different than at the start of the incubation period.

Gleadthorpe

Despite significant (p <0.05) decreases in C_{mic} after 2 weeks, in untreated soil and soil receiving the undigested control, no significant changes in C_{mic} were observed over the course of the incubation period (**Figure 5.7**), nor were any significant differences in C_{mic} observed between untreated soil or any of the sludge amended soils (**Table 5.3**).

Hartwood

Similarly to Auchincruive, an initial decrease in C_{mic} was observed over the first 2 weeks of the incubation period in all soils at Hartwood, with the exception of soil receiving the Zn sludge treatment (**Figure 5.7**); however this was only statistically significant in the untreated soil (p <0.05). After 1 month C_{mic} had begun to increase in all soils, however in no instance was C_{mic} at 1 month significantly higher than at either of the previous time points. A dramatic increase in C_{mic} occurred in all soils following 2 months of incubation (**Figure 5.7**), with statistically significant increases in C_{mic}, in comparison to the previous month, observed in soils receiving the Zn (793.24 µg C_{mic} g soil^{-1}; p <0.001) and Cu (580.55 µg C_{mic} g soil^{-1}; p <0.001) sludge treatments, as well as in the untreated soil (770.97 µg C_{mic} g soil^{-1}; p <0.01). However, similar mean values for C_{mic} in soils receiving the digested (701.24 µg C_{mic} g soil^{-1}) and undigested (817.48 µg C_{mic} g soil^{-1}) controls, no significant difference was observed due to large variation between incubated samples (**Table 5.3**). No significant change was observed in any of the soils at 3 months, in comparison to the previous month, though C_{mic} in soils receiving the Zn (787.24 µg C_{mic} g soil^{-1}) and Cu (586.91 µg C_{mic} g soil^{-1}) sludge treatments were significantly (p <0.001) higher than at the start of the incubation period. At no point during the incubation period were any significant differences in C_{mic} observed between any of the sludge amended or untreated soils (**Table 5.3**), however it should be noted that C_{mic} in soil receiving the Cu sludge treatment was persistently lower in comparison to soil receiving the undigested control for the duration of the incubation period (**Figure 5.6**).

Woburn

Apart from C_{mic} in soil receiving the undigested control being significantly (p <0.05) lower at the end of the incubation period (113.90 µg C_{mic} g soil^{-1}) than at the start (166.07 µg C_{mic} g soil^{-1}), no significant changes in C_{mic} were observed in any of the soils over the course of three months (**Figure 5.6**).

In comparison to soil receiving the digested control (197.20 µg C_{mic} g soil^{-1}), C_{mic} was only significantly (p <0.05) lower in soil receiving the Zn sludge treatment (112.77 µg C_{mic} g soil^{-1}) at the start of the incubation period (**Table 5.3**). Whereas, although C_{mic} in soil receiving the Cu sludge treatment (147.49 µg C_{mic} g soil^{-1}) and undigested control (158.63 µg C_{mic} g soil^{-1}) were significantly (p <0.01) higher in comparison to untreated soil (103.33 µg C_{mic} g soil^{-1}) after 2 weeks, no significant differences in C_{mic} were observed between the two soils over the course of the incubation period (**Table 5.3**).

5.2.7. Change in Microbial Biomass Carbon Overview: Incubation Study (2014)

A distinct difference in the response of C_{mic} to a fresh application of sewage sludge was observed between the Scottish and English sites, with significant increases in C_{mic} observed at both of the Scottish

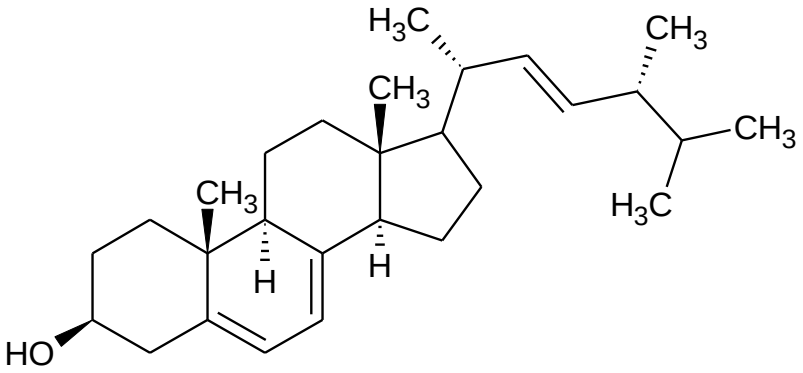


Figure 5.8:- Chemical structure of the fungal biomarker Ergosterol.

field sites after a period of 2 months (**Figure 5.7**); whereas, in general, no change in C_{mic} was observed for the English sites over the course of the incubation period. This difference in response is likely to be due to the differences the in moisture content and water holding capacity of the untreated and sludge amended soils at each site. In each case, the soil moisture content was significantly higher, during the 2014 sampling event, in samples collected at the Scottish sites in comparison to the English sites (**See Section 2.5**). Hence the incubated samples from the Scottish field sites contained more water at the start of the incubation period which could have potentially led to a priming effect, as the bioavailability of labile organic matter within the applied sewage sludge would be increased, thereby promoting microbial activity and the proliferation of C_{mic} (Fontaine et al., 2003; Marschner & Kalbitz, 2003). In addition, it should be noted that the moisture content of the incubated samples was only replenished once per week, hence the moisture content for each of the soils is likely to have fluctuated over the course of the incubation period. The drying and rewetting of soils can cause significant decreases in the rate of organic matter mineralisation, particularly C and N (Fierer et al., 2002; Rodrigo et al., 1997), and soil microbial biomass (Van Gestel et al., 1993) within the soil environment. Therefore since the incubated samples collected from the Scottish field sites had both a higher SOC content (**See Section 2.7**), as well as a higher clay content (**Table 2.1**), in comparison to the English field sites, it is likely that due to a greater water holding capacity the moisture content of the Scottish sites has remained relatively stable over the course of the incubation period. In contrast, samples from the English field site are likely to have undergone frequent drying and rewetting, which could have inhibited, or disrupted, the microbial response. Nevertheless, it is interesting to note that a similar response was observed for the two Scottish sites with no apparent interference from the presence of Zn or Cu.

5.3. Ergosterol

Found predominantly in the membranes of fungal species (Weete, 1989), ergosterol is a sterol compound (**Figure 5.8**) frequently used as a biomarker to determine the size of fungal biomass in a range of environmental samples (Djajakirana et al., 1996; Ruzicka et al., 2000; Seitz et al., 1979; Stahl & Parkin, 1996; Wallander et al., 2013). The analysis of ergosterol can therefore be used to determine

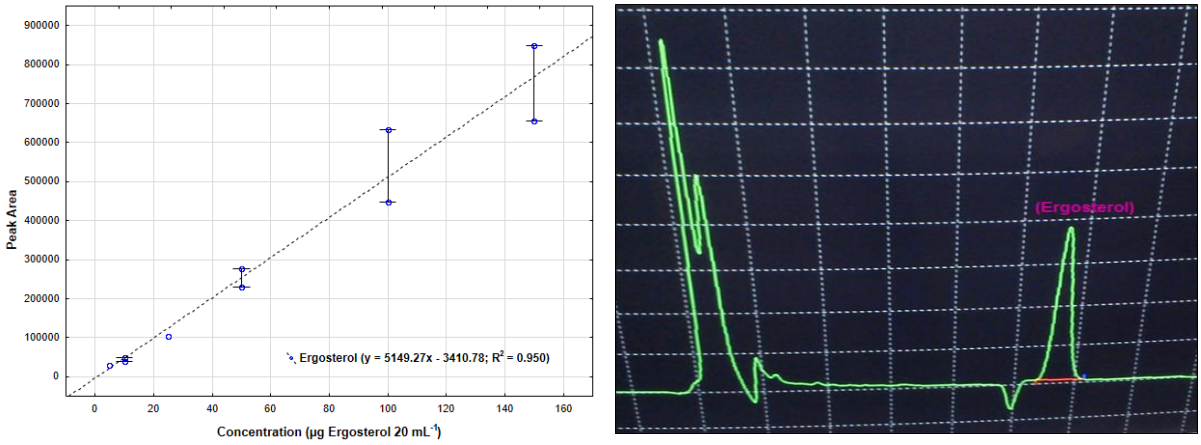


Figure 5.9:- Calibration graph (left) used to determine ergosterol concentration (µg Ergosterol 20 mL^{-1}) by UV/visible spectroscopy following separation by HPLC. Chromatogram peak area (right) is proportional to ergosterol concentration. Error bars represent standard error (n = 2).

the proportion of soil microbial biomass, within samples from the LTSE field sites, that is comprised of fungal species. Any significant changes in the proportion of fungal biomass, in soils receiving the Zn and Cu sludge treatment, will indicate whether the presence of Zn and Cu has caused a lasting change in microbial community structure.

5.3.1. Ergosterol Method

Ergosterol was determined using a method of ultrasonic extraction based on that described by Ruzicka et al. (1995). Approximately 5 g of freeze dried soil was weighed out in duplicate, rehydrated by adding 2 mL of deionised water and allowed to stand for 15 minutes. One of the duplicates was spiked with 1 mL of 2.5 mM ergosterol (100 µg mL^{-1}) standard solution and allowed to stand for a further 15 minutes. Each sample was then suspended in 10 mL of methanol:ethanol (4:1; v/v) and stored at 4°C for ≤ 2 hours. An additional 20 mL (19 mL for spiked samples) of n-hexane:2-propanol (98:2, v/v) was added to each sample before sonicating for approximately 3.5 minutes. A 2mL aliquot of the upper organic layer was then centrifuged at 7000 RCF for 10 minutes and transferred to a clean HPLC vial for analysis.

Ergosterol was analysed using an LC-10AD Liquid Chromatograph (Shimadzu) running CLASS-VP software (Version 6.10). Samples (100 µL) were passed through a LiChrosorb® Si-60 (Phenomenex) HPLC column (150 mm, 4.6 mm ID, 10 µm particle size) by n-hexane:2-propanol (98:2; v/v) mobile phase (1.5 mL min^{-1}) and ergosterol detected by UV absorbance at 282 nm (SPD-10A UV-Vis Detector, Shimadzu). The concentration of ergosterol in spiked and unspiked samples was calculated by referecnce to a calibration graph (**Figure 5.9**). The percentage of the ergosterol internal standard recovered from the spiked samples was then used to adjust the concentrations of ergosterol observed in the unspiked soil samples as follow:

$$Recovery_{\%} = \left(\frac{[Erg._{spiked}] - [Erg._{soil}]}{[Erg._{added}]}\right) \quad (\mathbf{E.5.3})$$

$$[Erg.] = \frac{[Erg._{Soil}]}{m} \times \frac{1}{Recovery_{\%}} \quad (\mathbf{E.5.4})$$

where [Erg.$_{spiked}$] and [Erg.$_{soil}$] are the concentrations of ergosterol (μg Ergosterol 20 mL^{-1}) measured in spiked and unspiked soil samples, respectively, [Erg.$_{added}$] is the mass of ergosterol added as an internal standard (100 μg), and *m* is the mass (g) of unspiked soil used for analysis. Ergosterol concentration was then used to estimate the size of fungal biomass carbon (C_{fungi}) in each sample as follows:

$$C_{fungi} = [Erg.] \times K_f \times F_F \quad (\mathbf{E.5.5})$$

where, K_f and F_F are conversion factors equal to 250 mg fungal biomass μg Ergosterol^{-1} and 0.43 (the average ratio of C_{fungi} to fungal biomass), respectively (Montgomery et al., 2000). **Table 5.4** shows the concentrations of ergosterol and C_{fungi} measured over two consecutive years, 2013 (2012 for Woburn) and 2014, in each of the soils sampled from the LTSE field sites.

5.3.2. Ergosterol Results (2012-2014)

Auchincruive

No significant differences in the concentration of ergosterol or C_{fungi} were observed between sludge amended and untreated soils at the Auchincruive field site at any time during the current investigation (**Table 5.4**). With the exception of soil receiving the Zn sludge treatment in 2013, the concentration of ergosterol and C_{fungi} was greater in soils receiving the Zn and Cu sludge treatments in comparison to their respective controls, however at no point were the differences statistically significant (**Table 5.4**).

In 2013, C_{fungi} accounted for approximately 25-27 % and 18-22 % of total C_{mic} in the soils receiving the digested and undigested sludge treatments, respectively, and approximately 17 % in untreated soil. This percentage decreased to approximately 14 % in soils receiving the digested and undigested controls in 2014, however the decrease was not statistically significant, nor were the values significantly different in comparison to untreated soil or the respective contaminated soils (**Table 5.4**).

Gleadthorpe

In comparison to untreated soil (73.08 ± 10.54 μg C_{fungi} g soil^{-1}), the concentration of ergosterol and C_{fungi} in soils receiving the Zn (185.62 ± 35.62 μg C_{fungi} g soil^{-1}) and Cu (154.43 ± 28.11 μg C_{fungi} g soil^{-1}) sludge treatments were significantly higher (p <0.05) at Gleadthorpe in 2013. These values were also higher than the respective uncontaminated controls (Ctrl1 (133.90 ± 17.61 μg C_{fungi} g soil^{-1}) and Ctrl2 (124.09 ± 25.09 μg C_{fungi} g soil^{-1}) but were not significantly different in comparison

Table 5.4:- Concentrations of ergosterol fungal biomarker (µg Ergosterol g soil^{-1}), and fungal biomass carbon (µg C_{fungi} g soil^{-1}) measured over two consecutive years (2013 and 2014) at the Long-Term Sludge Experiment field sites.

	2013[1]			2014		
Sludge Treatment	**Ergosterol (µg g soil^{-1})**	**Fungal Biomass (µg C_{fungi} g soil^{-1})**	**Percent C_{mic} (%)**	**Ergosterol (µg g soil^{-1})**	**Fungal Biomass (µg C_{fungi} g soil^{-1})**	**Percent C_{mic} (%)**
AUC/Zn	0.81 (0.09)$^{a[2][3]}$	86.69 (9.31)^{a}	25.33 (3.28)^{a}	0.64 (0.09)^{a}	69.02 (10.17)^{a}	25.58 (7.59)^{a}
AUC/Ctrl1	1.22 (0.56)^{a}	130.78 (60.23)^{a}	27.54 (8.40)^{a}	0.42 (0.07)^{a}	44.62 (7.33)^{a}	14.04 (1.99)^{a}
AUC/Cu	0.81 (0.10)^{b}	86.94 (10.98)^{b}	22.65 (2.14)^{b}	0.56 (0.06)^{b}	60.46 (6.08)^{b}	22.90 (3.62)^{b}
AUC/Ctrl2	0.67 (0.11)^{b}	72.18 (11.76)^{b}	18.71 (3.28)^{b}	0.40 (0.09)^{b}	43.50 (10.15)^{b}	14.07 (3.20)^{b}
AUC/NS	0.53 (0.02)^{ab}	57.26 (1.77)^{ab}	16.89 (0.78)^{ab}	0.43 (0.06)^{ab}	46.29 (6.98)^{ab}	16.42 (2.80)^{ab}
GLE/Zn	1.73 (0.33)^{a}	185.62 (35.62)^{a}	87.59 (13.24)^{a}	1.91 (0.24)^{a}	205.17 (25.94)^{a}	95.57 (18.32)^{a}
GLE/Ctrl1	1.25 (0.16)^{ab}	133.90 (17.61)^{ab}	49.00 (5.53)^{b}	2.10 (1.14)^{a}	225.58 (122.44)^{a}	74.87 (28.54)^{a}
GLE/Cu	1.44 (0.26)^{c}	154.43 (28.11)^{c}	61.86 (10.34)^{c}	1.90 (0.50)^{b}	204.26 (53.66)^{b}	92.42 (13.96)^{b}
GLE/Ctrl2	1.15 (0.23)^{cd}	124.09 (25.09)^{cd}	36.08 (7.54)^{cd}	1.16 (0.13)^{b}	124.73 (14.41)^{b}	47.40 (3.95)^{b}
GLE/NS	0.68 (0.10)^{bd}	73.08 (10.54)^{bd}	22.55 (3.49)^{bd}	1.61 (0.38)^{ab}	173.11 (40.60)^{ab}	89.45 (22.79)^{ab}
HAR/Zn	1.31 (0.39)^{a}	141.17 (41.47)^{a}	24.33 (4.78)^{a}	2.19 (0.52)^{a}	235.89 (56.25)^{a}	43.67 (5.72)^{a}
HAR/Ctrl1	1.35 (0.39)^{a}	144.89 (41.70)^{a}	21.39 (3.94)^{a}	0.96 (0.25)^{b}	102.94 (27.02)^{b}	16.93 (2.40)^{b}
HAR/Cu	1.33 (0.28)^{b}	142.65 (29.75)^{b}	29.58 (5.76)^{b}	1.14 (0.30)^{c}	122.03 (32.01)^{c}	23.92 (7.42)^{c}
HAR/Ctrl2	1.17 (0.30)^{b}	125.46 (32.43)^{b}	36.59 (20.43)^{b}	1.54 (0.60)^{c}	165.15 (64.53)^{c}	27.66 (6.34)^{c}
HAR/NS	1.05 (0.36)^{ab}	112.81 (38.51)^{ab}	18.41 (5.50)^{ab}	0.64 (0.01)^{bc}	68.68 (1.08)^{bc}	10.95 (0.81)^{bc}
WOB/Zn	1.41 (0.08)^{a}	151.67 (8.29)^{a}	N/A[4]	1.16 (0.35)^{a}	125.08 (37.68)^{a}	85.09 (23.86)^{a}
WOB/Ctrl1	1.19 (0.13)^{a}	127.63 (14.39)^{a}	N/A	0.77 (0.09)^{a}	82.78 (9.78)^{a}	53.30 (8.92)^{a}
WOB/Cu	1.45 (0.11)^{b}	156.17 (12.18)^{b}	N/A	0.99 (0.02)^{b}	106.70 (2.67)^{b}	78.73 (7.40)^{b}
WOB/Ctrl2	0.97 (0.07)^{c}	103.90 (7.41)^{c}	N/A	0.78 (0.08)^{c}	83.68 (8.75)^{c}	52.11 (7.28)^{b}
WOB/NS	1.44 (0.10)^{ab}	154.31 (10.29)^{ab}	N/A	0.66 (0.04)^{ac}	71.05 (3.96)^{ac}	58.60 (5.85)^{ab}

[1]Ergosterol data for the Woburn field site is for year 2012. [2]Values in parenthesis are standard errors (n = 3). [3] Values without corresponding letters denotes statistical significance at α = 0.05 for individual sampling events, note comparisons between different sludge types (i.e. digested (Zn and Ctrl1) and undigested (Cu and Ctrl2)) have not been made. [4]Not Applicable.

(**Table 5.4**). No significant differences in ergosterol and C_{fungi} content were observed between soils receiving digested and undigested sludge treatments in 2014, nor were any significant differences seen in comparison to untreated soil. However C_{fungi} in soil receiving the Cu sludge treatment remained higher in comparison to the undigested control, whereas C_{fungi} in soil receiving the Zn sludge treatment was now lower than in the digested control (**Table 5.4**).

As a percentage of C_{mic}, C_{fungi} in soil receiving the Zn (88 %) sludge treatment was significantly higher in comparison to the untreated soil (23 %; p <0.01) and soil receiving the digested control (49 %; p <0.05) in 2013. Whereas C_{fungi} in soil receiving the Cu (62 %) sludge treatment was only significantly (p <0.05) higher than the untreated soil; though the percentage of C_{mic} comprised of C_{fungi} was greater in comparison to soil receiving the undigested control (36 %). No significant differences in C_{fungi}, as a percentage of C_{mic}, were observed between soils receiving corresponding sludge types or untreated soil in 2014 (**Table 5.4**); though again the percentage of C_{mic} comprised of C_{fungi} was highest in soils receiving the Zn and Cu sludge treatments.

Hartwood

At Hartwood, the concentration of ergosterol and C_{fungi} was significantly (p <0.05) higher in soil receiving the Zn sludge treatment (235.89 ± 56.25 µg C_{fungi} g soil^{-1}) in 2014, in comparison to both

untreated soil (68.68 ± 1.08 μg C_{fungi} g $soil^{-1}$) and soil receiving the digested control (102.94 ± 27.02 μg C_{fungi} g $soil^{-1}$). For the duration of the current investigation fungal biomass was higher in the sludge amended soils in comparison to the untreated soil, however no other significant differences were observed (**Table 5.4**).

Similarly, the percentage of C_{mic} comprised of C_{fungi} was significantly higher in soil receiving the Zn (44 %) sludge treatment in comparison to the untreated soil (11 %; p <0.001) and soil receiving the digested control (17 %; p <0.01) in 2014 (**Table 5.4**); this was also higher in 2013 though the difference was not significant. In contrast, C_{fungi} as a percentage of C_{mic} was lower in soil receiving the Cu sludge treatment, in comparison to soil receiving the undigested control for the duration of the current investigation, and in both cases was higher than in the untreated soil, however no significant differences were observed (**Table 5.4**).

Woburn

Although at the Woburn field site ergosterol and C_{fungi} were higher in soil receiving the Zn sludge treatment in comparison to soil receiving the digested control, no significant differences were observed between the two soils at any point during the current investigation, nor were they significantly different to that in the untreated soil (**Table 5.4**). However, in 2012, ergosterol and C_{fungi} were significantly (p <0.05) lower in soil receiving the undigested control (103.90 ± 7.41 μg C_{fungi} g $soil^{-1}$) in comparison to the untreated soil (154.31 ± 10.29 μg C_{fungi} g $soil^{-1}$) and soil receiving the Cu sludge treatment (156.17 ± 12.18 μg C_{fungi} g $soil^{-1}$). The concentration of ergosterol subsequently decreased in each of these soils in 2014, with C_{fungi} in the untreated soil now lower in comparison to soil receiving the undigested control, whereas C_{fungi} in soil receiving the Cu sludge treatment remained significantly (p <0.05) higher in comparison to both soils (**Table 5.4**).

For the Woburn field site, analysis of ergosterol was carried out on freeze dried samples collected in 2012, for which C_{mic} was not determined. Therefore it was not possible to calculate the percentage of C_{mic} comprised of C_{fungi} for the year 2012. However, similar to Gleadthorpe, the percentage of C_{mic} as C_{fungi} in 2014 was higher in the contaminated soils in comparison to both untreated soil and soils receiving the respective uncontaminated controls, though the differences were not statistically significant (**Table 5.4**).

5.3.2. Ergosterol Overview (2012-2014)

The overall mean values for C_{fungi} in untreated soil were highest at the English field sites, with the lowest value seen at Auchincruive (**Figure 5.10**); however no significant differences were found between any of the sites. In addition, the proportion of C_{mic} comprised of C_{fungi} appeared to be inversely proportional

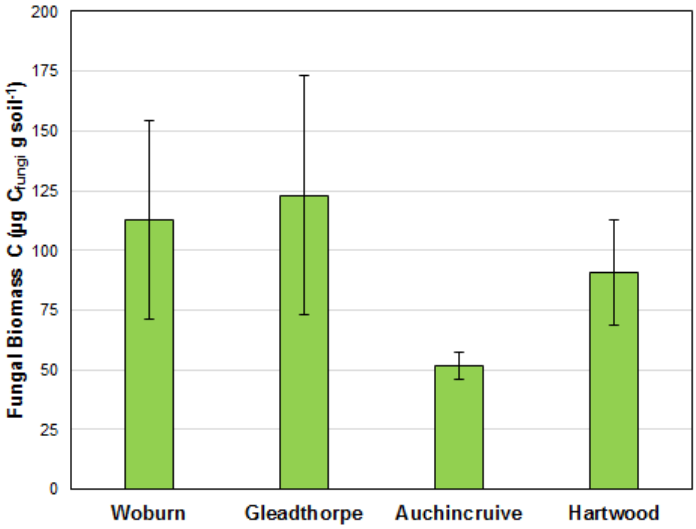


Figure 5.10:- Mean values for fungal biomass carbon (µg C_{fungi} g $soil^{-1}$) in untreated soil (NS) at each of the Long-Term Sludge Experiment field sites, over two consecutive years (2013-2014). Values for Woburn are for 2012 and 2014. Error bars represent standard error (n = 2).

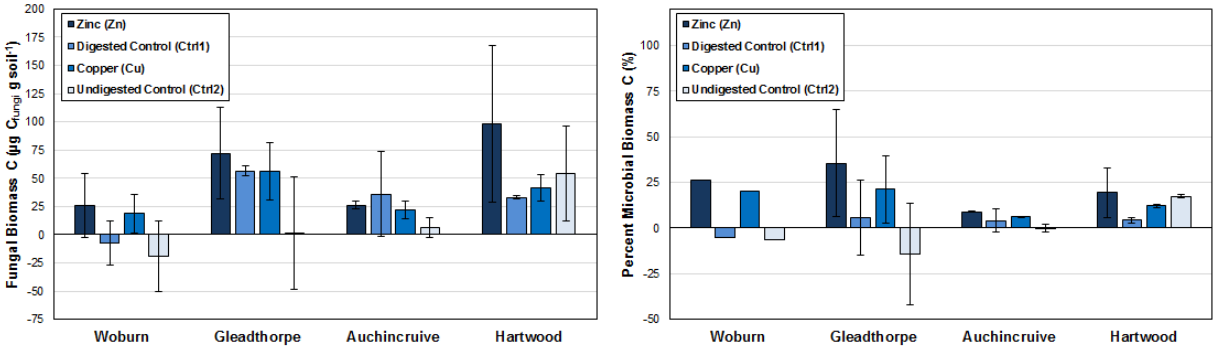


Figure 5.11:- Change in fungal biomass carbon (µg C_{fungi} g $soil^{-1}$; left) and percentage of C_{mic} comprised of C_{fungi} (right) in soils receiving digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments relative to the untreated soil at each of the Long-Term Sludge Experiment field sites. Values are mean fungal biomass carbon over two consecutive years (2013-2014). Values for Woburn are 2012 and 2014; percentage of C_{mic} comprised of C_{fungi} was not determined for Woburn samples collected in 2012. Error bars represent standard error (n = 2).

to SOC, with higher values at the English field sites where SOC was lowest:

HAR (14.7 ± 3.7 %) < AUC (16.7 ± 0.2 %) < GLE (56.0 ± 33.5 %) < WOB (58.6 %)

With the exception of soils receiving the undigested sludge treatments (Cu and Ctrl2) at Woburn, C_{fungi} in the sludge amended soils remained higher in comparison to the untreated soil at each of the LTSE sites (**Figure 5.11**); however no significant differenecs were observed between sites for any of the sludge amended soils. In addition, with the exception of soil receiving the Cu sludge treatments at Hartwood, application of the contaminated sludge treatments caused a greater increase in the percentage of C_{mic} comprised of C_{fungi} in comparison to the respective uncontaminated controls (**Figure 5.11**). This may be an indication that the presence of Zn and Cu has caused a decrease in bacterial species, hence C_{fungi} now accounts for a greater proportion of the total C_{mic} in soils receiving the contaminated sludge treatments.

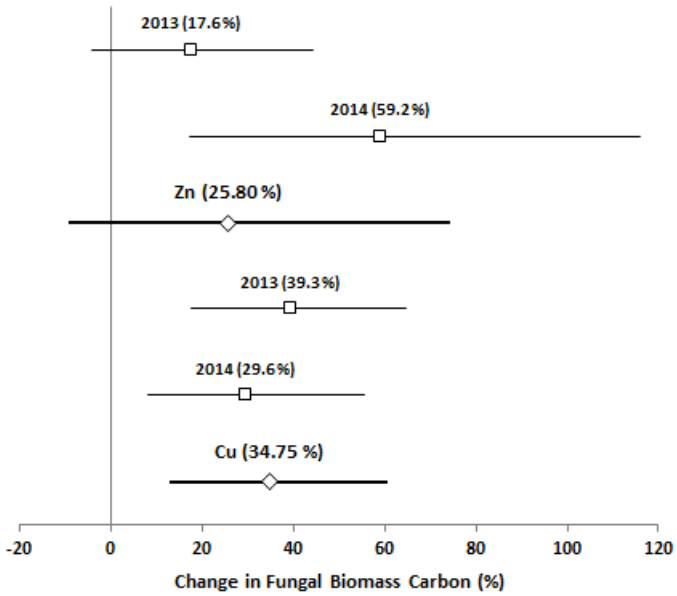


Figure 5.12:- Forest plot showing the change in fungal biomass carbon (µg C_{fungi} g $soil^{-1}$) in soils receiving the Zn and Cu sludge treatments, in comparison to uncontaminated controls (Ctrl1 and Ctrl2), across all four LTSE fields sites for years 2013 and 2014 (2013 group contains values for Woburn soil sampled in 2012). Horizontal lines represent 95 % confidence intervals. Effects are not statistically significant (p <0.05) if the 95 % confidence interval crosses the centre line.

Combining the results using meta-analysis (**See Section 2.4.2**) indicated that application of the contaminated sludge treatments caused an increase in C_{fungi} of approximately 25-35 % in comparison to the uncontaminated controls (**Figure 5.12**). These results are in contrast to the overall effect observed for C_{mic} (**See Section 5.2.3**) and again may be an indication that Zn and Cu are predominantly affecting bacterial species in the soil. Assuming Zn and Cu do not promote the growth of fungi, then the observed increase in C_{fungi} is possibly due to a lack of competition from bacteria in the contaminated soils. Therefore if fungal species are more resistant to heavy metal contamination the observed decreases in C_{mic} can be attributed to the loss of bacterial biomass from the contaminated soils. However, due to considerable variation in the detected quantities of ergosterol (**Table 5.4**), these conclusions should be made with caution.

5.4. Phospholipid Fatty Acids

The use of phospholipid fatty acids as a means of investigating the changes in microbial community structure in response to changing environmental factors, such as the presence of heavy metal contamination, has increased steadily over the past two decades (Frostegård et al., 2011; Zelles, 1999). Phospholipid fatty acids (PLFA) are essential structural components within the membrane of all living cells (Zelles, 1999). Microorganisms produce a wide range of PLFA compounds and in some cases specific PLFA compounds can be used to indicate the presence of certain microorganisms within the

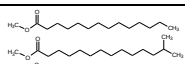
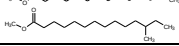
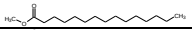
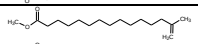
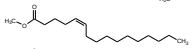
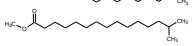
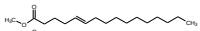
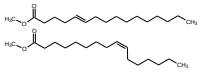
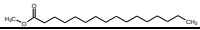
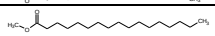
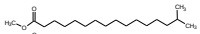
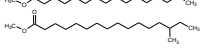
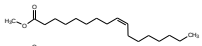
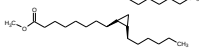
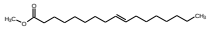
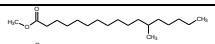
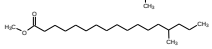
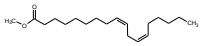
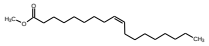
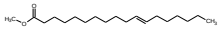
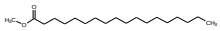
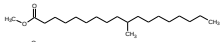
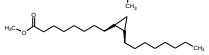
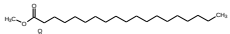
soil environment. However, in general, changes in the PLFA profile can only be considered qualitatively, as a means of identifying overall differences in the microbial community structure between samples (Frostegård et al., 2011; Zelles, 1999).

5.4.1. Phospholipid Fatty Acids Method

For the current investigation PLFA were determined using a method of lipid extraction based on that described by Frostegård et al. (1991), which in turn was based on the methods described by Bligh and Dyer (1952) and White et al. (1979). A monophasic mixture (1:2:0.8; v/v/v) of chloroform ($CHCl_3$), methanol (MeOH), and citrate buffer solution (0.15 M citric acid; 0.17 M trisodium citrate), known as 'Bligh and Dyer' solvent, was used to extract phospholipids from the cell membrane. Approximately 10 g of freeze dried soil sample was weighed into a glass vial and extracted with 20 mL of 'Bligh and Dyer' solvent. Samples were sonicated for 30 minutes, then centrifuged at 700 RCF for 10 minutes before decanting the supernatant into a clean glass vial. Extracts were then separated into organic and aqueous phases by adding 4 mL of $CHCl_3$, plus 4 mL of citrate buffer solution; samples were left to stand overnight to allow phase separation. Following separation, the upper aqueous layer was removed by vacuum pump, and the remaining organic phase evaporated to dryness under a stream of nitrogen (N_2). Approximately 0.5 g of sodium sulphate (Na_2SO_4) was added to a clean solid phase extraction (SPE) cartridge before conditioning with 2 mL each of MeOH, acetone, and $CHCl_3$; residual solvent was allowed to evaporate before adding a further 2 mL of $CHCl_3$. Lipid extracts were reconstituted in 1 mL $CHCl_3$, loaded onto the pre-conditioned SPE cartridges, and separated into neutral (sterols), glycol, and polar (incl. phospholipids) lipid fractions by elution with 5 mL $CHCl_3$, 12 mL acetone, and 8 mL MeOH, respectively. The polar lipid fraction was then evaporated to dryness under a stream of N_2. At this point 200 µL of 0.08 mM (5.12 µg 200 μL^{-1}) methyl nonadecaonate (**Table 5.5**) solution was added as an internal standard.

Phospholipids were derivatised to fatty acid methyl esters (FAME) by mild alkaline methanolysis (Dowling et al., 1986) prior to analysis by gas chromatography (GC). Polar lipid fractions were reconstituted in 1 mL of toluene:MeOH (1:1; v/v), before adding 1 mL of 0.2 M potassium hydroxide (KOH) solution. Samples were then incubated at 37°C and mechanically shaken to allow derivatisation to take place. Methylation was stopped after 30 minutes by the addition of 0.25 mL of 1 M acetic acid. Samples were then made up to approximately 10 mL final volume by adding 5 mL of hexane:$CHCl_3$ (4:1; v/v), followed by 3 mL of deionised water, before sonicating for 30 minutes and allowing organic and aqueous phases to separate overnight. The upper organic phase was filtered through glass wool topped with Na_2SO_4 and collected in a clean glass vial before evaporating to dryness under a stream of N_2. Samples were then reconstituted in 200 µL hexane and transferred to a GC vial for analysis.

Table 5.5:- Details of phospholipid fatty acid (PLFA) compounds detected in sludge amended and untreated soil at each of the LTSE field sites.

Peak	Ret. Time[1]	Name	Formula	Notation	Structure
1	0 minutes	Methyl Tetradecanoate	$C_{15}H_{30}O_2$	14:0	
2	+ 2.1 min	(13 Me.) Methyl Tetradecanoate	$C_{15}H_{30}O_2$	15:0i	
3	+ 2.4 min	(12 Me.) Methyl Tetradecanoate	$C_{15}H_{30}O_2$	15:0ai	
4	+ 3.5 min	Methyl Pentadecanoate	$C_{16}H_{32}O_2$	15:0	
5	+ 4.9 min	-	$C_{17}H_{32}O_2$	16:1i	
6	+ 5.7 min	Methyl-cis-5-Hexadecenoate	$C_{17}H_{32}O_2$	16:1ω11c	
7	+ 5.9 min	(14 Me.) Methyl Pentadecanoate	$C_{17}H_{34}O_2$	16:0i	
8	+ 6.3 min	Methyl-trans-5-Hexadecenoate	$C_{17}H_{32}O_2$	16:1ω11t	
9	+ 6.5 min	Methyl-cis-9-Hexadecenoate	$C_{17}H_{32}O_2$	16:1ω7c	
10	+ 6.9 min	-	$C_{17}H_{32}O_2$	16:1ω5	-
11	+ 7.3 min	Methyl Hexadecanoate	$C_{17}H_{34}O_2$	16:0	
12	+ 8.9 min	Methyl Heptadecanoate	$C_{18}H_{36}O_2$	17:0 isomer	
13	+ 9.1 min	Methyl Heptadecanoate	$C_{18}H_{36}O_2$	17:0 isomer 2	-
14	+ 9.4 min	(15 Me.) Methyl Hexadecanoate	$C_{18}H_{36}O_2$	17:0i	
15	+ 9.9 min	(14 Me.) Methyl Hexadecanoate	$C_{18}H_{36}O_2$	17:0ai	
16	+ 10.3 min	-	$C_{18}H_{36}O_2$	17:0br	-
17	+ 10.5 min	Methyl-cis-9-Heptadecenoate	$C_{18}H_{34}O_2$	17:1ω8c	
18	+ 10.9 min	Methyl-9,10-methylene-Hexadecanoate	$C_{18}H_{34}O_2$	cy17:0	
19	+ 11.2 min	Methyl-trans-9-Heptadecenoate	$C_{18}H_{34}O_2$	17:1ω8t	
20	+ 11.4 min	-	$C_{18}H_{34}O_2$	17:1ω7	-
21	+ 11.6 min	(12 Me.) Methyl Heptadecanoate	$C_{19}H_{38}O_2$	17:0 (12 Me)	
22	+ 13.2 min	(10 Me.) Methyl Heptadecanoate	$C_{19}H_{38}O_2$	17:0 (10 Me)	
23	+ 13.9 min	-	$C_{19}H_{32}O_2$	18:3(5,10,12)	-
24	+ 14.3 min	Methyl-cis-9,12-Octadecadienoate	$C_{19}H_{34}O_2$	18:2ω6,9c	
25	+ 14.6 min	Methyl-cis-9-Octadecenoate	$C_{19}H_{36}O_2$	18:1ω9c	
26	+ 14.8 min	Methyl-trans-11-Octadecenoate	$C_{19}H_{36}O_2$	18:1ω7t	
27	+ 15.3 min	-	$C_{19}H_{36}O_2$	18:1ω10 or 11	-
28	+ 15.7 min	Methyl Octadecanoate	$C_{19}H_{36}O_2$	18:0	
29	+ 16.0 min	-	$C_{20}H_{36}O_2$	19:1ω6	-
30	+ 17.3 min	(10 Me.) Methyl Octadecanoate	$C_{20}H_{38}O_2$	18:0 (10 Me)	
31	+ 19.4 min	Methyl-cis,9,10-methylene-Octadecanoate	$C_{20}H_{38}O_2$	cy19:0	
32	**+ 19.7 min**	**Methyl Nonadecanoate[2]**	**$C_{20}H_{40}O_2$**	**19:0**	
33	+ 23.1 min	Methyl-cis-11-Eicosenoate	$C_{21}H_{40}O_2$	20:1ω9c	
34	+ 23.4 min	-	$C_{21}H_{42}O_2$	20:01	-
35	+ 24.2 min	Methyl Eicosanoate	$C_{21}H_{42}O_2$	20:0	

[1]Retention times are given relative to that of Methyl Tetradecanoate (t = 18.5 minutes). [2]Methyl Nonadecanoate was used as an internal standard.

Fatty acid methyl ester compounds were analysed using an Agilent 6890N Network Gas Chromatograph running Agilent G2070 ChemStation software. Samples (1 µL) were loaded onto the GC column by splitless injection (310^{o}C) and passed through an HP-5 capilliary column (30 m, 0.32 mm ID, 0.25 µm film) by helium carrier gas (1 mL min^{-1}). Fatty acid methyl esters were then separated by temperature programe (50^{o}C for 1 minute, then increasing by 25^{o}C min^{-1} to 160^{o}C, followed by 2^{o}C min^{-1} to 240^{o}C and 25^{o}C to 310^{o}C) and detected using a flame ionisation detector (FID). Fatty acid methyl ester compounds were identified from chromatograms (**Figure 5.13**) by retention time (**Table 5.5**) and quantified (µg FAME g soil^{-1}) against the methyl nonadecanoate (19:0) internal standard, as follows:

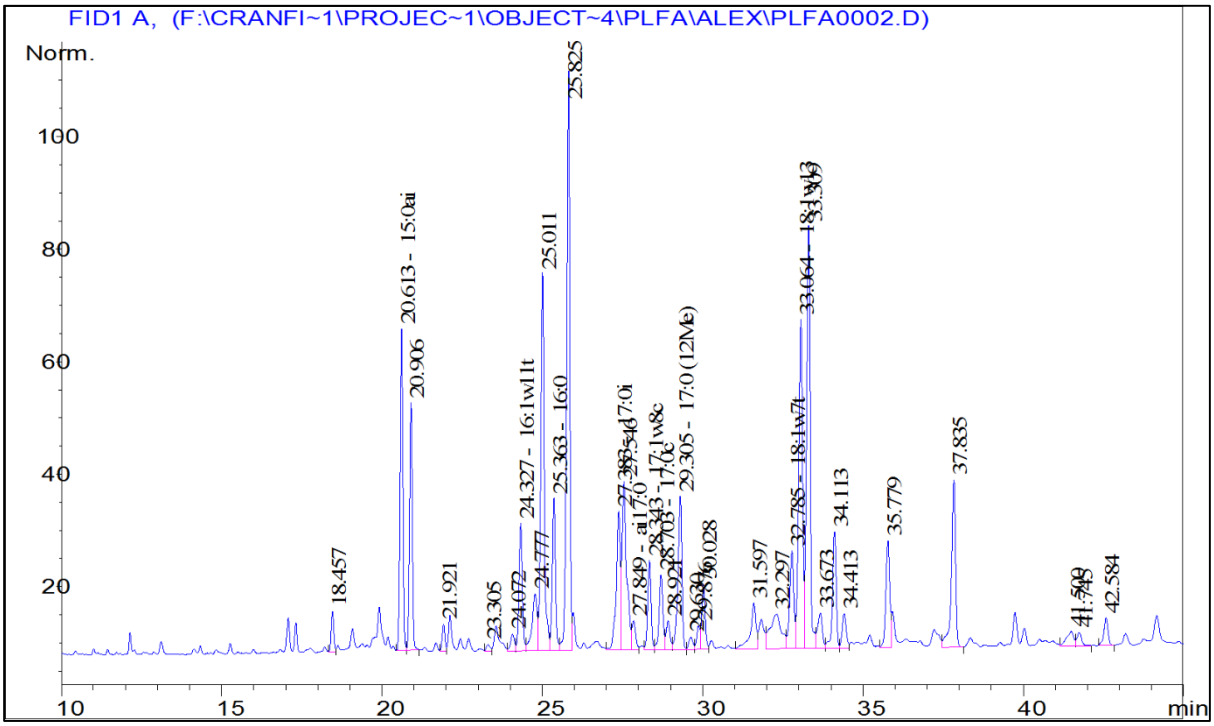


Figure 5.13:- Gas chromatogram showing phospholipid fatty acid (PLFA) peaks detected during the analysis of untreated and sludge amended soils collected from the LTSE field sites.

$$[FAME_n] = \frac{A_{FAME_n}}{A_{19:0}} \times \frac{m_{19:0}}{m} \qquad (\mathbf{E.5.6})$$

where A_{FAME_n} is the chromatogram peak area corresponding to the *n*th FAME (**Table 5.5**), $A_{19:0}$ and $m_{19:0}$ are the chromatogram peak area and the mass (µg) of methyl nonadecanoate internal standard, respectively, and *m* is the mass of soil (g) analysed. Phospholipid fatty acid analysis was carried out on samples collected from each of the LTSE field sites during years 2013 (with the exception of Woburn, where samples from 2012 were used) and 2014.

Phospholipid fatty acids are described using the following notation (Zelles, 1999):

$$C_n : n_{C=C}\ \omega_n\ x$$

where C_n is the fatty acid chain length, $n_{C=C}$ is the number of double bonds in the fatty acid chain, ω_n is the position of the double bond relative to the terminal C, and *x* denotes the stereochemistry of the double bond (i.e. *x* = c (cis), *x* = t (trans)). Branched fatty acids with a terminal methyl group are denoted by i (iso) or ai (anti-iso),according to stereochemistry, otherwise the position of methyl groups (nMe) are specified (e.g. (10Me)); unknown branced structures are denoted by br. Fatty acids containing a cyclopropane group are denoted cy. Notation for PLFA compounds is summaried in **Table 5.5**, however it should be noted that in some cases the position and sterochemistry of double bonds along the fatty acid chain have not been specified.

A total of 34 readily identifiable PLFA compounds (**Table 5.5**), representative of both bacterial and

Table 5.6:- Summary of statistically significant differences found between phospholipid fatty acid (PLFA) profiles along Principal Component Factor 1, Factor 2, and Factor 3, in soils receiving Zn and Cu sludge treatments, digested (Ctrl1) and undigested (Ctrl2) controls, and untreated soil (NS) at each of the LTSE field sites.

	2013[1]											
	PCA Factor 1				PCA Factor 2				PCA Factor 3			
AUC	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**
Ctrl1	0.412	-			**<0.05**	-			0.556	-		
Cu	0.966	0.436	-		**<0.05**	0.424	-		**<0.01**	**<0.01**	-	
Ctrl2	**<0.05**	0.161	**<0.05**	-	0.165	0.141	0.463	-	**<0.05**	0.075	0.255	-
NS	**<0.05**	0.142	**<0.05**	0.935	**<0.05**	0.433	0.986	0.453	**<0.05**	0.132	0.150	0.734
GLE	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**
Ctrl1	**<0.001**	-			0.466	-			0.430	-		
Cu	0.363	**<0.001**	-		0.167	0.481	-		**<0.05**	**<0.05**	-	
Ctrl2	**<0.001**	**<0.01**	**<0.001**	-	0.678	0.747	0.313	-	**<0.05**	**<0.01**	0.795	-
NS	**<0.001**	**<0.05**	**<0.001**	0.290	0.221	0.597	0.857	0.401	0.857	0.338	0.061	**<0.05**
HAR	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**
Ctrl1	0.232	-			**<0.05**	-			0.798	-		
Cu	0.877	0.183	-		0.394	0.140	-		0.304	0.431	-	
Ctrl2	0.480	0.601	0.393	-	**<0.01**	0.390	**<0.05**	-	0.616	0.804	0.584	-
NS	0.283	0.891	0.226	0.699	**<0.05**	0.613	0.059	0.715	0.091	0.059	**<0.05**	**<0.05**
WOB	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**
Ctrl1	0.391	-			0.238	-			0.151	-		
Cu	**<0.05**	**<0.01**	-		0.550	0.538	-		**<0.01**	0.091	-	
Ctrl2	0.645	0.068	**<0.01**	-	**<0.01**	0.070	**<0.05**	-	0.123	0.902	0.112	-
NS	**<0.01**	**<0.05**	**<0.001**	**<0.05**	0.248	0.978	0.557	0.066	**<0.001**	**<0.05**	0.266	**<0.05**
	2014											
	PCA Factor 1				PCA Factor 2				PCA Factor 3			
AUC	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**
Ctrl1	**<0.001**	-			0.468	-			0.930	-		
Cu	**<0.05**	**<0.01**	-		0.775	0.655	-		0.733	0.669	-	
Ctrl2	**<0.001**	**<0.05**	**<0.001**	-	0.493	0.173	0.338	-	0.823	0.893	0.575	-
NS	**<0.001**	**<0.05**	**<0.001**	0.553	0.081	0.264	0.131	**<0.05**	0.687	0.624	0.950	0.534
GLE	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**
Ctrl1	**<0.001**	-			0.762	-			0.489	-		
Cu	**<0.01**	**<0.001**	-		0.278	0.423	-		0.252	0.628	-	
Ctrl2	**<0.001**	0.341	**<0.001**	-	0.337	0.501	0.893	-	0.468	0.971	0.654	-
NS	**<0.001**	0.805	**<0.001**	0.239	0.102	0.167	0.527	0.446	0.400	0.876	0.742	0.905
HAR	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**
Ctrl1	**<0.01**	-			0.469	-			0.123	-		
Cu	0.581	**<0.05**	-		0.251	0.650	-		0.063	0.693	-	
Ctrl2	0.093	0.160	0.228	-	**<0.05**	0.087	0.184	-	0.675	0.239	0.129	-
NS	**<0.05**	0.350	0.098	0.602	**<0.01**	**<0.05**	0.062	0.519	0.613	0.273	0.148	0.930
WOB	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**	**Zn**	**Ctrl1**	**Cu**	**Ctrl2**
Ctrl1	**<0.05**	-			0.087	-			0.757	-		
Cu	0.509	**<0.01**	-		0.139	0.778	-		**<0.01**	**<0.05**	-	
Ctrl2	**<0.01**	0.377	**<0.01**	-	**<0.05**	0.254	0.165	-	0.918	0.836	**<0.01**	-
NS	**<0.001**	**<0.05**	**<0.001**	0.080	0.811	0.058	0.094	**<0.01**	**<0.01**	**<0.05**	0.822	**<0.05**

[1]PLFA data for the Woburn field site is for year 2012.

fungal species (Zelles, 1997, 1999; Frostegård & Bååth, 1996), were selected for principal component analysis (PCA) to determine changes in microbial community structure as a response to the application of contaminated sludge treatments (Zn and Cu). The concentration of each FAME was normalised and expressed in mol%, as follows:

$$[FAME_n]\ \text{mol\%} = \frac{A_{FAME_n}}{\sum A_{FAME_n}} \times 100 \qquad \textbf{(E. 5. 6)}$$

where A_{FAME_n} is the chromatogram peak area corresponding to the *n*th FAME as above, and $\sum A_{FAME_n}$ is the total sum of peak areas in each chromatogram (**Figure 5.13**). Principal component analysis was based on correlations between variables and was carried out using STATISTICA (64-bit) Version 12 (StatSoft Inc., 2014). Mean factor scores obtained for PCA Factor 1 (F_1), Factor 2 (F_2), and Factor 3 (F_3), were compared using one way ANOVA to determine statistically significant changes in the PLFA profile between soils; a summary of significant differences is given in **Table 5.6**. In each case, between 62-72 % of the observed variation in the PLFA profiles at each field site could be accounted for by the first three principle component factors.

5.4.2. Phospholipid Fatty Acids Results (2012-2014)

Auchincruive

The first PCA factor accounted for approximately 37 % of the observed variation during 2013 and 2014 at the Auchincruive field site. Regression of F_1 scores against the total concentrations of Zn and Cu indicated the observed differences along PCA F_1 during 2013 (**Figure 5.14**) were explained partly by changes in total Cu ($p = 0.057$; $R^2 = 0.194$) though the correlation was not significant. Nevertheless, the PLFA profile of soil receiving the Cu sludge treatment was significantly ($p < 0.05$) different to soil receiving the undigested control and the untreated soil along PCA F_1. Significant correlations were found between total Zn concentration and the F_2 ($p < 0.01$; $R^2 = 0.377$) and F_3 ($p < 0.05$; $R^2 = 0.214$) scores, along which the PLFA profile of soil receiving the Zn sludge treatment was significantly different to soil receiving the digested control, the untreated soil, as well as soils receiving the undigested (Cu and Ctrl2) sludge treatments (**Table 5.6**; **Figure 5.14**).

The following year both total Zn ($p < 0.01$; $R^2 = 0.529$) and total Cu ($p < 0.05$; $R^2 = 0.364$) were significantly correlated to the F_1 scores, with the PLFA profile in soils receiving the contaminated sludge treatments (Zn and Cu) both significantly different from their respective uncontaminated controls (**Table 5.6**; **Figure 5.15**). In addition, the PLFA profile of soil receiving the digested control was also signifcantly different from that in the untreated soil and soil receiving the undigested control (**Table 5.6**). However, in this case no further significant differences in PLFA profile were observed along PCA factors F_2 and F_3, although they accounted for approximately 20 % and 10 % of the total variation, respectively.

For both years, the variable loadings along F_1 indicate an increase in the saturated PLFAs 14:0, 15:0, 16:0, and 20:0 in soils receiving the contaminated sludge treatments (Zn and Cu), whereas the unsaturated PLFA 16:1ω5 appears to have a strong infuence on the F_1 scores for the untreated soil and soil receiving the undigested control, particularly in 2014 (**Figure 5.14** and **Figure 5.15**). In addition, the 2013 variable loading for the fungal biomarker 18:2ω6,9c indicates a possible decrease in fungal

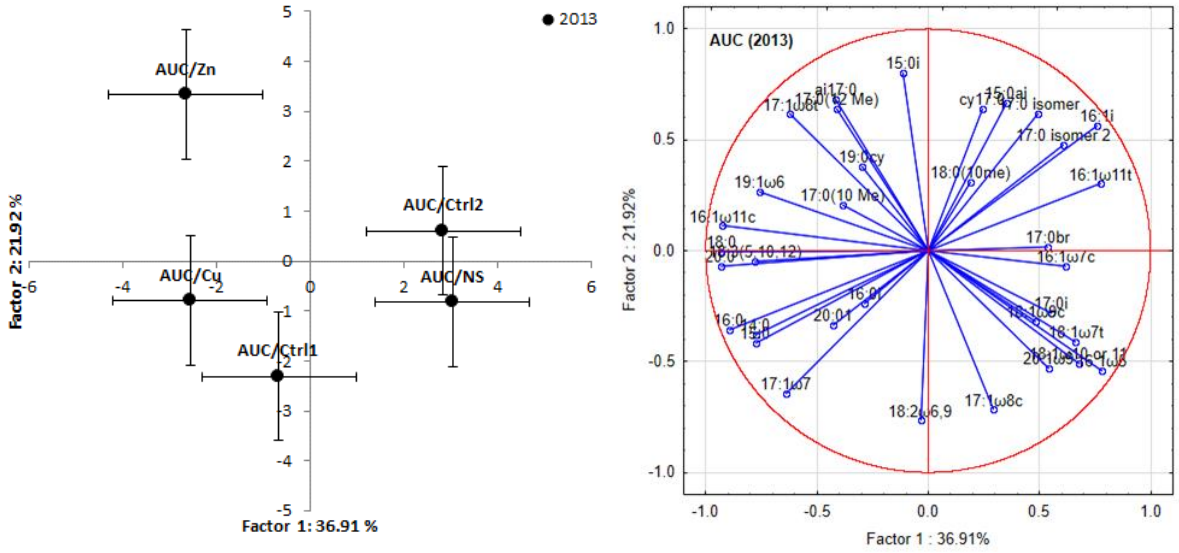


Figure 5.14:- Projection of mean factor scores (left) and PLFA variable loadings (right) on the PCA factor plane (Factor 1 and Factor 2) for soils receiving Zn and Cu sludge treatments, digested (Ctrl1) and undigested (Ctrl2) controls, plus untreated soil (NS) at the Auchincruive (AUC) field site (2013). Error bars represent pooled standard error (n = 3).

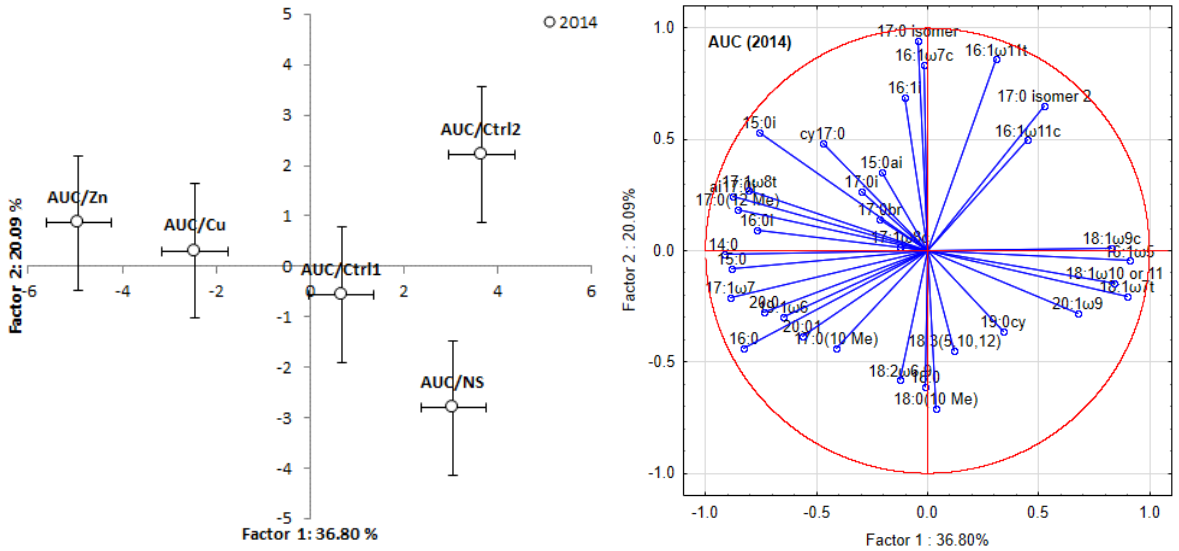


Figure 5.15:- Projection of mean factor scores (left) and PLFA variable loadings (right) on the PCA factor plane (Factor 1 and Factor 2) for soils receiving Zn and Cu sludge treatments, digested (Ctrl1) and undigested (Ctrl2) controls, plus untreated soil (NS) at the Auchincruive (AUC) field site (2014). Error bars represent pooled standard error (n = 3).

biomass in soil receiving the Zn sludge treatment, in comparison to the digested control (**Figure 5.14**). However, the following year, with the possible exception of untreated soil, 18:2ω6,9c appears to have little influence on the F_2 factor scores (**Figure 5.15**).

Gleadthorpe

At Gleadthorpe, the differences in PLFA profile along PCA F_1 for soils receiving the contaminated sludge treatments (Zn and Cu) were highly significant (p <0.001) in comparison to both of the respective controls, and untreated soil, for the duration of the current investigation (**Figure 5.16** and **Figure 5.17**).

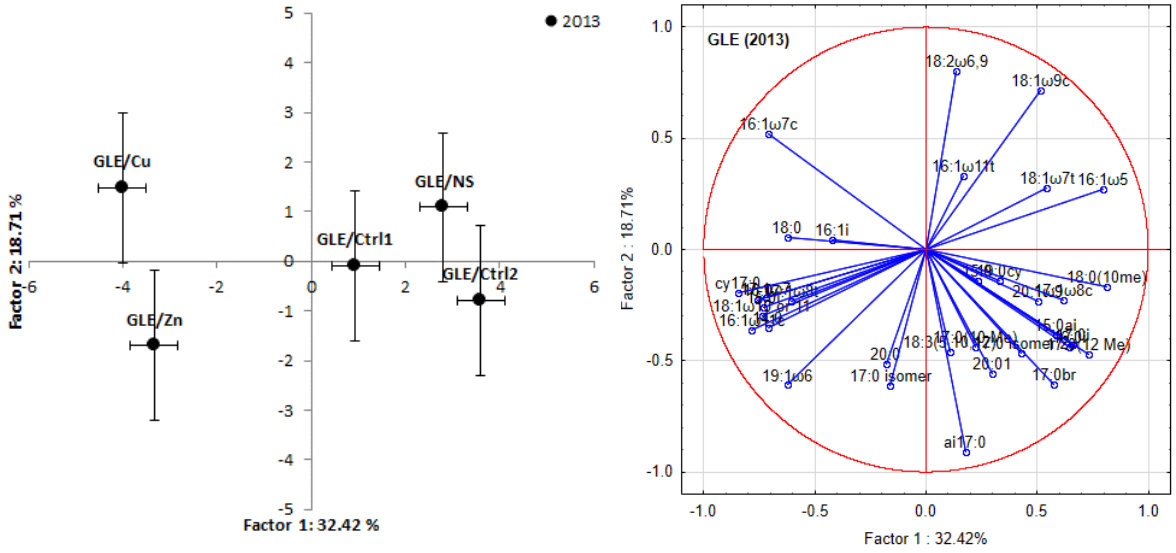


Figure 5.16:- Projection of mean factor scores (left) and PLFA variable loadings (right) on the PCA factor plane (Factor 1 and Factor 2) for soils receiving Zn and Cu sludge treatments, digested (Ctrl1) and undigested (Ctrl2) controls, plus untreated soil (NS) at the Gleadthorpe (GLE) field site (2013). Error bars represent pooled standard error (n = 3).

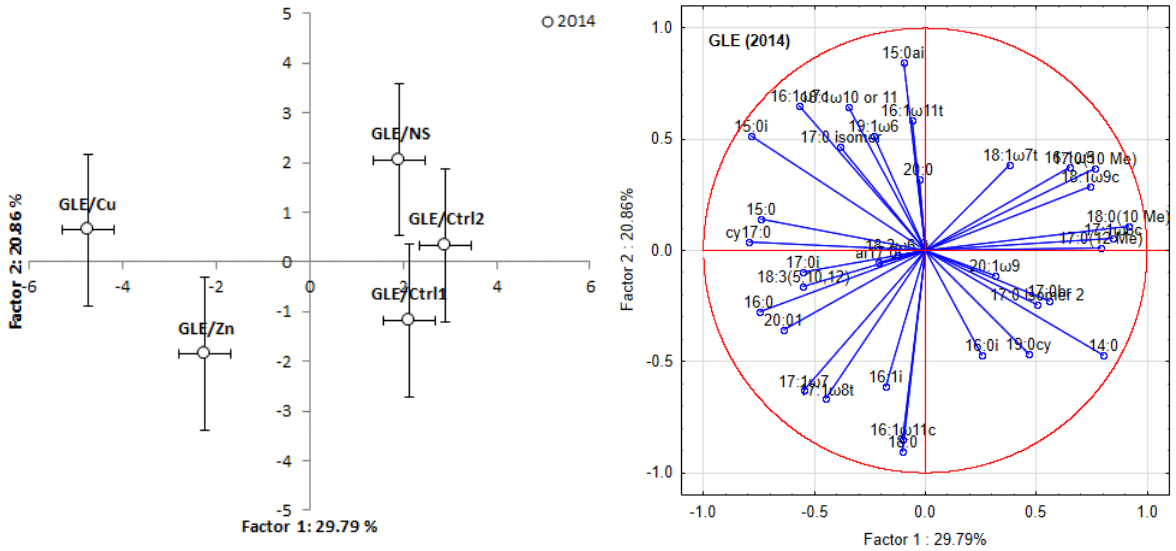


Figure 5.17:- Projection of mean factor scores (left) and PLFA variable loadings (right) on the PCA factor plane (Factor 1 and Factor 2) for soils receiving Zn and Cu sludge treatments, digested (Ctrl1) and undigested (Ctrl2) controls, plus untreated soil (NS) at the Gleadthorpe (GLE) field site (2014). The direction of Factor 2 scores (left) have been inverted to correspond to those for year 2013. Error bars represent pooled standard error (n = 3).

For each year, PCA F_1 accounted for approximately 30-32 % of the total variation and in both cases the F_1 scores were significantly (p <0.001) correlated to total Cu concentration (2013 R^2 = 0.634; 2014 R^2 = 0.728). No significant correlation was found for total Zn, although approximately 20 % and 12 % of the observed varance in F_1 scores was accounted for by total Zn, for years 2013 and 2014, respectively. No significant differences in PLFA profile were observed between any of the soils along PCA F_2 for the duration of the experiment (**Table 5.6**), depite accounting for approximately 20 % of the total variation in each case. Similarly for PCA F_3, no significant difference in PLFA profile was seen in any

of the soils during 2014, however, the PLFA profile of soils receiving digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments were significantly different in 2013 (**Table 5.6**), possibly indicating an effect of sludge type on the PLFA profile of sludge amended soils.

Variable loadings for years 2013 and 2014, indicate that saturated PLFAs 15:0i and 16:0, as well as cyclic PLFA cy17:0, have strongly influenced the F_1 scores of soils receiving the contaminated sludge treatments (Zn and Cu) for the duration of the current experiment (**Figure 5.16** and **Figure 5.17**). Whereas branched PLFAs 17:0 (12Me) and 18:0 (10Me) have influenced the F_1 scores of untreated soil and soils receiving the uncontaminated controls.

Hartwood

The first principal component accounted for approximately 34 % of the total variation in PLFA profiles at Hartwood in 2013. However, no significant differences were observed between any of the soils (**Table 5.6**; **Figure 5.18**), despite a significant correlation between F_1 scores and the total concentration of Cu (p <0.05; R^2 = 0.213). Approximately 22 % of the total PLFA variation was subsequently accounted for by PCA F_2 and in this case the total concentrations of both Cu (p <0.05; R^2 = 0.261) and Zn (p <0.05; R^2 = 0.340) were significantly correlated to F_2 scores. In this case the PLFA profiles of soils receiving the Zn and Cu sludge treatments were significantly different to their respective uncontaminated controls (**Figure 5.18**), with the PLFA profile of soil receiving the Zn sludge treatment also significantly different to that in the untreated soil (**Table 5.6**). The only significant (p <0.05) differences in PLFA profile observed along PCA F_3 were between soils receivng the undigested sludge treatments (Cu and Ctrl2) and the untreated soil.

The following year PCA F_1 accounted for approximately 31 % of the total variation in PLFAs, with the PLFA profile of soil receiving the Zn sludge treatments significantly different to that seen in untreated soil and soil receiving the digested control (**Figure 5.19**). Regression of total Zn (R^2 = 0.276) and Cu (R^2 = 0.337) concentration on F_1 scores indicated a significant (p <0.05) correlation in both cases, however no significant difference in the PLFA profile of soils receiving the undigested sludge treatments (Cu and Ctrl2) was observed. The second principal component accounted for approximately 23 % of the total PLFA variation in 2014. The correlations between total metal concentration and F_2 scores were not significant, though total Zn (R^2 = 0.179) accounted for approximately 20 % of the observed variance between factor scores. However, the only significant differences in PLFA profiles along PCA F_2 were between soils receiving the digested sludge treatments (Zn and Ctrl1) and untreated soil (**Table 5.6**), again indicating a possible influence of sludge type on soil PLFA profiles; the PLFA profile in soil receiving the Zn sludge treatment was also significantly (p <0.05) different in comparison to soil receiving the undigested control. Approximately 15 % of the total PLFA variation in 2014 was accounted for by PCA F_3, however no significant differences in PLFA profile were observed between any of the soils along this factor (**Table 5.6**).

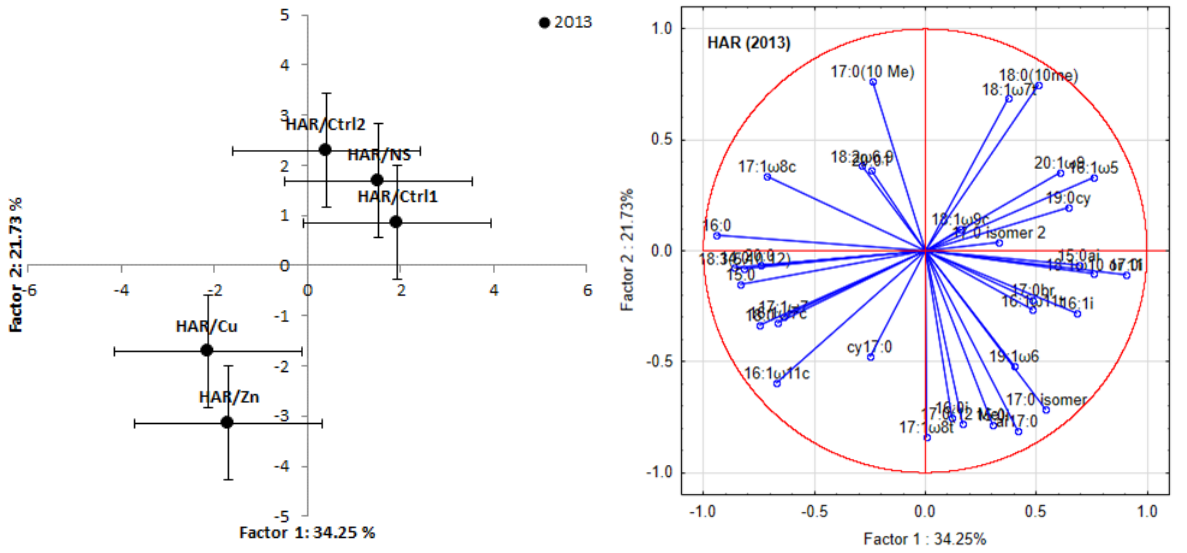


Figure 5.18:- Projection of mean factor scores (left) and PLFA variable loadings (right) on the PCA factor plane (Factor 1 and Factor 2) for soils receiving Zn and Cu sludge treatments, digested (Ctrl1) and undigested (Ctrl2) controls, plus untreated soil (NS) at the Hartwood (HAR) field site (2013). Error bars represent pooled standard error (n = 3).

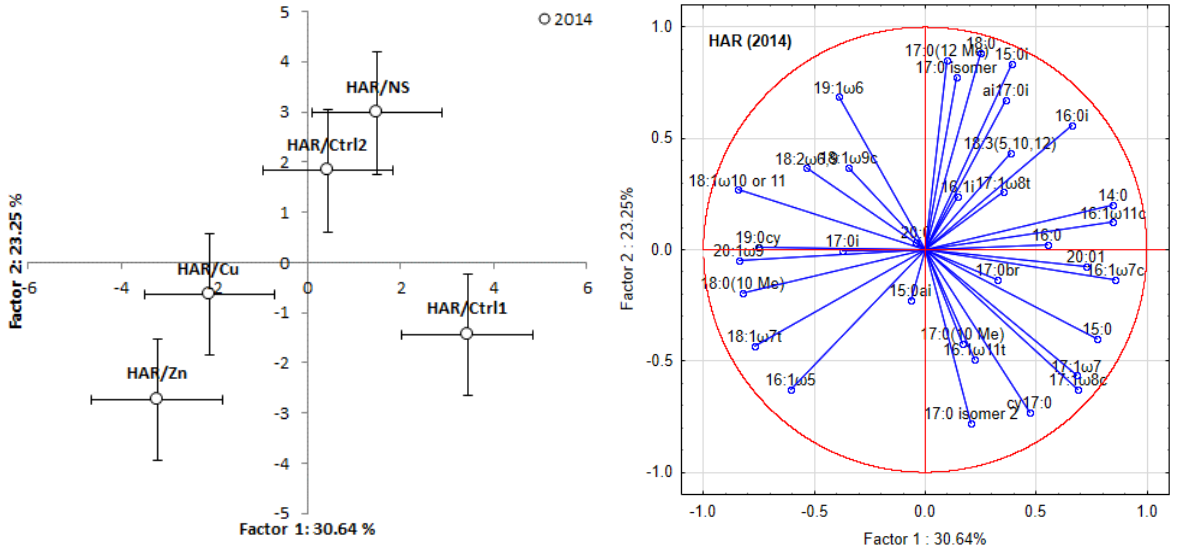


Figure 5.19:- Projection of mean factor scores (left) and PLFA variable loadings (right) on the PCA factor plane (Factor 1 and Factor 2) for soils receiving Zn and Cu sludge treatments, digested (Ctrl1) and undigested (Ctrl2) controls, plus untreated soil (NS) at the Hartwood (HAR) field site (2014). The direction of scores for Factor 1 and Factor 2 (left) have been reversed to correspond to those for year 2013. Error bars represent pooled standard error (n = 3).

Similar to Auchincruive, the saturated PLFAs 14:0 and 15:0 appear to have strongly influenced the F_1 scores for soils receiving the contaminated sludge treatments (Zn and Cu) over the course of the current investigation (**Figure 5.18** and **Figure 5.19**). Whereas the unsaturated PLFA 18:1ω10 or 11 appears to be influencing the F_1 score for the untreated soil and soils receiving the uncontaminated controls (Ctrl1 and Ctrl2); particularly for soil receiving the digested control in 2014 (**Figure 5.19**). In contrast to Gleadthorpe, the branched PLFA 17:0 (12Me), plus Me17:0 isomer, now appear to influence the F_2

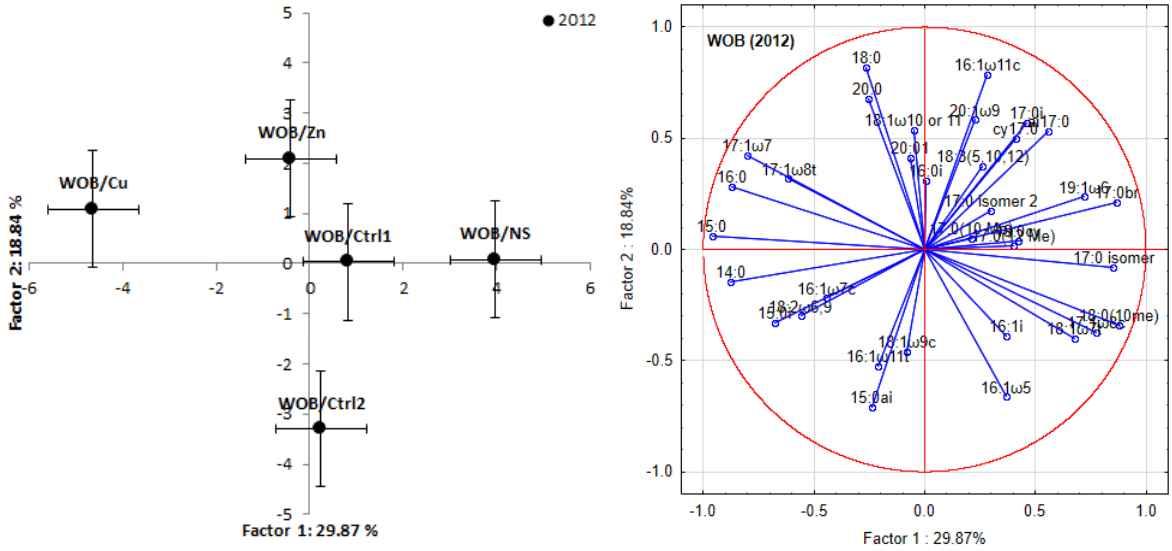


Figure 5.20:- Projection of mean factor scores (left) and PLFA variable loadings (right) on the PCA factor plane (Factor 1 and Factor 2) for soils receiving Zn and Cu sludge treatments, digested (Ctrl1) and undigested (Ctrl2) controls, plus untreated soil (NS) at the Woburn (WOB) field site (2012). Error bars represent pooled standard error (n = 3).

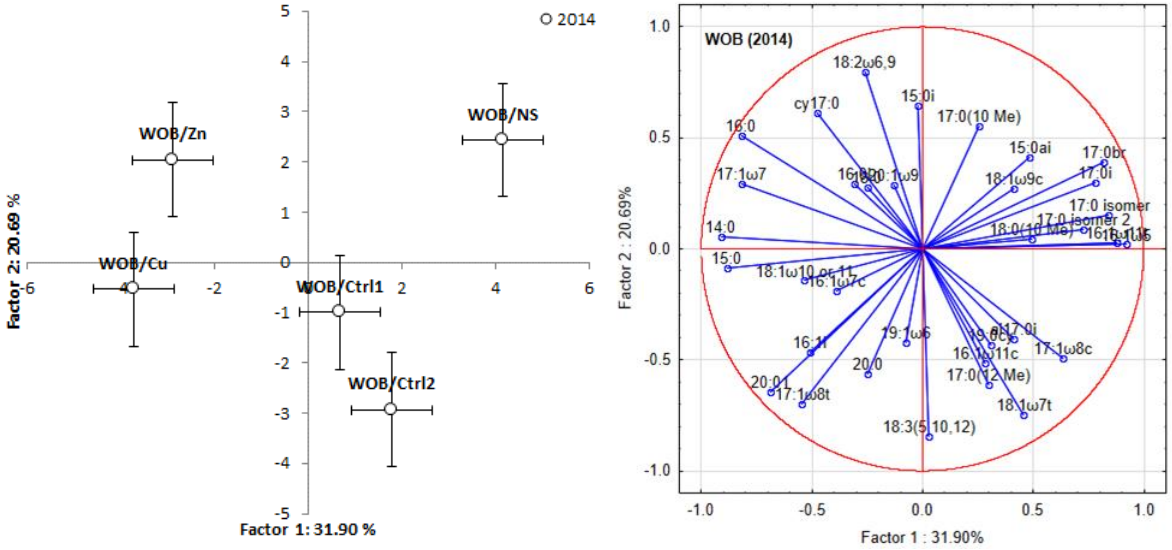


Figure 5.21:- Projection of mean factor scores (left) and PLFA variable loadings (right) on the PCA factor plane (Factor 1 and Factor 2) for soils receiving Zn and Cu sludge treatments, digested (Ctrl1) and undigested (Ctrl2) controls, plus untreated soil (NS) at the Woburn (WOB) field site (2014). Error bars represent pooled standard error (n = 3).

score of the contaminated soils at Hartwood. Though at both sites, the saturated PLFA 15:0i appears to influence factor scores for contaminated soils, in this case for PCA F_2. No consistent trend could be seen in the magnitude of variable loadings that would indicate an influence on F_2 scores for the uncontaminated soils.

Woburn

Principal component F_1 accounted for 30 % and 32 % of the total PLFA variation at Woburn, for years 2012 and 2014, respectively. With the exception of soil receiving the undigested control in 2014, the

PLFA profiles of sludge amended soils were significantly different from that of untreated soil along PCA F_1, for the duration of the current investigation (**Table 5.6**). For both years, the PLFA profile of soil receiving the Cu sludge treatment was significantly (p <0.01) different to that of soil receiving the undigested control (**Figure 5.20** and **Figure 5.21**), with the total concentration of Cu strongly and significantly (p <0.001) correlated to F_1 scores (2012 (R^2 = 0.596) and 2014 (R^2 = 0.701)). The PLFA profiles of soils receiving the undigested sludge treatments were also significantly different along PCA F_2 and PCA F_3 for years 2012 and 2014 respectively (**Table 5.6**). The PLFA profile of soil receiving the Zn sludge treatment was only significantly (p <0.05) different in comparison to soil receiving the digested control along PCA F_1 in 2014 (**Figure 5.21**). No significant correlation was found between the factor scores for PCA F_1 and F_2 and the total concentration of Zn, however in 2012 total Zn was correlated to the F_3 scores, and in this case the PLFA profiles of the contaminated soils were significantly (p <0.01) different (**Table 5.6**). In addition, although the total concentration of Zn and Cu had no apparent influence on the F_3 scores in 2014, the PLFA profile of soil receiving the Cu sludge treatment was significantly different to both of the soils receiving the digested sludge treatments (Zn and Ctrl1), again indicating a possible effect of sludge type on soil PLFA profile (**Table 5.6**).

Again, the variable loadings for saturated PLFAs 14:0, 15:0, and 16:0, plus the unsaturated PLFA 17:1ω7, appeared to strongly influence the F_1 scores for the contaminated soils, whereas in this case the branched PLFAs 17:0Me isomer, and 17:0br influenced the F_1 scores for uncontaminated soils, particularly the untreated soil (**Figure 5.20** and **Figure 5.21**). No apparent trends could be seen in the magnitude of variable loadings that would suggest the influence of specific PLFAs on F_2 or F_3 scores.

5.4.3. Phospholipid Fatty Acids Overview (2012-2014)

Phospholipid fatty acid data from each of the LTSE field sites was subsequently combined and re-analysed using PCA in order to investigate the differences in soil microbial communities between field sites. Factorial ANOVA was carried out using factors scores for PCA F_1, F_2 and F_3, to determine the effects of field site location and sludge treatment (including untreated soil) on the PLFA profile in each soil and identify significant differences between field sites.

For the data collected in 2013 (2012 for Woburn), PCA F_1 accounted for 27.95 % of the total PLFA variation, with each of the factor scores for the PLFA profiles determined in the Woburn soils significantly (p <0.001) different to the other sites (**Figure 5.22**). In each case the effect of site location and sludge treatment was statistically significant (p <0.001), with a significant (p <0.01) interaction also observed between the two variables. The observed difference in the PLFA profiles of the Woburn soils may be due to the fact that the soil analysed was originally collected in 2012; whereas for the remaining sites the samples analysed were collected in 2013. Hence there may also be some temporal

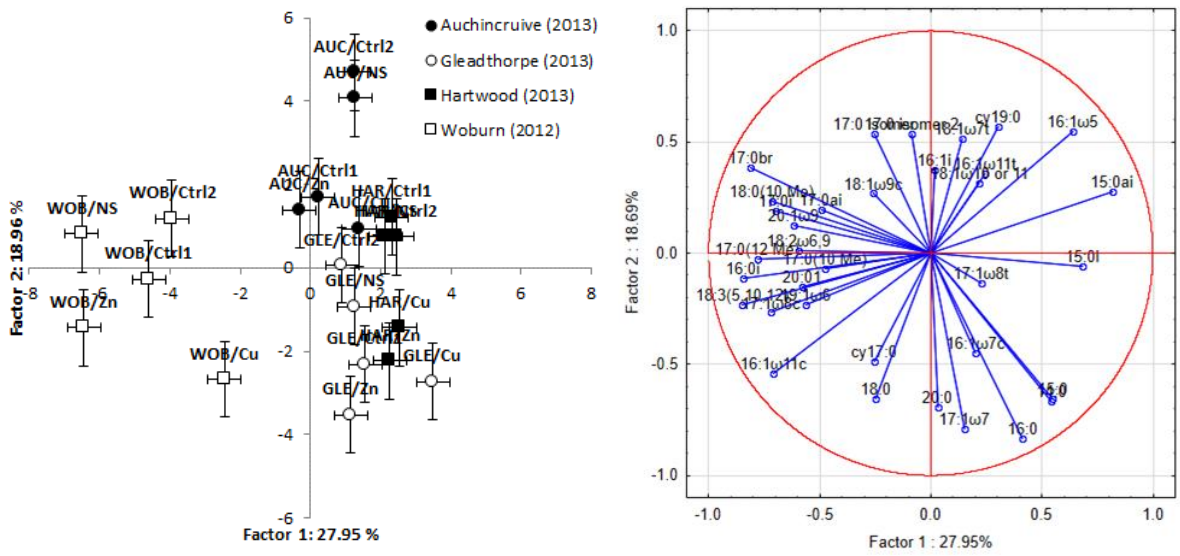


Figure 5.22:- Projection of mean factor scores (left) and PLFA variable loadings (right) on the PCA factor plane (Factor 1 and Factor 2) for soils receiving Zn and Cu sludge treatments, digested (Ctrl1) and undigested (Ctrl2) controls, plus untreated soil (NS) at each of the LTSE field sites (Auchincruive (AUC), Gleadthorpe (GLE), Hartwood (HAR), and Woburn (WOB)) in 2013. Data for Woburn is from 2012. Error bars represent pooled standard error (n = 3).

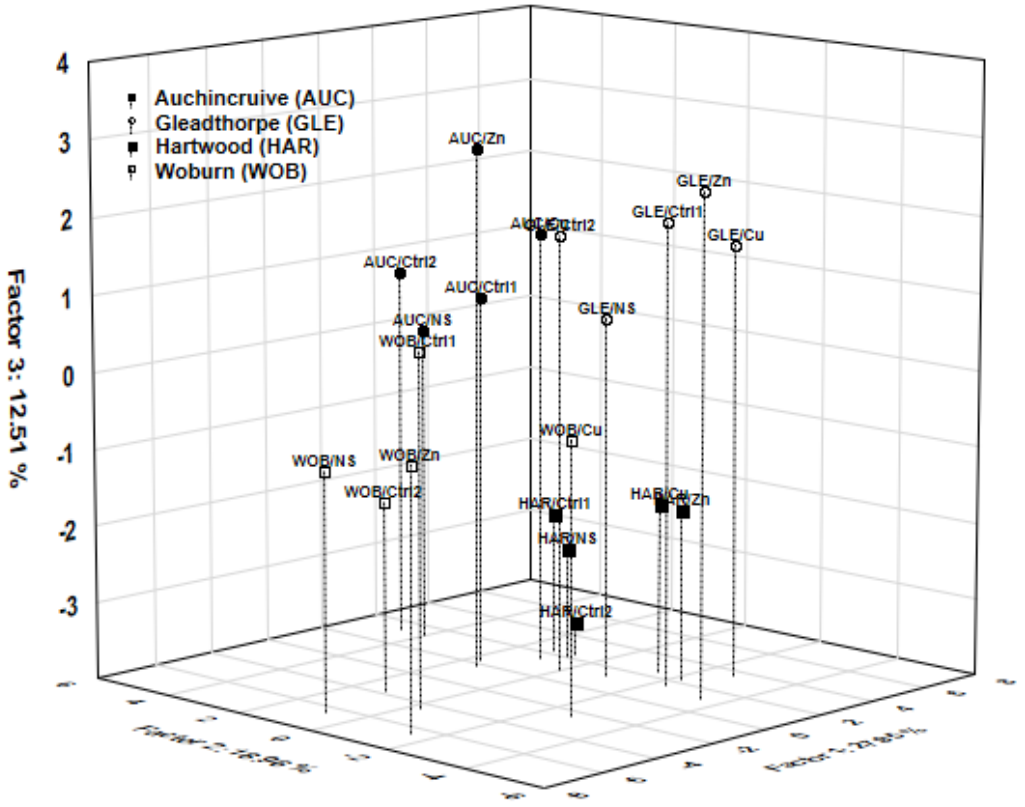


Figure 5.23:- Projection of mean factor scores along PCA Factor 3 for soils receiving Zn and Cu sludge treatments, digested (Ctrl1) and undigested (Ctrl2) controls, plus untreated soil (NS) at each of the LTSE field sites (Auchincruive (AUC), Gleadthorpe (GLE), Hartwood (HAR), and Woburn (WOB)) in 2013. Data for Woburn is from 2012.

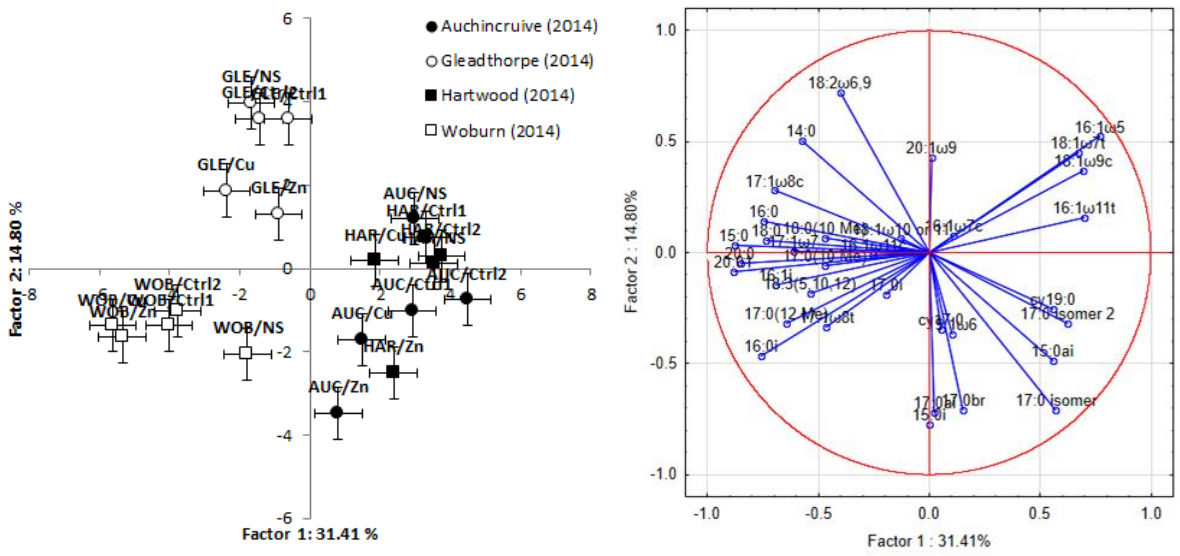


Figure 5.24:- Projection of mean factor scores (left) and PLFA variable loadings (right) on the PCA factor plane (Factor 1 and Factor 2) for soils receiving Zn and Cu sludge treatments, digested (Ctrl1) and undigested (Ctrl2) controls, plus untreated soil (NS) at each of the LTSE field sites (Auchincruive (AUC), Gleadthorpe (GLE), Hartwood (HAR), and Woburn (WOB)) in 2014. Error bars represent pooled standard error (n = 3).

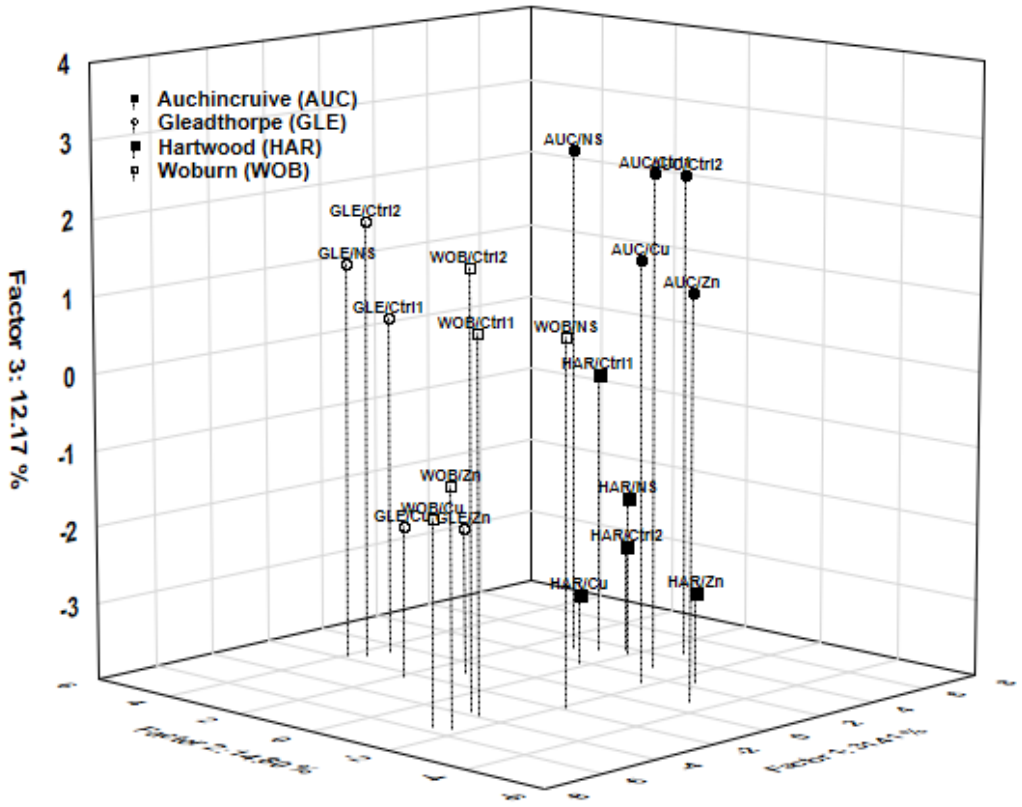


Figure 5.25:- Projection of mean factor scores along PCA Factor 3 for soils receiving Zn and Cu sludge treatments, digested (Ctrl1) and undigested (Ctrl2) controls, plus untreated soil (NS) at each of the LTSE field sites (Auchincruive (AUC), Gleadthorpe (GLE), Hartwood (HAR), and Woburn (WOB)) in 2014.

influence on the PLFA profile, though, it may also be due to soil texture as the Woburn site has both the highest sand content (**Table 2.1**), and lowest organic matter content (**See Section 2.7**) of the four sites. In most cases the differences between PCA F_1 scores for the remaining sites were not statistically significant (**Figure 5.22**). However, the soils receiving the digested sludge treatments (Zn (p <0.001) and Ctrl1 (p <0.01)) at Auchincruive were significantly different to each of the soils at Hartwood. In addition, soils receiving the Cu sludge treatment and undigested control at Gleadthorpe were significantly different to each of the sludge amended soils at Auchincruive (p <0.01), and Hartwood (p <0.05), respectively.

Principal component factor 2 accounted for 18.96 % of the total PLFA variation, with significant differences predominantly found between the untreated soil, and soil receiving the undigested control at Auchincruive; both significantly (p <0.01) different to each of the soils at the remaining sites (**Figure 5.22**). With a few exceptions for the untreated soil and soil receiving the undigested control, each of the F_2 scores for Gleadthorpe were significantly (p <0.01) different to those of Auchincruive. A significant difference in the PLFA profiles of soils from the Scottish sites was also observed along PCA F_3, with the PLFA profiles in the sludge amended soils at the Hartwood site significantly (p <0.001) different to those seen at Auchincruive (**Figure 5.23**). The PLFA profile of the untreated soil at Auchincruive was also significantly (p <0.05) different to the untreated soil at Hartwood, as well as soils receiving the uncontaminated controls. Similarly, the PLFA profiles observed in each of the soils at Hartwood were significantly (p <0.01) different to those at Gleadthorpe along PCA F_3. Hence along the first three PCA factors, statistically significant differences can be observed between the PLFA profiles of each of the four LTSE field sites indicating distinct differences in the soil microbial communities of untreated and sludge amended soils at each site.

For year 2014 a marked difference in the PLFA profiles of the English and Scottish sites can be seen along PCA F_1, now accounting for 31.41 % of the total PLFA variation, with the F_1 scores for each of the soils at Woburn significantly (p <0.001) different to those for both Auchincruive and Hartwood (**Figure 5.24**). Similarly, at Gleadthorpe the F_1 scores for untreated soil and soils receiving the undigested sludge treatments (Cu and Ctrl2) were significantly (p <0.001) different in comparison to the respective soils at both of the Scottish sites. The PLFA profiles of soils receiving the digested sludge treatments (Zn and Ctrl1) at Gleadthorpe were also significantly (p <0.05) different to each of the soils at the Scottish sites, with the exception of soil receiving the Zn sludge treatment at Auchincruive (**Figure 5.24**).

A difference in the PLFA profile of the English sites was observed along PCA F_2, with the F_2 scores of both the untreated and sludge amended soils at the Gleadthorpe site significantly different to those for the respective soils at Woburn (**Figure 5.24**). In addition, the PLFA profiles of the untreated soil and soils receiving the uncontaminated controls at the Gleadthorpe site were also significantly different to

both of the Scottish sites (Auchincruive ($p < 0.01$) and Hartwood ($p < 0.001$)). This was also the case for the soils receiving the Zn and Cu sludge treatments which were significantly ($p < 0.01$) different to each of the soils at Auchincruive along PCA F_2, excepting the untreated soil. However, in contrast, the contaminated soils at Gleadthorpe were only significantly ($p < 0.001$) different to the Hartwood soil receiving the Zn sludge treatment. In agreement with the results for the previous year, a significant difference in the PLFA profiles of the Scottish sites was also observed along PCA F_3, which accounted for 12.17 % of the total variation in PLFA. In each case, the F_3 scores for the Auchincruive site were significantly ($p < 0.001$) different to those at Hartwood (**Figure 5.25**). In addition, the PLFA profiles of soils receiving the contaminated sludge treatments at both of the English sites were significantly ($p < 0.001$) different to each of the soils at Auchincruive along PCA F_3.

In summary, both contaminated and uncontaminated sewage sludge treatments appear to have caused significant long-term changes the PLFA profile of the receiving soils, in comparison to untreated soil, at each of the LTSE field sites. Additional changes in the PLFA profiles have also occurred due to the presence of heavy metal contamination, particularly Cu. Furthermore, comparison of the PLFA profiles of both sludge amended and untreated soils at each of the four field sites, indicates distinct differences in the soil microbial communities. The most prominent difference appears to be due to location, with significant differences observed between the PLFA profiles of the English and Scottish sites, particularly in 2014. Further differences in PLFA profile were also detected between sites of the same region, therefore it appears that each of the LTSE field sites represents a distinct soil microbial community, which have now been altered locally due to the application of the sludge treatments. However, it should be noted, that although the changes observed can be attributed to the relative differences in the concentration of specific PLFAs, it cannot be said for certain whether this is due to macroscopic changes in microbial community structure (i.e. the proliferation of metal tolerant species) or a response of the indigenous microbial population (i.e. the production of additional PLFAs) to the presence of heavy metals (Frostegård et al., 2011).

5.4.4. Phospholipid Fatty Acids as a Function of Microbial Biomass Carbon

Table 5.7 shows the mean sum of PLFA compounds in each of the soils sampled from the LTSE field sites for year 2014. The total PLFA concentration in soils receiving the Zn sludge treatment was significantly ($p < 0.05$) higher in comparison to untreated soil at both Auchincruive and Gleadthorpe, whereas at Woburn, both of the soils receiving digested sludge treatments (Zn and Ctrl1) had significantly ($p < 0.01$) higher PLFA concentrations in comparison to untreated soil. In addition, the total PLFA concentration in soil receiving the undigested control was also significantly higher in comparison to soil receiving the Cu sludge treatment ($p < 0.05$), and untreated soil ($p < 0.01$). No significant differences in total PLFA concentration were observed at the Hartwood field

Table 5.7:- Sum of phospholipid fatty acid (Σ PLFA) concentrations (μg n-FAME g soil^{-1}) measured during year 2014 at the Long-Term Sludge Experiment field sites.

Sludge Treatment	Σ PLFA (μg n-FAME g soil^{-1}) 2014
AUC/Zn	17.98 (0.31)[a][1][2]
AUC/Ctrl1	15.78 (1.13)[ab]
AUC/Cu	18.48 (2.37)[c]
AUC/Ctrl2	16.92 (2.50)[c]
AUC/NS	13.67 (0.93)[bc]
GLE/Zn	11.36 (0.98)[a]
GLE/Ctrl1	9.84 (1.35)[ab]
GLE/Cu	9.44 (2.31)[c]
GLE/Ctrl2	8.51 (1.00)[c]
GLE/NS	5.79 (1.74)[bc]
HAR/Zn	18.08 (1.48)[a]
HAR/Ctrl1	18.37 (1.58)[a]
HAR/Cu	16.81 (0.99)[b]
HAR/Ctrl2	17.18 (1.31)[b]
HAR/NS	17.97 (1.13)[ab]
WOB/Zn	9.30 (0.72)[a]
WOB/Ctrl1	8.71 (0.58)[a]
WOB/Cu	6.16 (0.53)[c]
WOB/Ctrl2	7.50 (0.19)[d]
WOB/NS	5.26 (0.08)[bc]

[1]Values in parenthesis are standard errors (n = 3). [2] Values without corresponding letters denotes statistical significance at α = 0.05, note comparisons between different sludge types (i.e. digested (Zn and Ctrl1) and undigested (Cu and Ctrl2)) have not been made.

site (**Table 5.7**). With the exception of soil receiving the Cu sludge treatment, regression of total PLFA concentration on C_{mic} showed significant correlations between the two variables in both sludge amended and untreated soil (**Figure 5.26**). In each case, the regression coefficients were similar (**Table 5.8**) suggesting both the application of sewage sludge, and the presence of heavy metals, has not affected the overall quantity of PLFAs produced by microorganisms in the receiving soils in comparison to the indigenous population in untreated soil.

5.5. Chapter Discussion

5.5.1. Previous Findings (1997-2011)

Following the final applications of sewage sludge at the end of experimental Phase I, a marked increase in C_{mic} was observed in the receiving soils, particularly in soils receiving the undigested sludge treatments. However, only at Gleadthorpe was C_{mic} significantly lower in the contaminated soils in comparison to the uncontaminated controls (Gibbs et al., 2006). Soil microbial biomass subsequently declined over the course of experimental Phase II and Phase III but generally remained higher in sludge amended soils than in untreated soil (Defra, 2002, 2007a). By the end of experimental Phase III, C_{mic} in soils receiving the Zn and Cu sludge treatment at Woburn and Gleadthorpe, respectively, were reported to be significantly lower than in their corresponding controls (Defra 2007a). Overall it was concluded

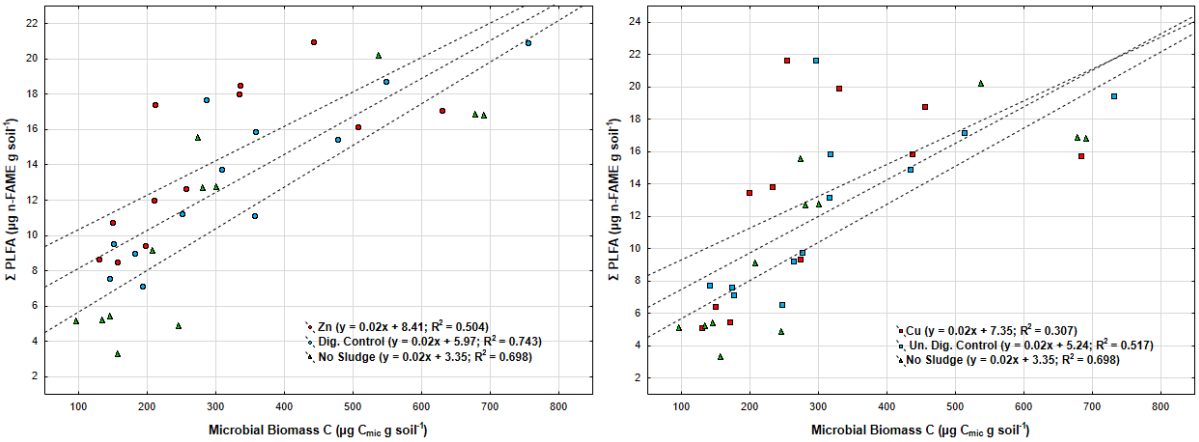


Figure 5.26:- Plot of total PLFA concentration (µg n-FAME g soil^{-1}) as a function of microbial biomass carbon (µg C_{mic} g soil^{-1}). Values are total PLFA concentration and microbial biomass carbon measured in each of the samples taken from the LTSE field sites during 2014.

Table 5.8:- Regression and correlation coefficients from regression analysis of total PLFA concentration (Σ PLFA) on microbial biomass carbon (C_{mic}).

	Regression of Σ PLFA on Biomass C					
Sludge Treatment	**Slope (C_{mic})**	***p***	**Intercept**	***p***	**R**	**$R^{2[2]}$**
Zinc (Zn)	0.02 (0.01)[1]	<0.01	8.41 (2.03)	<0.01	0.710	0.455
Digested Control (Ctrl1)	0.02 (0.004)	<0.001	5.97 (1.51)	<0.01	0.862	0.717
Copper (Cu)	0.02 (0.01)	0.077	7.35 (3.34)	0.055	0.554	0.230
Undigested Control (Ctrl2)	0.02 (0.01)	<0.01	5.24 (2.49)	0.061	0.719	0.463
No Sludge (NS)	0.02 (0.004)	<0.001	3.35 (1.81)	0.093	0.836	0.668

[1] Values in parenthesis are standard error (n = 12). [2]Values are adjusted R^2.

that the presence of Cu caused a 20 % decrease in C_{mic} in comparison to the uncontaminated control, whereas a 6 % decrease was seen in soils contaminated with Zn (Defra, 2008).

5.5.2. Current Investigation (2012-2014)

For the current investigation, values for C_{mic} in sludge amended soil no longer appeared to be significantly different from that in untreated soil, with a few exceptions at Woburn and Gleadthorpe. In addition the only significant difference in C_{mic} between contaminated and uncontaminated soil was seen at Gleadthorpe in 2013; C_{mic} in soil receiving the Cu sludge treatment was lower than in soil receiving the undigested control. Therefore, in contrast to the findings at other long-term field experiments, no significant long-term impact on C_{mic} was detected at any of the LTSE field sites during the current investigation; now almost 20 years since the final sludge applications were made. However, combining the results using meta-analysis did show an overall negative effect on C_{mic} due to the presence on metal contamination, particularly Zn; though this may also include the confounding Cu contamination (**See Section 5.2.3**).

As described in **Chapter 1** (**See Section 1.4**), significant long-term decreases in C_{mic} have been observed at a number of field sites where historical applications of contaminated sewage sludge have

been made. However, in most of these cases, the observed decreases are seen in soils where the applied sludge contained metal concentrations above the current UK/EU limits, which in turn has increased the total concentration of metals in the receiving soils above the current limits. Furthermore the soils often contain a mixture of heavy metals which could potentially exacerbate toxic effects. For instance, the 50 % decreases in C_{mic} observed by Brookes and McGrath (1984) at the Woburn Market Garden Experiment were seen in soils where Zn (469 mg kg^{-1}), Cu (163 mg kg^{-1}) and Cd (15.4 mg kg^{-1}) were all present at concentrations above the current UK limits (McGrath et al. 1988). Similarly, the decreases in C_{mic} reported by Abaye et al. (2005) were still observed in soils where the concentration of Cd was approximately twice the UK limit. In addition, the decreases in C_{mic} at Luddington, Lee Valley, and Gleadthorpe, observed by Chander and Brookes (1991, 1993) also occurred in soils where total metal concentrations were above UK Statutory limits. This was also the case for the long-term sludge experiment at Brunswick (**See Section 1.4**), Germany, where Fließbach et al. (1994) observed a 26 % decrease in C_{mic} in arable and ex-woodland soils containing 345 mg Zn kg^{-1} and 404 mg Zn kg^{-1}, respectively. No significant effect of metal contamination was observed on C_{mic} at each of the LTSE field sites when considered as separate cases. However combining the results did suggest an overall negative effect. Therefore it may be that the total concentrations of Zn and Cu encountered in the contaminated soils at the LTSE sites are approaching a toxicity threshold (coinciding with the UK statutory limits) with regards to C_{mic}, above which more drastic decreases in C_{mic} would be observed. However, as mentioned above, measurements of C_{mic} are a gross undifferentiated estimate of the size of a microbial population, and do not give any indication of microbial community structure. Clearly the application of both contaminated and uncontaminated sludge treatments has caused significant changes in micorbial community structure at the LTSE field sites.

The results reported in **Section 5.3.1.** show a general increase in the percentage of soil microbial biomass comprised of fungal species in soils receiving the contaminated sludge treatments (Zn and Cu), particularly at the English arable sites. This observation is in agreement with the results of several investgations. For instance, Chander et al. (2001a, 2001b) saw an increase in the ratio of ergosterol to C_{mic} as the total concentratons of Zn, Cu, and Pb increased across a range of contaminated field sites (including the sludge amended soils at Brunswick), indicating an increase in the proportion of the microbial community comprised of fungal species. Concentrations of ergosterol in the sludge amended soils ranged from 1.13-3.40 µg ergosterol g $soil^{-1}$ (plus an outlier of 7.30 µg ergosterol g $soil^{-1}$) and were broadly in agreement with the values reported in **Table 5.4** (Chander et al., 2000b). Whereas concentrations of Zn, Cu and Pb ranged from 76-459 mg kg^{-1}, 14-95 mg kg^{-1}, and 26-108 mg kg^{-1}, respectively; only at two of the sites was the total concentration of Zn over the UK limit. In addition, the ergosterol to C_{mic} ratio was found to be significantly correlated to the concentrations of Zn extractable by NH_4NO_3 (in addition to total and NH_4NO_3 extractable Pb). Frostegård et al. (1996) also observed an increase in fungal species, determined as the PLFA 18:2ω6,9c, over the course of 18

months in an arable soil spiked with 128 mmol Zn kg^{-1} (8368.6 mg kg^{-1}). Although the concentration of Zn used far exceeds the UK statutory limits for sludge amended soils, it does indicate that fungal species are more tolerant to heavy metal contamination in comparison to bacteria (Doleman, 1985). Again this is in agreement with the overall effect observed in sludge amended soils at the LTSE field sites as C_{fungi} increased in the contaminated soils, although in this case Cu seemed to have a greater effect (**Figure 5.11**).

Several changes in the PLFA profiles of sludge amended soils have been observed at a number of long-term field experiments around Europe. In 1996, Witter et al., (2000) investigated the PLFA profile of sludge amended soils at the arable field site in Brunswick, which had previously received applications of 'uncontaminated' and contaminated (spiked with metal salts) sludge over the course of 9 years (1980-1989). With the exception of Zn in soils that received 300 m^3 ha^{-1} yr^{-1} of contaminated sludge, total concentrations of Zn, Cd, Cu, and Ni, were now all below the maximum EU and UK levels, for the respective soil pH values. Significant differences in PLFA profiles were observed along PCA F_1 (40 % variation) between untreated soil, and soils receiving the contaminated and 'uncontaminated' sludge treatments at a rate of 300 m^3 ha^{-1} yr^{-1}. In agreement with the data presented above (**See Section 5.4.2**), particularly for Auchincruive, increases in the PLFAs 14:0, 15:0i, 15:0a, 16:0, and 19:0cy were observed in the contaminated soils, while 16:1ω5, 18:1ω7, and 18:0 (10Me) were more prominent in untreated soil. Overall it was concluded that significant changes in microbial community structure could occur in sludge amended soils, at metal concentrations are below the current EU maximum, even if the total size of microbial biomass is unaffected (Witter et al., 2000). As mentioned previously, Abaye et al. (2005), investigated changes in microbial community structure at the Woburn Market Garden Experiment in 1998, almost 40 years since the finals applications of sludge were made. Total PLFA concentration was found to be correlated (R = 0.910) to C_{mic}, but was significantly (p <0.05) lower in sludge amended soils, containing Cd (6.0-6.3 mg kg^{-1}) at twice the UK limit, in comparison to soil amended with farmyard manure. Increases in mono-unsaturated, cyclopropyl, and hydroxyl PLFAs, indicative of Gram-negative bacteria, were significantly (p <0.05) higher in the sludge amended soils in comparison to branched PLFAs, indicative of Gram-positive bacteria, and indicated that sludge amended soils contained 62-68 % more Gram-negative bacteria, in comparison to Gram-positive. These results appear to be in agreement with the PLFA data for Woburn given above, as mono-unsaturated and branched PLFAs had the most influence on F_1 scores for contaminated and untreated soils, respectively, though this was not observed for the other LTSE field sites. Similarly, in 1993, Dahlin et al. (1997) investigated the PLFA profile of sludge amended soils at a field site in Brunnby, Sweden, receiving sludge applications at varying rates (0, 5, 10, and 20 t ha^{-1}), over the course of 23 years (1966-1989). Total concentrations of Cd (0.52-0.78 mg kg^{-1}), Cu (34.3-60.2 mg kg^{-1}), Pb (22.8-24.8 mg kg^{-1}), Ni (22.1-23.4 mg kg^{-1}), and Zn (91-117 mg kg^{-1}) were all below the UK statutory limits, and, with the exception of Cu (60.2 mg kg^{-1}), were also below the lower limits specified by the EU Sludge Directive

(**Table 1.1**). A significant change in PLFA profile was observed along PCA F_3 between untreated soil and soil receiving sludge at 20 t ha^{-1} yr^{-1}, however in contrast to the ergosterol data given above, Cu appeared to cause a decrease in the PLFA 18:2ω6,9c, indicating a decrease in fungal species. More recently, Börjesson et al. (2012) investigated the PLFA profile of sludge amended soils at the 'Long-Term Soil Organic Matter Experiment' in Ultuna, Sweden. Samples were collected in 2009 from soils which had received 26 biennial applications of sludge (4 t C ha^{-1}) over the course of 53 years. The mean total PLFA content of the sludge amended soil was 89.2 ± 22.4 nmol g $soil^{-1}$, considerably higher than that found in the LTSE field sites (**Table 5.7**), and contained high conentrations of branched PLFAs (16:0i, 17:0i, 17:0cy, 18:0br, 17:0 (10Me), and 19:0cy), whereas the concentrations of mono-unsaturated PLFAs (16:1ω9, 16:1ω7c, 16:1ω9) were relatively low. Hence in contrast to the results of Abaye et al. (2005), the sludge amended soils at Ultuna now appeared to be dominated by Gram-positive bacteria. However it should be noted that although these changes were attributed to the presence of heavy metals, the total concentration in sludge amended soils was not reported. Clearly, there is no universal response of soil microorganisms to the presence of heavy metals in soils, therefore observed changes in the PLFA profiles of sludge amended soils should be considered on a site by site basis.

5.6. Conclusions

For the current investigation, values for C_{mic} in sludge amended soil no longer appeared to be significantly different from that in untreated soil, with some exceptions at Woburn and Gleadthorpe. In addition the only significant difference in C_{mic} between contaminated and uncontaminated soil was seen at Gleadthorpe in 2013, where C_{mic} in soil receiving the Cu sludge treatment was lower than in soil receiving the undigested control. Hence, in general, no significant differences were observed between untreated soil, and soils receiving contaminated (Zn and Cu) and uncontaminated (Ctrl1 and Ctrl2) sludge treatments. Therefore, with regards to **Research Question 1**, it can be concluded that application of contaminated sludge treatments does not cause a long-term decrease in C_{mic}, contrary to the posed hypothesis. This is also contrary to the hypothesis posed for **Research Question 4**, as the current statutory limits seem sufficient to prevent long-term decrease in C_{mic} at the LTSE field sites. However, it should be noted that combining the results using meta-analysis, did indicate an overall negative effect on C_{mic}, particularly for Zn, whereas the effect of Cu, although negative, was not statistically significant. Therefore it is expected that any further increase in the total metal concentration at the LTSE field sites would cause noticeable, significant, decreases in soil microbial biomass. However, although the overall amount of C_{mic} appears to be relatively unaffected at concentrations close to the UK statutory limits, clearly there have been significant changes in the microbial community structure due to the application of the contaminated sludge treatments.

Analysis of the fungal biomarker ergosterol indicated that the proportion of the soil microbial biomass comprised of fungal species had increased in soils receiving the contaminated sludge treatments (Zn and Cu) in comparison to those receiving the uncontaminated controls; however the only significant differences occurred at Woburn. This was also confirmed by meta-analysis, which suggested that Cu had a greater effect on the amount of C_{fungi} present in the sludge amended soils. Hence, as fungal species are generally considered to be more tolerant of heavy metal contamination, these results suggest the observed decreases in C_{mic} over the course of experimental Phases I-III are primarily due to the loss of bacterial biomass from the soil.

Significant changes in the PLFA profiles of sludge amended soils, in comparison to untreated soil, were seen at each of the LTSE field sites, with further differences observed between the contaminated and uncontaminated soils. Significant differences were also observed in the PLFA profiles between field sites, indicating that the overall response of the soil microbial communities, to the application of sewage sludge, was not uniform. Again this gives further indication that although the size of the microbial communities (i.e. C_{mic}) in sludge amended soils appear to be returning to that of untreated soil, the historical application of contaminated sewage sludge has caused long-term changes in the soil microbial community structure. This does have implications for **Research Question 4**, if the observed changes in microbial community structure correspond to the loss of species responsible for specific functions within the soil environment, i.e. the production of phosphatase enzymes and the mineralisation of organic phosphorus as an example. In this case the current UK limits do not appear to be sufficient to prevent such changes occurring thereby risking a potential decline in soil quality; this is discussed further in **Section 7.4**. However, although it appears the microbial communities in the contaminated soils at each of the LTSE field sites have become tolerant to Zn and Cu contamination, it cannot be determined whether the observed changes are due to the proliferation of metal tolerant species, or if the indigenous microbial community has now become metal tolerant due to the synthesis of additional PLFAs within the cell membrane.

Cranfield UNIVERSITY

CHAPTER 6

SOIL ENZYMOLOGY

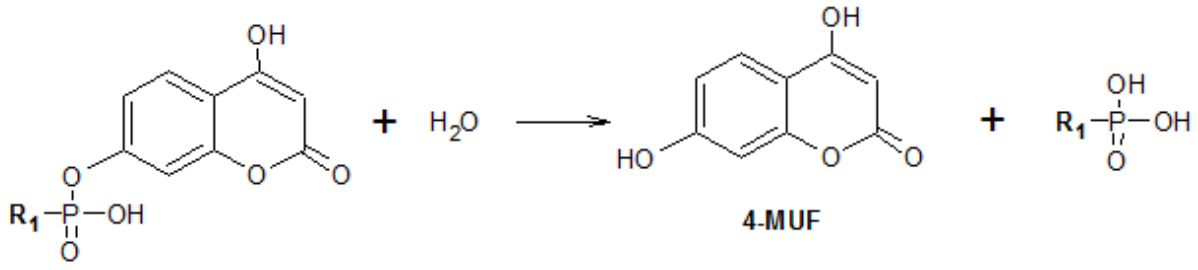
6. SOIL ENZYMOLOGY

6.1. Introduction

The objective of this chapter is to characterise the activities of both phosphomonoesterase and phosphodiesterase enzymes present in untreated and sludge amended soils at each of the Long-Term Sludge Experiment field sites. A series of fluorimetric enzyme assays were carried out in the laboratory in order to measure enzyme pH optima and kinetic parameters (K_M and V_{MAX}) and hence determine the effects of Zn and Cu on phosphatase enzyme activity. The short-term synthesis of phosphatase enzymes following the fresh application of organic phosphorus (in the form of liquid sludge) in contaminated and uncontaminated soils is also discussed.

6.2. Fluorometric Micro-Plate Assay

Phosphomonoesterase and phosphodiesterase enzyme activities (v) were determined as the respective rates of hydrolysis of 4-methylumbelliferyl-phosphate (4-MUF-P), and *bis*-4-methylumbelliferyl-phosphate (*bis*-4-MUF-P), to the highly fluorescent compound 4-methylumbelliferone (4-MUF; **Figure 6.1**). Assay parameters were based on the method described by Marx, et al. (2001). Approximately 1 g (dry matter basis) of field moist soil was weighed out and incubated at 25°C ± 1°C for at least 16 hours. Soils were then suspended in 100 ml of autoclaved deionised water and magnetically stirred for ≥ 5 minutes to ensure a homogenous mixture (10 µg soil μL^{-1}). All assays were carried out using modified universal buffer to control pH. Buffers were prepared by dissolving tris(hydroxymethyl)aminomethane (THAM; 0.01 M), maleic acid (0.01 M), citric acid (0.07 M), and boric acid (0.1 M) in 488 mL of 1 M sodium hydroxide (NaOH) before diluting to 1 L final volume with deionised water. A 50 mL aliquot of buffer was then adjusted to the desired pH by titrating with either 0.2 M hydrochloric acid (HCl) or 0.2 M NaOH. Fluorescence intensity was measured using a



R_1 = OH (4-methylumbelliferyl-phosphate (**4-MUF-P**)); R_1 = 4-MUF (*bis*-4-methylumbelliferyl-phosphate (***bis*-4-MUF-P**))

Figure 6.1:- Hydrolysis of fluorogenic substrate to 4-methylubelliferone (4-MUF).

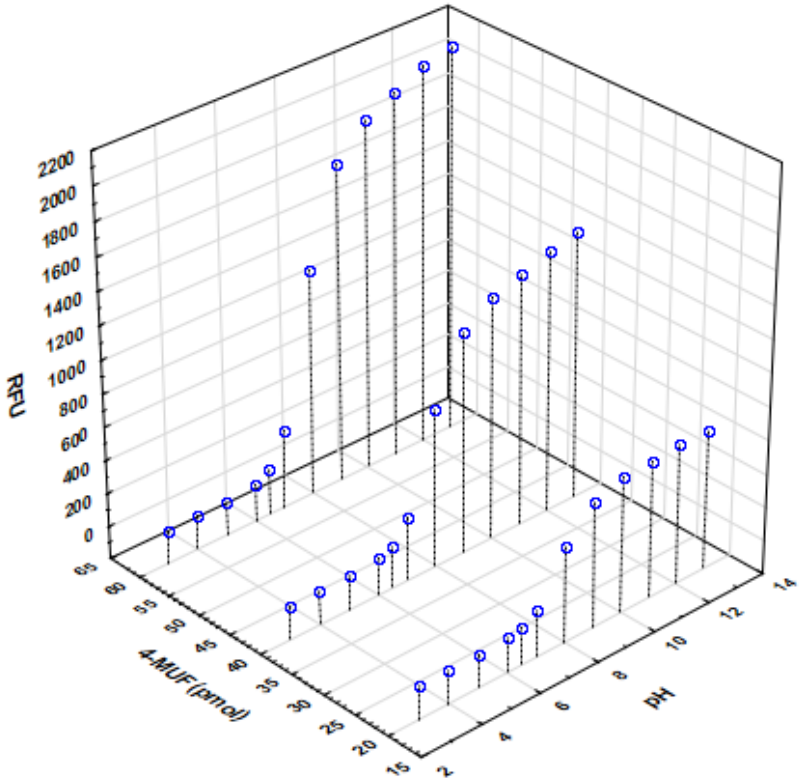


Figure 6.2:- Relationship between 4-MUF fluorescence intensity (RFU) and pH at concentrations of 20, 40, and 60 pmol.

fluorometric micro-plate reader (SpectraMAX – GeminiEM, Molecular Devices) connected to a PC running SoftMax® Pro software (v5.0.1). Measurements were taken every 30 seconds over a period of 30 minutes (n = 61) using excitation and emission wavelengths of 365 and 460 nm, respectively, whilst the temperature of the assay mixture was kept constant at 25°C. Increases in fluorescence intensity (measured in relative fluorescence units (RFU)) were then plotted against time (s) to give the overall rate of hydrolysis (RFU s^{-1}).

6.2.1. Calibration

At constant pH the relationship between fluorescence intensity and 4-MUF concentration is linear, however, 4-MUF fluorescence is extremely pH dependent and a more complex relationship is observed across the pH scale (**Figure 6.2**). Although an approximately linear relationship occurs within the range pH 7-9, fluorescence intensity approaches asymptotes at very acidic (pH < 3) and very alkaline (pH > 10) pH values (Chrost & Krambeck, 1986). Therefore separate calibration graphs were produced for the following pH values: 3, 4, 5, 6, 6.5, 7, 8, 9, 10, 11, 12, and 13. A 10 mM stock solution of 4-MUF was prepared by dissolution in 100 mL methanol. Standard solutions (1 µM) were then prepared by diluting 2.5 µL aliquots to 25 mL final volume in modified universal buffer, adjusted to the required pH. Aliquots (n = 12) of 0, 10, 20, 30, 40, 50, 60, and 70 µL were then dispensed to a clean 96 well (8

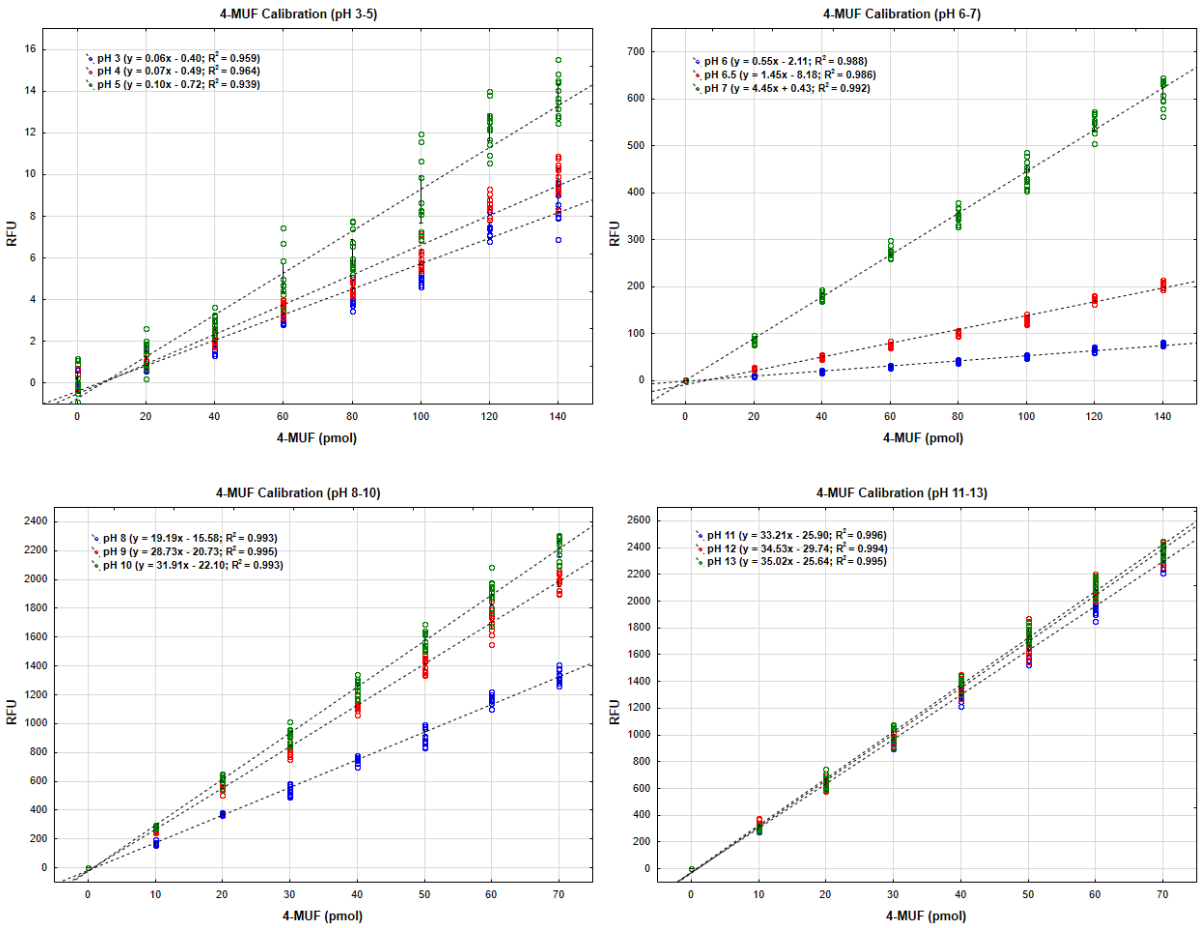


Figure 6.3:- Calibration graphs used for fluorometric micro-plate assay, plotting fluorescence intensity (RFU) against 4-MUF Concentration (pmol) over a range of pH values (pH 3-13). Error bars represent standard error (n = 12).

Table 6.1:- Summary of regression coefficients used to determine phosphatase enzyme activity.

pH	Slope	Intercept	R^2
3	0.061 (0.001)[1]	-0.388 (0.111)	**0.959**
4	0.071 (0.001)	-0.491 (0.120)	**0.964**
5	0.100 (0.003)	-0.720 (0.221)	**0.939**
6	0.553 (0.006)	-2.110 (0.529)	**0.988**
6.5	1.464 (0.019)	-8.178 (1.637)	**0.986**
7	4.450 (0.041)	0.427 (3.420)	**0.992**
8	19.189 (0.166)	-15.579 (6.962)	**0.993**
9	28.734 (0.220)	-20.726 (9.150)	**0.995**
10	31.905 (0.272)	-22.103 (11.345)	**0.993**
11	33.213 (0.231)	-25.908 (9.668)	**0.996**
12	34.530 (0.275)	-29.744 (11.621)	**0.994**
13	35.023 (0.252)	-25.6381 (10.696)	**0.995**

[1]Values in parenthesis are standard errors (n = 12).

× 12) micro-plate and made up to 200 µL final volume with buffer. Final concentrations for calibration were 0, 10, 20, 30, 40, 50, 60, 70 pmol 4-MUF 200 μL^{-1}; however, concentrations were doubled below pH 7 due to low fluorescence. Fluorescence of 4-MUF was measured at 60 second intervals for a period of 10 minutes, then averaged. Each RFU value was background corrected, by subtracting the mean RFU measured at 0 pmol 4-MUF 200 μL^{-1}, RFU was then plotted against 4-MUF concentration (**Table 6.1**; **Figure 6.3**). Estimates of fluorescence quenching (i.e. the reduction in the observed fluorescence

Table 6.2:- Percent of total fluorescence (quenching) observed from bis-4-MUF-P hydrolysis at pH 11, 12, and 13. Values were used to derive mean quenching factors (QF) for each of the LTSE field sites.

	Quenching (%)			
	pH 11	pH 12	pH 13	QF
Auchincruive	50.01 (3.02)	44.42 (2.63)	60.64 (3.47)	0.52
Gleadthorpe	64.16 (3.52)	63.68 (3.25)	64.35 (4.32)	0.63
Hartwood	36.90 (3.69)	32.02 (3.64)	36.80 (3.78)	0.34
Woburn	54.80 (2.58)	47.97 (1.41)	63.02 (3.85)	0.60

[1]Values in parenthesis are standard errors (n = 15).

intensity (RFU) due to the presence of soil particles in the assay mixture (Freeman et al., 1995)) were determined as the mean percentage difference in RFU between *bis*-4-MUF-P hydrolysis in blank (ν_{Blank}) and sample (ν_{Soil}) assay mixtures at pH 11, 12, and 13. Above pH 10, *bis*-4-MUF-P was unstable and readily underwent hydrolysis, hence the differences in the observed rates are primarily due to fluorescence quenching; these measurements were incorporated into assays of phosphodiesterase pH Optima (**Figure 6.4**). Fluorescence quenching was calculated for each soil sample as follows:

$$\text{Quenching (\%)} = \left(\frac{\nu_{Soil}\ (\text{pmol 4-MUF min}^{-1})}{\nu_{Blank}\,(\text{pmol 4-MUF min}^{-1})} \times 100 \right) \qquad (\mathbf{E.6.1})$$

Following which a mean quenching factor (QF) was determined for each field site, in order to adjust the measured activities:

$$\text{QF} = \left(\frac{\sum Quenching\ (\%)}{n} \right) \times \frac{1}{100} \qquad (\mathbf{E.6.2})$$

Quenching factors used for each field site are given in **Table 6.2**. Phosphatase enzyme activity (nmol 4-MUF min^{-1} g soil^{-1}) for subsequent assays was determined using the calibration regression coefficients in **Table 6.1**:

$$\nu\left(\text{RFU 30 min}^{-1}\, m\ \text{g soil}^{-1}\right) = \frac{\nu\left(\text{RFU s}^{-1}\, m\ \text{g soil}^{-1}\right) \times 1800\ \left(\text{s 30 min}^{-1}\right)}{QF} \qquad (\mathbf{E.6.3})$$

$$\nu\left(\text{pmol 4-MUF 30 min}^{-1}\ m\ \text{g soil}^{-1}\right) = \frac{\left(\nu\left(\text{RFU 30 min}^{-1}\, m\ \text{g soil}^{-1}\right) - \text{Intercept (RFU)}\right)}{\text{Slope (RFU pmol 4-MUF}^{-1})} \qquad (\mathbf{E.6.4})$$

$$\nu\left(\text{pmol 4-MUF min}^{-1}\, m\ \text{g soil}^{-1}\right) = \frac{\nu\left(\text{pmol 4-MUF 30 min}^{-1}\, m\ \text{g soil}^{-1}\right)}{30\ (\text{min 30 min}^{-1})} \qquad (\mathbf{E.6.5})$$

$$\nu\left(\text{pmol 4-MUF min}^{-1}\ \text{g soil}^{-1}\right) = \nu\left(\text{pmol 4-MUF min}^{-1}\right) \times \left(\frac{1\ (\text{g soil})}{m\ (\text{g soil})}\right) \qquad (\mathbf{E.6.6})$$

$$\nu\left(\text{nmol 4-MUF min}^{-1}\ \text{g soil}^{-1}\right) = \frac{\nu\left(\text{pmol 4-MUF min}^{-1}\ \text{g soil}^{-1}\right)}{1000\ (\text{pmol nmol}^{-1})} \qquad (\mathbf{E.6.7})$$

where m is the mass of soil used in the assay (500 µg; 0.0005 g). Maximum enzyme activity is proportional to the total concentration of enzyme present in a soil sample (Tabatabai, 1994), however

it was not possible to determine the total concentration of phosphatase enzyme per gram of soil. As a result of this, the true nature of any observed enzyme inhibition could not be determined (**See Section 6.4.2**). There are two possible scenarios that could explain decreases in enzyme activity in soil contaminated with heavy metals. Either the total enzyme concentrations in sludge amended soils are equal and a percentage of that in the contaminated soil is directly inhibited due to binding of heavy metals, or the total enzyme concentration in contaminated soil is reduced due to a reduction in microbial biomass and enzyme synthesis. Enzyme activities were therefore normalised and expressed per mg of C_{mic} (**Section 5.2**), as follows, in order to allow direct comparison between untreated soil and soils receiving contaminated and uncontaminated sludge treatments, as well as between field sites (Johnson et al., 1998; Turner & Haygarth, 2005):

$$\nu\left(\text{nmol 4-MUF min}^{-1}\ \text{mg } C_{mic}{}^{-1}\right) = \frac{\nu\left(\text{nmol 4-MUF min}^{-1}\ \text{g soil}^{-1}\right)}{C_{mic}\ (\text{mg C}_{\text{mic}}\ \text{g soil}^{-1})} \qquad (\mathbf{E.6.8})$$

Expressing enzyme activity per mg of C_{mic} can give an indication as to how enzyme synthesis has changed due to the application of heavy metals. In this case, higher enzyme activity per mg of C_{mic} suggests that phosphorus may be a limiting nutrient in the soil (Johnson et al., 1998). Therefore due to the possible inhibition of phosphatase enzymes by Zn and Cu, enzyme activity in contaminated soils may have to increase in order to sustain an equal quantity of C_{mic}.

6.3. pH Optima Assay

Enzyme activity is markedly influenced by pH, due to changes in the ionisation state of the protein structure which can alter the conformation of the active site, as a result of this each enzyme has an optimum pH value at which maximum enzyme activity is observed (Tabatabai, 1994). However, within the soil environment, pH can also influence the solubility of enzyme and substrate, and the adsorption of the enzyme onto clay particles (Quiquampoix, 2000; Theng 2012). Hence the pH optima of an enzyme can vary with soil properties, therefore it is often recommended that enzyme pH optima is determined for each soil under investigation (Malcolm, 1983; Turner, 2010).

The pH optima of phosphomonoesterase and phosphodiesterase were determined using a method based on that described by Turner (2010). Stock solutions of 4-MUF-P (10 mM) and *bis*-4-MUF-P (10 mM) were prepared by dissolving the respective substrates in either 20 mL deionised water or 20 mL 2-methoxyethanol. Assay solutions (500 µM) were then prepared by diluting 1.25 mL aliquots of the 4- MUF-P/*bis*-4-MUF-P stock solutions to 25 mL final volume in modified universal buffer. Micro-plates were prepared by dispensing 50 µL buffer, 50 µL soil suspension (500 µg soil), and 100 µL assay solution to each micro-plate well, giving a final substrate concentration of 250 µM. Each sample was

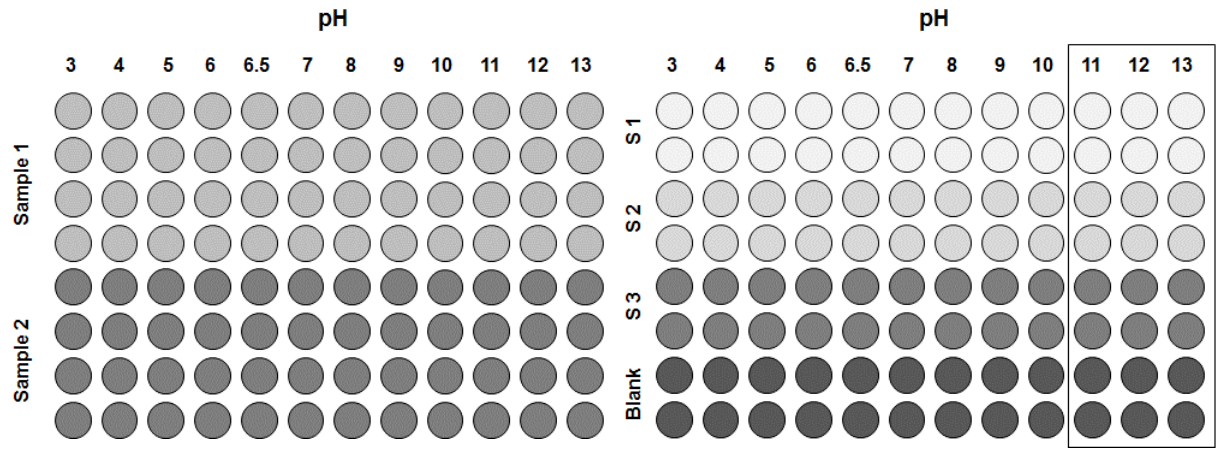


Figure 6.4:- Micro-plate layout used to determine phosphatase enzyme (Phosphomonoesterase (left), Phosphodiesterase (right)) pH optima. Fluorescence quenching was determined as the difference in bis-4-MUF-P hydrolysis between blank and sample assay mixtures at pH 11, 12, and 13.

replicated four times (n = 4) for each pH value (**Figure 6.4**); blank samples were also analysed by dispensing 50 µL deionised water instead of soil suspension.

6.3.1. Phosphomonoesterase pH Optima Results (2014)

In all cases soils showed considerable acid phosphomonoesterase activity below pH 7, with negligible activity in the alkaline pH region; although activity in this region was greater at the English sites in comparison to the Scottish sites managed as grassland. Maximum enzyme activity (v_1) was predominantly between pH 3-5, with a secondary pH optima (v_2) at pH 6.5; although, in some cases v_2 was not discernible at the English sites. The relative magnitude between activities (v_1/v_2) ranged from approximately 2.20 in the untreated soils at Auchincruive and Gleadthorpe to 1.01 in soils receiving the Cu sludge treatment at Hartwood (**Table 6.3**), with an overall mean value of 1.6 ± 0.1. However, it was not possible to determine whether the two pH optima observed were due to conformational changes, caused by the immobilisation of acid phosphatase on soil organic matter and clay particles (Quiquampoix, 2002), the presence of additional isozymes catalysing the same reaction (Nannipieri et al., 1982), or both.

Auchincruive

At pH 6.5 the activity of phosphomonoesterase at Auchincruive was significantly (p <0.05) greater in soil receiving the Cu sludge treatment in comparison to the untreated soil (**Figure 6.5**). Enzyme activity was also higher in soil receiving the undigested control, suggesting an increase in activity due to sludge application, however the measured activity was not significantly different to that in the untreated soil or soil receiving the Cu sludge treatment (**Table 6.3**). No significant differences in activity were observed between soils receiving the digested sludge treatments (Zn and Ctrl1) and the untreated soil;

Table 6.3:- pH optima of phosphomonoesterase enzyme activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) measured in sludge amended and untreated soil at each of the LTSE field sites in 2014.

	Phosphomonoesterase Activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1})			
Sludge Treatment	**pH 3**	**pH 4**	**pH 6.5**	**Ratio**
AUC/Zn	ND[1]	99.49 (16.07)[a][2][3]	64.13 (16.46)[a]	1.63 (0.17)
AUC/Ctrl1	ND	118.51 (11.43)[a]	85.08 (8.60)[a]	1.36 (0.22)
AUC/Cu	ND	109.25 (10.63)[b]	83.37 (12.05)[b]	1.39 (0.02)
AUC/Ctrl2	ND	100.92 (1.38)[b]	59.86 (6.52)[bc]	1.73 (0.21)
AUC/NS	ND	105.54 (10.44)[ab]	49.16 (6.44)[ac]	2.17 (0.18)
GLE/Zn	ND	100.59 (3.11)[a]	56.56 (6.08)[a]	1.81 (0.15)
GLE/Ctrl1	ND	87.57 (15.36)[a]	54.43 (4.10)[a]	2.03 (0.10)
GLE/Cu	ND	82.02 (3.11)[bc]	40.62 (3.17)[b]	1.59 (0.20)
GLE/Ctrl2	ND	53.99 (2.39)[b]	43.25 (2.77)[b]	1.26 (0.12)
GLE/NS	ND	98.69 (14.10)[ac]	45.50 (3.60)[ab]	2.18 (0.33)
HAR/Zn	101.36 (2.46)[a]	ND	99.84 (13.56)[a]	1.40 (0.13)
HAR/Ctrl1	76.84 (9.61)[a]	ND	67.63 (9.50)[ab]	1.01 (0.13)
HAR/Cu	85.71 (4.74)[cd]	ND	92.84 (15.63)[c]	1.78 (0.28)
HAR/Ctrl2	116.48 (10.82)[d]	ND	77.90 (9.42)[c]	1.51 (0.04)
HAR/NS	74.41 (6.89)[bc]	76.13 (4.91)	54.93 (5.90)[bc]	1.40 (0.09)
WOB/Zn	106.22 (13.33)	ND	78.09 (11.16)[a]	1.44 (0.28)
WOB/Ctrl1	ND	116.06 (16.84)[a]	84.29 (13.58)[a]	1.56 (0.17)
WOB/Cu	ND	122.98 (33.74)[b]	77.23 (13.35)[b]	1.39 (0.05)
WOB/Ctrl2	ND	97.90 (10.94)[b]	50.70 (2.15)[b]	1.94 (0.23)
WOB/NS	ND	95.63 (3.22)[ab]	62.84 (6.60)[ab]	1.56 (0.18)

[1]Not Detected. [2]Values in parenthesis are standard errors (n = 3). [3] Values without corresponding letters denotes statistical significance at $\alpha = 0.05$, note comparisons between different sludge types (i.e. digested (Zn and Ctrl1) and undigested (Cu and Ctrl2)) have not been made.

though in both cases enzyme activity had increased. Nor was the difference in activity observed between the two soils statistically significant, despite the mean activity in soil receiving the Zn sludge treatment being almost 30 % lower (**Figure 6.5**).

At pH 4, no significant differences were observed between any of the treatments (**Table 6.3**), although, again, the mean activity in soil receiving the Zn sludge treatment was almost 25 % lower than activity in soil receiving the digested control (**Figure 6.5**).

Gleadthorpe

As mentioned previously, no discernible pH optima could be seen at pH 6.5 in either the untreated soil, or soil receiving the Cu sludge treatment (**Figure 6.6**). Nevertheless, no significant differences in phosphomonoesterase activity were observed between any of the soils analysed at this pH value (**Table 6.3**). Most noticeable however, is the apparent lack of enzyme activity at pH 4 in soil receiving the undigested control (**Figure 6.6**), which was significantly ($p < 0.01$) lower than in the untreated soil. In addition, the enzyme activity in soil receiving the Cu sludge treatment was approximately 17 % lower in comparison to the untreated soil, however the difference was not significant. Similarly, phosphomonoesterase activity appeared to be lower in soil receiving the digested control in comparison to untreated soil and soil receiving the Zn sludge treatment however in this case none of the differences were statistically significant (**Table 6.3**).

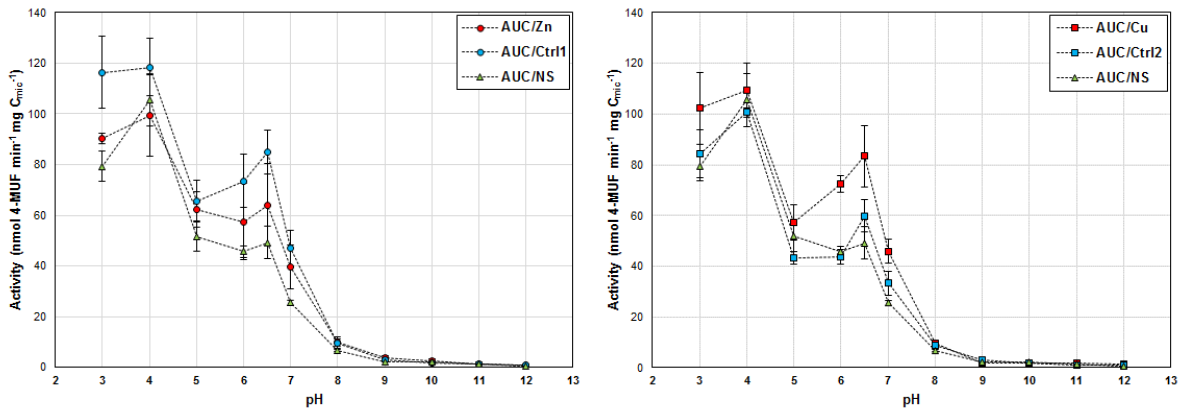


Figure 6.5:- Change in phosphomonoesterase activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) with pH (3-13) in soils receiving digested (left) and undigested (right) sludge treatments at the Auchincruive (AUC) field site in 2014. Error bars represent standard error (n = 3).

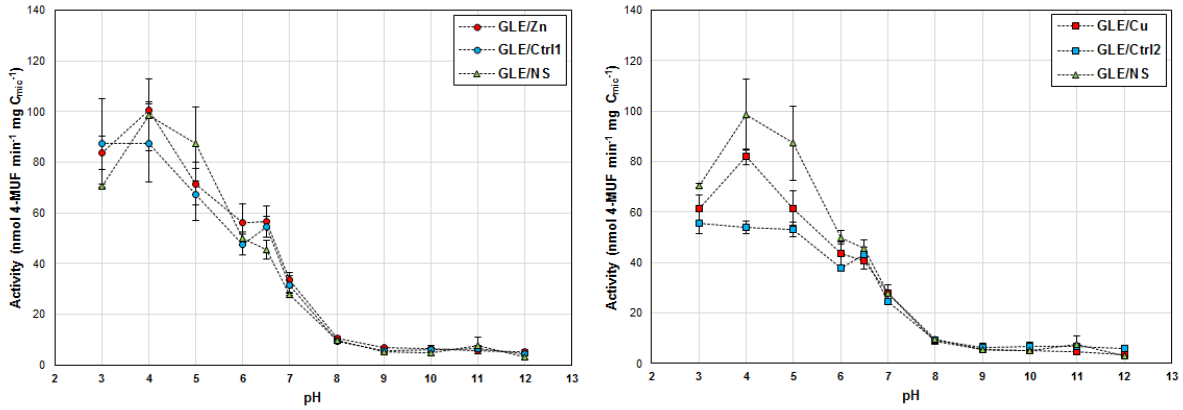


Figure 6.6:- Change in phosphomonoesterase activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) with pH (3-13) in soils receiving digested (left) and undigested (right) sludge treatments at the Gleadthorpe (GLE) field site in 2014. Error bars represent standard error (n = 3).

Hartwood

With the exception of the untreated soil, the predominant pH optima in soils receiving the sludge treatments at Hartwood appear to have shifted to pH 3 (**Figure 6.7**). At this pH, phosphomonoesterase activity was significantly higher in both soils receiving the digested sludge treatments (Zn (p <0.01) and Ctrl1 (p <0.05)) in comparison to the untreated soil, however the difference between them was not significant (**Table 6.3**). Similarly, enzyme activity was higher in soils receiving the undigested sludge treatments, however only in soil receiving the undigested control was the difference in activity significant (p <0.05). The activity measured in soil receiving the Cu sludge treatment was approximately 25 % lower in comparison to soil receiving the undigested control, but again the difference between the sludge amended soils was not significant (**Table 6.3**).

At pH 6.5, the activity of phosphomonoesterase was greater in both soils receiving the contaminated sludge treatments (Zn and Cu), in comparison to untreated soil and soils receiving the uncontaminated controls (**Figure 6.7**). However the only significant (p <0.05) difference observed was between the untreated soil and the soil receiving the Zn sludge treatment (**Table 6.3**).

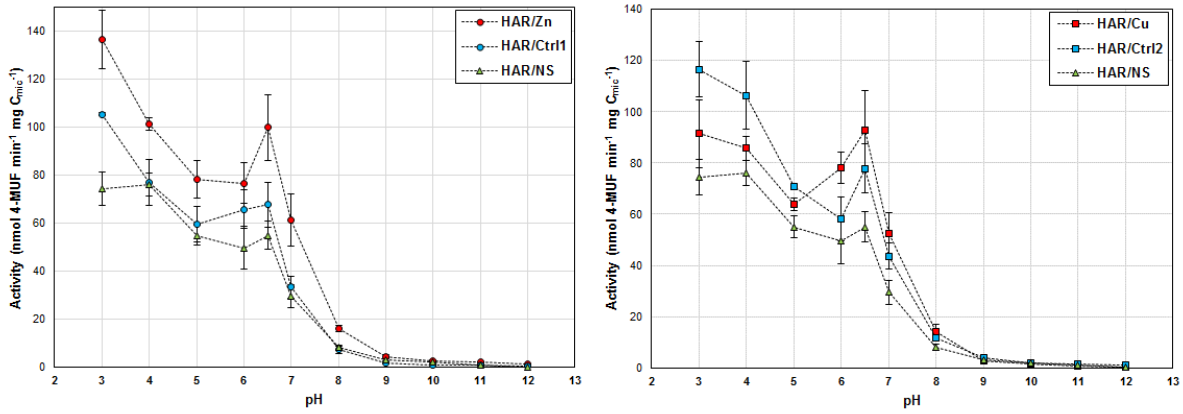


Figure 6.7:- Change in phosphomonoesterase activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) with pH (3-13) in soils receiving digested (left) and undigested (right) sludge treatments at the Hartwood (HAR) field site in 2014. Error bars represent standard error (n = 3).

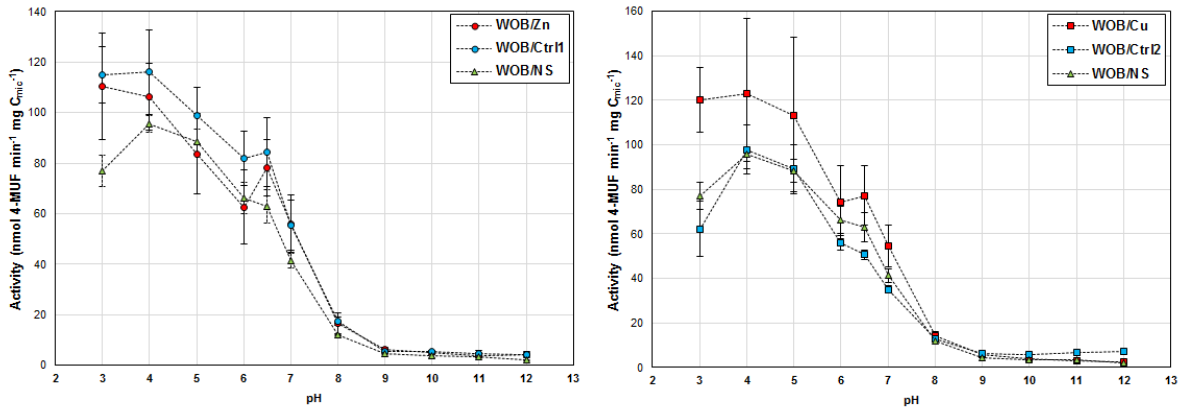


Figure 6.8:- Change in phosphomonoesterase activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) with pH (3-13) in soils receiving digested (left) and undigested (right) sludge treatments at the Woburn (WOB) field site in 2014. Error bars represent standard error (n = 3).

Woburn

As with Gleadthorpe, no discernible pH optima could be seen at pH 6.5 in the either the untreated soil or soils receiving the undigested sludge treatments (Cu and Ctrl2) at the Woburn field site. In the case of soil receiving the Zn sludge treatment the predominant pH optima had also shifted to pH 3, whereas for the remaining soils maximum enzyme activity was seen at pH 4 (**Figure 6.8**). However, no significant differences in enzyme activity were observed between any of the soils at either of the pH values (**Table 6.3**).

6.3.2. Phosphodiesterase pH Optima Results (2014)

Below pH 7, phosphodiesterase activity steadily increased, at all of the field sites, to an apparent maximum at pH 3 (**Figure 6.9**). No significant differences in enzyme activity were observed between any of the soils at Auchincruive or Gleadthorpe. At Hartwood, phosphodiesterase activity appeared to have increased markedly in soil receiving the Zn sludge treatment and was significantly

Table 6.4:- pH optima of phosphodiesterase enzyme activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) measured in sludge amended and untreated soil at each of the LTSE field sites in 2014.

	Phosphodiesterase Activity (nmol 4-MUF min_{-1} mg C_{mic}^{-1})
Sludge Treatment	**pH 3**
AUC/Zn	115.12 (20.14)[a][1][2]
AUC/Ctrl1	113.67 (11.87)[a]
AUC/Cu	98.70 (10.09)[b]
AUC/Ctrl2	98.09(3.28)[b]
AUC/NS	94.04 (11.97)[ab]
GLE/Zn	107.48 (12.58)[a]
GLE/Ctrl1	97.03 (8.85)[a]
GLE/Cu	86.14 (13.31)[b]
GLE/Ctrl2	87.49 (17.07)[b]
GLE/NS	78.18 (7.86)[ab]
HAR/Zn	193.64 (25.82)[a]
HAR/Ctrl1	125.75 (11.22)[b]
HAR/Cu	77.34 (9.13)[c]
HAR/Ctrl2	110.32 (1.26)[d]
HAR/NS	83.88 (6.64)[bc]
WOB/Zn	166.10 (9.04)[a]
WOB/Ctrl1	182.93 (37.29)[a]
WOB/Cu	121.19 (13.39)[c]
WOB/Ctrl2	110.79 (17.54)[c]
WOB/NS	74.26 (3.02)[bc]

[1]Values in parenthesis are standard errors (n = 3). [2] Values without corresponding letters denotes statistical significance at α = 0.05, note comparisons between different sludge types (i.e. digested (Zn and Ctrl1) and undigested (Cu and Ctrl2)) have not been made.

higher in comparison to untreated soil (p <0.01) and soil receiving the digested control (p <0.05; **Table 6.4**). Whereas, the application of undigested sludge appears to have had the opposite effect, i.e. phosphodiesterase activity in soil receiving the Cu sludge treatment was significantly (p <0.05) lower in comparison to soil receiving the undigested control, in which phosphatase enzyme activity was significantly (p <0.05) greater than in the untreated soil (**Table 6.4**). Similar increases were seen in soils receiving the digested sludge treatments (Zn and Ctrl1) at the Woburn field site, with phosphodiesterase activity in soil receiving the Zn sludge treatment and in soil receiving the digested control both significantly (p <0.05) greater than the untreated soil. In addition, although phosphodiesterase activity was higher in soils receiving the undigested sludge treatments (Cu and Ctrl2), no significant differences were observed between any of the soils (**Table 6.4**).

However, due to the heterogeneous nature of soil, the assay mixtures in this case contained both phosphodiesterase and phosphomonoesterase enzymes. Although it is often assumed that the contribution of phosphomonoesterase in assays of phosphodiesterase activity is negligible, Sirová et al. (2013) give convincing evidence that indicates the use of artificial phosphodiester substrates, in this case *bis*-4-MUF-P, may lead to an overestimation of phosphodiesterase activity, due to subsequent hydrolysis by phosphomonoesterase of the 4-MUF-P reaction product (**Figure 6.1**). Any phosphorus present in the assay mixture, following incubation, can only be due to phosphomonoesterase activity, therefore, it is suggested that the ratio of products (4-MUF:P) is determined, and the measured activities of phosphodiesterase be adjusted accordingly to control for the activity of

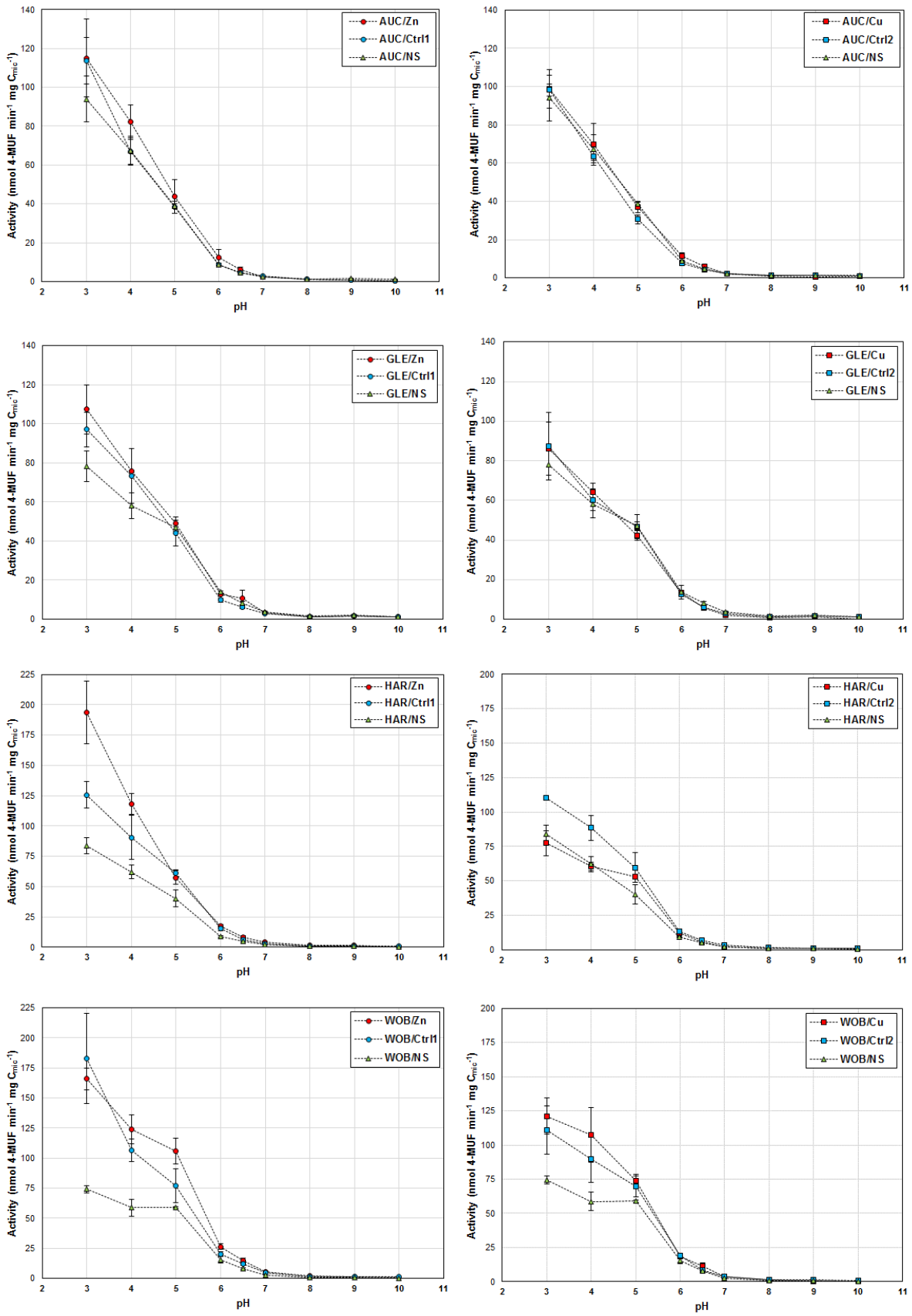


Figure 6.9:- Change in phosphodiesterase enzyme activity with pH (3-13) at each of the Long-Term Sludge Experiment Sites: Auchincruive (AUC; August 2014), Gleadthorpe (GLE; June 2014), Hartwood (HAR; July 2014), and Woburn (WOB; April 2014). Error bars represent standard error (n = 3).

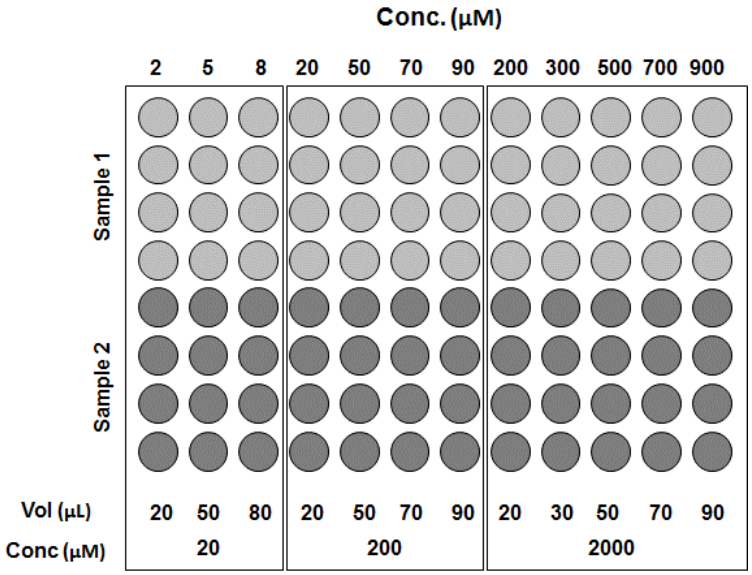


Figure 6.10:- Micro-plate layout used to determine phosphatase enzyme kinetics.

phosphomonoesterase. It should therefore be noted that the results presented here do not take into account the activity of phosphomonoesterase, and therefore potentially overestimate the activity of phosphodiesterase at the LTSE field sites; especially since both enzymes appear to show considerable activity at acidic pH values. Hence further discussion of these results must be done with a degree of caution.

6.4. Kinetic Assay

Assay solutions (2000, 200, and 20 µM) were prepared by diluting 5000, 500, and 50 µL aliquots of 10 mM 4-MUF-P stock solution to 25 mL final volume in modified universal buffer, adjusted to pH 6.5. Micro-plates were prepared by dispensing 50 µL soil suspension (500 µg soil), followed by aliquots of increasing volume of each assay solution (**Figure 6.10**); blank samples were also analysed by dispensing 50 µL deionised water instead of soil suspension. Assay mixtures were then made up to 200 µL final volume with modified universal buffer (pH 6.5). Final concentrations for assay mixtures were as follows: 2, 5, 8, 20, 50, 70, 90, 200, 300, 500, 700, and 900 µM.

6.4.1. Michaelis-Menten Model of Enzyme Kinetics

The rate of enzyme activity in soils, such as phosphate ester hydrolysis (**Figure 6.1**), is largely determined using the Michaelis-Menten model of enzyme kinetics (Michaelis & Menten, 1913). This model assumes that an enzyme (E) and substrate (S) combine to form a stable intermediate complex (ES) before the formation of reaction products (P) occurs. Formation of the ES complex is assumed to

be reversible and in equilibrium with the formation of the free enzyme (E_f) and free substrate (S_f). Therefore the overall reaction proceeds as follows:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (\mathbf{E.6.9})$$

where k_1, k_{-1}, and k_2, are the rate constants used to determine reaction velocities (ν):

$$\nu_1 = k_1[E_f][S_f] \qquad (\mathbf{E.6.10})$$

$$\nu_{-1} = k_{-1}[ES] \qquad (\mathbf{E.6.11})$$

$$\nu_2 = k_2[ES] \qquad (\mathbf{E.6.12})$$

Assuming the total substrate concentration is in excess of the total enzymes concentration ([S] >> [E]), and remains constant throughout the reaction ($[S_f] = [S]$), and that the reaction has reached a '*steady state*', i.e. the enzyme is completely saturated with substrate and the rate of ES formation (**E. 6.10**) is equal to the rate of ES disappearance (**E. 6.11** and **E. 6.12**), then:

$$k_1[E_f][S_f] = [ES](k_{-1} + k_2) \qquad (\mathbf{E.6.13})$$

An expression for [ES] can then be obtained by rearranging to give:

$$[ES] = \frac{[E_f][S_f]}{\left(\frac{k_{-1} + k_2}{k_1}\right)} = \frac{[E_f][S]}{K_M} \qquad (\mathbf{E.6.14})$$

where K_M is the 'Michaelis constant' with the same units as the substrate concentration. The free enzyme concentration is equal to:

$$[E_f] = [E] - [ES] \qquad (\mathbf{E.6.15})$$

which can be substituted into **E. 6.14** and rearranged to give:

$$[ES] = \frac{[E][S]}{[S] + K_M} \qquad (\mathbf{E.6.16})$$

Substituting this expression into **E. 6.12** then gives:

$$\nu_2 = k_2 \frac{[E][S]}{[S] + K_M} \qquad (\mathbf{E.6.17})$$

The assumptions of '*steady state'* and [S] >> [E] means the contribution of K_M to **E. 6.17** can be considered negligible and [S] now cancels from the equation (Copeland, 2000). Hence the rate of product formation now follows zero order kinetics and is no longer dependent on substrate

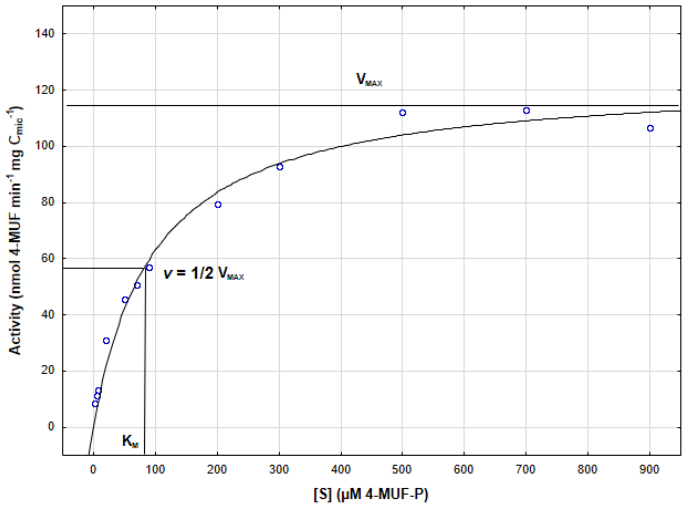


Figure 6.11:- Michaelis-Menten plot showing enzyme activity (v) as a function of substrate concentration ([S]). At concentrations far greater than K_M the reaction follows zero order kinetics and v is independent of [S].

concentration. At this point it can be assumed that the enzyme is completely saturated with substrate and the reaction has reached its maximum velocity (V_{MAX}):

$$V_{MAX} = k_2[E] \qquad (\mathbf{E.6.18})$$

Substituting **E. 6.18** into **E. 6.17** then gives the Michaelis-Menten equation

$$v = \frac{V_{MAX}[S]}{[S] + K_M} \qquad (\mathbf{E.6.19})$$

The Michaelis-Menten equation describes the rate of product formation by enzyme activity as a function of substrate concentration (Copeland, 2000). Plotting v against [S] gives a curve showing the change in reaction velocity as the substrate concentration is increased (**Figure 6.11**). At lower substrate concentrations ([S] << K_M) the reaction follows pseudo first order kinetics and v increases linearly with [S]. At the point where [S] is equal to K_M, the Michaelis-Menten equation becomes:

$$v = \frac{V_{MAX}[S]}{2[S]} = \frac{V_{MAX}}{2} \qquad (\mathbf{E.6.20})$$

Therefore K_M can be defined as the substrate concentration at which half of the maximum reaction velocity is obtained (**Figure 6.11**), giving an indication of the binding affinity between the enzyme and substrate (Copeland, 2000).

6.4.2. Enzyme Inhibition

Enzyme inhibitors (I), such as heavy metals, can affect enzyme kinetics in a number of ways. Competitive inhibition occurs when an inhibitor binds to an enzyme molecule in such a way that

prevents the binding of the substrate, often due to steric hindrance or distortion of the active site (Copeland, 2000; Tabatabai, 1994). Therefore, rather than proceeding as shown in **E. 6.9** enzyme activity may proceed as follows:

$$E + I \underset{K_i = k_1/k_{-1}}{\rightleftharpoons} EI \qquad (\mathbf{E.6.21})$$

where K_i is the inhibitor constant, and determines the relative concentrations of free enzyme (E), and the enzyme-inhibitor complex (EI). Since both substrate and inhibitor are competing to bind to the free enzyme the extent of inhibition is determined by the relative concentrations and binding affinities of the substrate and inhibitor. Hence when the inhibitor concentration is less than the total enzyme concentration a proportion of free enzyme will still remain active. In this situation V_{MAX} is unaffected at high substrate concentrations, however the concentration of substrate required to reach half of the maximum velocity (K_M) increases, due to the competition between substrate and inhibitor for the free enzyme (Coepland, 2000; Tabatabai, 1994).

Non-competitive inhibition occurs when the binding of an inhibitor does not prevent the formation of an enzyme-substrate complex, but does distort the enzyme in such a way that formation of reaction products is prevented. Only when the inhibitor dissociates to leave an enzyme-substrate complex (ES) can the reaction proceed. Therefore, enzyme activity (**E. 6.9**) proceeds as follows:

$$E + I \underset{K_i = k_1/k_{-1}}{\rightleftharpoons} EI + S \underset{K_S = k_2/k_{-2}}{\rightleftharpoons} EIS \underset{K_i = k_3/k_{-3}}{\rightleftharpoons} ES + I \qquad (\mathbf{E.6.21})$$

where EIS is the enzyme-inhibitor-substrate complex, and K_S is the equilibrium constant for enzyme substrate binding. Since substrate binding is unaffected there is no change observed in K_M. As a result of this, increasing substrate concentration does not change the extent of inhibition, therefore in contrast to competitive inhibition, a decrease in V_{MAX} is observed in proportion to the amount of total enzyme present as ESI complexes (Copeland, 2000; Tabatabai, 1994). Uncompetitive inhibition can also occur if the inhibitor is only able to bind with the enzyme once an enzyme-substrate complex has already formed. In this case, both K_M and V_{MAX} are decreased (Copeland, 2000).

However, as mentioned above, V_{MAX} is also proportional to the total enzyme concentration ([E]) present within the system under investigation (**E. 6.18**). Therefore determining the mechanisms of inhibition requires that the two systems being compared, inhibited and uninhibited, contain the same total concentration of enzyme. Unfortunately, there is currently no reliable way to quantify the total concentration of an enzyme within the soil environment; although extracting enzymes from soil is possible (Fornasier et al. 2011). Therefore as mentioned previously, there are two possible scenarios that could explain decreases in enzyme activity in soil contaminated with heavy metals. Either the total enzyme concentration in sludge amended soils are equal and a percentage of that in the contaminated

Table 6.5:- Kinetic parameters (K_M and V_{MAX}) determined for phosphomonoesterase activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) measured in sludge amended and untreated soil at each of the LTSE field sites in 2014.

	Michaelis-Menten Model		
Sludge Treatment	**K_M (µM 4-MUF-P)**	**V_{MAX} (nmol 4-MUF min^{-1} mg C_{mic}^{-1})**	**R^2**
AUC/Zn	135.32 (30.06)[a][1][2]	103.27 (15.00)[a]	0.960 (0.01)
AUC/Ctrl1	185.20 (27.97)[a]	128.68 (8.11)[a]	0.971 (0.01)
AUC/Cu	132.65 (6.91)[b]	96.41 (9.52)[b]	0.982 (0.002)
AUC/Ctrl2	154.56 (13.07)[c]	72.13 (3.56)[b]	0.981 (0.004)
AUC/NS	189.51 (18.76)[ac]	90.54 (11.01)[ab]	0.977 (0.006)
GLE/Zn	124.50 (20.82)[a]	105.10 (13.96)[a]	0.956 (0.004)
GLE/Ctrl1	159.47 (12.95)[a]	91.59 (6.67)[a]	0.976 (0.01)
GLE/Cu	131.08 (18.52)[b]	71.59 (1.52)[b]	0.966 (0.007)
GLE/Ctrl2	135.98 (16.02)[b]	74.53 (5.63)[b]	0.981 (0.003)
GLE/NS	148.95 (18.77)[ab]	72.95 (4.47)[ab]	0.961 (0.010)
HAR/Zn	157.31 (27.70)[a]	167.71 (31.33)[a]	0.979 (0.002)
HAR/Ctrl1	166.44 (12.95)[a]	148.15 (11.47)[a]	0.963 (0.02)
HAR/Cu	172.66 (33.63)[b]	176.29 (35.73)[b]	0.978 (0.01)
HAR/Ctrl2	115.44 (19.33)[b]	106.01 (13.37)[b]	0.988 (0.002)
HAR/NS	164.97 (8.38)[ab]	132.02 (18.92)[ab]	0.978 (0.01)
WOB/Zn	ND[3]	ND	ND
WOB/Ctrl1	76.22 (20.55)[a]	171.16 (4.69)[a]	0.982 (0.01)
WOB/Cu[1]	107.74[b]	152.58[b]	0.988
WOB/Ctrl2	117.16 (0.07)[b]	139.94 (7.61)[b]	0.972 (0.002)
WOB/NS	97.48 (8.31)[ab]	124.31 (11.83)[ab]	0.980 (0.001)

[1]Values in parenthesis are standard errors (n = 3; except for WOB/Cu where n = 1). [2] Values without corresponding letters denotes statistical significance at α = 0.05, note comparisons between different sludge types (i.e. digested (Zn and Ctrl1) and undigested (Cu and Ctrl2)) have not been made. [3]Not Determined.

soil is directly inhibited due to non-competitive inhibition, or the total enzyme concentration in contaminated soil is reduced due to a reduction in microbial biomass and enzyme synthesis.

The data presented in **Chapter 3** shows that application of the Cu sludge treatment has caused significant increases in organically bound Cu, whereas concentrations of exchangeable, soluble, Cu appear to be negligible (**Section 3.5.4**). Hence, it is more likely that Cu will interact directly with phosphatase enzymes within the soil environment. Similarly, significant increases in organically bound Zn were also observed in soils receiving the Zn sludge treatment, though these increases were lower in comparison to Cu. However, significant increases in exchangeable Zn, in comparison to untreated soil and soil receiving the digested control, were observed in soils receiving the Zn sludge treatment (**Section 3.5.2**). Furthermore, application of the Zn sludge treatment appears to have caused an overall decrease in C_{mic} (**Section 5.2.3**), hence Zn could potentially inhibit the activity of phosphatase enzymes via both of the scenarios mentioned above. However, given that phosphatase enzyme are stabilised and remain active within the soil environment (**See Section 1.5**) it is more likely that organically bound metals will cause greater inhibition. Therefore with regards to **Research Question 2**, it appears that Cu will have a greater effect on phosphatase enzyme activity in comparison to Zn.

6.4.3. Phosphomonoesterase Enzyme Kinetics: Michaelis-Menten Model (2014)

Kinetic parameters (K_M and V_{MAX}) were determined for both sludge amended and untreated soils at each of the LTSE field sites by plotting enzyme activity (ν nmol 4-MUF min^{-1} mg C_{mic}^{-1}) against substrate

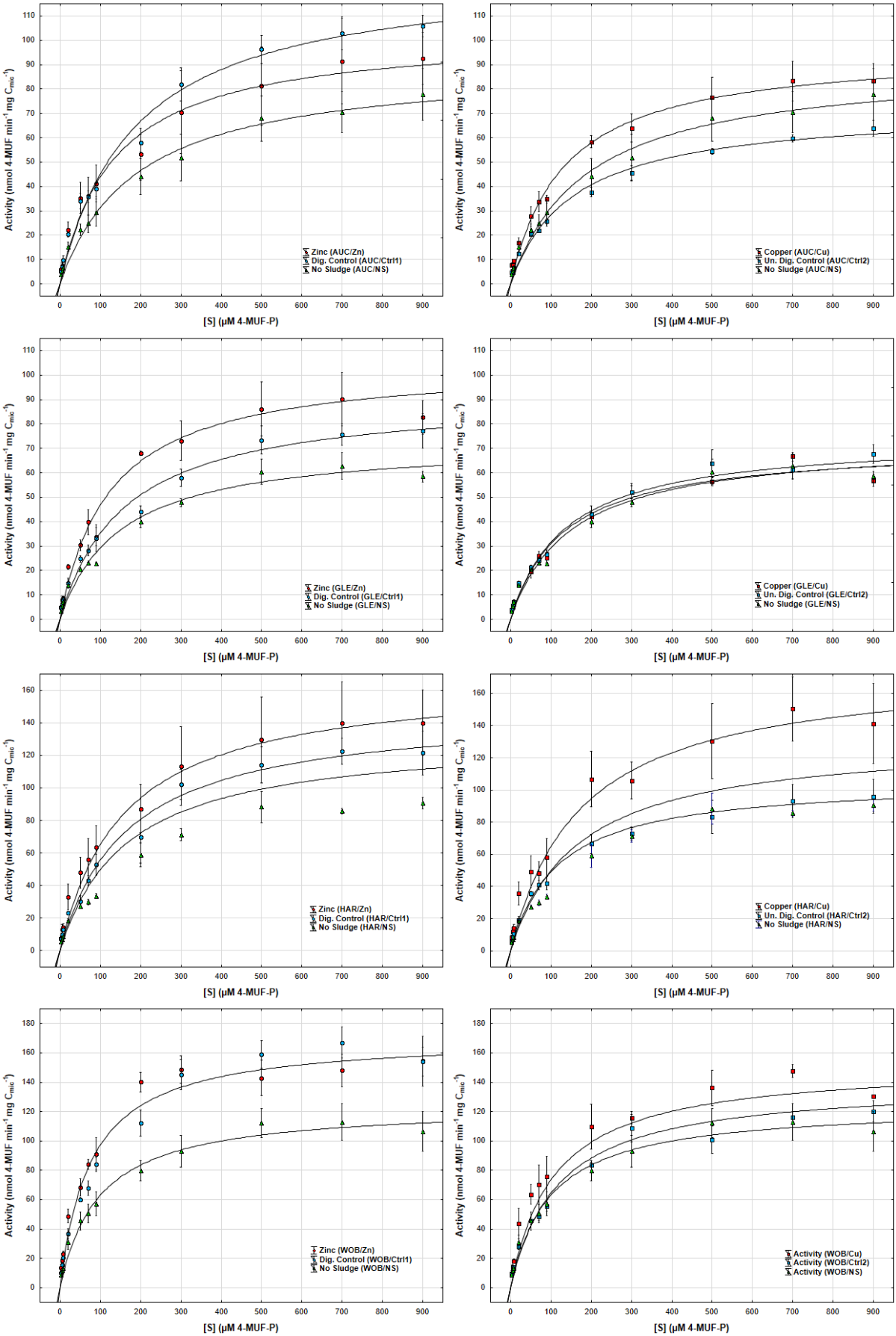


Figure 6.12:- Michaelis-Menten plots showing change in phosphomonoesterase enzyme activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) with increasing substrate concentration (µM 4-MUF-P) in soils receiving digested (left) and undigested (right) sludge treatments at each of the Long-Term Sludge Experiment Sites in 2014. Error bars represent standard error (n = 3).

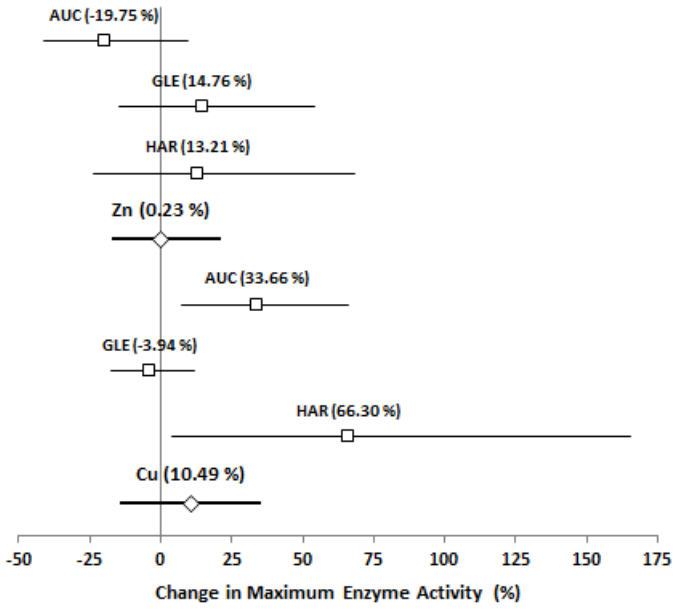


Figure 6.13:- Forest plot showing the change in maximum enzyme activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) in soils receiving the Zn and Cu sludge treatments, in comparison to uncontaminated controls (Ctrl1 and Ctrl2), across three of the LTSE fields sites (Auchincruive (AUC), Gleadthorpe (GLE), and Hartwood (HAR)) during 2014. Horizontal lines represent 95 % confidence intervals. Effects are not statistically significant (p <0.05) if the 95 % confidence interval crosses the centre line.

concentration ([S] μM) and fitting the Michaelis-Menten equation to the data using Statistica (v12.5.192.7; StatSoft, Inc.). Kinetic parameters for each site are summarised in **Table 6.5**.

At each of the LTSE field sites, higher values for V_{MAX} were observed in soils receiving the digested sludge treatments (Zn and Ctrl1) in comparison to untreated soil (**Figure 6.12**); however at no point were any of the increases statistically significant (**Table 6.5**). At Auchincruive, V_{MAX} was higher in soil receiving the digested control in comparison to soil receiving the Zn sludge treatment, whereas the opposite effect was observed at Hartwood and Gleadthorpe (**Figure 6.12**). It should be noted that the Michaelis-Menten equation could not be fitted to the enzyme activity data obtained for soil receiving the Zn sludge treatment at Woburn. With the exception of the Gleadthorpe site, application of the Cu sludge treatment caused an increase in V_{MAX} in comparison to untreated soil at each of the LTSE field sites (**Figure 6.12**). An increase in V_{MAX} was also seen in soil receiving the undigested control at Woburn, whereas at the Scottish field sites, V_{MAX} was lower in comparison to untreated soil; however again, no significant differences were observed. In addition, the only significant difference for the Michaelis constant (K_M) was seen at Auchincruive, where K_M values in soil receiving the Cu sludge treatment were significantly (p <0.05) lower in comparison to the untreated soil (**Table 6.5**).

Maximum enzyme activity in the untreated soil was significantly (p <0.05) greater at Hartwood in comparison to that at Auchincruive and Gleadthorpe, with V_{MAX} determined for the untreated soil at Woburn also significantly (p <0.05) greater in comparison to Gleadthorpe. The Michaelis constant determined at Woburn was also significantly (p <0.05) lower in comparison to the remaining field sites,

indicating a greater binding affinity of phosphomonoesterase for the 4-MUF-P substrate at this site. No significant difference in kinetic parameters were seen in soils receiving the Zn sludge treatment, whereas for soil receiving the digested sludge treatment, V_{MAX} at Woburn (p <0.001), as well as both of the Scottish sites (Auchincruive (p <0.05) and Hartwood (p <0.01)) was significantly greater in comparison to Gleadthorpe; V_{MAX} at Woburn was also significantly (p <0.05) greater in comparison to Auchincruive. Again the lowest value for K_M in soil receiving the digested control was seen at Woburn, which was significantly (p <0.05) lower in comparison to the other sites. For soils receiving the undigested sludge treatments (Cu and Ctrl2), V_{MAX} in soil receiving the Cu sludge treatment at Hartwood was significantly (p <0.05) greater in comparison to that of Auchincruive and Gleadthorpe. Whereas for soil receiving the undigested control, values for V_{MAX} determined for both Woburn (p <0.01) and Hartwood (p <0.05), were significantly greater in comparison to Auchincruive and Gleadthorpe; V_{MAX} at Woburn was also significantly (p <0.05) higher in comparison to Hartwood. No significant differences in K_M were observed for soils receiving the undigested sludge treatments.

Where possible, results were combined using meta-analysis (**See Section 2.4.2**) to determine the overall effects of Zn and Cu contamination on V_{MAX}. In this case, the effect of Cu on V_{MAX} was statistically significant at the Scottish sites, however the overall effect was not significant. Whereas Zn appeared to have no overall effect on V_{MAX} in soils receiving the Zn sludge treatment (**Figure 6.13**).

Possible deviations from the Michaelis-Menten model of enzyme kinetics were investigated using the 'Eadie-Hofstee' linear transformation of **E. 6.8**:

$$\nu = V_{MAX} - K_M\left(\frac{\nu}{[S]}\right) \qquad (\mathbf{E\ 6.21})$$

Plotting ν against $\frac{\nu}{[S]}$ should give a straight line from which it is possible to determine K_M (slope) and V_{MAX} (intercept). However, in all cases, plots of ν against $\frac{\nu}{[S]}$ were non-linear (**Figure 6.14**), indicating that the Michaelis-Menten model is too simplistic to describe phosphomonoesterase kinetics at the LTSE field sites (Irving & Cosgrove, 1976; Walter, 1974). In addition, intermediary plateaus (between substrate concentrations of 50 to 200 µM) were observed in several of the Michaelis plots for individual soil samples, indicating a change in the binding affinity between enzyme and substrate as the substrate concentration increases (Teipel & Koshland, Jr., 1969); this can be due to presence of several binding sites.

6.4.4. Phosphomonoesterase Enzyme Kinetics: Multi-Enzyme Model (2014)

Since, in the majority of cases, phosphomonoesterase activity showed two pH optima (**Section 6.3.1**), the possibility of there being two enzymes present that catalyse phosphomonoester hydrolysis was

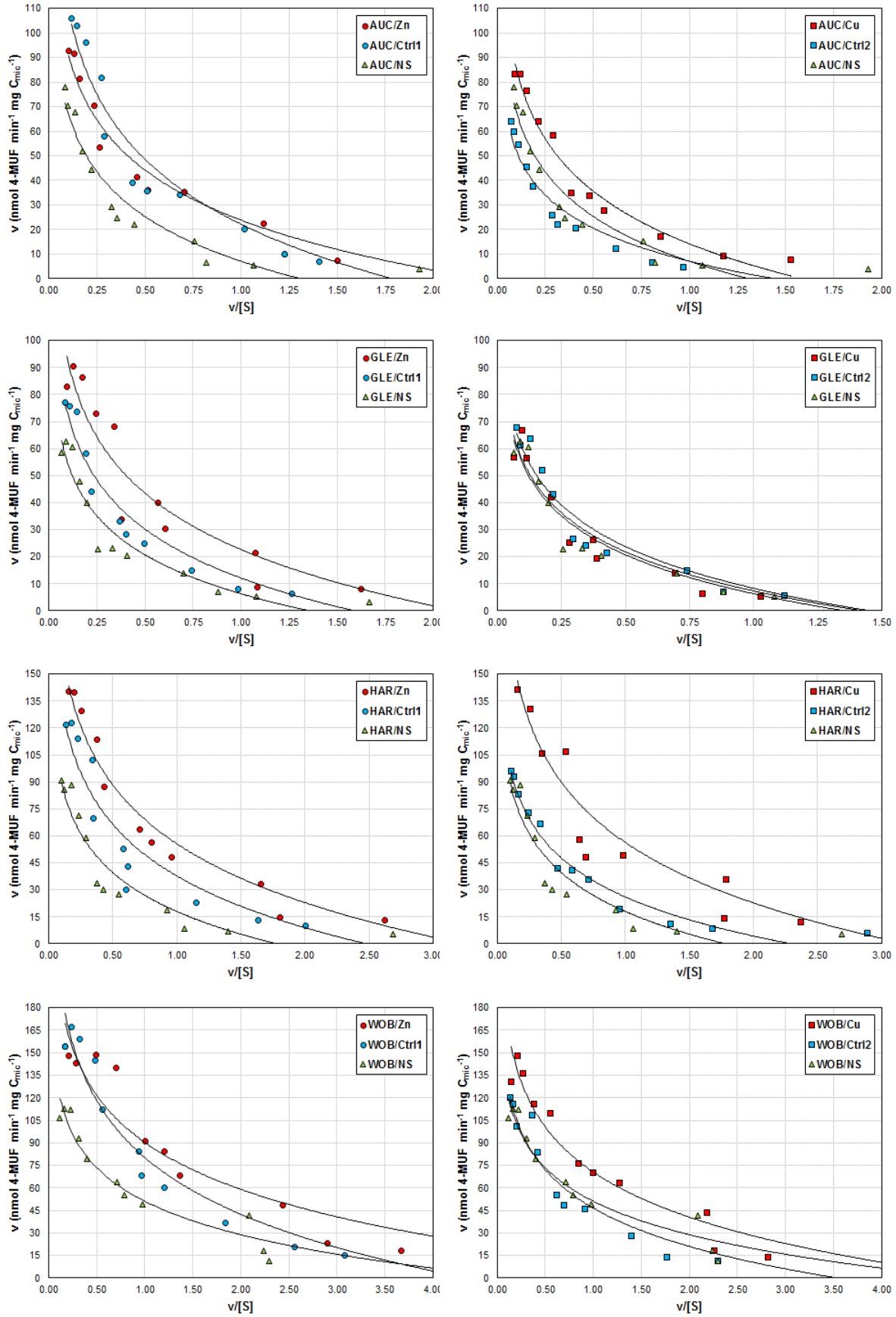


Figure 6.14:- Eadie-Hoftsee linear transformations of the Michaelis-Menten equation for soils receiving digested (left) and undigested (right) sludge treatments at each of the LTSE field sites (2014). Enzyme activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) is plotted against activity per unit of substrate (nmol 4-MUF min^{-1} mg $Cmic^{-1}$ µM 4-MUF-P^{-1}). Non-linearity indicates deviation from the Michaelis-Menten kinetic model.

Table 6.6:- Kinetic parameters determined for phosphomonoesterase activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) measured in sludge amended and untreated soil at each of the LTSE field sites in 2014 assuming two enzymes are present with high (K_M^H and V_{MAX}^H) and low (K_M^L and V_{MAX}^L) substrate binding affinities.

	Multi-Enzyme Model				
	High Affinity		Low Affinity		
Sludge Treatment	K_M^H	V_{MAX}^H	K_M^L	V_{MAX}^L	R^2
AUC/Zn	19.99 (9.61)[a][1][2]	32.61 (9.43)[a]	1308.06 (889.15)[a]	152.29 (24.17)[a]	0.986 (0.004)
AUC/Ctrl1	6.69 (1.13)[a]	14.22 (1.68)[a]	330.78 (76.86)[a]	130.56 (12.31)[a]	0.980 (0.014)
AUC/Cu	0.23 (0.53)[b]	6.38 (1.99)[b]	179.13 (13.74)[b]	94.55 (9.45)[b]	0.991 (0.001)
AUC/Ctrl2	6.32 (2.89)[b]	9.67 (1.88)[b]	303.24 (50.76)[b]	72.13 (4.16)[b]	0.993 (0.002)
AUC/NS	12.84 (7.81)[ab]	16.77 (8.57)[ab]	675.05 (264.27)[ab]	103.21 (12.02)[ab]	0.992 (0.001)
GLE/Zn	1.98 (2.43)[a]	7.17 (4.27)[a]	154.46 (36.79)[a]	98.14 (9.57)[a]	0.960 (0.003)
GLE/Ctrl1	9.05 (7.26)[a]	14.06 (7.38)[a]	372.30 (152.48)[a]	93.50 (9.60)[a]	0.987 (0.004)
GLE/Cu	48.54 (45.57)[b]	10.92 (4.53)[b]	189.51 (62.01)[b]	70.81 (2.72)[b]	0.973 (0.01)
GLE/Ctrl2	5.42 (3.89)[b]	8.74 (2.86)[b]	241.26 (42.62)[b]	75.10 (3.82)[b]	0.972 (0.02)
GLE/NS	3.66 (1.67)[ab]	7.27 (1.48)[ab]	240.09 (42.82)[ab]	71.68 (5.39)[ab]	0.971 (0.01)
HAR/Zn	11.81 (6.72)[ab]	29.39 (9.92)[a]	540.66 (337.33)[a]	171.13 (12.16)[a]	0.989 (0.004)
HAR/Ctrl1	0.58 (0.82)[a]	7.60 (1.16)[a]	227.26 (27.30)[a]	149.17 (12.75)[a]	0.970 (0.02)
HAR/Cu[[1]	3.49 (3.72)[c]	12.18 (5.15)[b]	213.18 (43.27)[b]	142.18 (15.09)[c]	0.974 (0.01)
HAR/Ctrl2	7.37 (2.78)[c]	15.07 (4.72)[b]	206.61 (58.59)[b]	100.01 (11.94)[cd]	0.994 (0.003)
HAR/NS	110.66 (52.66)[bc]	33.88 (16.23)[ab]	222.05 (59.83)[ab]	80.11 (18.92)[bd]	0.983 (0.010
WOB/Zn[1]	0.47 (1.75)[a]	9.57 (6.73)[a]	85.29 (23.17)[a]	160.26 (21.20)[ab]	0.972 (0.02)
WOB/Ctrl1[1]	1.13 (0.81)[a]	10.99 (1.07)[a]	138.38 (5.29)[a]	173.15 (14.39)[a]	0.978 (0.02)
WOB/Cu[1]	8.27 (0.20)[b]	31.83 (5.68)[b]	142.91 (49.34)[b]	128.52 (8.58)[b]	0.982 (0.01)
WOB/Ctrl2	0.43 (0.34)[b]	8.33 (0.89)[b]	132.00 (10.70)[b]	127.50 (9.50)[b]	0.979 (0.001)
WOB/NS	5.03 (3.60)[ab]	19.51 (10.14)[ab]	154.28 (24.78)[ab]	111.32 (6.04)[bc]	0.987 (0.003)

[1]Values in parenthesis are standard errors (n = 3; except for HAR/Cu, WOB/Zn, WOB/Ctrl1, and WOB/Cu where n = 2).
[2] Values without corresponding letters denotes statistical significance at α = 0.05, note comparisons between different sludge types (i.e. digested (Zn and Ctrl1) and undigested (Cu and Ctrl2)) have not been made.

investigated. A second kinetic model was fitted to the data (**Figure 6.15**) which assumes the observed enzyme activity is the sum of activities of two enzymes with different affinities (high (H) and low (L)) for the substrate (Nannipieri et al., 1982):

$$v = \left(\frac{V_{MAX}{}^{H}[S]}{[S] + K_M{}^{H}}\right) + \left(\frac{V_{MAX}{}^{L}[S]}{[S] + K_M{}^{L}}\right) \qquad (\mathbf{E\ 6.22})$$

Kinetic parameters determined using the multi-enzyme model are summarised in **Table 6.6**. With few exceptions, the proportion of variance explained by the multi-enzyme kinetic model was greater in comparison to the Michaelis-Menten model, although in most cases the increase in R^2 was only slight. The only significant differences observed between kinetic parameters were seen at Woburn and Hartwood. At Hartwood, $V_{MAX}{}^{L}$ was significantly higher in both soils receiving the digested sludge treatments (Zn ($p < 0.01$) and Ctrl1 ($p < 0.05$)) in comparison to the untreated soil, whereas at Woburn $V_{MAX}{}^{L}$ was only significantly higher in soil receiving the digested control (**Table 6.6**). In addition, $K_M{}^{H}$ was significantly ($p < 0.05$) higher in the untreated soil at Hartwood in comparison to soil receiving the digested control. The only significant differences in $K_M{}^{H}$ and $V_{MAX}{}^{H}$ observed between field sites were at Hartwood and Woburn, where at Hartwood $K_M{}^{H}$ determined for soil receiving the Cu sludge treatment, and at Woburn $V_{MAX}{}^{H}$ determined for the untreated soil, were significantly ($p < 0.01$) greater in comparison to the remaining sites. It should be noted, however, that the kinetic parameters (i.e. regression coefficients) determined using the multi-enzyme model, particularly $K_M{}^{H}$ and $V_{MAX}{}^{H}$, were not

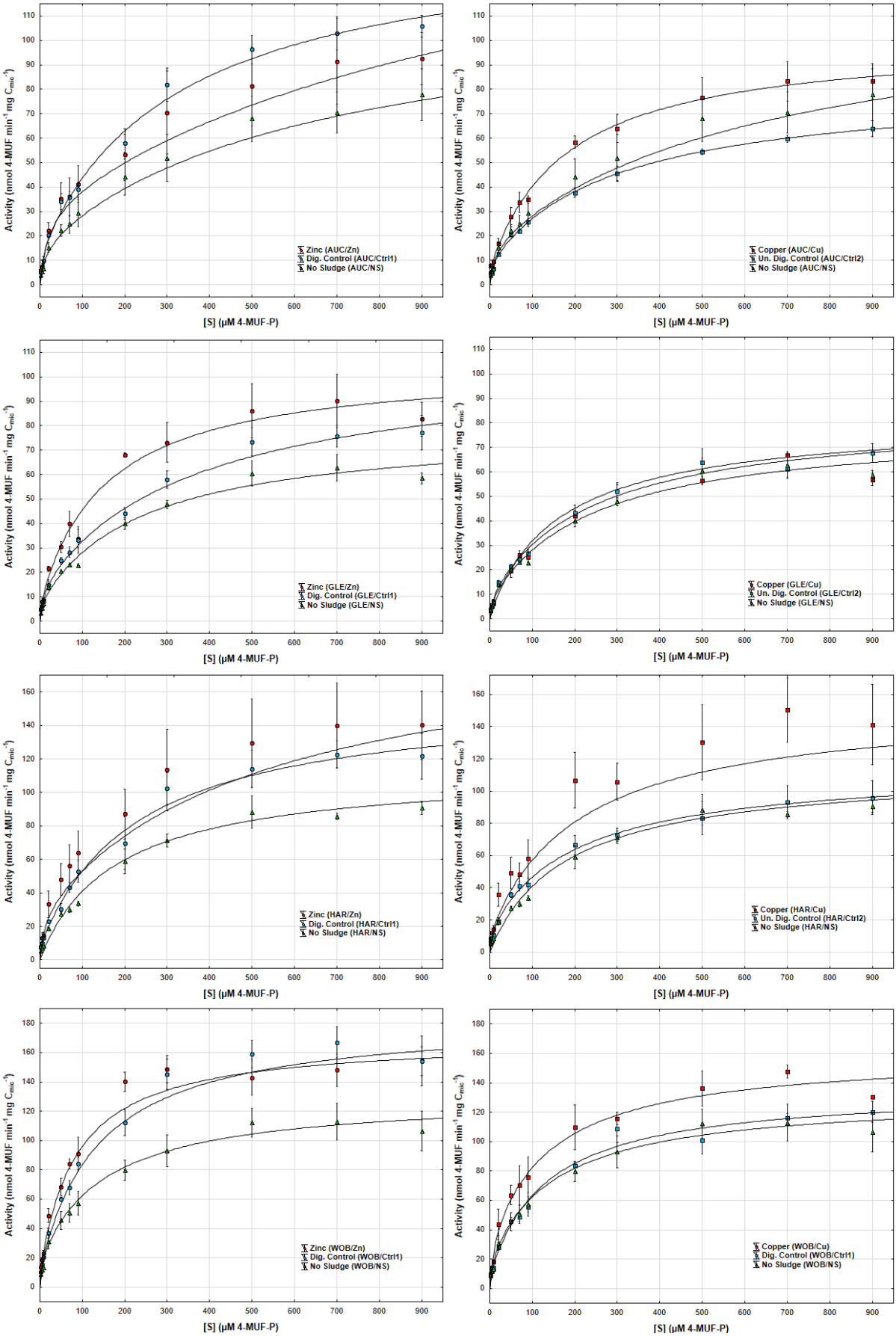


Figure 6.15:- Kinetic pots showing change in phosphomonoesterase enzyme activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) assuming two enzymes are present with high (K_M^H and V_{MAX}^H) and low (K_M^L and V_{MAX}^L) substrate binding affinities in soils receiving digested (left) and undigested (right) sludge treatments at the LTSE sites in 2014. Error bars represent standard error (n = 3).

Table 6.7:- Regression and correlation coefficients from regression analysis of V_{MAX} (nmol 4-MUF min^{-1} g $soil^{-1}$) on microbial biomass carbon (µg C_{mic} g $soil^{-1}$) and available phosphorus (µg P g $soil^{-1}$).

	Regression of V_{MAX} on C_{mic}					
Sludge Treatment	**Slope (C_{mic})**	***p***	**R**	**Intercept**	***p***	**$R^{2[2]}$**
Zinc (Zn)	0.15 (0.02)[1]	<0.001	0.894	-7.92 (8.46	0.371	**0.779**
Dig. Control (Ctrl1)	0.13 (0.02)	<0.001	0.907	-2.97 (7.78)	0.711	**0.806**
Copper (Cu)	0.21 (0.04)	<0.001	0.893	-28.08 (12.26)	<0.05	**0.774**
Un. Dig. Control (Ctrl2)	0.10 (0.02)	<0.01	0.821	-3.13 (7.88)	0.699	**0.641**
No Sludge (NS)	0.11 (0.01)	<0.001	0.937	-7.10 (5.08)	0.193	**0.866**
	Regression of V_{MAX} on Available P					
	Slope (Available P)	***p***	**R**	**Intercept**	***p***	**R^2**
Zinc (Zn)	4.92 (1.29)	<0.01	0.769	-59.97 (26.77)	<0.05	**0.550**
Dig. Control (Ctrl1)	8.06 (2.71)	<0.01	0.685	-107.16 (51.44)	0.064	**0.416**
Copper (Cu)	-2.29 (2.93)	0.453	0.240	68.79 (41.74)	0.130	**-0.04**
Un. Dig. Control (Ctrl2)	0.53 (2.54)	0.838	0.066	22.20 (39.05)	0.582	**-0.10**
No Sludge (NS)	-6.92 (1.66)	<0.01	0.796	80.04 (12.65)	<0.001	**0.598**

	Regression of V_{MAX} on C_{mic} and Available P								
	Slope (C_{mic})	***p***	**R**	**Slope (Avail. P)**	***p***	**R**	**Intercept**	***p***	**R^2**
Zinc (Zn)	0.12 (0.02)	<0.001	0.680	2.76 (0.61)	<0.01	0.431	-52.4 (10.9)	<0.001	**0.926**
Dig. Control (Ctrl1)	0.11 (0.01)	<0.001	0.754	4.52 (0.98)	<0.01	0.384	-80.1 (17.3)	<0.01	**0.936**
Copper (Cu)	0.22 (0.04)	<0.001	0.926	1.58 (2.22)	0.496	0.115	-51.4 (35.0)	0.180	**0.761**
Un. Dig. Control (Ctrl2)	0.02 (4.48)	<0.01	0.832	1.09 (1.49)	0.485	0.135	-20.2 (24.8)	0.436	**0.624**
No Sludge (NS)	0.13 (0.03)	<0.01	1.068	1.28 (2.12)	0.560	0.148	-21.5 (24.4)	0.400	**0.857**

[1] Values in parenthesis are standard error (n = 12) [2]Values are adjusted R^2.

statistically significant in most cases, whereas in each case the values for K_M and V_{MAX} determined by the Michaelis-Menten model were significant. In addition, it cannot be determined whether the low affinity enzyme was fully saturated during the assay (Marx et al., 2005), therefore although the activity of phosphomonoesterase is likely to follow a more complex kinetic model, due to limitations of the current data set only the Michaelis-Menten model will be considered when discussing the influence of additional environmental factors on phosphomonoesterase activity.

6.4.5. Phosphodiesterase Enzyme Kinetics

It was not possible to determine kinetic parameters for phosphodiesterase using the method of Marx et al. (2001). Phosphodiesterase activity is often reported to be lower in comparison to that of phosphomonoesterase (Browman & Tabatabai, 1978; Turner & Haygarth, 2005; Turner, 2010) therefore it is possible that the enzyme was saturated across the entire range of substrate concentrations used in the assay. An alternative colourimetric method, described by Öhlinger et al. (1996), uses substrate concentrations which differ by a factor of more than 20 to prepare assay solutions for the determination of phosphomonoesterase (115 mM *p*-nitrophenol phosphate) and phosphodiesterase (5 mM *bis-p*-nitrophenol phosphate) activity, which would suggest the maximum enzyme activity for these enzymes differs markedly. However, in contrast, the colourimetric method described by Tabatabai (1994) uses equal concentrations of these substrates (0.05 M). Hence, the use of bis-4-MUF-P as a substrate for phosphodiesterase needs to be investigated further in order to determine the optimum parameters for kinetic assay.

Furthermore, it was unclear as to which pH the kinetic assay should be conducted at. As described above (**See Section 6.3.2**) maximum phosphodiesterase activity appeared to occur at pH 3, however determining enzyme activity at this pH would not be relevant to the current investigation as the target pH values for the LTSE field sites ranged from 5.8-6.5 (**See Section 2.5**). In addition, both the EU Sludge Directive and UK Sludge (Use In Agriculture) Regulations prohibit the application of sewage sludge to soil with pH lower than 5, hence the investigation of enzyme inhibition by heavy metal contamination at this pH would be unrealistic. Investigation of phosphodiesterase activity at a higher pH would also be difficult due to the presence of phosphomonoesterase in the assay mixture (Sirová et al., 2013).

6.4.6. Phosphomonoesterase Activity as a Function of Soil Phosphorus Content

Using un-normalised values for V_{MAX} (nmol 4-MUF-P min^{-1} g $soil^{-1}$), a preliminary regression model was established to investigate the relationship between phosphomonoesterase activity, soil microbial biomass carbon (C_{mic}), and the concentration of available phosphorus. Regression of V_{MAX} on C_{mic} showed highly significant correlations between the two variables in both untreated and sludge amended soils (**Table 6.7**). Hence per gram of soil the activity of phosphomonoesterase was greatest at Hartwood, with the lowest values for V_{MAX} observed at Woburn. Similarly, for soils receiving the digested sludge treatments (Zn and Ctrl1), and hence the greater phosphorus loadings (**See Section 4.2**), regression of V_{MAX} on available phosphorus showed significant positive correlations (**Table 6.7**), whereas no significant correlation was seen in soils receiving the undigested sludge treatments (Cu and Ctrl2). However, in contrast a significant negative relationship was observed for untreated soil, therefore, per gram of soil the maximum enzyme activity observed at Hartwood now corresponded to the lowest concentrations of available phosphorus (**See Section 4.2.3**). Combining C_{mic} and available phosphorus into a single regression model (**Figure 6.16** and **Figure 6.17**) only increased the proportion of variance explained for soils receiving the digested sludge treatments, however in each case the F-test results were statistically (p <0.01) significant indicating both C_{mic} and available phosphorus influence the activity of phosphomonoesterase.

Regression of V_{MAX} (nmol 4-MUF min^{-1} mg $Cmic^{-1}$) on available phosphorus, using the normalised values given in **Table 6.4**, also showed significant positive correlations between the two variables in soils receiving the digested sludge treatments (Zn and Ctrl1), whereas for untreated soil the relationship was no longer statistically significant (**Table 6.8**; **Figure 6.18**). As mentioned above, the normalised values for V_{MAX} determined for untreated soil at Woburn and Hartwood were not significantly different, therefore despite significant differences in C_{mic} between the two sites (**See Section 5.2**), the activity of phosphomonoesterase per mg of C_{mic}, again in soils with significantly different concentrations of available phosphorus (**See Section 4.2.4**), appear to be equal. This could potentially indicate two

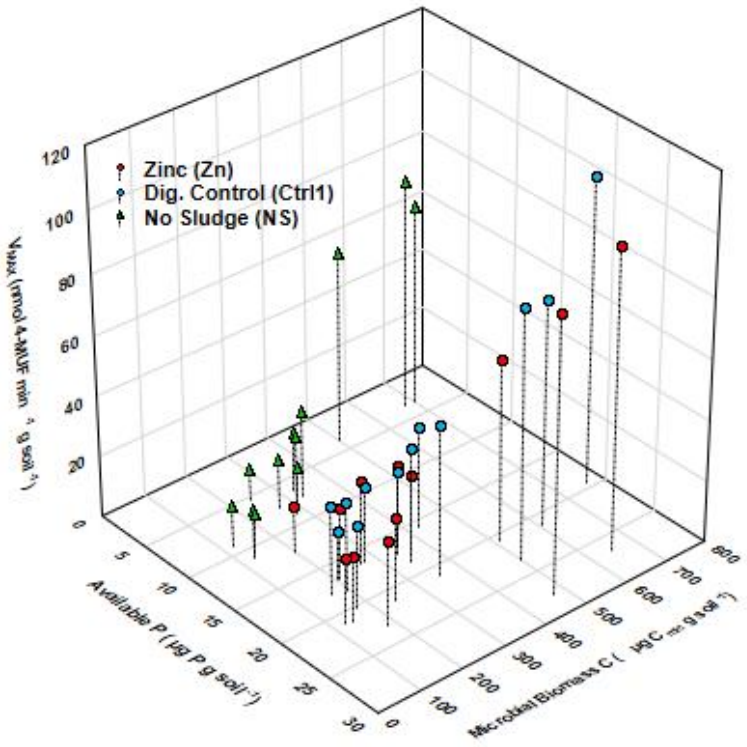


Figure 6.16:- Plot of V_{MAX} (nmol 4-MUF min^{-1} g $soil^{-1}$), determined for phosphomonoesterase as a function of soil microbial biomass carbon (µg C_{mic} g $soil^{-1}$) and available phosphorus concentration (µg P g $soil^{-1}$), in untreated soil (NS), and soils receiving digested sludge treatments (Zn and Ctrl1), taken from each of the LTSE fields sites in 2014.

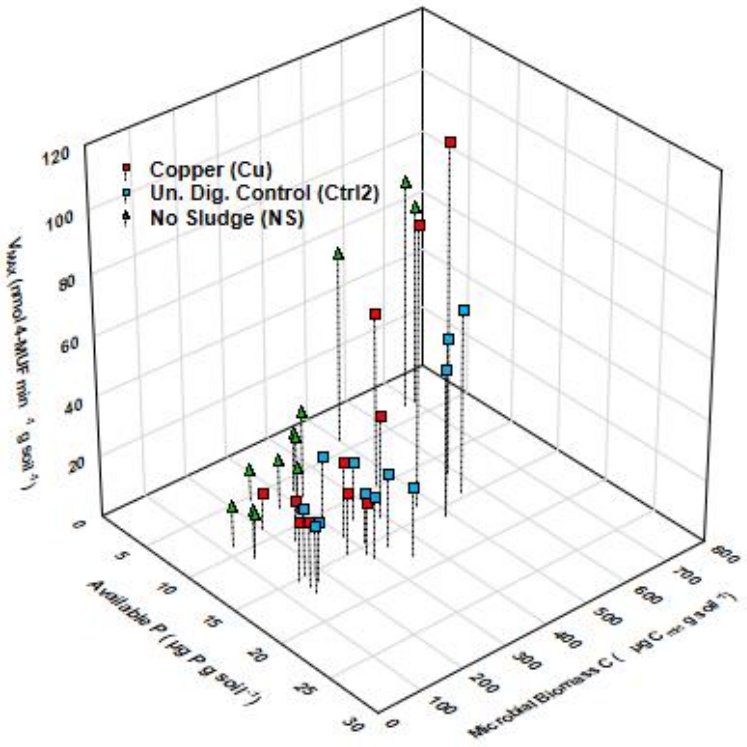


Figure 6.17:- Plot of V_{MAX} (nmol 4-MUF min^{-1} g $soil^{-1}$), determined for phosphomonoesterase as a function of soil microbial biomass carbon (µg C_{mic} g $soil^{-1}$) and available phosphorus concentration (µg P g $soil^{-1}$), in untreated soil (NS), and soils receiving undigested sludge treatments (Cu and Ctrl2), taken from each of the LTSE fields sites in 2014.

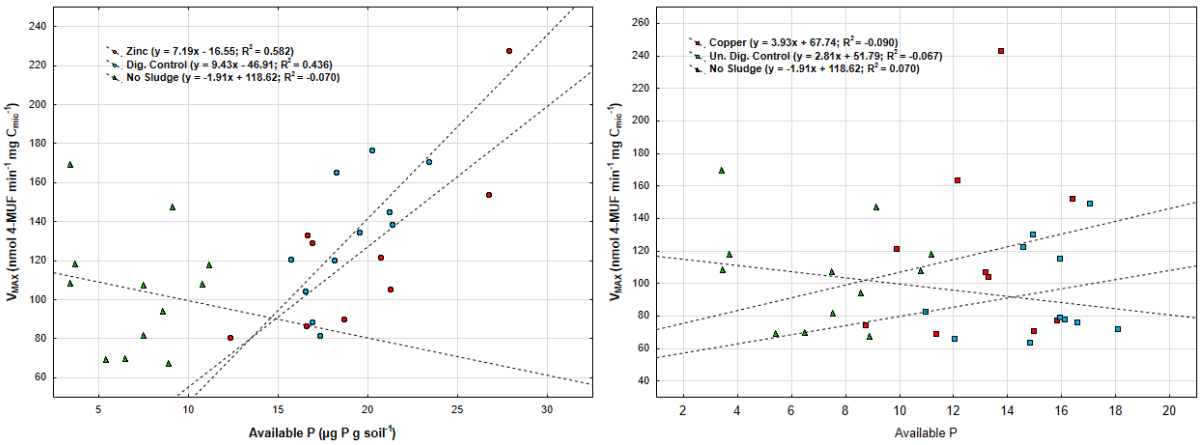


Figure 6.18:- Plot of V_{MAX} (nmol 4-MUF min^{-1} mg C_{mic}^{-1}), determined for phosphomonoesterase, as a function of available phosphorus concentration (µg P g $soil^{-1}$), in untreated and sludge amended soils taken from each of the LTSE fields sites in 2014.

Table 6.8:- Regression and correlation coefficients from regression analysis of V_{MAX} (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) on available phosphorus (µg P g $soil^{-1}$).

	Regression of V_{MAX} on Available P					
Sludge Treatment	**Slope (Avail. P)**	***p***	**Intercept**	***p***	**R**	**$R^{2[2]}$**
Zinc (Zn)	7.19 (2.07)	<0.05	-16.55 (41.90)	0.705	0.796	**0.582**
Digested Control (Ctrl1)	9.43 (3.20)	<0.05	-46.91 (61.00)	0.462	0.701	**0.435**
Copper (Cu)	3.93 (7.77)	0.627	67.74 (102.17)	0.526	0.176	**-0.090**
Undigested Control (Ctrl2)	2.81 (4.59)	0.556	51.79 (70.18)	0.479	0.200	**-0.067**
No Sludge (NS)	-1.91 (3.59)	0.607	118.62 (27.36)	<0.01	0.166	**-0.070**

[1] Values in parenthesis are standard error (n = 12). [2]Values are adjusted R^2.

different scenarios occurring at the two field sites, where fewer microorganisms at Woburn, stimulated by relatively larger concentrations of available phosphorus, are producing more enzyme activity per mg C_{mic}, in comparison to a greater number of microorganisms at Hartwood, which presumably share the burden of phosphorus limitation and cumulatively produce enzyme activity equal to that at Woburn. In soils receiving the digested sludge treatments (Zn and Ctrl1) the enzyme activity produced per mg of C_{mic} was proportional to the concentration of available phosphorus present in the soil. In this case the greatest enzyme activity per mg C_{mic} was seen at Hartwood in soil receiving the Zn sludge treatment, which also has the highest concentration of available phosphorus remaining in the soil following sludge application (**Figure 4.13**). The correlation between the normalised values of V_{MAX} and available phosphorus for soils receiving the undigested sludge treatments (Cu and Ctrl2) were again not statistically significant, although in this case both regression coefficients were now positive (**Table 6.8**). Since soils receiving the undigested sludge treatments received the lowest phosphorus loadings (**See Section 4.2**), the influence of available phosphorus on the production of phosphatase enzymes may be returning to a situation similar to that of the untreated soil.

6.5. Change in Phosphatase Enzyme Activity: Incubation Study (2014)

Phosphatase enzyme activity was measured over the course of three months as part of an investigation into the response of microorganisms in sludge amended soils to fresh applications of organic matter.

Table 6.9:- Changes in phosphomonoesterase enzyme activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) measured over the course of 3 months following a fresh application of liquid sludge (See Section 5.2.5). Samples were incubated at 20ºC.

Sludge Treatment	Phosphomonoesterase Enzyme Activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1})				
	<24 Hours	2 Weeks	1 Month	2 Months	3 Months
AUC/Zn	68.93 (8.10)[a][1][2]	105.59 (5.32)[a]	126.63 (26.42)[a]	73.57 (1.39)[a]	59.63 (6.36)[a]
AUC/Ctrl1	61.12 (4.71)[ab]	75.54 (8.41)[b]	95.73 (5.34)[a]	80.21 (1.27)[a]	85.61 (3.13)[a]
AUC/Cu	54.29 (2.69)[c]	76.33 (6.90)[c]	121.65 (11.78)[b]	84.33 (4.14)[b]	55.14 (17.08)[b]
AUC/Ctrl2	54.53 (3.79)[c]	75.18 (3.65)[c]	93.24 (9.22)[bc]	76.76 (3.86)[b]	69.83 (8.48)[b]
AUC/NS	49.51 (0.84)[bc]	80.13 (1.37)[bc]	87.36 (7.96)[ac]	69.39 (14.92)[ab]	63.03 (14.44)[ab]
GLE/Zn	59.84 (8.00)[a]	67.83 (5.61)[a]	74.86 (18.10)[a]	73.53 (4.43)[a]	91.16 (2.62)[a]
GLE/Ctrl1	46.39 (3.10)[ab]	64.30 (1.73)[a]	49.22 (4.33)[a]	71.92 (1.83)[a]	79.73 (1.65)[b]
GLE/Cu	38.32 (0.81)[c]	47.24 (3.50)[c]	47.63 (6.78)[b]	48.41 (5.31)[c]	71.27 (16.12)[d]
GLE/Ctrl2	43.29 (5.38)[c]	86.55 (9.23)[d]	60.67 (5.72)[b]	64.47 (1.90)[c]	74.40 (3.10)[d]
GLE/NS	35.81 (1.32)[bc]	51.27 (2.00)[bc]	53.15 (7.23)[ab]	51.08 (8.28)[bc]	64.38 (4.20)[cd]
HAR/Zn	149.38 (29.56)[a]	161.71 (20.93)[a]	153.29 (24.42)[a]	112.82 (20.33)[a]	164.43 (27.49)[a]
HAR/Ctrl1	94.74 (8.69)[ab]	122.11 (6.22)[ab]	127.46 (8.66)[a]	104.70 (2.36)[ab]	102.50 (12.29)[a]
HAR/Cu	110.72 (16.61)[c]	135.10 (14.79)[c]	134.09 (13.26)[b]	121.14 (22.04)[c]	160.51 (19.13)[b]
HAR/Ctrl2	59.98 (5.86)[d]	110.57 (18.31)[c]	114.42 (22.04)[b]	84.87 (12.47)[cd]	121.46 (25.19)[b]
HAR/NS	56.87 (5.15)[bd]	113.71 (6.64)[bc]	122.86 (2.61)[ab]	68.74 (6.53)[bd]	140.06(19.38)[ab]
WOB/Zn	139.08 (12.96)[a]	125.37 (13.07)[a]	110.89 (12.71)[a]	141.09 (22.93)[a]	141.58 (9.38)[a]
WOB/Ctrl1	83.22 (6.68)[b]	76.61 (4.92)[b]	102.73 (12.04)[a]	118.97 (20.53)[a]	107.98 (22.50)[a]
WOB/Cu	73.03 (10.97)[c]	77.66 (10.72)[c]	89.24 (4.87)[b]	128.79 (15.58)[b]	123.83 (19.72)[b]
WOB/Ctrl2	68.59 (6.92)[c]	71.13 (5.94)[c]	88.22 (5.05)[b]	85.45 (11.42)[b]	106.58 (15.20)[b]
WOB/NS	68.04 (9.15)[bc]	82.88 (8.95)[bc]	103.53 (22.69)[ab]	91.99 (20.91)[ab]	123.53 (22.90)[ab]

[1]Values in parenthesis are standard error (n = 3). [2]Values without corresponding letters denotes statistical significance at α = 0.05 for individual sampling events, note comparisons between different sludge types (i.e. digested (Zn and Ctrl1) and undigested (Cu and Ctrl2)) have not been made.

Incubated samples were analysed for phosphomonoesterase and phosphodiesterase activities at periods of < 24 hours, 2 weeks, 1 month, 2 months, and 3 months, following a fresh application of anaerobically digested liquid sludge to each of the soils sampled from the LTSE field sites (**See Section 5.2.5**). The assay parameters used were the same as those previously described for the determination of enzyme pH optima (**See Section 6.3**), with phosphomonoesterase activity determined at pH 6.5 and phosphodiesterase activity determined at pH 3.

6.5.1. Change in Phosphomonoesterase Activity Results (2014)

Auchincruive

In both soils receiving the contaminated sludge treatments at Auchincruive, phosphomonoesterase activity increased significantly (Zn (p <0.01) and Cu (p <0.001)) over the course of one month reaching approximately 124 nmol 4-MUF min^{-1} mg C_{mic}^{-1} in each case (**Table 6.9**; **Figure 6.19**). However, enzyme activity subsequently declined in both soils and for the remainder of the incubation period was no longer significantly different to the activity measured at the start of the experiment. Significant increases in activity were also observed in soils receiving the uncontaminated controls (Ctrl1 (p <0.001) and Ctrl2 (p <0.01)) and the untreated soil (p <0.05) over the course of the first month (**Figure 6.19**). Again, phosphomonoesterase activity subsequently declined in each of these soils, but in soil receiving the digested control remained significantly higher (p <0.05) than that observed at the

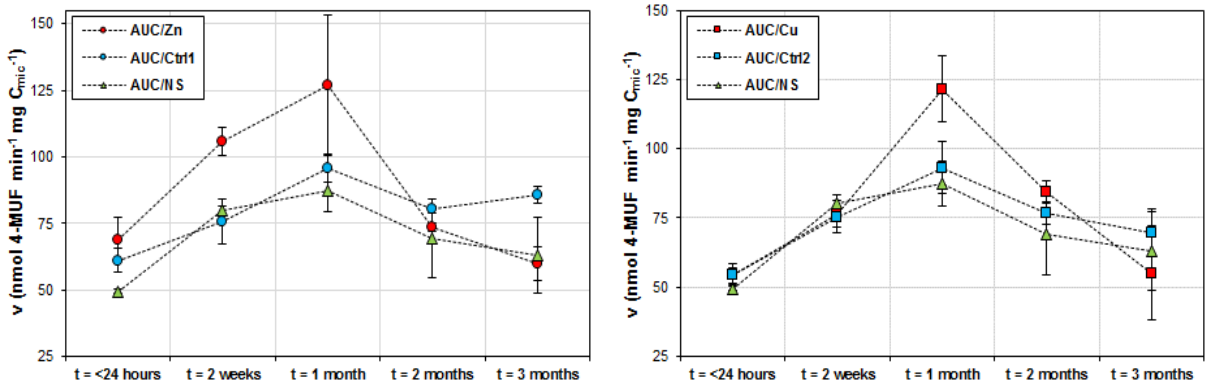


Figure 6.19:- Changes in phosphomonoesterase activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) following a fresh application of liquid sludge (See Section 5.2.5). Samples of soil receiving digested (Zn and Ctrl1; left) and undigested (Cu and Ctrl2; right) sludge treatments, plus untreated soil (NS), were taken from the Auchincruive (AUC) field site during 2014 and incubated at 20°C for 3 months. Error bars represent standard error (n = 3).

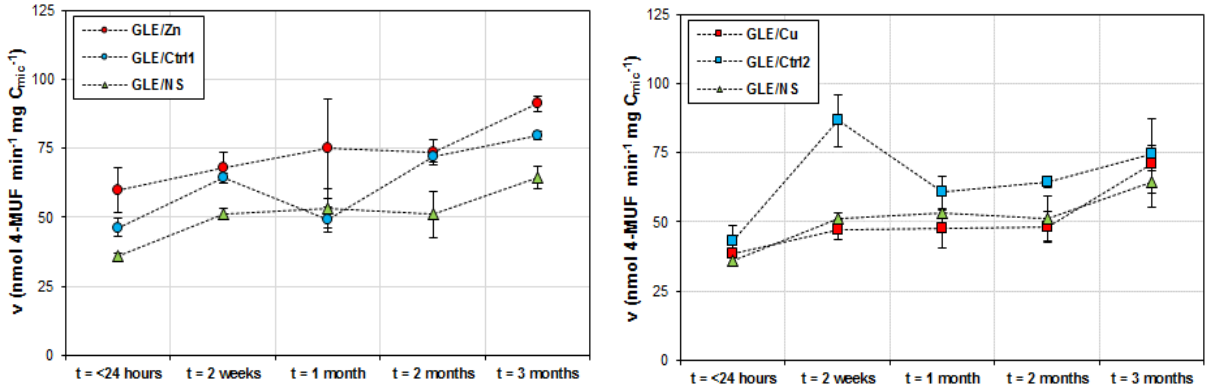


Figure 6.20:- Changes in phosphomonoesterase activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) following a fresh application of liquid sludge (See Section 5.2.5). Samples of soil receiving digested (Zn and Ctrl1; left) and undigested (Cu and Ctrl2; right) sludge treatments, plus untreated soil (NS), were taken from the Gleadthorpe (GLE) field site during 2014 and incubated at 20°C for 3 months. Error bars represent standard error (n = 3).

start of the incubation period. Enzyme activity in soil receiving the undigested control was also significantly (p <0.05) higher at two months in comparison to activity at the start of the incubation, but was no longer significantly different after 3 months (**Figure 6.19**).

In comparison to untreated soil, phosphomonoesterase activity was significantly (p <0.05) higher in soil receiving the Zn sludge treatment for the first two weeks of the incubation period; as well as soil receiving the digested control in the second week (**Table 6.9**). However, no significant differences were observed between these soils for the remainder of the incubation period. After one month phosphomonoesterase activity in soil receiving the Cu sludge treatment was also significantly (p <0.05) higher in comparison to the untreated soil (**Table 6.9**), though no other significant differences were observed between soils receiving the undigested sludge treatments (Cu and Ctrl2) or untreated soil at any time during the incubation period.

Gleadthorpe

At Gleadthorpe, phosphomonoesterase activity steadily increased over the course of the incubation

period in both soils receiving the contaminated sludge treatments (Zn and Cu), as well as in the untreated soil (**Figure 6.20**). In each case the activity measured after three months was significantly (p <0.05) higher in comparison to the activity determined at the start of the incubation period. A significant (p <0.001) increase in enzyme activity was seen after two weeks in both soils receiving the uncontaminated controls (Ctrl1 and Ctrl2), reaching values of 64.3 ± 1.73 and 86.5 ± 9.23 nmol 4-MUF min^{-1} mg C_{mic}^{-1}, respectively (**Table 6.9**; **Figure 6.20**). After one month, enzyme activity was no longer significantly different to that at the start of the experiment, however activity continued to increase in both soils and was significantly (Ctrl1 (p <0.001) and Ctrl2 (p < 0.05)) higher for the remainder of the incubation period.

With the exception of the activity at one month, phosphomonoesterase activity was significantly (p <0.05) higher in soil receiving the Zn sludge treatment, in comparison to untreated soil, for the duration of the incubation period (**Table 6.9**); and was significantly (p <0.05) higher than in the digested control after three months (**Figure 6.20**). Enzyme activity in soil receiving the digested control was also significantly (p <0.05) higher in comparison to the untreated soil at two weeks, two months, and three months (**Table 6.9**). After two weeks enzyme activity in soil receiving the undigested control was significantly (p <0.01) higher in comparison to both the untreated soil and soil receiving the Cu sludge treatment (**Figure 6.20**), however no other significant differences were observed between these soils at any time during the incubation period (**Table 6.9**).

Hartwood

No significant changes in phosphomonoesterase activity were seen in soils receiving the contaminated sludge treatments (Zn and Cu) at Hartwood for the duration of the incubation period. Significant (p <0.05) increases in the enzyme activity of soil receiving the digested control were seen at two weeks and one month, however activity subsequently declined and was no longer significantly different to that at the start of the experiment (**Figure 6.21**). Similarly, enzyme activity in the untreated soil was also significantly higher at two weeks (p <0.01), and one month (p <0.001), before declining. However another significant (p <0.001) increase in the enzyme activity of untreated soil was seen after three months (**Figure 6.21**). The change in phosphomonoesterase activity over the course of the incubation period in soil receiving the undigested control was similar to that of the untreated soil, however only after three months was the measured activity significantly (p <0.05) higher than at the start of the incubation period.

Phosphomonoesterase activity was significantly (p <0.05) higher in soil receiving the Zn sludge treatment, in comparison to the untreated soil at the start of the incubation period, at two weeks and at two months (**Table 6.9**). Whereas no significant differences were observed between soils receiving the digested sludge treatments (Zn and Ctrl1), or between untreated soil and soil receiving the digested control at any point during the incubation period. Enzyme activity was also significantly (p <0.05)

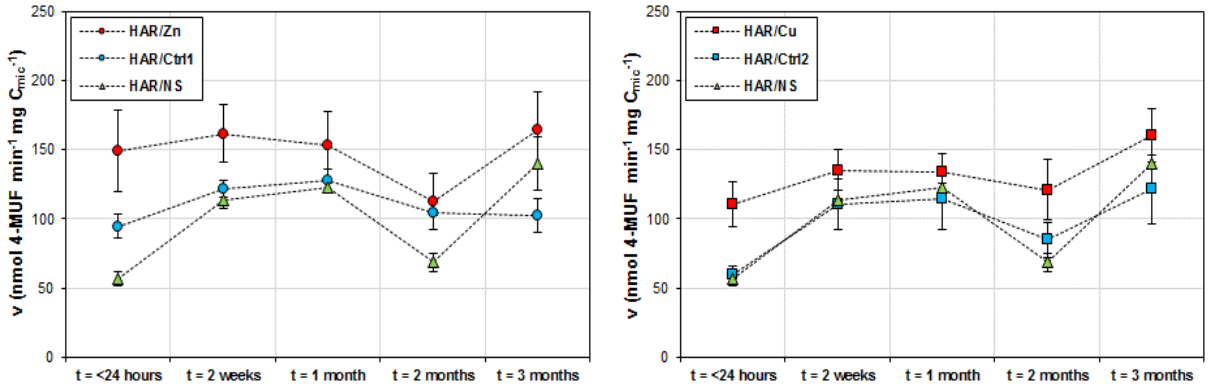


Figure 6.21:- Changes in phosphomonoesterase activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) following a fresh application of liquid sludge (See Section 5.2.5). Samples of soil receiving digested (Zn and Ctrl1; left) and undigested (Cu and Ctrl2; right) sludge treatments, plus untreated soil (NS), were taken from the Hartwood (HAR) field site during 2014 and incubated at 20ºC for 3 months. Error bars represent standard error (n = 3).

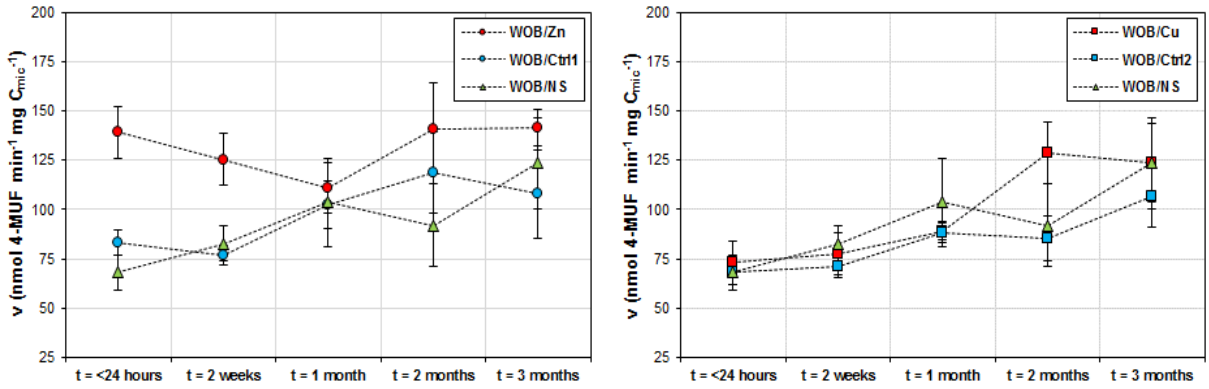


Figure 6.22:- Changes in phosphomonoesterase activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) following a fresh application of liquid sludge (See Section 5.2.5). Samples of soil receiving digested (Zn and Ctrl1; left) and undigested (Cu and Ctrl2; right) sludge treatments, plus untreated soil (NS), were taken from the Woburn (WOB) field site during 2014 and incubated at 20ºC for 3 months. Error bars represent standard error (n = 3).

higher in soil receiving the Cu sludge treatment at the start of the incubation period in comparison to untreated soil and soil receiving the undigested control (**Table; 6.9**; **Figure 6.21**). No other significant differences in enzyme activity were observed between soils receiving the undigested sludge treatments (Cu and Ctrl2), though enzyme activity in soil receiving the Cu sludge treatment was again significantly (p <0.05) higher in comparison to the untreated soil after two months.

Woburn

No significant changes in phosphomonoesterase activity were observed in soils receiving the digested sludge treatments (Zn and Ctrl1) at Woburn over the course of the incubation period. Whereas, enzyme activity increased steadily in both the untreated soil and soils receiving the undigested sludge treatments (Cu and Ctrl2) and after three months was significantly (p <0.05) higher, in each of the soils, than at the start of the experiment (**Figure 6.22**).

For the first two weeks phosphomonoesterase activity was significantly (p <0.05) higher in soil receiving the Zn sludge treatment in comparison to both the untreated soil and soil receiving the digested control (**Table 6.9**); however no significant differences were observed between these soils after one month. At no point throughout the incubation period were any significant differences in enzyme activity observed between soils receiving the undigested sludge treatments (Cu and Ctrl2) and the untreated soil.

6.5.2. Change in Phosphomonoesterase Activity Overview (2014)

In general, the activity of phosphomonoesterase per mg of C_{mic} was higher in the contaminated soils, in comparison to soils receiving the uncontaminated controls for the duration of the incubation period (**Table 6.9**). Furthermore, the short term response of microorganisms to the application of fresh organic phosphorus differed in contaminated soils, particularly at Auchincruive where phosphomonoesterase activity increased significantly over the first month of incubation (**Figure 6.19**). This may be an indication that enzyme activity is inhibited by the presence of heavy metals (**See Section 6.4.2**) as the microorganisms in contaminated soils are repeatedly required to synthesise more phosphomonoesterase, whereas microorganisms in uncontaminated soils can rely on uninhibited extracellular enzymes stabilised in the soil environment (**See Section 1.5**). In general, the production of phosphomonoesterase in soils receiving the uncontaminated sludge treatments followed that of the untreated soil, with the exception of soil receiving the undigested control at Gleadthorpe. Though as mentioned above, low phosphomonoesterase activity was observed in this soil at pH 4 (**Figure 6.6**), hence application of fresh organic matter may have prompted enzyme synthesis. However, it should also be noted that significant differences in the PLFA profile of soil microorganisms were detected between soils receiving different sludge treatments at each site, plus between the field sites themselves (**See Section 5.3**), indicating differences in the soil microbial communities. Therefore it is to be expected that soil microorganisms in different soils will react differently, i.e. produce enzymes at different rates, in response to the application of fresh organic matter.

6.5.3. Change in Phosphodiesterase Activity Results (2014)

Auchincruive

Similar to phosphomonoesterase activity, the activity of phosphodiesterase in soils receiving the Zn and Cu sludge treatments at Auchincruive increased significantly (p <0.05) after one month, reaching respective maxima of 221.25 ± 33.17 and 125.60 ± 12.51 nmol 4-MUF min^{-1} mg C_{mic}^{-1} (**Figure 6.23**). Again, in both cases enzyme activity subsequently declined and after three months was not significantly

Table 6.10:- Changes in phosphodiesterase activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) measured over the course of 3 months following a fresh application of liquid sludge (See Section 5.2.5). Samples were incubated at 20ºC.

	Phosphodiesterase Enzyme Activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1})				
Sludge Treatment	**<24 Hours**	**2 Weeks**	**1 Month**	**2 Months**	**3 Months**
AUC/Zn	114.68 (19.92)$^{a[1][2]}$	152.80 (8.93)^{a}	221.25 (33.17)^{a}	74.01 (8.61)^{a}	128.14 (32.22)^{a}
AUC/Ctrl1	97.86 (7.14)^{a}	120.26 (14.06)^{a}	125.34 (10.38)^{b}	82.05 (6.82)^{a}	110.80 (24.14)^{a}
AUC/Cu	89.23 (5.98)^{b}	99.02 (7.99)^{b}	125.60 (12.51)^{c}	83.93 (10.23)^{b}	115.81 (7.73)^{b}
AUC/Ctrl2	94.09 (9.54)^{b}	116.44 (7.30)^{b}	111.75 (12.35)^{c}	74.85 (3.65)^{b}	142.91 (29.50)^{b}
AUC/NS	83.32 (5.86)^{ab}	112.33 (12.03)^{ab}	139.52 (16.31)^{bc}	73.64 (12.02)^{ab}	ND[3]
GLE/Zn	114.62 (19.68)^{a}	150.10 (14.97)^{a}	164.92 (35.81)^{a}	114.85 (13.15)^{a}	173.89 (16.27)^{a}
GLE/Ctrl1	99.82 (4.84)^{a}	158.96 (16.11)^{a}	128.47 (27.31)^{a}	108.03 (9.51)^{a}	115.73 (5.64)^{b}
GLE/Cu	91.82 (4.03)^{b}	123.91 (8.56)^{b}	92.85 (7.07)^{b}	91.44 (10.17)^{b}	158.39 (41.23)^{c}
GLE/Ctrl2	83.10 (7.97)^{b}	199.50 (48.85)^{b}	156.46 (33.97)^{b}	103.16 (11.53)^{b}	126.16 (15.92)^{c}
GLE/NS	77.25 (9.75)^{ab}	132.58 (3.95)^{ab}	123.96 (36.54)^{ab}	116.68 (5.97)^{ab}	111.65 (20.30)^{bc}
HAR/Zn	291.74 (37.78)^{a}	240.70 (46.60)^{a}	267.40 (16.08)^{a}	119.11 (11.07)^{a}	129.37 (11.32)^{a}
HAR/Ctrl1	220.41 (16.95)^{a}	251.49 (8.24)^{a}	244.36 (8.24)^{a}	133.30 (17.61)^{a}	102.54 (16.37)^{a}
HAR/Cu	234.79 (32.97)^{c}	180.45 (12.47)^{b}	186.58 (28.63)^{c}	121.49 (21.87)^{c}	140.98 (17.51)^{b}
HAR/Ctrl2	153.30 (7.54)^{d}	164.59 (26.93)^{b}	204.03 (24.81)^{c}	97.50 (15.45)^{cd}	120.20 (14.26)^{b}
HAR/NS	115.43 (7.91)^{bd}	213.34 (3.93)^{ab}	197.69 (13.33)^{bc}	58.37 (0.31)^{bd}	98.92 (19.82)^{ab}
WOB/Zn	210.24 (32.05)^{a}	202.87 (22.80)^{ab}	161.29 (41.56)^{a}	208.55 (39.22)^{a}	180.56 (25.35)^{a}
WOB/Ctrl1	123.85 (33.33)^{ab}	70.63 (65.75)^{a}	183.51 (5.63)^{a}	196.78 (31.01)^{a}	191.43 (14.62)^{a}
WOB/Cu	114.75 (25.87)^{c}	113.00 (14.02)^{c}	136.90 (5.15)^{b}	283.21 (44.52)^{b}	180.63 (26.03)^{b}
WOB/Ctrl2	106.76 (19.31)^{c}	141.48 (30.21)^{cd}	141.65 (12.21)^{b}	139.26 (26.63)^{c}	163.81 (11.84)^{b}
WOB/NS	50.28 (10.60)^{bc}	242.38 (42.85)^{bc}	148.82 (56.19)^{ab}	145.57 (35.62)^{ac}	147.72 (50.63)^{ab}

[1]Values in parenthesis are standard error (n = 3). [2]Values without corresponding letters denotes statistical significance at α = 0.05 for individual sampling events, note comparisons between different sludge types (i.e. digested (Zn and Ctrl1) and undigested (Cu and Ctrl2)) have not been made. [3]Not Detected.

different to the activity observed at the start of the experiment. In addition, phosphodiesterase activity was also significantly (p <0.05) higher in soil receiving the undigested control at the end of the incubation period. Phosphodiesterase activity also appeared to increase in the untreated soil over the course of the first month, however no significant changes were observed (**Figure 6.23**). Nor were any significant changes in enzyme activity observed over time in soil receiving the digested control.

After one month phosphodiesterase activity was significantly (p <0.05) higher in soil receiving the Zn sludge treatment in comparison to the untreated soil and soil receiving the digested control (**Table 6.10**). Otherwise no significant differences in enzyme activity were observed either between soils receiving the digested (Zn and Ctrl1) and undigested (Cu and Ctrl2) sludge treatments or sludge amended and untreated soil.

Gleadthorpe

With the exception of soil receiving the Zn sludge treatment, phosphodiesterase activity increased significantly (p <0.05) to a maximum after two weeks in the sludge amended soils from the Gleadthorpe site (**Figure 6.24**); particularly in soil receiving the undigested control which reached a maximum of 199.50 ± 48.85 nmol 4-MUF min^{-1} mg C_{mic}^{-1}. In each case enzyme activity subsequently declined, and for soils receiving the uncontaminated controls was no longer significantly different to that at the start of the incubation period. However a significant (p <0.05) increase was observed in soil receiving the Cu sludge treatment at three months (**Figure 6.24**). Enzyme activity in soil receiving the Zn sludge treatment also reached maximum values of 164.92 ± 35.81 and 173.89 ± 16.27 nmol 4-MUF min^{-1} mg

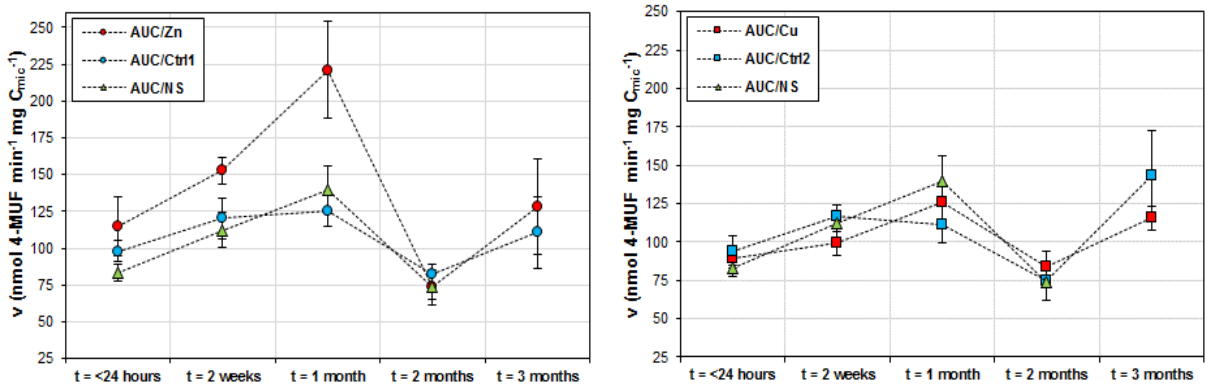


Figure 6.23:- Changes in phosphodiesterase activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) following a fresh application of liquid sludge (See Section 5.2.5). Samples of soil receiving digested (Zn and Ctrl1; left) and undigested (Cu and Ctrl2; right) sludge treatments, plus untreated soil (NS), were taken from the Auchincruive (AUC) field site during 2014 and incubated at 20ºC for 3 months. Error bars represent standard error (n = 3).

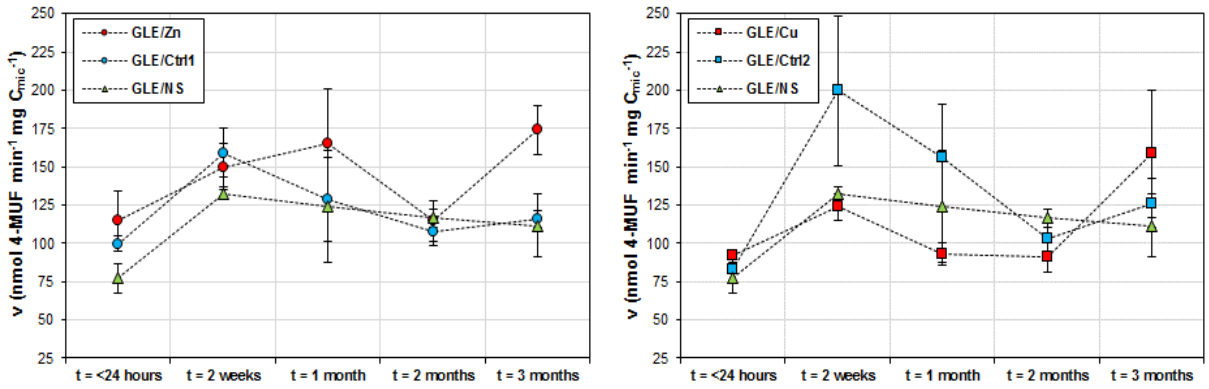


Figure 6.24:- Changes in phosphodiesterase activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) following a fresh application of liquid sludge (See Section 5.2.5). Samples of soil receiving digested (Zn and Ctrl1; left) and undigested (Cu and Ctrl2; right) sludge treatments, plus untreated soil (NS), were taken from the Gleadthorpe (GLE) field site during 2014 and incubated at 20ºC for 3 months. Error bars represent standard error (n = 3).

C_{mic}^{-1} at one and three months respectively, however these values were not significantly different in comparison to that at the start of the incubation period. Activity in the untreated soil also reached a maximum of 132.58 ± 3.95 nmol 4-MUF min^{-1} mg C_{mic}^{-1} after two weeks, before steadily declining, though again these changes were not statistically significant in comparison to that at the start of the incubation period.

With the exception of soil receiving the Zn sludge treatment, where after three months phosphodiesterase activity was significantly (p <0.05) higher in comparison to untreated soil and soil receiving the digested control (**Table 6.10**), no significant differences in enzyme activity were observed between any of the soils from Gleadthorpe for the duration of the incubation period.

Hartwood

Phosphodiesterase activity appeared to decline steadily over the course of the incubation period in each of the sludge amended soils from Hartwood, and with the exception of soil receiving the undigested

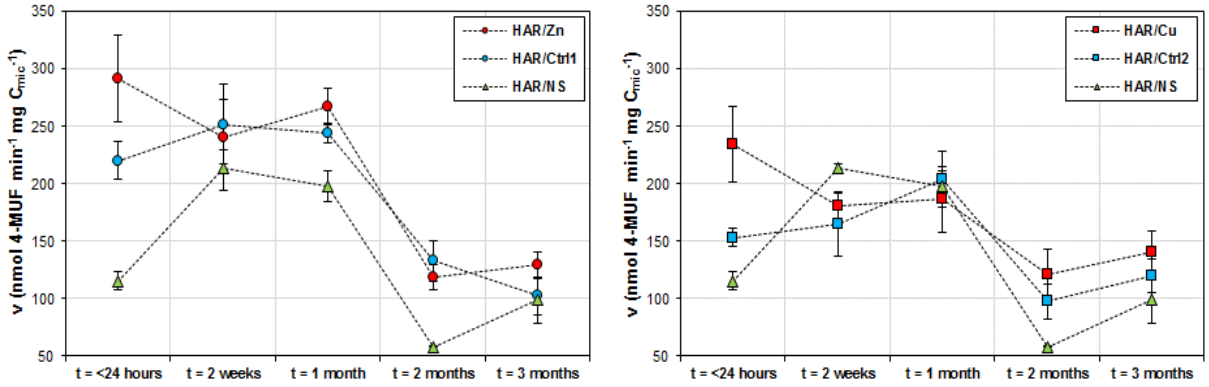


Figure 6.25:- Changes in phosphodiesterase activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) following a fresh application of liquid sludge (See Section 5.2.5). Samples of soil receiving digested (Zn and Ctrl1; left) and undigested (Cu and Ctrl2; right) sludge treatments, plus untreated soil (NS), were taken from the Hartwood (HAR) field site during 2014 and incubated at 20°C for 3 months. Error bars represent standard error (n = 3).

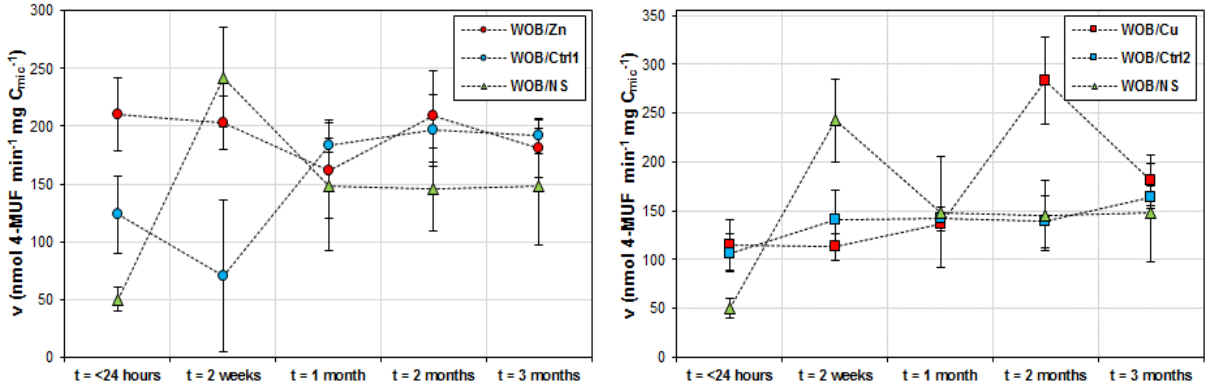


Figure 6.26:- Changes in phosphodiesterase activity (nmol 4-MUF min^{-1} mg C_{mic}^{-1}) following a fresh application of liquid sludge (See Section 5.2.5). Samples of soil receiving digested (Zn and Ctrl1; left) and undigested (Cu and Ctrl2; right) sludge treatments, plus untreated soil (NS), were taken from the Woburn (WOB) field site during 2014 and incubated at 20°C for 3 months. Error bars represent standard error (n = 3).

control was significantly lower at two and three months in comparison to the activity measured at the start of the experiment (**Figure 6.25**). In contrast, phosphodiesterase activity in the untreated soil increased significantly (p <0.001) to a maximum of 213.34 ± 3.93 nmol 4-MUF min^{-1} mg C_{mic}^{-1} after two weeks, and remained significantly (p <0.001) higher at one month (**Figure 6.25**). However, enzyme activity subsequently declined and by the end of the incubation period was not significantly different to that at the start.

At the start of the incubation period, then again at one month and two months, phosphodiesterase activity was significantly higher in soils receiving the digested sludge treatments (Zn (p <0.01) and Ctrl1 (p <0.05)) in comparison to the untreated soil. Whereas only in soil receiving the Cu sludge treatment, at the start of the experiment (p <0.01), and at two months (p <0.05), was enzyme activity in soils receiving the undigested sludge treatments significantly higher in comparison to the untreated soil (**Table 6.10**); activity was also higher in comparison to soil receiving the undigested control at the start of the experiment (p <0.05).

Woburn

In soil receiving the Zn sludge treatment at Woburn, values for phosphodiesterase activity remained relatively similar for the duration of the incubation period, and no significant change in activity was observed over time. Similarly, despite a decrease in enzyme activity after two weeks, no significant change was observed over time in soil receiving the digested control (**Figure 6.26**). In soils receiving the undigested sludge treatments, phosphodiesterase activity increased significantly (p <0.01) to a maximum of 283.21 ± 44.52 nmol 4-MUF min^{-1} mg C_{mic}^{-1} after two months in soil receiving the Cu sludge treatment, whereas activity in soil receiving the undigested control reached a maximum of 163.81 ± 11.84 nmol 4-MUF min^{-1} mg C_{mic}^{-1} after three months, significantly (p <0.05), higher than at the start of the experiment. In the untreated soil, phosphodiesterase activity increased (p <0.01) markedly to 242.38 ± 42.85 nmol 4-MUF min^{-1} mg C_{mic}^{-1} after two weeks, before declining again to approximately 147 nmol 4-MUF min^{-1} mg C_{mic}^{-1} for the remainder of the incubation period (**Figure 6.26**), though the measured activity at three months was again significantly (p <0.05) higher than at the start of the experiment.

In comparison to the untreated soil, phosphodiesterase activity was significantly (p <0.01) higher in soil receiving the Zn sludge treatment at the start of the experiment, however no significant differences were observed between these soils for the remainder of the incubation period (**Table 6.10**). Similarly, activity in the untreated soil was also significantly (p <0.05) higher than in soil receiving digested control at two weeks, following which no other significant differences were observed; at two weeks, activity in the untreated soil was also significantly (p <0.05) higher than in soil receiving the Cu sludge treatment. Whereas at two months enzyme activity in soil receiving the Cu sludge treatment was significantly (p <0.05) higher in comparison to the untreated soil and soil receiving the undigested control (**Table 6.10**).

6.5.4. Change in Phosphodiesterase Activity Overview (2014)

In some instances the production of phosphodiesterase followed that seen for phosphomonoesterase, particularly in soil receiving the Zn sludge treatment at Auchincruive, and in soil receiving the uncontaminated control at Gleadthorpe. In addition, although the differences in activity were not significant, the production of phosphodiesterase at Gleadthorpe appeared to be delayed in soil receiving the Zn sludge treatment in comparison to that in soil receiving the digested control, reaching maximum values at one month, and two weeks, respectively (**Figure 6.24**). The apparent decline in enzyme activity per mg C_{mic} at Hartwood is likely to be due to the increase in C_{mic} seen after two months (**See Section 5.2.3**). Hence it is possible that production of phosphodiesterase has remained relatively constant in Hartwood soil over the course of the incubation period.

6.6. Chapter Discussion (2014)

The results presented in **Section 6.3**, for the pH optima of phosphomonoesterase and phosphodiesterase, are in agreement with those reported by Turner (2010) for three acidic rain forest soils with pH ranging from 3.3-3.6. In these soils, the pH optima of phosphomonoesterase and phosphodiesterase were determined to be pH 4-5 and pH 3-5, respectively; however unlike the LTSE soils only one pH optima was observed for phosphomonoesterase. In addition, phosphomonoesterase activity (per gram of soil) was seen to be inversely proportional to total phosphorus concentration, with the highest activity seen in soil with the lowest total phosphorus content. This is also in agreement with the above regression analysis for untreated soil (**Table 6.7**), although in this case, phosphomonoesterase activity was inversely correlated to 'available' phosphorus. However, in contrast to the results presented above, phosphatase enzyme activity in rain forest soils with pH comparable to the LTSE field sites (pH 5.4-6.4) showed pH optima of 9.5-11.5, and 8.5-10 for phosphomonoesterase and phosphodiesterase, respectively. Similarly, Niemi and Vepsäläinen (2005) observed pH optima of pH 5 and pH 4 for phosphomonoesterase and phosphodiesterase, respectively, in two Finnish soils with pH 3.7-3.8. Whereas, in a third soil with pH 7.4, the phosphomonoesterase pH optima decreased to pH 4, while that for phosphodiesterase increased to pH 7.5. A further two soils were investigated with pH 6.5-6.4, comparable to the English LTSE field sites, for which the phosphomonoesterase pH optima was pH 6-6.5 (phosphodiesterase = pH 4.5). Herbian and Neal (1990) have also reported phosphomonoesterase pH optima of approximately pH 5-5.5 in a forest and grassland soil with pH 4.9 and 6.6, respectively; whereas, the phosphodiesterase pH optima were reported to be pH 4 and pH 6. However, in this case chromogenic substrates were used during the enzyme assay (*p*-nitrophenol-phosphate; *bis*-*p*-nitrophenol-phosphate), so these results are not directly comparable to those in **Table 6.3**; although good correlation between methods has been reported (Droullion & Merckx, 2005). In contrast to the results presented above, an arable soil, with pH 7.2 was seen to have maximum phosphomonoesterase activity at pH 11 (although a secondary optima was seen at pH 4.8), while phosphodiesterase activity was greatest at pH 8 (Herbian & Neal, 1990).

The pH optima of phosphomonoestarse is generally reported to be pH 6.5, or pH 11 for alkaline phosphomonoesterase, while that for phosphodiesterase is regularly reported to be pH 8 (Browman & Tabatabai, 1978; Eivazi & Tabatabai, 1976; Tabatabai, 1994; Frankenberger, Jr. & Johanson, 1982), whereas multiple pH optima are not often reported. With regards to phosphomonoesteras activity, the results described here appear to be in agreement with those presented in **Table 6.3**, therefore it is entirely possible that the two pH optima seen in the majority of LTSE soils are due to the presence of phosphomonoesterase isozymes. However, as mentioned above, due to limitations of the micro-plate method, it was not possible to distinguish between the activity of immobilised enzymes and the activity

of isozymes within the soil. Interestingly, Turner (2010), citing Tabatabai (1994), suggests that fungal phosphomonoesterases have a lower pH optima in comparison to those produced by bacteria, therefore soils where maximum phosphomonoesterase activity is observed at acidic pH values are considered to have a larger fungal population. Although tentative, the overall observed increase in V_{MAX} (per mg of C_{mic}) at pH 6.5 (**Figure 6.13**) also correspond to the observed increases in C_{fungi} (**Figure 5.11**), which may indicate that fungal species now play a more prominent role with regards to the synthesis of phosphomonoesterase in the contaminated soils at the LTSE field sites. However, an increase in V_{MAX} per mg of C_{mic} may also be due to the inhibition of extracellular enzymes within the soil environment.

In general, it is thought that metal ions inhibit enzymes through interactions with thiol (SH) groups present within the enzyme protein structure (Huang & Shindo, 2000b; Juma & Tabatabai, 1977; Shaw, 1954). Therefore the extent of enzyme inhibition by metal ions depends on the binding affinities of each metal for SH groups and the stability of the sulphide-metal complex formed (Huang & Shindo, 2000b; Shaw, 1954). For the metals legislated for by the EU Sludge Directive and UK Sludge (Use In Agriculture) Regulations, the relative chelate stabilities decrease as follows: $Hg^{2+} > Cu^{2+} > Ni^{2+} > Pb^{2+} > Zn^{2+} > Cd^{2+}$ (Shaw, 1954). Therefore, with regards to **Research Question 2**, it can be expected that Cu will have a greater inhibitory effect on the activity of phosphatase enzymes at the LTSE field sites in comparison to Zn, this is supported by the metal speciation data presented in **Chapter 3**.

Huang and Shindo (2000a, 2000b, 2001) have investigated the inhibition mechanisms for Zn and Cu on phosphomonoesterase present as free enzyme in solution, and immobilised on clay surfaces. For Zn, the mechanism of enzyme inhibition was predominantly uncompetitive (**See Section 6.4.2**) over the pH range 5-6 (although non-competitive inhibition was observed for phosphomonoesterase adsorbed to kaolinite at pH 5-5.5), as both K_M and V_{MAX} were seen to decrease with increasing Zn concentration (Huang & Shindo, 2000b). This would imply that Zn only interacts with phosphomonoesterase during the mineralisation process, as uncompetitive inhibition can only occur once an enzyme-substrate complex has already formed. Inhibition of phosphomonoesterase by Cu, again over pH range 5-6 was non-competitive (**See Section 6.4.2**), which implies Cu is able to bind with both free enzyme, and enzyme-substrate complexes. Given the high binding affinity of Cu for soil organic matter discussed in **Chapter 3** (**See Section 3.5.4**), it is assumed that soils receiving the Cu sludge treatment, at each of the LTSE field sites, will now contain the greatest proportion of extracellular enzyme-metal complexes, and therefore the extent of inhibition should be greatest in these soils; although this is yet to be demonstrated. The reason for the overall observed increase in V_{MAX} (per mg of C_{mic}) remains unclear. However, this may be due to enzyme inhibition within the soil, which would mean microorganisms in the Cu contaminated soil are frequently required to produce new enzymes (*de novo* synthesis) in order to mineralise organic matter, hence increasing activity per unit of C_{mic} (**Figure 6.27**). Whereas those in untreated soil and soils receiving uncontaminated sludge treatments can still rely on uninhibited extracellular enzymes within the soil environment and, as described by Burns (1982), can proliferate

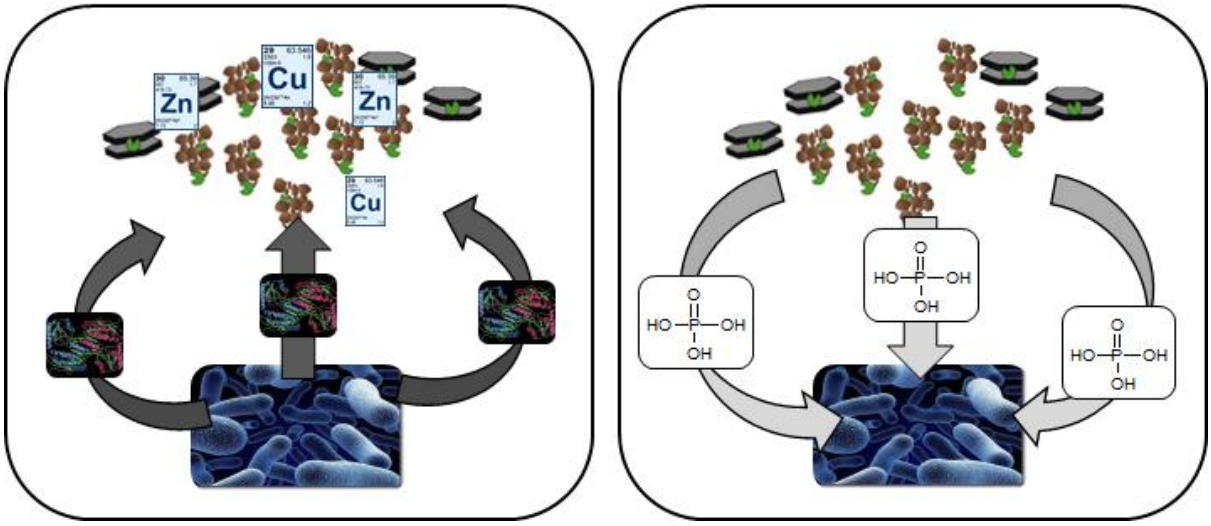


Figure 6.27:- Schematic showing possible differences in enzyme activity within contaminated (left) and uncontaminated (right) soil. In contaminated soil extracellular enzymes are inhibited, therefore *de novo* enzyme synthesis is required in order for microorganisms to actively mineralise fresh organic matter. In uncontaminated soils extracellular enzymes remain uninhibited facilitating passive mineralisation of organic matter.

C_{mic} without necessarily the need for enzyme synthesis (**See Section 1.5**). The short-term changes in enzyme activity observed during incubation, particularly at Auchincruive may also be an indication of this scenario (**See Section 6.5**). However, further investigation is required to test this hypothesis.

6.7. Conclusions (2014)

At each of the LTSE field sites, phosphomonoesterase and phosphodiesterase activity appeared to increase with decreasing pH. In general, phosphomonoesterase showed two pH optima, with maximum activity observed between pH 3-4, and a secondary pH optima at pH 6.5, whereas the activity of phosphodiesterase increased to an apparent maximum at pH 3. It is thought that the phosphatase enzymes produced by fungi generally have lower pH optima, hence this may be an indication that the proportion of the soil microbial community comprised of fungal species has increased, in agreement with the ergosterol data presented in **Chapter 5**. With the exception of soil receiving the undigested control, the activity of phosphomonoesterase per mg of C_{mic} at pH 6.5, remained higher in soils receiving the digested sludge treatments (Zn and Ctrl1) in comparison to the untreated soil, with significant differences observed at Hartwood and Auchincruive, in soils receiving the Zn and Cu sludge treatments respectively. Similarly, phosphodiesterase activity at pH 3 was significantly higher in soils receiving the Zn sludge treatment at Hartwood and Woburn.

The presence of two pH optima for phosphomonoesterase activity indicates that either the soil contains more than one form of phosphomonoesterase, or the activity of phosphomonoesterase has been altered due to the stabilisation of the protein within the soil environment. Phosphomonoesterase activity was

investigated further using the Michaelis-Menten model of enzyme kinetics, which did indicate that other factors, such as the presence of isozymes, were influencing enzyme activity. An additional kinetic model, incorporating the activity of a second enzyme, was therefore used to interpret the data. However, although a greater proportion of the observed variance was explained by the model, indicating the presence of isozymes, it was not possible to accurately determine the relative activities of the two enzymes. Using the Michaelis-Menten model, no significant differences in the maximum activity of phosphomonoesterase were observed in sludge amended soils. Therefore with regards to **Research Question 1,** there appears to be no detectable long-term decrease in the activity of phosphomonoesterase, contrary to the posed hypothesis. However combining the results using meta-analysis indicated that in general phosphomonoesterase activity per mg of C_{mic} was greater in soils receiving the Cu sludge treatment. Although the reason for this remains unclear. This may be an indication that Cu is inhibiting extracellular phosphatase enzyme activity, as more enzyme is now required per unit of C_{mic}. This would be in agreement with the metal speciation results presented in **Chapter 3**, as a significant fraction of the total Cu concentration, in soils receiving the Cu sludge treatment, was bound to soil organic matter; which would include extracellular enzymes. Though although this hypothesis is plausible, it has not been sufficiently tested, therefore **Research Question 2** remains unanswered.

In contrast, Zn appeared to have no effect on phosphomonoesterase activity, although activity per mg C_{mic} was again greater in soils receiving the Zn sludge treatment. However, in this case, the difference may be due to the higher concentration of available phosphorus in soil receiving the Zn sludge treatment. In soil receiving the digested sludge treatments, phosphomonoesterase activity per g of soil was seen to increase in proportion to both C_{mic} and the concentration of orthophosphate; whereas no correlation to available phosphorus was seen in soils receiving the undigested sludge treatments. Similarly, the activity of phosphomonoesterase per mg of C_{mic} also increased in proportion to the concentration of available phosphorus. The response of soil microorganisms to the fresh application of sewage sludge varied considerably between field sites, presumably due to differences in the soil microbial communities between soils. This may also be an indication of an indirect effect of heavy metal contamination on the activity of phosphomonoesterase, if the rate of enzyme synthesis is altered due to changes in the microbial community. In general, phosphomonoesterase activity per mg of C_{mic} was higher in the contaminated soils for the duration of the incubation period, whereas the production of phosphomonoesterase in soils receiving the uncontaminated sludge treatments generally followed that of the untreated soil. Again, this may be an indication that enzyme activity is inhibited by the presence of heavy metals. Phosphodiesterase appeared to be less affected over the course of the incubation period, and with few exceptions, no significant differences in enzyme activity were observed between soils receiving the contaminated sludge treatments (Zn and Cu) and soils receiving the respective uncontaminated controls (Ctrl1 and Ctrl2).

CHAPTER 7

INTEGRATED DISCUSSION

7. INTEGRATED DISCUSSION

7.1. Introduction

The aim of this chapter is to consider the results presented in **Chapters 2-6** with regards to both the research questions and hypotheses given in **Chapter 1**, and determine how the results may influence each other, while discussing them in a wider context. The accuracy and reliability of the results obtained, and the analytical methods used to obtain them, are also discussed. Recommendations for future work are also suggested.

7.2. Accuracy and Statistical Power

On several occasions throughout the current investigation, and often within environmental science as a whole, conversion factors have been applied in order to estimate the magnitude of one variable by measuring another. However the derivation of such factors and the assumptions upon which they are based need to be considered carefully, particularly with regards to heterogeneous materials such as soil, in order to ensure the calculated results are as accurate as possible. For instance in **Chapter 2**, SOC was determined for soils sampled in years 2013 and 2014 by converting measured values for SOM using a conversion factor (F_C). It is generally assumed that soil organic matter is comprised of 58 % organic carbon, therefore conversion of SOM to SOC is achieved by multiplying values for SOM by 0.58. However, Pribyl (2010) has recently criticised this assumption, stating that the percent of organic carbon present within SOM varies considerably between soils and suggests values for F_C could range from 0.40-0.71, therefore the use of a universal conversion factor for all soils is questionable. For the current investigation values for F_C were calculated for each soil (**E. 2.13**) sample using measurements of SOC and SOM determined for samples collected in 2012 (**See Section 2.7.1**). Although this is more accurate than assuming SOM is comprised of 58 % organic C, an improvement would be to measure SOC directly in samples collected during 2013 and 2014.

Similarly, for the measurements of C_{mic} and C_{fungi} conversion factors were used to derive estimates of both forms of microbial biomass (**See Section 5.2.1** and **Section 5.3.1**). For C_{mic}, the conversion factor K_{EC} (equal to 0.45) was applied (**E. 5.2**), based on the method described by Vance et al. (1987), that assumes the extracted organic C only constitutes 45 % of C_{mic} present within the soil. However, although the value of 0.45 is widely used for K_{EC}, estimates of its value range from 0.10-0.66 (Martens, 1995). The appropriate value for K_{EC} still remains undetermined, however Jenkinson et al. (2004) have recently stressed that there are sufficient similarities between the microbial biomass of different soils to warrant

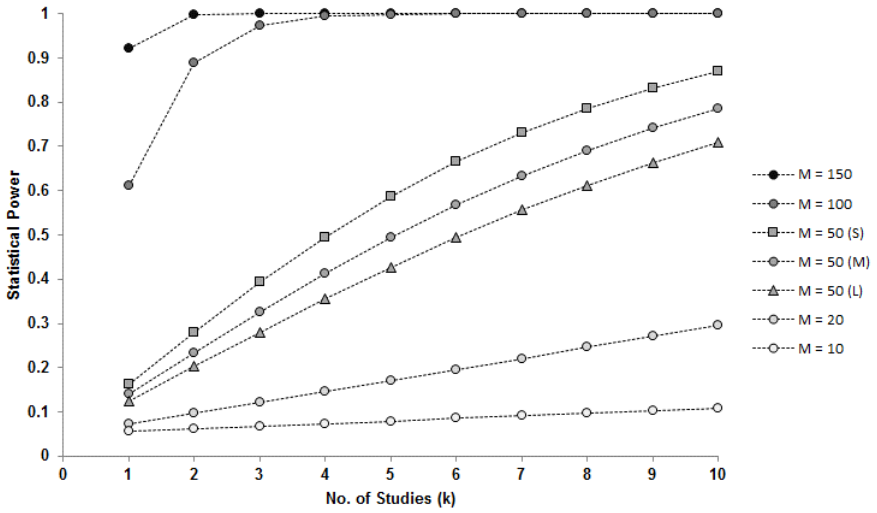


Figure 7.1:- Power analysis, showing statistical power (%) of meta-analysis as a function of effect size (M) and No. of studies (k). Here M represents the observed difference in Microbial Biomass Carbon (µg C_{mic} g $soil^{-1}$) between soils treated with contaminated sludge and uncontaminated controls. The influence of varying effect sizes (S = Small, M = Medium, L = Large) between studies on statistical power is also shown for M = 50.

the use of a single conversion factor; although they acknowledge that an up-to-date review of the literature is required. Fungal biomass C was calculated from measurements of ergosterol using the conversion factors K_F and F_F (**E. 5.5**), based on the method of Montgomery et al. (2000), which respectively assume that 1µg ergosterol corresponds to 250 mg of fungal biomass, and that fungal biomass is comprised of 43 % organic carbon (C_{fungi}). However, in the same year Ruzicka et al. (2000) criticise the use of a universal ergosterol:fungal biomass ratio due to the varying quantities of ergosterol produced by different species of fungus, along with a range of systematic errors encountered in deriving values for the ratio. Furthermore, Ruzicka et al. (2000) suggest that measurements of ergosterol may be more suited to determining the surface area of the fungal biomass rather than its mass. Therefore, although the relative differences in ergosterol observed between untreated and sludge amended soils at the LTSE field sites should remain unchanged following conversion to C_{fungi}, the estimated values for the percentage of C_{mic} comprised of C_{fungi} should be treat with caution, as their accuracy remains to be determined. Nevertheless, they provide an overall indication as to the changes in microbial structure that have occurred in soils receiving the contaminated sludge treatments.

Scaling factors were also used to account for fluorescence quenching encountered during the assay of phosphatase enzyme activities (**See Section 6.2.1**). These were calculated as the percentage of total fluorescence observed in assay mixtures containing soil samples (**E. 6.1**), by comparison to blank assay mixtures, before averaging to give a mean quenching factor for each of the LTSE field sites (**Table 6.2**). However, values for enzyme activity were calculated by reference to calibration graphs for which fluorescence quenching was not taken into account. Although application of QF should still produce accurate estimates of soil enzyme activity, it is recommended by Marx et al. (2001) that individual calibration graphs be produced for each soil sample under investigation. However, given that a total of

60 samples were collected during the current investigation (**See Section 2.3**), which were to be analysed for enzyme activity across 10 pH units (**See Section 6.3**), this would require 600 separate calibration graphs. This would be extremely time consuming, therefore the use of QF in the current investigation is justifiable. Nevertheless, accuracy should not be sacrificed for the sake of convenience, therefore an improvement of the micro-plate method would be to incorporate the measurement of calibration standards into the micro-plate layout for each soil sample.

With the exception of the total concentrations determined for Zn, Cu, and P where clear significant differences remain detectable in sludge amended soils, the data sets obtained for other variables such as SOC, C_{mic}, ergosterol, and phosphatase enzyme activity appear to lack statistical power. Often it is assumed that the absence of statistical significance provides evidence for the null hypothesis, e.g. Zn and Cu have no long-term effect on C_{mic}. However non-significant results can simply be due to low statistical power where a small sample size has been used during an investigation (Borenstein, 2000). For instance, assuming the application of contaminated sludge causes a decrease in C_{mic} of 50 µg C_{mic} g $soil^{-1}$ relative to soils receiving uncontaminated sludge, and the standard deviation of C_{mic} in both soils is 55 µg C_{mic} g $soil^{-1}$, then the statistical power (i.e. the probability of obtaining a statistically significant result) based on a sample size of n = 3 is only 15 % (**Figure 7.1**). Whereas, using the same assumptions, combining k = 10 studies, each reporting results based on a sample size of n = 3, gives a statistical power of 80 % (**Figure 7.1**). Combining studies in this way markedly increases statistical power and can help reduce the 'noise' produced by random sampling error (Hedges & Pigott, 2001). However, it should be noted that standard error is a function of sample size, therefore increasing n will eventually produce a statistically significant result. For this reason, meta-analysis is primarily concerned with the overall magnitude and direction, or practical significance, of the observed effects, rather than statistical significance. For instance, combining the data for C_{mic} across the four LTSE field sites showed that the overall long-term effect of applying the Zn sludge treatment was to decrease C_{mic} in the receiving soil by approximately 16 % relative to soil receiving the digested control (**Figure 5.4**). However, with regards to the activity of phosphomonoesterase this appears to have little practical significance as no overall effect on V_{MAX} was observed (**Figure 6.13**). In contrast, the Cu sludge treatments appeared to have a lesser effect on C_{mic}, causing a decrease of approximately 8 %, but a greater effect on the activity of phosphomonoesterase, as V_{MAX} per mg C_{mic} increased by approximately 10 % (**Figure 6.13**), however the practical significance of this effect remains to be determined.

7.3. Current Perspectives on EU/UK Sludge Policy

As described in **Chapter 1**, sewage sludge often contains concentrations of heavy metals which could potentially lead to dangerous levels of heavy metals accumulating in the environment (**See Section 1.3**), thus posing a risk to soil microorganisms (**See Section 1.4**) and the overall quality of agricultural soils.

Table 7.1:- Maximum permissible concentrations (mg kg^{-1}) for heavy metals in sewage sludge and sludge amended soils adopted by EU member states and the United States (Adapted from MAFF/DoE (1993b), McGrath et al. (1994), and Smith (1996)).

	Total Metal Concentration (mg kg^{-1})										
	Sludge										
	France	Germany	Italy	Spain	The Netherlands	UK[1]	Denmark	Finland	Norway	Sweden	USA
Cd	20	10	20	20	1.25	N/A[2]	1.2	1.5	4	2	5
Cu	1000	800	1000	1000	75	N/A	1000	600	1000	600	4300
Pb	800	900	750	750	100	N/A	120	100	100	100	840
Hg	10	8	10	16	0.75	N/A	1.2	1.0	5	2.5	57
Ni	200	200	300	300	30	N/A	45	100	80	50	420
Zn	3000	2500	2500	2500	300	N/A	4000	1500	1500	800	7500
	Soil[3]										
Cd	2	1.5	3	1	0.8	3	0.5	0.5	1	0.5	20
Cu	100	60	100	50	36	135	40	100	50	40	750
Pb	100	100	100	50	85	300	40	60	50	40	150
Hg	1	1	1	1	0.3	1	0.5	0.2	1	0.5	8
Ni	50	50	50	30	35	75	15	60	30	15	210
Zn	300	200	300	150	140	300	100	150	150	100	1400

[1]The UK Sludge (Use in Agriculture) Regulations do not set maximum limits for heavy metals in sewage sludge. Instead metal loadings (kg ha^{-1} yr^{-1}) are to be kept below a specified annual average over a 10 year period (**Table 1.2**). [2]Not Applicable. [3]Concentration in soils with pH 6-7.

The approach to mitigating the accumulation of heavy metals in sludge amended soils varies between EU member states and depends on the soil usage priorities and the extent of environmental impact tolerated by each country (McGrath et al., 1994). McGrath et al. (1994) examined the scientific approaches taken by different countries and identified three principal methods for determining statutory limits for the concentration of heavy metals in sewage sludge and sludge amended soils. The first approach is to carry out an environmental risk assessment and analysis of exposure pathways, as done by the United States Environmental Protection Agency (USEPA). A number of potential exposure routes, whereby metal toxicity may occur in plants, animals, and humans, were considered by the USEPA resulting in some of the highest metal loadings permitted with regards to the application of sewage sludge to agricultural land (**Table 7.1**); noticeably the only consideration of soil biota was the potential bioaccumulation of heavy metals in earthworms (McBride, 1995; McGrath et al., 1994). As mentioned previously (**See Section 1.3**), the majority of EU member states do permit a certain degree of metal accumulation and have adopted statutory limits based on the observed impacts to soil microorganisms (**Table 7.1**); with the statutory limits set by the UK Sludge (Use In Agriculture) Regulations amongst, if not the highest, in Europe. In this case, the approach is to adopt statutory limits below the minimum concentrations of heavy metals know to have an adverse effect on soil microorganisms (McGrath et al., 1994). Whereas, in contrast, the most cautious method is the 'metal-balance' approach adopted by countries such as Sweden, Denmark, and the Netherlands. This approach recognises the extreme persistence of heavy metals within the soil environment and tries to match the rate of sludge application with that of natural attenuation and removal processes in order to prevent a net accumulation of heavy metals. As a result of this the statutory limits set by these countries are the lowest in Europe (**Table 7.1**) and significantly restrict the recycling of sewage sludge to agricultural

land. Although there is little evidence to support the need for such conservative limits, Witter (1996) suggests this approach provides more of an incentive to reduce metal emissions to wastewater thus improving the overall quality of sewage sludge. This is clearly a desirable situation and an obvious solution to the problem of heavy metals accumulating in agricultural land due to the application of sewage sludge.

Given the varying approaches adopted by EU member states, with regard to the use of sewage sludge in agriculture, a review of the EU Sludge Directive is currently ongoing in order to re-evaluate the existing regulatory framework (CEC, 2003, 2010; Gendebien et al., 2010). A 'Working Document on Sludge' (CEC, 2000b) was drafted in 2000, coinciding with the implementation of the Water Framework Directive 2000/60/EC (CEC, 2000a), which proposes several reductions in the concentrations of heavy metals currently permitted in sewage sludge, and sludge amended soils (**Table 7.2**); along with additional legislation for organic micro-pollutants and microbial pathogens, which are not currently regulated by the EU Sludge Directive (CEC, 2000b). The following year a list of 'Priority Substances' (including Cd, Pb, Hg, and Ni) was compiled as an amendment to the Water Framework Directive (CEC, 2001), which aims to phase out and cease all emissions of the listed pollutants to water bodies by 2020. Although, Zn and Cu are not listed as priority substances, both the 'Working Document on Sludge' and Water Framework Directive provide incentive to reduce emissions of heavy metals to wastewater steams where possible, thereby improving the overall quality of sewage sludge.

A steady decline in the concentration of heavy metals has been seen in sewage sludge, over the past 35 years, due to a reduction in heavy metal emissions from domestic, commercial, and industrial sources, as well as urban run-off (Gendebien et al., 1999, 2010; MAFF/DoE, 1993b; Smith, 1996; Thornton et al., 2001). At the time the UK Sludge (Use In Agriculture) Regulations were enacted in 1990, a decrease of approximately 25 % was observed in the median concentrations of both Zn and Cu measured in UK sewage sludge in comparison to values recorded 8 years previously, with further decreases of 37 % and 21 % seen for both metals, respectively, over the next 5 years (**Table 7.3**). Therefore at the time the Long-Term Sludge Experiment was established the contaminated sludge treatments (Zn and Cu; **Table 3.2**) applied contained more than 10 times the median concentration (8 × mean) of Zn and Cu typically found in UK sewage sludge. Similarly, the confounding Cu contamination present in the Zn sludge treatment (**See Section 3.3**) was also 3 times the median concentration (2.5 × mean), whereas that of Zn in the Cu sludge treatment was 1.5 times greater. Comparison to contemporary sludge quality data shows the concentrations of Zn and Cu in the contaminated sludge treatments were respectively 10 and 17 times the mean concentration of Zn and Cu now seen in UK sewage sludge. Hence, the contaminated sludge treatments are clearly exceptional cases and their application to agricultural land would not be permitted within any of the EU countries mentioned in **Table 7.1**; although application of the Zn sludge

Table 7.2:- Proposed changes to the maximum concentrations of heavy metals in sludge, sludge amended soils, and average annual rate of application over a 10 year period, permitted by the EU Sludge Directive given in the EU 'Working Document on Sludge 3rd Draft' (CEC, 2000).

	Total Metal Concentration (mg kg^{-1})				Annual Mean over 10 Years (kg ha^{-1} yr^{-1})
		Soil			
	Sludge	pH 5.0<6.0	pH 6.0-7.0	pH >7.0	
Cadmium (Cd)	10	0.5	1	1.5	30
Copper (Cu)	1000	20	50	100	3000
Lead (Pb)	750	70	70	100	2250
Mercury (Hg)	10	0.1	0.5	1	30
Nickel (Ni)	300	15	50	70	900
Zinc (Zn)	2500	60	150	200	7500

Table 7.3:- Mean (weighted) and median concentrations (mg kg^{-1}) of heavy metal contaminants measured in UK sewage sludge over the course of 15 years (1982-1996). Data obtained from CEC (2010), Gendebien et al. (1999), MAFF/DoE (1993b), Smith (1996), and Thornton et al. (2001).

	Total Metal Concentration (mg kg^{-1})						
	Median			Mean (Weighted)			
	1982/83	1990/91	1995/1996	1982/83	1995/1996	2006	Overall Decrease (%)
Cadmium (Cd)	9	3.2	1.6	9	3.3	1.3	82.2 (85.5)[1]
Copper (Cu)	625	473	373	589	568	295	40.3 (50.1)
Lead (Pb)	418	217	99	398	221	112	76.3 (71.9)
Mercury (Hg)	3	3.2	1.5	4	2.4	1.2	50.0 (70.0)
Nickel (Ni)	59	37	20	61	57	30	66.1 (50.8)
Zinc (Zn)	1205	889	559	1144	792	574	53.6 (50.2)

[1]Values in parenthesis are weighted mean.

treatment would be permitted in the US. Furthermore, the total metal loadings applied over the course of experimental Phase I would not be permitted within UK or EU agricultural practice, and represents a case of acute exposure to heavy metal contamination, rather than a chronic accumulation, which may influence both the short-term and long-term response of soil microorganisms. In addition, the concentrations of Zn and Cu present in the uncontaminated controls (Ctrl1 and Ctrl2; **Table 3.2**) appear to be typical of UK sludge produced at the time, particularly in the digested control where the concentrations of Zn and Cu were respectively equal to and above the median values reported in **Table 7.3**, whereas only Cu was above the median value in the undigested control; though in both cases metal concentrations were below the reported means. However, it should be noted that application of either of the uncontaminated controls would not be permitted in the Netherlands, due to such a low statutory limit set for Cu (**Table 7.1**), and application of the digested control in Finland or Sweden would only just be passable.

Following experimental Phase I, the total concentrations of Zn and Cu in soils receiving the contaminated sludge treatments increased above the respective UK statutory limits for these metals in sludge amended soils at each of the 'Long-Term Sludge Experiment' field sites. At the Scottish sites, Auchincruive and Hartwood, total metal concentrations have only decreased by approximately 9-12% over the past 17 years, and respectively remain equal to and above the current UK limits. Whereas at the two English sites the total concentrations of Zn and Cu have now decreased by approximately 29-37 %, and 30-35 %, respectively, and are now below the UK statutory limits; although in both cases Zn

still remains above the advisory limit of 200 mg kg^{-1}. However, if the EU Sludge Directive is revised, and the limits proposed in the 'Working Document on Sludge' are implemented (**Table 7.2**), then the concentrations of Zn and Cu at Woburn and Gleadthorpe, present in the soils receiving the respective sludge treatments, would again be above the permitted maximum. Furthermore, the confounding Cu contamination present in soils receiving the Zn sludge treatment (**See Section 3.3**) at each of the LTSE field sites, would be above the proposed statutory limit (**Table 7.2**). Therefore, given the trends in both sludge quality and sludge policy, i.e. a decline in sludge metal content, and a reduction in permitted metal content, it is becoming increasingly unlikely that the situations presented at the LTSE field sites, with regards to heavy metal contamination, would be encountered under current UK and EU agricultural practices. However, as EU member states work towards the targets imposed by the Water Framework Directive, increasing controls over diffuse sources of pollution will be implemented, which include nitrates and phosphates (CEC 2000a). Often the ratio of P to N in sewage sludge is such that applications of sludge based on the N requirements of crops will exceed the P requirement (Gendebien et al., 2010), which, as shown in **Chapter 4**, could potentially lead to an accumulation of P in the receiving soils. Therefore, it is likely that in future sludge P content will become the predominant factor limiting the use of sewage sludge in agriculture, however this has serious implications for sludge recycling as the quantities of sludge applied per annum would be considerably reduced (Gendebien et al., 2010).

7.4. Do the Limits Need to Change?

The results presented in **Chapter 5** show that, despite being able to sustain a microbial biomass of size comparable to untreated soil, significant changes in microbial community structure have occurred in the sludge amended soils, at each of the LTSE fields, due to the application of contaminated sludge treatments (Zn and Cu). Even in soils receiving the uncontaminated controls (Ctrl1 and Ctrl2) significant changes in the PLFA profiles could be seen in comparison to the untreated soil in some cases (**Table 5.5**). Hence, with regards to the LTSE field sites, the current UK statutory limits do not appear to be sufficient to prevent changes in soil microbial community structure. As mentioned in **Section 1.4**, two questions have been posed with regards to soil microorganisms and their significance in determining statutory limits for heavy metals in sludge and sludge amended soils, these are: 'do microbes matter?' (Giller et al., 1999) and if so 'where's the limit?' (Dahlin et al., 1997).

The problem encountered by legislators, when deciding to set statutory limits for sludge amended soils, is trying to balance waste management with environmental protection. Therefore, as described above, statutory limits are generally set at the maximum tolerable metal concentration, thus providing some degree of protection to soil microorganisms, whilst facilitating the recycling of sewage sludge (Giller et al., 1999; McGrath, 1994). However, as described in **Chapter 5**, significant changes in soil microbial community structure can occur in soils where the total metal concentration is below current EU/UK

limits. Hence, the question posed by Dahlin et al. (1997) emphasises the point that there is no distinct 'universal' toxicity threshold and in some situations the proposed limits will not suffice. Witter (2000) gives three hypotheses which predict how soil microbial communities will respond to heavy metal contamination over time, these are:

- "*Metal toxicity exerts a selective pressure on soil-microorganisms thus changing the relative competitive advantage between microbial groups."*
- *"This results in changes in microbial community structure and in the diversity of soil microorganisms."*
- *"These "unseen" effects precede and are the cause of most of the more visible effects seen at the functional level."*

As mentioned in **Chapter 1**, such a response can lead to the loss of important processes within the soil environment, for example the fixation of nitrogen by *Rhizobium* (Chaudri et al., 1993; Giller et al., 1989; McGrath et al., 1988). Therefore, both Giller et al. (1999) and Witter (2000) stress the importance of long-term field experiments in determining the response of soil microbial communities to heavy metal contamination.

With regards to the first two hypotheses posed by Witter (2000), the results from **Chapter 5** are in agreement. However, the current investigation is primarily concerned with the ability of soil microorganisms to mineralise organic P, through the activity of phosphatase enzymes. The results presented in **Chapter 4** and **Chapter 6** suggests that, despite significant changes in soil microbial community structure, the microorganisms that remain are still capable of producing phosphatase enzymes, thereby facilitating the cycling of phosphorus as a nutrient; this can be seen in the ^{31}P-NMR spectra of untreated and sludge amended soils at Woburn and Hartwood (**See Section 4.4**). Hence, in this case, it cannot be said that there is a long-term impact on soil microorganisms with regards to soil processes. Though, it should be stressed that the current investigation only focussed on the mineralisation of organic P, therefore it is still possible that other enzymes, such as urease which mineralises organic N, may be inhibited by the presence of Zn and Cu, or their synthesis may have ceased due to the loss of specific microbial species. Due to the uncertain relationship between microbial diversity and soil processes, Witter (2000) emphasises that microbial diversity should be protected and therefore suggests a more precautionary approach in setting statutory limits (Witter 1996; 2000). Hence, with regards to **Research Question 4**, although the UK limits appear sufficient to protect C_{mic} and phosphatase enzyme activity, contrary to the hypothesis posed, the ergosterol and PLFA data presented in **Chapter 5** suggest the current UK statutory limits are not sufficient to prevent changes in microbial community structure, thereby risking the loss or disruption of essential soil processes such as nitrogen fixation, or the mineralisation of organic matter. Therefore, it would be advisable to reduce the current UK limits, in line with those proposed in the 'Working Document on Sludge' (CEC, 2000b). Given that

sludge quality is continuing to improve, i.e. sludge metal content is decreasing (**Table 7.3**), such a reduction in the statutory limits would not severely restrict the use of sludge in agriculture nor decrease the overall longevity of sludge applications.

7.5. Implications for the Use of Sewage Sludge as a Source of Phosphorus

This investigation provides a comprehensive overview of the factors that may potentially limit the use of sewage sludge as a sustainable source of phosphorus. Incorporation of meta-analysis into the statistical analysis signifies a novel approach in the interpretation of experimental data, and allows a more accurate overview of the trends and relationships observed between separate studies, in this case between field sites. Furthermore, the discussion of phosphatase enzyme activity normalised per unit of microbial biomass carbon, provides new insights into the relationship between soil microorganisms and extracellular enzymes within the soil environment. So far comparison of normalised enzyme activities in soils contaminated with Zn and Cu have not been reported.

Contrary to the hypothesis posed for **Research Question 3**, no significant accumulation of organic phosphorus compounds was observed in any of the sludge amended soils at the LTSE field sites. Further investigation of the forms of organic phosphorus present within the sludge amended soils at Woburn and Hartwood, showed that the more labile forms of organic phosphorus, phosphodiesters and phosphonates, as well as *scyllo*-inositol-pentakisphosphate, present in the applied sludge treatments, had now been mineralised with no apparent interference from Zn and Cu. However, the relative rates at which organic phosphorus was mineralised over time in soils receiving the contaminated and uncontaminated sludge treatments has not been determined. It may be that the mineralisation of organic compounds in soils receiving the contaminated sludge treatments occurred over a longer period of time. No significant difference in the activity of phosphomonoesterase could be detected, however the presence of Cu appeared to increase enzyme activity per mg of C_{mic}, possibly indicating phosphorus limitation due to enzyme inhibition. Significant changes in the microbial communities of contaminated soils were observed, indicating long-term adaptation to the presence of Zn and Cu. However, these changes did not appear to affect organic phosphorus mineralisation in the long-term.

No detectable difference could be seen in the ^{31}P-NMR of soils receiving contaminated or uncontaminated sludge treatments, and in both cases, the spectra of sludge amended soils were comparable to the spectra for untreated soil. Therefore, with regards to the use of sewage sludge as a sustainable source of phosphorus, this investigation has found no long-term impact on the mineralisation of organic phosphorus through the activity of phosphatase enzymes. Given the current decline in the heavy metal content of sewage sludge, there appears to be no reason why sewage sludge should not be used as an alternative source of phosphorus, in place of mineral fertilisers derived from

phosphate rock, so long as current agricultural best practices are adhered to. However in most cases the application of digested and undigested sludge treatments has caused significant increases in the total concentration of phosphorus in the receiving soils. As mentioned above, this may eventually prevent the application of sewage sludge in some soils, as this represents a diffuse source of phosphate which can infiltrate water bodies. Therefore, in future there must be increasing consideration to the forms of phosphorus present within sewage sludge. Given that a significant proportion of phosphorus in sewage sludge is in organic form, removal of the inorganic component would reduce the risk of phosphate emissions, whilst still providing plants and soil microorganisms, via the process of mineralisation, a substantial phosphorus feedstock.

7.6. Recommendations for Future Work

As mentioned above, some of the data sets obtained during the current investigation appear to have low statistical power due to a sample size of n = 3. Hence it may be that the long-term effects of applying contaminated sludge treatments are now too small to detect given the natural variation of soil properties at each of the LTSE field sites. As mentioned in **Section 2.2**, the LTSE was originally established at nine field sites around the UK. Three of these sites have since been decommissioned, however the remaining two sites, at Rosemaund (Herefordshire, UK) and Bridgets (Hampshire, UK), are still maintained by ADAS. Therefore, the current investigation could be extended to include these two sites, providing additional data which could be incorporated into the meta-analyses presented above. Furthermore, including data from an additional two sites into the above regression models, may reduce the collinearity observed between variables such as SOC and the total concentrations of Zn and Cu (**See Section 3.5.4**), allowing more accurate determination of the relationship between heavy metals and soil properties such as C_{mic}.

As suggested in **Chapter 4**, a proportion of the soil organic phosphorus at each of the LTSE field sites is immobilised within the microbial biomass (P_{mic}), however this remains undetermined. Another extension of the current investigation would be to measure P_{mic} in both untreated and sludge amended soils at the LTSE field sites. Preliminary investigation into P_{mic} indicated the C_{mic}/P_{mic} ratio had decreased at the Woburn field site in soils receiving the Zn and Cu sludge treatments which could be an indication that phosphorus availability is reduced due to the presence of heavy metals.

Also as described in **Chapter 6**, the activity of phosphodiesterase at the LTSE field sites could not be accurately determined. The analysis of phosphodiesterase using the fluorogenic substrate *bis*-4-MUF-P was subject to several limitations. Therefore further investigation is needed in order to develop a suitable method of analysis; particularly with regards to determinng the kinetic parameters K_M and V_{MAX}, as the extent of phosphodiesterase inhibition by heavy metals in soils has not been reported. This may

require the use of molecular biology techniques (Nannipieri et al., 2012; Wallenstein & Weintraub, 2008). Furthermore, the reason for an observed increase in the maximum activity of phosphomonoesterase per mg of C_{mic} remains unclear. Extraction of the enzymes from soil could be attempted, allowing the concentration of enzyme within a soil to be estimated. From this it would be possible to determine a ratio of enzyme concentration to activity which would give a more accurate indication of the effects of heavy metals on extracellular enzymes within the soil environment.

CHAPTER 8

CONCLUSIONS

8. CONCLUSIONS

8.1. Introduction

This chapter presents the overall conclusions of the current investigation. The research questions posed in **Chapter 1** are reiterated at the start of each section, with the overall conclusion presented in ***bold***. In each case a summary of the results and discussion that led to each conclusion is given.

8.2. Conclusion 1

- *Does the presence of residual heavy metal contamination (Zn and Cu) cause a lasting decrease in soil microbial biomass and phosphatase enzyme activity, or can soil microbial communities become tolerant and recover?*

Application of the Zn and Cu sludge treatments during experimental Phase I, at 'Level 3' of the dose response curve, increased the total concentration of each metal, in the receiving soils at each field site, above the respective UK statutory limits. Almost 20 years since the final sludge applications were made, the total concentrations of Zn and Cu at the Scottish grassland sites, Auchincruive and Hartwood, remained equal to and above the UK statutory limits, respectively. In contrast, at the English arable sites the total concentrations of Zn and Cu at Gleadthorpe had now decreased below the UK limits, though total Zn remained above the UK advisory limit of 200 mg Zn kg^{-1}. Whereas, in general, the total concentrations of Zn and Cu at Woburn were now below both statutory and advisory limits. However, at each site, the total concentrations of Zn and Cu remained significantly higher in soil receiving the contaminated sludge treatments in comparison to untreated soil and soils receiving the uncontaminated controls. Therefore, the microbial communities present in soils receiving the contaminated sludge treatments have experienced prolonged exposure to elevated levels of heavy metal contamination.

For the current investigation, values for C_{mic} in soils receiving both contaminated and uncontaminated sludge treatments no longer appeared to be significantly different from that in untreated soil. In addition, the presence of heavy metals appeared to have little effect on the short-term proliferation of soil microorganisms over the course of three months. However, combining the results using meta-analysis showed that Zn and Cu contamination did have an overall negative effect on C_{mic}. Similarly, no significant differences were observed in the maximum activity of phosphomonoesterase in soils receiving the Zn and Cu sludge treatment. Though again, meta-analysis indicated that the overall effect of Cu contamination was to increase the maximum activity of phosphomonoesterase activity per mg of

C_{mic}; whereas Zn appeared to have no effect. Analysis of the fungal biomarker ergosterol, and in particular soil PLFA profiles, clearly showed significant changes in the microbial community due to prolonged exposure to Zn and Cu contamination.

It was therefore concluded that prolonged exposure to Zn and Cu contamination, due to the historical application of contaminated sewage sludge, does not necessarily cause a persistent decrease in soil microbial biomass or phosphatase enzyme activity but does cause significant changes in the microbial community. In addition, since the contaminated soils are still capable of sustaining a microbial community of size comparable that of untreated soil, the microbial communities must have adapted and become tolerant to Zn and Cu contamination.

8.3. Conclusion 2

- *What role does metal speciation play in the inhibition of phosphatase enzymes and toxicity to soil microorganisms?*

Exchangeable Zn and organically bound Cu metal species still remain significantly higher in soils receiving the respective contaminated sludge treatments, 20 years following the final sludge applications. Despite significant losses of organic C over the course of the Long-Term Sludge Experiment, the relative concentrations of Zn and Cu species in sludge amended soils remain unaffected and are largely determined by the total concentration of each metal present. A significant correlation between exchangeable and total metal concentration could still be seen in soils contaminated with Zn. Whereas, the solubility of Cu appeared to be very low, with less than 1 % of the total concentration observed present in exchangeable form; although a significant percent of the total concentration remained bound to organic matter. These results are in agreement with those reported for a number of long-term sludge experiments. However, at each site, application of the Zn sludge treatment also caused increases in the total concentration of Cu in the receiving soils. These were significantly greater in comparison to soils receiving the digested control, hence the observed changes in microbial community are likely due to a combination of Zn and Cu toxicity. This may also explain why the overall negative effect on C_{mic}, determined using meta-analysis, was greater for the Zn sludge treatment in comparison to that for the Cu sludge treatment. In contrast, meta-analysis showed that the overall effect of Cu on phosphomonoesterase was to increase enzyme activity per mg of C_{mic}. Copper has been seen to bind with both free enzyme, and enzyme-substrate complexes within the soil environment. Therefore, given the high binding affinity of Cu for soil organic matter it is likely that soils receiving the Cu sludge treatment, at each of the LTSE field sites now contain the greatest proportion of extracellular enzyme-metal complexes, and therefore the extent of inhibition is greatest in these soils. However, the reason for the overall observed increase in V_{MAX} (per mg of C_{mic}) remains unclear.

It was therefore concluded that although Cu is likely to have a greater inhibitory effect on phosphatase enzymes, the role of metal speciation in enzyme inhibition could not be determined due to limitations within the data set. Several possible extensions to the current investigation have been suggested which could improve the data set.

8.4. Conclusion 3

- *What is the long-term fate of organic phosphorus compounds derived from sewage sludge in sludge amended soils?*

Although the organic phosphorus content of sludge amended soils was higher in comparison to untreated soil, in most cases the increase was no longer significantly different, nor were the differences between sludge amended soils significantly different. Analysis of the sludge treatments by ^{31}P-NMR showed that a range of organic phosphorus compounds were applied to each of the receiving soils during experimental Phase I. Orthophosphate, phosphomonoesters, and pyrophosphate were the predominant forms of phosphorus within the applied sludge treatments, with the greatest range of organic phosphorus forms seen in the uncontaminated sludge treatments (Ctrl1 and Ctrl2). However, in each case, analysis of the sludge amended soils at Woburn and Hartwood, showed that the signals for the respective organic phosphorus species, with the exception of inositol phosphates, were now comparable to those seen in the spectra for untreated soil. At both sites, the inositol phosphate signals were greater in the spectra for sludge amended soils, in comparison to untreated soil, showing an increase in these compounds. The quantities of organic phosphorus determined in sludge amended soils are now likely to be comprised of microbial biomass phosphorus, phosphorus incorporated into the soil humus fraction, and inositol phosphates derived from the applied sludge treatments.

It was therefore concluded that the organic phosphorus compounds applied to soil in both contaminated and uncontaminated sludge treatments have now effectively been mineralised and the organic phosphorus contents of the sludge amended soils are returning to that of untreated soil, with no long-term interference caused by the presence of Zn and Cu.

8.5. Conclusion 4

- *Are the current limits specified for Zn and Cu in the UK Sludge (Use in Agriculture) Regulations sufficient to protect microorganisms and phosphatase enzyme activity in sludge amended soils?*

Significant long-term decreases in C_{mic} have been observed at a number of long-term field experiment sites where the historical application of contaminated sewage sludge has increased the total concentration of heavy metals in the receiving soils above the current EU/UK statutory limits. However, no significant effect of Zn or Cu contamination was observed on C_{mic} at any of the LTSE field sites when considered as separate cases. Combining the results using meta-analysis did show an overall negative effect. Therefore it is possible that the total concentrations of Zn and Cu present in the contaminated soils at the LTSE sites are approaching a toxicity threshold (coinciding with the UK statutory limits) above which more drastic decreases in C_{mic} may be observed. However, significant changes in microbial community structure can occur in sludge amended soils where total metal concentrations are below the current EU/UK maximum limits, even if the size of microbial biomass is unaffected. As mentioned above, application of both contaminated and uncontaminated sludge treatments at the LTSE field sites has clearly caused significant changes in the microbial communities of the contaminated soils.

It was therefore concluded that the current UK statutory limits may not be sufficient to prevent changes in the microbial communities of sludge amended soils which could potentially lead to the loss or disruption of essential soil processes. It is therefore recommended that the current UK limits are reduced in order to protect microbial diversity. Given that sludge quality is continuing to improve, and the metal content of sewage sludge is decreasing, such a reduction in the statutory limits is not expected to restrict the use of sludge in agriculture nor decrease the overall longevity of sludge applications.

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