DIVERSITY AND ACTIVITY OF FREE-LIVING DIAZOTROPHIC AND TOTAL BACTERIAL COMMUNITIES IN ORGANIC AND CONVENTIONALLY MANAGED SOIL.

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Abstract

Agricultural soils are heterogeneous environments in which conditions affecting microbial growth and diversity fluctuate widely in space and time. This study aims to test the hypothesis that the use of organic farming practices (fertility management and crop protection) enhances the diversity and activity of free-living N fixers as well as the bacterial community as a whole. The effects of seasonal variability, and crop rotation, on the diversity and activity of free-living N fixers and the total bacterial community were also tested. Soils were taken from the Nafferton factorial systems comparison (NFSC) study in North East England, and were sampled in March, June and September of 2007, 2008 and 2009. PCR-DGGE and qPCR analysis of the *nifH* and 16S rRNA genes were utilized as well as sequence analysis and community level substrate utilization in the form of BIOLOG plates.

Overall, season and crop rotation produced the most community variability. Diversity and activity of both genes were decreased in June after perturbation, regardless of management type. On average conventional fertility management led to increased bacterial and diazotrophic gene copy number, and both communities were significantly influenced by pH, carbon and nitrogen availability. Crop protection protocols affected the two communities differently with organic crop protection promoting the diazotrophic community and conventional crop protection promoting the total bacterial community. The presence of legumes in the organic rotation had a detrimental effect on activity and diversity of the diazotrophic community as excess nitrogen remained in the soil restricting the development of the nitrogen fixing community.

To our knowledge the effects of organic and conventional farming systems on free-living diazotrophs have never been studied, particularly with respect to the effects of crop rotation and crop protection protocols. An increased understanding of the impacts of management practices on free-living N fixers could allow modifications in soil

management practices to optimize the activity of these organisms. It is also hoped that fully understanding nitrogen fixation will help farmers adapt a more rational fertility management system in turn reducing some of the negative environmental impacts of unused nitrogen species.

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List of Accompanying Material.

A journal article and conference proceedings with the references:

> Orr, C.H., James, A., Leifert, C., Cooper, J.M. & Cummings, S.P. (2011) 'Diversity and Activity of Free-Living Nitrogen-Fixing Bacteria and Total Bacteria in Organic and Conventionally Managed Soils', *Applied and Environmental Microbiology*, 77 (3), pp. 911-919.
>
> Orr, C., James, A., Cummings, S. P., Leifert, C. & Cooper, J. (2009) 'The effect of organic and conventional farming practices on the soil microbial community carbon substrate utilization', *International Conference on Positive Plant Microbial Interactions in Relation to Plant Performance and Ecosystem Function*. Grantham, UK. 16-19 December 2009. Warwick, UK.: the Association of Applied Biologists, pp. 67-74.

respectively are attached as accompanying material at the end of the thesis.

List of Abbreviations.

ANOVA = analysis of variance

APS=ammonium persulphate

ATP= adenosine triphosphate

AWCD= average well colour density

Bp = Base pair(s)

BSA = Bovine Serum Albumin

°C = degrees Celsius

CCA = canonical correspondence analysis

CLSU = community level substrate utilization

Con = conventional

CP = crop protection

DCA = detrended correspondence analysis

DGGE=denaturing gradient gel electrophoresis

DNA = deoxyribonucleic acid

dNTPs = deoxynucleotriphosphate

FM = fertility management

g = gram(s)

H’ = Shannon diversity index

Ha = hectare(s)

IPTG = Isopropyl-β-D-Thiogalactopyranoside

L = litre(s)

M = Molar

Min = minute(s)

mm = Millimetre(s)

mA = milliamps

NFSC = Nafferton Factorial Systems Comparison

OD = optical density

Org = organic

PC= previous crop

PCA = principal component analysis

PCR=polymerase chain reaction

Pot/wb = winter barley followed by potatoes

Pot/beans= beans followed by potatoes

qPCR = quantitative polymerase chain reaction

RDA = redundancy analysis

SBR = soil basal respiration

SD = sample date

sp. = species

TEMED = N,N,N',N'-tetramethylethylene diamine

UV = Ultraviolet

V = Volt(s)

v/v= volume by volume

w/v = weight by volume

X-Gal = 5-bromo-4-chloro-3-indoyl-β-D-galactoside

Y = year

α = alpha

β = beta

γ = gamma

µ = micro

3' = Three prime

5' = Five prime

Dedication

This thesis is dedicated to my Mam, Jean Orr. You did the hard part this year by getting better. I can never thank you enough for all the help and support you have given me even when you have been struggling yourself.

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Firstly I would like to thank my supervisor Prof. Stephen Cummings for his support, molecular biology expertise and much needed patience throughout this process. I also appreciate the constructive criticism, which helped in the preparation of this thesis.

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Declaration

I declare that the work contained in this thesis has not been submitted for any other award and that it is all my own work. The work was done in collaboration with Nafferton Ecological Farming Group, Newcastle University.

Name:

Signature:

Date:

1. Introduction

1.1 Brief review of soil microbial diversity and the molecular methods often used.

Soil is an incredibly heterogeneous environment supporting a higher population of prokaryotes than any other habitat (Delmont *et al*., 2011; Tiedje *et al*., 1999). There are many different methods used to investigate bacterial diversity within soil environments. A basic summary of these methods is provided in table 1.1. By using these methods some basic characteristics of general soil microbial diversity have been deduced.

One gram of soil is thought to harbour thousands of different species of bacteria, most of which are unknown and unculturable (Handelsman *et al*., 1998). It is estimated that, of these thousands of species, only 1% of the soil bacterial community is currently culturable. It is possible that this 1% does not well represent the bacterial community as a whole (Kirk *et al*., 2004). It is this reason which makes molecular biology necessary to study the functioning and processing of this environment. Our understanding of microbial diversity has been greatly expanded by the use of molecular methods including: denaturing gradient gel electrophoresis (DGGE), phospholipid fatty acid/phospholipid ether lipid analysis (PLFA/PLEL), terminal restriction fragment length polymorphism analysis (T-RFLP) and pyrosequencing. For example, when a culture-dependent-DGGE approach and culture-independent DGGE approach were compared 32% of the profiles identified were unique to the culture-independent approach (Edenborn and Sexstone, 2007). However, there are still problems associated with the use of molecular methods due to a general lack of taxonomic understanding of unculturable microorganisms (Kirk *et al*., 2004). As so many members are unculturable the majority of soil taxa are relatively unstudied, with a few exceptions of taxa such as the ammonia-oxidizing *Nitroso*- genera which have a specific physiological capability (Fierer *et al*., 2007).

Table 1.1. Brief summary of common methods used to study soil microbial diversity (adapted from Kirk *et al*., 2004).

Technique	Brief description	Positives	Drawbacks	Example of study using this technique to measure soil microbial diversity
Plate counts	Bacteria are grown on selective media and identified using biochemical tests	Fast and inexpensive	Unable to detect unculturables and is baised towards fast growers	Tabacchioni *et al*., 2000
Community level physiological profiling (CLPP)	Bacterial communities are exposed to various carbon sources and the level of substrate utilization is measured.	Fast and can be tailored so that carbon source match specific environments	Biased towards fast growing heterotrophs and doesn't detect unculturable bacteria	Fleißbach and Mäder, 2004
Fatty acid methyl ester (FAME) analysis (e.g. Phospholipid fatty acid analysis (PLFA))	Biochemical method which measures changes in fatty acid content of microbial communities using gas chromatography	Do not need to directly culture microorganisms	Can be influenced by external factors	Esperschütz *et al*., 2007
Nucleic acid hybridization	DNA is extracted, denatured and allowed to anneal in the presence of probes. The rate the DNA reanneals is proportional to the complexity (diversity) of the community.	DNA or RNA can be studied. Does not suffer from PCR bias.	Not very sensitive and requires high copy numbers	Cho and Tiedje, 2001
DNA microarrays	Microarrays contain genes of interest. DNA is denatured and allowed to anneal. The presence of probes allows any DNA annealing to genes on the microarray to be identified.	Thousands of genes can be used	Only accurate in low diversity environments	Greene and Voordouw, 2003

Table 1.1 (continued) Brief summary of common methods used to study soil microbial diversity (adapted from Kirk *et al*., 2004).

Technique	Brief description	Positives	Drawbacks	Example of study using this technique
Denaturing gradient gel electrophoresis (DGGE)/Temperature gradient gel electrophoresis (TGGE)	Genes of interest are amplified using PCR. A community 'fingerprint' showing the number of individual organisms carrying the gene is obtained by separating according to the G/C content of the organisms sequence.	Reproducible and reliable	PCR bias means dominant species are favoured. One band can represent more than one species	Hayden *et al*., 2010
Terminal restriction fragment length polymorphism (T-RFLP) analysis	Genes of interest are amplified using PCR with fluorescently labelled tags. Fragments are then digested with restriction enzymes and individual organisms are identified by the resulting fragment lengths.	Reproducible and capable of automation	Similar to DGGE/TGGE	Hartmann *et al*., 2006
Ribosomal intergenic spacer analysis (RISA)/automated ribosomal intergenic spacer analysis (ARISA)	Intergenic spacer regions are amplified using PCR and sequence polymorphisms are detected by separating on a polyacrylamide gel.	Highly reproducible	Need large quantities of nucleic acid	Tiedje et al., 1999
Clone library	Gene of interest is amplified using PCR. The gene is then cloned and the clones sequenced to identify individual members of the community	Individual organisms can easily be identified by sequencing clones.	Labour intensive and dominant members are favoured	Coelho *et al*., 2009
Pyrosequencing	PCR is carried out using a bar code label system. All PCR products are then sequenced and identified.	Provides large amounts of sequence data showing exactly what members are present	Expensive and dominant members are favoured.	Acosta-Martinez *et al*., 2008

Residing within the soil there are thought to be approximately 52 bacterial and 4 Archaeal phyla (Swift *et al*., 2008). A recent study (Coleman *et al*., 2010) analysing total soil bacteria within Australian vertisols found that, of those that could be identified, *Proteobacteria*, particularly *β-Proteobacteria* is the most abundant phyla, followed by *Firmicutes*, *Acidobacteria* and *Bacteroidetes* respectively, with *Planctomycetes*, *Verrucomicrobia* and *Gemmatimonadetes* being other reasonable well represented phyla. However, many of these abundant taxa, for example *Acidobacteria*, are particularly difficult to culture and therefore little is known about their physiological capabilities or habitat preferences (Edenborn and Sexstone, 2007; Fierer *et al*., 2007).

1.2 Soil management and its effects on the total bacterial community.

In general, land use can alter the soil microbial community. Environmental differences, changes in the plant species grown, and the associated management practices leads to changes in soil carbon, soil structure, nutrient contents and pH which can in turn alter the soil microbial community (Lauber *et al.,* 2008). This has been repeatedly demonstrated when comparisons are made between the microbial community of forest, pasture and crop land soil, with changes in soil pH, carbon mineralization rates and soil nutrient status given as the cause (Lauber *et al*., 2008; Jangid *et al*., 2008). As agricultural land is subject to the most anthropogenic change, in the form of application of fertilizers and tillage, it is often found to have a different bacterial community structure to other soils. For example, when comparisons were made between soils under different land-uses, Jangid *et al.* (2008) found cropland had increased *Delta-* and *Gamma-proteobacteria*, and decreased *Firmicutes* and *Alpha-proteobacteria*, when compared to forest and pasture soil.

There is also evidence that historical soil characteristics brought about by agriculture continue to influence the soil microbial community for decades after such activity has ceased. For example, agricultural soil which had been abandoned for 9 years was more

similar, in terms of the seven most common members of the soil bacterial community, to current agricultural field soil than soil which had never been managed (Philippott *et al*., 2010; Buckley and Schmidt, 2003). Suggesting that, although soil microbial communities are dynamic they may still exhibit patterns due to past and current management, and that the anthropogenic changes observed in agricultural systems clearly affect the soil microbial community.

Crop production can be carried out either organically or conventionally. Organic farming is regulated in the UK by guidelines laid down by the Soil Association (www.soilassociation.org). A strong emphasis is placed on protecting the environment by severely restricting the use of synthetic chemical fertilizers and pesticides. In order to maintain soil fertility, composts and animal manures are applied to soil, and diverse crop rotations are used. Pests and disease are also kept under control by the use of carefully thought out crop rotations, natural pesticides and good cultivation practice.

The Organic food and farming action plan outlined by DEFRA (http://www.defra.gov.uk/farm/organic/policy/actionplan/annex3.htm) combining studies carried out by MAFF, English Nature, The European Commission and the Soil Association found that on average organic farming improves biodiversity (of both macro- and microorganisms), energy efficiency, pesticide pollution, and reduces carbon dioxide emissions as it complies with strict regulations on the amount of chemicals and other inputs that can be added to the land.

Conventional farming is harder to define as it is not controlled by EU standards. It focuses on increasing productivity by the use of chemical fertilisers, pesticides and monocultures. As the planet's population increases the need for high levels of production is increasingly important. For example, it is estimated that by 2030 40% more rice will have to be grown to meet demand (Khush, 2005). However, there are a number of environmental problems associated with conventional farming. These include water pollution through nutrient leaching, soil erosion and decreases in landscape quality, food safety and biodiversity

(Hole, 2005). An extreme example of the detrimental effects of conventional agriculture is the oxygen starved “dead zone” in the Gulf of Mexico, an almost lifeless area which is a result of an accumulation of leached nutrients from farms across the Mississippi (Adesemoye *et al*., 2009).

For these reasons, agricultural policy laid down by the EU has changed over the last 10 years and farmers are now encouraged to focus on environmental benefits, such as countryside quality and reduced pesticide residues in food, as well as increased production (Firbank, 2005; Hole, 2005).

The overall effect of organic and conventional farming systems on the total microbial community has been previously studied. Mäder *et al.* (2002) compared organic and conventional systems and found increased soil diversity in the form of: increased microbial biomass, increased dehydrogenase activity, increased alkaline phosphatase and increased area of root length colonized by mycorrhizal fungi associated with organic farming. A separate study found no difference in microbial biomass and activity between two organic and conventional systems but did find differences in the soil microbial community structure (Donnison *et al*., 2000). As there are many differences between the two farming systems, changes to the soil microbial community could be due to differences in fertility management, crop protection protocols and crop rotations between the soil management regimes. The effect of all three processes has been studied in the complex DOK experiment in Switzerland (Esperschütz *et al*., 2007). All three factors were found to influence the microbial community with fertility management having the most profound effect followed by pesticide application and crop rotation respectively.

Currently the factor most thought to influence the soil microbial community is the application of fertilizers. Conventional farms rely on synthetic fertilizers such as ammonium sulphate, ammonium nitrate and superphosphate. Whereas, organic farms rely on compost, green manures and manure from livestock (Bulluck *et al.,* 2002). It was assumed the overriding effect of fertility management was due to the fact that farmyard

manure released a diverse mixture of organic compounds stimulating a complex microbial community. The application of farmyard manure has been shown to affect the soil biota in terms of total biomass (Esperschütz *et al*., 2007; Bossio *et al*., 1998) and microbial activity (Widmer *et al*., 2006). It has also been shown to change the microbial community structure in terms of diversity (Hartmann et al., 2006; van Diepeningen *et al*., 2006) and activity (Wessén *et al*., 2010). These differences are mainly attributed to differences in levels of nitrogen, carbon and pH between the two management systems (Wessén *et al*., 2010). The organic substrates and increased soil carbon provided by farmyard manure may stimulate microbial growth when compared with soils exposed to no fertilizer and conventional mineral fertilizer (Jangid *et al*., 2008; Hartmann *et al*., 2006).

A study compiling results from 39 different field trials found that on average organic farming produces 28% higher soil carbon levels compared to non-organic farming, in northern Europe (Azeez *et al*., 2009). The study also demonstrated a positive association with soil carbon levels and soil quality, soil structure, and microbial biomass. R strategists are opportunistic bacteria which grow quickly in response to favourable conditions; whereas, k strategists are slow growing microorganisms, which proliferate best when conditions are less favourable to competition (Sarathchandra *et al*., 2001). When organic fertilizers are first applied fast growing r strategists are mainly found, presumably utilizing the readily available carbon. However, when conventional fertilizers are used less carbon is available and the microbial community shifts towards those k strategist microorganisms (Esperschütz *et al*., 2007).

There are also secondary effects due to the input of different fertilizers. Pagliai *et al*. (2004) found that manure and compost react with the soil matrix enhancing pore size and soil porosity.

The main secondary effect, however, could be changes in soil pH, as pH is considered a predictor of soil microbial community composition (Fierer and Jackson, 2006). In general

bulk soil has a stable pH. However, perturbations such as liming and changes in plant community can suddenly change pH forcing the microbial community to adapt (Fernandez-Calvino and Bååth, 2010). The addition of both organic and chemical fertilizers lowers soil pH, this is more apparent after the addition of chemical fertilizers such as ammonium sulphate (Hao *et al*., 2008). Hallin *et al.* (2009) found that pH affected community composition between soils treated with different fertilizers. *Acidobacteria*, for example, are more tolerant of acidic environments and are therefore observed in higher numbers after fertilizers are added (Wessén *et al*., 2010; Jangid *et al*., 2008). As most microorganisms proliferate best within 1 pH unit of neutral, bacterial richness and diversity plateaus at near neutral pH (Fierer and Jackson, 2006). Meaning, soil pH can be used to predict the diversity and composition of the soil bacterial community.

Crop protection measures could also potentially affect the soil microbial community. Conventional farmers can use a complex mixture of herbicides, insecticides, fungicides, growth regulators and desiccants to protect their crops. Globally around 3 $x10^9$ kg of pesticides is used in conventional farming annually. It is estimated that only 0.1% of this actually reaches the target organism (Hussain *et al*., 2009). As organic farmers are severely restricted on the use of chemicals, they must rely on: mechanical weeding and flaming to combat weeds; crop rotations; natural plant extract-based fungicides and copper based treatments to protect against disease; and netting and the use of predators/competitors to avoid damage from pests (Litterick *et al*., 2002).
When applying chemicals to the soil it is possible that they may affect non-target organisms. As soil microorganisms are small in size and large in number they can provide a large surface for chemicals from the surrounding soil to interact with (Cycoń and Piotrowska-Seget, 2009). Individual groups of microorganisms will react differently to the presence of pesticide some will proliferate as they will be able to degrade pesticides and use them as an energy source, while others will find the pesticide toxic which may relieve some species of the stress of competition (Hussain *et al*., 2009; Johnsen *et al*., 2001). For

example, soil basal respiration and community level substrate utilization (CLSU) were used to compare the bacterial community within potato soil before and after the application of a defoliant spray. Initially increased soil basal respiration was observed following application of the pesticide suggesting high C turnover possibly due to the toxic action of the pesticide. Microbial biomass and activity was also reduced although this returned to normal after 135 days. Interestingly community level substrate utilization showed continued increased heterotrophic activity possibly due to increased catabolic capabilities as the pesticide is degraded, and adaption to environmental change (Flieβbach and Mäder, 2004).

The majority of studies into the effects of chemical pesticides have found that they do not significantly affect the soil microbial community when used at the correct dose (Spyrou *et al*., 2009; Cycon and Piotrowska-Seget, 2009; Bending *et al*., 2007). However, in most instances the pesticides are tested singularly and the effects measured against a sub-set of the bacterial community. In realistic agricultural situations, pesticides are often used in combination (Flieβbach and Mäder, 2004). Cycon and Piotrowska-Seget (2009) found that although there was no significant change to the microbial community at recommended dosages of herbicides, fungicides and insecticides fast growing microorganisms (r strategists) seemed to become more dominant after exposure as they are more suited to the less stable environment. They also found that cfu counts were higher in low doses following insecticide and herbicide treatment probably due to the microorganisms degrading the chemical and using it as a carbon source (Cycoń and Piotrowska-Seget, 2009). It is the unculturable bacteria, in particular the methanotrophs and ammonia oxidizers, which are thought to be most greatly affected by long term pesticide application (Seghers *et al*., 2003; El Fantroussi *et al*., 1999).

Organic crop protection methods can also possibly affect the soil microbial community. Copper oxychloride was found to reduce soil metabolic potential using BIOLOG techniques and significantly affect protist numbers when used in concentrations greater

than 100 mg/kg (Du Plessis *et al*., 2005). However this greatly exceeds the recommended dosage.

Organic systems include crop rotations and ley periods which allow the soil to recover lost nutrients whereas, conventional systems mainly rely on monocultures and more intensive crop rotations containing more cash crops. The crop growing in the soil can influence the soil microbial community as different plant species will contribute different quantities and qualities of carbon to the rhizosphere (Ladygina and Hedlund, 2010). However, it is unclear if this is significant, especially in bulk soil, due to the influence of other soil environmental factors (Ostle *et al*., 2003). It is also unclear whether the effect persists once other crops are grown.

The presence of a crop rotation has a positive effect on the soil microbial community. Acosta-Martinez *et al.* (2008) used pyrosequencing to show microbial diversity was significantly lower in soils from a cotton monoculture compared to soils from a cotton-wheat-corn rotation. In soil from the monoculture 181 bacterial species were detected, compared to 285 species in soil from the rotation. Soils under the rotation also had higher Gram negative species and lower Gram positive species than the soil from the monoculture. It is thought that this is due to the positive influence of quality organic materials deposited in the soil from the different vegetation. However, conventional farmers also often use crop rotations as they are known to optimize yield by breaking disease patterns (Larkin and Honeycutt, 2006).

The crops present within a rotation may also affect the microbial community. Legumes are thought to have a positive effect on the diversity and activity of soil microorganisms as they have root exudates rich in nitrogen (Ladygina and Hedlund, 2010; Wardle *et al*., 2003; Warembourg *et al*., 2003). When comparing winter wheat soil, which had previously grown clover and peas, with winter wheat monoculture soil Lupwayi *et al*. (1998) found

microbial diversity was significantly higher under the legume based crop rotation. Pascault *et al*. (2010) showed that incorporation of alfalfa, rape and wheat residues all affected the microbial community differently as they degraded to offer variable carbon sources. Alfalfa and rape residues led to higher soil respiration and higher numbers of *Proteobacteria*. Wheat residues led to increased *Actinobacteria* and *Bacteroidetes*.

Previous crops in a rotation can affect the soil microbial community. However, the effect is often overshadowed by the effect of the application of manures and composts (Esperschütz *et al*., 2007; Hartmann *et al*., 2006). The effect of preceding crop was measured using PLFA/PLEL (Esperschütz *et al*., 2007) and T-RFLP (Hartmann *et al*., 2006) for the same field trial in Switzerland. PLFA and T-RFLP results did change significantly due to preceding crop but this mainly corresponded to small changes in the fungal and eukaryotic communities rather than the bacterial community.
In contrast, Larkin (2003), and Larkin and Honeycutt (2006) compared microbial activity in 2 and 3-year cropping systems and a potato monoculture using BIOLOG and FAME (fatty acid methyl ester) and found the immediate preceding crop to be the primary influential factor. Grain crops, such as wheat, barley and oats, tended to support greater microbial activity and biomass possibly due to the fact that, in general, grain residues double organic C content and the C to N ratio, when used in rotation, and compared with crops like potato and soybean (Meriles *et al*., 2009). The structure of the microbial community, and fungal-bacteria ratios, also changed in response to rotation. For example, barley rotations led to increased *Actinomycetes* and fluorescent pseudomonads (Larkin, 2003). In general the application of farm yard manure, the restriction of pesticides and the use of diverse crop rotations are thought to have a positive effect on microbial diversity and activity. However, results are equivocal and studies mostly focus on small sections of farm management rather than full systems. Part of this study will examine the effect of full farm management systems on the soil microbial community which inhabit bulk soil to try and add clarity to the discussion above.

In this study soil samples will be taken from 3 years (2007-2009) and at 3 time points within each year (March, June and September). It is possible that sample date could affect the bacterial community. Although they offer no explanation as to why, Jangid *et al*. (2008) found the diversity of the microbial community in winter to be almost double that of the summer when analysing microbial communities in cropland and pasture of Georgia, USA. They also found Gram positive organisms to favour winter and Gram negatives to favour summer and suggest this is due to changes in numbers of Gram negative *Proteobacteria*. Wakelin *et al*. (2009) also saw significant changes in the diversity of the bacterial community over the growing season. They suggested this was due to changes in water availability but also suggested changes in temperature and changes in quality and quantity of plant root exudates could have an effect.

Clearly changes to soil management can affect the soil microbial community in many ways (Stark *et al*., 2008). Any changes in soil microbial diversity and activity could lead to changes in carbon and nitrogen cycling and organic matter decomposition, as bacteria are responsible for 90-95 % of nutrient cycling (He *et al*., 1997). This can affect the crops growing in the soil as nitrogen and carbon are two common limiting factors.

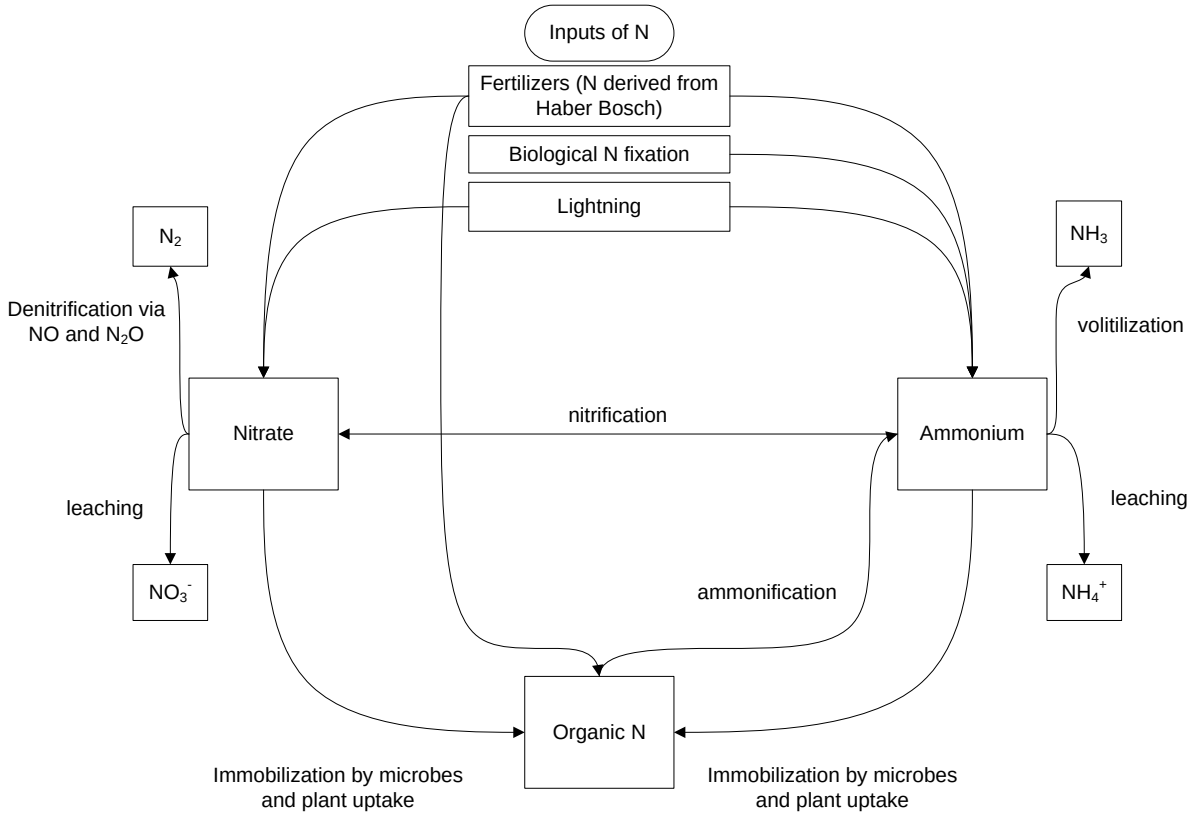
1.3 Nitrogen fixation and the effect of farm management on the free-living diazotrophic community.

Nitrogen is essential to all living-organisms. Although there is approximately 5 billion metric tons of nitrogen contained in the Earth's atmosphere, oceans and rocks as well as the terrestrial, marine and soil biota, less than 2% is available to organisms (Galloway, 1998). After water, nitrogen is often the limiting factor in crop growth, and therefore, plays a crucial role in the organization and functioning of the world's ecosystem (Vitousek *et al.*, 2000).

There are 3 main pools of nitrogen; gaseous N (N_2, NO, NH_3 and N_2O), inorganic N (NH_4^+, NO_3^- and NO_2^-) and organic N (in organic matter and plants). The nitrogen cycle is basically a series of steps which links the N pools.

To be utilized the triple bond of di-nitrogen (N_2) must be broken and the nitrogen must be 'fixed' in a more useable form. Before human involvement this was carried out either by biological nitrogen fixation (BNF) carried out by certain microorganisms (90-130 $TgNyear^{-1}$) or by lightning (3-5 Tg N $year^{-1}$) (Galloway *et al*., 1995). Nitrogen can also be fixed chemically using the Haber-Bosch process, used to make chemical fertilizers. Nitrogen fixation will be discussed further later in this chapter. Once fixed, ammonium can also be converted to nitrate in the soil via nitrification. Nitrification is carried out by members of 3 genera of *Proteobacteria*; *Nitrosomonas, Nitrosococcus* and *Nitrosospira* and ammonia-oxidizing archaea (Di *et al*., 2010; Rotthauwe *et al*., 1997).
Ammonium and nitrate can then be used above ground via plant uptake or below ground via immobilization by microbes.

Figure. 1.1. Simplified diagram of the nitrogen cycle.



When inorganic N species are in excess they are often leached out of the soil into streams, ground water and the atmosphere. This can lead to environmental concerns as excess nitrogen can pollute an ecosystem. Leaching adversely affects soil fertility due to the fact that nitrate ions are negatively charged and will therefore associate with positively charged ions such as calcium, magnesium and potassium (Vitousek *et al*., 2000). Nitrogen is also lost from systems in gaseous forms. Ammonium can be converted to ammonia via volitization, however, the most studied loss of gaseous N is the conversion of nitrate back to N_2 via the anaerobic microbial redox process denitrification (Zumft, 1997). This step wise process involves the conversion of nitrate to nitric oxide, then nitrous oxide, before finally being converted to di-nitrogen. Nitrous oxide depletes ozone from the stratosphere leading to global warming, and nitric oxide causes acid rain. The emissions of both of these gases, as well as the leaching of nitrate, are affected directly and indirectly by the use of N fertilizers (Pang and Letey, 2000; Smith *et al*., 1997). By further understanding the microbes responsible for these processes it may be possible to minimize the excess N, which leads to environmental problems.

Nitrogen is vital for all living organisms and is required in relatively large concentrations by most agricultural crops. Crops such as wheat, rice and maize need 20 to 40 kg soil N Ha^{-1} over a period of 3 to 5 months to satisfy the N requirements for each tonne of grain produced (Peoples *et al*., 1995). It is essential for crop production as it is a vital component of proteins, nucleic acids, porphyrins and alkaloids (Schulten and Schnitzer, 1998).

Although the atmosphere is 79% dinitrogen it is unavailable to the vast majority of organisms in this triple bonded form and needs to be ‘fixed’ into ammonium. In the production of chemical fertilizers nitrogen is fixed via the Haber-Bosch method. This chemically fixed N is a convenient and relatively cheap way of providing bioavailable N. However, the Haber-Bosch process relies on non-renewable, and increasingly expensive, fossil fuels (the production of 1 kg N-fertiliser requires 38,000 kJ of fossil energy)

(Refsgaard *et al*., 1998) and results in significant emissions of greenhouse gases (IPCC, 2006).

The vast majority of biological nitrogen fixation is carried out by microorganisms. Microorganisms which possess the ability to fix nitrogen are known as diazotrophs. Diazotrophs were first cultured in 1888 by Martinus Beijerinck (Fred *et al*., 1932). As nitrogen free media was developed, more nitrogen fixers could be cultured and studied leading to Allen and Senoff isolating the first nitrogenase enzyme in 1965 and discovered that the nitrogenase enzyme, responsible for catalyzing the reduction of atmospheric dinitrogen to ammonia, was composed of two protein subunits (dinitrogenase and dinitrogenase reductase) (Mortenson, 1965). Between the 1970s and the 1990s the equation of nitrogen fixation was deduced and studies focussed on isolating the nitrogenase enzyme and studying its amino acid, and in turn, crystal structure (Simpson and Buris, 1984; Hausinger and Howard, 1983; Howard *et al.,* 1989; Morgan *et al.,* 1990; Georgiadis *et al.,* 1992; Kim and Rees, 1992).

Once the nitrogenase enzyme was more fully understood focus turned to studying nitrogen fixing communities and their environments. There are several different ways to study the action of nitrogen fixing bacteria, the most commonly used is the acetylene-ethylene assay developed by Hardy *et al.* in 1968. As well as nitrogen nitrogenise reduces other targets such as acetylene, which is reduced to ethylene. The acetylene reduction assay measures the amount of ethylene produced when N-fixers are exposed to acetylene. This rate is directly proportional to the rate of nitrogen fixation (Duc e*t al,.* 2009; Hardy *et al*., 1968). $^{15}N_2$ can also be used to assess the rate at which N is fixed in a culture/environment (Buckley *et al*., 2007).

The reaction involved in nitrogen fixation is summarized in the following equation.

$$N_2 + 8e^- + 8H^+ + 16\ MgATP \rightarrow 2\ NH_3 + H_2 + 16MgADP + 16P_i$$

Each transferred electron ‘costs’ the cell 2 MgATP, a considerable energy input. The enzyme also has a slow turnover, and there are a large number of genes required for nitrogenase regulation and assembly (Zehr *et al*., 2003), meaning that for a microorganism to rely solely on nitrogen fixation as the source of nitrogen it would need to dedicate 20% of its protein to nitrogenase production and regulation (Dixon and Kahn, 2004). Nitrogen fixation is therefore tightly regulated from the transcription to translational protein modification stages (Zehr *et al*., 2003). The nitrogenase enzyme is oxygen sensitive and the *nif*LA operon regulates at the transcriptional level in response to fixed nitrogen levels, to stop unnecessary production (Yan *et al*., 2010; Dixon and Kahn, 2004; Pedrosa *et al*., 2001; Gussin *et al*., 1986).

Initially studies were carried out using N free media on culturable diazotrophs (See Table 1.2 for a list of all culturable diazotrophs (adapted from Zehr *et al*. (2003)). Diazotrophs are highly diverse and are found in almost all bacterial, and some archael, phylogenetic groups including all subdivisions of *Proteobacteria*. They have a wide range of physiologies, there are examples of diazotrophs which are: aerobic (*Azotobacter*), anaerobic (*Clostridium*) or facultatively anaerobic (*Klebsiella*) heterotrophs; anoxygenic (*Rhodobacter*) and oxygenic (*Anabaena*) phototrophs; and chemolithotrophs (Dixon and Kahn, 2004). Of the 49 diazotrophic groups only 22 have members which can be cultured (Buckley *et al*., 2007). As a result, molecular studies are essential to assess full diazotrophic diversity. As diazotrophs are spread across so many different groups, primers must be designed specifically for *nif* genes, rather than 16S based primers, for use in PCR. The *nif* genes encode the nitrogenase enzyme. The nitrogenase enzyme consists of a Molybdenum-Iron (MoFe) and Iron (Fe) protein (Roeselers *et al*., 2007). The *nifH* gene encodes one of the subunits of the Fe protein and is often studied as it is the most conserved of the *nif* genes, is not constitutively expressed, is regulated in response to factors that control nitrogen fixation, and levels correspond to nitrogenase activity

Table 1.2. Summary of all culturable diazotrophs (adapted from Zehr *et al*., 2003).

Cluster	Group	Genera
I	*Alpha-proteobacteria*	*Azospirillum*
		Gluconacetobacter
		Mesorhizobium
		Rhodobacter
		Rhodospirillum
		Rhizobium
		Sinorhizobium
		Beijerinckia
		Methylocella
		Methylosinus
		Methylocystis
		Rhizobium
		Xanthobacter
	Beta-proteobacteria	*Burkholderia*
		Herbaspirillum
		Azoarcus
		Alcaligenes
	Epsilon-proteobacteria	*Arcobacter*
	Gamma-proteobacteria	*Vibrio*
		Acidothiobacillus
		Klebsiella
		Marichromatium
		Methylomonas
		Azotobacter (vnfH)
		Methylobacter
		Azomonas
		Pseudomonas
	Cyanobacteria	*Anabaena*
		Chlorogloeopsis
		Calothrix
		Cyanothece
		Dermacarpa
		Fischerella
		Gloeothece
		Lyngbya
		Myxosarcina
		Nostoc
		Oscillatoria
		Phormidium
		Plectonema
		Pseudanabaena
		Scytonema
		Symploca

Table 1.2 (continued). Summary of all culturable diazotrophs (adapted from Zehr *et al*., 2003).

Cluster	Group	Genera
I	Cyanobacteria	*Synechococcus (Cyanothece)*
		Synechocystis (marine)
		Tolypothrix
		Trichodesmium
		Xenococcus
	Firmicutes	*Frankia*
		Paenibacillus
II	*Alpha-proteobacteria*	*Rhodobacter*
	Delta-proteobacteria	*Desulfobacter*
	Gamma-proteobacteria	*Azotobacter*
	Firmicutes	*Paenibacillus*
		Clostridium
	Spirochaetes	*Spirochaeta*
		Treponema
	Archaea	*Methanobrevibacter*
		Methanococcus
		Methanothermobacter
		Methanosarcina
III	*Delta-proteobacteria*	*Desulfobacter*
		Desulfomicrobium
		Desulfovibrio
		Desulfotomaculum
		Desulfonema
		Desulfovibrio
	Firmicutes	*Clostridium*
		Acetobacterium
		Desulfosporosinus
	Spirochaetes	*Spirochaeta*
		Treponema
	Archaea	*Methanosarcina*
	Green sulphur	*Chlorobium*
		Pelodictyon
IV	*Spirochaetes*	*Treponema*
	Archaea	*Methanobrevibacter*
		Methanocaldococcus
		Methanococcus
		Methanopyrus
		Methanosarcina
		Methanothermobacter
		Methanosarcina

(Levitan *et al*., 2010; Zehr *et al*., 2003). Due to the conserved nature of the *nifH* gene there has been considerable work creating primer sets so that the soil community can be analysed using a PCR-DGGE based system (Bürgmann *et al*., 2005; Burgmann *et al*., 2004; Burgmann *et al*., 2003; Poly *et al*., 2001a; Poly *et al*., 2001b; Widmer *et al*., 1999; Rosado *et al*., 1998).From 1992-1996 Young compiled lists of all known nitrogen fixing bacteria and began to assemble phylogenetic trees. It was found that when the *nifH* gene was used the trees were consistent with 16S rRNA based studies (Young, 1996; Young, 1992).

These studies, along with many others, have meant that diazotrophic diversity has been studied across many different environments such as; oligotrophic oceans (Zehr *et al*., 2001), lakes, rivers and estuaries (Zehr and McReynolds, 1989), mats and sediments (Zehr *et al*., 2001), soils (Poly *et al* 2001b) and termite guts (Ohkuma *et al*., 1999).

Phylogenetic studies of diazotrophs have found that they separate into 4 clusters. Certain genera belong to more than one cluster as they have more than one homologue of the *nifH* gene (Raymond *et al*., 2004; Zehr *et al*., 2003). Diazotrophs belonging to cluster I are the most widespread and are mostly cyanobacteria and *Proteobacteria* containing the largest *nif* gene operons. Diazotrophs of cluster II are similar to those of cluster I but are mostly anaerobic and are found in environments of low oxygen such as sediments and microbial mats. Cluster III contains diazotrophs which have alternative vanadium or iron nitrogenases. Diazotrophs of cluster IV are rare and are mostly found in invertebrate guts (Raymond *et al*., 2004; Zehr *et al*., 2003).

Studies from the last few years have continued to use techniques such as denaturing gradient gel electrophoresis (DGGE) and quantitative polymerase chain reaction (qPCR), and many have also incorporated sequencing of key organisms. These techniques have been used to study land use effects (Hayden *et al*., 2010), crop effects (Hauggaard-Nielsen *et al*., 2009; Coelho *et al*., 2009), N management effects (Coelho *et al*., 2009),

seasonal effects (Gamble *et al*., 2010) and the functional significance of diazotrophs (Hsu *et al*., 2009).

This study aims to extend this work by studying the effects of different organic and conventional farm management practices on the free-living diazotrophic community. Generally the most widely studied, nitrogen-fixers are those that are in symbiosis with legumes. These have been extensively investigated as they are accountable for around 80% of biological nitrogen fixed in arable agriculture (Peoples and Craswell, 1992). The plant has a nitrogen source and the bacteria receive energy from the photosynthates and amino acids in the root (Fischer, 1994).

However, under specific conditions, bacteria which are free-living in soil (e.g. *Frankia*, cyanobacteria, *Pseudomonas, Azospirillum* and *Azotobacter*) may also fix significant amounts of nitrogen (0-60 kg N ha^{-1} $year^{-1}$) (Burgmann *et al*., 2004; Kahindi *et al*., 1997). Due to their large diversity it could be expected that free-living diazotrophs would be found in all soils however in a recent survey of soils in South East Australia Hayden *et al*. (2010) found, using qPCR, that nitrogen-fixers were only present in half the soils due to the specific conditions they need to thrive. Free-living nitrogen fixing bacteria may be particularly important in organically managed soils, which could have lower nitrogen content. There is also evidence that free-living N fixation is important for sustaining soil fertility, particularly in arable soils which have a low abundance of leguminous plants (Patra *et al*., 2007).

As nitrogen fixation is energy-expensive it is reliant on a carbon source. Free-living diazotrophs can generally be stimulated locally by providing an easily degradable carbon source, or by increasing the C/N ratio (Hayden *et al*., 2010). Keeling *et al*. (1998) found long term glucose application increased the diazotroph population by 300% in one field but had little effect in another due to an increase in nitrogen.

Nitrogen fixation is adversely affected by acidic pH < 5.3 (Nelson and Melle, 2006; Schubert *et al*., 1990). Although some strains have developed a tolerance, acidic and alkaline pH constrain nitrogen fixing bacteria in soils and reduce symbiotic nodulation of roots (Zahran, 1999). Schubert *et al*. (1990) used the acetylene reduction assay to show that the optimum pH for nitrogen fixation was between 6.2 and 7. Nitrifiers and denitrifiers are also affected by acidity and alkalinity (Philippot *et al*., 2007).

Optimal conditions for nitrogen fixation are more likely to be found in organically managed soils as increased organic C is added in the form of manure, there is on average less readily-available nitrogen and the pH is, on average, closer to neutral (Fernandez-Calvino and Bååth, 2010; Mäder *et al*., 2002). The diazotrophic community structure and diversity has been shown to respond to changes in grazing, liming, the nature of nitrogen added, and the incorporation of crop residues (Patra *et al*., 1996; Wakelin *et al*., 2007). They are also especially sensitive to chemical inputs such as pesticides (Omar *et al*., 1992).

When looking at the effect of land use type (agricultural vs. remnant) on the nitrogen fixing community Hayden *et al*. (2010) looked at 60 sites from three different geomorphic zones in South East Australia. Although *nifH* was more abundant in neutral-alkaline Calcarosols, they found no significant difference between land use type or geomorphic zone and their interaction with the nitrogen fixing community. However, they found DNA levels of *nifH* were very low and could only amplify *nifH* from 50% of soils. They also suggested that the drivers of *nifH* expression were microbial biomass carbon and nitrogen, total nitrogen and total potassium (Hayden *et al*., 2010).

It is unclear how inorganic fertilizers affect the N fixing community. It could be assumed that an increase in fertilizer leads to an increase in soil inorganic N levels which in turn would lead to a reduction in fixation (Patra *et al*., 2007). This effect could be reasonably rapid. Tan *et al*. (2003) found a significant decrease in diversity and activity of N fixation 15 days after fertilizer application. Coelho *et al*. (2009; 2008) found that an increase in

fertilizer N led to a reduction in *nifH* density and that the cultivar of sorghum growing in the soil also directly affected the nitrogen fixing community. However, it could also be assumed that low input treatments may lead to more mineralizable, rather than inorganic N, which could also inhibit fixation (DeLuca *et al.,* 1996). There have also been studies which have found little correlation between inputs of nitrogen and a reduction in fixation. When looking at nitrogen fixation in the rhizosphere Piceno and Lovell (2000) suggested that increased nitrogen fertilizer could result in decreased numbers of diazotrophs as nitrogen fixers would lose their evolutionary advantage and have to compete with other bacteria for a carbon source. They also suggested that increased nitrogen levels in the short term could increase nitrogen fixation in the long term due to increased plant production in turn leading to increased carbon in the soil.

Many studies investigate the effects of applying different levels of nitrogen (Coelho *et al*., 2009; Coelho *et al*., 2008; Deslippe *et al*., 2005; Tan *et al*., 2003). However, in different systems it is often the form of nitrogen applied that changes rather than the amount of nitrogen. DeLuca *et al*. (1996) looked at similar levels of nitrogen applied to fields in different forms (green manure, cattle manure and urea fertilizer). They found that in soils 0-20cm deep, there was a higher rate of nitrogen fixation in urea fertilized soils as the pH was closer to neutral in these plots. Conversely, when qPCR was used to study the effect of manure, urea and straw application on the diazotrophic community in the rhizosphere of sorghum, it was found that the application of manure, and manure plus urea, led to the most *nifH* expression, and that urea applied on its own led to the least *nifH* expression. This was attributed to the elevated concentration of total C, N and P following the manure application (Hai *et al*., 2009).

There may be a difference between the amount of available carbon between organic and conventionally fertilized plots. Organically managed soils often have higher levels of soil organic carbon and retain the carbon for longer than conventional soils (Pimental *et al*., 2005). Increases in carbon have been shown to stimulate nitrogen fixation although this is

inconsistent (Hsu and Buckley, 2009; Bürgmann *et al*., 2005; Hartley and Schlesinger, 2002; Keeling *et al*., 1998). The activity of diazotrophic populations have been shown to be enhanced by 300% when measured 2 months after carbon inputs were increased. This was accompanied by a 100% increase in plant nitrogen uptake (Keeling *et al*., 1998). However, studies showing this positive effect applied carbon in the form of sugar containing substances such as glucose as they are trying to mimic the effect of photosynthetically assimilated carbon, given off as rhizodeposits, rather than increased carbon associated with the use of organic manures (Bürgmann *et al*., 2005; Hartley and Schlesinger, 2002).

As well as applying ammonium and nitrate to the soil conventional soil management also involves the application of phosphorus and potassium. Phosphorus can also stimulate nitrogen fixation as it is required for energy production. Reed *et al*. (2007) observed doubling of nitrogen fixation in response to the addition of phosphorus. It has been suggested that the N:P ratio could be a good predictor for the rate of nitrogen fixation (Hartley and Schlesinger, 2002). However, there was no noticeable effect of phosphorus addition to the diazotrophic community structure and activity when management effects were investigated in the rhizosphere of *Spartina alterniflora* and in arid grassland (Hartley and Schlesinger, 2002; Piceno and Lovell, 2000).

Nitrogen fixing bacteria are thought to be especially sensitive to pesticides (Omar *et al*., 1992). A recent review (Lo, 2010) discussing the effect of pesticides on soil microorganisms demonstrated how different chemicals and different environments can lead to different responses in the diazotrophic community. Certain chemicals have an inhibitory affect (e.g. diflubenzuron) and others have a stimulatory effect (e.g. methylpyrimitos). Most of the work looking at the effect of pesticides has been carried out on symbiotic diazotrophs such as *S. meliloti*. Fox *et al*. (2007; 2004) have showed both *in vitro* and *in vivo* that around 30 different pesticides have a negative effect on the relationship between *S. meliloti* and alfalfa probably due to a disruption in the chemical

signalling between the bacteria and its host. The *in vitro* study showed that all chemicals significantly reduced N fixation for at least 6 weeks post pesticide application (Fox *et al*., 2007). Most chemicals only affected bacterial nitrogen fixation ability and did not affect bacterial growth.

The limited studies on the free-living diazotrophic community have led to very varied results. Different chemicals can lead to different responses. For example, in flooded soil *Azospirillum* and other anaerobic diazotrophs responded positively to Butachlor, negatively to carbofuran and were not affected at all by carbaryl herbicides (Jena *et al*., 1987). There are even discrepancies in results when looking at the effect of one pesticide. The response of the nitrogen fixing community to glyphosphate pesticides has been studied several times. *In vitro* studies observed that application of glyphosphate had no effect on growth of *Azotobacter* and *Azospirillum* species even when dosage was doubled, although there was a decrease in cell size of *Azotobacter* species when the dosage was exceeded (Santos and Flores, 1995). The effect of the pesticide within soybean fields found that nitrogen fixation was inhibited in response to glyphosphate when N uptake was measured using ^{15}N (Bohm *et al*., 2009) but, found no response to glyphosphate even when they doubled the recommended dose, when nitrogen fixation was measured using acetylene reduction assay (Zablotowicz and Reddy, 2007).

When DGGE was used to study the effects of acetochlor, methamidophos, and their combination on the nitrogen fixing community in soil it was found that different bands, and therefore different organisms, reacted differently with four bands disappearing in response to the pesticide, four bands showing resistance and five bands actually proliferating in response to the pesticide (Hussain *et al*., 2009; Su *et al*., 2007). Clearly not all organisms will respond in a similar way leading to a possible change in overall community structure in response to pesticides.

Crop rotation can alter microbial activity and diversity, break disease patterns and can increase soil N and C by around 25% (Lupwayi and Kennedy, 2007; Kelley *et al*., 2003). It

is also possible that crop rotation can affect the soil diazotrophic community as rotation can lead to carry over of diazotrophic endophytes (Roesch *et al*., 2008). This is particularly true when legumes are involved in the rotation. When the diazotrophic community of soil from a continuous soybean rotation was compared with soil from a soybean-corn rotation, significantly different diversity was observed (Xiao *et al*., 2010). Also, once diazotrophs have been introduced to soil there is evidence that they can persist. For example, the population, diversity and activity of *Bradyrhizobium* species was measured in soybean soil in Brazil 15 years after it had been inoculated with 16 *Bradyrhizobium* species. Crop rotations, which included soybean, had higher populations, diversity and activity of *Bradyrhizobium*, and *Bradyrhizobium* was still present even when soybean was not (Ferreira *et al*., 2000).

The crop present in the rotation could have a strong effect on diversity and activity of diazotrophs (Tan *et al*., 2003). Although not as strong as the influence of fertility management, different grass species have been found to significantly affect the activity, but not the diversity, of free-living nitrogen fixers (Patra *et al*., 2006).

If the current crop can affect the soils nitrogen fixing community it could be possible that influences of preceding crops could influence communities in the current soil. When soil factors were measured in 3 soils, all under spring wheat but which had previously had either Faba beans, lupins, peas or oats present, it was found that although N leaching was higher following legumes, there was more soil mineral N remaining in the soil following faba beans due to their unusually high uptake of nitrogen due to nitrogen fixation. This difference did not lead to any changes in the growth of the subsequent crop but it is possible that the increased N remaining in the soil could lead to changes in the diazotrophic community (Hauggaard-Nielsen *et al*., 2009).

Seasonal effects such as increased temperature can lead to increased rates of metabolic processes and increased nitrogen fixation (Deslippe *et al*., 2005). The optimum temperature for diazotroph growth and activity is between 10 °C and 25 °C (Beauchamp

et al., 2006; Petterson and Bååth, 2003) meaning, in temperate latitudes, nitrogen fixation is often diminished in winter. When nitrogen fixation associated with *Spartina* was measured it was found to be highest in September and lowest in February, also correlating with changes in organic matter (Gamble *et al*., 2010). Heterotrophic diazotrophs may also be lower in numbers in the winter but will increase with temperature and increases in labile organic carbon throughout the growing season (Kirchman *et al*., 2010).

There are exceptions to this. Shaffer *et al*. (2000) found no difference in *nifH* community composition over a 16 month period in forest soil and litter in Oregon, USA. These results are consistent with other studies of forest soil. However these studies look at DNA rather than RNA so it is possible that although the overall community remains the same its activity, and therefore *nifH* expression, changes seasonally.

In summary, the effect of crop rotation, fertility management and crop protection associated with organic and conventional farming on the diazotrophic community will be studied using qPCR and DGGE. The soil used will be bulk soil. It is important to note the difference between microbial communities of the rhizosphere and those of bulk soil. Rhizosphere bacteria are well studied as in general they are easier to culture (Dennis *et al*., 2010). Biomass and activity is often enhanced in the rhizosphere as the microorganisms here are under the control of the plant root system and can, therefore, take advantage of increased carbon and energy obtained from root exudates (Berg *et al*., 2009; Piceno and Lovell, 2000). However, due to its stability the rhizosphere is often less diverse than bulk soil (Dennis *et al*., 2010). A large proportion of studies into nitrogen fixation occur in the rhizosphere as, under similar conditions, nitrogen fixation will always be higher in the rhizosphere than in bulk soil (Patra *et al*., 2007).

Considering the results of the previous studies discussed above it may be assumed that organic farming would have a positive effect on the diazotrophic community due to the diverse crop rotation, decreased N input and increased C availability. It is possible that

any changes seen in the diazotrophic community could simply be a reflection of changes to the overall bacterial community. By combining results from studies of both communities it can be assured that changes reported in the diazotrophic community are due to a specific influence on their capacity to fix nitrogen.

1.4 General statement of aims.

This study aims to measure the diversity of nitrogen fixing bacteria within agricultural soil and observe changes in diversity and expression of the *nifH* gene brought about by changes in farm management. The changes in farm management which will be studied will be the effect of organic and conventional rotations, fertility management and crop protection regimes.

In this study molecular techniques such as PCR, DGGE, real-time PCR and sequencing are used to study the free-living nitrogen fixing community and the bacterial community as a whole. Molecular biology is necessary to study soil functioning and processes as so many soil microorganisms are unculturable. Analysis of *nifH* gene fragments has shown that in many soil systems it is the unculturable, rather than culturable N-fixers that are dominant (Hsu and Buckley, 2009; Tan *et al*., 2003). While trying to assess the extent to which *Azoarcus* strains could provide nitrogen to Kaller grass, Hurek *et al.* (2002) found that although *nifH* transcripts were high the bacteria could not be isolated, suggesting ecological dominance of unculturable diazotrophs in the grass ecosystem.
DNA and RNA will be extracted from soils over the course of a 3 year period (2007-2009) at 3 time points (March, June and September). Sampling over consecutive years, and in more than one month, will hopefully mean that any conclusions are not the result of a one year phenomenon and mean that changes can be seen across the growing season. In order to analyse the nitrogen fixing community the *nifH* gene will be amplified using PCR. DGGE and qPCR will be used to analyse diversity and expression of the gene

respectively. The use of DGGE will allow organisms of interest to be identified via sequencing.

In the past, after amplification using PCR, genes of interest would be cloned into vectors. The analysis of these clone libraries is not suitable for this study as the scale of the project means that it would be time-consuming and labour intensive. A recent similar study required analysis of 349 clones to accurately analyse *nifH* diversity (Hsu and Buckley, 2009). This study has 3 times more sample dates and 3 times more sample years suggesting over 3,000 clones would need to be generated to accurately analyse the organisms of the nitrogen fixing community.

As well as looking at differences in community structure, differences in activity are analysed using quantitative PCR. QPCR uses fluorescence to quantify amounts of DNA in a sample during the exponential phase of the PCR cycle. This technique makes it possible to extrapolate back and quantify the amounts of DNA present in the initial sample (Ginzinger, 2002). For example, Fierer *et al*. (2005) used qPCR to estimate the relative abundance of major soil bacterial taxonomic groups using a range of primers. QPCR has also been used to detect functional genes in soil, such as genes involved in denitrification and nitrification (Kandeler *et al*., 2006).

In order to ensure that farm management is directly influencing nitrogen fixing communities rather than the bacterial community as a whole the 16S rRNA gene will also be targeted to show how farm management affects diversity and activity of the total bacterial community. DGGE and qPCR will be used to measure changes in the 16S rRNA gene as well as BIOLOG plates which will give some indication how farm management may affect the utilization of carbon substrates.

Throughout the sample years environmental variables such as: pH, available carbon, nitrogen etc will be measured using a wide variety of techniques. This will allow

comparisons to be made, and any correlations observed between changes in key soil factors and expression and diversity of the *nifH* and 16S rRNA genes.

2. Methods

A list of all chemicals used throughout this study can be found in the appendix (section A1).

2.1 Nafferton Ecological Farming Group

The soils used in this study are taken from the Nafferton factorial systems comparison (NFSC) study, a field trial based at Nafferton Farm in the Tyne valley, North East England. The aim of the farming group is to carry out research into the effects of agronomic practices on sustainability, the environment, and food quality and safety, however, the main objective is to address the problems in 'low-input' and organic food production systems.

The NFSC trial was established in 2001. Crop rotation is the key variable with the main plot having two levels, organic (diverse, rich in leguminous crops) and conventional (arable crop-dominated rotation typical of conventional systems). In turn, each main plot is divided into two crop protection subplots (6 x 48 m) in which crop protection is carried out according to conventional farming practice (British Farm Assured standards; CON CP) or to organic crop protection standards (Soil Association organic farming standards; ORG CP). Finally, each of these subplots is divided into two fertility management sub-subplots (6 x 24 m) in which fertilization is either carried out according to conventional farming practice (CON FM) or organic farming standards (ORG FM) (See Table 2.1 and 2.2 for details). The arrangement of crop protection subplots and fertilization sub-subplots within sub-blocks is randomised. Unplanted separation strips (10 m) are established between crop protection subplots and 5 m unplanted separation strips between fertilization sub-subplots. There are four experiments following this design within the NFSC trial, each starting at a different stage in the crop rotation, so that a diversity of crops can be studied

Table 2.1. Crop protection protocols and fertility management used in the NFSC experiments for 2006, 2007, 2008 and 2009 under organic crop protection (ORG CP) or conventional crop protection (CON CP) and organic fertility management (ORG FM) or conventional fertility management (CON FM).

Current crop	
Potatoes (2007-9)	Treatment
ORG CP	mechanical weeding (ridging); copper-oxychloride[b] (23 kg/ha)
CON CP	aldicarb[d] (33.5 kg/ha); linuron[a] (3.5 L/ha); fluazinam[c] (1.5 L/ha); mancozeb and metalaxyl-M[c] (4.7 kg/ha); oiquat[e] (2 L/ha)
ORG FM	composted cattle manure (equivalent to 180 kg N/ha)
CON FM	0:20:30 (134 kg P_2O_5/ha; 200 kg K_2O/ha); Nitram (180 kg N/ha)
Previous crop	
Beans (2006)	Treatment
ORG CP	No amendment
CON CP	Battalion[a] (2.8 L/ha); Bravo 500[b] (1.5 L/ha)
ORG FM	No amendment
CON FM	0:20:30 (60 kg P_2O_5/ha; 90 kg K_2O/ha)
Winter barley (2006)	
ORG CP	mechanical weeding (finger weeder)
CON CP	Pendimethalin[a] (2.5 L/ha); isoproturon[a] (1.5 L/ha); Duplosan[a] (1 L/ha); Acanto[b] (0.4 L/ha); Proline[b] (0.4 L/ha); Corbel[b] (0.5 L/ha); Fluroxypyr[b] (0.75 L/ha); Amistar[b] (0.25 L/ha); Bravo 500[b] (0.5 L/ha); Cleancrop EPX[b] (0.4 L/ha)
ORG FM	no amendment
CON FM	0:20:30 (64 kg P_2O_5/ha; 96 kg K_2O/ha); Nitram (170 kg N/ha)
Winter Wheat (2007-8)	
ORG CP	mechanical weeding (finger weeder)
CON CP	isoproturon[a] (6 L/ha); Optica[a] (1 L/ha); Pendimethalin[a] (1.5 L/ha); Corbel[b] (0.2 L/ha); Cleancrop EPX[b] (1.25 L/ha); Bravo 500[b] (1.75 L/ha); chlormequat[c] (2.3 L/ha); Tern[b] (0.15 L/ha); Twist[b] (0.25 L/ha)
ORG FM	no amendment
CON FM	0:20:30 (64 kg P_2O_5/ha; 96 kg K_2O/ha); Nitram (210 kg N/ha)

[a] herbicide; [b] fungicide; [c] growth regulator; [d] nematicide; [e] desiccant

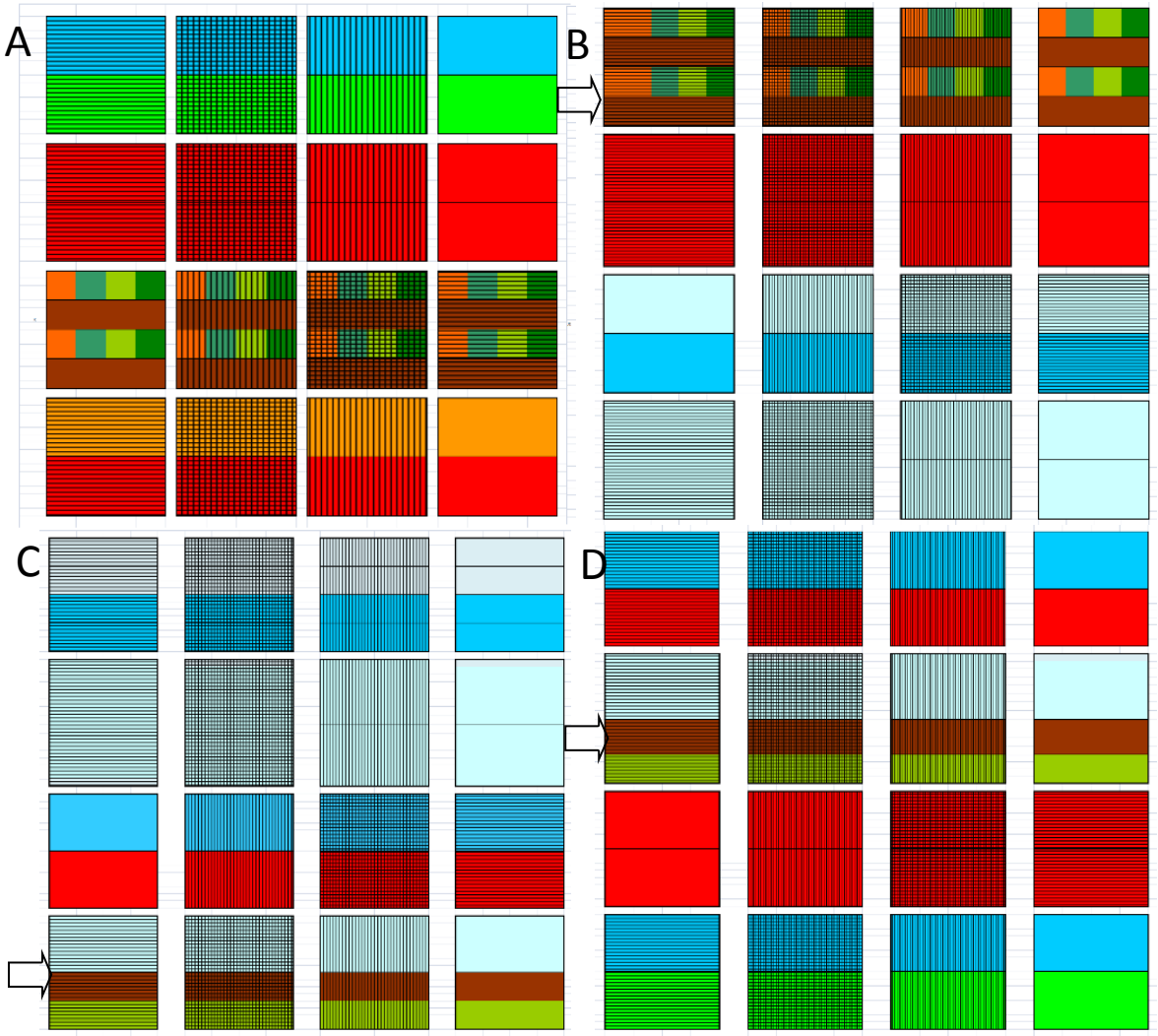
Table 2.2. Summary of the dates fertilizers and pesticides were applied to plots

Component	Date added (2007)	Date added (2008)	Dated added (2009)
Fertilizer (Nitram/Manure)	25-Apr	07-May	17-Mar (Org), 21-Apr (Con)
Superphosphate	12-Apr	22-Apr	115-Apr
Nematicide and growth regulator	25-Apr	07-May	21-Apr
Herbicide	02-May	14-May	29-Apr
Fungicide (conventional)	20-Jun, 02-Jul, 12-Jul, 19-Jul, 27-Jul, 01-Sep, 08-Sep, 13-Sep	24-Jun, 04-Jul, 17-Jul, 27-Jul, 31-Jul, 07-Aug, 15-Aug, 26-Aug	22-Jun, 30-Jun, 08-Jul, 16-Jul, 23-Jul, 31-Jul, 07-Aug, 19-Aug
Fungicide (organic)	20-Jun, 09-Jul, 18-Jul, 24-Jul, 31-Jul	01-Jul, 17-Jul, 24-Jul, 04-Aug, 15-Aug	24-Jun, 02-Jul, 10-Jul, 20-Jul, 28-Jul, 07-Aug
Desiccant		26-Aug, 05-Sep	24-Aug

Table 2.3. Details of the organic and conventional crop rotation in the Nafferton Factorial Systems Comparison trial

Crop rotation	**Year**							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Con	Winter wheat	Winter wheat	Winter barley	Veg/ Potatoes	Winter wheat	Winter barley	Grass/ clover	Grass
Org	Winter wheat	Veg/ Potatoes	Spring beans	Veg/ Potatoes	Winter barley	Grass/ clover	Grass/ clover	Grass/ clover

Figure 2.1. Block 1 of NFSC in 2006-2009.



	ORG FM, CON CP		Winter barley		Carrrots		Potatoes
	CON FM, CON CP		Spring beans		Lettuce		Winter wheat
	CON FM, ORG CP		Grass/ clover		Cabbage		
	ORG FM, ORG CP		Grass		Onions		

Figure 2.1. Block 1 of the Nafferton Factorial Systems Comparison trial in 2006 (A), 2007 (B), 2008 (C) and 2009 (D). Arrow indicates plots from which soil was sampled. Legend below indicates crop grown (coloured cells) and management practices (cell hatching).

Figure 2.2. Representation of the split of crop treatment in each sub-plot.

	Conventional Crop Protection (CP)		Organic Crop Protection (OP)			
	Organic fertility (OF)	Conventional fertility (CF)	Conventional fertility (CF)	Organic fertility (OF)	12 m	24 m
Conventional Rotation (CR)	Low input 1	Conventional	Low input 2	Organic*		
Organic Rotation (OR)	Low input 1	Conventional*	Low input 2	Organic		
	24m (ssp-l)					
	48m (subplot length)					
	96m (main plot length)					

*except for rotational design

in the trial each year. Figure 2.1 is an example of the layout for the field trial in one of the blocks. The design allows the effect of 4 production systems to be compared within each level of crop rotation (a) organic ORG FM-ORG CP, (b) low input 1 ORG FM-CON CP, (c) low input 2 CON FM-ORGCP and (d) conventional CON FM-CON CP (Figure 2.2). The soil used in this study was sampled on 3 dates in 2007 (Experiment 2, crop rotation year 4), 2008 (Experiment 3, crop rotation year 2) and 2009 (Experiment 4, crop rotation year 2). Table 2.3 shows the sequence of crops grown in the organic and conventional rotation in each year. In each year the soil was planted with potatoes (Santé variety). The preceding crop in 2007 was faba beans (Fuego variety) in the organic crop rotation and winter barley (Pearl variety) in the conventional crop rotation. In 2008 and 2009 potato soils, in the organic crop rotation only, were studied. The preceding crop in both years was winter wheat (Malacca variety).

2.2 Soil sampling

Five cores of soil (0-30 cm) were sampled from each plot, using an auger, and mixed to form one composite sample per plot, on 3 dates in 2007, 2008 and 2009. On 2nd March, 11th June and 24th September 2007 samples were taken from potato/winter barley and potato/bean plots. On 17th March, 26th June and 1st October 2008 and 13th March, 22nd June and 21st September 2009 samples were taken from potato/winter wheat plots. Soils were sieved fresh (4mm) and a portion (about 10 g) was frozen immediately and stored at -80 ºC before extraction of nucleic acids. Another portion (about 500 g fresh) was stored at 4ºC before measurements of soil basal respiration were taken using the Sensomat-Measurement-System (Robertz *et al.*, 2000). Approximately 100 g was air dried prior to analysis for total C and N, and pH in water (1:1). Finally, approximately 100 g was stored at -20 ºC for measurement of available nitrate and ammonium using potassium chloride extraction.

2.3 Potassium chloride extraction of nitrate and ammonium.

Nitrate and ammonium were extracted from soil using the method described by Keeney and Nelson (1982). Frozen soils were thawed and 50 ml of 2M KCl were immediately added to 6-8 g of the fresh soil. The soil-KCl mixtures were then shaken for 50 minutes at 250 rpm and allowed to settle for 1 hour. A 20 ml aliquot of the solution was filtered through a glass-microfibre (GF/A) filter and stored at -20^{o}C until analysis for nitrate and ammonium content using a Brann and Leubbe Autoanalyzer 3. Nitrate-N was analyzed using the hydrazine reduction method (Magill and Aber, 2000; Technicon Industrial Systems, 1977) and ammonium-N was analyzed by the salicylate method (Nelson, 1983). Lists of reagents used to extract ammonium-N and nitrate-N are listed in the appendix section B3.

2.4 Measuring soil basal respiration

Approximately 300 g of soil was weighed into a bottle and the weight was recorded. An adaptor was fixed to the bottle so a quiver containing 5 drops of potassium hydroxide could be placed in the neck. An Aqualytic head (Sensomat, Darmstadt, Germany) was then screwed on and activated. The bottles were left in an incubator at 22 ºC in the dark for 7 days. Data was then collected and basal respiration is worked out in the following way:

The volume of soil and water in the jar = (mass of dry soil/2.65) + (mass of wet soil – mass of dry soil)

Vfr = (610-(volume of soil + water/610))/1000

The aqualytic head records the change in pressure over time. Results were taken from the first few days of incubation where the change in pressure over time is linear. Results are normalized using the following equation.

$$R[CO_2] = \frac{MR(CO_2)}{R \times T} \times \frac{V_{fr}}{m_{Bt}} \times \Delta p$$

$R[CO_2]$ = Soil respiration (CO_2 evolution) [mg kg^{-1}]

$MR[CO_2]$ = molecular mass of CO_2 [=(15999.4 × 2 + 12011.15) mg/mol]

R = gas constant [83.14 L × hPa × mol^{-1} * K^{-1}]

T = incubation temperature [=(273+15)ºK = 288ºK]

V_{fr} = free gas volume in bottle (calculated according to equation above)

m_{Bt} = dry mass of incubated soil sample = moist mass / (1+GWC) = 0.250 / (1+GWC) [kg]

Δp = recorded pressure difference [hPa]

This formula assumes a respiratory quotient of 1 i.e. 1 mol O_2 is turned into 1 mol CO_2. The slope of the line of results over time gives the soil basal respiration in mg CO_2/Kg/h.

2.5 Measuring soil pH

20 g of dried sieved soil was added to 20 ml distilled water and shaken for 10 minutes (250 rpm). Soil was left to settle for 10 minutes before the pH was taken using a Jenway 3340 ion meter.

2.6 Soil total C and N and extractable nutrients (P, K, Fe, Mo)

Total C and N and extractable soil nutrient data was procided by Nafferton Ecological Farming Group. Total C and N were measured by Dumas combustion (LECO Corporation, USA). Extractable soil nutrients were measured using a Mehlich-3 extractant and analysed using inductively coupled plasma (ICP).

2.7 Nucleic Acid Extraction

2.7.1 DNA Extraction from soil using the MoBio UltraClean Soil DNA extraction kit.

Please see appendix section A5 for details of each solution. Soil (0.25 g) was loaded into the provided bead tube and mixed by vortexing. Solution S1 was heated to 60 ºC and 60 µl was added to the bead tube along with 200 µl of Solution IRS. The tubes were then attached to a vortex using a vortex adaptor and vortexed for 10 minutes at maximum speed. Tubes were centrifuged for 30 seconds at 10,000 x g and the supernatant was transferred to a clean microcentrifuge tube. 250 µl of solution S2 was added before the tubes were incubated at 4 ºC for 5 minutes. Tubes were centrifuged for 1 minute at 10,000 x g. The supernatant was then added to 1.3 ml of solution S3 and mixed. 700 µl of the solution was loaded to a spin filter and centrifuged at 10,000 x g for 1 minute before the flow through was discarded and another 700 µl of solution was added. This was repeated until all of the solution had passed through the spin filter. 300 µl of solution S4 was added to the spin filter before it was centrifuged at 10,000 x g for 30 seconds. The flow through was discarded and the tube was centrifuged again for 1 minute. The spin filter was then placed into a new microcentrifuge tube and 50 µl of solution S5 was added directly to the membrane. The tube is centrifuged for 30 seconds to elute any DNA. DNA was then stored at -80 ºC.

2.7.2 RNA extraction from soil using the MoBio UltraClean Microbial RNA Isolation kit.

Please see appendix section A5 for details of each solution. Before starting solution MR1 and MR2 were heated to 65 ºC. 0.25 g of soil was loaded into the provided bead tube with 300 µl of solution MR1 and 15 µl of solution MR2. Bead tubes were vortexed briefly before being incubated at 65 ºC for 10 minutes. The tubes were then attached to a vortex using a

vortex adaptor and vortexed for 10 minutes at maximum speed. Tubes were centrifuged for 30 seconds at 10,000 x g. The supernatant was transferred to a clean microcentrifuge tube and 500 µl of solution MR3 was added. Tubes were vortexed for 5 seconds and then 250 µl of solution MR4 was added. Tubes were then incubated at 4 ºC for 5 minutes before being centrifuged at 10,000 x g for 1 minute. The supernatant was removed from the tube and 650 µl was added to a spin filter and centrifuged for 30 seconds at 10,000 x g. The flow through was discarded and the process repeated until all the supernatant was filtered. 300 µl of solution MR5 was added to the spin filter and centrifuged for 30 seconds at 10,000 x g. The flow through was discarded and the spin filter centrifuged again for 1 minute. The spin filter was then added to a clean microcentrifuge tube and 50 µl of RNase-free water was added to the white filter membrane. The tubes were then centrifuged for 30 seconds to elute the RNA.

Once extracted RNA was reverse transcribed using Superscript II Reverse transcriptase kit. Remaining RNA was stored at -80 ºC.

After nucleic acid extraction yield and purity was checked using a spectrophotometer at absorbencies of 260 nm and 280 nm. For further tests and controls please see results chapter 1.

2.8 Reverse transcription of RNA

Any RNA extracted was reverse transcribed using Superscript II reverse transcriptase kit (Invitrogen).

Extracted RNA (10 µl) was mixed with 1 µl random hexamers (Qiagen) and 1 µl dNTPs (NE Biolabs) before being heated to 65 ˚C for 5 minutes. The reaction was then chilled on ice and 4 µl of 5 x first-strand buffer, 2 µl 0.1 M DTT and 1 µl RNase OUT added. Tubes are incubated at 25 ˚C for 2 minutes before 1 µl of Superscript II RT was added. The

reaction was then heated at 25 ˚C for 10 minutes, 42 ˚C for 50 minutes and finally 70 ˚C for 15 minutes. Reverse transcribed RNA was then stored at -20 ºC.

2.9 Detecting genes of interest – Polymerase Chain Reaction.

All PCR reactions were carried out using a Eppendorf Mastercycler gradient. All PCR products were visualised using agarose gel electrophoresis. 2 µl of bromophenol blue was mixed with 10 µl PCR product and ran on a 1% agarose gel. DNA bands were compared with Hyperladder (Bioline) to check size.

2.9.1 Total bacterial population (V3 PCR)

The 16S rRNA gene is essential and occurs at least once in a bacterial genome making it an ideal candidate for phylogenetic study. The structure of rRNA gene products is complex with highly conserved loop regions and variable regions which can be used for species identification (Wang and Qian, 2009). In this study the total bacterial population is assessed by amplification of a 193 base pair sequence of the V3 variable region of the 16S rRNA gene, using V3 primers (Muyzer *et al.*, 1993). If the PCR products needed to be analysed using denaturing gradient gel electrophoresis (DGGE) clamped forward primers (V3-fc) were used instead of unclamped (V3-f) as described by Baxter and Cummings (2006). See Table 2.4 for details of all primers (all primers Eurofins).

To carry out the reaction 1 µl of nucleic acids were added to 0.5 µM of both primers, 1x amplification buffer, 25 mM of each dNTP, 50 mM $MgSO_4$, 1 x PCR enhancement solution and 1.25 U Platinum Pfx polymerase (Invitrogen). The reaction mixture was made up to 50 µl with sterile water.

PCR was carried out according to the conditions in Table 2.5. For Further information regarding PCR controls please see results chapter 1 (section 3.2.2).

Table 2.4. Sequence of primers used in PCR and qPCR reactions

primer name	Gene target	sequence (5'-3')	reference
PolF	*nifH*	TGC GA(CT) CC(GC) AA(AG) GC(GCT) GAC TC	Poly *et al.*, 2001
PolR	*nifH*	AT(GC) GCC ATC AT(CT) TC(AG) CCG GA	Poly *et al.*, 2001
AQER-GC30	*nifH*	CGC CCG CCG CGC CCC GCG CCC GGC CCG CCC GAC GAT GTA GAT (CT)TC CTG	Wartiainen *et al.*, 2008
PolFI	*nifH*	TGC GAI CC(GC) AAI GCI GAC TC	Wartiainen *et al.*, 2008
V3R	16S rRNA	ATT ACC GCG GCT GCT GG	Muyzer *et al.*, 1993
V3FC	16S rRNA	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG	Muyzer *et al.*, 1993
Eub338	16S rRNA	ACT CCT ACG GGA GGC AGC AG	Lane *et al.,* 1991

2.9.2 *nifH* (PolF/R and PolFI/AQERGC-30)

A 360 bp fragment of the *nifH* gene was amplified using a nested PCR reaction adapted from Wartiainen *et al.,* (2008). The first reaction used primers PolF and PolR (Poly *et al.*, 2001a). In order to clamp the products for DGGE a second round of PCR was needed using AQER-GC30 and PolFI primers (Wartiainen *et al.*, 2008).

The reaction mix was as follows: 1 µl of reverse transcribed RNA, 0.5 µM of each primer, 25mM of each dNTP, 50 mM $MgCl_2$ (25 mM when using AQER-GC30/PolFI), 5X taq buffer, 5U taq polymerase (New England Biolabs) (2.5 U when using AQER-GC30/PolFI), 0.1 mg BSA (Promega) and made up to 50 µl using sterile water.

PCR conditions for first and second round reactions were taken from Poly *et al.,* (2001) (Table 2.6). For further information regarding PCR controls please see results chapter 1 (section 3.2.2).

Table 2.5. V3 PCR reaction conditions

temperature	time	no. of cycles
95 °C	5 minutes	1 cycle
94 °C	1 minute	20 cycles
65 °C - 0.5 °C each cycle	1 minute	
68 ºC	1 minute	
94 °C	1 minute	15 cycles
55 °C	1 minute	
68 °C	3 minutes	
68 °C	10 minutes	1 cycle

Table 2.6. PCR reaction conditions used to amplify the *nifH* gene.

Temperature	Time	No. of cycles
94 °C	5 minutes	1 cycle
94 °C	1 minute	30 cycles
55 °C	1 minute	
72 °C	2 minutes	
72 °C	5 minutes	1 cycle

2.10 Denaturing gradient gel electrophoresis (DGGE).

DGGE was carried out using the D-Code system (Bio-Rad Laboratories). The apparatus was set up according to manufacturer's instructions.

The gels had a gradient of 35-55 % denaturing solution this was achieved using 16 ml of two denaturing solutions. Recipes for denaturing solutions can be found in the appendix section B4.

Clamped PCR (15 µl) product was mixed with 15 µl loading dye prior to loading onto a gel. Gels were run at 60 °C at 200 V. As *nifH* and V3 PCR results in fragments of different sizes, V3 fragment gels were run for 4 ½ hours, and *nifH* fragment gels were run for 6 hours. To visualise the gels were stained in 250 ml of 1 x TAE (see appendix section B2) with 25 µl of SYBR green I nucleic acid gel stain (Invitrogen) and left for 30 minutes before destaining in distilled water for 10 minutes. Bands were identified and relative intensities were found showing percentage intensity of each band in the lane, using Quantity One software (Bio-Rad Laboratories).

Replication experiments were carried out to ensure gel variability did not confound the results (please see preliminary results in results chapter 1).

2.11 Sequencing

All sequencing was carried out on a 3130 genetic analyzer (Applied Biosystems).

To determine the sequence of individual PCR products, DGGE bands of interest were cut from the gel using a clean scalpel. The gel piece was then left in 10 µl of sterile water, at 4 ˚C, overnight. A 1 µl aliquot of the water was then used as the template in the *nifH* PCR reaction described in section 2.9.2. New products were visualized on DGGE gels and the process was repeated until the band of interest was the only band in the lane. To purify

the sample 2 µl of ExoSAP-IT (GE healthcare) was added to every 5 µl of PCR product. The reactions were then heated to 37 ºC for 15 minutes and then 80 ºC for 15 minutes. The sequencing reaction was set up according to table 2.7 using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and PCR was set up according to Table 2.8.

Sequencing products were then purified using ethanol precipitation to remove excess primers and nucleotides. 2.5 µl of 125 mM EDTA and 30 µl of 95% ethanol were added to the sequencing reaction products. Tubes were inverted and left at room temperature for 15 minutes. The tubes were then centrifuged at 13,000 rpm for 10 minutes. The supernatant was removed and 60 µl of 70% ethanol was added. The tubes were centrifuged again at 13,000 rpm for 10 minutes. The supernatant was removed and excess ethanol was allowed to evaporate by leaving the tubes at room temperature in the dark for 2 minutes. The purified products were dried in a vacuum centrifuge for 5 minutes at 60 °C before 10 µl Hi-Di™ formamide was added. A 10 µl aliquot of this was then added to the sequencing plate and placed inside the sequencer. In the sequencer samples are drawn up using 4 capillary arrays and subjected to capillary electrophoresis. Bases are called using the sequencing analysis 5.2 software.

Sequence data was compared with the nucleotide collection database of the BLAST database using the Blastn suite and the megablast algorithm (Altschul *et al*., 1990) in order to try and identify organisms expressing *nifH*. The closest match was recorded if this was an unidentified unculturable isolate the closest cultured match was also recorded. Statistical analysis was carried out using DGGE gel data for each band which was identified. ANOVA was carried out to investigate if the presence/absence of the band was determined by treatment, previous crop, sample date, year and nucleic acid used.

Sequence data was aligned using the default settings of ClustalW and then trimmed to allow better matching and realigned. Phylogenetic trees were then created using the dendrogram tree option in ClustalW.

Table 2.7. Sequencing reaction set-up.

Reagents	test (µl)	negative control (µl)	positive control (µl)
BigDye mix	0.25	0.25	0.25
5 x seq buffer	1.875	1.875	1.875
Template*	10-40 ng	0	0.5
Primer (1pmol/µl)*	3.2	2	2
H_2O	Up to 10	5.875	5.375
Total	10	10	10

*In the test and negative control tubes the primer used was the primer specific for the gene of interest. In the positive control the M13 primer included in the Big Dye Terminator kit was used. In the test and negative control tubes purified PCR product was used as the template. In the positive control tube the template used was the plasmid included in the kit.

Table 2.8. Reaction conditions for sequencing PCR.

temperature	time	no. of cycles
96 ˚C	1 min	1
96 ˚C	1 min	25
50 ˚C	5 sec	
60 ˚C	4 min	

2.12 Making chemically competent cells.

A starter culture of JM109 cells was inoculated onto M9 minimal media plates and left overnight at 37 ˚C. A single colony was then inoculated into 50 ml of LB broth and incubated aerobically overnight (37 ˚C; 150 rpm). Fresh LB broth (50 ml) was then inoculated with the overnight culture (500 µl) so that it was 1% v/v. The culture was then incubated (37 ˚C; 200 rpm) until it reached an OD_{550} (around 2 ½ hours). The culture was put on ice for 30 minutes before pelleting by centrifugation at 2000 x g for 10 minutes at 4 ˚C. The supernatant was discarded and the pellet re-suspended in 4 ml of ice cold FSB solution before being left on ice for a further 15 minutes. The culture was centrifuged (2000 x g; 10 min; 4 ˚C) again and the supernatant discarded. Cells were re-suspended in 720 µl ice-cold FSB and 26 µl DMSO before being incubated on ice for a further 15 minutes and another 26 µl DMSO added. The cells were either used immediately or 15 µl of 50% sterile glycerol was added before storage at – 80 ˚C. Instructions on how to make LB broth, M9 minimal media and FSB solution can be found in Appendix section B1 and B2.

2.13 Cloning using the pGEM-T easy vectors (Promega)

As in section 2.11, 5 µl of PCR product was purified using the ExoSAP-IT.
Ligation reactions were set up according to table 2.9 and left at room temperature for 1 hour. After ligation, 2 µl of each solution was added to sterile microcentrifuge tubes on ice. To check transformation efficiency of the competent cells another tube was set up containing 0.1 ng uncut plasmid. Competent JM109 cells (50 µl) were then transferred to each tube. The tubes were gently flicked to mix and then placed on ice for 20 minutes.

Table 2.9. Ligation reaction set-up.

Reagent	Positive control (µl)	Test (µl)
2 x ligation buffer	5	5
pGEM-T vector	1	1
PCR product	0	X
Control Insert DNA	2	0
T4 DNA ligase	1	1
Water	up to 10	up to 10

The cells were then heat shocked for 45-50 seconds in a water bath at exactly 42 ˚C before being immediately returned to ice for 2 minutes. Next, 950 µl of SOC medium (see appendix section B1) at room temperature, was added to each ligation tube and 900 µl added to each control tube. Tubes were then incubated for 90 mins at 37 ˚C.

A 100 µl aliquot of each transformation culture was then plated onto duplicate LB plates containing 10 mg/ml ampicillin, 0.1M IPTG and 50 mg/ml X-Gal. For the transformation control, a 1:10 dilution with SOC medium is recommended for plating. Plates were incubated at 37 ˚C for 24 hours. Single colonies were then inoculated into a 10 ml LB broth containing 1ml of ampicillin (10mg/ml) per 100ml broth and left to grow at 37 ˚C overnight.

Promega PureYield Plasmid Miniprep system was used to extract the plasmid DNA from *E. coli*. Details of the Plasmid Miniprep kit can be found in Appendix section A5.

Firstly, 1.5 ml of bacterial culture grown in LB medium was added to a microcentrifuge tube and centrifuged at maximum speed for 30 seconds. The supernatant was discarded and a further 600 µl of culture was added. The tube was vortexed to re-suspend the pellet, before 100 µl of cell lysis buffer was added and mixed by inverting until the solution became opaque. Cold neutralization solution (350 µl) was added and mixed thoroughly by inversion until a yellow precipitate was formed. The solution was then centrifuged at maximum speed for 3 minutes. The supernatant was transferred to a PureYield Minicolumn and collection tube and centrifuged for 15 seconds. The flow through was discarded. Next, 200 µl of endotoxin removal wash was added to the minicolumn and centrifuged for 15 seconds. Then 400 µl of column wash solution was added and the tubes were centrifuged for 30 seconds. The minicolumn was then transferred to a clean microcentrifuge tube and 30 µl of elution buffer was added directly to the minicolumn matrix. Tubes were left to stand for 1 minute at room temperature before the plasmid was eluted by centrifuging for 15 seconds. Eluted plasmid was stored at -20 ºC.

To carry out the restriction digest the absorbance of the DNA was measured at 260 nm. For each µg of DNA, 5U of EcoR1 was added. 1 x buffer and 0.1 mg/ml BSA was also added. Care was taken to make sure that the glycerol EcoR1 was suspended in did not make up more than 10 % of the total of the solution. The tubes were then left at 37 ˚C for 1 ½ to 2 hours before being visualized on a 1% agarose gel.

2.14 Quantitative PCR (qPCR)

2.14.1 Making qPCR standards using the pGEM-T easy vectors

In order to quantify the gene copy number present in soil samples, a standard curve was set up using dilutions of DNA of known copy number. To make the standards the gene of interest was first cloned into a pGEM-T easy vector plasmid. The *nifH* gene of *Rhizobium* sp. IRBG74 bacterium was used for *nifH* qPCR and the 16S rRNA gene of *Pseudomonas aeruginosa* NCTC10662 was used for eubacterial qPCR. As in section 2.11, 5 µl of PCR product was purified using ExoSAP-IT. Ligations and transformations were carried out according to section 2.13.

Colony PCR was carried out on any white transformed colonies. Colonies with a positive result were grown overnight in 10 ml of LB containing 1ml ampicillin (10mg/ml) per 100 ml broth, at 37 ºC overnight. Plasmid was then extracted from the broth using the Promega PureYield Plasmid Miniprep System (see section 2.13) to quantify the amount of plasmid present the absorbance was measured at 260 nm. PCR was carried out on the plasmid using qPCR conditions (described in section 2.14.2) and visualized on an agarose gel to ensure a single band of the correct size was amplified. The plasmid DNA was then mixed with an equal volume of 50 % sterile glycerol and stored at -80 ºC.

2.14.2 qPCR protocol

All qPCR reactions were set up according to Baxter and Cummings (2008) using the Rotor-Gene RG 3000 (Corbett Research). Prior to the PCR set-up test DNA/reverse transcribed RNA was diluted 1 in 2 with sterile water and denatured for 10 mins at 95ºC. The standard DNA was also denatured for 10 mins at 95 °C before being serially diluted so that 5 standards were obtained with copy numbers of 300,000, 30,000, 3,000, 300 and 30.

Each PCR reactions were set up in triplicate. Each tube contained 12.5 µl of SYBR green qPCR mix (Thermo Fisher Scientific), 1.75 µl of each primer (10 µM), 1.25 µl of BSA (10mg/ml), 2.75 µl of sterile water and 5 µl of diluted DNA. No template control reactions are also set up containing sterile water instead of diluted DNA. PolF and PolR primers were used for *nifH* qPCR and Eub338 and V3R were used for total bacteria qPCR (see Table 2.4 for details).

The reaction tubes were heated to 95 ºC for 15 minutes to activate the SYBR green contained within the qPCR mix before completing 50 PCR cycles comprising of a denaturation of 95 ºC for 15 seconds, annealing step of 55 ºC (*nifH*) or 65 ºC (16S rRNA) for 15 seconds and an extension of 72 ºC for 15 seconds. After a 45 second wait, this was followed by a melting step where the temperature was raised by 1˚C every 5 seconds from 72 ˚C to 95 ˚C.

In order to ensure qPCR results were reproducible and reliable the suggestions made in Karlen *et al.,* (2007) were followed. During the qPCR cycle the increase in fluorescence and, therefore, the amplification of DNA, should become linear. The cycle number at which this happens is known as the threshold. The Ct value relates to the point at which each sample crosses the threshold. The higher this value the smaller the amount of DNA in the sample. To ensure reproducibility, standard deviation should be below 0.4 between replicate Ct scores. If standard deviation of replicate scores was over 0.4 the results for that sample were rejected and repeated. To ensure results were reliable Ct scores should

be below 30 (this corresponds to 1.0 x 10^4 copies per g of soil) any sample which does not fit this criterion has a copy number below reasonable levels of detection. Optimization of qPCR is further discussed in results chapter 1.

2.15 BIOLOG plates

Community level substrate utilisation (CLSU) was determined for soils collected in 2007 using EcoPlates™ from the Biolog™ system. EcoPlates contain 31 carbon substrates and a water control, in triplicate, in the presence of a tetrazolium dye (see appendix section A8 for further details). The protocol for Ecoplate analysis was adapted from methods employed by Kashama *et al.,* (2009), Prévost *et al.,* (2006) and Widmer *et al.* (2001).

Soils (3g dry weight) that had been previously frozen at -80 ºC were pre-conditioned at 20 °C in a water saturated atmosphere for 7 days to standardise analysis for each sample. Bottles were weighed regularly and any reduction in weight due to moisture loss was made up with sterile distilled water. After 7 days, 30ml of 0.9% NaCl (w/v) was added to the soil samples and the bottles were shaken for 1 hour (300 rpm) to ensure homogeneous dispersion of the soil particles. The bottles were then left to settle for 30 minutes. The turbid supernatant was diluted to a final dilution of 10^{-3} with 0.9% NaCl (w/v) and 150 µL inoculated into each well of the EcoPlate™. Plates were then incubated at 20 °C in the dark. After 54 hours absorbance readings were taken (540nm) using a microplate reader (Bio-tek). All substrate absorbancy readings were corrected by subtracting the water control well value and normalized by dividing by the average well colour development (AWCD). AWCD is the sum of differences between the control (water) well and substrate wells divided by the number of substrates.

AWCD = $\sum OD_i/31$, where OD_i is the optical density value, at 540 nm, from each well.

Functional diversity was measured using Shannon's diversity index:

$$H' = - \sum p_i \ln(p_i)$$

where p_i is the ratio of colour development of well i to the sum of normalized colour development of all positive wells, and ln refers to the natural log (Farnet *et al*., 2008; Zak *et al*., 1994).

2.16 Statistical analysis

In all tests significant effects/interactions were those with a *P* value of <0.05. All univariate data was analyzed using the linear mixed effects (lme) function in the nlme package of R (Crawley, 2007; R Development Core Team, 2006). Each year is analyzed separately. The combined data for all three dates was analyzed first and where interaction terms were significant, further analyses were conducted at each level of the interacting factor. The hierarchical nature of the split-split plot design was reflected in the random error structures that were specified as block/date/pre-crop/crop protection. Where analysis at a given level of a factor was carried out, that factor was removed from the random error term. The normality of the residuals of all models was tested using QQ-plots and data was log transformed when necessary to meet the criteria of normal data distribution (Clough *et al*., 2007). Differences between main effects were tested using analysis of variance. Differences between the four crop management strategies within each level of crop rotation were tested using Tukey contrasts in the general linear hypothesis testing (glht) function of the multcomp package in R. A linear mixed effects model was used for the Tukey contrasts containing a treatment main effect with four levels with the random error term specified as block/crop protection.

Relative intensities from DGGE data were first analysed indirectly using R followed by direct ordination with Monte Carlo permutation testing using CANOCO. Indirect ordination was first carried out using detrended correspondence analysis (DCA) if the 1st axis was shorter than 3.5 principal component analysis (PCA) was used instead. DCA/PCA was

carried out using the decorana and pca commands in the vegan package of R. Plots were created by pasting R read-outs into Minitab (Minitab, 2006). Direct ordination was either by canonical correspondence analysis (CCA) or redundancy discriminate analysis (RDA) depending on the length of the DCA axis (axis >3.5 = CCA, axis <3.5 = RDA). CANOCO for windows 4.5 and CANODRAW for windows were used to carry out CCA and RDA.

ANOVA and Pearson's rank correlation analysis were used to analyse all background data. Background data was then used as factors in DCA, CCA and RDA analysis when investigating DGGE data. ANOVA was carried out as above. Pearson's product-moment correlations were calculated using the cor.test function in R.

Relative intensities from DGGE analysis were converted to univariate indicators of diversity i.e. Shannon's diversity index (H'). H' was calculated using the following formula:

$$H' = - \sum p_i \ln(p_i)$$

Where p_i was the ratio of relative intensity of the band i compared with relative intensity of the lane. In refers to the natural log. Univariate diversity data was analyzed as described above using lme in R.

3. Results chapter 1- Preliminary data and analysis of environmental variables throughout the sample years.

3.1. Introduction

The molecular tools used in this study have the potential to be very powerful as long as they are efficient and reproducible (Park and Crowley, 2005). Therefore, all techniques require careful optimization. Two identical samples could have significantly different results if techniques, such as nucleic acid extraction, are not standardized, and the correct statistical analysis is not applied. For example, a recent study which received the Science 'Breakthrough of the year 2005' award (Huang *et al*., 2005) had to be completely retracted (Bohlenius *et al*., 2007) due to inappropriate use of qPCR and statistical analysis (Bustin *et al*., 2009).

However, as with all fast moving fields, certain techniques, which could have been used to further optimise and improve the work, were not widely discussed when the experiments were designed. This chapter looks at the optimization of molecular techniques and discusses potential flaws in the data and areas which could be improved if the experiments were revisited.

This chapter also examines the environmental variables which were measured throughout the three sample years. NFSC routinely measure changes in soil temperature, soil pH, soil basal respiration, soil carbon and nitrogen, concentrations of ammonium and nitrate, as well as changes in trace elements such as P, K, and Fe. Some changes to environmental variables (for example pH) could result in changes to the bacterial communities of interest. It is also possible that some of the variables (e.g. soil basal respiration) could change as a result of the changes to the communities themselves. Many of the variables will also change as a result of treatment. For example, levels of ammonium and nitrate will differ between plots as greater concentrations were added to the conventional plots, as opposed to the organic plots. It is therefore important to try and

understand the nature of the environment the bacterial communities find themselves in to try and deduce if changes observed are due to the treatments themselves or an overriding soil condition. Examining the soil variables could also show if conditions remain similar across sample dates and sample years.

3.2. Results and Discussion

3.2.1. Checking RNA integrity and quantity.

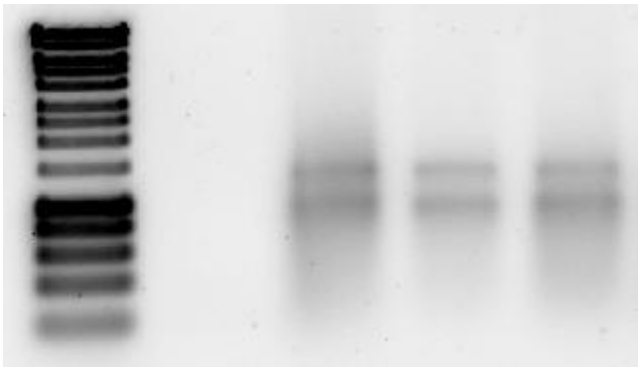
RNA is susceptible to degradation due to the ubiquitous presence of RNase enzymes. Degradation can have effects on downstream processes, in particular qPCR (Schroeder *et al*., 2006). In order to check RNA was of acceptable integrity 500 ng of RNA was electrophoresed through 1% agarose gels (Figure 3.1). Intact RNA should show 23S and 16S rRNA in a ratio of 2:1 (Mohanty *et al*., 2006). RNA was also quantified by measuring optical density at 260 and 280 nm to quantify RNA and check for protein contamination. RNA without protein contamination should have an A_{260}/A_{280} ratio of 1.8-2.0 (Mohanty *et al*., 2006).

The protocol used could have been optimized further by using more reliable quantification techniques such as the Nanodrop system (Thermo Fisher Scientific) which requires only 1 µl of sample to determine RNA, DNA and protein concentrations. There are also problems with using agarose gels to assess RNA integrity as the result is subjective. A more reliable method would be use of the fully automated Agilent 2100 bioanalyzer which calculates an RNA integrity number (Schroeder *et al*., 2006)

3.2.2. Optimisation of *nifH* and 16S rRNA gene amplification.

PCR was carried out as described in methods section 2.9. Initial experiments were carried out to ensure that the protocols gave the sharpest single bands. This included altering

Figure 3.1. Gel image showing 23S and 16S bands of intact RNA.



Lane 1 = hyperladder, Lane 2 = blank, Lane 3, 4 and 5 contain examples of intact RNA. Bands correlate to 16S and 23S subunits.

concentrations of template DNA, $MgCl_2$ and primers, as well as the inclusion, or exclusion, of BSA and DMSO. The protocol used resulted in the best amplification.

PCR was successful for all samples from 2007 and 2009. However, a large proportion of samples from 2008, including all of the September sample date, were unsuccessful in the amplification of the *nifH* gene. Several attempts were made as well as the re-extraction of RNA but all attempts were unsuccessful. As the 16S rRNA gene could still be extracted from these samples (and appeared to have no reduced diversity) it was assumed that the *nifH* gene was not present in these samples. For this reason DNA was also extracted from 2008 and 2009 samples to allow study of the (apparently inactive) N fixing community.

In order to ensure no DNA contaminated the RNA samples PCR was carried out, using the usual protocol, on RNA samples before reverse transcription occurred (see Fig. 3.2). This is recommended by the manufacturer of the RNA extraction kit.

Figure 3.2. Agarose gels showing examples of DNA contamination tests of RNA.

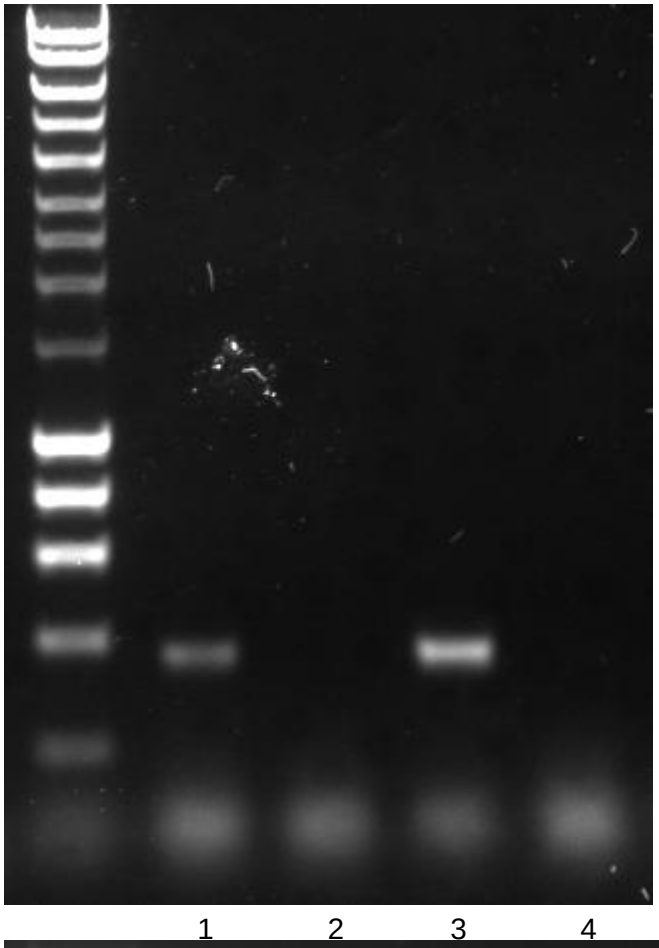


Fig 3.2a Agarose gel image showing an example of a *nifH* DNA contamination test. Lane 1 = Hyperladder, lane 2 = reverse transcribed RNA from soil, lane 3 = RNA from soil, lane 4 = *nifH* positive control, lane 5 = negative control (sterile water as template).

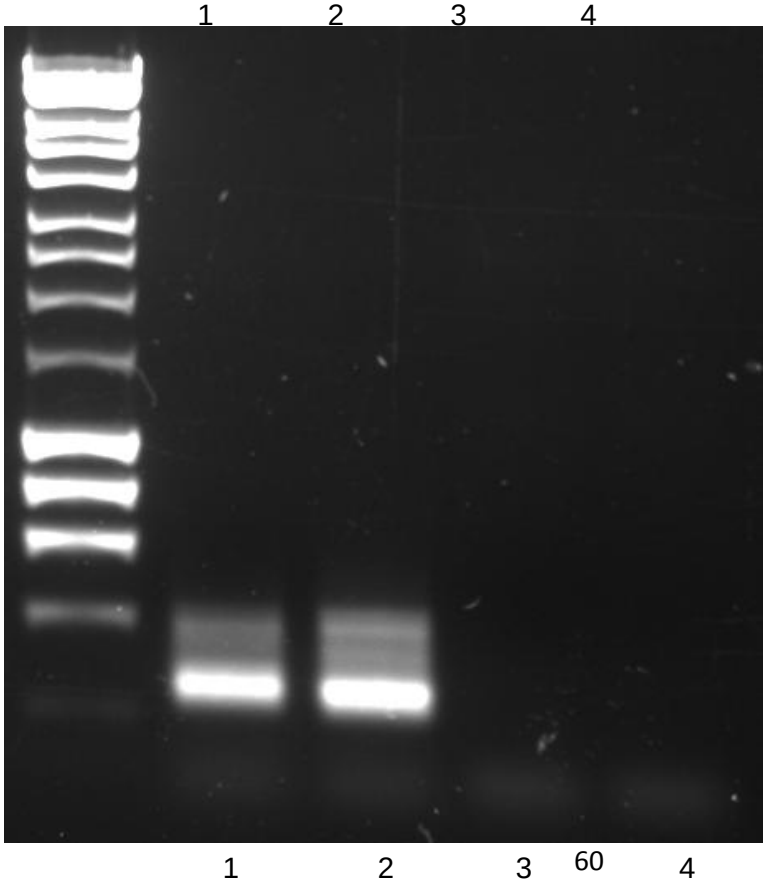


Fig 3.2b Agarose gel image showing an example of a 16S rRNA DNA contamination test. Lane 1 = Hyperladder, lane 2 = reverse transcribed RNA from soil, lane 3 = 16SrRNA positive control, lane 4 = RNA from soil, lane 5 = negative control (sterile water as template).

3.2.3. DGGE optimisation including replication of DGGE results.

The DGGE protocol is described in methods section 2.10. The 16S rRNA gene DGGE protocol was already routinely used in the laboratory (Baxter *et al*., 2006). DGGE for the separation of the *nifH* gene was optimised by altering denaturing gradients and run times.

The DGGE apparatus used (Bio-Rad DCODE universal mutation detection system) allows the comparison of 16 lanes on each gel. Due to variation it is often unreliable to compare between 2 or more gels without the use of a standard (Park and Crowley, 2005). As there are 16 plots in each sample date, each gel contains a different sample date. In order to check for variability between samples, due to differences in reverse transcription, and both stages of PCR, preliminary DGGE gels were produced, the results of which are discussed in the section below.

3.2.4. An example of technical replication.

Three RNA samples were taken from plots 2, 3 and 4 from the September 2007 sample date of the conventional rotation. Reverse transcription and PCR was carried out to test the variance due to:

a) second round PCR

b) first and second round PCR

c) reverse transcription and both rounds of PCR.

Figure 3.3 provides a summary of the experiment. For each sample reactions labelled ‘A’ are from the same reverse transcription and the same 1st round PCR step, therefore, any variation between the two samples would be caused by the second round PCR reaction. Each reaction labelled ‘B’ comes from one reverse transcription reaction, therefore, any variance in the sample is due to 1st and 2nd round PCR steps. Each

Figure 3.3. Schematic to show the set up of the DGGE replicate experiment.

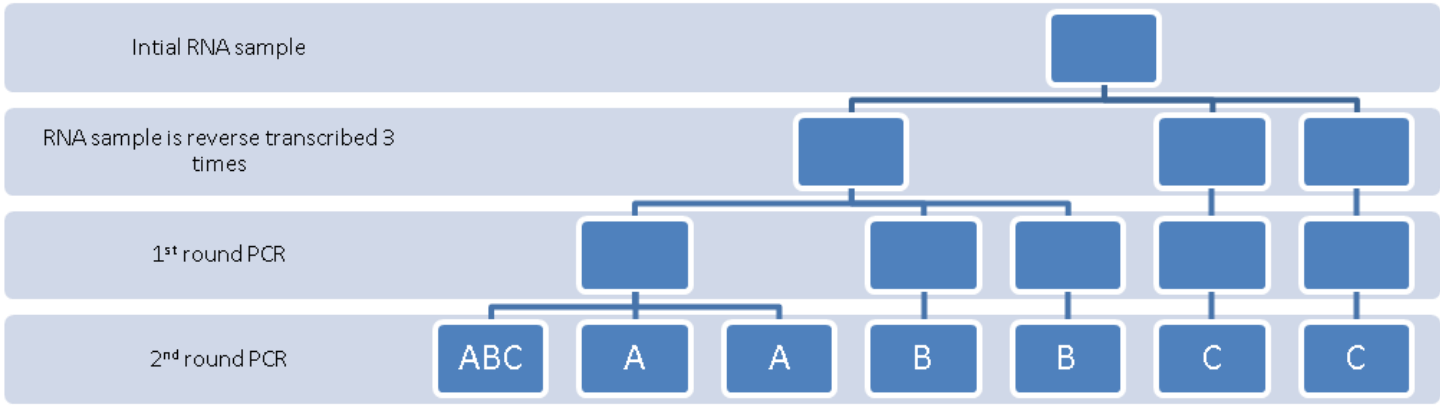
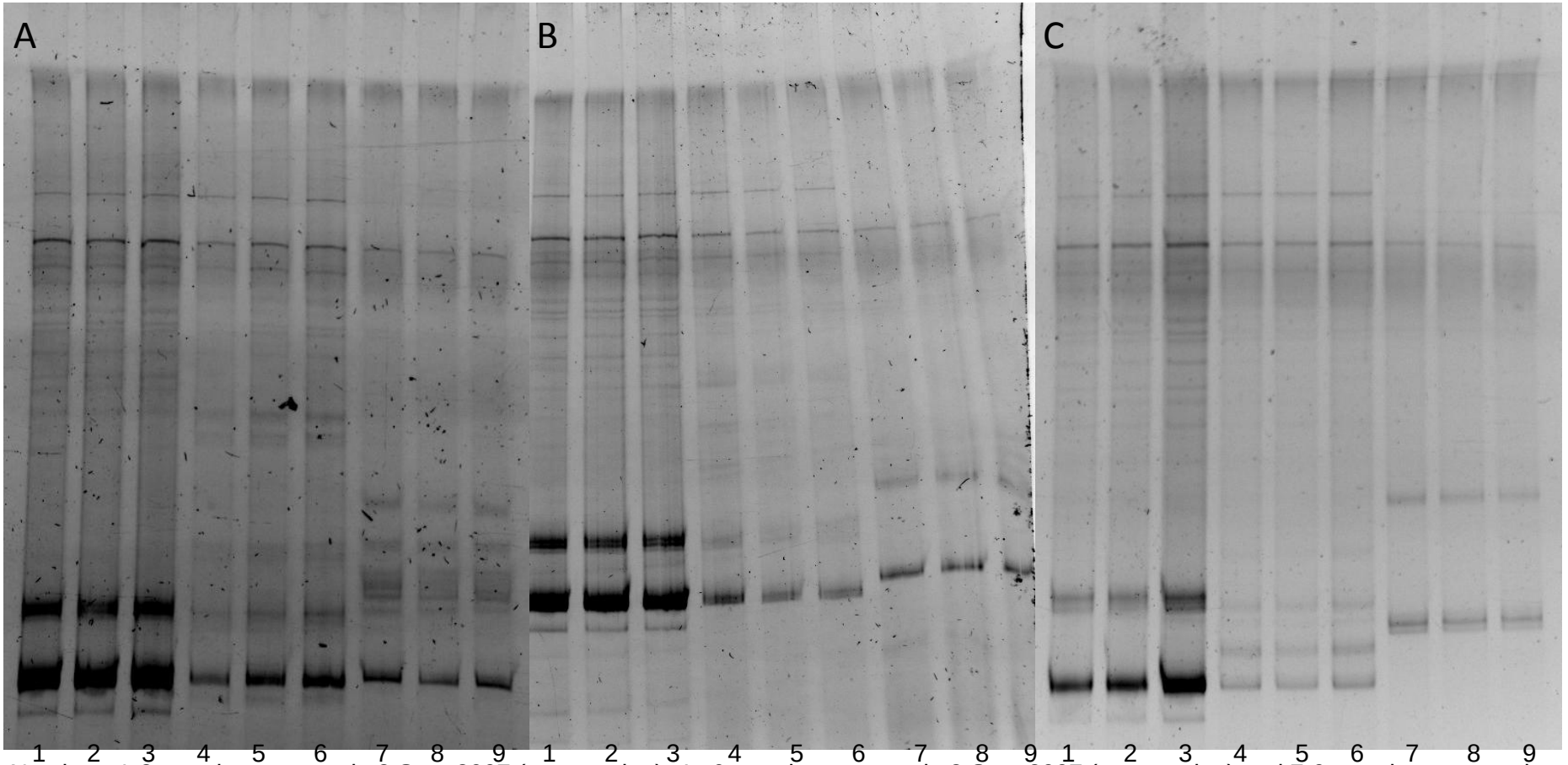


Figure 3.3. Samples labelled A show in triplicate any variation caused by 2nd round PCR. Samples labelled B show in triplicate any variation caused by 1st and 2nd round PCR. Samples labelled C show in triplicate any variation caused by reverse transcription and 1st and 2nd round PCR. This was carried out for 3 soil samples.

Figure 3.4 DGGE gels showing the effect of technical replication



Numbers 1-3 correlate to sample 2 Sept 2007 (con rotation), 4– 6 correlate to sample 3 Sept 2007 (con rotation) and 7-9 correlate to sample 4 Sept 2007 (con rotation). Gel A shows variation due to 2nd round PCR, Gel B shows variation due to 1st and 2nd round PCR and Gel C shows variation due to reverse transcription, and 1st and 2nd round PCR.

Figure 3.5 Results of Raup-Crick similarity distance indices analysis of technical replicate gels.

A	1	2	3	4	5	6	7	8	9
1		**0.999**	**1.000**	0.670	0.766	0.486	0.083	0.067	0.159
2			**1.000**	0.502	0.606	0.592	0.027	0.029	0.093
3				0.662	0.760	0.478	0.074	0.080	0.176
4					**1.000**	**1.000**	0.115	0.129	0.261
5						**1.000**	0.251	0.238	0.443
6							0.072	0.059	0.174
7								**1.000**	**1.000**
8									**1.000**
9									

B	1	2	3	4	5	6	7	8	9
1		**1.000**	**0.999**	0.635	0.393	0.320	0.015	0.017	0.007
2			**1.000**	0.514	0.250	0.201	0.004	0.006	0.003
3				0.502	0.262	0.175	0.005	0.006	0.000
4					**1.000**	**0.990**	0.609	0.599	0.449
5						**0.999**	0.786	0.785	0.670
6							0.862	0.867	0.766
7								**1.000**	**1.000**
8									**1.000**
9									

C	1	2	3	4	5	6	7	8	9
1		**0.971**	**0.986**	0.377	0.317	0.352	0.023	0.015	0.011
2			**0.998**	0.730	0.653	0.711	0.170	0.118	0.064
3				0.618	0.556	0.627	0.110	0.077	0.047
4					**1.000**	**1.000**	0.865	0.753	0.561
5						**1.000**	0.926	0.815	0.647
6							0.871	0.753	0.576
7								**1.000**	**0.999**
8									**0.998**
9									

Numbers correspond with lanes on gels. Statistically significant values are those above 0.95. A = results from gel A where the only difference between sets of samples was the 2nd round PCR. B = results from gel B where the difference between sets of samples was 1st and 2nd round PCR. C = results from gel C where the difference between sets of samples was reverse transcription, 1st and 2nd round PCR.

reaction labelled ‘C’ is from a separate reverse transcription reaction, therefore, any variance between the samples is due to variance caused by reverse transcription and both rounds of PCR. All PCR and reverse transcription was carried out according methods section 2.8 and 2.9.2.

DGGE was carried out on each set of samples according to methods section 2.10 (see Fig. 3.4 for gel images). Raup-Crick similarity and distance indices analysis was then carried out on DGGE data using the PAST palaeontology tool (Hammer *et al*., 2001) (Fig. 3.5). Results show no significant variation due to the methodology and therefore DGGE gels used in the study only contain one sample per plot.

This experiment also shows that although the banding pattern is similar between the three gels the relative intensities of a particular band can differ between gels. For band intensities to be reproducible between gels standard error should be low (Diez *et al*., 2001). However, in these gels standard error shows around a 20% variation in band intensity for the same bands. For this reason comparisons are only made between gels when they involve comparisons of band patterns (Shannon diversity indices) rather than band intensities (direct and indirect ordination).

3.2.5. Optimisation of qPCR

MIQE guidelines outlined by Bustin *et al*. (2009) aim to make qPCR as accurate and reproducible as possible. These guidelines were not published until after qPCR analysis was underway. However, the guidelines were followed retrospectively wherever they were possible and relevant. As described in the methods section 2.14 standards were created by cloning gene fragments and creating samples of known concentration. To obtain the most accurate qPCR results a standard curve must be produced where samples of interest fall within the limits of the curve and are in the linear phase. Initial experiments involved dilution of RNA and standards so that this was possible. The optimum standard

concentrations were found to be between 30-300,000 copies (*nifH*) and 30-3,000,000 copies (16S rRNA). Optimum dilution of RNA was found to be 1 in 40.

qPCR is a powerful technique which can theoretically detect very low concentrations of amplified product. However, in practice this is not always the case. Therefore, a limit of detection must be found which is equal to the lowest concentration that can be detected with reasonable certainty (Bustin *et al*., 2009). Following the advice of Karlen *et al.* (2007) the limit of detection for the set up used in this study was 1.0×10^4 copies per g of soil. This relates to a C_t score of over 30. In some cases *nifH* values fell below this threshold and therefore, it had to be assumed that, in these samples, *nifH* could not be detected. Each sample was set up in triplicate. To ensure qPCR was set up accurately results were rejected and repeated if the standard deviation of the C_t scores within a sample was above 0.4 (Karlen *et al*., 2007).

A major risk with any molecular biology technique, particularly when looking at the 16S rRNA gene, is contamination. In order to avoid contamination all qPCR was set up in a sterile PCR hood away from the main laboratory and all plastic ware (e.g. tips, eppendorfs etc) was double autoclaved. Filter tips were used and the pipettes used were limited to qPCR work only.

No-template controls (NTCs) were also set up. NTCs are set up identically to samples but the template is replaced with sterile water. Due to the nature of SYBR green a small number of copies may be detected in the NTCs but this number should be low and fall below the limit of detection outlined above. The use of NTCs also indicates that primer dimer is kept to a minimum as any rise in NTC copy number could be down to this.

Burgmann *et al.* (2003) highlighted that checking for DNA contamination of RNA using agarose gels (as discussed above) is often not sensitive enough to pick up small amounts of contamination. For this reason samples of non-reverse transcribed RNA were also ran

using qPCR as a second negative control. Here the template used in setting up samples is replaced with non-reverse transcribed RNA.

The standard curve should provide a linear range and therefore have an r^2 value close to 1. Occasionally curves may be non-linear due to samples at the lower or higher end of the curve. Samples should only be used if they fall within the linear range of the curve. The rotor-gene software also calculates a PCR efficiency which, if copy number doubled at each cycle, should be equal to 1 (Bustin *et al*., 2009). PCR efficiency is affected by the presence of inhibitors, poor primer design and pipetting errors. Standard curves giving efficiencies between 0.9 and 1.10 are widely considered to be acceptable, however, as long as the samples of interest fall within a linear range the efficiency can be greater than 1.10 (Rebrikov and Trofirnov, 2006).

See Figures 3.6, 3.7 and Table 3.1 for examples of qPCR standard curves. Figure 3.6 shows the increase in fluorescence caused by the increase in double stranded DNA created during the PCR reaction. Curves related to the samples rise in between the standard range and curves for NTCs and non-reverse transcribed controls rise after the standards and samples. Figure 3.7 shows the standard curve created by the fluorescence pattern shown in Figure 3.6, and Table 3.1 shows the copy numbers calculated by the standard curve in Figure 3.6.

Figure 3.6. Graph showing the increase in fluorescence during *nifH* qPCR

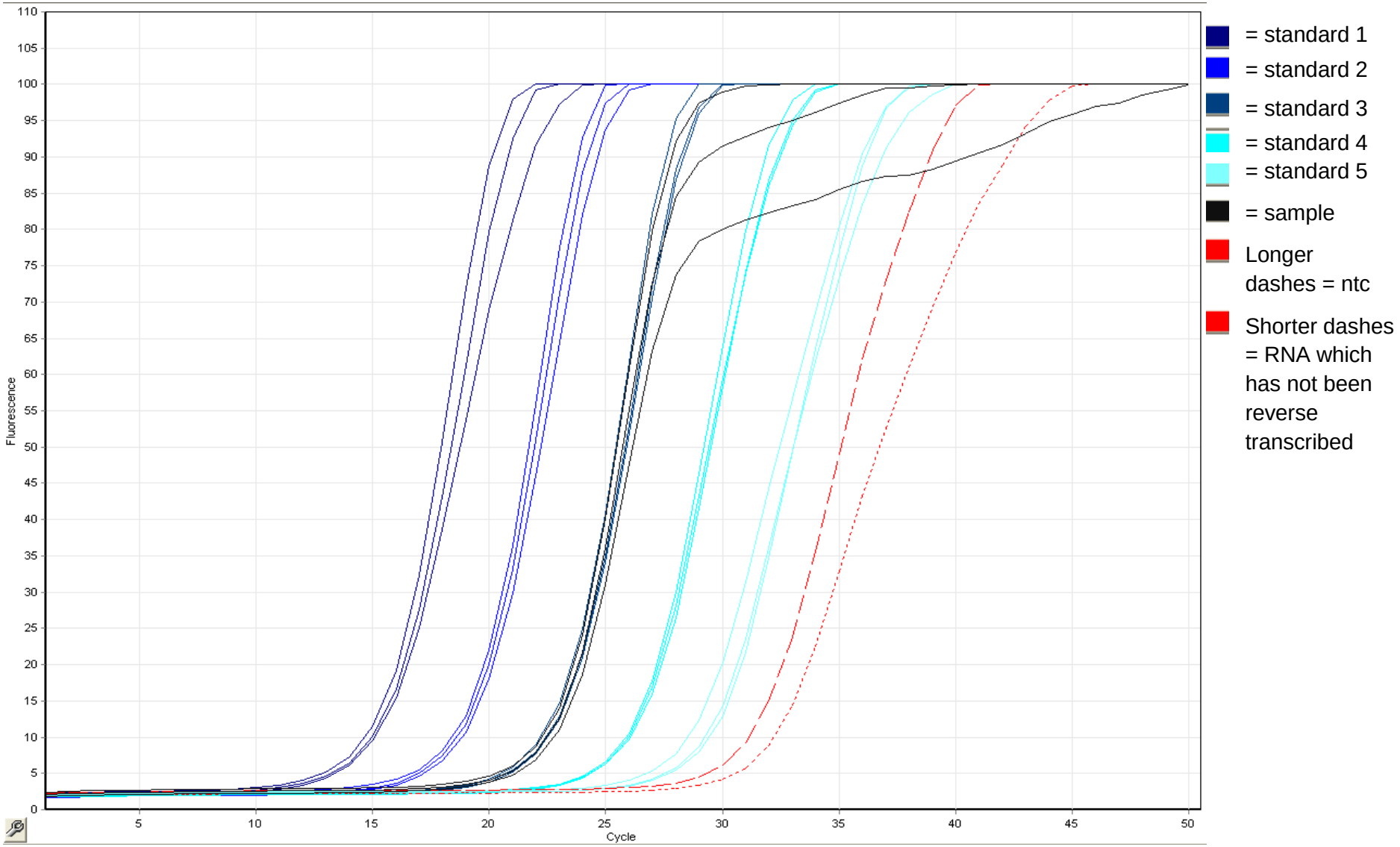
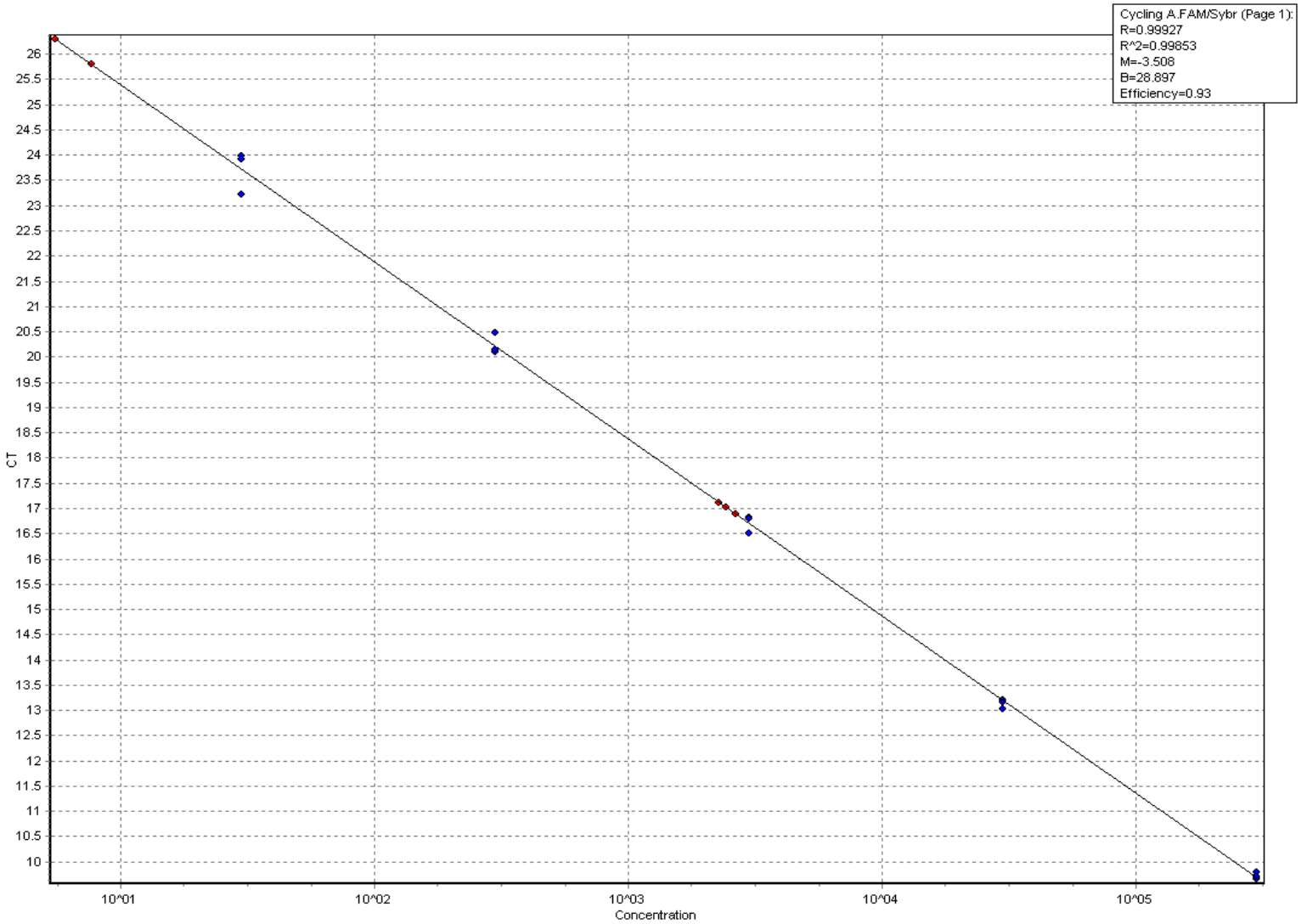


Figure 3.7. Example qPCR standard curve



qPCR standard curves should be linear (r^2 close to 1) and efficiency should be > 0.9.

Blue markers show the standards, red markers at low concentrations show the negative controls and at higher concentrations show the sample.

Table 3.1. Calculated copy number from standard curve.

No.	C	Name	Type	Ct	Given Conc (cop	Calc Conc (copie	% Var	Rep. Ct	Rep. Ct Stc	Rep. Calc. Conc.
1		std1	Standard	9.66	300,000.00000	305,155.11204	1.7%	9.71	0.07	294,019.10166
2		std1	Standard	9.79	300,000.00000	280,109.79729	6.6%			
3		std1	Standard	9.70	300,000.00000	297,356.67366	0.9%			
4		std2	Standard	13.21	30,000.00000	29,674.80299	1.1%	13.13	0.09	31,144.53185
5		std2	Standard	13.16	30,000.00000	30,561.59703	1.9%			
6		std2	Standard	13.03	30,000.00000	33,310.52919	11.0%			
7		std3	Standard	16.83	3,000.00000	2,755.96359	8.1%	16.71	0.18	2,984.99569
8		std3	Standard	16.80	3,000.00000	2,813.01425	6.2%			
9		std3	Standard	16.50	3,000.00000	3,430.72351	14.4%			
10		std4	Standard	20.10	300.00000	322.07748	7.4%	20.24	0.20	293.54692
11		std4	Standard	20.47	300.00000	252.61417	15.8%			
12		std4	Standard	20.15	300.00000	310.89556	3.6%			
13		std5	Standard	23.22	30.00000	41.57772	38.6%	23.70	0.42	30.28501
14		std5	Standard	23.98	30.00000	25.25305	15.8%			
15		std5	Standard	23.91	30.00000	26.45505	11.8%			
17		ntc	NTC	25.80		7.65160		26.05	0.35	6.49957
18		ntc	Negative	26.29		5.52098				
34		sample	Unknown	17.12		2,273.65150		17.01	0.12	2,444.13975
35		sample	Unknown	17.03		2,420.16814				
36		sample	Unknown	16.89		2,653.43630				

3.2.6. Study of environmental variables.

All environmental variables were compared across all sample years and raw data can be found in appendix section D. When the data set was analyzed whole, using ANOVA, year and pre-crop was a significant factor for all environmental variables (Table 3.2) even though the same amounts of fertilizers and pesticides were applied each year. To investigate this, 2007 variables were removed from analysis due to the difference in previous crop in this year. When 2008 and 2009 variables were analyzed together year was still significant for every variable except extractable iron (Table 3.3). For this reason each year was analyzed separately as shown in Tables 3.4, 3.5 and 3.6. Correlations were also investigated between environmental variables using Pearson's correlation coefficient analysis (Table 3.7).

A recent report by the Soil Association (Azeez, 2009) compiled 39 published studies of organic and conventional farming and found that on average organic farming produced 28% higher soil carbon levels than conventional farming, in Northern Europe. This is attributed to the farm yard manure and composts added to the soil creating humus. However, analysis of the environmental variables found no significant difference in levels of total C and organic N between the organic and conventionally managed plots (Table 3.2). Although there was negative correlation between %C (increased C would normally be expected in organically fertilized soils (Clark *et al*., 1998)) and nitrogen species (increased nitrogen is associated with conventionally fertilized soils in this study) (Table 3.7). This mirrors a study by Gosling and Shepherd (2005) who rationalized their findings by concluding that although animal and green manures led to increased input of organic matter this may be equalled in conventional soils by increased crop residues resulting from increased crop yields.

Gosling and Shepherd (2005) also found significant differences in the concentrations of extractable phosphorus following the theory that organic systems rely on reserves of soil

Table 3.2. ANOVA analysis showing the effect of farm management and year on all environmental variables

	Total C (%)	Organic N (%)	pH	Soil basal respiration (mg CO_2 kg^{-1} h^{-1})[z]	P (mg kg^{-1})	Fe (mg kg^{-1})	available NO_3^- (March) (kg ha^{-1})	available NO_3^- (June) (kg ha^{-1})	available NO_3^- (Sep) (kg ha^{-1})	available NH_4^+ (March) (kg ha^{-1})	available NH_4^+ (June) (kg ha^{-1})	available NH_4^+ (Sep) (kg ha^{-1})
Mean±SE year (PC)												
2007 (barley)	1.81 ± 0.14	0.27 ± 0.04	6.27 ± 0.2	1.24 ± 0.4	51.28 ± 3.7	330.2 ±11.5	5.81 ± 0.8	279.3 ± 43.3	27.05 ± 2.3	0.94 ± 0.7	7.54 ± 3.2	6.42 ± 1.9
2007 (beans)	1.83 ± 0.17	0.28 ± 0.03	6.10 ± 0.1	1.24 ± 0.5	54.95 ± 3.6	342.4 ± 10.3	12.47 ± 0.7	234.4 ± 31.0	22.46 ± 1.7	5.95 ± 1.0	1.46 ± 1.2	0.00 ± 0.0
2008 (wheat)	2.72 ± 0.15	0.24 ± 0.02	6.80 ± 0.3	0.86 ± 0.1	47.88 ± 4.2	373 ± 16.9	5.31 ± 0.5	73.2 ± 13.8	9.23 ± 1.0	1.28 ± 1.2	4.88 ± 4.8	0.00 ± 0.0
2009 (wheat)	2.24 ± 0.15	0.22 ± 0.02	6.31 ± 0.3	1.89 ± 0.5	63.24 ± 2.5	412.4 ± 13.4	0.00 ± 0.0	234.1 ± 38.0	21.58 ± 4.4	0.29 ± 1.2	15.68 ± 18.2	2.65 ± 2.2
Crop protection												
ORG	2.14 ± 0.10	0.26 ± 0.01	6.41 ± 0.1	1.31 ± 0.1	53.18 ± 3.6	367.5 ± 16.5	5.75 ± 1.2	216.5 ± 40.3	23.06 ± 3.1	2.23 ± 0.6	7.33 ± 2.7	2.40 ± 0.8
CON	2.16 ± 0.10	0.25 ± 0.01	6.34 ± 0.1	1.37 ± 0.2	55.49 ± 3.9	361.5 ± 13.9	6.05 ± 1.3	194.0 ± 36.3	17.10 ± 3.0	1.99 ± 0.6	7.45 ± 2.7	2.13 ± 0.7
Fertility management												
ORG	2.17 ± 0.11	0.26 ± 0.01	6.49 ± 0.09	1.51 ± 0.1	50.33 ± 3.3	361.2 ± 15.2	5.34 ± 1.2	91.6 ± 11.6	17.35 ± 3.7	2.37 ± 0.7	3.20 ± 0.6	2.49 ± 0.8
CON	2.13 ± 0.10	0.25 ± 0.01	6.26 ± 0.08	1.17 ± 0.1	58.34 ± 3.9	367.7 ± 15.3	6.46 ± 1.3	318.9 ± 34.0	22.81 ± 2.2	1.86 ± 0.6	11.58 ± 3.5	2.05 ± 0.7
ANOVA *P*-values												
Y+PC	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.031**	**0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
CP	0.558	0.719	0.223	0.576	0.532	0.674	0.579	*0.063*	**0.010**	0.366	0.948	0.466
FM	0.354	0.664	**<0.001**	**0.002**	**0.031**	0.650	**0.042**	**<0.001**	**0.017**	*0.061*	**<0.001**	0.239
Y+PC*FM	0.479	0.840	0.509	0.129	0.685	0.849	*0.076*	**<0.001**	**0.003**	0.992	**<0.001**	0.214
Y+PC*CP	0.798	0.917	0.228	0.609	0.980	0.667	0.514	0.121	0.242	0.286	0.981	0.834
CP*FM	0.991	0.832	0.506	0.312	0.802	0.812	0.235	0.728	0.125	0.665	0.664	0.705
Y+PC*FM*CP	0.808	0.938	0.674	0.753	0.861	0.875	0.425	0.456	0.397	0.776	0.914	0.489

Table 3.3. ANOVA analysis showing the effect of farm management and year on environmental variables in 2008 and 2009.

	Total C (%)	Organic N (%)	pH	Soil basal respiration (mg CO_2 kg^{-1} h^{-1})[z]	P (mg kg^{-1})	Fe (mg kg^{-1})	available NO_3^- (March) (kg ha^{-1})	available NO_3^- (June) (kg ha^{-1})	available NO_3^- (Sep) (kg ha^{-1})	available NH_4^+ (March) (kg ha^{-1})	available NH_4^+ (June) (kg ha^{-1})	available NH_4^+ (Sep) (kg ha^{-1})
Mean±SE												
year												
2008	2.72 ± 0.15	0.24 ± 0.02	6.80 ± 0.3	0.86 ± 0.1	47.88 ± 4.2	373 ± 16.9	5.31 ± 0.5	73.2 ± 13.8	9.23 ± 1.0	1.28 ± 1.2	4.88 ± 4.8	0.00 ± 0.0
2009	2.24 ± 0.15	0.22 ± 0.02	6.31 ± 0.3	1.89 ± 0.5	63.24 ± 2.5	412.4 ± 13.4	0.00 ± 0.0	234.1 ± 38.0	21.58 ± 4.4	0.29 ± 1.2	15.68 ± 18.2	2.65 ± 2.2
Crop protection												
ORG	2.46 ± 0.07	0.23 ± 0.01	6.59 ± 0.1	1.43 ± 0.1	55.07 ± 3.6	403.9 ± 17.1	2.81 ± 0.8	150.3 ± 33.7	16.1 ± 3.1	0.71 ± 0.3	9.85 ± 3.7	1.39 ± 0.6
CON	2.51 ± 0.08	0.23 ± 0.01	6.53 ± 0.1	1.47 ± 0.2	56.04 ± 4.4	381.5 ± 14.4	2.50 ± 0.8	156.9 ± 36.9	14.71 ± 4.0	0.86 ± 0.3	10.71 ± 3.6	1.26 ± 0.4
Fertility management												
ORG	2.51 ± 0.07	0.23 ± 0.01	6.71 ± 0.08	1.50 ± 0.2	50.30 ± 3.5	383.9 ± 17.1	2.69 ± 0.8	58.2 ± 9.1	10.31 ± 1.3	1.03 ± 0.4	3.15 ± 0.6	1.31 ± 0.4
CON	2.46 ± 0.08	0.23 ± 0.01	6.41 ± 0.09	1.41 ± 0.2	60.83 ± 3.9	401.5 ± 14.6	2.62 ± 0.8	249.0 ± 34.6	20.51 ± 4.5	0.55 ± 0.2	17.40 ± 4.4	1.34 ± 0.6
ANOVA *P*-values												
Y	**0.004**	**<0.001**	**<0.001**	**<0.001**	**0.005**	0.101	**<0.001**	**<0.001**	**0.007**	**0.030**	**0.009**	**<0.001**
CP	0.932	0.366	0.570	0.820	0.845	0.344	0.588	0.558	0.743	0.743	0.821	0.839
FM	0.299	0.340	**0.005**	0.561	**0.043**	0.456	0.897	**<0.001**	**0.023**	0.273	**0.001**	0.963
Y*FM	0.904	0.631	0.856	0.967	0.993	0.978	0.897	**<0.001**	**0.037**	0.815	**0.009**	0.963
Y*CP	0.653	0.787	0.236	0.898	0.946	0.786	0.588	0.946	0.915	0.315	0.955	0.839
CP*FM	0.787	0.926	0.318	0.785	0.422	0.534	0.843	0.310	0.833	0.473	0.726	0.380
Y*FM*CP	0.397	0.640	0.806	0.599	0.859	0.938	0.843	0.359	0.961	0.536	0.644	0.380

Table 3.4. ANOVA analysis showing the effect of farm management on environmental variables in 2007

	Total C (%)	Organic N (%)	pH	SBR (mg CO_2 kg^{-1} h^{-1})[z]	P (mg kg^{-1})	Fe (mg kg^{-1})	available NO_3^- (March) (kg ha^{-1})	available NO_3^- (June) (kg ha^{-1})	available NO_3^- (Sept) (kg ha^{-1})	available NH_4^+ (March) (kg ha^{-1})	available NH_4^+ (June) (kg ha^{-1})	available NH_4^+ (Sept) (kg ha^{-1})
Mean±SE												
Pre-crop												
Barley	1.81±0.03	0.27±0.01	6.14±0.05	1.15±0.03	51.28±3.7	330.1±11.1	5.81±0.8	279.34±43.2	27.08±2.3	0.94±0.2	7.58±0.8	6.47±0.5
Beans	1.83±0.04	0.28±0.01	6.23±0.04	1.00±0.04	54.95±3.6	342.3±10.3	12.47±0.7	234.42±31.2	22.51±1.7	5.95±0.3	1.51±0.3	0±0
Crop protection												
ORG	1.82±0.05	0.28±0.01	6.20±0.05	1.08±0.04	51.29±3.8	331.0±9.5	8.68±1.1	282.75±40.5	30.07±1.9	3.75±0.1	4.85±1.0	3.44±1.0
CON	1.82±0.03	0.28±0.01	6.17±0.04	1.07±0.04	54.93±3.5	341.4±11.9	9.60±1.1	231.01±34.3	19.52±1.3	0.18±0.9	4.24±1.0	3.03±0.8
Fertility management												
ORG	1.83±0.05	0.28±0.01	6.26±0.03	1.08±0.04	50.37±3.2	338.4±10.8	7.98±1.2	125.04±6.9	24.44±1.3	3.71±0.1	3.29±0.6	3.68±1.0
CON	1.81±0.02	0.28±0.01	6.11±0.05	1.06±0.04	55.85±4.0	333.9±10.8	10.31±0.9	388.72±26.7	25.14±2.7	3.18±0.9	5.80±1.2	2.79±0.7
ANOVA *P*-values												
PC	0.700	*0.090*	0.250	**0.047**	0.507	0.466	**<0.001**	**0.042**	**0.004**	**<0.001**	**<0.001**	**<0.001**
CP	0.905	0.255	0.603	0.844	0.510	0.538	0.327	**0.021**	**<0.001**	*0.051*	0.399	0.349
FM	0.670	0.992	**<0.001**	0.645	0.324	0.791	**0.018**	**<0.001**	0.632	*0.095*	**0.002**	**0.049**
CP*FM	0.900	0.615	0.177	0.569	0.693	0.635	0.209	0.352	**<0.001**	0.787	0.619	0.574
FM*PC	*0.087*	0.111	0.524	0.986	0.344	0.606	0.216	**0.045**	**0.001**	0.87	**0.014**	**0.049**
CP*PC	0.509	0.342	0.657	0.810	0.840	0.629	0.448	0.874	0.748	0.464	0.542	0.349
FM*CP*PC	0.308	0.772	0.290	0.586	0.948	0.764	0.271	0.573	**0.009**	0.914	0.526	0.574

Table 3.5. ANOVA analysis showing the effect of farm management on environmental variables in 2008

	Total C (%)	Organic N (%)	pH	Soil basal respiration (mg CO_2 kg^{-1} h^{-1})[z]	P (mg kg^{-1})	Fe (mg kg^{-1})	available NO_3^- (March) (kg ha^{-1})	available NO_3^- (June) (kg ha^{-1})	available NO_3^- (Sep) (kg ha^{-1})	available NH_4^+ (March) (kg ha^{-1})	available NH_4^+ (June) (kg ha^{-1})	available NH_4^+ (Sep) (kg ha^{-1})
Mean±SE												
Crop protection												
ORG	2.68±0.03	0.24±0.007	6.89±0.1	0.86±0.04	43.63±4.5	363.42±20.6	4.92±0.4	64.44±20.8	10.28±1.6	1.43±0.5	4.70±1.4	6.07±1.2
CON	2.78±0.07	0.24±0.008	6.72±0.1	0.87±0.03	52.12±7.1	382.48±27.7	5.70±1.0	67.46±19.4	8.18±1.3	1.13±0.3	5.95±2.0	14.17±5.6
Fertility management												
ORG	2.74±0.04	0.25±0.008	6.95±0.1	0.90±0.01	42.63±5.2	363.86±27.6	5.39±0.8	25.89±3.5	8.75±1.4	1.47±0.6	2.92±0.6	12.14±5.2
CON	2.71±0.06	0.24±0.007	6.66±0.1	0.82±0.04	53.12±6.4	382.04±20.9	5.24±0.7	120.47±12.9	9.71±1.6	1.09±0.1	7.74±2.0	8.09±3.1
ANOVA *P*-values												
CP	0.257	0.720	0.225	0.796	0.339	0.615	0.473	0.428	0.353	0.445	0.594	0.205
FM	0.733	0.555	*0.058*	0.181	0.242	0.631	0.890	**<0.001**	0.667	0.531	*0.056*	0.516
CP*FM	0.604	0.435	0.383	0.512	0.894	0.829	0.200	0.706	0.555	0.270	0.800	0.569

Table 3.6. ANOVA analysis of the effect of farm management on environmental variables in 2009

	Total C (%)	Organic N (%)	pH	SBR (mg CO_2 kg^{-1} h^{-1})[z]	P (mg kg^{-1})	Fe (mg kg^{-1})	available NO_3^- (March) (kg ha^{-1})	available NO_3^- (June) (kg ha^{-1})	available NO_3^- (Sep) (kg ha^{-1})	available NH_4^+ (March) (kg ha^{-1})	available NH_4^+ (June) (kg ha^{-1})	available NH_4^+ (Sep) (kg ha^{-1})
Mean±SE												
Crop protection												
ORG	2.20±0.04	0.22±0.01	6.31±0.1	1.80±0.06	62.92±3.7	420.4±20.6	0.00±0.0	230.39±36.4	22.50±3.57	0.00±0.0	15.35±4.9	2.78±0.7
CON	2.27±0.06	0.22±0.01	6.34±0.1	1.98±0.15	63.56±3.6	404.5±17.9	0.00±0.0	237.78±41.9	20.66±5.4	1.16±0.6	15.89±4.5	2.53±0.3
Fertility management												
ORG	2.28±0.04	0.22±0.01	6.47±0.1	1.94±0.14	57.95±3.1	404.0±19.4	0.00±0.0	90.59±4.6	11.86±1.46	1.16±0.6	3.06±0.7	2.62±0.3
CON	2.20±0.04	0.21±0.01	6.16±0.1	1.84±0.10	68.53±3.1	420.9±19.2	0.00±0.0	377.57±11.1	31.31±5.1	0.00±0.0	28.18±4.6	2.67±0.7
ANOVA *P*-values												
CP	0.394	0.672	0.921	0.487	0.892	0.590	N/A	0.674	0.823	0.337	0.941	0.841
FM	0.301	0.362	**0.037**	0.707	**0.040**	0.569	N/A	**<0.001**	**0.033**	0.337	**0.004**	0.963
CP*FM	0.682	0.645	0.406	0.661	0.506	0.666	N/A	0.223	0.894	0.337	0.661	0.388

Table 3.7. Correlation between environmental variables

	pH	%N	%C	SBR	P	Fe	NH4
%N	-						
%C	**+++**	**---**					
SBR	-	**---**	-				
P	**--**	**--**	**++**	**+++**			
Fe	**++**	**---**	**+++**	**+++**	**+++**		
NH4	-	+	**---**	+	+	-	
NO3	**---**	**++**	**---**	-	+	-	**+++**

+/- = not significant
++/-- = $P < 0.05$
+++/--- = $P < 0.001$

P and K left over from previous conventional management. At NFSC levels of extractable P were always higher under conventional management (although this is not significant when 2007 and 2008 are analyzed separately (Table 3.2, Table 3.4 and Table 3.5).

Mäder *et al*. (2002) found that organic farming was associated with higher levels of biological activity by showing increases in microbial biomass, dehydrogenase activity, alkaline phosphatase, saccharase and root length colonization by mycorrhizal fungi. Soil basal respiration was significantly affected by previous crop (Table 3.3) as well as fertility management with SBR always recorded higher after organic fertility management. However, a change in soil basal respiration does not necessarily mean that the structure of the bacterial community changes and SBR is not a significant factor when each sample year is analyzed separately.

Across all sample years, pH is increased after organic fertility management (significant in 2007 and 2009). There is also positive correlation associated with pH and %C (increased C normally expected in organically fertilised soil) and negative correlation with nitrogen species (increased N normally associated with conventionally fertilised soil) (Table 3.7). Compost additions will lead to increased mineralization of organic N which lowers pH (Bulluck *et al*., 2002). However, other studies have found that despite this pH is increased after compost additions due to the presence of basic cations in manure and increased base saturation due to aluminium saturation (Bulluck *et al*., 2002; Bossio *et al*., 1998). This could possibly lead to changes in the microbial community residing in the soil as pH is often considered to be the best indicator of bacterial diversity. In a study sampling 98 soils across North and South America, pH was found to greatly affect diversity and richness of the bacterial communities with neutral soils having the highest diversity and acidic soils having the lowest (Fierer and Jackson, 2006). It is also possible that, although the overall numbers may stay the same, different species more suited to living at different pH could proliferate more in the different soils.

Most of the significant differences seen between environmental variables occurred in 2007, the only year where previous crop was a factor. The organic rotation has increased nitrate and ammonium in March. This is probably due to the nature of the beans crop. As it is a legume it will remove less nitrogen from the soil. However, this effect is short lived as the conventional rotation then has increased nitrate and ammonium in June and September.

Fertility management also had significant effects on levels of nitrate and ammonium across all of the sample years. Where conventional fertility management resulted in increased nitrate in March and June 2007, June 2008, and June and September 2009, and increased ammonium in June of all years (2008, $P = 0.056$). This is very much in line with the expected effects of fertility management as much more nitrate and ammonium has been applied to the fields in the conventional plots. Interestingly, in 2007 and 2009, there is more nitrate present in the conventional plots than has been applied to the fields suggesting mineralization is occurring. Nitrogen can be assimilated via mineralization. This occurs due to the microbial breakdown of organic N to ammonia (ammonification) and conversion of ammonium to nitrate (nitrification). The increased values suggest an active soil community converting forms of nitrogen within the soil. Future studies to continue the work of this thesis could investigate these communities to build up a more complete picture of nitrogen cycling within the soils.

It is unclear why, in certain circumstances (June 2007-CP-nitrate, September 2007-CP-nitrate, September 2007-FM-ammonium and September 2008- FM-ammonium) organic management shows increased levels of nitrate and ammonium. Organic management can lead to reduced N leaching due to changes in soil organic matter (Knudsen *et al*., 2006). The Nitram (chemical fertilizer used) added to the conventional soils here is also more

Table 3.8 Average soil temperature (˚C) for each month of the growing season (±SE).

	2007	2008	2009
Feb	4.42 ± 0.3	3.79 ± 0.3	3.15 ± 0.5
Mar	5.93 ± 0.2	4.52 ± 0.2	5.81 ± 0.2
Apr	10.50 ± 0.3	6.79 ± 0.3	9.30 ± 0.2
May	12.89 ± 0.2	12.07 ± 0.2	11.95 ± 0.4
Jun	14.88 ± 0.3	14.59 ± 0.2	14.98 ± 0.4
Jul	15.66 ± 0.1	15.93 ± 0.3	16.09 ± 0.3
Aug	15.52 ± 0.2	15.53 ± 0.3	15.36 ± 0.2
Sep	13.31 ± 0.3	13.08 ± 0.1	14.27 ± 0.2

soluble than the organic fertilizers and therefore will be more readily removed from the soil either through crop uptake or nutrient leaching. It is also likely that a lot of nitrate-N is lost via denitrification, although, the denitrifying community is thought to be more active and more efficient in organic soils (Kramer *et al*., 2006). Changes in soil temperature could also affect the bacterial communities residing within the soil (Jangid *et al*., 2008). Table 3.8 gives average soil temperatures across the growing season for each year. ANOVA analysis showed that there was no difference between the years in terms of soil temperature ($P = 0.935$). Therefore temperature differences cannot explain any variation between sample years. However, the changes in soil temperature between different sampling months could have an effect as the temperature is obviously highest in summer and lowest in winter. Soils from the first sample date have also been exposed to prolonged low temperature whereas soils from the final sample date have had months at higher temperatures.

To summarize, it may be expected that organic and conventional farming will affect the nitrogen fixing and total bacterial community as it is fertility management which appears to affect the environmental variables studied in this preliminary chapter. Organic farming leads to increased soil basal respiration and a more neutral pH, which are both factors usually associated with increased bacterial diversity (Lauber *et al*., 2008). Other factors such as available nitrate, ammonium and phosphorus are also significantly affected by fertility management. Other factors which may impact on the two communities are previous crop and year as they also affect many of the environmental variables.

4. Results Chapter 2 – The effect of crop management on the nitrogen fixing community

4.1. Introduction

The application of organic and conventional fertilizers affects the soil in different ways. The addition of organic fertilizers will, in general, lead to increased carbon availability and a more neutral pH, whereas, the addition of conventional fertilizers will lead to increased nitrogen and phosphorus availability (Fernandez-Calvino and Bååth, 2010; Azeez, 2009; Hartley and Schlesinger, 2002). Carbon and pH have been shown to be strong drivers of bacterial diversity across various soil types (Fierer *et al*., 2007). Carbon in particular strongly influences the nitrogen fixing community with additions being reported to occasionally increase nitrogen fixation by 300% (Keeling *et al*., 1998).

Changes in nitrogen availability has also been shown to drive *nifH* expression (Hayden *et al*., 2010) as nitrogen fixing bacteria are inhibited by increased nitrogen species (Coelho *et al*., 2009; Coelho *et al*., 2008; Vintila and El-Shehawy, 2007). Tan *et al*., (2003) observed a decrease in N fixation following fertilizer addition most probably as a result of the increase in inorganic N.

Diazotrophs are also particularly sensitive to pesticides (Fox *et al*., 2007; Omar *et al*., 1992). Different chemicals and different environments can lead to different responses in the diazotrophic community (Lo, 2010) as certain chemicals have an inhibitory affect (e.g. diflubenzuron) and others have a stimulatory effect (e.g. methylpyrimitos).

Crop rotation has been shown to alter microbial activity and diversity (Lupwayi and Kennedy, 2007; Kelley *et al*., 2003). The diazotrophic community may be affected, particularly when legumes are used, as rotation can lead to carry over of diazotrophic endophytes (Roesch *et al*., 2008). These diazotrophs can then persist in the soil (Ferreira *et al*., 2000). The crop present in the rotation could have a strong effect on diversity and

activity of diazotrophs as different crops will release exudates with different quantities of carbon and nitrogen (Tan *et al*., 2003).

The objectives of this results chapter are:

1. To evaluate the overall effect of crop management on the nitrogen fixing community.
2. To evaluate the effect of previous crop on the nitrogen fixing community.
3. To correlate diazotrophic diversity and *nifH* copy number with key environmental variables including ammonium and nitrate concentration, total carbon, pH and concentrations of phosphorus.

The overall effect of crop management will be examined by looking at the response of the nitrogen fixing community over all three sample years. We hypothesise that the nitrogen fixing community may be enhanced by organic fertility management and crop protection due to the presence of lower amounts of nitrate and ammonium, and decreased chemical pesticides. Factors such as carbon and pH, associated with organic farming, could also positively influence nitrogen fixers.

Results from 2007 could be different from 2008 and 2009, as soils from 2007 are from a later phase of the farms 8 year rotation. In 2007, half of the soils come from the organic rotation (and were previously under beans) and the remaining half are from the conventional rotation (and were previously under winter barley). The presence of the beans could negatively affect the nitrogen fixing community as increased nitrogen remaining in to soil could decrease diversity and expression of *nifH*. For the same reasons increased nitrogen fixation could be associated with 2008 and 2009 as the rye catch crop which precedes the potato crop may take up any excess nitrogen from the soil and could therefore be associated with an increase in nitrogen fixation.

4.2. Results

4.2.1. Amplification of the *nifH* gene from RNA and DNA extracted from soils.

A single band of 360 bp, corresponding to the expected *nifH* gene product, was successfully amplified from RNA extracted from all 2007 and 2009 plots. However, the *nifH* gene could not be amplified from all 2008 plots. Acceptable copy numbers of the 16S rRNA gene were successfully amplified from these samples (discussed in results chapter 3) suggesting that the *nifH* gene was not being expressed in certain dates in 2008. Therefore, DNA was also extracted from all soils in 2008 and 2009, and the conventional rotation in 2007. The clamped products were electrophoresed through 35-55% DGGE gels. As each gel contained 16 lanes, PCR products were split according to sample date and pre-crops. All DGGE was successful (Figures 4.1-4.7).

As discussed in the methods section relative intensities were recorded for each band and recorded in spreadsheets. Data was initially subject to univariate analysis. To do this data is transformed using Shannon diversity index. An example of this is shown in Figure 4.8 and Table 4.1-4.2. For Shannon diversity results from other gels please see the appendix section E. Univariate analysis allows comparisons to be made between DGGE gels and therefore effects such as sample date, year and previous crop can be measured. However, in order to look at management effects and the effects of factors associated with management, such as carbon and pH in more detail, each gel (and therefore sample date) is analysed separately using multivariate analysis.

Figure 4.1. DGGE gels showing *nifH* amplified from RNA from soils in the organic rotation (potatoes/beans)

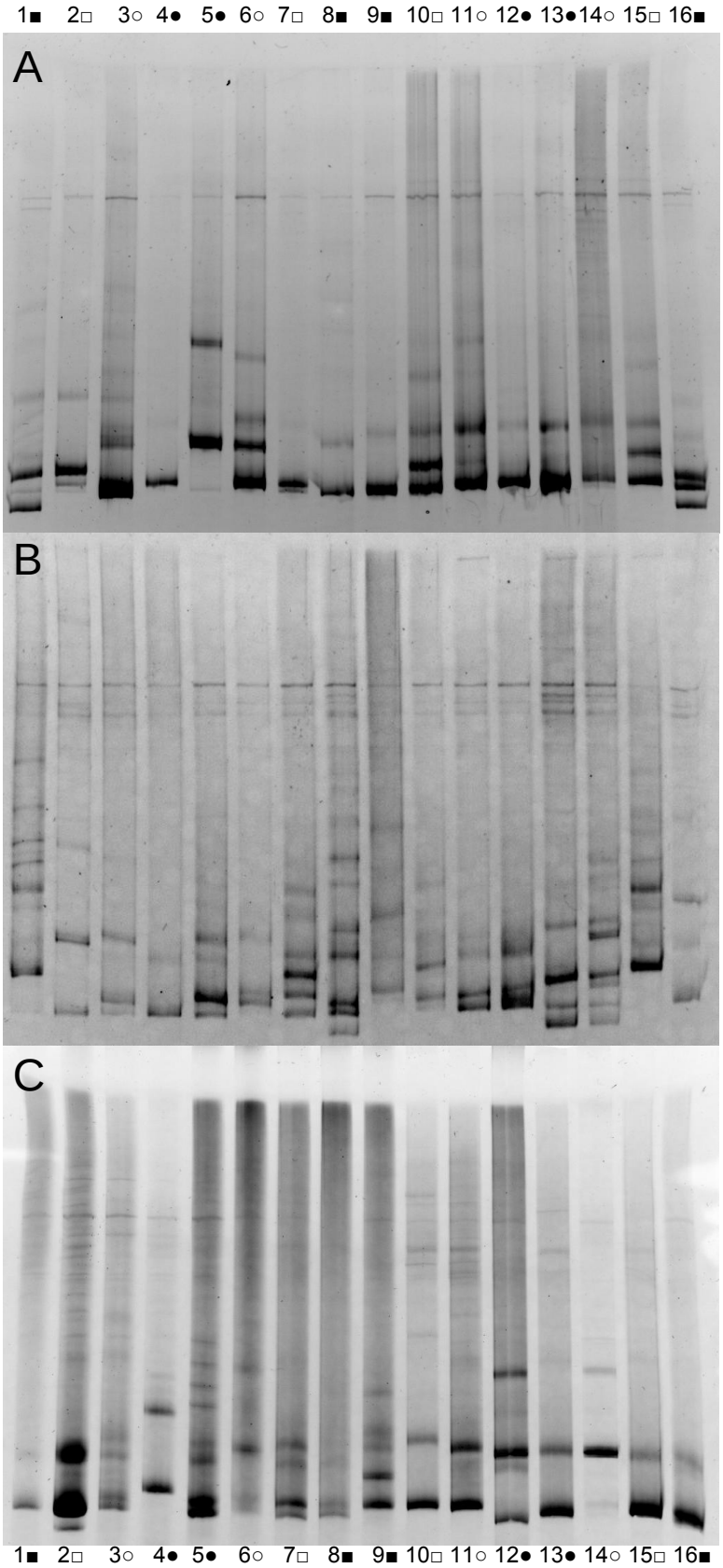


Figure 4.1. a) *nifH* DGGE image for March 2007 from the organic rotation, b) *nifH* DGGE image for June 2007 from the organic rotation and c) *nifH* DGGE image for September 2007 from the organic rotation. For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

Figure 4.2. DGGE gels showing *nifH* amplified from RNA from soils in the conventional rotation (potatoes/winter barley)

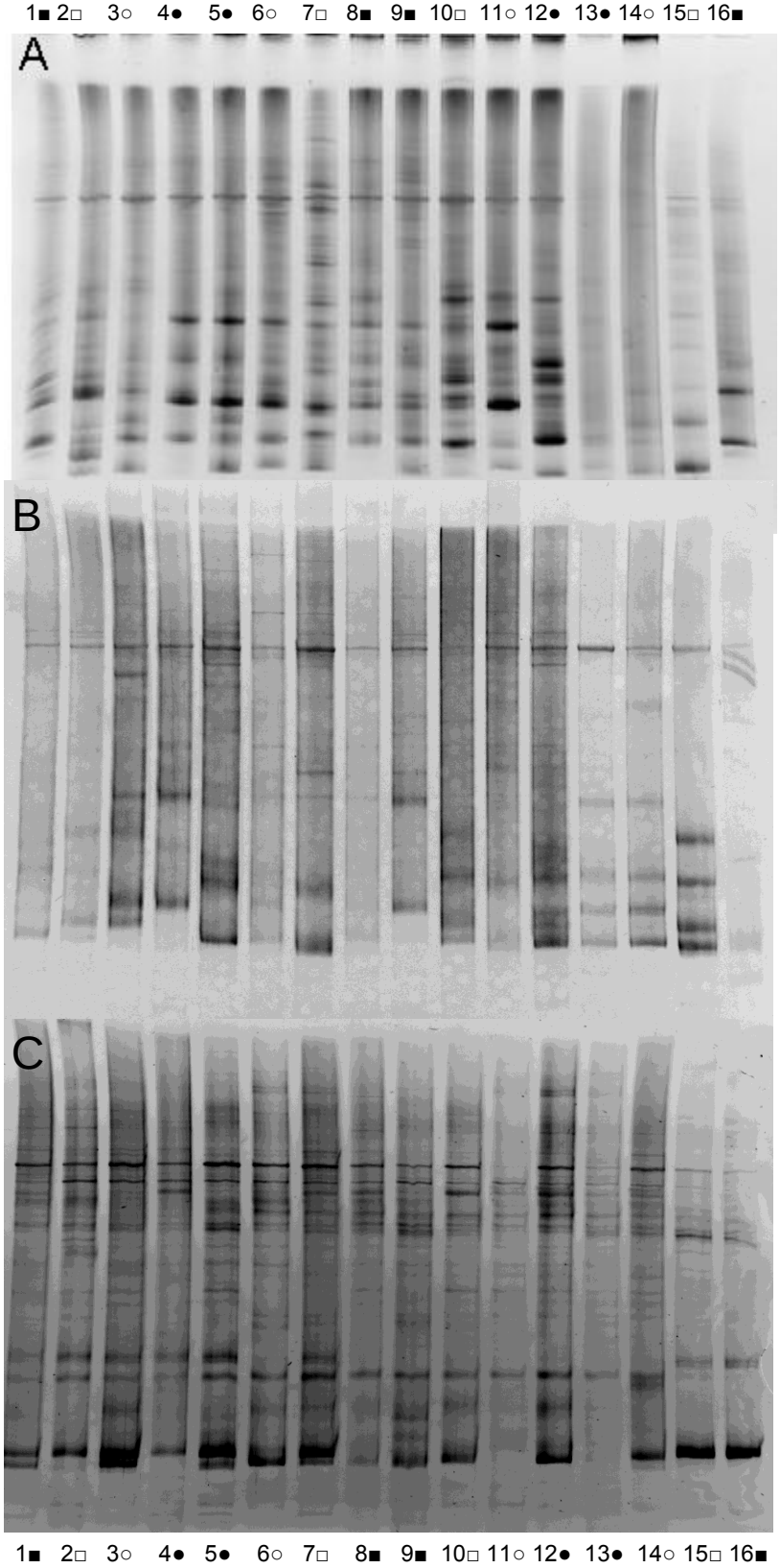


Figure 4.2. a) *nifH* DGGE image for March 2007 from the conventional rotation, b) *nifH* DGGE image for June 2007 soil from the conventional rotation, c) *nifH* DGGE image for September 207 soil from the conventional rotation. For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

Figure 4.3. DGGE gels showing *nifH* amplified from DNA from 2007 soils in the conventional rotation

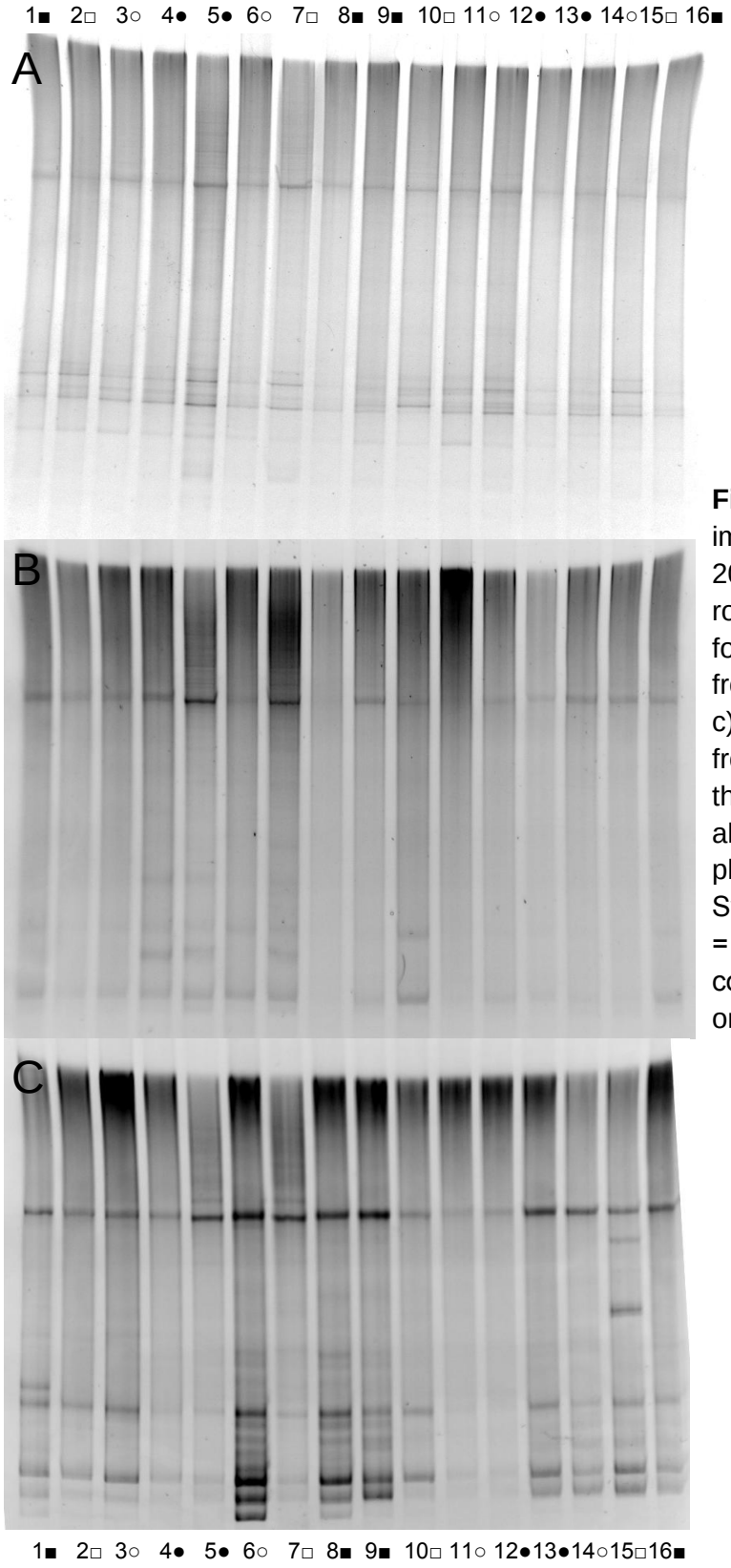


Figure 4.3. a) *nifH* DGGE image for DNA from March 2007 soil from the conventional rotation, b) *nifH* DGGE image for DNA from June 2007 soil from the conventional rotation, c) *nifH* DGGE image for DNA from September 2007 soil from the conventional rotation. For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

Figure 4.4. DGGE gels showing *nifH* amplified from RNA from 2008 soils

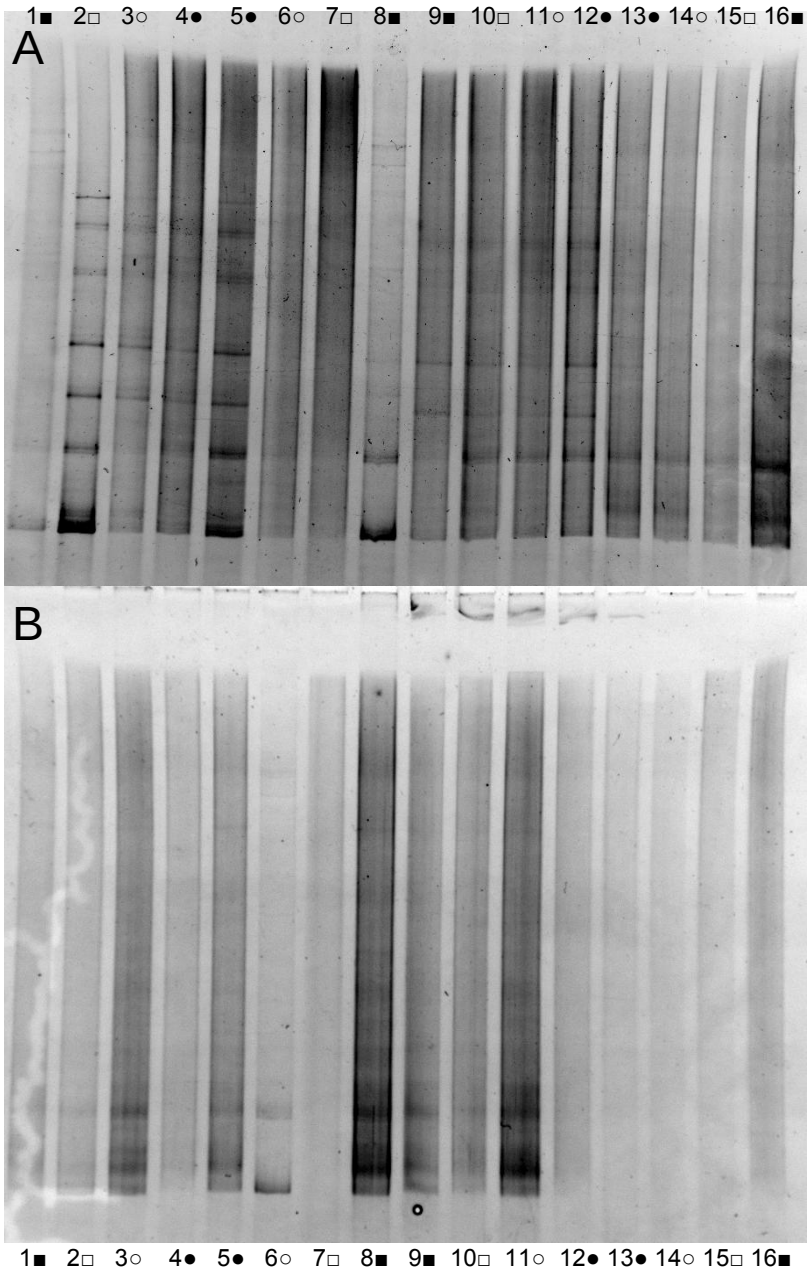


Figure 4.4. a) *nifH* DGGE image for RNA from March 2008 soil, b) *nifH* DGGE image for RNA from June 2007 soil. For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

Figure 4.5. DGGE gels showing *nifH* amplified from RNA from 2009 soils

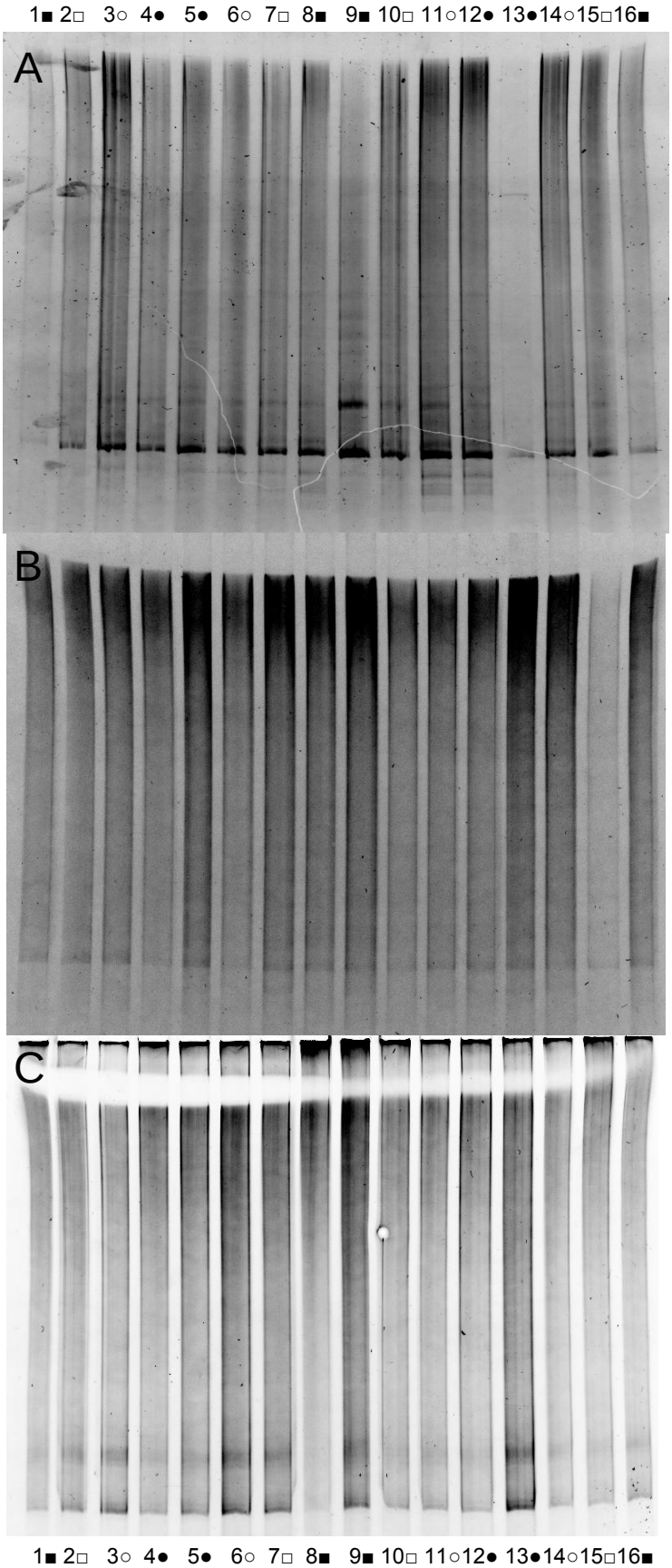


Figure 4.5. a) *nifH* DGGE image for RNA from March 2009 soil, b) *nifH* DGGE image for RNA from June 2009 soil, c) *nifH* DGGE image for RNA from September 2009 soil. For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

Figure 4.6. DGGE gels showing *nifH* amplified from DNA from 2008 soils

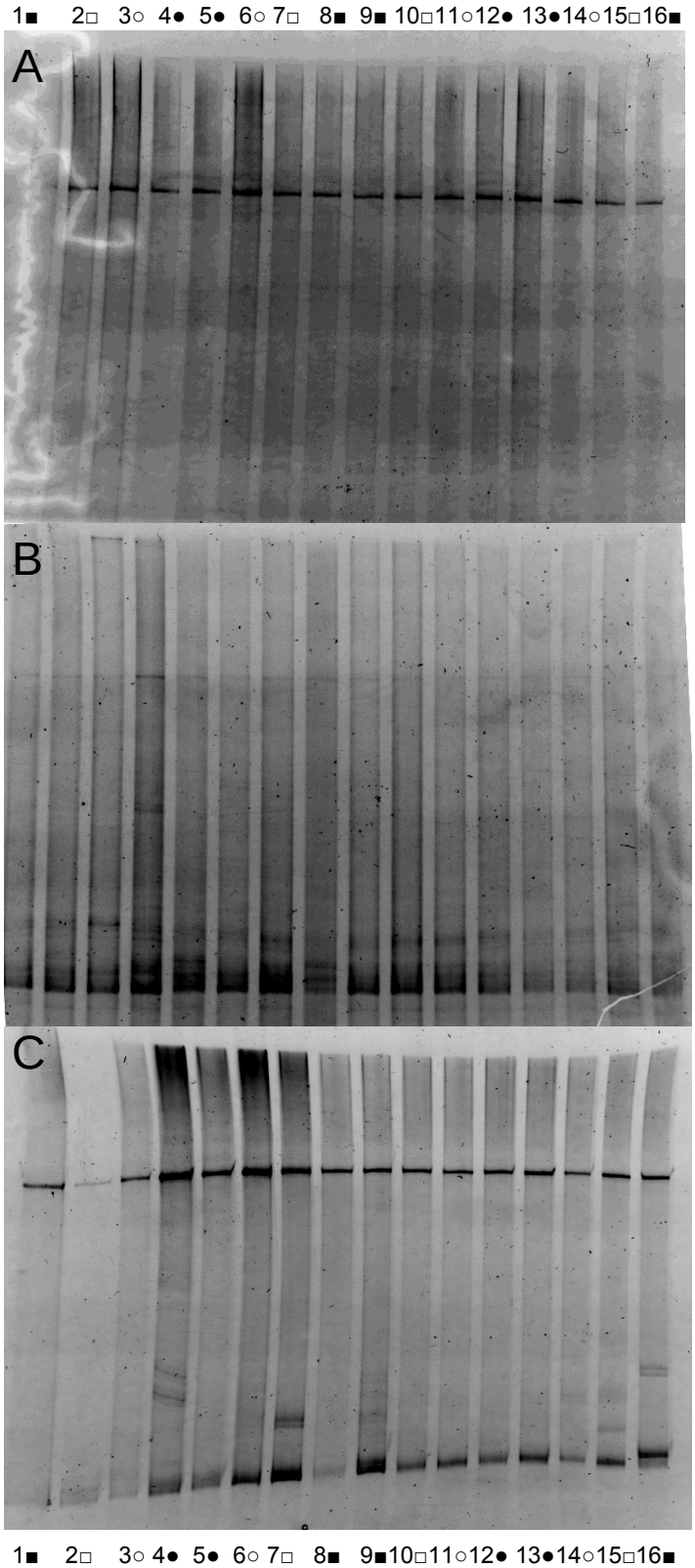


Figure 4.6. a) *nifH* DGGE image for DNA from March 2008 soil, b) *nifH* DGGE image for DNA from June 2008 soil, c) *nifH* DGGE image for DNA from September 2008 soil. For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

Figure 4.7. DGGE gels showing *nifH* amplified from DNA from 2009 soils

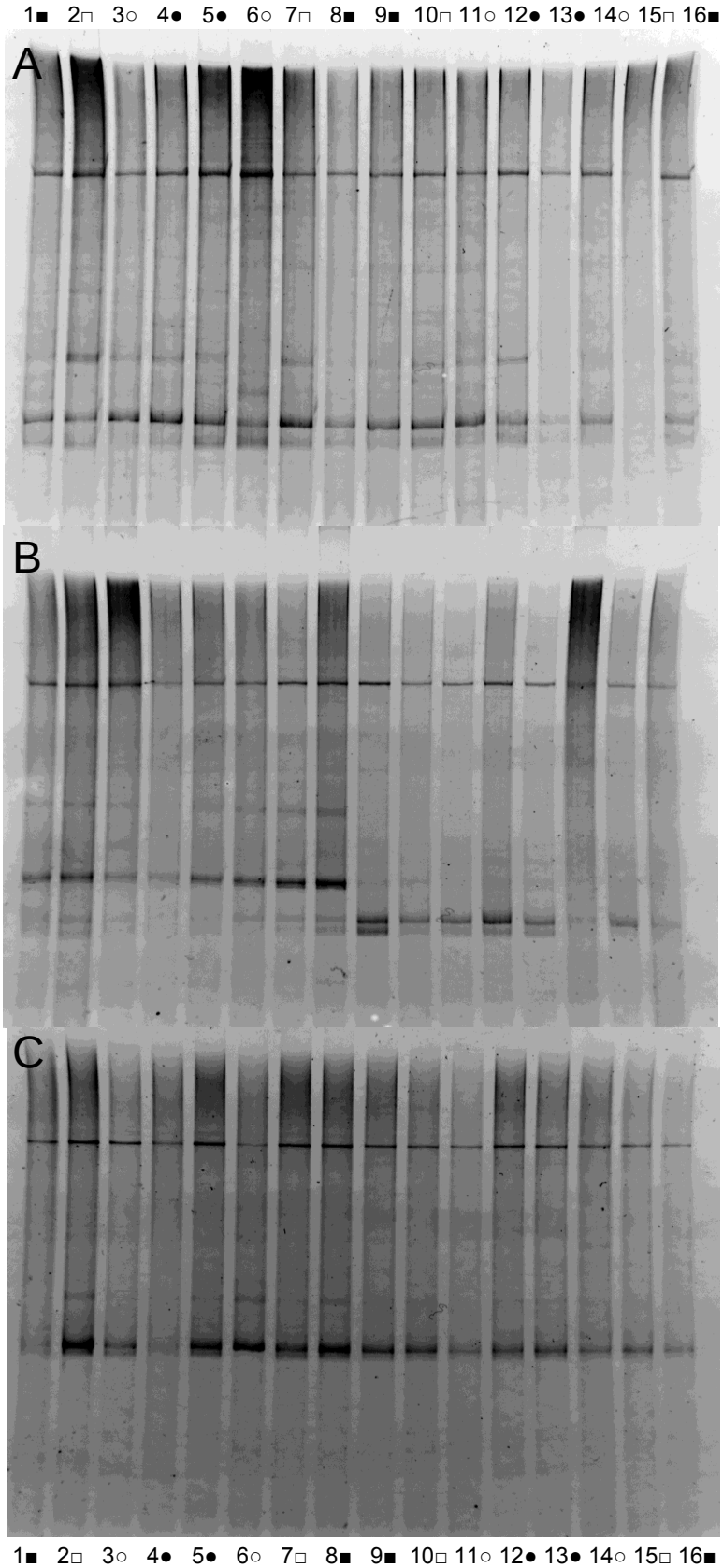
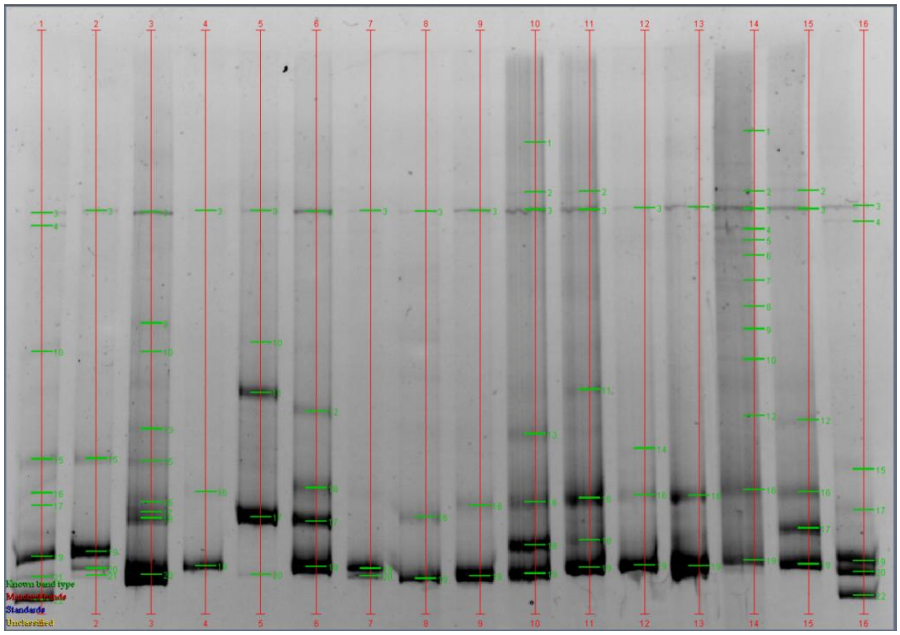


Figure 4.7. a) *nifH* DGGE image for DNA from March 2009 soil, b) *nifH* DGGE image for DNA from June 2009 soil, c) *nifH* DGGE image for DNA from September 2009 soil. For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

Figure 4.8. Gel image for DGGE gel showing the *nifH* gene in March 2007 soil from the organic rotation, showing band matchings.



Green lines show matched bands

Table 4.1 Relative quantities of each *nifH* DGGE band for March 2007 from the organic rotation.

Lane	band number																					
	b1	b2	b3	b4	b5	b6	b7	b8	b9	b10	b11	b12	b13	b14	b15	b16	b17	b18	b19	b20	b21	b22
1	0.00	0.00	0.04	0.04	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.13	0.06	0.06	0.00	0.27	0.00	0.09	0.25
2	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.52	0.15	0.08	0.00
3	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.05	0.05	0.00	0.00	0.05	0.00	0.07	0.10	0.07	0.11	0.00	0.45	0.00	0.00
4	0.00	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.16	0.00	0.00	0.70	0.00	0.00	0.00
5	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.30	0.00	0.00	0.00	0.00	0.00	0.51	0.00	0.00	0.07	0.00	0.00
6	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.17	0.24	0.00	0.42	0.00	0.00	0.00
7	0.00	0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.65	0.26	0.00	0.00
8	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.69	0.00	0.00	0.00
9	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.19	0.00	0.00	0.72	0.00	0.00	0.00
10	0.36	0.06	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.12	0.00	0.16	0.19	0.00	0.00	0.00
11	0.00	0.06	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.31	0.00	0.12	0.35	0.00	0.00	0.00
12	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.00	0.17	0.00	0.00	0.71	0.00	0.00	0.00
13	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.27	0.00	0.00	0.67	0.00	0.00	0.00
14	0.03	0.04	0.07	0.03	0.04	0.04	0.04	0.05	0.05	0.05	0.00	0.04	0.00	0.00	0.00	0.21	0.00	0.00	0.30	0.00	0.00	0.00
15	0.00	0.04	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.21	0.30	0.00	0.34	0.00	0.00	0.00
16	0.00	0.00	0.18	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.06	0.00	0.27	0.17	0.00	0.22

All band quantities in a lane are normalized.

Table 4.2. Table showing how Shannon diversity index (H') was calculated for *nifH* DGGE data from RNA sample taken from the organic rotation in March 2007.

	Band number										
	1	2	3	4	5	6	7	8	9	10	11
1	0.000	0.000	-0.120	-0.132	0.000	0.000	0.000	0.000	0.000	-0.159	0.000
2	0.000	0.000	-0.193	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
3	0.000	0.000	-0.153	0.000	0.000	0.000	0.000	0.000	-0.144	-0.143	0.000
4	0.000	0.000	-0.267	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	0.000	0.000	-0.150	0.000	0.000	0.000	0.000	0.000	0.000	-0.185	-0.362
6	0.000	0.000	-0.169	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
7	0.000	0.000	-0.208	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
8	0.000	0.000	-0.175	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
9	0.000	0.000	-0.216	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
10	-0.368	-0.170	-0.133	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
11	0.000	-0.162	-0.188	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-0.212
12	0.000	0.000	-0.161	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
13	0.000	0.000	-0.170	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
14	-0.112	-0.138	-0.188	-0.111	-0.137	-0.138	-0.139	-0.141	-0.144	-0.149	0.000
15	0.000	-0.134	-0.137	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
16	0.000	0.000	-0.310	-0.137	0.000	0.000	0.000	0.000	0.000	0.000	0.000

	12	13	14	15	16	17	18	19	20	21	22	Sum	H'
1	0.000	0.000	0.000	-0.273	-0.173	-0.173	0.000	-0.353	0.000	-0.210	-0.347	-1.940	1.940
2	0.000	0.000	0.000	-0.303	0.000	0.000	0.000	-0.340	-0.283	-0.208	0.000	-1.327	1.327
3	0.000	-0.156	0.000	-0.180	-0.240	-0.189	-0.243	0.000	-0.360	0.000	0.000	-1.806	1.806
4	0.000	0.000	0.000	0.000	-0.300	0.000	0.000	-0.249	0.000	0.000	0.000	-0.816	0.816
5	0.000	0.000	0.000	0.000	0.000	-0.345	0.000	0.000	-0.190	0.000	0.000	-1.232	1.232
6	-0.237	0.000	0.000	0.000	-0.304	-0.344	0.000	-0.365	0.000	0.000	0.000	-1.418	1.418
7	0.000	0.000	0.000	0.000	0.000	0.000	0.000	-0.278	-0.351	0.000	0.000	-0.837	0.837
8	0.000	0.000	0.000	0.000	-0.347	0.000	0.000	-0.259	0.000	0.000	0.000	-0.781	0.781
9	0.000	0.000	0.000	0.000	-0.314	0.000	0.000	-0.234	0.000	0.000	0.000	-0.764	0.764
10	0.000	-0.192	0.000	0.000	-0.250	0.000	-0.294	-0.317	0.000	0.000	0.000	-1.723	1.723
11	0.000	0.000	0.000	0.000	-0.364	0.000	-0.258	-0.367	0.000	0.000	0.000	-1.551	1.551
12	0.000	0.000	-0.189	0.000	-0.298	0.000	0.000	-0.245	0.000	0.000	0.000	-0.892	0.892
13	0.000	0.000	0.000	0.000	-0.353	0.000	0.000	-0.268	0.000	0.000	0.000	-0.791	0.791
14	-0.128	0.000	0.000	0.000	-0.326	0.000	0.000	-0.361	0.000	0.000	0.000	-2.213	2.213
15	-0.171	0.000	0.000	0.000	-0.327	-0.361	0.000	-0.367	0.000	0.000	0.000	-1.498	1.498
16	0.000	0.000	0.000	-0.162	0.000	-0.175	0.000	-0.352	-0.302	0.000	-0.332	-1.770	1.770

Values from Table 4.1 are converted into Shannon diversity index values using the formula discussed earlier. Values are added together and multiplied by -1 to obtain H'.

4.2.2. Measuring copy number of *nifH* using qPCR

For DNA, all samples were above the limit of detection apart from plots 1 of the March sample date in 2009. For RNA samples, all 2008 samples were below the limit of detection as were a number of 2009 samples (plots 3, 7, 11, 14, and 15 from sample date 1, and plots 3, 6, 7, 8, 11, 12, 14, and 16 from sample date 2). All qPCR is done in triplicate and an average of the 3 results is taken. A master sheet containing all of these triplicate results and averages can be found in the appendix (section E). Results needed to be log transformed before further analysis in order to create a normal distribution. Initially the data set was analysed whole using ANOVA.

4.2.3. Univariate analysis of DGGE and qPCR results from all sample years.

H' from *nifH* RNA, and DNA, DGGE gels from all years was compiled and ANOVA was carried out (Table 4.3 and 4.4 respectively). The terms year and previous crop had to be combined in order to make the test balanced and valid. ANOVA indicated that, for RNA data, year combined with previous crop, and sample date, were significant factors. Analysis of the DNA results showed that year is not a significant factor but year and sample date (which is also significant) form a significant interaction.

The number of gaps in the qPCR data set meant that the RNA data could not be analyzed whole and was immediately broken up into sample years. However, DNA data could be analyzed as a whole data set. Across the three years, analysis of the DNA data set showed that year and crop protection were significant factors (Table 4.5).

Looking at these initial findings three factors have been highlighted as significant and shall be further explored in this chapter; year, sample date and crop protection. With the exception of DNA qPCR results, year is a significant factor affecting *nifH* diversity and

Table 4.3 Results of Shannon diversity index and subsequent ANOVA analysis showing the effect of farm management, year and sample date when the whole RNA data set is analysed together.

	Shannon diversity index for *nifH* DGGE (RNA) band data (mean + SE)
year (+ previous crop) (Y+PC)	
2007 (after Barley)	2.202 ± 0.08
2007 (after Beans)	1.674 ± 0.07
2008 (after wheat)	0.759 ± 0.11
2009 (after wheat)	0.979 ± 0.10
sample date (SD)	
March	1.641 ± 0.07
June	1.134 ± 0.11
September	1.437 ± 0.13
Crop protection (CP)	
ORG	1.411 ± 0.09
CON	1.397 ± 0.09
Fertility management (FM)	
ORG	1.374 ± 0.09
CON	1.434 ± 0.09
ANOVA *P*-values	
Y+PC	**<0.001**
SD	**<0.001**
CP	0.827
FM	0.384
Y+PC*SD	**<0.001**
Y+PC*CP	0.724
Y+PC*FM	0.807
SD*FM	0.618
SD*CP	0.375
CP*FM	0.693
Y+PC*SD*FM	0.663
Y+PC*SD*CP	0.543
Y+PC*FM*CP	0.823
SD*FM*CP	0.688
Y+PC*SD*FM*CP	0.683

Table 4.4. Results of Shannon diversity index and subsequent ANOVA analysis showing the effect of farm management, year and sample date on the whole DNA data set is analysed together

	Shannon diversity index for *nifH* DGGE (DNA) band data (mean + SE)
year (+ previous crop) (Y)	
2007 (after Barley)	1.294 ± 0.10
2008 (after wheat)	1.243 ± 0.07
2009 (after wheat)	1.429 ± 0.07
sample date (SD)	
March	1.484 ± 0.08
June	1.297 ± 0.09
September	1.184 ± 0.07
Crop protection (CP)	
ORG	1.374 ± 0.06
CON	1.270 ± 0.07
Fertility management (FM)	
ORG	1.278 ± 0.06
CON	1.365 ± 0.07
ANOVA *P*-values	
Y	0.128
SD	**0.007**
CP	0.177
FM	0.258
Y*SD	**<0.001**
Y*CP	0.613
Y*FM	0.282
SD*FM	0.949
SD*CP	0.823
CP*FM	0.162
Y*SD*FM	0.998
Y*SD*CP	0.787
Y*FM*CP	0.509
SD*FM*CP	0.396
Y*SD*FM*CP	0.356

Table 4.5. Results of ANOVA analysis of all DNA qPCR copy numbers showing the effect of farm management, year and sample date.

	average copies of *nifH* per g of soil DNA all years(mean + SE)
year (+ previous crop) (Y)	
2007 (after Barley)	$5.688 \times 10^5 \pm 6.81 \times 10^4$
2008 (after wheat)	$3.885 \times 10^6 \pm 2.62 \times 10^5$
2009 (after wheat)	$2.995 \times 10^5 \pm 6.31 \times 10^4$
sample date (SD)	
March	$1.450 \times 10^6 \pm 2.66 \times 10^5$
June	$1.430 \times 10^6 \pm 2.51 \times 10^5$
September	$1.771 \times 10^6 \pm 3.44 \times 10^5$
Crop protection (CP)	
ORG	$1.746 \times 10^6 \pm 2.43 \times 10^5$
CON	$1.355 \times 10^6 \pm 2.27 \times 10^5$
Fertility management (FM)	
ORG	$1.645 \times 10^6 \pm 2.31 \times 10^5$
CON	$1.455 \times 10^6 \pm 2.41 \times 10^5$
ANOVA *P*-values	
Y	**<0.001**
SD	0.249
CP	**0.039**
FM	0.309
Y*SD	0.421
Y*CP	0.092
Y*FM	0.526
SD*FM	0.541
SD*CP	0.446
CP*FM	0.867
Y*SD*FM	0.373
Y*SD*CP	0.541
Y*FM*CP	0.946
SD*FM*CP	0.687
Y*SD*FM*CP	0.619

expression. It is possible that treatments are significant within years and, data was therefore split up by year and analysed separately. Soils in 2008 and 2009 have been subjected to identical management as both are from the organic rotation and have a previous crop of wheat. Soils in 2007 are different as half are from the conventional rotation (with a previous crop of barley) and half from the organic rotation (with a previous crop of beans). However, even when 2007 data was removed from the DGGE data set, and the 2008 and 2009 data sets analyzed together, year, sample date, and year x sample date were found to be significant for both RNA and DNA gels (Tables 4.6 and 4.7). Copy number of *nifH* in the DNA data set was also highest in 2008, and lowest in 2009, with 2007 results being between the two (Table 4.5). For this reason, for all data sets, results were separated so that each year could be analysed separately (Tables 4.8-4.11).

Sample date was also a significant factor throughout the analysis. The general trend was that *nifH* gene copy number, both in the RNA and the DNA data set, increased throughout the year. This was always significant in the RNA data set (Tables 4.10) but was only significant in 2007 for the DNA data set, although there were significant interactions with management and sample date in other years (Tables 4.11). Sample date always significantly affected diversity of the diazotrophic community with both RNA and DNA exhibiting dips in diversity in June. For the RNA data set diversity was highest in March (Table 4.3) and for the DNA data set diversity was highest in September (Table 4.4).

As sample date is always a significant factor, data from each sample date was separated and analysed individually. This allows management affects, which may only be significant immediately after fertilizer/pesticide addition, to be looked at in more detail and is also important when moving on to multivariate analysis as between gel variation makes it

Table 4.6. ANOVA analysis of Shannon diversity indices for the 2008 and 2009 RNA DGGE data set showing the effect of farm management, year and smaple date

	Shannon diversity index for *nifH* DGGE (RNA) band data (mean + SE)
year	
2008	0.759 ± 0.11
2009	0.979 ± 0.10
sample date	
March	1.484 ± 0.13
June	0.494 ± 0.12
September	0.630 ± 0.13
Crop protection	
ORG	0.846 ± 0.11
CON	0.893 ± 0.11
Fertility management	
ORG	0.863 ± 0.11
CON	0.876 ± 0.10
ANOVA *P*-values	
Y	**0.039**
SD	**<0.001**
CP	0.653
FM	0.899
Y*SD	**<0.001**
Y*CP	0.723
Y*FM	0.755
SD*FM	0.531
SD*CP	0.489
CP*FM	0.957
Y*SD*FM	0.59
Y*SD*CP	0.854
Y*FM*CP	0.761
SD*FM*CP	0.768
Y*SD*FM*CP	0.512

Table 4.7. ANOVA analysis of Shannon diversity indices for the 2008 and 2009 DNA DGGE data set showing the effect of farm management, year and sample date

	Shannon diversity index for *nifH* DGGE (DNA) band data (mean + SE)
year	
2008	1.243 ± 0.07
2009	1.429 ± 0.07
sample date	
March	1.322 ± 0.10
June	1.537 ± 0.07
September	1.149 ± 0.07
Crop protection	
ORG	1.369 ± 0.07
CON	1.302 ± 0.07
Fertility management	
ORG	0.863 ± 0.07
CON	0.876 ± 0.07
ANOVA *P*-values	
Y	**0.028**
SD	**0.001**
CP	0.422
FM	0.995
Y*SD	**<0.001**
Y*CP	0.421
Y*FM	0.921
SD*FM	0.979
SD*CP	0.572
CP*FM	0.579
Y*SD*FM	1.000
Y*SD*CP	0.677
Y*FM*CP	0.764
SD*FM*CP	0.106
Y*SD*FM*CP	0.443

Table 4.8. ANOVA analysis of each individual year of the RNA DGGE data set showing the effect of previous crop, sample date and farm management

	Shannon diversity index for *nifH* DGGE (RNA) band data (mean + SE)		
year	**2007**	**2008**	**2009**
previous crop (PC)			
Barley	2.202 ± 0.08		
Beans	1.674 ± 0.07		
sample date (SD)			
March	1.797 ± 0.12	1.507 ± 0.77	1.461 ± 0.12
June	1.774 ± 0.07	0.772 ± 0.20	0.216 ± 0.10
September	2.245 ± 0.10	0.000 ± 0.00	1.260 ± 0.12
Crop protection (CP)			
ORG	1.977 ± 0.09	0.717 ± 0.15	0.956 ± 0.16
CON	1.900 ± 0.09	0.801 ± 0.18	0.984 ± 0.14
Fertility management (FM)			
ORG	1.886 ± 0.08	0.769 ± 0.17	0.956 ± 0.16
CON	1.991 ± 0.08	0.750 ± 0.16	1.002 ± 0.14
ANOVA *P*-values			
PC	**<0.001**		
SD	**<0.001**	**<0.001**	**<0.001**
CP	0.381	0.587	0.944
FM	0.231	0.900	0.746
PC*SD	**<0.001**		
PC*CP	0.520		
PC*FM	0.486		
SD*FM	0.384	0.851	0.348
SD*CP	0.349	0.898	0.448
CP*FM	0.582	0.209	0.854
PC*SD*FM	0.647		
PC*SD*CP	0.150		
PC*FM*CP	0.358		
SD*FM*CP	0.510	0.447	0.950
PC*SD*FM*CP	0.439		

Table 4.9. ANOVA analysis of each individual year of the DNA DGGE data set showing the effect of sample date and farm management

	Shannon diversity index for *nifH* DGGE (DNA) band data (mean + SE)		
year	**2007**	**2008**	**2009**
sample date (SD)			
March	1.810 ± 0.11	0.933 ± 0.08	1.710 ± 0.13
June	0.918 ± 0.24	1.733 ± 0.05	1.342 ± 0.12
September	1.254 ± 0.20	1.062 ± 0.10	1.236 ± 0.09
Crop protection (CP)			
ORG	1.253 ± 0.15	1.310 ± 0.09	1.429 ± 0.10
CON	1.335 ± 0.12	1.176 ± 0.10	1.429 ± 0.10
Fertility management (FM)			
ORG	1.327 ± 0.13	1.238 ± 0.10	1.433 ± 0.10
CON	1.261 ± 0.14	1.247 ± 0.09	1.425 ± 0.10
ANOVA *P*-values			
SD	**<0.001**	**<0.001**	**0.020**
CP	0.620	0.159	0.999
FM	0.689	0.926	0.855
SD*FM	0.728	0.988	0.990
SD*CP	0.547	0.244	0.979
CP*FM	0.289	0.821	0.607
SD*FM*CP	0.327	0.512	0.152

Table 4.10. ANOVA analysis of each individual year RNA *nifH* qPCR results showing the effect of previous crop, sample date and farm management

	average copies of *nifH* per g of soil RNA 2007 (mean + SE)	
year	**2007**	**2009**
previous crop (PC)		
barley	$9.286 \times 10^6 \pm 2.85 \times 10^6$	
beans	$1.002 \times 10^5 \pm 3.48 \times 10^4$	
sample date (SD)		
March	$9.161 \times 10^5 \pm 4.98 \times 10^5$	$4.312 \times 10^4 \pm 1.17 \times 10^4$
June	$6.765 \times 10^5 \pm 2.32 \times 10^5$	$6.508 \times 10^3 \pm 5.25 \times 10^2$
September	$1.249 \times 10^7 \pm 4.16 \times 10^6$	$6.283 \times 10^4 \pm 5.95 \times 10^3$
Crop protection (CP)		
ORG	$6.280 \times 10^6 \pm 1.74 \times 10^6$	$3.212 \times 10^4 \pm 6.69 \times 10^3$
CON	$3.106 \times 10^6 \pm 1.19 \times 10^6$	$4.285 \times 10^4 \pm 8.23 \times 10^3$
Fertility management (FM)		
ORG	$5.013 \times 10^6 \pm 2.59 \times 10^6$	$4.615 \times 10^4 \pm 8.079 \times 10^3$
CON	$4.373 \times 10^6 \pm 1.53 \times 10^6$	$2.882 \times 10^4 \pm 6.75 \times 10^3$
ANOVA *P*-values		
PC	**<0.001**	
SD	**<0.001**	**<0.001**
CP	0.174	0.215
FM	0.783	**0.049**
PC*SD	**<0.001**	
PC*FM	0.794	
PC*CP	0.171	
SD*FM	0.644	0.174
SD*CP	*0.068*	*0.066*
CP*FM	0.186	0.744
PC*SD*FM	0.629	
PC*SD*CP	*0.073*	
PC*FM*CP	0.174	
SD*FM*CP	0.278	0.972
PC*SD*FM*CP	0.286	

Table 4.11. ANOVA analysis of each individual year DNA *nifH* qPCR results showing the effect os sample date and farm management

	average copies of *nifH* per g of soil DNA 2007 (mean + SE)		
year	**2007**	**2008**	**2009**
sample date (SD)			
March	$3.311 \times 10^5 \pm 1.08 \times 10^5$	$3.719 \times 10^6 \pm 3.16 \times 10^5$	$2.987 \times 10^5 \pm 1.82 \times 10^5$
June	$5.654 \times 10^5 \pm 1.20 \times 10^5$	$3.547 \times 10^6 \pm 3.45 \times 10^5$	$1.771 \times 10^5 \pm 6.12 \times 10^4$
September	$8.098 \times 10^5 \pm 7.15 \times 10^6$	$4.389 \times 10^6 \pm 6.31 \times 10^5$	$1.152 \times 10^5 \pm 2.22 \times 10^4$
Crop protection (CP)			
ORG	$5.754 \times 10^5 \pm 9.46 \times 10^4$	$4.367 \times 10^6 \pm 2.73 \times 10^5$	$2.958 \times 10^5 \pm 1.22 \times 10^5$
CON	$5.621 \times 10^5 \pm 1.00 \times 10^5$	$3.404 \times 10^6 \pm 4.30 \times 10^5$	$9.813 \times 10^4 \pm 1.84 \times 10^4$
Fertility management (FM)			
ORG	$7.617 \times 10^5 \pm 1.08 \times 10^5$	$3.739 \times 10^6 \pm 4.25 \times 10^5$	$1.427 \times 10^5 \pm 4.24 \times 10^4$
CON	$3.758 \times 10^5 \pm 6.45 \times 10^4$	$4.031 \times 10^6 \pm 3.12 \times 10^5$	$2.513 \times 10^5 \pm 1.18 \times 10^5$
ANOVA *P*-values			
SD	**0.012**	0.398	0.628
CP	0.915	*0.077*	**0.008**
FM	**0.003**	0.583	**0.019**
SD*FM	0.927	0.405	**<0.001**
SD*CP	0.497	0.471	**0.040**
CP*FM	0.715	0.924	**0.039**
SD*FM*CP	0.984	0.593	0.189

inappropriate to compare between DGGE gels (Please see Results Chapter 1 for more information on this). The significant interactions of sample date and management also suggest that significant management effects could be overlooked by only analysing data sets as whole years.

4.2.4. DGGE and qPCR analysis of 2007 data – the effect of previous crop

When the effect of changes in management on environmental variables was examined in Results Chapter 1, it was found that previous crop was often a significant factor. In order to explore the effect of previous crop on the diversity and expression of *nifH*, within the NFSC soils, the 2007 RNA data set must be used.

Analysis of the *nifH* DGGE Shannon's diversity index values for the whole year (Table 4.3) indicated that sample date and crop rotation (pre-crop) significantly affected the diversity of the nitrogen fixing community (Sample date $P < 0.001$, pre-crop $P < 0.001$ and sample date × pre-crop $P < 0.001$), with the soil following the barley crop having increased diazotrophic diversity. When split by sample date (Table 4.12) soils with a previous crop of barley showed significantly higher diazotrophic diversity than soils with a previous crop of beans in March and September (average H' was 2.203 for the barley pre-crop and 1.674 for the beans pre-crop).

Analysis of the qPCR data set showed that for the whole year and at each sample date increased copy number was seen in the conventional rotation (barley pre-crop) compared to the organic rotation (beans pre-crop) (Table 4.10 and 4.13).

Table 4.12. ANOVA analysis of Shannon diversity indices for each years RNA DGGE gels at each different sample date.

year	Shannon diversity index for *nifH* DGGE band data							
	2007			2008		2009		
	March	June	Sept	March	June	March	June	Sept
Pre-crop (PC)								
Barley	2.259 ± 0.11	1.675 ± 0.12	2.675 ± 0.08					
Beans	1.335 ± 0.08	1.873 ± 0.12	1.815 ± 0.11					
Crop protection (CP)								
ORG	1.752 ± 0.08	1.826 ± 0.17	2.354 ± 0.14	1.468 ± 0.11	0.684 ± 0.22	1.494 ± 0.17	0.087 ± 0.09	1.342 ± 0.17
CON	1.842 ± 0.11	1.722 ± 0.17	2.136 ± 0.15	1.546 ± 0.14	0.859 ± 0.34	1.428 ± 0.17	0.346 ± 0.19	1.178 ± 0.19
Fertility management (FM)								
ORG	1.661 ± 0.10	1.781 ± 0.18	2.215 ± 0.16	1.574 ± 0.09	0.733 ± 0.31	1.404 ± 0.22	0.087 ± 0.09	1.379 ± 0.15
CON	1.933 ± 0.09	1.767 ± 0.15	2.274 ± 0.14	1.439 ± 0.15	0.810 ± 0.27	1.519 ± 0.10	0.346 ± 0.19	1.142 ± 0.20
ANOVA *P-values*								
PC	**<0.001**	0.154	**<0.001**					
CP	0.597	0.450	0.153	0.456	0.860	0.805	0.232	0.547
FM	0.121	0.916	0.695	0.663	0.690	0.665	0.232	0.389
CP*FM	0.381	0.537	0.546	0.316	0.502	0.929	0.682	0.953
FM*PC	0.381	0.731	0.599					
CP*PC	0.558	**0.037**	0.859					
FM*CP*PC	0.170	0.951	0.943					

Table 4.13. ANOVA analysis of all years RNA qPCR results at each different sample date

	Average copies of *nifH* per g of soil					
year	**2007**			**2009**		
Mean±SE	**March**	**June**	**Sept**	**March**	**June**	**Sept**
Pre-crop (PC)						
Barley	$1.647 \times 10^6 \pm 9.71 \times 10^5$	$1.315 \times 10^6 \pm 4.10 \times 10^5$	$2.490 \times 10^7 \pm 7.15 \times 10^6$			
Beans	$1.851 \times 10^5 \pm 1.01 \times 10^5$	$3.765 \times 10^4 \pm 8.02 \times 10^3$	$7.794 \times 10^4 \pm 1.972 \times 10^4$			
Crop protection (CP)						
ORG	$4.090 \times 10^5 \pm 1.29 \times 10^5$	$5.024 \times 10^5 \pm 2.24 \times 10^5$	$1.793 \times 10^7 \pm 7.56 \times 10^6$	$2.381 \times 10^4 \pm 1.01 \times 10^4$	$4.527 \times 10^3 \pm 5.48 \times 10^2$	$6.802 \times 10^4 \pm 9.72 \times 10^3$
CON	$1.423 \times 10^6 \pm 9.862 \times 10^5$	$8.506 \times 10^5 \pm 4.10 \times 10^5$	$7.044 \times 10^6 \pm 3.24 \times 10^6$	$6.242 \times 10^4 \pm 1.81 \times 10^4$	$8.488 \times 10^3 \pm 8.25 \times 10^2$	$5.764 \times 10^4 \pm 7.041 \times 10^3$
Fertility management (FM)						
ORG	$3.858 \times 10^5 \pm 1.22 \times 10^5$	$3.100 \times 10^5 \pm 1.24 \times 10^5$	$1.434 \times 10^7 \pm 7.37 \times 10^6$	$3.160 \times 10^4 \pm 1.79 \times 10^4$	$4.140 \times 10^3 \pm 8.13 \times 10^2$	$3.348 \times 10^4 \pm 7.53 \times 10^3$
CON	$1.446 \times 10^6 \pm 9.85 \times 10^5$	$1.043 \times 10^6 \pm 4.35 \times 10^5$	$1.063 \times 10^7 \pm 4.11 \times 10^6$	$2.303 \times 10^4 \pm 1.58 \times 10^4$	$4.736 \times 10^3 \pm 7.49 \times 10^2$	$5.870 \times 10^4 \pm 9.50 \times 10^3$
ANOVA *P-values*						
PC	**0.012**	**0.006**	**<0.001**			
CP	0.426	0.877	**0.014**	0.675	0.790	0.814
FM	0.164	**0.032**	0.448	*0.078*	0.409	**0.050**
CP*FM	0.540	0.735	0.719	0.972	0.688	0.931
FM*PC	0.194	0.341	0.536			
CP*PC	0.103	0.157	0.267			
FM*CP*PC	*0.096*	0.737	0.270			

4.2.5. DGGE and qPCR analysis of all sample years – the effect of fertility and health management.

As mentioned previously, when sample years are combined and analysed together, treatment only significantly affects *nifH* copy number in the DNA data set (*P*=0.039) with organic crop protection leading to increased copy number (Table 4.5). Organic crop protection also leads to increased diazotrophic diversity (in both RNA and DNA data sets), although this is not significant. In order to explore the effect of treatment further the data sets are initially split up into sample years.

Diversity as determined by DGGE community profile analyses remained unaffected by fertility and health management (Tables 4.8-4.9). In contrast, quantitative data was significantly affected by both fertility and health management. Organic fertility management led to increased *nifH* copy number in 2009 (RNA data set, Table 4.10) and 2007 (DNA data set, Table 4.11), conventional fertility management lead to increased *nifH* copy number in 2009 (DNA data set, Table 4.11) and organic crop protection lead to increased *nifH* copy number in 2008 (DNA data set, Table 4.11) and 2009 (DNA data set, Table 4.11).

As discussed above it is also necessary to split the results up by sample date (Tables 4.12-4.15). This leads to very mixed observations, with significant effects being very point specific, making it very difficult to draw any conclusions from them. Treatment only significantly affects DGGE data in March of 2008 (DNA data set, Table 4.14) when conventional crop protection leads to increased diazotrophic diversity. Treatment significantly affects qPCR data at every date with very varied results. These results are summarized in Table 4.16.

Table 4.14. ANOVA analysis of Shannon diversity indices for each years DNA DGGE gels at each different sample date.

	Shannon diversity index for *nifH* DGGE band data								
	2007			**2008**			**2009**		
	March	**June**	**Sept**	**March**	**June**	**Sept**	**March**	**June**	**Sept**
Crop protection (CP)									
ORG	1.886 ± 0.12	0.764 ± 0.24	1.110 ± 0.25	0.777 ± 0.12	1.814 ± 0.04	1.025 ± 0.12	1.728 ± 0.12	1.326 ± 0.12	1.232 ± 0.08
CON	1.734 ± 0.11	0.871 ± 0.25	1.398 ± 0.14	1.090 ± 0.06	1.651 ± 0.09	1.099 ± 0.17	1.691 ± 0.16	1.357 ± 0.10	1.239 ± 0.10
Fertility management (FM)									
ORG	1.782 ± 0.14	0.941 ± 0.24	1.258 ± 0.20	0.919 ± 0.12	1.734 ± 0.10	1.063 ± 0.17	1.700 ± 0.11	1.352 ± 0.11	1.247 ± 0.12
CON	1.839 ± 0.09	0.695 ± 0.24	1.250 ± 0.21	0.948 ± 0.10	1.732 ± 0.04	1.062 ± 0.12	1.720 ± 0.16	1.331 ± 0.11	1.224 ± 0.06
ANOVA *P*-values									
CP	0.382	0.762	0.362	**0.050**	0.117	0.741	0.899	0.892	0.975
FM	0.740	0.487	0.981	0.841	0.983	0.996	0.945	0.927	0.911
CP*FM	0.408	0.177	0.724	0.888	0.268	0.494	0.243	0.204	0.419

Table 4.15. ANOVA analysis of DNA qPCR results for all years at each different sample date.

	Average copies of *nifH* per g of soil								
	2007			2008			2009		
Mean±SE	**March**	**June**	**Sept**	**March**	**June**	**Sept**	**March**	**June**	**Sept**
Crop protection (CP)									
ORG	$2.421 \times 10^5 \pm 7.43 \times 10^4$	$5.849 \times 10^5 \pm 1.66 \times 10^5$	$8.991 \times 10^5 \pm 1.56 \times 10^5$	$4.626 \times 10^6 \pm 3.34 \times 10^5$	$3.975 \times 10^6 \pm 4.58 \times 10^5$	$4.498 \times 10^6 \pm 6.14 \times 10^5$	$5.352 \times 10^5 \pm 3.44 \times 10^5$	$2.584 \times 10^5 \pm 1.15 \times 10^5$	$9.391 \times 10^4 \pm 1.78 \times 10^4$
CON	$4.200 \times 10^5 \pm 2.05 \times 10^5$	$5.458 \times 10^5 \pm 1.83 \times 10^5$	$7.206 \times 10^5 \pm 1.29 \times 10^5$	$2.813 \times 10^6 \pm 2.88 \times 10^5$	$3.118 \times 10^6 \pm 4.96 \times 10^5$	$4.280 \times 10^6 \pm 1.15 \times 10^6$	$6.360 \times 10^4 \pm 1.79 \times 10^4$	$9.575 \times 10^4 \pm 3.13 \times 10^4$	$1.365 \times 10^5 \pm 4.08 \times 10^4$
Fertility management (FM)									
ORG	$2.458 \times 10^5 \pm 1.97 \times 10^5$	$3.830 \times 10^5 \pm 1.94 \times 10^5$	$5.138 \times 10^5 \pm 1.32 \times 10^5$	$2.068 \times 10^6 \pm 4.85 \times 10^5$	$1.966 \times 10^6 \pm 5.02 \times 10^5$	$2.013 \times 10^6 \pm 6.85 \times 10^5$	$3.695 \times 10^4 \pm 2.18 \times 10^4$	$1.096 \times 10^5 \pm 1.16 \times 10^5$	$6.825 \times 10^4 \pm 4.21 \times 10^4$
CON	$1.705 \times 10^5 \pm 6.04 \times 10^4$	$3.647 \times 10^5 \pm 1.11 \times 10^5$	$5.921 \times 10^5 \pm 1.11 \times 10^5$	$3.302 \times 10^6 \pm 3.80 \times 10^5$	$3.163 \times 10^6 \pm 4.64 \times 10^5$	$4.752 \times 10^6 \pm 1.09 \times 10^6$	$5.249 \times 10^5 \pm 3.46 \times 10^5$	$1.350 \times 10^5 \pm 4.51 \times 10^5$	$9.389 \times 10^5 \pm 1.46 \times 10^4$
ANOVA *P-values*									
CP	0.413	0.647	0.374	**0.004**	0.691	0.367	**0.034**	0.242	0.471
FM	**0.031**	0.220	*0.084*	**<0.001**	0.152	0.406	**<0.001**	0.160	0.136
CP*FM	0.254	0.863	0.392	0.893	0.164	0.664	0.309	**0.010**	0.734

Table 4.16. Summary of all occasions where treatment significantly affects *nifH* copy number

	RNA		DNA	
year	sample date	treatment with highest copy no.	sample date	treatment with highest copy no.
2007	June	con FM	March	org FM
	September	org CP		
2008	N/A		March	con FM
				org CP
2009	March	con FM	September	con FM
		con CP		

4.2.6. Multivariate analysis of DGGE results to further explore the effect of management.

The DGGE results discussed in the above section were found using univariate analysis. This analysis found treatments to be significant if the number of bands present in lanes of the gels changed notably. However, relative intensity scores from DGGE gels can also be analysed using multivariate statistics. These tests look for differences in band patterns and intensities rather than the presence or absence of a particular band. The data was first subjected to indirect analysis to visualise variance between the profiles of each gel. This was done using PCA or DCA depending on the length of the DCA axis (axis >3.5 = DCA, axis <3.5 = PCA). PCA and DCA were carried out using the vegan library in the R package. Scores for each axis were taken from R and plots were generated using Minitab (Figs 4.9 and appendix section E). Scores were also subject to ANOVA to see if treatment had any effect on variance among each axes.

Direct analysis was also carried out so that relative intensity data could be compared with environmental variables. As above the test used was determined by the length of the DCA axis (axis>3.5 = CCA, axis <3.5 RDA). CCA, RDA and Monte Carlo permutation testing were carried out using CANOCO and the results are presented in Figs 4.10-4.15.

A summary of the results of this analysis is provided in Fig. 4.16. In general, indirect analysis only revealed a significant impact of treatment in the RNA data set with fertility management affecting diversity in 2007 and 2009 and crop protection affecting diversity in 2007 only. Direct analysis did not report fertility management as a significant factor at any of the sample dates. However, crop protection significantly affected diversity in 2 of the 2008 sample dates (DNA only) and 1 of the 2007 sample dates (RNA only). Environmental variables total C, total N, available Fe, available P, ammonium concentration, nitrate concentration, soil basal respiration and pH, all affected

Figure 4.9. PCA showing variation between *nifH* DGGE lanes for pot/beans in 2007

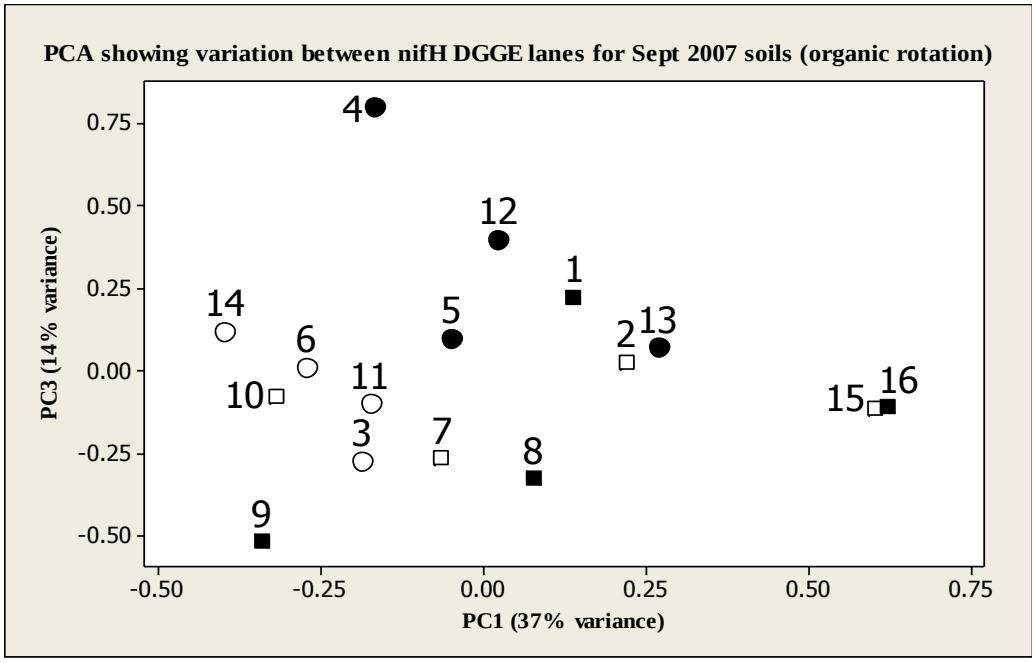


Figure 4.9. Numbers relate to plot numbers. Treatments are represented by the following symbols; orgFM orgCP (●), conFM conCP (□), orgFM conCP (■) and conFM orgCP (○). The Y axis shows significant variation due to health management (*P* = 0.044). *P* values are according to ANOVA.

Figure 4.10. RDA of *nifH* 2007 RNA DGGE gels showing variation between treatments

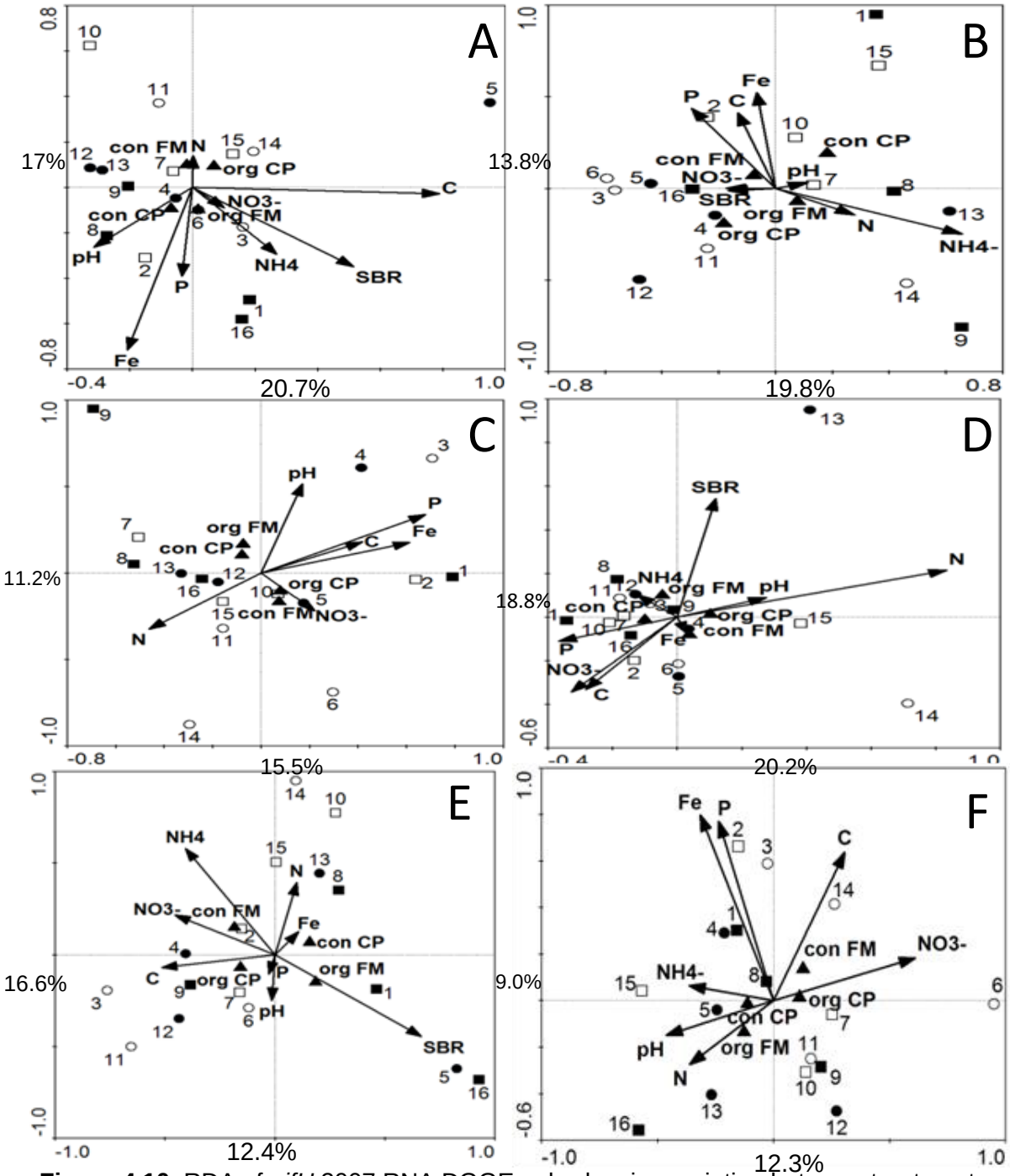


Figure 4.10. RDA of *nifH* 2007 RNA DGGE gels showing variation between treatments: orgFM orgCP (●), conFM conCP (□), orgFM conCP (■) and conFM orgCP (○). Arrows represent environmental variables. N = total nitrogen, C = total organic carbon, SBR = Soil Basal Respiration, NH4 = available ammonium, NO3- = available nitrate, P = phosphorus and Fe = iron. Triangles represent centroids for management treatments. A = March soil after beans. C (P = 0.024) and Fe (P = 0.020) are significant factors. B = June after beans. CP (P = 0.006) and NH4 (P = 0.008) are significant factors. C = September after beans. P (P = 0.024) is a significant factor. D = March after barley. N (P = 0.002) is a significant factor. E = June after barley. Fe (P = 0.050) and SBR (P = 0.008) are a significant factors. F = September after barley. P values are according to Monte Carlo permutation testing.

Figure 4.11. RDA of *nifH* DNA (2007) DGGE gels showing variation between treatments

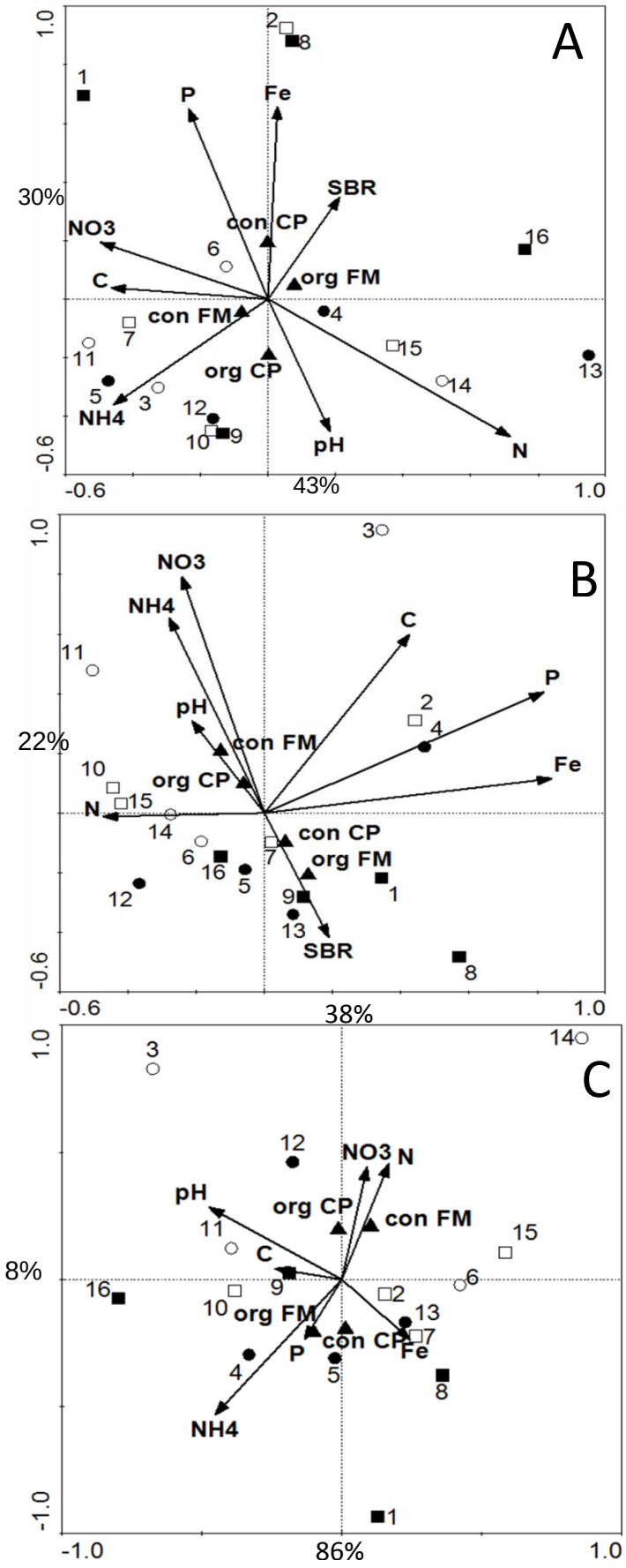


Figure 4.11. RDA of *nifH* DNA (2007) DGGE gels showing variation between treatments: orgFM orgCP (●), conFM conCP (□), orgFM conCP (■) and conFM orgCP (○). Arrows represent environmental variables. N = total nitrogen, C = total organic carbon, SBR = Soil Basal Respiration, NH4 = available ammonium, NO3- = available nitrate, P = phosphorus and Fe = iron. Triangles represent centroids for management treatments. A = March soil after barley. N (*P* = 0.004) is a significant factor. B = June after barley. P (*P* = 0.002) and NO3 (*P* = 0.014) are significant factors. C = September after barley. *P* values are according to Monte Carlo permutation testing.

Figure 4.12. RDA and CCA analysis of *nifH* RNA DGGE gels for 2008 soils

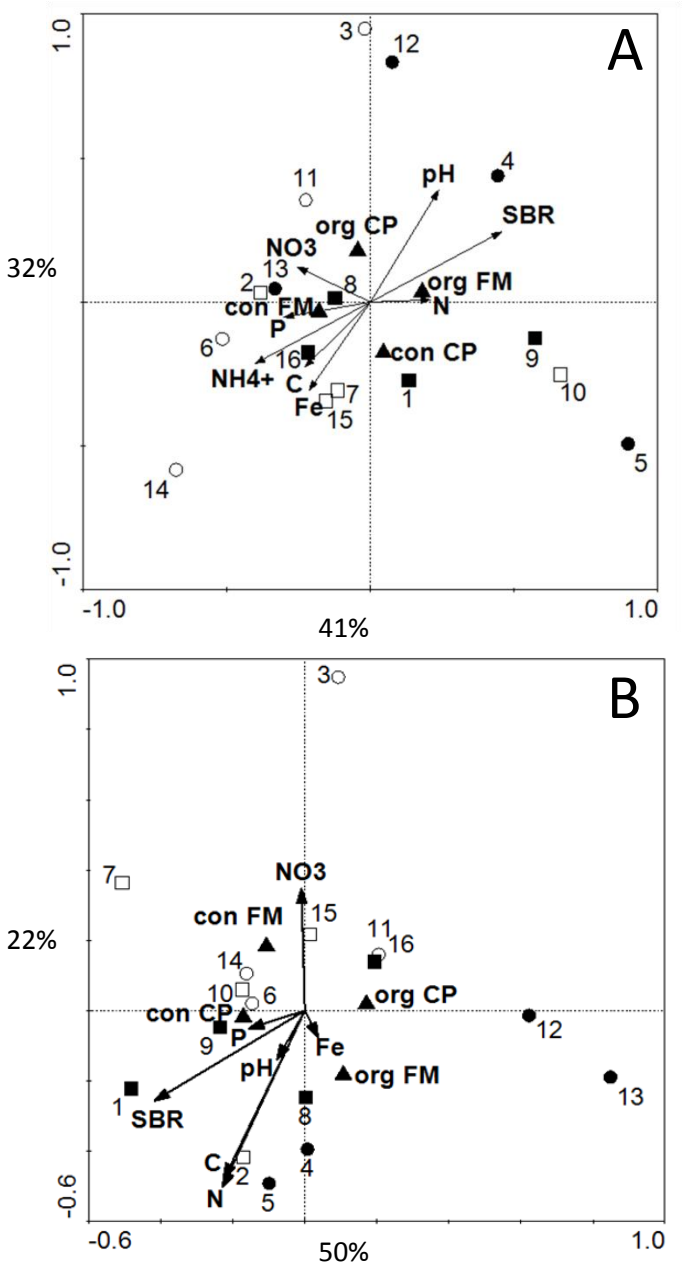


Figure 4.12. RDA of *nifH* RNA DGGE gels showing variation between treatments: orgFM orgCP (●), conFM conCP (□), orgFM conCP (■) and conFM orgCP (○). Arrows represent environmental variables. N = total nitrogen, C = total organic carbon, SBR = Soil Basal Respiration, NH4 = available ammonium, NO3- = available nitrate, P = phosphorus and Fe = iron. Triangles represent centroids for management treatments. A = March 2008 soil. N (P = 0.044) is a significant factors. B = June 2008 soil. P values are according to Monte Carlo permutation testing.

Figure 4.13. RDA and CCA of *nifH* DNA DGGE gels showing variation between treatments for 2008 soils.

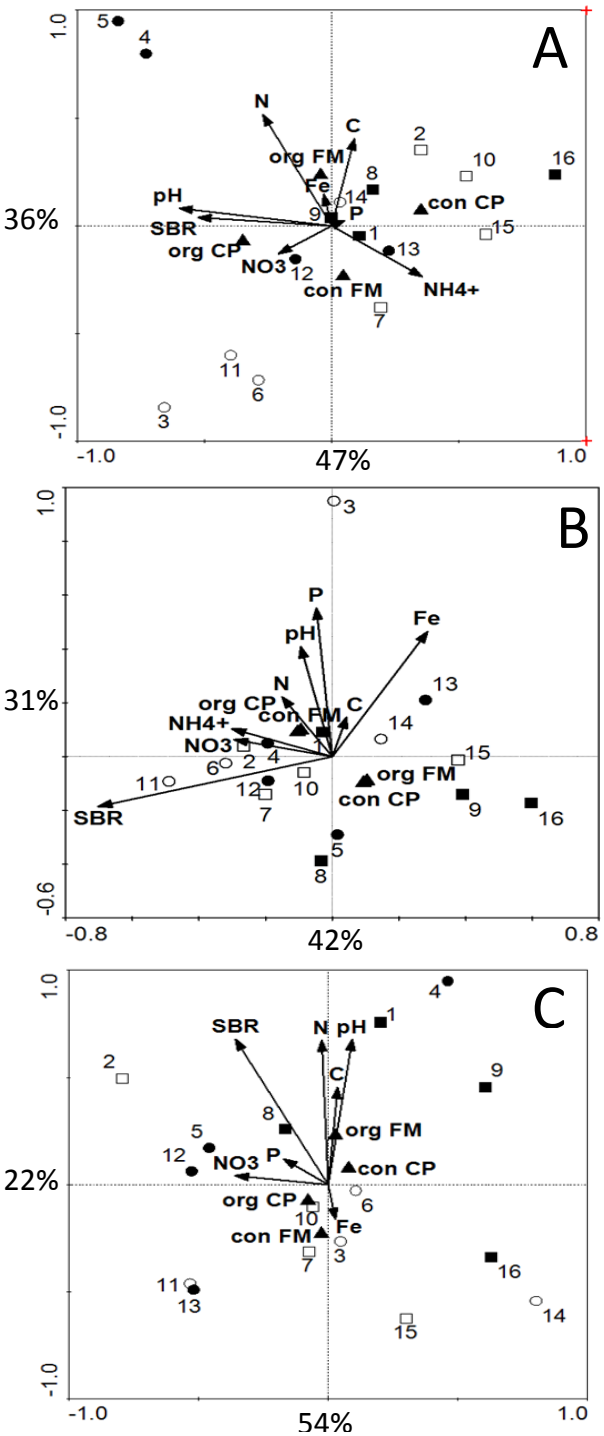


Figure 4.13. RDA of *nifH* DNA DGGE gels showing variation between treatments: orgFM orgCP (●), conFM conCP (□), orgFM conCP (■) and conFM orgCP (○). Arrows represent environmental variables. N = total nitrogen, C = total organic carbon, SBR = Soil Basal Respiration, NH4 = available ammonium, NO3- = available nitrate, P = phosphorus and Fe = iron. Triangles represent centroids for management treatments. A = March 2008 soil. CP (*P* = 0.006), SBR (P = 0.032) and NO3- (P = 0.012) are significant factors. B = June 2008 soil. CP (*P* = 0.042), SBR (P = 0.032) and pH (P = 0.034) are significant factors. C = September 2008 soil. *P* values are according to Monte Carlo permutation testing.

Figure 4.14. RDA and CCA analysis of *nifH* RNA DGGE gels for 2009 soils

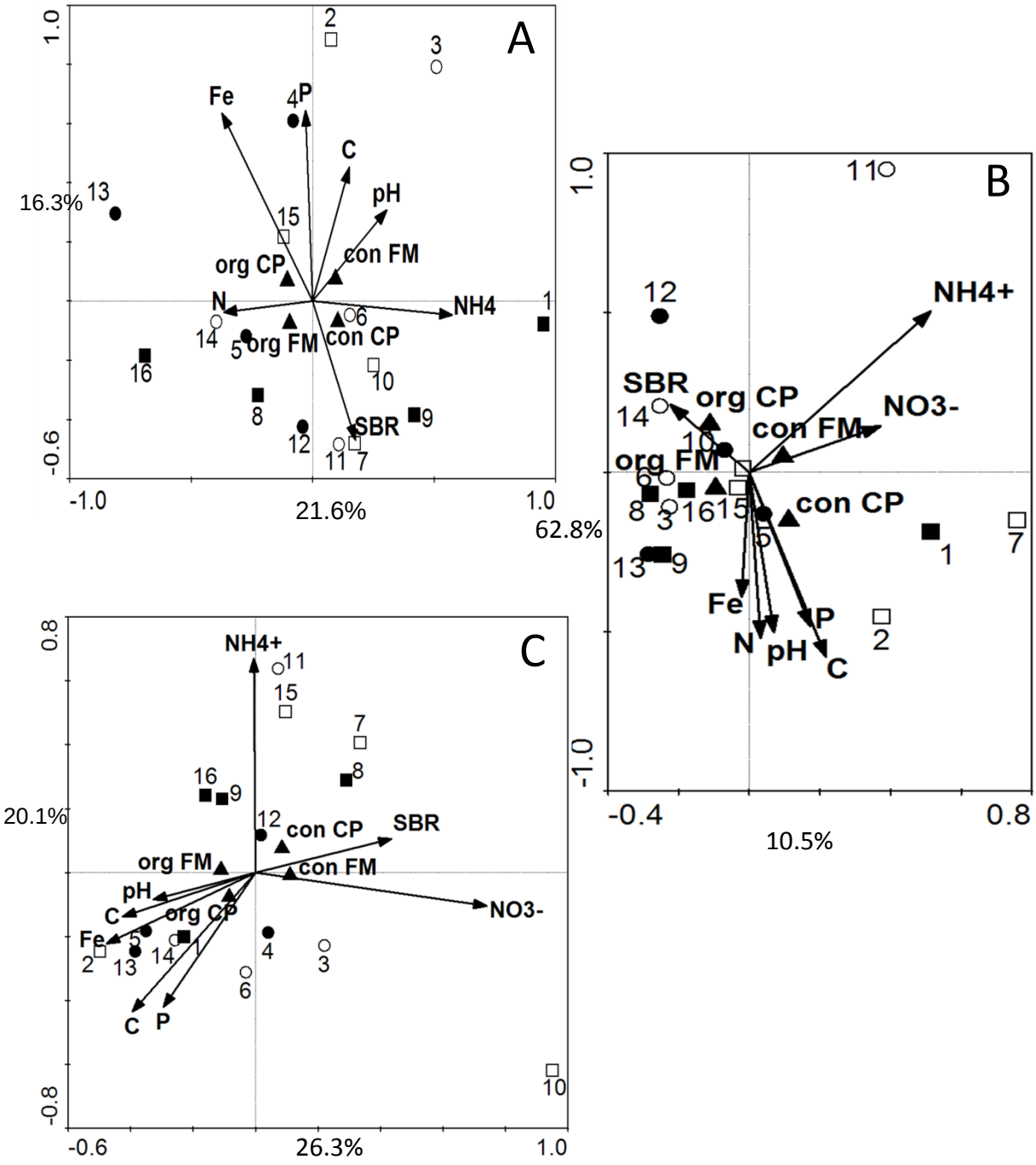


Figure 4.14. RDA of *nifH* RNA DGGE gels showing variation between treatments: orgFM orgCP (●), conFM conCP (□), orgFM conCP (■) and conFM orgCP (○). Arrows represent environmental variables. N = total nitrogen, C = total organic carbon, SBR = Soil Basal Respiration, NH4 = available ammonium, NO3- = available nitrate, P = phosphorus and Fe = iron. Triangles represent centroids for management treatments. A = March 2009 soil. Fe (P = 0.050) is a significant factor. B = June 2009 soil. C (P = 0.016), Fe (P = 0.044), NO3 (P=0.038) and NH4 (P=0.036) are significant factors. C = September 2009. NO3 (P=0.03) is a significant factor. *P* values are according to Monte Carlo permutation testing.

Figure 4.15. RDA and CCA of *nifH* DNA DGGE gels showing variation between treatments for 2009 soils.

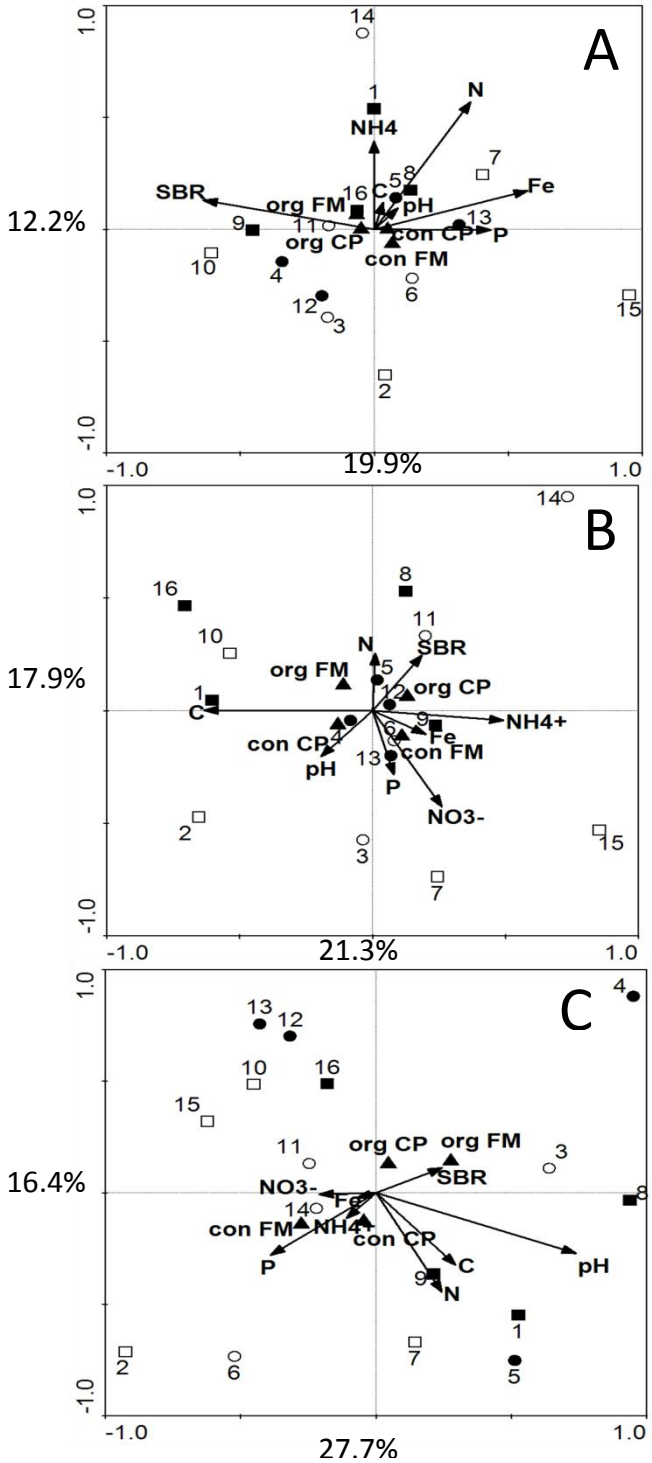


Figure 4.15. RDA of *nifH* DNA DGGE gels showing variation between treatments: orgFM orgCP (●), conFM conCP (□), orgFM conCP (■) and conFM orgCP (○). Arrows represent environmental variables. N = total nitrogen, C = total organic carbon, SBR = Soil Basal Respiration, NH4 = available ammonium, NO3- = available nitrate, P = phosphorus and Fe = iron. Triangles represent centroids for management treatments. A = March 2009 soil. B = June 2009 soil. C = September 2009 soil. pH (P = 0.006) is a significant factor. *P* values are according to Monte Carlo permutation testing.

Figure 4.16. Statistically significant factors affecting results of molecular analysis of the nitrogen fixing community.

		2007 RNA		
		March	June	Sept
direct ordination (RDA and CCA)	beans	**C and Fe**	**CP and NH4**	**P**
	barley	**N**	**Fe and SBR**	n/s
indirect ordination (PCA and DCA)	beans	**FM**	**CP**	**CP**
	barley	n/s	**FM**	n/s
qPCR	both	n/s	**FM**	**CP**

		2007 DNA		
		March	June	Sept
direct ordination (RDA and CCA)	barley	**N**	**P and NO3**	n/s
indirect ordination (PCA and DCA)	barley	n/s	n/s	n/s
qPCR	barley	**FM**	n/s	**FM**

		2008 RNA		
		March	June	Sept
direct ordination (RDA and CCA)	wheat	**N** and *P*	n/s	n/a
indirect ordination (PCA and DCA)	wheat	n/s	n/s	n/a
qPCR	wheat	n/a	n/a	n/a

		2008 DNA		
		March	June	Sept
direct ordination (RDA and CCA)	wheat	**CP, SBR and NO3**	**CP, SBR and pH**	*FM*
indirect ordination (PCA and DCA)	wheat	n/s	n/s	n/s
qPCR	wheat	**CP and FM**	n/s	n/s

		2009 RNA		
		March	June	Sept
direct ordination (RDA and CCA)	wheat	*Fe*	**C, NH4, NO3 and Fe**	**NO3 and NH4**
indirect ordination (PCA and DCA)	wheat	n/s	n/s	**FM**
qPCR	wheat	n/s	n/s	**FM**

		2009 DNA		
		March	June	Sept
direct ordination (RDA and CCA)	wheat	n/s	n/s	**pH**
indirect ordination (PCA and DCA)	wheat	n/s	n/s	n/s
qPCR	wheat	**CP and FM**	n/s	n/s

Figure 4.16. Summary of all *nifH* molecular analysis. n/s = not significant. n/a = not applicable. Factors in bold are significant and have a *P* value < 0.05. Factors in italics have a *P* value of <0.1.

diazotrophic diversity at some point over the sampling period, although results were sporadic meaning few conclusions could be drawn.

4.2.7. Analysing correlations between diazotrophic diversity, *nifH* copy number, and environmental variables.

Direct multivariate analysis of DGGE data suggests that environmental variables affect *nifH* diversity. Therefore, Pearson's product moment correlation was used to see if environmental variables correlated with changes in *nifH* diversity and copy number. All years were analyzed together.

Percentage organic N had a strong significant correlation overall, with organic N being positively correlated to *nifH* copy number and diversity (Table 4.17). There were many differences seen between how variables correlated with RNA results and DNA results. Nitrate and ammonium concentration positively correlated with RNA expression but negatively correlated with DNA expression. Available iron and percentage carbon were negatively correlated with RNA expression but positively correlated with DNA expression.

Pearson's product moment correlation also allowed comparisons to be made between RNA and DNA results and between *nifH* copy number and diversity. Results between analysis of RNA and DNA of the same plots seem to give dramatically different significance values to different treatments. H' of *nifH* RNA DGGE gels for 2007 of the organic rotation was removed as DNA diversity was not studied in these plots. As shown in Table 4.18 no significant positive or negative correlation was seen between levels of *nifH* RNA and DNA within the same plots.

Significant positive correlation was observed between H' and copy number for the RNA data set (Table 4.19). This was not observed in the DNA data set. Significant negative correlation was reported between DNA and RNA copy number. However, this is mostly due to the plots from which the *nifH* gene could not be amplified.

Table 4.17. Summary of results of Pearson's correlation analysis for all years.

	H' RNA *nifH*	qPCR RNA *nifH*	H' DNA *nifH*	qPCR DNA *nifH*
qPCR RNA *nifH*	**+++**			
H' DNA *nifH*	+	+		
qPCR DNA *nifH*	-	**---**	-	
pH	**---**	**---**	-	**+++**
%N	**+++**	**+++**	+	**++**
%C	**---**	**---**	-	**+++**
SBR	-	**++**	+	**---**
P	-	+	+	**---**
Fe	**---**	**---**	+	-
NH4	+	**+++**	-	-
NO3	+	+	-	-

+/- = non-significant correlation, ++/— = correlation with $P \leq 0.05$, +++/—- = correlation with $P \leq 0.001$.

Table 4.18. Table showing correlation between H' for RNA and DNA *nifH* DGGE gels

	correlation coefficient (ρ)	*P* value
whole data set	0.076	0.366
2007	0.222	0.129
2008	-0.163	0.268
2009	0.192	0.191

Table 4.19. Table showing correlation between *nifH* diversity and *nifH* copy number from DNA and RNA data sets.

comparison	ρ	*P* value
H' RNA and qPCR RNA	0.623	<0.001
H' DNA and qPCR DNA	-0.111	0.187
qPCR DNA and qPCR RNA	-0.569	<0.001

4.3. Discussion.

4.3.1 The effect of crop rotation on the diversity of diazotrophs and *nifH* copy number.

Organic farming practices rely on the addition of organic material to the soil and it was expected that organic fertility management would promote more activity and diversity in the soil microbial community (Shannon *et al*., 2002). However, in the 2007 sample date, fertility management affected microbial populations to a lesser extent than crop rotation. Rotation (identified by the previous crop in this study) had a strong affect on both free-living N fixing bacterial population structure (measured by DGGE profiles) and activity (measured by RNA gene copy numbers) and was the dominant management factor affecting microbial population structure and function in 2007.

The different crop species grown in each rotation in the previous three years (beans, potatoes and winter wheat in the organic rotation, and winter barley and two years of winter wheat in the conventional rotation) resulted in fundamental changes to the structure and activity of the free-living N fixing bacterial community (Table 2.3 – Methods chapter). A considerably more active and diverse diazotrophic community was seen in soils previously under barley (conventional rotation) (Table 4.3, 4.8, 4.10). Even on the final sample date of 2007 differences in the composition of the *nifH* community (between organic and conventional rotation) was evident (Table 4.12-4.13).

Most research into the effect of crop species on the soil's microbial community has been carried out on rhizosphere soils. Any changes to the microbial community are attributed to changes in organic root exudates affecting microbial activity in a species-specific manner (Funnell-Harris *et al.*, 2008; Wieland *et al*., 2001). In this study, these changes were detected in the bulk soil, and were apparent even though the crop was the same in both rotations in the sampling year.

These findings support those of Larkin and Honeycutt (2006) who suggested that plant effects i.e. crop rotation, are the most important drivers of soil microbial community characteristics within a given site and soil type. Crop rotational effects on populations of free-living N fixing bacteria were also reported by Chunleuchanon *et al* (2003), who found that when rice was grown in rotation there was higher diversity of nitrogen-fixing cyanobacteria than when it was grown in monoculture. However, to our knowledge, this is the first study which documents increased free-living diazotroph *nifH* expression and diversity following a rotation containing non-legumes when compared with a rotation containing legumes.

One explanation of the dramatic effect of crop rotation on the free-living nitrogen fixing community in this study is due to the fundamental differences between the two rotations. Faba beans can derive 90% of their N from N_2 fixation (Funnell-Harris *et al*., 2008), therefore, beans do not have as high an N demand as barley (Jensen *et al*., 2005). For this reason, even under conventional fertility management, no N fertilizer is applied to the beans in the NFSC experiments. Cereal crops such as barley efficiently utilize available N in the soil depleting mineral N during crop growth (Jensen *et al*., 2005). Therefore, the low mineral N levels in soils under cereal crops may make it a more suitable environment for free-living diazotrophs resulting in the increased numbers seen in the soil in the conventional rotation. Indeed, even in March of the following year (2007), there was still more mineral N in the soil under potatoes following a crop of beans in the previous year (Table 3.2), compared with soil under potato following barley (*P*=0.0895). This suggests that higher levels of mineral N throughout the season, in the soil after a legume pre-crop, may be suppressing the activity of free-living N fixing bacteria.

Nitrogen species are significant drivers of diversity in two of the 6 RDA plots (Figures 4.10 B and D). Also, in terms of the correlation coefficients for 2007, nitrate and ammonium had non-significant negative correlation with RNA H' and copy number (Table 4.20).

Table 4.20. Summary of results of Pearson's correlation analysis for 2007.

	H' RNA	copy number RNA
copy number RNA	+++	
%N	--	-
%C	+	-
pH	+	+++
SBR	+	-
NO3-	-	-
NH4+	-	+++
Fe	-	-
P	-	-

+/- = non-significant correlation, ++/— = correlation with $P \leq 0.05$, +++/—- =correlation with $P \leq 0.001$.

However, available ammonium has a significant positive correlation with *nifH* copy number. It would also be assumed that if available nitrate and ammonium were affecting the nitrogen fixing community with respect to the change in previous crop, that there would also be significant affects due to fertility management, which provides more nitrate and ammonium to the plots (Table 3.2 – Results Chapter 1).

Taking this into account it is assumed that it is organic N (%N) which is driving the nitrogen fixing community to become more active and diverse after a crop of winter barley. Although %N is only a significant driver in one of the RDA plots, there is significant negative correlation throughout 2007 between %N and H' (Table 4.20). The %N was only measured from soils in November 2007 and was found to still be lower in plots which had barley growing in the previous year (*P*=0.090) (Table 3.2).

In order to make this conclusion more robust more than one year would have to be analysed. This was not possible in this study as year 4 of the rotation was not replicated in NFSC until 2010. It would also be interesting to see how long-lived the 'negative' (in terms of diversity and expression of *nifH*) effect of the beans crop was. The diversity and expression of *nifH* do not seem to increase more throughout the season in the beans crop as oppose to the barley crop suggesting that the effect would continue on into later sample years within these plots. This would be interesting to see as this would then become clearer as a rotational effect rather than the direct effect of the previous crop.

4.3.2. The effect of treatment on diversity of diazotrophs and *nifH* copy number.

Fertility management did not significantly affect expression and diversity of the *nifH* gene. When looking at the DNA data set fertility management was not a significant driver of diazotrophic diversity. Occasional significant results were found when looking at indirect ordination of DGGE banding patterns, when RNA was used as the nucleic acid (March 2007, organic rotation; June 2007, conventional rotation; September 2009) (Figure

Table 4.21a. Summary of percentage variance explained by fertility management in each DNA RDA/CCA plot.

	2007 (Barley)	2008	2009	Average
March	2.9	10.5	3.1	5.5
June	13.4	6.4	3.3	7.7
September	2.4	4.8	9.1	5.4
Average	6.2	7.2	5.2	6.2

Table 4.21b. Summary of percentage variance explained by fertility management in each RNA RDA/CCA plot.

	2007 (Beans)	2007 (Barley)	2008	2009	Average
March	8.0	7.5	7.8	4.8	7.0
June	6.1	9.9	7.8	8.0	8.0
September	7.2	7.0	N/A	9.1	7.8
Average	7.1	8.1	7.8	7.3	7.6

4.16), although the inconsistency means that no conclusions could be drawn. Table 4.21 summarizes this and shows that fertility management explained on average 7.6% of the variance observed in *nifH* RNA RDA/CCA plots, and 6.2% of the variance observed in *nifH* DNA RDA/CCA plots. Percentage was marginally higher in June and September, after fertilizers were applied. The general trend seemed to be that conventional fertility management led to a slight increase in H' (Tables 4.3 and 4.4). However, this was never significant and values are always very similar between fertility management treatments.

Analysis of *nifH* expression, using *nifH* copy number from qPCR data, showed that for all years, when using the DNA data set, conventional fertility results in increased *nifH* expression once fertilizers have been applied to the soil (Table 4.15). Although, fertility management was often a significant factor, both organic and conventional management appeared to have positives effects in different years and sample dates. For example, in March of all years of the DNA data set fertility management was significant. However, in 2007 increased *nifH* copy number is associated with organic fertility management, and in 2008 and 2009 increased *nifH* copy number is associated with conventional fertility management (Tables 4.10).

It was hypothesised that fertility management would have a more significant effect on the nitrogen fixing community due to the decreased amount of nitrate and ammonium seen in organically managed soils (Table 3.2). Although fertility management does not have a consistent affect on *nifH* diversity and expression the general trend seemed to be that conventional fertility management resulted in increased nitrogen fixation compared to organic fertility management. %N, available ammonium and available nitrate also seemed to have an overall positive correlation with diazotrophic diversity and *nifH* copy number, particularly when it came to the RNA data set (Table 4.31).

This is contrary to other studies which have suggested that the amount of nitrogen applied to soils has a significant effect on the nitrogen fixing community (Coelho *et al*., 2009;

Coelho *et al*., 2008). However, most studies that have reported the effect of levels of N on the *nifH* community have been conducted on free-living N fixing bacteria in the rhizoplane or rhizosphere soils. Coelho *et al.* (2008) found higher levels of nitrogen fertilizer decreased N fixation in rhizosphere soils but found it had no effect in bulk soil. Rhizosphere soils are very different to bulk soils as the bacterial community is stimulated by root exudates (Smalla *et al*., 2001). It is estimated that 64-86% of carbon released as root exudates is respired by microorganisms resulting in a 10 to 100 fold increase in the size of the microbial community compared to bulk soils (Burgmann *et al*., 2005). Actual soil structure can also be different between bulk and rhizosphere soils with rhizosphere soil pores allowing better water drainage (Whalley *et al*., 2005).

Previous studies also seem to focus on particular sections of farm management for example nitrogen addition rather than whole farming systems. In the NFSC experiments the different fertility management regimes do not just involve application of different forms of nitrogen; the conventionally managed plots receive superphosphate and potassium chloride whereas the organic plots receive only compost (which contains varying amounts of P and K as well as other macro- and micronutrients). Changes in fertilizer management also led to changes in pH and soil basal respiration, which can also affect the nitrogen fixing community. Most of the environmental variables, for examples total C, total N, available P, pH, concentrations of ammonium and concentrations of nitrate, were responses to changes in fertility management. If we look at the effect of all variables associated with fertility management on the *nifH* DGGE results, the average amount of variance in diversity explained by fertility management was 50.8% in RNA gels and 36.8% in DNA gels (Table 4.22). This combined with *nifH* qPCR results (Tables 4.14, 4.15) could suggest that fertility management does have some effect on the nitrogen fixing community. These factors also cause far more variation in the RNA data set than the DNA data set. Reed *et al.* (2007) found that the addition of phosphorus to soil more than doubles nitrogen fixation. Concentrations of phosphorus in organically managed soils

Table 4.22a. Summary of percentage variance explained by fertility management and associated fertility management variables in each RNA RDA/CCA plot.

	2007 (Beans)	2007 (Barley)	2008	2009	Average
March	59.2	45.3	49.6	54.0	52.0
June	49.3	52.4	44.4	69.8	54.0
September	43.4	46.2	N/A	48.4	34.5
Average	50.6	48.0	47.0	57.4	50.8

Table 4.22b. Summary of percentage variance explained by fertility management and associated fertility management variables in each DNA RDA/CCA plot.

	2007 (Barley)	2008	2009	Average
March	25.7	27.9	27.3	27.0
June	54.2	45.1	49.3	49.5
September	21	38.6	41.7	33.8
Average	33.6	37.2	39.4	36.8

can be half that of conventionally managed soils, with organically fertilised soils relying on phosphorus remaining in the soil from past conventional management (Gosling and Shepherd, 2005). In the NFSC experiment overall conventional plots have significantly more available P (Figure 3.2). Nitrogen fixation requires a lot of energy and therefore, has increased phosphorus requirements. The activation energy required to break the triple bond of dinitrogen is high and requires large amounts of ATP (LaRoche and Breitbarth, 2005). Therefore, it is possible that the positive effect of conventional fertility management, seen at certain dates, has nothing to do with the nitrogen applied to the field but more to do with the increased availability of phosphorus in this treatment. Phosphorus is highlighted as a significant driver in two of the 2007 RDA plots (Figure 4.16). However, correlation coefficients (Table 4.17 and Table 4.20) show there is little significant correlation between available phosphorus and *nifH* copy number and diversity. In the one instance that there is significant correlation (between phosphorus and DNA copy number all years) the correlation was negative. When looking at a soil solution only a very small amount of phosphorus will be present as PO_4^{3-} ions, and therefore freely available. Therefore, phosphorus is the least mobile major nutrient as it is very easily adsorbed onto Fe and Al oxides (Hinsinger, 2001). This could suggest why phosphorus is detected in soils but is not positively influencing the nitrogen fixing community.

Iron is an essential element for nitrogen fixation as it is a component of the key proteins in nitrogenase. Although diazotrophs need 30-300 times more P than Fe there is evidence that both co-limit N fixation (Mills *et al.,* 2004). Mills *et al.* (2004) found that adding Fe and P simultaneously increased N fixation 2-3 fold. This effect was not seen when Fe and P were added individually. Although not significant, iron was always higher after conventional management (Figure 3.2).There was also significant positive correlation between amounts of available phosphorus and iron in the present study. However correlation between iron and *nifH* diversity and copy number varied between dates and was never significant. Iron is a significant driver of *nifH* diversity in the RNA data set on 3

occasions (Figure 4.16) and significant negative correlation was seen between diversity and copy number of *nifH* in the RNA 2007 data set. However, when looking at the RDA plots (Figure 4.26 and 4.30) these effects seem to be related to the location of the plots within the field, rather than the individual treatments, suggesting that within field variability in soil chemical characteristics may also play an important role in the diversity and activity of free-living N fixing bacteria.

Soil is a major reserve of organic carbon. Organic farming, on average, produces 28% higher carbon levels than conventional farming in Northern Europe (Azeez, 2009). When available carbon concentrations are low bacterial growth and diversity can be limited (Monard *et al*., 2008).Carbon is a significant driver of variance in March 2007 and June 2009 (Figure 4.16). However, overall correlation between carbon and *nifH* diversity and *nifH* copy number was negative for the DNA data set and positive for the RNA data set (Table 4.17). A number of studies have shown that soil carbon generally increases N fixation (Rogers *et al*., 2009; Heimann and Reichstein, 2008; Burgmann *et al*., 2005). Increased carbon dioxide has been shown to be directly correlated with increased nitrogen fixation when phosphorus, iron and molybdenum are also present, due to changes in the C:N ratio (van Groenigen *et al*., 2006; Zanetti *et al*., 1996). Our results are similar to those of Hsu and Buckley (2009) who reported that carbon was a driver of nitrogen fixation but that carbon was not significantly affected by the change in management type.

4.3.3. The effect of crop protection on diazotrophic diversity and *nifH* copy number.

Crop protection significantly affects *nifH* expression when DNA is used as the template (Table 4.5). Over all the years crop protection was significant and increased in organic health management plots. When broken down into dates this was significant in 2009 (and was close to being significant in 2008 $P = 0.077$).

NifH diversity (as measured by DGGE) was not affected significantly by changes in crop protection protocols. In the DNA data set crop protection drives differences in diversity in March and June 2008 according to direct ordination. H' is never significantly affected by crop protection although the average for all years is higher after organic health management (Table 4.4). The overall average percentage variance explained by crop protection according to RDA/CCA was only 5.9% and on average the percentage variance decreased throughout the sample year (Table 4.23).

In the RNA data set crop protection significantly affected diversity in the organic rotation in June and September 2007 (indirect ordination only) (Figure 4.16). However, crop protection never significantly affected H' and is responsible for on average only 7.6% of the variation within the DGGE RDA/CCA data set. The average percentages are higher in June directly after pesticides have been applied than other dates (Table 4.23). *NifH* copy number is also affected in September 2007 where conventional management results in increased expression (Table 4.15).

Conventional crop protection of potatoes and the preceding crops, involves the use of a variety of synthetic pesticides (Table 2.1). The ideal pesticide should be toxic only to target organisms. However, this is rarely the case. Approved pesticides should have been shown to affect activities of; nitrate and ammonium transformations, oxygen uptake, carbon dioxide release, or carbon and nitrogen mineralization by less than 25% (Černohlávkoá *et al*., 2009; Johnsen *et al*., 2001). However, this does not mean that the overall structure of the bacterial community has not changed in response to the pesticide. Some microorganisms may proliferate by using the pesticide as an energy source, and some may find the pesticide toxic (Johnsen *et al*., 2001).

It was hypothesised that conventional crop protection would have a negative effect on *nifH* diversity, and expression, as studies into the environmental impacts of pesticides have shown that they can significantly affect the bacterial community as a whole and that

Table 4.23a. Summary of percentage variance explained by crop management in each DNA RDA/CCA plot.

	2007 (Barley)	2008	2009	Average
March	4.9	23.4	2.8	10.4
June	4.2	9.3	2.7	5.4
September	1.1	3.4	1.6	2.0
Average	3.4	12.0	2.4	5.9

Table 4.23b. Summary of percentage variance explained by crop management in each RNA RDA/CCA plot.

	2007 (Beans)	2007 (Barley)	2008	2009	Average
March	6.4	5.8	7.1	5.1	6.1
June	11.6	6.4	11.1	10.1	9.8
September	7.4	5.2	N/A	5.5	4.5
Average	8.5	5.8	9.1	6.9	7.6

diazotrophs could be particularly affected. Cycon and Piotowska-Seget (2007) found that bacteria involved in nitrogen turnover, particularly nitrogen fixation and nitrification were particularly sensitive to a range of pesticides. For example, the fungicide mancozeb was found to exert an inhibitory effect on aerobic dinitrogen fixers in soil (Doneche *et al*., 1983). Our results suggest that, like the effect of fertility management, crop protection has an affect although the changes in diversity it causes are not consistent. Diversity of diazotrophs appeared to be significantly affected on some dates although this affect was neither positive nor negative and simply resulted in a changed community structure as some bacteria proliferate and some are suppressed.

In every instance that crop protection significantly affected *nifH* copy number conventional management suppressed *nifH* expression. This appeared to be more significant in the DNA data set compared to the RNA data set. This suggests that, although numbers of bacteria capable of nitrogen fixation are reduced, the bacteria are still capable of increasing their nitrogen fixation activity. This could also explain why the soil in the organic rotation is more affected than soil in the conventional rotation in 2007. As the diazotrophic community is better established after the barley crop it will be better equipped to handle stress meaning when one species struggles to fix nitrogen another can take its place. In the less diverse organic rotation this may not happen as readily.

A range of pesticides are applied to the potato crops in the NFSC experiment, at several dates from April onwards (Table 2.2). The herbicide used is linuron. Miloševiã and Govedarica (2002) found that *Azotobacter* species were particularly sensitive to pesticides and were reduced in number by 78% 14 days after application. They also suggest that results are varied depending on the crop grown but do not suggest a reason for this. Fluazinam and mancozeb are used as growth regulators. As mentioned above mancozeb has previously been found to affect aerobic diazotrophs (Doneche *et al*., 1983). Fluazinam is a fungicide used to control potato blight as it disrupts eukaryotic

mitochondria by protonating amino groups (Leroux, 1996). No previous studies could be found testing its action against nitrogen fixing bacteria. Aldicarb is a nematicide which has been shown to suppress the plant growth promoting bacteria which contribute to potato growth, including the N-fixers, mineral solubilizers and phytohormone producers (Sturz and Kimpinski, 1999). To our knowledge the effect the desiccant Oiquat has on nitrogen-fixers has never been studied.

Although diazotrophic diversity does not seem to have been affected by the pesticides used in this study, organic crop protection led to increased copies of the *nifH* gene in the DNA data (Table 2.5). Many previous studies looking at the effect of pesticides on the diazotrophic community have focussed on nitrogen fixers which are symbiotic with legumes. *Bradyrhizobium japonicum*, for example, has been found to be particularly susceptible to the effects of glyphosphate due to the sensitivity of its phosphate synthase enzyme (Bohm *et al*., 2009; Zablotowicz and Reddy, 2007). Other studies have found that herbicides will affect nitrogenase activity, nodule formation, nodule biomass and leghaemoglobin concentrations (Bohm *et al*., 2009; Zablotowicz and Reddy, 2007; Reddy and Zablotowicz, 2002). However, it is unclear whether this is due to direct changes in the rhizobia, in direct physiological changes in the plant, or both (Zablotowicz and Reddy, 2007; Vierra *et al*., 2007). Fox *et al*. (2007) suggested that pesticides can disrupt the molecular interactions between rhizobia and their host plant and demonstrated this between *Sinorhizobium meliloti* and alfalfa. This does not suggest why we see significant changes to the free-living nitrogen fixing community.

4.3.4. Seasonal effects on diversity of diazotrophs and *nifH* copy number.

Sample date was a significant factor throughout the study and often had significant interactions with crop management factors (this has been summarised in Figures 4.18-4.19). There are many factors associated with the change in season that could affect microbial community structure and function, but the main ones that could be important in

this study are temperature and management regime, i.e. how the sample dates relate to the plant growth stage and the application of treatments.

Temperature is one of the most important environmental factors affecting the soil bacterial community (Pettersson and Bååth, 2003). The microbial community will adapt to suit the minimum and maximum temperatures of its environment. Figures 4.17-4.19 show there is an almost inverse relationship between temperature, and *nifH* diversity and copy number. However, optimum temperature for nitrogen fixers' growth and activity is between 10 and 25 °C (this is the temperature in the field between June and September) (Beauchamp *et al*., 2006; Eckford *et al*., 2002) (Figure 4.17 and Table 3.8). The temperature in the field on the March sampling date was approximately 4.5 °C and had not been above 7 °C for the month before that. When looking at the qPCR data it seems likely that expression of *nifH* (in terms of RNA copy number) of the free-living N fixing population was suppressed by temperature at this time. However, by September the population had increased by 18 fold from on average 3.42×10^5 g^{-1} soil in June, to 6.28×10^6 copies g^{-1} soil in September (Figure 4.34). However, if anything there is an inverse relationship with diazotrophic diversity and soil temperature (Figure 4.17 and 4.19). It is suspected that this is due to application of pesticides and fertilizers to the plots. Time scales are summarized in Table 2.2. In March there is little nitrate and ammonium present, on average, in soils (Figure 4.20) as fertilizers have not yet been applied and potatoes are yet to be planted. The majority of fertilizer and pesticides have been applied by the June sample date and increased nitrate and ammonium is seen in the plots. This is where significant decreases in *nifH* diversity and copy number are seen. As nitrate and ammonium decrease again in September and temperatures remain optimum the diazotrophic community recovers. It is possible that although there is no particularly significant effect attributed to one farming practice the action of applying fertilizers in general has some negative effect of excess nitrogen to the nitrogen fixing community. It is possible that the expected correlation is not seen between nitrate and ammonium and the RNA data set due to the surprisingly low

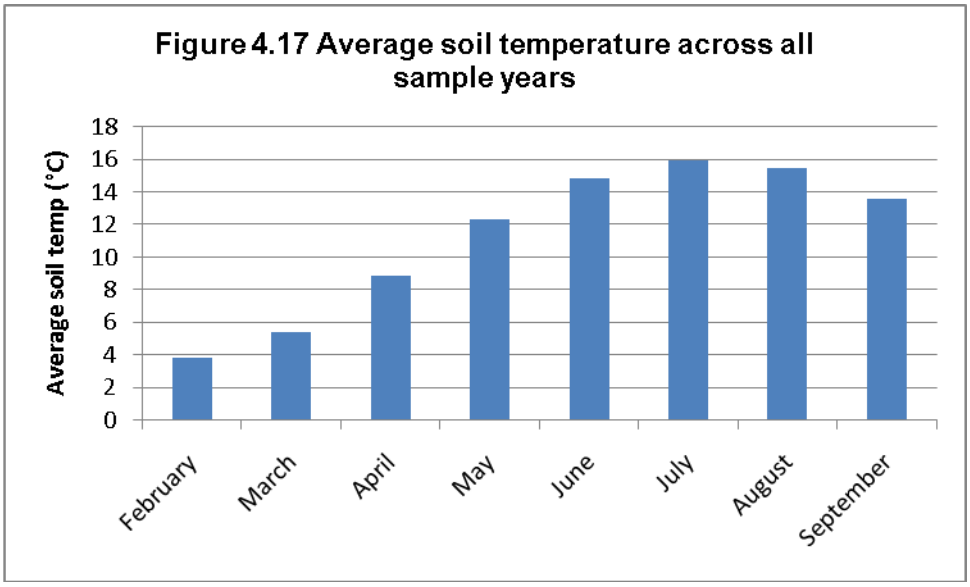


Figure 4.17 Average soil temperature across all sample years
Average soil temp (°C)
18
16
14
12
10
8
6
4
2
0
February
March
April
May
June
July
August
September

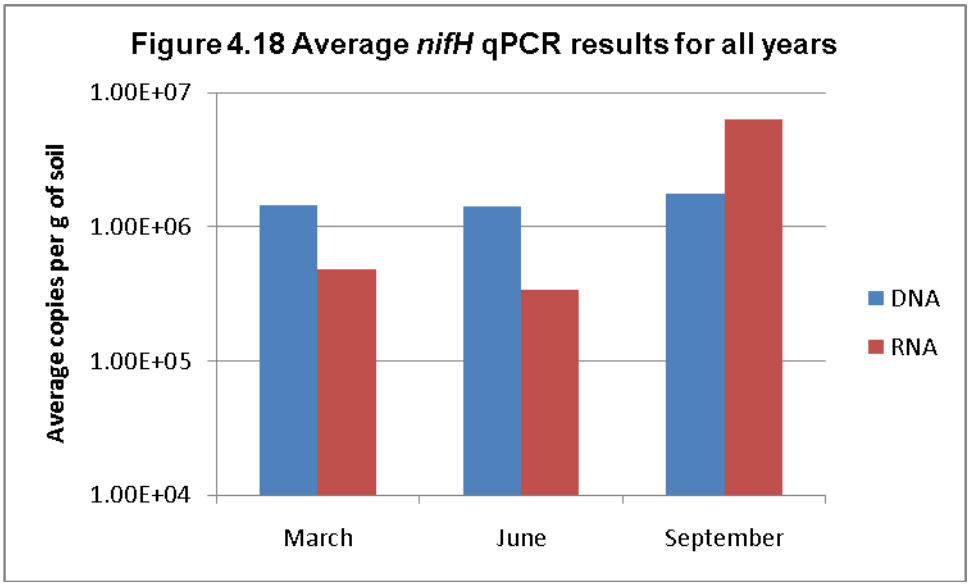


Figure 4.18 Average nifH qPCR results for all years
Average copies per g of soil
1.00E+07
1.00E+06
1.00E+05
1.00E+04
March
June
September
DNA
RNA

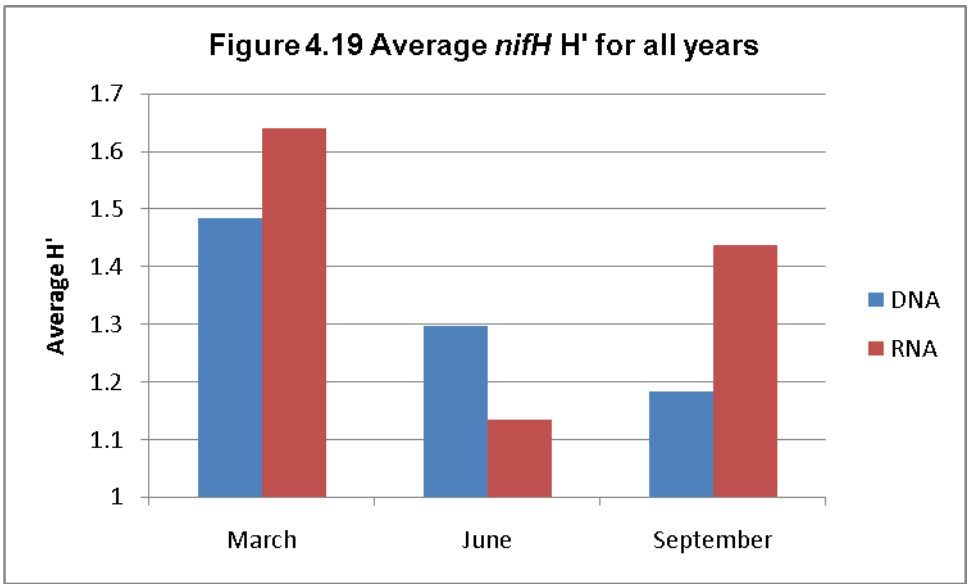


Figure 4.19 Average nifH H' for all years
Average H'
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
March
June
September
DNA
RNA

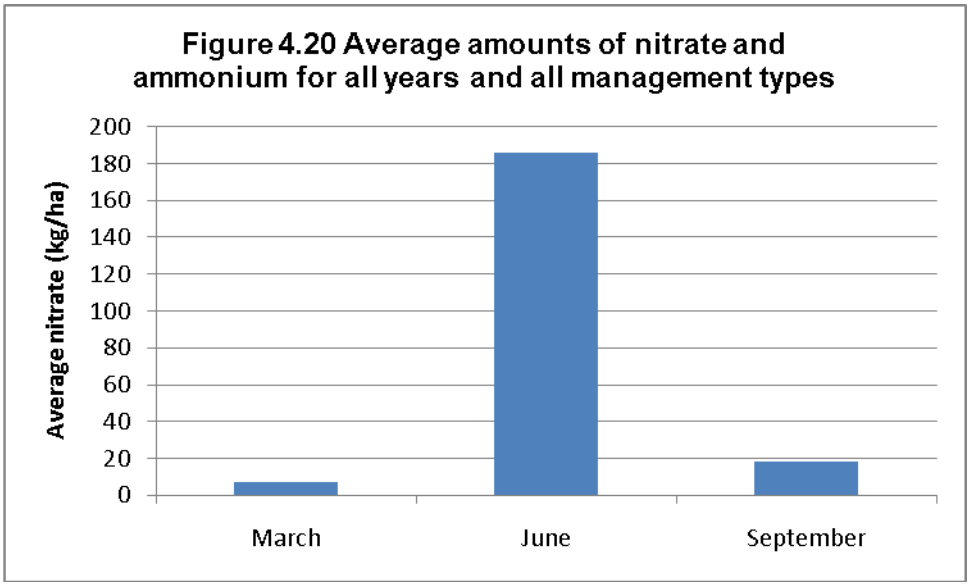


Figure 4.20 Average amounts of nitrate and ammonium for all years and all management types
Average nitrate (kg/ha)
200
180
160
140
120
100
80
60
40
20
0
March
June
September

nitrate levels observed in 2008 (65.95 kg/ha in 2008 compared with 256.88 and 234.08 kg/ha in 2007 and 2009) and the low RNA diversity and lack of *nifH* expression in 2008.

4.4. Conclusion

In conclusion the main factors affecting diazotrophic diversity and activity within the soils at NFSC are year, sample date, crop rotation and crop protection. Although fertility management did impact the nitrogen fixing community on some dates the effects were subtle/short lived. There is also limited evidence that the variables associated with changes in fertility management may affect the nitrogen fixing community. The effects of year, sample date and previous crop produced the most consistent significant results. The reason for the variation between the years remains unclear. However, the effect of sample date is most probably due to the community responding to the perturbation of the soil regardless of management type. The effect of crop rotation can be attributed to the previous crop used. The increase in nitrogen uptake by the barley crop is likely to create conditions more favourable to free-living nitrogen fixation. Although the increased nitrate and ammonia found in the soil following the bean crop is only apparent in March, the free-living diazotrophs are more likely established in the soil following barley and the community appears more diverse and abundant throughout the growing season.

The results show that in the DNA data set conventional crop protection led to increased copy number of *nifH*. As this effect is not seen in the RNA data set it is suggested that this is due to crop protection leading to long tern changes in expression of the *nifH* gene as RNA data is simply a measure of expression in the soil at the exact moment of sampling.

5. Results Chapter 3 - The effect of fertility and health management on the total bacterial community.

5.1. Introduction.

This results chapter aims to answer three main questions regarding diversity and activity of the total bacterial community.

1. What is the overall effect of crop management on the total bacterial community, and how do environmental variables correlate to these changes?
2. What is the effect of previous crop on the total bacterial community?
3. Is there any correlation between changes in the diversity and copy number of the diazotrophic community and changes in the diversity and copy number of the total bacterial community?

As discussed in the introduction the effect of crop management on the total bacterial community has been studied in the past. However, these studies often give varied results and are mostly based on DNA profiles rather than RNA. By extracting RNA from all sample years of the NFSC experiment it is hoped that a picture of how the treatments directly affect active bacterial communities will be gained. One hypothesis is that the organic management will result in increased bacterial diversity due to the increased carbon and nutrients applied to the soil in the form of manure or compost, and the reduction in the use of potentially toxic pesticides (Jangid *et al*., 2008; Hartmann *et al*., 2006). However, as we observed for the diazotrophic community, in the NFSC experiment there was no 'positive' effect of organic fertility management and it is possible that this could also apply to the whole bacterial community. Significant attention has focussed on the impact of pesticides on key groups of bacteria, such as the nitrogen fixers, as they perform important processes and could not be easily replaced by other microorganisms. However, this can sometimes provide a very group specific and restrictive view of the microbial community (Johnsen *et al*., 2001).

Results chapter 2 indicated that previous crop significantly affected the diazotrophic community. This chapter will compare diversity and activity of the total bacterial community between the organic and the conventional rotation at NFSC. Increased plant diversity, associated with organic rotations, tends to lead to increased microbial diversity (Hartmann *et al*., 2006). However, the significant effect observed in the diazotrophic community was attributed to the changes in soil nitrogen, brought about by the two different previous crops, affecting nitrogen fixation (Jensen *et al*., 2005). Therefore, previous crop may not affect the total microbial community as significantly.

Molecular analysis will be carried out on RNA extracted from all soils to examine the diversity and expression (as estimated using copy number) of the 16S rRNA gene. In addition, physiological diversity of the heterotrophic community will be measured using BIOLOG plates. Correlations will also be examined between the environmental variables: pH, total C, total N, soil basal respiration, available P, ammonium concentration and nitrate concentration, and the total bacterial community. Previous work has indicated that pH, soil basal respiration and available carbon will have the most marked effects (Hallin *et al*., 2009; Lauber *et al*., 2009; Jangid *et al*., 2008).

The final section of this results chapter will discuss the correlation between changes in the diazotrophic community presented previously (results chapter 2) and the total bacterial community. A lack of correlation between the results would suggest that the diazotrophic community is independently affected by the different crop managements. Although correlation could simply show that the communities are influenced by the same stimuli it could also suggest that any reduction in diversity or copy number of the nitrogen fixing community is simply due to a reduction in total bacterial number.

5.2. Results

5.2.1. Univariate analysis of the total microbial community across all sample years.

A band of around 120 bp was amplified from reverse transcribed RNA from every plot in all years. Successfully amplified PCR products were resolved on 35-55% denaturing gradient gels (Figures 5.1-5.4). Normalized relative intensities were first analysed using Shannon diversity index (H'). H' values are available in the appendix section F.

H' from 16S RNA gene DGGE gels from all years was compiled and ANOVA was carried out (Table 5.1). The terms year and previous crop had to be combined in order to make the test balanced and valid. ANOVA indicated that year combined with previous crop, and sample date, were significant factors. There was also a significant interaction between fertility and health management.

Quantitative PCR was carried out on all samples using the protocol described in the methods section and results chapter 1. All samples were above the limit of detection. Master sheets containing all triplicate results and averages can be found in the appendix section F.

Results needed to be log transformed before further analysis in order to create a normal distribution. Initially the data set was analysed whole using ANOVA. When the data set was analysed whole, year and previous crop were significant with 2009 having the greatest copy number (Table 5.2). Sample date also had a low *P* value (*P* = 0.054).

For both data sets it is possible that treatments are significant within years and, therefore, data was split up by year and analysed separately. Soils in 2008 and 2009 have been subjected to identical management as both are from the organic rotation and have a previous crop of wheat. Soils in 2007 are different as half are from the conventional rotation (with a previous crop of barley) and half from the organic rotation (with a previous

Figure 5.1. DGGE gels showing 16S rRNA gene amplified from RNA from 2007 soils from the conventional rotation

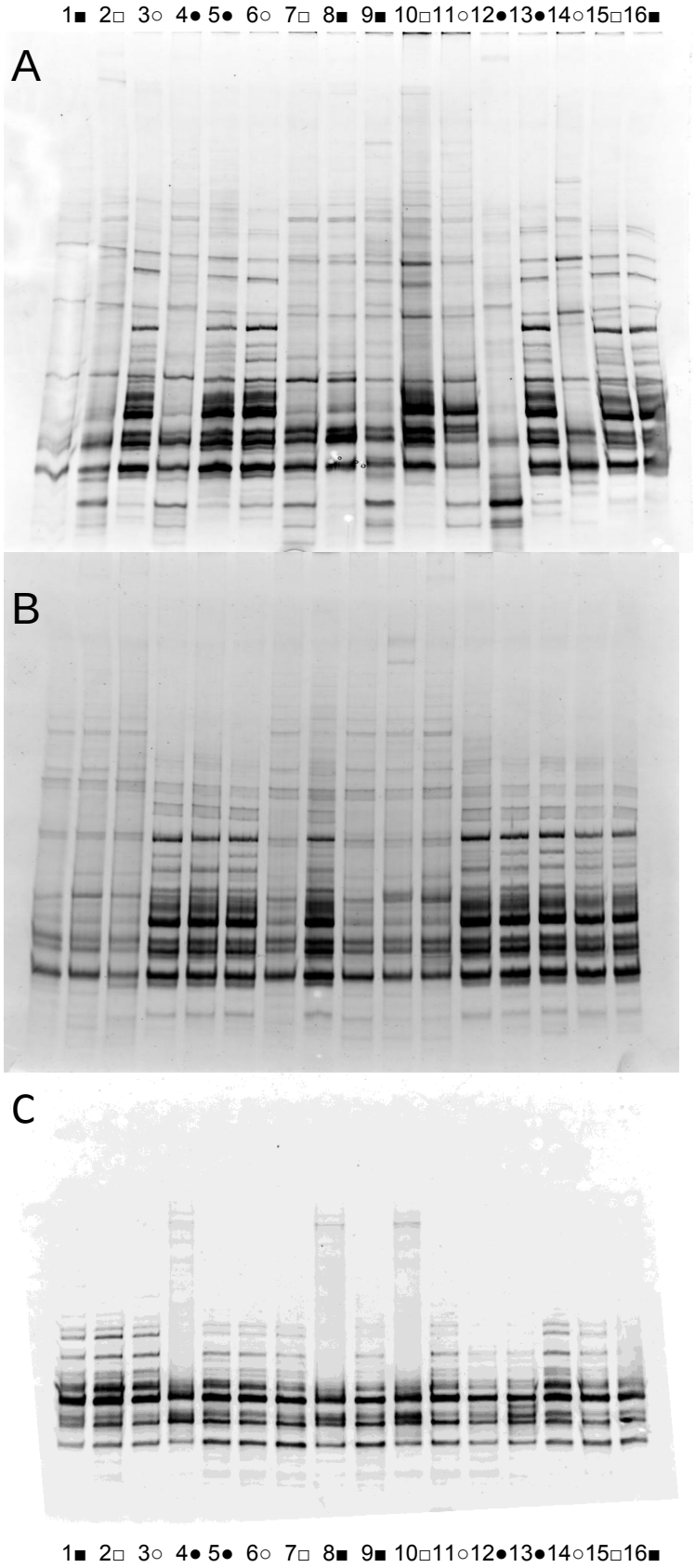


Figure 5.1. A = 16S DGGE image for RNA from March 2007 soil for the conventional rotation, B= 16S DGGE image for RNA from June 2007 soil for the conventional rotation, C = DGGE image for RNA from September 2007 soil for the conventional rotation. For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

Figure 5.2. DGGE gels showing 16S rRNA gene amplified from RNA from 2007 soils from the organic rotation

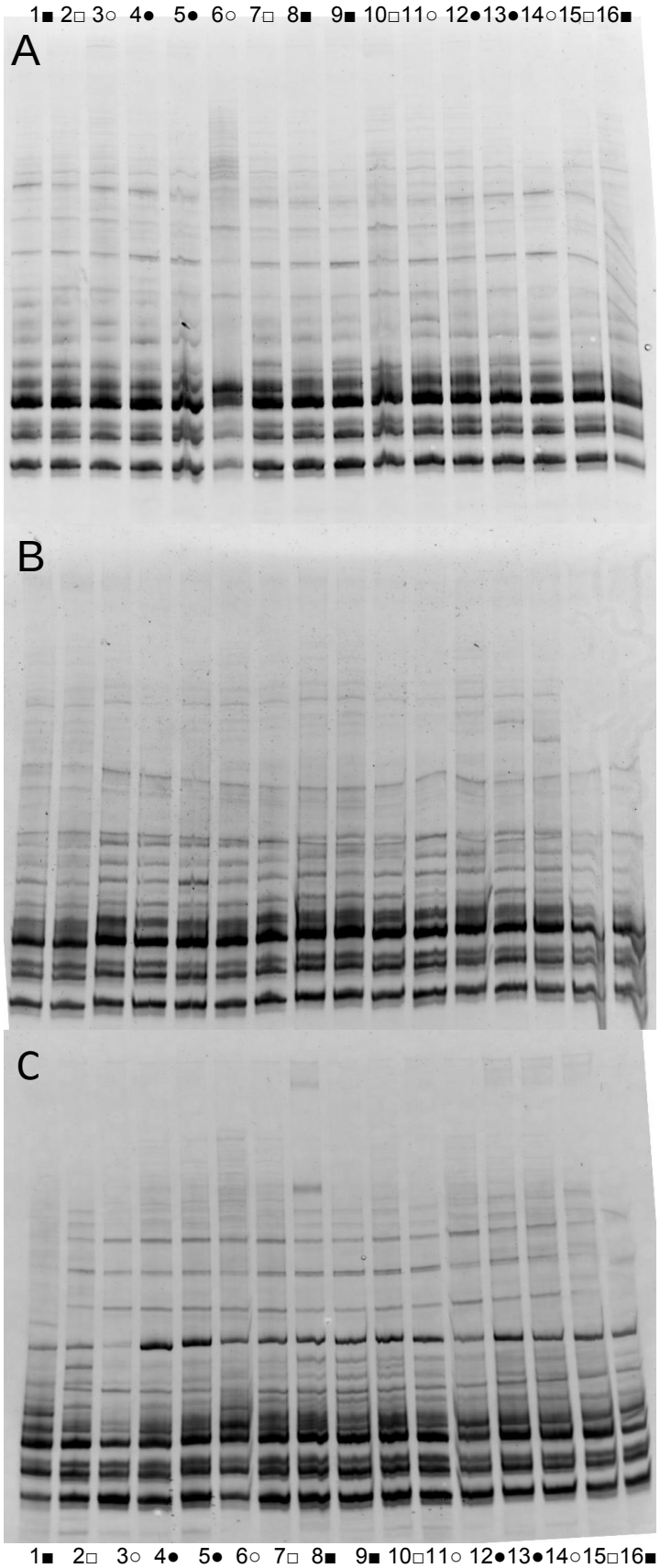


Figure 5.2. A = 16S DGGE image for RNA from March 2007 soil for the organic rotation, B = 16S DGGE image for RNA from June 2007 soil for the organic rotation, C = 16S DGGE image for RNA from September 2007 soil for the organic rotation. For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

Figure 5.3. DGGE gels showing 16S rRNA gene amplified from RNA from 2008 soils

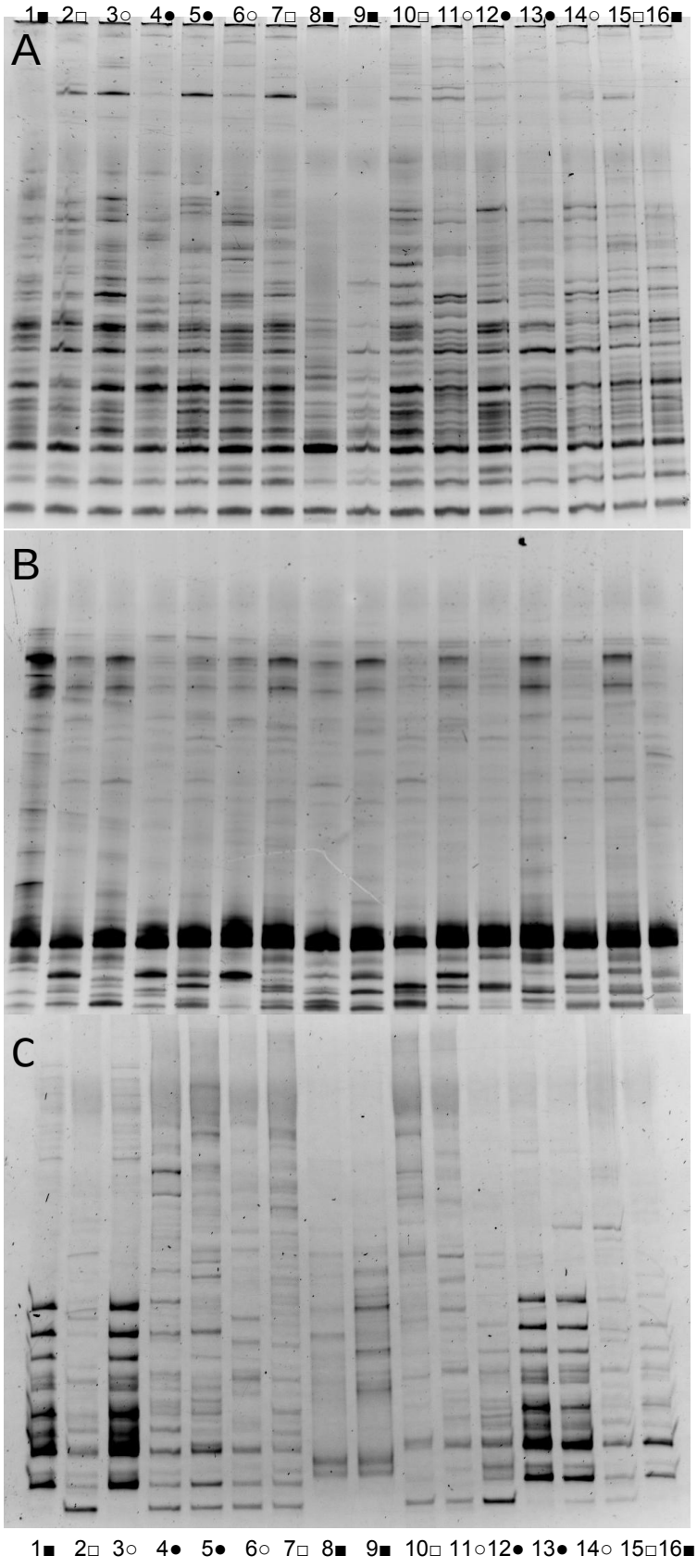


Figure 5.3. A = 16S DGGE image for DNA from March 2008 soil, B = 16S DGGE image for DNA from June 2008 soil, C = 16S DGGE image for DNA from September 2008 soil. For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

Figure 5.4. DGGE gels showing 16S rRNA gene amplified from RNA from 2009 soils

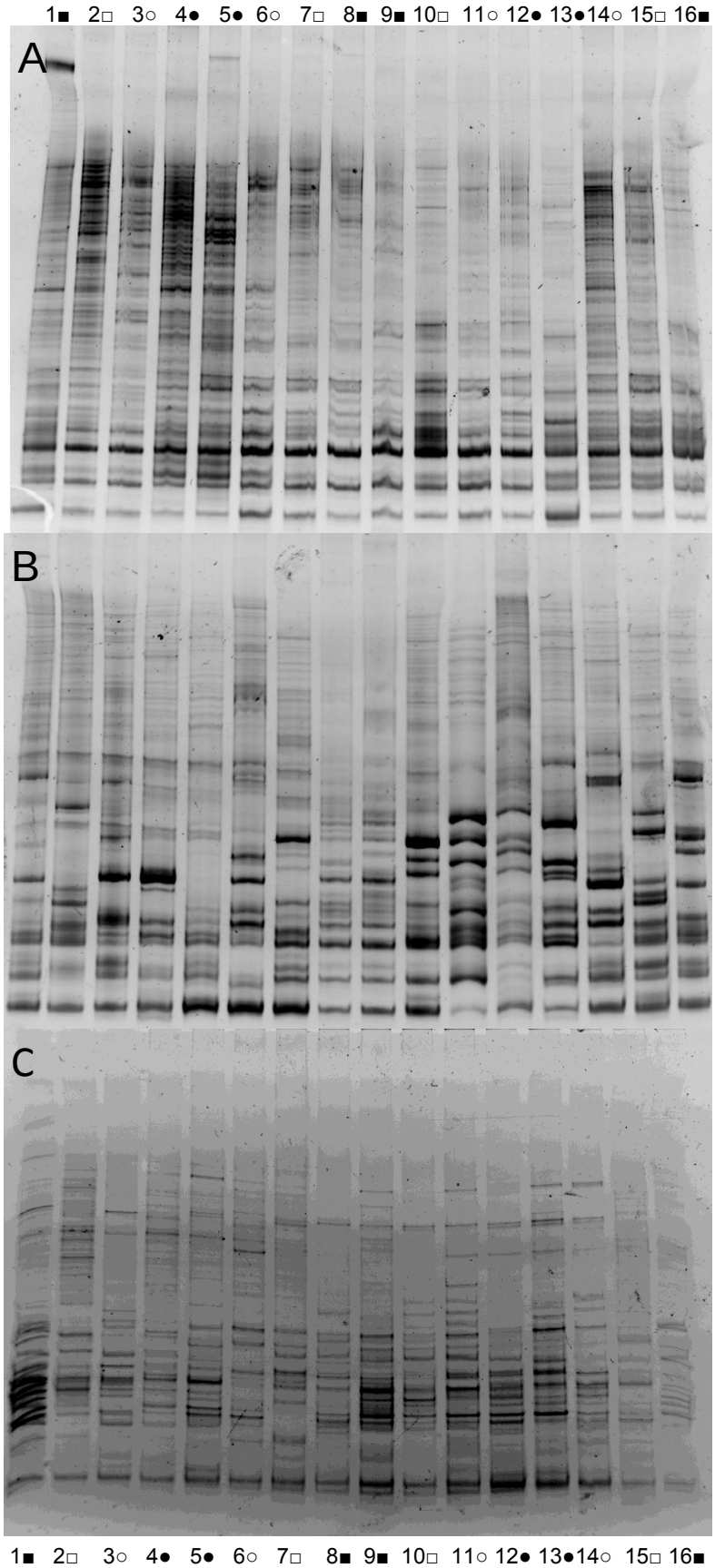


Figure 5.4. A = 16S DGGE image for DNA from March 2009 soil, B = 16S DGGE image for DNA from June 2009 soil, C = 16S DGGE image for DNA from September 2009 soil. For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

Table 5.1. ANOVA results for Shannon diversity indices of 16S rRNA DGGE gels across all sample years showing the effect of year, sample date and farm management.

	Shannon diversity index for 16S rRNA DGGE band data (mean + SE)
year and pre-crop (Y + PC)	
2007 (barley)	2.579 ± 0.06
2007 (beans)	2.634 ± 0.02
2008 (wheat)	2.812 ± 0.05
2009 (wheat)	3.063 ± 0.04
sample date (SD)	
March	2.935 ± 0.04
June	2.753 ± 0.03
September	2.628 ± 0.05
Crop protection (CP)	
ORG	2.780 ± 0.04
CON	2.763 ± 0.05
Fertility management (FM)	
ORG	2.748 ± 0.04
CON	2.795 ± 0.04
ANOVA *P*-values	
Y+PC	**<0.001**
SD	**<0.001**
CP	0.592
FM	0.139
Y+PC*SD	**<0.001**
Y+PC*FM	0.632
Y+PC*CP	0.714
SD*FM	0.677
SD*CP	0.273
CP*FM	**0.021**
Y+PC*SD*FM	0.994
Y+PC*SD*CP	0.928
Y+PC*FM*CP	0.184
SD*FM*CP	*0.061*
Y+PC*SD*FM*CP	0.617

Table 5.2. ANOVA analysis of qPCR data for copy number of the 16S rRNA gene across all sample years showing the effect of year, sample date and farm managment

	16S rRNA copies per g of soil (mean + SE)
year and pre-crop (Y+PC)	
2007 (barley)	$8.054 \times 10^7 \pm 1.17 \times 10^7$
2007 (beans)	$5.041 \times 10^7 \pm 8.91 \times 10^6$
2008 (wheat)	$5.255 \times 10^7 \pm 2.30 \times 10^7$
2009 (wheat)	$2.959 \times 10^8 \pm 1.29 \times 10^8$
sample date (SD)	
March	$2.310 \times 10^8 \pm 9.74 \times 10^7$
June	$4.587 \times 10^7 \pm 6.82 \times 10^6$
September	$8.238 \times 10^7 \pm 1.86 \times 10^7$
Crop protection (CP)	
ORG	$8.846 \times 10^7 \pm 2.70 \times 10^7$
CON	$1.512 \times 10^8 \pm 2.70 \times 10^7$
Fertility management (FM)	
ORG	$1.040 \times 10^8 \pm 2.97 \times 10^7$
CON	$1.360 \times 10^8 \pm 6.01 \times 10^7$
ANOVA *P*-values	
Y+PC	**0.024**
SD	*0.054*
CP	0.340
FM	0.634
Y+PC*SD	**<0.001**
Y+PC*FM	0.646
Y+PC*CP	0.798
SD*FM	0.637
SD*CP	0.838
CP*FM	0.918
Y+PC*SD*FM	0.849
Y+PC*SD*CP	0.816
Y+PC*FM*CP	0.890
SD*FM*CP	0.824
Y+PC*SD*FM*CP	0.997

Table 5.3. ANOVA results for Shannon diversity indices of 16S rRNA DGGE gel data showing each year individually.

	Shannon diversity index for 16S rRNA DGGE band data (mean + SE)		
year	**2007**	**2008**	**2009**
previous crop (PC)			
barley	2.579 ± 0.06		
beans	2.634 ± 0.02		
sample date (SD)			
March	2.755 ± 0.03	3.127 ± 0.06	3.102 ± 0.11
June	2.692 ± 0.03	2.571 ± 0.06	3.055 ± 0.04
September	2.372 ± 0.07	2.736 ± 0.07	3.031 ± 0.04
Crop protection (CP)			
ORG	2.629 ± 0.04	2.814 ± 0.07	3.049 ± 0.07
CON	2.584 ± 0.05	2.810 ± 0.07	3.076 ± 0.05
Fertility management (FM)			
ORG	2.577 ± 0.05	2.786 ± 0.07	3.052 ± 0.05
CON	2.636 ± 0.04	2.837 ± 0.07	3.073 ± 0.07
ANOVA *P*-values			
PC	*0.056*		
SD	**<0.001**	**<0.001**	0.794
CP	0.107	0.959	0.757
FM	**0.040**	0.496	0.814
PC*SD	**<0.001**		
PC*FM	*0.055*		
PC*CP	0.296		
SD*FM	0.499	0.729	0.992
SD*CP	0.613	0.309	0.802
CP*FM	0.867	**0.038**	0.145
PC*SD*FM	0.916		
PC*SD*CP	0.950		
PC*FM*CP	0.940		
SD*FM*CP	0.482	0.201	0.335
PC*SD*FM*CP	0.350		

Table 5.4. ANOVA analysis of qPCR data for copy number of the 16S rRNA gene showing each year analysed separately.

	16S rRNA copies per g of soil (mean + SE)		
	2007	**2008**	**2009**
previous crop (PC)			
barley	$8.050 \times 10^7 \pm 1.17 \times 10^7$		
beans	$5.040 \times 10^7 \pm 8.91 \times 10^6$		
sample date (SD)			
March	$4.453 \times 10^7 \pm 1.02 \times 10^7$	$1.658 \times 10^7 \pm 4.24 \times 10^6$	$8.196 \times 10^8 \pm 3.58 \times 10^8$
June	$6.316 \times 10^7 \pm 1.13 \times 10^7$	$1.518 \times 10^7 \pm 3.09 \times 10^7$	$4.198 \times 10^7 \pm 1.20 \times 10^7$
September	$8.873 \times 10^7 \pm 1.59 \times 10^7$	$1.259 \times 10^8 \pm 6.652 \times 10^7$	$2.616 \times 10^7 \pm 4.49 \times 10^6$
Crop protection (CP)			
ORG	$5.715 \times 10^7 \pm 8.83 \times 10^6$	$3.118 \times 10^7 \pm 8.73 \times 10^6$	$2.084 \times 10^8 \pm 1.04 \times 10^8$
CON	$7.380 \times 10^7 \pm 1.21 \times 10^7$	$7.393 \times 10^7 \pm 4.53 \times 10^7$	$3.834 \times 10^8 \pm 2.37 \times 10^8$
Fertility management (FM)			
ORG	$6.753 \times 10^7 \pm 1.09 \times 10^7$	$7.326 \times 10^7 \pm 4.54 \times 10^7$	$2.085 \times 10^8 \pm 1.07 \times 10^8$
CON	$6.342 \times 10^7 \pm 1.04 \times 10^7$	$3.185 \times 10^7 \pm 8.70 \times 10^6$	$3.834 \times 10^8 \pm 2.36 \times 10^8$
ANOVA *P*-values			
PC	0.222		
SD	0.665	**0.010**	**0.005**
CP	0.444	0.318	0.241
FM	0.269	0.558	*0.068*
PC*SD	0.571		
PC*FM	0.515		
PC*CP	0.333		
SD*FM	*0.089*	0.435	**0.027**
SD*CP	*0.059*	0.624	0.486
CP*FM	**0.035**	0.159	0.225
PC*SD*FM	**<0.001**		
PC*SD*CP	0.525		
PC*FM*CP	*0.092*		
SD*FM*CP	0.406	0.263	0.288
PC*SD*FM*CP	0.709		

crop of beans). Therefore, 2007 data was separated first (Tables 5.3 and 5.4). However, when 2008 and 2009 data was subjected to ANOVA analysis (Tables 5.5 and 5.6) as one data set, year was still a significant factor for the DGGE data set, with 2009 having the highest bacterial diversity and activity.

Sample date was a significant factor throughout. For both DGGE and qPCR data sets diversity and copy number decreased throughout the sample dates (Tables 5.1 and 5.2). Therefore, in order to ensure no significant interactions were missed, the data was split up by sample date and re-analysed.

5.2.2. The effect of previous crop on the total bacterial community.

To explore the impact of the previous crop on the total bacterial community, the 2007 data set was analysed (Table 5.3 and 5.4). Although, diversity and copy number of the 16S rRNA gene was higher after the barley crop, previous crop was not a significant factor. However, there was a significant interaction with previous crop and sample date in the DGGE data set. When the data was split into sample dates (Table 5.7 and 5.8), previous crop was significant at each date, for the DGGE data set. In March and June diversity was highest following barley and in September diversity was highest following beans. In the qPCR data set barley led to an increase in 16S rRNA gene copy number at every date, although, this was never significant.

5.2.3. Univariate analysis of the effect of fertility and health management, on the total bacterial community, across all sample years.

When sample years are combined fertility and health management do not significantly affect diversity or expression of the total bacterial community. When sample years are analyzed separately (Table 5.3 and 5.4) fertility management significantly affects diversity in 2007 with increased diversity following conventional fertility management.

Table 5.5. ANOVA results for Shannon diversity indices of 16S rRNA gene DGGE gels from 2008 and 2009.

	Shannon diversity index for 16S rRNA DGGE band data (mean + SE)
year (Y)	
2008 (wheat)	2.812 ± 0.05
2009 (wheat)	3.063 ± 0.04
sample date (SD)	
March	3.115 ± 0.06
June	2.813 ± 0.05
September	2.884 ± 0.05
Crop protection (CP)	
ORG	2.931 ± 0.05
CON	2.943 ± 0.05
Fertility management (FM)	
ORG	2.919 ± 0.05
CON	2.955 ± 0.05
ANOVA *P*-values	
Y	**<0.001**
SD	**<0.001**
CP	0.839
FM	0.534
Y*SD	**0.002**
Y*FM	0.793
Y*CP	0.787
SD*FM	0.908
SD*CP	0.409
CP*FM	**0.014**
Y*SD*FM	0.837
Y*SD*CP	0.696
Y*FM*CP	0.801
SD*FM*CP	0.114
Y*SD*FM*CP	0.622

Table 5.6. ANOVA analysis of qPCR data for copy number of the 16S rRNA gene in 2008 and 2009.

	16S rRNA gene copies per g of soil (mean + SE)
year (Y)	
2008 (wheat)	$5.255 \times 10^7 \pm 2.30 \times 10^7$
2009 (wheat)	$2.959 \times 10^8 \pm 1.287 \times 10^8$
sample date (SD)	
March	$4.181 \times 10^8 \pm 1.90 \times 10^8$
June	$2.858 \times 10^7 \pm 6.56 \times 10^6$
September	$7.603 \times 10^7 \pm 3.40 \times 10^7$
Crop protection (CP)	
ORG	$1.198 \times 10^8 \pm 5.33 \times 10^7$
CON	$2.287 \times 10^8 \pm 1.22 \times 10^8$
Fertility management (FM)	
ORG	$1.409 \times 10^8 \pm 5.82 \times 10^7$
CON	$2.076 \times 10^8 \pm 1.196 \times 10^8$
ANOVA *P*-values	
Y	0.154
SD	**0.001**
CP	*0.079*
FM	0.885
Y*SD	**<0.001**
Y*FM	**0.015**
Y*CP	0.905
SD*FM	*0.063*
SD*CP	0.483
CP*FM	0.158
Y*SD*FM	0.429
Y*SD*CP	0.715
Y*FM*CP	**0.004**
SD*FM*CP	0.716
Y*SD*FM*CP	**0.021**

Table 5.7. ANOVA analysis of Shannon diversity indices from 16S rRNA gene gels showing each sample date.

	Shannon diversity index for 16S rRNA gene DGGE band data								
	2007			2008			2009		
	March	June	Sept	March	June	Sept	March	June	Sept
Pre-crop (PC)									
Barley	2.882 ± 0.03	2.821 ± 0.03	2.034 ± 0.04						
Beans	2.627 ± 0.03	2.562 ± 0.04	2.711 ± 0.03						
Crop protection (CP)									
ORG	2.777 ± 0.04	2.698 ± 0.04	2.413 ± 0.10	3.120 ± 0.10	2.508 ± 0.06	2.813 ± 0.10	3.052 ± 0.19	3.045 ± 0.07	3.051 ± 0.04
CON	2.733 ± 0.05	2.686 ± 0.05	2.332 ± 0.09	3.135 ± 0.09	2.634 ± 0.09	2.660 ± 0.11	3.135 ± 0.12	3.065 ± 0.05	3.011 ± 0.07
Fertility management (FM)									
ORG	2.709 ± 0.04	2.685 ± 0.03	2.337 ± 0.10	3.098 ± 0.08	2.584 ± 0.09	2.677 ± 0.13	3.088 ± 0.11	3.041 ± 0.07	3.028 ± 0.07
CON	2.801 ± 0.05	2.699 ± 0.06	2.408 ± 0.09	3.157 ± 0.06	2.559 ± 0.07	2.795 ± 0.08	3.117 ± 0.20	3.069 ± 0.04	3.033 ± 0.04
ANOVA *P*-values									
PC	**<0.001**	**<0.001**	**<0.001**						
CP	0.342	0.813	0.108	0.904	0.305	0.295	0.667	0.823	0.649
FM	**0.057**	0.796	0.154	0.644	0.835	0.414	0.902	0.755	0.957
CP*FM	0.584	0.407	0.514	*0.066*	0.788	*0.091*	0.202	0.941	0.425
FM*PC	0.391	0.191	0.268						
CP*PC	0.696	0.441	0.539						
FM*CP*PC	0.684	0.425	0.271						

Table 5.8. ANOVA analysis of qPCR data for the copy number of the 16S rRNA gene at each individual sample date.

	Shannon diversity index for 16S rRNA DGGE band data								
	2007			2008			2009		
	March	June	Sept	March	June	Sept	March	June	Sept
Pre-crop (PC)									
Barley	$6.188 \times 10^7 \pm 1.88 \times 10^7$	$7.211 \times 10^7 \pm 1.54 \times 10^7$	$1.080 \times 10^8 \pm 2.50 \times 10^7$						
Beans	$2.719 \times 10^7 \pm 5.69 \times 10^6$	$5.421 \times 10^7 \pm 1.66 \times 10^7$	$6.982 \times 10^7 \pm 1.94 \times 10^7$						
Crop protection (CP)									
ORG	$5.407 \times 10^7 \pm 1.86 \times 10^7$	$4.559 \times 10^7 \pm 1.18 \times 10^7$	$7.178 \times 10^7 \pm 1.50 \times 10^7$	$1.449 \times 10^7 \pm 5.91 \times 10^6$	$1.293 \times 10^7 \pm 3.23 \times 10^6$	$6.612 \times 10^7 \pm 2.11 \times 10^7$	$5.713 \times 10^8 \pm 2.80 \times 10^8$	$3.394 \times 10^7 \pm 1.31 \times 10^7$	$1.995 \times 10^7 \pm 5.77 \times 10^6$
CON	$3.500 \times 10^7 \pm 8.24 \times 10^6$	$8.072 \times 10^7 \pm 1.86 \times 10^7$	$1.057 \times 10^8 \pm 2.80 \times 10^7$	$1.868 \times 10^7 \pm 6.39 \times 10^6$	$1.743 \times 10^7 \pm 5.38 \times 10^6$	$1.857 \times 10^8 \pm 1.32 \times 10^8$	$1.068 \times 10^9 \pm 6.73 \times 10^8$	$5.002 \times 10^7 \pm 2.07 \times 10^7$	$3.236 \times 10^7 \pm 6.50 \times 10^6$
Fertility management (FM)									
ORG	$5.455 \times 10^7 \pm 1.75 \times 10^7$	$6.948 \times 10^7 \pm 1.93 \times 10^7$	$7.856 \times 10^7 \pm 2.02 \times 10^7$	$1.554 \times 10^7 \pm 5.67 \times 10^6$	$1.574 \times 10^7 \pm 3.989 \times 10^6$	$1.885 \times 10^8 \pm 1.32 \times 10^8$	$5.632 \times 10^8 \pm 2.92 \times 10^8$	$3.218 \times 10^7 \pm 1.35 \times 10^7$	$3.001 \times 10^7 \pm 5.76 \times 10^6$
CON	$3.452 \times 10^7 \pm 1.03 \times 10^7$	$5.683 \times 10^7 \pm 1.21 \times 10^7$	$9.890 \times 10^7 \pm 2.50 \times 10^7$	$1.762 \times 10^7 \pm 6.68 \times 10^6$	$1.462 \times 10^7 \pm 4.98 \times 10^6$	$6.329 \times 10^7 \pm 2.15 \times 10^7$	$1.076 \times 10^9 \pm 6.67 \times 10^8$	$5.178 \times 10^7 \pm 2.02 \times 10^7$	$2.230 \times 10^7 \pm 6.70 \times 10^6$
ANOVA *P*-values									
PC	0.100	0.919	0.280						
CP	0.153	*0.080*	0.572	0.731	*0.097*	0.513	0.852	0.431	*0.088*
FM	*0.080*	**0.016**	0.311	0.812	0.150	*0.094*	**0.049**	*0.070*	*0.061*
CP*FM	*0.088*	**0.005**	0.854	**0.035**	0.629	**0.005**	0.244	0.749	**0.002**
FM*PC	**0.007**	**0.001**	**0.034**						
CP*PC	0.234	0.746	0.370						
FM*CP*PC	0.796	*0.080*	0.161						

Although conventional crop protection leads to higher copy number of 16S rRNA gene each year, this is never significant. When data is split by sample date (Table 5.7 and 5.8) fertility management is significant on 3 occasions: March 2007 (DGGE), June 2007 (qPCR) and March 2009 (qPCR). In March 2007 and 2009 conventional fertility management led to increased diversity and copy number respectively, and in June 2007 organic fertility management led to increased expression.

5.2.4. Multivariate analysis of DGGE results to further explore the effect of management.

The DGGE results discussed in the above section were found using univariate analysis. This analysis found treatments to be significant if the number of bands present in lanes of the gels changed notably. However, relative intensity scores from DGGE gels can also be analysed using multivariate statistics. These tests look for differences in band patterns and intensities rather than the presence or absence of a particular band. The data was first subjected to indirect analysis to visualise variance between the plots of each gel. This was done using PCA or DCA depending on the length of the DCA axis (axis >3.5 = DCA, axis <3.5 = PCA). PCA and DCA were carried out using the vegan library in the R package. Scores for each axis were taken from R and plots were generated using Minitab (results are summarised in Table 5.9 and full plots can be found in the abstract section F). Scores were also subject to ANOVA to see if treatment had any effect on variance among each axis.

Direct analysis was also carried out so that relative intensity data could be compared with environmental variables. As above the test used was determined by the length of the DCA axis (axis>3.5 = CCA, axis <3.5 RDA). CCA, RDA and Monte Carlo permutation testing were carried out using CANOCO and the results are presented in Figs 5.5-5.6.

Figure 5.5. RDA and CCA analysis of 16S rRNA gene DGGE gels for 2007 soils.

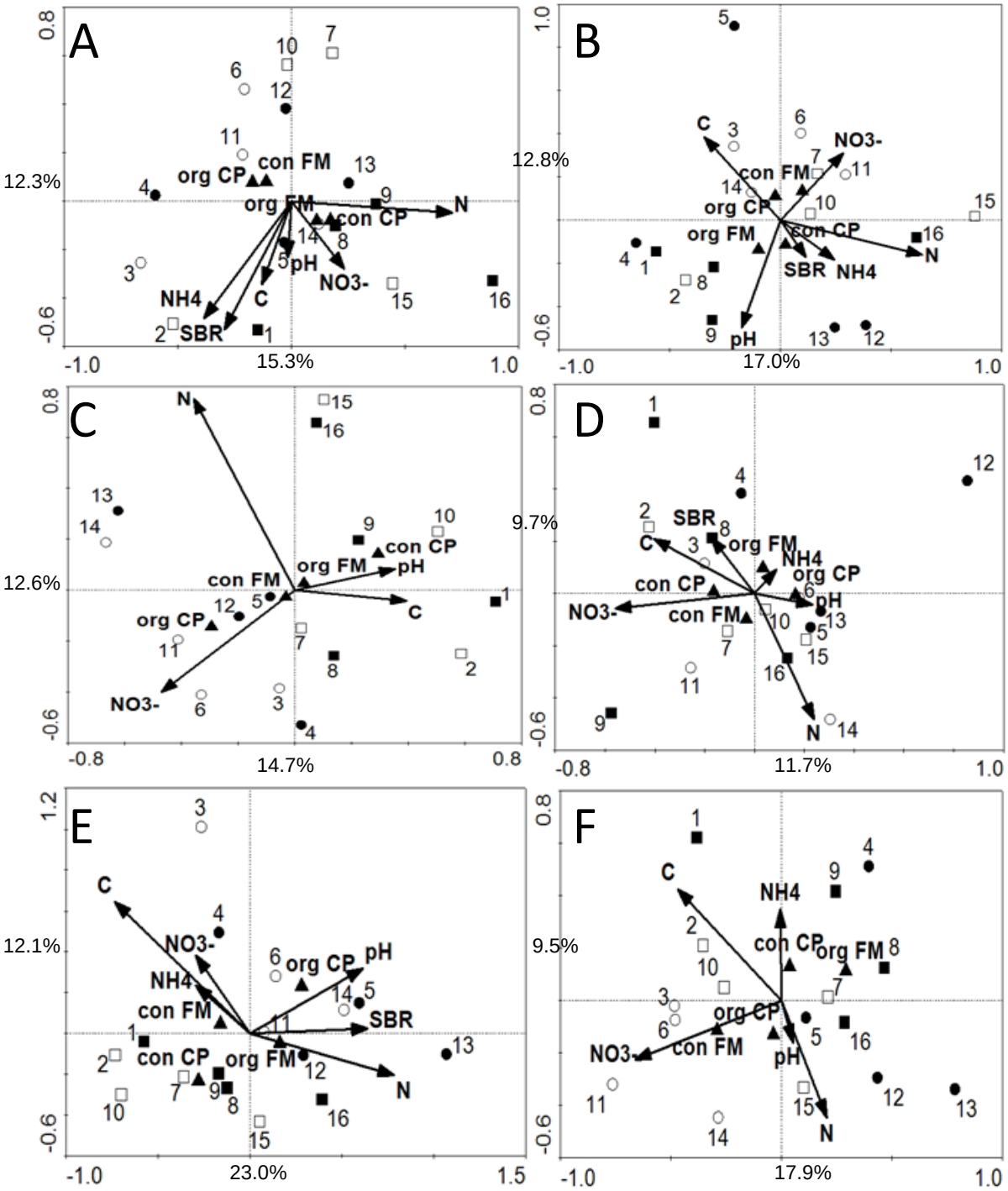


Figure 5.5. RDA of 16S rRNA DGGE gels showing variation between treatments: orgFM orgCP (●), conFM conCP (□), orgFM conCP (■) and conFM orgCP (○). Arrows represent environmental variables. N = total nitrogen, C = total organic carbon, SBR = Soil Basal Respiration, NH4 = available ammonium, and NO3- = available nitrate. Triangles represent centroids for management treatments. A = March soil after beans. B = June soil after beans. N (P=0.010) and pH (P=0.046) are significant factors. C = September soil after beans. N (P=0.018) and CP (P=0.028) are significant factors. D = March soil after barley. E = June soil after barley. C (P=0.032) and CP (P=0.022) are significant factors. F = September soil after barley. FM (P=0.076) is a significant factor. P values are according to Monte Carlo permutation testing.

Figure 5.6. RDA and CCA analysis of 16S rRNA gene DGGE gels for 2008 and 2009 soils.

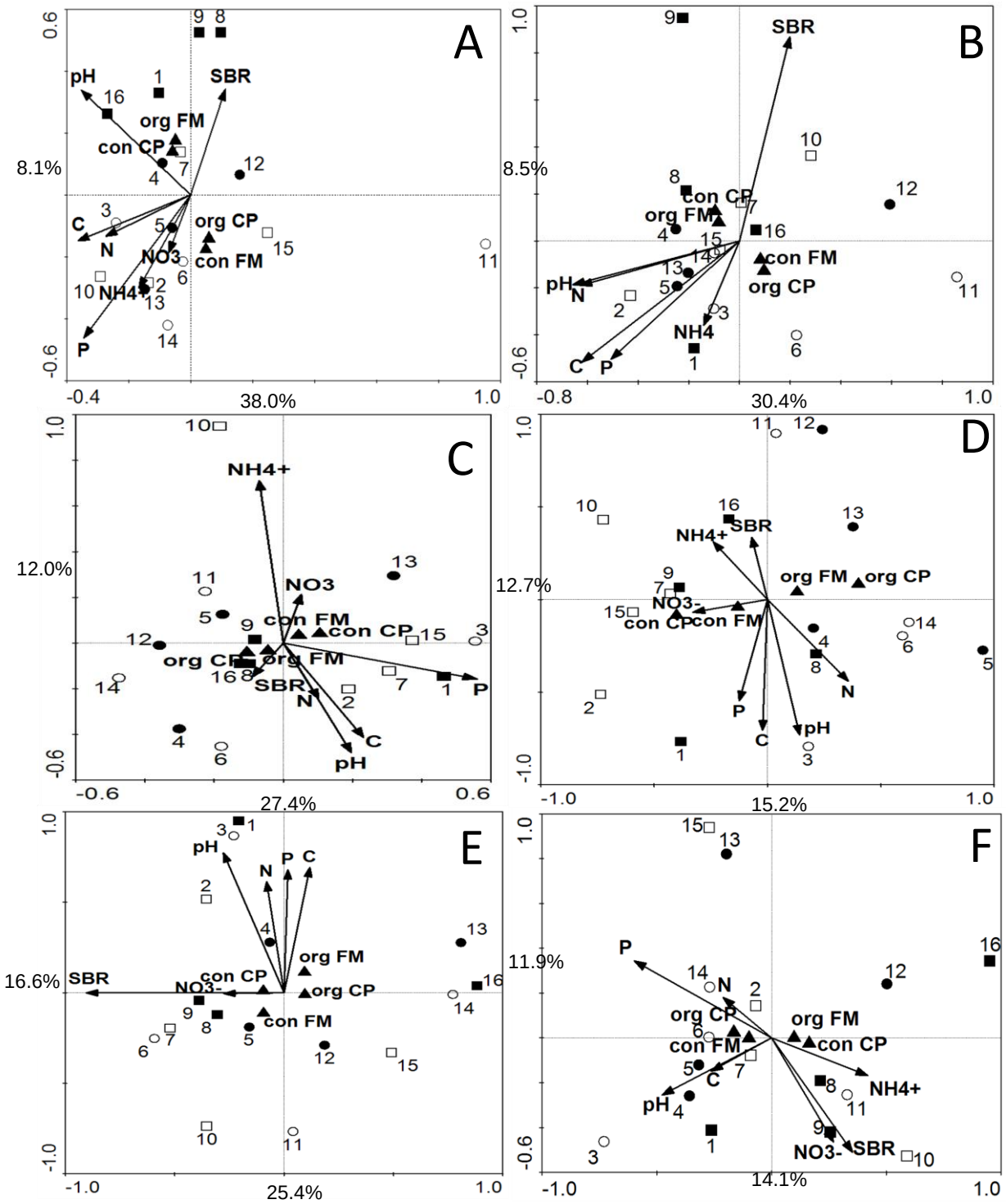


Figure 5.6. RDA of 16S rRNA DGGE gels showing variation between treatments: orgFM orgCP (●), conFM conCP (□), orgFM conCP (■) and conFM orgCP (○). Arrows represent environmental variables. N = total nitrogen, C = total organic carbon, SBR = Soil Basal Respiration, NH4 = available ammonium, and NO3- = available nitrate. Triangles represent centroids for management treatments. A = March 2008 soil. NH4 (P = 0.014) is a significant factor. B = March 2009 soil. C = June 2008 soil. D = June 2009 soil. CP (P = 0.034) is a significant factor. E = September 2008 soil. SBR P=0.002) and pH (P=0.002) are significant factors. F = September 2009 soil. FM (P=0.076) is a significant factor. P values are according to Monte Carlo permutation testing.

Table 5.9. Summary of significant results from all 16S rRNA gene molecular analysis.

		2007		
		March	June	September
direct ordination (RDA and CCA)	Beans	n/s	**N and pH**	n/s
	Barley	n/s	**C and CP**	FM
indirect ordination (PCA and DCA)	Beans	n/s	**FM** and *CP*	n/s
	Barley	n/s	**CP**	n/s
qPCR	Both	**FM**	**FM** and *CP*	n/s

		2008		
		March	June	September
direct ordination (RDA and CCA)	wheat	**NH4**	*C and P*	**SBR, pH** and *P*
indirect ordination (PCA and DCA)	wheat	**FM**	**CP**	**FM**
qPCR	wheat	n/s	*CP*	**FM**

		2009		
		March	June	September
direct ordination (RDA and CCA)	wheat	**C**	**C** and *pH*	*SBR, C and NO3*
indirect ordination (PCA and DCA)	wheat	n/s	**CP**	n/s
qPCR	wheat	**FM**	*FM*	*FM and CP*

Table 5.9. Summary of all significant results to *nifH* molecular analysis. FM = fertility management, CP = crop protection, N = total nitrogen, C = total organic carbon, SBR = Soil Basal Respiration, NH4 = available ammonium, NO3- = available nitrate and P = phosphorus. Bold variables have a *P* value of < 0.05, italicised variables have a *P* value of < 0.1 and n/s shows no significant results were found.

Table 5.10. Summary of Pearson's product moment correlation analysis.

	All years		2007		2008		2009	
	qPCR 16S	H' 16S	qPCR 16S	H' 16S	qPCR 16S	H' 16S	qPCR 16S	H 16S
H' 16S	+		+		-		+	
pH	-	**++**	+	+	+	+	+	+
%N	-	**---**	**---**	-	-	+	+	+
%C	-	**+++**	**++**	+	-	+	+	+
SBR	+	**++**	+	-	+	+	**--**	-
P	**++**	**+++**	**++**	-	-	+	**++**	+
Fe	+	**+++**	**++**	-	-	+	+	+
NH4	-	**--**	+	-	**--**	-	**--**	-
NO3	-	**---**	+	-	-	**---**	**---**	-

+/- = not significant
++/-- = $P < 0.05$
+++/--- = $P < 0.001$

A summary of the results of this analysis is provided in Table 5.9. Indirect ordination revealed crop protection to significantly affect diversity in June of each year. Fertility management significantly affected diversity at sporadic points throughout the 3 years. Direct ordination found the environmental variables total N, total C, pH, soil basal respiration and available ammonium to be significant at different dates with total C and pH being significant on more than one occasion.

5.2.5. Correlation between 16S rRNA gene diversity and copy number, and environmental variables.

Pearson's correlation coefficient was used to examine correlations between copy number and diversity of the 16S rRNA gene and the environmental variables (Table 5.10). When all years were looked at together the most number of significant correlations was seen, especially when looking at the DGGE results. Positive correlations were found between H' and pH, percentage carbon, soil basal respiration and extractable phosphorus and iron. Negative correlations were found between H' and percentage nitrogen, available nitrate and available ammonium. When each date is looked at separately correlations vary and lose significance.

Pearson's correlation coefficient was also calculated to see if there was a link between diversity and expression of *nifH* and diversity and expression of the 16S rRNA gene (Table 5.11). Negative correlation was observed between H' *nifH* and H' 16S rRNA but positive correlation was found between qPCR copy numbers of the two genes.

5.2.6. Community level substrate utilization using BIOLOG plates.

CLSU using Biolog^{TM} microplates was first developed by Garland & Mills in 1991 in order to study the functional diversity of a microbial community in an environmental sample

Table 5.11. Table showing possible correlation between *nifH* results and 16S rRNA gene results.

	H' 16S rRNA	qPCR 16S rRNA	H' *nifH* (RNA)
qPCR 16S rRNA	0.016 0.829		
H' *nifH* (RNA)	**-0.289** **<0.001**	**0.204** **0.004**	
qPCR *nifH* (RNA)	**-0.226** **0.002**	**0.269** **<0.001**	**0.624** **<0.001**

(Klimek & Niklińska, 2007; Garland & Mills, 1991). EcoPlates contain 31 carbon substrate wells in triplicate, and a tetrazolium dye (Klimek and Niklińska, 2007). Growth of aerobic, heterotrophic microorganisms within the wells, indicated by substrate utilisation, results in formazan production due to reduction of the tetrazolium dye. This produces a colour change. The specific pattern of colour change on the plate provides a metabolic fingerprint for the community which allows the effects of different soil management practices on the metabolic diversity to be studied. The CLSU patterns were significantly altered by sample date, pre-crop and crop protection however results were inconsistent. When looking at Shannon's diversity index on the whole data set no significant differences were found between results at different dates or after different pre-crops, although a significant interaction between sample date and crop protection was found (Table 5.12). A significant effect due to crop protection (P=0.016) was seen with soils which were conventionally protected having a higher H'. When the data set is broken up into dates, conventional protection only has a significantly higher H' in September (Table 5.13). A table showing all H' CLSU results can be found in appendix section F.

When analysing data using RDA the only factors affecting the CLSU were the amount of available ammonium in March (P=0.01) and the pre-crop in June (P=0.002) (Figure 5.7).

5.2.7. Correlation between H', copy number and CLSU.

There is no real correlation between 16S rRNA diversity (as measured by H') and 16S rRNA gene expression (as measured by copy number). Pearson's correlation coefficient was very weakly positive ($\rho = 0.016$) with a P value of 0.829. When the years are looked at individually all show no correlation except 2009 which has strong positive correlation ($\rho = 0.378$, $P = 0.008$). 2007 correlations also included CLSU data. There was positive correlation between CLSU data and H' for DGGE, and copy number although this was very weak ($\rho = 0.006$ and 0.105 respectively).

Table 5.12. ANOVA analysis of CLSU data for all sample dates showing the effect of previous crop, sample date and farm management

	Shannon diversity index for CLSU data
Previous crop	
Barley	2.833 ± 0.02
Beans	2.793 ± 0.02
sample date	
March	2.615 ± 0.02
June	2.639 ± 0.01
September	2.646 ± 0.03
Crop protection	
ORG	2.781 ± 0.02
CON	2.845 ± 0.02
Fertility management	
ORG	2.828 ± 0.02
CON	2.799 ± 0.02
ANOVA P-values	
sample date	0.469
pre-crop	0.132
fertility	0.264
crop protection	**0.016**
SD*PC	0.750
SD*FM	0.632
SD*CP	*0.060*
PC*FM	0.500
PC*CP	0.394
FM*CP	0.822
SD*PC*FM	0.972
SD*PC*CP	0.970
SD*FM*CP	0.875
PC*FM*CP	0.249
SD*PC*CP*FM	0.842

Table 5.13. ANOVA of CLSU data showing the effect of previous crop and farm management at each sample date.

	Shannon diversity index for CLSU data		
	March	**June**	**September**
Previous crop			
Barley	2.825 ± 0.02	2.833 ± 0.02	2.840 ± 0.04
Beans	2.758 ± 0.04	2.803 ± 0.02	2.815 ± 0.04
Crop protection			
ORG	2.762 ± 0.04	2.823 ± 0.02	2.757 ± 0.04
CON	2.820 ± 0.03	2.814 ± 0.02	2.902 ± 0.03
Fertility management			
ORG	2.818 ± 0.04	2.837 ± 0.02	2.828 ± 0.04
CON	2.764 ± 0.03	2.832 ± 0.04	2.832 ± 0.04
ANOVA P-values			
PC	0.201	0.217	0.704
FM	0.295	0.134	0.930
CP	0.267	0.709	**0.013**
PC*FM	0.693	0.719	0.666
PC*CP	0.678	0.552	0.574
FM*CP	0.781	0.600	0.762
PC*FM*CP	0.440	0.728	0.443

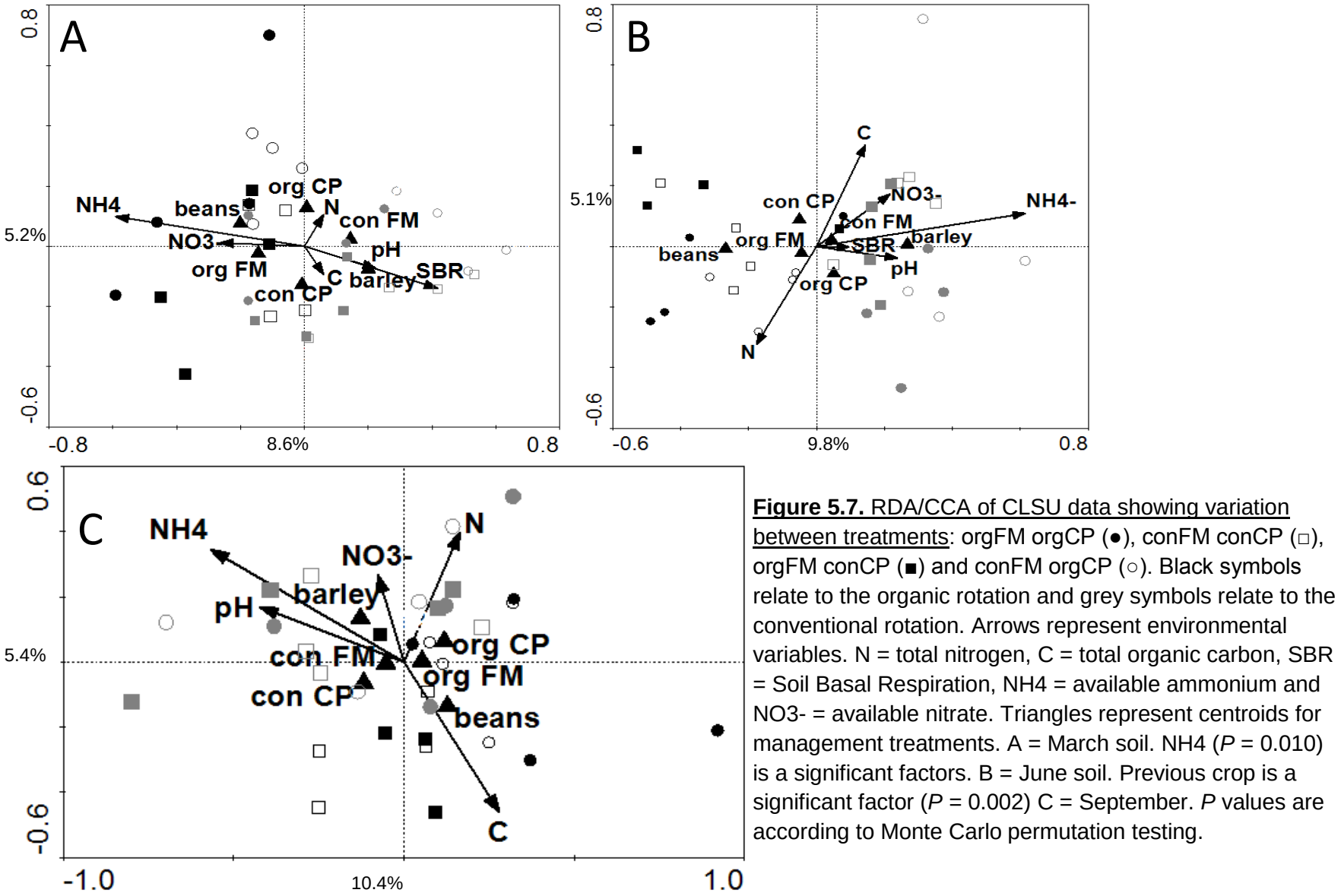


Figure 5.7. RDA/CCA of CLSU data showing variation between treatments: orgFM orgCP (●), conFM conCP (□), orgFM conCP (■) and conFM orgCP (○). Black symbols relate to the organic rotation and grey symbols relate to the conventional rotation. Arrows represent environmental variables. N = total nitrogen, C = total organic carbon, SBR = Soil Basal Respiration, NH4 = available ammonium and NO3- = available nitrate. Triangles represent centroids for management treatments. A = March soil. NH4 (P = 0.010) is a significant factors. B = June soil. Previous crop is a significant factor (P = 0.002) C = September. P values are according to Monte Carlo permutation testing.

5.3. Discussion

5.3.1. The effect previous crop has on the total bacterial community.

The 2007 data set details changes in the total bacterial community under two different rotations. The organic rotation soil previously had beans growing on it and the conventional rotation soil previously had barley growing on it. We hypothesised that the soil community would be affected by the previous crop and the different rotations, and that increased diversity and expression would be seen in the organic rotation. Changes in the plant species growing in soil have been shown to lead to changes in the soil bacterial community as they release different quantities and qualities of carbon (Ladygina and Hedlund, 2010). However, this may only be significant in the rhizosphere as the effect of the plant could be masked by a whole host of other environmental factors such as the effects of fertilizers and pesticides, in bulk soil (Ostle *et al*., 2003).

Organic rotations in general are thought to have a 'positive' effect on microbial diversity due to the presence of ley periods allowing soil nutrients to recover (Acosta-Martinez *et al*., 2008). The presence of legumes within the rotation has repeatedly been shown to lead to increased microbial diversity and activity, using BIOLOG and other culture-dependent methods, due to their root exudates being rich in nitrogen (Wardle *et al*., 2003; Warembourg *et al*., 2003; Lupwayi *et al*., 1998).

When looking at the diversity of the soil bacterial community across the whole of 2007 (using Shannon diversity index of DGGE gels) previous crop is almost a significant factor (P=0.056, Table 5.3) with H' in the organic rotation being higher than the conventional rotation. When each date is analyzed separately (Table 5.7) previous crop is significant at every date with the beans crop leading to increased H' in September and the barley crop leading to increased H' in March and June.

When qPCR and BIOLOG data was analysed it was found that the barley crop led to increased 16S rRNA copy number and increased heterotrophic activity (Table 5.4, 5.8 and 5.12), disagreeing with our hypothesis. Although the only occasion this is significant is when CLSU data is analyzed in June (Figure 5.7).

Clearly the effect of previous crop on the total bacterial community is not as evident as its effect on the diazotrophic community showing diazotrophs in particular were affected by the differences between the barley and beans crops as discussed in results chapter 2 (Tables 4.8 and 4.10). The fact that the effect of previous crop is often non-significantly affects the activity of the microbial community is probably due to the fact the bulk soil was investigated rather than soil of the rhizosphere. Previous studies have found, when looking at bulk soil, that although changing the previous crop leads to small changes in the microbial community, this effect is often overshadowed by changes due to factors such as fertility management (Esperschutz *et al*., 2007; Hartmann *et al*., 2006).

Larkin (2003) and Larkin and Honeycutt (2006) did observe differences in bacterial communities due to previous crop in bulk soil. Their results also supported increased bacterial diversity and activity after a barley crop compared to other cropping systems including rotations containing legumes (although the barley containing rotation was not significantly higher than the legume containing rotation). This mirrors results from our study (Tables 5.7 and 5.8). It is suggested that grain crops lead to increased diversity and support greater biomass as microorganisms here utilized more amino acids and amines than carbohydrates (Larkin, 2003). It is thought that the barley crop alters soil physical, chemical or biological characteristics which, in turn leads to stimulation of the microbial community and a lower incidence of plant disease (Larkin and Honeycutt, 2006).

5.3.2. The effect of treatments on the diversity and activity of the total bacterial community

5.3.2.1. Fertility management

Diversity of the soil microbial community was not affected significantly by fertility management on many occasions. When looking at Shannon diversity indices for DGGE results, conventional management had a higher H' at all dates except June 2008 (Tables 5.7). Although, the only occasion this was significant was in March of 2007 (Table 5.7). Table 5.14 shows that fertility management was not responsible for large percentage of variation within the direct ordination plots and that, on average, fertility management caused a higher percentage variance in September than other months.

Fertility management seemed to result in more changes in the expression of the 16S rRNA gene (as measured by qPCR). Although results were not consistent, on average, across all of the sample dates, conventional fertility management did appear to be a driver for increased 16S rRNA gene expression (Table 5.2). The CLSU also showed conventional fertility management to result in slight increased H' although results for both fertility managements were very similar (Table 5.12).

We hypothesised that organic fertility management would result in increased soil microbial diversity and activity, due to the more neutral pH seen in organic plots and the increased organic substrates provided by manure (Hartmann *et al*., 2006). However, overall it would have to be concluded that no trend could be seen towards increased diversity and expression of 16S rRNA gene due to either management as both were significant on different occasions (Tables 5.8).

When looking at Table 5.10 it is clear that there are many correlations, particularly with 16S rRNA gene diversity and environmental variables associated with fertility

Table 5.14. Table showing summary of percentage variance in all 16S rRNA gene RDA/CCA plots caused by fertility management in direct ordination plots.

	March	June	September	Average
2007 (Beans)	6.6	6.9	4.2	5.9
2007 (Barley)	3.1	6.2	11.3	6.9
2008	5.8	4.5	9.3	6.5
2009	4.7	4.4	5.8	5.0
Average	5.1	5.5	7.7	6.1

Table 5.15. Summary of percentage variation in all 16S rRNA gene RDA/CCA plots caused by fertility management and associated variables.

	March	June	September	Average
2007 (Beans)	39.1	46.5	35.1	40.2
2007 (Barley)	34.6	43.3	38.8	38.9
2008	55.2	56.4	45.8	52.5
2009	42.5	43.7	48.6	44.9
Average	42.9	47.5	42.1	44.1

management, such as; total C, total N, pH, soil basal respiration, available phosphorus, and concentrations of ammonium and nitrate. This is also reflected in RDA/CCA plots. Table 5.15 shows that on average fertility management and associated environmental variables were responsible for 44.1% of diversity variance.

Total C, pH soil basal respiration, available phosphorus and available iron all had positive correlation with 16S rRNA gene diversity (Table 5.10). Meaning, when these variables were increased, bacterial diversity increased. Increased pH, percentage carbon and soil basal respiration are all associated with organic fertility management (Table 3.2). A study looking at 98 soils across North and South America found that pH was the biggest predictor of bacterial diversity and that on average bacterial diversity was higher in soils which had a near neutral pH (Fierer and Jackson, 2006). This is said to be mainly attributed to changes in 3 of the most common groups of soil organisms; *Acidobacteria*, *Actinobacteria* and *Bacteroidetes* (Lauber *et al*., 2009).

Carbon was a significant driver of variance in 4 of the 9 sample dates and is positively correlated to 16S rRNA gene diversity. Several studies have suggested that an increase in organic carbon is related to an increase in soil diversity and suggest this is the reason why organic fertility management results in increased microbial diversity as increased degradation occurs (Jangid *et al*., 2008; Lejon *et al*., 2007; Hartmann *et al*., 2006). There is a positive correlation with carbon and bacterial diversity (Table 3.10). However, in NFSC percentage carbon is not significantly higher in organically fertilised soils. This is possibly one of the reasons increased microbial diversity is not seen in the organic plots of NFSC. This is supported by Hallin *et al*., (2009) who suggests that the microbial community structure changes in response to pH, soil C and soil N rather than management practices.

Soil basal respiration is also positively associated with 16S rRNA gene copy number and diversity and is a significant factor in September of 2008 and 2009 (Table 5.9). This is

logical as increased soil basal respiration shows increased carbon dioxide emitted from the soil by microorganism and macroorganisms (Vanhala *et al*., 2005). Soil basal respiration is significantly higher under organic fertility management compared to conventional fertility management in NSFC. However, this is not mirrored when looking at results of diversity and activity of the 16S rRNA gene suggesting perhaps increased soil basal respiration in organic fertility management is due to changes in the fungal and plant community (Moyano *et al*., 2007).

Increased available phosphorus and iron are associated with conventional fertility management (Gosling and Shepherd, 2005) (Table 3.2). Phosphorus is a significant driver of diversity in June and September of 2008 and both phosphorus and iron are positively correlated with 16S rRNA gene diversity (Table 5.9 and 5.10). Increases in soil nutrients often lead to distinct microbial communities (Hartmann *et al*., 2006). This is seen in this study as, regardless of fertility management type, the application of fertilizers leads to a change in the bacterial community from June onwards (Table 5.1-5.4). Phosphorus is an important resource to bacteria as it is required to produce energy in the form of ATP and is therefore often a growth limiting factor (Zavaleta-Pastor *et al*., 2009). Sugiyama *et al*., (2008) suggest that phosphorus influences soil bacterial diversity more than vegetation, as it more directly affects soil fertility and drives changes in bacterial richness.

Past studies have found that, like carbon, increased nitrogen increases microbial diversity as increased degradation occurs (Jangid *et al*., 2008; Lejon *et al*., 2007). However, when organic nitrogen is converted to percentage nitrogen a negative correlation with microbial diversity can be observed, possibly due to microorganisms struggling to decompose organic matter with a high C:N ratio (Lejon *et al*., 2007). This is also seen in our study (Table 5.9), nitrogen species (Percentage nitrogen, available ammonium and available nitrate) are negatively associated with 16S rRNA gene diversity and copy number, and

are drivers of diversity in June 2007 (Percentage N), March 2008 (ammonium) and September 2009 (nitrate).

Available ammonium and nitrate are significantly higher in plots under conventional fertility management (Table 3.2). Decreases in bacterial numbers have been observed when increased nitrogen was applied to crops, with bacterial numbers being most greatly reduced after N addition of 180 kgN/ha (Dong *et al*., 2008). Soil at NFSC also receives 180 kgN/ha. This could be the reason why bacterial diversity and activity is reduced in June (Table 5.1 and 5.2) following the application of fertilizers.

In summary the two fertility managements appear not to significantly affect the microbial community. However, factors associated with fertility management do influence bacterial diversity and activity. It is possible that different factors within one management type are antagonistic and therefore no overall effect is observed. For example within the conventional fertility management system there is increased phosphorus, which has a 'positive' effect on the bacterial community, and a more acidic pH, which has a 'negative' effect on the bacterial community, so overall no significant change is observed.

5.3.2.2. Crop protection

When looking at indirect and direct ordination analysis (Table 5.9) crop protection is a significant factor in June of all years. Although overall crop protection is only responsible for 7.6% of the variation within the RDA/CCA plots this does increase to an average of 10.4% in June (Table 5.16). Overall analysis of Shannon diversity indices of DGGE data shows that organic crop protection leads to slightly increased bacterial diversity although this is not significant (Table 5.1). This suggests that although crop protection is causing a change in the structure of the bacterial community there is neither an increase nor decrease in diversity. However, CSLU (Table 5.12 and 5.13) and qPCR analysis (Table 5.8) show significant positive effects due to conventional health management.

Table 5.16. Summary of the percentage variance in 16S rRNA gene RDA/CCA plots caused by crop protection.

	March	June	September	Average
2007 (Beans)	5.7	7.7	11.4	8.3
2007 (Barley)	6.2	14.1	4.7	8.3
2008	6.1	8.0	3.9	6.0
2009	5.9	11.7	5.3	7.6
Average	6.0	10.4	6.3	7.6

Examining all the results together it would have to be concluded that crop protection may affect bacterial community structure immediately after pesticides are applied (June sample dates), but that this is never significant. This is supported by work carried out by Bending *et al*. (2007), which found, by analyzing 16S rRNA and 18S rRNA DGGE profiles, that pesticides resulted in the removal of certain Eukaryotes but did not alter the bacterial community structure. It is also possible that diversity did not change significantly as although certain bacteria were diminished by the toxicity of the fertilizer, others proliferated in the vacant niches (Johnsen *et al*., 2001).

The hypothesis that organic crop protection would lead to increased diversity and expression of the bacterial community due to the toxic effect of pesticides is not supported as conventional health management led to a more active bacterial community (as measured by qPCR and BIOLOG). Previous studies have observed an increase in cfu directly following the application of pesticides (Cycon and Piotrowska-Seget, 2007). Pesticides can stimulate heterotrophic bacteria as they can be used as a source of energy, carbon and nutrients as the bacteria decompose the chemicals. The death of more sensitive members of the bacterial community could provide increased access to resources, including organic C, for the non-sensitive members (Cycon and Piotrowska-Seget, 2007). This suggests why BIOLOG data showed a positive response to conventional crop protection as BIOLOG studies select for fast growing, heterotrophs.

It is also possible that the organic fungicide copper oxychloride used in the organic plots has had a negative effect on 16S rRNA gene expression. However, studies on copper oxychloride have found that it only significantly affects bacterial communities in concentrations over 100 mg/kg (Du Plessis *et al*., 2005). Annual rates of application total 6 kg Cu ha^{-1} or approximately 3 mg Cu kg^{-1} soil in the NFSC experiments, suggesting that Cu levels in the system are well below safe limits for bacterial communities.

Finally, it is possible that the application of both fertilizers and pesticides could have resulted in the decreases in diversity and copy number particularly in June (Table 5.9). Girvan *et al*. (2004) showed that although the effect of fertilizer and pesticide application was not significant soil activity and diversity was initially decreased after application. This could suggest why negative correlation was observed with percentage nitrogen, available ammonium and available nitrate, and diversity and expression of the 16S rRNA gene.

5.3.3. The effect of seasonal variation on the diversity and activity of the total bacterial community

Sample date is often a significant factor when analyzing the 16S rRNA gene data and is summarised in Figures 5.8 and 5.9. As discussed in results chapters 1 and 2 soil temperature changes throughout the sample year (Figure 4.17). Pearson's correlation coefficient analysis showed significant negative correlation between diversity (as measured by H' from DGGE gels) and temperature (ρ=-0.151 *P* =0.037). Weak negative correlation was also observed between 16S rRNA gene copy number and temperature (ρ=-0.013 and *P* = 0.857).

When looking at the effect of fertility management Jangid *et al*. (2008) also found that diversity seemed to trail off from winter to summer. They also found that, in general, Gram positive organisms favoured winter and Gram negatives proliferated in summer. This was also observed in alpine environments by Lipson and Schmidt (2004). They observed higher microbial biomass in winter and early spring due to changes in substrate availability and temperature. Winter communities comprised of large numbers of cold tolerant *Actinobacteria* and summer communities contained more phototrophs and oligotrophs (Lipson and Schmidt, 2004). However, as mentioned in the above section it is more likely that the seasonal effects are due to the application of fertilizers and pesticides, regardless of management type, before June.

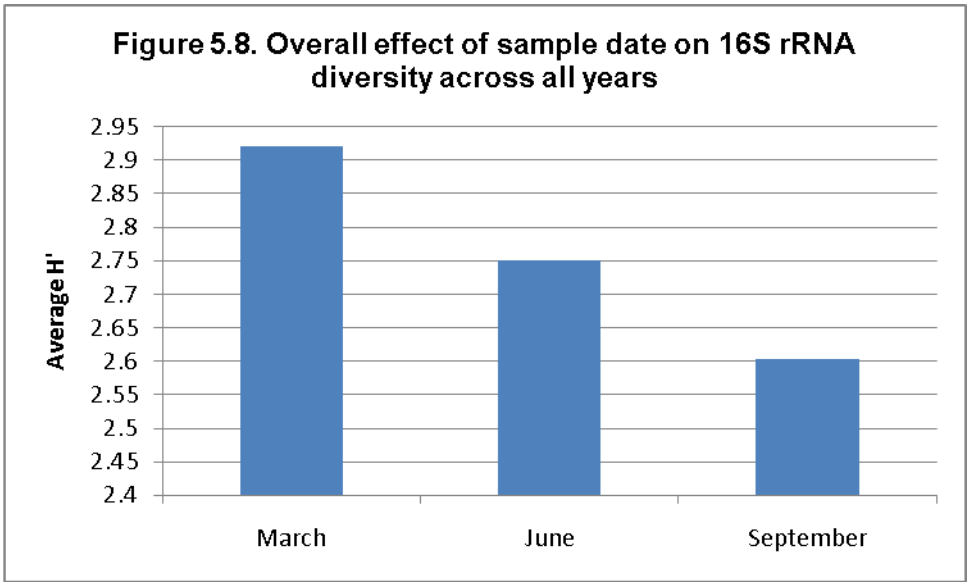


Figure 5.8. Overall effect of sample date on 16S rRNA diversity across all years
Average H'
2.95
2.9
2.85
2.8
2.75
2.7
2.65
2.6
2.55
2.5
2.45
2.4
March
June
September

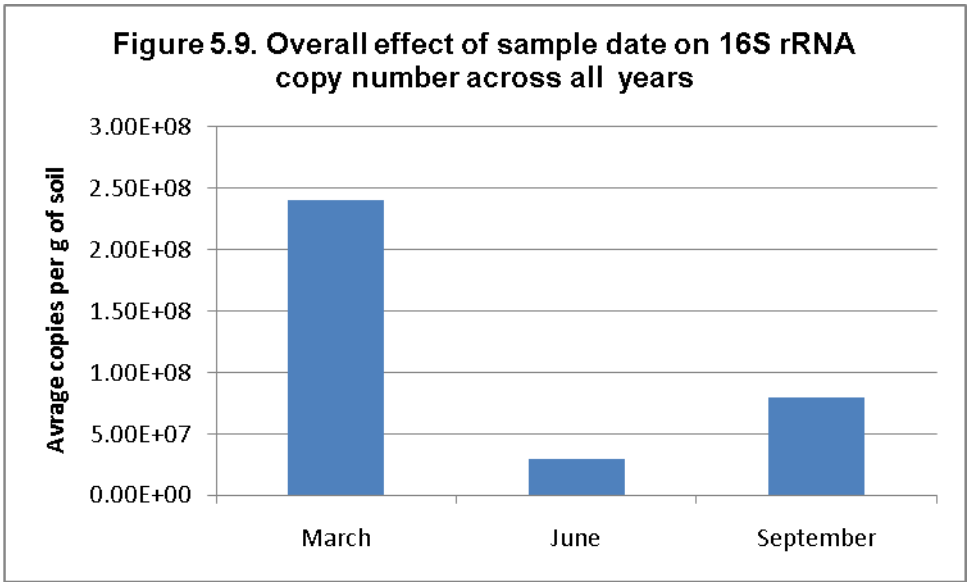


Figure 5.9. Overall effect of sample date on 16S rRNA copy number across all years
Avrage copies per g of soil
3.00E+08
2.50E+08
2.00E+08
1.50E+08
1.00E+08
5.00E+07
0.00E+00
March
June
September

5.3.4. Correlation between activity and diversity of the *nifH* and 16S rRNA genes.

Table 5.11 shows there is negative correlation between *nifH* and 16S rRNA diversity, and positive correlation between *nifH* and 16S rRNA gene copy number. However, the overall findings of the two studies were different. The *nifH* community was slightly positively affected by conventional fertility management and organic crop protection. The 16S rRNA community was not affected by fertility management, but was positively affected by conventional crop protection. There is clearly some correlation between the two communities probably driven by general factors such as pH and available carbon. Hallin *et al*. (2009) observed a direct comparison between changes in the 16S rRNA community and denitrifying genes when looking at the effect of fertility management. However, the difference in findings suggests that results described in results chapter 2, for example the positive effect of organic crop protection and the barley pre-crop, are due to influences of the treatments on the nitrogen fixing community only and are not simply a result of changes to the whole bacterial community.

5.4. Conclusion.

In conclusion the total bacterial community are more significantly affected by environmental variables normally associated with fertility management for example pH and organic carbon than the different fertility managements as whole treatments. Positive effects of both organic and conventional fertility managements cancelled out any overall effect on the community as a whole. Pesticide application and seasonal changes did have significant affects on the soil bacterial community with the soils in March being the most active and diverse. Conventional pesticide application resulted in increased soil bacterial activity (as measured by qPCR and BIOLOG plates). Suggesting the majority of organisms within the soil are capable of degrading the pesticides applied and utilizing them as a carbon and energy source.

It is also important to note the difference between results in results chapter 2, diazotrophs react positively to organic crop protection and the barley previous crop, and results chapter 3, the total bacterial community responds positively to conventional crop protection, which suggest that although there are similarities overall the two communities react independently to the different treatments.

6. Results chapter 4 – Analysing sequence data to investigate the structure of the nitrogen fixing community.

6.1. Introduction.

This results chapter aims to support results from DGGE gels discussed in results chapter 2 by analysing the structure of the diazotrophic community using sequencing. It is possible that the amount of diversity present is neither higher nor lower within two samples but that the members of the community present may change. By sequencing bands which appear to be significant within the DGGE gel the members of the community which are causing the most change within a sample date may be identified.

Previous studies looking at diazotrophs present within soil environments have shown that it is necessary to use sequencing techniques to attempt to identify specific organisms, as of the 49 groups of bacteria which contain diazotrophs only 22 of these contain culturable species (Buckley *et al*., 2007). It has also been suggested that it is the uncultured members of the diazotrophic community which are responsible for the most nitrogen fixation and are predominant within agricultural soils (Hsu and Buckley, 2009; Roesch *et al*., 2008; Buckley *et al*., 2007).

It could be expected that the soils taken from the Nafferton field trial would contain a diverse group of diazotrophs. Previous studies suggest that the identifiable example may mainly belong to the *Alpha* and *Beta-proteobacteria* as well as examples of *Actinobacteria*, *Gamma-proteobacteria* and *Firmicutes* (Buckley *et al*., 2007; Demba Diallo *et al*., 2004). Predominant species may include: *Azospirillum*, *Azotobacter*, *Azoarcus*, *Bradyrhizobium*, *Rhizobium* and *Frankia* (Xiao *et al*., 2010; Wartiainen *et al*., 2008; Coelho *et al*., 2008; Knauth *et al*., 2005).

Although there are suggestions that the overall diazotrophic community reacts positively to low levels of nitrogen fertilizer as opposed to high levels of nitrogen fertilizer (Coelho *et*

al., 2009; Coelho *et al*., 2008), there is limited evidence of how individual species of bacteria may react to changes in management. There is some evidence that members of the *Azotobacter* genus may be inhibited by increased levels of N fertilizer and soil amendments, but this is by no means unequivocal (Ogilvie *et al*., 2008; Burgmann *et al*., 2005). It is hoped that by sequencing DGGE bands responsible for variation in band patterns within the sample dates key members of the community which are influenced by the different management techniques can be identified.

6.2. Results.

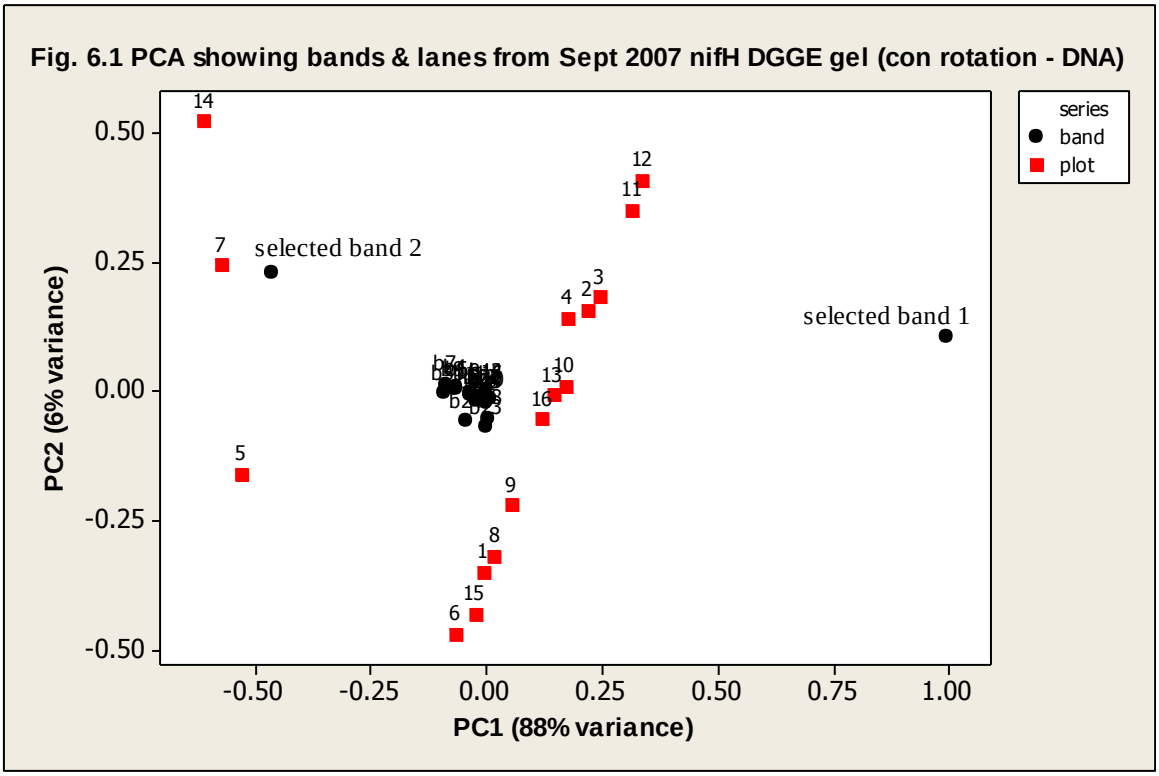
6.2.1. Obtaining sequence data from bands of interest.

Due to time constraints and the number of bands present in the DGGE gels it was not possible to sequence all *nifH* bands. PCA analysis was carried out showing each individual band on the gel (An example of this is shown in Fig. 6.1). It was found that in each gel there were a number of bands which would separate from the main cluster of bands. It is assumed that these bands caused the most variation within lanes of the gel. These bands were therefore chosen for sequence analysis. Bands which were chosen for analysis are shown in Figs 6.2-6.21. Several of the bands of interest could not be sequenced and are circled in red. The ones which could be sequenced are circled in black.

In order to gain bands for sequencing PCR products had to be separated on DGGE gels several times. During this process it was noted that some of the bands of interest were also present in other gels, although in other gels they are not outliers according to PCA. These bands are circled in blue in Figs 6.2-6.21.

In total 22 different sequences were found across all of the soil samples. These sequences are shown in Table 6.1. Table 6.2 also shows the closest match according to the BLAST database as of 18/11/2010. If this match did not belong to an identifiable

Figure 6.1. PCA analysis of all bands in September 2007 *nifH* DGGE gel (conventional rotation - DNA)



This figure shows principal componenet analysis of September 2007 *nifH* DGGE gel from the conventional rotation when DNA was used as the nucleic acid. This figure correlates with figure 6.15. Two bands separate from the overall band cluster. It is assumed that these two bands are responsible for the most amount of variance and are therefore chosen for sequencing. Referring to figure 6.15 selected band 1 is the red highlighted band which could not be reamplified and selected band 2 is the black highlighted band 'B1'.

DGGE gels showing *nifH* amplified from RNA from soils in the organic rotation (potatoes/beans)

Fig. 6.2 *nifH* DGGE image for March 2007 soil from the organic rotation

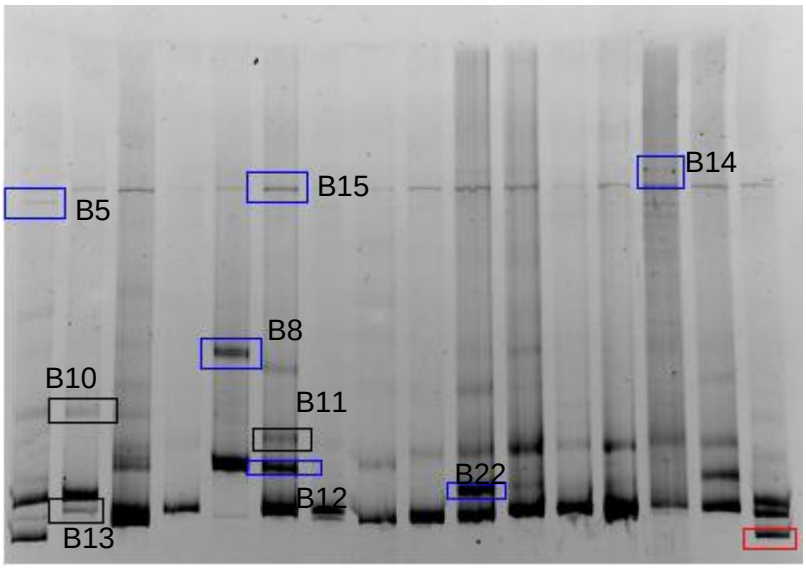
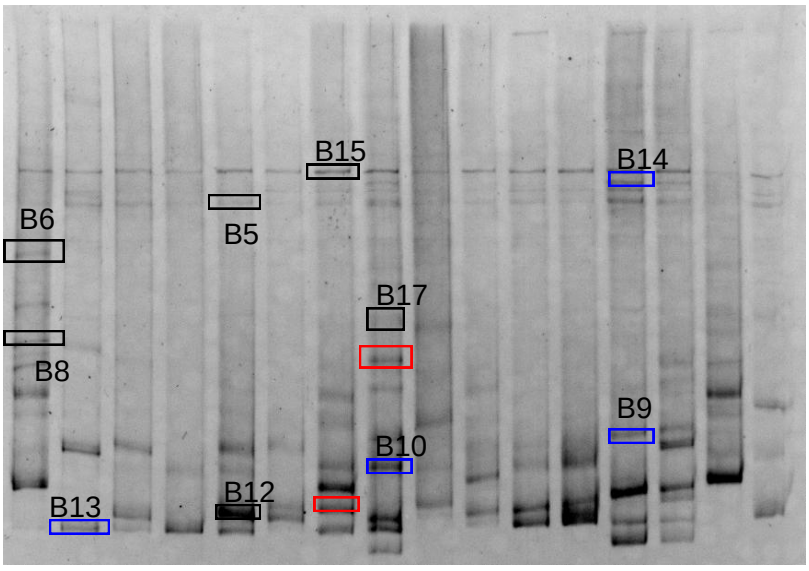


Fig. 6.3 *nifH* DGGE image for June 2007 soil from the organic rotation



For all images

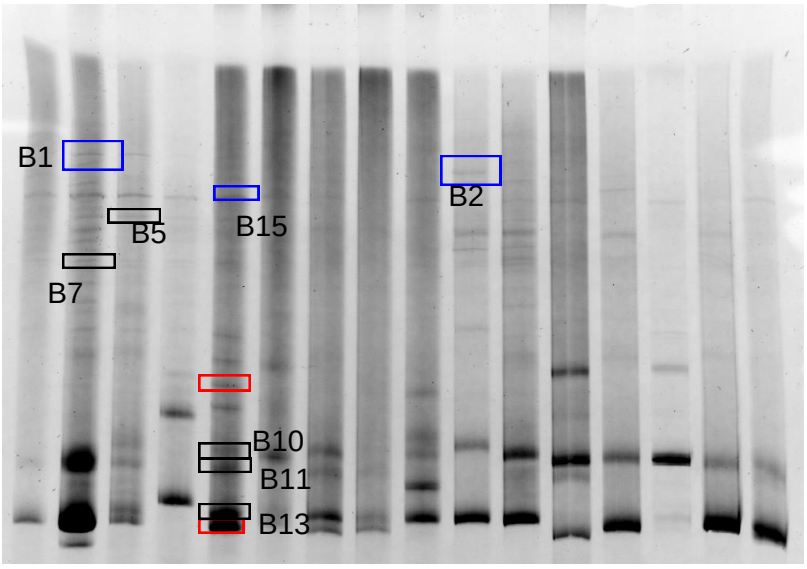
Significant band which has been sequenced

Sequenced band which is not significant in the gel

Significant band which could not be sequenced

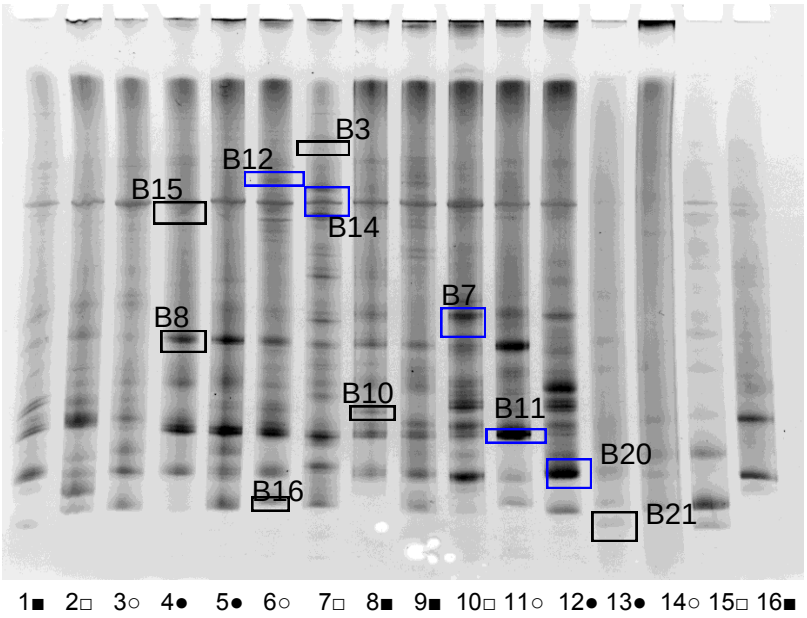
For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

Fig. 6.4 *nifH* DGGE image for September 2007 soil from the organic rotation



DGGE gels showing *nifH* amplified from RNA from soils in the conventional rotation (potatoes/winter barley)

Fig. 6.5 *nifH* DGGE image for March 2007 soil from the conventional rotation



For all images

Significant band which has been sequenced

Significant band which could not be sequenced

Sequenced band which is not significant in the gel

For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

Fig. 6.6. *nifH* DGGE image for June 2007 soil from the conventional rotation

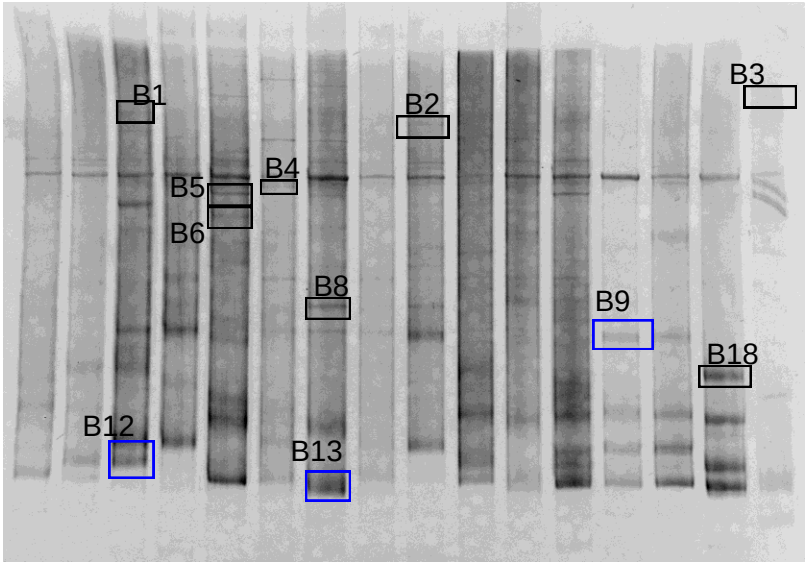
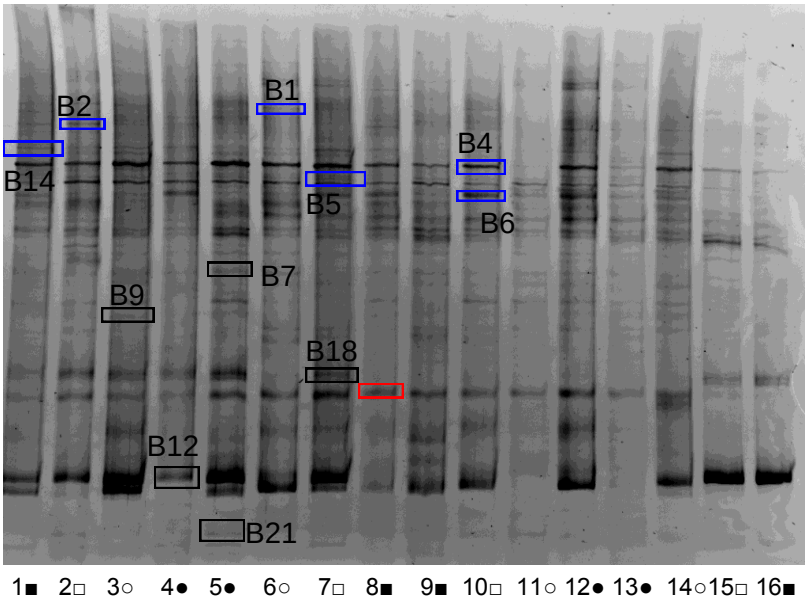


Fig. 6.7. *nifH* DGGE image for September 2007 soil from the conventional rotation



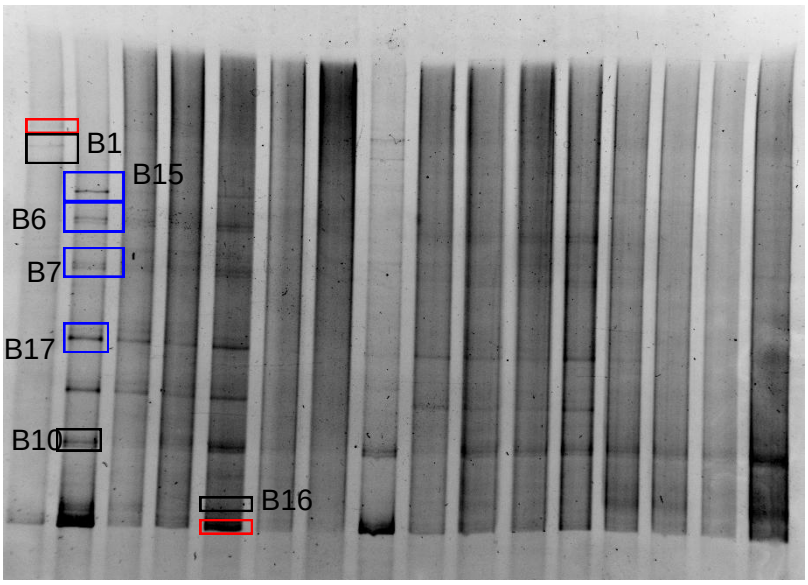
For all images

Significant band which has been sequenced	Significant band which could not be sequenced	Sequenced band which is not significant in the gel of interest

For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

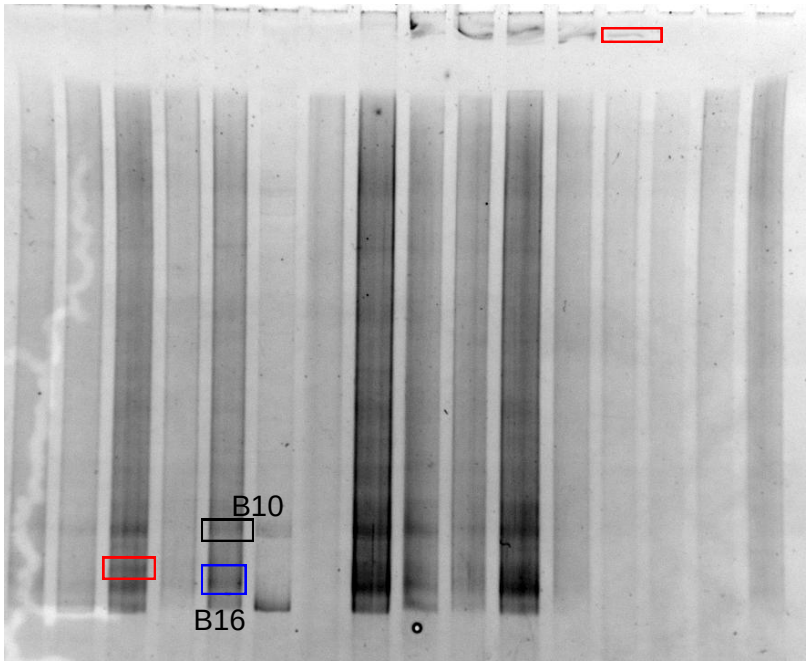
DGGE gels showing *nifH* amplified from RNA from 2008 soils

Fig. 6.8. *nifH* DGGE image for RNA from March 2008 soil



1■ 2□ 3○ 4● 5● 6○ 7□ 8■ 9■ 10□ 11○12● 13●14○15□16■

Fig. 6.9. *nifH* DGGE image for RNA from June 2008 soil



1■ 2□ 3○ 4● 5● 6○ 7□ 8■ 9■ 10□ 11○ 12● 13● 14○ 15□ 16■

For all images

Sequenced band which is not significant in the gel	Significant band which could not be sequenced	Significant band which has been sequenced

For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

DGGE gels showing *nifH* amplified from RNA from 2009 soils

Fig. 6.10. *nifH* DGGE image for RNA from March 2009 soil

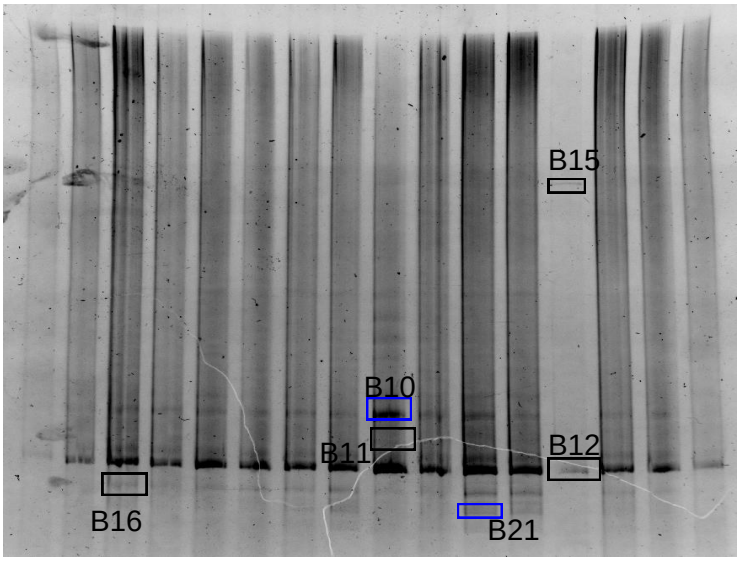
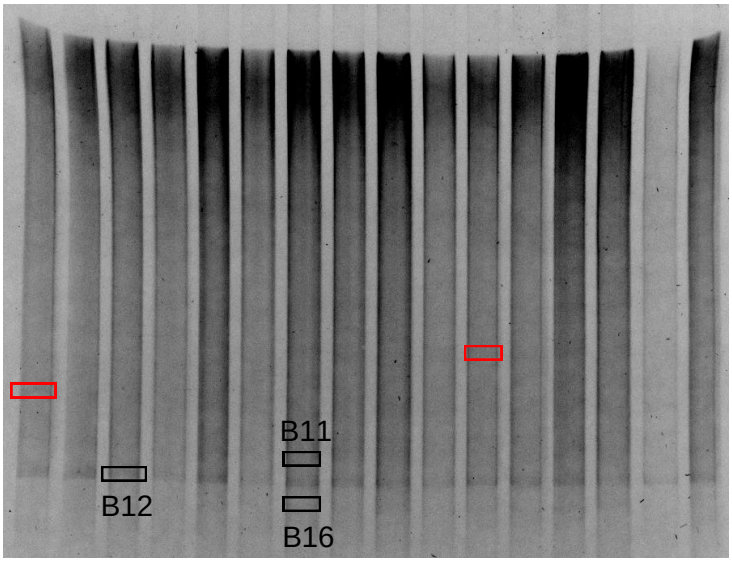


Fig. 6.11. *nifH* DGGE image for RNA from June 2009 soil



For all images

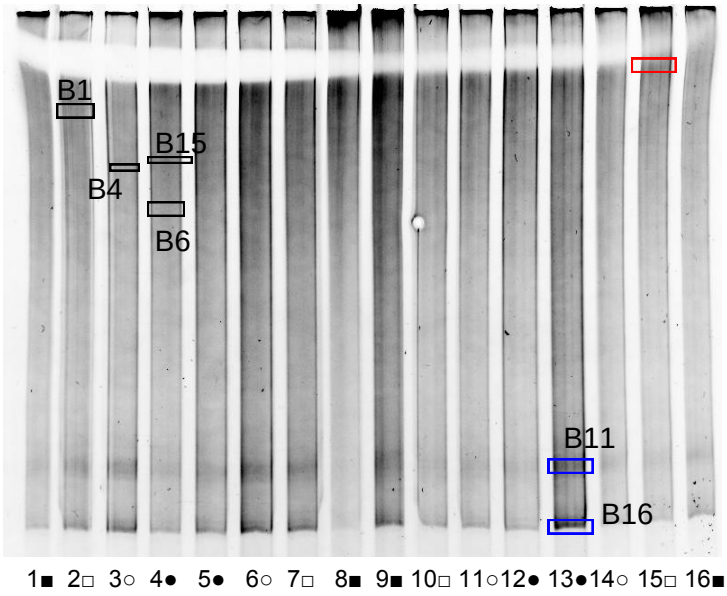
Significant band which has been sequenced

Significant band which could not be sequenced

Sequenced band which is not significant in the gel of interest

For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

Fig. 6.12. *nifH* DGGE image for RNA from September 2009 soil



For all images

Significant band which has been sequenced

Significant band which could not be sequenced

Sequenced band which is not significant in the gel of interest

For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

DGGE gels showing *nifH* amplified from DNA from 2007 soils in the conventional rotation

Fig. 6.13. *nifH* DGGE image for DNA from March 2007 soil from the conventional rotation

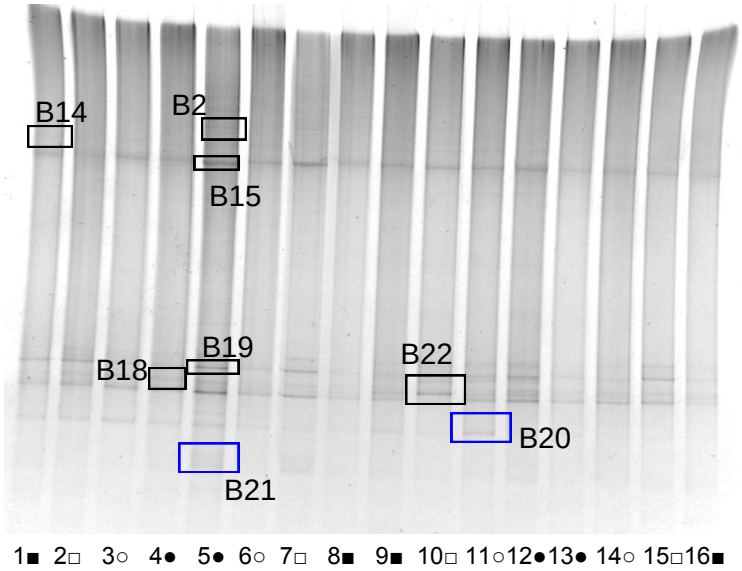
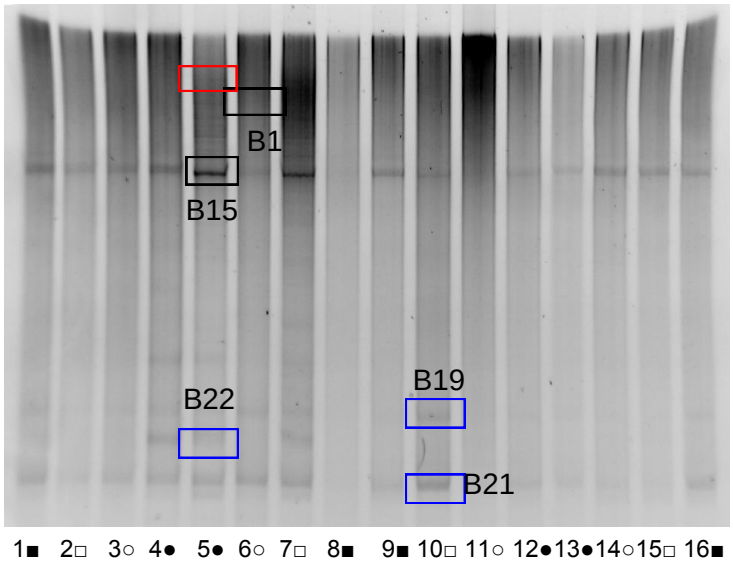


Fig. 6.14. *nifH* DGGE image for DNA from June 2007 soil from the conventional rotation



For all images

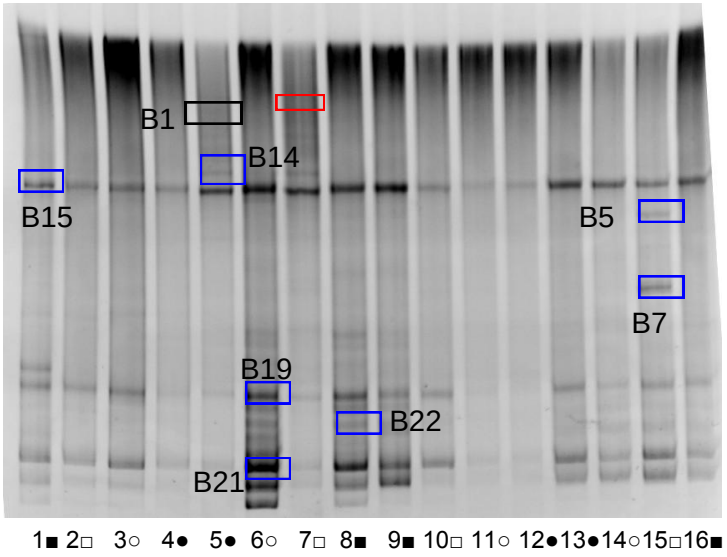
Significant band which has been sequenced

Significant band which could not be sequenced

Sequenced band which is not significant in the gel of interest

For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

Fig. 6.15 *nifH* DGGE image for DNA from September 2007 soil from the conventional rotation



For all images

Significant band which has been sequenced

Significant band which could not be sequenced

Sequenced band which is not significant in the gel of interest

For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

DGGE gels showing *nifH* amplified from DNA from 2008 soils

Fig. 6.16. *nifH* DGGE image for DNA from March 2008 soil

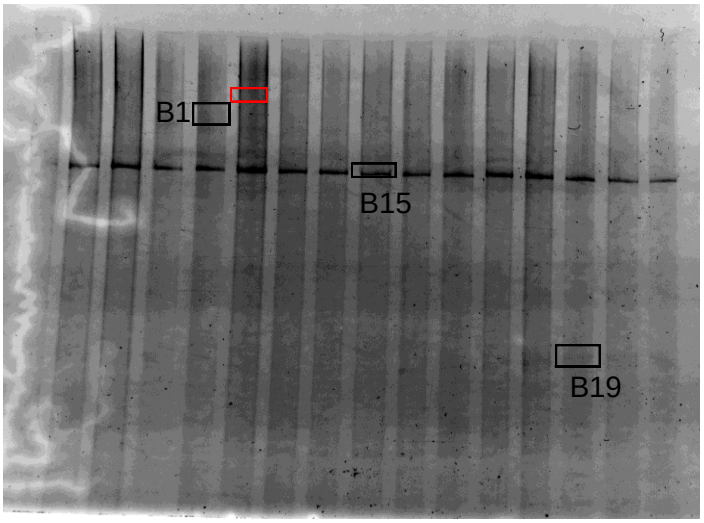
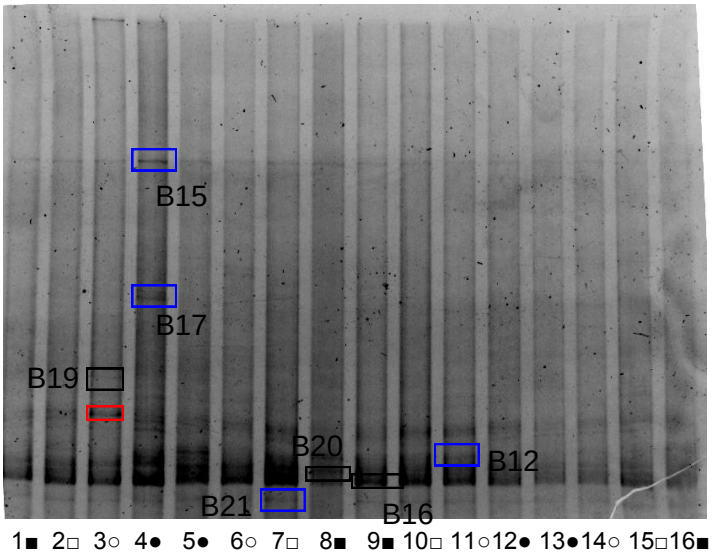


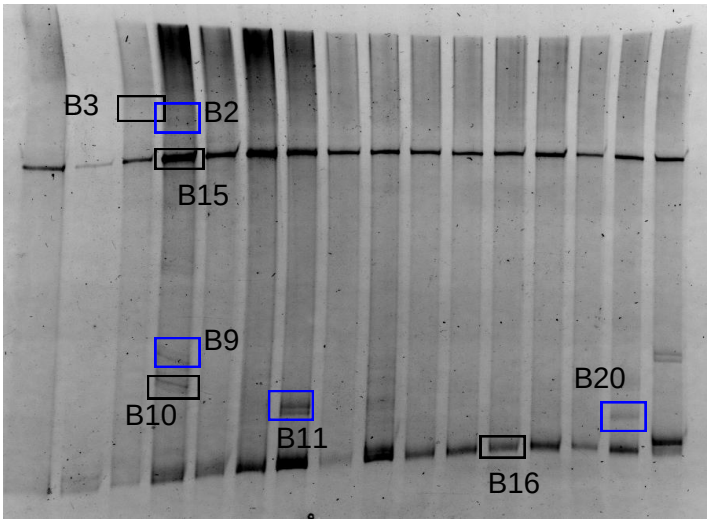
Fig. 6.17. *nifH* DGGE image for DNA from June 2008 soil



Significant band which has been sequenced	For all images Significant band which could not be sequenced	Sequenced band which is not significant in the gel of interest

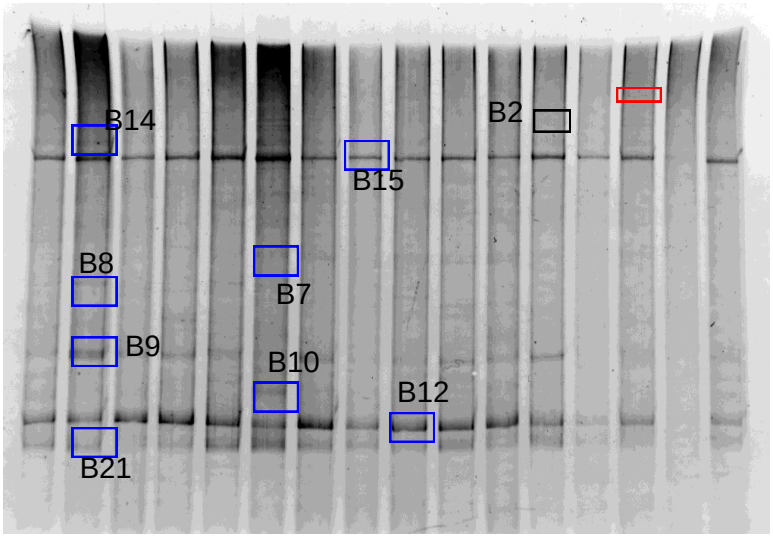
For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

Fig. 6.18. *nifH* DGGE image for DNA from September 2008 soil



DGGE gels showing *nifH* amplified from DNA from 2009 soils

Fig. 6.19. *nifH* DGGE image for DNA from March 2009 soil



For all images

Significant band which has been sequenced

Significant band which could not be sequenced

Sequenced band which is not significant in the gel of interest

For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

Fig. 6.20. *nifH* DGGE image for DNA from June 2009 soil

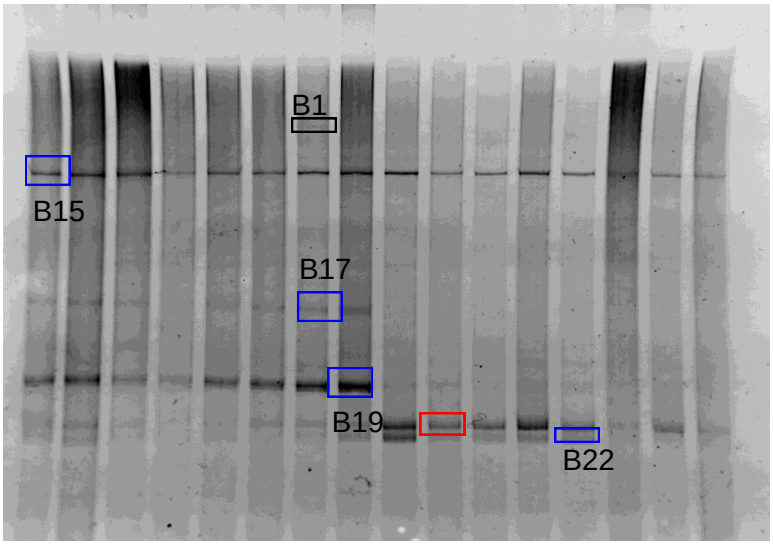
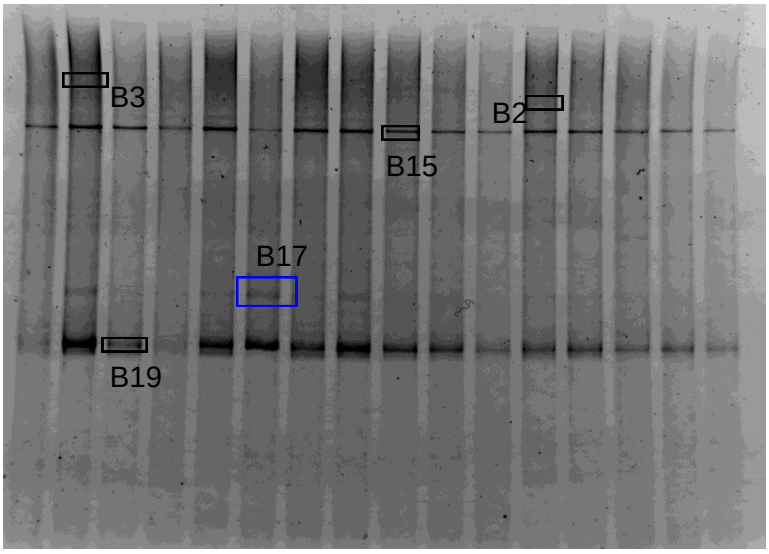


Fig. 6.21. *nifH* DGGE image for DNA from September 2009 soil



For all images

Significant band which has been sequenced	Significant band which could not be sequenced	Sequenced band which is not significant in the gel of interest

For all images numbers relate to plot numbers in the field. Symbols indicate treatments; ● = orgFM conCP, □ = conFM conCP, ○ = conFM orgCP, ■ = orgFM conCP.

Table 6.1. All sequence data obtained from *nifH* DGGE bands

Band label	Sequence
B1	CTCGCGTCGCAAGCGCCGAGTTGCTCGAGCAGGTTGATGCTCGTAATGATGCCCC GGCCCGCGCACCCGACGCCCGGCTCCGGCCCGCCTGATTCCACGCACATCGTCTT GCCGTAACCGGGCGAGCGAATGTCGGCCAGGTCGACGTCCTCGCCCTCTTCGCGC AGCGTGTCGAGCACTGATTTCTGCGCCAGCCCGCCCAGCAATAGCCGCGTGGAGT CCGCCTTGGGCTCGCAA
B2	AGGGAGAGATGGGGTGAGACCCCTCCTTAAAAGGAAAAGCAACGGGGGCGGGGA CGCCGGACTCGAAGCACTTGACGCCGCCGTAGCCGACCGACAACATCTTCTTCCA GTTCCACGTCCTCGACGCTGGCGGCTTCAGCCGGCGGTTTCATGACGGAGTGCTG CCCCGCCGAGTGAAAGAACGGGCGGGTGGATTTCGCCTTGGGCTCACAAGGGGG GGTGGCTGTTTGTTTTGGAACAACCCCCACCCCCGCGGTTG
B3	AGATTTTGAGCGACGTGATTGGAAAGGACCCCCCTGTTAGAGGATCTGGGCCGCC GACTCCACGCATTTAAAGCTTTGTAACGACCTTCACACGTCTCCTCCTCGCGAGGTC TTCCACCGAACCCTCCTTCGCCCGAGATGGTGGTTTGTGTCCTGCGCCTTCAGTCC AGGATGAGGCGAGTGGAGTCGCCTTCGGCCCCAAAA
B4	AGGGGAGAAAGCAGGTCGAGTCCGCCTTCGGCTCGCAACCTTTTAAAGAACCGACT TGGGGCAGCAAATGTCCCGGTAGCCCACCTTCATGACCTCTTCAATTTCCAAGGTC TTCCCCTCTGCCGGCCTCTGCCGCCAGGCAGAGAACTGGTGTCCTGGGCCTTGGC TTGGAGGATTAGACGAGTTGAAGTCCGCCTTCG
B5	CNNNTCGCAGAGTCCGCCTTCGGCTCGCATGTATCGCCATCATTTCGCCGGAGTCC GCCTTCGGCTCGCATCTATGGCCATCATTTCACCGGAGTCCGCCTTCGGCTCGCAA AGTCCGCT TTGGGATCGCAGAGTCCGCC
B6	GCGNANCCGCCGCACACCACGTCGCCGAGCACGTCGTAGGAGACGTAGTCGACAT CATCGTAGGCGCCGTTTTCTTCCAGAAAGTTGATCGACGTGATTACGCCGCGCCCG GCGCAACCGACGCCCGGCTCGGGGCCGCCGGACTCCACGCATTTGATGCCTCTGT AACCGACTTTCAGCACGTCCTCCACCTCGAGGTCTTCCACAGAACCCTCCTTTGCC GCGAGATGCAGTACTGTGTCCTGCGCCTTCGAGTTCAGGATCAGGCGGGTGGAGT CCGCCTTCGGCTCGCAANCGGANCTGCTGATCTCGC
B7	CATCATGTCGTAGGAGACGGACTCGAGGTCGTCATAAAAGAAAAAGTCTTCTTCGA GGAAGATCAAGGTGGTGACGCCGCCCCCGACGGAACCACCAACCCGTGGCTGCG GGCCACCGGATTCGAAGCAGTTGACGCCGCCGAAACCGACCGACACGACGTCCTC GAGTTCGAGGTCTTCCACAGCGCCGGCTTCAGCCGCCAGGTGCATCGGGGTCTTG TGCCTCTTGGTGTGGAAGATCACGCGGGGAGAGTGCGTCCTGCTCTC
B8	GCACCGACGCCCGGCTCGGGACCGCCCGACTCCACGCATTTGATGCCTTTGTAAC CGACTTTCAGCACGTCCTCCACCTCCAGGTCTTCCACCGAACCCTCCTTTGCCGCG AGATGCAGCACCGTGTCCTGCGCCTTCGAGTTCAGGATCAGGCGGGTGGAGTCCG CCTTGGGCTCGCAA
B9	ATGGAATCGGCTGTAGGATCGACCTTTACCGTCATTCACCATTTGGTACAAACGAAG TCCAGGTCTTCGTCATACGCGCCTTCTTCTTCGAGGAAGTTGATGGCGGTGATGAC GCCGCGGCCTGCGCAACCGACGCCAGGCTCAGGACCACCGGACTCGACGCACTT CACGCCGCCGTAACCAACCGACAGCACGTCTTCGAGTTCCAGGTCTTCCACGCTAC CGGCTTCAGCGGCCAGTTCCATCACGGAGTTCTGCGCCTTGGAGTGCAGGATCAA GCGGGTGGAGTCCGCCTTCGGCTCGCAACATTTCCCCGGGGTTCCCCCTTGGCCC CCAAAGTTTGCCAAGGATTTGCCCCGC
B10	GTTTTGAGGCCTCGACGTAGCCGCCGCAACCACGTCGCCGAGCACGTCGTAGGAG ACGTAGTCGACATCATCGAGGCGGGGGGGGGTCCAGAAAGTTGATCGACGTGATT ACGCCGCGCAAGGAAACAACCGACGCCCGGCTCGGGGCCGCCGGACTCCACGCA TTTGATGCCTCTGTAACCGACTTTCAGCACGTCCTCCACCTCGAGGTCTTCCACAGA ACCCTCCTTTGCCGCGAGATGCAGTACTGTGTCCTGCGCCTTCGAGTTCGGATCAG GCGGGTGGAGTCCGCCTTCGTGAAT
B11	GCTCCGGACCGCCGGACTCGACGCATTTGATACCGCCGTAACCGACGGACAGGAA GTGTTCGAGTTCGAGGTCTTCCACACTGCCGGCTTCCGCCGCCAGTTCCATGACCG AGTTCTGGGCCTTGGCGTGGAGGATCAGGCGTGTTGAGTCCGCCTTCGGCTCGCA AATCG

Table 6.1 (continued). All sequence data obtained from *nifH* DGGE bands

Band label	Sequence
B12	CTTGAGATAGACTTCGAGCAAGCCAAGTCCACCACGTCGCCGAGCACGTCGTAGGAGA CGTAGTCGACATCATCGTAGGGAAAAGGGAATCCAGAAAGTTGATCGACGTGATTACGC CGCGCCCTTTAGCAACCGACGCCCGGCTCGGGGCCGCCGGACTCCACGCATTTGATG CCTCTGTAACCGACTTTCAGCACGTCCTCCACCTCGAGGTCTTCCACAGAACCCTCCTT TGCCGCGAGATGCAGTACTGTGTCCTGCGCCTTCGAGTTCAGGATCAGGCGGGTGGAG TCCGCCTTCGGCTCGCAA
B13	CACGTCGTAGGAGACGTAGTCGACATCATCGTAGGCGCCATTCTCTTCCAGGAAGTTGA TCGACGTGATTACGCCGCGCCCGGCGCAACCGACGCCCGGCTCTGGGCCGCCAGACT CCACGCATTTAATGCCTTTGTAACCGACCTTCAGCACGTCCTCCACCTCGAGGTCTTCC ACCGAACCCTCCTTCGCCGCGAGATGCAGTACCGTGTCCTGCGCCTTCGAGTCCAGGA TGAGGCGAGTGGAGTCCGCCTTCGGCTCGCAAAGTTTT
B14	ATGGGGTGAGACCCCTCCTTAAAAGGAAAAGCAACGGGGGCGGGGACGCCGGACTCG AAGCACTTGACGCCGCCGTAGCCGACCGACAACATCTTCTTCCAGTTCCACGTCCTCGA CGCTGGCGGCTTCAGCCGGCGGTTTCATGACGGAGTGCTGCCCCGCCGAGTGAAAGA ACGGGCGGGTGGATTTCGCCTTGGGCTCACAA
B15	GAGTTGGCGAGGAGGGGGCCTAAGCGAGGAAATGACGAGGTGAGACGCCTCGGTAAG AGAAAAGAGACACCCGGCTCGGGGCCGCCGGACTCCACACACTTGATGTCTTTGTAGC CGATCTTGAGCACGTCCTGCCGTTCGAGGTCTTCCACAGAACCTTCCTGGGCGGCGAG ATGCGGAACCGTCTCCTGCGCCTTTGCGTTCATGATTTTGCGGGTGGAGCCCGCCCTC GCCCGCAATT
B16	AGATCAGGAAGGTGAGGGCGGGGTGACCTTCTAAAAGGGAACAACCGACGCCGGGCT CCGGACCGCCGGACTCGACGCACTTGATGCCGCCGTAACCGACGGACAACACGTCTTC GAGTTCGGGTCCTCCACACTGCCGGCTTCCGCCGCCAGTTCCATGACGGAGTTCTGGG CCTTGGCGTGGAGGACCAGGCGTGTTGAGTCCGCCTTCGGCTCAAAAGGG
B17	CTTACATTAGACCTTTAGGCTTCAAATTGACCCTCTAATCGCAAGTAGGCTCGAAGGTCG CCGGAGTCGGCGGAAGGGGGGGGGCGCGAAGAGGAGTTGATCGAGTATCACGCCATT TACAAAAAAAAAAAACACCTGGCTCTGGACCACCGGATTCAACGCAACGGATGTCGCGA TATCCCACCTTCATGACGTCTTCCAGTTCCAGATCCTCGACGGAACCGGCTTCAGCGGC CAGCGACAGGATGGTGTCCTGGGCCTTGGCGTGCAGGATAGACGTGTGGAGTCCGCC TTCGCTCCAATT
B18	CTCGATTGCCTGTAGGTTTCGCACGGCTACCCGTCGCCGAGCAGTCGTAGGAGACGTA GTCGACATCATCGTAGGGGACAGTAGATTCCAGGAAGTTGATCGACGTGATTACGCCG CGCCCGGCGCAACCGACGCCCGGCTCTGGGCCGCCAGACTCCACGCATTTAATGCCTT TGTAACCGACCTTCAGCACGTCCTCCACCTCGAGGTCTTCCACCGAACCCTCCTTCGCC GCGAGATGCAGTACCGTGTCCTGCGCCTTCGAGTCCAGGATGAGGCGAGTGGAGTCC GCCTTCGGCTCGCAAAA
B19	CAGGGCCGCCAGACTCGAGGCACTTAACGCCGCCGTAACCAAGAAAGAGGGGGTTCC GGATCCCCGTCCGCGACGCTACCGGCTTCGGCAGCCTGTTTCCATCACGGAGTTCTGA GCCTTCGAGTGCAGAATCAGGCGAGC
B20	CAGGGGGCCGCCCTCGGGTCGCACCATTCACCCGGCAGTACGCATTGTGGTAGAAAA GAACTCGAGGTCGCCGGTGAAGGCGCCCACTTCCTCAGGAAGTTGATGGCGGTCATGA CGCCCCTACCGGAACAACCAACCCCTGGCTCAGGGCCACCCGACTCGACGCACTTGAT ACCGCCGTAACCGACGGACAGCACGTCTTCAAGTTCGAGGTCTTCCACGCTGCCGGCT TCCGCCGCCAGTTCCATGACCGAGTTCTGAGCCTTGGCGTGCAGGATCAGACGTGTTG AGTCCGCCTTCGGCTCGAGAATTCCGGCTGGGTTCCCCGGAGGGTCAAATCGGGTGAG TAGGGTCCCTTTTTTTCCCCATTGGGTTATTTGCCCCCAGAGGTTAAAAGGG
B21	CAATGGGCTCGCAACTGAGGAGGGGACGGGGGGATGTCGCAACGGCCGGCGCAGCC GACGCCAGGCAAAGAAAAGACGGACTCGACGCAGCGGATATCCTGGTAGCCGACCTTC ATGACCTCTTCGATTTCGAGGTCTTCGACGCTGCCGGCCGCCGCCGCCAGGCTGAGAA TGGTGTCCTGCGCCTTGGTTGCAGGATCAGGCGGGTCGAGTCCGTTTCGGCTCCAATT
B22	GGTGACCTTCTAAAAGGGAACAACCGACGCCGGGCTCCGGACCGCCGGACTCGACGC ACTTGATGCCGCCGTAACCGACGGACAACACGTCTTCGAGTTCGGGTCCTCCACACTG CCGGCTTCCGCCGCCAGTTCCATGACGGAGTTCTGGGCCTTGGCGTGGAGGACCAGG CGTGTTGAGTCCGCCTTCGGCTCAAAAGGG

Table 6.2. Summary of BLAST results for all *nifH* sequences

Band label	Closest match in BLAST as of 18/11/10	E value	% max. id	number of query nts which match BLAST hit	Closest identified match	E vaule	% max. id	number of query nts which match BLAST hit	Closest match class	closest match order
B1	Uncultured soil bacterium clone (AY796013)	3.0E-43	81%	186/227	*Bradyrhizobium japonicum* (GQ289567.1)	9.0E-45	78%	181/232	*Alphaproteobacteria*	*Rhizobiales*
B2	Uncultured soil bacterium clone (EU331531)	3.0E-22	81%	127/156	*Aeromonas* sp. IPPW-29 (FJ687521)	3.0E-19	74%	121/162	*Gammaproteobacteria*	*Aeromonadales*
B3	Uncultured bacterium clone (HM063717)	2.0E-33	84%	138/163	*Mesorhizobium alhagi* strain DB7 (GU083829.1)	9.0E-32	79%	130/163	*Alphaproteobacteria*	*Rhizobiales*
B4	Uncultured bacterium clone (GQ464112.1)	4.0E-30	84%	113/133	*Azospira oryzae strain* 6a3 (U97115.2)	8.0E-26	81%	114/140	*Betaproteobacteria*	*Rhodocyclales*
B5	Uncultured bacterium clone (HM063793.1)	4.8E-01	100%	23/23	*Desulfitobacterium hafniense* DCB-2	4.8E-01	92%	26/28	*Clostridia*	*Clostridiales*
B6	*Rhizobium huautlense* strain CCBAU 65679 (EU622086.1)	6.0E-141	99%	277/278					*Alphaproteobacteria*	*Rhizobiales*
B7	Uncultured bacterium clone (EU241558.1)	9.0E-44	79%	132/166	*Azonexus fungiphilus* strain LMG 19178 (DQ029204)	3.0E-33	77%	172/221	*Betaproteobacteria*	*Rhodocyclales*
B8	Uncultured bacterium clone (HM063717.1)	2.0E-73	96%	165/171	*Rhizobium etli* strain CCBAU 65830 (EU622089.1)	1.0E-71	94%	168/177	*Alphaproteobacteria*	*Rhizobiales*
B9	Uncultured bacterium isolate (GU097353.1)	3.0E-104	93%	244/261	*Sideroxydans lithotrophicus* ES-1 (CP001965)	2.0E-77	87%	229/262	*Betaproteobacteria*	*Gallionellales*
B10	*Rhizobium huautlense* strain CCBAU 65679 (EU622086.1)	6.0E-116	93%	273/293					*Alphaproteobacteria*	*Rhizobiales*
B11	Uncultured bacterium clone (FJ008540)	3.0E-52	92%	137/148	*Azoarcus communi* strain Swub3 (U97116)	4.0E-40	85%	141/165	*Betaproteobacteria*	*Rhodocyclales*
B12	*Rhizobium huautlense* strain CCBAU 65679 (EU622086.1)	4.0E-113	94%	256/271					*Alphaproteobacteria*	*Rhizobiales*
B13	Uncultured bacterium clone (HM063739.1)	7.0E-110	94%	248/258	*Rhizobium etli* strain CCBAU 65830 (EU622089.1)	1.0E-71	94%	249/264	*Alphaproteobacteria*	*Rhizobiales*

Table 6.2 (continued). Summary of BLAST results for all *nifH* sequences

Band label	Closest match in BLAST as of 18/11/10	E value	% max. identity	number of query nts which match BLAST hit	Closest identified match	E vaule	% max. identity	number of query nts which match BLAST hit	Closest match class	closest match order
B14	Uncultured bacterium clone (EU331528)	1.0E-26	81%	127/156	*Azovibrio restrictus* (U97119.1)	4.0E-11	74%	118/158	*Betaproteobacteria*	*Rhodocyclales*
B15	Uncultured bacterium clone (FJ263748)	4.0E-62	94%	149/157	*Mesorhizobium loti* MAFF303099 (BA000012.4)	4.0E-62	94%	149/157	*Alphaproteobacteria*	*Rhizobiales*
B16	Uncultured bacterium clone (HM063828.1)	2.0E-54	90%	150/165	*Azoarcus communi* strain Swub3 (U97116)	1.0E-37	83%	141/125	*Betaproteobacteria*	*Rhodocyclales*
B17	Uncultured bacterium clone (DQ776446.1)	4.0E-78	92%	195/210	*Ideonella* Sp. Long 7 (AY231580.1)	3.0E-44	91%	125/137	*Betaproteobacteria*	*Burkholderiales*
B18	Uncultured bacterium clone (HM063739.1)	2.0E-105	92%	253/273	*Rhizobium etli* strain CCBAU 65830 (EU622089.1)	2.0E-105	92%	257/279	*Alphaproteobacteria*	*Rhizobiales*
B19	Uncultured bacterium clone (EU331531.1)	2.0E-105	82%	115/139	*Sideroxydans lithotrophicus* ES-1 (CP001965)	2.0E-17	78%	107/136	*Betaproteobacteria*	*Rhodocyclales*
B20	Uncultured bacterium clone (FJ008540)	4.0E-79	89%	218/244	*Pseudomonas* sp. IPPW-3 (FJ687518.1)	5.0E-53	81%	214/262	*Gammaproteobacteria*	*Pseudomonadales*
B21	Uncultured bacterium clone (GU727691)	1.0E-62	90%	173/192	*Bradyrhizobium* sp. MAFF 210318 (AB079620)	3.0E-48	86%	159/191	*Alphaproteobacteria*	*Rhizobiales*
B22	Uncultured bacterium clone (HM063828.1)	2.0E-54	90%	150/165	*Azoarcus communi* strain Swub3 (U97116)	9.0E-38	83%	136/166	*Betaproteobacteria*	*Rhodocyclales*

organism the closest cultured isolate is also listed. Of the 22 sequences, 19 matched unculturable bacteria. The remaining 3 bands were identified as belonging to *Rhizobium huautlense*. When using the closest cultured matches, it was found that potentially 10 sequences belong to *Alpha-Proteobacteria*, 9 belong to *Beta-Proteobacteria*, 2 belong to *Gamma-Proteobacteria* and 1 belongs to the order *Clostridia*.

ClustalW was used to create the phylogenetic trees shown in Figs 6.22-6.23.

6.2.2. The effect of previous crop, year, sample date and nucleic acid used on the presence of sequenced bands.

Each of the 22 bands sequenced was present in more than one DGGE gel. Table 6.3 shows the distribution of the sequenced bands throughout the DGGE gels. Within each gel the band of interest may be present in more than one lane. Table 6.4 shows the number of lanes the band of interest is present in, in each gel. Tabulating data in this way allowed ANOVA to be carried out to see if distribution of taxa was affected by previous crop (for the 2007 RNA samples), sample date, sample year and the nucleic acid used. The results of this are shown in Table 6.5.

Previous crop did not significantly affect the presence of any of the taxa. However, it did appear to be a driver affecting the distribution of band B15, which was most prevalent in the organic rotation (P=0.067).

Sample date and year were often significant factors affecting *nifH* community diversity and *nifH* copy number (Results Chapter 2). Although some bands, for example B4 did not appear in any soils sampled in March and B8 and B20 did not appear in any soils in September, sample date was never a significant factor when it came to the distribution of the sequenced bands. Results were similar when looking at the effect of year, so although bands B5 and B13 were only present in soils sampled in 2007, this was not significant

Figure. 6.22. Phylogenetic tree showing the phylogenetic relationship between the bands sequenced.

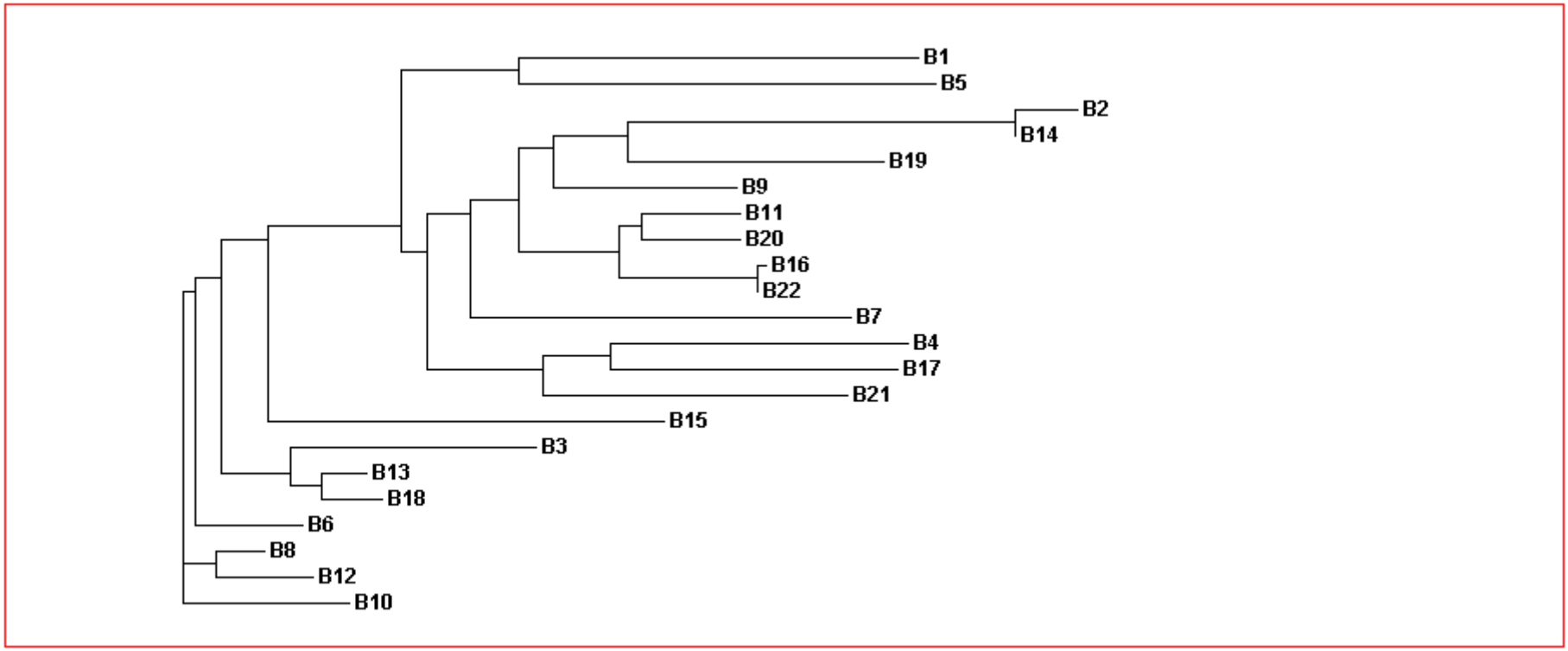


Figure. 6.23. Phylogenetic tree showing the phylogenetic relationship between the bands sequenced and labelled assuming that bands correlate to their closest cultured match in BLAST

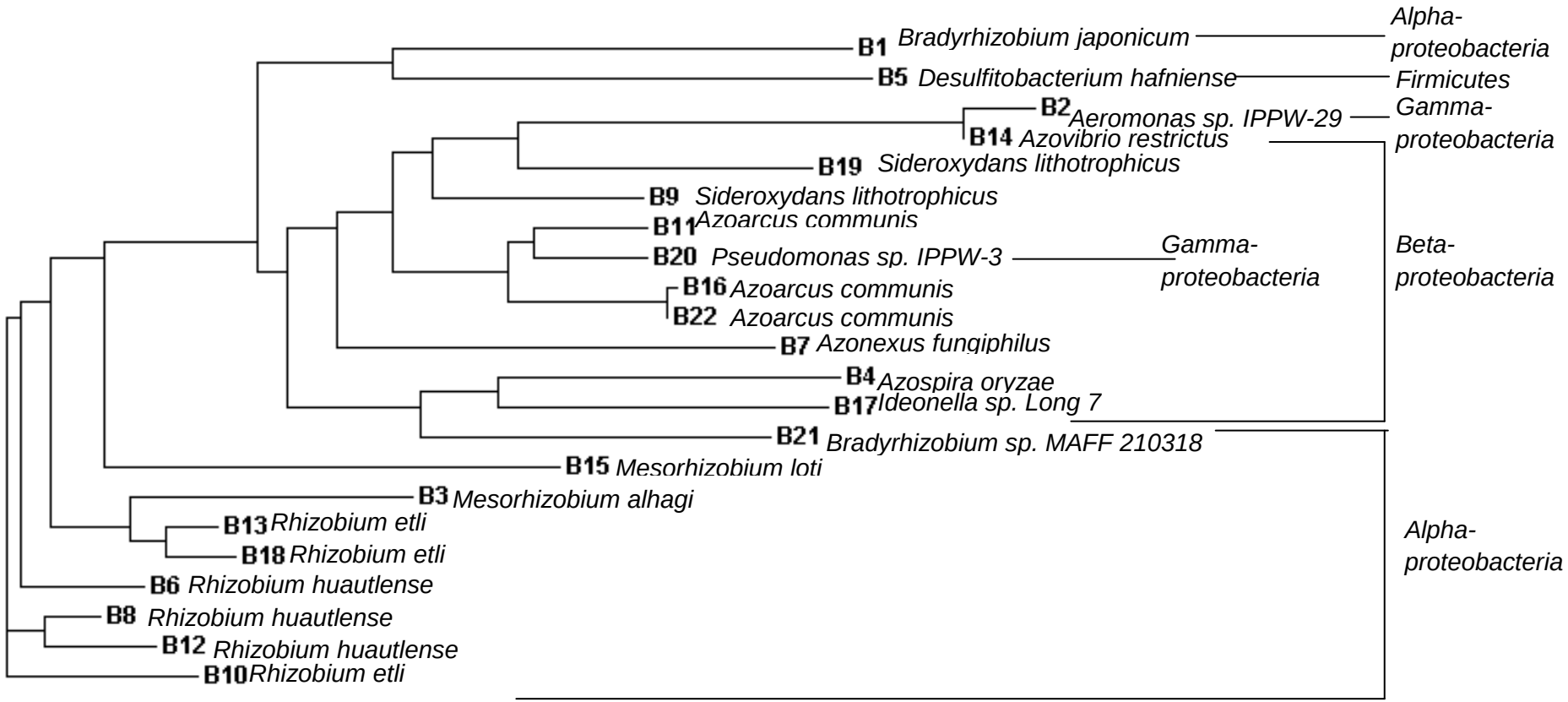


Table 6.3. Distribution of sequenced bands throughout the sampling dates.

Band label	RNA											DNA								
	2007 organic rotation			2007 conventional rotation			2008		2009			2007			2008			2009		
	Mar	Jun	Sep	Mar	Jun	Sep	Mar	Jun	Mar	Jun	Sep	Mar	Jun	Sep	Mar	Jun	Sep	Mar	Jun	Sep
B1			X		X	X	X				X		X	X	X				X	
B2			X		X	X						X					X	X		X
B3				X	X												X			X
B4					X	X					X									
B5	X	X	X		X	X								X						
B6		X			X	X	X				X									
B7			X	X		X	X							X				X		
B8	X	X		X	X													X		
B9		X			X	X											X	X		
B10	X	X	X	X			X	X	X								X	X		
B11	X		X	X					X	X	X						X			
B12	X	X		X	X	X			X	X						X		X		
B13	X	X	X		X															
B14	X	X		X		X						X		X				X		
B15	X	X	X	X			X		X		X	X	X	X	X	X	X	X	X	X
B16				X			X	X	X	X	X					X	X			
B17		X					X									X			X	X
B18					X	X						X								
B19												X	X	X	X	X			X	X
B20				X								X				X	X			
B21				X		X			X			X	X	X		X		X		
B22	X											X	X	X					X	

Table 6.4. Distribution of sequenced bands within lanes of each gel.

Band label	RNA											DNA								
	2007 organic rotation			2007 conventional rotation			2008		2009			2007			2008			2009		
	Mar	Jun	Sep	Mar	Jun	Sep	Mar	Jun	Mar	Jun	Sep	Mar	Jun	Sep	Mar	Jun	Sep	Mar	Jun	Sep
B1	0	0	4	0	3	9	4	0	0	0	6	0	7	2	3	0	0	0	5	0
B2	0	0	3	0	4	8	0	0	0	0	0	2	0	0	0	0	4	6	0	5
B3	0	0	0	3	2	0	0	0	0	0	0	0	0	0	0	0	4	0	0	3
B4	0	0	0	0	5	16	0	0	0	0	7	0	0	0	0	0	0	0	0	0
B5	3	16	5	0	4	15	0	0	0	0	0	0	0	2	0	0	0	0	0	0
B6	0	4	0	0	1	12	7	0	0	0	3	0	0	0	0	0	0	0	0	0
B7	0	0	3	4	0	16	11	0	0	0	0	0	0	1	0	0	0	5	0	0
B8	2	2	0	12	4	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0
B9	0	7	0	0	8	2	0	0	0	0	0	0	0	0	0	0	2	13	0	0
B10	4	9	11	9	0	0	15	10	12	0	0	0	0	0	0	0	3	3	0	0
B11	12	0	10	11	0	0	0	0	4	1	13	0	0	0	0	0	4	0	0	0
B12	6	7	0	7	3	11	0	0	16	4	0	0	0	0	0	15	0	15	0	0
B13	2	8	9	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B14	4	4	0	2	0	10	0	0	0	0	0	7	0	4	0	0	0	6	0	0
B15	16	16	12	15	0	0	3	0	4	0	5	16	15	16	16	16	16	16	16	16
B16	0	0	0	12	0	0	15	7	14	2	16	0	0	0	0	16	16	0	0	0
B17	0	3	0	0	0	0	9	0	0	0	0	0	0	0	0	4	0	0	8	11
B18	0	0	0	0	2	6	0	0	0	0	0	13	0	0	0	0	0	0	0	0
B19	0	0	0	0	0	0	0	0	0	0	0	16	5	14	7	2	0	0	10	16
B20	0	0	0	15	0	0	0	0	0	0	0	11	0	0	0	12	2	0	0	0
B21	0	0	0	2	0	11	0	0	3	0	0	2	4	16	0	16	0	14	0	0
B22	3	0	0	0	0	0	0	0	0	0	0	16	12	13	0	0	0	0	13	0

Table 6.5. ANOVA results for the analysis of the distribution of sequenced bands between gels

ANOVA *P* values	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11
sample date	0.299	0.113	0.442	0.184	0.460	0.572	0.319	0.233	0.755	0.338	0.186
year	0.672	0.732	0.926	0.582	*0.058*	0.719	0.735	0.381	0.698	0.628	0.566
RNA/DNA	0.720	0.653	0.586	0.153	0.086	*0.077*	0.216	0.349	0.942	**0.009**	**0.043**
pre-crop (2007 RNA only)	0.331	0.445	0.218	0.292	0.573	0.342	0.178	0.467	0.957	0.687	0.355

ANOVA *P* values	B12	B13	B14	B15	B16	B17	B18	B19	B20	B21	B22
sample date	0.404	0.441	0.381	0.697	0.828	0.907	0.668	0.745	0.443	0.868	0.908
year	0.712	*0.092*	*0.075*	0.826	**0.038**	0.259	0.266	0.764	0.479	0.944	0.282
RNA/DNA	0.558	*0.077*	0.960	**0.001**	0.446	0.363	0.624	**0.001**	0.515	*0.096*	**0.017**
pre-crop (2007 RNA only)	0.797	0.381	0.917	*0.067*	0.208	0.673	0.286	N/A	0.437	0.343	0.286

overall. The only band significantly affected by sample date was B16 which was present in all years but in much higher numbers in 2008.

The nucleic acid used in the analysis (DNA or RNA) was a factor which most affected the presence/absence of sequenced bands. Although not significant, bands B4, B6 and B13 were only present when RNA was used. Bands B11 (*P*=0.009), B12 (*P*=0.043) and B15 (*P*=0.001) were more common when RNA was used as the template, and bands B19 (*P*=0.001) and B22 (*P*=0.017) were more common when DNA was used as a template.

6.2.3. The effect of management type on the presence of sequenced bands.

For each band all relative intensity data was incorporated into a spreadsheet. This data was used to carry out ANOVA analysis. ANOVA results are presented in Table 6.6. Management type did not significantly affect the presence or intensity of any of the sequenced bands.

Table 6.6. ANOVA results for the analysis of the distribution of sequenced bands within each gel.

	B1	B2	B3	B4	B5	B6	B7	B8
fertility	0.679	0.277	0.502	0.419	0.663	0.143	0.648	0.786
health	*0.092*	0.674	0.423	0.329	0.806	0.338	0.223	0.672
FM*CP	0.754	**0.023**	0.300	0.418	0.412	*0.073*	0.541	0.675

	B9	B10	B11	B12	B13	B14	B15	B16
fertility	0.872	0.586	0.742	0.735	0.238	0.344	0.380	0.347
health	0.720	0.894	0.160	0.507	0.488	0.606	*0.063*	0.341
FM*CP	*0.088*	0.388	0.195	0.878	*0.067*	0.613	0.920	0.426

	B17	B18	B19	B20	B21	B22
fertility	0.910	0.424	0.523	0.746	0.627	0.909
health	0.896	0.551	0.577	0.855	0.592	0.800
FM*CP	0.387	0.172	0.972	0.856	0.523	0.284

6.3. Discussion.

Of the 22 *nifH* genes which have been sequenced in this study 19 appear to belong to unculturable bacteria. This is a problem which has been reported by many others especially when looking at diazotrophs residing in the soil rather than within plant roots (Hsu and Buckley, 2009; Roesch *et al*., 2008; Coelho *et al*., 2008; Buckley *et al*., 2007). For example, Beauchamp *et al*. (2006) cultured soils exposed to fertilizer for a week, tested isolates ability to fix nitrogen then analysed the isolates using fatty acid analysis and 16S ribosomal DNA sequencing. Only 2 isolates survived the culturing and purification process.

There has been some progress in beginning to identify unculturable diazotrophs. More specific PCR primers which only amplify members of a particular group or genera have been developed enabling the researcher to get a better idea of the species present without the need to sequence (Burgmann *et al*., 2005). However, these primers often also amplify up similar bacteria from other genera (Burgmann *et al*., 2005). Another possible technique is $^{15}N_2$-DNA stable isotope probing. Here the 16S rRNA gene is sequenced in all organisms which have incorporated ^{15}N from the environment. 16S rRNA sequence data reveals more about the phylogeny of the nitrogen fixers than the *nifH* gene and therefore allows more organisms to be identified. In a recent study, $^{15}N_2$-DNA stable isotope probing identified that the most common diazotrophs in soil belonged to three groups; *Rhizobiales*, unclassified *Beta-proteobacteria* and unclassified *Actinobacteria* (Buckley *et al*., 2007).

For purposes of the discussion it can be assumed that the sequenced diazotrophs are reasonably similar to their closest identified match. Following this assumption, of the 22 bands sequenced 10 belong to *Alpha-proteobacteria*, 9 belong to *Beta-proteobacteria*, 2 belong to *Gamma-proteobacteria* and 1 belongs to the *Clostridia*. This is in agreement with recent studies of soil environments which have suggested that the most common

diazotrophs belong to *Alpha*- and *Beta-proteobacteria* with *Gamma-proteobacteria* and *Firmicutes* present in smaller numbers (Hsu and Buckley, 2009; Ogilvie *et al*., 2008; Demba Diallo *et al*., 2004).Many genera and groups have been reported as being particularly dominant in soil environments these include: *Azoarcus* (including *Azovibrio*, *Azospira* and *Azonexus*), *Azospirillum*, *Azotobacter*, *Bradyrhizobium*, *Rhizobiales* and *Rhizobium* (Xiao *et al*., 2010; Coelho *et al*., 2009; Coelho *et al*., 2008; Roesch *et al*., 2008; Buckley *et al*., 2007; Demba Diallo *et al*., 2004).

All 10 of the *Alpha-proteobacteria* matches also belonged to the order *Rhizobiales* which have been previously shown to be dominant in agricultural soils (Coelho *et al*., 2009). Within the 10 sequences there are 6 organisms which match most closely. Demba Diallo *et al*. (2004) observed that bands from two different positions within a DGGE gel were matched to a single phylotype and attributed this to the fact that good quality sequence was only obtained for a section of the *nifH* gene and that the remaining part of the sequence could differ between the two bands.

B1 and B21 are closely matched to *Bradyrhizobium* species. B1 is *Bradyrhizobium japonicum* and B21 is *Bradyrhizobium* sp. MAFF 210318 a slow growing non-phototroph which is a very close relative to *B. japonicum* (Cantera *et al*., 2004). Although *Bradyrhizobium* is often observed in symbiosis with plants it can survive well in soils and have been observed as one of the most abundant genera in soils under maize (Roesch *et al*., 2008). In the present study, band B21 was much more common when DNA was used as the template (P = 0.096) suggesting this taxa is possibly not actively expressing *nifH* in soils.

The remaining *Alpha-proteobacteria* belong to the *Mesorhizobium* and *Rhizobium* genera. The only 3 bands (B6, B10 and B12) which were matched immediately to cultured bacteria were all identified as *Rhizobium huautlense,* Bands B8, B13 and B18 are similar to *Rhizobium etli*, B3 is similar to *Mesorhizobium alhagi* and B15 is similar to

Mesorhizobium loti. These bacteria belong to the family *Rhizobiaceae* and are characterized by their ability to interact with higher plants (Segovia *et al*., 1991). Nevertheless, in this study they have been amplified from bulk soil. *Rhizobium huautlense* is a relatively unstudied symbiote of *Sesbania herbacea* (Wang *et al*., 1998). However, there is evidence that bacteria, such as *Mesorhizobium loti*, are predominantly free-living but have acquired some symbiotic genes from *Rhizobium loti* (Ferreira *et al*., 2000; Sullivan *et al*., 1995). In fact, in most *Rhizobium* species, genes required for symbiotic nitrogen fixation are contained in plasmids that are often spontaneously lost or rearranged leading to loss of nodulating ability (Segovia *et al*., 1991) .

Of the 9 *Beta-proteobacteria*, 6 taxa were most similar to species of the genera *Azoarcus*, *Azospira*, *Azonexus* or *Azovibrio*. Before being split into 4 genera each was originally grouped under the genus *Azoarcus* (Demba Diallo *et al*., 2004). Bands B11, B16 and B22 are all similar to *Azoarcus communis*, B4 is similar to *Azospira oryzae*, B7 is similar to *Azonexus fungiphilus* and B14 is similar to *Azovibrio restrictus*. Originally *Azoarcus* was thought to only survive within the roots of kallar grass and rice (Demba Diallo *et al*., 2004; Hurek *et al*., 2002).However, all of the species listed above have been previously identified to be predominant member of bulk and rhizosphere soils, under a range of different crops (Hsu and Buckley, 2009; Ogilvie *et al*., 2008; Wartiainen *et al*., 2008; Knauth *et al*., 2005). The remaining *Beta-proteobacteria* was most closely matched to *Sideroxydans lithotrophicus* (B1 and B19) and *Ideonella* sp. Long 7 (B17). Members of the *Ideonella* genus are thought to be more dominant in the interior of plants and relatively rare in soils. However, when looking in the rhizosphere of maize, Roesch *et al*. (2008) found *Ideonella* to be one of the most abundant genera.

Band B5 was most closely matched to the *Firmicute Desulfitobacterium hafniense*. The percentage homology is very low so there is very little confidence in this identification. B2 and B20 were most closely matched to the *Gamma-proteobacteria Aeromonas* sp. IPPW-

29 and *Pseudomonas* sp. IPPW-29 both identified as being dominant genera in carbon rich wastewater. It has previously been noted that the *nifH* genes of the two bacteria are more closely related to *Alpha* and *Beta-proteobacteria* than *Gamma-proteobacteria* (Addison *et al*., 2010). In the phylogenetic tree (Fig. 6.23) created using the sequences in this study B2 and B20 cluster with the bands most closely matched to *Beta-proteobacteria*.

Other well-studied diazotrophs which were not detected were *Frankia* and cyanobacteria. Although it has been previously noted that although common in other environments *Frankia* and cyanobacteria are often only represented in small numbers in agricultural soil (Wartiainen *et al*., 2008; Buckley *et al*., 2008). This study is not a completely comprehensive study of all of the organisms present. Firstly, not all of the DGGE bands were sequenced. Due to time constraints only bands which appeared to cause variation within gels were chosen. Among the bands of interest there were examples of bands which could not be re-amplified, or which were re-amplified but produced sequence data of very poor quality. Often a single band on a DGGE gel does not represent a single bacterial strain (Sekiguchi *et al*., 2001). This is due to more than one phylotype possessing very similar electrophoretic mobilities (Diez *et al*., 2001). When the band of interest is excised, purified and sequenced the multiple phylotypes mean poor quality sequence is obtained.

Even if all bands were sequenced some sequences would be missed as DGGE gel will only show dominant bands. Even in complex environmental samples the number of DGGE bands will rarely exceed a few dozen (Nikolausz *et al*., 2005). PCR will also cause bias as preferential priming and differences in elongation rates mean different phylotypes will be preferentially amplified during PCR (Diez *et al*., 2001). This problem could be overcome with the use of molecular cloning. However, cloning is also subject to bias. Therefore, in order to get an accurate representation of the organisms present in a

community, hundreds of isolates would need to be sequenced making cloning of complex environmental samples an expensive and laborious process (Hsu and Buckley, 2009).

The nucleic acid used in the analysis significantly affected the presence of bands B10, B11, B15, B19 and B22. This shows there is a place for analysis of both RNA and DNA, with RNA representing short term change (changes in gene expression) and DNA representing long term change (changes in community structure and composition) (Morales *et al*., 2010).

As sample date, year and previous crop significantly affected diazotrophic diversity, it may have been expected that more significant differences would have been observed in the presence of the sequenced bands. Only limited differences were seen. Year only significantly affected B16. Sample date and previous crop did not significantly affect the presence of any of the bands. Previous studies have reported a change in the structure of the diazotrophic community in response to the species of crop grown and seasonal changes. Using in field and artificial conditions the incorporation of different root exudates has been shown to affect *Azotobacter* and *Sinorhizobium* species (Burgmann *et al*., 2005; Demba Diallo *et al*., 2004). However, these species of bacteria were not among the sequenced taxa in this study. It has also been suggested that a barley crop leads to increased numbers of *Actinomycetes* and *Pseudomonas* (Pascault *et al*., 2010) and a beans crop leads to increased numbers of *Actinobacteria* and *Bacteroidetes* (Larkin and Honeycutt, 2006). The only group from this list represented within the sequenced data was band B21 which was matched to a pseudomonad. This band did only appear following the barley crop, but numbers were low meaning results were not significant (Table 6.5).

It has also been suggested that it is mainly anaerobic diazotrophs, such as pseudomonads and *Gamma-proteobacteria*, which respond to seasonal changes within a soil environment (Gamble *et al*., 2010). Of the taxa sequenced B2 and B20 fell into this

category. B2 appeared sporadically across all sample dates. However, although not significant, B20 mostly only appeared in March and never appeared in September.

Management also had no affect on the distribution of sequenced bands. There have been several previous studies looking at the effect of high and low levels of nitrogen fertilizer on the structure of the diazotrophic community. In these studies the predominant taxa, for example; *Bradyrhizobium, Idoenella* and *Rhizobium etli* were present in all agricultural soils regardless of the amount of nitrogen fertilizer used (Coelho *et al*., 2008; Ogilvie *et al*., 2008; Knauth *et al*., 2005). It has been suggested that, while the predominant OTUs remain unaffected by the amount of nitrogen fertilizer applied, minor members of the community may be affected and are more likely to appear under higher levels of nitrogen than low levels (Knauth *et al*., 2005).

This kind of statistical analysis involves the use of multiple ANOVA. When carrying this out the user must be aware that even with a randomly generated data set 5% of the results will be observed as significant. For this reason care must be taken not to rely too heavily on single significant *P* values. However, the results of this analysis generated very few significant results leading to the assumption that for the bands sequenced, year, sample date, previous crop, nucleic acid used and management type did not affect the presence or absence of particular diazotrophs. It is possible that if every band was sequenced and analysed more significant results would have been found in line with the significant effects of year, sample date and previous crop observed when analysing diversity in the DGGE gels.

7. Discussion and Conclusion.

7.1. The impact of farm management on the total bacterial community.

In this study the total bacterial community was analysed in organic and conventionally managed soil using DGGE and qPCR analysis of the 16S rRNA gene, and CLSU analysis using BIOLOG plates. It was found that on average, at the majority of sample dates, the organic crop rotation, in particular, the previous crop of beans, led to increased bacterial diversity and activity. Fertility management and crop protection also affected the community. The effects of both on overall bacterial diversity (as measured by DGGE) were subtle with an overall trend of conventional fertility management and organic crop protection leading to increased diversity. Although overall diversity was not changed significantly by fertility management, factors associated with fertility management had strong correlations with DGGE data. Specifically, we showed total C, pH, soil basal respiration and phosphorus were all positively correlated with an increase in bacterial diversity. In contrast, total N, available nitrate and available ammonium were negatively correlated with bacterial diversity. Bacterial activity (as measured by qPCR and CLSU) was more significantly affected by treatment with both conventional fertility management and crop protection having 'positive effects'.

In the past there have been multiple studies looking at the effect of farm management on the bacterial community. When the search terms 'organic farming' and 'bacteria' were inputted into the 'web of science' 187 hits were found. The results from all relevant studies, found from this search, have been summarised in Table 7.1. On average, these studies focus on the effects of fertility management on the diversity and structure of the bacterial community. In particular, the impact of the use of farmyard manure and compost has been studied. With the exception of Moreno *et al*. (2009), even though different pesticides are used between different treatments in a large proportion of the studies,

Table 7.1. Summary of all past studies comparing the impact of different farm managements on the soil bacterial community.

Farming types compared	Community of interest	Methods used	Results	Reference
Mineral fertilizer vs. high low and medium amounts of cattle manure	Total bacterial and AM fungi	PLFA	Soil microbial biomass and AM fungi biomass was enhanced by the application of manure. The ratio of fungi to bacteria was increased when mineral fertilizer was applied	Ngosong *et al*., 2010
Long term organic and conventional farming systems, short term mineral and organic fertility management systems and a reference site. (experiments are in 3 different European countries)	Bacteria capable of causing soil- and air-borne disease	culture-dependent bioassay	Long term organic amendments led to the promotion of soil microorganism which induced disease resistance in plants. This also occurred, but to a lesser extent, in the short term trials.	Tamm *et al*., 2010
Four treatments testing the effects of cover crops and the use of herbicides	Total bacteria	DGGE and qPCR of 16S rRNA gene and analysis of activity of 6 enzymes	Covered soils exhibited greater bacterial biomass and diversity, as well as higher microbial functional diversity than non-covered soils. Herbicides reduced the microbial functional diversity in covered soil only. The effect of cover crop was the strongest	Moreno *et al*., 2009
Compared a grazed grassland field, 2 conventionally managed fields with 2 levels of chemical input and 5 organically managed fields which had been organically managed for between 2 and 14 years.	Total bacteria	GN BIOLOG plates	Diversity was higher in organic fields. The time fields had been under organic management did not make a significant difference. Seasonal and plant growth stage effects were greater than management effects.	Papatheodorou *et al*., 2008

Table 7.1 (continued). Summary of all past studies comparing the impact of different farm managements on the soil bacterial community.

Farming types compared	Community of interest	Methods used	Results	Reference
Mineral fertilizer and pesticides vs. cattle manure	Total bacteria	Direct cfu counting and PLFA	Fungal and bacterial PLFAs were increased by 17-18% in the plots receiving cattle manure.	Birkhofer *et al*., 2008
Ten organic farms in the Netherlands	Total bacterial and fungal	DGGE	C/N ratio and pH were significantly positively correlated with microbial biomass.	Postma *et al*., 2008
Organic vs. mineral fertilizers	Total bacterial and AM fungi	T-RFLP, cloning and sequencing	The fungal and bacterial community structure was changed by the different fertilizer regimes. This was mainly caused by changes in pH, but was also linked to changes in phosphate levels and soil carbon content	Toljander *et al*., 2008
Farmyard manure vs. mineral fertilizers vs. no fertilizer control	Total bacterial and fungal	PLFA	Farmyard manure application revealed the strongest influence on microbial community structures. The highest fungal biomass was found in the two organic systems, their contribution to the differentiation of community structures according to the management regime was relatively low.	Esperschutz *et al*., 2007
Different compost rates vs. mineral fertilizers vs. no fertilizer control	Total bacteria, *Actinomycetes* and fungi	Direct cfu counting, chloroform fumigation and soil enzyme activities	Organic compost application resulted in a general increase in soil microbial numbers and soil enzyme activities	Chang *et al*., 2007

Table 7.1 (continued). Summary of all past studies comparing the impact of different farm managements on the soil bacterial community.

Farming types compared	Community of interest	Methods used	Results	Reference
Farmyard manure vs. mineral fertilizers vs. no fertilizer control	Total bacteria	T-RFLP, direct cfu counting and BIOLOG	Cfu and microbial biomass were higher following organic fertilizers. However soil microbial structure did not change as a results of farmyard manure application.	Widmer *et al*., 2006
Various organic and conventionally managed farms	Total bacteria and nematodes	Direct cfu counting and DGGE	Although organic management in general led to higher bacterial numbers, soil type was a more significant factor as it had a stronger influence on pH, organic C, phosphorus, nitrate and ammonium	van Diepeningen *et al*., 2006
Various carbon amendments; compost vs. vetch vs. no amendment	Total bacteria	PLFA	PLFA higher when carbon is added but this is not significant	Drenovsky *et al*., 2004
Three separate farms with different management strategies	Total bacteria	DGGE and T-RFLP of 16S rRNA gene and BIOLOG	Soil type caused the biggest change in community structure with management type having a much smaller effect.	Girvan *et al*., 2003
Two farms, one organically managed and one conventionally managed	Total bacterial and fungal	Direct cfu counts and FISH	Higher levels of bacteria and fungi following organic management. However, the differences are relatively subtle	Shannon *et al*., 2002
Conventional management vs. low input vs. organic management	Total bacteria	Respiration rates and PLFA	Organic and low input soils had a different bacterial composition to conventional soils	Lundquist *et al.,* 1999

the associated effects are not discussed, or are seen as secondary to the effects of fertility management.

The main findings of the studies summarised in Table 7.1 are:

- Microbial biomass, diversity, activity and community structure are enhanced by organic fertility management.
- The application of herbicides reduced microbial diversity.
- Total carbon, pH and phosphorus are significantly correlated with increased bacterial diversity.
- Changes to bacterial structure and diversity due to management are often subtle.
- Seasonal and plant growth effects often have a greater influence than management.

Overall the results from our study agree with the results of previous studies in that changes due to management are relatively subtle, as far as change in bacterial diversity are concerned, and that pH, carbon and phosphorus are positively correlated to increases in bacterial diversity. However, our study stands apart from previous studies in that the effect of the application of pesticides have been looked at in detail, as well as the effects of fertility management. In our study effects of season, previous crop and crop protection affected the bacterial community to a greater extent than fertility management. This is probably due to the nature of the NFSC experiment. Changes in pH, carbon and nitrogen availability brought about by changes in long-term fertilization can significantly change microbial community structure and function (Campbell *et al*., 2010). The application of farmyard manures can significantly increase soil carbon content, in turn increasing both bacterial and fungal biomass (Ngosong *et al*., 2010; Birkhofer *et al*., 2008). However, when the impact of management on nitrogen mineralization was investigated it was found

that soil carbon in conventionally managed soil is more accessible to microbes, and therefore, increased microbial activity is often seen here (Birkhofer *et al*., 2008). It is also possible that increased N availability may result in carbon loss as the microbial community proliferates and decomposition of soil organic matter increases (Huang *et al*., 2010). In the short term, N addition, in the form of fertilizers, relieves N limitation and can lead to increased bacterial growth and activity. However, in the long term this could deplete pools of soil organic carbon resulting in further change to the soil bacterial community (Campbell *et al*., 2010; Huang *et al*., 2010). This could explain why previous studies see changes to the bacterial community in their experiments. As discussed in results chapter 1, levels of nitrogen and carbon at NFSC do not differ significantly between the two fertility management types. This may explain why significant correlations are seen between factors associated with fertility management (e.g. carbon and nitrogen) and bacterial diversity, while diversity does not change significantly between the organic and conventional soil when management is looked at as a whole.

7.2. The impact of farm management on the free-living nitrogen fixing community.

In this study, the free-living diazotrophic community was analysed in organic and conventionally managed soil using DGGE and qPCR analysis of the *nifH* gene. Both DNA and RNA were extracted from soils. With the exception of the effect of previous crop, where winter barley resulted in increased diversity and activity, the RNA data set gave very varied results from which few conclusions could be drawn. The DNA data set revealed that conventional fertility management led to increased *nifH* copy number. Although overall fertility management had no affect on the diversity of the diazotrophs, the factors pH, total N and total C were positively correlated with *nifH* diversity, and soil basal respiration and phosphorus were negatively correlated with *nifH* diversity. Organic crop protection resulted in increased diversity and activity of *nifH*.

There are very few studies on the impact of organic farming on the free-living diazotrophic communities in agricultural soil. Of these only 2 studies related to soil management activities, one of which compared the effects of stubble and tillage management (Gupta *et al*., 2006) and the other compared between the use of cattle manure and urea fertilizers (DeLuca *et al*., 1996). The study by DeLuca *et al*. (1996) found results which were comparable with those of this study, in that both fertilizer types inhibited nitrogen fixation and pH was correlated to nitrogen fixation ability. The methods used in this study, however, analysed nitrogen fixing activity using the acetylene reduction assay and by counting colony forming units. Therefore, our results complement this study, as we also take into account the influence of non-culturable diazotrophs, which have been shown to be significant in the soil environment (Hsu and Buckley, 2009).

There has been other work looking at the effect of individual attributes of farm management on the nitrogen fixing community. In general, as far as fertility management is concerned, previous studies suggest that the application of increased amounts of nitrogen fertilizer (normally associated with conventional fertility management) would result in decreased diazotrophic diversity and activity (Coelho *et al*., 2009; Coelho *et al*., 2008). Our findings contradict this conclusion and, suggest that many different factors affect the nitrogen fixing community and that the combined effects of nitrogen level, pH, carbon availability, phosphorus and crop protection, mean that one particular type of management is not more appropriate than the other.

To our knowledge this is the first study which fully investigates the effects of previous crop and realistic crop protection protocols on free-living nitrogen fixation. We found these factors to have more impact than fertility management and suggest that free-living diazotrophs are particularly sensitive to the application of pesticides and that a history of crop rotation can also significantly affect the community.

7.3. The impact of season on both the free-living nitrogen fixing community and the total bacterial community.

As soil microbial communities are dynamic they are capable of significant change due to seasonal and temporal effects (Buckley and Schmidt, 2003). For both the free-living nitrogen fixers and the total bacterial community sample date was repeatedly a significant factor, influencing both diversity and activity. In general, diversity and activity of both communities was decreased in June. As discussed in results chapter 2 and 3 it is possible, but unlikely that this is due to temperature changes (Figure 4.17). The effect of season has been observed in previous studies and is possibly due to one of two factors or the combination of them both. Firstly, the decrease in diversity and activity coincides with the application of fertilizers and pesticides. Certain members of the soil bacterial community, particularly *Acidobacteria*, *Bacteroidetes* and *Alpha*-, *Beta*-, and *Gamma-proteobacteria*, have previously been observed to be diminished in summer in crop land (Jangid *et al*., 2008). Although it was often difficult to discriminate between the two management types it is possible that the action of perturbing the soil led to a decrease in diversity and activity regardless of management type as both will cause significant perturbations of the soil environment. Secondly, it is possible that the community changes due to plant growth effects. It has been demonstrated that potato growth stage and seasonal effects significantly affect diversity in potato soil (Diallo *et al*., 2010; van Overbech and van Elsas, 2008). When culture dependent and independent (cloning and DGGE) methods were used to assess bacterial diversity in bulk and rhizosphere soil in 3 species of potato, bacterial communities were observed to change as the plant developed. Less diversity was observed at growth stage 9 (140 days after planting (July in NFSC)) compared to growth stage 1 (25 days after planting (March in NFSC)) and growth stage 6 (65 days after planting (April in NFSC)) (van Overbech and van Elsas, 2008). This was also observed in maize where bacterial activity, as measured by PLFA and BIOLOG,

changed as maize went through five leaf stage, flowering and maturity (Griffiths *et al*., 2006). A change in community structure has also been observed, with numbers of Gram positive bacteria increasing as the plant is ready to be harvested, probably due to the amount and quality of root exudates changing as the plant reaches maturity (Ngosong *et al*., 2010).

7.4. Comparison between the nitrogen fixing and total bacterial community.

As expected, diversity and copy number of the 16S rRNA gene were always higher than diversity and copy number of the *nifH* gene. Ratios of the *nifH* gene to the 16S rRNA gene were similar to ratios seen between the 16S rRNA gene and genes used in nitrogen cycling found in other studies (Morales *et al*., 2010; Kandeler *et al*., 2006). As most bacteria respond to factors such as pH and available carbon in similar ways (Fierer and Jackson, 2006), it would be expected that there would be similarities between the two communities. Positive correlation was seen when comparing *nifH* and 16S rRNA gene copy number. However, it was also important to show that any changes in the nitrogen fixing community were not simply an artifact of changes in the whole bacterial community. This was demonstrated in this study with respect to the different results found when looking at the impact of farm management on the two different communities.

7.5. A discussion of the molecular techniques used.

Table 7.1 shows how a wide range of techniques can be utilized to examine bacterial communities within the soil. In this study we chose to use PCR-DGGE, qPCR, sequencing and BIOLOG plates. These techniques were chosen so that both diversity and activity of the total bacterial and the free-living nitrogen fixing community can be examined. As far as investigating diversity and expression of the chosen genes the study was successful and informative. Since DGGE was introduced (Muyzer *et al*., 1993), it has been the most widely used community fingerprinting technique as it allows easy

comparison between different samples (Anderson and Cairney, 2004). One main advantage of the technique, which was exploited in this study, was the ability to cut out and sequence DGGE bands of interest. This allowed additional information about the structure of the nitrogen fixing community in the soils at NFSC.

Measuring copy number of the two genes as a proxy for gene expression using qPCR was also successful. Although compared to some soil environments 16S rRNA gene copy number in our study was low (Whitmann *et al*., 1998), values in the region of 10^7-10^8 copies per g of soil appears to be average for potato soil (Diallo *et al*., 2010). Copy numbers obtained from qPCR are meant to allow comparisons between soils in this study and should not be used as absolute quantifications.

To gain an accurate representation of a community it is vital that the RNA/DNA extracted from soils is pure and of good quality. Humic acids are often co-extracted with nucleic acids and can inhibit polymerases used in PCR (Anderson and Cairney, 2004). In this study, we removed humic acids by using nucleic acid extraction kits designed for the soil environment and including BSA in all PCR reactions. However, the use of the different nucleic acids did lead to different results when looking at the nitrogen fixing community. In general, when DNA was used as a template results appeared to be more consistent and trends could be seen more clearly. When RNA was used as the template results were often erratic and very time point specific. It is accepted that, as the majority of the bacterial community within soil is inactive, studies analysing DNA are obtaining a historical perspective of community change, rather than the 'snap shot' of the active bacterial community you would obtain from an RNA study (Girvan *et al*., 2003). RNA profiles therefore, often show greater discrimination than DNA profiles showing short term changes rather than sustained community change (Girvan *et al*., 2003). For example, Morales *et al*. (2010) suggested that carbon had a major role in shaping long term total community structure and that this was best demonstrated using DNA-based experiments. However, they also suggest that treatment practices such as changes in weather and

fertilizer application could lead to short term responses in bacterial activity which would be detected using RNA-based experiments. This suggests that it is appropriate to study both nucleic acids in this kind of study and that DNA results should be considered when looking for long term community change due to farm management.

Analysis of the active bacterial and diazotrophic community could be improved by expanding the range of experiments. The quantification of gene copy number using qPCR allowed us to quantify the amount of expression of each gene in NFSC soil. However, this does not tell us how much nitrogen is actually being fixed as it does not take into account any post-translational modifications (Bustin *et al*., 2010). Two possible approaches could be used to quantify nitrogen fixation. The most commonly used is the acetylene reduction assay. The other possibility is to measure how much ^{15}N is fixed into soils when they are left in a ^{15}N atmosphere.

BIOLOG plates were used to assess overall community functional diversity which is assumed to increase as substrate utilization increases (Klimek and Niklinska, 2007). However, BIOLOG provides a relatively narrow and biased view of the soil microbial community as the substrates used are selective. The use of BIOLOG also introduces a bias towards bacteria which are culturable as the plates involve growth in liquid medium (Yin *et al*., 2000). More recently microtiter plates which contain an O_2 sensitive fluorophore have been introduced which can be up to 100-fold more sensitive to changes in soil basal respiration and substrate-induced responses (Garland *et al*., 2010).

7.6. Conclusion- what does this mean for farmers?

Legumes are often included in crop rotations as they, reduce energy costs, improve soil quality and decrease pest and weed populations (Rubiales, 2010). In this study we found that overall the presence of Faba beans and the organic rotation increased overall soil bacterial diversity and activity. However, we also found that this leads to a diminished N-

fixing community as Faba beans have a high reliance on symbiotic N_2 fixation and subsequently release N-rich residues and do not take N from the soil (Jensen *et al*., 2010). This absence of free-living diazotrophs will not be of immediate detriment to the soil; however, this effect can last throughout the following year. This research highlights the importance of crop rotation and the choice of crops within the rotation and shows that the crop grown not only affects symbiotic microorganisms but also microorganisms in surrounding soil (Ngosong *et al*., 2010).

While changes to fertilizer applications to agricultural land are understood to alter the bacterial communities of soil, the effect it has on specific soil communities remains largely unknown (Jangid *et al*., 2008). In this study we found fertility management to have a relatively subtle affect on the diversity of both the nitrogen fixing and the total bacterial community. However, in terms of copy number of the *nifH* and 16S rRNA genes conventional fertility management had a positive effect. The combination of our study and the work of others suggests that rather than the bacterial communities being affected directly by the nature of the fertilizers applied they are more likely to respond to the pH, carbon and nitrogen levels in the soil (Campbell *et al*., 2010; Postma *et al*., 2008; Toljander *et al*., 2008; van Diepeningen *et al*., 2006). By controlling these factors it may be possible to optimise the activity of the bacterial and diazotrophic communities regardless of fertility management type.

Crop protection protocols affected both communities differently with the total bacterial community proliferating in response to the increased carbon obtained from degrading the pesticides, and the nitrogen fixing community suffering from the toxic effects of the chemicals. In general, this suggests that, when used at recommended dosages, pesticides will not have a detrimental affect on the microbial community as a whole. However, for unknown reasons, bacteria which are often plant growth promoting are more sensitive to the effects of pesticides. Therefore, it may be necessary to test the response

of all bacteria involved in nitrogen cycling when approving pesticides, rather than just bacteria involved in nitrogen mineralization, to understand the impact on soil health.

In general, this study shows that bacterial communities in agricultural soil have a high degree of functional stability, meaning overall diversity will often remain unchanged while activity levels and relative abundances change (Bagwell and Lovell, 2000). This complex structure can protect the environment from declines in functionality and allow the bacterial community to adapt to a change in conditions, as when a function is common within the community it is more likely to remain regardless of perturbation (Wittebolle *et al*., 2009). To further test the impact of farm management on the nitrogen fixing community the amount of nitrogen fixed in the soil would have to be measured. This would give an indication of how changes to the diversity and copy number of *nifH* actually relate to fixed nitrogen in the soil and would give an idea of how stable the community was to change. Expanding the study to look at the effect of farm management on other members of the nitrogen cycling community would also allow farmers to gain a greater understanding of nitrogen dynamics within their soil.

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Appendix

Appendix A (Chemicals, reagents and kits)

A1. List of chemicals.

Acros

- dichloro-isocyanuric acid sodium salt
- N-1-Naphthylethylenediamine di-HCL (NEDD)
- Sodium phosphate

Aldrich

- Hexamine cobalt chloride

Applied Biosystems

- HiDi Formamide

BDH

- Bromophenol Blue

BioRad

- Ethidium Bromide
- Manganese chloride

Duchefa

- Urea (electrophoresis grade)

Fisher

- Acetic acid (glacial)
- Dimethylsulphoxide (DMSO)
- Glycerol
- Ortho-phosphoric acid
- Potassium hydroxide
- Sodium hydroxide

GE healthcare

- Exo-SAP-IT

Hayman Ltd

Absolute alcohol (ethanol)

Invitrogen

SYBR green I nucleic acid gel stain

SYBR safe

Melford

Agarose

Sodium chloride

Tris Base Ultrapure

Merck

Tetra-sodium-diphosphate

New England Biolabs

dNTPs

Promega

Bovine Serum Albumin

Qiagen

Random hexamers

Sigma

40 % (v/v) solution (37.5:1 acrylamide:bisacrylamide)

Ammonium chloride

Ammonium persulphate

Ampicillin

Calcium chloride

Ethylene diamine tetraacetic acid (EDTA)

Formamide (deionised)

Glucose

Glycerol

IPTG

Isopropanol

Magnesium chloride

Sigma

- Magnesium sulphate
- Potassium acetate
- Potassium chloride
- Potassium phosphate
- Sodium nitroprusside
- Sodium salicylate
- Sucrose
- Thiamine-HCl (vit B_1)
- TEMED
- X-Gal
- Xylene cyanol

Sigma-Aldrich

- Sulphanilamide
- Tri-sodium citrate

Thermo Fisher Scientific

- Absolute qPCR SYBR green mix
- Brij-35

A2. Media

Oxoid

- Agar (Bacteriological agar no. 1)
- Yeast extract
- Tryptone

A3. List of enzymes

Applied Biosystems.

- BigDye Terminator v3.1 ready reaction mix. Reaction buffer is patented.

New England Biolabs

Taq (DNA polymerase). Buffer contains 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl.

GE Healthcare

ExoSAP-IT contains two enzymes Exonuclease I and Shrimp Alkaline phosphatase.

Invitrogen.

Pfx (DNA polymerase). Reaction buffer is patented.

Superscript II reverse transcriptase. Reaction buffer contains 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$.

Promega.

EcoRI. Buffer H contains 90 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, pH 7.5.

T4 DNA ligase. Buffer contains 30 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1mM ATP, 5% PEG.

Thermo Fisher Scientific

Absolute qPCR SYBR green mix contains the patented Hot Start enzyme Thermo-Start (DNA polymerase) and a patented buffer containing SYBR green I.

A4. DNA size standard.

Bioline.

Hyperladder I (10,000, 8,000, 6,000, 5,000, 4,000, 3,000, 2,500, 1,500, 1,000, 800, 600, 400, 200 bp)

A5. Kits.

Cambio.

MoBio UltraClean Soil DNA extraction

Table A5i. Solutions used to extract DNA from soil using the MoBio UltraClean Soil DNA extraction kit (Absolute components are patented).

Solution	Solution contains	Role of solution
S1	Sodium dodocyl sulphate and proprietary salts	Aids cell lysis
IRS	not disclosed	removes humic acids
S2	Acetate and proprietary salts	precipitates proteins
S3	Guanidine hydrochloride, isopropanol and proprietary salts	binds DNA to the silica membrane
S4	Ethyl alcohol and proprietary salts	removes excess salt
S5	10 mM Tris (pH 8.0)	elutes DNA

MoBio UltraClean Microbial RNA isolation kit.

Table A5ii. Solutions used to extract RNA using the MoBio UltraClean Microbial RNA isolation kit (Absolute components are patented).

Solution	Solution contains	Role of solution
MR1	Guanidine thiocyanate	aids cell lysis
MR2	dithiothreitol and proprietary salts	prevents oxidation of RNA and inhibits RNase activity
MR3	Lauryl sulphate sodium	aids cell lysis and protein disruption
MR4	Sodium chloride and proprietary salts	causes RNA precipitation
MR5	ethanol	removes excess salt

Promega

Pure Yield plasmid mini prep system

Table A5iii. Components of Pure Yield plasmid mini prep system kit (Absolute components are patented).

Pure Yield plasmid mini prep kit
Cell lysis buffer (CLC)
Neutralization solution (NSC)
Endotoxin Removal Wash (ERB)
Column wash solution (CWC)
Elution buffer (EBB)

A6. Bacterial Strains.

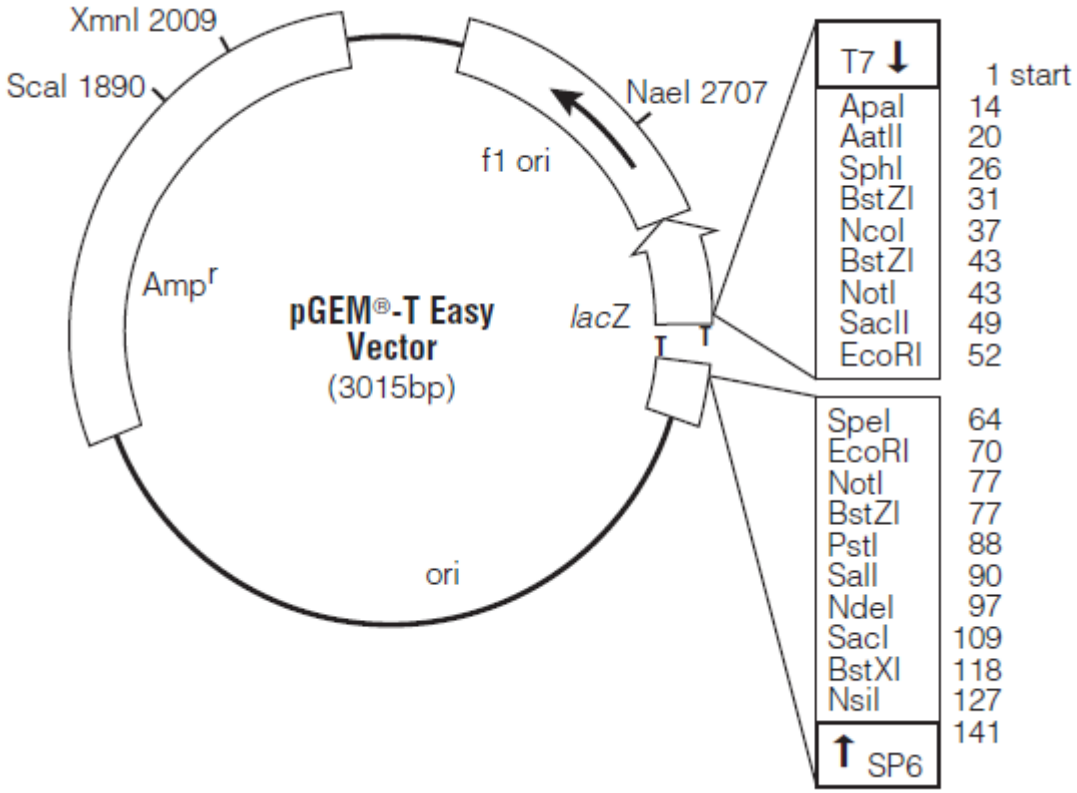
JM109 competent cells were purchased from Promega.

Rhizobium sp. IRBG74 was isolated from root nodules of *S. cannabina* by the International Rice Research Institute.

Pseudomonas aeruginosa was obtained from the National Collection of Type Cultures (NCTC 10662).

A7. Vector information.

Figure A7i. Map of the pGEM-T Easy vector (Promega) used in this study.



BIOLOG

Microbial Community Analysis

EcoPlate™

A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine
B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine
C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine
D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine
E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine
F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid
G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine
H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine

Appendix B (Media, solutions and buffers)

B1. Media.

Table B1i. Chemicals required to make M9 minimal media + thiamine HCl

reagent	amount per L
5 x M9 salts (see table B1i)	200 ml
1 M $MgSO_4$	2 ml
20 % w/v glucose	20 ml
1M $CaCl_2$	0.1 ml
Agar	15 g
thiamine	100 uL

Table B1ii. 5 x M9 salts required to make M9 minimal media

reagent	Amount g/L
sodium phosphate	64
Potassium phosphate	15
sodium chloride	2.5
Ammonium chloride	5

Table B1iii. Reagents needed to make LB Broth.

reagent	g per L
tryptone	10
yeast extract	5
NaCl	5
pH 7.0	

To make LB agar add 14 g/L agar

Table B1iv. Reagents needed for SOC medium.

reagent	Amount per 100 ml
Bactrtryptone	2 g
yeast extract	0.5 g
1M NaCl	1 ml
1M KCl	0.25 ml
2M Mg^{2+}	1 ml
2M glucose	1 ml

B2. Buffers

Table B2i. Reagents needed for 50 x TAE

reagent	amount per L
Tris	242 g
glacial acetic acid	57.1 ml
500 mM EDTA pH 8.0	100ml

Table B2ii. Reagents needed for FSB solution

reagent	g/100ml
1 mM potassium acetate	0.0098
45 mM $MnCl_2$	0.8905
10 mM $CaCl_2$	0.147
10 mM KCl	0.7455
3 mM Hexamine cobalt chloride	0.08
10 % v/v Glycerol	10 ml
18.2 MQ water	up to 100 ml

Store at 4 ˚C.

B3. Solutions for analyzing available nitrate and ammonium using the hydrazine reduction method:

Nitrate-N solutions

Table B3i. Colour reagent used to measure nitrate-N.

reagent	amount/500 ml
Sulphanilamide	5 g
N-1-Naphthylethylenediamine di-HCL (NEDD)	0.25 g
ortho-phosphoric acid	50 ml
distilled water	up to 500 ml

Table B3ii. Sodium hydroxide solution used to measure nitrate-N.

reagent	amount/500 ml
NaOH	20 g
diluted Brij-35	0.5 ml
distilled water	up to 500 ml

Table B3iii. Phosphoric acid solution used to measure nitrate-N.

reagent	amount/500 ml
ortho-phosphoric acid	1.5 ml
tetra-sodium-diphosphate x $10H_2O$	2 g
diluted Brij-35	0.5 ml
distilled water	up to 500 ml

Table B3iv. Hydrazine sulphate solution used to measure nitrate-N.

reagent	amount/500 ml
stock Cu solution[1]	7 ml
stock Zn solution[2]	5 ml
hydrazine sulphate	3 g
distilled water	up to 500 ml

[1]stock Cu solution = 0.5 g cupric sulphate in 500 ml distilled water.

[2]stock Zn solution = 5 g zinc sulphate in 500 ml distilled water.

Ammonium-N solutions

Table B3v. Buffer used to measure ammonium-N.

reagent	amount/500 ml
tri-sodium citrate	20 g
diluted Brij-35	0.5 ml
distilled water	up to 500 ml

Table B3vi. Sodium salicylate used to measure ammonium-N.

reagent	amount/500 ml
Na salicylate	20 g
Na nitroprusside	0.5 g
distilled water	up to 500 ml

Table B3vii. DCI solution used to measure ammonium-N.

reagent	amount/500 ml
NaOH	10 g
dichloro-isocyanuric acid Na salt	1.5 g
distilled water	up to 500 ml

B4. Reagents for DGGE

Table B4i. Reagents needed for 12% acrylamide denaturing solution with 35% denaturant

reagent	amount per 100 ml
40 % (v/v) solution (37.5:1 acrylamide:bisacrylamide)	30 ml
50 x TAE	2 ml
Formamide (deionised)	14 ml
Urea (electrophoresis grade)	14.7 g

Table B4ii. Reagents needed for 12% acrylamide denaturing solution with 55% denaturant

reagent	amount per 100 ml
40 % (v/v) solution (37.5:1 acrylamide:bisacrylamide)	30 ml
50 x TAE	2 ml
Formamide (deionised)	22 ml
Urea (electrophoresis grade)	23.1 g

Solutions are stored at 4 °C. For different percentage denaturant add an extra 2 ml formamide and 2.1 g urea for each increase of 5 %.

Solution 1:

25 ml 55% denaturing solution, 21.6 µl TEMED, 216 µl 10 % APS (ammonium persulphate), 100 µl Dcode dye

Solution 2:

25 ml 35 % denaturing solution, 21.6 µl TEMED, 216 µl 10% APS.

Table B4iii. Reagents needed to make Dcode dye.

reagents	Amount added (10 ml)
Bromophenol blue	0.05 g
Xylene cyanol	0.05 g
50 x TAE	10 ml

Table B4iv. Reagents needed to make 2 x Loading dye for DGGE.

reagents	Amount added (10 ml)
2% Bromophenol blue	0.25 ml
2% xylene cyanol	0.25 ml
100% glycerol	7.0 ml
water	2.5 ml

B5. Miscellaneous

Table B5i. Reagents needed to make Bromophenol Blue loading dye

Reagent	Amount added (10 ml)
Sucrose	4 g
Bromophenol blue	0.025 g
water	10 ml

Appendix C (Equipment)

Agarose gel kits

Agarose gel electrophoresis was carried out in BioRad Mini horizontal (HU6) and Midi horizontal (HU13) gel units and a BioRad Power-Pac Basic.

Autoanalyzer

Ammonium and nitrate concentrations were found using a Brann and Leubbe Autoanalyzer 3.

Autoclaving

Sterilization was carried out in a Tactoral 2 Priorclave or Prestige® Medical 2100 Classic benchtop autoclave at 121 °C, 32 lb/inch^2 pressure for 20 mins.

Centrifugation

A Sigma 1-15 benchtop micro-centrifuge was used to carry out all centrifugation steps.

DGGE kits

DGGE was carried out using a BioRad Dcode Universal Mutation Detection system and BioRad Power-Pac Basic.

Gel documentation

Gels were visualised using a BioRad Universal hood II and analysed using BioRad Quantity one ID analysis software.

Incubators

Static incubation was carried out in a GallenKamp static incubator. Orbital incubation was carried out in a GallenKamp orbital shaker.

PCR machine

All PCR was carried out using an Eppendorf Mastercycler gradient.

pH meter

A Jenway Ion Meter 3340 was used to adjust pH of all media and solutions, and to measure soil pH.

Plate reader

Absorbance readings of BIOLOG plates were found using a Bio-tek ELx800 microplate reader.

qPCR

Gene copies numbers were quantified using a Corbett Rotor-Gene RG-3000 and associated computer software.

Sequencer

Sequence data was obtained using an Applied Biosystems 3130 genetic analyzer.

Soil basal respiration heads

Soil basal respiration measurements were recorded using Sensomat Aqualytic heads and associated computer software.

Spectrophotometer

A Heλios spectronic unicam spectrophotometer was used to measure all OD and absorbance readings.

Reaction vessels

Unless otherwise stated, volumes up to 0.2 ml were contained in 0.2 ml micro-centrifuge tubes (Starstedt), 0.2-1.5 ml volumes in 1.5 ml micro-centrifuge tubes (Starstedt) and for volumes greater than 1.5 ml, 28 ml sterile plastic universal containers were used (Fisherbrand®).

Appendix D (results RC1).

Table Di. Environmental variables recorded across all sample years and used in multivariate analysis.

year	previous crop	plot no.	% C	% N	pH	SBR March	SBR June	Fe	P	NO_3^- March	NO_3^- June	NO_3^- Sept	NH_4^+ March	NH_4^+ June	NH_4^+ Sept
2007	barley	1	1.91	0.22	6.24	1.29	1.45	382	66	6.6	109.7	29.1	1.0	3.9	9.6
2007	barley	2	1.91	0.25	6.10	1.24	0.76	400	78	9.8	389.4	25.1	0.0	8.5	5.8
2007	barley	3	2.03	0.26	6.43	1.23	1.22	372	76	9.8	656.1	34.8	1.8	13.0	6.5
2007	barley	4	2.07	0.26	6.45	1.16	1.25	375	65	6.3	152.9	16.2	1.3	7.2	10.2
2007	barley	5	1.74	0.26	6.46	1.07	1.97	354	56	4.0	150.5	29.3	2.5	3.5	9.2
2007	barley	6	1.90	0.24	6.04	1.00	1.11	303	44	7.9	388.3	42.7	0.6	6.1	5.3
2007	barley	7	1.79	0.24	5.93	1.01	0.93	314	51	6.4	376.5	16.3	0.9	6.3	4.5
2007	barley	8	1.68	0.24	6.02	1.19	1.49	387	66	4.0	103.6	22.5	0.9	6.4	5.1
2007	barley	9	1.89	0.28	6.23	1.04	1.06	291	35	6.8	88.1	23.7	1.1	3.3	5.4
2007	barley	10	1.84	0.27	6.17	1.02	1.01	263	40	6.4	347.2	23.1	0.3	14.2	5.7
2007	barley	11	1.80	0.29	6.44	1.28	0.88	275	42	11.2	419.9	42.0	1.3	9.6	7.5
2007	barley	12	1.68	0.25	6.39	1.07	1.37	274	33	0.0	110.2	29.0	1.5	6.9	6.3
2007	barley	13	1.62	0.33	6.37	1.38	1.60	334	42	1.2	134.4	21.5	1.2	7.5	6.3
2007	barley	14	1.80	0.33	6.32	1.09	0.94	345	42	5.4	442.9	41.2	0.7	10.7	3.4
2007	barley	15	1.65	0.33	6.31	1.24	0.82	316	38	3.7	446.3	15.6	0.0	9.4	5.4
2007	barley	16	1.63	0.30	6.43	1.08	2.00	298	46	3.8	153.3	20.7	0.0	4.2	6.5
2007	beans	1	2.08	0.26	6.27	1.11	1.38	414	68	14.1	71.4	21.4	7.6	0.8	0.0
2007	beans	2	1.81	0.25	6.13	1.11	1.28	416	79	15.1	214.5	16.1	6.5	0.9	0.0
2007	beans	3	1.85	0.25	6.08	1.27	1.14	381	83	9.3	344.9	24.3	6.9	0.3	0.0
2007	beans	4	1.92	0.24	6.27	1.06	1.17	374	66	9.7	127.5	29.0	7.3	0.3	0.0
2007	beans	5	2.32	0.28	6.03	1.15	1.26	276	42	12.3	143.4	29.5	6.7	0.3	0.0
2007	beans	6	1.73	0.27	5.91	0.94	1.14	364	62	11.2	360.8	30.7	5.4	0.9	0.0
2007	beans	7	1.81	0.25	5.85	0.85	0.86	346	66	13.1	292.8	12.5	4.8	0.9	0.0
2007	beans	8	1.76	0.25	6.09	0.86	1.39	368	54	15.8	144.1	21.4	6.3	0.9	0.0
2007	beans	9	1.84	0.29	6.24	1.16	1.40	303	48	7.9	106.4	15.7	4.9	2.1	0.0
2007	beans	10	1.81	0.29	6.16	0.82	0.79	311	52	12.5	427.3	16.1	5.0	2.1	0.0
2007	beans	11	1.65	0.29	5.98	0.90	0.75	312	51	13.5	426.7	27.8	5.5	1.9	0.0
2007	beans	12	1.67	0.30	6.31	0.98	1.41	304	36	7.3	143.6	33.7	4.4	0.9	0.0
2007	beans	13	1.66	0.33	6.23	0.73	1.22	345	39	14.7	165.3	25.1	7.6	3.2	0.0
2007	beans	14	1.71	0.32	5.90	0.94	0.81	310	40	15.3	356.4	23.6	5.5	4.6	0.0
2007	beans	15	1.86	0.33	6.05	1.07	0.88	316	48	14.5	329.1	9.8	5.9	2.6	0.0
2007	beans	16	1.80	0.35	6.17	1.02	2.93	338	43	13.3	95.9	22.6	5.2	0.6	0.0

Table Di (continued). Environmental variables recorded across all sample years and used in multivariate analysis.

year	previous crop	plot no.	% C	% N	pH	SBR March	SBR June	Fe	P	NO_3^- March	NO_3^- June	NO_3^- Sept	NH_4^+ March	NH_4^+ June	NH_4^+ Sept
2008	wheat	1	2.99	0.28	7.28	n/a	0.90	420	66	9.7	16.4	10.7	2.9	6.9	0.0
2008	wheat	2	3.08	0.28	6.78	n/a	0.87	435	89	8.8	58.4	9.6	1.1	1.2	0.0
2008	wheat	3	2.66	0.24	7.26	n/a	0.66	421	66	6.8	85.4	14.0	0.8	7.4	0.0
2008	wheat	4	2.87	0.28	7.26	n/a	0.93	358	47	5.1	15.8	8.4	0.0	2.3	0.0
2008	wheat	5	2.72	0.26	6.95	n/a	0.97	434	44	5.2	21.9	7.9	0.7	3.8	0.0
2008	wheat	6	2.67	0.23	6.87	n/a	0.96	356	44	5.7	115.1	6.1	1.8	2.7	0.0
2008	wheat	7	2.61	0.22	6.63	n/a	0.94	318	40	3.8	154.3	14.3	0.3	7.0	0.0
2008	wheat	8	2.67	0.22	6.81	n/a	0.94	280	31	3.9	34.0	9.8	0.0	4.1	0.0
2008	wheat	9	2.63	0.24	6.96	n/a	0.84	321	31	4.3	21.3	4.9	0.7	4.5	0.0
2008	wheat	10	2.58	0.24	6.36	n/a	0.74	332	41	*2.2*	128.7	7.2	0.7	19.6	0.0
2008	wheat	11	2.55	0.23	6.45	n/a	0.77	312	34	5.2	160.6	15.8	0.7	8.7	0.0
2008	wheat	12	2.57	0.23	6.85	n/a	0.85	265	26	4.1	36.6	16.5	1.1	1.7	0.0
2008	wheat	13	2.79	0.24	6.82	n/a	n/a	489	61	8.0	19.7	2.7	4.6	1.8	0.0
2008	wheat	14	2.87	0.25	6.66	n/a	n/a	464	56	4.9	106.8	6.2	1.7	6.4	0.0
2008	wheat	15	2.71	0.23	6.30	n/a	n/a	417	54	4.5	154.4	4.5	1.5	0.0	0.0
2008	wheat	16	2.71	0.23	6.63	n/a	n/a	344	34	2.8	41.3	9.1	1.8	0.0	0.0
2009	wheat	1	2.58	0.23	6.89	n/a	1.34	425	67	0.0	77.8	5.9	4.7	2.3	2.5
2009	wheat	2	2.44	0.22	6.31	n/a	1.35	458	81	0.0	373.0	8.9	0.0	14.7	1.8
2009	wheat	3	2.28	0.20	6.58	n/a	1.83	438	68	0.0	410.6	22.9	0.0	5.2	0.6
2009	wheat	4	2.29	0.21	6.64	n/a	2.06	437	60	0.0	112.3	10.4	0.0	6.2	0.9
2009	wheat	5	2.35	0.25	6.62	n/a	1.74	423	61	0.0	79.7	12.5	0.0	1.7	2.5
2009	wheat	6	2.22	0.22	6.17	n/a	1.57	403	68	0.0	358.3	34.8	0.0	7.7	2.6
2009	wheat	7	2.14	0.23	6.27	n/a	2.07	404	64	0.0	444.7	34.2	0.0	48.9	3.5
2009	wheat	8	2.31	0.24	6.62	n/a	2.01	399	53	0.0	72.0	16.0	0.0	1.7	2.2
2009	wheat	9	2.08	0.21	6.42	n/a	3.11	324	52	0.0	109.8	18.8	0.0	3.8	4.6
2009	wheat	10	2.16	0.20	5.80	n/a	2.44	344	63	0.0	351.0	68.1	0.0	21.0	1.0
2009	wheat	11	2.03	0.19	5.86	n/a	2.01	354	53	0.0	385.9	50.0	0.0	53.8	9.6
2009	wheat	12	2.04	0.19	6.14	n/a	2.15	327	45	0.0	115.3	19.8	0.0	8.3	4.3
2009	wheat	13	2.24	0.23	6.22	n/a	1.44	487	72	0.0	84.3	6.2	11.7	1.5	1.1
2009	wheat	14	2.18	0.24	6.04	n/a	2.10	495	77	0.0	296.7	23.4	0.0	38.5	0.6
2009	wheat	15	2.11	0.21	6.22	n/a	1.38	472	74	0.0	400.4	8.1	0.0	35.6	1.7
2009	wheat	16	2.35	0.23	6.23	n/a	1.66	410	55	0.0	73.5	5.3	0.0	0.0	2.8

Appendix section E (results RC2)

Table Ei. Shannon diversity indices (H') for each DGGE lane at each sample date (RNA)

	2007 (organic rotation)			2007 (conventional rotation)			2008		2009		
lane	March	June	Sept	March	June	Sept	March	June	March	June	Sept
1	1.940	2.111	1.909	2.151	1.385	2.800	1.530	0.000	1.055	0.693	0.755
2	1.327	1.702	2.425	2.237	1.604	2.910	2.141	1.083	1.286	0.692	1.004
3	1.806	1.524	2.351	2.628	1.876	2.780	1.599	0.693	1.559	0.000	1.446
4	0.816	1.510	1.931	2.105	1.333	2.304	2.024	0.938	1.596	0.000	1.515
5	1.232	1.707	2.633	2.514	2.126	3.154	1.478	1.054	1.511	0.000	1.915
6	1.418	1.480	2.007	2.728	1.944	2.909	1.047	1.428	1.244	0.000	1.733
7	0.837	2.110	1.530	2.842	1.965	2.921	1.066	0.000	1.565	1.385	1.784
8	0.781	2.545	1.556	2.482	1.098	2.599	1.870	2.128	1.733	0.000	1.329
9	0.764	1.896	1.713	2.617	1.750	2.776	1.387	1.747	2.170	0.000	1.613
10	1.723	1.670	2.034	2.768	0.658	2.631	1.894	1.915	1.707	0.000	0.163
11	1.551	1.786	2.178	2.197	2.078	2.515	1.495	1.362	2.130	0.693	1.186
12	0.892	1.646	1.804	2.588	2.361	2.958	1.453	0.000	2.024	0.000	0.784
13	0.791	2.212	1.266	1.383	1.429	2.909	1.554	0.000	0.567	0.000	1.661
14	2.213	2.345	1.329	1.166	1.851	2.635	1.094	0.000	1.322	0.000	0.500
15	1.498	2.122	1.214	1.992	1.553	2.015	1.179	0.000	1.339	0.000	1.321
16	1.770	1.599	1.155	1.749	1.787	1.977	1.301	0.000	0.573	0.000	1.457

Table Eii. Shannon diversity indices (H') for each DGGE lane at each sample date (DNA)

	2007 (conventional rotation)			2008			2009		
lane	March	June	Sept	March	June	Sept	March	June	Sept
1	1.890	0.920	1.635	0.693	1.891	0.670	2.249	1.257	1.824
2	1.554	0.586	0.783	0.746	1.541	0.692	2.109	1.868	1.324
3	1.823	0.406	0.749	0.801	1.901	1.024	2.016	1.146	1.406
4	1.744	1.241	0.725	0.603	1.977	1.477	1.378	0.930	0.744
5	2.532	2.210	2.124	0.792	1.898	0.983	2.175	2.105	1.740
6	1.730	0.714	1.998	1.310	1.700	1.211	2.566	1.417	1.215
7	2.298	2.292	1.949	1.072	1.698	1.238	2.012	1.934	1.392
8	1.304	0.000	1.617	0.661	1.730	0.693	1.233	1.393	1.280
9	1.754	1.458	1.402	1.068	1.111	1.840	1.903	1.533	1.581
10	1.902	0.769	0.955	0.643	1.846	0.674	1.622	1.037	1.240
11	2.027	0.000	0.405	1.319	1.720	0.679	1.453	1.532	1.274
12	2.069	0.360	0.312	1.532	1.862	0.677	1.517	1.650	1.369
13	1.370	0.724	1.073	1.334	1.749	0.655	1.103	1.262	0.792
14	1.790	0.462	1.493	1.032	1.708	1.494	1.615	0.568	1.318
15	1.584	0.330	1.671	0.663	1.741	1.480	0.363	1.148	0.624
16	1.593	0.611	1.174	0.666	1.654	1.507	2.038	0.688	0.643

Table Eiii. QPCR master sheet showing copy numbers of all replicates of 2007 soil from the conventional rotation (RNA).

		March			June			September		
	rep	copies	Average copies	copies per g of soil	copies	Average copies	copies per g of soil	copies	Average copies	copies per g of soil
1	1	227.10	412.89	4.01E+05	19.46	36.53	3.52E+04	22699.51	23321.61	2.26E+07
	2	833.56			28.08			18409.00		
	3	178.00			89.16			30354.89		
2	1	359.04	744.09	7.36E+05	4080.17	3574.57	3.41E+06	24159.10	50727.76	4.79E+07
	2	957.78			2995.28			64039.37		
	3	1198.03			3737.29			84374.07		
3	1	1669.79	1348.90	1.29E+06	48.11	97.22	9.24E+04	6698.84	8496.95	8.11E+06
	2	1475.37			130.32			9962.98		
	3	996.28			146.58			9191.80		
4	1	714.98	432.52	4.17E+05	557.48	224.55	2.14E+05	24867.89	29334.60	2.80E+07
	2	482.98			233.83			31557.63		
	3	234.31			868.51			32166.03		
5	1	614.40	517.16	4.88E+05	321.34	343.67	3.23E+05	132042.48	124519.63	1.18E+08
	2	425.61			435.30			136834.00		
	3	528.96			290.19			106857.68		
6	1	158.34	184.74	1.84E+05	148.17	374.81	3.51E+05	12540.09	9486.16	9.10E+06
	2	180.11			121.66			5452.46		
	3	221.07			2920.86			12484.72		
7	1	386.13	490.37	4.84E+05	6646.79	6391.91	5.97E+06	41052.32	19691.13	1.86E+07
	2	533.30			5098.36			15422.51		
	3	572.61			7706.37			12059.23		
8	1	282.07	289.37	2.91E+05	11186.20	1956.07	1.81E+06	7582.49	3255.25	3.08E+06
	2	249.89			1352.81			2043.91		
	3	343.76			494.57			2225.76		
9	1	756.35	1390.16	1.41E+06	136.41	124.06	1.18E+05	555.85	915.03	8.88E+05
	2	1441.16			241.43			691.07		
	3	2464.68			57.98			1994.46		
10	1	14805.97	16531.68	1.61E+07	594.51	479.11	4.49E+05	3145.20	4046.51	3.80E+06
	2	19079.33			367.28			6398.76		
	3	15993.78			503.68			3292.29		
11	1	944.75	1005.50	9.90E+05	2585.48	2342.27	2.21E+06	40415.25	46226.92	4.35E+07
	2	1001.73			2053.51			52801.69		
	3	1074.18			2420.31			46290.48		
12	1	275.58	147.78	1.44E+05	1412.22	976.37	9.30E+05	24642.79	21607.03	2.04E+07
	2	189.17			1539.54			18565.89		
	3	61.90			428.10			22048.53		
13	1	255.31	359.57	3.63E+05	543.83	477.79	4.48E+05	31969.69	26814.66	2.54E+07
	2	203.82			639.10			27773.66		
	3	893.40			313.81			21714.26		
14	1	1254.97	1755.60	1.81E+06	2204.45	3343.31	3.14E+06	32831.90	35280.01	3.39E+07
	2	1543.90			3857.45			48868.40		
	3	2792.67			4394.70			27369.19		
15	1	642.47	667.67	6.77E+05	1345.04	778.18	7.36E+05	2953.03	4690.44	4.44E+06
	2	600.91			616.57			3607.44		
	3	770.93			568.23			9686.65		
16	1	470.90	590.82	5.82E+05	473.25	864.61	8.19E+05	9306.17	11621.91	1.11E+07
	2	769.16			1356.91			14857.22		
	3	569.42			1006.50			11353.35		

Table Eiv. QPCR master sheet showing copy numbers of all replicates of 2007 soil from the organic rotation (RNA).

		March			June			September		
	rep	copies	Average copies	copies per g of soil	copies	Average copies	copies per g of soil	copies	Average copies	copies per g of soil
1	1	38.54	37.94	3.83E+04	17.04	17.37	1.72E+04	21.26	20.72	2.00E+04
	2	44.82			17.68			22.64		
	3	31.62			17.41			18.48		
2	1	44.95	46.85	4.63E+04	96.20	81.71	7.98E+04	77.75	69.01	6.52E+04
	2	55.12			62.50			58.71		
	3	41.50			90.72			71.98		
3	1	75.85	47.80	4.69E+04	62.07	66.28	7.07E+04	900.01	360.35	3.41E+05
	2	31.15			62.30			114.63		
	3	46.21			75.30			453.55		
4	1	33.42	23.12	2.33E+04	24.14	21.26	2.11E+04	26.91	25.30	2.41E+04
	2	18.44			25.83			19.13		
	3	20.05			15.41			31.46		
5	1	71.31	45.80	4.57E+04	126.61	133.77	1.29E+05	111.17	142.99	1.34E+05
	2	48.91			144.19			482.35		
	3	27.55			131.13			54.52		
6	1	150.74	162.17	1.60E+05	26.41	25.51	2.54E+04	52.39	72.34	6.92E+04
	2	197.61			24.28			64.10		
	3	143.18			25.90			112.73		
7	1	157.10	77.76	7.62E+04	28.98	62.42	6.00E+04	22.66	22.15	2.09E+04
	2	102.29			88.25			13.92		
	3	29.26			95.11			34.43		
8	1	105.78	67.55	6.69E+04	18.85	24.48	2.53E+04	33.06	23.29	2.17E+04
	2	74.83			27.99			19.46		
	3	28.94			27.79			19.64		
9	1	111.17	115.16	1.15E+05	18.35	20.44	1.97E+04	20.60	36.58	3.47E+04
	2	110.57			12.51			3.60		
	3	124.25			37.18			52.57		
10	1	77.46	43.92	4.35E+04	34.44	34.37	3.41E+04	64.06	88.61	8.34E+04
	2	35.44			43.89			108.32		
	3	30.86			26.86			100.27		
11	1	205.60	202.26	2.00E+05	17.35	15.69	1.53E+04	42.27	66.13	6.26E+04
	2	183.95			10.75			69.40		
	3	218.76			20.69			98.56		
12	1	68.28	67.08	6.53E+04	5.49	10.14	1.09E+04	121.73	97.85	9.24E+04
	2	57.04			15.76			62.88		
	3	77.49			12.05			122.40		
13	1	50.73	53.90	5.34E+04	34.64	25.02	2.49E+04	140.78	99.10	9.46E+04
	2	40.83			22.33			98.01		
	3	75.59			20.25			70.54		
14	1	315.61	271.14	2.73E+05	32.12	38.40	3.80E+04	182.37	124.05	1.17E+05
	2	245.83			37.61			125.81		
	3	256.91			46.87			83.19		
15	1	33.94	36.75	3.64E+04	22.26	19.46	1.89E+04	14.21	31.48	2.97E+04
	2	34.36			18.56			35.98		
	3	42.54			17.84			61.05		
16	1	1581.42	1681.38	1.67E+06	9.75	12.42	1.20E+04	63.97	38.78	3.69E+04
	2	1716.51			15.98			34.23		
	3	1751.08			12.31			26.64		

Table Ev. QPCR master sheet showing copy numbers of all replicates of 2009 soil (RNA).

		March			June			September		
	rep	copies	Average copies	copies per g of soil	copies	Average copies	copies per g of soil	copies	Average copies	copies per g of soil
1	1	66.19	73.19	6.48E+04	14.24	16.41	1.67E+04	68.87	73.00	8.35E+04
	2	90.49			18.31			59.78		
	3	65.44			16.93			94.49		
2	1	67.96	58.88	8.20E+04	12.05	13.67	1.45E+04	105.45	104.09	5.04E+04
	2	54.03			13.18			106.82		
	3	55.58			16.09			100.11		
3	1	66.29	63.01	*	13.71	15.21	*	28.66	38.11	4.66E+04
	2	53.18			16.91			41.18		
	3	70.97			15.17			46.88		
4	1	61.92	65.73	1.33E+04	17.29	17.50	1.08E+04	87.20	90.24	5.76E+04
	2	68.50			17.13			52.81		
	3	66.94			18.11			159.55		
5	1	80.70	69.45	6.77E+04	15.00	11.88	1.38E+04	53.79	110.52	6.62E+04
	2	61.41			10.56			228.38		
	3	67.86			10.60			109.89		
6	1	93.45	79.29	1.03E+04	13.46	13.74	*	80.48	108.65	1.18E+05
	2	79.79			15.03			164.91		
	3	66.86			12.83			96.63		
7	1	6.32	6.07	*	6.47	5.72	*	57.20	54.20	4.30E+04
	2	5.34			5.08			56.07		
	3	6.64			5.72			49.07		
8	1	10.09	13.70	1.95E+04	6.13	7.36	*	124.68	132.64	7.66E+04
	2	13.36			6.70			128.57		
	3	19.05			9.71			145.58		
9	1	9.24	8.19	6.70E+04	6.65	6.74	1.33E+04	45.59	50.50	2.69E+04
	2	7.76			6.83			48.02		
	3	7.66			6.75			58.83		
10	1	6.68	8.15	9.19E+04	10.68	12.71	1.30E+04	47.72	48.85	7.68E+04
	2	8.29			13.72			51.81		
	3	9.77			14.02			47.15		
11	1	8.81	8.42	*	5.13	5.56	*	38.40	44.06	3.58E+04
	2	9.37			6.35			45.88		
	3	7.23			5.27			48.54		
12	1	9.59	9.21	3.08E+04	3.07	2.94	*	49.76	45.72	6.67E+04
	2	8.38			3.13			41.55		
	3	9.73			2.64			46.21		
13	1	12.86	11.95	6.84E+04	11.13	8.53	1.17E+04	60.10	64.16	9.93E+04
	2	11.83			5.74			69.70		
	3	11.21			9.73			63.05		
14	1	30.68	34.48	*	5.09	5.12	*	71.53	73.71	5.40E+04
	2	39.26			4.55			71.51		
	3	34.03			5.79			78.31		
15	1	170.14	169.03	*	4.47	4.88	1.03E+04	62.61	53.94	4.50E+04
	2	163.07			4.77			43.56		
	3	174.05			5.45			57.53		
16	1	18.72	19.95	1.74E+05	3.64	4.11	*	78.75	71.34	5.89E+04
	2	13.45			4.88			64.62		
	3	31.54			3.83			*		

Table Evi. QPCR master sheet showing copy numbers of all replicates of 2007 soil from the conventional rotation (DNA).

		March			June			September		
	rep	copies	Average copies	copies per g of soil	copies	Average copies	copies per g of soil	copies	Average copies	copies per g of soil
1	1	722.10	740.07	2.63E+05	1687.46	1468.95	1.41E+06	1051.61	859.10	8.31E+05
	2	784.24			1459.32			879.49		
	3	715.77			1287.18			685.57		
2	1	760.91	735.46	4.85E+05	255.76	249.78	2.38E+05	678.53	740.80	7.00E+05
	2	646.89			253.94			762.64		
	3	808.18			239.95			785.62		
3	1	454.77	447.95	1.56E+05	350.59	342.16	3.25E+05	496.11	528.05	5.04E+05
	2	488.18			371.58			534.16		
	3	404.87			307.49			555.62		
4	1	695.50	655.70	6.71E+04	1680.78	1550.09	1.48E+06	1504.79	1572.92	1.50E+06
	2	618.31			1457.21			1520.36		
	3	655.56			1520.67			1700.98		
5	1	1749.66	1888.06	5.73E+05	531.08	499.87	4.70E+05	529.03	510.76	4.82E+05
	2	1982.85			500.15			569.95		
	3	1940.00			470.22			441.91		
6	1	575.92	543.82	8.07E+04	246.14	228.25	2.14E+05	667.69	779.97	7.48E+05
	2	489.44			191.57			850.58		
	3	570.58			252.18			835.50		
7	1	963.49	851.79	2.52E+04	237.65	253.56	2.37E+05	502.88	521.49	4.92E+05
	2	826.57			276.50			507.26		
	3	776.00			248.10			555.95		
8	1	976.38	968.25	2.70E+05	401.90	386.36	3.58E+05	816.55	771.39	7.30E+05
	2	897.22			358.75			803.33		
	3	1036.21			400.01			699.76		
9	1	605.76	641.19	1.80E+06	1252.46	1243.51	1.18E+06	1349.38	1312.69	1.27E+06
	2	666.72			1262.93			1240.50		
	3	654.97			1215.64			1351.29		
10	1	505.50	429.19	3.72E+05	47.68	89.15	8.35E+04	649.17	654.90	6.15E+05
	2	442.63			117.18			639.31		
	3	353.34			126.85			676.79		
11	1	291.75	295.43	1.69E+05	122.64	118.83	1.12E+05	472.77	475.57	4.47E+05
	2	314.22			114.75			524.50		
	3	281.26			119.24			433.76		
12	1	672.94	631.31	4.13E+05	291.34	282.31	2.69E+05	1595.36	1588.39	1.50E+06
	2	586.44			312.19			1613.38		
	3	637.56			247.38			1556.96		
13	1	723.07	684.89	4.63E+05	1030.81	951.38	8.92E+05	881.18	888.30	8.42E+05
	2	655.73			991.10			879.12		
	3	677.58			842.89			904.84		
14	1	855.89	807.68	1.47E+04	1002.01	981.01	9.21E+05	1228.09	1217.63	1.17E+06
	2	735.89			1002.61			1184.36		
	3	836.55			939.77			1241.17		
15	1	780.13	724.53	6.23E+04	919.17	832.53	7.88E+05	63.87	65.26	6.18E+04
	2	683.97			753.63			62.89		
	3	712.80			833.00			69.20		
16	1	1049.51	981.35	8.65E+04	71.49	73.60	6.97E+04	1337.57	1108.87	1.06E+06
	2	1018.84			85.12			904.35		
	3	883.86			65.51			1127.16		

Table Evii. QPCR master sheet showing copy numbers of all replicates of 2008 soil (DNA).

		March			June			September		
	rep	copies	Average copies	copies per g of soil	copies	Average copies	copies per g of soil	copies	Average copies	copies per g of soil
1	1	3021.33	2915.81	3.03E+06	2264.46	2344.86	2.46E+06	2195.42	2146.42	2.12E+06
	2	2840.72			2277.56			2341.71		
	3	2888.37			2499.84			1923.52		
2	1	2202.32	2113.09	2.18E+06	2036.70	1728.36	1.76E+06	1074.55	1056.12	1.04E+06
	2	2030.94			1563.32			1065.88		
	3	2109.47			1621.53			1028.53		
3	1	3778.10	3835.68	3.95E+06	7018.52	5694.88	5.78E+06	4423.58	4767.91	4.75E+06
	2	3763.10			5733.68			4971.70		
	3	3969.26			4589.61			4928.40		
4	1	6216.35	5671.12	5.88E+06	5395.16	5495.30	5.61E+06	6698.18	6476.60	6.42E+06
	2	5377.65			5516.99			6370.54		
	3	5456.04			5575.51			6366.62		
5	1	4591.87	4238.49	4.32E+06	6410.94	4688.84	4.75E+06	2628.63	2698.59	2.66E+06
	2	4709.46			5556.39			2696.37		
	3	3521.05			2893.88			2772.69		
6	1	4101.17	4153.29	4.29E+06	2273.65	2444.14	2.48E+06	5151.94	4823.46	4.72E+06
	2	4071.25			2420.17			4601.77		
	3	4290.28			2653.44			4733.47		
7	1	2006.03	2213.82	2.24E+06	1953.49	1929.91	1.92E+06	8439.62	8856.19	8.54E+06
	2	2521.47			1906.61			9310.51		
	3	2145.05			1929.62			8839.83		
8	1	3847.57	4218.05	4.31E+06	2944.38	2444.04	2.43E+06	3950.46	4208.50	4.08E+06
	2	4231.61			2151.79			3990.33		
	3	4609.38			2304.26			4728.52		
9	1	1836.43	1762.22	1.80E+06	4664.54	5556.39	5.67E+06	1798.59	1962.83	2.01E+06
	2	1746.88			5815.86			2153.22		
	3	1705.87			6323.45			1952.65		
10	1	2273.94	2483.14	2.52E+06	2865.97	2546.07	2.54E+06	1216.93	1222.19	1.26E+06
	2	2534.59			2212.83			1185.84		
	3	2656.54			2602.49			1265.09		
11	1	3197.99	3131.45	3.16E+06	3113.80	3432.13	3.43E+06	2224.15	2341.51	2.37E+06
	2	2925.08			3987.60			2463.35		
	3	3282.62			3256.02			2343.15		
12	1	4135.13	4326.10	4.38E+06	2392.42	2472.39	2.50E+06	2583.67	2604.98	2.63E+06
	2	4285.88			2704.05			2514.58		
	3	4568.37			2336.14			2720.57		
13	1	5483.48	5684.92	5.85E+06	2819.23	3156.63	3.26E+06	5992.84	5968.89	6.19E+06
	2	6091.45			3330.36			5523.06		
	3	5500.43			3350.03			6424.93		
14	1	4698.47	5034.80	5.17E+06	4360.17	3863.04	3.98E+06	6706.43	6304.81	6.25E+06
	2	5178.48			3278.47			6138.96		
	3	5245.50			4032.87			6087.37		
15	1	2775.71	2863.00	2.90E+06	3679.24	3362.11	3.42E+06	8839.29	8873.67	9.09E+06
	2	3121.47			3684.66			9228.85		
	3	2708.51			2803.37			8565.34		
16	1	3581.45	3423.61	3.51E+06	5045.58	4650.07	4.75E+06	6542.37	6091.62	6.11E+06
	2	3215.74			4264.93			6119.70		
	3	3484.29			4672.56			564.89		

Table Eviii. QPCR master sheet showing copy numbers of all replicates of 2009 soil (DNA).

		March			June			September		
	rep	copies	Average copies	copies per g of soil	copies	Average copies	copies per g of soil	copies	Average copies	copies per g of soil
1	1	0.82	0.76	1.15E+04	75.79	82.65	8.00E+04	375.18	417.05	3.99E+05
	2	0.83			94.19			444.27		
	3	0.65			79.09			435.20		
2	1	64.12	71.30	7.25E+04	241.39	315.41	3.05E+05	74.65	74.65	7.00E+04
	2	69.94			331.48			74.65		
	3	80.84			392.13			74.65		
3	1	21.13	19.31	1.94E+04	59.17	59.17	5.73E+04	117.19	123.14	1.15E+05
	2	17.23			59.17			155.71		
	3	19.78			59.17			102.32		
4	1	43.59	43.59	4.51E+04	955.42	1032.00	9.99E+05	203.55	215.72	2.07E+05
	2	43.59			1204.07			196.61		
	3	43.59			955.42			250.83		
5	1	66.08	131.98	1.35E+05	261.75	345.63	3.35E+05	88.56	60.54	5.82E+04
	2	194.09			387.38			52.76		
	3	179.24			407.21			47.48		
6	1	4508.33	2780.20	2.83E+06	314.77	372.35	3.60E+05	102.51	102.51	9.71E+04
	2	1797.82			300.35			102.51		
	3	2651.34			546.06			102.51		
7	1	63.43	70.31	7.18E+04	17.12	17.12	1.66E+04	170.33	30.25	1.61E+05
	2	98.52			17.35			170.33		
	3	55.61			17.12			170.33		
8	1	11.11	11.11	1.15E+04	56.45	59.33	5.74E+04	86.76	87.01	8.47E+04
	2	11.11			50.50			77.77		
	3	11.11			73.25			97.61		
9	1	20.89	19.25	1.95E+04	58.09	51.94	5.03E+04	54.72	44.66	4.18E+04
	2	20.68			70.89			30.47		
	3	15.65			34.03			53.41		
10	1	96.46	115.16	1.13E+05	108.73	108.73	1.05E+05	58.26	53.26	5.08E+04
	2	116.58			108.73			45.00		
	3	135.80			108.73			57.61		
11	1	171.72	132.89	1.31E+05	32.56	37.73	3.65E+04	67.55	67.55	6.29E+04
	2	123.46			32.68			67.55		
	3	110.69			50.47			67.55		
12	1	122.49	145.03	1.46E+05	90.86	90.86	8.80E+04	98.82	98.82	9.22E+04
	2	137.29			90.86			98.82		
	3	181.41			90.86			98.82		
13	1	64.20	69.77	7.02E+04	76.74	76.74	7.43E+04	69.34	68.20	6.42E+04
	2	67.21			76.74			64.23		
	3	78.72			76.74			71.23		
14	1	1072.71	883.69	9.02E+05	114.66	120.77	1.17E+05	64.36	58.00	5.47E+04
	2	1095.60			127.86			56.04		
	3	587.16			120.16			54.09		
15	1	55.84	55.84	5.63E+04	85.29	84.23	8.15E+04	148.21	148.21	1.40E+05
	2	55.84			93.96			148.21		
	3	55.84			74.56			148.21		
16	1	168.24	148.96	1.53E+05	71.95	71.95	6.96E+04	153.89	153.89	1.45E+05
	2	153.22			71.95			153.89		
	3	128.24			71.95			153.89		

Figure Ei. PCA and DCA showing variation between *nifH* DGGE lanes for 2007 RNA

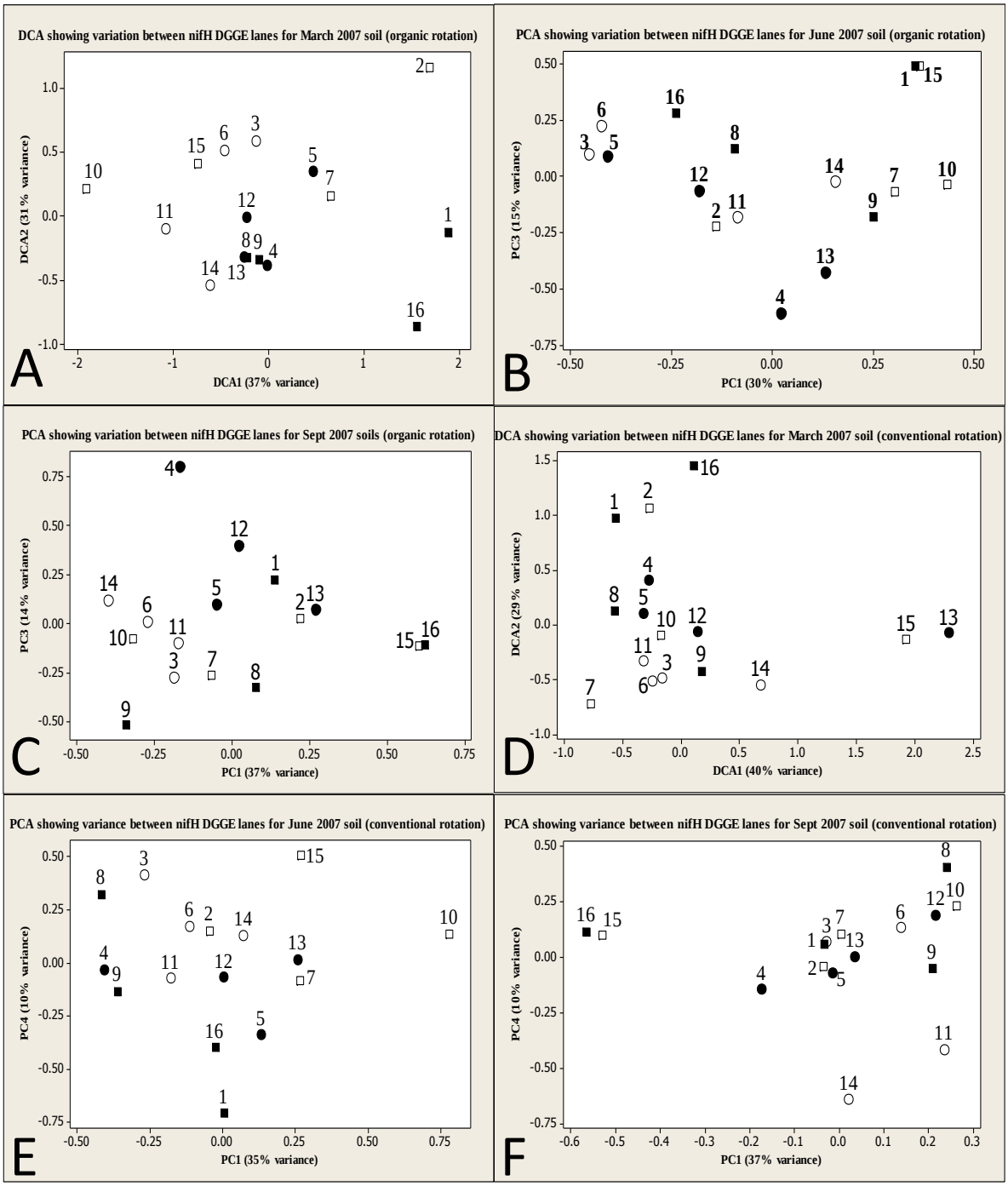


Figure Ei. Numbers relate to plot numbers. Treatments are represented by the following symbols; orgFM orgCP (●), conFM conCP (□), orgFM conCP (■) and conFM orgCP (○). A = March soil after beans. The Y axis shows significant variation due to fertility management (P = 0.022). B = June after beans. The X axis shows significant variation due to health management (P = 0.039). C = September after beans. The Y axis shows significant variation due to health management (P = 0.044).D = March after barley. E = June after barley. The X axis shows significant variation due to the fertility x health interaction (P = 0.033) and the Y axis shows significant variation due to fertility management (P = 0.033). F = September after barley. P values are according to ANOVA.

Figure Eii. PCA and DCA showing variation between *nifH* DGGE lanes for 2007 DNA gels.

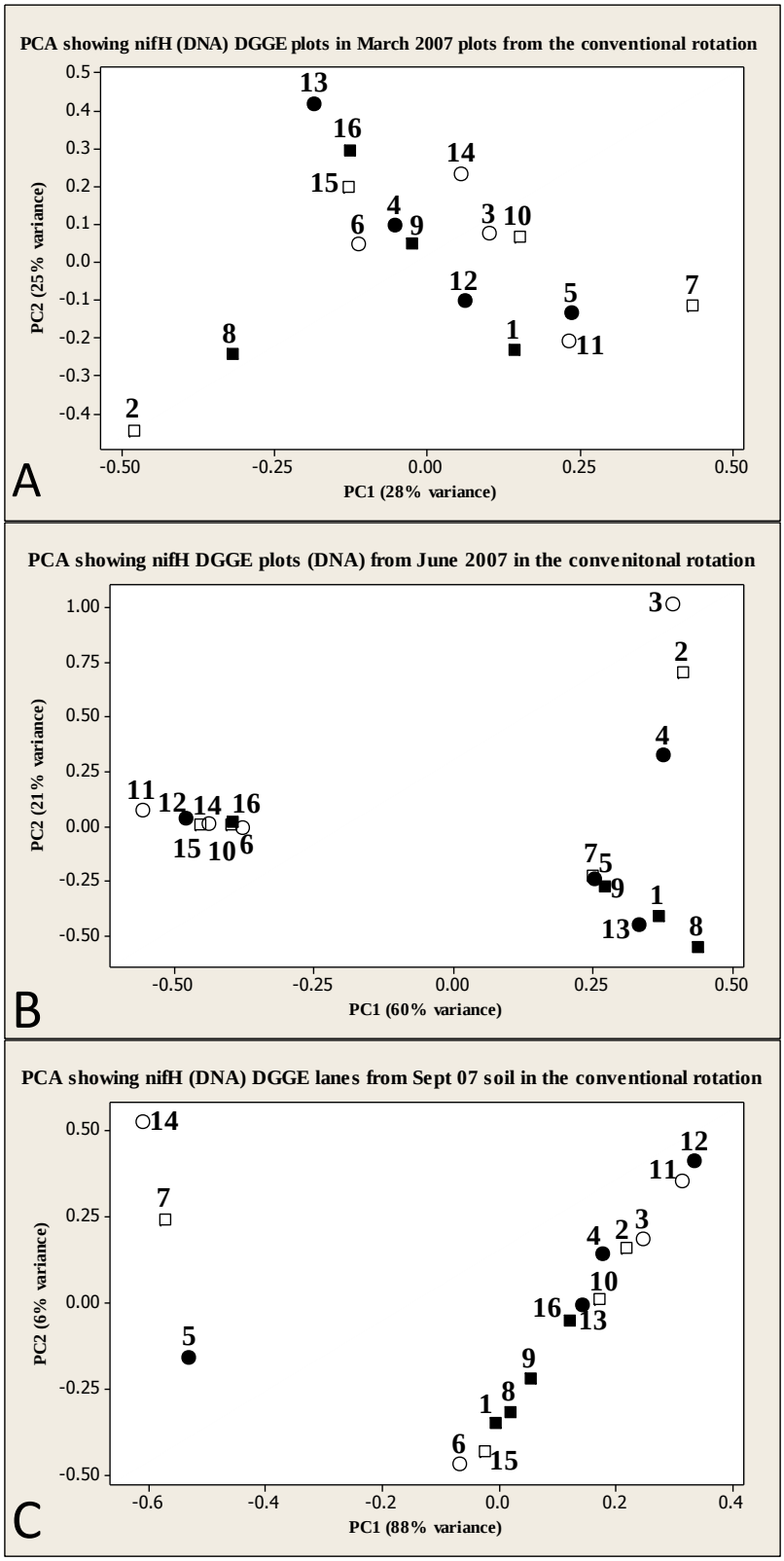


Figure Eii. Numbers relate to plot numbers. Treatments are represented by the following symbols; orgFM orgCP (●), conFM conCP (□), orgFM conCP (■) and conFM orgCP (○). A = March soil after barley. B = June after barley. The Y axis shows significant variation due to fertility management ($P = 0.050$). C = September after barley. *P* values are according to ANOVA.

Figure Eiii. PCA and DCA showing diversity between lanes of *nifH* RNA DGGE gels in 2008 and 2009.

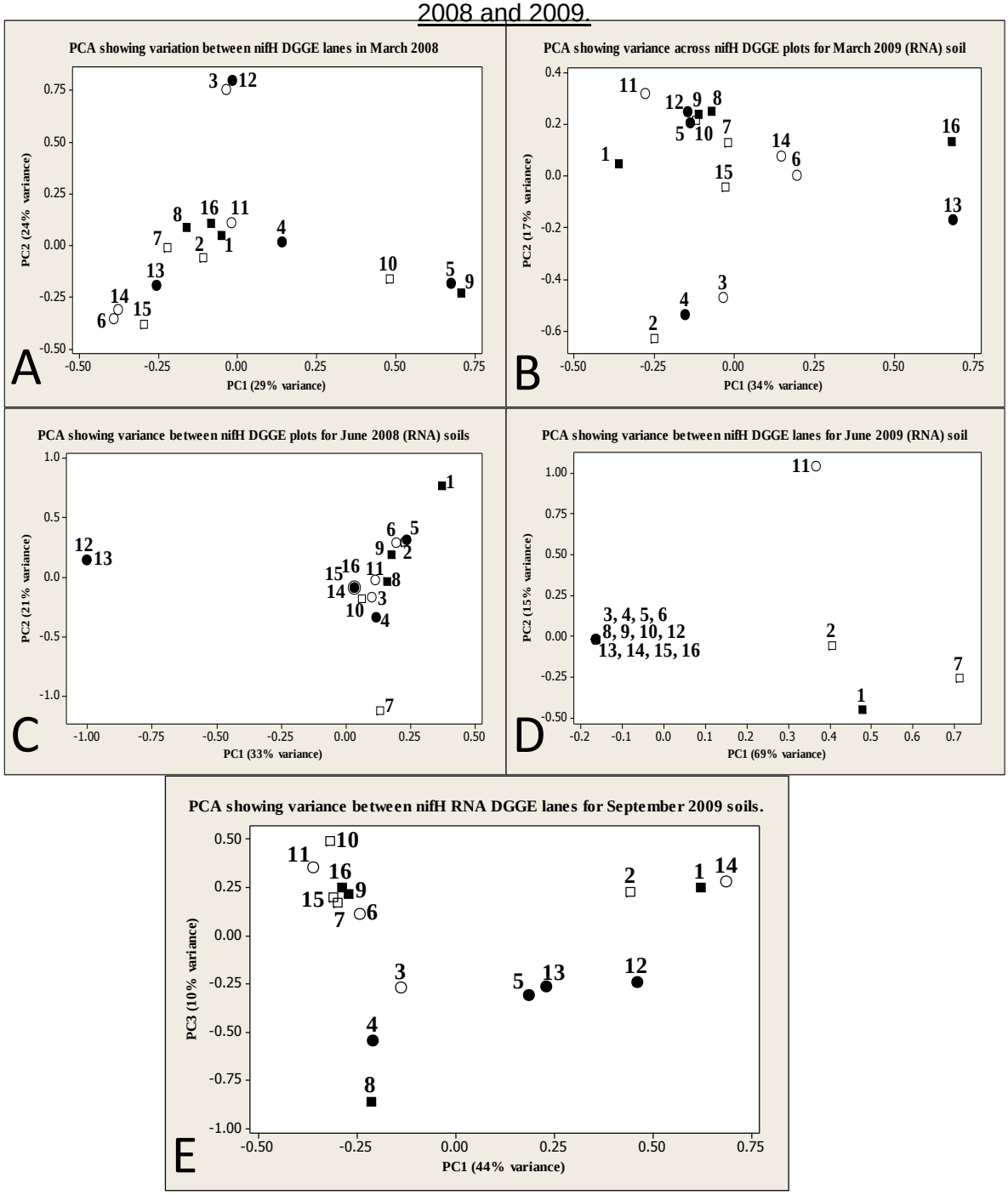


Figure Eiii. Numbers relate to plot numbers. Treatments are represented by the following symbols; orgFM orgCP (●), conFM conCP (□), orgFM conCP (■) and conFM orgCP (○). A = March 2008. B = March 2009. C = June 2008. D = June 2009. E = September 2009. The Y axis shows significant variation due to fertility management (*P* = 0.036). *P* values are according to ANOVA.

Figure Eiv. PCA and DCA showing variation between *nifH* DGGE lanes for DNA 2008 and 2009.

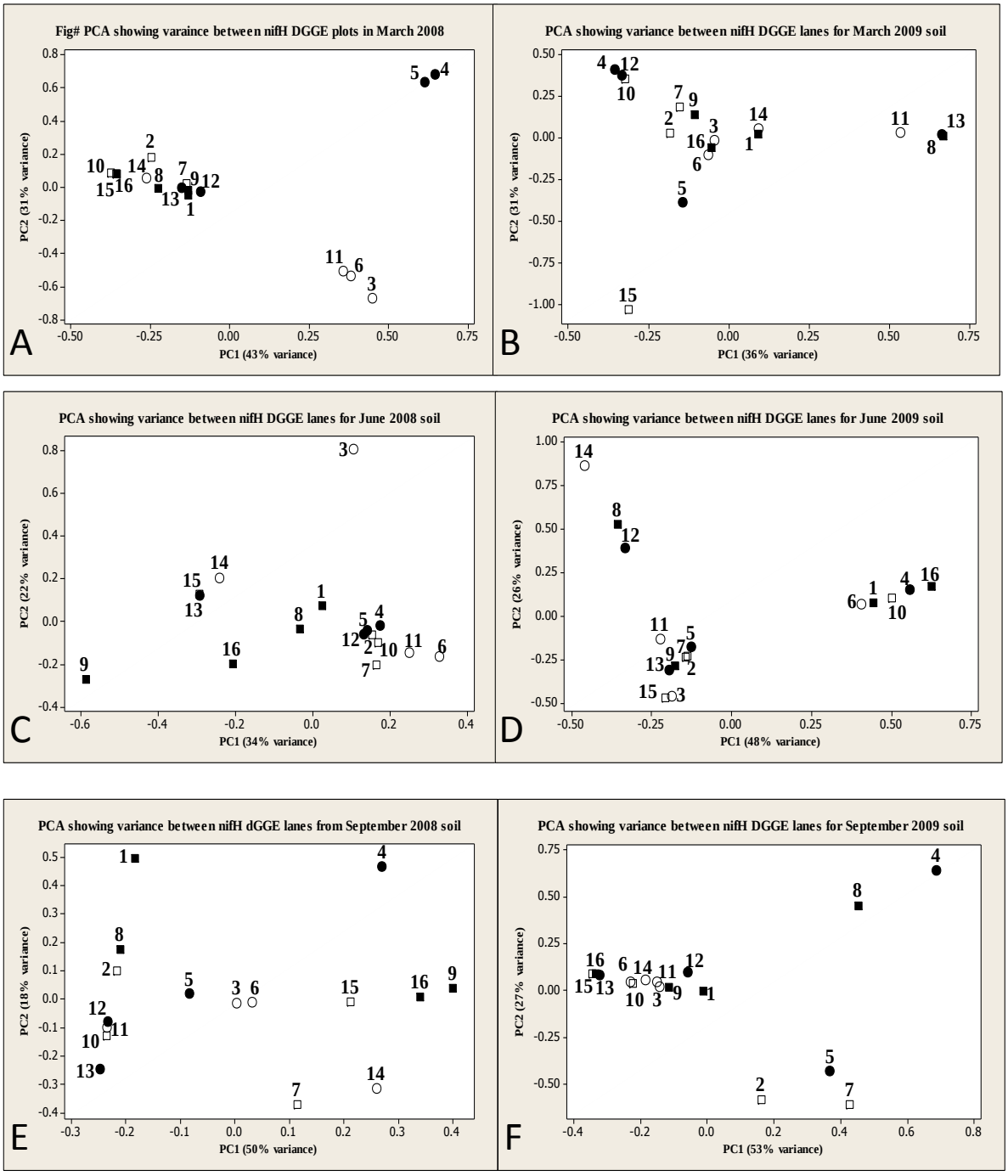


Figure Eiv. Numbers relate to plot numbers. Treatments are represented by the following symbols; orgFM orgCP (●), conFM conCP (□), orgFM conCP (■) and conFM orgCP (○). A = March 2008 soil. The X axis shows significant variation due to health management (*P* = 0.005).The Y axis shows significant variation due to fertility management (*P* = 0.026) and a significant interaction of fertility and health management (*P* = 0.007). B = March 2009 soil. C = June 2008 soil. D = June 2009 soil. E = September 2008 soil. F = September 2009 soil. *P* values are according to ANOVA.

Appendix section F (RC3).

Table Fi. Shannon diversity indices (H') for each 16S rRNA gene DGGE lane at each sample date (RNA)

	2007 (organic rotation)			2007 (conventional rotation)			2008			2009		
lane	March	June	Sept	March	June	Sept	March	June	Sept	March	June	Sept
1	2.683	2.702	2.592	2.668	2.577	2.135	3.163	3.158	2.836	3.352	3.347	3.201
2	2.733	2.666	2.789	2.921	2.911	2.299	3.189	2.773	2.660	3.585	3.066	3.250
3	2.909	2.628	2.503	2.954	2.881	2.200	3.225	2.766	2.747	3.228	3.231	2.925
4	2.745	2.501	2.775	2.927	2.789	1.817	3.120	2.513	3.140	3.530	3.073	3.219
5	2.491	2.524	2.780	2.873	2.722	2.133	3.390	2.508	3.228	3.340	2.706	2.933
6	2.560	2.707	2.866	2.922	2.768	2.108	3.270	2.432	2.814	3.202	3.137	3.118
7	2.680	2.376	2.769	2.944	2.947	2.153	3.412	2.800	3.211	3.492	3.110	3.075
8	2.546	2.641	2.694	2.762	3.058	1.788	2.789	2.550	2.169	3.196	2.898	2.889
9	2.563	2.786	2.660	3.080	2.885	2.113	2.704	2.396	2.553	2.560	2.959	3.163
10	2.581	2.522	2.583	3.140	2.975	1.844	3.201	2.324	2.759	3.030	3.097	2.923
11	2.659	2.432	2.695	2.991	2.949	2.103	2.472	2.450	2.955	1.800	2.837	3.119
12	2.795	2.669	2.850	2.740	2.812	1.951	3.261	2.294	2.605	3.043	3.260	3.092
13	2.515	2.602	2.798	2.867	2.739	2.039	3.091	2.703	2.553	2.856	3.098	3.092
14	2.596	2.646	2.861	2.890	2.799	2.121	3.128	2.402	2.458	3.414	3.019	2.908
15	2.507	2.300	2.686	2.826	2.669	1.946	3.357	2.526	2.759	3.183	3.058	2.946
16	2.476	2.297	2.471	2.614	2.659	1.793	3.264	2.547	2.333	2.825	2.988	2.638

Table Fii. QPCR master sheet showing 16S rRNA gene copy numbers of all replicates in 2007 (conventional rotation).

		March			June			September		
	rep	copies	Average copies	copies per g of soil	copies	Average copies	copies per g of soil	copies	Average copies	copies per g of soil
1	1	119331.30	213563.60	2.08E+08	74244.68	71835.66	6.91E+07	330560.87	229219.20	2.22E+08
	2	234194.10			65363.32			206740.39		
	3	348538.90			76387.25			176228.52		
2	1	33836.89	61657.61	6.10E+06	112973.44	115190.00	1.10E+08	43897.25	67075.55	6.34E+07
	2	72525.57			121376.26			106753.04		
	3	95516.54			111463.93			64398.42		
3	1	163555.53	157903.20	1.50E+08	35031.03	34030.91	3.23E+07	164180.42	98083.30	9.36E+07
	2	141969.50			32110.54			67324.38		
	3	169555.62			35036.44			85367.29		
4	1	297137.60	315797.30	3.05E+08	41899.81	44799.85	4.27E+07	51928.37	61457.23	5.86E+07
	2	225036.28			47347.14			102397.46		
	3	470993.68			45323.56			43654.11		
5	1	74607.96	96670.56	9.13E+07	166592.72	174853.30	1.64E+08	69605.77	42635.72	4.03E+07
	2	55638.35			179996.63			25719.85		
	3	217632.23			178279.38			43291.93		
6	1	20107.03	21832.94	2.13E+08	83225.28	80040.76	7.50E+07	85248.83	42596.27	4.08E+07
	2	21804.87			78567.35			20425.12		
	3	23737.53			78421.71			44387.59		
7	1	15929.47	27748.47	2.74E+07	64261.03	66164.91	6.18E+07	144090.72	76675.52	7.24E+07
	2	47189.49			62681.10			43231.86		
	3	28423.03			71911.60			72365.28		
8	1	3698.77	3543.50	3.56E+06	173990.31	175268.30	1.62E+08	36444.38	24796.40	2.35E+07
	2	31814.83			184690.59			30089.26		
	3	3780.62			167548.51			13903.48		
9	1	100302.95	65684.37	6.65E+07	83476.59	81327.76	7.71E+07	90871.58	45029.07	4.37E+07
	2	55709.99			73923.95			21651.70		
	3	50715.33			87169.91			46404.36		
10	1	18186.95	22887.71	2.23E+07	21272.98	23593.71	2.21E+07	28861.61	20749.09	1.95E+07
	2	21973.86			25123.57			31282.15		
	3	30001.35			24574.17			9894.18		
11	1	50584.98	57977.10	5.71E+07	56718.46	67699.94	6.38E+07	20396.78	20115.52	1.89E+07
	2	64428.75			77559.52			26115.71		
	3	59795.45			70535.10			15280.24		
12	1	25808.27	27183.03	2.64E+07	43379.76	43085.64	4.10E+07	123337.74	84502.44	7.99E+07
	2	27915.59			40081.14			64487.90		
	3	27879.70			46001.33			75863.60		
13	1	26017.02	28116.71	2.84E+07	9642.33	10036.55	9.41E+06	78561.45	76510.34	7.25E+07
	2	28650.73			10817.92			80799.73		
	3	29819.52			9692.35			70577.14		
14	1	52640.01	56293.37	5.79E+07	765.38	511.68	4.80E+05	39780.23	37329.13	3.58E+07
	2	55340.23			431.77			35587.37		
	3	61237.14			405.38			36743.50		
15	1	74522.11	77918.69	7.90E+07	45767.49	43294.30	4.10E+07	323420.19	24946.05	2.36E+08
	2	85175.32			44209.18			33905.69		
	3	74528.83			40107.22			14156.73		
16	1	3256.73	3163.79	3.11E+06	9668.12	9976.19	9.45E+06	15639.67	169332.97	1.62E+07
	2	3135.29			10370.65			17111.99		
	3	3101.43			9902.53			18141.49		

Table Fiii. QPCR master sheet showing 16S rRNA gene copy numbers of all replicates in 2007 (organic rotation).

		March			June			September		
	rep	copies	Average copies	copies per g of soil	copies	Average copies	copies per g of soil	copies	Average copies	copies per g of soil
1	1	86697.63	63699.09	6.43E+07	280269.52	269275.10	2.67E+08	194864.78	198846.60	1.92E+07
	2	47587.81			269662.30			188468.33		
	3	62646.50			258340.57			214083.33		
2	1	65674.99	53326.69	5.27E+07	209746.02	213831.40	2.09E+08	214299.96	159259.20	1.50E+08
	2	51794.17			217780.87			168114.06		
	3	44581.33			214043.07			112121.18		
3	1	8091.37	10408.12	1.02E+07	97893.62	95675.61	1.02E+08	35773.71	39414.68	3.73E+07
	2	12238.46			90643.19			37686.42		
	3	11385.94			98699.33			45417.69		
4	1	54222.69	48882.61	4.92E+07	50347.79	51822.88	5.14E+07	18157.34	16345.88	1.46E+07
	2	148588.47			53623.36			13412.18		
	3	14497.63			51550.18			14839.65		
5	1	37133.42	36531.44	3.65E+07	37334.37	35295.72	3.41E+07	79215.12	82931.67	7.77E+07
	2	35168.19			33010.36			76305.55		
	3	37332.35			35678.54			94361.96		
6	1	24301.12	13388.22	1.32E+07	83356.45	87519.27	8.71E+07	12770.15	15643.62	1.50E+07
	2	9348.41			92050.29			18434.91		
	3	10563.43			87366.86			16262.04		
7	1	6806.82	13967.40	1.37E+07	57431.76	55481.18	5.33E+07	53341.41	67345.68	6.34E+07
	2	3696.01			52121.57			58929.10		
	3	10830.77			57051.55			97170.61		
8	1	10770.78	15482.49	1.53E+07	114925.43	112379.70	1.16E+08	71812.76	71498.72	6.66E+07
	2	31725.56			114204.95			75499.50		
	3	10860.89			108134.21			67413.83		
9	1	8818.81	16765.43	1.68E+07	10772.33	10108.18	9.75E+06	19519.02	19542.07	1.85E+07
	2	23374.45			11767.77			20876.98		
	3	22860.87			8147.31			18314.11		
10	1	71944.72	68209.61	6.76E+07	31268.90	33487.29	3.32E+07	17088.26	22280.53	2.10E+07
	2	59305.22			34284.60			30956.54		
	3	74378.06			35029.04			20908.68		
11	1	9283.73	22524.77	2.23E+07	10959.60	11082.13	1.08E+07	58661.51	54092.95	5.12E+07
	2	10231.40			10670.75			55515.54		
	3	120316.08			11638.03			48602.01		
12	1	14337.48	21062.68	2.05E+07	5761.66	13894.95	1.36E+07	20562.65	20673.87	1.95E+06
	2	48927.88			53247.74			21271.28		
	3	13320.23			8744.24			20201.91		
13	1	62126.74	91225.98	9.04E+07	13039.35	20810.78	2.07E+07	2601468.98	738094.12	6.59E+06
	2	100724.49			22092.78			3920330.46		
	3	121322.65			21427.06			39427.02		
14	1	11338.61	12925.98	1.30E+07	10665.47	10901.93	1.08E+07	196966.15	82059.58	7.74E+07
	2	8979.45			11223.72			22394.08		
	3	21209.42			10824.15			125274.60		
15	1	2948.59	3791.09	3.76E+06	22453.19	21260.15	2.06E+07	14599.98	26661.13	2.51E+07
	2	4708.02			24100.57			36476.70		
	3	3925.01			17757.99			35585.08		
16	1	56135.13	52547.86	5.22E+07	10115.67	9715.42	9.39E+06	7744.46	6912.73	6.58E+06
	2	51458.20			9882.52			6804.06		
	3	50231.45			9173.23			6268.87		

Table Fiv. QPCR master sheet showing 16S rRNA gene copy numbers of all replicates in 2008.

		March			June			September		
	rep	copies	Average copies	copies per g of soil	copies	Average copies	copies per g of soil	copies	Average copies	copies per g of soil
1	1	25757.15	22977.61	1.84E+07	31971.00	50368.52	4.03E+07	805110.05	178355.50	1.43E+08
	2	16584.17			64158.40			300336.01		
	3	28400.30			62297.11			71483.97		
2	1	8209.01	9798.28	7.84E+06	5135.61	5835.70	4.67E+06	37352.87	81919.33	6.55E+07
	2	11695.24			8236.52			217102.63		
	3	10465.80			4698.34			67790.68		
3	1	10876.35	16031.38	1.28E+07	22651.96	17652.86	1.41E+07	7326.59	52649.94	4.21E+07
	2	18532.36			21185.92			84077.51		
	3	21242.70			11462.83			236925.11		
4	1	8280.72	12741.01	1.02E+07	2711.72	44485.25	3.56E+07	133429.42	44485.25	3.56E+07
	2	6884.42			678.34			65095.69		
	3	36280.77			700.13			10135.48		
5	1	11758.01	12063.49	9.65E+06	8426.04	8377.32	6.70E+06	83034.57	90239.78	7.22E+07
	2	6916.74			8608.39			319677.23		
	3	21586.57			8105.31			27683.65		
6	1	11041.27	32699.35	2.62E+07	18482.83	7140.89	5.71E+06	1640001.30	133152.10	1.07E+08
	2	18681.76			4085.19			35980.02		
	3	169504.30			4822.55			40007.25		
7	1	27028.36	50876.59	4.07E+07	40597.05	19744.19	1.58E+07	9762.96	21702.84	1.74E+07
	2	34114.80			11601.72			130139.72		
	3	142820.76			16341.84			8045.60		
8	1	20135.53	40164.76	3.21E+07	35894.44	18059.05	1.44E+07	28919.87	45231.80	3.62E+07
	2	78841.21			10706.62			44763.75		
	3	40518.94			15325.16			71483.97		
9	1	2640.90	3945.50	3.16E+06	3346.71	9253.48	7.40E+06	1318193.50	1556888.00	1.25E+09
	2	5483.64			28667.58			3990987.56		
	3	4241.16			8258.59			717318.95		
10	1	7641.85	5958.01	4.77E+06	6845.35	58057.86	4.64E+07	292931.24	58057.86	4.64E+07
	2	6675.75			7437.64			22113.01		
	3	4145.77			4073.88			30211.30		
11	1	37065.95	69584.26	5.57E+07	2705.28	4399.16	3.52E+06	66741.19	245965.20	1.97E+08
	2	48181.89			7641.85			906469.94		
	3	188657.49			4118.12			58542.99		
12	1	13549.95	8105.31	6.48E+06	16672.66	16672.66	1.33E+07	131718.70	53290.44	4.27E+08
	2	7282.43			15573.24			36418.91		
	3	5396.28			18681.76			31548.12		
13	1	3206.42	6160.70	4.93E+06	12109.32	26174.10	2.09E+07	11287.54	15558.24	1.24E+07
	2	13262.91			17472.67			10872.68		
	3	5498.33			84749.33			30686.37		
14	1	1130.56	2813.87	1.75E+06	1661.28	18128.67	1.45E+07	21233.92	18128.67	1.45E+07
	2	4129.16			1661.28			14947.50		
	3	2231.13			1661.28			18771.51		
15	1	3882.65	4434.63	3.55E+06	15740.86	19621.27	1.57E+07	22044.12	13897.82	1.12E+06
	2	8901.26			26455.81			19640.38		
	3	2523.43			18139.78			6200.09		
16	1	12605.34	69646.37	5.57E+07	3872.27	4434.63	3.55E+06	46689.78	51780.93	4.14E+07
	2	41922.05			404.53			44277.51		
	3	639290.49			55673.67			67159.02		

Table Fv. QPCR master sheet showing 16S rRNA gene copy numbers of all replicates in 2009.

		March			June			September		
	rep	copies	Average copies	copies per g of soil	copies	Average copies	copies per g of soil	copies	Average copies	copies per g of soil
1	1	3233982.92	3811430.00	3.05E+09	36282.58	38389.79	3.07E+07	28099.49	35834.69	2.87E+07
	2	3958476.33			37281.83			40063.28		
	3	4325118.12			41826.52			40875.79		
2	1	6963296.61	7191903.00	5.75E+09	142140.03	170724.70	1.37E+08	31223.16	36861.74	2.95E+07
	2	8344469.83			184678.58			36398.68		
	3	6402031.27			189564.01			44072.24		
3	1	2290699.26	3084131.00	2.47E+09	44916.43	42932.99	3.43E+07	5344.04	5011.11	4.01E+06
	2	3859341.07			38935.44			4114.07		
	3	3318310.45			45250.47			5723.50		
4	1	845792.19	897696.10	7.18E+08	34424.39	38985.85	3.12E+07	11170.60	13756.34	1.10E+07
	2	1203154.35			40220.06			14518.34		
	3	710981.70			42796.87			16051.50		
5	1	422267.02	375321.60	3.00E+08	715.04	592.54	4.74E+05	77281.32	44840.84	3.59E+07
	2	324953.65			423.31			26017.94		
	3	385302.68			687.34			46951.32		
6	1	320798.08	318539.20	2.55E+08	44837.25	50155.27	4.01E+07	38572.34	31217.36	2.50E+07
	2	333679.49			51415.50			31066.83		
	3	301944.80			54728.89			25837.24		
7	1	360741.57	361288.20	2.89E+08	16072.53	17068.99	1.37E+07	12756.26	18012.45	1.44E+07
	2	336724.83			11042.06			20561.68		
	3	388230.86			28021.35			22281.14		
8	1	274472.23	348262.60	2.79E+08	14729.35	13682.25	1.09E+07	26516.01	28658.71	2.29E+07
	2	474485.32			12858.44			19338.16		
	3	324339.17			13523.86			45903.60		
9	1	76410.07	96891.36	7.75E+07	60092.99	57696.67	4.62E+07	15891.16	15265.69	1.22E+07
	2	105652.10			53412.56			16231.54		
	3	112674.69			59839.09			13792.19		
10	1	157693.87	143817.10	1.15E+08	1804.20	2092.39	1.67E+06	36754.20	4294.59	3.44E+06
	2	153744.61			2447.25			58846.11		
	3	122692.13			2074.75			46993.26		
11	1	71865.02	107440.20	8.60E+07	1360.49	1678.70	1.34E+06	6102.62	8278.03	6.62E+06
	2	50596.15			1575.59			16560.73		
	3	341086.70			2206.89			5612.86		
12	1	29770.85	26594.97	2.13E+07	1159.53	1231.36	9.85E+05	6470.67	6250.75	5.00E+06
	2	25471.69			1233.82			6702.06		
	3	24805.60			1305.01			5631.68		
13	1	1301717.76	1033053.00	8.26E+08	94298.43	117730.50	9.42E+07	18473.68	34212.56	2.74E+07
	2	1025519.61			146467.52			46764.95		
	3	825859.91			118146.65			32339.73		
14	1	194488.16	169368.20	1.35E+08	32592.29	31785.95	2.54E+07	4455.62	4455.62	3.56E+06
	2	232888.59			27244.05			4455.62		
	3	107264.05			36167.55			4455.62		
15	1	98394.47	63939.96	5.12E+07	117730.52	118536.40	9.48E+07	49080.88	49714.53	3.98E+07
	2	29725.80			123648.46			49714.53		
	3	89374.32			114413.45			63423.48		
16	1	9034.97	15532.45	1.24E+07	2886.80	1966.80	1.57E+06	76851.51	48284.47	3.86E+07
	2	14294.91			1516.95			46939.15		
	3	29014.29			1737.37			31205.75		

Figure Fi. PCA showing variation between 16S rRNA gene DGGE lanes for 2007.

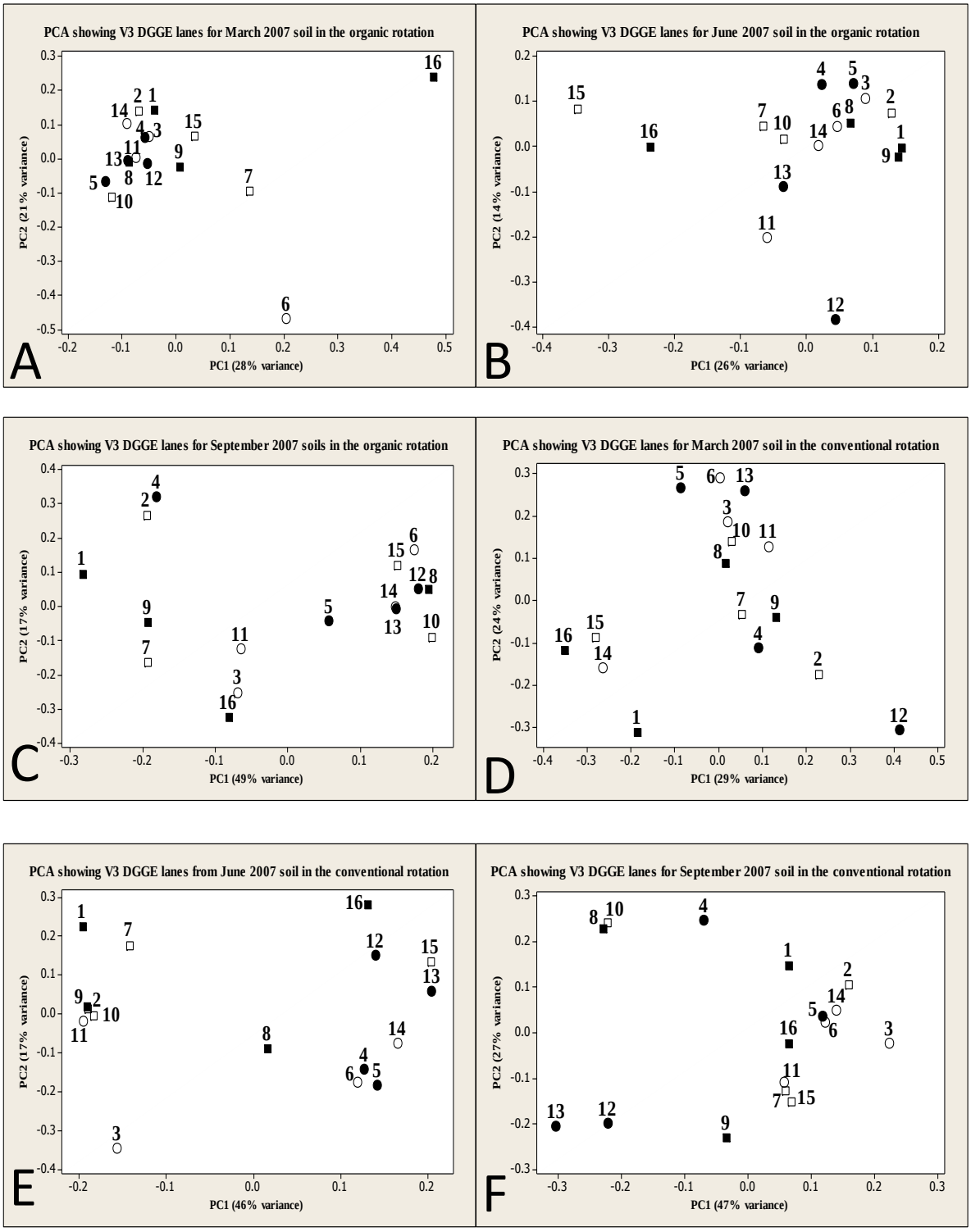


Figure Fi. Numbers relate to plot numbers. Treatments are represented by the following symbols; orgFM orgCP (●), conFM conCP (□), orgFM conCP (■) and conFM orgCP (○). A = March soil after beans. B = June after beans. C = September after beans. D = March after barley. E = June after barley. The Y axis shows significant variation due to the health management (P = 0.024). F = September after barley. P values are according to ANOVA.

Figure Fii. PCA of 16S rRNA gene DGGE gels for 2008 and 2009

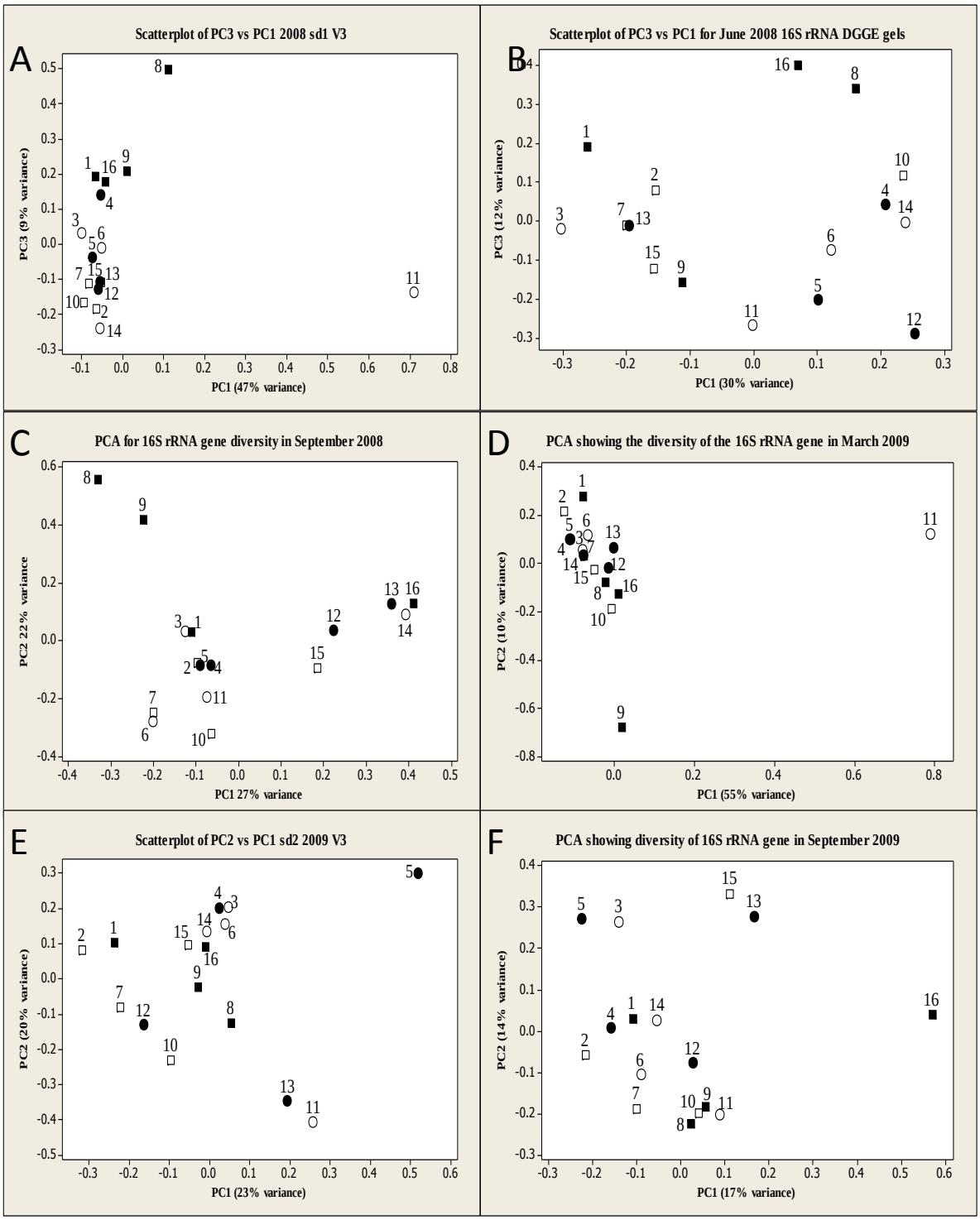


Figure Fii. Numbers relate to plot numbers. Treatments are represented by the following symbols; orgFM orgCP (●), conFM conCP (□), orgFM conCP (■) and conFM orgCP (○). A = March 2008 The Y axis show significant variation due to fertility management (*P* = 0.002) and a significant fertility x health interaction (*P* = 0.010). B = June 2008. The Y axis shows significant variation due to health management (*P* = 0.029). C = September 2008. The Y axis shows significant variation due to fertility management (*P* = 0.007) and a significant fertility x health interaction (*P* = 0.044).D = March 2009. E = June 2009. The X axis shows significant variation due to health management (*P* = 0.026)F = September 2009. *P* values are according to ANOVA.

Table Fvi. Shannon diversity indices of BIOLOG data for each 2007 plot.

	2007 (conventional rotation)			2007 (organic rotation)		
plot	March	June	Sept	March	June	Sept
1	2.812	2.812	2.936	2.676	2.798	2.842
2	2.796	2.712	2.599	3.006	2.920	2.923
3	2.819	2.898	2.979	2.647	2.785	2.913
4	2.769	2.817	3.015	2.764	2.667	2.964
5	2.814	2.803	2.878	2.744	2.766	2.750
6	2.840	2.820	2.875	2.533	2.829	2.831
7	2.837	2.826	2.871	2.721	2.771	2.895
8	2.776	2.869	2.683	2.674	2.697	2.363
9	2.788	2.856	2.994	2.769	2.815	2.867
10	3.017	2.752	3.000	2.810	2.829	3.031
11	2.948	2.873	2.763	2.881	2.759	2.741
12	2.797	2.856	2.893	2.829	2.870	2.977
13	2.786	2.939	2.887	2.894	2.798	2.687
14	2.887	2.951	2.889	2.842	2.803	2.729
15	2.681	2.831	2.712	2.296	2.914	2.922
16	2.833	2.731	2.471	3.043	2.823	2.680

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Diversity and Activity of Free-Living Nitrogen-Fixing Bacteria and Total Bacteria in Organic and Conventionally Managed Soils[▽]†

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Agricultural soils are heterogeneous environments in which conditions affecting microbial growth and diversity fluctuate widely in space and time. In this study, the molecular ecology of the total bacterial and free-living nitrogen-fixing communities in soils from the Nafferton Factorial Systems Comparison (NFSC) study in northeast England were examined. The field experiment was factorial in design, with organic versus conventional crop rotation, crop protection, and fertility management factors. Soils were sampled on three dates (March, June, and September) in 2007. Total RNA was extracted from all soil samples and reverse transcribed. Denaturing gradient gel electrophoresis (DGGE) and quantitative PCR (qPCR) were used to analyze *nifH* and 16S rRNA genes in order to study free-living diazotrophs and the total bacterial community, respectively. Crop rotation was shown to have a significant effect on total bacterial diversity (and that of free-living N fixers) ($P \leq 0.001$). On all three dates, *nifH* activity was higher in the conventional crop rotation. In contrast, qPCR analysis of free-living N fixers indicated significantly higher levels of activity in conventionally fertilized plots in June ($P = 0.0324$) and in plots with organic crop protection in September ($P = 0.0143$). To our knowledge, the effects of organic and conventional farming systems on free-living diazotrophs have never been studied. An increased understanding of the impacts of management practices on free-living N fixers could allow modifications in soil management practices to optimize the activity of these organisms.

After water, nitrogen is most often the limiting factor for plant growth (45a). Crops such as wheat, rice, and maize need 20 to 40 kg soil N ha^{-1} over a period of 3 to 5 months to satisfy the N requirements for each tonne of grain produced (34). To meet such high demand, farmers must either apply inorganic synthetic N fertilizers to their land or rely on biological nitrogen fixation (BNF) and the input of recycled organic wastes, such as manure.

Adding nitrogen in the form of synthetic fertilizers can have negative environmental impacts since inorganic N, particularly nitrate; can be dispersed into surface and groundwater, leading to eutrophication (45a). In addition, the manufacture of N fertilizers relies on nonrenewable fossil fuels (the production of 1 kg N fertilizer requires 38,000 kJ of fossil energy) (39) and results in significant emissions of greenhouse gases (20). These environmental concerns, coupled with increasing fuel costs and a desire for improved sustainability have led some farmers to seek alternative N management strategies (34).

N cycling in natural ecosystems and traditional agricultural production relies on biological N fixation primarily by diazotrophic bacteria. Diazotrophs are highly diverse and are widely distributed across bacterial and archael taxa (13). Most (~80%) of biological nitrogen fixation (BNF) is carried out by diazotrophs in symbiosis with legumes (33). However, under specific conditions bacteria which are free-living in soil (e.g., cyanobacteria, *Pseudomonas*, *Azospirillum*, and *Azotobacter*) may fix significant amounts of nitrogen (0 to 60 kg N ha^{-1} $year^{-1}$) (5, 24). This may be particularly important in organically managed soils, which typically have a lower proportion of nitrogen in available forms (43).

The effects of crop management on diversity and function of the soil microbial community are equivocal. Many authors report an increase in total biomass and microbial activity when organic matter inputs are increased and chemical amendments are reduced (4, 7, 10). In contrast, Donnison et al. (15) found that a change in management had no effect on soil nutrient status, soil microbial biomass, and soil microbial activity. However, they did find that management practices significantly affected the soil microbial community structure and suggested that this was due to changes in plant composition and the form and quantity of fertilizer applied (29). Diazotrophic community structure and diversity have been shown to respond to changes in grazing, liming, the nature of the nitrogen added, and incorporations of crop residues (32, 46, 47). They are also especially sensitive to chemical inputs, such as pesticides (31).

The nitrogenase enzyme catalyzes the reduction of atmospheric dinitrogen to ammonia. This process is very energy expensive and is, therefore, tightly regulated (13). At neutral pH, low levels of fixed N and increased levels of C will allow more optimal conditions for free-living N fixation (12). These conditions are more likely to be found in organically managed soils as increased organic C is added in the form of manure and on average less readily available nitrogen is applied.

In this study, the diazotrophic population was monitored by PCR-denaturing gradient gel electrophoresis (DGGE) exploiting the *nifH* gene. The *nifH* gene is the most conserved gene in

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TABLE 1. CP protocols and FM used in the NFSC experiments for 2006 and 2007[a]

Crop, rotation type, and CP/FM protocol (yr)[a]	Management treatment(s)
Conventional rotation	
Winter barley (2006)	
ORG CP	Mechanical weeding (finger weeder)
CON CP	Pendimethalin[b] (2.5 liters/ha), isoproturon[b] (1.5 liter/ha), Duplosan[b] (1 liter/ha), Acanto[c] (0.4 liter/ha), Proline[c] (0.4 liter/ha), Corbel[c] (0.5 liter/ha), Fluroxypyr[c] (0.75 liter/ha), Amistar[c] (0.25 liter/ha), Bravo 500[c] (0.5 liter/ha), Cleancrop EPX[c] (0.4 liter/ha)
ORG FM	No amendment
CON FM	0:20:30 (64 kg P_2O_5/ha, 96 kg K_2O/ha), Nitram (170 kg N/ha)
Potatoes (2007)	
ORG CP	Mechanical weeding (ridging), copper-oxychloride[c] (23 kg/ha)
CON CP	Aldicarb[d] (33.5 kg/ha), linuron[b] (3.5 liters/ha), fluazinam[e] (1.5 liter/ha), mancozeb and metalaxyl-M[e] (4.7 kg/ha), oiquat[f] (2 liters/ha)
ORG FM	Composted cattle manure (equivalent to 180 kg N/ha)
CON FM	0:20:30 (134 kg P_2O_5/ha, 200 kg K_2O/ha), Nitram (180 kg N/ha)
Organic rotation	
Beans (2006)	
ORG CP	None
CON CP	Battalion[b] (2.8 liters/ha), Bravo 500[c] (1.5 liter/ha)
ORG FM	None
CON FM	0:20:30 (60 kg P_2O_5/ha, 90 kg K_2O/ha)
Potatoes (2007)	See conventional rotation for management treatments

[a] Shown are results for crops under organic crop protection (ORG CP) or conventional crop protection (CON CP) and organic fertility management (ORG FM) or conventional fertility management (CON FM).
[b] Herbicide.
[c] Fungicide.
[d] Nematicide.
[e] Growth regulator.
[f] Dessicant.

the *nif* operon and encodes the Fe subunit of the nitrogenase enzyme (41). Due to the conserved nature of the *nifH* gene, it has been possible to identify primer sets that can be used for analysis of diazotrophs so that this community can be analyzed by a PCR-DGGE-based technique (5, 36, 42, 50).

In this study we have tested the hypothesis that the use of organic farming practices (crop rotation, fertility management [FM], and crop protection) enhances the diversity and activity of free-living N fixers and the total bacterial population. We also investigated the seasonal variability of the diversity (as measured by changes in DGGE expression fingerprints) and activity (as measured by changes in expression of genes measured by quantitative PCR [qPCR]) of free-living N fixers and total bacteria.

MATERIALS AND METHODS

Soil sampling. The soil used in this study was taken from the Nafferton Factorial Systems Comparison (NFSC) study, a field trial based at Nafferton Farm in the Tyne Valley, northeast England. The objective of the field trial is to study the effects of "low-input" and organic food production systems on crop productivity, sustainability, environmental impacts, and food quality and safety.

The NFSC was established in 2001 and consists of a series of four field experiments established within four replicate blocks: plots 1 to 4 in block 1, plots 5 to 8 in block 2, plots 9 to 12 in block 3, and plots 13 to 16 in block 4. The experiment is a split split-plot design with three factors. The main factor is crop rotation. An 8-year, conventional cereal intensive rotation is compared to an 8-year, diverse legume intensive organic crop rotation. Each main plot is split to compare two levels of crop protection: organic (ORG CP; according to Soil Association organic farming standards [45]) and conventional (CON CP; following British Farm Assured practice). Each crop protection subplot is further split into two fertility management sub-subplots: organic (ORG FM; using compost as a fertility amendment [applied 26 March]) and conventional (CON FM; using mineral NPK fertilizer as a fertility amendment [applied 12 and 25 April]). This design also allows the experiment to be analyzed within each level of crop rotation, as four separate production systems: fully organic (ORG), organic crop protection and conventional fertility management (ORG CP-CON FM), conventional crop protection and organic fertility management (CON CP-ORG FM), and fully conventional (CON).

Compost was applied to ORG FM plots on 26 March, and NPK and Nitram were applied to CON FM plots on 12 and 25 April, respectively. ORG CP plots received copper fungicide weekly between 20 June and 31 July. CON CP plots received pesticide on 25 April, herbicide on 2 May, and fungicide weekly between 20 June and 13 August. Full details of the organic and conventional fertility management and crop protection practices used in the potato crop and the preceding year are shown in Table 1. The soil used in this study is a uniform sandy loam (alluvial deposit) and was sampled from experiment 2 of the NFSC trial in year 4 of both crop rotations, when potatoes (cv. Santé) were grown. The previous crops (PCs) in the organic and conventional rotations were faba beans (cv. Fuego) and winter barley (cv. Pearl), respectively.

In order to allow for within-plot variability, five cores of soil (0 to 30 cm) were randomly sampled within a plot and immediately mixed to form one composite sample per plot, on 2 March (prior to planting), 11 June (potatoes in growth stage 30—elongation) and 24 September (after harvesting) 2007. There are 4 plots for each treatment, giving a total of 16 plots. Soils were sieved fresh (4 mm), and a portion (about 10 g) was frozen immediately and stored at −80°C before extraction of RNA. Another portion (about 500 g fresh) was stored at 4°C before measurements of soil basal respiration with the Sensomat measurement system (40). A further portion of fresh soil was frozen at −20°C until extraction for nitrate and ammonium-N (2 M KCl). Concentrations of NO_3^--N and NH_4^+-N in the KCl extracts were determined with a Brann-Luebbe autoanalyzer 3 and the hydrazine reduction method for nitrate and the salicylate method for ammonia. The September soil samples were used for pH analysis (1:1 in water). Representative samples from the plots were taken in November 2007, dried, and sieved (2 mm) before analysis for total C and N by Dumas combustion (LECO Corporation) and Mehlich-3 extractable macro- and micronutrients.

RNA extraction and PCR. RNA was extracted from 0.25 g of soil with the UltraClean microbial RNA isolation kit (MoBio) and reverse transcribed with the Superscript II reverse transcriptase kit (Invitrogen).

The *nifH* gene was amplified by a nested-PCR method adapted from Wartiainen et al. (48). The first reaction used primers PolF and PolR (36) to amplify a 360-bp fragment. In order to clamp the products for DGGE, a second round of PCR was needed using AQER-GC30 and PolFI primers (48). PCR and the

TABLE 2. PCR and qPCR primers used in this study

Primer	Sequence (5′→3′)	Reference
PolF	TGC GA(CT) CC(GC) AA(AG) GC (GCT) GAC TC	36
PolR	AT(GC) GCC ATC AT(CT) TC(AG) CCG GA	36
AQER-GC30	CGC CCG CCG CGC CCC GCG CCC GGC CCG CCC GAC GAT GTA GAT (CT)TC CTG	48
PolFI	TGC GAI CC(GC) AAI GCI GAC TC	48
V3R	ATT ACC GCG GCT GCT GG	30
V3FC	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG	30
Eub338	ACT CCT ACG GGA GGC AGC AG	26

qPCR primers are summarized in Table 2. The reaction mixture contained 1 μl of reverse-transcribed RNA, 0.5 μM each primer, 25 mM each deoxynucleoside triphosphate (dNTP), 50 mM $MgCl_2$ (25 mM when using AQER-GC30/PolFI), 5× *Taq* buffer, 5 U *Taq* polymerase (New England Biolabs) (2.5 U when using AQER-GC30/PolFI), and 0.1 mg bovine serum albumin (BSA) and made up to 50 μl with sterile water. PCR conditions were taken from Poly et al. (36). The annealing temperature for both rounds was 55°C. In order to amplify the total bacterial community, the V3FC and V3R primers were used as described by Baxter and Cummings (2).

DGGE. DGGE was carried out using the D-Code system (Bio-Rad Laboratories) as described by Baxter and Cummings (2). Gels were electrophoresed at a current of 200 V for 6 h (*nifH*) or 200 V for 4.5 h (16S rRNA) at a constant temperature of 60°C. Bands were identified and relative intensities were calculated based on the percentageof intensity of each band in a lane. This was done with Quantity One software (Bio-Rad). Shannon's diversity index (H') was calculated by the formula $H' = -\sum p_i \ln(p_i)$, where p_i is the ratio of relative intensity of band i compared with the relative intensity of the lane.

qPCR. Reactions were set up using SYBR green (Thermo Fisher Scientific) according to Baxter and Cummings (1) with the Rotor-Gene RG 3000 (Corbett Research). All DNA was denatured at 95°C for 10 min prior to reaction setup. Reaction mixtures were heated to 95°C for 15 min to activate the SYBR green before completing 50 cycles of denaturation (95°C, 15 s), annealing (55/65°C [*nifH*/16S rRNA], 15 s), and extension (72°C, 15 s). PolF and PolR primers were used for *nifH* qPCR, and Eub338 and V3R were used for total bacteria qPCR. A standard curve was set up using 10-fold dilutions of pGEM-T Easy vector plasmid DNA containing either the *nifH* gene of *Rhizobium* sp. strain IRBG74 bacterium (isolated from root nodules of *Sesbania cannabina* by the International Rice Research Institute) (11) or the 16S rRNA gene of *Pseudomonas aeruginosa* NCTC10662. Each soil extraction, no-template control, and standard curve dilution was replicated three times. Average copy number was converted into copies of the gene per g of soil.

Standard deviation was determined (by the Rotor-Gene 6 software [Corbett Research]) on the replicate threshold cycle (C_T) scores. qPCR was repeated if the deviation was above 0.4. Samples were considered to be below reasonable limits of detection if the C_T score was above 30 (25). In the system used in this study, this would equate to results below 1.0×10^4 copies per g of soil being rejected. Although certain *nifH* copy numbers were low, none fell below this threshold. All no-template control results fell below this threshold (35.4 ± 2.8). The standard curve produced was linear ($r^2 = 0.98$), and the PCR efficiency was >0.9.

Statistical analysis. In all tests, significant effects/interactions were those with a P value of <0.05. All univariate data were analyzed using the linear mixed effects (lme) function in the nlme package of R (37). The combined data for all three dates were analyzed first, and where interaction terms were significant, further analyses were conducted at each level of the interacting factor. The hierarchical nature of the split split-plot design was reflected in the random error structures that were specified as block/date/precrop/crop protection. Where analysis at a given level of a factor was carried out, that factor was removed from the random error term. The normality of the residuals of all models was tested with QQ plots, and data were cube root transformed when necessary to meet the criteria of normal data distribution (2). Differences between main effects were tested by analysis of variance (ANOVA). Differences between the four crop management strategies within each level of crop rotation were tested with Tukey contrasts in the general linear hypothesis testing (glht) function of the multcomp package in R. A linear mixed effects model was used for the Tukey contrasts, containing a treatment main effect with four levels and with the random error term specified as block/crop protection.

Pearson's product-moment correlations were calculated using the cor.test function in R.

DGGE data were analyzed by detrended correspondence analysis (DCA) on relative intensities followed by direct ordination with Monte Carlo permutation testing. Direct ordination was either by canonical correspondence analysis (CCA) or redundancy discriminate analysis (RDA), depending on the length of the DCA axis (where an axis of >3.5 = CCA and an axis of <3.5 = RDA). CANOCO for Windows 4.5 and CANODRAW for Windows were used to carry out DCA, CCA, and RDA (28).

TABLE 3. Shannon diversity index values for 16S rRNA DGGE and *nifH* DGGE data sets and results of ANOVA carried out on these data

Crop management or statistical significance parameter	Shannon diversity index value for[b]:					
	nifH DGGE band data			16S rRNA DGGE band data		
	March	June	September	March	June	September
Precrop						
Barley	2.26	1.67	2.67	2.88	2.82	2.03
Beans	1.33	1.87	1.81	2.63	2.56	2.71
CP						
ORG	1.75	1.82	2.35	2.78	2.70	2.41
CON	1.84	1.72	2.14	2.73	2.69	2.33
FM						
ORG	1.66	1.78	2.22	2.71	2.69	2.34
CON	1.93	1.77	2.27	2.80	2.70	2.41
ANOVA P values[a]						
PC	**<0.0001**	0.154	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
CP	0.597	0.450	0.153	0.342	0.813	0.108
FM	0.121	0.916	0.695	0.057	0.796	0.154
CP × FM	0.381	0.537	0.546	0.584	0.407	0.514
FM × PC	0.381	0.731	0.599	0.391	0.191	0.268
CP × PC	0.558	**0.037**	0.859	0.696	0.441	0.539
FM × CP × PC	0.170	0.951	0.943	0.684	0.425	0.271

[a] PC, previous crop; FM, fertility management; CP, crop protection.
[b] Boldface is used for a significance (P) of <0.05.

RESULTS

Diversity and expression of *nifH*. (i) DGGE of *nifH*. DGGE gels are shown in Fig. S1 in the supplemental material. Analysis of the *nifH* DGGE Shannon's diversity index values for the whole data set (data not shown) indicated that sample date and crop rotation (precrop) significantly affected the *nifH* diversity (sample date, $P < 0.001$; precrop, $P < 0.001$; and sample date × precrop, $P < 0.001$). For these reasons, a separate analysis of variance (ANOVA) of the *nifH* DGGE Shannon's diversity index values was conducted at each date.

Soils in the conventional rotation with a previous crop (PC) of barley showed significantly higher *nifH* diversity than soils in the organic rotation with a previous crop of beans in March and September (the average H' values were 2.203 for the barley precrop and 1.674 for the beans precrop) (Table 3). Pearson's product-moment correlation found a significant negative correlation between nitrate and ammonium and *nifH* H' in March and a significant positive correlation between nitrate and ammonium and *nifH* H' in September (Table 4).

Table 5 summarizes results of CCA and RDA (for plots, see Fig. S3 in the supplemental material). Crop management effects were found to be significant in June in the organic rotation. Total carbon and nitrogen, available ammonium, and

TABLE 4. Changes to pH, available nitrate, and available ammonium across the field trial and Pearson's product-moment correlation analysis comparing data to *nifH* diversity and gene expression

Crop management or significance parameter	pH	Available NO_3^- (kg ha^{-1}) in[d]:			Available NH_4^+ (kg ha^{-1}) in[d]:		
		March	June[a]	September	March	June	September
Crop management[b]							
Precrop							
Barley	6.14 ± 0.05	5.81 ± 0.8	279.34 ± 43.2	27.08 ± 2.3	0.94 ± 0.2	7.58 ± 0.8	6.47 ± 0.5
Beans	6.23 ± 0.04	12.47 ± 0.7	234.42 ± 31.2	22.51 ± 1.7	5.95 ± 0.3	1.51 ± 0.3	0 ± 0
CP							
ORG	6.20 ± 0.05	8.68 ± 1.1	282.75 ± 40.5	30.07 ± 1.9	3.75 ± 0.1	4.85 ± 1.0	3.44 ± 1.0
CON	6.17 ± 0.04	9.60 ± 1.1	231.01 ± 34.3	19.52 ± 1.3	0.18 ± 0.9	4.24 ± 1.0	3.03 ± 0.8
FM							
ORG	6.26 ± 0.03	7.98 ± 1.2	125.04 ± 6.9	24.44 ± 1.3	3.71 ± 0.1	3.29 ± 0.6	3.68 ± 1.0
CON	6.11 ± 0.05	10.31 ± 0.9	388.72 ± 26.7	25.14 ± 2.7	3.18 ± 0.9	5.80 ± 1.2	2.79 ± 0.7
Statistical significance							
ANOVA *P* values							
PC	0.250	**<0.001**	**0.042**	**0.004**	**<0.001**	**<0.001**	**<0.001**
CP	0.603	0.327	**0.021**	**<0.001**	*0.051*	0.399	0.349
FM	**<0.001**	**0.018**	**<0.001**	0.632	*0.095*	**0.002**	**0.049**
ρ[c]							
Correlation with *nifH* DGGE *H′*	NS	−−	NS	+++	−−−	NS	+++
Correlation with *nifH* copy no.	+++	−−	NS	+++	−−−	+++	++

[a] Amounts of nitrate are often larger than amounts added to the soil due to mineralization of organic N to inorganic N by soil microorganisms.
[b] PC, previous crop; CP, crop protection; FM, fertility management.
[c] ρ, Pearson's product-moment correlation coefficient; NS, not significant; ++/−−, significant positive or negative correlation at $P < 0.05$; +++/−−−, significant positive or negative correlation at $P < 0.01$.
[d] Boldface is used for a significance (*P*) of <0.05, and italics are used for a significance of <0.1.

extractable phosphorus, as well as soil basal respiration, all significantly affect *nifH* diversity at some point over the sampling season, although the effects were not consistent across dates and between precrops.

(ii) qPCR of *nifH*. Analysis of the full set of data across sample dates indicated that there was a decrease in *nifH* copy number in June compared to March (average numbers of copies per g of soil, 5.70×10^5 in June versus 7.45×10^5 in March), followed by an increase in September (1.05×10^7 copies per g soil) which exceeded the March levels. There was a significant interaction between sample date and crop rotation (precrop effect) ($P = 0.0005$); therefore, the results of the ANOVA of the *nifH* qPCR are shown for each date in Table 6. On all sample dates, increased *nifH* copy number was seen in the conventional rotation (barley precrop) compared to the organic rotation (beans precrop). In the conventional rotation, the *nifH* copy number was 10 times higher in March and June than that in September. In June, increased *nifH* copy number was associated with conventional fertility management. In September, crop protection was a significant factor with increased *nifH* copy number when organic crop protection was used.

Pearson's product-moment correlation found a positive correlation between *nifH* copy number and pH, available ammonium in June and September, and available nitrate in September. There was a negative correlation between available nitrate and ammonium and *nifH* copy number in March (Table 4).

Total bacterial diversity and function. There are clearly differences, in the nitrogen-fixing community, between dates and between treatments. In order to ensure these factors are affecting the nitrogen-fixing community specifically and not the bacterial community as a whole, the 16S rRNA gene diversity and abundance were also analyzed.

DGGE of the 16S rRNA gene. DGGE gels showing diversity of total bacteria are shown in Fig. S2 in the supplemental material. As with the free-living nitrogen-fixing community, ANOVA results for the Shannon diversity index for the 16S rRNA gene indicated that date ($P < 0.001$) and date × crop rotation ($P < 0.001$) were significant factors (data not shown); therefore, each sample date was analyzed separately. This indicated that soils with a previous crop of barley (conventional rotation) showed significantly higher 16S rRNA diversity than soils following beans (organic rotation) in March and June; however, the situation was reversed in September (Table 3).

RDA and Monte Carlo permutation testing of the V3 DGGE profiles indicated that crop protection (June conventional rotation and September organic rotation) and fertility management (September conventional rotation) were significant drivers of bacterial community structure (Table 5). Total soil nitrogen and total soil carbon are also significant drivers of bacterial diversity in some cases. However, in all instances the differences in soil nitrogen and carbon are associated with different locations of the plots in the field (block effect) rather than the treatments themselves (see Fig. S4 in the supplemental material). The only significant driver which seems to be related to both crop management and soil diversity is pH in the

TABLE 5. Summary of CCA and RDA showing significant variables

Gene of interest	Previous crop	Sample date	Variable(s) tested	Significant variable(s) selected by forward selection[c]	Variance of DGGE data explained by the model (%)
nifH	Beans	March	FM		8.0
			CP		6.4
			Associated variables[a]	C	65.3
			Associated variables,[a] FM, CP[b]	C	76.3
		June	FM		6.1
			CP	CP	11.6
			Associated variables[a]	NH_4^+	48.2
			Associated variables,[a] FM, CP	CP, NH_4^+	65.7
		September	FM		7.2
			CP		7.4
			Associated variables[a]	P	36.9
			Associated variables,[a] FM, CP	P	48.3
	Barley	March	FM		7.5
			CP		5.8
			Associated variables[a]	N	51.0
			Associated variables,[a] FM, CP	N	61.9
		June	FM		9.2
			CP		6.4
			Associated variables[a]	SBR	53.7
			Associated variables,[a] FM, CP	SBR	68.1
		September	FM		7.0
			CP		5.2
			Associated variables[a]		40.9
			Associated variables,[a] FM, CP		51.7
16S rRNA	Beans	March	FM		6.6
			CP		5.7
			Associated variables[a]		41.8
			Associated variables,[a] FM, CP		55.0
		June	FM		6.9
			CP		7.7
			Associated variables[a]		46.6
			Associated variables,[a] FM, CP	N, pH	57.8
		September	FM		4.2
			CP	CP	11.4
			Associated variables[a]	N	30.7
			Associated variables,[a] FM, CP	CP, N	42.4
	Barley	March	FM		3.1
			CP		6.2
			Associated variables[a]		35.4
			Associated variables,[a] FM, CP		44.0
		June	FM		6.2
			CP	CP	14.1
			Associated variables[a]	C	42.0
			Associated variables,[a] FM, CP	CP, C	57.4
		September	FM	*FM*	11.3
			CP		4.7
			Associated variables[a]		31.3
			Associated variables,[a] FM, CP	*FM*	45.1

[a] These associated variables include available carbon (C) and nitrogen (N), pH, soil basal respiration (SBR), available phosphorus (P), ammonium (NH_4^+), and nitrate.

[b] These associated variables, including FM and CP, show the total effect of treatments and variables.

[c] All variables shown are significant at a P value of <0.05 unless shown in italics ($P < 0.1$).

TABLE 6. ANOVA of qPCR results

Crop management factor or significance parameter[a]	Avg no. of copies of *nifH*/g of soil[b]			Avg no. of copies of 16S rRNA/g of soil[b]		
	March	June	September	March	June	September
Precrop						
Barley	$1.3 \times 10^6 \pm 7.9 \times 10^5$ A	$1.1 \times 10^6 \pm 3.5 \times 10^5$ A	$2.1 \times 10^7 \pm 6.1 \times 10^6$ B	$6.2 \times 10^7 \pm 1.7 \times 10^7$ C	$5.2 \times 10^7 \pm 1.1 \times 10^7$ C	$4.8 \times 10^7 \pm 1.0 \times 10^7$ C
Beans	$1.5 \times 10^5 \pm 8.1 \times 10^4$ D	$3.0 \times 10^4 \pm 6.6 \times 10^3$ D	$6.6 \times 10^4 \pm 1.7 \times 10^4$ D	$2.7 \times 10^7 \pm 5.2 \times 10^6$ E	$5.3 \times 10^7 \pm 1.5 \times 10^7$ E	$4.4 \times 10^7 \pm 1.1 \times 10^7$ E
CP						
ORG	$3.3 \times 10^5 \pm 1.0 \times 10^5$ F	$4.3 \times 10^5 \pm 1.9 \times 10^5$ F	$1.5 \times 10^7 \pm 6.4 \times 10^6$ G	$5.1 \times 10^7 \pm 1.6 \times 10^7$ H	$3.9 \times 10^7 \pm 8.9 \times 10^6$ H	$3.9 \times 10^7 \pm 5.8 \times 10^6$ H
CON	$1.2 \times 10^6 \pm 8.1 \times 10^5$ I	$7.2 \times 10^5 \pm 3.5 \times 10^5$ I	$5.9 \times 10^6 \pm 2.7 \times 10^6$ I	$3.8 \times 10^7 \pm 1.0 \times 10^7$ J	$6.6 \times 10^7 \pm 1.6 \times 10^7$ J	$5.4 \times 10^7 \pm 1.4 \times 10^7$ J
FM						
ORG	$3.1 \times 10^5 \pm 9.8 \times 10^4$ K	$2.6 \times 10^5 \pm 1.1 \times 10^5$ K	$1.2 \times 10^7 \pm 6.2 \times 10^6$ K	$5.5 \times 10^7 \pm 1.7 \times 10^7$ L	$5.7 \times 10^7 \pm 1.5 \times 10^7$ L	$5.0 \times 10^7 \pm 1.3 \times 10^7$ L
CON	$1.2 \times 10^6 \pm 8.1 \times 10^5$ M	$8.9 \times 10^5 \pm 3.7 \times 10^5$ M	$8.9 \times 10^6 \pm 3.5 \times 10^6$ N	$3.4 \times 10^7 \pm 7.8 \times 10^6$ O	$4.8 \times 10^7 \pm 1.1 \times 10^7$ O	$4.3 \times 10^7 \pm 7.6 \times 10^6$ O
ANOVA *P* values						
PC	**0.012**	**0.006**	**<0.001**	**0.003**	0.897	0.366
CP	0.426	0.876	**0.014**	0.195	**0.027**	*0.079*
FM	0.163	**0.032**	0.447	**0.039**	0.524	0.982
CP × FM	0.539	0.734	0.718	0.372	0.608	0.728
FM × PC	0.194	0.340	0.530	0.488	0.297	0.406
CP × PC	0.103	0.156	0.266	0.285	0.314	0.700
FM × CP × PC	*0.096*	0.736	0.270	0.881	0.610	0.508

[a] PC, previous crop; CP, crop protection; FM, fertility management.

[b] The values shown for management factors are means ± standard errors. Values followed by the same letter in the same row at each level of management factor are not significantly different (Tukey's honestly significant difference; $P > 0.05$). Boldface is used for a significance (P) of <0.05, and italics are used for a significance of <0.1.

organic rotation in June. Here an increase in pH is associated with organic fertility management.

qPCR of 16S rRNA gene. The aim of the qPCR analysis was to compare differences between plots rather than absolute quantification. There was a significant interaction between sample date and crop protection when the 16S bacterial populations were quantified using qPCR ($P = 0.017$). For this reason, a separate analysis was conducted at each date (Table 6). In contrast with the DGGE results for the 16S rRNA gene, the previous crop was the only significant factor in March. On the same date, a history of organic fertilization also resulted in higher numbers of the 16S rRNA gene copy. In June, crop protection affected 16S rRNA gene copy numbers, with higher numbers where conventional crop protection was used. This positive effect of conventional crop protection continued into September, although it was no longer significant ($P = 0.079$).

Pearson's product-moment correlation showed that there is no link between increased expression of the 16S gene and increased expression of the *nifH* gene (data not shown).

DISCUSSION

This study allowed a detailed analysis of the effects of key components of organic and conventional farming systems on soil bacterial and free-living N-fixing bacterial population structure and gene expression. Expression of *nifH* and 16S rRNA genes was compared using Pearson's product-moment correlation, and no link was found between the levels of expression of both genes. This suggests that factors which affect the free-living N fixers do not necessarily affect the community as a whole. Most molecular studies looking at the *nifH* gene use DNA rather than RNA. The numbers of copies of *nifH* per g of soil seem low in some instances and suggest *nifH* is not always transcribed. This was also observed in pine forest soil by Izumi et al. (21), who found although diverse populations of nitrogen-fixing organisms were found using DNA, *nifH* could often not be amplified from RNA samples.

16S rRNA copy numbers are also are lower than would be expected in agricultural soil and are more similar to numbers observed in forest soil (49). This is possibly due to the efficiency of RNA extraction/reverse transcription. The purpose of qPCR in this experiment is to compare between sample plots not absolute quantification.

The dominant management factor affecting microbial population structure and function in this study was crop rotation. Rotation (identified by the previous crop in this study) had a strong effect on both total bacterial and free-living N-fixing bacterial population structure (measured by DGGE profiles) and activity (measured by gene copy numbers).

Most research into the effect of crop species on the soil's microbial community has been carried out on rhizosphere soils. Any changes to the microbial community are attributed to changes in organic root exudates affecting microbial activity in a species-specific manner (18, 51). In this study, these changes were detected in the bulk soil and were apparent even though the crop was the same in both rotations in the sampling year. The different crop species grown in each rotation in the previous 3 years had resulted in fundamental changes to the structure and activity of both the free-living N-fixing bacteria and the broader bacterial community.

A considerably more active and diverse diazotroph community was seen in soils previously under barley (conventional rotation). Even on the final sample date, differences in the composition of the *nifH* community (between organic and conventional rotation) were evident (Procrustes rotation of the PCA axes; $P = 0.003$) (data not shown). These findings support those of Larkin (27), who suggested that plant effects (i.e., crop rotation) are the most important drivers of soil microbial community characteristics within a given site and soil type. Crop rotational effects on populations of free-living N-fixing bacteria were also reported by Chunleuchanon et al. (6), who found that when rice was grown in rotation there was higher diversity of nitrogen-fixing cyanobacteria than when it was grown in monoculture. However, to our knowledge, this is the first study which documents increased *nifH* expression and diversity of a free-living diazotroph following a rotation containing nonlegumes compared with a rotation containing legumes.

It could be hypothesized that the dramatic effect of crop rotation on the free-living nitrogen-fixing community in our study is due to the fundamental differences between the two rotations. Faba beans can derive 90% of their N from N_2 fixation (19); therefore, beans do not have as high an N demand as barley (22). For this reason, even under conventional fertility management, no N fertilizer is applied to the beans in the NFSC experiments. Cereal crops such as barley efficiently utilize available N in the soil, depleting mineral N during crop growth. The low N levels in soils under cereal crops may make the soil a more suitable environment for free-living diazotrophs, resulting in the increased numbers seen in the soil in the conventional rotation. Indeed, even in March of the following year (2007), there was still more available nitrate and ammonium (Table 4) in the soil under potatoes following a crop of beans in the previous year than there was in soil under potatoes following barley ($P = 0.0895$). This suggests that higher levels of mineral N throughout the season in the soil after a legume precrop may be suppressing the activity of free-living N-fixing bacteria.

Organic farming practices rely on the addition of organic material to the soil, and it was expected that organic fertility management would promote more gene expression and diversity in the soil microbial community (44). However, in this study fertility management affected microbial populations to a lesser extent than crop rotation, influencing the activity of the *nifH* gene on one date (June 2007) and the activity of the 16S rRNA gene on one date (March 2007).

However, some fertility-related factors did affect the nitrogen-fixing community according to Pearson's product-moment correlation (Table 4), although the factors affected the community differently at different sampling dates. Levels of N in bulk soils may not affect the *nifH* community consistently. Most studies that have reported this effect have been conducted on free-living N-fixing bacteria in the rhizoplane or rhizosphere soils. Coelho et al. (8) found higher levels of nitrogen fertilizer decreased N fixation in rhizosphere soils but found it had no effect in bulk soil.

In the NFSC experiments, the different fertility management (FM) regimens do not just involve application of different forms of nitrogen: the conventionally managed plots receive superphosphate and potassium chloride, whereas the organic plots receive only compost (which contains various amounts of P and K as well as other macro- and micronutrients) (Table 1). Reed et al. (38) found that the addition of phosphorus to soil more than doubles nitrogen fixation, due to the energy requirements of nitrogen fixation. While it is possible that the positive effect of conventional fertility management (see the number of copies of the *nifH* gene for June 2007) has nothing to do with the nitrogen applied to the field but more to do with the increased availability of phosphorus in this treatment, this is not supported by CCA/RDA analysis (Table 5).

Organic and conventional crop protection practices were also shown to have an impact on the bacterial community. Conventional crop protection of potatoes and the preceding cereal crops involves the use of a variety of synthetic pesticides (Table 1). These chemicals can have a marked effect on the bacterial community structure and function as some microorganisms may be suppressed and some will proliferate in the vacant ecological niches (23). Organic farming practices have been criticized for relying on copper products to control disease: for example, copper oxychloride is used for control of fungal diseases in the NFSC experiments. Studies of copper oxychloride have found that it only significantly affects bacterial communities in concentrations over 100 mg/kg (16). Annual rates of application total 6 kg Cu ha^{-1}, or approximately 3 mg Cu kg^{-1} soil in the NFSC experiments, suggesting that Cu levels in the system are well below safe limits for bacterial communities. In September, there was increased expression of *nifH* after organic crop protection. This could be a result of the cumulative effect of crop protection over the season (and the previous 3 years), possibly inhibiting the free-living N-fixing community where conventional crop protection was used. Studies into the environmental impacts of pesticides have shown that they can significantly affect the bacterial community as a whole and that diazotrophs could be particularly affected. For example, the fungicide mancozeb was found to exert an inhibitory effect on aerobic dinitrogen fixers in soil (14).

Strong seasonal effects and interactions between the sample date and crop management factors were detected. Temperature is one of the most important environmental factors affecting the soil bacterial community (35). The optimum temperature for nitrogen fixers' growth and activity is between 10 and 25°C (this is the temperature in the field between June and September) (3, 17). The temperature in the field on the March sampling date was approximately 4.5°C. It seems likely that the activity of the free-living N-fixing population was suppressed by temperature at this time. The average numbers of copies of the *nifH* gene on the March sampling date were 7.45×10^5 g^{-1} soil, and even on the June sampling date, copy number had not yet recovered (5.7×10^5 copies g^{-1} soil); however, by September the population had increased 14-fold to 1.1×10^7 copies g^{-1} soil. Eckford et al. (17) suggested that the free-living diazotrophs may only be active seasonally *in situ*. Interestingly, the populations of the total metabolically active bacteria were not affected by seasonal variations in temperature, with populations only ranging between 4.5×10^7 and 5.2×10^7 copies g^{-1} soil on the three sampling dates.

Management factors may also have played a role in the seasonal variations in *nifH* activity. Soil mineral N also varies seasonally with levels, being generally highest in June, after fertilizers have been applied but before the crop's root system has developed sufficiently to take up the available N. Coelho et

al. (8, 9) found that 30% more free-living diazotrophs could be isolated from soil in the presence of low levels of nitrogen fertilizer compared with high levels of nitrogen fertilizer. In our study, June soil samples were very high in mineral N, even in the organic fertility management treatments (~200 kg mineral N ha^{-1} for ORG FM and ~400 kg mineral N ha^{-1} for CON FM in June 2007), and exceeded the amounts added to the soil, suggesting mineralization of organic N is occurring in the soil. The relatively high soil available N levels in June could, therefore, have suppressed the activity of the free-living N-fixing bacteria. By September, levels of mineral N in the potato soils were approximately 30 kg N ha^{-1}, regardless of the fertility treatment.

Conclusions. The effect of crop rotation was consistent. The increase in nitrogen uptake by the barley crop is likely to create conditions more favorable to free-living nitrogen fixation. Although the increased amounts of nitrate and ammonia found in the soil following the bean crop are only apparent in March, the free-living diazotrophs are more likely established in the soil following barley and the community appears more diverse and abundant throughout the growing season.

The results show that the management regimen clearly affects both the total bacterial community and the free-living diazotroph community. However, the communities are not always affected in the same way and the effects are often subtle/short-lived. When looking at the total bacterial community in this study, significant differences were found as a result of changing management. However, although this gives a greater understanding of the structure of the community, it does not necessarily tell us anything about function. The effect of fertility management and crop protection on free-living N fixation was not consistent. However, organic crop protection and conventional fertility management often had positive effects on *nifH* diversity and activity. This study supports the work of Bossio et al. (4) and suggests that although management does affect community structure/activity, it could be secondary to other factors such as time of sampling and previous crop. Current work aims to make these findings more robust by studying further sample years.

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Positive plant microbial interactions in relation to plant performance and ecosystem function

The effect of organic and conventional farming practices on the soil microbial community carbon substrate utilization

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Summary

Understanding the response of the soil microbial community to organic and conventional farming practices could be important in the long term for understanding how key nutrients are cycled within agricultural soils. Here the effects of three aspects of soil and crop management: crop rotation, crop protection protocols and fertility management on microbial community function were investigated using BIOLOG Ecoplates. The data indicated that the soil microbial community level substrate utilisation was significantly affected by organic and conventional crop rotations and crop protection methods but was unaffected by different fertility management regimes.

Key words: BIOLOG, Ecoplates, CLSU, organic farming

Introduction

Organic farming relies on crop rotations, green manure crops and organic amendments (compost and manure) to maintain soil fertility, and excludes the use of mineral fertilisers, and other chemicals such as synthetic pesticides (Elfstrand *et al*., 2007). It is well known that soils amended with organic matter such as plant residues, manures and compost have improved physical, chemical and biological properties and exhibit increased functional stability of the microbial community (Toyota & Kuninaga, 2006). Similarly, crop rotation can increase soil biodiversity due to the differences in root exudates released by the different crops.

Community level substrate utilisation (CLSU) using BiologTM microplates was first developed by Garland & Mills (1991) in order to study the functional diversity of a microbial community in an environmental sample (Klimek & Niklińska, 2007). Ecoplates contain 31 carbon (C) substrate wells in triplicate, and a tetrazolium dye (Klimek & Niklińska, 2007). Growth of aerobic, heterotrophic microorganisms within the wells, indicated by substrate utilisation, results in formazan production due to reduction of the tetrazolium dye. This produces a colour change. The specific pattern of colour change on the plate provides a metabolic fingerprint for the community which allows the effects of different soil management practices on the metabolic diversity to be studied.

The objectives of this study were to find out how organic and conventional farming practices affect the function of the microbial community residing in agricultural soil using CLSU. Although the effects of crop rotations, fertility management and crop protection protocols have been studied previously (Young & Ritz, 2000; Cycoń & Piotrowska-Seget, 2009), the factorial design of this

study allows investigation of the effects of each individual factor, as well as the interactions among the factors. This will provide further information on how specific aspects of farming practice impact on microbial community function.

Materials and Methods

Soil sampling

The soil used in this study was taken from the Nafferton factorial systems comparison (NFSC) study, a field trial based at Nafferton Farm in the Tyne valley, North East England.

The main plot factor is crop rotation with two levels: organic (an 8 year, diverse rotation) and conventional (an 8 year cereal-intensive rotation). Each main plot is split to compare two levels of crop protection: organic (ORG CP, Soil Association organic farming standards (Soil Association, 2005)) and conventional (CON CP, following British Farm Assured practice). Each crop protection sub-plot is further split into two fertility management sub sub-plots: organic (ORG FM, using compost as a fertility amendment) and conventional (CON FM, using mineral nitrogen, phosphorus and potassium (NPK) fertiliser as a fertility amendment). This design also allows the experiment to be analyzed within each level of crop rotation, as four separate production systems: fully organic (ORG), organic crop protection and conventional fertility management (ORG CP-CON FM), conventional crop protection and organic fertility management (CON CP-ORG FM) and fully conventional (CON). The soil used in this study was sampled from experiment 2 of the NFSC trial in year 4 of both crop rotations, when potatoes (*Solanum tuberosum* cv. Santé) are grown. The previous crop in the organic and conventional rotations was spring faba beans (*Vicia faba* cv. Fuego) and winter barley (*Hordeum vulgare* cv. Pearl) respectively.

Community level substrate utilisation

Five cores of soil (0–30 cm) were sampled from each plot and mixed to form one composite sample per plot on 2 March, 11 June and 24 September 2007. Soils were sieved fresh (4 mm) and stored at -20°C.

Community level substrate utilisation (CLSU) was determined using EcoPlatesTM from the BiologTM system. The protocol for Ecoplate analysis was adapted from methods employed by Kashama *et al*. (2009), Prévost *et al*. (2006) and Widmer *et al*. (2001).

Soils (3 g dry weight) that had been previously frozen at -20°C were pre-conditioned at 20ºC in a saturated atmosphere for 7 days to standardise analysis for each sample. Bottles were weighed regularly and any reduction in weight due to moisture loss was made up with sterile distilled water.

Plates were incubated at 20ºC in the dark for 54 h before absorbance readings were taken (540 nm using a microplate reader (Bio-tek)). All substrate absorbancies were corrected by subtracting the control well value and normalized by dividing by the average well colour development (AWCD). Functional diversity was measured using Shannon's diversity index:

$$H' = - \sum p_i \ln(p_i)$$

where p_i is the ratio of colour development of well i to the sum of colour development of all positive wells, and ln refers to the natural log (Farnet *et al*., 2008; Zak *et al*., 1994).

Additional soil analyses

Soil basal respiration was measured in fresh soils using the Sensomat-Measurement-System (Robertz *et al*., 2000). Approximately 100 g of soil was air dried prior to analysis for total carbon (C) and nitrogen (N) by Dumas combustion (LECO Corporation, USA) and pH in water (1:1). Inorganic N in frozen soil samples for each date was extracted (2 M potassium chloride)

and analyzed using a Bran+Luebbe Continuous Flow Auto-analyzer 3 (salicylate method for ammonium-N and hydrazine reduction method for nitrate-N).

Statistical analysis

In all tests significant effects/interactions were those with a P value of < 0.05. All univariate data were analyzed using the linear mixed effects (lme) function in the nlme package of R (R Development Core Team, 2006; Crawley, 2007). The combined data for all three dates were analyzed first and where interaction terms were significant, further analyses were conducted at each level of the interacting factor. The hierarchical nature of the split-split plot design and repeated measures sampling pattern was reflected in the random error structures that were specified as block/date/pre-crop/crop protection. Differences between main effects were tested using analysis of variance.

CLSU AWCD data were analysed using detrended correspondence analysis (DCA) followed by direct ordination with Monte Carlo permutation testing. Direct ordination was by redundancy discriminate analysis (RDA). CANOCO for Windows 4.5 and CANODRAW for Windows were used to carry out DCA and RDA.

Differences between sample dates were tested using procrustes rotation of PCA ordinations in the vegan package in R version 2.8.1 (Oksanen *et al*., 2009). Procrustes rotation can be used to compare PCA ordinations by looking for the maximum similarity between data sets by comparing the sum of the squared differences from the axis in principal component plots.

Results

The CLSU patterns were significantly altered by sample date, pre-crop and crop protection however results were inconsistent. Date was found to be a significant factor when using procrustes rotation. When soil had previously been under faba beans there was a statistically significant difference between CLSU in June and September (P = 0.05). Procrustes rotation found no differences at other dates or between pre-crops.

When Shannon's diversity indices for the whole dataset were analyzed there were no significant differences between dates or after different pre-crops; there were also no significant interactions between the main effects. There was a significant effect due to crop protection (P = 0.016) with soils where conventional crop protection was used having a higher H' than soils from organic crop protection treatments (Table 1). Although not significant, there was a trend (P = 0.06) towards an interaction between sample date and crop protection suggesting that the effect of crop protection may be more relevant at different sample dates. When the dataset was split by sample date to investigate this it was found that crop protection was only significant in September with higher diversity measured under organic crop protection compared to conventional crop protection (P = 0.013, data not shown).

RDA was carried out for all sample dates however only the June RDA plot is shown here as an example (Fig. 1). RDA ordinations show the variation in principal component analysis plots which are due to pre-crop (crop rotation), fertility management and crop protection, and also how the results are affected by other environmental variables such as soil total N and organic C. When analysing data using RDA the only significant factors (found by Monte Carlo permutation testing) affecting the CLSU were the amount of available ammonium in March (P = 0.01) and the pre-crop in June (P = 0.002) (Fig. 1). When looking at the March plots an increased ammonium level seemed to be associated with a previous crop of beans and organic fertility management. In the June plot the significant effect of pre-crop seems to be driven by increased ammonium, pH and soil basal respiration (SBR) associated with the barley pre-crop. In September increased ammonium and pH are also associated with the barley pre-crop although the effect is not significant.

Table 1. *Shannon diversity index values for whole data set and results of ANOVA carried out on these data*

H' for CLSU Main effect means			ANOVA	
			Effect	*P*-value
Pre-crop			SD	0.469
	Barley	2.833	PC	0.132
	Beans	2.793	FM	0.264
Crop protection			CP	0.016
	ORG	2.828	SD*PC	0.75
	CON	2.799	SD*FM	0.632
Fertility management			SD*CP	0.06
	ORG	2.781	PC*FM	0.5
	CON	2.845	PC*CP	0.394
			FM*CP	0.822
			SD*PC*FM	0.972
			SD*PC*CP	0.97
			SD*FM*CP	0.875
			PC*FM*CP	0.249
			SD*PC*CP*FM	0.842

SD=sample date; PC=previous crop; FM=fertility management; CP=crop protection.

Discussion

The effects of four factors: sampling date, previous crop, fertility management and crop protection methods, on CLSU were tested. With the exception of fertility management, all factors were shown to have an effect at certain points although results were inconsistent.

The non-effect of fertility management may be surprising as its effects on the structure and activity of the microbial community have been widely discussed, with most authors showing a positive effect associated with organic practices (O'Donnell *et al*., 2001; van Diepeningen *et al*., 2006; Mäder *et al*., 2002). However the majority of these reported changes are speculated to be a consequence of changes in soil organic C and so far there have been no significant effects of management practices on soil organic C in the NFSC.

Although fertility management did not affect the CLSU, an ANOVA of the Shannon diversity index shows that, in September, there was an increased diversity in the plots which have had organic crop protection. This was supported by molecular analysis of the 16S rRNA gene using denaturing gradient gel electrophoresis (DGGE) (data not shown).

Applying chemicals, in the form of herbicides and pesticides, to the soil can have a marked effect on the bacterial community structure and function as some microorganisms will be suppressed and others will proliferate in the vacant ecological niches (Johnsen *et al*., 2001). Different chemicals can also have different effects. For example, copper oxychloride is used in organic treatments and has been found to have no effect on bacterial communities when in concentrations under 100 mg kg^{-1} (Du Plessis *et al*., 2005). However, linuron and mancozeb (used in the conventional plots) have been shown to significantly reduce plate counts of bacteria (Cycon & Piotrowska-Seget, 2007). The increase in diversity in September associated with organic crop protection could be a result of a cumulative effect of crop protection over the season.

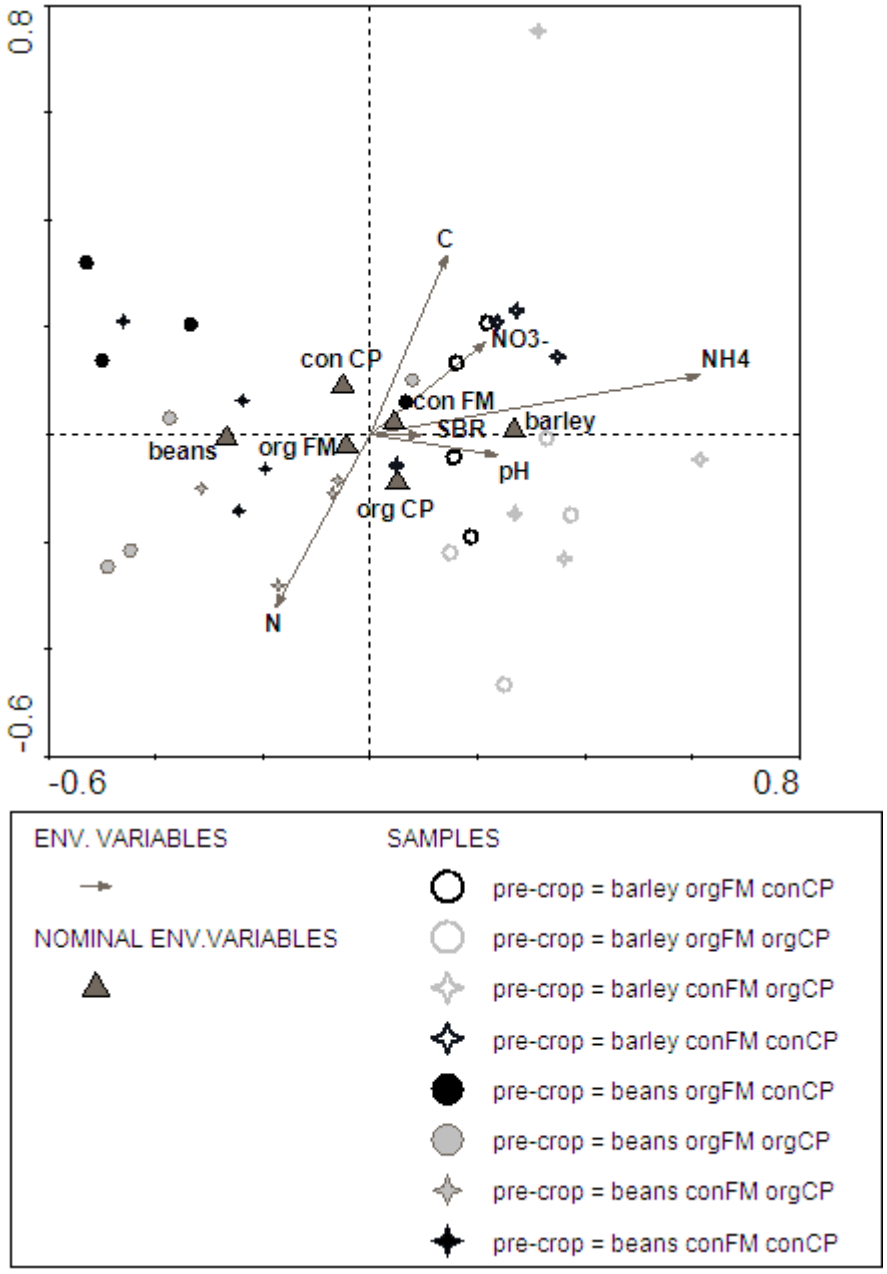

Fig. 1. Redundancy discriminate analysis (RDA) showing effects of drivers on the microbial community in June 2007. Further details of 'ENV(IRONMENTAL) VARIABLES' and SAMPLES' are given in the Materials and Methods.

The effect of sample date could be explained by seasonal variations or differences in how the plots are treated at different times of the year if the effect was seen after both pre-crops. The fact it is only seen after faba beans suggests that there is an interaction between crop rotation and sample date. Crop rotation can affect the soil microbial community as different plants growing in soil will remove different nutrients from the soil, as well as, releasing different forms of organic root exudates in different quantities. These root exudates will influence the microbial community in the rhizosphere and can also influence the community in bulk soil. When looking at crop type Wieland *et al*. (2001) found that the crop species had a more significant effect on the microbial population than soil type and crop development stage. The effect of pre-crop is seen when looking at RDA analysis of CLSU but only in June. Barley requires more N from the soil than faba beans and could, therefore, reduce the availability of N to the microbial community. The presence of barley as a pre-crop is correlated with increased levels of ammonium, soil basal respiration

and soil pH. The ammonium and pH could affect the community's structure and the SBR effect indicates a change in function after the barley crop. It is possible that the changes to the microbial community caused by a crop can remain into the next phase of the rotation.

Different approaches to analysis of the same data can provide additional information about the results. Shannon's diversity index is a measure of substrate diversity and encompasses substrate richness and substrate evenness. Two sites could exhibit identical H' but catabolise different substrates (Zak *et al*., 1994). In contrast, a multivariate analysis such as RDA, takes all factors into account to provide a qualitative assessment of the relationships among the factors. The results of the multivariate analysis are therefore complementary to the univariate analysis conducted on the H' values.

The Biolog method gives an indication of the function of the culturable fraction of the bacterial community which has been inoculated onto the plate; however, the patterns of substrate utilisation cannot be related directly to population composition (Widmer *et al*., 2001). As such, this method should be employed in conjunction with molecular analysis. Future publications will report on parallel studies that were conducted using DGGE and qPCR of key functional groups in the same soils.

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