The Effects of Copper on the Degradation of Atrazine and Indoxacarb in a New Zealand Soil

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Abstract

Pesticides are an important component of New Zealand's primary production sectors. Infestation of pests and diseases can affect crop yield, crop value and damage the country's export reputation, resulting in economic losses. Repeat applications of pesticides, however, can result in contamination of land and water. Therefore, it is important to understand the fate of pesticides in the environment. Factors which can affect pesticide persistence include soil properties (pH, SOM, CEC), leaching and run-off, volatilisation and co-contamination with heavy metals. Many soils in New Zealand contain high levels of copper from historical applications of copper-based pesticides. Co-contamination of soils may lead to the persistence of some synthetic organic pesticides.

An investigation was undertaken to determine the effects of co-contamination with copper on the biodegradation of atrazine and indoxacarb in a New Zealand soil. A Templeton sandy loam soil was spiked with $CuSO_4$ to achieve concentrations of 0, 100, 250, 500 and 1000 mg kg^{-1} Cu. The spiked soils were field aged for six months prior to pesticide spiking with either atrazine or indoxacarb. The aged Cu-spiked soils were spiked with either atrazine or indoxacarb at a rate of 2 mg kg^{-1}. A glasshouse study was conducted to determine if copper inhibited the degradation of the pesticides. The pesticide-spiked soils were sampled at the time of spiking (t_0), at the estimated half-lives (t_1) and at twice the estimated half-lives (t_2) of the individual pesticides. The estimated half-lives were based on literature values.

The bioavailability and subsequent adverse effects of copper on the soil microbial community was investigated. Total and bioavailable copper concentrations, phosphatase and urease enzyme activities, microbial biomass, and pesticide residue concentrations were all measured in the experimental soil. Methods were developed for the extraction of atrazine, atrazine metabolite and indoxacarb residues from the experimental soil.

Total copper concentrations extracted ranged from 4–1060 mg kg^{-1} in the experimental soils and were consistent throughout the pesticide degradation studies. The bioavailability of copper was a maximum of 2% of the total copper concentration. Bioavailable copper concentrations were positively correlated to total copper ($p<0.01$).

Soil biological properties were investigated to determine the effects of copper on the soil microbial community. Phosphatase and urease enzyme activities, as well as microbial biomass concentrations, were negatively correlated with total copper ($p<0.05$). Total copper was a better indicator of effects on microorganisms than bioavailable copper. The soil biological properties began showing adverse effects above a total copper concentration of 100 mg kg^{-1}. This concentration also corresponds to New Zealand's copper limit in biosolids, which is protective of human, plant and microorganism health.

Phosphate buffer extraction methods were developed for the analysis of atrazine and indoxacarb residues in the experimental soil by HPLC-UV. Elevated copper concentrations did not inhibit the degradation of atrazine or indoxacarb in the experimental soil. The half-lives of both atrazine (≤19.4 d) and indoxacarb (≤18.8 d) were lower in the spiked experimental soils than the means reported in previous New Zealand and international studies, but were within the reported ranges. This study provided the first data on the fate of indoxacarb in New Zealand.

Hydroxyatrazine was the only metabolite detected in the atrazine-spiked experimental soils. Significant differences between the control (Cu-1) and copper levels above 100 mg kg^{-1} were observed for hydroxyatrazine at t_2. Significant negative correlations were observed between hydroxyatrazine and the microbiomass at t_1 and phosphatase activity at t_2 ($p<0.05$). These significant relationships suggest that elevated copper concentrations may alter the degradation of this metabolite in the experimental soils due copper toxicity of the soil microbial community.

The results of this thesis indicate that elevated levels of copper above 100 mg kg^{-1} negatively impact the soil microbial community and may reduce the overall health of the soil. Biodegradation is a key mechanism for the degradation of atrazine and indoxacarb in the soil, so it is important that the health of the soil microbial community is maintained. Therefore, it is recommended that atrazine and indoxacarb are only applied to soils with a total copper concentration less than 100 mg kg^{-1}. This will protect the health of the soil microbial community and prevent the potential adverse effects of copper on the degradation of pesticide metabolites in the soil.

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"Do not be anxious about anything, but in everything, by prayer and petition, with thanksgiving, present your requests to God. And the peace of God, which transcends all understanding, will guard your hearts and your minds in Christ Jesus."

-Philippians 4:6–7

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Abbreviations

ABP	Atrazine Breakdown Products
ACN	Acetonitrile
ANOVA	Analysis of Variance
ATZ	Atrazine
BDL	Below Detection Limit
CEC	Cation Exchange Capacity
CRM	Certified Reference Material
DCM	Dichloromethane
DDT	Dichlorodiphenyltrichloroethane
DEA	Desethylatrazine
DEHA	Desethylhydroxyatrazine
DGT	Diffusive Gradient Thin film
DIA	Desisopropylatrazine
%DIFF	percent Difference
DIHA	Desisopropylhydroxyatrazine
EDC	Endocrine Disrupting Compound
EtOH	Ethanol
FAAS	Flame Atomic Absorption Spectroscopy
HA	Hydroxyatrazine
HADP	Hydroxyatrazine Degradation Product

HFBA	Heptafluorobutyric Acid
HPLC	High Performance Liquid Chromatography
HPLC-UV	High Pressure Liquid Chromatography UV/Vis spectroscopy
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IND	Indoxacarb
IPA	Isopropanol
K_{EC}	microbiomass correction coefficient
K_{OC}	Organic Carbon-water distribution coefficient
MeOH	Methanol
PAS	Pesticide Analytical Standard
PSS	Pesticide Stock Standard
PWS	Pesticide Working Standard
%RSD	percent Relative Standard Deviation
SAX	Strong Anion Exchange
SCX	Strong Cation Exchange
SOM	Soil Organic Matter
SPE	Solid Phase Extraction

Chapter 1: Introduction

1.1 PESTICIDES

The use of pesticides (herbicides, insecticides, fungicides, plant growth regulators, etc.) is an important component of the New Zealand primary production sector (Manktelow *et al.*, 2005; Sarmah *et al.*, 2004). Pesticides control weeds, disease and insects in the agricultural farming, forestry and horticulture industries (Manktelow *et al.*, 2005). The New Zealand economy relies on the use of pesticides to optimise its agricultural industry in order to produce high quality goods which meet international trading standards (Sarmah *et al.*, 2004). In 2003, New Zealand's export earnings for primary production was worth NZ$18 billion (Manktelow *et al.*, 2005). Infestation by pests and diseases can affect both crop yield and quality. This can result in reduced export earnings (Manktelow *et al.*, 2005), as well as damage the country's export reputation (Self, 2003).

1.1.1 New Zealand Trends in Pesticide Use

Pesticides were traditionally used in New Zealand to control agricultural pests and diseases (Sarmah *et al.*, 2004). Increasingly they are also being used to control other pests such as the Australian bush-tail possum (*Trichosurus vulpecula*), wasps (*Vespoidea germanica*), invasive exotic weeds such as gorse (*Ulex europea*) and aquatic organisms (Sarmah, 2004). Pesticide trend data compiled by Holland and Rahman (1999) for the period 1984–1998 showed that herbicides dominated pesticide use, followed by fungicides and insecticides The data showed that there were sectoral differences in pesticide use. Arable farming, pastoral farming and forestry industries predominantly used herbicides, while the horticulture and vegetable crop industries predominantly used synthetic fungicides. In 1998 the use of phosphonyl herbicides (e.g. glyphosate), triazine herbicides (e.g. atrazine), sulfonylurea herbicides and pyrethroid insecticides was increasing in use by 1998, while organophosphorus insecticide and dicarboximide fungicide usage was declining. By 2004, organophosphate insecticides, dithiocarbamate and inorganic fungicides, and glyphosate and triazine herbicides were the major classes most commonly used in New Zealand (Manktelow *et al.*, 2005). The amount of pesticides being used has also increased. Over the period of 1999–2003, total pesticide

imports into New Zealand increased by 17% to 8.3 million kg (gross product) (Manktelow *et al.*, 2005).

1.1.2 Environmental Fate of Pesticides

Many different physical, biological and chemical processes influence the fate of pesticides in the environment (Sarmah *et al.*, 2004). Pesticide degradation can occur by microbial, chemical and/or photolysis mechanisms. Soil characteristics, including soil organic matter (SOM) content, pH and cation exchange capacity (CEC) can further influence these mechanisms. Biodegradation of pesticides can be inhibited by co-contamination with heavy metals (Sandrin and Maier, 2003) (refer to Section 1.3.1 below). Sorption mechanisms, surface run-off, leaching through the soil profile, uptake by plants and volatilisation also affect the fate of a pesticide in the environment (Sarmah *et al.*, 2004; van der Werf, 1996). Sorption to SOM can reduce biodegradation (Sarmah *et al.*, 2004) and leaching capabilities (Aislabie *et al.*, 2004). Pesticides can migrate into surface water through run-off and leaching (Sarmah *et al.*, 2004; van der Werf, 1996). They can enter the atmosphere by spray drift during application and through volatilisation (Sarmah *et al.*, 2004; van der Werf, 1996). As New Zealand soils are unlike many soils overseas (Sarmah *et al.*, 2004), a better understanding of the fate and transport of pesticides under New Zealand conditions is needed (Close *et al.*, 2008). There is very little information on the sorption and half-lives of pesticides in New Zealand soils (Sarmah *et al.*, 2009). Therefore, long-term field studies are necessary to provide the information essential to make reliable risk assessments (Bunemann *et al.*, 2006).

Pesticide use can result in negative environmental effects, including contamination and exposure to non-target organisms such as humans (van der Werf, 1996). Runoff and leaching may result in contamination of surface and groundwater sources (Close, 1993; van der Werf, 1996). Water contamination is of concern, especially if the source is used for drinking water, or if the pesticide is toxic to aquatic organisms, because adverse health effects can occur in non-target organisms upon exposure to the pesticide. Volatilisation followed by deposition on plants is another exposure route of pesticides to humans and other non-target organisms (van der Werf, 1996). Volatilisation is greatest in moist environments, especially when pesticides are applied to soil surfaces or plants (Sarmah *et al.*, 2004; van der Werf, 1996). Volatilisation can be reduced if pesticides are incorporated into the soil (van der Werf, 1996).

Many chlorinated compounds are stable in the environment (vanLoon and Duffy, 2005). Organochloride pesticides in particular have a history of environmental persistence and adverse effects on humans and other organisms (Crinnion, 2009; Van Zwieten *et al.*, 2003). Many of these pesticides have lipophilic and xenobiotic properties, making them endocrine disrupting compounds (EDC) that bioaccumulate in organisms (Crinnion, 2009). Persistent organochloride pesticides (e.g. DDT) are somewhat volatile and can travel long distances within the atmosphere (vanLoon and Duffy, 2005). They have been detected in regions with no history of use at significant levels in water. These pesticides can then bioaccumulate in the fatty tissues of aquatic organisms, representing an exposure route to humans if the organisms are consumed (vanLoon and Duffy, 2005). DDT, DDE, dieldrin and chlordane (and metabolites) are associated with some of the greatest documented adverse effects on human health (Crinnion, 2009).

1.1.3 Atrazine and Indoxacarb

The two pesticides investigated in this study were atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine) and indoxacarb ((S)-methyl-7-chloro-2,5-dihydro-2-[[(methoxycarbonyl)[4-(trifluoromethoxy)phenyl]amino]carbonyl]-indeno-[1,2-e][1,3,4]-oxadiazine-4a(3H)-carboxylate). These were chosen because they are both registered pesticides in New Zealand (Young, 2005) and are both chlorinated compounds (Figure 1.1). Atrazine is an herbicide that controls broad leaf and grassy weeds, mainly in the production of maize and sweet corn crops (Mandelbaum *et al.*, 1995; Meyer *et al.*, 2009; Silva *et al.*, 2004; Topp *et al.*, 2000). Atrazine interferes with the photosynthesis of the target weeds, initially causing discoloration and stunted growth, followed by death (Dean *et al.*, 1996; Peterson *et al.*, 2001; Young, 2005). Because atrazine can be applied pre- or post-emergence (Silva *et al.*, 2004), it must have a natural selectivity between the target weeds and the crop plant (Dean *et al.*, 1996). Non-target plants are able to metabolise the triazine herbicide into non-toxic substances (Peterson *et al.*, 2001), so are not susceptible to atrazine toxicity. The effectiveness of atrazine depends on the soil structure, organic matter content, moisture content and particle size (Dean *et al.*, 1996).

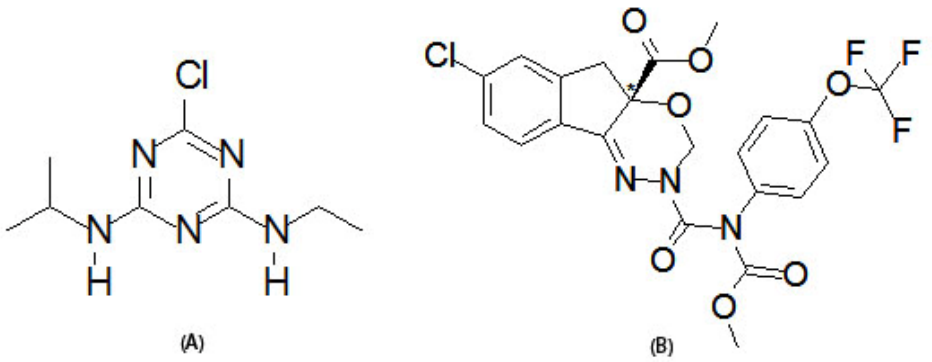

Figure 1.1: Structural diagrams of (A) atrazine and (B) indoxacarb. * indicates the chiral centre in the indoxacarb molecule.

In the environment, atrazine has a half-life of 13–402 d, with a median of 41 d in New Zealand soils (Close *et al.*, 2008). It is a moderate to highly mobile pesticide, with an average K_{OC} value of 72 mL g^{-1} in New Zealand soils (Close *et al.*, 2008). This indicates that atrazine could easily leach through the soil profile and contaminate drinking water. Trace levels of atrazine have been detected in groundwater in the US (Barbash *et al.*, 2001) and in some wells in New Zealand, with the highest concentrations in areas of intense spraying (Close, 1993). The Ministry of Heath has set the maximum acceptable value of atrazine in groundwater at 2 μg L^{-1} (Sarmah *et al.*, 2004). Atrazine concentrations in New Zealand groundwater generally range from 0.02–0.9 μg L^{-1} (Sarmah *et al.*, 2004). However, an atrazine concentration of 37 mg m^{-3} (37 μg L^{-1}) was measured in a well in Poverty Bay (Close, 1993). As atrazine is an EDC (Topp *et al.*, 2000; U.S. EPA, 2006), human exposure from drinking water is of concern. Therefore, care has to be taken when drinking well water in areas where atrazine has been applied frequently (Close, 1993).

Indoxacarb is a member of the new class of oxidiazine insecticides, controlling Lepidoptera insects (Campbell *et al.*, 2005; Xu *et al.*, 2008) in the production of fruits, vegetables, soybeans, alfalfa and peanuts (Xu *et al.*, 2008) and cotton (Dias, 2006). Indoxacarb is metabolised to its *N*-decarbomethoxyllated metabolite in the gut of the insect (Wing *et al.*, 2000) (refer to Figure 4.4, pathway 2). This metabolite interferes with the sodium channel of target pests, blocking the flow of sodium ions into nerve cells. Cessation of feeding in target pests occurs within 2–8 h after ingestion and death follows within a few days (Dias, 2006). This mode of action distinguishes indoxacarb from any other commercial insecticides on the market (Wing *et al.*, 2000). Indoxacarb has been found to be highly effective against insects

that had developed resistance to other insecticides (McCann *et al.*, 2001). Indoxacarb is a chirally active, with only the S-enantiomer showing insecticidal activity (Dias, 2006). Indoxacarb has a half-life in the environment ranging from <1 d (Dias, 2006) to 693 d (U.S. EPA, 2000a), making it a moderately persistent pesticide. Microbial degradation is the key process for the mineralization of indoxacarb in soil (Dias, 2006). It is considered to be immobile in soil, with K_{OC} values ranging from 3,300 to 9,600 mL g^{-1} (U.S. EPA, 2000a).

The degradation mechanisms of atrazine and indoxacarb are discussed in Chapter 4.

1.2 HEAVY METALS

Heavy metals are defined as elements which have a density greater than 6 g cm^{-3} (Naidu *et al.*, 2001). The essential heavy metals include cobalt (Co), copper (Cu), manganese (Mn) and zinc (Zn). These trace elements, also known as micronutrients, are required in low concentrations for biological processes for most organisms (Naidu *et al.*, 2001). The non-essential heavy metals include cadmium (Cd), lead (Pb), mercury (Hg) and arsenic (As). These non-essential elements are toxic to plants, animals and microorganisms (Naidu *et al.*, 2001). While heavy metals have been demonstrated to have a range of negative effects, this thesis focused on the effect of copper on the microbial degradation of pesticides. Copper is an important trace element for the development of plants, animals and microorganisms (Baker and Senft, 1995; Mouta *et al.*, 2008; van der Lelie and Tibazarwa, 2001); both too much or too little can result in adverse effects on organisms (Baker and Senft, 1995; Mouta *et al.*, 2008; van der Lelie and Tibazarwa, 2001). Microorganisms require trace amounts of copper as protein cofactors (van der Lelie and Tibazarwa, 2001). The movement of copper within microbial cells is tightly controlled, because Cu^{2+} is very toxic to microorganisms (van der Lelie and Tibazarwa, 2001). In plants, Cu is a structural element in proteins, is involved in the electron transfer process in photosynthesis and is a co-factor of many enzymes (Mouta *et al.*, 2008). In animals, copper is involved in the functioning of a wide range of enzymes (Bolan *et al.*, 2003), such as cytochrome *c* oxidase and Cu/Zn superoxide dismutases (Nies, 1999). Cu deficiency can cause bone dysfunction, anaemia, and heart and gastrointestinal difficulties (Mouta *et al.*, 2008). However, Cu toxicity can also occur to high levels of Cu exposure, and result in lung fibrosis and liver cancer (Mouta *et al.*, 2008). Wilson's disease affects Cu-binding ligands, allowing toxic levels to accumulate in several tissues (Baker and Senft, 1995).

1.2.1 Sources of Heavy Metals in the Environment

Heavy metals in terrestrial and aquatic environments either originate from natural sources or anthropogenic activities (Naidu *et al.*, 2001; Spurgeon *et al.*, 2008). Weathering of soil parent material leads to mineral breakdown and redistribution of the products in a process known as pedogenesis (Naidu *et al.*, 2001; Spurgeon *et al.*, 2008). Soil formation is influenced by the parent rock material, time, climate, topography, and organisms (Boggs, 2006; Marshak, 2005). Thus, the concentrations of naturally occurring heavy metals released during the pedogenic process are related to the origin and nature of the parent material. Anthropogenic source of heavy metals include industrial processes, mining, manufacturing, waste and refuse disposal from domestic and industrial sources, and agricultural practices (Alloway, 1995; Naidu *et al.*, 2001).

Mining and smelting, refining processes, industrial wastes (Alloway, 1995; Trevors and Cotter, 1990) and timber treatments with Cu-Cr-As (Alloway, 1995; Robinson *et al.*, 2006) are all anthropogenic sources of Cu. Copper is also widely used in the agricultural and horticultural industries (Alloway, 1995; Komarek *et al.*, 2010; Mouta *et al.*, 2008; Speir *et al.*, 2007). Copper-based products were traditionally and are presently being used as fungicides in New Zealand (Gaw *et al.*, 2006). $CuSO_4$ has been used as an herbicide (Gaw *et al.*, 2006) and is commonly used as a footbath in dairy milking yards to prevent lameness (Bolan *et al.*, 2003). Residual $CuSO_4$ from milking yards is flushed into effluent ponds and then applied to land in many New Zealand regions (Bolan *et al.*, 2003). The application of effluent to land helps to remove many contaminants. However, repeat applications, even low copper concentrations, can result in high levels of copper contamination (Wang *et al.*, 2009). Chronic copper poisoning in sheep may occur if they are allowed to graze on the land (Bolan *et al.*, 2003). Vineyard soils can also contain high levels of copper, due to repeat applications of fungicides (Komarek *et al.*, 2010). Some vineyard topsoils become so contaminated that new vines cannot be planted (Komarek *et al.*, 2010).

1.2.2 Copper Concentrations in New Zealand Soils

The average soil concentration of copper is between 20–30 mg kg^{-1} globally (Baker and Senft, 1995). In New Zealand, natural background levels are between 7.2–27 mg kg^{-1} Cu, while pastoral lands contain levels of copper between 9–32 mg kg^{-1} (Roberts *et al.*, 1996).

However, copper levels in New Zealand have been reported as high as 523 mg kg^{-1} in orchard soils with long-term applications of copper fungicides (Gaw *et al.*, 2006).

1.2.3 Fate of Heavy Metals in Soil

Heavy metals have very long residence times in soils and are not degraded over time (Alloway, 1995; Spurgeon *et al.*, 2008). Metal loss in soils can only come from slow processes such as erosion, leaching and possibly cropping (Spurgeon *et al.*, 2008). For example, Cd is estimated to persist for 75–380 y and Hg 500–1000 y. The more strongly sorbed elements such as As, Cu, Pb, and Zn can persist for 1000–3000 y, depending on soil conditions (Alloway, 1995). Copper is strongly adsorbed by soil organic matter, making it one of the least mobile trace elements in soil (Baker and Senft, 1995; Bolan *et al.*, 2003; Komarek *et al.*, 2010).

Some heavy metals, including As, Cd, Cu, Hg, Pb and Zn, tend to concentrate in the topsoil due to vegetation cycling, atmospheric deposition and adsorption by the SOM (Alloway, 1995). Recently contaminated soils will often have higher concentrations of heavy metals in the topsoil, because the pedogenic process will not have had enough time to redistribute them through the soil profile.

Generally, heavy metals associated with the parent ores have low bioavailability, so are not taken up by plants, and have minimum impacts on soil organisms (Komarek *et al.*, 2010; Naidu *et al.*, 2001). In comparison, heavy metals added to soils by anthropogenic activities can be highly bioavailable (Komarek *et al.*, 2010; Naidu *et al.*, 2001), because they are not as strongly associated with soil components as the naturally occurring heavy metals (Komarek *et al.*, 2010).

As metal-contaminated soils age, the metals adsorb to SOM and diffuse into micropores within clay particles (Kim *et al.*, 2008; McLaughlin *et al.*, 2000), reducing availability to organisms (Ma *et al.*, 2006b). Stronger bonds are formed with soil particles as contact time increases (Komarek *et al.*, 2010). Therefore, methods used to extract the "total" metal concentration tend to overestimate the amount that is available to organisms for uptake (Giller *et al.*, 1998), so should not be used to determine regulatory limits (Menzies *et al.*, 2007). Instead the labile, or bioavailable fraction, should be investigated (Menzies *et al.*, 2007).

Soil metal concentrations can be divided into six pools: 1) soluble ions and inorganic/organic complexes in the soil solution; 2) exchangeable metals; 3) stable organic complexes in humus; 4) metals adsorbed by Mn-, Fe, and Al-hydrous oxides; 5) metals adsorbed on clay-humus colloidal complexes; and 6) metals bound in the soil crystal lattice (Baker and Senft, 1995). The total metal concentration of a soil includes metals from all six pools. The bioavailable fraction includes metals from the labile pools only (1 and 2). The bioavailable metal concentration can be difficult to measure, because it can vary with environment and organism (Sandrin and Maier, 2003). Therefore, the definition of "bioavailable" used in this thesis is the metal concentration found in the labile pools, as discussed above.

Metal concentrations in the solution phase are often used to approximate the bioavailable metal fraction (Sandrin and Maier, 2003), though not all of the metal in the soil solution is bioavailable (Giller *et al.*, 1998). Metals may be in forms which cannot be directly taken up by organisms. For example, chelation by organic molecules can render metals inaccessible (Giller *et al.*, 1998). It is usually assumed that the free ion species of a metal in solution is what causes acute toxicity to organisms at high concentrations (Giller *et al.*, 1998; McLaughlin *et al.*, 2000). Neutral salt extractions, such as 0.01 *M* $CaCl_2$, are common methods used to measure the metal bioavailability in soil (Nolan *et al.*, 2005). $CaCl_2$ extractions have been used to determine the potential bioavailability of Cu to plants (Nolan *et al.*, 2005) and the potential toxicity to soil microorganisms (Merrington *et al.*, 2002).

Soil properties can influence the bioavailability of heavy metals in soil. Organic matter in highly weathered soils has been shown to reduce copper concentration in the soil solution (Komarek *et al.*, 2010; Mouta *et al.*, 2008). The soil pH is one of the most important factors that determines metal bioavailability to organisms (Giller *et al.*, 1998). The pH controls the speciation of the metals in the soil solution phase (Giller *et al.*, 1998; Sandrin and Maier, 2003; Sarmah *et al.*, 2004). As the pH increases, cationic metals will preferentially form insoluble metal oxides and phosphates, thus reducing the metal concentration in the soil solution (Sandrin and Maier, 2003). Most soils in New Zealand tend to be acidic (Sarmah *et al.*, 2004), which would favour the free metal species. SOM and clay content are also important factors that can reduce metal concentrations in the soil solution (Sandrin and Maier, 2003; Sarmah *et al.*, 2004). SOM has sites which can bind to metals, making them inaccessible to organisms. Clays, especially those with high CEC, are effective at reducing metal bioavailability and toxicity (Sandrin and Maier, 2003).

Most copper in aged soils is insoluble, though part is reversibly bound to soil particles (Suave *et al.*, 1997). Desorption of copper from soils can be quite slow and is dependant on soil conditions (Ma *et al.*, 2006b). Desorption mechanisms include oxidisation of SOM which releases bound Cu, and the depletion of Cu from the labile pool by plant and microbial uptake or leaching, causing non-labile copper to migrate to the labile pool to maintain equilibrium (Bolan *et al.*, 2003; Ma *et al.*, 2006b).

1.3 MICROORGANISMS

The soil microbial community is very diverse (Giller *et al.*, 1998), with estimates of up to 10^6 bacterial species present per gram of pristine topsoil (van der Meer, 2006). Soil microorganisms include bacteria, fungi and protozoa. Microorganisms are essential to the functioning of ecosystems (van der Meer, 2006). They are involved in the cycling of nutrients (e.g. C, N, P) and other elements, the mineralisation of organic matter to carbon dioxide and water, decomposition reactions which purify air and water, and the biodegradation of organic pollutants (Bunemann *et al.*, 2006; van der Meer, 2006).

1.3.1 Heavy Metal Toxicity to Microorganisms

Microbial populations differ in sensitivity to heavy metal contamination in soils (Giller *et al.*, 1998). Exposure to high metal concentrations can result in immediate cell death from disruption of essential functions, and long-term exposure can alter the diversity of the soil microbial community. Decreasing microbial diversity may diminish resilience of the soil ecosystem to new stressors (Giller *et al.*, 1998), such as the addition of a pesticide. Cu-based fungicides are the most toxic pesticides to soil microorganisms (Bunemann *et al.*, 2006). The presence of heavy metals can inhibit a broad range of microbial processes, including the dehalogenation process, which is essential to the mineralisation of many organic pollutants in soil (Sandrin and Maier, 2003). There are, however, metal tolerant bacterial species, which can survive in highly metal contaminated soils (Giller *et al.*, 1998). These bacteria use mechanisms to reduce toxic effects such as binding the metals to proteins, extracellular polymers or the cell wall, compartmentation of metals within the cells, forming insoluble metal sufides, decreasing metal uptake, enhancing metal export from cells, and volatilisation of metals (Giller *et al.*, 1998). Microorganisms may also alter metal availability in the soil by

acidifying the area or producing compounds which can form complexes with metals (Giller *et al.*, 1998).

High levels of heavy metals in soil, including Cu, have been reported to inhibit litter decomposition, methanogenesis, acidogenesis, nitrogen transformation, biomass generation, and enzyme activities of soil microorganisms (Sandrin and Maier, 2003). Changes in the microbial community can result in altered biomass concentrations (Kandeler *et al.*, 2000), a reduction in microbial diversity (van der Meer, 2006) and nutrient cycling (Kirk *et al.*, 2004; van der Meer, 2006), as well as diminished soil fertility (Kim *et al.*, 2008; Kirk *et al.*, 2004). These changes may result in an overall decline in soil health.

1.3.1.1 Measurements of adverse effects on soil microorganisms

The effects of agricultural inputs, such as heavy metals, pesticides or fertilisers, can be measured by changes in individual microorganisms, the microbial community as a whole, by defined groups (e.g. the biomass), or in microbial activity (Bunemann *et al.*, 2006). Common measurement techniques include enzyme assays (Bunemann *et al.*, 2006; Kim *et al.*, 2008; Sannino and Gianfreda, 2001; Speir *et al.*, 2007), phospholipid fatty acid (PLFA) analysis (Bunemann *et al.*, 2006; Wilke *et al.*, 2005), microbiomass, soil respiration (Bunemann *et al.*, 2006; Speir *et al.*, 2007), and molecular techniques such as rDNA analysis (Kandeler *et al.*, 2000) or denaturing gradient gel electrophoresis (DGEE) (Bunemann *et al.*, 2006; Kandeler *et al.*, 2000), among many others.

Microbial enzymes are commonly used as indicators of soil microbial health (Wang *et al.*, 2009), because they are sensitive to a range of metals, and assays are simple, rapid to run, and cost-effective (Kim *et al.*, 2008). Invertase, urease and phosphatase enzymes are important in the carbon, nitrogen and phosphorous cycles, respectively (Sannino and Gianfreda, 2001). Phosphatases are ectocellular enzymes (Megharaj *et al.*, 1999; Sannino and Gianfreda, 2001) responsible for hydrolysing organic phosphorus to inorganic phosphorous (Megharaj *et al.*, 1999). Stable phosphatases have been reported to significantly contribute to overall soil phosphatase activity (Sannino and Gianfreda, 2001). Native soil ureases are mainly extracellular and are persistent due to associations with inorganic and organic soil matter (Sannino and Gianfreda, 2001). Therefore, interpreting enzyme activities in soil can be difficult, because enzymes can remain active when stabilised on organic matter or mineral

surfaces in the soil (Bunemann *et al.*, 2006). In order to overcome this, multiple assays should be run to assess the overall soil health. The soil microbiomass is also a commonly measured microbial variable in soil, though it seems to be less sensitive than enzyme activities to changes in the soil (Bunemann *et al.*, 2006).

1.3.1.2 Short-term vs. long-term studies

Though there have been many studies on the effects of heavy metals to soil microorganisms, there are inconsistencies between short-term laboratory trials and long-term field trials (Giller *et al.*, 1998; Ma *et al.*, 2006a; Oorts *et al.*, 2006). Short-term laboratory studies measure the response to immediate acute toxicity to heavy metal exposure (Giller *et al.*, 1998). Acute toxicity results in a selection of microorganisms with either greater heavy metal tolerances or lead to development of metal-resistant microorganisms. Long-term field studies, on the other hand, measure the response to long-term chronic toxicity from gradual accumulation of heavy metals in soils. Chronic toxicity can result in subtle changes in competitive abilities in the microbial community, which may lead to changes in community structure with a potential risk of functionality loss. There is also evidence that changes in the microbial community structure may not necessarily increase the metal tolerance of the overall community (Giller *et al.*, 1998). Though chronic exposure was not measured in this thesis, it is important to consider differences in methodologies when comparing results to previous studies.

1.3.1.3 Effects of pesticide amendments

Pesticides themselves can affect the activity of the soil microbial community through direct or indirect effects (Bunemann *et al.*, 2006; Sannino and Gianfreda, 2001). Direct effects due to toxicity can result in a decline in microbial populations and/or activity (Bunemann *et al.*, 2006). These effects can be seen within the first season of pesticide application or over long-term application of pesticides. Indirect effects on the soil microbial community are due to changes in the soil pH, changes in the soil properties (aggregation, porosity, SOM levels), changes in productivity or changes in pesticide inputs (Bunemann *et al.*, 2006). These effects are usually seen after a few seasons of pesticide application. However, comparison between studies is complicated because contradictory results are often reported (Sannino and Gianfreda, 2001) and there is a general lack of method standardisation (Bunemann *et al.*, 2006).

1.4 EFFECTS OF HEAVY METALS ON PESTICIDE DEGRADATION

Heavy metals can affect the degradation of synthetic organic pollutants in the environment in a variety of ways, such as decreasing degradation rates by impairing soil microbial functions (Gaw *et al.*, 2006; Van Zwieten *et al.*, 2003) or complexation (Morillo *et al.*, 2000), and enhancing photocatalysis (Liu *et al.*, 1999; Liu *et al.*, 2007; Sancho *et al.*, 1997). Gaw *et al.* (2006) observed increased persistence of DDT in New Zealand horticultural soils co-contaminated with elevated levels of trace elements (As, Cd, Cu and Pb). Two proposed mechanisms to explain the increased persistence of DDT in the soils were: 1) copper-induced changes to the soil microbial community inhibited biodegradation of DDT and 2) constant grass cover on the sites prevented photodegradation of DDT (Gaw *et al.*, 2003). Van Zwieten *et al.* (2003) also observed persistence of DDT in Australian soils co-contaminated with As and partially attributed the persistence of DDT to inhibited microbial activity from As and DDT toxicity.

Liu *et al.* (2007) investigated the effects of copper on the degradation of two pyrethroid pesticides, cypermethrin and cyhalothrin, in soil and in solution. In soil, the presence of 10 mg kg^{-1} copper increased the half-lives of both pesticides. The authors concluded that copper adversely impacted the soil microbial activity, resulting in increased persistence of the pesticides. When the pesticides were in solution and irradiated with light, their photodegradation rates were accelerated when in the presence of copper, indicating copper had catalytic properties. This study showed that copper had both inhibiting and enhancing effects on the degradation of pesticides, depending on the environmental media (soil or solution).

Copper can also inhibit pesticide degradation by stabilising the molecule, preventing mineralisation. Sancho *et al.* (1997) observed that copper formed a stable complex with metamitron, a triazine pesticide, in solution at pH 5.6. The Cu-metamitron complex reduced the photodegradation rate in solution by 15% within the first 30 d when compared to the photodegradation of metamitron in solution alone. Morillo *et al.* (2000) observed that the adsorption of glyphosate to soil was enhanced by the presence of copper. Proposed mechanisms included: 1) copper strongly coordinated to glyphosate, and these complexes had a higher affinity for adsorption onto the soils than free glyphosate; 2) glyphosate adsorbed to

soil sites where copper was previously adsorbed, thus copper acted as a bridging agent; and 3) copper lowered the pH of the soil solution, leading to the formation of glyphosate with a lower negative charge, which could adsorb more easily onto the negatively charged soil surfaces (Morillo *et al.*, 2000). In contrast, Kools *et al.* (2005) observed that glyphosate degradation rates increased with increasing heavy metal concentrations in the soil.

1.5 THESIS OBJECTIVES

The objectives of this thesis were to:

- Determine the effects of copper co-contamination on the degradation of atrazine and indoxacarb.
- Determine if elevated copper concentrations were bioavailable to the soil microorganisms and had adverse effects on the soil microbial biomass and enzyme activities.

1.6 THESIS STRUCTURE

A glasshouse study was undertaken to determine the effects of co-contamination with copper on the degradation of atrazine and indoxacarb. A Templeton sandy loam soil (bulk soil) was spiked with $CuSO_4$ to achieve concentrations of 0, 100, 250, 500 and 1000 mg kg^{-1} Cu and field aged for six months (aged Cu-spiked soils). The copper spike rates chosen were consistent with the range measured in New Zealand horticultural soils. The aged Cu-spiked soils were then spiked with either atrazine or indoxacarb at a rate of 2 mg kg^{-1} (pesticide-spiked experimental soils). The pesticide-spiked soils were sampled twice at intervals based on published half-lives for atrazine and indoxacarb. The soil microbiomass and enzyme activities were measured to determine the effects of Cu on the soil microbial community.

Chapter 2 describes the methods used in the investigation. The results of the enzyme assays, microbiomass, total and bioavailable copper extractions and soil properties are presented in Chapter 3. The results of the pesticide studies are presented in Chapter 4. Chapter 5 contains a summary of the key findings and discusses the possible implications, and concludes with recommendations for further research.

Chapter 2: Methods

2.1 INTRODUCTION

This chapter outlines the methods used in this thesis, including soil sampling and storage procedures, copper spiking and analysis, enzyme assays, and pesticide spiking and analysis. Each sub-section contains details for preparation of reagents as required and a description of the method. The final section of this chapter outlines the different cleaning procedures used.

2.2 SAMPLE COLLECTION AND PREPARATION

2.2.1 Sample Site

The bulk soil was collected from the Lincoln University Dairy Farm, Canterbury, New Zealand (43°38′40″S, 172°28′7″E; Figure 2.1) on 23 April 2008. The sample site had not been sprayed with pesticides or fertilised for at least 12 years. Grass seed was applied approximately 3 years prior to collection and there was a possibility of fertiliser drift from application to adjacent pastureland (Clucas, 2008). The soil was a Templeton sandy loam soil and was originally planted in rye grass and clover pasture.

2.2.2 Sample Collection

Soil was collected from a plot approximately 2 m^2 in size to a nominal depth of 15 cm below the grass line. The grass layer was removed before collection and replaced after. Soil was collected in 10–15 kg batches and placed in high density polyethylene Glad Drawstring Kitchen and Tidy Bags and stored in black Payless rubbish liners for transport. Fourteen bags were collected, containing a total of approximately 130 kg soil. The soil was transported to the Department of Biological Sciences Glasshouses at the University of Canterbury for initial sieving, homogenisation and weighing.

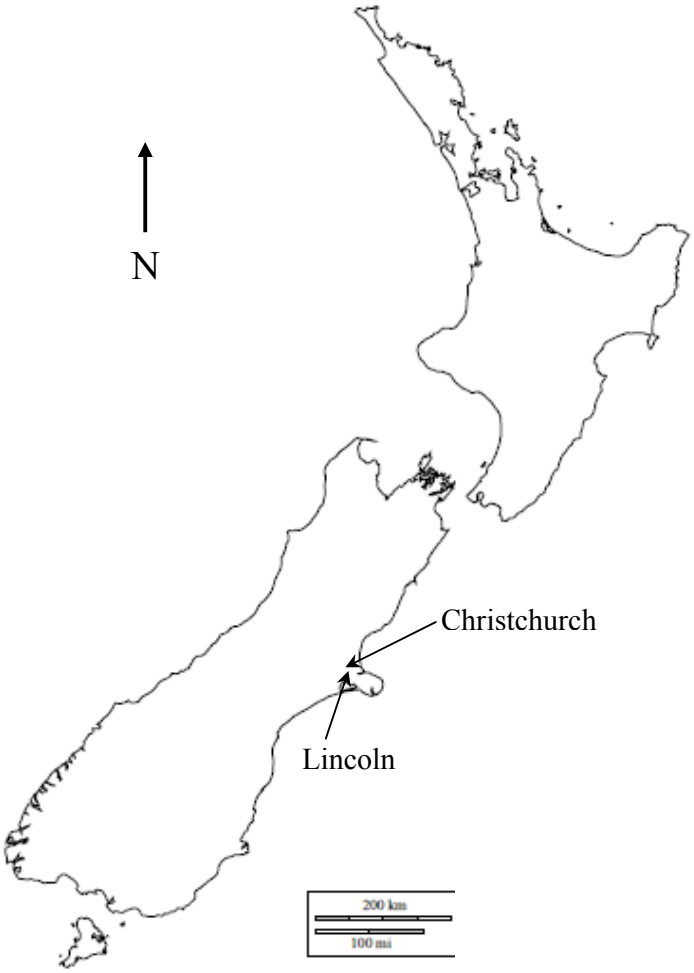

Figure 2.1: Locations of the sampling and experimental sites (from http://d-maps.com).

2.2.3 Initial Sieving, Homogenisation and Weighing

Each bag of soil was sieved to <5 mm. Green glass fragments, rib and spine bones, rocks and worms were removed from the soil while sieving. The sieved soil was homogenized periodically by hand with a clean shovel, by mixing and overturning the soil multiple times. Once the clear containers were full, they were emptied into a 200 L gray plastic bin (type unknown) and thoroughly homogenised with shovels. A moisture content test was conducted on three sub-samples, in preparation for weighing on a dry weight (DW) basis. The soil was weighed into heavy-duty plastic bags in five 6 kg and ten 12 kg batches (Figure 2.2), wet weight (25 kg DW= 30 kg wet weight), on a FG-30 KAM balance. The bags were sealed and

transported to the Department of Chemistry for copper spiking. The Cu concentration of the bulk soil was measured prior to Cu-spiking.

Figure 2.2: Weighing out of homogenised bulk soil, in preparation for copper spiking.

2.3 COPPER SPIKING, AGING AND SAMPLING

2.3.1 Copper Spiking

The soils were Cu-spiked in the 5th floor undergraduate laboratory in the Department of Chemistry, at the University of Canterbury. Two tables were joined and covered with two layers of plastic sheeting. Analytical grade $CuSO_4 \cdot 5H_2O$ (BioLab, Australia) spiking solutions were prepared to provide final concentrations of 0, 100, 250, 500 and 1000 mg Cu kg^{-1} soil. The soils were spiked in order of increasing concentration. These Cu concentrations were chosen as they are consistent with the range measured in New Zealand horticultural soils (Gaw *et al.*, 2006). The 100 mg kg^{-1} spike rate was also chosen as it is the

copper limit in New Zealand biosolids guidelines (NZWWA, 2003). Solid $CuSO_4$ was dissolved in 1 L of Milli-Q water (18.2 MΩ) (Table 2.1) and the solution was poured into a clean 2 L Spray Maker hand-held pump sprayer. Twenty-five kg of soil (DW) was poured onto the tables and spread out to approximately 2 cm depth. Each 25 kg batch was sprayed with a Cu-spiking solution and was turned over by hand after every pass, with direction of spraying and turnover varied regularly. It took approximately 1 h to spike each soil batch. There was 8% of the 0 mg kg^{-1} spiking solution remaining in the pump sprayer, which was taken into account for the remaining spiking solutions. The freshly spiked soils were returned to their labelled plastic bags and sealed. A sub-sample was taken from each spiking level, with a portion stored frozen at -20ºC and a portion dried (refer to Section 2.5), for analysis.

Table 2.1: Copper spiking rates.

Target Cu spike rate (mg kg^{-1})	***Equiv. g $CuSO_4$ kg^{-1} soil***	***Equiv. g $CuSO_4$ 25 kg^{-1} soil***	***g $CuSO_4$ added***	***Calc. spike (mg kg^{-1})***
0	0	0	0	**0**
100	0.393	9.83	10.6	**99.4**
250	0.983	24.6	26.7	**250**
500	1.97	49.1	53.2	**498**
1000	3.93	98.3	106	**994**
Note: spike rate includes 8% allowance for solution remaining in hand-held sprayer.				

Once the spiking was complete, the soils were transferred into black polypropylene "soil aging bins" labelled 1–5, in preparation for aging. The soil aging bins consisted of a lower bin to collect leachate and an upper bin to hold the soil. A layer of green plastic garden mesh followed by a layer of white mesh was laid over the drainage holes in the upper bin to prevent the soil from passing through (Figure 2.3). The soil aging bins were pre-leached overnight twice with distilled water before addition of soil.

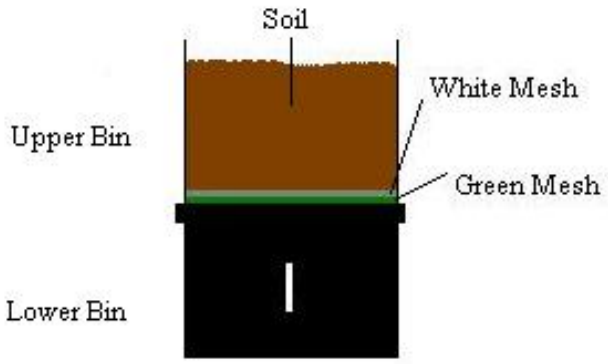

Figure 2.3: Soil aging bin diagram, consisting of upper and lower bins, with mesh to prevent soil loss.

2.3.2 Soil Aging

The soil aging bins were transported to the UC/ESR Lysimeter facility on Creyke Rd on 5 May 2008 for the duration of the aging process (Figure 2.4). The bins were locked inside a chain linked enclosure with limited access. Each bin was covered with white netting anchored by plastic pegs to prevent disturbance by birds and other animals. Each bin was bunded with a 60 L clear plastic container (catch bins) to collect any leachate overflow. The leachate levels were checked weekly during the 6 month aging process (194 d total), with more frequent monitoring during and after significant storm events (Appendix A). Leachate collected in the lower soil aging bin was emptied into white plastic waste containers and was returned to the Department of Chemistry for disposal. The soil samples were weeded with plastic tweezers, to prevent variation in the soil microbial community between the spike levels due to the presence of plants.

2.3.3 Pesticide Spiking

Analytical-grade atrazine and indoxacarb were spiked into the soils instead of commercial formulations to minimise the effects of components in the formulations that could interfere with degradation.

Figure 2.4: Copper spiked soil samples aging in the UC/ESR lysimeter facility on Creyke Rd., Christchurch, after a hail storm.

2.3.3.1 Materials

Analytical grade atrazine (97.2% pure) and 50:50 isomer mix of indoxacarb (93.9% pure) were purchased from Sigma-Aldrich (Germany). ChromAR acetone was purchased from BioLab (New Zealand).

2.3.3.2 Spiking

The soil aging bins were transported back to the Department of Chemistry's 5th floor undergraduate laboratory on 17 November 2008. The soils were sieved to <4 mm. The soil moisture content, pH and water holding capacity (WHC) were measured in duplicate for each Cu-spiked soil (Section 2.6). Eight 2 kg (DW) sub-samples of soil at each Cu-level were weighed into labelled sealable plastic bags. The same pesticide spiking procedure was used for both indoxacarb and atrazine. Analytical grade indoxacarb or atrazine spiking solutions were prepared in ChromAR acetone at a concentration of 80 mg L^{-1} in 1 L volumetric flasks on the day of spiking. One 2 kg soil sub-sample at each Cu-level was poured into 5 L beakers and weighed. These soil sub-samples were spiked with 200 mL of the appropriate pesticide

spiking solution in 5 mL increments. The final spiking rates were 2 mg kg^{-1} after blending with the remaining soil (Section 2.3.3.3 below). Atrazine and indoxacarb spike rates were chosen in order to balance field application rates and method detection limits. The atrazine spike rate was consistent with field application rates (750 g ha^{-1}) (AGPRO, 2008), while the indoxacarb spike rate was 6.5 times the field application rate of 400 g ha^{-1} (Du Pont, 2005). Spiking took place in a fume hood. The spiked soil was thoroughly mixed with a stainless steel spoon after each increment was added. The spiked soils were allowed to stand in the fume hood overnight to evaporate the acetone and were thoroughly mixed the following morning. The spiked soils were re-weighed and made up to their original weight with Milli-Q water. The spiked soils were thoroughly mixed again. In this way, only one quarter of the soil was spiked with the pesticide to minimise enhancing and inhibiting effects on soil microbial activity due to addition of solvent during spiking (Brinch *et al.*, 2002). Spiking with indoxacarb and atrazine was staggered so that sampling days did not overlap. The indoxacarb samples were spiked on 20 November 2008, while the atrazine samples were spiked on 27 November 2008.

2.3.3.3 Homogenisation

Each pesticide-spiked soil sub-sample and its three corresponding non-spiked equivalents were blended in a large plastic drum (type unknown). The soil was homogenised by rolling the drum on the floor covered in plastic sheeting (Figure 2.5) for approximately 10 mins. Soil homogenisation was performed in the order of increasing Cu-spike level, with the indoxacarb soils processed before the atrazine soils. Once thoroughly homogenised, each soil was poured into a large plastic bag. The plastic drum was thoroughly wiped out with 90% drum ethanol to prevent cross-contamination of microorganisms between the Cu-levels.

2.3.3.4 Pesticide degradation study

For each Cu-spike level, four replicates (equivalent to 1.8 kg DW) of the homogenised pesticide-spiked soil were weighed into 4 L polypropylene liver pails to a depth of approximately 70 mm. Soils were made up to 60% WHC by weight with Milli-Q water. The remaining soil was divided into labelled sealable plastic bags and either stored frozen at -20°C, dried or at 4°C (t_0; refer to section 2.5 for storage procedures). The soil containers were covered in plastic sheeting and transported to the University of Canterbury's Biological

Sciences Glasshouses. Atrazine and Indoxacarb samples were placed on separate benches in the glasshouse. Samples were monitored three times a week and maintained at 60% WHC with Milli-Q water and were weeded as necessary. The containers were randomised weekly. The internal temperature of the glasshouse was monitored over the sampling period with an Onset HOBO Pro temperature logger, housed in a radiation shield, provided by the Department of Geography. The average temperature over the sampling period was 21ºC, with a maximum of 43ºC and a minimum of 10ºC.

Figure 2.5: Homogenising the pesticide-spiked soils in the plastic drum.

2.3.4 Pesticide Sampling

The pesticide-spiked soils were sampled twice at intervals based on published half-lives: t_1 (atrazine: 40 d; indoxacarb: 60 d) and t_2 (atrazine: 80 d; indoxacarb: 120 d). At the appropriate sampling times, the soil containers were adjusted to 60% WHC with Milli-Q water and allowed to equilibrate for 2 h. Sixteen soil cores were collected from each container and were placed in labelled sealable plastic bags. The soil corers (22 mm diameter)

were manufactured from stainless steel pipes, by the Department of Chemistry's Mechanical Workshop. The soil cores were approximately 70 mm in depth and sampled the whole soil profile (i.e. from the soil surface to the bottom of the containers; Figure 2.6). Sample bags were stored on ice in a Styrofoam chilly bin, with a layer of cardboard between the samples and ice. Each sample was sieved (<2 mm) into large plastic bags with stainless steel sieves (refer to section 2.5.1). The soils were weighed into labelled sealable plastic bags according to storage method (refer to section 2.5.3), and stored until analysis. Outer gloves were changed between sieving each replicate, to prevent microbial cross-contamination. Core samples from t_1 were weighed and the 60% WHC equivalent weight was re-calculated for each replicate container. After core sampling, each soil replicate was redistributed evenly by hand.

Figure 2.6: Pesticide spiked soil after cores were taken, showing sampling of the whole soil profile.

2.4 SAMPLE PRE-TREATMENT

2.4.1 Sieving

The soil samples collected at t_1 and t_2 were sieved to <2 mm with stainless steel sieves before storage (Figure 2.7). The soils were sieved in the Department of Geography's physical laboratory. After sieving, a sub-sample was taken from each replicate to calculate the moisture contents.

Figure 2.7: Sieving the experimental soil to <2 mm at the University of Canterbury.

2.4.2 Drying

Soil sub-samples were oven dried in aluminium tins at 30ºC for 5 d prior to storage. Dried soils include sub-samples of the bulk soil, aged Cu-spiked soils, and pesticide-spiked soils at t_0, t_1 and t_2.

2.4.3 Sample Storage for Analysis

Representative sub-samples of the experimental soils were stored according to the analysis method. Four sub-samples from aged Cu-spiked soils and pesticide-spiked soils sampled at t_0, t_1 and t_2 were stored in labelled sealable plastic bags according to the storage procedure. Enzyme-labelled sub-samples were stored at 4ºC and were analysed within a week for

phosphatase activity. Pesticide- and micro-labelled sub-samples were stored at -20°C and were thawed before analysis of pesticide residues and urease activity, respectively. Dry-labelled sub-samples were stored in a cupboard after drying and were analysed for total and bioavailable metal concentrations. Sub-samples from each Cu-spiked soil (pre-aged) were stored both as frozen (-80°C) and dried.

2.5 SOIL CHARACTERISATION

Moisture content, pH and CEC of the soil samples were determined using NZ Soil Bureau methods (Blakemore *et al.*, 1987).

2.5.1 Moisture Content

For each replicate, field moist soil (5–10g, <2 mm) was weighed into labelled aluminium pie tins and placed in a 105ºC oven overnight. Samples were removed from oven, cooled and re-weighed. The moisture factor (MF) and percent dry matter (%DM) were calculated.

2.5.2 Water-Holding Capacity

The water holding capacity (WHC) of the aged Cu-spiked soils was determined using the method outlined in Rothamsted lab manual (Grace *et al.*, 2006). Short lengths of rubber tubing were attached to the stems of clean 100 mm glass funnels. Screw clips were positioned on the tubing below the stem to seal off the tubing. Cotton wool (0.25–0.30g) was packed in each funnel and tamped down firmly. Fifty g of field moist soil was placed into each funnel. Blanks contained only cotton wool. Fifty mL of distilled water was poured into each funnel and allowed to stand for 30 min to saturate the soil. A stopwatch was used to accurately measure the time. After 30 min, the screw clips were loosened and water was allowed to drain into measuring cylinders for an additional 30 min. The final volume was noted and the 100% WHC (mL water 100 g^{-1} dry soil) was calculated. The WHC was measured in duplicate for each aged Cu-spiked level and blanks. The %DIFF for the duplicates ranged from 3.2–17.3%.

2.5.3 Soil pH

Dried soil samples (10 g) were weighed into 70 mL polypropylene specimen containers fitted with polyethylene caps. Twenty-five mL of distilled water was added to each with a dispenser. Samples were stirred vigorously for 15 s with a high speed electric stirrer and left to stand overnight. The stirrer was rinsed thoroughly with distilled water in between samples. A Hanna Instruments HI 8424 microcomputer pH meter (U.O.C. #7999) was used to measure the pH of each sample. The pH meter was calibrated daily with LabServ buffer solutions at pH 4 and 7. The sample pH was recorded after 2 min. Duplicates were analysed every 10 samples and a bulk soil sample was analysed with each batch to insure inter-batch reproducibility. If the soil duplicates varied by more than 0.1 pH unit, the whole batch was re-run (Blakemore *et al.*, 1987). Replicates of the bulk soil were measured initially to determine method reproducibility and had a mean of 4.99, with a %RSD of 0.46 (n=6). The mean pH of the bulk soil run in each batch was 4.97 with a %RSD of 1.9 (n=10). The %DIFF of pH measurements for duplicate soil samples ranged from 0–2%.

2.5.4 Cation Exchange Capacity

The soil cation exchange capacity (CEC) was determined for the bulk soil and aged Cu-spiked soils (dried, <2 mm). A solution blank and an in-house reference soil were included with each batch, and samples were measured in duplicate. Soils (0.80 +/- 0.05 g) were weighed into 50 mL polypropylene screw cap centrifuge tubes. Forty mL of 0.01 *M* silver thiourea (AgTU) was added and the tubes shaken end-over-end for 16 h. The AgTU solution was prepared by dissolving 7.5 g thiourea (TU) and 0.8495 g $AgNO_3$ in 500 mL distilled water. Samples were centrifuged at 2000 rpm for 10 min. Standards were prepared at concentrations of 0, 0.0025, 0.005, 0.0075 and 0.01 mol L^{-1} AgTU. A 0.625 mL aliquot of each standard and sample solution was diluted to 50 mL with CsCl (1000 µg Cs mL^{-1}). Solutions were analyzed for silver by Varian SpectrAA 220FS Atomic Absorption Spectrometer (FAAS) at 328.1 nm using an air-acetylene flame. A standard curve was prepared and the CEC values for the samples were calculated from this. The method reproducibility was calculated from data from an in-house reference soil (14.8 meq. 100 g^{-1}) with a %RSD of 1.7% (n=3) and a soil sample (10.2 meq. 100 g^{-1}) with a %RSD of 5.88% (n=6). The CEC was 96% of reference value (n=3). The limit of detection (LOD) for this method was calculated at 1.17 meq. 100 g^{-1} from three times the standard deviation of procedural blanks.

2.5.5 Total Organic Carbon

Total organic carbon (TOC) contents were analysed by Hills Laboratory, Hamilton, New Zealand. A sub-sample of the dried bulk soil, the pre-pesticide spike soils (Cu Nov08), and two soil replicates at each Cu-level for atrazine and indoxacarb at t_1 were analysed. Samples were treated with acid to remove carbonates, if present and then analysed with an Elementar Combustion Analyser for TOC content. The units were g C 100 g^{-1} soil (DW).

2.5.6 Particle Size

The particle size distribution was analysed by Chris Grimshaw in the Geological Sciences Department at the University of Canterbury. A sub-sample of the fresh bulk soil (stored at 4ºC) was analysed by the Fraunhofer method on a Micromeritics Saturn DigiSizer 5200 laser sizer. Particle size was determined on a %volume basis and was reported as %sand, %silt and %clay.

2.6 COPPER ANALYSIS

2.6.1 Total Copper

The total copper concentration was measured for the dried pesticide-spiked soils collected at t_0, t_1 and t_2 by acid digestion. This method was adapted from the US EPA Method 200.2 (Martin *et al.*, 1994). One g of each soil (dried, <2 mm) was weighed into 50 mL acid washed polycarbonate vials, pre-marked at 20 mL. Tracepur HNO_3 and HCl were purchased from Merck (New Zealand). Four mL of 50% HNO_3 and 10 mL of 20% HCl were added to each vial and placed in a stainless steel heating block fitted to a hot plate. The dilute acid solutions were prepared in Milli-Q water. Samples were heated to 85°C and refluxed for 30 min. Samples were cooled, made up to 20 mL with Milli-Q water and centrifuged on a BTL Bench Centrifuge for 10 min at 2000 rpm. Cu-1 and Cu-2 samples were measured directly by a Varian SpectrAA 220FS Atomic Absorption Spectrometer at 324.7 nm, with an air-acetylene flame. Cu-3 and Cu-4 samples were diluted 5-fold, and Cu-5 was diluted 10-fold into acid-washed 35 mL polypropylene vials with 2% HNO_3 before analysis. A procedural blank and an in-house reference soil were included with each batch. One duplicate was analysed for every ten samples. Standards were prepared from a Cu stock solution

(CentiPUR, 1001 +/ 2 mg L^{-1}) at concentrations of 0, 0.1, 0.2 0.5, 1, 2, 5 and 10 mg L^{-1} in 2% HNO_3. Method reproducibility was determined from repeat measurements of a soil sample (%RSD 2.75, n=6) and the in-house reference soil (%RSD 2.78, n=10). The %DIFF values for duplicate samples ranged from 0.01–4.4% (n=2) and the %RSD values for replicate samples varied between 1.0–6.9% (n=4). The LOD was calculated at 3.76 µg Cu g^{-1} soil, from three times the standard deviation of procedural blanks.

This method was verified by analysing two certified reference soils in duplicate, Montana I (2710) and Montana II (2711a), alongside duplicates of the in-house reference soil and selected test soils, analysed by FAAS and Agilent 7500 cx. inductively coupled plasma mass spectrometer (ICP-MS). Certified soils were also analysed for As, Cd, Pb and Zn concentrations by ICP-MS. The extracted metal concentrations for the certified soils are summarised in Table 2.2. The %DIFF of the duplicate certified soils ranged from 0.05–1.65% for each of the metals analysed. The recovery of total concentrations of the analysed heavy metals in the in-house reference soil and two soil CRMs was excellent with replicate analyses providing acceptable levels of accuracy and precision by both Flame AA and ICP-MS. The mean values of Cu from the Montana I certified soil (2710) were 91 and 92% of the certified value when analysed by Flame AA and ICP-MS, respectively (n=2). The mean values of Cu from the Montana II certified soil (2711a) were 97 and 88% of the certified value when analysed by Flame AA and ICP-MS, respectively (n=2). The recovery of additional heavy metals (As, Cd, Pb and Zn) ranged from 88–97% in the Montana I certified soil and from 83–94% in the Montana II certified soil, when analysed by ICP-MS.

Table 2.2: Mean total metal concentrations of certified reference soils, analysed by FAAS and ICP-MS.

CRM	*Metal*	*Flame AA (mg kg^{-1})*	*ICP-MS (mg kg^{-1})*	*Certified value (mg kg^{-1})*
Montana I (2710)	Cu	2683	2722	2950
	As	–	607	626
	Cd	–	20.6	21.8
	Pb	–	5304	5532
	Zn	–	6109	6952
Montana II (2711a)	Cu	136	123	140
	As	–	97.0	107
	Cd	–	50.5	54.1
	Pb	–	1318	1400
	Zn	–	345	414

The mean value of Cu from the in-house reference soil was 104% of the reference value when analysed by FAAS (n=10; Table 2.3). The recovery of additional heavy metals ranged from 65–121% of the reference value when analysed by ICP-MS (n=2).

Table 2.3: Mean values of in-house standard replicates, compared to in-house standard reference values. Total metals are measured in mg kg^{-1}. Bioavailable Cu is presented as % of total Cu.

	Total Cu	***% Bioavailable Cu***	***Total As***	***Total Cd***	***Total Pb***	***Total Zn***
In-house reference value	237	0.1%	4.9	0.74	45	114
Experimental value	248	0.1%	5.93	0.77	29.3	94.7
% recovery	104%	100%	121%	104%	65%	83%

2.6.2 Bioavailable Copper

The 0.01 *M* $CaCl_2$ extraction of Cu was determined using a method modified from Gray *et al*. (1999). Five g of soil (dried, <2 mm) was weighed into separate 50 mL polycarbonate vials and 25 mL of 0.01 *M* $CaCl_2 \bullet 2H_2O$ was added to each vial. Vials were shaken for 16 h on an end-over-end mixer. Samples were then centrifuged for 10 min at 2000 rpm and an aliquot of the supernatant was transferred into 35 mL polypropylene vials. Samples were acidified to pH <2 with concentrated HNO_3 and stored at 4°C until analysis. A procedural blank and an in-house reference soil were analysed with each batch. One duplicate was included per ten soil samples (at least one per batch). Samples were diluted 10-fold with 1% HNO_3 in acid washed 35 mL polypropylene vials. Samples were mixed thoroughly before either analysing on an Agilent 7500 cx. ICP-MS straight away or being stored at 4ºC overnight until analysis the following day. Method reproducibility was determined from repeat measurements of a soil sample (%RSD 3.22, n=6). %DIFF for duplicate measurements ranged from 1.0–19.9%. The ICP-MS detection limit for copper was calculated at 0.07 mg kg^{-1} (3 times the standard deviation of solution blanks).

2.7 MICROORGANISM ASSAYS

The phosphatase and urease enzyme activities for each soil sample were determined using methods adapted from Speir *et al.* (1984). The phosphatase assay was completed within one week of sampling, using the Enzyme-labelled sub-samples (stored at 4ºC) at t_1 and t_2. Micro- and frozen-labelled samples were thawed overnight at 4ºC before being sub-sampled for the urease enzyme activity analysis. The remaining soil was re-frozen after it was sub-sampled (-20ºC). Urease enzyme activity is stable in soils frozen at -20ºC for at least 5 months (Kandeler and Gerber, 1988). Moisture contents were measured for thawed samples and compared to moisture contents measured at time of sampling, to determine if the moisture content changed during storage. The soil microbiomass was calculated using the Microbial Biomass Carbon method as outlined in Lincoln University's Methods of Soil Analysis (Cresswell and Hassall, n.d.).

2.7.1 Phosphatase Activity Assay

For each soil sample, four replicates of 0.5 g (+/- 0.005) field most soil were weighed into glass 28 mL vials. Soil clumps were dispersed before weighing. Two mL of Tris/Malate Buffer was added to each vial and gently swirled to mix. The buffer was prepared by dissolving 60.57 g tris(hydroxymethyl)-aminomethane (Tris) and 58.03 g maleic acid in 900 mL distilled water. The pH was adjusted to 6.5 with drop-wise addition of 50% NaOH and then made up to 1 L with distilled water. A 0.5 mL aliquot of 100 *mM* sodium *p*-nitrophenyl phosphate (enzyme substrate) was quickly added to three vials for each sample, leaving the fourth untreated to act as a control. The enzyme substrate was prepared by dissolving 1.8558 g in 50 mL of the buffer. The vials were incubated for 1 h in a 30ºC water bath. After incubation, reagents were quickly added in the following order: 0.5 mL enzyme substrate to untreated vials; 0.5 mL 1 *M* $CaCl_2$, followed by 2 mL 0.5 *M* NaOH to all vials. All samples were filtered through Whatman 540 filter papers into clean glass vials. A 0.5 mL aliquot of each filtrate was transferred into 50 mL high density polyethylene centrifuge tubes, made up to 25 mL with distilled water, and mixed well. The absorbance of each sample was measured at 400 nm on a Biochrom Libra S4 Visible spectrophotometer (U.O.C. #8054). Absorbances were recorded and phosphatase activity (nmol g^{-1} s^{-1}) was calculated using Beer's Law with an extinction coefficient of 17000. The %RSD for replicate measurements

ranged from 0.15–10.4% (n=3). The LOD was calculated at 0.060 nmol g^{-1} s^{-1}, from three times the standard deviation of procedural blanks.

2.7.2 Urease Activity Assay

For each sample, four replicates of 0.5 g (+/- 0.005) of thawed soil were weighed into 35 mL polypropylene vials. Soil clumps were dispersed before weighing. Phosphate buffer (pH 6.8, 1.6 mL) was added to each vial and then gently swirled to disperse the soil. The phosphate buffer was prepared by dissolving 17.908 g $Na_2HPO_4 \bullet 12H_20$ and 7.411 g sodium $NaH_2PO_4 \bullet 2H_20$ in 1 L of Milli-Q water. The enzyme substrate (1 *M* urea; 0.4 mL) was quickly added to three of the vials for each sample, leaving the fourth untreated to act as a control. The enzyme substrate was prepared daily by dissolving 3.003 g urea in 50 mL of phosphate buffer. All vials were incubated for 4 h at 30ºC in a water bath. After incubation, reagents were quickly added in the following order: 0.4 mL enzyme substrate to untreated vials; 8 mL 2 *M* KCl to all vials. Two solution blanks were analysed with each batch. Samples were filtered through Whatman 540 filter papers into fresh plastic vials.

Samples were analysed colourimetrically for $NH4^+$-N. Standards were prepared from a stock NH_4Cl standard (1.909 g NH_4Cl in 500 mL Milli-Q water) at concentrations of 0, 1, 2, 5, 10, 15 and 20 μg mL^{-1}. A 0.5 mL aliquot of the standards and sample filtrates were transferred into separate fresh labelled plastic vials. Reagents were added to vials in the following order: 4 mL complexing agent, 6 mL sodium salicylate, 3 mL hypochlorite solution and 1 mL sodium nitroprusside. The complexing agent was prepared by adding 6 g tri-sodium citrate, 18 g sodium hydrogen tartrate and 25 g NaOH in 1 L Milli-Q water. The hypochlorite solution was prepared by dissolving 5 g NaOH in 200 mL Milli-Q water, followed by addition of 3 mL Janola bleach (regular; 4.2% hypochlorite) and was made up to 250 mL with Milli-Q water. The sodium salicylate solution was prepared by dissolving 15 g sodium salicylate in 1 L Milli-Q water. The sodium nitroprusside solution was prepared by dissolving 1.2 g sodium nitroprusside in 500 mL Milli-Q water. Standards and samples were allowed to develop for 30 mins in the dark. The absorbance of the standards and samples was measured at 660 nm on a Biochrom Libra S4 Visible spectrophotometer (U.O.C. #8054). A standard curve was prepared and used to calculate sample urease activity (nmol g^{-1} s^{-1}). Replicate measurements ranged in %RSD from 0.28–17.8% (n=3). The LOD was calculated at

0.028 nmol g^{-1} s^{-1}, from three times the standard deviation of procedural blanks. Standards and reagents were stored in the dark when not in use to prevent photodegradation.

2.7.3 Soil Microbiomass

The soil microbiomass was measured in Lincoln University's soil laboratory by the fumigation-extraction method. The chloroform had been purified with 5% H_2SO_4 by Roger Cresswell before analysis. Five g field moist soil (<2 mm) was weighed in duplicate into clean 50 mL beakers in preparation for fumigation with chloroform. These beakers were placed in a vacuum box in a fume hood, containing two shelves with holes. Care was taken not to completely cover the holes, in order to allow circulation of chloroform. A beaker containing approximately 25 mL purified chloroform and a few boiling chips was placed into the centre of the vacuum box below the soil beakers. The box was evacuated until the chloroform boiled for two minutes. The vacuum tap was closed and the box was left in the dark at 25ºC for 18–24 h. After fumigation, air was slowly allowed into the vacuum box chamber and the beaker of chloroform was removed. The box was evacuated repeatedly under vacuum until residual chloroform vapour was removed from the box. Fumigated samples were transferred into 70 mL polypropylene specimen containers fitted with polyethylene caps.

Five g field moist soil (<2 mm) was weighed in duplicate into fresh specimen containers (non-fumigated). Fumigated and non-fumigated samples were extracted with 20 mL 0.5 *M* K_2SO_4 for 30 min with shaking on a home-made end-over-end shaker. Extracts were filtered through Whatman 540 filter papers into fresh polypropylene specimen containers and stored at 4ºC until analysis. Sample extracts were analysed for TOC on an automated Shimadzu 5000A total organic C analyser. The limit of detection for the TOC analyser was 1.2 mg L^{-1} (three times the standard deviation of blanks). The microbiomass was corrected with a K_{EC} factor of 0.35, as in Sparling *et al.* (1990) for New Zealand soils.

2.8 PESTICIDE ANALYSIS

The pesticide analyses were conducted at Plant and Food Research, Ruakura, New Zealand. Frozen pesticide-labelled soil samples were thawed at 4ºC overnight before analysis. The remaining soil was re-frozen after it was sub-sampled (-20ºC). Moisture contents were

measured for each sample and compared to the moisture contents measured at time of sampling. The moisture contents measured at time of analysis were used for calculations of pesticide concentrations.

2.8.1 Materials

Atrazine (99.0% pure) was purchased from the Institute of Organic Industrial Chemistry (Warsaw, Poland). Desethylatrazine (DEA, 99.9% pure) and desisopropylatrazine (DIA, 98.7% pure) were purchased from Promochem (Middlesex, United Kingdom). Desethyldesisopropylatrazine (DDA, 98.0% pure), hydroxyatrazine (HA, 99.9% pure), desethylhydroxyatrazine (DEHA, 99.5% pure) and desisopropylhydroxyatrazine (DIHA, 98.5% pure) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Indoxacarb (99.3% pure) was purchased from Du Pont (Wilmington, Delaware). UltimAR Acetonitrile (ACN) and ChromAR Methanol (MeOH) were purchased from BioLab (New Zealand). Heptafluorobutyric acid (HFBA) was purchased from Sigma-Aldrich (New Zealand).

2.8.2 Atrazine

The atrazine analysis method was modified from Lerch and Li (2001) and adapted for this particular soil type. Soil samples were analysed for atrazine, DEA, DIA, DDA, HA, DEHA and DIHA, however only atrazine and HA were detected in the soil. Matrix-spiked experiments were undertaken to determine the efficiency of this method for this particular soil type, and amendments were made as necessary. These amendments included reducing the amounts of soil and solvents used and adding a water wash to prevent precipitate formation. A more detailed description of the method development is presented in Chapter 4 (Section 4.2).

2.8.2.1 Soil Extraction

An extractant mixture was prepared from 0.5 *M* KH_2PO_4 (pH 7.5) and ACN (75:25, v/v) and was heated to 60°C in a water bath. The pH of the buffer was adjusted to 7.5 with 50% KOH before mixing with ACN. Thawed soil samples (equivalent to 10 g DW) were weighed into 50 mL Teflon centrifuge tubes. Only three of the four replicates were analysed (samples A-C), due to time constraints and solvent availability. The soil samples were extracted twice

with 20 mL of the extractant mixture by sonication on a Bandelin Sonorex Digital 10P sonicator for 30 min at 50°C, followed by shaking at 400 rpm for 30 min at room temperature (~22°C) on an IKA KS-500 orbital shaker and then by centrifuging for 30 min at 4000 rpm and room temperature in a Hettich Rotanta 460R centrifuge. The combined supernatants were collected in a 50 mL graduated boiling tubes and the total extracted volume was recorded (range 36–38 mL). After mixing, a 20 mL aliquot was transferred into a 40 mL Turbo Evaporator (TurboVap) vial and the ACN was evaporated by a Caliper LifeSciences TurboVac LV turbo evaporator (20 mL to 15 mL). TurboVap vials were pre-marked at the 15 mL level and evaporation was stopped once the solution reached the mark. Combined sample extracts were mixed and then stored at 4°C overnight, as this was a suitable pause point before beginning clean-up and analysis.

2.8.2.2 Sample Clean-up and Analysis

Samples were cleaned up by passage through solid phase extraction (SPE) using strong anion exchange cartridges (SAX; Varian, 1 g, 6 mL). The cartridges were pre-conditioned with methanol and Milli-Q water as outlined in Table 2.4. The breakthrough solutions were collected separately in 20 mL glass vials. The SAX cartridges were rinsed with 80% MeOH, followed by Milli-Q water (Table 2.4), and rinses were collected with the breakthrough solutions (~20 mL total). The cartridges were allowed to run dry and vacuum was applied until sputtering stopped. The total combined breakthrough solutions were mixed and acidified to pH 2.5 with concentrated H_3PO_4 to protonate the hydroxyatrazine degradation products (HADPs).

Atrazine and its degradation products were isolated and concentrated using strong cation exchange (SCX) cartridges. The SCX (Varian, 2 g, 12 mL) cartridges were pre-conditioned with MeOH, Milli-Q water and 0.05 *M* KH_2PO_4 (pH 2.5) as described in Table 2.2. The 0.05 *M* phosphate buffer was adjusted to pH 2.5 with concentrated H_3PO_4. The acidified sample breakthrough solutions from the SAX clean-up were passed through the SCX cartridges and the eluate passed to waste. Each cartridge was then washed with 3 mL Milli-Q water which prevented the formation of a precipitate. The wash was discarded and the cartridges were allowed to run dry. The cartridges were then placed under vacuum for 2–3 min until sputtering stopped. Atrazine and degradation products were eluted with 10 mL MeOH:NH_4OH:Milli-Q water (8:1:1, v/v/v) and collected in 40 mL TurboVap vials. Once

the eluent solutions had reached the cartridge bed, vacuum was applied until sputtering stopped.

Table 2.4: SPE cartridges used for extraction and elution of atrazine and metabolites.

Cartridge	*Conditioning*	*Flow Rate*	*Sample Flow Rate*	*Rinses*	*Elution*	*Flow Rate*
SAX	6 mL MeOH 2x5 mL Milli-Q water	Gravity	5-10 mL min^{-1}	2.5 mL 80% MeOH 2.5 mL Milli-Q water	–	–
SCX	12 mL methanol 12 mL Milli-Q water 12 mL 0.05 *M* KH_2PO_4 (pH 2.5)	Gravity	3-5 mL min^{-1}	3 mL Milli-Q water	10 mL MeOH:NH_4OH: Milli-Q water (8:1:1, v/v/v)	1 mL min^{-1}
Oasis HLB	5 mL ACN 2x5 mL MeOH 5 mL Milli-Q water	Gravity	5-10 mL min^{-1}	5 mL 10% ACN	5 mL ACN	Gravity

These eluent solutions, which contained any atrazine and metabolites, were evaporated to dryness with the turbo evaporator at 45-50°C (~70 min). Samples were reconstituted with 1 mL of 40% methanol. Solutions were swirled to dissolve residue, taking special care to dissolve residue adhering to the sides of the vials. The concentrated samples were filtered through 0.45 µm syringe filters, transferred to high pressure liquid chromatography (HPLC) auto sampler vials and stored at 4°C until analysis. Samples and standards were analysed by reverse-phase HPLC using a Phenomenex Luna 5u C18 (2) column (150 x 4.60 mm) held at 35ºC on a Shimadzu LC10A Liquid Chromatograph fitted with a SIL-10AF auto injector, a SPD-M10A Diode Array Detector, and a Shimadzu Class VP operating and quantification software. Detection wavelengths used for analysis were 230 and 245 nm.

Soil samples were analysed for atrazine and its metabolites DEA, DIA, DDA, HA, DEHA and DIHA at either a single wavelength or at both wavelengths, as appropriate. The 230 nm wavelength gave the best response for atrazine and the chloro-metabolites, with the 245 nm

wavelength used to assist with identification of chromatographic peaks by comparing the ratios of peak-heights at each wavelength. The 245 nm wavelength gave the best response for HA and the other hydroxy-metabolites. Approximate retention times for target compounds are listed in Table 2.5. Retention times did shift slightly from run to run as mobile phase solvents were replenished. These changes were accounted for during each run by bracketing every six samples with an indoxacarb standard solution injection. Retention times of the standards were compared to those of the samples for data processing. Examples of chromatograms for the atrazine breakdown products (ABP) standard (10 µg mL^{-1}) and a spiked solution blank are shown in Figure 2.8. There were minimum interferences from solvents in the standard and extraction mixtures.

Table 2.5: Approximate retention times for atrazine and metabolites.

Compound (230 nm)	*Retention time (min)*	*Compound (245 nm)*	*Retention time (min)*
Atrazine	24.6	HA	14.2
DEA	10.1	DIHA	3.4
DIA	5.3	DEHA	4.8
DDA	2.2		

The mobile phases were MeOH (B), 20% MeOH plus 0.1% formic acid (C) and 20% MeOH plus 0.1% HFBA (D). These were held for 4 min at a ratio of 20:40:40, respectively, with a flow rate of 1 mL min^{-1}. A linear gradient over 15 min altered solvent concentrations to 30% B, 30%C and 40% D. This was held for 8 min. A linear gradient over 1 min returned solvents to their original ratios (20:40:40). This was held for 17 min to re-equilibrate the column before the next injection. The total run time was 45 min. Samples were analysed for the first 30 min for atrazine and metabolites. The column was maintained at 35ºC for the duration of each run.

Calibration was based on a single point forced through the origin. 7.5 µg mL^{-1} of the mixed atrazine standard was used to calibrate for atrazine and metabolites (r^2=1.000). A 10 µg mL^{-1} mixed atrazine standard was analysed multiple times with each batch, in order to determine if the calibration held. These all gave accurate and consistent results (atrazine: average 9.40 µg mL^{-1}, %RSD 7.96, n=24; HA: average 10.1 µg mL^{-1}, %RSD 8.33, n=24).

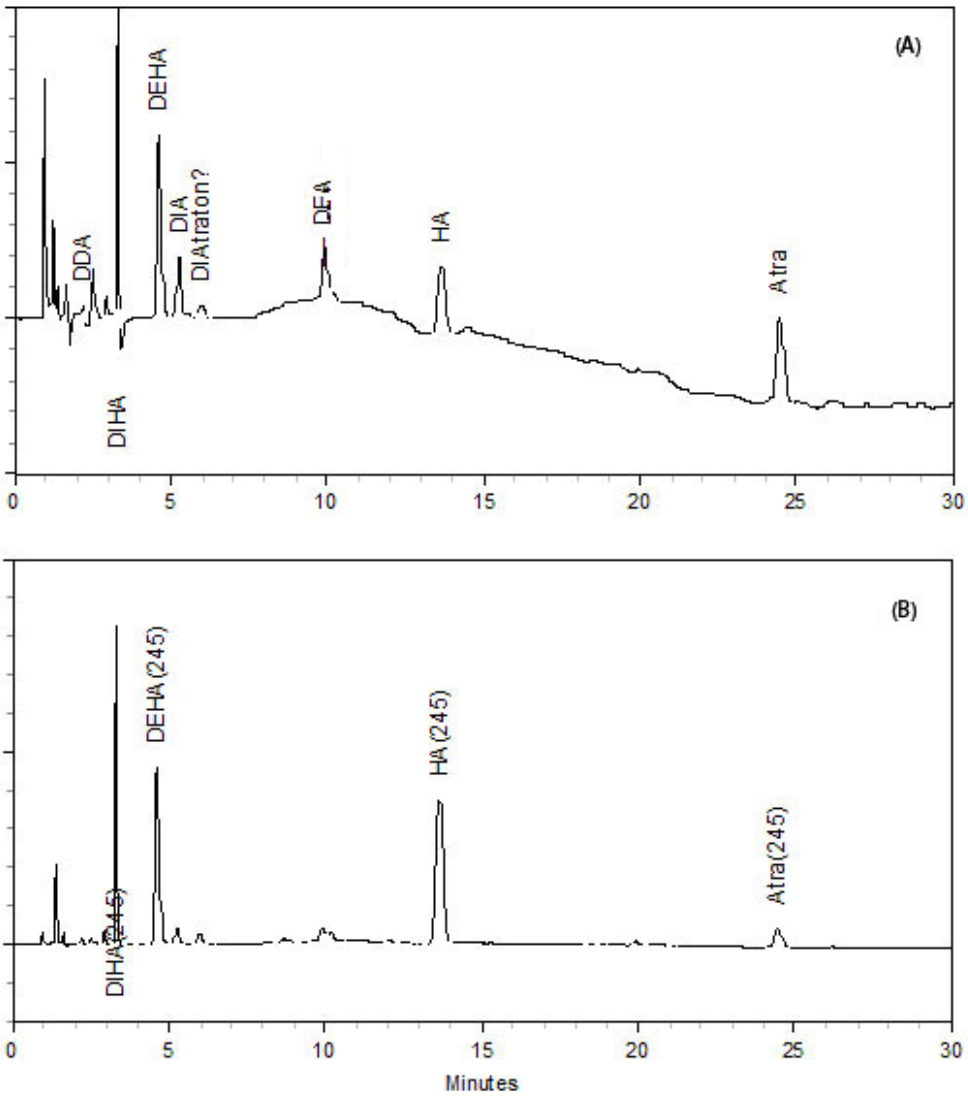

Figure 2.8: HPLC chromatograms of (A) 10 µg mL^{-1} atrazine mixed standard and (B) ACN/buffer solution blank spiked with atrazine mixed standard.

2.8.2.3 Preparation of standards

Pesticide analytical standards (PAS) were used to prepare pesticide stock standards (PSS) for atrazine, DEA, DIA, DDA, HA, DEHA and DIHA as outlined in Table 2.6. Atrazine, DEA and DIA were prepared in ACN, DDA and hydroxyatrazine breakdown products (HABPs) were made up in MeOH with four molar equivalents of HFBA to aid in dissolution. Pesticide working standards (PWS) were prepared from PSS for solutions of Atrazine, DEA and DIA, all ABPs and all HABPs, as outlined in Table 2.7.

Table 2.6: Pesticide stock standard (PSS) preparation.

PSS	*From PAS*	*Compound*	*Purity (%)*	*Conc. (µg mL^{-1})*	*solvent*
1079	566	Atrazine	99.0	**200.2**	ACN
1080	34	DEA	99.9	**201.6**	ACN
1081	33	DIA	98.7	**199.8**	ACN
1087	866	DDA	98.0	**99.4**	MeOH
1088	42	HA	99.9	**100.4**	MeOH/HFBA*
1089	865	DEHA	99.5	**100.0**	MeOH/HFBA*
1090	867	DIHA	98.5	**99.6**	MeOH/HFBA*
1086	786	Indoxacarb	99.3	**199.8**	ACN
*4 molar equivalents of heptafluorobutyric acid (HFBA) added to help with dissolution.					

2.8.2.4 QA/QC

A spiked soil blank (Cu-1 Nov08) was included in each batch to determine method recovery (Table 2.8). Soil blanks were spiked with either 100 µL or 25 µL of the mixed chloro- and hydroxy-metabolite standards (PWS 677-1 and PWS 690-1) before SPE sample clean-up, with final concentrations of 2 µg mL^{-1} and 0.5 µg mL^{-1} (respectively). At least one comparative standard was also included in each batch, prepared with 100 µL or 25 µL of the mixed chloro- and hydroxy-metabolite standards (PWS 677-1 and PWS 690-1), and made up to 1 mL with 40% MeOH (final concentrations of 2 µg mL^{-1} and 0.5 µg mL^{-1}, respectively). Sample duplicates were measured over different days, to assess reproducibility. The %RSD for repeat measurements ranged from 2.33–51.04% for atrazine and 0.30–47.61% for HA (n=2 or 3). The high %RSD values occurred when extracted atrazine or HA concentrations were close to the detection limit. The method detection limits for atrazine and HA were determined from the signal-to-noise ratio of low-level standards and matrix-spiked blanks. The instrument detection limits for the standards were 0.25 µg mL^{-1} for atrazine and 0.2 µg mL^{-1} for HA (Figure 2.). Matrix-spiked blanks gave method detection limits of 0.5 µg mL^{-1} (0.1 mg kg^{-1} in soil) for atrazine and 0.3 µg mL^{-1} (0.06 mg kg^{-1} in soil) for HA (Figure 2.). The 95% confidence intervals for mean recoveries of atrazine and HA from spiked soil blank extracts at 2 µg mL^{-1} were 70 ± 19% and 94 ± 6%, respectively (n=5). Mean recoveries of atrazine and HA from spiked soil blank extracts at 0.5 µg mL^{-1} were 105% and 69%, respectively (n=2).

Table 2.7: Pesticide working standard (PWS) preparation.

PWS	*From PSS*	*Compound Mix*	*Concentration (µg mL^{-1})*	*solvent*
677-1	1079 1080 1081	Atrazine DEA DIA	**20**	ACN
686-1 **686-2**	1079 1080 1081 1087 1088 1089 1090	ABPs	**10**	MeOH/ HFBA*
690-1	1088 1089 1090	HABPs	**20**	MeOH/ HFBA*
680-1	1086	Indoxacarb	**20**	ACN
680-2	1086	Indoxacarb	**20**	50% MeOH
680-3	PWS 680-1	Indoxacarb	**2**	50% MeOH
*MeOH:H_2O (20:80) + 0.1% HFBA.				

Table 2.8: Recovery of atrazine, HA and indoxacarb from matrix-spiked soil blanks.

Compound	*100 µL spike*			*25 µL spike*		
	Measured conc. (µg mL^{-1})	*%RSD/ %DIFF*	*Recovery (%)*	*Measured conc. (µg mL^{-1})*	*%RSD/ %DIFF*	*Recovery (%)*
Atrazine	1.39*	30.9	69.7	0.53**	38.6	105
HA	1.69*	23.7	84.4	0.35**	0.20	69.3
Indoxacarb	–	–	–	4.71***	4.80	94.1
*n=5; **n=2; ***n=4; %RSD calculated for n≥3, %Diff calculated for n=2.						

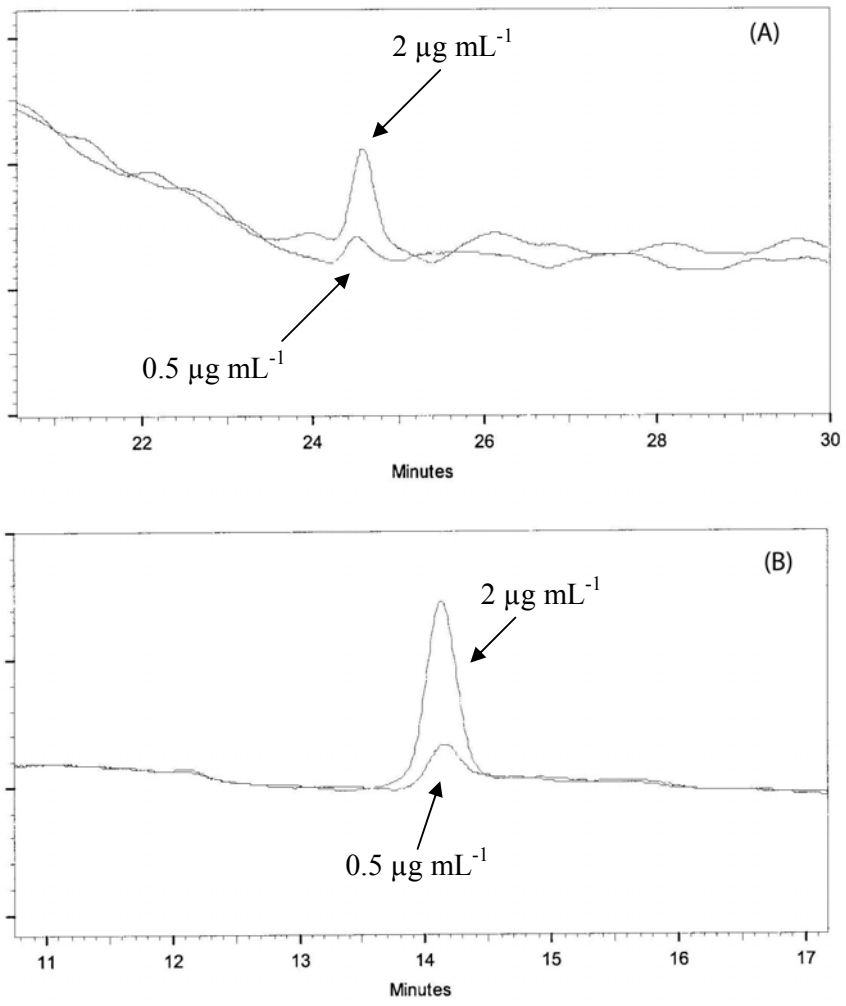

Figure 2.9: HPLC chromatograms used to determine instrument detection limits for (A) atrazine and (B) hydroxyatrazine from mixed standards at 0.5 and 2 µg mL^{-1}.

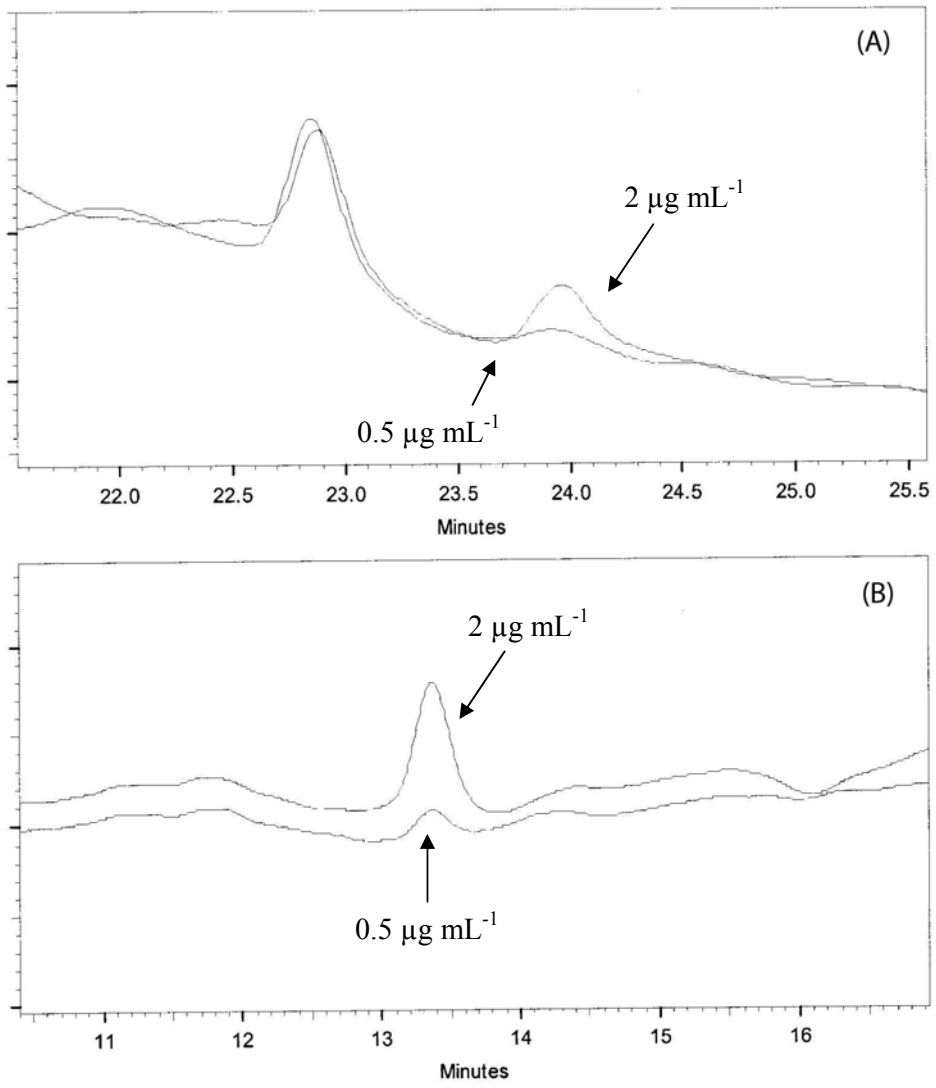

Figure 2.10: HPLC chromatograms used to determine method detection limits for (A) atrazine and (B) hydroxyatrazine by spiking a soil blank with a mixed standard at 0.5 and 2 µg mL^{-1}.

2.8.3 Indoxacarb

The indoxacarb analysis method was adapted from methods published by Campbell *et al.* (2005) and Xu *et al.* (2008). Matrix-spiked experiments were undertaken to determine the efficiency of this method for this particular soil type, and amendments were made as necessary. The extraction procedure used was the same as with Atrazine (sonication, orbital shaking, centrifuging) and Oasis HLB cartridges were used to clean-up and concentrate

samples. A more detailed description of the method development is presented in Chapter 4 (Section 4.2.2).

2.8.3.1 Soil Extraction

Indoxacarb soil samples were extracted twice with 20 mL of a 0.5 *M* KH_2PO_4 (pH 7.5):ACN solution (75:25, v/v) and 10 mL MeOH. The buffer/ACN solution was heated to 60ºC in a water bath before extraction, but the methanol was not heated due to its low boiling point. Indoxacarb soil samples were extracted as described in Section 2.8.2.1. As with the atrazine samples, only three of the four indoxacarb replicates were analysed (samples A-C), due to time constraints and solvent availability. The combined supernatants were collected in 50 mL graduated boiling tubes. The volume of the first extraction was recorded, in order to calculate the total volume collected. The total volumes ranged from 55.5–59 mL. Combined sample extracts were mixed and then stored at 4°C overnight, as this was a suitable pause point before beginning clean-up and analysis.

2.8.3.2 Sample Clean-up and Analysis

The extracted samples were cleaned up by SPE with Oasis HLB cartridges (Waters, 500 mg, 6 mL). The cartridges were conditioned as described in Table 2.4. Care was taken to ensure the columns did not run dry. The combined sample extracts were transferred into 250 mL Schott bottles and diluted to approximately 240 mL by adding 180 mL of Milli-Q water to give approximately 6% organic solvent. A portion of the diluted sample was added to the cartridges with a Pasteur pipette before connecting Teflon plastic tubing with Teflon connector plugs to each cartridge. The tubing was inserted into the corresponding diluted sample solutions. Parafilm was placed over the bottles to prevent contamination and to hold tubing in place during aspiration. The extracted samples were passed through the cartridges at a flow rate of 5–10 mL min^{-1} under vacuum to keep the flow rate consistent. The flow was stopped when the solutions reached the column beds and were not allowed to run dry. The cartridges were rinsed with 5 mL of 10% ACN, with the first 1 mL added and taken down to the column bed, followed by the final 4 mL which was allowed to run dry. The cartridges were pulled under full vacuum until sputtering stopped, about 1–2 min. The samples were eluted with 5 mL ACN into 40 mL TurboVap vials under gravity. The flow was stopped when about half of the solution passed through and allowed to equilibrate for 2 min. Taps

were re-opened and allowed to resume flow under vacuum. Once the flow stopped, full vacuum was used to continue to dry cartridges until sputtering stopped. Samples were evaporated to dryness with the turbo evaporator at 45–50°C and were reconstituted in 1 mL 50% MeOH. The solution was swirled to dissolve all of the residue, taking care to dissolve residue on sides of the vials. Reconstituted samples were filtered through Pasteur pipettes loosely packed with glass wool and collected in HPLC autosampler vials. Vials were stored at 4°C until analysis.

The samples and standards were analyzed by reverse-phase HPLC using a Phenomenex Luna 5u C18 (2) column (150 x 4.60 mm) held at 35ºC on a Shimadzu LC10A Liquid Chromatograph fitted with a SIL-10AF auto injector, a SPD-M10A Diode Array Detector, and a Shimadzu Class VP operating and quantification software. Detection wavelengths used for analysis were 310 and 225 nm. The 310 nm wavelength gave the best response for indoxacarb, with the 225 nm wavelength used to assist with identification of chromatographic peaks by comparing the ratios of peak-heights at each wavelength.

Soil samples were analysed for indoxacarb only, due to time constraints and availability of metabolite standards. The retention time of indoxacarb was approx. 9.1 min. As with atrazine and metabolites, this peak did shift as mobile phase solvents were replenished. These changes were accounted for during each run by bracketing every six samples with an indoxacarb standard solution injection. Retention times of the standards were compared to those of the samples for data processing. Figure 2.11a is an example of the indoxacarb standard, PWS 680-2 (20 $\mu g\ mL^{-1}$). Figure 2.11b is an example of a typical spiked soil blank chromatogram. The peak between 1–4 min is due to co-extractives from the soil, as are the peaks between 12–15 min during the column flush. Neither of these interfered with the indoxacarb peak (Figure 2.11b).

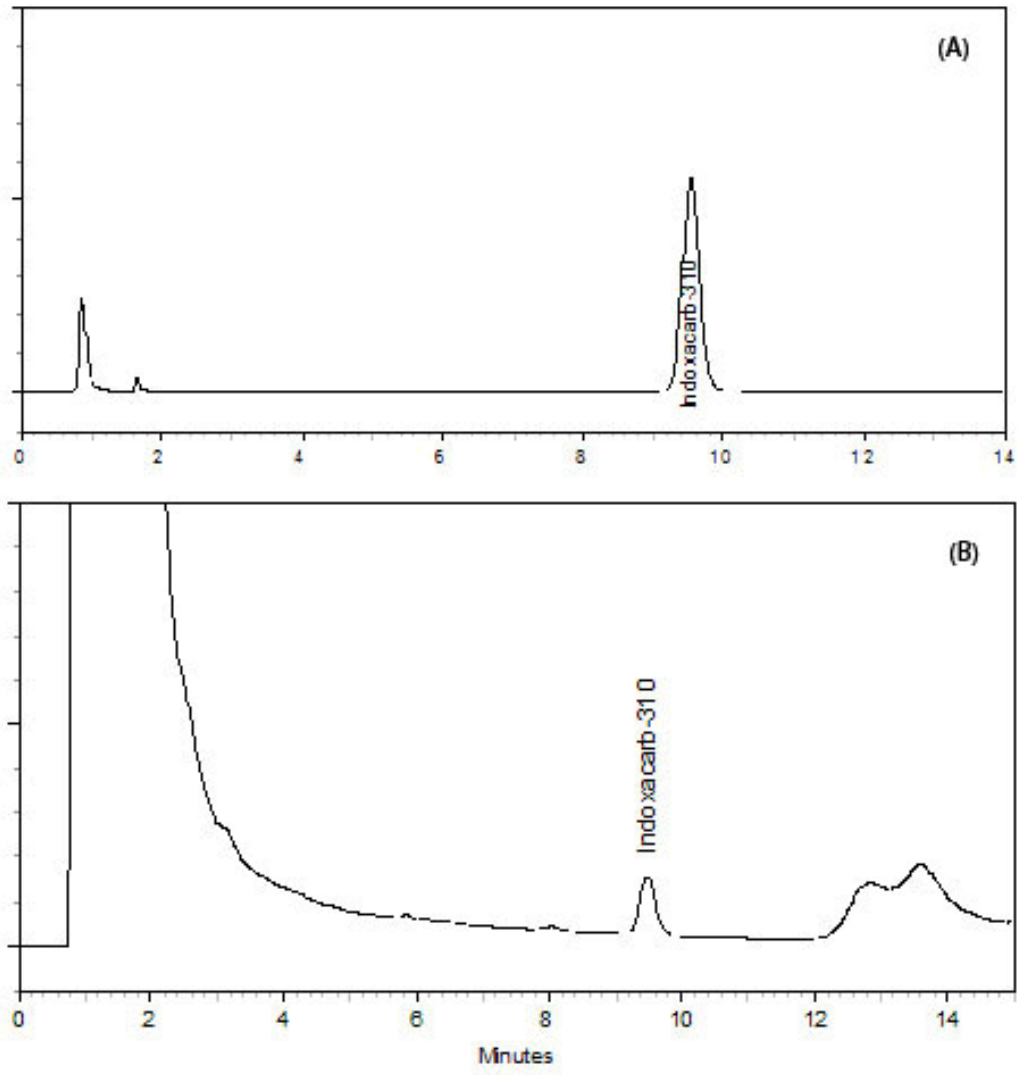

Figure 2.11: HPLC chromatograms of (A) 20 µg mL^{-1} indoxacarb standard and (B) soil blank spiked with 5 µg mL^{-1} of the indoxacarb standard, both measured at 310 nm.

The mobile phases were 20% MeOH (A) and 100% MeOH (B). For the indoxacarb standards, the solvents were held isocratically at 30% A and 70% B for 14 min, at a flow rate of 1 mL min^{-1}. The mobile phases for the soil samples were held at the same concentrations as the standards for 9 min. Solvent B then increased to 100% over 1 min and was held for 9 min to flush the column. After the flush, solvent B was reduced back to 70% over 1 min and this was held for a further 15 min to re-equilibrate the column before the next injection. The total run time was 35 min, with samples analysed for the first 14 min. The column was kept at 35ºC for the duration of each run.

Calibration was based on a single point forced through the origin. Twenty μg mL^{-1} of the indoxacarb standard was used for calibration (r^2=1.000). A 20 μg mL^{-1} and 2 μg mL^{-1} indoxacarb standards were analysed multiple times with each batch, in order to determine if the calibration held. These all gave accurate and consistent results (20 μg mL^{-1}: mean 19.9 μg mL^{-1}, %RSD 2.16, n=21; 2 μg mL^{-1}: mean 1.92 μg mL^{-1}, %RSD 1.75, n=19).

2.8.3.3 Preparation of Standards

Standards were prepared in ACN from analytical-grade Indoxacarb (PAS 786) at 200 μg mL^{-1} (PSS 1086), 20 μg mL^{-1} (PWS 680-1 and -2) and 2 μg mL^{-1} (PWS 680-3), as described in Table 2.6.

2.8.3.4 QA/QC

A spiked soil blank (Cu-1 Nov08) was included in each batch to determine method recovery. Soil blanks were spiked with 25 μL of the indoxacarb standard (PSS 1086, 200 μg mL^{-1}) before the dilution step, with an expected final concentration of 5 μg mL^{-1}. The recovery was 94.1% and the %RSD was 4.80 (n=4; Table 2.8). A comparative standard was also included in each batch and prepared from 25 μL of PSS 1086 (200 μg mL^{-1}) and made up to 1 mL with 50% MeOH. Sample duplicates were measured over multiple days to assess method reproducibility (**Table 2.9**). The %RSD for repeat measurements ranged from 0.69–10.55% (n= 2 or 3). The method detection limit for Indoxacarb was determined from the signal-to-noise ratio of matrix-spiked blanks and was 0.1 μg mL^{-1} (0.0025 mg kg^{-1} in soil) (Figure 2.). The 95% confidence interval for mean recoveries of indoxacarb from spiked soil blank extracts at 5 μg mL^{-1} was 94 ± 4% (n=4).

Table 2.9: Results for replicate analysis of indoxacarb, to assess method reproducibility.

Sampling time	*Sample*	*Average (µg mL^{-1})*	*Standard deviation*	*%RSD*
t_0	IND Cu-1	14.77*	0.81	5.49
	IND Cu-2	14.50*	1.18	8.13
	IND Cu-3	15.38*	1.31	8.49
	IND Cu-4	14.78*	1.45	9.81
	IND Cu-5	15.01*	0.73	4.86
t_1	IND Cu-1A	1.61**	0.078	4.85
	IND Cu-2A	1.68**	0.064	3.78
	IND Cu-3A	1.84**	0.014	0.77
	IND Cu-4A	1.48**	0.035	2.40
	IND Cu-5A	1.80**	0.12	6.70
t_2	IND Cu-1A	1.03**	0.0071	0.69
	IND Cu-2A	1.14**	0.028	2.48
	IND Cu-3A	1.14**	0.064	5.61
	IND Cu-4A	1.01**	0.11	10.55
	IND Cu-5A	1.09**	0.071	6.49
* n=3; **n=2				

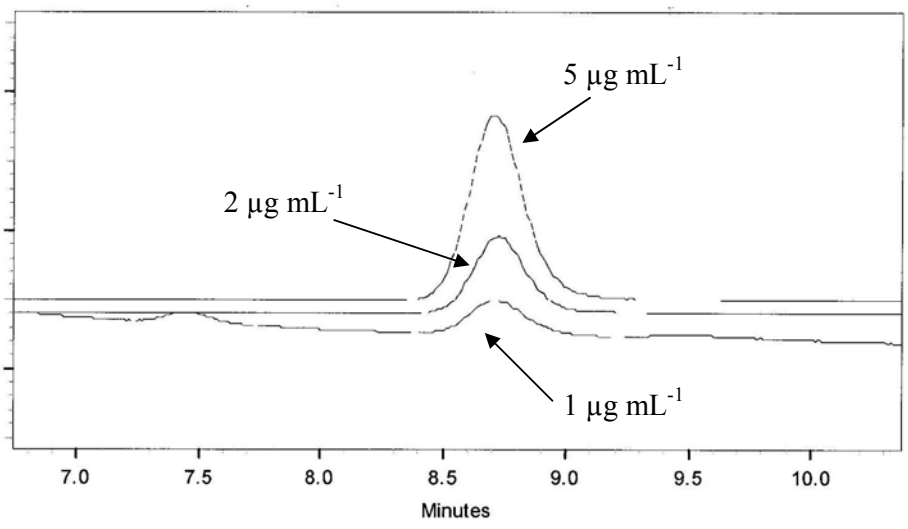

Figure 2.12: HPLC chromatogram used to determine the method detection limit for indoxacarb with a 5 µg mL^{-1} comparative standard, a 2 µg mL^{-1} standard and a 1 µg mL^{-1} low-level sample.

2.9 STATISTICAL ANALYSES

Pearson's correlation analyses were used to determine if there were significant relationships between total and bioavailable Cu, and selected experimental data. Significant differences were calculated using one-way analysis of variance (ANOVA). When significant differences occurred, differences between the control (Cu-1) and the other Cu-spike levels were determined by Dunnett's test ($p<0.05$). If values were less than the detection limit, values of half the detection limit were used for the statistical analyses.

2.10 CLEANING PROCEDURES

The soil aging bins were triple-rinsed with distilled water before filling with distilled water and allowed to leach overnight. Bins were covered in plastic sheeting to prevent contamination. This process was repeated. The bins were dried upside-down on plastic sheeting.

All glassware, plasticware, shovels, mesh, the hand-held spray pump and liver pails were washed with a dilute Decon-90 solution, allowed to soak overnight and then triple-rinsed with distilled water. Glassware and plasticware used for copper and pesticide spiking, and the urease assay were thoroughly rinsed with Milli-Q water before use. Glassware for pesticide spiking was triple rinsed with ChromAR acetone before making up the spiking solutions.

Sample corers and sieves were washed in the following order: dilute Decon-90 wash and scrub to remove soil particles; tap water rinse to remove wash solution; Milli-Q water rinse; drum ethanol rinse to prevent microbial cross-contamination; second Milli-Q water rinse to prevent adverse effects on soil microorganisms from ethanol toxicity. Corers were then patted dry with paper towels. Sieves were dried in a 60ºC oven on aluminium foil.

Plastic spoons to weigh fresh and frozen soil samples were rinsed with drum EtOH before use and in between soil replicates to prevent microbial cross-contamination between replicates. Plastic spoons used to weigh dry soil samples were rinsed with distilled water before use and in between soil replicates. Spoons were allowed to dry on paper towels. Soil samples were always weighed in order of increasing Cu-concentration, to prevent Cu-contamination in lower Cu levels.

Glassware and plasticware used for the total and bioavailable copper analyses were soaked in 10% HNO_3 at least overnight, and then were thoroughly rinsed with Milli-Q water before drying upside-down on paper towels. The acid bath was prepared from AnalAR HNO_3 purchased from Merck (New Zealand) and diluted to 10% with Milli-Q water.

Glassware used for standard preparation in the pesticide studies were solvent rinsed before use with 3x MeOH, 3x dichloromethane (DCM), followed by triple-rinsing with solvent used to make up standards (i.e. MeOH or ACN). After use, all glassware and plastics were triple-rinsed with EtOH to remove pesticide residues, followed by soaking in a dilute Decon-90 solution overnight. After soaking, glassware was scrubbed (if able) and rinsed thoroughly with warm tap water, triple-rinsed with MeOH and dried either in a warm oven (non-volumetric) or in racks on top of the oven (volumetric).

Chapter 3: Effects of Copper on Soil Microorganisms

3.1 INTRODUCTION

The results for the total and bioavailable Cu concentrations in soil, microbial biomass, and phosphatase and urease enzyme assays are presented in this chapter. These soil biological properties were investigated in order to determine if the copper spiked into the soil was bioavailable and had adverse effects on the soil microbial community.

Heavy metals including Cu can negatively impact the soil microbial community by either exterminating the soil microorganisms, lowering diversity by favouring tolerant species, (Giller *et al.*, 1998), or inhibiting essential microbial processes (Sandrin and Maier, 2003). Enzyme activities are one indicator of soil health (Wang *et al.*, 2009). Heavy metals can interact with microbial enzymes directly involved in the biodegradation processes of organic contaminants, or through interactions with enzymes involved in general metabolism (Sandrin and Maier, 2003). More specifically, copper can inhibit enzyme activities by either: 1) reducing the production of enzymes through toxic effects to the microorganisms; 2) combining with the active protein groups of the enzyme; 3) complexing with the substrate required by the enzyme; or 4) reacting with the enzyme-substrate complex (Wang *et al.*, 2009). As copper is known to interfere with the dehydrogenase activity assay (Giller *et al.*, 1998) and inhibit the urease enzyme (Tyler, 1974), multiple assays should be run on soils contaminated with copper to assess microbial effects. Heavy metals are also known to decrease the soil microbiomass and this can occur at moderate to low levels of metal loading (Giller *et al.*, 1998).

Phosphatase and urease enzymes are important in the cycling of phosphorus and nitrogen in the soil (Sannino and Gianfreda, 2001). The phosphatase enzyme is responsible for hydrolysing organic phosphorus to inorganic phosphorous in the soil (Megharaj *et al.*, 1999), making it available for plant uptake (Troeh and Tompson, 1993). The phosphatase assay measures the amount of inorganic phosphate released by the microorganisms (Tabatabai and Bremner, 1969). The urease enzyme is responsible for catalysing the hydrolysis of urea to ammonia (Kim *et al.*, 2008). Ammonia is able to absorb an additional hydrogen ion in the soil, making it NH_4^+, which enters the soil solution and becomes available for uptake by

plants (Troeh and Tompson, 1993). Adverse effects on these enzymes from heavy metal contamination could potentially reduce the fertility of agricultural soils (Kim *et al.*, 2008).

The soil microbiomass is another commonly measured soil biological property (Bunemann *et al.*, 2006). Heavy metals, including Cu, are known to reduce the soil microbiomass (Knight *et al.*, 1997; Sandrin and Maier, 2003). The microbiomass to organic C ratio (C_{mic}:C_{org}) can also be used as an indicator of change in the microbial community due to heavy metal contamination (Merrington *et al.*, 2002). Decreasing the microbial diversity may diminish the resilience of the soil ecosystem to new stressors (Giller *et al.*, 1998), such as amendments with pesticides.

Total and bioavailable Cu concentrations were investigated in order to determine the concentrations of Cu available to microorganisms for uptake in the Templeton experimental soil. Copper is strongly adsorbed by soil organic matter, making it one of the least mobile trace elements in soil (Baker and Senft, 1995; Bolan *et al.*, 2003; Komarek *et al.*, 2010), thus total metal concentrations tend to overestimate the amount of metal available for uptake by organisms (Giller *et al.*, 1998). Even if the total copper concentration is high, the bioavailable fraction may be quite low (Giller *et al.*, 1998). Neutral salt extractions, such as 0.01 *M* $CaCl_2$, are common methods used to measure the metal bioavailability in soil (Nolan *et al.*, 2005). $CaCl_2$ targets the solution phase and exchangeable heavy metal fractions in soil (Gupta *et al.*, 1996). The soil is flooded with the desorbing cation (Ca^{2+}), removing the metal from the soil surface and bringing it into solution (McLaughlin *et al.*, 2000). The Cl-ion forms moderately-strong complexes with metal ions, promoting additional desorption of the metal from the soil surface (McLaughlin *et al.*, 2000). $CaCl_2$ extractions have been used to determine the potential bioavailability of Cu to plants (Nolan *et al.*, 2005) and the potential toxicity to soil microorganisms (Merrington *et al.*, 2002).

3.1.1 Objectives

The objectives of the work in this chapter were to:

- Determine if the aged Cu in the experimental soils was bioavailable to the soil microorganisms and had adverse effects on the soil microbial community.
- Measure the total and bioavailable concentrations of copper in the experimental soils.

- Determine if there were significant relationships between copper and the measured soil biological properties.

Where appropriate, the results have been compared to a previous study of the effects of heavy metals on the soil microbial community undertaken at the same site (Speir *et al.*, 2007). In their study, heavy metal-spiked sludge was applied to soil plots to determine the toxicity threshold concentrations of heavy metals in soil. Applied concentrations of Cu ranged from 0–200 mg Cu kg^{-1} soil. Total metal, soil solution metal, total C, soil pH, soil respiration, soil microbiomass, phosphatase activity and sulphatase activity were all monitored over the six year study period. The soil plots were limed half-way through the study to increase the soil pH.

The numerical data for bioavailable Cu and the phosphatase and urease enzyme assays are presented in Appendix A.

3.2 RESULTS AND DISCUSSION

3.2.1 Soil Characterisation

The properties of the bulk soil and the aged experimental soils before pesticide spiking are summarised in Table 3.1. The pH, CEC and TOC were all measured on a dry weight basis. These soil properties remained consistent over time. The pH-values of the atrazine- and indoxacarb-spiked soils are presented in Table 3.2. The bulk soil was a sandy loam soil (60% sand, 30.7% silt and 9.3% clay) (McLaren and Cameron, 1996). The soil properties for the bulk soil are consistent with those reported by Speir *et al.* (2007) for the same site. The only significant difference for pH was between the control (Cu-1) and indoxacarb Cu-5 at t_2 (p <0.05), indicating that Cu spiking and the addition of pesticides did not alter the soil pH. Kim *et al.* (2008) also observed that metal and pesticide loading of their soil had little effect on the soil properties after aging.

Table 3.1: Soil properties of the bulk soil and the aged Cu-spiked samples prior to pesticide spiking.

Sampling time	*Sample*	*pH*	*CEC (meq. 100g^{-1})*	*%TOC*
Pre-pesticide spike	Bulk Soil	5.0	9.7	3.8
	Cu-1	4.7	10.0	3.8
	Cu-2	4.7	9.1	3.8
	Cu-3	4.7	9.2	3.8
	Cu-4	4.6	10.7	3.9
	Cu-5	4.5	9.8	4.1
pH: n=1; CEC: bulk n=3, Cu soil n=2; %TOC: n=1				

Table 3.2: Mean pH-values of atrazine and indoxacarb spiked soils at t_0, t_1 and t_2 (n=4).

Pesticide	*Sample*	*Sampling Time*		
		t_0	*t_1*	*t_2*
Atrazine	Cu-1	4.7	4.5	4.4
	Cu-2	4.7	4.6	4.5
	Cu-3	4.7	4.6	4.4
	Cu-4	4.6	4.5	4.4
	Cu-5	4.5	4.6	4.4
Indoxacarb	Cu-1	4.7	4.5	4.6
	Cu-2	4.6	4.6	4.6
	Cu-3	4.6	4.6	4.5
	Cu-4	4.5	4.5	4.4
	Cu-5	4.4	4.5	4.3

3.2.2 Total Copper

The results for total Cu concentrations in the experimental soil are presented in Table 3.3. The total Cu concentration extracted from the bulk soil was 3.74 mg kg^{-1}. The total Cu concentrations at each Cu-level were consistent across both pesticides and the three sampling times, as they were within 10% of the t_0-value for each Cu-level. This indicated that the Cu spiking procedure was successful; the soils were homogeneous and very little Cu leached from the soil during the aging process. Total Cu concentrations greater than 100% of the initial spike rate were due to the amount of Cu originally present in the bulk soil and the addition of 8% more $CuSO_4$ during spiking to account for the volume of the spiking solution remaining in the hand-held pump sprayer.

Table 3.3: Total Cu concentrations (mg kg^{-1}) for aged atrazine- and indoxacarb-spiked soils (n=4).

Cu-level	*Spike rate (mg kg^{-1})*	*Atrazine samples*			*Indoxacarb samples*		
		t_0	t_1	t_2	t_0	t_1	t_2
Cu-1	0	4.09	4.50	4.54	4.54	5.10	4.75
Cu-2	100	86.8	86.1	87.9	84.2	90.0	89.9
Cu-3	250	254	259	254	245	255	257
Cu-4	500	538	545	566	540	556	563
Cu-5	1000	986	1060	1030	1010	1060	1020

The bulk soil and pesticide-spiked soils at t_0 were analysed for total As, Cd, Pb and Zn concentrations by ICP-MS, to determine if these metal concentrations were altered in the experimental soil during Cu and pesticide spiking (Table 3.4). The metal concentrations in the pesticide-spiked experimental soils were consistent with the bulk soil indicating that the Cu and pesticide spike solutions did not alter the concentrations of these metals in the experimental soils. The samples were also analysed for Cu by ICP-MS (Table 3.4). The Cu concentrations measured by ICP-MS were generally within 10% of the FAAS concentrations.

Table 3.4: Total metal concentrations (mg kg^{-1}) in the bulk soil and pesticide-spiked experimental soil at t_0, analysed by ICP-MS (bulk: n=2; pesticide-spiked soils: n=1).

Sample	*Total Cu*	*Total As*	*Total Cd*	*Total Pb*	*Total Zn*
Bulk Soil	4.31	3.09	0.17	11.7	53.2
ATZ Cu-1	4.57	2.65	0.16	10.7	48.1
ATZ Cu-2	85.3	2.68	0.16	11.4	48.7
ATZ Cu-3	238	3.46	0.19	14.2	55.3
ATZ Cu-4	500	3.23	0.17	13.7	52.2
ATZ Cu-5	949	2.77	0.16	12.3	55.5
IND Cu-1	4.47	2.93	0.15	11.0	46.4
IND Cu-2	83.4	2.94	0.16	12.0	51.2
IND Cu-3	233	3.11	0.18	13.1	54.0
IND Cu-4	531	2.84	0.19	12.6	51.7
IND Cu-5	1052	3.26	0.16	14.1	52.8

3.2.3 Bioavailable Copper

Bioavailable Cu concentrations ranged from 0.01–20.1 μg g^{-1} (Figure 3.1). The % bioavailable Cu ranged from 0.37–1.97% (Figure 3.2) and was positively correlated with total Cu concentrations (p<0.01). This relationship indicates that as the Cu spike increased the concentration of Cu in the labile pool increased. Speir *et al.* (2007) reported a similar positive relationship between bioavailable and total Cu concentrations for their study. Studies in the United States (Kim *et al.*, 2008), Germany (Wilke *et al.*, 2005) and China (Wang *et al.*, 2009) have also observed significant relationships between the bioavailable metal fraction and total metal concentration.

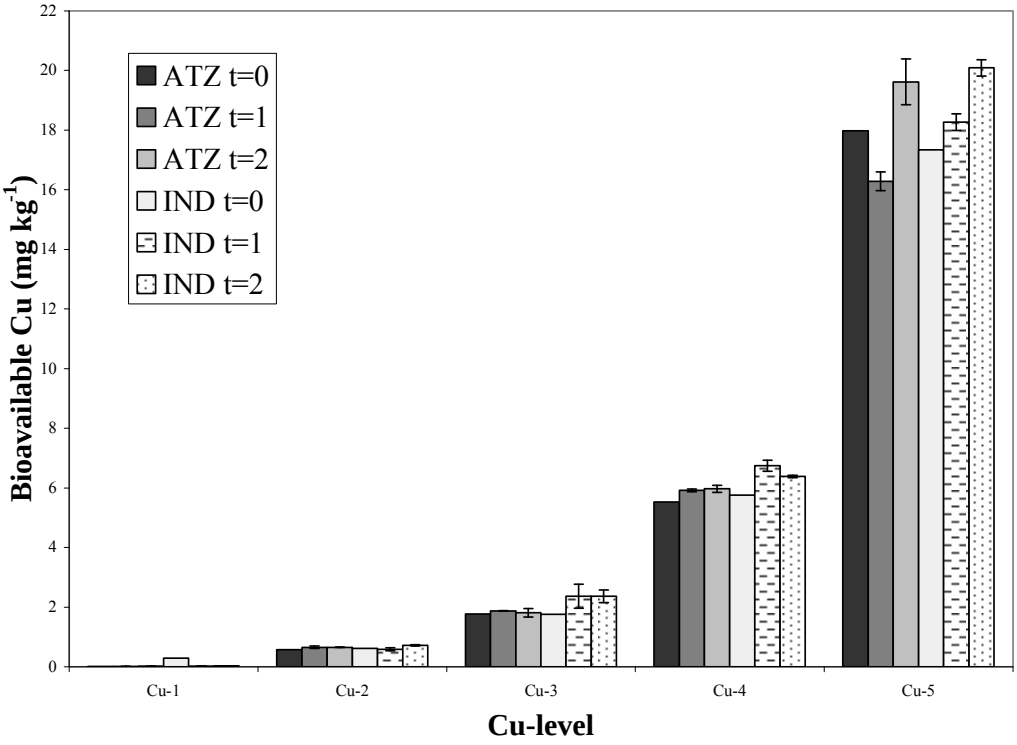

Figure 3.1: Mean bioavailable Cu concentrations extracted (mg kg^{-1}) from the experimental soils. Error bars represent the standard error (n=4).

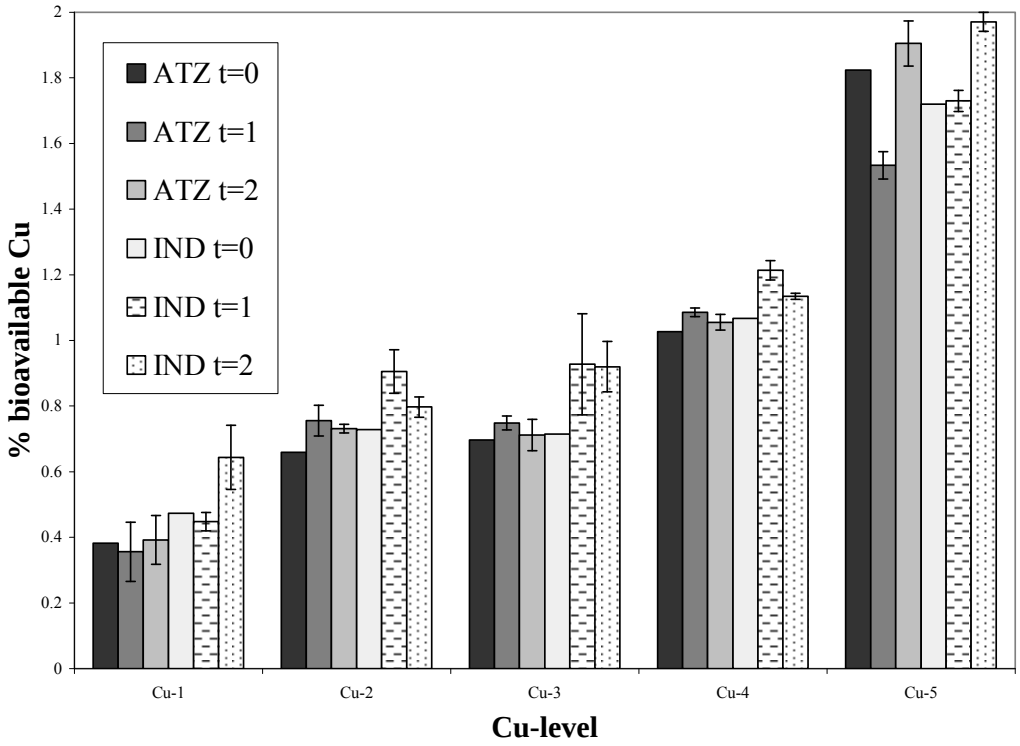

Figure 3.2: The % bioavailable Cu extracted by 0.01 *M* $CaCl_2$. Error bars represent the standard error (n=4).

The Cu-spiked experimental soils were aged for a period of 6 months to simulate field-aging prior to pesticide application. During the aging period, the applied Cu equilibrated between the solid and solution phases, as the bioavailable Cu concentrations are similar across the sampling times at each Cu-level (Figure 3.1). Ma *et al.* (2006a) observed that for European soils with pH <6.0, approximately 40% of applied Cu was removed from the labile pool by 30 d. It is reasonable assume that a similar equilibration process occurred in the Templeton experimental soil as the pH was ≤5.0 and the experimental soils were aged for 6 months. From their study, Ma *et al.* (2006b) concluded that this initial partitioning period was followed by a slow continuous reaction of soluble Cu, reducing metal bioavailability. The slow partitioning was not observed in the experimental soil after aging, as the bioavailable concentrations were consistent over the pesticide degradation studies.

3.2.4 Microbiomass

The soil microbiomass was only measured at t_1 for the pesticide-spiked soils due to budget constraints. The microbiomass ranged from 235–415 µg C g^{-1} soil for the atrazine-spiked soils and 359–645 µg C g^{-1} soil for the indoxacarb-spiked soils (Table 3.5, Figure 3.3). The microbiomass for Cu-3, Cu-4 and Cu-5 were significantly less than the control (Cu-1; $p<0.05$) for both pesticides. Cu-1 and Cu-2 had similar microbiomass for both pesticides, suggesting that the presence of Cu at concentrations greater than 100 mg kg^{-1} in the experimental soil had a negative effect on the soil microorganisms. For both pesticides, there was a significant negative correlation between microbiomass and total Cu concentration ($p<0.05$). The microbiomass of the atrazine-spiked soils was also negatively correlated with bioavailable Cu ($p<0.05$). The corresponding decrease in the C_{mic}:C_{org} ratio with increasing Cu concentration was expected as the %TOC remained constant (Table 3.5). The decrease in the C_{mic}:C_{org} ratio indicates a change in the soil microbial community structure occurred in the presence of elevated Cu concentrations (Merrington *et al.*, 2002).

Table 3.5: %TOC and microbiomass of atrazine and indoxacarb samples at t_1 (n=4).

Sample	*%TOC*	*Microbiomass (µg C g^{-1} soil)*	*C_{mic}:C_{org} (%)*
ATZ Cu-1	3.8	397.2	10.5
ATZ Cu-2	3.9	414.5	10.6
ATZ Cu-3	3.8	340.9	9.0
ATZ Cu-4	3.9	271.5	7.0
ATZ Cu-5	4.0	235.2	5.9
IND Cu-1	4.0	644.7	16.1
IND Cu-2	4.0	548.1	13.7
IND Cu-3	4.0	496.9	12.4
IND Cu-4	4.0	404.0	10.1
IND Cu-5	4.0	359.1	9.0

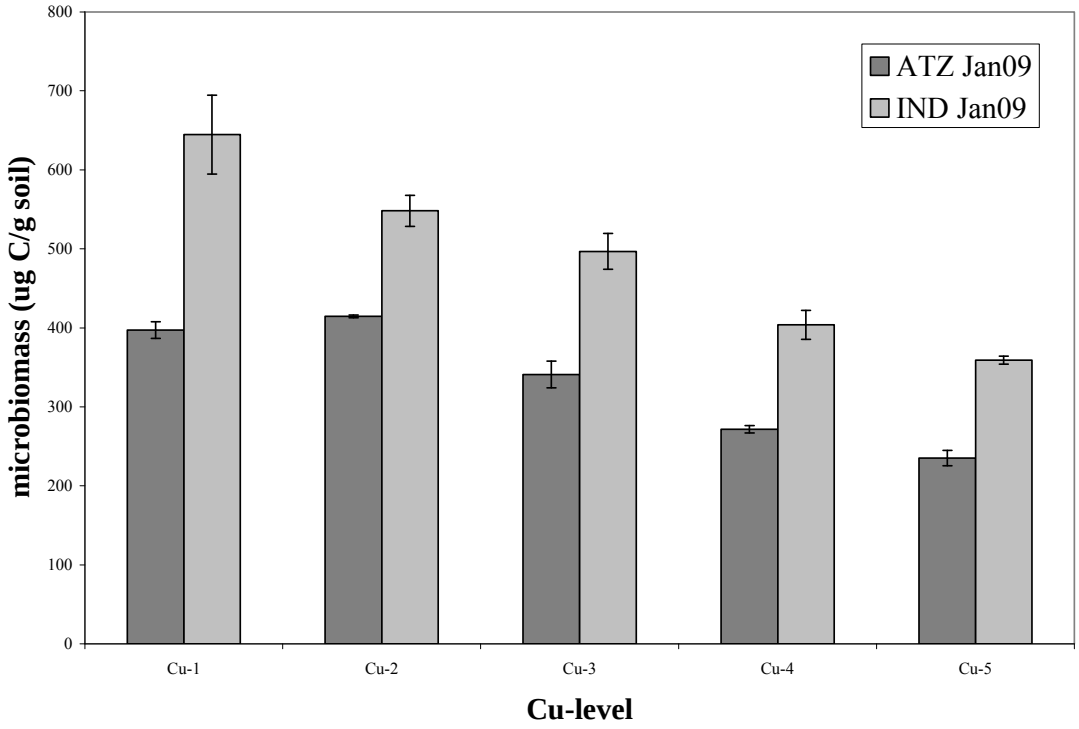

Figure 3.3: Microbiomass concentration of atrazine and indoxacarb samples at t_1. Error bars represent the standard error (n=4).

The microbiomass for the indoxacarb-spiked soils were greater than the atrazine-spiked soils. The difference between the two pesticides may be due to different sampling times and the nature of the pesticides (the atrazine-spiked soils were sampled before the indoxacarb-spiked soils at t_1). The addition of solvents to soil can result in an initial decline in total microorganism numbers due to toxic effects, followed by a re-growth of the microorganism populations (Brinch *et al.*, 2002). The population re-growth can result in larger microorganism numbers than in control soils (Brinch *et al.*, 2002). The indoxacarb-spiked soils had a greater estimated half-life and were sampled after the atrazine-spiked soils. Therefore, the indoxacarb-spiked soils had more time (20 additional days) for the microorganism populations to recover from the spiking procedure.

Atrazine is an herbicide, and its long-term use can reduce the amount of organic matter that reaches the soil, altering microbial activity (Voets *et al.*, 1974). Both pesticide-spiked soils, however, were weeded regularly, so both would have received minimum additions of organic matter. In previous studies, microbiomass were not found to be modified by low atrazine

treatments (Mahia *et al.*, 2008), and in some cases, the microbiomass was enhanced (Moreno *et al.*, 2007). As atrazine was applied to the atrazine-spiked experimental soils at the same rate (2 mg kg^{-1}), the decrease in microbiomass of the atrazine-spiked soils was most likely due to the increasing Cu concentrations. There is no published data on the effects of indoxacarb on soil microbiomass concentrations. It is probable that the difference in microbiomass between atrazine- and indoxacarb-spiked soils is due to the time elapsed between pesticide spiking and sampling, and the recovery of the microbial populations.

The decrease in microbiomass of the spiked experimental soils with increasing Cu concentration is consistent with previous studies. Knight *et al.* (1997) observed a significant reduction in microbiomass for an aged soil with a total Cu concentration of 150 mg kg^{-1} at pH 4.5 when compared to a control plot at the same pH. Merrington *et al.* (2002) reported significant decreases in microbiomass in soils from an Australian orchard with total Cu concentrations ranging from 280–345 mg kg^{-1} when compared to the control sites (total Cu 13–14 mg kg^{-1}). Similarly, Wang *et al.* (2009) observed that the microbiomass negatively correlated with bioavailable Cu ($p<0.01$) in aged Cu-contaminated horticultural soils. They also observed a significant decrease in microbiomass at 103 mg kg^{-1} Cu, which is consistent with the observations in the Templeton experimental soil.

3.2.5 Phosphatase Activity

The phosphatase activities ranged from 2.85–6.14 nmol g^{-1} s^{-1} at t_1 and 2.40–5.21 nmol g^{-1} s^{-1} at t_2 in the experimental soil (Figure 3.4). In general, the phosphatase activity was lower at t_2 than t_1 for each pesticide, with the exception of Cu-4 where the activity was similar across both pesticides and both sampling times. The phosphatase activities of the indoxacarb-spiked soils were also generally lower than those of the atrazine-spiked soils. As with the microbiomass, this difference in activity may be due to the time elapsed between pesticide spiking and sampling, and the recovery of the microorganisms.

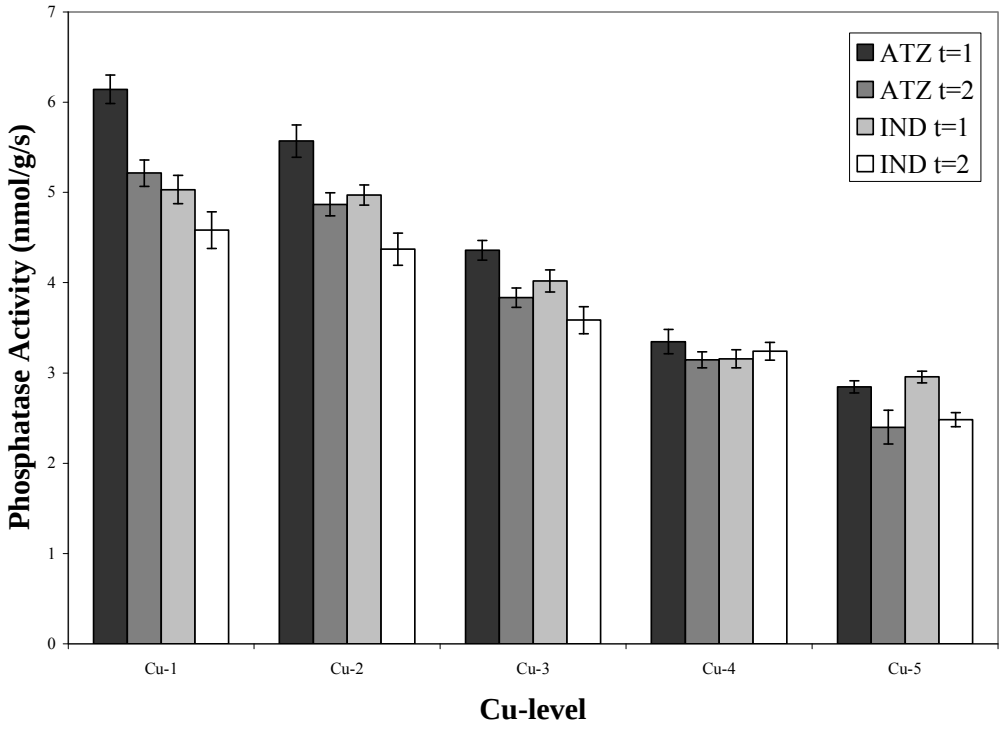

Figure 3.4: Mean phosphatase activity (nmol g^{-1} s^{-1}) for atrazine and indoxacarb soils at t_1 and t_2. Error bars represent the standard error (n=4).

The phosphatase activity decreased as the Cu-concentration increased ($p<0.05$, Figure 3.4). There was only a significant relationship between bioavailable Cu and phosphatase activity for indoxacarb at t_2 ($p<0.05$, Table 3.6). The phosphatase activity was significantly lower than the control (Cu-1) at all Cu-levels for atrazine-spiked soils, and at Cu-3, Cu-4 and Cu-5 for indoxacarb-spiked soils ($p<0.05$) at both t_1 and t_2. These significant differences in enzyme activities between the Cu-levels indicated that elevated total Cu concentrations in the soil had negative effects on the soil microorganisms.

Table 3.6: Pearson's correlation coefficients for relationships between total and 0.01 *M* $CaCl_2$ extractable Cu, and phosphatase activity at t_1 and t_2 for atrazine and indoxacarb.

Sampling time	*Total extractable Cu*		*0.01 M $CaCl_2$ extractable Cu*	
	Atrazine	*Indoxacarb*	*Atrazine*	*Indoxacarb*
t_1	-0.924*	-0.924*	NS	NS
t_2	-0.962**	-0.973**	NS	-0.907*
*$p<0.05$; **$p<0.01$; NS = not significant				

The phosphatase activities in the Templeton experimental soil were of the same order of magnitude as the biosolids study by Speir *et al.* (2007) using soil from the same site. The same inverse relationship between phosphatase and total Cu concentrations (0–200 mg kg^{-1}) was reported (Speir *et al.*, 2007). The negative relationship between phosphatase activity and Cu concentration is consistent with previous studies. Spier *et al.* (1999) reported that phosphatase activity was inhibited by the addition of metal salts, including Cu. Similar negative relationships have also been reported in international soils: in two Chinese soils, one contaminated by a local Cu smelter (Wang *et al.*, 2007) and the other a horticultural soil contaminated by long-term application of a copper fungicide (Wang *et al.*, 2009); and in a Swedish soil contaminated by Cu and Zn from a local brass foundry (Tyler, 1974). Wang *et al.* (2007) observed significant decreases in phosphatase activity at a total Cu concentration of 245 mg kg^{-1}. However, the lowest Cu concentration in their study was greater than the lowest Cu-spike in the Templeton experimental soil (100 mg kg^{-1}), so it is unclear if a significant decrease would be observed at 100 mg kg^{-1} compared to natural background levels in their soil.

3.2.6 Urease Activity

The urease activities ranged from 0.31–0.71 nmol g^{-1} s^{-1} at t_0, 0.31–0.66 nmol g^{-1} s^{-1} at t_1 and 0.21–0.57 nmol g^{-1} s^{-1} at t_2 in the experimental soil. As with the phosphatase activities, the urease activities decreased as the Cu-concentration was increased for each sampling period (Figure 3.5). The urease activity was lower for t_2 than t_1 for both atrazine- and indoxacarb-spiked soils. However the activity increased or remained the same between t_0 and t_1, with the exception of atrazine Cu-1 soils. The urease activities for the aged Cu soils were comparable with both pesticide-spiked soils at t_0, indicating that neither of the pesticides had an acute toxic effect on the urease enzyme activity. Even though the pesticides were spiked into the soils using acetone, only one-quarter of the soil was spiked and the acetone was allowed to evaporate before blending (Chapter 2, Section 2.3.3). Studies show that spiking a fraction of the soil with acetone minimises negative effects on the soil microorganisms (Brinch *et al.*, 2002), as was seen in the experimental soil.

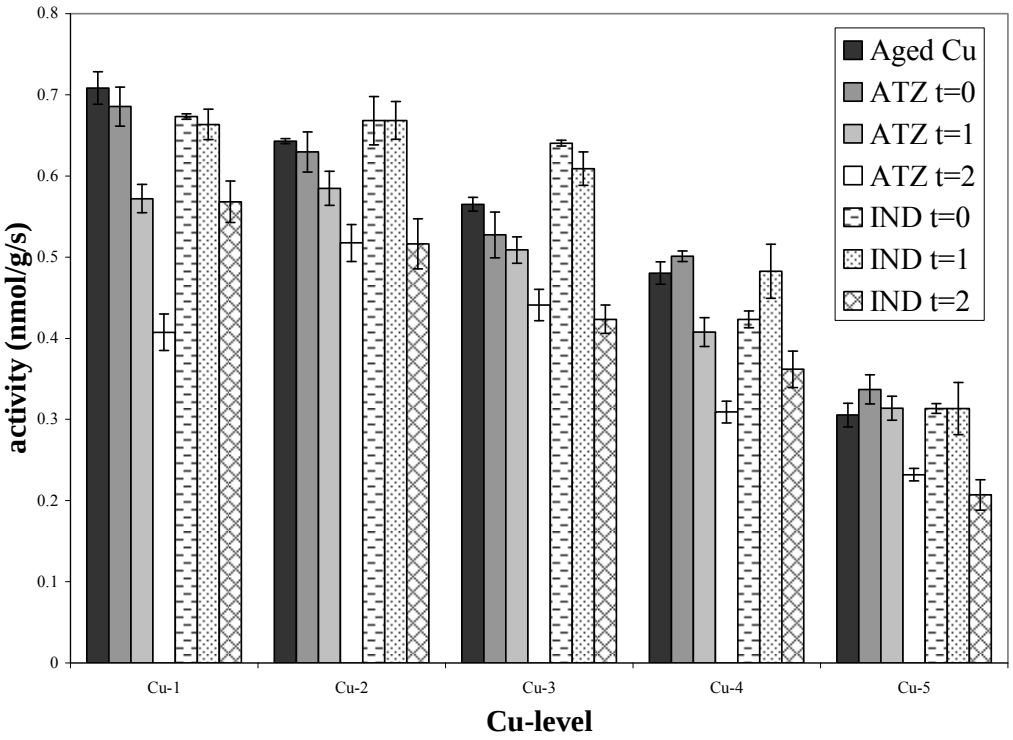

Figure 3.5: Urease activity for aged Cu soils, and atrazine and indoxacarb soils at t_0, t_1 and t_2. Error bars represent the standard error (n=4).

The urease activity in the aged Cu-spiked soils were all significantly lower than the control (Cu-1; $p<0.05$). Many of the urease activities in the soils at the Cu-2 and Cu-3 spike levels were not significantly different than the controls (Cu-1). Only the atrazine-spiked Cu-2 (t_2) and Cu-3 (t_0) soils, and the indoxacarb-spiked Cu-3 (t_2) soil had significant differences to the control (Cu-1; $p<0.05$). In addition, the activities of the pesticide-spiked Cu-4 and Cu-5 soils were significantly lower than that of the control (Cu-1; $p<0.05$). Although there were overall significant negative relationships between urease activity and both total and bioavailable Cu concentrations ($p<0.05$; Table 3.7), the lower Cu spike levels (100 mg kg^{-1} and 250 mg kg^{-1}) were not significantly different to the control. Comparable negative relationships between urease activity and both total and bioavailable metal concentrations in soil have been reported previously (Chaperon and Sauve, 2008; Kim *et al.*, 2008).

Table 3.7: Pearson's correlation coefficients for relationships between the urease activities and both the total and 0.01 *M* $CaCl_2$ extractable Cu concentrations in the experimental soil.

Sampling time	*Total extractable Cu*		*0.01 M $CaCl_2$ extractable Cu*	
	Atrazine	*Indoxacarb*	*Atrazine*	*Indoxacarb*
t_0	-0.976**	-0.971**	-0.951*	-0.933*
t_1	-0.983**	-0.996***	-0.948*	-0.986**
t_2	-0.893*	-0.988**	NS	-0.945*
*p<0.05; **p<0.01; ***p<0.001; NS = not significant				

3.2.7 Relationships between Copper and Soil Biological Properties

As discussed in Chapter 1, the bioavailable fraction of Cu should decrease as the soil ages, reducing its toxic effects on soil microorganisms (Ma *et al.*, 2006a). Kim *et al.* (2008) observed that the urease activity of soils freshly spiked with 200 mg kg^{-1} Cu was lower than in aged soils amended with the same total Cu concentration. This indicated that as metal-contaminated soils age, they become less toxic to the soil microorganisms. If the Templeton experimental soils were still in the process of aging, the enzyme activities would be expected to increase as the bioavailable Cu fraction became less available. The bioavailable Cu concentrations remained relatively constant over the sampling period in the experimental soil, thus the enzyme activities would be expected to remain constant as well. However, the phosphatase and urease activities in the experimental soils both continued to decrease over the sampling period. A possible explanation for this continued decrease over time could be due to a lack of available food/substrates for the soil microorganisms. Since the experimental soils were regularly weeded during the aging and sampling periods, very little organic matter would have been added to the soil over the duration of the investigation.

Time effects were not observed in the microbiomass, because only one sampling time was analysed (t_1). However, the microbiomass strongly correlated with phosphatase and urease activities at t_1 (p<0.05). It is probable, then, that the microbiomass would continue to decrease over time, as with the phosphatase and urease activities, due to a lack of organic matter added to the experimental soil.

It was observed in many of the experimental soils that the soil biological properties at Cu-2 were not significantly lower than Cu-1 (the control): the phosphatase activities for

indoxacarb-spiked soils at t_1 and t_2; the urease assay for atrazine-spike soils at all sampling times and indoxacarb-spiked soils at t_1 and t_2; and the microbiomass concentrations for both pesticide-spiked soils at t_1. The Cu concentration of the soils at the Cu-2 level ranged between 84.2–90.0 mg kg $^{-1}$ (Table 3.3), which is close to the New Zealand biosolids guideline value for Cu (100 mg kg^{-1}) (NZWWA, 2003). This guideline is protective of plant, human and soil microorganism health (NZWWA, 2003). It would be expected that the soil biological properties would not exhibit negative effects due to Cu at this spike level, as was observed in the experimental soils.

3.3 CONCLUSIONS

The copper applied to the soils remained consistent over time, indicating that the soils were homogeneous and very little Cu leached over the aging period. The total and bioavailable Cu concentrations were positively correlated in the experimental soil ($p<0.01$). The pH, CEC and %TOC remained consistent for the experimental soils over time, indicating that Cu- and pesticide-spiking did not affect these soil properties.

Copper negatively impacted the soil microorganisms. Significant decreases in phosphatase and urease enzyme activities, and microbiomass were observed as the Cu concentration increased ($p<0.05$). The decrease in the C_{mic}:C_{org} ratio also indicates a change in the soil microbial community structure occurred in the presence of elevated Cu concentrations. Overall, total Cu was a better indicator of enzyme activities and microbiomass than bioavailable Cu in the Templeton experimental soil.

Chapter 4: The Effects of Copper on the Degradation of Atrazine and Indoxacarb

4.1 INTRODUCTION

This chapter contains the results for the atrazine and indoxacarb degradation studies and the effects of copper on their degradation in the Templeton experimental soil. It includes an introduction summarising soil degradation mechanisms for atrazine and indoxacarb, followed by method development and results and discussion sections.

4.1.1 Atrazine

Biotic and abiotic degradation are the two main environmental degradation processes for atrazine (Hartenbach *et al.*, 2008; Mandelbaum *et al.*, 1993; Meyer *et al.*, 2009; Sarmah *et al.*, 2009). The mechanisms of these two processes have been extensively investigated. Both biotic and abiotic processes lead to the same degradation products through de-halogenation of the triazine ring and de-amination of the side-chains (Figure 4.1). DEA, DIA, HA, DEHA and DIHA are the first degradation products of atrazine. The formation of cyanuric acid follows and degradation is completed with mineralization to NH_3 and CO_2. The degradation of atrazine to HA in the environment is important as this metabolite does not retain herbicidal activity (Mandelbaum, 1993).

Abiotic degradation can occur by direct or indirect photolysis (Hartenbach *et al.*, 2008), as well as by chemical hydrolysis (Behki and Khan, 1986; Mandelbaum *et al.*, 1993; Wackett *et al.*, 2002). As light penetrates the soil surface to a maximum depth of 1 cm (depending on soil conditions), photodegradation of atrazine can only occur in the very top surface layer of the soil (Sarmah *et al.*, 2004). Therefore, microorganisms largely control the fate of atrazine in soil (van der Meer, 2006; Wackett *et al.*, 2002). As microorganisms are sensitive to heavy metal contamination, it is plausible that co-contamination with metals could inhibit atrazine degradation in soil (Gaw *et al.*, 2003; Sandrin and Maier, 2003).

Prior to 1993, N-dealkylation of atrazine was thought to be the major biodegradation pathway in soils, because DEA and DIA were routinely detected in treated soils (Wackett *et al.*, 2002). Previous studies suggested that DEA and DIA formation is solely due to the biodegradation of atrazine (Behki and Khan, 1986; Meyer *et al.*, 2009; Wackett *et al.*, 2002). De-chlorination of atrazine to the hydroxy-metabolites was believed to result from either chemical processes in the soil or photolysis. However, in the 1990's, atrazine de-chlorinating bacteria were independently isolated in a number of laboratories (Mandelbaum *et al.*, 1995; Topp *et al.*, 2000; Wackett *et al.*, 2002), all of which predominantly produced HA through hydrolytic de-chlorination reactions. Topp *et al.* (2000) isolated an atrazine degrading bacterial species, *Nocardioides* sp. from a Canadian soil. HA was detected in the soil containing this species, whereas DEA and DIA were not. They concluded that atrazine was initially degraded by a chlorohydrolase mechanism to form HA. The rate of microbial degradation of atrazine is faster than for chemical hydrolysis. The second order rate constant for the enzyme atz-A (atrazine chlorohydrolase), which catalyses the microbial hydrolysis of atrazine to HA, is 10^{10} fold greater than the rate constant for chemical hydrolysis (Wackett *et al.*, 2002). This indicates that only a few microorganisms containing the atz-A enzyme are necessary to catalyse the hydrolysis of atrazine to HA in soil. Further evidence that HA is formed biologically was reported by de Souza *et al.* (1998), with the identification of the atz-ABC enzymes in isolated atrazine de-chlorinating bacteria.

The microbial enzymes responsible for catalysing the hydrolysis of atrazine to cyanuric acid (through an HA step) have been identified as atz-A, -B and -C (de Souza *et al.*, 1998; Seffernick *et al.*, 2002). The enzymes trz-A, tri-A, trz-C and trz-N are alternative enzymes involved in the hydrolysis reactions of atrazine to cyanuric acid (Seffernick *et al.*, 2002). Figure 4.2 shows the steps at which the atz-A, -B and -C enzymes catalyse the degradation reactions of atrazine to cyanuric acid. Recently, Meyer *et al.* (2009) have been able to determine the biotic hydrolysis mechanism of atrazine in soil. From their study of stable C and N isotopic fractionation patterns of the Atz-A and Trz-N enzymes, they determined that the biodegradation of atrazine undergoes acidic hydrolysis as it is converted to HA (Figure 4.3). The slopes of dual isotope plots ($\delta^{13}C$:$\delta^{15}N$) are characteristic of a particular chemical reaction and can be used to help differentiate between biotic and abiotic degradation mechanisms of atrazine in the soil.

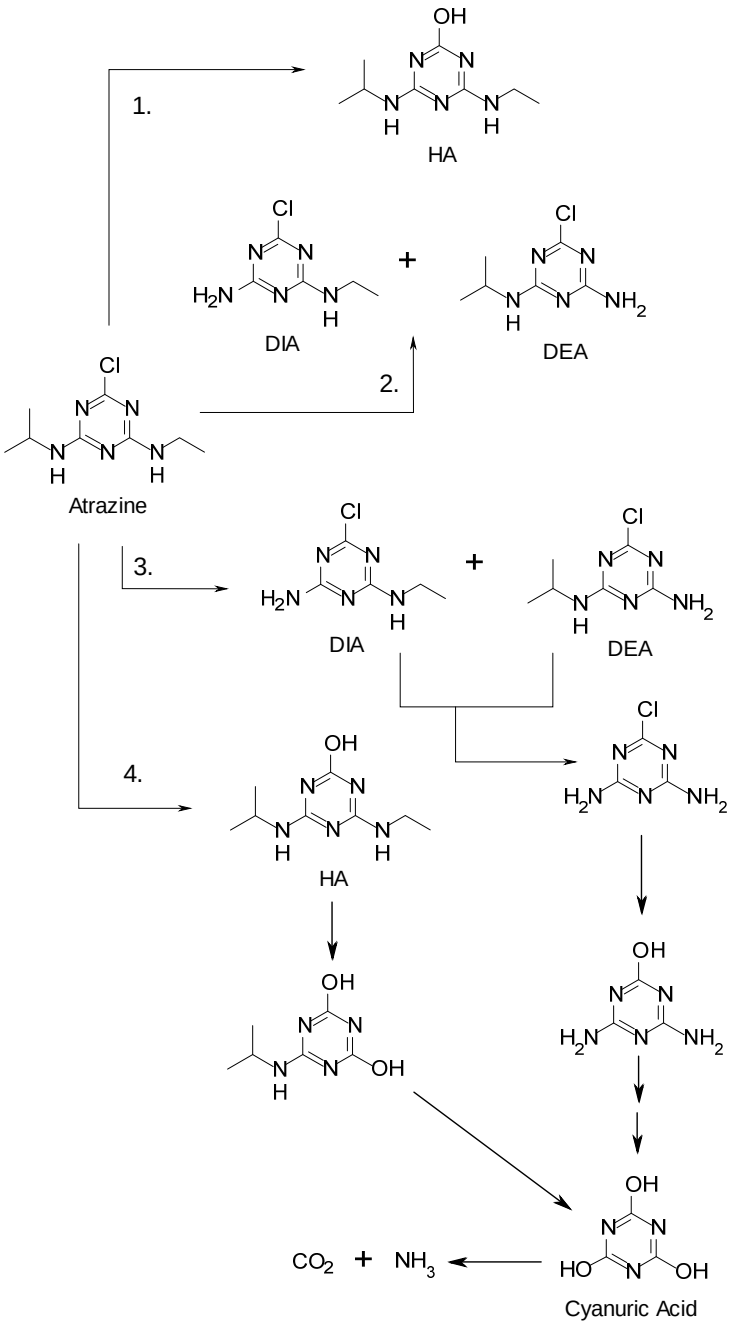

Figure 4.1: Mechanisms of atrazine degradation by: 1) direct photodegradation; 2) indirect photodegradation; 3) microbes without hydrolysis catalysing enzyme; and 4) microbes with hydrolysis catalysing enzyme (adapted from Hartenbach *et al.*, 2008; Wackett *et al.*, 2002). All pathways ultimately produce cyanuric acid that is metabolised to CO_2 and NH_3.

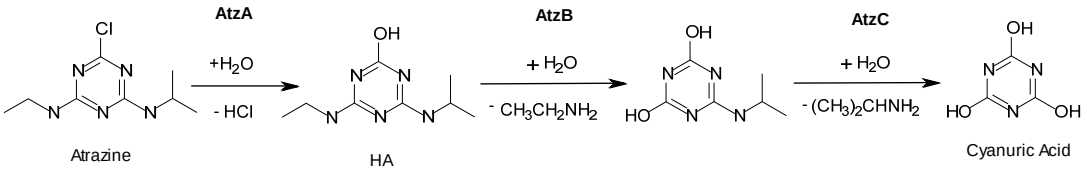

Figure 4.2: Catabolic pathway for degradation of atrazine to cyanuric acid via the Atz-A, Atz-B and Atz-C enzymes (re-drawn from de Souza *et al.*, 1998; Seffernick *et al.*, 2002).

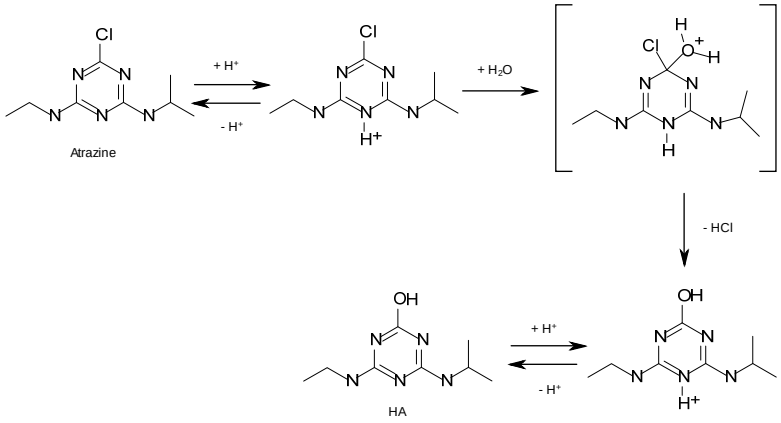

Figure 4.3: Biodegradation of atrazine to HA by the acidic hydrolysis mechanism (redrawn from Meyer *et al.*, 2009).

4.1.2 Indoxacarb

There was limited published data on the degradation of indoxacarb in the environment, as it was only registered for use in 2000 (Dias, 2006). Indoxacarb can be degraded biotically in the soil, or abiotically in solution (Du Pont, 2006). Photolysis occurs rapidly for indoxacarb in solution. The biodegradation of indoxacarb in soil can follow three major pathways: 1) ester hydrolysis (IN-KT413); 2) cleavage of the N-carboxylate ester (IN-JT333); or 3) oxadiazine ring cleavage (IN-KG433) (Du Pont, 2006) (Figure 4.4). These three primary degradation products are further degraded until indoxacarb has been mineralised to CO_2 and bound residues (Dias, 2006).

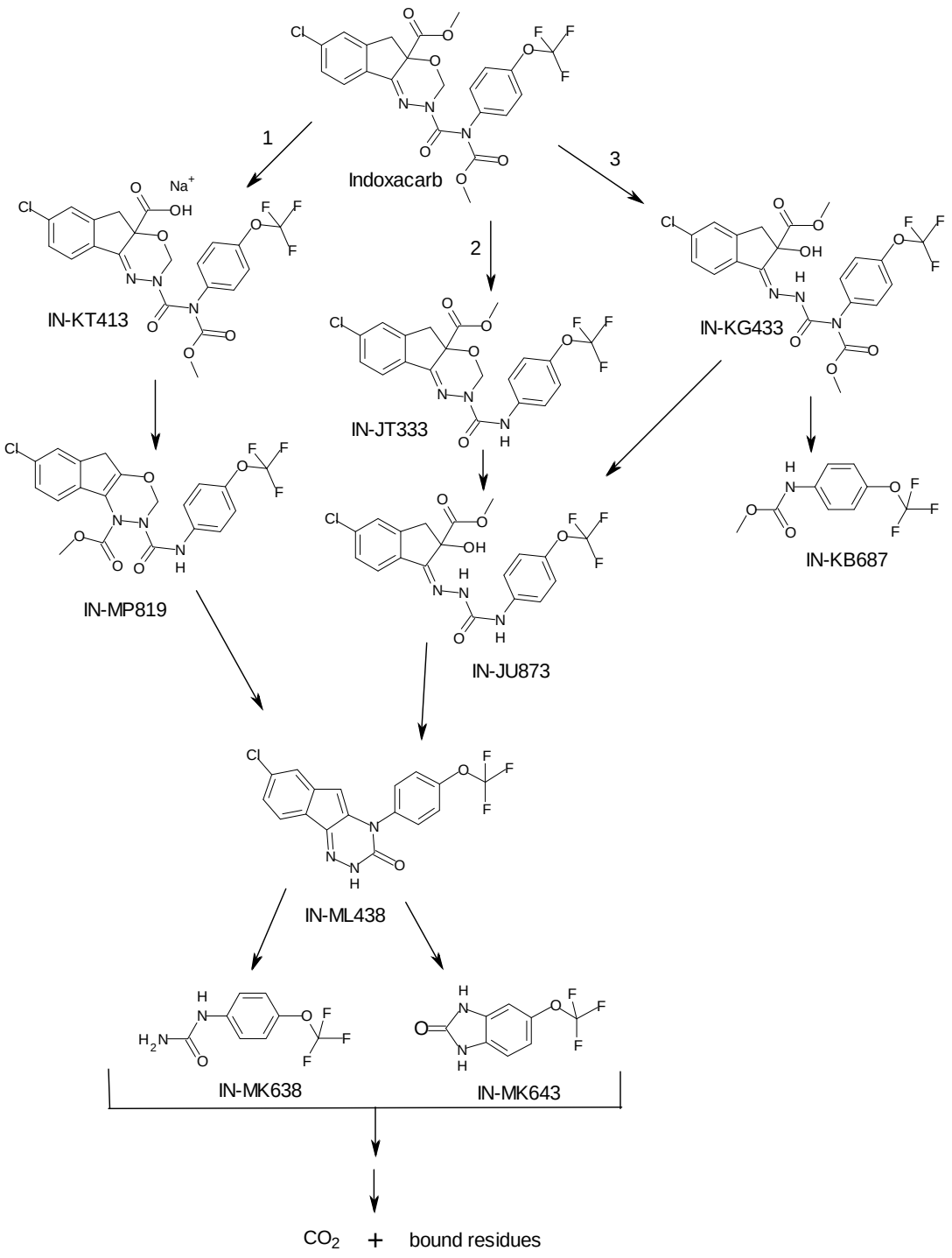

Figure 4.4: Proposed degradation routes of indoxacarb in aerobic soils (redrawn from Dias, 2006). CAS names for indoxacarb metabolites are presented in Table 4.1.

Table 4.1: CAS names corresponding to indoxacarb metabolite codes (from Dias, 2006).

Code	*CAS name*
IN-JT3333	methyl 7-chloro-2,5-dihydro-2-[[[4-(trifluoromethoxy)phenyl]amino]carbonyl] indeno[1,2-*e*][1,3,4]oxadiazine-4a(3*H*)-carboxylate
IN-KG433	methyl 5-chloro-2,3-dihydro-2-hydroxy-1-[[[(methoxycarbonyl)[4-(trifluoromethoxy) phenyl]amino]carbonyl]hydrazono]-1H-indene-2-carboxylate
IN-KT413	sodium 7-cholor-2,5,-dihydro-2-[[methoxycarbonyl)[4(trifluoromethoxy)phenyl] amino]carbonyl]indeno[1,2-e][1,3,4]oxadiazine-4a(3H)-carboxylic acid
IN-MP819	N/A
IN-JU873	methyl 5-chloro-2,3-dihydro-2-hydroxy-1-[[[[4-trifluoromethoxy)phenyl]amino] carbonyl]hydrazono]-1H-indene-2-carboxylate
IN-ML438	7-chloro-2,4-dihydro-4-[4(trifluoromethoxy)phenyl]3H-indeno[2,1-e]-1,2,4-triazin-3-one
IN-KB687	methyl [4-(trifluoromethoxy)phenyl]carbamate
IN-MK643	1,3-dihydro-5-(trifluoromethoxy)-2Hbenzimidazol-2-one
IN-MK638	[4-(trifluoromethoxy)phenyl]urea

4.1.3 Objectives

The main objectives of the pesticide degradation studies were to:

- Develop analytical methods to extract pesticide residues from the experimental soil.
- Determine if there was a relationship between the copper spike concentration and the degradation of the pesticides in the experimental soil.

4.2 METHOD DEVELOPMENT FOR PESTICIDE ANALYSES

4.2.1 Atrazine

This section outlines the modifications to the method published by Lerch and Li (2001) that were incorporated into the final method for extracting and analysing atrazine and its metabolites. The final method for extracting atrazine and metabolites is presented in Chapter 2 (Section 2.8.2). Recovery data for target pesticide compounds from the soil matrix were obtained by spiking blank soil samples (Cu-1 Nov08) with pesticide working standards (PWS). Recoveries for spiked solution and sample blanks were used to determine the final extraction mixture, evaporation instrument, wash solution, and validate the recovery of atrazine and metabolites.

4.2.1.1 Sample and cartridge sizes

The method published by Lerch and Li (2001) was modified to suit the equipment available in the lab and to minimise the quantities of reagents, solvents and other materials used. The quantity of soil was reduced from 25 g to 10 g because of the amount of sample available for analysis. Smaller SAX cartridges were used for clean-up. However, atrazine and metabolites were not retained by the SAX cartridge and were found in the breakthrough solution, so a smaller cartridge size was not a concern. The SCX cartridges had the same capacity as in Lerch and Li (2001) and the same volume was used at the elution step. Therefore, even though the initial clean-up was modified, the critical elution step remained unchanged.

4.2.1.2 Extraction mixture study

Three different extraction mixtures were investigated to identify the most suitable extractant to optimise the recovery of atrazine and metabolites and reduce soil co-extractant interferences. The first mixture investigated was 0.5 *M* KH_2PO_4 (pH 7.5)/ACN (3:1, v/v). This mixture had previously been reported for the extraction of hydroxyatrazine degradation products (HADPs) from soil incubation studies (Lerch and Li, 2001). A number of other published methods have employed MeOH mixtures to extract atrazine and metabolites with good recoveries (Juracek and Thurman, 1997; Lerch *et al.*, 1999; Mills and Thurman, 1992; Schewes *et al.*, 1993). Therefore a 0.5 *M* KH_2PO_4 (pH 7.5)/MeOH mixture (3:1, v/v) was also evaluated. The final solution evaluated in this assessment was an isopropanol (IPA)/H_2O mixture (80:20, v/v). The mixtures were heated to 60°C prior to extraction as the extractability of HADPs is reported to increase with increasing temperature (Lerch and Li, 2001). At higher temperatures, there is the possibly of atrazine hydrolysis (Lerch and Li, 2001), so 60ºC was selected as the temperature for these tests since this is below the boiling point of MeOH (64.7ºC).

Blank soil samples (equivalent to 10 g DW, 1 per extraction mixture) were extracted twice in Teflon centrifuge tubes with 20 mL of the extraction mixtures by sonication at 50°C for 30 min, followed by orbital shaking at room temperature (22°C) for 30 min and centrifuging for 30 min at 4000 rpm and 10°C. The cool temperature was reported to harden the Teflon tubes to prevent tube deformation and spillage of the sample extracts (Lerch and Li, 2001). In later extractions, the centrifugation step was performed at room temperature. The robust

Teflon centrifuge tubes used had already cooled to room temperature during orbital shaking and did not require further cooling. Sonication occurred at 50ºC because it was easier to maintain the water bath at this temperature. The combined supernatants were collected into 50 mL graduated test tubes and the final volumes were recorded. Total volumes ranged from 36–39 mL. The solutions were left to stand overnight after thorough mixing, because there was not enough time to complete the extraction and clean-up in one day.

Ten mL of the buffer/ACN and buffer/MeOH soil extracts were transferred to 10 mL graduated Kimax tubes and the organic solvent was evaporated under N_2 and mild heat (<30ºC) on a Pierce ReactiTherm heating block and nitrogen blow down unit. Ten mL of the IPA extract was transferred into a 250 mL Schott Bottle and made up to 200 mL with KH_2PO_4 (pH 2.5), in order to dilute the IPA to a concentration that could be run on the SAX cartridge (4% organic solvent). Samples were cleaned-up by passage through SAX cartridges, conditioned as described in Chapter 2 (Section 2.8.2.2), and the breakthrough solutions were collected and acidified to pH 2.5 with concentrated H_3PO_4. The acidified breakthrough solutions were passed through the conditioned SCX cartridges (24 mL MeOH, 24 mL Milli-Q water and 24 mL 0.05 *M* KH_2PO_4 (pH 2.5)) at a rate of 3–5 mL min^{-1}. Atrazine and metabolites were recovered into graduated conical tubes by elution with 10 mL of $MeOH/H_2O/NH_4OH$ (8:1:1, v/v/v) at a flow rate of 1 mL min^{-1}. A milky-coloured precipitate, assumed to be ammonium phosphate, was observed in all sample eluents. Eluents were stored at 4°C overnight and were dried to almost dry under N_2 at 40–50°C the following morning. The persistence of the precipitate prevented the samples from evaporating to dryness. To overcome this, two mL of MeOH was added and the vials were vortexed, and evaporated under N_2 again, to no avail. The samples evaporated to dryness only after addition of 1 mL toluene and 2 mL ethanol (EtOH), though the precipitate remained. One full day was needed to evaporate samples to dryness under N_2.

The samples were reconstituted in 1 mL of 40% MeOH (40:60 $MeOH/H_2O$, v/v), sonicated for 5 min, followed by 30 s of vortexing. Samples were filtered through 0.45 µm Whatman glass microfiber syringe filters into auto sampler vials for analysis by HPLC. None of the three blank soil extracts showed indications of the presence of atrazine or metabolites, as would be expected in soil that had never been treated with atrazine. The interferences present in the HPLC chromatograms were similar for the three extractant mixtures, so the buffer/ACN

mixture was selected for the final extraction method to maintain consistency with the reference method (Figure 4.5).

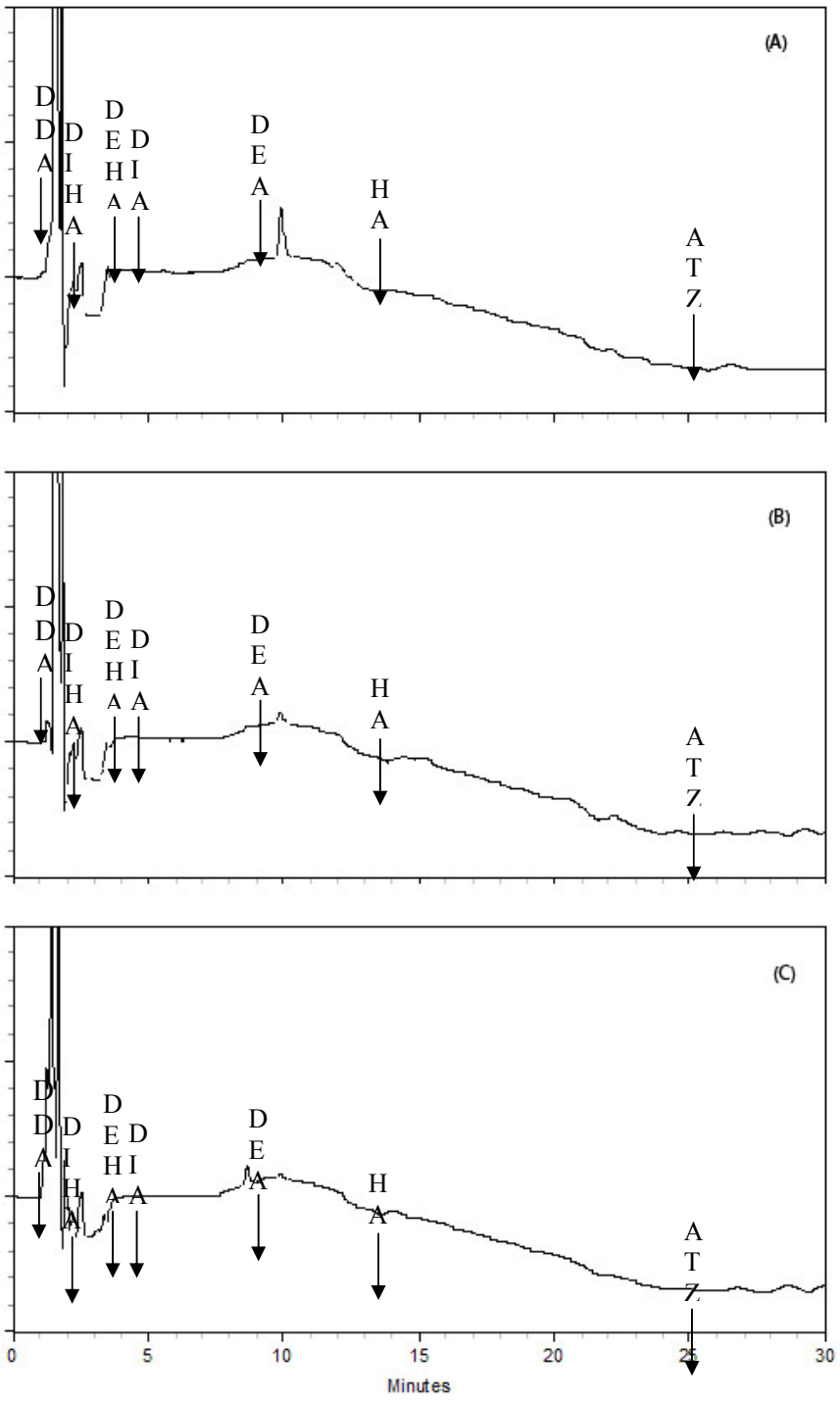

Figure 4.5: HPLC chromatograms for extractions of blank soil samples at 230 nm: (A) ACN/buffer, (B) MeOH/buffer, (C) 80% IPA.

4.2.1.3 Evaluation of rotary evaporation for concentration of SCX eluent

As evaporating eluents under N_2 was very time consuming, a Buchi R-200 rotary evaporator with a V-800 Vacuum Controller was evaluated as an alternative way to reduce the SCX eluent solutions to dryness. Twenty-five mL of a toluene/EtOH mixture (1:1, v/v) was added to the round bottom flasks at the start of evaporation to form an azeotrope with water, and therefore aid in its removal. However, when the samples were analysed by HPLC, large interference peaks between 8.5–11.5 min were observed, due to residues of toluene or EtOH (Figure 4.6a). These interference peaks coincided with the DEA retention time range (see Chapter 2, Table 2.5). It was not clear why the use of toluene and/or EtOH led to these interferences, since both solvents were of high quality. When samples were reduced to dryness without addition of toluene/EtOH, this interference was not present (Figure 4.6b). However, rotary evaporation led to other problems. The samples could only be evaporated one at a time, which again became time consuming when processing multiple samples. Also, extreme care was needed when using the rotary evaporator, since the soil extracts bumped easily under vacuum and this could both lower recoveries and introduce interferences. In conclusion, the rotary evaporation method of evaporating and concentrating sample extracts proved to be impractical, therefore, a turbo evaporator was investigated to dry eluents and was found to be successful (refer to Section 4.2.1.5 below).

4.2.1.4 Water wash

In order to prevent formation of the presumed ammonium phosphate precipitate found after the SCX eluent concentration step described in Section 4.2.1.2 above, a 3 mL water wash of the SCX cartridge was included before the elution step. The SAX breakthrough solution contains phosphate from the initial extractant buffer. Presumably some of this could remain within the dried SCX cartridge and be removed with the SCX eluent. This additional water wash step was designed to remove any phosphate salts from the cartridge, thus preventing the formation of the milky white precipitate upon elution with $MeOH/H_2O/NH_4OH$. This step was successful; therefore the filtering step before evaporating solutions to dryness was no longer necessary and was removed.

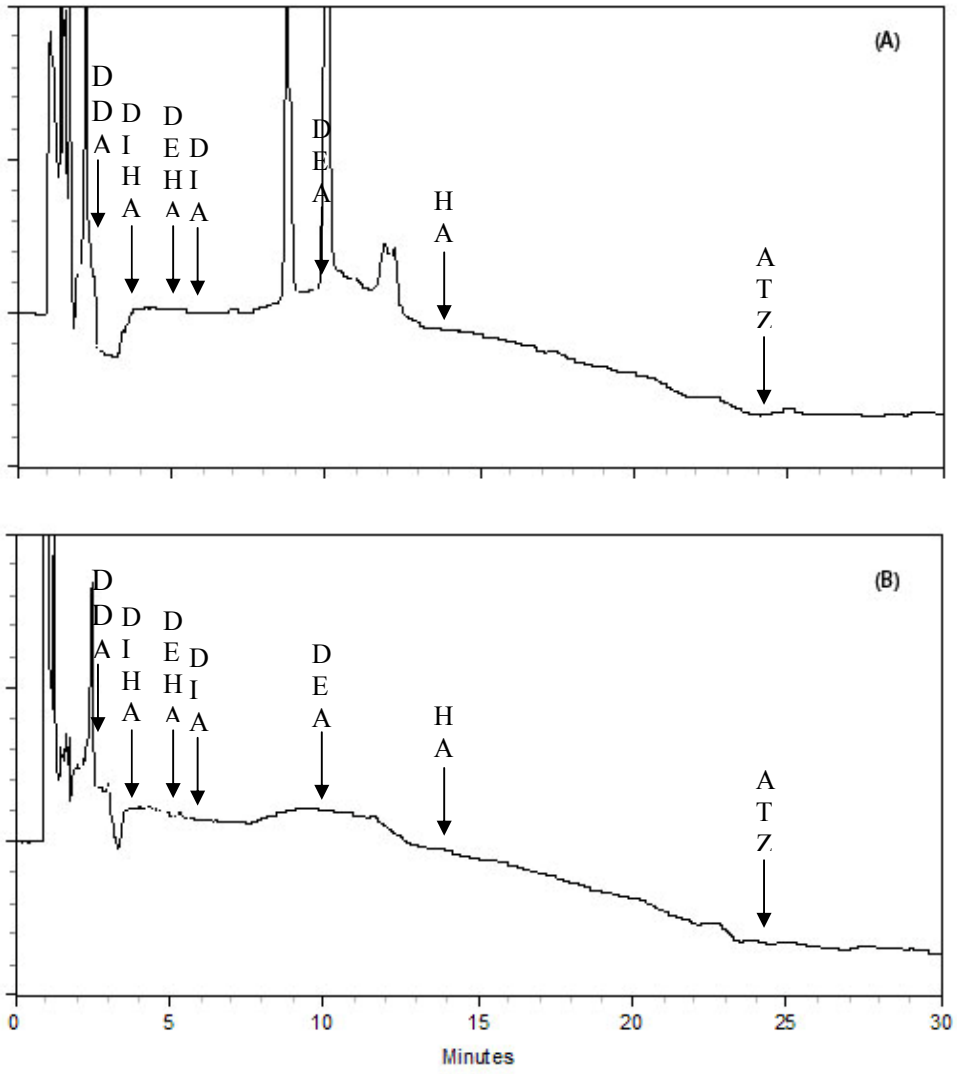

Figure 4.6: HPLC chromatograms of blank soil samples at 230 nm with EtOH/toluene interference (A) and after removing EtOH/toluene step (B) for rotary evaporation of samples.

4.2.1.5 Method Recovery

Spiked solution blanks were used to assess the recovery efficiencies of the pesticides from the cartridges and compare their recoveries between the rotary evaporator and turbo evaporator concentration steps (Table 4.2). Only the chlorinated metabolite recoveries were investigated, because the original method was designed for hydroxy metabolites (Lerch and Li, 2001) and the chlorinated metabolites demonstrated the lowest recoveries using this method in preliminary experiments. A 7.5 mL aliquot of the buffer was spiked with 0.5 mL of a mixed

atrazine standard (PWS 677-1, 20 µg mL^{-1}), to simulate the extraction solution after evaporation, before SAX clean-up. This solution was then passed through the SAX cartridge and washed as described in Chapter 2 (Section 2.8.2.2), with a final volume of 14 mL (breakthrough solution plus washes). One mL of this solution, with an expected final concentration of 0.75 µg mL^{-1}, was analysed by HPLC. A second 7.5 mL buffer aliquot of the buffer was spiked with 0.5 mL of a mixed atrazine standard (PWS 677-1, 20 µg mL^{-1}). The wash solutions (MeOH, Milli-Q water) were added to simulate the solution post-SAX clean-up (14 mL total). One mL of this solution was analysed HPLC, with an expected final concentration of 0.75 µg mL^{-1}.

Table 4.2: Recovery data for solution blanks spiked with atrazine, DEA and DIA pre- and post-SAX and SCX cartridges, to assess recovery of cartridges and evaporation step.

Spike Treatment	*Expected conc. (µg mL^{-1})*	*Atrazine*		*DEA*		*DIA*	
		Conc. (µg mL^{-1})	*% recovery*	*Conc. (µg mL^{-1})*	*% recovery*	*Conc. (µg mL^{-1})*	*% recovery*
Pre-SAX (no evaporation)	0.75	0.534	**71**	0.877	**116**	0.650	**93**
Post-SAX (no evaporation)	0.75	0.559	**75**	0.829	**111**	0.689	**92**
Pre-SCX, Rotary evaporator	10	7.342	**73**	5.242	**52**	4.365	**44**
Post-SCX, Rotary evaporator	10	8.24	**82**	8.417	**84**	8.546	**85**
Post-SCX, Turbo evaporator	10	10.7	**107**	8.973	**90**	8.884	**89**

A post-SAX spike solution was prepared (buffer plus wash solutions), spiked pre-SCX with 0.5 mL of a mixed atrazine standard (PWS 677-1, 20 µg mL^{-1}), pH adjusted to 2.5 and then passed through the SCX cartridge as described in Chapter 2 (Section 2.8.2.2). This solution was reduced to dryness by rotary evaporation, reconstituted in 1 mL 40% MeOH and analysed by HPLC with an expected final concentration of 10 µg mL^{-1}. Two 10 mL aliquots of the SCX cartridge eluent solution ($MeOH/NH_4OH/H_20$) were spiked with 0.5 mL of a mixed atrazine standard (PWS 677-1, 20 µg mL^{-1}), to simulate the collection of post-SCX solutions. One of these solutions was reduced to dryness by rotary evaporation at 60ºC, while the second was dried by turbo evaporation at 45ºC. Both were reconstituted with 1 mL 40% MeOH and analysed by HPLC with a maximum expected concentration of 10 µg mL^{-1}. All solutions in this recovery study were analysed at a wavelength of 230 nm.

Atrazine and DIA were lost during the SAX, SCX and rotary evaporation steps (Table 4.2). The recovery of atrazine spiked into the pre-SAX solution and passed through the SAX cartridge had a recovery of 71%. In comparison, the recovery of atrazine spiked directly into the post-SAX solution, without passing through the SAX cartridge was 75%, 4% greater than that obtained for the solution spiked pre-SAX. The post-SAX solution only came in contact with the glass vial it was contained within and transferred to for analysis. These results suggest that only 4% of atrazine was lost directly during SAX cartridge extraction step, and the remaining atrazine that was lost (25%) could have adsorbed to the surfaces of the glass vial before analysis. DEA recoveries were over 100% for both the pre- and post-SAX spiked solutions, indicating that this metabolite was not retained by the SAX cartridge and did not adsorb to the surfaces of the glass vial. The recoveries of DIA pre- and post-SAX were within 1% of each other.

The SCX cartridge was investigated for loss of atrazine and metabolites. Solutions spiked pre-SCX and evaporated by rotary evaporation provided a 73% recovery for atrazine, 52% recovery for DEA and 44% recovery for DIA. In comparison, the solution spiked post-SCX and evaporated by rotary evaporation provided a recovery of 82% for atrazine, 84% recovery for DEA and 85% recovery for DIA. These results suggest that 9% of atrazine, 30% of DEA and 41% of DIA were lost during the SCX cartridge extraction step. The remaining atrazine and metabolite losses (18, 16 and 15% for atrazine, DEA and DIA, respectively) were attributed to rotary evaporating the solution to dryness. The turbo evaporator was investigated to determine if it increased the recoveries of atrazine and metabolites compared with rotary evaporation. The turbo evaporator had a maximum loss of 11% (DIA) when the solution was spiked post-SCX, demonstrating it provided superior recovery to rotary evaporation. Overall, the data shows that the turbo evaporator has better recoveries than the rotary evaporator and SPE cartridge steps cause losses of atrazine and metabolites. Thus, the turbo evaporator was incorporated in the final method for the evaporation of SCX eluent solutions. The turbo evaporator also provided the advantages of being less time consuming to concentrate extraction solutions prior to SPE clean-up and than rotary evaporation or blown down with N_2. The overall recoveries of atrazine and HA obtained using the final optimised method are reported in Chapter 2 (Section 2.8.2).

4.2.2 Indoxacarb

This section outlines the development of the method to extract and analyse indoxacarb, adapted from methods published by Campbell *et al.* (2005) and Xu *et al.* (2008). The final method for extraction and analysis of indoxacarb in the soil samples is presented in Chapter 2 (Section 2.8.3). Recovery of indoxacarb from the soil matrix was obtained by spiking blank soil samples (Cu-1 Nov08) with pesticide working standards (PWS). Recoveries for spiked solution and sample blanks were used to determine the final wash and eluent solutions, and reconstitution and extractant mixtures. A solubility study was also undertaken to determine the optimal proportion of ACN in the extraction solution to ensure indoxacarb remained soluble in the solution.

4.2.2.1 Indoxacarb solubility study

The optimal proportion of ACN necessary to retain indoxacarb in solution was investigated. The results of this study were incorporated into the preparation of the final extractant mixture. 0.5 *M* KH_2PO_4 (pH 7.5)/ACN solutions were prepared at concentrations of 0, 5, 10, 20, 25. Mixtures were spiked with the indoxacarb standard (PSS 1086, 200 μg mL^{-1}) to provide a concentration of 2 μg mL^{-1} indoxacarb and were vortexed to mix. Three aliquots were removed from each individual mixture, 0.5 mL at the time of mixing (1st aliquot), and 1 mL at 1 h (2nd aliquot) and 5 h (3rd aliquot) after mixing, with the solutions remaining static at room temperature during this time. Final concentrations were expected to be 1 μg mL^{-1} for the 1st sampled aliquot (0.5 mL made up to 1 mL with ACN), and 2 μg mL^{-1} for both the 2nd and 3rd aliquots (1 mL directly analysed). Care was taken to take samples from the centre of the upper two-thirds of the spiked solutions, to avoid any indoxacarb that may have adsorbed to the sides of the vials. Recovery of indoxacarb from the 0% ACN solution was low (≤3.5%), indicating that ACN is necessary to dissolve indoxacarb in the buffer solution (Table 4.3). Maximum recovery of indoxacarb for the 1st aliquot was achieved at 25% ACN. Indoxacarb was most stable in 25% ACN over time, as indicated by the highest consistent recoveries across all the three analysed aliquots for this %ACN. Higher ACN concentrations were briefly investigated, without improved results. These were not investigated further as a large dilution would be needed in order to pass the solutions through the Oasis HLB cartridge for clean-up. Therefore, the 3:1 buffer/ACN solution (25% ACN) was used for extraction in the final method (Section 2.8.3).

Table 4.3: Indoxacarb recovery for solubility study.

%ACN	*Concentration (µg mL^{-1})*			*% recovery*		
	Aliquot 1	*Aliquot 2*	*Aliquot 3*	*Aliquot 1*	*Aliquot 2*	*Aliquot 3*
0	0	0.07	0.07	0	3.5	3.5
5	0.70	0.31	0.35	70	15.5	17.5
10	0.77	0.65	0.63	77	32.5	31.5
20	1.05	1.54	1.53	105	77	76.5
25	1.18	1.76	1.71	118	88	85.5

4.2.2.2 Wash solution study

The solvent concentration in the buffer/ACN extractant solution needed to be reduced prior to loading the extract onto the Oasis HLB cartridge for clean-up. In addition, a wash solution was necessary to preferentially remove soil co-extractants from the Oasis HLB cartridge before elution of indoxacarb. This wash step was incorporated to remove potential interferences for the HPLC analysis. Wash solutions containing 0, 5 and 10% ACN were investigated to see which gave the best indoxacarb recovery. A 0.5 *M* KH_2PO_4 (pH 7.5)/ACN (3:1, v/v) extraction solution was prepared as previously described (Section 2.8.2.1). Three 10 mL aliquots of the extraction solution were transferred into separate test tubes and each was spiked with 100 µL of the indoxacarb standard (PSS 1086, 200 µg mL^{-1}). One aliquot was diluted to 50 mL with Milli-Q water (5% ACN) and the second was diluted to 25 mL with Milli-Q water (10% ACN). The third was placed under N_2 and the ACN was evaporated using mild heat (0% ACN). Oasis HLB cartridges (6 mL/500 mg, Waters) were conditioned as previously described (Chapter 2, Table 2.4) and the spiked solution blanks were passed through at a rate of 5–10 mL min^{-1} under low vacuum to keep the rate constant. The 0 and 5% ACN solution blank cartridges were washed with 5 mL of 5% ACN and the 10% ACN solution blank with 10% ACN. The blanks were then eluted with two aliquots of 2 mL ACN, with each aliquot collected in separate vials for direct analysis by HPLC. The maximum expected concentration of indoxacarb each 2 mL aliquot was 10 µg mL^{-1}. Using 10% ACN for both the spiked loading solution and for the wash solution provided the highest recovery for indoxacarb at 79% (Table 4.4) and was adopted into the final method Chapter 2 (Section 2.8.3). The reduced recoveries of the 0 and 5% ACN loading solutions (with 5% wash solution) indicate the poor solubility of indoxacarb in low %ACN solutions. This agrees with the indoxacarb solubility study above.

Table 4.4: Indoxacarb recoveries for the wash solution study.

Final %ACN in solution	*Wash solution*	*Concentration (µg mL^{-1})*		*Total recovered (µg)*	*% recovery*
		Aliquot 1	*Aliquot 2*		
0%	5% ACN	1.12	0.36	2.96	15
5%	5% ACN	2.06	4.02	12.2	61
10%	10% ACN	6.64	1.25	15.8	79

4.2.2.3 Eluent solution study

Three solutions were assessed for their efficiency to elute the indoxacarb from the Oasis HLB cartridges. These solutions were ACN, ACN/dichloromethane (DCM) (1:1, v/v), and ACN/MeOH (1:1, v/v). Three 4 mL aliquots of the 3:1 buffer/ACN buffer solution were spiked with 100 µL of the indoxacarb standard (PSS 1086, 200 µg mL^{-1}) and diluted to 10 mL with Milli-Q water to give approximately 10% ACN. These spiked solution blanks were passed through conditioned Oasis HLB cartridges and eluted with either two 4 mL aliquots of ACN, ACN/DCM, or ACN/MeOH. The aliquots were collected separately and 1 mL of both of the ACN and ACN/MeOH aliquots were analysed directly by HPLC. The DCM in the ACN/DCM eluents could not be analysed directly by HPLC, so both 4 mL aliquots were reduced to dryness on the turbo evaporator. These dried aliquots were reconstituted in 4 mL ACN and 1 mL of both aliquots were analysed by HPLC. The maximum expected concentration of indoxacarb each 4 mL aliquot was 5 µg mL^{-1}. The recovery of indoxacarb ranged from 85–92% (Table 4.5). Elution with 100% ACN provided the highest recovery for indoxacarb (92%) and was adopted in the final method in Chapter 2 (Section 2.8.3).

Table 4.5: Indoxacarb recoveries for eluent solution study.

Eluent	*Recovery (µg mL^{-1})*		*Total (µg)*	*% recovery*
	Aliquot 1	*Aliquot 2*		
ACN	4.50	0.10	18.4	92
ACN/DCM	4.25	0	17.0	85
ACN/MeOH	4.08	0.15	16.9	85

4.2.2.4 Reconstitution mixture study

In order to attain greater sensitivity in the final analysis method, the ACN used to elute indoxacarb from the Oasis HLB cartridges needed to be evaporated to dryness and the residue reconstituted for analysis. It was essential that this reconstitution solution fully re-solubilised indoxacarb before analysis by HPLC. Three organic solvent mixtures, ACN, MeOH and 50% ACN (1:1 ACN/H_2O, v/v) were investigated for their suitability to reconstitute indoxacarb from the dried eluent. Solution blanks and soil blank extracts were spiked with the indoxacarb standard either before or after passing the extract solutions through Oasis HLB cartridges. This study allowed the simultaneous investigation of which reconstitution mixture provided the highest recovery of indoxacarb, and where the potential losses of indoxacarb occurred during this method.

One solution blank (20 mL 3:1 buffer/ACN extraction mixture), and 20 mL aliquots of three soil blank extracts (extracted as described above in Section 4.2.1.2) were spiked with 25 µL of the indoxacarb standard (PSS 1086, 200 µg mL^{-1}) before passing through conditioned Oasis HLB cartridges (pre-cartridge). A second solution blank, consisting of 5 mL ACN to represent the Oasis HLB column eluent, and 20 mL aliquots of three additional soil blank extracts (extracted as described above in Section 4.2.1.2) were spiked with the indoxacarb standard after passing through the conditioned Oasis HLB cartridges and before drying on the turbo evaporator (post-cartridge). The first spiked solution blank and all six soil blank extracts were passed through the Oasis HLB cartridges, eluted with 5 mL ACN and were collected in 40 mL turbo evaporator vials. All spiked eluents were then evaporated to dryness by turbo evaporation. Both solution blanks were reconstituted with 1 mL ACN. Spiked soil blanks from each set (pre- and post-cartridge) were reconstituted with either 1 mL ACN, MeOH or 50% ACN. The final concentration of indoxacarb after reconstitution was expected to be 5 µg mL^{-1}.

The recovery of indoxacarb ranged from 0–102% (Table 4.6). The highest recovery was attained with 50% ACN as the reconstitution mixture (recovery pre- and post-cartridge 98–102%). The residue left after turbo evaporation of the soil extracts completely dissolved into this reconstitution solution. MeOH recovered 57–78% of indoxacarb, while ACN alone did not recover any of the indoxacarb spikes from the soil blank extracts. Both MeOH and ACN only partially dissolved the residue. The remaining un-dissolved residue must have retained

some indoxacarb, as is evident by the low recoveries obtained for these solutions. The water present in the 50% ACN solution helped to dissolve the residue retaining indoxacarb, aiding in the re-dissolution of indoxacarb in this solution. Based on this information, 50% MeOH (1:1 MeOH/H_2O, v/v) was chosen as the reconstitution mixture, because MeOH is a weaker solvent than ACN, and would allow larger injections on the HPLC if required to gain extra sensitivity during analysis. In addition, MeOH alone had re-dissolved 60–78% of the indoxacarb present in these evaporated spike solutions, compared with 0% for ACN alone. It was reasoned that 50% MeOH should give results at least as good as those obtained with 50% ACN.

Table 4.6: Indoxacarb recoveries for reconstitution study.

Spiking step	*Sample*	*Reconstitution mixture*	*Recovery (µg mL^{-1})*	*% recovery*
Pre-Cartridge	Solution Blank	ACN	4.04	80.8
	Soil Blank	ACN	0	0
	Soil Blank	MeOH	2.87	57.4
	Soil Blank	50% ACN	4.92	98.4
Post-Cartridge	Solution Blank	ACN	5.17	103
	Soil Blank	ACN	0	0
	Soil Blank	MeOH	3.88	77.6
	Soil Blank	50% ACN	5.12	102

The efficiency of the 50% MeOH (1:1 MeOH/H_2O, v/v) as the reconstitution solution was investigated further by analysing spiked soil blank extracts and also t_0 indoxacarb soil samples in duplicate. These soil samples were extracted twice with 20 mL of the 3:1 buffer/ACN extraction solution as described above in Section 4.2.1.2. Twenty mL of the soil blank extract was spiked with 25 µL of the indoxacarb standard (PSS 1086, 200 µg mL^{-1}), diluted to 40 mL with Milli-Q water, passed through an Oasis HLB cartridge, eluted with 5 mL ACN, evaporated to dryness on the turbo evaporator, and reconstituted with 1 mL of 50% MeOH. The expected final concentration of indoxacarb in the reconstituted soil blank was 5 µg mL^{-1}. Indoxacarb t_0 soil samples were treated the same as the soil blank, but were not spiked. Based on the original spike rate of 2 mg kg^{-1}, the expected concentration of the t_0 samples in the final reconstituted solutions was 10 µg mL^{-1}.

The sample blank recovery was 94% (Table 4.7). However, recoveries determined for the t_0 samples were low and ranged from 46–52%. This suggested that the 3:1 buffer/ACN mixture

was not strong enough to extract all of the indoxacarb from the soil. In order to increase the recovery of indoxacarb from soil samples, and keeping in mind the superior performance of MeOH over ACN for re-dissolving indoxacarb in evaporated extracts, 10 mL MeOH was added to each future extraction in addition to the 20 mL of 3:1 buffer/ACN mixture. To test this new extraction mixture, duplicate samples of the t_0 indoxacarb soils were extracted with 3:1:2 buffer/ACN/MeOH, cleaned-up as above, and reconstituted in 1 mL 50% MeOH. Recovery for the t_0 indoxacarb soil samples was increased to 69–77% of the theoretical value (Table 4.8), indicating that the modified extractant mixture was successful. There were no chromatographic interferences observed due to the different Cu-spike levels.

Table 4.7: The recovery of indoxacarb from t_0 soil samples extracted with 3:1 buffer/ACN and reconstituted with 50% MeOH.

Sample	*Spike/ Expected conc. (µg mL^{-1})*	*Duplicate 1*		*Duplicate 2*	
		Conc. (µg mL^{-1})	*% recovery*	*Conc. (µg mL^{-1})*	*% recovery*
Soil blank	5	4.72	94	N/A	N/A
IND Cu-1	10	5.10	51	4.61	46
IND Cu-2	10	5.22	52	4.95	50
IND Cu-3	10	5.03	50	5.05	51
IND Cu-4	10	5.06	51	5.25	52
IND Cu-5	10	5.09	51	4.61	46

Table 4.8: Recoveries of indoxacarb from t_0 soil samples extracted with 3:1:2 buffer/ACN/MeOH and reconstituted with 50% MeOH.

Sample	*Spike/ Expected conc. (µg mL^{-1})*	*Duplicate 1*		*Duplicate 2*	
		Conc. (µg mL^{-1})	*% recovery*	*Conc. (µg mL^{-1})*	*% recovery*
Soil blank	5	4.63	93	N/A	N/A
IND Cu-1	20	13.85	69	15.39	77
IND Cu-2	20	13.70	69	13.94	70
IND Cu-3	20	14.48	72	14.79	74
IND Cu-4	20	13.97	70	13.91	70
IND Cu-5	20	14.24	71	15.10	76

In order to test the completeness of the two-times indoxacarb extraction method (Chapter 2, Section 2.8.3.1), the t_0 indoxacarb soil samples extracted in duplicate above were extracted for a third time using the 3:1:2 buffer/ACN/MeOH solution. These soils were extracted a third time to determine if an additional extraction would increase recovery of indoxacarb.

These third extractions recovered an additional 4–6% of the total indoxacarb expected from the initial spike (Table 4.9). While this would increase overall recovery of the target compound slightly, this increase was deemed trivial when compared to the time and additional materials needed for a third extraction. Therefore, it was decided to use the method with a double extraction and 69–77% recovery. The final method used for analysis of the samples in this study is presented in Chapter 2 (Section 2.8.3).

Table 4.9: Recovery of indoxacarb from third extractions of t_0 soil samples.

Sample	*Spike/ Expected conc. (µg mL^{-1})*	*Duplicate 1*		*Duplicate 2*	
		Conc. (µg mL^{-1})	*% recovery*	*Conc. (µg mL^{-1})*	*% recovery*
Soil blank	(not spiked)	0.18	N/A	N/A	N/A
IND Cu-1	20	1.02	5	0.90	5
IND Cu-2	20	0.92	5	0.86	4
IND Cu-3	20	1.04	5	0.98	5
IND Cu-4	20	1.21	6	1.19	6
IND Cu-5	20	1.16	6	1.23	6

4.3 RESULTS AND DISCUSSION

4.3.1 Atrazine

4.3.1.1 Atrazine and metabolite concentrations in soil

In the course of these studies, atrazine and HA were the only compounds detected using the extraction method reported above. Although high levels of most atrazine metabolites (except DDA) could have been determined, determination of trace levels of these compounds other than atrazine and HA would have been difficult due to the presence of soil co-extractants, such as those having the same retention times as DDA, DEHA and DIHA (Figure 4.7; Table 2.5). Hence, these compounds were not able to be differentiated from these interferences and quantified by this method. The Lerch and Li (2001) method developed specifically for the HADPs was also reported to have difficulty in quantifying DEHA by LC-UV. They did not report any soil co-extractives interfering with the remaining HADPs.

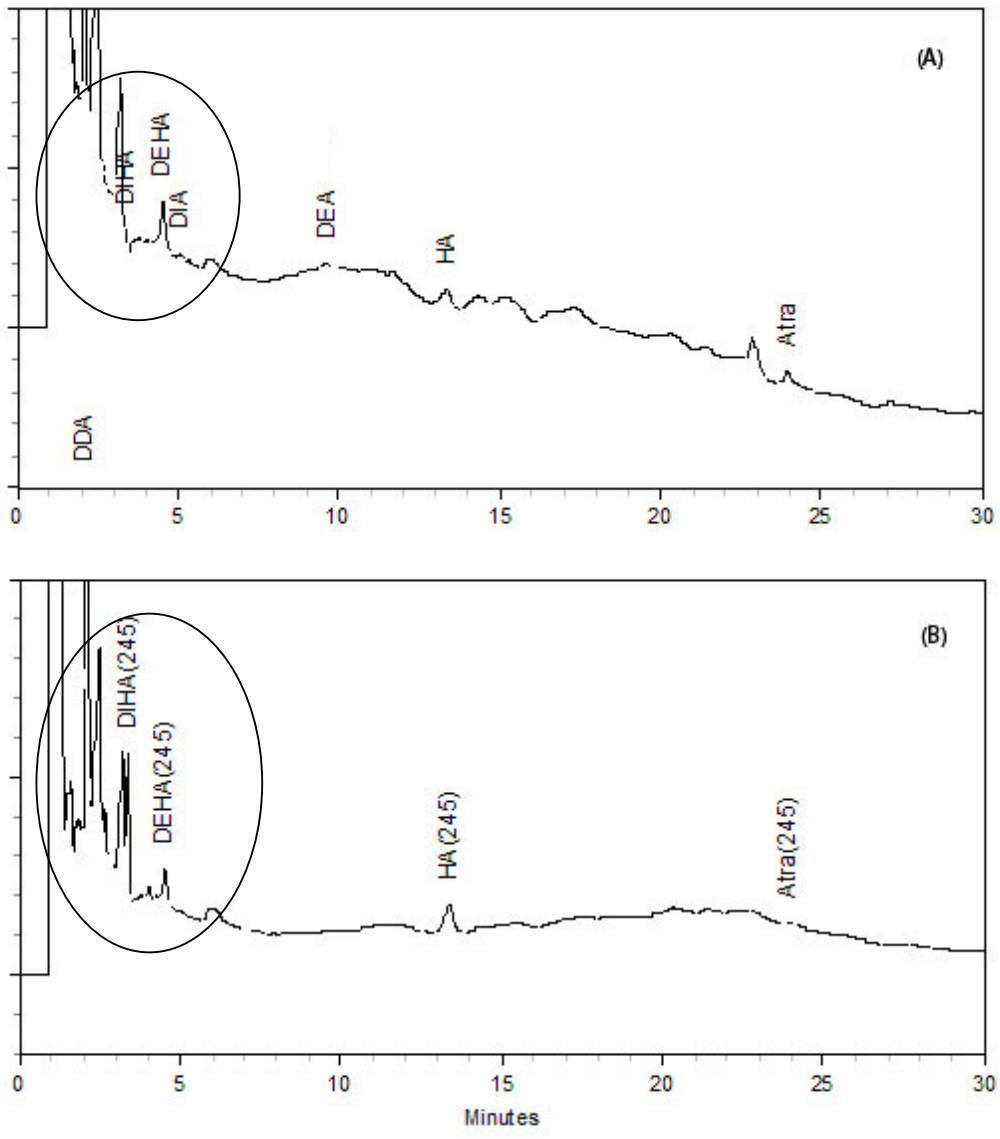

Figure 4.7: HPLC chromatogram of a spiked soil blank (2 µg mL^{-1}) showing interferences of soil co-extractants (circled) at (A) 230 nm and (B) 245 nm.

In these experiments, atrazine concentrations ranged between 1.02–1.29 mg kg^{-1} at t_0, 0.28–0.40 mg kg^{-1} at t_1, and <0.1–0.14 mg kg^{-1} at t_2 (Table 4.10; Figure 4.8). Atrazine concentrations decreased with time in the soil samples as expected, due to degradation. The %RSD-values for the replicate samples increased as residual atrazine concentrations approached the method detection limit (Table 4.10), where quantification is less certain.

Table 4.10: Concentration (mg kg^{-1}) of atrazine and hydroxyatrazine extracted from soil samples (n=3).

Sampling time	*Sample*	*Atrazine*			*Hydroxyatrazine*		
		Average (mg kg^{-1})	*Standard deviation*	*%RSD*	*Average (mg kg^{-1})*	*Standard deviation*	*%RSD*
t_0	ATZ Cu-1	1.12	0.14	12.7	0.06	0.02	29.4
	ATZ Cu-2	1.29	0.08	6.15	0.08	0.03	43.9
	ATZ Cu-3	1.02	0.15	14.4	0.08	0.04	48.1
	ATZ Cu-4	1.04	0.33	31.4	0.08	0.01	16.2
	ATZ Cu-5	1.11	0.18	16.0	0.09	0.03	32.4
t_1	ATZ Cu-1	0.28	0.11	38.5	0.36	0.01	3.69
	ATZ Cu-2	0.31	0.06	18.2	0.33	0.01	2.93
	ATZ Cu-3	0.21	0.10	47.9	0.38	0.01	2.53
	ATZ Cu-4	0.39	0.18	47.0	0.43	0.02	5.50
	ATZ Cu-5	0.40	0.10	24.2	0.41	0.02	5.80
t_2	ATZ Cu-1	<0.1	–	–	0.17	0.03	17.6
	ATZ Cu-2	<0.1	–	–	0.17	0.03	20.2
	ATZ Cu-3	<0.1	–	–	0.35	0.03	7.77
	ATZ Cu-4	<0.1	–	–	0.49	0.03	6.92
	ATZ Cu-5	0.14	0.24	173	0.47	0.02	3.35
detection limits: 0.1 mg kg^{-1} ATZ; 0.06 mg kg^{-1} HA							

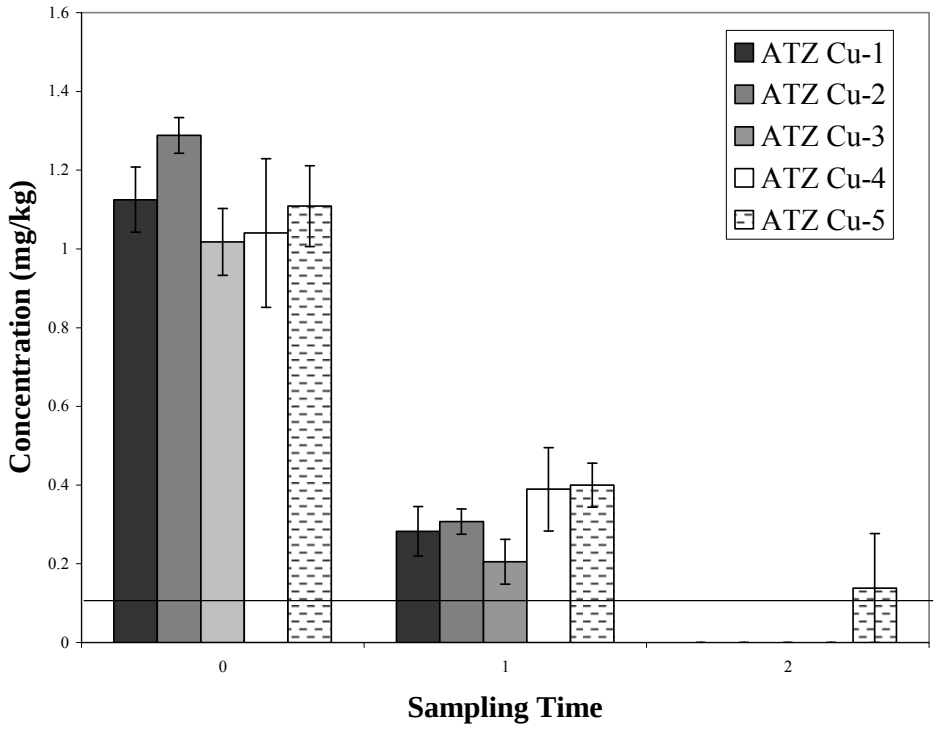

Figure 4.8: Soil concentration of atrazine over the sampling period. Error bars represent standard error of replicate measurements (n=3). The line represents the sample detection limit.

The corresponding HA concentrations ranged between 0.06–0.09 mg kg^{-1} at t_0, 0.33–0.43 mg kg^{-1} at t_1, and 0.17–0.49 mg kg^{-1} at t_2 (Table 4.10; Figure 4.9). HA concentrations increased markedly in the soil samples between t_0 and t_1 as expected as atrazine is degraded to HA. This matched the corresponding decrease in the concentration of residual atrazine in the soil. The %RSD-values for HA were high at t_0, because the concentrations were close to the method detection limit, and decreased as the concentration of HA in the soil increased.

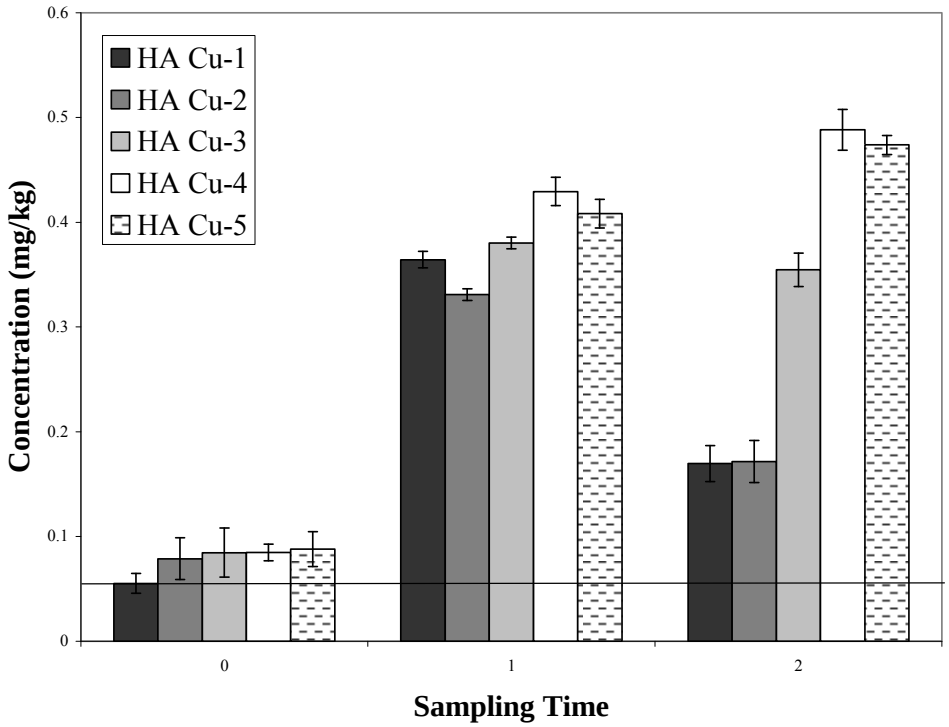

Figure 4.9: Hydroxyatrazine concentration at each sampling time. Error bars represent standard error of replicate measurements (n=3). The line represents the sample detection limit.

DEA and DIA were not detected in any of the atrazine-spiked soil samples (Figure 4.10). Other studies detected very little, if any, of the chlorinated degradation products in aged atrazine soils (Juracek and Thurman, 1997; Lerch and Li, 2001; Lerch *et al.*, 1999; Mahia *et al.*, 2008). HADPs were the major metabolites in field-aged soils from the Mid-Western United States, making up 91% of the total atrazine residues in the soils studied (Lerch and Li, 2001). HA was the predominant metabolite in those soils, which agrees with the results of these experiments at the later sampling times, after the soils were aged (refer to Section 4.3.1.1 above).

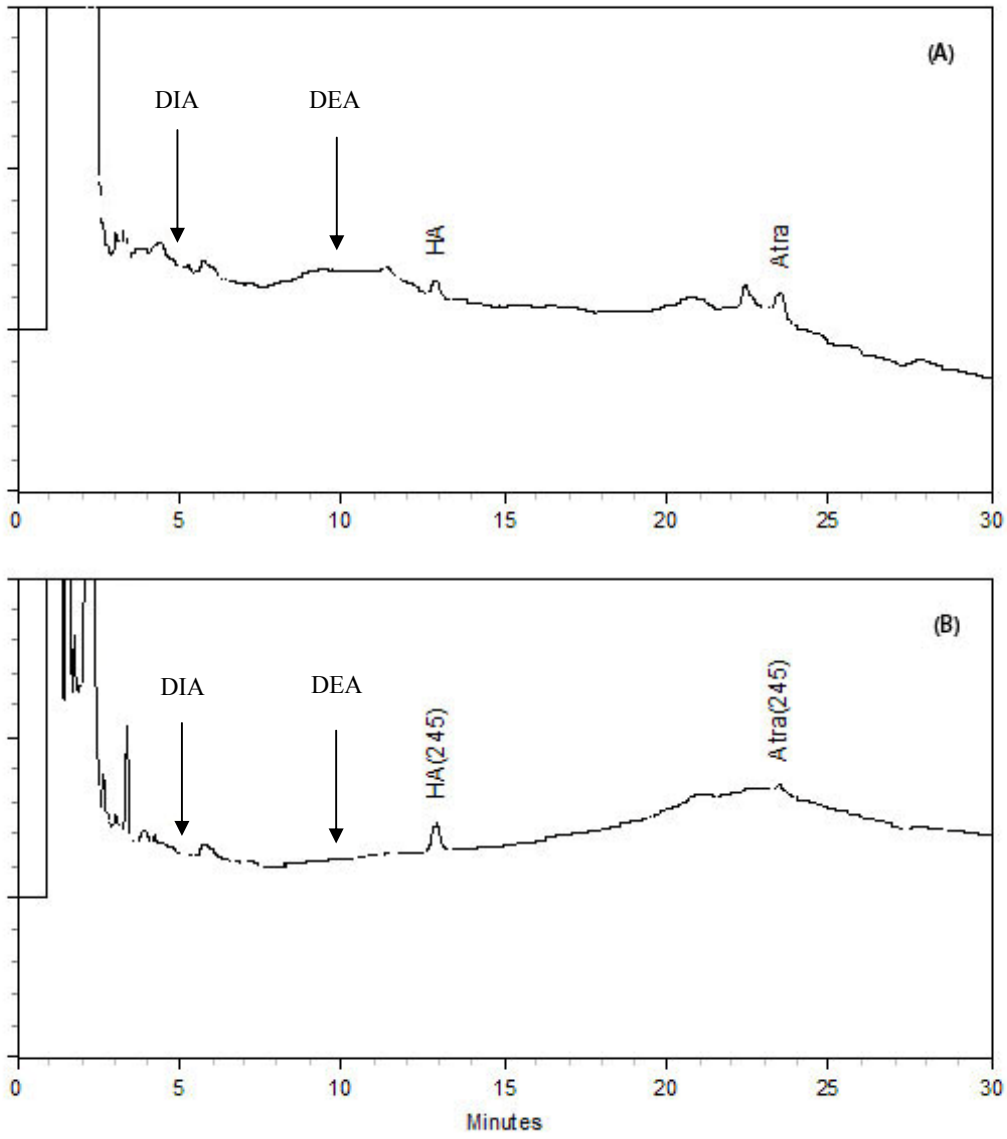

Figure 4.10: HPLC chromatogram of sample ATZ Cu-4A Jan09, showing lack of DEA and DIA peaks at both (A) 230 nm and (B) 245 nm.

The chlorinated metabolites are more mobile than HA in soil (Lerch and Li, 2001), so DEA and DIA could potentially migrate down the soil profile, making them undetectable if only the top soil was sampled and analysed. However, Juracek and Thurman (1997) sampled soil at three depths ranging from 0–3 m. DEA was only detected in two sites sampled, at concentrations of 1.4 µg kg^{-1} (between 1.2–1.8 m) and 1.5 µg kg^{-1} (between 0–1.2 m), while DIA was not detected at all. In contrast, the atrazine-spiked soils in this study were contained in liver pails and the whole soil profile was sampled. The pails did not have drainage holes,

preventing metabolite loss from the containers through leaching. Possible explanations for the lack of chlorinated metabolites in this thesis study could be: 1) the formation of HA was favoured over DEA and DIA in the experimental soils (Lerch and Li, 2001; Lerch *et al.*, 1999); 2) HA is more persistent in soils than DEA, DIA or atrazine (Clay and Koskinen, 1990; Lerch and Li, 2001; Mahia *et al.*, 2008), especially for soils with pH<6 (Loiseau and Barriuso, 2002); 3) DEA and DIA may have degraded very rapidly in the experimental soils so were not detected; or 4) the method was not sensitive enough to detect the presence of these metabolites in the soil. The pH-values of the Cu-spiked Templeton experimental soils were all <5.0, so it is reasonable to assume that HA was more persistent in the experimental soil than DEA, DIA or atrazine. To investigate (3), metabolites from the next step of the degradation process after DEA and DIA would need to be analysed.

4.3.1.2 Effects of copper on the degradation of atrazine

There were no significant relationships between atrazine or HA and either total or bioavailable Cu. Significant differences (p<0.05) were detected for HA at t_2 between the control and treatments Cu-3, Cu-4 and Cu-5, indicating that Cu did inhibit the degradation of HA at concentrations greater than 100 mg Cu kg^{-1} soil. There were also significant differences between the control and Cu-2, Cu-4, and Cu-5 at t_1 for HA, indicating that the presence of elevated Cu concentrations in the soil potentially had effects on the degradation of HA at this sampling time as well. It is uncertain why the HA concentration for Cu-2 was less than Cu-1 at t_1. The differences at t_1 could be random, as Cu-2 was significantly lower, Cu-3 was not significantly different and Cu-4 and Cu-5 were significantly higher in HA concentration than the control. There were no significant differences between HA concentrations in the soil at t_0. To conclusively determine if total Cu does impact metabolite degradation, the next step of the degradation process after HA would have to be investigated, by analysing the soils for these metabolites (i.e. DEHA, DIHA). Since DEHA and DIHA were not detected in the soils with this method, potentially due to interferences from the soil co-extractants, cyanuric acid would be the next logical metabolite to extract from the soil and analyse (Figure 4.1).

Copper has been shown to inhibit the atz-A enzyme responsible for catalysing the microbial hydrolysis of atrazine to HA (Seffernick *et al.*, 2002). This did not appear to be the case in the Templeton experimental soil, as there was no statistically relevant relationship between

Cu concentration and atrazine. It has also been reported that heavy metals can alter the soil microbial community by favouring metal-resistant populations and inhibiting sensitive ones (van der Meer, 2006). In the case of the experimental soil, the microorganisms that express the atz-A enzyme could be favoured in the elevated copper soils over the microorganisms that did not express this enzyme (i.e. those that would preferentially degrade atrazine through the DEA/DIA pathway, Figure 4.1). Though this could explain why DEA and DIA were not present in the experimental soil, the control soil (Cu-1) which had an average background Cu concentration of 4.59 mg kg^{-1} (Table 3.3), did not have measurable concentrations of DEA or DIA extracted. To confirm whether the atz-A enzyme-expressing microorganisms were favoured in the experimental soil, the soil microorganisms would have to be isolated and examined for this enzyme.

Abiotic factors such as photolysis (e.g. Hartenbach *et al.*, 2008; Lackhoff and Niessner, 2002), presence of humic acids (e.g. Martin-Neto, 1994), and heavy metals (e.g. Lackhoff and Niessner, 2002; Liu *et al.*, 1999) have been reported to enhance the degradation of organic pesticides in soil. To determine the role of abiotic processes in the degradation of atrazine in soils, degradation rates of microbially-active soils must be compared to rates of sterilised soils. Two New Zealand soils, sterilised by repeated autoclaving, were found to degrade 5–15% of applied atrazine by 120 d, indicating that some abiotic hydrolysis of atrazine could be expected in microbially-active (non-sterilised) soils (Sarmah *et al.*, 2009). Based on the low degradation reported for sterilised soils, it is probable that the majority of atrazine degraded in the present studies was degraded biotically. Purified humic acids can enhance HA formation at pH<5 (Martin-Neto, 1994). Organic soils are also known to tightly bind Cu^{2+} ions, which prevented rapid catalytic hydrolysis of organic phosphate pesticides (Mortland and Raman, 1967). Liu *et al.* (1999) observed that the hydrolysis of a non-halogenated s-triazine algecide was catalysed by Hg^{2+}, but not by Cu^{2+} or other divalent metals. Pure metal oxides are known to photocatalyse the degradation of atrazine (Lackhoff and Niessner, 2002), though in environmental samples (soot, sand, dust, volcanic ash, fly ash), metal concentrations were not high enough to photocatalytically degrade atrazine (Lackhoff and Niessner, 2002).

The abiotic degradation of atrazine was not specifically studied in these experiments, but from the literature it is reasonable to assume that some abiotic degradation would have occurred throughout the duration of the glasshouse experiment. The Cu in the experimental soils was bound tightly as indicated by the similar total Cu concentrations obtained for each sampling

time, at each Cu-level (refer to Table 3.3). Therefore, Cu-catalysed hydrolysis is unlikely to have occurred in the Templeton experimental soil. Photolysis or photocatalysis will only occur in the very top layer of soil (Gong *et al.*, 2001), and the experimental soils were well-mixed after spiking, so only a small percentage of the total atrazine applied to the soil would have been exposed to light in the top layer. Therefore, photodegradation would have made a very small contribution of the abiotic degradation of atrazine in the experimental soils. The organic carbon content of one of the sterilised New Zealand soils (Sarmah *et al.*, 2009) was similar to that of the Templeton experimental soil. In addition, the Templeton experimental soil had pH<5.0, so it is reasonable to conclude that humic acids may have contributed to the abiotic degradation of atrazine to HA. Based on all of these potential degradation pathways, it is reasonable to conclude that microbial degradation of atrazine would have been the predominant degradative process in the Templeton experimental soil.

As discussed in Chapter 1, copper has been observed to complex with pesticides, inhibiting their degradation (Morillo *et al.*, 2000; Sancho *et al.*, 1997). Sancho *et al.* (1997) observed that complex formation to a triazine pesticide was stabilised in solution at pH 5.6, decreasing the photodegradation rate by 15% within the first 30 d. Morillo *et al.* (2000) observed that copper enhanced glyphosate adsorption to soil, potentially acting as a bridge between the pesticide and soil, which would increase persistence. If copper was complexing atrazine in the Templeton experimental soil, there potentially would have been a significant difference between the measured HA extracted with increasing Cu concentration: Cu-5 would have the lowest HA concentrations, while Cu-1 would have highest HA concentrations. Extractable atrazine concentrations could vary, depending if the complexed atrazine could be extracted by the method in Chapter 2. As discussed above, there were significant differences between copper and HA concentrations in the experimental soils, especially at t_2, suggesting that copper could have been stabilising this metabolite, preventing its degradation in the experimental soils. Mechanisms are uncertain, but could include direct complexation of HA with copper or copper bridging HA adsorption to the SOM.

4.3.1.3 Half-life of Atrazine

The half-life of a pesticide is an important parameter for assessing its fate in the environment (Sarmah *et al.*, 2009). The half-life of atrazine for the Templeton experimental soil was calculated from the exponential degradation of extracted residues from t_0 to t_2. Since there

were no significant differences in residual atrazine concentrations between the Cu-levels at each sampling time (refer to Section 4.3.1.1 above), the residual concentrations obtained at each sampling time were averaged in order to determine the degradation equation ($y = 1.3579e^{-0.0462x}$; $r^2 = 0.9673$; Figure 4.11). Half the residual amount of atrazine extracted at t_0 was calculated to be 0.558 mg kg^{-1}. This value was inserted into the degradation equation and atrazine's half-life was estimated to be 19.4 d for the Templeton experimental soil. However, more sampling between t_0 and t_1 would be needed in order to validate this estimated half-life value. Therefore, 19.4 d is only an approximate value, and atrazine's half-life can only be reported as ≤19.4 d within Templeton experimental soil and under the conditions of this experiment.

This estimated half-life for atrazine in the experimental soil was lower than the median reported for atrazine in a field study of seven New Zealand soils of 41 d (Close *et al.*, 2008), but is still within the reported national (16–188 d) and international (13–402 d) ranges (Close *et al.*, 2008). In another study, Muller *et al.* (2003) determined a half-life of 77 d for atrazine in a New Zealand soil near Hamilton. This value also falls within the range reported by Close *et al.* (2008).

The soil temperature can influence the degradation rate (Aislabie *et al.*, 2004). Since this atrazine degradation study was conducted in a glasshouse, the soil temperature will have most likely exceeded that of the field study by Close *et al.* (2008). This could account for the increase in degradation rate of atrazine obtained in the Templeton experimental soil as compared to the national median of 41 d (Close *et al.*, 2008).

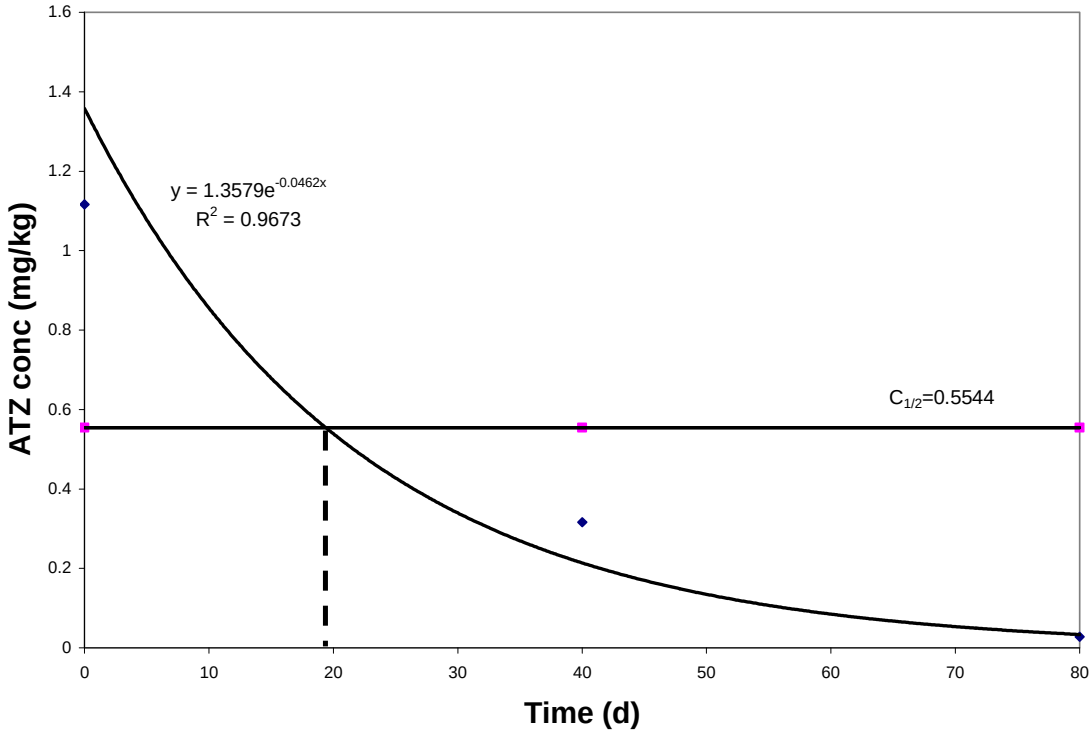

Figure 4.11: Estimated half-life of atrazine in the Templeton experimental soil, calculated from the point where half the initially applied atrazine concentration ($C_{1/2}$) crossed the exponential degradation of atrazine from t_0 to t_1.

4.3.2 Indoxacarb

4.3.2.1 Indoxacarb concentrations in soil and effects of copper

Indoxacarb concentrations measured in the experimental soils ranged from 1.51–1.59 mg kg^{-1} at t_0, 0.16–0.18 mg kg^{-1} at t_1 and 0.10–0.11 mg kg^{-1} at t_2 (Table 4.11, Figure 4.12). Indoxacarb concentrations in the experimental soils decreased over time, as was expected as indoxacarb degrades. There were no significant differences in indoxacarb concentration between the different Cu-spike levels at each sampling time, indicating increasing Cu concentration did not affect the degradation of indoxacarb. Since indoxacarb metabolites were not analysed in this experiment, it cannot be confirmed if Cu impacted their formation or persistence in the experimental soil.

Table 4.11: Concentration of indoxacarb extracted from soil samples (n=3).

Sampling Time	*Sample*	*Average (mg kg^{-1})*	*Standard deviation*	*%RSD*
t_0	IND Cu-1	1.51	0.09	6.07
	IND Cu-2	1.51	0.06	4.04
	IND Cu-3	1.58	0.03	1.64
	IND Cu-4	1.53	0.04	2.93
	IND Cu-5	1.59	0.03	2.16
t_1	IND Cu-1	0.16	0.007	4.06
	IND Cu-2	0.17	0.007	4.22
	IND Cu-3	0.18	0.01	5.68
	IND Cu-4	0.16	0.02	13.8
	IND Cu-5	0.17	0.008	4.74
t_2	IND Cu-1	0.10	0.003	3.44
	IND Cu-2	0.11	0.007	6.63
	IND Cu-3	0.12	0.007	5.83
	IND Cu-4	0.11	0.014	12.9
	IND Cu-5	0.11	0.007	6.69
Detection limit = 0.0025 mg kg-$^{-1}$				

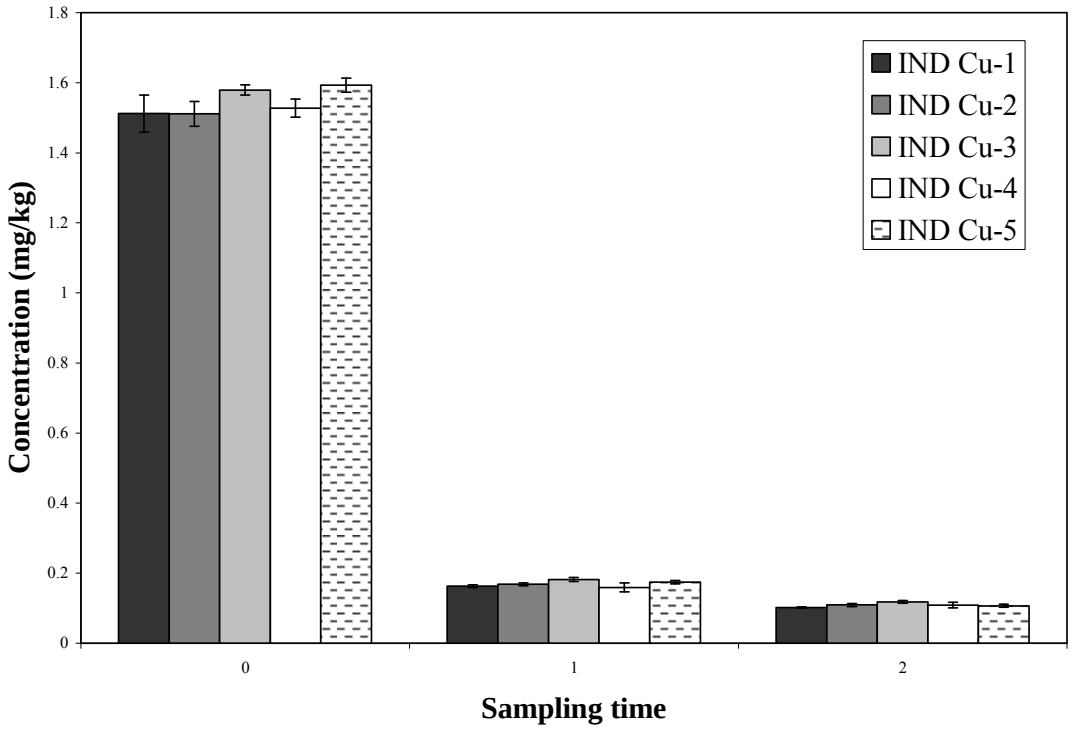

Figure 4.12: Indoxacarb concentrations at each sampling time. Error bars represent standard error of replicate samples (n=3). The detection limit for indoxacarb was 0.0025 mg kg^{-1} (not shown).

4.3.2.2 Half-life of Indoxacarb

Indoxacarb is known to exhibit bi-phasic degradation in soil, characterised by an initial rapid degradation period, followed by a slower degradation period (Dias, 2006). Thus, the first rapid phase of indoxacarb's half-life in the Templeton experimental soil was calculated from the exponential degradation of extracted indoxacarb residues from t_0 to t_1 (Figure 4.13). Since there were no significant differences in residual indoxacarb concentrations between the Cu-levels at each sampling time (refer to Section 4.3.2.1 above), the residual concentrations obtained at t_0 and t_1 were averaged in order to determine the degradation equation ($y = 1.5445e^{-0.0368x}$; $r^2=1$, because there were only two points; Figure 4.13). Half the averaged residual amount of indoxacarb extracted from the experimental soil at t_0 was calculated to be 0.772 mg kg^{-1}. This concentration was inserted into the equation and the resulting estimated half-life of indoxacarb in the experimental soil was 18.8 d. The bi-phasic degradation characteristic of indoxacarb was evident in the experimental soil; over 90% of the initial spike concentration (2 mg kg^{-1}) degraded within the first sampling time of 60 d (Figure 4.12). Therefore, the estimated half-life of indoxacarb in the Templeton experimental soil and under the specific experimental conditions was ≤18.8 d for the first rapid degradation phase. It was not known exactly where the first rapid degradation phase ended and the second slower degradation phase began. For this reason, the half-life of indoxacarb between t_1 and t_2 had not been calculated. A better estimate of indoxacarb's half-life in the Templeton experimental soil could have been obtained if samples had been taken and analysed multiple times between t_0 and t_1. With a higher sampling frequency, the residue data and resulting fit into the degradation equation would more accurately represent the first rapid phase of degradation for indoxacarb. This would also have provided an improved estimate of where the second degradation phase began, so the half-life of indoxacarb in the second slower phase of degradation could be calculated.

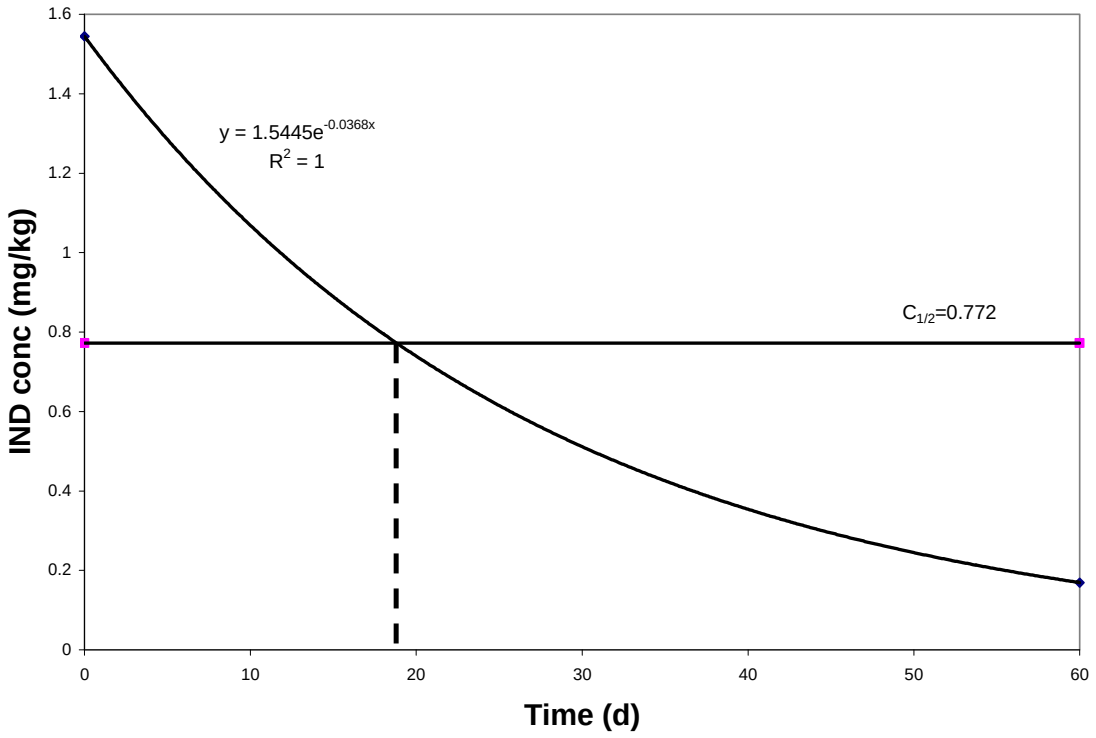

Figure 4.13: Estimated half-life of indoxacarb for first degradation phase in the Templeton experimental soil, calculated from the point where half the initially extracted indoxacarb concentration ($C_{1/2}$) crossed the exponential degradation of indoxacarb between t_0 and t_1.

As indoxacarb is a relatively new insecticide, there is no data available on its fate in New Zealand soils. This study provides the first data on its fate in New Zealand. Few international studies have investigated or published findings on the environmental fate of indoxacarb and the majority of the available data has been generated by Du Pont, the manufacturer and marketer for indoxacarb. Many of the published studies, for example Campbell *et al.* (2005) and Rose *et al.* (2009), only investigated the concentrations of parent pesticide residues in various media (soils, grapes, etc.) and not metabolite concentrations. Du Pont (2006) reported that indoxacarb was rapidly degraded in aerobic soils under laboratory and field conditions (<1–12 d and 9–27 d, respectively). However, the US EPA reported its half-life ranged between 3–693 d in aerobic soils (U.S. EPA, 2000b). A half-life of 7.6 d has been reported for indoxacarb under field conditions in China (Zhou and Li, 2008). The half-life of indoxacarb obtained these experiments for the Templeton experimental soil falls within ranges reported by both Du Pont and US EPA, but is higher than that reported for the Chinese study.

4.3.3 Relationships between Pesticides and Soil Biological Properties

The soil biological properties investigated in the experimental soils began showing significant decreases in activity and microbiomass above a total Cu concentration of 100 mg kg^{-1}. However, the both parent pesticides did not correlate with either total Cu or the soil biological properties in the experimental soils. This suggests that the soil biological properties investigated may not be sufficient to predict the degradation of the parent pesticide compounds. The activities of other enzymes that are more closely associated with the degradation of these pesticides may be more appropriate indicators of microbial activity in relation to pesticide degradation. In contrast to atrazine, HA began showing increased persistence above a total copper concentration of 100 mg kg^{-1}, which corresponds to the Cu-level where decreases in microbial activity and microbiomass were observed. HA negatively correlated with the microbiomass at t_1 ($p<0.05$) and phosphatase activity at t_2 ($p<0.05$) in the experimental soils. These significant relationships indicate that the soil biological properties investigated were better predictors of metabolite concentrations than parent pesticide concentrations in the experimental soils.

4.4 CONCLUSIONS

Atrazine and indoxacarb concentrations in the experimental soils declined over time as they degraded. HA was the only metabolite of atrazine detected in the spiked experimental soils using the developed analytical method and will likely be the predominant metabolite detected in similar soils amended with atrazine. Indoxacarb metabolites were not investigated.

Copper did not inhibit the degradation of indoxacarb or atrazine in the experimental soil. Total Cu, bioavailable Cu and the soil biological properties did not have significant relationships with parent pesticide concentrations. Significant differences ($p<0.05$) for HA at t_2 between Cu-1 and Cu-3, Cu-4, and Cu-5 suggest that Cu may have negatively impacted the degradation of this metabolite in soil. This is confirmed by negative correlations between HA and the microbiomass at t_1 and phosphatase activity at t_2 ($p<0.05$). These relationships indicate that the soil biological properties investigated may be used to predict the persistence of HA in Cu-contaminated soils. However, more research is necessary in order to confirm the mechanisms (abiotic or biotic).

The half-lives of both atrazine (≤19.4 d) and indoxacarb (≤18.8 d) were lower in the Cu-spiked experimental soils than the medians reported in previous New Zealand and international studies. However, they were still within the national and international ranges.

Chapter 5: Final Conclusions

This chapter brings together the main conclusions from the overall investigation. Implications of the key findings are discussed along with recommendations for future research.

5.1 SUMMARY OF KEY FINDINGS

Total and bioavailable copper concentrations were strongly correlated in the experimental soil ($p<0.05$). The concentrations of both the total and bioavailable copper fractions did not change over time, suggesting that the soils were homogeneous, very little copper leached during aging, and that the soils were sufficiently aged before pesticide amendments. Additions of copper and pesticides did not alter the soil properties (pH, CEC, %TOC).

Elevated copper concentrations adversely affected the soil microbial community. The phosphatase and urease activities, and microbiomass concentrations were negatively correlated with total copper concentrations ($p<0.05$). Total copper was a better indicator of enzyme activities and microbiomass concentrations than bioavailable copper in the experimental soil. The soil biological properties showed adverse effects above a level of 100 mg Cu kg^{-1} in the experimental soils. This was the limit set by the New Zealand biosolids guidelines in order to protect plant, human and microorganism health (NZWWA, 2003). The continued decrease in enzyme activities over time could be explained by a lack of organic matter added to the experimental soil over the course of the aging and sampling periods, and may not have been due to copper toxicity.

Extraction methods were developed to determine the concentrations of atrazine and indoxacarb residues in the pesticide-spiked soils. Elevated concentrations of copper did not inhibit the degradation of either atrazine or indoxacarb in the experimental soils. The half-lives of both atrazine (≤19.4 d) and indoxacarb (≤18.8 d) were lower in the spiked experimental soils than the means reported in previous New Zealand and international studies.

HA was the only metabolite detected in the atrazine-spiked soils with the extraction method utilised. Negative correlations were observed between HA and the microbiomass at t_1 and phosphatase activity at t_2 ($p<0.05$). These negative relationships indicate that elevated copper

concentrations in the experimental soil may have inhibited the degradation of this metabolite by negatively impacting the soil microbial community. They also suggest that the soil biological properties investigated may be used to predict the persistence of HA in copper-contaminated soils.

5.2 IMPLICATIONS

Total copper was a better indicator of impaired microbial activity than bioavailable copper in this study. Though 0.01 *M* $CaCl_2$ has been used as an indicator of microbial activity in previous studies (Merrington *et al.*, 2002), $CaCl_2$ extractable copper concentrations did not correlate with phosphatase activity or with the microbiomass for indoxacarb. Since only one bioavailable copper method was investigated, it is unknown if other bioavailable methods (e.g. NH_4NO_3 or diffusive gradient thin film (DGT) devices) would show more consistent correlations to the soil biological properties. Therefore, multiple bioavailable techniques should be investigated in order to determine significant correlations with soil biological properties under the experimental conditions of the study.

Co-contamination of atrazine- and indoxacarb-spiked soils with copper did not affect the degradation of these parent compounds in the experimental soils. However, elevated copper concentrations did show negative effects on the soil microbial community. Many of the soil biological properties began showing adverse effects above a concentration of 100 mg kg^{-1} Cu in the experimental soils. This suggests that elevated copper concentrations exceeding this value could reduce the overall health of the soil. Since many synthetic organic pesticides, including atrazine and indoxacarb, rely on biodegradation mechanisms in soils, it is important that the health of the microorganisms is maintained to prevent persistence of the pesticides in soils. The persistence of atrazine is especially of concern as it is an EDC. Although elevated copper levels did not affect the persistence of atrazine in this study, care should still be taken when applying atrazine to soils previously contaminated with heavy metals, especially near waterways and shallow aquifers.

The main focus of previous studies has been on the effects of copper on the degradation of parent pesticide compounds (e.g. Gaw *et al.*, 2006; Liu *et al.*, 2007; Morillo *et al.*, 2000). They have shown that copper can inhibit parent pesticide degradation by impairing soil microbial functions (Gaw *et al.*, 2006; Liu *et al.*, 2007) or by increasing adsorption to SOM

(Morillo *et al.*, 2000). However, the persistence of metabolites may also be enhanced in the presence copper. The results from this thesis suggest that elevated copper concentrations may inhibit the degradation of HA in soil, most likely as a result of copper toxicity to the soil microbial community. The formation of HA in the environment is significant as it is non-herbicidal, but little is known about its toxicity to other organisms. HA is more persistent in soils than atrazine or its chloro-metabolites, thus inhibited degradation would result in further persistence. Other studies should also investigate the effects of copper on metabolites, or at the very least, include the detection of metabolites in their investigations. This is especially true for persistent metabolites, such as DDE.

Copper has been shown to inhibit the atz-A enzyme responsible for catalysing the microbial hydrolysis of atrazine to HA (Seffernick *et al.*, 2002). However, it is unknown if copper also inhibits the atz-B or -C enzymes, which catalyse the reactions of HA to cyanuric acid. Inhibition of these enzymes, especially atz-B, would confirm that microbial copper toxicity was the mechanism for HA persistence in the experimental soils.

This thesis provided the first investigation into the degradation of indoxacarb in a New Zealand soil. Little is known to date about the long-term effects of indoxacarb in the environment. As there is a large half-life range for indoxacarb (<1–693 d), it is unlikely that this pesticide will behave exactly as expected based on the manufacturer's instructions. Therefore, in order to prevent unwanted adverse effects on the environment, a precautionary approach should be followed when applying this pesticide to crops until more research is completed.

Based on the results from this thesis, from previous studies and the New Zealand biosolids limit for copper, it is recommended that atrazine and indoxacarb should only be applied to soils with a total copper concentration less than 100 mg kg^{-1}. This would protect the health of the soil microbial community and prevent potential adverse effects copper may have on the degradation of pesticide metabolites in the soil. As many soils in New Zealand and internationally contain elevated levels of heavy metals, it is also recommended that pesticide regulating agencies should consider the effects of heavy metals on the degradation of pesticides and their metabolites when assessing their fate and behaviour in the environment.

5.3 RECOMMENDATIONS FOR FUTURE RESEARCH

The investigations in this thesis have provided insight into the effects of copper contamination on the microbial community and co-contamination effects on atrazine degradation in a New Zealand soil. This thesis also provided the first data for degradation of indoxacarb in a New Zealand soil.

Additional soil biological properties could be investigated to further determine the effects of copper on the soil microbial community, such as PLFA analysis, invertase, sulphatase and lacase enzyme assays, and molecular techniques such as rDNA and DGEE. PLFA analysis can be used to determine compositional changes in the soil microbial community due to the addition of stressors (e.g. metals or pesticides) (Bunemann *et al.*, 2006).

$CaCl_2$-extractable copper is only one method to determine the bioavailable copper fraction in soil. Other methods to determine bioavailable copper fractions and potential exposure to soil microorganisms could also be investigated. These could include different neutral salt extractions (e.g. NH_4NO_3) and DGT devices. These methods could be compared to give a better understanding of the bioavailability of copper in the experimental soil.

As there was evidence that the soil microorganisms may have had decreased activity over time due to insufficient organic matter inputs, an organic matter substrate could be added to the soils to prevent this effect.

Recoveries of residual atrazine and its chlorinated metabolites extracted from the soil may be increased by utilising a buffer/ACN/MeOH extraction mixture, as was used to extract residual indoxacarb from the experimental soil.

In order to conclusively determine if elevated levels of copper effect the degradation of HA, in soil, cyanuric acid residues should be extracted and analysed. DEHA and DIHA were not detected with the method used and cyanuric acid is the next logical metabolite to investigate. Indoxacarb metabolites should also be investigated to determine if copper affects their degradation in the soil.

To accurately determine the half-lives of atrazine and indoxacarb in the experimental soil, additional sampling was needed between t_0 and t_1. This would help determine where the first rapid phase of indoxacarb degradation ended and its second slower phase began.

In order to better understand the effects of copper co-contamination on the degradation of pesticides in New Zealand, other commonly used pesticides should also be investigated. These could include other triazine herbicides (e.g. simazine), glyphosate, dithiocarbamate fungicides and pyrethroid insecticides, and their associated metabolites.

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Appendix A

Table A.1: Significant storm events during field aging of the Cu-spiked soils.

Date	*Storm description*
02.05.08	Heavy weather warning for weekend- didn't start field-aging soils until 06.05.08
16-23.06.08	Frosts followed by warm, sunny days (freeze-thaw)
25.06.08	Hail
05.07.08	Snow
31.07.08	MetService reported 145mm rain fell by 3pm in previous 24 hrs

Table A.2: Mean phosphatase activity (nmol g^{-1} s^{-1}) for the pesticide-spiked soils at t_1 and t_2 (n=4).

Cu-level	*Phosphatase Activity*			
	ATZ t_1	ATZ t_2	IND t_1	IND t_2
Cu-1	6.14	5.21	5.03	4.58
Cu-2	5.57	4.87	4.97	4.37
Cu-3	4.36	3.84	4.02	3.59
Cu-4	3.35	3.15	3.16	3.24
Cu-5	2.85	2.40	2.96	2.48

Table A.3: Mean urease activity (nmol g^{-1} s^{-1}) for the aged Cu-spiked soils and pesticide-spiked soils at t_0, t_1 and t_2 (n=4).

Cu-level	*Urease Activity*						
	Aged Cu	ATZ t_0	ATZ t_1	ATZ t_2	IND t_1	IND t_2	IND t_3
Cu-1	0.708	0.685	0.572	0.407	0.673	0.663	0.568
Cu-2	0.643	0.629	0.585	0.517	0.668	0.668	0.516
Cu-3	0.565	0.527	0.509	0.441	0.640	0.609	0.423
Cu-4	0.480	0.501	0.408	0.309	0.423	0.483	0.362
Cu-5	0.305	0.337	0.314	0.232	0.313	0.313	0.207

Table A.4: Bioavailable Cu extracted by 0.01 *M* $CaCl_2$ with ICP-MS analysis.

Sampling time	Sample	Bioavailable Cu ($\mu g\ g^{-1}$)	% of Total Cu extracted
Pre-spike	Bulk Soil	0.014	0.39
t_0	ATZ Cu-1	0.016	0.38
	ATZ Cu-2	0.572	0.66
	ATZ Cu-3	1.77	0.70
	ATZ Cu-4	5.52	1.03
	ATZ Cu-5	18.0	1.82
	IND Cu-1	0.017	0.37
	IND Cu-2	0.613	0.73
	IND Cu-3	1.75	0.72
	IND Cu-4	5.76	1.07
	IND Cu-5	17.3	1.72
t_1	ATZ Cu-1	0.016	0.36
	ATZ Cu-2	0.651	0.76
	ATZ Cu-3	1.93	0.75
	ATZ Cu-4	5.92	1.09
	ATZ Cu-5	16.3	1.53
	IND Cu-1	0.022	0.45
	IND Cu-2	0.815	0.91
	IND Cu-3	2.36	0.93
	IND Cu-4	6.75	1.21
	IND Cu-5	18.3	1.73
t_2	ATZ Cu-1	0.018	0.39
	ATZ Cu-2	0.655	0.74
	ATZ Cu-3	1.81	0.71
	ATZ Cu-4	5.97	1.06
	ATZ Cu-5	19.6	1.90
	IND Cu-1	0.031	0.65
	IND Cu-2	0.717	0.80
	IND Cu-3	2.36	0.92
	IND Cu-4	6.39	1.13
	IND Cu-5	20.1	1.97