EFFECTS OF A CONSORTIA PROBIOTIC INOCULANT ON SOIL MICROORGANISMS AND IMPACTS ON AUTOINDUCERS EMPLOYED IN QUORUM SENSING

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

EFFECTS OF A CONSORTIA PROBIOTIC INOCULANT ON SOIL MICROORGANISMS AND IMPACTS ON AUTONIDUCERS EMPLOYED IN QUORUM SENSING

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candidate for the degree of Master of Science

and hereby certify that, in their opinion, it is worthy of acceptance.

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DEDICATION

To my dad and grandfather.

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Dziękuję.

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LIST OF ABBREVIATIONS

ACN – Acetonitrile

AMP - Antimicrobial oligopeptide

AHL - N-(3-oxododecanoyl)-homoserine lactone

AHLEc - N-(3-oxohexanoyl)-L-homoserine lactone

AI-2 - 3A-methyl-5,6-dihydro-furo[2,3-D][1,3,2] dioxaborole-2,2,6,6A-tetrol; Autoinducer-2

BC – Biological control

BHL - N-(butryl)-homoserine lactone (BHL)

CE – Competitive exclusion

CP - Consortia probiotics

CS – Cultivated soil under minimum-till soybean-corn rotation

DGGE - Denaturizing gradient gel electrophoresis

DI – Deionized

E – Species evenness

FAME - Fatty acid methyl ester

FDA – Fluorescein diacetate

GC – Gas chromatograph

GC/MS - Gas chromatography/mass spectrometry

GIT – Gastrointestinal tract

H'- Shannon's diversity index

ISC – Intact soil core

MEOH – Methanol

NAHL – N-acyl homoserine lactone

OHL - N-(3-oxooctanoyl)-L homoserine lactone

PBP - SCD ProBio Balance Plus™

PCR - Polymerase chain reaction

PGPR – Plant growth promoting rhizobacteria

PLFA - Phospholipid fatty acid

QS – Quorum sensing

RG – Soil restored native grassland

S – Species richness

SCD – SCD Probiotics, LLC 1627 Main St #700, Kansas City, MO 64108

SOM – Soil organic matter

TPF - 1,3,5-triphenylformazan

UPGMA - Unweighted pair group method with mathematical averages

ABSTRACT

Disease control in plant agriculture is largely achieved through application of various antimicrobials. Manipulation of indigenous soil microbial communities received attention as it promises improved pathogen control and enhanced plant outputs. Another promising tool in disease control is disrupting quorum sensing (QS)-controlled pathogenicity expression. In this work, efficacies of a consortia probiotic soil amendment (CP) were evaluated: (1) impact on soil microbial diversity; (2) capacity to degrade autoinducers involved in QS in soil-borne pathogens. The first efficacy was assayed in Central Missouri during growing season of 2012. Soils under two management situations were studied: cultivated soil and restored grassland. Samples from cultivated soils were also studied under environmentally controlled conditions. Analysis of soil microbial community diversity was carried out with 16s rDNA PCR DGGE. To evaluate the second efficacy, two autoinducers were incubated in a controlled, triplicated study. Pre- and post-incubation autoinducer concentrations were verified using GC-MS. Under field conditions, CP was found to produce no impact (beneficial or adverse) on soil microbial community diversity. Under controlled conditions, depending on particular treatment, CP had no impact on soil microbial diversity or enhanced it. The CP studied was capable of degrading one of two autoinducers, however the capacity varied and it apparently depended on CP batch or storage conditions. Results suggest that CP studied has benign profile against soil microbial communities and under certain circumstances may enhance community diversity. Furthermore, by disrupting QS autoinducers, CP revealed the potential to control pathogenicity. Future studies are required to fully evaluate CP impacts on agricultural produce.

1. Chapter 1. Literature review

1.1. Disease control in plant agriculture

The struggle to achieve maximum yields in plant agriculture is probably as old as agriculture itself. Plant growth can be compromised by a plethora of biotic and abiotic factors (Figure 1.1). Among the biotic factors, fungal and bacterial pathogens that cause crop disease can considerably reduce yields (Oerke, 2006). Over the course of history, a multitude of unorthodox practices were exercised with the intent to repel the disease varying from religious worship (Hewitt, 1998) to applying inorganic inputs (i.e. sulfur, brine or copper sulfate) (Lamberth, 2004; Morton and Staub, 2008; Russell, 2005; Tweedy, 1981). Microorganisms were not recognized as causative agents of disease until the 19th century. Kelman and Peterson (2002) refer to the works of Pr vost (1807) and De Bary (1853) as the first documents noting the relationship between microorganisms and disease. Currently, the adverse impact of microorganisms is offset primarily through the application of biochemical substances (Oerke and Dehne, 2004), prominently chemicals whose development started in the early to mid-20th century (McManus and Stockwell, 2001; Russell, 2006). These substances are collectively referred to as antimicrobials and are often divided into two categories: (1) antibiotics and (2) fungicides. The former term is attributed to substances that combat bacteria (McManus et al., 2002), the latter describes substances that eradicate fungi (Hewitt, 1998).

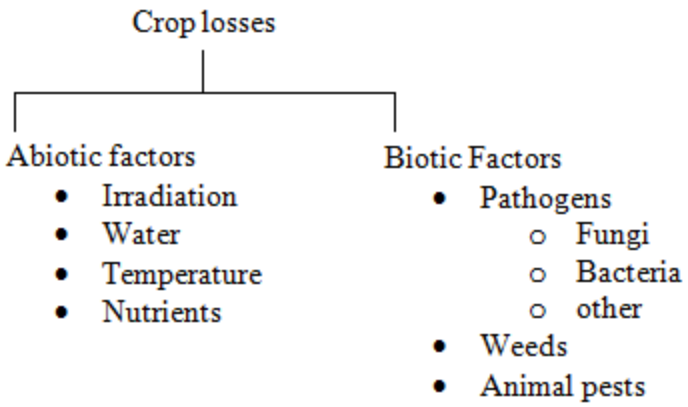


Figure 1.1 Abiotic and biotic factors causing crop losses (after Oerke, 2006)

Fungicides play a key role in securing crop yields. It is estimated that on a global scale, fungal and bacterial pathogens could potentially lead to a 14.9% reduction of overall yields among leading crops; however, thanks to antimicrobials, this reduction is only 9.9% (Oerke and Dehne, 2004). Grube (2011) estimated that the global fungicide market value was $9.216 billion (USD) in the year 2007. Grube et al. (2011) do not specify what share of the global market is comprised by crop protection fungicides; however, the authors report that $1.375 billion (USD) worth of fungicides was purchased in the U.S. in 2007 (15% of global market), 78% of which was consumed by agriculture. Other reports suggest that substantial fungicide consumption in the U.S. is not an isolated case and, on the global scale, agriculture is the predominant consumer of fungicide products (McDougall, 2006; McDougall, 2008; Morton and Staub, 2008). Unlike the market for fungicides, the demand for antibiotics in plant agriculture does not contribute so markedly to overall sales. For instance, in the U.S., it is estimated that the consumption of antibiotics in plant agriculture ranges from 0.1% to 0.5% of the nation-

wide use of antibiotics for all purposes (Levy, 1992; McManus et al., 2002; Vidaver, 2002).

1.2. Disease control in livestock

Just as agricultural producers have struggled to reduce plant disease, animal husbandry has historically struggled with overcoming livestock disease (Swabe, 1999). Modern disease control in food producing animals is thought to have originated in the period of time marked by the invention of penicillin and the outbreak of the Second World War (Gustafson and Bowen, 1997). Shortly after antibiotics were first used for therapeutic purposes in livestock, it was recognized that these products can effectively control disease as well as deliver other benefits. It was found that antibiotics, if administered to animals regularly and in subtherapeutic doses, allowed for improved animal growth rates. The enhanced growth is explained by: (1) suppression of infections; (2) reduced production of growth-inhibiting microbial metabolites; (3) suppressed nutrient use by gut microflora; and (4) amplified nutrient uptake through a thinner intestinal wall found in animals that are fed antibiotics (Gaskins et al., 2002). Nowadays livestock breeders regularly use antibiotics for therapeutic and nontherapeutic purposes. Currently, U.S. livestock operations appear to use the majority of antibiotics that are sold nationwide. According to the Food and Drug Administration as much as 79% of antibiotics sold in the US were used in livestock operations (1.3067×10^7 kg) (FDA, 2009), while 21% (3.316×10^6 kg) were sold for human use (FDA, 2010). In contrary, European Union has banned the non-therapeutic use of antibiotics in animal husbandry (SANCO, 2006).

1.3. Concerns regarding the use of antimicrobials

The widespread use of antimicrobials has been associated with a heated dispute over its hazardous impact on humans and the environment. Concerns regarding the effects of chemical use in general appear to have been raised in public debates since the 1960s. Numerous researchers indicate that the work of Dr. Rachel Carson (1962) was a turning point in presenting chemicals as factual hazards. Although antimicrobials were not the focus of Carson's publication, a more critical approach to chemical usage (including antimicrobials) has become more common. Swann (1969) discussed the widespread use of antibiotics in livestock in terms of its possible contribution to public health hazards due to promoting drug-resistance in human-related pathogens (McDermott et al., 2002). Nearly concurrently, the question of harmful properties of fungicides was raised (Domsch, 1964). Since then, the dispute has been evolving and current concerns are far too diverse and complex to be discussed in depth in this document. Therefore, the discussion will be limited to four primary concerns: (1) human and environmental toxicity, (2) depletion of soil fertility, (3) public health risks due to drug-resistance development in human pathogens, and (4) possible reduction in disease control potential in agriculture.

1.3.1. Human and environmental toxicity of antimicrobials

Antimicrobials are found to be among the chemicals that cause hazards to humans such as skin burns and other injuries (Reigart and Roberts, 1999). Certain antimicrobials are also discussed as causative agents of health conditions in children including brain and

nervous system impacts, childhood cancers and other disorders (Schafer et al., 2012). Other authors find antimicrobials (along with other agrochemicals) as factors that contribute to elevated levels of certain cancers in farmers (Blair and Zahm, 1995). Numerous reports discuss negative environmental consequences that result from antimicrobial applications. Some studies reveal contamination of runoff and groundwater in areas where antimicrobials are applied (markedly in agricultural zones) (Reilly et al., 2012). Others discuss adverse impacts of antimicrobials on aquatic and biphasic species (Belgers et al., 2009; Costanzo et al., 2005; Falfushinska et al., 2008; Gustafsson et al., 2010; Kümmerer, 2009a; Kümmerer, 2009b; Liess and Ohe, 2005; Sch fer et al., 2011; Seeland et al., 2012). There are also reports that focus on the impact of antimicrobials on terrestrial species (Anway et al., 2006; Berkett et al., 1995; Schreck et al., 2008).

1.3.2. Depletion of soil fertility

Soil microbial communities are central to biogeochemical transformations in soils (Aponte et al., 2010; Nannipieri et al., 2003). Therefore the adverse influence that antimicrobials might exert on these communities is seen as a threat to soil fertility (Pal et al., 2005). There is a growing body of evidence that the use of antimicrobials can be linked to alterations of soil microbial communities. Numerous reports are available that discuss the impact of fungicides (Ahemad and Khan, 2012; Bending et al., 2007; Bünemann et al., 2006; Smith et al., 2000; Wang et al., 2009; Yang et al., 2011) and antibiotics on soil environment (Demoling et al., 2009; Hammesfahr et al., 2008; Kong et al., 2006; Thiele-Bruhn and Beck, 2005; Westergaard et al., 2001).

However, the soil microbial community may overcome initial effects of antibiotics and functional traits quickly return to levels prior to exposure to antibiotics (Unger et al., 2013). Similar observations were made by Liu et al. (2012) who found that soil microbial community functions may quickly recover or improve after single antibiotic application to the soil, however it is unclear whether similar effects would be observed after prolonged antibiotic application to soil.

1.3.3. Public health risks due to drug-resistance development in human pathogens

Antimicrobials exert pressure on pathogenic microorganisms and might enhance the development of antibiotic resistance in these organisms (Levy, 1992). Once resistant pathogens emerge, a pronounced risk of deleterious infections in humans occurs. Especially in the case of antibiotics, this risk raises major concerns for public health. Administering antibiotics (Table 1.1) in livestock is found to greatly aggravate these concerns. In particular, the common use of subtherapeutic doses of antibiotics as growth promoters in livestock feed is considered one of main factors enhancing the development of antibiotic resistance in pathogenic bacteria.

There are considerable difficulties associated with providing the exact data on use antibiotic growth promoters in the US (GAO, 2011), however various estimates can be consulted. Reports can be found indicating that as little as 17% of all antibiotics are used as growth promoters (Animal Health Institute, 2000). Others suggest 80% (Harrison and Lederberg, 1998), or even more than 90% (Mellon et al., 2001) to be the actual share

comprised by antibiotic growth promoters. These substances in feed are highly relevant to the development of antibiotic resistance in bacteria. As much as 30% to 90% of AGPs ingested can be excreted in an unchanged, bioactive form (Alcock et al., 1999; Elmund et al., 1971; Feinman and Matheson, 1978). These compounds are likely to be discharged into the environment (e.g., during manure application to cropland) and might migrate through diverse pathways (Figure 1.2). In livestock operations and in the environment, antibiotics facilitate drug-resistance development in pathogens and in other microbial groups which can transmit genes that code for antibiotic resistance to an even larger array of pathogens (Bezoen et al., 1999). A dangerous phenomenon associated with these situations is that multitudinous channels for transfer of drug-resistant bacteria to humans emerge (Figure.1.3). It is also worth mentioning that antibiotics, if released into environment, might impair the characteristics of soil microbial communities and therefore add to the aforementioned issue of degrading soil fertility (Sarmah et al., 2006; Thiele-Bruhn, 2003).

Table 1.1 Types of antibiotics use in food animals (after McEwen and Fedorka-Cray, 2002).

Type of antibiotic use	Purpose	Route or vehicle of administration	Administration to individuals or groups	Diseased animals
Therapeutic	Therapy	Injection, feed, water	Individual or group	Diseased individuals, may include animals that are not diseased or subclinical ones
Metaphylactic	Disease prophylaxis, therapy	Injection, feed, water	Group	Some
Prophylactic	Disease prevention	Feed	Group	Non evident, although some animals might be subclinical
Subtherapeutic	Growth promotion	Feed	Group	None
	Feed efficiency	Feed	Group	None
	Disease prophylaxis	Feed	Group	None

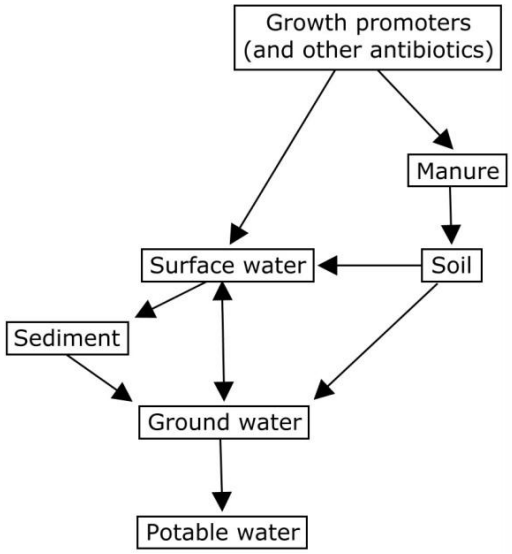


Figure 1.2 Distribution of antibiotics in the environment upon release from livestock operations; after Kümmerer (2003).

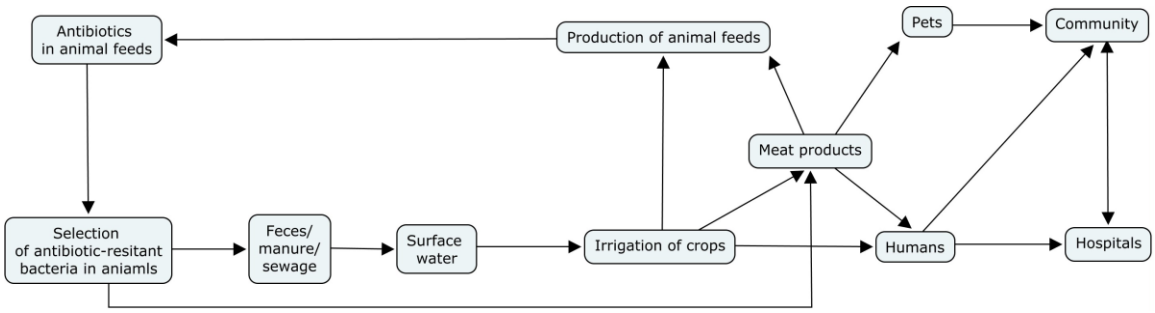


Figure 1.3 Routes by which resistant bacteria can migrate through the environment as well as pools from which infections can be transmitted to humans (after Khachatourians, 1998).

1.3.4. Possible reduction of disease control potential in agriculture

In the light of key contributions of fungicides to production in plant agriculture, pathogen resistance appears as critical concern because of possible reduction in the safeguarding potential provided by antimicrobials. This reduction might likely compromise agricultural outputs. In 2007, approximately 150 fungicidal compounds were present on market and several still in the development phase. However, it is usually the case that not more than four active compounds provide effective treatments for a particular crop fungal disease, therefore the development of resistance to even one compound might heavily compromise management options available for defense against pathogens (Brent and Hollomon, 2007; Knight et al., 1997).

Resistance development to antibiotics is clearly observed in plant pathogens (Vidaver, 2002). However, antibiotics only minimally contribute to crop protection and other means of crop protection seem to be capable of providing effective alternatives,

hence the phenomenon of antibiotic resistance does not seem to be a major threat to productivity in plant agriculture (McManus et al., 2002).

With respect to antibiotics in livestock, there are indications that relevant pathogens become increasingly resistant to antibiotics and there is a possibility that difficulties with maintaining good livestock health and high productivity will emerge (Catry et al., 2003). However, the realization of such a possibility has not been observed (Aarestrup, 2005; Mathers et al., 2011).

1.3.5. Public perception

The use of antimicrobials in food production is associated with strong reactions from the general public in numerous countries worldwide (Duffy et al., 2005; GAO, 2011; Goetz, 2011; Gullino and Kuijpers, 1994; McCauley, 2012; Ragsdale and Sisler, 1994). In developed countries, there is pronounced public desire for produce that promotes health (Siró et al., 2008) and high environmental quality (Sundrum, 2001). This desire motivates policies and stimulates customers' choice of food and other products. In those countries, the general public associates the use of antimicrobials with less healthy or harmful food products. Customers there often refuse to purchase food products produced with the use of antimicrobials. Public demand for what is perceived as more healthy produce and meats in those countries has also served as the driving force behind policies that put stringent regulations on antimicrobials some of which entirely ban the use of certain compounds (Willer et al., 2008).

1.4. Future perspective for disease control

Significant efforts from science, industry and government will be required to ensure that disease control is achievable in general (Freire-Moran et al., 2011; Knight et al., 1997; Sonderholm, 2009). Currently, research on fungicides is motivated by the desire to meet: (1) public demand for crop protection agents with low use rates and minimal toxicity to humans and wildlife, (2) the challenge posed by microorganisms that have been poorly controlled with products currently available on the market, and (3) the need for novel modes of antimicrobial action so that antimicrobial-resistant pathogens can be controlled (Brent and Hollomon, 2007)

With respect to antibiotics, it is likely that increasingly stringent control measures on the use of antibiotics will be implemented to maintain high efficacy of this class of compounds (Aarestrup, 2005; Whittier et al., 2012). The rate of introduction of novel antibiotics has been exceptionally low (Freire-Moran et al., 2011), and it is desirable that the microbial exposure to antibiotics be as low as possible so that the rate of resistance development in pathogens may be controlled.

In general, it appears that antimicrobials will continue to play a key role in ensuring consistent levels of agricultural productivity (Casewell et al., 2003; Russell, 2005). However, their severe non-target impacts on the planet's health create a pronounced need to increase dependence on alternative means of disease control (Montesinos, 2003).

1.5. Alternative means of disease control

According to Verschuere et al. (2000), Ojiambo and Schrem (2006), Pal and McSpadden Gardener (2006), multiple non-antimicrobial-based disease control measures are currently being practiced or studied. Many of the new measure rely on activities of living microorganisms to suppress pathogen growth. The use of microorganisms for disease control is known as Biological Control (BC) (although BC may involve other strategies as well). Biological control strategies are adopted both in plant agriculture and in animal husbandry, and the principles that underlie them are similar. Pathogen control resulting from the activity of living microorganisms is a highly complex phenomenon that emerges from a plethora of biological interactions. These interactions can occur directly between (1) pathogens and BC microorganisms and in (2) BC microorganisms interacting with the crop or livestock host. In the first case, interactions may involve antagonism and competition. In the second case, the reaction of host organisms to BC can bring results such as defense induction against pathogens. Moreover, organisms used in BC can synthesize substances that promote a better condition in the host and, therefore, contribute to higher resistance against pathogens. Furthermore, BC can exert its benefits indirectly by (1) causing desirable changes in the ecology of host-associated microbial communities and by (2) balancing microbial communities in the ambient environment thus contributing to better environment quality. It is also very likely that more than one type of interaction is involved at any given time.

The interplay of interactions as well as the inherent complexity of any particular interaction makes it very challenging to categorize biological processes that underlie BC efficacy. Often, BCs are categorized based on (1) the type of microorganism that is

employed or (2) by interactions that are found to dominate efficacy modes of the product employed. In plant agriculture, these categories are: (1) competitive exclusion (CE) and saprophytic antagonists, (2) plant growth promoting rhizobacteria (PGPR), (3) saprophytic organisms, and (4) bacteriophages.

In livestock, BC is largely accomplished by the use of probiotics. According to one definition, probiotics are living microorganisms that when administered in sufficient amount with feed can produce desirable health effects in their host through balancing intestinal microflora (Fuller, 1990). Probiotics are found to be (1) promoters of good health in an animal's gastrointestinal tract (GIT) and (2) factors that improve the quality of an animal's environment. Furthermore, BC in animal production might use products and strategies that are similar to those found in plant agriculture, namely: (1) bacteriophages and (2) competitive exclusion. A brief outline of strategies and products is provided below.

1.5.1. Competitive exclusion

The phenomenon of antagonism between microbial pathogenic and non-pathogenic strains and its relation to disease in agriculture has been widely discussed and CE has been coined to name this type of antagonism. The principle for CE has been spelled out by Gause (1934) who stated that organisms whose niches fully overlap cannot exist at the same time. According to (Duclezau and Raibaud, 1979), CE appears as a hindered establishment of a microbial population in an ecological niche, and that

hindrance is caused by another population that had already claimed the niche (Gabriel et al., 2006).

These tendencies have been utilized to control bacterial disease in plant agriculture and in animal husbandry. In some plant-agriculture related cases, non-virulent strains of otherwise pathogenic organisms were administered to plants and were found to populate plants and subsequently precluded plant infestation with pathogenic strains (Frey et al., 1994). Elsewhere, non-pathogenic strains are reported to contribute to disease control by eliciting a plant defensive response (Feng et al., 2012).

Similar results were achieved in livestock, where the introduction of a non-pathogenic strain of a pathogenic species in swine reduced disease symptoms associated with the presence of the strain's pathogenic counterpart (Songer et al., 2007). In poultry production, it was found that the infestation of the GIT caused by certain pathogens in young birds can be precluded if the GIT is inoculated with microbial preparations from the GIT of older, pathogen-free individuals (Schneitz, 2005).

1.5.2. Plant-growth promoting rhizobacteria

The rhizosphere is a region of soil that harbors numerous microorganisms and some of these organisms are known to boost plant growth. The latter are termed plant growth-promoting rhizobacteria (PGPR) and numerous members of PGPR have been extracted, cultured and applied in plant agriculture to support the control of certain diseases (Ji et al., 2006).

There are several modes of action that have been found to drive the beneficial impact of PGPR. A large body of scientific work exists on the induction of systemic resistance in plants (Zehnder et al., 2001). Researchers also find PGPR-associated benefits to stem from: (1) the synthesis of organic compounds that further result in improved plant nutrition or pathogen suppression and (2) by excluding pathogens via CE-like mechanisms (van Loon, 2007; Vessey, 2003). Currently, numerous commercially available formulations of PGPR are being offered and utilized in agriculture (Nakkeeran et al., 2006).

1.5.3. Saprophytes

In plant agricultural settings, some saprophytic organisms can control disease and it appears that underlying biological processes are similar to those found in CE as well as to ones that drive beneficial impacts of PGPR (Baker, 1987). It was found that plants amended with certain saprophytic fungi developed fewer fungal diseases, and this observation was attributed to the saprophytic fungus being able to preclude subsequent pathogen infestation (Chand-Goyal and Spotts, 1997; Köhl et al., 1998). Similar results were found using fungal and bacterial saprophytes to control fungal disease (Beasley et al., 2001). Certain saprophytes (prominently bacteria) are known to synthesize antibiotics and other substances that contribute to the disease control properties of these organisms (Pal and McSpadden Gardener, 2006).

1.5.4. Bacteriophages

Bacteriophages are viruses that attack bacteria for subsequent proliferation (Carlton, 1999). Since their discovery in early 20th century, bacteriophages have been successfully used for the control of bacterial diseases in plant agriculture (Jones et al., 2007) as well as in animal husbandry (Kropinski, 2006). Although the use and research on bacteriophages in disease control was abandoned as soon as successes with the first antibiotics were reported, bacteriophages are being now reevaluated and they seem to promise development of a whole new arsenal for disease control (Barrow, 2001). There are certain key advantages that characterize bacteriophages. Firstly, bacteriophages are capable of rapid proliferation in the presence of pathogens and are swiftly degraded upon pathogens' removal. Hence, they are highly potent in establishing deleterious infections in target organisms, and they appear to remain viable in the environment for periods that are too short to produce undesired, non-target effects. Secondly, bacteriophages are highly discriminatory, therefore they are benign to non-target organisms – most importantly humans, animals and plants (Jones et al., 2007).

1.5.5. Probiotics

Probiotics,, prominently lactobacilli, streptococci and bifidobacteria are able to populate animal GITs and their presence results in enhanced resistance to enteric diseases, improved feed utilization and better growth rates in host animals (Collins et al., 2009). These benefits are found to result from diverse traits: (1) metabolism or co-metabolism of organic nutrients, (2) stimulation of digestion-related metabolic pathways

in a host, (3) alteration of toxin receptors and attenuation of toxin-induced health conditions, and (4) preclusion of GIT infestation with pathogenic microorganisms (Gupta and Garg, 2009).

In addition to being used in livestock, probiotics are applied at a considerable scale in aquacultures and they are found to deliver similar benefits as in animal agriculture (Irianto and Austin, 2002). Interestingly, the research on probiotics in aquaculture has resulted in a definition that surpasses the one from animal husbandry. In aquaculture it is recognized that apart from delivering GIT-associated benefits, probiotics improve the wellbeing of aquatic organisms by enhancing the quality of their environment (Verschuere et al., 2000). Numerous authors suggest that the use of probiotics promises an alternative to the use of antibiotics as growth promoters (Irianto and Austin, 2002; Kritas and Morrison, 2005; Patterson and Burkholder, 2003; Reid and Friendship, 2002).

1.6. Biological control and the need for more research

Alternative means of disease control abound; however, they frequently deliver inconsistent results when applied in non-laboratory, real world settings. The inherent variability of results is largely due to complexity of biological interactions that drive the efficacy of aforementioned alternative tools of disease control. Further research is necessary to improve understanding of such complexity and to optimize BC processes to achieve more reliable results. Despite challenges associated with their application, alternative means of disease control have been consistently referred to as an attractive

and viable response to the needs of modern agriculture (Alamri et al., 2012; Garcia-Gutierrez et al., 2013; McSpadden Gardener and Fravel, 2002; Stirling and Stirling, 1997).

1.7. Studies on microbial interactions and premises for novel disease control methods

Biological processes that underlie pathogen control represent a field requiring additional, in depth investigations and it is likely that new research will result in discoveries of novel methods to combat disease. According to Miller and Bassler (2001) and Hentzer and Givskov (2003), one particular field of study seems especially promising, the one where it was found that microbial populations can express different genes at the population level in response to varying population density. This phenomenon has been termed quorum sensing (QS). Population density-dependent gene expression is regulated by biochemicals (referred to as autoinducers) that microorganisms release to the environment which subsequently bind to receptors in other microorganisms within the population. Population growth is followed by an increase in an autoinducer to a threshold value (signaling that the microbial population is quorate). Subsequently, altered gene expression occurs in the entire microbial population. This allows microbial communities to orchestrate their actions and it is argued that this strategy allows for more effective competition for ecological niches and resources. In many pathogens, the expression of genes that code for virulence is controlled by QS, and researchers see the disruption of

QS as possible strategy to control disease (Defoirdt et al., 2012; Hurley et al., 2012; Stacy et al., 2012).

1.8. Microbial-based inoculants and SCD Probiotics

The soil microbial community is critically important in nutrient cycling, decomposition, and plant growth, therefore numerous efforts have been directed at manipulating indigenous microbial communities by inoculating with selected microorganisms to supplement or alter these processes. Several microorganisms including plant growth promoting rhizobacteria and plant disease suppressive bacteria and fungi have been evaluated for plant growth stimulation and biocontrol properties. These types of microorganisms constitute products known as microbial-based inoculants that are currently on the market. A class of microbial-based inoculants include those prepared with probiotic microorganisms that, in general, may be applied as single-species inoculants; as blends of multiple microorganisms individually cultured (Fuller, 1990; Gupta and Garg, 2009; Kesarcodi-Watson et al., 2008; Reid and Friendship, 2002); or as complex cultures where numerous microbial species are allowed to grow together (consortia probiotics or CP). Consortia probiotics have been applied in agricultural settings in countries worldwide and benefits of CP application translated as yield increase, enhanced yield quality, pathogen control or improved breakdown of organic residues in composting (Heo et al., 2008; Javaid, 2006; Javaid and Bajwa, 2011; Kremer et al., 2000).

SCD Probiotics is an example of a microbial-based inoculant that falls into the CP category and consists of following species: *Bacillus subtilis, Bifidobacterium animalis, Bifidobacterium bifidum, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus bulgaricus, Lactobacillus fermentum, Lactobacillus plantarum, Lactococcus lactis, Rhodopseudomonas palustris, Rhodopseudomonas sphaeroides, Saccharomyces cerevisiae*, and *Streptococcus thermophilus.* Cultures are manufactured in a proprietary fermentation process where microorganisms are cultured collectively for extended periods of time in an aqueous medium with sugarcane molasses used as carbon source. Upon Quality Assurance/Quality Control release, the product is decanted into appropriate containers and prepared for distribution in bulk or in retail packages.

Two types of CP manufactured by SCD are Mother Cultures and Secondary Products (ready to use products). In present work both types of cultures were studied. Mother Cultures are starter cultures used in manufacturing process of Secondary Products. Depending on manufacturing process, Secondary Products gain unique properties distinct from Mother Cultures and are used for variety of applications. For instance, Secondary Products are often applied soil amendments or can be used in foliage sprays.

Consortia probiotics manufactured by SCD reportedly produced certain benefits in agriculture (SCD, 2000; SCD, 2002a; SCD, 2002b; SCD, 2013) and other systems (SCD, 2010a; SCD, 2010b). However, peer reviewed studies on the efficacy of these CPs as well as detailed information on associated modes action associated are scarce.

1.9. Quorum sensing

Numerous bacterial species have been found to monitor their populations and to alternate gene expression upon reaching a threshold population; this phenomenon has been termed Quorum Sensing (QS). In general, the regulation of gene expression is mediated by diffusible molecules (referred to as autoinducers). However, processes, signaling molecules and receptors involved in QS can vary greatly between bacterial genera. Especially marked differences occur between Gram negative and Gram positive species. In Gram negative bacteria, QS is largely found to be mediated by N-acyl homoserine lactones (NAHL), although other signaling systems that use different molecules are present as well. In Gram positive bacteria, QS depends on oligopeptides as autoinducers. Also, a signaling mechanism is known that operates in Gram negative and Gram positive species (the system that uses Autoinducer-2) (Miller and Bassler, 2001). Key concepts associated with QS in bacteria are reviewed below.

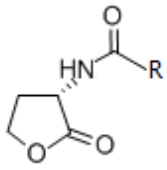
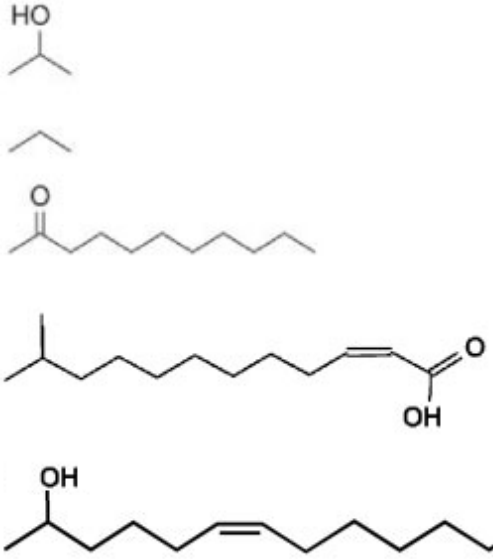
1.9.1. Quorum sensing in Gram negative species: N-Acyl homoserine lactones-mediated systems

The research on QS advanced over four decades ago through studies on bioluminescence in bacteria performed by Nealson and colleagues (1970) and additional studies by Nealson and Hastings (1979). These studies revealed that *Vibrio fischeri* and *Vibrio harveyi* (Gram negative marine species) activate genes that respond for bioluminescence only when cultured populations are sufficiently high. It was also found that transferring cell-free supernatants from highly populated cultures to low-population

cultures resulted in the latter producing light as if populations were sufficiently high (Nealson and Hastings, 1979). This finding suggested that a soluble factor is involved with inducing bioluminescence in the bacteria (Nealson and Hastings, 1979) Eberhard et al. (1981) later found that the factor is an NAHL. Several NAHLs and species whose gene expression is altered by these NAHLs are presented in Table 1.2.

Later studies revealed that numerous NAHLs mediate gene expression in a variety of Gram negative species (eg. Bainton et al., 1992; Piper et al., 1993; Throup et al., 1995). These findings were groundbreaking in understanding prokaryotic interactions at the molecular level – they indicated that prokaryotes employ intercellular communication signaling systems, which had been thought to be an exclusive trait of eukaryotes (Miller and Bassler, 2001). Quorum Sensing in Gram negative species controls numerous traits some of which are listed in Table 1.3.

Table 1.2 Examples of QS mediated traits in Gram negative species; after Williams (2007)

<table>
<tr><td colspan="2">General structure of NAHL</td></tr>
<tr><td colspan="2"> 
After: Ryan and Dow (2008)</td></tr>
<tr><td>Example R Groups</td><td>Relevant Species</td></tr>
<tr><td>  </td><td>Vibrio harveyi
(Ryan and Dow, 2008)

Pseudomonas aeruginosa
(Ryan and Dow, 2008)

Xanthomonas fastidosa
(Ryan and Dow, 2008)

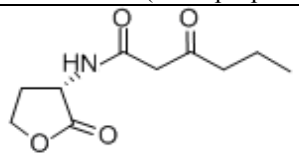
Rhizobium leguminosarum
(Taga and Bassler, 2003)</td></tr>
<tr><td colspan="2">Structure of N-(3-oxopropanoyl)-L-homoserine lactone, NAHL identified by Eberhard et al. (1981)</td></tr>
<tr><td colspan="2"> 
After: http://www.chemicalbook.com/ProductChemicalPropertiesCB7222683_EN.htm</td></tr>
</table>

Table 1.3 Examples of QS mediated traits in Gram negative species after Williams (2007)

Species	QS-mediated trait
Aeromonas hydrophila	• Biofilm formation, exoproteases synthesis, virulence
Aeromonas salmonicida	• Exoproteases synthesis
Agrobacterium tumefaciens	• Plasmid conjugation
Agrobacterium vitis	• Virulence
Burkholderia cenocepacia	• Exoenzymes syntheis, biofilm formation, swarming motility, siderophore synthesis, virulence
B. pseudomallei, B. mallei	• Exoenzyme synthesis, virulence • Virulence
Chromobacterium violaceum	• Synthesis of exoenzymes and violacein; cyanide formation
Erwinia carotovora	• Sythesis of Carbapenem antibiotics and exoenzymes; virulence
E. stewartii	• Exopolysaccharide synthesis
Pseudomonas aeruginosa	• Synthesis of exoenzymes, toxins; protein secretion, biofilm formation, swarming motility, virulence
P. aureofaciens	• Synthesis of phenazine antibiotics and protease; aggregation, root colonization, colony morphological changes
P. chlororaphis	• Phenazine-1-carboxamide synthesis
P. putida	• Biofilm development
P. fluorescens	• Mupirocin synthesis
Rhizobium leguminosarum	• Root nodulation and symbiosis, plasmid transfer, growth inhibition, stationary phase adaptation
Rhodobacter sphaeroides	• Aggregation
Serratia sp. ATCC 39006	• Synthesis of antibiotics, exoenzymes and pigments
S. liquefaciens	• Exoprotease and biosurfactants synthesis, biofilm development
S. marcescens	• Sliding motility, biosurfactants and pigments synthesis
S. proteamaculans	• Exoenzyme synthesis
Sinorhizobium meliloti	• Root nodulation and symbiosis, exopolysaccharide synthesis
Yersinia enterocolitica	• Swimming and swarming motility
Y. pseudotuberculosis	• Motility, aggregation

1.9.2. Quorum sensing in Gram positive species – oligopeptide circuits

Expression of certain traits in numerous Gram positive species are controlled via QS (Table 1.4) and QS circuits are highly similar among species (Figure. 1.4). Autoinducers are post-transcriptionally altered peptides (see Figure. 1.3 for an example) whose secretion is mediated by dedicated exporters. Once in extracellular space at a threshold concentration, peptides activate a two-component system in recipient cells. The system consists of a membrane-bound sensor kinase and response regulator protein. Upon detecting the cognate peptide, the sensor kinase undergoes autophosphorylation and activates cognate response in the regulator protein that activates transcription of the target gene (Kleerebezem et al., 1997).

Interestingly in some species, oligopeptides act not only as autoinducers but they exhibit pronounced antimicrobial properties as well. Furthermore, in species that use antimicrobial oligopeptides (AMPs) as autoinducers, positive feedback loops of AMP secretion are found where AMPs stimulate rapid AMP synthesis in quorate populations (Kleerebezem, 2004).

Table 1.4 Traits whose expression is controlled by oligopeptide-mediated QS.

Microorganism or group	QS controlled trait
Bacillus subtilis	genetic competence (Kleerebezem et al., 1997) and synthesis of antimicrobial peptides (Kleerebezem, 2004)
Streptococus pneumoniae	genetic competence (Kleerebezem et al., 1997)
Staphylococcus aureus	virulence (Kleerebezem et al., 1997)
Lactic acid bacteria	synthesis of antimicrobial peptides (Kuipers et al., 1998)

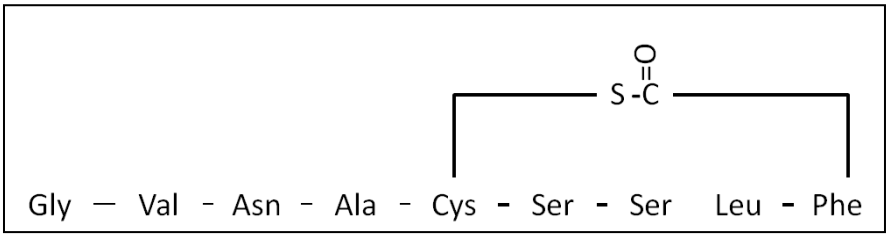


Figure 1.3 Cyclic thiolactone - autoinducer found in *S. aureus* (Zhang and Dong, 2004)

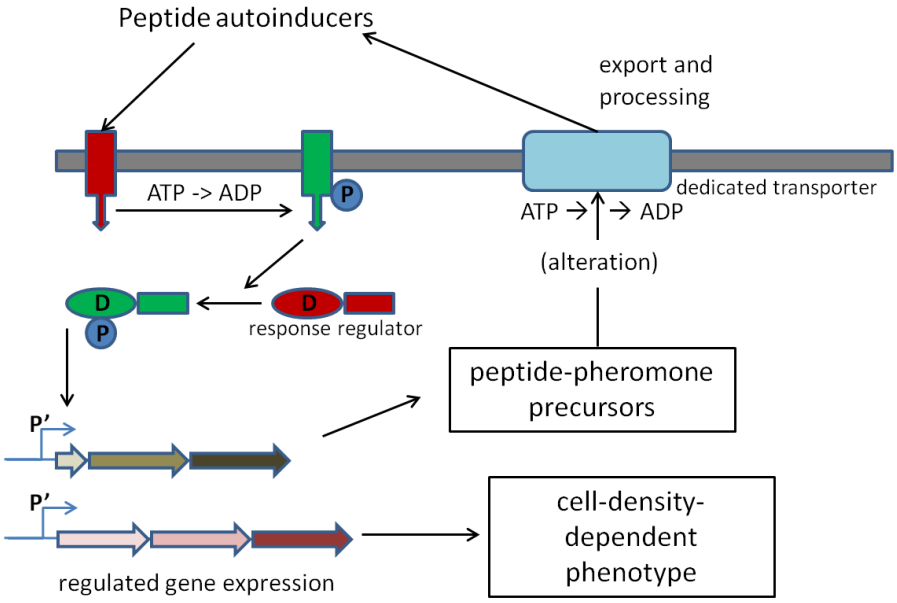


Figure 1.4 Example of a QS circuit from a Gram positive species; after Kleerebezem (1997).

1.9.3. Common signaling systems between Gram negative and Gram positive species

Studies on microbial communication have resulted in identifying another QS system that is found across many bacterial genera (including Gram negative and Gram positive). The system, initially found in *V. harveyi* has been dubbed LuxS/AI-2 system (Swift et al., 2008). LuxS is the signal synthase that synthesizes 3A-methyl-5,6-dihydro-furo[2,3-D][1,3,2] dioxaborole-2,2,6,6A-tetrol, a furanosyl borate diester (Figure. 1.5) also referred to as autoinducer-2 (AI-2) to signify that it is involved in the second signaling system and AI-2 compliments the NAHL based signaling system in *V. harveyi*. Later studies have shown that LuxS genes are conserved in many bacterial species and

that AI-2 mediated QS controls numerous traits in bacteria (Table 1.5) (Swift et al., 2008).

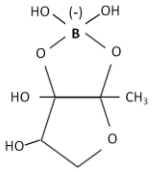


Figure 1.5 Autoinducer-2 from *V. harveyi*; after from Taga and Bassler (2003)

Table 1.5 Traits whose expression is under control of AI-2-mediated QS (after Taga and Bassler, 2003)

Microorganism	AI-2 controlled trait
V. Harveyi	bioluminescence
Escherichia coli	virulence
Vibrio cholerae	
Clostridium perfingens	
Streptococcus pyogenes	
Poprphyromonas gingivalis	iron acquisition
Actinobacillus actinomycetemcomitans	
Photorhabdus luminescens	antibiotic production
Campylobacter jejuni	motility
P. gingivalis and *Streptococcus gordonii*	mixed-species biofilm formation

Autoinducer-2 may act on various types of receptors in bacteria. For example, in *V.harveyi* AI-2 binds to a periplasmic receptor that then initiates a signal cascade and alters gene expression in recipient cells (Taga and Bassler, 2003). In contrast, in *Salmonella typhimurum* AI-2 is first transported into the cell by a transporter protein (Taga and Bassler, 2003). An example of LuxS/AI-2 system with periplasmic receptor is depicted in Figure 2.4 (Taga and Bassler, 2003).

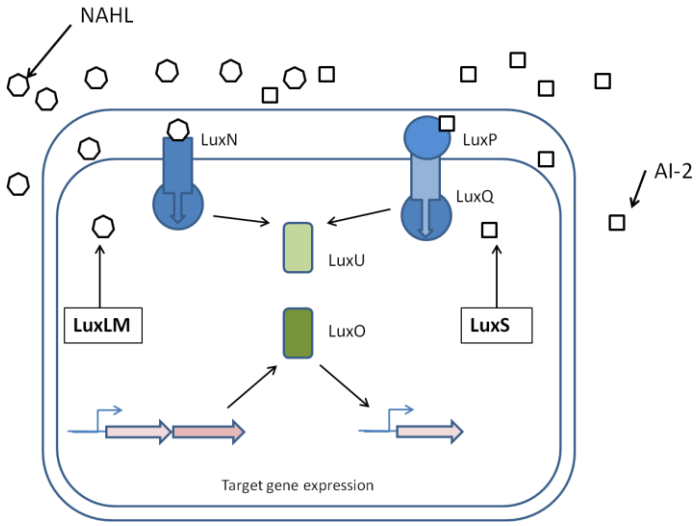


Figure 1.6 Example LuxS/AI-2 system. LuxP, LuxQ, LuxU and LuxO are proteins that are involved in signal transduction [after Taga and Bassler (2003)].

1.9.4. Molecular details of QS circuits in Gram negative species

In many Gram negative species, QS circuits that use NAHLs are highly similar to those studied in *V. fischeri.* These systems are referred to as LuxI/LuxR (Figure. 1.7); LuxI and LuxR are proteins found inside bacterial cells. The former is autoinducer synthase enzyme while the latter acts to (1) bind the autoinducer and (2) activate transcription of respective operons (*luxICDABE* in case of *V.fischeri*). Autoinducers freely diffuse through cell membranes to extracellular space and then permeate to other cells. Upon cell entry, autoinducers bind with LuxR proteins and trigger target gene expression. Variations and more complex versions of such systems can be found in many species (Miller and Bassler, 2001). For the purpose of this study, two such systems will

be explained in more detail: (1) a virulence system found in *Pseudomonas aeruginosa* and (2) a virulence and antibiotic synthesis system present in *Erwinia carotovora.*

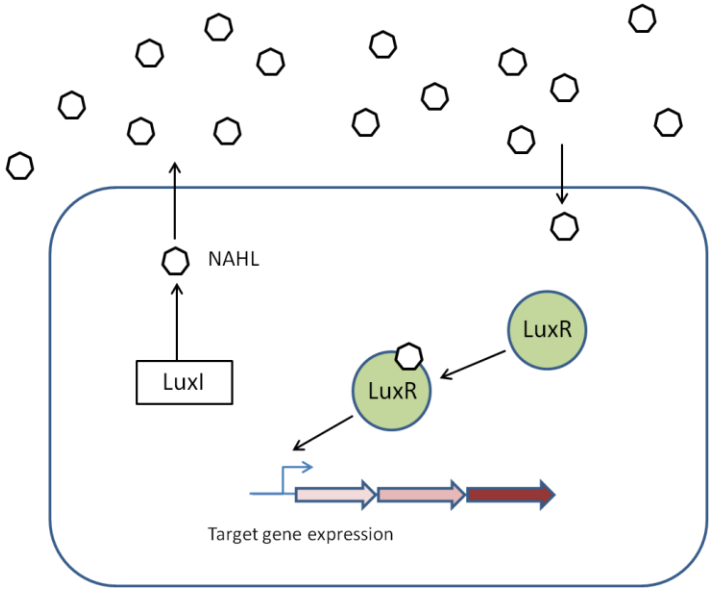


Figure 1.7 Canonical quorum-sensing circuit found in most Gram-negative bacteria; after Xavier and Bassler (2003)

1.9.4.1. *P. aeruginosa* virulence system

There are over 300 genes found to be controlled by QS in *P. aeruginosa,* and QS in this species is among the most thoroughly studied. For the purpose of this review, the discussion will be limited to only two examples of QS in this species. In *P. aeruginosa*, synthesis of numerous virulence factors responsible for tissue destruction in host organisms is controlled by LasI/LasR and RhlI/RhlR systems (Fig. 1.8). LasI and RhlI are autoinducer synthases that synthesize N-(3-oxododecanoyl)-homoserine lactone (AHL) and N-(butryl)-homoserine lactone (BHL), respectively. Complexes of LasR and RhlR are cognate receptors of AHL and BHL respectively. Besides controlling the expression

of virulence, these systems are interweaved in a feedback loop that allows for orchestrated gene expression (Venturi, 2006).

More specifically, in quroate populations, LasI/LasR activates the synthesis of elastase as well as activates biofilm formation. Furthermore, this system activates genes that code for RhlI and RhlR proteins, which sets the second QS system in place. Upon sufficient BHL concentration, the autoinducer is bound by RhlR and second gene expression cascade is turned on. Virulence factors whose expression is controlled by RhlI/RhlR include cytotoxic lectin and hemolysins. Furthermore RhlI/RhlR is involved in pyocyanin antibiotic synthesis. It is noteworthy that the interplay between these two systems extends further, i.e., it was found that AHL may prevent the binding of BHL to RhlR. This likely ensures that a proper order of gene expression exists; namely that RhlR controlled genes are expressed in consequence of successful LasI/LasR system activity (Venturi, 2006).

Additionally, in *P. aeruginosa* another signaling system exists, one that uses 2-heptyl-3-hydroxy-4-quinolone. This non-NAHL compound creates another loop between aforementioned QS systems in *P. aeruginosa.* Specifically the compound is found to facilitate the expression of RhlR genes and is suspected to do so only after LasI/LasR-mediated cascade is successfully established (Venturi, 2006). Vast amounts of regulatory systems that allow for step-by-step gene expression make it apparent that an appropriate order for virulence factors synthesis is vital to *P. aeruginosa;* however, the advantages of the cascade of gene expression remain unclear (Venturi, 2006).

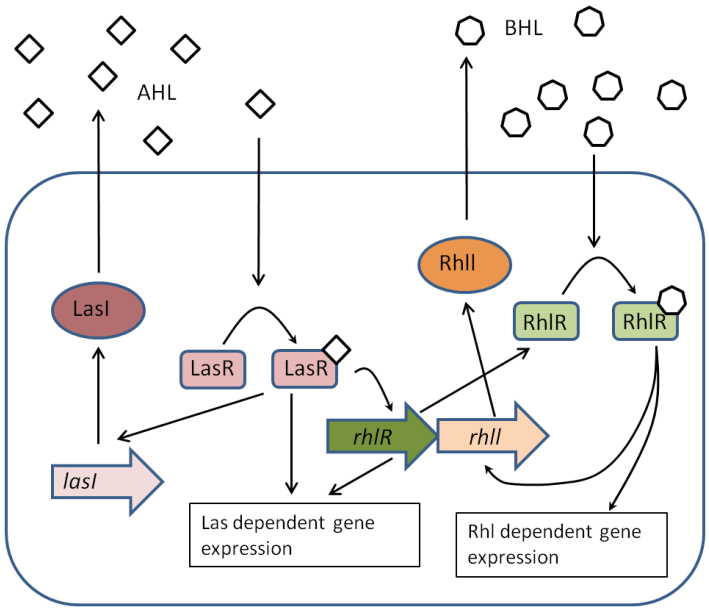


Figure 1.8 The *Pseudomonas aeruginosa* LasI/LasR-RhlI/RhlR quorum sensing system (2-heptyl-3-hydroxy-4-quinolone loop is not included); after Waters and Bassler (2005)

1.9.4.2. *E. carotovora* virulence system

A vast array of pathogenic phenotypes in *E. carotovora* is known to be controlled by QS. In orchestrating their phenotypes, these species utilize circuits that are highly similar to LuxI/LuxR systems of *V. fischeri*; however, different strains are characterized with different synthases and receptors of NAHL-signals. There are two autoinducers that are found to mediate QS in *E. carotvora*: N-(3-oxohexanoyl)-L-homoserine lactone (AHLEc), and/or N-(3-oxooctanoyl)-L homoserine lactone (OHL). Based on the NAHLs employed in QS, Erwiniae are divided into Class I and Class II Erwiniae. Organisms in Class I synthesize primarily OHL and some quantities of AHLEc, while those in Class II produce primarily AHLEc and no appreciable amounts of OHL are known to be synthesized. The summary of NAHL QS systems in *E. carotovora* strains can be found in

Table 1.6 Furthermore it has been found that AI-2 controls expression of virulence factors in some stains of *E. carototvora*, however precise role of AI-2 dependent systems in Erwiniae may vary (Barnard and Salmond, 2007).

Table 1.6 Example QS systems in *E. carotovora* (after Barnard and Salmond, 2003).

Erwinia carotovora strain	Major QS signal(s)	NAHL synthase (LuxI homologue)	NAHL receptor (LuxR homologue)	QS regulated trait
Class I Erwiniae				
EC153	OHL and	AhlI	ExpR	Production of exoenzymes
SCC3193	AHLEc	ExpI	ExpR1, ExpR2	Virulence, production of exoenzymes
Class II Erwiniae				
ATCC39048	3-oxo-C6-HSL	CarI	CarR, ExpR, VirR	Carbapenem antibiotic production
SCRI193		ExpI	ExpR, VirR,	virulence, production of exoenzymes
71		AhlI	ExpR1, ExpR2	Virulence, production of exoenzymes
				Virulence, production of exoenzymes

1.9.5. Bacterial benefits associated with QS

In general, it is argued that QS provides diverse advantages to bacteria that possess this system (Chhabra et al., 2005; Swift et al., 2008). In Erwiniae, it has been suggested that QS allows for (1) frugal allocation of resources and energy that are involved in the formation of virulence factors or antibiotics, and (2) improved inhibitory activity of secreted antibiotics (Barnard and Salmond, 2007). Specifically, Barnard and Salmond (2007) discuss that the expression of related traits prior to securing the quorate

population could: (1) insufficiently damage host tissue and thwart microbial development by not allowing for the satisfactory release of nutrients; (2) prematurely alarm host tissue and trigger immune response that might remove microorganisms; and (3) compromise otherwise effective competition for a niche because of synthesizing insufficient amounts of antibiotics. In pseudomonads, benefits of employing QS to control population phenotypes are discussed in similar terms (Darch et al., 2012).

Numerous studies on QS conclude that the exact functionality of QS circuits and benefits that they bring to particular populations may vary greatly in natural settings from what had been described under controlled conditions. Furthermore, questions are raised whether QS mechanisms exist solely as signaling circuits (that are used by bacteria to benefit each other) and whether some QS systems are in fact utilized by bacteria to exploit others (Keller and Surette, 2006).

1.9.6. QS disruption – a promise for disease control

Currently a fundamental change in the approach to disease control is being observed. For a long time, disease control depended on the use of antimicrobials; however, with findings on QS, a new class of tools for combating disease is expected to appear, i.e., antipathogenic drugs (Hentzer and Givskov, 2003).

While antimicrobials debilitate the growth, or eradicate microorganisms, antipathogenic drugs prevent microorganisms from acting as pathogens without directly harming them. As such, they seem unlikely to promote resistance in target species. This novel mode of action would make antipathogenic drugs especially promising in the light

of pathogen resistance to known antimicrobial drugs. The following general strategies are proposed for QS disruption: (1) inhibition of signal generation; (2) inactivation of signal molecules; and (3) inhibition of signal reception - receptor competition/blocking (Hentzer and Givskov, 2003). And additional means of achieving disease control via QS disruption-like strategy is to increase autoinducer concentrations (Williams, 2007). Exploration of these strategies has resulted in successful treatment methods, many of which have been patented. A comprehensive review of QS disruption methods and patents is provided by Pan and Ren (2009). This review narrows its scope to only a brief discussion of three aspects of QS disruption: (1) inactivation of signaling molecules; (2) inhibition of signal reception; and (3) signal amplification.

1.9.6.1. Inactivation of signaling molecules

Inactivation of signal molecules can be achieved by chemical or enzymatic degradation; furthermore, molecules can be metabolized. N-acyl-homoserine lactones can be deactivated simply by increasing pH. For NAHLs, at greater than pH 7, lactonolysis occurs (a process of opening of the lactone ring). Interestingly, one of the first reactions in plants infected with *E. carotovora* is to increase the pH in the lesion associated with infection (Rasmussen and Givskov, 2006).

A large share of research on QS interference has been conducted on enzymes that are capable of degrading NAHLs. Two classes have been identified: (1) NAHL lactonases which catalyze lactone ring opening; and (2) NAHL acylases, which catalyze amide bond breakage and release homoserine and the corresponding fatty acid. Initially, a NAHL degrading enzyme (NAHL lactonase) was found in *Bacillus* species isolates

from soil. Subsequently, NAHL-degrading enzymes (lactonases and acylases) have been characterized in numerous bacteria and it is now apparent that these enzymes are widespread among prokaryotes (Dong and Zhang, 2005).

Interestingly, NAHL-degrading enzymes have been also found in eukaryotic organisms including humans. Specifically it was found that epithelial cells in the human airway secrete NAHL lactonase that was able to degrade autoinducers from *P. aeruginosa* (Dong and Zhang, 2005).

1.9.6.2. Inhibition of signal reception

It has been shown that alteration in NAHL structures may render them potent blockers of receptors. Several synthetic NAHL mimics were shown to produce such results. Modification of NAHL structures that are conducive to receptor blocking include: (1) substitution of C-3 atom in acyl side chain with sulfur; and (2) addition of aryl residues at the end of the acyl chain. Other modifications of NAHL molecules were attempted, however, with varying results.

Receptor blockers involved in QS disruption are not limited to only NAHL mimics. For example, 4-nitro-pyridine-N-oxide can act as receptor blocker of LasR and RhlR receptors. Furthermore a vast array of naturally occurring compounds have been found to deliver similar effects. These molecules include penicillic acid and patulin being secondary metabolites of *Penicillium radicola*. Furthermore high efficiency in receptor blocking has been shown for brominated furanones from a macroalga, *Delisea pulchra* (Rasmussen and Givskov, 2006).

1.9.6.3. Signal amplification

An alternative approach to controlling pathogenicity could be increasing the concentration of autoinducers. In particular, it has been argued that external inputs of respective NAHL could attenuate the infectiousness of *E. carotovora*. It has been hypothesized that due to concentration increases in autoinducers, the bacterium might synthesize virulence factors prematurely, thus alarming the plant host and inducing an overwhelming immune response (Williams, 2007).

Such an approach has been pursued and demonstrated using transgenic tobacco plants (*Nicotiana tabacum* cv. Samsun) genetically engineered to synthesize *E. carotovora* NAHLs, which were more resistant to infection caused by *E. carotovora*. It has been hypothesized that enhanced resistance resulted from the effects of premature synthesis of plant cell wall degrading enzymes by *E. carotovora*. More specifically, it was concluded that products of cell wall degradation may have alarmed plant cells and trigger an immune response that was effective against non-quorate populations of *E. carotovora*. Similar effects were achieved when NAHLs were added directly to *E. carotovora* infection sites (Mae et al., 2001). A similar study was conducted on potato plants (*Solanum tuberosum* cv. Desirée). However, in this case, genetically engineered plants were more susceptible to *E. carotovora* than the control, non-modified plants.

1.9.6.4. Methods of studying NAHLs

Studies on NAHLs utilize two general methods of NAHL identification: (1) the use of reporter organisms (Chu et al., 2011; McClean et al., 1997; Molina et al., 2003;

Shaw et al., 1997); and (2) detection through the use of molecular methods (Cataldi et al., 2008; Cataldi et al., 2004; Shaw et al., 1997).

There are multiple studies that have assessed NAHL degradation that frequently utilize synthetic NAHLs and subsequently adopted the NAHL identification methods to assess the degradation (Byers et al., 2002; Dong et al., 2004; Medina-Martínez et al., 2007; Ramos et al., 2012; Wang and Leadbetter, 2005). Recently, Gas Chromatography/Mass Spectrometry (GC/MS) analysis was used in profiling NAHLs and GC/MS appears as another useful tool for direct assessment of treatment effects on NAHLs (Cataldi et al., 2004).

1.10. Microorganisms and agroecosystem functioning

It has been said that the activities of microorganisms are central to the very existence of the biosphere (Balser et al., 2010; Pace, 1997; Whitman et al., 1998). Existence of the biosphere is sustained by inputs of solar energy (Makarieva et al., 2008) and the recycling of matter (Field et al., 1998). Microorganisms play a pivotal role in the cycle of matter—they release nutrients from inorganic as well as organic sources and synthesize the biomass that becomes available for higher trophic levels and virtually all nutrients on the planet pass through microbial biomass before uptake by organisms in higher trophic webs. Furthermore, numerous essential components of ecosystems are the results of microbial metabolism. For example the presence of oxygen in the early Earth's atmosphere, which set the premise for furthering the evolution of life on Earth, was the result of microbial metabolism (Giri et al., 2005). Another example that is much more

closely related to recent, everyday life is the production of soil organic matter (SOM). Microorganisms partially utilize organic residues that are deposited onto the soil thereby releasing nutrients in the residue for cycling within the biosphere. At the same time, microbes alter the physical and chemical properties of organic residues subsequently resulting in SOM formation (Baldock, 2007; Guggenberger, 2005). Organic matter in the form of SOM contributes immensely to soil quality, which is related to broad life processes (i. e., soil quality is linked to agricultural outputs and the availability of food and other products) (Bot and Benites, 2005). Furthermore, microorganisms can remove or neutralize numerous toxic substances (Jeanne and George, 2005; Johnson and Hallberg, 2005; Valls and de Lorenzo, 2002).

On the other hand, microorganisms may be viewed as the menace of the biosphere (Adler et al., 2011; Dean et al., 2012; Mansfield et al., 2012). They possess the unmatched ability of breaking down organic matter (Torsvik, 2007), and some microorganisms have the ability to attack and degrade substances that are associated with living tissues. When this ability is activated, deleterious changes may occur in a living organism affected by a microorganism. This is particularly true in plant agriculture where major crop losses are attributed to the activity of pathogenic microorganisms (Oerke, 2006).

Although recent studies have greatly advanced knowledge about the biology of microorganisms and their role in the biosphere, microorganisms still remain a poorly understood biological group (Rajendhran and Gunasekaran, 2011; Torsvik, 2007). Our advanced knowledge has been achieved largely due to the adaptation of molecular tools (Oros-Sichler et al., 2007). It is now appreciated that microorganisms are likely the most

genetically diverse of all known life forms (Schloss and Handelsman, 2004; Torsvik et al., 2002) and the links between microbial communities and ecosystem functions they provide are the focus of numerous studies (Nannipieri et al., 2003; Schimel et al., 2007; Van Der Heijden et al., 2008; Zak et al., 2003). This review aims at presenting the key concepts associated with: (1) ecosystem functions that microorganisms provide with respect to cycling of C and the nutrients, N and P; (2) the relation between the diversity of microbial communities and the stability of ecosystem functions; (3) soil microbial diversity and disease suppression in agriculture, and (4) current analytical methods employed in studies on microbial diversity.

1.10.1. The cycling of C and nutrients N and P

The ways in which microorganisms are involved in cycles of chemical elements are enormously complex and diverse. For the purpose of this review, the discussion of microbial functions in terms of microbial impact on the cycle of elements is narrowed down to key concepts associated with the (1) carbon cycle, (2) nitrogen cycle, and (3) phosphorus cycle.

1.10.1.1. Carbon cycle

Major global reservoirs of carbon include: (1) sedimentary rocks which are estimated to contain more than 60×10^6 Pg of the element (Falkowski et al., 2000); (2) the ocean, which comprises 38,000 Pg of C; (3) fossil fuels (coal, oil and gas) with 5000 Pg of C (Lal, 2004); (4) soil inorganic carbon (carbonates in soil), which accounts for as

much as 1,738Pg of carbon to the depth of 1m; (4) soil organic carbon (SOC); this pool may contain as much as 1,555 Pg of C to the depth of 1m (Eswaran et al., 1995.); (5) the atmosphere with 760 Pg of C; (6) biota (living organisms) which comprise 560 Pg of C (Lal, 2004). It seems that the importance of microorganisms in the C cycle largely stems from their ability to alter the formation of carbon bonds in organic molecules, especially those that are released from the biota (decaying plant and animal tissue and/or detritus and excreta). This is particularly evident in case of SOM. Soil organic matter emerges chiefly as a result of the decomposing activity of microorganisms (Lal, 2009; Schnitzer, 1991). Microorganisms attack the organic matter and as a result, the vast majority of its constituents are metabolized and released as CO_2. The remaining, more resistant substance undergoes chemical alterations (i.e., formation of hydroxyl carboxyl, and ketone groups followed by polymerization). As a result of these alterations, organic molecules may take on new properties such as the enhanced ability to attach to other substances via hydrogen bonds (Lichtfouse et al., 1998; Sanderman and Amundson, 2003). An example is the transformation of lignin and other biomolecules which results in the formation of humic substances that contribute beneficially to the functioning of the soil (i.e., humic substances improve the cation exchange capacity and water holding capacity) (Sanger et al., 1997). Lignin is an important constituent of plant tissue; as much as 30% of all carbon in biomass may exist as lignin (Boerjan et al., 2003). Without microbial metabolism, the beneficial impacts associated with lignin transformation would not be observed. Other organic molecules that are in abundant supply in the biosphere and are attacked by microorganisms for subsequent SOM formation include cellulose, hemicelluloses and chitin (Sanderman and Amundson, 2003). Notably, byproducts of

microbial metabolism of organic C include biologically relevant forms of numerous other elements (key examples associated with nutrients N and P are reviewed below), and conceivably no life could be sustained on the planet without microbial ability to process the organic, carbon-based structures. Figure 1.9 summarizes key concepts associated with C cycle.

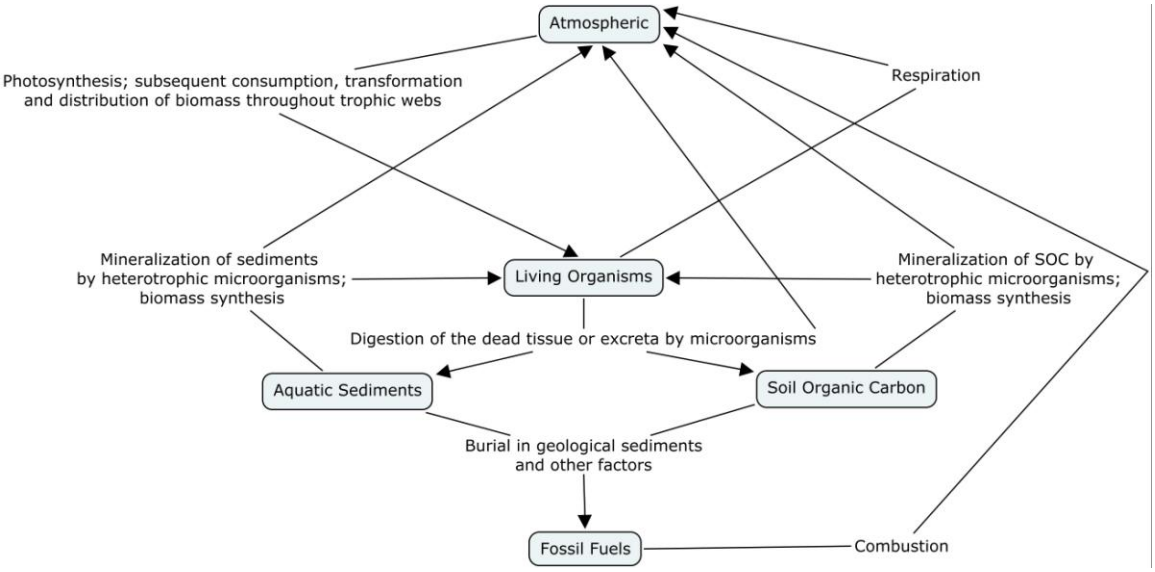


Figure 1.9 Cycle of Carbon; blue boxes indicate major carbon pools (after Eswaran et al., 1995.)

1.10.1.2. Nitrogen cycle

Although N is an abundant element in the biosphere it is largely unavailable to primary producers of trophic chains. Particularly, as much as 78% of the atmosphere near Earth's surface consists of highly stable dinitrogen gas [N_2 (g)] (Leigh, 2004). Other abundant sources of nitrogen (namely SOM and freshly deposited organic residues) do not readily contribute N forms that are available to plants, thus microbial activity is

necessary for the release of plant-available forms of N from these sources. Plants absorb N as: (1) ammonium (Chaillou and Lamaze, 1997), (2) nitrate and nitrite (Darwinkel, 1975), and (3) simple amino acids (Raab et al., 1999). One of the key processes that microorganisms facilitate in the N cycle is ammonification—the release of ammonium (NH_4^+) from organic substrates; numerous, non-specific soil microorganisms are involved in the process (McNeill and Unkovich, 2007). Another process in which microorganisms play a major role is nitrification—a process where NH_4^+ is oxidized to nitrate (NO_3^-) by organisms such as *Nitrosomonas* (Wood, 1990). Additionally, products of N metabolism can be recycled to dinitrogen gas in the process called denitrification, mediated by bacteria such as *Pseudomonas* spp. (Ye et al., 1994).

Furthermore, there are microorganisms capable of fixing dinitrogen gas in the process that is collectively referred to as the Biological Nitrogen Fixation (BNF). Biological nitrogen fixation is a process carried out by diverse bacteria collectively referred to as diazotrophs. The enzymatic machinery possessed by diazotrophic bacteria contains the nitrogenase enzyme complex, which is an assembly of proteins that enable the bacteria to synthesize ammonia using dinitrogen as a substrate. The ammonia may then be used in the synthesis of amino acids (Dilworth and Glenn, 1991). Diazotrophs include free-living bacteria (i.e., *Acetobacter* or *Azospirillum)* (James, 2000) as well as those that form symbiotic relationships with plants. A prominent example of the symbiotic relationship is that between leguminous plants (such as: *Glycine, Acacia, Trifolium*) and various bacteria (i.e., *Azorhizobium, Bradyrhizobium, Sinorhizobium*) (Sprent, 2001).

Approximately 25% of the global budget of biologically available N is due to BNF (40 million tons out of the total 160 million tons) (Galloway et al., 1995). Conceivably, the flux of N provided by BNF was the major tributary to the global N-budget over millions of years. However, its relative contribution has dropped sharply in the last century, largely due to the expansion of industrial N-fixation and the release of N-containing compounds during fossil fuel combustion (Leigh, 2004). A conceptual overview of processes involved in N cycling in soil is provided in Figure 1.10.

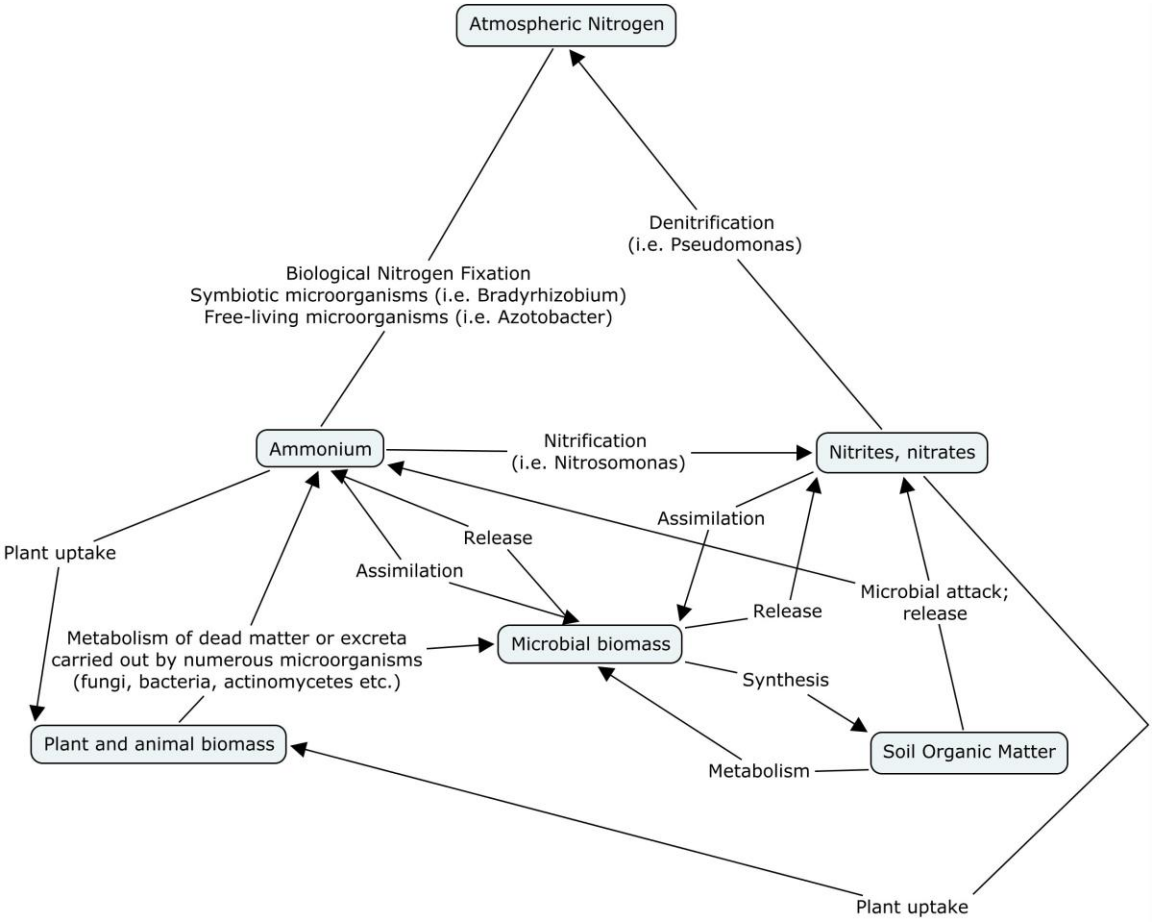


Figure 1.10 A graphic overview of processes involved in N cycle in soil

1.10.1.3. Phosphorus cycle

Unlike other essential elements (C, N and others such as S), P does not form gaseous compounds that are in appreciable supply in the atmosphere. This largely restricts the P cycle within the biosphere and, unless time scales larger than 10^8 are considered, global transfer of the element appears as a one-way route that ends with P immobilization in sediments in water reservoirs (i.e., marine or oceanic). Phosphorus reaches these deposits via various pathways (i.e., transport with runoff from soils in the form of P-bearing secondary minerals) (Smil, 2000). Filippelli (2008) may also be consulted for an interesting review of processes involved in global P cycling. Once buried in sediments, P is virtually lost from the cycle and it remains so until the tectonic uplifts reshape Earth's surface and expose the P-enriched formations to the erosion. The cycle may require as many as 10^8 years to close (Smil, 2000).

Because P is an essential nutrient and needs to be in permanent supply for adequate biological activity, life processes are entirely dependent on transformations within the long-term P cycle that operate on a relatively short time scale (less than one year to several years). These transformations include: (1) immobilization and release of P from minerals and clay lattices in soils and (2) assimilation of P by living organisms and its subsequent release from dead tissue or excreta. These processes allow sequestration of P in the biosphere where microorganisms play a prominent role (Bünemann and Condron, 2007; Smil, 2000).

Phosphorus is an abundant constituent of living tissue (Schlesinger, 1997; Westheimer, 1987). More specifically, it is contained by adenosine phosphates, the universal energy transporters in living cells and as such, P saturates nearly all parts of

living organisms. Furthermore nucleic acids, necessary components of all living organisms, are rich sources of P. Another important organic source of P that is frequently mentioned is phytic acid, a storage form of the nutrient, synthesized by numerous plants.

Upon tissue death, these sources may replenish the supply of available P; however, in order to do so, they must first be subjected to microbial attack and subsequent P release. The form of P that is available for plant uptake is the phosphate ion, and it needs to be cleaved from organic molecules. This role is played by a wide group of phosphatase enzymes synthesized by numerous microorganisms (Bünemann and Condron, 2007). Once liberated from the organic material, P may be assimilated by microorganisms and may not be readily available to plants. However, due to the high turnover rate of the microbial biomass (sometimes ranging from 40 to 160 days depending on environmental conditions and soil management options) (Oehl et al., 2004), the nutrient may be eventually released from microbial biomass to the environment.

In general, microorganisms compete with plants for available phosphates and the competition may result in a decrease in nutrient availability to plants. At the same time it is recognized that the microbial immobilization of P is an important factor in maintaining the long term P supply in soils and several explanations of the phenomenon are provided (Olander and Vitousek, 2004; Parton et al., 2005; Seeling and Zasoski, 1993). More specifically, these explanations start with a rationale that besides phosphate, P in soils exists as (1) P in mineral phase (such as apatite) and (2) P adsorbed to clay minerals (apart from these, P is also found in organic matter) (Bünemann and Condron, 2007). These forms exist in complex equilibria that are affected by factors such as ionic strength or pH of soil solution and are characterized with varying availability to plants (i.e., low

pH may favor adsorption of phosphate to iron and aluminum oxides, while under alkaline conditions, phosphate may precipitate with calcium and form hydroxyapatite; under both circumstances the availability of P to plants decreases). The sequestration of P in microbial tissue may protect excessive amounts of phosphate from leaching or forming strong complexes within the soil matrix that prohibit the P uptake by plants and that can be lost due to erosion (Olander and Vitousek, 2004; Seeling and Zasoski, 1993).

Microorganisms may also greatly support plants in accessing P that is associated with the mineral fraction of soils (Oberson and Joner, 2005). This may be due to microbial synthesis of organic and inorganic acids and/or release of protons to the soil by microbial cells. Local acidification of the soil that follows from these processes modifies the equilibria of P sources in soil and low molecular weight organic acids can enhance P release through ligand promoted dissolution (Goyne et al., 2006). Together, acidification and ligand promoted release may allow for enhanced release of plant available P. Figure 1.11 summarizes key pathways involved in P cycling in soil.

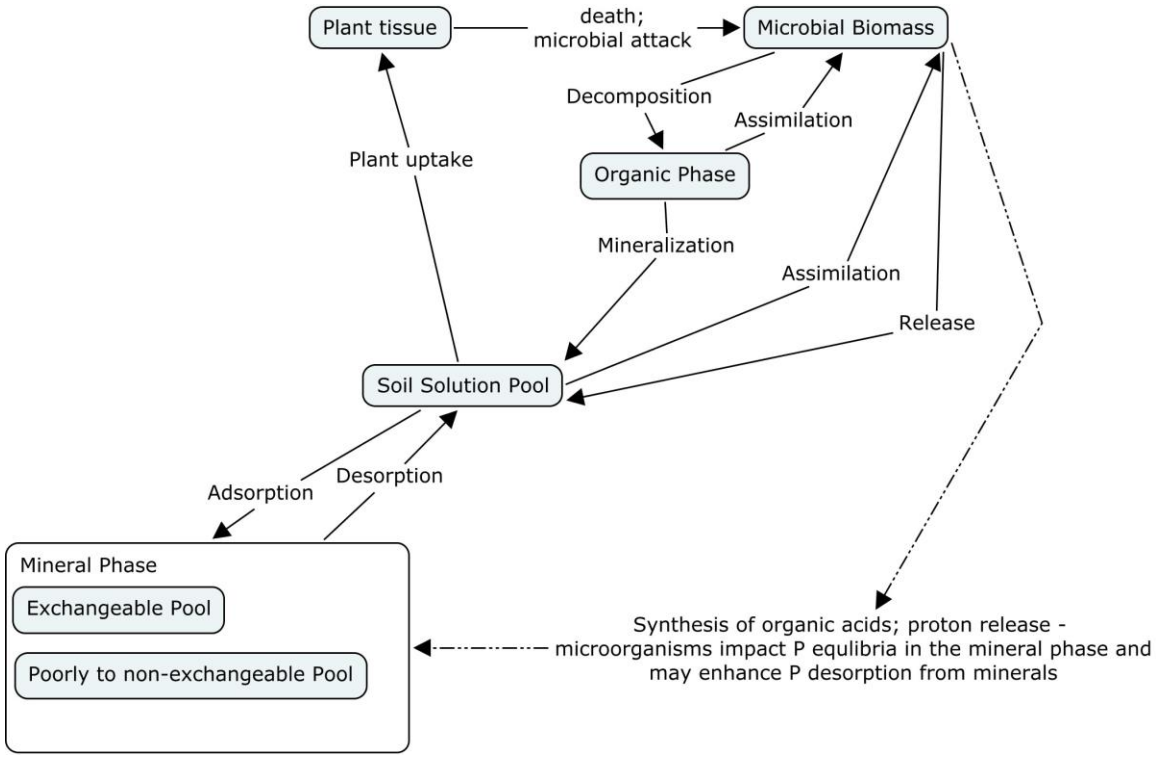


Figure 1.11 The cycle of P in soil, after Bünemann and Condron (2007)

A noteworthy example of microbial involvement in P cycling is the one that exists in mycorrhiza. In mycorrhiza, a mutualistic relationship occurs between plant roots and mycelia formed by certain groups of mycorrhizal fungi (prominently *Glomus*) (Schwarzott et al., 2001). This relationship allows the plant to achieve numerous benefits such as improved penetration of the bulk soil by the mycorrhizal association rather than by exclusive dependence on plant roots and enhanced P uptake due to the synthesis of organic acids and phosphatases by the fungal symbiont (Bolan, 1991).

1.10.2. Soil microbial diversity and functions of agroecosystems

Studies on ecosystems in general (i.e., marine or forest ecosystems) have frequently found that the levels of biological diversity in ecosystems correlates with the stability of ecosystem functioning (Downing et al., 2012; Elmqvist et al., 2003; Loreau et al., 2001). Mechanisms that underlie the ecosystem diversity-ecosystem stability relation are believed to apply to the diversity of soil microbial communities and agroecosytem functioning (Balser et al., 2002; Nannipieri et al., 2003)

There has been a pronounced interest in unraveling the relationship between soil microbial diversity and ecosystem stability. For example, as early as the 1960s, Hairston et al. (1968) studied the stability of an artificial, small-scale ecosystem as a function of the diversity of microorganisms in the ecosystem. Contrary to the wide preference for a diverse ecosystem when looking for ecosystem stability, results of the Hairston et al. (1968) study show that ecosystem stability is higher in a less diverse ecosystem. Authors of the study concluded that some complex, secondary interactions may exist between species and these interactions govern ecosystem stability and that more research is required to fully understand these processes. Examinations of the links between the microbial diversity and ecosystem stability have continued; currently, their focus includes the contributions of microbial diversity to the stability of agroecosytem functions(i.e., stability under abiotic and biotic stresses as well as enhancement of water and nutrient use efficiencies by plants) (Altieri, 1999; Brussaard et al., 2007).

Numerous studies have explored the link between microbial diversity and ecosystem resistance and resilience, which are factors that eventually determine the stability of an ecosystem function (Nannipieri et al., 2003). Resistance is defined as the

ability to withstand perturbations while resilience labels the ecosystem's ability to regain functionalities that were lost due to perturbations (McNaughton, 1994). For example, Griffiths et al. (2000b) studied the stability of soil microbial activity (measured by soil respiration) upon application of an abiotic stress (fumigation) and found that even though the stress caused a decline in microbial diversity it had no impact on the measured microbial function. However, when an additional stress was applied (addition of $CuSO_4$ or heat treatment) the decline in soil respiration was observed in soils whose microbial diversity had been diminished. Also, these soils required a longer period to regain their pre-stress functionality. These findings suggest that the microbial diversity is related positively to the ecosystem's resistance and resilience.

In a follow-up experiment, Griffiths et al. (2001) studied (1) how microbial communities in sterilized soils regain functionality (measured by thymidine and leucine incorporation as well as soil respiration) as a result of the reestablishment of the microbial biodiversity (addition of serially diluted suspensions from a parent soil) and (2) how functionality in these communities responded to stresses (addition of $CuSO_4$ or heat treatment). The Griffiths et al. (2001) study showed no interaction between microbial diversity and the regaining of functions and stability. These experiments indicate that the decline in functions may depend on the type of stress and not necessarily on shifts in microbial diversity (Brussaard et al., 2007).

Over the years, a body of evidence accumulated indicating that soil microbial communities are characterized by functional redundancy, which means that numerous microbial groups may perform similar tasks and that the removal of microbial groups does not readily affect the functioning of the whole community (Nannipieri et al., 2003).

An interesting example of the phenomenon was reported by Yin et al. (2000) who examined the microbial species richness and functional traits of microbial communities (measured by the incorporation of various carbon sources) in soils from and around a tin mine. Yin et al. (2000) found that the functional redundancy of microbial communities increased as a function of decreasing contamination from the mine waste in studied soils (i.e., denuded mine spoil deposits to forest floor). It was concluded that this functional redundancy may be a highly characteristic property of natural microbial communities, and that it may be a good indicator of soil quality.

Contrary findings have been reported as well (Balser et al., 2002). For example, Balser (2000) found that mineralization of N in soil was more affected by changes in microbial communities than by environmental factors (temperature or moisture content). Furthermore, Hunt et al. (1988) and Sugai and Schimel (1993) reported that organic residues (leaf litter) are more readily decomposed in soil in their natural environment than in substitute soils unaccustomed to those residues. This indicates that the diversity of microbial communities does affect the functions they provide. There is clearly considerably more information to be learned about the nuances of relationships between microorganisms and functions they provide in the environment (Torsvik, 2007)

1.10.3. Soil microbial diversity and pathogen suppression

The studies on the impact of soil microbial communities on pathogen suppressiveness encompass a notable research area. Numerous researchers have found that soil microbial diversity and abundance inversely relate to the activity of pathogenic,

microbial species (Irikiin et al., 2006; Matos et al., 2005; Messiha et al., 2009; van Elsas et al., 2012; Van Elsas et al., 2007). However, processes that underlie the pathogen suppressiveness exhibited by some microbial communities still need to be fully described. Currently, researchers simplify the discussion of phenomena associated with pathogen suppressiveness by using to two terms: (1) Specific Suppressiveness and (2) General Suppressiveness. In this review, several key concepts are reviewed that link to Specific and General Suppressiveness.

1.10.3.1. Specific suppressiveness

Over the years, compelling evidence indicates that native soil microbial communities in numerous soils inhibit disease development in plants (i.e., the inhibition of *Fusarium oxysporum* or *Gaeumannomyces graminis*) (Weller et al., 2002). Specific suppressiveness results, at least partially, from individual groups of microorganisms influencing pathogen activity (Weller et al., 2002).

Particularly, in case of suppressiveness to *F. oxysporum*, it was found that microbial species or groups such as *Bacillus* and *Trichoderma* (Sivan and Chet, 1989), *Pseudomonas* (Kloepper et al., 1980; Lemanceau and Alabouvette, 1993; Scher and Baker, 1982), as well as nonpathogenic *F. oxysporum* (Alabouvette, 1986; Larkin and Fravel, 1998; Larkin and Fravel, 1999) are suppressive against pathogenic *F. oxysporum*. Detailed research has been offered to argue that mechanisms such as competition or induced systemic resistance as shown by the suppressive activity of non-pathogenic *F. oxysporum* and *Pseudomonas*, contribute to the overall disease-control effect (Alabouvette, 1990; Duijff et al., 1998; Kloepper et al., 1980; Scher and Baker, 1982).

Gaeumannomyces graminis is the causative agent of take-all disease in wheat and other cereals, and studies have shown that in some soils, microbial groups develop that may cause the decline of the severity of take-all (Weller et al., 2002). A prominent example of a microorganism that contributes to the decline of take-all is *Pseudomonas spp*.; studies have found that *Pseudomonas spp*. produces the antibiotics 2,4-diacetylphloroglucinol (Harrison et al., 1993; Pierson and Weller, 1994; Vincent et al., 1991) or phenazine-1-carboxylic acid (Thomashow and Weller, 1988; Weller and Cook, 1983) that are implicated in the suppression of *G. graminis*.

1.10.3.2. General suppressiveness

General suppressiveness has been described to occur in some soils and, unlike Specific Suppressiveness, it is related to microbial biomass and not the activity of particular microbial groups (Weller et al., 2002). It is reasoned that in soils where high microbial biomass is present, there exists a pronounced competition for nutrients. This results in a state of permanent starvation which inhibits pathogenic microorganisms (Weller et al., 2002). It has long been recognized that the addition of organic materials to soils, which leads to an increase in microbial biomass and activity in soils, has a potential to reduce the prevalence of disease in crops (Litterick et al., 2004; van Bniggen and Termorskuizen, 2003). For example, Lumsden et al. (1983) studied the development of plant pathogens in plants grown in soils amended with composted wastewater sludge and found reductions in symptoms of several pathogens (i.e. *Aphanomyces, Rhizoctonia* or *Fusarium*). Similar results were found by Cotxarrera et al. (2002) who amended soils with composts from wastewater sludge for control of *Fusarium* wilt in tomato. The use of

composts made from other materials (dairy solids or vegetable refuse) as soil amendments have also been reported to suppress the root wilt caused by *Fusarium* (Kannangara et al., 2000).

1.10.3.3. Microbial communities for pathogen suppression – inconsistencies

Pathogen suppressiveness in soil is produced by an interplay between Specific Suppressiveness and General Suppressiveness (Weller et al., 2002). Processes that are involved in suppressiveness (interaction between microbial groups, fluctuations of availability of resources) in any given soil are extremely complex and therefore disease control as provided by soil microbial communities may produce variable results (Brussaard et al., 2007). The inconsistency of results may be particularly evident in the case of strategies that aim at enhancing the microbial component of soils (i.e., application of organic amendments). For example, Bonanomi et al. (2007), after reviewing a major volume of case studies (n= 2423), concluded that the application of organic inputs to soils may produce highly variable results in terms of pathogen control (e.g., instances of pathogen suppression and pathogen activation as well as examples of no impact on pathogens were observed).

Nonetheless, the interest in the maintenance of soil microbial communities for disease control is still greatly pronounced. For example, it was found that the decline in the natural diversity of microbial communities may be conducive to disease development (Bailey and Lazarovits, 2003; Hoitink and Boehm, 1999). Some researchers conclude that strategies that aim at preserving the microbial component of soils help control disease in agriculture (Bailey and Lazarovits, 2003). Studies on the relationship between the

diversity of soil microbial communities and the stability of the functions that they provide encounter considerable difficulties and many of these owe to an impressive abundance of microorganisms in soil (Nannipieri et al., 2003).

1.10.4. Methods in studies of microbial diversity and functions

A recent survey of soil microbial diversity reports that one gram of soil may be inhabited by as many as 2.4×10^4 to 5.4×10^4 of microbial species (Roesch et al., 2007), and it is widely accepted that as many as 10^9 microbial cells may thrive in one gram of soil (Whitman et al., 1998). Although microbiology possesses a vast array of analytical methods (some of which are reviewed below), many of them are ill-suited for studying ecosystems as diverse as soils. This is particularly true in the case of culture-dependent methods traditional to microbiology because likely only about 1% of all microorganisms can be harvested and subsequently studied via culture dependent methods (Kirk et al., 2004; Torsvik et al., 2002).

1.10.4.1. Culture dependent methods and substrate utilization assays

These methods depend on microbial ability to utilize (or proliferate in) a given medium (aqueous, solid) under certain growth conditions (temperature, pH, availability of oxygen, nutrients, etc.). An example method–plate counts, uses environmental samples or their extracts for subsequent transfer to culture plates filled with growth medium where microorganisms are allowed to grow. The amount of cultures that develop in the medium and their appearance allow for easy identification and enumeration (Kirk et al.,

2004). However, this method has severe limitations: (1) it allows for the identification of only those organisms that are capable of rapid growth in the medium (Dix and Webster, 1995); and (2) results may depend on the ability and efficiency of removal of microbial cells from physical niches they occupy in the environment (i.e., biofilms or micropores) (Tabacchioni et al., 2000).

Another way to identify microbial communities is to evaluate microbial ability for utilization of different carbon sources. A particular application of that method is used in BIOLOG microplates. Wells in BIOLOG microplates contain various carbon substrates (i.e., glycogen, D-mannitol, γ -hydroxybutyric acid), and their utilization is indicated by color development (Choi and Dobbs, 1999). The method has frequently been adapted for microbiological studies in characterization of microbial communities (Irikiin et al., 2006; Kirk et al., 2004; Zhang et al., 2013)

1.10.4.2. Fatty acid methyl ester and phospholipid fatty acid analysis

Fatty acid methyl ester analysis (FAME) does not depend on culturing microorganisms and provides the information about the composition of microbial communities based on fatty acids that comprise a relatively constant component of microbial biomass. Certain signature fatty acids found only in certain microbial groups allow for differentiation between groups within microbial communities, and screening for these fatty acids allows researchers to survey the structure and diversity of microbial communities. In FAME analysis, fatty acids are extracted directly from soil and methylated for subsequent analysis by gas chromatography (Ibekwe and Kennedy, 1999).

Similar principles apply to phospholipid fatty acid (PLFA) analysis. Namely, the rationale for the analysis is based on the assumption that PLFAs make up a constant proportion of microbial biomass, and that different microbial groups synthesize different PLFAs as constituents of their biomass. The analysis of PLFAs is carried out by gas chromatography following a direct extraction of PLFAs from soil (Ibekwe and Kennedy, 1998) and has been frequently applied in microbiological studies (Frostegård et al., 2011).

1.10.4.3. Genetic fingerprinting

Recent advances in soil microbiology have been largely attributed to the adaptation of molecular, culture-independent methods of study. A method that has been widely adopted is targeting microbial 16s mRNA gene for polymerase chain reaction (PCR) and resolving of PCR product using denaturizing gradient gel electrophoresis (DGGE) (16s rDNA PCR DGGE) (Ahn et al., 2009; Kirk et al., 2004; Torsvik and Øvreås, 2002).

The gene termed 16s rDNA codes for the RNA component of small ribosomal unit and is a common constituent of prokaryotic genomes. Their gene is comprised of two distinct types of fragments (1) those that are highly conserved among numerous prokaryotes, and (2) those whose sequence is highly variable between taxa. Primers have been devised that target the conserved gene sequence and allow subsequent PCR-based amplification of the sequence. The separation of amplified DNA is then carried out using DDDE which separates DNA based on differences in properties of PCR products. In DGGE, the separation occurs as a result of immobilization of DNA in a polyacrylamide

gel that is saturated with denaturants (formamide and urea). Although widely adopted, this method usually allows for only coarse conclusions to be made regarding the composition of microbial communities (Valášková and Baldrian, 2009).

More specifically, 16s rDNA PCR DGGE involves direct extraction of microbial DNA from soils, which is subsequently amplified via PCR. Aliquots of PCR products are loaded on the polyacrylamide gel and an electric current is applied that forces the movement of native DNA from PCR products through the gel. As native DNA fragments travel in the gel, they encounter an increasing concentration of denaturants (formamide and urea) and once the denaturant concentration is sufficient, the native DNA strands denature and single DNA strands are formed that stop in the gel. As a result a pattern of "bands" form, where each band consists of DNA fragments denatured at a certain position and each band signifies different members of microbial communities that contributed to the DNA comprising the band (Valášková and Baldrian, 2009).

2. Chapter 2: Hypotheses and Objectives

It is likely that in case of consortia probiotic, processes that drive its efficacy are complex. In our work we hypothesized that the efficacy of consortia probiotic stems from its capacity to: (1) enhance microbial diversity in soils and (2) interfere with pathogen quorum sensing by degrading autoinducers mediating infectious traits in pathogens.

Objective 1

To examine the diversity of soil microbial communities in two settings where CP was applied: (1) in the field and (2) under controlled conditions in a growth chamber

Objective 2

To evaluate the impact of CP on transformation or inactivation of the autoinducers AHL and AHLEc.

2.1. Objective 1, the Study 1 and Study 2 overview

The field experiment was performed at University of Missouri Bradford Research and Extension Center (38° 53' 48" N, 92° 12' 23.5" W) located 12 km from Columbia, MO. The experiment was conducted on soils (Mexico silt loam) from contrasting management situations: (1) cultivated soil under corn and soybean rotation and (2) restored, native grassland. Six experimental plots (3m x 5m) were established in each management situation. Three plots in each management situation served as treatment plots and the other three served as control plots. Over the course of the experiment (from March 2012 to October 2012), treatment plots received 100 L ha^{-1} of SCD ProBio Balance Plus™

(PBP), a particular CP. The product was diluted with DI water up to 100 times administered in three applications: (1) 40 L ha^{-1} on 03/13/2012; (2) 20 L ha^{-1} on 06/16/2012; (3) 40L ha^{-1} on 10/04/2012. Soils in control plots received corresponding amounts of water. Soils were sampled eight times over the course of the experiment.

The growth chamber experiment employed intact soil cores (ISC) that were taken up from control plots that had been established in the cultivated soil. Intact soil cores were planted to tomatoes (*Solanum lycopersicum* cv. Brandywine) and treated with PBP (five different treatment regimes; 100L ha^{-1} total per ISC applied as 1% solution in DI water at different times and in varying volumes depending on treatment regime) or only water (controls). Soils were sampled eleven times.

Soil DNA was extracted from soil samples for subsequent PCR amplification and resolving of the PCR product using DGGE. After DGGE, gels were photographed and software-enhanced analysis of images was carried out to reveal the microbial diversity in samples.

2.2. Objective 2, Study 3 overview

Two autoinducers N-(3-Oxodecanoyl)-L-Homoserine Lactone (AHL) and N-(3-oxohexanoyl)-L-homoserine lactone (AHLEc) were suspended in aqueous solutions containing two CP (PBP and SCD BioAg™) (treatments) or only water (controls). Solutions were incubated for 0, 2, 4 or 8 hours and after the incubation they samples were extracted with chloroform. Chloroform extracts were then analyzed using a GC/MS to quantify AHL and AHLEc concentrations. Detection and quantification of autoinducers

in extracts was performed based on comparison with data obtained from GC/MS analysis of autoinducer standard solutions.

3. Chapter 3: Study 1 - Impacts of a Consortia Probiotic Inoculant on Soil Microbial Diversity and Enzymatic Activity under Two Management Practices

Abstract

Soil microbial communities are key drivers of soil processes and contribute crucially to agricultural productivity. The goal of optimized agricultural productivity has long been fulfilled through manipulation of indigenous microbial communities for example by applying microbial inoculants. We hypothesized that application of SCD ProBio Balance™ Plus (PBP), a particular consortia probiotic product, may enhance soil microbial diversity, thus improving pathogen control and amplifying crop residue turnover. To test the hypothesis, field experiment was established in Central Missouri on Mexico silt loam soils under two different management situations: (1) restored grassland; (2) standard corn/soybean rotation. During the 2012 growing season, soils were amended with the PBP product at the rate of 100L ha^{-1} (treatment) or water (control). The treatment was replicated in triplicate and soils were sampled to the depth of 10cm and sampling was repeated eight times throughout the experiment. Soil microbial diversity was surveyed through 16srDNA DGGE. Furthermore, soil enzymatic activity was examined with respect to dehydrogenase activity and the capacity to digest fluorescein diacetate. Application of PBP correlated with no major changes in soil enzymatic activity or soil microbial diversity. Certain limitations may have obscured the actual impacts of

PBP on soil microbial communities (i.e. short study duration, narrow array of enzymatic activates studied, severe drought conditions). However, it seems unlikely that PBP studied may improve pathogen control and amplify crop residue turnover by enhancing microbial diversity.

3.1. Introduction

Humans depend on ecosystem services and arguably the most fundamental of these is the provision of food products and fiber by agricultural ecosystems (Norris et al., 2010). An important role in the functionality of those ecosystems is played by soil microorganisms (Buscot and Varma, 2005; Kennedy, 1998) . Microorganisms drive soil functions largely by perpetuating the cycling of carbon and nutrients - they are efficient in releasing essential elements from inorganic sources as well as releasing carbon and nutrients during the breakdown of organic material (Bünemann and Condron, 2007; McNeill and Unkovich, 2007; Stotzky and Pramer, 1972). The latter ability may appear as both beneficial and deleterious. It is desirable that microorganisms degrade organic residues deposited onto the soil; by doing so they facilitate nutrient release and contribute to synthesis of soil organic matter. However, at the same time certain microorganisms may deteriorate tissues of living organisms (i.e., cause diseases) and this undesirable activity may compromise outputs in agroecosystems (Goto, 1992; Lane et al., 2012). Indeed, major crop losses worldwide are attributed to the pathogenic activity of microorganisms (Oerke and Dehne, 2004).

Over last several decades, the correlation between biological diversity and stability of ecosystem functions has encouraged numerous studies. General conclusions that stem from this research are that biologically diverse ecosystems may be: (1) more resistant to perturbations; (2) capable of regaining their functionality more rapidly if the functionality was damaged by perturbations; and (3) more productive (Downing et al., 2012; Holling, 1973; Levin and Lubchenco, 2008; Peterson et al., 1998; Tilman, 1999;

Vallina and Le Quere, 2011; Wilson, 1988). Furthermore, with respect to soil microbial diversity arguments have been made that it confers higher resistance to pathogens (Brussaard et al., 2007; van Elsas et al., 2002).

Soil ecosystems are among the most complex on the planet and there exists an immense diversity of relationships between organisms in the soil (Pierzynski et al., 2005). Some interactions may result in pathogen suppression and have received a considerable scientific attention. Studies show that the soil microbial diversity and abundance are inversely correlated with pathogen development (Irikiin et al., 2006; Matos et al., 2005; Messiha et al., 2009; van Elsas et al., 2012; Van Elsas et al., 2007). The detailed processes that drive these effects still remain to be understood. Currently, processes that underlie the suppressiveness of soils to pathogens are discussed in two wide categories: (1) general suppressiveness; (2) specific suppressiveness.

Brussaard et al. (2007) explains that general suppressiveness is correlated with high soil microbial biomass. Under such conditions, ample competition for nutrients exists and as a result a state of permanent starvation occurs which inhibits pathogen proliferation. General suppressiveness is favored under conditions where copious inputs of organic matter (OM) are supplied, which is associated with a high diversity of microbial communities (Bulluck and Ristaino, 2002; Hoitink and Boehm, 1999; Tuitert et al., 1998; van Bniggen and Termorskuizen, 2003). It is argued that no specific group of microorganisms is responsible for general suppressiveness. (Weller et al., 2002).

Specific suppressiveness refers to the activity of individual groups of organisms. Analytical differences between general and specific suppressiveness types include: (1) transferability – unlike the former, the latter may be transferred from a soil to another by

means of transferring soil samples; (2) specific suppressiveness is often removed by harsh treatments which are only seldom inhibitory to general suppressiveness. It is likely that in natural settings, pathogen suppression results from an interplay between both types of suppressiveness (Weller et al., 2002).

Although it has been found that the loss in natural composition of microorganisms in soil may be followed by an amplified activity of pathogens (Bailey and Lazarovits, 2003; Hoitink and Boehm, 1999), enhancing the composition of soil microbial communities does not necessarily correlate with improved pathogen control (Brussaard et al., 2007) . For example, a soil management option for enhancing soil microbial composition is to provide inputs of organic matter to soils. However, Bonanomi et al. (2007) after thoroughly reviewing the volume of research on organic matter inputs (n=2423 articles) concluded that the strategy may produce highly variable results in terms of pathogen control (i.e. pathogen activity may be thwarted, unaffected or fostered). Nonetheless, strategies aimed at maintaining high microbial abundance and diversity are considered potential promoters of disease control (Bailey and Lazarovits, 2003).

Stability of ecosystem function is a result of resistance and resilience, and both characteristics are positively related biodiversity. Ecosystem resistance is described as the capacity to withstand perturbations, while resilience indicates the rate at which ecosystems regain functionality after a disturbance event (McNaughton, 1994). However, soil ecosystems and their microbial component are exceptionally hard to study and little knowledge is available about the link between specific microbial taxa and the functions they perform in the soil environment (Allison and Martiny, 2008; Hollister et al., 2010; Lynch et al., 2004; Nannipieri et al., 2003). Particularly, this difficulty stems

from the abundance of microorganisms in soils as well as from analytical challenges associated with the identification of microorganisms (Kirk et al., 2004; Torsvik and Øvreås, 2002).

A general conclusion arising from studies on soil microbial communities is that microbial communities appear to be characterized with functional redundancy (Andrén and Balandreau, 1999; Brussaard et al., 2007). This is suspected to be an important feature for the natural functionality of the soil –when subjected to perturbation, redundancies provide continued expression of functions that could be lost otherwise (Lin, 2011). Interestingly, Yin et al. (2000) found that in regions where soil contamination occurs, the functional redundancy increases toward areas that are less affected by contamination. They suggested that the redundancy is a natural and desirable characteristic of soil microbial communities and may be important in evaluating soil health and quality.

The link between microbial diversity and functionality has also been assessed by Griffiths et al (2000b) who found that exposure to a harsh treatment (fumigation), which caused a decrease in soil microbial diversity, had no major impact on soil function, as assessed by soil respiration. However upon exposure to another stress (addition of $CuSO_4$ or heat treatment), soils in which diversity had been diminished, lost their function more noticeably than those soils where diversity had not been reduced. Furthermore, soils with diminished microbial diversity required longer periods to regain pre-stress functionality than did soils with higher microbial diversity. These results suggest that the soil microbial diversity has a positive impact on soil ecosystem resistance and resilience to stresses caused by various perturbations.

In another experiment Griffiths et al. (2001) examined how sterilized soils regain functionality upon artificially increasing their microbial biodiversity. Authors found that changes in biodiversity did not relate to the rate at which the soils regained their function. Brussaard et al. (2007) suggested that these contrasting results indicate the different stability response may owe to the type of stress and not to the change of the biodiversity itself and they further concluded that the link between microbial diversity and soil functionality needs more evaluation for complete understanding. Even though analytical difficulties exist, the importance of diversity of soil microbial communities in maintaining soil functions seems to be a well-accepted paradigm (Domsch, 1964; Dorr de Quadros et al., 2012; Garbeva et al., 2004; Kennedy and Smith, 1995; Zak et al., 2003).

Recent advances in soil microbiology have been achieved largely due to the adaptation of molecular, culture-independent methods of study. Such methods, particularly those that focus on the screening of genetic material contained within microbial communities, have been widely adopted in studies of soil microbial diversity. A method that has been widely employed is targeting of the bacterial16s mRNA gene for amplification using the polymerase chain reaction (PCR) and resolving of PCR products using denaturizing gradient gel electrophoresis (DGGE) (16s rDNA PCR DGGE) (Ahn et al., 2009; Kirk et al., 2004; Torsvik and Øvreås, 2002).

The gene termed 16s rDNA codes for the RNA component of the small ribosomal unit and is widespread among prokaryotes. The gene contains two major types of fragments (1) those that are highly conserved among microbial groups and (2) others whose sequence is highly variable among taxa. Primers have been devised that target conserved fragments of the gene and allow for PCR-based amplification of specific

fragments of genetic material. Amplified DNA may be separated afterwards due to differences in properties of variable gene fragments. The separation pattern, which is produced through the DGGE process, conveys information about the diversity of microbial genera. However this method usually allows for only very coarse conclusions about the composition of microbial communities (Valášková and Baldrian, 2009).

Probiotics are wide range of microbial species that have been traditionally defined as organisms that may augment host species condition by improving its digestive health (Gupta and Garg, 2009). Recently however, beneficial effects of using products containing probiotics have been examined beyond the domain of animal or human digestive health. For example, in aquaculture the beneficial impact of probiotics has been resulted in improved production. Thus, it has been argued that the definition of probiotics be extended to include organisms that may affect other species well-being by improving the species' environment (Kesarcodi-Watson et al., 2008).

Some probiotic species have long been recognized as antagonists of certain plant pathogens (Visser et al., 1986) and attributes of probiotic species may include antagonism towards pathogens (i.e. synthesis of antibiotic-like substance by lactic acid bacteria). A pursuit for microbial products that deliver benefits to plant and soil has resulted in formulation of multi-microbial inoculants that consist of numerous probiotic microorganisms cultured collectively in aqueous medium. This type of multi-microbial probiotic cultures (or consortia probiotics) has been applied in agricultural settings and produced satisfactory results in terms of improved yields, pathogen control or crop residue breakdown(Fatunbi and Ncube, 2009; Javaid, 2006; Javaid and Bajwa, 2011; Khaliq et al., 2006; Shah et al., 2001). Unfortunately, these studies offer no in-depth

explanations regarding the avenues by which probiotic products deliver their beneficial impacts.

Recently another consortia-probiotic type of product was introduced into the market under brand name SCD Probiotics®. Application of the product reportedly allowed for achieving certain benefits in agriculture such as improved yields, reduced pathogen outbreaks, or enhanced cropping residue breakdown (SCD, 2000; SCD, 2002a; SCD, 2002b). However, there is paucity of data that describes modes of action associated with the product.

The overall goal of the study was to elucidate modes of action associated with SCD consortia probiotic cultures. The research hypotheses were (a) upon application to soil, microorganisms present in cultures enhance the diversity of soil microbial communities and/or stimulate enzymatic activities of microbial communities and (b) such impacts could, in turn, improve pathogen resistance and enhance breakdown of crop residues, which may contribute to improved plant growth and better yields.

To test these hypotheses, a probiotic culture manufactured by SCD (SCD ProBio Balance Plus™) was surveyed for its impact on several characteristics of soil microbial communities and evaluated under field conditions. Soil microbial characteristics examined in this study were: (1) soil microbial diversity (assessed by16s rDNA PCR DGGE); and (2) soil enzyme activity including dehydrogenase and fluorescein diacetate (FDA) hydrolase (representing microbial potential to degrade complex substances). A complimentary study was performed under controlled conditions (soils microcosms from field evaluated in an environmental growth chamber) and is presented in Chapter 4.

3.2. Material and Methods

3.2.1. Field study design

The study area was located at Bradford Research and Extension Center in Boone County, Missouri (38° 53’ 48” N, 92° 12’ 23.5” W). Experimental plots (n=12) were sized 3 m x 5 m. Plots were localized on Mexico silt loam (fine, smectitic, mesic Vertic Albaqualf) under contrasting management systems: (1) minimum-till soybean-corn rotation (CS); and (2) restored native grassland (RG). There were six plots established per management system, and plots were separated with 1-m wide buffers (See Figure 3.1). Three plots per management system were designated as control plots and three were designated as treatment plots.

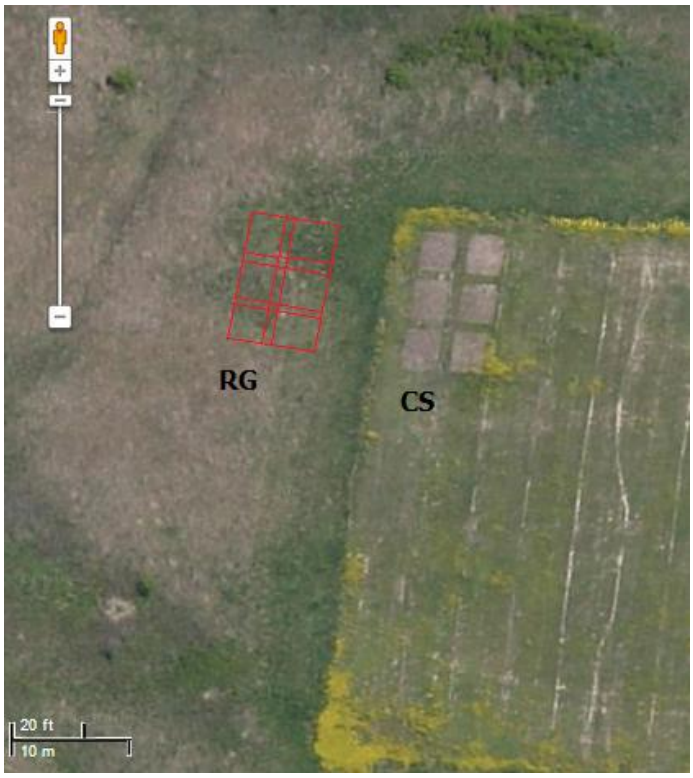


Figure 3.1 Aerial photo showing the field experiment site. Six cultivated soil (CS) plots are visible as gray, rectangular zones (upper right from center). Six plots in the restored grassland (RG) plots are marked with red grid. Picture modified from https://maps.google.com/.

3.2.2. Treatments, controls and application outline

SCD ProBio Balance Plus™ (PBP) was the consortia probiotic product chosen for study. This product consists of the following species: *Bacillus subtilis, Bifidobacterium animalis, Bifidobacterium bifidum, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus bulgaricus, Lactobacillus fermentum, Lactobacillus plantarum, Lactococcus lactis, Rhodopseudomonas palustris, Rhodopseudomonas sphaeroides, Saccharomyces cerevisiae*, and *Streptococcus thermophilus*. A proprietary fermentation process is used where microorganisms are cultured collectively with

sugarcane molasses serving as the primary carbon source. Upon Quality Assurance/Quality Control release, the product is decanted into appropriate containers and prepared for sale. According to manufacturer's guidelines this product is a "Mother Culture" designed for manufacturing ready to use products (Secondary Products) and normally Secondary Products, not Mother Cultures are suggested for use as soil amendments or foliar sprays. This product was applied three times to treatment plots on the following dates and in the following quantities: (1) March 18, 2012, 40 L ha^{-1} (0.06 L per plot) (2) June 16, 2012, 20 L ha^{-1} (0.03 L per plot); (3) October 5, 2012, 40 L ha^{-1} (0.06 L per plot). SCD ProBio Balance Plus™ was diluted in deionized water (DI) and solution was sprayed onto soil with a hand sprayer. Volumes of DI used for applications were 1.24, 2.97 and 1.94 L, respectively. Soils in control plots received only DI in corresponding amounts, that is, 1.3 L, 3 L and 2 L, on: March 18, 2012; June 16, 2012 and October 5, 2012, respectively.

To comply with AG manufacturer's guidelines, applications were performed during evening hours in order to prevent exposure of AG to excessive solar radiation. Furthermore, on March 18, 2012, immediately after the first application, the soil in CS control plots and treatment plots was tilled in order to incorporate AG better into the soil.

3.2.3. Soil sampling outline

Soils in experimental plots were sampled eight times on following days: March 13, 2012; March 21, 2012; April 27, 2012; June 16, 2012; June 18, 2012; July 23, 2012; October 4, 2012; October 8, 2012. Soil samples were collected with stainless steel bore to

a 10 cm depth. One sample was collected per plot per sampling day. The sampling bore was carefully cleaned after collecting a sample. The following cleaning procedure was adopted: (1) rinsing with DI; (2) spraying with 70% ethanol; (3) rinsing with DI; (4) pushing the bore head into soil in target plot to let soil particles remove water residues before removing actual sample. Samples were placed in separate plastic bags and placed on ice until arrival to the storage area where they were kept in a freezer at -16^{o}C.

3.2.4. Soil DNA and AG DNA extraction technique

Total bacterial DNA was extracted from air-dried soil samples (0.25 g) using the Power Soil DNA® Isolation Kit (MO BIO Laboratories, Inc) following procedures provided by manufacturer. Bacterial DNA was also extracted from 250µl aliquots of AG. Extractions yielded 100-ul DNA extracts per sample and extracts were stored at -60°C. Optical density of the DNA was examined to determine DNA concentration in extracts (28 to 100.67 µg DNA g^{-1} of air dry soil).

3.2.5. Polymerase chain reaction

Polymerase chain reaction was run using two types of templates: (1) DNA extracts from individual replicates and (2) composite DNA extracts (extracts from three replicates were pooled to make one composite DNA extract). Amplification of 16S rDNA fragments was carried out using the F984GC-R1378 pair of bacteria-specific primers. Mixtures for PCR reactions contained each primer's (5µl) DNA template and: (1) soil or

AG DNA (4µl); or (2) marker DNA (1µl) and REDTaq ReadyMix (35µl of REDTaq ReadyMix was combined with soil and AG DNA, while 50µl was added with marker DNA). Marker DNA contained DNA from the species: *Bacillus cereus*; *Bacillus pumilus*; *Flavobacterium balustinum* and *Pseudomonas fluorescens.* An Eppendorf Mastercycler Thermal Cycler (Perkin-Elmer, Waltham, MA) was used to carry out PCR reaction. The reaction program was initiated at 94 °C for 4 min followed by 35 cycles in which temperatures were kept at 94 °C for 1 min, then at 55 °C for 1 min and at 72 °C for 2 min. In the final step, the temperature was held at 72 °C for 10 min and the program went into standby (4 ^{o}C). DNA extracts were subjected to PCR twice. First, PCR amplification was carried out separately for DNA extracts from each replicate soil sample. Second, DNA extracts from replicate samples were combined and composite DNA extracts were amplified (Scholz-Starke et al., 2013; Singh and Ramaiah, 2011).

3.2.6. Denaturing gradient gel electrophoresis conditions

The DGGE separation was conducted using 8% (wt/vol) polyacrylamide gel with a linear denaturing gradient of concentration from 37 – 57% (100% denaturant defined as 7M urea in 40% formamide). Aliquots of soil DNA PCR products and marker DNA PCR products (30ul and 12ul, respectively) were loaded into wells in the gel so that marker DNA PCR products occupied external wells in each gel. When PCR products from composite samples were analyzed one well was also loaded with PCR products from PBP DNA extracts. Gel electrophoresis was run at 60 ^{o}C at 130 V for 6 h in 1x TAE with the Bio-Rad Dcode System (Bio-Rad Laboratories, Hercules, CA). Gels were stained with

SYBR green I (1:10,000 dilution) in 1x TAE buffer for 20 min to visualize DNA, and gels were photographed with a GeneGenius Gel Document System (Syngene, Frederick, MD) using the shortwave band filter. Gene Snap software (Syngene) was used to quantify the band intensities to interpret differences between gel patterns from the various soil treatments.

3.2.7. Assessment of richness, diversity, evenness and similarity of microbial communities

Digitized gel images were enhanced using the Sharpen function (repeated three times for each gel) within the GeneGenius Gel Document System software. Images were further analyzed with Phoretix 1D Pro (TotalLab Ltd., Newcastle, UK) and each lane was converted into a densitometric curve; background subtraction was applied (Rolling Ball mechanism, radius = 10) and band positions were converted to Rf values. Band positions and intensities (peak volume) were exported to Excel spreadsheets for subsequent survey of Richness (S); Diversity (H) and Evenness (E) of microbial community profiles. Richness was defined as the number of bands detected in each lane. Diversity was calculated using Shannon's diversity index [1] using peak height of each band as the input value. Evenness was defined as ln S/H. Similarity between DGGE profiles (Dice's similarity) was calculated using the Phoretix 1D Pro software package. The same package was used for cluster analysis of DGGE profiles using the unweighted pair group method with mathematical averages (UPGMA) (Ibekwe et al., 2010).

$$H' = \sum_{i=1}^{i=n} pi \ln pi \qquad [1]$$

In the Shannon's diversity index (Garcia-Teijeiro et al., 2009), pi is defined as peak height corresponding with band i compared to the sum of peak heights for all detected bands in a lane (Ibekwe et al., 2001)

3.2.8. Enzyme assays

Soil samples from five sampling events (March13, April 27, June 18, July 23, October 8) were selected for assessing soil enzymatic activity. Soil samples were removed from storage at -4°C and allowed to equilibrate under room temperature, in the dark for 24h. Subsamples (approx. 6 g each) were removed and oven-dried to determine gravimetric soil moisture.

Subsamples of moist soil (1 g) were transferred to a plastic tubes; suspended in 0.6 ml of 0.2M $CaCO_3$ and 0.2 ml of 0.09M 2, 3, 5-triphenyltetrazolium chloride. Tubes were capped and suspensions incubated at 37°C for 24h. After the incubation, contents of tubes were extracted with 20ml of methanol and filtered (Grade P8 Fisherbrand™ filter paper, Fisher Scientific, Pittsburgh, PA). Aliquots were taken from filtrates and absorbance read on an UltroSpec 2100 spectrophotometer (Amersham Biosciences, Piscataway, NJ) at 485nm wavelength. Absorbance values were converted to μg 1,3,5-triphenylformazan (TPF) produced per g of oven dried soil per hour using a standard

curve, linear regression equation. Standards for calibration curve were prepared by dissolving TPF in methanol and preparing a range of concentrations.

Moist soil subsamples (1g) were placed in 50-mL Erlenmeyer flasks, amended with 20 ml of sodium phosphate buffer (pH = 7.6), capped and shaken for 15 min at 100 rpm. Subsequently, 100 ml of 4.8mM FDA (in acetone) was added to each flask. Mixtures were incubated for 1.75h at room temperature and shaken at 100 rpm. After incubation, mixtures were agitated and filtered (Grade P8 Fisherbrand™ filter paper). Absorbance of filtrates was read on a UltroSpec 2100 spectrophotometer (Amersham Biosciences) at a wavelength of 490nm. Absorbance values were converted into μg of fluorescein converted per g of oven-dried soil per hour using a standard curve, linear regression equation. Standards for calibration curve preparation contained known concentrations of fluorescein dissolved in sodium phosphate buffer.

3.2.9. Statistical analyses

A single factor ANOVA was carried out using SAS Enterprise® 9.3 software (SAS Institute Inc., Cary, NC). Shannon's diversity index values representing control and treated soils' microbial communities were compared at each point in time; CS and RG soils were compared separately, so that eight comparisons were made for each management situation. Values different at $p<0.05$ were assumed to be statistically different. Tukey's HSD multiple comparison test at alpha = 0.05 was used for comparing soil enzymatic activity of all soils. Separate comparisons were performed for each point in time.

3.3. Results

3.3.1. Analysis of DGGE profiles – S, H' and E values

Figure 3.2 depicts S, H' and E values related to DGGE profiles of soil microbial communities from the field experiment (these profiles were produced from replicate DNA extracts). Values describing microbial profiles from field experiment soil samples using composite DNA are presented in Figure 3.3 When replicate soil DNA extracts were used for DGGE profiling of microbial communities, H' values showed apparent, increasing trends until April 27. Afterwards, H' values appeared to decline until June 18, seemingly increased again until July 23, and by October 8 returned to levels comparable to those revealed in samples removed on March 13. Soil microbial profiles prepared from composite DNA extracts revealed that H' values generally fluctuated around similar levels for all microbial communities until April 27. From June 18 until July 23, H' values appeared to decline. Subsequently, on October 4 and October 8 H' values appeared to return to levels comparable with those encountered in March.

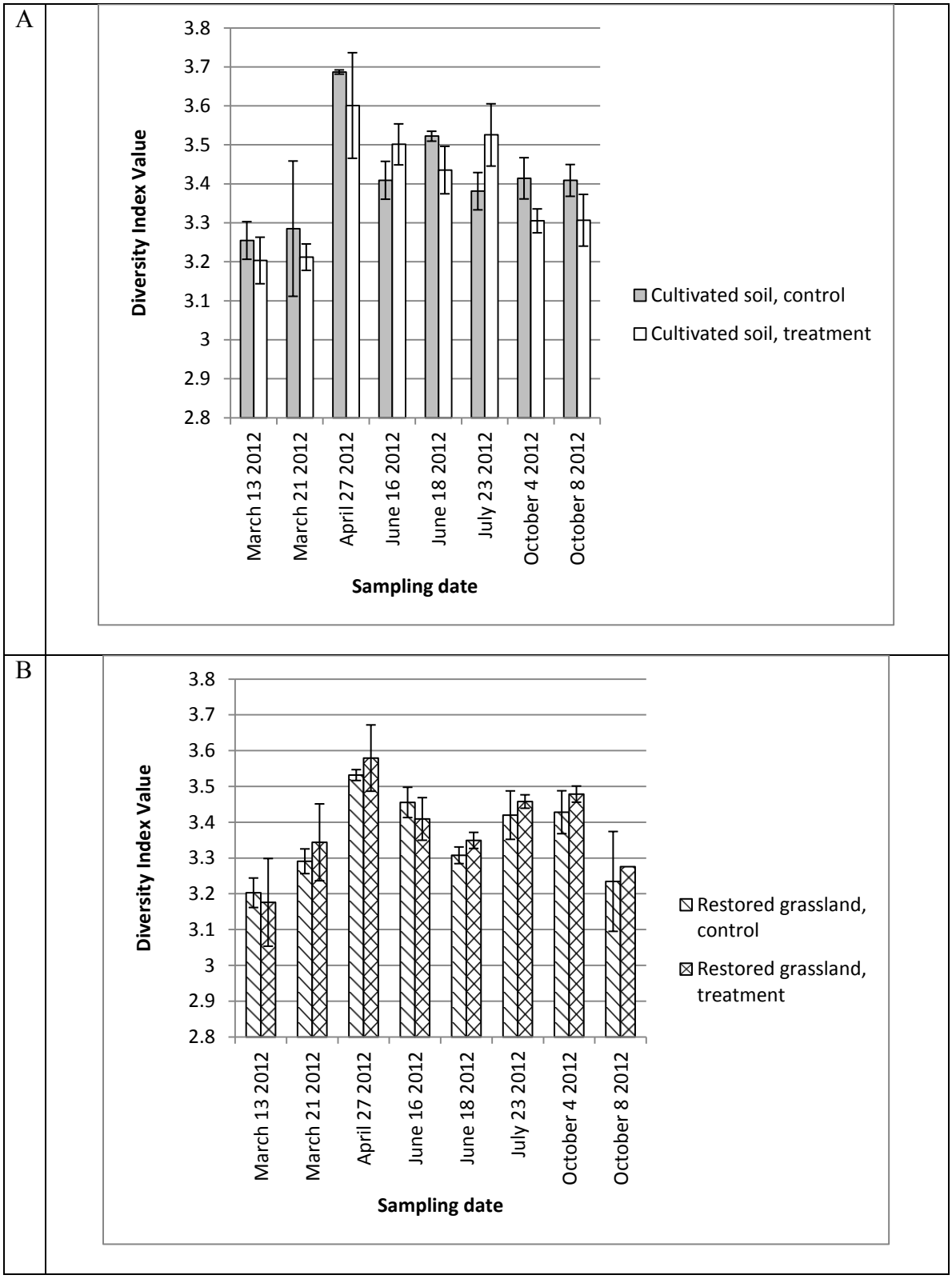


Figure 3.2 Microbial community diversity indices revealed in DDGE profiles of soil microbial communities in the field experiment: (A) cultivated soil; (B) restored grassland. Mean values representative of replicated samples (n=3) are shown.

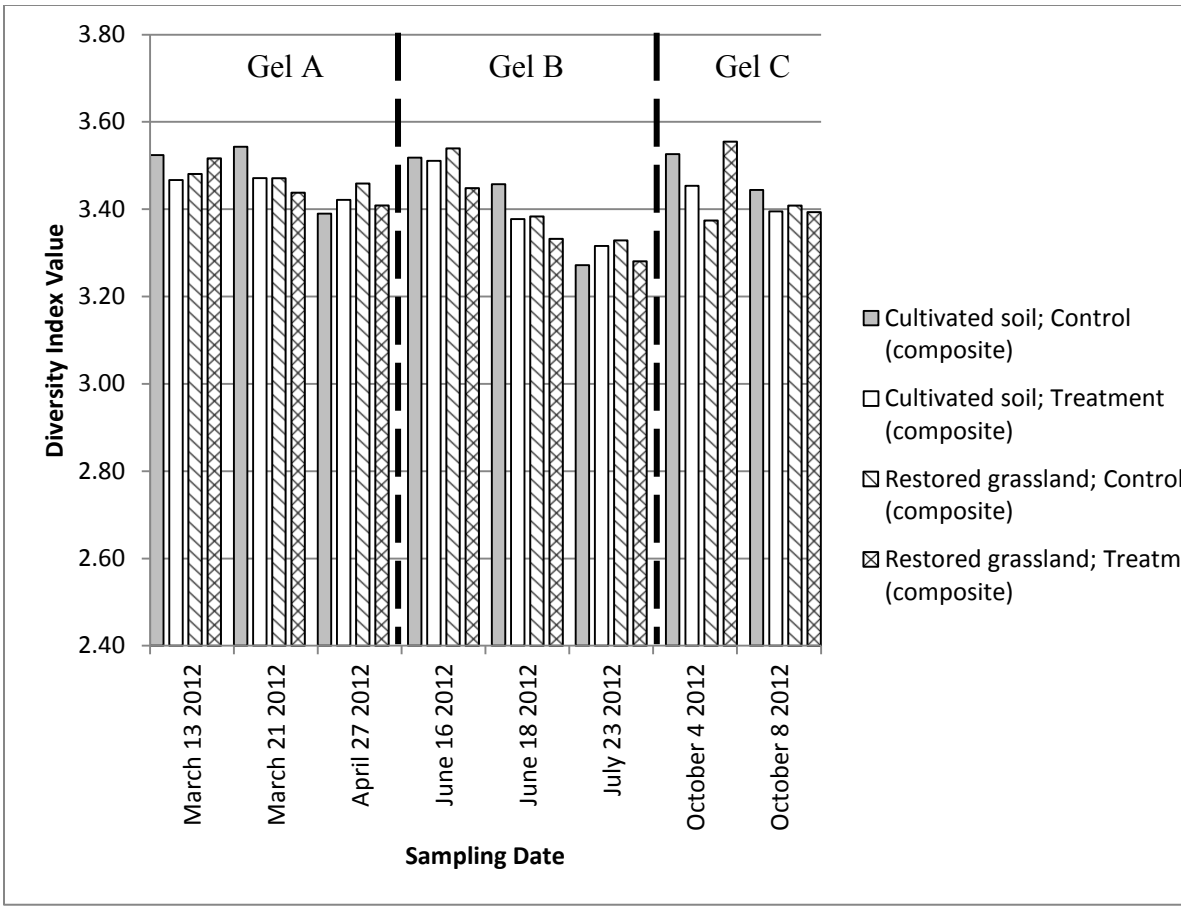


Figure 3.3 Chart displaying Shannon's diversity index values (H') observed in DGGE profiles prepared using composite DNA extracts from the field experiment soils. Dashed lines separate values retrieved from DGGE profiles resolved on different gels (Gel A, Gel B, Gel C).

3.3.2. Similarities between microbial communities analyzed from composite soil DNA samples

Figures 3.4 through 3.6 present UPGMA dendrograms prepared from Dice similarity matrix for all DGGE profiles revealed from field experiment soil samples. Dendrograms suggest that at the onset of the experiment (March 13, 2012 sampling), soil microbial communities were very similar regardless of soil management. Profiles that

corresponded March 21 and April 27, 2012 samples were found on increasingly distant branches. For instance, the RG -associated community at April 27 was dissimilar enough to form a separate dendrogram branch. However, CS communities from March 21 were relatively similar to communities from previous sampling. Additionally, on March 21, communities from treated and control soils showed a high level of similarity within the same soil management. Communities revealed on April 27 appeared to form an increasingly distant relationship with communities observed in earlier sampling dates.

Further dendrograms (Figure 3.5) consisted of two major branches, each chiefly composed of soil microbial communities from the same management regime (CS or RG). The branch that consisted of CS profiles further divided into two smaller sub-branches, each representing profiles from treated or control soils. Such a clear division (treated versus control soil) was not the case for the RG-dominated branch.

Dendrograms revealed for the October 4, 2012 sample date (Figure 3.6) suggest a close relationship between microbial communities and land management. With respect to October 8, 2012 samples such a relationship was far less evident (i.e. RG profiles were only very remotely related).

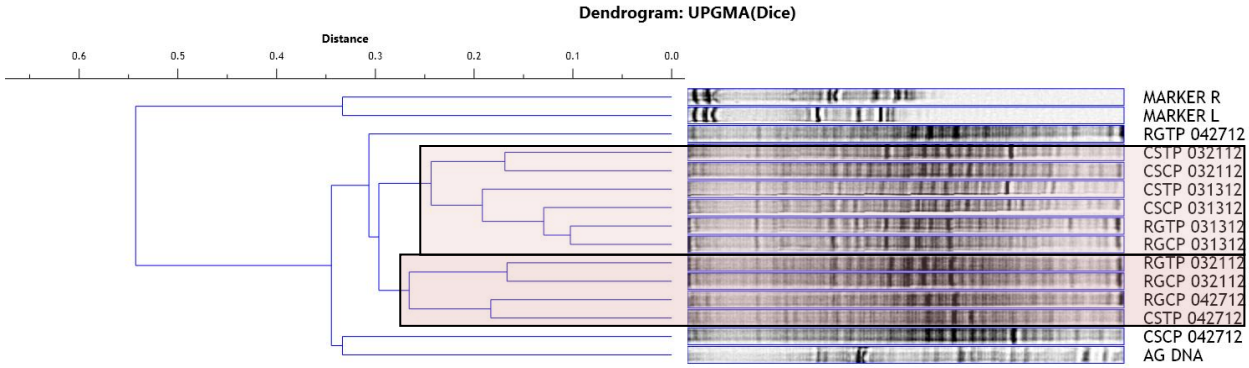


Figure 3.4 Dendrogram highlighting similarity between microbial profiles revealed in soil samples harvested between March 13, 2012 and April 27, 2012. First two letters of lane names indicate management situations (CS for cultivated soil and RG for restored grassland); remaining two letters denote control (CP) or treatment (TP). Numbers after lane names denote sampling dates (i.e., 03212012 is equivalent to March 21, 2012); L and R in case of Marker names denote left-hand side or right-hand side gel marker, respectively. Boxes indicate two particular branches. See discussion section for details.

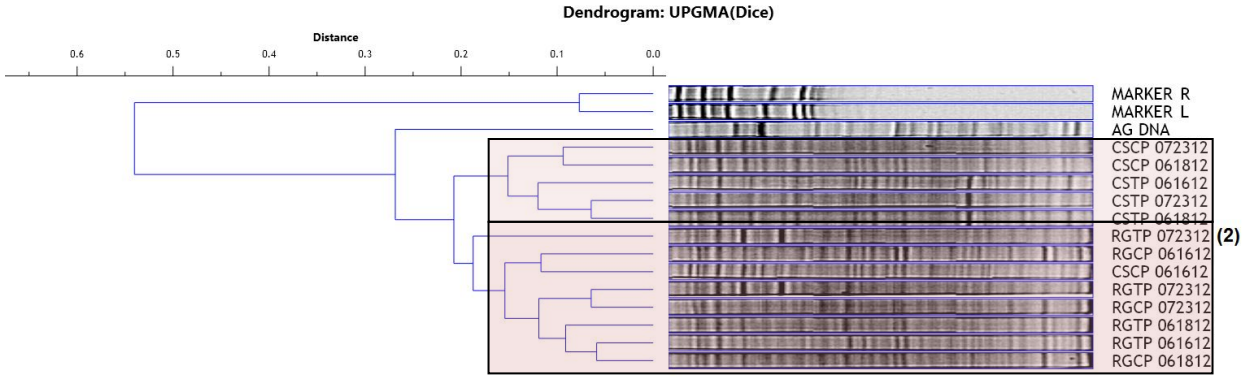


Figure 3.5. Dendrogram highlighting similarity between microbial profiles revealed in soil samples harvested between June 16, 2012 and July 27, 2012. . First two letters of lane names indicate management situations (CS for cultivated soil and RG for restored grassland); remaining two letters denote control (CP) or treatment (TP). Numbers after lane names denote sampling dates (i.e., 06182012 is equivalent to June 6, 2012); L and R in case of Marker names denote left-hand side or right-hand side gel marker, respectively. Boxes indicate two particular branches, see Discussion section for details.

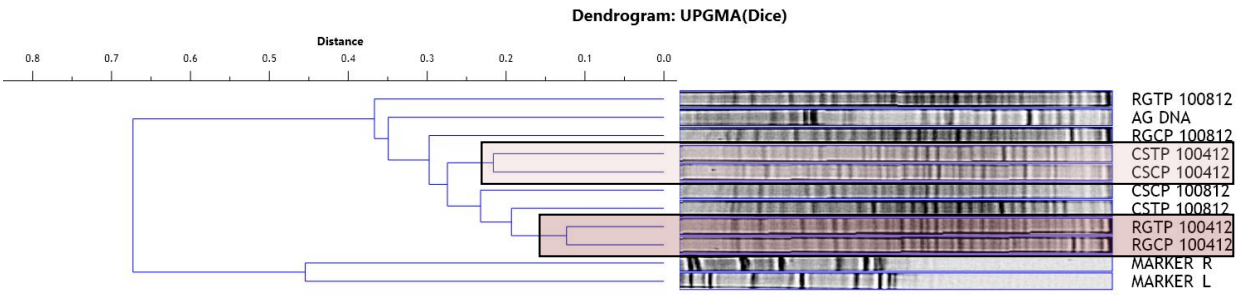


Figure 3.6 Dendrogram highlighting similarity between microbial profiles revealed in soil samples harvested between October 4, 2012 and October 8, 2012. . First two letters of lane names indicate management situations (CS for cultivated soil and RG for restored grassland); remaining two letters denote control (CP) or treatment (TP). Numbers after with lane names denote sampling dates (i.e., 04102012 is equivalent to October 4, 2012); L and R in case of Marker names denote left-hand side or right-hand side gel marker respectively. Boxes indicate two particular branches. See discussion section for details.

3.3.3. Similarities between soil microbial communities and microbial community associated with PBP

Figure 3.7 provides Dice similarity values between PBP microbial community and soil microbial communities studied in the field experiment. Dice similarity ranges from 0 to 100 where 0 indicates that analyzed communities were entirely unrelated while value of 100 is indicative of identical communities.. Similarity values linking soil and PBP-associated microbial communities appear to follow uniform trends regardless of soil management regime or treatment. In all cases a trend appeared - similarity increased in control soils as well as in treated soils from March 13 until June 16, 2012 and then decreased until the end of the experiment.

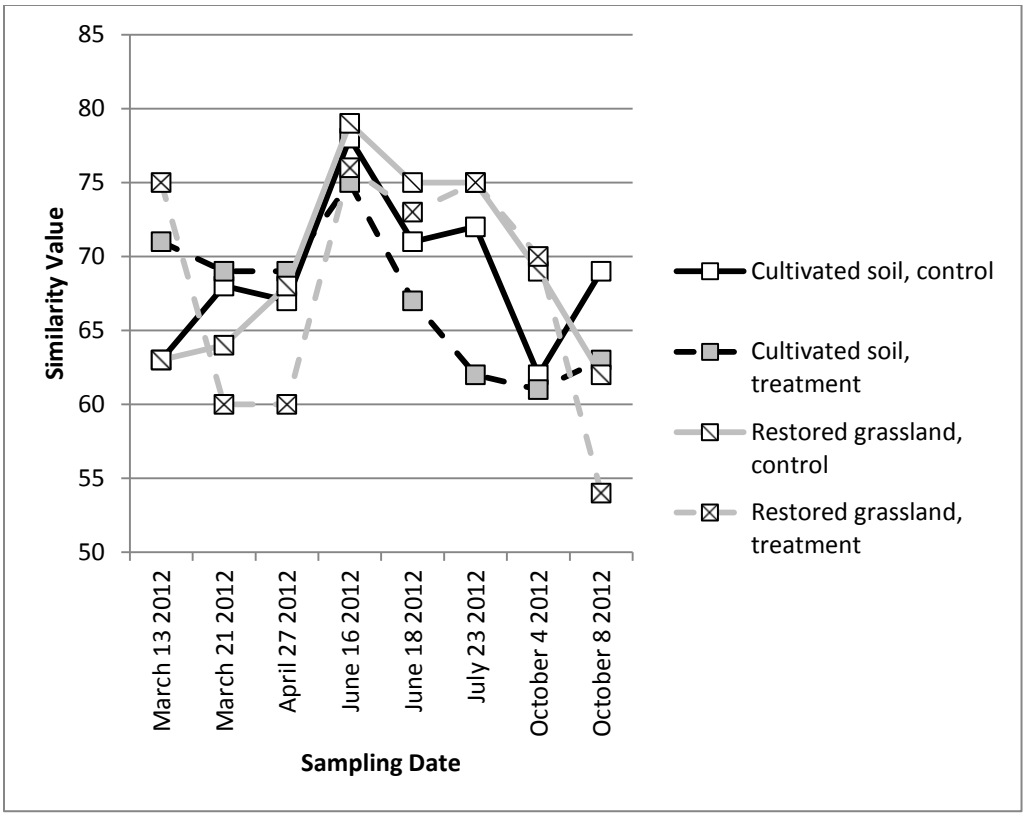


Figure 3.7 Dice similarity values representing similarity between PBP microbial community and soil microbial communities revealed in soils from field experiment. Microbial community profiles were revealed from composite DNA extracts.

3.3.4. Soil enzymatic activity

Figure 3.8 and Figure 3.9 present values for formation of TPF resulting from activity of dehydrogenase and fluorescein products released due to FDA hydrolase activity, respectively. Field-collected soils showed no statistically significant difference in terms of dehydrogenase activity (no difference within or among management situations). There also existed an apparent reduction of enzymatic activity after April in all soils. Similar observations apply to fluorescein diacetate degradation, however, the activity reduction appears less evident.

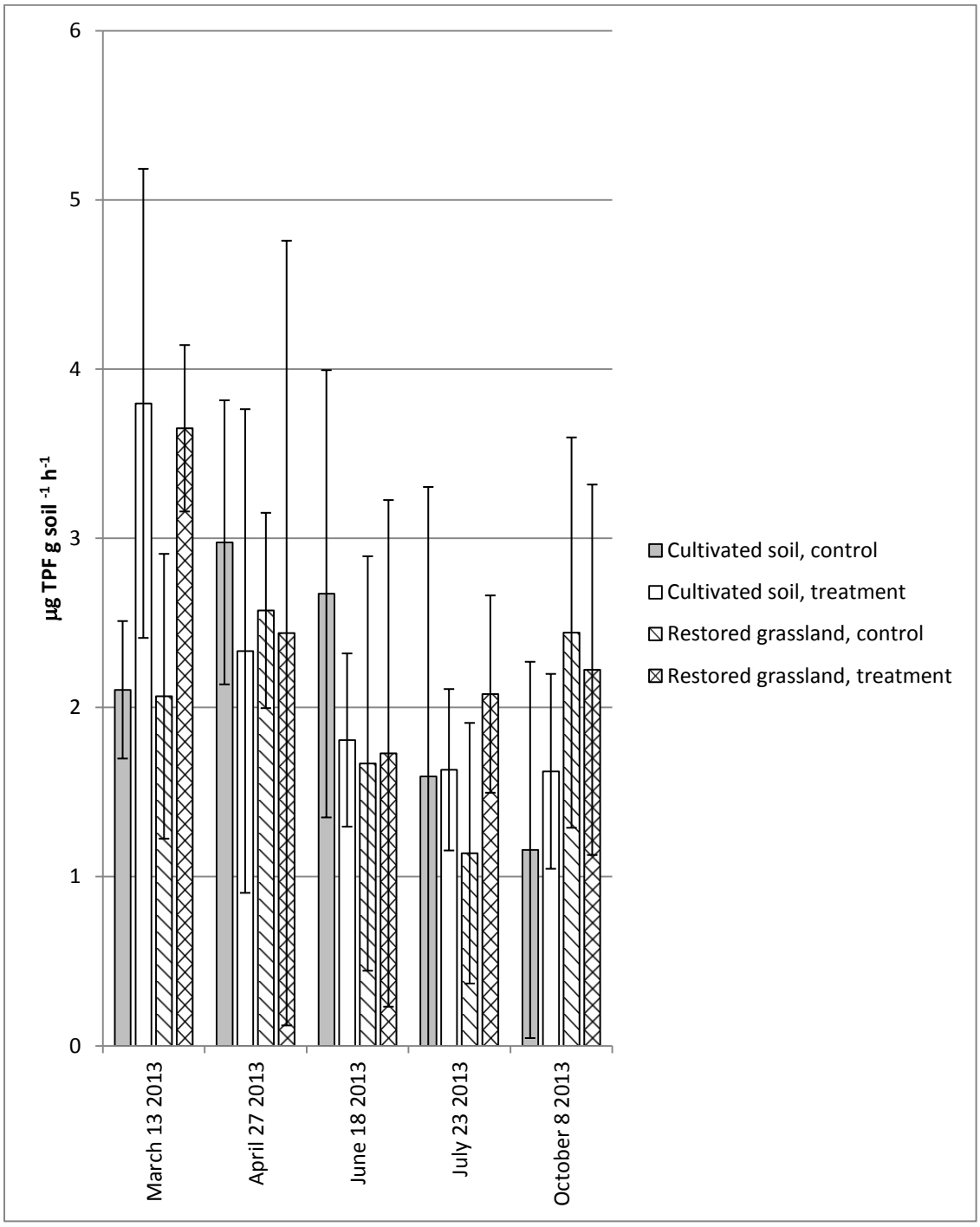


Figure 3.8 Dehydrogenase activity in soils from field experiment expressed in terms of 1,3,5-triphenylformazan (TPF) released per gram of dry soil per hour . Error bars indicate standard deviation intervals.

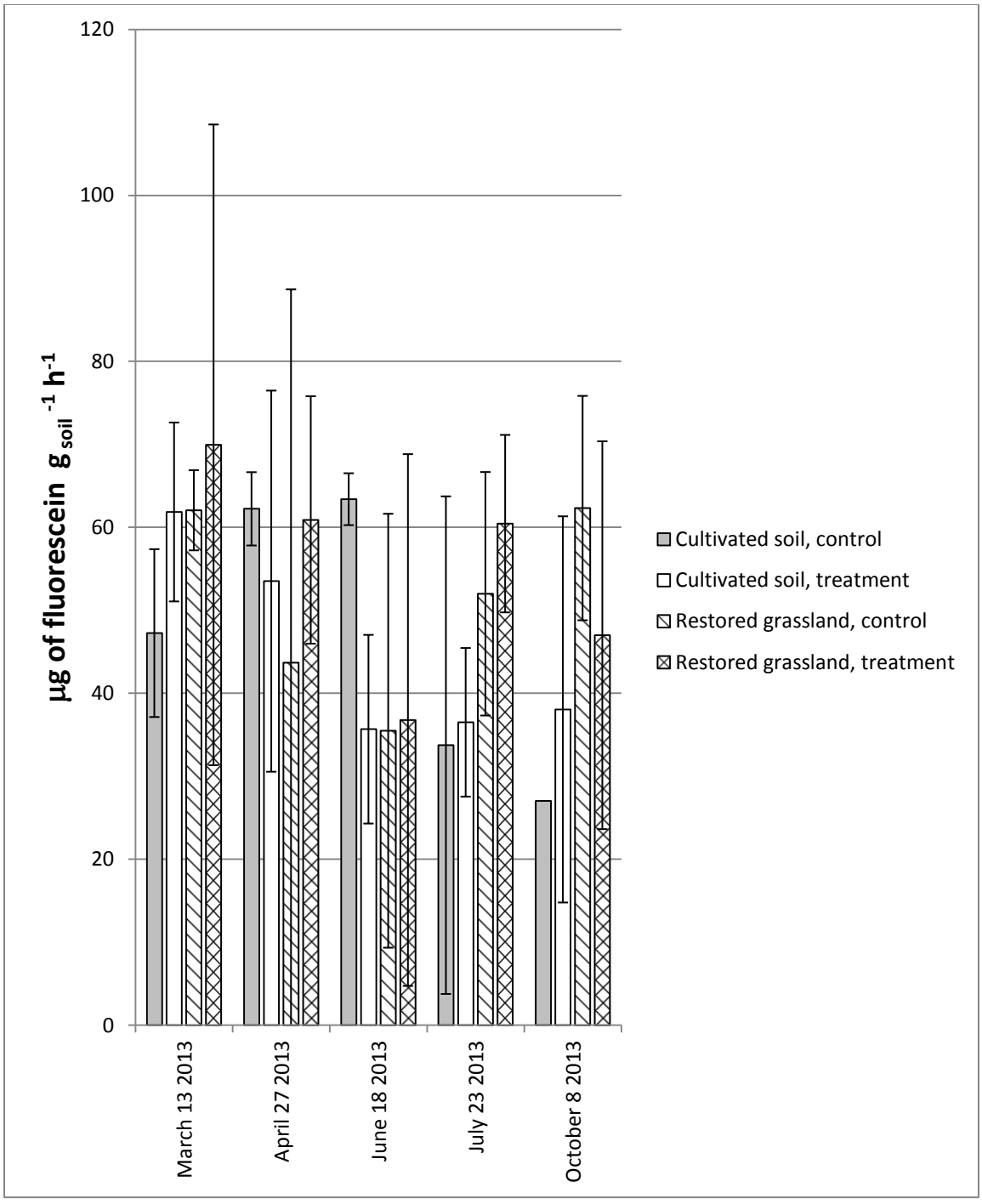


Figure 3.9 Capacity to degrade fluorescein diacetate in soils from field experiment expressed fluorescein released per gram of dry soil per hour. Error bars indicate standard deviation intervals.

3.4. Discussion

3.4.1. Soil DNA analysis

Quality of gels used for DGGE was variable and comparisons between microbial profiles using different gels were highly variable. Most likely, soil microbial diversity trends encountered while replicate DNA extracts were analyzed resulted from analytical issues (see appendix) and not from actual changes in microbial community structure. Similar issues have been reported elsewhere (Ibekwe et al., 2001). In order to circumvent this problem, replicate DNA extracts representing a sampling event were composited and analyzed as one sample so that the amount of profiles analyzed on a gel was maximized (Scholz-Starke et al., 2013; Singh and Ramaiah, 2011). This analytical strategy allowed for more accurate observations to be made and final conclusions regarding changes in soil microbial diversity are drawn from analysis based on composite DNA extracts.

3.4.2. Similarity between microbial profiles

Analysis of community profiles (Figure 3.4) provides no clear insights into factors that govern the similarity between soil microbial communities. Specifically, microbial communities were highly similar between: (1) control and treated soils; (2) soils under different management; (3) soils sampled at different times. With respect to samples removed later (Figure 3.5) it appears that soil management was a major controlling factor for microbial community structure and that AG application was a secondary controlling factor for microbial community structure in CS. Later in the experiment (Figure 3.6) soil

management appeared to be the key determinant for microbial community structure. Relationships between soil microbial communities seemed to have been unaffected by treatment with AG.

Interestingly, the observation that microbial communities appear highly similar early in the growing season agrees with findings reported elsewhere (Dunfield and Germida, 2003; Griffiths et al., 2000a). Furthermore, considerable seasonal variability of microbial communities found in this work is concurrent with findings from other studies (Meier et al., 2008; Smit et al., 2001). Table 3.5 summarizes factors that appeared to influence (as "controlling factors") microbial community structure in soils sampled at different times

Table 3.1 Summary of factors that appeared to control the structure of microbial communities surveyed at different times.

Sampling date	Apparent controlling factor	AG application date and quantity
March 13 2012	none evident	March 18, 2012 40L ha^{-1}
March 21 2012	none evident	
April 27 2012	none evident	
June 16 2012	management situation, AG application in CS	June 16, 2012, 20 L ha^{-1}
June 18 2012	management situation, AG application in CS	
July 23 2012	management situation, AG application in CS	
October 4 2012	management situation	October, 5 2012, 40 L ha^{-1}
October 8 2012	management situation (less evident)	

3.4.3. Similarity between profiles representative of soil microbial communities and AG-associated community

Based on similarity of DGGE profiles, it appears that microbial genera common to both soils and PBP may have been present, however, the prevalence of these genera in soils was apparently unaffected by PBP treatment. The apparent failure of PBP microorganisms to establish in soils was likely linked to insufficient competence to overcome unfavorable environmental conditions. Adverse conditions that microorganisms introduced to soils encounter include: predation by soil organisms and low nutrient levels; furthermore, microorganisms need to compete for physical niches (i.e. micropores between clay particles) (van Veen et al., 1997). Such factors often preclude successful establishment in soils (Savka et al., 2002; van Veen et al., 1997). Moreover, PBP applied translated as only very small amount of microbial cells. Specifically, lactic acid bacteria count in PBP according to its manufacturer amounted to 7×10^6 cells/ml and given that 15 ml PBP were applied to each m^2 of treated soil, only 7×10^9 cells m^{-2} were applied –an amount that is possibly to minute to affect indigenous soil microbial communities. Furthermore, according to its manufacturer, PBP is not optimized for soil applications and should be converted into a ready to use product that is better suited for this use type.

3.4.4. Soil enzymatic activity

The apparent trend of dehydrogenase activity may be associated with microbial response to severely dry conditions experienced during the 2012 growing season (Figure 3.10). Despite the environmental stress of drought conditions, it appears that application of AG to field soils did not disrupt the microbial community function as revealed by analyses of the selected soil enzyme activities.

3.4.5. Weather conditions and changes in soil microbial community characteristics

The apparent reduction of microbial diversity observed from June 16 through July 23 coincided with apparent reduction of dehydrogenase activity. This suggests that the microbial community could have been under some environmental stress due to the severe drought conditions that occurred in 2012. These apparent trends coincided with reduced rainfall and sharply rising average monthly temperatures (presented in Figure 3.10). Correlation between dry conditions and adverse effects on characteristics of soil microbial communities has frequently been reported (Alster et al., 2013; Hueso et al., 2012; Sardans and Peñuelas, 2005).

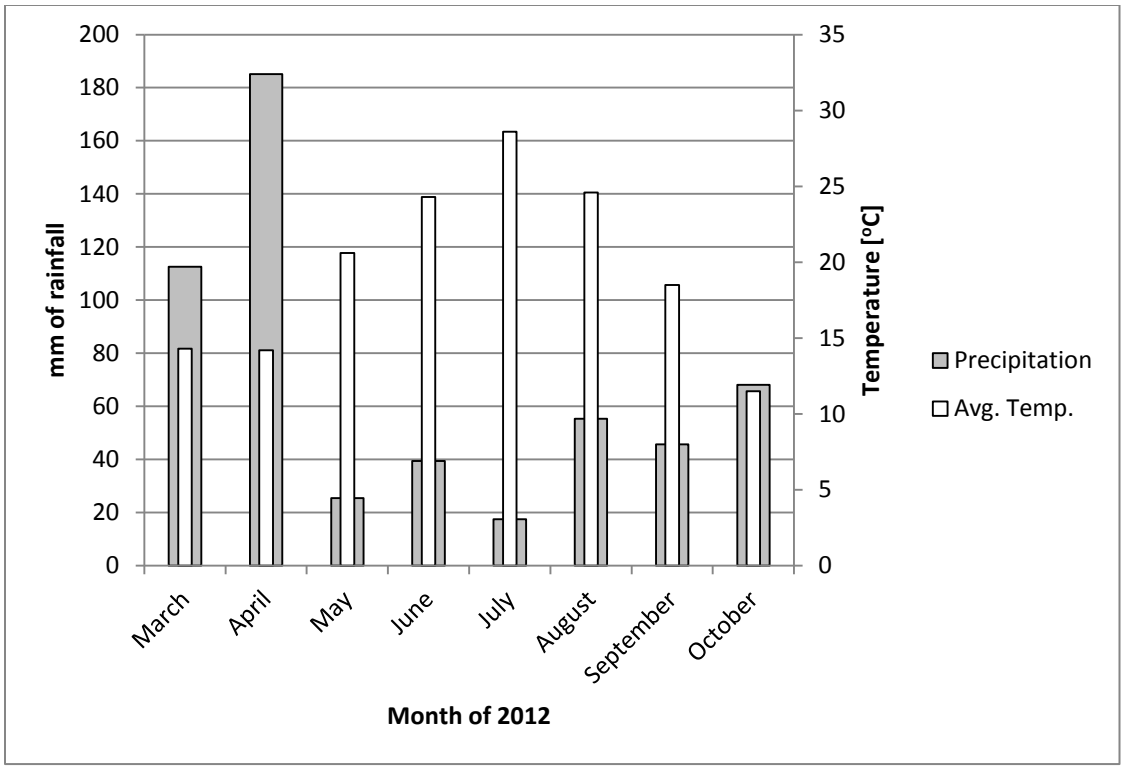


Figure 3.10 Total monthly precipitation and average monthly temperatures recorded at Bradford Research and Extension Center, Boone County, Missouri (field experiment site) between March and October, 2012 (field experiment duration).

3.5. Conclusions

Under this experiment's conditions, treatment with PBP did not impact soil microbial diversity. Based on observation of changes in similarity between soil and AG-associated microbial communities it seemed unlikely that microorganisms present in PBP were capable of establishing viable populations in soil. However, treatment with PBP correlated with formation of a distinct soil microbial community structure in CS between June 16 and July 23, 2012, suggesting that some short-term establishment may have occurred. Soil enzymatic activity was unaffected by treatment.

Based on findings from this study it seems unlikely that PBP could deliver its effects in agriculture by means of enhancing soil microbial diversity or activity. However, this point may be questionable due to weather conditions in 2012, which were unfavorable for soil biological activity and could have obscured treatment effects. Additionally, it has been shown previously under some circumstances that DGGE may detect microbial populations only if they constitute more than 1-2% of total microbial population (MacNaughton et al., 1999; Muyzer et al., 1993; Stephen et al., 1999). It is not certain whether surveying only some soil microbial groups and leaving others behind is sufficient for an exhausting discussion of AG-mediated impacts on soil microbial composition.

Furthermore, only a very narrow range of enzyme assays was employed, thus limiting observations to only a small subset of soil metabolic activities. In this work, PBP was evaluated to see how the inoculant functions in "as manufactured" form (without the necessity to make another preparation out of it prior to application). However, according

to the PBP manufacturer's guidelines, PBP should be used as a Mother Culture for manufacturing Secondary Products which are ready to use and much better suited for soil applications. Further studies will be needed to elucidate the mode of action associated with consortia probiotics such as AG.

3.6. Future Work

Future studies should allow for better discernment of uncertainties associated with the current study design (drought, DGGE procedure, enzyme assays) and to better elucidate modes of action that drive the efficacy of consortia probiotics. Furthermore, such studies would contribute observations necessary for better understanding of soil microbial communities functioning in general.

Future studies should adopt a longer temporal scale that would most likely provide more insightful observations. A study spanning at least two to three growing seasons would prove robust enough to compensate for extreme weather conditions such as those experienced in the present work. Application of the 16s rDNA PCR DGGE procedure appeared to be a powerful analytical tool for future analyses; however, techniques associated with DGGE would need to be improved to yield more accurate observations. For instance DNA extractions should be replicated and PCR products could be analyzed to reveal whether and to what extent extraction and PCR procedures contribute to variability within a dataset. Furthermore, PCR product samples would need to be resolved on multiple gels to compensate for variability of profiles produced with DGGE. Furthermore reference ladders (markers) for DGGE profiles should be prepared from microbial communities indigenous to study sites (Ponnusamy et al., 2008).

Enzymatic assays are an efficient method of screening general soil microbial activity and could be adopted. However, a more robust array of assays should be employed in the future. Possibly, broader analysis will provide far more informative insights into soil microbial activity and will help to elucidate the impact of consortia probiotics on soil processes.

Future studies should examine microbial communities not only in soils in natural settings but also analyze soils that were transferred to controlled environments (such as growth chambers). The amount of variables that affect the experiment in natural settings (water availability, temperatures, transmission of microorganisms by water or soil organisms such as earthworms, etc.) may impair clarity of treatment effects, if any, and analysis performed under controlled environments may provide auxiliary measurements essential for deeper understanding of treatment effects on soil microbial communities.

4. Chapter 4: Study 2 - Impacts of a Consortia Probiotic Inoculant on Soil Microbial Diversity and Tomato (*Solanum Lycopersicum*) Growth using Intact Soil Microcosms

Abstract

Microorganisms are potent modulators of plant growth (i.e. they may augment or thwart plant growth). Application of microbial inoculants has been frequently adopted to meet that goal and one particular type of microbial inoculants are those that consist of numerous probiotics microorganisms collectively cultured (consortia probiotics; CP). There is insufficient data that describes principles that drive CP efficacy. In the present work, we hypothesized that CP enhance soil microbial diversity. To test the hypothesis, we conducted a study on soils maintained under controlled temperature, humidity and light conditions for six months. Soils (Mexico silt loam) were removed as intact soil cores (ISCs). In treatment, soil received a SCD ProBio Balance Plus™ (PBP), a CP-type product at the rate of up to 100L ha^{-1} (applied as 1% aqueous solution; four treatment regimes were adopted). Control soils received only water. Soils were planted to tomato (*Solanum lycopersicum* cv. Brandywine). Soils were sampled 11 times and soil microbial community profiles were prepared using 16s rDNA DGGE and surveyed to reveal microbial community diversity and shifts in community structures. Upon experiment end tomato plant tissues were harvested; their biomass, C and N contents were analyzed. Soil microbial diversity decline was observed in control soils as well as in two out of four

PBP treatment regimes; diversity decline in treated soils was less pronounced than in control soils. Tomato plants under one treatment regime had increased nitrogen content. Overall, results demonstrate that even though indigenous soil microbial diversity was not enhanced, PBP either suppressed or did not contribute to declines in microbial diversity, which suggests a benign attribute of PBP when applied to soils as a plant growth-promoting product.

4.1. Introduction

Microorganisms are ubiquitous in nature and they likely influence all living organisms. That is particularly evident in plants – microorganisms may benefit plants as well as present considerable potential for suppressing plant growth (Maheshwari, 2012; Oerke, 2006). The soil microbial community is especially involved in modulating plant growth – it is known to be critically important in nutrient cycling, decomposition, and protecting plants from pathogenic organisms (Maheshwari, 2012; Marschner and Rengel, 2007). There is a pronounced global need to reduce the dependence on chemical means of crop and soil management (Dayan et al., 2009; Spadaro and Gullino, 2005). Numerous efforts have been directed at manipulating indigenous microbial communities by inoculating with selected microorganisms to supplement crop and soil management while reducing chemical inputs. For instance, organisms such as plant growth promoting rhizobacteria (Ji et al., 2006; Nautiyal et al., 2013; Niranjan Raj et al., 2005) and plant disease suppressive bacteria and fungi have been evaluated for plant growth stimulation and biocontrol properties (Alamri et al., 2012; Feng et al., 2012; Fravel and Keinath,

1991; Gutierrez et al., 2009; Pal and McSpadden Gardener, 2006; Weller, 1988). These types of microorganisms constitute products known as microbial-based inoculants that are currently on the market (McSpadden Gardener and Fravel, 2002).

Consortia probiotics are class of microbial inoculants that: (1) are characterized as complex cultures where numerous microbial species are allowed to grow together; (2) contain species previously defined as probiotics (Fuller, 1992; Gupta and Garg, 2009). Consortia probiotics have been applied to achieve certain agricultural benefits (i.e. enhanced yield quality, pathogen control or improved breakdown of organic residues in composting) (Heo et al., 2008; Javaid, 2006; Javaid and Bajwa, 2011; Kremer et al., 2000).

Detailed reports discussing modes driving the efficacy of consortia probiotics are scarce. Elucidating principles underlying efficacies of consortia probiotics may allow for optimized use of this type of microbial inoculant and enlarge the array of crop and soil management options available to end users.

In the present work, efficacy of SCD ProBio Balance Plus™ (PBP), which is a representative consortia probiotic product, was studied. The hypothesis of the present research was that application of PBP to the soil enhances soil microbial diversity, which in turn could be linked to improved plant growth. Positive relationships between enhanced soil microbial diversity and improved plant growth have been reviewed previously (Van Der Heijden et al., 2008).To test this hypothesis, soil microcosms collected from cultivated soil in Central Missouri were studied under controlled temperature, humidity and light conditions for six months..

4.2. Material and Methods

4.2.1. Sampling site and Core Removal Procedure

Fifteen intact soil cores (ISCs) were collected from cultivated (minimum-till, soybean-corn rotation) soil plots (Mexico silt loam; fine, smectitic, mesic Vertic Albaqualf) at the University of Missouri Bradford Research and Extension Center in Boone County, Missouri (38° 53' 48" N, 92° 12' 23.5" W). Intact soil cores were collected using a metal cylinder (diameter: 153 mm; height: 127 mm). The inner surface of the cylinder was lined with a polyethylene plastic liner and soil was forced up into the cylinder as the cylinder was driven into soil. The cylinder was immediately retrieved after each plunge and the plug of soil captured therein (the actual ISC) was pushed out with removable liner surrounding it for protection. Because of highly heterogeneous soil cover, the upper layer of organic debris (approximately 1cm) and plant seedlings were removed prior to collecting the ISCs. To ensure optimal protection during transportation, ISCs were wrapped tightly in an additional layer of liner that secured the lower and upper ISC surfaces.

Upon arrival at the laboratory, the protective plastic layers were removed from the ISCs; in cases were the primary liner layer became damaged or did not provide satisfactory enclosure, ISCs were re-lined using new liner sheets. Intact soil cores were transferred to plastic containers (height: 167mm; width 305mm; length: 392mm) (one ISC per container), which were prepared with a 3-cm layer of coarse sand covering the container bottom. With the ISCs resting on the sand layer, additional sand was dispensed around the ISC so that the final sand level was even with the upper surface of each ISC.

Intact soil cores stabilized within pots in this manner were allowed to equilibrate for one week under ambient temperature in the dark.

4.2.2. Planting intact soil cores to tomatoes

Tomato seeds (*Solanum lycopersicum* var. Brandywine) were sterilized in 6% sodium hypochlorite solution and rinsed in sterile distilled water. Seeds were then placed on agar in Petri dishes to germinate. Upon germination (≥ 2mm radicle protrusion), seeds were transferred to biodegradable coconut fiber seedling cups. The seedling cups (~ 50 mm diameter and 15 mm height) were prepared for the experiment by first filling them with soil (same soil as in the ISCs) followed by autoclaving. Seedlings were propagated in cups for 14 days. For the first 5 days, cups and seedlings were placed in plastic bags to prevent excessive loss of water. Throughout the propagation period, seedlings were kept under ambient temperature in an area adjacent to a window that provided access to natural sunlight.

After the propagation period, seedlings in cups were transferred to ISC. Intact soil cores, were placed on growth chamber floor. Small holes were created in the middle of each ISC to accommodate the planting cups and cups with seedlings were placed into the holes (one cup per ISC). Transparent plastic cups were placed upside down on the seedlings to prevent excess evaporation. Cups were installed several millimeters above soil surface in order to ensure fresh air entry. Cups were removed after several days. In the early stage of growth, plants in ISCs were watered with 0.075 L of water every other day which was increased to as much as 0.2 L in later plant growth stages.

4.2.3. Growth chamber settings

The experiment was performed in an environmentally controlled growth chamber (Environmental Growth Chambers GC72 walk-in unit, Chagrin Falls, OH). The day/night period was set to 16 h and 8 h. The daytime was further divided into five sub-periods (0.5 h, 0.5 h, 14 h, 0.5 h, 0.5 h), each with different light, relative humidity or temperature settings. Temperature was set to 23°C for the first, second, fourth and fifth sub-periods and 25°C for the third sub-period. Incandescent lamps were turned on for all sub-periods; 1/3 of fluorescent lamps were turned on during the second and fourth sub-periods; all of the fluorescent lamps were turned on during the third sub-period. Relative humidity was maintained at 75% throughout all sub periods. Temperature and humidity were kept at 18°C and 85% when lights were shut off.

4.2.4. Treatments, controls and the application schedule

SCD ProBio Balance Plus™ was the consortia probiotic product (PBP) chosen for study. This product consists of the following species: *Bacillus subtilis, Bifidobacterium animalis, Bifidobacterium bifidum, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus bulgaricus, Lactobacillus fermentum, Lactobacillus plantarum, Lactococcus lactis, Rhodopseudomonas palustris, Rhodopseudomonas sphaeroides, Saccharomyces cerevisiae*, and *Streptococcus thermophilus*. Cultures are manufactured in a proprietary fermentation process where microorganisms are cultured collectively during specified incubation period in an aqueous medium with sugarcane molasses serving as the primary carbon source. Upon Quality Assurance/Quality Control

release, the product is decanted into appropriate containers and is ready for purchase. According to the manufacturer, this PBP should be used as a "Mother Culture" to produce ready to use products (Secondary Products). Secondary Products and not Mother Cultures are suggested to be used as soil inoculants.

Intact soil cores were randomly organized in five groups of three within the growth chamber. For treatments, PBP was applied as a 1% solution in DI water (solutions were poured directly onto soil in ISCs, spreading solution uniformly on soil surface); four treatment regimes were adopted, each with different application rates or application times (to be referred to as Group I, Group II, Group III and Group IV). The control group received a similar amount of only DI water.

Solutions of 1% AG were applied in varying volumes on the following days: (1) on April 6, 2012, 28.3 ml was applied in Group I, IV and VI; (2) on May 3, 2012, 14.1 ml was applied in Group I and 42.4 ml was applied in Group IV; (3) on September 15, 2012, 14.1 ml was applied in Group I and 42.4 ml was applied in Group II; (4) on October 2, 2012, 14.1 ml was applied in Group I and 42.4 ml was applied in Group III. Consult table 4.1 for summary of amounts applied and dates.

Intact soil cores that were amended with AG solution on a particular day received a lower dose of DI water during the watering stage, while ISCs that were not treated received the full amount of DI water during the watering stage (full amount ranged from 0.075L at the experiment onset to 0.2L at later plant growth stages).

Table 4.1 SCD ProBio Balance Plus™ (PBP) application schedule. Numbers in parentheses represent corresponding rates of PBP in terms of L ha^{-1}.

	Application date			
	04.06.2012	05.03.2012	09.15.2012	10.02.2012
Treatment	Amount of 1% SCDPBP solution in DI water [ml]			
Group I	28.3 (40)	14.1 (20)	14.1 (20)	14.1 (20)
Group II	28.3 (40)		42.4 (60)	
Group III				42.4 (60)
Group IV	28.3 (40)	14.1 (60)		

4.2.5. Tomato yield and C and N content analysis

Upon termination of the growth chamber experiment (October 4, 2012), above-ground biomass of the tomato plants was harvested by severing the stem at the soil surface. Mass of the fresh biomass was recorded and plants were placed in paper bags and oven dried for 48h at 65 °C. Dry plant biomass was determined and dry biomass contents were calculated (dry biomass/fresh biomass). Furthermore dry biomass was analyzed for total organic C and total N contents. Total organic carbon and total nitrogen were determined by dry combustion at 900°C (Nelson and Sommers, 1996) using a carbon – nitrogen analyzer (LECO Tru-Spec C/N Analyzer, LECO Corporation, St. Joseph, MI, USA).

4.2.6. Soil sampling schedule and technique

Soil in ISCs was sampled with a cork borer (8-mm diam.) to a depth of 10 cm. A small, stainless steel rod was used for removing soil samples from the borer. Both borer and rod were carefully cleaned after each sampling by removing soil residues, rinsing in DI water, followed by rinse in 6% sodium hypochlorite, and a final rinse in DI water.

Holes left behind in ISCs after samples were removed were gently collapsed to prevent excess soil aeration and evaporation. Soil samples were stored at -16^{o}C in separate plastic bags. Soil in ISCs was sampled 11 times throughout the experiment: April 6, 2012; April 9, 2012; April 26, 2012; May 8, 2012; June 12, 2012; June 15, 2012; July 23, 2012; September 12, 2012; September 17, 2012; October 2, 2012; October 4, 2012.

4.2.7. Soil DNA and AG DNA extraction technique

Total bacterial DNA was extracted from air-dried soil samples (0.25 g) using the Power Soil DNA® Isolation Kit (MO BIO Laboratories, Inc) by following standard procedures provided by manufacturer. Bacterial DNA was also extracted from 250µl aliquots of AG. Extractions yielded 100ul DNA extracts per sample and extracts were stored at -60°C. The DNA concentration in each purified extract was quantified by UV spectroscopy at 260 nm (GeneQuant *pro*, Amersham Biosciences, Piscataway, NJ) and expressed as mg DNA g^{-1} dry soil. Extracts contained from 14.35 to 117.20 µg DNA g^{-1} of dry soil.

4.2.8. Polymerase chain reaction

Polymerase chain reaction was run using two types of templates: (1) DNA extracts from individual replicates and (2) composite DNA extracts (extracts from three replicates were pooled to make one composite DNA extract).Amplification of 16S rDNA fragments was carried out using the F984GC-R1378 pair of bacteria-specific primers.

Mixtures for PCR reactions contained each primer's (5µl) DNA template and: (1) soil or AG DNA (4µl); or (2) marker DNA (1µl) and REDTaq ReadyMix (35µl of REDTaq ReadyMix was combined with soil and AG DNA, while 50µl was added with marker DNA). Marker DNA contained DNA from the following species; *Bacillus cereus*; *Bacillus pumilus*; *Flavobacterium balustinum* and *Pseudomonas fluorescens.* An Eppendorf Mastercycler Thermal Cycler (Perkin-Elmer, Waltham, MA) was used to carry out PCR reaction. The reaction program was initiated at 94°C for 4 min; initial step was followed by 35 cycles in which temperatures were kept at 94 °C for 1 min, then at 55°C for 1 min and at 72°C for 2 min. In the final step, the temperature was held at 72°C for 10 min and the program went into standby (4°C).

DNA extracts were subjected to PCR twice. First, PCR amplification was carried out separately for DNA extracts from each replicate soil sample. Second, DNA extracts from replicate samples were combined and composite DNA extracts were amplified (Scholz-Starke et al., 2013; Singh and Ramaiah, 2011).

4.2.9. Denaturing gradient gel electrophoresis conditions

DGGE was conducted with 8% (wt/vol) polyacrylamide gel with a linear denaturing gradient of concentration from 37 – 57% (100% denaturant defined as 7M urea in 40% formamide). Aliquots of soil DNA PCR products and marker DNA PCR products (30ul and 12ul, respectively) were loaded into wells in the gel so that marker DNA PCR products occupied external wells in each gel. When PCR products from composite samples were analyzed: (1) 11 wells were loaded with PCR products

representing treated soils; (2) four wells were loaded with PCR products representing control soils sampled on April 6, 2012; May 8, 2012, September 12, 2012; October 4, 2012 (co-analyzed controls); (3) one well was loaded with PCR products from PBP DNA extracts.

Gel electrophoresis was run at 60°C at 130 V for 6 h in 1x TAE with the Bio-Rad Dcode System (Bio-Rad Laboratories, Hercules, CA). Gels were stained with SYBR green I (1:10,000 dilution) in 1x TAE buffer for 20 min to visulize DNA and were photographed with GeneGenius Gel Document System (Syngene, Frederick, MD) using the shortwave band filter. Gene Snap software (Syngene) was used to quantify the band intensities to interpret differences between gel patterns from the various soil treatments.

4.2.10. Assessment of richness, diversity, evenness and similarity of microbial communities

Digitized gel images were enhanced using the Sharpen function (repeated three times for each image) of the GeneGenius Gel Document System. Images were further analyzed with Phoretix 1D Pro (TotalLab Ltd.) and each lane was converted into a densitometric curve; background subtraction was applied (Rolling Ball mechanism, radius = 10) and band positions were converted to Rf values. Band positions and intensities (peak volume) were exported to Excel spreadsheets for subsequent survey of Richness (S); Diversity (H′) and Evenness (E) of microbial community profiles. Richness was defined as the number of bands detected in a soil sample within each lane. Diversity, calculated using Shannon's diversity index [Eq. 1] using peak height for each band as the

input value, was used to compare changes in diversity of microbial communities within all soil samples. Evenness was defined as lnS/H´, and reflects how evenly bands are distributed in a given soil sample. Similarity between DGGE profiles was expressed using a band-based similarity coefficient, Dice's similarity, which was calculated using Phoretix 1D Pro software package. The same package was used for cluster analysis of DGGE profiles using unweighted pair group method with mathematical averages (UPGMA) (Ibekwe et al., 2010).

$$H' = \sum_{i=1}^{i=n} pi \ln pi \qquad [1]$$

In the Shannon's Diversity index (Garcia-Teijeiro et al., 2009), pi is defined as peak height corresponding with band i compared to the sum of peak heights for all detected bands in a lane (Ibekwe et al., 2001)

4.2.11. Statistical analyses

A single factor ANOVA was carried out using SAS Enterprise® 9.3 software. Tukey's HSD multiple comparison test at $\alpha = 0.05$ was performed on tomato tissue characteristics. Similarly, diversity of soil microbial communities was analyzed using Tukey's HSD multiple comparison test at $\alpha = 0.05$. Comparisons were made for all groups at each point in time (11 separate tests were performed). Furthermore, regression analyses approximating H' distribution over time in Group I, Group IV and Control were

compared using GLM procedure and slope coefficients different at $p<0.05$ were assumed to be significantly different.

4.3. Results

4.3.1. Tomato biomass yields; C and N contents

Table 4.2 presents dry matter contents exhibited by tomatoes grown in ISCs in experimental groups. Table 4.3 summarizes C and N plant tissue contents. There were no statistically significant differences ($p<0.05$) in terms of dry matter content among plants grown in ISCs. There were no statistically significant differences in terms of carbon content between treated and untreated plants. However, plants grown in Group I and Group III ISCs exhibit higher C-contents than Group IV. Relative to plant tissue nitrogen content, plants grown in Group IV ISC exhibit significantly higher N-contents than plants from control and Group II ISCs.

Table 4.2 Dry matter content values representative of tomato plants grown in ISCs in the growth chamber study.

Experimental Group	Dry matter content	SD
Control	18.81%	0.62%
Group I	19.19%	1.19%
Group II	19.45%	1.19%
Group III	18.66%	0.24%
Group IV	17.06%	0.96%

Table 4.3 Plant tissue C and N content within the tomato plants on dry mass basis. Asterisk (*) indicates that statistically significant differences were found between marked values and control ($p<0.05$).

Experimental Group	C- Content	SD		N- Content	SD	
Control	42.57%	0.23%		2.07%	0.10%	
Group I	42.67%	0.45%		1.90%	0.10%	
Group II	42.47%	0.55%		1.92%	0.10%	
Group III	43.03%	0.06%		2.05%	0.15%	
Group IV	41.83%	0.31%		2.60%	0.41%	*

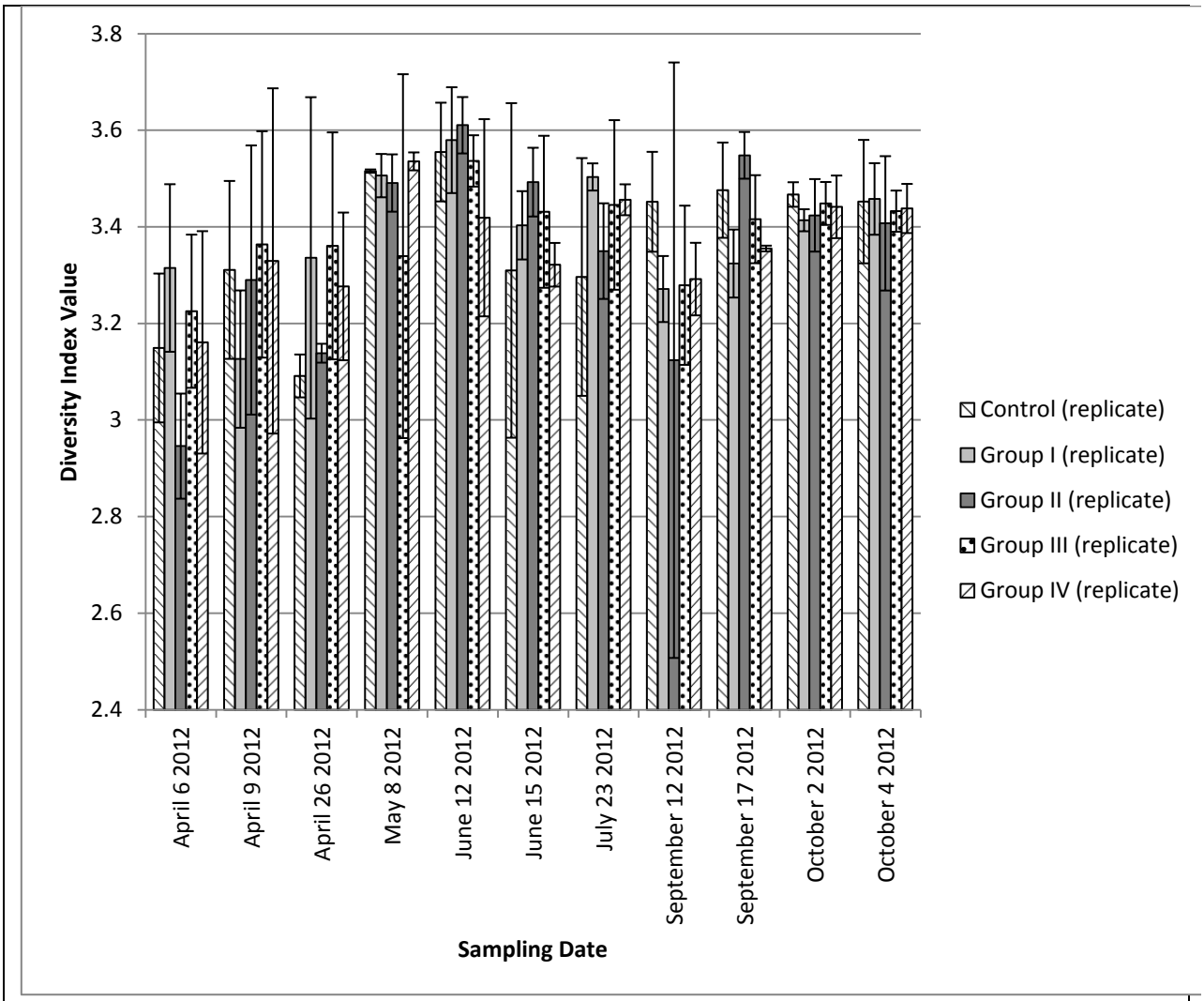
4.3.2. Analysis of DGGE profiles – S, H´and E values

Figure 4.1 summarizes richness (S), diversity (H´), and evenness (E) values representative of DGGE profiles of soil microbial communities (based on replicate soil DNA extracts). Values of S, H', and E related to soil microbial community DGGE profiles produced from composite soil DNA extracts are presented in Figure 4.2.

When replicate soil DNA extracts were used for DGGE profiling of microbial communities, diversity appeared fairly consistent, with an apparently increasing trend until June 12. At later dates, diversity appeared to decline until September 12, and then it seemingly increased again until October 4 and returned to levels comparable with those encountered on June 12. Statistical analysis of H'-values revealed that on September 17,

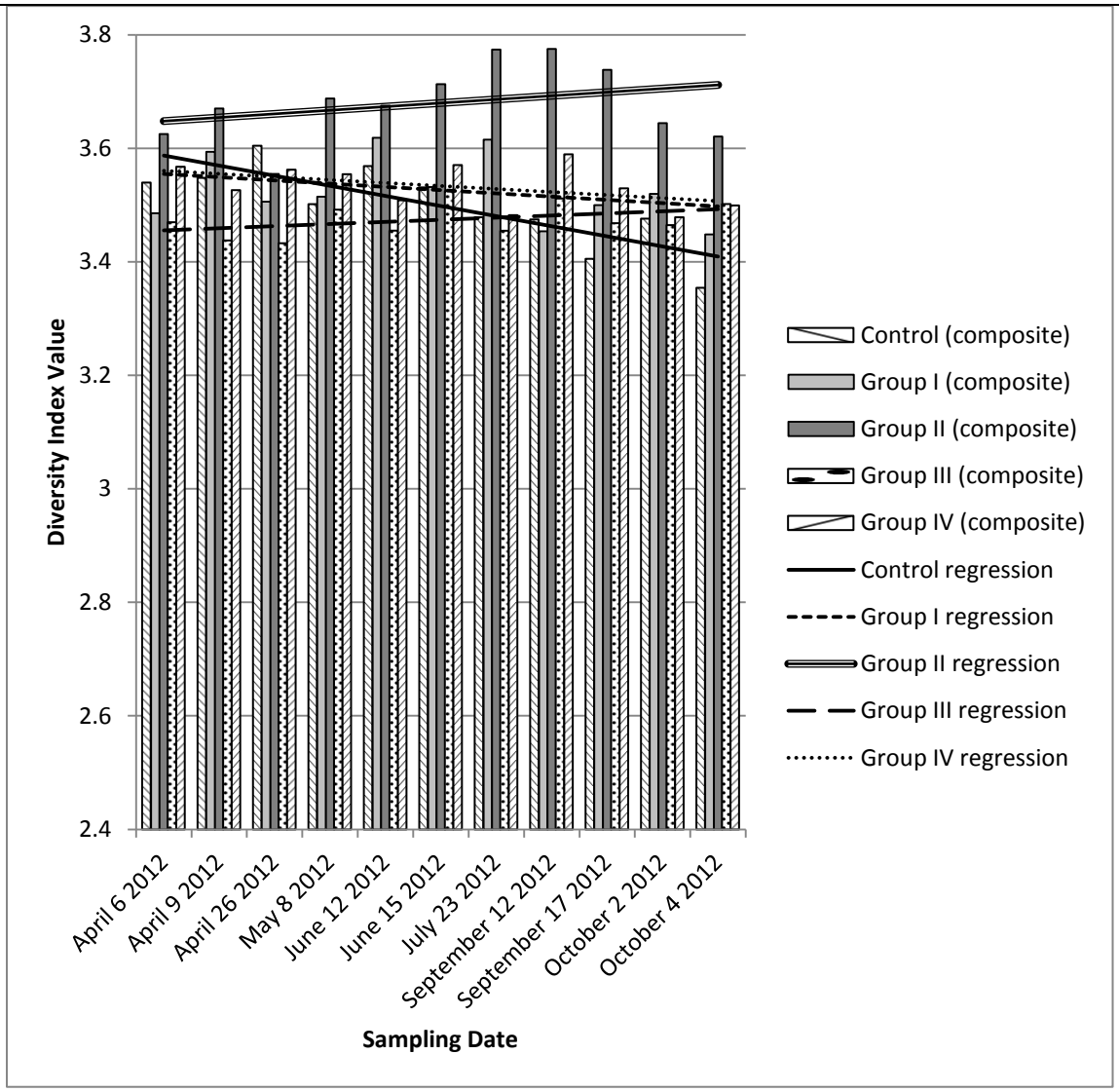
2012, soil microbial communities were less diverse in Group I than in Group II. Furthermore, on September 17, 2012 Group IV has a lower H'-value than Group II. No other significant differences were detected.

In contrast, DGGE profiles produced from composite DNA extracts revealed that diversity stagnated at comparable levels throughout the experiment. However, trends associated with diversity were markedly different among groups. For instance, decline in diversity was observed in control soils as well as in soils from Group I and Group IV. Slopes of the linear regression analyses were significantly less for control samples than for Group IV. Regression coefficients for Group I and the control samples were not statistically different. In contrast, H' values for Group II- and Group III showed increasing trends and differences were statistically significant for each group slope coefficients compared to control.



	IV 6 2012	IV 9 2012	IV 26 2012	V 8 2012	VI 12 2012	VI 15 2012	VII 23 2012	IX 12 2012	IX 17 2012	X 2 2012	X 4 2012
Group I	A	A	A	A	A	A	A	A	B	A	A
Group II	A	A	A	A	A	A	A	A	A	A	A
Group III	A	A	A	A	A	A	A	A	AB	A	A
Group IV	A	A	A	A	A	A	A	A	B	A	A
Control	A	A	A	A	A	A	A	A	AB	A	A

Figure 4.1 Diagram representing diversity index values revealed in DGGE profiles of soil microbial communities studied in growth chamber experiment (replicate soil DNA samples were used). Treatment values followed by different letter within sample dates in accessory table are significantly different ($p<0.05$)



Trend line equation		R^2
Group I	y = -0.0057x + 3.5604	0.1017
Group II	y = 0.0064x + 3.6416 regression different from Control (p =0.0069)	0.0963
Group III	y = 0.0038x + 3.4514 [regression different from Control (p =0.0005)	0.1734
Group IV	y = -0.0053x + 3.5655 [regression different from Control (p= 0.0338)]	0.2168
Control	y = -0.0178x + 3.6052	0.6644

Figure 4.2 Summary of soil microbial diversity (H´) values revealed from composite DNA samples in growth chamber experiment . The accessory table provides regression equations as well corresponding R^2 related to H´values revealed in each group

4.3.3. Similarities between microbial communities analyzed from composite soil DNA samples

Figures 4.3 through 4.7 present UPGMA dendrograms derived from Dice similarity matrices. Each dendrogram was prepared from DGGE profiles resolved on individual gels (composite DNA extracts were used). The dendrogram produced from the control soil microbial community profiles contained two major branches (Figure 4.3). One branch was comprised of profiles representing samples removed between April 6 and June 12, 2012, while the other branch represents samples that were harvested later. Additionally, the profile produced from the last sample (October 4, 2012) falls into a separate, minor branch. This indicates that microbial communities were gradually becoming dissimilar (compared to time zero community).

On Figure 4.4, a dendrogram is shown representing similarity between control soil microbial communities and PBP-treated soil microbial community (Group I). Three clear branches are depicted; each branch incorporates the control data as well as PBP-treated soil associated microbial profiles. Branches link profiles that are temporally related related. For instance, one of branches contains profiles related to samples removed between May 8 and July 23, 2012; while the other cumulates profiles related to samples harvested between September 17 and October 4, 2012. The temporal relation between profiles revealed on the third branch is less clear (treated soils from April 9 and April 26, 2012 as well as well as control soil profile from September 12, 2012 are linked together). Observations indicate that: (1) despite some initial dissimilarity, treated and control soil microbial communities were similar for a large part of the experiment; (2)

time-zero microbial communities and microbial communities revealed at later experiment stages gradually became dissimilar.

Figure 4.5 presents a dendrogram that links control soil microbial communities and PBP-treated soil microbial communities (Group II). The dendrogram reveals two major branches: (1) a branch consisting of control soil microbial profiles as well as AG-treated soil microbial profiles corresponding to samples removed between March 6 and May 8, 2012; (2) a branch composed of treated soil microbial profiles from samples removed between June 12 and October 4, 2012. Within the first branch, two distinct sub-branches are formed; one consisting exclusively of control soil microbial profiles and the other containing treated soil microbial profiles. This demonstrates that control and treated soil microbial communities were dissimilar at time zero and remained so throughout the course of the experiment. Furthermore, the dendrogram indicates that similarity between time-zero microbial community and other communities revealed in the treated soil gradually declined.

Figure 4.6 is a dendrogram that presents similarity between treated soil microbial communities (Group III) and control soil microbial communities. Two major branches are formed that are composed of treated soil microbial profiles differing due to sample period. One branch consist of profiles representing microbial communities encountered in soil samples removed between April 6 and July 23, 2012; the other cumulates profiles corresponding to samples removed between September 12 and October 4, 2012. Profiles related to control soils are found on branches distant from those linking treated soil microbial profiles. This reveals that treated and control soil microbial community profiles were only modestly similar at time zero and remained so for the whole experiment.

Secondly, indications are that microbial communities in treated soil became gradually dissimilar with time-zero soil microbial community.

Figure 4.7 provides a dendrogram where treated soil (Group IV) and control soil microbial communities are linked and two major branches are depicted. One branch consists of treated soil microbial community profiles revealed throughout the entire experiment as well as control soil, time-zero microbial community profile is present on that branch. The other branch combines profiles representing soil microbial communities from control soils encountered at later experiment stages. This suggests that despite initial similarity between treated and control soil microbial communities these microbial communities became dissimilar as time advanced.

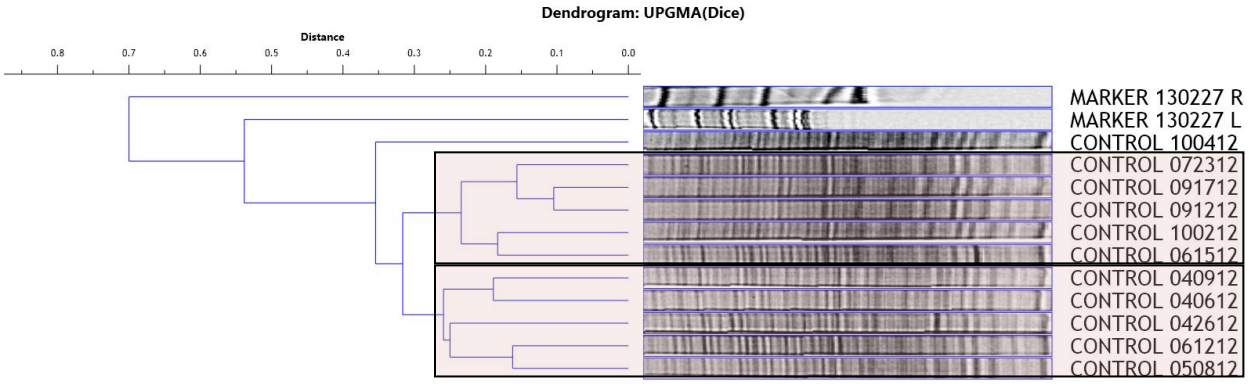


Figure 4.3 Dendrogram representing similarity between microbial communities from control soils. Numbers associated with lane names denote sampling dates (i.e. 04062012 is equivalent to April 6, 2012); L and R in case of Marker names denote left-hand side and right-hand side gel marker, respectively. Boxes indicate distinct branches revealed by UPGMA algorithm.

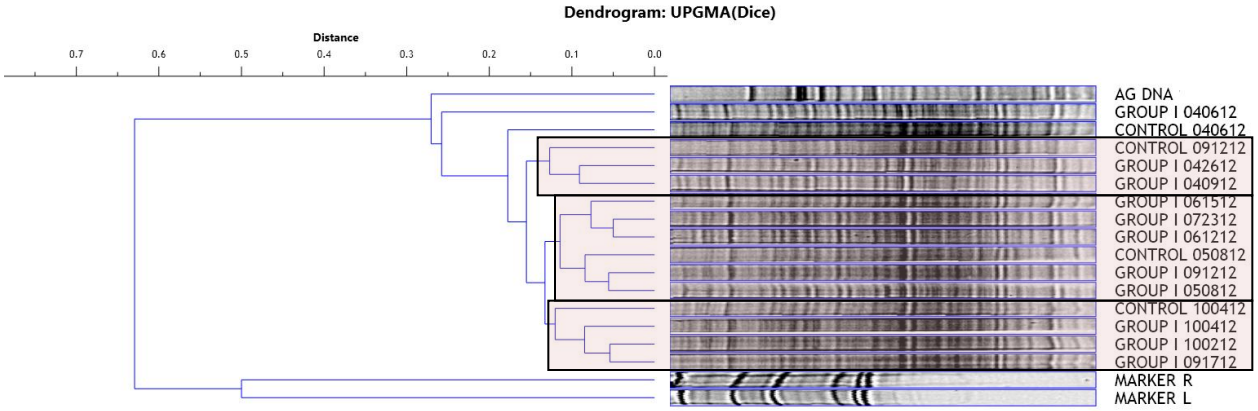


Figure 4.4 Dendrogram representing similarity between Group I and control soil microbial communities. Numbers associated with lane names denote sampling dates (i.e., 04062012 is equivalent to April 6, 2012); L and R in case of Marker names denote left-hand side and right-hand side gel marker, respectively. Boxes indicate distinct branches revealed by UPGMA algorithm.

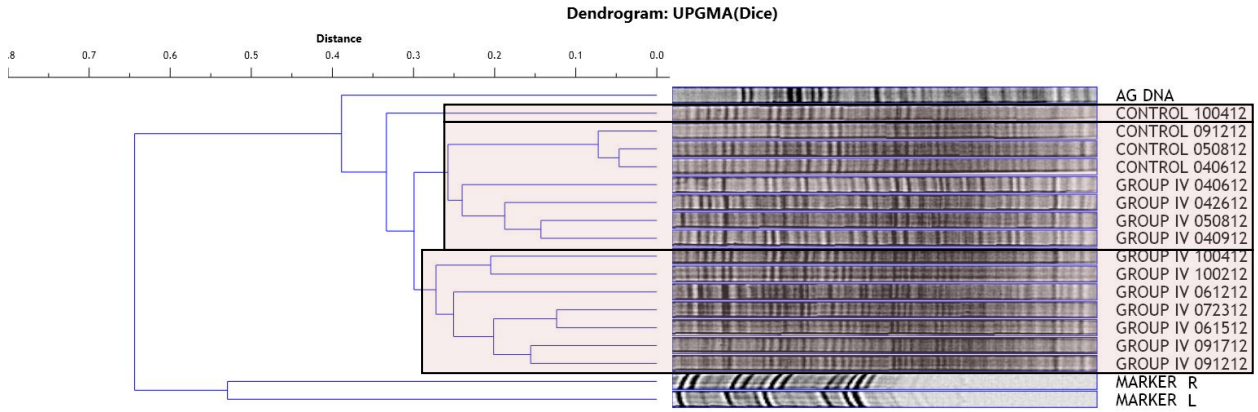


Figure 4.5 Dendrogram representing similarity between Group II (marked as Group IV) and control soil microbial communities. Numbers associated with lane names denote sampling dates (i.e., 04062012 is equivalent to April 6, 2012); L and R in case of Marker names denote left-hand side or right-hand side gel marker, respectively. Boxes indicate distinct branches revealed by UPGMA algorithm

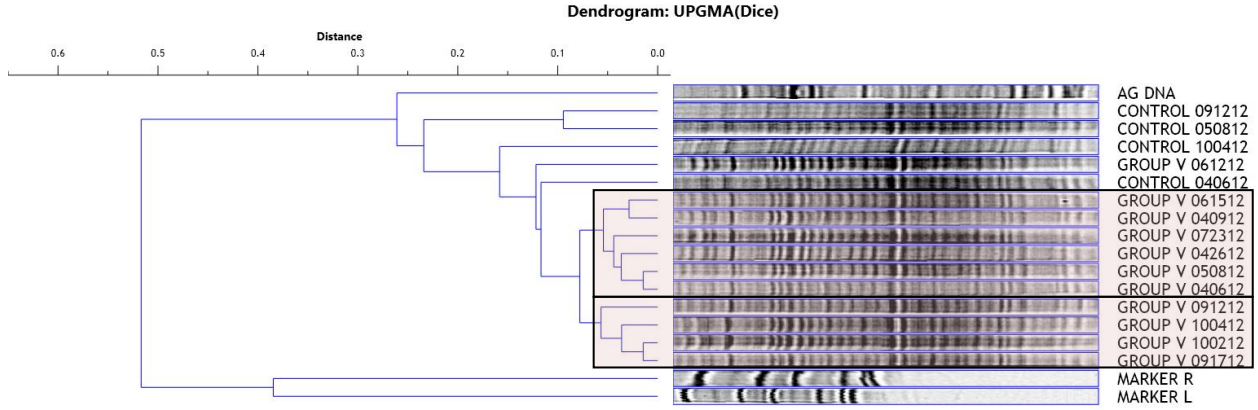


Figure 4.6 Dendrogram representing similarity between Group III (marked as Group V) and control soil microbial communities. Numbers associated with lane names denote sampling dates (i.e., 04062012 is equivalent to April 6, 2012); L and R in case of Marker names denote left-hand side or right-hand side gel marker, respectively. Boxes indicate distinct branches revealed by UPGMA algorithm.

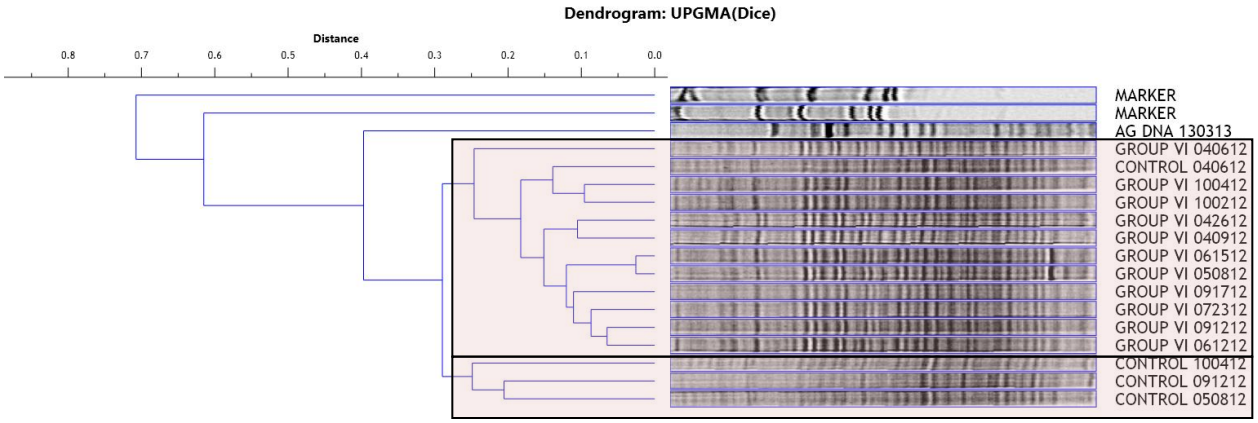


Figure 4.7 Dendrogram representing similarity between Group IV (marked as Group VI) and control soil microbial communities. Numbers associated with lane names denote sampling dates (i.e., 04062012 is equivalent to April 6, 2012). Boxes indicate distinct branches revealed by UPGMA algorithm.

Figures 4.8 through 4.11 depict values representing similarity, based on the Dice similarity coefficient, between soil microbial community profiles revealed from composite soil DNA extracts and AG profiles. As assessed by linear regression (Figure 4.8), the similarity between soil and AG communities increased in Group I as well as in control group. With respect to Figure 4.9 (Group II), a decline in similarity was observed. A decline in similarity was revealed between AG and soil microbial communities from the control soil as well. Relative to Group III (Figure 4.10), a trend of increasing similarity between the treated soil and AG community was revealed. Control soil microbial communities co-analyzed with Group III were gradually less similar to the AG community. On the other hand, microbial communities in soils from Group IV (Figure 4.11) were increasingly dissimilar to AG microbial community. At the same time, the similarity linking control soil and AG microbial communities was found to increase. Therefore, the similarity between soil and AG microbial communities appears to be unrelated to the AG application.

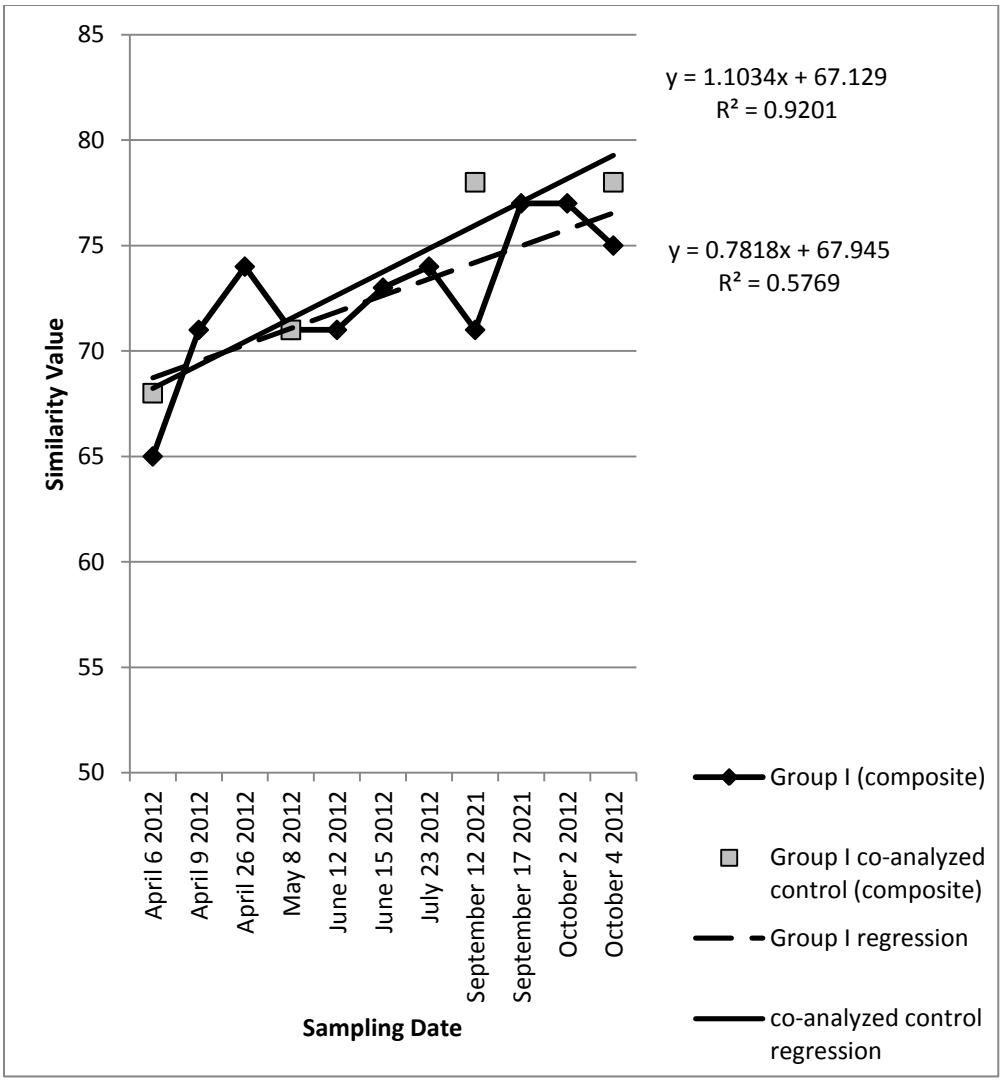


Figure 4.8 Dice similarity values representing similarity between soil and AG microbial communities (Group I and co-analyzed control). Trend lines for each data series as well corresponding equations are given.

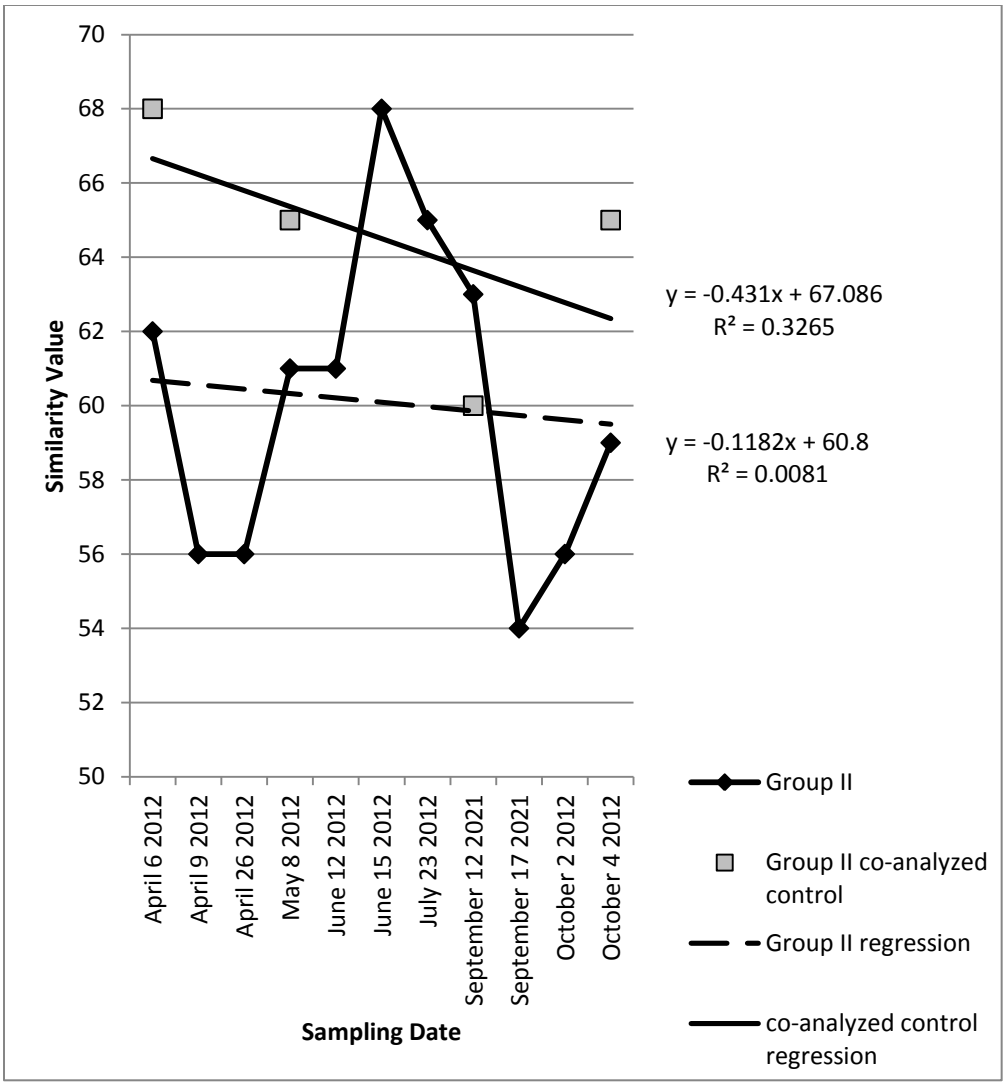


Figure 4.9 Dice similarity values representing similarity between soil and AG microbial communities (Group II and co-analyzed control). Trend lines for each data series as well corresponding equations are given

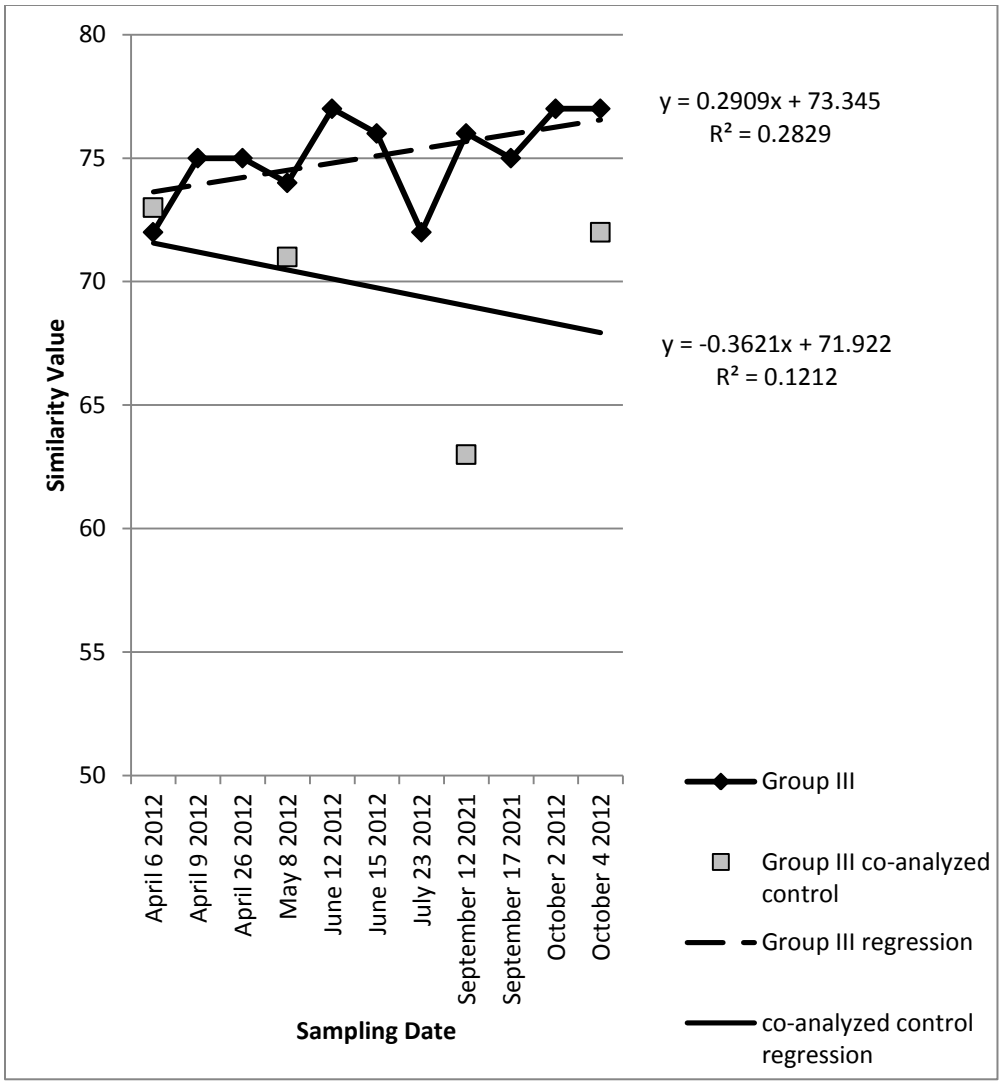


Figure 4.10 Dice similarity values representing similarity between soil and AG microbial communities (Group III and co-analyzed control). Trend lines for each data series as well corresponding equations are given.

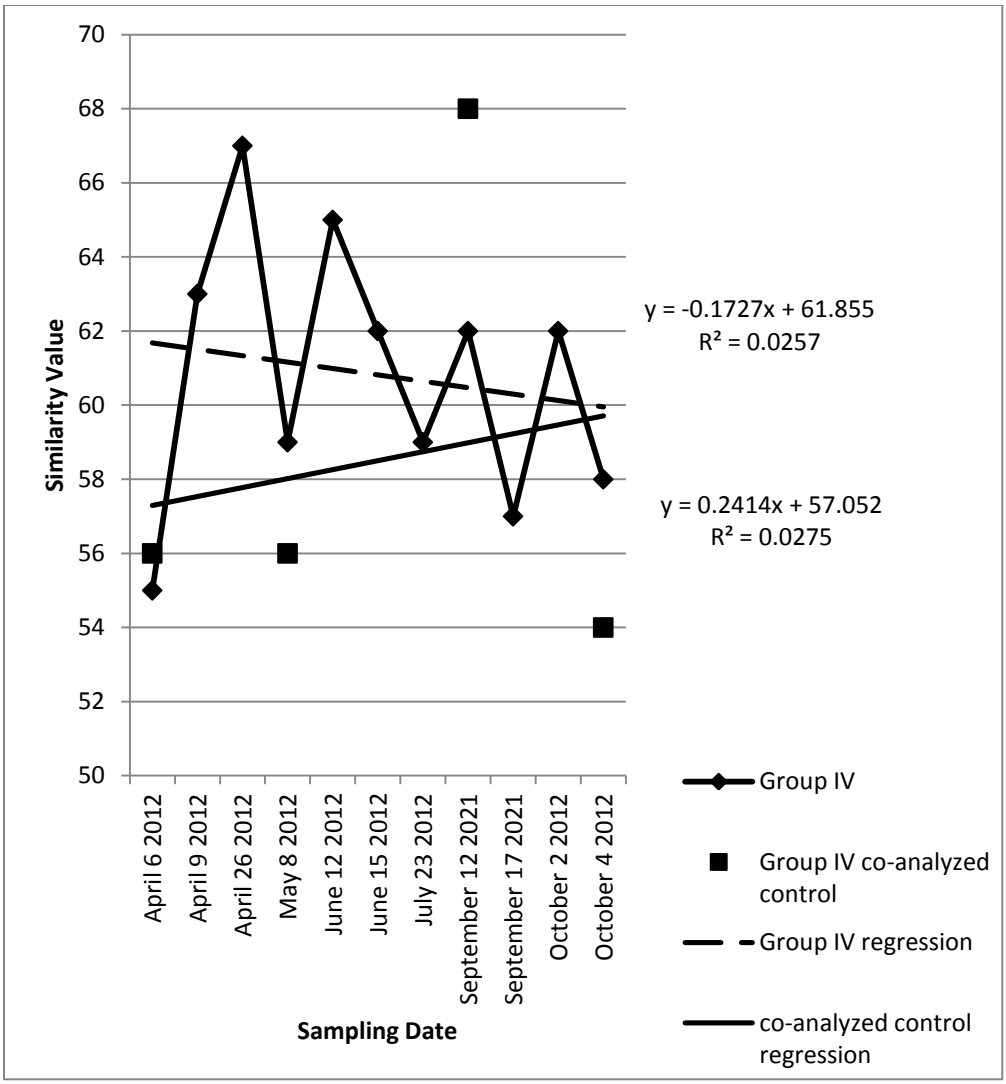


Figure 4.11 Dice similarity values representing similarity between soil and AG microbial communities (Group IV and co-analyzed control). Trend lines for each data series as well corresponding equations are given

4.4. Discussion

4.4.1. Tomato yields; C and N content

Results (Table 4.2) suggest that treatment with AG had no impact on plant tissue dry matter contents Similarly,(Table 4.3) treated and control plants did not differ in terms of plant tissue C-content, however within treatments, Group IV has significantly lower C-contents than those in Group I and Group III. With respect to plant tissue N-content, plants in Group VI have higher levels than the control and Group II plants. Treatment with AG in Group IV (40 L ha-1 and 60L ha-1 applied on April 6 and May 3, 2012) resulted in the lowest plant tissue C-content among all treated plant as well as significantly increased nitrogen content (as compared to control and Group II).

Soils in Group III might have been naturally more supportive for tomato growth contributing to increased C content in plant tissue. Group III was amended with AG only at the experiment's end and therefore it is highly unlikely that AG interacted significantly with tomato growth. It has been shown previously that factors involved in growth chamber experiments (particularly air movement employed for air temperature and humidity control) severely impeded tomato growth and overshadowed treatment effects on tomato growth (Liptay, 1992). Thus, stress associated with air circulation could have heavily influenced results reported in current work.

4.4.2. Inconsistent gel quality and the need for the analysis of composite soil DNA samples

Comparisons between microbial profiles revealed from individually analyzed replicates (Figure 4.1) were highly problematic. Because of large sample set, profiles had to be resolved on numerous. Therefore, varying H´ values observed when replicate soil DNA extracts were analyzed may result from issues associated with variable gel quality or inconsistencies contributed by the PCR procedure (see appendix). They most likely overshadowed actual changes in microbial community structure. Similar issues have been previously reported (Ibekwe et al., 2001). In order to circumvent this problem, replicate DNA extracts representing a sampling event were composited and analyzed as one sample so that the number of profiles analyzed on a gel was maximized (Scholz-Starke et al., 2013; Singh and Ramaiah, 2011). Therefore, in the present work, final conclusions were drawn from observations made using composite sample strategy.

4.4.3. Impact of PBP treatment on diversity of soil microbial communities as revealed from composite sample-based profiles

Trends associated with soil microbial diversity distribution seemed to vary in relation to PBP application. Presumably, application of PBP improved chances of survival of certain soil microbial groups, which were otherwise removed from soils (that would be the case in Group I and Group II). Possibly, microbial utilization of nutrients or organic compounds provided with PBP and subsequent improved microbial growth could

explain less steep decline in diversity in soils in Group I and II. On the other hand, AG-borne microorganisms could have perished in soils and cell constituents could have been utilized as energy sources by indigenous soil microorganisms. An extensive review of events involved in establishment of inoculant-borne microorganisms in soils has been provided by Van Veen et al. (1997). Factors such as predation by soil organisms, soil type, and availability of substrates for microbial growth were discussed as factors that affect and limit the efficacy of microbial inoculants. Such factors may have played an important role in the current experiment. In Groups II and III, H´ values showed an increasing trend, however it is not certain whether this can be attributed to AG. These particular observations are discussed in the upcoming section.

4.4.4. Changes in similarity between soil microbial profiles and soil microbial diversity

Results (Figure 4.3) suggest that the indigenous soil microbial community gradually changed during incubation compared with the time-zero community. This observation coincided with a microbial diversity decline in the control soil (Figure 4.2). Presumably, relatedness between microbial communities changed due to loss of some members of soil microbial community.

With respect to the treated soil (Group I), microbial community structure plausibly changed due to loss of some microbial groups that had been present at time zero. This was indicated by increased similarity between microbial communities from

treated and control soils (Figure 4.4) analyzed at later experiment stages as well as a slight reduction in soil microbial diversity (Figure 4.2).

Microbial diversity revealed in soils in Group II appeared to increase after PBP treatment (Figure 4.2). The diversity enhancement could possibly be attributed to inoculation with AG microorganisms; however, such conclusion may be premature. For instance, very similar observations can be made in Group III, where AG was applied only at the very end of the experiment. Furthermore, the similarity between soil microbial communities from Groups II and III and control remained comparatively low throughout the whole experiment (Figure 4.5.and Figure 4.6, respectively). This suggests that soils studied in the experiment varied in terms of characteristics of their microbial communities and due to this variability, soils from Group II and III were not comparable to control. Soils in these groups could have been populated by dormant or inactive microorganisms whose presence was undetected in initial samples. However, over the course of the experiment, these microorganisms may have become stimulated and reached populations necessary for detection with DGGE. Substances associated with tomato root exudates and/or organic matter in the soil could have increased certain microbial populations. Root exudates have been previously found to be capable of modulating soil microbial community structure (Haichar et al., 2008; Shi, 2009). Others have shown that root exudates facilitate mobilization of soil organic matter and hypothesized that by secreting exudates, plants may influence biophysicochemical events in the soil (Nardi et al., 2000). A comprehensive review of root exudate-mediated impacts on soil microorganisms is provided elsewhere (Bais et al., 2006). On the contrary, soil amendment with AG could have improved soil microbial diversity (for instance, dormant

or inactive microorganisms could have been stimulated due to AG applications). However, due to lack of appropriate controls (soil microbial communities in these groups appeared highly dissimilar to control soil microbial communities), principles underlying changes observed in Group II and Group III cannot be resolved.

Soil from Group IV (Figure 4.7) and control soils at time zero appeared to be comparatively similar. However, similarity diminished as time advanced suggesting soil microbial communities evolved divergently. Concurrently, microbial diversity decline was observed in soils in Group IV (trend was less steep in Group IV than in control) (Figure 4.2). Possibly, treatment with AG selected for improved survival or enhanced growth of certain microbial genera that were otherwise removed from control soil or whose growth was impeded.

4.4.5. Similarity between soil and AG microbial communities

These observations are highly contradictory; for instance inconsistent trends (increasing or decreasing) were found for control soil microbial communities revealed on different gels. This suggests that analytical issues played a considerable role in the process. For instance, false positive observations could have resulted from inconsistent gel quality. On some gels, bands produced by impurities rather than actual soil microbial DNA may have formed (see appendix for details). Similar issues have been reported elsewhere (Ibekwe et al., 2001). On the other hand, some microbial communities associated with AG may be native to the analyzed soils, yet amending soils with AG apparently did not influence the expression of these communities.

4.4.6. Discussion summary

For better clarity, observations made in this study are summarized concisely in Table 4.7. Observations regarding tomato plant tissue characteristics are listed: (1) dry matter content; (2) carbon content and (3) nitrogen content (indicating whether the quantity was less, equal or higher than control). Furthermore, changes in soil microbial diversity are summarized (shown as increasing or decreasing trend). Additionally, similarity between control and treated soil microbial communities is summarized qualitatively (similar or dissimilar). Lastly, trends associated with similarity between soil and PBP microbial communities are compiled (presented whether increasing or decreasing).

Table 4.4 Summary of observations made in the experiment.

	DMC[q]	%C[b]	%N[c]	SMD[d]	Similarity (t_0)[e]	Similarity (end)[f]	PBP Similarity[g]
Control	equal	equal	equal)	↓	N/A	N/A	N/A
Group I				↓	+	+	↑ (↑)
Group II				↑	-	-	↓ (↓)
Group III				↑	-	-	↑ (↓)
Group IV			↑	↓*	+	-	↓ (↑)

[a] Dry Matter Content of tomato plants; downward arrow indicates reduced DMC (p=0.05)
[b] Carbon content of tomato plant tissue; arrows indicate increased (upward arrow) or decreased (downward arrow) C content compared to control(p=0.05)
[c] Nitrogen content of tomato plant; upward arrow indicates increased N content
[d] Trend based on soil microbial diversity (Shannon's diversity index); arrows indicate diversity increase (upward arrow) or decrease (downward arrow), asterisk indicates Group IV where diversity decline (as assessed by H' over time regression) was significantly less than in control. Group I and Control were not statistically significant different. Group II and V were not tested vs. control.
[e] Similarity between soil microbial community from a given group and control revealed at the experiment's beginning . "+" indicates that microbial communities were comparatively similar; "-" indicates otherwise.
[f] Similarity between soil microbial community from a given group and control revealed at later experiment stages. "+" indicates that microbial communities were comparatively similar; "-" indicates otherwise
[g] Similarity between soil and PBP-microbial communities. Arrows indicate increasing (upward arrow) or decreasing (downward arrow) trend in similarity revealed in treated soils; arrows in parentheses correspond to trends revealed from co-analyzed control soil microbial community profile.

4.5. Conclusions

All soil microbial communities studied appeared to be dynamic entities and community dynamics could be explained in terms of diversity decline as well as diversity enhancement. In this study, application of PBP did not drastically disrupt soil microbial community structure. Therefore biological and ecological functions mediated by soil microorganisms were most likely unaffected. However, one treatment (Group IV) have significantly reduced soil microbial diversity decline and significantly enhanced tomato tissue nitrogen content. Probably, PBP may influence plant growth and tissue characteristics through balancing the stability of soil microbial communities. Some microorganism present in PBP (or closely related) may have naturally existed in soils, however their presence was unaffected by PBP treatment. These results will need to be verified in further studies employing other consortia probiotic products in order to investigate if a wider class of probiotic products is similarly efficacious or whether they are PBP-specific..

4.6. Future work

Future studies should thoroughly examine the impact of PBP as well as other consortia probiotics on soil microbial communities. For instance, this impact should be studied on different soil types and/or under various management situations. Moreover, plants should be selected based on their immunity to stresses induced by growth chamber conditions. Additionally, experiment duration should be extended and cover the periods

corresponding to several growing seasons. Such design would provide insightful observations into efficacies exhibited in diverse soil and plant settings. Possibly, these observations could be useful from the perspective of managing probiotic applications for achieving best results in crop and soil management.

Application of the 16s rDNA PCR DGGE procedure appeared to be a powerful analytical tool and could be used in future analyses; however, procedures would need to be improved to yield more accurate observations. For instance replicated DNA extractions should be adopted and PCR products from replicated DNA extracts should be resolved to reveal the extent of biases introduced by extraction and PCR procedures. Furthermore reference ladders (markers) for DGGE profiles should be prepared from microbial communities indigenous to study sites. This could allow for more accurate expression of band positions in terms of Rf values and alleviate ambiguities associated with surveying DGGE profiles for microbial community similarities (Ponnusamy et al., 2008; Valášková and Baldrian, 2009).

Moreover, future studies should employ improved protocols for selecting ISCs for the study. In more detail, ISCs should be equilibrated for longer periods and analyzed to reveal the similarity between microbial communities. Intact soil cores should be carefully chosen for treatment or control, based on whether they tested positively for satisfying levels of soil microbial communities. This may require limiting the number of treatment regimes. Furthermore, the initial ISC amounts harvested should be sufficiently higher than the amount needed for the actual experiment. That would increase the chance of finding soils whose microbial communities are adequately similar. On the other hand, another approach suggested by van Elsas et al. (2012) could be employed. Briefly, in that

study, soils were sterilized and subsequently populated with soil microorganisms (microbial isolates were suspended in water and applied to sterile soils). It is believed that such an approach would ensure satisfactory similarity between microbial communities in soils prior to experiment onset.

5. Chapter 5: Study 3 - Impacts of a Consortia Probiotic Inoculant on N-Acyl Homoserine Lactone Components Involved in Quorum Sensing By Soilborne Bacterial Phytopathogens

Abstract

Numerous bacteria control their phenotypes in a cell density-dependent manner and this phenomenon has been termed quorum sensing (QS). Signals employed in QS (N-Acyl Homoserine Lactones or NAHLs) can be targeted for subsequent degradation and this can be utilized in disease control for preventing virulent phenotypes. Two particular consortia probiotic products SCD ProBio Balance Plus™ (PBP) and SCD BioAg™ were evaluated for their impact on NAHLs utilized by pathogenic species: N-(3-oxododecanoyl)-homoserine lactone (AHL) and N-(3-oxohexanoyl)-L-homoserine (AHLEc). Mixtures of NAHLs and either probiotic cultures or their fractions concentrated by centrifugation were incubated under room temperature for up to 8 hours. Following incubation, NAHLs were extracted with trichloromethane and NAHL concentrations in extracts were monitored and quantified using gas chromatography-mass spectrometry. SCD ProBio Balance Plus™, its supernatant and the concentrated, washed cells showed significant reduction of AHL concentration (69.16%, 34.09% and 51.68%, respectively).However, when another PBP batch was used no AHL degradation occurred. Furthermore, SCD BioAg™ removed 100% of AHL within 8h. Consortia probiotic products employed in this study did not degrade AHLEc. Results indicate that consortia

probiotic cultures may exert their impacts by interfering with the mechanism involved in QS, a necessary virulence step for pathogenic activity.

5.1. Introduction

5.1.1. Quorum sensing

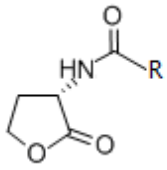
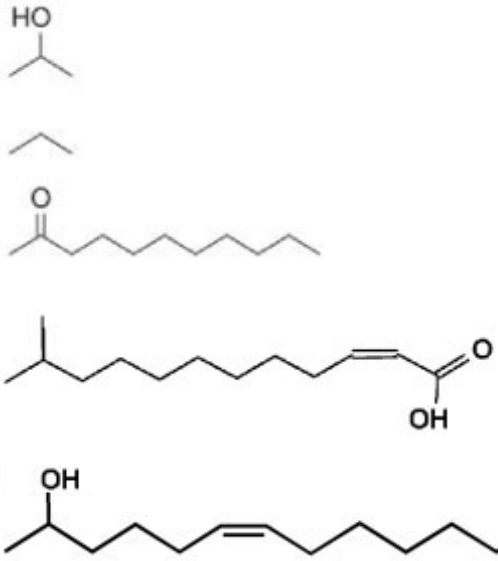
The phenomenon of quorum sensing (QS) refers to the ability of bacteria to monitor their populations and to alternate gene expression upon reaching a threshold population. Numerous bacterial species depend on QS in mediating population behavior. Key elements of QS are similar for many bacteria; for example, QS is mediated by diffusible molecules (referred to as autoinducers). However, signaling molecules and receptors involved in QS vary between bacterial genera. In Gram negative bacteria, QS uses N-acyl homoserine lactones (NAHL). In contrast, QS in Gram positive bacteria depends on oligopeptides that act as autoinducers (Miller and Bassler, 2001).

5.1.2. N-Acyl homoserine lactones-mediated QS systems in Gram negative species

Studies conducted in 1970s and 1980s revealed that marine bacteria use N-acyl homoserine lactones (NAHLs) (see Table 5.1) for synchronizing expression of genes responsive for bioluminescence (Eberhard et al., 1981; Nealson et al., 1970; Nealson and Hastings, 1979). Further research has shown that numerous NAHLs are employed in gene expression regulation in a variety of Gram negative species (eg. Bainton et al., 1992; Piper et al., 1993; Throup et al., 1995). These findings had pronounced impact on understanding prokaryotic interactions at the molecular level. Unlike it had been hypothesized before, prokaryotic organisms were utilizing intercellular communication

signaling systems; a trait previously thought to be exclusive to eukaryotes (Miller and Bassler, 2001). Quorum sensing in Gram negative species controls numerous traits some of which are listed in Table 5.2.

Table 5.1 Examples of QS mediated traits in Gram negative species after Williams (2007)

<table>
<tr><td colspan="2">General structure of NAHL</td></tr>
<tr><td colspan="2"> 
After: Ryan and Dow (2008)</td></tr>
<tr><td>Example R Groups</td><td>Relevant Species</td></tr>
<tr><td>  </td><td>Vibrio harveyi
(Ryan and Dow, 2008)

Pseudomonas aeruginosa
(Ryan and Dow, 2008)

Xanthomonas fastidosa
(Ryan and Dow, 2008)

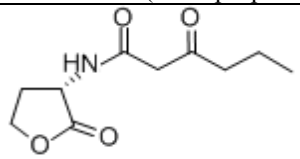
Rhizobium leguminosarum
(Taga and Bassler, 2003)</td></tr>
<tr><td colspan="2">Structure of N-(3-oxopropanoyl)-L-homoserine lactone, NAHL identified by Eberhard et al. (1981)</td></tr>
<tr><td colspan="2"> 
After: http://www.chemicalbook.com/ProductChemicalPropertiesCB7222683_EN.htm</td></tr>
</table>

Table 5.2 Examples of QS mediated traits in Gram negative species after Williams (2007)

Species	QS-mediated trait
Aeromonas hydrophila	• Biofilm formation, exoproteases synthesis, virulence
Aeromonas salmonicida	• Exoproteases synthesis
Agrobacterium tumefaciens	• Plasmid conjugation
Agrobacterium vitis	• Virulence
Burkholderia cenocepacia	• Exoenzymes syntheis, biofilm formation, swarming motility, siderophore synthesis, virulence
B. pseudomallei, B. mallei	• Exoenzyme synthesis, virulence • Virulence
Chromobacterium violaceum	• Synthesis of exoenzymes and violacein; cyanide formation
Erwinia carotovora	• Sythesis of Carbapenem antibiotics and exoenzymes; virulence
E. stewartii	• Exopolysaccharide synthesis
Pseudomonas aeruginosa	• Synthesis of exoenzymes, toxins; protein secretion, biofilm formation, swarming motility, virulence
P. aureofaciens	• Synthesis of phenazine antibiotics and protease; aggregation, root colonization, colony morphological changes
P. chlororaphis	• Phenazine-1-carboxamide synthesis
P. putida	• Biofilm development
P. fluorescens	• Mupirocin synthesis
Rhizobium leguminosarum	• Root nodulation and symbiosis, plasmid transfer, growth inhibition, stationary phase adaptation
Rhodobacter sphaeroides	• Aggregation
Serratia sp. ATCC 39006	• Synthesis of antibiotics, exoenzymes and pigments
S. liquefaciens	• Exoprotease and biosurfactants synthesis, biofilm development
S. marcescens	• Sliding motility, biosurfactants and pigments synthesis
S. proteamaculans	• Exoenzyme synthesis
Sinorhizobium meliloti	• Root nodulation and symbiosis, exopolysaccharide synthesis
Yersinia enterocolitica	• Swimming and swarming motility
Y. pseudotuberculosis	• Motility, aggregation

5.1.3. Aspects of QS circuits in Gram negative species

The structure of QS signals systems in *V. fischerii* provides the paradigm for studying QS in Gram negative species in general. In these species, QS circuits are referred to as LuxI/LuxR (Fig. 5.1); LuxI and LuxR are proteins found in bacterial cytoplasm. The former is an autoinducer synthase while the latter acts to (1) bind the autoinducer and (2) activate transcription of respective operons (*luxICDABE* in case of *V.fischeri*). Autoinducers are released from cytosol by means of free diffusion and then permeate to other cells where they may bind with LuxR proteins and trigger target gene expression. Many species exhibit QS systems that vary from the paradigm *V. fischerii* system (Miller and Bassler, 2001). For the purpose of this study, two such systems will be explained in more detail: (1) a virulence system found in *Pseudomonas aeruginosa* and (2) a virulence and antibiotic synthesis system present in *Erwinia carotovora.*

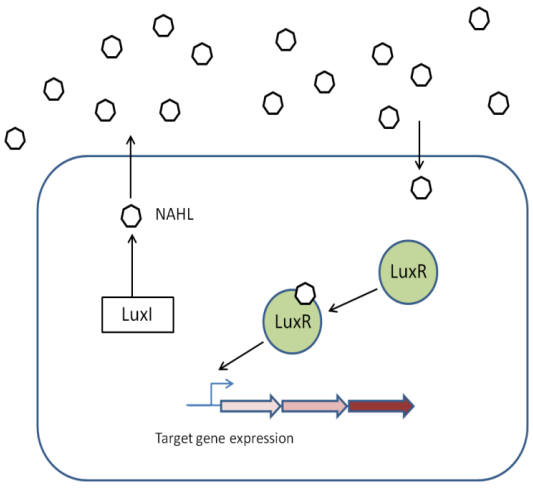


Figure 5.1 Canonical quorum-sensing circuit found in most Gram-negative bacteria (after Xavier and Bassler, 2003)

5.1.4. *P. aeruginosa* virulence system

Pseudomonas aeruginosa employs QS to control expression of as many as 300 genes. For example, synthesis of numerous virulence factors responsible for tissue destruction in host organisms is controlled by LasI/LasR and RhlI/RhlR systems (Fig. 5.2). LasI and RhlI are autoinducer synthases that synthesize N-(3-oxododecanoyl)-homoserine lactone (AHL) and N-(butryl)-homoserine lactone (BHL), respectively. Complexes of LasR and RhlR are cognate receptors of AHL and BHL respectively. Besides controlling the expression of virulence independently, these systems are interweaved in a feedback loop that allows for controlled release of virulence factors to maximize chances of successful attack on a host (Venturi, 2006).

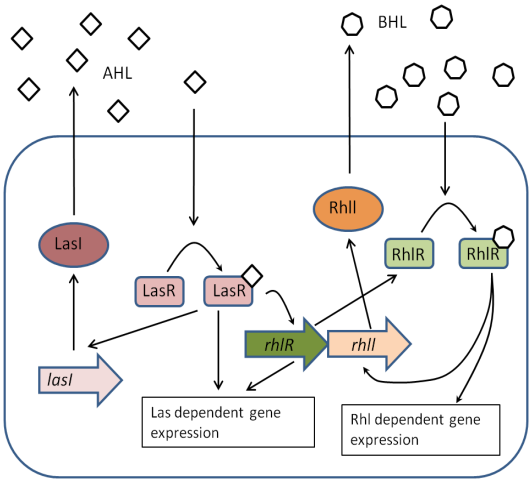


Figure 5.2 The Pseudomonas aeruginosa LasI/LasR-RhlI/RhlR quorum sensing system (after Waters and Bassler, 2005)

5.1.5. *E. carotovora* virulence system

Vast arrays of pathogenic phenotypes in *E. carotovora* are known to be controlled by QS and there are several marked differences in receptors and NAHL signal R groups among strains. Two autoinducers are found to play major role in QS in *E. carotvora*: N-(3-oxohexanoyl)-L-homoserine lactone (AHLEc), and/or N-(3-oxooctanoyl)-L homoserine lactone (OHL). Based on the NAHLs employed in QS, Erwiniae are divided into Class I and Class II Erwiniae. Organisms in Class I synthesize primarily OHL and some quantities of AHLEc, while those in Class II produce primarily AHLEc and no appreciable amounts of OHL are known to be synthesized (Barnard and Salmond, 2007). The summary of NAHL QS systems in *E. carotovora* strains can be found in Table 5.3.

Table 5.3 Example QS systems in E. carotovora (after Barnard and Salmond, 2003).

Erwinia carotovora strain	Major QS signal(s)	NAHL synthase (LuxI homologue)	NAHL receptor (LuxR homologue)	QS regulated trait
Class I Erwiniae				
EC153	OHL and	AhlI	ExpR	Production of exoenzymes
SCC3193	AHLEc	ExpI	ExpR1, ExpR2	Virulence, production of exoenzymes
Class II Erwiniae				
ATCC39048	3-oxo-C6-HSL	CarI	CarR, ExpR, VirR	Carbapenem antibiotic production virulence, production of exoenzymes
SCRI193		ExpI	ExpR, VirR,	Virulence, production of exoenzymes
71		AhlI	ExpR1, ExpR2	Virulence, production of exoenzymes

5.1.6. Bacterial benefits associated with QS

Numerous authors discuss that QS provides diverse advantages to bacteria that possess this system (Chhabra et al., 2005; Swift et al., 2008). Particularly, in case of Erwiniae, it has been suggested that QS allows for: (1) frugal allocation of resources and energy that are consumed by the formation of virulence factors or antibiotics, and (2) enhanced inhibitory activity of secreted antibiotics (Barnard and Salmond, 2007). In detail, Barnard and Salmond (2007) argue that without successful QS control, traits would be exhibited before securing the quorate population level and that could: (1) insufficiently damage host tissue, prevent satisfactory release of nutrients and thwart microbial development; (2) prematurely elicit host immune response and eradicate microorganisms; and (3) bring insufficient microbial populations to synthesize amounts of antibiotics that are inadequate to effectively compete for a niche. In pseudomonads, similar arguments are put forward to discuss benefits of adopting QS to regulate population phenotypes (Darch et al., 2012).

5.1.7. QS disruption - a promising strategy for disease control

Recently, a fundamental change in the approach to disease control has been observed. For a long time, disease control was provided almost exclusively by antimicrobials, however, recent findings on QS promise invention of new class of drugs in disease control - antipathogenic drugs (Hentzer and Givskov, 2003).

While antimicrobials debilitate the growth, or remove microorganisms, antipathogenic drugs prevent microorganisms from expressing virulent traits and produce no direct harm to microorganisms. Such a mode of action makes antipathogenic drugs unlikely candidates for promoting drug-resistance in target species and, given the context of antimicrobial resistance development (Freire-Moran et al., 2011) in pathogenic microorganisms, makes antipathogenic drugs especially promising agents. The following strategies have been proposed for QS disruption: (1) inhibition of signal generation; (2) inactivation of signal molecules; and (3) inhibition of signal reception by receptor competition/blocking (Hentzer and Givskov, 2003). Another disease control strategy that depends on QS disruption is to increase autoinducer concentrations (Williams, 2007).These strategies have been extensively explored and it resulted in development of disease treatment methods, some of which have been patented. Pan and Ren (2009) may be consulted for a comprehensive review of patents and methods associated with QS disruption. Present review narrows its scope to only a brief discussion of one of aspects of QS disruption, which is inactivation of signaling molecules.

5.1.7.1. Inactivation of signaling molecules

Signal molecules can be inactivated by chemical or enzymatic degradation and molecules can be metabolized. N-acyl-homoserine lactones undergo structural changes at pH greater than 7. Under such conditions, lactonolysis occurs (a process of lactone ring opening) and the signalling molecule is inactivated (Rasmussen and Givskov, 2006).

A considerable amount of research on QS interference has focused on enzymes that are capable of degrading NAHLs. Two classes of enzymes have been identified: (1)

NAHL lactonases whose catalytic properties facilitate lactone ring opening; and (2) NAHL acylases, which liberate homoserine and the corresponding fatty acid by breaking the amide bond in NAHLs. First reports on NAHL degrading enzymes (NAHL lactonase) came from studies on *Bacillus* species isolated from soil. Subsequent investigations have shown that NAHL-degrading enzymes (lactonases and acylases) are widespread among prokaryotes (Dong and Zhang, 2005). .

5.1.8. Methods of studying NAHLs

Studies on NAHLs employ two general methods of NAHL identification: (1) the use of reporter organisms (Chu et al., 2011; McClean et al., 1997; Molina et al., 2003; Shaw et al., 1997); and (2) NAHL with molecular methods (Cataldi et al., 2008; Cataldi et al., 2004; Shaw et al., 1997). With respect to studies on QS signal degradation, numerous researchers found it advantageous to (1) utilize synthetic NAHLs for degradation trials and (2) apply identification methods for subsequent assessment of degradation levels (Byers et al., 2002; Dong et al., 2004; Medina-Martínez et al., 2007; Ramos et al., 2012; Wang and Leadbetter, 2005). Recently, gas chromatography/mass spectrometry (GC/MS) analysis has been used in profiling NAHLs and GC/MS appears as another useful tool for direct assessment of treatment effects on NAHLs (Cataldi et al., 2004).

5.1.9. Probiotics

The term probiotics defines a range of microbial species that may improve host species condition upon ingestion by improving its digestive health (Gupta and Garg, 2009). Recently however, scientists propose to expand such a definition of probiotics to include areas of probiotics use that go beyond digestive health. For example, with respect to aquacultures, it has been argued that the definition of probiotics be extended to include organisms that may affect aquatic species' well-being by improving their environment conditions (Kesarcodi-Watson et al., 2008).

5.1.9.1. SCD Probiotics, a consortia probiotic product

Some probiotic organisms have long been recognized as antagonists of certain plant pathogens (Visser et al., 1986) and several attributes of probiotic organisms are believed to underlie pathogen control efficacy (i.e. synthesis of antibiotic-like substance by lactic acid bacteria). Numerous probiotic inoculants consist of single species cultures or blends of microorganisms individually cultured. Another type of probiotic inoculants includes complex, multimicrobial media where microorganisms are cultured collectively for extended periods of time. The latter type is referred to as consortia probiotics. Such products have been tested in field experiments and instances were recorded where application of consortia probiotics allowed for improved production results (Fatunbi and Ncube, 2009; Javaid, 2006; Javaid and Bajwa, 2011; Khaliq et al., 2006; Shah et al., 2001). These studies offer no in-depth discussion of avenues by which probiotic products deliver their beneficial impacts.

Recently, new formulations of consortia probiotics have been introduced on the market by SCD Probiotics. These products are manufactured following a proprietary procedure by which numerous species of probiotic and other microorganisms are cultured collectively in aqueous media for extended periods of time. There are two types of cultures manufactured by SCD are (1) "Mother Cultures" and (2) Secondary Products (ready to use products). In the present work both types of cultures were studied. Mother Cultures are cultures used in the manufacturing process of Secondary Products. Depending on the manufacturing process, Secondary Products gain unique properties that distinguish them from Mother Cultures and make them better suited for application (i.e. field applications). Application of the product has reduced pathogen outbreaks in agriculture (SCD, 2000; SCD, 2002a; SCD, 2002b). However, there is a lack of basic scientific investigation for elucidating modes of action associated with the product.

In the present work, two probiotic cultures; SCD ProBio Balance Plus™ (PBP) and BioAg™ (a mother culture and a secondary product, respectively) were surveyed for their impact on autoinducers involved in QS in some Gram-negative bacteria species. Improved understanding of the efficacies of the two products in particular and consortia probiotics in general could enhance the array of pathogen control options for the end user's disposal.

The objective of this study was to elucidate modes of action associated with consortia probiotics. The particular types of cultures studied were SCD probiotic cultures: PBP and BioAg™.. It was hypothesized that the SCD probiotic cultures could synthesize enzymes that degrade QS signals of the pathogens tested. To test the hypothesis, NAHL-

containing mixtures were incubated with or without probiotic amendment and GC/MS was employed to quantify the extent of NAHL degradation.

5.2. Material and Methods

5.2.1. Experimental Design

The impact of probiotic cultures on autoinducers was studied in triple replicates using autoinducers AHL and AHLEc. For treatments, autoinducers were combined with either (1) 10% aqueous solution of probiotic culture or (2) 10% aqueous solutions of culture fractions retrieved by centrifugation or (3) whole probiotic cultures. In controls, autoinducers were combined with de-ionized water (DI) or with DI that was first adjusted to pH 3.5 with acetic acid. Autoinducers were analyzed separately. After the exposure, autoinducers were extracted with chloroform, and chloroform extracts were analyzed by GC/MS to reveal their concentrations.

5.2.2. Stock solutions and calibration curve development

An analytical stock solution of AHL was prepared with HPLC-grade acetonitrile (ACN) to achieve a final concentration of 100 mg L^{-1} (371 μM). A bioassay stock solution of AHL was prepared with HPLC-grade methanol (MEOH) to achieve the concentration of 5392 mg L^{-1} (20 mM). With respect to AHLEc, a general purpose stock solution was prepared with MEOH and yielded the concentration of 4000 mg L^{-1} (18.9 mM).

Analytical stock solution of AHL and AHLEc stock solution were adjusted separately with ACN or HPLC-grade chloroform to yield standard dilutions. Concentrations ranging up to 100 mg L^{-1} were employed for standard curve preparation.

5.2.3. First Bioassay setup and sample preparation for GC/MS analysis

SCD ProBio Balance Plus™ (lot code: AG10154-14) was centrifuged at 10,000 rpm at 4 ^{o}C for 10 min. Pellet was resuspended in phosphate buffered saline (pH=7.4) (PBS) and centrifuged repeating the previous method. Cell pellets were sonicated on ice for one minute, resuspended in 250ml of PBS and centrifuged again. Cell pellets and supernatants were stored in separate containers at 4 ^{o}C. Before being used for the bioassay, a loopful of cell pellet was mixed with 2 ml of PBS and vortexed. According to the manufacturer, this PBP batch contained $7x10^{6}$ lactic acid bacteria cells per ml (data provided by SCD quality assurance/quality control department). Centrifugation allowed to concentrate cells by the factor of 12.5 and cell density of supernatant corresponded to that of culture diluted by the factor of 109.7 (consult appendix for details).
Mixtures for treatments consisted of: (1) 100 µl of PBP, PBP supernatant or resuspended PBP pellet; (2) 10 µl of AHL bioassay stock solution; (3) 890 µl of DI. The control mixture contained 10 µl of AHL bioassay stock solution and 990 µl of DI. Final concentration of AHL was 54 mg L^{-1} or 200 µM. Mixtures were accommodated in plastic, 1.5 ml centrifuge tubes, sealed and incubated at room temperature for 2 h.

After the incubation, solutions were transferred to separate glass tubes. The autoinducer compound and its degradation products in solutions were extracted repeatedly with chloroformliquid/liquid extraction three times. During each extraction step, a fresh, 5 ml volume of chloroform was added to each tube, contents were shaken vigorously for 30 s and the chloroform fraction was collected from the bottom of the flask using glass pipette while an aqueous fraction was left behind. All the chloroform

fractions were combined. Fractions were stored at -16 ^{o}C until analysis. Gas chromatography/mass spectrometry analysis of chloroform fractions was performed using a Varian 3400cx GC (Varian Inc., Walnut Creek, CA) with a cross-linked methylsiloxane DB-5 capillary column (30 m x 0.25 mm I.D.) (Hewlett Packard, Palo Alto, CA) combined with a Varian Saturn 2000 ion-trap mass selective detector (Varian Inc.). The detailed GC/MS procedure is described below. Concentrations reported by GC/MS analysis were multiplied by a factor of 15 to compensate for dilution associated with the liquid/liquid extraction procedure.

5.2.4. Second Bioassay setup and sample preparation for GCMS analysis

Biodegradation of AHL and AHLEc was analyzed with 10% solutions of probiotic cultures PBP (lot code: AG12183-10-T9) or BioAg (lot code: BA 11223-01). Furthermore, biodegradation assay for AHLEc was performed with undiluted PBP and BioAg. Mixtures for bioassay with 10% culture solutions consisted of: (1) 60 μl of probiotic culture; (2) 12 μl of AHL bioassay stock solution or AHLEc stock solution; (3) 528 μl of DI water. Undiluted culture bioassay mixtures contained 588 μl of PBP or SCD BioAg™and 12 μl of AHLEc srock solution. Control mixtures comprised of 12μl of AHL bioassay stock solution or AHLEc stock solution and 588 μl of DI that had been adjusted to pH 3.5 with acetic acid to match with the pH of cultures used. Final concentrations of autoinducers were 108 mg L^{-1}or 400 μM for AHL and 80 mg L^{-1} or 375

μM for AHLEc. Twelve samples were prepared per treatment or control and samples were accommodated in separate glass tubes and sealed with Parafilm®. Mixtures were incubated for 0, 2, 4 or 8 hours at room temperature and agitated at 100 rpm during the incubation. Tubes were wrapped in aluminum foil to prevent the access of light.

After each incubation period, three tubes from each treatment or control were removed from the incubator and amended with 600 μl of chloroform. Tubes were sealed with aluminum foil and vortexed vigorously for 15 s. Chloroform fractions were collected and transferred to injection vials. The extraction process was repeated three times. Chloroform fractions were analyzed by GC/MS directly or after filtration. Filtration was carried on Whatman Anotop™ 10, 0.2μm filters (GE Healthcare, Buckinghamshire, UK). Chloroform extracts were forced through filters using NORM-JECT® 10ml syringes (Henke-Sass, Wolf GmbH, Tuttlingen, Germany). Syringe needles (Precision Glide 18G™; BD, Franklin Lakes, New Jersey) were used to guide filtered fractions into GC/MS injection vials. Concentrations reported by GC/MS analysis were multiplied by a factor of three to compensate for the dilution factor associated with liquid/liquid extraction procedure.

5.2.5. GC/MS conditions, Method 1 (AHL analysis)

The identification and quantification of AHL and AHLEc from the extracts obtained in trials were performed using a Varian 3400cx GC with a Hewlett Packard cross-linked methylsiloxane DB-5 capillary column (30 m x 0.25 mm I.D.) combined with a Varian Saturn 2000 ion-trap mass selective detector (Varian Inc., Walnut Creek,

CA). The gas chromatograph (GC) program was initiated at 35 ºC with temperature kept unchanged for 10 min, then increased by 10 ^{o}C min^{-1} up to 200 ºC; after which, it was ramped to 260 ºC at 3 ºC min^{-1} where it was kept unchanged for 6 min. Injector temperature was held at 260 ºC for 5 min. Split injection mode with split ratio of 1:100 was used. Helium was used as the carrier gas and the flow rate was 1 mL min^{-1}. The transfer line between the GC and mass spectrometer was maintained at 280 ºC and the ion trap manifold was set to 250 ^{o}C.

5.2.6. Identification and quantification of AHL

Table 5.4 summarizes parameters that were used in identification and quantification of AHL in extracts produced in the First Bioassay. These parameters did not allow for satisfactory analyses of AHL in the Second Bioassay and new values needed to be utilized. Table 5.5 provides parameters utilized for the Second Bioassay AHL analysis.

Table 5.4 First Bioassay: GC/MS Parameters for the identification and quantification of AHL

Parameter	Value	Unit
Quantification Ions	58+143	m/z
Retention time	16.642	min
Curve Fit Type	quadratic	
Origin Point	ignore	
Regression weighting	$1/nx^2$	
Integration Window	0.5	min
Peak Width	5	
Slope Sensitivity	20	
Tangent	99	%
Peak Reject	100	
Peak Smoothing	None	
Search Window	0.2	min
Search Type	Spectrum	
Match Threshold	450	
Minimum Abundance	1	

Table 5.5 Second Bioassay: GC/MS parameters for the identification and quantification of AHL.

Parameter	Value	Unit
Quantification Ions	143+101	m/z
Retention time	23.226	min
Curve Fit Type	quadratic	
Origin Point	ignore	
Regression weighting	$1/nx^2$	
Integration Window	0.07	min
Peak Width	5	
Slope Sensitivity	25	
Tangent	10	%
Peak Reject	30	
Peak Smoothing	Mean; 5 Point Smooth	
Search Window	0.2	min
Search Type	Spectrum	
Match Threshold	1	
Minimum Abundance	1	

5.2.7. GC/MS conditions, Method 2 (AHLEc analysis)

Method 2 was run on the same equipment as Method 1 and similar analytical parameters were utilized. However, in Method 2, the GC program was initiated at 50 ^{o}C, the temperature was kept constant for 0.5 min and then allowed to increase at the rate of 10 ^{o}C min^{-1} until 260 ^{o}C was reached and this temperature was held for 3 min. Identification and quantification parameters for AHLEc are listed in Table 5.6.

Table 5.6 Identification and Quantification Parameters: **Summary of parameters for the identification and quantification of AHLEc with GC/MS Method 2**

Parameter	Value	Unit
Quantification Ions	71+ 101+ 129	m/z
Retention time	9.63	min
Curve Fit Type	quadratic	
Origin Point	ignore	
Regression weighting	$1/nx^2$	
Integration Window	0.2	min
Peak Width	10	
Slope Sensitivity	15	
Tangent	10	%
Peak Reject	500	
Peak Smoothing	none	
Search Window	0.4	min
Search Type	Spectrum	
Match Threshold	700	
Minimum Abundance	1	

5.2.8. Statistical analyses

Single factor ANOVA was carried out using SAS Enterprise® 9.3 software. Tukey's HSD multiple comparison test at alpha = 0.05 was used for data comparison in the First Bioassay. Single comparison tests (treatment vs. control) were carried out on data from the Second Bioassay. Tests were repeated for each incubation period (total of four comparisons were made per treatment at 0, 2, 4 and 8h).

5.3. Results

5.3.1. Identification of AHL in standards; First Bioassay

Table 5.7 summarizes parameters for signals revealed in AHL standards analyzed during the First Bioassay. The calibration curve prepared from these standards was described with the following equation: $y = -2.1422x2 + 494.0374x$; $R^2 = 0.9987$. Figure 5.3 presents AHL mass spectrum revealed in present work.

Table 5.7 Signal parameters associated with AHL response revealed in standards prepared for the First Bioassay

AHL Standard Concentration	Ret. Time [min]	Area
100	16.456	27588
10	16.457	5460
5	16.457	2067
2.5	16.46	1220
Average Retention Time	16.457	
Std. Deviation:	0.0014	
Rel. Std. Deviation:	0.01%	

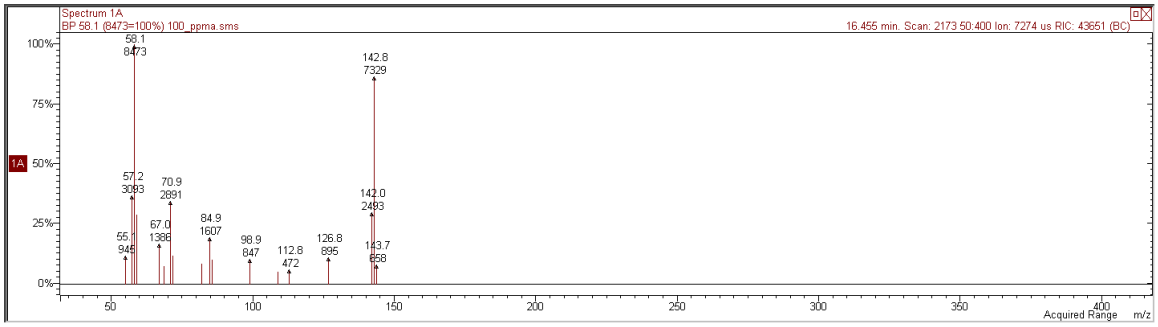


Figure 5.3 Mass spectrum of AHL revealed in the First Bioassay

5.3.2. Identification and quantification of AHL; First Bioassay

Table 5.8 summarizes AHL concentrations revealed in the First Bioassay. All treatments showed significant reduction (p=0.05) of AHL. Treatment with the intact PBP product showed significantly greater reduction than treatments with PBP supernanatant or the PBP cell pellet.

Table 5.8 Concentrations of AHL revealed after 2 h incubation. All treatments have shown AHL concentrations significantly different from control (p<0.05)

Compound: AHL; First Bioassay			
	Concentration [mg L^{-1}]		% Reduction[2]
Treatment or control	Mean	SD	
Control[1]	56.63	3.97	N/A
PBP Supernatant	37.32	8.85	34.09%
PBP Pellet	27.36	2.62	51.68%
PBP Culture	17.47	2.66	69.16%

[1] In control only DI was used.
[2] Percent reduction refers to reduction observed in a treatment compared to control.

5.3.3. Identification of AHL in standards; Second Bioassay

Table 5.9 summarizes parameters of signals revealed in AHL standards analyzed during the Second Bioassay. The calibration curve prepared from these standards was described with following equation: $y = 0.0925x2 + 83.2186x – 594.0575$; $R^2 = 0.9942$.

Table 5.9 Signal parameters associated with AHL response revealed in standards prepared for the Second Bioassay

Compound: AHL		
AHL Standard Concentration	Ret. Time [min]	Area
100	23.228	8899
50	23.228	5836
50	23.227	3382
25	23.233	1626
25	23.231	1729
10	23.237	324
10	23.237	232
Average Retention Time	23.231	
Std. Deviation:	0.0042	
Rel. Std. Deviation:	0.02%	

5.3.4. Identification and quantification of AHL; Second Bioassay

Figure 5.4 presents data on concentration levels of AHL revealed in experimental mixtures prepared with 10% solutions of PBP (Figure 5.4A) and SCD BioAG™ (Figure 5.4B). Each treatment was accompanied by a control run and data for both treatment and control are presented.

Concentrations of AHL in the treatment with 10% PBP were not significantly different from the respective control at any point in time ($p=0.05$). The treatment with 10% SCD BioAG™ significantly reduced AHL concentrations after 2 h of incubation (as compared to control). After 4 h of incubation, concentrations in treatment and control were statistically indifferent. However, after 8 h of incubation, AHL presence in

treatment with 10% SCD BioAG™ was undetected while appreciable amounts of AHL were still detected in the control (29.41 +/- 2.00 mg L^{-1}).

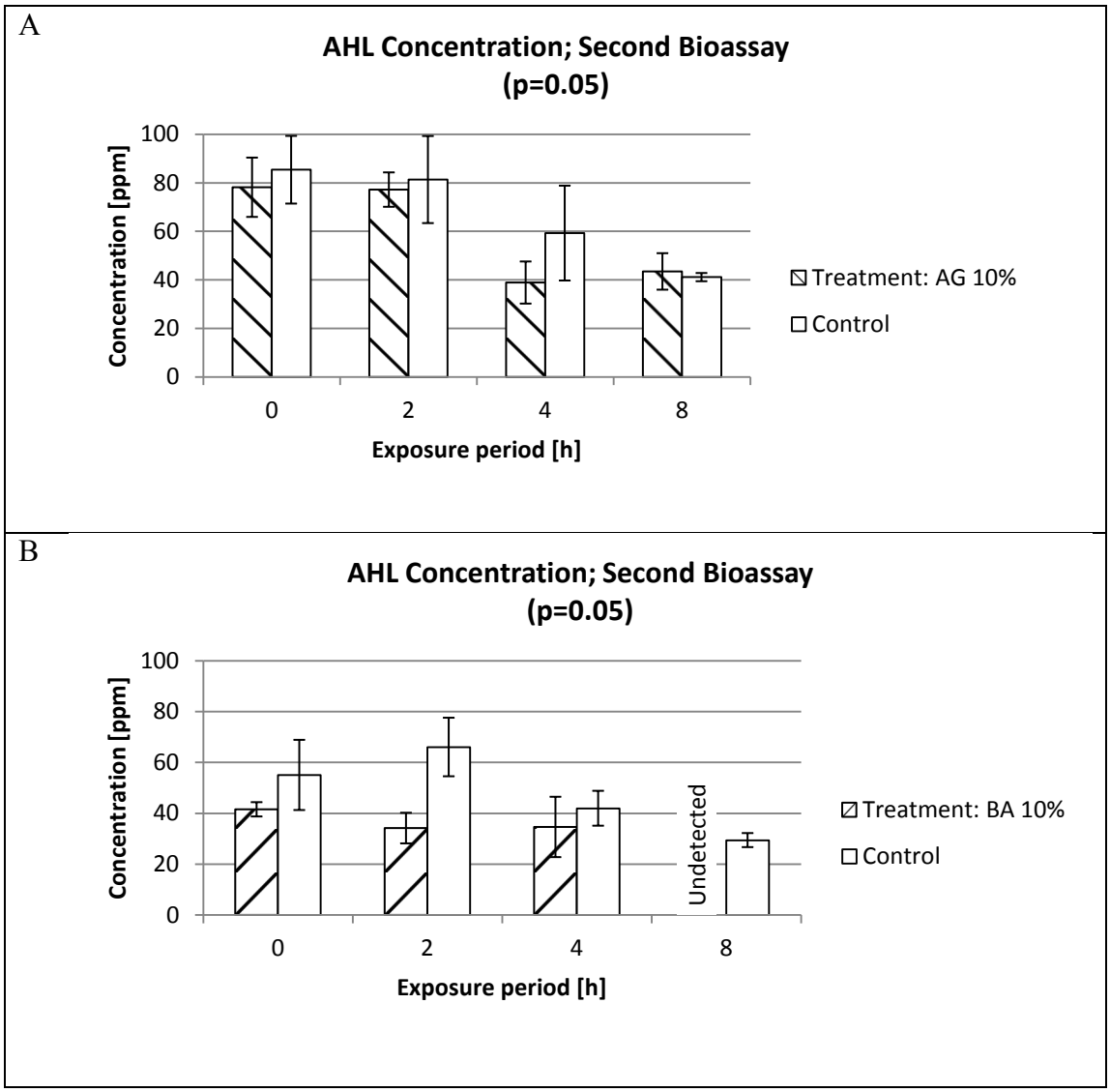


Figure 5.4 Concentrations of AHL revealed in Second Bioassay when treatment with PBP (A) and BioAg (B) was applied.

5.3.5. Identification of AHLEc in standards

Table 5.10 summarizes results representative of AHLEc signals from standards analyzed with Method 2. The calibration curve was produced with these data and was described by the equation $y = -10.55x^2 + 1440.81x - 4250.49$; $R^2 = 0.99$. Figure 5.5 provides a spectrum representative of AHLEc.

Table 5.10 Summary of signal parameters associated with AHLEc GCMS response

Compound: AHLEc		
Concentration [mg L^{-1}]	Ret. Time [min]	Area
50	9.628	35457
50	9.624	41509
25	9.625	21593
25	9.625	25048
10	9.627	11323
10	9.632	9155
5	9.627	3575
5	9.633	2681
Average:	9.628	
Std. Deviation:	0.0034	
Rel. Std. Deviation:	0.04%	

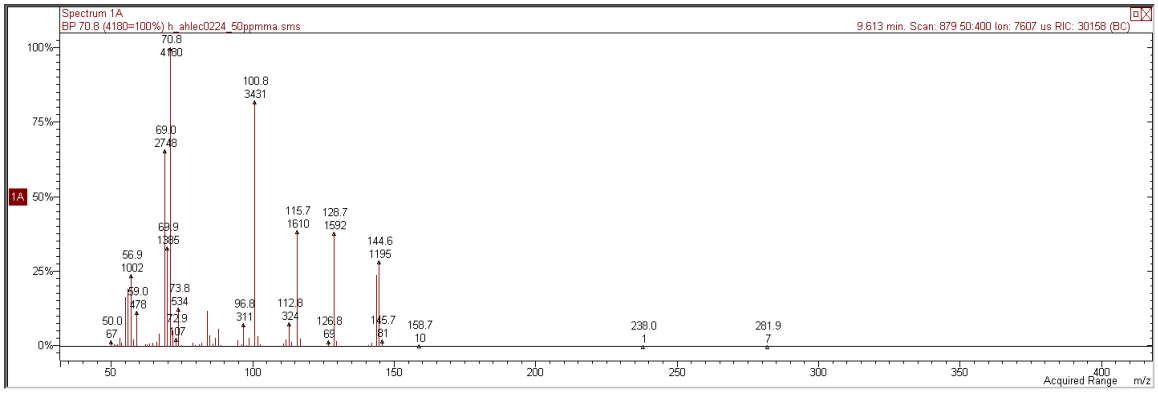


Figure 5.5 Spectrum recorded for AHLEc with Method 2

5.3.6. Identification of AHLEc in actual samples

Figure 5.7 presents signal intensities associated with AHLEc response from actual samples. Signal intensities of AHLEc in treatments were not significantly different from control.

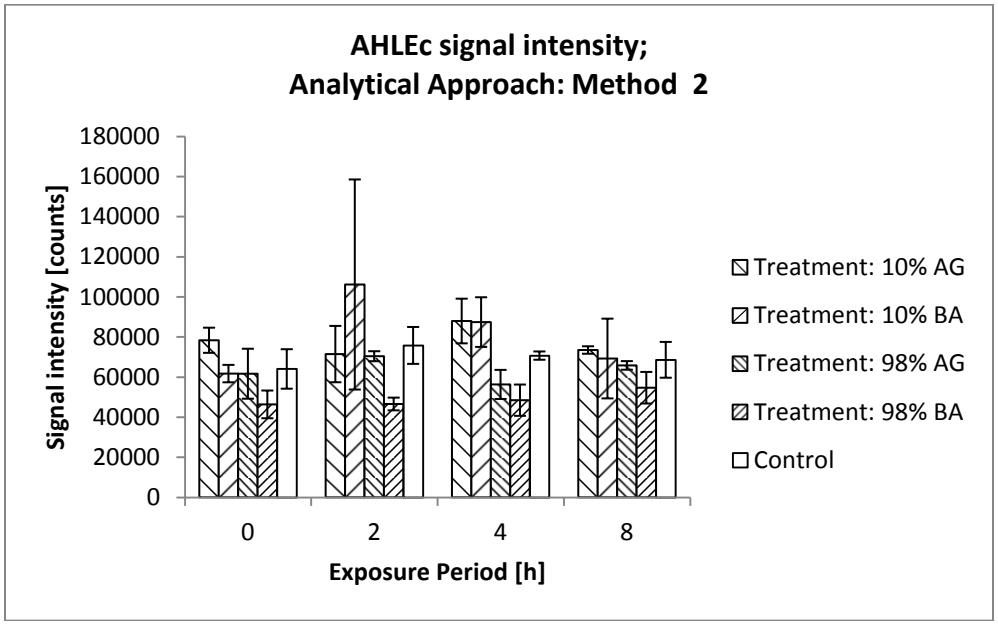


Figure 5.6 Signal intensities associated with signals representative of AHLEc as revealed in actual samples. Error bars indicate standard deviation intervals.

5.4. Discussion

5.4.1. Analytical issues and method choice

Extraction efficiency of AHL and AHLEc and signal peak quality (i.e. narrow or wide peaks) depended on several factors: dilution of probiotic cultures, type of solvent used (i.e. ACN, chloroform, MEOH, with AHL and AHLEc). These effects were particularly problematic in AHLEc analysis where it was impossible to make accurate correlation between signal intensity and concentration. Hence results shown in Figure 5.6 are expressed in terms of signal intensities, not concentrations. Additionally, these issues could have contributed to results variability. For instance, apparent increase in AHL concentration in Control can be seen on Figure 5.4B, which likely stems from problems associated with AHL extraction.

Furthermore, it appeared that GC program temperature ramp affected signal quality. For instance, when Method 2 was used AHL signals appeared stronger than when Method 1 was employed allowing for more accurate observations. Furthermore, Method 1 did not allow for accurate analysis of AHLEc. See appendix for detailed discussion of solvent, culture dilution and GC program temperature ramp effects on AHL and AHLEc signal quality. Consult Appendix for further details on effects of factors: dilution of probiotic cultures; type of solvent used and GC program temperature ramp.

5.4.2. Identification of AHL and AHLEc

The mass spectra and the mass to charge ratios (m/z) of the molecular ions reported by GCMS analysis of AHLEc appear to be in agreement with the values and mass spectra reported by other researchers. In particular, Cataldi et al. (2004) reported that spectra representative of several NAHLs were largely comprised of molecular ions with m/z ratio 143 and accompanied by other major ions (i.e., 125, 101, 71 and 57 m/z). In our work, for AHL, a strong response at 143 m/z was found. Although AHLEc mass spectrum exhibits only minor response at 143 m/z, other molecular ions (i.e., 71 and 101 m/z) agree with previous findings. Some differences in mass spectra may have resulted from different structures of NAHLs studied by Cataldi et al. (2004). Precisely, NAHLs studied in the present work contained two ketone groups, while those studied in referenced works contained only one ketone group. The presence of one more oxygen atom could have caused a local increase in electronegativity which may have modified the charge distribution in the molecule following electron impact. This could produce a different fragmentation pattern and bring some dissimilarity between mass spectra representative of different NAHLs. Figure 5.9 presents a spectrum reported by Cataldi et al. (2004) as well as provides spectra representative of NAHLs studied in the present work.

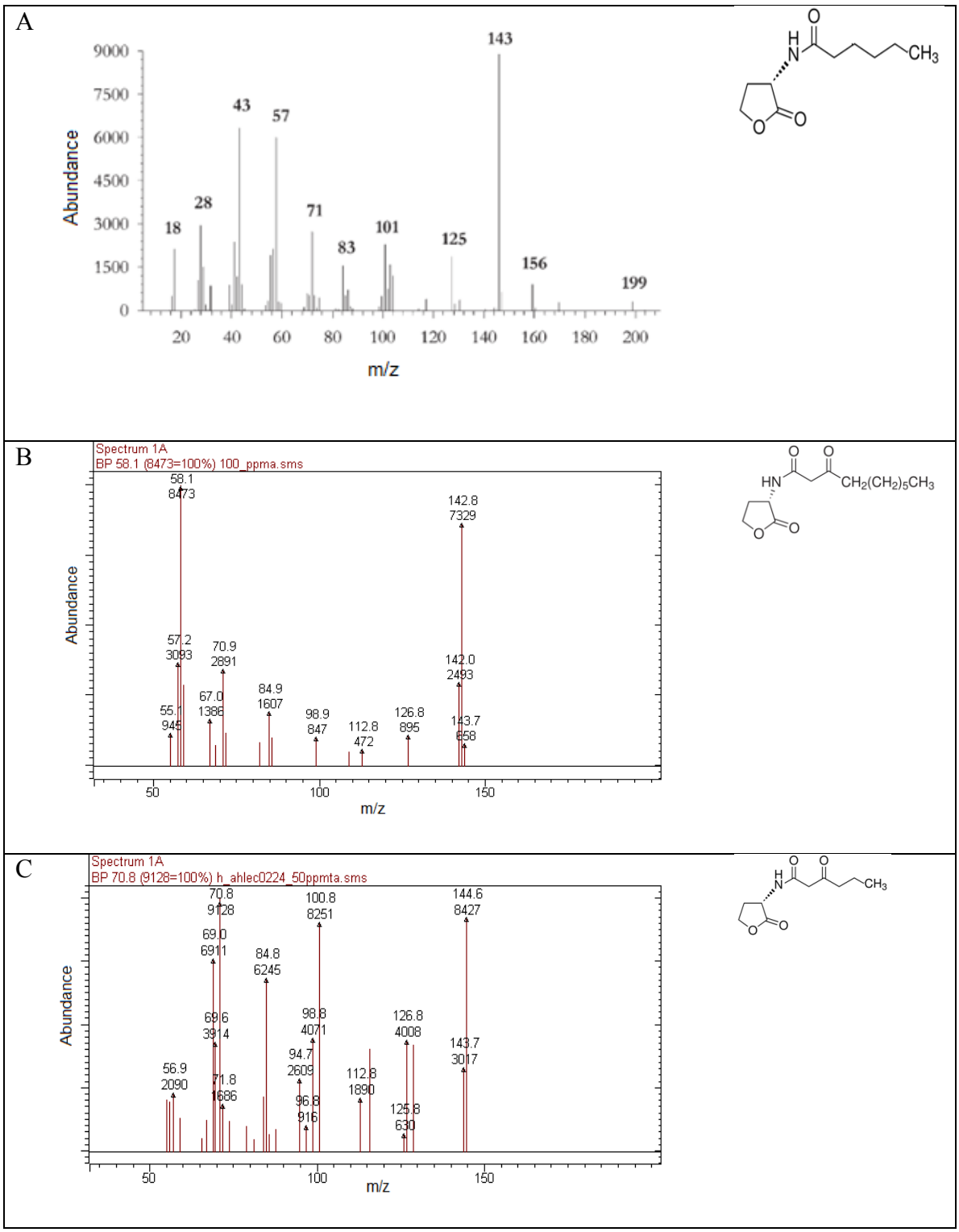


Figure 5.7 Mass spectra representative of several NAHLs: (A) Hexanoyl-L-homoserine lactone [after Cataldi et al.(2004)]; (B) AHL; (C) AHLEc. Representations of chemical structures are provided as well (after http://www.sigmaaldrich.com/).

5.4.3. Degradation of AHL: First Bioassay

Degradation of AHL appeared to occur over a wide pH range (3.3 to 6.8). Specifically, pH of 10% solution of PBP ranged from 3.3 to 3.5 while that of supernatant varied from 6.4 to 6.8; pH values for cell pellet suspension were between 6.6 and 6.8. The control mixture pH ranged from 6.6 to 6.8.

It has been shown previously that NAHLs are more stable at lower pH (Yates et al., 2002); therefore, lower concentration of AHL in treatment with PBP suggests that reduction was caused by enhanced biochemical degradation – likely by enzymatic degradation. Furthermore, similar AHL degrading capacity was shown by cell-free PBP supernatant and PBP cell pellet suspension. This suggests that extracellular AHL-degrading enzymes are secreted by PBP cultures. Reduction of AHL in cell pellet suspensions suggests that some microorganisms in PBP could have captured extracellular AHL for subsequent degradation or that the washing was inefficient and failed to remove extracellular AHL-degrading enzymes.

5.4.4. Degradation of AHL; Second Bioassay

In the Second Bioassay, control mixtures were acidified (pH 3.5) with acetic acid to stabilize NAHLs by preventing lactonolysis (Yates et al., 2002). Findings suggest that PBP was not capable of degrading AHL, which is in contrast to results of First Bioassay. However, this lack of efficiency could have resulted from unique properties of PBP cultures from different batches or storage conditions. Different batches were used in

experiments and factors associated with culture age and storage (nutrient depletion, gene expression and protein profile, activity of proteolytic enzymes etc.) could have compromised putative enzymatic activity of PBP batch used for Second Bioassay. On the other hand, results of Second Bioassay showed that SCD BioAG™ was capable of enhancing AHL degradation. Given the fact that SCD BioAG™ is manufactured using PBP as an ingredient, this observation supports the stipulation that probiotic cultures are capable of expressing AHL-degrading enyzmes.

5.4.5. Degradation of AHLEc

Findings reveal that probiotic cultures were unable to degrade the AHLEc molecule. Researchers have previously reported that the activity of NAHL-degrading enzymes is related to acyl chain length of NAHLs (activity is lower for short-acyl chain NAHLs) (Lin et al., 2003; Park et al., 2005). Furthermore, Park et al. (2005) have shown that NAHLs containing two ketone groups were more resistant to enzymatic attacks than single ketone group-containing NAHLs. Therefore, the observation that PBP and SCD BioAG™ failed to degrade AHLEc may suggest that consortia probiotic cultures studied still have the enzymatic ability to degrade NAHLs, however the ability depends on particular structure of the molecule.

Furthermore, pH could have significant effects on the NAHL-degradation. Most previous research was conducted with NAHL-degradation bioassays under nearly neutral to alkaline pH (6.5 to 10) (Dong et al., 2000; Huang et al., 2003; Lee et al., 2002; Lin et al., 2003; Park et al., 2005; Ramos et al., 2012; Xu et al., 2003; Zhang et al., 2004),

which indicates that this pH range was optimal for NAHL-degrading activity. In the present work, assays were run at generally lower pH (with exception of two treatments in the First Bioassay that were run under nearly neutral pH), which was likely unfavorable for optimal enzymatic activity. Arguably, a combination of: (1) greater than optimal pH, (2) short length of side acyl chain, and (3) presence of additional ketone groups likely played a pronounced role in our present experiments. These factors could have contributed to compromised degradation of not only AHLEc but AHL as well.

5.5. Conclusions

Probiotic cultures examined in this study appear to contain enzymes that are capable of degrading some NAHLs (particularly AHL was degraded while AHLEc was unaffected). However, AHL-degrading activities appeared to be highly variable. This variability could have resulted from differences between product batches, storage conditions or product age as well as low pH of reaction solutions.

Probiotic cultures examined in this study may have a potential to interfere with pathogen QS. This potential, however, may be removed due to inconsistent quality of probiotic cultures or handling. Furthermore, the potential may depend on environmental variables such as pH. In order to assure that microorganisms have consistent effects on pathogenicity mechanisms, more stringent quality control of consortia probiotic cultures would need to be developed by the manufacturer.

5.6. Future work

In future work, inconsistencies in NAHL-degradation should be examined more thoroughly. Specifically, bioassays should be run at different pH values to elucidate the optimal pH value for NAHL degradation. Furthermore, it should be examined whether there is a correlation between probiotic culture age, batch or storage conditions and the culture's NAHL-degradation capacity. For instance, NAHL degradation should be analyzed with aliquots from fresh batches and repeated after batches were allowed to age and/or were stored under different conditions (i.e., different temperature regimes). The study design involving synthetic NAHLs and GC/MS analysis proved to provide for efficient identification, and future studies could adopt a similar design. However, in order to solve whether NAHL degradation is due to enzymatic activities (particularly whether NAHL-acylase and/or lactonase enzymes are involved), future studies should employ methods that allow for identification of products of NAHL degradation (i.e., acyl homoserine and corresponding fatty acids can be screened for detecting NAHL-acylase activity). In previous studies, liquid chromatography coupled with mass spectrometry has been applied for detection of fatty acid byproducts of NAHL degradation (Huang et al., 2003; Lin et al., 2003) and this approach could be replicated in future investigations. Activity of a NAHL lactonase was determined previously using high performance liquid chromatography and mass spectrometry (Dong et al., 2001). Possibly, this scheme could be replicated in the future as well. Additionally, bioassay-guided enzyme purification could be performed on order to identify enzymes responsible for degradation.

6. Chapter 6: Conclusions

Consortia probiotic cultures used as soil inoculants did not enhance soil microbial diversity or enzymatic activity. Therefore it seems unlikely that PBP deliver their benefits in agriculture by stimulating soil microbial diversity. On the other hand severe drought conditions experienced in 2012 and may have obscured treatment effects. It appeared that PBP is non-harmful to indigenous microbial communities.

Microbial communities in soils maintained under controlled conditions exhibited declining diversity and the decline was less in treated than in control soils. In one treatment regime, reduced microbial diversity decline correlated with significant changes in tomato plant tissue characteristics (reduced dry biomass content and C-content as well increased N-content). In other groups, treatment with PBP correlated with no significant impacts on plant tissue characteristics. Possibly, PBP efficacy may owe to stabilizing impacts on soil microbial communities.

Two CP studied (PBP and SCD BioAG™) degraded AHL over a wide range of pH, however, they failed to reduce AHLEc concentration. Consortia probiotic cultures may exert their impacts by disrupting signaling pathways involved in QS, a necessary step for pathogenic activity. Most likely, QS-disrupting capacity is due to enzymatic activity associated with CP studied and the activity depends on the structure of signaling molecules involved in QS. The AHL degrading capacity varied and appeared to depend on PBP batch and/or storage conditions. In order to assure consistent effects on QS-controlled pathogenicity mechanisms, improved quality control would need to be implemented by the manufacturer of CP studied.

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7. APPENDIX

7.1. Study 1 annd Study 2 Appendix

Table 7.1(represented in Figure 3.2 A) mean values and standard deviation (SD) of microbial community richness (S), Shannon's diversity index (H') and Evenness (E) related to DGGE profiles of soil microbial communities in cultivated soil. Asterisks indicate H' values that significantly differ from control ($p<0.05$). Values were based on observations made in triplicates.

	S		H'			E	
Sampling Date	Mean	SD	Mean	SD		Mean	SD
	Cultivated soil, control						
March 13 2012	29.00	1.73	3.25	0.04		0.967	0.006
March 21 2012	29.33	3.79	3.29	0.15		0.974	0.007
April 27 2012	43.00	0.00	3.69	0.00		0.980	0.001
June 16 2012	34.33	1.53	3.41	0.04		0.964	0.000
June 18 2012	37.33	0.58	3.52	0.01		0.973	0.003
July 23 2012	32.67	1.15	3.38	0.04		0.970	0.004
October 4 2012	34.00	2.00	3.41	0.05		0.969	0.003
October 8 2012	32.67	1.53	3.41	0.04		0.978	0.004
	Cultivated soil, treatment						
March 13 2012	27.00	1.00	3.20	0.05		0.972	0.005
March 21 2012	27.33	1.15	3.21	0.03		0.971	0.005
April 27 2012	40.00	4.36	3.60	0.12		0.977	0.003
June 16 2012	36.33	2.08	3.50	0.05		0.975	0.005
June 18 2012	34.00	1.41	3.44	0.04		0.974	0.001
July 23 2012	38.00	2.65	3.53	0.07	*	0.970	0.006
October 4 2012	29.67	1.15	3.31	0.03	*	0.975	0.005
October 8 2012	29.33	1.15	3.31	0.06		0.979	0.006

Table 7.2 (Represented in Figure 3.2 B) mean values and standard deviation (SD) of microbial community richness (S), Shannon's diversity index (H') and Evenness (E) related to DGGE profiles of soil microbial communities in restored grassland soil. Asterisks indicate H' values that significantly differ from control ($p<0.05$). Values were based on observations made in triplicates.

Sampling Date	S		H'			E	
	Mean	SD	Mean	SD		Mean	SD
	Restored grassland, control						
March 13 2012	27.67	0.58	3.20	0.04		0.965	0.006
March 21 2012	29.33	1.53	3.29	0.03		0.974	0.006
April 27 2012	38.00	0.00	3.53	0.01		0.971	0.003
June 16 2012	35.33	1.53	3.46	0.04		0.970	0.006
June 18 2012	29.33	0.58	3.31	0.02		0.979	0.001
July 23 2012	34.33	1.53	3.42	0.06		0.967	0.007
October 4 2012	33.33	2.52	3.43	0.05		0.978	0.006
October 8 2012	27.33	3.21	3.23	0.12		0.979	0.006
	Restored grassland, treatment						
March 13 2012	27.00	3.00	3.18	0.11		0.965	0.004
March 21 2012	30.33	2.08	3.34	0.09		0.980	0.008
April 27 2012	39.67	3.06	3.58	0.08		0.973	0.002
June 16 2012	33.67	1.15	3.41	0.05		0.970	0.006
June 18 2012	30.67	0.58	3.35	0.02		0.978	0.005
July 23 2012	35.33	1.15	3.46	0.02		0.970	0.004
October 4 2012	34.67	0.58	3.48	0.02		0.981	0.004
October 8 2012	28.00	N/A	3.28	N/A		0.983	N/A

Table 7.3 (Represented in Figure 3.7) dice similarity values related to PBPmicrobial community profiles and profiles of soil microbial communities studied in the field experiment.

	Dice similarity value (sample microbial community to PBPmicrobial community)			
Sampling Date	CSCP	CSTP	RGCP	RGTP
March 13 2012	63	71	63	75
March 21 2012	68	69	64	60
April 27 2012	67	69	68	60
June 16 2012	78	75	79	76
June 18 2012	71	67	75	73
July 23 2012	72	62	75	75
October 4 2012	62	61	69	70
October 8 2012	69	63	62	54

Table 7.4 (Presented in Figure 3.8 and 3.9) mean soil enzyme activities (n=3) and associated standard deviations (SD) revealed in field experiment, expressed in terms of substrate degradation products formed per gram of dry soil, per hour. Degradation products are 1,3,5-triphenylformazan (TPF) (dehydrogenase activity assay) and fluorescein (fluorescein diacetate degradation assay).

	μgTPF g_{soil}^{-1} h^{-1}		μgFluorescein g_{soil}^{-1} h^{-1}	
Sampling date	Mean	SD	Mean	SD
Cultivated soil; control				
March 13, 2012	2.10	0.41	47.25	10.11
April 27, 2012	2.98	0.84	62.23	4.42
June 18, 2012	2.67	1.32	63.39	3.13
July 23, 2012	1.59	1.71	33.75	29.98
October, 8 2012	1.16	1.11	27.03	25.60
Cultivated soil; treatment				
March 13, 2012	3.80	1.39	61.85	10.78
April 27, 2012	2.33	1.43	53.51	22.97
June 18, 2012	1.81	0.51	35.67	11.37
July 23, 2012	1.63	0.48	36.51	8.95
October, 8 2012	1.62	0.58	38.06	23.27
Restored grassland; control				
March 13, 2012	2.07	0.84	62.05	4.83
April 27, 2012	2.57	0.58	43.69	44.99
June 18, 2012	1.67	1.22	35.49	26.15
July 23, 2012	1.14	0.77	51.99	14.67
October, 8 2012	2.44	1.15	62.32	13.52
Restored grassland; treatment				
March 13, 2012	3.65	0.49	69.94	38.60
April 27, 2012	2.44	2.32	60.89	14.91
June 18, 2012	1.73	1.50	36.78	32.04
July 23, 2012	2.08	0.58	60.45	10.69
October, 8 2012	2.22	1.09	47.00	23.37

Table 7.5 (Presented in Figure 3.10) total monthly precipitation and average monthly temperatures recorded at Bradford Research and Extension Center, Boone County, Missouri (filed experiment site) between March and October, 2012 (field experiment duration). Obtained from http://agebb.missouri.edu/weather/history/index.asp?station_prefix=bfd

	March	April	May	June	July	August	September	October
Precipitation [mm]	112.52	185.17	25.4	39.37	17.53	55.38	45.72	68.08
Avg. Temp. [^{o}C]	14.3	14.2	20.6	24.3	28.6	24.6	18.5	11.5

Table 7.6 (Presented in Figure 4.1) mean richness (S), Shannon's diversity index (H´), and evenness values (E) and associated standard deviations representative of DGGE profiles of soil microbial communities studied in growth chamber experiment (replicate DNA extracts were analyzed).

	S		H´		E		S		H´		E	
Sampling Date	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Group I							Group IV					
April 6 2012	30.67	3.21	3.31	0.15	0.97	0.02	25.67	4.62	3.16	0.20	0.98	0.01
April 9 2012	25.67	3.06	3.13	0.13	0.97	0.01	32.33	9.07	3.33	0.32	0.97	0.01
April 26 2012	31.67	8.50	3.34	0.29	0.97	0.00	29.33	4.04	3.28	0.14	0.97	0.01
May 8 2012	36.67	0.58	3.51	0.04	0.97	0.01	37.33	0.58	3.54	0.02	0.98	0.00
June 12 2012	41.00	5.29	3.58	0.10	0.97	0.01	34.67	6.51	3.42	0.18	0.97	0.00
June 15 2012	32.67	1.53	3.40	0.06	0.98	0.01	30.33	0.58	3.32	0.04	0.97	0.01
July 23 2012	36.33	1.15	3.50	0.02	0.98	0.00	34.00	1.41	3.46	0.02	0.98	0.01
September 12 2012	29.33	1.53	3.27	0.06	0.97	0.00	29.50	0.71	3.29	0.05	0.97	0.01
September 17 2012	30.75	1.89	3.32	0.07	0.97	0.00	31.33	0.58	3.36	0.01	0.97	0.01
October 2 2012	34.67	1.15	3.41	0.02	0.96	0.00	34.67	2.52	3.44	0.06	0.97	0.01
October 4 2012	34.33	2.52	3.46	0.07	0.98	0.00	34.33	2.08	3.44	0.05	0.97	0.00
Group II							Control					
April 6 2012	21.00	2.00	2.95	0.10	0.97	0.00	26.00	3.00	3.15	0.14	0.97	0.01
April 9 2012	30.50	8.35	3.29	0.28	0.97	0.01	31.00	5.60	3.31	0.19	0.97	0.01
April 26 2012	26.33	0.58	3.14	0.02	0.96	0.01	24.33	0.58	3.09	0.04	0.97	0.01
May 8 2012	35.33	2.08	3.49	0.05	0.98	0.00	36.00	0.00	3.52	0.00	0.98	0.00
June 12 2012	41.33	2.89	3.61	0.05	0.97	0.01	39.00	3.46	3.55	0.09	0.97	0.00
June 15 2012	35.33	2.31	3.49	0.06	0.98	0.00	32.00	6.93	3.31	0.31	0.96	0.03
July 23 2012	31.67	3.06	3.35	0.09	0.97	0.00	30.33	6.51	3.30	0.22	0.97	0.01
September 12 2012	27.67	11.85	3.12	0.54	0.96	0.02	35.00	2.65	3.45	0.09	0.97	0.01
September 17 2012	38.33	2.52	3.55	0.04	0.97	0.01	36.33	4.04	3.48	0.09	0.97	0.01
October 2 2012	33.67	2.89	3.42	0.07	0.97	0.01	35.67	0.58	3.47	0.02	0.97	0.01
October 4 2012	33.00	4.36	3.41	0.12	0.98	0.00	34.67	3.21	3.45	0.11	0.97	0.01
Group III												
April 6 2012	28.00	3.46	3.23	0.14	0.97	0.01						
April 9 2012	32.33	6.66	3.36	0.21	0.97	0.01						
April 26 2012	32.25	6.75	3.36	0.24	0.97	0.01						
May 8 2012	31.00	9.64	3.34	0.33	0.98	0.00						
June 12 2012	38.67	2.52	3.54	0.05	0.97	0.01						
June 15 2012	34.00	4.58	3.43	0.14	0.98	0.01						
July 23 2012	34.00	5.29	3.45	0.16	0.98	0.00						
September 12 2012	30.00	4.24	3.28	0.12	0.97	0.01						
September 17 2012	33.67	2.31	3.42	0.08	0.97	0.00						
October 2 2012	35.33	0.58	3.45	0.04	0.97	0.01						
October 4 2012	34.00	1.73	3.43	0.04	0.97	0.01						

Table 7.7(Presented in Figure 4.2) Richness (S), diversity (H´), and evenness (E) representing soil microbial communities studied in growth chamber experiment (composite DNA extracts were analyzed).

Sampling Date	S	H´	E	S	H´	E
Group I				Group IV		
April 6 2012	35	3.49	0.98	40	3.57	0.967
April 9 2012	40	3.59	0.974	38	3.53	0.969
April 26 2012	37	3.51	0.971	38	3.56	0.979
May 8 2012	37	3.51	0.973	39	3.55	0.97
June 12 2012	40	3.62	0.981	37	3.51	0.972
June 15 2012	38	3.53	0.971	39	3.57	0.975
July 23 2012	40	3.62	0.98	36	3.48	0.972
September 12	35	3.45	0.971	40	3.59	0.973
September 17	37	3.5	0.969	38	3.53	0.97
October 2 2012	37	3.52	0.975	36	3.48	0.971
October 4 2012	34	3.45	0.978	37	3.5	0.969
Group II				Control		
April 6 2012	40	3.63	0.983	37	3.54	0.98
April 9 2012	42	3.67	0.982	37	3.55	0.983
April 26 2012	38	3.55	0.977	39	3.6	0.984
May 8 2012	42	3.69	0.987	36	3.5	0.977
June 12 2012	42	3.67	0.983	38	3.57	0.981
June 15 2012	43	3.71	0.987	37	3.53	0.978
July 23 2012	46	3.77	0.986	34	3.48	0.986
September 12	46	3.78	0.986	35	3.47	0.977
September 17	44	3.74	0.988	32	3.41	0.983
October 2 2012	42	3.64	0.975	34	3.48	0.986
October 4 2012	41	3.62	0.975	30	3.35	0.986
Group III						
April 6 2012	34	3.47	0.984			
April 9 2012	34	3.44	0.975			
April 26 2012	34	3.43	0.973			
May 8 2012	35	3.49	0.982			
June 12 2012	35	3.45	0.972			
June 15 2012	36	3.52	0.984			
July 23 2012	34	3.45	0.98			
September 12	36	3.51	0.98			
September 17	34	3.47	0.983			
October 2 2012	35	3.46	0.975			
October 4 2012	36	3.5	0.977			

Table 7.8(Presented on Figures 4.8 through 4.11) Dice similarity values linking soil microbial communities from growth chamber experiment and PBP microbial communities .

	Similarity with PBPprofile (Dice similarity)			
Sampling Date	Group I (composite)	Group I co-analyzed control (composite)	Group III (composite)	Group III co-analyzed control (composite)
April 6 2012	65	68	72	73
April 9 2012	71		75	
April 26 2012	74		75	
May 8 2012	71	71	74	71
June 12 2012	71		77	
June 15 2012	73		76	
July 23 2012	74		72	
September 12 2021	71	78	76	63
September 17 2021	77		75	
October 2 2012	77		77	
October 4 2012	75	78	77	72
		Mean 73.75		Mean 69.75
		SD 5.06		SD 4.57
	Group II (composite)	Group II co-analyzed control (composite)	Group IV (composite)	Group IV co-analyzed control (composite)
April 6 2012	62	68	55	56
April 9 2012	56		63	
April 26 2012	56		67	
May 8 2012	61	65	59	56
June 12 2012	61		65	
June 15 2012	68		62	
July 23 2012	65		59	
September 12 2021	63	60	62	68
September 17 2021	54		57	
October 2 2012	56		62	
October 4 2012	59	65	58	54
		Mean 64.5		Mean 58.5
		SD 3.32		SD 6.4

7.1.1. Gel quality and profiles of microbial communities

Distribution of bands that were most likely representing similar bacterial genera was found to vary from gel to gel (Figure 7.1). Changeable distribution of bands could have resulted from inconsistent distribution of denaturant in gels. Factors such as different rates at which gels were cast and turbulent and diffuse transmission of denaturant in cast medium could have contributed to the observed lack of uniform quality. Because of differences in band distribution, it was exceptionally difficult to compare microbial profiles resolved on different gels. This problem was to a large extent minimized when DNA extracts representing a sampling event were composited and analyzed as one sample. As a result the amount of soil microbial profiles analyzed on a gel was maximized.

Microbial community profiles prepared from composite extracts provided for another insight into the issue of gel quality and comparison capacity of DGGE profiles. The dendrogram depicted in Figure 7.2 suggests that similarity-based clusters form according to the gel from which a profile was made (recall that lanes belonging to same group were found on same gel). Similar observations can be drawn from the dendrogram seen on Figure 7.3. In there, clusters are chiefly comprised of profiles revealed on the same gel. This suggests that differences between gels could overwhelm any similarities between profiles.

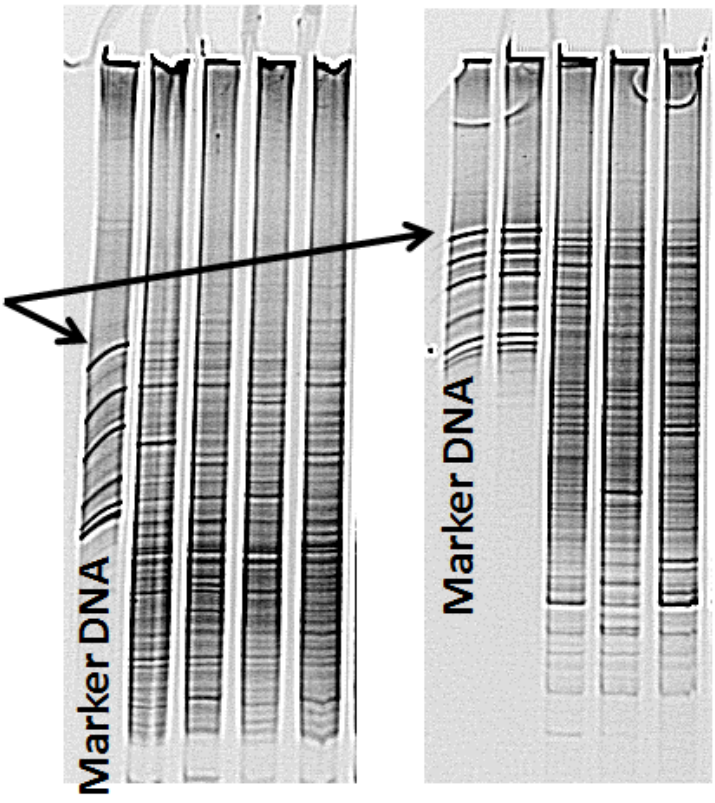

Figure 7.1 Fragments of two different gel images enhanced to improve band clarity. Arrows indicate bands that most likely represent the same microbial genera. Notice the difference in position of bands.

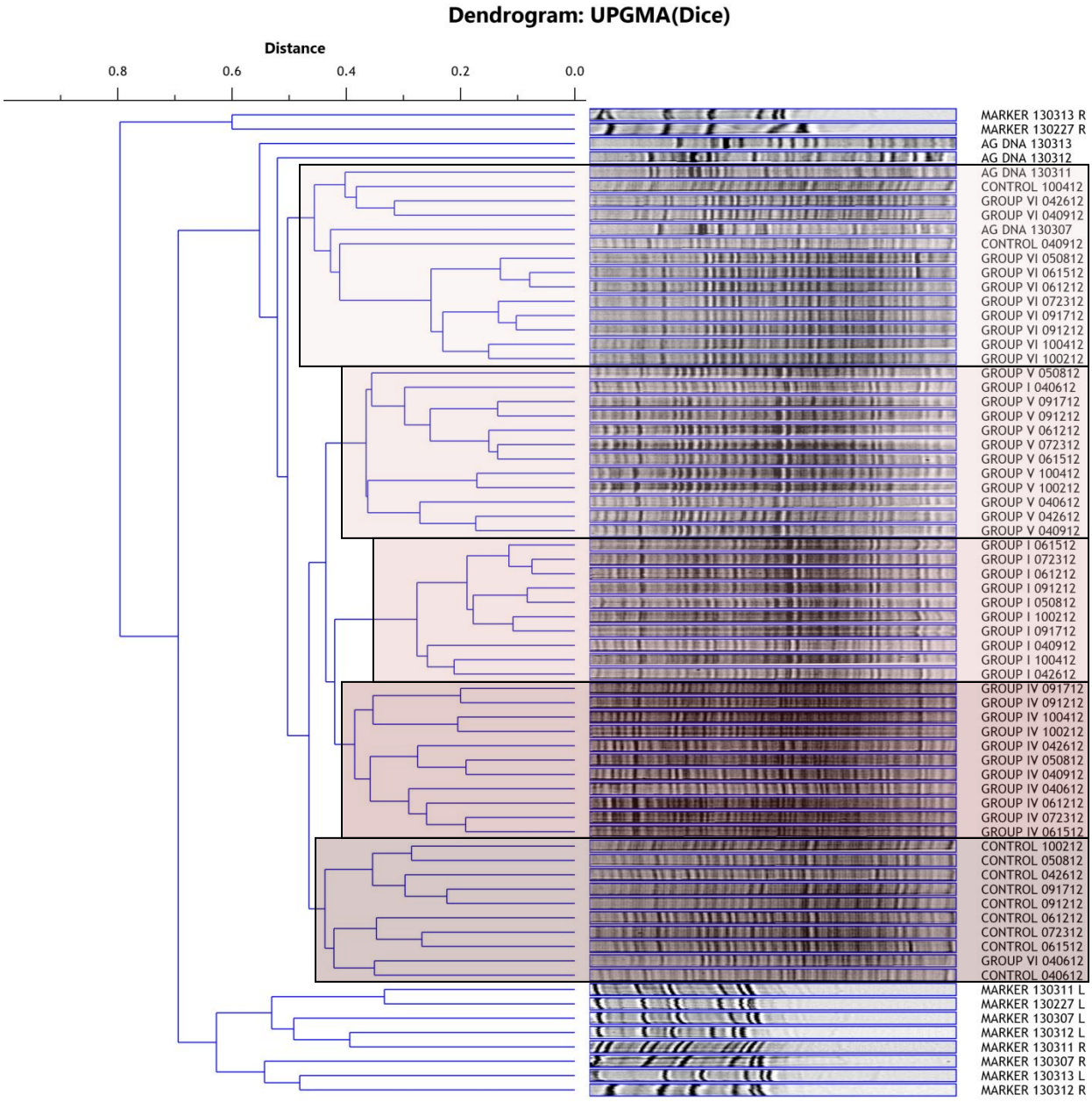

Figure 7.2 Dendrogram representing similarity between microbial community profiles revealed in composite soil DNA samples from Growth Chamber Experiment for soils receiving different PBPtreatments. Boxes indicate particular clusters formed by UPGMA algorithm. First two letters of lane names indicate management situations (CS for cultivated soil and RG for restored grassland); remaining two letters denote control (CP) or treatment (TP)..Numbers associated with lane names denote sampling dates (i.e. 04062012 is equivalent to April 6, 2012). Lanes marked as "AG DNA" as well as those labeled as "Marker" were captured from the same gel if followed by the same number. Furthermore, in case of Marker names, "L" and "R" denote left-hand side and right-hand side gel marker respectively.

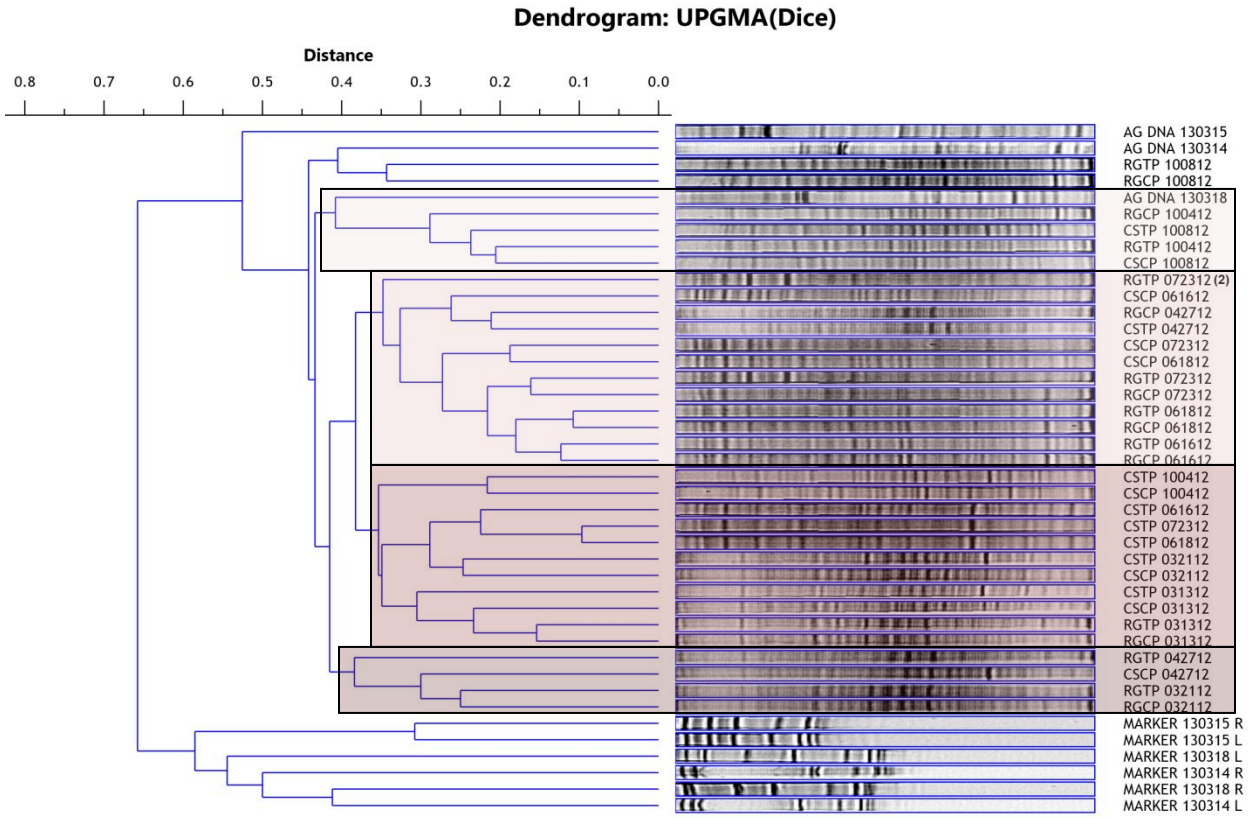

Figure 7.3 Dendrogram representing similarity between microbial community profiles revealed in composite soil DNA samples removed from Field Experiment. Boxes indicate particular clusters formed by UPGMA algorithm. . First two letters of lane names indicate management situations (CS for cultivated soil and RG for restored grassland); remaining two letters denote control (CP) or treatment (TP). Numbers associated with lane names denote sampling dates (i.e., 03132012 is equivalent to March 13, 2012). Lanes marked as "AG DNA" as well as those labeled as Marker followed by the same number were captured on the same gel. Furthermore, in case of Marker names, "L" and "R" denote left-hand side and right-hand side gel marker, respectively.

7.1.2. Variability of H' values among gels

Differences existing between H' values related to "replicate" and "composite" microbial profiles revealed for corresponding profiles suggested that gel quality or other factors may have influenced identification of these values. Consult Table 7.9 for the

summary of H' values representing soil microbial communities analyzed using replicated samples and corresponding composite samples from Field Experiment. Table 7.10 summarizes respective data related to Growth Chamber experiment. Values are also represented on Figures 7.4 and 7.5.

As can be seen, with respect to Growth Chamber Experiment (Figures A.4), nearly all H' values revealed with composite samples either appear to be higher or are significantly higher than their replicate-related counterparts. On the other hand, in profiles corresponding to Field Experiment (Figure 7.5), a tendency for "composite profiles" to exhibit superior H' values is not as evident as it was in the former case and instances are relatively common where H' values observed for composite samples are significantly lower than those reported for replicates.

The variability of H' values could have resulted from inconsistent distribution of denaturant (for example, higher or lower local concentrations of denaturant could have retained more or less DNA fragments and enhanced or reduced band intensity). Additionally some variability may be due to the PCR procedure. Possibly quality and quantity of DNA fragments synthesized during PCR depended on contaminants in samples that were left behind despite filtration procedure. If so, quality and quantity of bands formed during DGGE could have been affected as well

Table 7.9 Summary of Shannon's diversity index (H') values revealed in microbial soil microbial community profiles in the field experiment. The column titled "Mean" represents mean H' value based on replicated soil DNA samples (n=3); 95% confidence intervals (CI) associated with means are presented as well. Furthermore, the column titled "Observed value" represents H' values revealed when replicate samples were pooled. Asterisks in Columns "A" highlight the observed values that lay beyond confidence intervals associated with mean H' of replicate samples.

Sampling Date	H values			
	Mean	CI	Observed value	
	Cultivated soil, control			A
March 13 2012	3.25	0.05	3.52	*
March 21 2012	3.29	0.17	3.54	*
April 27 2012	3.69	0.01	3.39	
June 16 2012	3.41	0.05	3.52	*
June 18 2012	3.52	0.01	3.46	
July 23 2012	3.38	0.05	3.27	
October 4 2012	3.41	0.05	3.53	*
October 8 2012	3.41	0.04	3.44	
	Cultivated soil, treatment			
March 13 2012	3.20	0.06	3.47	*
March 21 2012	3.21	0.03	3.47	*
April 27 2012	3.60	0.14	3.42	
June 16 2012	3.50	0.05	3.51	
June 18 2012	3.44	0.06	3.38	
July 23 2012	3.53	0.08	3.32	
October 4 2012	3.31	0.03	3.45	*
October 8 2012	3.31	0.07	3.40	*
	Restored grassland, control			
March 13 2012	0.04	3.20	3.48	*
March 21 2012	0.03	3.29	3.47	*
April 27 2012	0.02	3.53	3.46	
June 16 2012	0.04	3.46	3.54	*
June 18 2012	0.02	3.31	3.38	*
July 23 2012	0.07	3.42	3.33	
October 4 2012	0.06	3.43	3.37	
October 8 2012	0.14	3.23	3.41	*
	Restored grassland, treatment			
March 13 2012	3.18	0.12	3.52	*
March 21 2012	3.34	0.11	3.44	
April 27 2012	3.58	0.09	3.41	
June 16 2012	3.41	0.06	3.45	
June 18 2012	3.35	0.02	3.33	
July 23 2012	3.46	0.02	3.28	
October 4 2012	3.48	0.02	3.55	*

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Table 7.10 Summary of Shannon's diversity index (H') values revealed in microbial soil microbial community profiles in the growth chamber experiment. Columns "Mean" represent mean H' value based on replicated observations (n=3); 95% confidence intervals (CI) associated with means are presented as well. Columns titled "Observed value" represent H' values revealed when replicates were pooled. Asterisks in Columns "A" highlight the observed values that lay beyond confidence intervals associated with mean H' of replicate samples.

Sampling Date	H' value			A	H' value			A	H' value			A
	Mean	CI	Observed value		Mean	CI	Observed value		Mean	CI	Observed value	
	Group I				Group III				Control			
	Group I (comp.)			A	Group III (comp.)			A	Control (comp.)			
IV 6	3.31	0.17	3.49		3.23	0.16	3.47	*	3.15	0.15	3.54	*
IV 9	3.13	0.14	3.59	*	3.36	0.23	3.44		3.31	0.18	3.55	*
IV 26	3.34	0.33	3.51		3.36	0.24	3.43		3.09	0.04	3.6	*
V 8 2012	3.51	0.04	3.51		3.34	0.38	3.49		3.52	0	3.5	
VI 12	3.58	0.11	3.62		3.54	0.05	3.45		3.55	0.1	3.57	
VI 15	3.4	0.07	3.53	*	3.43	0.16	3.52		3.31	0.35	3.53	
VII 23	3.5	0.03	3.62	*	3.45	0.18	3.45		3.3	0.25	3.48	
IX 12	3.27	0.07	3.45	*	3.28	0.16	3.51	*	3.45	0.1	3.47	
IX 17	3.32	0.07	3.5	*	3.42	0.09	3.47		3.48	0.1	3.41	
X 2 2012	3.41	0.02	3.52	*	3.45	0.04	3.46		3.47	0.03	3.48	
X 4 2012	3.46	0.07	3.45		3.43	0.04	3.5	*	3.45	0.13	3.35	
	Group II				Group IV							
	Group II (comp.)				Group IV (comp.)							
IV 6	2.95	0.11	3.63	*	3.16	0.23	3.57	*				
IV 9	3.29	0.28	3.67	*	3.33	0.36	3.53					
IV 26	3.14	0.02	3.55	*	3.28	0.15	3.56	*				
V 8 2012	3.49	0.06	3.69	*	3.54	0.02	3.55	*				
VI 12	3.61	0.06	3.67	*	3.42	0.2	3.51					
VI 15	3.49	0.07	3.71	*	3.32	0.05	3.57	*				
VII 23	3.35	0.1	3.77	*	3.46	0.03	3.48					
IX 12	3.12	0.62	3.78	*	3.29	0.08	3.59	*				
IX 17	3.55	0.05	3.74	*	3.36	0.01	3.53	*				
X 2 2012	3.42	0.08	3.64	*	3.44	0.06	3.48					
X 4 2012	3.41	0.14	3.62	*	3.44	0.05	3.5	*				

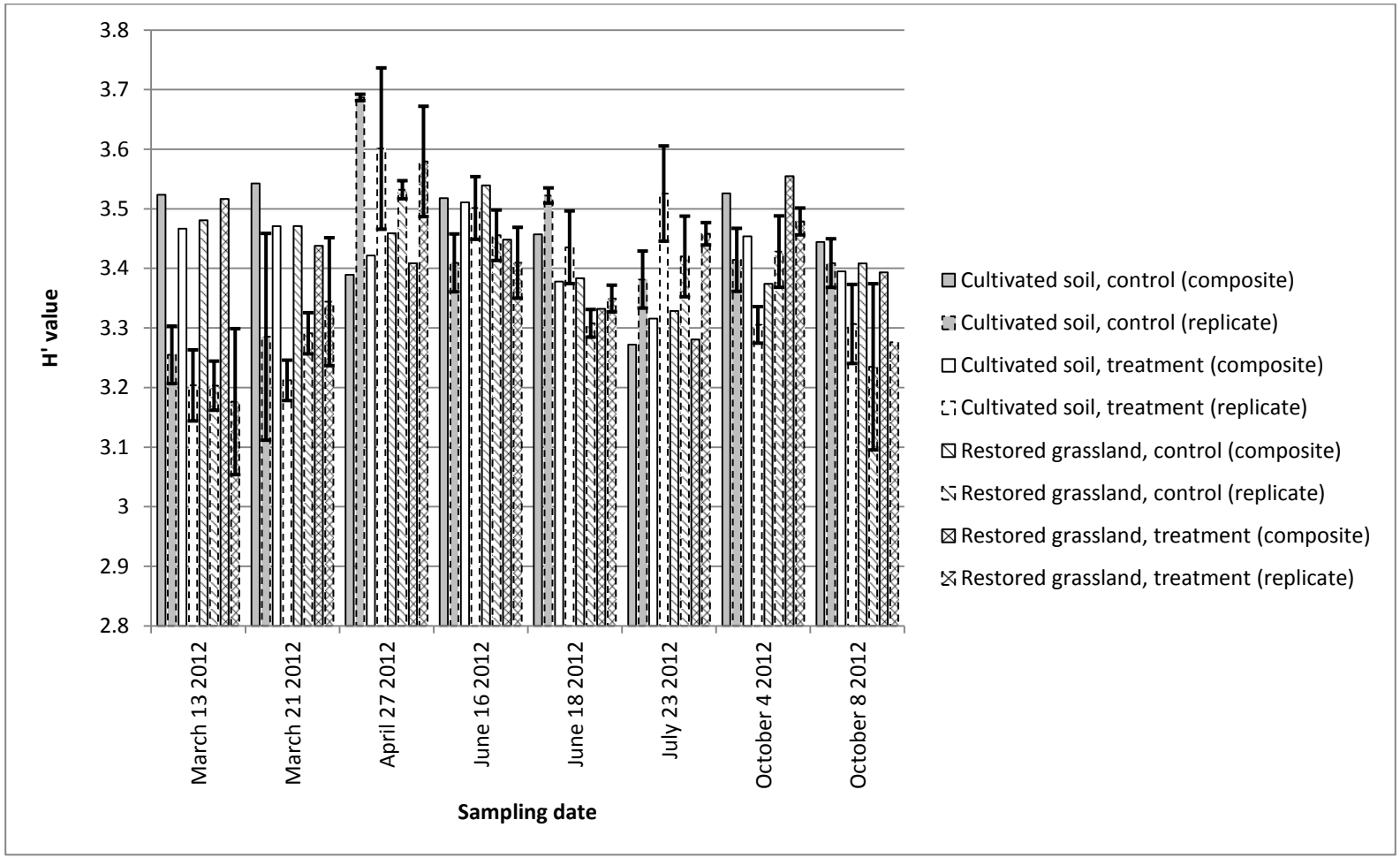

Figure 7.4 Graphic summary of Shannon's diversity index (H') values revealed in DGGE profiles produced from replicate and composite soil DNA extracts from soils in the field experiment; error bars represent 95% confidence intervals on population mean representing replicate samples..

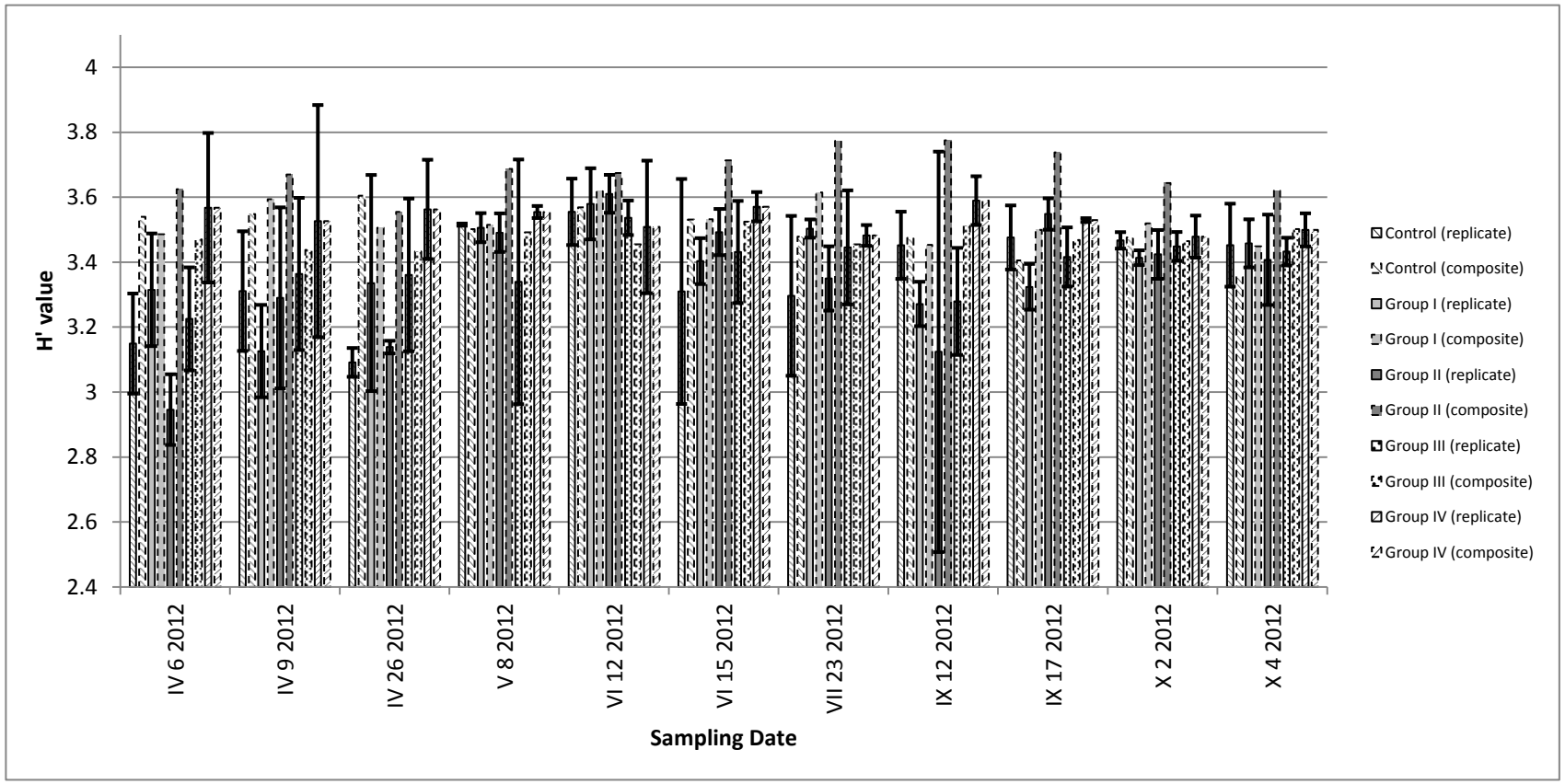

Figure 7.5 Graphic summary of Shannon's diversity index (H') values revealed in DGGE profiles produced from replicate and composite soil DNA extracts from soils in the growth chamber experiment; error bars represent 95% confidence intervals on population mean representing replicate samples.

7.2. Study 3 Appendix

7.2.1. Estimations of cell densities in PBP, supernatnat and cell pellet used in the First Bioassay

Cell densities were quantified after the study was completed and a PBP batch was used whose lactic acid bacteria cell count amounted to 4 x 10^6. This PBP was centrifuged and cells were washed as described in Study 3 Material and Methods section. PBP volumes of 250 ml were used and yielded 1g (0.004 g of cells ml^{-1} of PBP). The cell pellet and supernatant were collected. Loopful of cell pellet was found to be 0.1 g (equivalent of cells suspended in 25ml of culture). Therefore, cell pellet resuspended in 2 ml of PBS in the First Bioassay, could have contained 12.5 times more cells than PBP.

Cell densities in PBP and supernatant were compared by measuring absorbance at 600 nm wavelength (UltroSpec 2100 spectrophotometer ; Amersham Biosciences, Piscataway, NJ). Standard curve was prepared with PBP samples diluted in PBS; PBP (see Figure 7.6). Supernatant absorbance at 600 nm amounted to 0.017 suggesting that the optical density of the supernatant used in the First Bioassay could have corresponded to that of PBP diluted by the factor of 109.7.

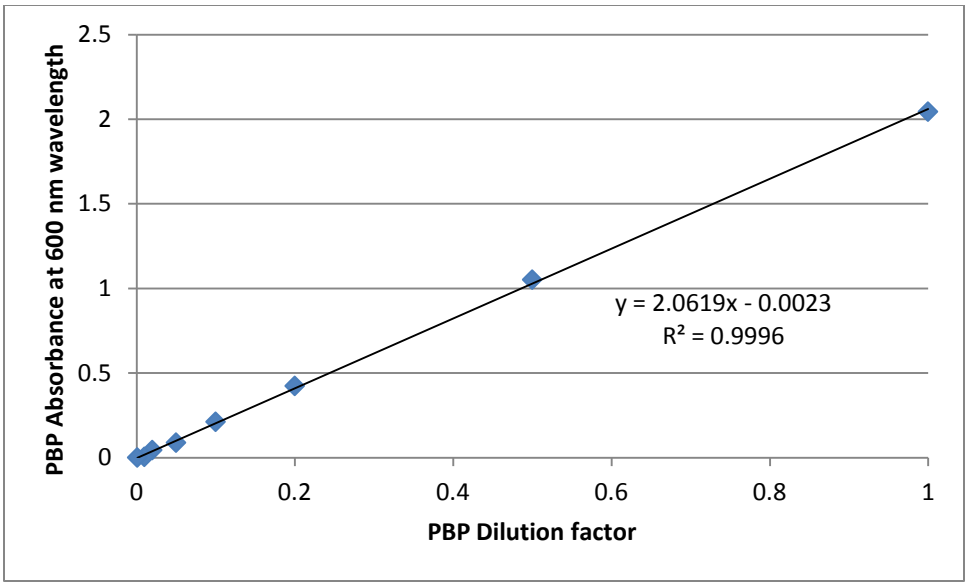

Figure 7.6 Calibration curve prepared by plotting SCD ProBio Balnce Plus™ (PBP) absorbance at 600nm wavelength against PBP dilution factor. Calibration equation and R2 are provided.

Table 7.11 (Presented in Figure 5.4)Concentrations of AHL revealed in mixtures from the Second Bioassay. Means followed by asterisks were significantly different than control.

Compound: AHL; Second Bioassay				
Exposure Period	Concentration [mg L^{-1}]			
	Mean	SD	Mean	SD
	Control		Treatment: CP 10%	
0h	85.40	12.33	78.18	10.79
2h	81.35	15.86	77.22	6.26
4h	59.30	17.26	38.93	7.67
8h	41.15	1.23	43.50	5.42
	Control		Treatment: SCD BioAG™ 10%	
0h	55.03	12.20	41.52	2.48
2h	66.02	10.17	34.15 *	5.33
4h	41.94	6.06	34.59	10.48
8h	29.41	2.00	0.00 *	N/A

Table 7.12(Presented in Figure 5.6) AHLEc signal intensities revealed in samples for AHLEc biodegradation assay.

Compound: AHLEc; Second Bioassay		
Exposure Period	Area	
	Mean	SD
Treatment: CP 10%		
0 h	78324.33	6265.63
2 h	71434.00	14051.5
4 h	87927.67	11136.51
8 h	73463.67	1864.184
Treatment: SCD BioAG™ 10%		
0 h	61701.00	4339.01
2 h	106141.3	52386.02
4 h	87384.67	12380.78
8 h	69216.67	19889.89
Treatment: CP 98%		
0 h	61642.00	12462.24
2 h	70372.33	2490.134
4 h	56295.67	7256.651
8 h	65782.00	2151.019
Treatment: SCD BioAG™ 98%		
0 h	46340.33	6914.16
2 h	46542.33	3180.944
4 h	48437.00	7795.997
8 h	54655.67	7875.83
Control		
0 h	64012.00	9852.585
2 h	75754.00	9202.087
4 h	70679.00	2064.347
8 h	68575.67	8925.882

7.2.2. Differences in RT between bioassays

There has been considerable change in RT observed for AHL in bioassays (from 16.42 in the First Bioassay to 23.23 min in the Second Bioassay) and this was most likely due to different physicochemical properties of GC columns that were employed for the analysis. Between bioassays, GC column was changed, so that extracts obtained in Study 3 Second Bioassay were analyzed using a new column.

7.2.3. Filtration procedure contributed an impurity and compromised AHL response

When chloroform extracts from bioassays with AHL were filtered and analyzed it was found that AHL response was heavily compromised and that another peak had formed at RT close to the RT previously recorded for AHL (See Figure 7.7). Figure 7.8 provides mass spectrum related to the new signal that appeared only after filtration. Similar signal was revealed in AHLEc bioassay extracts after filtration but no such signal was revealed earlier. This signal was most likely produced by an impurity added in the filtration process. It appeared that this impurity coeluted with AHL when Method 2 was applied which compromised accuracy of AHL analysis and results were discarded.

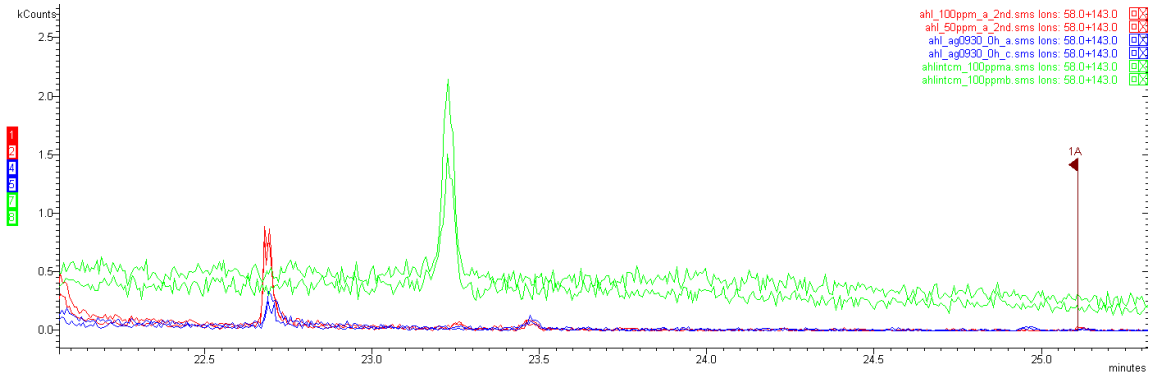

Figure 7.7 Chromatograms produced during analysis of AHL extracts: (1) filtered – blue and red; (2) unfiltered – green. Strong green peak indicates AHL response; peaks to left of AHL peak marked response from an impurity that was introduced during filtration process. Filters with m/z values of 58 and 143 were applied.

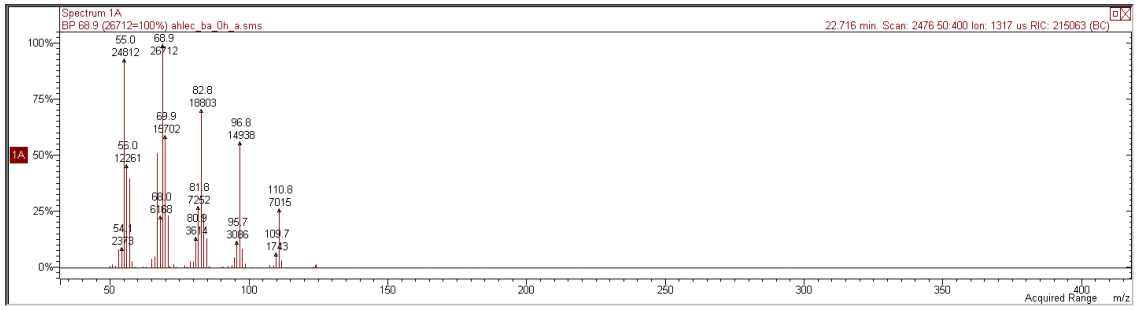

Figure 7.8 Mass spectrum associated with an impurity that was added to extracts during filtration procedure.

7.2.4. Extraction of AHL was heavily compromised by whole cultures (Second Bioassay)

Second Bioassay included an experiment where AHL was exposed to whole microbial cultures. However, when AHL was analyzed it was found that whole cultures may have heavily compromised AHL extraction efficiency (see Table 7.13 for AHL concentrations). Notably, AHL concentration in analytical mixtures was theoretically 108

mg L^{-1} and almost instantaneous, more than threefold reduction of AHL is highly unlikely. In the future, an improved extraction strategy will need to be developed to achieve satisfactory recovery of AHL from whole cultures.

Table 7.13 AHL Concentrations revealed in the second Bioassay when undiluted cultures were used.

Treatment: CP, undiluted			Treatment: SCD BioAg™, undiluted		
Exposure period [h]	Replicate	Observed value [mg L^{-1}]	Exposure period [h]	Replicate	Observed value [mg L^{-1}]
0	A	27.786	0	A	undetected
	B	30.378		B	undetected
	C	31.59		C	undetected
2	A	23.316	2	A	undetected
	B	undetected		B	undetected
	C	undetected		C	undetected
4	A	undetected	4	A	undetected
	B	undetected		B	undetected
	C	undetected		C	undetected
8	A	undetected	8	A	undetected
	B	undetected		B	undetected
	C	undetected		C	24.558

7.2.5. Quality of AHLEc GCMS response depended on the analytical solvent type

Initially, AHLEc was analyzed using a program similar to the one employed for AHL analysis, and it was found that the quality of signals produced by AHLEc (signal peak shape) were dependent on: (1) solvent that AHLEc was injected with and (2) the medium that AHLEc was associated with in bioassays (water or probiotic cultures). Particularly, when MEOH was used as solvent, peaks appeared wider than when

chloroform was used. A similar signal enhancement/suppression effect was observed in actual samples. Precisely, when extracts from control runs were analyzed, representative peaks were wider than when extracts from treatments with cultures were analyzed. This observation suggested that matrix effects played a considerable role in GCMS analysis. In an attempt to reduce matrix effects, the GC program was changed from the method used for AHL analysis to another, with more rapid temperature increase (for simplicity these methods are referred to as Method 1 and Method 2). Consult Figures 7.9 and 7.10 for depiction of signal peaks representative of AHLEc revealed in different media. As can be seen, quality of signals representative of AHLEc revealed with Method 2 was generally more uniform than when Method 1 was applied (peak shape is similar despite different media that AHLEc was associated with). However, AHLEc response when injected with MEOH was characterized by wide and low peaks in either method. The impact of MEOH on AHLEc signal peak shape could have been reduced by means of adding chloroform to AHLEc mixtures in MEOH. When AHLEc standards with MEOH were combined with chloroform, peak shape became similar to the one previously recorded for chloroform only standards as shown in Figure 7.11.

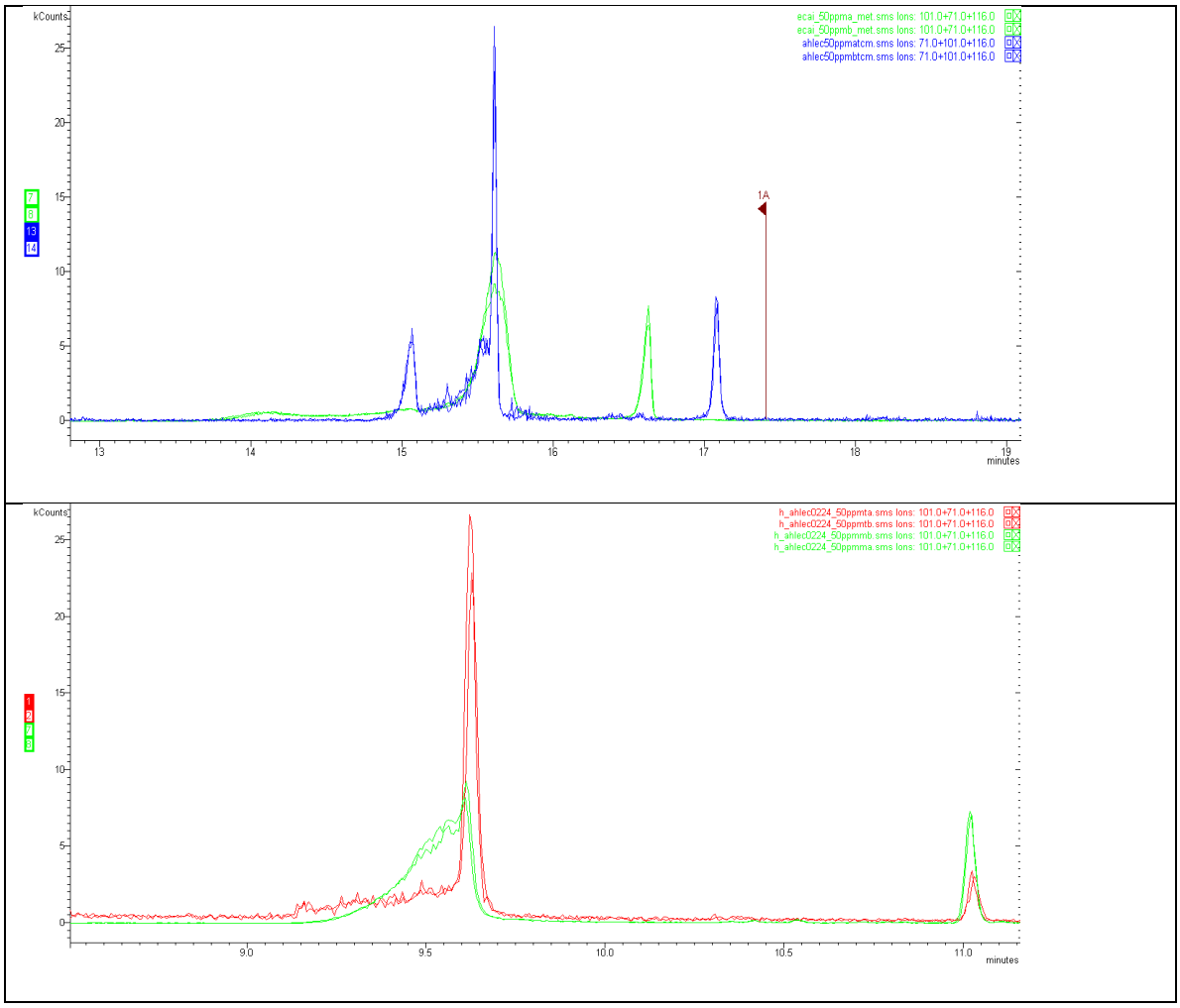

Figure 7.9 Chromatograms presenting AHLEc signals in standard dilutions containing equal amounts of AHLEc (50 mg L^{-1}). Upper graph relates to data reported by Method 1, while lower graph depicts signals recorded with Method 2. Blue and red lines represent signals produced by AHLEc when analyzed in chloroform; green lines refer to signals detected when AHLEc solutions in MEOH were analyzed. Ion filters with m/z values of m/z 71, m/z 101, and m/z 116 were applied.

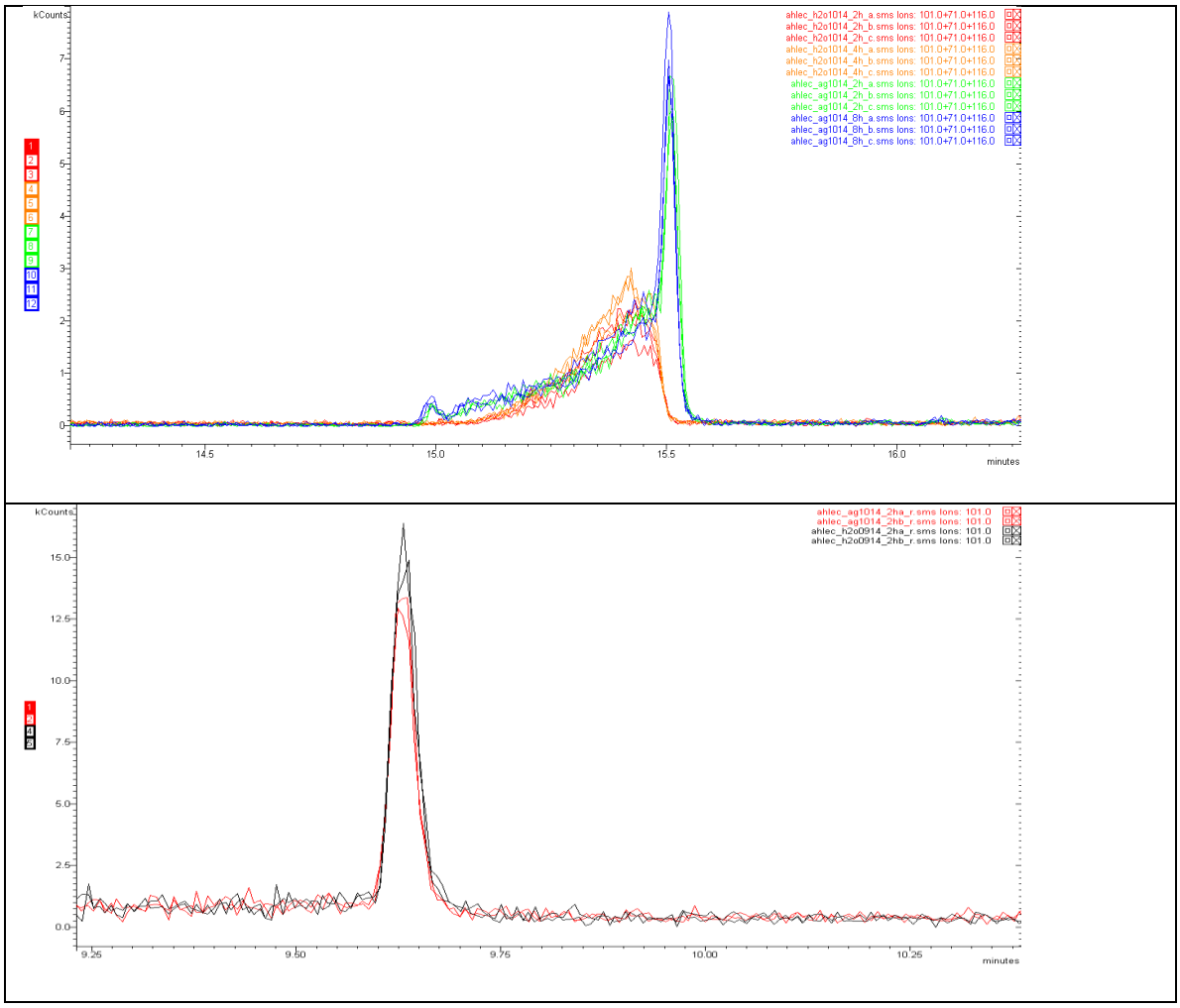

Figure 7.10 Chromatograms depicting AHLEc signals in actual samples as revealed by Method 1 (upper graph) and Method 2 (lower graph). On the upper graph, colors green and blue represent signals captured in a treatment (AHLEc and whole culture); while red and orange signify AHLEc response that was revealed in controls (when ion filter values m/z 71, m/z 101, and m/z 116 were applied). Lower graph's black lines represent signals captured from control while red lines indicate AHLEc response to treatment with whole culture using ion filter with m/z value of m/z 101

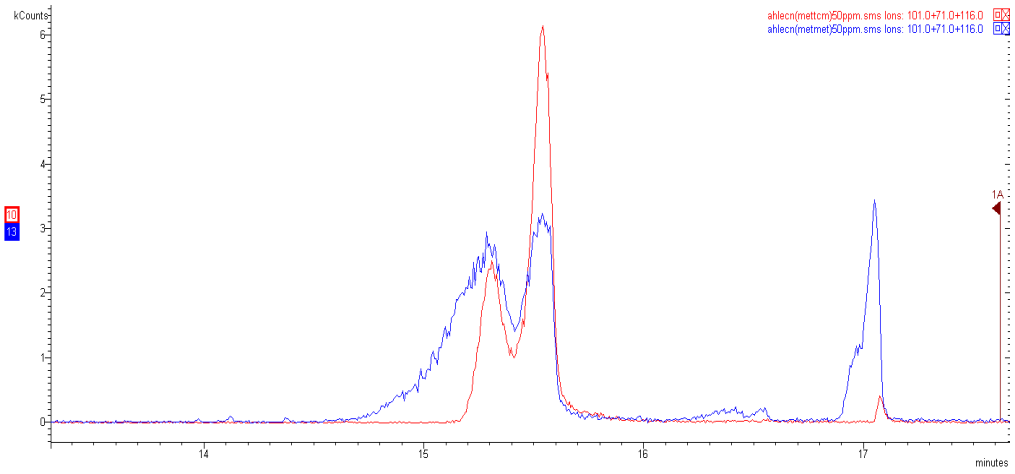

Figure 7.11 GCMS signal peaks recorded for AHLEc dilutions in MEOH (blue) and 50% chloroform in MEOH (v/v) (red)

7.2.6. Modification of standard dilutions to offset signal suppression/enhancement

When Method 2 was finally developed and applied for GCMS analysis of AHLEc, it appeared that standards with chloroform could have not provided for precise AHLEc quantification. Volumes of standard dilutions were compromised due to evaporation and vapor loss which most likely escaped through holes left in caps by GCMS injector needle. The method development lasted for over two months which apparently was long enough for the solvent to evaporate. A new set of standards was prepared – AHLEc solution in MEOH (100 mg L^{-1}, 50 mg L^{-1} and 25 mg L^{-1})—and suspensions of AHLEc in MEOH were diluted with chloroform (50% dilution v/v). This improved AHLEc signal quality as seen before. However, when AHLEc+MEOH+chloroform mixtures were analyzed by Method 1 and AHLEc was quantified on average, AHLEc response corresponded with 53% of nominal

concentration in these solutions (see Table 7.14) .Unsurprisingly, when calibration algorithm for Method 2 was tentatively fed with values representative of AHLEC+MEOH+chloroform standards and actual samples were analyzed, concentration values were returned that were as much as two times higher than theoretical concentrations in samples. Hence, Method 2's calibration algrorithm was chosen to use data representative of signals produced by AHLEc in MEOH/chloroform for verification of AHLEc response and quantification of AHLEc signal but not for drawing a relation between AHLEc signal intensity and AHLEc concentrations.

Table 7.14 AHLEc response in MEOH/chloroform standards

Nominal Concentration (mg L^{-1})	Actual Concentration	
	mg L^{-1}	Nominal Concentration Percentage
50	22.54	0.45
50	34.77	0.70
25	11.95	0.48
25	14.47	0.58
12.5	5.56	0.45
	Average	0.53
	SD	0.11

7.2.7. Improved analytical power of Method 2

Signal intensity revealed during analysis of the same standard dilutions was as much as 3.46 (+/- 0.73) times higher for Method 2 than Method 1. This indicates that Method 2 was more powerful than Method 1 and became the standard (preferred method) for surveying AHLEc in this research.

7.2.8. Tentative explanation of AHLEc signal enhancement/suppression

Results suggest that hydrophilicity and hydrophobicity of AHLEc matrix had a pronounced impact on AHLEc signal profiles. That may have been because AHLEc formed an emulsion with water. Application of chloroform for extractions could have removed virtually all water molecules when AHLEc was extracted from samples that contained probiotic cultures. However, some amount of water could have remained in chloroform extracts from samples that contained only water. These contrasting outcomes could have resulted from varied ionic strength of solutions that AHLEc was extracted from. In detail, in case of samples that contained probiotic cultures, solutions most likely had strongly ionic environments (conditions included: low pH, presence of salts, organic acids and other byproducts of microbial metabolism), while in control samples, ionic strength was conceivably lower (only small quantity of acetic acid was added in order to acidify deionized water). Interestingly, modification of ionic strength of aqueous solutions (by means of adding salts or organic ions) prior to extraction is a known method to reduce emulsification in extraction processes (Jansson, 1992). Furthermore, it is likely that application of a more rapid temperature increase in GC program could have facilitated water removal and loss of the emulsion-like character of AHLEc giving an enhanced signal in comparison to that realized under a slower temperature increase.

However, peak shapes recorded for AHLEc in conjunction with MEOH indicated that some signal suppression was present despite application of more rapid temperature increase, which suggested that principles underlying the suppression were more complex. Supposedly, a cluster of AHLEc could have formed due to hydrogen bonding between AHLEc and media (water or MEOH). This proposition is drawn on the basis of findings

reported by Kaufmann et al. (2005) who found that products of degradation of NAHLs can form clusters that represent siderophore-like properties (see Figure 7.12). Arguably, when AHLEc interacted with non-ionic, hydrophilic moieties (MEOH for instance), similar clusters could have formed.

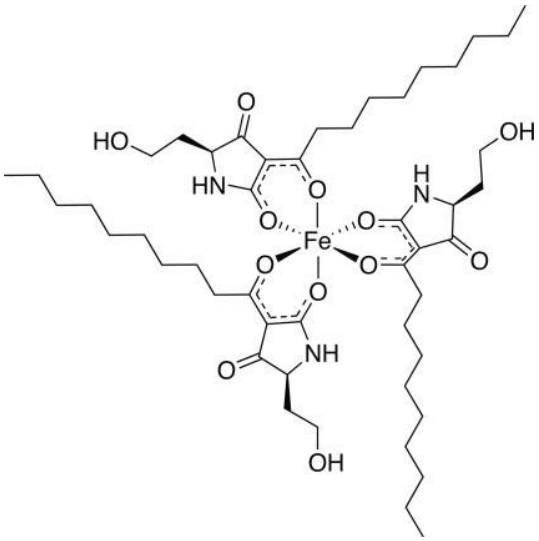

Figure 7.12 A cluster formed by tetramic acid (a product of degradation of an NAHL) associated with Fe^{3+} ion (Kaufmann et al., 2005)