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CHANGES IN MICROBIAL COMMUNITY RESPONSES TO GRADIENTS OF CARBON, NITROGEN, AND WETTING CYCLES IN CONCENTRIC LAYERS OF SOIL MACRO-AGGREGATES

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By

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ABSTRACT

CHANGES IN MICROBIAL COMMUNITY RESPONSES TO GRADIENTS OF CARBON, NITROGEN, AND WETTING CYCLES IN CONCENTRIC LAYERS OF SOIL MACRO-AGGREGATES

By

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This study examined the effect of carbon (C), nitrogen (N), and wetting cycle gradients within soil aggregates on microbial communities and populations. New methods of bacterial extractions and identification were developed for this study. Direct microscopic counts of bacterial populations were 35% higher in moist than air dry aggregates. Bacterial populations from contrasting management systems and soil types were native forest on silt loam soils > conventional-till on silt loam soils ≅ native grassland on loam soils > conventional-till planted to continuous alfalfa on sandy loam soils. A 17% and 32% increase in populations occurred in the interior regions of aggregates exposed to 3 and 6 W/D cycles. Populations in the exterior regions increased 23% with 3 W/D cycles and decreased 29% with 6 W/D cycles. A decrease in polar tensile strength was observed in aggregates exposed to 6 W/D cycles. This decrease in strength may be caused by decreased populations in the exterior regions, leading to fewer microbial by-products produced and lower aggregate strength. C and N concentrations and other factors appear to contribute to bacterial populations. Direct relationships exist between aggregate stability, porosity, and bacterial populations. As greater energy sources are available to soil bacterial populations, those populations increase in biovolume and density. This increase in size and population leads to greater bacterial by-products that in turn lead to increased aggregate formation and stabilization.

This work is dedicated to my parents, Bruce and Kimberly Dopp, for their unending love and support in all that I do, and instilling in me the confidence to accomplish my goals and dreams.

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INTRODUCTION

Microorganisms are present in nearly every habitat found in the soil ecosystem. Soil bacteria are highly numerous and diverse. On average, a gram of soil contains 10^9 bacteria and some 20,000+ different species (Paul and Clark, 1996; Brady and Weil, 2002). A major role of soil bacteria is the breakdown of organic material, thus releasing nutrients for uptake by other organisms. Soil bacteria also directly release compounds to the soil through excretions, which benefit other microbial communities and help bind soil particles together (Tisdall and Oades, 1982; Kandeler and Murer, 1993; Oades 1993; Tisdall, 1994; Paul and Clark, 1996; Monreal and Kodama, 1997; Hillel, 1998; Brady and Weil, 2002).

Microbial diversity within aggregates seems to be as large as the microbial diversity identified across extensive geographical areas (Grundmann and Normand, 2000). This localized diversity provides a huge resource of multifunctional microbial communities which may be modified for a specific function (Loeffler et al., 1999). Communities of microorganisms located in the surface and interior regions of aggregates can rapidly alter their metabolisms, thereby resulting in completely different microbial communities (Blackwood, 2001). External more robust bacterial communities are thought to contribute to the function and strength of soil aggregates through carbon (C) mineralization, while more C mineralization and sequestration appears to be associated with microbial populations within internal regions associated with intra-aggregate micro-porosities (Kandeler and Murer, 1993; Smucker et al., 1996, 1998; Bending et al., 2002; Dell et al., 2004; Blackwood et al.).

Soil aggregation processes and wetting/drying (W/D) cycles modify microhabitats by changing pore networks and sequestering C within newly formed microsites associated with interiors of aggregates. Previous approaches for identifying bacterial communities and populations include soil dispersion, bacterial extraction, and fluorescent staining and microscopy. These techniques have been optimized for bulk soil samples (Bloem, 1995; Paul et al., 1999). Image analysis procedures of bacterial communities within concentric layers of soil aggregates can be used to quantify bacterial populations associated with microhabitats located on surfaces and within central regions of macro-aggregates. Biovolume distributions can also be used to identify gradients of bacterial biomass from aggregate surfaces to their interiors.

The objectives and hypotheses of this study were:

1. To quantify changes in bacterial populations and communities within concentric layers of soil aggregates containing measured gradients of C and nitrogen (N) compounds.

   Hypothesis 1: Large concentrations of C and N compounds contained in the surface layers of aggregates contribute to greater aggregate stability by promoting larger populations of bacterial communities.

   Corollary 1.1: The highest positive correlations between C and N concentrations and bacterial communities will be observed in the surface layers of aggregates.

2. To determine if uniform distributions of C and N compounds, generated by more frequent wetting-drying cycles, within aggregates contribute to more uniform populations of bacteria and more water stable aggregates.

Hypothesis 2: The absence of C and N gradients will increase the uniformity of bacterial distributions and communities within aggregates.

Corollary 2.1: More uniform concentrations of total C and N will increase the water stability of whole aggregates.

Corollary 2.2: Increased concentrations of C and N compounds will be positively correlated with greater water stability of whole aggregates.

Corollary 2.3: There will be a positive correlation between increased uniformity of bacterial populations and the increased water stability of whole aggregates.

New methods of bacterial extractions and identification, including the expanded application of the Center for Microbial Ecology Image Analysis System (CMEIAS), a computer-assisted image analysis program, for soil bacteria were developed to verify the above objectives. Combinations of a newly developed soil aggregate erosion (SAE) method with conventional C and N analyses were integrated to prove the objectives and hypotheses listed above.

CHAPTER 1

LITERATURE REVIEW

Soil functions as a reservoir for water, nutrients, gases, soil organic matter (SOM), and microorganisms. Stable soil aggregate formation is crucial for the sustainability of food production systems, sequestration of carbon (C), and the remediation of wastes and pollutants (Tisdall and Oades, 1982; Beare et al, 1994; Monreal and Kodama, 1997; Hillel, 1998; Brady and Weil, 2002). Increased stability of macro-aggregates increases C sequestration by soil aggregates. This leads to better water infiltration and aeration porosity, reduced water and wind erosion, and decreased crust formation on aggregates and the soil surface (Kandeler and Murer, 1993; Edgerton et al, 1995; Monreal and Kodama, 1997; Hillel, 1998; Park and Smucker, 2003). Aggregate formation and stability results from the interactions of many factors, including C and nitrogen (N) content, microbial and mesofaunal activities, root exudates, clay minerals, and wetting/drying (W/D) frequencies.

Soil Aggregate Formation

Understanding what occurs within soil aggregates may explain many of the processes beneficial for food and fiber production, and how aggregates contribute to remediation processes that sequester soil C and detoxify anthropogenic pollutants. In addition to functioning as a reservoir for water, nutrients, gases, microorganisms, and SOM, soils facilitate massive quantities of micro- and macro-organismal activities.

The soil matrix, a blend of solid and porous phases, results from the flocculation and cementation of soil textural particles, ions, SOM, and associated microbial and mesofaunal communities into organized and aggregated structures (Hillel, 1998). These aggregation processes are dependent on the interactions of C and N compounds, microbial and mesofaunal activities, root exudates, types of clay minerals, parent materials, W/D frequencies, time, and other factors (Oades, 1993). Total C, soil bulk density and porosity, saturated hydraulic conductivity (K_s), and root activities are not significantly correlated with increased water stabilities of aggregates (Rasse et al., 2000). However, they demonstrated that other factors, such as C sources from shoot mulch and root decomposition, had positive effects on aggregate stability.

Soil management practices have a profound effect on soil structure and aggregate stability (Tisdall and Oades, 1982; Kandeler and Murer, 1993; Beare at al., 1994; Hillel, 1998; Brady and Weil, 2002). Conventional tillage (CT) practices incorporate crop residues and fertilizers into the plow layer using a moldboard plow to lift, twist, and invert the soil. These soils are subjected to extreme changes in soil water content and temperature. During these extremes, weaker aggregates are frequently broken along planes of weakness and substantial quantities of SOM are lost to accelerated mineralization by microbial respiration. Further geochemical decomposition, such as weathering, leads to reduced SOM, lower particulate organic matter (POM) content (Beare et al., 1994), and diminished energy resources for soil microbial activities (Monreal and Kodama, 1997). Reduced or no-tillage (NT) practices use minimal or no plowing, leaving plant residues on the soil surface. These soils generate higher quantities of stable soil aggregates at or near the soil surface. These surface soils have nearly

double the amount of total C sequestered within soil aggregates associated with at least 10 years under NT management (Smucker et al., 1996; Dell et al., 2004). Plant residues on the surfaces of NT soils also protect the soil from erosion by sheltering aggregates from wind and rain, thereby preserving soil aggregate structure.

Clay and SOM Effects on Aggregation

Clay mineralization ratios and total C are higher in the external regions of aggregates in CT, NT, and grassland (GL) soils (Smucker et al., 1996). As aggregate size increased, accumulations of clay in the external increased and clay content of internal regions of aggregates decreased (Kavdir, 1996). External clay accumulations may be due to more numerous W/D cycles which align the particles into uniform orientations, leading to a build up of highly oriented clay particles on the surfaces of the aggregates. Accumulations may also be due to increased microbial populations in the external layers of aggregates. Higher levels of C in the external regions of aggregates, which provide energy sources for microbial communities, and higher aeration in the internal regions lead to increased microbial populations (Sexstone et al., 1985).

Microbial Effects on Aggregation

Aggregate stability is dependent on soil microorganisms (Tisdall, 1994). Edgerton et al. (1995) demonstrated a positive linear correlation between stable macro-aggregates and microbial biomass C. Changes in the soil environment that affect microbial communities are also thought to affect soil structure (*e.g.*, aggregation and aeration) (Kandeler and Murer, 1993; Edgerton et al., 1995). Bacterial biomass C

increased in the presence of macro-aggregates, and decreased where no macro-aggregates were present (Denef et al., 2001). Wetting and drying cycles of soils with no macro-aggregate formation led to a reduction in the bacterial activity.

Microbial activities can promote the development of anaerobic regions in soil aggregates when soil aeration porosities are low, leading to the process of denitrification. It is expected that greater microbial populations and different microbial communities would be located in the exterior regions of aggregates from a majority of improved soil management systems. Results obtained in the Soil Biophysics Lab at Michigan State University (MSU) showed lower bulk densities in the exterior layers than the interior layers of aggregates in the 4-6.3mm size fraction across 3 management systems, native forest (NF), no-till (NT), and conventional-till (CT) for Wooster silt loam soil, sampled in 1998 (Park et al., 2001). These data demonstrated higher porosities in the exterior layers of aggregates and presumably accelerated the diffusion of atmospheric oxygen (O_2) and increased microbial respiration rates of the sequestered C and N compounds. Additional results comparing bulk densities from three aggregate size fractions (2-4, 4-6.3, and 6.3-9.5mm) showed consistent bulk densities through all size fractions for CT systems (Park et al., 2001). The 2-4mm size fraction of NT systems had higher bulk densities than the 4-6.3 and 6.3-9.5mm fractions. Bulk densities of the forest soil aggregates increased as the aggregate size fractions decreased (*e.g.*, the lowest bulk density for the 6.3-9.5mm aggregate fractions and highest bulk density for the 2-4mm fraction).

Microbial diversity within aggregates seems to be as large as the microbial diversity identified across extensive geographical areas (Grundmann and Normand, 2000). This localized diversity provides a huge resource of multifunctional microbial

communities which may adapt for specific functions (Loeffler et al., 1999). Communities of microorganisms located in the surface and interior regions of soil aggregates often alter the net production of metabolic products altering the communities within aggregates (Blackwood, 2001). Microorganisms detoxify their surroundings various ways; for example through electron consumption during different processes of dechlorination (Loeffler et al., 1999), by developing pH and ion concentration gradients within soil aggregates (Horn, 1990; Smucker et al., 1998), by altering oxygen concentrations (Sexstone et al., 1985), and by utilizing multiple sources of C and N compounds within single aggregates (Santos et al., 1997; Smucker et al., 1998; Dell et al., 2004). These activities result in the biogeochemical processes associated with the advective-dispersive flow of solutes, the production of specific greenhouse gases (GHG), C sequestration potentials, plant nutrient sources, and aggregate stability.

Blackwood (2001) reported no change in the composition of microbial populations due to aggregate size. However, the location and community structures of the microbial populations appeared to change within individual aggregates. This was shown by differences in the terminal-restriction fragment length polymorphism (T-RFLP) composition of microbial populations in aggregates between their internal and external concentric layers. T-RFLP is a polymerase chain reaction (PCR) based method that produces electrophoretic bands with lengths representative of the different forms of the gene of interest (Ogram and Sharma, 2002). Analysis for the dissimilarity of the 16s rDNA gene determines the diversity of microbial communities in soil.

Sissoko (1997) reported that soluble plant root C and N increased soil microbial biomass (SMB) and the water stability of soil aggregates. Uniquely different microbial

communities (Blackwood et al), and specific gradients of C and N (Smucker et al., 1998) from external layers toward internal regions of soil aggregates have been observed among aggregates from 3 different soil types.

Microorganisms fulfill many important roles in the soil profile. They promote soil aggregation processes by providing cementing agents, promote plant growth through symbiotic relationships (*e.g.*, N-fixing bacteria, mycorrhizal fungi, and plant growth-promoting rhizobacteria), control root diseases (biocontrol agents), fix N (free-living N-fixing bacteria), assimilate plant nutrients bound in organic matter (OM) or soil minerals (P from rock phosphate), and degrade pollutants (Oades, 1993; Tisdall, 1994; Paul and Clark, 1996).

Carbon in Aggregates

Total C within soil aggregates is higher in the external layers than the internal layers (Smucker et al., 1996; Horn, 1990; Park et al., 2001; Dell et al., 2004). Most of this additional C found in the external layers is part of the labile or active C pools associated with the microbial biomass, or is dissolved organic C (DOC) and mineralizable POM (Dell et al., 2004). Soil tillage, periodic W/D cycles, oxygen diffusion, and other abiotic processes are projected to contribute to gradients of C and N substrates within aggregates (Smucker et al., 1996). Concentrating greater quantities of C and N compounds, available to microbes in external regions, leads to specific locations for accelerated microbial growth and their associated contributions to increased soil aggregation.

Wetting/Drying Cycles

Aggregate stability and formation is influenced by W/D cycles. Six W/D cycles have been shown to increase aggregate stability (Utomo and Dexter, 1982). Sissoko (1997) found aggregate stability, mean weight diameter (MWD), was not affected by three W/D cycles, but was enhanced with nine W/D cycles when C and N compounds were added during the wetting phase.

Dissolved organic matter (DOM) and other C and N substrates are delivered to the internal regions of soil aggregates by repeated W/D cycles of soil aggregates (Utomo and Dexter, 1982). Each re-hydration of dry aggregates transports and distributes additional soil organic carbon (SOC) into the intra-aggregate pore networks of stable soil aggregates. Microbial communities located in the exterior layers of aggregates appear to be able to alter these C-sources and produce specific metabolites that contribute to aggregate stability (Christensen et al., 1990).

Denef et al. (2001) witnessed a continuous cycle in regards to W/D cycles, aggregate stability, and microbial activity. After a couple W/D cycles the OM became physically protected and was no longer accessible for microbial attack. This led to decreased microbial activity, decreased production of binding agents, and decreased aggregate stability. Upon additional W/D cycles, the less stable aggregate falls apart, freeing up previously inaccessible C for microbes. This leads to increased microbial activity, increased production of binding agents, and increased aggregate formation and stability. When the C source becomes depleted, the cycle of aggregate stability and microbial activity begins again.

Organic Matter and Aggregate Stability

Organic matter is crucial for forming and stabilizing soil aggregates (Brady and Weil, 2002). Organic compounds act as cementing agents and provide the energy substrates needed for most bacterial and fungal activities (Tisdall and Oades, 1982). As organic residues are decomposed, gels and other viscous microbial by-products promote aggregate formation by acting as gluing agents for particles and OM. These organic polymers consist of polysaccharides, polyuronides, and glomalin (a glycoprotein produced by arbuscular mycorrhizal fungi) (Paul and Clark, 1996; Hillel, 1998; Brady and Weil, 2002). Polysaccharides, polyuronides, and other nonhumic compounds originating from plants and microorganisms constitute a large group of semi-flexible molecules capable of forming multiple bonds with several mineral particles simultaneously. These complex organic polymers chemically bond with particles of silicate clays and iron and aluminum oxides, orienting and forming bridges between individual soil particles and cementing them together into water-stable aggregates (Brady and Weil, 2002). The polymers attach to clay surfaces by means of hydrogen bonding, anionic and cationic interactions, and van der Waals forces (Hillel, 1998). In cases where organic polymers are unable to penetrate individual clay micelles, they form protective films around micro-aggregates. In other cases, solutions of labile organic compounds penetrate the interior of soil aggregates, forming co-precipitate cementing agents that can be further mineralized biologically. Increasing the quantity of organic substrate within the aggregate enables microbial communities to redesign and strengthen the interior regions of the aggregate.

Functional diversity of soil microbial communities is affected by crop residues and the quality of SOM (Bending et al., 2002). The quality of SOM directly affects microbial communities by nutrient substrates available and indirectly by soil aggregation and aeration. Bending et al. (2002) also found that increased enzyme activity, which indicates larger microbial communities, increased as native SOM increased. Root exudates include aliphatic amino and aromatic acids, amides, and sugars (Paul and Clark, 1996). Soil management practices that result in accumulation of organic C (*e.g.*, NT, manure application, cover crop use), result in increased microbial biomass levels and changes in microbial community structure (Peacock et al., 2001). Paul and Clark (1996) reported that additions of simple organic compounds to soils changed their metabolic profiles and altered the diversity of soil microbial communities. Community response characteristics vary according to the nature of the compound added (Degens, 1998). Various applications of manure and N lead to different microbial communities. Peacock et al. (2001) found manure applications led to increased microbial biomass, activity, and diversity, while ammonium nitrate (inorganic N) applications led to decreased microbial biomass, activity, and diversity. The response of the microbial community to manure applications is similar to a rhizosphere response where the community is highly active metabolically and rapidly responding to additions of C (Peacock et al., 2001).

Concentric Aggregate Layers and Aggregate Stability

Soil aggregate erosion (SAE) chambers developed by Smucker et al. (1998) allow for surface layers to be eroded from soil aggregates and kept for further soil analyses, retaining sample purity (Figure 1.1). Individual soil aggregates in single SAE chambers

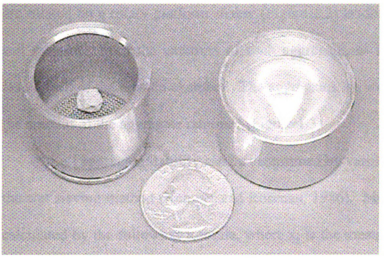

Figure 1.1. Soil aggregate erosion (SAE) chamber.

are rotated on a rotary platform shaker (Figure 1.2) leading to the mechanical erosion of soil materials from the exteriors of each aggregate, as it revolves against the knurled inside wall of the erosion chamber. The aggregates are weighed at intervals to determine the amount of the aggregate removed (Figure 1.3).

Soil aggregate stability can be determined by various destructive methods. One is the wet sieving method (Kemper and Rosenau, 1986). Mean weight diameter (MWD) is calculated by the following formula, where x_i is the mean diameter of the aggregate size ranges separated by sieving, and w_i is the weight fraction based on the total oven dry weight of the original aggregates that were recovered in that size range:

$$MWD = \sum_{i=1}^{n} x_i w_i$$

Slow hydration of dry aggregates by nebulization, prevents the breakage of most soil aggregates during the wetting process (Kemper and Rosenau, 1986).

Another destructive method for determining aggregate stability is polar tensile strength (PTS). The PTS of a soil aggregate is the force per unit area that is required to break the aggregate (Dexter and Kroesbergen, 1985; Hillel, 1998). In this method, the force applied to the aggregate, required to break the aggregate, is incorporated to the following equation to calculate PTS, where dc is the effective diameter of each aggregate (mm), dm is the mean aggregate diameter (mm), m is the dry mass of the aggregate (kg), m_o is the mean mass of the aggregates in the subsample (kg), F is the crushing force required to break the aggregate (kg m s^{-2}), b is the measured balance reading (kg), γ is the acceleration force due to gravity (9.807 m s^{-2}), and Y is the tensile strength (kPa):

Figure 1.2. Rotary shaker containing soil aggregate erosion (SAE) chambers.

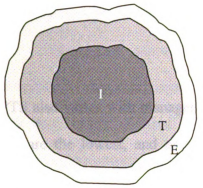

Figure 1.3. Concentric layers of a soil aggregate.
External (E), transitional (T), and internal (I) layers.

$$d_c = dm\ (m/m_o)^{1/3}$$

$$F = b\gamma$$

$$Y = 0.567F/(d_c)^2$$

PTS also varies with management practices. Usually, NF aggregates are the highest and CT are the lowest, and as aggregate size increases, PTS decreases (Aditjandra et al., 2002).

Bacterial Extraction from Soil

Soil dispersion and slide preparation techniques for extracting bacteria from bulk soil samples have been developed by Paul et al. (1999) and Bloem (1995). Modifications of these techniques can provide adequate extraction of bacteria for analysis of air dry and moist soil aggregates. Lindahl (1996) showed increased soil dispersion and bacterial counts when pyrophosphate, a chemical dispersing agent, replaced distilled water in the dispersion and extraction procedure, leading to increased extraction efficiency. Pyrophosphate disrupts soil aggregates by deflocculating the clay present in the aggregate. Pyrophosphate displaces the cations adsorbed to the clay with sodium, which increases the hydration of the clay micelles leading to repulsion of the micelles (Hillel, 1998). Although the use of pyrophosphate increases soil dispersion and the extraction efficiency of bacteria there are disadvantages of its use. Fluorescent stains behave differently with samples dispersed with pyrophosphate, leading to changes in the cell color (Lindahl, 1996). Pyrophosphate also increases the pH of the soil solution from 6.9 with distilled water to 7.9. This pH change is thought to alter the physiology of bacteria thus leading to metabolic changes of the bacteria modifying staining results (Lindahl, 1996).

Fluorescence Microscopy and Image Analysis

Fluorescent stains and microscopy interfaced with image processing make it possible to determine the size, shape, and numbers of organisms in the soil matrix (Harris and Paul, 1994). Several stains may be used for bacterial biomass estimates. 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF) is a fluorescent, anionic stain which gives low levels of background staining, producing images with good contrast (Bloem, 1995). DTAF binds covalently to neutral amino groups of proteins, specifically to cell surfaces. One disadvantage of using DTAF is that it is unstable and needs to be prepared fresh daily. Acridine orange (AO) is a fluorescent, cationic stain which gives high background staining, especially in clay and loam soils (Bloem, 1995; Paul and Clark, 1996). Phenolic aniline blue (PAB) is also a fluorescent stain which gives high background staining. For fluorescent stains, filters need to be used on the light microscope, for the fluorochromes to fluoresce. The excitation and emission wavelengths for light filters for DTAF, AO, and PAB are located in Table 1.1 (Paul et al., 1999; Carl Zeiss Microscopy, Germany).

The best method for viewing soil microbes is by soil smears. This is because there is less background staining and fading of fluorochromes, and the smears are flat allowing for better image analysis when compared to a filter. Smears can also be stored for up to a year in the dark at 2°C (Bloem, 1995).

Bacteria can be analyzed by computer-assisted image analysis or manual observation. Some benefits to computer-assisted image analysis include higher accuracy in enumeration of bacteria and higher consistency. Reduced photo-bleaching of the fluorochromes occurs by faster digital acquisition of the image (Paul et al., 1999). The

Table 1.1. Light filter requirements for fluorescent staining (Paul et al., 1999; Carl Zeiss Microscopy, Germany).

Stain	Excitation (nm)	Emission (nm)	Color
DTAF	450-490	525	green
AO	440-480	526	orange
PAB	455	600	blue

Center for Microbial Ecology at Michigan State University has a computer-assisted microscopy program, Center for Microbial Ecology Image Analysis System (CMEIAS). CMEIAS analyses can determine microbial abundance (bacterial cell density, biovolume, biomass C), morphological diversity (11 predefined cell shapes, 5 undefined cell shapes, cell size), and spatial distribution (nearest neighbor distances, cells/area, percent microbial cover) (Lui et al., 2001; Dazzo et al., 2003). It is believed that CMEIAS can be efficiently used to quantify bacterial morphotypes more accurately with less time required than by manual analyses.

REFERENCES

Aditjandra, K., E.J. Park, and A.J.M. Smucker. 2002. Tensile Strength Domains within Soil Aggregates from Contrasting Ecosystems. ASA Abstracts, Indianapolis, IN.

Beare, M.H., P.F. Hendrix, and D.C. Coleman. 1994. Water-stable aggregates and organic matter fractions in conventional- and no-tillage soils. Soil Sci. Soc. Am. J. 58:777-786.

Bending, G.D, M.K. Turner, and J.E. Jones. 2002. Interactions between crop residue and soil organic matter quality and the functional diversity of soil microbial communities. Soil Bio. Biochem. 34:1073-1082.

Blackwood, C.B. 2001. Spatial organization in soil bacterial communities. Ph.D. Dissertation. Michigan State University, East Lansing.

Blackwood, C.B., C.J. Dell, A.J.M. Smucker, and E.A. Paul. Eubacterial community response to position within soil macroaggregates and soil management. (*in preparation*).

Bloem, J. 1995. Fluorescent staining of microbes for total direct counts. Molecular Microbial Ecology Manual 4.1.8:1-12.

Brady, N.C. and R.R. Weil. 2002. The Nature and Properties of Soils. 13th ed. Prentice Hall, Upper Saddle River, NJ.

Christensen, S., S. Simkins, and J.M. Tiedje. 1990. Spatial variation in denitrification: Dependency of activity centers on the soil environment. Soil Sci. Soc. Am. J. 54:1608-1613.

Dazzo, F.B., J. Lui, A. Prabhu, C. Reddy, M. Wadekar, R. Peretz, R. Bollempalli, D. Trione, E. Marshall, J. Zurdo, H. Hammoud, J. Wang, M. Li, D. McGarrell, A. Gore, J. Maya-Flores, S. Gantner, and N. Hollingsworth. 2003. CMEIAS: Integrative software package to strengthen microscopy-based approaches for understanding microbial ecology. Kellogg Biological Station-Long Term Ecological Research All Scientists Meeting (KBS-LTER ASM), September 12, 2003. http://lter.kbs.msu.edu/Meetings/2003_All_Inv_Meeting/Abstracts/dazzo.htm.

Degens, B.P. 1998. Microbial functional diversity can be influenced by the addition of simple organic substrates to soil. Soil Biol. Biochem. 30:1981-1988.

Dell, C.J., A.J.M. Smucker, and E.A. Paul. 2004. Carbon mineralization from concentric layers of soil aggregates. (*submitted*).

Denef, K., J. Six, H. Bossuyt, S.D. Frey, E.T. Elliot, R. Merckx, and K. Paustian. 2001. Influence of dry-wet cycles on the interrelationship between aggregate, particulate organic matter, and microbial community dynamics. Soil Biol. Biochem. 33:1599-1611.

Dexter, A.R., and B. Kroesbergen. 1985. Methodology for determination of tensile strength of soil aggregates. J. Agric. Eng. Res. 31:139-147.

Edgerton, D.L., J.A. Harris, P. Birch, and P. Bullock. 1995. Linear relationship between aggregate stability and microbial biomass in three restored soils. Soil Bio. Biochem. 27:1499-1501.

Grundmann, G.L and P. Normand. 2000. Microscale diversity of the genus *Nitrobacter* in soil on the basis of analysis of genes encoding rRNA. Appl. Environ. Microbiol. 66:4543-4546.

Harris, D. and E.A. Paul. 1994. Measurement of bacterial growth rates in soil. Appl. Soil Eco. 1:277-290.

Hillel, D. 1998. Soil Structure and Aggregation. p. 101-125. *In* Environmental Soil Physics. Academic Press, San Diego, CA.

Horn, R. 1990. Aggregate characterization as compared to bulk soil properties. Soil Tillage Res. 17:265-289.

Kavdir, Y. 1996. Some physical and mineralogical characteristics of soil aggregates. Special Topic Research Project.

Kandeler, E. and E. Murer. 1993. Aggregate stability and soil microbial processes in a soil with different cultivation. Geoderma 56:503-513.

Kemper, W.D and R.C. Rosenau. 1986. Aggregate stability and size distribution. p. 425-442. *In* A. Klute (ed.) Methods of Soil Analysis. Part 1, Physical and Mineralogical Methods, 2nd edition. Soil Sci. Soc. Am., Madison, WI.

Lindahl, V. 1996. Improved soil dispersion procedures for total bacterial counts, extraction of indigenous bacteria and cell survival. J. Microbiol. Methods 25:279-286.

Loeffler, F.E., J.M. Tiedje, and R.A. Sanford. 1999. Fraction of electrons consumed in electron acceptor reduction and hydrogen thresholds as indicators of halorespiratory physiology. Appl. Environ. Microbiol. 65:4049-4056.

Lui, J., F.B. Dazzo, O.Glagoleva, B. Yu, and A.K. Jain. 2001. CMEIAS: A computer aided system for the image analysis of bacterial morphotypes in microbial communities. Microbial Ecology 41:173-194.

Monreal, C.M. and H. Kodama. 1997. Influence of aggregate architecture and minerals on living habitats and soil organic matter. Can. J. Soil Sci. 77:367-377.

Oades, J.M. 1993. The role of biology in the formation, stabilization, and degradation of soil structure. Geoderma 56:377-400.

Ogram, A. and K. Sharma. 2002. Methods of Soil Microbial Community Analysis. p. 554-563. *In* Manual for Environmental Microbiology, 2nd edition. American Society for Microbiology, Washington, D.C.

Park, E.J. and A.J.M. Smucker. 2003. Intra-aggregate porosity and saturated hydraulic conductivity of single aggregates in conventional-till, no-till agroecosystems, and native forest soils. Soil Sci. Soc. Am. J. *(in preparation).*

Park, E.J., K.L. Aditjandra, and A.J.M. Smucker. 2001. Spatial variability of porosity, tensile strength, and mineralogy within soil aggregates. ASA Abstracts, Charlotte, NC.

Paul, E.A and F.E. Clark. 1996. Methods for studying soil microorganisms. p. 35-68 *In* Soil Microbiology and Biochemistry, 2nd edition. Academic Press, San Diego, CA.

Paul, E.A., D. Harris, M.J. Klug, and R.W. Ruess. 1999. The determination of microbial biomass. p. 291-317. *In* Standard Soil Methods for Long-Term Ecological Research. Oxford University Press, New York, NY.

Peacock, A.D., M.D. Mullen, D.B. Ringelberg, D.D. Tyler, D.B. Hedrick, P.M. Gale, and D.C. White. 2001. Soil microbial community responses to dairy manure or ammonium nitrate applications. Soil Bio. Biochem. 33:1011-1019.

Rasse, D.P., A.J.M. Smucker, and D. Santos. 2000. Alfalfa root and shoot mulching effects on soil hydraulic properties and aggregation. Soil Sci. Soc. Am. J. 64:725-731.

Santos, D., S.L.S. Murphy, H. Taubner, A.J.M. Smucker, and R. Horn. 1997. Uniform separation of concentric surface layers from soil aggregates. Soil Sci. Soc. Am. J. 61:720-724.

Sexstone, A.J., N.P. Revsbech, T.B. Parkin, J.M. Tiedje. 1985. Direct measurement of oxygen profiles and denitrification rates in soil aggregates. Soil Sci. Soc. Am. J. 49:645.

Sissoko, F. 1997. Enhancement of soil aggregation by the combined influences of soil wetting and drying and root-microbial associations. M.S. Thesis. Michigan State University, East Lansing.

Smucker, A.J.M., D. Santos, and Y. Kavdir. 1996. Concentric layering of carbon, nitrogen, and clay minerals within soil aggregates from tilled and non-tilled agroecosystems and natural grasslands. Third Eastern Canada Soil Structure Workshop Proceedings. p. 129-140.

Smucker, A.J.M., D. Santos, Y. Kavdir, and E. Paul. 1998. Concentric gradients within stable soil aggregates. Proceedings of the 16th World Congress of Soil Science, Montpellier, France.

Tisdall, J.M. 1994. Possible role of microorganisms in aggregation of soils. Plant Soil 159:115-121.

Tisdall, J.M. and J.M. Oades. 1982. Organic matter and water-stable aggregates in soils. J. Soil Sci. 33:141-163.

Utomo, W.H. and A.R. Dexter. 1982. Changes in soil aggregate water stability induced by wetting and drying cycles in non-saturated soil. J. Soil Sci. 33:623-637.

CHAPTER 2

DEVELOPMENT AND RESULTS OF METHODS

In recent years, bacterial extraction techniques have included soil dispersion and preparation of samples on slides for viewing through microscopes. These techniques focused on bulk soil samples and required a 10g sample (Bloem, 1995; Paul et al., 1999). When working with soil aggregates, especially concentric layers of aggregates, the amount of soil per aggregate is limited in quantity (*e.g.*, on average a 4-6.3mm aggregate weighs 0.15g). This amount is decreased even more when identifying bacterial populations in concentric layers of the aggregate. Therefore, modifications of the above techniques are needed to allow for adequate extraction of bacteria from the small sample sizes associated with the exterior and interior regions of soil aggregates.

Soil Dispersion and Bacterial Extraction

Modifications to the bulk soil bacterial extraction process by Bloem (1995) and Paul et al. (1999) which required 10g samples was initiated by determining the minimal quantity of soil required to extract representative numbers of bacteria from concentric layers of aggregates. The aggregate size fraction selected for comparing bacterial and soil C analyses was 4-6.3mm across. Therefore, the average weight of whole aggregates was approximately 0.15g. Since the minimal weight needed was 10g for conventional extraction methods, 67 aggregates would be required. The number of aggregates needed to complete bacterial analyses on concentric aggregate layers (exterior 33% and interior 33%) would be 200 aggregates. Extracting bacteria from these many soil aggregates

created difficulties as too many samples, having contrasting and highly variable bacterial populations would be combined. Combining samples from large numbers of aggregates forfeited the possibility of identifying spatial variabilities of bacteria among soil aggregates.

A reasonable amount of soil from aggregates was defined as the amount that would give the best representation of the variability of the soil aggregates from that specific soil type. For instance, assuming that a 4-6.3mm aggregate weighs 0.15g, 3 whole aggregates would be needed to run statistical evaluations on the data collected. The total amount of soil would therefore be 0.45g. To make calculations simpler the weight was rounded up to 0.5g. So on average, 3-4 whole aggregates or 9-12 aggregates sub-divided into 3 concentric layers would supply the amount of soil needed for bacterial analyses on aggregates.

A dilution study was conducted to determine if the dilution rate of 1:200 of soil:distilled water was adequate for the smaller amount of soil processed. The efficacies of dilution rates of 1:100, 1:150, and 1:200 were determined by image analysis. The quantity of bacteria was used to calculate the extracted bacteria per gram of soil at each rate. These data are found in Figure 2.1 and show that the dilution study was linear. Another important part of the study was to observe the amount of overlapping of cells in the images acquired. In order for the image analysis system to accurately identify cells, the image must be segmented so that the individual cells are not touching. This overlapping of cells will result, for instance, in 2 cells being analyzed as one. To prevent this, the cells need to be separated during the editing step, before image analysis. This increases the amount of time spent on editing images. In the 1:200 dilution, little to no

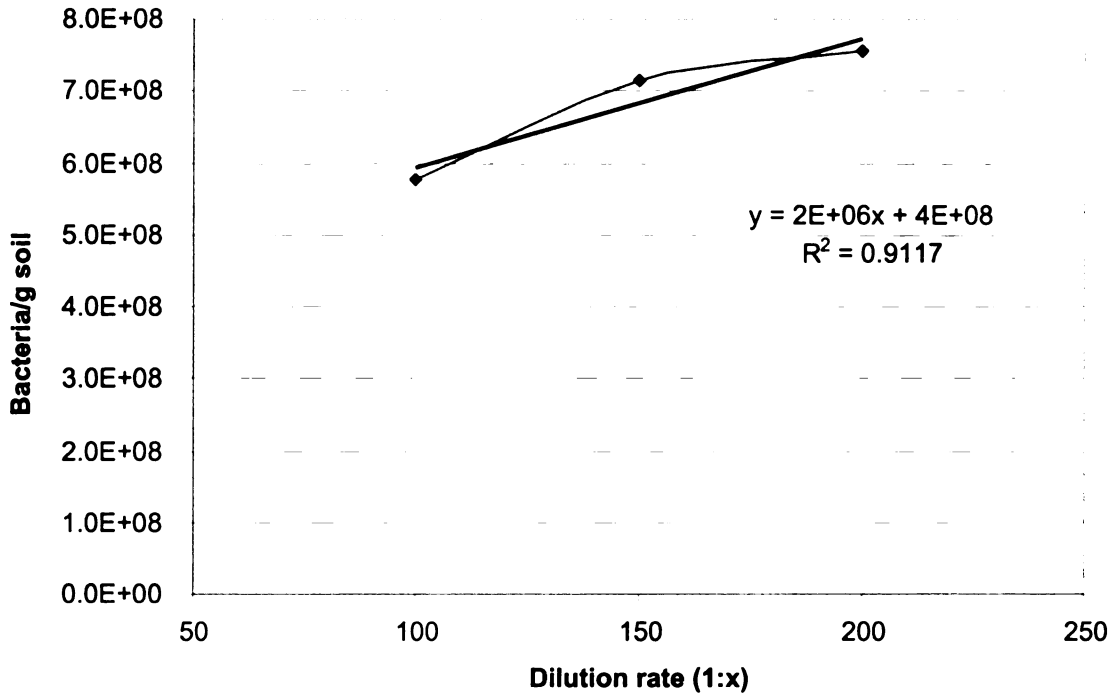

Figure 2.1. Extracted bacteria per gram of soil at three dilution rates (1:100, 1:150, 1:200) for 6.3-9.5mm native forest (NF) soil aggregates from a Hoytville clay loam (Fine, illitic, mesic Mollic Epiqualfs); n=3.

overlapping of cells was observed. However, with the lesser diluted samples (1:100 and 1:150) there were increased numbers of cells overlapping. The last parameter looked at in the dilution study was the mean biovolume of cells analyzed by CMEIAS. No significant differences in the standard errors of cell biovolumes per gram were observed across the three dilution rates (Figure 2.2). Therefore, dilutions of 1:200 were used in the soil dispersion and extraction of bacteria for identifying the spatial distributions of bacteria in soil aggregates.

The next step in adapting the soil bacterial extraction procedure of Bloem (1995) and Paul et al. (1999) was to replace the soil dispersion step of using a blender with another method such as vortexing or sonication. Dispersion of the small volumes of soil and water was not possible with a blender. Vortexing did a good job of mixing and suspending the soil particles into solution. However, vortexing did not break up the macro- and micro- aggregates and therefore did not adequately disperse the soil nor break apart microbe clusters. Dispersion by sonication was tested using the cup-holder attachment on the W-385 Sonicator (Heat Systems-Ultrasonics, Inc.). Sonicator settings were the following: continuous cycle time, 90% duty cycle, and output level 3. Sonication times of 0, 1, 10, 20, 30, 40, 50s were tested to evaluate the efficacy of sonication on soil dispersion. Through conversations with colleagues it was determined that continuous sonication for 2-3 minutes kills all microorganisms. The maximum number of whole, intact bacteria, with the least number of soil particles, was the desired result. By direct, manual counts on the microscope it was determined that the highest bacterial cell to soil particle ratio (by counting stained bacteria and stained soil particles) was by sonication for 10s (Figure 2.3). However, upon further observation it appeared

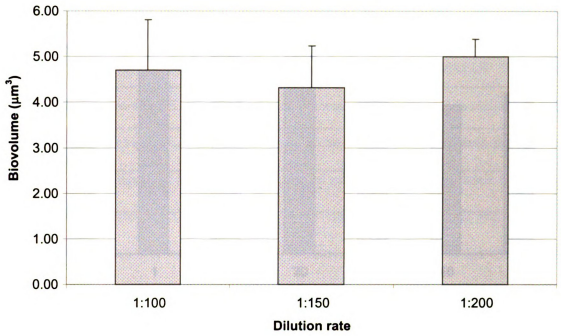

Figure 2.2. Mean cell biovolume measurements determined by CMEIAS-1 biovolume formula at three dilution rates (1:100, 1:150, 1:200 soil:distilled water) for 6.3-9.5mm native forest (NF) soil aggregates from a Hoytville clay loam (Fine, illitic, mesic Mollic Epiqualfs); n=3 (aggregates and images).

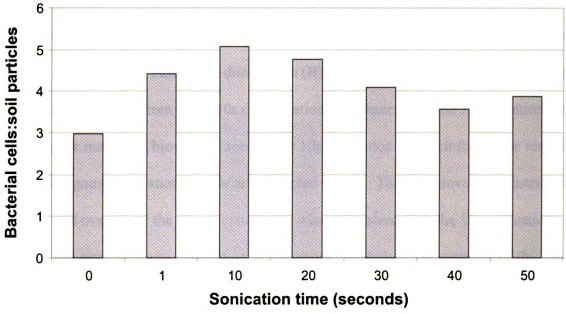

Figure 2.3. Ratio of bacterial cells to soil particles by direct count for a Kalamazoo loam (Fine-loamy, mixed, mesic Typic Hapludalf); n=3.

that some of the cells were disrupted. Therefore, an optimized set of data were collected after 5 and 10s of sonication to determine the highest mean cell biovolume measurements (Figure 2.4A) and cell biovolume distribution (B) as calculated by CMEIAS. The largest mean cell biovolume from 5 and 10s of sonication was observed at the 5s sonication time. The smaller mean cell biovolume seen at the 10s sonication time reinforces the idea that cells undergoing sonication for 10s are disrupted in part. The cell biovolume distribution is increased overall for the 5s sonication time when compared with the 10s sonication.

The next step was conducting a sedimentation study. The purpose of this study was to determine when the majority of soil particles had settled out of the top 5mL of solution. According to Stoke's law, $t = 6.97\eta/g(\rho_s-\rho_l)$, 64 minutes was required for soil particles 2μm in diameter to settle 1.55cm in distilled water at 25°C. It was assumed that the density of bacterial cells was less than the density of soil particles. A range of sedimentation times (15, 30, 45, 60, 64, 75, 90s) and one sonication time (5s) were evaluated (Figure 2.5). The best bacterial cell to soil ratio was observed at the 60 minute sedimentation with 5s sonication.

Staining

Several biological stains could be used in this experiment, however, the best stain requires a low background staining of soil particles and will adequately stain the bacteria present. Three stains were evaluated for the above characteristics: 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF), acridine orange (AO), and phenolic aniline blue (PAB). All are fluorescent stains. By direct microscopic, manual counts it was determined that DTAF gave the highest bacterial cell to soil particle ratio (Figure 2.6). The modified

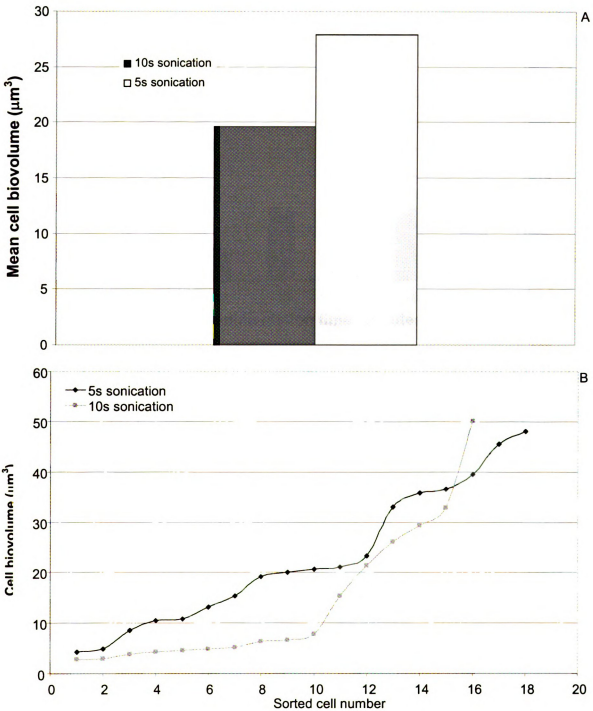

Figure 2.4. Mean cell biovolume measurements (A) and cell biovolume distribution (B) as determined by CMEIAS at 1:200 dilution and two sonication times (5s, 10s) for 6.3-9.5mm native forest (NF) soil aggregates from a Hoytville clay loam (Fine, illitic, mesic Mollic Epiqualfs); n=1 aggregate, n=3 images.

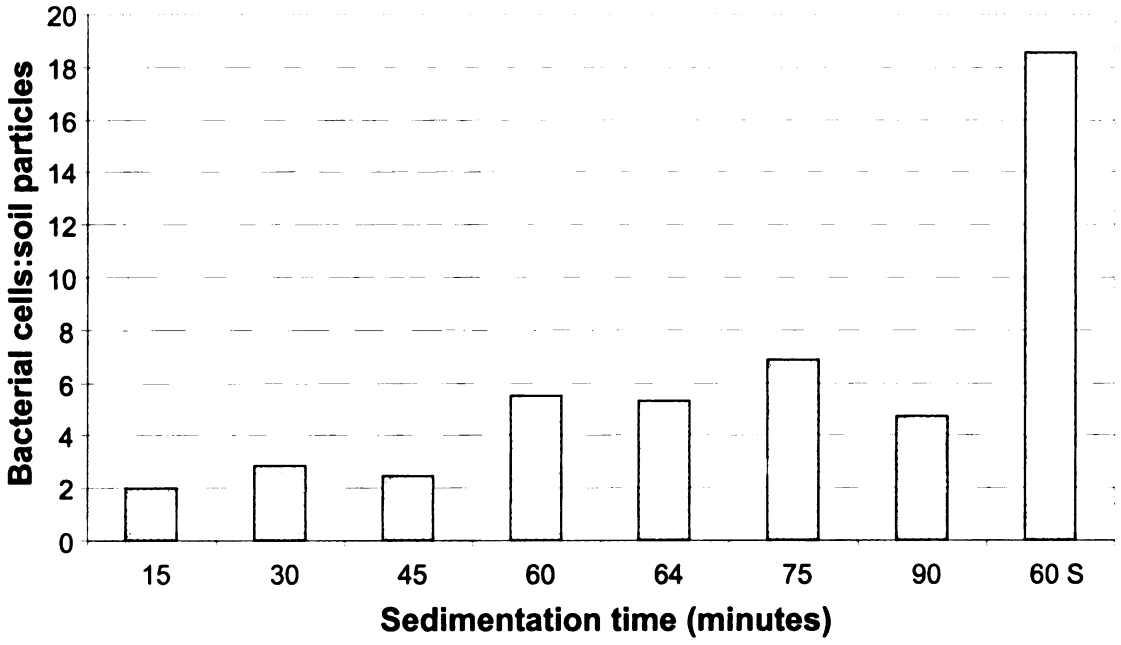

Figure 2.5. Effects of sedimentation time (15-90 minutes) and 5 second sonication (60 S) on the ratio of bacterial cells to soil particles for a Kalamazoo loam soil (Fine-loamy, mixed, mesic Typic Hapludalf); n=3. The 60S is the bacteria to soil ratio after sample has been exposed to 5s of sonication and sedimentation time is 60 minutes.

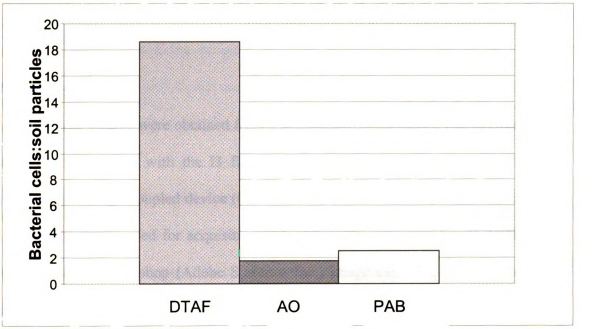

Figure 2.6. Ratio of bacterial cells to soil particles for a Kalamazoo loam (Fine-loamy, mixed, mesic Typic Hapludalf). Aggregates are of size fraction 4-6.3mm across, stained with 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF), acridine orange (AO), and phenolic aniline blue (PAB). Dilution of 1:200, sedimentation time of 60 min, sonication time of 5s; n=3 aggregates and images.

bacterial extraction from soils and staining procedures are located in Appendix A (Figure A.1).

Microscopy

Digital images were obtained from a fluorescence microscope (Leitz Orthoplan 2, Leitz, Inc., Germany) with the I3 filter for FTIC (fluorescein) stain and a 100x oil objective. A charge-coupled device (CCD) camera (Leica DC 500, Leica Microsystems AG, Germany) was used for acquisition of high resolution images (up to 3090 x 3600 pixels). Adobe Photoshop (Adobe Systems, Inc.) image capturing software on a Dell Pentium 4 PC, allowed for viewing the image before collection, and then saving the image as a Tif file before further editing. The image capture settings include the following: 8 bit color, 1030 x 1300 1 shot, always live, 20-30% brightness, and shutter speeds of 0.1 to 0.3 seconds.

Image Analysis (pre-CMEIAS)

Before image analysis with CMEIAS can occur, the original microscope image must undergo an editing process. This process has many steps (*e.g.*, changing brightness and contrast, making image grayscale, marking and outlining bacterial cells, embedding 10μm bar, increasing pixel resolution) which are outlined in Appendix A (Figure A.2). The amount of time spent on editing the images and preparing them for analysis with CMEIAS can range from 15 to 30 minutes, depending on the number of bacteria present in the image.

Image Analysis with CMEIAS

The Center for Microbial Ecology Image Analysis System (CMEIAS) is a computer-assisted microscopy program that can analyze digital images of microbial communities. CMEIAS v3.0 was used to analyze the images collected for this research. CMEIAS v3.0 has multiple analyses, some of which include: microbial abundance (*e.g.*, cell density, biomass C), morphological diversity (16 different cell shapes: 11 predefined and 5 undefined), and spatial distribution (*e.g.*, nearest neighbor distances, percent microbial cover) (Lui et al., 2001; Dazzo et al., 2003a). Recent developments with the object analysis measurement features include biovolume measurements. For the morphotypes recognized by CMEIAS v3.0, eight out of 18 microbial biovolume formulas have been identified by comparison to ground truth data obtained by volume displacement as most accurate to measure this parameter in all the morphotypes classified by CMEIAS. The most accurate biovolume formula (called the CMEIAS-1 biovolume formula) is adaptive to microbial cell shape (computed as object roundness) and also contains cell length, width, and area parameters whose computations are all described by Liu et al. (2001). Summary results of this formula are located in Table 2.1. CMEIAS morphotype analysis has an accuracy of 94.1% overall with individual accuracies located in Table 2.2.

CMEIAS has some requirements that need to be met for the best analyses and compilations of data. One of these requirements is a minimum of 30 objects per image. This requirement is to determine if adequate sampling has occurred. Another requirement is that the minimum pixel density of each object is 30 pixels. This requirement must be met for correct morphotype analysis. To be sure this requirement

Table 2.1. Summary results of CMEIAS-1 biovolume formula (Dazzo et al., 2003a; personal communication). Morphotype populations that were tested include cocci, spiral, U/curved rod, regular rod, unbranched filament, branched filament, rudimentary branched rod, prosthecate, ellipsoid, and club.

	Accuracy (%)
Overall morphotype populations	92.4
Overall communities with representation of all morphotypes	95.8
Computed overall accuracy	94.1

Table 2.2 Classification accuracy of specific morphotypes using CMEIAS (Lui et al., 2001). Accuracy test results of the CMEIAS-2 classifier using a large dataset of ground truth images (from Table 4 of Lui et al., 2001). Rows indicate true class labels; columns indicate CMEIAS classifier-assigned labels and numbers of correctly and incorrectly classified cells in each morphotype class. For example, CMEIAS classified 1212 of 1217 cocci correctly (99.6% accuracy), and incorrectly classified 4 cocci as regular rods and 1 cocci as an ellipsoid.

	Morphotype	A	B	C	D	E	F	G	H	I	J	K	Total	Accuracy (%)
A	Coccus	**1212**	0	0	0	4	0	1	0	0	0	0	**1217**	99.6
B	Spiral	0	**399**	1	0	0	6	0	0	1	0	1	**408**	97.8
C	Curved Rod	0	0	**156**	6	2	0	0	0	0	0	0	**164**	95.1
D	U-Shaped Rod	0	0	1	**132**	0	11	0	0	1	3	0	**148**	89.2
E	Regular Rod	24	0	13	0	**1100**	4	1	15	1	0	0	**1158**	95.0
F	Unbranched Filament	0	1	0	0	1	**240**	0	0	1	0	0	**243**	98.8
G	Ellipsoid	3	0	0	0	4	0	**130**	0	0	0	0	**137**	94.9
H	Club	0	0	0	0	3	0	4	**298**	0	4	0	**309**	96.4
I	Prostecate	0	0	0	0	0	0	0	0	**206**	0	0	**206**	100
J	Rudimentary Branched Rod	0	0	1	0	0	0	0	11	0	**138**	0	**150**	92.0
K	Branched Filament	0	0	0	0	0	0	0	0	0	0	**130**	**130**	100

was being met, one representative image from each soil type and treatment was analyzed for object area (pixels) and object sizes were greater than 30 pixels each and all objects found in each image were analyzed.

Image Analysis (post-CMEIAS)

After the collection of data from image analysis with CMEIAS, the data are available for statistical analysis. Two CMEIAS macros were used, working in Microsoft Excel, to prepare and compute the data obtained from the CMEIAS analyses (Dazzo et al., 2003a). The first macro used was the data preparation macro. This macro is used for both object analysis and classification data. It prepares the data for ecological statistical analyses by compiling, concatenating, and graphically displaying the data. Examples of the output data of this macro include the frequency distribution (Figure 2.7A), % frequency distribution (B), and % cumulative frequency distribution (C). The second macro used was the sampling statistics macro. This is the analysis where the 30 objects per image minimum is required. This macro analyzes datasets each containing multiple images, to determine whether the sampling sizes (cumulative number of objects) are sufficient for computing the morphological diversity indices in the microbial communities. Examples of the output data of this macro include the operational morphological unit (OMU) richness by % abundance (Figure 2.8), and Shannon's and Simpson's diversity indices (Towner, 1999) and 95% tolerance envelopes (Dazzo et al., 2003).

The number of bacteria extracted per gram soil can be calculated with a minimum amount of information from the microscopy and image analysis processes (Paul and

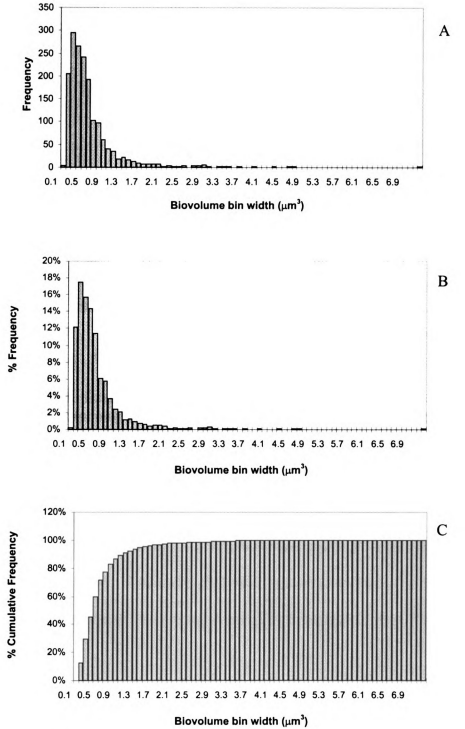

Figure 2.7. Example of CMEIAS data preparation macro; A=frequency distribution, B=% frequency distribution, and C=% cumulative frequency distribution. Cell biovolume frequency distributions of the exteriors of two field replications of Wooster native forest (NF) aggregates of size fractions 4-6.3mm; n=16-17 aggregates, 10 images each replication.

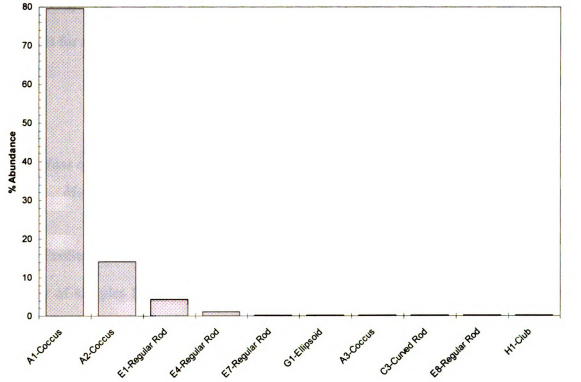

Figure 2.8. Example of CMEIAS sampling statistics macro. Relative rank abundance plot of morphological diversity in Wooster native forest (NF) aggregates of size fractions 4-6.3mm. The letter-number label corresponds to the operational morphological unit (morphotype size) or morphological diversity classified by CMEIAS (Dazzo et al., 2003); n=16 aggregates, 10 images.

Clark, 1996). The following formulas were used to calculate the number of bacteria per gram soil for a 1:200 dilution and microscopy with 1000x total magnification:

$$\textit{Area of image: (0.135mm)} \times \textit{(0.106mm)} = 0.01431mm^2$$

$$\textit{Area of smear (well): } \pi r^2 = 28.27mm^2$$

$$\textit{Image area/smear (well) area: (0.01431)/(28.27)} = 0.00051$$

$$\textit{Mass of soil/smear (well): (4/1000)} \times \textit{(0.495/99.495)} = 0.00002g \textit{ soil/smear}$$

$$\textit{Mass of soil/image: (0.00002)} \times \textit{(0.00051)} = 1.01 \times 10^{-8} g \textit{ soil/image}$$

$$\textit{Bacteria/g soil: \# per image/1.01} \times 10^{-8}$$

EcoStat, an ecological analysis program (Towner, 1999), analyzes community diversity of samples from the data computed from the above CMEIAS macros. Several diversity indices can be used when comparing two or more communities (*e.g.*, proportional similarity (%), Jaccard coefficient, Sorenson coefficient, Morisita index, Horn index, Sneath/Sokal distance, Bray/Curtis distance, and Chord distance). The community similarity index selected for these studies was the proportional similarity (%) index, based on the OMU richness and the % frequency biovolume distributions of abundance. The proportional similarity (%) index is a simple measure which incorporates information on species abundances based on the proportion or percentage of total community abundance that each species comprises. It gives the percentage of similarity between the communities under comparison (Figure 2.9).

Conclusion

Extraction of bacteria from concentric aggregate layers include efficient sequential removal of exterior vs. interior aggregate soil layers by the SAE chamber system, sonication of soil samples for 5 seconds, sedimentation of soil particles to remove clay-sized minerals, diluting the bacterial solution to 1:200, and preparing the

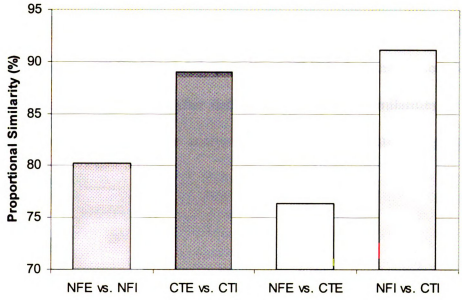

Figure 2.9. Example of CMEIAS data analyzed in EcoStat; proportional similarity (%) of interiors (I) and exteriors (E) of Wooster native forest (NF) and conventional-till (CT) aggregates of size fractions 4-6.3mm across; n=13-17 aggregates, 10 images each treatment.

slides and staining samples with DTAF for microscopic evaluation. Each of these parameters was optimized for this study. Additional current innovations with image analysis systems (CMEIAS) and data analyses (CMEIAS macros) allow for more data to be acquired and analyzed. After determining that data, similarities or differences of bacterial communities can be analyzed with ecological statistical programs, such as EcoStat.

REFERENCES

Aditjandra, K., E.J. Park, and A.J.M. Smucker. 2002. Tensile Strength Domains within Soil Aggregates from Contrasting Ecosystems. ASA Abstracts, Indianapolis, IN.

Bloem, J. 1995. Fluorescent staining of microbes for total direct counts. Molecular Microbial Ecology Manual 4.1.8:1-12.

Dazzo, F.B., J. Lui, A. Prabhu, C. Reddy, M. Wadekar, R. Peretz, R. Bollempalli, D. Trione, E. Marshall, J. Zurdo, H. Hammoud, J. Wang, M. Li, D. McGarrell, A. Gore, J. Maya-Flores, S. Gantner, and N. Hollingsworth. 2003a. CMEIAS: Integrative software package to strengthen microscopy-based approaches for understanding microbial ecology. Kellogg Biological Station-Long Term Ecological Research All Scientists Meeting (KBS-LTER ASM), September 12, 2003. http://lter.kbs.msu.edu/Meetings/2003_All_Inv_Meeting/Abstracts/dazzo.htm.

Dazzo, F., J. Liu, B. Yu, O. Glagoleva, A. Jain, F.I. Liu, E. Marshall, D. Trione, and J. Zurdo. 2003b. CMEIAS Version 1.27 Operator Manual: Computer-Assisted Microscopy and Digital Image Analysis Software to Strengthen Microscopy-Based Approaches for Understanding Microbial Ecology

Lui, J., F.B. Dazzo, O.Glagoleva, B. Yu, and A.K. Jain. 2001. CMEIAS: a computer-aided system for the image analysis of bacterial morphotypes in microbial communities. Microbial Ecology 41:173-194.

Paul, E.A., D. Harris, M.J. Klug, and R.W. Ruess. 1999. The determination of microbial biomass. p. 291-317. *In* Standard Soil Methods for Long-Term Ecological Research. Oxford University Press, New York, NY.

Towner, H. 1999. EcoStat Ecological Analysis Program for Windows, Ver 1.02, Trinity Software, Compton, NH, Internet site: http://www.trinitysoftware.com/lifesci/index.html.

CHAPTER 3

SOIL BACTERIAL COMMUNITY DIVERSITY IN CONCENTRIC AGGREGATE LAYERS AS INFLUENCED BY SOIL TYPE AND MANAGEMENT PRACTICE

INTRODUCTION

Microorganisms promote soil aggregation processes by providing glue and cementing agents, promote plant growth through symbiotic relationships (*e.g.*, N-fixing bacteria, mycorrhizal fungi, and plant growth-promoting rhizobacteria), control root diseases (biocontrol agents), fix N (free-living N-fixing bacteria), assimilate plant nutrients bound in organic matter (OM) or soil minerals (P from rock phosphate), and degrade pollutants. Microbial diversity within aggregates has been reported to be as large as the microbial diversity identified across extensive geographical areas (Grundmann and Normand, 2000). This localized diversity provides a huge resource of multifunctional microbial communities. Soil aggregation processes modify microhabitats located in soil aggregates which are thought to further modify the microbial communities located in these regions. Bacterial extraction along with fluorescence microscopy and computer-assisted image analysis allow for quantification of changes in bacterial populations and communities associated with the above microhabitats in external and internal regions of soil aggregates.

The objective of this study was to quantify changes in bacterial populations and community diversities within concentric layers of soil aggregates from three tillage and plant management systems.

MATERIALS AND METHODS

Site Description and Soils

Samples of soil were collected from surface soils of a native forest (NF) site, long-term conventional-tillage (CT) site, native grassland (NG) succession site, and a conventional tillage planted to continuous alfalfa (CT-CA) site. The NF site and long-term CT site are located on a Wooster silt loam (Fine-loamy, mixed, mesic Oxyaquic Fragiudalfs) at the Ohio Agricultural Research and Development Center (OARDC) near Wooster, OH. The long-term CT site has been under CT management for the past 100 years. The NG succession site is located on a Kalamazoo loam (Fine-loamy, mixed, mesic Typic Hapludalf) at the Michigan State University Kellogg Biological Station (KBS), a NSF Long Term Ecological Research Station in Hickory Corners, MI. The CT-CA site is located on a Ternary sandy loam (Sandy loam, mixed, frigid Alfic Haplorthodes) at the Michigan Agricultural Experiment Station in Chatham, MI. Samples were taken for the NF and CT sites in the spring of 2003 from the surface 0-5cm. Samples were taken from the NG and CT-CA sites in the summer of 2002 from the surface 0-10cm. Additional samples were taken from the NG site in September of 2003 for comparison analyses of moist and air-dry aggregates. All samples were stored field moist in plastic containers prior to arrival in the lab. Soils were stored at 4°C until processing occurred. Soils were air-dried and manually sieved prior to further analyses. Sieved aggregate size fractions include >9.5, 6.3-9.5, 4-6.3, 2-4, 1-2, 0.5-1, <0.5mm in diameter. The aggregate sizes from all field sites used in this study were from the 4-6.3mm fraction.

Bacterial Analyses (Preparation, Microscopy, and Image Analysis)

Bacterial extraction techniques for bulk soil samples have been developed to allow for soil dispersion and preparation of bacterial samples on slides for computer-assisted microscopy and image analysis (Bloem, 1995; Paul et al., 1999). Modifications of the above techniques were needed before small temporal and spatial changes in bacterial populations and community diversities, modified by intra-aggregate microhabitats at external and internal regions within soil macro-aggregates were quantified (Appendix A, Figure A.1). Soil dispersion and bacterial extraction procedures were modified by first decreasing the amount of soil used from 10g to 0.5g. Soil dispersion by blending was replaced with continuous sonication (W-385 Sonicator, Heat Systems-Ultrasonics, Inc.) for 5s. Sedimentation was optimized by Stoke's law to determine that after 42 minutes, 2μm soil particles had settled out of the top 5mL of solution used to prepare microscope slides. It was assumed the density of bacteria was less than the density of soil particles at the same size. The biological fluorescent stain 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF) was determined to give the least background staining by having the highest bacterial cell to soil particle ratio when compared with acridine orange (AO) and phenolic aniline blue, also fluorescent stains. Digital images were obtained from a fluorescence microscope (Leitz Orthoplan 2, Leitz Inc., Germany) with the I3 filter for FTIC (fluorescein) stain and a 100x oil objective. A charge-coupled device (CCD) camera (Leica DC 500, Leica Microsystems AG, Germany) was used for acquisition of high resolution images (up to 3090 x 3600 pixels). Adobe Photoshop (version 7, Adobe Systems, Inc.) image capturing software on a Dell Pentium 4 PC allowed for viewing the image before collection, and then saving the image

as a Tif file before further editing. The image capture settings include the following: 8 bit color, 1030 x 1300, single shot, always live, at a brightness of 20-30%, and a shutter speed of 0.1-0.3 seconds. Editing of images in Photoshop was required before image analysis to obtain a binary image with the background being lighter than the foreground objects (Appendix A, Figure A.2). Image analysis with the Center for Microbial Ecology Image Analysis System (CMEIAS), a computer-assisted microscopy program, analyses digital images of microbial communities for individual cell size and shape. Biovolume measurements were calculated using the CMEIAS-1 biovolume formula, which is adaptive to microbial cell shape (computed as object roundness) and also contains cell length, width, and area parameters whose computations are all described by Liu et al. (2001). CMEIAS-1 biovolume formula has an overall accuracy of 94.1% with all CMEIAS morphotypes represented (Dazzo et al., 2003). The data preparation macro developed for CMEIAS, working in Microsoft Excel, prepares the data collected for ecological statistical analyses by compiling, concatenating, and graphically displaying the data (Dazzo et al., 2003). Output data of this macro includes frequency distribution, % frequency distribution, and cumulative frequency distribution graphs of the data selected. For this study, cell biovolume distributions were identified. Extracted bacteria per gram soil were calculated from microscope images and cell counts from CMEIAS analysis (Paul and Clark, 1996).

Aggregate Analyses

Soil aggregate analyses parameters for this study include wet sieving water stability, polar tensile strength (PTS), intra-aggregate porosity, and dry combustible total

C and N concentrations. Aggregate stability, mean weight diameter (MWD), was determined by the wet sieving technique with nebulization of dry aggregates to prevent the breakage of most aggregates during the wetting process (Kemper and Rosenau, 1986). Aggregate strength was determined by the polar tensile strength technique. The polar tensile strength of a soil aggregate is the force per unit area that is required to break the aggregate (Dexter and Kroesbergen, 1985; Hillel, 1998). Porosity was calculated from determining the bulk densities of soil aggregates by the Saran method (Blake and Hartge, 1986). Total C and N analyses were determined by combustion at 800°C of small quantities of soil (30mg soil) in a Carlo-Erba CHN analyzer, model NA1500.

Statistical Analysis

Bacterial community diversity was analyzed using EcoStat (Towner, 1999), an ecological analysis program that is commonly used to perform many statistical tests including the evaluation of species diversity and community similarity analyses of many ecosystems. The input data from CMEIAS included the % frequency distributions of cell biovolumes.

RESULTS AND DISCUSSION

Bacterial Populations

Greater populations of bacteria (23%, 24%, 18%, and 7%) were observed in the exterior regions of NF, CT, NG, and CT-CA aggregates when compared with the corresponding interiors (Figure 3.1). Exterior regions of NF aggregates contained the highest numbers of bacteria (8.35E+10), while the CT-CA interiors had the least

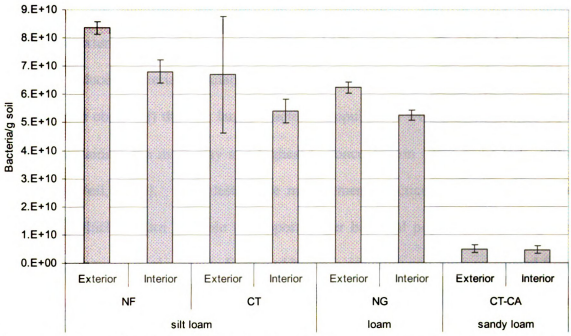

Figure 3.1. Declining populations of bacteria per gram of exterior and interior soil layers as soil textures become more coarse. Soil samples from a Wooster silt loam (Fine-loamy, mixed, mesic Oxyaquic Fragiudalfs) native forest (NF) were sampled from the surface 0-5cm, a Kalamazoo loam (Fine-loamy, mixed, mesic Typic Hapludalf) native grassland (NG) succession and a Ternary sandy loam (Sandy loam, mixed, frigid Alfic Haplorthodes) conventional-till planted to continuous alfalfa (CT-CA) were sampled from the surface 0-10cm, from soil aggregate size fractions 4-6.3mm across; n=7-21 aggregates, n=20-30 images per layer (dependent on number of field replications), n=446-1687 cumulative cells sampled. Bars indicate standard deviations.

(4.66E+09). No significant differences in the quantities of bacteria were observed between the exterior and interior regions of CT and NF aggregates. One hundred years of tillage has reduced bacterial populations to 25% below that of the native forest. It was expected (and observed) that the largest bacterial populations would be associated with the soils containing the most clay and highest C concentration (Paul and Clark, 1996; Brady and Weil, 2002). Less destructive management practices lead to more stable aggregates, which in turn are able to support larger bacterial populations (Young and Ritz, 2000; Lupwayi et al., 2001). Extracted bacteria per gram soil were calculated from microscope images and cell counts from CMEIAS analysis. A 35% increase in bacteria per gram soil was observed in the moist vs. air dry NG aggregates (Figure 3.2). This indicates that air drying significantly reduces bacterial populations extracted from soil aggregates.

Cell Biovolume Measurements

Microbial activity and aggregate stability are linearly related (Edgerton et al, 1995). Microbial biovolume evaluations give insight into the strength and stability of soil aggregates. Mean cell biovolume measurements were calculated by CMEIAS using CMEIAS-1 biovolume formula developed by Dazzo et al. (2003). This formula is adaptive to cell shape using roundness shape measurements and also contains cell length, width, and area parameters as described by Liu et al. (2001). CMEIAS-1 biovolume formula has an overall accuracy of 94.1% with all CMEIAS morphotypes represented (Dazzo et al., 2003). In contrasts to populations, no differences were observed between the mean cell biovolumes of moist and air dry soil aggregates from NG treatments

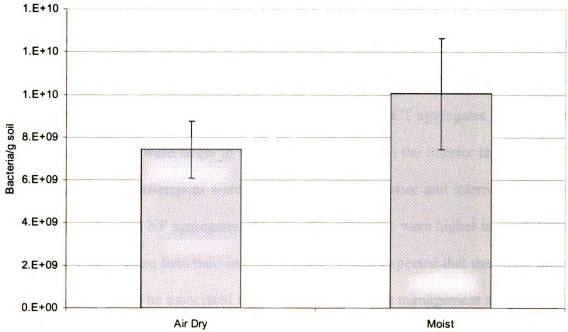

Figure 3.2. Extracted bacteria are significantly lower in cell density from air dry vs. moist whole aggregates from a Kalamazoo loam (Fine-loamy, mixed, mesic Typic Hapludalf) native grassland (NG) succession from soil aggregate size fractions 4-6.3mm across, sampled from the surface 0-10cm; n=3 aggregates, n=20 images, n=446-1687 cumulative cells sampled. Bars indicate standard deviations.

(Figure 3.3). These results indicate that sampling air dry aggregates or moist aggregates at field water contents had minimal affects on the bacterial community when considering their cell biovolumes. Mean cell biovolumes were largest in the NG aggregates, next largest in the CT-CA aggregates, and smallest in the NF and CT aggregates (Figure 3.4). Mean cell biovolumes were larger in the exterior regions than the interior regions of NG aggregates, while no differences were observed between exterior and interior regions of CT-CA aggregates and NF aggregates. Mean cell biovolumes were higher in the exterior regions of CT aggregates than their interior regions. It was expected that the larger mean cell biovolumes would be associated with the least disruptive management system, (*e.g.*, NF), finer soil type, (*e.g.*, clay loam), and exterior regions of soil aggregates.

Cell Biovolume Distributions

The distribution of cell biovolume frequencies present bacterial community comparisons based on cell size compositions among bacterial communities. Cell biovolume frequencies are calculated by the CMEIAS data preparation macro, that compiles, concatenates, and graphically presents the CMEIAS data. Sorted cell biovolume distributions allow for comparisons of numbers of cells analyzed and the biovolume of those cells. Increased numbers of cells were observed in the moist NG aggregates, while the biovolumes of those cells were smaller than the cell biovolumes observed in the air-dry NG aggregates (Figure 3.5). Cell numbers varied between the exterior and interior regions of NG aggregates from 3 field replications (Figure 3.6) and CT-CA aggregates from 2 field replications (Figure 3.7), though no significant differences were seen in the corresponding cell biovolumes. Cell biovolumes were

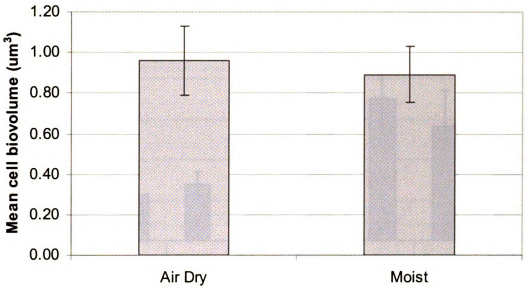

Figure 3.3. Mean cell biovolume measurements determined by CMEIAS-1 biovolume formula for air dry and moist whole aggregates from Kalamazoo loam (Fine-loamy, mixed, mesic Typic Hapludalf) native grassland (NG) succession from soil aggregate size fractions 4-6.3mm across, sampled from the surface 0-10cm,; n=3 aggregates, n=20 images, n=750-1014 cumulative cells sampled. Bars indicate standard deviations.

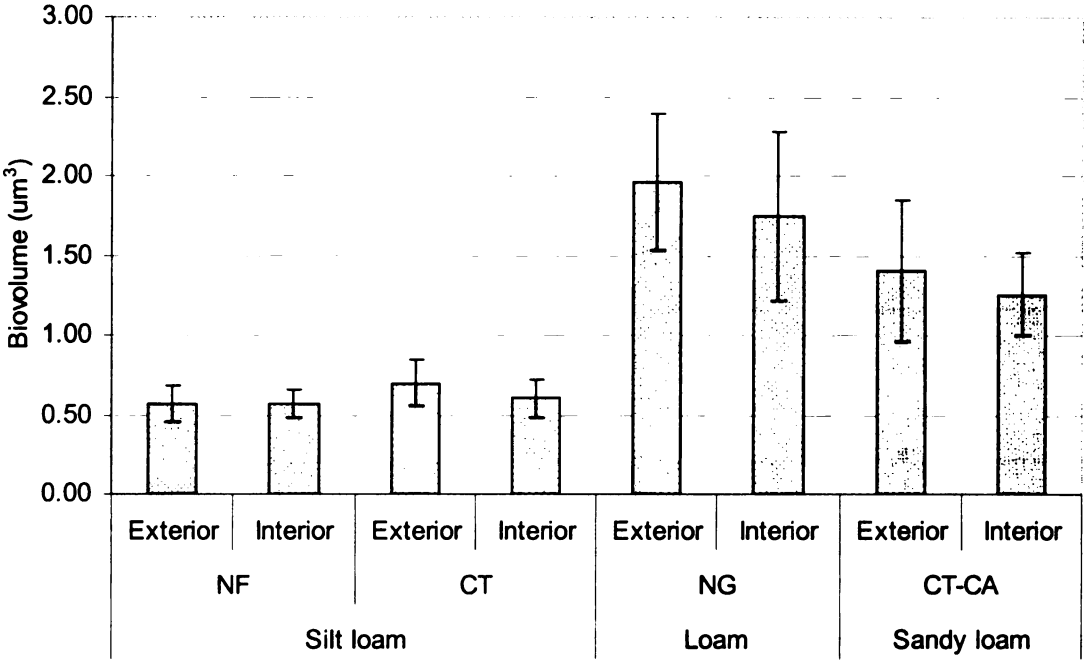

Figure 3.4. Mean cell biovolume measurements determined by CMEIAS-1 biovolume formula for exterior (E) and interior (I) layers of Wooster silt loam (Fine-loamy, mixed, mesic Oxyaquic Fragiudalfs) native forest (NF) aggregates, taken from the surface 0-5cm, Kalamazoo loam (Fine-loamy, mixed, mesic Typic Hapludalf) native grassland (NG) succession aggregates and Ternary sandy loam (Sandy loam, mixed, frigid Alfic Haplorthodes) conventional-till planted to continuous alfalfa (CT-CA) aggregates from soil aggregate size fractions 4-6.3mm across, sampled from the surface 0-10cm; n=7-21 aggregates, n=20-30 images per layer (dependent on number of field replications), n=446-1687 cumulative cells sampled. Bars indicate standard deviations.

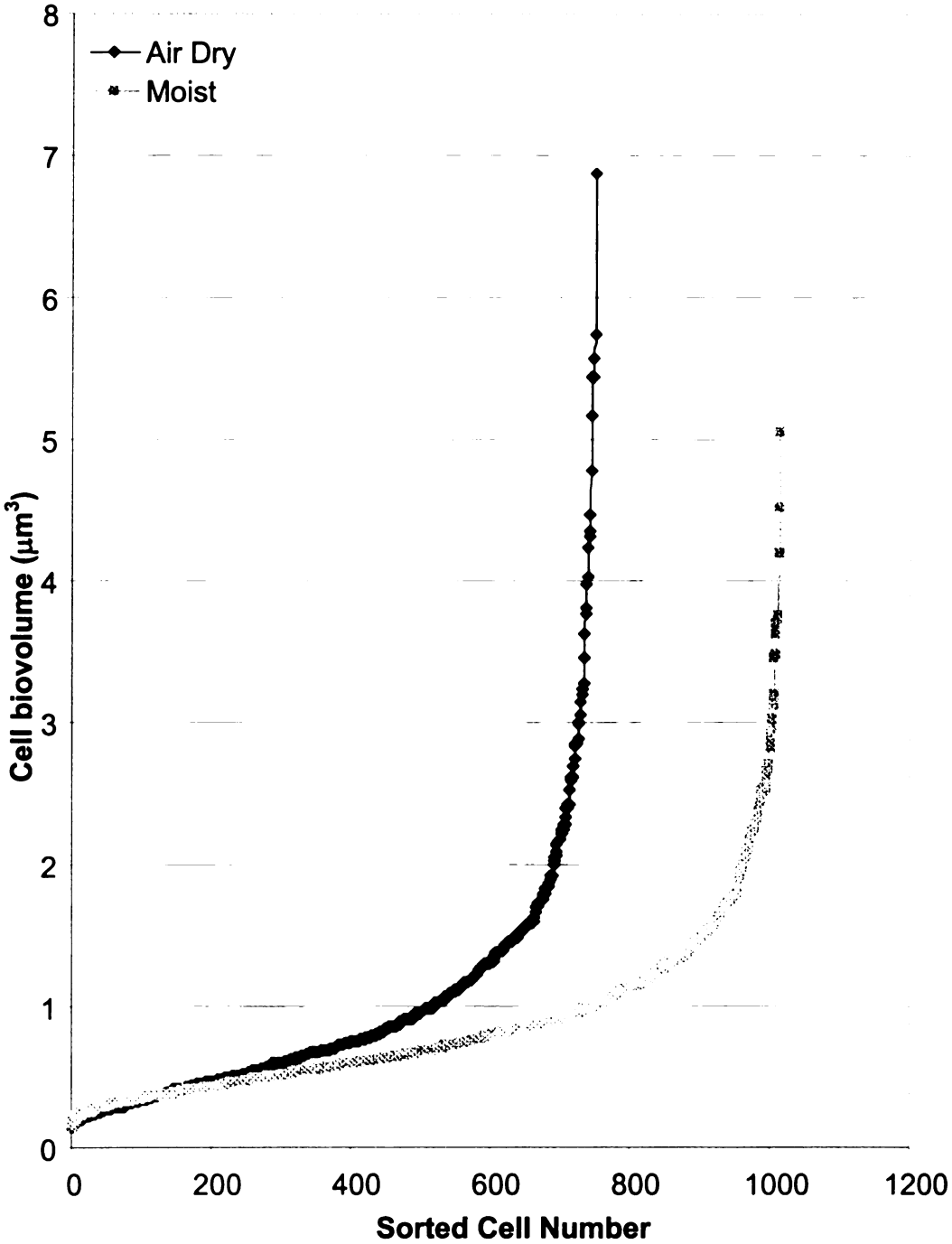

Figure 3.5. Increased numbers of cells were observed in moist aggregates, while a larger biovolume was observed in the air dry aggregates. The distribution of cell biovolumes were calculated by the CMEIAS data preparation macro for moist and air dry whole aggregates from Kalamazoo loam (Fine-loamy, mixed, mesic Typic Hapludalf) native grassland (NG) succession from soil aggregate size fractions 4-6.3mm across, sampled from the surface 0-10cm; n=3-4 aggregates, n=10 images per treatment, n=750-1014 cumulative cells sampled.

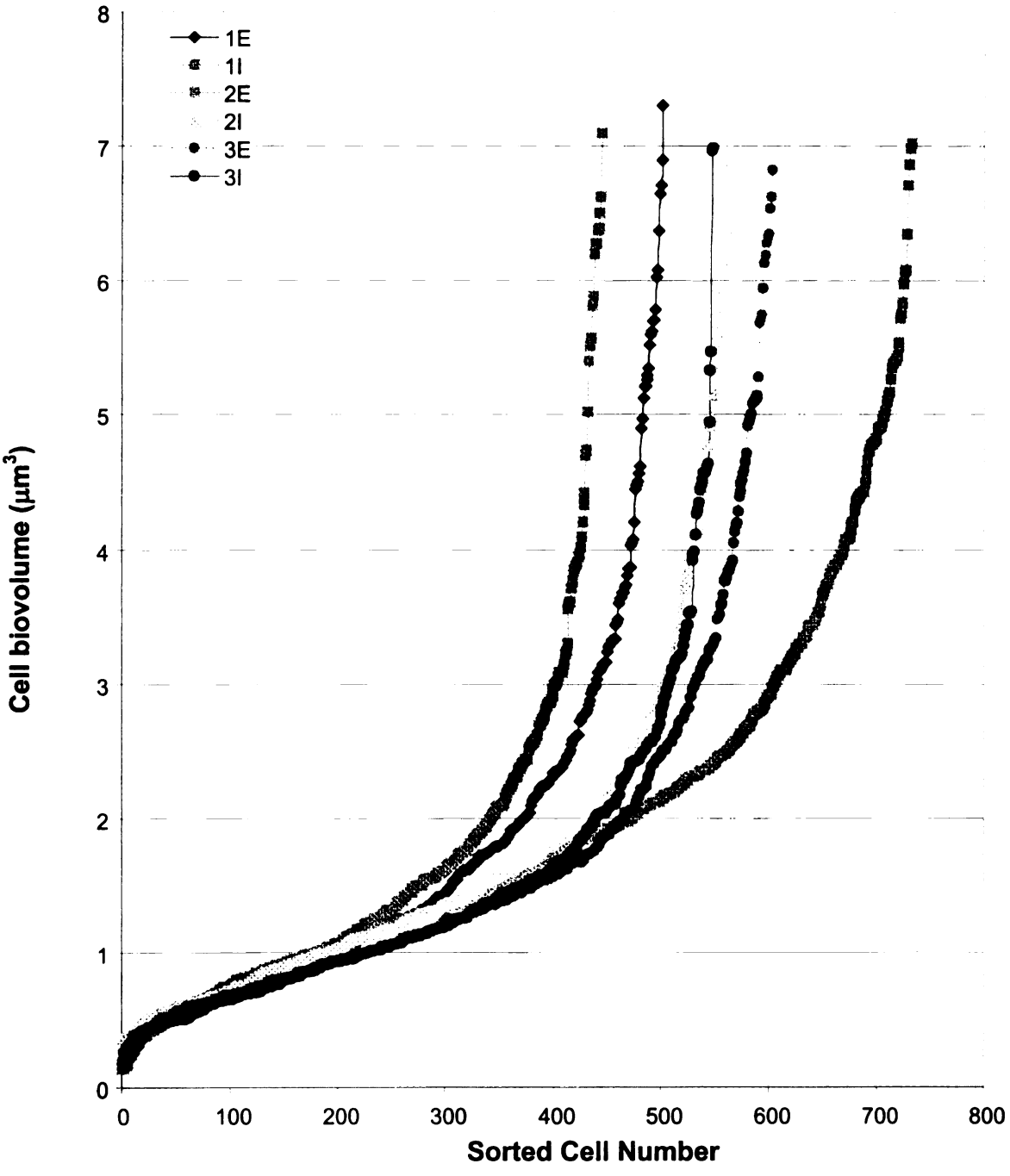

Figure 3.6. Increased numbers of cells were observed in the exteriors of aggregates from 3 field replications when compared to their interiors. Biovolumes of these cells showed no significant differences between regions of the aggregate or field replication. The distribution of cell biovolumes were calculated by the CMEIAS data preparation macro for exterior (E) and interior (I) layers of aggregates from 3 field replications (1,2,3) of Kalamazoo loam (Fine-loamy, mixed, mesic Typic Hapludalf) native grassland (NG) succession from soil aggregate size fractions 4-6.3mm across, sampled from the surface 0-10cm; n=16-21 aggregates, n=10 images per replication and layer, n=446-732 cumulative cells sampled.

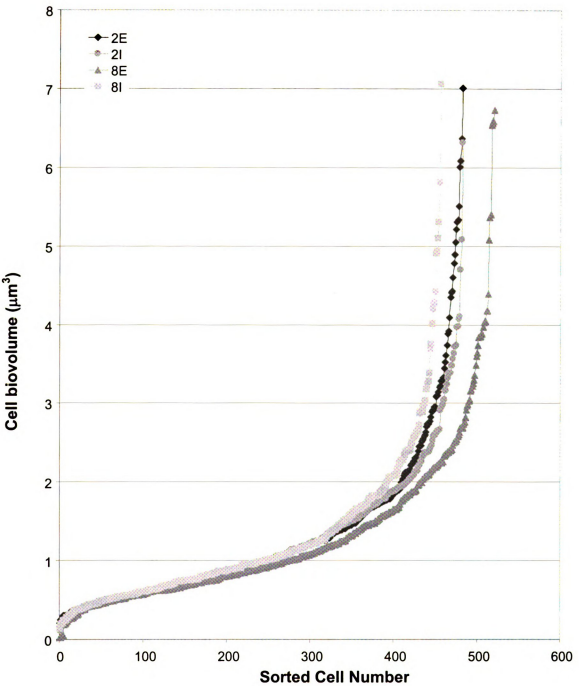

Figure 3.7. Biovolumes of these cells showed no significant differences between regions of the aggregate or field replication. The distribution of cell biovolumes were calculated by the CMEIAS data preparation macro for exterior (E) and interior (I) layers of aggregates from 2 field replications (2,8) of Ternary sandy loam (Sandy loam, mixed, frigid Alfic Haplorthodes) conventional-till planted to continuous alfalfa (CT-CA) from soil aggregate size fractions 4-6.3mm across, sampled from the surface 0-10cm; n=7-10 aggregates, n=10 images per replication and layer, n=457-520 cumulative cells sampled.

similar for NF aggregates and the exterior regions of CT aggregates, with the interior regions of CT aggregates having smaller cell biovolumes (Figure 3.8).

Community diversity statistics include various indices for comparing and contrasting bacterial communities. For this study proportional similarity (%) was determined, based on the OMU richness and the % frequency biovolume distributions of abundance. The proportional similarity (%) index is a simple measure which incorporates information on species abundances based on the proportion or percentage of total community abundance that each species comprises.

comparison. Proportional similarity (%) was calculated using the cell biovolume frequencies calculated by the CMEIAS data preparation macro. Community similarities around 85% or more were not considered to be significantly different in morphological diversity. The differences between proportional similarities among compared communities cannot be labeled as increased or decreased cell biovolumes. For this interpretation and summary, the bacterial cell biovolume frequencies give greater insight as bacterial populations and their potential metabolic activities are compared, as metabolically active cells are larger.

Bacterial cell biovolume frequency distributions, using percent abundance, give reasonable estimates of bacterial community comparisons of the most abundant size class or mode (personal communication with Dr. Dazzo). Cell biovolumes of bacterial communities were 13.5% greater in moist vs. air dry NG aggregates (Figure 3.9). Therefore it appears that community diversities determined by cell biovolumes can possibly be used to identify accumulative or historic changes in soil water content changes within soil aggregates.

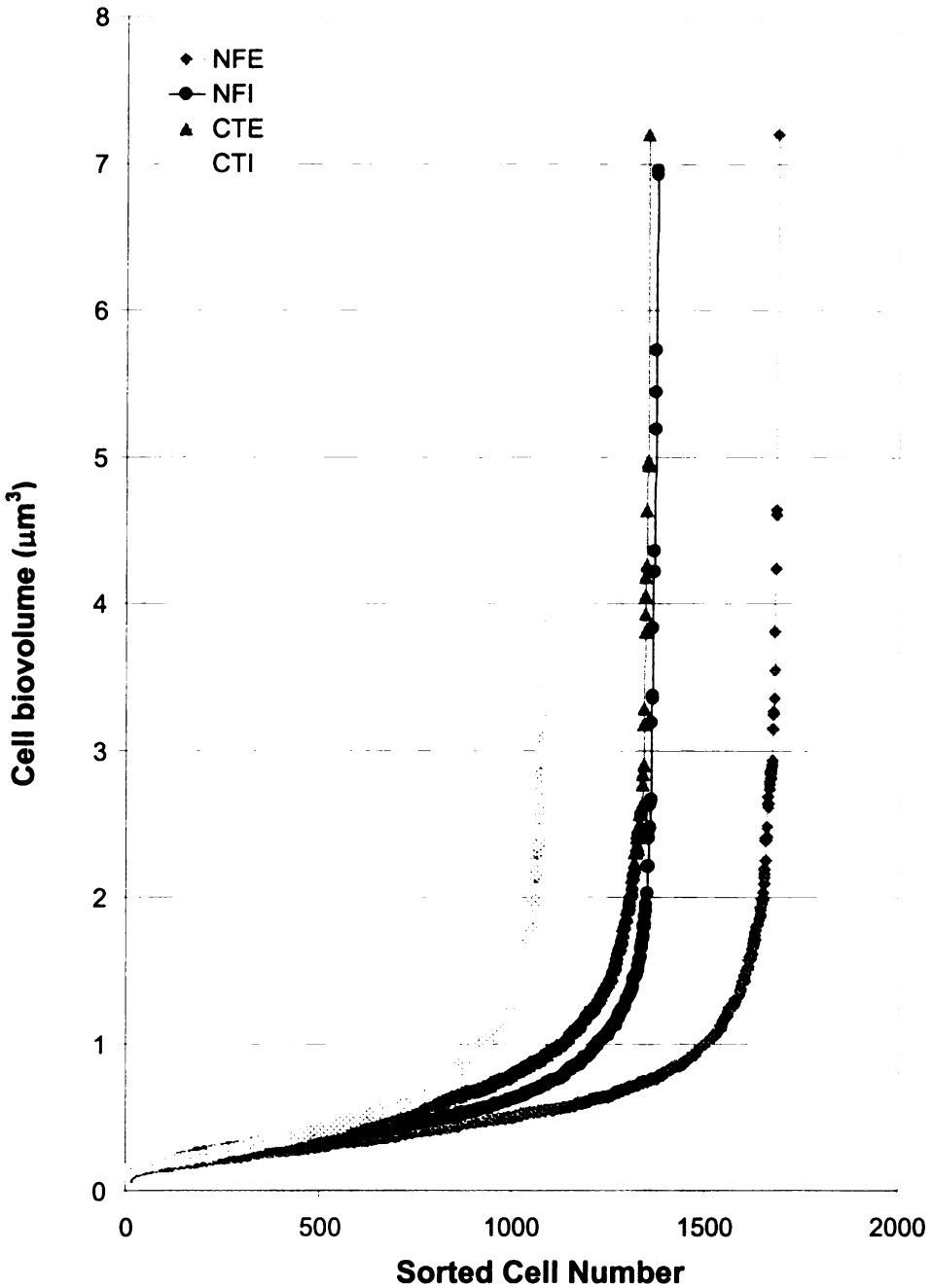

Figure 3.8. Increased numbers of cells were observed in NF aggregates compared to CT aggregates, with the exteriors of the aggregates having increased numbers than their interiors. Cell biovolumes were decreased in the interior region of CT aggregates. The distribution of cell biovolumes were calculated by the CMEIAS data preparation macro for exterior (E) and interior (I) layers of aggregates from 2 field replications (2,8) of Wooster silt loam (Fine-loamy, mixed, mesic Oxyaquic Fragiudalfs) native forest (NF) and conventional-till (CT) from soil aggregate size fractions 4-6.3mm across, sampled from the surface 0-5cm; n=13-17 aggregates, n=10 images per management system and layer, n=1091-1687 cumulative cells sampled.

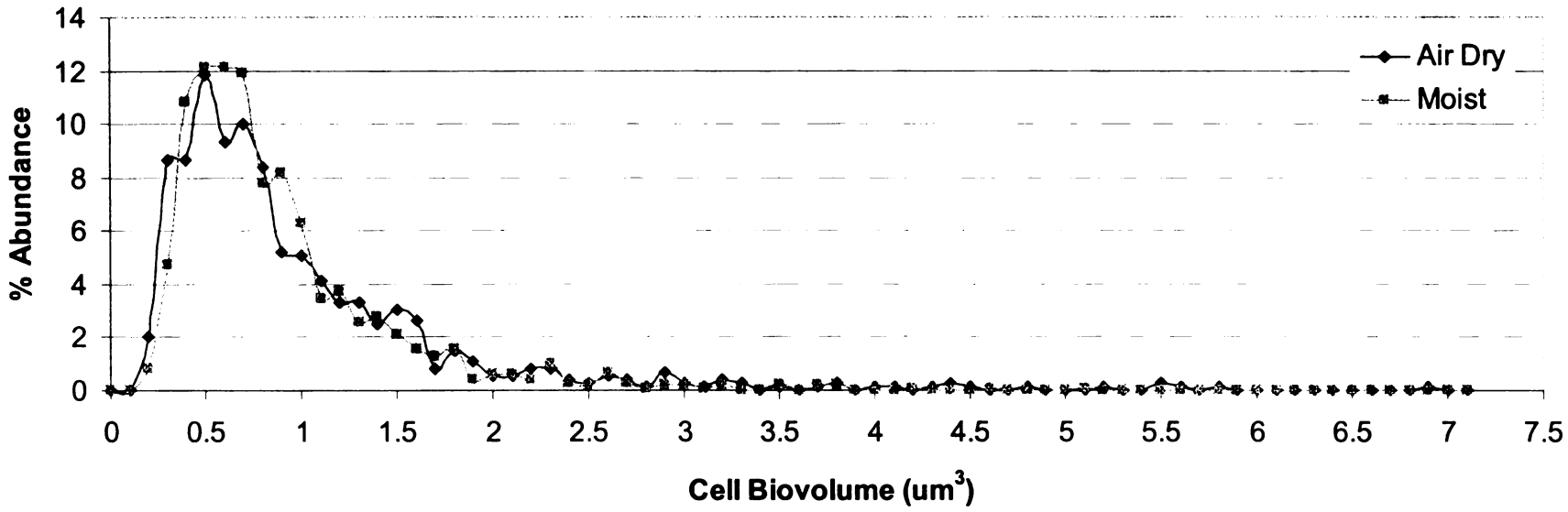

Figure 3.9. Increased cell biovolumes were observed in the moist vs. air dry whole aggregates from Kalamazoo loam (Fine-loamy, mixed, mesic Typic Hapludalf) native grassland (NG) succession from soil aggregate size fraction 4-6.3mm across, sampled from the surface 0-10cm. Biovolume frequency distributions were calculated using a constant bin width of $0.1um^3$ by the CMEIAS data preparation macro and by EcoStat; n=3 aggregates, n=20 images, n=750-1014 cumulative cells sampled.

Bacterial cell biovolume distributions in exterior layers were 15%, 18%, and 16% smaller than interior regions of NG aggregates from three field replications, respectively (Figure 3.10). Bacterial cell biovolume distributions were 21%, 16%, and 21% different in external layers between field replications, 1-2, 1-3, and 2-3, respectively, while cell biovolume diversities for interior layers between field replications were contrasted by 19%, 21%, and 20%. Cell biovolumes located in the exterior regions followed the trend (largest to smallest) of rep3 > rep1 > rep2, while cell biovolumes located in the interior regions followed the trend of rep3 > rep2> rep1. Field replication 2 was least similar to replications 1 and 3. This indicates that the cell size distribution within bacterial communities could differ considerably across the landscape as indicated by these contrasts among soil aggregates from three field replications, and supports the idea that microbial diversity within aggregates are as great as the microbial diversity identified across extensive geographical areas (Grundmann and Normand, 2000).

Bacterial cell biovolume distributions in exterior layers were the same (*e.g.*, 13%) as the interior regions of CT-CA aggregates from two field replications, 2 and 8 (Figure 3.11). Bacterial cell biovolume distributions contrasted by 12% within exterior layers and 16% within interior layers between these two field replications, demonstrating small spatial variabilities in the bacterial community between field replications.

Bacterial cell biovolume distributions were 23% larger in exterior layers (Figure 3.12A) and not significantly larger (9%) in the interior regions (Figure 3.12B) of CT and NF aggregates, indicating greater differences in exterior layer communities between management systems. Bacterial cell biovolume distributions in exterior layers were 20% smaller than interior regions of NF aggregates (Figure 3.12C) and 11% larger in exteriors

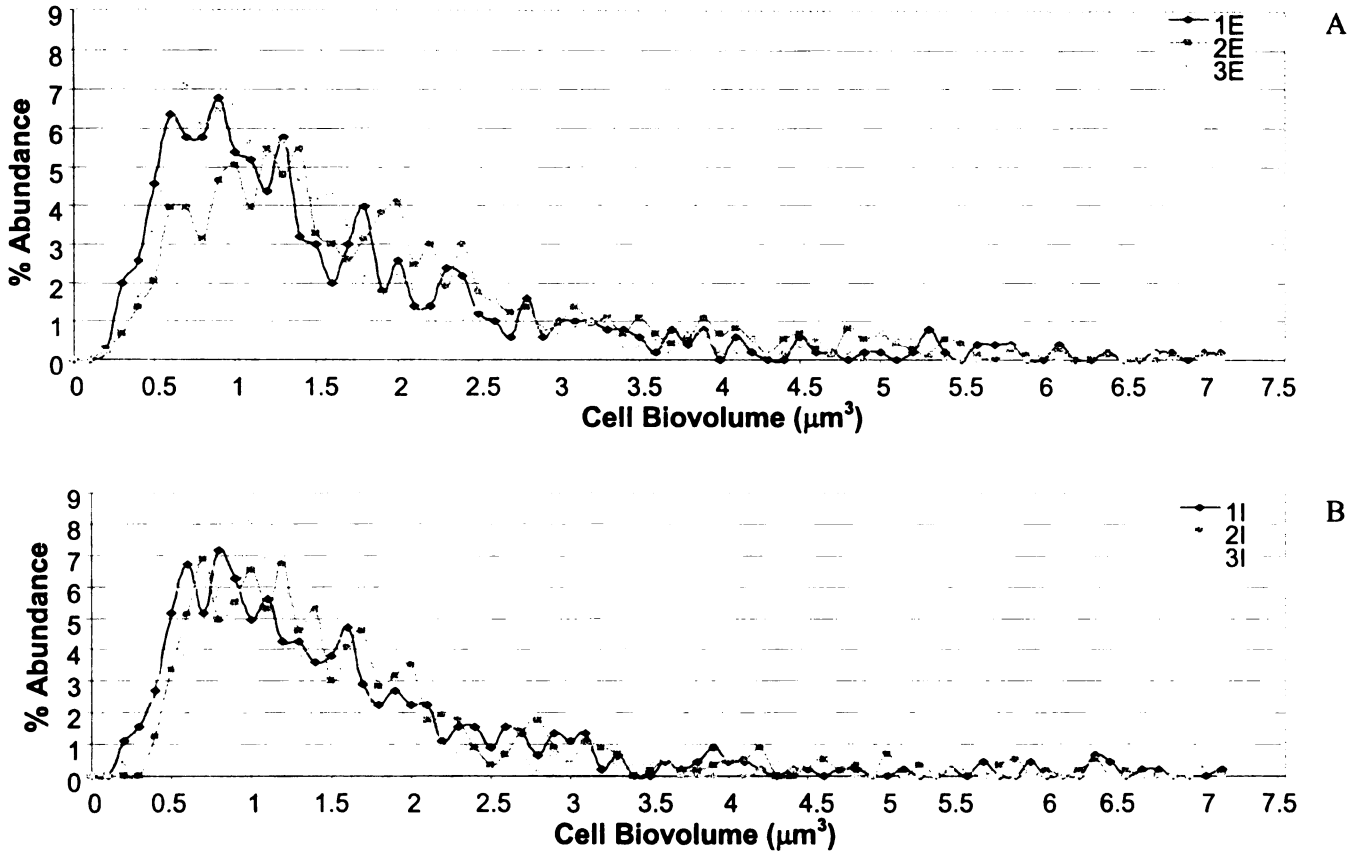

Figure 3.10. Increased cell biovolume frequencies in the exteriors (E) (A) of aggregates from 3 field replications (1,2,3) were observed following the trend (largest to smallest) rep3 > rep1 > rep2, while interiors (I) (B) were rep3 > rep2> rep1, from Kalamazoo loam (Fine-loamy, mixed, mesic Typic Hapludalf) native grassland (NG) succession from soil aggregate size fraction 4-6.3mm across, sampled from the surface 0-10cm. Biovolume frequency distributions were calculated using a constant bin width of 0.1um^3 by the CMEIAS data preparation macro and by EcoStat; n=16-21 aggregates, n=10 images per replication and layer, n=446-732 cumulative cells sampled.

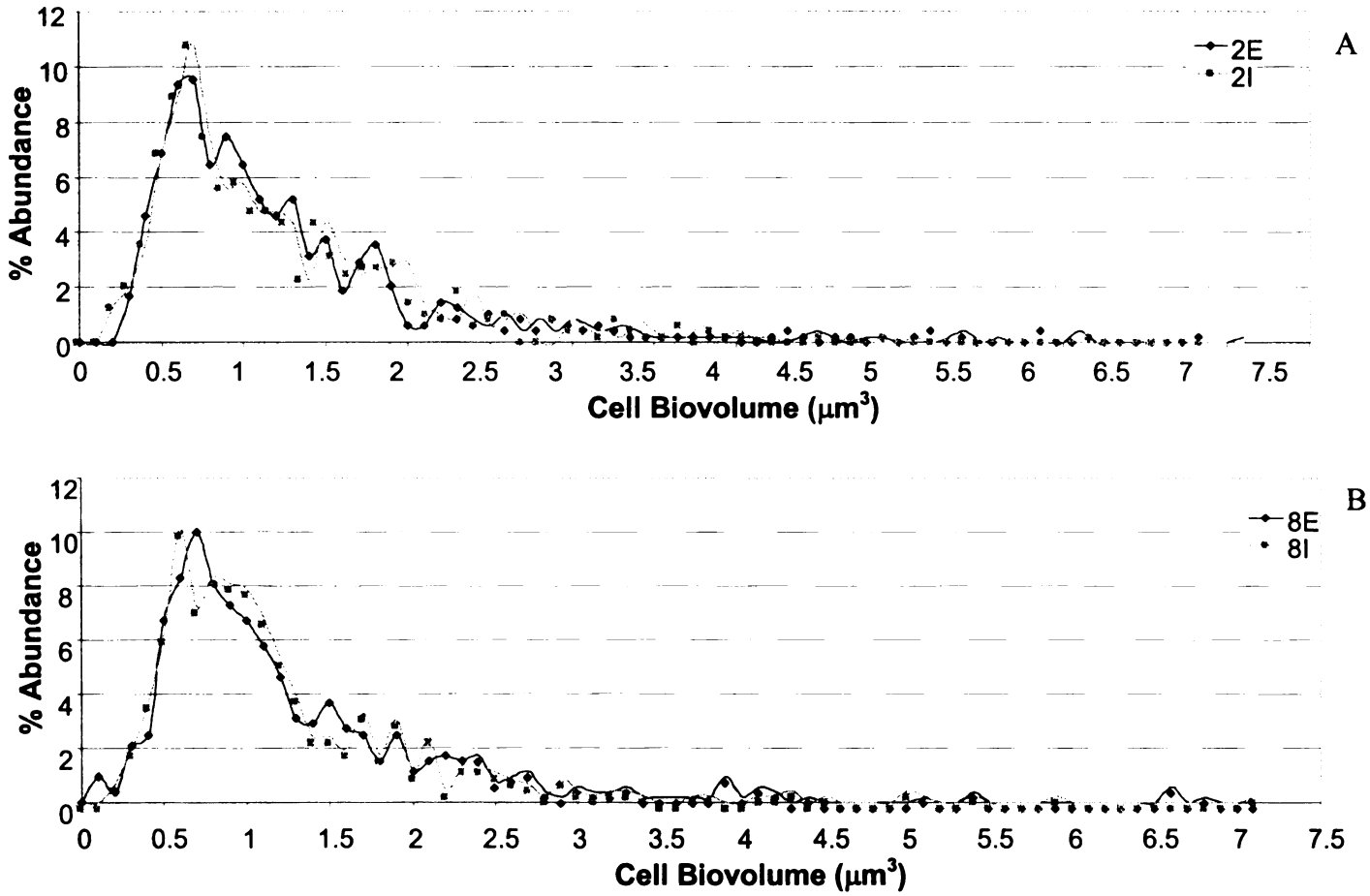

Figure 3.11. Increased cell biovolume frequencies were observed in the interiors (I) vs. exteriors (E) of 2 field replications [2(A),8(B)] from Ternary sandy loam (Sandy loam, mixed, frigid Alfic Haplorthodes) conventional-till planted to continuous alfalfa (CT-CA) from soil aggregate size fractions 4-6.3mm across, sampled from the surface 0-10cm. Biovolume frequency distributions were calculated using a constant bin width of 0.1um^3 by the CMEIAS data preparation macro and by EcoStat; n=7-10 aggregates, n=10 images per replication and layer, n=457-520 cumulative cells sampled.

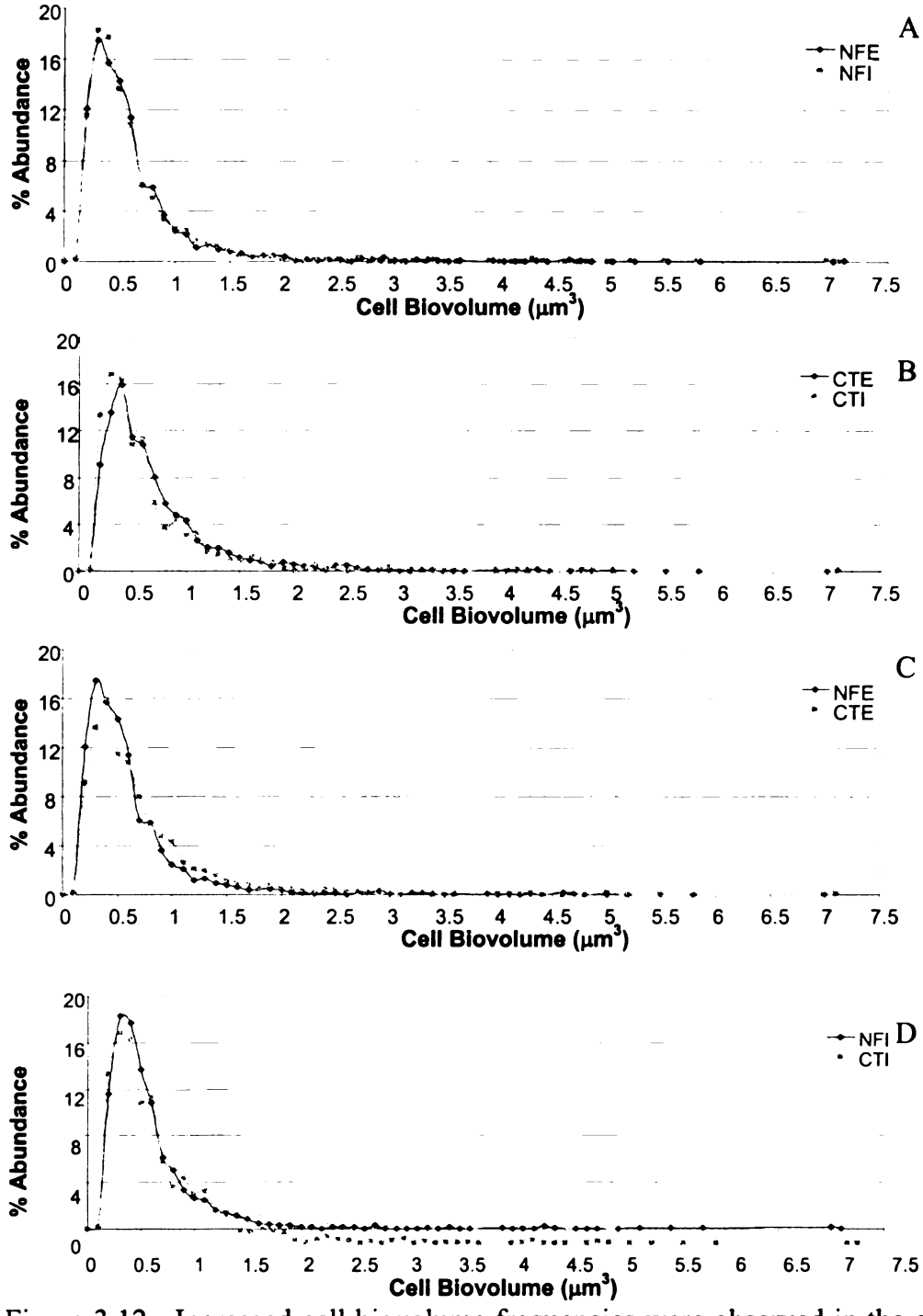

Figure 3.12. Increased cell biovolume frequencies were observed in the exteriors (E) of CT vs. NF aggregates (C), while slight increases were seen in the interiors (D). Decreased cell biovolume frequencies were observed in the exteriors vs. interiors (I) of NF aggregates and increased for CT aggregates from Wooster silt loam (Fine-loamy, mixed, mesic Oxyaquic Fragiudalfs) native forest (NF) (A) and conventional-till (CT) (B) from soil aggregate size fractions 4-6.3mm across, sampled from the surface 0-5cm. Biovolume frequency distributions were calculated using a constant bin width of 0.1um^3 by the CMEIAS data preparation macro and by EcoStat; n=13-17 aggregates, n=10 images per management system and layer, 1091-1687 cumulative cells sampled.

vs. interiors of CT aggregates (Figure 3.12D). This indicates that the cell size distributions reflect modifications in the structure and/or physiological state of these bacterial communities *in situ*. These modifications among bacterial communities within soil aggregates are thought to contribute to the stability of the aggregate. These reported spatial gradients of changes in the distribution of biovolume diversities of bacteria within soil aggregates contributes to previously reported contributions by bacteria and their associated metabolic byproducts (Tisdall, 1994; Paul and Clark, 1996). Native forest aggregates were observed to have similar cell biovolume distributions between field replications (1,2) of exterior and interior regions (Figure 3.13A and B), while increased variability of field replications was observed for exterior and interior regions of CT aggregates (Figure 3.13C and D).

A summary of the bacterial data for these soils is located in Appendix A (Table A.1). These data include object counts per soil and management practice, extracted bacteria per gram of soil, and biovolume measurements of mean, median, mode, skewness, minimum, and maximum.

Aggregate Stability

Mean weight diameter evaluations, by wet sieving, were much higher for soil aggregates from NF treatments than CT treatments of the Wooster silt loam (Figure 3.14). Therefore it was concluded that 100 years of tillage significantly reduced the water stability of soil aggregates. Additional wet sieving comparisons of soil aggregates from native grasslands (NG) on Kalamazoo loam soils demonstrated similar MWD with NF treatments on Wooster silt loams soils. Conventional tillage (CT) treatments of a

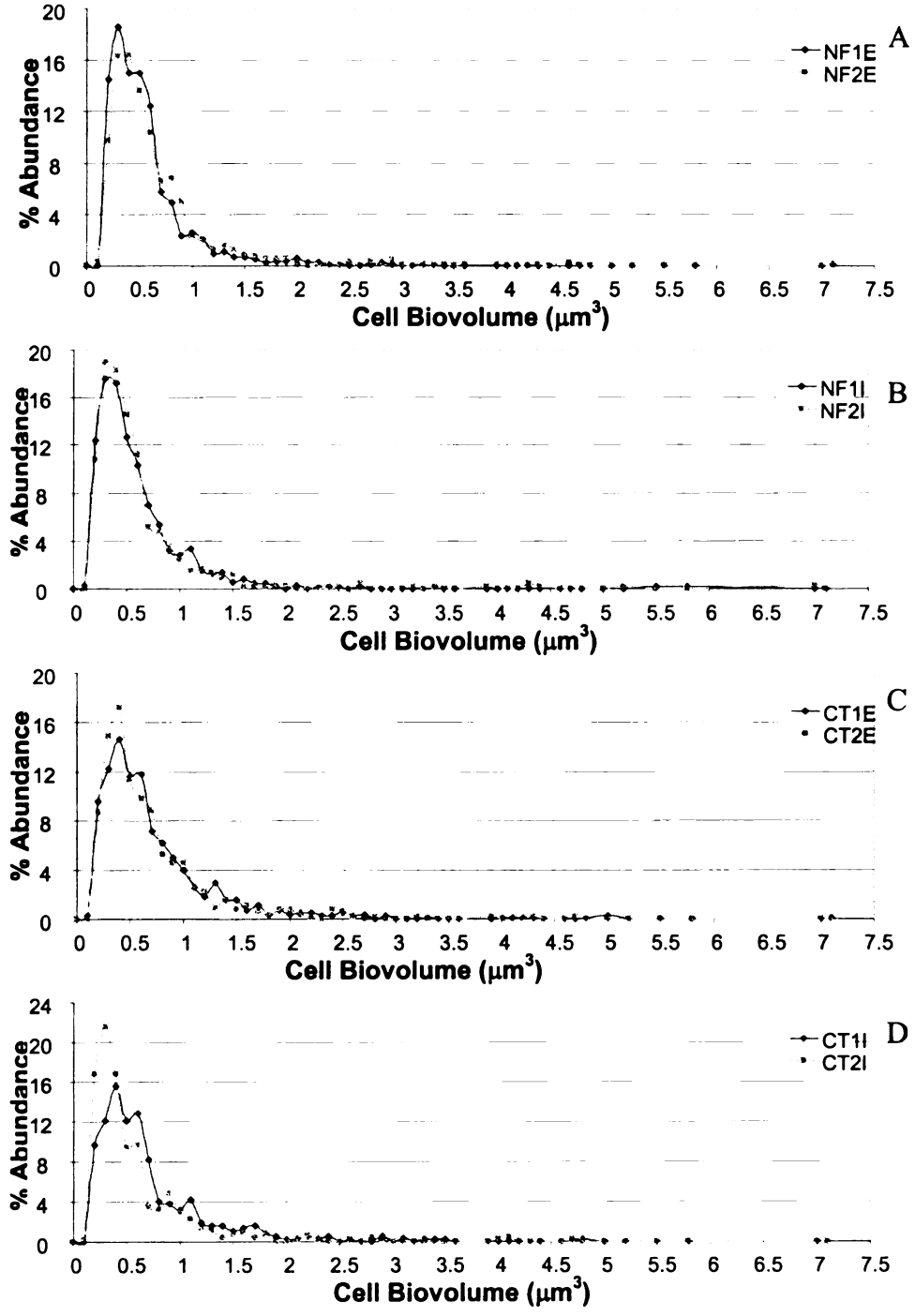

Figure 3.13. Similar cell biovolume distributions were observed between field replications (1,2) of exterior (E) and interior (I) regions of NF aggregates (A,B), while increased variability of field replications was observed for exterior and interior regions of CT aggregates (C,D) from Wooster silt loam (Fine-loamy, mixed, mesic Oxyaquic Fragiudalfs) native forest (NF) (A,B) and conventional-till (CT) (C,D) from soil aggregate size fractions 4-6.3mm across, sampled from the surface 0-5cm. Biovolume frequency distributions were calculated using a constant bin width of 0.1um^3 by the CMEIAS data preparation macro and by EcoStat; n=13-17 aggregates, n=10 images per management system and layer, n=1091-1687 cumulative cell sampled.

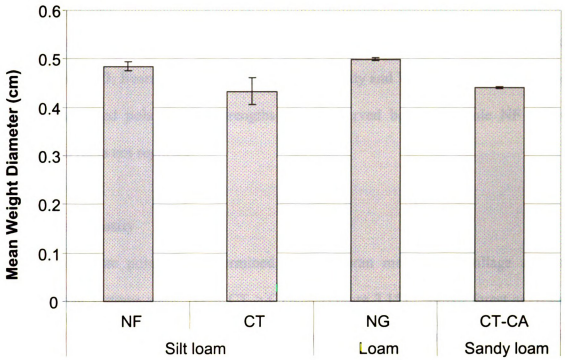

Figure 3.14. Mean weight diameter (MWD) by wet sieving for Wooster silt loam (Fine-loamy, mixed, mesic Oxyaquic Fragiudalfs) native forest (NF) aggregates, sampled from the surface 0-5cm, Kalamazoo loam (Fine-loamy, mixed, mesic Typic Hapludalf) native grassland (NG) succession aggregates and Ternary sandy loam (Sandy loam, mixed, frigid Alfic Haplorthodes) conventional-till planted to continuous alfalfa (CT-CA) aggregates, sampled from the surface 0-10cm. Samples are from soil aggregate size fractions 0.4-0.63cm across; n=3. Bars indicate standard errors.

Wooster silt loam and a Ternary sandy loam (CT-CA) had the lowest MWDs. These data support the concept that disruptive management decreases aggregate stability (Kandeler and Murer, 1993; Beare et al., 1994; Hillel, 1998; Brady and Weil, 2002). No differences among measured polar tensile strengths were observed between whole NF and CT aggregates (data not reported).

Aggregate Porosity

Aggregate porosities, determined by the Saran method, for tillage and crop management systems were: NF > CT > CT-CA (Figure 3.15). Native forest aggregates were 19% more porous than CT aggregates from the Wooster silt loam series. Aggregates from CT Wooster silt loam were 26% more porous than CT-CA aggregates from sandy loam soils. Soil aggregates from NF Wooster silt loam were 51% more porous than CT-CA aggregates from sandy loam soils, indicating that soil type and management practices together have a large affect on the porosity of aggregates. It has been reported that as the soil porosity increases, soil bacterial populations also increase (Young and Ritz, 2000).

C and N Concentrations

A strong positive correlation (R^2=0.996, $p<0.002$) was observed between C and bacterial populations of CT-CA and NG aggregates (Figure 3.16A) sampled three years earlier. It is expected that these data represent C and N values in the 2003 aggregates. As expected C and N concentrations and bacterial populations were positively correlated. Nitrogen concentrations and bacterial populations of the same aggregates, however, were

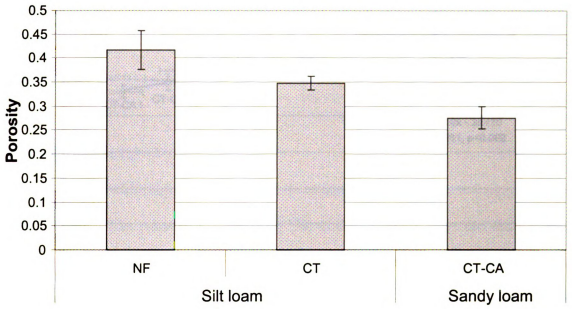

Figure 3.15. Porosity by Saran method for Wooster silt loam (Fine-loamy, mixed, mesic Oxyaquic Fragiudalfs) native forest (NF) and conventional-till (CT) aggregates, taken from the surface 0-5cm, and Ternary sandy loam (Sandy loam, mixed, frigid Alfic Haplorthodes) conventional-till planted to continuous alfalfa (CT-CA). Soil aggregate size fractions 4-6.3mm across were sampled from the surface 0-10cm; n=10. Bars indicate standard errors.

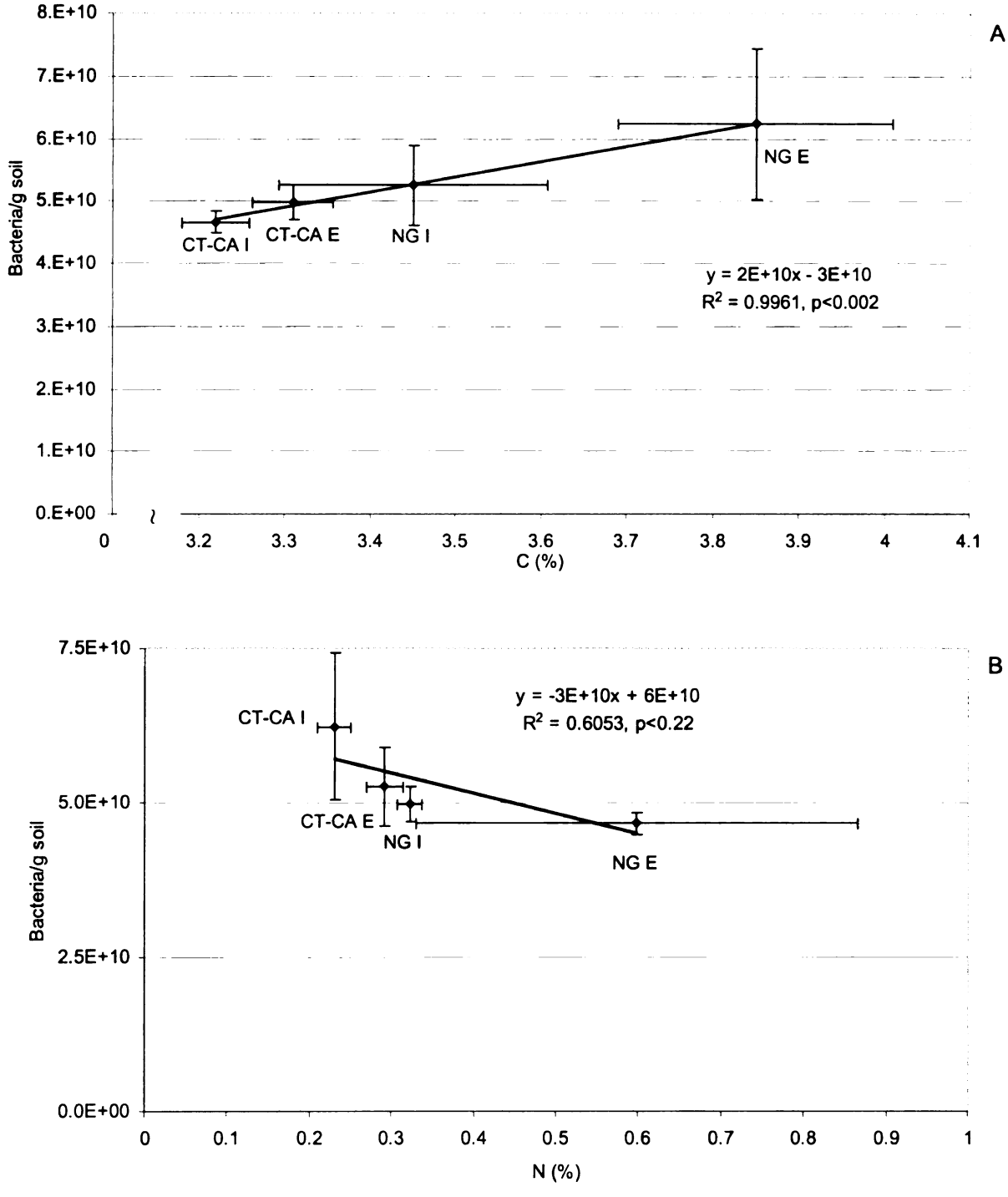

Figure 3.16. Extracted bacteria per gram soil and C (A) and N (B) concentrations for exterior (E) and interior (I) layers of Kalamazoo loam (Fine-loamy, mixed, mesic Typic Hapludalf) native grassland (NG) succession aggregates and Ternary sandy loam (Sandy loam, mixed, frigid Alfic Haplorthodes) conventional-till planted to continuous alfalfa (CT-CA). Soil aggregate size fractions 4-6.3mm across were sampled from the surface 0-10cm; n=7-21 aggregates, n=10 images per management system and layer, n=446-732 cumulative cells sampled, n=2 replications for C and N data. Bars indicate standard deviations.

not as strongly correlated nor significant (R^2=0.605, p<0.22) (Figure 3.16B). Relatively high, yet non-significant correlations were observed between C (R^2=0.586, p<0.23) and N (R^2=0.568, p<0.25) concentrations and bacterial populations of CT and NF aggregates (Figure 3.17 A and B).

Bacterial Morphotype Analysis

CMEIAS v3.0 morphotype analyses were conducted on moist and air dry whole NG aggregates. The proportional similarity (%) of the data concludes that the bacterial communities were 97.3% similar in morphotype diversity. These data indicate that the change in environment due to air drying the aggregates, led to minimal changes in the structure of the bacterial community revealed by this characteristic. The proportional similarity of morphological diversity for exterior and interior layers of NG and CT-CA aggregates were 98% and 98%, indicating little to no change in the morphological diversity of microbial communities throughout the aggregates. The proportional similarity of morphological diversity for exteriors of NG and CT-CA aggregates was 88% and for interiors was 86%, indicating some contrast among microbial community diversities between different management practices and soil types. The proportional similarity of the morphological diversity for the exterior and interior layers of NF aggregates was 97% similar, indicating only small changes in the characteristic of bacterial communities throughout aggregate interiors.

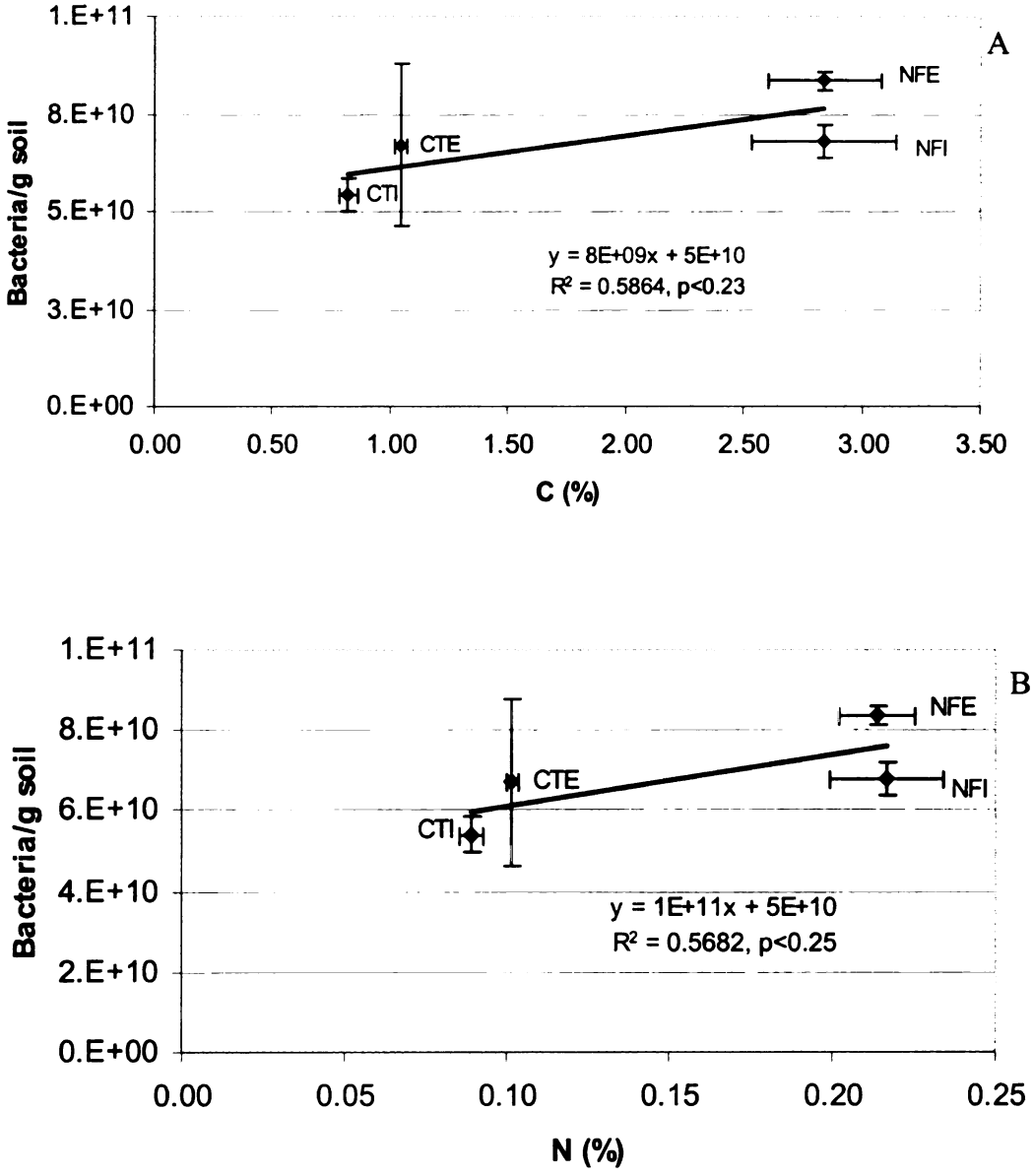

Figure 3.17. Extracted bacteria per gram soil and C (A) and N (B) concentrations for exterior (E) and interior (I) layers of Wooster silt loam (Fine-loamy, mixed, mesic Oxyaquic Fragiudalfs) native forest (NF) and conventional-till (CT). Soil aggregate size fractions 4-6.3mm across were sampled from the surface 0-5cm; n=13-17 aggregates, n=10 images per management system and layer, n=1091-1687 cumulative cells sampled, n=2 replications for C and N data. C and N data from samples in 2000. Bars indicate standard deviations.

SUMMARY AND CONCLUSIONS

Soil bacterial communities and populations undergo changes throughout the season and with changes in soil water contents. Morphologically the community changes are small, 3%, while cell biovolume changes are larger, 14%, correlating well with slightly larger abundance size classes in the moist aggregates. Bacterial density was higher in moist aggregates than air dry aggregates, and no differences were observed in the mean cell biovolumes.

Highest bacterial densities were seen in the NF aggregates and the smallest bacterial populations were observed in the CT-CA aggregates. The largest mean cell biovolumes were observed in the air dry NG aggregates and the smallest in the air dry NF aggregates. The highest diversity in the distribution of cell biovolumes between exterior and interior regions, was observed in the NF aggregates and the lowest in the CT-CA aggregates. The highest diversity in the distribution of cell biovolumes was observed in the exteriors of NF vs. CT aggregates and the smallest diversity among bacterial biovolumes in the interiors of the same aggregates. These differences indicate that the cell size distributions reflect contrasting structures and/or physiological conditions of these bacterial communities. Small differences in morphological diversity were seen in NF, NG and CT-CA aggregates between the aggregate exterior and interior. Larger differences were observed in exteriors and interiors of aggregates from differing soil types and management practices.

Carbon and N concentrations appear to contribute to bacterial populations, as hypothesized. However, since these correlations were relatively small, other factors seem

to exert greater influences on the population sizes in the communities located in soil aggregates. Data presented here demonstrate direct relationships between aggregate stability, porosity, and bacterial populations.

Increased soil bacterial populations were positively related to C and N concentrations which could be correlated to increased aggregate stability (MWD). As greater energy sources become available and are readily accessible to soil bacterial populations, those populations appear to increase in biovolume and density. These increased sizes and populations lead to greater bacterial by-products that, in turn, lead to greater formations of more stable aggregates.

REFERENCES

Beare, M.H., P.F. Hendrix, and D.C. Coleman. 1994. Water-stable aggregates and organic matter fractions in conventional- and no-tillage soils. Soil Sci. Soc. Am. J. 58:777-786.

Blake, G.R. and K.H. Hartge. 1986. Bulk Density. *In* A. Klute (ed.). Methods of Soil Analysis: Part 1-Physical and Mineralogical Methods. p.363-375. American Society of Agronomy, Inc. and Soil Science Society of America, Inc., Madison, WI.

Bloem, J. 1995. Fluorescent staining of microbes for total direct counts. Molecular Microbial Ecology Manual 4.1.8:1-12.

Brady, N.C. and R.R. Weil. 2002. The Nature and Properties of Soils. 13th ed. Prentice Hall, Upper Saddle River, NJ.

Dazzo, F.B., J. Lui, A. Prabhu, C. Reddy, M. Wadekar, R. Peretz, R. Bollempalli, D. Trione, E. Marshall, J. Zurdo, H. Hammoud, J. Wang, M. Li, D. McGarrell, A. Gore, J. Maya-Flores, S. Gantner, and N. Hollingsworth. 2003a. CMEIAS: Integrative software package to strengthen microscopy-based approaches for understanding microbial ecology. Kellogg Biological Station-Long Term Ecological Research All Scientists Meeting (KBS-LTER ASM), September 12, 2003. http://lter.kbs.msu.edu/Meetings/2003_All_Inv_Meeting/Abstracts/dazzo.htm.

Dexter, A.R., and B. Kroesbergen. 1985. Methodology for determination of tensile strength of soil aggregates. J. Agric. Eng. Res. 31:139-147.

Edgerton, D.L., J.A. Harris, P. Birch, and P.Bullock. 1995. Linear relationship between aggregate stability and microbial biomass in three restored soils. Soil Bio. Biochem. 27:1499-1501.

Grundmann, G.L and P. Normand. 2000. Microscale diversity of the genus *Nitrobacter* in soil on the basis of analysis of genes encoding rRNA. Appl. Environ. Microbiol. 66:4543-4546.

Hillel, D. 1998. Soil Structure and Aggregation. p. 101-125. *In* Environmental Soil Physics. Academic Press, San Diego, CA.

Kandeler, E. and E. Murer. 1993. Aggregate stability and soil microbial processes in a soil with different cultivation. Geoderma 56:503-513.

Kemper, W.D and R.C. Rosenau. 1986. Aggregate stability and size distribution. p. 425-442. *In* A. Klute (ed.) Methods of Soil Analysis. Part 1, Physical and Mineralogical Methods, 2nd edition. Soil Sci. Soc. Am., Madison, WI.

Lui, J., F.B. Dazzo, O.Glagoleva, B. Yu, and A.K. Jain. 2001. CMEIAS: A computer aided system for the image analysis of bacterial morphotypes in microbial communities. Microbial Ecology 41:173-194.

Lupwayi, N.Z., M.A. Arshad, W.A. Rice, and G.W. Clayton. 2001. Bacterial diversity in water-stable aggregates of soils under conventional and zero tillage management. Applied Soil Ecology 16:251-261.

Paul, E.A and F.E. Clark. 1996. Methods for studying soil microorganisms. p. 35-68 *In* Soil Microbiology and Biochemistry, 2nd edition. Academic Press, San Diego, CA.

Paul, E.A., D. Harris, M.J. Klug, and R.W. Ruess. 1999. The determination of microbial biomass. p. 291-317. *In* Standard Soil Methods for Long-Term Ecological Research. Oxford University Press, New York, NY.

Towner, H. 1999. EcoStat Ecological Analysis Program for Windows, Ver 1.02, Trinity Software, Compton, NH, Internet site: http://www.trinitysoftware.com/lifesci/index.html.

Young, I.M. and K. Ritz. 2000. Tillage, habitat space and function of soil microbes. Soil Tillage Res. 53:201-213.

CHAPTER 4

WETTING AND DRYING MODIFICATIONS OF SOIL MACRO-AGGREGATE STABILITIES: BIOPHYSICAL MECHANISMS

INTRODUCTION

The soil functions as a reservoir for water, nutrients, gases, soil organic matter (SOM), and microbial populations. The formation of stable soil aggregates increases terrestrial C sequestration within the soil profile. Greater aggregate stability, especially of surface soils, leads to increased water infiltration and aeration porosity, reduced water and wind erosion, and decreased crust formation. Aggregation processes depend on several factors, such as: the interaction of carbon (C), nitrogen (N), and SOM, microbial and mesofaunal activities, wetting/drying (W/D) frequencies, and management practices (Oades, 1993; Tisdall, 1994; Edgerton et al., 1995).

Organic matter is the primary stabilizing ingredient that is essential for the formation and stabilization of soil aggregates. SOM is the primary energy substrate needed for most bacterial activities (Tisdall and Oades, 1982; Brady and Weil, 2002). Until recently, the measurement of SOM has been limited to bulk soils. However, total C and N contents have been reported to be higher in the external layers of soil aggregates than their internal layers (Horn et al., 1990; Smucker et al., 1996). Most of the additional C located in the external layers is thought to be part of the more labile C pool associated with the microbial biomass, or dissolved organic C.

Changes in the soil environment that affect microbial communities are also thought to affect soil structure (*e.g.*, aggregation and aeration) (Kandeler and Murer, 1993; Edgerton et al., 1995). A positive linear correlation between stable macro-

aggregates and microbial biomass C was demonstrated by Edgerton et al. (1995). Bacterial biomass C increased in the presence of macro-aggregates and decreased where no macro-aggregates were present (Denef et al., 2001). Denef et al. (2001) also demonstrated a reduction in bacterial activity associated with W/D cycles of soils with no macro-aggregates present. Microbial diversity within aggregates seems to be as large as the microbial diversity identified across extensive geographical areas (Grundmann and Normand, 2000).

Wetting/drying cycles (W/D) influence aggregate formation and stability. Six W/D cycles have been shown to increase aggregate stability (Utomo and Dexter, 1982). Sissoko (1997) demonstrated that aggregate stability, as determined by the mean weight diameters (MWD) from wet sieving data, was not affected with three W/D cycles, however, aggregate stabilization was enhanced when C and N compounds were added during the wetting phase of 9 W/D cycles.

Conventional-tillage (CT) management practices incorporate crop residues and fertilizers into the plow layer using a moldboard plow to lift, twist, and invert the soil. Surface soils of CT management practices are subjected to extreme changes in water content and temperature. Extreme changes frequently break or destroy weaker aggregates exposing additional quantities of SOM to microbial mineralization. Further weathering leads to continued SOM losses and decreased particulate organic matter (POM) contents (Beare et al., 1994), reducing energy resources for soil microbial activities (Monreal and Kodama, 1997). Reduced or no-tillage (NT) practices use minimal or no plowing, leaving plant residues on the soil surface, which shelter aggregates from wind, rain, and temperature extremes, preserving aggregate structure.

These soils generate higher quantities of stable soil macro-aggregates which have accumulated nearly doubled the intra-aggregate quantities of total C stored within the exterior regions of macro-aggregates sampled from loam soils converted from CT to NT management systems for periods of at least 10 years (Smucker et al., 1996).

The objective of this study was to determine how the frequency of W/D cycles affects bacterial populations and communities located in the external and internal regions of soil aggregates sampled from a native forest ecosystem.

MATERIALS AND METHODS

Site Description and Soils

Samples of soil were collected from a native forest (NF) site located on a Wooster silt loam (Fine-loamy, mixed, mesic Oxyaquic Fragiudalfs) at the Ohio Agricultural Research and Development Center (OARDC) near Wooster, OH. Samples were taken in the spring of 2003 from the surface 0-5cm. All field samples were transported to the laboratory in rigid plastic containers to prevent packaging pressures on natural aggregates. Soils were stored at 4°C until processing. Soil samples were manually broken into their most stable aggregate size fractions during the air drying and manually sieved to separate into multiple size fractions without further abrasion of natural aggregates. Sieved aggregate size fractions included >9.5, 6.3-9.5, 4-6.3, 2-4, 1-2, 0.5-1, <0.5mm in diameter. Individual size fractions were stored in air-dried conditions within rigid plastic containers until further analyses. The aggregate size used in this study was the 4-6.3mm fraction.

Wetting and Drying Cycles

Ninety uniform soil aggregates, 4.0 to 6.3mm across, were subjected to 0, 3, and 6 wetting and drying (W/D) cycles (16 hours wetting/16 hours drying) before quantification for polar tensile strength, soil C and N contents, and soil bacterial populations and biovolume distributions.

Bacterial Analyses (Soil preparation, Microscopy, and Image analysis)

Bacterial extraction techniques for bulk soil samples have been developed to allow for soil dispersion and preparation of bacterial samples on slides for computer-assisted microscopy and image analysis (Bloem, 1995; Paul et al., 1999). Modifications of the above techniques were needed before small temporal and spatial changes in bacterial populations and community diversities, modified by intra-aggregate microhabitats at external and internal regions within soil macro-aggregates were quantified (Appendix A, Figure A.1). Soil dispersion and bacterial extraction procedures were modified by first decreasing the amount of soil used from 10g to 0.5g. Soil dispersion by blending was replaced with continuous sonication (W-385 Sonicator, Heat Systems-Ultrasonics, Inc.) for 5s. Sedimentation was optimized by Stoke's law to determine that after 42 minutes, 2μm soil particles had settled out of the top 5mL of solution used to prepare microscope slides. It was assumed that the bacterial cell density was less than the density of soil particles at the same size. The biological fluorescent stain 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF) was determined to give the least background staining by having the highest bacterial cell to soil particle ratio when compared with acridine orange (AO) and phenolic aniline blue, also fluorescent stains.

Digital images were obtained from a fluorescence microscope (Leitz Orthoplan 2, Leitz Inc., Germany) with the I3 filter for FTIC (fluorescein) stain and a 100x oil objective. A charge-coupled device (CCD) camera (Leica DC 500, Leica Microsystems AG, Germany) was used for acquisition of high resolution images (up to 3090 x 3600 pixels). Adobe Photoshop (version 7, Adobe Systems, Inc.) image capturing software on a Dell Pentium 4 PC allowed for viewing the image before collection, and then saving the image as a Tif file before further editing. The image capture settings include the following: 8 bit color, 1030 x 1300, single shot, always live, at a brightness of 20-30%, and a shutter speed of 0.1-0.3 seconds. Editing of images in Photoshop was required before image analysis to obtain a binary image with the background being lighter than the foreground objects (Appendix A, Figure A.2). Image analysis with the Center for Microbial Ecology Image Analysis System (CMEIAS), a computer-assisted microscopy program, analyses digital images of microbial communities for individual cell size and shape. Biovolume measurements were calculated using the CMEIAS-1 biovolume formula, which is adaptive to microbial cell shape (computed as object roundness) and also contains cell length, width, and area parameters whose computations are all described by Liu et al. (2001). CMEIAS-1 biovolume formula has an overall accuracy of 94.1% with all CMEIAS morphotypes represented (Dazzo et al., 2003). The data preparation macro developed for CMEIAS, working in Microsoft Excel, prepares the data collected for ecological statistical analyses by compiling, concatenating, and graphically displaying the data (Dazzo et al., 2003). Output data of this macro includes frequency distribution, % frequency distribution, and cumulative frequency distribution graphs of the data selected. For this study, cell biovolume distributions were identified. Extracted bacteria per gram

soil were calculated from microscope images and cell counts from CMEIAS analysis (Paul and Clark, 1996).

Aggregate Analyses

Strengths of soil aggregates were measured by the polar tensile strength (PTS) technique and reported as the force per unit area required to disrupt air dry soil aggregates. Average aggregate diameters are determined by measuring effective diameters at three locations around each aggregate, by an external-dimension caliber. A constant force is applied to one polar end of an aggregate placed on a stationary platform. The applied forces are graphed with significantly large inflection points identified. Graphic disruptions are viewed and the largest is selected as the force required to break the major plane of weakness within each aggregate (Dexter and Kroesbergen, 1985; Hillel, 1998). Another parameter for comparing aggregate stability are the total C and N contents, determined by the combustion at 800°C of small quantities of soil (30mg) in a Carlo-Erba CHN analyzer, model NA1500.

Statistical Analysis

Bacterial community diversity was analyzed using EcoStat (Towner, 1999), an ecological analysis program that is commonly used to perform many statistical tests including the evaluation of species diversity and community similarity analyses of many ecosystems. The input data from CMEIAS included the % frequency distributions of cell biovolumes.

RESULTS AND DISCUSSION

Aggregate Strength

Increased polar tensile strengths (PTS) were observed when W/D cycles increased from 0 to 3 for NF aggregates from Wooster silt loam soils (Figure 4.1). PTS decreased as frequencies of W/D were increased from 3 to 6 cycles. Although neither of the increases or decreases in PTS were significant, changes in aggregate strength were observed.

Bacterial Populations

A 23% increase in bacterial populations was observed in the exterior layers of the more stable aggregates exposed to 3 W/D cycles, with a 29% decrease in bacterial populations as W/D cycles increased from 3 to 6. (Figure 4.2). A 17% increase in bacterial populations was observed in the interior layers of aggregates exposed to 3 W/D cycles, with an additional 32% increase with 3 more W/D cycles. These data indicate that bacterial populations in the exteriors of aggregates increase with 3 W/D and then decrease to lower than initial populations with 6 W/D cycles. In the interiors of aggregates, however, bacterial populations continue to increase through 6 W/D cycles. This may be due to increased pore networks with each wetting phase, which increases the movement of C into interior regions of aggregates. The C is then more accessible to these populations located in the interior of aggregates. This potential movement of C from the exterior to interior regions of soil aggregates with 3 to 6 W/D cycles may explain the decrease in PTS with 6 W/D cycles. The microbial populations in the exteriors of aggregates exposed to 6 W/D cycles significantly decrease from 3 W/D

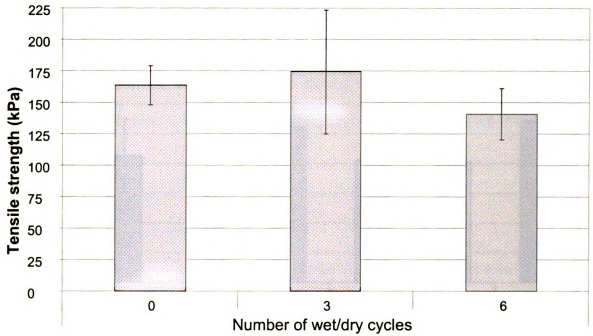

Figure 4.1. Increased polar tensile strengths (PTS) with 3 W/D cycles and decreased PTS with 6 W/D cycles for native forest (NF) aggregates from Wooster silt loam (Fine-loamy, mixed, mesic Oxyaquic Fragiudalfs) of aggregate size fraction 4-6.3mm across, sampled from the surface 0-5cm, exposed to 0, 3, and 6 wetting-drying (W/D) cycles; n=10 aggregates. Bars indicate standard errors.

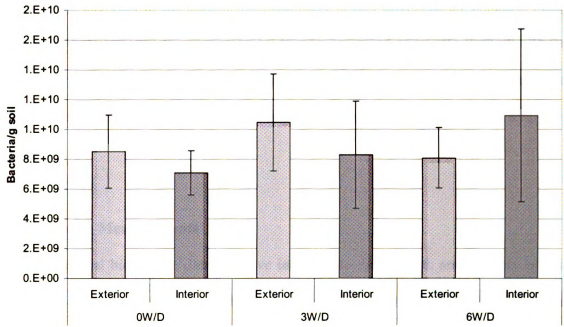

Figure 4.2. Increased populations of bacteria were observed in the exteriors and interior of aggregates exposed to 3 W/D cycles. The increase in populations continued in the interior regions through 6 W/D cycles, while a decrease was observed in the exteriors of aggregates exposed to 6 W/D cycles. Extracted bacteria per gram soil for exterior and interior layers of native forest (NF) aggregates from Wooster silt loam (Fine-loamy, mixed, mesic Oxyaquic Fragiudalfs) of aggregate size fraction 4-6.3mm across, sampled from the surface 0-5cm, exposed to 0, 3, and 6 wetting-drying (W/D) cycles; n=13-16 aggregates, n=10 images per WD cycle and layer, n=717-1141 cumulative cells sampled. Bars indicate standard errors.

cycles. This decrease in population indicates there is less microbial by-products being produced to help stabilize the aggregate, leading to decreased aggregate strength. These data clearly support the accentuation of microbial community activities by repeated hydrations/dehydrations of intra-aggregate regions and support previous reports by Denef et al. (2001).

Cell Biovolume Measurements

Microbial biovolume data can give insight into the strength and stability of soil aggregates. Biovolume measurements were calculated using the CMEIAS-1 biovolume formula, which is adaptive to microbial cell shape (computed as object roundness) and also contains cell length, width, and area parameters whose computations are all described by Liu et al. (2001). CMEIAS-1 biovolume formula has an overall accuracy of 94.1% with all CMEIAS morphotypes represented (Dazzo et al., 2003). Increased mean cell biovolumes were observed in the interior regions of NF aggregates from Wooster silt loam soils, exposed to 3 W/D cycles than other interior regions exposed to 0 and 6 W/D cycles or exterior region of aggregates exposed to 0, 3, or 6 W/D cycles (Figure 4.3). A decrease, though not significant, was observed in the exterior regions of aggregates exposed to 6 W/D cycles. No differences were observed between exterior and interior regions of NF aggregates exposed to 0 and 6 W/D cycles. Cell size and numbers of bacteria both increased in interior regions of aggregates exposed to 3 W/D cycles. However, cell size decreased in the interior regions of aggregates exposed to 6 W/D cycles while populations increased. These data support previously reported information that microbial activity and aggregate stability are linearly

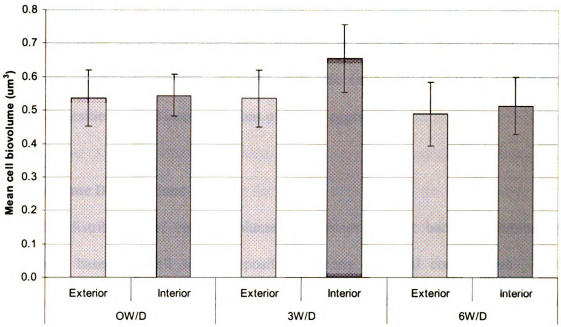

Figure 4.3. Increased cell biovolumes were observed in the interior regions of aggregates exposed to 3 W/D cycles. The mean cell biovolume was calculated by CMEIAS-1 biovolume formula for exterior and interior layers of native forest (NF) aggregates from Wooster silt loam (Fine-loamy, mixed, mesic Oxyaquic Fragiudalfs) of aggregate size 4-6.3mm across, taken from the surface 0-5cm, exposed to 0, 3, and 6 wetting-drying (W/D) cycles; n=13-16 aggregates, n=10 images per WD cycle and layer, n=717-1141 cumulative cells sampled. Error bars indicate standard deviations.

related (Edgerton et al, 1995). Additionally, our data demonstrate that aggregate stabilities increase as bacterial diversities increase in exterior regions of aggregates. Furthermore, aggregate stability declined with increasing W/D cycles transferred bacterial diversities toward interior regions of macro-aggregates.

Cell Biovolume Distributions

The distribution of cell biovolume frequencies present bacterial community comparisons based on cell size compositions among bacterial communities. Cell biovolume frequencies are calculated by the CMEIAS data preparation macro, that compiles, concatenates, and graphically presents the CMEIAS data.

Community diversity statistics include various indices for comparing and contrasting bacterial communities. For this study proportional similarity (%) was determined, which is a simple measure which incorporates information on species abundances based on the percentage of total community abundance that each species comprises. Proportional similarity (%) was calculated using the cell biovolume frequencies calculated by the CMEIAS data preparation macro. Community similarities around 85% or more were not considered to be significantly different in morphological diversity. The differences between proportional similarities among compared communities cannot be labeled as increased or decreased cell biovolumes. For this interpretation and summary, the bacterial cell biovolume frequencies give greater insight as bacterial populations and their potential metabolic activities are compared, as metabolically active cells are larger. Bacterial cell biovolume frequency distributions,

using percent abundance, give reasonable estimates of bacterial community comparisons of the most abundant size class or mode (personal communication with Dr. Dazzo).

Bacterial cell biovolume distributions in exterior layers were not significantly different (9%, 15%, and 8%) than interior regions of NF aggregates exposed to 0, 3, and 6 W/D cycles. The cell biovolumes in the exterior regions of aggregates exposed to 3 W/D cycles were actually smaller than the interior cell biovolumes. The proportional similarity (%) indicates that bacterial communities become more diverse following 3 short term W/D cycles and less diverse with 6 W/D cycles. A significant increase in the % difference of % abundance in cell biovolume distribution was observed with 3 W/D cycles for biovolume size $0.3\mu m^3$. Bacterial cell biovolume distributions were 8% smaller, 12% smaller, and 8% smaller in exterior layers for 0 vs. 3, 0 vs. 6, and 3 vs. 6 W/D cycles, and 12% larger, 8% smaller, and 16% smaller in interior regions. This indicates that W/D cycles influence microbial communities in both the exterior and interior regions of aggregates. The diversity of exterior regions of aggregates, increased with both 3 and 6 W/D cycles; however, the diversity of interior regions increased with 3 W/D cycles and decreased with 6 W/D cycles. A significant increase in the % difference of % abundance in cell biovolume distributions was observed with 3 W/D cycles at $0.3\mu m^3$ cell biovolume (Figure 4.4). Increases were also observed in the exteriors of 0 and 6 W/D cycles, and in the interiors of 3 and 6 W/D cycles. These data show that W/D cycles do affect cell size composition of bacterial communities in soil aggregates.

A summary of the bacterial data for these soils is located in Appendix A (Table A.1). These data include object counts per soil and management practice, extracted

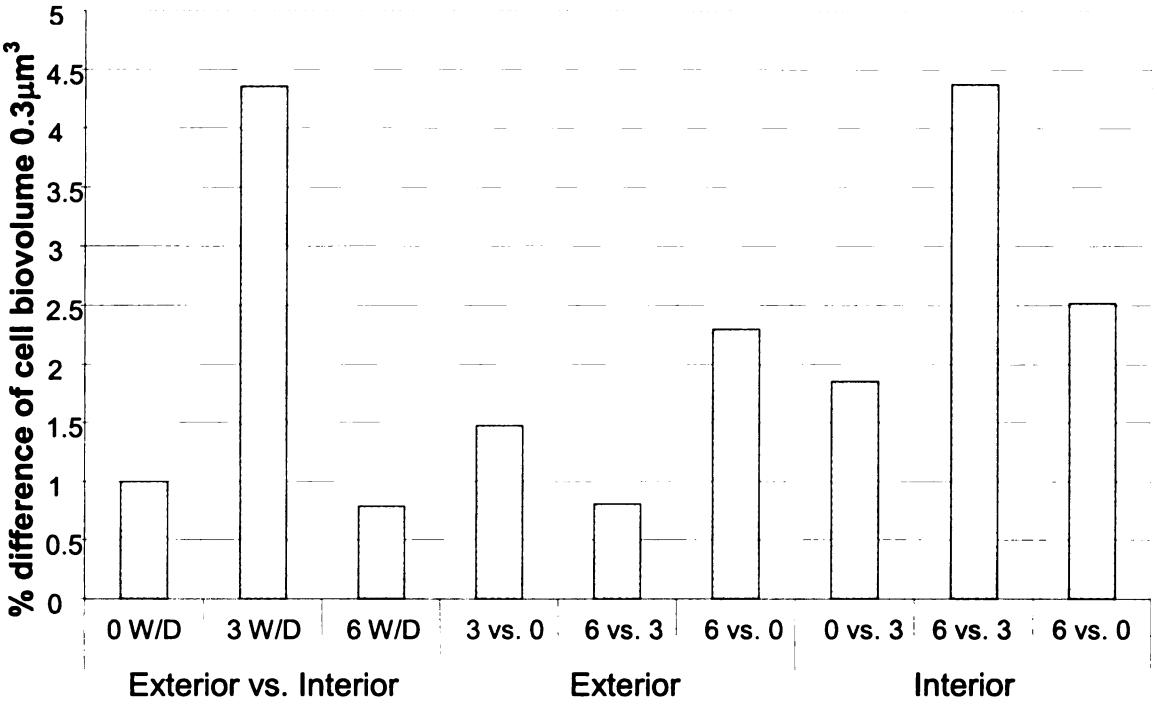

Figure 4.4. Changes among the % abundances of cell biovolume distributions were observed with 3 W/D cycles at 0.3µm^3 cell biovolume. Increases were also observed in the exteriors of 0 and 6 W/D cycles, and in the interiors of 3 and 6 W/D cycles. The biovolume frequency distributions were calculated using a constant bin width of 0.1um^3 by the CMEIAS data preparation macro and by EcoStat for native forest (NF) aggregates from Wooster silt loam (Fine-loamy, mixed, mesic Oxyaquic Fragiudalfs) of aggregate size fraction 4-6.3mm across, sampled from the surface 0-5cm, exposed to 0, 3, and 6 wetting-drying (WD) cycles; n=13-16 aggregates, n=10 images per WD cycle and layer, n=717-1141 cumulative cells sampled.

bacteria per gram of soil, and biovolume measurements of mean, median, mode, skewness, minimum, and maximum.

C and N Concentrations

Carbon and nitrogen concentrations from the soil aggregates exposed to 0, 3, and 6 W/D cycles are currently being analyzed.

SUMMARY AND CONCLUSION

Soil bacterial communities and populations undergo changes throughout the season. Wetting-drying cycles influence bacterial communities in the exterior and interior regions of soil aggregates. These influences include desiccation and osmotic stresses. The cell biovolumes of bacterial communities were observed to become more diverse with 3 W/D cycles and less diverse with 6 W/D cycles. The diversity of cell biovolume distributions in exterior regions of aggregates, increased with both 3 and 6 W/D cycles; however, the diversity in interior regions increased with 3 W/D cycles and decreased with 6 W/D cycles. Significant changes to the bacterial community composition seem to occur with 3 W/D cycles. Mean cell biovolumes were larger in the interior regions of aggregates exposed to 3 W/D cycles than any other exterior region of aggregates exposed to 0, 3, and 6 W/D cycles, or interior region of aggregates exposed to 0 and 6 W/D cycles.

Bacterial density increased in the exterior regions of aggregates with 3 W/D cycles and decreased with 3 additional W/D cycles. Bacterial density increased in the

interior regions of aggregates through 3 and 6 W/D cycles. Differences in the PTS of soil aggregates exposed to 0, 3, and 6 W/D cycles were observed though not significant. PTS increased with 3 W/D cycles and decreased with 6 W/D cycles. This difference may be explained by changes in soil bacterial populations as aggregates were exposed to increasing W/D cycles. The bacterial populations in soil aggregates increased in the interior regions of aggregates through 6 W/D cycles. However, the populations increased only through 3 W/D cycles for the exterior regions and then decreased with 6 W/D cycles. This may be due to increased pore networks with each wetting phase, which increases the movement of C into interior regions of aggregates. The C is then more accessible to these populations located in the interior of aggregates. This potential movement of C from the exterior to interior regions of soil aggregates with 3 to 6 W/D cycles may explain the decrease in PTS with 6 W/D cycles. The microbial populations in the exteriors of aggregates exposed to 6 W/D cycles were significantly lowered as W/D cycles increased from 3 to 6. This decrease in population indicates there are fewer microbial by-products produced with increasing W/D cycles, leading to decreased strength among soil aggregates.

REFERENCES

Aditjandra, K., E.J. Park, and A.J.M. Smucker. 2002. Tensile Strength Domains within Soil Aggregates from Contrasting Ecosystems. ASA Abstracts, Indianapolis, IN.

Beare, M.H., P.F. Hendrix, and D.C. Coleman. 1994. Water-stable aggregates and organic matter fractions in conventional- and no-tillage soils. Soil Sci. Soc. Am. J. 58:777-786.

Bloem, J. 1995. Fluorescent staining of microbes for total direct counts. Molecular Microbial Ecology Manual 4.1.8:1-12.

Brady, N.C. and R.R. Weil. 2002. The Nature and Properties of Soils. 13th ed. Prentice Hall, Upper Saddle River, NJ.

Dazzo, F.B., J. Lui, A. Prabhu, C. Reddy, M. Wadekar, R. Peretz, R. Bollempalli, D. Trione, E. Marshall, J. Zurdo, H. Hammoud, J. Wang, M. Li, D. McGarrell, A. Gore, J. Maya-Flores, S. Gantner, and N. Hollingsworth. 2003. CMEIAS: Integrative software package to strengthen microscopy-based approaches for understanding microbial ecology. Kellogg Biological Station-Long Term Ecological Research All Scientists Meeting (KBS-LTER ASM), September 12, 2003. http://lter.kbs.msu.edu/Meetings/2003_All_Inv_Meeting/Abstracts/dazzo.htm.

Denef, K., J. Six, H. Bossuyt, S.D. Frey, E.T. Elliot, R. Merckx, and K. Paustian. 2001. Influence of dry-wet cycles on the interrelationship between aggregate, particulate organic matter, and microbial community dynamics. Soil Biol. Biochem. 33:1599-1611.

Dexter, A.R., and B. Kroesbergen. 1985. Methodology for determination of tensile strength of soil aggregates. J. Agric. Eng. Res. 31:139-147.

Edgerton, D.L., J.A. Harris, P. Birch, and P.Bullock. 1995. Linear relationship between aggregate stability and microbial biomass in three restored soils. Soil Bio. Biochem. 27:1499-1501.

Grundmann, G.L and P. Normand. 2000. Microscale diversity of the genus *Nitrobacter* in soil on the basis of analysis of genes encoding rRNA. Appl. Environ. Microbiol. 66:4543-4546.

Hillel, D. 1998. Soil Structure and Aggregation. p. 101-125. *In* Environmental Soil Physics. Academic Press, San Diego, CA.

Horn, R. 1990. Aggregate characterization as compared to bulk soil properties. Soil Tillage Res. 17:265-289.

Kandeler, E. and E. Murer. 1993. Aggregate stability and soil microbial processes in a soil with different cultivation. Geoderma 56:503-513.

Lui, J., F.B. Dazzo, O.Glagoleva, B. Yu, and A.K. Jain. 2001. CMEIAS: A computer aided system for the image analysis of bacterial morphotypes in microbial communities. Microbial Ecology 41:173-194.

Monreal, C.M. and H. Kodama. 1997. Influence of aggregate architecture and minerals on living habitats and soil organic matter. Can. J. Soil Sci. 77:367-377.

Oades, J.M. 1993. The role of biology in the formation, stabilization, and degradation of soil structure. Geoderma 56:377-400.

Paul, E.A and F.E. Clark. 1996. Methods for studying soil microorganisms. p. 35-68 *In* Soil Microbiology and Biochemistry, 2nd edition. Academic Press, San Diego, CA.

Paul, E.A., D. Harris, M.J. Klug, and R.W. Ruess. 1999. The determination of microbial biomass. p. 291-317. *In* Standard Soil Methods for Long-Term Ecological Research. Oxford University Press, New York, NY.

Santos, D., S.L.S. Murphy, H. Taubner, A.J.M. Smucker, and R. Horn. 1997. Uniform separation of concentric surface layers from soil aggregates. Soil Sci. Soc. Am. J. 61:720-724.

Sissoko, F. 1997. Enhancement of soil aggregation by the combined influences of soil wetting and drying and root-microbial associations. M.S. Thesis. Michigan State University, East Lansing.

Smucker, A.J.M., D. Santos, and Y. Kavdir. 1996. Concentric layering of carbon, nitrogen, and clay minerals within soil aggregates from tilled and non-tilled agroecosystems and natural grasslands. Third Eastern Canada Soil Structure Workshop Proceedings. p. 129-140.

Tisdall, J.M. 1994. Possible role of microorganisms in aggregation of soils. Plant Soil 159:115-121.

Tisdall, J.M. and J.M. Oades. 1982. Organic matter and water-stable aggregates in soils. J. Soil Sci. 33:141-163.

Towner, H. 1999. EcoStat Ecological Analysis Program for Windows, Ver 1.02, Trinity Software, Compton, NH, Internet site: http://www.trinitysoftware.com/lifesci/index.html.

Utomo, W.H. and A.R. Dexter. 1982. Changes in soil aggregate water stability induced by wetting and drying cycles in non-saturated soil. J. Soil Sci. 33:623-637.

CHAPTER 5

CONCLUSIONS

Soil bacterial communities and populations undergo changes throughout the season and with increased cycling soil water contents. This study has shown that increased bacterial populations and proportionally larger cell biovolumes exist in moist soil aggregates than in air dry aggregates. Therefore, it seems that water stresses (desiccation and osmotic) in the soil adversely affect microbial communities and populations. Wetting and drying cycles influence bacterial communities in the exterior and interior regions of soil aggregates. Bacterial populations increased in both the exterior and interior regions of aggregates exposed to 3 W/D cycles, however, populations decreased in the exterior regions of aggregates exposed to 6 W/D cycles while continuing to increase in the interiors. Polar tensile strength of aggregates increased with 3 W/D cycles and decreased with 6 W/D cycles. This change in aggregate strength may be explained by the changes in soil bacterial populations with W/D cycles. The increase in bacterial populations in the interiors of aggregates exposed to 6 W/D cycles may be due to increased pore networks with each wetting phase, which increases the movement of C into interior regions of aggregates. The C is then more accessible to these populations located in the interior of aggregates, hence the increase in bacterial populations in the interior regions and decrease in exterior regions. This potential movement of C from the exterior to interior regions of soil aggregates with 3 to 6 W/D cycles may also explain the decrease in polar tensile strength observed with 6 W/D cycles. The decrease of bacterial populations in the exterior regions of aggregates

indicates there is less microbial by-products being produced to help stabilize the aggregate, leading to decreased aggregate strength.

Soil type and management practices influence soil bacterial communities and populations. The largest bacterial populations were associated with the soils containing the most clay and highest C concentration (silt loam > loam > sandy loam), with the exterior regions of aggregates having increased populations over the interior regions. Increased bacterial populations were observed with soil aggregates under the least destructive management practice. These aggregates also had higher stabilities, leading them to be able to support the larger populations of bacteria observed. The native grassland loam aggregates had larger biovolumes than the conventional-till planted to continuous alfalfa sandy loam aggregates, which in turn had larger biovolumes than the native forest and conventional-till silt loam aggregates. This indicates that cell biovolumes were not significantly influenced by soil type (clay content) or management practice. The highest diversity in the distribution of cell biovolumes was observed in the exteriors of native forest vs. conventional-till aggregates and the smallest diversity in the interiors of the same aggregates. These differences indicate that the cell size distributions reflect differences in the structure and/or physiological conditions of these bacterial communities.

Carbon and N concentrations appear to contribute to bacterial populations, however, since some correlations are relatively low, other factors seem to be contributing to the population estimates of the communities located in soil aggregates. Results from this study demonstrate direct relationships between aggregate stability, porosity, and bacterial populations. Increased soil bacterial populations were positively related to C

and N concentrations and increased aggregate stability as determined by MWD. As greater energy sources are made available and accessible to soil bacterial populations, those populations increase in biovolume and density. This increase in size and population leads to greater bacterial by-products that in turn lead to increased aggregate formation and stabilization.

APPENDIX

Figure A.1. Modified bacterial extraction and staining procedure.

Direct Microscopy Counts for Bacteria using Soil Smears and DTAF
(Bloem, 1995; Paul et al., 1999; *modified by Dopp, 2003*)

Reagents

1. Buffer: Phosphate Buffered Saline – 0.05M Na_2HPO_4 (7.8g l^{-1}) and 0.15M NaCl (8.8 g l^{-1}) adjusted to pH 9.0
2. Stain: DTAF (5-(4,6-dichlorotriazin-2-yl) aminofluorescein) – 2mg in 10ml buffer
3. Water: filtered, distilled

Filter buffer, stain, and water through 0.22μm membrane. Stain solution is good for 1 day.

Vortex for 10 sec per time.

Sample and Slide preparation

1. Combine 0.5g eroded soil and 10mL filtered distilled water in sedimentation tube.
2. *Sonicate for 5 sec continuous with cup holder attachment.*
3. *Vortex and allow coarse particles to sediment for 42 minutes (Stoke's law). Transfer top 5mL sample to disposable centrifuge tube.*
4. *Increase dilution from 1:20 to 1:200 (soil:water) (vortex and transfer 0.5ml and add 4.5ml water).*
5. Add 0.1mL formaldehyde. (can stop here and store at 4°C until slide preparation)
6. Vortex sample before subsampling to prevent further sedimentation for replicate slides.
7. Place 4μL of soil suspension onto 6mm wells on slides. Spread suspension around well with pipette tip without touching the tip to the slide surface.
8. Allow smears to dry completely (*e.g.*, overnight)

Staining

1. Flood each well with 8μL of stain.
2. Store slide(s) in covered container with wet tissue to prevent drying.
3. Stain for 30 minutes.
4. Place slide(s) in staining jar containing buffer for 30 minutes, three times.
5. Place slide(s) in staining jar containing distilled water for final rinse for 30 minutes.
6. Remove slide(s) from final rinse and air dry.
7. *Add small drop of VECTASHIELD Mounting medium (Vector Laboratories) to each smear and cover with large (50mm) cover glass. Add immersion oil to top of cover slip before microscopy.*

Counting

Observe bacteria on a fluorescence microscope using a 10x ocular, a 100x objective and "I 3" filter.

Figure A.2. Editing procedure for CMEIAS analysis of DTAF stained images.

1. Open image in Adobe Photoshop
2. Resize image and increase pixel resolution (Image<image size, uncheck resample image box, increase resolution to 300 pixels/inch – size automatically adjusts)
3. Decrease brightness to 15; increase contrast to 15 (Image<adjust<brightness/contrast)
4. Duplicate image (Image<duplicate); change name (delete 1 and add e)
5. Change to grayscale (Image<mode<grayscale)
6. Invert image(Image<adjust<invert)
7. Increase brightness to 5-10 (depends on image – increase to reduce background color and noise, but don't want to lose cells)
8. Mark each cell (pencil tool) with a black dot; constantly look between original image (color) and edited image
9. Zoom in (300-400%) and outline cells with pencil tool; may need to compare with original image
10. Use paint tool to fill in cells
11. Use eraser (change color so white is on top) to fill in edges of cells; change colors back to black on top
12. Adjust levels to decrease background to white (move almost all the way to the left of the box) (Image<adjust<levels)
13. Move cells that are close together apart or are at edge of image in
14. Decrease noise of the image (smoothes out cells some – decreases size slightly) (Filter<noise<median)
15. Add bar scale (10μm) from microscope calibration scale acquired from same magnification
16. Flatten image (Layer<flatten image)
17. Save edited image

Table A.1. Overall summary of data for soils compared.

		Biovolume						
	Bacteria/g soil	mean	median	mode	skewness	minimum	maximum	Object count
KBS air dry	7.34E+09	0.946	0.70	0.67	2.884	0.14	6.87	750
KBS moist	1.01E+10	0.851	0.68	0.61	2.398	0.14	5.05	1014
KBS E	6.16E+09	1.730	1.33	1.02	1.629	0.14	7.30	1837
KBS I	5.22E+09	1.531	1.19	0.65	2.021	0.14	7.10	1560
UP E	4.98E+09	1.280	0.96	0.62	2.343	0.03	7.01	1002
UP I	4.66E+09	1.213	0.96	0.68	2.137	0.11	7.06	938
NF E	8.35E+10	0.568	0.44	0.17	4.149	0.10	7.20	1687
NF I	6.81E+10	0.562	0.42	0.33	5.390	0.09	6.95	1375
CT E	6.7E+10	0.671	0.51	0.33	3.547	0.10	7.20	1353
CT I	5.4E+10	0.609	0.43	0.27	3.130	0.09	4.73	1091
NF 0W/D E	8.51E+09	0.525	0.42	0.17	5.239	0.11	7.20	859
NF 0W/D I	7.10E+09	0.547	0.42	0.33	4.658	0.09	5.73	717
NF 3W/D E	1.04E+10	0.528	0.40	0.23	3.799	0.09	5.80	1141
NF 3W/D I	8.28E+09	0.653	0.50	0.27	5.132	0.11	7.10	846
NF 6W/D E	8.07E+09	0.472	0.36	0.33	4.167	0.10	5.41	814
NF 6W/D I	1.09E+10	0.496	0.38	0.27	4.677	0.09	5.55	1105