AN ABSTRACT OF THE THESIS OF

Iêda de Carvalho Mendes for the degree of Doctor of Philosophy in Soil Science presented on August 7, 1997. Title: MICROBIOLOGY OF SOIL AGGREGATES RECOVERED FROM DIFFERENT CROP MANAGEMENT SYSTEMS.

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Peter J. Bottomley

My thesis research examined the relationships between soil structure and soil microorganisms. I examined the distribution of microbial biomass and its activities across soil aggregates and also the distribution across aggregates of a specific soil bacterium, *Rhizobium leguminosarum* bv *trifolii*. These distributions were evaluated in a summer vegetable cropping system in which three winter cover-crop treatments are practiced (fallow; cereal and legume). Five aggregate size classes (<0.25, 0.25 to 0.5, 0.5 to 1.0, 1.0 to 2.0 and 2.0 to 5.0 mm) were obtained from each treatment by a dry sieving procedure. The distributions of total organic C (TOC), total Kjeldahl N (TKN), total soil bacterial population, microbial biomass C (MBC), mineralizable C and N (Min.C and Min.N), and two enzyme activities (β-glucosidase, FDA-hydrolysis) were measured after cover crop incorporation at seedbed preparation (June), and after harvest of the sweet-corn or broccoli summer crop (September). Immunofluorescence direct count microscopy (IFDC) and the plant infection soil dilution technique (MPN) were

used to examine the seasonal distribution of *R. leguminosarum* bv *trifolii* across the same soil aggregate preparations. Despite the cover crop treatments having been in place for seven years, they have not influenced the overall level of TOC, TKN, and aggregate size distribution. Cover crop effects on microbial soil interactions were subtle, often being seen at specific sampling times and interacting with aggregate size. *Rhizobium* serotype AR-18, total soil bacteria, MBC and its activities were heterogeneously distributed across soil aggregates. Temporal changes in *Rhizobium* densities, MBC, and Min. N occurred in the microaggregate size class indicating the latter size class to be biologically active and dynamic in this soil. The distribution of MBC and its activities across aggregates was complex. Size classes were identified in which high levels of MBC were associated with low levels of activities and vice-versa, suggesting that substrate does not always exist in the vicinity of a microbial population sufficient to sustain microbial activity.

MICROBIOLOGY OF SOIL AGGREGATES RECOVERED FROM DIFFERENT CROP MANAGEMENT SYSTEMS

by

Iêda de Carvalho Mendes

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Iêda de Carvalho Mendes, Author

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MICROBIOLOGY OF SOIL AGGREGATES RECOVERED FROM DIFFERENT CROP MANAGEMENT SYSTEMS

Chapter 1. INTRODUCTION TO THE THESIS

Soil is composed of a complex mixture of mineral particles of varying sizes, organic matter in varying stages of decomposition, and a tremendous diversity of living organisms. Despite this complexity, some organization of these materials can be ascertained. Soil aggregates are composed of primary particles and organic matter that bind to each other more strongly than to other surrounding soil particles (Nikiforoff, 1941; Kemper and Rosenneau, 1986). Aggregates represent microhabitats which provide a place for attachment of microorganisms, a source of nutrients, and afford protection against predation and desiccation. Soil aggregation also facilitates the physical protection and accumulation of soil organic matter (SOM) through isolation of potentially labile organic compounds from microorganisms and their enzymes (Miller and Jastrow, 1992; Hassink et al., 1993; Ladd et al., 1993; Beare et al., 1994a; Foster, 1994).

During the past 60 years a considerable amount of research effort has been expended to examine if a relationship exists between changes in aggregate stability and changes in soil structure associated with cultivation (Yoder, 1936; Klodny and Jeffe, 1939, Rovira and Greacen, 1957; Doormar, 1983; Dalal and Meyer, 1986; Elliott, 1986; Gupta and Germida, 1988; Singh and Singh, 1995). Many of these studies showed that conventional tillage practices associated with cultivation disrupt soil aggregates, expose otherwise inaccessible organic matter (OM) to microbial attack, and accelerate OM

mineralization (Russel, 1950; Low 1954; Tisdall and Oades 1980 and 1982; Elliott, 1986; Gupta and Germida, 1988; Coleman et al., 1994). As a result, interest has focused on gaining a more precise understand of how soil structure influences soil biotic processes such as SOM cycling and microbial activity, and how soil biotic processes influence soil structure (Jastrow and Miller, 1991)

Most of the studies associated with soil aggregation have examined either the distribution of SOM in the soil fabric, and/or focused upon how changes in aggregation under different tillage and residue management regimes contribute to accumulation and loss of SOM (Dormaar, 1983; Christensen, 1986; Elliott, 1986; Jastrow, 1987, 1996, Cambardella and Elliott, 1993; Beare et al., 1994a; Buyanovsky et al., 1994; Puget et al., 1995; Jastrow et al., 1996). Fewer studies have looked at the spatial distribution of microorganisms and their activities across aggregates (Gupta and Germida, 1988; Seech and Beauchamp, 1988; Miller and Dick, 1995a and b; Singh and Singh, 1995). Soil quality and soil health have received considerable attention from scientists and the general public since the U.S. National Academy of Sciences published its 1993 report called: "Soil and water quality: an agenda for agriculture". The increased publicity given to the soil resource has provided the impetus for soil scientists to generate more information about the factors that influence changes in soil properties (Doran et al., 1994). As part of this national effort, Professor Richard Dick and colleagues at Oregon State University initiated studies in 1989 to examine the benefits of using legume and non-legume species as winter cover crops for intensive summer vegetable cropping systems in the Willamette valley of western Oregon. As a soil microbiologist, I was interested in getting a better understanding of the distribution of microorganisms and

their activities associated with the soil structure under these different cover-crop management systems. Furthermore, the treatments provided an opportunity to examine microbial activities and population dynamics at different times of the year, namely right after seedbed preparation and at the end of the summer growing season. The presence of a legume (red clover, *Trifolium pratense* L.) in the rotational system, provided a unique opportunity to study how a specific bacterium, namely *Rhizobium leguminosarum* bv *trifolii*, was distributed across soil aggregates, and how its ecology would respond to seasonal changes in soil conditions, in the presence and absence of the host plant.

Chapter 2. LITERATURE REVIEW

AGGREGATE FORMATION AND SOIL STRUCTURE

The concept of soil structure involves three components: the size distribution of primary particles, their spatial arrangement into aggregates of various sizes, and the geometry of the pores and channels that result (Payne, 1988; Jastrow and Miller, 1991). Soil structure has a major impact on processes as diverse as crop growth, water holding capacity and infiltration, transport of agricultural chemicals, soil erosion and loss and accumulation of SOM (Boyle et al., 1989; Kay, 1990; Oades and Waters, 1991). The dynamic nature of soil structure is associated with biotic (plant root growth, soil microbes and soil fauna) and abiotic factors (wetting and drying, freezing and thawing, compaction and soil tillage) (Tisdall and Oades, 1982; Kay, 1990; Oades, 1993).

The relative importance of biotic and abiotic influences on soil structure depends on the soil being considered. Biotic factors are thought to play a major role in the stabilization of soil aggregates (Lynch and Bragg, 1985), and their importance is expected to be greatest in soils with low shrink-swell capacity (sands and loams), and less in clay soils where inorganic cementing agents provide stabilization (Oades, 1993). Microorganisms and plant roots can be involved in the formation and stabilization of soil aggregates by mechanically binding soil particles together, and producing effective binding agents either by biosynthesis or through the decomposition of organic materials (Tisdall and Oades, 1979 and 1982; Lynch and Bragg, 1985; Angers and Mehuys, 1989; Miller and Jastrow, 1990; Edgerton et al., 1995; Chantigny et al., 1997).

Several models have been proposed to describe the ways in which mineral particles are held together to form soil aggregates (Edwards and Bremmer,1967; Tisdall and Oades, 1982; Oades, 1984; Elliott and Coleman, 1988; Oades and Waters, 1991). By recognizing that different mechanisms of aggregate formation and stabilization occur at various spatial and temporal scales, the hierarchical model proposed by Tisdall and Oades (1982) stimulated mechanistic studies of aggregate formation, stabilization and degradation (Elliott, 1986; Gupta and Germida, 1988; Miller and Jastrow, 1990, 1992; Jastrow et al., 1996). With the Tisdall and Oades model, an aggregate of soil is thought to be composed of structural units of different sizes, held together by various binding agents. The first hierarchical order is represented by primary particles and clay aggregates (< 2.0 μm and 2.0 - 20 μm) which are cemented together to form the second hierarchical order, microaggregates (20 - 250 μm), which are bound together into the third order, macroaggregates (> 250 μm). The binding agents involved in aggregate formation and stabilization are classified into three categories: i) persistent; ii) temporary, and iii) transient. Persistent binding agents are involved primarily in the stabilization of microaggregates, as described originally by Edwards and Bremmer, (1967). They include complexes of aromatic humic material associated with polyvalent inorganic cations (such as Ca, Mg, Fe, Al and aluminosilicates) and clay. The center of the aggregate is believed to be organic matter, with particles of fine clay sorbed onto it (Tisdall and Oades, 1982). Temporary binding agents consist of roots, fungal hyphae (particularly vesicular-arbuscular (VA) mycorrhizal hyphae), bacterial cells and algae. Roots and fungal hyphae develop quickly in soil as a plant grows and provide the framework around which macroaggregates can be formed. Transient binding agents are

organic materials which are decomposed rapidly by microorganisms, namely polysaccharides produced by bacteria, fungi and plant roots. The persistence of transient agents is considered to be on the order of weeks, whereas temporary agents may exist for periods varying from months to a few years. Persistent materials remain in the soil for much longer periods (Tisdall and Oades, 1982). The size of the stabilizing material influences the scale at which the association between particles takes place. As pointed out by Kay (1990), inorganic materials and organic molecules are capable of bonding clay particles, and cementing larger mineral grains together at their points of contact. Polysaccharides for example, are involved mainly in the stabilization of aggregates < 50 μm (Tisdall and Oades, 1980). However where grains are separated by greater distances, binding involves materials such as roots and fungal hyphae.

The process of packaging soil particles into aggregate units has been identified as a means by which soils can accrue organic matter (Oades, 1984; Jastrow and Miller, 1992; Hassink et al., 1993; Jastrow, 1996; Sollis et al.,1996). This occurs because organic residues are protected from the action of microbes when they are encapsulated by clays and silts during the aggregation processes, and also because of their location in small pores.

Oades and Waters (1991) demonstrated aggregate hierarchy in a Mollisol and an Alfisol (aggregates breakdown in a stepwise fashion) but not in an Oxisol (aggregates breakdown to release silt and clay size particles). Oades (1993) speculated that aggregate hierarchy would be found in soils in which aggregate stability is controlled by organic materials, and which have a long history of exploration by the fine root systems

of grasses. Aggregate hierarchy may not apply to young soils, or to highly weathered soils such as oxisols, where inorganic cementing agents are dominant.

In contrast to the hierarchical model proposed by Tisdall and Oades (1982), an alternative model for the formation of macro- and microaggregates of soil has been suggested (Oades, 1984; Elliott and Coleman, 1988; Beare et al., 1994a). In this alternative view of aggregate organization, the growth of roots and hyphae serve to physically form macroaggregates. Microaggregates are then formed and stabilized within these macroaggregates as a result of decomposition of organic debris, deposition of microbial products, reorientation of clay platelets, and physicochemical reactions between polyvalent cations and clays. As pointed out by Beare et al., (1994a) microaggregates formed in this way, and subsequently released from macroaggregates, should be relatively stable and enriched in SOM.

INFLUENCE OF CROPPING SYSTEMS AND TILLAGE PRACTICES ON SOIL AGGREGATION

Changes in soil structure that result from intensive cultivation often are accompanied by a decline in SOM content (Dalal and Meyer, 1986; Elliott, 1986; Tisdall and Oades, 1982; Kay, 1990), and a shift in aggregate size distribution and stability (Dormaar, 1983; Elliott, 1986; Gupta and Germida, 1988; Angers et al., 1992; Miller and Dick, 1995 a). Some of the factors that contribute to SOM decline are listed by Angers et al., (1992) and include: i) accelerated mineralization due to tillage

operations; ii) increased erosion, iii) lower amounts of C input, and iv) dilution of SOM through mixing of the OM-rich surface layer with subsurface soil lower in SOM.

Unstable aggregates will slake when wetted, and the detached microaggregates and clay particles may migrate into pores thereby obstructing and narrowing them (Foster, 1994). This process can lead to the formation of crusts that inhibit the movement of water and air into the soil and increase the chance of surface erosion (Tisdall and Oades, 1982). Because the stability of macroaggregates depends largely on roots and hyphae (temporary binding agents), conceptually they are the least stable, and as a result are most heavily impacted by management practices. Management systems which promote greater root activity and C input (such as no-till, cover-cropping, inter-cropping and pasture) have a better chance of increasing and stabilizing macroaggregates (Jastrow, 1987, 1996; Carter, 1992; Latif et al., 1992; Beare et al., 1994b; Miller and Dick, 1995a; Chantigny et al., 1997) than conventional tillage systems with lower C inputs (Angers et al., 1992; Beare et al., 1994 b). Not surprisingly, therefore, the proportion of macroaggregates has been reported to decrease (Elliott, 1986; Gupta and Germida, 1988; Beare et al., 1994 b; Angers et al., 1992) and to increase (Jastrow, 1987, 1996; Miller and Dick, 1995a) in response to different management practices. The impact of different cropping systems and management practices on soil structural characteristics can vary with soil type and climate, as well as with depth in the profile (Kay, 1990; Beare et al., 1994 b).

The repeated mechanical disruption of macroaggregates in conventional tillage (CT) practice lowers their stability and leaves them more susceptible to the disruptive forces of drying and rewetting, raindrop impact, and freezing and thawing (Tisdall and

Oades, 1982; Angers et al., 1992; Beare et al., 1994). As a result CT practices expose more of the physically protected SOM to microbial attack and mineralization (Low, 1954; Rovira and Greacen, 1957; Tisdall and Oades, 1982; Coleman et al., 1994).

A significant increase in the percentage of microaggregates (< 250 μm) with a concurrent decrease in macroaggregates (> 250 μm) was observed by Elliott, (1986) when he compared a cultivated soil with a native sod, after slaking air-dried aggregates recovered from the two soils. The results indicated that the OM binding microaggregates into macroaggregates (intermicroaggregate OM) was particularly sensitive to cultivation. Based on the fact that the amounts of roots and hyphae present in the soil account for only a small amount of the total SOM, he pointed out that the large losses of SOM due to long-term cultivation should come from another SOM fraction, such as fine clay associated organic C.

In a study comparing the effects of long-term (13 years) CT and NT (no-tillage) management on water stable aggregates (WSA) and aggregate associated OM, Beare et al., (1994b) observed that macroaggregates (> 250 μm) were more abundant and more stable in surface soils (0 - 5.0 cm) of NT than in CT. Although, there were no differences in size distribution of WSA at a greater depth (5.0 - 15.0 cm), the largest macroaggregates (> 2.0 mm) of CT were less stable than in NT. Nearly all of the differences between tillage in macroaggregate C were found in surface soil. CT soils retained much less macroaggregate-protected SOM (defined as the difference in C mineralized from crushed and intact aggregates) than NT soils and, the unprotected pools of C (C mineralized in intact aggregates) also made up a higher percentage of whole soil aggregate C in soils of CT than in NT.

Using electron microscopy, Foster (1994) studied the distribution of soil minerals, OM, and microorganisms in and on microaggregates from virgin soils and from nearby field trials exposed to conventional and reduced tillage. No differences were observed in the ultrastructure of microaggregates from either conventionally or reduced tilled plots. However, differences in the distribution of minerals and SOM were observed between aggregates from the tilled soils and virgin soils. Fine pores within microaggregates tended to be open in aggregates recovered from the virgin sites, but filled with dispersed clay in aggregates from tilled sites. SOM was more degraded in aggregates from the tilled plots than from the virgin sites. Microscopic observations reported by Gupta and Germida, (1988), showed that macroaggregates (> 1.0 mm) from native grassland soil had extensive growth of fungal mycelium, whereas little fungal growth was detected in macroaggregates from a cultivated soil.

Indeed, the link between grasslands and improved soil aggregation has been observed on several occasions. For example, the establishment of a pasture, or of a forage species is a successful method of manipulating soil structure (Tisdall and Oades, 1982; Lynch and Bragg, 1985; Drury et al., 1991). Jastrow (1987) and Miller and Jastrow, (1991) reported an increase in macroaggregates associated with the restoration of cultivated soil to tallgrass prairie. The formation of WSA was found to be related to the quantity of root biomass and the plant community composition (C_4 prairie grasses and perennial species of Compositae with their fine and very fine root length exhibited the strongest effects on macroaggregate formation compared to C_3 eurasian grasses). By comparing monthly changes in water stable aggregates in a clay soil under continuous silage corn, alfalfa and barley stands over the course of two growing seasons, Angers

and Mehuys (1988) measured a significant increase in water stable aggregates under alfalfa and barley. Three years later (Angers 1992), the mean weight diameter of wet-sieved aggregates (MWD) under alfalfa had increased from 1.5 to 2.2 mm and then leveled off. The increase in MWD under alfalfa was attributed primarily to an increase in aggregates > 2.0 mm. Under silage corn and fallow (bare-soil control), changes in MWD were minimal throughout the five years of the study. During a 3 year study, Perfect et al., (1990) conducted monthly field measurements of changes in dispersible clay (DC) and wet aggregate stability under conventional corn, bromegrass, alfalfa and red clover. All of the forage treatments resulted in highly significant changes in soil structural stability, as determined by a combination of wet-aggregate sieving and dispersible clay analysis. Some of these studies showed that increases in organic C lagged behind increases in macroaggregates (Angers 1992, Jastrow, 1996). For example, Jastrow (1996) observed that the rate constant for the increase in macroaggregation was more than 35 times that for TOC accumulation.

Plant species that regenerate the structural form and stability of soil can be incorporated into intensive crop-production systems through their use as intercrops (interseeded in the commercial crop) and cover crops (Scott - Smith et al., 1987). In a 3 year study, Latif et al., (1992) observed that intercropping legumes (alfalfa, clover, hairy vetch) with maize promoted increases in the mean weight diameter (MWD) and stability of soil aggregates in the two September samplings. In the two May samplings stability and size of soil aggregates were at a minimum, and no treatment effects were observed. Miller and Dick (1995a) observed a 35% increase in the percentage of macroaggregates in a Willamette silt loam soil after 3 years of an alternative rotation in which a legume

winter-cover-crop (red clover) was added to a summer vegetable crop/winter fallow system. In the fifth season of a field experiment which examined the effects of winter cover crops and fertilizer N rates on an irrigated tomato crop system, Roberson et al., (1995) demonstrated that the production of extracellular polysaccharides (EPS) by microorganisms affected soil structure, and that N availability strongly controlled EPS production. A vetch winter cover-crop performed better than either a non N-fixing cover crop or moderate N fertilization (168 kg N ha^{-1}) in stimulating microbial EPS production and increasing aggregate slaking resistance and saturated hydraulic conductivity. High N fertilization rates (280 kg N ha^{-1}) depressed EPS production and consequently did not improve soil structure.

In addition to cropping practices, soil tillage also can affect soil aggregation. Carter (1992) reported that minimum-tillage systems with direct drilling and chisel plowing conducted for 3 to 5 years, increased the stability in the surface (0 to 5 cm) of a fine sandy-loam by 43 to 84 % in comparison with moldboard plowing. Interestingly he observed that MWD calculated after dry-sieving was not as sensitive as MWD calculated after wet sieving to discriminate between the different tillage systems.

Kay et al., (1988), proposed the concept of half-life, referring to the time required to improve the stability of a soil to a point midway between the initial level and the maximum possible stability. Values have been reported that range from 2.3 to 7.2 years for a silt-loam soil under different forages (Perfect et al., 1990) and 0.9 years for a clay soil under alfalfa (Angers, 1992). However, claims that changes in soil structure may be achieved within a relatively short time should be interpreted with caution. Changes may only be temporary (Malope, 1988; Kay, 1990; Drury et al., 1991; Latif et

al., 1992; Mulla et al., 1992). For example, Perfect et al., (1990) conducted a study on the rates of change in soil structural stability under forages and corn and observed that temporal fluctuations in soil stability over the growing season were as large, or larger than the changes observed between treatments over a three year period. In their study fluctuations in soil water content explained 63 and 46% of the total variation in dispersible clay and water stable aggregates, respectively. The improvement in structural stability with time explained up to 43% of the remaining variability, depending on cropping treatment.

RELATIONSHIPS BETWEEN MICROBES AND SOIL STRUCTURE

The importance of soil structure in determining soil habitability and the availability of SOM to microorganisms was emphasized by Elliott and Coleman (1988). In their view, biotic factors bind the different sizes of aggregates together and in doing so, create pore networks, such that intra- and intermicroaggregate pores are connected to intermacroaggregate pores and macropores. They also pointed out that pore sizes limit the kinds of organisms that can occur within them. Because of size restrictions, only bacteria would be found in intramicroaggregate pore space. Fungal hyphae, protozoa, small nematodes, very fine roots and root hairs occupy the intermicroaggregate pore space. Microarthropods, worms and coarse roots are restricted to macropores.

More evidence supporting this view has been obtained from electron microscopic studies. Fungi, actinomyces and amoebae were observed on the surfaces of microaggregates, whereas bacteria were not found in this region (Kelbertius, 1980;

Foster, 1988, 1994). The absence of bacteria may be related to the fact that they are susceptible to predation, whereas fungi are more tolerant of water stress and rapid rewetting conditions (Shipton, 1986). Bacteria are observed in the ultrathin sections of aggregates, occurring as isolated cells or as small colonies in pores within the clay matrix.

Substrate and water availability, as well as protection against predators are among the major factors driving microbial distribution in soil. The effects of substrate availability on the spatial distribution of microorganisms in soils can be fully appreciated in the rhizosphere surrounding plant roots, and/or on the surface of plant and animal residues where larger populations of microorganisms can be found (Paul and Clark, 1996).

Based on the ability of pores between 2 and 6 μm in diameter to retain water (capillary pores), Hattori and Hattori (1976) suggested these pores could be the most favorable microhabitats of soil bacteria. Postma and Van Veen (1990) proposed the classification of soil pore spaces into five categories: total, accessible, habitable, protective and inaccessible. The habitable pore space was defined as the part of the accessible pore space which is suitable for the survival and establishment of bacterial cells. Using *Rhizobium leguminosarum* bv *trifolii* cells as a model, protective pore space was defined as the part of the habitable pore space that protects bacteria from predation by protozoa (between 0.8 and 3.0 μm). Mean diameter for some protozoa are: 5 μm for flagellates, 10 μm for amoebae, and 20 μm for ciliates (Hattori and Hattori, 1976). Pores < 0.8 μm in diameter were considered to be inaccessible to rhizobial cells. The importance of the clay content, as another factor influencing the growth and spatial

distribution of microbial communities in soils was reported by Rutherford and Juma, (1992). Clay particles generally impart a fine texture and a heavy nature to soil. The fine texture produces a large amount of total pore space, but pore sizes are small. As a result, bacteria are better protected from protozoan predation in fine-textured soils than in coarse textured ones (Rutherford and Juma, 1992; Hassink et al., 1993).

RELATIONSHIPS BETWEEN MICROBES AND SOIL PARTICLE SIZE FRACTIONS

The location of microorganisms in the soil matrix has also been studied by physical soil fractionation (Kanazawa and Filip, 1986; Jocteur-Monrozier et al., 1991; Lensi et al., 1991; Van Gestel et al., 1996). These studies provide information on the association between soil minerals, SOM, and microorganisms and help to identify suitable microniches for microbial survival and activity. The results obtained from fractionation studies are dependent upon the soils studied, and are influenced by the procedures used to obtain the different particle size-classes. For example, in one study by Kanazawa and Filip, (1986), the coarse organic particles (> 5.0 mm) and the silt clay fraction (< 50 μm) represented the sites with the highest concentration of microorganisms, ATP content and enzyme activities in a brown soil from Central Europe. In Australian soils, Jocteur-Monrozier et al., (1991) found that most of the microbial biomass C (MBC) in a Vertisol was concentrated in the 2.0 to 20.0 μm fraction, which also contained the higher concentration of soil organic C and N. In contrast, the biomass C of an Alfisol was most heavily concentrated in the 2.0 to 0.1 μm size class. In another study, Lensi et al., (1995), evaluated fractions recovered from the

upper layer of two Mollisols under contrasting land use. They observed that in both soils the 2.0 to 20.0 μm fraction exhibited the highest concentrations of MBC, organic N, and a population density of denitrifiers greater than in the unfractioned soil; enumeration of total bacteria by an MPN procedure revealed that the highest density was found in the < 2.0 μm. In a silty-loam Alfisol, Van Gestel et al., (1996) observed that the variation in MBC contents among size fractions was largely explained (> 99%) by the clay and OM content of the size fractions. More than 50% of the MBC content of the total soil was associated with the 2.0 to 20.0 μm and the clay size fraction (< 2.0 μm) which represented 19 and 5% of the total soil weight, respectively. These observations fit with the concept of the hierarchical model proposed by Tisdall and Oades (1982) which suggests that such associations are important to initiate soil aggregation.

Although studies with soil fractions have helped to define the associations between microorganisms and the mineral components of soil, procedures that minimize dispersion of soil should be used when information is needed on soil structure and biological activities (Ladd et al., 1993).

RELATIONSHIPS BETWEEN MICROBES AND SOIL AGGREGATES

The separation of soil into different aggregate size classes by wet and dry sieving techniques has been used to study the spatial distribution of microorganisms and their activities in the soil matrix (Gupta and Germida, 1988; Seech and Beauchamp, 1988; Miller and Dick, 1995a and b; Singh and Singh, 1995).

Gupta and Germida (1988) demonstrated that the amount of MBC, length of fungal hyphae, soil enzymes (arylsulfatase and acid phosphatase), respiratory activities and mineralization rates of C, N and S, were lower in microaggregates size classes (0.25 to 0.10 and < 0.10 mm) than in macroaggregates (0.25 to 0.50, 0.50 to 1.0, and > 1.0 mm). In contrast, bacterial counts were greatest in the microaggregate size class < 0.10 mm. Other studies have not always found that microbial parameters were greater in macroaggregates than in microaggregate size classes. For example, Miller and Dick (1995a and b) reported higher concentrations of MBC and amidase activity in macroaggregates (0.25 to 0.50; 0.50 to 1.0, and 1.0 to 2.0 mm), and higher activities of l-asparaginase and β-glucosidase in microaggregate size classes (< 0.10 and 0.10 to 0.25 mm). Singh and Singh (1995) reported greater contents of MBC in macroaggregates (> 0.30 mm), whereas microbial biomass N and P were higher in microaggregates (< 0.30 mm).

Seech and Beauchamp (1988), found that the contents of MBC and mineralizable carbon (basal respiration) in aggregates recovered from a silt loam cultivated continuously with corn for 15 years, were greatest in microaggregates and decreased with increasing aggregate size. They suggested that microaggregates were more biologically dynamic than macroaggregates. Similarly, in a study to investigate turnover and inputs of organic C in WSA of different sizes, Jastrow et al., (1996) observed that although net inputs of "new" C increased with aggregate size, the net input rate for microaggregates was equal to the rates for small macroaggregates. They pointed out that, the formation and degradation of microaggregates may be more

dynamic than originally predicted by their stability in cultivated soils (Tisdall and Oades, 1982) or by the observed turnover times for "old" C.

Few studies have examined the distribution across aggregates of specific groups of microorganisms and/or their related processes. Among these are studies of denitrification rates (Sextone et al., 1985; Myrold and Tiedje, 1985; Seech and Beauchamp, 1988; Beauchamp and Seech, 1990), and the distribution of soil protozoa (Vargas and Hattori, 1990). The studies conducted by Sextone et al., (1985) and Myrold and Tiedje, (1985) were carried out with individual soil aggregates. Larger aggregates (radius > 10.0 mm) often had measurable anaerobic centers, whereas aggregate < 6.0 mm were generally oxic. Anaerobic centers were present in all aggregates that denitrified, but not all aggregates with anaerobic zones denitrified (Sextone et al., 1985). Other factors such as the supply of carbon substrates and limitations on NO_3 diffusion also influenced the denitrification rates in individual soil aggregates (Myrold and Tiedje, 1985). Seech and Beauchamp (1988) and Beauchamp and Seech, (1990) evaluated the denitrification potential in different aggregate size classes of a silt loam as a possible way to explain spatial variability of denitrification. They observed a large spatial variability in denitrification rates associated with aggregates of different sizes and water stability. Denitrification rates generally decreased as the size of dry-sieved aggregates increased, whereas in wet-sieved aggregates denitrification rates increased as the aggregate size increased. The results suggested that the large spatial variability of the denitrification rates could be associated with the availability of C substrates in each aggregate size class and also that the microbiological characteristics associated with soil

aggregates were largely dependent upon the procedures used to sample and prepare them.

Vargas and Hattori (1990), evaluated the distribution of five groups of protozoan cells in 330 individual soil aggregates (1.0 to 2.0 mm) from an arable field. Some aggregates did not contain representatives of all five groups studied, indicating that the protozoan groups conglomerate in some aggregates while scarcely residing in others. It was suggested that the heterogeneous patterns of distribution of protozoan groups across aggregates could have implications not only for the interactions between protozoa and bacteria, but also for the interactions between different protozoan groups and the interactions between protozoa and other soil organisms.

As it can be seen from this review, although several studies have examined the spatial distribution of microorganisms and their activities in soil aggregates, comparisons are difficult because of variations in soil type, plant species, method of aggregate preparation and the uncertainty of intra-seasonal effects. In particular, studies are lacking where the seasonal dynamics of the distribution of soil aggregates, microbial biomass and activities have been evaluated under different cropping systems. The work presented in this thesis was carried out to contribute to our understanding of the distribution of microorganisms, their activities, and their dynamics across soil aggregates recovered from a western Oregon summer vegetable cropping system in which winter cover cropping has recently been integrated.

SPATIAL DISTRIBUTION OF *RHIZOBIUM* IN SOIL

In recent years the search for useful indicators of soil quality changes has led researchers to examine whether changes in the overall microbial community composition of soil can be related to management practices (Bååth, et al., 1995; Zelles et al., 1995; Frostegard et al., 1996; 1997; Reichardt et al., 1997). To date, no studies have been conducted to examine the influence of management practices on the distribution of a specific bacterium among aggregate size classes. In this context, bacteria of the genus *Rhizobium* and *Bradyrhizobium* are of particular interest. Despite being recognized primarily for its ability to form symbiotic associations with leguminous plants, *Rhizobium* is a bona fide soil saprophyte with dynamic soil populations ranging from 10^8 cells g^{-1} soil in the rhizosphere of its legume host to < 100 cells g^{-1} in the absence of the host plant (Moawad et al., 1984; Bottomley, 1990). As a result of their build up in legume rhizospheres and their release from senescent nodules, large populations of rhizobia exist in cultivated fields with a legume cropping history compared to fields where legumes have not been cultivated (Weaver et al., 1972; Elkins et al., 1976 and Mahler and Wollum, 1982). Information on the spatial distribution of *Rhizobium* is limited and studies have ranged across a wide scale from the spatial variability associated with 0.3 m increments across apparently uniform field sites (Wollum and Cassel, 1984), to the association with soil particles (Ozawa and Yamaguchi, 1986) and variable pore sizes (Postma et al., 1990; Postma and Van Veen, 1990).

Wollum and Cassel (1984) observed that *Rhizobium* populations ranged from 4.67×10^2 to 1.07×10^7 cells g^{-1} soil in an apparent uniform 18 m by 18 m area, illustrating that *Rhizobium* populations vary widely over short distances. Moreover, in the two sites evaluated the variance structure for the most probable number (MPN) counts was found to be directional. In one site there was more variance in the direction parallel to the soybean row than in the direction perpendicular to it, whereas in the other site the opposite trend was observed. The reasons for these apparent inconsistencies between the two sites were not known, but the authors suggested that they could be associated with variations in root densities and inherent soil properties.

Ozawa and Yamaguchi (1986) reported that soil-borne *Rhizobium* exist attached and unattached to soil particles. The fractionation studies showed that more than 90% of the indigenous rhizobia in an agricultural field were attached to soil particles, and it was suggested that adsorption to soil particles influenced the susceptibility of rhizobial cells to predation by protozoa and their movement in soils. Using a soil washing procedure Postma et al., (1989) observed that after 5 consecutive washings the number of *Rhizobium* cells which were unattached or attached to particles < 50 μm decreased faster than the cells in aggregates > 50 μm. The results indicated that rhizobial cells attached to, or enclosed in soil particles or aggregates larger than 50 μm had a more favorable microhabitat than unattached cells or cells attached to smaller particles.

The impact of soil physical features on the fate of introduced rhizobial strains was examined in a series of studies conducted by van Veen and colleagues which demonstrated the importance of colonization of soil particles and aggregates to the survival of introduced strains in soil (Postma et al., 1988, 1989 and 1990; Postma and

van Veen, 1990; van Veen et al., 1997). Postma et al., (1989) showed that introduced rhizobial cells survived better in soils with a low moisture content than in soils with a higher initial moisture content. Under high moisture content, water in soil pores prevents introduced rhizobial cells from penetrating smaller pores, and leaves them more accessible to predators than cells introduced into dry soils. Early studies in a silt loam and in a loamy sand soils, showed that habitable pore space (pore neck diameter > 3.0 μm) and protective pore spaces (0.8 to 3.0 μm) were not limiting factors for the survival of rhizobial strains in a sterilized soil (Postma and van Veen, 1990). Nevertheless, the addition of bentonite-clay (large swelling capacity) to a loamy sand soil did improve the survival of introduced rhizobia, presumably through the creation of more protective microhabitats ranging between 0.8 to 6.0 μm (Heijnen and van Veen, 1991).

Because of the decline in population that follows the introduction of bacteria in soils, Postma et al., (1990) also evaluated the influence of the presence of other soil bacteria (competitors) and protozoa (predators). The presence of other soil bacteria hindered rhizobial colonization of soil particles and aggregates, and reduced the number of soil-particle associated rhizobia. The presence of predators caused an increase in the percentage of particle-associated rhizobia. A synergistic effect was observed when both predators and competitors were present, and the populations of introduced rhizobia decreased drastically.

Because no one has examined the distribution across soil aggregates of an indigenous population of any bacterial species, the presence of red clover (*Trifolium pratense*, L) in the rotational systems at the North Willamette Extension and Research

Center (NWERS), Aurora, Oregon, provided an opportunity to examine the dynamics and distribution across soil aggregates of a particular member of the soil community, namely *Rhizobium leguminosarum* bv *trifolii*, both in the presence and absence of its host plant.

Chapter 3. DYNAMICS, ACTIVITY AND DISTRIBUTION OF MICROBIAL BIOMASS IN SOIL AGGREGATES RECOVERED FROM DIFFERENT COVER CROP SYSTEMS

ABSTRACT

The effects of three winter cover-crop treatments (fallow, cereal, and legume) in a summer vegetable cropping system were evaluated for their impacts on soil aggregate size distribution, and on the spatial distribution of soil microorganisms and their activities in a Willamette silt loam. Five aggregate size classes (< 0.25, 0.25 to 0.5, 0.5 to 1.0, 1.0 to 2.0, and 2.0 to 5.0 mm) were obtained by a dry sieving procedure, and the distribution of total organic C (TOC), total Kjeldahl N (TKN), microbial biomass C (MBC), mineralizable C and N (Min.C and Min.N), and two enzyme activities (β-glucosidase, FDA-hydrolysis) were evaluated at seedbed preparation (June) and after harvest of sweet-corn or broccoli summer crops (September). Despite the cover crop treatments having been in place for 7 years, they have not influenced the overall level of TOC, TKN, and aggregate size distribution. More often, cover crop effects on biomass and the other activity measurements were subtle, being seen only at specific times of the year or interacting with aggregate size. MBC and its activities were heterogeneously distributed across soil aggregates, and the distributions of MBC, Min. N, β-glucosidase and FDA-hydrolysis were dynamic and changed between June and September. Furthermore, temporal changes in MBC and Min. N occurred in the < 0.25 mm size class in all treatments indicating that microaggregates are biologically active and dynamic in this soil. The profiles of C and N mineralization and enzyme activities

across aggregate size classes were often different. Further work is required to determine if this is a reflection of differences in substrate quality and/or community composition.

INTRODUCTION

By contributing organic matter (OM) both directly through decomposition of root and shoot residues, and indirectly through stimulation of microbial activity in their rhizospheres, legume and non-legume cover crops have shown some potential for improving soil structure (Havlin et al., 1990; Kuo et al., 1997). Reductions in bulk density and soil resistance, increases in water retention, infiltration properties, and in the stability and amount of macroaggregates, have been reported to occur in relatively short periods of time after the introduction of cover crops into cropping systems (Wilson et al., 1982; McVay et al., 1989; Latif et al., 1992; Miller and Dick , 1995a; Roberson et al., 1995). In addition, while a legume winter cover crop can be a significant source of N for the summer crop (Ebelhar et al., 1984; Hargrove, 1986), non-legume cover crops can sequester residual NO^-_3 and prevent leaching to ground water (Wagger and Mengel, 1988; Martinez and Guiraud, 1990; Brandi-Dorn et al., 1997).

Further refinement of our understanding of the processes contributing to accretion of soil organic matter (SOM) and development of soil structure will require an examination of the compartmentalization of microorganisms and their activities in soil aggregates. Several studies have shown that soil bacteria and fungi, microbial biomass, soil protozoa, C and N mineralization, denitrification rates and soil enzyme activities are

not evenly distributed across soil aggregates (Gupta and Germida, 1988; Hattori, 1988; Seech and Beauchamp, 1988; Beauchamp and Seech, 1990; Vargas and Hattori, 1990; Miller and Dick, 1995a and b; Singh and Singh, 1995). Some studies have shown greater microbial biomass and activity in macroaggregates compared to microaggregates (Gupta and Germida, 1988; Miller and Dick, 1995a and Singh and Singh, 1995), whereas others have reported similar and even higher levels in microaggregates compared to macroaggregates (Seech and Beauchamp, 1988; Miller and Dick, 1995 b and Jastrow et al., 1996). These findings indicate that soil aggregates represent environments of varying suitability for microorganisms, and that different soils and cropping systems may respond differently in how microorganisms and their activities are distributed. In this context, no studies have been reported where the impacts of different cover crops on soil aggregation and on the spatial distribution of soil microorganisms have been examined simultaneously at different times of the year. The objectives of the present study were to examine the aggregation state of soil and the dynamics, activity and distribution of microbial biomass in whole soil and soil aggregates recovered from a vegetable crop rotational system in which either a winter fallow, or a red clover or cereal winter cover crop were used.

MATERIALS AND METHODS

Experimental site

Soil samples were collected from a vegetable crop rotation experiment initiated in 1989 at the North Willamette Research and Extension Center (NWREC), Aurora, Oregon. The soil is a Willamette silt loam (Pachic Ultic Argixeroll), the climate is Mediterranean characterized by cool wet winters and warm dry summers. The mean annual precipitation is 1040 mm with 70% occurring between November and April. The mean air temperature is 11.1°C and the average soil temperature in the winter and summer at a depth of 5 cm is 7.2°C and 19.3°C, respectively (Miller and Dick, 1995b). General characteristics of soil from NWERC are presented in Table 3.1. The site had been in a winter wheat-fallow rotation for the previous ten years, before the experiment was initiated.

Field treatments included three winter management systems in a vegetable crop rotation that alternates two summer crops, sweet corn (*Zea Mays* L. cv. Jubilee) and broccoli (*Brassica oleracea* L. var Italica cv. Gem) (Table 3.2). The three winter treatments are: (1) winter fallow, (2) red clover cover crop (*Trifolium pratense* L var. Kenland), and (3) cereal rye cover crop (*Secale cereale* L. var. Wheeler). The cover crops are established by the "relay' method in which they are seeded under the summer crop in late July to take advantage of the irrigation. The strategy behind the relay procedure is to have the cover crops established prior to the onset of fall rains. Beginning in 1995 cereal rye was replaced with Celia trititicale (*X Triticosecale*

Table 3.1. General characteristics of soils from the North Willamette Extension and Research Center (NWERC).

Soil characteristics	Depth (cm) 0-10	10 - 20
pH†	5.60	6.18
Total C (g kg^{-1} soil)†	15.0	15.0
Total N (mg kg^{-1} soil)†	1058.0	1008.0
Total P (mg kg^{-1} soil)†	2612.0	2632.0
Bulk density (Mg m^{-3})‡	1.24	
K sat (x 10^{6} ms-1) ‡	60	

† Data collected in September 1989 (Kauffman, S.M., Masters Thesis, OSU, 1994)
‡ Brandi-Dohrn et al., 1997

Table 3.2. Crop rotations at North Willamette Extension and Research Center (NWERC).

Year	Season	Winter Fallow	Relay cover crop Cereal	Legume
1989	Fall	Fallow	Rye	Red Clover
1990	Spring	Corn	Corn	Corn
	Fall	Fallow	Rye	Red Clover
1991	Spring	Broccoli	Broccoli	Broccoli
	Fall	Wheat	Rye	Red Clover
1992	Spring	Wheat	Corn	Corn
	Fall	Fallow	Rye	Red Clover
1993	Spring	Broccoli	Broccoli	Broccoli
	Fall	Fallow	Rye	Red Clover
1994	Spring	Corn	Corn	Corn
	Fall	Fallow	Rye	Red Clover
1995	Spring	Broccoli	Broccoli	Broccoli
	Fall	Fallow	Celia Triticale	Red Clover
1996	Spring	Corn	Corn	Corn
	Fall	Fallow	Celia Triticale	Red Clover

Wittmack). Hereafter, these treatments will be referred as fallow, cereal, and legume respectively.

Experimental design

The experimental design was a randomized complete block split-plot with four replications. The plots are 18.0 m x 9.0 m, with cropping systems as the main plot and N rate as the subplot. The main plots are divided into 3 equal sub-plot areas of 54 m^2. Nitrogen fertilizer (urea) rates are: zero, medium (56 and 140 kg of N ha^{-1} for sweet-corn and broccoli, respectively) and recommended (224 and 280 kg of N ha^{-1} for sweet-corn and broccoli, respectively). Samples were taken only from the zero N rate.

Soil sampling

Soil samples were collected with a shovel to a depth of 20 cm in early September 1995 (post broccoli harvest), early June 1996 (one month after the incorporation of the cover-crops and 5 days after the germination of the sweet-corn), and early September 1996 (post sweet corn harvest). Twenty cores from each replicate were composited, mixed by hand in a bucket and placed in large Ziploc bags for transportation to the laboratory. For the purpose of the studies involving aggregate size distribution, samples were also collected in June 1995, and composited before dry-sieving. The June samplings were conducted to evaluate soil properties at the time of seed bed preparation, and prior to the summer crop growing period. In contrast, the

September samplings were taken to evaluate soil properties after the summer cropping period, and immediately prior to fall rains.

Soil sieving and aggregates distribution

Before sieving, large soil clods in field moist soil samples were gently crushed by hand. Soil samples were dried at 4°C until they reached a gravimetric water content between 5 and 14% (w/w). The field moisture contents of the samples collected in June and September 1995, June and September 1996 ranged between 14.5 to 20.5, 10.6 to 16.4, 13.8 to 20.1 and 12.0 to 17.6 % (w/w) respectively.

Aggregates were prepared by placing portions (200 g) of soil in the top of a nest of sieves on a Tyler Ro-Tap testing sieve shaker (Combustion Engineering Inc., Mentor, OH). Soil was sieved for three minutes into the following size classes: < 0.25, 0.25 to 0.50, 0.50 to 1.00, 1.00 to 2.00, and 2.00 to 5.00 mm. All samples were stored at 4°C. Results of a preliminary experiment indicated that sieving > 3 minutes increased the amount of soil present in the < 0.25 mm fraction, probably as a consequence of abrasion of larger aggregates (see Appendix 1). Sieving periods < 3 minutes were insufficient to promote a good separation of the 0.25 to 0.5 mm and < 0.25 mm size classes (data not shown). For the purpose of the studies examining aggregate distribution, portions (100 g) of soil were sieved, and the amount of aggregates retained in each sieve determined by weight. The procedure was repeated on another 100 g portion from the same field treatment. The percentage of soil in each aggregate size class was calculated after excluding the material > 5.0 mm. The mean weight diameter (MWD) of aggregates was

calculated based on the sum of the products of the mean diameter of each size fraction and the proportion of total sample weight occurring in that corresponding size fraction as described by Kemper and Rosenneau (1986).

In September 1995, experiments were conducted on composite aggregate samples that were prepared by mixing equal amounts of aggregates from a specific size class prepared from each field replication. The aggregates obtained from soil recovered in June and September 1996 were treated separately as field replications. Whole soil samples were sieved to pass a 5.0 mm sieve. Aggregates and whole soil samples were stored in polyethylene bags at 4°C until the time of the analysis.

Soil chemical analyses

Whole soil and aggregate samples were ground with a mortar and pestle to pass a 0.25 mm sieve. Total organic carbon (TOC) was determined by direct combustion to CO_2 in a DC-80 Dohrmann carbon analyzer (Dohrmann Inc., Santa Clara, CA) equipped with an infra-red detector. Less than 8 mg of soil samples were used for TOC analysis.

Total Kjeldahl Nitrogen (organic N and NH_4^+) was determined as described by Bremner and Mulvaney, (1982). Soil samples (0.5 g) were digested in 10 ml of concentrated H_2SO_4 and 1 g of Kjeldahl reagent (100:10:1 (w/w) K_2SO_4: $CuSO_4$: Se) for three hours until they cleared (slight blue-green color). After digestion, distilled water was added to a final weight of 30 g (equivalent to a 1:20 dilution). Ammonia was distilled from aliquots (1 ml) in an all glass micro-distillation unit for five minutes after

addition of 2 ml of 10 M NaOH. The distillate was collected in H_3BO_3 indicator and titrated with standardized H_2SO_4 (0.0025 M) using a micro-burette.

Total organic C was determined on soil samples collected in September 1995, June and September 1996. Total Kjeldahl N was determined only on June and September 1996 samples.

Physical analyses

Five aggregate size fractions and whole soil samples recovered from the fallow (June 1996) and legume treatments (June and September 1996) were analyzed for texture and particle size distribution using the pipette method as described by Gee and Bauder, (1986). All samples were analyzed without any pretreatment for removal of organic matter.

Ten gram portions of either whole soil or aggregates were placed in 500 ml plastic bottles. Ten ml portions of 5% (w/v) sodium hexametaphosphate and 250 ml of water were added to each bottle and the samples were shaken overnight at 20 rpm at room temperature. The sand fraction was removed by washing the soil suspension with distilled water through a 270 mesh sieve that was fitted into a large funnel sitting on top of a 1000 ml glass cylinder. After the soil suspensions had been transferred through the sieve, the sand retained on the sieve was further washed to remove silts and clays. All wash water was collected in the cylinder and the final volume completed to 1000 ml. The sand content was determined after drying at 105°C for 72 hours.

The cylinders were covered with a glass watch to prevent evaporation losses and allowed to stand in the constant temperature room (20°C) overnight before the beginning of the sampling. Sampling times for this temperature are 4 minutes and 48 seconds for the first pipetting and 7 hours 59 minutes and 51 seconds for the second pipetting. Each cylinder was mixed for 30 seconds, at five minute consecutive intervals with a plastic plunger and a smooth up-and-down motion. The exact time that mixing ceased was recorded. About one minute prior to sampling, a 20 ml pipette was lowered to a 10 cm depth in the solution. The pipette was steadily filled over 15 seconds, using a pistol handle attached to a vacuum pump. The sample was released into a tared glass vial, and the pipette rinsed with distilled water. The rinse water was added to the clay suspension in the glass vial as well. After the pipette sampling was done, the glass vials and contents were oven-dried at 105°C for 72 hours, cooled in a desiccator and weighed. The proportions of silt and clay were determined by the difference in weight between the two sampling times.

Microbiological analyses

With the exception of September 1995, when only microbial biomass and mineralizable C were determined, the following parameters were measured in whole soil and soil aggregate size fractions recovered in June and September 1996.

Microbial Biomass Carbon (MBC)

MBC was determined by the chloroform -fumigation-incubation method described by Jenkinson and Powlson (1976). Plant debris and roots were carefully removed from the soil samples before determination of MBC. The water content of dried soil samples (20g) was raised with distilled water to 30% (w/w) and samples were incubated in the dark for 4 days at 25°C. Then, one half of the samples were fumigated (F) at room temperature for 48 hours in a desiccator containing 20 ml of ethanol-free chloroform (HPLC grade, Mallinckrodt). In the meantime, the unfumigated controls (UF) were kept at 25°C. After fumigation, F and UF soil samples were transferred to canning jars (500 ml volume) containing a scintillation vial with 10 ml of 0.3 M KOH and incubated in the dark for 10 days at 25°C. The amount of CO_2 evolved was determined after titration with 0.1 M HCl using 1% (w/v) phenolphthalein as indicator. Before the titration, carbonates were precipitated to Ba_2CO_3 with 3 ml of $BaCl_2$ 20 % (w/w). The amount of MBC was determined from the difference between CO_2 evolved by the F and UF controls using a Kc of 0.41 (Anderson and Domsch, 1978). Four and two analytical replicates per sample were used in September 1995 and in both of the 1996 samples, respectively.

Mineralizable C

Mineralizable C was calculated from the amount of CO_2 by the UF controls of the biomass determination experiments. The quantity of CO_2 evolved was determined as described in the previous section.

Mineralizable N

Mineralizable N (NH_4-N and NO_3- N) was calculated from the difference between the amounts of NH_4-N and NO_3- N found in the UF controls after a 16 day incubation period, and the amounts found in those samples before the addition of water (time zero). Nitrate and ammonium were extracted from one gram samples of soil with 5 ml of 1 M KCl. The tubes were mixed in a vortex, shaken for 30 minutes, and centrifuged at 10,000 rpm (7166 x*g*) for 15 minutes. The supernatant was collected and used in the NH_4- N and NO_3- N analysis. The amount of NO_3- N was determined with Szechrome NAS (Polysciences, Inc.). An aliquot (0.5 ml) of the KCl supernatant was mixed in a vortex with 5 ml Szechrome NAS reagent solution (5g of Szechrome NAS in one liter of a mixture of equal volumes of nitrate-free concentrated H_3PO_4 and concentrated H_2SO_4). The samples were incubated for one hour. The absorbance at 570 nm was measured with a visible light spectrophotometer. The amounts of NO_3 were determined from a standard curve prepared with known concentrations of KNO_3 (4.2; 8.4; 12.6 and 16.8 μg of NO_3^-- N ml^{-1}). Two analytical replicates were used per sample.

Ammonium was determined using the procedure described by Solorzano (1969). A one ml aliquot of KCl supernatant was mixed with 2 ml of a 10% (v/v) phenol solution, 2 ml of 0.5% (w/v) sodium nitroprusside, and 5 ml of a solution prepared after mixing 2 parts of commercial bleach with 3 parts of a solution containing 100 g trisodium citrate and 5 g of NaOH in 500 ml of water. The samples were incubated for one hour and the absorbance at 620 nm was measured. NH_4- N was determined from a standard curve (0.5; 1.0; 2.0; 3.0 and 4.0 μg NH_4- N ml^{-1}). Two analytical replicates

were used per sample. Because the amounts of NH_4- N found in the samples were always < 0.5 μg of NH_4- N, they were not considered in the calculations of the total amount of N mineralized.

Enzyme assays

As a continuation of the studies initiated by Bandick, A. (Masters Thesis, OSU, 1997), β-glucosidase activity and FDA (fluorescein diacetate)-hydrolysis were determined in the soil samples collected in September 1996. β-Glucosidase activity was determined by the method of Tabatabai (1994) except that toluene was omitted. The method is based on a colorimetric determination of the *p*-nitrophenol released by β-glucosidase when one gram of soil is incubated with one ml of a buffered (pH 6.0) 0.05 M *p*-nitrophenyl β D-glucoside (PNG) solution (0.377g PNG in 50 ml MUB pH 6.0). Four ml of a modified universal buffer (MUB) pH 6.0 are added and the soil solution is incubated at 37°C for one hour. The MUB solution is prepared by mixing 200 ml of a MUB stock (12.1 g of THAM (tris(hydroxymethyl) aminomethane); 11.6 g of maleic acid; 14.0 g of citric acid; 6.3 g of boric acid; 488 ml 1 M NaOH; final volume adjusted to 1000 ml), and adjusting to pH 6.0 with 0.1 M HCl, to a final volume of 1000 ml. The *p*-nitrophenol released is extracted by filtration (Whatman No. 2) after addition of 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.1 M THAM buffer pH 12 (12.2 g of THAM in 700 ml of distilled water; pH adjusted to 12 with 0.5 M NaOH; final volume adjusted to 1000 ml). The intensity of the yellow color of the filtrate was measured at 420 nm with a visible light spectrophotometer.

FDA hydrolysis was determined by a modification of the procedure described by Zelles et al., (1991). The method is based on the colorimetric determination of the fluorescein released after the hydrolysis of FDA by diverse enzymes such as proteases, lipases and esterases (Schnürer and Rosswall, 1982). One gram of soil and 20 ml of a 60 mM sodium phosphate buffer (pH 7.6) were placed in a 125-ml Erlenmeyer flask and shaken at 100 rpm on a rotary shaker at 25°C. After 15 minutes, 100 µl of a 4.8 mM FDA solution were added and the suspension was shaken for an additional 1.75 h. The reaction was stopped by addition of 20 ml acetone. The suspension was centrifuged for 5 minutes at 4300 x*g* and filtered through Whatman No. 4 filters; absorbance of the filtrate was measured at 499 nm with a visible light spectrophotometer. Three analytical replicates plus a control, were used per sample.

Results of all microbiological, chemical and physical analyses are reported on an oven-dry-weight basis, determined by drying the soils for 24 hours at 105°C.

Statistical Analysis

Data were analyzed using the SAS statistical package (SAS Institute, Cary, NC). Within each season, the data were analyzed using a repeated measures analysis of variance, aggregate size being the repeated term (see Appendix 2). The appropriate covariance structure was determined using a sphericity test. This test determines if it is acceptable to treat the data as a univariate analysis, and as a result, if the experiment can be modeled as a split-plot. For those parameters that did not satisfy the Huynh-Feldt condition (i.e., failed the sphericity-test), significance levels associated with adjusted F

tests, employing either the Greenhouse and Geiser, and Huynh-Feldt epsilon adjustments were used (Freund et al., 1986). Because the soil samples collected in September 1995 were composited, the structure of the repeated measures ANOVA was different (the block effect was not considered in the analysis). At each sampling time, whole soil samples were analyzed as a sixth level in the repeated term.

For the purpose of comparing sampling time, only data collected in June and September 1996 were considered. A two repeated measures factors analysis of variance was used, the two repeated terms being sampling time (with two levels: June and September) and aggregate size (with six levels: 5 corresponding to each aggregate size class, plus the whole soil samples). Sources of variation included treatment, aggregate size, sampling time and their interactions. Main effects means were separated using a LSD at $P = 0.05$ level.

Covariance analyses were used to model the relationships among the variables evaluated (see Appendix 2). Individual analyses using the SAS mixed models procedure were conducted using one of the variables as the covariate and the other as the response after accounting for the design structure (treatments, blocks, aggregate size).

RESULTS

The probability levels associated with the one and two factor repeated measures analyses of variance are presented in Tables 3.3 and 3.4. Because the block effect was not taken into account in the samples collected in September 1995, those data were not used in the analysis of sampling time effects.

Table 3.3. Summary of the repeated measures analysis of variance, for treatment and aggregate size effects within each sampling date.

Source of variation	Probability level (P>F)†							
	Aggr.	TOC	TKN	MBC	Min. C	Min. N	FDA	β Gluc.
				September 1995				
Treat.	0.4144	0.0302	-	0.0343	0.3922	-	-	-
Size	0.0001	0.0007	-	0.0296	0.0001	-	-	-
Treat. x Size	0.8528	0.3533	-	0.0001	0.3941	-	-	-
				June 1996				
Treat.	0.7035	0.5291	0.1937	0.0758	0.7018	0.0058	0.0552	0.0443
Size	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0007	0.0001
Treat. x Size	0.3367	0.4174	0.0262	0.0905	0.0041	0.0059	0.0011	0.5429
				September 1996				
Treat.	0.1239	0.3187	0.1768	0.2647	0.0455	0.1336	0.1534	0.0028
Size	0.0001	0.0001	0.0001	0.0001	0.0013	0.0001	0.0001	0.0020
Treat. x Size	0.0368	0.1308	0.1511	0.7947	0.0897	0.0064	0.0003	0.2005

† Abbreviations are: Aggr. = aggregate size distribution; TOC = total organic C; TKN = total Kjeldahl N; MBC = microbial biomass; Min. C = mineralizable C; Min. N = mineralizable N; FDA = fluorescein diacetate hydrolysis; β Gluc. = β glucosidase

Table 3.4. Summary of the two-factors repeated measures analysis of variance for treatment (Treat.), aggregate size (Size) and sampling time (Time) effects †.

Source of variation	Probability level (P>F) ‡							
	Aggr.	TOC	TKN	MBC	Min. C	Min. N	FDA	β Gluc.
Treat.	0.3686	0.3895	0.1519	0.1646	0.2439	0.0038	0.0807	0.0006
Size	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Treat. x Size	0.0172	0.6252	0.0218	0.2123	0.0657	0.0010	0.0010	0.0772
Time	0.2515	0.3107	0.1066	0.0365	0.7462	0.0063	0.0001	0.0001
Time x Treat.	0.3992	0.4310	0.9054	0.0710	0.4124	0.0147	0.6534	0.3406
Time x Size	0.0001	0.0990	0.1717	0.0001	0.0701	0.0001	0.0001	0.0008
Time x Size x Treat.	0.4410	0.2041	0.1448	0.1245	0.0114	0.0025	0.0001	0.6310

† Sampling times were June and September 1996.
‡ Abbreviations are the same used in Table 3.3.

Aggregate Size Distribution

Throughout the study, aggregate size distribution was not influenced by crop rotational treatment (Table 3.3 and Figs. 3.1 and 3.2). However, the interaction between sampling time and aggregate size distribution was highly significant ($P < 0.0001$) (Table 3.4). The shift in aggregate size distribution was most pronounced among smaller aggregate size classes (< 1.0 mm) (Figs. 3.1, 3.2 and 3.3a). From June to September 1996, the amount of soil present in the < 0.25 mm size class increased by 40%, with concurrent reductions of 30 and 17 % in the amounts of soil contained in the size classes 0.25 to 0.5 and 0.5 to 1.0 mm, respectively. Although the sampling time analysis could not be conducted with data collected in 1995, the same trend was observed (Fig. 3.1).

The change in aggregate size distribution with sampling time was accompanied by a change in the dry-aggregate mean weight diameter (MWD) between June and September 1996 (Table 3.5). The small change in MWD (1.46 and 1.39 for June and September 1996, respectively) is a consequence of the fact that the shift in aggregate size distribution was observed primarily in the microaggregate size fraction (< 0.25 mm). By definition the latter size class is associated with the smallest weighting factor (0.125) in the calculation of MWD. No changes in MWD were observed between June and September 1995 (Table 3.5).

At both sampling times in 1996, microaggregates from the legume and fallow treatments contained more sand and less silt than the other aggregate sizes (Table 3.6). Nevertheless, considering the narrow range of sand contents across the dry-sieved

Figure 3.1. Effect of cover crop systems on the distribution of aggregate size classes in June and September of 1995.

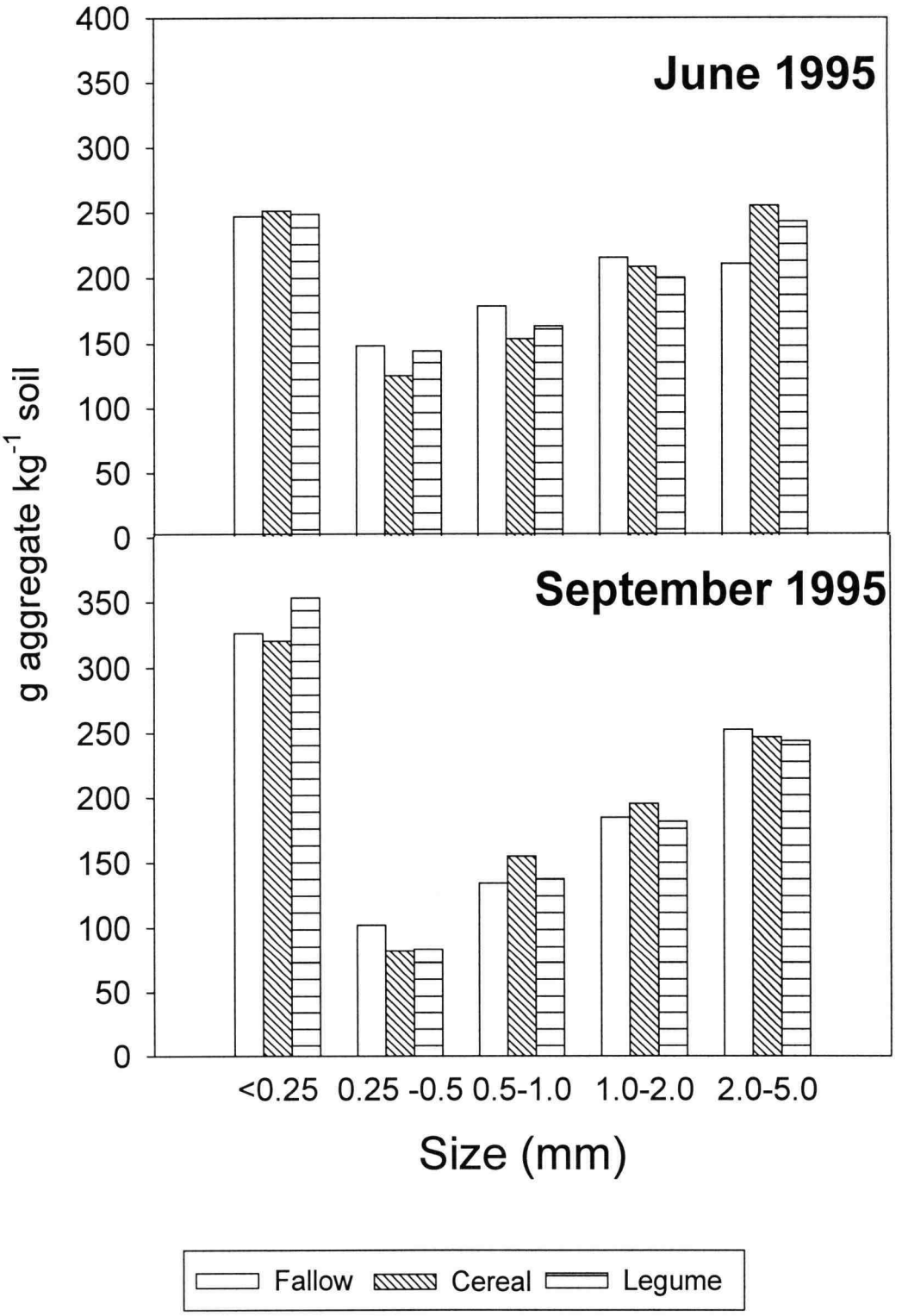
June 1995
September 1995
g aggregate kg-1 soil
400
350
300
250
200
150
100
50
0
<0.25
0.25 -0.5
0.5-1.0
1.0-2.0
2.0-5.0
Size (mm)
Fallow
Cereal
Legume

Figure 3.1.

Figure 3.2. Effect of cover crop systems on the distribution of aggregate size classes in June and September of 1996.

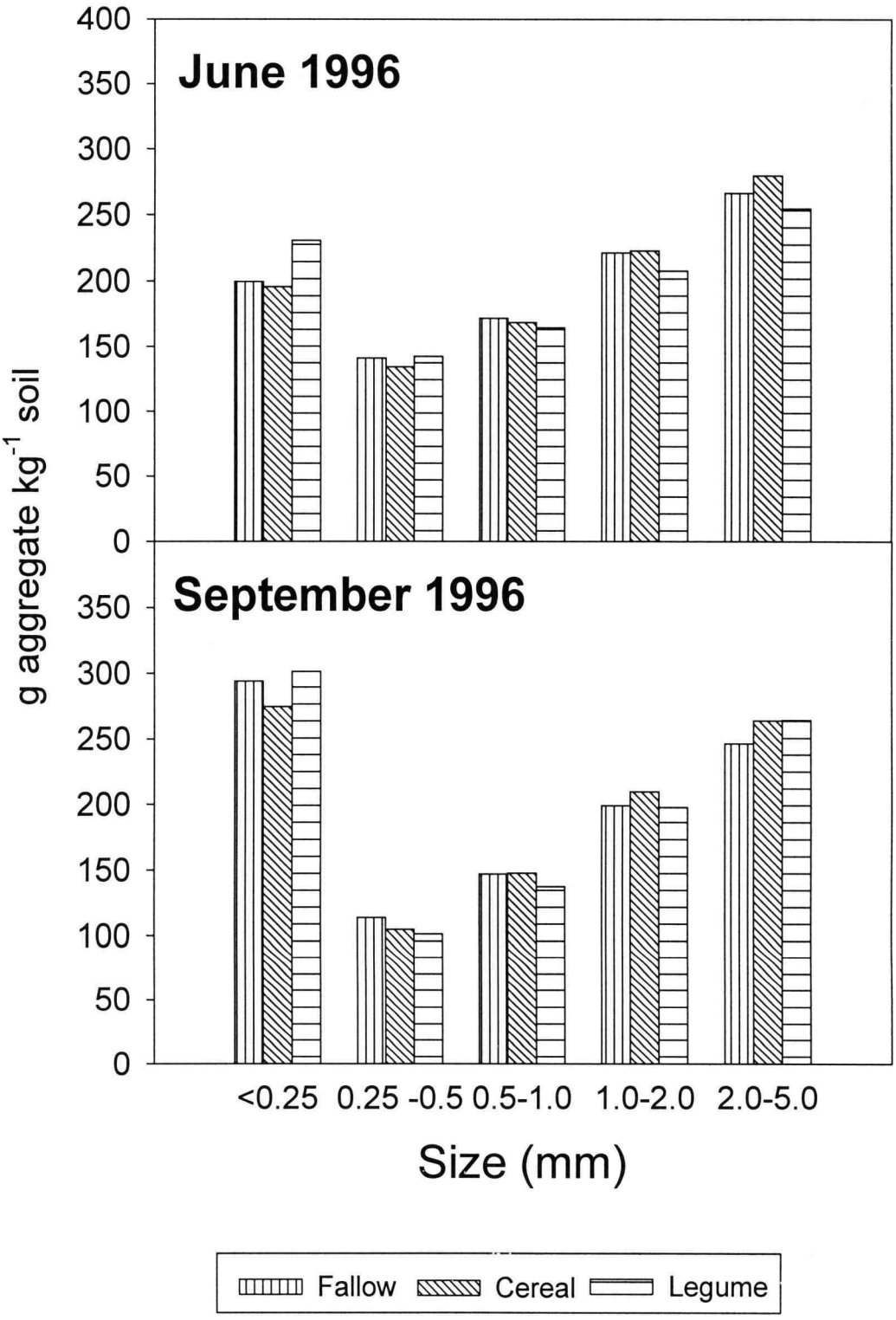
June 1996
September 1996
g aggregate kg-1 soil
400
350
300
250
200
150
100
50
0
<0.25
0.25 -0.5
0.5-1.0
1.0-2.0
2.0-5.0
Size (mm)
Fallow
Cereal
Legume

Figure 3.2.

Figure 3.3. Seasonal changes in aggregate size distribution (A), microbial biomass carbon (B), and mineralizable nitrogen (C). Letters refer to comparisons between June and September 1996. Within the same aggregate size class, values followed by the same letter are not significantly different at $P < 0.05$.

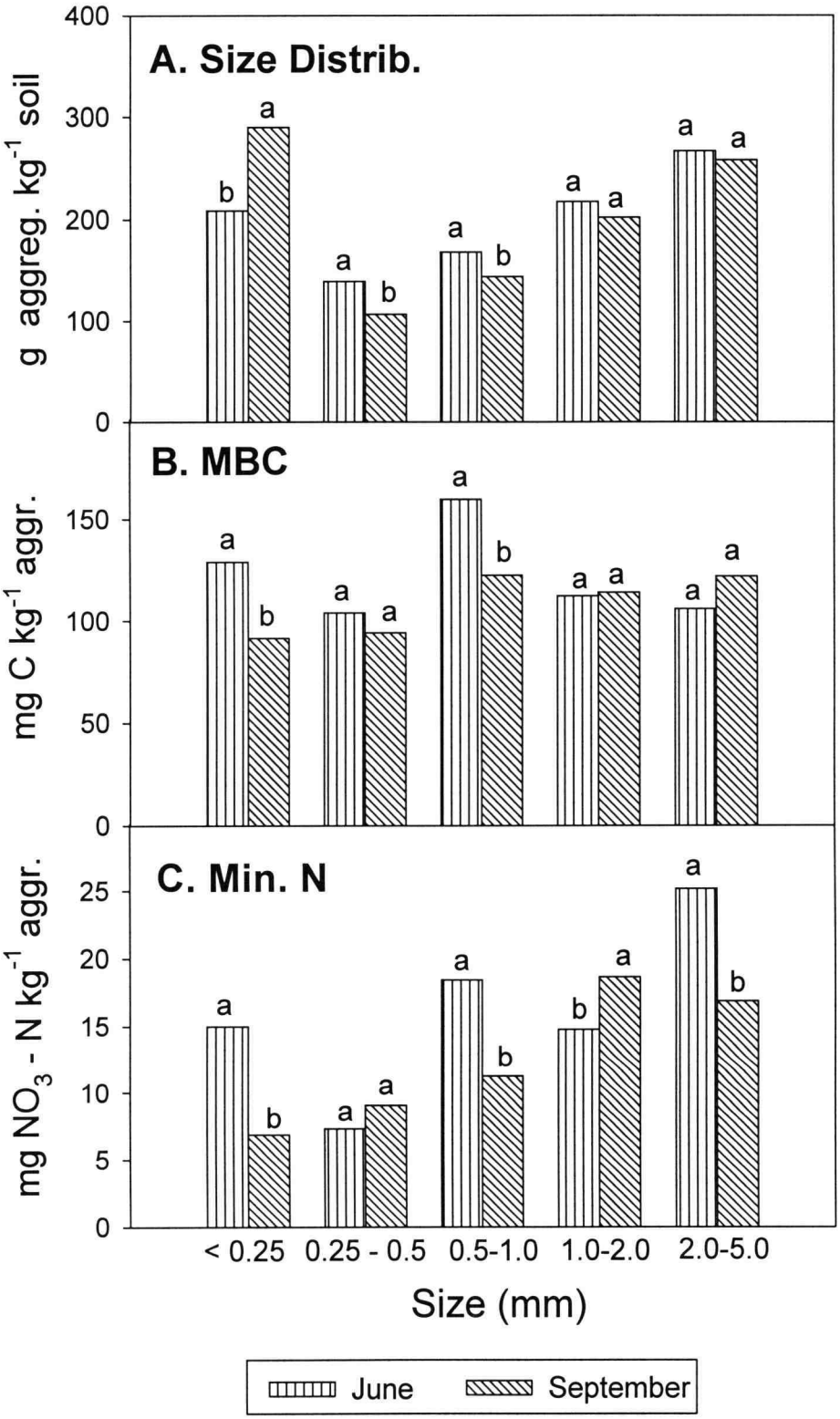
A. Size Distrib.
g aggreg. kg-1 soil
400
300
200
100
0
B. MBC
mg C kg-1 aggr.
150
100
50
0
C. Min. N
mg NO3 - N kg-1 aggr.
25
20
15
10
5
0
< 0.25
0.25 - 0.5
0.5-1.0
1.0-2.0
2.0-5.0
Size (mm)
June
September

Figure 3.3.

Table 3.5. Mean weight diameter (MWD) of dry-sieved aggregates prepared from different cover crop systems at different times of the year.

	MWD			
Treatment	June 1995	September 1995	June 1996	September 1996
Fallow	1.28 a	1.34 a†	1.47 a	1.35 a
Cereal	1.40 a	1.34 a	1.51 a	1.42 a
Legume	1.36 a	1.30 a	1.41 a	1.40 a
Average	1.35	1.33	1.46 A‡	1.39 B

† Lower case letters indicate comparisons within sampling times. Values followed by the same letter within a column are not significantly different at $P < 0.05$.

‡ Upper case letters indicate comparisons between sampling times in 1996. Values followed by the same letter within a row are not significantly different at $P < 0.05$.

Table 3.6. Particle size analysis of aggregates and whole soil recovered from the fallow and legume treatments in June and September 1996.

	Fallow - September 1996			Legume - June 1996			Legume - September 1996		
Aggregate (mm)	Clay	Silt	Sand	Clay	Silt	Sand	Clay	Silt	Sand
	----------	----------	----------	----------	g kg^{-1} ----------	----------	----------	----------	----------
< 0.25	135	521	344	139	488	373	127	484	389
0.25 - 0.5	140	558	302	151	528	322	154	513	333
0.50 - 1.0	128	554	318	149	539	312	147	532	321
1.0 - 2.0	148	546	306	141	526	333	146	518	336
2.0 - 5.0	121	561	318	138	518	345	135	533	332
Whole Soil	134	535	331	147	496	357	128	539	332

aggregate size classes I concluded that it was not necessary to normalize the subsequent data on chemical and biological parameters to a sand-free aggregate weight.

Total Organic C and Total Kjeldahl N

Although the cover crop treatments have been established since 1989, significant treatment differences in total soil organic carbon (TOC) and total Kjeldahl nitrogen (TKN) were not detected in whole soil sampled in 1995 and 1996 (Table 3.7). However, it was noticeable that the TOC and TKN levels were consistently lower in the cereal treatment than in both fallow and legume treatments. Significant differences in TOC were detected in specific aggregate size classes across treatments in September 1995 (Table 3.8). TOC levels in < 0.25 and 0.25 to 0.5 mm size classes were significantly lower in the cereal treatment, than in the legume (< 0.25 mm) and fallow (0.25 to 0.5 mm) treatments.

With the exception of the legume treatment in September 1995, TOC content varied significantly ($P < 0.0001$) among aggregate sizes (Table 3.8). Several trends were apparent. First, the lowest TOC levels were almost always found in both the smallest (< 0.25 mm) and in either of the largest 1.0 to 2.0 and 2.0 to 5.0 mm aggregate size classes regardless of treatment. Second, the highest TOC levels were always found in the 0.25 to 0.5 mm size class regardless of treatment. The interaction between treatment x aggregate size was not significant on any of the three sampling times (Table 3.3). Although the time of sampling x treatment, and time of sampling x aggregate size interactions were not statistically significant (Table 3.4), there were tendencies for TOC

Table 3.7. Effect of cover cropping systems on whole soil parameters in June and September 1996.†

Treatment	TOC‡	TKN	MBC	Min. C	Min. N	FDA	β- Gluc
				September 1995			
Fallow	18.3 a§	-	135 b	62.10 a	-	-	-
Cereal	16.6 a	-	196 a	59.30 a	-	-	-
Legume	17.6 a	-	206 a	62.10 a	-	-	-
				June 1996			
Fallow	16.4 a	839 a	109a	30.30 a	10.35 a	18.34 b	51.87 b
Cereal	14.6 a	718 a	91 a	47.59 a	16.59 a	30.13 a	64.80 ab
Legume	16.3 a	753 a	104 a	45.38 a	13.40 a	28.58 a	73.11 a
				September 1996			
Fallow	17.5 a	843 a	112 a	43.30 b	7.11 a	53.46 a	77.41 b
Cereal	14.3 a	792 a	115 a	48.43 ab	8.29 a	48.94 a	109.19 a
Legume	17.3 a	809a	124 a	59.01 a	9.69 a	57.50 a	115.21 a

† Abbreviations are the same used in Table 3.3.

‡ Units are: TOC = g C kg^{-1} soil; TKN = mg N kg^{-1} soil; MBC and Min.C = mg of C kg^{-1} soil; mineralizable N = mg NO^{-}_{3}-N kg^{-1} soil; FDA =, μg fluorescein released g^{-1} soil h^{-1}, and β-gluc. = μg p-nitrophenol released g^{-1} soil h^{-1}

§ Lower case letters indicate comparisons among treatments within seasons. Values followed by the same letter within columns are not significantly different at $P < 0.05$.

Table 3.8. Distribution of total organic carbon (TOC) across aggregate size classes in September 1995, June and September 1996.

	September 1995			June 1996			September 1996		
Aggregate (mm)	Fallow	Cereal	Legume	Fallow	Cereal	Legume	Fallow	Cereal	Legume
	----------TOC (g C kg $^{-1}$)- ----------								
< 0.25	16.6bc† AB‡	13.7b B	17.5a A	14.0c	13.6b	14.8c	13.9c	13.4c	14.7cd
0.25 - 0.5	21.8a A	17.5a B	19.5a AB	17.9a	16.2a	19.0a	19.4a	18.3a	20.8a
0.50 - 1.0	19.0b A	16.3ab A	17.7a A	17.9a	15.5ab	17.6ab	18.0ab	16.0b	17.6b
1.0 - 2.0	17.5bc A	16.5a AB	19.3a A	16.3ab	14.5ab	17.1ab	16.8b	15.0bc	14.1d
2.0 - 5.0	16.3c A	15.7ab A	17.9a A	15.3bc	16.3a	16.1bc	16.0bc	13.4c	16.4bc

† Lower case letters indicate comparison within aggregates recovered from one treatment. Values followed by the same lower case letters within each column are not significantly different at $P < 0.05$.

‡ Upper case letters indicate comparisons between treatments for one same size fraction. Values followed by the same uppercase letters are not significantly at $P< 0.05$.

levels to change within specific aggregate size classes between the two sampling times. For example, the level of TOC increased by 1.5 to 2.1 g C kg^{-1} soil in the 0.25 to 0.5 mm size class between June and September whereas TOC levels declined about 3.0 g C kg^{-1} soil in the 1.0 to 2.0 mm (legume) and 2.0 to 5.0 mm (cereal) size classes over the same time period.

Although the whole soil TKN levels did not change between June and September, the levels of TKN varied significantly ($P < 0.0001$) as a function of aggregate size at each sampling time (Table 3.3). In June 1996, the interaction between treatment x aggregate size was statistically significant (Table 3.3). At both sampling times, levels of TKN in all aggregate size classes > 0.25 mm of the legume treatment were significantly greater than the level in the < 0.25 mm size class (Table 3.9). In the case of the cereal and fallow treatments, several macroaggregate size classes contained TKN levels not significantly different from the < 0.25 mm size class.

Even though the effect of sampling time on TKN levels was not statistically significant (Table 3.4), there was evidence for TKN levels changing within specific aggregate size classes of the treatments between June and September. In June, the size class 0.5 to 1.0 mm of the fallow treatment contained significantly more TKN than the 0.25 to 0.50 mm size class (961 compared to 810 mg N kg^{-1}). However, in September the TKN content of the 0.25 to 0.5 mm size class had increased substantially (909 mg N kg^{-1}), and was accompanied by a substantial decline in the level of TKN in the 0.5 to 1.0 mm size class (961 to 841 mg N kg^{-1}). Between June and September, coincident with the changes in TOC levels, TKN levels declined substantially in the 1.0 to 2.0 and 2.0 to 5.0 mm size classes of the cereal and legume treatments, respectively.

Table 3.9. Distribution of total kjeldahl N (TKN) across aggregate size classes in June and September 1996.

Aggregate (mm)	June			September		
	Fallow	Cereal	Legume	Fallow	Cereal	Legume
	----------TKN (mg N kg^{-1} soil)----------					
< 0.25	726 c†	710 b	775 b	723 c	672 b	743 b
0.25 - 0.5	810 bc	889 a	979 a	909 a	810 a	936 a
0.50 - 1.0	961 a	817 ab	934 a	841 ab	810 a	948 a
1.0 - 2.0	877 ab	766 b	1010 a	820 ab	772 ab	896 ab
2.0 - 5.0	810 bc	892 a	936 a	753 bc	724 ab	940 a

† Lower case letters indicate comparison within aggregates recovered from one treatment. Values followed by the same lower case letters within each column are not significantly different at $P < 0.05$.

MBC

In September 1995, microbial biomass C (MBC) contents of whole soil samples recovered from the legume and cereal treatments (206 and 196 mg C kg^{-1}, respectively), were found to be significantly higher than the fallow treatment (135 mg C kg^{-1}) (Table 3.7). At that sampling time, the interaction between treatment and aggregate size was significant (Table 3.3). For example, the amount of biomass C determined in the 5.0 to 2.0 mm size class of the legume treatment (148 mg C kg^{-1}) was low compared with the same size class from the fallow treatment (215 mg C kg^{-1}). In contrast, significantly more MBC was found in the size classes 0.25 to 0.5 mm and < 0.25 mm from the legume treatment (210 and 262 mg C kg^{-1}, respectively) than in the corresponding size fractions from fallow (114 and 172 mg C kg^{-1}) and cereal (185 and 163 mg C kg^{-1}) treatments.

In the samples obtained in 1996, MBC level did not change in response to cover crop treatments, nor was there a significant interaction between treatment x aggregate size (Tables 3.3 and 3.10). Nevertheless, the effect of aggregate size on MBC was highly significant ($P < 0.001$). In June samples, the cover crop treatments contained the greatest MBC levels in the size classes < 0.25 and 0.5 to 1.0 mm. Samples collected in September generally contained significantly more MBC in aggregate size classes > 0.5 mm. Even though only small changes occurred in the overall amount of MBC in soil aggregates between June (118 mg C kg^{-1}) and September 1996 (110 mg C kg^{-1}), the difference was statistically significant. The interaction between season x aggregate size

Table 3.10. Distribution of microbial biomass carbon (MBC) across aggregate size classes in September 1995, June and September 1996.

Aggregate (mm)	September 1995			June 1996			September 1996		
	Fallow	Cereal	Legume	Fallow	Cereal	Legume	Fallow	Cereal	Legume
	MBC (mg C kg $^{-1}$)								
< 0.25	114c† C‡	185ab B	262a A	118b	137a	132b	86b	88b	101b
0.25 - 0.5	172bc B	163b B	210ab A	111b	102b	98cd	91b	93b	98b
0.50 - 1.0	214ab A	157b B	206b A	166a	148a	165a	117a	118a	132a
1.0 - 2.0	247a A	219a A	222ab A	117b	97b	123bc	113a	118a	111b
2.0 - 5.0	215ab A	195ab AB	148c B	130b	93b	94d	122a	114a	130a

† Lower case letters indicate comparison within aggregates recovered from one treatment. Values followed by the same lower case letters within each column are not significantly different at $P < 0.05$.

‡ Upper case letters indicate comparisons between treatments for one same size fraction. Values followed by the same uppercase letters are not significantly at $P< 0.05$.

also was significant (Table 3.4). These changes were statistically significant ($P < 0.001$) for the size classes < 0.25 and 0.5 to 1.0 mm (Fig. 3.3b).

Mineralizable C

With the exception of mineralizable C levels determined in whole soil and the 1.0 to 2.0 mm aggregate size class of the legume treatment from September 96, the amounts of C mineralized did not change as a function of cover crop treatment (Table 3.7 and Fig. 3.4). However, in all sampling periods the levels of mineralizable C changed as a function of aggregate size. Furthermore, for the samples collected in June 1996, the interaction treatment x aggregate size also was significant (Table 3.3).

In September 1995, the lowest levels of mineralizable C (on average 25.2 mg C kg^{-1}) were found in the 2.0 to 5.0 mm aggregate size class, regardless of treatment (Fig. 3.4). While similar amounts of mineralizable C (75.5 mg C kg^{-1}) were determined in all size classes < 2.0 mm from the legume treatment, significant differences were observed among those size classes of the fallow and cereal treatments.

The overall amounts of C mineralized in June and September 1996 did not change (no sampling time effect) (Table 3.4). In the same way the interactions of sampling time x treatment and sampling time x aggregate size were not statistically significant. Nevertheless, the distribution of mineralizable C across aggregates within each treatment differed at both sampling times (i.e., the interaction season x treatment x aggregate size was significant). In June 1996, all aggregates > 0.5 mm recovered from the fallow treatment showed significantly higher mineralizable C than aggregates < 0.5

Figure 3.4. Distribution of mineralizable C across aggregate size classes under different cover cropping systems. Letters indicate significant differences in means within treatments at $P < 0.05$.

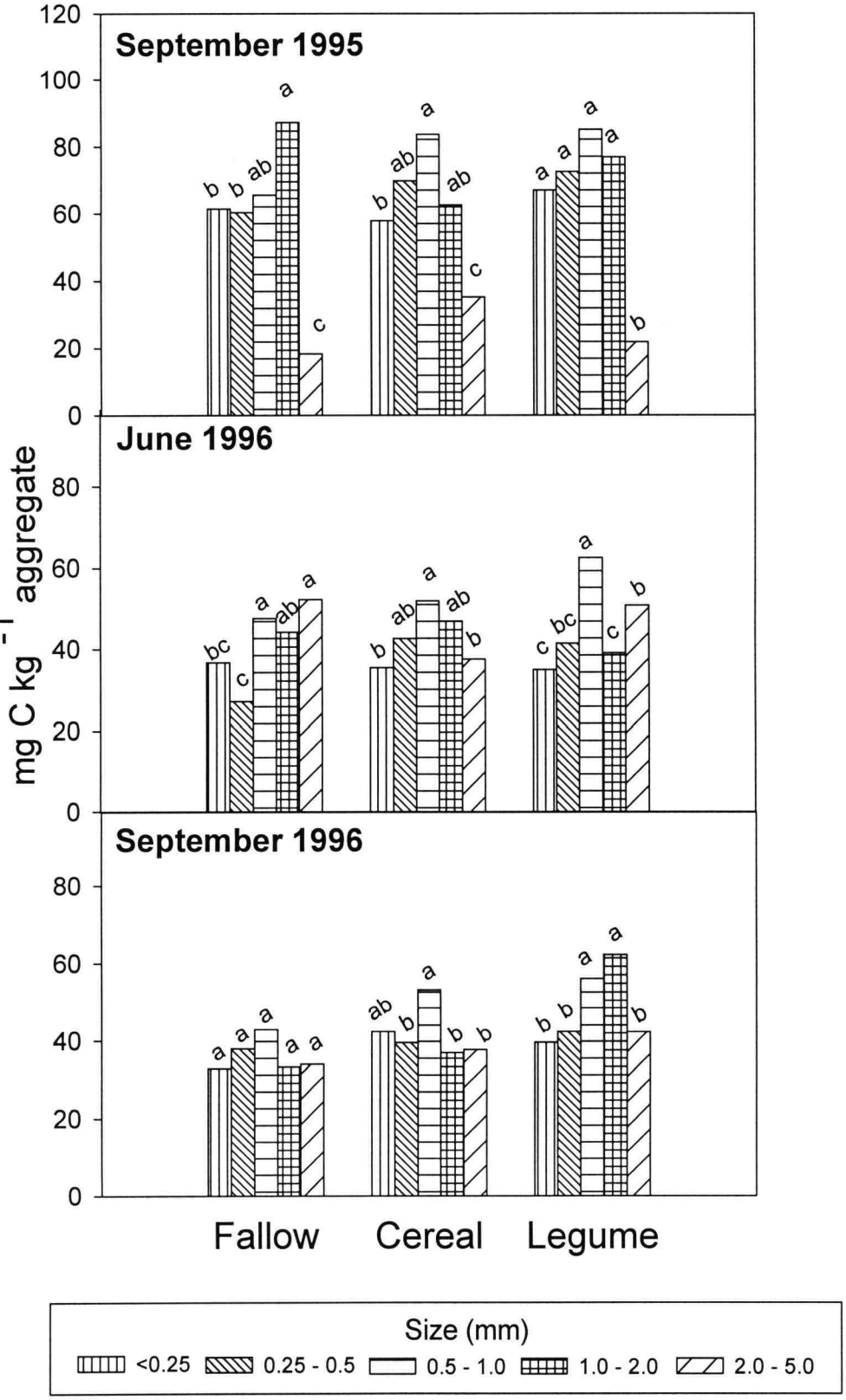
September 1995
June 1996
September 1996
mg C kg -1 aggregate
120
100
80
60
40
20
0
Fallow
Cereal
Legume
Size (mm)
<0.25
0.25 - 0.5
0.5 - 1.0
1.0 - 2.0
2.0 - 5.0

Figure 3.4.

mm, whereas in the case of the cover crop treatments, macroaggregates > 1.0 mm contained levels of mineralizable C not significantly different from the <0.5 mm size classes (Fig. 3.4). In September 1996, no significant differences were found in the mineralizable C levels across aggregate size classes recovered from the fallow treatment (Fig. 3.4), whereas in the cover crop treatments substantially greater amounts of mineralizable C were found in the size classes 0.5 to 1.0 mm of the cereal, and 0.5 to 1.0 and 1.0 to 2.0 mm of the legume treatments.

Mineralizable N

Although no statistically significant differences were observed in the amounts of N mineralized in whole soil samples from the three treatments (Table 3.7), there was a substantial reduction in the amount of mineralizable N available between the June and September 1996 sampling times. Also, the amounts of N mineralized at the two sampling times changed as a function of treatment (Fig. 3.5). The levels determined in the cereal and legume treatments in June (16.80 and 18.9 mg NO_3-N kg^{-1}) were greater than those measured in September (13.0 and 12.0 mg NO_3-N kg^{-1}). No significant differences could be detected in the amounts of N mineralized at the two sampling times in the fallow treatment (11.5 and 10.6 mg NO_3-N kg^{-1}, respectively).

Distinct patterns of mineralizable N were found across aggregate size classes at each sampling time (Fig. 3.6). In June, mineralizable N varied in response to treatments, aggregate size and their interaction. Although a main treatment effect was not apparent

Figure 3.5. Effect of cover crop systems on the amounts of mineralizable N, in June and September, 1996. Letters indicate significant differences in means within treatments at $P < 0.05$.

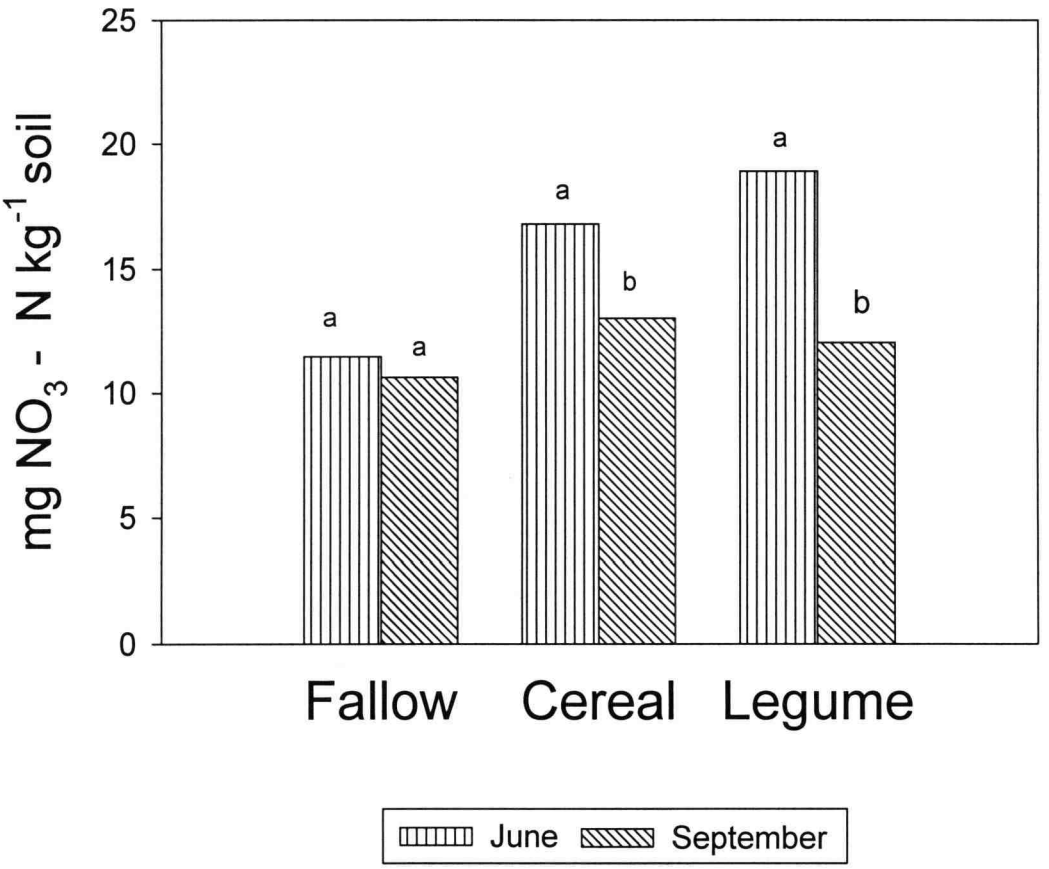
25
20
15
10
5
0
mg NO3 - N kg-1 soil
a
a
a
b
a
b
Fallow
Cereal
Legume
June
September

Figure 3.5.

Figure 3.6. Distribution of mineralizable N across aggregate size classes under different cover cropping systems. Letters indicate significant differences in means within treatments at $P < 0.05$. The * refers to comparisons of the same size class between treatments (bars followed by * were significantly lower ($P < 0.05$) compared to those without *).

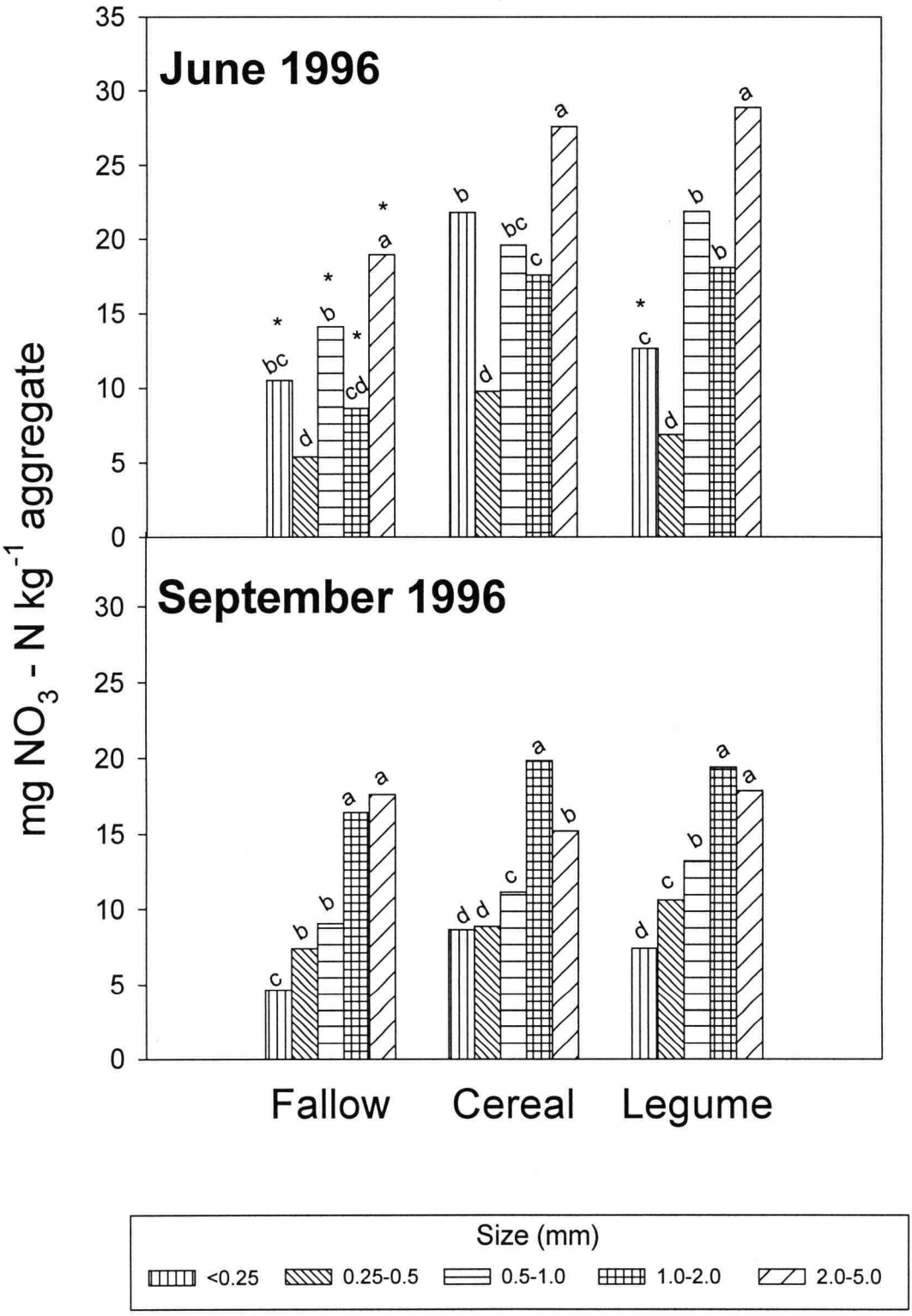
June 1996
September 1996
mg NO_3 - N kg^{-1} aggregate
0
5
10
15
20
25
30
35
Fallow
Cereal
Legume
Size (mm)
<0.25
0.25-0.5
0.5-1.0
1.0-2.0
2.0-5.0

Figure 3.6.

in September, the amount of mineralizable N varied as a function of aggregate size and its interaction with treatments (Table 3.3).

In June, significantly larger amounts of N were mineralized from all aggregate sizes > 0.5 mm in the cover crop treatments than in the fallow treatment (Fig.3.6). Similar amounts of N were mineralized in the size fraction 0.25 to 0.5 mm in all three treatments, whereas the < 0.25 mm size class from the cereal treatment mineralized significantly larger amounts of N than the same size classes from fallow and legume (these differences are indicated by an * in Fig. 3.6). The largest amounts of mineralizable N were found in the size class 5.0 to 2.0 mm in all three treatments.

In September, the distribution of mineralizable N across aggregate size classes was relatively simple (Fig. 3.6). Regardless of treatment, the largest amounts of mineralizable N were found in the aggregate size classes > 1.0 mm. The size classes 1.0 to 2.0 and 2.0 to 5.0 mm, represent on average 46% of the soil, and contributed 64% of the total N mineralized. In contrast, the microaggregate size fraction which contained almost 30% of the soil, contributed only 15% of the total N mineralized.

The amounts of mineralizable N changed significantly ($P < 0.0001$) between the two periods as a function of aggregate size (Fig. 3.3c). In June, greater amounts of mineralizable N were found in the size classes < 0.25; 0.5 to 1.0 and 2.0 to 5.0 mm than in September.

Soil enzyme activities

On both sampling dates in 1996, a significant treatment effect was observed for the rates of β-glucosidase activity (Table 3.3). The rates determined in aggregates and whole soil from the legume and cereal treatments averaged 37 and 30% higher than the fallow treatment (Table 3.7 and Fig. 3.7). With the exception of aggregates from fallow in September, the rates of β-glucosidase activity changed significantly across aggregate size classes. The interaction between treatment x aggregate size was not significant in any of the two periods evaluated (Table 3.3). At both sampling times, the highest rates of activity were determined in the size class 0.25 to 0.5 mm.

The rates of β-glucosidase activity determined in September were 1.6 times greater than the June rates. Furthermore, the distribution patterns of activities across aggregates also changed between the two sampling times (the interaction sampling time x aggregate size was statistically significant) (Table 3.4). Aggregates recovered in June showed the lowest rates of activity in the < 0.25 mm size class, whereas in September the lowest activities were found in the 2.0 to 5.0 mm size class.

In June 1996, FDA-hydrolysis rates determined in whole soil from the cover crops treatments and in the size class 2.0 to 5.0 mm from the legume treatment, were greater than the rates determined in the fallow and cereal treatments (Tables 3.3 and 3.7). In September 1996, although the differences between treatments were not statistically significant, the rates found in the size classes < 0.25 and 0.25 to 0.5 mm from cereal and legume treatments and 2.0 to 5.0 mm from the legume treatment, tended to be greater than the fallow treatment rates (Fig. 3.8).

Figure 3.7. Distribution of β-glucosidase activity across aggregate size classes under different cover cropping systems. Letters indicate significant differences in means within treatments at $P < 0.05$.

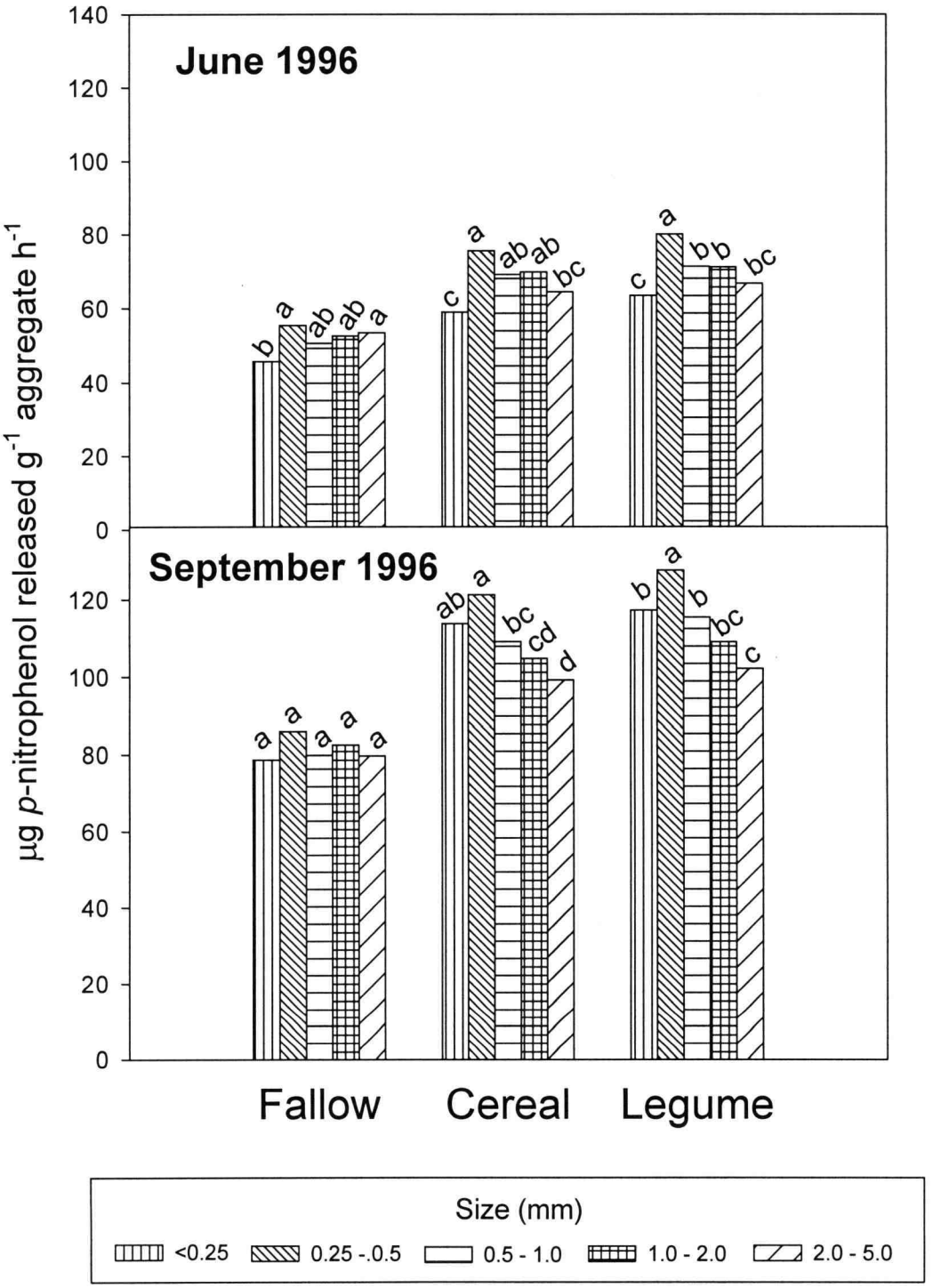
June 1996
September 1996
µg p-nitrophenol released g-1 aggregate h-1
140
120
100
80
60
40
20
0
b a ab ab a
c a ab ab bc
c a b b bc
a a a a a
ab a bc cd d
b a b bc c
Fallow
Cereal
Legume
Size (mm)
<0.25
0.25 -.0.5
0.5 - 1.0
1.0 - 2.0
2.0 - 5.0

Figure 3.7

Figure 3.8. Distribution of FDA-hydrolysis across aggregate size classes under different cover cropping systems. Letters indicate significant differences in means within treatments at $P < 0.05$.

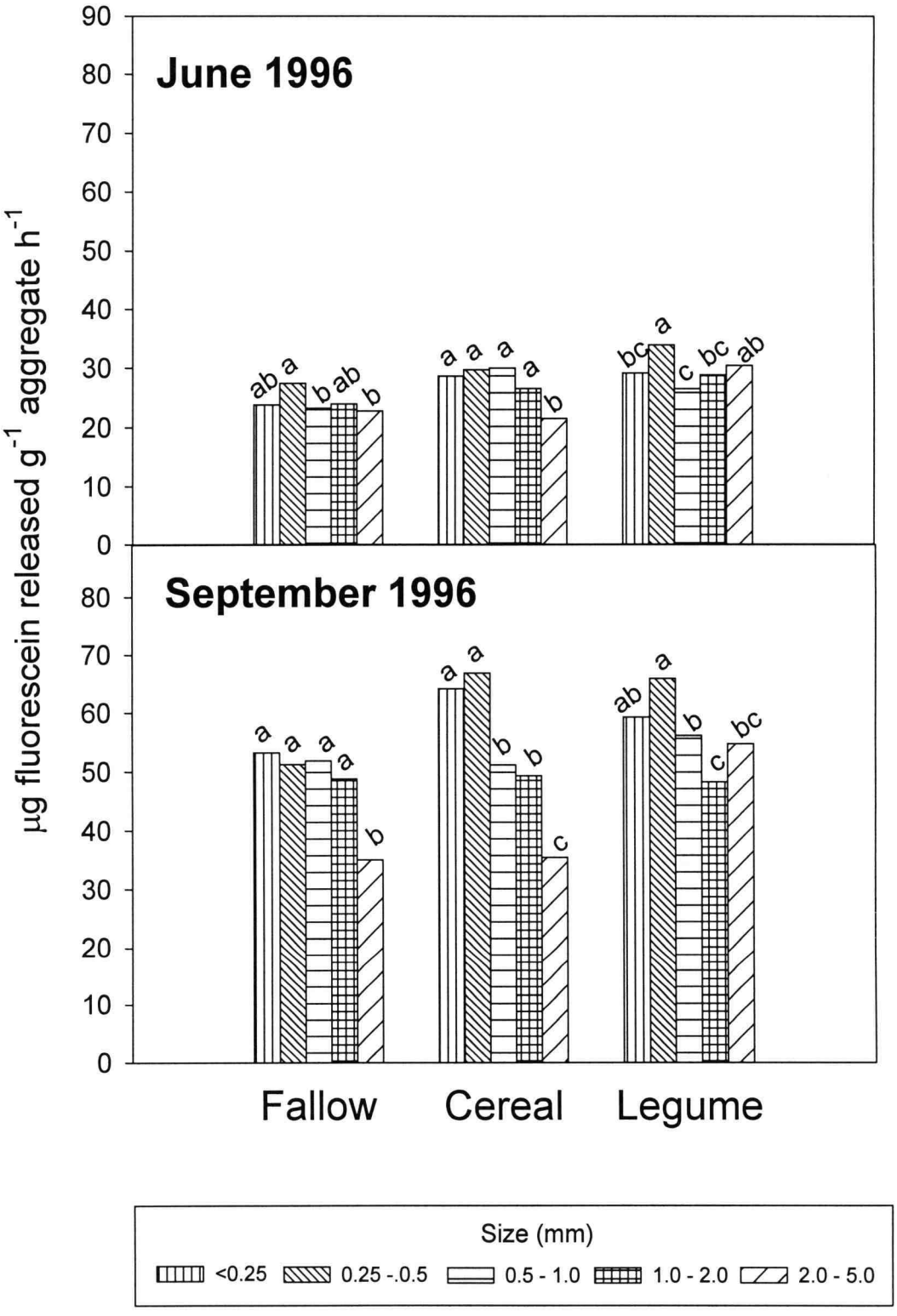
June 1996
September 1996
µg fluorescein released g-1 aggregate h-1
0
10
20
30
40
50
60
70
80
90
ab a b ab b
a a a a b
bc a c bc ab
a a a b b c
ab a b c bc
Fallow
Cereal
Legume
Size (mm)
<0.25
0.25 -.0.5
0.5 - 1.0
1.0 - 2.0
2.0 - 5.0

Figure 3.8.

In both sampling periods, the rates of FDA hydrolysis changed significantly as a function of aggregate size and its interaction with treatment (Table 3.3 and Fig. 3.8). In the June sampling, the size class 0.25 to 0.5 mm from the fallow treatment contained significantly greater rates of hydrolysis than the size classes 0.5 to 1.0 and 2.0 to 5.0 mm. In the legume treatment, the hydrolysis rate determined in the size class 0.25 to 0.5 was significantly greater than the rates found in the size classes < 0.25, 0.5 to 1.0, 1.0 to 2.0, and 2.0 to 5.0 mm. In the cereal (June) and fallow (September) treatment, aggregates between 2.0 to 5.0 mm presented the lowest rates of hydrolysis, whereas similar amounts were found in all the other size classes. In September, aggregates < 0.5 mm from the cover crop treatments showed the greatest rates of hydrolysis. The rates of FDA-hydrolysis determined in September were on average 2.0 times greater than in June and the differences found in hydrolysis rates across aggregates were more pronounced (Table 3.4). Furthermore, the distribution of hydrolysis rates across aggregates changed between the two sampling times. Similar overall rates of hydrolysis were determined in macroaggregates between 0.5 and 5.0 mm in the June sampling, whereas in September, those rates increased with a decrease in aggregate size. The interaction sampling time x aggregate size x treatment was statistically significant, indicating that at each sampling time the distribution of FDA hydrolysis across aggregates changed as a function of treatment.

Covariance analyses

At both sampling times, statistically significant relationships were determined between MBC and mineralizable C, and mineralizable N and β-glucosidase by the covariance analyses (Table 3.11). Even though the patterns of MBC and mineralizable C were not identical (i.e., some of the size classes with high MBC did not necessarily correspond with the size classes containing high levels of mineralizable C), the size classes where those levels did coincide were sufficient to show evidence for a positive relationship between the two variables. At both sampling times, regardless of treatments, the aggregate size class 0.5 to 1.0 mm contained high levels of both MBC and of C mineralization whereas the size class 0.25 to 0.5 mm contained low levels of MBC and C mineralization. The remaining size classes possessed either high levels of MBC and low levels of C mineralization or vice versa.

On the other hand, because the patterns of distribution of mineralizable N and β-glucosidase were exactly the opposite of each other (i.e., aggregate size classes with high levels of mineralizable N contained low levels of β-glucosidase, and vice-versa), a negative relationship between these two variables emerged from the covariance analysis.

No other significant relationships between chemical and or microbiological variables were observed.

Table 3.11. Probability levels associated with the Type III sum of squares in the covariance analyses†.

	Covariate					
Response	TKN	MBC	Min. N	Min. C	FDA	β- Gluc.
			June 1996			
TOC	0.7899	0.9084	0.9680	0.1039	0.6194	0.0980
TKN		0.0529	0.1382	0.3608	0.5203	0.0963
MBC			0.4455	0.0003	0.6075	0.9587
Min. N				0.0869	0.0644	0.0038
Min. C					0.1260	0.9735
FDA						0.9432
			September 1996			
TOC	0.1770	0.3684	0.2188	0.2385	0.2930	0.8757
TKN		0.2859	0.4663	0.4959	0.1345	0.9195
MBC			0.8688	0.0016	0.6923	0.5705
Min. N				0.2164	0.8861	0.0053
Min. C					0.3361	0.3919
FDA						0.3541

† Abbreviations are the same used in Table 3.3.

DISCUSSION

My research findings provide some new insights at the interface between soil microbiology and soil aggregation, and to the current interest in understanding how cover crops enhance soil quality in cropping systems.

Heterogeneous distribution of microbial biomass and its activities across aggregates has been observed previously (Gupta and Germida, 1988; Hattori, 1988; Seech and Beauchamp, 1988; Beauchamp and Seech, 1990; Miller and Dick, 1995a and b; Singh and Singh, 1995) and indicates the existence of microniches in soil structure that have variable ability to support and/or protect microbial growth. However, by sampling soil on different occasions during the crop year, I gained some additional insights into the dynamics of the interactions between microorganisms and the soil structure. For example, recent studies have indicated that microorganisms inside of microaggregates (< 0.25 mm) might be more biologically active and involved in processing soil carbon than was originally predicted (Jastrow et al., 1996). In this context, I observed that microbial biomass levels declined by approximately one fifth, and mineralizable N levels by at least one half in both the < 0.25 and 0.5 to 1.0 mm size classes between the June and September 1996 sampling times (Figs. 3.3b and c). In contrast, during the same time interval microbial biomass levels increased by approximately 20% in the 2.0 to 5.0 mm size classes of both the cereal and legume treatments and the level of mineralizable N declined (Table 3.10 and Fig. 3.6). Further studies are needed to examine the rates of biomass turnover in these particular aggregate size classes during the June September interval. In addition, it would be extremely

interesting to determine if the decline in the mineralizable N pool of the smaller aggregate sizes is directly related to the microbial biomass decline, and to what extent the decline in the mineralizable N pool in the 2.0 to 5.0 mm size class between June and September can be accounted for by the increase in microbial biomass N.

Several studies conducted with whole soil have reported positive correlations between the level of microbial biomass and microbial activities such as C and N mineralization and enzyme activities (Frankerberger et al., 1983; Nannipieri et al., 1983; Seech and Beauchamp, 1988; Perucci, 1992; Franzluebbers et al., 1996). A more complex picture emerges from my studies. For example, even in the case of MBC and Min.C where a statistically significant relationship was observed, the distribution patterns were not identical. At both sampling times, regardless of treatment, aggregate size classes were identified that showed high levels of both MBC and of C mineralization (0.5 to 1.0 mm), and low levels of MBC and C mineralization (0.25 to 0.5 mm). Other size classes possessed either high levels of MBC and low levels of C mineralization. Therefore, at the time of sampling, situations existed where substrates were available in the immediate vicinity of the microbial population to support their activity. In the other cases it seems reasonable to conclude that the microbial population had exhausted the suply of mineralizable substrates in its immediate vicinity.

Another particularly interesting example relates to the observation that aggregate size classes with the highest levels of mineralizable C and N do not coincide at either the June or September sampling dates. It is possible that different types of substrate exist in the different aggregate sizes thereby influencing the "net" rate of mineralization by modifying the "gross" rates of N immobilization and mineralization. Further studies

are required using isotope dilution procedures (Hart et al., 1994) to determine if the gross rates of immobilization and mineralization of N differ sufficiently across the different aggregate size classes.

Although soil enzyme activities have been used for many years as indicators of biological activity in soil, it is unclear if they reflect upon the current microbial communities, or the history of past microbial colonization. In my study, the greatest rates of enzyme activities were found in September samples and in the smallest aggregate size classes (< 0.25 and 0.25 to 0.5 mm) that possessed lower levels of biomass and mineralizable C and N. Further work should be carried out to examine more closely the microbiological and chemical dynamics of the 0.25 to 0.5 mm size class in this soil.

Studies have shown that aggregate size distribution can be significantly influenced by the soil water content at the time of sampling (Perfect et al., 1990; Angers, 1992), water content at the time of sieving (Klodny and Jeffe, 1939; Gerard, 1987; Gollany et al., 1991), prewetting methods prior to wet sieving (Elliott, 1986; Gollany et al., 1991), and whether or not the sieving procedure is carried out under dry or wet conditions (Puget et al., 1995; Beauchamp and Seech, 1990). Although various sieving procedures were not compared in my study, I was able to achieve consistent distribution of soil in the different aggregate size classes over the two year study period, at the specific sampling times. Furthermore, it is worth noting that the field water content of the soil sampled throughout this study from the seedbed and after the summer-crop harvest ranged between 11 and 20.1 % (w/w) and averaged 14.7 % (w/w). I observed that soil water contents ranging between air dry (5% w/w) and 14% at the

time of sieving did not influence the aggregate size distribution in the Willamette silt loam. Furthermore, while distinctly different patterns of aggregate size distribution were observed in the June and September samplings, the percentage of soil in the size classes > 1.0 mm remained remarkably constant regardless of the different times of sampling.

A few comments should be made about my findings in the context of cover crops. In this study, I showed that the cover crop influence on soil biology was often subtle, being seen only in specific aggregate size classes, at specific times of the year, or interacting with the aggregate size. However, it should be pointed out that managing cover crops in the Pacific Northwest is not a trivial matter, where fall weather conditions can be extremely variable and establishment of the cover crop can be erratic. For example, in 1995 and 1996 the dry matter yield of the cover crop averaged 1640 and 593 kg ha^{-1} for the cereal, and 1376 and 897 kg ha^{-1} for the legume treatment, respectively (Richard Dick and Del Hemphill, unpublished data). As a result of this type of variability, it is difficult to discriminate between short and long term effects of cover crops on soil properties in our rotational systems. Therefore, more effort will be required to determine if my observations have any relevance to soil quality indicators. On a final note, I observed that the levels of TOC and TKN were consistently lower in the cereal than in the fallow treatment. Presumably, the stimulation of biological activity by this treatment has resulted in increased mineralization of soil organic matter. It should be pointed out that I only sampled the treatments that received zero inputs of N fertilizer, nevertheless indiscriminate use of cover crops could lead to a decline in soil quality if traditional soil fertility issues are not also taken into consideration.

Chapter 4. DISTRIBUTION AND DYNAMICS OF *Rhizobium leguminosarum* bv *trifolii* SEROTYPES AMONG SOIL AGGREGATE SIZE CLASSES

ABSTRACT

A combination of the plant infection soil dilution technique (MPN) and immunofluorescence direct count microscopy (IFDC) were used to examine the distribution of a soil population of *Rhizobium leguminosarum* bv. *trifolii* in five different size classes of soil aggregates (< 0.25, 0.25 to 0.5, 0.5 to 1.0, 1.0 to 2.0 and 2.0 to 5.0 mm). The aggregates were prepared from a Willamette silt loam sampled out of replicated field plots on three different occasions (two immediate post harvests of broccoli and sweet corn in September 1995 and 1996, and one immediate pre-plant of sweet corn in June 1996). Prior to the summer crop, the plots had been winter fallowed, or had grown a red clover or triticale winter cover crop. Regardless of sampling time, the *Rhizobium* populations in all aggregate size classes were stimulated to varying degrees (5 to 284 fold) by the presence of the red clover cover crop. However, the distribution of the population across the aggregate size classes changed between sampling times. At the September samplings, the microaggregate size class (< 0.25 mm) recovered from the red clover plots carried between 30 and 70% of the total nodulating *R. leguminosarum* population, while the same size class from the June sampling only carried 6% of the soil population. IFDC also showed that the distribution of one of the most successful nodule occupying serotypes (AR-18) changed distinctly in the soil that

was cover cropped with red clover between the June and September 1996 samplings. In June, the population density found in the 1.0 to 2.0 mm size class was significantly larger than the densities in < 0.25 and > 2.0 mm size classes, whereas in September, the population density of AR-18 in the 0.25 to 0.5 mm size class was significantly larger than the population densities found in aggregates < 0.25 and >1.0 mm. The populations of two other *Rhizobium* serotypes (AR-6 and AS-36) followed the same trends of change in distribution between the June and September samplings. An immunofluorescence cell elongation assay showed that varying percentages of the IF visualized cells were viable in whole soil recovered from the three treatments (19 to 45%). Further studies are required to determine if percent viability differs across the aggregate size classes, and if the distribution of viability is influenced by sampling time.

INTRODUCTION

Recently, considerable interest has been shown in determining if changes in microbial community composition of soils can be brought about by either abusive or improved management practices (Bååth, et al., 1995; Zelles et al., 1995; Frostegård et al., 1996; 1997; Reichardt et al., 1997). In addition, interest has been shown in the phenomenon of soil aggregation and the distribution and activities of soil microorganisms in the latter (Hattori and Hattori, 1976; Hattori, 1988; Gupta and Germida, 1988; Seech and Beauchamp, 1988; Beauchamp and Seech, 1990; Miller and Dick, 1995a and b). I wished to examine if there was any relationship between soil structural features such as aggregates and the population dynamics of soil bacteria under

different soil management regimes. In this particular situation I chose to examine the distribution of an individual bacterial species, *Rhizobium leguminosarum* bv *trifolii*. Despite being recognized primarily for its ability to form symbiotic associations with leguminous plants, *Rhizobium* is a bona fide soil saprophyte with dynamic soil populations ranging from 10^8 cells g^{-1} in rhizosphere soil to < 100 cells g^{-1} soil in the absence of the host plant (Moawad et al., 1984; Bottomley, 1990). Furthermore, well-documented methods exist for enumerating *R. leguminosarum* bv *trifolii* populations with fluorescent antibodies (Demezas and Bottomley, 1986; Bottomley and Dughri, 1989).

Although several studies were conducted previously to examine the impact of soil physical features on the fate of introduced rhizobial strains (Postma, et al., 1989, 1990; Postma and van Veen, 1990; Heijnen and van Veen, 1991), no one has examined the distribution across soil aggregates of an indigenous population of any bacterial species. The presence of red clover (*Trifolium pratense*, L) in the winter cover crop rotational system at the North Willamette Extension and Research Center (NWERS), Aurora, western Oregon, provided an opportunity to examine the dynamics and distribution across soil aggregates of *Rhizobium leguminosarum* bv *trifolii*, both in the presence and absence of the host plant. In the present study, immunofluorescence direct counts and the plant infection soil dilution technique, were used to examine the seasonal distribution of *R. leguminosarum* bv *trifolii* across soil aggregates under a summer crop of corn or broccoli followed by a winter-fallow, or a legume/non legume winter cover crop.

MATERIAL AND METHODS

Experimental site

Soil samples were collected from a vegetable crop rotation experiment initiated in 1989 at the North Willamette Research and Extension Center (NWREC), Aurora, Oregon. The soil is a Willamette silt loam (Pachic Ultic Argixeroll), the climate is Mediterranean characterized by cool wet winters and warm dry summers. The mean annual precipitation is 1040 mm with 70% occurring between November and April. The mean air temperature is 11.1°C and the average soil temperature in the winter and summer at a depth of 5 cm is 7.2°C and 19.3°C, respectively (Miller and Dick, 1995b). General characteristics of soil from NWERC are presented in Table 4.1. The site had been in a winter wheat-fallow rotation for the previous ten years, before the experiment was initiated.

Field treatments included three winter management systems in a vegetable crop rotation that alternates two summer crops, sweet corn (*Zea Mays* L. cv. Jubilee) and broccoli (*Brassica oleracea* L. var Italica cv. Gem) (Table 4.2). The three winter treatments are: (1) winter fallow, (2) red clover cover crop (*Trifolium pratense* L var. Kenland), and (3) cereal rye cover crop (*Secale cereale* L. var. Wheeler). Beginning in 1995 cereal rye was replaced with Celia trititicale (*X Triticosecale Wittmack*). Hereafter, these treatments will be referred as fallow, cereal and legume respectively

Table 4.1. General characteristics of soils from the North Willamette Extension and Research Center (NWERC).

Soil characteristics	Depth (cm) 0-10	10 - 20
pH†	5.60	6.18
Total C (g kg^{-1} soil)†	15.0	15.0
Total N (mg kg^{-1} soil)†	1058.0	1008.0
Total P (mg kg^{-1} soil)†	2612.0	2632.0
Bulk density (Mg m^{-3})‡	1.24	
K sat (x 10^{6} ms-1) ‡	60	

† Data collected in September 1989 (Kauffman, S.M., Masters Thesis, OSU, 1994)

‡ Brandi-Dohrn et al., 1997

Table 4.2. Crop rotations at North Willamette Extension and Research Center (NWERC).

Year	Season	Winter Fallow	Relay cover crop Cereal	Legume
1989	Fall	Fallow	Rye	Red Clover
1990	Spring	Corn	Corn	Corn
	Fall	Fallow	Rye	Red Clover
1991	Spring	Broccoli	Broccoli	Broccoli
	Fall	Wheat	Rye	Red Clover
1992	Spring	Wheat	Corn	Corn
	Fall	Fallow	Rye	Red Clover
1993	Spring	Broccoli	Broccoli	Broccoli
	Fall	Fallow	Rye	Red Clover
1994	Spring	Corn	Corn	Corn
	Fall	Fallow	Rye	Red Clover
1995	Spring	Broccoli	Broccoli	Broccoli
	Fall	Fallow	Celia Triticale	Red Clover
1996	Spring	Corn	Corn	Corn
	Fall	Fallow	Celia Triticale	Red Clover

Experimental design

The experimental design was a randomized complete block split-plot with four replications. The plots are 18.0 m x 9.0 m, with winter cover cropping systems as the main plots and N rates as the subplots. Samples were taken only from the zero kg N ha^{-1} subplots to avoid interactions between N fertilizer and legume nodulation on the dynamics of the indigenous *Rhizobium* populations.

Soil sampling

Soil samples were collected to a depth of 20 cm, in June 1994, September 1995, June and September 1996. Twenty cores from each replicate were composited and thoroughly homogenized.

Soil sieving and aggregates distribution

Different size classes of soil aggregates were prepared from soil samples collected in September 1995, and June and September 1996. Field moist soil samples were gently broken up by hand and dried at 4°C until they reached a gravimetric water content ranging between 5 and 14% (w/w). Subsamples (200 g) of soil were placed in the top of a nest of sieves and sieved for three minutes on a Tyler Ro-Tap shaker (Combustion Engineering Inc., Mentor, OH) into the following size classes: < 0.25, 0.25 to 0.50, 0.50 to 1.00, 1.00 to 2.00, and 2.00 to 5.00 mm.

Determining the magnitude of the viable nodulating population of *Rhizobium leguminosarum* bv *trifolii* in Willamette silt loam with a most probable number (MPN) procedure

Seeds of *Trifolium pratense* cv Kenland, were surface sterilized by consecutive immersions in ethanol 95% (v/v) for 30 seconds, and 25% (v/v) commercial Chlorox bleach for 5 minutes, followed by several rinses with sterile distilled water. Seeds were germinated on inverted water agar plates for two days in the dark at room temperature. Seedlings were transferred aseptically to sterile test tubes (20 by 2.5 cm) containing 20 ml of a N-free Jensen's seedling nutrient medium solidified with 15 g of Bacto-agar per liter (Difco). The concentrations of the constituents were (in grams per liter): $CaCl_2$, 0.6; $MgSO_4$. $7H_2O$, 0.2; NaCl, 0.1; K_2HPO_4, 0.5; KH_2PO_4, 0.25; ferric citrate, 0.05; and 10 ml of a trace element mixture (Evans, 1974). Ferric citrate was dissolved in a small quantity of 0.1 M HCl before addition to the growth medium. In order to prevent precipitation, the phosphate salts were dissolved in 100 ml of water, autoclaved separately and added to the remainder of the growth medium after cooling to 50°C. The final pH ranged between 6.5 - 7.0.

Portions (5g) of soil were suspended in 47.5 ml of a mineral salts solution (NaCl, 0.1; $CaCl_2$, 0.05; $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 0.34, and KH_2PO_4, 0.16 grams per liter), in 160 ml volume milk bottles containing 5g portions of 5 mm diameter glass beads. The suspensions were shaken by a wrist-action shaker for 15 minutes, and five-fold dilution series made to final dilutions 1: 156,250; 1: 781,250 and 1: 19,531,250 of the fallow, cereal and legume treatments, respectively. One milliliter portions of each dilution were pipetted onto each of four replicate tubes containing red clover seedlings.

Each of the tubes inoculated with the highest soil dilutions contained two red clover seedlings. Plants were grown in a completely randomized design in a greenhouse using natural day-light supplemented with artificial illumination (Dughri and Bottomley, 1983). Non-inoculated, inoculated, and mineral N-supplemented seedlings were included as controls in all experiments. Beginning two weeks after inoculation, the plants were scored for nodulation at four day-intervals and the final evaluation occurred six weeks after inoculation. The MPN per gram of dry soil, was calculated using the MPNES program (Woolmer et al., 1990). In this program, the upper and lower confidence limits (P=0.05) of the population size are obtained by either multiplication or division of the population estimate with a confidence factor.

The first MPN determination of population size (June 1994) was conducted with whole soil samples collected from all four field replicates of each treatment, to assess the spatial variability of viable rhizobial populations throughout the experimental field. Subsequent MPN determinations were carried out in September 1995 and June 1996 on composite samples of either aggregates or whole soil from the three treatments. In September 1996, only the aggregates recovered from the legume treatment and whole soil samples representing the three treatments were evaluated.

Isolation of representatives from the indigenous population of *Rhizobium leguminosarum* bv *trifolii* in Willamette silt loam

Two collections of isolates were obtained. In the first group, nodules were recovered from plants in the highest nodulation positive soil dilutions of the first MPN (June 1994). One hundred and eighty isolates of *R. leguminosarum* bv *trifolii* were

obtained from the three treatments (60 isolates per treatment). The second group of isolates was obtained in April 1996 from field nodules of red clover growing in the legume cover crop treatment. One hundred and twenty isolates were obtained from field plants sampled across the four replicates (eight plants were sampled per replicate). Nodules were detached, rinsed in sterile distilled water, and surface sterilized by consecutive immersions in 95 % ethanol (v/v) for 50 seconds, 15 minutes in 25% (v/v) Chlorox bleach, followed by several rinses with sterile distilled water. The nodules were crushed onto plates of YEM agar (Vincent, 1970) containing 20 mg l^{-1} of cycloheximide, and contents streaked to purity. Single colonies were picked, restreaked to purity for a second time, and transferred onto slants of YEM agar, grown for 72 hours at 27°C, and stored at 4°C.

Serological analyses of *R. leguminosarum* bv *trifolii* in Willamette silt loam

In an initial attempt to characterize the *Rhizobium* strains from Willamette silt loam, isolates recovered from the MPN terminal positive soil dilutions and field nodules were examined by immunofluorescence microscopy using fluorescein-labeled immunoglobulin-conjugates (FAs). Cultures were grown in YEM (Vincent, 1970) for 3 days, centrifuged, washed once with 0.5% (w/v) NaCl and once with sterile distilled water. Cell pellets were resuspended in distilled water to a final optical density of 0.1 at 660 nm, equivalent to about 10^8 cells/ml. Microscope smears (10 µl) were prepared, air-dried, heat fixed, pre-stained with one drop of a rhodamine-gelatin conjugate to suppress non-specific fluorescence (Bohlool and Schmidt, 1968), and dried at 55°C for

20 minutes. Each smear was stained for 30 minutes in a dark humidified container with one drop of the appropriate FA solution diluted 25X in 0.02 M phosphate buffer saline (PBS), pH 7.2. Smears were mounted in FA mounting fluid (Difco) and examined under a Zeiss epifluorescent microscope describe elsewhere (Demezas and Bottomley, 1987).

Purification of antibodies from antiserum for the indirect assays with goat-anti-rabbit labeled IgG

An analysis of two serotypes (AR-6 and AR-18) was conducted with indirect immunofluorescence using goat-anti-rabbit-fluorescein-labeled IgG (Sigma). Immunoglobulin G (IgG) was enriched from the two antisera mentioned above with a procedure provided by Dr. Michael Sadowsky (University of Minnesota). One ml of antiserum was added to 2 ml of 0.06 M sodium acetate buffer, pH 4.8 and dialyzed overnight against the same buffer. The antiserum was transferred to a 20 ml scintillation vial and 0.082 ml of octanoic acid was added dropwise and stirred for 30 minutes at room temperature. The suspension was centrifuged for 10 minutes at 8000 rpm and the supernatant dialyzed against 0.02 M PBS pH 7.2 for 6 hours at 4°C. The material was transferred to a 50 ml beaker and diluted with distilled water to a volume of 8 ml. While stirring at room temperature, 8 ml of saturated $(NH_4)_2SO_4$ was added and stirred for 30 minutes. The solution was centrifuged for 10 minutes at 6000 rpm, the sediment resuspended in 2 ml of 0.02 M PBS pH 7.2, and dialyzed against PBS at 4°C overnight. The concentrated IgG was transferred to a scintillation vial, merthiolate (0.1% w/v) added to a final concentration of 0.01%, and stored at -20°C. When necessary, another centrifugation step (10 minutes at 6000 rpm) was performed to clarify the supernatant.

The concentration of IgG was determined by measuring the absorbances at 260 and 280 nm and using the formula: mg/ml protein = (1.4 x A280) - (0.7 x A260).

Appropriate working dilutions of the two antisera and of the goat-anti-rabbit-fluorescein-labeled IgG were determined on smears from broth-grown cultures of a variety of antigenically distinct *R. leguminosarum* bv *trifolii* isolates. Smears were prepared as described above, pre-stained with rhodamine-gelatin, and stained in a humidified container for 30 minutes with one drop of a specific dilution of the purified antiserum (diluted 10X, 25X, 50X and 100X). After washing with 0.02 M PBS pH 7.2, one drop of fluorescein labeled goat-anti-rabbit IgG (diluted 100X, 150X and 500X) was added, the smears stained for an additional 30 minutes, and rinsed with 0.02 M PBS pH 7.2. Smears were mounted in mounting fluid (Difco) and viewed under a Zeiss microscope as described above.

These experiments established the working dilutions for the IgG preparations (25X for AR-18 and AR-6) and for the goat-anti-rabbit fluorescein labeled IgG (100X). All the dilutions were made in 0.02 M PBS pH 7.2.

Enumeration of serotypes *R. leguminosarum* bv *trifolii* in North Willamette soil

Bacteria were extracted from soil by the method of Demezas and Bottomley (1986). Five gram portions of soil were suspended in 47.5 ml of 0.1 M $(NH_4)_2HPO_4$ in 160 ml milk bottles containing 5g portions of 5 mm diameter glass beads. Two drops of tween 20 and one drop of antifoam B were added and the suspensions shaken in a wrist-action shaker for 15 minutes. Soil was flocculated by adding a mixture of 0.4 g $CaCl_2$

and 0.25 g $MgCO_3$. The suspensions were shaken for an additional two minutes, allowed to settle for 20 minutes, and approximately 25 ml of supernatant were collected from each bottle and transferred to sterile Erlenmeyer flasks.

Direct immunofluorescence analysis was carried out as described by Leung et al., (1994). Ten ml portions of the appropriate dilution of the flocculated supernatants were filtered through a 0.4 μm pore size blackened 25 mm diameter polycarbonate membrane filter under -0.05 MPa suction pressure. The membrane was transferred to a microscope slide, covered with one drop of gelatin-rhodamine conjugate to suppress non-specific fluorescence (Bohlool and Schmidt, 1968) and dried at 55°C. Under subdued light the membranes were covered with one drop of the appropriate fluorescent antibody and incubated for one hour in a humidified container. The membranes were destained by passing 80 ml of filter-sterilized 0.02 M PBS pH 7.2. To retard the fading of the fluorescence, one drop of *p*-phenylenediamine mounting fluid (100 mg phenylenediamine in 100 ml glycerol, pH adjusted to 8.0 with 0.5 M carbonate-bicarbonate buffer) was placed on top of the filter. The slides were covered with #1 cover slips and viewed under a Zeiss epifluorescent microscope as described above.

For the assays involving indirect immunofluorescence analysis, the filters were stained for one hour with the unconjugated rabbit IgG, rinsed with 40 ml of 0.02 M PBS pH 7.2, and stained for one hour with one drop of goat-anti-rabbit labeled IgG in a humidified chamber under dim light. Membranes were transferred to a filter support, destained with 40 ml 0.02 M PBS under -0.05 MPa suction pressure, mounted on slides, and viewed as described previously. Twenty fields of view were counted per slide.

Population densities of *Rhizobium* serotypes AS-6, AS-36, AR-6 and AR-18 were determined from aggregate size fractions collected from each of the four field replicates in June and September 1996. For the enumeration of serotypes AS-36 , AR-6 and AR-18, the supernatant recovered after soil extraction was further diluted 10X with 0.1 M $(NH_4)_2HPO_4$ (final dilution of 100X). For enumeration of serotype AS-6, the supernatant was diluted 2X (final dilution 20X). The distribution of serotype AR-18 was evaluated in aggregate size classes recovered from the three treatments, whereas the distribution of the other serotypes was evaluated only in aggregates recovered from the legume treatment.

Determination of viability within populations of serotypes AR-18 by immunofluorescence and cell elongation

The viability of populations of serotype AR-18 was evaluated by an immunofluorescence cell elongation assay (Bottomley and Maggard, 1990). For the purpose of these analyses, composites of whole soil samples from the three treatments were prepared. Triplicate portions (3 g) of soil were suspended in 27.0 ml of 0.15M NaCl in 160 ml milk bottles containing 5g portions of 5 mm diameter glass beads. Suspensions were shaken on a wrist-action shaker for 10 minutes, allowed to settle for 5 minutes, and a 10 ml portion of the supernatant was recovered and transferred to 85 ml of filter-sterilized (0.2 μm pore size) YEM mineral salts. The soil suspension was filtered consecutively through 8.0- and 3.0 μm pore-size filters. Five ml of yeast extract stock solution (800 mg $liter^{-1}$) were added to give a final concentration of 40 mg $liter^{-1}$. A thirty ml portion was dispensed in a 100 ml pre-sterilized Erlenmeyer flask, and 300

μl of a stock solution of nalidixic acid (10 mg ml^{-1} in 0.01 M NaOH) was added to give a final concentration of 10 μg ml^{-1}. Samples were incubated for 24 hours and the percentage of elongated cells (> 5.0 μm) in the population of *R. leguminosarum* bv *trifolii* AR-18 enumerated by immunofluorescence.

Total bacterial counts with DAPI

The DNA-specific stain, DAPI - diphenylamidino indophenol- (Sigma), was used for the enumeration of the total bacterial population in the five aggregate size classes. One milliliter of a DAPI stock solution (10 mg of DAPI in 100 ml of filter-sterilized (0.02 μm) distilled water) was added to 10 ml of a 10X dilution of the supernatant recovered after soil extraction (1 ml of supernatant and 9 ml of 0.1 M $(NH_4)_2PO_4$), incubated for 30 minutes and passed through a 0.4 μm blackened polycarbonate filter (25 mm diameter) under -0.05 MPa suction pressure. The membranes were destained under dim light with 20 ml of 0.02 M PBS pH 7.2, and placed on microscope slide on top of a drop of Cargille immersion oil type B. The same oil was applied on the upper side of the filter and covered with a #1 cover slip. The slides were viewed under a Zeiss epifluorescent microscope using a BP365110 excitation filter, FT 390 chromatic beam splitter, and a LP395 cut-off barrier filter (Zeiss filter set: 447701). A neofluor (100/1.30) oil objective, without flat field correction was used. The bacterial cells fluoresce a light blue color. Cells within the grid (35 μm x 35 μm) of a whipple disc were counted. Fifteen fields of view were counted

per slide. The density of total bacterial populations in aggregate size fractions recovered from all three treatments was determined in June and September 1996 samplings.

Determination of rhizosphere and nonrhizosphere rhizobial and total bacterial populations

Rhizobial and total bacterial populations in rhizospheres of field-grown red clover and triticale were determined in April, 1996. A total of eight plants were taken randomly from each of the four replications in the field. Plant roots were gently shaken to remove the loosely attached soil and allowed to air-dry overnight. On the next day the soil adhering firmly to the root surface was brushed off with a tooth-brush. In the case of red clover, care was taken to minimize the detachment and rupturing of nodules. In addition, fifteen soil samples were also randomly recovered from each plot (including the four plots of the fallow treatment), from locations in the field that lacked plant cover (referred to as non-rhizosphere (NR) soil hereafter). Immunofluorescence and DAPI were used to enumerate the populations of *Rhizobium* serotypes.

Statistical Analysis

Within each season, the distribution of AR-18 and total bacterial populations, across aggregate size classes recovered from the three treatments was analyzed using a repeated measures analysis of variance, aggregate size being the repeated term. The appropriate covariance structure was determined using a sphericity-test. This test

determines if it is acceptable to treat the data as a univariate analysis, and therefore, if the experiment can be modeled as a split-plot (Freund et al., 1986).

The seasonal comparisons were carried out with data collected in June and September 1996. A two repeated measures factors analysis of variance was used; the two repeated terms being sampling time (with two levels: June and September) and aggregate size (with five levels corresponding to each aggregate size class). Main effects means were separated using a LSD at P = 0.05 level. Data were analyzed using the SAS statistical package (SAS Institute, Cary, NC).

To compare the distribution of different *Rhizobium* serotypes across aggregates from the legume treatment, individual ANOVA were conducted for each serotype by comparing the densities found in the five aggregate size classes. Main effect means were separated using a LSD at P = 0.05.

RESULTS

Characterization of the indigenous populations of *R. leguminosarum* bv *trifolii* from Willamette silt loam

The serological analysis of *R. leguminosarum* bv *trifolii* isolates recovered from nodules on field-grown plants, and from terminal soil dilutions of the MPN analysis, identified specific serotypes of the biovar in the Willamette silt-loam (Tables 4.3 and 4.4). Fifty-four of 120 field nodule isolates reacted positively with six different FAs, and 41 of those reacted against FA-AR 18 (Table 4.3). In addition, a substantial

percentage of isolates recovered from the MPN soil dilutions of fallow (38%), cereal (19%), and legume (23%) treatments (Table 4.4) belonged to serotype AR-18.

Rhizosphere studies

Immunofluorescence analysis confirmed the existence of several serotypes in soil samples recovered from the fallow, cereal, and legume treatments. The population densities of five serotypes (AR-21, AR-6, AR-18, AS-6 and AS-36) in non-rhizosphere (NR) soil of the legume treatment ranged from 10^4 to 10^6 cells g^{-1} soil (Table 4.5). Populations of serotypes AR-21, AS-6 and AS-36 were found at similar densities in NR soil in all three treatments (4.4×10^4, 1.2×10^5 and 6.8×10^6 cells g^{-1} soil, respectively), whereas the population densities of serotypes AR-6 and AR-18 averaged 8 to 10 times greater in the legume treatment compared with fallow and cereal NR soil. The R/NR ratios for *Rhizobium* serotypes and the total soil bacterial population, were highest in the legume treatment and ranged between 2.8 and 41 (Table 4.5). Statistically significant differences between rhizosphere (R) and non-rhizosphere soil were observed for AR-6, AR-18, and total bacteria in the legume treatment, and for AR-18 and total bacteria in the cereal treatment.

Rhizobium distribution across aggregates as determined by MPN

The MPN analysis of whole soil showed that *Rhizobium* population densities in soil samples recovered from the legume treatment were greater than in soil from the fallow and cereal treatments (Fig. 4.1). The magnitude of this difference varied from

Table 4.3. Serological characterization of *Rhizobium leguminosarum* bv *trifolii* isolates recovered from field nodules of red clover.

Serogroup	Isolates in serotype	
	No.	%
AR-6	1	0.8
AR-23	1	0.8
AS-36	1	0.8
AS-21	2	1.6
WR-27	8	6.7
AR-18	41	34.2
Total of positive reactions	54	45
Total of unknowns	66	55

Table 4.4. Serological characterization of *Rhizobium* isolates obtained from nodules of MPN terminal positive dilutions of soil from the fallow, cereal and legume treatments, as determined by immunofluorescence (FA).

Serotype	Treatment		
	Fallow	Cereal	Legume
	---------No. of isolates †---------		
AS-36	1	0	2
AS-6	1	0	0
AR-23	6	0	3
AR-6 ‡	0	1	22
AR-18 ‡	18	9	14
Total of positive reactions	26(55.3) §	10(20.8)	41(66.1)
Total of unknowns	21(44.7)	38(79.2)	21(33.9)

† A total of 47, 48 and 62 isolates recovered from fallow, cereal and legume treatments, were used for FA tests.
‡ Analyses conducted by indirect immunofluorescence
§ Number in parentheses represent the percentage of the total

Table 4.5. Population densities (cells g^{-1} soil) of *Rhizobium* serotypes in rhizosphere (R) and non-rhizosphere (NR) soil recovered from the cover crop treatments.

	Fallow	Cereal			Legume		
		R	NR	R/NR	R	NR	R/NR
AR-21	3.00×10^4	8.56×10^4 a †	5.51×10^4a	1.55	3.40×10^5 a	4.71×10^4 a	7.21
AS-6	6.52×10^4	3.75×10^5 a	1.36×10^5a	2.76	1.48×10^6 a	1.51×10^5 a	9.79
AS-36	5.88×10^5	1.19×10^6 a	6.87×10^5a	1.73	2.14×10^6 a	7.53×10^5a	2.84
AR-6	1.61×10^5	1.13×10^6 a	4.33×10^5a	2.61	7.66×10^7 a	1.86×10^6b	41.23
AR-18	5.63×10^5	3.19×10^6a	6.64×10^5b	4.81	6.29×10^7 a	5.96×10^6 b	10.55
Total ‡	5.57×10^7	3.89×10^8a	7.36×10^7b	5.30	1.39×10^9 a	9.43×10^7 b	12.10

† Letters refer to comparisons between R and NR soil.

‡ Total population of soil bacteria as determined by direct counts with DAPI.

year to year ranging between five to 50 and between two to 34 times greater than the fallow and cereal populations, respectively. Aggregates recovered from the legume treatment carried *Rhizobium* population densities that ranged between 19 to 284 and five to 77 times greater than the fallow and cereal aggregate populations, respectively. (Fig. 4.2). There were two exceptions in the cereal treatment (size classes 0.25 to 0.5 mm, and 1.0 to 2.0 mm), from September 1995 which contained *Rhizobium* densities (5.9 x 10^4 cells g^{-1} soil) similar to their counterparts in the legume treatment (8.2 x 10^4 and 10.2 x 10^4 cells g^{-1} soil, respectively). Despite the large confidence limits associated with MPN population determinations, there were indications that population densities differed among aggregate size classes within each treatment. In September 1995 and June 1996, the microaggregate size class (< 0.25 mm) from both fallow and cereal was among the size classes with the lowest *Rhizobium* populations. In contrast, microaggregates recovered from the legume treatment in September 1995 and September 1996 contained *Rhizobium* population densities similar to, and sometimes greater than some of the larger aggregate size classes. In contrast, in June 1996, the lowest densities were found in the size classes < 0.25 and 0.25 to 0.5 mm of the legume treatment. These differences in population distribution can be expressed in terms of the percentage contribution that each aggregate class represents of whole soil (Table 4.6). For example, in September 1995, even though a similar percentage of soil was found in the < 0.25 mm size class of the three treatments (33.3%, on average), the proportion of the nodulating *Rhizobium* population found in that aggregate size class differed greatly among treatments (18.7, 12.1, and 69.5% for fallow, cereal, and legume, respectively). In contrast, in June 1996, the proportion of the nodulating rhizobial populations found

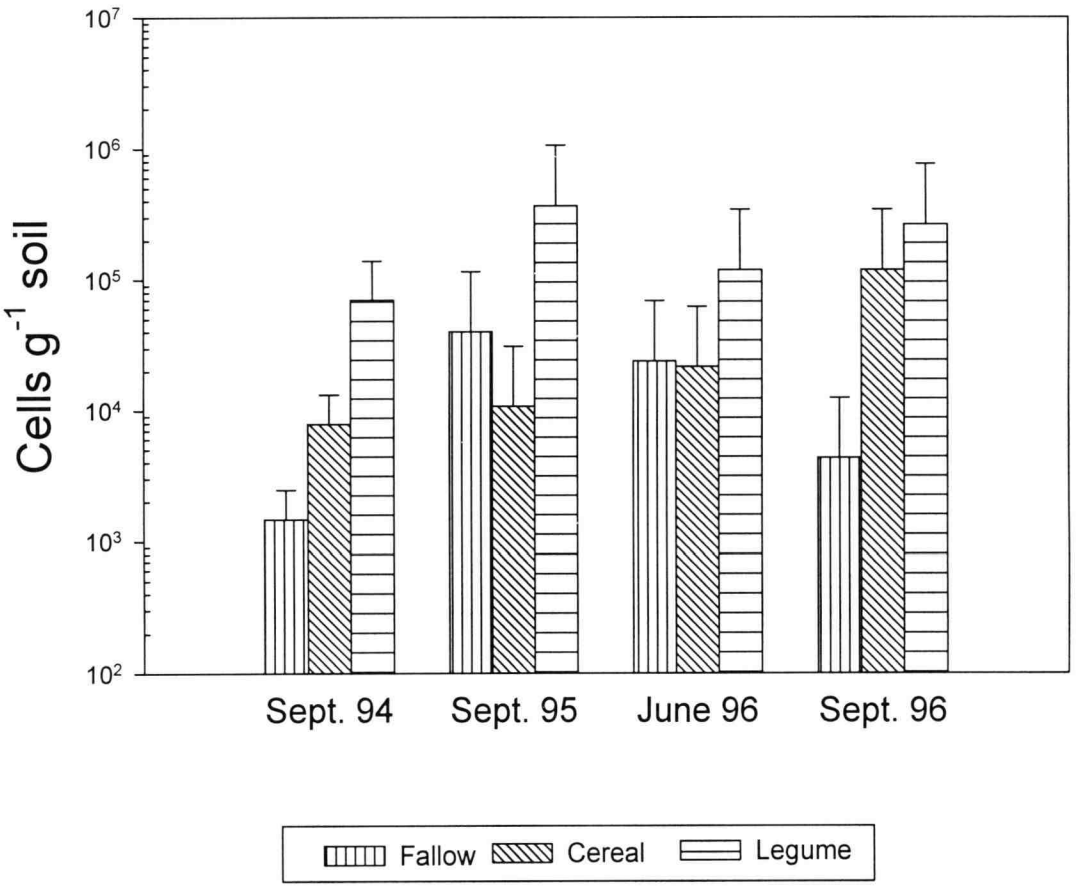

Figure 4.1. *Rhizobium* distribution in whole soil as determined by the most probable number (MPN). For June 1994, the error bars represent the standard errors of MPN determinations conducted on each of the four field replicates. For the other seasons, error bars represent the upper limits of the confidence limits ($P < 0.05$).

Figure 4.2. Distribution of nodulating rhizobial populations across aggregate size classes under different cover cropping systems in September 1995, June and September 1996. Error bars represent the upper limits of the confidence limits ($P < 0.05$).

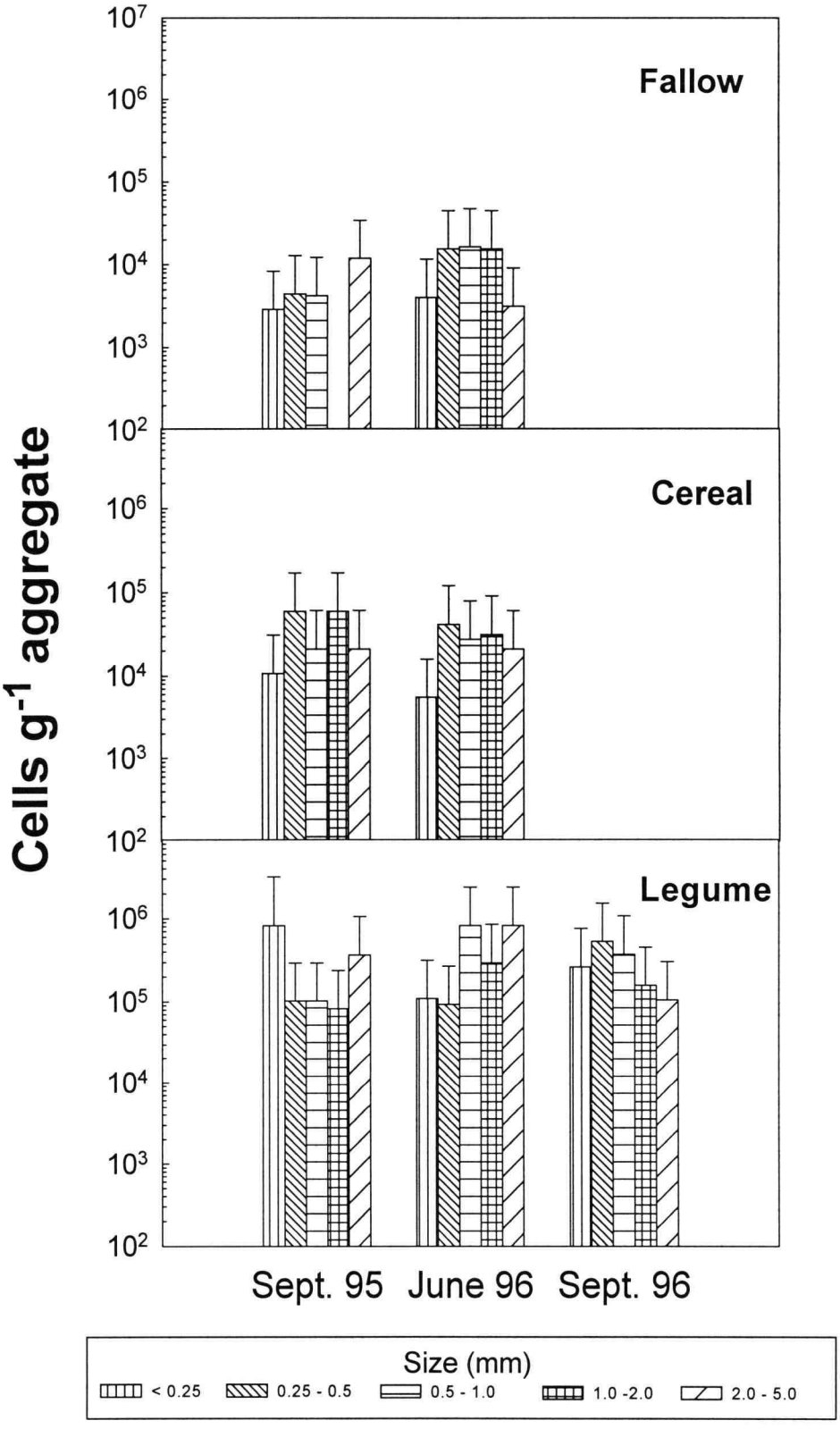
Fallow
Cereal
Legume
Cells g-1 aggregate
10^7
10^6
10^5
10^4
10^3
10^2
Sept. 95
June 96
Sept. 96
Size (mm)
< 0.25
0.25 - 0.5
0.5 - 1.0
1.0 -2.0
2.0 - 5.0

Figure 4.2.

Table 4.6. Aggregate distribution and relative contribution (%) of each aggregate size class in terms of the total viable nodulating *Rhizobium* populations as determined by MPN.

	Fallow		Cereal		Legume	
Aggregate (mm)	Aggr. Distrib.	Rhizobium	Aggr. Distrib.	Rhizobium	Aggr. Distrib.	Rhizobium
	----------	----------	%----------	----------	----------	----------
			September 1995			
<0.25	32.6	18.7	32.1	12.1	35.2	69.5
0.25 - 0.50	10.4	9.0	8.2	17.2	8.4	2.1
0.50 - 1.00	13.4	11.2	15.5	11.5	13.8	3.4
1.00 - 2.00	18.5	nd†	19.5	40.8	18.2	3.6
2.00 - 5.00	25.2	59.4	24.7	18.3	24.4	21.4
			June 1996			
<0.25	20.0	8.0	19.6	4.5	23.1	5.7
0.25 - 0.50	14.1	21.7	13.4	23.1	14.2	3.0
0.50 - 1.00	17.2	27.9	16.83	19.1	16.5	30.51
1.00 - 2.00	22.1	34.0	22.3	28.9	20.8	13.8
2.00 - 5.00	26.6	8.4	28.0	24.3	25.5	47.1
			September 1996			
<0.25					30.1	32.6
0.25 - 0.50					10.1	22.1
0.50 - 1.00					13.7	21.0
1.00 - 2.00					19.8	12.8
2.00 - 5.00					26.4	11.5

† nd non-determined

in the < 0.25 mm size class was similar in all three treatments (4.5 to 8.0%). In September 1996, only aggregates recovered from the legume treatment were evaluated. Compared to the June 1996 sampling, there was an increase in the proportion of the total *Rhizobium* populations found in the aggregate size classes < 0.5 mm (from 8.7% in June to 54.7 % in September). In September 1995 and 1996 legume macroaggregates > 0.5 mm, contained 28.4 and 45.3% of the total rhizobial population, in contrast to 91.3 % in June 1996 (Table 4.6).

Distribution of serotype AR-18 across aggregates

Because of the success of serotype AR-18 as a nodule occupant of field grown plants and its ability to develop large populations in both rhizosphere and non-rhizosphere soil in the legume treatment, we compared its distribution across aggregates of the three treatments during the June and September 1996 samplings (Fig. 4.3). At both sampling times, the population density of AR-18 varied significantly as a function of aggregate size. Furthermore, the interaction between aggregate size x treatment was significant, indicating that AR-18 distribution among aggregate size classes of the legume treatment differed from the distribution found in the fallow and cereal aggregates (Table 4.7). In June, a significantly greater density of AR-18 (2.3×10^6 cells g^{-1} soil) was found in the aggregate size class 0.5 to 1.0 mm from the fallow treatment, than in 0.25 to 0.5 and 2.0 to 5.0 mm size classes. Although similar trends were observed in the cereal treatment, the differences across aggregates were not significant. In contrast, the aggregate size class 1.0 to 2.0 mm of the legume treatment contained a significantly greater density of AR-18 than the size classes < 0.25, 0.25 to 0.5, and 2.0

Figure 4.3. Distribution of *Rhizobium* serotype AR-18 across aggregate size classes under different cover cropping systems. Letters indicate significant differences in means within treatments at $P < 0.05$.

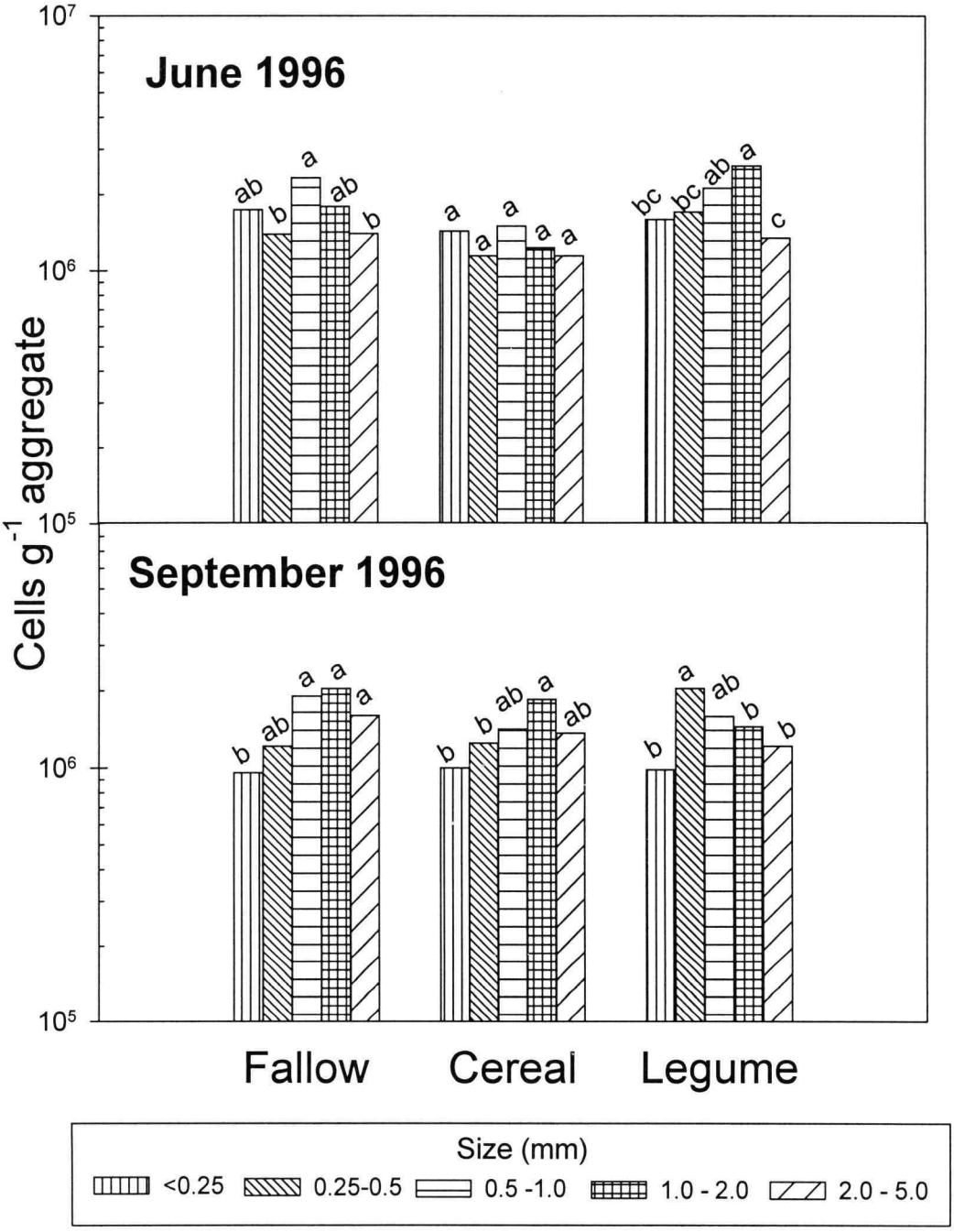
June 1996
September 1996
Cells g-1 aggregate
10^7
10^6
10^5
Fallow
Cereal
Legume
Size (mm)
<0.25
0.25-0.5
0.5 -1.0
1.0 - 2.0
2.0 - 5.0

Figure 4.3.

Table 4.7. Summary of the repeated measures analysis of variance within each season, for treatment and aggregate size effects on AR-18 and total soil bacterial population.

	Probability level (P>F)			
	June 1996		September 1996	
Source of variation	AR-18	Total	AR-18	Total
Treatment	0.0028	0.0097	0.2631	0.0672
Agg. Size	0.0095	0.0001	0.0001	0.0001
Treat x Agg. Size	0.0163	0.0053	0.0007	0.0010

Table 4.8. Summary of the two-factors repeated measures analysis of variance for treatment (Treat.), aggregate size (Size) and sampling time (Time) effects on AR-18 and total soil bacteria.

		Probability (P>F)	
Source of variation	df	AR-18	Total Bacteria
Treatment	2	0.0083	0.0090
Agg. Size	5	0.0044	0.0001
Treat. x Agg. Size	10	0.0009	0.0003
Error (a)	30		
Time	1	0.4442	0.1104
Time x Treat.	2	0.0347	0.0123
Time x Size	5	0.0220	0.1910
Time x Size x Treat.	10	0.0804	0.0329
Error (b)	30		

to 5.0 mm. In September, AR-18 densities in the size fractions > 0.5 mm of the fallow treatment and 1.0 to 2.0 mm of the cereal treatment were greater than those found in the < 0.5 mm size classes. In contrast, aggregates recovered from the legume treatment contained the highest density of AR-18 in the size fraction 0.25 to 0.5 mm, and significantly lower densities in the size fractions < 0.25, 1.0 to 2.0 mm, and 2.0 to 5.0 mm (Fig. 4.3).

Although, the overall population density of serotype AR-18 did not change between June and September, the interactions of sampling time x aggregate size and of sampling time x treatment on population density were statistically significant (Table 4.8). Significantly greater population densities were observed in aggregates < 0.25 mm in June than in September. Furthermore, the AR-18 densities in the legume treatment were significantly greater in June than in September.

Distribution of serotypes AS-6, AS-36, AR-6 and AR-18 across aggregates recovered from the legume treatment

In June 1996, the population densities of serotypes AS-6, AS-36 and AR-6 did not differ significantly (Fig. 4.4) among the different aggregate size classes, although there was a trend for the lowest densities to be in the < 0.25 mm size class. In September 1996, however, significant differences had developed for populations of serotypes AS-36 and AR-6 across aggregates. As with AR-18, a shift had occurred in population distribution with the highest densities of AS-36 and AR-6 being found in the size classes 0.25 to 0.5 and 0.25 to 1.0 mm, respectively.

Figure 4.4. Distribution of distinct *Rhizobium* serotypes across aggregates recovered from the legume treatment. Letters indicate significant differences in means within treatments at $P < 0.05$.

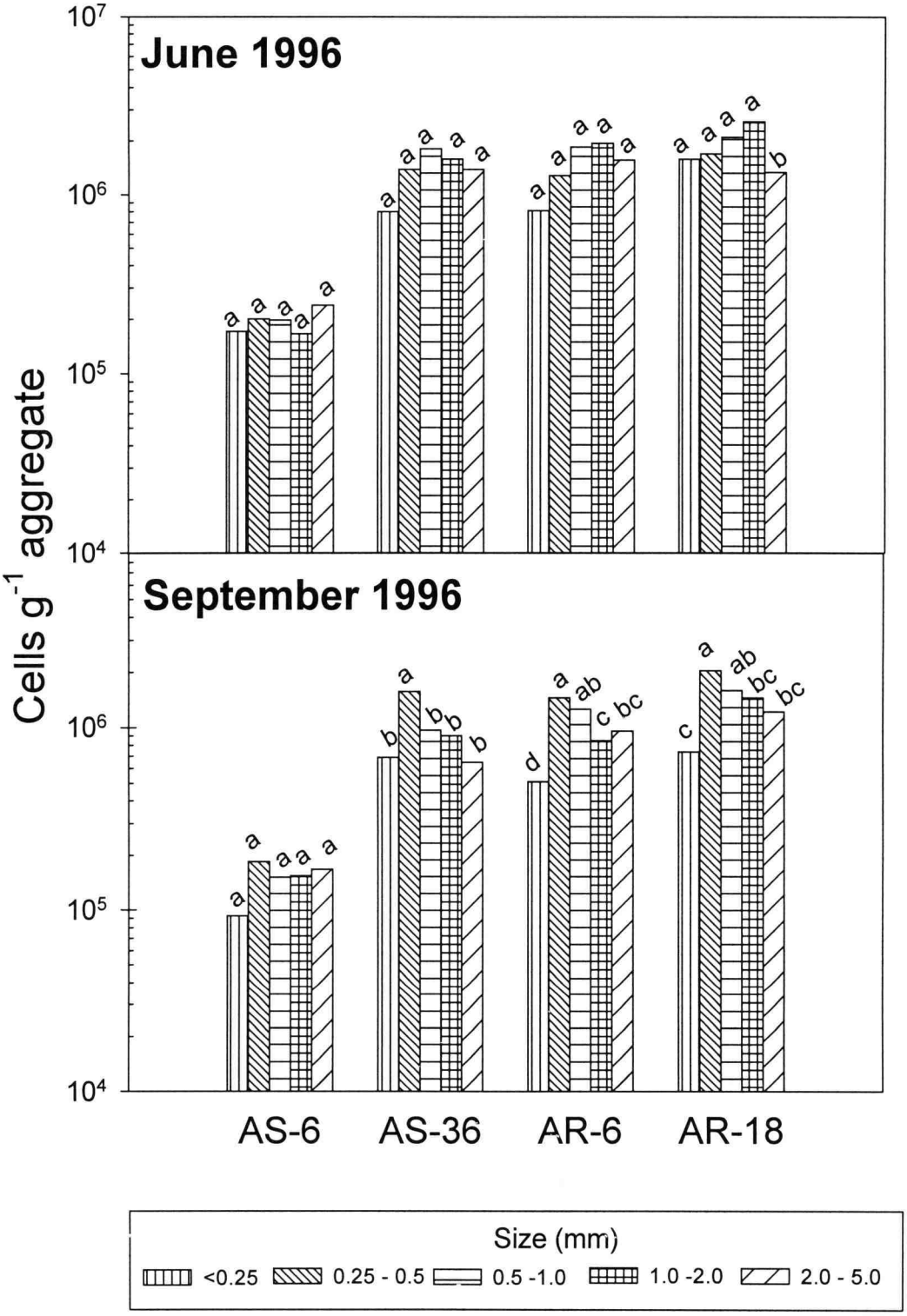
June 1996
September 1996
Cells g-1 aggregate
10^7
10^6
10^5
10^4
AS-6
AS-36
AR-6
AR-18
Size (mm)
<0.25
0.25 - 0.5
0.5 -1.0
1.0 -2.0
2.0 - 5.0

Figure 4.4.

Distribution of total bacterial populations across aggregates

The density of the total soil bacterial population in aggregates from fallow, cereal and legume treatments was evaluated in June and September 1996 (Fig. 4.5). At both sampling times the total soil bacterial population varied significantly as a function of aggregate size. Two distinct patterns of soil bacterial population distribution across aggregates were observed. In the fallow treatment (in June and September) and in the legume treatment (September), the two extremes of aggregate size classes (< 0.25 and 2.0 to 5.0 mm), contained the lowest densities of bacteria. In the cereal treatment (both seasons) and in the legume treatment (June) the < 0.25 mm size class contained significantly lower bacterial densities than all larger size classes.

The overall densities of soil bacterial population did not change from June to September (Table 4.8), nor as a function of aggregate size (the interaction sampling time x size was not significant). The interaction between sampling time x treatment was statistically significant, as a consequence of the higher populations observed in the fallow treatment in the June sampling.

DISCUSSION

Some of my findings have significance to the field of *Rhizobium* ecology, specifically, and to more general aspects of soil microbiology.

A few features of the distribution of *R. leguminosarum* bv *trifolii* across soil aggregate size classes were particularly interesting. First, although one expects the

Figure 4.5. Distribution of total soil bacteria across aggregates size classes under different cover cropping systems in June and September 1996. Letters indicate significant differences in means within treatments at $P < 0.05$.

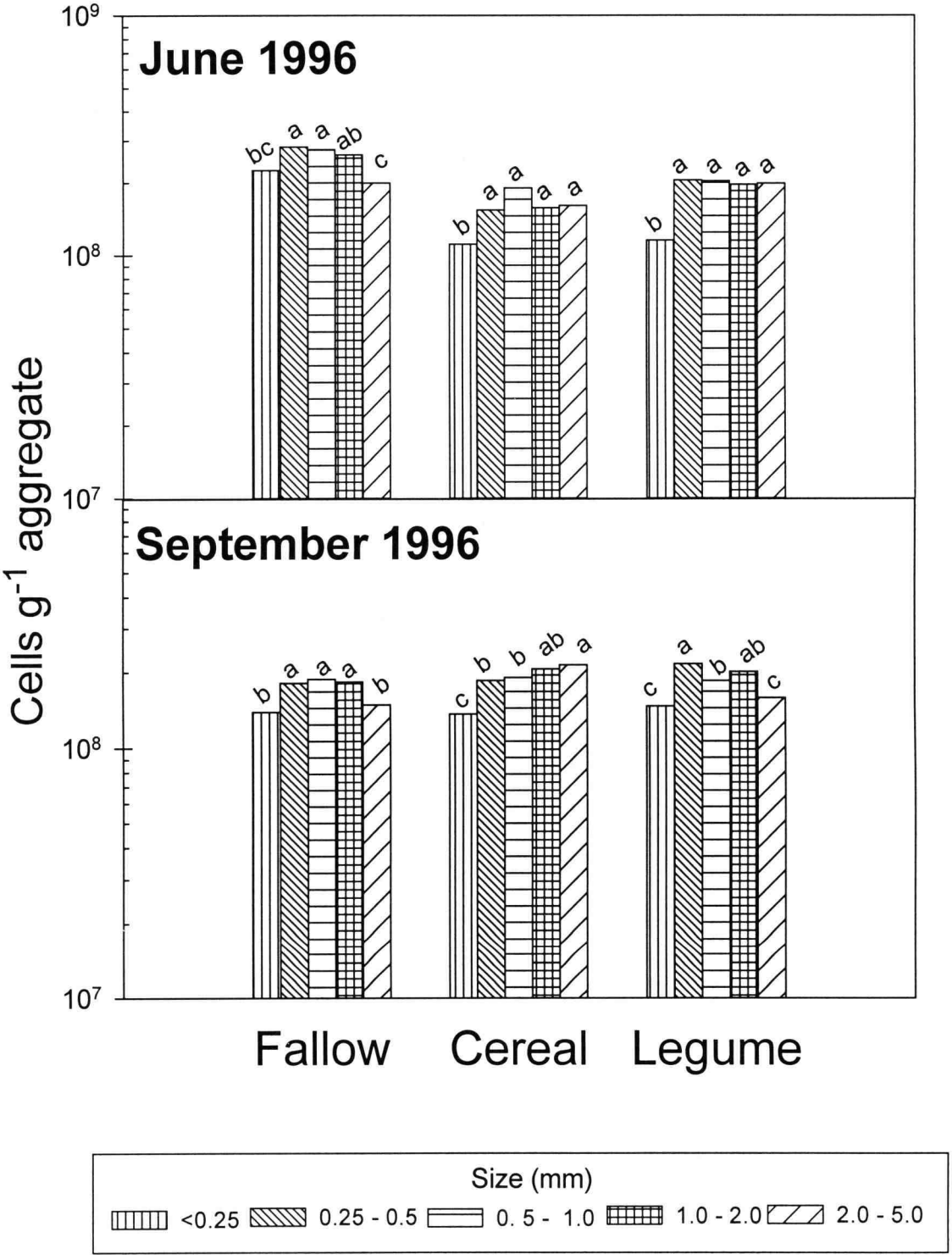
June 1996
September 1996
Cells g-1 aggregate
10^9
10^8
10^7
bc a a ab c
b a a a a
b a a a a
b a a a a
c b b ab a
c a b ab c
Fallow
Cereal
Legume
Size (mm)
<0.25
0.25 - 0.5
0. 5 - 1.0
1.0 - 2.0
2.0 - 5.0

Figure 4.5.

Table 4.9. Density of viable and non-viable populations of *Rhizobium* serotype AR-18, and percentage of viable cells. †

	Density (10^6 cells g^{-1} soil)		
Treatment	Viable	Total	% Viability
Fallow	0.52	2.37	22.1 (4.16) ‡
Cereal	0.29	1.52	19.1 (5.68)
Legume	1.18	2.56	45.4 (8.32)

† Whole soil samples were prepared from composites of the four field replicates. Each value is an average of three analytical replicates.
‡ Values in parentheses represent the standard error of the mean.

population of nodulating *Rhizobium* to be greater in a legume cropped soil than in either the cereal or fallow soil, aggregate size classes were identified in which population densities were similar in the cereal and legume treatments. Second, although data exist in the literature indicating that microaggregates are a less favorable habitat than macroaggregates for microbial activity (Tisdall and Oades, 1982; Gupta and Germida, 1988; Singh and Singh, 1995), several reports indicate that microaggregates can be very active (Seech and Beauchamp, 1988; Beauchamp and Seech, 1990; Jastrow, 1996; Jastrow et al., 1996). Certainly our *Rhizobium* MPN data from the legume cover crop (Fig. 4.2 and Table 4.6) indicate that the < 0.25 mm size class is dynamic, influenced by the soil management practice, and is a favorable habitat for an organism such as *Rhizobium* at certain times of the year. Further studies will be needed to gain a better understanding of this phenomenon.

The change in distribution of *Rhizobium* across aggregate size classes in the legume treatment between the June and September samplings was also observed in the immunofluorescence analysis of a specific serotype, AR-18 (Fig. 4.3). In this case, while the population density in June was significantly greater in the 1.0 to 2.0 mm size class than others, the population of the 0.25 to 0.5 mm size class was significantly higher in September. Again, these data can be placed in context with the counts of total bacteria (Fig. 4.5). In June, the total bacteria were generally greater in > 0.5 mm size classes of the legume treatment, while in September, total bacterial counts were significantly larger in the 0.25 to 0.5 mm size class than either of the extremes (< 0.25 or the 2.0 to 5.0 mm) of the size classes. Data presented earlier in the thesis showed that the aggregate size class 0.25 to 0.5 mm shows increased enzyme activity, and greater

accumulation of carbon between June and September than other size classes. Further studies are required to elucidate why certain size classes of soil aggregates house greater or lesser bacterial populations, and how the cover crop influences the dynamics of populations among the aggregate size classes over the growing season.

Although the differences in immunofluorescence direct count (IFDC) estimates of AR-18 across treatments were not as pronounced as the MPN estimates, the fact that at both sampling times the distribution of AR-18 across aggregate size classes from the legume treatment was different from those observed in the fallow and cereal treatments illustrates the influence that a specific cover crop can promote on microbial-soil structure interactions.

A comparison of IFDC and MPN estimates of the population size of AR-18 showed that MPN estimates ranged between 5 to 65 and 10 to 30% of IFDC from June and September soil samples from the legume treatment. These values agree reasonably well with each other if some latitude is given for the large confidence intervals of the MPN procedure, and by the preliminary data from a cell elongation assay that < 50% of IFDC cells are viable (Table 4.9). However, in the case of the cereal and fallow soils (September 1996), the discrepancies between MPN and IFDC estimates are greater, MPN estimates of AR-18 are substantially lower, representing only 0.4 to 4% and 0.2 to 2.0 % of the cereal and fallow IFDC, respectively. A variety of possibilities might account for the discrepancy between IFDC and MPN estimates. First, unrelated bacteria carrying similar surface antigens to rhizobia are mistakenly being enumerated by the FAs, and they are enriched in the cereal and fallow treatments. For example, Bohlool and Schmidt (1970) reported a cross reaction by an FA raised to *B. japonicum* against a

soil actinomycete. Second, dead rhizobial cells might persist in the soil entrapped in microsites that are inaccessible to bacterial predators (Postma et al., 1990; Rutherford and Juma, 1992). Third, IFDC could be exposing nonsymbiotic rhizobia that are identical with, or closely related to *R. leguminosarum* serotypes. The existence in soils of non-symbiotic forms of *Rhizobium* species has been shown on several occasions over the past few years (Soberon-Chavez and Najera, 1989; Segovia et al., 1991, Laguerre et al., 1993; Sullivan et al., 1996), and nonsymbiotic cells have been reported to be 40 times more numerous than the symbiotic forms (Segovia et al., 1991).

Finally, a cell elongation assay (Table 4.9) carried out with AR-18 in whole soil samples collected in April 1997, indicated that only a percentage of the IF-detectable cells were viable and that the percent viability differed across the soil treatments (22.1, 19.1 and 45.4% for the fallow, cereal and legume treatments, respectively). These observations add credence to the idea that protected pore space exists in soil that can be occupied by bacteria and is inaccessible to predators (Postma and van Veen, 1990; Postma et al., 1990; Heijnen and van Veen, 1991). Further studies are necessary to determine why the percent viability of the rhizobia serotypes differs among treatments, and if the percentage of viable cells varies across the aggregate sizes among the treatments.

Chapter 5. SUMMARY

There is much current interest in identifying microbiological changes (transient and permanent) that occur when crop management is modified to improve soil physical and fertility properties. Although I found that a significant literature already exists on some aspects of this subject, it seemed that the emphasis was focused mainly upon practices which promote minimum disturbance for relatively long periods of time, e.g., planting perennial species and/or minimum tillage operations. Situations like the one described in my thesis have been less well studied, and represent a form of intensive agriculture in which improvements in soil quality are needed.

It was the premise of my thesis that cover cropping would change the structural properties of the Willamette silt loam, and this would be reflected in a change in aggregate size distribution. By sampling soil at the time of seed bed preparation, and after the summer crop was harvested, I could examine the changes that occurred in biomass distribution and activities during this period of intense biological activity. Furthermore, I hoped that this sampling schedule would provide an opportunity to identify which aggregate size classes might contain "labile" nutrients, and be majors providers of minerals to the summer crop. In addition, I would identify the aggregate size classes in which biomass and nutrients were protected throughout the summer crop growth period, and the aggregate size classes that might be the primary source of mineral N upon the return of the fall rain.

Despite the cover crop treatments have been in place for seven years, it was disappointing to find that they have not resulted in a measurable change in aggregate

size distribution, nor increased the level of soil TOC and TKN. Obviously, it is not a trivial matter to achieve accretion of SOM, and cause change in soil physical properties in this kind of cropping system. However, since I did obtain evidence for aggregate size distribution to be influenced by sampling time, it is entirely possible that other methods of aggregate preparation might be required to reveal treatment effects in this soil. Nevertheless, I did obtain evidence that microbial biomass and some indicators of its activity were influenced by cover crop treatment, and interactions with aggregate size and sampling time were evident.

The finding that biomass and its activities are distributed heterogeneously across aggregates of different size implies the existence of niches within the aggregate structure that vary in their ability to support microbial growth and to protect the latter from predation. Furthermore, the profiles of C and N mineralization, and enzyme activities across aggregate size classes were often different. This observation indicates that substrate quality might differ across the aggregate size classes. Further work is required to obtain evidence for this possibility, and to determine if it has ramifications to mineralization/immobilization potentials, and to microbial community composition of different aggregate size classes.

Most recent studies aimed at examining soil microbial community composition in the context of management changes have used biochemical or molecular techniques capable of detecting relatively gross changes in the microbiology. Furthermore, many of these techniques cannot discriminate between living or dead cells, nor can they tell if the indicator is still part of an intact cell. In addition, situations exist in which interest might be directed at examining changes within the population of an individual species

of agricultural importance such as a symbiont, a pathogen or biodegrader. Immunofluorescence provides an opportunity to examine directly bacterial cells recovered from soil without the need for culturing. In addition, when combined with the cell elongation procedure, dead and live cells can be distinguished. From my studies on the distribution of *R. leguminosarum*, I obtained additional evidence for the < 0.25 mm aggregate size class being microbiologically dynamic, and for soil conditions changing sufficiently between June and September samplings to result in a change in the distribution of the *R. leguminosarum* population across the aggregate size classes. Further work is required to determine what caused this population shift to occur. The observation illustrating differential viability of *R. leguminosarum* serotype AR-18 across the cover crop treatments indicates that niches exist where dead cells are protected from predation. Further studies are required to determine why the percent viability of serotype AR-18 differed among treatments, and if viability of this species differs across different aggregate size classes and at different sampling times.

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APPENDICES

Appendix 1

SIEVING TIME AND AGGREGATE SIZE DISTRIBUTION

Sieving time (minutes)	Aggregate size (mm)				
	< 0.25	0.25 - 0.5	0.5 - 1.0	1.0 - 2.0	2.0 - 5.0
	----------g aggregate kg^{-1} soil----------				
3.0	203	112	167	189	288
6.0	251	125	154	209	255

Aggregates were recovered from the cereal treatment after sieving for 3.0 minutes, and the amounts retained in each sieve were weighed. Subsequently, the same aggregates were placed on the top of the nest of sieves and sieved for an additional 3.0 minutes.

Appendix 2

STATISTICAL APPROACH

Repeated Measures Analysis of variance

When several measurements are taken on each subject or experimental unit (person, plant, plot) they tend to be correlated with each other. When these measurements represent **qualitatively** different things such as, weight, width, and length this correlation is taken into account by use of multivariate methods, such as multivariate analysis of variance (MANOVA). When the measurements can be thought of as responses to levels of an experimental factor of interest such as time or treatment, the correlation can be taken into account by performing a repeated measures analysis of variance. The repeated measures design also is needed, when questions arise about changes across the various measurements taken on an individual subject or experimental unit (i.e., when there is interest in testing the effects within subject). This is exactly the situation observed in my experiments where I wanted to study the distribution of soil biological parameters across aggregates within each cover-crop treatment, and also the interactions of aggregate size and treatment (interaction within subject x between subject). In contrast, when there is no interest in testing hypothesis within subjects nor in the interaction within subjects x between subject, the MANOVA is the statistical approach to be used.

The univariate analysis of repeated measures designs is similar to the analysis of a split-plot. However, a split-plot analysis assumes that each observation is independent

of every other observation. In repeated measures designs, observations on the same experimental unit are correlated. Therefore certain conditions regarding the covariance structure of the repeated measures have to be met. These conditions are known as Huynh-Feldt (H-F) conditions and are tested by a test called Sphericity-Test. By testing the hypothesis that the orthogonal components are **uncorrelated** and have equal variance, the sphericity test determines if it is acceptable to treat the data as a univariate analysis, i.e., if the repeated measurements design can be modeled as a split-plot.

For those parameters that do not satisfy the Huynh-Feldt condition (i.e., failed the sphericity-test), significance levels associated with adjusted F tests are used with either the Greenhouse and Geiser, or the Huynh-Feldt epsilon adjustments. These adjusted probabilities attempt to control for the correlation among the repeated measures. Both of the tests are carried out by estimating a quantity know as epsilon (ε) and multiplying the numerator and denominator degrees of freedom by this estimate, before determining the significance level for the adjusted F-test. In cases where the sphericity test is rejected dramatically ($p<0.001$), the univariate test should be interpreted cautiously.

Covariance Analysis

The blocking principle is one of the most common ways to eliminate the effect of controllable nuisance factors. The analysis of covariance is another technique that also is useful for improving the precision of an experiment. Traditionally the covariance analysis is used when the response variable y, is linearly related to another variable, x.

However, although x can be observed along with y it cannot be controlled by the experimenter. The variable x is called the covariate or the concomitant variable. The covariance analysis is a method of adjusting for the effects of the uncontrollable nuisance variable x, and the procedure is a combination of ANOVA and regression analysis.

In my studies, covariance analysis was used to model the relationships between the distribution of TOC, TKN, MBC and its activities, after accounting for the design structure (treatments, blocks, aggregate size). The relationship was modeled as:

y = x(block, treatment, aggregate size);

where y and x are the response and the covariate, respectively. The significance of the relationship between the two variables was tested by using the p-value of the Type III sum of squares of the covariate in the SAS output. If the Type III test of the covariate has a small p-value, there is a linear relationship between the two variables even after adjusting for treatment, block and aggregate size effects.

Appendix 3

REP-PCR FINGERPRINTS OF *Rhizobium leguminosarum* bv *trifolii* ISOLATES

All AR-18 isolates recovered from field nodules and MPN terminal positive soil dilutions were fingerprinted using REP-PCR. I will describe the protocol used for those analyses and the results obtained.

Cultures were grown in 9 ml of liquid YEM (Vincent, 1970) for 24 hours. Portions (1.2 ml) of cells were transferred to an Eppendorf tube, centrifuged for 10 minutes at 10,000 rpm, washed once with 1 M NaCl and once with sterile distilled water. Cell pellets were resuspended in 100 µl of distilled water. The preparation of genomic DNA was carried out by adding 100 µl of InstaGene matrix (Biorad) to 30µl of the cell suspension. The mixture was incubated at 56°C for 30 minutes, vortexed at high speed for 10 seconds, incubated at 100°C for an additional 8 minutes, vortexed, centrifuged at 10,000 rpm for 3 minutes, and stored at -20°C .

The oligonucleotides (REP) primers used were synthesized by the OSU Center for Gene Research. The DNA sequence of primer 1 is 5" ICG ICT TAT CIG GCC TAC 3" and the DNA sequence of primer 2 is 5'III ICG ICG ICA TCI GGC 3", where I, C, G, and T denote inosine, cytosine, guanine and tiamine.

Each PCR reaction was carried out in clean, sterile 250 µl thin-wall PCR tubes. The final volume of each reaction was 50 µl (15 µl of genomic DNA (template); 25 µl of a master mix solution; and 10 µl of Taq solution).

The composition of the master mix solution was:

Primer 1	2 µl (100 pmol)
Primer 2	2 µl (100 pmol)
dNTP solution	8 µl (200 µM of each)
$MgCl_2$	4 µl (2 mM)
DMSO	5 µl (10%)
10X buffer	4 µl

The stock solution of dNTPs was prepared by adding 25 µl of each 10 mM dNTP (Gene Amp DNTPs, Perkin Elmer) and 100 µl of sterile distilled water. The stock solution was stored at -20°C, and 8 µl were used per reaction.

Fresh stock solutions of Taq polymerase were prepared by adding 82 µl of sterile distilled water, 10 µl of 10X PCR buffer II (Perkin Elmer) (containing 100 mM tris-HCl, pH 8.3 and 500 mM KCl) and 8 µl Taq (AmpliTaq DNA Polymerase, Perkin Elmer). Ten µl of this stock were used per PCR reaction, to a final concentration of 3 units per reaction.

The amplifications were performed in a DNA thermocycler (Amplytron II Thermolyne, Barnsted, Dubuque, IA). The cycles used were as follows: 1 cycle at 95°C for 6 minutes, 30 cycles at 94°C for 1 minute, and at 65°C for 8 minutes, 1 cycle at 65°C for 16 minutes, and a final soak at 4°C. After the reactions, 10 µl of each REP-PCR product were separated on 1.5% agarose mini-gels, stained with ethidium bromide, and photographed by using a Gel print 2000i station (Biophotonics Corporation, Ann Arbor, MI).

A total of 74 AR-18 isolates were analyzed and visually classified into nine groups. All the nine groups share at least one band. With the exception of groups 1a and 2 where all the isolates are identical, the other groups contain isolates with a high percent similarity. A list of the isolates in each group is given below.

Group 1: a: 14, 15, 16, 17, 25, 26, 53, 58, 64, 65, 67, 68, 72, 75, 77, 78

b: 4, 36, 35, 50, 52, 55, 69 59, 60, 73

Group 2: 5, 6, 7, 8, 9, 44

Group 3: 29,31, 32, 33

Group 4: 27, 37, 39, 40, 41, 42, 56, 63, 70, 71, 74

Group 5: 54, 46, 61, 62

Group 6: 22, 23, 24

Group 7: 10, 11, 12, 18, 19, 20, 49, 51, 57 and AR-18 (parental strain)

Group 8: 2, 3, 30, 45, 47

Group 9: 38, 43

Strains with unique band patterns (miscellaneous): 13, 48, 66, 76

Table 1- Origin (MPN terminal positive soil dilutions and/or field nodules) of the AR-18 isolates in each group.

Group	MPN			Field nodules				Total
	Fallow	Cereal	Legume	Plot #1	Plot #18	Plot #26	Plot # 34	
1a	4	2		1			9	16
1b	1			2	2	1	4	10
2	5				1			6
5					2		2	4
3			4					4
6		3						3
4			1	5		1	4	11
7	3	3			2	1		9
8	2		1		2			5
9				2				2
Miscel.	1				1		2	4