BIOAVAILABILITY OF METOLACHLOR AND GLYPHOSATE IN AEROBIC AND ANAEROBIC SOILS

BY

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DISSERTATION

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Metolachlor and glyphosate are two extensively used herbicides in the USA and throughout the globe. Despite the prevalence of anaerobic wet situations in the soil, the predictive capability for the environmental fate and bioavailability of these herbicides is based primarily on well-drained soil conditions. Anoxic events and flooding ubiquitous to agroecosystems may pose a threat for persistence and transport or conversely may facilitate herbicide degradation. This study was undertaken to explore the knowledge gap in the microbial bioavailability and degradation of metolachlor and glyphosate in aerobic and anaerobic soil conditions.

Metolachlor retention pattern in the soils were significantly influenced by anaerobic conditions. Anaerobic soil incubations induced better degradation and mineralization of metolachlor in the range of soils evaluated, despite the differences in soil properties. The findings also confirmed the activity of microorganisms in the degradation and mineralization of metolachlor in the soils in spite differences in their soil properties and redox conditions. The anaerobic degradation and mineralization of metolachlor coincided with the iron (Fe) reducing conditions in soil namely Fe$^{2+}$ formation and suggested a probable role of Fe in the microbial fate of metolachlor under such soil environmental conditions. Stable isotope probing (SIP) facilitated the identification of microorganisms responsible for the mineralization of metolachlor in aerobic and anaerobic soils. The 16S rRNA gene sequences of clones implied the role of organisms closely related to *Bacillus* spp. in aerobic and *Acidobacteria* in anaerobic mineralization of metolachlor in soils.

Glyphosate also was influenced by soil redox conditions for bioavailability and mobility in soils. Contrary to metolachlor, the degradation and mineralization of glyphosate exhibited a slower kinetics in anaerobic soils compared to corresponding aerobic soils in all the soil types.
investigated. Glyphosate degradation was also deduced as a purely microbiological process as almost no degradation or mineralization occurred in sterile control soils. The addition of phosphate suppressed the adsorption of glyphosate in both aerobic and anaerobic soils and confirmed the widespread competition between glyphosate and phosphate for adsorption sites in soils.

In summary, the results from this dissertation research clearly highlight the significance of aerobic versus anaerobic soil conditions as an important factor affecting the bioavailability of metolachlor and glyphosate in soils. The information generated from the current study could be applied towards efficacious use of metolachlor and glyphosate in soils and also for framing a viable strategy for the efficient clean-up of soils contaminated by these herbicides.
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CHAPTER 1

General Introduction

1.1 Introduction

Herbicides are a group of compounds that, in spite of their benefits may produce a wide range of toxic side effects which pose a potential threat to the environment. The concept that ‘any substance in the wrong place, at the wrong time, or in the wrong amount is a pollutant’ has also proved relevant for the existence of herbicides in the environment (Bunce et al., 1993). Herbicides can enter the soil environment from direct spraying onto the soil surface during pre-emergent or post-harvest applications, irrigation runoff or leaching from the dead vegetation resulting in concentrations varying from a few µg to mg Kg\(^{-1}\) of soil (Zabaloy et al., 2011). The persistence of herbicides in the environment is a matter of dire concern given that only a small fraction of them reach the intended target leading to potential impacts of residual herbicides in soil and water (Pimentel, 1995). There is an array of possible toxicological implications of residual herbicides on the ecosystem (Buchanan et al., 2009). As a result, a wealth of research has been published on the occurrence, fate and effects on human health and the environment from the use of herbicides in weed control (Kolpin et al., 1998; Devos et al., 2008; Hiller et al., 2008). However, the bulk of the knowledge on the key processes determining the fate and bioavailability of herbicides in the soil is based on assumed aerobic conditions, which leaves a dearth of knowledge in saturated soil conditions (Sims & Kanissery, 2012). Herbicide degradation processes may be directly or indirectly coupled and may respond differently to varying environmental conditions, thus making the net effect on overall fate challenging to predict (Sims & Cupples, 1999). Furthermore, amongst the many factors contributing to
herbicide fate, those mediated by microorganisms tend to be the most difficult to understand. Hence the current research project was undertaken to unravel the ambiguities in microbial bioavailability of popular herbicides in soils enduring aerobic and anaerobic conditions.

The immense diversity of herbicides commercially available today makes it impossible to explore all of them. Hence, this dissertation is focused on two popular herbicides glyphosate and metolachlor that have been used extensively in the USA and throughout the globe. While these herbicides have been the subjects of much research, their fate remains understudied amidst conditions of high microbial diversity and varying soil redox conditions, in the milieu of a multifaceted agroecosystems. Most work on the environmental fate and bioavailability of these herbicide compounds has focused on aerobic environmental conditions. Although there is probably more aerobic biotransformation possible, this does not mean that there are no anaerobic conversions of potential use. Despite the incidence of anaerobic wet situations in the soil, the predictive capability for the fate of these herbicides is based primarily on well-drained soil conditions. Hence, it is imperative to evaluate the bioavailability and biodegradation of the herbicides glyphosate and metolachlor in soils under varying redox conditions. Anoxic events and flooding ubiquitous to agroecosystems may pose a threat for herbicide persistence and transport or, conversely, may facilitate herbicide degradation. Treatment of soils under diverse environmental conditions like reduced (anaerobic) and oxidized (aerobic) regimes would give an opportunity to explore the knowledge gap in the microbial bioavailability and metabolism of popular herbicides like metolachlor and glyphosate. Then, that knowledge could be applied towards efficient herbicide use and frame a viable strategy for the efficient clean-up of soils contaminated by spills of these herbicides.
1.2 Microbial bioavailability of herbicides in soil

The microbial bioavailability of a herbicide is the measure of its accessibility to microorganisms in the environment (Wu et al., 2011). It serves as an important consideration in the risk assessment of soil contaminants and hence aids in tailoring a viable remediation strategy for the cleanup of herbicides from the contaminated soils. The distribution and retention of herbicides in soil is determined by an array of chemical and biological processes (Figure 1.1). These include adsorption, desorption, biodegradation and non-extractable residue formation. All these processes are interdependent, occurring in parallel and regulating the availability of the herbicide that can be used by microorganisms in the environment (Cheng, 1990).

1.2.1 Adsorption

Adsorption to soil is of critical importance for the regulation of herbicide persistence throughout the soil environment as it controls the amount of herbicide present in the soil solution. The importance of this process is determined in part by the physical and chemical properties of the herbicide and in part by soil characteristics such as mineral composition, organic matter content and soil solution chemistry. Soil bound herbicides are temporarily non-bioavailable for microbial degradation because most microbes may not be able to utilize the herbicides in the adsorbed state (Ainsworth et al., 1993). To relate the bioavailability to adsorption, it is helpful to acknowledge the complexity in the composition of soils. Typically soil contains 50% solid material and 50% pore space (Zabaloy et al., 2011). The solid component of the soil is formed by primary and secondary minerals along with the organic matter. These materials provide the specific sites for herbicide adsorption (Dixon & Weed, 1990). Between the solid components of soil is the space forming pores that play a major role in movement of water, solutes and air.
The bioavailability of herbicide in the soil depends on the partitioning of herbicide between the soil solution and the soil solid phase. The appropriate term for defining such processes is adsorption equilibrium which is often used for describing the interaction between any herbicide and the soil components like solid surface sites and soil solution. Surface sites where the herbicides can be adsorbed are numerous and varied in soils. The bulk of these sites are provided by soil minerals (clays, Fe and Mn oxides) as well as by organic matter. The specific mechanisms by which herbicides adsorb to these soil solid sites involve the formation of surface complexes, electrostatic interactions, hydrophobic interactions and ion exchange.

Characterizing the bioavailability of a herbicide requires a thorough understanding of its strength of interaction with particular solids and herbicide concentration in the soil solution. Bioavailability is characterized using adsorption isotherms. An adsorption isotherm shows the relationship between the herbicide’s concentration in the soil solution and the amount adsorbed to the soil surface sites at a constant temperature after equilibrium was reached. The fit of experimental data with theoretical and/or empirical equations for adsorption isotherms is very useful for providing information on the strength of the soil-herbicide interaction, which will give an idea of the bioavailability of the herbicide in a particular soil. However, isotherms are only a description of macroscopic data and do not conclusively shed any light on the specific adsorption mechanisms (Sparks, 2003).

1.2.2 Desorption

Adsorption may be either reversible or irreversible depending on the properties of both herbicide and soil. Reversibility of the adsorbed herbicide back into the soil solution is termed desorption. Desorption is determined in continuation with the adsorption isotherm coefficient test, where the amount of adsorbed herbicide released back into the soil solution is calculated.
Adsorbed herbicides are assumed less accessible to attached or suspended microorganisms, which preferentially utilize herbicides in the soil solution phase. In this view, the herbicide is available for biodegradation only after desorption into the soil solution. The adsorbed fraction remains protected from microbial degradation as a result of physical sequestration of the herbicide into the soil matrix and/or their chemical stabilization on the soil surface sites (Ainsworth et al., 1993). Reduction of herbicide concentration from the soil solution to levels that do not sustain microbial growth, emphasizes the importance of herbicide desorption in the microbial bioavailability of herbicides. Although desorption has been considered a prerequisite for biodegradation of soil-bound herbicides, there is increasing evidence that adsorbed herbicides may still be degraded by the attached microbial cells (Park et al., 2001). However, there is still considerable work ahead for researchers to understand the mechanisms and populations intervening in these processes.

1.2.3 Biodegradation

One of the most evident indications for the bioavailability of a certain herbicide in soil is its biologically mediated degradative by-products. Biodegradation is the primary attenuation process for herbicides in soil and is principally facilitated through the enzymatic transformations by living microbial cells. Biodegradation is controlled by biotic factors, for instance microbial activity and a number of physiochemical processes such as adsorption and desorption, diffusion and dissolution (Chen et al., 2009). Generally, biodegradation of a herbicide can be limited by its bioavailability in soil as the compounds may be adsorbed to soil solids, physically entrapped in micropores, or simply distributed through a much greater portion of the pore volume of the soil solution than are the degraders, thus leading to diffusion limited degradation kinetics (Sims et al., 1992 & 2009).
Microorganisms do extremely well in thriving on herbicide compounds in the soil by utilizing them as a supply of nutrients and energy. Many herbicides serve as good carbon and/or nitrogen sources for soil microorganisms (Qiu et al., 2009). Evidence for their remarkable range of degradative abilities can be seen in the recycling rather than accumulation of vast quantities of biomass that have been produced throughout the history of life on earth (Dua et al., 2002). Herbicide biodegradation by microorganisms that are capable of using the chemical as a source of carbon and energy for growth is called mineralization (Zabaloy et al., 2011). Mineralization results in the complete aerobic or anaerobic degradation of the herbicide compounds to form carbon dioxide, water and some other inorganic elements. The biomass of the microbial degraders increases at the expense of the herbicide substrate. Further, mineralization has been used as a sensitive indicator of pesticide and heavy metal bioavailability and is generally accepted as a measure of total soil microbial activity (Anderson, 2003). Conversely, the incomplete transformation of a herbicide by microorganisms that yields no carbon or nutrients and energy is called co-metabolism. In most co-metabolic processes, a co-susubstrate provides carbon and energy for microbial growth and the herbicide itself does not support microbial growth. However, some herbicides may be partially transformed by co-metabolism; their intermediate metabolites may be completely mineralized by other soil microorganisms.

The biodegradation rate of the herbicide in the soil can be described by their half-life $T_{1/2}$, the time required for 50% of the compound to degrade or dissipate. In addition to degradation, non-biological field dissipation of the herbicide includes, leaching losses, photo degradation and volatilization. However, the presence of microorganisms in the soil system is crucial as they can significantly affect the $T_{1/2}$ of herbicides in soil. An active soil microbial population is considered a key component of good soil quality (Anderson, 2003). Microbes are expected to be more
effective indicators of the soil quality and health than physical and chemical parameters as they are able to respond immediately to environmental changes. When predicting the potential in situ biodegradation of herbicides, additional factors other than presence of potential degraders and bioavailability must be considered. These include the presence of other contaminants that can compete for adsorption sites and the availability of nutrients and co-factors necessary for the degrader’s growth and activity (Haws et al., 2006). Intrinsic soil environmental factors like oxygen concentration, water availability, temperature and pH can also have great impact on the biodegradation of herbicides in the agro-ecosystem.

1.2.4 Non-Extractable Residue (NER) formation

NER represent compounds in the soil which persist in the matrix in the form of the parent substance or its metabolite(s) after laboratory chemical extraction. NER formation is typically considered a process decreasing the bioavailability of the herbicides, as consistent data suggests that only a small percentage of the total amount of NER can be released from the soil solid surfaces. Hence, NER formation is often accounted as the irreversible sink in pesticide risk assessment procedures. Typically, strongly bound herbicide molecules on soil particles retard the microbial degradation of herbicides and lead to its accumulation in soil. These irreversibly bound herbicide molecules are considered as the non-extractable fraction of the chemicals. The factors governing the formation of NER are not clear-cut yet, however, herbicides or metabolites supporting free reactive chemical groups, such as aniline or phenol, have a tendency to create a larger proportion of NER during degradation (Bollag et al., 1980). Furthermore, if it is the parent compound which is involved in the NER formation, then rapid degradation competes with the NER formation. On the contrary, if it is the metabolite which is involved in NER formation, then rapid degradation may lead to an extensive formation of NER (Barriuso et al., 2008). From an
eco-toxicological view point NER formation can reduce the toxicity of a compound by decreasing its interaction with soil biota (Gevao et al., 2000).

Among the various factors affecting the formation of NER, microbial activity has a direct and significant effect. The accumulation of NER for most herbicides is usually correlated to the soil biological activity and to the amount of organic matter in the soil. The total microbial activity has a direct effect on the NER formation as observed from herbicide incubation experiments. NER formation was shown to be low from incubation experiments involving soil samples taken from deeper depths which usually have low microbial activity (Schiavon, 1988).

Most environmental factors affecting the microbial bioavailability of herbicide in the soil such as temperature or moisture content are likely to have an influence on the NER formation. Generally, NER formation varied with an increase in soil moisture, and in most cases it increased with soil saturation (Rice et al., 2002; Kruger et al., 1993)

1.3 Relevance of the current study

Terrestrial soil environments can be considered as being dominated by aerobic conditions, but there are situations when anaerobiosis prevails. Anaerobiosis occurs in soil when the oxygen consumption rate exceeds the supply rate. The rate of oxygen consumption depends primarily on the amount of available carbon for respiration. The oxygen supply rate depends on the moisture content and the physical characteristics of the soil, especially porosity which is influenced by structure and texture. A change in either the rate of oxygen consumption or supply can bring about anaerobiosis, for example, by a large application of manure or by compaction of the soil. Most soil anaerobiosis is caused by high soil moisture resulting from a high water table or heavy rains. The dramatic effect of water on the aeration status of soil is due to several factors like much lower oxygen diffusion coefficient of water filled pores than of air filled pores and a much
smaller reservoir of oxygen in soils with a high proportion of water filled pores (Tiedje et al., 1984). Even normal soils have significant periods of anoxia caused by a high water table in the spring and following heavy rainfalls resulting in temporary anaerobic sites (Sexstone et al., 1985).

Although many studies have been published on the effects of soil physical and chemical properties on the adsorption of herbicides, few attempts have been made to create adsorption models to predict herbicide adsorption and transport in anaerobic soil environments. Adsorption is often considered a process that governs and regulates herbicide degradation in soil. Lack of information on the adsorption and desorption patterns of herbicides in anaerobic soil environments means we cannot explain patterns in the degradation and fate of herbicide compounds in soils enduring such environments. For instance, soil organic matter has been implicated in the adsorption process of herbicides (Benoit et al., 1999) and therefore the chemical modifications in soil organic groups in anoxic soils could alter their availability for microbial degradation (Clausen et al., 2004). To understand the microbial fate of herbicide in soil it is critical to study both aerobic and anaerobic conditions experimentally.

In unsaturated soil, solutes, such as herbicides exhibit limited availability due in part to the requirement to diffuse through limited pore spaces and thin or discontinuous water films. Under saturated conditions, aqueous diffusion is maximized for solutes, however, movement through the vapor phase ceases. Among the most profound consequences of this transition, is a 10,000-fold decrease in the diffusive supply of oxygen (Sims & Kanissery, 2012). After soil is inundated with water, biological and chemical oxygen demand will result in the rapid depletion of oxygen. The result is succession of microbial heterotrophic communities adapted to utilize a variety of potential electron acceptors other than oxygen as conditions become suitable for their
activities. For example, in flooded soil microcosms the reduced product Fe\(^{2+}\) was detected immediately after nitrate depletion (Tor et al., 2000).

The occurrence of unexpected anaerobiosis in soil highlights the importance of anaerobic microbial processes occurring in soil such as Fe\(^{3+}\) and Mn\(^{4+}\) reduction, denitrification, fermentation, sulfate reduction and methanogenesis according to their approximate sequence of occurrence as the redox of soil decreases. These processes are those which might be expected in any soil with temporary anaerobic microsites. Microorganisms can anaerobically oxidize many contaminants with alternative electron acceptors such as nitrate, sulfate and Fe-oxides (Anderson & Lovely, 1997; Lovely, 2001). Fe\(^{3+}\) is often the most abundant potential electron acceptor in anaerobic conditions for the oxidation of organic matter (Lovely, 1991). Enhancing the availability of Fe\(^{3+}\) for microbial reduction can greatly stimulate the anaerobic degradation of organic contaminants (Lovely et al., 1994 &1996).

The presence of obligate anaerobes in a soil cannot be used to indicate the prevalence of sustained anaerobic conditions since Clostridia sp., sulfate-reducing bacteria, and methanogens can survive well in the absence of anaerobic growth conditions. The anaerobic processes that most likely occur in cultivated soils are those carried out by facultative anaerobes. Of these, Fe\(^{3+}\) and Mn\(^{4+}\) reduction and denitrification require the aerobic generation of the oxidized ions first, which suggests that these processes should be more significant wherever there are more aerobic-anaerobic interfaces. The anaerobic front in a soil aggregate is thought to expand and contract in response to the supply and respiratory consumption of oxygen. This concept suggests that denitrification, for example, should be more extensive in habitats where the area of the aerobic – anaerobic interface is greatest and where the movement of this zone is periodic (Sexstone et al., 1985).
1.4 Aims of the study

The main aim of this dissertation was to test the effects of anaerobic and aerobic conditions on the microbial bioavailability of herbicides metolachlor and glyphosate, so that their biogeochemical transport in the environment may be more accurately assessed. Based on soil type, adsorption-desorption properties and oxidation–reduction conditions, herbicide transport/movement may be decreased or enhanced which suggests that, given enough time, under the different environmental conditions, non-available material may become bioavailable. The more detailed objectives of the studies were:

- **To investigate the bioavailability factors of metolachlor in soils under aerobic and anaerobic conditions.** In this study, the adsorption and desorption patterns of the herbicide metolachlor were evaluated using a range of redox conditions and soils with different properties. The current project also analyzed the microbial degradation and the mineralization of metolachlor in aerobic and anaerobic conditions.

- **To identify the microorganisms responsible for aerobic and anaerobic mineralization of the herbicide metolachlor in soil by using stable isotope probing (SIP).** Gathering information on the microbial degraders of metolachlor is imperative for understanding the bioavailability of the herbicide in soils. SIP was utilized to determine whether the same or different organisms functioned in aerobic versus anaerobic mineralization of metolachlor in soil.

- **To assess the impact of aerobic and anaerobic conditions on the microbial bioavailability of glyphosate in soils.** The purpose of this objective was to examine the effects of aerobic and anaerobic soil environments on bioavailability factors like adsorption, desorption, degradation and mineralization of glyphosate in a range of soils. Phosphate has been shown to influence glyphosate mobility in the soil (Gimsing & Borggaard, 2002). Hence, the influence of soil phosphate on bioavailability factors of glyphosate in aerobic and anaerobic soils was also determined.
1.5 Dissertation orientation

This dissertation is a compilation of three manuscripts and is organized into six chapters. Chapter 2 is the literature review and provides background information for the dissertation research. Chapter 3 and 5 respectively, are manuscripts based on the findings from the metolachlor and glyphosate bioavailability studies, and are currently under preparation for publication. Chapter 4 is written as a manuscript based on the stable isotope probing of metolachlor mineralizing microorganisms. Chapter 6 encompasses the concluding remarks and future directions of this dissertation work.
1.6 Figures

**Figure 1.1:** Schematic representation of chemical and biological processes involved in the distribution and retention of herbicide in soil.
1.7 References


CHAPTER 2

Literature Review

2.1 Metolachlor

2.1.1 General Information and physiochemical characteristics

Metolachlor [2-chloro-N-(2-ethyl 6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide], a selective chloroacetamide herbicide, is extensively used throughout the globe for the pre-emergent control of broadleaf and annual grassy weeds in a wide range of crops such as corn, soybean, peanut, potato, and tobacco (Extoxnet, 2000). When absorbed through the roots and shoots of target weed or seeds, metolachlor inhibits the synthesis of chlorophyll, proteins, fatty acids and lipids (Rivard, 2003). Selected physico-chemical characteristics of metolachlor are summarized in Table 2.1. Metolachlor has the potential to leach into groundwater because of its relatively high water solubility and relatively low adsorption to the soil particles. Adding to the concern, a recent water monitoring program acknowledged metolachlor among the prominent chemical contaminants in drinking water throughout the United States (EWG, 2009). Field and laboratory experiments have provided evidence of long-term persistence in soils (Accinelli et al., 2003 & 2004a). The fate of metolachlor has attracted worldwide concern due to its prolonged persistence in soil, high water solubility and significant toxicological properties (USEPA, 1988).

2.1.2 Metolachlor degradation in soil

Degradation of metolachlor in soil is primarily through microbial processes (Miller et al., 1997; Accinelli et al., 2001). Field and laboratory investigations have shown that degradation of metolachlor is related to soil microbial biomass and activity (Staddon et al., 2001; Accinelli et al., 2003). There is an apparent fluctuation in the degradation half-life of metolachlor, depending
on the soil type and environmental conditions, and is estimated to be between 15 – 132 days (USDA, 1995; Kollman & Segawa, 2000). The breakdown of any herbicide in the soil is affected by temperature, moisture, microbial activity, soil type, nitrification, oxygen concentration, sunlight, and amount of leaching (Extoxnet, 2000). Furthermore, timing and intensity of field applications may also influence herbicide degradation in soil. For instance, Accinelli et al. (2004a) illustrated that short-term repeated applications of metolachlor caused enhanced degradation of the active ingredient, though the effect was transient.

Rice et al. (2002) studied the influence of soil depth, soil moisture, and concentration on the persistence and degradation of metolachlor in soil. They found that mineralization of metolachlor to CO₂ was minimal in surface and subsurface soils. Increased metolachlor concentrations did not inhibit microbial activity; however, the greater rate of application did result in a reduction in the percentage of applied metolachlor that was bound to surface or subsurface soil. A significant reduction in the quantity of extractable metolachlor degradates and non-extractable soil bound residues in sterile soil revealed the significance of biodegradation to the dissipation of metolachlor in soil. Miller et al. (1997) had similar observations and showed that metolachlor degraded slower and adsorbed less to the soil with increasing soil depth, especially below 25 cm where microbial activity was lowest.

Mulbah et al. (2000) studied the effect of soil redox conditions on the degradation of metolachlor in two Mississippi soils. Herbicide was added to soil microcosms and incubated either under oxidized (aerobic) or reduced (anaerobic) conditions. The authors concluded that the herbicide degraded faster in the soil under aerobic conditions as compared to anaerobic conditions. Konopka (1994) observed the anaerobic microbial transformations of various chloroacetanilide herbicides, including metolachlor, and found modest microbial transformations
(in most cases 30-60% losses) of these herbicides in agricultural soils incubated under anaerobic conditions. Accinelli et al. (2001) also provided evidence for anaerobic degradation of metolachlor.

Seybold et al. (2001) determined the $T_{1/2}$, degradation rates, and metabolite formation patterns of metolachlor in an anaerobic wetland soil. Results clearly indicated that metolachlor showed substantial degradation under the strongly reducing conditions found in wetland soils. However, known metolachlor metabolites like ethanesulfonic acid or oxanilic acid were not significantly formed under anaerobic conditions. Mersie et al. (2004) conducted a laboratory level metolachlor degradation study in a sediment system. The metolachlor metabolites ethanesulfonic acid and oxanilic acid were detected in the water-sediment system and in soil from tilted beds. They concluded that metolachlor can be degraded in sediments and moisture level accelerated its breakdown in such environments.

One of the most interesting reactions under anaerobic xenobiotic metabolism is reductive dehalogenation of an aromatic ring (Sulfita, 1982). The anaerobic dechlorination represents a mechanism by which the Cl can be removed before ring cleavage, thereby making the compound more susceptible to further aerobic or anaerobic degradation. Metolachlor is a potential xenobiotic candidate to undergo such a transformation in soil by the displacement of chlorine atoms (Stamper & Tuovinen, 1998).

2.1.3 Microbial degraders and potential enzymatic pathways of metolachlor degradation

Studies involving mixed and pure cultures have confirmed that microbial transformations are the essential mechanisms responsible for the degradation of metolachlor. Krause et al. (1985) successfully analyzed the transformation of metolachlor with an actinomycete strain isolated from metolachlor contaminated soil. Metolachlor was completely degraded within 16 days in the
culture media and metabolites of the herbicide were obtained and identified from enrichment culture after incubation. Saxena et al. (1987) used various inocula and enrichment culture techniques to describe the microbial degradation of metolachlor and reported the efficacy of bacterial strains Bacillus circulans and B. megaterium in substantial degradation of the herbicide. Liu et al. (1989) explored the possibility of using bacterial mixed cultures to degrade metolachlor. They employed a mixed community of bacteria to transform the herbicide in liquid media. They found that about 80% of the added metolachlor disappeared from the medium. High performance liquid chromatography of the microbial biomass extracts confirmed the transformation of the herbicide by the bacterial community. However, pure cultures isolated from the bacterial mixed culture were less effective for degrading metolachlor.

Additionally, some studies also suggest the potential involvement of fungi in degradation and transformation of metolachlor in soil. Fungal degradation of metolachlor was first reported by McGahen & Tiedje (1978) who found 45% disappearance of metolachlor after 144 h of incubation using resting cells of Chaetomium globosum. Liu et al. (1991a) reported the degradation of metolachlor by the white-rot fungus Phanerochaete chrysosporium. Metolachlor was degraded by 99% within 48 h by the fungus Cunninghamella elegans (Pothuluri et al., 1997). The contribution of fungi in metolachlor degradation in situ has not been established.

Metolachlor transformation by soil microorganisms to its primary degradates (metolachlor ESA - ethane sulfonic acid and metolachlor OA - oxanilic acid) has been suggested to occur as a result of displacement of the chlorine atom of the parent compound by glutathione, followed by the formation of the ESA and OA degradates by different enzymatic pathways (Barbash et al., 1999). In the laboratory, a half-life ($T_{1/2}$) of 67 days for aerobic microbial metabolism of metolachlor in sandy loam soil (at 25°C) has been reported resulting in the
production of (2-ethyl-6-methylphenyl)(2-methoxy-1-methylethyl) amino oxo-acetic acid as the major degradate (WSSA, 1994). A laboratory $T_{1/2}$ of 81 days was reported for anaerobic microbial metabolism that produced the same degradates (WSSA, 1994).

### 2.1.4 Bioavailability factors of metolachlor

Adsorption in soils and sediments is an important factor controlling the migration and bioavailability of acetanilide herbicides, including metolachlor. Yet microbial degradation is the most important factor in determining their overall fate in the environment (Stamper & Tuovinen, 1998). Metolachlor is considered to be moderately adsorbed to the soil and the adsorption results were best explained by the Freundlich isotherm (Si et al., 2009). Metolachlor is a neutral non-ionizable herbicide dominated by apolar groups and the adsorption mechanism has been frequently described by the interactions of the herbicide with soil organic matter (Ghosh & Singh, 2012). Accordingly, the adsorption of metolachlor to the soil is enhanced with increased organic matter content, ultimately hindering its movement in the soil (Bedmar et al., 2011).

Rice et al., (2004) reported that particulate matter and sediment can alter the bioavailability of herbicide to organisms and therefore influence the herbicide’s toxicity and availability for microbial degradation. In their study, sediment significantly reduced metolachlor concentrations in the surface water as a result of greater degradation. The $T_{1/2}$ values for metolachlor in the surface water-sediment incubation were almost four times less than the $T_{1/2}$ calculated for the sediment-free systems.

The influence of other environmental chemicals on the bioavailability of metolachlor has also been explored. Concentrations of the antimicrobial agent sulfamethazine in soil did not induce significant effects on metolachlor degradation and adsorption, and thus was ruled out as a factor affecting the environmental fate of the herbicide in soil (Accinelli et al., 2006).
2.2 Glyphosate

2.2.1 General Information and physiochemical characteristics

Glyphosate [N-(phosphonomethyl)glycine] has been one of the world's most widely applied herbicides since it came on the market in 1974. It is a systemic, broad-spectrum herbicide that is first absorbed by foliage and then translocated throughout the target plant. It suppresses the weed growth by inhibiting the production of essential aromatic amino acids (Franz et al., 1997). Glyphosate is non-volatile, does not degrade photo-chemically and is stable in air. Selected physico-chemical properties of glyphosate are presented in Table 2.1. Glyphosate has been considered as environmentally safe due to its rapid inactivation in soil, both by degradation and by adsorption (Quinn et al., 1988). However, extensive use of glyphosate has stimulated numerous studies on its behavior and persistence in the environment.

2.2.2 Glyphosate degradation in soil

The route of degradation of glyphosate in the laboratory is well studied (Torstensson, 1985) and is mainly governed by microbial processes (Sprankle, 1975; Gimsing et al., 2004a). Glyphosate degrades relatively rapidly in soils, with an estimated T_{1/2} of 7-60 days (Giesy et al., 2000), predominately by microbial-mediated processes (Torstensson, 1985; Accinelli, 2004b). Several studies have shown that glyphosate can stimulate microbial activity (Haney et al., 2000; Bussey et al., 2001) while a few studies are available indicating toxic effects on soil microorganisms (Araujo et al., 2003).

Degradation of glyphosate takes place under both aerobic and anaerobic conditions, although the degradation under anaerobic conditions is normally slower than under aerobic conditions (Rueppel et al., 1977). In fact, Sorensen et al. (2006) found negligible mineralization
of glyphosate in anoxic soil layers. Lancaster et al. (2009) reported that the total amount of $^{14}$CO$_2$ produced as a result of $^{14}$C-glyphosate mineralization was reduced with repeated herbicide applications compared to a single application, which suggested that the microbial community was not well adapted to long histories of herbicide treatment. Biodegradation is influenced by adsorption/desorption but also by edaphic and climatic factors that control the activity of microflora. For instance, Gimsing et al. (2004a) showed that the mineralization rate of glyphosate correlated with abundance of *Pseudomonas* spp. bacteria in the soil. They also observed stimulation in mineralization after the addition of phosphate to the soil. Also, temperature may be an important factor determining the degradation of glyphosate (Stenrod, 2005).

Glyphosate biodegradation appears to be a co-metabolic process (Accinelli et al., 2005) as microorganisms are not able to use glyphosate as a carbon source (Dick & Quinn, 1995). The fact that degradation of glyphosate has been correlated with the general microbial activity of the soil also implies co-metabolic involvement in the metabolic degradation of the herbicide. The observation that degradation of glyphosate takes place without a lag phase in soil has also been seen as evidence for co-metabolic degradation (Franz et al., 1997), because the enzymes used in the degradation must be present before the application of glyphosate. Nevertheless, a few studies have shown that some organisms are capable of using glyphosate as a carbon substrate (Haney et al., 2002; Bussey et al., 2001), a source of nitrogen (Haney et al., 2000) or phosphate (P) (Dick & Quinn, 1995).

2.2.3 Microbial degraders and potential enzymatic pathways of glyphosate degradation

Microbial degradation of glyphosate has been extensively explored and several glyphosate degrading bacteria have been isolated and characterized (Kononova & Nesmeyanova, 2002). The
degradation kinetics of glyphosate, when used a source of P, from pure culture studies is found to be different from degradation in soil. Apart from that, it has also been demonstrated that the degradation rate of glyphosate can exhibit great variability among the microorganisms studied (Quinn et al., 1989). Contrary to degradation in soil, degradation of glyphosate by a pure culture starts with a lag phase with very slow degradation, leading to an accelerating phase resulting in a very fast degradation of the glyphosate (Quinn et al., 1989). Such observations indicate that, although experiments with pure cultures may give useful information on the degrading potentials of different microorganisms, application of the results to in situ conditions needs more exploration. Even though bacteria are considered the main biological degrader of glyphosate in soils, the importance of fungi has also been acknowledged (Singh & Walker, 2006).

Microbial degradation of glyphosate proceeds through two pathways (Dick & Quinn, 1995). One pathway leads to the intermediate formation of aminomethylphosphonic acid (AMPA) and the other leads to the formation of sarcosine and glycine. However, the most important metabolite of glyphosate is AMPA accounting for more than 90% of the reported metabolites. In AMPA formation, the first step is the cleavage of the C–N bond by the bacterial enzyme glyphosate oxidoreductase, producing AMPA and glyoxylate (Liu et al., 1991b). The involvement of glyphosate oxidoreductase enzyme, employing flavine adenine dinucleotide (FAD) as a co-factor, is critical in the degradation pathways of glyphosate. The mechanism is purported to involve the reduction of (FAD) at the active site by glyphosate. Glyphosate tolerance in Roundup Ready® crops is induced by inserting this glyphosate oxidoreductase enzyme into appropriate plant genomes (Barry & Kishore, 1998).
2.2.4 Bioavailability factors of glyphosate

Mineralization/ degradation of glyphosate in soil has been found to be inversely correlated with the glyphosate adsorption capacity of the soil (Sorensen et al., 2006). If adsorption is strong, then the mineralization/degradation will be low, possibly because bioavailability is low. Despite its high water solubility, glyphosate is strongly adsorbed to soil particles and consequently has low mobility through the soil profile (Zaranyika, 1993). Organic matter, clay content, and aluminum and iron oxides play important roles in the adsorption of glyphosate to soil (Glass, 1987; Gerritse, 1996). Strong adsorption by solids, such as aluminum and iron oxides may indeed protect organic compounds like glyphosate against microbial degradation, and adsorption/desorption processes may control the degradation rate (Scow & Hutson, 1992; Eberbach, 1998). Despite numerous studies having addressed the adsorption behavior of glyphosate, few experimental studies have considered the effect of adsorption on the bioavailability of glyphosate in the soil.

The vertical distribution of the adsorption, desorption and mineralization of glyphosate was examined in samples from two contrasting soil and subsurface profiles, obtained from a sandy agricultural site and a non-agricultural clay rich site. In the deeper parts of the sandy profile high adsorption and low desorption of glyphosate coincided with no or minor mineralization, indicating a limited glyphosate bioavailability in those depths (Sorensen et al., 2006).

Morillo et al. (1997) conducted an adsorption-desorption study of the herbicide glyphosate onto the clay mineral montmorillonite in the presence of copper, in order to clarify the effects that this strongly complexing metal could have on the bioavailability and mobility of the herbicide in the soil environment. In contrast with previous findings, glyphosate adsorption
on montmorillonite decreased in the presence of copper, in solution or adsorbed on the mineral, due to the formation of copper-glyphosate complexes that have a lower tendency to be adsorbed on montmorillonite than the free glyphosate. These experiments lead to the conclusion that, in relation to glyphosate release from soil, it is necessary to take into account not only the type of soil to which it is applied (e.g., whether it has high clay mineral content) but also whether there is any element in the soil solution or adsorbed on the soil capable of forming strong complexes with the herbicide compound.

Recently, Schnurer et al. (2006) tested the effect of adsorption on the bioavailability and biodegradation of glyphosate in soils. The aim of this study was to test the effect of surface adsorption on microbial utilization of the herbicide glyphosate as a source of phosphorus, nitrogen, or carbon. Geothite was added to a humus soil to manipulate the soil’s glyphosate adsorption capacity. Data from this study suggested that adsorbed glyphosate is microbially degradable despite the reduction in the microbial activity in the presence of the herbicide.

### 2.2.5 Role of phosphate on the adsorption and availability of glyphosate in soil

Glyphosate possesses unique adsorption characteristics in soil when compared to other herbicides. Other types of herbicide molecules are dominated by apolar groups (aliphatic and/or aromatic carbon) and often have only one functional group. Hence, these compounds are weakly adsorbed to soils, mainly by soil organic matter (Oliveira et al., 2001; Borggaard & Elberling, 2004). Conversely, glyphosate is a small molecule with three polar functional groups (carboxyl, amino and phosphonate groups) and is strongly adsorbed by soil minerals (Gimsing et al., 2004b). In most soils within the pH range of 4 to 8, glyphosate forms mono- and divalent anions with high affinity for trivalent cations such as $\text{Al}^{3+}$ and $\text{Fe}^{3+}$ (Gimsing & Borggaard, 2007). Because phosphate reacts similarly, glyphosate and phosphate may compete for the surface sites,
which may affect glyphosate adsorption, and hence mobility, in phosphate-rich soils (Gimsing et al., 2004b).

The impact of phosphate on glyphosate adsorption in soil was described shortly after the herbicide launch into the market (Sprankle et al., 1975). Later, several studies have confirmed the competitive adsorption of glyphosate and phosphate as well as its substantial variability in various soils (Hance, 1976; Wang et al., 2005). A relevant study by Gimsing & Borggaard (2001) on the comparative adsorption of glyphosate and phosphate by iron oxide minerals clearly demonstrated competition and adsorption preference for phosphate. The preceding adsorption of phosphate onto the minerals not only eliminated glyphosate adsorption but also resulted in enhanced mobility (desorption) of the herbicide following the addition of phosphate to soil. Competition for adsorption sites between glyphosate and phosphate therefore seems apparent and may have a severe impact on glyphosate mobility, and hence bioavailability. This is particularly important in agricultural soils in the USA and elsewhere that are saturated or nearly saturated with phosphate as a result of excessive phosphorus fertilization over many years (Hart et al., 2004). Glyphosate/phosphate competition is of environmental concern because the suppressed glyphosate adsorption on phosphate enriched soil may lead to an increased risk of glyphosate leaching to the aquatic environment.

2.3 Similar research approaches with other compounds

This section will briefly present some related research approaches that have been successfully used to study the bioavailability and degradation of other xenobiotic compounds including herbicides in aerobic and anaerobic soil environmental conditions.

The degradation of the herbicide 2,4-D and two chlorophenols, adsorbed on different organic materials (wood chips, straw, lignin, humic acids) and aluminum oxide in soil
incubations was explored by Benoit et al. (1999). They observed that mineralization of these compounds, when incubated in direct contact with soil, varied greatly according to the nature of the adsorbent, but was generally higher in more humified organic matter (humic acid) than in raw organic matter (wood, lignin and straw).

A few studies focused on the involvement of clay minerals in the soil on the biodegradation and availability of herbicides. For instance, Xu et al. (2001) looked at the influence of oxidation state of structural iron in clay minerals on the adsorption and degradation of herbicides in the environment. They found that increased reduction potential of the reduced clay surfaces may promote degradation of the herbicides. In a similar study Tor et al. (2000) examined the fate of the herbicide trifluralin in anoxic environments and the contribution of reduced iron (Fe$^{2+}$) to its anaerobic degradation in soil. They noticed that reduced but not oxidized or reoxidized forms of purified ferruginous smectite catalyzed rapid transformation (72% of applied in 30 h) of trifluralin to polar products with a concomitant reoxidation of structural iron in the clay. Results from their study indicated that trifluralin is subject to reaction with Fe$^{2+}$ associated minerals in anoxic environments.

Hermosín et al. (2006) evaluated the bioavailability of organoclay-based formulations of the herbicide 2,4–D for bacterial degradation in pure culture and leaching potential in soil columns. The rate of mineralization of 2,4–D from the organoclay complexes was related to the rate of release from the complexes, suggesting that desorption into the aqueous phase was the limiting step for biodegradation.

O’Loughlin et al. (2000) determined the effect of adsorption on the bioavailability of 2-methylpyridine and concluded that adsorption of the pollutant on clay minerals reduced the rate of biodegradation and bioavailability. Sorensen et al. (2006) studied the adsorption and
biodegradation of phenoxic acid herbicide 4-chloro-2-methylphenoxy-acetic acid (MCPA) in soil and subsurface samples from a sandy agricultural site and a clay rich till in Denmark. The authors observed that MCPA adsorbed to a minor extent and was mineralized rapidly in most samples, except in the deepest layers at both sites. No relation was found between adsorption and mineralization for this herbicide. Interestingly, the highest extent of mineralization of MCPA occurred in the top soil which coincided with the largest adsorption and lowest desorption. These results described new evidence that adsorbed herbicides may be accessible for biodegradation.

The influence of varying redox environments on the fate of herbicides in the soil have also been explored adequately. Pravecek et al. (2006) monitored the changes in bioavailability of pyrene in three uncontaminated soils under aerobic and anaerobic conditions. The results showed that, under aerobic conditions, microorganisms mineralized 58–82% of the added pyrene. Additionally, the soils amended with nitrate were seen to have enhanced aerobic mineralization rates. In one of the studied soils, non-extractable pyrene residue was seen to decrease over the course of the study probably due to desorption and mineralization. These observations have important implications relative to the influence of microbial electron acceptors on bioavailability and transport of xenobiotic compounds. Similarly, the dissipation of dimethenamid was also influenced significantly by the anaerobic redox conditions that may develop in the soil environment (Crawford et al. 2002). Anaerobic degradation of the herbicide occurred in all non-sterile treatments, with a T_{1/2} of 13-14 days. More than half of the applied parent compound was eventually incorporated into soil-bound residue. Vink & van der Zee (1997) showed that the insecticide aldicarb was transformed more rapidly under anaerobic than under aerobic conditions.
Conversely, Harrison et al. (2003) found that methanogenic, sulphate-reducing or iron-reducing conditions in the soil have no impact on the biodegradation of the herbicide mecoprop. Larsen & Amand (2001) also showed a non-substantial mineralization of mecoprop, isoproturon, atrazine, and metsulphuron-methyl in denitrifying, sulphate-reducing, and methanogenic soil environmental conditions when compared to well-aerated situations.

Overall, these studies show that while adsorption-desorption processes affect bioavailability and degradation in the soil; there is a clear indication of the influence of soil redox regime and microbial electron acceptors on bioavailability and fate of xenobiotic compounds in the environment.
### 2.4 Tables

**Table 2.1: Selected physical and chemical properties of metolachlor and glyphosate**

<table>
<thead>
<tr>
<th>Properties</th>
<th>Metolachlor</th>
<th>Glyphosate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical Structure</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td><img src="image" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>CAS number</td>
<td>51218-45-2</td>
<td>1071-83-6</td>
</tr>
<tr>
<td>Chemical name</td>
<td>2-Chloro-N-(2-ethyl-6-methyl-phenyl)-N-(1-methoxypropan-2-yl)acetamide</td>
<td>N-(phosphonomethyl) glycine</td>
</tr>
<tr>
<td>Empirical formula</td>
<td>C$<em>{15}$H$</em>{22}$ClNO$_2$</td>
<td>C$_3$H$_8$NO$_5$P</td>
</tr>
<tr>
<td>Molecular weight (g mol$^{-1}$)</td>
<td>283.80</td>
<td>169.08</td>
</tr>
<tr>
<td>Water solubility (mg L$^{-1}$ at 25 °C)</td>
<td>488 to 550$^a$</td>
<td>10,000 to 15,700$^a$</td>
</tr>
<tr>
<td>Vapor pressure (mm Hg at 25 °C)</td>
<td>3.1 x 10$^{-5}a$</td>
<td>4.3 x 10$^{-10}a$</td>
</tr>
<tr>
<td>Octanol-water coeff. ($K_{ow}$)</td>
<td>2.6 to 3.28$^a$</td>
<td>-4.6 to -1.6$^a$</td>
</tr>
<tr>
<td>Freundlich adsorption coeff. ($K_{ads}$) (L Kg$^{-1}$)</td>
<td>1.3 to 26.7$^b$</td>
<td>0.6 to 303$^c$</td>
</tr>
<tr>
<td>Photolysis half-life (days)</td>
<td>8 to 37$^d$</td>
<td>Not substantial</td>
</tr>
<tr>
<td>Degradation half-life in soil (T$_{1/2}$) (days)</td>
<td>15 to 132$^d$</td>
<td>7-60$^e$</td>
</tr>
<tr>
<td>EPA maximum contamination level (µg L$^{-1}$)</td>
<td>100$^a$</td>
<td>700$^a$</td>
</tr>
</tbody>
</table>

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2.5 References


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redox conditions." Chemosphere **44**: 231-236.


CHAPTER 3

Impact of aerobic and anaerobic soil conditions on the microbial bioavailability of

$^{14}$C-metolachlor

3.1 Abstract

Although there has been extensive information on bioavailability factors of the globally used herbicide $^{14}$C-metolachlor under aerobic soil conditions such properties in strict anaerobic conditions have not been investigated thoroughly. This study was undertaken to unravel the ambiguity associated with the effects of aerobic and anaerobic soil conditions on the availability and degradation of the herbicide $^{14}$C-metolachlor in three soils with varying soil properties. Anaerobic conditions in the soil significantly reduced the adsorption of $^{14}$C-metolachlor and improved its desorption and thereby release of the herbicide from soil. Furthermore, first order degradation and mineralization kinetics of $^{14}$C-metolachlor were distinctively enhanced in anaerobic conditions in all the soils tested. Degradation and mineralization rate of $^{14}$C-metolachlor in non-sterilized versus sterilized soil microcosms clearly implicate microbial activity in the degradation of metolachlor in soil. Increased detection of reduced Fe over the course of the microcosm incubations provided suggestive evidence for the involvement of biologically mediated iron reducing organisms in the anaerobic fate of metolachlor. Soil redox conditions do impact the herbicide metolachlor from the perspective of its bioavailability and environmental transport.

*Key words:* metolachlor herbicide, bioavailability, adsorption, desorption, biodegradation, mineralization, aerobic soil, anaerobic soil
3.2 Introduction

Despite growing awareness of soil redox status in driving soil processes, little progress has been made in characterizing the influence of anaerobic conditions and soil saturation on bioavailability factors like adsorption, desorption, and biodegradation of the extensively used herbicide metolachlor. These factors serve as an important consideration in the risk assessment of soil applied chemicals like herbicides as only a small fraction reach the intended target (Pimentel, 1995). Excess herbicide can have adverse effects on human, animal and crop health (Devos et al., 2008; Hiller et al., 2008). Adsorption of herbicide onto soil particles reduces their release into the soil solution, but simultaneously limits herbicide availability to microorganisms for degradation. Degradation affects the concentration of pesticide residues in soils, thereby controlling their persistence in soils. Herbicide adsorption and degradation is determined in part by the physical and chemical properties of the herbicide, such as organic-carbon and mineral-surface adsorption coefficients, aqueous solubility, and soil dissipation half-life (Savoca et al., 2000). Different processes determining the persistence and bioavailability of herbicides in the soil may be directly or indirectly coupled and may respond differently to varying environmental conditions like redox status, making the net effect on overall herbicide fate challenging to predict (Sims & Cupples, 1999).

Terrestrial soil environments are typically considered to be dominated by aerobic conditions, but there are situations when anaerobiosis prevails. Most soil anaerobiosis is caused by high soil moisture resulting from a high water table or heavy rains. Such that, even normal soils have significant periods of anoxia caused by a high water table in the spring and following heavy rainfalls resulting in temporary anaerobic sites (Sextone et al., 1985). Understanding the effects of anaerobic conditions is critical so that the bioavailability of a compound and its
biogeochemical transport in the environment may be more accurately assessed. Based on soil type, adsorption-desorption properties and oxidation–reduction conditions, the herbicide transport in soil may be decreased or enhanced and previously non-available material may become bio-available.

Metolachlor [2-chloro-N-(2-ethyl 6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide] is a soil-applied chloracetanilide herbicide widely used for pre-emergence and post-emergence weed control in a variety of crops including corn and soybean. Environmental fate studies of metolachlor have attracted worldwide apprehension due to its prolonged persistence in soil (Accinelli et al., 2003), high water solubility and the significant toxicological properties (USEPA, 1988). Adding to the concerns, a recent water monitoring program acknowledged metolachlor among the prominent chemical contaminants in drinking water throughout the United States (EWG, 2009). In spite of the pressing demand, research on the environmental behavior of this universally used herbicide has largely focused on its fate and degradation in aerobic soil environments (Rice et al., 2002). Like several other homocyclic herbicides containing halogens, metolachlor tends to undergo slower degradation kinetics under aerobic conditions than anaerobic conditions (Alexander & Lustigman, 1966). Conversely, in anaerobic soils, reductive dehalogenation may initiate a more complete degradation process for metolachlor (Stamper & Tuovinen, 1998). These established facts necessitate the need for a more thorough investigation on the degradation and mineralization of metolachlor under anaerobic soil environmental conditions.

Metolachlor is a neutral non-ionizable herbicide dominated by apolar groups. The adsorption mechanism has been frequently described by the interactions of the herbicide with soil organic matter (Ghosh & Singh, 2012). Although many studies have been published on the
adsorption of metolachlor to soils (Krutz et al., 2004; Si et al., 2009; Bedmar et al., 2011), few attempts were made to elucidate the models to predict herbicide adsorption and transport in anaerobic soil environments. Adsorption is often considered a critical process that governs and regulates herbicide degradation in soil (Ainsworth et al., 1993). Lack of information on the adsorption and desorption patterns of metolachlor in anaerobic soil environments means we cannot explain the patterns in the degradation and fate of metolachlor in soils. Therefore, to understand the microbial fate of metolachlor in soils it is critical to study both aerobic and anaerobic conditions experimentally.

When soil becomes saturated, increased biological and chemical oxygen demand will result in the rapid depletion of O2. Anaerobiosis results in a succession of microbial heterotrophic communities adapted to utilize a variety of potential electron acceptors as conditions become suitable for their activities. Nitrate, one such electron acceptor in the anaerobic soil is generally available in relatively limited quantities, even in fertilized soils, and can be depleted within one or two days of submergence of a surface soil. Typically, after complete depletion of nitrate, soils and sediments undergo iron-reducing conditions (Tor et al., 2000). Mounting evidence suggests that that many soil processes are the result of coupled microbial functions (Lovely, 2001). Microbial process occurring in anoxic zones in soil, such as iron reduction may be coupled to herbicide degradation. Several dinitroaniline herbicides (Tor et al., 2000), as well as alachlor and atrazine (Xu et al., 2001) appear to be degraded by microbially reduced iron minerals. The prospect of utilizing biologically mediated iron reduction for the accelerated detoxification and degradation of metolachlor in soils requires immediate research.

Treatment of different soils under anaerobic and aerobic conditions would give an opportunity to explore the uncertainty in the bioavailability and metabolism of popular herbicide
like metolachlor. Then, that knowledge could be applied towards more efficient metolachlor use and frame a viable strategy for the efficient clean-up of soils contaminated by spills of this herbicide. The present study was undertaken to i) investigate the bioavailability determinant factors like adsorption and desorption of $^{14}$C-metolachlor in different types of soils treated under aerobic and anaerobic environmental conditions ii) examine the microbial bioavailability indicators like degradation and mineralization of $^{14}$C-metolachlor in aerobic and anaerobic soils and iii) determine the role of iron reduction in the microbial fate of the herbicide in soil.

3.3 Materials and methods

3.3.1 Chemicals

Uniformly ring labeled $^{14}$C-metolachlor (specific activity: $2.04 \times 10^9$ Bq mmol$^{-1}$) was obtained (Moravek Biochemicals and Radiochemicals, Brea, CA). Unlabeled metolachlor (chemical purity: 99%) was also procured (Sigma chemical company, St. Louis, MO). Organic solvents and water were of Optima grade (Fisher Scientific, Pittsburgh, PA) and used without further purification.

3.3.2 Soils

Three types of soil (Catlin, Flanagan and Drummer) belonging to a soil catena sequence were used for the study. The soils were collected (to a depth of 15 cm) from University of Illinois Crop Sciences Research and Education Center in Urbana, IL. All soil samples were sieved through a 2 mm screen and stored at 4°C for duration of four weeks. Relevant physical and chemical properties of the three soil types used in this study were listed in Table 3.1.
3.3.3 Adsorption-desorption study

Adsorption isotherms of $^{14}$C-metolachlor were determined using the batch equilibrium method for three soil types. The initial concentrations of $^{14}$C-metolachlor (0.1, 1, 5 and 10 mg L$^{-1}$) were prepared in 0.01 M aqueous CaCl$_2$ solution as recommended by the EPA guidelines for adsorption studies (USEPA, 2008). 2 g of air dried soil was equilibrated with 10 ml of $^{14}$C-metolachlor solution in 20 ml Teflon centrifuge tubes in a horizontal shaker (150 rpm) for 24 hr at room temperature (25 ± 1°C). For anaerobic treatment, similar 2 g soil contained in the Teflon centrifuge tubes was flooded with sterile-deoxygenated water. The tubes were flushed with N$_2$ gas, sealed and incubated in an anaerobic chamber, at room temperature (25°C) for 2 weeks to allow complete reduction. To study the adsorption, $^{14}$C-metolachlor was added to the reduced soil (anaerobic system) to attain the final concentrations of 0.1, 1, 5 and 10 mg L$^{-1}$. Sealed tubes were equilibrated on a shaker inside the anaerobic chamber for 24 h, where the oxygen was maintained at zero concentration.

At the end of the equilibration period, the soil suspension was centrifuged (15 min, 12000g) and the aliquots removed from the each tube for radioactivity assay using a Packard Tri-Carb (1900TR) scintillation counter. Controls (treatment without herbicide) were included for calibration and background correction purposes. The amount adsorbed was calculated from the difference between the initial and final concentrations of herbicide in solution. All the experiments were performed in triplicate.

Following equilibration and removal of 5 ml of the initial 10 ml of supernatant, herbicide desorption from soil was estimated by adding equal amounts of fresh 0.01 M CaCl$_2$ solution to the centrifuge tubes, dispersing the soil aggregates by vibration and re-shaking for 24 h. Sampling from the anaerobic soil treatments were handled inside an anaerobic glove box.
chamber. Soil samples were centrifuged (15 min, 12000g) and an aliquot of the supernatant was removed and analyzed by radioactivity assay. The desorption process was repeated 4 times. Desorption is estimated by determining the amount of herbicide in the soil solution following equilibration and calculated by subtracting the amount still adsorbed to soil (Si et al., 2009).

### 3.3.4 Degradation study

**Microcosm incubation**

Soil incubations were performed under reduced (anaerobic) or oxidized (aerobic) conditions using serum bottle microcosms to determine the degradation kinetics of $^{14}$C-metolachlor.

**Anaerobic incubations**: Microcosms, consisting of serum bottles (60 ml) were amended with soil (10 g) and were spiked with ring labeled $^{14}$C-metolachlor (specific activity of 2.37 x $10^3$ Bq µmol$^{-1}$, diluted with unlabeled metolachlor) in 50 µl of methanol to produce a final concentration of 5 mg Kg$^{-1}$ of soil. This concentration corresponded to the recommended agriculture application rate. The metolachlor-spiked soils were agitated on a reciprocating shaker for 24 h at room temperature to ensure thorough mixing and to evaporate the solvent. The soil was then flooded with 20 ml of sterile-deoxygenated water. The microcosm headspace was flushed with N$_2$ gas, immediately crimp sealed with a butyl stopper that was fitted with a vial containing 1 ml of 0.5 M NaOH to trap the mineralized $^{14}$CO$_2$. These microcosms were incubated in a dark, temperature controlled chamber at 25°C. Sterilized soil microcosms were included as a control for each soil types. Sterilization was achieved by autoclaving the soils twice at 121°C for 1 h.

**Aerobic incubations**: Soil microcosms were built from serum bottles as described above. Sterile distilled water was added to the metolachlor-spiked soils to adjust the moisture content to about 60% of the field water-holding capacity (WHC). The serum bottles were lightly capped with a
butyl stopper fitted with NaOH trap and stored in dark at 25°C. Soil moisture content was adjusted weekly by weighing and adding sterile distilled water.

**Sampling and analysis**

Anaerobic and aerobic microcosms were destructively sampled on consecutive intervals by removing the NaOH trap, followed by agitating the microcosm for 1 min, and transferring the contents to a 50 ml Teflon centrifuge tube. Quantification of $^{14}$CO$_2$ in NaOH traps were accomplished by direct liquid scintillation spectrometry (LSS) using a Packard Tri-Carb (1900TR) scintillation counter. The solid and liquid phases of the soil slurry were then separated by centrifugation (15 min, 12000g). Aqueous samples were removed, filtered (0.2 µm) and total aqueous radioactivity was estimated using LSS. A portion of the aqueous aliquot was stored at -20°C for subsequent nitrate and aqueous Fe$^{2+}$ analyses. Soil was extracted with 20 ml acetone/water mixture (90/10, v/v) in a Teflon centrifuge tube with horizontal shaking for 24 h. Extracts were centrifuged at 12,000 rpm for 15 min, an aliquot was removed for LSS (to quantify extractable radioactivity) and the supernatant was retained for analysis of metolachlor and degradation products.

For all treatments, triplicate soil extracts from each sampling day were combined, evaporated and re-suspended in methanol for the HPLC analysis. Soil extract samples containing $^{14}$C-metolachlor were analyzed using HPLC equipped with a Hewlett-Packard 1050 series autosampler instrument, using flow scintillation detector (Packard Radiomatic Flo-one/beta). Separation was achieved with an isocratic elution of the mobile phase composed of acetonitrile: water (50:50, v/v) through a 4.6 x 150 mm, 5 µm particle size, C$_{18}$ column (Prontosil, Chadds Ford, PA). Metolachlor had a reproducible retention time of 13.4 min at a flow rate of 1 ml min$^{-1}$ under these conditions.
Non-extractable radioactivity (bound residue) was determined on samples that had been already extracted with acetone/water mixture (90/10, v/v). After the soil was dried and pulverized with a mortar and pestle, non-extractable $^{14}$C-labeled residues were quantified by combustion at 900°C (Biological Oxidizer, OX500, R. J. Harvey Instruments, Hillsdale, NJ).

**Analysis of nitrate and Fe$^{2+}$**

Nitrate was determined by improvising the method of Sims et al. (1995). Triplicate aliquots (250 µl) of the aqueous sample were added to a 96-well plate (Corning No. 25860, Corning, NY) containing 15-25 mg of Devarda’s alloy (mesh size 0.05-0.2 mm, EM Science, Gibbstown, NJ). To avoid volatilization loss of NH$_3$, 10 µl of 0.2 M H$_2$SO$_4$ were added to samples in each well containing the alloy. Plates were sealed and incubated up to 5 h to facilitate the reduction of nitrate to ammonia. After being reduced, sample aliquots of 25 µl from each well (avoiding Devarda’s alloy) received 25 µl of citrate reagent followed by 50 µl of salicylate reagent and 25 µl of hypochlorite reagent. The plate was agitated for 30 min for color development and absorbance was measured at 650 nm with a CERES Model UV900HDi plate reader (Bio-Tek Instruments, Winooski, VT).

Aqueous Fe$^{2+}$ formation (indicator of Fe$^{3+}$ reduction) was estimated with a microplate modification of the Lovely & Philips (1987) ferrozine method. A 250 µl aliquot of an aqueous sample was pipetted into a 96 well plate, and 15 µl of the ferrozine reagent (Sigma Chemical Co., St. Louis, MO) was added. The plate was mixed for 1 min on a vortex mixer fitted with a micro plate adapter and allowed 10 min for color development. The absorbance was measured at 590 nm.
3.3.5 Data analysis

Adsorption and desorption parameters of metolachlor in aerobic and anaerobic conditions for each soil type were calculated using the transformed Freundlich equation: \( \log C_s = \log K + \frac{1}{n} \log C_e \), where \( C_s \) is the amount of glyphosate adsorbed to the soil (mg Kg\(^{-1}\)), \( C_e \) is the equilibrium concentration in the soil solution (mg L\(^{-1}\)) and \( K \) and \( 1/n \) are empirical constants that reflect the affinity of the soil for the herbicide and the degree of linearity between the amount adsorbed and the solution concentration, respectively. Regression analysis was performed on adsorption and desorption isotherms to calculate \( K \) (intercept) and \( 1/n \) (slope) values of metolachlor in aerobic and anaerobic soils. Hereafter, \( K_{ads} \) and \( 1/n_{ads} \) will indicate Freundlich parameters for adsorption, \( K_{des} \) and \( 1/n_{des} \) will refer to desorption parameters.

The degradation data of metolachlor in soils were fitted into the first-order kinetics model \( C_t = C_0 e^{-kt} \) where \( C_0 \) is the initial concentration (mg Kg\(^{-1}\) soil) of the herbicide in the soil, \( C_t \) is the herbicide concentration (mg Kg\(^{-1}\) soil) detected in the soil at time \( t \), and \( k \) is the first-order rate constant. Degradation rate constants were calculated by linear regression of the natural logarithm of the percentage of herbicide remaining against the time. Aerobic and anaerobic degradation half-life (\( T_{1/2} \)) for each soil type was calculated using the equation \( T_{1/2} = \ln 2/k \). The statistical program SAS v.9.3 was used to calculate the treatment means and standard errors (n = 3). The differences between treatments were evaluated using one-way analysis of variance (ANOVA) followed by least significant difference test at \( p < 0.05 \).
3.4 Results

3.4.1 Adsorption - desorption

The Freundlich adsorption coefficient ($K_{ads}$) and slope ($1/n_{ads}$) along with the corresponding coefficients of determination ($R^2$) are given in Table 3.2. The results from the experiment adequately fit into the Freundlich isotherm ($R^2 > 0.96$) for the range of herbicide concentrations from 0.1 to 10 mg L$^{-1}$ in all the soil types under both aerobic and anaerobic soil conditions. The $1/n_{ads}$ values ranged from 0.69 to 0.91 among the soils and the treatments. $K_{ads}$ of the metolachlor in the soils kept under aerobic and anaerobic conditions ranged from 1.23 to 5.85 and 1.04 to 4.54 L Kg$^{-1}$ respectively. The anaerobic soil environment had significantly lower ($p < 0.05$) $K_{ads}$ values throughout the studied soil types. Further, $K_{ads}$ was lowest in Catlin and highest for Drummer soils for aerobic and anaerobic conditions.

Desorption isotherms for metolachlor in all the soils and environmental conditions also fit well with the Freundlich equation ($R^2 > 0.80$). The calculated Freundlich coefficients for desorption ($K_{des}$) of metolachlor in the aerobic and anaerobic soils are presented in Table 3.3. $K_{des}$ values of metolachlor were lower ($p < 0.05$) in anaerobic soils when compared to the aerobic soil treatments. Among the three soils tested, $K_{des}$ values of metolachlor were highest for Drummer soil irrespective of the redox conditions.

3.4.2 Degradation

Degradation parameters of $^{14}$C-metolachlor in the Catlin, Flanagan and Drummer soil microcosms incubated under aerobic and anaerobic environmental conditions are summarized in Table 3.4. The coefficients of regression ($R^2$) of natural logarithm of the percentage of initial compound remaining in the soil against time ranged from 0.82 to 0.95 and were statistically
significant \( (p < 0.05) \) demonstrating that the degradation of metolachlor followed the first-order reaction kinetics model. Generally, in all the soils studied, the half-life \( (T_{1/2}) \) for metolachlor in anaerobic soils (65 to 69 days) was significantly lower than the corresponding aerobic half-lives (117 to 154 days). The degradation kinetics of \(^{14}\)C-metolachlor in non-sterilized soils incubated under aerobic and anaerobic conditions in Catlin, Flanagan and Drummer soils is depicted in Figure 3.1 a, b, c.

Among the different soil types used in the experiment, Drummer had the lowest \( T_{1/2} \) for metolachlor both under aerobic and anaerobic soil conditions (Table 3.4). Although the \( T_{1/2} \) of the herbicide in Catlin and Flanagan were comparable for anaerobic incubation, metolachlor disappearance was marginally faster in Catlin soil under aerobic incubation. Altogether the microcosms with non-sterilized soils exhibited substantially greater degradation of the metolachlor than the corresponding sterilized control microcosms despite their dissimilarities in the soil type and redox conditions.

3.4.3 Microbial mineralization

Figure 3.1 d, e, f illustrates the microbial mineralization pattern of metolachlor measured as the amount of \(^{14}\)CO\(_2\) evolved from aerobic and anaerobic non-sterilized soils over time in Catlin, Flanagan and Drummer soils. Under both soil incubations, there was a lag phase of at least 8 weeks before the evolution of mineralized \(^{14}\)CO\(_2\). About 8-11% of the applied herbicide was mineralized in the soils maintained under aerobic incubation, whereas 13-16% of the metolachlor was converted to \(^{14}\)CO\(_2\) in the anaerobic microcosm after 140 days of incubation (Table 3.4). No significant evolution of \(^{14}\)CO\(_2\) was measured in any of the sterilized control soils. Amongst the various soil types used, Drummer exhibited the highest mineralization rate under aerobic and anaerobic soil environmental conditions.
3.4.4 Non extractable residue (NER) formation

Non-extractable residues (NER) were formed in all the microcosms despite the soil redox conditions (Figure 3.2). At the end of the incubation period an appreciably larger amount (47-62%) of the applied parent compound was incorporated as NER into anaerobic soils than into the aerobic soils (27 - 39%). NER formation in the non-sterilized soil microcosms exceeded their corresponding sterilized control microcosms irrespective of the soil type and environmental conditions. However, this disparity was more distinct in the soils kept under anaerobic conditions. Drummer soil exhibited maximum NER accumulation at the end of the incubation period in both aerobic and anaerobic soils.

3.4.5 Nitrate and iron flux in soil

The amount of nitrate and Fe$^{2+}$ in the soil during the degradation of the $^{14}$C-metoachlor was quantified at regular intervals. The accumulation of Fe$^{2+}$ was observed immediately following the disappearance of nitrate in all the anaerobic microcosms irrespective of soil types. Depletion of nitrate or accumulation of Fe$^{2+}$ was never observed in any of the aerobic soil treatments. In anaerobic soils, metolachlor exhibited biphasic kinetics with an increase in degradation and mineralization rate coinciding with Fe$^{2+}$ accumulation (Figure 3.3).

3.5 Discussion

Adsorption - desorption

The $K_{ads}$ values of the metolachlor from this study were within the range of published metolachlor adsorption coefficients of 1.0 to 26.7 L Kg$^{-1}$ (Seybold & Mersie, 1996; Zhu & Selim, 2000; Patakioutas & Albanis, 2002; Krutz et al., 2004). Lower $K_{ads}$ indicate less adsorption affinity of the herbicide to soils. The isotherm slopes ($1/n_{ads}$) of less than 1 for all the
soils, implies a non-linearity between the amount of herbicide adsorbed and the concentration in the applied solution. This clearly suggests the potential for metolachlor leaching particularly at higher application rates.

In both aerobic and anaerobic soil environmental conditions, metolachlor adsorption to soils was highest in Drummer followed by Flanagan and was lowest in the Catlin soil. Such adsorption increase corresponds to an increase in the soil organic matter content (Table 3.1) and emphasizes the involvement of organic matter in the adsorption process of metolachlor, which has been already communicated in previous studies (Wu et al., 2011; Ghosh & Singh, 2012). Moreover, the difference in adsorption between anaerobic and aerobic soils was more prominent in the soils with relatively higher organic matter content.

Another remarkable observation made from the study is the noticeable reduction in the adsorption of $^{14}$C-metolachlor to the anaerobic soils as evident from their respective low $K_{des}$ values compared to the aerobic incubations. There are some explanations proposed for reduced adsorption for herbicides in anaerobic soils. One theory suggests that modifications to functional groups on soil organic matter under anoxic soil conditions causes reduced adsorption (Clausen et al., 2004). Another explanation for less herbicide adsorption refers to the decrease in inorganic surface area caused by the reduction of ferric to ferrous ions (Wahid & Sethunathan, 1980). However, the fact that the decline in the anaerobic adsorption was greater in the soil with higher organic matter tends to support the former explanation.

The calculated desorption coefficient ($K_{des}$) of metolachlor from the current study was in agreement with the previous values of 1.3 to 6.5 L Kg$^{-1}$ (Seybold & Mersie, 1996) and 5.9 to 51.6 L Kg$^{-1}$ (Zhu & Selim, 2000). Relatively lower $K_{des}$ values in anaerobic treatments compared to aerobic treatments in all the tested soils indicate that the release of the chemical following the
adsorption step was enhanced in the anaerobic/reduced soils. Such desorption patterns of the herbicide could improve its bioavailability and subsequently expedite the degradation or bioactivity of the herbicide in soil. Furthermore, metolachlor desorption decreased with increase in organic matter content, regardless of the soil environmental conditions again, suggesting a role for soil organic matter in desorption kinetics of metolachlor in soils.

The intriguing observation of the adsorption - desorption patterns of metolachlor in anaerobic soils suggests that soil redox is one of the key factors governing these processes in soil. The decreased adsorption of metolachlor to anaerobic soils may have significant implications from the standpoint of its bioavailability and environmental transport. For instance, weak adsorption suggests that metolachlor may have a high potential to degrade on site under anaerobic conditions or is more likely to be released into the solution if soil sediments enter the lakes or other water bodies.

**Degradation**

Results from the present study suggests an enhanced degradation of $^{14}$C-metolachlor under anaerobic conditions when compared to aerobic conditions in all the soil types despite differences in soil properties, as evident from the remaining fraction of applied parent compound in the soil at the end of the study as well as their degradation half-life ($T_{1/2}$) values (Table 3.4). Chesters et al. (1989) reported half-lives of metolachlor ranging from 36 to 182 days under aerobic soils conditions and is in agreement with the aerobic half-life for the herbicide in the current experiment. Seybold (2001) reported a 62 day half-life for metolachlor in anaerobic sediments, which is on par with the anaerobic $T_{1/2}$ values obtained from the present study. Other studies report an apparent fluctuation in the degradation half-life of metolachlor depending on
the soil type and environmental conditions and are estimated to be between 15 – 132 days (Kollman & Segawa, 2000).

One of the earliest reports of the impact of soil aeration on the degradation of metolachlor was from Walker and Brown, where they observed a reduction in the half-life of the herbicide in soil with an increase in soil saturation (Walker & Brown, 1985). More recently, Rice et al. (2002) reported a similar trend in the degradation rate of metolachlor, where the first order $T_{1/2}$ was substantially shorter for the saturated surface soils when compared to the unsaturated soils. Accinelli et al. (2005) also observed a reduction in the half-life of metolachlor in flooded soil (24.1 days) when compared to non-flooded soils (32.2 days). In yet another study, faster degradation of metolachlor was observed in flooded soils with partial anaerobic soil conditions as compared to aerobic soils (Sanyal et al., 2000). Conversely, there are also reports of a slight suppression of degradation of the metolachlor under anaerobic or reduced soil conditions (Mulbah et al., 2000).

The enhanced degradation of metolachlor from anaerobic or flooded microcosms may be explained by microbial contributions to the degradation mechanisms. Flooding is characterized by strongly reducing conditions, which are compatible with the activity of strict anaerobic microorganisms (Accinelli et al., 2005). Such an anoxic environment in the soil creates a conducive habitat for the microorganisms to perform specific reactions, whereby the metolachlor is actively degraded. Stamper & Tuovinen (1998) suggested anaerobic dehalogenation as one such viable microbial mechanism for the transformation of certain chloroacetanilide herbicides in soils incubated under anaerobic conditions. Additionally, the significant decrease of degradation rates in the autoclaved control soils reiterates the dominance of microbial pathways.
in the degradation and metabolism of metolachlor in soils of various properties and environmental conditions.

Metolachlor tends to be more persistent in the soils with lower organic matter content as observed from the negative correlation between the organic matter content of the soil and the degradation half-life of the herbicide in both the aerobic and anaerobic microcosms. This result highlights the importance of organic matter in determining the fate of applied herbicide regardless of the soil environmental conditions. The enhanced degradation of metolachlor in soils with increasing organic matter content has been summarized in previous studies (Rice et al., 2002). Researchers attribute this aspect to the augmentation of herbicide degrading microbial populations benefitting from an increase in organic matter content in the soil.

**Microbial mineralization**

Mineralization results in the complete aerobic or anaerobic degradation of the herbicide compounds to form carbon dioxide, water and some other inorganic elements. Anaerobic soil environmental conditions induced higher mineralization of $^{14}$C-metolachlor irrespective of the types of soils used as evident from the increasing $^{14}$CO$_2$ measurements from the soil microcosms. Mineralization data of the $^{14}$C-metolachlor from the aerobic microcosms in the present study were in agreement with the observations from Kotoula-Syka et al. (1997) where they reported $\leq 8\%$ cumulative mineralization of $^{14}$C-metolachlor in cultivated soils. Krutz et al. (2006) reported a higher cumulative mineralization of 17.6 % during the degradation of metolachlor in cultivated soils. Contrary to the current observation, Rice et al. (2002) noted that the mineralization of $^{14}$C-metolachlor was minimal (<2%) in both saturated and unsaturated soils. Similar conclusions of very low mineralization of the metolachlor herbicide have been reported previously (Henderson et al., 2007).
Some researchers suggest that metolachlor is not readily mineralized in soil, and requires a lag period before the initiation of herbicide mineralization (Miller, 1992; Rice, 1996). Researchers agree on the fact that repeated application of herbicides could abate such lag periods from occurring during their mineralization (Fenlon et al., 2007). Long term application history of a herbicide in the field could enable the survival and acclimation of herbicide degrading microbial populations in soil assuring the rapid mineralization of the applied herbicide without any delay (Cheyns et al., 2012).

The observations on the evolution of $^{14}$CO$_2$ during the mineralization of $^{14}$C-metolachlor confirm the cleavage of the labeled phenyl ring in the metolachlor molecule during the microbial metabolism of the herbicide. However, to the best of our knowledge, no efforts have been done yet to establish the identity of the microorganisms able to mineralize metolachlor in anaerobic soil. Gathering such information on the microorganisms involved in the substrate utilization of metolachlor is imperative for developing a better understanding of the dissimilarities in the degradation and mineralization kinetics observed in aerobic and anaerobic soils.

**Non-extractable residue (NER) formation**

NER represent compounds in the soil which persists in the matrix in the form of the parent substance or its metabolite(s) after extraction. NER formation is typically considered as a process decreasing the bioavailability of the herbicides, as consistent data suggests that only a small percentage of the total amount of NER can be released. Hence, NER formation is often considered as the irreversible sink in herbicide risk assessment procedures.

Nevertheless, massive NER accumulation in anaerobic microcosms from this study, could be ascribed to the enhanced degradation of $^{14}$C-metolachlor under saturated soil conditions and the subsequent binding of degradates to the soil. Considering the rapid degradation of the
herbicide resulted in a vast formation of NER, it could be deduced that it is the metabolite that has been incorporated into the soil matrix as irreversibly bound residue (Barriuso et al., 2008). Rice et al. (2002) mentioned a significantly higher formation of the $^{14}$C-metolachlor bound residues in the saturated soil when compared to unsaturated soil for both surface and subsurface soils. Furthermore, Miller et al. (1997) found that metolachlor degradation in the aerobic top soil coincided with an increase in the bound residue formation and accounted for about 30% of the applied herbicide at the end of experiment. The occurrence of significantly higher NER in the non-sterilized soils further reinforce this conclusion and suggests the involvement of microorganisms in the metabolism of $^{14}$C-metolachlor in both aerobic and anaerobic soils.

**Role of Fe$^{3+}$ reducing conditions**

The accumulation of Fe$^{2+}$ subsequent to depletion of nitrate was anticipated in the anaerobic soil environment (Tor et al., 2000). The enhanced degradation of metolachlor in the presence of Fe$^{2+}$ in anaerobic soils suggests a probable role (direct or indirect) of Fe in fate of metolachlor under anaerobic soil environmental conditions. This possibility is further validated by the rapid mineralization of $^{14}$C-metolachlor corresponding with the appearance of Fe$^{2+}$ in anaerobic soils (Figure 3.3). Similar findings signifying the role of Fe$^{2+}$ on the transformation of organic pollutants has been shown previously (Klausen et al., 1995; Tor et al., 2000). These new findings necessitate further investigations for determining whether the active organisms involved in the herbicide degradation are iron-reducing bacteria, or other microorganisms with some other reduction reaction capable of degrading metolachlor.

**3.6 Conclusions and future directions**

The influence of soil environmental conditions like aeration on the microbial bioavailability factors of the widely used herbicide metolachlor was evaluated in the present study. Results from
the study provided ample evidence for the enhanced bioavailability and microbial biodegradation of metolachlor in the anaerobic soil systems. Adsorption of metolachlor to the soils was noticeably reduced in anaerobic soils, as evident from their respective Freundlich adsorption coefficients. Similarly, relatively lower Freundlich desorption constants for metolachlor in the anaerobic soils irrespective of their varying soil properties suggested a greater release of the adsorbed herbicide from the soil under anaerobic conditions. These results demonstrated that anaerobic conditions significantly influenced the metolachlor retention pattern in the soils and should be considered when designing a remediation plan for the clean-up of this herbicide in the environment. Anaerobic soil incubations induced better degradation and mineralization of metolachlor in the range of soils evaluated, despite differences in soil properties. Among the various soil types used in the experiment, Drummer soil with the highest organic matter content exhibited the highest rate of degradation and mineralization for metolachlor both under aerobic and anaerobic soil conditions. The non-sterilized soil microcosms exhibited substantial degradation and mineralization of the metolachlor compared to the corresponding sterilized control microcosms in spite of incubation conditions. These facts accentuate the dominance of microbial pathways in the degradation and metabolism of metolachlor in the soils in spite of differences in soil properties and environmental conditions. An extensive quantity of non-extractable residues (NER) was formed in all the soil microcosms regardless of the soil type or incubation environment and was attributed to metolachlor metabolite formation.

The anaerobic degradation and mineralization of metolachlor coincided with the iron reducing conditions in soil. Although, these new findings were suggestive of the role of iron reducing conditions on the anaerobic fate of metolachlor in soils, it necessitates additional information for determining whether the active organisms involved in the herbicide metabolism
are iron-reducing bacteria. Additionally, distinctive differences in the degradation and mineralization kinetics of metolachlor in aerobic and anaerobic soil environments suggest the need for a thorough investigation of microbial involvement in the fate of the herbicide when soil undergoes changes in redox status.
3.7 Tables

**Table 3.1:** Selected properties of the soils used in the experiment

<table>
<thead>
<tr>
<th>Soil</th>
<th>pH</th>
<th>Texture (%)</th>
<th>WHC (%)</th>
<th>CEC (Cmol·Kg⁻¹)</th>
<th>Organic matter (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sand</td>
<td>Silt</td>
<td>Clay</td>
<td></td>
</tr>
<tr>
<td>Catlin</td>
<td>7.6</td>
<td>10</td>
<td>58</td>
<td>32</td>
<td>31.4</td>
</tr>
<tr>
<td>Flanagan</td>
<td>6.5</td>
<td>12</td>
<td>56</td>
<td>32</td>
<td>32.0</td>
</tr>
<tr>
<td>Drummer</td>
<td>7.1</td>
<td>14</td>
<td>46</td>
<td>40</td>
<td>33.0</td>
</tr>
</tbody>
</table>

**WHC** water holding capacity @ 1/3 bar  
**CEC** cation exchange capacity
Table 3.2: Adsorption (Freundlich model) of $^{14}$C-metolachlor in different soil types under aerobic (Aer) and anaerobic (An) environmental conditions

<table>
<thead>
<tr>
<th>Soil</th>
<th>$K_{ads}$</th>
<th>$1/n_{ads}$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aer</td>
<td>An</td>
<td>Aer</td>
</tr>
<tr>
<td>Catlin</td>
<td>1.23$^e$ (±0.04)$^*$</td>
<td>1.04$^f$ (±0.02)</td>
<td>0.69 (±0.03)</td>
</tr>
<tr>
<td>Flanagan</td>
<td>3.50$^c$ (±0.05)</td>
<td>2.16$^d$ (±0.03)</td>
<td>0.82 (±0.03)</td>
</tr>
<tr>
<td>Drummer</td>
<td>5.85$^a$ (±0.08)</td>
<td>4.54$^b$ (±0.07)</td>
<td>0.78 (±0.03)</td>
</tr>
</tbody>
</table>

$K_{ads}$ Freundlich adsorption coefficient
$1/n_{ads}$ adsorption isotherm slope
$R^2$ goodness of fit for Freundlich model
$^*$ mean values followed by the same letter superscripts are not significantly different ($p < 0.05$)
$^*$ values in the parentheses are the 95% confidence intervals
Table 3.3: Desorption (Freundlich model) of $^{14}$C-metolachlor in different soil types under aerobic (Aer) and anaerobic (An) environmental conditions

<table>
<thead>
<tr>
<th>Soil</th>
<th>$K_{des}$</th>
<th>$1/n_{des}$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aer</td>
<td>An</td>
<td>Aer</td>
</tr>
<tr>
<td>Catlin</td>
<td>3.11$^c$† (±0.04)$^*$</td>
<td>2.75$^f$ (±0.03)</td>
<td>0.21 (±0.002)</td>
</tr>
<tr>
<td>Flanagan</td>
<td>7.63$^c$ (±0.03)</td>
<td>5.52$^d$ (±0.03)</td>
<td>0.21 (±0.006)</td>
</tr>
<tr>
<td>Drummer</td>
<td>11.13$^a$ (±0.04)</td>
<td>9.95$^b$ (±0.03)</td>
<td>0.16 (±0.002)</td>
</tr>
</tbody>
</table>

$K_{des}$ Freundlich desorption coefficient  
$1/n_{des}$ desorption isotherm slope  
$R^2$ goodness of fit for Freundlich model  
† mean values followed by the same letter superscripts are not significantly different ($p < 0.05$)  
*$ values in the parentheses are the 95% confidence intervals
Table 3.4: Degradation (first-order kinetics) and mineralization of $^{14}$C-metolachlor in different soil types under aerobic (Aer) and anaerobic (An) environmental conditions

<table>
<thead>
<tr>
<th>Soil</th>
<th>$k$ (day$^{-1}$)</th>
<th>$T_{1/2}$ (days)</th>
<th>$R^2$</th>
<th>Degradation$^+$</th>
<th>Mineralization$^#$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aer</td>
<td>An</td>
<td>Aer</td>
<td>An</td>
<td>Aer</td>
</tr>
<tr>
<td>Catlin</td>
<td>0.0048 (0.0018)*</td>
<td>0.0093 (0.0017)</td>
<td>144$^{b\dagger}$ (388)</td>
<td>75$^{d}$ (415)</td>
<td>0.88 (0.85)</td>
</tr>
<tr>
<td>Flanagan</td>
<td>0.0045 (0.0014)</td>
<td>0.0088 (0.0010)</td>
<td>154$^{a}$ (487)</td>
<td>79$^{d}$ (675)</td>
<td>0.92 (0.57)</td>
</tr>
<tr>
<td>Drummer</td>
<td>0.0059 (0.0022)</td>
<td>0.0107 (0.0024)</td>
<td>117$^{c}$ (314)</td>
<td>65$^{e}$ (288)</td>
<td>0.82 (0.86)</td>
</tr>
</tbody>
</table>

$k$ rate constant
$T_{1/2}$ degradation half-life
$R^2$ goodness of fit for first order degradation model
$^+\%$ of applied herbicide remaining after 140 days of incubation
$^#$% of applied herbicide evolved as $^{14}$CO$_2$ after 140 days of incubation
*values in the parentheses in each column represent the corresponding values for sterilized soil control
$^\dagger$mean values followed by the same letter superscripts are not significantly different ($p < 0.05$)
ND: not detected
3.8 Figures

Figure 3.1: Degradation kinetics (a, b, c) and mineralization pattern (d, e, f) of $^{14}$C- metolachlor in aerobic (Aer) and anaerobic (An) soil conditions in Catlin, Flanagan and Drummer soils. Error bars represents standard errors (n=3).
**Figure 3.2:** Non-extractable residue accumulated during the degradation of $^{14}$C- metolachlor in aerobic (Aer) and anaerobic (An) soil conditions in different soil types. Error bars represents standard errors (n=3).
Figure 3.3: Time sequence for the flux of electron acceptors (a), $^{14}$C-metolachlor degradation (b) and mineralization (c) in aerobic (Aer) and anaerobic (An) soil (Catlin). Error bars represents standard errors (n=3).
3.9 References


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CHAPTER 4

Identification of metolachlor mineralizing bacteria in aerobic and anaerobic soils using DNA-stable isotope probing

4.1 Abstract

Soil microcosms were incubated aerobically or anaerobically and received herbicide treatments with unlabeled metolachlor, $^{13}$C-metolachlor or $^{14}$C-metolachlor. Maximum mineralization of metolachlor was observed from the $^{14}$C-metolachlor treated microcosms after 28 days of incubation and clearly demonstrated the active utilization of the herbicide as a carbon source with increased degradation in anaerobic incubations. Terminal restriction fragment length polymorphisms (T-RFLP) bacterial community profiling performed on soil DNA extracts after 28 days indicated that fragment 307 bp from aerobic soil and 212 bp from anaerobic soil were detected only in the herbicide treated (both $^{12}$C-metolachlor and $^{13}$C-metolachlor) soils when compared to the un-treated control microcosms. The extracted DNA from unlabeled ($^{12}$C-metolachlor) and labeled ($^{13}$C-metolachlor) herbicide treated soil samples were subjected to ultracentrifugation, followed by fractionation of the centrifuged samples. T-RFLP profiles from the ultracentrifugation fractions illustrated that these characteristic fragments experienced an increase in relative abundance at higher buoyant density (BD) in the labeled fractions, when compared to the unlabeled herbicide amendment fractions. The shift in BD of characteristic T-RFLP fragments in the density resolved fractions suggested the incorporation of $^{13}$C from labeled herbicide into the bacterial DNA and enabled the identification of organisms responsible for metolachlor uptake from the soil. Subsequent cloning and 16S rRNA gene sequencing of the $^{13}$C-enriched fractions implicated the role of organisms closely related to *Bacillus* spp. in aerobic and members of *Acidobacteria* phylum in anaerobic mineralization of metolachlor in soils.
Key words: metolachlor herbicide, stable isotope probing, mineralization, aerobic, anaerobic

4.2 Introduction

The influence of soil environmental factors such as aeration on the ecology of microorganisms involved in the mineralization and degradation of a popular herbicide like metolachlor is unknown. To address this knowledge gap, we used stable isotope probing (SIP). SIP is a novel approach that exploits microbial biomarkers to link the identity of microorganisms to specific functions like the degradation of soil contaminants or herbicides. The most common biomarkers utilized in SIP include DNA, RNA, and lipids (Sims, 2008). However, SIP has been generally targeted at DNA and accordingly the term SIP is synonymously used by some researchers for the probing of nucleic acids (Radajewsky et al., 2003). Most nucleic acid based SIP studies have targeted the small ribosomal subunit (16S rRNA) gene (Radajewski et al., 2000; Padmanabhan et al., 2003), though a growing body of evidence supports the utility of this method for specific functional genes (Morris et al., 2002, Miller et al., 2004).

SIP utilizes growth substrates labeled with a heavier isotope of carbon (typically $^{13}$C). Carbon naturally occurs in three isotopes: $^{12}$C at approximately 98.9% abundance, stable $^{13}$C at approximately 1.1% abundance, and radioactive $^{14}$C at abundance several orders of magnitude less than $^{12}$C. The SIP method typically involves incubation of an environmental sample harboring a complex microbial community with an exogenously added $^{13}$C labeled substrate. Organisms that are capable of growth on the labeled substrate during the incubation will produce nucleic acid labeled with $^{13}$C. DNA is then extracted from organisms in the environmental sample. The extracted DNA is subjected to ultracentrifugation which separates labeled (‘heavy’) from unlabeled (‘light’) nucleic acids by differences in buoyant density in a density gradient medium. From recovered DNA fractions, microbial gene targets can then be amplified by PCR.
based methods for 16S rRNA genes or functional genes. A variety of molecular techniques are available to characterize the resulting labeled DNA from the density resolved fractions. Cloning, then sequencing the 16S rRNA genes for identifying substrate-degrading organisms is the most popular method (Radajewski et al., 2000; Morris et al., 2002; Padmanabhan et al., 2003; Hutchens et al., 2004). Whole community profiling of amplified 16S rRNA genes have also been accomplished by denaturing gradient gel electrophoresis (DGGE) (Whitby et al., 2001; Singleton et al., 2005) and terminal restriction fragment length polymorphisms (T-RFLP) (Kunapuli et al., 2007). However T-RFLP, followed by cloning and sequencing is gaining wide acceptance for the identification of microbial degraders (Lueders et al., 2004). With T-RFLP, whole communities from soil are displayed as a series of DNA fragments, which correspond to specific bacterial populations. Populations degrading the labeled substrate can be determined by observing the shift in the buoyant density (BD) of the same fragment in the samples spiked with $^{12}$C versus $^{13}$C- labeled substrate.

A significant advantage to SIP, unlike many other nucleic acid based methods, is that only active organisms are targeted. The method was first introduced to investigate methanol-utilizing microorganisms in soil (Radajewski et al., 2000), since then, the method has been successfully utilized to investigate the active organisms in the aerobic degradation of an array of compounds including phenol (DeRito et al., 2005), pentachlorophenol (Mahmood et al., 2005) and herbicides like 2,4-D (Cuples & Sims, 2007) and atrazine (Shaffer et al., 2010). The utility of this technique in diverse environmental samples like soils (Radajewski et al., 2000; Padmanabhan et al., 2003), sediments (Lin et al., 2004) and ground water (Hutchens et al., 2004) has been successfully demonstrated. Recently, Cupples (2011) reviewed the methods and key
results of the studies that have used SIP to specifically investigate anaerobic degradation of environmental contaminants.

Metolachlor [2-chloro-N-(2-ethyl 6-methylphenyl)-N-(2-methoxy-1-methylethyl) acetamide], a selective chloroacetamide herbicide, is extensively used throughout the globe for the control of broadleaf and annual grassy weeds in a wide range of crops such as corn, soybean, peanut, potato, and tobacco (Extoxnet, 2000). Studies involving mixed and pure cultures have confirmed that microbial transformations are the essential mechanisms responsible for the degradation of metolachlor (Krause et al., 1985; Liu et al., 1989). Scanty attempts were made to date to assess the microbial responses in soil during the degradation of metolachlor, using molecular approaches. Recently, Si et al., (2009) utilized 16S rRNA gene DGGE to observe changes in microbial communities with varying soil depth and duration of metolachlor incubation. In contrast to the considerable knowledge on aerobic metolachlor degradation (Saxena et al., 1987), much less is known about microbial metabolisms involved in anaerobic metolachlor degradation. To date no microorganisms have been isolated or characterized with the ability to anaerobically transform metolachlor.

Despite the limited information on metolachlor degrading microorganisms, many researchers have reported metolachlor degradation under a range of soil types and environmental conditions (Kollman & Segawa 2000; Rice et al., 2002). Moreover, our recent investigation on the metolachlor fate in soil clearly indicated the enhanced degradation and mineralization of the herbicide in anaerobic soil incubations when compared to aerobic incubations in a range of soils, despite the differences in their physico-chemical properties (Kanissery & Sims, unpublished data). Gathering information on the microbial degraders of metolachlor is critical for understanding the dissimilarities in the degradation and mineralization kinetics observed in
aerobic versus anaerobic soils. SIP may aid in determining whether the same or different organisms function in aerobic versus anaerobic conditions during the degradation of metolachlor. Any such information could be used for predicting herbicide efficacy and persistence in agricultural soils enduring such environmental conditions. Previous research in our group successfully demonstrated the use of SIP for the identification of microorganisms involved in the degradation of various isotopically enriched compounds (Cupples & Sims 2007; Shaffer et al., 2010). Here we investigate the microorganisms responsible for the aerobic and anaerobic mineralization of the herbicide metolachlor in soil by using $^{13}$C-DNA targeted stable isotope probing (SIP).

4.3 Materials and methods

4.3.1 Chemicals

Uniformly ring labeled $^{13}$C-metolachlor (99%, 100 µg ml$^{-1}$ in nonane) was obtained from Cambridge Isotope Laboratories, Inc (Andover, MA). Unlabeled metolachlor (chemical purity: 99%) and $^{14}$C-labeled metolachlor (ring-$^{14}$C, >98%, 100µCi ml$^{-1}$ in ethanol) were procured from Sigma Chemical Company, St. Louis, MO and Moravek Biochemicals (Brea, CA) respectively. Organic solvents and water were of Optima grade (Fisher Scientific, Pittsburgh, PA) and used without further purification.

4.3.2 Practical considerations for the SIP of metolachlor

The success of SIP relies on the labeling efficiency, which, in turn depends on the rate of biomass synthesis and extent of label incorporation into the nucleic acids. High concentrations of the labeled substrate are often used to maximize the extent of label incorporation and therefore the chances of detecting labeled biomass may be enhanced. The documented maximum
intentional field application rate for metolachlor is 9 mg Kg$^{-1}$ soil (Rice et al., 2002), assuming incorporation throughout the top 15 cm of the soil profile. Since metolachlor is surface-applied onto the soil and not typically incorporated, the concentration can be much higher than this in the surface 7.5 cm early in the growing season. We designed a surface applied application that was meant to mimic metolachlor that has been moved by rain into the top 7.5 cm in an area of overlap between spray applications, resulting in a soil concentration of 50 mg Kg$^{-1}$ soil for the SIP incubation. Though elevated, this concentration could realistically occur at some point of overlap between spray applications early in the growing season.

Such a high application rate for the microcosm studies of metolachlor is not unusual. For instance, prior reports point towards the incorporation of metolachlor to the tune of 90 mg Kg$^{-1}$ soil in a microbial degradation study of metolachlor. The authors did not observe any activity inhibition of metolachlor degrading microorganisms at this high level of concentration (Rice et al., 2002). Moreover, incubations involving large concentrations of the herbicides provide valuable insights into herbicide degrading microbes in adverse soil situations ranging from uneven application to mishandling and spills of herbicides (Dzantor & Felsot, 1991). Furthermore, Shaffer et al. (2010) observed a consistent relationship between substrate concentration and detectable increase in DNA buoyant density and established a minimum threshold of labeled substrate enrichment necessary for the separation of light and heavy DNA.

4.3.3 Soil Incubations

Soil was collected (to a depth of 15 cm) from the University of Illinois Crop Sciences Research and Education Center in Urbana, IL and stored at 4°C for duration of four weeks. Soil samples (Drummer soil, 5mm sieved) were incubated aerobically and anaerobically in serum bottles with either $^{13}$C-metolachlor, $^{14}$C-metolachlor or unlabeled metolachlor at the rate of 50 mg Kg$^{-1}$ soil.
Triplicate microcosms were incubated in a dark, temperature controlled chamber at 25°C. Control microcosms without the herbicide amendments were also incubated correspondingly. Moisture content in the soil was adjusted to 60% water-filled pore space for aerobic incubations. Deoxygenated water was used to flood the soil in anaerobic microcosms. The microcosm head-space was flushed with oxygen free N₂ gas, then immediately sealed with a butyl septum to achieve anaerobic conditions. Triplicate ¹⁴C metolachlor microcosms were destructively sampled at regular intervals (based on preliminary studies) for monitoring mineralization rates by trapping ¹⁴CO₂ in a vial containing 1 ml of 0.1 M NaOH, which was subsequently analyzed using liquid scintillation spectrometry (Tor et al., 2000). The microbial mineralization of herbicide was routinely measured from the ¹⁴C-metolachlor microcosms maintained under aerobic and anaerobic incubation conditions. DNA extractions from ¹³C-metolachlor and unlabeled metolachlor soil microcosms were performed when the herbicide mineralization rates approached maximum for the corresponding incubations.

4.3.4 DNA extraction, ultracentrifugation and fractionation

Triplicate soil microcosms were destructively sampled from the control, labeled (¹³C-metolachlor) and unlabeled (¹²C-metolachlor) microcosms near maximal mineralization rates, which occurred at day 28 for both aerobic and anaerobic soil incubation. The FastDNA SPIN Kit for soil (MP Biomedicals, Santa Ana, CA) was used to extract DNA from 0.5 g of soil following manufacturer’s instructions. DNA extracted from the labeled and unlabeled incubations were pooled and added to Quick seal polyallomer centrifuge tubes (13 x 51mm, 5.1 ml, Beckman Coulter, Brea, CA), along with CsCl solution (Tris-EDTA, pH 8.0). Ultracentrifugation was performed on each DNA sample using an Optima LE-80K preparative ultracentrifuge (Beckman Instruments) equipped with a VTi 65.2 vertical tube rotor for 48 h at an acceleration of 184,000
$g_{av}$ (20°C). After centrifugation, water was introduced with a precision pump (model PHD 2000, Harvard Apparatus, Holliston, MA) into the top of the centrifuge tube and fractions (350 µl each) were collected from the bottom of the tube (Cupples & Sims, 2007). Buoyant densities (BD) of each fraction were then measured with a model AR200 digital hand-held refractometer (Leica Microsystems Inc.). Subsequently, CsCl from the fractions was removed using glycogen-assisted ethanol precipitation (Cupples & Sims, 2007) and purified DNA from the fractions was stored at -20°C.

4.3.5 T-RFLP analysis and sequencing

Both the total soil DNA extracts and fractions obtained after ultracentrifugation were analyzed by 16S rRNA gene terminal restriction fragment length polymorphism (T-RFLP) following a standard protocol (Liu et al., 1997). DNA was amplified using the PCR primers 27F-FAM, 5’ end labeled with carboxyflourescein (5’-AGAGTTTGATCMTGGCTCAG-3’) and 1492R (5’-GGTTACCTTGGTTACGACTT-3’) (Cupples & Sims, 2007). The PCR reaction mix (50 µl) included the following: 1µl DNA template; 1X PCR buffer (Clontech Laboratories Inc., Mountain View, CA, USA), 25 µg/mL T4 gene 32 protein (Roche Applied Science, Indianapolis, IN, USA), 0.25 mM of each dNTP, 0.4 µM each primer (27F-FAM and 1492R), 0.025 U/µL TaKaRa Ex Taq DNA polymerase (Clontech). The PCR thermal cycling program was: 94°C (5 min); 94°C (30 s); 55°C (30 s); 72°C (1.5 min) (25cycles); 72°C (10 min). The resulting PCR products were purified with the QIAquick® PCR purification kit (Qiagen Inc, Valencia, CA). The purified PCR products from all the fractions were digested with the restriction enzyme HaeIII following the manufacturer’s guidelines (New England Biolabs, Ipswich, MA). Additionally, the $^{13}$C fractions of soil DNA extracts were digested with HhaI and RsaI (New England Biolabs). The digested DNA samples were analyzed using capillary
electrophoresis (model 3730xl Genetic Analyzer, W.M. Keck Center for Comparative and Functional Genomics, Urbana, IL). The percent abundance of fragments present in the labeled and unlabeled fraction was determined using GeneMarker (Version 2.4.1) software (Soft Genetics, State College, PA). Only fragment peaks that underwent a shift in the presence of $^{13}$C-metolachlor, as compared to buoyant density observed with $^{12}$C-metolachlor, were considered further.

The DNA fractions containing the specific fragment showing the shift for $^{12}$C and $^{13}$C aerobic and anaerobic fractions (n=4) were PCR amplified for 16S rRNA genes as described above except using a forward primer in PCR without the FAM label (27F). The amplicons were gel purified (Ultra Clean 15 DNA Purification Kit, MoBio, Carlsbad, CA), ligated into pGEM$^\text{T-Easy}$ vector (Promega, Madison, WI) and transformed into E. coli JM109 competent cells (Promega). 96 clones were grown from each fraction. The 16S rRNA gene was amplified directly from each clone using the FAM labeled primers. Clones were screened for the T-RF of interest by T-RFLP analysis as performed for the soil fractions. Those clones with the peaks of interest were screened by RFLP of the 16S rRNA gene (Sambrook & Russel, 2001). Clones with unique RFLP patterns for each peak of interest were sequenced on 2 strands using the vector primers T7 and Sp6 (W. M. Keck Center). The sequences obtained were analyzed using GENEIOUS 5.3.4 (Biomatters Ltd, Auckland, New Zealand) and sequence homology was compared with the NCBI GenBank sequence database (Benson et al., 2011). The Ribosomal Database Project was used to align clone sequences with closely related pure cultures (Cole et al., 2009; Meadow et al., 2014). Distance matrices and neighbor joining phylogenetic analyses with 1000 bootstrap replicates were generated using MEGA v4 (Tamura et al., 2007). The clone sequences were deposited in GenBank with accession numbers KJ606962 to KJ606968.
4.4 Results

4.4.1 Metolachlor mineralization

Anaerobic soil environmental conditions induced higher mineralization of $^{14}$C-metolachlor as evident from the $^{14}$CO$_2$ measurements from the microcosms (Figure 4.1). About 20% of the applied herbicide was mineralized in the soils maintained under aerobic incubation, whereas 31% of the metolachlor was converted to $^{14}$CO$_2$ in the anaerobic soil after 35 days of incubation. After day 28, the mineralization rate of the herbicide started showing signs of plateauing irrespective of the incubation conditions. Another noteworthy observation is the absence of a lag phase prior to the emergence of $^{14}$CO$_2$.

4.4.2 T-RFLP of soil DNA

Subsequent to the metolachlor mineralization, DNA was extracted from the aerobically and anaerobically incubated soil microcosms amended with labeled ($^{13}$C-metolachlor) and unlabeled ($^{12}$C-metolachlor) herbicide after 28 days incubation. Similarly, DNA was extracted from the corresponding control microcosms to which no herbicide is added. Terminal restriction fragment length polymorphism (T-RFLP) was performed on these DNA extracts and the T-RFLP data from the HaeIII enzyme digestion were then used to compare the relative abundance of fragments from the herbicide amended and control treatments. In aerobic soil microcosms, the fragment 307 was found to be abundant only in the herbicide amended soils when compared to un-amended control soil. Likewise, in anaerobic soil microcosms, fragment 212 was detected only in the herbicide added soils (Figure 4.2).

4.4.3 Stable Isotope Probing (SIP)

The extracted DNA from labeled ($^{13}$C-metolachlor) and unlabeled ($^{12}$C-metolachlor) herbicide amended soil samples were pooled and subjected to ultracentrifugation, followed by
fractionation of the centrifuged samples. Subsequently, T-RFLP was performed on each fraction, and relative abundance of each fragments were determined. Most of the fragments were distributed identically throughout the gradient profile for both labeled and unlabeled metolachlor microcosms and displayed no characteristic peak shift indicative of incorporation of the label (Figure 4.3 a, b). However, fragment 307 from the aerobic soil treatment experienced an increase in relative abundance at higher buoyant density (BD) in the labeled fractions, when compared to the unlabeled herbicide amendment fractions (Fig 4.3 c). Similar shifts in the BD due to the incorporation of $^{13}$C from the labeled metolachlor into the DNA was also observed in anaerobic soil treatments when fragment 212 showed a shift in peak abundance at a heavier BD (Fig 4.3 d).

4.4.4 Phylogenetic identification of metolachlor degraders

The specific microbial degraders of metolachlor represented by the detected T-RFLP fragments within unlabeled and labeled DNA fractions were subsequently identified by cloning and sequencing. The closest relatives of these clone sequences from the aerobic and anaerobic soil incubations were determined using BLAST in the GeneBank database and independent clone libraries were constructed from aerobic and anaerobic incubations (Figure 4.4). Clones from the aerobic incubations were most similar to members of phylum Firmicutes (Bacillus spp.). Clones from anaerobic incubations exhibited close similarity to members of the phylum Acidobacteria. Sequence similarity of the 16S rRNA gene of 97% or greater generally indicates bacteria of the same species (Stackebrandt et al., 2002). Most clones from the aerobic incubations could be assigned to specific bacterial species with 97 to 99% 16S rRNA gene similarity to described species (Figure 4.4). No clones, however, from the anaerobic incubations could be confidently assigned to specific species based on 16S rRNA gene sequence similarity with clone
An1_12C_T212 95% similar to bacterium Ellin 371 and clone An10_13C_T212 only 86% similar to *Holophaga foetida* (Figure 4.4). Clones from $^{12}$C and $^{13}$C incubations with the same T-RFLP in aerobic incubations were 91-99% similar to each other and from anaerobic incubations clones were 79% similar.

$^{13}$C enriched soil DNA heavy fractions from the aerobic and anaerobic incubation treatments were further digested using additional restriction enzymes (HhaI and Rsal) and yielded characteristic fragments for each enzyme. These fragments were compared to the *in silico* digests of the 16S rRNA gene sequences of clones from $^{13}$C heavy fractions to validate the clones they represented (Table 4.1). *In silico* restriction fragments from soil clones were easily identified in T-RFLP peaks in $^{13}$C DNA fractions from soil microcosms confirming the presence of organisms representing these clones in the metolachlor degrading soil community. The slight differences between the observed fragment size in soil DNA terminal restriction fragments (T-RFs) and those predicted from the *in silico* digests of the clone sequence data has been reported previously (Osborn et al., 2000; Cupples & Sims, 2007).

### 4.5 Discussion

**Mineralization**

SIP requires the microbial DNA biosynthesis and cell replication for labeling to occur, hence necessitates the active utilization of labeled moiety of the substrate as carbon source. The effectiveness of metolachlor as a carbon source has been confirmed previously by several authors following the evolution of $^{14}$CO$_2$ from soils applied with radioactively ring labeled herbicide. Liu et al. (1988) observed that 18.4% of the added $^{14}$C metolachlor was mineralized from the soil as $^{14}$CO$_2$. Krutz et al. (2006) also reported a cumulative mineralization of 17.6% during the degradation of metolachlor in cultivated soils. Similar observations on the evolution
of $^{14}$CO$_2$ corroborate the involvement of labeled phenyl ring cleavage of metolachlor during the microbial metabolism of the herbicide.

Many reports in the literature suggest that metolachlor is not readily mineralized in soil, clearly demonstrating a lag period before the initiation of herbicide mineralization (Miller, 1992; Rice, 1996). However, the lack of lag phase in the current study could be attributed to the elevated concentration of the metolachlor in the soil incubations. The addition of high concentrations of substrate can make the incubation, in fact, an enrichment assuring the rapid microbial mineralization of the applied herbicide without any delay (Radajewski et al., 2002).

**Stable Isotope Probing (SIP)**

The shift in buoyant density of characteristic T-RFLP fragments in the density resolved fractions allowed the detection of label incorporation into the DNA and hence enabled the identification of organisms responsible for metolachlor uptake from the soil. Previously, several authors have effectively demonstrated the utilization of T-RFLP profiles of 16S rRNA genes from ultracentrifugation fractions to identify the putative degraders of xenobiotic compounds in soil (Cuppies & Sims, 2007) and enrichment cultures (Kunapuli et al., 2007). The detection of the target fragments in metolachlor amended soils compared to un-amended control soils implied the positive response of the organisms represented by these fragments to the herbicide amendments. The trend was similar for the identified fragments in both aerobic and anaerobic incubations. Interestingly, the specific fragment from the anaerobic soils exhibited a higher relative abundance than the corresponding target fragment from the aerobic soil incubations. This is most likely due to the limited diversity of microorganisms and fewer number of T-RFLP peaks in the presence of anaerobic growth conditions (Tiedje et al., 1984).
Clones with the same T-RFLP peak in both $^{12}\text{C}$ and $^{13}\text{C}$ incubations had very similar sequence suggesting that the isotopic signature of the metolachlor amendments did not affect the community response to treatment. Recovering sequences from similar organisms in both $^{12}\text{C}$ and $^{13}\text{C}$ amended microcosms supports the assertion that these organisms degrade metolachlor. 16S rRNA gene sequences obtained from clones from the aerobic incubations with $^{13}\text{C}$-metolachlor and $^{12}\text{C}$-metolachlor exhibited close sequence similarity to microorganisms from the genus *Bacillus*. This observation validates the involvement of same or closely related organisms in the utilization of metolachlor as a carbon source from both labeled and unlabeled herbicide incubations. The organisms from the soils treated with labeled metolachlor had a higher DNA buoyant density owing to the incorporation of $^{13}\text{C}$ into their biomass due to the uptake and mineralization of $^{13}\text{C}$-metolachlor. Additionally, in line with our findings *Bacillus circulans* and *B. megaterium* were found to have substantial role in the degradation of metolachlor in pure culture experiments (Saxena et al., 1987).

The 16S rRNA gene sequences of metolachlor degrading organisms identified from anaerobic incubations belonged to phylum *Acidobacteria* and were not associated previously with metolachlor degradation. However, *Holophaga foetida*, the closest relative of the clone sequence from anaerobic soil incubations treated with $^{13}\text{C}$-metolachlor has been shown to oxidize benzene in Fe reducing conditions (Anderson et al., 1998). Moreover, increasing evidence suggests that members of *Acidobacteria* play an important role in iron (Fe) redox reactions in soil (Ward et al., 2009) as they have been found to dominate 16S rRNA gene sequence libraries from Fe-rich mine environments (Blothe et al., 2008). This information provides evidence to link the anaerobic mineralization of metolachlor to Fe reduction in the soil and corroborates our previous findings that a Fe reducing regime in soils apparently enhanced
the degradation and mineralization kinetics of metolachlor in anaerobic soils (Kanissery & Sims, unpublished).

Some potential caveats need to be acknowledged in this SIP study. One important concern is that long incubation times can increase the risk of cross-feeding of $^{13}$C from the primary consumers to the rest of the community. We are hopeful that transfer of $^{13}$C between species may not have occurred by day 28. This is supported by the fact that we did recover sequences similar to *Bacillus* spp. shown to be metolachlor degraders (Saxena et al., 1987) in both $^{12}$C and $^{13}$C treatments. Another SIP technique concern is to make sure the identified DNA is indeed $^{13}$C-incorporated and not simply G-C rich (which also increases the density of DNA) or the result of contamination. Nevertheless, this effect is expected to only occur in incubations using very low $^{13}$C – substrate concentrations that may impede the successful labeling of the microbial biomass (Dumont & Murrell, 2005). Contrarily, the relatively large dose of labeled herbicide added to the soil in the current experiment could select the populations not representative at natural substrate concentrations. Additionally, the clear shifts in T-RFLP peak abundance with increasing buoyant density in $^{12}$C versus $^{13}$C amended microcosms (*Figure 4. 3 c, d*) strongly support the incorporation of the $^{13}$C into microbial DNA.

The 16S rRNA gene sequences of aerobic and anaerobic metolachlor mineralizing microorganisms identified in our study belong to known pure culture degraders of the herbicide as well as novel organisms not previously associated with metolachlor degradation. Similar identification of previously undiscovered bacteria for an array of compounds has been achieved by utilizing SIP (Kasai et al., 2006; Borodina et al., 2005; Cupples & Sims, 2007). To best of our knowledge, this is the first attempt to investigate the microorganisms assimilating metolachlor directly in soils. Hence, the current study remains profoundly pertinent, since any information on
the microorganisms utilizing metolachlor in aerobic or anaerobic soils would offer unprecedented insights into the herbicide metabolism in soils. Future research can make use of these data for tailoring a viable remediation strategy for the soils contaminated by metolachlor or other allied chloracetanilide herbicides.
### 4.6 Tables

**Table 4.1**: Comparison of 16S rRNA gene clone T-RF cut sites predicted (*in silico*) from sequence analysis to the T-RFLP fragments in the $^{13}$C fractions from soil microcosms.

<table>
<thead>
<tr>
<th>Clones*</th>
<th>Clone <em>in silico</em> T-RF cut sites (bp)</th>
<th>Soil DNA fraction T-RFLP (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HaeIII† HhaI RsaI</td>
<td>HaeIII HhaI RsaI</td>
</tr>
<tr>
<td><strong>Aer</strong> incubation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aer11_13C_T307 and Aer12_13C_T307</td>
<td>309 240 474</td>
<td>307 237 473</td>
</tr>
<tr>
<td><strong>An</strong> incubation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>An10 _13C_T212</td>
<td>214 221 487</td>
<td>212 224 489</td>
</tr>
</tbody>
</table>

*Clones from the $^{13}$C-metolachlor enrichment fractions
† Restriction enzymes used
4.7 Figures

Figure 4.1: Time course of $^{14}$CO$_2$ evolution during the degradation of $^{14}$C-metolachlor in aerobic (Aer) and anaerobic (An) soils. Arrows indicate the days of sampling for stable isotope probing analysis. Error bars represent standard errors (n=3).
Figure 4.2: Comparison of the relative abundance of T-RF fragments in metolachlor amended and un-amended microcosm soils incubated under aerobic (Aer) and anaerobic (An) conditions.
**Figure 4.3**: The distribution of specific T-RF fragments with increasing buoyant density (BD) in ultracentrifuged fractions of DNA extracted from microcosms amended with either labeled (\(^{13}\)C-metolachlor) or unlabeled (\(^{12}\)C-metolachlor) herbicide incubated under aerobic (Aer) and anaerobic (An) soil environments. Fig a & b shows the general trend in distribution of the fragments throughout the gradient profile with no shift in characteristic fragments with increasing BD. Fig c & d depicts the shift in characteristic fragments across the BD due to the incorporation of \(^{13}\)C from the labeled metolachlor.
Figure 4.4: Phylogenetic trees showing the affiliation of representative bacterial 16S rRNA gene clone sequences from $^{12}$C-metolachlor and $^{13}$C-metolachlor enrichments generated from the density gradient fractions of DNA from aerobic (Aer) and anaerobic (An) soil incubations. Clone sequences are indicated in bold and are named for soil incubations (Aer or An), herbicide treatments ($^{12}$C or $^{13}$C) and T-RF peaks of interest (307 or 212 bp). Numbers on branches indicate bootstrap support measures for that branch. The scale bar represents 1% and 10% sequence divergence, respectively. GenBank accession numbers of reference sequences are indicated.
4.8 References


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CHAPTER 5

Effect of soil aeration and phosphate addition on the microbial bioavailability of

\(^{14}\text{C}\)-glyphosate

5.1 Abstract
The consequences of aerobic and anaerobic soil conditions on the key microbial bioavailability factors of the universally applied herbicide glyphosate were investigated in the current study. The impact of phosphate addition on these factors was also explored under these diverse soil conditions. The adsorption, desorption, degradation and mineralization of \(^{14}\text{C}\)-glyphosate was examined in Catlin, Flanagan and Drummer soils under aerobic and anaerobic soil conditions. With the exception of the Drummer soil, soil aeration did not significantly alter the adsorption pattern of \(^{14}\text{C}\)-glyphosate to soils. Herbicide desorption was generally enhanced with anaerobiosis in all the soil types. Anaerobic soils demonstrated slower microbial degradation and mineralization kinetics of \(^{14}\text{C}\)-glyphosate than aerobic soils across the range of soil types studied. Phosphate additions significantly reduced the adsorption of \(^{14}\text{C}\)-glyphosate to soils irrespective of the soil aeration and confirmed the well-established competitive adsorption theory. The addition of soil phosphate only stimulated degradation in anaerobic soils. The results from this research highlight the importance of soil redox conditions as an important factor affecting the bioavailability and mobility of glyphosate in soils.

Key words: glyphosate herbicide, adsorption, desorption, biodegradation, mineralization, phosphate addition, aerobic soil, anaerobic soil
5.2 Introduction

The investigations on the bioavailability factors of the universally used herbicide glyphosate have largely focused on an aerated soil environment. However, factors like adsorption, degradation and mineralization of glyphosate fluctuate with the soil aerobic and anaerobic conditions, properties and composition (Sorensen et al., 2006; Laitinen et al., 2006). Although terrestrial soil environments can be considered to be dominated by aerobic conditions, there are situations when anaerobiosis prevails. For instance, a large application of manure or compaction of the soil could change the rate of oxygen consumption or supply and bring about anaerobiosis (Tiedje et al., 1984). Most soils have significant periods of anoxia caused by a high water table in the spring and following heavy rainfalls resulting in temporary anaerobic sites (Sextone et al., 1985). Moreover, a growing body of evidence suggests that anaerobic soil conditions could alter the mineralogical and surface properties of soil (Stucki et al., 2002). These alterations could affect the bioavailability and mobility of herbicides in such soil conditions (Xu et al., 2001). These fluctuations in bioavailability warrant a more thorough investigation into the chemical and biological fate of glyphosate under both aerobic and anaerobic soil conditions.

Glyphosate [N-(phosphonomethyl)glycine] has been one of the world's most widely applied herbicides since it originated on the market in 1974 (Franz et al., 1997). The continued extensive use of glyphosate places concern on its environmental safety and have stimulated numerous studies on its behavior in the soil. The route of degradation of glyphosate on the laboratory scale is well documented and is mainly determined by microbial processes (Gimsing et al., 2004). In contrast to other non-ionic herbicides, glyphosate possesses unique adsorption characteristics in soil. It has three polar functional groups (carboxyl, amino and phosphonate groups) and hence, soil minerals rather than organic matter play a significant role in the strong
adsorption of glyphosate to soils. In most soils glyphosate forms mono and divalent anions exhibiting high affinity for trivalent cations such as Al$^{3+}$ and Fe$^{3+}$ in the soil (Sheals, 2002). Glyphosate has been considered as environmentally safe due to its rapid inactivation in soil, both by degradation and by adsorption (Quinn et al., 1988). Despite copious information presenting glyphosate as environmentally benign, some newer investigations indicate possible leaching and toxicity problems associated with the persistent usage of this herbicide (Borggaard & Gimsing 2008).

Soil phosphate plays an important role in determining the bioavailability and fate of glyphosate in soil. Researchers attribute this phenomenon to the phosphonate group in the glyphosate molecule and similarity in mechanisms by which glyphosate and phosphate are adsorbed to the soil solids (Gimsing & Borggaard, 2002). The impact of phosphate on glyphosate adsorption in soil was communicated shortly after the herbicide’s launch into the market (Sprankle et al., 1975). Later several studies have confirmed the competitive adsorption of glyphosate and phosphate as well as its substantial variability in various soils (Hance, 1976; Wang et al., 2005). Competition for adsorption sites between glyphosate and phosphate therefore seems vital and may have severe consequences on glyphosate mobility, and hence bioavailability. Competition may be especially important in many agricultural soils in the USA and elsewhere that are saturated or nearly saturated with phosphate as a result of excessive phosphorus fertilization over many years (Hart et al., 2004). Nevertheless, the impact of such competitions on the microbial bioavailability of glyphosate in aerobic and anaerobic soil redox conditions has not been adequately addressed.

This project investigated the effects of aerobic and anaerobic soil environments on bioavailability factors like adsorption, desorption, degradation and mineralization of $^{14}$C-
glyphosate in a range of soils. Additionally, the influence of soil phosphate on bioavailability factors of 14C-glyphosate in aerobic and anaerobic soils was determined.

5.3 Materials and methods

5.3.1 Chemicals

14C-Glyphosate (phosphonomethyl-14C) (specific activity: 1.85 x 10⁹ Bq mmol⁻¹) was obtained from American radiolabeled chemicals, St. Louis, MO. Unlabeled glyphosate (chemical purity: 99%) was procured from Sigma chemical company, St. Louis, MO. Organic solvents and water were of Optima grade (Fisher Scientific, Pittsburgh, PA) and used without further purification.

5.3.2 Soils

Three types of soil (Catlin, Flanagan and Drummer) belonging to a soil catena sequence were used for the study. The soils were collected (to a depth of 15 cm) from University of Illinois Crop Sciences Research and Education Center in Urbana, IL. All soil samples were sieved through a 2 mm screen, and stored at 4°C for duration of four weeks. Relevant physical and chemical properties of the three soil types used in this study were listed in Table 5.1.

5.3.3 Adsorption-desorption Study

Adsorption isotherms of 14C-glyphosate were determined using the batch equilibrium method for the three soil types. The initial concentrations of 14C-glyphosate (0.1, 1, 5 and 10 mg L⁻¹) were prepared in 0.1 M KCl solution (Gimsing & Borggaard, 2001) and adsorption experiment was followed as per the EPA guidelines for adsorption studies (USEPA, 2008). 2 g of air dried soil sample was equilibrated with 10 ml of 14C-glyphosate in 20 ml Teflon centrifuge tubes in a horizontal shaker (150 rpm) for 24 hr at room temperature (25 ± 1°C). For the anaerobic treatment, 2 g portions of the soil samples contained in the Teflon centrifuge tubes were flooded
with sterile-deoxygenated water. The tubes were flushed with N₂ gas, sealed and incubated in an anaerobic chamber, at room temperature (25°C) for 2 weeks to allow reduction. To study the adsorption, ¹⁴C-glyphosate was added to the reduced soil (anaerobic system) to attain the final concentrations of 0.1, 1, 5 and 10 mg L⁻¹. Sealed tubes were equilibrated on a shaker inside the anaerobic chamber for 24 h, where the oxygen was maintained at zero concentration. The effect of phosphate addition on glyphosate adsorption in aerobic and anaerobic soils was examined by incorporating CaHPO₄ into Catlin, Flanagan and Drummer soils at concentration of 500 mg Kg⁻¹, followed by thorough mixing of the soils prior to the addition of herbicide.

At the end of the equilibration period, the soil suspension was centrifuged (15 min, 12,000 × g) and aliquots removed from each tube for a radioactivity assay using a Packard Tri-Carb (1900TR) scintillation counter. Controls (treatment without herbicide) were included for calibration and background correction purposes. The amount ¹⁴C-glyphosate adsorbed to soil was calculated based on the difference between the initial and final concentrations of herbicide in solution. All the experiments were performed in triplicate.

Following equilibration and removal of 5 ml of the initial 10 ml of supernatant, herbicide desorption from soil was estimated by adding equal amounts of fresh 0.1 M KCl solution to the centrifuge tubes, dispersing the soil aggregates by vibration and re-shaking for 24 h. Sampling from the anaerobic soil treatments were handled inside an anaerobic glove box chamber. Soil samples were centrifuged (15 min, 12000g) and an aliquot of the supernatant was removed and analyzed utilizing radioactivity assay. The desorption process was repeated 4 times. Desorption was estimated by determining the amount of herbicide in the soil solution following equilibration and calculated by subtracting the amount still adsorbed to soil.
5.3.4 Degradation study

Microcosm preparation

Soil incubations were performed under reduced (anaerobic) or oxidized (aerobic) conditions using serum bottle microcosms to determine the degradation kinetics of $^{14}$C-glyphosate.

Anaerobic incubations: Microcosms, consisting of serum bottles (60 ml) were amended with soil (10 g) and were spiked with phosphonomethyl-C labeled $^{14}$C-glyphosate (specific activity of $3.33 \times 10^3$ Bq µmol$^{-1}$, diluted with unlabeled glyphosate) in 50 µl of methanol to produce a final concentration of 2 mg Kg$^{-1}$ of soil that corresponded to the recommended agriculture application rate. The glyphosate-spiked soils were agitated on a reciprocating shaker for 24 h at room temperature to ensure thorough mixing and to evaporate the solvent. To determine the effect of soil phosphate on glyphosate degradation, CaHPO$_4$ was uniformly mixed into the soils at a concentration of 500 mg Kg$^{-1}$ soil prior to the addition of herbicide. The soil was then flooded with 20 ml of sterile-deoxygenated water. The microcosm headspace was flushed with N$_2$ gas, immediately crimp sealed with a butyl stopper fitted with a vial containing 1 ml of 0.5 M NaOH to trap the mineralized $^{14}$CO$_2$. These microcosms were incubated in a dark, temperature controlled chamber at 25°C. Sterilized soil microcosms were included as control for each soil type. Sterilization was achieved by autoclaving the soils twice at 121°C for 1 h.

Aerobic incubations: Soil microcosms were built from serum bottles as described above. Sterile distilled water was added to the glyphosate-spiked soils to adjust the moisture content to about 60% of the field water-holding capacity (WHC). The serum bottles were lightly capped with a butyl stopper fitted with NaOH trap and stored in dark at 25°C. Soil moisture content was adjusted weekly by weighing and adding sterile distilled water.
Sample extraction and analysis

Anaerobic and aerobic microcosms were destructively sampled on consecutive intervals by removing the NaOH trap, followed by agitating the microcosm for 1 min, and transferring the contents to a 50 ml Teflon centrifuge tube. Quantification of $^{14}\text{CO}_2$ in NaOH traps was accomplished by direct liquid scintillation spectrometry (LSS) using a Packard Tri-Carb (1900TR) scintillation counter. The solid and liquid phases of the soil slurry were then separated by centrifugation (15 min, 12000g). Aqueous samples were removed, filtered (0.2 µm) and total aqueous radioactivity was estimated using LSS. Soil was extracted with 20 ml NaOH (0.1 M) in a Teflon centrifuge tube with horizontal shaking, by following the method described by Druart et al., (2011). Extracts were centrifuged at 12,000 rpm for 15 min, an aliquot was removed for LSS (to quantify extractable radioactivity) and the supernatant was retained for analysis of glyphosate and degradation products. Soil extract samples containing $^{14}\text{C}$-glyphosate were analyzed using HPLC equipped with a Hewlett-Packard 1050 series auto-sampler instrument, using flow scintillation detector (Packard Radiomatic Flo-one/beta). Separation was achieved with an isocratic elution of mobile phase composed of acetonitrile: water (10:90, v/v) through a 4.6 x 150 mm, 5 µm particle size, C$_{18}$ column (Prontosil, Chadds Ford, PA). Glyphosate had a reproducible retention time of 4.1 min at a flow rate of 1 ml min$^{-1}$.

5.3.5 Data analysis

Adsorption and desorption parameters of metolachlor in aerobic and anaerobic conditions for each soil type were calculated using the transformed Freundlich equation: $\log C_s = \log K + l/n \log C_e$, where $C_s$ is the amount of glyphosate adsorbed to the soil (mg Kg$^{-1}$), $C_e$ is the equilibrium concentration in the soil solution (mg L$^{-1}$) and $K$ and $l/n$ are empirical constants that reflect the affinity of the soil for the herbicide and the degree of linearity between the amount
adsorbed and the solution concentration, respectively. Regression analysis was performed on adsorption and desorption isotherms to calculate $K$ (intercept) and $1/n$ (slope) values of glyphosate in aerobic and anaerobic soils. Hereafter, $K_{ads}$ and $1/n_{ads}$ will indicate Freundlich parameters for adsorption and $K_{des}$ and $1/n_{des}$ will refer to desorption parameters.

The degradation data of glyphosate in soils were fitted into the first-order kinetics model $C_t = C_0 e^{-kt}$ where $C_0$ is the initial concentration (mg Kg$^{-1}$ soil) of the herbicide in the soil, $C_t$ is the herbicide concentration (mg Kg$^{-1}$ soil) detected in the soil at time $t$, and $k$ is the first-order rate constant. Degradation rate constants were calculated by linear regression of the natural logarithm of the percentage of herbicide remaining against the time. Aerobic and anaerobic degradation half-life ($T_{1/2}$) for each soil type was calculated using the equation $T_{1/2} = \ln 2/k$. The statistical program SAS v.9.3 was used to calculate the treatment means and standard errors ($n = 3$). The differences between treatments were evaluated using one-way analysis of variance (ANOVA) followed by least significant difference test at $p < 0.05$.

5.4 Results

5.4.1 Adsorption-desorption

Adsorption data from the experiment fit perfectly into the Freundlich isotherm ($R^2 = 1$) for the range of herbicide concentrations (0.1 to 10 mg L$^{-1}$) and soils tested regardless of the soil redox conditions. Table 5.2 depicts the calculated Freundlich adsorption coefficient ($K_{ads}$), slope ($1/n_{ads}$) and the corresponding coefficients of determination ($R^2$) for the glyphosate adsorption in aerobic and anaerobic soils. Among the different soils and the treatments, the $1/n_{ads}$ values ranged from 0.76 to 0.93 and $K_{ads}$ from 62.21 to 103.46. Soil redox conditions did not alter the glyphosate adsorption in Catlin and Flanagan soils as evident from their statistically insignificantly different $K_{ads}$ values. However, the herbicide exhibited a significantly lower ($p <$
0.05) $K_{ads}$ value in the anaerobically treated Drummer soil versus the aerobic Drummer soil incubations. Further, $K_{ads}$ was observed to be lowest for Catlin and highest for Drummer regardless of the soil redox conditions. Higher $K_{ads}$ indicate higher adsorption affinity of the herbicide to the soils.

Desorption isotherms for glyphosate in all the soils fit ($R^2 > 0.92$) into the Freundlich model. The calculated desorption parameters of glyphosate in the aerobic and anaerobic soils are presented in Table 5.3. Freundlich desorption coefficient ($K_{des}$) values of glyphosate were significantly lower ($p < 0.05$) in anaerobic soils when compared to the corresponding values from the aerobic soils. Among the three soils tested, the highest $K_{des}$ was observed in Drummer soil irrespective of the soil redox conditions. Higher $K_{des}$ indicate slower release kinetics of the adsorbed herbicide back into soil solution.

5.4.2 Degradation and mineralization

The first-order parameters including rate constant ($k$) and degradation half-life ($T_{1/2}$) of the $^{14}$C-glyphosate in different soil types and redox conditions are presented in Table 5.4. $^{14}$C-glyphosate degradation followed the first-order kinetics in all the non-sterile aerobic and anaerobic soils as obvious from their $R^2$ values (0.83 - 1.00). The loss of herbicide from the sterile soil control microcosms was not substantial from either aerobic or anaerobic incubations. In all the three soil types studied, the aerobic $T_{1/2}$ values (15 - 18 days) calculated for glyphosate were significantly lower than the corresponding anaerobic values (42 – 51 days). The $T_{1/2}$ of the herbicide in Catlin, Flanagan and Drummer were about the same in aerobic incubations. On the other hand, compared to other soils, glyphosate degradation was relatively slow in Flanagan soil for anaerobic incubation. Figure 5.1 a, b, c depicts the degradation pattern of $^{14}$C-glyphosate in Catlin, Flanagan and Drummer soils incubated under aerobic and anaerobic conditions.
More than half (53 – 63%) of the applied radioactivity in $^{14}\text{C}$-glyphosate was mineralized as $^{14}\text{CO}_2$ from the aerobic soils, and only 38 - 41% of the applied $^{14}\text{C}$-glyphosate mineralized in the anaerobic microcosms by the end of incubation (Table 5.4). Conversely, aerobically or anaerobically incubated sterilized microcosms had little or no mineralization of herbicide in all the soil types considered. Figure 5.1 d, e, f illustrates the comparative microbial mineralization trends of glyphosate amendments observed as the amount of $^{14}\text{CO}_2$ measured from the alkali trap from aerobic and anaerobic soil microcosms. Another interesting observation from the study is the absence of lag phase before the evolution of $^{14}\text{CO}_2$ from the soils. The evolution of $^{14}\text{CO}_2$ from soils was evident immediately after the day zero of incubation in both aerobic and anaerobic soils. Glyphosate mineralization in aerobic soils was initially rapid followed by a gradually decreasing rate. However, in anaerobic soils mineralization of the glyphosate started out slowly and steadily improved towards the end of incubation.

5.4.3 Effect of phosphate addition on adsorption and degradation

Addition of phosphate to Catlin, Drummer and Flanagan soils significantly reduced the $^{14}\text{C}$-glyphosate adsorption to aerobic and anaerobic soils (Figure 5.2 a, c). Moreover, the extent of reduction in the herbicide adsorption was more pronounced in the aerobic soils. Phosphate additions did not improve or had no effect on the degradation of $^{14}\text{C}$-glyphosate in the aerobic soils as observed from the degradation half-life values ($T_{1/2}$) of the herbicide in the respective soils (Figure 5.2 b). Conversely, the presence of soil phosphate significantly enhanced the anaerobic degradation of $^{14}\text{C}$-glyphosate in all the three soil types studied (Figure 5.2 d).
5.5 Discussion

Adsorption-desorption

Greater $K_{ads}$ values of $^{14}$C-glyphosate from the present study clearly indicate greater adsorption affinity of the herbicide to the soil. The results obtained from this study were comparable to the reported $K_{ads}$ values (33 to 152.9) for glyphosate (Glass, 1987; Piccolo et al., 1994; Al-Rajab, 2008). Studies attribute the variability in glyphosate adsorption to the physical and chemical heterogeneity of the soils (Al-Rajab, 2008). The non-linearity ($I/n_{ads} < 1$) between the amount of herbicide adsorbed and the concentration in the applied solution suggests the potential for glyphosate leaching at higher application rates.

The suppression in the glyphosate adsorption to anaerobically treated Drummer soil may be attributed to the changes in the mineralogical and surface properties of the soil brought about by the anaerobic soil redox conditions. Strong adsorption of glyphosate by clay minerals, such as aluminum and iron oxides has been shown previously (Gimsing & Borggaard, 2002; Sheals et al., 2002). Anaerobic conditions could alter the mono- and divalent cations $\text{Al}^{3+}$ and $\text{Fe}^{3+}$ (Stucki et al., 2002) in the soil to their respective reduced forms, causing them to lose their affinity for glyphosate. Moreover, relatively higher clay content in the Drummer soil corroborates this argument (Table 5.1).

The greatest extent of $^{14}$C-glyphosate desorption was observed in the soils having the lowest adsorption. Relatively lower $K_{des}$ values in anaerobic treatments corresponding to aerobic ones in all the tested soils indicate that desorption of the herbicide was enhanced in the anaerobic/reduced soils. The possible involvement of reduced clay minerals in the reduced affinity of the herbicide in anaerobic soil conditions may account for this desorption trend in anaerobic soils. These increased desorption patterns of the herbicide may result in enhanced
bioavailability of glyphosate subsequently expediting the degradation or bioactivity of the herbicide in anaerobic soil conditions.

**Degradation and mineralization**

Degradation of $^{14}$C-glyphosate occurred more rapidly in aerobically incubated Catlin, Flanagan and Drummer soils than in the corresponding anaerobic incubations, as evident from the significantly lower aerobic T$_{1/2}$ values. Concurrent with our findings, previous reports show that glyphosate degrades relatively rapidly in aerobic soils, with an estimated T$_{1/2}$ of 7-60 days (Giesy, 2000). Rueppel et al. (1977) also found that degradation of the herbicide glyphosate in anaerobic soils is normally less than aerobic conditions. In contrast to other studies, (Sorensen et al., 2006; Laitinen et al., 2006) our soils did not exhibit a lot variability in their ability to degrade glyphosate.

Glyphosate degradation could be inferred as a purely microbially mediated process as practically no degradation or mineralization occurred in sterile control soils in any soil type or redox condition. The observation that mineralization of glyphosate takes place without a lag phase in the soil could be seen as an indication for co-metabolic processes involved in the glyphosate uptake and metabolism, as the enzymes involved must be present in the soil before the herbicide application (Franz et al., 1997). The possibility of co-metabolic processes in the degradation of glyphosate has been suggested in various studies (Franz et al., 1997; Accinelli et al., 2005), as microorganisms were not able to utilize glyphosate as a source of carbon. Nevertheless, a few studies have shown that some organisms are capable of using glyphosate as a carbon substrate (Haney et al., 2002; Bussey et al., 2001) and a source of nitrogen (Haney et al., 2000). At the same time, increasing evidence also suggests microbial utilization of glyphosate is as a potential phosphate source (Dick & Quinn, 1995; Hove-Jensen et al., 2014). Contrary to
previous observations (Sorensen et al., 2006), substantial mineralization of glyphosate was found in the anaerobic soil environment. The slow start in the anaerobic mineralization may be ascribed to the acclimation of specialized herbicide degrading microbial populations in the anaerobic soil.

**Impact of soil phosphate**

Suppression of glyphosate adsorption in both aerobic and anaerobic soils with phosphate addition explicitly demonstrated the competition for adsorption sites between glyphosate and phosphate despite differences in redox conditions. Several studies have confirmed similar competitive adsorption of glyphosate and phosphate on $\text{Al}^{3+}$ and $\text{Fe}^{3+}$ surface sites in soils (Dion et al., 2001; Gimsing & Borggaard, 2002). The variability in the extent of suppression of glyphosate adsorption between aerobic and anaerobic soils was not well understood. One plausible explanation would be the diminished availability of the $\text{Al}^{3+}$ and $\text{Fe}^{3+}$ adsorption sites in anaerobic soils due to mineralogical changes occurring due to reduced conditions in soil.

The effect of phosphate addition on the enhanced bioavailability of glyphosate was found only in the anaerobic soils, where the $T_{1/2}$ of glyphosate was noticeably reduced in all the soil types treated anaerobically with phosphate. Phosphate addition did not stimulate the glyphosate degradation in aerobic soils. Previous studies have shown that available phosphate may have a positive effect, no effect or even a negative effect on glyphosate degradation and mineralization (Gimsing et al., 2004; Moshier & Penner, 1978). These differences in behavior may be explained by the existence of two different adsorption sites in the soil. The common surface sites with phosphate preference and the specific adsorption sites that are available for either glyphosate or phosphate (Borggaard & Gimsing, 2008).
5.6 Conclusion

The current study examined the significance of soil aerobic and anaerobic conditions on microbial bioavailability of glyphosate in soils. Although $^{14}$C-glyphosate was highly adsorbed to the soils regardless of the soil type and redox conditions, among the three soils tested, the adsorption affinity between aerobic and anaerobic/reduced soils was only different in the Drummer soil. Desorption or release of adsorbed herbicide was enhanced in anaerobic soils when compared to aerobic ones regardless of the soil type. The degradation and mineralization of $^{14}$C-glyphosate exhibited slower kinetics in anaerobic soils compared to corresponding aerobic soils in all the soil types investigated. Glyphosate degradation was deduced to be a purely microbiological process as practically no degradation or mineralization occurred in sterile control soils across the range of soils and redox conditions studied. The observation that mineralization of glyphosate in soil took place without a lag phase pointed towards the involvement of co-metabolic processes in the microbial uptake and metabolism of the herbicide. The addition of phosphate to the soil suppressed the adsorption of glyphosate in both aerobic and anaerobic soils, though to a variable extent, which confirmed the competition between glyphosate and phosphate for adsorption sites in diverse soil conditions. However, compared to anaerobic soils, phosphate amendment failed to improve the degradation rate in aerobic soils. This conflicting observation between aerobic and anaerobic soil environments on the behavior of glyphosate in the presence of soil phosphate, requires additional research attention.
5.7 Tables

Table 5.1: Selected properties of the soils used in the experiment

<table>
<thead>
<tr>
<th>Soil</th>
<th>pH</th>
<th>Texture (%)</th>
<th>WHC (%)</th>
<th>CEC (Cmol Kg(^{-1}))</th>
<th>Organic matter (%)</th>
<th>Phosphorus (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sand</td>
<td>Silt</td>
<td>Clay</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catlin</td>
<td>7.6</td>
<td>10</td>
<td>58</td>
<td>32</td>
<td>31.4</td>
<td>13.9</td>
</tr>
<tr>
<td>Flanagan</td>
<td>6.5</td>
<td>12</td>
<td>56</td>
<td>32</td>
<td>32.0</td>
<td>17.5</td>
</tr>
<tr>
<td>Drummer</td>
<td>7.1</td>
<td>14</td>
<td>46</td>
<td>40</td>
<td>33.0</td>
<td>23.8</td>
</tr>
</tbody>
</table>

*WHC* water holding capacity @ 1/3 bar
*CEC* cation exchange capacity
Table 5.2: Adsorption (Freundlich model) of $^{14}$C-glyphosate in different soil types under aerobic (Aer) and anaerobic (An) environmental conditions

<table>
<thead>
<tr>
<th>Soil</th>
<th>$K_{ads}$</th>
<th>$1/n_{ads}$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aer</td>
<td>An</td>
<td>Aer</td>
</tr>
<tr>
<td>Catlin</td>
<td>62.21$^{c†}$ (±1.71)$^*$</td>
<td>72.38$^c$ (±5.80)</td>
<td>0.92 (±0.02)</td>
</tr>
<tr>
<td>Flanagan</td>
<td>78.14$^c$ (±2.05)</td>
<td>69.64$^c$ (±4.02)</td>
<td>0.90 (±0.02)</td>
</tr>
<tr>
<td>Drummer</td>
<td>103.46$^a$ (±5.11)</td>
<td>84.82$^b$ (±4.36)</td>
<td>0.93 (±0.03)</td>
</tr>
</tbody>
</table>

$K_{ads}$ Freundlich adsorption coefficient  
$I/n_{ads}$ adsorption isotherm slope  
$R^2$ goodness of fit for Freundlich model  
† mean values followed by the same letter superscripts are not significantly different ($p < 0.05$)  
* values in the parentheses are the 95% confidence intervals
Table 5.3: Desorption (Freundlich model) of $^{14}$C-glyphosate in different soil types under aerobic (Aer) and anaerobic (An) environmental conditions

<table>
<thead>
<tr>
<th>Soil</th>
<th>$K_{des}$</th>
<th>$1/n_{des}$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aer</td>
<td>An</td>
<td>Aer</td>
</tr>
<tr>
<td>Catlin</td>
<td>46.52$^{a\dagger}$ (±0.03)*</td>
<td>42.85$^{b}$ (±0.20)</td>
<td>0.02 (±0.002)</td>
</tr>
<tr>
<td>Flanagan</td>
<td>17.08$^c$ (±0.03)</td>
<td>5.81$^c$ (±0.02)</td>
<td>0.09 (±0.002)</td>
</tr>
<tr>
<td>Drummer</td>
<td>17.95$^c$ (±0.04)</td>
<td>7.75$^d$ (±0.05)</td>
<td>0.02 (±0.003)</td>
</tr>
</tbody>
</table>

$K_{des}$ Freundlich desorption coefficient
$1/n_{des}$ desorption isotherm slope
$R^2$ goodness of fit for Freundlich model
$\dagger$ mean values followed by the same letter superscripts are not significantly different ($p < 0.05$)
* values in the parentheses are the 95% confidence intervals
Table 5.4: Degradation (first-order kinetics) and mineralization of $^{14}$C-glyphosate in different soil types under aerobic (Aer) and anaerobic (An) environmental conditions

<table>
<thead>
<tr>
<th>Soil</th>
<th>$k$ (day$^{-1}$)</th>
<th>$T_{1/2}$ (days)</th>
<th>$R^2$</th>
<th>Degradation$^\dagger$</th>
<th>Mineralization$^#$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aer</td>
<td>An</td>
<td>Aer</td>
<td>An</td>
<td>Aer</td>
</tr>
<tr>
<td>Catlin</td>
<td>0.038</td>
<td>0.016</td>
<td>18$^a$</td>
<td>42$^c$</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>(0.003)*</td>
<td>(0.005)</td>
<td>(209)</td>
<td>(154)</td>
<td>(0.73)</td>
</tr>
<tr>
<td>Flanagan</td>
<td>0.048</td>
<td>0.014</td>
<td>15$^a$</td>
<td>51$^b$</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>(0.003)</td>
<td>(0.005)</td>
<td>(228)</td>
<td>(140)</td>
<td>(0.84)</td>
</tr>
<tr>
<td>Drummer</td>
<td>0.038</td>
<td>0.015</td>
<td>18$^a$</td>
<td>45$^c$</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>(0.003)</td>
<td>(0.004)</td>
<td>(210)</td>
<td>(200)</td>
<td>(0.85)</td>
</tr>
</tbody>
</table>

$k$ rate constant
$T_{1/2}$ degradation half-life
$R^2$ goodness of fit for first order degradation model
$^\dagger$ % of applied herbicide remaining after 56 days of incubation
$^#$ % of applied herbicide evolved as $^{14}$CO$_2$ after 56 days of incubation
* values in the parentheses in each column represent the corresponding values for sterilized soil control
$^\dagger$ mean values followed by the same letter superscripts are not significantly different ($p < 0.05$)
ND: not detected
5.8 Figures

Figure 5.1: Degradation kinetics (a, b, c) and mineralization patterns (d, e, f) of $^{14}$C- glyphosate in aerobic (Aer) and anaerobic (An) soil conditions in Catlin, Flanagan and Drummer soils. Data from aerobic (Aer Ster) and anaerobic (An Ster) sterilized control soils are also shown.
Figure 5.2: Effect of phosphate addition on the adsorption and degradation of $^{14}$C-glyphosate in aerobic (Aer) and anaerobic (An) soils. The comparison of adsorption coefficient (Figure a, c) and degradation half-life (Figure b, d) of glyphosate in aerobic and anaerobic soils without (No P added) and with (Soil + P) phosphate amendment is shown here. Bars in each soil category displaying (*) sign are significantly different from each other ($p < 0.05$). Error bars represent standard errors ($n=3$).
5.9 References


CHAPTER 6
General Conclusions

6.1 Principal conclusions

The results from this dissertation research clearly highlight the significance of aerobic versus anaerobic soil conditions as an important factor affecting the bioavailability and mobility of the herbicides metolachlor and glyphosate in soils. Figure 6.1 and Table 6.1 respectively, illustrate a schematic and tabular representation of the summary of metolachlor and glyphosate bioavailability in aerobic and anaerobic soil conditions.

Metolachlor retention patterns in the soils were significantly influenced by the anaerobic conditions in soils. Adsorption of metolachlor to the soils was noticeably reduced and greater release of the adsorbed herbicide was observed, in soils that received anaerobic treatments. Additionally, anaerobic soil incubation induced better degradation and mineralization of metolachlor in the range of soils evaluated, despite the differences in soil properties. The findings also confirmed the dominance of microorganisms in the degradation and mineralization of metolachlor in the soils in spite of their soil properties and redox conditions. A high percentage of non-extractable residues (NER) were formed in all the microcosms regardless of the soil type or incubation conditions and was attributed to the metabolite formation. The anaerobic degradation and mineralization of metolachlor coincided with the iron (Fe) reducing conditions in soil and suggested a probable role of Fe in microbial fate of metolachlor under such soil environmental conditions. In a nutshell, results from this study provided evidence for the enhanced bioavailability and microbial biodegradation of metolachlor in anaerobic soil systems.
Stable isotope probing (SIP) facilitated the identification of microorganisms responsible for the mineralization of metolachlor in aerobic and anaerobic soils. The 16S rRNA gene sequencing of clones implied the role of organisms closely related to Bacillus in aerobic and Acidobacteria in anaerobic mineralization of metolachlor in soils. The microorganisms identified from this study belonged to known pure culture degraders of metolachlor as well as novel organisms not associated with metolachlor degradation. Furthermore, involvement of known Fe reducers in the metolachlor assimilation established during the SIP provides a link between the anaerobic mineralization of metolachlor and Fe reduction in soil.

Glyphosate also was influenced by redox conditions for bioavailability and mobility in soils. Glyphosate showed a substantially higher adsorption affinity to the soils, in comparison to metolachlor, regardless of the soil redox conditions (Table 6.1). However, differences in the glyphosate adsorption affinity between aerobic and anaerobic/reduced soils were only found in the Drummer soil. Desorption or release of adsorbed herbicide was generally enhanced in all the anaerobic soils. Contrary to metolachlor, the degradation and mineralization of glyphosate exhibited a slower kinetics in anaerobic soils compared to corresponding aerobic soils in all the soil types investigated. Glyphosate degradation was also deduced as a pure microbiological process as practically no degradation or mineralization occurred in sterile control soils across the range of soils and redox conditions studied. The addition of phosphate suppressed the adsorption of glyphosate in both aerobic and anaerobic soils and confirmed the consistent competition between glyphosate and phosphate for adsorption sites in soils. Additionally, phosphate amendment improved the degradation rate only in anaerobic soils.
6.2 Future directions

Future experiments should be addressed towards better understanding of microbial pathways involved in the metabolism of metolachlor and glyphosate, with special emphasis on the anaerobically mediated pathways. The body of evidence from this dissertation research necessitates deep insights into the thermodynamic processes occurring during the microbial assimilation of metolachlor, particularly in concert with iron reduction. Despite demonstrating the potential for the uptake of metolachlor in the SIP study, the current findings bid for more work in the prospect of using iron reducing microorganisms as a dependable candidate for the bioremediation of metolachlor in soils. Additionally, the contradictory behavior of glyphosate in aerobic and anaerobic soil conditions after the addition of soil phosphate requires additional research attention.

Although utilization of the varied soil redox environment could be considered under the broad definition of biostimulation, manipulating soil aeration in situ is mostly impractical due to both logistics and cost. However, incorporation of this concept into ad situ and ex situ bioremediation techniques, such as bioreactors could be viewed as a more suitable alternative. For instance, the feasibility of utilizing iron reducing bioreactors should be assessed for the removal of metolachlor from contaminated soils. Achieving the same result in a field scale as in the lab is another challenge to face in implementing a bioremediation strategy. Hence, it is imperative to test the efficacy of the observations from the current microcosm study. The addition of phosphate to improve the anaerobic glyphosate degradation should be tested in mesocosms or pilot scale remediation projects. The information generated from the current study could be applied towards efficacious use of metolachlor and glyphosate in soils and also for framing a viable strategy for more efficient clean-up of soils contaminated by these herbicides.
### Table 6.1: Bioavailability parameters of metolachlor and glyphosate in aerobic (Aer) and anaerobic (An) soils

<table>
<thead>
<tr>
<th>Properties</th>
<th>Metolachlor*</th>
<th>Glyphosate*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aer</td>
<td>An</td>
</tr>
<tr>
<td>Freundlich adsorption coeff. ($K_{ads}$) (L Kg$^{-1}$)</td>
<td>1.23 - 5.85</td>
<td>1.04 - 4.54</td>
</tr>
<tr>
<td>Freundlich desorption coeff. ($K_{des}$) (L Kg$^{-1}$)</td>
<td>3.11 - 11.13</td>
<td>2.75 - 9.95</td>
</tr>
<tr>
<td>Degradation half-life (T$_{1/2}$)(days)</td>
<td>117 - 154</td>
<td>65 - 75</td>
</tr>
<tr>
<td>Mineralization (%) #</td>
<td>9 – 11</td>
<td>13 – 17</td>
</tr>
</tbody>
</table>

* values obtained from the range of soils used in the study
# % of applied $^{14}$C evolved as $^{14}$CO$_2$ after 140 days of incubation for metolachlor and 56 days of incubation for glyphosate
6.3 Figures

Figure 6.1: Schematic representation of the summary of metolachlor (Met) and glyphosate (Gly) bioavailability in aerobic (Aer) and anaerobic (An) soil conditions.