Abiotic and Biotic Influences on Acetochlor Fate in Pristine Soils and Subsoils

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by

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Abbreviations

In this study, the names of chemical compounds and elements are abbreviated using standard accepted chemical symbols and formulae, unless noted below.

2,4-D	2,4-dichlorophenoxyacetic acid
2,4-DCP	2,4-dichlorophenol
acetochlor	2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl) acetamide
ac-	acetochlor-based
alachlor	2-chloro-**N**-(2,6-diethylphenyl)-*N*-(methoxymethyl) acetamide
al-	alachlor-based
aldrin	1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-*exo*-1,4-*endo*-5,8-dimethanonaphthalene
allidochlor	2-chloro-*N*,*N*-di-2-propenylacetamide
atrazine	2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine
ARP	Acetochlor Registration Partnership
butachlor	*N*-(butoxymethyl)-2-chloro-*N*-(2,6-diethylphenyl) acetanilide
butylate	S-ethyl diisobutylthiocarbamate
bq	becquerel(s)
carbofuran	2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate
CCPR	Codex Committee on Pesticide Residues
CEC	cation exchange capacity
CFUs	colony forming units
chlorpyrifos	O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate
chlorthal dimethyl	dimethyl tetrachloroterephthalate
CPM	counts per minute
cyanazine	2-([4-chloro-6-(ethylamino)-8-triazin-2-yl] amino)-2-methylpropionitrile
d	days(s)
DC	direct count
dieldrin	1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-endo,exo-1,4:5,8-dimethanonaphthalene
DNA	deoxyribonucleic acid
DOM	dissolved organic matter
DPM	disintegrations per minute
DPS	disintegrations per second
DT	dissipation time
d wt	dry weight
EPTC	S-ethyl dipropylthiocarbamate
ESA	ethane-sulphonic acid
FAME	fatty acid methyl ester

FDA	fluorescein diacetate
(f, p, n, μ, k)g	(femo, pico, nano, micro, milli, kilo) gram(s)
x *g*	relative centrifugal force
GSH	glutathione
GST	glutathione S-transferase
h	hour(s)
ha	hectare(s)
HPLC	high performance liquid chromatography
INT	2 (*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride
INTF	iodonitrotetrazolium formazan
isoproturon	3-(4-isopropylphenyl)-1,1-dimethylurea
isoxaben	*N*-[3-(1-ethyl-1-methylpropyl)-5-isoxazolyl]-2,6-dimethoxybenzamide
IUPAC	International Union of Pure and Applied Chemistry
K_d	adsorption distribution coefficient (ml/g)
K_F	Freundlich adsorption distribution coefficient (ml/g)
K_{Fd}	Freundlich desorption distribution coefficient (ml/g)
K_{OC}	organic-carbon-based sorption coefficient
LSC	liquid scintillation counting
linuron	*N*'-(3,4-dichlorophenyl)-*N*-methoxy-*N*-methylurea
(μ, m, c, k)m	(micro, milli, centi, kilo) metre
(n, μ, m)M	(nano, micro, milli) molar
metazachlor	2-chloro-N-(2,6-dimethylphenyl)-N-(1*H*-pyrazol-1-ylmethyl) acetamide
metolachlor	2-chloro-N-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl) acetamide
min	minute(s)
MRL	maximum residue levels
mRNA	messenger ribonucleic acid
N/a	not applicable
ND	not determined
NMR	nuclear magnetic resonance
OD	optical density
OXA	oxanilic acid
PGW	prospective groundwater site
picloram	40amino-3,5,6-trochloro-2-pyridinecarboxylic acid
PMT	photomultiplier tube
POM	particulate organic matter
ppb	parts per billion
pretilachlor	2-chloro-*N*-(2,6-diethylphenyl)-N-(2-propoxyethyl) acetamide
propachlor	2-chloro-*N*-(1-methylethyl)-*N*-phenylacetamide
propyzamide	3,5-dichloro-*N*-(1,1-dimethyl propynyl) benamide

rev min^{-1}	revolutions per minute
Rf	retardation factor
S	second(s)
simazine	2-chloro-4,6-bis(ethylamino)-s-triazine
SOM	soil organic matter
thiazafluron	1,3-dimethyl-1-(5-trifluoromethyl-1,3,4-thiadiazol-2-yl)urea
TLC	thin-layer chromatography
TOC	total organic carbon
USEPA	United States Environmental Protection Agency
UV	ultraviolet
VBNC	viable but not-culturable
VLCFA	very long chain fatty acid(s)
v/v	volume for volume
wet wt	wet weight
w/w	weight for weight

All other abbreviations are explained where appropriate in the text.

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Abstract

Soil exists as an intricate matrix in which a wide variety of biotic (e.g. enzymes, macro-, micro- fauna) and abiotic (e.g. clay minerals, oxides, humic substances, organo-mineral composites) factors interact, forming a highly dynamic and heterogeneous environment. Upon release into this complex environment, pesticides are subject to a number of processes that result in sorption to soil surfaces, biodegradation/transformation, or leaching. Pesticides leaching through a soil profile to groundwater will be exposed to changing environmental conditions as different horizons with distinct physical and chemical properties are encountered. The way these divergent soil properties influence pesticide degradation and retention needs to be assessed to allow accurate predictions of environmental fate and more efficient management practices.

To address this issue, soil cores were taken from two soil profiles (surface textures: silty clay loam and loamy sand), and samples taken from 0-30 cm (surface), 1.0-1.3 m (mid) and 2.7-3.0 m (deep; clay) and 3.9-4.2 m (deep; sand). A variety of soil biotic (microbial numbers, microbial biomass and enzyme activities) and abiotic (pH, organic matter content, texture, CEC) properties were measured for each soil. Microbial numbers and enzyme activities were found to decrease significantly with soil depth and were positively correlated to the organic matter content. An exception was urease activity in the clay soil, under buffered conditions, where a 2.9-fold greater activity was exhibited in the mid soil compared to the surface soil. Although microbial numbers did decrease with soil depth substantial numbers of bacteria were still isolated from the deep soils (direct counts: 5.6 x 10^8 sand, 4.5 x 10^8 clay) despite only representing 4.7 and 1.7 % of those in the respective surface soils.

Equilibrium sorption and desorption isotherms of ^{14}C-ring-labelled acetochlor revealed that the sorptive behaviour of this pesticide varied with soil depth. The difference in retention capacity with soil depth was strongly correlated to soil organic carbon content. Differential desorption characteristics were also apparent between different particle size fractions, highlighting the influence of microsite variation on pesticide fate in soil, and this was also related to the soil organic carbon content of the fractions.

Degradation and sorption processes were coupled in a long-term (100 d) fate study of acetochlor, under laboratory conditions. Acetochlor was shown to dissipate under biotic and sterile conditions, with the formation of a number of environmentally stable metabolites including ethanesulphonic acid and oxanilic acid derivatives. Mineralization was not a major fate process with less than 5 % of the initially applied acetochlor recovered as 14CO2. Nonextractable residue formation occurred instantly and rapidly progressed over the initial 21 d of incubation. Nonextractable residues were unevenly distributed between soil size factions, concentrated in the macroaggregate fractions. Nonextractable residue formation was enhanced under biotic conditions for those fractions. Under biotic conditions, DT50 values of 9.32, 12.32 and 12.56 d were determined for acetochlor in clay surface, mid and deep soil, respectively. Further experiments are needed to generate more data, to enable accurate modelling of pesticide fate.

Chapter 1. General Introduction

1.1. Herbicide fate in the environment

1.1.1. Introduction

A prerequisite of modern conventional agronomic cropping practices is the use of pesticides to maximise productivity and to maintain economic viability. Pesticides, where used appropriately, can save up to 40 % in crop losses; however, when pesticides are mal-, mis-, or over-used the environmental and public health consequences can be considerable (Richards, 1998). The inherent biocidal nature of these chemicals, and their general broad selectivity (Conway and Pretty, 1991), has roused great public and scientific concern over the potential health hazards posed by their persistence in the environment. The resultant tightening of water quality regulations, triggered also by advances in detection methodologies, has led to the implementation of routine groundwater monitoring programmes. The outcome of these programmes has been an increase in the frequency of reported cases of groundwater contamination by agricultural chemicals over the last decade (Goodrich *et al.*, 1991; Potter and Carpenter, 1995; Kolpin *et al.*, 1996; Kolpin *et al.*, 2000). The most extensively used (and most widely found) pesticides are herbicides. A common occurrence reported in the Kolpin *et al.* (2000) study was the detection of multiple herbicide compounds (e.g. acetochlor [2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl) acetamide], alachlor [2-chloro-N-(2,6-diethylphenyl)-*N*-(methoxymethyl) acetamide], atrazine [2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine], cyanazine [2-([4-chloro-6-(ethylamino)-8-triazin-2-yl] amino)-2-methylpropionitrile], metolachlor [2-chloro-N-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl) acetamide]) from a single water sample, which could potentially produce synergistic toxicity effects (Pape-Lindstrom and Lydy, 1996).

Soil exists as an intricate matrix in which a wide variety of biotic (e.g., enzymes, macro- and micro- fauna) and abiotic (e.g., clay minerals, oxides, humic substances, organo-mineral composites) factors interact, forming a highly dynamic and heterogeneous environment (Chenu and Stotzky, 2002). Once pesticides are released into the environment their fates become inextricably linked to a number of naturally occurring processes, which can be broadly divided into the following categories: degradation, sorption, leaching, volatilisation and albeit a small component, plant uptake (Figure 1.1). Soil half-lives for compounds in

widespread use range from 10 days to several years, but for most mobile pesticides they are normally less than 100 days (Chilton *et al.*, 1998).

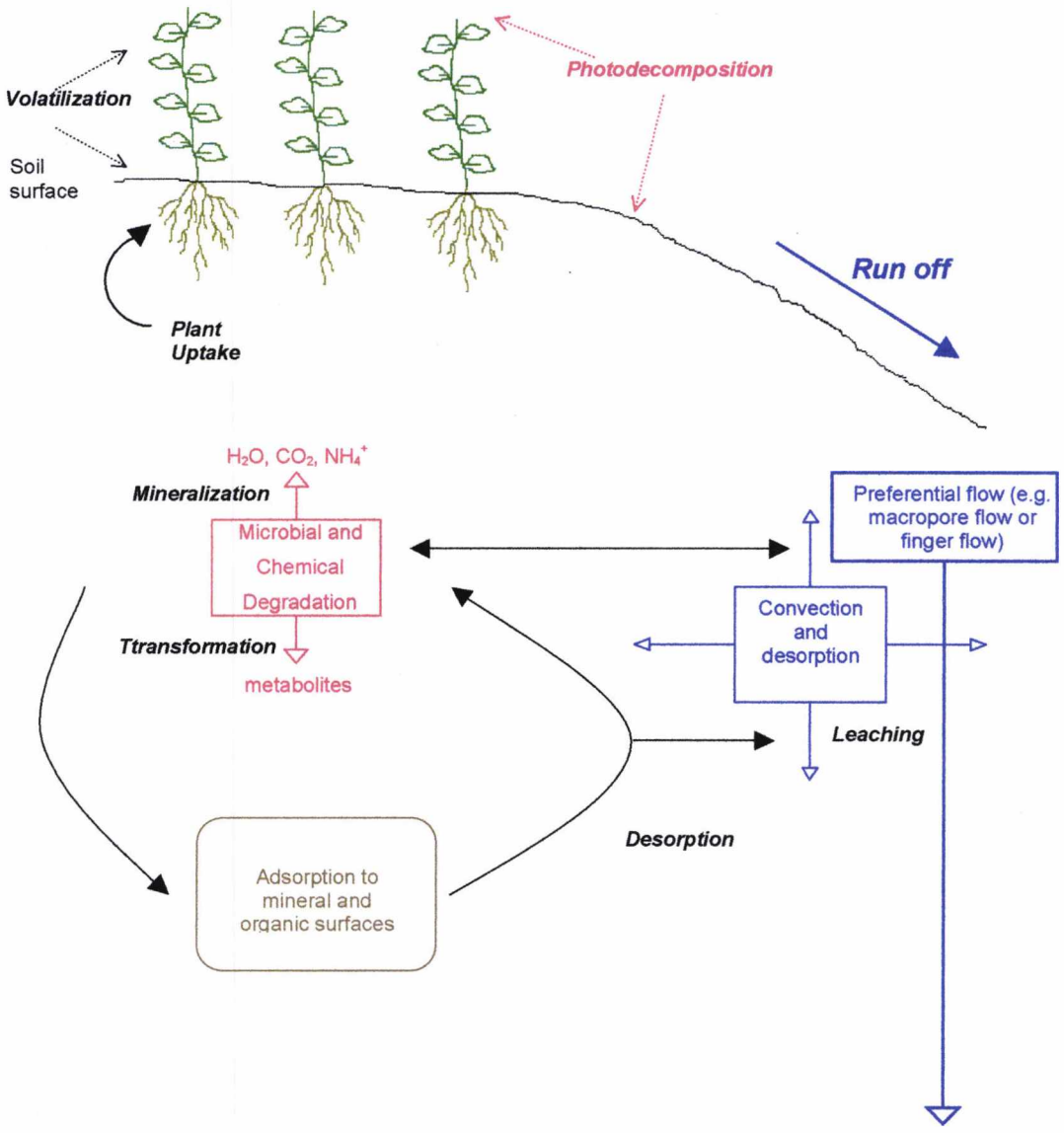


Figure 1.1. Fate processes affecting pesticides once released into soil

1.1.2. Degradation and transformation processes

Pesticides are degraded through photolytic, chemical and biological processes, yielding metabolites or, after several transformation reactions, simpler products such as ammonia and carbon dioxide. Phototransformation takes place when irradiative sunlight causes the decomposition or disassociation of the pesticide molecule. The rate of photolysis is dependent on the energy of incident sunlight, the adsorption spectrum of the chemical and the presence of photosensitizers, such as humic and fulvic acids (Richard *et al.*, 1997; Mansour *et al.*, 1999). Chemical degradation occurs when the molecule is unstable in the conditions of its environment. For example, several organophosphate and *s*-triazine pesticides have been degraded on clays by surface catalysis due to the acidity (Brown and White, 1969; Mingelgrin *et al.*, 1977), and humic acids and fulvic acids have been shown to catalyse the dechlorohydroxylation of atrazine (Li and Felbeck Jr., 1972; Khan, 1978). Abiotic degradation has been reviewed elsewhere by Wolfe *et al.* (1990).

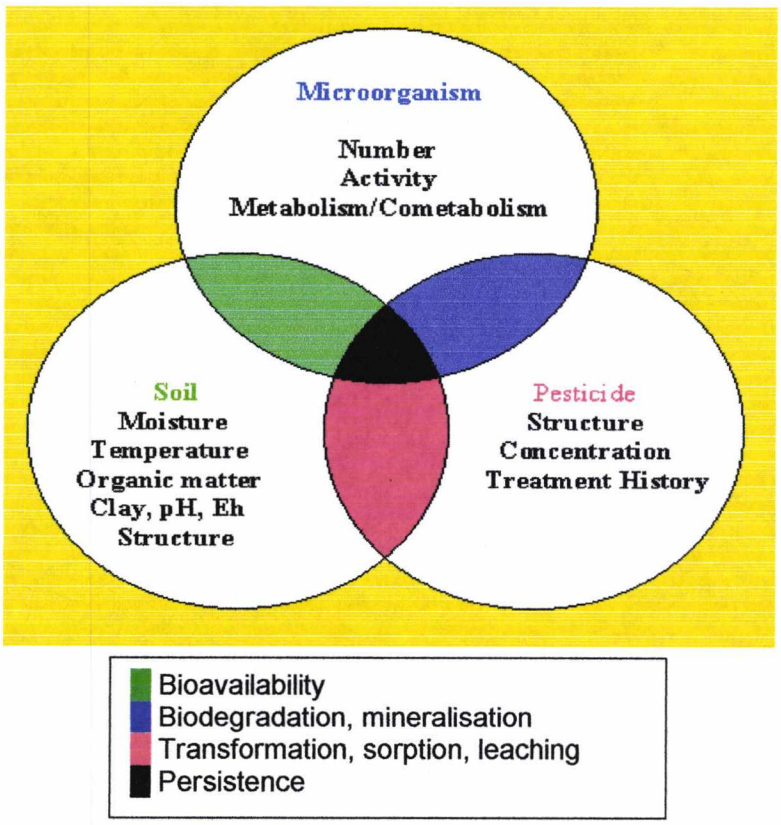


Figure 1.2. Factors affecting pesticide persistence in the environment (adapted from Topp *et al.*, 1997).

The dominant cause of pesticide loss in soil is of biological origin, with the metabolic multiplicity and ubiquitous distribution of microorganisms key to the destruction of these and other organic compounds. This has been proven numerous times with studies comparing degradation rates in sterile and non-sterile soils (e.g. Slade *et al.*, 1992; Dinelli *et al.*, 1998; Taylor-Lovell *et al.*, 2002). However, the rates and routes of biodegradation are ultimately dependent upon the physical nature of the chemical, the soil and the catabolic potential of the indigenous microbial populations, none of which can be considered in isolation when assessing the environmental fate of a particular compound (Figure 1.2.). For example, it has been demonstrated in artificial saturated subsoil that degradation can occur under methanogenic, sulphate-reducing, denitrifying and aerobic conditions, with different degradation rates for different compounds (Nay *et al.*, 1999).

Organic compounds can be rapidly mineralized, releasing simple inorganic compounds such as CO_2, H_2O and mineral elements, whereas others can only be partially mineralized yielding terminal degradation products. Mineralization is generally associated with growth-linked degradation, i.e. the compound is being used as an energy source or to produce cell components (Kunc and Rybarova, 1983).

Xenobiotics are subject to fortuitous metabolism or cometabolism, where degradation occurs coincidently to the general metabolic activities of the soil community, generating no energy for the organisms involved. Cometabolic transformation commonly generates terminal degradation products, which may be further altered by secondary physical and chemical reactions. The creation of these reactive metabolites (e.g. epoxides, dihydrodiols, aromatic diols, aromatic amines) can be disadvantageous, in terms of environmental quality. Transformation products may be retained in the soil matrix by adsorption reactions and misrouting of contaminants into unproductive metabolic routes In addition, generation of toxic intermediates (Belfroid *et al.*, 1998) capable of inhibiting the biodegradative processes taking place may occur (Knackmuss, 1996). In natural environments, complex interactions take place within the indigenous microbial communities, which can lead to syntrophy, where cometabolically produced metabolites from one species are utilised as carbon and energy sources by other members of the community. This can, therefore, lead to extensive degradation or even mineralization of a compound (Park *et al.*, 1999).

For the biotreatment of pollutants in high concentrations, in industrial effluents, conditions can be tailored to accentuate transformation and detoxification processes. In contrast, the diffuse nature of agrochemical contamination and the low concentrations of such deliberately released compounds found in the environment, render this clean-up approach untenable

(Rieger *et al.*, 2002). Unfortunately, despite the immense catabolic potential of microbes to breakdown various natural and synthetic compounds and their evolutionary potential to develop new catabolic traits (Timmis and Pieper, 1999), they are not a universal remedy. For example, dieldrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-endo,exo-1,4:5,8-dimethanonaphthalene), an epoxide metabolite of the insecticide aldrin (1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-*exo*-1,4-*endo*-5,8-dimethanonaphthalene) and pesticide in its own right, was detected on sites that had not been exposed to aldrin or this chemical for over 20 years (Alexander, 1999).

This lack of observable biodegradation could be due to a number of factors. Firstly, low concentrations of a chemical may fail to induce the formation of enzymes necessary for its degradation and, even if the enzymes are induced, steric hindrance could prevent the enzyme from reaching its substrate (Brandl *et al.*, 1995). Secondly, certain substituents are rare (e.g. halo- and nitro- groups) or virtually unknown (e.g. fluoro-, sulpho- and azo- groups) in natural compounds (Rieger *et al.*, 2002), and thus enzymes that have the capacity to breakdown molecules structured in this way may not exist naturally. Under field conditions, the evolutionary processes that occur may necessitate long periods of time before such catabolic traits can develop. Lastly, the proliferation of the potentially small population of degrading microbes (Jayachandran *et al.*, 1997) or the dissemination of catabolic genes into other non-degrading populations (De Souza *et al.*, 1998) may be required before noticeable mineralization can occur.

Despite the small quantities of pesticides in the environment, which are generally not toxic, the potential for long-term exposure affecting susceptible species or individuals as well as the threat of biomagnification renders the removal of these compounds preferable. In order to minimise the risk of environmental contamination, thorough appraisals of the risks of individual compounds and the prerequisite controls need to have been carried out prior to the use of any agrochemical. The outcome of such an appraisal is the basis for the registration criteria set for a given agrochemical, with Section 1.3.3, detailing the conditions imposed on the registration and use of acetochlor.

1.1.3. Sorption

The magnitude of pesticide sorption to soils is generally expressed using the sorption or distribution coefficient, K_d (ml/g). The value of this coefficient is dependent on the properties of the both the chemical and the soil, and the relationship is commonly expressed as:

$$[1] \quad S = K_d C$$

where S is the mass of sorbed pesticide (μg/g) and C is the equilibrium solution concentration (μg/ml). For non-ionic chemicals, the sorption coefficient can be normalised for soil organic carbon content to yield a chemical specific constant:

$$[2] \quad K_{oc} = \frac{K_d}{f_{oc}}$$

where K_{oc} is the organic-carbon-based-sorption coefficient and f_{oc} is the fraction of organic carbon in soil. The linear relationship depicted in equation [1] is especially applicable for pesticides in the soil solution at low concentrations, as would result from normal agricultural use. However, it has been stated that linearity is often assumed without the construction of an adsorption isotherm and that the widespread lack of marked curvature has led to 'intrinsically non-linear' isotherms being mistaken for linear (Pignatello, 1998).

The description of sorption has two distinct approaches: sorption site theory and partitioning theory. The difference between these is the degree to which the sorbate molecule interacts with or is free to migrate within the sorbent phase (Weber *et al.*, 1991). The influence of water on sorbent properties is critical; contaminant molecules have to traverse water or a water-swollen phase to reach sorption sites, even in the unsaturated zone of the soil column. This implies that solution-solid interactions dominate sorption processes in soil (Pignatello, 1998). Soil porosity is also of paramount importance for the mechanism of sorption, with capillary forces inside small pores reducing the vapour pressure as well as the liquid solubility (Corley *et al.*, 1996). Xing and Pignatello (1997) proposed a dual-mode sorption model incorporating partitioning and hole-filling to account for non-linear sorption. Soils contain both fixed-pore micrographitic substances (such as hard coals, soot and charcoal), which are associated with adsorption and pore-filling (pores or holes being nanometer-size voids or cavities), and flexible-pore macromolecular substances (such as fulvic and humic acids) which are capable of absorbing small molecules (Chen *et al.*, 1997).

Although sorptive behaviours, such as non-linearity (Xing and Pignatello, 1997; Xia and Ball, 1999), competitive effects (Xing and Pignatello, 1998; Xia and Ball, 2000) and sorption-desorption hysteresis (Kan *et al.*, 1998) have been frequently observed, the mechanisms for such non-ideal behaviour are not yet fully understood. The assumption that organic matter acts as a rubbery polymer implies a linear and non-competitive sorption process (Pignatello,

1998), but the aforementioned non-ideal behaviour suggests more than just a dissolution mechanism. The presence of fixed-pore substances has been suggested to be the causal agent due to hole-filling (Xia and Ball, 1999), but recently the deformation of flexible pores in soil organic matter has been implicated in the hysteretic and slow desorption behaviours (Xia and Pignatello, 2001).

Sorption processes control the persistence and environmental fate of agrochemicals, defining their transport, reactivity and bioavailability. Sorption occurs through both physical and chemical phenomena, including van der Waals forces, ionic bonding, hydrogen bonding, ligand exchange, charge-transfer complexes, hydrophobic partitioning, covalent bonding and sequestration (see reviews by Weber *et al.*, 1991; Gevao *et al.*, 2000). Such binding mechanisms usually act in concert, with the relative importance of each being dependent on the given environmental conditions. The most persistent complexes result from direct covalent binding of chemicals and their metabolites to soil humic substances or clays (Bollag *et al.*, 1992). The constituents of humus undergo humification through oxidative coupling and it is likely that this process leads to the incorporation of organic contaminants and their breakdown products, especially if these compounds are structurally similar to humic material, i.e. phenolic in character (Bollag *et al.*, 1992). Oxidative coupling can occur through the action of abiotic and biotic catalysts, including enzymes of both microbial and plant origin (e.g., peroxidases, laccases, tyrosinases), inorganic chemicals and clay (Wang *et al.*, 1986; Dec and Bollag, 1997). Such chemical linkages will also result in the loss of a compounds chemical identity (Calderbank, 1989; Dec and Bollag, 1997).

Although organic matter is recognized as the principal factor involved in the sorption of many organic chemicals, the roles played by clay and other mineral colloids are also significant; in many cases both major soil constituents are involved. The soil clay fraction, with its high cation exchange capacity, has been shown to be responsible for the sorption of numerous polar herbicides (Roldan *et al.*, 1993; Cox *et al.*, 1996). The extent of sorption to clay surfaces and organic matter-coated clays is dependent on the structure of the herbicide, the type of clay and the nature of the coating, e.g., alachlor sorption to Na-montmorillonite decreased proportionally to the amount of natural organic matter sorbed to the clay (Torrents and Jayasundera, 1997). Surface complexation, ion exchange and hydrophobic partitioning are all potential sorption mechanisms to bind contaminants to clay and colloidal fractions. Soil colloids, with their high surface area and small particle size, are also important for the adsorption of polar organic compounds, especially in the case of smectites where internal surfaces are accessible to these compounds. Thiazafluron [1,3-dimethyl-1-(5-trifluoromethyl-

1,3,4-thiadiazol-2-yl) urea] was adsorbed in this interlayer space in smectites and montmorillonite (Cox *et al.*, 1995, 1997a). The amino functional groups on polar organic compounds such as atrazine can protonate to form a cationic compound that can be adsorbed by ion exchange to clay minerals. The organic (humic substances) and inorganic fractions (clays and oxyhydroxides) can contribute to adsorption both separately and in combination (Moreau-Kervévan and Mouvet, 1998).

Binding to soils reduces the amount of compound available to interact with the biological components of soil and, as the amount of available chemical diminishes, so does its relative toxicity (Ogram *et al.*, 1985). The degradation of these chemicals may also be reduced, as it has been reported that only aqueous phase compounds are available to microorganisms. For example, 2,4-dichlorophenoxyacetic acid (2,4-D) could only be degraded by a species of *Flavobacterium*, when it was dissolved in water with no degradation of 2,4-D occurring when it was adsorbed to soil or clay (Ogram *et al.*, 1985). This study also showed that biodegradation could only take place after the 2,4-D desorbed. However, there is evidence that sorbed chemicals can be degraded directly. Chlorinated phenols, covalently bound to humic acid via enzyme-mediated coupling, underwent partial mineralization (up to 10 % in 12 weeks) by mixed cultures of soil bacteria (Dec and Bollag, 1988; Dec *et al.*, 1990). The mineralization rate was actually higher for sorbed 2,4-dichlorophenol (2,4-DCP) than for the free chemical over this time period. This degradation of sorbed phase chemicals could be due to adsorbed molecules being transported directly into cells adhered to the same surface (Alexander, 1999).

Apparent increases in the adsorption distribution coefficient, K_d, as the residence time of the chemical in the soil increases has pointed to sorption hysteresis. Such increases in this coefficient have been observed for a variety of pesticides including picloram [40amino-3,5,6-trochloro-2-pyridinecarboxylic acid] (McCall and Agin, 1985), linuron [*N*'-(3,4-dichlorophenyl)-*N*-methoxy-*N*-methylurea)], isoxaben [*N*-(3-[1-ethyl-1-methylpropyl]-5-isoxazolyl)-2,6-dimethoxybenzamide] and propyzamide [3,5-dichloro-*N*-(1,1-dimethyl propynyl) benamide] (Walker, 1987), carbofuran [2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate] (Shelton and Parkin, 1991), atrazine and metolachlor (Pignatello and Huang, 1991) and isoproturon [3-(4-isopropylphenyl)-1,1-dimethylurea] (Gaillardon and Sabar, 1994; Cox *et al.*, 1998b; Oi *et al.*, 1999). This hysteresis has been attributed to preferential degradation in the soil solution accompanied by limited re-equilibration from the adsorbed phase and has been observed for a number of sulphonylurea herbicides (Duffy *et al.*, 1993). This phenomena has been linked to redistribution of the chemical from weaker to stronger adsorption sites as

preferential degradation of readily available chemical occurs, leaving residues inaccessible in nanopores and bound chemicals relatively untouched (Hatzinger and Alexander, 1995; Dec *et al.*, 1997a; Koskinen *et al.*, 2001).

These bound residues are classed as such, according to their lack of extractability after exhaustive solvent extraction and it has been shown that aging residues become less toxic with time (Hatzinger and Alexander, 1995; Kelsey *et al.*, 1997; White *et al.*, 1997). However, despite the extensive documentation of non-extractable residue formation, the long-term environmental risk of such bound residues remains a contentious issue. Although soil bound residues are highly stabilised, there is potential for their release from the soil particles into the solution phase where they can be subject to transformation or leaching into groundwater and therefore may have long-term ecotoxicological significance. From a more pragmatic perspective it could be argued that the bound residues are effectively trapped chemical molecules, slowly degraded to products that pose no short- or long- term environmental risk. Although released residues may be bioavailable while in solution, it is also likely that the residue may become bound to soil particles again. Scheunert *et al.* (1995) concluded that the consequent effects on biological systems would only be noticeable if the concentration of the bound residues was sufficiently high and that it would be highly unlikely that such concentrations would exist in agricultural soils. However, accidental spillages could release pesticides into the environment in larger concentrations than would be expected from normal field application rates, producing greater bound residue formation.

1.1.4. Leaching

A general macroscopic theory for water movement in unsaturated soils led to Richards` equation, which as analytical and numerical advances have occurred has been extended into many forms, to incorporate such phenomena as rigid and non-rigid soils (reviewed by Raats, 2001). Water movement is generally assumed to follow either a Darcy-type flow or equations for tubular flows are applied (Marshall *et al.*, 1996), whereas solutes or chemicals are moved by mass transport. Field studies have shown that small amounts of pesticides and their metabolites can move rapidly through the soil profile, while the bulk of the pesticides remain in the soil surface (Kladivko, 1991). Layering, aggregation, cracks, fissures, and biopores such as earthworm burrows or root channels, can have a large impact on the movement of water and transport of solutes in soil, resulting in macropore or preferential flow (Flury *et al.*, 1995). However, preferential flow, thought to be the cause for chemicals reaching subsoils

and groundwater (albeit in low concentrations), can also be caused by non-homogenous infiltration and wetting front instabilities (Sollins and Radulovich, 1988; Selker *et al.*, 1992).

Dual porosity models, such as those incorporating the advective-dispersive equation, are routinely used to describe chemical transport in soil and take into account both macropore and micropore flow. They are based on distinguishing a mobile and a stagnant phase, roughly corresponding to networks of large and small pores, respectively (Raats, 2001). Application of these models to soils with no significant macroporosity effectively defaults the transport pattern to the standard Richards` equation. Other factors taken into account by these models include the mechanisms of transport in the mobile phase and the nature of the storage capacities of the phases and the associated exchanges between the phases (Raats, 2001). For non-equilibrium transport the conventional advective-dispersive equation can be amended with a term to describe rate-limited adsorption (Guo *et al.*, 1997).

The potential association of contaminant compounds with colloids may lead to facilitated transport (McCarthy and Zachara, 1989). The colloidal phase is mainly composed of clay minerals, amorphous silica, metal oxyhydroxides, carbonates, fragments of primary minerals, (e.g., quartz or feldspars) and associated organic matter (Elimelech and Ryan, 2002), with their abundance dependent on chemical and hydrodynamic perturbations. For colloidal facilitated transport to occur, the binding mechanism must be extensive and irreversible (Seta and Karathanasis, 1997b), with slow desorption kinetics (Roy and Dzombak, 1998). The mobility of the colloidal fraction itself is governed by filtration or adsorption by the soil matrix and is likely to be affected by surface charge heterogeneity (Song, 1994; Johnson *et al.*, 1996). For example, size exclusion phenomena within the soil matrix will reduce the tortuousity of flow enhancing colloid transport, whilst exposure to soil aggregate surfaces will enhance colloidal deposition (Grolimund *et al.*, 1998).

The enhanced transport of colloids due to macroporosity and preferential flow is, therefore, self-evident with such a transport mechanism reducing the potential for attachment due to the increased hydraulic conductivity and the minimization of colloid collision (Toran and Palumbo, 1992; Seta and Karathanasis, 1997a). It has also been shown that when present in larger concentrations, pesticides can influence the migratory behaviour of dispersed clay minerals, affecting viscosity and the yield of dispersion both positively or negatively. Penner and Lagaly (2000) found that the addition of multivalent cationic pesticides strongly increased the viscosity and yield value of montmorillonite dispersions. However, despite colloid-facilitated transport being commonplace in laboratory studies, there are very few field studies where such transport processes have been proven conclusively (Elimelech and Ryan, 2002).

It is generally recognized that soils containing small amounts of organic carbon have a low capacity for retarding pesticide mobility, with humic substances being the principal adsorbing sink for these compounds (Piccolo *et al.*, 1992). This has led to the suggestion that the incorporation of exogenous organic matter could be used as part of a remediation strategy to decontaminate soils (Bollag *et al.*, 1992). However, this also introduces exogenous dissolved organic matter (DOM) to the system, which can have a negative impact on pesticide retention, enhancing the transport potential (Williams *et al.*, 2001). DOM has been shown both to associate with pesticide molecules and to also compete with them for sorption sites on soil surfaces (Celis *et al.*, 1998; Cox *et al.*, 2000) resulting in another facilitated transport pathway.

The physical nature of some soils means that preferential flow and leaching are virtually inevitable, whilst for other soils management practices will have a profound impact on leaching of herbicides (Droogers *et al.*, 1996). Novak *et al.* (2001) found the physical characteristics of a Vertic Cambisol, which contained wide and deep shrinkage cracks, favoured preferential flow and facilitated transport, whereas the small and less continuous fissures found with a loamy Stagnic Luvisol had a much reduced capacity preferential flow. Movement often occurs after heavy rainfall or irrigation within a few days or weeks of application (Cogger *et al.*, 1998). Tillage practices can influence leaching directly (Weed *et al.*, 1998) or by influencing the activity and abundance of microbial populations and impact on organic matter content, which will consequently govern the extent of sorption and concurrently the amount available to leach (Fermanich and Daniel, 1991).

1.2. Microbiological and biodegradative potential of the subsurface

1.2.1. Introduction

Subsurface microbial life has been extensively documented (Ghiorse and Balkwill, 1983; Federle *et al.*, 1986; Colwell, 1989), and has been found at depths in excess of 5000 m (Szewzyk *et al.*, 1994). The presence of microbial populations in subsoils and subsurfaces may result from the survival of or derivation from microbes present at the time of deposition or they may have been transported to deeper layers through groundwater movement or events

that occur over shorter geological time periods (Lawrence *et al.*, 2000). Bacterial transport is dependent on the nature of the substratum, the solute, the hydrodynamics and the cell size, shape and surface characteristics (Lawrence and Hendry, 1996). Persistence and survival of these populations is dependent on a number of factors including the pore-size distribution and the structure of the geological formation (Fredrickson *et al.*, 1997) and the temperature and hydrology (Colwell *et al.*, 1997). It has also been estimated that doubling times for deep subsurface microbial populations could be in the order of centuries (Parkes *et al.*, 1990). Although microbes are present at such depths they may be dormant (Russell *et al.*, 1994) or their level of activity may be low (Palumbo *et al.*, 1994). In either case the consequent impact on the environment may be minimal.

However, it has been well established that subsurface microbial populations can have a direct impact on their surrounding environment driving mineral degradation, diagenesis, authigenesis and precipitation reactions (Ehrlich, 1998) and thus are inextricably involved in groundwater chemistry (Hiebert and Bennett, 1992; Stevens *et al.*, 1993). The affect of such subsurface microbial activity on interstitial water chemistry has long been of concern regarding the long-term fate of geologically contained radionuclides. However, the degradation potential of chemicals in the vadose zone has been thought to be low because of the associated low organic matter content, low microbial activity and low temperatures in contrast to surface soil (Alexander, 1977). More recent studies have shown that there are abundant and active microbial populations in this zone (Federle *et al.*, 1986; Konopka and Turco, 1991), suggesting that the potential for degradation may have been underestimated.

1.2.2. Microbial abundance and activity

High numbers of microorganisms have been found in a variety of different groundwater, aquifer sediments and vadose zone samples (Wallis and Ladd, 1983; Beloin *et al.*, 1988; Lawrence *et al.*, 2000). Other than photosynthetic organisms that are found in surface environments, subsurface environments can contain active microorganisms of all physiological types (Lovley and Chappelle, 1995), depending on their interstitial porosity, with bacteria, fungi and protozoa all being recovered from a variety of shallow and deep aquifers (Hirsch and Rades-Rohkohl, 1983; Beloin *et al.*, 1988; Konopka and Turco, 1991). However, the vast majority of microbes detected in the subsurface are bacteria (Beloin *et al.*, 1988; Sinclair and Ghiorse, 1989). The population densities of heterotrophic microorganisms will be limited by the low concentrations of metabolizable organic carbon, as well as N and P limitations. Ammonia, nitrate and phosphate could potentially be at very low concentrations in

particular locations (Parkin *et al.*, 1987). Redox conditions will govern microbial activities, with transformation controlled by the presence of oxygen consuming biotic and abiotic oxidative reactions (Ghiorse and Wilson, 1988).

The abundance of microbes has generally been shown to decrease rapidly in the first few metres of a soil profile (Federle *et al.*, 1986; Beloin *et al.*, 1988; Colwell, 1989; Veeh *et al.*, 1996), although after this initial decline numbers tend to be more or less constant (Ghiorse and Wilson, 1988). However, there are exceptions. Beloin *et al.* (1988), in some cases, found numbers in the saturated zone to be similar to those found at the surface. As well as microbial abundance and biomass concentrations, microbial activities and other process parameters can be stratified with depth. Murphy *et al.* (1998) observed rapid declines in gross N mineralization, NH_4^+ consumption and soil inorganic N levels below 10 cm. Similar trends have been reported in other studies (van Gestel *et al.*, 1992; Gupta *et al.*, 1994). The different environmental stimuli and nutrient sources in subsurface systems compared to surface systems may result in the development of divergent microbial communities. Denaturant gradient gel electrophoresis (DGGE) analysis indicated that bacterial diversity was highest in samples collected from the top 0 - 30 cm compared to those collected from deeper horizons of an aquitard system (Lawrence *et al.*, 2000). The banding patterns displayed in this study indicate that the dominant organisms in all the zones analysed were similar, but also showed that certain bands (and therefore organisms) were absent or only present at specific depths in the aquitard. Land use can also impact on subsurface microbial populations. Dodds *et al.* (1996) noted a shift in subsurface microbial community composition after cultivation, with denitrifiers becoming more abundant.

The types of organic compounds found in subsurface zones will be significantly influenced by: the parent material of the formation, the time recharge water takes to reach the zone, and the biological activity in the recharge zones (Ghiorse and Wilson, 1988). The most labile organic compounds will be preferentially degraded by the surface soil microflora before subsurface microflora ever encounter them in the unsaturated horizons (Alexander, 1977). However, DOM, an intermediate decomposition product of soil organic matter (SOM) that is rich in functional polar groups (Guggenberger *et al.*, 1994), may be transported to subsoil environs before it can be mineralized. ^{13}C-nuclear magnetic resonance (NMR) indicated that organic carbon that had accumulated in a mineral soil horizon bore a strong similarity to the organic C in DOM leached from the surface forest floor (Kaiser and Guggenberger, 2000).

Wallis and Ladd (1983) determined that the DOM in their study consisted of 67 % fulvic acids, 20 % humic acids, 6 % carbohydrates, 4 % tannins and lignins and 2 % phenols,

amino acids and proteins. DOM transport into subsoil horizons not only involves C cycling but is also critical to the cycling of N, P and S. Schoenau and Bettany (1987) reported N, P and S enriched fulvic acids in certain subsoil horizons in comparison to the humic and fulvic acids in the surface horizons. The downward leaching of DOM is a significant pedogenic process (Schoenau and Bettany, 1987) and is the driving force in heterotrophic subsurface processes (Gron *et al.*, 1992).

The distribution of microorganisms is dependent upon the energy substrates and nutrients needed for their survival; therefore there must be a relationship between the microbiology of an environment and its chemical and physical properties. Natural environments are extremely complex with overlapping, spatially heterogeneous distributions of numerous interacting physical, chemical and biological properties. The matrix formed will contain niches that can be optimal, sub-optimal and potentially inhibitory for particular microbes and their activities. These niches have the potential to occur at the pore, laminae, stratum and ecosystem scales (Kieft *et al.*, 1995; Brockman and Murray, 1997). Beeman and Suflita (1987) found distinct zones in an anoxic aquifer, which were sulphate-reducing or methanogenic. The availability of electron acceptors governed the C and energy flows in this study. Greater numbers of microorganisms and associated activities were found in paleosols than in neighbouring sediments (Brockman, 1992; Kieft *et al.*, 1993). This disparity was linked to the negligible soil development in the neighbouring sediments because of lower initial microbial numbers and the lack of residual organic matter nutrient sources.

This variation will also be more evident in subsurface systems compared to surface systems. Stevens and Holbert (1995), using 1-cm^3 samples, found that the coefficient of variance for cultured aerobic heterotrophs was up to 40x greater in unsaturated sediment than in nearby surface soil. Numerous studies of subsurface microbial life, especially those in low hydraulic conductivity materials, have displayed this irregular distribution (Boivin-Jahns *et al.*, 1996; Fredrickson *et al.*, 1997; Krumholtz, 1997). Lawrence *et al.* (2000) demonstrated this 'patchy' distribution using fatty acid methyl ester (FAME) profiling, microscopy, viable counts and molecular techniques. For example, a nitrate reductase gene (*nar*H) was amplified from every sample, whereas genes involved further downstream in the denitrification pathway, i.e. nitrite reductase (*nir*S) and nitrous oxide reductase (*nos*Z), were only amplified in a fractured till. Studies of denitrification have shown that, unless readily available C is present, subsurface denitrification will not occur (Lind and Eiland, 1989; Sotomayer and Rice, 1996). Parkin *et al.* (1987) found denitrification to be isolated in soil

microsites with high organic matter contents. To account for the variation between replicates, these authors suggested 10 - 15 kg of soil should be used to obtain a representative sample.

1.2.3. Environment fate of pesticides in the soil profile

Studies usually report degradation rates for pesticides in the surface (0 - 20 cm) soil (Ghadiri *et al.*, 1984, Brejda *et al.*, 1988, Winkleman and Klaine, 1991, Topp *et al.*, 1994). However, the rates of degradation found for surface soils will not be directly comparable to those of subsoils for many reasons. For example, degradation of 2,4-D to its metabolites is favoured by the presence of sunlight more than by microbial action (Crespin *et al.*, 2001) and photodegradation will, obviously, be absent in subsoils. Furthermore, while some studies report the loss of the parent chemical (Konopka and Turco, 1991; Kruger *et al.*, 1993; Konda and Pásztor, 2001), they often neglect to investigate the fate of metabolites or report the extent of bound residue formation. Pesticides can rapidly enter subsurface soil and shallow groundwater by transportation with infiltrating water through preferential flow paths or through finger flow (Flury, 1996).

The pesticides leached into subsoil will be exposed to very different environmental conditions to that of the surface soil. Microbial biomass as well as available nutrients will vary between surface and subsurface environments (Federle *et al.*, 1986; Brockman *et al.*, 1992). However, the prevailing environmental conditions in the subsurface could select for biochemically and ecologically divergent microbial populations contrasting with the surface soil, which could result in distinct biodegradative processes (or even none at all). For example, Fe (II) minerals produced in subsurface environments as a result of Fe (III) reducing-bacteria may abiotically donate electrons to react with chlorinated contaminants (Glass, 1972) or nitroaromatics (Heijman *et al.*, 1993). Vanderheyden *et al.* (1997) also noted that biodegradation did not occur in subsoil materials, except in a few samples where significant numbers of bacteria were sheltered by stones and iron components. A number of soil properties have been implicated in the retention and mobility of herbicides in soil profiles including organic matter content, soil texture, soil acidity, Fe and Al oxide content and clay mineralogy (Johnson and Sims, 1993).

Although it has been assumed that pesticide degradation occurs at slower rates in subsoil compared to surface soil, some studies have shown variable rates of microbial degradation of xenobiotics depending on the properties of the soil and the pesticide compound (Federle *et al.*, 1986; Pothuluri *et al.*, 1990, Konopka and Turco 1991; Bolan and Baskaran, 1997; Di et al 1998; Karpouzas *et al.*, 2001; Mills *et al.*, 2001). Predicting real groundwater flow conditions

and behaviour of pesticides in subsoils is difficult because local subsurface environments can be very heterogeneous (e.g. grain size, clay lenses, organic matter content, presence of iron- or manganese- oxides) with specific microsites altering over space and time, which may be distinct from the bulk soil.

Some studies have shown that degradation rates do decrease uniformly with depth as organic matter and biomass decrease (Kördel *et al.*, 1995; Veeh *et al.*, 1996; Bolan and Baskaran, 1997; Anderson *et al.*, 2001; Vinther *et al.*, 2001), whereas others have shown no correlation between degradation rate and biomass. For example, Di *et al.* (1998) reported higher rates of degradation for chlorpyrifos (*O*,*O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate), chlorthal dimethyl (dimethyl tetrachloroterephthalate), linuron and propyzamide in some subsoils compared to their respective surface horizons. Other studies have also found no correlation with biomass (Sparling *et al.*, 1998; Karpouzas *et al.*, 2001). Di et al (1998) attributed the nonuniform degradation rates with soil depth to interactive effects of changes in soil microbial activities and in organic matter and their consequent effects on pesticide sorption, and therefore availability, in the different soil layers. Soil profile degradation of atrazine was shown to correlate with the numbers of specific atrazine degraders, giving higher degradation rates in the 60 - 90 cm layer compared to the surface layer (Sparling *et al.*, 1998).

The discussion in the previous section (1.2.3.) highlighting microsite variation in microbial abundance and activities caused by a variety of environmental factors, suggests that the degradation of contaminants in subsoils may also concentrate in particular niches. Studies of carbofuran and 2,4-D degradation in soil profiles have noted high levels of variability between replicates. For example, in two replicates from a depth of 40 - 50 cm, approximately 56 % of the initial radiolabelled 2,4-D had been evolved as $^{14}CO_2$ within 83 d, whereas in another two replicates at the same depth, only about 5 % had been mineralized (Shaw and Burns, 1998) . In another study, carbofuran had completely disappeared after 18 d in one replicate from 70 - 80 cm depth, whilst it took 50 d in another replicate from this depth (Karpouzas *et al.*, 2001). Both studies attributed the difference between replicates to low and variant numbers of specific pesticide degrading organisms and an uneven distribution of these microbes in subsoils at a greater scale than in the respective surface soils.

Another source of heterogeneity and microbial 'hot spots' in soil profiles are preferential flow paths. Preferential flow paths reduce the interaction between solutes and the soil matrix, thereby increasing the mobility of the chemicals and the potential for entrance into deeper layers of the soil profile and also ground and surface water contamination. However, the

sorption and degradation rates of chemicals entering these flow paths may be different compared to those in the bulk soil because of differences in microbial populations (Pivetz and Steenhuis, 1995; Mallawantri *et al.*, 1996). For example, greater populations of microbial functional groups including actinomycetes, *Pseudomonas* spp., aerobic spore-forming bacteria, cellulolytic bacteria, cellulolytic fungi, NH_4 oxidising bacteria and NO_2 oxidising bacteria were found in macropore wall linings compared to the bulk soil (Pankhurst *et al.*, 2002). The increase in microbial activity has been attributed to the increased nutrients released through root decomposition, excretion by earthworms or lateral diffusion of leached nutrients through the macropore wall (Pankhurst *et al.*, 2002).

Physico-chemical characteristics of preferential flow paths, which may persist for decades (Bundt *et al.*, 2000), are distinct from those of the soil matrix. Pierret *et al.* (1999) found higher concentrations of C, N, P, Fe and Mn in macropore sheath soil, along with more abundant populations of bacteria and fungi. Higher concentrations of C and N and higher microbial activities have been found in the walls of root-induced macropores and earthworm burrows compared to the bulk soil (Tiunov and Scheu, 1999; Bundt *et al.*, 2001; Pankhurst *et al.*, 2002). Tiunov and Scheu (1999) also found that the populations in the macropore wall had adapted to the continuous nutrient addition from earthworm mucus and faeces with a more rapid growth response. The increased microbial numbers in macropore sheath soil was indicated as the causal factor for the faster biodegradation of 2,4-D in macropore soil compared to bulk soil (Pivetz and Steenhuis, 1995).

In summary, the microbiological and biodegradative potentials of subsoils and subsurface environments are dependent on soil structure controlling the spatial relationships between nutrient resources and microbial populations. Soil structure will also govern the migration of chemicals through soil profiles and the potential exposure of organic contaminants to microbial consortia with the capacity to bring about their biodegradation.

Section 1.3. Chloroacetanilide Herbicides

1.3.1. Introduction

Chloroacetanilides are among the most widely used herbicides for protection of corn, soybeans, sorghum and rice (Dearfield *et al.*, 1999), but are also used to protect a variety of other crops including sunflower, peanut, cotton, sugar cane, and coffee (Extension

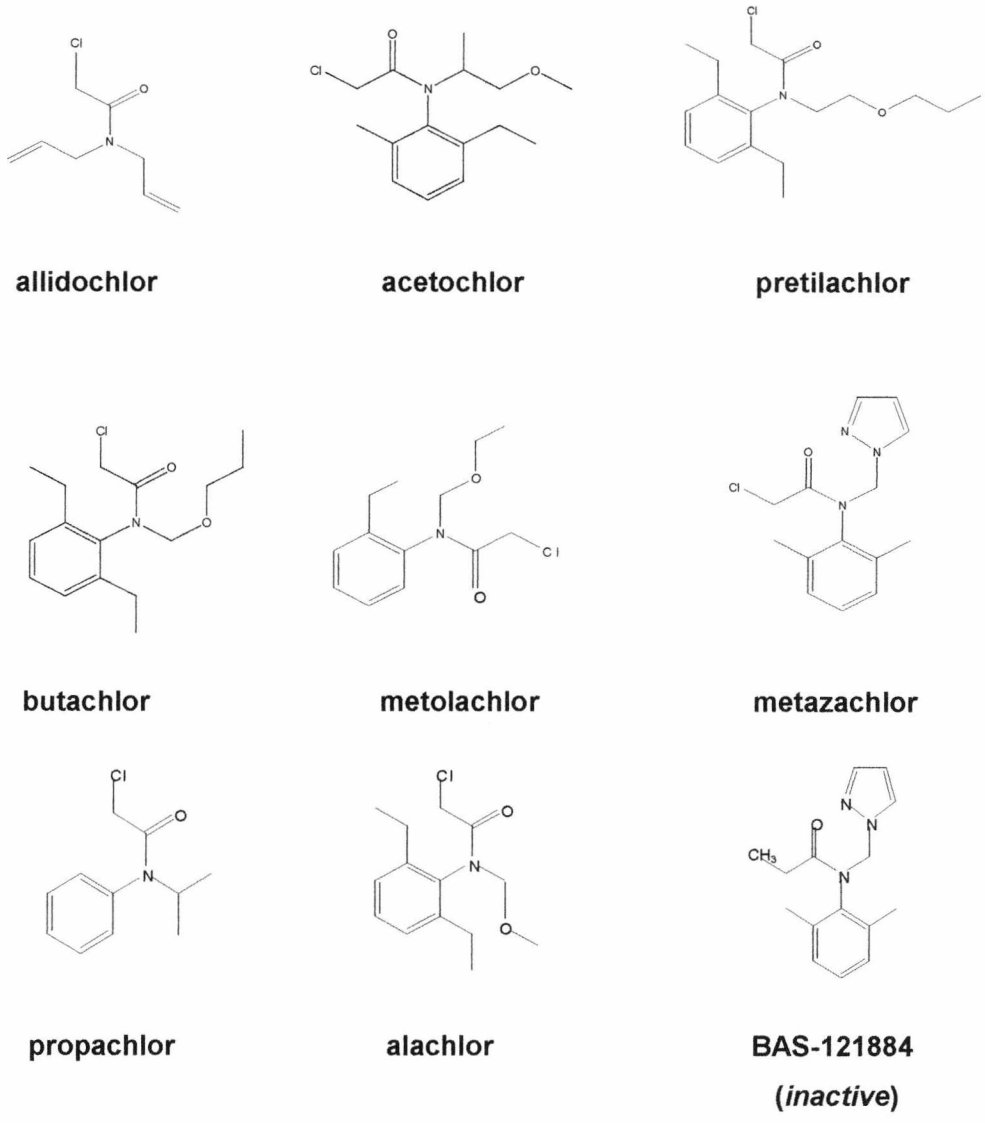


Figure 1.3. Examples of chloroacetanilide herbicides and an analog of metazachlor, BAS-121884.

Toxicology Network, 1998). The most prominent herbicides from this class are acetochlor, alachlor, metolachlor and propachlor [2-chloro-*N*-(1-methylethyl)-*N*-phenylacetamide] (Figure 1.3.). Chloroacetanilide herbicides are used world wide in crop protection strategies in countries in Latin America, South Africa and Eastern Europe. However, the main market is in

the North Central United States. It has been estimated that over 100 million pounds of chloroacetanilide active ingredient per year is used in the United States (United States Environmental Protection Agency, 1997).

1.3.2. Mode of Action

1.3.2.1. Lipid Biosynthesis

This class of herbicides is primarily used as a preemergent control against grasses and some dicotyledonous weeds, which absorb the compound through roots, shoots or cotyledons, suppressing the early development of susceptible weeds. They are then xylem transported by acropetal movement. A multitude of physiological processes in higher plants have been reported to be inhibited by chloroacetanilides including lipid, protein, gibberellin syntheses, cell division, mineral uptake and an increase in cell permeability (Wilkinson, 1982; Fuerst, 1987; Le Baron *et al.*, 1988).

High activity of these herbicides at submicromolar concentrations (Fuerst, 1991; Couderchet and Böger, 1993) *in vivo* suggested the involvement of a single target site in their phytotoxic action, although direct evidence for such a target site has only recently been found. Studies have indicated that lipid biosynthesis is the essential function targeted by these herbicides, with the role of acetyl-CoA first being implicated by Jaworski (1956). In plants, fatty acids are synthesized by the fatty acid synthase localized in the plastid producing C_{16} and C_{18} length precursors. These chains can be further modified upon export to the cytosol, where fatty acid elongases, for production of very-long-chain-fatty-acids (VLCFAs) are present (Schmalfuβ *et al.*, 2000). Further studies noted that production of VLCFAs was inhibited in the presence of chloroacetanilides (Ebert and Ramsteiner, 1984; Weisshaar and Böger, 1991, Takahashi *et al.*, 2001).

1.3.2.2. Inhibition of VLCFA synthesis

VLCFAs are stabilizing constituents of the plasma membrane, essential for the function of cuticular waxes, which protect plants against a number of biotic and abiotic stresses including desiccation, mechanical damage, and pathogenic insects and fungi (Kerstiens, 1996). However, although VLCFA synthesis is inhibited by these herbicides, the actual role VLCFAs have on cell membrane stability and plant seedling growth, as well as the effect of elongases, thioesterases, reductases and transacylases, on regulation of acyl-CoA elongation and the

final cuticular wax composition, has yet to be discovered. VLCFA's are thought to be involved, directly or indirectly, with stabilization of the highly curved membranes around the nuclear pores, which are required for the proper assembly of the nuclear envelope (Schneiter and Kohlwein, 1997).

The high sensitivity of the elongase reaction complex against these compounds is thought to be due to an accumulative inhibition process as the concentration of the activated acyl-primer decreases with each elongation step. Chloroacetanilides are known to be alkylating agents, and bind to nucleophiles like glutathione and cysteine *in vitro* (Field and Thurman, 1996) as originally suggested by Jaworski (1956) for allidochlor [2-chloro-*N*,*N*-di-2-propenylacetamide]. It has been postulated that an alkylating reaction occurs that is specific for the highly sensitive condensing enzyme of the plant microsomal elongase system; presumed to be by nucleophilic attack leading to covalent attachment to the conserved cysteine in the reactive site (Böger *et al.*, 2000).

The inhibition of VLCFA synthesis by chloroacetanilides has been shown, along with their biocidal and biodegradative capacities, to be stereospecific. For example, using leek seedlings, only the S-enantiomer of metolachlor, and not the R-enantiomer was shown to inhibit all the acyl elongation steps (Böger *et al.*, 2000). Using an analog of metazachlor [2-chloro-N-(2,6-dimethylphenyl)-N-(1*H*-pyrazol-1-ylmethyl) acetamide], BAS-121884 (Figure 1.3.), these authors also demonstrated that the location of the binding site for covalent attachment between the herbicide and the target enzyme is the Cl substituent. A methyl group in the analog, which left neither a leaving group nor an activated C atom to react in a nucleophilic attack, had replaced the Cl; unsurprisingly, the analog was inactive.

1.3.2.3. Resistance to chloroacetanilides

Conjugation with glutathione or its homologue homoglutathione has long been established as the main metabolic reaction causing detoxification of these commercially important herbicides in higher plants (Jablonkai and Hatzios, 1991). In fact, glutathione (GSH) levels mediate resistance. GSH is a core component of plant tissue antioxidation and detoxification defence, with elevated concentrations found in plants induced by exposure to a number of environmental stressors and also microbial infections (Fodor *et al.*, 1997; Noctor and Foyer, 1998). Plants containing propitious concentrations of glutathione S-transferases (GSTs; EC 2.5.1.18) with suitably high activity for these herbicides are tolerant, whereas species with unfavourably low affinities for these substrates are inclined to be susceptible. GSTs could therefore be the key underlying elements governing herbicide selectivity (Edwards and Cole,

1996). Predictably, GST enzymes with activity towards chloroacetanilides are present in tolerant plant species such as maize (*Zea mays*) (Mozer *et al.*, 1983; Rossini *et al.*, 1996), sorghum (Gronwald and Plaisance, 1998), wheat (*Triticum aestivum*) (Edwards and Cole, 1996) and soybean (*Glycine max*) (Skipsey *et al.*, 1997). The expression of GSTs is an area actively researched in attempts at phytoremediation of herbicide-contaminated land. Untransformed *Populus spp.* (Poplar) has been used for the phytoremediation of soils contaminated by the herbicide atrazine, which is detoxified by the formation of GSH-conjugates (Burken and Schnoor, 1997). Transgenic poplar plants, overexpressing γ-glutamylcysteine synthetase in response to chloroacetanilides has been proposed as potential tool for phytoremediation (Gullner *et al.*, 2001).

However, the chances of resistance developing in the weeds targeted by this class of herbicide are thought to be slight for a number of reasons. Sequence data has indicated that the cysteine residue in the active site of the condensing enzyme is highly conserved (Millar *et al.*, 1999). An exchange of the cysteine, which is the likely point of interaction, with the inhibitor would abolish both binding and catalytic activity, resulting in a lethal toxic insult to the plant (Böger *et al.*, 2000). It has also been suggested that chloroacetanilides may target other condensing synthases in other biosynthetic pathways and that metabolic resistance would require increased expression of more than one gene so as to not only maintain GSH concentrations, but also to keep it in its reduced form (Böger *et al.*, 2000).

1.3.2.4. Toxicological profile of chloroacetanilides

Exposure to chloroacetanilides, or indeed any other herbicide, can occur through a number of routes including intake of residues on treated raw agricultural commodities and from groundwater used for drinking water resources. To minimize exposure, herbicide tolerance levels have to be established, which are considered safe. The United States Environmental Protection Agency (USEPA) defines safe as "*a reasonable certainty of that no harm will result from aggregate exposure to the pesticide chemical residue, including all anticipated dietary exposures and all other exposures for which there is reliable information*" according to FFDCA section 408. Mutagenic and carcinogenic effects are two of the principal criteria used in toxicological appraisals of the potential risk of pesticide exposure to humans.

Genotoxic carcinogens usually induce tumour formation in multiple target tissues, multiple species and both sexes (Ashby and Tennant, 1991). This scenario is evident for at least acetochlor and alachlor. Acetochlor was shown to induce tumours at multiple sites in both sexes of rats and mice (Office of Pesticide Programs, 1992). In addition, there is evidence for

mutagenic activity, especially clastogenicity (Hozier *et al.* 1985). The processes altered by these chemicals may exacerbate the clastogenicity and tumourigenicity, causing hormonal imbalance, protein alterations and increased cell proliferation (Dearfield *et al.*, 1999) thus non-genotoxic tumour formation may be more important. A key to predicting the potential for health risks of these chemicals is the consideration of their extensive biotransformations and reactive intermediates. For example, rats treated with propachlor yielded at least 11 metabolites in their urine and several *in vivo* metabolites of alachlor have been identified (Bakke and Price, 1979; Office of Pesticide Programs, 1997).

The relevant structures of these chloroacetanilides, with regard to their potential for creation of reactive intermediates, include the active Cl on the methylene carbon α to the carbonyl group, the reactive quinonimine intermediate, formaldehyde and other aldehydes (Dearfield *et al.*, 1999). As has been discussed previously, the electrophilic Cl will react preferentially with nucleophiles such as GSH and other SH-containing moieties, which means these compounds are expected to react with and deplete GSH concentrations. This depletion could prove to be harmful in tissues where low levels of endogenous GSH are present (e.g. blood, nasal tissue, stomach), increasing their susceptibility to toxic and reactive intermediates (Dearfield *et al.*, 1999). After *in vivo* exposures to alachlor, butachlor [*N*-(butoxymethyl)-2-chloro-*N*-(2,6-diethylphenyl) acetanilide], acetochlor and metolachlor in male Sprague-Dawley rats, direct evidence for quinonimine metabolites has been found (Jefferies *et al.*, 1998).

It is thought that quinonimine may account for the mutagenic activity (Dearfield *et al.*, 1999). Although clear *in vitro* evidence for clastogenicity has been reported in Chinese hamster cells, human lymphocytes and the mouse lymphoma assay (Hozier *et al.*, 1985) at concentrations of 200 μg/ml, it has not been seen using *in vivo* studies. The reasons for this disparity are thought to be due to detoxification of the parent compounds and their intermediates by GSH (Dearfield *et al.*, 1999). These authors also suggest that the small concentrations needed to cause a toxic insult *in vitro* may be relevant *in vivo* where small amounts of DNA damage may be difficult to quantify, but may contribute to the development of adverse effects such as tumours and dominant lethal effects.

The importance of multi-species testing is also apparent with this class of herbicides. Research on the metabolism of alachlor (Heydens *et al.*, 1999) proposed that quinonimines produced after metabolism in the liver and nasal epithelium lead to cell death and compensatory hyperplasia causing the formation of nasal adenomas. However, the induction of adenomas in rats, which are in fact non-life threatening, does not occur in mice (Ashby *et al.*, 1996). The species differences lie in the processing of the herbicides in their respective

livers. Mice metabolize acetochlor primarily through oxidative pathways and glucuronidation reactions, whereas rats principally utilise the GSH pathway (Ashby *et al.*, 1996). In the case of acetochlor, the causal factor in adenoma production is the metabolite acetochlor sulphoxide, which is produced in the GSH pathway (Green *et al.*, 2000). This study also showed that acetochlor sulphoxide could not be metabolized by human nasal tissues, indicating that this pathway is not a nasal hazard for humans.

An initial toxicity screen of the main metabolites produced in soil, chloroacetanilide-ethane sulphonic acid (ch-ESA) and chloroacetanilide-oxanilic acid (ch-OXA), showed that they are poorly absorbed and rapidly excreted, exhibiting a low degree of toxicity to mammals and aquatic organisms and are considered not to be of toxicological concern and not to pose a significant risk to human health or the environment (Acetochlor Registration Partnership [ARP], 2001).

1.3.2.5. Biodegradation and environmental fate of chloroacetanilides

1.3.2.5.1. Biodegradation and transformation

The main structural differences, between the various chloroacetanilides are in the non-chlorinated alkyl group attached to the anilide moiety and in the distribution of ethyl and methyl substituents on the benzene ring (Figure 1.3.). These factors regulate selectivity and water solubility (Chesters, 1989). Biodegradation is thought to be the driving force behind the dissipation of this class of herbicides in the environment. However, groundwater monitoring, soil and mixed culture studies have shown that chloroacetanilide herbicides undergo complex transformations of ring substitutions (Tiedje and Hagedorn, 1975; Saxena *et al.*, 1987; Potter and Carpenter, 1995; Stamper and Tuovinen, 1998), which makes it difficult to ascertain the relative importance of abiotic and biotic influences on the formation of these transformants (Figure 1.4.).

The hydrolytic, abiotic degradation of chloroacetanilides has been suggested to be of minor importance at pH values typically recorded in soil and water (Stamper and Tuovinen, 1998), although granular iron metal has been shown to cause the reductive dechlorination of two important chloroacetanilide herbicides, alachlor and metolachlor (Eykholt and Davenport, 1998). These herbicides are relatively resistant to direct photolytic decomposition, but can be degraded by indirect nitrate-induced OH-mediated photolysis (Brekken and Brezonik, 1998). However, DOM and inorganic ions inhibit this indirect process considerably (Brekken and

Brezonik, 1998; Zheng and Ye, 2001), which suggests that its potential in soils is limited. It has been demonstrated that the dominant electron acceptor conditions present will also influence the rate of degradation, with sulphate-reducing conditions being significantly more conducive to transformation than aerobic and nitrate-reducing conditions for alachlor and propachlor (Wilber and Wong, 1997).

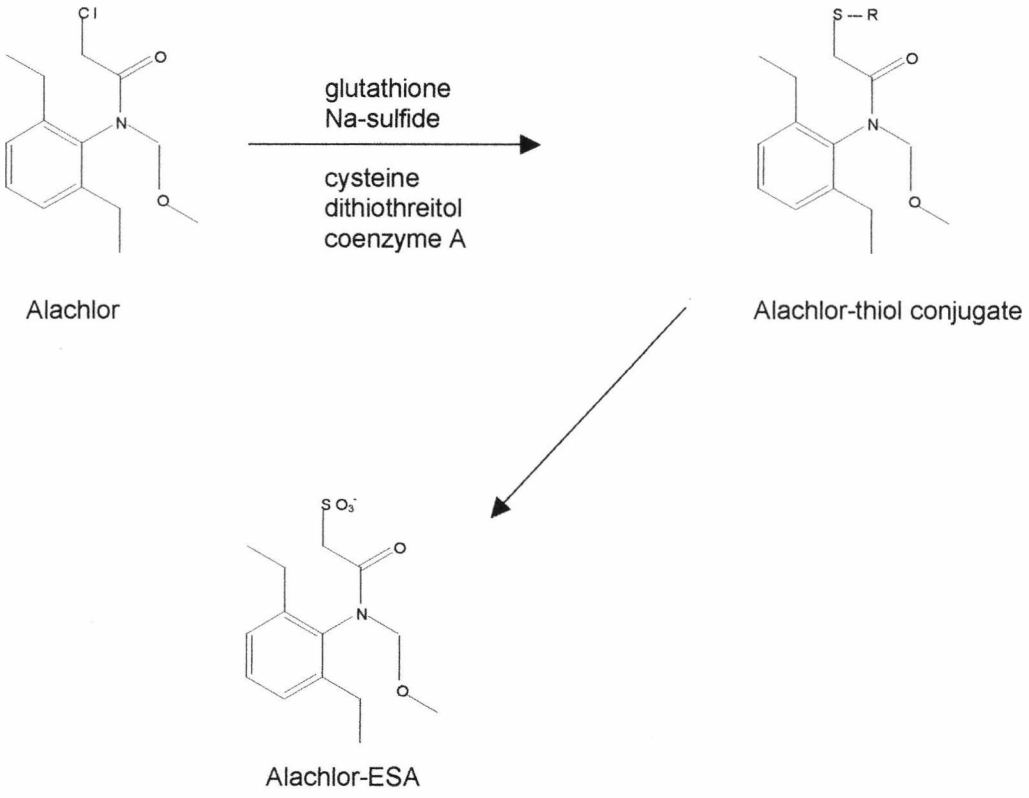


Figure 1.4. Formation of alachlor ethane-sulphonic acid (al-ESA) through enzyme-mediated conjugation (i.e. glutathione) and abiotic conjugation (Stamper and Tuovinen, 1998)

Numerous attempts at isolating microbes that can mineralize chloroacetanilides have failed (e.g., [alachlor] Saxena *et al.*, 1987; [metolachlor] Liu *et al.*, 1991 and Villarreal *et al.*, 1991). However, studies involving propachlor have led to the isolation of microbial consortia that can bring about its mineralization either through cleavage of the amide bond (Novick *et al.*, 1985) or by cleavage of the bond between the N atom and the aromatic ring (Villarreal *et al.*, 1991; Martin *et al.*, 1999). A fundamental structural difference between propachlor and other acetanilide herbicides is the lack of ring substituents in the *ortho*-positions (2'-, 6'-). The recalcitrance of alachlor and metolachlor has been attributed to the combined presence of *N*-

alkyl groups and 2', 6'-dialkyl substituents on the aniline ring sterically hindering the cleavage of the bonds to the N-atom (Kaufman, 1974; Saxena *et al.*, 1987; Villarreal *et al.*, 1991). The strains from the Villarreal (1991) study, although growing on propachlor, could not metabolize alachlor or metolachlor, which supports this hypothesis. Aniline, a putative intermediate in the chloroacetanilide degradative pathways, is readily mineralized (Konopka *et al.*, 1989) suggesting that the rate limiting steps in the degradation of chloroacetanilides are associated with the initial transformation steps, which involve recalcitrant structures with xenobiotic motifs.

A soil fungus, *Chaetomium globosum*, was isolated that could utilize alachlor a sole C and energy source but no ring cleavage occurred (Tiedje and Hagedorn, 1975), despite the production of several metabolites. White rot fungi (*Ceriporiopsis subvermispora, Phlebia tremellosa, Phanerochaete chrysosporium*) were shown to mineralize between 6 - 14 % of the applied alachlor over a 122 d period, but transformed over 90 % to polar and non-extractable residues (Ferrey, 1994). In another study, a mixed culture dominated by three bacterial species mineralized up to 12 % of the applied alachlor, even though alachlor was depleted relatively rapidly (Sun *et al.*, 1990). Similar partial transformation patterns have been observed for metolachlor (Liu *et al.*, 1989). From these studies, it can be concluded that mineralization is not a major fate process for most chloroacetanilides, rather that biodegradation results from cometabolic transformations.

The main metabolites of chloroacetanilides are produced by a combination of processes including reductive dechlorination (via dehydrogenolysis), dealkylation and oxidation (Eykholt and Davenport, 1998). The slower rates of degradation recorded for alachlor in subsurface sediments compared to surface soil were attributed to the C limitation of these sediments and to the greatly reduced fungal populations at the depths studied (Clay *et al.*, 1997). This is consistent with degradation of alachlor being cometabolic in character. Half-lives for the main chloroacetanilides are shown in Table 1.1. In contrast, Mills *et al.* (2001) conducted a number of field and laboratory investigations into acetochlor fate in soil profiles and observed variable degradation rates, with subsoils often displaying much shorter half-lives than the corresponding surface soil. For example, a 1997 Wisconsin field study showed a dissipation time for 50 % of the originally applied chemical (DT_{50}) of 8 d at a depth of 30 - 76 cm, 14 d at a depth of 260 - 305 cm and 18 d in the surface horizon of 0 - 30 cm. Soil texture was different at all the depths analysed and none of the studies showed correlations between biomass and degradation rates.

Table 1.1. Reported half-life ranges for transformation of acetochlor, alachlor and metolachlor in aerobic surface soils. Data taken from Barbash *et al.* (2001) and Mills *et al.* (2001).

Herbicide	Half-life (d)
Acetochlor	2-110
Alachlor	14-21
Metolachlor	26, 67

The focal point of transformation for chloroacetanilides is the chlorine-bearing carbon through which acidic metabolites such as oxanilic, sulphonic and sulphinylacetic acids can be formed. These compounds have been identified as significant soil metabolites of acetochlor (Feng, 1991), alachlor (Sharp, 1988) and propachlor (Lamoureux and Rusness, 1989). The presence of contaminant metabolites in the environment allows for a greater understanding of transformation pathways and their accompanying enzymes, especially if these metabolites contain elements, such as S, which are not a constituent of the parent molecule (Field and Thurman, 1996). Identification of sulphonated metabolites of chloroacetanilides led to the implication of GSH conjugation in the transformation of chloroacetanilides. The role of GSH conjugation in soil metabolism of chloroacetanilides was first reported for propachlor (Lamoureux and Rusness, 1989) and later demonstrated for acetochlor (Feng, 1991). GSH conjugation is catalysed by GSTs, the gene expression of which can be induced by a wide variety of both endogenous and exogenous electrophilic compounds and can lead to detoxification of a broad range of agricultural and industrial chemicals including polyaromatic hydrocarbons, nitroaromatic compounds, polychlorinated biphenyls, hydroquinones, insecticides (e.g., fosfomycin), and herbicides [e.g., triazine, sulphonyl urea, diphenyl ether and chloroacetanilide] (Vuilleumier, 1997).

Sulphonated compounds are difficult to detect using conventional analytical techniques because of their associated low volatilities, which hinders their analysis by gas chromatography and mass spectrometry. The presence of false positives in an alachlor immunoassay led to the identification of its sulphonated metabolite; alachlor was not detected

by gas chromatographic analysis of the same samples (Baker, 1993). Evidence for the formation of S-containing metabolites of chloroacetanilides being a soil rather than plant derived process was provided by Lamoureux and Rusness (1989). In this study, hydroponically grown soybean did not yield sulphonated metabolites from propachlor detoxification, whilst soil-grown soybean and, more importantly, soil alone produced sulphonated and sulphinylacetic metabolites. Although this suggests that these metabolites are formed in soil and then taken up by the plants, the relative contribution of plants and soil microorganisms to the formation of S and non-S containing metabolites has yet to be verified. Aga and Thurman (2001) point out that although an increased concentration of alachlor (al)-ethane sulphonic acid (ESA) occurred after corn harvesting, it could be linked to environmental stimuli such as changes in hydrology or soil-disturbances post-harvest.

GSTs are present in plants, bacteria, fungi, protozoa, yeast and algae (Fahey *et al.*, 1978; Lau *et al.*, 1980; Zablotowicz *et al.*, 1995). It would, therefore, appear reasonable to assume that glutathione conjugation of electrophilic chemicals could be commonplace in the terrestrial environment. The conjugation reaction between GSH and chloroacetanilides involves nucleophilic substitution with the electrophilic functional group, chlorine, displaced by a nucleophilic thiol group. The transformation pathway for acetochlor was elucidated by Feng (1991) who found that the degradation of the acetochlor-glutathione complex was instigated by γ-glutamyl transpeptidase and that cysteine-β-lyase was responsible for the degradation of the conjugate to S-containing metabolites (Feng, 1991). The herbicide-GSH conjugates are generally much less toxic and more water-soluble than the original herbicide molecules (Edwards *et al.*, 2000). However, as previously stated some sulphide-containing molecules (Figure 1.4) have produced similar results through abiotic transformation of these herbicides (Leavitt and Penner, 1979; Stamper *et al.*, 1997).

The stereochemistry of chloroacetanilides can also influence the potential for degradation, with transformation shown to be stereoselective and/or enantioselective (Müller and Buser, 1995) and also their biocidal character. The hindered rotation about the phenyl-N bond of these compounds with the unsymmetrical substitution of the aromatic ring leads to the formation of atropisomers (Aga *et al.*, 1999). Two stereoisomers can exist for alachlor, four for acetochlor and eight for metolachlor. The authors postulate that the different stereoconfigurations may be more or less biodegradable with potentially different affinities for glutathione and that a differential distribution of these compounds with soil constituents may occur. They also suggest that further research is needed to ascertain whether the formation of the various ESA and OXA derivatives is stereospecific.

1.3.2.5.2. Leaching and adsorption potential of chloroacetanilides.

The adsorption of chloroacetanilide herbicides on soil has been extensively studied (Kozak *et al.*, 1983; Peter and Weber, 1985; Senesi *et al.*, 1994; Crisanto *et al.*, 1995; Wang *et al.*, 1999; Liu *et al.*, 2000). Sorption to clays and organic matter is likely to play an important role in influencing the bioavailability and retarding the migration of these compounds in and through subsurface environments. Metolachlor and alachlor bind to organic matter and clay minerals through hydrogen bonding and hydrophilic and lipophilic interactions (Peter and Weber, 1985, Bosetto *et al.*, 1993; Senesi *et al.*, 1994). Soil organic C content has also been implicated in butachlor adsorption (Sato *et al.*, 1997). Senesi *et al.* (1994) demonstrated, using infra-red analysis, that the adsorption of alachlor to humic acid was concentration dependent with H-bonding and charge-transfer processes dominating at low concentrations, whilst at high concentrations, hydrophobic binding occurred with the aliphatic zones of humic acid. The importance of H-bonding and charge transfer bonding of alachlor with humic acid was further corroborated in a later study using infrared spectra and electron spin resonance (Wang *et al.*, 1999).

That study also suggested that the hydrophilic character influences the relative sorptivity of chloroacetanilides, with the terminal alkyls of the ether bonds extending gradually from metolachlor to butachlor, thus becoming weaker and less water-soluble in that order; the higher the water solubility the lower the likelihood of adsorption. However, a later study (Liu *et al.*, 2000), using a different range of chloroacetanilides, found that the relative sorptivity could not be correlated to either the water solubility or the octanol-to-water coefficient of these herbicides. These authors suggested that relative sorptivity was related to the impact of the respective side chain substitutions and their spatial arrangement on nitrogen electron density and the consequent reactivity of the functional groups participating in the binding reactions. Hysteretic sorption behaviour has been displayed by chloroacetanilides, with initial binding of alachlor shown to be rapid but desorption from soil to be slow and potentially irreversible (Bosetto *et al.*, 1993; Xue and Selim, 1995).

The most frequently detected pesticides in the USA are those herbicides that have been used extensively: triazines (atrazine, cyanazine, and simazine [2-chloro-4,6-bis(ethylamino)-s-triazine]), acetanilides (metolachlor, acetochlor, and alachlor) and the phenoxyacetic acid, 2,4-D. Scribner *et al.* (2000) compared the overall use of corn herbicides in the Midwest of

the United States, with the concentrations of these herbicides found in Midwestern streams. The authors observed that the reduction in concentrations for several herbicides including alachlor and metolachlor did not reflect changes in the amount used. These authors cite split herbicide applications, decreased per acre application rates, increased post-emergent use of pre-emergent compounds and improved herbicide management practices as partially responsible for the changes in concentrations. However, as acetochlor usage increased, the concentrations found in these streams also increased. Maximum residue levels (MRL) are set by the European Union for pesticides in environmental and drinking water, according to the mandate of the Codex Committee on Pesticide Residues (CCPR). The MRL are 0.1 μg/l for individual compounds (80/778/ECC). Konda and Pásztor (2001) detected concentrations of acetochlor one order of magnitude above the MRL two weeks after the initial application. The rapid movement of acetochlor to the sampling point was attributed to facilitated transport and rainfall events.

Although the soil from the Konda and Pásztor (2001) study met the USEPA requirements for acetochlor use (sandy loam, 1.26 % organic matter content), the herbicide was only transported to a depth of 60 cm before it was collected. However, USEPA standards require that acetochlor is not used if the water table is within 30 metres of the surface. Further to this, although acetochlor has been classified as a 'leacher' (Balinova, 1997), its degradation products and also the degradation products of other chloroacetanilides are detected with greater frequency than the parent compounds (Potter and Carpenter, 1995; Barbash *et al.*, 2001); Konda and Pásztor (2001) did not analyse for degradation products in their study. Potter and Carpenter (1995) found 20 degradation products of alachlor or products derived from alachlor. The total concentration of degradation products exceeded the concentration of alachlor by at least 2x. This study also highlighted the substantial potential for the formation of stable environmental degradation products. Of the twenty products detected in the initial analyses, only one (7-ethylindoline) was not detected 30 months later, with the two data sets nearly qualitatively identical. However, as this study was a water quality survey it is not possible to ascertain the origin of these metabolites, i.e. soil produced or groundwater produced. The lower molecular weights or oxidised character of these transformation products does suggest that they have more potential to leach than the parent compounds due to their enhanced water solubility. For any informative analysis of the environmental fate of chloroacetanilides, the behaviour of their transformation products needs to be taken into account.

Other studies have looked at degradation in groundwater. Cavalier *et al.* (1991) recorded half-lives for alachlor, incubated at 15 °C and 22 °C, in groundwater samples from different locations, ranging from 808 to 1518 d. Pothuluri *et al.* (1990) reported half-lives of between 320 to 324 d in other aquifer samples. These studies indicate that the degradative potential of subsurface microorganisms for alachlor is limited. Alachlor and its metabolites may, therefore, remain in groundwater unaltered for extended periods.

1.3.3. Acetochlor

1.3.3.1. Use and restriction

Chloroacetanilide herbicides have been in use for over 40 years (Böger *et al.*, 2000), but in 1994, the change in the EPA's stance towards preventing pollution problems resulted in criteria of unprecedented stringency for the conditional registration of acetochlor (USEPA, 2000). The criteria included that a complete registration application was made by March 7, 2001 and that acetochlor achieved the "*reasonable certainty of no harm*" health standard. The following is an overview of these criteria.

1.3.3.1.1. Concentration cancellation triggers and environmental monitoring

The ARP (Monsanto & Zeneca Agrochemicals) was required to provide a continuous ground and surface water-monitoring program in affected areas, with use restricted to certified applicators. The registration of acetochlor would be automatically cancelled if of the eight sites where prospective groundwater (PGW) studies were being conducted, four sites indicated a pattern of movement of acetochlor towards groundwater resulting from use according to label directions, or from widespread and commonly recognised practice. Further to PGW studies, the ARP was made responsible for monitoring 177 wells across Iowa, Illinois, Indiana, Kansas, Minnesota, Nebraska and Wisconsin. The concentrations of acetochlor for which registration would be cancelled were set at: 0.10 parts per billion (ppb) in over 20 of the wells or 0.2 ppb in over 150 of the wells, followed by 2 subsequent detections over the following 6 months at this concentration (USEPA, 2000). The ARP was also obliged to monitor 175 sites as part of a surface water-monitoring program. The cancellation trigger for this program was set at 2.0 ppb as an annual time-weighted mean concentration if the surface water is used as a primary source of community water supply. In addition if a single peak of 8.0 ppb was detected the ARP would be responsible for biweekly sampling of that

water system throughout the following 12 months to determine if the 2.0 ppb annual concentration had been exceeded (USEPA, 2000).

1.3.3.1.2. Displacement of other herbicides

One of the main potential benefits of using acetochlor would be the consequent reduction in use of other high-risk corn herbicides. For this reason one of the registration criteria necessitated reductions in the use of alachlor, metolachlor, atrazine, EPTC (S-ethyl dipropylthiocarbamate), 2,4-D and butylate (S-ethyl diisobutylthiocarbamate) by 4 million pounds within 18 months, 22.6 million pounds within 3 years and 66.3 million pounds or 33.0 % within 5 years (USEPA, 2000).

1.3.3.1.3. Soil type

For further protection of groundwater and surface water resources extensive soil mapping of all major corn-growing areas in the United States has been carried out to ensure vulnerable areas are not exposed to this herbicide. The criteria have set out that acetochlor cannot be applied to sand soils, loamy sand soils, or sandy loam soils with less than 3.0 %, 2.0 % or 1.0 % organic matter content, respectively, when groundwater is within 30 feet of the soil surface (ARP, 2001). Acetochlor cannot be applied to frozen or snow-covered soils, or highly compacted soil because of the accentuated risk for run-off. For example, users in Iowa and a number of other states are advised that applications need to be made when the "sustained soil temperature at 4-inch depth is less than 50 degrees Fahrenheit, but before ground freezes" (ARP, 2001). Restrictions on tillage protocols have also been imposed.

1.3.3.1.4. Current registration status of acetochlor

The usage targets have been met, with the cumulative reduction in the use of other corn herbicides of 66.3 million pounds exceeded in 1998. The EPA also decreed that the registration of acetochlor would be terminated on March 7, 2004 if the ARP, with all the necessary data, did not submit a complete application for registration by March 7, 2001.

1.4. Soil structure

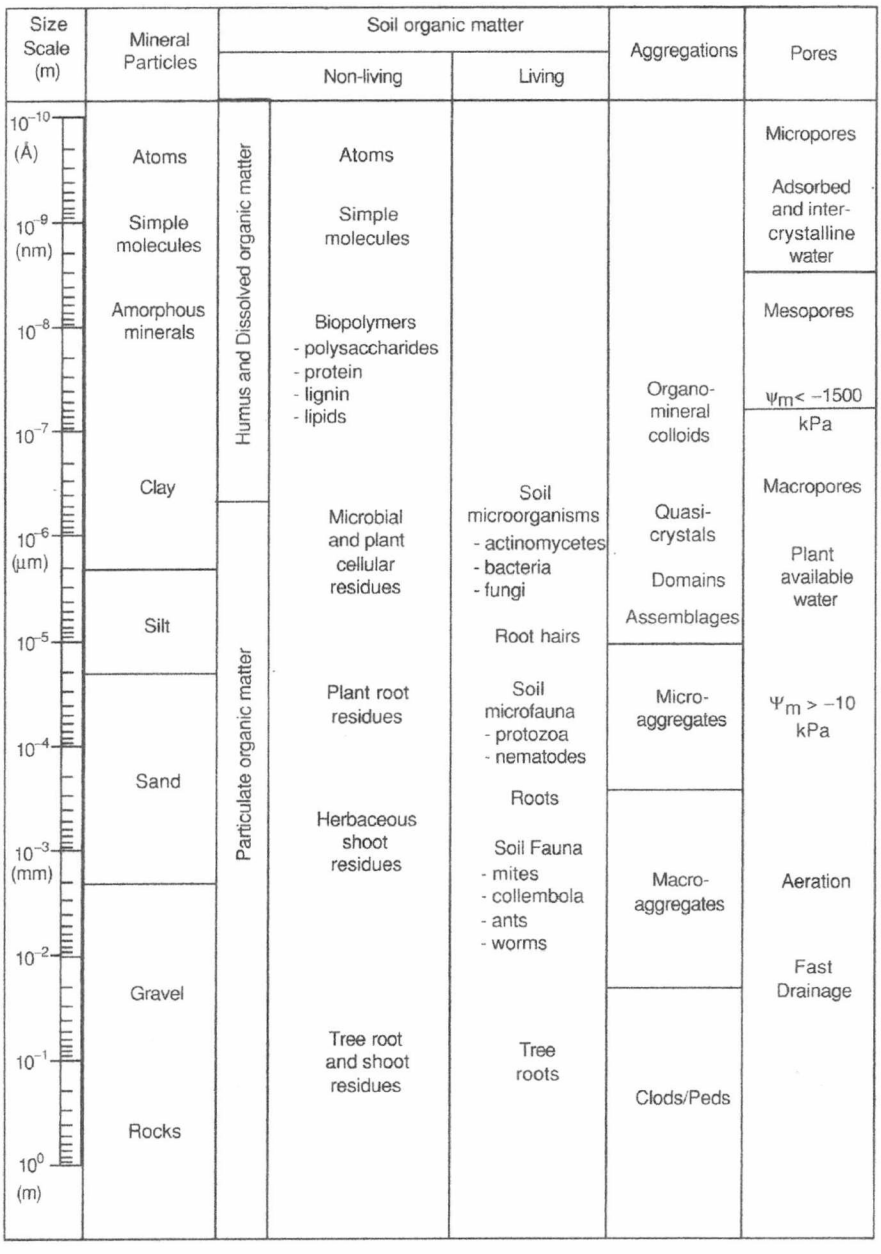
1.4.1. Introduction

Soil is a highly complex and heterogeneous system composed of mineral material, the roots of plants, microbial and animal biomass, organic matter in its various states of decomposition, and atmospheric gases (Killham, 1995). Some of these products are recognisable in the form of stones, sand grains and leaf litter, whereas others, such as clay minerals and humic fractions result from the intense chemical changes that occur in both inorganic and organic material during soil formation processes (Marshall *et al.*, 1996). The resulting soil can range in texture from coarse sands to fine clays, with an organic matter content ranging from less than 5 % by weight to around 80 % in peaty soils (Marshall *et al.*, 1996).

These soil fractions of clay, silt, sand and humic substances are all unevenly distributed (Smiles, 1988) and can complex together to form aggregates (Tisdall and Oades, 1982). Although soil can occur as a collection of individual grains, such as in sands, they usually are linked into clusters or aggregates of varying sizes, compositions (Table 1.2) and stabilities (van Elsas *et al.*, 1998, Baldock, 2002). The smallest structural units recognised when aggregates breakdown along surfaces of weaknesses are microaggregates. Edwards and Bremner (1967) concluded, from experiments using sonic vibration to disperse and then reaggregate soil, that these microaggregates were smaller than 250 μm in diameter (diam.). and were composed of clay and organic matter joined by polyvalent metals (e.g. Ca, Mg, Fe). Microaggregates are generally characterized as containing older, more humified or recalcitrant SOM, and have a greater persistence in the environment than macroaggregates (Beare *et al.*, 1994).

Aggregation determines the pore distribution of a soil, with approximately half the volume of a well-aggregated soil consisting of pore space (Killham, 1995). This porosity, in turn, controls the distribution and activity of the soil biota. Porosity and soil microstructure define the local physico-chemical conditions that microorganisms have to survive in and therefore their distribution and interactions, by controlling, amongst other factors, the availability of water and oxygen. Hydrophilic soil particles, such as clays and humic substances adsorb water to their surfaces or retain them within pores by capillary forces. However, this retained water is not always available to microorganisms, as it can be held in interlayer surfaces in Ca-saturated smectites at energies much too high for microbes to utilise (Robert and Chenu, 1992). It is, therefore, evident that particle size distribution and shrink/swell capacities both need to be when considering the mechanics of soil structure (Baldock, 2002). For example,

soils dominated by either kaolinite or illite clay, but which contain small amounts of smectites, are dispersive (Stern *et al.*, 1991). A more recent study also showed that soils with smectites and vermiculite have weak aggregation (Igwe *et al.*, 1999).



Ψ_m = soil water matric potential

Table 1.2. Size scales associated with soil mineral particles, organic components, pores and aggregations of mineral and organic components (taken from Baldock [2002]).

1.4.2. Macroaggregates, microaggregates and soil size fractions

The physical interactions between the various components of the soil matrix are dependent on a number of factors including pore size distribution, water retention, aggregate stability, and the mechanical properties of the soil, as well as the surface characteristics of the soil particles (e.g., surface area, electrostatic charge, surface free energy and functional groups; Chenu and Stotzky, 2002). Tisdall and Oades (1982) proposed a conceptual model for soil structure based on an aggregate hierarchy in mineral grassland soils. The model distinguishes three basic levels of structural organisation: free primary particles (sand, silt, clay), microaggregates, and macroaggregates. Further evidence for this hierarchical classification has been documented (Oades and Waters, 1991; Golchin *et al.*, 1994). According to this model, macroaggregates (> 2000 μm) consist of enmeshed microaggregates and soil particles held together by a fine network of roots, fungal hyphae and other transient binding agents and microaggregates consist of 2 - 20 μm diameter particles bound together by a number of additive forces, such as persistent organic materials and crystalline oxides and highly disordered aluminosilicates, making them highly stable. For example, clay minerals, such as montmorillonite and kaolinite are known to adsorb and catalyse the polymerisation of polyphenols and the copolymerisation of amino acids and polyphenols (Wang and Huang, 1989; Wang, 1991; Bosetto *et al.*, 1997) producing clay-organic complexes.

However, in soil it is not yet known if preferential adsorption of specific components of the organic matter is taking place on different clay minerals or whether clay mineralogy is impacting on the humification process (Laird *et al.*, 2001). Tisdall and Oades (1982) also indicated that this model would only be appropriate for soils where organic matter was the main binding agent, citing the study of Collis-George and Lal (1970), where slaking of 1000 μm to 2000 μm aggregates, of a black earth soil, directly into water-stable particles of 30 μm diameter was observed. Slaking occurs when air pressure builds up as water rapidly enters soil pores causing the disruption of the soil structure. Slaking can overcome the binding force of soil organic matter causing aggregate breakdown (Zhang and Horn, 2001). Bossuyt *et al.* (2001) observed an increase in macroaggregate formation over time, concurrent with a decrease in microaggregate abundance and attributed this to the aggregate hierarchy model.

Oades (1993) further specified that aggregate hierarchy would only exist in soil with extensive rhizosphere development. However, an alternative concept of aggregate organisation has been proposed on the basis of plant and root debris, the main source of particulate organic matter in soil (Six *et al.*, 1999). In this theory, macroaggregates form as

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particulate organic matter (POM) becomes colonized by microbes and encrusted by mineral materials which, as decomposition of the POM occurs, causes structural instability and eventual breakdown into newly formed microaggregates containing the residual POM (Golchin *et al.*, 1994, 1998). Gale *et al.* (2000) noted, over a 90 d period, that significant increases in the amount of new, ^{14}C-labelled root-derived POM in microaggregates (53 - 250 μm) were released when unstable macroaggregates (> 250 μm) underwent slaking, which corroborates this second theory. Similar observations were made by Angers *et al.* (1997). Macroaggregates can be disrupted by crop management, rapid wetting or even raindrop impacts (Tisdall and Oades, 1982).

Particle size fractionation has been used to distinguish between different pools of organic matter and their turnover rates (Christensen, 1992). The SOM contained within sand fractions is predominantly composed of fresh or slightly decomposed plant material or debris with high concentrations of carbohydrates and is readily degradable (Guggenberger *et al.*, 1994; Amelung *et al.*, 1998). The SOM found in clay- and silt-sized fractions tends to be at more advanced stage of decomposition, consisting mainly of aromatic and aliphatic structures (Guggenberger *et al.*, 1995). It is also generally more recalcitrant to microbial degradation (Dalal and Mayer, 1986; Angers and Giroux, 1996). It is known that silt and clay particles occur together as microaggregates (Anderson and Paul, 1984) and these have been shown to contain the most stable organic matter in soil (Skjemstad *et al.*, 1993). Adsorption of SOM on clays or in microaggregates induces considerable physical protection against microbial degradation (Tisdall and Oades, 1982). Puget *et al.* (1995) showed that young SOM content increased with water-stable aggregate size and that it was likely to play a role in macroaggregate stabilization.

An increasing organic carbon and total nitrogen concentration has been observed with decreasing particle-size (Ahmed and Oades, 1984; Stemmer *et al.*, 1998). Stemmer *et al.* (1998) found the greatest concentration of organic C and total N in the clay-sized fraction, although Jocteur-Monrozier *et al.* (1991) found the highest organic C and N in the silt-sized fractions. The C-to-N ratios are commonly found to decrease from coarser to finer-sized fractions, reflecting the changes in the mineralization and humification status of the SOM (Hassink *et al.*, 1995; Stemmer *et al.*, 1998), and this ratio has been linked to the differences in the concentrations of plant debris in the size-fractions.

Soil aggregation is strongly affected by cultivation (Young and Ritz, 2000). Cultivation typically reduces the organic matter content of soils with a corresponding decrease in aggregate stability (Angers and Mehuys, 1989). The disruption of macroaggregates through

tillage of soil exposes labile organic carbon sources that would not have usually been available, depleting SOM (Beare *et al.*, 1994) and causing deterioration of soil structure (Lupwayi *et al.*, 2001). Aggregate sizes have typically been found to be greater in no-till compared to tilled soils (Drees *et al.*, 1994).

1.4.3. Microbiology of soil structure

Soil structure has been defined as a *"juxtaposition of a multitude of microenvironments or microhabitats, characterised by a variety of physical and chemical conditions"* (Chenu and Stotzky, 2002). The major determinants of aggregate stabilization are organic materials, which in addition to the microorganisms include the decomposition products of plant, animal and microbial remains and biosynthetic products such as polysaccharides. Fungal colonization of particulate organic matter is crucial for aggregate formation (Six *et al.*, 1999). The stabilization of microaggregates within macroaggregates is governed by a number of processes. These include microbial activity and the deposition of adhesive biosynthetic products, which are in turn influenced by the accumulation of organic matter and the pore size distribution (Miller and Dick, 1995). Microorganisms, therefore, are critical to the dynamic process of aggregate formation and degradation (Sollins *et al.*, 1996; Wright and Upadhyaya, 1996) and variations in their distribution by aggregate size (Drążkiewicz, 1994) suggests a positive relationship between microbial populations and the stability of soil structure.

Fungi are thought to be important in soil aggregate formation, colonizing and enmeshing POM with hyphae and thus may be concentrated in larger pores of > 20 μm diam. (Degens *et al.*, 1996), whilst exopolysaccharides produced by bacteria and unicellular algae as well as fungi are more dominant at smaller scales (< 20 μm) and may be critical to cementing together clay particles and domains to form stable microaggregates (Foster, 1988; Dorioz *et al.*, 1993; Falchini *et al.*, 1997). Fungal hyphae are thought to strengthen pre-existing arrangements of soil particles and aggregates rather than to start macroaggregate formation (Baldock, 2002) and microorganisms have been shown to reorganise fine clay particles to align them to external cellular surfaces (Lünsdorf *et al.*, 2000). Dorioz *et al.* (1993) observed that fungal hyphae could compact and reorientate clays 20 μm away from hyphal surfaces and bacterial cells are also thought to be able to induce such reorientation through the presence of polysaccharide mucilage (Baldock, 2002). For example, the addition of xanthan or dextran, both bacterial polysaccharides, accentuated bridging between clay particles, producing stronger cohesion and enhanced water stability (Chenu, 1993).

Microbial growth also decreases the wettability of aggregates in soil (Hallett and Young, 1999) limiting the extent of slaking. The lipid content of soils has been correlated with aggregate stability (Capriel *et al.*, 1990), as has the presence of glomalins, which are glycoproteins produced in copious quantities by vesicular arbuscular mycorrhizal fungi (Wright and Upadhyaya, 1998). This was suggested to be due to the glomalins making clay surfaces more hydrophobic (Chenu and Stotzky, 2002). These authors argue that although qualitative *ex situ* observations suggest an interactive bi-directional relationship between soil architecture and soil microbes, the actual knowledge of these interactions *in situ* is too little to quantify the real importance of each mechanism. It is also apparent that all components of SOM involved in soil structural stabilization are transient, in that they too will be the focus of decomposition processes.

1.4.4. Distribution of microbes within different aggregate sizes and activities

The spatial relationship between microbial biomass and the aggregate hierarchy has been estimated using fumigation methods, biomass indicators (e.g., ATP, dehydrogenase) or microbial counting (Ahmed and Oades, 1984; Kanazawa and Filip, 1986; Jocteur-Monrozier *et al.*, 1991; Lensi *et al.*, 1995). The biomass concentrations were correlated with organic carbon contents and were generally highest in the silt- and clay- sized fractions. A comparison of bacterial populations in aggregated and disaggregated soils showed higher numbers of bacteria associated with the former, suggesting these structures yielded a more favourable environment for bacteria (Elliott, 1988; Andrade *et al.*, 1998).

The heterogeneous and non-uniform structure of soil produces a variety of distinct or '*temporary discrete microhabitats*' (Wright *et al.*, 1993) each containing a variety of microorganisms, which are randomly distributed across aggregate size classes. On a microsite level, five main groups of protozoa (flagellates, amoeba, small ciliates, *Colpoda* spp., and large ciliates other than *Colpoda* spp.) were found unevenly distributed amongst 330 aggregates (Vargas and Hattori, 1990). Other studies have highlighted the differential distribution of microbes in aggregates for specific populations, i.e. nitrifying bacteria (Nishio and Furusaka, 1970), denitrifying bacteria (Seech and Beauchamp, 1988), cells of amoebae, flagellates and ciliates (Vargas and Hattori, 1991) and *Rhizobium leguminosarum* bv. trifolii. (Mendes and Bottomley, 1998). Hattori and Hattori (1993) concluded, "*aggregates have divergent composition of microbial groups*" indicating that "*microbial processes in soil are carried out by a diverse array of microbial assemblages in each aggregate*". Community functional diversity has also been demonstrated at an aggregate scale using BIOLOG

fingerprints of soil communities associated with different size fractions (Winding, 1994). This study showed that a more detailed description of bacterial communities was obtained when the bulk soil was fractionated into different size classes, revealing that the bacterial communities of microaggregates differed in different soils and were distinct from both bacterial communities of macroaggregates, and free and loosely associated bacteria (< 2 μm). It has also been demonstrated that operationally defined outer and inner compartments of aggregates also have divergent populations (Hattori, 1988; Drążkiewicz, 1994; Ranjard *et al.*, 1997).

Foster (1988), using electron microscopy, found that porosity determined microbial distribution. This author found that fungi were restricted to large diam. interaggregate pores, whilst bacteria were located within aggregates in smaller pores. This pore-size separation or exclusion of microbial populations is also evident for predator-prey relationships (Heijnen and van Veen, 1991; Wright *et al.*, 1993), whereby bacterial populations are protected in pores with small neck diameters, as they are inaccessible to their protozoan predators. However, other studies have concluded that it is the larger distances, and enhanced tortuousity, that protozoa have to travel in fine textured soil that limits their populations and predation (Young *et al.*, 1994). Thus, it seems likely that other factors may also contribute to microbial distribution in soils.

Other studies that have incorporated measurements of microbial activity also showed an uneven distribution in the aggregate size fractions. Silt- or clay- sized fractions displayed the highest invertase activities and correlated with the biomass and organic carbon content of the size fractions (Stemmer *et al.*, 1998). Filip *et al.* (2000) also found highly concentrated proteinase activity in a silt and clay fraction of a Haplic Luvisol. However, this study found reduced β-glucosidase and β-acetylglucosaminidase activities in this fraction. This disparity was linked to these enzymes adsorbing onto silt-clay particles, which resulted in diminished activities (Burns, 1982). However, despite all these studies no definite trends can be reached since the fractionation methods used, varied in each of the studies and thus the force of dispersion the soil was exposed to also varied. The factors that control the spatial distribution of microbes have not yet been fully realized (Chenu and Stotzky, 2002).

1.5. Aims and objectives

Microorganisms are known to be present in surface and subsurface environs. The presence of microbes in subsoils could have a direct impact on the environmental fate of pesticides and other organic contaminants, yet in these environs knowledge of their relative abundance and their associated activities is limited. The initial aim of this project was to ascertain whether microbes were present in the subsoils under investigation and to determine whether these indigenous populations could be actively involved in nutrient cycling.

One of the main factors limiting the availability of a chemical to biodegradative processes is its sorption to soil surfaces. Although a number of chloroacetanilide herbicides have been studied with regard to this phenomenon, published information on the sorption-desorption behaviour of acetochlor is scant, especially in subsoils. The second objective was, therefore, to generate detailed information on the potential of the surface soils and subsoils to retain acetochlor.

The presence of active microorganisms in surface soils and subsoils, along with reactive soil surfaces can lead to the dissipation of pesticides from terrestrial environments. The primary aim of this project was to determine the relative contribution of biological and chemical degradation and nonextractable residue formation to understand the principal routes affecting the environmental fate of acetochlor.

Chapter 2. Materials and Methods

2.1. Soil analysis

Unless stated otherwise all soil analyses was carried out in triplicate.

2.1.1. Physical and chemical analysis

Soil cores (approx. 30 kg; one from each site) were collected from agricultural fields at two Prospective Groundwater (PGW) study sites (ARP, 2001) in Iowa and Michigan, USA. These soils were chosen because of the distinct chemical and physical properties displayed by these soils. The soils at both sites had undergone identical cropping practices: soils were tilled conventionally, crops (maize and soybean) were rotated annually and the sites were neither irrigated nor drained. PVC tubes (10 cm $\times$ 45 cm^2) were pushed vertically into the soils at the field sites using a hydraulic drilling rig and cores sampled using colour and texture as a guide to different horizons. The sample depths were 0.0-0.3 (surface), 1.0-1.3 (mid), 2.7-3.0 (clay deep) and 3.9-4.2 m (sand deep). Soils were stored field moist in sealed polythene bags at 4°C until required. Syngenta provided these soils and samples were collected prior to the start of this PhD studentship.

Particle size distribution was classified according to the USDA scheme; organic matter (as percentage of soil weight) was determined using the Walkley-Black oxidation method and a factor of 1.724 (Walkley and Black, 1934) was used to convert organic C to organic matter. Cation exchange capacity (milliequivalents 100 g^{-1} air dried soil) was determined by sodium saturation at pH 7.0 and flame photometry. Natural Resource Management Ltd supplied this data.

2.1.2. Soil size fractionation

The physical fractionation procedure and fractions obtained are summarised in Table 2.1. Soil (100 g d wt) was placed on a 2.0 mm aperture sieve and lowered into a container containing 150 ml sterile distilled water (4°C). The soil was left in the water for 15 min to allow saturation to take place. The sieve was then raised and lowered 60 times over a period of 5 min. The soil retained by the sieve was stored at 4°C in a sterile container. The soil slurry, which passed through the 2.0 mm diam. aperture sieve, was passed through a 250 μm diam. aperture sieve followed by a 53 μm diam. aperture sieve. The wet sieving

was carried out, as before with the sieves being raised and lowered for 5 min, after which the soil fractions were stored at 4°C. The 20-53 μm fraction was isolated using sedimentation (Appendix 1). The 2-20 μm and 0-2 μm fractions were obtained by centrifugation at 90 x *g* and 2500 x *g*, respectively. The sieve wash was made up to 10 mM $CaCl_2.2H_2O$ by direct addition of this compound into the wash. This allowed the colloidal clay fraction to flocculate (24 h, 4°C), prior to centrifugation (2500 x *g*, 5 min) and separation from the remaining aqueous mineral phase. All sieves used were constructed from stainless steel and brass. All centrifuge tubes were autoclaved (121°C and 0.2 MPa, 15 min) prior to use

Table 2.1. Soil size separates obtained after a sequence of physical fractionation procedures

diameter, μm	*Soil size fraction*	*Procedure*
>2000	Macroaggregate	Wet sieving
250 – 2000	Macroaggregate	Wet sieving
53 – 250	Microaggregate	Wet sieving
20 – 53	Coarse silt	Sedimentation
2 – 20	Fine silt	Centrifugation
0 – 2	Clay	Centrifugation
0 – 1	Colloidal clay	Flocculation

2.1.3. Total Organic Carbon content

A modified Walkley-Black method (Jackson, 1958) was used to determine the total organic carbon (TOC) of air-dried bulk soils and soil particle size fractions. Air-dried soil was homogenised with a pestle and mortar and 1.000 g weighed into a 100 ml Erlenmeyer flask. A 10 ml aliquot of 5 % potassium dichromate was added to the soil and shaken gently to dissolve the soil. A 20 ml aliquot of concentrated sulphuric acid (98 %) was pipetted into the soil slurry and shaken gently. The acid-mixture was allowed to cool and then 50 ml of 0.5 M $BaCl_2$ was added. The solution was allowed to cool once more and centrifuged (9464 x *g*, 5 min). The absorbance of the supernatant was recorded at 600 nm (UNICAM 5625 spectrophotometer, UNICAM, UK). Blanks were carried out as before without the addition of soil. A calibration curve was obtained, using glucose as the carbon source, over a range of 0 - 25 mg C. Aliquots of the aqueous glucose standards were pipetted into 100 ml Erlenmeyer flasks and dried at 105°C. The oxidation procedure

as outlined above was performed and the absorbance of the resulting solutions measured. A factor of 0.74 was used to correct for the incomplete digestion (Jackson, 1958).

2.1.4. Water-holding capacity

Water-holding capacity (WHC) was determined volumetrically. Glass wool (approx. 300 mg) was tamped down into the top of a glass funnel stem. Attached to the base of the stem was a piece of flexible tubing. A clip was placed on the tube to close it completely. Field moist soil (50 g) was placed into the funnel, which was secured into place using a clamp stand. A 50 ml volume of water was added to the soil and was allowed to stand for 30 min to ensure saturation. Upon saturation, the clip on the tubing was opened and the draining water collected in measuring cylinders. After 30 min the volume of the water in the measuring cylinder was noted. Controls were run without soil to allow measurement of the water retained by the glass wool. Triplicate samples were used for each soil and for the controls. Separate portions of soil (50 g) were oven-dried (105°C) to determine the soil moisture content. The WHC (ml water held at 100 % WHC per 100 g d wt soil) was determined thus:

A = 50 – (volume of water retained by glass wool + volume of water collected) ml

B = 2A + moisture content (%)

WHC = B / soil d wt

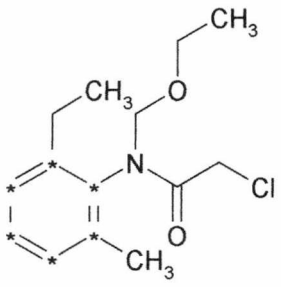
2.1.5. Soil pH

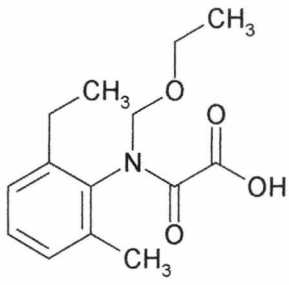
Soil (25 g) was weighed into a 100 ml Erlenmeyer flask and 50 ml of 10 mM $CaCl_2.2H_2O$ added. The suspension was shaken and then left to stand for 2 h. The pH of the soil solution was then taken using an electronic probe.

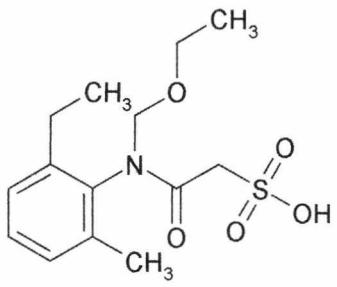
2.2. Chemicals

Acetochlor is a non-ionic herbicide, with a solubility of 223 mg l^{-1} at 25°C, and has a Henry's Law constant (the relative degree of partitioning between gas and aqueous phases in the unsaturated zone) of 0.00709 Pa m^3 mol^{-1}. Non-labelled and ^{14}C-labelled acetochlor were synthesized and supplied by Syngenta (formerly Zeneca Agrochemicals;

Jealott's Hill Research Station, UK). The structure, position of radiolabel and specific activities are shown in Figure 2.1. The purity of the stock radiochemical solution was determined using thin layer chromatography (TLC), as described in Section 2.3.3.1, and was found to be 99.8 %. The metabolites, ac-ESA [2{(ethoxymethyl)(2-ethyl-6-methylphenyl) amino}-2-oxo-ethanesulphonic acid] and ac-OXA [{(ethoxymethyl)(2-ethyl-6-methylphenyl)amino} oxo-acetic acid] (Figure 2.1) were also supplied by Syngenta. The non-radiolabelled acetochlor, ac-ESA and ac-OXA were all authentic reference standards, above 99.8% purity. All of the chemicals were dissolved in acetonitrile:Milli-Q water (75:25 v/v) and stored frozen until needed. The ^{14}C-ring labelled acetochlor was isotopically diluted using the non-radiolabelled acetochlor reference standard as required. All the reference standards were used for co-chromatography with sample extracts, during characterization of the extracts by TLC analysis (Section 2.3.3.1).







CAS Name	**2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl) acetamide**	**2[(ethoxymethyl)(2-ethyl-6-methylphenyl) amino] oxo-acetic acid**	**2[(ethoxymethyl)(2-ethyl-6-methylphenyl) amino]-2-oxo-ethanesulphonic acid**
IUPAC Name	2-chloro-*N*-ethoxymethyl-6`-ethylacet-*o*-toluidide	*N*-ethoxymethyl-*N*-(2-ethyl-6-methylphenyl) oxamic acid	*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl) carbamoylmethanesulphonic acid

Figure 2.1. Structures and nomenclatures of acetochlor and its two principal metabolites: acetochlor-oxanilic acid and acetochlor-ethanesulphonic acid, respectively.

2.3. Analytical Techniques

Unless otherwise stated all procedures were carried out in triplicate.

2.3.1. Extraction of acetochlor and metabolites from soil

Acetochlor was extracted exhaustively from soil using aqueous 10 mM $CaCl_2.2H_2O$ solution. Each extraction was conducted at 25±4°C for 30 min, with supernatants decanted following centrifugation (10 min, 1528 x *g*). This extraction procedure was repeated until negligible amounts of radioactivity were recovered (which was approximately 4 - 5 cycles). The extracts were combined and made up to a defined volume using the extraction solvent mixture. The aqueous extracts were analysed by liquid scintillation counting (LSC; Section 2.3.7), and for the bulk soil extracts were subject to TLC analysis (Section 2.3.3). All glassware was rinse-checked before use to confirm that no residual radioactivity was present.

2.3.2. Acetochlor analysis by High Performance Liquid Chromatography for determination of specific activity

High Performance Liquid Chromatography (HPLC) was used to measure the specific activity of the treatment solutions prepared for the environmental fate study (Section 2.3.8.). The chromatography system used was a Hewlett Packard 1100, operating in the isocratic mode with a flow rate of 1.0 ml min^{-1}, a variable wavelength UV/VIS detector set at 216 nm and the injection volume set at 20 µl. The HPLC column was a 250 mm by 4.6 mm (i. d.) Spherisorb Excel C18. To avoid temperature induced shifts in retention time the column was maintained at 20°C. The mobile phase was 85:15 (v/v) acetonitrile/Milli-Q water (acidified to pH 2.0 with concentrated H_3PO_4). This protocol is a standard operating procedure carried out for acetochlor analysis by Syngenta (Verity, pers. com, 2000).

2.3.3. TLC analysis

2.3.3.1. Determination of acetochlor purity and metabolite profile.

TLC was used to measure the purity of the dispensed radiochemical. TLC was also used to determine the concentrations of acetochlor, and its metabolites, in solvent extracts. Authenticated reference markers (Figure 2.1) and samples from soil extracts were applied to TLC tracks both individually and admixed. The reference markers were compared with the radioactive components by co-chromatography. The reference markers were all

detected on developed plates by the quenching of gel fluorescence under short wave ultraviolet light (Section 2.3.3.2.). The plates were activated prior to use by immersing in methanol. Samples were applied with micropipettes in 1 cm bands to pre-coated plates without clean up of the sample extract. The types of plates used (including manufacturers) and the solvent systems they were developed in are shown in Table 2.2.

The percentage of parent compound and metabolites was determined from cross-referencing silica plates developed in at least two normal phases and one reversed phase solvent system. This number of TLC plates was required per sample as not all plates provided acceptable separation of parent and metabolites. Overall values are derived from the separations of individual compounds, and data where distinct separations are not resolved. Values for individual compounds can be elucidated from co-chromatographic data as the range of separations observed is different in each solvent system. This method of combining information from several plates and cross-referencing the data moderates the final values.

Table 2.2. Solid and mobile phases used for determination of acetochlor purity and for extract separations

Purity determination:

Whatman KC18F (reversed phase)	methanol:water 80:20 v/v
Merck Si60 F254 (normal phase)	chloroform:methanol 95:5 v/v
Merck Si60 F254 (normal phase)	n-hexane:acetone 50:50 v/v

Extract separations:

Merck RP18F (reversed phase)	water:acetonitrile:acetic acid 50:45:5 v/v/v
Merck Si60 F254 (normal phase)	ethyl acetate:propan-1-ol:water 60:40:10 v/v/v
Merck Si60 F254 (normal phase)	chloroform:methanol:formic acid:water 70:25:3:3 v/v/v/v

After samples were applied to the TLC plates they were allowed to dry for at least 24 hours before development in the solvent systems. The actual development process took approximately 90 minutes at 22±2°C

2.3.3.2. Imaging TLC plates

For the detection of acetochlor and its metabolites an amount containing 15 bq of radioactivity was spotted onto the TLC plates. This was calculated from LSC analysis of the solvent extracts. After development and drying, the radioactive regions on the TLC plates were detected by exposure against a storage phosphor plate (Fuji Type Bas-III).

The exposure necessary for imaging was 7 d during which the plates were kept in a lead box to minimise background contamination. The imaging plate was a flexible image sensor comprised of bundles of very small (grain size ≈ 5 μm) photosensitive phosphor crystals from barium fluorobromide, containing trace amounts of europium as a luminescence centre (formulated as $BaFBr:Eu^{2+}$), uniformly coated onto a polyester support film. A Fuji BAS2000 Bio-imaging analyser system was used to obtain autoradiograms of the TLC plates. The exposed imaging plate was scanned with a He-Ne laser beam of red light (633 nm) while the plate was being processed in the phosphor reader. A bluish-purple (400 nm) photostimulated luminescence, released upon laser excitation, was collected through the light collecting guide to the photomultiplier tube (PMT), and converted there to analog signals in chronological order. Subsequently, these were converted to digital signals. The Bio-imaging analyser system gives very high sensitivity, high resolution (100 or 200 μm), superior linearity with much shorter exposure times (up to 50 times quicker) compared to X-ray film autoradiography and additionally because of the increased linear dynamic range quantitative data from the resulting autoradiogram equivalents can be obtained (Vaughan, personal communication, 1999). BAS Reader 2.13 and AIDA 2.11 software were used for the analysis of the autoradiograms.

For identification of acetochlor and the metabolites, acetochlor-ESA and acetochlor-OA, authenticated reference makers were also spotted onto the TLC plates. After analysis of the plates with the bio-imager, ultraviolet (UV) light was used to allow detection of the markers. Comparing the Rf values for the bands detected on the autoradiogram with the corresponding bands of the markers allowed identification of these compounds. No other metabolite standards were available.

2.3.4. Combustion analysis

All solvent extracted soils (Section 2.3.1.) were air-dried and homogenised using a pestle and mortar. Duplicate sub-samples (approximately 250 mg each) were taken for combustion analysis (4 min; manual biological oxidiser, Harvey, UK) from each extracted bulk soil sample. For analysis of soil size fractions only one sub-sample (approximately 250 mg) was taken from each replicate. Two cellulose combustion cones (Packard Limited, UK) were combusted and the $^{14}CO_2$ evolved used as backgrounds for LSC. The efficiency of the combustor was determined before and after each batch of samples by combustion of a known quantity of radioactivity spiked into a combustion cone. The

efficiency of the combustion process, i.e. recovery of the applied radiochemical, was always greater than 95.0 % and was used as a correction factor for the combusted samples. Efficiency checks were also carried out every 20 samples. The $^{14}CO_2$ evolved from the oxidation of samples was trapped in 2-methoxyethylamine - part of a combustion cocktail consisting of 'Optiphase Safe':2-methoxyethylamine:distilled water, 50:25:2 (v/v/v) - and then analysed by LSC.

2.3.5. Mineralization

For entrapment of $^{14}CO_2$, (as a measure of mineralization) sodium hydroxide (2.0 M) traps were used and were changed at every 3-5 d. NaOH (200 µl) was analysed directly by LSC to determine the levels of radioactivity present.

2.3.6. Volatile production

Suba-seals (see 2.3.8.) were removed at the end of the incubation period, flushed with methanol, and the wash analysed by LSC. No volatile production was detected during the study.

2.3.7. LSC

All solutions containing ^{14}C-labelled acetochlor were checked routinely for the radioisotope concentration before, during and post application. The actual amount of radioisotope added to a sample was checked, by measuring the activity contained within the same size aliquot from the same pipette by LSC. LSC was used to analyse carbon dioxide traps for mineralization, solvent extracts for available radiochemical, and the solutions produced by combustion analysis.

For the combustion analysis (Section 2.3.4.) liquid scintillation counting was carried out using a 1409 Wallac scintillation spectrometer. The 1409 counter uses the entire spectrum with no fixed window, a counts per minute/disintegrations per minute (CPM/DPM) counting mode and a sample-quench monitoring using the external spectrum generated from a ^{152}Eu source (SQP[E]). Quench correction spectra were set up using a series of quenched standards and a digital overlay technique was used to adjust the spectra to

enable automatic calculation of DPS values. The 1409 automatically adjusts the SQP[E] values to compensate for colour, chemical, and physical quenching within the samples. Counts due to chemiluminescence are monitored and automatically subtracted to provide counts per minute values. The replicate aliquots of each sample were used to obtain an average DPS value. Batches of samples were preceded by two suitable background samples from which a mean was calculated. The mean background count was automatically subtracted from subsequent count rates in the batch to provide net CPM values. LSC was carried out for 5 min for each sample.

All other samples analysed by LSC were carried out on a Beckman-Coulter scintillation counter (Beckman-Coulter, UK). The mode of operation of this model resulted in read-outs in disintegrations per minute (DPM). Samples were mixed with Ultima Gold in a ratio of at least 1:4 prior to LSC and were also analysed over a 5 min period.

2.3.8. Experimental set-up for environmental fate study (Iowa clay profile only; Michigan sand profile omitted)

Soils (3 replicates x 3 soil depths x 6 time points x 2 sterility treatments (autoclaved versus non-autoclaved); 100 g d wt. per sample) were weighed into sterile 250 ml Erlenmeyer flasks (108 altogether), in a category II microbiological safety cabinet to maintain aseptic conditions, using a sterile spatula. One glass tube containing sterile distilled water, to maintain humidity, and another containing 2.0 M NaOH, to absorb CO_2, were placed into each flask. The flask was then sealed with a suba-seal, which also served as a volatile trap The CO_2 traps were changed every 3-5 d, which also allowed a flux of fresh oxygen into the system. Prior to the application of acetochlor (95:5 non-radiolabelled-to-^{14}C-labelled acetochlor (w/w), the soils were allowed to incubate for two weeks (22±4°C at a WHC of 40 %) to allow equilibration. The recommended field application rate (3.5 kg ha^{-1}) of acetochlor was then added drop-wise to the surface soil, with sufficient sterile distilled water to make the soils up to 60 % WHC. The mid and deep soils were exposed to 50 % and 25 % of this application rate, respectively. The rates of application for the subsoils were chosen arbitrarily, with 100 % of the field application being unlikely to reach the subsoils (Mills, pers. com., 2000). All soil samples were maintained at 60.0 % water holding capacity throughout the incubation and were kept at 22±4°C. Flasks were destructively sampled over a 100 d time course. No loss in mass occurred over the 100 d exposure time for any of the flasks. Sterile soil controls were set-

up in the same way. Sterilization was achieved by autoclaving (121°C and 0.2 MPa, 1 h) on three consecutive days.

2.4. Sorption and Desorption Isotherms

All soil bulk and size fraction samples were air-dried and homogenised by grinding in a pestle and mortar, prior to use for determination of adsorption and desorption isotherms. The herbicide added as a spike consisted of a ratio of 95:5 non-radiolabelled-to-^{14}C-labelled acetochlor (w/w). Unless stated otherwise all procedures were carried out in triplicate.

2.4.1. Sorption isotherms

Soil (500 mg) was suspended in 1.0 ml 10 mM $CaCl_2.2H_2O$ and spiked with the recommended field application rate of acetochlor for the surface soil. The soils from the clay profile were spiked with 3.5 kg acetochlor ha^{-1} and soils from the sand profile were spike with 2.2 kg acetochlor ha^{-1}. The samples were then shaken on a rotary shaker at 25°C over a time course of 48 h. Samples were centrifuged (2500 x *g*, 5 min) and the supernatant fraction analysed by LSC. After 24 h, equilibrium had been reached for all soils. Controls were run to measure adsorption of acetochlor to microfuge tubes over this 48 h time period. Aliquots were taken from the soil slurry and analysed by LSC.

A 24 h equilibration time was used for the generation of adsorption isotherms. Soil (500 mg) was suspended in 1.0 ml 10 mM $CaCl_2.2H_2O$ containing 0.2, 0.75, 2.5, 5, 10, 15, 25, 50 and 100 μg acetochlor ml^{-1} and the samples shaken on a rotary shaker at 25°C for 24 h. Samples were then centrifuged (2500 x *g*, 5 min) and the supernatants analysed by LSC. Controls were run at the different concentrations and the soil slurry analysed by LSC.

2.4.2. Desorption

Autoclaved bulk soil (500) mg was suspended in 1.0 ml 10 mM $CaCl_2.2H_2O$, spiked with the field application rate, and shaken on a rotary shaker. After 24 h, the soils were

centrifuged (2500 x *g*, 5 min) and the supernatant collected and analysed by LSC. The pellet was then resuspended in 1.0 ml 10 mM $CaCl_2.2H_2O$. This desorption step was carried out at 24 h intervals for 5 d, with each supernatant being analysed by LSC. This procedure was carried out for soil size fractions from the clay profile. Size fractions from the sand profile were not analysed due to a sieving pre-treatment at Syngenta, prior to despatch, which would have disrupted the soil structure. To account for swelling properties of the clay minerals in the 0 - 2 and 0 - 1 μm diam. fractions, a ratio of 1-to-10 (w/v) was used to allow effective separation of solid and liquid phases. Controls as described in Section 2.4.1 were run in conjunction with this experiment for the bulk soil and soil size fractions.

2.5. Microbiological analysis

Unless otherwise stated all procedures were carried out in triplicate.

2.5.1. Enumeration of microorganisms

2.5.1.1. Direct Microscopic Counts

After aliquots were removed for viable counts, 50 ml portions of each diluted soil extract were filtered through 8.0 μm (Millipore, Bedford, UK) and 3.0 μm membrane filters (Whatman, Wallingford, UK). Aliquots (1.9 ml) were removed from the filtrate, admixed with 100 μl formalin, and DAPI (Sigma, Poole, UK) added to give a final concentration of 5.0 μg ml^{-1}. Samples were incubated in the dark for 10 min at 20±2°C. A 250 μl aliquot was taken and added to 8.0 ml of filter-sterilized 100 mM NaCl. Each sample was filtered through a gridded, blackened polycarbonate membrane filter (25 mm diam., 0.45 μm; Millipore, Bedford, UK). These filters were washed with 20 ml of filtered 100 mM sodium citrate buffer (three cycles: pH 6.6, pH 5.5 and pH 4.0) (Sigma, Poole, UK) and once with distilled water. Bacteria were viewed using an epifluorescence microscope (Model DMRB, Leica Microsystems, Wetzlar, Germany). Every square was counted and the mean calculated. The numbers are expressed as cells per g^{-1} d wt soil.

2.5.1.2. Plate counts

Bacteria were extracted from soil using a multi-stage dispersion and differential centrifugation technique (Hopkins *et al.*, 1991). Ten-fold dilutions of the soil suspensions were prepared in phosphate-buffered saline (Sigma, Poole, UK). The dilution series were

used to inoculate two solid culture media: R2A and SEA. R2A was composed of 0.5 g yeast extract; 0.5 g proteose peptone; 0.5 g casamino acids; 0.5 g glucose; 0.5 g soluble starch; 0.3 g sodium pyruvate; 0.3 g potassium dihydrogen orthophosphate; 0.05 g magnesium chloride per litre of distilled water (Reasoner and Geldreich, 1985) and acts as a copiotrophic medium. SEA is an oligotrophic medium containing soluble organic matter extracted from soil (Fredrickson and Balkwill, 1998). A separate SEA was made up for each of the six soil samples (two soils at three depths) by suspending 100 g (wet wt.) of the appropriate soil (dry wt equivalents: 81.0 g [clay surface], 78.2 g [clay mid], 75.2 g [clay deep], 96.9 g [sand surface], 93.0 g [sand mid] and 97.2 g [sand deep]) in 200 ml tap water and autoclaving (121°C and 0.2 MPa, 1 h) every 24 h for three consecutive days. Solid particles were allowed to settle and the fluid decanted and centrifuged (3500 × *g* for 10 min). The supernatant fractions were frozen, thawed and passed through filter paper (No. 4; Whatman, Wallingford, UK) and the filtrate made up to 200 ml with tap water. Filtrate (50 ml) was added to tap water (950 ml) containing 15.0 g Technical agar (No. 3) (Oxoid Ltd., Basingstoke, UK) and autoclaved before pouring. Three plates of each culture medium were inoculated per dilution and the cultures were incubated at 20°C for 7 d (R2A) or 21 d (SEA) before counting.

2.5.1.3. Biomass-C

Microbial biomass was determined by substrate-induced respiration (Anderson and Domsch, 1978) using an infra-red gas analyser [Data supplied by Syngenta]. A conversion factor of 80.8 fg C bacterial cell^{-1} was used to calculate bacterial numbers. This is an average of the figures produced by Watson *et al.* (1977), Kroer (1994) and Moser *et al.* (1996), i.e. 103.75, 56.45, 82.0 fg C cell^{-1}, respectively and assuming a C-to-cell dry weight ratio of 0.5.

2.5.1.4. Enumeration of fungi

Fungi were counted from soil using a soil-plate method (Warcup, 1950). Soil, (0.5 g (wet wt.) was dispersed in 20 ml warm Rose-Bengal agar containing 30 μg streptomycin ml^{-1} and incubated for 7 d at 20°C, with the plates covered by foil to minimize inhibitory effects on fungal growth from photo-oxidation of the dye (Chilvers *et al.*, 1999) and sporulation. These counts were carried out in triplicate.

2.5.2. Soil Enzyme Assays

2.5.2.1. Arylsulphatase, β-glucosidase and phosphomonoesterase activity

Arylsulphatase (EC 3.1.6.1), β-glucosidase (EC 3.2.1.21) and phosphomonoesterase (EC 3.1.3) assays were all based on *p*-nitrophenol release after cleavage of a synthetic substrate (*p*-nitrophenyl sulphate, *p*-nitrophenyl glucoside and *p*-nitrophenyl phosphate, respectively). For the arylsulphatase assay (adapted from Tabatabai and Bremner, 1970) 1.0 g soil (wet wt.) was mixed with 4.0 ml 500 mM acetate buffer (pH 5.8) and 1.0 ml substrate (25 mM). Controls contained 4 ml acetate buffer and 1 ml sterile distilled water. The soils were vortexed briefly and then incubated (20°C, 200 rev min^{-1}) on an orbital shaker (Gallenkamp, Loughborough, UK) for 2 h. Then, 1.0 ml sterile distilled water was added to the samples and 1.0 ml of substrate to the controls, before terminating the reaction with the addition of 1.0 ml 500 mM $CaCl_2$ and 4.0 ml 500 mM NaOH. The suspensions were shaken on an orbital shaker (20°C, 200 rev min^{-1}) for 30 min. Aliquots (1.5 ml) were centrifuged (5 min, 9464 × *g*) and the colour intensity of extracted *p*-nitrophenol measured at 400 nm (5625 UV/VIS spectrophotometer, UNICAM, UK). The acetate buffer was replaced by distilled water for assays conducted at the natural pH of the soil. A standard curve was plotted using a range of p-nitrophenol (Sigma, Poole, UK) concentrations between 0 and 50 μg ml^{-1} distilled water.

β-Glucosidase (Tabatabai, 1982) and phosphomonoesterase (Eivazi and Tabatabai, 1977) assays differed from the above only in the choice of buffer (β-glucosidase: modified universal buffer (pH 6.0); phosphomonoesterase: modified universal buffer (pH 4.0 and pH 9.0)). The pH values for the buffer used were chosen to optimise the activity of acid- and alkaline-phosphomonoesterases (4.0 - 6.5 and 9.0 - 10.0 as pH optima respectively) (Speir and Ross, 1978). The substrate concentration for the phosphomonoesterase assays was 15 mM. The extracting solvent used in the β-glucosidase assay was Tris buffer (pH 12.0) and the substrate concentration was 25 mM.

2.5.2.2. Fluorescein Diacetate Hydrolysis

FDA hydrolysis was measured according to the method of Schnürer and Rosswall (1982). Soil, 3.0 g (wet wt.), was suspended in 50.0 ml phosphate-buffered saline and 250 μl FDA (Sigma, Poole, UK) (2.0 mg ml^{-1} in acetone) added. Controls contained 250 μl of distilled water. The soil suspensions were incubated (20°C, 200 rev min^{-1}) for 4 h. After incubation, 250 μl of distilled water were added to the samples and 250 μl of FDA added to the

controls. The suspensions were vortexed and the reaction terminated by removing 5 ml subsamples and placing these into test tubes containing 5 ml acetone. Aliquots (1.5 ml) were centrifuged (9464 × *g*, 5 min) and the optical density of the supernatant was measured at 490 nm (5625 UV/VIS spectrophotometer, UNICAM, UK). Values for FDA hydrolysis were obtained using a calibration curve relating optical density and fluorescein concentration (ranging from 0 - 10 μg ml^{-1}).

2.5.2.3. Urease activity

A modified assay for urease (EC 3.5.1.5) activity based on that of Kandeler and Gerber (1988) was used. Soil (5.0 g wet wt.) was mixed with 2.5 ml urea (80 MM) and 20 ml (75 MM) borate buffer (pH 10.0). The mixture was reacted for 4 h in an orbital shaker (20°C, 200 rev min^{-1}). Controls were prepared by addition of 2.5 ml sterile distilled water and 20 ml borate buffer. After 4 h, 2.5 ml sterile distilled water was added to the treatment and 2.5 ml urea to the controls, before extraction with 30 ml acidified 2 M KCl (Naseby and Lynch, 1997). The suspensions were shaken on an orbital shaker (20°C, 200 rev min^{-1}) for 30 min. Aliquots (1.5 ml) were centrifuged (9464 × *g*, 5 min) and 1.0 ml of the supernatant fraction was mixed with 9.0 ml distilled water, 5.0 ml sodium salicylate/NaOH solution and 2.0 ml dichloroisocyanuric acid (Na^+ salt). The colour intensity of the solution, after standing at 20 ± 2 °C for 1 h, was measured at 690 nm (5625 UV/VIS spectrophotometer, UNICAM, UK). The borate buffer was replaced by distilled water for the natural soil pH assay. Ammonium concentrations were determined using a calibration curve of ammonium chloride standard solutions from 0 to 2.5 μg ml^{-1}.

2.5.2.4. Dehydrogenase activity

INT (2 (*p*-iodophenyl) -3- (*p*-nitrophenyl) -5-phenyl tetrazolium chloride) reductase activity (i.e. dehydrogenase activity) was determined according to von Mersi and Schinner (1991). Briefly, 1.0 g (wet wt.) soil was placed in foil-wrapped universal bottles and mixed with 1.5 ml 1 M Tris buffer pH 7.0 and 2 ml INT (5 mg ml^{-1} in 2.0 % v/v N,N-dimethylformamide). The control soils received 1.5 ml Tris buffer and 2.0 ml distilled water. The samples were incubated in an orbital shaker (20°C, 200 rev min^{-1}) for 24 h. Then, 2.0 ml distilled water were added to the sample soils and 2 ml INT added to the control soils. The reaction was stopped by adding 10 ml N,N-dimethylformamide/ethanol (1:1 ratio) extractant and shaking (20°C, 200 rev min^{-1}) for 1 h. Aliquots (1.5 ml) were removed and centrifuged (9464 × *g*, 5 min) and the absorbance of the supernatants measured at 464 nm. The Tris buffer was replaced by distilled water for the natural soil pH assay. A standard curve was

obtained using INTF (iodonitrotetrazolium formazan; Sigma, Poole, UK) at a concentration range of 0 - 27 $\mu g\ ml^{-1}$ extractant.

2.6. Statistical Analysis

Minitab 12 and Microsoft Excel 97 were used in the statistical processing of the data (Student`s *t*-test, correlation analysis and ANOVA). SigmaPlot 5 was used to fit equations to data and to ascertain statistical validity of these plots.

Chapter 3. Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques

3.1. Introduction

Subsurface microbial populations have a direct impact on their surrounding environment driving mineral diagenesis, authigenesis and precipitation reactions (Ehrlich, 1998), and thus are inextricably involved in groundwater chemistry (e.g., Hiebert and Bennet, 1992). As a consequence, the genotypic diversity and the metabolic activity of subsoil microbes need to be better understood in order to quantify their influence on the transformation and degradation of both natural and xenobiotic compounds as they pass down through the soil profile.

The presence and activity of the microbial component of surface- and sub- soils can be detected and measured in many ways, including microbial numbers (Kästner *et al.*, 1994), microbial biomass (Lovell *et al.*, 1995), functional activity (such as respiration and N mineralization; Murphy *et al.*, 1998) and enzyme activities (Bandick and Dick, 1999). In addition, newer molecular-based approaches, including probing for specific genotypes (Sayler *et al.*, 1995) and monitoring mRNA expression (Wilson *et al.*, 1999) have an increasingly important part to play in advancing our understanding of microbial ecology.

Quantitative and representative recovery of microorganisms from environmental samples is essential in understanding ecosystem function. A number of binding forces, including electrostatic and van der Waals forces, hydrogen bonding and physical entrapment (Marshall, 1976) need to be overcome in order to reduce cell-soil associations and allow extraction of cells. Chemical (anionic detergents, ion-exchange resins) and physical (shaking, blending, ultrasonication) dispersion treatments are often used but even with exhaustive multi-stage extractions, large proportions of bacterial populations remain associated with soil particles (Hopkins *et al.*, 1991). A further cause of under estimating numbers is that many of the dislodged bacteria cannot be grown on conventional media (Bakken, 1997). The use of vital stains (e.g. DAPI) and microscopy show that bacterial populations counted as CFUs significantly underestimate the total extracted from the soil. A potentially more accurate method of enumerating all the

microbes contained in a soil sample is suggested by calculations based on biomass-C (Watson *et al.*, 1977) and extracted DNA (Sandaa *et al.*, 1998).

Enzyme activities in soil can be associated with active cells (animal, plant, microbial), entire dead cells and cell debris as well as being complexed with clay minerals and humic colloids (Burns, 1982). While the activity of many extracellular hydrolases is probably a result of enzymes associated with some or all these components, dehydrogenase assays measure intracellular catalysis and are more likely to be correlated with the activity of extant cells (Dick, 1997). As with all enzyme assays, the incubation conditions determine the rate of substrate catalysis and the design of soil enzyme measurements and the interpretation of the resulting data are controversial. For example, the use of buffers is vigorously debated (Gianfreda and Bollag, 1996) although they are mainly used to poise and maintain pH at the optimum for activity (Kandeler and Gerber, 1988). A second approach is to rely on the inherent buffering capacity of the soil itself using purified water (Gong, 1997) or to use buffer solely to maintain the pH at the value of the bulk soil (Sinsabaugh *et al.*, 2000). The first approach provides a measure of enzyme potential while the second will often measure activity at a sub-optimal pH producing a lower rate of substrate catalysis but one that is more likely to equate to that shown in the natural environment.

In the experiments reported in this chapter, a number of methods were evaluated to measure and compare microbial presence and activity in surface and subsurface soils. The methods include culturable bacterial and fungal counts, direct counts of total bacteria, biomass-C and arylsulphatase, dehydrogenase, fluorescein diacetate hydrolysis, β-glucosidase, phosphomonoesterases and urease activities.

3.2. Results

3.2.1. Soil physicochemical properties

For the Iowa clay soil (Table 3.1), organic matter decreased with depth with an 85 % reduction from the surface to the mid soil and a further 9 % reduction from the mid to the deep soil. Organic matter was much less in the Michigan sand profile and decreased by 88 % from the sand surface to the mid soil (Table 3.1). There was no further decline from

the mid soil to the deep soil. Cation exchange capacity (CEC) was much higher in the clay soil and decreased with depth in both soils, but the most prominent changes were seen in the sand profile with a 31 % reduction of CEC from the surface to the mid soil and a further 18 % decrease from the mid to the deep soil. With increasing depth, the sand soil was more alkaline increasing by 2.3 pH units from the surface to the mid soil.

Table 3.1. Chemical and physical properties of the Iowa clay and Michigan sand profiles selected for study.

	Iowa Clay Soil			Michigan Sand Soil		
	Surface	Mid	Deep	Surface	Mid	Deep
Depth (m)	0-0.3	1-1.3	2.7-3.0	0-0.3	1-1.3	3.9-4.2
pH (10 mM $CaCl_2$)	5.3	6.0	5.8	5.5	6.6	7.8
Organic Matter (% w/w)*	2.8	0.43	0.17	0.83	0.1	0.1
Cation Exchange Capacity (meq $100g^{-1}$)*	23.4	21.7	19.5	4.5	3.1	0.6
Textural Classification*	Silty Clay Loam	Silty Clay	Silty Clay Loam	Loamy Sand	Sand	Sand

*Data generated by Natural Resource Management Ltd. (Coopers Bridge, Braziers Lane, Bracknell, Berkshire, RG42 6NS).

3.2.2. Microbial Numbers

For both soils, independent of growth media, the numbers of culturable bacteria declined with depth (Table 3.2.). Comparable numbers were obtained for R2A media after a 7 d incubation and the SEA after 21 d incubation. The exception was the clay surface soil in which bacterial numbers on R2A were significantly greater ($P < 0.01$) than on SEA. Fungal counts were significantly greater ($P < 0.01$) in the surface than in the mid soil samples, but were not detected in either of the deep soils (Table 3.2.). Direct counts were much larger but these also declined with depth for the clay soil ($P < 0.01$) but there was no difference between the mid and deep sand soils.

Biomass-C contents (and therefore calculated microbial numbers) decreased with depth for both profiles (Table 3.2.). The clay mid and deep soils contained 33 % and 37.4 % less biomass than that in the surface soil, respectively. In the sand profile there was a 70 % reduction in biomass from the surface to the mid soil. The bacterial numbers indicated by this method increased as a percentage of those counted by direct counts with depth (Table 3.2.). In the clay deep soil, the numbers estimated were approximately 200 %

greater than those determined by microscopic analysis. This difference could potentially be related to the sensitivity of the carbon analysis.

Table 3.2. Microbial abundance measurements and their relationship to direct counts of bacteria: DC = direct count; ND = not determined; [1]calculated using a factor of 80.8 fg C $cell^{-1}$; [2]DNA concentration multiplied by a factor of 8.4 fg DNA $cell^{-1}$. For each soil letters in rows refer to significant differences ($P < 0.05$) with depth. *DNA concentration data provided by Bryan Wilson (pers comm., 2000).

Measure of microbial abundance	Iowa clay			Michigan sand		
	surface (0.0-0.3m)	mid (1.0-1.3m)	deep (2.7-3.0m)	surface (0.0-0.3m)	mid (1.0-1.3)	deep (3.9-4.2)
Direct counts of bacteria (cells x 10^7 g^{-1} d wt soil)	2740 ± 1.44	163 ± 5.44	45 ± 0.1	1170 ± 5.47	50 ± 1.05	56 ± 0.01
Culturable bacteria on R2A (CFUs x 10^7 g^{-1} d wt soil)	10.70 ± 1.04	1.86 ± 0.29	1.21 ± 0.13	4.47 ± 0.27	0.62 ± 0.04	0.26 ± 0.04
Bacterial numbers from CFUs on R2A as % DC	*(0.39)*	*(1.1)*	*(2.7)*	*(0.38)*	*(1.3)*	*(0.46)*
Culturable bacteria on SEA (CFUs x 10^7 g^{-1} d wt soil)	6.19 ± 0.30	1.93 ± 0.12	0.96 ± 0.12	4.25 ± 0.15	0.40 ± 0.03	0.25 ± 0.01
Bacterial numbers from CFUs on SEA as % DC	*(0.23)*	*(1.18)*	*(2.14)*	*(0.36)*	*(0.80)*	*(0.44)*
Culturable fungi (CFUs x 10^4 g^{-1} d wt soil)	10.30 ± 1.2	1.55 ± 0.73	0.00 ± 0	16.00 ± 2.6	2.00 ± 0.24	0.00 ± 0
Biomass (mg C 100 g $soil^{-1}$)	11.63	7.78	7.28	7.77	2.36	ND
Bacterial numbers: Biomass basis[1] (cells x 10^7 g^{-1} d wt soil)	144	96.2	90.1	96.1	29.2	ND
Bacterial numbers from biomass as % DC	*(5.26)*	*(59.02)*	*(200.22)*	*(8.21)*	*(58.4)*	
DNA concentration (μg g^{-1} d wt soil)*	1.34 ± 0.38	0.13 ± 0.07	0.01 ± 0.00	1.23 ± 0.10	0.06 ± 0.03	0.02 ± 0.01
Bacterial numbers: DNA basis[2] (cells x 10^7 g^{-1} d wt soil)	16.00	1.55	0.12	14.6	0.71	0.24
Bacterial numbers from DNA as % DC	*(0.58)*	*(0.95)*	*(0.27)*	*(1.25)*	*(1.44)*	*(0.42)*

3.2.3. Soil enzyme activities

3.2.3.1. Arylsulphatase activity

Arylsulphatase activity (Figure 3.1) decreased with depth ($P < 0.01$) in both profiles in both buffered and non-buffered assays. The buffered assay gave significantly ($P < 0.05$) higher rates of arylsulphatase activity compared to the non-buffered treatment at all depths in both soils, with the exception of the sand deep soil, were the presence of buffer made no difference to the activity. Increases in activity in presence of buffer, in comparison to the non-buffered assays were greatest in the clay soil (surface +58.4 %, mid +96.1 %, deep +21.2 %).

3.2.3.2. β-Glucosidase activity

In non-buffered assays, β-glucosidase activity decreased significantly with soil depth, in both soils ($P < 0.01$) (Figure 3.2). In comparison, when a buffer was used there was significantly greater activity in both surface soils compared to the mid soils. However, no activity was detected from the deep samples of either profile in the presence or absence of buffer. Using a buffer actually decreased activity in the clay surface and mid soils by 12.2 % and 13.8 %, respectively.

3.2.3.3. Phosphomonoesterase activity

Under both buffered (pH 4.0) and non-buffered conditions phosphomonoesterase activity (Figure 3.3) declined with soil depth ($P < 0.01$). At pH 9.0, although there was a significant reduction in activity ($P < 0.01$) with depth in the clay soil and between the sand surface soil and the mid soil there was no difference between sand mid and sand deep soil. For both soils, phosphomonoesterase activities declined with depth, with non-buffered assays displaying greater activities than the buffered (pH 4.0) assays, which were equivalent to the buffered (pH 9.0) assays. For all cases (except pH 4.0 sand deep) the buffer reduced measured activity significantly ($P < 0.01$).

3.2.3.4. Urease activity

For the sand soil, urease activity decreased down the profile using buffered and non-buffered assays (Figure 3.4). Non-buffered soils always showed greater activity and were 2.9, 8.9, 4.7-fold higher for the surface, mid and deep soils, respectively. Under buffered conditions, in the clay soil (Figure 3.4), there was no significant difference in urease activity between the surface and the mid soil and there was no urease measured in the

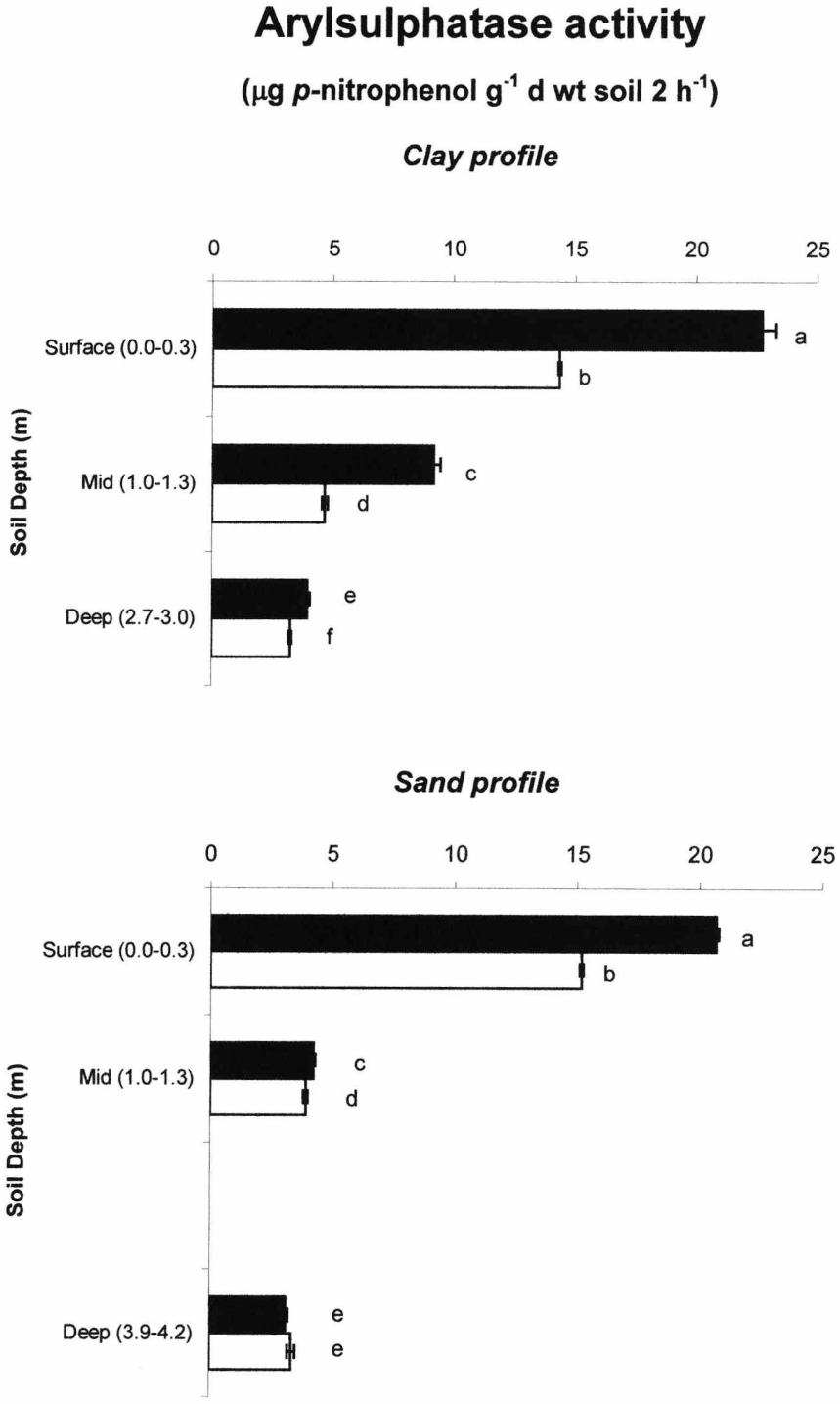


Figure 3.1. Changes in buffered ■ and non-buffered □ arylsulphatase activity with soil depth in an Iowa clay and a Michigan sand profile (all bars represent ± 1 s.e.). For each soil, different letters alongside columns refer to significant differences ($P < 0.05$) between buffered and non-buffered activities with depth.

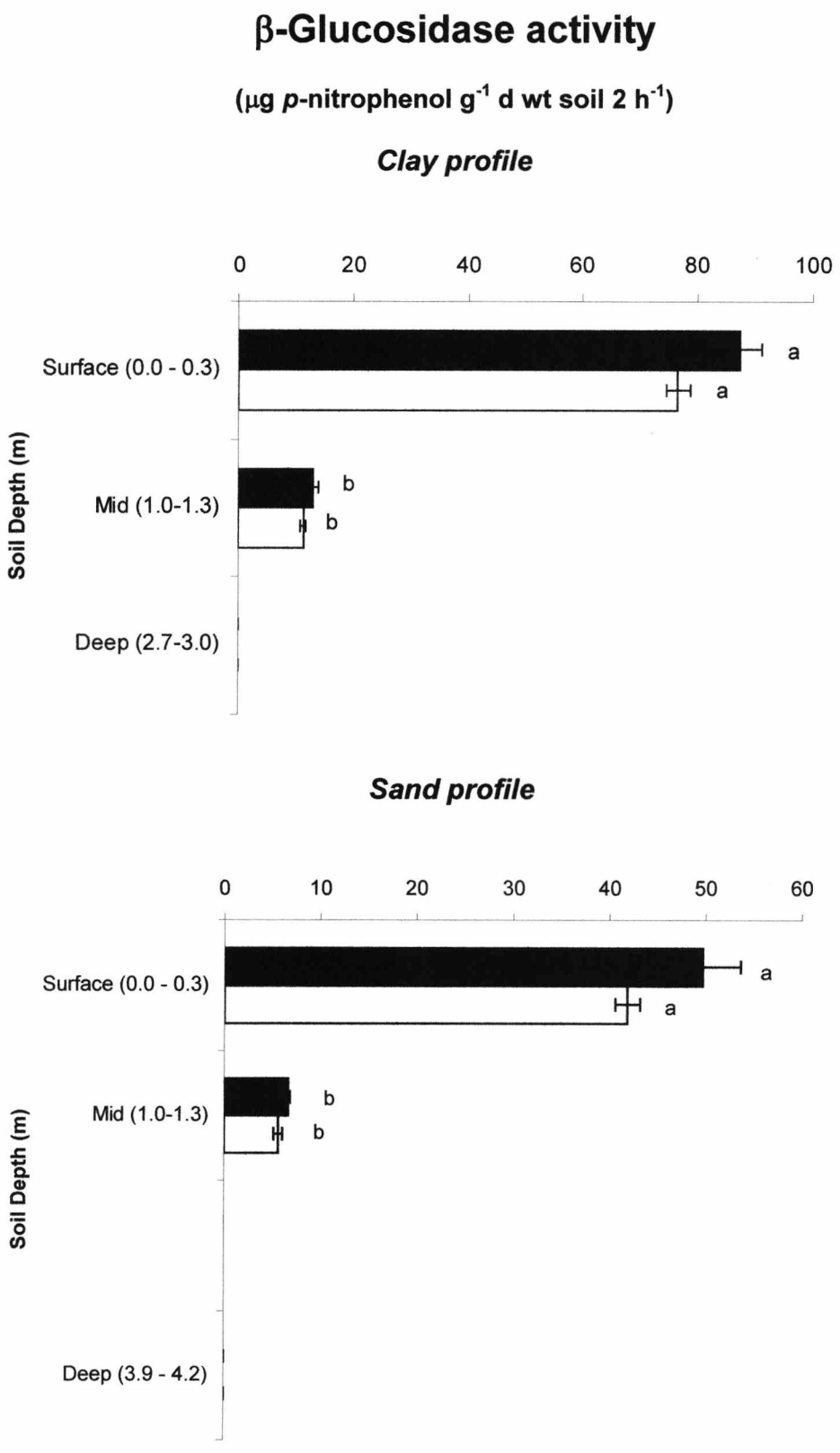


Figure 3.2. Changes in buffered ■ and non-buffered □ β-glucosidase activity with soil depth in an Iowa clay and a Michigan sand profile (all bars represent ± 1 s.e.). For each soil, different letters alongside columns refer to significant differences ($P < 0.05$) between buffered and non-buffered activities with depth.

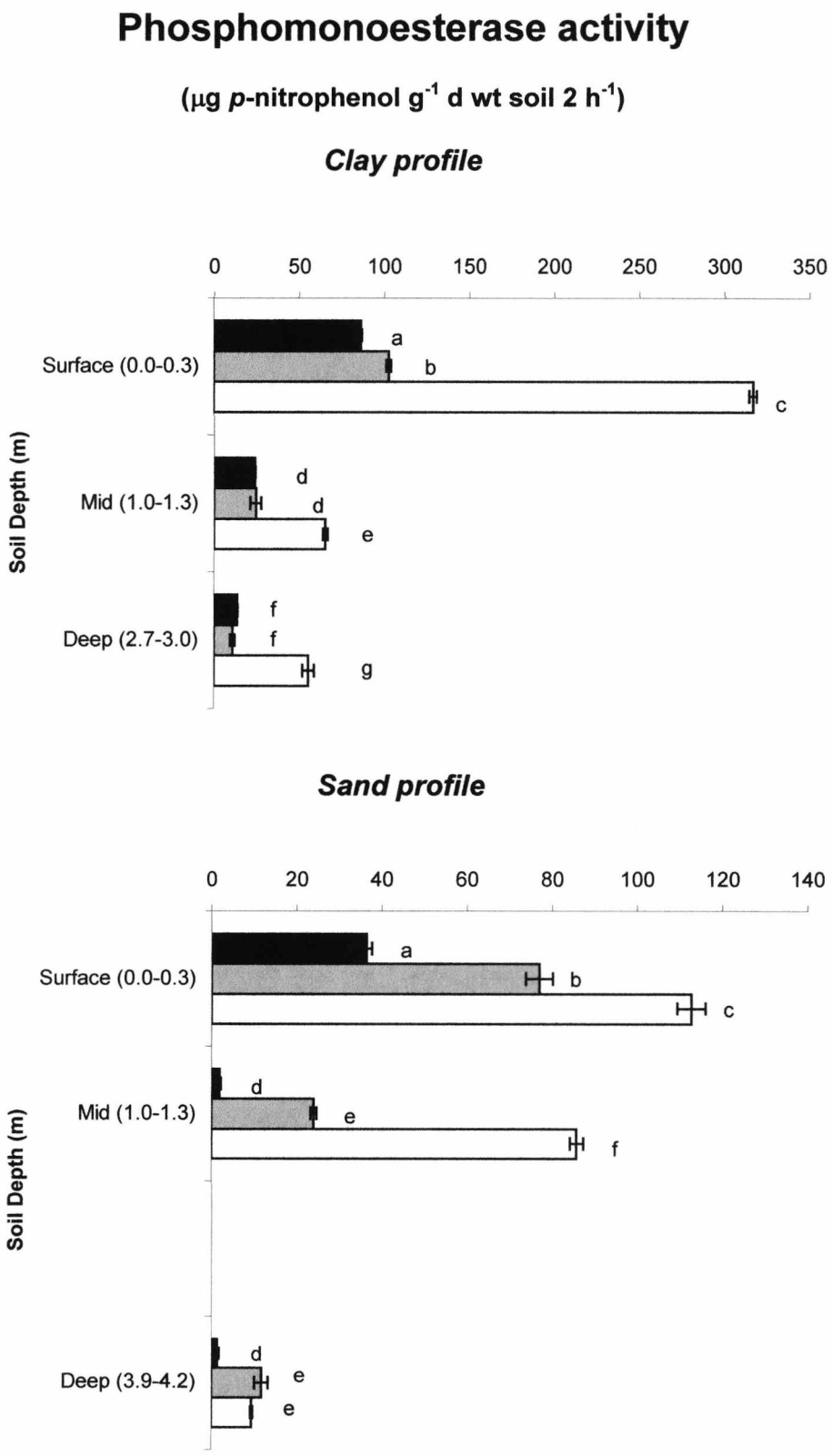


Figure 3.3. Changes in buffered (pH 9.0) ■, buffered (pH 4.0) ■ and non-buffered □ phosphomonoesterase activity with soil depth in an Iowa clay and a Michigan sand profile (all bars represent ± 1 s.e.). For each soil, different letters alongside columns refer to significant differences ($P < 0.05$) between buffered and non-buffered activities with depth.

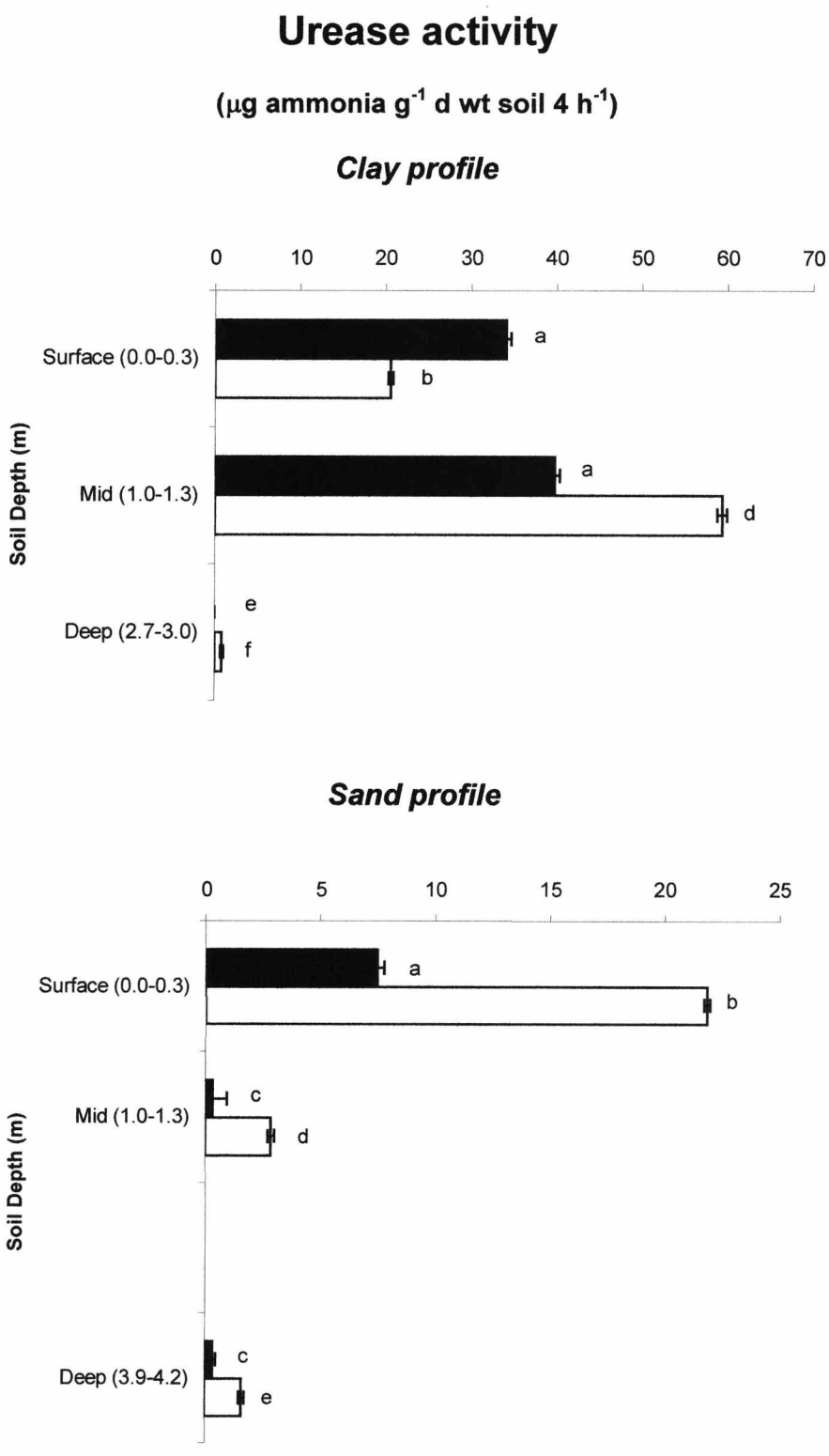


Figure 3.4. Changes in buffered ■ and non-buffered □ urease activity with soil depth in an Iowa clay and a Michigan sand profile (all bars represent ± 1 s.e.). For each soil, different letters alongside columns refer to significant differences ($P < 0.05$) between buffered and non-buffered activities with depth.

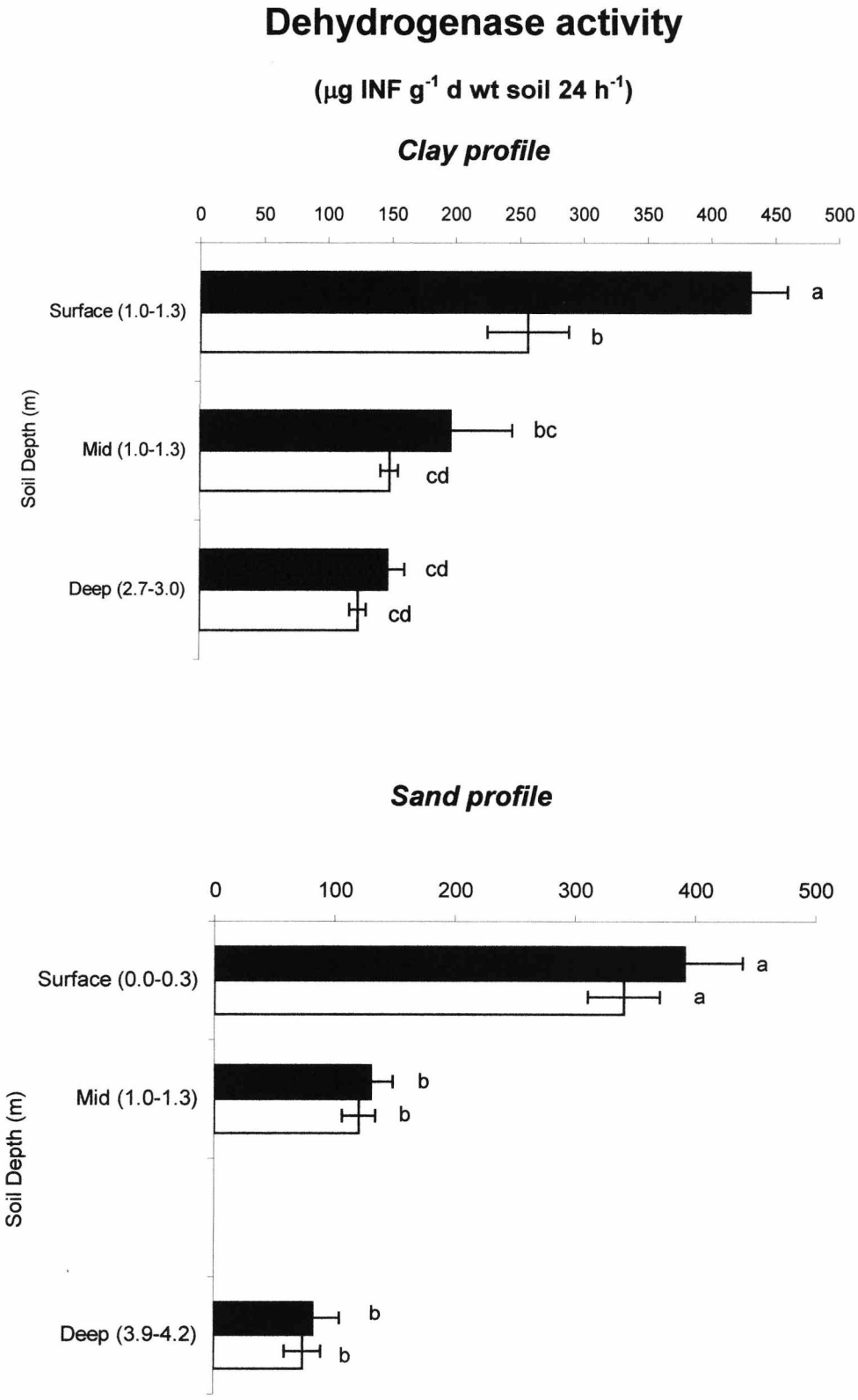


Figure 3.5. Changes in buffered ■ and non-buffered □ dehydrogenase activity with soil depth in an Iowa clay and a Michigan sand profile (all bars represent ± 1 s.e.). For each soil, different letters alongside columns refer to significant differences ($P < 0.05$) between buffered and non-buffered activities with depth.

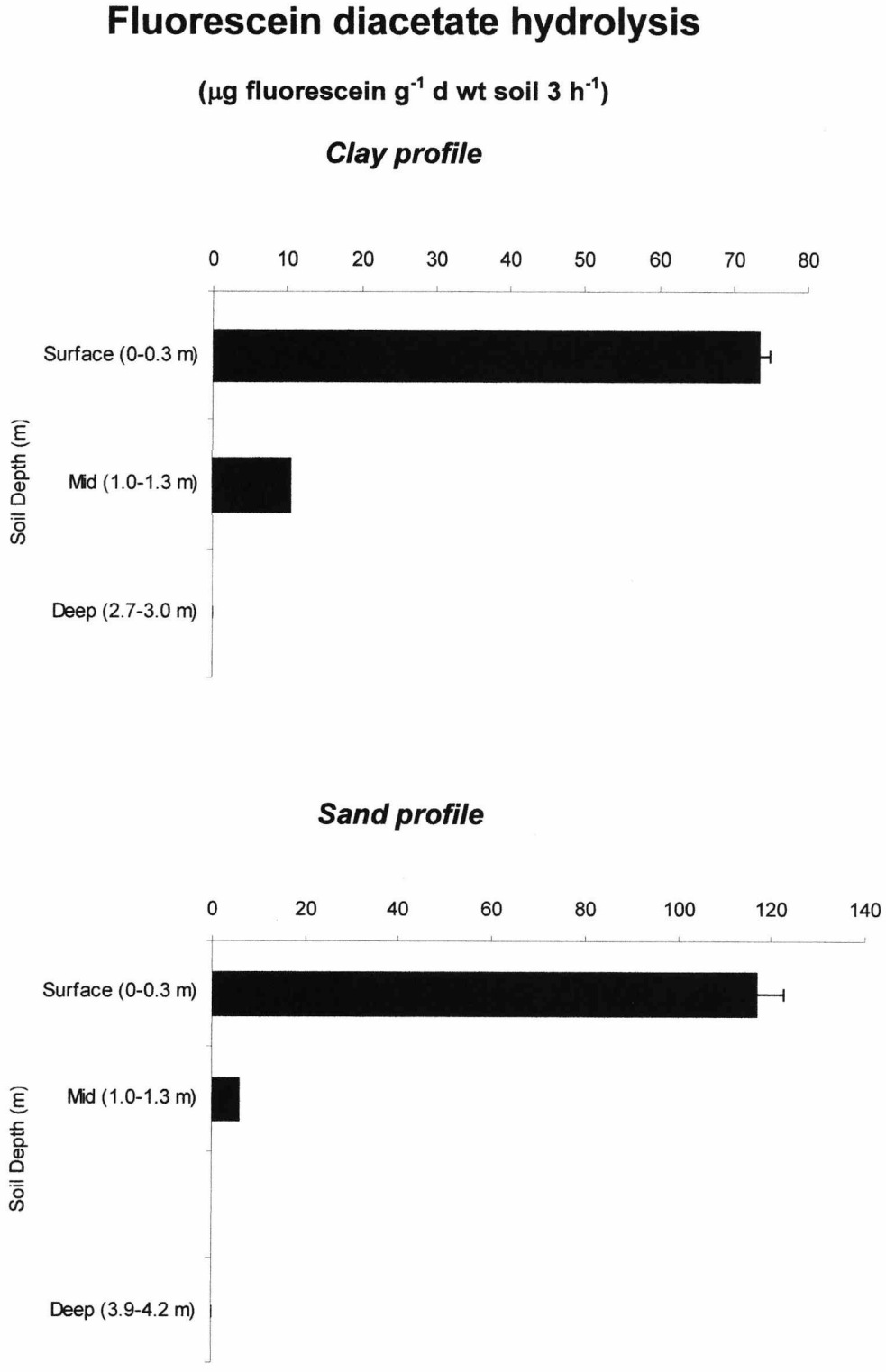


Figure 3.6. Changes in fluorescein diacetate hydrolysis with soil depth in an Iowa clay and a Michigan sand profile (all bars represent ± 1 s.e.). For each soil, different letters alongside columns refer to significant differences ($P < 0.05$) in activity with depth.

deep soil. In the absence of buffer significantly greater activity ($P < 0.01$) in the mid soil was found than in the surface soil. Urease activity, although significantly lower ($P < 0.01$) than in either the surface or mid soils, was also detected in the deep soil.

3.2.3.5. Dehydrogenase activity

In buffered assays, dehydrogenase activity (Figure 3.5) decreased with depth in both soils ($P < 0.05$). In non-buffered assays significantly greater activities were measured in the surface soils compared to the mid soils (clay 1.7-fold decrease, sand 2.8-fold decrease) but there was no difference between mid and deep soils. Activity in the sand profile did not increase significantly in the presence of buffer. For the clay soil significantly higher activities ($P < 0.05$) were observed in the surface (1.7-fold) and mid (0.75-fold) soil samples in the presence of buffer, although no significant effect was observed for the deep soil.

3.2.3.6. FDA hydrolysis

FDA hydrolysis was only measured under buffered conditions due to chemical hydrolysis occurring outside the pH 7.0 - 8.0 range (Alef, 1995). FDA (Figure 3.6) rapidly and significantly ($P < 0.01$) decreased with soil depth for both soils. Mid soil activities were only 14.1 % (clay) and 4.8 % (sand) of the surface soils.

3.3. Discussion

The combination of physical and chemical processes employed in the dispersion technique disrupts soil particles and releases into the aqueous phase a proportion of the bacteria that are attached to clays and humates or trapped within aggregates (Hopkins *et al.*, 1991). The extent of adsorption of microorganisms is dependent on the surface properties of the cells and on their physiological state (Grasso *et al.*, 1996) and will affect their ease of extraction. However, even if large and representative numbers are extracted, their culturability, and therefore their enumeration, will be determined by the nutritional and growth requirements of the individual species. Use of a number of different selective media, including those targeted at identifying C substrate utilisation patterns, should provide conditions favourable for the growth of a high proportion of those bacteria extracted (Balestra and Misaghi, 1997). However, even if a range of growth media are

used, the so-called viable but not-culturable (VBNC) bacteria will not be detected (Bakken, 1997) and these may need treatment to resuscitate them prior to extraction (McDougald *et al.*, 1998). Only two media were used in our experiments; one copiotrophic (R2A), the other (SEA) a low nutrient minimal salts medium containing only the soluble organics found in the soils under study (Frederickson and Balkwill, 1998). The CFUs counted after using R2A were similar in numbers to those using SEA, although it took 21 d to produce visible colonies on SEA in comparison to 7 d on R2A. Of course, in the absence of identification, comparable counts may conceal major differences in species composition. However, in the work reported here, almost 90 % of the colonies isolated on SEA could be cultured on the nutrient-rich R2A (data not shown).

Direct counts, using epifluorescence microscopy, proved to be the most sensitive method for determining bacterial cell numbers in soil extracts and, on this basis, CFUs accounted for < 3 % of the total. Even if we assume that the growth media supported the development of different species, culturable bacteria are still < 6 % of the direct count. However, direct counting also suffers from the same problems encountered in viable cell counts: the numbers recorded are determined by the extraction efficiency of bacteria from the soil. This technique does not differentiate between living and dead cells so overestimations can also occur (Bloem *et al.*, 1995).

The decrease in microbial biomass with soil depth has been noted in other studies (e.g. Ekelund *et al.*, 2001). The conversion factors used to estimate bacterial numbers from the biomass-C are dependent on cell size. Carbon content per unit volume increases with decreasing size (Lee and Fuhrman, 1987) and could shift due to changes in microbial community composition (Kroer, 1994). The calculated bacterial numbers are likely to be overestimated, as carbon from fungal cells would contribute to the biomass-C measurement. The proportion of bacterial numbers accounted for by the biomass estimate increases relative to the direct counts with depth. This suggests that either the extractability of bacteria from the soil matrix decreased considerably with depth in both soils or that the proportion of fungal biomass decreased substantially, which would be in agreement with the numbers of fungal CFUs obtained (Table 3.2).

As an alternative, Marstorp *et al.* (2000) suggested that the DNA concentration of a soil could have potential as an estimator of microbial biomass. They reported concentrations of DNA and chloroform-labile C in eight soils to be highly correlated (R = 0.96). Comparing the biomass concentrations obtained from this study with the amounts of DNA extracted from the same soils (Wilson, pers. comm., 2000) showed a strong positive correlation between biomass and DNA (R > 0.95; Table 3.3 a, b, c, d).

The cellular DNA content of soil bacteria has been reported to be as low as 2 fg DNA $cell^{-1}$ when considering dwarf cells (Bakken and Olsen, 1989), with higher values reported for more commonly known bacteria, such as 8.4 fg $cell^{-1}$ (Torsvik and Goksøyr, 1978), 8.8 - 11.5 fg $cell^{-1}$ (Sandaa *et al.*, 1998) and 9 fg DNA $cell^{-1}$ (Holben, 1997). Even when using the figure of 2 fg DNA cell-1 to estimate bacterial numbers, the values generated underestimated the numbers found using direct counts, generally by the same degree as plate counts (i.e., 2 - 3 orders of magnitude). The resistance of dwarf cells to bead mill homogenisation and the suggestion that the majority of non-culturable bacteria are dwarf cells (Lindahl *et al.*, 1997) could explain this disparity.

There are a number of other possible inaccuracies that arise when using soil DNA to calculate bacterial population size, which may result in underestimates or overestimates. In terms of methodology, it has been shown that different direct extraction procedures will result in different DNA yields (Stach *et al.*, 2001) and indeed, Wilson (pers. comm., 2000) noted that not all the DNA had actually been eluted from the binding columns during the extraction procedure. Wilson (pers. comm., 2000) also quantified DNA using PicoGreen fluorescent dye, which according to Sandaa *et al.* (1998) permits the measurement of pg quantities without interference from co-extracted humic matter. However, a more detailed study of the effects of quenching by humic substances (Bachoon *et al.*, 2001) showed that Hoechst dye was more reliable than PicoGreen for DNA quantification.

The efficiency of the extraction will also be reduced by the retention of DNA due to binding by clays (Ogram *et al.*, 1988) and organic matter (Crecchio *et al.*, 1998), which could take place during the extraction procedure (Holben, 1997). The high clay content of the Iowa soils may, therefore, have influenced the yield of DNA. The total DNA extracted from soil will contain DNA from a number of sources including fungi, algae, plant and protozoa as well as bacteria (Harris, 1994) and extracellular sources (Steffan, 1988), therefore potentially leading to an overestimate of bacterial numbers. A more accurate method of estimating bacterial numbers from soil DNA would be to quantify universal bacteria-specific sequences by either competitive PCR (Phillips *et al.*, 2000) or dot-blot hybridisation (Frostegård *et al.*, 1999), eliminating the interference from unwanted eukaryotic and plant DNA.

All methods used in this study and the estimates of bacterial numbers through DNA quantities showed strong positive correlations ($R > 0.95$; Table 3.3 a, b, c, d) with each other. Numbers always declined significantly with depth in both soils and this was positively correlated to the decrease in organic matter. In contrast, the proportion of the direct counts that were culturable increased with depth in the clay soil as well as from the sand surface to the mid soil.

Conventional soil enzyme assays are performed under defined conditions using temperatures, pH values, excess substrates and other reaction conditions that generate a near maximum rate of substrate catalysis (Schnürer and Rosswall, 1982; Kandeler and Gerber, 1988). This approach gives a reliable and reproducible measure of potential activity but one that is rarely, if ever, realised in soil (Burns, 1978). The assumptions and decisions that must be made when designing soil enzyme assays and interpreting the data have been discussed at length by Tabatabai and Dick (2002).

An alternative approach is to measure enzyme activity at the natural pH of the soil, which will give a more realistic indication of the activities likely to be expressed *in situ* (Kandeler and Berger, 1988). Here, both non-buffered and buffered assays were performed to allow comparisons of 'optimal' and 'natural' enzyme activities. In addition, assays were measured at 20°C (well below the optimum for each), closer to the soil temperatures that are likely to exist in surface and subsoils. This necessitated some assays to be prolonged in order to take account of slow rates of catalysis.

Whilst the non-buffered assay may provide a more realistic measure of activity in the field, it does not allow comparisons between activities at different depths (which have different pH values). In order to assess the likely effects of different natural soil pH on activity, the sand surface (pH 5.5), mid (pH 6.6) and deep (pH 7.8) soils were buffered to bring their pH to that of the surface, mid or deep soil pH. Under these conditions phosphomonoesterase activity still significantly ($P < 0.05$) decreased with soil depth (data not shown).

Arylsulphatase, β-glucosidase, phosphomonoesterase and urease are enzymes that carry out specific hydrolyses and were selected in these experiments because they catalyse reactions involved in the biogeochemical transformations of S, C, P and N, respectively. They are likely to be an essential component of any assessment of soil microbial activity and substrate mineralization. Dehydrogenase is present in all microorganisms (von Mersi and Schinner, 1991; Dick, 1997). Therefore, assays are considered to be an accurate measure of the microbial oxidative activity of the soil and should have a direct relationship to total viable microorganisms. Strong correlations ($R > 0.95$; Table 3.3 a, b, c, d) were found between dehydrogenase and all bacterial abundance measures (viable counts, direct counts, biomass-C, DNA). However, dehydrogenase activity may have been underestimated due to competition from alternative hydrogen acceptors within soil (Dick, 1997).

Table 3.3 Correlations between buffered and non-buffered enzyme activities and physical, chemical and biological properties of the clay and sand soil profiles. Values in bold are significant ($P < 0.05$). Values in italics are negative values. (a) Clay soil under non-buffered conditions, (b) clay soil under buffered conditions, (c) sand soil under non-buffered conditions, (d) sand soil under non-buffered conditions.

(a) Clay soil under non-buffered conditions

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*	*M*	*N*	*O*	*P*	*Q*
Organic matter **(A)**	**1.000**																
pH **(B)**	*-0.966*	**1.000**															
Cation exchange capacity **(C)**	0.871	*-0.968*	**1.000**														
Sand (0.05-2.0 mm) **(D)**	*-0.276*	0.515	*-0.713*	**1.000**													
Silt (0.002-0.05 mm) **(E)**	0.532	*-0.296*	0.048	0.666	**1.000**												
Clay (<0.002 mm) **(F)**	*-0.329*	0.074	0.177	*-0.817*	*-0.975*	**1.000**											
Biomass **(G)**	**1.000**	*-0.970*	0.879	*-0.291*	0.519	-0.314	**1.000**										
DNA **(H)**	**1.000**	*-0.964*	0.867	*-0.268*	0.539	*-0.337*	**1.000**	**1.000**									
Direct counts **(I)**	**0.999**	*-0.952*	0.845	*-0.227*	0.575	*-0.377*	**0.998**	**0.999**	**1.000**								
CFUs (R2A) **(J)**	**1.000**	*-0.958*	0.857	*-0.249*	0.556	*-0.356*	**0.999**	**1.000**	**1.000**	**1.000**							
CFUs (SEA) **(K)**	**0.996**	***-0.985***	0.910	*-0.357*	0.458	*-0.247*	**0.998**	**0.996**	**0.991**	**0.994**	**1.000**						
CFUs (fungi) **(L)**	**0.999**	*-0.978*	0.895	*-0.324*	0.489	*-0.281*	**0.999**	**0.998**	**0.995**	**0.997**	**0.999**	**1.000**					
arylsulphatase **(M)**	**1.000**	*-0.973*	0.884	*-0.302*	0.509	*-0.303*	**1.000**	**0.999**	**0.997**	**0.998**	**0.998**	**1.000**	**1.000**				
dehydrogenase **(N)**	**0.996**	***-0.984***	0.910	*-0.357*	0.459	*-0.248*	**0.998**	**0.996**	**0.991**	**0.994**	**1.000**	**0.999**	**0.998**	**1.000**			
glucosidase **(O)**	**0.999**	*-0.977*	0.894	*-0.322*	0.491	*-0.283*	**0.999**	**0.998**	**0.995**	**0.997**	**0.999**	**1.000**	**1.000**	**0.999**	**1.000**		
phosphomonoesterase **(P)**	**0.998**	*-0.950*	0.842	*-0.221*	0.580	*-0.383*	**0.997**	**0.999**	**1.000**	**1.000**	**0.990**	**0.994**	**0.996**	**0.990**	**0.994**	**1.000**	
urease **(Q)**	0.461	*-0.674*	0.837	*-0.980*	*-0.506*	0.686	0.475	0.454	0.415	0.436	0.535	0.505	0.485	0.534	0.503	0.409	**1.000**

Table 3.3 continued

(b) Clay soil under buffered conditions

Soil properties	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
Organic matter (% w/w) **(A)**	**1.000**																		
pH **(B)**	*-0.966*	**1.000**																	
Cation exchange capacity **(C)**	0.871	*-0.968*	**1.000**																
Sand (0.05-2.0 mm) **(D)**	*-0.276*	0.515	*-0.713*	**1.000**															
Silt (0.002-0.05 mm) **(E)**	0.532	*-0.296*	0.048	0.666	**1.000**														
Clay (<0.002 mm) **(F)**	*-0.329*	0.074	0.177	*-0.817*	*-0.975*	**1.000**													
Biomass **(G)**	**1.000**	*-0.970*	0.879	*-0.291*	0.519	*-0.314*	**1.000**												
DNA **(H)**	**1.000**	*-0.964*	0.867	*-0.268*	0.539	*-0.337*	**1.000**	**1.000**											
Direct Counts **(I)**	**0.999**	*-0.952*	0.845	*-0.227*	0.575	*-0.377*	**0.998**	**0.999**	**1.000**										
CFUs (R2A) **(J)**	**1.000**	*-0.958*	0.857	*-0.249*	0.556	*-0.356*	**0.999**	**1.000**	**1.000**	**1.000**									
CFUs (SEA) **(K)**	**0.996**	*-0.985*	0.910	*-0.357*	0.458	*-0.247*	**0.998**	**0.996**	**0.991**	**0.994**	**1.000**								
CFUs (Fungi) **(L)**	**0.999**	*-0.978*	0.895	*-0.324*	0.489	*-0.281*	**0.999**	**0.998**	**0.995**	**0.997**	**0.999**	**1.000**							
Arylsulpahatase (pH 5.8) **(M)**	**0.983**	***-0.997***	0.946	*-0.446*	0.370	*-0.152*	0.986	0.982	0.973	0.978	0.995	**0.991**	**1.000**						
Dehydrogenase **(pH 7.0)(N)**	**0.997**	*-0.982*	0.905	*-0.346*	0.469	*-0.259*	**0.998**	**0.997**	**0.992**	**0.995**	**1.000**	**1.000**	**0.994**	**1.000**					
FDA (pH 7.6) **(O)**	**0.999**	*-0.976*	0.890	*-0.315*	0.498	*-0.290*	**1.000**	**0.999**	**0.996**	**0.998**	**0.999**	**1.000**	**0.990**	**0.999**	**1.000**				
Glucosidase (pH 6.0) **(P)**	**0.999**	*-0.978*	0.895	*-0.325*	0.489	*-0.281*	**0.999**	**0.998**	**0.995**	**0.997**	**0.999**	**1.000**	**0.991**	**1.000**	**1.000**	**1.000**			
Phosphatase (acid; pH 4.0) **(Q)**	**0.999**	*-0.977*	0.894	*-0.322*	0.491	*-0.283*	**0.999**	**0.998**	**0.995**	**0.997**	**0.999**	**1.000**	**0.991**	**1.000**	**1.000**	**1.000**	**1.000**		
Phosphatase (alkali; pH 9.0) **(R)**	**0.999**	*-0.976*	0.891	*-0.317*	0.496	*-0.289*	**1.000**	**0.999**	**0.996**	**0.997**	**0.999**	**1.000**	**0.990**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	
Urease (pH 10.0) **(S)**	*-0.096*	*-0.164*	0.405	*-0.930*	*-0.894*	0.972	*-0.081*	*-0.104*	*-0.147*	*-0.124*	*-0.011*	*-0.046*	*0.086*	*-0.023*	*-0.056*	*-0.046*	*-0.048*	*-0.054*	**1.000**

Table 3.3 continued

(c) sand soil under non-buffered conditions

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*	*M*	*N*	*O*	*P*	*Q*
Organic matter **(A)**	**1.000**																
pH **(B)**	*-0.853*	**1.000**															
Cation exchange capacity **(C)**	0.774	***-0.991***	**1.000**														
Sand (0.05-2.0 mm) **(D)**	*-0.966*	0.960	*-0.912*	**1.000**													
Silt (0.002-0.05 mm) **(E)**	**0.988**	*-0.924*	0.863	***-0.994***	**1.000**												
Clay (<0.002 mm) **(F)**	0.866	***-1.000***	**0.987**	*-0.966*	0.933	**1.000**											
Biomass **(G)**	0.955	*-0.969*	0.927	***-0.999***	**0.990**	0.975	**1.000**										
DNA **(H)**	**1.000**	*-0.868*	0.792	*-0.973*	**0.992**	0.880	0.963	**1.000**									
Direct counts **(I)**	**1.000**	*-0.851*	0.771	*-0.964*	**0.987**	0.864	0.954	**0.999**	**1.000**								
CFUs (R2A) **(J)**	**0.997**	*-0.891*	0.821	*-0.983*	**0.997**	0.902	0.975	**0.999**	**0.997**	**1.000**							
CFUs (SEA) **(K)**	**0.999**	*-0.870*	0.795	*-0.974*	**0.992**	0.882	0.964	**1.000**	**0.999**	**0.999**	**1.000**						
CFUs (fungi) **(L)**	**0.993**	*-0.907*	0.842	***-0.989***	**0.999**	0.918	0.983	**0.996**	**0.993**	**0.999**	**0.997**	**1.000**					
arylsulphatase **(M)**	**0.999**	*-0.874*	0.800	*-0.975*	**0.993**	0.886	0.967	**1.000**	**0.999**	**0.999**	**1.000**	**0.997**	**1.000**				
dehydrogenase **(N)**	**0.988**	*-0.924*	0.864	***-0.994***	**1.000**	0.934	**0.990**	**0.992**	**0.987**	**0.997**	**0.992**	**0.999**	**0.993**	**1.000**			
glucosidase **(O)**	**0.993**	*-0.910*	0.846	***-0.990***	**0.999**	0.920	0.984	**0.996**	**0.992**	**0.999**	**0.996**	**1.000**	**0.997**	**0.999**	**1.000**		
phosphomonoesterase **(P)**	0.701	*-0.970*	**0.994**	*-0.863*	0.804	0.964	0.881	0.722	0.698	0.754	0.725	0.778	0.730	0.804	0.783	**1.000**	
urease **(Q)**	**1.000**	*-0.852*	0.773	*-0.965*	**0.988**	0.865	0.955	**1.000**	**1.000**	**0.997**	**0.999**	**0.993**	**0.999**	**0.987**	**0.992**	0.700	**1.000**

Table 3.3 continued

(d) Sand soil under buffered conditions

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
Organic matter (% w/w) **(A)**	**1.000**																		
pH **(B)**	*-0.853*	**1.000**																	
Cation exchange capacity **(C)**	0.774	***-0.991***	**1.000**																
Sand (0.05-2.0 mm) **(D)**	*-0.966*	0.960	*-0.912*	**1.000**															
Silt (0.002-0.05 mm) **(E)**	**0.988**	*-0.924*	0.863	***-0.994***	**1.000**														
Clay (<0.002 mm) **(F)**	0.866	***-1.000***	**0.987**	*-0.966*	0.933	**1.000**													
Biomass **(G)**	**1.000**	***-1.000***	**1.000**	***-1.000***	**1.000**	**1.000**	**1.000**												
DNA **(H)**	**1.000**	*-0.868*	0.792	*-0.973*	**0.992**	0.880	**1.000**	**1.000**											
Direct Counts **(I)**	**1.000**	*-0.851*	0.771	*-0.964*	**0.987**	0.864	**1.000**	**0.999**	**1.000**										
CFUs (R2A) **(J)**	**0.997**	*-0.891*	0.821	*-0.983*	**0.997**	0.902	**1.000**	**0.999**	**0.997**	**1.000**									
CFUs (SEA) **(K)**	**0.999**	*-0.870*	0.795	*-0.974*	**0.992**	0.882	**1.000**	**1.000**	**0.999**	**0.999**	**1.000**								
CFUs (Fungi) **(L)**	**0.993**	*-0.907*	0.842	***-0.989***	**0.999**	0.918	**1.000**	**0.996**	**0.993**	**0.999**	**0.997**	**1.000**							
Arylsulphatase (pH 5.8) **(M)**	**0.998**	*-0.882*	0.809	*-0.979*	**0.995**	0.893	**1.000**	**1.000**	**0.998**	**1.000**	**1.000**	**0.998**	**1.000**						
Dehydrogenase **(pH 7.0)(N)**	**0.985**	*-0.931*	0.873	***-0.996***	**1.000**	0.940	**1.000**	**0.989**	0.984	**0.995**	**0.990**	**0.998**	**0.993**	**1.000**					
FDA (pH 7.6) **(O)**	**1.000**	*-0.860*	0.782	*-0.969*	**0.990**	0.872	**1.000**	**1.000**	**1.000**	**0.998**	**1.000**	**0.995**	**0.999**	**0.987**	**1.000**				
Glucosidase (pH 6.0) **(P)**	**0.993**	*-0.910*	0.846	***-0.990***	**0.999**	0.920	**1.000**	**0.996**	**0.992**	**0.999**	**0.996**	**1.000**	**0.998**	**0.999**	**0.994**	**1.000**			
Phosphatase (acid; pH 4.0) **(Q)**	0.984	*-0.932*	0.874	***-0.996***	**1.000**	0.941	**1.000**	**0.989**	0.983	**0.995**	**0.990**	**0.998**	**0.993**	**1.000**	**0.986**	**0.998**	**1.000**		
Phosphatase (alkali; pH 9.0) **(R)**	**1.000**	*-0.860*	0.782	*-0.969*	**0.990**	0.872	**1.000**	**1.000**	**1.000**	**0.998**	**1.000**	**0.995**	**0.999**	**0.987**	**1.000**	**0.994**	**0.986**	**1.000**	
Urease (pH 10.0) **(S)**	**0.998**	*-0.881*	0.808	*-0.979*	**0.995**	0.892	**1.000**	**1.000**	**0.998**	**1.000**	**1.000**	**0.998**	**1.000**	**0.993**	**0.999**	**0.998**	**0.993**	**0.999**	**1.000**

FDA hydrolysis was used as a general indicator of soil hydrolytic activity, as it measured the activities of proteases, lipases and esterases that are all capable of cleaving fluorogenic FDA (Dick, 1997). FDA hydrolysis, like dehydrogenase activity, is regarded by some as a reliable measure of total microbial activity although, unlike dehydrogenases, these enzymes can function outside of the cell and form stable complexes with soil colloids (Schnürer and Rosswall, 1982). In addition, FDA hydrolysis cannot be considered a specific measure of bacterial and fungal hydrolytic activity within soil, as the reaction can be catalysed by a range of other soil organisms, including algae and protozoa (especially in surface soils) (Barak and Chet, 1986). The values obtained for FDA activity could be underestimates due to the choice of solvent used to stop the reaction. A recent study (Adam and Duncan, 2001) showed that the sensitivity of the assay could be enhanced by 37 % using chloroform/methanol (2:1 v/v). The use of this solvent system may be of benefit for the deep soil samples, where the activity found was bordering on the sensitivity of the assay using acetone.

Strong positive correlations ($R > 0.95$; Table 3.3 a, b, c, d) were observed between all but two enzyme activities under both buffered and non-buffered conditions. The first exception was urease activity in the clay profile (under buffered and non-buffered conditions), which was weakly correlated with the other enzyme activities. This could be explained by the strong positive correlations with the clay content of this profile (R values of 0.972 and 0.686 for non-buffered and buffered assays, respectively; Table 3.3 a, b) and the strong negative correlations with sand content ($R > 0.95$; Table 3.3 a, b). This strong negative correlation with sand content was also evident with the Michigan profile. This suggests the clays in the Iowa mid soil have a capacity to retain (and protect) urease in an active extracellular form or through protection of an ureolytic microbial biomass. This protective property of clays for hydrolases is well known (Burns, 1982) and soil fractionation studies have shown microbial biomass is strongly associated with clays (Ladd *et al.*, 1996). The other exception was non-buffered phosphomonoesterase activity in the sand profile. This also correlated strongly ($R = 0.96$; Table 3.3 d) with clay content, although no such correlation was seen with the clay profile.

Landi *et al.* (2000) used the ratio of enzyme activity-to-biomass-C to compare cadmium treatments of soil and this calculation was applied to all enzyme assays in this study. In general, there was a decrease in the ratio of enzyme activity-to-biomass-C with depth. The two exceptions, again, were urease activity and non-buffered phosphomonoesterase activity in the clay mid and sand mid soils, respectively. Both had far greater ratios of enzyme activity-to-biomass-C compared to their respective surface soils. For example, the non-buffered urease activity had a urease-to-biomass-C ratio of 0.763 (clay mid) and

0.177 (clay surface), a 4.3-fold difference in the ratios, and the non-buffered phosphomonoesterase assay had an activity-to-biomass-C ratio of 3.63 (sand mid) and 1.45 (sand surface), a 2.5-fold difference in ratios. This further suggests, along with the lack of correlation with biomass-C for those two assays, that a significant component of the total urease and phosphomonoesterase activity at these depths is due to a stabilised extracellular enzyme fraction and is not directly associated with the extant biomass.

Strong positive correlations ($R > 0.90$; Table 3.3 a, b, c, d) were found between bacterial abundance and enzyme activities and between enzyme activities and organic matter content; again with the two exceptions of urease and phosphomonoesterase activity. Explanations for the generally lower rates of enzymatic activity in the subsoil samples include, the lower number of microbes (Swenson and Bakken, 1998) and the decrease in organic matter content (Table 3.1).

3.4. Conclusions

This study shows that two soils from as deep as 3.0 m (clay) and 4.2 m (sand), with sharply contrasting physical and chemical composition and properties, are metabolically active and contain substantial numbers of microorganisms. This has important implications for our understanding and modelling of the transformation of downward moving natural and synthetic organics. A greater understanding of subsoil processes is needed to assess the contribution of subsoil biogeochemistry to the cycling of elements, as well as further developments and harmonisation of methods in order to account for potential changes in surface-applied chemicals and the metabolites as they move down to groundwater.

Chapter 4. Sorption and desorption behaviour of acetochlor in surface, subsoil and size fractionated soils

4.1. Introduction

Interactions between soil surfaces and dissolved pesticides control the mobility and bioavailability of these chemicals (Weber *et al.*, 1991). Soil exists as a matrix of intimately associated conglomerations of organo-minerals, rather than discrete organic and mineral phases. The organic (humic substances) and inorganic (clays and oxyhydroxides) fractions can contribute to sorption both separately and in combination (Moreau-Kervévan and Mouvet, 1998). The various soil components have different affinities for sorption of organic contaminants (Kohl and Rice, 1998; Herwig *et al.*, 2001), due to the diverse chemical properties of these components. For example, polycyclic aromatic hydrocarbons (PAHs) strongly associate with hydrophobic soot material and are considered non-bioavailable due to slow desorption kinetics (Gustafsson *et al.*, 1997), which could lead to herbicides being continually transported with leachate into groundwater over prolonged periods of time (Herwig *et al.*, 2001). In contrast, the solubility of polar herbicides can be accentuated due to associations with water-soluble fulvic and humic acids (Chiou and Kile, 1994).

Sorption of herbicides to individual soil components, such as humic acids and clay minerals, has been studied to elucidate their relative importance to this process in soil (Cox *et al.*, 1997a; Torrents and Jayasundera, 1998). However, physicochemical interactions between soil components frequently lead to changes in properties such as pH, specific surface area and functional group availability (Cernini-Silva *et al.*, 2000; Cox *et al.*, 2000; Herwig *et al.*, 2001). Soils separated into sand-, silt- and clay- size fractions have shown that the character of soil organic matter (e.g. total organic matter, total nitrogen content, aromaticity and functional groups) differs between these fractions (Guggenberger *et al.*, 1995; Stemmer *et al.*, 1998). Sorptive behaviour exhibited by complexes of constituents is, therefore, likely to be distinct from that expected from the sum of the individual components (Pusino *et al.*, 1992; Celis *et al.*, 1996; Cox *et al.*, 2000).

Organic contaminants can become unevenly distributed among these soil particle-size fractions (Barriuso and Koskinen, 1996; Müller *et al.*, 2000; Krauss and Wilcke, 2002). The differences in sorption strength between the different particle-size fractions can influence the potential bioavailability of these compounds (Krauss and Wilcke, 2002) as

well as the mobility and amount available for leaching. The differences in bioavailability in different particle-size fractions could have serious implications for those residues associated with the smaller particle-size fractions, which are a nutritional resource for certain organisms, such as earthworms (e.g. *Lumbricus terrestris*; Zhang and Schrader, 1993; Cortez and Hameed, 2001).

Understanding the influence of soil properties and specific soil components on the sorption and desorption characteristics of herbicides is vital for predicting and explaining their mobility and retention in soils, and to give an indication of bioavailability. The potential mobility of herbicides in soils has been modelled in several different ways. Freundlich, Langmuir and linear (equilibrium) approaches are commonly used to predict pesticide distribution/partitioning between the solid matrix and the aqueous phase (Xing and Pignatello, 1998; Carrizosa *et al.*, 2001; Hinz, 2001) and have been widely applied to describe sorption to surface soils (0.0-0.3 m). However, few studies have quantified sorption and desorption processes in subsoils (greater than 0.3 m depth). The soil properties that influence herbicide sorption (including organic matter and clay mineralogy) change with depth and, therefore, the sorptive behaviour of herbicides are likely to change with depth (Jenks *et al.*, 1998). In the research described in this chapter, sorption and desorption isotherms were generated to compare the behaviour of acetochlor in surface soils, subsoils and different soil size fractions (macroaggregate-, microaggregate-, silt- and clay- size separates) to assess herbicide retention in the soils. The implications for leaching and potential bioavailability are discussed.

4.2. Results

4.2.1. Adsorption

The bulk physical and chemical properties for the soils are characterized and discussed in Section 2.1 and the methods used to measure sorption and desorption are described in Section 2.4. Figure 4.1 shows the sorption isotherms of acetochlor in the clay and sand profiles. Based on the Giles *et al.* (1960) classification, for which the main categories are briefly summarised in Figure 4.2, L-shaped isotherms were generated for the sand profile and S-shaped isotherms for the clay profile, which reflects the difference in the Freundlich *n* values for these two profiles; *n* denoting the degree of non-linearity and representing the energy distribution of sorption sites (Farrell and Reinhard, 1994).

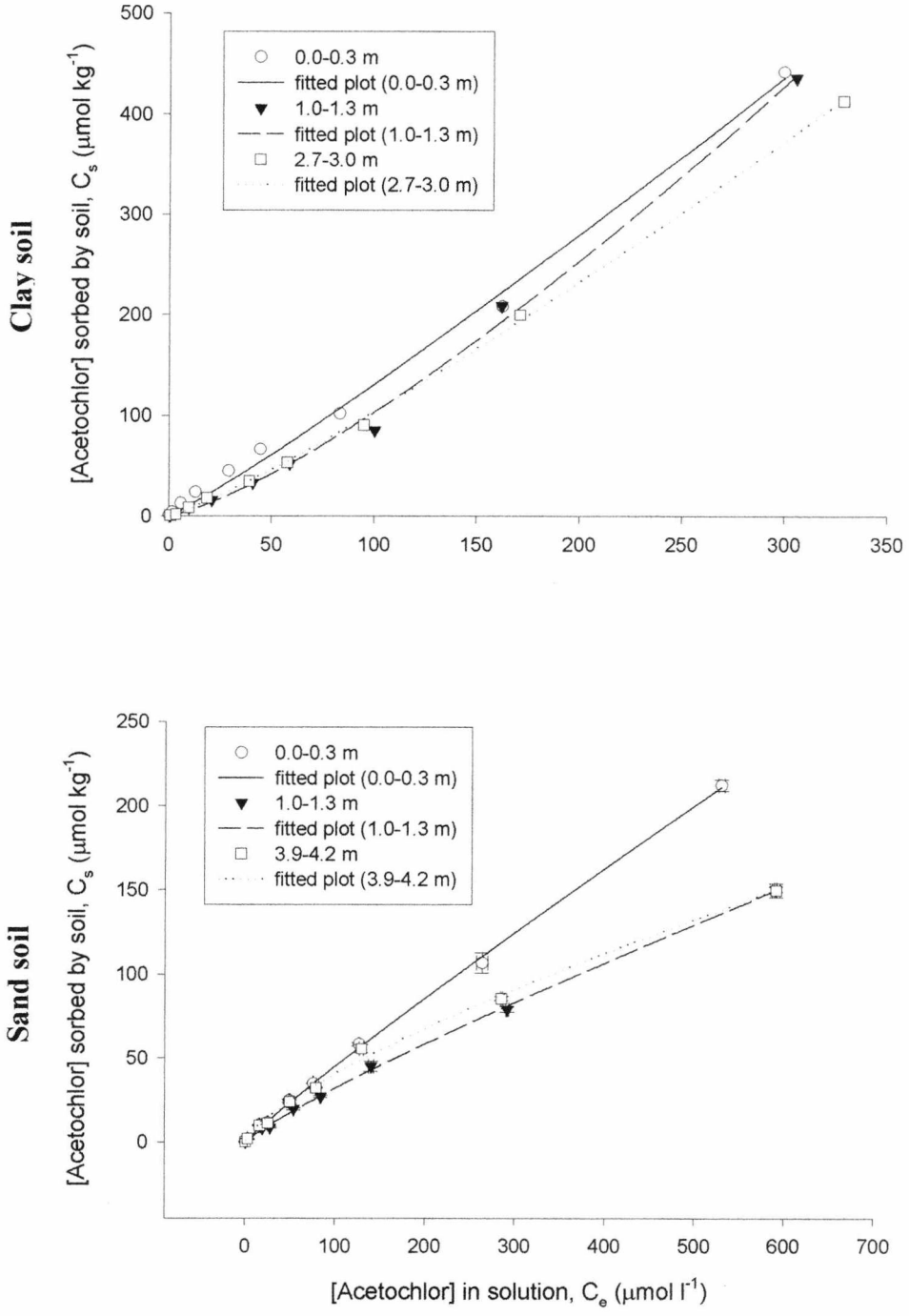


Figure 4.1. Differences in Freundlich sorption isotherms with soil depth for an Iowa clay profile and a Michigan sand profile (all bars represent ± 1 standard error).

The S-type curve shows increasing interaction between the solute and the sorbate as solute concentration increases.

The L-type curve shows that the solute has a high affinity for the sorbate, and has a finite number of sorption sites

The H-type curve shows that the solute has been completely sorbed or that no measurable amount remains in solution.

The C-type curve shows constant partitioning of the solute between the solid and solution phases.

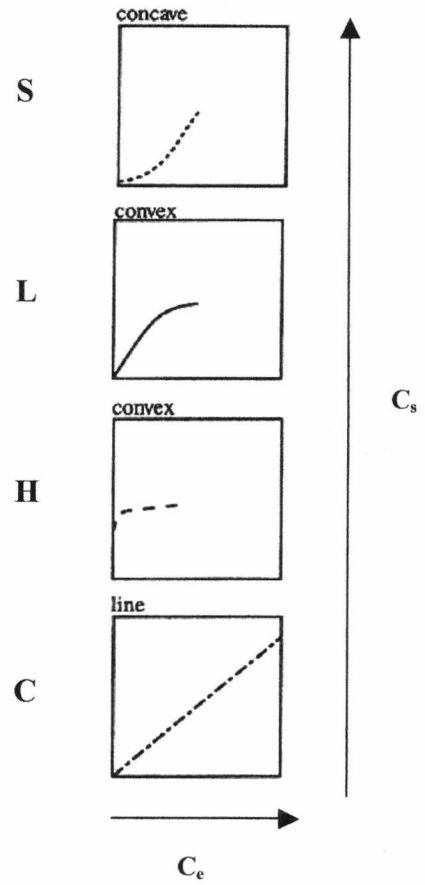


Figure 4.2. Basic isotherm classification proposed by Giles et al., (1960). The plots are examples of the main classification types. C_s is the concentration on the solid and C_e is the equilibrium concentration in the solution phase.

Table 4.1. Changes in the Freundlich coefficients (K_F, n, K_{Fd}, n_d) for the sorption and desorption isotherms of acetochlor with soil depth for an Iowa clay and a Michigan sand profile.

	Sorption			Desorption		
	K_F (ml g^{-1})	n	R	K_{Fd} (ml g^{-1})	n_d	R
Iowa clay						
Surface (0.0-0.3 m)	0.832±0.214	1.098±0.050	0.998	6.533±0.017	0.3219±0.007	0.999
Mid (1.0-1.3 m)	0.262±0.073	1.297±0.050	0.998	3.734±0.06	0.334±0.019	0.995
Deep (2.7-3.0 m)	0.481±0.052	1.166±0.011	1.000	3.201±0.073	0.406±0.024	0.995
Michigan sand						
Surface (0.0-0.3 m)	0.633±0.056	0.9264±0.015	1.000	1.153±0.048	0.270±0.031	0.981
Mid (1.0-1.3 m)	0.597±0.049	0.866±0.013	1.000	0.410±0.016	0.164±0.023	0.971
Deep (3.9-4.2 m)	1.411±0.198	0.731±0.023	0.998	0.424±0.012	0.123±9.015	0.978

The linear equilibrium and Freundlich equilibrium models were used to describe acetochlor sorption and desorption. The Langmuir equilibrium approach was not suitable for modelling acetochlor sorption or desorption. Linear partitioning is described using the K_d partitioning constant. The Freundlich equilibrium model is based on the equation:

$$C_s = K_F C_e^n$$

where C_s is the sorbed concentration of acetochlor (μmol kg^{-1}), C_e is the concentration of acetochlor in solution at equilibrium (μmol l^{-1}) and K_F and n are constants. K_F denotes the extent of adsorption, giving the amount adsorbed per unit equilibrium concentration. Both of these constants are widely used to model the sorption and transport of pesticides (Cox *et al.*, 1997b; Jenks *et al.*, 1998; Wang *et al.*, 1999). Freundlich fittings are thought to be best for describing sorption onto heterogeneous surfaces, with several different types of sorptive interactions taking place at once (Calvet, 1989). As a number of soil constituents (organic matter, clays, iron oxides [Johnson and Sims, 1993]) have been implicated in pesticide sorption, with potentially different sorptive mechanisms (Cox *et al.*, 1998; Lagaly, 2001), it appears that soil systems meet this assumption. The adsorption isotherms generated for acetochlor fitted the Freundlich equation well in all the soils ($R \geq 0.998$; $P < 0.01$). Best-fit values for the parameters K_F and n are given in Table 4.1. Sorption is linear when $n = 1$, and the distribution coefficient K_d is the appropriate parameter. However, n values ranged from 1.1 to 1.3 for the clay profile and from 0.731 to 0.926 for the sand profile. All these values were significantly different to unity ($P < 0.01$) showing a Freundlich non-linear isotherm was more suitable than a linear isotherm.

For the clay surface soil, the K_d values, i.e. C_s/C_e for a specific concentration rather than for the whole isotherm, decreased with increasing initial acetochlor concentration, with the K_d for the highest initial concentration (100 μg g^{-1}) being 45.2 % of the K_d for the lowest initial concentration (0.2 μg g^{-1}). However, for the clay mid and deep soils, the trend was reversed. The K_d values for the lowest initial concentrations were 62.7 % and 67.6 % of the K_d values for the highest initial concentration for the mid and deep soils, respectively. For the sand profile, the K_d values decreased as the initial concentration increased, with values for the highest initial concentration being 68.2 %, 82.1 % and 52.2 % of the K_d values for the lowest initial concentration (surface, mid and deep soils, respectively).

4.2.2. Desorption of acetochlor from bulk soils

Figure 4.3 shows desorption isotherms of acetochlor for the bulk soils of the clay and sand profiles. Although there are no equations specifically designed to model desorption, the Freundlich approach has been used for this purpose (Moorman *et al.*, 2001). The Freundlich equilibrium model was therefore applied to the desorption process, with the constants as K_{Fd} and n_d (to allow differentiation between the constants for sorption and the constants for desorption). The Freundlich equation fitted the data well ($R \geq 0.971$; $P < 0.01$), with the best-fit values for the parameters K_{Fd} and n_d given in Table 4.1. All the n_d values were significantly ($P < 0.01$) lower than unity, ranging from 0.123 to 0.406 for both soil profiles. All the n values were significantly higher than the n_d values ($P < 0.01$). For the clay profile and the sand surface soil the K_{Fd} values were significantly ($P < 0.01$) greater than the respective K_F values. For the sand mid and deep soils the K_{Fd} values were significantly lower than the respective K_F values.

For the clay profile, of the initial 2.50 μg acetochlor g^{-1} applied to the soil samples, 82.36 %, 62.26 % and 59.9 % remained sorbed to the surface, mid and deep soils, respectively after the 24 h equilibration step. For the sand profile, of the initial 1.57 μg acetochlor g^{-1} applied to the soil samples, 34.17 %, 24.41 % and 24. 6 % remained sorbed to the surface, mid and deep soils, respectively. For both profiles, the herbicide residues left in the surface samples was significantly greater ($P < 0.01$) than in the respective subsoils. The residues remaining in the mid soils of each profile were not significantly different ($P > 0.05$) from the residues remaining in the respective deep soils. For all soils, although progressively smaller amounts of acetochlor were desorbed, the process was not complete after four desorption steps. More acetochlor could potentially have been desorbed after further desorption steps. The amount of acetochlor desorbed (%) by each desorption step, relative to the amount initially sorbed is shown in Figure 4.4 for the clay and the sand soil profile.

In terms of the relative percentage concentration sorbed and desorbed, the clay profile exhibited significantly ($P < 0.01$) greater rates of desorption as depth increased, as well as a decreasing sorption affinity for the acetochlor. In contrast, desorption rates decreased with soil depth for the sand profile; although significantly ($P < 0.01$) more acetochlor was sorbed by the sand surface soil than the deeper layers, the rate of desorption was significantly ($P < 0.01$) greater. The K_d values all increased with increasing acetochlor concentration, but this trend was more prominent for the clay profile. The K_d values for the first desorption step were 35.1 %, 28.55 % and 49.23 % of the K_d values for the final desorption step for the clay surface, mid and deep soils, respectively. The difference

between these values for the sand surface soil was less than 10 % and for the mid and deep soils, less than 5 %.

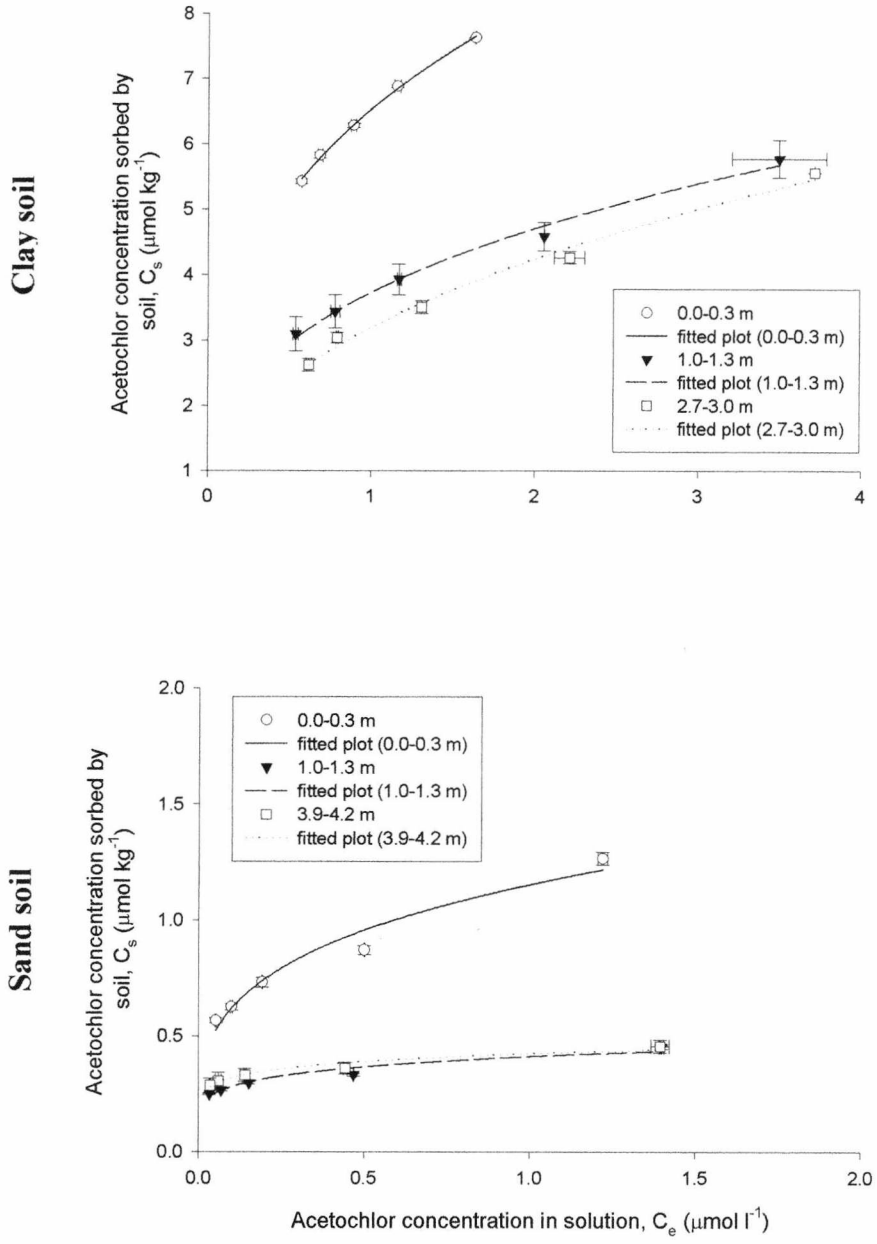


Figure 4.3. Differences in Freundlich-fitted desorption isotherms with soil depth for an Iowa clay profile and a Michigan sand profile (all bars represent ± 1 standard error).

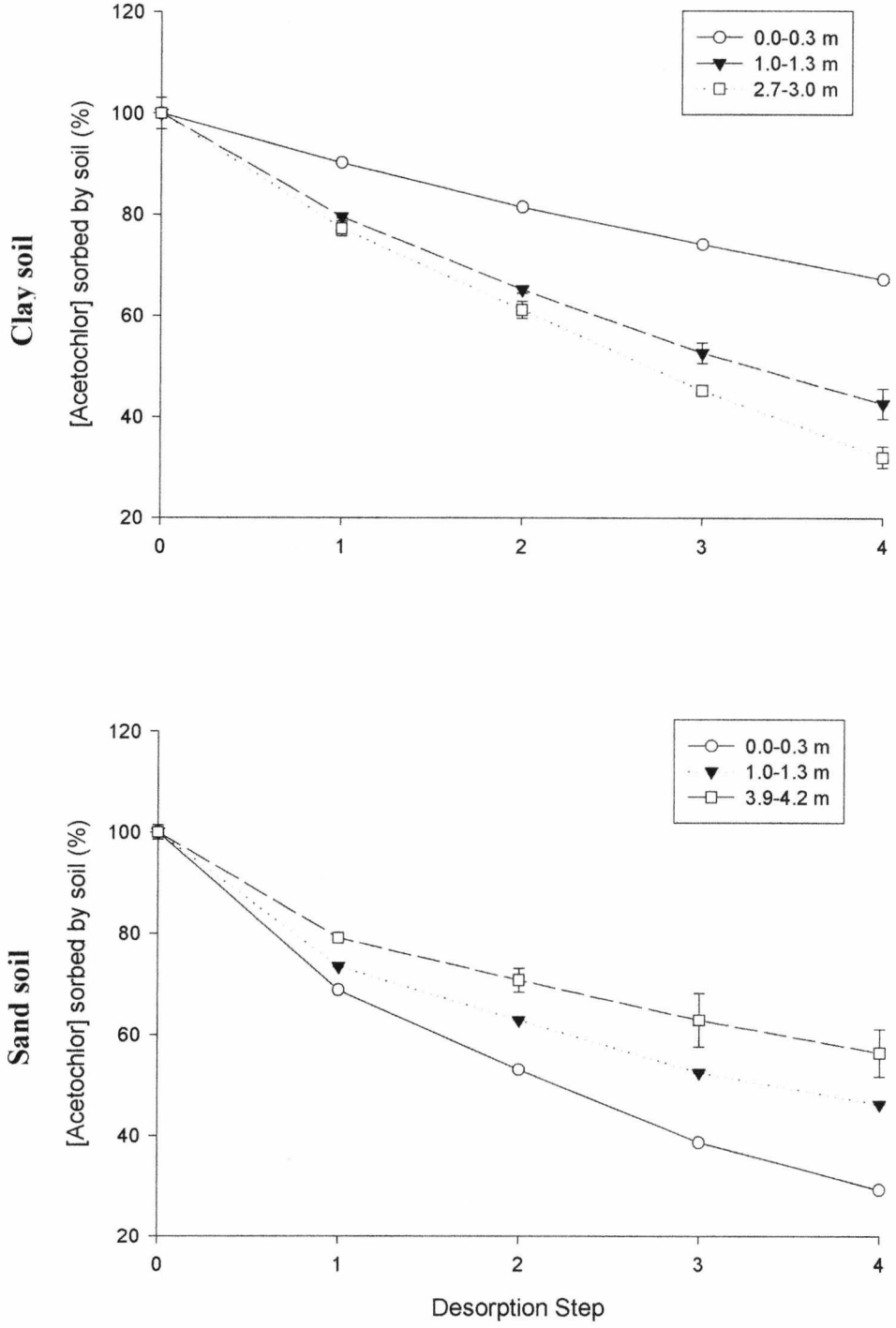


Figure 4.4. Soil depth differences in the amount of acetochlor (%) desorbed by each desorption step, relative to the amount initially sorbed, for an Iowa clay and Michigan sand profile (all bars represent ± 1 standard error).

4.2.3. Desorption of acetochlor from soil size fractions of the clay profile

Figures 4.5, 4.6 and 4.7 show desorption isotherms for the aggregate and particle size fractions obtained from the clay surface, mid and deep soils, respectively. The linear and Freundlich equilibrium models were used to describe the acetochlor desorption process in the soil fractions. The Freundlich equation modelled the behaviour well ($R \geq 0.957$; $P < 0.05$). Although the Freundlich equation appropriately described all the data, a linear equation was more statistically significant for many of the samples. These included all the size fractions isolated from the clay deep soil, the 53-250, 2-20, 0-2 and 0-1 μm diam. fractions of the clay mid soil and the 20-53 and 0-1 μm diam. fractions of the clay surface soil. The deviations from the Freundlich equation are clearly shown in Figures 4.5 - 4.7, where the plotted data is distinct from the calculated line of the Freundlich isotherm. It is, therefore, evident that the distribution coefficient K_d, is a more appropriate parameter for describing the desorption characteristics of these fractions. The best-fit parameters for K_{Fd}, n_d and K_d values, where appropriate, are given in Table 4.2.

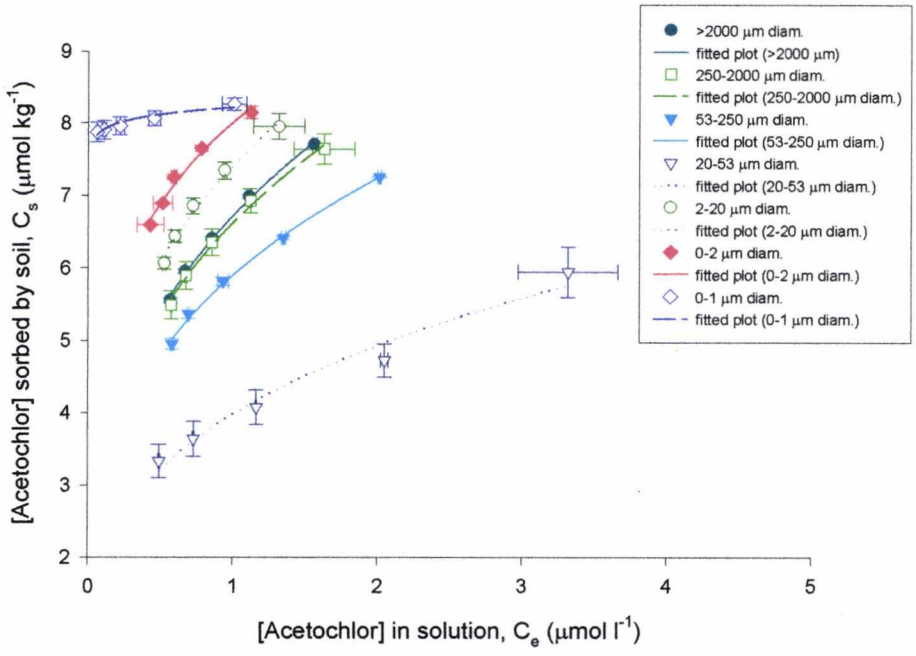


Figure 4.5. Differences in Freundlich-fitted desorption isotherms of acetochlor from different soil size fractions obtained from the surface (0.0-0.3 m) depth (all bars represent ± 1 standard error).

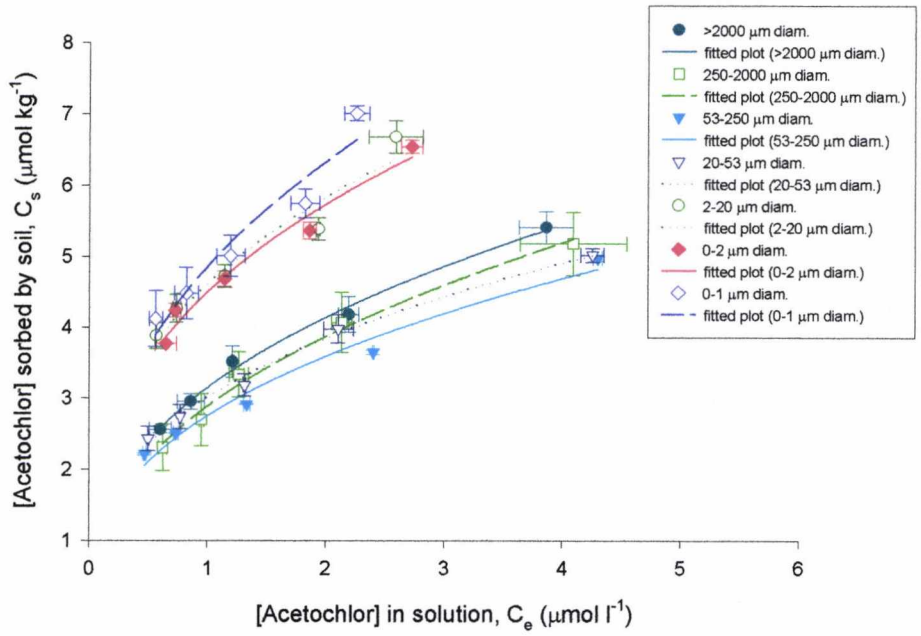


Figure 4.6. Differences in Freundlich-fitted desorption isotherms of acetochlor from different soil size fractions obtained from the mid (1.0-1.3 m) depth (all bars represent ± 1 standard error).

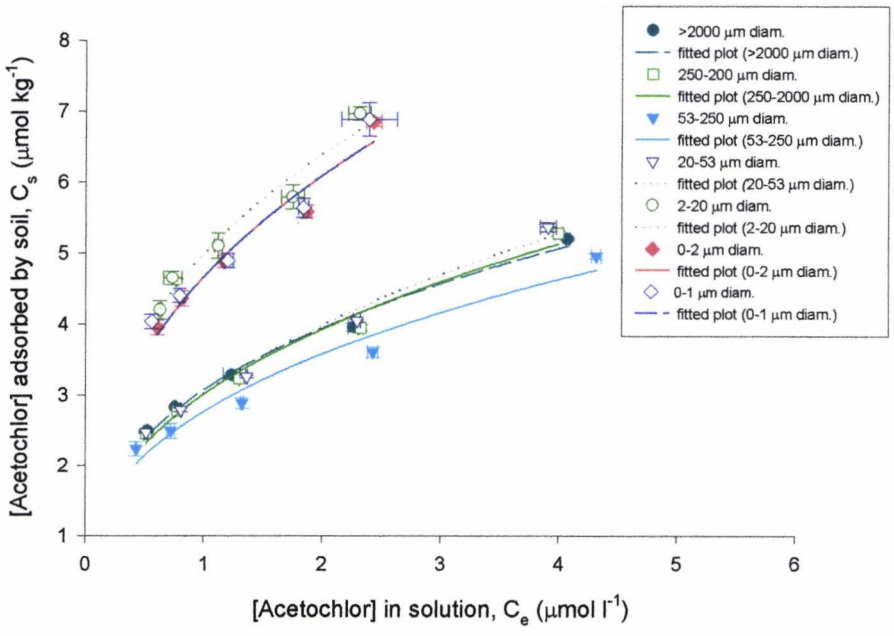


Figure 4.7. Differences in Freundlich-fitted desorption isotherms of acetochlor from different soil size fractions obtained from the deep (2.7-3.0 m) depth (all bars represent ± 1 standard error).

Table 4.2. Differences in the Freundlich coefficients (K_{Fd}, n_d) for the desorption isotherms of acetochlor from soil size fractions obtained from an Iowa clay profile. Linear equation parameters are shown where a linear distribution of sorbed and aqueous phase acetochlor was found. The significance (*P*) is shown where both Linear and Freundlich equations were applicable to allow statistical comparison. N/a = not applicable.

Soil Fraction (μm diam.)	Desorption parameters			Linear parameters	
	K_{Fd} (ml g^{-1})	n_d	R(*P*)	K_d (ml g^{-1})	R(*P*)
Surface (0.0-0.3 m)					
>2000	6.72±0.02	0.32±0.01	0.999	N/a	N/a
250-2000	6.69±0.04	0.31±0.01	0.997	N/a	N/a
53-250	5.90±0.03	0.30±0.01	0.998	N/a	N/a
20-53	3.98±0.10	0.31±0.03	0.986 (0.0019)	2.95±0.06	0.998 (<0.0001)
2-20	7.41±0.05	0.28±0.02	0.994	N/a	N/a
0-2	8.01±0.07	0.22±0.02	0.991	N/a	N/a
0-1	8.21±0.04	0.02±0.03	0.957 (0.0107)	7.86±0.01	0.997 (0.0002)
Mid (1.0-1.3 m)					
>2000	3.14±0.05	0.40±0.02	0.997	N/a	N/a
250-2000	2.88±0.07	0.43±0.03	0.995	N/a	N/a
53-250	2.74±0.11	0.39±0.04	0.987 (0.0017)	1.94±0.03	0.999 (<0.0001)
20-53	3.00±0.05	0.35±0.02	0.997	N/a	N/a
2-20	4.60±0.15	0.34±0.05	0.972 (0.0057)	3.20±0.20	0.986 (0.0019)
0-2	4.49±0.11	0.35±0.04	0.986 (0.002)	3.17±0.16	0.990 (0.0011)
0-1	4.85±0.18	0.39±0.06	0.966 (0.0076)	3.12±0.25	0.984 (0.0024)
Deep (2.7-3.0 m)					
>2000	3.07±0.07	0.36±0.02	0.995	N/a	N/a
250-2000	3.01±0.10	0.39±0.03	0.990 (0.0011)	2.13±0.052	0.999 (<0.0001)
53-250	2.77±0.13	0.37±0.04	0.981 (0.0032)	1.96±0.02	1.000 (<0.0001)
20-53	3.01±0.10	0.40±0.03	0.990 (0.0012)	2.09±0.03	1.000 (<0.0001)
2-20	4.98±0.12	0.36±0.04	0.980 (0.0035)	3.37±0.19	0.989 (0.0014)
0-2	4.66±0.14	0.39±0.05	0.979 (0.0038)	3.08±0.19	0.990 (0.0013)
0-1	4.66±0.14	0.39±0.05	0.979 (0.0038)	3.08±0.19	0.990 (0.0013)

The range of n_d values found for the clay surface soil show that non-linearity was more prominent at this depth. No significant ($P > 0.05$) difference was observed in n_d values through the macroaggregate to 20-53 μm diam. size fractions, whereas the extent of retention, reflected by the increasing K_{Fd} value, did increase significantly ($P < 0.05$). For the finer fractions (0-20 μm diam.) of this depth, the retention of acetochlor was significantly ($P < 0.01$) greater than the larger fractions, as was the non-linearity suggested by the n_d values. The clay mid and deep soils had n_d values that were similar throughout the size classes. As with the surface horizon, the finer size fractions (0-20 μm diam.) had the greatest retentive capacity ($P < 0.05$) according to the K_{Fd} parameter.

The percentage of acetochlor retained by the different soil size fractions of the clay profile is shown in Table 4.3. As with the desorption study of the bulk soils, progressively smaller amounts of acetochlor were desorbed at each desorption step from all the soil size fractions. Further desorption steps may have yielded more desorption of acetochlor, with none of the isotherms reaching plateaux. Figures 4.8, 4.9 and 4.10 show the percentage desorption of acetochlor with each desorption step for the soil size fractions of the clay surface, mid and deep soils.

Table 4.3. Percentage sorption (%) of acetochlor after a 24 h equilibration step, previous to desorption, for soil size fractions obtained from an Iowa clay profile.

Size Fraction (μm diam.)	Sorption of acetochlor (%)		
	Surface (0.0-0.3 m)	Mid (1.0-1.3 m)	Deep (2.7-3.0 m)
>2000	83.14±0.19	58.37±2.45	56.10±0.18
250-2000	82.40±2.27	55.91±4.85	56.96±0.40
53-250	78.28±0.13	53.71±0.41	53.50±0.43
20-53	64.15±3.67	54.18±1.05	57.89±0.72
2-20	85.79±1.92	72.06±2.45	75.06±2.02
0-2	87.90±0.27	70.62±0.99	73.79±0.70
0-1	89.15±0.92	75.62±1.15	74.18±2.55

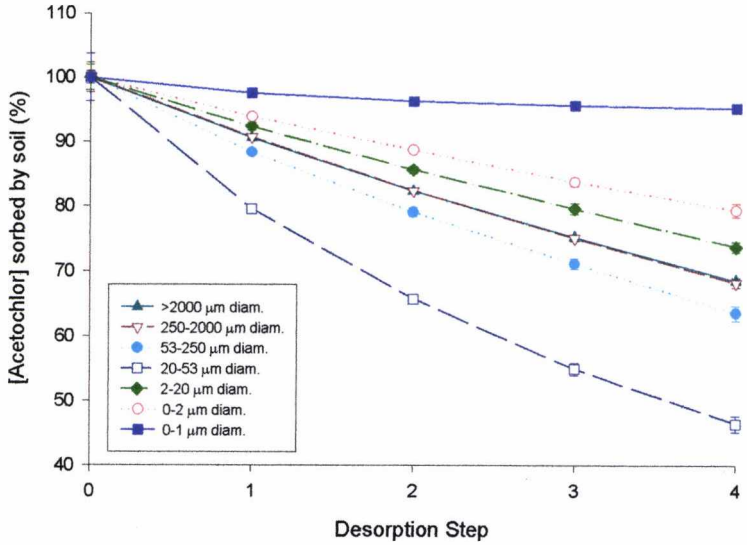


Figure 4.8. The amount of acetochlor desorbed (%) by each desorption step for soil size fractions obtained from the surface (0.0-0.3 m) depth, relative to the amount initially sorbed (all bars represent ± 1 standard error).

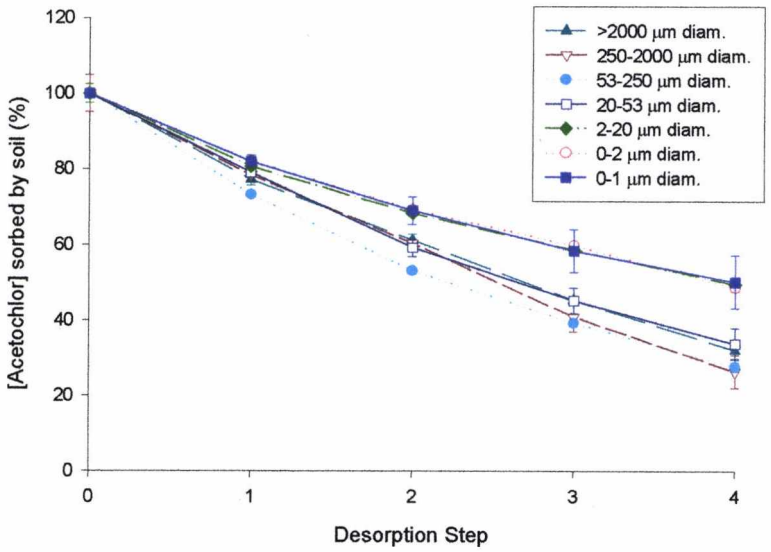


Figure 4.9. The amount of acetochlor desorbed (%) by each desorption step for soil size fractions obtained from the mid (1.0-1.3 m) depth, relative to the amount initially sorbed (all bars represent ± 1 standard error).

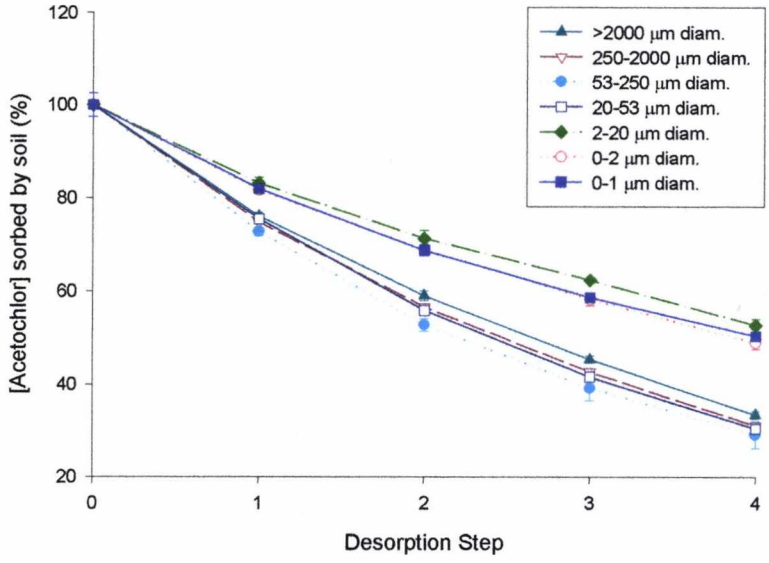


Figure 4.10. The amount of acetochlor desorbed (%) by each desorption step for soil size fractions obtained from the deep (2.7-3.0 m) depth, relative to the amount initially sorbed (all bars represent ± 1 standard error).

The total organic carbon content (TOC %) of the different fractions is shown in Table 4.4. TOC contents for the surface soil fractions were highest in the clay fractions (0-2 and 0-1 μm diam.) and were not significantly ($P > 0.05$) different from each other. The coarse silt (20-53 μm diam.) fraction contained the least amount of organic carbon and was significantly ($P < 0.01$) lower than in the other fractions for this horizon. For the clay mid soil, the TOC values were more evenly spread between the fractions with the macroaggregate (>2000 μm diam.) and coarse silt (20-53 μm diam.) containing the greatest amounts of organic carbon. The microaggregate (53-250 μm diam.) fraction contained the least amount of organic carbon. However, the TOC values were all significantly ($P < 0.01$) lower than the value for the bulk clay mid soil. The TOC measurements taken from the fractions of the clay deep layer displayed values the same as for the blanks. Substantial portions of the TOC content of the clay deep layer could originate from DOM sources, which could have leached away during the wet sieving procedure into the mineral solution phase, due to a low sorption potential of this subsoil

for DOM binding (Kaiser *et al.*, 1996). The mineral phase which was not assessed for TOC content.

Table 4.4. Total organic carbon (TOC) content determined for bulk soils and soil size fractions of an Iowa clay profile.

Soil Size Fraction (μm diam.)	Total Organic Carbon (% w/w)		
	Surface (0.0-0.3 m)	Mid (1.0-1.3 m)	Deep* (2.7-3.0 m)
Bulk	5.70±0.21	0.80±0.03	0.17±0.01
>2000	4.28±0.11	0.35±0.03	0
250-2000	4.16±0.08	0.11±0.01	0
53-250	3.06±0.03	0.01±0.01	0
20-53	1.38±0.02	0.33±0.02	0
2-20	5.28±0.07	0.07±0.01	0
0-2	6.54±0.02	0.30±0.01	0
0-1	7.38±0.06	0.28±0.02	0

* Method used to determine TOC not appropriate for analysis of fractions from deep (2.7-3.0 m) samples – all samples gave lower TOC readings than the controls.

4.3. Discussion

4.3.1. Sorption

For both profiles, sorption of acetochlor was non-linear and fitted well to the Freundlich equilibrium model (Table 4.1). It did not fit well to the Langmuir approach and the strength of the relationship was reduced when modelled using the linear approach. The L and S type isotherms obtained for acetochlor were also obtained, in soils with differing physicochemical properties, by other authors (Wang *et al.*, 1999) and for other chloroacetanilides (Crisanto *et al.*, 1995). The retention of acetochlor was significantly greater ($P < 0.01$) in the Iowa clay profile than the Michigan sand profile. The sorptive behaviour of acetochlor was clearly different between the two profiles. Although sorption for both sets of soils was non-linear, as reflected by the significant disparity between n and unity (Table 4.1), the fit of the Freundlich equation was distinct between the two profiles. The clay profile yielded n values significantly ($P < 0.01$) greater than unity, with the isotherms curving noticeably upwards for the mid and deep soils at the higher concentrations (Figure 4.1). This is indicative of interactions occurring between the soil surface and acetochlor that augment the affinity of these surfaces for this compound

(Homenauth and McBride, 1994). Extrapolation of the isotherm for higher applied concentrations of acetochlor shows a greater affinity for this herbicide in the clay mid soil than the clay surface soil.

In contrast, the sand profile produced *n* values significantly ($P < 0.01$) smaller than unity, with the isotherms approaching plateaux at the higher concentrations for the mid and deep soils. This suggests that as the initial concentration of acetochlor increases, the percentage sorption to the sand soils will decrease and the mobility and potential for leaching will increase (Clay and Koskinen, 1990), especially once acetochlor enters the subsoils of this profile. Further support for this hypothesis comes from analysis of the K_d values defined for a single concentration along the isotherm. These K_d values decreased as the initial concentration of acetochlor increased in the sand soils. For example, the K_d for the highest initial acetochlor concentration (370 μmol l^{-1}) was 0.401 g ml^{-1} which was considerably smaller than that measured at 0.73 μmol l^{-1} (K_d = 0.587 g ml^{-1}). The diminishing K_d values indicate that the acetochlor sorption sites were becoming increasingly saturated.

Non-linear sorptive behaviours are reported frequently (Xing and Pignatello, 1997; Xia and Pignatello, 1998; Chiou *et al.*, 2000). This is suggestive of a combination of partitioning (which alone would yield linear sorptive behaviour) and adsorptive mechanisms (Xing and Pignatello, 1997) being involved in the sorption process. The reasons for this non-linear behaviour are not well understood but hole-filling in the presence of fixed-pore substances or high-surface-area carbonaceous material (e.g. charcoal), competition, and specific interactions of a particular chemical with the active groups of soil organic matter have all been implicated (Xing and Pignatello, 1998; Xia and Ball, 1999; Chiou *et al.*, 2000). The acetochlor was dissolved in a solution containing 75-to-25% acetonitrile: distilled water. The non-linearity of the isotherm could, therefore, be a reflection of competitive interactions between acetonitrile molecules and acetochlor molecules for the same adsorption sites (Martins and Mermoud, 1998).

The sorption of some chloroacetanilide herbicides (e.g. alachlor and metolachlor) has been extensively documented (Peter and Weber, 1985; Pusino *et al.*, 1992; Senesi *et al.*, 1994, Xue and Selim, 1995; Zhu and Selim, 2000), but information regarding other herbicides in this class, such as acetochlor, is scant (Wang *et al.*, 1999; Liu *et al.*, 2000). No significant ($P > 0.05$) correlations were found between soil properties (organic matter content [%], cation exchange capacity, sand, silt and clay content and pH) and the K_F values for either soil (Table 4.5). Significant correlations were found between CEC and the *n* parameter (R = 0.998) in the sand profile (Table 4.6). Cation exchange capacity was also implicated in the sorption and retention of metolachlor in soil (Johnson and Sims,

1993). The small number of soil profiles tested in this research hinders any statistically robust assessment of the relationships between soil properties and acetochlor sorption. However, in surface soils physicochemically dissimilar to those investigated here, acetochlor and other chloroacetanilides have been shown to bind to organic matter and clay minerals through hydrogen bonding and hydrophilic and lipophilic interactions (Peter and Weber, 1985, Bosetto *et al.*, 1993; Senesi *et al.*, 1994, Liu *et al.*, 2000). These data suggest that several processes are involved in the sorption of acetochlor in soil further supporting the use of the Freundlich equilibrium approach for describing the sorption process.

Table 4.5. Correlation coefficients of a number of sorption parameters with soil properties for the Iowa clay profile. None of the R-values were significant ($P > 0.05$).

	Organic matter (%)	pH	CEC	Sand	Silt	Clay	K_F	*n*
Organic matter (%)	1.000							
pH (10 mM $CaCl_2.2H_2O$)	-0.970	1.000						
Cation exchange capacity	0.878	-0.968	1.000					
Sand (0.05-2.0 mm)	-0.290	0.515	-0.713	1.000				
Silt (0.002-0.05 mm)	0.520	-0.296	0.048	0.666	1.000			
Clay (<0.002 mm)	-0.315	0.074	0.177	-0.817	-0.975	1.000		
K_F	0.880	-0.737	0.546	0.199	0.863	-0.728	1.000	
n	-0.690	0.493	-0.260	-0.492	-0.977	0.904	-0.951	1.000

Table 4.6. Correlation coefficients of a number of sorption parameters with soil properties for the Michigan sand profile. The R-value highlighted in bold was statistically significant ($P < 0.05$).

	Organic Matter (%)	pH	CEC	Sand	Silt	Clay	K_F	*n*
Organic matter (%)	1.000							
pH (10 mM $CaCl_2.2H_2O$)	-0.853	1.000						
Cation exchange capacity	0.774	-0.991	1.000					
Sand (0.05-2.0 mm)	-0.966	0.960	-0.912	1.000				
Silt (0.002-0.05 mm)	0.988	-0.924	0.863	-0.994	1.000			
Clay (<0.002 mm)	0.866	-1.000	0.987	-0.966	0.933	1.000		
K_F	-0.466	0.859	-0.921	0.680	-0.598	-0.846	1.000	
n	0.738	-0.982	**0.998**	-0.888	0.834	0.977	-0.941	1.000

4.3.2. Desorption from bulk soils and size fractionated soils

For both profiles, desorption of acetochlor was non-linear and, again, application of the Freundlich equilibrium model was appropriate ($P < 0.01$; Table 4.1; Figure 4.3). For both profiles, n_d values were small and significantly ($P < 0.01$) less than unity. This has also been observed for other studies involving desorption of chloroacetanilides (Zhu and Selim, 2000) and indicates that, as the initial concentration of acetochlor increases, so will the mobility and leaching potential. Substantially greater ($P < 0.01$) K_{Fd} values for the surface soils compared to their respective subsoils imply that the extent of sorption is greater in the surface soils. This suggests that once acetochlor reaches the subsoils of these two profiles the potential for leaching is increased. Although this behaviour is evident in the clay profile with significantly ($P < 0.01$) greater rates of desorption (on a percentage basis of that initially sorbed) from the subsoils than the surface soil (Figure 4.4), the same is not true, however, for the sand profile. Figure 4.4 shows that the reverse is actually occurring. For the sand profile, the rates of desorption (% basis) decrease significantly ($P < 0.01$) with soil depth. This indicates that although the extent of sorption is greater in the sand surface soil, the strength of association is greater in the subsoils, i.e. the sorptive capacity is not a function of the strength of binding (Carrizosa *et al.*, 2001).

Correlations of K_{Fd} and n_d with various soil properties (Table 4.7 and Table 4.8) suggest desorption in both profiles is significantly ($P < 0.05$) influenced by soil organic matter content, with greater amounts of organic matter reducing the desorption rate. The importance of the influence of organic matter on desorption rates has been previously documented (Cornelissen *et al.*, 1998). Increasing desorption rates were also significantly ($P < 0.05$) correlated with the increasing sand content of the Michigan profile. The correlations for the organic matter and sand contents of the sand soil profile imply that the mobility of acetochlor should increase with soil depth for this profile, yet desorption rates are significantly ($P < 0.01$) greater for the surface horizon. This indicates that other factors not taken into consideration (e.g. iron oxyhydroxides) may be affecting the desorptive behaviour of acetochlor in this profile.

Table 4.7. Correlation coefficients of a number of desorption parameters with soil properties for the Iowa clay profile. Significant ($P < 0.05$) R value shown in bold.

	Organic Matter (%)	pH	CEC	Sand	Silt	Clay	K_{Fd}	n_d
Organic matter (%)	1.000							
pH (10 mM $CaCl_2.2H_2O$)	-0.966	1.000						
Cation exchange capacity	0.871	-0.968	1.000					
Sand (0.05-2.0 mm)	-0.276	0.515	-0.713	1.000				
Silt (0.002-0.05 mm)	0.532	-0.296	0.048	0.666	1.000			
Clay (<0.002 mm)	-0.329	0.074	0.177	-0.817	-0.975	1.000		
K_{Fd}	**0.998**	-0.980	0.899	-0.333	0.481	-0.272	1.000	
n_d	-0.679	0.846	-0.952	0.893	0.260	-0.470	-0.722	1.000

Table 4.8. Correlation coefficients of a number of desorption parameters with soil properties for the Michigan sand profile. Significant ($P < 0.05$) R values are shown in bold.

	Organic Matter (%)	pH	CEC	Sand	Silt	Clay	K_{Fd}	n_d
Organic matter (%)	1.000							
pH (10 mM $CaCl_2.2H_2O$)	-0.853	1.000						
Cation exchange capacity	0.774	-0.991	1.000					
Sand (0.05-2.0 mm)	-0.966	0.960	-0.912	1.000				
Silt (0.002-0.05 mm)	0.988	-0.924	0.863	-0.994	1.000			
Clay (<0.002 mm)	0.866	**-1.000**	0.987	-0.966	0.933	1.000		
K_{Fd}	**1.000**	-0.844	0.764	-0.961	0.985	0.858	1.000	
n_d	0.963	-0.962	0.917	**-1.000**	0.993	0.969	0.958	1.000

It is also difficult to understand the lack of correlation between sorption and retentive potential with the clay content of the Iowa profile, considering the significantly ($P < 0.01$) greater capacity for retention of acetochlor in the clay deep soil compared to the sand surface soil, which has a significantly ($P < 0.01$) greater organic matter content. The sorption and desorption of acetochlor from these two profiles may be better linked to differences in the composition of organic matter (Seybold and Mersie, 1996) and clay

mineralogy (Cox *et al.*, 1997a) rather than considering these properties *en masse*. For example, Na-montmorillonite was shown to adsorb more metolachlor than kaolinite on a clay weight basis, with site-limited adsorption behaviour displayed by the kaolinite (Torrents and Jayasundera, 1998). Separation of organic phase from clay phase and further purification of clay fractions to identify the different clay minerals could allow a greater insight into the sorption and desorption influences on acetochlor.

Desorption isotherms for size fractioned soils from the Iowa clay profile were generated to assess the relative contributions of these fractions to the retention of acetochlor and to the observed hysteresis. The chemical and physical characteristics of soil size fractions have been shown to be different in terms of their organic matter composition (e.g. humification status, plant debris, aromatic and aliphatic structures), total carbon and total nitrogen content and texture (Jocteur-Monrozier *et al.*, 1991; Guggenberger *et al.*, 1995; Stemmer *et al.*, 1998). These differences could have a significant influence on the desorptive behaviour of pesticides in soils. The general characteristics of different soil size fractions are shown in Figure 1.4.

For all size fractions, from all depths tested, the Freundlich equation suitably described the data ($P < 0.01$). For all three horizons n_d values were significantly smaller than unity ($P < 0.01$). For all three horizons, the fine silt, clay and colloidal clay fractions dominate the retention of acetochlor, whereas desorption is most prominent for acetochlor bound to the microaggregate and coarse silt fractions. The differences between the fractions are more pronounced in the surface profile (Figure 4.5). Correlation of the K_{Fd} desorption parameter with the TOC (% w/w) for the bulk soils and the soil fractions of all the soil horizons, shows a significant ($P < 0.01$) positive influence of soil organic carbon on the retention of acetochlor ($R = 0.939$). The different rates of desorption found for the different size classes of this profile suggest that the non-linear sorption and desorption isotherms found for the bulk soil were influenced by the different retentive capacities of the size fractions. A distribution of slower and faster desorption kinetics corresponding to differential contaminant-binding strengths has been noted in other studies (Cornelissen *et al.*, 1997; Schelbaum *et al.*, 1999). The extensive sorption of acetochlor and slower desorption rates from the colloidal clay fractions, for example, could account for observed hysteresis in the bulk soils of the Iowa clay profile.

The strong retention of acetochlor in the colloidal clay fractions also has significance for its transport potential. Mobile colloids can facilitate transport of organic contaminants through subsurfaces (McCarthy and Zachara, 1989, Magee *et al.*, 1991; Vilks *et al.*, 1996) and the main conditions needed for this to occur are extensive association of the chemical to the colloidal matter and a sufficient amount of colloidal matter to compete with subsoil materials for contaminant sorption (Elimelech and Ryan, 2002). The potential for

extensive association of acetochlor to colloidal matter has been demonstrated here for the Iowa surface soil, but this fraction only accounts for approximately 1.3 % of the bulk soil (Chapter 5) and, therefore, acetochlor sorption in bulk soil may be mediated to a greater extent by the more profuse fractions. This is supported by the respective sorption isotherms for the different soil fractions when their abundance in soil is taken into account. The isotherm for the bulk soil is significantly ($P < 0.01$) different from the isotherm for the colloidal fraction, whereas it is most similar to the more abundant macroaggregate (>2000 μm diam.) fraction.

A number of factors may have contributed to desorption non-linearity. In terms of methodology, equilibrium may not have been reached or the ionic strength of the desorption solution may have altered between desorption steps. However, data (not shown) indicates that 24 h was sufficient for equilibration, and similar findings have been observed elsewhere (Wang *et al.*, 1999). The ionic strength of the desorption solution was maintained using 10 mM $CaCl_2.2H_2O$, so changes in the ionic strength should have been minimal. However, no measurements were taken to validate this. Although the desorption process was kept under sterile conditions (no bacteria were isolated using plate counts from the resuspended soil pellet after the centrifugation steps), there was still the potential for abiotic transformation of acetochlor. Metabolite formation was not investigated and only total ^{14}C was quantified during desorption experiments. Competition between acetochlor, acetochlor metabolites and acetonitrile (co-solvent) could have contributed to the non-linearity. Degradation data (Chapter 5) indicates that, over the time-course used for desorption, production of acetochlor metabolites would be negligible. Non-linearity could have occurred due to the formation of non-extractable residues during the desorption study (Moreau-Kervévan and Mouvet, 1998) or due to deformation of flexible pores in soil organic matter hindering desorption (Xia and Pignatello, 2001). A more exhaustive desorption protocol, involving more desorption steps and/or the use of other solvents (e.g. acetonitrile or methanol), followed by combustion analysis could validate the hypothesis of non-extractable residue formation.

4.3.3. Sorption-desorption hysteresis

The Freundlich n values for sorption were significantly ($P < 0.05$) larger than the corresponding Freundlich n_d values for desorption, in all cases. This indicates that hysteresis is taking place. Values of n_d less than n suggest that the sorption process is not completely reversible. Cox *et al.* (1997b) used the percentage difference between n and

n_d, [i.e. (n_d/n) x 100] as a measure of hysteresis. For the sand profile, the hysteresis coefficient (29.3) was significantly smaller ($P < 0.05$) in the surface soil than in the subsoils (18.9, 16.83 for mid and deep soils, respectively), which were not significantly different ($P > 0.05$) from each other. Hysteresis was significantly ($P < 0.05$) different between the clay profile depths studied. The degree of hysteresis was lowest in the clay deep soil (34.82), with hysteresis coefficients of 29.3 and 25.75 for the clay surface and clay mid soils, respectively. The increase in hysteresis with soil depth (with the exception of the clay deep soil in this study) has been noted elsewhere (Moorman *et al.*, 2001).

The hysteresis coefficients correlated positively with organic matter content ($R = 0.988$) and clay content ($R = 0.934$) for the sand profile, but the relationships were not significant ($P > 0.05$). No such correlations were seen for the clay profile. As previously discussed for sorption and desorption isotherm linearity, the same factors could be involved in the hysteresis. Incomplete equilibrium, changes in solution composition with each desorption step, non-reversible binding to or slow desorption from soil constituents and experimental error have all been suggested as potential sources of hysteresis (Jenks *et al.*, 1998; Mitra *et al.*, 2000; Moorman *et al.*, 2001; Xia and Pignatello, 2001).

Desorption coefficients are not as widely reported as sorption coefficients. As a result, sorption-desorption isotherm hysteresis, is not completely understood (Mitra *et al.*, 2000; Moorman *et al.*, 2001). Desorption studies typically use short equilibrium times, such as 24 h (Carrizosa *et al.*, 2001). However, the relationship between sorption and desorption is recognised as being time-dependent (Pignatello and Huang, 1991; Kookana *et al.*, 1992; Cox *et al.*, 1998; Zhu and Selim, 2000), with desorption being less likely to occur as time progresses. For prediction of sorption behaviours under field conditions, where contact times between contaminants and soil surfaces will be much longer than in traditional laboratory sorption studies, it may be better to increase the contact time previous to the desorption process for modelling purposes. Zhu and Selim (2000) used such an approach to study the behaviour of metolachlor in soil, and produced a more realistic model for hysteresis of this compound. These authors also constructed their desorption isotherms using a range of initial concentrations, rather than the single initial concentration used in our research and elsewhere (Carrizosa *et al.*, 2001), and showed that desorption is concentration dependent. This relationship has also been shown for atrazine (Moorman *et al.*, 2001).

The low binding potential, along with the high desorption rates for acetochlor indicates that this herbicide would have rapidly leached through the sand soil profile, to potentially contaminate groundwater resources. Not surprisingly, this soil does not meet the pre-requisite standards needed for the safe use of acetochlor, i.e. the organic matter content of this profile is too low (Section 1.3.3.1.3). For the clay profile, the higher binding

potentials would limit leaching to a certain extent but all the acetochlor was not sorbed and, thus, leaching to groundwater would still be possible. The greater degree of acetochlor retention in the clay profile may lead to a decrease in degradation because of diffusional limitations and restricted accessibility to microbes and their extracellular enzymes, whilst in the sand profile, bioavailability, and therefore biodegradation, may occur to a greater extent because of this much lower sorption capacity. Biodegradation and bioavailability of acetochlor are discussed in greater detail in Chapter 5, which examines the rates of acetochlor mineralization and transformation, and bound residue formation over time under aerobic laboratory-incubated conditions.

4.4. Conclusion

The results of this study show that the sorption and desorption characteristics of acetochlor vary with soil depth and between soil size fractions. Sorption-desorption hysteresis was evident for both clay and sand profiles at all depths indicating irreversible sorption or differential rates of desorption from different soil size fractions. Different sorption strengths and rates of desorption from the various soil components and fractions could have serious implications for the long-term bioavailability of chemicals in soil, as well as on the long-term migration of chemicals through soil profiles to groundwater.

Chapter 5. Influence of biotic and abiotic factors on the dissipation of acetochlor in pristine soil profiles

5.1. Introduction

The interaction between the intrinsic properties of a pesticide (water solubility, functional groups and structure) and the extrinsic properties of the soil environment (temperature, pH, organic matter content) will govern the potential for sorption and degradation of a compound (Veeh *et al.*, 1996; Moreau-Kervevan and Moreau, 1998; Sannino *et al.*, 1999). These extrinsic properties will vary both temporally and spatially as a consequence of soil heterogeneity (Vinther *et al.*, 2001). As a consequence, chemicals that are transported horizontally or vertically (even over micrometer distances) in a soil profile can encounter very different environmental conditions from those first encountered (Johnson and Sims, 1993; Brockman and Murray, 1997; Jenks *et al.*, 1998). The variation in soil characteristics, including changes in microbial community composition, abundance and function, could have an important influence on the ultimate fate of agrochemicals applied to soil (Bending *et al.*, 2001; Vinther *et al.*, 2001).

The influence of subsoil microbes and soil properties need to be better understood in order to ascertain their impact on the retention and degradation/transformation of pesticides and other xenobiotic compounds as they pass through a soil profile. The biodegradative capacity of subsoils has been shown to be variable, depending on both the compound and the soils in question (Di *et al.*, 1998; Shaw and Burns, 1998; Mills *et al.*, 2001). Mineralization and transformation of a number of pesticides including 2,4-D (Shaw and Burns, 1998), sulphonylurea herbicides (Anderson *et al.*, 2001), chloroacetanilides (Clay *et al.*, 1997; Mills *et al.*, 2001) and atrazine (Miller *et al.*, 1997) have been demonstrated in subsoil horizons. In certain cases, the rate of degradation is greater in the subsoil layer than in the respective surface layer (Karpouzas *et al.*, 2001; Mills *et al.*, 2001).

The bioavailability of a pesticide in soil is restricted by the extent of adsorption to soil/soil components (Cox *et al.*, 1997b) and by sequestration within nanopores that exclude microbes and create diffusional barriers to degradation (Alexander, 1999). A number of binding forces are involved in the associations between pesticide and soil particles (e.g. van der Waals forces, charge-transfer mechanisms, ligand exchange, covalent binding, and partitioning [Gevao *et al.*, 2000]). These associations can lead to non-reversible

binding and so-called bound residue formation (Gevao *et al.*, 2000), which has been shown to increase the longer a chemical resides within soil (Dec *et al.*, 1997a; Kelsey *et al.*, 1997; Koskinen *et al.*, 2001). Bound residues are operationally defined as those residues that are not extractable using mild solvents, i.e. solvents that do not affect the nature of the chemical residues (Gevao *et al.*, 2000). The increasing rate of nonextractable residue formation over time generally coincides with a decrease in bioavailability and the subsequent degradation of a compound (Cox *et al.*, 1998b; Koskinen *et al.*, 2001).

In order to improve recommendations that will limit or eliminate groundwater contamination by acetochlor, the fate of this compound in both surface soil and subsurface materials must be understood. As noted in the previous chapter, the exposure of herbicides to soil systems is likely to occur over long time periods, with certain herbicides remaining in soil several years after their initial application (Alexander, 1999). This study compared the sorption and degradation of acetochlor in surface soil and subsoils of the clay profile up to 100 days after application, with the distribution of nonextractable ^{14}C residues among particle-size fractions was also examined.

5.2. Results

5.2.1. Environmental fate of acetochlor in the clay soil profile

A preliminary study carried out at Syngenta (Jealott`s Hill Research Station) showed that acetochlor was transformed at all the depths sampled from the Iowa clay profile and the Michigan sand profile after 5 days. TLC (Section 2.3.3) revealed the presence of acetochlor-ethanesulphonic acid (ac-ESA) and acetochlor oxanilic acid (ac-OXA) in addition to traces of other metabolites that could not be identified. However, due to experimental limitations no other meaningful data was generated and is, therefore, not reported here. As mentioned in the previous chapter, the Michigan sand profile is inappropriate for the use of acetochlor due to the high leaching potential caused by its low organic matter content. For this reason, as well as logistical ones, acetochlor environmental fate was only studied using the Iowa clay profile.

^{14}C-labelled residues were detected in three pools: (i) extractable ^{14}C, recovered from the soil by exhaustive extractions with 10 mM $CaCl_2.2H_2O$, (ii) Bound or nonextractable ^{14}C, not removed by this mild aqueous extraction but recovered by oxidation, and (iii) $^{14}CO_2$ produced by mineralization to CO_2 and H_2O. The extractable residues were further separated into pools of parent compound (acetochlor), ac-ESA, ac-OXA, and acetochlor-

transformants of unknown character (ac-UI). The acetochlor extraction efficiencies, i.e. those extractions carried out at time zero (2 h after application), for both biotic and sterile samples, were approximately 100 % with no significant differences in efficiency between the depths.

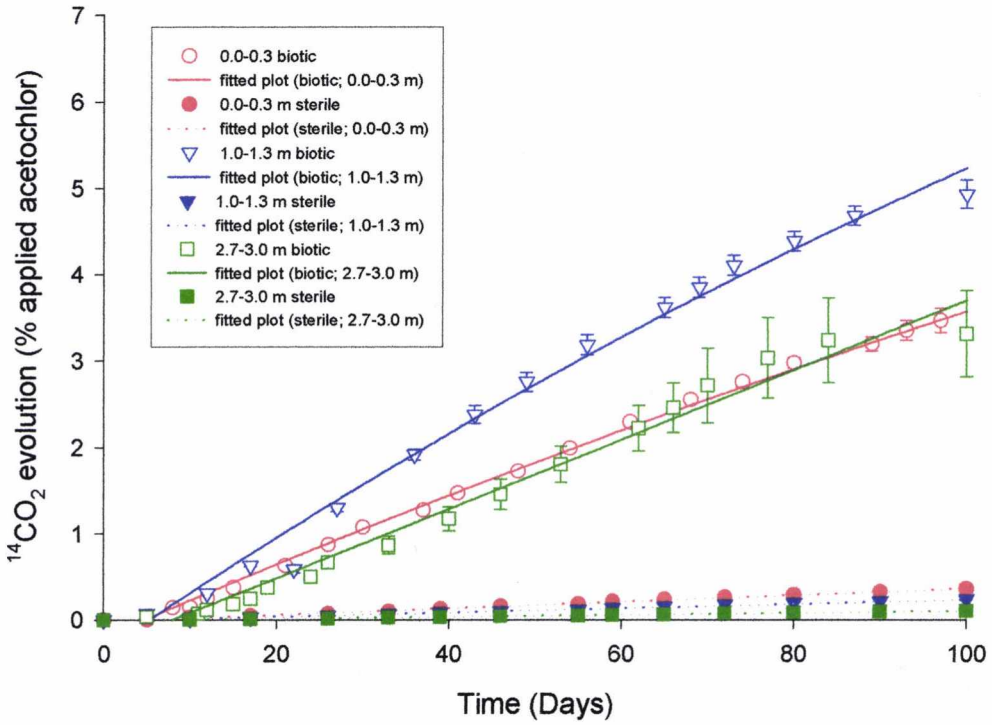


Figure 5.1. Soil depth related differences in cumulative evolution of $^{14}CO_2$, expressed as a percentage of the total applied acetochlor, over time. Fitted data plot according to the relationship, $y = yo + a(1-e^{-bx})$ for the biotic soils, which represents an exponential rise to a maximum. A linear equation was fitted to the sterile soil data. All bars represent ± 1 standard error.

5.2.1.1. Mineralization

The cumulative evolutions of $^{14}CO_2$ from soil samples, obtained from depths of 0.0-0.3, 1.0-1.3, and 2.7-3.0 m, after inoculation with ^{14}C-labelled acetochlor are shown in Figure 5.1 for both biotic and sterile soil samples. The curves produced by the biotic soil samples are typified by an initial lag phase, followed by a fast mineralization rate and then a gradual reduction until the end of the incubation. The length of the lag phase was

significantly ($P < 0.05$) different for the different depths, with the deep soil displaying the longest lag phase (0-32 d) followed by the mid soil (0-24 d) and surface soil (0-10 d).

Despite the difference in lag phases between the surface and deep soils, no significant ($P > 0.05$) difference in cumulative evolution of $^{14}CO_2$ was evident at the end of the 100 d incubation. In contrast, after the initial 24 d of incubation, the mineralization rate observed for the mid soil increased to a significantly ($P < 0.01$) greater rate than for the other depths. The cumulative evolution of $^{14}CO_2$ in the biotic samples was significantly ($P < 0.01$) greater than those seen in the sterile controls: 0.36, 0.23 and 0.1 % of the total ^{14}C applied accumulated in the sterile surface, mid and deep soils, respectively, compared to the 3.6, 4.93 and 3.31 % recovered from the biotic surface, mid and deep soils, respectively, over the same 100 d time period. The mineralization data for the biotic soil samples (at all depths) could be modelled appropriately with a 3-parameter exponential equation (Equation 5.1; $R \geq 0.99$; $P \leq 0.0001$) and with a power equation, which is of the same form as the Freundlich equation (Equation 5.2; $R \geq 0.99$; $P \geq 0.0001$). The mineralization data obtained from the sterile soils followed a linear pattern.

Equation 5.1. $y = yo + a(1-e^{-bx})$

Equation 5.2. $y = ax^b$

5.2.2. Biological and abiotic transformation of acetochlor

The changes in the amount of extractable ^{14}C over time are shown for the surface, mid and deep biotic soils in Figure 5.2. Similar trends were observed for the sterile soils (data not shown). The loss of ^{14}C from the aqueous phase was rapid, but the rate of loss decreased significantly ($P < 0.05$) as the incubation progressed. No significant ($P > 0.05$) differences in extractable ^{14}C were observed between the biotic and sterile samples at each of the sample points over the incubation period. The changes in extractable ^{14}C residues over time were appropriately modelled using an exponential decay equation (Equation 5.3; $R \geq 0.98$; $P \leq 0.0076$), with the relationship significantly stronger for the sterile soils ($R \geq 0.99$; $P \leq 0.0021$). After 100 d, the mean recovery of ^{14}C residues was 20.3, 24.2, and 18.4 % of that initially applied for the biotic surface, mid and deep samples, respectively, with similar percentages recovered for the sterile soils.

Equation 5.3 $y = yo + ae^{-bx}$

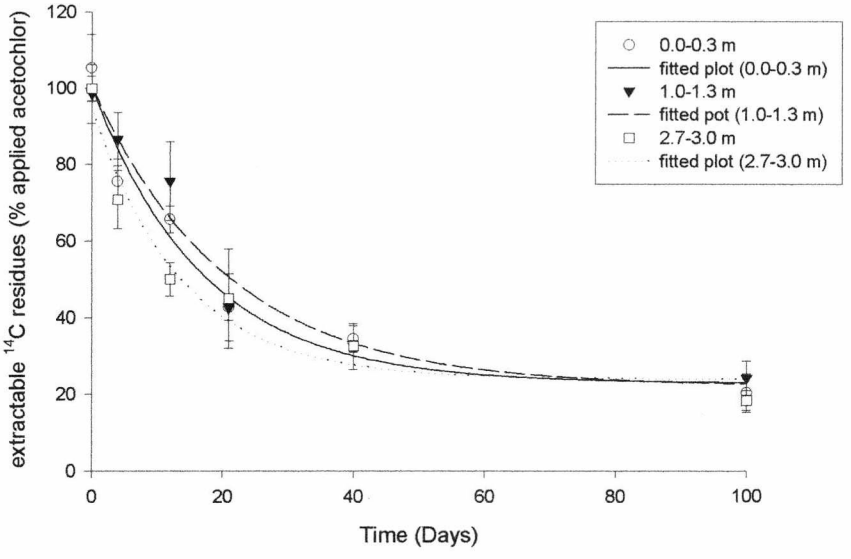


Figure 5.2. Soil depth related differences in extractable ^{14}C residues for biotic soils, expressed as a percentage of the initially applied acetochlor, over time. Fitted data plot according to the relationship $y = yo + ae^{-bx}$, which represents exponential decay. All bars represent ± 1 standard error.

The dissipation of acetochlor in the layers from the clay profile analysed are shown in Figure 5.3 for the biotic samples. TLC analysis revealed that the acetochlor concentration decreased significantly ($P < 0.01$) over time, as a component of the extractable ^{14}C residues in these samples (Figure 5.3) and followed a similar exponential decay pattern ($R \geq 0.99$; $P \leq 0.0029$) as observed for the total extractable ^{14}C residues (Equation 5.3). Dissipation half-lives, DT_{50}'s, were determined from the fit of the exponential decay equation to the data, using approximate iterations to achieve the value closest to the time point where 50 % of the total applied acetochlor had become unavailable. In this study, the DT_{50} values did not include losses through plant uptake, leaching or photolysis, as these fate processes could not occur due to the nature of the system used (Section 2.3.8). Only the processes of sorption and degradation/mineralization were influential in producing these values. The DT_{50}'s were 9.32, 12.32 and 12.58 d for the biotic surface, mid and deep soils. The dissipation of acetochlor in the sterile controls was significantly ($P < 0.01$) slower than in the biotic soils, although degradation was still shown to occur with TLC analysis. Dilution plating and CFU analysis indicated that these samples were still

sterile. Abiotic degradation of acetochlor was, therefore, evident at all depths. DT_{50} values for acetochlor in the sterile soils were 20.92, 23.5, and 23.99 d for the surface, mid and deep soils, respectively (Figure 5.4).

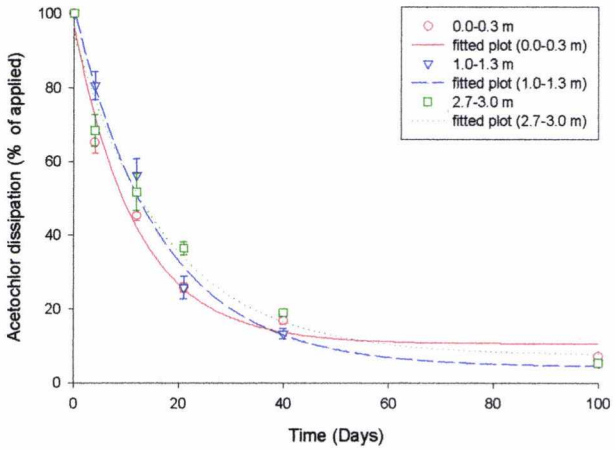


Figure 5.3. Soil depth related differences in extractable acetochlor residues for biotic soils, expressed as a percentage of the initially applied acetochlor, over time. Fitted data plot according to the relationship $y = yo + ae^{-bx}$, which represents exponential decay. All bars represent ± 1 standard error.

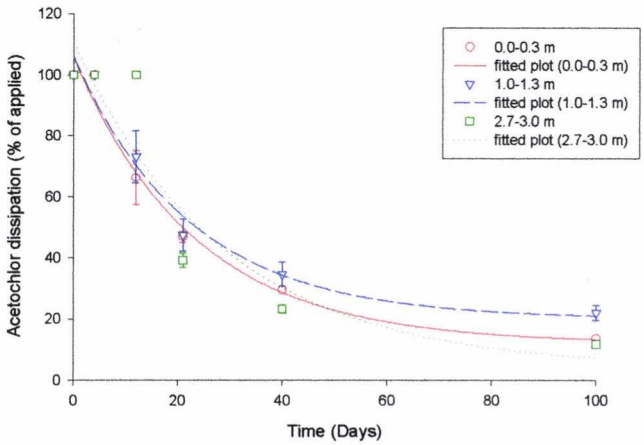


Figure 5.4. Soil depth related differences in extractable acetochlor residues for sterile soils, expressed as a percentage of the initially applied acetochlor, over time. Fitted data plot according to the relationship $y = yo + ae^{-bx}$, which represents exponential decay. All bars represent ± 1 standard error.

Acetochlor transformation profiles are shown in Figures 5.5, 5.6, and 5.7 for the surface, mid and deep soils, respectively, under biotic conditions. For the biotic samples, TLC analysis showed that ac-ESA, ac-OXA and traces of several unidentified metabolites were formed, although one of the unidentified metabolites was not evident in the deep soil sample. The degree of resolution achieved with the TLC analysis was not sufficient for separation of all metabolites from each other, making it impossible to identify individual transformants in such cases. The levels of these compounds increased significantly over the initial 21 d of incubation after which metabolite levels were relatively constant. For the sterile controls, ac-OXA, ac-ESA, and the same unidentified metabolites were evident. The formation of metabolites occurred after 4 d for the biotic soils, 12 d for the sterile surface and mid soils and 21 d for the sterile deep soil.

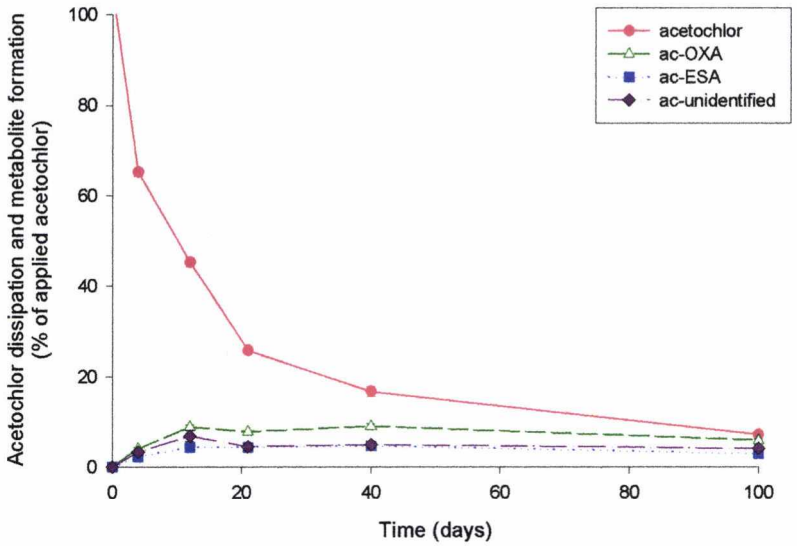


Figure 5.5. Changes in extractable acetochlor and metabolite residues for biotic surface soil (0.0-0.3 m), expressed as a percentage of the initially applied acetochlor, over time. All bars represent ± 1 standard error.

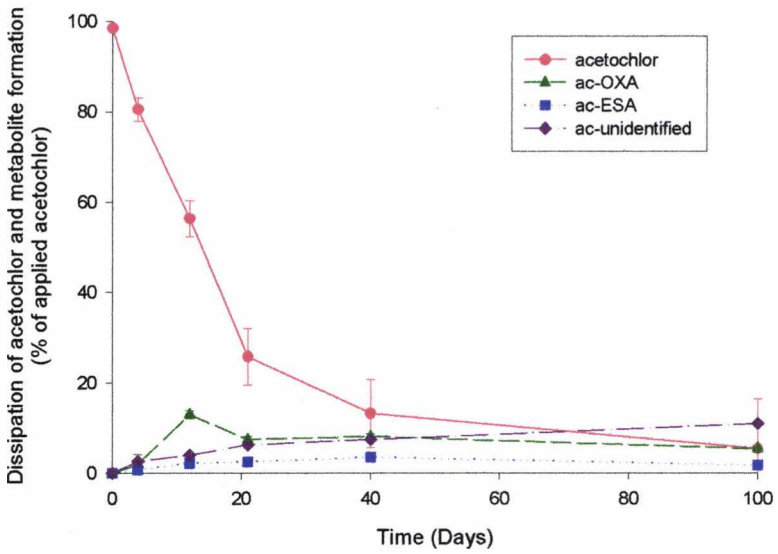


Figure 5.6. Changes in extractable acetochlor and metabolite residues for biotic mid soil (1.0-1.3 m), expressed as a percentage of the initially applied acetochlor, over time. All bars represent ± 1 standard error.

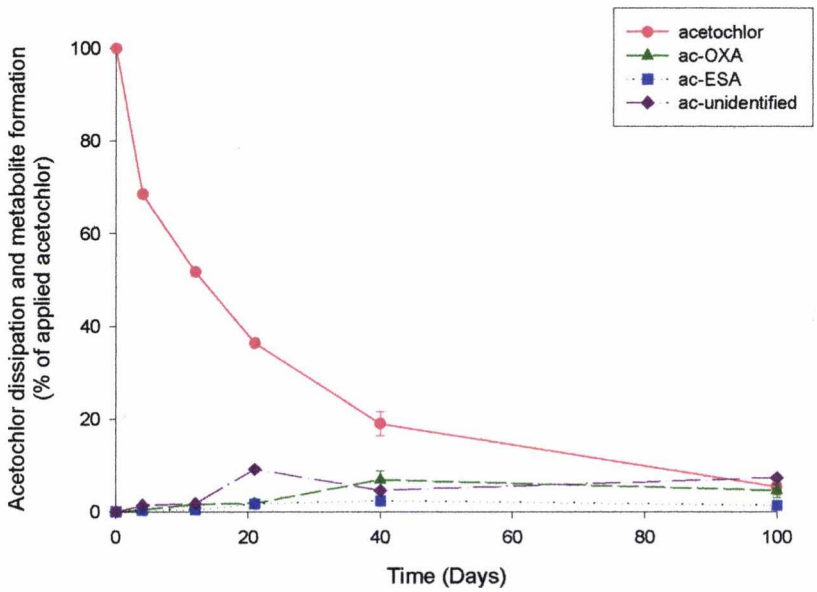


Figure 5.7. Changes in extractable acetochlor and metabolite residues for biotic deep soil (2.7-3.0 m), expressed as a percentage of the initially applied acetochlor, over time. All bars represent ± 1 standard error.

5.2.3. Bound/nonextractable residue formation

Nonextractable residue formation was modelled with a 3-parameter exponential equation [equation 5.1], which fitted the data from all the soils well ($R \geq 0.97$; $P \leq 0.013$) Changes in nonextractable residue formation in the bulk soils are shown in Figures 5.8, 5.9 and 5.10, for the surface, mid, and deep soils, respectively. Nonextractable residues formed immediately after acetochlor application, with approximately 11.7, 3.94 and 2.04 % of the acetochlor initially applied in the surface, mid and deep soils, respectively, associated with this ^{14}C pool. For the biotic surface soil, nonextractable residue formation increased significantly ($P < 0.05$) over the first 21 d, after which the concentrations remained constant. In contrast, the sterile surface soil displayed two phases of significantly ($P < 0.05$) increased nonextractable residue formation: over the first 12 d and then over the period spanning 21 d to 100 d, with levels constant in between.

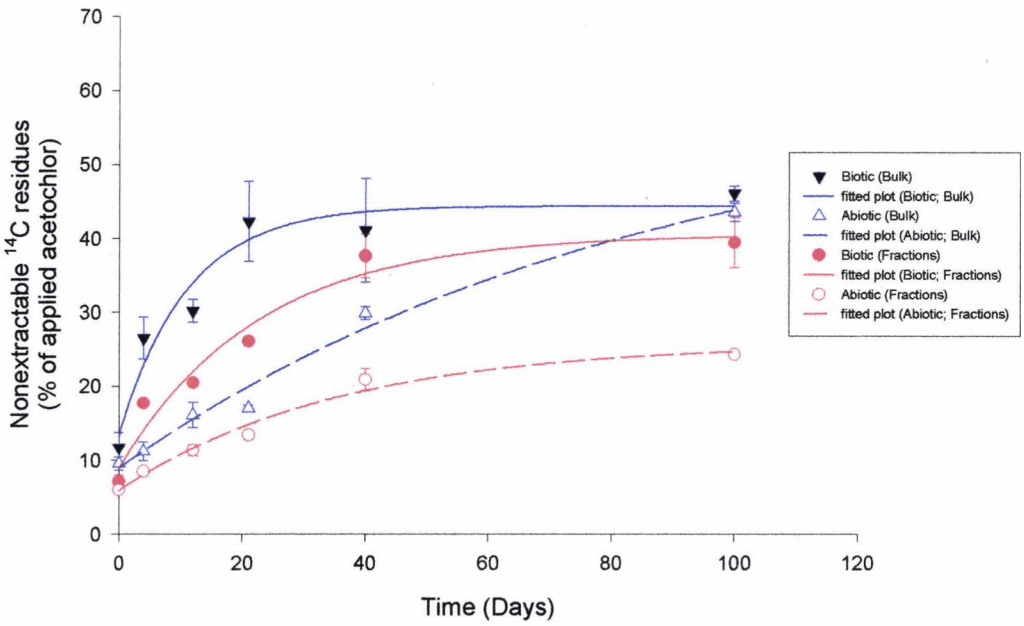


Figure 5.8. Change in nonextractable ^{14}C residues in bulk and fractionated surface (0.0-0.3 m) soil, expressed as a percentage of the initially applied acetochlor. All bars represent ± 1 standard error.

Significant ($P < 0.05$) greater rates of nonextractable residue formation were apparent in the biotic surface soil compared to the sterile control until the final 100 d sampling point. For the mid soil, the nonextractable residues increased significantly ($P < 0.05$) over time, with no significant ($P > 0.05$) differences after 100 d displayed between biotic and sterile treatments, but significant ($P < 0.05$) differences were observed at the 12 d and 21 d time points. For the deep soils, nonextractable residue formation was also time-dependent but significantly ($P < 0.05$) greater nonextractable residues were formed in the biotic treatment than in the sterile controls after 100 d, and were generally significantly ($P < 0.05$) higher throughout the incubation.

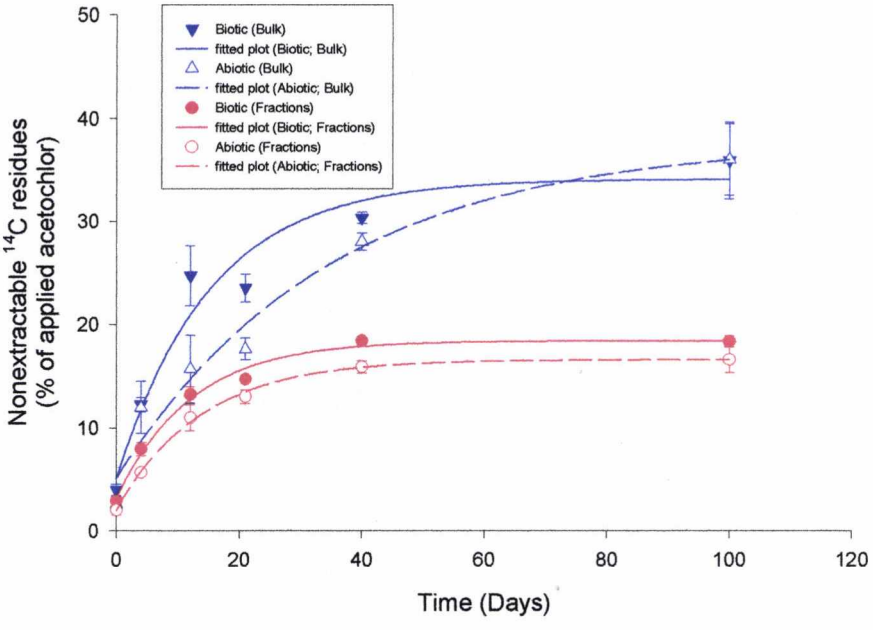


Figure 5.9. Change in nonextractable ^{14}C residues in bulk and fractionated mid (1.0-1.3 m) soil, expressed as a percentage of the initially applied acetochlor. All bars represent ± 1 standard error.

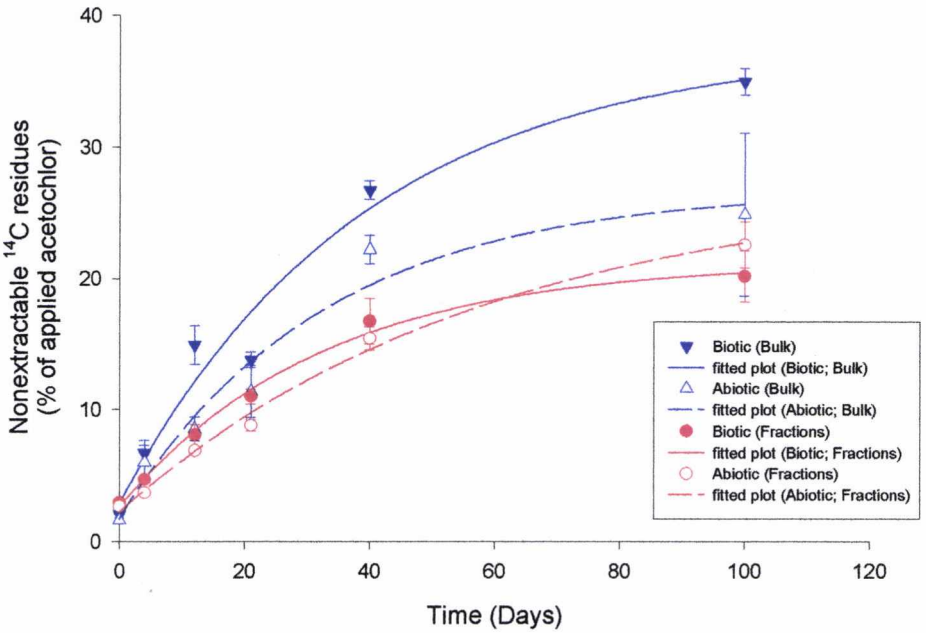


Figure 5.10. Change in nonextractable ^{14}C residues in bulk and fractionated deep (2.7-3.0 m) soil, expressed as a percentage of the initially applied acetochlor. All bars represent ± 1 standard error.

For the biotic soil samples, the initial rates of nonextractable residue formation (0-4 d) decreased significantly ($P < 0.05$) with soil depth, with clear differences evident in Figures 5.8, 5.9, and 5.10). Nonextractable residue formation was significantly ($P < 0.05$) greater in the surface soil compared to the mid and deep soils, which were not significantly ($P > 0.05$) different from each other after the 100 d. In the sterile controls, nonextractable residue formation significantly ($P < 0.05$) decreased with increasing depth. After the 100 d incubation approximately 46.1, 35.9, and 35 % of the initially applied ^{14}C had become non-reversibly bound to the surface, mid and deep soils, respectively.

In general, time-dependent formation of nonextractable residues was exhibited by all fractions, but this relationship was not significant in all cases (Figures 5.11, 5.12, and 5.13.). For comparison with the bulk soils, at each time point the nonextractable residues were summed together, relative to their proportion in the bulk soil and the data modelled with equation 5.1, which fitted the data well ($R \geq 0.98$; $P \leq 0.0076$; Figures 5.8, 5.9 and 5.10). The relative proportions of each fraction from the respective depths are given in

Table 5.1. Relative composition of soil, in terms of aggregates and particle sizes, as a % of the total amount of soil undergoing the fractionation procedure.

		Soil fraction diam. (μm)						
		>2000	**250-2000**	**53-250**	**20-53**	**2-20**	**0-2**	**0-1**
Surface								
Biotic	Mean	50.00	13.97	8.50	19.35	4.08	2.77	1.32
	standard error	*1.17*	*1.01*	*0.60*	*1.47*	*0.33*	*0.17*	*0.15*
	coefficient of variance (%)	2.34	7.21	7.04	7.61	7.98	6.00	11.11
Sterile	Mean	39.97	19.51	6.14	23.72	4.94	4.63	1.10
	standard error	*0.78*	*0.55*	*0.49*	*1.03*	*0.48*	*0.34*	*0.16*
	coefficient of variance (%)	1.96	2.82	8.02	4.32	9.74	7.28	14.45
Mid								
Biotic	Mean	60.14	11.36	4.73	15.38	4.17	2.87	1.35
	standard error	*1.42*	*0.40*	*0.58*	*0.96*	*0.36*	*0.26*	*0.09*
	coefficient of variance (%)	2.37	3.55	12.23	6.34	8.68	9.17	6.87
Sterile	Mean	56.54	8.97	4.49	20.11	4.61	2.58	2.71
	standard error	*1.04*	*0.42*	*0.50*	*0.80*	*0.36*	*0.19*	*0.18*
	coefficient of variance (%)	1.85	4.74	11.17	3.97	7.86	7.20	6.76
Deep								
Biotic	Mean	41.57	2.69	12.70	31.57	4.86	3.20	3.40
	standard error	*1.23*	*0.19*	*0.84*	*1.01*	*0.45*	*0.20*	*0.17*
	coefficient of variance (%)	2.96	6.95	6.63	3.20	9.23	6.12	5.07
Sterile	Mean	41.89	5.15	11.88	27.79	4.33	5.66	3.32
	standard error	*1.44*	*0.58*	*0.89*	*0.78*	*0.34*	*0.42*	*0.29*
	coefficient of variance (%)	3.43	11.27	7.47	2.79	7.82	7.35	8.69

Table 5.1 for both biotic and sterile soils. The total obtained from the sum of the nonextractable residues in the fractions was significantly ($P < 0.05$) lower than residues found in the bulk soil after 4 d and throughout the incubation afterwards until the 100 d time point where no significant ($P > 0.05$) difference was seen for the surface and mid soils. For the sterile soils, the sum of the nonextractable residues in the fractions was significantly ($P < 0.01$) less than in the bulk soil for the surface depth (approximately 55 % of the bulk soil value), with the exception of 0 d, whilst in the mid and deep soils there was no significant ($P > 0.05$) difference.

For the surface soil at 0 d, nonextractable ^{14}C residue formation decreased in the following order: macroaggregate (>2000 μm diam.) > macroaggregate (250-2000 μm diam.) > 53-250 μm diam. ~ 20-53 μm diam. > 2-20 μm diam. ~ 0-2 μm diam. > 0-1 μm diam. (Table 5.2). The amounts of nonextractable residues increased significantly (P < 0.05) over the initial 21 - 40 d (depending on the soil fraction), after which the levels of bound residues remained constant. The exception to this was the colloidal clay fraction, where nonextractable residues fluctuated considerably over the 100 d.

For the mid soil at 0 d, nonextractable ^{14}C decreased in the following order: > 2000 μm diam. > 250-2000 μm diam. ~ 20-53 μm diam. ~ 2-20 μm diam. > 0-2 μm diam. >53-250 μm diam. > 0-1 μm diam. For the first four fractions the relationship stayed the same over the 100 d, whilst for the remaining fractions the order varied (Table 5.3). For the deep soil at 0 d, nonextractable 14C decreased in the following order: 20-53 μm diam. > macroaggregate (> 2000 μm diam.) > 0-2 μm diam. > 0-1 μm diam. > 2-20 μm diam. > 53-250 μm diam. > 250-2000 μm diam. After 4 d the order changed for the first two fractions, but the otherwise remained the same during the 100 d (Table 5.4). Similar distributions were observed for the respective fractionated sterile soils (Table 5.1, 5.2, and 5.3 for the sterile surface, mid and deep soils, respectively).

Table 5.2. Distribution of nonextractable residues in surface soil over a 100 d incubation period, as found after fractionation. Expressed as a percentage of the initially applied acetochlor. Significant (P < 0.05) differences in nonextractable residues over time are denoted by different letters in the rows of each individual column. For example, the figures marked with an 'a' are significantly different to figures marked with all other letters (b - g) in that column. Values based on weight of soil fraction per 100 g soil.

	Surface (0.0-0.3 m)						
	Soil fraction diam. (μm)						
Time (days)	<2000	250-2000	53-250	20-53	2-20	0-2	0-1
Biotic							
0	2.73±0.50^{a}	1.77±0.29^{a}	0.68±0.05^{a}	0.85±0.08^{a}	0.44±0.08^{a}	0.44±0.13^{a}	0.15±0.05^{a}
4	9.83±0.31^{b}	4.07±0.35^{b}	1.23±0.21^{b}	1.60±0.51^{a}	0.26±0.06^{a}	0.43±0.01^{a}	0.27±0.09^{a}
12	10.52±0.72^{b}	3.48±0.26^{b}	2.59±0.34^{c}	1.96±0.38^{a}	0.69±0.06^{b}	0.75±0.06^{b}	0.42±0.07^{b}
21	15.25±1.09^{c}	3.08±0.78^{b}	1.76±0.33^{b}	3.44±0.46^{b}	1.34±0.08^{c}	0.99±0.06^{b}	0.20±0.05^{a}
40	22.78±3.32^{d}	6.30±0.12^{c}	2.96±0.47^{c}	2.84±1.73^{a,b}	1.36±0.34^{c}	1.25±0.06^{c}	0.14±0.01^{a}
100	23.44±2.56^{d}	5.13±0.22^{c}	3.30±0.84^{c}	4.89±0.66^{c}	1.15±0.31^{d}	0.98±0.15^{b}	0.55±0.19^{b}
Sterile							
0	2.77±0.19^{a}	0.93±0.23^{d}	0.28±0.04^{d}	1.11±0.05^{d}	0.37±0.09^{a}	0.43±0.04^{a}	0.06±0.03^{c}
4	3.80±0.78^{a}	1.76±0.05^{a}	0.45±0.10^{e}	1.65±0.13^{a}	0.41±0.11^{a}	0.33±0.06^{a}	0.05±0.01^{c}
12	5.43±0.69^{e}	1.93±0.19^{a}	0.60±0.11^{e}	2.14±0.08^{a}	0.66±0.20^{a}	0.50±0.11^{a}	0.07±0.04^{c}
21	6.98±0.60^{f}	2.35±0.18^{a}	0.87±0.10^{f}	2.11±0.14^{a}	0.52±0.10^{a}	0.51±0.19^{a}	0.06±0.01^{c}
40	9.44±0.48^{b}	3.84±0.15^{e}	0.83±0.21^{f}	3.97±1.10^{b}	1.87±0.24^{d}	0.77±0.17^{a}	0.13±0.06^{a}
100	12.79±0.66^{g}	3.22±0.17^{b}	1.32±0.41^{g}	4.78±0.37^{c}	1.53±0.70^{d}	0.57±0.11^{a}	0.10±0.01^{a}

Table 5.3. Distribution of nonextractable residues in mid soil over a 100 d incubation period, as found after fractionation. Expressed as a percentage of the initially applied acetochlor. Significant ($P < 0.05$) differences in nonextractable residues over time are denoted by different letters in the rows of each individual column. For example, the figures marked with an 'a' are significantly different to figures marked with all other letters (b - g) in that column. Values based on weight of soil fraction per 100 g soil.

	Mid (1.0-1.3 m)						
Time (days)	<2000	250-2000	53-250	20-53	2-20	0-2	0-1
Biotic							
0	1.64±0.43^{a}	0.56±0.08^{a}	0.07±0.02^{a}	0.21±0.04^{a}	0.26±0.05^{a}	0.12±0.02^{a}	0.02±0.01^{a}
4	4.69±0.79^{b}	1.07±0.07^{b}	0.22±0.05^{b}	0.84±0.08^{b}	0.89±0.10^{b}	0.14±0.02^{a}	0.07±0.01^{b}
12	8.76±0.60^{c}	1.26±0.05^{c}	0.34±0.14^{b}	1.46±0.31^{c}	1.09±0.17^{b}	0.18±0.02^{b}	0.09±0.01^{c}
21	9.14±0.30^{c}	1.86±0.13^{d}	0.41±0.12^{b}	1.51±0.35^{c}	1.18±0.20^{b}	0.40±0.09^{c}	0.16±0.02^{d}
40	9.20±0.54^{c}	2.21±0.35^{e}	0.54±0.10^{b}	2.61±0.19^{d}	2.56±0.71^{c}	0.91±0.16^{d}	0.36±0.08^{e}
100	11.67±0.79^{d}	1.25±0.09^{c}	0.80±0.14^{c}	2.18±0.29^{d}	1.73±0.24^{d}	0.41±0.06^{c}	0.30±0.03^{e}
Sterile							
0	1.18±0.50^{a}	0.27±0.02^{e}	0.03±0.01^{d}	0.25±0.01a	0.12±0.02^{e}	0.07±0.01^{e}	0.06±0.04^{a}
4	2.69±0.55^{b}	0.84±0.02^{f}	0.29±0.11^{b}	1.05±0.27^{b}	0.57±0.03^{f}	0.09±0.01^{f}	0.09±0.01^{c}
12	6.73±0.69^{c}	1.15±0.10^{b}	0.32±0.11^{b}	1.66±0.46^{b}	0.69±0.10^{b}	0.22±0.06^{b}	0.21±0.02^{f}
21	8.21±0.70^{c}	1.18±0.32^{b}	0.51±0.13^{b}	1.50±0.24^{b}	0.80±0.15^{b}	0.41±0.05^{ce}	0.41±0.17^{e}
40	9.31±0.54^{c}	2.12±0.67^{e}	0.65±0.20^{b}	2.24±0.55^{d}	0.85±0.19^{b}	0.36±0.09^{c}	0.33±0.03^{e}
100	10.55±0.93^{c}	1.32±0.30^{b}	0.57±0.09^{b}	2.57±0.30^{d}	0.66±0.15^{b}	0.26±0.04^{c}	0.78±0.16^{g}

Table 5.4. Distribution of nonextractable residues in deep soil over a 100 d incubation period, as found after fractionation. Expressed as a percentage of the initially applied acetochlor. Significant ($P < 0.05$) differences in nonextractable residues over time are denoted by different letters in the rows of each individual column. For example, the figures marked with an 'a' are significantly different to figures marked with all other letters (b - g) in that column. Values based on weight of soil fraction per 100 g soil.

	Deep (2.7-3.0 m)						
Time (days)	<2000	250-2000	53-250	20-53	2-20	0-2	0-1
Biotic							
0	0.52±0.09^{a}	0.05±0.01^{a}	0.20±0.02^{a}	1.06±0.06^{a}	0.23±0.06^{a}	0.48±0.12^{a}	0.38±0.11^{a}
4	1.79±0.03^{b}	0.14±0.02^{b}	0.31±0.02^{b}	1.27±0.13^{a}	0.27±0.09^{a}	0.37±0.08^{a}	0.55±0.15^{a}
12	2.55±0.03^{c}	0.32±0.02^{c}	0.49±0.09^{c}	3.10±0.93^{b}	0.62±0.29^{b}	0.41±0.08^{a}	0.58±0.09^{a}
21	6.08±0.43^{d}	0.29±0.07^{c}	0.40±0.04^{c}	2.83±0.18^{b}	0.20±0.12^{a}	0.43±0.03^{a}	0.77±0.03^{b}
40	7.76±0.43^{d}	0.45±0.03^{d}	0.80±0.14^{d}	4.99±2.18^{b}	0.94±0.39^{b}	0.86±0.26^{b}	0.95±0.22^{b}
100	9.62±1.96^{d}	0.59±0.07^{e}	0.98±0.16^{d}	4.65±0.19^{c}	1.50±0.06^{c}	1.29±0.13^{c}	1.54±0.33^{b}
Sterile							
0	0.48±0.11^{a}	0.11±0.02^{b}	0.23±0.01^{a}	0.70±0.04^{d}	0.18±0.04^{a}	0.52±0.18^{a}	0.42±0.16^{a}
4	0.94±0.10^{b}	0.25±0.04^{c}	0.28±0.04^{b}	0.76±0.16^{d}	0.55±0.11^{b}	0.54±0.11^{a}	0.35±0.14^{a}
12	2.56±0.34^{c}	0.56±0.21^{c}	0.33±0.03^{b}	2.09±0.31^{e}	0.22±0.04^{a}	0.61±0.11^{a}	0.52±0.20^{a}
21	4.70±0.21^{e}	0.87±0.29^{d}	0.56±0.10^{c}	1.58±0.22^{e}	0.32±0.06^{a}	0.54±0.14^{a}	0.24±0.06^{c}
40	8.69±0.55^{d}	1.61±0.95^{d}	0.67±0.13^{c}	2.34±0.35^{e}	0.58±0.06^{b}	0.87±0.12^{b}	0.68±0.16^{b}
100	8.79±1.62^{d}	1.05±0.38^{d}	1.91±0.24^{d}	5.15±1.25^{b}	1.29±0.39^{c}	2.92±0.28^{d}	1.46±0.18^{b}

5.3. Discussion

5.3.1. Mineralization and degradation of acetochlor

The pattern of acetochlor mineralization exhibited by all the biotic soil samples, consisted of an initial lag phase before a rapid phase of $^{14}CO_2$ evolution, followed by a gradual slowing of $^{14}CO_2$ production. This behaviour has been reported for other pesticides (Miller *et al.*, 1997; Cox *et al.*, 1998b; Shaw and Burns, 1998). The observed increase in lag phase as the soil depth increased (Figure 5.1) has also been noted in other studies (Veeh *et al.*, 1996; Shaw and Burns, 1998). There are several factors that may contribute to the lag phase duration, including the presence of an appropriate catabolic capacity for a compound, the response time for induction of the necessary enzyme systems and the proliferation of degrading-microorganisms to a number sufficient to cause detectable losses of compound; the size of degrading microbial communities have been suggested to be as low as tens or hundreds of individuals per gram of soil (Fournier, 1980; Helweg, 1993; Jayachandran *et al.*, 1997). The duration of the lag phases (10, 24 and 32 d for the surface, mid and deep soils) correlated significantly ($R = 1$; $P < 0.05$) with the total numbers of microbes counted by epifluorescence microscopy (Table 3.2), suggesting that the number of microbes with the capacity to transform acetochlor is a limiting factor in this process. Further evidence for this comes from the lack of correlation between mineralization and microbial abundance. It is likely, the duration of the lag phases in acetochlor degradation were controlled by increases in microbial growth, caused by increasing the soil from 40 % WHC to 60 % WHC, allowing proliferation of acetochlor-transforming microbes to sufficient numbers that could produce detectable transformation. As the soils in this study were pristine and never exposed to acetochlor, repeated applications of this compound may eventually allow acclimation of the indigenous microbial communities to this herbicide and increase the soils capacity for its degradation, as has been demonstrated for a number of other pesticides (Mallawantri *et al.*, 1996; Jenks *et al.*, 1998). This acclimation is regarded to be a long-term process, especially in subsoils (Wilson *et al.*, 1985; Aelion *et al.*, 1989). However, the low rates of mineralization, along with the deceleration towards the end of the incubation period points to an initial cometabolic transformation of acetochlor, with availability to microbial communities limited by sorption and irreversible binding as time progresses.

The decelerating rate of $^{14}CO_2$ evolution in the latter stages of the 100 d incubation was presumably as a result of the increasing ^{14}C associated with the solid phase (Figures 5.8-5.10) and the concomitant decrease in ^{14}C available in the solution phase (Figure 5.2).

Radosevich *et al.* (1996) inversely correlated mineralization with sorption, supporting this relationship. The effect of sorption in restricting biodegradation potential has been widely recognized (Ogram *et al.*, 1985; Mihelcic *et al.*, 1993). A further compounding factor that could contribute to the reduction in mineralization rate is localised exhaustion of pesticide substrates around the degrading microbial communities, creating diffusional limitations (Scow and Hutson, 1992). Considering the nonextractable residue formation was most likely to form on the macroaggregate fractions, the localised exhaustion of acetochlor could have occurred. It has also been suggested that the stabilization of the final mineralization rate results from biomass turnover, with ^{14}C released from decomposing storage polysaccharides dominating over the mineralization of the remaining slowly degradable nonextractable residues (Soulas, 1993).

Several studies have reported a decrease in mineralization with soil depth (Radosevich *et al.*, 1996; Shaw and Burns, 1998; Andersen *et al.*, 2001), whereas others have found there to be no link between mineralization and soil depth (Willems *et al.*, 1996; Andersen *et al.*, 2001). No association between soil depth and mineralization was evident in this study, with the mid soil displaying significantly ($P < 0.01$) higher rates of mineralization than the surface and deep soils. The differences in mineralization behaviour within a soil profile are likely to be as a result of differing soil characteristics at different depths in a soil profile (Di *et al.*, 1998) and on the nature of pesticide (Andersen *et al.*, 2001).

Mineralization was low (< 5 % of total applied ^{14}C recovered after 100 d) in all the soils, and suggests that this process is not a major factor in its environmental fate. This low rate of mineralization has been reported for other chloroacetanilides, in addition to acetochlor (Mills *et al.*, 2001). For example, Clay *et al.* (1997) reported a recovery of 2.5 % of the applied ^{14}C as $^{14}CO_2$ over 112 d of incubation. The lack of mineralization found in this study indicates that biodegradation of acetochlor is only partial and principally cometabolic, with insignificant ring cleavage. Clay *et al.* (1997) noted that mineralization of alachlor was C-limited, which also supports this view of cometabolic breakdown of chloroacetanilides. However, no correlation between the accumulated mineralization of acetochlor at d 100 and TOC content of the soils was found in this study. The lack of ring cleavage found for most chloroacetanilides has been attributed to the steric hindrance produced by the ring substituents in the *ortho* positions (2'-, 6'-) and also the presence of N-alkyl groups (Villarreal *et al.*, 1991). This has been substantiated by the complete mineralization of propachlor, a chloroacetanilide herbicide that does not possess *ortho* ring substituents (Novick *et al.*, 1985; Villarreal *et al.*, 1991; Martin *et al.*, 1999). It is also reflected by the ring-intact metabolites identified here and elsewhere (Feng, 1991)

The extractability of herbicide residues from soil can be used to assess their availability. For example, all solvent extractable residues are considered to be available, whereas

nonextractable residues are regarded as unavailable or very slowly available (Barriuso *et al.*, 1996). As used here, aqueous extractions, if repeated enough times, are thought to be able to recover all extractable residues (Raman and Rao *et al.*, 1988; Barriuso *et al.*, 1992). Aqueous $CaCl_2$ solution-extractable ^{14}C decreased rapidly during the first 40 d of incubation, but at 0 d approximately 100 % of the applied acetochlor was recovered. At the end of the 100 d incubation, the extractable residues were only 44.03, 67.47 and 52.65 % of those residues bound to the surface, mid and deep soils, respectively. Alongside the diminishing extractable residues was a concurrent and significant ($P < 0.05$) reduction in acetochlor concentration in this pool (Figure 5.3).

Although many studies focus on the disappearance of various chemicals in soils and subsoils, they do not report the formation of metabolites and/or the extent of bound residue formation (Pothuluri *et al.*, 1990; Shaw and Burns, 1998; Mills *et al.*, 2001). However, risk assessments are required for metabolites that account for more than 10 % of the initially applied active ingredient, and exhibit pesticidal or ecotoxicological properties (Beulke and Brown, 2001). The loss of acetochlor over time and production of metabolites is shown in Figures 5.5, 5.6, and 5.7 for the surface, mid and deep biotic soils, respectively. Several metabolites were distinguished using TLC analysis, of which only two could be identified with certainty: ac-OXA and ac-ESA. No further reference standards were available. Due to the resolution of the TLC analysis it cannot be determined whether the separation of all metabolites was complete (Appendix 2). Furthermore, none of the metabolites amounted to more than 10 % of the total ^{14}C added, which means they would not be considered as important breakdown products, in terms of registration requirements.

Isolation of both ac-OXA and ac-ESA indicate that GSH-based transformation pathways were active in the microbial components of each soil depth. However, abiotic production of these metabolites also occurred albeit at a significantly ($P < 0.05$) slower rate. The GSH-based transformation pathway and abiotic production of these two metabolites are discussed in more detail in Section 1.3.2.5.1. It is also possible that the same reaction pathway causing ac-OXA and ac-ESA formation could be responsible for the creation of the other isolated metabolites (Feng, 1991). Another potential cause of metabolite production in the sterile soils would be the presence of microbial contaminants or nonculturable micorbes. Although the soils were tested using dilution-plating and no CFUs were isolated, this does not guarantee the sterility of the soils. The presence of non-culturable bacteria would not be detected by this method and it would be unlikely that all bacteria would grow on the media used for isolation. The rapid losses of acetochlor from the deep soil, in excess of both the surface and mid depths, after 21 d suggest nonculturable bacteria survived the autoclaving procedure and were degrading acetochlor.

The rapid loss of acetochlor from the soil systems was not matched by rapid production of metabolites and mineralization. This suggests that dissipation of acetochlor is principally affected by soil sorption rather than microbial/abiotic degradation. This was further demonstrated by the pattern of nonextractable residue formation in these soils. The decrease in metabolite formation could have been as a result of depleting levels of acetochlor, from its adsorption to soil surfaces, failing to elicit metabolic responses to its presence (Alexander, 1999), thus preventing its transformation.

5.3.2. Nonextractable residue formation

Soil-herbicide associations are not completely reversible (Jenks *et al.*, 1998; Moorman *et al.*, 2001) and have been shown to be time-dependent (Kookana *et al.*, 1992; Zhu and Selim, 2000). This time-dependent non-reversible binding was demonstrated for acetochlor, and was implicated by the generation of nonlinear sorption and desorption isotherms (Chapter 4). For all three depths, irrespective of biotic or abiotic status, the formation of nonextractable residues in the bulk soils was nonlinear and fitted well to an exponential equation (Equation 5.1; $R \geq 0.972$; $P = 0.01$). Differences in capacity to form nonextractable residues were evident immediately after acetochlor application, but the range varied with incubation time, depth and fraction.

The biphasic nature of the nonextractable residue formation is consistent with an increasing saturation of binding sites, which cause irreversible or slowly reversible binding over time. The isotherms generated in Chapter 4 also displayed this hole-filling behaviour, as proposed by Xing and Pignatello (1996). The decreasing rate of nonextractable residue formation could also reflect changes in acetochlor concentration, with this compound being continually degraded as the incubation progresses. The metabolites produced could have different characteristics to acetochlor; ac-OXA and ac-ESA both have greater water solubilities (Field and Thurman, 1996). This would reduce the potential for nonextractable residue formation due to the decreased affinity for the solid matrix of these molecules compared to acetochlor. The change in structure caused by the transformation could also allow molecular interactions with soil surfaces that are different to the parent compound, or they could compete directly with acetochlor for binding sites. The subtle structural differences between chloroacetanilide herbicides have been shown to influence their sorption behaviours (Wang *et al.*, 1999; Liu *et al.*, 2000).

The formation of bound residues was most closely associated with the macroaggregate (>2000 μm diam. and 250-2000 μm diam.) fraction in all the depths, with the exception of

the deep soil, where the 250-2000 μm diam. fraction contained the least nonextractable residues. This particular fraction was considerably darker and granular in appearance, in contrast to the yellowy-brown, amorphous appearance of the other fractions. The structural differences may account for the disparity. This differential binding capacity of soil size fractions has been found for atrazine (Barriuso *et al.*, 1996). The formation of nonextractable residues over the 100 d period was not uniform throughout the fractions. Modelling of the data revealed that no single equation was appropriate for describing bound residue formation in all the fractions. This suggests that different processes are involved in the associations of acetochlor and/or acetochlor metabolites in the different fractions.

The TOC content of the fractions significantly correlated ($R = 0.782$; $P < 0.05$) with the nonextractable residue concentrations of the surface and mid soils at 100 d. The correlation with TOC content, along with the potential for different fractions associating with acetochlor in different ways, could be related to the nature of the organic carbon residues present in the fractions. As soil particle size decreases, the character of the organic matter changes. For example, carbohydrate concentrations decreased and lignin and aliphatic C contents increased (Baldock *et al.*, 1997). This shows that the more recalcitrant organic matter is contained in the finer fractions, whilst the more labile and reactive non-humified organic matter is present in the coarser fractions. The reactivity of the organic matter contained in macroaggregate fractions could account for nonextractable residue formation being significantly greater in these fractions.

For the surface soil, nonextractable residues associated in significantly ($P < 0.05$) greater amounts with the silt size fractions compared to the clay fractions. The aromaticity and molecular dimensions of organic constituents are generally greater in silt size fractions than clay size fractions (Christensen, 1992), which could favour interactions with acetochlor and its metabolites, especially through electron donor processes that generate charge transfer bonds (Senesi *et al.*, 1994; Wang *et al.*, 1999). In the surface soil, the capacity for acetochlor stabilisation, as bound residues, decreased as particle size decreased. This is probably due to the influence of organic matter content and composition, and a reduction in accessibility (Barriuso *et al.*, 1996). In marked contrast to the bound residue formation in this study, Barriuso *et al.* (1996) found that bound residue formation was favoured in the finer 0.2 – 20 μm diam. fractions, whilst macroaggregates were the favoured site of bound residue formation in the soil used here. However, the fractionation procedure used by Barriuso *et al.* (1996) was considerably harsher than the procedure used here. The largest fraction category in their study was > 200 μm diam., whereas > 2000 μm diam. is the upper limit in this study. The gentle fractionation

procedure employed here was designed to simulate pressures and stresses that would occur naturally, such as through rainfall impact, whereas Barriuso *et al.* (1996) deliberately dispersed macroaggregates using shaking. Employing the same procedure would likely enrich the smaller fraction sizes as disaggregation into microaggregates and erosion of particles from the micro- and macro- aggregate surfaces occurs. Operational differences, therefore, limit any meaningful comparisons between this study and the Barriuso *et al.* (1996) study. As far as I am aware no other studies have looked at the distribution of organic contaminants in size-fractionated soils.

The contribution of microorganisms to nonextractable residue formation is also evident in the macroaggregate fractions, with significantly ($P < 0.05$) enhanced formation occurring in biotic soils compared to sterile soils. Bacteria associated with macroaggregates have been found to be distinct from those associated with microaggregates (Winding, 1994) and it has also been shown that aggregate-surface associated microbial populations are divergent from those contained within the aggregate (Ranjard *et al.*, 1997). The potentially different metabolic activities of the microbial populations in the macroaggregate fractions could also contribute to the nonextractable residue formation. Different zones of activity for a given function could contribute to or diminish the capacity for degradation and the potential for formation of nonextractable residues. Stemmer *et al.* (1998) found that soil enzyme activities were unevenly distributed between soil fractions and that different soil enzyme activities were concentrated in different fractions. For example, they found that xylanase activity was mainly associated with the coarser soil fractions, which is where less mineralized POM was concentrated. The processes inducing nonextractable residue formation or transformation of acetochlor could only be present in specific microsites within a soil and they could be limited in number.

5.4. Dissipation

Degradative processes are traditionally modelled using first-order rate reaction kinetics, which assume that the pesticide concentration regulates its biodegradation (Beulke and Brown, 2001) and that the relevant microbes are present in excess. However, studies have suggested that the size of the degrading community can limit the rate of degradation (Jayachandran *et al.*, 1997; Shaw and Burns, 1998) and non-linear rate kinetics are required to model degradation (Beulke and Brown, 2001). Bi-phasic dissipation of pesticide residues was apparent for acetochlor, showing an initial rapid decline and low levels persisting at the end of the incubation (Figure 5.3).

However, in this study acetochlor dissipation was the culmination of mineralization and transformation, as well as losses through sorption to the soil matrix. Monitoring ^{14}C concentrations does not allow differentiation between the acetochlor and its metabolites that form nonextractable residues, which means that degradative losses of acetochlor cannot be measured with any accuracy. For this reason, the half-life of acetochlor could not be determined. Contrasting the rate of acetochlor loss with the rates of metabolite formation show that the dissipation of acetochlor is influenced to a greater degree by other factors, i.e. sorption. The importance of sorption in the environmental fate of chloroacetanilides has been noted elsewhere (Aga and Thurman, 2001). The laboratory-derived DT_{50} values obtained were concordant with the average values obtained elsewhere (Mills *et al.*, 2001), but were smaller than those reported by Mills *et al.* (2001) for a similar soil profile (i.e. Iowa, silty clay loam). The DT_{50} for the surface soil in this study was 9.32 d and the specific Iowa surface soil in their study was 12 d. However, the disparity between the DT_{50}'s for the subsoils is considerable. DT_{50}'s of 12.3 and 12.6 d were found for acetochlor in the subsoils here, whereas Mills *et al.* (2001) found values of 35 and 53 d. The differences are probably due to slight differences in sampling depth between the two studies, with distinct physical and chemical properties from those sampled for this study.

5.5 Conclusion

Acetochlor dissipated rapidly from the surface and subsoil layers, predominantly through sorption to soil surfaces, but also through biodegradation and transformation. Several metabolites were produced including ac-OXA and ac-ESA, further confirming the critical importance of GSH-mediated removal of chloroacetanilides from soil. Upon entrance to soil, the extensive macroaggregate surfaces provide substantial areas for acetochlor sorption, causing localisation of acetochlor residues and a non-homogenous distribution. This could have serious consequences for the biodegradation of this compound in soil, removing it from potential degrading microorganisms that may only survive in finer soil fractions, and could also lead to facilitated transport to less microbially active groundwater. The DT_{50} values reported are within the range of DT_{50} values previously published for acetochlor in surface soils and subsoils (Mills *et al.*, 2001)

Chapter 6. General Discussion

6.1. Interactions between soil characteristics and biological functions

In general, this work indicates that microbial activities are dependent on the number of microorganisms and the concentration of nutrients, in terms of organic matter (Chapter 3). Numerous studies (e.g. Parkin *et al.*, 1987), including this one (Chapter 3), have found statistically significant relationships between soil characteristics and biological function, they do not allow identification of the actual interactions taking place between the soil components and microorganisms. For example, clays can stimulate microbial activity (growth, respiration and uptake of substrates) (Anderson *et al.*, 1990), with montmorillonite shown to enhance glucose uptake and melanin formation in a number of fungi (Filip *et al.*, 1971). However, in the latter study, correlation analysis showed that there was no relationship between the clay contents of the soils and glucose uptake. A similar lack of correlation between the clay contents and organic matter contents of the Iowa clay profile and urease activity was observed here, yet substantial urease activity was measured in the Iowa mid soil, in excess of the surface soil. This suggests that a substantial quantity of extracellular urease is present in this subsoil layer. Correlation analysis does not aid in identifying where this source of extracellular enzyme is located. Specific clay minerals or organic matter constituents may be responsible for this elevated urease activity. This urease anomaly and the Filip *et al.* (1971) study show that a more detailed assessment of soil characteristics and microbial function is required to fully understand *in situ* biogeochemistry.

Soil characteristics control the activity and distribution of indigenous microorganisms exerting considerable influence on microbially driven processes, such as organic matter turnover and those involved in the biodegradation and transformation of organic chemicals. The spatial arrangement of soil particles defines the local physicochemical conditions that microbes are exposed to, and governs biogeochemical cycling in soil through its influence on substrate accessibility/availability (Alexander, 1999) and microbial interactions (e.g. predator-prey dynamics [Wright *et al.*, 1995]).

Batch systems, such as those used here (Chapter 3) are often used to investigate microbial interactions and activities. However, suspending soil in a solution phase increases the ratio of particle surface area to the volume of solution due to disaggregation of the soil. This will alter the accessibility/availability of substrates from those experienced under normal aggregated soil conditions, with greater surface areas available for sorption of substrates, extracellular enzymes and of the microbes themselves. The biodegradation

rates of small organic molecules were shown to decrease when particles such as clay minerals or aluminium hydroxides were present (Jones *et al.*, 1998). The values here, and in other studies (e.g. Kandeler and Gerber, 1988) will, therefore, underestimate the potential of surface and subsoil microbial populations for nutrient cycling. However, this work does show that nutrient cycling is taking place in subsoils and that the potential for degradation of a wide variety of organic contaminants may also be possible in these environs. The influence of subsoil microbial populations should not be overlooked in assessing the environmental fate of pesticides.

Studies of the enzyme activities and microbial biomass in bulk soils are common, yet only a few have assessed these properties relative to soil particle size fractions (Gupta and Germida, 1988; Jocteur-Monrozier *et al.*, 1991; Stemmer *et al.*, 1998, 1999). Stemmer *et al.* (1998) found that enzyme activities were concentrated in certain fractions, but the location varied according to the enzyme. This suggests that preferential sorption of enzymes occurs according to their structure and the soil surface properties. This microsite variation in specific microbial functions could have implications for degradation of organic contaminants in soil. Limited accessibility of pesticide molecules to finer soil fractions (Johnson *et al.*, 1999), where the necessary catabolic enzyme activities could be present may preclude or restrict their degradation. In conjunction with the dominance of sorption processes in acetochlor environmental fate over biodegradative processes, nonextractable ^{14}C residues were found to be concentrated in the macroaggregate fractions, which would also support this view. It is likely that a closer analysis of macroaggregate surfaces would be of benefit in producing a realistic depiction of acetochlor fate, rather than the bulk soil.

It is also recognised that agricultural practices can alter soil structure, potentially resulting in a loss of SOM through the breakdown of macroaggregates (Gupta and Germida, 1988). Beare *et al.* (1994a) found that macroaggregate-protected SOM accounted for 18.8 and 19.1 % of the total mineralizable C and N, respectively. Gupta and Germida (1988) concluded that the loss of SOM was related to the fungal biomass, which has an important role in macroaggregate formation, being disrupted during cultivation and utilised as a labile C source. It can, therefore, be seen that the disruptive procedures commonly used to assess biological functions in soil may not provide environmentally relevant measures of *in situ* activity due to changes in spatial relations between substrates and enzymes (Stemmer *et al.*, 1998).

The relative importance of interactions at the scales of both surface and microstructure needs to be addressed to allow a comprehensive view of the functioning of soil *in situ*. Definition and integration of these *in situ* processes could be beneficial to bioremediation strategies. It is known that 'hot spots' of specific activity in soil can occur naturally or can

be induced by external stimuli. For example, Christensen *et al.* (1990) stimulated denitrification activity in selected zones by addition of particulate organic matter, and these regions of accentuated activity were seen to persist over a range of days and weeks. This spatial and temporal distribution of biological activities is implicated by the greater concentration of urease activity in the Iowa mid soil compared to the respective surface and deep soils.

The spatial variance of biological activities in soil, exemplified by studies on denitrification (Parkin *et al.*, 1987; Lensi *et al.*, 1995), is likely to be accentuated by the widely documented reduction in size of microbial populations with soil depth (Chapter 3; Federle *et al.*, 1986, Colwell, 1989; Veeh *et al.*, 1996) and the parallel reduction in organic matter and other nutrient sources (Chapter 3; Kieft *et al.*, 1993). For example, Vanderheyden *et al.* (1997) observed that biodegradative processes in subsoils only took place where microbes were sheltered by stones and iron components. Assessment of bulk subsoil characteristics (as carried out in Chapter 3) may not reflect the actual activities present in locations that are directly affecting subsoil contaminants. Studies using macropore sheath soil, have shown greater numbers of microbes and enriched activities exist in these preferential flow paths (Tiunov and Scheu, 1999; Pankhurst *et al.*, 2002), and their influence on the environmental fate of subsoil contaminants has also been implicated comparative to the bulk matrix soil (Pivetz and Steenhuis, 1995).

Given the array of components and processes involved in biogeochemical cycling in soils and subsoils, the design of relevant microcosms to assess the multitude of interactions taking place between physical and biological systems and how they mediate processes such as organic matter turnover or attenuation of organic chemicals will be vital but challenging.

6.2. Soil characteristics and the fate of environmental contaminants

Upon entrance to the soil environment, a chemical may be exposed to degrading microbial communities, which will be comprised of different strains and species acting as functional entities combining individual, diverse catabolic capacities to achieve a complete sequence of degradative steps leading ultimately to mineralization of the chemical or production of recalcitrant metabolites (Chapter 5). Complicating this situation further, not only will a wide array of biodegradative systems be acting on the pesticide, vast and reactive soil surfaces will be available for the pesticide molecules to interact with.

Furthermore, some interactions will be reversible whilst others will not. All of these competing forces will contribute to the final fate of a pesticide, and was demonstrated here for acetochlor (Chapter 5). Significant amounts of acetochlor were immediately removed from the environment by sorption, which will immediately reduce the efficacy of acetochlor as a biocide and will also reduce the amount that is degraded. However, the concentration remaining in the aqueous phase was subject to biodegradation, producing transformation products. Although irreversible binding, principally to macroaggregate and other soil surfaces, essentially renders acetochlor nonbioavailable, the long-term potential for release cannot be discounted and remediation attempts should be encouraged.

A detailed understanding of these biological, chemical and physical processes and their influence on a pesticide is crucial in order to develop realistic models for environmental fate. Soil persistence and leaching models are very sensitive to the parameters governing sorption and degradation (Boesten and van der Linden, 1991). Modelling can be beneficial for agricultural management practices by adapting pesticide type and application rates to the characteristics of the area where particular biocidal requirements are needed. Locally tailored application rates taking into account soil properties are a potential source of economic and environmental benefits (Sylvester-Bradley *et al.*, 1999). Acetochlor use is restricted to certain soil types because of its rapid leaching characteristics in sandy soils (Section 1.3.3.1.3), as was shown by the sorption-desorption isotherms presented in Chapter 4 for the Michigan sand soil, where acetochlor use would not be allowed.

At a microscale level, all the theoretical descriptions of microbial degradation assume that pesticides and microorganisms are uniformly distributed throughout the soil. This is unlikely considering the multitude of distinct microenvironments present in soil systems and their influence on the resident microflora within these niches. The heterogeneity of soil surfaces will also result in an uneven distribution of pesticide within the soil matrix (Chapter 5) because of the differential capacity of the wide spectrum of soil components and conglomerates for retention of these molecules (Chapter 4) and on the influence of the soil structure on the leaching of pesticides in interstitial soil pore- water.

Microbial communities are likely to exist as biofilms (Stach and Burns, 2002) as well as discrete planktonic units, which will have some consequences for the transformation of pesticides. The physiology of bacteria in biofilms has been shown to be considerably different from that of their planktonic counterparts (Costerton *et al.*, 1994). The intimacy of mixed microbial populations in the biofilm mode of growth (Costerton *et al.*, 1995) may allow coordination of metabolic processes to mineralize organic contaminants that no individual member of the community can achieve in isolation. A metabolically interdependent mixed population in a biofilm is likely to yield a relatively concentrated and sustained supply of nutrients (Ehrlich, 2002). Signal transduction and cell-to-cell signalling

has been suggested to occur between microbial species within biofilms (Caldwell *et al.*, 1997). Biofilm growth may also result in attenuation of inhibitory effects from toxic compounds (Cowan *et al.*, 2000).

In addition to soil microsite variation, microenvironments are known to exist in biofilms, with gradients in internal pH and redox potentials formed by differential diffusion of nutrients and metabolic products (Ehrlich, 2002). Spatial colony distribution and biofilm formation and growth are likely to be major influences on the rates of biodegradation in soil. Modelling biofilm mediated pesticide degradation kinetics may allow insights into the effects of commensal interactions on the environmental fate of these agrochemicals. The use of molecular methodologies, such as fluorescent *in situ* hybridisation (FISH) for tagging specific microbial populations (Sayler *et al.*, 1995), in conjunction with techniques such as microautoradiography may allow the distribution of the ^{14}C-labelled herbicide to be assessed relative to the proximity of microorganisms.

6.3. Pesticide monitoring in the environment

Environmental monitoring of pesticide fate concentrates, to a certain extent, on the parent compound. This is understandable, since they are inherently biocidal and can persist in the environment for considerable periods of time, even decades (Alexander, 1999). Pesticide residues are commonly found in ground water and surface water (Potter and Carpenter, 1995; Barbash *et al.*, 2001). However, during environmental screening for pesticide compounds, their associated transformation products are also frequently detected. In some cases, the concentrations of the metabolites found are greater than that of the parent compound (Potter and Carpenter, 1995). Pesticide intermediates can also be environmentally stable. Several acetochlor metabolites were seen to persist during the environmental fate study, with unidentified metabolites in the deep Iowa clay (2.7-3.0 m) layer actually still increasing in concentration at the 100 d point of the incubation.

The multitudes of metabolites that can be formed from a single pesticide (Chapter 5; Potter and Carpenter, 1995; Stamper and Tuovinen, 1998) have to be addressed to fully understand and quantify the environmental fate of a pesticide. In the case of acetochlor, the two major metabolites, ac-ESA and ac-OXA, should be directly monitored in any fate study of acetochlor. Several studies on environmental fate of pesticides in the literature fail to monitor/report metabolite formation or the extent of bound residue formation (Shaw and Burns, 1998; Mills *et al.*, 2001). Pesticide metabolites are commonly not as toxic as the parent compound, although this is not true in all cases (Belfroid *et al.*, 1998), and as

such, they may constitute a potentially serious long-term environmental risk. One of the difficulties with quantitative determination of environmental contaminants is the sensitivity of analytical techniques. Registration requirements necessitate a full ecotoxicological screen for metabolites that are produced in concentrations of 10 % and above the initial applied concentration of parent compound, but the metabolites produced that account for less than 10 % are not investigated. None of the metabolites in this study reached 10 % of the initially applied acetochlor. The route of pesticide degradation may vary between soils, according to the metabolic capacity of the microbial communities present. Although similar bands were found using TLC analysis, the metabolites produced at each depth were not fully resolved in this research; the metabolites formed in surface soil may not necessarily be the same metabolites produced in subsoil and vice versa given exposure to a different set of environmental conditions..

Methods of detection are improving all the time. Baker *et al.* (1993) found al-ESA whilst monitoring for alachlor. The TLC analysis carried out in this study could not separate certain metabolites (Appendix 1). The application of techniques such as nuclear magnetic resonance (NMR; Aga *et al.*, 1999) and solid phase extraction used in conjunction with HPLC coupled to a mass spectrometer (Ferrer *et al.*, 1999; Hostetler and Thurman, 2000) in acetochlor analysis, may lead to a more detailed examination of the full range of metabolites produced. The greater the range of techniques used to analyse for degradation products, the greater the scope for finding out the degradation pathway of a compound, which is important for pesticides like acetochlor which have not had their catabolic breakdown characterised.

6.4. Future work

6.4.1. Degradation under transport conditions

In the research presented in this thesis, static laboratory experiments were employed. This discounts the influence of transport conditions on the degradation of acetochlor. Laboratory studies need to simulate field conditions as closely as possible, including, therefore, the process of leaching needs to be addressed in degradation studies, to accurately predict environmental fate. It is widely recognised that sorption has a considerable impact on the potential for biodegradation of a compound. However, static systems encourage equilibrium sorption conditions, which would not reflect the non-equilibrium sorption interactions that tend to dominate during transport (Guo and

Wagenet, 1999). Guo and Wagenet (1999) found that the rate of alachlor degradation was increased under transport conditions compared to static incubation studies. Guo *et al.* (2000) determined that nonequilibrium sorption will initially favour degradation until desorption becomes a limiting factor in the degradation process, unless there is a lag phase that allows considerable diffusion of a chemical into sorption sites. In that scenario, degradation will be impeded by nonequilibrium sorption. The applicability of degradative parameters derived from static incubation studies to simulate degradative potential during transport and non-equilibrium sorption has not been established (Beulke and Brown, 2001). More realistic degradation parameters may be achieved by coupling degradation, sorption and leaching in column experiments, with soils subject to acetochlor over a varying time scale.

As discussed previously, the biofilm mode of growth could have profound influences on the fate of chemicals in soil. The system employed by Stach and Burns (2002) could be useful to ascertain the influence of biofilms on acetochlor degradation. Flow of an aqueous phase containing acetochlor over a biofilm may mimic degradation under transport conditions. Direct observation of the biofilm community, in conjunction with microautoradiography, could allow visualization of the fate of acetochlor within the biofilm matrix. Furthermore, the use of molecular probes to detect genes encoding for relevant metabolic reactions, such as for GST may allow analysis of a community-level response to acetochlor exposure.

6.4.2. Routes of degradation and biogeochemical cycling

The environmentally stable transformants of chloroacetanilides, such as the sulphonated derivatives, are routinely discovered in ground waters and surface waters, and often in concentrations in excess of the parent compound (Potter and Carpenter, 1995). The environmental fate of these molecules should, therefore, be assessed in greater detail than they have been so far. Other than identification of these metabolites in groundwater/surface water, information on the environmental fate of these metabolites in soil is either scant, or nonexistent. Laue *et al.* (1996) found a gram-negative bacterial isolate that could use the sulphonated derivative of metazachlor as a sole source of S for growth. Clay *et al.* (1997) found that mineralization of alachlor was inhibited by the addition of exogenous N sources. Using strategies that use acetochlor as a sole source of N, or its metabolites as sole sources of S, may be helpful in elucidating the next steps in the biodegradative sequence of these compounds.

Cometabolism is likely to be the main degradative process affecting acetochlor (Chapter 5). This could be verified by stimulating indigenous microorganisms with nutrient amendments such as simple sugars (e.g. glucose) or more complex nutrient sources such as particulate organic matter. Observation of an increase in mineralization with a concurrent increase in microbial activity would provide further evidence for this route of degradation. However, evidence for metazachlor suggests that different supplemental nutrients could have either positive or negative impacts on degradation (Clay *et al.*, 1997). It could be useful to monitor acetochlor degradation under varying nutrient regimes, to assess the effect on transformation rates, and on the metabolites formed. The addition of fertilisers during the growing season could influence acetochlor environmental fate.

In addition, different analytical techniques may be necessary to allow detection of other metabolites. The TLC system used was not specifically developed for analysis of acetochlor and its intermediates (P. Vaughan, pers. comm., 2000). Fine tuning through changes in the composition of the solvent phases, or even the use of other solvents, may improve the resolution of the analysis and allow better separation of metabolites. As previously mentioned, the use of other techniques such as solid-phase microextraction coupled with HPLC - mass spectrometry, or the use of NMR-based techniques may provide insights into potential acetochlor intermediates.

6.4.3. Sorption to soil

As shown in Chapter 5, acetochlor tends to be associated with the macroaggregate fractions and is not uniformly distributed. Macroaggregates are the least stable structural units in soil and can be disrupted by forces, such as rainfall impact (Tisdall and Oades, 1982). The erosion of macroaggregates can produce fragmentation into finer particles, with the result that these finer fractions are transported to sediments, with the attached pesticide. The peeling action on the aggregate by raindrop action, which removes the surface layer, produces fine particles that are enriched in sorbed chemicals relative to the original fractions (Ghadiri and Rose, 1991). As such, the influence of macroaggregate breakdown in this way could have serious implications for the fate of acetochlor and should be addressed, not only in terms of the potentially enhanced rate of transport to water resources but also on the availability of these residues once the finer particles are released from the macroaggregate surface. Degradation potentials for pesticide contained on these eroded particles may be different to those for pesticides associated with static soil surfaces or those in solution.

As with the statistically significant relationships found between soil properties and enzyme activities or microbial abundance (Chapter 3), the correlation analysis of soil characteristics with sorption capacity (Chapter 4), does not allow identification of the actual interactions taking place. The importance of interactions with soil components has been shown by a number of studies (Cox *et al.*, 1997a; Torrents and Jayasundera, 1997). The use of defined clay minerals and organic matter constituents such as humic acids, fulvic acids and humin could allow a better prediction of the sorption processes affecting acetochlor in soil. It would also be interesting to see how competition between acetochlor and its metabolites for sorption sites affects its degradation and leaching potential. The continual reduction of solution phase acetochlor throughout the 100 d incubation and the build-up of metabolites, especially in the deep soil is behaviour that needs to be addressed to predict the leaching potential of these metabolites.

6.5. Perspectives

In terms of environmental fate, the processes of sorption and biodegradation are inextricably linked. All attempts to model environmental fate should include components for both sorption and degradation. They also need to take into account the route of degradation. Different mathematical relationships are needed for describing growth-linked degradation compared to cometabolic degradation. It is likely both processes are occurring at once, so models may need to couple these processes for accurate simulation of biodegradative fate. This research demonstrates that active subsoil microbial communities have the capacity to transform and mineralize acetochlor, and joins the growing body of evidence that indicates subsoil processes need to be addressed to allow accurate environmental fate modelling. Further to this, the impact acetochlor has on these subsoil microbial communities, in terms of diversity and potentially more importantly, in terms of their function needs to be clarified.

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Appendix 1. Stoke's Law: determination of sedimentation time to obtain 20 μm diam. particle size range

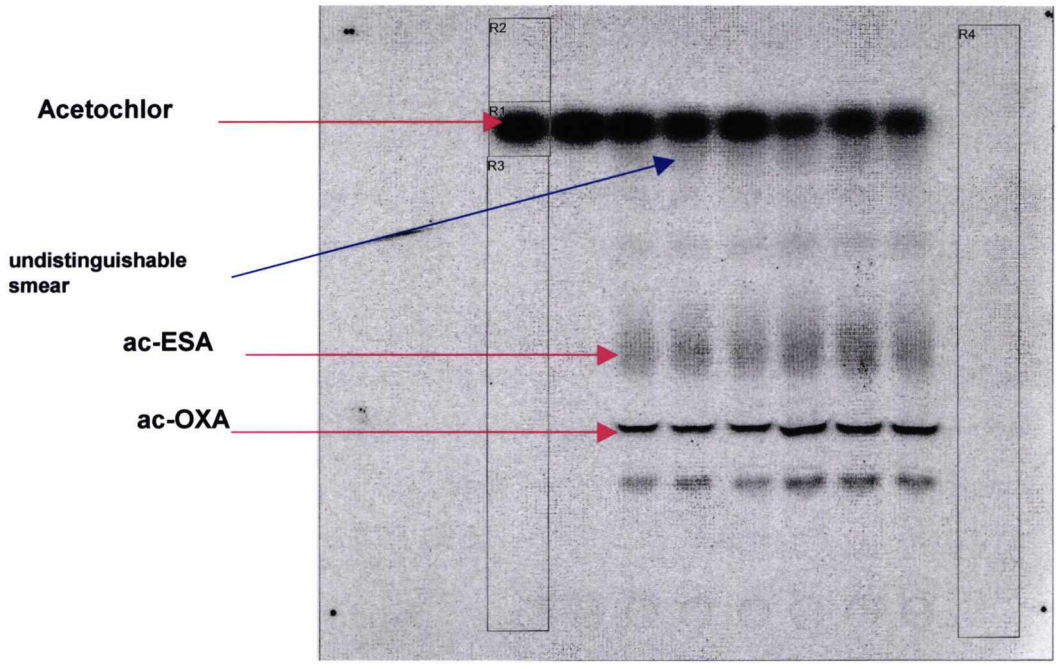
Settling velocity, V =

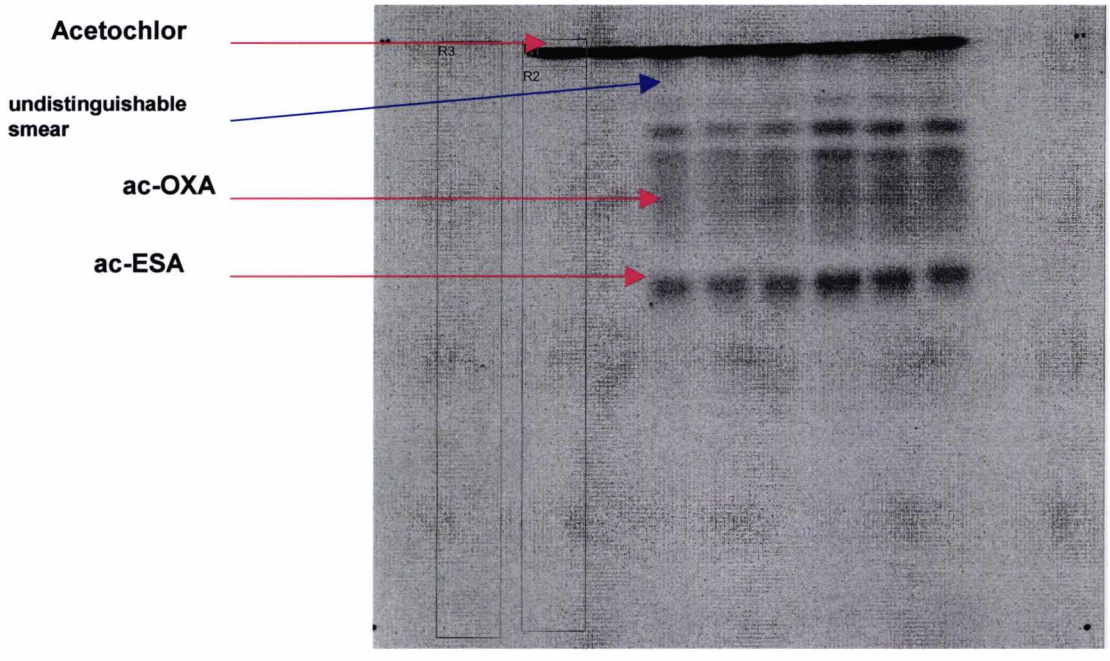
$$(2 \times g \times r^2 \times [Dp - Di]) / (9 \times \eta)$$

where
- g = acceleration due to gravity (cm/s^2)
- r = particle radius
- Dp = particle density
- Di = liquid density
- η = liquid viscosity

values:
- g = 980 cm/s^2
- Dp = 2.65 g/cm^3
- Di = 1 g/cm^3
- η =1.0019 x 10^{-3} g/cm^3

Appendix 2. Example TLC plates





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Comparison of microbial numbers and enzymatic activities in surface soils and subsoils using various techniques

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Abstract

Knowledge of microbial numbers and activity in subsoils is essential for understanding the transformation and downward movement of natural and synthetic organics. Soil cores were taken from two soil profiles (surface textures: silty clay loam and loamy sand), and samples extracted from the 0–30 cm (surface), 1.0–1.3 m (mid) and 2.7–3.0 m (deep; clay) and 3.9–4.2 m (deep; sand) layers. A variety of soil biotic (microbial numbers, microbial biomass, enzyme activities) and abiotic properties (pH, organic C, texture, CEC) were measured. Bacterial numbers decreased with depth as indicated by viable counts and by calculations based upon biomass carbon and extracted DNA. Direct microscopic counts were the most sensitive method of enumeration and gave bacterial numbers between 37 and 442× greater than colony forming units and those calculated from DNA extracted from soil. DNA extracted from soil ranged from 1.23 (sand surface) and 1.34 (clay surface) $\mu g\ g^{-1}$ d wt soil to 0.02 (sand deep) and 0.01 (clay deep) $\mu g\ g^{-1}$ d wt soil. Bacterial numbers, estimated from biomass-C measurements, were comparable to direct counts. Large numbers of bacteria were recorded in the subsoils (direct counts: 5.6×10^8 sand, 4.5×10^8 clay) even though this was equivalent to only 4.7 and 1.7% of those in the surface soils. Fungi were isolated from surface and mid-depth layers of both soils but were absent from the deep soil samples. Enzymatic activities (arylsulphatase, β-glucosidase, phosphomonoesterases, urease, dehydrogenase, FDA hydrolysis), assayed with or without buffers, also decreased with depth. The exception was urease activity in the clay soil where no difference was seen between mid and deep in non-buffered assays but a 2.9-fold greater activity was exhibited in the mid than in the surface soil when buffered. Strong positive correlations ($R > 0.95$) were observed between all enzyme activities (except with urease activity in clay soil and non-buffered phosphatase activity in sand soil) and between all methods of estimating bacterial abundance. Strong positive correlations ($R > 0.90$) were also found between bacterial abundance and enzyme activities and between enzyme activities and organic matter content.

Keywords: Surface soils; Subsoils; Microbial numbers; Soil DNA; Biomass-C; Soil enzymes

1. Introduction

Ground water chemistry is directly influenced by subsurface microbiology as a consequence of diagenesis, dissolution and precipitation reactions (Hiebert and Bennett, 1992). As a consequence, the genotypic diversity and the metabolic activity of subsoil microbes need to be better understood in order to quantify their influence on the transformation and degradation of both natural and xenobiotic compounds as they pass down through the soil profile.

The presence and activity of the microbial component of surface and subsoil can be detected and measured in many ways, including microbial numbers (Kästner et al., 1994), microbial biomass (Lovell et al., 1995), functional activity (such as respiration and N mineralisation; Murphy et al., 1998) and enzyme activities (Bandick and Dick, 1999). In addition, newer molecular approaches, including probing for specific genotypes (Sayler et al., 1995) and monitoring mRNA expression (Wilson et al., 1999) have an increasingly important part to play in advancing our understanding.

Quantitative and representative recovery of microorganisms from environmental samples is essential in understanding ecosystem function. A number of binding forces, including electrostatic and van der Waals forces, hydrogen bonding and physical entrapment (Marshall, 1976) need to be overcome in order to reduce cell–soil associations and allow extraction of cells. Chemical (anionic detergents, ion-exchange resins) and physical (shaking, blending, ultrasonication) dispersion treatments are often used but even with exhaustive multi-stage extractions, large proportions of bacterial populations remain associated with soil particles (Hopkins et al., 1991). A further cause of underestimating

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numbers is that many of the dislodged bacteria cannot be grown on conventional media (Bakken, 1997). The use of vital stains (e.g. 4′6-diamidoino-2-phenylidole; DAPI) and microscopy will show that bacteria counted as colony-forming units (CFUs) significantly underestimates the total extracted from soil. A potentially more accurate method of enumerating all the microbes contained in a soil sample is suggested by calculations based on biomass-C (Watson et al., 1977) and extracted DNA (Sandaa et al., 1998).

Enzyme activities in soil can be associated with active cells (animal, plant, microbial), entire dead cells and cell debris as well as being complexed with clay minerals and humic colloids (Burns, 1982). While the activity of many extracellular hydrolases is probably a result of enzymes associated with some or all these components, dehydrogenase assays measure intracellular catalysis and are more likely to be correlated with the activity of extant cells (Dick, 1997). As with all enzyme assays, the incubation conditions determine the rate of substrate catalysis and the design of soil enzyme measurements and the interpretation of the resulting data are controversial. For example, the use of buffers is vigorously debated (Gianfreda and Bollag, 1996) although they are mainly used to poise and maintain pH at the optimum for activity (Kandeler and Gerber, 1988). A second approach is to rely on the inherent buffering capacity of the soil itself using purified water (Gong, 1997) or to use a buffer solely to maintain the pH at the value of the bulk soil (Sinsabaugh et al., 2000). The first approach provides a measure of enzyme potential while the second will often measure activity at a sub-optimal pH producing a lower rate of substrate catalysis but one that is more likely to equate to that shown in the natural environment.

In the experiments reported here, a number of methods were evaluated to measure and compare microbial presence and activity in surface and subsurface soils. The methods included culturable bacterial and fungal counts, direct counts of total bacteria, DNA extraction and quantification, and arylsulphatase, dehydrogenase, fluorescein diacetate hydrolysis, β-glucosidase, phosphomonoesterases and urease activities.

2. Materials and methods

2.1. Soils: physical and chemical analysis

Soil cores were collected from agricultural fields at two Prospective Groundwater Study sites (Acetochlor Registration Partnership, 2001) in Iowa and Michigan, USA. The soils at both sites had undergone identical cropping practices: soils were tilled conventionally, crops (soybean and corn) were rotated annually and the sites were neither irrigated nor drained. PVC tubes (10×45 cm^2) were pushed vertically into the Iowa clay and Michigan sand soils using a hydraulic drilling rig and cores sampled using colour and texture as a guide to different horizons. The sample depths are given in Table 1. Soils were stored field moist in sealed polythene bags at 4 °C until required.

Particle size distribution was classified according to the USDA scheme; organic matter (as percentage of soil weight) was determined using the Walkley–Black oxidation method and a factor of 1.724 (Walkley and Black, 1934) was used to convert organic C to organic matter. Cation exchange capacity (meq 100 g^{-1} air dried soil) was determined by sodium saturation at pH 7.0 and flame photometry.

For the Iowa clay soil (Table 1) organic matter decreased with depth with an 85% reduction from the surface to the mid soil and a further 9% reduction from the mid to the deep soil. Organic matter was much less in the Michigan sand soil and decreased by 88% from the sand surface soil to the mid soil (Table 1). There was no further decline from the mid soil to the deep soil. Cation exchange capacity was much higher in the clay soil and decreased with depth in both soils, but the most prominent changes were seen in the sand profile with a 31% reduction of CEC from the surface to the mid soil and a further 18% decrease from the mid to the deep soil. With increasing depth, the sand soil was more alkaline increasing by 2.3 pH units from the surface to the deep soil.

2.2. Extraction and enumeration of culturable microbes

Bacteria were extracted from soil using a multi-stage dispersion and differential centrifugation technique (Hopkins et al., 1991). Ten fold dilutions of the soil suspensions were prepared in phosphate-buffered saline (Sigma, Poole, UK). The dilution series were used to inoculate two solid culture media: R2A (0.5 g yeast extract; 0.5 g proteose peptone; 0.5 g casamino acids; 0.5 g glucose; 0.5 g soluble starch; 0.3 g sodium pyruvate; 0.3 g potassium dihydrogen orthophosphate; 50 mg magnesium chloride) (Reasoner and Geldreich, 1985) and soil extract agar (SEA) (Fredrickson and Balkwill, 1998). The first functions as a copiotrophic media and the second as an oligotrophic medium containing soluble organic matter extracted from each soil sample. A separate SEA was made up for each of the six soil samples (two soils at three depths) by suspending 100 g (wet weight) of the appropriate soil (dry weight equivalents: 81.0 g (clay surface), 78.2 g (clay mid), 75.2 g (clay deep), 96.9 g (sand surface), 93.0 g (sand mid) and 97.2 g (sand deep)) in 200 ml tap water and autoclaving (121 °C and 0.2 MPa, 1 h) for three consecutive days. Solid particles were allowed to settle and the fluid decanted and centrifuged ($3500 \times g$, 10 min). The supernatant fractions were frozen, thawed and passed through filter paper (No. 4; Whatman, Wallingford, UK) and the filtrate made up to 200 ml with tap water. Filtrate (50 ml) was added to tap water (950 ml) and Technical agar (No. 3) (Oxoid Ltd, Basingstoke, UK) (15 g) and autoclaved before pouring. The cultures were incubated at 20 °C for 7 d (R2A) or 21 d (SEA) before counting.

Table 1
Chemical and physical properties of the Iowa clay and Michigan sand profiles selected for study

	Iowa clay soil			Michigan sand soil		
	Surface	Mid	Deep	Surface	Mid	Deep
Depth (m)	0–0.3	1–1.3	2.7–3.0	0–0.3	1–1.3	3.9–4.2
pH (10 mM $CaCl_2$)	5.30	6.00	5.80	5.50	6.60	7.80
Organic matter (%w/w)	2.80	0.43	0.17	0.83	0.10	0.10
Cation exchange capacity (meq 100 g^{-1})	23.40	21.70	19.50	4.50	3.10	0.60
Textural classification	Silty clay loam	Silty clay	Silty clay loam	Loamy sand	Sand	Sand

Fungi were counted using a soil-plate method (Warcup, 1950). Soil (0.5 g wet weight) was dispersed in 20 ml warm Rose-Bengal agar containing 30 μg streptomycin ml^{-1} and incubated for 7 d at 20 °C, with the plates covered by foil to minimise inhibitory effects on fungal growth from photo-oxidation of the dye (Chilvers et al., 1999) and sporulation. Three plates of each culture medium were inoculated per dilution.

2.3. Direct enumeration by epifluorescence microscopy

After aliquots were removed for viable counts, 50 ml portions of each diluted soil extract were passed through 8.0 (Millipore, Bedford, UK) and 3.0 μm membrane filters (Whatman, Wallingford, UK). Aliquots (1.9 ml) were removed from the filtrate, mixed with 0.1 ml formalin and DAPI (Sigma, Poole, UK) added to give a final concentration of 5.0 μg ml^{-1}. Samples were incubated in the dark for 10 min at 20 ± 2 °C. A 250 μl aliquot was taken and added to 8.0 ml of filter-sterilised 100 mM NaCl. Each sample was then filtered through a gridded, blackened polycarbonate membrane filter (25 mm diam., 0.45 μm; Millipore, Bedford, UK). These filters were washed with 20 ml of filtered 100 mM sodium citrate buffer (three cycles: pH 6.6, pH 5.5 and pH 4.0) (Sigma, Poole, UK) and once with distilled water. Bacteria were then viewed using an epifluorescence microscope (Model DMRB, Leica Microsystems, Wetzlar, Germany). Every square was counted and the mean calculated. The numbers were expressed as cells g^{-1} dry weight soil.

2.4. Enumeration by extraction and quantification of soil DNA

DNA was extracted from samples using the Ultraclean Soil DNA Isolation kit (Mo Bio Laboratories, Inc., CA, USA). Soils (0.5 g surface and mid, 1.0 g deep) were mixed in a 2 ml microfuge tube containing lysis buffer and glass beads, and the extraction performed (as described by the manufacturer) by heating the samples in a waterbath at 70 °C for 10 min. The DNA concentration was measured with an LS50B fluorescence spectrophotometer (Perkin Elmer, Inc., Seer Green, UK) using PicoGreen dye (Sandaa et al., 1998) according to the manufacturer's instructions (Molecular Probes, Inc., Eugene, OR). A DNA standard curve over a concentration range of 0–1000 ng ml^{-1}, was prepared with bacteriophage lambda DNA provided with the PicoGreen kit. A figure of 8.4 fg DNA bacterial $cell^{-1}$ (Torsvik et al., 1990) was used to calculate bacterial numbers.

2.5. Biomass-C and enumeration

Microbial biomass was determined by substrate-induced respiration (Anderson and Domsch, 1978) using an infra-red gas analyser. A conversion factor of 80.8 fg C bacterial $cell^{-1}$ was used to calculate bacterial numbers. This is an average of the figures produced by Watson et al. (1977), Kroer (1994), and Moser et al. (1996) (103.75, 56.45, 82.0 fg C $cell^{-1}$, respectively) assuming a C-to-cell dry weight ratio of 0.5.

2.6. Enzyme assays

2.6.1. Arylsulphatase, β-glucosidase and phosphomonoesterase activity

Arylsulphatase (EC 3.1.6.1), β-glucosidase (EC 3.2.1.21) and phosphomonoesterase (EC 3.1.3) assays were all based on *p*-nitrophenol release after cleavage of a synthetic substrate (*p*-nitrophenyl sulphate, *p*-nitrophenyl glucoside and *p*-nitrophenyl phosphate, respectively). For the arylsulphatase assay (adapted from Tabatabai and Bremner, 1970) 1 g soil (wet weight) was mixed with 4 ml 500 mM acetate buffer (pH 5.8) and 1 ml substrate (25 mM). Controls contained 4 ml acetate buffer and 1 ml sterile distilled water. The soils were vortexed briefly and then incubated (20 °C, 200 rev min^{-1}) on an orbital shaker (Gallenkamp, Loughborough, UK) for 2 h. Then, 1 ml sterile distilled water was added to the samples and 1 ml of substrate to the controls, before terminating the reaction with the addition of 1 ml 500 mM $CaCl_2$ and 4 ml 500 mM NaOH. The suspensions were shaken on an orbital shaker (20 °C, 200 rev min^{-1}) for 30 min. Aliquots (1.5 ml) were centrifuged (9464 × *g*, 5 min) and the colour intensity of extracted *p*-nitrophenol measured at 400 nm (UNICAM 562 UV/VIS spectrophotometer). The acetate buffer was replaced by distilled water for assays conducted at the natural pH of the soil. A standard curve was plotted using a range of *p*-nitrophenol (Sigma, Poole, UK) concentrations between 0 and 50 μg ml^{-1} distilled water.

Table 2
Microbial abundance measurements and their relationship to direct counts of bacteria. DC = direct count; ND = not determined; [1]calculated using a factor of 80.8 fg C cell^{-1}; [2]DNA concentration multiplied by factor of 8.4 fg DNA cell^{-1}. For each soil letters in rows refer to significant differences ($P < 0.05$) with depth

Microbial abundance measure	Iowa clay			Michigan sand		
	Surface (0.0–0.3 m)	Mid (1.0–1.3 m)	Deep (2.7–3.0 m)	Surface (0.0–0.3 m)	Mid (1.0–1.3)	Deep (3.9–4.2)
Direct counts (DC) of bacteria (cells × 10^7 g^{-1} d wt soil)	2740 ± 1.44[a]	163 ± 5.44[b]	45 ± 0.1[c]	1170 ± 5.47[a]	50 ± 1.05[b]	56 ± 0.01[b]
Culturable bacteria on R2A (CFUs × 10^7 g^{-1} d wt soil)	10.70 ± 1.04[a]	1.86 ± 0.29[b]	1.21 ± 0.13[c]	4.47 ± 0.27[a]	0.62 ± 0.04[b]	0.26 ± 0.04[c]
Culturable bacteria on R2A as % DC	0.39	1.1	2.7	0.38	1.3	0.46
Culturable bacteria on SEA (CFUs × 10^7 g^{-1} d wt soil)	6.19 ± 0.30[a]	1.93 ± 0.12[b]	0.96 ± 0.12[c]	4.25 ± 0.15[a]	0.40 ± 0.03[b]	0.25 ± 0.01[c]
Culturable bacteria on SEA as % DC	0.2	1.18	2.14	0.36	0.8	0.44
Culturable fungi (CFUs × 10^4 g^{-1} d wt soil^{-1})	10.30 ± 1.2[a]	1.55 ± 0.73[b]	0.00 ± 0[c]	16.00 ± 2.6[a]	2.00 ± 0.24[b]	0.00 ± 0[c]
Biomass (mg-C 100 g soil^{-1})	11.63	7.78	7.28	7.77	2.36	ND
Bacterial numbers: biomass basis[1] (cells × 10^7 g^{-1} d wt soil)	144	96.2	90.1	96.1	29.2	ND
Bacterial numbers from biomass as % DC	5.26	59.02	200.22	8.21	58.4	
DNA concentration (μg g^{-1} d wt soil)	1.34 ± 0.38[a]	0.13 ± 0.07[b]	0.01 ± 0.00[b]	1.23 ± 0.10[a]	0.06 ± 0.03[b]	0.02 ± 0.01[b]
Bacterial numbers: DNA basis[2] (cells × 10^7 g^{-1} d wt soil)	16.00	1.55	0.12	14.6	0.71	0.24
Bacterial numbers from DNA as % DC	0.58	0.95	0.27	1.25	1.44	0.42

β-Glucosidase (Tabatabai, 1982) and phosphomonoesterase (Eivazi and Tabatabai, 1977) assays differed from the above only in the choice of buffer (β-glucosidase: modified universal buffer (pH 6.0); phosphomonoesterase: modified universal buffer (pH 4.0 and 9.0)). The pH values for the buffer used were chosen to optimise the activity of acid- and alkaline-phosphomonoesterases (4.0–6.5 and 9.0–10.0 as pH optima, respectively) (Speir and Ross, 1978). The substrate concentration for the two phosphomonoesterase assays was 15 mM. The extracting solvent used in the β-glucosidase assay was Tris buffer (pH 12.0) and the substrate concentration was 25 mM.

2.6.2. Urease activity

A modified assay for urease (EC 3.5.1.5) activity based on that of Kandeler and Gerber (1988) was used. Soil (5 g wet weight) was mixed with 2.5 ml urea (80 mM) and 20 ml 75 mM borate buffer (pH 10.0). The mixture was reacted for 4 h in an orbital shaker (20 °C, 200 rev min^{-1}). Controls were prepared by addition of 2.5 ml sterile distilled water and 20 ml borate buffer. After 4 h, 2.5 ml sterile distilled water was added to the treatment and 2.5 ml urea to the controls, before extraction with 30 ml acidified 2 M KCl (Naseby and Lynch, 1997). The suspensions were shaken on an orbital shaker (20 °C, 200 rev min^{-1}) for 30 min. Aliquots (1.5 ml) were centrifuged (9464 × *g*, 5 min) and 1 ml of the supernatant fraction was mixed with 9 ml distilled water, 5 ml sodium salicylate/NaOH solution and 2 ml dichloroisocyanuric acid (Na^+ salt). The colour intensity of the solution, after standing at 20 ± 2 °C for 1 h, was measured at 690 nm using a UNICAM 5625 UV/VIS spectrophotometer. The borate buffer was replaced by distilled water for the natural soil pH assay. Ammonium concentrations were determined using a calibration curve of ammonium chloride standard solutions from 0 to 2.5 μg ml^{-1}.

2.6.3. Dehydrogenase activity

INT (2 (*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride) reductase activity (i.e. dehydrogenase activity) was determined according to Von Mersi and Schinner (1991). Briefly, 1 g (wet weight) soil was placed in foil-wrapped universal bottles and mixed with 1.5 ml 1 M Tris buffer pH 7.0 and 2 ml INT (5 mg ml^{-1} in 2% v/v *N,N*-dimethylformamide). The control soils received 1.5 ml Tris buffer and 2 ml distilled water. The samples were incubated in an orbital shaker (20 °C, 200 rev min^{-1}) for 24 h. Then, 2 ml distilled water were added to the sample soils and 2 ml INT added to the control soils. The reaction was stopped by adding 10 ml *N,N*-dimethylformamide/ethanol (1:1 ratio) extractant and shaking (20 °C, 200 rev min^{-1}) for 1 h. Aliquots (1.5 ml) were removed and centrifuged (9464 × *g*, 5 min) and the absorbance of the supernatants measured at 464 nm. The Tris buffer was replaced by distilled water for the natural soil pH assay. A

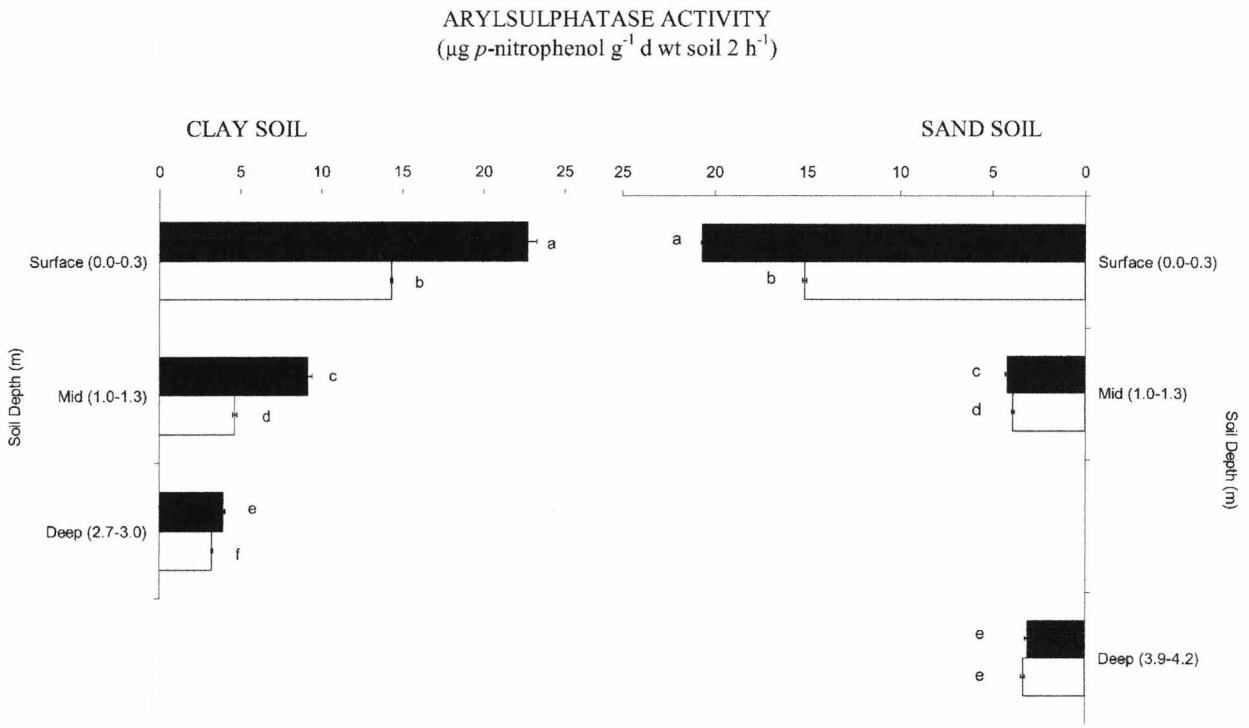

Fig. 1. Changes in buffered □ and non-buffered ■ arylsulphatase activity with soil depth in an Iowa clay and a Michigan sand soil (all bars represent mean ± 1 s.e.). For each soil, different letters alongside columns refer to significant differences ($P < 0.05$) between buffered and non-buffered activities and with depth.

standard curve was obtained using INTF (iodonitrotetrazolium chloride) (Sigma, Poole, UK) at a concentration range of 0–27 μg ml^{-1} extractant.

2.6.4. *Fluorescein diacetate hydrolysis*

FDA hydrolysis was measured according to the method of Schnürer and Rosswall (1982). Soil (3 g wet weight) was suspended in 50 ml phosphate-buffered saline and 250 μl FDA (Sigma, Poole, UK) (2 mg ml^{-1} in acetone) added. Controls contained 250 μl of distilled water. The soil suspensions were incubated (20 °C, 200 rev min^{-1}) for 4 h. After incubation, 250 μl of distilled water were added to the samples and 250 μl of FDA added to the controls. The suspensions were vortexed and the reaction terminated by removing 5 ml subsamples and placing these into test tubes containing 5 ml acetone. Aliquots (1.5 ml) were centrifuged (9464 × *g*, 5 min) and the optical density of the supernatant was measured at 490 nm. Values for FDA hydrolysis were obtained using a calibration curve relating optical density and fluorescein concentration (ranging from 0 to 10 μg ml^{-1}).

2.7. *Statistical analysis*

Minitab 12 and Microsoft Excel 97 were used in the statistical processing of the data (Student's *t*-test, correlation analysis and ANOVA).

3. Results

3.1. *Microbial numbers*

For both soils, independent of growth media, the numbers of culturable bacteria declined with depth (Table 2). Comparable numbers were obtained for R2A media after a 7 d incubation and the SEA after 21 d incubation. The exception was the clay surface soil in which bacterial numbers on R2A were significantly greater ($P < 0.01$) than on SEA. Fungal counts were significantly greater ($P < 0.01$) in the surface than in the mid soil samples, but were not detected in either of the deep soils (Table 2). Direct counts were much larger but these also declined with depth for the clay soil ($P < 0.01$) but there was no difference between the mid and deep sand soils.

The amount of DNA extracted from soil (and calculations of bacterial numbers based on the data) (Table 2) suggested the numbers of bacteria (and all microorganisms) were significantly reduced from the surface to the mid samples ($P < 0.01$) in both soils. However, there was no significant difference ($P < 0.05$) between the mid and deep soil samples in either soil.

Biomass-C contents (and therefore calculated microbial numbers) decreased with depth for both profiles (Table 2). The clay mid and deep soils contained 33 and 37.4% less biomass, respectively, than that in the surface soil. In the sand profile there was a 70% reduction in biomass from the surface to the mid soil. The bacterial numbers indicated by

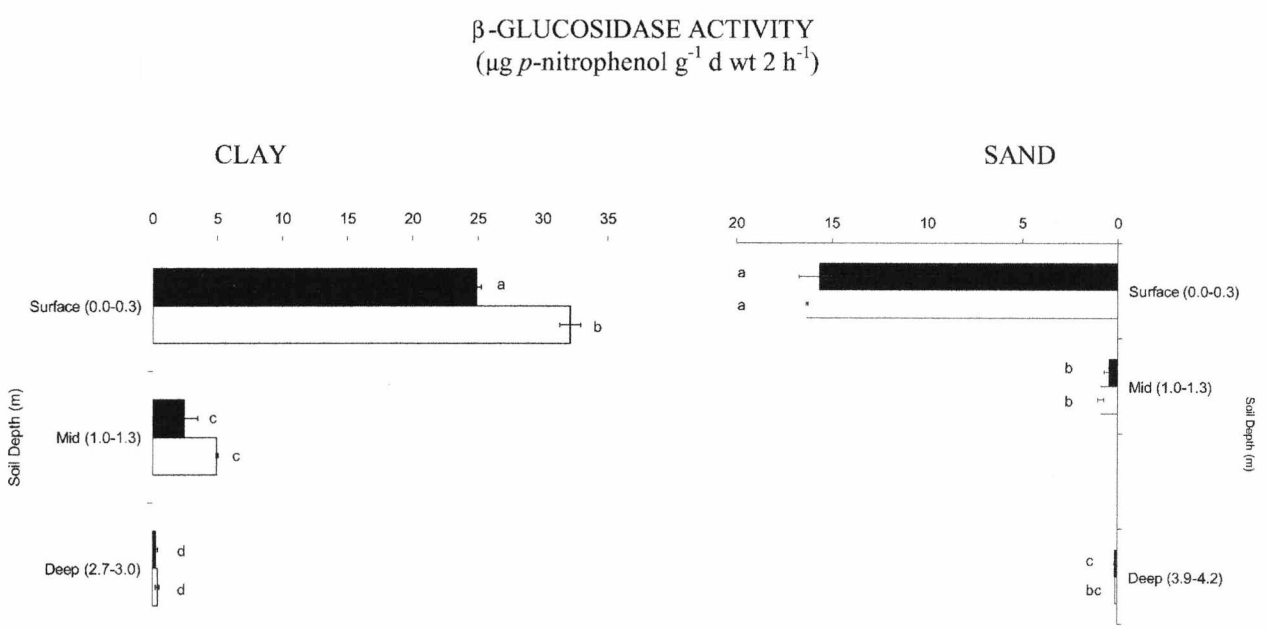

Fig. 2. Changes in buffered □ and non-buffered ■ β-glucosidase activity with soil depth in an Iowa clay and a Michigan sand soil (all bars represent mean ± 1 s.e.). For each soil, different letters alongside columns refer to significant differences ($P < 0.05$) between buffered and non-buffered activities and with depth.

this method increased as a percentage of those counted by direct counts, with depth (Table 2). In the clay deep soil, the numbers estimated were approximately 200% greater than those determined by microscopic analysis.

3.2. Enzyme activities

3.2.1. Arylsulphatase activity

Arylsulphatase activity (Fig. 1) decreased with depth ($P < 0.01$) in both soils in both buffered and non-buffered assays. The buffered assay gave significantly ($P < 0.05$) higher rates of arylsulphatase activity compared to the non-buffered treatment at all depths in both soils, with the exception of the sand deep soil, where the presence of buffer made no difference to the activity. Increases in activity in presence of buffer, in comparison to the non-buffered assays were greatest in the clay soil (surface +58.4%, mid +96.1%, deep +21.2%).

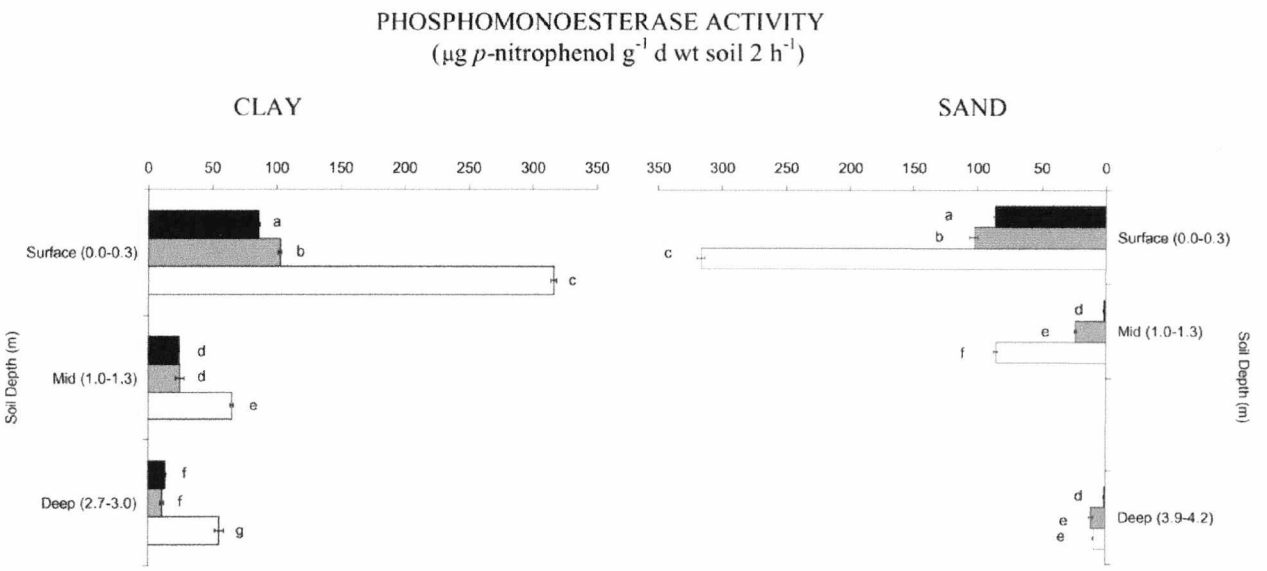

Fig. 3. Changes in buffered pH 4.0 ■, pH 9.0 ▨ and non-buffered □ phosphomonoesterase activity with soil depth in an Iowa clay and a Michigan sand soil (all bars represent mean ± 1 s.e.). For each soil, different letters alongside columns refer to significant differences ($P < 0.05$) between buffered and non-buffered activities and with depth.

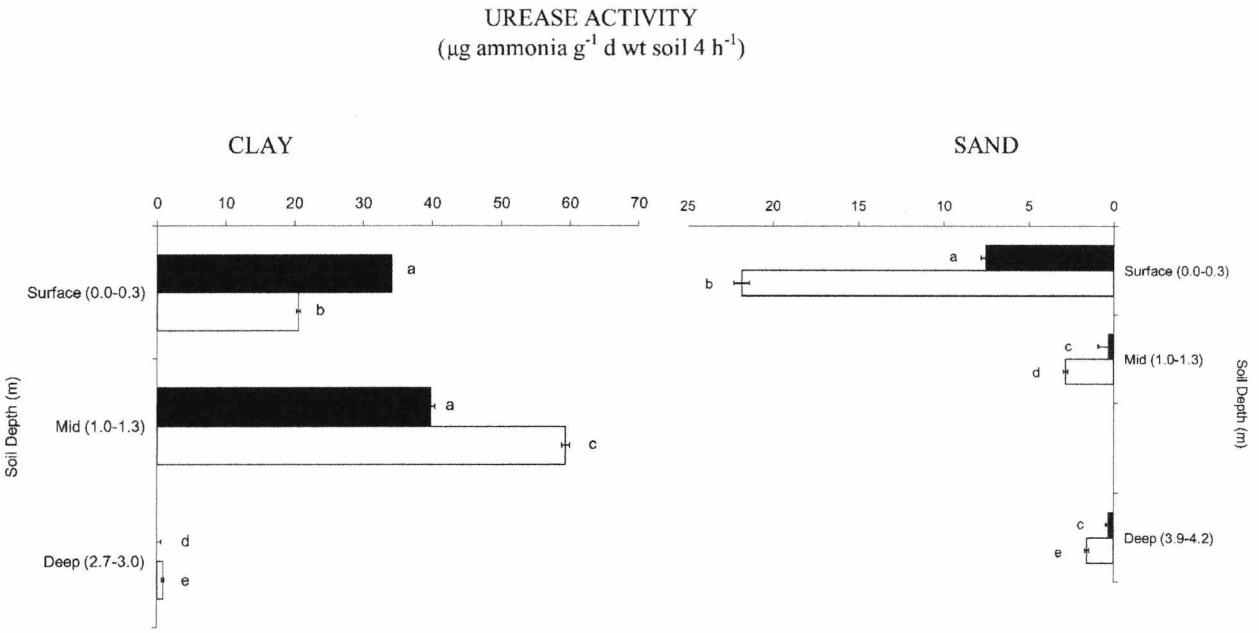

Fig. 4. Changes in buffered □ and non-buffered ■ urease activity with soil depth in an Iowa clay and a Michigan sand soil (all bars represent mean ± 1 s.e.). For each soil, different letters alongside columns refer to significant differences ($P < 0.05$) between buffered and non-buffered activities and with depth.

3.2.2. β-Glucosidase activity

In non-buffered assays, β-glucosidase activity decreased significantly with soil depth, in both soils ($P < 0.01$) (Fig. 2). In comparison, when a buffer was used there was significantly greater activity in both surface soils compared to the mid and deep soils but no difference in activity between the mid and deep soil samples. Using a buffer actually decreased activity in the clay surface and mid soils by 22.4 and 50.6%, respectively.

3.2.3. Phosphomonoesterase activity

Under both buffered (pH 4.0) and non-buffered conditions, phosphomonoesterase activity (Fig. 3) declined with soil depth ($P < 0.01$). At pH 9.0, although there was a significant reduction in activity ($P < 0.01$) with depth in the clay soil and between the sand surface soil and the mid soil there was no difference between sand mid and sand deep soil. For both soils, phosphomonoesterase activities declined with depth, with non-buffered assays having

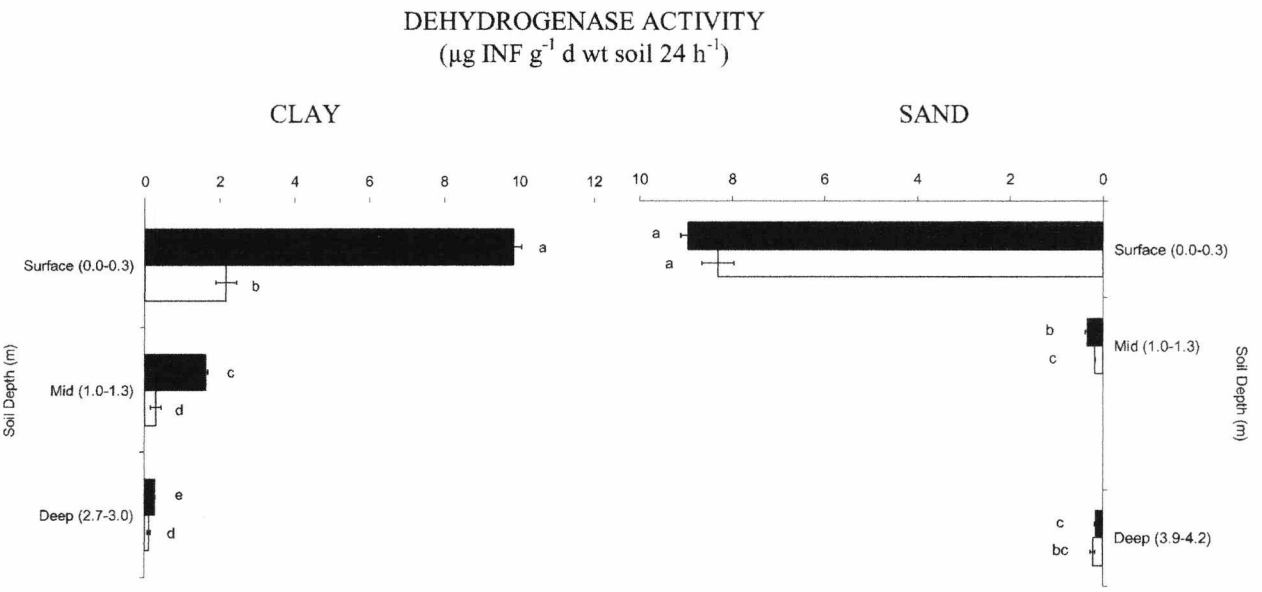

Fig. 5. Changes in buffered □ and non-buffered ■ dehydrogenase activity with soil depth in an Iowa clay and a Michigan sand soil (all bars represent mean ± 1 s.e.). For each soil, different letters alongside columns refer to significant differences ($P < 0.05$) between buffered and non-buffered activities and with depth.

Fig. 6. Changes in buffered fluorescein diacetate hydrolysis with soil depth in an Iowa clay and a Michigan sand soil (all bars represent mean ± 1 s.e.). For each soil, different letters alongside columns refer to significant differences ($P < 0.05$) between buffered and non-buffered activities and with depth.

greater activities than both buffered (pH 4.0, 9.0) assays. For all cases (except pH 4.0 sand deep), the buffer reduced measured activity significantly ($P < 0.01$).

3.2.4. Urease activity

For the sand soil, urease activity decreased down the profile using buffered and non-buffered assays (Fig. 4). Non-buffered soils always showed greater activity and were 2.9, 8.9 and 4.7-fold higher for the surface, mid and deep soils, respectively. Under buffered conditions, in the clay soil (Fig. 4), there was no significant difference in urease activity between the surface and the mid soil and there was no urease measured in the deep soil. In the absence of buffer significantly greater activity ($P < 0.01$) in the mid soil was found than in the surface soil. Urease activity, although significantly lower ($P < 0.01$) than in either the surface or mid soils, was also detected in the deep soil.

3.2.5. Dehydrogenase activity

In buffered assays, dehydrogenase activity (Fig. 5) decreased with depth in both soils ($P < 0.05$). In non-buffered assays, significantly greater activities were measured in the surface soils compared to the mid soils (clay 7.3-fold decrease, sand 49.9-fold decrease) but there was no difference between mid and deep soils. Buffering in the sand profile increased the activity only in the mid soil and had no effect in the other soils. For the clay soil significantly higher activities ($P < 0.05$) at all depths were measured when using the buffer (surface 4.5-fold, mid 5.5-fold, deep 2.4-fold).

3.2.6. FDA hydrolysis

FDA hydrolysis was only measured under buffered conditions due to chemical hydrolysis occurring outside the pH 7.0–8.0 range (Alef et al., 1995. FDA (Fig. 6) rapidly and significantly ($P < 0.01$) decreased with soil depth for both soils. Mid soil activities were only 10 (clay) and 2.2% (sand) of the surface soils.

4. Discussion

4.1. Viable and direct counts

The combination of physical and chemical processes employed in the dispersion technique disrupts soil particles and releases into the aqueous phase a proportion of the bacteria that are attached to clays and humates or trapped within aggregates (Hopkins et al., 1991). The capacity of some species to adhere to soil particles more strongly than others (Mehmannavaz et al., 2001) will affect their ease of extraction. However, even if large and representative numbers are extracted, their culturability, and therefore their enumeration, will be determined by the nutritional and growth requirements of the individual species. Use of a number of different selective media, including those targeted at identifying C substrate utilisation patterns, should provide conditions favourable for the growth of a high proportion of those bacteria extracted (Balestra and Misaghi, 1997). Nonetheless, even if a range of growth media are used, the so-called viable but non-culturable (VBNC) bacteria will not be detected (Bakken, 1997) and these may need a treatment to resuscitate them prior to

extraction (McDougald et al., 1998). Only two media were used in our experiments: one copiotrophic (R2A), the other (SEA) a low nutrient minimal salts medium containing only the soluble organics found in the soils under study (Fredrickson and Balkwill, 1998). The CFUs counted after using R2A were similar in numbers to those using SEA, although it took 21 d to produce visible colonies on SEA in comparison to <7 d on R2A. Of course, in the absence of identification, comparable counts may conceal major differences in species composition. In the work reported here, almost 90% of the colonies isolated on SEA could be cultured on the nutrient-rich R2A (data not shown).

Direct counts, using epifluorescence microscopy, proved to be the most sensitive method for determining bacterial cell numbers in soil extracts and, on this basis, CFUs accounted for <3% of the total. Even if we assume that the growth media supported the development of different species, culturable bacteria are still <6% of the direct count. However, direct counting also suffers from the same problems encountered in viable cell counts: the numbers recorded are determined by the extraction efficiency of bacteria from soil. In addition, this technique does not differentiate between living and dead cells and so overestimations can also occur (Bloem et al., 1995).

4.2. DNA extraction and microbial biomass

Another approach to assess microbial abundance is to extract DNA from soil, either directly or indirectly. Indirect extraction involves washing bacterial cells from soil prior to lysis and extraction of genomic DNA. This technique benefits by providing high purity bacterial intracellular DNA but is again limited by the number of cells extracted into the supernatant fraction. In the direct method, used in this study, microbial cells are lysed in situ. The technique allows rapid extraction and processing of the total DNA and eliminates cell extraction bias. Sandaa et al. (1998) compared extraction procedures and showed that the amount of DNA extracted by the direct method (26.2 $\mu g\ g^{-1}$ soil) was greater than using indirect methods (6.4 $\mu g\ g^{-1}$ soil). It has also been shown that different direct extraction procedures will result in different DNA yields (Stach et al., 2001).

Extracted DNA was quantified using PicoGreen fluorescent dye, which permits the measurement of DNA in pg quantities, without interference from co-extracted humic matter (Sandaa et al., 1998). The cellular DNA content of soil bacteria has been reported as 8.4 fg $cell^{-1}$ (Torsvik and Goksøyr, 1978), 8.8–11.5 fg DNA $cell^{-1}$ (Sandaa et al., 1998) and 9 fg DNA $cell^{-1}$ (Holben, 1997). We used the figure of 8.4 fg DNA $cell^{-1}$ to estimate bacterial numbers (Table 2) although even this might be above the average. Dwarf cells, which may account for the majority of non-culturable cells (Lindahl et al., 1997), have been reported to contain approximately 2 fg DNA $cell^{-1}$ (Bakken and Olsen, 1989). Dwarf cells are also extremely resistant to bead-mill homogenisation (Bakken, 1997).

There are a number of other possible inaccuracies using soil DNA to calculate bacterial populations that may result in overestimates or underestimates. Microbial numbers may be overestimated because DNA will be extracted, not only from living cells but also from extracellular sources (Steffan et al., 1988), including that bound to clays (Lorenz and Wackermagel, 1987). RNA, which is degraded rapidly in soils once released from cells, would better reflect the extant bacterial community. On the other hand, the capacity of clays and organic matter to rapidly bind DNA (Ogram et al., 1988; Pietramellara et al., 1997) may actually reduce the recovery of released DNA from lysed cells during the extraction procedure (Holben, 1997), thereby giving underestimates. The high clay content of the Iowa soils may have influenced the yield of DNA. This potential source of error may be overcome by blocking the DNA-binding sites prior to cell lysis and Frostegård et al. (1999) attempted to saturate the adsorption sites by treating soils with RNA, before adding lambda phage DNA. Although they reported no positive effect of RNA treatment on DNA recovery in sandy loam soils, a decrease in the adsorption of DNA in clay soils was measured. Adding excess phosphate and altering soil pH may also help in blocking DNA adsorption sites (Holben, 1997). Further investigations into the extraction efficiency of the commercial kit also showed that a small amount of DNA remained associated with binding columns following extraction from soil (data not shown). The limited provision of reagents in the kit meant that it was not possible to elute all DNA bound and this would have contributed to the underestimates.

Any DNA extracted from soil may also be derived from other sources such as plant debris, fungi, algae and protozoa, although Harris (1994) considered that the contribution to total DNA content of soils from these sources may be less than 0.1 $\mu g\ g^{-1}$. Fungal hyphae are widespread throughout soils and may contribute substantially to the total DNA extracted by direct lysis (Sandaa et al., 1998). However, relating an amount of fungal DNA to total numbers or biomass is difficult because of the greater variability in DNA content of fungal populations compared to bacterial. In a soil containing 200 m hyphae g^{-1}, Harris (1994) found that the DNA content of the isolated hyphae was approximately 1 $\mu g\ g^{-1}$. However, the extraction procedure used was much more harsh than that usually applied to soil, suggesting that fungal hyphal DNA is not usually extracted with bacterial DNA. No fungi were recorded in either clay or sand deep soils, but in the surface and mid soils where populations of up to 1.6×10^5 fungal CFUs were recorded (Table 2), even if DNA were extracted a very small proportion of the total DNA would be attributed to fungi. We used fungal-specific 5.8S (Clapp, 1999) and ITS4 primers (White et al., 1990), but failed to show the presence of fungi in any of the soils (data not shown). A more accurate method of estimating bacterial numbers from soil DNA would be to quantify universal bacteria-specific sequences by either competitive PCR (Phillips et al., 2000) or dot-blot

Table 3
Correlations ($P < 0.05$) between buffered and non-buffered enzyme activities and physical, chemical and biological properties of the clay and sand soil profiles. Values in bold are above 0.90 or below −0.90. Values in italic are negative values. (a) Clay soil under buffered conditions, (b) clay soil under non-buffered conditions, (c) sand soil under buffered conditions, (d) sand soil under non-buffered conditions

Soil properties	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
(a) Clay soil under buffered conditions																			
Soil depth (**A**)	**1.000**																		
pH (**B**)	0.621	**1.000**																	
Organic matter (**C**)	*−0.863*	***−0.932***	**1.000**																
Cation exchange capacity (**D**)	***−1.000***	*−0.638*	0.874	**1.000**															
Sand (**E**)	0.724	*−0.091*	*−0.276*	*−0.709*	**1.000**														
Silt (**F**)	*−0.032*	*−0.803*	0.532	0.054	0.666	**1.000**													
Clay (**G**)	*−0.193*	0.649	*−0.329*	0.172	*−0.817*	***−0.975***	**1.000**												
Biomass (**H**)	*−0.871*	***−0.926***	**1.000**	0.881	*−0.291*	0.519	*−0.314*	**1.000**											
Direct counts (**I**)	*−0.836*	***−0.949***	**0.999**	0.848	*−0.227*	0.575	*−0.377*	**0.998**	**1.000**										
DNA (**J**)	*−0.859*	***−0.935***	**1.000**	0.870	*−0.268*	0.539	*−0.337*	**1.000**	**0.999**	**1.000**									
CFUs (R2A) (**K**)	*−0.848*	***−0.942***	**1.000**	0.860	*−0.249*	0.556	*−0.356*	**0.999**	**1.000**	**1.000**	**1.000**								
CFUs (SEA) (**L**)	***−0.903***	*−0.898*	**0.996**	**0.912**	*−0.357*	0.458	*−0.247*	**0.998**	**0.991**	**0.996**	**0.994**	**1.000**							
Arylsulphatase (pH 5.8) (**M**)	***−0.940***	*−0.851*	**0.983**	**0.948**	*−0.446*	0.370	*−0.152*	**0.986**	**0.973**	**0.982**	**0.978**	**0.995**	**1.000**						
Dehydrogenase (pH 7.0) (**N**)	*−0.883*	***−0.916***	**0.999**	0.893	*−0.316*	0.497	*−0.289*	**1.000**	**0.996**	**0.999**	**0.998**	**0.999**	**0.990**	**1.000**					
FDA (pH 7.6) (**O**)	*−0.860*	***−0.934***	**1.000**	0.871	*−0.271*	0.537	*−0.334*	**1.000**	**0.999**	**1.000**	**1.000**	**0.996**	**0.982**	**0.999**	**1.000**				
Glucosidase (pH 6.0) (**P**)	*−0.858*	***−0.936***	**1.000**	0.869	*−0.267*	0.541	*−0.338*	**1.000**	**0.999**	**1.000**	**1.000**	**0.995**	**0.981**	**0.999**	**1.000**	**1.000**			
Phosphatase (alkali) (pH 9.0) (**Q**)	*−0.883*	***−0.916***	**0.999**	0.894	*−0.316*	0.496	*−0.289*	**1.000**	**0.996**	**0.999**	**0.998**	**0.999**	**0.990**	**1.000**	**0.999**	**0.999**	**1.000**		
Phosphatase (acid) (pH 4.0) (**R**)	***−0.908***	*−0.892*	**0.995**	**0.917**	*−0.369*	0.447	*−0.235*	**0.997**	**0.989**	**0.994**	**0.992**	**1.000**	**0.996**	**0.998**	**0.995**	**0.994**	**0.998**	**1.000**	
Urease (pH 10.0) (**S**)	*−0.846*	*−0.108*	0.461	0.834	***−0.980***	*−0.506*	0.686	0.475	0.415	0.454	0.436	0.535	0.615	0.498	0.456	0.452	0.498	0.546	**1.000**
(b) Clay soil under non-buffered conditions																			
Soil depth (**A**)	**1.000**																		
pH (**B**)	0.621	**1.000**																	
Organic matter (**C**)	*−0.863*	***−0.932***	**1.000**																
Cation exchange capacity (**D**)	***−1.000***	*−0.638*	0.874	**1.000**															
Sand (**E**)	0.724	*−0.091*	*−0.276*	*−0.709*	***1.000***														
Silt (**F**)	*−0.032*	*−0.803*	0.532	0.054	*0.666*	**1.000**													
Clay (**G**)	*−0.193*	0.649	*−0.329*	0.172	*−0.817*	***−0.975***	**1.000**												
Biomass (**H**)	*−0.871*	***−0.926***	**1.000**	0.881	*−0.291*	0.519	*−0.314*	**1.000**											
Direct counts (**I**)	*−0.836*	***−0.949***	**0.999**	0.848	*−0.227*	0.575	*−0.377*	**0.998**	**1.000**										
DNA (**J**)	*−0.859*	***−0.935***	**1.000**	0.870	*−0.268*	0.539	*−0.337*	**1.000**	**0.999**	**1.000**									
CFUs (R2A) (**K**)	*−0.848*	***−0.942***	**1.000**	0.860	*−0.249*	0.556	*−0.356*	**0.999**	**1.000**	**1.000**	**1.000**								
CFUs (SEA) (**L**)	***−0.903***	−0.898	**0.996**	**0.912**	*−0.357*	0.458	*−0.247*	**0.998**	**0.991**	**0.996**	**0.994**	**1.000**							
Arylsulphatase (**M**)	*−0.877*	***−0.921***	**1.000**	0.887	*−0.303*	0.508	*−0.302*	**1.000**	**0.997**	**0.999**	**0.998**	**0.998**	**1.000**						
Dehydrogenase (**N**)	*−0.860*	***−0.934***	**1.000**	0.871	*−0.270*	0.538	*−0.335*	**1.000**	**0.999**	**1.000**	**1.000**	**0.996**	**0.999**	**1.000**					
Glucosidase (**O**)	*−0.884*	***−0.915***	**0.999**	0.894	*−0.318*	0.495	*−0.288*	**1.000**	**0.996**	**0.999**	**0.997**	**0.999**	**1.000**	**0.999**	**1.000**				
Phosphatase (**P**)	*−0.833*	***−0.951***	**0.998**	0.845	*−0.221*	0.580	*−0.383*	**0.997**	**1.000**	**0.999**	**1.000**	**0.990**	**0.996**	**0.999**	**0.995**	**1.000**			
Urease (**Q**)	*−0.420*	0.450	*−0.096*	0.400	***−0.930***	*−0.894*	**0.972**	*−0.081*	*−0.147*	*−0.104*	*−0.124*	*−0.011*	*−0.068*	*−0.102*	*−0.053*	*−0.153*	**1.000**		

Table 3 (*continued*)

Soil properties	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
(*c*) *Sand soil under buffered conditions*[~]																			
Soil depth (**A**)	**1.000**																		
pH (**B**)	**0.969**	**1.000**																	
Organic matter (**C**)	*−0.698*	*−0.853*	**1.000**																
Cation exchange capacity (**D**)	***−0.994***	***−0.991***	0.774	**1.000**															
Sand (**E**)	0.860	**0.960**	***−0.966***	***−0.912***	**1.000**														
Silt (**F**)	*−0.801*	***−0.924***	**0.988**	0.863	***−0.994***	**1.000**													
Clay (**G**)	***−0.963***	***−1.000***	0.866	**0.987**	***−0.966***	**0.933**	**1.000**												
Biomass (**H**)	*−0.879*	***−0.969***	**0.955**	**0.927**	***−0.999***	**0.990**	**0.975**	**1.000**											
Direct counts (**I**)	*−0.695*	*−0.850*	**1.000**	0.771	***−0.964***	**0.987**	0.863	**0.954**	**1.000**										
DNA (**J**)	*−0.719*	*−0.868*	**1.000**	0.793	***−0.973***	**0.992**	0.880	**0.963**	**0.999**	**1.000**									
CFUs (R2A) (**K**)	*−0.752*	*−0.891*	**0.997**	0.821	***−0.983***	**0.997**	**0.902**	**0.975**	**0.997**	**0.999**	**1.000**								
CFUs (SEA) (**L**)	*−0.722*	*−0.870*	**0.999**	0.795	***−0.974***	**0.992**	0.882	**0.964**	**0.999**	**1.000**	**0.999**	**1.000**							
Arylsulphatase (pH 5.8) (**M**)	*−0.738*	*−0.882*	**0.998**	0.809	***−0.979***	**0.995**	0.893	**0.970**	**0.998**	**1.000**	**1.000**	**1.000**	**1.000**						
Dehydrogenase (pH 7.0) (**N**)	*−0.711*	*−0.862*	**1.000**	0.786	***−0.970***	**0.990**	0.875	**0.960**	**1.000**	**1.000**	**0.998**	**1.000**	**0.999**	**1.000**					
FDA (pH 7.6) (**O**)	*−0.707*	*−0.860*	**1.000**	0.782	***−0.969***	**0.990**	0.872	**0.959**	**1.000**	**1.000**	**0.998**	**1.000**	**0.999**	**1.000**	**1.000**				
Glucosidase (pH 6.0) (**P**)	*−0.712*	*−0.863*	**1.000**	0.787	***−0.971***	**0.991**	0.876	**0.961**	**1.000**	**1.000**	**0.998**	**1.000**	**0.999**	**1.000**	**1.000**	**1.000**			
Phosphatase (alkali) (pH 9.0) (**Q**)	*−0.707*	*−0.860*	**1.000**	0.782	***−0.969***	**0.990**	0.872	**0.959**	**1.000**	**1.000**	**0.998**	**1.000**	**0.999**	**1.000**	**1.000**	**1.000**	**1.000**		
Phosphatase (acid) (pH 4.0) (**R**)	*−0.814*	***−0.932***	**0.984**	0.874	***−0.996***	**1.000**	**0.941**	**0.992**	**0.983**	**0.989**	**0.995**	**0.990**	**0.993**	**0.987**	**0.986**	**0.988**	**0.986**	**1.000**	
Urease (pH 10.0) (**S**)	*−0.697*	*−0.852*	**1.000**	0.773	***−0.965***	**0.988**	0.865	**0.955**	**1.000**	**1.000**	**0.997**	**0.999**	**0.998**	**1.000**	**1.000**	**1.000**	**1.000**	**0.984**	**1000**
(*d*) *Sand soil under non-buffered conditions*																			
Soil depth (**A**)	**1.000**																		
pH (**B**)	**0.969**	**1.000**																	
Organic matter (**C**)	*−0.698*	*−0.853*	**1.000**																
Cation exchange capacity (**D**)	***−0.994***	***−0.991***	0.774	1.000															
Sand (**E**)	0.860	**0.960**	***−0.966***	***−0.912***	**1.000**														
Silt (**F**)	*−0.801*	***−0.924***	**0.988**	0.863	***−0.994***	**1.000**													
Clay (**G**)	***−0.963***	***−1.000***	0.866	**0.987**	***−0.966***	**0.933**	**1.000**												
Biomass (**H**)	*−0.879*	***−0.969***	**0.955**	**0.927**	***−0.999***	**0.990**	**0.975**	**1.000**											
Direct counts (**I**)	*−0.695*	*−0.850*	**1.000**	0.771	***−0.964***	**0.987**	0.863	**0.954**	**1.000**										
DNA (**J**)	*−0.719*	*−0.868*	**1.000**	0.793	***−0.973***	**0.992**	0.880	**0.963**	**0.999**	**1.000**									
CFUs (R2A) (**K**)	*−0.752*	*−0.891*	**0.997**	0.821	***−0.983***	**0.997**	**0.902**	**0.975**	**0.997**	**0.999**	**1.000**								
CFUs (SEA) (**L**)	*−0.722*	*−0.870*	**0.999**	0.795	***−0.974***	**0.992**	0.882	**0.964**	**0.999**	**1.000**	**0.999**	**1.000**							
Arylsulphatase (**M**)	*−0.727*	*−0.874*	**0.999**	0.800	***−0.975***	**0.993**	0.886	**0.967**	**0.999**	**1.000**	**0.999**	**1.000**	**1.000**						
Dehydrogenase (**N**)	*−0.695*	*−0.851*	**1.000**	0.772	***−0.964***	**0.987**	0.864	**0.954**	**1.000**	**0.999**	**0.997**	**0.999**	**0.999**	**1.000**					
Glucosidase (**O**)	*−0.728*	*−0.874*	**0.999**	0.800	***−0.976***	**0.994**	0.886	**0.967**	**0.999**	**1.000**	**0.999**	**1.000**	**1.000**	**0.999**	**1.000**				
Phosphatase (**P**)	***−1.000***	***−0.970***	0.701	**0.994**	*−0.863*	0.804	**0.964**	0.881	0.698	0.722	0.755	0.725	0.730	0.698	0.731	**1.000**			
Urease (**Q**)	*−0.737*	*−0.881*	**0.998**	0.808	***−0.979***	**0.995**	0.892	**0.970**	**0.998**	**1.000**	**1.000**	**1.000**	**1.000**	**0.998**	**1.000**	0.740	**1.000**		

hybridisation (Frostegård et al., 1999), eliminating the interference from unwanted eukaryotic or plant DNA.

The decrease in microbial biomass with soil depth has been noted in other studies (Ekelund et al., 2001). The conversion factors used to estimate bacterial numbers are dependent on cell size. Carbon content per unit volume increases with decreasing size (Lee and Fuhrman, 1987) and could shift due to changes in microbial community composition (Kroer, 1994). The proportion of bacterial numbers accounted for by the biomass estimate increases relative to the direct counts with depth. This suggests the extractability of bacteria from the soil matrix decreased considerably with depth in both soils. Total DNA has been described by Marstorp et al. (2000) as having potential as an estimate of microbial biomass. They reported concentrations of DNA and chloroform-labile C in eight soils to be highly correlated ($R = 0.96$; Table 3). In our study, a strong positive correlation was also found between biomass and DNA ($R > 0.95$; Table 3) for the two soils. Although the decreases in bacterial abundance are similar (Table 2), microbial numbers based on extracted DNA underestimated the number generated by direct counts by the same degree as the plate counts (i.e. 2–3 orders of magnitude).

All methods used showed strong positive correlations ($R > 0.95$; Table 3) with each other. Numbers always declined significantly with depth in both soils and this was positively correlated to the decrease in organic matter. In contrast, the proportion of the direct counts that were culturable, increased with depth in the clay soil as well as from the sand surface to the mid soil.

4.3. Enzyme activities

Conventional soil enzyme assays are performed under defined conditions using temperatures, pH values, excess substrates and other reaction conditions that generate a near maximum rate of substrate catalysis (Schnürer and Rosswall, 1982; Kandeler and Gerber, 1988). This approach gives a reliable and reproducible measure of potential activity but one that is rarely, if ever, realised in soil (Burns, 1978). Assumptions and decisions that must be made when designing soil enzyme assays and interpreting the data have been discussed at length by Tabatabai and Dick (2002).

An alternative approach is to measure enzyme activity at the natural pH of the soil, which will give a more realistic indication of the activities likely to be expressed in situ (Kandeler and Gerber, 1988). In our experiments, both non-buffered and buffered assays were performed to allow comparisons of 'optimal' and 'natural' enzyme activities. In addition, assays were measured at 20 °C (well below the optimum for each), but closer to the soil temperatures likely to exist in surface and subsoils. This necessitated some assays to be prolonged in order to take account of slow rates of substrate catalysis.

While the non-buffered assay may provide a more realistic measure of activity in the field, it does not allow comparisons between activities at different depths (which have different pH values). In order to assess the likely effects of different natural soil pH on activity, the sand surface (pH 5.5), mid (pH 6.6) and deep (pH 7.8) soils were buffered to bring their pH to that of the surface, mid or deep soil pH. Under these conditions phosphomonoesterase activity still significantly ($P < 0.05$) decreased with soil depth (data not shown).

β-Glucosidase, urease, phosphatase and arylsulphatase are enzymes that carry out specific hydrolyses and were selected in these experiments because they catalyse reactions involved in the biogeochemical transformations of C, N, P and S and are likely to be an essential component of any assessment of soil microbial activity and substrate mineralisation.

Dehydrogenase is present in all microorganisms (Von Mersi and Schinner, 1991; Dick, 1997). Therefore, assays are considered to be an accurate measure of the microbial oxidative activity of the soil and should have a direct relationship to total viable microorganisms. Strong correlations ($R > 0.95$; Table 3) were found between dehydrogenase and all bacterial abundance measures (viable counts, direct counts, biomass-C, DNA). The pH of the soils at each depth (and therefore the pH at which the non-buffered assays were performed) vary widely from the conditions imposed by the buffer (pH 7.0). The use of buffer increased the activity measured significantly ($P > 0.05$) (with the exception of sand deep soil). The buffered assay results showed there was a significant difference between dehydrogenase activity in the surface and that in deeper soils, but no difference between the mid and the deep soils. However, dehydrogenase activity may have been underestimated due to competition from alternative hydrogen acceptors within soil (Dick, 1997).

FDA hydrolysis was used as a general indicator of soil hydrolytic activity, as it measured the activities of proteases, lipases and esterases that are all capable of cleaving the fluorogenic FDA (Dick, 1997). FDA hydrolysis, like dehydrogenase activity, is regarded by some as a reliable measure of total microbial activity although, unlike dehydrogenases, these enzymes can function outside of the cell and form stable complexes with soil colloids (Schnürer and Rosswall, 1982). In addition, FDA hydrolysis cannot be considered a specific measure of bacterial and fungal hydrolytic activity within soil as the reaction can be catalysed by a range of other soil organisms, including algae and protozoa (especially in surface soils) (Barak and Chet, 1986).

Strong positive correlations ($R > 0.95$; Table 3) were observed between all but two enzyme activities under both buffered and non-buffered conditions. The first exception was urease activity in the clay soil (under buffered and non-buffered conditions), which was weakly correlated with the other enzyme activities. This could be explained by the strong positive correlations with the clay content of this

profile (R values of 0.972 and 0.686 for non-buffered and buffered assays, respectively; Table 3) and the strong negative correlations with sand content ($R > 0.95$; Table 3a and b). This strong negative correlation with sand content was also evident with the Michigan profile (Table 3c and d). This suggests the clays in the Iowa mid soil have the capacity to retain (and protect) urease either in an active extracellular form or through protection of an ureolytic microbial biomass. This protective property of clays for hydrolases is well known (Burns, 1982) and soil fractionation studies have shown that microbial biomass is strongly associated with clays (Ladd et al., 1996). The other exception was non-buffered phosphomonoesterase activity in the Michigan profile. This also correlated strongly ($R = 0.96$;; Table 3d) with clay content, although no such correlation was seen with the clay profile. Landi et al. (2000) used the ratio of enzyme activity-to-biomass to compare cadmium treatments of soil and this calculation was applied to all our enzyme assays. In general, there was a decrease in the ratio of enzyme activity-to-biomass-C with depth. The two exceptions, again, were urease activity and non-buffered phosphomonoesterase activity in the clay mid and sand mid soils, respectively. Both had far greater ratios of enzyme activity-to-biomass-C compared to their respective surface soils. For example the non-buffered urease assay had a urease: biomass-C ratio of 0.763 (clay mid) and 0.177 (clay surface), a 4.3-fold difference in the ratios, and the non-buffered phosphomonoesterase assay had an activity:biomass-C ratio of 3.63 (sand mid) and 1.45 (sand surface), a 2.5-fold difference in the ratios. This further suggests, along with the lack of correlation with biomass-C for those two assays, that a significant component of the total urease activity and phosphomonoesterase activity at these depths is due to the stabilized extracellular enzyme fraction and is not directly associated with the extant biomass. Strong positive correlations ($R > 0.90$; Table 3) were found between bacterial abundance and enzyme activities and between enzyme activities and organic matter content; again with the two exceptions of urease and phosphomonoesterase activity. Explanations for the generally lower rates of enzymatic activity in the subsoil samples include the lower number of microbes (Swenson and Bakken, 1998) and the decrease in organic matter content (Table 1).

5. Conclusions

This study shows that two soils from as deep as 3.0 m (clay) and 4.2 m (sand), with sharply contrasting physical and chemical composition and properties, are metabolically active and contain substantial numbers of microorganisms. This has important implications for our understanding and modelling of the transformation of downward moving natural and synthetic organics. A greater knowledge of subsoil processes is needed to assess the contribution of subsoil biogeochemistry to the cycling of elements, as well as further developments and harmonisation of methods in order to account for potential changes in surface-applied chemicals and their metabolites as they move down to ground water. This is the subject of current research.

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