

STUDIES OF GENERA CYTOPHAGA-FLAVOBACTERIUM
IN CONTEXT OF THE SOIL CARBON CYCLE

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Microbiology and Molecular Genetics

2011

ABSTRACT

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There is a great need to understand the global carbon cycle; with specific interest in the cycling of soil carbon. This need is two-fold and interconnected. The global climate change is in-part due to the loss of soil organic carbon and the reduced ability of soils to sequester carbon. Also, to maintain sustainable, high yielding agriculture, carbon loss from soil must be mitigated and in turn this can restore the ability of soils to sequester carbon.

A specific group of bacteria, the *Cytophaga-Flavobacterium* (CF), are of interest in these contexts because evidence suggests that they have the potential to exacerbate the loss of organic carbon from soil. The CF are globally distributed, often detected as organisms responsible for degradation of organic compounds. Other research has demonstrated their active presence in soil, especially the zone around plant roots. Most importantly, these bacteria produce a large assortment of hydrolytic enzymes that can degrade complex carbohydrates.

The first studies in this dissertation were intended to investigate the phylogenic diversity and distribution of CF in soil. CF specific clone libraries were generated to investigate a possible link between the CF and soils under different land management and use history, and thus different carbon contents. Also in this dissertation, we tested the hypothesis that *Flavobacterium* soil isolates would weaken, or degrade, remolded aggregates generated with xylan, a complex carbohydrate, in an effort to describe how *Flavobacterium* can be responsible for the loss of organic carbon from soil.

While the initial studies were exploratory in design, some specific comparisons were made. The CF community from the agricultural soil was determined to be a subset of the communities found in the non-agricultural soils. When the CF communities from deciduous forest soil and fertilized deciduous forest soil were compared, no differences in phylogenetic diversity or distribution were identified. A significant difference in the phylogenetic distribution was detected when the CF community from conventionally tilled agricultural soils was compared to the community from a no-till agricultural management. The diversity of the community from the tilled soil was greater than the community from the no-till soil.

Remolded aggregates composed with xylan were challenged with nine *Flavobacterium* spp. isolated from soil to test the hypothesis that they would degrade the xylan causing a reduction in the erosive strength of the remolded aggregates. In natural soils, increased breakage can lead to a loss of carbon from the soil. The erosive strength is a measure of how readily aggregates will break apart. These values are determined by eroding the aggregates in precision-machined soil aggregate erosion chambers and recording the length of time and amount of force required to remove layers of the aggregate. Using two measures of erosive strength, the highest recorded value, or peak erosive strength, and the total erosive strength for all layers of the aggregate, the aggregates treated with *Flavobacterium* spp. were compared to uninoculated controls. It was determined that six of the nine species significantly reduced the peak erosive strength and four species also reduced the total erosive strength. These data support the hypothesis that *Flavobacterium* spp. contribute to a loss of organic carbon from soil. These works can now be extended, using the same techniques, by investigating the role of *Flavobacterium* as part of the whole bacterial community, as well as investigating the influence of temperature on their degradation activity.

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Chapter 1

Consequences of Loss of Soil Organic Carbon and Reasons to Study Soil Dwelling *Cytophaga-Flavobacterium* in this Context

Introduction

To better understand the role of *Cytophaga-Flavobacterium* in the cycling of soil organic carbon, the studies in this dissertation first observed their diversity and distribution in soils of different land managements and history of usage; thus different organic carbon contents. In addition, soils were sampled in an effort to observe possible specific land management techniques, addition of inorganic fertilizer and soil tillage, that may influence the diversity and distribution of the *Cytophaga-Flavobacterium*. Following, we attempted to describe the role of *Flavobacterium* species in the cycling of soil organic matter by performing *in vitro* studies of remolded soil aggregates. As an introductory chapter to this dissertation, the following is a brief discussion describing why there is a need to study the cycling of soil organic matter, and why we believe that the *Cytophaga-Flavobacterium* are worthy of studying in this context.

Soil Carbon

The loss of soil organic carbon (SOC) has global impacts both on global climate change, and the sustainability of agriculture. The soil carbon pool, containing approximately 2700 gigatons (Gt) of carbon (C), is larger than both the atmospheric (780 Gt) and biotic (650 Gt) pools, and is second only to the oceanic carbon pool (38,000 Gt) (44). Of the global soil carbon, greater than half is organic C (1550 Gt) and the remaining 950 Gt is inorganic C (44). Around the globe, at a depth of 1 meter, there is a range of SOC from 30 tons/ha in arid climates, to 800 tons/ha in organic soils in cold regions. The chief range is between 50 and 150 tons/ha (46). In the United States, the soil carbon pool is approximately 5% of the global soil carbon pool (45).

Current estimates show that the SOC pools are declining and the atmospheric carbon pool is increasing at a rate of 0.5% per year (45). The conversion of natural soils to agricultural use, especially those used for crop growth in contrast to grazing lands, has been credited for both the reduction of SOC and the increased C in the atmospheric pool (25, 46, 74, 96). Conversion of natural soils to agriculture has caused an exhaustion of as much as 75% of the SOC in cultivated tropical soils and up to 60% in the soils of temperate regions (46). In the United States the SOC pool has been lessened by a total of between 0.3 to 0.5 tons, and most agricultural soils in Midwestern United States have lost 25 to 40 tons C per hectare, leaving them well below their carbon-holding capacity (45).

The loss of SOC degrades soil quality, reduces biomass productivity, and adversely impacts water quality in agricultural croplands (47). The global human population is increasing, and yet the amount of arable land, and quality water is decreasing (43). Additionally, these circumstances exacerbate the global warming effect, as some soils have lost 20 to 80 tons C per hectare, most of which is released into the atmosphere (46, 74).

Aboveground and Belowground Dependence

The loss of soil carbon directly affects crop productivity and thus the production of food for the growing human population. In addition, because plants fix carbon dioxide into biomass causing atmospheric carbon to be sequestered in the soil, the loss of soil carbon also exacerbates the global climate change. If there is less plant productivity, less carbon is sequestered.

However, plants are only a portion of the total ecosystem. Plants are the primary producers that deposit complex carbon on the soil surface in the form of plant litter and also exude a variety of nutrient-rich compounds from their roots. The extent to which this root exudate, called *rhizodeposition*, diffuses through the soil is the defining edge of the *rhizosphere*.

Many soil organisms actually colonize the surface and subsurface of the plant roots. These organisms, such as mycorrhizal fungi that can act as an extended root system, and bacteria that can fix nitrogen, are widely studied for their beneficial effects on plants (94).

Not to be overlooked are the organisms that are not root-associated, rhizosphere-dwelling organisms. They too can have dramatic effects on plants. This is because many soil microorganisms, especially bacteria and fungi, are decomposers that release the organic matter in labile forms for the plant roots to take up. Even soil macrofauna, such as species that prey on bacteria, can have an indirect effect on plant health through trophic cascades (94). It is argued that the belowground food web is the key regulator of plant productivity because the amount and rate of belowground decomposition dictates the availability of nutrients to the aboveground plant community (93, 94).

Soil Aggregate Environment

The belowground environment has a gaseous phase and liquid phase, but is dominated by the solid phase that consists mostly of sand, silt, clay, and soil organic matter. It is the assembly of these components into *soil aggregates* that dictates the microenvironment for soil bacteria and other microorganisms. A soil aggregate is a physical assembly of sand, silt and clay that is held together by the binding capacity of clay, fungal mycelia, bacterially produced exopolysaccharides, and other soil organic matter.

Within an aggregate there can be an array of environmental conditions depending upon the location within the aggregate (Figure 1.1). The location within an aggregate can dictate the protection from predators, the air content, and the availability of water and nutrients. The number of pores and the diameter of the pores is the *porosity* of the aggregates. In general, pore size

decreases into the interior of aggregates. It is the size of each pore that physically dictates whether predators can access microorganisms within an aggregate.

Air diffuses into aggregates through pores and the porosity governs how quickly air can move into the interior. Certainly, if there is moisture blocking these openings the diffusion through the water will greatly reduce the inflow of air. Many aggregates have a gradient of oxygen conditions with anaerobic interiors because respiration within depletes the oxygen faster than it can be replenished.

The amount of moisture within and between aggregates also directs the pH of the soils, the sorption of organic compounds, the availability of nutrients, and microbial translocation. If the porosity of aggregates is high, moisture can evaporate from the interior reducing bacterial movement and respiration.

Soil Aggregate Cycling and Carbon Sequestration

In practicality, aggregates are living units and indeed they do have a life cycle (Figure 1.2) (84). An aggregate is formed when new organic matter, older organic matter, and soil minerals are held together through salt bridging, and the binding capacity of clay and the soil organic matter itself. In the interior of aggregates, soil microbes decompose the organic matter. New organic matter becomes old, and old organic matter become particulate organic matter. This particulate organic matter can be rebound into smaller *microaggregates* within the interior of the primary *macroaggregate*. The ratio of bacteria to fungi is higher in microaggregates and lower in the outer macroaggregates suggesting that it is bacteria that are primarily responsible for the formation of microaggregates, while fungi bind the macroaggregates (13). Eventually, microbial decomposition degrades the binding agents of macroaggregate and it is disbanded. The contents,

microaggregates and particulate organic matter, are released to be incorporated into the new macroaggregates and repeat the cycle.

SOC is lost from agricultural soils through the acceleration of aggregate cycling. The loss can come about in two ways; carbon leaves the soil in the form of respired carbon dioxide, and unbound particulate carbon is leached from the soil. The net carbon loss occurs when aggregates are mineralized faster than new organic matter can be incorporated (84). Alternatively, carbon is sequestered when the rate of aggregate cycling is slow enough to allow more incorporation of organic matter into the aggregates than what is released.

Agricultural Management Effects

Tillage can accelerate the aggregate cycling and contribute to a net carbon loss (84). The influence of tillage on arable soils is multifaceted. Perhaps most obvious is the fact that when soils are tilled the aggregates are physically broken. Smaller aggregates have more surface area that is exposed to decomposers (31). Indeed, there is a burst of oxidative respiration directly following a tillage event, and the amount and variability of both carbon and nitrogen in soils that have been tilled repeatedly is significantly reduced (32, 41).

In addition, without the physical protection of the macroaggregate, stable microaggregates are less likely to be formed, as it has also been shown that tillage disturbs the soil bacterial communities that are responsible for aggregation (13, 83). When stable microaggregates are not formed, not only is less carbon sequestered in the soil, but there is an increased chance for additional carbon to be lost when unstable aggregates break (1). In fact, the *tensile strength* (a common measure of resistance to crush force) of aggregates in tilled soils is reduced (1).

Along with reduced tensile strength, tilled soils also have less aggregate porosity, and saturated hydraulic conductivity (a description of how easily water can flow) (66). It has been shown that tilled soils have significantly reduced biological diversity (36). It stands to reason that because tillage generates a more uniform soil aggregate environment, the diversity of the soil dwelling organisms would be more uniform, or less diverse (85). It is certain that the loss of soil carbon in tilled soils is facilitated by the manipulation of soil microorganisms.

Other management practices, such as the use of fertilizer, strive to improve the soil quality, carbon content, and ultimately plant productivity. Fertilizer can take many forms each of which has a different effect on soil microbes and their cycling of soil aggregates resulting in different effects upon soil carbon sequestration. The effects are absolutely dependent upon the quality and quantity of the fertilizer additions, along with the timing with respect to tillage events and weather conditions, as well as the individual soil traits.

Inorganic fertilizers such as the conventional nitrogen-phosphorous-potassium can induce an increase in SOC but only when it is timed with the addition of crop residue and only when compared to unfertilized soils (13, 36). When compared to organically fertilized soils there is less microbial biomass, the least stable macroaggregates and thus less SOC (13, 98). Any improvement in SOC is likely an indirect result of improved crop yield that is returned to the soil which stimulates the slight improvement of the soils by the soil microorganisms.

The change in ionic content of the soils affects the dispersive qualities of the clay. Because inorganically fertilized soil aggregates are smaller and less porous, they are more stable when dry, but in this case the stability does not increase the sequestration of carbon. This influence is especially great with regard to ammonium fertilizers which not only affects the clay

nature but also direct changes in the microbial community responsible for nitrification rather than those responsible for aggregation (13, 55, 62, 88).

Soil Bacteria of Interest

There are many bacterial groups that are studied for their relationship to, and effects on, soil, especially with regard to agriculture and global climate change. The studies included in this dissertation focused on bacteria belonging primarily to the genus *Flavobacterium*, but also of interest are bacteria belonging to genus *Cytophaga*. These Gram-negative, rod-shaped bacteria comprise a heterogeneous collection of which the taxonomy and nomenclature are still being clarified (8). At present, while the bacteria all belong to the phylum *Bacteroidetes*, the *Cytophaga* are in a completely different class (*Sphingobacteria*) from *Flavobacterium* (class *Flavobacteria*) (29). Currently, there are 40 recognized *Flavobacterium* species, 6 of which have been reassigned from the genus *Cytophaga* (29). The latter genus contains only 5 recognized species (29). Because the classification of these organisms has been rearranged numerous times since 1923 when the *Flavobacterium* genus was first described, many investigations have often grouped them as a single unit for study (8, 9, 29). They have been called the ‘flavobacter-bacteroides’ phylum, the *Flavobacterium-Cytophaga* complex, the *Cytophaga-Flavobacterium-Bacteroides* group, and *Cytophaga-Flexibacter-Bacteriodes* (8, 61).

In the studies herein, one of the PCR primers used to describe the diversity of these organisms in soils was named to be specific for ‘the *Cytophaga* group’ (89). During analysis, it was found that the majority of sequences amplified using this primer were of the genus *Flavobacterium*, with a few sequences detected from the *Cytophaga* genus. For these reasons, the group will be referred to as *Cytophaga-Flavobacterium* in the phylogenetic survey that follows.

Perhaps most importantly, and regardless of current taxonomy, these bacteria are well known for their production of a wide variety of hydrolytic enzymes. Both *Cytophaga* and *Flavobacterium* species produce many extracellular enzymes, with one of the current physiological distinctions between the two genera being the ability to degrade crystalline cellulose, such as filter paper (8). While *Flavobacterium* species can degrade cellulose derivatives using enzymes other than *cellulase*, only *Cytophaga* species are truly cellulolytic (8, 90). Species from these two genera have been shown to degrade; cell walls, cellulose, cellobiose, carboxymethylcellulose, chitin, starch, inulin, keratin, xylan, pectin, pectate, agarose, agar, agarose, alginate, laminarin, porphyran, lipids, DNA, RNA and casein (8, 18, 22, 26, 38, 48, 78, 97). The variety of enzymes produced by these bacteria shows that they can degrade many of the complex carbohydrates found in soil organic matter and may be influential in the cycling of soil carbon.

Cytophaga-Flavobacterium are globally distributed, as species belonging to these genera have been isolated from both aquatic and terrestrial environments (97). Many environmental surveys have detected the *Cytophaga-Flavobacterium* in competition for carbon sources, further supporting their potential to influence carbon cycling (7, 34, 40, 50). Of particular interest to these studies is their presence in the rhizosphere, where the cycling of soil organic carbon is most likely to influence plant productivity. Multiple investigations have shown that they are present in the rhizosphere, and that they are well adapted to colonizing plant roots (35, 37, 58, 63, 64). In addition, it has been found that the CFF comprised up to 70% of the bacteria isolated from the rhizosphere that were in possession of enzymes involved in carbon turnover (37). In one survey, '*Flavobacterium* and *Cytophaga* spp. were predominant in the most improved soil', further suggesting that they have a role in the cycling of soil organic matter (79). Another experiment

demonstrated that inoculation of the rhizosphere with a *Flavobacterium* species increased the activities of other enzymes that degrade complex carbohydrates; α - and β -galactosidase, and α - and β -glucosidase (57). Collectively these pieces of research suggest that *Cytophaga-Flavobacterium* spp. are involved in the degradation of polymeric carbon compounds on rhizosphere soil, however no investigations have specifically examined these bacteria.

Brief Summary

The loss of soil carbon has two major consequences that are not exclusive; global climate change, and loss of global food productivity. To counteract these negative consequences, the biotic and abiotic components of the soil matrix must be understood as well as the effects of anthropogenic manipulations upon them. Of particular interest is the role of the bacteria belonging to the genera *Cytophaga* and *Flavobacterium*. Their well-known production of enzymes that can degrade complex carbohydrates in combination with their active presence in the rhizosphere allude to a significant role in the cycling of soil organic matter and thus the sequestration or loss of soil carbon.

Brief Dissertation Overview

The main objective of these studies was to specifically investigate the *Cytophaga-Flavobacterium* in soils with an understanding of their potential role to influence the cycling of soil organic carbon. While many investigations have detected *Cytophaga-Flavobacterium* using indirect methods, no known studies have focused solely on the *Cytophaga-Flavobacterium*. The clone library studies described in Chapter 2 of this dissertation provide a survey of the distribution and diversity of *Cytophaga-Flavobacterium* in selected soils. It was expected that because the *Cytophaga-Flavobacterium* are likely involved in the cycling of soil organic carbon, that different *Cytophaga-Flavobacterium* communities might be detected within soils that have

varied land use history and thus different carbon contents. In these studies, soils of different land use history and management were sampled and used to construct clone libraries that only contained *Cytophaga-Flavobacterium* sequences. This was accomplished by the use of a reverse PCR in the initial DNA amplification that is considered specific to *Cytophaga-Flavobacterium* (89). The cloned sequences, representing different soil communities, were compared by soil treatment in an effort to detect patterns. These studies were purely exploratory in nature.

Further, evidence points to the potential for the *Cytophaga-Flavobacterium* to be involved in the cycling of soil organic matter. However, no studies have attempted to determine or confirm their role in this cycle. The experiments in Chapter 3 of this dissertation were designed to describe the effect of *Flavobacterium* isolates on remolded soil aggregates *in vitro*. The expectation is that should *Flavobacterium* spp. decrease the strength of soil aggregates *in vivo*, the rate of soil aggregate cycling would be accelerated and carbon would be lost from the less stable soil aggregates. In these studies, nine *Flavobacterium* soil isolates were used to inoculate remolded soil aggregates. After incubation, the aggregates were eroded using precision-machined chambers that allowed for the calculation of the *erosive strength* of the aggregates, or the force required to erode the aggregates. This value can be used to describe how readily an aggregate would break apart *in vivo*; accelerating the cycling of soil aggregates. It was hypothesized that inoculation of the aggregates with *Flavobacterium* spp. would result in a decrease of the erosive strength of the aggregates when compared to an uninoculated control group.

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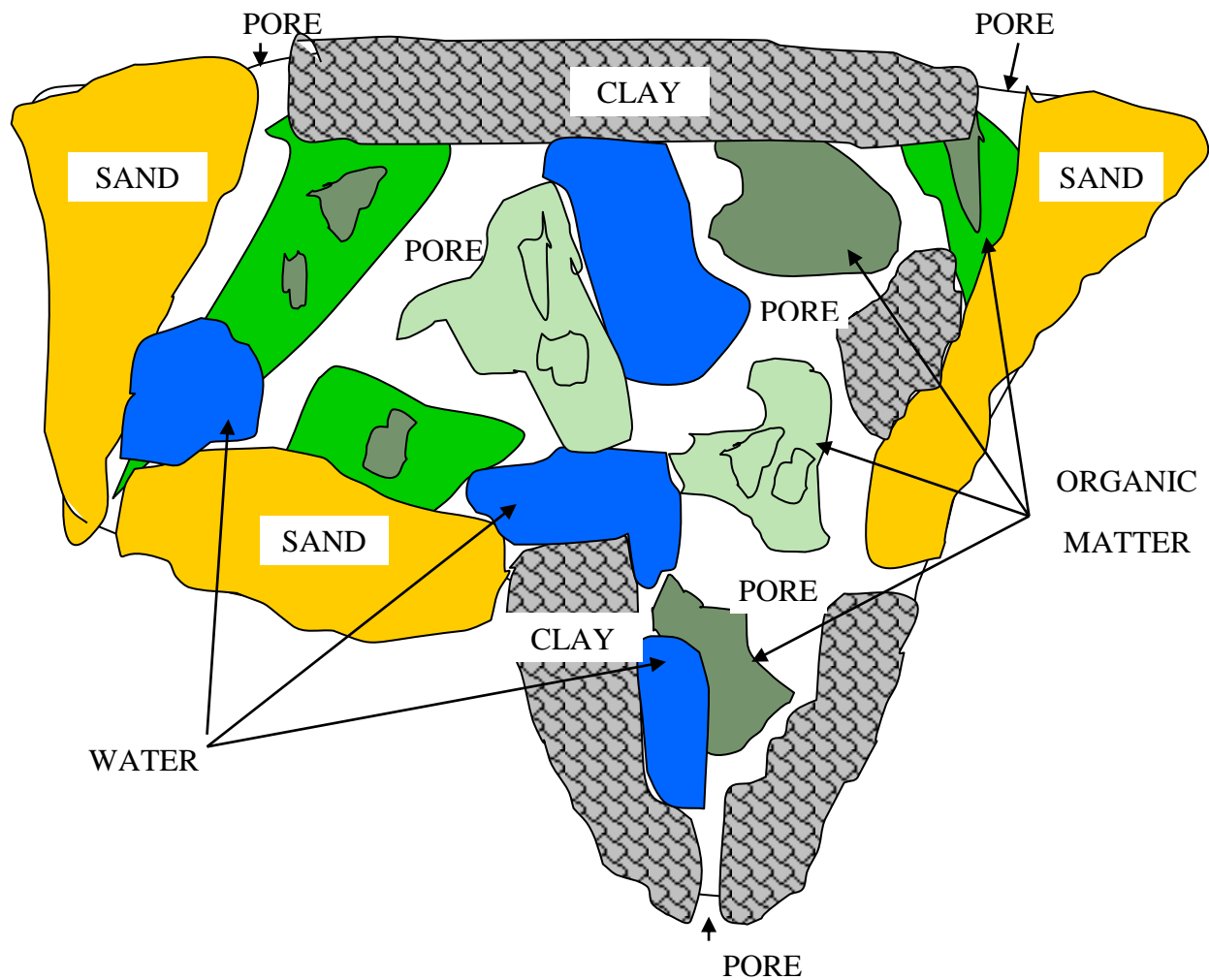


Figure 1.1 Diagram of a soil aggregate. A soil aggregate is composed of sand, silt and clay bound by fungal mycelia, bacterially produced polysaccharides, and the ionic binding of clay micelles and particulate organic matter. The openings into the interior of the aggregate are call pores. The number and size of pores determines the aggregate porosity. The presence of water in an aggregate determines the diffusion of oxygen into the aggregate as well as the translocation of bacteria. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

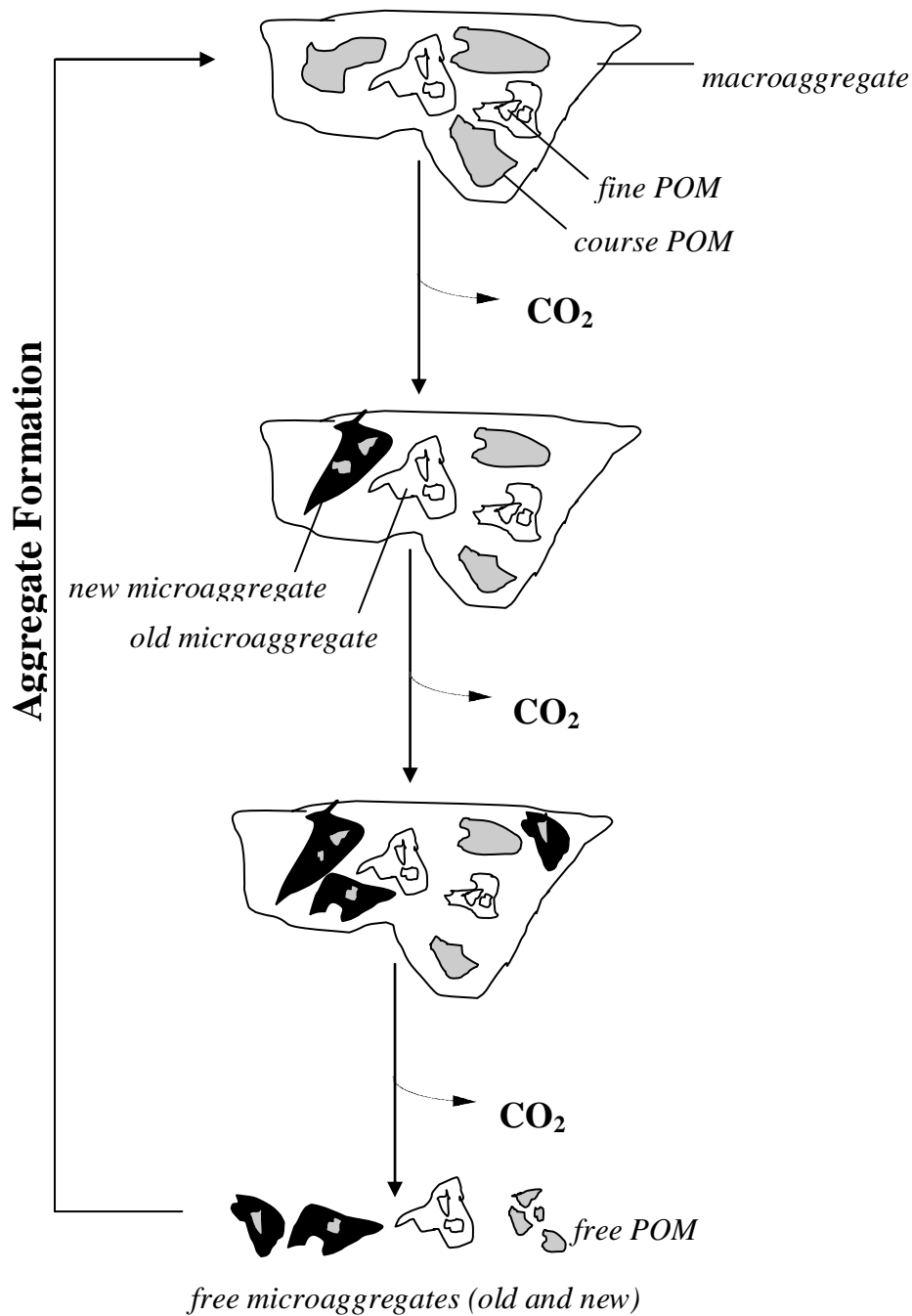


Figure 1.2 The cycling of soil organic matter in aggregates. This diagram depicts the formation of soil macroaggregates through the binding of new organic matter with old and soil minerals. Plant root hairs, fungal mycelia, and bacterial exopolysaccharides serve as binding agents in the formation. Over time, microbial degradation converts macroaggregates into microaggregates, and microaggregates into coarse particulate organic matter (POM) and then fine POM. The microbial activity causes carbon to be lost from the soil in the form of respired carbon dioxide. Sometimes, within the protection of the macroaggregate, POM is rebound into microaggregate form. Eventually the adhesive components binding macroaggregates are degraded and the contents are released to be bound into new macroaggregates or to be leached from the soil. Adapted from a figure by Six *et. al.* 2000

Chapter 2

Taxa Partitioning of the *Cytophaga-Flavobacterium* in Agricultural and Non-Agricultural; Fertilized and Unfertilized; and Till and No-Till Soil Communities

Abstract

The *Cytophaga-Flavobacterium* (CF) genera of bacteria are capable of influencing soil organic matter; a parameter that governs crop yield and sustainability. In this study the phylogenetic distribution of CF was compared between agricultural and non-agricultural soils at the Michigan State University, Kellogg Biological Station, Long Term Ecological Research Site. Using a CF-specific primer, 648 cloned 16S rRNA gene sequences were derived from agricultural, early successional, late successional, and deciduous forest soils. The agricultural community had an asymmetrical distribution when compared to the non-agricultural community ($p = \leq 0.006 - 0.012$). The species richness and diversity indices were lower than the non-agricultural samples. No more than 35% of the operational taxonomic units (OTUs) found in the agricultural soil were unique when compared to the non-agricultural soil. A second sampling was for the purpose of comparing soils that differed only in the application of fertilizer or in annual tillage. Unfertilized soils harbored only slightly different CF phylogenetic distributions ($p = 0.554$). The richness and diversity was similar with over 60% of the OTUs being shared between them. The communities from tilled soils had significantly different distributions ($p = \leq 0.001$). While many OTUs were shared, the calculated richness and diversity was lower in the no-till soil.

Introduction

Many studies have focused on the impact of soil organic matter and how it affects agricultural yield and sustainability (25, 46, 76, 99). It is known that the reduction of soil carbon has a consequential, and often irreversible, loss of crop production. With the understanding that the soil microbial communities are inextricable members of the agricultural food web (68, 93), research has often focused on the effects of agricultural management practices on soil microbes (10, 14, 73). It has often been noted that conventional management practices such as tillage, fertilizer, and the conversion from high plant diversity to monoculture crop rotations has measurable impacts on the microbial communities (16, 19, 24).

The *Cytophaga-Flavobacterium* (CF) are a group of globally distributed bacteria that have potential to influence the cycling of organic matter (7, 23, 34, 40, 50). These bacteria are often characterized by their ability to degrade complex carbohydrates (18, 22, 26, 38). In addition they have been found both in the *rhizosphere*, or the plant root zone, (37, 63, 64), and are well adapted to colonizing plant roots (57, 64, 79). Moreover, the CF were found to comprise 70% of the bacteria isolated from the rhizosphere that were in possession of enzymes involved in carbon turnover (37).

The primary goal of this study was to examine the phylogenetic distribution and diversity of CF species in an agricultural soil and in non-agricultural soils to determine if differences could be detected. Using 16S rRNA clone libraries, significant differences in the richness, diversity, and phylogenetic distribution between agricultural and non-agricultural soils were found. Soils sampled were from Michigan State University's Kellogg Biological Station Long Term Ecological Research Site where the conventionally managed agricultural system included the use of herbicides and pesticides; the application inorganic fertilizer; annual tillage and soil

finishing; as well as the annual crop rotation between corn, soybean and wheat. This study further examined the two specific management practices of soil tillage, and the use of fertilizer, to determine if they could be cited as potential driving forces that select for different CF communities.

Methods

KBS LTER

Michigan State University Kellogg Biological station (KBS) is centrally located in the lower Michigan peninsula (42° 24' N, 85° 24' W, elevation 288 m). Established in 1988, the long term ecological research site (LTER) is 48 hectares, on which there are seven different cropping systems (Treatments 1-7). Important to this study were: treatment 1, a conventional agricultural rotation of corn, soybean, and wheat; treatment 2, a conventional agricultural rotation of corn, soybean and wheat that is not tilled; and treatment 7, an early successional field site that had not been tilled since 1989. Treatments 1, 2, and 7 are replicated in six, one hectare blocks on the LTER main site. Treatment 8 is replicated in 4 blocks of 15m x 40m that are located 200m off of the main LTER site. There are three replicate 1ha sampling sites within the deciduous forest, all located within 5 km. The T1 agricultural treatment is chisel plowed annually in spring at a depth of 6-7 inches. It is soil finished (4 inches) and rotary hoed as necessary. Also included in this study are treatment 8, a never-tilled late successional treatment; three old-growth deciduous forest sites, and their corresponding fertilized subplots. The fertilized forest subplots are 2 square meters and received 3g N as ammonium nitrate from their creation in 1995 to 1998. For the two years that followed, no fertilizer was applied. Starting again in 2001 20-30g N was applied as either urea or ammonium nitrate. Then, in the year of sampling, only 10g N was applied as urea.

The dominant soil type is Kalamazoo (fine-loamy, mixed, mesic Typic Hapludalfs) and Oshtemo (coarse-loamy, mixed, mesic Typic Hapludalfs). The average annual temperature at KBS is 9.7°C. The annual precipitation is 890 mm/y on average; about half is snow.

Sampling procedures

Agricultural vs. Non-Agricultural Comparison. All samples were collected as indicated in Table 2.1. Soil cores from treatments 1, 7, 8 and the deciduous forest sites were collected on December 9, 2003. At all replicate plots, for all treatments sampled, there are five sampling sites from which soil cores were extracted. The five soil cores were 0-10 cm in depth and 2.5 cm in diameter. All cores from a replicate plot were pooled and homogenized. The soil was transported on ice (no more than two hours) then transferred to -80°C for storage.

Fertilizer Comparison. On January 5, 2008, five soil cores 0-10cm and 2.5 cm in diameter were taken from each sampling site at each of the three replicate plots of the unfertilized forest soil (DF). Because the area of the fertilized sub-plots was significantly smaller than that of the forest replicate plots, only three soil cores were taken from each of the three replicate fertilized plots (DFF). Samples were stored on ice for no more than two hours during transport to the laboratory. Upon arrival at the lab, soil cores were stored at -20° until the DNA could be extracted.

Tillage Comparison. On January 7, 2008 five soil cores were taken from each of the six replicate plots for both soil treatments 1 and 2. Samples were handled in the same manner as the fertilizer samples.

DNA Extraction

The DNA was extracted from the soils of the first sampling event using an SDS-based DNA extraction method (102). After extraction, DNA was run on a 0.7% agarose gel overnight at 30V, 4C and electroeluted. This was done to further purify the DNA by removal of humic

acids. DNA from all 6 replicate plots was extracted separately, then pooled for PCR amplification. Also prior to use for PCR, the DNA was heated to 65°C for 1 hour to further eliminate inhibitors to the PCR.

Both subsequent soils were extracted using a MO BIO Laboratories, Inc. (Solana Beach, CA) UltraCleanTM Soil DNA Isolation Kit. The lysis step 6 in the standard protocol was modified to use a mini bead beater (BioSpec Products) for 1 minute. DNA from all 6 replicate plots was extracted separately, then pooled for PCR amplification. The DNA was not further treated.

PCR Amplification and Cloning

PCR The PCR reactions were 25µL and contained 1x PCR reaction buffer (Invitrogen, Carlsbad, CA), 25µg bovine serum albumin (Invitrogen), 1.6mM MgCl₂ (Invitrogen), deoxynucleotide triphosphate at a concentration of 0.2mM (Invitrogen), 27F primer (5'AGA GTT TGA TCM TGG CTC AG 3'), 1349R (5' GGA TCA TGG CTG ATA TCC GAT 3') primer each at a concentration of 0.5µM (Integrated DNA Technologies, Inc., Coralville, IA), and 5U *Taq* DNA polymerase (Invitrogen). The 27F primer is considered general for the bacterial 16S ribosomal RNA gene, and the 1349R primer is specific for the genera *Cytophaga-Flavobacterium* (89).

The reactions were conducted in a Perkin Elmer GeneAmp PCR System 2400. Initial denaturation of DNA at 94°C for 5 minutes was followed by 30 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 56.5°C for 30 seconds, extension at 72°C for 1.5 minutes. There was a final extension period at 72°C for 7 minutes, and then the samples were incubated at 4°C until they could be retrieved for agarose gel analysis (1% agarose and ethidium bromide staining).

Transformation. An InvitrogenTM TOPO TA Cloning[®] Kit was used according to the vendor's recommended protocol. The PCR product (1.5 μ L) was inserted into the pCR 2.1 TOPO[®] Vector, transformed into TOP10 One Shot[®] chemically competent DH5 α *Escherichia coli* and screened on LB supplemented with kanamycin and X-gal. White colonies with suspected inserts were picked for sequencing. The efficiency of cloning was evaluated by checking 6 colonies each for inserts using restriction digest.

Sequencing. For the first sampling event, a total of 648 clones were successfully sequenced by Michigan State University's Research Technology Support Facility (MSU RTSF). Sequences have been submitted to Genbank. T1 sequences from 2003 are EF377707-EF377877, T7 are EF377878-EF378060, T8 are EF378061-EF378264, and DF are EF378265-EF378428. For the fertilizer comparison total of 210 clones were successfully sequenced by MSU RTSF. Sequences have been submitted to GenBank. DF-2 is FJ532799-FJ532978 and DFF is FJ532979-FJ533152. The clones from the tillage comparison were sequences by Macrogen, Inc. (Seoul, Korea); totaling 228. Sequences have been submitted to GenBank. Accession numbers for T1-2 are FJ53250-FJ532649. Accession numbers for T2 are FJ32650-FJ53798.

Ecological Indices and Statistical Methods

Tree Construction. Sequences were aligned in ARB (54) using the SILVA database. At least 14 *Bacteroidetes* type strain sequences (named by Bergey's Taxonomic Outline) that were not included in this database were downloaded from GenBank and added (29). Initially, the sequences were entered by parsimony into a pre-cast neighbor-joining tree that contained 381 *Bacteroidetes* type strain sequences (named by Bergey's Taxonomic Outline; obtained from GenBank). Forty nine type strain sequences that neighbored cloned sequences were chosen.

From the first soil sampling, a total of 152 T1, 193 T7, 168 T8 and 152 DF sequences that were a minimum of 500 bases in a common region were chosen along with 49 type strain sequences. A filter was calculated that considered 377 valid nucleotide positions (vnps). Using this filter, a neighbor-joining tree was cast that included all of the mentioned sequences.

A total of 211 DF and DFF sequences (108 DF and 103 DFF) that were at least 550 bases long in a common region were used along with the 49 *Bacteroidetes* type strains to calculate a new filter. The filter considered 451 vnps and was used to cast the neighbor-joining tree.

A total of 117 T1 and 111 T2 sequences that were at least 500 bases long in a common region were chosen along with the same 49 *Bacteroidetes* type strain sequences to generate a new filter. The filter considered 389 vnps and was used to cast a new neighbor-joining tree.

DOTUR. Distance matrices from each tree were exported from ARB using the same filters used to construct the trees. The matrices were used for assignment to operational taxonomic units (OTUs), and calculation of richness and diversity indices by DOTUR (80). The number of OTUs, and Chao1, ACE, Shannon and Simpson indices are listed in Table 1. The threshold distance for OTU assignment was chosen as 0.03.

SONS. To analyze the number of OTUs shared between treatments, new distance matrices were exported from ARB that contained only the pairwise comparisons intended for analysis in SONS (81). The same filter used to generate the tree in which the sequences were cast, was also used to filter the distance matrix.

UniFrac. For the purpose of analyzing phylogenetic distribution of the cloned sequences and the environmental distance between treatments, UniFrac was used. New neighbor-joining trees were constructed that included only KBS sequences and the root *Bacillus cereus* because type strains

were not wanted for the analysis. The same filters used previously were also used to cast the new trees.

Newick formatted trees were exported from ARB for use in the web-based UniFrac analysis tool (52). UniFrac was used to calculate the environmental distance. Unweighted UniFrac Significance analyses were performed for each pair of environments as well as each environment individually with 1000 permutations. More than one analysis was performed and obtained p-values were different, however, categorization of significant or not remained unchanged.

Results and Discussion

Agriculture vs. Non-Agriculture

To compare the phylogeny of the *Cytophaga-Flavobacterium* from soils of several land usages, including agricultural and non-agricultural plots, 648 cloned sequences were generated. First, all sequences that were at least 500 nucleotides in length in a common region were used to construct a phylogenetic tree that also contained 381 type strains from the phylum *Bacterioidetes* to determine the phylogenetic breadth of the sampling. When the cloned sequences were compared to type strain sequences, it was found that nearly all clustered with the *Flavobacterium* species confirming the specificity of the reverse primer in the set used to generate the clones.

Forty nine type strain sequences that surrounded the cloned sequences were chosen to construct a smaller phylogenetic tree (Figure 2.1) for the purpose of comparing the sequences from different soil treatments. When the tree was cast it was noted that several of the sequences from the agricultural treatment did not group with sequences from the non-agricultural treatments. The percentage of sequences contributed by each treatment to each clade was

calculated based upon the total number of sequences per treatment. Many of the clades are dominated ($\geq 5\%$ difference between contributions) by sequences from either agricultural or non-agricultural clones (indicated by arrows in Figure 2.1). In other terms, one treatment or treatments contributed greater than or equal to 5% more of the total number of sequences sampled from that treatment. Clades 2 and 3 are dominated by sequences obtained from the conventionally managed agricultural plot (T1) with clade 2 containing 43% of the 152 T1 sequences used in this study. Alternatively, clades 1, 4, 5, 6, and 7 are dominated by sequences from the non-agricultural plots with nearly equal proportions from each treatment. Clade 5 is dominated by just the deciduous forest (DF) sequences.

To determine if the asymmetrical distribution of the sequences was statistically significant, the treatments were compared based upon the branch lengths of Figure 2.1 using UniFrac (52). Environmental distance values are a measure of how alike two treatments are based upon the branch lengths of shared sequences. The environmental distance values (Table 2.2) demonstrate that the agricultural treatment (T1) is most distant from the other three treatments.

It might be expected that T1 would be more alike to the early successional (T7) as T7 has a history of tillage, but of the three non-agricultural treatments, T1 was less distant from the never-tilled late successional treatment (T8; 0.801) than T7 (0.747). It was not unanticipated, however, that the successional treatments are more alike to each other (0.600) than either is to the deciduous forest (DF) sequences.

The significance values presented for the pairwise comparisons in Table 2.2 are the corrected (Bonferroni) p-values for the UniFrac significance test. This test is a pairwise comparison of the distribution of the treatments in the phylogenetic tree (Figure 1.1) based upon

a random redistribution of the sequences to treatments. The significance values verify that the distribution of the T1 sequences significantly differs from that of both T7 and DF ($p = \leq 0.006$). However, as shown by the environmental distances, Treatments 1 and 8 are less different from each other with only a tendency toward significance ($p = 0.012$).

The probability values for the individual UniFrac Significance test allows for a better understanding as to which specific treatment is directing the significance of the differences seen in the pairwise comparisons. According to these values, DF has the most unique branch lengths when compared to chance ($p = 0.001$), followed by T7 ($p = 0.202$), meaning that it is likely these two treatments that are driving the significant difference seen when compared to T1. This also means that T1 has very few unique branch lengths when compared to DF and T7. In fact, the distribution of T1 is equal to that which would be found if the sequences were randomly distributed ($p = 0.928$). The distribution of T1 has no unique features when compared to that of the other treatments, while unique branches are found in the distribution of the other treatments. This shows that there is little to no phylogenetic diversity found in T1 that is not also found in the other treatments; the CF community in T1 is a subset of the communities in the other treatments.

To further examine the idea that T1 is a subset of the phylogenetic diversity found in the three non-agricultural communities, the sequences were then compared based upon their phylogenetic content. To do so, an operational taxonomic unit (OTU) had to be defined based upon the percentage of sequence similarity. The similarity of 97% was chosen because it is a commonly accepted species level threshold (86), although it should be noted that the relationships between the treatments remains the same at 96 and 98 percent sequence similarity.

Sequences that were 97% similar to one another were binned into the same OTU using the programs DOTUR (80). The number of OTUs, as well as richness estimators, and diversity indices are presented in Table 1. Immediately it can be seen that T1 has the least number of OTUs. Because of this, both the Chao1 and ACE richness estimators are the lowest for T1, predicting only 57 and 63 total OTUs possible in the T1 soils. These values strongly suggest that there is little species richness found in the agricultural treatment when compared to the other treatments.

The Shannon and Simpson diversity indices are also the lowest for T1. These indices consider not only the OTU richness, but also the evenness, or species abundance; the frequency of distribution of sequences into the OTUs. The 151 sequences from T1 binned into 35 OTUs but one OTU contained 66 sequences or 43% of all T1 sampled sequences (data not shown) demonstrating an *uneven* frequency of distribution. (It is interesting to note that all but 9 of these sequences are from clade 2.) The unevenness of this distribution along with the low species richness contributed to the low diversity index values.

The T8 and DF sequences also binned into OTUs with one OTU containing an uneven number of sequences. However, these OTUs contained only 17% and 16% of the T8 and DF sequences respectively, not 43% as in T1. These non-agricultural treatments are more even than the agricultural treatment. The T7 sequences showed the most diversity with 56 OTUs as well as the most evenness with no more than five sequences difference between OTUs (data not shown). This is demonstrated by T7 being assigned the highest richness estimates and diversity values.

Once the sequence data was binned into OTUs, pairwise comparisons could be made between treatments based upon the number of OTUs each treatment shared with another. A program called SONS (81) was used to perform this comparison and the relationships are

represented in Figure 2.2. When compared to the non-agricultural treatments, T1 has the least percentage of unique, or not-shared, OTUs in all comparisons. This lack of unique OTUs further illustrates that the CF community in T1 is likely a subset of one or all of the other treatments. Specifically, when compared to T8, T1 has only 3 OTUs (just 3 unique sequences) that are not shared with T8 (data not shown). Alternatively, T8 shares 17 OTUs with T1, but has 25 sequences in 12 OTUs (46%) that remain unique to T8. This relationship between T1 and T8 also corroborates that which was demonstrated by the UniFrac environmental distance and significance values in Table 2.2.

The relationship of early successional, late successional, and deciduous forest treatments represented by the analysis in Figure 2.2 is what might be expected due to succession. The early (T7) and late (T8) successional treatments are highly similar based upon the number of shared OTUs. In addition, the OTUs in T8, and the 40-60 year old-growth forest treatment (DF) are similar, although somewhat less so. Lastly, T7 and DF share the least number of OTUs in these three pairwise comparisons. This also reflects the relationship represented by the environmental distance values in Table 2.2.

Since members of the CF have been found to be prominent in the rhizosphere, it is possible that the CF community diversity is the greatest in the early successional soil (T7) because the plant community diversity is also the greatest in that treatment. Greater plant diversity leads to greater rhizodeposition (nutrients deposited by plant roots in the rhizosphere), as well as leaf litter deposited on the soil surface, both of which may lead to a greater diversity of bacteria, such as the CF, feeding upon the organic matter (5, 95). Perhaps while an early successional soil (T7) has the greatest CF diversity, this diversity decreases with time as the plant community becomes more uniform or with the conversion to monoculture crop growth (51, 101).

Over time changes in the bacteria will be clonally replicated eventually shifting toward a population with different diversity. This is one possible explanation for the differences seen with the T1 sequences.

Comparison of Fertilizer Addition Management

To compare the distribution of CF sequences from soils that are fertilized to CF sequences from soils that are not, 107 sequences from the 40-60 year old late successional forest, and 103 sequences from a subplot of the successional forest were compared. The fertilized forest subplots are 2 square meters and received 3g N as ammonium nitrate from their creation in 1995 to 1998. For the two years that followed, no fertilizer was applied. Starting again in 2001 20-30g N was applied as either urea or ammonium nitrate. Then, in the year of sampling only 10g N was applied as urea.

First, sequences that were at least 550 bases in common were added to the large tree containing 381 type strains from the phylum *Bacteroidetes*. They clustered mostly with the *Flavobacterium* type strain sequences, further confirming the specificity of the reverse primer used in the set. The type strains that clustered most closely with the sequences were chosen to cast a new, smaller tree (Figure 2.3).

The percentage of sequences contributed by each treatment to each clade was calculated based upon the total number of sequences per treatment. Of the 17 clades of sequences, there are four that are dominated ($\geq 5\%$ difference) by sequences from one soil treatment or the other. These clades are indicated by arrows in Figure 2.3. Two clades are from the unfertilized deciduous forest (DF) and two are from the fertilized deciduous forest (DFF). The separation of the sequences into such unevenly distributed clades initially suggested there is a difference in the CF communities sampled from the two soil types, and thus a potential for fertilization to be

influential. However, the UniFrac significance test (Table 2.2) assigned the treatments a non-significant probability value (0.554) indicating that there is no true difference in the phylogenetic distribution of the two communities based upon branch lengths.

To compare each soil treatment using another method, sequences that were at least 97% similar were sorted into the same OTU using DOTUR (80). The number of OTUs found in each treatment was nearly the same with 55 and 53 OTUs for DF and DFF respectively (Table 2.1). When the number of OTUs that are shared between the two treatments was calculated (Figure 2.2) it was found that that each treatment maintains at most only one third of the total number of OTUs as unique, demonstrating that each community represented nearly the same species richness.

Other studies have found that in fertilized soils there is less microbial biomass when compared to undisturbed soils and concurrently, the measured abundance of cellulase and amylase producers was also lower in fertilized soils suggesting that there was an effect of fertilizer upon the CF community in that investigation; although the CF was not directly quantified or examined (55). In this study, the calculated diversity indices that consider both species richness and species abundance in each OTU were compared. The diversity indices for both treatments were nearly the same and no effect of fertilizer upon the CF community could be detected.

Other studies have reported a shift in the nitrifying bacterial community in soils under the application of fertilizer (42, 88, 100). It is possible that the byproducts from nitrification, and the resultant change in soil pH, are the causative agents driving this conversion of the bacterial community structure. In the first soil sampling comparison, a statistically significant difference in the CF community is seen. The respective pH for T1 and DF is 6.3 and 5.6 (Kellogg

Biological Station data). Unfortunately, the pH of the soil in the DFF plot has not been calculated. Also, while the T1 plot has been established in the LTER since 1988 making the comparison between T1 and the 60 year old DF one of long-term soil management, the DFF subplot had only been fertilized irregularly for 8 years at the time of sampling. It is possible that there had not been enough time for measurably statistically significant changes to occur.

A potential explanation for changes in the CF community as a result of fertilization, is that the passage of unique metabolic species from the nitrifying bacteria to the soil community influences the CF. Studies have demonstrated close interactions between nitrifiers and the CF population through the passage of metabolites and labeled cell wall components (39, 62). Another study demonstrated the dependence of one CF species upon another soil organism. The CF species was found to co-culture with *Bacillus cereus*, and the former was respiring the peptidoglycan of the latter (26, 69). It stands to reason that changes in the nitrifier community diversity as a response to fertilizer will change the available substrate diversity for the CF species, supporting a different CF population. It is possible that the indirect effects of fertilizer on the CF population would be more pronounced had the soils been sampled during plant growing season instead of winter dormancy.

Comparison of Tillage Management

Tillage was also considered as a possible force driving the CF community differences in agricultural versus non-agricultural soils. To compare the effects of tillage, 117 sequences from a sampling of the T1 agricultural plot (T1) compared to 111 sequences from the no-till agricultural plot (T2). The same 49 *Bacterioidetes* type strains used in Figures 2.1 and 2.3 were also used to construct a phylogenetic tree (Figure 2.4).

There are eight clusters indicated by arrows in Figure 2.4 that are dominated by sequences from one of the soil treatments, three are dominated by sequences from T1, and five are dominated by sequences from T2. The UniFrac significance test (Table 2.2) verified that the distribution of the sequences from each treatment are different from one another with high significance ($p = \leq 0.001$). The significance is due to branches contributed by sequences from the tilled soil treatment (T1) as shown by the individual significance values ($p = \leq 0.001$).

Not only is there a difference in the phylogenetic distribution of the CF between tilled and no-tilled soils, but there is also a difference in the richness and diversity values generated by DOTUR (Table 2.1). Despite the large overlap of shared OTUs demonstrated by the SONS data (Figure 2.2), Table 2.1 shows that the no-till soils (T2) have fewer OTUs than the tilled treatment and therefore the assigned richness estimates are also less. In addition, the diversity indices were also lower for T2 than T1.

With all this in consideration, it appears that tillage is a potential selective force upon the CF community. However, it is worthy of noting that while the CF community from the tilled agricultural soil in this sampling had greater richness and diversity than the no-tilled agricultural soil community, in the first soil comparison the CF community in the agricultural soil was the least rich and diverse when compared to other not-tilled non-agricultural soils.

The difference is likely because the soils were sampled after different crops rotations. The first sampling of the tilled soil was following soybean cultivation, and the second followed the harvest of wheat. As discussed previously, there is an association between the root exudate and leaf litter deposition, and the belowground microbial community (5, 95). If the soils were sampled after different crop rotations, it would be expected that different CF communities might be present.

Focusing just on the act of tillage, there are a number of explanations for how the physical turnover of soil could affect the CF diversity. This manipulation aerates the soil and reduces the moisture content. The CFF are motile by gliding and a reduction in soil moisture could restrict their translocation, thereby affecting the distribution of CFF in the soil. In addition, after a tillage event there is an increase in oxidative respiration by soil microorganisms, which is reflected by a community shift to aerobic species (16, 21, 33). This increase in respiration further dries the soils because the soil temperature is increased. Miscellaneous evidence alludes to a CF preference for cold temperatures (11, 14, 18, 34, 50, 60, 87) and it is not inconceivable that a rapid or dramatic temperature increase would change the diversity of any bacterial community, including the CF community.

Lastly, it is known that CF species are aerobic organisms (18). In consideration of the last soil sampling for the comparison of effects of tillage, it is possible that the aeration of the soil would select for CF that are able to out-compete other bacterial species during the rapid increase in respiration. Such competition could come at the expense of the less competitive species, such as those that are less aerotolerant; have less enzymatic capabilities or slower enzymatic rates; or those that are less metabolically efficient. The greater 'fitness' of the CF species could result in the observed greater diversity in T1-2 tilled agricultural soils than the T2 no-tilled agricultural soils.

Acknowledgements

The author would like to thank Kristen Huizinga for generously providing the extracted DNA from December 2003. The author would also like to thank Natasha Isaacs-Cosgrove for generating five of the nine clone libraries used in the first sampling comparison. Thank you very much; the foundation of this study lies on your efforts.

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Table 2.1 Description of samples according to treatment, sample date, and number of sequences. The number of OTUs, and richness estimators, and alpha diversity indices were calculated using DOTUR.

Soil Treatment	Sampling Date	Abbrev.	No. of Seqs.	No. of OTUs	Chao1	ACE	Shannon	Simpson (1-D)
Standard Chemical Input corn/soybean/wheat Rotation Conventionally Tilled (corn/soybean prior to 1992) Crop Prior to Sampling: Soybean	Dec. 9, 2003	T1	152	35	57	63	2.5	0.80
Early Successional Community Historically Tilled Soil (Ended 1989)	Dec. 9, 2003	T7	176	56	104	113	3.5	0.96
Late Successional Community Never-Tilled Soil	Dec. 9, 2003	T8	168	53	83	92	3.4	0.95
40-60 Year Old Late Successional Deciduous Forest	Dec. 9, 2003	DF	152	45	64	75	3.3	0.95
40-60 Year Old Late Successional Deciduous Forest	Jan. 5, 2008	DF-2	107	55	93	98	3.7	0.98
Sub-Plot of Deciduous Forest Receiving Conventional Agricultural Fertilizer Input	Jan. 5, 2008	DFF	103	53	127	134	3.7	0.97
Standard Chemical Input corn/soybean/wheat Rotation Conventionally Tilled Crop Prior to Sampling: Wheat	Jan. 7, 2008	T1-2	117	47	76	72	3.6	0.97
Standard Chemical Input corn/soybean/wheat Rotation No-Till Crop Prior to Sampling: Wheat	Jan. 7, 2008	T2	111	29	33	35	3.0	0.94

Table 2.2 Environmental distances and significance of phylogenetic distribution calculated using UniFrac. For the UniFrac significance test, sequences are randomly reassigned to a treatment within the phylogenetic tree. This can be done as a pairwise comparison or an examination of each treatment individually. The UniFrac significance test is a measure of the number of reassigned trees that have a UniFrac value greater than or equal to that assigned to the original tree. The p-values represent the probability that the treatment in question has more unique branch lengths than what would be found by chance alone. The p-values are corrected using the Bonferroni adjustment.

Treatment	Environmental Distance				Significance of Distribution			
					Pairwise		Individual	
	DF	T1	T7	T8	DF	T1	T7	T8
DF	▪				▪			0.001
T1	0.882	▪			≤0.006	▪		0.928
T7	0.733	0.801	▪		0.018	≤0.006	▪	0.202
T8	0.723	0.747	0.600	▪	0.534	0.012	1.00	0.591
DF-2							0.243	
DFF	0.561				0.554		0.786	
T1-2							≤0.001	
T2	0.711				≤0.001		0.250	

Figure 2.1a

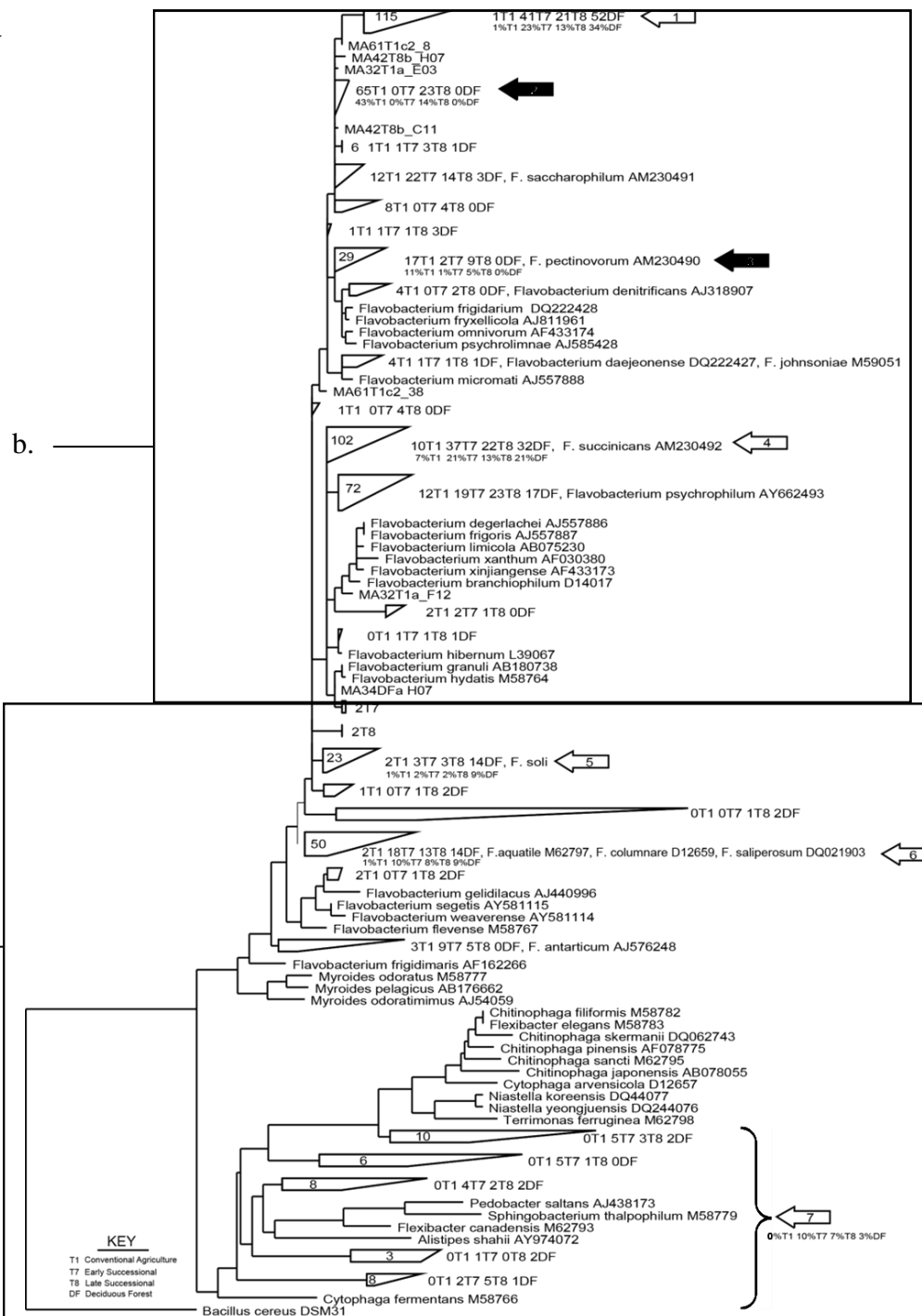


Figure 2.1 Neighbor-joining phylogenetic tree of sequences derived from agricultural (T1), early successional (T7), late successional (T8) and deciduous forest (DF) soils. Figure 2.1 is divided into two sections such that text is legible. a.) Boxes denote how the figure is divided into portions b and c. Parts b.) and c.) insets are expanded clusters. Arrows note the clades that have an asymmetrical proportion ($\geq 5\%$ difference) of agricultural or non-agricultural sequences. Percentages noted are based upon the total number of sequences sampled from that treatment. For example, in clade 1 there are 52 DF sequences, or 34% of the 152 sequences samples from DF.

Figure 2.1b

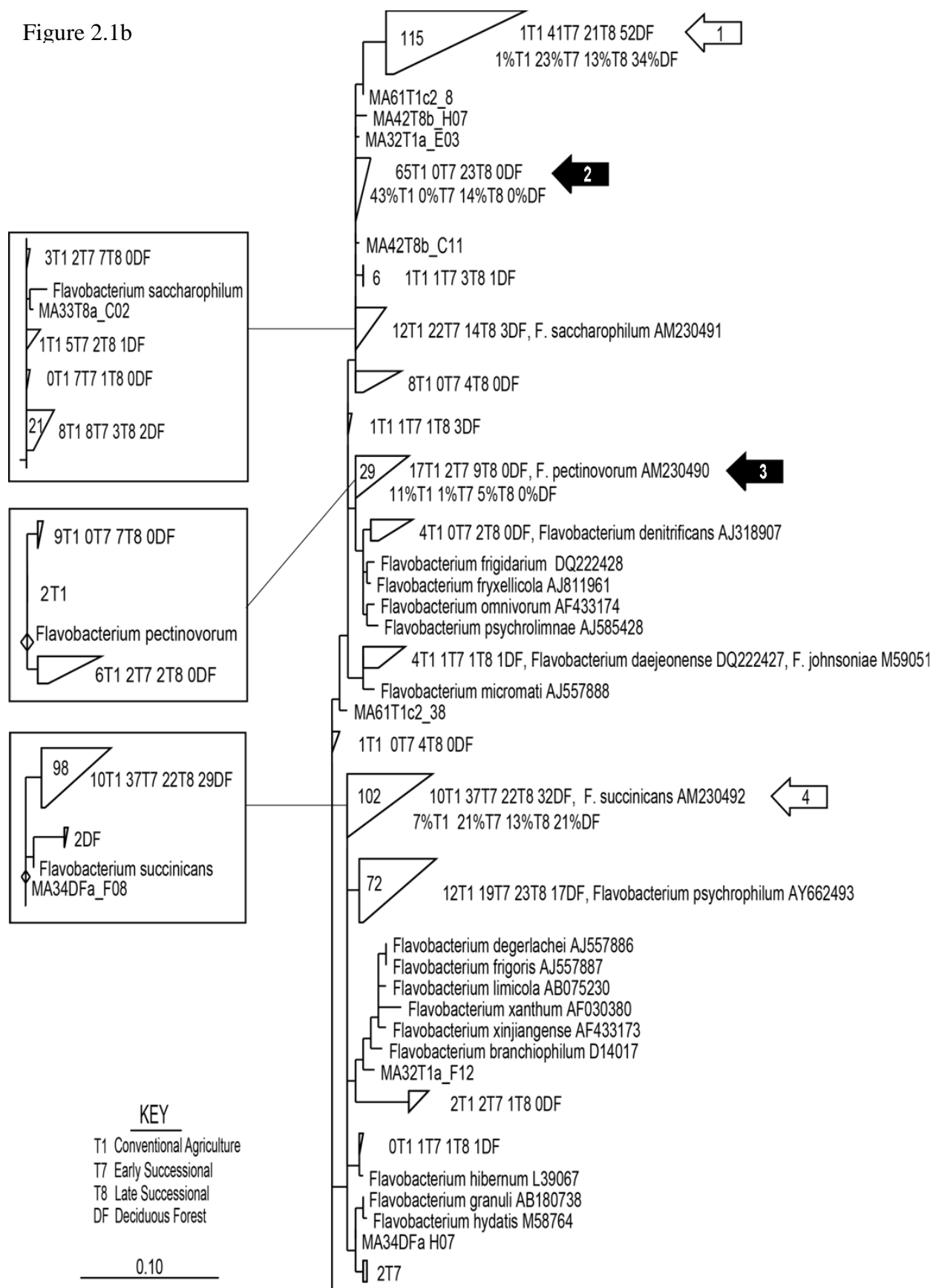
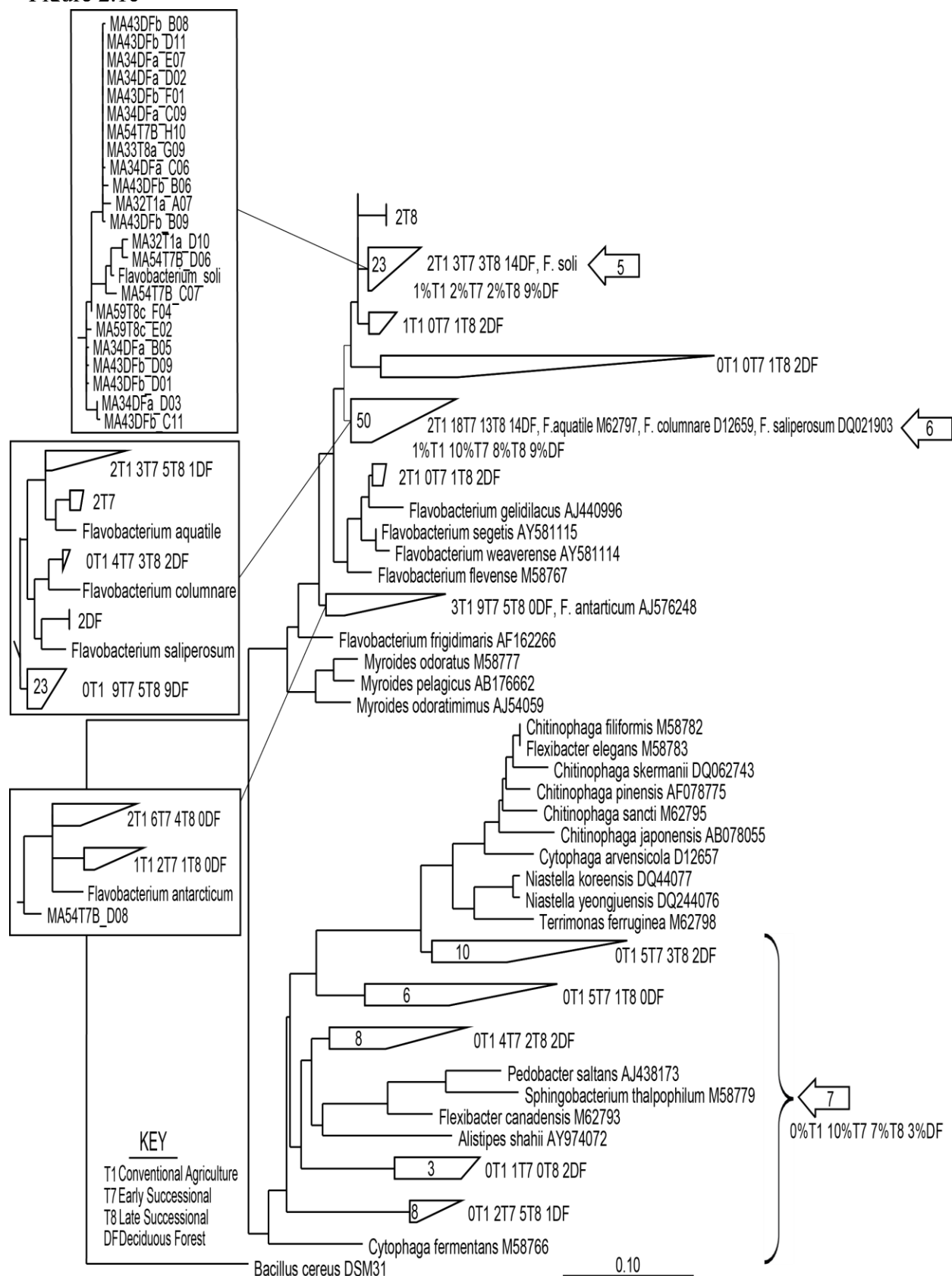


Figure 2.1c



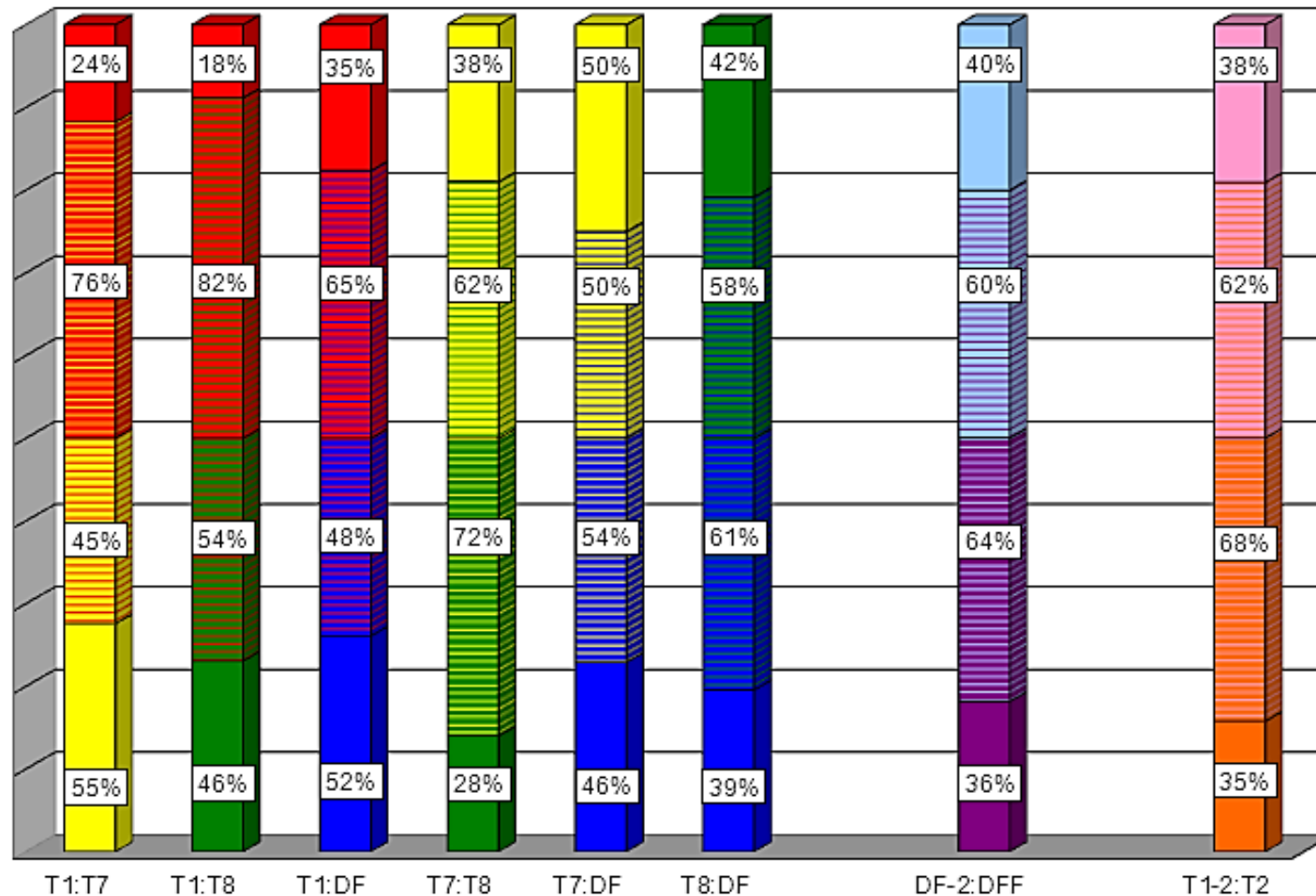


Figure 2.2 Percentage of DOTUR assigned operational taxonomic units (assigned at 97% sequences similarity) that are shared between sampled treatments. Percentages are based upon the total number of OTUs assigned to each treatment individually. Pairwise comparisons were performed using DOTUR and SONS and are noted on the x axis; the first listed treatment is represented on top (Top:Bottom). For example, in the comparison of T1 to T7, 76% of the total OTUs assigned to T1 were also represented by sequences in the T7 sample; 26% of the T1 sequences were not.

Figure 2.3a



Figure 2.3 Neighbor-joining phylogenetic tree sequences derived from fertilized (DFF) and unfertilized (DF-2) deciduous forest soils. Figure 2.3 is divided into two sections such that text is legible. a.) Boxes denote how the figure is divided into portions b and c. Parts b.) and c.) insets are expanded clusters. Arrows note the clades that have an asymmetrical proportion ($\geq 5\%$ difference) of fertilized and unfertilized soil community sequences. Percentages noted are based upon the total number of sequences sampled from that treatment.

Figure 2.3b

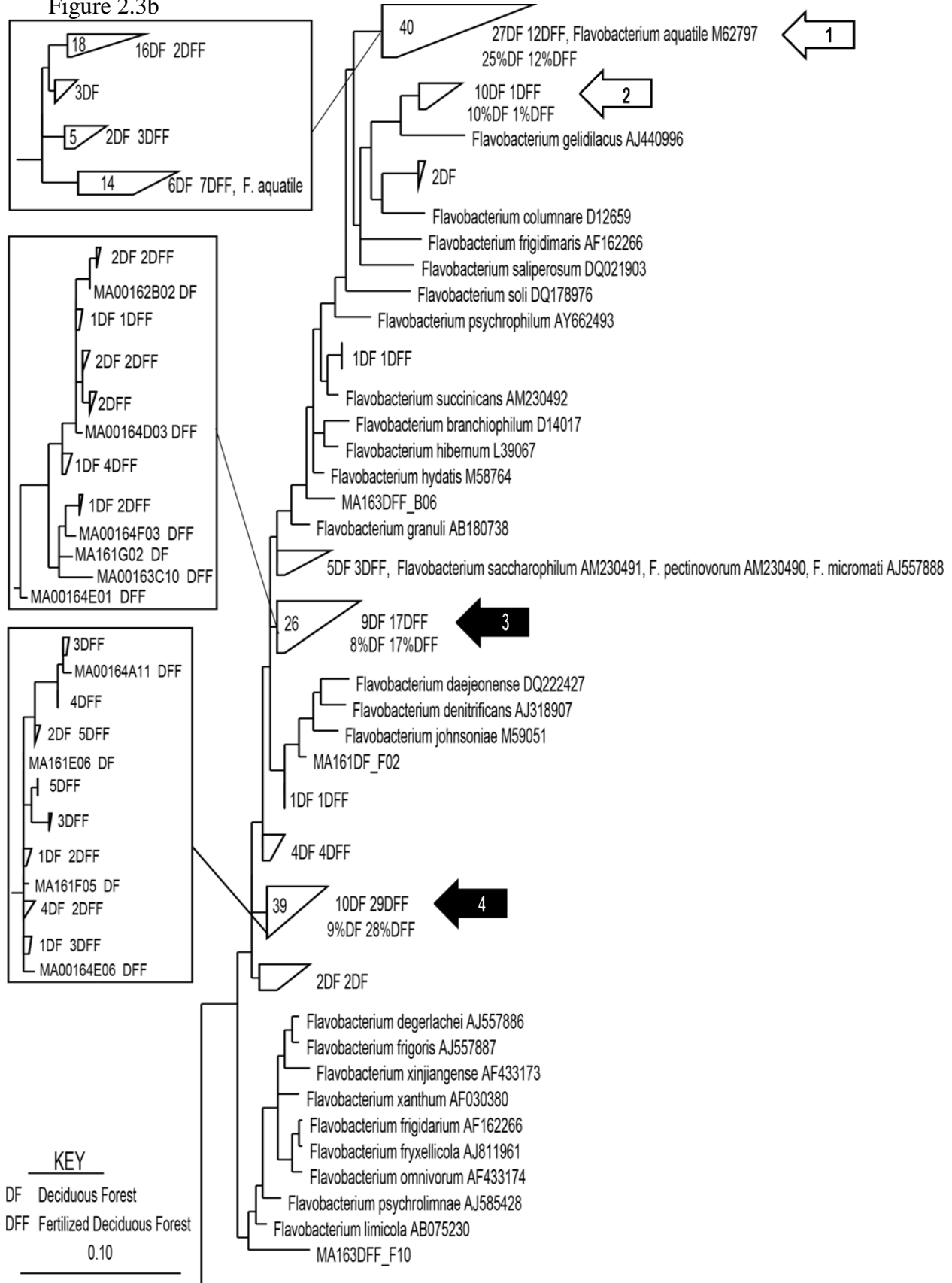


Figure 2.3c

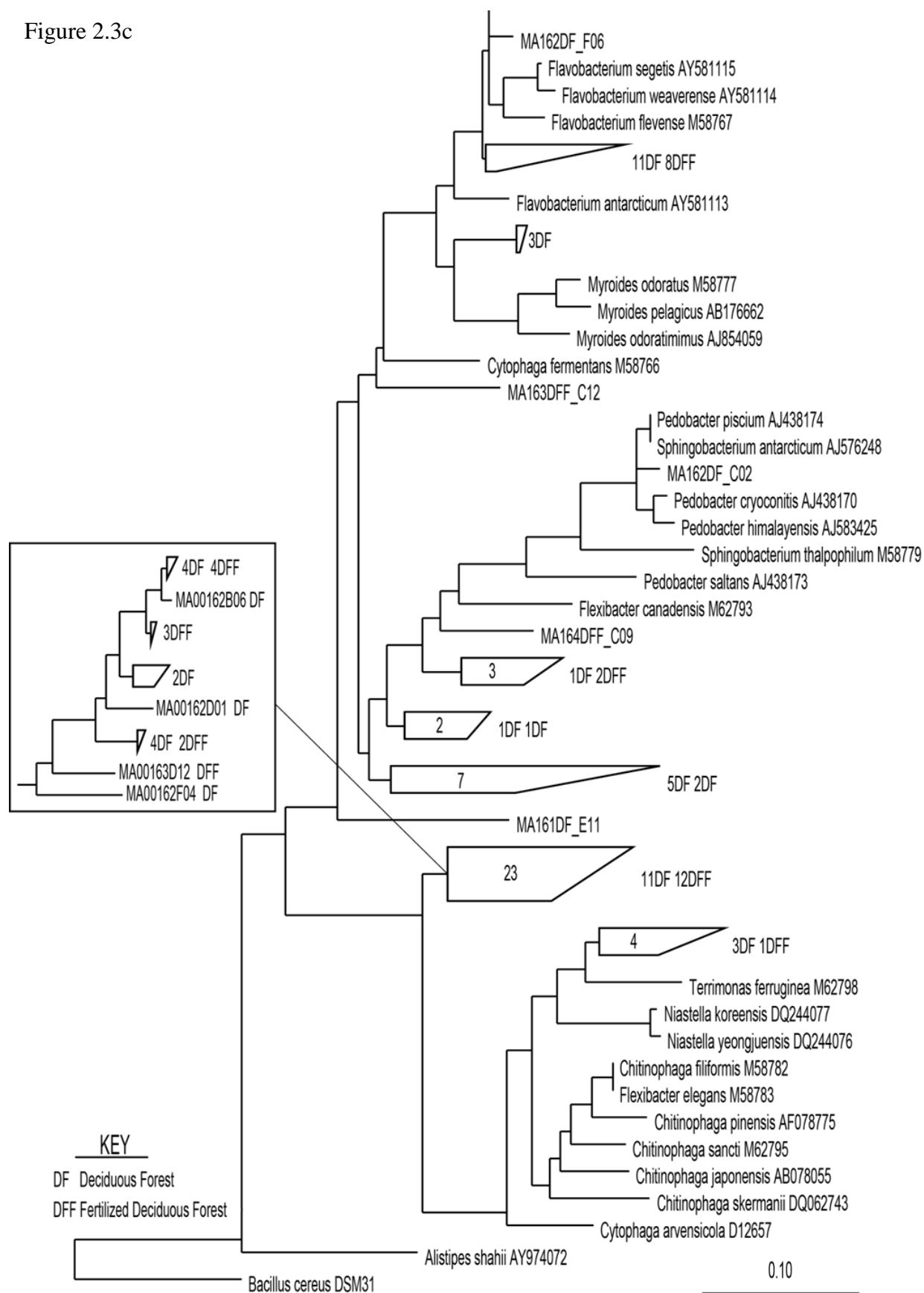


Figure 2.4a

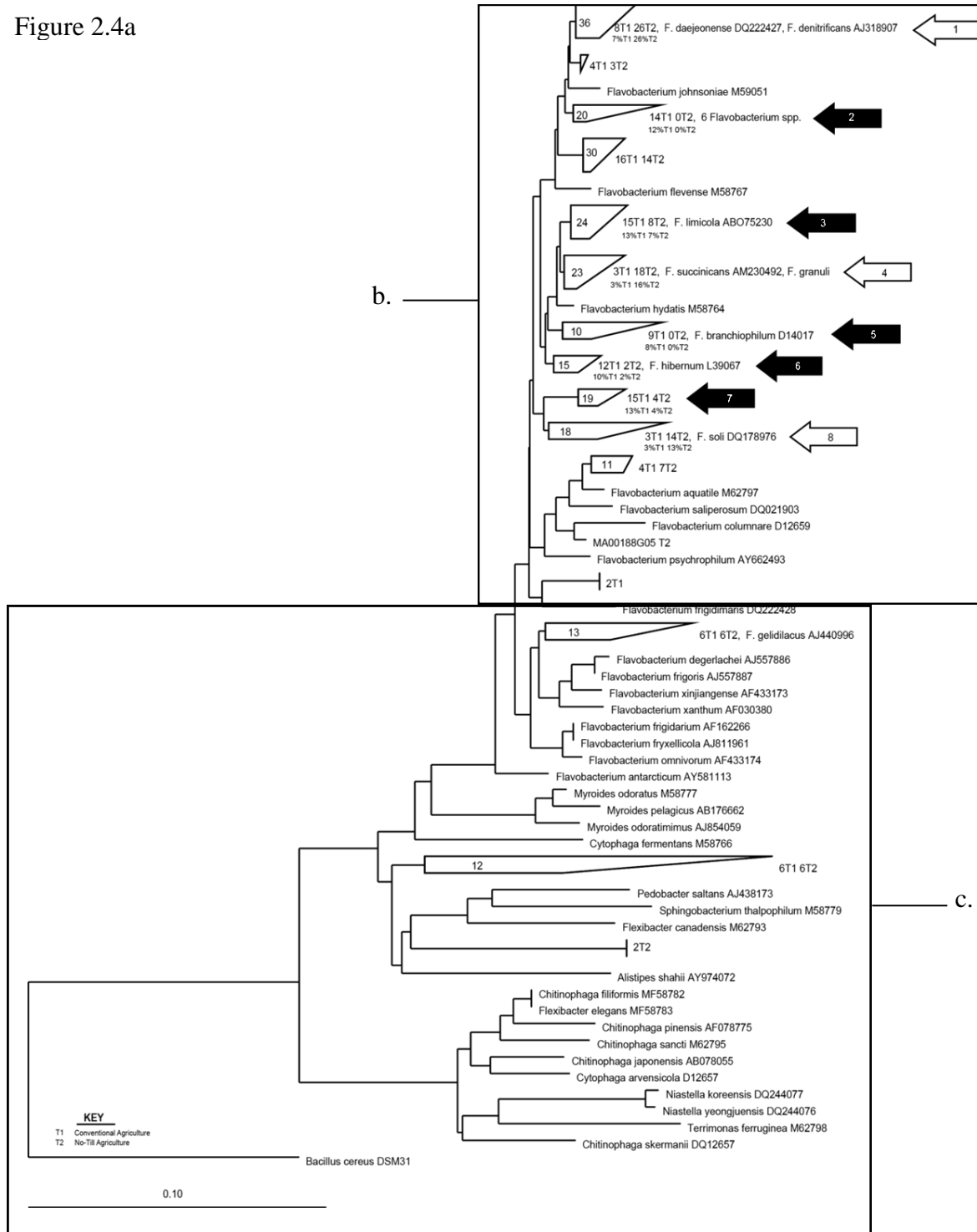


Figure 2.4 Neighbor-joining phylogenetic tree of sequences derived from the tilled (T1-2) and no-till agricultural soils. a.) Boxes denote how the figure is divided into portions b and c. Parts b.) and c.) insets are expanded clusters. Arrows note the clades that have an asymmetrical proportion ($\geq 5\%$ difference) of tilled and no-till soil community sequences. Percentages noted are based upon the total number of sequences sampled from that treatment.

Figure 2.4b

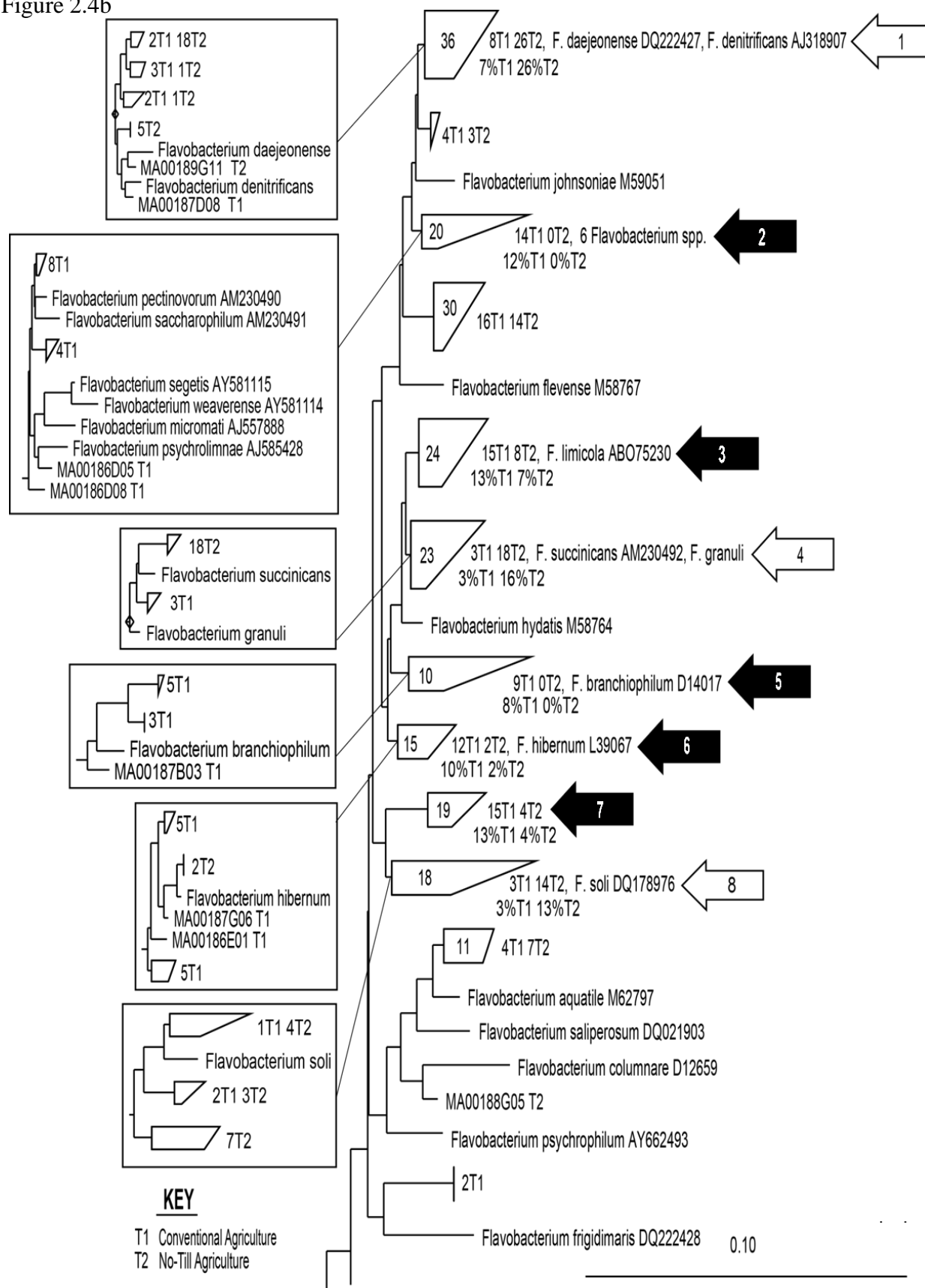
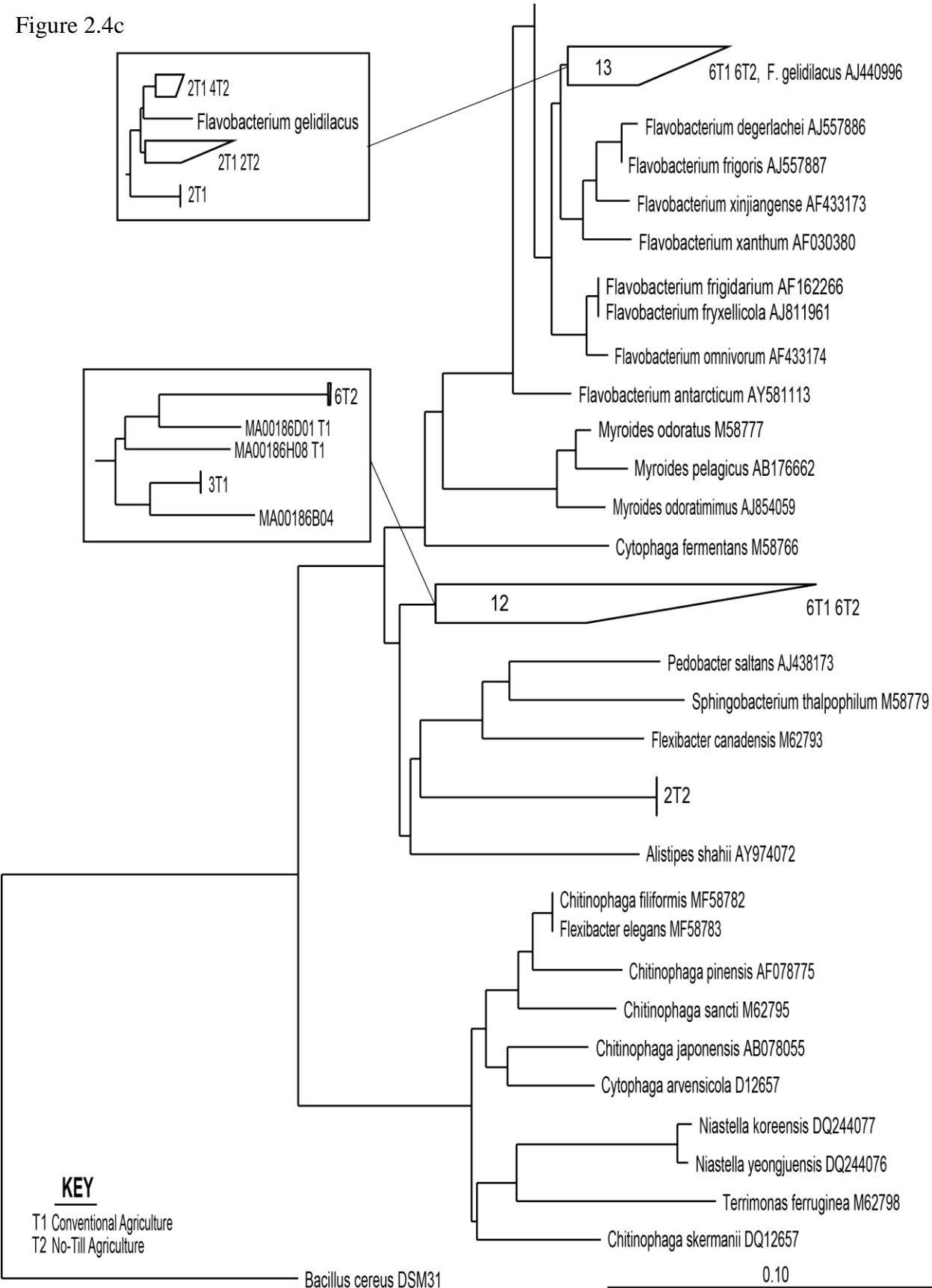


Figure 2.4c



Chapter 3

***Flavobacterium* Species Decrease Strength of Remolded Soil Aggregates *in vitro* and may Contribute to the Loss of Soil Organic Carbon in Native Systems**

Abstract

The stability of soil aggregates dictates their rates of formation, decomposition and associated carbon loss, and recycling directly controlling the amount of carbon sequestered by soil. Bacteria belonging to the genus *Flavobacterium* have the potential to influence the stability of soil aggregates because they produce a variety of enzymes that degrade the complex carbohydrates known to bind the mineral fractions within soil aggregates. *Flavobacterium spp.* were isolated from soil and described for their potential to degrade xylan, to produce extracellular polysaccharides, and their gliding motility on soil extract. Remolded soil aggregates amended with xylan were treated with the *Flavobacterium* isolates to determine the bacterial effect on aggregate strength. Carbon respiration, during incubation was monitored to describe this activity. We measured the erosive strength of the aggregates (N/mg/min) using precision-machined soil aggregate erosion chambers; a highly sensitive technique for examining the strength of concentric layers within individual aggregates. Of the 9 *Flavobacterium* isolates tested, 8 degraded the xylan, reducing the erosive strength of aggregate layers. The erosive strength of aggregates from 6 treatments were significantly reduced at $p < 0.05$ when compared to the uninoculated control. One isolate appeared to increase the erosive strength, yet was not statistically significant. A *Sphingobacterium sp.* was included in this study to confirm the sensitivity of these techniques to detect significant increases in erosive strength. These studies indicate that *Flavobacterium spp.* are likely responsible for the accelerated erosion of native soil aggregates that can lead to a loss of organic carbon from soils.

Introduction

The amount of soil organic carbon directly affects soil structure, plant growth and crop yield (83, 94). Plants provide carbon sources to the belowground organisms in three major pathways; soluble root exudates, litter decomposition products, and partially decomposed and recalcitrant organic matter (75). Soil microorganisms decompose these organic constituents, releasing bound nutrients as labile forms more readily available to plants (72).

Microbial decomposition of soil organic matter (SOM) results in an assortment of forms. During the formation of soil aggregates, newly deposited organic matter combines with older recalcitrant organic matter and soil minerals. SOM stabilization of soil aggregates is facilitated by binding agents such as plant root hairs, fungal mycelia, extracellular polysaccharides, and cations which further cement mineral constituents together during frequent wetting and drying cycles (67). Microbial microsites located within macroaggregates, are frequently separated from most of the plant residue and associated particulate organic matter (POM). Therefore, until these stable larger soil volumes are broken down into smaller microaggregates (<250µm), by mechanical tillage, freezing/thawing, wetting/drying and other natural forces much of the sequestered POM is protected from microbial degradation. As soil respiration continues, more of the polysaccharides and other cementing compounds are sorbed to clay surfaces forming new microaggregates which continue reforming into larger macroaggregates.

In this way, soil organic matter (SOM) has a cycle (84). Greater soil sequestration of carbon occurs when the rate of aggregate breakdown and re-assembly, or recycling is slow enough to allow more incorporation of SOM in the forms of particulate organic matter (POM) or dissolved organic matter (DOM) released by microbial mineralization, into aggregate interiors

(1). Aggregate recycling results from a plethora of water-based environmental variables, inorganic binding agents, roots, soil fauna, and soil microorganisms (83).

Bacteria belonging to the genus *Flavobacterium* have the potential to influence the stability and recycling of soil aggregates. *Flavobacterium* are globally distributed, including terrestrial environments (14, 50, 64). Perhaps most importantly, *Flavobacterium* spp. are often characterized by their ability to degrade complex carbohydrates (18, 22, 26, 38). Species belonging to this genus are often found in competition for carbon sources (37, 39, 62). Because of their diverse enzymatic capabilities, *Flavobacterium* may be responsible for the degradation of large polymeric carbohydrates that serve as aggregate bonding agents; reducing POM-sized SOM to DOM. During these catabolic processes, carbon is lost from the soil in the form of respired carbon dioxide, often reducing the strength of aggregates as the bonding agents are degraded. Degradation of adhesive agents accelerates the rate of aggregate recycling by increasing their breakage; exposing sequestered POM to further decomposition and leaching. Therefore, we believed *Flavobacterium* isolates, applied to remolded soil aggregates will reduce their surface strength that leads to aggregate destabilization, breakdown and recycling.

To determine if *Flavobacterium* species, in pure culture, would indeed contribute to aggregates instability, sterile remolded aggregates enriched with xylan, the most abundant polysaccharide on Earth, were inoculated with pure *Flavobacterium* cultures. After incubation, the aggregate surfaces were eroded using precision-machined soil aggregate erosion chambers to determine the erosive strength (E_s) of concentric layers removed from aggregate surfaces (65). Calculated erosive strengths (N/mg/min) of aggregate surfaces removed were statistically compared to uninoculated controls to determine bacterial contributions to aggregate stability.

Flavobacterium isolates were characterized in an effort to describe their potential to decrease the E_s of remolded aggregates enriched with xylan. Growth of the isolates on xylan and in soil extract media enriched with xylan was determined both qualitatively and quantitatively. In addition, while it was predicted that the *Flavobacterium* isolates would decrease the E_s of concentric surfaces of remolded aggregates enriched with xylan, we also investigated the potential for isolates to increase stabilities of aggregate surfaces. In some studies *Flavobacterium* species have been associated with ‘slime layers’ or biofilms (4, 6, 70). Contributory roles of extracellular polysaccharide production by soil bacteria are known to facilitate the production and strengthening of macroaggregates (83, 84). *Flavobacterium* isolates selected for this study were screened for their production of a capsule and for the formation of biofilms to describe their potential to generate such exopolysaccharides. A *Sphingobacterium* soil isolate reported to aggregate soil was also tested to serve as a potential positive control (15).

Methods

Enrichment for Flavobacterium spp.

Soil cores (0-10cm) were sampled from the NSF-LTER at the Michigan State University Kellogg Biological Station (KBS) on November 15, 2008. Cores were sampled from several cropping systems; early and late successional soils, and deciduous forest soils. They were homogenized, transported on ice, and stored at 4°C for no more than 7 days.

Approximately 0.25g of each soil was used to inoculate enrichment media consisting of mineral base and trace element solution SL-6 (3); 100µg/mL cyclohexamide; and 1% w/v of one of the following; agar, agarose, cellulose, chitin, gelatin, starch, or xylan. One culture received no additional carbon. Enrichments were incubated at 16°C. After 62 days, cultures were

sampled, serially diluted, and spread onto R2A agar. Plates were incubated at room temperature for 2 days then transferred to 4°C for 7 days. Using a dissecting microscope and needle, colonies that demonstrated yellow pigmentation and motility were purified on fresh R2A.

Isolate Screening and Sequencing Identity

Isolates were screened by whole-cell PCR using a 1349R primer, reported to be specific for strains belonging to the genera *Cytophaga* and *Flavobacterium* (89). Pure cultures were sequenced at the MSU Research Technology Support Facility using a 27F primer. GenBank accession numbers are HQ917614-HQ917677. A *Sphingobacterium* isolate, generously donated by Caesar-TonThat was added to the culture collection (15).

Sequences were aligned in ARB (Ludwig, *et al.*, 2004) using the SILVA database. At least 14 *Bacteroidetes* type strain sequences (Bergey's Taxonomic Outline (29)) that were not included in this database were downloaded from GenBank and added to confirm the identity of the isolates as *Flavobacterium spp.* A phylogenetic tree was constructed for comparison of sequences. Distance matrices were exported from ARB using the same filter made to cast the tree (389 valid nucleotide positions). DOTUR was used to group the isolates into operational taxonomic units at 97% sequence similarity (80).

Growth on Xylan

Isolates were inoculated onto xylan agar (0.1%, 0.2%, 1%, 2%, 5% w/v); 5x mineral base with trace element solution SL-6 (3) or M9 minimal media; with 15g agar and xylan from oat spelts or birchwood (Sigma, St. Louis, MO). Plates were incubated at approximately 25°C for 2-3 days then qualitatively scored for growth.

For quantitative analysis, soil extract media (SEM) was generated using soil from the unmanaged rim of the KBS LTER as follows. Dry soil was sieved (2mm) and wetted with 2x

deionized water then autoclaved for 1 hour (121°C, 15psi). The slurry was cooled, decanted and frozen at -20°C in a frost-free freezer overnight. The extract was gravity filtered through a Whatman No 1 cellulose filter and 25mL aliquots were autoclaved again for 20 minutes. Soil extract was considered 20X and diluted accordingly for subsequent inoculations. Xylan broth (0.1% w/v xylan in M9), selected because it was the amount of carbon used in the enrichments for isolation, was also inoculated and scored.

Isolates were grown in R2A broth overnight then diluted 1:10 into soil extract broth, soil extract broth with 0.1% w/v xylan, and M9 broth with 0.1% w/v xylan in a microtiter plate. The plate was incubated at room temperature without shaking for 7 days. The optical density was recorded at 600nm in a Spectramax M2 microtiter plate reader (Molecular Devices). Uninoculated controls were used to zero the spectrophotometer. Tests were performed in triplicate (n = 3 wells). Optical densities were averaged. Because the media used was inherently opaque, the optical densities were normalized with the uninoculated controls.

Production of Exopolysaccharides

Twenty one isolates, selected for their maximum growth on xylan and the *Sphingobacterium sp.* were screened for the production of capsules. Capsule stains using India Ink were examined microscopically and scored for presence or absence of capsule-like material. Isolates used in this study were also scored for biofilm formation (6). Following the measurement of optical density described above, cultures were aspirated, the wells were stained with crystal violet for 5 minutes, and then aspirated again. The optical density of adherent stained cells was recorded at 530nm in a Spectramax M2 microtiter plate reader (Molecular Devices). Tests were performed in triplicate (n = 3 wells). The optical densities were normalized

with the uninoculated controls which still demonstrated some adherence of media components to the wells.

Motility

Cultures were grown overnight in R2A broth with 1% SEM and 10 μ L were spotted on three replicate agar plates containing 1% v/v SEM. The diameter of the spots was marked and the plates were sealed and incubated at room temperature for 7 days. After each 24 hour period, the edge of the growth front was marked. After the total 7 day incubation the maximum change in diameter and the maximum distance traveled for each 24 hour period was measured, and averaged for each treatment.

Generation and Analysis of Remolded Soil Aggregates

Michigan State University's Kellogg Biological station (KBS) is centrally located in the lower Michigan peninsula (42° 24' N, 85° 24' W, elevation 288 m). The dominant soil type is Kalamazoo (fine-loamy, mixed, mesic Typic Hapludalfs) and Oshtemo (coarse-loamy, mixed, mesic Typic Hapludalfs). Soil (0-10cm) from the non-treatment space around the edge of the KBS Long Term Ecological Research Site was sieved (53 μ m) to extract the silt and clay. The calculated soil texture was consistent with the reported values (lter.kbs.msu.edu) of 43% sand and 57% silt/clay.

In a mortar, 57g of silt/clay were mixed with 43g of clean silica sand (mesh -50 +70, Sigma) and wetted with approximately 30mL deionized water containing 1% w/v xylan. The slurry was pipetted into a 12.7mm spherical silicone mold (Chicago School of Mold Making, Chicago, IL). The mold was warmed at 65°C to dry the remolded aggregates overnight. The mold was weighed both prior to and after drying to determine the intraggregate pore space of the average aggregates. A CT scan of five aggregates showed between 0.73-2.5% of the pores were

greater than 11 μ m (92). Aggregates were wrapped in aluminum foil and autoclaved for 50 minutes to sterilize.

Aggregate Inoculation and Incubation

Pure overnight culture of *Flavobacterium* sp. and the *Sphingobacterium* sp. were resuspended in M9 with 1% w/v xylan broth to a cell density such that inoculation of each aggregate with 160 μ L of broth culture would equate to 10⁹ cells per gram dry weight soil; the same cell density determined by quantitative PCR (data not shown) and a correct volume to fill 80% of the pore spaces. Using a pipetteman, each sterile aggregate was uniformly wetted with 4 x 40 μ L of broth culture. Pilot study tests demonstrated that the wetting front would move to the center of the aggregates within 2 minutes of addition but it was not known if the bacteria could also migrate to aggregate interiors. Aggregates were suspended on a sterile wire tray inside a sterile glass container and sealed for incubation.

The duration of incubation was 7 days, during which 1-3 aggregates were aseptically removed daily and sacrificed for determination of cell density by spread plate counts. Also daily, the headspace of each glass microcosm was flushed with sterile air and sampled through a septum. Samples were analyzed using a LI-COR CO₂ Analyzer (model 6252) and the accompanying Hewlett Packard 3390^A Reporting Integrator. Three to five samples for each microcosm, at each time point, were averaged then compared to a standard curve to determine the amount of carbon dioxide produced per gram of soil per hour.

Measuring Erosive Strength (E_s)

At the end of the 168 hour incubation, the microcosms were flushed continuously overnight with dry, sterile, air to air-dry the aggregates and kill the cultures by desiccation. Dried

aggregates were weighed and placed into a soil aggregate erosion chamber (65). This method was chosen over soil crushing strength primarily because we wanted to measure concentric surface strengths in contrast to the whole aggregate. Because the *Flavobacterium* isolates were added to the surface of sterilized remolded aggregate without multiple wetting and drying cycles which increase the connectivity of soil pores, it was not known if they could migrate to aggregate interiors. It was expected that their strengthening/weakening activities would be restricted to thresholds of their initial penetration depths of the aggregate.

Aggregates were enclosed before being eroded on a rotary shaker at rates between 200 and 400 rpms. Every 5 to 10 minutes the mass of the aggregate eroded and passed through the 352 μ m screen was weighed and the concentric surface was eroded until at least 33% of the total aggregate mass was eroded. The erosive strength (E_s) was calculated using the following formulas (65):

$$E_s = C / \left[\frac{m(t_n) - m(t_{n-1})}{t_n - t_{n-1}} \right] \quad [1]$$

Where:

$m(t_n)$ and $m(t_{n-1})$ are the masses of the aggregates at removal times

t_n and t_{n-1} are the removal times (min)

C is the centrifugal force (Newtons) defined by equation 2:

$$C = m(t)[R_{SAE} - R_{aggregate}]\omega^2 \quad [2]$$

The original radius (R) of the aggregate, prior to erosion, was 6.35cm. During erosion the diminishing aggregate radii were calculated, assuming it retained the shape of a uniform sphere, using the percent mass eroded the volume was calculated using the soil bulk density of the partially eroded aggregates. All reformed aggregates were of a uniform spherical volume conforming to the spherical mold and they eroded uniformly. The angular velocity (ω), expressed as (rad m^{-1}) is calculated by equation 3 using rotational speed of the shaker per minute (rpm).

$$\omega = 2\pi \times \frac{\text{rpm}}{60} \quad [3]$$

Statistical Analysis of Erosive Strength (E_s)

Plots of the E_s versus PME were generated with a cut-off of 15% aggregate mass eroded for $n = 9$ aggregates per treatment. The area under the generated curve was determined by piece-wise integration. The treatment effects on the area under the curve, highest E_s value and percent mass eroded location of the highest E_s values were tested using ANOVA. The assumption of normality of the residuals was tested by examining normal probability plots and stem-and-leaf plots of the residuals. The homogeneity of variances assumption was assessed visually by examining the side-by-side box plots and checked using Levene's test for equal variances. When the homogeneity of variances assumption was questionable, the model with heterogeneous variances was fitted and compared to the model with equal variances using AIC values. The model that produced the lowest AIC was used for further analyses. The analyses were carried out using PROC MIXED (SAS, Inc. 2006). When the effect of treatments was found to be statistically significant at either $p < 0.1$ for the areas under the curve and at $p < 0.05$ for the average

peak heights, the treatments were compared among each other and to the uninoculated control aggregates using t-tests.

Results

Isolation and Specific Characterization of Flavobacterium spp.

Flavobacterium strains were isolated from several soil treatments and enrichment conditions with xylan-enriched, tilled early successional soil generating the most success (Table 1). All isolates were gold or yellow in color, translucent, and motile on R2A agar. All isolates grew well or best at room temperature and produced pigment most when incubated at 4°C. A phylogenetic analysis of the 16S rRNA gene sequences from each isolate confirmed that they were of the genus *Flavobacterium* (Figure 1) although no isolate sequences were identical to any of the *Flavobacterium* type strain sequences. Further analysis showed that at 97% sequence similarity threshold there were 15 operational taxonomic units (OTU). This similarity threshold can be considered a species defining unit, therefore, in this study, it was determined that the 65 isolates represented 15 total species (86).

All 65 *Flavobacterium* isolates were able to grow on xylan as a sole carbon source. Nine isolates representing different species that grew best for each OTU were chosen for further examination. The normalized optical density of isolates grown in soil extract with no added carbon produced the least growth for all isolates tested (Table 1). Isolates incubated in soil extract with 0.1% w/v xylan added had the greatest growth; roughly one order of magnitude greater than without xylan. Cultures in broth M9 media with 0.1% xylan were more dense than soil extract alone (approximately 2-fold), but far less dense than soil extract with xylan added.

Isolates were also investigated for the potential to produce capsules (Table 2). Capsule staining was not conclusive, nor quantitative. The only isolates with capsule production that obviously matched that of the positive *Klebsiella pneumoniae* control were the numbers 35, 52 and the *Sphingobacterium sp.*. Isolate numbers 11 and 12 demonstrated some extracellular substance between groups of cells when examined microscopically.

Another technique was employed to attempt to quantitate the potential for the selected *Flavobacterium* isolates to aggregate soil. This involved staining cultures adherent to a microtiter plate and measuring the optical density of the stained culture (Table 2). Cultures grown in minimal media with 0.1% w/v added xylan were the least adherent to the wells of the plate. Cultures grown on soil extract alone and with added xylan were more adherent. With the exception of *Flavobacterium* isolate number 57, all were more adherent in the presence of xylan. *Flavobacterium* isolate 57 was the most adherent in both soil extract medias, with and without xylan, but most adherent without the addition of xylan (145.2-fold greater than control) than without (81.3-fold greater than control).

While all *Flavobacterium* isolates were motile on R2A agar, it was not known to which depth the isolates would penetrate the remolded aggregates upon inoculation and during incubation. To help describe penetration, a comparative motility test was performed (Table 3). All *Flavobacterium* isolates were motile on agar with 1% v/v SEM and the *Sphingobacterium sp.* was non-motile (Table 3). The average distance isolates traveled per day was between 0.9mm and 2.6mm. *Flavobacterium* isolates #35 and #36 traveled farther (12.2mm), during the 7 day incubation, than the other isolates.

Remolded Aggregates as a Model System

Remolded aggregates were composed of the same mineral content as the KBS soil from which the *Flavobacterium* species were isolated. The soil was air-dried, sieved and the percentage of sand ($>53\mu\text{m}$) to clay and silt ($<53\mu\text{m}$) was calculated to be 43.9% based upon three replicate sifting events. This is consistent with reported the soil fraction composition for Kalamazoo type soil (kbs.msu.edu). Clay and silt fractions were not further separated to preserve the ionic content in an effort to truly represent the native soil.

Remolded aggregate diameters were 12.7mm which were within the range of 1 to 20 mm diameters for native KBS soil aggregates (77). Since *Flavobacterium* were applied to surfaces of remolded aggregates, it was assumed greater surface areas of these aggregates would enhance detectable effect of the *Flavobacterium*. The average bulk density of the remolded aggregates was 1.51g/cm^3 . This is consistent with the native aggregates at KBS which ranged from 1.2- 1.8g/cm^3 (85). It is also consistent with the lower intraggregate porosity (27%) which is somewhat lower than the 34 to 37% porosity range for native soil aggregates (91). A CT scan of five aggregates showed between 0.73-2.5% of the pores were greater than $11\mu\text{m}$ (Figure 2) (92). Although it was anticipated the inoculum would penetrate little farther than the outer third of the aggregate, this lower total porosity, relative to native aggregates, was not a concern.

Lastly, the addition of xylan was at a calculated final concentration of approximately 0.0035% (w/w). Since total carbon content of the KBS Kalamazoo soil type is 1.2 % (w/w) the addition of xylan did not greatly change the aggregate environment. However, in pilot studies we discovered these additions of xylan concentrations increased the E_s nearly 3.5 fold, from 1.34×10^6 N/mg/min to 4.26×10^6 N/mg/min. This E_s increase provided a new opportunity to identify

the potential for *Flavobacterium* isolates to reduce soil aggregate strength by degrading xylan during metabolism.

Respiration During Incubation

Soil respiration rates were measured by infra-red gas analyses of the carbon dioxide in the headspace gases in the gas-tight incubation vessel. Most treatments demonstrated two peaks in carbon dioxide production (Figure 3). There were also two corresponding peaks in colony forming unit counts when aggregates were sacrificed daily for dilution spread plates (data not shown). *Flavobacterium sp.* #52 generated the most carbon dioxide on most days of the incubation period. The theoretical total carbon respired (calculated based upon the rate of carbon dioxide produced when sampled daily) was greatest for this isolate (Figure 3 inset). *Flavobacterium sp.* #36 produced the least carbon dioxide for most days, and the least theoretical total amount of carbon respired. *Flavobacterium sp.* #12 demonstrated the greatest increase in carbon dioxide production between day 2 and day 3. *Flavobacterium sp.* #28 demonstrated the greatest decrease in carbon dioxide production between days 4 and 5. Four isolates; #8, #12, #35 and #36, stopped respiring on or before day 7. Four *Flavobacterium* isolates (#8, #11, #12, and #27) showed little to no respiration on one or more days during the seven-day incubation period, but subsequently increased carbon dioxide production before the experiment was terminated on day 7. The fluctuations in respiration of these cultures correspond to the fluctuations in growth (data not shown).

Analysis of E_s of Remolded Aggregates

After the 7 day incubation, the remolded aggregates were air-dried overnight with sterile air before being concentrically eroded by the soil aggregate erosion chamber method (65). In an effort to view the changes in E_s through the aggregate, the E_s was plotted against the percent

aggregate mass that was eroded (Figure 4). It was first observed that most aggregates from all treatments demonstrated a peak in E_s between 0 and 2% PME. This peak in E_s is a result of the addition of xylan to the surface of the aggregates at the time of inoculation. Cultures were resuspended in xylan broth and the uninoculated control aggregates had sterile broth applied to the surface. We know the peak in E_s is due to the application of xylan because there was an E_s peak in the uninoculated control aggregates (Figure 4b). Also, there was no peak in the aggregates used in the pilot study (data not shown). The aggregates used in the pilot study were made with xylan throughout (as all aggregates in these studies were made) but did not have additional xylan added to the surface as they were not inoculated prior to erosion.

The second prominent observation was that while the E_s of the aggregates from nearly all treatments formed E_s peaks, the height of the peaks (pE_s) were much lower than those of the uninoculated control (Table 4). Aggregates from eight of the ten bacterial treatments (*Flavobacterium sp.* #8, #12, #27, #28, #35, #36, #52, and #57) displayed reduced pE_s . At a probability threshold of 0.05, six of the nine *Flavobacterium* treatments (#27, #28, #35, #36, #52, and #57) resulted in significant reductions in pE_s heights when compared to the control aggregates.

Only two treatments demonstrated increased pE_s values *Flavobacterium sp.* #11 and *Sphingobacterium sp.* At a probability threshold of 0.05 neither treatment was considered statistically greater than the control treatment.

The mean total area under the E_s curves (tE_s) for all treatments, which represent the most global sum of aggregate strength, was also calculated and compared to the control aggregates

(Table 4). Only four of the ten isolates displayed reduced tE_s and none were significantly different at a probability threshold of 0.01. Four isolates displayed reduced tE_s values that were less, however not significantly, than controls. Five *Flavobacterium* isolates and the *Sphingobacterium* isolate displayed greater total aggregate strengths than uninoculated control aggregates. These isolates demonstrated a statistically greater increase in tE_s beyond the controls.

Discussion

Because all but one of the nine *Flavobacterium sp.* isolate treatments of remolded soil aggregates resulted in reduced pE_s of the aggregates (Table 4), it can be concluded that these species have the potential to increase the rate of aggregate recycling in native soil systems. Additions of xylan throughout the remolded aggregates, increased erosive strengths of aggregates by 3.5-fold beyond those constructed without xylan. Xylan additions to surfaces of remolded aggregates at the time of inoculation also increased their erosive strength creating a peak in E_s (pE_s). However, the pE_s was significantly reduced following inoculation with the *Flavobacterium* isolates. If *Flavobacterium spp.* degrade the xylan that strengthens soil aggregates, they are likely to increase the rate at which aggregates break and subsequently decompose. Therefore, when native soil aggregates are weakened by the invasion of *Flavobacterium*, they are more likely to break down, losing soil carbon before being recycled into new aggregates. Breakage of aggregates also increases the exposure of sequestered soil carbon to microbial respiration (71). The *Flavobacterium* are also capable of contributing to the subsequent decomposition of the organic matter contained within the aggregates. Furthermore, because much of the organic matter is respired as carbon dioxide, or lost due to soil leaching,

these results also suggest that *Flavobacterium spp.* have the potential to promote soil carbon losses.

Another measurement of aggregate strength, identified in this study was the comparison of total area under the curve comparing Newtonian erosive strength to the percent aggregate mass eroded (Table 4). Total erosive strength (tE_s) is a new parameter for comparing microbial modifications of aggregates beyond their surface layers. Aggregates from four treatments with *Flavobacterium* isolates had reduced tE_s , although not significantly. This is likely due to the lower concentration of xylan in the interior portions of aggregates. Had there been more xylan in the interior portions of the aggregates as in the outer layer, a decrease in tE_s after treatment should have been more readily detected.

It is also possible that the bacteria did not penetrate the interior layer of the aggregates. It is known that *Flavobacterium spp.* are motile by gliding (59). We attempted to describe the specific potential of the isolates to translocate by gliding on soil extract agar (Table 3). No additional carbon source was added to the agar plates for that test because during isolation it was observed that the *Flavobacterium* isolates displayed no gliding motility on agar plates with added xylan. When nutrient sources are readily available, there is no factor driving the bacteria to translocate. In this study, when the aggregates were inoculated, the bacteria were in a xylan suspension. It is probable that their close proximity to a carbon nutrient source discouraged translocation into interior portions of the aggregates.

Alternatively, aggregates from other *Flavobacterium* treatments, even those that had significantly reduced the pE_s , increased tE_s , although the increase in tE_s was not significant. No *Flavobacterium* treatments resulted in statistically significant overall changes in tE_s . There are

two possible explanations for an increase in tE_s . The xylan degradation could have been incomplete resulting in intermediate polysaccharide compounds that were stronger binding agents than xylan or the *Flavobacterium* could have produced some extracellular polysaccharide that served as a binding agent similar to xylan.

Even though we anticipated that additions of *Flavobacterium* isolates would decrease erosive strengths of remolded soil aggregates, we did investigate their potential to produce extracellular polysaccharides (Table 2) because bacterially produced extracellular polysaccharides are known to bind soil aggregates (83, 84). Five isolates demonstrated a non-significant increase in tE_s when the area under the curve was compared to the uninoculated control. Four of these isolates, when grown in soil extract media with added xylan, were found to adhere to the walls of a microtiter plate with a measured optical density that was between 58 and 82-fold greater than the uninoculated control (Table 2). However, one isolate (#28) that non-significantly increased the tE_s was the least adherent to the microtiter plate (14.3-fold) when compared to all isolates tested.

Alternatively, the *Flavobacterium* isolate #52, calculated to be the most adherent isolate, with an 81.3-fold increase, when grown in soil extract media with xylan added, did not significantly increase the tE_s of the remolded soil aggregates. Furthermore, treatment of remolded aggregates with this isolate resulted in a significantly reduced pE_s . Taken as a whole, these data demonstrate that production of extracellular polysaccharides does not coincide with an increase in tE_s , nor does it appear to counterbalance the weakening effect of xylan degradation.

Therefore, we believe it is unlikely that *Flavobacterium spp.* contribute to the stabilization of natural soil aggregates by this mechanism.

Uniquely, of the nine *Flavobacterium* isolates tested, treatment of remolded aggregates with one isolate (#11) was shown to increase not only tE_s , but also the pE_s (Table 4). Neither increase was found to be statistically significant from the uninoculated control aggregates and therefore it is probable that this isolate would not contribute to the stabilization of natural soil aggregates. Based upon the lowest C losses by respiration for isolate #11 (Figure 3) one plausible explanation for this non-significant increase in E_s is the binding capacities of xylan intermediates. This suggests that there was very little, or weak, catabolism of xylan by *Flavobacterium* isolate #11. Also, this isolate was found to produce a capsule when examined microscopically, as well as to be 58.9-fold more adherent to the microtiter plate than the uninoculated controls. A combination of these three factors, may account for the non-significant increase in erosive strength.

Since nearly all *Flavobacterium* isolates tested reduced the erosive strength of the remolded soil aggregates *in vitro* it is likely that they will also be an influential factor in the acceleration of aggregate cycling *in situ*. Other studies have targeted similar questions about microbial influence in soil aggregation (2, 4, 15). However, these studies have all focused on the ability of bacteria to increase aggregate strength, not destabilize aggregates. One study determined that seeds inoculated with a *Pantoea* species produced plants that had more root adherent aggregates, and those aggregates had a greater diameter than control plants (2). These conclusions were not meant to address sequestration of carbon in soil, but rather the indirect influence of stable aggregate-facilitation of water retention capacity by rhizosphere soil.

Another study, found that remolded aggregates made with *Sphingobacterium sp.* in the wet slurry had a greater tensile strength than those generated without bacteria (15). Although the aggregates were not incubated with the bacteria to allow for physiological activities to affect the aggregate strength, aggregates amended with the *Sphingobacterium sp.* were reported to have greater tensile strength than unammended aggregates and aggregates amended with other bacterial species. In our study we used the same *Sphingobacterium sp.* with similar results. Treatment of remolded aggregates with this strain was the only treatment determined to significantly increase the pE_s and the tE_s (Table 4). While it was determined this *Sphingobacterium* strain can indeed metabolize xylan as a sole carbon source, suggesting that it could degrade the strengthening components of the remolded aggregates, it is expected the production of extracellular polysaccharides by this species are the primary source of the net increase in aggregates strength (15). Inclusion of this organism in this study confirms the sensitivity of the E_s techniques and analyses to detect both statistically significant increases in aggregate strength responses along with the observed E_s decreases observed by *Flavobacterium spp.* which catabolized the aggregate-strengthening substrate xylan.

In a more recent study that focused on a *Flavobacterium johnsoniae* strain (4), the authors concluded additions of these bacteria to loose granular sand increased shear strength resulting from the bacterial production of biofilm. We also tested a *Flavobacterium johnsoniae* strain in this experiment for the attempted purpose of identifying the penetration of the aggregate by the inoculum. This strain carried a manufactured plasmid containing the gene for green fluorescent protein (17). We had hoped that after the incubation we could slice the remolded aggregates and visualize the fluorescence under a microscope to determine the bacterial

penetration. Unfortunately, the antibiotic selective force causing the strain to retain and express the plasmid containing the green fluorescent protein gene could not be maintained and no fluorescence could be seen after seven days. The strain demonstrated respiration throughout the incubation and could be cultivated from the aggregates afterward, so it was certain the bacteria remained viable and active. Because the strain had not served its intended purpose, and it was not a soil cultivar, it was not included in the final analysis. However, we did find that the average pE_s of aggregates from this treatment was 2.87×10^4 N/mg/min (data not shown). These Newtonian energies fell within the range of pE_s from other treatments that were indeed significantly lower when compared to the control treatment (Table 4). It is expected that while a *Flavobacterium johnsoniae* species would increase the strength of sand through the formation of biofilms, in an aggregate soil system the destabilizing effect of bacterial degradation of polymeric carbohydrates such as xylan would be greater, resulting in a net reduction in aggregate strength.

To conclude, we have determined that the majority of the *Flavobacterium* species isolated from soil in this study caused significant reductions in the erosive strengths of remolded soil aggregates where xylan penetrated the surface. In a native soil system this would likely result in weakened aggregates that are more likely to break with a subsequent loss of carbon from the soil. The data reported here were the results of live, monoculture, bacterial activity in a controlled environment. It would be of importance to compare the results to a secondary experiment in which the aggregates were incubated at lower temperatures that mimic seasonal differences. In these studies we observed what occurs during the summer season as incubations were at room temperature. However, during fall and winter months when there is a greater input of plant litter from crop residue and deciduous foliage these degrading effects on soil aggregate

strength, when combined with the frequent freeze/thaw cycling, may cause even greater reductions in soil aggregate strength and resultant soil C respiration losses during soil warming in early Spring. It is possible that the role of *Flavobacterium* during these seasons would have an even greater ecological impact on both the stabilization and soil C sequestration.

Acknowledgements

The authors would like to thank both the Ton-That lab and Michael Bagdasarian for their generous donations of *Sphingobacterium* and fluorescent *Flavobacterium johnsoniae* cultures to this study. We would also like to thank Wei Wang for his analysis of the remolded aggregate porosities using the CT scans, and for his many helpful conversations regarding native soil aggregate structures.

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Table 3.1 Isolation and specific characterization of *Flavobacterium* isolates. Growth on minimal agar with added xylan was scored based upon the sums of scores of the visible biomass on four different occasions. The optical density for three cultures grown in different media were averaged. To normalize for the natural opacity of the media, the optical density of the uninoculated control was subtracted from the average value and the net value was then divided by the control. Hereafter isolates are referred to by the last number of the identification, such that CoT2r3-A-G2-H1-08 is called *Flavobacterium* sp. #8.

Isolate identification	Isolate No.	Media enrichment	Soil used for enrichment inoculum	Qualitative score for growth on xylan (0-50, n=4)	Fold increases from control growth (OD ₆₀₀ , n=3)		
					Soil extract	Soil extract 0.1% Xylan	Minimal media 0.1% Xylan
CoT2r3-A-G2-H1-08	#8	Cellobiose	No-Till Conventional Agriculture	50	32.6	199.8	130.9
XT7Tr1-B-A6-C2-11	#11	Xylan	Early Successional, Tilled Sub-plot	23	52.1	421.0	98.3
XT7Tr1-B-A7-D2-12	#12	Xylan	Early Successional, Tilled Sub-plot	16	50.9	510.2	146.8
XT7Tr1-B-D9-C4-27	#27	Xylan	Early Successional, Tilled Sub-plot	27	42.1	382.7	132.9
XT4r3-B-E11-D4-28	#28	Xylan	No Chemical Input Agriculture	27	49.5	608.8	117.9
XT4Fr1-B-F4-C5-35	#35	Xylan	No Chemical Input Ag, Fertilized Sub-plot	17	40.8	688.3	116.5
XDFr2-B-F5-D5-36	#36	Xylan	Deciduous Forest	22	42.8	437.2	121.5
ChT7r1-C-D3-D7-52	#52	Chitin	Early Successional	24	45.2	584.8	126.6
GT7Tr1-C-E5-A8-57	#57	Gelatin	Early Successional, Tilled Sub-plot	5	52.7	440.7	54.9
<i>Sphingobacterium</i> sp.		Caesar-TonThat, <i>et al.</i> , 2007		37	71.5	572.2	158.1

Table 3.2 Screening *Flavobacterium* isolates for potential to produce exopolysaccharides. India ink capsule stains were compared to a *Klebsiella pneumonia* control using both bright field and phase contrast microscopy. The optical density for stained adherent cells from three cultures grown in listed media were averaged. To normalize for the natural opacity of the optical density of the uninoculated control was subtracted from the averages and the net value was then divided by the control. It was not possible to adjust the OD values to correct for more cells in the culture.

Isolate	Capsule staining observations	Fold-increases greater than the control Biofilm staining (OD ₅₃₀ , n=3)		
		Soil extract	Soil extract 0.1% xylan	Minimal media 0.1% xylan
<i>Flavobacterium</i> sp. #8	No capsule	19.2	40.6	0.4
<i>Flavobacterium</i> sp. #11	Capsule	20.3	58.9	4.2
<i>Flavobacterium</i> sp. #12	No capsule	10.0	49.9	8.3
<i>Flavobacterium</i> sp. #27	Capsule	34.0	46.2	9.8
<i>Flavobacterium</i> sp. #28	No capsule	5.9	14.3	6.2
<i>Flavobacterium</i> sp. #35	Capsule	7.1	20.8	0.0
<i>Flavobacterium</i> sp. #36	No capsule	36.7	60.8	0.7
<i>Flavobacterium</i> sp. #52	Capsule	145.2	81.3	3.5
<i>Flavobacterium</i> sp. #57	No capsule	32.2	65.1	5.0
<i>Sphingobacterium</i> sp.	Capsule	0.0	17.4	7.6

Table 3.3 Motility of isolates on soil extract agar. Isolates were spotted on three replicate soil extract agar plates and the distance traveled was measured daily. Because gliding motility was not uniformly concentric, the maximum distance was measured.

Isolate	Average maximum sum distance traveled (mm)	Average maximum distance traveled per day (mm)
<i>Flavobacterium sp. #8</i>	5.7	1.3
<i>Flavobacterium sp. #11</i>	10.8	2.1
<i>Flavobacterium sp. #12</i>	7.5	1.7
<i>Flavobacterium sp. #27</i>	6.3	1.7
<i>Flavobacterium sp. #28</i>	4.2	0.9
<i>Flavobacterium sp. #35</i>	12.2	2.6
<i>Flavobacterium sp. #36</i>	12.2	2.4
<i>Flavobacterium sp. #52</i>	7.2	2.1
<i>Flavobacterium sp. #57</i>	3.8	0.9
<i>Sphingobacterium sp.</i>	0.7	0.0

Table 3.4 Statistical analysis of the changes in erosive strength of treated remolded aggregates. Plots of the erosive strength (E_s) versus percent aggregate mass eroded were generated with a cut-off of 15% aggregate mass eroded for n=9 aggregates per treatment. Two characteristics of each curve were calculated; the highest measured E_s (N/mg/min), or peak E_s (pE_s), and the total area under the curve (tE_s). These parameters for each aggregate were recorded for n=9 aggregates per treatment. All treatments were compared among each other and to the uninoculated control aggregates using t-tests. Reported values have been averaged per treatment for presentation.

Mean peak height pE_s ($\times 10^4$)			Mean total area tE_s (151.5)*		
Treatment			Treatment		
<i>Sphingobacterium sp.</i>	11.4 (2.06)*	a**	<i>Sphingobacterium sp.</i>	2030	a**
<i>Flavobacterium sp.</i> #11	11.3 (2.31)	ab	<i>Flavobacterium sp.</i> #57	1957	ab
Uninoculated Control	7.90 (1.32)	abc	<i>Flavobacterium sp.</i> #11	1895	abc
<i>Flavobacterium sp.</i> #8	6.18 (1.03)	bcd	<i>Flavobacterium sp.</i> #36	1789	abcd
<i>Flavobacterium sp.</i> #12	5.17 (1.37)	cde	<i>Flavobacterium sp.</i> #52	1719	abcd
<i>Flavobacterium sp.</i> #36	3.16 (0.43)	de	<i>Flavobacterium sp.</i> #28	1645	abcd
<i>Flavobacterium sp.</i> #52	3.83 (0.55)	de	Uninoculated Control	1598	bcd
<i>Flavobacterium sp.</i> #57	3.80 (0.73)	def	<i>Flavobacterium sp.</i> #27	1575	bcd
<i>Flavobacterium sp.</i> #27	2.48 (0.70)	ef	<i>Flavobacterium sp.</i> #12	1475	cd
<i>Flavobacterium sp.</i> #28	2.91 (0.48)	ef	<i>Flavobacterium sp.</i> #35	1468	d
<i>Flavobacterium sp.</i> #35	1.85 (0.60)	f	<i>Flavobacterium sp.</i> #8	1438	d

*standard errors for the means of each characteristic are shown in parentheses

**means within the same column followed by the same letter are not significantly different at $p < 0.05$ for the mean peak heights and at $p < 0.1$ for the areas under the curve.

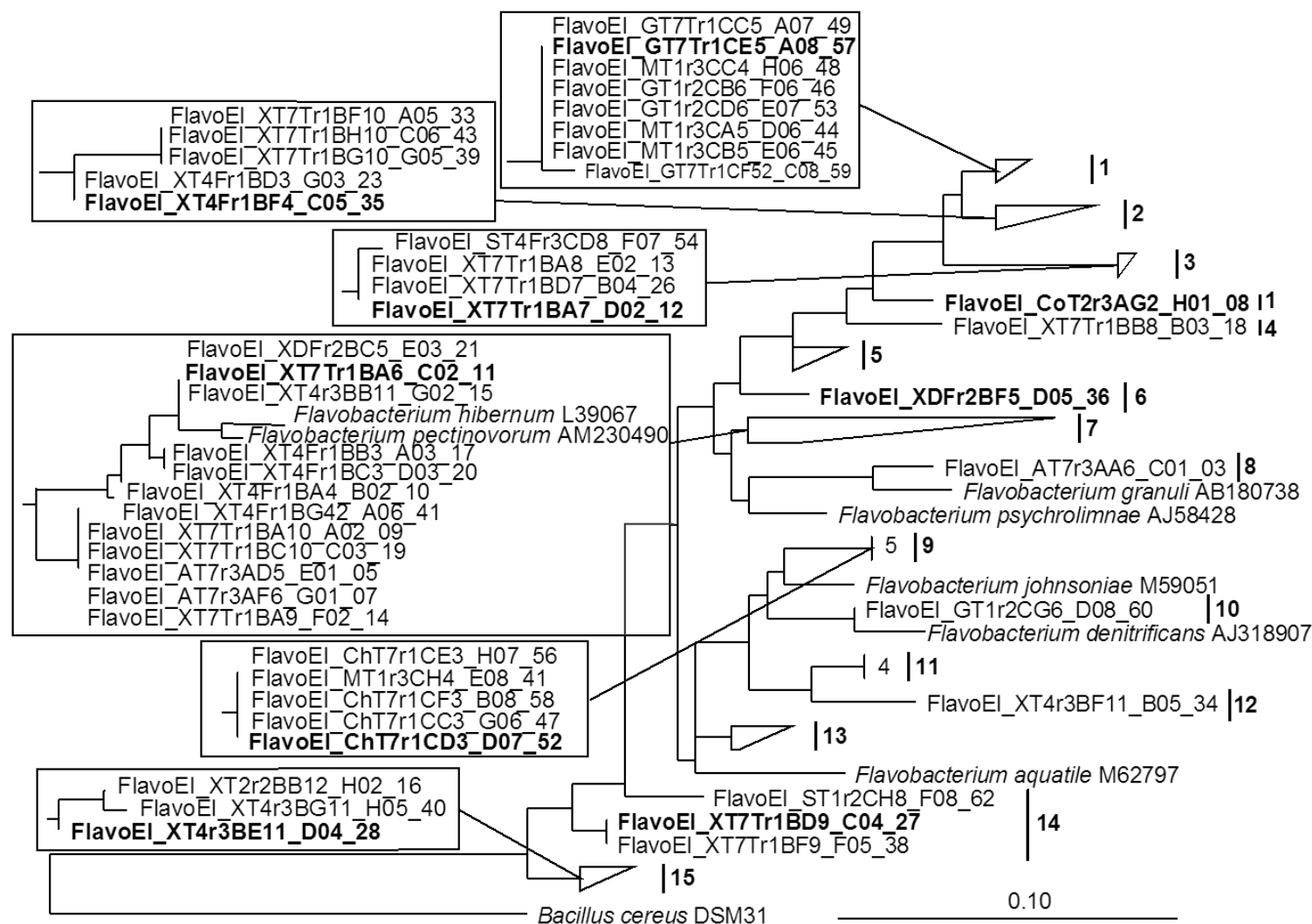


Figure 3.1 Phylogenetic tree of *Flavobacterium* environmental isolates with *Flavobacterium* type strain sequences. The filter used for construction had 386 valid nucleotide positions. Type strain sequences were added using parsimony and are noted with their respective GenBank accession number. All isolate sequences have been submitted to GenBank; accession numbers HQ917614-HQ917677. Vertical lines with numbers represent the operational taxonomic unit assignment using DOTUR at 97% sequence similarity threshold; there were 15 total. Isolates chosen for study are highlighted in bold. Insets represent expanded clades.

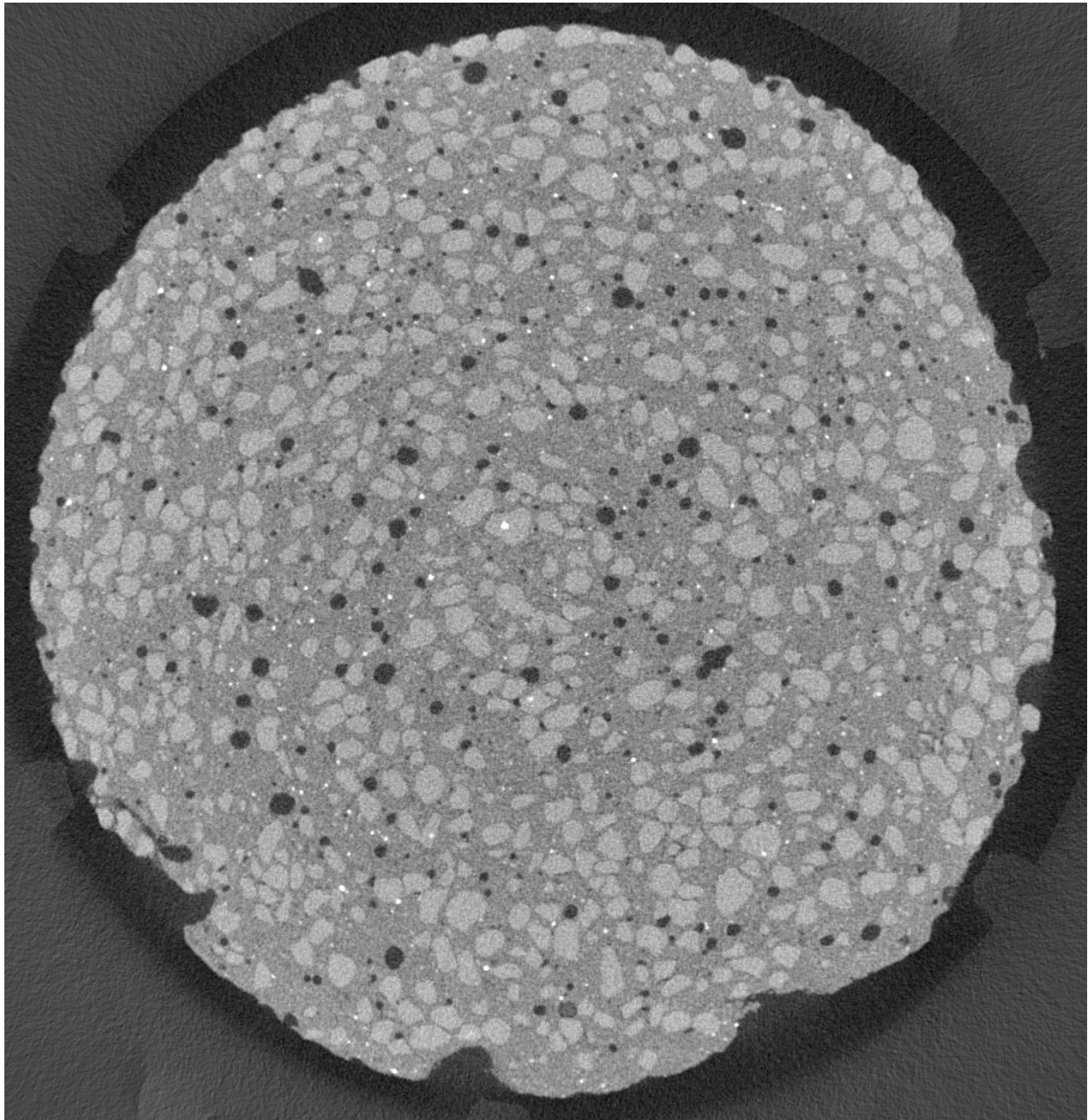


Figure 3.2 CT cross-section scan image of a remolded aggregate. Darkest areas are air filled pores, lighter areas are mineral silt and sand respectively, and white specks are heavy metals.

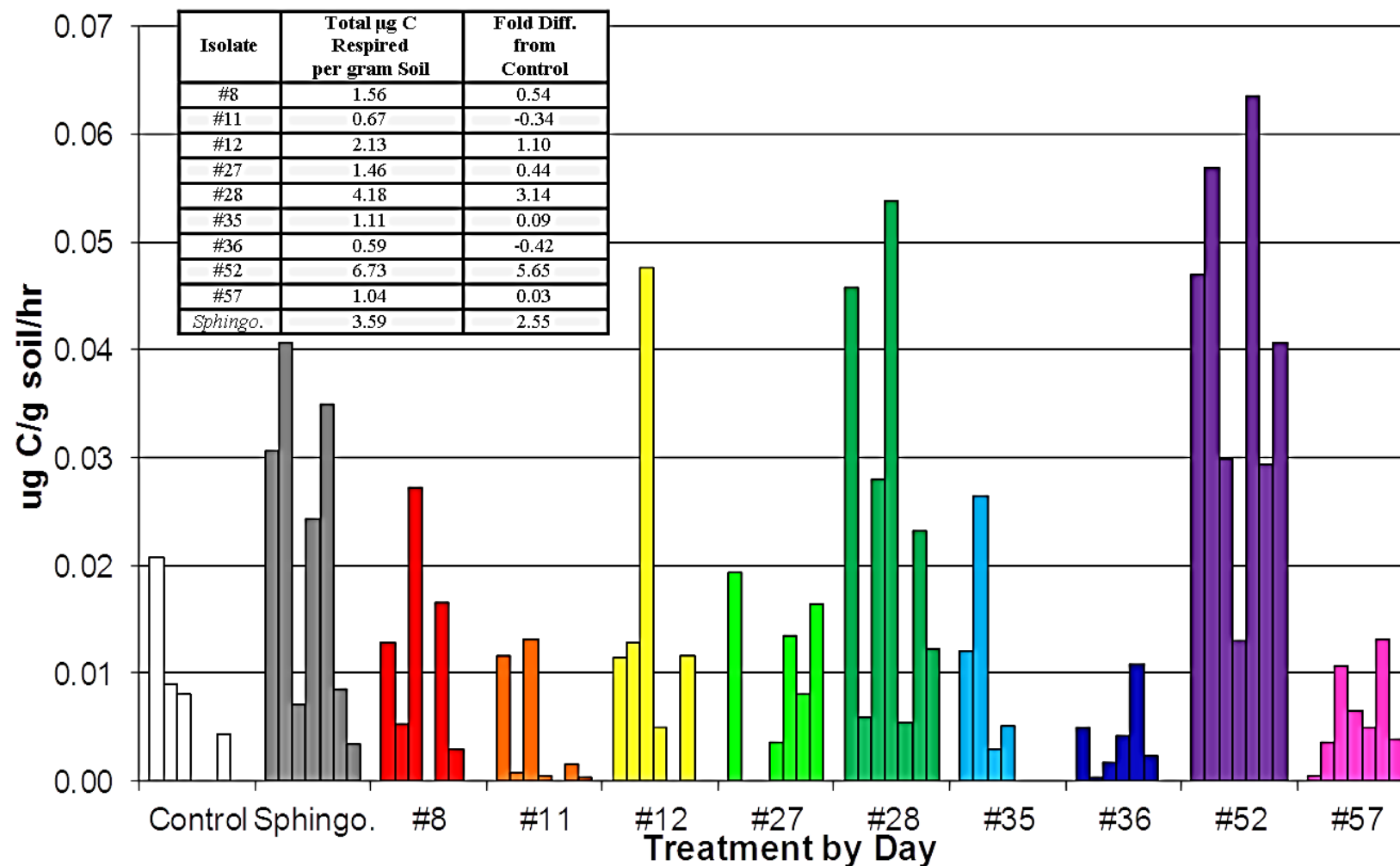


Figure 3.3 Respiration data for microcosm incubation. The headspace of microcosms containing aggregates for each treatment was sampled twice daily within one hour to determine the production of carbon dioxide. The respiration per gram was calculated using the average mass of the aggregates and the number of aggregates per microcosm on each day. Each bar represents the rate of carbon dioxide production per day. Inset: The total carbon respired was calculated by determining the theoretical total per day based upon the rate of respiration in the one hour sampling period. The fold difference from control was calculated by finding the difference between the averages and dividing by the control average.

Figure 3.3 Continued. Inset.

Isolate	Total µg C Respired per gram Soil	Fold Diff. from Control
#8	1.56	0.54
#11	0.67	-0.34
#12	2.13	1.10
#27	1.46	0.44
#28	4.18	3.14
#35	1.11	0.09
#36	0.59	-0.42
#52	6.73	5.65
#57	1.04	0.03
<i>Sphingo.</i>	3.59	2.55

Figure 3.4a

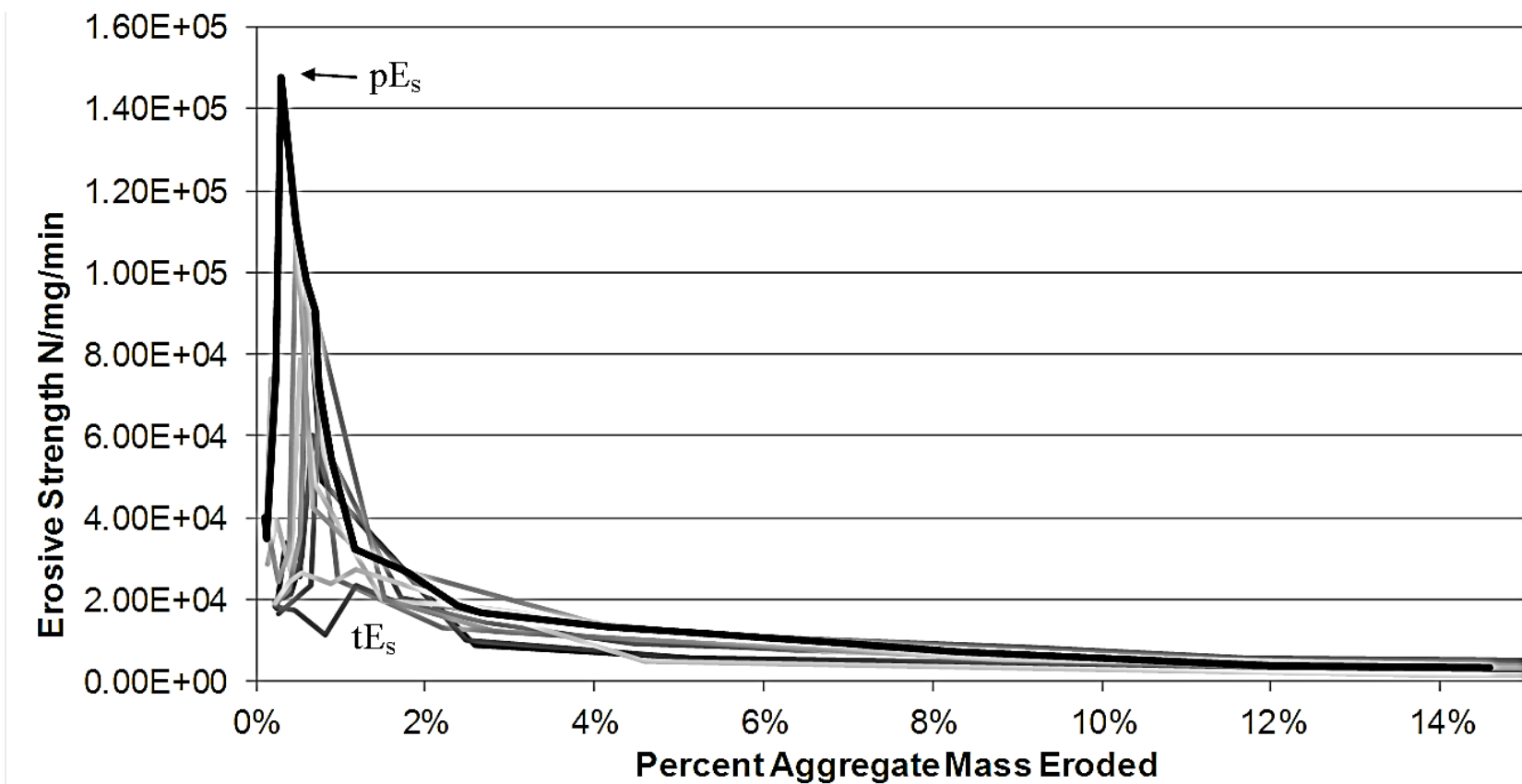


Figure 3.4 Erosive strength of remolded soil aggregates. The erosive strength of the aggregates changed as they were eroded. a.) Plot demonstrate the erosive strength of each control aggregate overlaid with an amended curve intended to represent all 9 aggregate curves (bold black). No mathematical calculations were used to generate the amended curve; each point was hand-selected. The locations of the peak erosive strength (pE_s) and total area under the curve (tE_s) are indicated. b.) Amended curves representing the control aggregates and aggregates from four bacterial treatments that either increased the erosive strength of aggregates, or caused no net change. c.) Amended curves representing the control aggregates and aggregates from the *Flavobacterium* treatments that reduced the erosive strength of the aggregates. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Figure 3.4b

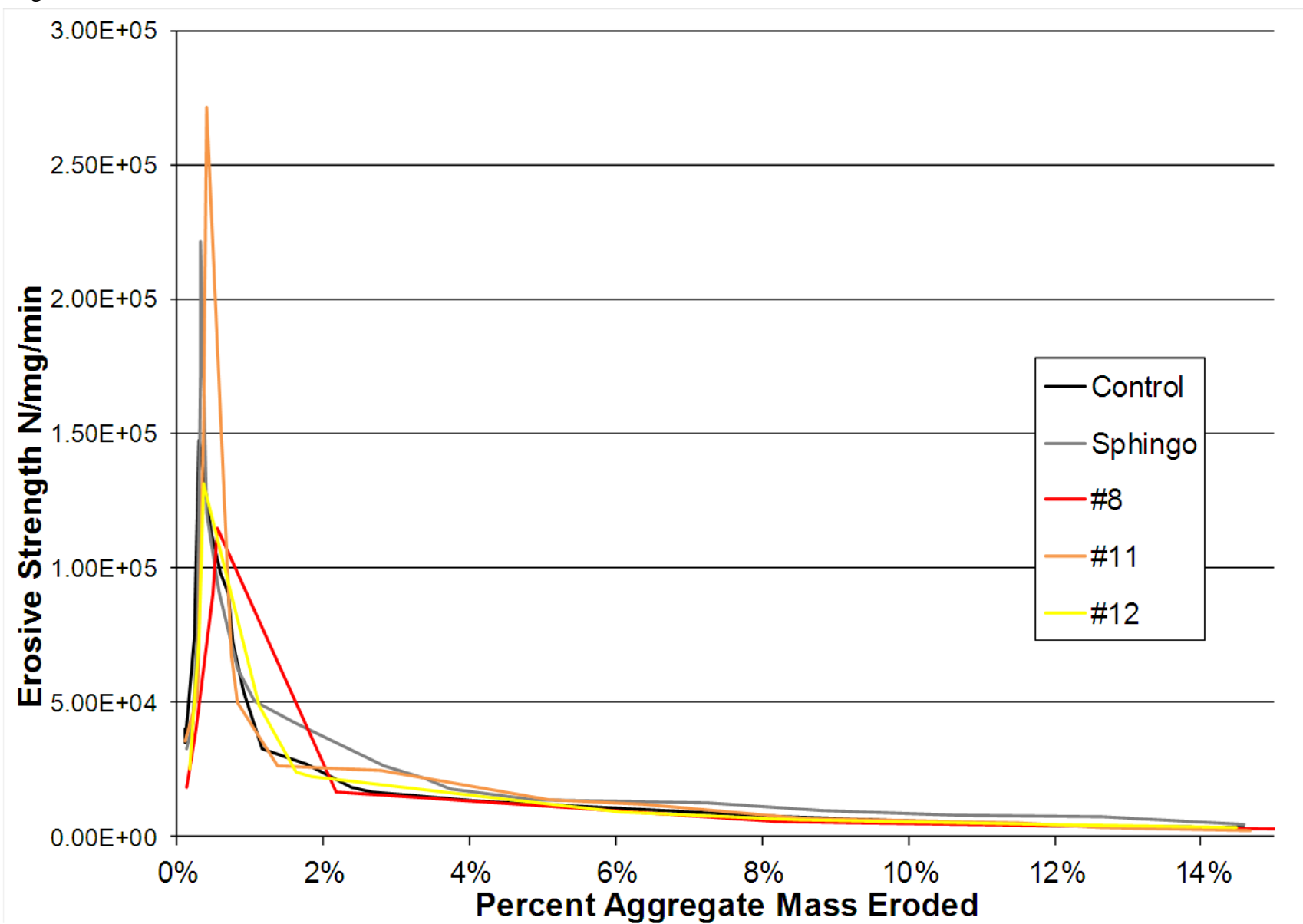
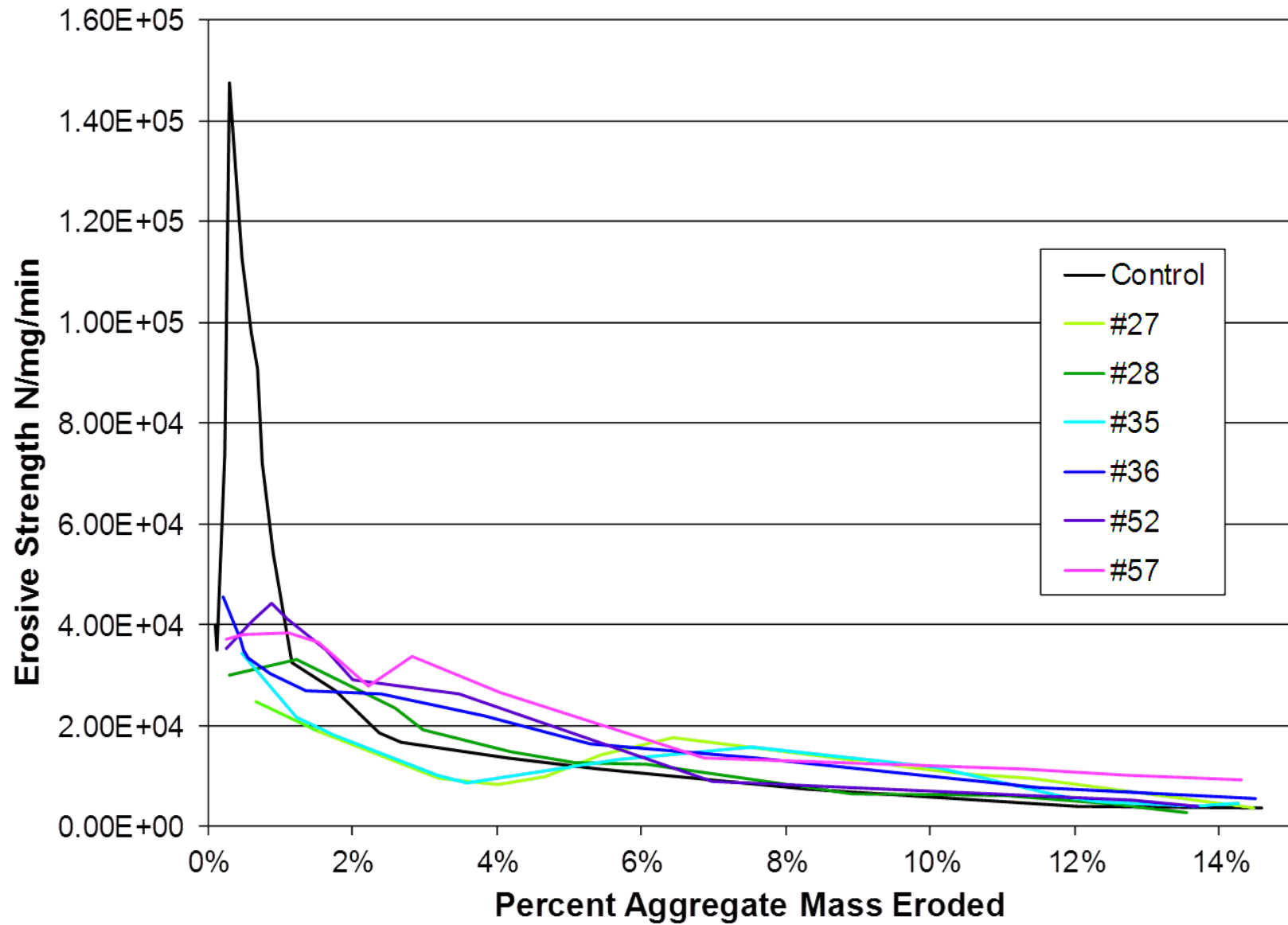


Figure 3.4c



Chapter 4

Conclusions and Future Research Directions

Brief Dissertation Conclusions

When the *Cytophaga-Flavobacterium* (CF) specific clone libraries that were generated from agricultural and non-agricultural soils were compared (Chapter 2), it was found that the CF community from the agricultural soil in that sample was a subset of the non-agricultural CF communities in this sampling. This conclusion is supported by the non-significant phylogenetic distribution of the cloned CF sequences derived from agricultural soils. In addition, the species richness estimates and diversity indices were the lowest for the agricultural community. Lastly, when the communities were compared on a pairwise basis, there were very few operational taxonomic units (representing species) that were unique to only the agricultural community and not also represented in the non-agricultural communities.

When CF-specific clone libraries from deciduous forest soils that differed only in fertilization were compared, no differences in the two communities were found. It is suggested that either fertilization is not a selective force upon the CF in soil, or that the soils had not been properly maintained for the effect of fertilization to be detected. Alternatively, when CF communities from tilled and no-till agriculture soils were compared, a significant difference in the phylogenetic distribution was noted. Many of the cloned sequences from the agricultural soil clustered separately from the non-agricultural cloned sequences. Also, the richness estimates and diversity indices were calculated to be greater for the tilled agricultural soil community than for the no-till community.

However, the significance in phylogenetic distribution and high richness and diversity in the tilled agricultural soil CF community is not consistent with the tilled agricultural soil CF

community from the first analysis. These discrepancies make it difficult to conclude that tillage is a selective force on the CF soil communities. A different crop was grown prior to each sampling and may be an uninvestigated selective force. Regardless, more sampling is required for solid conclusions to be made in all these comparisons.

In Chapter 3 of this dissertation remolded soil aggregates made with xylan (a common complex carbohydrate) were challenged with *Flavobacterium* soil isolates in an effort to determine if the *Flavobacterium spp.* would change the erosive strength of the aggregates. The erosive strength is a measure of how readily an aggregate degrades. In a native soil system, this degradation can lead to a loss of carbon from the soil. It was determined that the *Flavobacterium spp.* significantly decreased the peak in erosive strength when compared to the uninoculated control aggregates. Also, several isolates decreased the total erosive strength. These studies confirmed the potential for *Flavobacterium spp.* to contribute to the loss of organic carbon from soil.

Survey of *Cytophaga-Flavobacterium*

As discussed in Chapter 1, it is likely that the CF are involved in the carbon cycle because they produce a great assortment of hydrolytic enzymes that can degrade the complex carbohydrates found in soil organic matter. The CF have been observed in a wide assortment of ecological niches, often in competition for carbon sources (7, 11, 12, 28, 30, 53, 56, 60, 64, 70, 87). Other studies have detected the CF in soil as part of their research objectives finding that they represent between 0.1 to 0.4, but no more than 1% of the bacterial community (14, 27, 82). However, no known studies have focused solely on the CF. It is likely that despite their low relative abundance in soil, the CF could still have a significant impact on the cycling of soil

organic carbon. But, before research can address the nature of the CF involvement in the soil carbon cycle, it was necessary to describe their diversity and distribution in soil (Chapter 2).

The clone library studies in Chapter 2 utilized a reverse primer in the PCR amplification of soil DNA that is specific to the genera such that only sequences from these bacteria were obtained (89). In this way the survey focused only on the bacterial species of interest. The soils that were sampled for this survey were chosen to explore the idea that the CF were involved in the carbon cycle by attempting to link different soil management practices (and thus different carbon contents) to different CF communities. Although, it should be noted that these studies were performed as only directed exploration and not meant to test a hypothesis.

Should this research be hypothesis-testing some additions and changes are recommended. Foremost, more sampling is required if reliable conclusions are to be made. The studies in Chapter 2 described the CF communities that were sampled at only one date for each soil treatment. Although each replicate plot for each treatment was sampled, because the DNA extracted from each plot was pooled prior to PCR amplification, the clone libraries generated from each treatment can only be considered as one sample. Ideally, the soils would not only be sampled in the same season of multiple years, but each replicate plot would be used to generate separate clone libraries. With sampling replication, more solid conclusions could be formed regarding the diversity and distribution of the CF in agricultural and nonagricultural soils. Also, the CF communities from each replicate plot from the same soil treatment could be compared to one another. This approach would allow for a better interpretation of the internal variability of the CF communities and provide more information about the dynamic nature of CF communities under different soil managements.

Also in Chapter 2, two aspects of agricultural management practices were compared in an effort to detect a potential difference in the CF communities; fertilization and tillage. The CF communities were compared from soils that only differed by the application of these individual techniques. It was anticipated that if there was a strong difference between the two communities in each comparison, it would indicate that the management practice being examined is a selective force upon the CF in the soil. Statistically significant differences were observed when the phylogenetic distribution of cloned CF sequences from till and no-till soils were compared. In contrast, the calculated species richness and diversity was nearly equal. Additional sampling, as suggested above would confirm that the management practice of tillage is a driving influence on the CF communities in soil.

In the comparison concerning the use of inorganic fertilizer, soil CF communities from the deciduous forest and its fertilized subplot were compared and no differences were detected. The fertilized sub plot had not been consistently maintained for the 8 years since its inception and this is cited as one of the possible reasons no differences were detected. There are agricultural plots available that have been more consistently fertilized. It is possible that in an agricultural setting, and with a more consistently fertilized subplot, differences in the CF communities would be more apparent.

Although the application of fertilizer and the manipulation of tillage were the two soil management practices that were examined, the effect of crop rotation was not examined. Different plant species will exude different carbon compounds from their roots and deposit leaf litter of different composition. It is probable that soils with greater or different plant diversity aboveground will also have greater or different belowground diversity (5, 51, 95). Indeed, in the survey in Chapter 2, the early successional soil that has the greatest plant species diversity

yielded the CF community with the greatest diversity. Resampling of the early successional soil compared to soils with a plant monoculture would support these findings. Further research comparing the CF communities in agricultural soil that undergoes a yearly rotation of crops from corn to soybean to wheat would confirm the influence of plant species on the CF and also identify plant-specific CF species that can be assessed for their influence on rhizosphere activity, plant productivity and the cycling of carbon in soil.

Studies of Effect of *Flavobacterium spp.* on Aggregate Strength

While it is important to understand the diversity and distribution of CF in soil, determining the factors that drive their diversity and distribution does little without an understanding of their role in the cycling of soil organic carbon. Only with this combined information can we hope to manipulate the CF in an effort to conserve soil carbon. The studies in Chapter 3 were designed to assess the effects of *Flavobacterium* isolates on the stability of remolded aggregates. Aggregates that are more stable have a slower rate of recycling and thus sequester more carbon in the soil (84). It was determined that the *Flavobacterium spp.* reduced the strength of aggregate surfaces removed from remolded aggregates and are likely accelerating the cycling of soil aggregates resulting in a loss of organic carbon in soils. These studies also demonstrated that inoculating aggregates with bacteria and eroding them to determine their stability is a sensitive and useful technique for assessing the effects of the bacteria on aggregate strength.

The studies in Chapter 3 were performed in monoculture on remolded soil aggregates. A logical next pursuit would be to evaluate the effect of *Flavobacterium spp.* as part of the soil microbial community. As mentioned, the CF represent no more than 1%, of the bacterial community (14, 27, 82). Although *Flavobacterium* represent a small proportion of the bacterial

community, it is likely that they are largely responsible for the degradation of polymeric carbon compounds, especially at colder temperatures (49, 50, 82). The contribution of *Flavobacterium* to the cycling of organic matter in soil could have great ecological relevance regardless of their relative abundance.

In this same context, it would support the idea that *Flavobacterium* have a greater functional impact on soil carbon cycling at colder temperatures to compare the strength of remolded aggregates inoculated with *Flavobacterium* that are incubated at varied temperatures. The experiments performed in Chapter 3 only represented summer seasons as the aggregates were incubated at room temperature. There is much anecdotal evidence to suggest that the CF have increased activity in colder temperatures (11, 34, 50, 82). It would be interesting to discern whether the activity of the *Flavobacterium* actually increases, or it is more observable when the activity of some other decomposer group decreases.

Another important future endeavor would be to perform similar studies on native soil aggregates. While the aggregates used in this study were representative of native soil aggregates as a model, they did not undergo any biological processes (wetting and drying, root grow, organic matter decomposition, etc.) that give native soil aggregates their distinct structure and shape (13, 20, 66). Should *Flavobacterium* isolates also reduce the strength of native soil aggregates, it would further support the idea that they are involved in the acceleration of the cycling of organic matter in soil. At a minimum, remolded aggregates could be subjected to wetting and drying cycles that would mimic precipitation. With wetting and drying cycles, the pore connectivity of the soil aggregates would increase allowing the *Flavobacterium* to translocate toward the interior of the aggregates. This would potentially increase the portion of the aggregate in which the effect of the *Flavobacterium spp.* could be measured.

It is also known that soils that undergo tillage have aggregates with less porosity, accelerated recycling, and they sequester less carbon than no-till soils (66, 84). It is probable that the CF are involved in the accelerated loss of carbon in soil (Chapter 3). A comparison of the effects of *Flavobacterium* species from till and no-till soils on aggregate strength would also provide useful information connecting the *Flavobacterium* to the loss of organic carbon from soils. Ribosomal RNA gene sequences from the nine *Flavobacterium* isolates used in the Chapter 3 studies did not partition solely with the cloned CF sequences from either the tilled soil or the no till soil, but rather were evenly distributed (data not shown). However, a sample size of nine *Flavobacterium* sequences in comparison to cloned sequences from only one sampling of tilled and no till soils is not enough evidence to confirm that there is not a soil treatment-specific distribution of *Flavobacterium*. It is possible to generate more clone libraries and subsequently test more *Flavobacterium* species to determine if there is a connection between certain *Flavobacterium* species and tillage. A more direct approach would be to isolate a number of *Flavobacterium* species from tilled and no till soils and challenge remolded aggregates from each group to determine if there is an overall difference in their degradation activity.

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