

ABSTRACT

Title of Document: THE MICROBIAL ECOLOGY AND
HORTICULTURAL SUSTAINABILITY OF
ORGANICALLY AND CONVENTIONALLY
MANAGED APPLES.

Andrea R. Ottesen, PhD, 2008

Directed By: Professor, Dr. Christopher S. Walsh, Plant
Sciences and Landscape Architecture

Objectives Organically and conventionally managed apple trees (*Malus domestica* Borkh) were evaluated for three growing seasons (2005-2007) to examine the impact of organic and conventional pesticide applications on the microbial ecology of phyllosphere and soil microflora. An important objective was to establish if organic or conventional selection pressures contribute to an increased presence of enteric pathogens in phyllosphere microflora. The horticultural and economic sustainability of the organic crop was also compared to the conventional crop with regard to fruit yield and input costs.

Methods Microbial populations from phyllosphere and soil environments of apple trees were evaluated using clone libraries of 16S rRNA gene fragments. Clones were sequenced and software was used to assess diversity indices, identify shared similarities and compute statistical differences between communities. These

measurements were subsequently used to examine treatment effects on the microbial libraries.

Phyllosphere Results Eight bacterial phyla and 14 classes were found in this environment. A statistically significant difference between organically and conventionally managed phyllosphere bacterial microbial communities was observed at four of six sampling time points. Unique phylotypes were found associated with each management treatment but no increased human health risk could be associated with either treatment with regard to enteric pathogens.

Soil Results Seventeen bacterial phyla spanning twenty-two classes, and two archaeal phyla spanning eight classes, were seen in the 16S rRNA gene libraries of organic and conventional soil samples. The organic and conventional soil libraries were statistically different from each other although the sampling depth was not sufficient to make definitive inference about this environment.

Horticultural Results Fruit yields from organically managed apple trees were from one half to one third of the yields from conventionally managed trees. Based on input costs, organic fruit was about twice as expensive to produce. Asian pears (*Prunus serotina*) were also included in this horticultural analysis and showed greater field tolerance as an organic specialty niche crop than apples.

THE MICROBIAL ECOLOGY AND HORTICULTURAL SUSTAINABILITY OF
ORGANICALLY AND CONVENTIONALLY MANAGED APPLES.

By

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2008

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List of Abbreviations and Terms

Agrisphere, an environment impacted by agricultural practices
BLAST, Basic Local Alignment Search Tool
DGGE, Denaturing Gradient Gel Electrophoresis
Enteric pathogen, microbes that occur pathogenically in the intestine of humans or animals
FDA, Food and Drug Administration
GMO, genetically modified organism
IPM, Integrated Pest Management
NOP, National Organic Program
NOSP, National Organic Standards Board
OMRI, Organic Materials Review Institute
PCR, Polymerase Chain Reaction
Phylloplane, the environment of leaves
Phyllosphere, the aerial surfaces of plants
Phytosphere, synonym to Phyllosphere
rRNA, ribosomal RNA
SARE, Sustainable Agriculture Research and Education
USDA, United States Department of Agriculture
WREC, Wye Research and Education Center

Chapter 1: Introduction

Sustainable Agriculture, Food Safety and Microbial Ecology

Sustainable Agriculture

The dissertation work presented here spans multiple disciplines in the study of an organic and conventional crop of apples and Asian pears. In 2003, a one-hectare crop (2.5 acres) was planted at the Wye Research and Education Center in Queenstown, Maryland, in five replicated complete blocks of organic and conventional treatments. From a sustainable agricultural perspective, the experimental orchard was designed to support the investigation of the horticultural sustainability and economic viability of the crop of six cultivars of apples and three cultivars of Asian pears.

“Organic” is a rapidly growing trend in sustainable agriculture. Two and a half million acres were reported to be in organic production in 2002. Although this number is far less than one percent of all farmed lands in the United States (349 million acres), it is growing at an estimated rate of 20 percent annually (Delate, 2003; Wuerthner, 2002). U. S. sales of organic food and beverages were estimated at \$20 billion in 2007 according to the Organic Trade Association (www.ota.com).

“Organic” is in effect, the oldest form of agriculture on earth but has only recently become a certified practice (1990, Organic Food Production Act) that prohibits the use of synthetic pesticides, genetically modified organisms (GMOs), sewage sludges, irradiation and other practices deemed to be detrimental to society and the

environment. With the passing of the Organic Food Production Act (OFPA), the selling of produce labeled “organic” means that a set of prescribed practices has been followed. These practices and regulations are designed to minimize harmful practices associated with agricultural production.

Before World War II, agriculture did not rely upon petroleum-based chemicals, but post war, ammonium nitrate that had been used for ammunition evolved into ammonium nitrate fertilizer. Nerve gases made from organophosphates evolved into a highly effective class of insecticides (Delate, 2003). Some of the methods that have evolved in conventional agriculture in the last sixty years have brought about great increase in production yields but some are less efficient than the older systems they replace (Pimentel et al., 2005; Topp et al., 2007) and have serious detrimental costs to the environment.

Organic agriculture, as a brand of sustainable agriculture, aspires to take advantage of the progressive practices that have increased agricultural yields while excluding measures and materials that do not contribute to broader sustainability considerations such as economics, community and environment. The last sentence in the definition of “organic” provided by the National Organic Standards Board (NOSB) is;

“The primary goal of organic agriculture is to optimize the health and productivity of interdependent communities of soil life, plants, animals and people.”

(www.ams.usda.gov)

Our own experimental orchard provided an excellent test-system for the navigation of logistics associated with the development of an organic spray schedule for best management of pest pressures as well as effective maintenance of organic standards, required for legal certification. While traditional organic production relies on many holistic methods of farming that we were not able to incorporate into our study *because* of its experimental design, the orchard (Figure 1) provided a statistically significant replication, infrequently available in environmental studies of this nature.

Horticulturally, we aimed to provide practical recommendations for organic production based on our trials and errors. We also wanted to determine whether or not the crop selection itself (apples and Asian pears) can be sustainably managed in the hot humid growing seasons of the Mid-Atlantic, and if so, do any of the six cultivars of apples or three cultivars of Asian pears, have some inherent predisposition to thrive as organic specialty niche crops in Maryland?

An integral and inseparable component of agricultural sustainability is financial viability, so a complete financial analysis of the crop of apples was generated by Dr. Jim Hanson (Appendix 2) to examine this important component of the sustainability of the organic crop.

Food Safety

The dissertation work also provides an important contribution to food safety and public health initiatives. Food safety research, like plant pathology and other fields that focus on the health of a particular system, have ironically spent the past 100 years focusing primarily on the pathogens that impact these systems. This historical focus has provided a valuable understanding of pathogens and pathogenicity however, if it is not a pathogen, we really don't know that much about it. Only very recently have more ecological systems approaches been incorporated into food safety research initiatives.

The microbial ecology of the whole environment is now being examined in trace-back efforts to identify how the microbial members of a specific niche may be playing a role in both the source of a contamination event and the ability of a pathogen to survive in an environment once it has been introduced. Understanding the community dynamics of specific environmental niches - especially those associated with food plants will contribute to our overall ability to describe and manage health risks associated with crop environments.

Many of the primary pathogens that have been associated with produce-related health outbreaks in recent years (such as *Salmonella enterica* and *Escherichia coli*) (Heaton J.C. and Jones K., 2008) have demonstrated varying degrees of environmental fitness (Brandl and Mandrell, 2002; Brandl, 2006; Creel, 1912; Heaton J.C. and Jones K., 2008). The ability of enteric pathogens to survive in the agricultural environment of

food plants, has positioned the “phyllosphere” at an intersection of food safety, public health, microbial ecology and agricultural sustainability (Brandl, 2006). Research that focuses on the microbial ecology of food crops, especially those with a history of health associated outbreaks has become extremely relevant to food protection efforts.

Microbial Ecology

The microbial ecology of many biomes, both human and environmental, has become increasingly accessible to scientific study in the past thirty years. The use of the small subunit of the rRNA gene (referred to as 16S due to its sedimentation rate) in combination with dropping sequencing costs and more recently, newer sequencing technologies such as 454 pyrosequencing (Margulies, 2005), has provided a valuable set of tools with which to examine the diversity associated with a vast array of microbial environments.

The term metagenomic means “environmental genomics” or “community genomics.” It was coined by Dr. Jo Handelsman and refers to the use DNA that has been extracted directly from an environment so that all members of the environment, even those which we do not yet have the methods to culture, can be included in study (Handelsman et al., 1998). Estimates of organisms we fail to observe through the use of culturing methods can reach as high as 99% for specific environments (Handelsman, 2004). The term metagenomics also refers to all the genes in an environment. Many metagenomic studies take advantage of cheaper sequencing technologies such as 454 pyrosequencing (Margulies, 2005) to examine the genetic potential of a specific environment.

Phyllosphere

Phyllosphere environments have been the focus of very few culture-independent molecular studies. In 2001, only one study could be found in the literature that examined a phyllosphere environment using molecular methods (Yang et al., 2001). Today there is a growing body of work that has begun to describe the microbial species associated with natural and agricultural phyllosphere environments (deJager and Korsten, 2001;Heuer and Smalla, 1999;Jackson et al., 2006;Kadivar and Stapleton, 2003;Knief et al., 2008;Lambais et al., 2006;Yang et al., 2001).

The dissertation research presented here, if published today would be the largest 16S rRNA gene clone library data set currently available in the literature describing the microbial ecology of the phyllosphere environment and the only study to date to examine the impact of organic and conventional management on a food crop.

Soil

The microbial ecology of the soil of the organic and conventional orchard was also examined using 16S rRNA gene fragments. Because of the immense microbial diversity associated with soil environments (estimates of one billion cells per gram), and our limited resources for sequencing, we acknowledge that we will only be able to assemble preliminary data for future more quantitative methods or more comprehensive sequencing efforts.

The organic and conventional plots did not receive specific soil amendments associated with treatment, however the fact that both organic and conventional plots

were subjected to the diverse chemicals and materials associated with organic and conventional management for five continuous years may have influenced the soil microflora in currently undescribed ways (Table 1, Appendix 3). It is our hope that our results will generate the preliminary data to investigate questions associated with the microbial ecology of soil of agricultural systems – specifically potential treatment effects by organic or conventional management. We also hope to provide a valuable ecological description of the species in this agricultural soil environment.

The study of the microbial environments of phyllosphere and soil as well as production logistics of organic and conventional management of a crop of apples and Asian pears, provides a valuable platform for a very multidisciplinary investigation of research questions pertaining to food safety and public health, microbial ecology and sustainable agriculture.

Research Objectives

Sustainable Agriculture

From the perspective of sustainable agriculture and “organic” agriculture, our research objectives aim to:

- Assess the “sustainability” of organic apples and Asian pears as a specialty niche crop for Maryland.
- Develop practical recommendations for organic production of apples and Asian pears in Maryland.
- Evaluate the performance of six different cultivars of apples and three different cultivars of Asian pears.
- Evaluate the financial input associated with organic management of apples and Asian pears – compared to the financial input associated with conventional IPM.

Food Safety and Public Health

From the perspective of food safety and public health, our research aims to:

- Establish whether selection pressures associated with organic or conventional agricultural applications result in greater incidence of enteric pathogens.
- Establish whether or not organic or conventional management influences the composition or abundance of members of the family Enterobacteriaceae (home to *Salmonella* and *E. coli* pathovars).

Microbial Ecology: Phyllosphere

From the perspective of microbial ecology, our research objectives with regard to the phyllosphere are to:

- Establish whether selection pressures associated with organic or conventional agricultural applications result in a different bacterial composition associated with the phyllosphere of either treatment.
- Describe the Gram negative microbial consortia associated with an organic and conventional apple phyllosphere.

Microbial Ecology: Soil

With regard to the soil microbial ecology of the organic and conventional crop, our primary research objective was to;

- Provide preliminary data that may describe trends associated with treatment effect for future research with more quantitative methods or deeper sequencing efforts.

- Describe the microbial consortia associated with the soil of the organic and conventional apple tree plots.

Chapter 2: Horticultural Sustainability of Organic and Conventional Apples

Introduction

Agriculture in America

The greatest impact on American land comes not from urbanization but from agriculture. In 2001 approximately 349 million acres were planted in agricultural crops. Of that 349 million, 80 percent was planted primarily in “feeder corn” (80 million acres of usually transgenic corn, grown for livestock), soybeans (75 million acres – 95% of which is consumed by livestock), alfalfa hay (61 million acres), and wheat (62 million acres)(Vesterby and Krupa, 1997;Wuerthner, 2002).

This is approximately double the acreage that is comprised by all rural and residential lands in the U.S. It is an area the size of California, Montana, 2 Oregons and Maine put together. Agriculture has a huge impact on the pollution of natural waters (streams and rivers with pesticides and fertilizer run offs), species extinction, water scarcity, and fragmented and endangered ecosystems. Agricultural production is responsible for *the* largest consumption of water in the United States and ironically, the vast majority of our agricultural produce is grown to feed livestock rather than people (Wuerthner, 2002).

Consequently, efforts to develop more sustainable methods of land stewardship are fundamentally important for the sustainability of American agriculture. Conventional farming practices have done an enormous amount to improve crop yields, but components of this management have had serious costs to the environment, particularly effects of Nitrogen and Phosphorus run-off on natural watersheds such as the Chesapeake Bay (Kramer et al., 2006).

The industrialization of farming practices has also had a hypothesized impact on food safety and produce related health outbreaks (Brandl, 2006). Efforts to develop environmentally sustainable crops that can be grown locally and safely could contribute to a shift away from industrialized farming. Our research provides information about a variety of alternative, certified organic materials, (pesticides, fertilizers and herbicides), their efficacy and the overall functionality of organic production for apples and Asian pears in Maryland.

It may come as a surprise to some, how many applications of pesticides are actually applied to some organic crops. For apples grown in the mid-Atlantic, there can be up to 20 or more applications in a single growing season. It would also come as a surprise to people how many pests are competing with us for the nutrients provided by our crops. The more you understand about pest pressures associated with specific crops, the easier it is to understand the importance and the difficulties associated with protecting it. There are over 20 serious pests, bacterial, insect and fungal, that can damage an apple crop. In 2005, we monitored the orchard for five of the most

devastating pests in an effort to fine tune our spraying efforts and maximize the efficacy of organic materials that are often slightly less effective than their conventional counterparts.

Organic Agriculture

At its roots, “growing organic” is part of efforts to develop more sustainable models for agriculture. “Organic” is an aggregate of efforts to farm using environmentally, socially and economically sustainable practices (Helander and Delin, 2004; Pimentel et al., 2005; Topp et al., 2007). Its methods strive to improve crop management through the use of *natural* biological processes and materials. While this is sound philosophy, to arrive at efficient practice, considerable trial and error must take place as we design functional implementation of organic crop production. It is interesting to note that while the word “organic” is now defined by law, the term “natural” is not. Materials can still be described as “natural” even if they have synthetic components (Delate, 2003). There is still a lot of streamlining to do to maintain the integrity of the regulation of organic materials and practices.

Economics

A vital component of any sustainable business venture is of course economic viability. If a crop cannot be managed for a profit, there is no way to maintain it, (short of government subsidies) no matter how environmentally friendly its production may have been. In Europe, a lot of support from government has been directed to organic farmers, however the U.S. has yet to provide a similar level of support. There have been small per acre grants for transitional fields in Iowa and a

few cost share programs associated with organic production and other conservation practices such as riparian buffer strips and crop rotations, however the organic farmer is ultimately responsible for the majority of all expenses associated with their organic production. Organic premiums range from 20 percent to 400 percent according to the Organic Alliance (www.organicalliance.org) (Delate, 2003). To analyze the value of our crop of experimental organic and conventional apples, we used local fresh market prices applied to the yield statistics of our 2006 and 2007 harvests to develop a complete economic profile of the organic apples (Appendix 2).

Research Goals for Sustainable Agriculture

Establish whether or not organic apples and Asian pears are a sustainable venture in Maryland.

Identify the successes and failures associated with our organic management logistics and materials and develop recommendations based on our experiences to guide future organic production efforts.

Evaluate the performance of six different cultivars of apples and three different cultivars of Asian pears.

Evaluate the production costs associated with organic management of apples and Asian pears.

Materials and Methods

Experimental Design of the Organic and Conventional Orchard

Randomized complete blocks of apple trees were planted in 2003, in a one hectare (approx. 2.5 acres) plot at the Wye Research and Education Center in Queenstown, Maryland (Figure 1). Blocks were treated either with chemicals approved for certified organic management by the National Organic Program (NOP) of the USDA or with the most commonly applied chemicals in a standard commercial apple spray schedule for the Mid-Atlantic region (Table 1, Appendix 3).

Five replicates of each treatment were maintained for five years. Approximately 16 meters (50 feet) was maintained between plots to comply with Maryland Department of Agriculture regulation for proximity of conventional chemicals to certified organic lands (Figure 1).

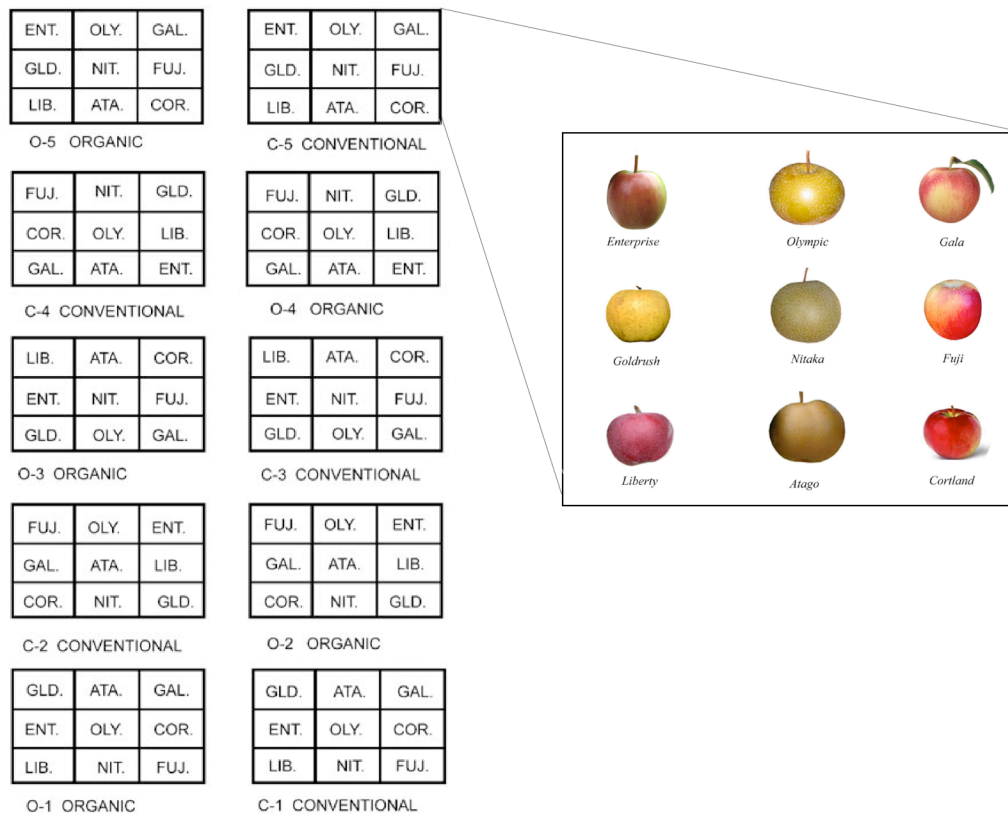


Figure 1. Experimental Design of the Organic and Conventional Orchard

For a larger view of the cultivars represented in each block, see Figure 4.

Organic and Conventional Materials

Certified organic materials from National Organic Program (NOP) lists were substituted at a one to one ratio with conventional materials used in a typical IPM management for commercial apples grown in the mid-Atlantic (*Spray bulletin*) (Maryland, 2003).

The following organic and conventional materials were used to manage the orchard (Table 1) at rates and dates provided in the full spray schedules for 2005-2007 (Appendix 3).

Treatment	Insecticides	Fungicides	Fertilizers	Bactericides	Herbicides
Organic	Kaolin, Pyrethrins, Spinosad, Azadirachtin	Copper, Sulfur	Kelp, fish emulsion, chicken manure, compost teas, 6-1-1 NPK 5-3-4 NPK	Streptomycin	Acetic acid, physical barriers (plastics)
Conventional	Pyrethroid, Carbamate, Organothio-phosphates	Carbamates	Calcium nitrate, 15-0-0 NPK	Streptomycin	glyphosate

Table 1. Organic and Conventional Materials

Monitoring of pest pressures associated with the crop was conducted in 2005 to assist with the planning for best application dates for materials. The five most frequently encountered pests of apples and pears were monitored throughout the 2005 growing season (Appendix 1).

One of the organic materials, brand name “Surround”, a preparation of kaolin clay that is used as an organic insecticide, is also known as a particle film. Plants use pubescence and cuticular waxes to reduce environmental stresses, disease and insect damage. The concept of the particle film builds on this strategy and functions as an insecticide, partially by disguising the tree and creating a reflective surface that repels insects (Figure 2). The normal smells and vision cues that insects react to are effectively masked by the particle film of kaolin clay (Glenn et al., 1999).

This material could obviously have a very big impact on the physical microenvironment of the leaves and fruits and also the microflora that are able to colonize this environment. It might even provide a selective advantage to microbial species due to the increased surface area and the abundance of protected niches. The Surround material is reported by its makers to have no adverse effects on photosynthetic capacity of the plants and is even ascribed a protective functionality against UV damage to the plant. Close up of the diverse physical micro-environments of organic and conventional leaves can be seen in Figure 2.

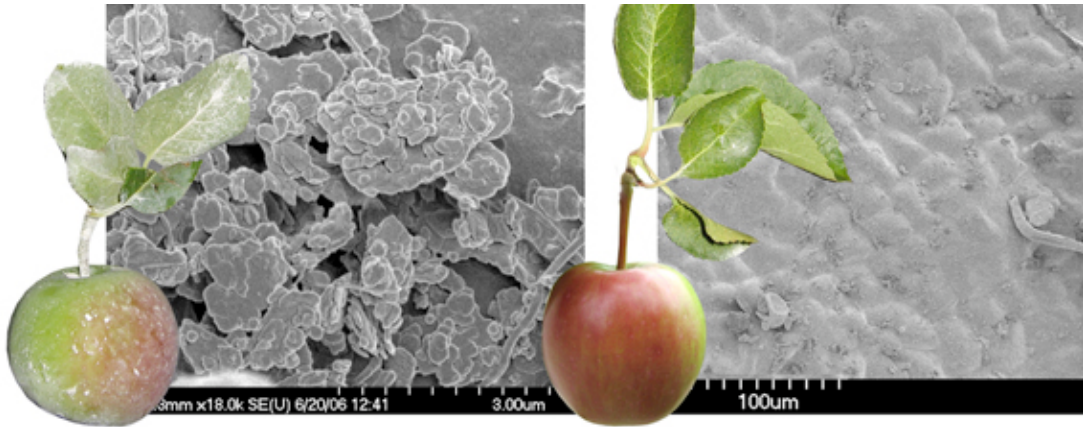


Figure 2. Scanning Electron Micrograph of the Diverse Physical Micro-environment of Organic and Conventional Leaf Surface.

Leaves from organic and conventional treatments were prepared for the scanning electron microscope and imaged at the University of Maryland. Images shown are at 3 and 100 micrometers. The leaf surface under organic management (left) is covered with the Surround kaolin clay insecticide and has an extremely diverse topography compared to the leaf under conventional management (right). It is not hard to imagine that a material that influences the physical microenvironment of leaf and fruit surfaces so profoundly may also influence the microbial ecology of this environment.

Cultivars

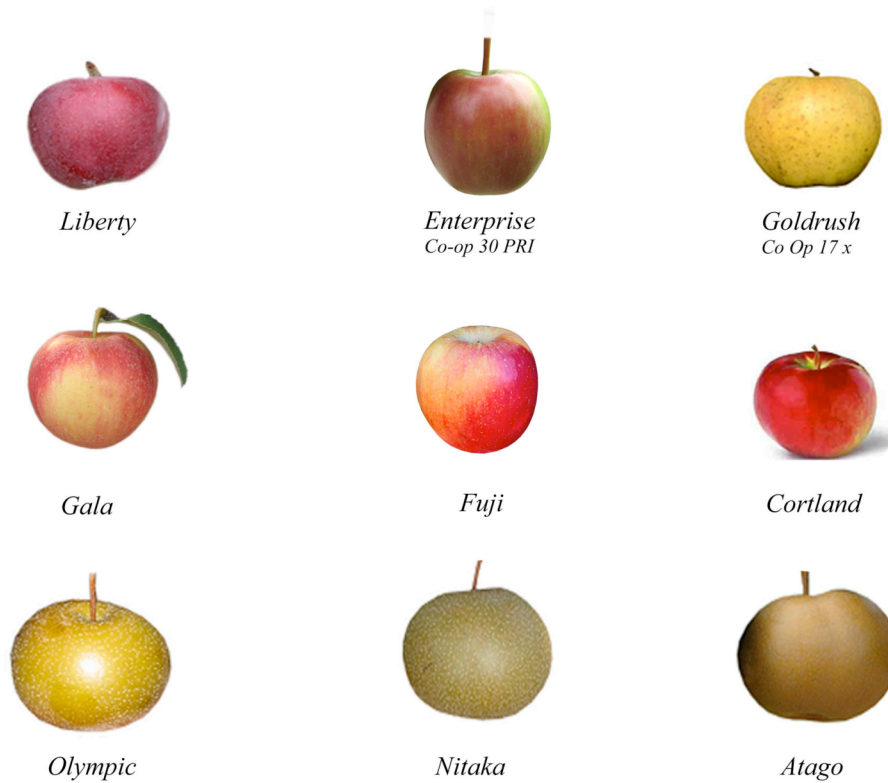


Figure 3. Cultivars of apples and Asian pears planted in the orchard.

Apples

The cultivars that were planted in the orchard were three cultivars of “disease resistant apples” ; ENT. (Enterprise), GLD. (Goldrush) and LIB. (Liberty) and three cultivars with popular commercial appeal were planted; FUJ.(Fuji), COR. (Cortland), and GAL. (Gala), and three cultivars of Asian pears; OLY. (Olympic), ATA. (Atago), and NIT. (Nitaka).

Enterprise cultivar was the only cultivar used for our microbiological and molecular work in an effort not to introduce variability among cultivars.

Enterprise is a late ripening fruit that was bred in a cooperative breeding program of the Indiana, Illinois and New Jersey Agricultural Experiment Stations. It, like the other disease-resistant cultivars has a field immunity to apple scab (*Venturia inaequalis*), a high resistance to Fire Blight (*Erwinia amylovora*), cedar-apple rust (*Gymnosporangium juniperi-virginianae*), and a moderate resistance to powdery mildew (*Podosphaera leucotrichia*). The letter “pri” in Enterprise commemorates the Purdue-Rutgers-Illinois cooperative breeding programs that contributed to the parental material for Enterprise and Goldrush cultivars.

Goldrush was the result of the breeding for disease resistance hybridized with Golden Delicious. Liberty was the result of two lines from the PRI breeding program. Gala is a popular commercial cultivar that was bred in New Zealand with the American born seedling, Golden Delicious and Kidd’s Orange Red, a New Zealand cultivar. Fuji was bred in Japan, grafted from Virginia Royalty, Rawls Jennet and Iowa’s Red Delicious.

Asian Pears

Unlike the “European pear” *Pyrus communis*, the Asian pear, which is primarily a result of selections from crosses between *Pyrus ussuriensis* (Ussuri pear) and *Pyrus serotina* (Japanese sand pear), previously *Pyrus pyrifolia*, is a fairly recent introduction to the “west”. Asian pears were not brought to America until the 1800s when they were introduced to the west coast of the U.S. by Chinese immigrants.

Because of the very recent exposure to the pest pressures of the Americas, the pears still perform extremely well with regard to their quality and tolerance to numerous pests and diseases.

Harvest

Due to the diverse harvest dates associated with different cultivars, trees were strip picked (the whole tree was harvested instead of selectively harvesting the ripest apples as they were ready). Strip picking was done by cultivar, usually two to three cultivars at each time-point in the harvest season.

Apples were graded in the field. They were separated into categories of insect damaged fruit, diseased damaged fruit and marketable “good fruit”.

All categories were counted, weighed and recorded.

Analysis of Variance

Analysis of variance associated with treatment and cultivar harvest data was analyzed using SAS ANOVA, proc mixed model with a Tukey-Kramer adjustment for multiple comparisons at an alpha of .05. Harvest statistics were given to Dr. James Hanson who used current local fresh market prices for apples and production costs to perform a full economic analysis (Appendix 2).

Results

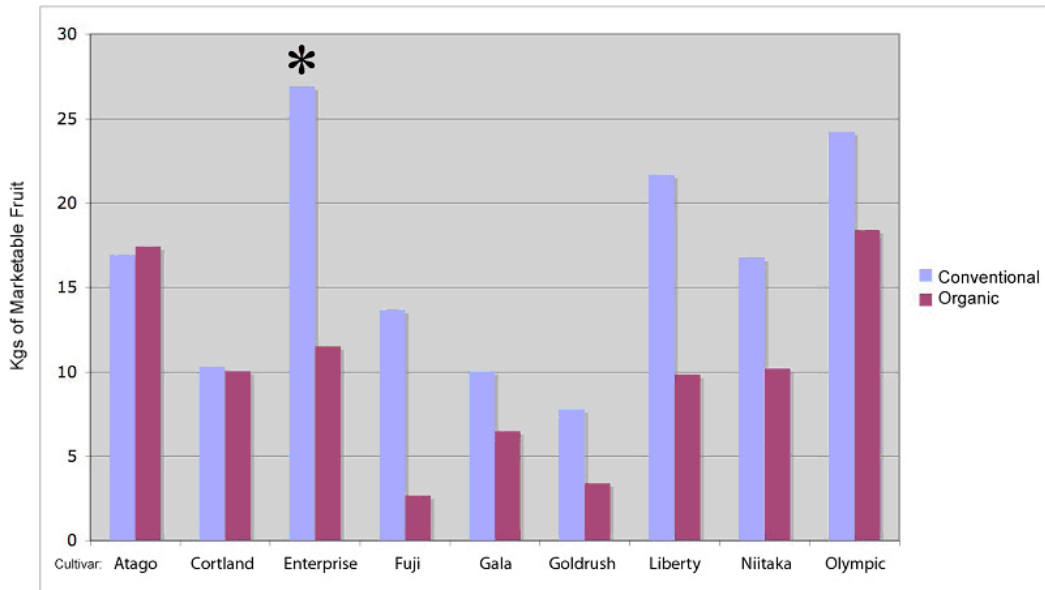
Yield

The yield of marketable fruit is presented for 2006, and 2007.

In 2006, which was actually the second crop of apples and pears, organic performed the best of previous and subsequent years (there was actually a small 2005 harvest and 2008 is in progress) (*data not shown*).

In 2006, there was only one significant interaction between cultivar and treatment with the Enterprise cultivar. By 2007, three significant interactions between cultivar and treatment can be seen with the cultivars Enterprise, Fuji and the Asian pear Nitaka.

2006 Yields of Total Marketable Fruit from each Organically and Conventionally Managed Cultivar



2007 Yields of Total Marketable Fruit from each Organically and Conventionally Managed Cultivar

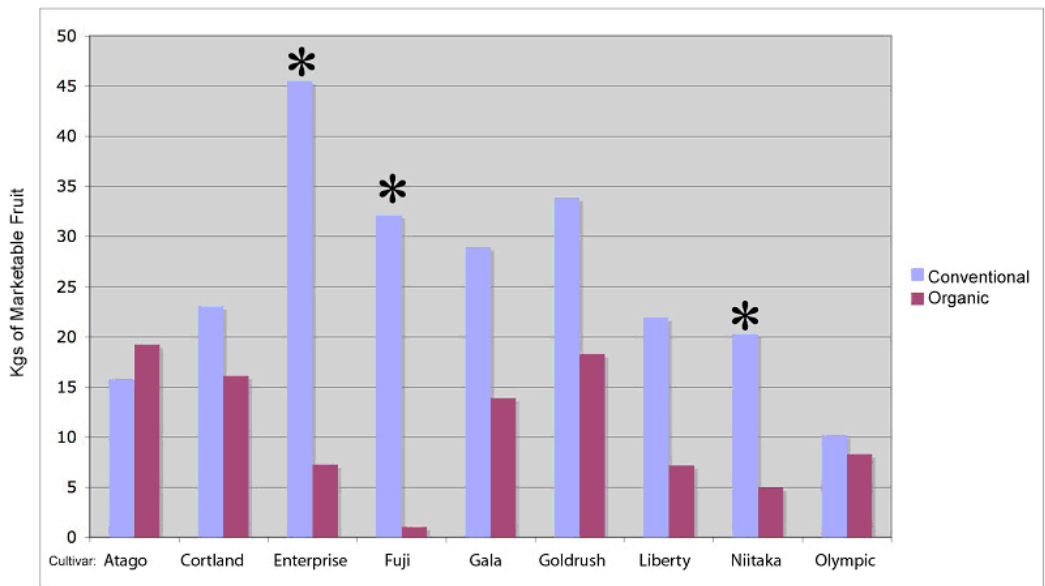


Figure 4. Total Yield of Good Fruit Yield for 2006 and 2007 in Kgs.

Starred cultivars indicate a significant interaction between cultivar and treatment ($p < .05$).

Discussion

With regard to our research objectives, we have effectively provided an enormous amount of data for the application of numerous practices associated with organic production of apples and Asian pears in Maryland. Our failures as well as our successes provide valuable information for future organic production efforts.

The organic crop had a difficult time from the beginning due to what we believe to be a limited nitrogen availability associated with organic fertilizers. In a sense, they never completely recovered from the combined effect of early nitrogen deficiency and competition for nutrients with weeds. Addressing the weed competition early is of paramount importance. The organic herbicide (acetic acid), used in the first couple of years was basically ineffective, based on observational data, and by the time we shifted to physical barriers such as plastic liners, it was probably too late.

One suggestion to avoid the stresses suffered by the organic crop due to nitrogen deficiency would be to transition a crop started under conventional management to organic management after it had a chance to establish itself with ample fertilizing requirements of Nitrogen. Organic certification transition takes three years. During that time, no unapproved materials can be applied to the farming system. Trees could be planted using conventional fertilizers and then the next year transitioned to organic.

Another way to approach the fertilizing issue would be the development of effective composting applications. Perhaps even addressing field preparation 1 to 3 years before an organic crop is introduced (using soil amendments and cover crops). We performed little research on the addition of soil amendments from biological sources of manure or other materials that have been shown to work well for organically managed crops (Kramer et al., 2006). Research of this nature would be very important to future efforts. Some natural fertilizing amendments were added in 2005 such as fish meal, kelp and compost tea but it was probably already too late at this date and more research needs to address the effective rates and methods of application to best take advantage of natural fertilizing materials.

With regard to the evaluation of cultivars suitable for organic management, Cortland apples seemed to be the most consistently able to thrive similarly to their conventional counterparts. An answer to the bigger question about how practical the selection of apples may be for organic production in Maryland is unfortunately, “not very”. Apples require a lot of material input to protect them from pest pressures and they are very susceptible to insect and disease damage.

The Asian pears on the other hand, did extremely well in both organic and conventional management. Both Olympic and Atago cultivars planted under organic management were consistently neck in neck with their conventional counterparts in terms of yield of “good fruit” (fruit graded for commercial sale). In fact, they did so well, Maryland initiatives plan to plant an entire orchard of Asian pears for further

analysis. It is likely however that with increased exposure to the diseases and pests of the Mid-Atlantic area, their natural pest tolerance will eventually decline. So in response to our objective to assess the sustainability of apples and Asian pears as potential organic crops for the Maryland area, apples would be less practical and pears showed great promise as a potentially sustainable organic specialty niche crop.

One hypothesis for the high performance of organic pears compared to organic apples is related to the sulfur applications made in the organic treatments as a fungicide. Lime-Sulfur and Sulfur applications have been reported to cause photosynthetic inhibition in apples and to even exert a thinning effect during bloom and perhaps throughout the season due to the high pH and osmotic dehydration effect of the Lime-Sulfur solution (Rom and Ela, 2002). This phenomenon is not known to occur with Asian pears.

Economics

In general, the organic crop was at least twice as expensive to manage as the conventional crop and in most cases the yield was much lower, so the premium for the fruit needs to be higher in order to balance the organic budget or an adjustment would need to be made to the inputs. The organic price premium for “break-even” pricing ranged from 167% to 322% (Appendix 2, Table 6). A full financial analysis was performed by agricultural economist Dr. James Hanson, and is available in Appendix 2.

Chapter 3: Microbial Ecology of the Phyllosphere of Organic and Conventionally Managed ‘Enterprise’ Apples

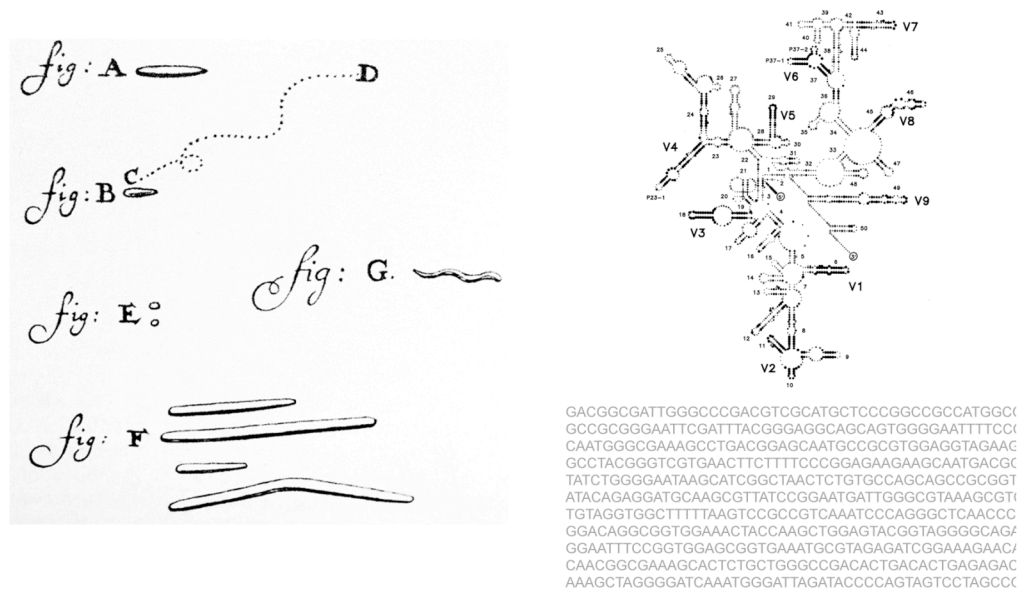


Figure 5. Drawings by Antony van Leeuwenhoek of “animalcules” seen under the microscope. Secondary structure of the 16S rRNA gene of *E. coli* and partial DNA sequence, taken from (Neefs et al., 1993; Perry et al., 2002).

Microbial Ecology, Historical Introduction

Despite the fact that microbial species have been on the planet for billions of years and have a biotic and physiological diversity that dwarfs that of all macro-organisms, the vast majority of community members from microbial environments have been essentially invisible to us until quite recently. This is true of microbial environments associated with crops, soils, the human body, deserts, and the deep sea. The past two decades have witnessed a virtual “awakening” in hundreds of fields with regard to

microbial ecology. The use of culture independent methods in the past two decades, has done as much to expand the understanding of microbial worlds as was achieved in the late 1600's when Antony van Leeuwenhoek directed the brand new tools of microscopy at the microbial world of oral and seawater samples. His drawings of "animalcules" (bacterial and protozoan species) were recorded in the scientific literature for the first time in 1683(Perry et al., 2002).

Not until approximately 200 years later, did we gain an understanding of anaerobic organisms, disprove the theory of "spontaneous generation"(Pasteur), and begin to describe "germ theory" and the responsible agents of many diseases (Koch and colleagues) (Perry et al., 2002). That only leaves an approximate one hundred additional years to arrive at the "modern" era. Which witnessed the suggestion (Zuckerkindl and Pauling, 1965) and application (Woese and Fox, 1977) of "molecules of heredity" (DNA) to organize all species according to their evolutionary history. Woese's work revolutionized our understanding of the diversity in the microbial world and identified a novel (to our understanding) "domain" of life –the Archaea - both evolutionarily and biologically distinct from Bacteria and Eukarya (Woese et al., 1990).

The human body has likely supported more bacterial cells than human cells for millions of years. This colonization begins with the first meals that an infant consumes (Gill et al., 2006d;Lotz et al., 2006). Examination of the microbial ecology associated with plant foods and ways in which we may influence this through the

growth and management of crops is a valuable field of research that will help us to guide both sustainable agricultural stewardship and the healthful stewardship of our own bodies.

Culture-dependent vs. Culture-independent

There had been a growing understanding in microbiology for some time, that “there was more going on than was meeting the eye” - or the culturing technique. A key event that drew the attention of science and the general public to a fuller understanding of unexplored microbial diversity and its potential importance to human health was the identification that ulcers are caused by the bacterium *Helicobacter pylori*. The earliest observations of this bacterium in the intestinal tract of animals and humans date back to 1893 and 1906 respectively (Buckley and O'Morain, 1998). However, the bacterium had never been successfully cultured so it was essentially invisible and thus unstudied. The great Bible of Bacterial Taxonomy, *Bergey's Manual of Determinative Bacteriology* stated that no organism could be classified without being cultured (American Society of Bacteriologists, 1923). It did not escape the notice of the public and science that a great deal of human suffering could have been avoided if study of the spiral *Helicobacter* bacterium had progressed from its earliest observations.

Metagenomics and 16S rRNA Gene Clone Libraries

Norman Pace and associates embarked on the use of rRNA genes as a tool for examining the genetic diversity present in various environments, without the need for a culturing step (Pace et al., 1985). Their work initiated a paradigm shift that subsequently took place in all fields of microbial ecology (Handelsman, 2004). The

use of the DNA of the rRNA gene as a means to explore microbial diversity in environments has become an invaluable standard and has been used for the past twenty some years.

The work by Pace's group was in a sense, the very first metagenomic study. The introduction of the word metagenomics is credited to Jo Handelsman, used in the late nineties, in a paper that examined the biological access to chemistry of unknown soil microbes through the use of molecular methods (Handelsman et al., 1998). Metagenomics is synonymous with "environmental genomics", or "community genomics" and while it began as a field of study that relied primarily on the use of a single gene, the 16S rRNA gene, to describe the microbial members of an environment, it has quickly evolved to include all the newest sequencing technologies and genomic strategies.

The Phyllosphere Biosphere: Life on a Leaf

The phyllosphere is a much larger environment than many people realize. Imagine every leaf on every tree in every forest, every shrub and weed along every highway, and every blade of grass in every field on earth. This environment has been estimated to span 10^{18} cm² of surface area and support between 10^4 and 10^8 cells per cm² of leaf tissue, an estimated 10^{26} organisms in total (Morris and Kinkel, 2004). The phyllosphere is a highly diverse physicochemical environment with huge fluctuations in nutrient availabilities, temperatures, water availability, wind pressures, exposure to pollutants, UV radiation and the variable biology of plant cuticles. The morphology of crystalline epicuticular waxes covering leaf surfaces and their associated diffusion

properties also play an important role in the geography that supports phyllosphere biota.

The term “phyllosphere” was first used by two independent studies in the same time period, Dr. Jakoba Ruinen and F. T. Last both used the term independently to describe their research environments (Last, 1955;Ruinen, 1956). Ruinen went on to publish an extensive body of research focusing on the phyllosphere, including the introduction of the term and concept of epiphytosis, host decline by epiphytes (Ruinen, 1953;Ruinen, 1956;Ruinen, 1970;Ruinen, 1974). One study in particular has been cited quite often and is more commonly associated with the introduction of the term simply because of its descriptive title; “*The phyllosphere: I. An ecologically neglected milieu*”(Ruinen, 1961).

Microorganisms in the phyllosphere contribute to the health and pathology of the plants they inhabit as well as to numerous other global processes in more ways than we realize (Lindow and Brandl, 2003;Morris and Kinkel, 2004). Bacteria in the phyllosphere have been shown to produce phytohormones that influence plant growth, they have been shown to cause diseases, contribute to plant health and even to prevent diseases of plants (Beattie, 2006;Holland et al., 2002;Patowska, 2003;Poppe et al., 2003;Stockwell et al., 2002;Wright et al., 2001). Phyllosphere bacteria have also been shown to fix atmospheric nitrogen (Bailey et al., 2002;Bentley and Carpenter, 1984;Freiberg, 1998;Ruinen, 1974), and degrade airborne pollutants such as monocyclic (toluene, phenol, ethylbenzene and xylene) and polycyclic aromatic hydrocarbons (PAHs). PAHs are by-products of burning

fuels such as oil and coal, some of which are likely human carcinogens (Darlington et al., 2001; DeKempeneer et al., 2004; Norramit et al., 2007; Sandhu et al., 2007; Waight et al., 2007). With a greater understanding of the numbers and the complexity of the organisms that exist in this environment, it is no longer surprising that bacterial members of the phyllosphere play an important role in various global processes. It has long been established that microorganisms play key roles in the earth's biogeochemical cycles (Ram et al., 2005).

Food Safety and the Phyllosphere

More than 200 diseases are thought to be transmitted through foods. In the United States alone, foodborne diseases are believed to cause an estimated 76 million illnesses annually, 325,000 hospitalizations and 5,000 deaths. What is even more intriguing about these estimates, assembled by the Center for Disease Control and Prevention, is that 62 million illnesses, 265,000 hospitalizations and 3,200 deaths are attributed to **unknown** disease agents associated with foods (Mead et al., 1999).

Enteric human pathogens such as *Salmonella enterica* have been the cause of health outbreaks linked to the consumption of fresh produce dating back to the early 1900s. As early as 1912, R.H. Creel published "Vegetables as a possible factor in the dissemination of Typhoid fever" in the *Public Health Reports*, linking celery to an outbreak of Typhoid fever – *Bacillus typhosus* (*Salmonella enterica* serovar *Typhi*) (Creel, 1912).

In the past ten years however, there has been a significant increase in the number of reported health outbreaks involving fresh produce. Strains of *Salmonella* have been associated with contamination of cauliflower, pepper, alfalfa sprouts, cantaloupe, lettuce, tomatoes, almonds, bean sprouts, basil, mung bean sprouts, unpasteurized orange juice, mixed bag salad, rocket salad, Spanish lettuce, and cilantro (Heaton J.C. and Jones K., 2008). The same time period has also witnessed outbreaks of human pathogenic *E.coli* associated with alfalfa sprouts, salad, fruit salad, coleslaw, clover sprouts, coriander, cucumber, spinach, parsley and unpasteurized apple juice (Heaton J.C. and Jones K., 2008). Other pathogens such as *Campylobacter jejuni*, Norovirus, and Hepatitis A, have also been associated with a large number of contamination incidents in the past ten years (Heaton J.C. and Jones K., 2008).

Why has this increase occurred in the past decade? Hypotheses include:

- Trends in industrialized agricultural production?
- Trends in industrialized distribution of foods? (Heaton J.C. and Jones K., 2008)
- Population pressures?
- Increased consumption of fresh produce in the average American diet?
- Increased levels of fecal material in close proximity to agricultural lands due to space constraints associated with population pressures or climate factors such as floods? (Brandl and Mandrell, 2002).
- Improved detection and surveillance? (Suslow, 2002)

- Pollution/climate impact on phyllosphere environment contributing to more favorable conditions for pathogen survival?

Few can provide definitive evidence for any specific epidemiology at this point but it is highly likely that one or all of these factors are contributing to the increased number of foodborne illnesses associated with fresh produce of the past ten years.

Research as early 1901 demonstrated that plants (and therefore foodstuffs) could be contaminated by infected soil. Work in the early 1900s established that enteric pathogens such as *Salmonella* are able to survive in soil for as long as 84 days, in water in a fish tank for up to 36 days, and in mud at the bottom of the tank, for approximately 60 days (Creel, 1912).

The observation that enteric pathogens have varying degrees of environmental fitness has been an important factor in the expansion of epidemiological trace-back efforts beyond processing environments, back to the growing environment of each crop. Thus, the preharvest environment of food crops has become an important new “field” (or phyllo-sphere) of research at the intersection of microbial ecology, food safety and medical microbiology (Brandl, 2006).

Research in the Phyllosphere

Research directed at the phyllosphere has focused largely on the ecological fitness of human pathogens on leaf surfaces, endophytic growth of these organisms, plant resource utilization by microbes, plant-microbe interactions, and microbe - microbe interactions (Brandl, 2006). The primary focus, however, has been afforded to plant

pathogens and consequently if it's not a plant pathogen, we probably don't know that much about it.

What is still surprisingly lacking, is a body of research that examines the more general ecology of the agricultural phyllosphere environment using non-culturing methods. The term "agrisphere" is suggested to describe the microbial ecology of agriculturally impacted environments. General questions of particular interest for study of the agrisphere are:

- *What species make up the epiphytic microbial population of a particular crop?*
- *Are the microbial consortia different for a crop grown in one location compared to the same crop grown in another location?*
- *How are the microbial consortia impacted by agricultural applications?*

This kind of information is still unavailable for most crops so the goal of my dissertation research is to make an important ecological contribution to fill this data gap.

Previous Phyllosphere Research

Phyllosphere microbial ecology has very few data sets derived from culture-independent methods that effectively survey and describe bacterial community members. Taking the *Helicobacter pylori* lesson into account along with the CDC data that describe an estimated 62 million illnesses caused by **unknown agents**, there is a certainly a lot to be learned about many aspects of food safety. With regard to plant foods, the microbiological continuum from the “field to the fork “ will become safer and healthier as we identify potential risks associated with the microbial dynamics in agricultural phyllospheres.

Citrus, Corn, Beans, Beets and Cotton

One of the very first groups (if not the first), to take a molecular approach to the study of the phyllosphere examined bacterial species on three species of citrus trees, corn, green beans, cotton and sugar beet in 2001. They demonstrated that bacterial species associated with the different plants and even different species of citrus (with the exception of corn) clustered together. This was presented as evidence to support the idea that there are unique phyllosphere microbial populations on different plants or different species of orange. The cluster analysis was based on a visual interpretation of bands in DGGE gels, not on sequence data, so further phylogenetic study would be of value. This group sequenced a total of 17 sequences (236 bp - V3 region of 16S rDNA gene) directly from DGGE bands (a total of 4,012 bps) (Yang et al., 2001).

Brazilian Forest Trees, *Trichilia* and *Campomanesia*

There have been several other molecular studies that have examined uncultured bacterial species associated with the phyllosphere. Research by Lambais et al. (2006) reported encountering the following bacterial phyla in the phyllosphere of three tree species (*Trichilia catigua*, *Trichilia clausenii* and *Campomanesia xanthocarpa*) from an Atlantic Forest in Brazil: Proteobacteriaceae – (Alpha, Beta, Delta and Gamma classes), Bacteroidetes, Firmicutes, Cyanobacteria, and Actinobacteria. This is one of the first molecular data sets to provide a picture of who may comprise the “usual suspects” if there even is a set of “usual suspects” in a phyllosphere environment. The work was published in *Science* in 2006 and is probably one of the largest molecular data sets reported for the phyllosphere to date –about 430 sequences that were approximately 481 bp long. This makes a data set of over 200,000 bps (Lambais et al., 2006).

Maize

The maize phyllosphere was also examined to study the impact of UV radiation on bacterial species composition. The total data set in this study was 72 sequences of the V3-16S rDNA fragment between 400 and 500 bps long, yielding a total of approximately 32,400 bps (Kadivar and Stapleton, 2003). Although their sample size was small and their statistical power was low, the authors described a trend in increased microbial diversity in response to UV exposure.

Potato – Transgenic and Nontransgenic

One study compared the bacterial microflora of transgenic and non-transgenic potato plants (*Solanum tuberosum*) (Heuer and Smalla, 1999). A variety of methods, including fatty acid analysis, PCR-DGGE and some 16S rRNA gene sequencing were used to determine if the bacterial species on a transgenic potato plant differed from those on a non-transgenic potato plant. Thirty BLAST identities based on a 200bp fragment of 16S were provided although identity scores associated with these taxonomies were extremely low for the majority of the sequences. Of the thirty identities reported in this study only five sequences were submitted to Genbank. No differences in microbial species of the transgenic and nontransgenic plants were reported.

Fern

An interesting “resurrection fern” phyllosphere study was recently reported (Jackson et al., 2006). Bacterial species associated with wet and dry periods (as the fern rehydrates from a desiccation-resistant physiologically inactive state to an actively growing plant) were examined. Fifty-five sequences of partial 16S rDNA gene fragments (550 bp) were submitted to Genbank and used for phylogenetic interpretations of the diversity in wet and dry fern environments.

The most common species in the library created from the dry fern sample were members of the Methylobacteriaceae and Acidobacteria. Members of the wet fern clone libraries were predominantly Methylobacteriaceae and Beijerinckiaceae.

Despite the same incidence of the family Methylobacteriaceae, the authors reported little overlap in bacterial ribotypes between these two libraries.

Essential Oils, Mango and Coffee

A study by Yadav et al. discovered that epiphytic bacterial species associated with plants with high levels of aromatic essential oils had a greater diversity of substrates they were capable of catabolizing (Yadav et al., 2008). Seasonal changes were demonstrated in the mango phyllosphere (deJager and Korsten, 2001) and species associated with a coffee phyllosphere demonstrated an interesting antifungal activity (Nair et al., 2002). More and more good research studies are beginning to address this “milieu”, with culture-independent molecular, hypothesis-driven, statistical and biochemical methods.

Agrisphere

The dissertation work presented here remains the first to address the impact that agricultural practices may have on microbial community species in the phyllosphere of organically and conventionally managed apples. Organic agriculture is an important growing trend that attempts to provide more sustainable methods with which to approach agricultural practices. The use of toxic synthetic pesticides and fertilizers, irradiation, sewage sludge and genetic engineering in anything that will have official organic certification are all prohibited (Organic Trade Association). Antibiotics are also prohibited in most livestock organic environments, however a few exceptions remain – such as the use of Streptomycin and Tetracycline to combat

the bacterial plant pathogen of apples and pears known as Fire Blight (*Erwinia amylovora*). The initial selection of apples as a crop to examine was a combination of sustainable agricultural objectives and food safety initiatives. The identification of small scale “niche” crops (such as low impact organic crops) is part of efforts to support local sustainable agricultural ventures and the streamlining of organic methodology and practice also provides valuable data for sustainability research.

From a food safety angle, health outbreaks that involved both *E. coli* O157 H:7 and *Salmonella* had been associated with unpasteurized apple cider at numerous time-points in the last twenty years, most recently in October of 2008 (Benedict, 2008; Luedke and Powell, 2000). Apples and pears also have the devastating bacterial pathogen that has been treated for many years with agricultural grades of antibiotics such as tetracycline, oxytetracycline and streptomycin. This pathogen, Fire Blight, (*Erwinia amylovora*) is in the same family as *E. coli* and *Salmonella*, which raises questions about risk potentially associated with broadcasting of antibiotics that target a species in the same family and genetically similar to both *Salmonella* and *E. coli*.

Questions surrounding potential horizontal gene transfer (HGT) of acquired antibiotic resistance in agricultural settings remain largely unanswered. Understanding the metagenomic microbial ecology of the crop environment will increase our ability to identify risks associated with agricultural applications and their selection pressures. HGT, for example, has been shown with increasing frequency to play a significant role in determining the mosaic structure of bacterial chromosomes. *Escherichia coli*

O157:H7, for example, an important human pathogen, has been documented to contain genetic “islands” derived from donor species that comprise almost 26% of its genes (Brown et al., 2003;Perna, 2001).

While our methodology will not provide the tools with which to examine HGT in the phyllosphere environment, we will be able to provide a description of the microbial ecology and hence the genetic pool that could be involved in possible HGT. Because of the food safety focus of this research (and also the sustainability focus from a plant pathology perspective, incidence of the Gram negative *Erwinia amylovora*), we did not want to miss bacterial members of the family Enterobacteriaceae in the phyllosphere of the apple and pear crop so we selected a Gram negative DNA extraction. Some researchers have hypothesized that with the heavy chemical lysing methods employed to examine Gram positive and archaeal members of certain environments, can degrade Gram negatives so they are not well represented in the resulting data sets (Gill et al., 2006c).

Research Objectives

Microbial Ecology of the Phyllosphere

We aim to establish whether selection pressures associated with organic or conventional agricultural applications result in a different bacterial microflora in the orchard phyllosphere.

We aim to make a significant contribution to the ecological description of microbial species associated with the agricultural phyllosphere of a food crop.

Phyllosphere and Food Safety

We aim to determine whether or not health risks associated with enteric pathogens increase under organic or conventional management primarily by examining whether or not members of the Enterobacteriaceae (*E. coli*, *Salmonella*) are more prevalent in organically or conventionally managed samples.

Materials and Methods

Phyllosphere Sampling

At multiple time-points throughout three growing seasons, 2005-2007, fruit and leaves of the cultivar 'Enterprise', were collected from 5 replicated blocks of organic and conventionally managed trees (see chapter 1 for details). Approximately 20 leaves plus two apples were placed in a sterile ziplock bag. Leaves were collected from around all sides of the tree and transported back to the lab in sealed bags in a cooler at 4° Celsius. Three hundred ml. of deionized water was added to the bags and samples were sonicated for five minutes to dislodge phyllosphere microbial species. The microfloral wash was transferred to centrifuge tubes and centrifuged at 30,000g for twelve hours at 4°C. Pellet was transferred to a small microcentrifuge tube and stored at -20° C until DNA extraction.

Microbiological Methods

Dilutions of the wash were plated in duplicate MacConkey agar plates and 3M Total Coliform Petrifilms. Plates and films were incubated at 37° for 48 hours and colonies were subsequently enumerated and analyzed for variance between treatments.

Molecular Methods

DNA Extraction

Protocols preferential for the extraction of Gram negative species (Promega Wizard DNA Extraction Kit) were used according to the manufacturer's specifications.

PCR for Clone Libraries

A 550 bp fragment of the V3 region of 16S rRNA gene was amplified for cloning and sequencing purposes; Forward primer: 5'-CCTACGGGAGGCAGCAG-3'. Reverse primer: 907R; 5'-CCCCGTCAATTCCTTTGAGTTT-3' (Muyzer et al., 1995; Teske et al., 1996). 50µl reactions were prepared with 5µl of 10x Buffer (Takara), 2µl of Mg Cl₂, 1 µl dNTPs, forward primer, and reverse primer at 25pmol and 39.8 µl water and .2µl taq (Takara) . PCR included a hot start of 95 °C for 5 min. Thirty cycles of denaturing at 94°C for 1 min., annealing at 55 °C for 1 min. and extension at 72 °C for 1 min., with a final extension of 72°C for 5 min. and storage at 4°C.

DGGE

Denaturing Gradient Gel Electrophoresis (DGGE) PCR. A 200 bp fragment of the V3 region of 16S rRNA gene with an added GC clamp was used for preliminary “community profiling” use with DGGE. (A GC clamp is a long series of G’s and C’s that serves as an “anchor” in the denaturing gradient gel. The band in DGGE gels is generated when the two strands of PCR product (in our case 16S rRNA gene fragment) denature. Without the heavy GC clamp, the denatured strands would continue to migrate through the gel and would not have generated the bands we use to get an understanding of the community profile). Primer sequences used in DGGE:
P3 (forward GC clamped) 5'CGCCCGCGCGGGCGGGGCGGGGGC
ACGGGGGGCCTACGGGAGGCAGCAG-3' P2 (reverse) 5'- ATTACCGCGGCT

GCTGG-3' (Muyzer et al., 1993). DGGE was performed using a D-Code Universal Mutation Detection System (Biorad) Hercules, CA.

Approximately 40 µl of PCR product was loaded into a 6% acrylamide gel with a linear gradient of (40 to 60%) urea and formamide. Gels were run at 60° for approximately 15 hours, at 60 volts. Gels were stained with SYBR green and imaged with UV light using Canon digital cameras associated with a photographic hood. When possible the STORM system (600 dpi flatbed densitometer) from Molecular Dynamics at the Center of Marine Biotechnology (COMB) in Baltimore was used.

Clone Library Construction

PCR products were cloned using the Promega T-Easy Kit, according to the manufacturers specifications (Promega) Madison, WI. Plasmids were initially isolated using the Wizard Plus Minipreps DNA Purification System (Promega) Madison, WI. A more rapid method was used in 2007 that involved growing *E. coli* clones in 200µl of Luria Broth (LB) and 20% glycerol stock in 96 well plates for exactly 12 hours at 37° and then freezing at -80°.

Sequencing

Frozen 200µl of *E. coli* clones in 20% glycerol and Luria Broth (LB) (Miller) solution, in 96 well plates, were shipped on dry ice to Agencourt Genomic Services in Beverly, MA where they were sequenced. Alternatively, approximately 20 µl of mini-prepped clones were shipped in 96 well plates to Genewiz in South Plainfield, NJ.

Bioinformatic Methods

Preprocessing of 16S Sequences

Quality scores of sequences were computed using Phred (Ewing et al., 1998). Sequences were subsequently trimmed for quality using LUCY (Chung and Holmes, 2008), an open source program developed by TIGR, and then trimmed for vector using NUCmer (Delcher et al., 2002). Trimmed sequences were filtered for short lengths and screened for vector, chloroplast, and 18S rDNA contaminants using BLASTN. Any sequences less than 400 bp were removed, as were those with BLASTN hits to contaminants (chloroplasts and 18 S rRNA gene fragments from Eukaryotes) with a bit score of greater than 300. Bellerophon was used to identify potential chimeras (Huber et al., 2007).

Taxonomic Assignment of 16S rDNA Gene Sequences

We downloaded the Ribosomal Database Project's (RDP II) unaligned release 9.57 (Cole et al., 2007; Wang et al., 2007) of approximately 471,000 rDNA sequences. From this we generated a database from RDP sequences containing at least 4 taxonomic identification levels. Each 16S sequence was assigned to its closest neighbor within that database using the BLASTN best bit score. The RDP Bayesian classifier was also used to check for consistency in classification. No major differences were found for high levels of taxa. BLASTN was used for final taxonomic identities because it provided closest possible species.

Alignment

Sequences were aligned using ARB (Ludwig et al., 2004). Alignment was also done with MUSCLE (Edgar, 2004) and NAST(DeSantis, Jr. et al., 2006) to ensure that observed differences were not due to artifacts of alignment programs (Wong et al., 2008). Finalized alignments were subsequently trimmed so that each sequence spanned the entire alignment. Columns with greater than 20% gaps were removed. Distance matrices were created using ARB with Olsen distance correction.

Assignment to Operational Taxonomic Units

DOTUR (Distance-Based OTU and Richness) assigns sequences to OTUs (operational taxonomic units) by nearest neighbor algorithm (Schloss and Handelsman, 2005a). Using the frequency at which each OTU is observed DOTUR generates “rarefaction” or “collectors” curves for designated measures of richness and diversity and to determine sampling depth needed to accurately represent community members of any given environment. We clustered OTU’s using the furthest neighbor algorithm for the recommended measurements of 3%, 5%, 10%, and 20% difference. DOTUR also calculates the ACE and Chao1 non-parametric estimators for each specified evolutionary distance along with the Shannon diversity index.

Shared Operational Taxonomic Units

Distance matrices were also analyzed using SONS, which implements nonparametric

estimators for the fraction and richness of OTU's shared between two communities (Schloss and Handelsman, 2006a).

Assigning a P Value to Observed Differences

f-Libshuff (Integral- Libshuff) The "integral" was added to the previous version of Libshuff to describe the addition of the exact and integral form of the Cramer von Mises statistic to the computation. The previous version of Libshuff uses the approximation of the statistic, which is also an option on the new version. *f*-Libshuff estimates the Cramer-von Mises statistic to test if two environments are drawn from the same underlying population using a Monte Carlo testing procedure. It evaluate differences between each community(Schloss, 2008). It is a phylogenetic approach because it measures the differences between communities based on the differences between sequences. The Monte Carlo testing procedure methods are particularly advantageous to our data set because significant differences can be detected even if libraries do not contain a large number of sequences. *f*-Libshuff reports p-values that measure the probability that the observed differences between two genetic libraries are due to chance. Significance levels were assessed through bootstrapping with 50,000 randomizations (Schloss et al., 2004).

C_x (coverage of x) and C_y (coverage of y) are the fraction of sequences that have at least one other sequence from the same library near them. Near is defined by the distance being considered. For a distance of .03, you would count the number of sequences that are within .03 distance of another sequence. To get C_x, you would

then divide by the total number of sequences in the library. To get C_{xy} (defined as “heterologous”), you would count the number of sequences from x that are within .03 from y . This number is then divided by the number of sequences in x . The program creates the following matrix:

xx xy

yx yy

Integral Libshuff pulls out xx (described as “homologous”) for each row and thus generates C_x as a function of Distance = .03. The same is then computed for C_y , C_{xy} , and C_{yx} . Then the program calculates $(C_x - C_{xy})^2$ for each distance such as .03, plots it and calculates the area under these curves (Schloss, 2007).

Phylogenetic Analysis

A random member of each shared and unique OTU at a distance of 0.03 was selected and imported into ARB. An Olsen-corrected distance matrix was generated, and this was used to create an unrooted neighbor-joining tree (Ludwig et al., 2004). The Interactive Tree of Life was employed for visualization (Letunic and Bork, 2007) and Figtree was also used to visualize a phylogenetic tree (<http://tree.bio.ed.ac.uk/>). An alignment created in NAST and then manually adjusted in MacClade was also used with GARLI to generate likelihood scores for the tree that best fit the data. GARLI performs heuristic phylogenetic searches under the General Time Reversible (GTR) model of nucleotide substitution (Zwickl, 2006).

Diversity Indices

Shannon Diversity Index is a measure of species richness and species evenness.

Richness is defined as the number of different species in a given environment and evenness is a term used to represent the relative abundance of species in an environment. This index can be increased by either the addition of unique species or greater species evenness. A Shannon Index typically falls between 1.5 and 4.5.

Chao1 Diversity Index

The Chao1 statistic is a nonparametric estimator that uses the frequencies of observed OTUs to estimate the richness of organisms in a community without having to sample every organism (Schloss and Handelsman, 2005a). Nonparametric Chao1 estimates, predict the point at which an accumulation curve will begin to level off. Because the Chao1 diversity estimate uses the relative proportions of singletons and doubletons for calculating estimated diversity, the abundance of rare sequences in phyllosphere samples leads to higher estimates of richness.

Results

Culture-dependent Microbiology

Using both MacConkey (selective media for Gram negative lactose fermenting species) (*data not shown*) and Total Coliform Petrifilms (3M) (also designed as a selective media to culture Gram negative lactose fermenting specie, reportedly with increased selectivity for coliforms). A coliform is defined as a bacterium that is found in the intestines of humans or animals but also in environmental spheres such as the soil.

This definition is so broadly inclusive, that it is slightly unclear what the exact range of taxonomic cultivation potential associated with this media may be. Both MacConkey media and Total Coliform Petrifilms (3M) have a long history of use as indicators of the possible presence of pathogenic organisms and were recommended for our preliminary research by Food and Drug Administration (FDA) microbiologists. They both contain lactose, Violet Red Bile or Crystal Violet with Bile Salts (3M and PML Microbiologicals Technical data sheets).

Colony Forming Unit Enumeration

Using both medias, no statistically significant differences between treatments could be identified associated with any of the sampling time-points. Colony forming units (CFUs) were enumerated analyzed for variance using SAS. A T-Test was also performed using excel functions. Both tests showed no significant differences between treatments at all time-points with an alpha of .05.

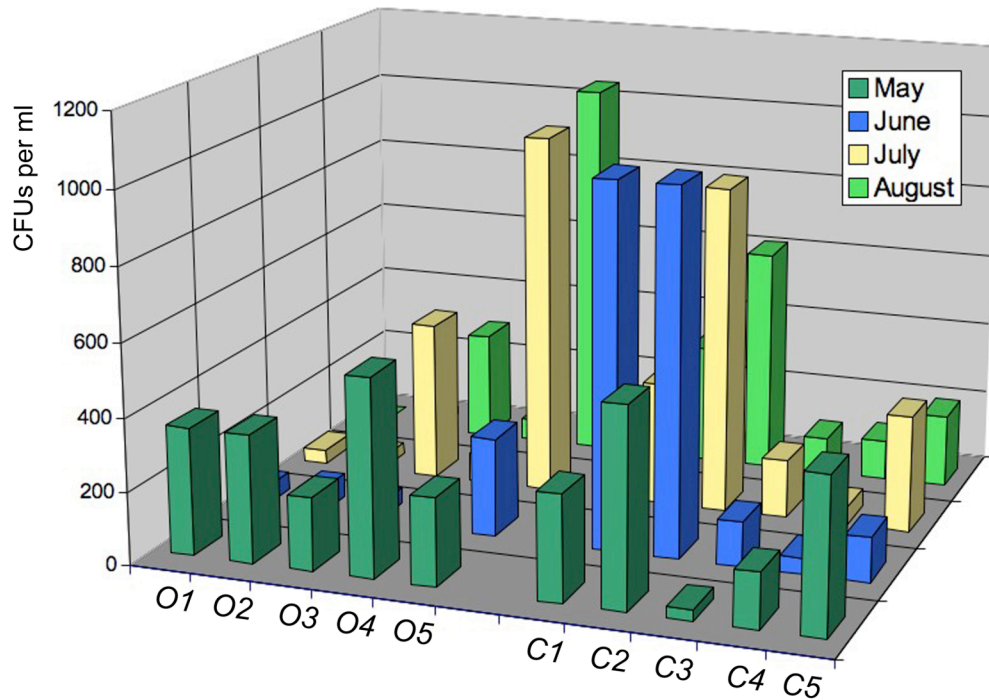


Figure 6. Enumerations of Colony Forming Units (CFUs) on Total Coliform Petrifilms (3M) of phyllosphere microbial species associated with apples and leaves of apple cultivar ‘Enterprise’ at time-points from May through August of 2005.

On the x axis, O1 through O5 represent five independent replications of the organic treatment and C1-C5 represent five independent replications of the conventional treatment (see Figure 1). On the y axis the enumerated colony forming units (CFUs) per ml are shown.

Molecular PCR-DGGE

With the switch to molecular methods, it was our hope that we might be able to get a higher resolution understanding of what was going on with bacterial species in the phyllosphere than what we were able to produce using the microbiological culturing techniques. If there was indeed a signal related to treatment, the variability or dispersion of the enumerations of CFUs may have created too much noise to allow us to identify it. Because molecular methods do not rely upon a culturing step, we hoped that with the molecular methods we could identify species or community dynamics that might be influenced by treatment impact but were not cultureable with the microbiological medias. Estimates of microbial species from the environment that scientists are unable to culture in laboratories are higher than 99% for some environments (Schloss and Handelsman, 2005b). Our first comparison of cultured to uncultured phyllosphere microbial diversity was extremely impressive (Figure 7). A much greater diversity was seen in the culture-independent molecular methods.

To generate the cultured organisms, we took a wash from apple leaves (May, 2005) and plated dilutions of the wash onto MacConkey agar (selective for Gram negative species). DNA was extracted from the cultured colonies with an extraction specific for Gram negative species (Promega, See Methods DNA extraction). For the culture-independent samples DNA was isolated directly from the microfloral wash of organic and conventional phyllosphere samples ***without the culturing step***. The same DNA extraction protocols were used and the same PCR primers for 16S rRNA gene

fragments were used. Resulting products were visualized using Denaturing Gradient Gel Electrophoresis (see Methods for more details) (Figure 7).

Our first DGGEs demonstrated a striking difference in the microbial diversity between culture-independent and culture-dependent phyllosphere samples.

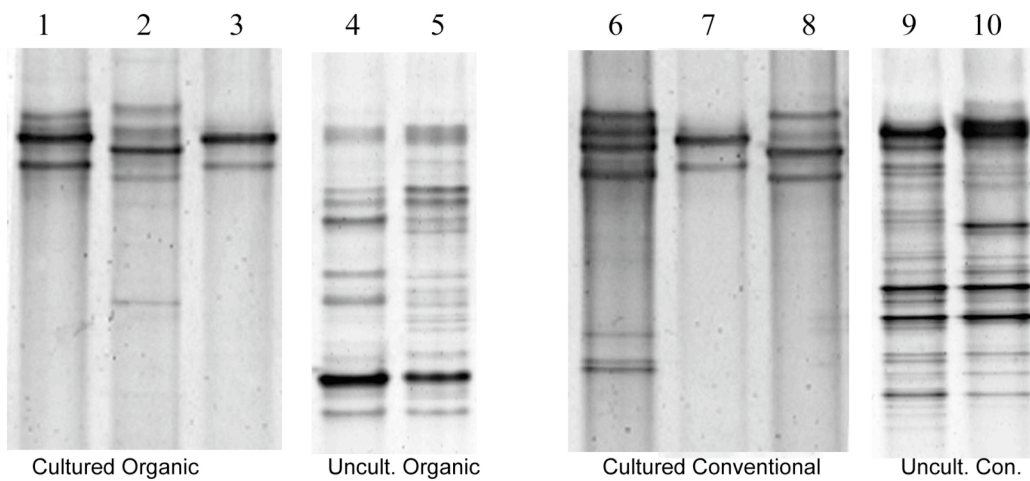


Figure 7. Denaturing Gradient Gel Electrophoresis Comparing Cultured and Uncultured Microflora from Organic and Conventional Phyllosphere Samples collected in May of 2005.

Lanes 1, 2, and 3 are cultured organic and Lanes 4 and 5 are uncultured organic.

Lanes 6, 7, and 8 are cultured conventional and Lanes 9 and 10 are uncultured conventional.

This preliminary DGGE work suggested that it might indeed be possible to identify a “signal” associated with treatment effect (organic and conventional) through the use

of culture-independent molecular methods. Subsequent DGGEs demonstrated an consistent increase in the diversity of Gram negative species associated with organic samples. Figure 8 shows four independent replications of organic (O1-4) and conventional (C1-4) treatments from July 2005. There is an increase in the number of bands seen in the middle section of the gel, perhaps illustrating a group of similar species associated only with the organic treatments.

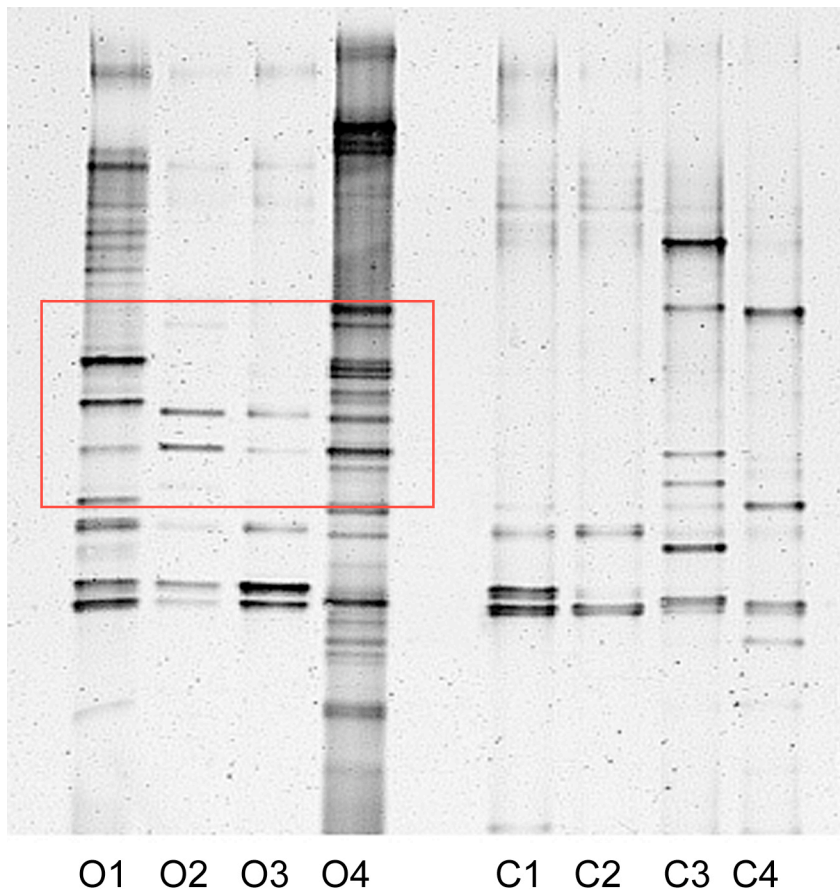


Figure 8. Organic and Conventional 16S rRNA Gene Fragment DGGE from July 2005.

O1 through O4 are independent replications of organically treated Enterprise apple samples created from 16S rRNA gene PCR of DNA extracted directly from the microfloral wash (See methods, Phyllosphere Sampling). C1 through C4 are independent replications of conventionally managed Enterprise apples also created from the same fragment of 16S rRNA gene PCR of DNA extracted directly from the microfloral wash. The increase in diversity associated with organic samples was observed at numerous subsequent time-points throughout 2006 (*data not shown*).

16S rRNA gene Clone Libraries

Over three years (2005 - 2007), at six different time-points, phyllosphere microflora was sampled and 16S rRNA gene clone libraries were generated and processed according to the protocols described in the Materials and Methods section. A total of eight hundred and eighty six sequences remained after removing contaminants such as chloroplast 16S, 18S, low quality sequences, vector and potential chimeras (see Methods). Four hundred and forty five sequences from the conventionally treated apple trees were generated and three hundred and eighty three sequences were generated from organically treated samples.

The taxonomic diversity represented in our metagenomic libraries spans 8 bacterial phyla and 14 classes. Despite the Gram negative extraction bias, two phyla of Gram positive organisms were identified, Firmicutes and Actinobacteria. The percentages of the eight phyla that were observed in the 16S rRNA gene clone libraries, based on BLAST NCBI taxonomic classifications are shown in Figure 9.

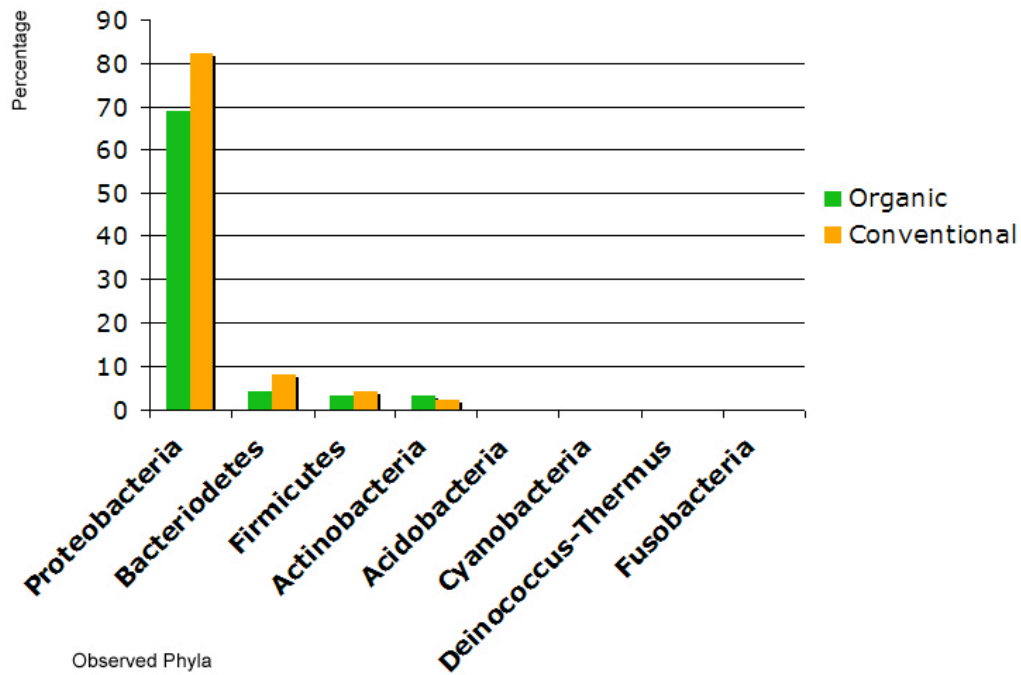


Figure 9. Percentages of Bacterial Phyla seen in Organic and Conventional Phyllosphere 16S rRNA gene clone libraries.

The x axis lists the observed phyla and the y axis is the percentage of each phylum represented in organic and conventional libraries.

By far, the most well represented bacterial phyla in the phyllosphere, was

Proteobacteria, followed by Bacteroidetes, Firmicutes, and Actinobacteria.

Acidobacteria, Cyanobacteria, Deinococcus-Thermus and Fusobacteria (Figure 9)

were only represented by a few sequences in the total library and amounted to less

than one percent in most cases. We encountered no species of Archaea, despite the use of additional Archaea specific primers for the 16S rRNA gene (as well as other

genes). It is highly likely that the one member of the phyla Fusobacteria is

contamination from the oral microflora of the researcher. It was left in the data set

due to the possibility that it originated from an environmental source of manure or the intestines or excrement of an insect. There are reports of Fusobacteria associated with manures and the intestines of many species as part of the normal flora (Woodbury, 2001).

The taxonomic representation by class delineation is broken down in Figure 10, which illustrates the eight most prevalent classes seen in the metagenomic phyllosphere data (14 classes were observed in total). Members of the classes Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria of the Proteobacteria were the dominant classes in the phyllosphere 16S rRNA gene clone libraries. Classes observed but not shown in the graph due to their singleton or low copy number status, include; Deltaproteobacteria, Flavobacteria, Acidobacteria, and Deinococci.

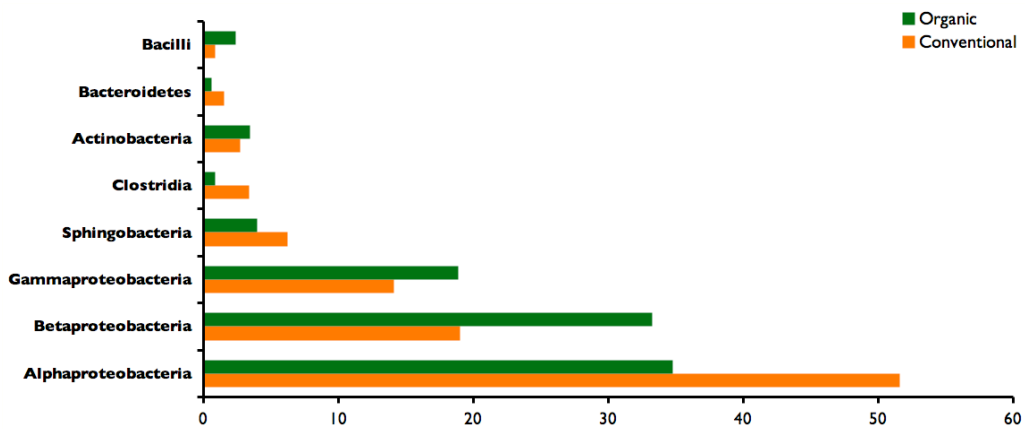


Figure 10. Percentages of Classes seen in Organic and Conventional 16S rRNA gene clone libraries.

Because we used such a small fragment of a highly conserved gene (16S rRNA gene – 550 bp), it is safest to use the higher orders of classifications to make inferences about bacterial taxonomic identities in this data set. We do however have very high similarity scores and very low E values for a number of sequences that are classified all the way to the species level (Full list with identity scores can be found in Appendix 6).

Table 2 shows a list of Phyla and Classes (in blue) and Families within these classes beneath (in black). (Reminder of Taxonomic hierarchy: [Phylum](#), [Class](#), Order, Family, Genus, and Species)

[Proteobacteria: Alphaproteobacteria](#)

Sphingomonadaceae
 Bradyrhizobiaceae
 Methylobacteriaceae
 Beijerinckiaceae
 Acetobacteriaceae
 Hyphomicrobiaceae
 Rhodobacteraceae
 Rhizobiaceae
 Bartonellaceae

[Proteobacteria: Betaproteobacteria](#)

Oxalobacteraceae
 Burkholderiaceae
 Comamondaceae

[Proteobacteria: Deltaproteobacteria](#)

Cystobacteriaceae

[Proteobacteria; Gammaproteobacteria](#)

Pseudomonadaceae
 Enterobacteriaceae
 Legionellaceae
 Moraxellaceae
 Pasteurellaceae
 Xanthomonadaceae
 Halomonadaceae

[Firmicutes: Clostridia](#)

Clostridiaceae
 Acidaminococcaceae
 Lachnospiraceae
 Bacillaceae

[Bacterioidetes: Flavobacteria](#)

Flavobacteriaceae

[Bacterioidetes: Sphingobacteria](#)

Flexibacteraceae
 Sphingobacteriaceae

[Bacterioidetes: Bacterioidetes](#)

Porphyromonadaceae
 Rickenellaceae

[Actinobacteria: Actinobacteria](#)

Microbacteriaceae
 Kineosporiaceae
 Actinomycetaceae
 Bifidobacteriaceae
 Nakamurellaceae

[Acidobacteria: Acidobacteria](#)

Acidobacteriaceae

[Cyanobacteria: Cyanobacteria](#)

Uncultured

[Deinococcus-Thermus: Deinococci](#)

Deinococcaceae

[Fusobacteria: Fusobacteria](#)

Fusobacteriaceae

Table 2. Families represented in the 16S rRNA gene clone libraries of organic and conventional phyllosphere bacteria.

Approaches for Comparing 16S rRNA Gene Clone Libraries

As shown in the previous figures and tables, a lot of information can be assembled by using a database to assign taxonomic delineation (databases such as NCBI BLAST or the Ribosomal Database Project (RDP)) based on reference genomes or reference sequences. There are however several other ways to approach the comparison of 16S rRNA gene clone libraries to test the simplest hypothesis associated with the microbial ecology investigation of the phyllosphere of organically and conventionally managed apples; *“Is there a difference?”*

Two other main approaches are currently accessible to test hypotheses associated with microbial communities represented by 16S rRNA gene data sets such as our own. The first is to use software tools such as software programs such as DOTUR and SONS (Schloss and Handelsman, 2005a; Schloss and Handelsman, 2006a) that assign sequences to operational taxonomic units (OTUs) based on the pairwise genetic distance between sequences. These programs also use observed OTUs to generate non-parametric diversity indices such as ACE, Chao1 and Shannon that can be used to examine species richness and evenness.

Values for OTUs, and the Shannon, ACE and Chao1 diversity indices were calculated for a distance value (D) set at .03 (percent different from each other or “dissimilarity”) with the 95% confidence interval shown in parentheses (Table 3).

Table 3. Phyllosphere OTUs and Diversity Indices at D = .03.

Distance of .03	# of sequences	OTUs	ACE	Chao1	Shannon
Organic	445	85	128(101,183)	123 (96, 185)	3.27 (3.12, 3.42)
Conventional	383	99	172(136,239)	225 (152, 390)	3.52 (3.39, 3.65)

The ACE (Abundance -based Coverage Estimator) index is a non-parametric measurement of diversity and species richness (basically the number of species in a sample). The ACE index uses the number of singletons and other rarely occurring sequences (sequences occurring up to 10 times) in a data set to estimate how many species are even rarer and didn't turn up in the data and to use this information to estimate species richness.

The Chao1 statistic is a non-parametric estimator that uses the *frequencies* of observed OTUs to estimate the richness of organisms in a community without the need to perform the impossible task of sampling every organism (Schloss and Handelsman, 2005a). Non-parametric Chao1 estimates also predict the point at which an accumulation curve will begin to level off. Because the Chao1 diversity estimate uses the relative proportions of singletons and doubletons for calculating estimated diversity, the abundance of rare sequences in phyllosphere samples leads to higher estimates of richness.

The Shannon Diversity index is another measure of species richness and species evenness. Richness is defined as the number of different species in a given environment and evenness is a term used to represent the *relative abundance* of each

of the species in the environment. This index can be increased by either the addition of unique species or greater species evenness (for example; there are five members of five phyla = high evenness). A Shannon Index typically falls between 1.5 and 4.5 with the upper end of the scale representing a greater diversity.

The program SONS (Schloss and Handelsman, 2006a) provides a nice examination of shared and unique OTUs from two libraries (Figure 11). The D (Distance of Dissimilarity) can be set at any percentage. The distance of .03 (97% similarity, 3% dissimilarity) is often as a cut off for species delineation. Though controversial, the following distances are considered to correspond to taxonomic delineations; less than .03 to a strain, .03 to a species, .05 to a genus, and between .30-.40 to a phylum (Schloss and Handelsman, 2006b) The shared and unique OTUs associated with the organic and conventional phyllosphere 16S rRNA gene clone libraries at a distance of .03 are shown in Figure 11.

Shared Similarities between OTUs of Organic and Conventional Bacteria

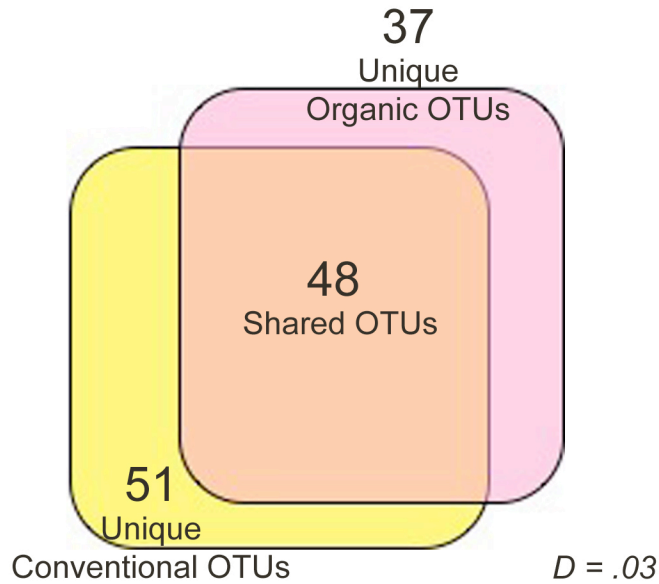


Figure 11. Shared OTUs and Similarities for Organic and Conventional Phyllosphere Bacteria at $D = .03$.

A total of 136 operational taxonomic units (OTUs) were found across both libraries, 48 of those OTUs were shared between organic and conventional, 51 were unique to conventional, and 37 were unique to organic. Despite the high number of unique OTUs associated with the organic and conventional treatments, these were actually very low abundance OTUs, comprising 5.3% and 9.3 % of the respective libraries. The 48 shared OTUs represented 85.4 % of all sequences. A list of the unique OTUs for each treatment can be found in Appendix 4 and 5.

Statistically Significant Differences between Organic and Conventional 16S rRNA
Gene Clone Libraries

A third method for examining the differences between libraries makes use of the Monte Carlo testing procedure to evaluate differences between each community. This approach is valuable because a large number of sequences is not required to detect significant differences, however the “precise nature of the hypotheses tested by these procedures is not clear” (Schloss, 2008). For the simple hypothesis; *is there a difference? Are the communities the same?* ; the Monte Carlo testing procedure used by the program β -Libshuff can examine our organic and conventional phyllosphere communities with a procedure that will optimize the information available in even small libraries.

When examining the sampling time-points from all six sampling dates over the three year period (2005 – 2007), we identified four of six time-points to be significantly different from each other with an alpha of ($p < .05$). Significant difference between organic and conventional libraries indicated with a star (*) (Table 3). β -libshuff generates two P-values for each comparison. If either P-value is significant, then the libraries are considered significantly different from each other.

Date	Conventional (as Homologous) P values	Organic (as Homologous) P values
July/August 2005(pooled)	0.2357	0.0005*
July 2006	0.1434	0.4717
August 2006	0.0032*	0.2322
September 2006	0.2909	0.167
July 2007	<0.0001	0.0955
August 2007	0.0002*	0.4756

Table 4. The Monte Carlo testing procedure used by J-Libshuff identified 4 sampling dates that demonstrated significant differences between organic and conventional 16S rRNA gene clone libraries.

Multiple P values are generated using J-Libshuff, so it is necessary to correct the experiment-wise false discovery error by dividing the alpha (.05) by the number of comparisons. For our situation, there are 6 time points and two comparisons for each time point (organic to conventional and conventional to organic) so using the Bonferroni correction (Abdi, 2007), our alpha is set at .05 and divided by 12 comparisons, which results in an alpha of (.004).

Results Important to Food Safety Objectives

Gammaproteobacteria is the taxonomic home to the family Enterobacteriaceae in which most of the enteric pathogens of concern in produce-related health outbreaks can be found. It is also however, home to thousands of environmental species that do not represent any known threat to human health. Examining Gammaproteobacteria is by no means the only way to try to estimate if microflora from either treatment may represent a greater human health risk. If there were however, significant differences between organic and conventional frequencies of Gammaproteobacteria and specifically within the family Enterobacteriaceae and the genera; *Escherichia* or *Salmonella*, only then, could we identify a potential trend that would still demand more precise diagnostic methods to make a definitive conclusion.

No differences, however, in presence of potential enteric pathogens could be associated with either treatment, in fact no *Salmonella* or *Escherichia* were found among any of the 868 sequences. There was no detectable abundance of compositional differences in the class Gammaproteobacteria or the family Enterobacteriaceae except at one time-point in 2005, which is the smallest library and is actually a pooled point of two sampling time-points from July and August. Observed Enterobacteriaceae genera in this time-point however were not specifically associated with increased health risks.

The Enterobacteriaceae plant pathogen, *Erwinia amylovora* was also never observed in the 16S rRNA gene libraries. This could be a by-product of the small fragment of 16S rRNA gene that does not contain enough information to definitively

identify this species, it could be a by-product of data-base issues (over 500 chloroplasts were found in our data set and *not one* was identified as *Malus domestica* or anything even close to apple – most were classified as tomato), or it could of course be a by-product of insufficient sample size.

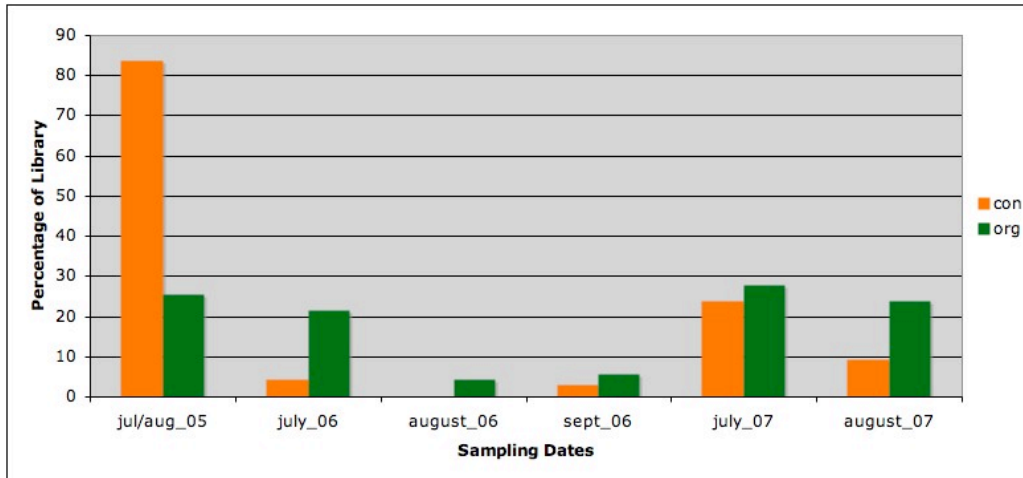


Figure 12. Percentage of Gammaproteobacteria in Organic and Conventional 16S rRNA Gene Libraries at 6 Sampling Dates during 2005-2007.

- o_05 is organic of 2005 and c_05 is conventional 2005 (pooled July & August)
- j06o is July 2006 organic and j06c is July 2006 conventional
- a06o is August 2006 organic and a06c is August 2006 conventional
- s06o is September 2006 organic and s06c is September 2006 conventional
- j07o is July 2007 organic and j07c is July 07 conventional
- a07o is August 2007 organic and a07c is August 2007 conventional

Species Accumulation and Rarefaction Curves

A collector's curve or species accumulation curve is generated from random sampling from a library. The number of OTUs encountered given x number of sequences have been sampled from the library is plotted on the graph. This results in a jagged line. Smooth curves are generated by shuffling the order of the samples a number of times (we used 1000) and then averaging the curves obtained. This allows us to interpolate which environment has more species given a certain number of samples. Estimating how many bacterial species we would expect to observe, if we had only sampled 100 (or x) number of samples is known as 'rarefaction'. Figure 13 shows the rarefaction curves for the Gram negative representatives of our library (excluding Firmicutes and Actinobacteria).

Species accumulation curves can also be used to determine whether or not the sample size of the organic and conventional libraries is actually big enough to make accurate inference about treatment effect on the Gram negative organisms in this environment. We can observe the number of sequences observed that is required to observe the "plateauing" of the species accumulation curves. By pooling all the sampling time points for both treatments our data set is estimated to have covered between 91 and 92 percent of the Gram negative species in this environment (Figure 13) however we still do not see a clear plateau. Our coverage at individual time-points is seriously insufficient to describe treatment impact on the organic and conventional 16S rRNA gene libraries (Figure 14).

Rarefaction Curves for Gram negative species at D = .03

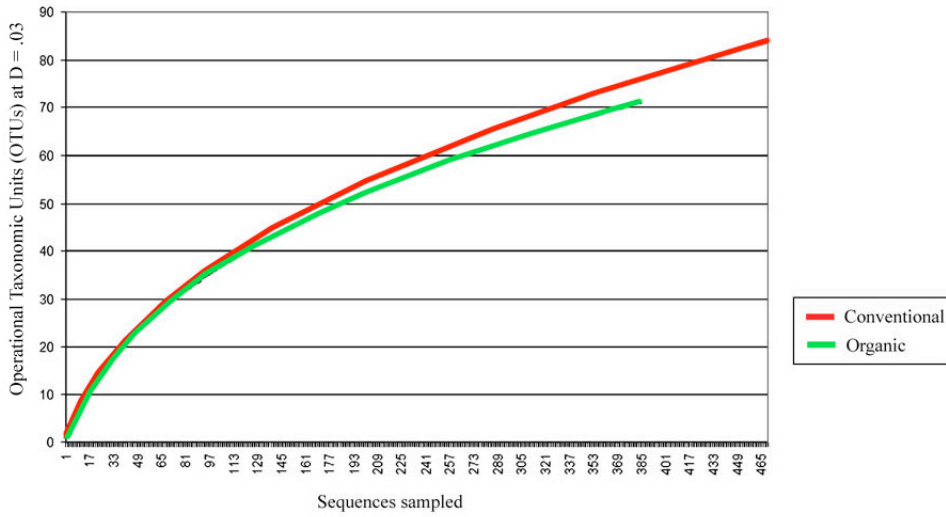


Figure 13. Rarefaction Curves for all organic and all conventional Gram negative sequences pooled.

Rarefaction Curves for Gram negative speics at D = .03

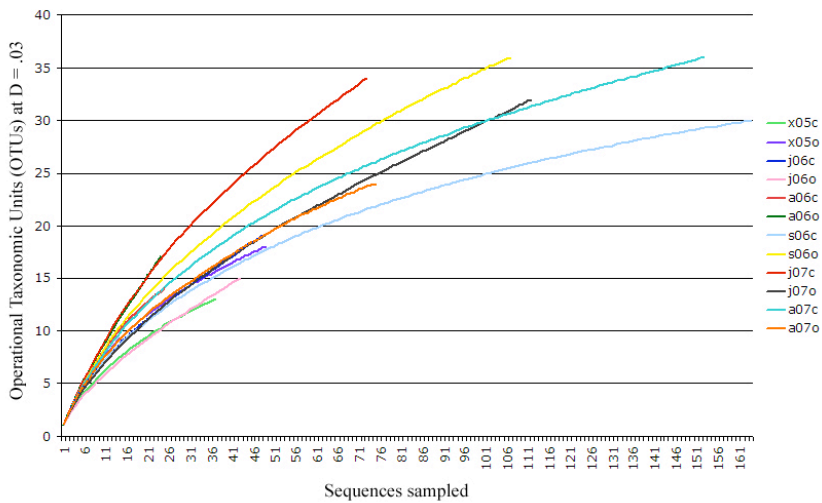


Figure 14. Rarefaction curves for sequences of organic and conventional Gram negative species from each sampling time-point.

- x_05c is conventional of 2005 and x_05o is organic 2005 (pooled July & August)
- j06o is July 2006 organic and j06c is July 2006 conventional
- a06o is August 2006 organic and a06c is August 2006 conventional
- s06o is September 2006 organic and s06c is September 2006 conventional
- j07o is July 2007 organic and j07c is July 07 conventional
- a07o is August 2007 organic and a07c is August 2007 conventional

Discussion

Culture-dependent & Culture-independent

The first comparison of the microbial diversity in the phyllosphere using DNA from culture-dependent phyllosphere microflora compared to culture-independent microflora was very striking (Figure 7). A much greater diversity was seen in the culture-independent samples despite the same DNA extraction protocols (See Materials and Methods Section of Chapter 3 for complete details). In the PCR-DGGE (16S rRNA gene fragments) (Figure 7), we could actually see what resembled a treatment “signal” in this first molecular examination of the organic and conventional microflora from a May, 2005 sampling time-point. At this time-point, both treatments in the orchard had already received approximately 5 applications of pesticides (Appendix 3).

The dramatic increase in diversity seen from cultured to uncultured and the visible contrast between organic and conventional treatments that the molecular methods were able to produce may not however, be associated exclusively with Gram negatives species. We know from our clone libraries that the Gram negative extraction method also resulted in Eukaryotic and Gram positive bacterial DNA. So, the observed increase in diversity may have been associated with yeasts, molds, and other fungal, protozoan or Gram positive bacterial species.

However, it is also likely that MacConkey media (selective for Gram negative species) does not culture *all* the Gram negatives in the phyllosphere environment and some of the increase in diversity is actually a result of Gram negative species that are not cultured by the standard medias.

Further sequencing of the exact species cultured by the Gram negative selective medias would be valuable to address the limitations and benefits associated with the use of these medias. They have a long history in food safety research as indicators of potential contamination by enteric pathogens.

Diversity found in the 16S rRNA Gene Clone Libraries from the Phyllosphere

A total library of 868 sequences was culled from over 1600 original sequences (many were chloroplasts, 18S, vector or potential chimeras). A total of 485 high quality bacterial sequences from the conventional treatment and a total of 383 were obtained from the organic samples. Eight Phyla of Bacteria were observed: Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria Acidobacteria, Cyanobacteria, Deinococcus-Thermus and Fusobacteria (Figure 9) (Phyla, Class and associated families Table 2). Archaea were never seen despite the use of archaeal-specific primer sets in addition to the universal 16S rRNA gene primer sets.

One of our most important goals was to identify whether or not the materials and practices associated with organic or conventional management might contribute to an

increased incidence of enteric pathogens seen in the phyllosphere microflora from either treatment.

This is perhaps the question that we are best able to answer with the data we generated. Using the complete (pooled libraries) of organic and conventional sequences, we estimated we covered approximately 91 and 92 percent (for organic and conventional respectively) of the Gram negatives estimated to be in the phyllosphere environment using DOTUR (Schloss and Handelsman, 2005a).

This gives us a reasonable degree of confidence when we say that no increased incidence of enteric pathogens could be associated with either treatment. There was one time-point in 2005 that had significant differences in the percentage of the library that was comprised by the family Enterobacteriaceae (Figure 12), however that library (2005) was extremely small and none of the observed members of Enterobacteriaceae were enteric pathogens.

With regard to our most elemental microbial ecology question:

Can organic management influence the bacterial microflora of an apple crop differently than conventional management? Is there a difference?

Although we observed significant differences at four of six time points between organic and conventional phyllosphere libraries using methods designed to optimize small data sets, not one of our data sets was large enough to definitively draw this conclusion. With an average library size of 80 sequences, we fall far short of a

number of sequences necessary to give us a comprehensive or even statistically accurate description of any differences associated with treatment observed in the metagenomic data sets.

When sequences from all organic and all conventional time-points were pooled, no statistically significant difference could be seen between the organic and conventional treatments. This may or may not dispute the significance associated with the individual time-points. By pooling everything together, the important dynamics associated with time-points may be lost. Temperature, water levels, winds, and insect pest levels - all could be having a significant impact on microbial communities in conjunction with treatment effect or on their own. It is possible that treatment effects are secondary to environmental and weather pressures. Figure 15 shows the overlapping OTUs of pooled organic and conventional treatments from three time points in the 2006 season. While there are shared OTUs, there are also just as many unique OTUs associated with each time-point, suggesting that environmental pressures may have as strong an impact on the shared and unique microflora as organic and conventional treatment effects do (Figure 15).

Timepoints of Pooled Organic and Conventional
July (A), August (C) and September (B) 2006

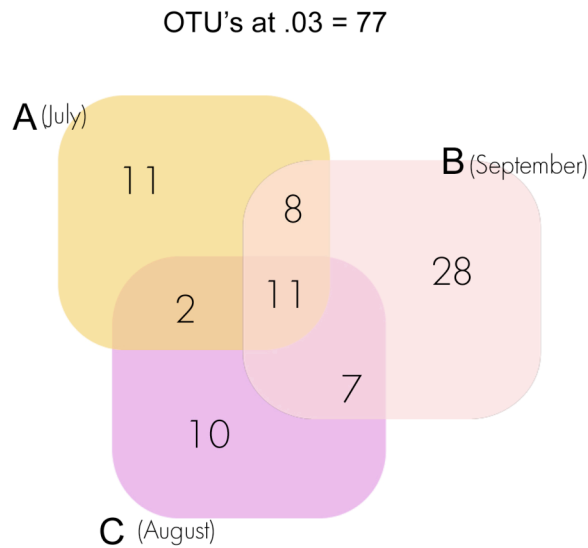


Figure 15. OTUs of Pooled Organic and Conventional Treatments Associated with three Different Time-points in July, August and September of 2006.

Technical biases may also play a role we have yet to fully understand.

The Gram negative DNA extraction method, for example should be compared to a Gram positive extraction method and also a chloroform extraction. It is also recommended to use physical methods of cell lysis rather than chemical methods to get a less biased representation of metagenomic DNA. Some researches, as previously mentioned, have hypothesized that recovery of Gram negatives in certain environments may not be effectively represented due to degradation that takes place from the lysing chemicals(Gill et al., 2006b).

Without exploring different extraction methods, we will not be able to describe how little of the environment we may have missed by having selected a Gram negative extraction method.

There may also be PCR biases associated with the primers, the PCR efficiency, number of amplification cycles, and the GC content of bacterial template strands. The melting temperature of rDNA templates with high GC content is higher than that of low GC templates so we may have inadequate representation of species with higher GC content such as Actinobacteria – which was moderately well represented in our libraries but perhaps was outperformed in PCR by the more prolific Proteobacteria.

It is interesting to note that the researchers studying the bacteria in the phyllosphere of maize in response to UV treatment described a trend towards increased diversity in the UV exposed phyllosphere samples based on approximately 15 sequences or less per treatment and the use of software to analyze the banding patterns from this DGGE gel.

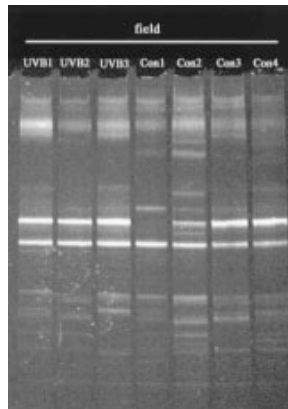


Figure 16. DGGE of Bacterial Microflora from UV and non-UV treated corn, taken from (Kadivar and Stapleton, 2003).

This demonstrates how data-poor many studies of the phyllosphere still are. Our libraries currently represent the largest molecular data assembled for the phyllosphere environment, but it is still seriously insufficient to definitely provide an answer for many of our research objectives. It is an extremely small study compared to 16S libraries currently being compiled in other environments, often with newer sequencing technologies such as 454 pyrosequencing (Margulies, 2005). In a study of the “rare biosphere” of the deep sea, 118,000 partial rRNA gene fragments were not enough to effectively describe the “rare biosphere” that exists in low abundance OTUs in sea water (Sogin et al., 2006). Granted there is a much higher cell count in this environment than there is in the phyllosphere.

Rare Biosphere

A very interesting component of the results of the organic and conventional phyllosphere study are the unique phylotypes associated with each treatment (Figure 11). This is the “rare biosphere” of our organic and conventional phyllosphere. Some of these under represented organisms may serve important functions in the

communities of this environment. These could be the products of ecological shifts that maintain the potential to become dominant in response to shifts in environmental conditions, (Sogin et al., 2006) or they could be keystone species in unidentified phyllosphere consortia (Peterson et al., 2008).

Clostridium spp., for example was found primarily with associated with conventional samples and *Legionella spp.* were found **only** among organic sequences. Does this have any epidemiological significance or import for human health considerations?

Could *Legionella* be one of the species that is observed in the increased diversity seen in the DGGEs of organic and conventional treatments? *Legionella* is a bacterium with 19 human pathogenic species and is known to be an endosymbiont of free living protozoa, primarily associated with fresh water environments. Water sources in both the organic and conventional treatments were the same so perhaps a protozoan association exists with one of the organic materials or its processing environment?

A member of the Enterobacteriaceae, *Pantoea agglomerans* that inhibits the plant pathogen *Erwinia amylovora* (Fire Blight) (Poppe et al., 2003; Wright et al., 2001) was observed more frequently in conventional samples compared to organic (4.0% and .7% respectively). Could conventional selection pressures be enabling a natural biological control for an important disease of apples and pears to dominate a phyllosphere niche? Radiation tolerant species were also found in conventional libraries – *Kineococcus radiotolerans* and *Deinococcus*. Could the unprotected UV

environment of the conventional samples sustain radiation tolerant species or speciation at a more rapid rate than the protected organic environment due to the differences in materials - primarily the kaolin clay insecticide “Surround” (Figure 2)

Organic and Conventional Food Safety

The present study identified an array of organisms in the phyllosphere of an apple crop with genera that include established or emerging human pathogenic species including *Haemophilus*, *Legionella*, *Mycobacterium*, *Enterococcus*, *Staphylococcus*, and *Enterobacter* (full list Appendix 7). *Enterobacter sakazakii*, for example, was recently associated with contaminated powdered milk formula products for infants. While environmental contamination for *E. sakazakii* was suspected, sources remain undocumented (Bowen and Braden, 2006b). *Enterobacter* is also a common Gram negative found in the environment that contains many nonpathogenic species.

Identification of genera with potentially pathogenic species does not necessarily define a human health risk, but it certainly suggests that further study would be of value. This is especially true when you consider the vast number of estimated foodborne illnesses that are of unknown origin (Mead et al., 1999).

Research Objective 2

With regard to our second objective to determine whether or not health risks associated with enteric pathogens increase under organic or conventional management, given our current data, it can be assumed that health risks do not increase under either management schedule.

No increased risks or presence of potential enteric pathogens could be identified with either treatment. No *Salmonella* or *Escherichia* were found among *any* of the 868 sequences in our 16S rRNA gene library. There was no detectable abundance of compositional shifts in the class Gammaproteobacteria or the family Enterobacteriaceae due to treatment, except at one time-point in 2005 (Figure 12).

If we had seen significant differences between organic and conventional representations of Gammaproteobacteria and specifically within the family Enterobacteriaceae and the genera; *Escherichia* and *Salmonella*, then perhaps we could identify a potential ecological trend but it is one that would still demand more precise diagnostic methods to make any definitive conclusions.

It might be of value to examine the genus *Enterobacter* more definitively in this environment using species specific probes or primers and quantitative methods to see if increased incidence of *Enterobacter sakazakii* may be associated with either treatment.

Deeper metagenomic sequencing of the agrisphere and its comparison to human microbiomes could help define and manage risks associated with the microbiological continuum “from the field to the fork.” It is likely that our present findings represent only the tip of the iceberg in terms of the value such metagenomic approaches may have for risk management and the development of sustainable crop management

practices attentive to their impact both on the agrisphere and on human and environmental health.

Newer sequencing technologies moving in synchronicity with bioinformatic methods have established a paradigm shift in the study of ecological (and human) biospheres. We can now “sequence environments” in order to define the complex communities and genetic potential of specific niches – both human and environmental (Gill et al., 2006a; Martin et al., 2006; Tyson et al., 2004; Woyke et al., 2006).

Our results indicate not just how complex the phyllosphere of a crop can be but also how agricultural inputs can significantly impact this diversity. The demonstration that organic and conventional crop management can impact phyllosphere microbial diversity differently at individual time-points opens new opportunities for higher resolution examination of the effects of specific agrichemicals on microbial biodiversity.

Chapter 4: Microbial Ecology of the Soil of Organic and Conventionally Managed Apples and Asian Pears

Introduction: Soil

The soil is one of the most, if not *the* most biodiverse environment on the planet. It is an environment with more “unknown” than “known” in terms of exactly who’s out there and what they’re doing. Estimates of total cells in one gram of soil range from 10^6 to 10^9 - varying of course, from soil to soil. The actual number of diverse archaeal and bacterial genomes represented in this same one gram of soil are estimated at numbers between 2,000 and 18,000 distinct species, and these estimates are not thought to include rare and under-represented members of the community, so true numbers might even be higher (Daniel, 2005). As of November 1st 2008, the National Center for Biotechnology Information had 21,700 bacterial genomes and 798 archaeal genomes in its database. This number has taken almost twenty years to assemble and it is not that different from the number of prokaryotic genomes that may exist in a single gram of soil (Daniel, 2005).

Soil is an extremely diverse habitat with water fluctuations that range from totally saturated to completely arid. There is enormous microscale (and macroscale) variability, including phase variations (such as gases) that contribute to a myriad of niche environments supporting a complex array of diverse biota.

Soil studies were some of the earliest work to contribute to our understanding that culturing methods do not accurately representing the full gamut of environmental microbial diversity. Work that employed DNA –DNA reassociation methods, demonstrated that the microbial diversity in soil was more than 100 times greater than that the diversity that could be estimated with culture-dependent methods (Torsvik et al., 1990). A recent study that demonstrates how much more there is to learn about soil microbial communities comes with the recent demonstration that nitrification activity in the soil, long attributed exclusively to bacteria, may be in fact carried out predominantly by archaeal species in certain soils (Leininger et al., 2006). This is not to say that bacterial species are not participating in nitrification but in some soils there may be greater nitrification activity at a rate of 3,000 to 1 being carried out by archaea (Leininger et al., 2006).

Soil Research Leading Towards a Census of Bacteria and Archaea

A set of “usual bacterial and archaeal suspects” is taking shape due to the efforts of numerous research endeavors (Borneman et al., 1996; Dunbar et al., 2002; Elshahed et al., 2008; Fierer et al., 2007; Janssen, 2006; Kuske et al., 21997; Rodon et al., 2000; Roesch et al., 2007; Schloss and Handelsman, 2006b). A “fairly typical phylum distribution pattern for soil” has been described to include Proteobacteria, Actinobacteria, Acidobacteria, Chloroflexi, Verrucomicrobia, Bacterioidetes, Planctomycetes, Gemmatimonadetes, and Firmicutes (Elshahed et al., 2008). Nitrospira is also frequently observed (as it was in our study) although most often on

the lower end of represented abundance (Dunbar et al., 2002; Rodon et al., 2000; Schloss and Handelsman, 2006b).

Organic and Conventional Soil

The challenge of successfully fertilizing a crop with organic fertilizers is a substantial one. Our own inputs for organic treatments were a variety of biological materials in the first year, kelp, fishmeal, and compost teas in 2005 and an organically approved (NPK) product in the last two years (2006 & 2007). While our main focus was not on organic soil amendments, the fact that five blocks were subjected to organic chemicals for five years and five were subjected to conventional chemicals for five years may have impacted soil microflora in some important undescribed ways. In addition, the heavy use of sulfur (as a fungicide) and aluminum (associated with the kaolin clay) in the organic blocks also could have had an impact on soil pH and soil microbiology. We hope that our soil data set will provide preliminary data to formulate more precise hypotheses about how these two treatments may have impacted soil microbial species.

No published molecular studies have examined the impact of organic and conventional management on the bacterial microflora of soil. A group of researchers associated with the Institute for Research on Environment and Sustainability from Newcastle University (www.ncl.ac.uk/environment/research/researchthemes/

[ActinobacterialCommunities.htm](#)) has investigated the impact of different agricultural practices, including the use of organic amendments on soil microflora but no published study is yet available.

One study examining the impact of organic and conventional management on soil, described higher potential denitrification rates, greater denitrification efficiency, more organic matter, and greater microbial activity associated with organically-farmed soils (Kramer et al., 2006). While this work did not use molecular methods for taxonomic or quantitative assessments of microbial species in their treatments, they were able to get valuable information using phospholipid fatty analyses (PLFA). PLFAs quantify fatty acids from microbial cell walls and can be used in conjunction with principal component analyses to visualize similarities and differences in microbial populations.

Research Objectives

Our main research objective for the soil research was to identify any differences in bacterial microflora that could possibly be attributed to organic and conventional pressures. We acknowledge that due to our small sample size, our results represent “preliminary data” that will hopefully provide the foundation for future research that will be able to apply more extensive sequencing methods or quantitative PCR methods to address the impact that organic and conventional pressure may have enacted on the soil microflora.

Materials and Methods

Soil Sampling

Soil core borers were used to take a total of twelve soil samples at depths between 15 to 22 cm (between 6 and 8 inches at the recommendation of Dr. Jeffery Buyer – Soil Microbial Systems Lab – USDA – ARS) at the drip line of ten Enterprise trees: five organic and five conventional. The twelve samples from each tree were pooled and well mixed. Ten grams from each pooled sample was used for subsequent DNA extractions.

DNA extraction

Total Genomic DNA of ten grams of soil was extracted using the MoBio Ultra Clean Soil Extraction Kit according to the manufactures specifications. Mo Bio, Carlsbad, CA.

PCR and Clone Library Construction

Primers to amplify the entire 16S region were used with the same conditions described in Phyllosphere Methods PCR conditions. Clone Libraries were also constructed with the Promega T-Easy Vector Cloning kit according to the manufacturers specifications. Details of this process are presented previously in Phyllosphere Methods. (Promega Corporation, Madison, WI).

Sequencing

All clones were sequenced by Agencourt Genomic Services Beverly, MA. Cloned 16S rRNA gene fragments in live *E. coli* (200 µl of 20% glycerol and LB broth) in 96 well plates were sent overnight on dry ice to Agencourt Genomic Services for Sanger sequencing.

Preprocessing of 16S Sequences

All sequences were screened for quality, contaminants, chimeras as described in Phyllosphere Methods. A total of 380 conventional and 462 organic sequences were used for further analysis.

Bioinformatic Methods

Alignment, assignment to operational taxonomic units, analysis of overlapping taxonomic units, generation of rarefaction curves and diversity indices were all calculated using the same programs and methods discussed in “Phyllosphere Methods”.

Results

Nineteen bacterial and archaeal phyla and one classification of “unknown” were represented among the 380 conventional and 462 organic soil sequences (Figure 16). Thirty-three classes were observed. Two phyla of archaea were represented in both treatments Crenarchaeota and Euryarchaeota. All other phyla were bacterial.

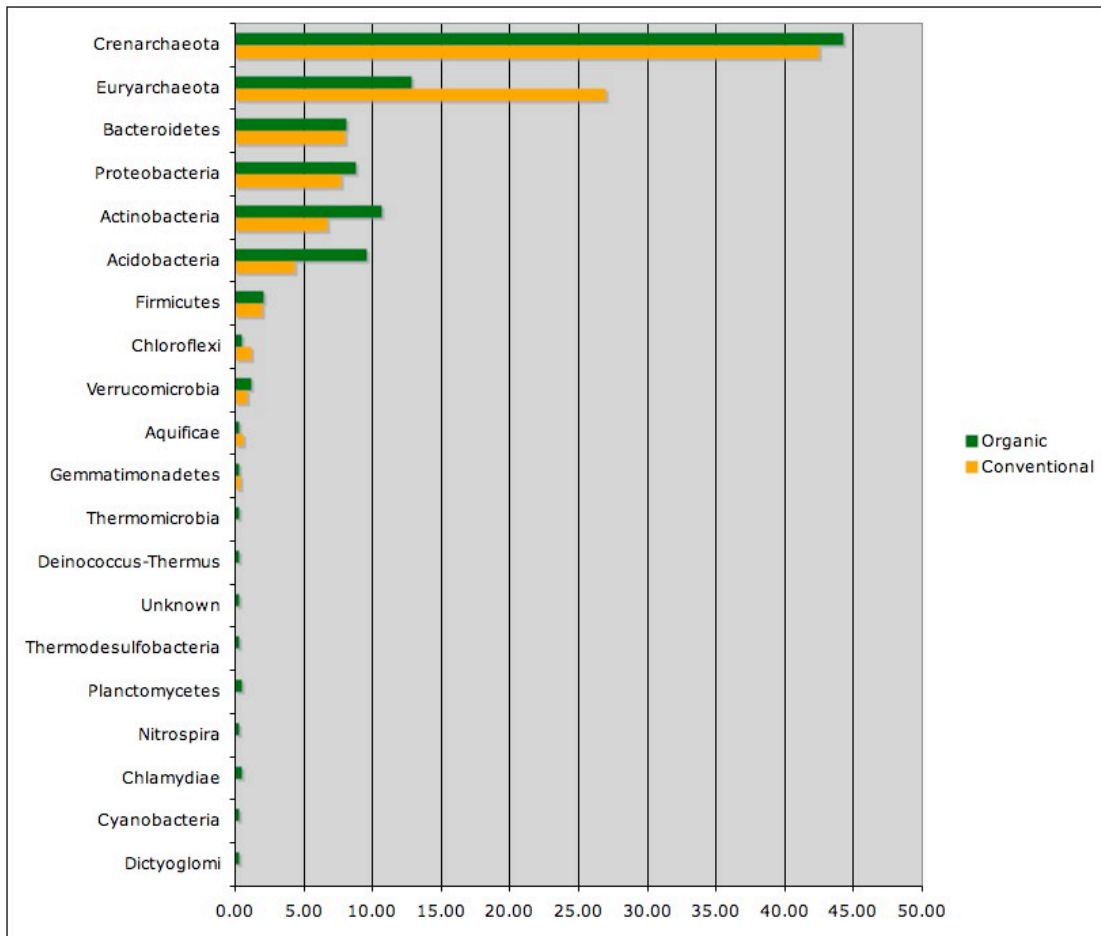


Figure 17. Percentages of Phyla represented in Organic and Conventional 16S rRNA gene Libraries from Soil Samples.

It is interesting to observe that six distinct phyla and one unknown classification were observed only in organic samples but not in conventional: Dictyoglomi, Cyanobacteria, Chlamydiae, Nitrospira, Planctomycetes, and Deinococcus-Thermus. This increase in diversity associated with the organic samples is likely a result of the larger organic library size (approximately 100 sequences more than in conventional), however it would be interesting to examine same sized libraries of the diverse treatments to see if any increase in phylogenetic diversity could be seen. The eleven phyla associated with the conventional samples are shown in a pie graph in Figure 17 and the 20 (including the unknown classification) associated with the organic samples are shown in the pie graph in Figure 18.

Phyla Observed in Conventional 16S rRNA Gene Clone Libraries

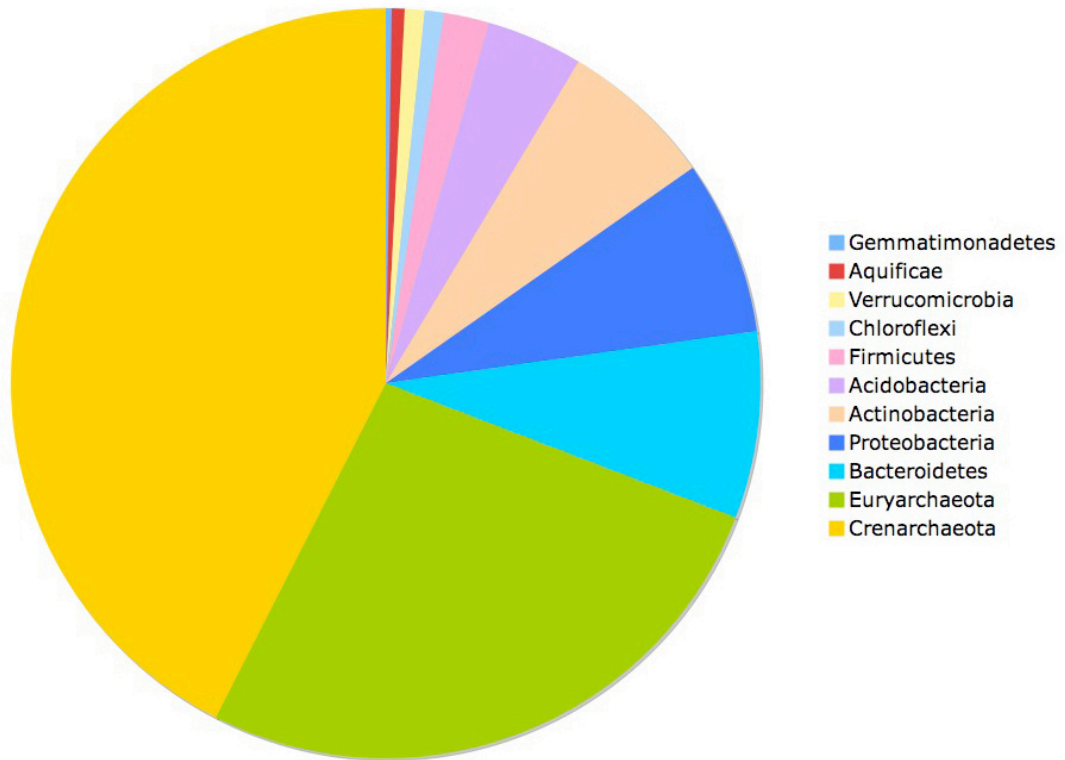


Figure 18. Percentages of the 11 Phyla seen in 16S rRNA Gene Clone Libraries from Conventional Soil Samples

Phyla Observed in Organic 16S rRNA Gene Clone Libraries

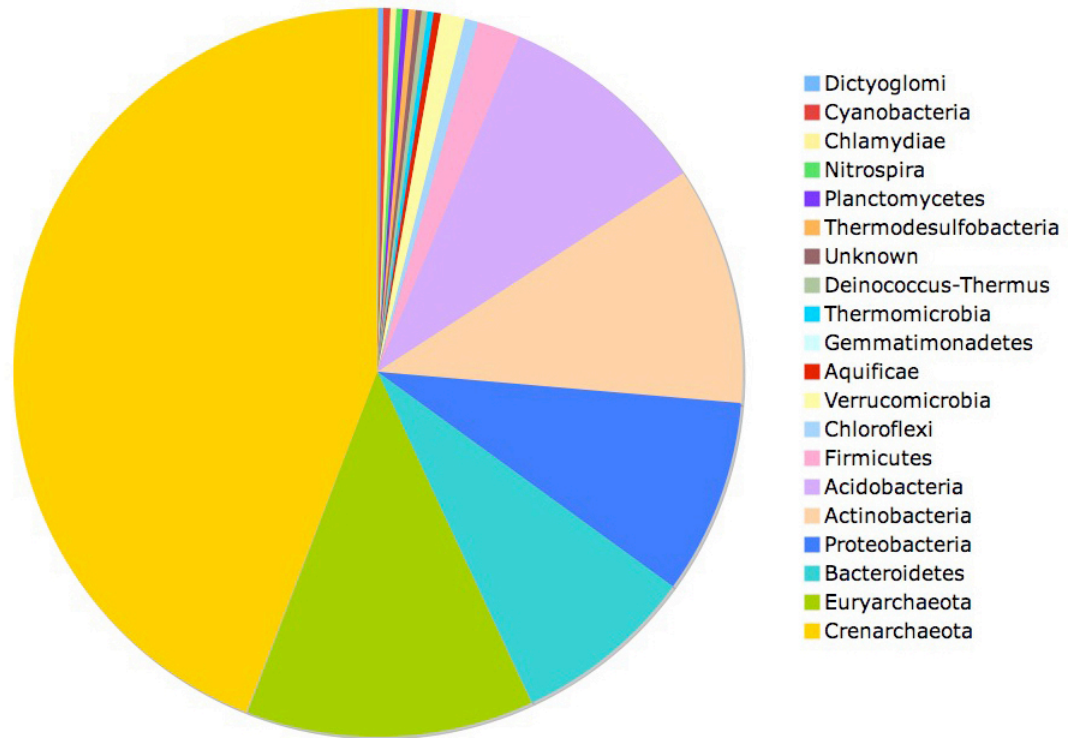


Figure 19. Percentages of the 19 Phyla seen in 16S rRNA Gene Clone Libraries from Organic Soil Samples.

Diversity Indices

The operational taxonomic units derived using DOTUR (Schloss and Handelsman, 2005a) are shown in Table 5 at a dissimilarity of .03, (the controversial but commonly used species delineation (Schloss and Handelsman, 2006b)).

Table 5. ACE, Chao1 and Shannon Diversity Indices for Soil at $D = .03$.

$D = 0.03$	Conventional	Organic
OTUs	138	199
ACE	567(394,858)	749(554,1053)
Chao 1	423(309,719)	582(434,824)
Shannon	3.99	4.49

As with the phyllosphere diversity indices, we cannot be sure that the increased diversity, this time associated with organic samples has anything to do with treatment. It is most likely that we have a skewed perception of our environment due to our insufficient sample sizes. The 95% confidence intervals for ACE and Chao 1, shown in parentheses are overlapping so they're in no significant difference that can be reported between the treatments. The fact that the OTUs, ACE and Chao1 estimates are only roughly double that of the phyllosphere OTUs and indices suggests that something is off. While the phyllosphere has an impressive biodiversity, it is not likely to be half as diverse as soil. In research designed to analyze the effect of sample size on various species richness estimates, subsets of 13,001 dataset were randomly drawn in subsets of 100, 500, 1000 and 3289 and estimates of species

richness were the same non-parametric indices ACE and Chao1 with the following results, taken from (Youssef and Elshahed, 2008):

Library size	Chao1	ACE
100	246	325
500	1127	2500
1000	1589	3011
3289	3827	4422

Table 6. Estimates of Species Richness Using Non-parametric Indices for Subset Clone Library Sizes 100, 500, 1000 and 3289 (Youssef and Elshahed, 2008).

This work demonstrates how enormous the diversity index variation can be based on variation in sample size.

Soil Rarefaction Sampling Curves

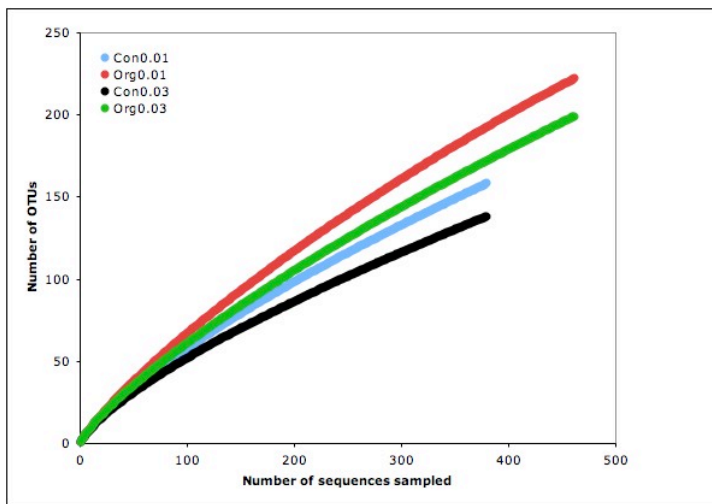


Figure 20. Rarefaction Curves for Organic and Conventional Soil Samples

The rarefaction curves generated for the soil libraries also demonstrate that the sampling size was nowhere near large enough to effectively describe the diversity of the communities present in this environment. Distance (or Dissimilarity) ($D = .03$) and ($D = .1$) are shown in Figure 20. One of reasons that rarefaction curves are used, as previously mentioned, is to interpolate how many species are present if we had only sampled for example, 200 species from each treatment. This allows us to compare the species richness of two libraries even if the sample size is different. If you were to draw a line up from the y axis at the 200 sequences sampled point, you still observe a greater species richness associated with the organic samples (red and green) than with the conventional samples (blue and black).

The species accumulation curves for both libraries have by no means reached a plateau and although the rarefaction curves allow us to examine both libraries, we have no definitive way of knowing (short of increasing our sample size) how the richness estimates will change as more sequence data is accumulated.

Overlapping OTUs in Organic and Conventional Soil Libraries

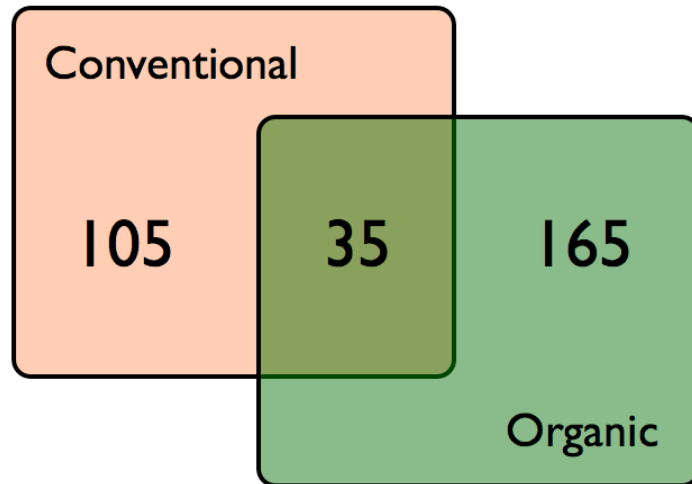


Figure 21. Overlapping OTUs for Organic and Conventional Soil Libraries

A much smaller number of the observed OTUs were actually shared by organic and conventional libraries than was observed in the phyllosphere libraries (Figure 11), but again this could change as our sample size increased.

Discussion

With regard to our research objectives;

To identify any trends or differences in bacterial microflora between organic and conventional soil microflora that may be related to treatment pressures and could serve as the preliminary data for future research with greater sequencing efforts or quantitative PCR or probing methods.

We were able to identify many interesting trends and questions that could be addressed with future research. Although, it may be a by-product of sampling deficiency once again, the increased diversity associated with the organic soil samples was an interesting trend that merits future study. Almost double the number of phyla that were seen in conventional libraries, were seen in the organic libraries. It is possible that this phenomenon may be associated with treatment effects. Using rarefaction curves to estimate the number of species associated with each library at same sample sizes, organic still seems to have an increased diversity however, previous work has demonstrated that with a rise in sample size, comes a rise in estimates of species richness (Dunbar et al., 2002; Youssef and Elshahed, 2008).

Archaea in the Organic and Conventional Soil

It was interesting to see that Crenarchaeota species were so well represented in soil from both treatments. This is extremely interesting given the recent discovery that archaea may be responsible for up to 3,000 times as much nitrification activity in certain soils when compared to bacteria (Leininger et al., 2006). Until this recent

discovery, it was assumed that nitrification in soils was carried out predominantly by Beta and Gamma subgroups of the Proteobacteria (which are almost four fold less prevalent in our soil 16S rRNA gene data set). Archaeal members of the phylum Crenarchaeota have been reported from many studies of soil environments (Fierer et al., 2007;Leininger et al., 2006;Roesch et al., 2007;Schleper et al., 2005) and interestingly in some work (including our own), they are reported in greater abundance associated with agricultural soils (Roesch et al., 2007). The 16s rRNA gene libraries from our organic and conventional experimental field, a sandy Metapax loam on the eastern shore of Maryland - long exposed to some kind of agricultural management, were comprised *predominantly* of Crenarchaeota (42 percent of the conventional library and 44 percent of the organic library). This was almost double the percentage of any other phyla represented in the library. The second most frequently observed phyla for soil from organic and conventional treatments was another Archaeal phyla - Euryarchaeota.

Although Euryarchaeota have been reported in oxic soils, to date, it has been with less frequency than reports of Crenarchaeota, and Euryarchaeota are frequently reported to be less abundant than sister phylum Crenarchaeota in most oxic soil environments (Bomberg and Timonen, 2007;Midgley et al., 2007;Yan et al., 2006). A very interesting trend in our 16S rRNA gene libraries was the diverse representation of members of Euryarchaeota. Six different classes of Euryarchaeota were seen among both organic and conventional sequences; Methanobacteria, Thermococci, Themoplasmata, Methanopyri, Methanococci and Halobacteria. Archaeoglobi, the

seventh class was only observed in the organic samples (again likely due to sample size of organic compare to conventional). Euryarchaeota, especially methanogenic species have been reported in numerous anoxic environments such as composts, peat bogs, wetlands, rice fields, (Brauer et al., 2006;Juottonen et al., 2005;Thummes et al., 2007;Utsumi et al., 2003;Wu et al., 2006) and halophilic species have been reported in saline soils(Miller et al., 1983;Valenzuela-Encinas C., 2008;Walsh et al., 2005) but their presence in oxic soils has been less commonly reported (Midgley et al., 2007;Roesch et al., 2007).

Several studies have described an increase in the diversity of archaeal species associated with agricultural soils (Bomberg and Timonen, 2007;Midgley et al., 2007;Roesch et al., 2007). It has even been suggested that if soil archaeal communities were consistently more diverse in disturbed soils, as several studies have demonstrated, they may represent an effective group of “indicator organisms” to assess disturbance in soils (Midgley et al., 2007).

Almost double the quantity of Euryarchaeota was seen in the conventional library compared to the organic (despite the smaller conventional library size). Could the conventional pressures be selecting for increased diversity and incidence of Euryarchaeota phyla? The more disturbed the soil, the greater the diversity is a current hypothesis (Midgley et al., 2007).

Bacteria in the Organic and Conventional Soil

Actinobacteria have long been established as a group important to soil health due to their roles in soil mineralization and carbon cycling. They are also quite famous for

their secondary metabolites and antibiotics of high pharmacological and commercial interest (streptomycin, for example) (Stackerbrandt et al., 1997). Another trend of interest, although very preliminary, is the slightly lower percentage of Actinobacteria that is seen in the conventional library compared to the organic library. This trend is similar to preliminary results from a study conducted by the Institute for Research on Environment and Sustainability, part of Newcastle University in the United Kingdom (www.ncl.ac.uk/environment/research/researchthemes/ActinobacterialCommunities.htm). Efforts to provide scientific evidence for the hypothesis that organic management may enhance nutrient cycling in soils fueled their examination of the long-term effects of organic and mineral applications of nitrogen to fields. Preliminary data from this work suggests that long term mineral fertilizer management has a profound effect on soil pH and this factor, more than others may be influencing levels and diversity of Actinobacteria found in these soils. Lower numbers and decreased diversity of Actinobacteria spp. have been associated with fields that have been managed with predominantly mineral sources of nitrogen (Jenkins et al., 2008). There have also however, been studies that have observed no impact on species and abundance of Actinobacteria in response to organic amendments (Piao et al., 2008). Given the complexity associated with trying to describe treatment effects of soil microflora, clearly many more studies are needed before any definitive trends can be identified. (It would also be advantageous to use Gram positive extraction methods).

We have definitely achieved our objective of identifying interesting preliminary data that may be associated with treatment impact that can be used to design future study.

Chapter 5: Summary, Conclusions and Future Work

Summary, Conclusions and Future Work

The dissertation research presented here spans numerous academic fields including environmental microbiology, medical microbiology, horticulture, plant pathology, sustainable and organic agriculture, and food safety and public health.

Newer and cheaper sequencing technologies are enabling “preliminary investigations” of previously unexplored niches of our microbial ecologies - both human and environmental. We will undoubtedly continue to shed light on the geobiochemical “rivers” that flow through all biotic and abiotic elements of the planet at a very rapid pace.

Recent work using pyrosequencing methods to assemble the “largest data set to date” to examine soil microbial diversity assembled between 26,140 and 53,533 reads of 16S rRNA genes fragments and never observed more than 10,000 OTUs in any of four soils, although ACE and Chao1 estimates of OTUs were both between 10,000 and 100,000 (Roesch et al., 2007).

An estimate of how many sequences would be necessary to observe 95% of the richness in a community of 5,000 members is about 15,000. To achieve a complete census, the number rises to 75,000 reads.

The more we know, the better we can control the impact of anthropogenic “rivers” on the global landscape. Nowhere is the intelligent and sustainable stewardship of our “footprint” more critical than with agriculture. Agriculture is crucial to feed the human population on this planet and the methods we have employed to do this in the past one hundred years have become obsolete. Modern intensive agricultural systems have been shown to be less efficient than the older systems they replace (Pimentel et al., 2005; Topp et al., 2007). They indirectly and directly impact thousands of ecosystems and do not even come close to providing humanity with even a small percentage of the biochemical cornucopia of nutrients that are available to us in edible plant biodiversity.

There is a full gamut of sustainability indices that were also not addressed in this study. A valuable direction for future work related to the sustainability objectives of this study would be to analyze the organic and conventional systems with the full range of sustainability indices that are being applied to sustainable agricultural experiments. Indices such as Energy Efficiency, Pesticide Index, Chemical Soil Analysis, Weed Survey, Nitrogen Available Reserves, Phosphorus Available Reserves, soil Quality, Nutrient Flow, Biological Diversity, Impact on Beneficial Insects, to name a few (Helander and Delin, 2004).

In future work, it would be advantageous to compile a much larger set of libraries that include eukaryotic species so we can make a more descriptive portrait of the impact of organic and conventional pressures on bacterial, fungal and archaeal species in

phyllosphere and soil environment. Plant species and their decomposition have been shown to drive the fungal species, who in turn play a significant role in the community dynamics of bacterial and archaeal communities (Beattie, 2006; Bomberg and Timonen, 2007; Borneman and Hartin, 2000; Garbeva et al., 2008; Pimentel et al., 2005). It might also be interesting to specifically examine how the impact of the numerous applications of sulfur and aluminum (that were associated with the organic pesticides) may have influenced the soil pH and how this may be playing a role in the microbial composition.

From a food safety and public health angle, it would be valuable to use more precise diagnostic methods to probe for potentially pathogenic species associated with some of our observed genera with known pathogenic members (we observed numerous genera that have known pathogenic species and pathovars but our 550 bp fragment of one highly conserved gene cannot suffice as a definitive diagnostic method, Appendix 7).

The confluence of research objectives relating to food safety and sustainable agriculture that initiated this dissertation research has resulted in a valuable contribution to the microbial ecology of the phyllosphere and soil of a food crop. Data generated from this work will be valuable to efforts to streamline sustainable agriculture (organic agriculture), and food safety and public health issues associated with the microbial ecology of a crop. Our results indicate just how complex the epiphytic agrisphere can be and that agricultural inputs may significantly impact this

diversity. It is likely these findings are only the proverbial tip of the iceberg with important ramifications for future assessment of risk and development of best practices to meet the world's urgent challenge of creating sustainable agricultural practices while ever mindful of their impact on public and environmental health.

Appendices

Appendix 1. 2005 Monitoring of Top Five Apple Pests in the Organic and Conventional Orchard.

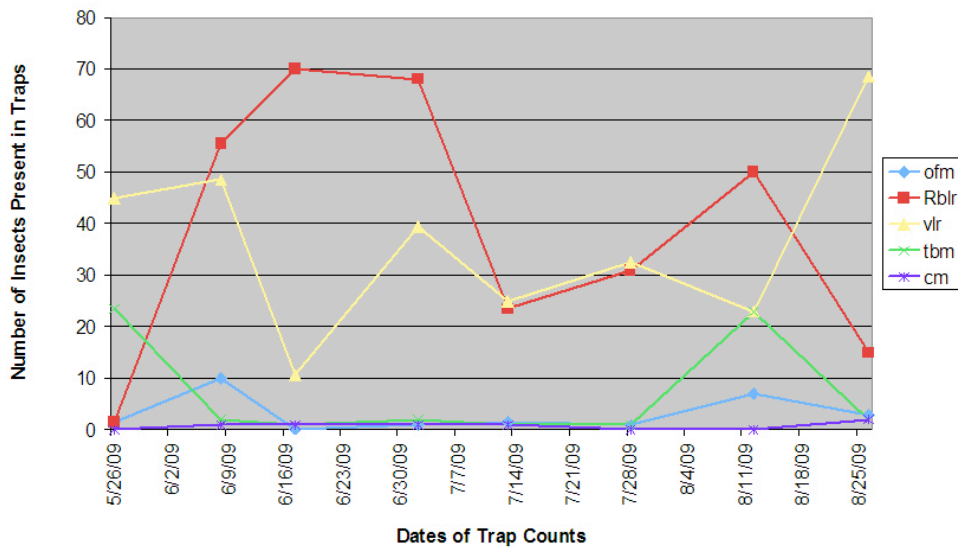


Figure 22. Monitoring of five top apple pests in 2005

(ofm) Oriental Fruit Moth

(Rblr) Redbanded Leaf Roller

(vlr) Varigated Leaf Roller

(tbm) Tufted Apple Budmoth

(cm) Codling Moth

Appendix 2. Economic Analysis of Organic and Conventional Apples by Jim Hanson
(Acta Hort 2008)

Table 7 summarizes net revenue over pest control and nutrient costs for the organic and conventional apple production systems. This analysis focuses on the differences between organic and conventional apple production. As a result, when costs associated with planting trees or harvesting fruit, which were assumed to be similar for both systems, are included, net revenue would be reduced for both types of operations. Total organic costs for the non-bearing years of 2003 and 2004 were \$12,624, while total conventional costs were \$5,874. When this difference in investment is amortized over 20 years with 5% real interest, the additional investment for the organic system is \$541 per ha. This investment cost is included as an annual cost in the organic orchard for the life of the orchard.

The analysis showed differences in net revenue when the price of apples was assumed to be \$2.64 per kg. Since the prices of organic apples are typically higher, the question was then asked, "*What price of organic apples would be required to equalize the net returns for the two production systems?*" The organic breakeven prices in 2005, 2006, and 2007 were \$5.06, \$4.40 and \$8.49 per kg and the organic price premiums were 192%, 166%, and 322%, respectively.

These breakeven organic price premiums were far greater than those reported previously in Washington State (Reganold et al., 2001). In that 6-year study the premiums for organic apple production required to breakeven with conventional apple production were 12 to 14%. Washington State organic apple price premiums ranged from +74% to +94% depending on the size of the apples (Granatstein and Kirby,

2007). Our organic price premiums were less than those in New York, however, where premiums of 400% would be required to produce pest-free apples organically (Jentsch, 1994). Clearly, producing organic fruit during hot, humid summers is problematic. If our expenses were lower, then the organic price needed to equalize the two systems would also be lower. When we reduced our organic expenses by 50% but kept the yields the same, break even prices were reduced a modest 33 to 48 cents per kg.

The greater issue in the relative profitability of organic systems in this study was the dramatic difference in marketable yields. If we could increase organic apple yields by 50%, the breakeven prices for organic production would fall to \$3.36, \$2.93, and \$5.65, respectively. These drops in the breakeven price were more significant at 67 cents to \$1.29 per kg. Similarly, when the top three organic yielding varieties in 2007 were compared with these three varieties grown conventionally, the organic breakeven price dropped considerably, suggesting that some cultivars are better suited to organic production than others.

Table 7. A comparison of yields, revenues and expenses between organic and conventional research orchards at Queenstown, MD (2005-2007).

Variable	2005		2006		2007	
	Org Conv	Conv	Org	Conv	Org	Conv
<i>Yields, revenues and expenses</i>						
Yield (kg/ha)	12,151	21,089	8,799	12,640	8,310	24,267
Price (\$/kg)	2.64	2.64	2.64	2.64	2.64	2.64
Revenue/\$ ha	32,077	55,676	23,230	33,369	21,938	64,064
Expenses/\$ ha	8,076	2,233	6,941	1,633	8,170	1,687
Net Revenue/\$ ha	24,001	53,443	16,289	31,736	13,768	62,377
<i>Calculated break-even values</i>						
Organic Premium	192%		167%		322%	
Organic Price	\$5.06		\$4.40		\$8.49	

Appendix 3. Spray Schedule for the Organic and Conventional Orchard (2005-2007).

Organic 2005	Spray Materials and Rates
April 7	Surround 25 lb/100gallon Kocide 4lb/100
April 21	Strep. 1.3 lb/acre
April 26	Strep. 1.3 lb/acre
April 29	Strep. 1.3 lb/acre
May 3	Pyganic 1pt/acre Neemix 8 oz/100 Sulfur 6lb/100 Surround 25lb/100 Strep.
May 10	Pyganic 1pt/acre Neemix 8 oz/100 Sulfur 6lb/100 Surround 25lb/100
May 17	Pyganic 1pt/acre Neemix 8 oz/100 Sulfur 6lb/100 Surround 25lb/100
May 27	Pyganic 1pt/acre Neemix 8 oz/100 Sulfur 6lb/100 Surround 25lb/100
June 6	Pyganic 1pt/acre Neemix 8 oz/100 Sulfur 6lb/100 Surround 25lb/100
June 11	Pyganic 1pt/acre Neemix 8 oz/100 Sulfur 6lb/100 Surround 25lb/100 Entrust 2.5 oz/acre
June 28	Pyganic 1pt/acre Neemix 8 oz/100 Sulfur 6lb/100 Surround 25lb/100 Entrust 2.5 oz/acre
July 6	Pyganic 1pt/acre Neemix 8 oz/100 Sulfur 6lb/100 Surround 25lb/100 Entrust 2.5 oz/acre

July 11	Pyganic 1pt/acre Neemix 8 oz/100 Sulfur 6lb/100 Surround 25lb/100 Entrust 2.5 oz/acre
July 21	Pyganic 1pt/acre Neemix 8 oz/100 Sulfur 6lb/100 Entrust 2.5 oz/acre
August 3	Pyganic 1pt/acre Neemix 8 oz/100 Sulfur 6lb/100 Entrust 2.5 oz/acre
August 13	Pyganic 1pt/acre Neemix 8 oz/100 Sulfur 6lb/100 Entrust 2.5 oz/acre
August 30	Pyganic 1pt/acre Neemix 8 oz/100 Sulfur 6lb/100 Entrust 2.5 oz/acre
September 13	Pyganic 1pt/acre Neemix 8 oz/100 Sulfur 6lb/100 Surround 25lb/100 Entrust 2.5 oz/acre
October 4	Pyganic 1pt/acre Neemix 8 oz/100 Sulfur 6lb/100 Surround 25lb/100 Entrust 2.5 oz/acre
Conventional 2005	Spray Materials and Rates
April 7	Lorsban 4e 1pt/100 Oil 2 gal/100 Kocide 4lb/100
April 21	Strep. 1.3 lb/acre
April 26	Strep. 1.3 lb/acre
April 29	Strep. 1.3 lb/acre
May 3	Imidan 3lb/acre Ziram 1 lb/100 Topsin-M 1 lb/acre
May 27	Imidan 3lb/acre Ziram 1 lb/100

	Topsin-M 1 lb/acre
June 11	Imidan 3lb/acre Ziram 1 lb/100 Topsin-M 1 lb/acre
June 28	Imidan 3lb/acre Ziram 1 lb/100 Topsin-M 1 lb/acre
July 11	Imidan 3lb/acre Ziram 1 lb/100 Topsin-M 1 lb/acre
August 3	Imidan 3lb/acre Ziram 1 lb/100 Topsin-M 1 lb/acre Lannate 2 pt/acre
August 13	Imidan 3lb/acre Ziram 1 lb/100 Topsin-M 1 lb/acre Lannate 2 pt/acre Nova 5 oz/acre
August 30	Imidan 3lb/acre Ziram 1 lb/100 Topsin-M 1 lb/acre Lannate 2 pt/acre
September 13	Imidan 3lb/acre Ziram 1 lb/100 Topsin-M 1 lb/acre Lannate 2 pt/acre
October 4	Imidan 3lb/acre Ziram 1 lb/100 Topsin-M 1 lb/acre Lannate 2 pt/acre

Organic 2006	Spray Materials and Rates
March 23	Copper 4lb/acre
April 10	Agri-Strep 1.2 lb/acre Lime-sulfur 2qt/100 gallon Sulfur 1.5 lb/100
April 14	Agri-Strep 1.2 lb/acre
April 18	Agri-Strep 1.2 lb/acre
April 21	Agri-Strep 1.2 lb/acre
April 25	Lime-sulfur 2qt/100 gallon Sulfur 1.5 lb/100 Surround 50 lb/acre

May 3	Lime-sulfur 2qt/100 gallon Sulfur 1.5 lb/100 Surround 50 lb/acre Pyganic 1pt/acre
May 13	Lime-sulfur 2qt/100 gallon Sulfur 1.5 lb/100 Surround 50 lb/acre Pyganic 1pt/acre
May 24	Lime-sulfur 2qt/100 gallon Sulfur 1.5 lb/100 Surround 50 lb/acre Pyganic 1pt/acre
June 4	Lime-sulfur 2qt/100 gallon Sulfur 1.5 lb/100 Surround 50 lb/acre Pyganic 1pt/acre
June 12	Lime-sulfur 2qt/100 gallon Sulfur 1.5 lb/100 Surround 50 lb/acre Pyganic 1pt/acre
June 22	Lime-sulfur 2qt/100 gallon Sulfur 1.5 lb/100 Surround 50 lb/acre Pyganic 1pt/acre
July 6	Lime-sulfur 2qt/100 gallon Sulfur 1.5 lb/100 Surround 50 lb/acre Pyganic 1pt/acre Entrust 3 oz/acre
July 20	Lime-sulfur 2qt/100 gallon Sulfur 1.5 lb/100 Surround 50 lb/acre Pyganic 1pt/acre Neemix 8 oz/100 Entrust 3oz/acre
July 28	Lime-sulfur 2qt/100 gallon Sulfur 1.5 lb/100 Surround 50 lb/acre Pyganic 1pt/acre Neemix 8 oz/100 Entrust 3oz/acre
August 11	Lime-sulfur 2qt/100 gallon Sulfur 1.5 lb/100 Surround 50 lb/acre Pyganic 1pt/acre Neemix 8 oz/100

	Entrust 3oz/acre
August 30	Lime-sulfur 2qt/100 gallon Sulfur 1.5 lb/100 Surround 50 lb/acre Pyganic 1pt/acre Neemix 8 oz/100 Entrust 3oz/acre
September 7	Sulfur 1.5 lb/100 Pyganic 1pt/acre
September 20	Lime-sulfur 2qt/100 gallon Sulfur 1.5 lb/100 Surround 50 lb/acre Pyganic 1pt/acre Neemix 8 oz/100 Entrust 3oz/acre
Conventional 2006	Spray Materials and Rates
March 23	Copper 4lb/acre
April 10	Ziram 1 lb/100 Topsin 0.25 lb/100
April 14	Agri-Strep 1.2 lb/acre
April 18	Agri-Strep 1.2 lb/acre
April 21	Agri-Strep 1.2 lb/acre
April 25	Ziram 1 lb/100 Topsin 0.25 lb/100 Imidan 1.3 lb/100
May 4	Ziram 1 lb/100 Topsin 0.25 lb/100 Imidan 1.3 lb/100
May 18	Ziram 1 lb/100 Topsin 0.25 lb/100 Imidan 1.3 lb/100
May 31	Ziram 1 lb/100 Topsin 0.25 lb/100 Imidan 1.3 lb/100
June 12	Ziram 1 lb/100 Topsin 0.25 lb/100 Imidan 1.3 lb/100
June 22	Ziram 1 lb/100 Topsin 0.25 lb/100 Imidan 1.3 lb/100

July 6	Ziram 1 lb/100 Topsin 0.25 lb/100 Imidan 1.3 lb/100 Provado 1.5 oz/100
July 20	Ziram 1 lb/100 Topsin 0.25 lb/100 Imidan 1.3 lb/100 Provado 1.5 oz/100
July 28	Ziram 1 lb/100 Topsin 0.25 lb/100 Imidan 1.3 lb/100 Lannate 4 oz/100
August 11	Ziram 1 lb/100 Topsin 0.25 lb/100 Imidan 1.3 lb/100 Lannate 4 oz/100
August 30	Pristine 0.8 lb/acre Imidan 1.3 lb/100
September 7	Imidan 1.3 lb/100 Topsin 0.25 lb/100 Sevin 4L 0.75qt/acre
September 20	Ziram 1 lb/100 Imidan 1.3 lb/100 Topsin 0.25 lb/100

Organic 2007	Spray Materials and Rates
March 31	JMS stylet oil Copper 4lb/acre
April 24	Agri-Strep 1.2 lb/acre
April 29	Agri-Strep 1.2 lb/acre Lime-sulfur 2qt/100 gallon Sulfur 1.5 lb/100
May 7	Lime-sulfur 2qt/100 gallon Sulfur 1.5 lb/100 Surround 50 lb/acre Pyganic 1pt/acre
May 16	Lime-sulfur 2qt/100 gallon Sulfur 1.5 lb/100 Surround 50 lb/acre Pyganic 1pt/acre
May 26	Lime-sulfur 2qt/100 gallon Sulfur 1.5 lb/100 Surround 50 lb/acre

	Pyganic 1pt/acre Neemix 8 oz/100 Entrust 3oz/acre
June 5	Pyganic 1pt/acre Neemix 8 oz/100 Entrust 3oz/acre Sulfur 1.5 lb/100 Surround 50 lb/acre
June 16	Pyganic 1pt/acre Neemix 8 oz/100 Entrust 3oz/acre Sulfur 1.5 lb/100 Surround 50 lb/acre
June 28	Pyganic 1pt/acre Neemix 8 oz/100 Entrust 3oz/acre Sulfur 1.5 lb/100 Surround 50 lb/acre
July 8	Pyganic 1pt/acre Neemix 8 oz/100 Entrust 3oz/acre Sulfur 1.5 lb/100 Surround 50 lb/acre
July 17	JMS oil
July 18	Pyganic 1pt/acre Neemix 8 oz/100 Entrust 3oz/acre Sulfur 1.5 lb/100 Surround 50 lb/acre
July 31	Pyganic 1pt/acre Neemix 8 oz/100 Entrust 3oz/acre Sulfur 1.5 lb/100 Surround 50 lb/acre
August 19	Pyganic 1pt/acre Neemix 8 oz/100 Entrust 3oz/acre Sulfur 1.5 lb/100 Surround 50 lb/acre
August 27	Sulfur 1.5 lb/100
September 11	Sulfur 1.5 lb/100 Neemix 8 oz/100

Conventional 2007	Spray Materials and Rates
March 31	Kocide 4lb/acre JMS oil
April 24	Agri-Strep 1.2 lb/acre
April 29	Agri-Strep 1.2 lb/acre Ziram 1 lb/100 Topsin 0.25 lb/100
May 7	Ziram 1 lb/100 Topsin 0.25 lb/100 Imidan 1.3 lb/100
May 16	Ziram 1 lb/100 Topsin 0.25 lb/100 Imidan 1.3 lb/100
June 5	Ziram 1 lb/100 Lannate 4 oz/100 Imidan 1.3 lb/100
June 16	Ziram 1 lb/100 Topsin 0.25 lb/100 Imidan 1.3 lb/100 Lannate 4 oz/100
June 28	Ziram 1 lb/100 Topsin 0.25 lb/100 Imidan 1.3 lb/100 Lannate 4 oz/100
July 8	Ziram 1 lb/100 Topsin 0.25 lb/100 Imidan 1.3 lb/100 Lannate 4 oz/100
July 17	Acaramite 1 lb/acre
July 28	Ziram 1 lb/100 Topsin 0.25 lb/100 Imidan 1.3 lb/100 Lannate 4 oz/100
July 31	Vydate 1pt/acre Imidan 1.3 lb/100 Pristine 0.8 lb/acre
August 19	Ziram 1 lb/100 Topsin 0.25 lb/100 Imidan 1.3 lb/100 Lannate 4 oz/100 Diazion 1pt/100
August 27	Pristine 0.8 lb/acre
September 3	Sevin 4L 0.75 qt/acre

Appendix 4. Phylotypes Found Uniquely Associated with Organic Samples.

_38; Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales;
Acetobacteraceae; Rhodopila; uncultured eubacterium WD271

a07o_22; Bacteria; Proteobacteria; Gammaproteobacteria; Legionellales;
Legionellaceae; Legionella; uncultured Legionella sp.

a07o_21; Bacteria; Proteobacteria; Gammaproteobacteria; Legionellales;
Legionellaceae; Legionella; Legionella donaldsonii

a07o_118; Bacteria; Proteobacteria; Gammaproteobacteria; Legionellales;
Legionellaceae; Legionella; uncultured bacterium

j07o_168; Bacteria; Proteobacteria; Gammaproteobacteria; Legionellales;
Legionellaceae; Legionella; uncultured bacterium

s06o_125; Bacteria; Proteobacteria; Deltaproteobacteria; Myxococcales;
Cystobacterineae; Cystobacteraceae; Anaeromyxobacter; uncultured bacterium

j07o_101; Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcaceae;
unclassified_Staphylococcaceae; uncultured Staphylococcaceae bacterium

a07o_111; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
Hyphomicrobiaceae; Hyphomicrobium; uncultured bacterium

s06o_4; Bacteria; Proteobacteria; Deltaproteobacteria;
unclassified_Deltaproteobacteria; uncultured bacterium

s06o_118; Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;
Burkholderiaceae; Ralstonia; bacterium HTCC4029

j07o_89; Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;
Burkholderiaceae; Ralstonia; bacterium HTCC4029

s06o_117; Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;
Burkholderiaceae; Ralstonia; bacterium HTCC4029

s06o_116; Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;
Burkholderiaceae; Ralstonia; bacterium HTCC4029

a07o_4; Bacteria; Acidobacteria; Acidobacteria; Acidobacteriales;
Acidobacteriaceae; Gp1; uncultured bacterium

j07o_135; Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
Enterobacteriaceae; Serratia; uncultured bacterium

j06o_79; Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
Enterobacteriaceae; unclassified_Enterobacteriaceae; Rahnella sp. 'WMR15'

j06o_78; Bacteria; Actinobacteria; Actinobacteria; Actinobacteridae;
Actinomycetales; Actinomycineae; Actinomycetaceae; Actinomyces; Actinomyces
radicidentis

x05o_15; Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
unclassified_Pseudomonadales; Moraxella sp. L70

s06o_20; Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae;
Flavobacterium; Flavobacterium sp. PR01

s06o_36; Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae;
Flavobacterium; Flavobacterium sp. PR01

s06o_78; Bacteria; Fusobacteria; Fusobacteria; Fusobacteriales; Fusobacteriaceae;
Leptotrichia; Leptotrichia sp. oral clone HE052

x05o_33; Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;
Oxalobacteraceae; Massilia; uncultured bacterium

a07o_60; Bacteria; Bacteroidetes; Sphingobacteria; Sphingobacteriales;
unclassified_Sphingobacteriales; uncultured bacterium

a07o_138; Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Sphingomonadaceae; Sphingomonas; Sphingomonas oligophenolica

x05o_22; Bacteria; Actinobacteria; Actinobacteria; Actinobacteridae;
Actinomycetales; Corynebacterineae; Corynebacteriaceae; Corynebacterium;
Corynebacterium accolens

x05o_17; Bacteria; Actinobacteria; Actinobacteria; Actinobacteridae;
Actinomycetales; Corynebacterineae; Corynebacteriaceae; Corynebacterium;
Corynebacterium accolens

x05o_10; Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;
Oxalobacteraceae; unclassified_Oxalobacteraceae; Massilia timonae

a07o_110; Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;
Oxalobacteraceae; Massilia; uncultured Oxalobacteraceae bacterium

s06o_101; Bacteria; Bacteroidetes; Flavobacteria; Flavobacteriales;
Flavobacteriaceae; Chryseobacterium; Chryseobacterium sp. RHA2-9

j07o_137; Bacteria; Actinobacteria; Actinobacteria; Actinobacteridae;
Actinomycetales; Corynebacterineae; Mycobacteriaceae; Mycobacterium;
Mycobacterium isoniacini

s06o_61; Bacteria; Proteobacteria; Deltaproteobacteria; Myxococcales;
Cystobacterineae; Cystobacteraceae; Anaeromyxobacter; uncultured bacterium

a07o_18; Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Pseudomonadaceae; Pseudomonas; Pseudomonas sp. 62AP4

a07o_56; Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Pseudomonadaceae; Pseudomonas; Pseudomonas sp. 62AP4

a06o_7; Bacteria; Bacteroidetes; Bacteroidetes; Bacteroidales; Porphyromonadaceae;
Dysgonomonas; uncultured Bacteroidetes bacterium

s06o_62; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
Bradyrhizobiaceae; Afipia; uncultured alpha proteobacterium

j07o_134; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
Bradyrhizobiaceae; unclassified_Bradyrhizobiaceae; uncultured Bradyrhizobium sp.

s06o_134; Bacteria; Actinobacteria; Actinobacteria; Actinobacteridae;
Actinomycetales; Frankineae; Nakamurellaceae; Quadrisphaera; Quadrisphaera
granulorum

a07o_129; Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Pseudomonadaceae; Pseudomonas; gamma proteobacterium RBE1CD-79

a07o_20; Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Sphingomonadaceae; Sphingomonas; uncultured bacterium

x05o_31; Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; uncultured
bacterium

x05o_9; Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus; uncultured
bacterium

s06o_139; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
unclassified_Rhizobiales; uncultured bacterium

a06o_33; Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Sphingomonadaceae; Sphingomonas; uncultured soil bacterium

a07o_75; Bacteria; Proteobacteria; Deltaproteobacteria; Myxococcales;
Cystobacterineae; Cystobacteraceae; Anaeromyxobacter; uncultured bacterium

s06o_121; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
Aurantimonadaceae; Aurantimonas; Aerobacter ureolyica

a06o_45; Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae;
Lactobacillus; uncultured Firmicutes bacterium

x05o_43; Bacteria; Cyanobacteria; Cyanobacteria; unclassified_Cyanobacteria;
uncultured chlorophyte

x05o_41; Bacteria; Cyanobacteria; Cyanobacteria; unclassified_Cyanobacteria;
uncultured chlorophyte

a06o_21; Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
unclassified_Pseudomonadales; uncultured bacterium

j07o_204; Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;
Oxalobacteraceae; unclassified_Oxalobacteraceae; uncultured Oxalobacteraceae
bacterium

j07o_64; Bacteria; Firmicutes; Clostridia; Clostridiales; Lachnospiraceae;
unclassified_Lachnospiraceae; uncultured bacterium

j06o_91; Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae;
Enterococcus; Enterococcus sp. MMZ60G

j06o_72; Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae;
Enterococcus; Enterococcus sp. MMZ60G

x05o_26; Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;
Oxalobacteraceae; unclassified_Oxalobacteraceae; uncultured beta proteobacterium

x05o_5; Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Pseudomonadaceae; Pseudomonas; unidentified bacterium

Appendix 5. Phylotypes Found Uniquely Associated with Conventional Samples.

j07c_72; Bacteria; Firmicutes; Clostridia; Clostridiales; unclassified_Clostridiales;
uncultured bacterium

j07c_3; Bacteria; Firmicutes; Clostridia; Clostridiales; unclassified_Clostridiales;
uncultured bacterium

j07c_26; Bacteria; Firmicutes; Clostridia; Clostridiales; unclassified_Clostridiales;
uncultured bacterium

j07c_63; Bacteria; Firmicutes; Clostridia; Clostridiales; unclassified_Clostridiales;
uncultured bacterium

s06c_21; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
Methylobacteriaceae; Methylobacterium; uncultured bacterium

a07c_92; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
Methylobacteriaceae; Methylobacterium; uncultured bacterium

s06c_108; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
Methylobacteriaceae; Methylobacterium; uncultured bacterium

a07c_178; Bacteria; Bacteroidetes; Sphingobacteria; Sphingobacteriales;
Sphingobacteriaceae; unclassified_Sphingobacteriaceae; uncultured bacterium

j07c_15; Bacteria; Firmicutes; Clostridia; Clostridiales; unclassified_Clostridiales;
uncultured bacterium

j07c_85; Bacteria; Firmicutes; Clostridia; Clostridiales; unclassified_Clostridiales;
uncultured bacterium

j07c_53; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Rhizobiaceae;
Rhizobium; Rhizobium sp. PSB16

s06c_55; Bacteria; Actinobacteria; Actinobacteria; Actinobacteridae;
Actinomycetales; Frankineae; Kineosporiaceae; Kineococcus; Kineococcus
radiotolerans SRS30216

a07c_102; Bacteria; Actinobacteria; Actinobacteria; Actinobacteridae;
Actinomycetales; Frankineae; Kineosporiaceae; Kineococcus; Kineococcus
radiotolerans SRS30216

a07c_174; Bacteria; Actinobacteria; Actinobacteria; Actinobacteridae;
Actinomycetales; Frankineae; Kineosporiaceae; Kineococcus; Kineococcus
radiotolerans SRS30216

a07c_11; Bacteria; Actinobacteria; Actinobacteria; Actinobacteridae;
Actinomycetales; Frankineae; Kineosporiaceae; Kineococcus; Kineococcus
radiotolerans SRS30216

a07c_35; Bacteria; Actinobacteria; Actinobacteria; Actinobacteridae;
Actinomycetales; Frankineae; Kineosporiaceae; Kineococcus; Kineococcus
radiotolerans SRS30216

s06c_43; Bacteria; Actinobacteria; Actinobacteria; Actinobacteridae;
Actinomycetales; Frankineae; Kineosporiaceae; Kineococcus; Kineococcus
radiotolerans SRS30216

s06c_194; Bacteria; Actinobacteria; Actinobacteria; Actinobacteridae;
Actinomycetales; Frankineae; Kineosporiaceae; Kineococcus; Kineococcus
radiotolerans SRS30216

j07c_113; Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
Enterobacteriaceae; Erwinia; Pantoea agglomerans

j07c_170; Bacteria; Bacteroidetes; Bacteroidetes; Bacteroidales;
Porphyromonadaceae; unclassified_Porphyromonadaceae; uncultured bacterium

j07c_123; Bacteria; Bacteroidetes; Bacteroidetes; Bacteroidales;
Porphyromonadaceae; unclassified_Porphyromonadaceae; uncultured bacterium

j07c_181; Bacteria; Bacteroidetes; Bacteroidetes; Bacteroidales;
Porphyromonadaceae; unclassified_Porphyromonadaceae; uncultured bacterium

j07c_111; Bacteria; Bacteroidetes; Bacteroidetes; Bacteroidales;
Porphyromonadaceae; unclassified_Porphyromonadaceae; uncultured bacterium

a06c_34; Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Sphingomonadaceae; Sphingomonas; Sphingomonas sp. Y57

j07c_157; Bacteria; Firmicutes; Clostridia; Clostridiales; Acidaminococcaceae;
Sporomusa; Desulfosporomusa polytropa

j07c_41; Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae; Sporobacter;
uncultured bacterium

x05c_40; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
Methylobacteriaceae; Methylobacterium; Methylobacterium sp. OSB1

s06c_180; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
Methylobacteriaceae; Methylobacterium; Methylobacterium sp. OSB1

s06c_173; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
Methylobacteriaceae; Methylobacterium; Methylobacterium sp. OSB1

s06c_73; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
Methylobacteriaceae; Methylobacterium; Methylobacterium radiotolerans

s06c_26; Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales;
Xanthomonadaceae; Schineria; uncultured bacterium

j07c_186; Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Sphingomonadaceae; Sphingomonas; uncultured bacterium

s06c_29; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
unclassified_Rhizobiales; uncultured bacterium

x05c_26; Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;

Oxalobacteraceae; Massilia; uncultured bacterium

j07c_11; Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales;

Xanthomonadaceae; Lysobacter; Xanthomonas sp. B05-08.04.0214

j07c_179; Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;

Pseudomonadaceae; Pseudomonas; Pseudomonas sp. Act34

j07c_103; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Rhizobiaceae;

Rhizobium; uncultured bacterium

a07c_43; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Rhizobiaceae;

Rhizobium; uncultured bacterium

a07c_53; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Bartonellaceae;

Bartonella; Bartonella tamiae

j07c_7; Bacteria; Proteobacteria; Gammaproteobacteria; Oceanospirillales;

Halomonadaceae; Zymobacter; Zymobacter palmae

j07c_93; Bacteria; Proteobacteria; Gammaproteobacteria; Oceanospirillales;

Halomonadaceae; Zymobacter; Zymobacter palmae

j07c_86; Bacteria; Firmicutes; Clostridia; Clostridiales; unclassified_Clostridiales;
uncultured earthworm cast bacterium

j07c_49; Bacteria; Firmicutes; Clostridia; Clostridiales; unclassified_Clostridiales;
uncultured bacterium

j07c_110; Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae; Clostridium;
Clostridium sp.

j07c_162; Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
Enterobacteriaceae; unclassified_Enterobacteriaceae; Pantoea ananatis

a06c_28; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
unclassified_Rhizobiales; uncultured alpha proteobacterium

s06c_47; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
unclassified_Rhizobiales; uncultured alpha proteobacterium

x05c_23; Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;
Oxalobacteraceae; unclassified_Oxalobacteraceae; uncultured beta proteobacterium

j07c_67; Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;
Oxalobacteraceae; unclassified_Oxalobacteraceae; uncultured Oxalobacteraceae
bacterium

a07c_57; Bacteria; Deinococcus-Thermus; Deinococci; Deinococcales;
Deinococcaceae; Deinococcus; uncultured bacterium

x05c_52; Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
Enterobacteriaceae; Pantoea; uncultured bacterium

x05c_54; Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
Enterobacteriaceae; Erwinia; rape rhizosphere bacterium tsb085

s06c_37; Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae;
Lactobacillus; uncultured Firmicutes bacterium

s06c_56; Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae;
Lactobacillus; uncultured Firmicutes bacterium

s06c_169; Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae;
Lactobacillus; uncultured Lactobacillus sp.

j07c_48; Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae;
unclassified_Clostridiaceae; uncultured Clostridiales bacterium

j07c_152; Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
Enterobacteriaceae; Pantoea; Pantoea ananatis

j07c_155; Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Pseudomonadaceae; Pseudomonas; Pseudomonas syringae pv. phaseolicola 1448A

j06c_43; Bacteria; Firmicutes; Clostridia; Clostridiales; Acidaminococcaceae;

Anaeroglobus; Anaeroglobus geminatus

j06c_76; Bacteria; Bacteroidetes; Sphingobacteria; Sphingobacteriales;

Flexibacteraceae; Dyadobacter; Dyadobacter sp. A54

s06c_1; Bacteria; Actinobacteria; Actinobacteria; Actinobacteridae;

Bifidobacteriales; Bifidobacteriaceae; Bifidobacterium; uncultured Bifidobacterium
sp.

s06c_202; Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;

Burkholderiaceae; Burkholderia; uncultured beta proteobacterium

s06c_39; Bacteria; Bacteroidetes; Sphingobacteria; Sphingobacteriales;

Flexibacteraceae; unclassified_Flexibacteraceae; uncultured Bacteroidetes bacterium

s06c_82; Bacteria; Actinobacteria; Actinobacteria; Actinobacteridae;

Actinomycetales; Micrococcineae; Microbacteriaceae;

unclassified_Microbacteriaceae; uncultured soil bacterium

j06c_70; Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;

Oxalobacteraceae; unclassified_Oxalobacteraceae; uncultured Duganella sp.

a06c_44; Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;
Oxalobacteraceae; unclassified_Oxalobacteraceae; uncultured Oxalobacteraceae
bacterium

x05c_53; Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;
Enterobacteriaceae; unclassified_Enterobacteriaceae; uncultured bacterium

j07c_17; Bacteria; Firmicutes; Bacilli; Lactobacillales; unclassified_Lactobacillales;
Lactococcus garvieae

x05c_27; Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;
Comamonadaceae; Acidovorax; uncultured bacterium

a07c_17; Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Sphingomonadaceae; Sphingomonas; Sphingomonas phyllosphaerae

a06c_61; Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Pseudomonadaceae; Pseudomonas; Pseudomonas sp. ISSDS-402

a07c_84; Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales;
Acetobacteraceae; Roseomonas; Roseomonas genomospecies 5

j06c_67; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
Hyphomicrobiaceae; Labrys; uncultured Phyllobacteriaceae bacterium

j06c_44; Bacteria; Actinobacteria; Actinobacteria; Actinobacteridae;
Actinomycetales; Micrococcineae; Micrococcaceae; Rothia; uncultured bacterium

a07c_152; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;
Hyphomicrobiaceae; Devosia; Devosia sp. IPL20

j07c_35; Bacteria; Bacteroidetes; Bacteroidetes; Bacteroidales; Rikenellaceae;
Alistipes; uncultured bacterium

j07c_143; Bacteria; Firmicutes; Clostridia; Clostridiales; unclassified_Clostridiales;
uncultured bacterium

j07c_90; Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Sphingomonadaceae; Sphingomonas; uncultured bacterium

a07c_147; Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;
Sphingomonadaceae; Sphingomonas; Sphingomonas oligophenolica

j07c_88; Bacteria; Firmicutes; Clostridia; Clostridiales; Clostridiaceae; Clostridium;
Clostridium methylpentosum

a07c_133; Bacteria; Acidobacteria; Acidobacteria; Acidobacteriales;

Acidobacteriaceae; Gp3; uncultured Acidobacteria bacterium

a07c_182; Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;

Oxalobacteraceae; Massilia; Janthinobacterium sp. WSH04-01

a07c_180; Bacteria; Acidobacteria; Acidobacteria; Acidobacteriales;

Acidobacteriaceae; Gp3; uncultured Firmicutes bacterium

x05c_47; Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales;

Enterobacteriaceae; unclassified_Enterobacteriaceae; Pantoea sp. BD 502

j07c_180; Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales;

Sphingomonadaceae; Sphingomonas; bacterium HTCC4155

j06c_17; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;

Methylobacteriaceae; Methylobacterium; Methylobacterium sp. OS-30A

a07c_75; Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;

Methylobacteriaceae; Methylobacterium; Methylobacterium radiotolerans

Appendix 6. List of Family, Genus and Species of all Phyllosphere Bacteria
 Represented in 16S r RNA Gene Clone Libraries with Identity Scores.

Family	Genus	Species	Identity (%)
Oxalobacteraceae	Unclass Oxalobacteraceae	Massilia sp. 62AD11	99.26
Sphingomonadaceae	Novosphingobium	uncultured bacterium	98.51
Sphingomonadaceae	Sphingomonas	uncultured bacterium	99.24
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
unclassified Rhizobiales	uncultured alpha proteobacterium		96.37
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Oxalobacteraceae	Massilia	uncultured Oxalobacteraceae bacterium	99.45
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. Y57	96.38
Flexibacteraceae	unclassified Flexibacteraceae	Bacteroidetes endosymbiont of Aspidiotus destructor	96.33
unclassified Rhizobiales	uncultured bacterium		97.89
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured Oxalobacteraceae bacterium	97.69
Flexibacteraceae	unclassified Flexibacteraceae	Bacteroidetes endosymbiont of Aspidiotus destructor	96.16
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	99.43
Flexibacteraceae	unclassified Flexibacteraceae	Bacteroidetes endosymbiont of Aspidiotus destructor	95.6
unclassified Flavobacteriales	Flavobacteriales endosymbiont of Leucaspis ohakunensis D039		95.45
unclassified Flavobacteriales	Flavobacteriales endosymbiont of Leucaspis ohakunensis D039		94.68
Oxalobacteraceae	Massilia	uncultured bacterium	99.64
Flexibacteraceae	Hymenobacter	uncultured bacterium	98.8
uncultured bacterium			89.45
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. ISSDS-402	89.2
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Flexibacteraceae	Hymenobacter	uncultured bacterium	98.15
Sphingomonadaceae	Novosphingobium	uncultured bacterium	99.43
Burkholderiaceae	Burkholderia	uncultured eubacterium WD296	98
Microbacteriaceae	Curtobacterium	Curtobacterium flaccumfaciens	100
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	98.47
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	99.81
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	99.81
Burkholderiaceae	Burkholderia	uncultured eubacterium WD296	98

Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	98.29
unclassified Pseudomonadales	uncultured bacterium		93.44
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	99.24
unclassified Rhizobiales	uncultured bacterium		95.8
Sphingomonadaceae	Sphingomonas	uncultured bacterium	95.81
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	97.14
unclassified Rhizobiales	uncultured bacterium		98.09
Sphingomonadaceae	Sphingomonas	uncultured soil bacterium	96
Sphingomonadaceae	Sphingomonas	uncultured soil bacterium	94.92
Beijerinckiaceae	unclassified Beijerinckiaceae	uncultured bacterium	99.04
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	100
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	99.81
Acetobacteraceae	unclassified Acetobacteraceae	uncultured bacterium	98.29
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	98.48
Lactobacillaceae	Lactobacillus	uncultured Firmicutes bacterium	99.46
Porphyromonadaceae	Dysgonomonas	uncultured Bacteroidetes bacterium	97.65
Sphingomonadaceae	Novosphingobium	uncultured alpha proteobacterium	99.81
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. 7056	99.81
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. 14 4K	100
Microbacteriaceae	Curtobacterium	Curtobacterium flaccumfaciens	99.81
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Kineosporiaceae	Kineococcus	Kineococcus radiotolerans SRS30216	99.81
Sphingomonadaceae	Sphingomonas	uncultured bacterium	99.81
Sphingomonadaceae	Sphingomonas	uncultured eubacterium WD252	99.12
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	100
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.27
Oxalobacteraceae	Massilia	Oxalobacteraceae	99.45
Kineosporiaceae	Kineococcus	Kineococcus radiotolerans SRS30216	99.81
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.27
Methylobacteriaceae	Methylobacterium	Methylobacterium brachiatum	99.43
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. G1016	97.82
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Sphingomonadaceae	Sphingomonas	uncultured eubacterium	99.16
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05

Comamonadaceae	Acidovorax	Acidovorax sp. G3DM-83	100
Sphingomonadaceae	Sphingomonas	Sphingomonadaceae bacterium KVD-1790-11	100
Comamonadaceae	Acidovorax	Acidovorax sp. G3DM-83	100
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	100
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	99.82
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Sphingomonadaceae	Sphingomonas	Sphingomonadaceae bacterium KVD-1790-11	100
Microbacteriaceae	Curtobacterium	Curtobacterium sp. K6-02	100
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.45
Acidobacteriaceae	Gp3	uncultured Acidobacteria bacterium	98.85
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	98.86
Oxalobacteraceae	Massilia	uncultured bacterium	99.64
Burkholderiaceae	Burkholderia	uncultured eubacterium WD296	98.18
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
unclassified Rhizobiales	uncultured bacterium		98.09
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. M60- VN10-2W	96.95
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	99.64
Pseudomonadaceae	unclassified Pseudomonadaceae	Pseudomonas graminis	99.76
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.27
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.27
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	90.29
Acetobacteraceae	unclassified Acetobacteraceae	uncultured bacterium	98.29
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	100
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. Alpha1-2	98.2
Hyphomicrobiaceae	Devosia	Devosia sp. IPL20	99.26
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.45
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.45
Rhodobacteraceae	Rhodobacter	uncultured bacterium	100
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	99.64
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	100
Pseudomonadaceae	Pseudomonas	uncultured bacterium	100

Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.45
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.45
Sphingomonadaceae	Sphingomonas	Sphingomonas phyllosphaerae	100
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	98.86
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Kineosporiaceae	Kineococcus	Kineococcus radiotolerans SRS30216	99.81
Burkholderiaceae	Burkholderia	uncultured eubacterium WD296	99.74
Sphingobacteriaceae	unclassified Sphingobacteriaceae	uncultured bacterium	96.86
Oxalobacteraceae	Massilia	Janthinobacterium sp. A1- 13	99.57
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	99.81
Acidobacteriaceae	Gp3	uncultured Firmicutes bacterium	100
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	93.81
Burkholderiaceae	Burkholderia	uncultured eubacterium WD296	98
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Sphingomonadaceae	Sphingomonas	Sphingomonas melonis	99.05
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Acetobacteraceae	unclassified Acetobacteraceae	uncultured bacterium	98.48
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	100
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	100
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. BAC302	99.81
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured Duganella sp.	98.36
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.43
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	99.24
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	100
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. 62AP4	100
Oxalobacteraceae	Massilia	Janthinobacterium sp. A1- 13	99.45
unclassified Rhizobiales	uncultured bacterium		98.28
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. 62AP4	99.81
Kineosporiaceae	Kineococcus	Kineococcus radiotolerans SRS30216	99.81
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	98.48
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured Duganella sp.	98.04
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	99.81
Rhizobiaceae	Rhizobium	Rhizobium soli	100

Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured beta proteobacterium	99.57
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	100
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	100
Rhizobiaceae	Rhizobium	uncultured bacterium	100
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Sphingomonadaceae	Sphingomonas	Sphingomonas melonis	99.05
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.43
Pseudomonadaceae	Pseudomonas	Pseudomonas syringae pv. phaseolicola 1448A	100
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	99.81
Sphingomonadaceae	Sphingomonas	Sphingomonas melonis	99.05
Bartonellaceae	Bartonella	Bartonella tamiae	98.67
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	100
Methylobacteriaceae	Methylobacterium	Methylobacterium brachiatum	100
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. 14 4K	99.81
Deinococcaceae	Deinococcus	uncultured bacterium	98.48
Comamonadaceae	Acidovorax	Acidovorax sp. G3DM-83	100
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured Duganella sp.	97.98
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	99.81
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	99.81
Sphingomonadaceae	Sphingomonas	uncultured bacterium	99.81
Pseudomonadaceae	Pseudomonas	Pseudomonas syringae pv. tagetis	98
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	100
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. 14 4K	99.81
Sphingomonadaceae	Sphingomonas	uncultured eubacterium WD252	99.1
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	99.81
Flexibacteraceae	unclassified Flexibacteraceae	uncultured Bacteroidetes bacterium	99.45
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	99.81
Methylobacteriaceae	Methylobacterium	Methylobacterium radiotolerans	88.04
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	100
Rhizobiaceae	Rhizobium	Rhizobium soli	99.62
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	98.86
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	99.81
Sphingomonadaceae	Sphingomonas	uncultured eubacterium WD252	99.05

Acetobacteraceae	Roseomonas	Roseomonas genomospecies 5	97.34
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. 14_4K	99.62
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. Act34	99.81
Rhizobiaceae	Rhizobium	Rhizobium soli	100
Sphingomonadaceae	Sphingomonas	Sphingomonadaceae bacterium KVD-1790-11	100
Sphingomonadaceae	Sphingomonas	Sphingomonadaceae bacterium KVD-1790-11	100
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.09
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured beta proteobacterium	99.82
Sphingomonadaceae	Sphingomonas	Sphingomonadaceae bacterium KVD-1790-11	100
Methylobacteriaceae	Methylobacterium	uncultured bacterium	100
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.43
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	99.81
Oxalobacteraceae	Massilia	uncultured soil bacterium	100
Comamonadaceae	Acidovorax	Acidovorax sp. G3DM-83	100
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.35
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.45
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Pseudomonadaceae	Pseudomonas	Pseudomonas syringae pv. phaseolicola 1448A	100
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. NARs1	100
Microbacteriaceae	Curtobacterium	Curtobacterium flaccumfaciens	99.81
Enterobacteriaceae	Pantoea	uncultured bacterium	99.64
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	100
Oxalobacteraceae	Massilia	uncultured Oxalobacteraceae bacterium	89.9
Hyphomicrobiaceae	Hyphomicrobium	uncultured bacterium	100
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea ananatis	99.64
Microbacteriaceae	Curtobacterium	Curtobacterium flaccumfaciens	99.81
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.27
Legionellaceae	Legionella	uncultured bacterium	94.81
Pseudomonadaceae	Pseudomonas	uncultured bacterium	99.82
Sphingomonadaceae	Sphingomonas	uncultured bacterium	99.81
Oxalobacteraceae	Massilia	Janthinobacterium sp. A1- 13	99.64
Sphingomonadaceae	Sphingomonas	uncultured bacterium	98.86
Pseudomonadaceae	Pseudomonas	gamma proteobacterium RBE1 CD-79	96.11
Sphingomonadaceae	Sphingomonas	uncultured eubacterium WD252	99.05
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. NARs1	99.82
Pseudomonadaceae	Pseudomonas	Pseudomonas syringae pv. phaseolicola 1448A	99.82
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea ananatis	100
Sphingomonadaceae	Sphingomonas	Sphingomonas	99.05

		oligophenolica	
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	94.7
Sphingomonadaceae	Sphingomonas	uncultured eubacterium WD252	99.24
Clostridiaceae	Hespellia	uncultured bacterium	97.99
Pseudomonadaceae	Pseudomonas	uncultured bacterium	100
Pseudomonadaceae	Pseudomonas	Pseudomonas syringae pv. phaseolicola 1448A	100
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.09
Sphingomonadaceae	Sphingomonas	uncultured bacterium	98.29
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. Alpha1-2	97.9
Microbacteriaceae	Frigoribacterium	Frigoribacterium sp. GIC6	99.09
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.45
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. 62AP4	95.68
Sphingomonadaceae	Sphingomonas	uncultured soil bacterium	99.43
Sphingomonadaceae	Sphingomonas	uncultured bacterium	98.1
Legionellaceae	Legionella	Legionella donaldsonii	96.14
Legionellaceae	Legionella	uncultured Legionella sp.	94.61
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. Alpha1-2	98.48
		uncultured bacterium	90.55
Sphingomonadaceae	Sphingomonas	uncultured bacterium	99.81
Flexibacteraceae	Cardinium	endosymbiont of Tetranychus urticae red form A	99.82
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured beta proteobacterium	99.64
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.27
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	99.81
Sphingomonadaceae	Sphingomonas	uncultured Sphingomonas sp.	98.67
Sphingomonadaceae	Sphingomonas	uncultured Sphingomonas sp.	98.48
Acidobacteriaceae	Gp1	uncultured bacterium	100
Flexibacteraceae	Cardinium	endosymbiont of Tetranychus urticae red form A	99.82
Oxalobacteraceae	Massilia	uncultured Oxalobacteraceae bacterium	98.91
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Microbacteriaceae	Frigoribacterium	Frigoribacterium sp. GIC6	99.09
Sphingomonadaceae	Sphingomonas	uncultured Sphingomonas sp.	98.1
Sphingomonadaceae	Sphingomonas	uncultured Sphingomonas sp.	98.48
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	99.81
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	99.09
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. 62AP4	95.68
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	99.81
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. Alpha1-2	98.1
unclassified_Sphingobacteriales		uncultured bacterium	100
Burkholderiaceae	Burkholderia	uncultured eubacterium	98

		WD296	
Microbacteriaceae	Leifsonia	actinobacterium KV-677	99.62
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.09
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea ananatis	99.82
uncultured bacterium			90.36
Sphingomonadaceae	Sphingomonas	uncultured bacterium	99.81
Cystobacterineae	Cystobacteraceae	Anaeromyxobacter	93.98
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	98.36
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	98.67
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Sphingomonadaceae	Sphingomonas	uncultured bacterium	99.81
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured Oxalobacteraceae bacterium	98.37
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.09
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea ananatis	99.82
Sphingomonadaceae	Sphingomonas	uncultured Sphingomonas sp.	98.1
Pasteurellaceae	Haemophilus	uncultured Haemophilus sp.	99.82
Flexibacteraceae	Cardinium	endosymbiont of Brevipalpus phoenicis	99.45
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.27
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	99.41
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured Oxalobacteraceae bacterium	98.18
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured beta proteobacterium	99.27
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OS-30A	97.61
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	98.72
Burkholderiaceae	Burkholderia	uncultured eubacterium WD296	98.18
Sphingomonadaceae	Sphingomonas	uncultured bacterium	98.67
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.64
Moraxellaceae	Acinetobacter	uncultured bacterium	98.74
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured beta proteobacterium	99.27
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Staphylococcaceae	Staphylococcus	Staphylococcus sp. DAN1	100
Oxalobacteraceae	Massilia	Massilia aerolata	100
Flexibacteraceae	Hymenobacter	uncultured bacterium	98.34
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Flexibacteraceae	unclassified Flexibacteraceae	uncultured Bacteroidetes bacterium	99.26

Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.45
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	99.81
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	99.62
Oxalobacteraceae	Massilia	uncultured bacterium	100
Acidaminococcaceae	Anaeroglobus	Anaeroglobus geminatus	99.82
Micrococcaceae	Rothia	uncultured bacterium	100
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured Oxalobacteraceae bacterium	98.18
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OS- 30A	100
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	100
Oxalobacteraceae	Massilia	Massilia timonae	99.45
Incertae sedis 5	Pelomonas	uncultured Comamonadaceae bacterium	100
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.09
Sphingomonadaceae	Sphingomonas	uncultured bacterium	98.48
Flexibacteraceae	Hymenobacter	uncultured bacterium	97.45
Sphingomonadaceae	Sphingomonas	uncultured bacterium	97.25
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.27
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.24
Hyphomicrobiaceae	Labrys	uncultured Phyllobacteriaceae bacterium	96.76
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium NR179	98.72
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured Duganella sp.	97.64
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	99.81
Sphingomonadaceae	Sphingomonas	uncultured bacterium	100
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OS- 30A	100
Flexibacteraceae	Dyadobacter	Dyadobacter sp. A54	98.71
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured beta proteobacterium	99.45
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Flexibacteraceae	unclassified Flexibacteraceae	uncultured Bacteroidetes bacterium	99.26
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Oxalobacteraceae	Massilia	Janthinobacterium sp. A1- 13	99.27
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.27
Oxalobacteraceae	Massilia	Janthinobacterium sp. A1- 13	99.09
Flexibacteraceae	unclassified Flexibacteraceae	Bacteroidetes endosymbiont of Aspidiotus destructor	93.68
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OS- 30A	100

Sphingomonadaceae	Sphingomonas	uncultured bacterium	97.93
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.43
Pasteurellaceae	Haemophilus	uncultured Haemophilus sp.	99.82
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea ananatis	99.64
Staphylococcaceae	Staphylococcus	Staphylococcus sp. MH37	100
Oxalobacteraceae	Massilia	uncultured Oxalobacteraceae bacterium	99.45
Oxalobacteraceae	Massilia	uncultured Oxalobacteraceae bacterium	99.27
Enterobacteriaceae	unclassified Enterobacteriaceae	bacterium 1-1	100
Oxalobacteraceae	Massilia	Janthinobacterium sp. A1-13	99.45
Oxalobacteraceae	Massilia	Janthinobacterium sp. A1-13	99.64
Oxalobacteraceae	Massilia	Janthinobacterium sp. A1-13	99.45
Moraxellaceae	Acinetobacter	uncultured bacterium	99.82
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. Act34	100
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	99.82
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.09
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	98.91
Oxalobacteraceae	Massilia	Janthinobacterium sp. A1-13	99.64
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OS-30A	100
Microbacteriaceae	Curtobacterium	Curtobacterium sp. 124NP18	99.81
Oxalobacteraceae	Massilia	uncultured Oxalobacteraceae bacterium	99.16
Oxalobacteraceae	Massilia	Janthinobacterium sp. A1-13	99.45
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	97.81
Oxalobacteraceae	Massilia	uncultured Oxalobacteraceae bacterium	98.91
Enterococcaceae	Enterococcus	Enterococcus sp. MMZ60G	98.73
Actinomycetaceae	Actinomyces	Actinomyces radidentis	99.27
Enterobacteriaceae	unclassified Enterobacteriaceae	Rahnella sp. 'WMR15'	99.82
Microbacteriaceae	Curtobacterium	Curtobacterium sp. 124NP18	99.44
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Oxalobacteraceae	Massilia	uncultured Oxalobacteraceae bacterium	99.27
Oxalobacteraceae	Massilia	uncultured Oxalobacteraceae bacterium	96.7
Enterobacteriaceae	unclassified Enterobacteriaceae	Enterobacter ludwigii	99.64
Enterococcaceae	Enterococcus	Enterococcus sp. MMZ60G	98.55
Clostridiaceae	Hespellia	uncultured bacterium	98.48

Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured beta proteobacterium	99.82
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea ananatis	100
Rhizobiaceae	Rhizobium	uncultured bacterium	100
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Xanthomonadaceae	Lysobacter	Xanthomonas sp. B05-08.04.0214	99.82
Clostridiaceae	Clostridium	Clostridium sp.	98.1
Porphyromonadaceae	unclassified Porphyromonadaceae	uncultured bacterium	94.83
Sphingomonadaceae	Sphingomonas	uncultured eubacterium WD252	99.05
Enterobacteriaceae	Erwinia	Pantoea agglomerans	96.55
Porphyromonadaceae	unclassified Porphyromonadaceae	uncultured bacterium	94.82
Pseudomonadaceae	Pseudomonas	Pseudo.syringae pv. phaseolicola 1448A	99.82
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	99.82
unclassified Clostridiales	uncultured bacterium		97.52
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea ananatis	99.64
unclassified Clostridiales	uncultured bacterium		90.67
Enterobacteriaceae	Pantoea	Pantoea ananatis	95.52
Pseudomonadaceae	Pseudomonas	Pseudomonas syringae pv. phaseolicola 1448A	98.73
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.45
Acidaminococcaceae	Sporomusa	Desulfosporomusa polytropa	94.84
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea ananatis	84.21
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. 14 4K	100
unclassified Lactobacillales	Lactococcus garvieae		100
Porphyromonadaceae	unclassified Porphyromonadaceae	uncultured bacterium	93.16
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea ananatis	100
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. Act34	95.86
Flexibacteraceae	Cardinium	endosymbiont of Tetranychus urticae red form A	99.82
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	90.32
Porphyromonadaceae	unclassified Porphyromonadaceae	uncultured bacterium	94.45
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	98.86
Sphingomonadaceae	Sphingomonas	uncultured bacterium	96.05
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	99.82
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. BAC302	100
Clostridiaceae	Coprobacillus	uncultured bacterium	96.18
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Flexibacteraceae	Cardinium	endosymbiont of Tetranychus urticae red form A	99.82
unclassified Clostridiales	uncultured bacterium		96.57
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. MC83	99.82
Flexibacteraceae	Cardinium	endosymbiont of Tetranychus urticae red form A	99.63
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45

unclassified Clostridiales	uncultured bacterium		96.57
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured Massilia sp.	99.82
Flexibacteraceae	Cardinium	endosymbiont of Tetranychus urticae red form A	99.64
Rikenellaceae	Alistipes	uncultured bacterium	96.51
Clostridiaceae	Sporobacter	uncultured bacterium	97.52
Clostridiaceae	unclassified Clostridiaceae	uncultured Clostridiales bacterium	96.78
unclassified Clostridiales	uncultured bacterium		97.9
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. 62AP4	99.81
Rhizobiaceae	Rhizobium	Rhizobium sp. PSB16	100
Oxalobacteraceae	Massilia	Janthinobacterium sp. A1-13	99.82
Oxalobacteraceae	Massilia	Janthinobacterium sp. A1-13	99.82
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
unclassified Clostridiales	uncultured bacterium		96.57
Oxalobacteraceae	unclassified Oxalobacteraceae	Uncult. Oxalobacteraceae bacterium	90.09
Flexibacteraceae	Cardinium	endosymbiont of Tetranychus urticae red form A	99.45
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Halomonadaceae	Zymobacter	Zymobacter palmae	98.18
Oxalobacteraceae	Massilia	Janthinobacterium sp. A1-13	99.45
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
unclassified Clostridiales	uncultured bacterium		96.57
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	99.81
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. G1016	98
Clostridiaceae	Coprobacillus	uncultured bacterium	96.18
Oxalobacteraceae	Massilia	Janthinobacterium sp. A1-13	99.82
Sphingomonadaceae	Sphingomonas	uncultured bacterium	98.67
unclassified Clostridiales	uncultured bacterium		90.67
unclassified Clostridiales	uncultured earthworm cast bacterium		99.4
Clostridiaceae	Clostridium	Clostridium methylpentosum	97.09
Sphingomonadaceae	Sphingomonas	uncultured bacterium	97.49
Halomonadaceae	Zymobacter	Zymobacter palmae	98.18
Flexibacteraceae	Cardinium	endosymbiont of Tetranychus urticae red form A	99.82
Flexibacteraceae	Cardinium	endosymbiont of Tetranychus urticae red form A	99.82
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. Act34	99.82
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. BAC302	99.81
Microbacteriaceae	Curtobacterium	Curtobacterium flaccumfaciens	99.28
Staphylococcaceae	Staphylococcus	Staphylococcus sp. MH37	99.82
Staphylococcaceae	unclassified Staphylococcaceae	uncultured Staphylococcaceae bacterium	99.64

Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.45
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.45
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Pseudomonadaceae	Pseudomonas	uncultured gamma proteobacterium	99.82
Pseudomonadaceae	Pseudomonas	uncultured gamma proteobacterium	99.64
Pseudomonadaceae	Pseudomonas	uncultured gamma proteobacterium	99.82
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.38
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.64
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.27
Pseudomonadaceae	Pseudomonas	Pseudomonas syringae pv. phaseolicola 1448A	100
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Pseudomonadaceae	Pseudomonas	uncultured gamma proteobacterium	99.42
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Pseudomonadaceae	Pseudomonas	uncultured gamma proteobacterium	99.82
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.27
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Bradyrhizobiaceae	unclassified Bradyrhizobiaceae	uncultured Bradyrhizobium sp.	100
Enterobacteriaceae	Serratia	uncultured bacterium	99.82
Mycobacteriaceae	Mycobacterium	Mycobacterium isoniacini	99.62
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	99.81
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.44
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.09
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured Oxalobacteraceae bacterium	99.64
Pseudomonadaceae	Pseudomonas	Pseudomonas syringae pv. phaseolicola 1448A	99.64
Microbacteriaceae	Curtobacterium	Curtobacterium flaccumfaciens	99.32
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea sp. BD 502	99.64

Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	99.62
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea ananatis	100
Sphingomonadaceae	Sphingomonas	uncultured Sphingomonas sp.	98.67
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. NARs1	100
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. 52AD24	98.91
Enterobacteriaceae	Pantoea	uncultured bacterium	99.82
Legionellaceae	Legionella	uncultured bacterium	95.01
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	98.86
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. BAC302	100
Enterobacteriaceae	Pantoea	uncultured bacterium	99.63
Enterobacteriaceae	Pantoea	uncultured bacterium	99.82
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. 52AD24	98.91
Enterobacteriaceae	Pantoea	uncultured bacterium	99.82
Rhizobiaceae	Rhizobium	Rhizobium radiobacter	94.64
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. 52AD24	98.91
Methylobacteriaceae	Methylobacterium	Methylobacterium brachiatum	99.62
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. BAC302	100
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured Oxalobacteraceae bacterium	99.45
Pseudomonadaceae	Pseudomonas	uncultured bacterium	97.64
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured Oxalobacteraceae bacterium	94.75
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. NARs1	100
Beijerinckiaceae	unclassified Beijerinckiaceae	uncultured bacterium	99.05
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.45
unclassified Burkholderiales	uncultured beta proteobacterium		94.66
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.09
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured Oxalobacteraceae bacterium	99.64
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea ananatis	100
Microbacteriaceae	Curtobacterium	Curtobacterium flaccumfaciens	99.81
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.45
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. 62AP4	99.81
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	98.36
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Sphingomonadaceae	Sphingomonas	Sphingomonadaceae bacterium KVD-1790-11	99.81
Flexibacteraceae	unclassified Flexibacteraceae	uncultured Bacteroidetes bacterium	97.79
Burkholderiaceae	Burkholderia	uncultured eubacterium WD296	97.42
Sphingomonadaceae	Sphingomonas	uncultured bacterium	98.29
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	99.62

Pseudomonadaceae	Pseudomonas	uncultured bacterium	100
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. BAC302	100
Pseudomonadaceae	Pseudomonas	uncultured gamma proteobacterium	99.27
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.45
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.27
Clostridiaceae	Coprobacillus	uncultured bacterium	96.55
Enterobacteriaceae	Pantoea	uncultured bacterium	99.64
Pseudomonadaceae	Pseudomonas	uncultured gamma proteobacterium	99.81
Lachnospiraceae	unclassified Lachnospiraceae	uncultured bacterium	100
Pseudomonadaceae	Pseudomonas	uncultured gamma proteobacterium	100
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.27
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.09
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.38
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured beta proteobacterium	99.45
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.45
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.27
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.45
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Pseudomonadaceae	Pseudomonas	Pseudomonas syringae pv. phaseolicola 1448A	99.82
Staphylococcaceae	Staphylococcus	Staphylococcus saccharolyticus	100
Staphylococcaceae	Staphylococcus	Staphylococcus sp. MH37	99.82
Pseudomonadaceae	Pseudomonas	uncultured gamma proteobacterium	99.82
Burkholderiaceae	Ralstonia	bacterium HTCC4029	99.82
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.27
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured Oxalobacteraceae bacterium	98.51
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Pseudomonadaceae	Pseudomonas	Pseudomonas syringae pv. phaseolicola 1448A	100
Pseudomonadaceae	Pseudomonas	uncultured gamma proteobacterium	100
Bifidobacteriaceae	Bifidobacterium	uncultured Bifidobacterium sp.	99.25
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	100

Flexibacteraceae	Hymenobacter	uncultured bacterium	98.15
Flexibacteraceae	Hymenobacter	uncultured bacterium	97.99
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Flexibacteraceae	Hymenobacter	uncultured bacterium	97.42
Methylobacteriaceae	Methylobacterium	uncultured bacterium	100
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
unclassified Rhizobiales	uncultured bacterium		98.28
Methylobacteriaceae	Methylobacterium	Methylobacterium aerolata	99.81
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Flexibacteraceae	Hymenobacter	uncultured bacterium	97.97
Flexibacteraceae	Hymenobacter	uncultured bacterium	99.08
Oxalobacteraceae	Massilia	Janthinobacterium sp. A1-13	99.45
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Beijerinckiaceae	unclassified Beijerinckiaceae	uncultured bacterium	98.1
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. Y57	98.86
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Oxalobacteraceae	Massilia	Janthinobacterium sp. A1-13	99.64
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	99.81
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	100
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	99.6
Acetobacteraceae	unclassified Acetobacteraceae	uncultured bacterium	98.45
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	99.8
Sphingomonadaceae	Sphingomonas	uncultured bacterium	100
Acetobacteraceae	unclassified Acetobacteraceae	uncultured bacterium	98.45
Methylobacteriaceae	Methylobacterium	Methylobacterium	100

		komagatae	
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	99.6
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Acetobacteraceae	unclassified Acetobacteraceae	uncultured bacterium	98.45
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	99.81
Acetobacteraceae	unclassified Acetobacteraceae	uncultured bacterium	98.45
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	99.81
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. 14_4K	100
Methylobacteriaceae	Methylobacterium	Methylobacterium brachiatum	99.62
Methylobacteriaceae	Methylobacterium	Methylobacterium brachiatum	99.62
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. 14_4K	99.81
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured Duganella sp.	98.36
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	99.43
Oxalobacteraceae	Massilia	uncultured soil bacterium	97.81
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Lactobacillaceae	Lactobacillus	uncultured Lactobacillus sp.	99.82
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	99.43
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	99.62
Methylobacteriaceae	Methylobacterium	Methylobacterium brachiatum	99.81
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	99.04
unclassified Rhizobiales	uncultured bacterium		98.28
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. M9-3	100
unclassified Rhizobiales	uncultured bacterium		98.28
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	99.24
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Methylobacteriaceae	Methylobacterium	Methylobacterium brachiatum	99.81
unclassified Rhizobiales	uncultured bacterium		98.09
Flexibacteraceae	Hymenobacter	uncultured bacterium	98.34
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. Alpha1-2	98.1
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Methylobacteriaceae	Methylobacterium	Methylobacterium aerolata	100
Kineosporiaceae	Kineococcus	Kineococcus radiotolerans SRS30216	99.81
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100

Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Methylobacteriaceae	Methylobacterium	Methylobacterium aerolata	99.81
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	98.86
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	99.62
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Burkholderiaceae	Burkholderia	uncultured beta proteobacterium	100
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Rhizobiaceae	Rhizobium	Rhizobium radiobacter	100
Methylobacteriaceae	Methylobacterium	uncultured bacterium	100
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
unclassified Rhizobiales	uncultured bacterium		99.05
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	99.81
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. Y57	99.05
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Xanthomonadaceae	Schineria	uncultured bacterium	100
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
unclassified Rhizobiales	uncultured bacterium		98.19
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	98.86
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	98.86
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Sphingomonadaceae	Sphingomonas	uncultured bacterium	98.67
Sphingomonadaceae	Sphingomonas	uncultured bacterium	98.67
Sphingomonadaceae	Sphingomonas	Sphingomonadaceae bacterium KVD-1790-11	100
Lactobacillaceae	Lactobacillus	uncultured Firmicutes bacterium	98.73
unclassified Rhizobiales	uncultured bacterium		98.28
Flexibacteraceae	unclassified Flexibacteraceae	uncultured Bacteroidetes bacterium	98.35
Methylobacteriaceae	Methylobacterium	Methylobacterium brachiatum	100
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.03
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05

Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	99.24
Kineosporiaceae	Kineococcus	Kineococcus radiotolerans SRS30216	99.62
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	99.81
unclassified Rhizobiales	uncultured alpha proteobacterium		99.53
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. 52AD24	99.6
Acetobacteraceae	unclassified Acetobacteraceae	uncultured bacterium	98.48
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	100
Flexibacteraceae	Hymenobacter	uncultured bacterium	99.63
Kineosporiaceae	Kineococcus	Kineococcus radiotolerans SRS30216	99.81
Lactobacillaceae	Lactobacillus	uncultured Firmicutes bacterium	99.09
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	99.81
Sphingomonadaceae	Sphingomonas	Sphingomonadaceae bacterium KVD-1790-11	99.81
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	98.86
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	98.86
unclassified Rhizobiales	uncultured bacterium		99.05
Sphingomonadaceae	Sphingomonas	uncultured bacterium	100
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Porphyromonadaceae	Dysgonomonas	uncultured bacterium	95.76
Methylobacteriaceae	Methylobacterium	Methylobacterium aerolata	99.81
Flexibacteraceae	Hymenobacter	uncultured bacterium	97.42
Pseudomonadaceae	Pseudomonas	Pseudomonas syringae pv. phaseolicola 1448A	100
Porphyromonadaceae	Dysgonomonas	uncultured bacterium	95.95
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. Y57	98.48
Sphingomonadaceae	Sphingomonas	Sphingomonadaceae bacterium KVD-1790-11	99.81
Methylobacteriaceae	Methylobacterium	Methylobacterium radiotolerans	99.62
unclassified Rhizobiales	uncultured bacterium		98.26
Rhizobiaceae	Rhizobium	Rhizobium soli	100
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	100
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	99.81
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	99.62
Microbacteriaceae	unclassified Microbacteriaceae	uncultured soil bacterium	99.25
Sphingomonadaceae	Novosphingobium	uncultured alpha proteobacterium	99.81
Flexibacteraceae	unclassified Flexibacteraceae	uncultured Bacteroidetes bacterium	97.97
unclassified Rhizobiales	uncultured bacterium		98.85

Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	98.87
Oxalobacteraceae	Massilia	uncultured Oxalobacteraceae bacterium	99.1
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. NARs1	100
Oxalobacteraceae	Massilia	Massilia aurea	99.64
Microbacteriaceae	unclassified Microbacteriaceae	Frigoribacterium sp. 73NP5	99.77
Methylobacteriaceae	Methylobacterium	Methylobacterium aerolata	99.81
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.45
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	99.37
unclassified Rhizobiales	uncultured bacterium		98.28
Sphingomonadaceae	Sphingomonas	Sphingomonadaceae bacterium KVD-1790-11	100
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
unclassified Rhizobiales	uncultured bacterium		98.28
unclassified Rhizobiales	uncultured bacterium		98.85
Flavobacteriaceae	Chryseobacterium	Chryseobacterium sp. RHA2-9	99.08
Flexibacteraceae	Hymenobacter	uncultured bacterium	97.97
Incertae sedis 5	Pelomonas	Uncult. Comamonadaceae	100
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	100
Incertae sedis 5	Pelomonas	uncultured Comamonadaceae bacterium	100
Burkholderiaceae	Ralstonia	bacterium HTCC4029	100
Burkholderiaceae	Ralstonia	bacterium HTCC4029	100
Burkholderiaceae	Ralstonia	bacterium HTCC4029	100
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	100
Flexibacteraceae	unclassified Flexibacteraceae	uncultured Bacteroidetes bacterium	98.52
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. M60-VN10-2W	97.14
Aurantimonadaceae	Aurantimonas	Aerobacter ureolyica	100
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.45
Sphingomonadaceae	Sphingomonas	uncultured eubacterium WD252	99.24
Cystobacterineae	Cystobacteraceae	Anaeromyxobacter	96.02
Beijerinckiaceae	unclassified Beijerinckiaceae	uncultured bacterium	99.05
Flexibacteraceae	unclassified Flexibacteraceae	uncultured bacterium	97.78
Beijerinckiaceae	unclassified Beijerinckiaceae	uncultured bacterium	98.85
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium KVD-1921-01	99.27
Staphylococcaceae	Staphylococcus	Staphylococcus sp. DAN1	99.64
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.64
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Rhizobiaceae	Rhizobium	Rhizobium soli	100

Nakamurellaceae	Quadrisphaera	Quadrisphaera granulorum	99.06
Flexibacteraceae	unclassified Flexibacteraceae	uncultured bacterium	97.97
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	99.24
Burkholderiaceae	Burkholderia	uncultured eubacterium WD296	98.18
unclassified Rhizobiales	uncultured bacterium		97.71
Sphingomonadaceae	Sphingomonas	uncultured bacterium	98.67
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	100
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	99.78
Oxalobacteraceae	Janthinobacterium	uncultured bacterium	99.64
Methylobacteriaceae	Methylobacterium	Methylobacterium rhodinum	99.05
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Flexibacteraceae	unclassified Flexibacteraceae	uncultured Bacteroidetes bacterium	99.08
Flavobacteriaceae	Flavobacterium	Flavobacterium sp. PR01	99.26
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.64
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. 52AD24	100
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	98.86
Flexibacteraceae	Hymenobacter	uncultured bacterium	99.44
Oxalobacteraceae	Massilia	Janthinobacterium sp.	99.45
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	99.81
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	98.86
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. Y57	99.05
Sphingomonadaceae	Sphingomonas	uncultured bacterium	100
Rhodobacteraceae	Rhodobacter	uncultured bacterium	100
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
unclassified Rhizobiales	uncultured bacterium		98.28
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea sp. 092305	99.79
unclassified Rhizobiales	uncultured bacterium		98.28
unclassified Rhizobiales	uncultured bacterium		98.1
Flavobacteriaceae	Flavobacterium	Flavobacterium sp. PR01	99.44
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Acetobacteraceae	Rhodopila	uncultured eubacterium WD271	98.1
Methylobacteriaceae	Methylobacterium	Methylobacterium brachiatum	99.81
uncultured bacterium			95.47
Enterobacteriaceae	unclassified Enterobacteriaceae	uncultured bacterium	100
Oxalobacteraceae	Massilia	Oxalobacteraceae bacterium NR179	98.72
Sphingomonadaceae	Sphingomonas	Sphingomonadaceae bacterium KVD-1790-11	100
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	99.81
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45

Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	99.62
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Flexibacteraceae	Hymenobacter	uncultured bacterium	97.97
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured Duganella sp.	97.92
Enterobacteriaceae	unclassified Enterobacteriaceae	uncultured bacterium	100
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Methylobacteriaceae	Methylobacterium	Methylobacterium brachiatum	100
Methylobacteriaceae	Methylobacterium	Methylobacterium aerolata	100
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Porphyromonadaceae	Dysgonomonas	uncultured bacterium	95.95
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	98.85
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Sphingomonadaceae	Sphingomonas	bacterium HTCC4155	100
Oxalobacteraceae	Massilia	bacterium NR179	98.72
Cystobacterineae	Cystobacteraceae	Anaeromyxobacter	97.21
Bradyrhizobiaceae	Afipia	uncultured alpha proteobacterium	100
Sphingomonadaceae	Novosphingobium	uncultured alpha proteobacterium	99.81
unclassified Rhizobiales	uncultured bacterium		96.37
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. NARs1	99.8
Oxalobacteraceae	Massilia	Massilia aurea	99.64
unclassified Rhizobiales	uncultured bacterium		98.09
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Oxalobacteraceae	Massilia	uncultured Oxalobacteraceae bacterium	99.09
Methylobacteriaceae	Methylobacterium	Methylobacterium aerolata	99.81
Enterobacteriaceae	unclassified Enterobacteriaceae	uncultured bacterium	100
Methylobacteriaceae	Methylobacterium	Methylobacterium aerolata	99.81
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Fusobacteriaceae	Leptotrichia	Leptotrichia sp. oral clone HE052	99.24
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	99.81
Flexibacteraceae	Hymenobacter	uncultured bacterium	97.97
Flexibacteraceae	Hymenobacter	uncultured bacterium	97.97
Flexibacteraceae	Hymenobacter	uncultured bacterium	97.97
Burkholderiaceae	Burkholderia	uncultured eubacterium WD296	98
Oxalobacteraceae	Massilia	Janthinobacterium sp. A1-	99.64

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Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Burkholderiaceae	Burkholderia	uncultured eubacterium WD296	98
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	100
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	100
Enterobacteriaceae	Enterobacter	uncultured gamma proteobacterium	99.45
Enterobacteriaceae	unclassified Enterobacteriaceae	uncultured bacterium	99.09
Enterobacteriaceae	Enterobacter	uncultured soil bacterium	99.27
Enterobacteriaceae	Enterobacter	uncultured soil bacterium	99.27
Enterobacteriaceae	Enterobacter	Enterobacter kobei	98.49
Enterobacteriaceae	Pantoea	uncultured bacterium	99.82
Enterobacteriaceae	Enterobacter	Enterobacter kobei	99.14
Enterobacteriaceae	Erwinia	Erwinia sp. CMG3059	97.64
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured beta proteobacterium	97.09
Enterobacteriaceae	Enterobacter	Enterobacter kobei	98.91
Oxalobacteraceae	Massilia	uncultured bacterium	95.22
Comamonadaceae	Acidovorax	uncultured bacterium	96.9
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. Nj-63	99.27
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea ananatis	99.82
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	100
Enterobacteriaceae	Pantoea	uncultured bacterium	98.18
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	98.12
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	99.55
Sphingomonadaceae	Sphingomonas	Sphingomonas sp. PA210	99.24
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	98.13
Enterobacteriaceae	unclassified Enterobacteriaceae	uncultured Pantoea sp.	99.08
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	98.6
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	99.16
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. 52AD24	99.64
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea sp. BD 502	96.26
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea stewartii	99.8
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. 52AD24	100
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	99.09
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	99.27
Enterobacteriaceae	Pantoea	uncultured bacterium	97.96
Enterobacteriaceae	unclassified Enterobacteriaceae	uncultured bacterium	92.01
Enterobacteriaceae	Erwinia	rape rhizosphere bacterium tsb085	98.06
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	99.64
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	99.18
Enterobacteriaceae	Enterobacter	Enterobacter hormaechei	99.72
Pseudomonadaceae	Pseudomonas	Pseudomonas syringae	97.39
Oxalobacteraceae	unclassified Oxalobacteraceae	Massilia timonae	97.1
Incertae sedis 5	Aquabacterium	uncultured bacterium	100

Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured beta proteobacterium	99.45
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea sp. 092305	99.45
unclassified Pseudomonadales	Moraxella sp. L70		100
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured beta proteobacterium	100
Corynebacteriaceae	Corynebacterium	Corynebacterium accolens	99.63
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. Nj-63	100
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured beta proteobacterium	99.64
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. Nj-63	100
Corynebacteriaceae	Corynebacterium	Corynebacterium accolens	98.69
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured beta proteobacterium	99.82
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. Nj-63	100
Pseudomonadaceae	unclassified Pseudomonadaceae	Pseudomonas graminis	99.77
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured beta proteobacterium	94.71
Bacillaceae	Bacillus	uncultured bacterium	98.01
Oxalobacteraceae	Massilia	uncultured bacterium	97.45
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured beta proteobacterium	98.91
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	100
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	98.91
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.09
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured beta proteobacterium	99.45
uncultured chlorophyte			90.6
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.27
uncultured chlorophyte			90.6
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.27
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
Sphingomonadaceae	Sphingomonas	uncultured bacterium	99.81
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	99.45
uncultured bacterium			90.36
Pseudomonadaceae	Pseudomonas	unidentified bacterium	94.97
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	98.91
Oxalobacteraceae	Massilia	Janthinobacterium sp. WSH04-01	98.91
Sphingomonadaceae	Sphingomonas	uncultured bacterium	100
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Methylobacteriaceae	Methylobacterium	Methylobacterium aerolata	99.81
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	99.43
Sphingomonadaceae	Sphingomonas	Sphingomonas oligophenolica	99.05
unclassified Rhizobiales	uncultured bacterium		98.09

Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea stewartii	99.45
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured beta proteobacterium	98.65
Methylobacteriaceae	Methylobacterium	Methylobacterium komagatae	99.25
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea agglomerans	99.27
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. 52AD24	99.79
Methylobacteriaceae	Methylobacterium	Methylobacterium sp. OSB1	100
Oxalobacteraceae	unclassified Oxalobacteraceae	uncultured beta proteobacterium	99.64
Pseudomonadaceae	Pseudomonas	Pseudomonas sp. 52AD24	99.27
Enterobacteriaceae	unclassified Enterobacteriaceae	Pantoea sp. 092305	99.45
Bacillaceae	Bacillus	uncultured bacterium	96.92

Appendix 7. Pathogenic Species Associated with Genera Documented in the Phyllosphere.

Only the **genera** of the following list were documented our study of the phyllosphere. We used a 550 bp fragment of a highly conserved gene. More definitive diagnostic methods would be necessary to identify a specific pathovar.

Alistipes finegoldii has been isolated from children with acute appendicitis and also from perirectal and brain abscess tissue. It is reported to have caused bacteremia in post operative patients(Fenner et al., 2007).

<http://www.cdc.gov/eid/content/13/8/1260.htm>

Acinetobacter baumannii (Krcmery and Kalavsky, 2007)

Bartonella spp. are vector-borne bacteria associated with numerous emerging infections in humans and animals. *Bartonella quintana*, *B. henselae*, *B. elizabethae*, and *B. vinsonii* subsp. *Berkhoffii* have been associated with cases of endocarditis.

<http://www.cdc.gov/ncidod/eid/vol8no2/01-0206.htm>

Burkholderia spp. are known to cause infections in immunocompromised persons and in cystic fibrosis (CF) patients. *Burkholderia* comprises more than 30 species, including the *Burkholderia cepacia* complex, *B. mallei*, and *B. pseudomallei*. The *B. cepacia* complex is a group of microorganisms composed of at least nine closely related genomovars – all causing infections, *B. cepacia* has also been reported to cause nosocomial infections in non-CF patients (Petrucca et al., 2004). CDC Emerging Infectious disease Vol. 10, No. 11 November 2004. *Burkholderia*

pseudomallei causes an infectious disease known as Melioidosis, which can present as acute localized infections, pulmonary infections and acute bloodstream infections.

http://www.cdc.gov/ncidod/dbmd/diseaseinfo/melioidosis_g.htm

Chryseobacterium meningosepticum, formerly known as *Flavobacterium meningosepticum* and CDC II-a, is a widespread environmental organism. *C. meningosepticum* causes meningitis in premature and newborn infants and pneumonia, endocarditis, bacteremia, and meningitis in immunocompromised adults.

<http://www.cdc.gov/ncidod/eid/vol6no5/chiu.htm>

Clostridium difficile is a bacterium that causes diarrhea and other intestinal conditions such as colitis and sepsis. ***Clostridium sordellii*** is a toxin-forming anaerobic bacteria that has been reported to cause fatal cases of toxic shock syndrome after medical abortion (McGregor et al., 1989; Sinave et al., 2002).

http://www.cdc.gov/ncidod/dhqp/id_Cdiff.html

Corynebacterium species (nondiphtheriae corynebacteria) are considered important emerging multiresistant nosocomial pathogens in the growing population of patients with immunocompromised disease.

Enterobacter sakazakii has been associated with contaminated powdered milk formula products for infants, but other environmental sources of contamination are suspected but still undocumented. *E. sakazakii* causes infections of the bloodstream and central nervous system, causing seizures; brain abscess; hydrocephalus;

developmental delay; and death in as many as 40%–80% of infants infected (Bowen and Braden, 2006a)

(Maurin et al., 2007). <http://www.cdc.gov/ncidod/eid/vol9no10/03-0218.htm>

Enterococcus faecium and faecalis are two of the most prevalent infection causing species in this genus. Enterococci are normal inhabitants of the gastrointestinal tract of humans and animals, but have become important pathogens recently with increased occurrence of antibiotic resistant strains of primarily *E. faecium* (Willems et al., 2005). <http://www.cdc.gov/ncidod/eid/vol5no3/wegener.htm>

Haemophilus influenzae type b (Hib) is the leading cause of invasive bacterial disease among children in the United States. Before vaccines were introduced, many children who developed invasive Hib disease died by the age of five with meningitis. <http://www.cdc.gov/ncidod/EID/vol12no06/05-1451.htm>

Legionella pneumophila is the causal agent of Legionellosis. The disease has two distinct forms: Legionnaires' disease - the more severe form characterized by infection including pneumonia and Pontiac fever – a milder illness (CDC, 2005). http://www.cdc.gov/ncidod/dbmd/diseaseinfo/legionellosis_g.htm

Moraxella catarrhalis causes acute, localized infections such as otitis media, sinusitis, and bronchopneumonia as well as more serious systemic diseases including endocarditis and meningitis. It is also reported to cause lower respiratory tract infections in elderly patients with chronic pulmonary diseases. <http://www.cdc.gov/std/Gonorrhea/lab/Mcat.htm>

Mycobacterium abscessus is distantly related to the *Mycobacterium spp.* that cause tuberculosis and leprosy. It can cause infections of the skin and the soft tissues. It has also been associated with lung infection in persons with chronic lung diseases.

http://www.cdc.gov/ncidod/dhqp/id_Mabscessus_faq.html

Pseudomonas aeruginosa is an increasingly prevalent opportunistic human pathogen associated with nosocomial infections. *P. aeruginosa* is responsible for 16% of nosocomial pneumonia cases, 12% of hospital-acquired urinary tract infections, 8% of surgical wound infections, and 10% of bloodstream infections.

Immunocompromised patients, such as neutropenic cancer and bone marrow transplant patients, are particularly susceptible to opportunistic infections (Van Delden and Iglewski, 1998). <http://www.cdc.gov/ncidod/eid/vol4no4/vandelden.htm>

Schineria larvae is reported to induce bacteremia in humans. The bacterium is associated with fly larvae, and in one human case, it is speculated that bacteremia originated from maggots that had infected a patient's wounds. Human cases of myiasis are less common than animal cases, animal myiasis is responsible for major economic losses to the livestock industry worldwide.

<http://www.cdc.gov/eid/content/13/4/657.htm>

Serratia marcescens has been reported to cause sepsis, blood stream infections and bacteremia in cardiovascular surgery patients.

<http://www2a.cdc.gov/HAN/ArchiveSys/ViewMsgV.asp?AlertNum=00224>

Staphylococcus aureus is one of the most problematic *Staphylococci*. It lives normally on skin and in human nasal passages but can cause serious problems by means of invasion or toxin production. It has been implicated in cases of toxic shock syndrome and septicemia. Methicillin-resistance *Staphylococcus aureus* (MRSA) has become a deadly and serious human pathogen usually associated with hospital settings. http://www.cdc.gov/ncidod/dhqp/ar_mrsa.html

Stenotrophomonas (formerly *Pseudomonas* and *Xanthomonas*) *maltophilia* is a widespread environmental microorganism that is an emerging nosocomial pathogen associated with opportunistic infections in patients with cystic fibrosis, cancer, and HIV. CDC.

Ralstonia pickettii has been associated with nosocomial outbreaks. Other *Ralstonia* species have been associated with the respiratory secretions of Cystic Fibrosis patients. Difficulty in accurately identifying *Ralstonia* species has hindered a full understanding of their clinical implications (Coenye et al., 2002).

<http://www.cdc.gov/ncidod/eid/vol8no7/01-0472.htm>

Xanthomonas, *Stenotrophomonas* (formerly *Pseudomonas* and *Xanthomonas*) *maltophilia* is a common environmental microorganism that has become an important opportunistic pathogen associated with nosocomial infections.

<http://www.cdc.gov/ncidod/eid/vol8no9/01>

Appendix 8. Actual Numbers of Sequences Observed in Each Taxonomic Class for
Organic and Conventional Soil Libraries

Class	Conventional	Organic
Clostridiales	0	1
Cyanobacteria	0	1
Chlamydiae	0	2
Anaerolineae	0	2
Bacillaceae	0	2
Nitrospira	0	1
Planctomycetacia	0	2
Deinococci	0	1
Thermomicrobia	0	1
Unknown	0	1
Archaeoglobi	0	1
Dictyoglomi	0	1
Thermodesulfobacteria	0	1
Methanobacteria	1	1
Gemmatimonadetes	1	1
Aquificae	2	1
Thermococci	2	3
Thermoplasmata	2	4
Verrucomicrobiae	3	5
Chloroflexi	4	0
Deltaproteobacteria	5	5
Gammaproteobacteria	5	4
Bacteroidetes	5	4
Clostridia	7	6
Sphingobacteria	8	14
Alphaproteobacteria	9	22
Methanopyri	9	6
Betaproteobacteria	10	9
Acidobacteria	16	44
Flavobacteria	17	19
Methanococci	20	8
Actinobacteria	25	49

Halobacteria	68	36
Thermoprotei	161	204

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