EFFECT OF H_2 ON SOIL BACTERIAL COMMUNITY STRUCTURE AND SOIL DENITRIFIER POPULATION

by

Xiang (Nancy) He

A Thesis Submitted to Saint Mary's University, Halifax, Nova Scotia in Partial Fulfillment of the Requirements for the Degree of Masters of Applied Science

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Examining Committee:

Approved:	Dr. Gefu Wang-Pruski, External Examiner Department of Plant and Animal Sciences Nova Scotia Agricultural College
Approved:	Dr. Zhongmin Dong, Senior Supervisor Department of Biology
Approved:	Dr. Russell Easy, Supervisory Committee Member Department of Biology
Approved:	Dr. Marc Lamoureux, Supervisory Committee Member Department of Chemistry
Approved:	Dr. Genlou Sun, Program Representative
Approved:	Dr. Pawan Lingras, Graduate Studies Representative

ABSTRACT

EFFECT OF H_2 ON SOIL BACTERIAL COMMUNITY STRUCTURE AND SOIL DENITRIFIERS POPULATION

By Xiang (Nancy) He

Previous studies have shown that H_2 from HUP^- legume nodules promotes plant growth and enhances N_2O emission from soil, possibly as the result of hydrogen-induced variation of rhizobacterial community structure. Using terminal restriction fragment length polymorphism and 16S rRNA clone library, the current study revealed that the major bacterial groups that responded to H_2 exposure belong to *Gammaproteobacteria* and *Bacteroidetes*. To understand the relationship between H_2 exposure and soil denitrification, the gene copy number of a key gene in the denitrification process, *nirK*, encoding copper nitrite reductase, was quantified in soil samples with different treatments using a real-time PCR technique. The result showed that the *nirK* gene copy numbers in soil increased significant after H_2 treatment. Several key genes in the denitrification pathway (such as *qnorB, nosZ*) were confirmed in some soil H_2-oxidizing bacterial isolates suggesting a connection between H_2 uptake and N_2O emission.

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LIST OF ABBREVATIONS

A	ampere
DRA	Amplified Ribosomal DNA Restriction Analysis
bp	base pair
BSA	Bovine Serum Albumin
BSF8/20	bacterial universal forward primer (5'-AGAGTTTGATCCTGGCTCAG-3')
BSR534/18	bacterial universal reverse primer (5'-ATTACCGCGGCTGCTGGC-3')
DC	Direct Current
DGGE	Denaturing Gradient Gel Electrophoresis
6-FAM	phosphoramidite fluorochrome 5-carboxyfluorescein
g	gram
HUP	uptake-hydrogenase
M	mol per litre
mA	milliampere
mg	milligram
mM	millimol per liter
PCR	Polymerase Chain Reaction
PGPR	Plant Growth Promotion Rhizobacteria

ppm	parts per million
RDP	Ribosomal Database Project
RE	Restriction Enzyme
RFLP	Restriction Fragment Length Polymorphism
rpm	revolutions per minute
TRF	Terminal Restriction Fragment
T-RFLP	Terminal Restriction Fragment Length Polymorphism
µl	microlitre
µmol	Micromole
µm	Micrometer
v/v	volume : volume

1 GENERAL INTRODUCTION

1.1 CROP ROTATION

About 2000 years ago, old crop rotation practice, "food, feed and fallow" was spread by the Romans throughout the Roman Empire (Harris, 1995). In China, the practice of crop rotation, intercropping and multiple cropping had been recorded in Qin and Han dynasties (221 B.C.-220 A.D.) (Gong *et al.*, 2000). By 1800, many European farmers had developed a four-year rotation system to keep fields always with either food or feed (Nicolini, 2003). Crop rotation is the practice of growing a series of different types of crops in the same area sequentially. For instance, the four-year system rotated wheat, barley, a root crop like turnips, and a nitrogen-fixing crop like clover. By this method, farmers can keep their fields under continuous production with less fertilizer.

Crop rotation has various benefits, such as increasing soil fertility and controlling pests (Roush *et al.*, 1990) and weeds (Doucet *et al.*, 1999) etc. The changing of crops in a sequence tends to decrease the population level of pests by breaking or limiting pest cycles (Finckh and Wolfe, 2006). In addition, rotation can increase the yield. For example, maize in a two-year rotation with soybean yielded about 5 to 20% more than continuous maize (Crookston *et al.* 1991).

In the Green Revolution, the traditional practice of crop rotation fell out of favor in some parts of the world and was replaced by applying the chemical inputs, such as pesticides, herbicides and synthetic nitrogen fertilizer to soil. Agricultural output increased as a result of the revolution. However, the disadvantages of this type of monoculture have become apparent, notably from the perspective of food safety and

environmental impacts, which led farmers to return to more natural practices like crop rotation in decades. In the future, the production of food will be increased possibly through improvement in soil productivity. Proper field management will become an important factor to achieving this objective (Sparks, 2005).

1.1.1 Legume Crops

Legumes, plants of the family Fabaceae, since the dawn of civilization, have been part of human food supply (e.g. soybean, bean, pea, peanuts and chickpea), edible oils (peanuts, soybean) and animal forage (alfalfa, clover) (Boerma *et al.*, 2004). All around world, food and forage legumes are planted on 12-15% of the arable land (about 180 million ha) and offer 33% of the nutritional nitrogen requirements of humans (Food and Agricultural Org. of the United Nations, 2003; Vance *et al.*, 2000).

Legumes also play an important role in crop rotation because of their symbiosis with bacteria that annually fix 40 to 60 million metric tons of atmospheric nitrogen (Smil, 1999). They contain the notable structure, root nodules which form a symbiotic relationship with certain bacteria known as rhizobia (the best-known belong to the genus *Rhizobium*). The enzyme in the bacteria called nitrogenase can catalyze the N-fixation process to convert nitrogen to ammonia. Legume crop can reduce dependence of farmers on expensive synthesis fertilizer and the depletion of nonrenewable resources, and improves soil and water quality (Graham *et al.*, 2003).

1.2 NITROGEN FIXATION – SOURCE OF H_2

Nitrogen (N) is essential for life - it is a component of amino acids which are the building blocks of proteins, nucleic acids which form the genetic material of cells and

enzymes which regulate cell metabolism. Thus, the growth of all organisms requires large amounts of nitrogen. Nearly 80% of the earth's atmosphere in the form of N_2 gas is the only source of new nitrogen for the biosphere. Most organisms are not able to use it until nitrogen is "fixed" in the form of ammonium or nitrate ions.

In agriculture, N is the main limiting nutrient for most crop species. Nitrogen fixation usually refers to the biological process by which dinitrogen (N_2) in the atmosphere is converted into ammonia. The overall formula is $N_2+8H^+ +8e^-+16ATP \rightarrow 2NH_3 + H_2+16ADP +16P_i$. The dinitrogen is normally very unreactive due to the very stable triple bond between two atoms. In industry, a catalyst at elevated pressures and temperatures as in the Haber-Bosch process makes N_2 reactive. By comparison, diazotrophic bacteria finish it at ordinary temperature and pressures under an atmosphere of oxygen. The biological enzyme, responsible for this process is termed nitrogenase.

Hydrogen (H_2) is the obligated byproduct in N-fixation process. An evaluation of the magnitude of energy loss in terms of the efficiency of electron transfer to nitrogen, via nitrogenase, in nodules suggested that H_2 production may severely reduce N_2 fixation in many legumes where photosynthetic supply is a factor limit in fixation. With most symbiosis, about 35% of the electron flow to nitrogenase was transferred to nitrogen (Hunt and Layzell, 1993). In a legume crop fixing 200kg N ha^{-1}, each hectare would produce about 240,000L H_2 (at STP) per season, the production of which costs the plant about 5% of net photosynthesis (Dong *et al.*, 2003).

1.2.1 HUP^+ and HUP^- Nodules

In some legume symbioses, the bacteria also produce an uptake hydrogenase (HUP) that is able to oxidize H_2 to recover the energy loss (Phelps and Wilson, 1941). However, many N_2 fixing legume nodules evolve H_2 due to the absence (HUP^-) or low activity of the uptake hydrogenase. In a HUP^- symbiosis, large amounts of H_2 can diffuse out of the nodule into the soil. For example, at peak growth every hectare of a N_2-fixation soybean field produces about 5000 L H_2 d^{-1}. This H_2 evolution represents an energy equivalent to about 5% of the crop's daily net photosynthetic C gain (Dong and Layzell, 2001). Schubert and Evans (1976) found that the majority of *Rhizobium japonicum* isolates are HUP^-. It has been reported that 75% of 1400 commercial isolates of *R. japonicum* from major soybean production areas in US are HUP^- (Uratsu *et al.*, 1982). And all known clover and alfalfa symbioses are HUP^-.

Albrecht *et al.* (1979) found that soybeans inoculated with *Rhizobium japonicum* strains that synthesized the hydrogenase system fixed significantly more nitrogen and produced greater yields than plants inoculated with strains lacking hydrogen-uptake capacity. Dixon (1972) suggested that hydrogenase may be of benefit to N-fixing bacteria in the following ways: prevention of nitrogenase reductase inhibition by removal of evolved H_2 during N_2 fixation; conservation of energy by combustion of H_2 through oxidative phosphorylation; subsequent removal of O_2 from the vicinity of the O_2-sensitive nitrogenase (La Favre and Focht, 1983). The existence of H_2 uptake capacity seems beneficial since it makes it possible for the symbiosis to recover at least a portion of the energy used for H_2 production (Postgate, 1998). Many attempts have been made to introduce the HUP genes into strains which lack the functional genes. However, the

expected benefits from HUP were not always apparent, especially in field studies and some studies showed negative effects of HUP on yield (Arp, 1992).

1.3 INTERACTION BETWEEN PLANTS AND RHIZOSPHERE MICROBIAL POPULATION

The rhizosphere is the portion of the soil under the direct influence of the roots of higher plants. It is considered the most intense ecological habitat in soil in which microorganisms are in direct contact with plant roots. Biochemical interactions and exchanges of signal molecules between plants and soil microorganisms have been reviewed by Pinton *et al.* (2001) and Werner (2001) (Antoun and Prevost, 2006). On one hand, plants absorb mineral nutrients and water from soil by roots to support growth. On the other hand, they release a lot of organic compounds including sugars, amino and organic acids, fatty acids and sterols, vitamins, etc (Rovira, 1979; Curl and Truelove, 1986). Rhizosphere microorganisms may have specific associations with plants through which they exert their influence on plant growth (Arshad *et al.*, 1998). *Pseudomonas*, *Flavobacterium, Alcaligenes* and *Agrobacterium* species have been shown to be particularly stimulated in the rhizosphere due to the release of exudates and lysates (Alexander, 1977; Curl and Truelove, 1986).

1.3.1 Plant Growth Promoting Rhizobacteria (PGPR)

Rhizobacteria are rhizosphere competent bacteria that aggressively colonize all the ecological niches found on the plant roots at all stages of plant growth, in the presence of a competing microflora (Antoun and Kloepper, 2001). Usually, rhizobacteria either exert a negative effect on plant growth as pathogenic bacteria (Lugtenberg *et al.*,

1991; Persello-Cartieaux *et al.*, 2003) or promote plant growth as plant growth promoting bacteria by plant root activities (Barea *et al.*, 2004). Only about 2 to 5% of rhizobacteria exert the beneficial influence, and some of them invade the tissues of living plants and cause asymptomatic infections (Sturz and Nowak, 2000).

PGPR may induce plant growth promotion by direct and indirect modes of action. Direct mechanisms include the production of stimulatory bacterial volatiles and phytohormones, lowing of the ethylene level in plant, improvement of the plant nutrient status, and stimulation of disease-resistance mechanisms. Indirect effects are for example when PGPR act like biocontrol agents reducing diseases, when they stimulate other beneficial symbioses, or when they protect the plant by degrading xenobiotics in inhibitory contaminated soils (Antoun and Prevost, 2006).

With the increasing concern about environment and climate, low input agricultural practices are focused on the management of soil microbial communities to enhance plant growth. In order to maximize the beneficial effects of microbial activity we need a greater understanding of the microbial diversity and activity (Grayston *et al.*, 1998).

1.3.2 Hydrogen-Oxidizing Bacteria

Despite large rates of H_2 diffusion by nodules, little or no H_2 evolve from the plant-soil ecosystem (Conrad and Seiler, 1979). Popelier *et al.* (1985) found a highly significant positive correlation between the microbial biomass of the soil and soil H_2 uptake rate. Despite numerous attempts, the microorganisms responsible for H_2 oxidation

in soil had yet to be identified and Conrad (1988) even questioned whether the H_2 oxidation was, in fact, biological (H_2-oxidizing bacteria) or chemical (soil enzymes).

Although some authors suggested soil bacteria as H_2-oxidizing soil organisms in the soil, no direct evidence had been found until recently (Haring and Conrad 1994). Autoclaving of soil had been reported to eliminate the H_2 uptake response (Conrad and Seiler 1981), indicating that physical or chemical adsorption of the H_2 to the soil cannot account for H_2 uptake in soil. Fyson and Oaks (1990) attempted to characterize this growth response of legume soil to non-legume crops by treating legume soil with several antimicrobial agents. They determined that the addition of certain fungicides such as benomyl and pentachloroitrobenzene significantly reduced the growth response, while the addition of antibiotics such as streptomycin did little to reduce the growth response. Based on their research, they concluded that the additional plant growth-promoting agent was fungal in nature. However, McLearn and Dong (2002) found in most cases, the addition of fungicides did not significantly affect the H_2 uptake ability of the soil, excluded the fungi as the H_2 uptake microorganisms in soil Addition of antibiotics affected the H_2 uptake ability of the soil suggesting the agent in soil is bacterial.

The groups of bacteria which are able to use molecular hydrogen as an electron donor in the energy-yielding process are termed as hydrogen-oxidizing bacteria. The key enzyme is a membrane-bound Ni/Fe-containing uptake hydrogenase. A few species also contain a cytoplasmic hydrogenase (Fridrich and Schwartz, 1993).

H_2-oxidizing bacteria can promote plant growth, so they're a kind of plant growth promotion Rhizobacteria (PGPR). Different from *rhizobia*, PGPR are beneficial free-live

soil bacteria which stimulate plant growth without developing symbiotic associations with plant roots (Bashan and Holguin 1998). They can survive without the supports of root exudates, but have the ability of efficiently utilize organic compounds released by roots in competition with other rhizosphere microbes (Tilak *et al.* 2005; Kloepper *et al.* 1991; Kloepper 1994). Maimaiti *et al.* (2007) presented that 17 H_2-oxidizing bacteria isolates enhanced (57-254%) roots elongation of spring wheat seedling. Using an *Arabidopsis thaliana* bioassay, plant biomass was increased by 11-27% when inoculated by one of four isolates of *V. paradoxus* or one isolate of *Burkholderia* that were selected for evaluation.

1.4 SOIL MICROORGANISMS

Soil microbes are successful in getting established in the soil ecosystem due to their high adaptability in a wide variety of environments, their faster growth rate and their biochemical versatility to metabolize a variety of natural and xenobiotic chemicals (Narasimhan *et al.*, 2003). Although microorganisms are perhaps the most diverse and abundant type of organism on Earth, the distribution of microbial diversity is poorly understood (Fierer and Jackson, 2006). Scientific understanding of microbial biogeography is particularly weak for soil bacteria, even though the diversity and composition of soil bacterial communities is thought to have a direct influence on a wide range of ecosystem processes (Fierer and Jackson, 2006). Most of the trace gas production and consumption processes in soil are probably due to microorganisms (Conrad, 1996).

Soil microorganisms are composed of the bacteria, archaea, fungi and protozoa (Conrad, 1996). Traditional soil microbiology has relied on the extraction of microorganisms from soils and their isolation in laboratory media for identification and enumeration (Foster, 1988). Eubacteria are prokaryotic microorganisms. They are the dominant group of microorganisms among the various kinds of soil.

1.4.1 Soil Bacterial Community

Much of the work in soil microbial ecology has focused on cataloging the diversity of soil bacteria and documenting how soil bacterial communities are affected by specific environmental changes or disturbances. As a result, we know that soil bacterial diversity is immense, and that the composition and diversity of soil bacterial communities can be influenced by a wide range of biotic and abiotic factors.

Investigation of soil bacterial is difficult due to community' size (typically 10^9-10^{12} bacterial cells per gram) (Whipps, 1990) and magnitude of species-level diversity. Based on DNA re-association kinetics, the estimated number of distinct genomes present in a gram of soil ranges from 2,000 to 18,000 (Dunbar *et al.*, 2002). In the past, diversity of bacterial communities was determined by phenotypic characterization of isolates, but most (99.5 to 99.9%) of the soil bacteria cannot be cultured on media (Torsvik *et al.*, 1990). Therefore, the taxonomy of bacterial domains is based on these less than 0.5% of known bacteria and our knowledge about them is very scant.

1.5 ASSESSMENT OF SOIL BACTERIA COMMUNITY STRUCTURE

The application of molecular biological methods to study the diversity and ecology of microorganisms in environments has been practiced since the mid-1980s (Head *et al.*, 1998). Lots of new insights about the composition of microbial communities have been gained since that time. Molecular techniques to characterize microbial ecology in many environments are achievable now. These techniques include fingerprinting approaches where either the whole community is characterized or clone libraries are generated from a single sample and individual clones are sequenced.

1.5.1 DNA Fingerprinting Approach

The microbial fingerprint is a drastically simplified representation of the microbial community, just like a DNA barcode. Fingerprinting is useful for readily monitoring changes in microbial communities. To understand at a community level the spatial and temporal variability of microbial community structure and function, it needs a rapid, simultaneous and reproducible analysis of multiple samples is required. Several molecular fingerprinting techniques are available for assessing the abundance or diversity of bacterial communities in ecosystems, such as fluorescence in situ hybridization (FISH) (Schramm *et al.*, 1999), immunofluorescence probing (Hastings *et al.*, 1998), single-strand conformation polymorphism (SSCP) (Lee *et al.*, 1993) and denaturing or temperature gradient gel electrophoresis (DGGE or TGGE) (Kowalchuk and Stephen, 2001).

FISH with rRNA-targeted oligonucleotide probes is a reliable tool for the direct identification and quantification of bacteria in their natural environment (Schramm *et al.*, 1999). A fluorescently labeled probe hybridizes specifically to its complementary target sequence within the intact cell (Moter and Gobel, 2000). SSCP analysis is based on the fact that a single-base modification can change the confirmation of single-strand DNA molecules leading to a different electrophoretic mobility in a non-denaturing gel (DGGE) (Lee *et al.*, 1996). DGGE or TGGE is a method by which fragments of DNA of the same length but different sequence can be resolved electrophoretically according to their melting properties. During electrophoresis the DNA fragment remains double-stranded until it reaches the conditions causing melting of the lower temperature melting domains.

1.5.1.1 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

T-RFLP is one of the most popular fingerprinting methods because of its relative simplicity. It has been widely used in recent years for the analysis of bacterial communities in different conditions (Moeseneder *et al.* 1999; Osborn *et al.*, 2000; Richardson *et al.*, 2002; Fierer *et al.* 2003) While it shares problems inherent to any PCR-based method, when coupled with clone library construction and clone sequencing, additional specific information on the composition of microbial communities can be obtained.

T-RFLP was firstly introduced by Liu *et al.* in 1997. The mechanism of the method is: first of all, amplify a selected region of the gene of interest with labeled primers at the 5' end by employing PCR; the PCR product is digested with restriction

enzymes, and the fluorescently labeled terminal restriction fragments are measured by using an automated DNA sequencer.

The same TRF length is predicted for various species of bacteria, but increased specificity can result from analysis of digests with multiple enzymes.

1.5.1.2 16S Ribosomal RNA (rRNA) Gene (rDNA) as an Intrinsic Marker

The most widely applied and significant marker genes in environmental microbiology to date are genes encoding for the RNA of the small subunit of ribosome (Head *et al.*, 1998). The ribosome, the protein manufacturing machinery of all living cells, is a complexe of RNA and protein. Ribosomal RNA (rRNA) is the central component of the ribosome, which provides a mechanism for decoding mRNA into amino acids and to interact with the tRNA during translation. Ribosomal RNA is the most conserved (least variable) gene in cells (Woese, 1987). For this reason, genes that encode the rRNA are sequenced to identify an organism's taxonomic group and calculate related groups.

The ribosome is composed of two subunits, named for how rapidly they sediment when subjected to centrifugation. The sizes of two subunits in prokaryotes and eukaryotes are different. The prokaryotic ribosome can be broken down into 50S and 30S subunits (the S represents Svedberg units). The small 30S subunits contain the 16S rRNA.

1.5.1.3 Preparation before analysis

Differences in the length and abundance of fluorescently labeled TRFs in a sample are usually determined by capillary or polyacrylamide gel electrophoresis wherein

the electrophoretic mobility of the TRFs are compared to those of known size in an internal standard. The actual sizes of TRFs are estimated by interpolation using algorithms. The abundance of each TRFs is determined based on fluorescence intensity and expresses as either peak height or peak area. Run-to-run variability (generally $\pm$1bp) always happens and results in small size discrepancies even among TRFs of the same bacterial populations and therefore fingerprints need to be aligned. Accurate fragment size determination is important because it affects the analysis result directly.

As a first step in the analysis of T-RFLP profiles, the signal has to be distinguished from electronic noise. In other words, the baseline has to be determined. And the true baseline must be determined by the researcher. Several approaches to define baselines have been developed including fixed threshold (Lueders and Friedrich 2003), proportional threshold (Osborne *et al.* 2006). These methods have advantages and disadvantages. Applications should be concerned according to different situations (Schutte *et al.*, 2008).

1.5.2 Analysis of PCR Product -- Clone Library and Sequencing

The pool of mixed PCR products generated from community DNA can be analyzed by cloning of amplified fragments followed by sequence analysis of the resulting clone library. Each clone within a library contains a 16S rDNA sequence amplified from a 16S rRNA gene in one bacterial genome. From the sequence data, for a clone library generated from DNA extracted from an environmental sample, inferences can be made on the composition and phylogenetic diversity of the microbial community within the sample (Parekh and Bardgen, 2002). Due to the increasing of efficiency and

accuracy and a decreasing of the cost, clone library construction and sequencing are widely used to study nonculturable microbe.

1.5.2.1 The Ribosomal Database Project (RDP-II)

RDP is a database that offers ribosome-related data, analysis services and associated computer programs. Until September 2008 (Release 10.3), the RDP maintained 643916 aligned and annotated public bacterial small subunit rRNA sequences. The collection is updated monthly from the International Nucleotide Sequence Database Collaboration (INSDC; DDBJ, EMBL and GenBank). New sequences are automatically aligned with the Infernal secondary structure based aligner, which is a stochastic context-free grammar based and provide a high-quality secondary structure aware alignment (Cole, 2009).

Though Genbank is the biggest repository of sequences, it's not easy to blast and identify sequences against multiple entries. Most of the time, you will get several entries which have the same similarity (>95%) with yours or just 'uncultured bacteria'. And RDP is a database that focuses on rRNA genes providing researchers with quality-controlled bacterial and archaeal small subunit rRNA alignments and analyze tool. Thus, the RDP is the ideal tool to analyze sequences from clone libraries.

1.6 OBJECTIVES

The objectives of my research are effects H_2 emission on soil bacteria community structure, in particular: 1) on overall soil bacterial community and 2) on populations of denitrifiers.

It is known that some changes happened in H_2-treated soil, such as the increase of biomass, soil moisture compared with air-treated soil. Growth enhancement of non-leguminous plants after crop rotation with legume crops was observed. Additionally, some H_2-oxidizing bacteria have been isolated from H_2-treated soil. These isolates can enhance root elongation of spring wheat seedlings (Maimaiti *et al.*, 2007). The bacterial community structure differences have been shown by T-RFLP between H_2- and air-treated soils, and soils adjacent to nodules inoculated with HUP^+ and HUP^- rhizobial strains (Zhang, 2006). The aim of the present study is to obtain more information about differences by combining results of T-RFLP and clone library.

It is found that when H_2 released from legume nodules, N_2O emission from soil increase not only during planting legumes, but also in subsequent year with non-leguminous plants (unpublished). The experiment done by Queen's University has shown that production of N_2O increased dramatically in H_2-treated soil. There are two assumptions to explain this observation: the increasing population of bacteria which are capable to produce N_2O and upregulation of the genes which charge for the N_2O production. However, the increase of N_2O emission doesn't happen immediately when the soil treated by H_2, which suggests the former explanation makes more sense, because it takes more time to grow and reproduce the offspring than to upregulate the genes in the existed individuals. The main process of N_2O production in soil is denitrification by denitrifier. We selected one key gene, nitrite reductase *nirK* in the process ($NO_2^- \rightarrow NO$) to investigate the abundance of denitrifying bacteria in different soil samples by quantification of the gene copies using real-time PCR technique.

2 PHYLOGENETIC ANALYSIS OF T-RFLP PROFILES BY PHYLOGENETIC ASSIGNMENT TOOL (PAT)

2.1 INTRODUCTION

2.1.1 Phylogenetic Assignment Tool (PAT)

Culture-independent DNA fingerprints such as T-RFLP are used to examine the diversity of a microbial community. They can offer rapid comparison of species richness among communities or different treatments. However, these methods require a trade-off between phylogenetic resolution and sample throughput (Kent *et al.*, 2003). The composition of the community is not assessed directly from profiles produced by fingerprint methods. Clone libraries provide the highest degree of phylogenetic resolution, but could be cumbersome for analysis of complex communities that was generated by studies of temporal or spatial variability of the community. Moreover, clone libraries composed of limited number of sequences only covers partial communities.

Web-based resources, such as RDP and Microbial community analysis (MiCA) allow prediction of TRFs from 16S rRNA gene sequences presently in the database based on user input of PCR primers and restriction enzymes. Users are able to compare fragments obtained from T-RFLP analysis to the fragment sizes predicted from known 16S rRNA gene sequences. This comparison is accomplished by manually scanning the predicted fragment sizes to find a subset of species that produce fragment sizes similar to one obtained experimentally. A species list can then be refined by comparison with additional digests. This is a reasonable procedure to carry out for uncomplicated profiles,

like individual unknown species or mixtures of very few species. However, such assignments are considerably more difficult in complex communities when each individual peak from each digest has the potential to represent multiple species. Phylogenetic assignment for complex community profiles involves finding the intersection of the species sets represented by each peak. This is a daunting task when an individual TRF may correspond to 15 or more species (Kent *et al.* 2003).

Using the phylogenetic assignment tool (PAT), the task of phylogenetic assignment from TRF profiles generated by multiple digests is done automatically, which makes this type of analysis accessible for analysis of complex communities.

T-RFLP has demonstrated its utility as a community fingerprint method for comparisons of bacterial communities' composition between environments or treatments. The phylogenetic assignment tool extends this utility by offering a rapid, automated approach for phylogenetic analysis of TRFs.

2.2 MATERIAL AND METHODS

All the bench work and data set collection were done by Zhang Ye in 2006. Here, I just review the whole project briefly.

To assess variation of soil bacterial community structure resulting from the metabolism of hydrogen gas, different soil samples from laboratory, green house and filed were compared by T-RFLP. The TRFs profiles include the size and area of TRFs. By comparison of T-RFLP profiles between soil adjacent to HUP^+ and HUP^- nodules, between H_2-treated and air-treated soil, between soil with and without plants, it was confirmed that H_2 induced soil bacterial community structure changes. Top 5 increased

TRFs for each restriction enzyme between soil adjacent to HUP^+ and HUP^- nodules and between air-treated and H_2-treated soil were obtained by Zhang Y. (Zhang, 2006). However, nothing is known what these increased or decreased peaks represent. PAT is used to shed light on the problem.

The standard data sets (sizes and areas) generated by three restriction enzymes (*Bst*UI, *Msp*I and *Hae*III) from all samples were compiled into 3 new Microsoft Excel files (separately by different enzymes). It was noticed that there is no *Hinf*I information in default database of PAT. Thus, we have to give up this enzyme in PAT analysis. Although the excel files contained 6 columns, the PAT only used information from columns 1, 3 and 5 in its analysis. They included the following information: sample and fragment ID (column 1), size (bp) (column 3) and peak area (column 5) were used in PAT analysis. Excel files were transferred into tab-delimited formatted text files and uploaded to the PAT website: https://secure.limnology.wisc.edu/trflp/. Following the step-by-step instruction, the data were analyzed automatically. The result was downloaded and imported in an excel worksheet.

The phylogenetic assignments were output at different taxonomic levels, with some are classified into genus, and other identified by family. The taxonomic groups were categorized into uniform taxonomic level, class or phylum and sorted by abundance.

2.3 RESULTS

After comparison with TRFs profiles of the groups, HUP^+ and HUP^- nodules soil communities; air- and H_2-treated soil communities, differences of TRFs intensity were acquired. All the increased and decreased TRFs and their percentage in total area were

listed in Zhang Y.'s thesis (2006).

The normalized data sets by Zhang Y. were used to do phylogenetic analysis using PAT. The obtained results from web-based tool were organized first. All the identified individuals were sorted by phylum and class, and then the abundance of each class and phylum were compared. The result of analysis (Table 1) showed that the dominant increased peaks represented *Firmicutes* and *Gammaproteobacteria* in the Phylum *Proteobacteria* in greenhouse soil samples, *Gammaproteobacteria* and *Betaproteobacteria* in lab treatment soils according to *Msp*I data sets. The data sets of the other two restriction enzymes, *Hae*III and *Bst*UI shared the same results. The decreased peaks appeared to be *Actinobacteria*, *Firmicutes* and *Alphaproteobacteria* in Phylum *Proteobacteria*.

2.4 DISCUSSION

Although PAT analysis extended the utility of T-RFLP as a powerful microbial community study method, it becomes more complicated when increasing the samples number or complexity of communities. Multiple restriction enzymes can increase the .specifity. However, the default database provided by PAT doesn't include a lot of enzymes. Thus, if PAT is the potential tool to analysis your T-RFLP data, which enzymes you plan to use for digestion should be concerned before starting. In addition, due to capillary electrophoresis, the obtained TRFs profiles may shift compared with real TRFs. The mechanism for PAT is compared the users' TRFs to fragment sizes predicted from known 16S rRNA gene sequences. It will make definitive prediction problematic. Thus, large clone libraries combined with T-RFLP are an effective way to confirm the result.

Table 1: PAT analysis result of top 5 increased TRFs between air- and H_2-treated soil, soil adjacent to HUP^+ and HUP^- nodules from Zhang Ye's T-RFLP data sets.

RE	Sample	TRFs(bp)	Differences	% differences	Represent	Dominant groups
*Mps*I	HUP^--HUP^+	62.9	1.75	10.1	N/A	*Gammaproteobacteria, Firmicutes*
		453.1	1.5	8.64	*Gammaproteobacteria, Firmicutes*	
		454.1	1.54	8.87	*Gammaproteobacteria, Firmicutes*	
		467.3	2.3	13.2	N/A	
		491.5	1.35	7.77	*Gamma-* and *Betaproteobacteria*	
	H_2-air	89.3	7.3	17.7	*Gamma-* and *Betaproteobacteria*	*Gamma- and Betaproteobacteria*
		143.2	5	12.1	*Gamma-* and *Betaproteobacteria*	
		153.9	2.7	6.53	*Gamma-* and *Betaproteobacteria*	
		184.1	7.5	18.1	*Firmicutes*	
		453.1	2.91	7.04	*Gammaproteobacteria, Firmicutes*	
*BstU*I	HUP^--HUP^+	109.5	0.97	6.75	*Bacteroidetes, Gamma-* and *Deltaproteobacteria*	*Firmicutes, Gamma- and Betaproteobacteria*
		198.6	1.5	10.4	*Alphaproteobacteria*	
		383.5	3.68	25.6	*Firmicutes, Gamma-* and *Betaproteobacteria*	
		385.2	1.05	7.3	*Firmicutes, Gamma-* and *Betaproteobacteria*	
		391.9	2.35	16.3	*Firmicutes, Gamma-* and *Betaproteobacteria*	
	H_2-air	280.7	1.92	4.87	*Actinobacteria*	*Firmicutes, Gamma- and Betaproteobacteria*
		369	9.76	24.7	*Firmicutes*	
		388.2	3.2	8.11	*Gamma-* and *Betaproteobacteria*	
		391.9	11.1	28.1	*Gamma-* and *Betaproteobacteria, Firmicutes*	
		460.1	1.7	4.31	*Gammaproteobacteria*	
*Hae*III	HUP^--HUP^+	191.3	1.42	6.73	*Alpha- and Gammaprotelbacteria*	*Gamma- and Betaproteobacteria*
		196	1.57	7.44	*Gamma-* and *Alphaproteobacteria*	
		209.5	3.34	16.3	*Gamma-* and *Betaproteobacteria*	
		218.6	2.48	11.7	*Firmicutes, Betaproteobacteria*	
		230.2	2.1	9.95	*Firmicutes, Actinobacteria*	
	H_2-air	167.6	7.33	21.9	*Bacteroidetes*	*Gammaproteobacteria, Firmicutes*
		201.6	5.2	15.57	*Beta-* and *Gammaproteobacteria, Firmicutes*	
		209.5	3.97	11.9	*Gammaproteobacteria, Firmicutes*	
		259.9	7.49	23.8	*Beta-* and *Gammaproteobacteria, Firmicutes*	
		320	2.43	7.28	*Frimicutes, Gammaproteobacteria*	

3 Effect of Hydrogen on soil bacterial community structure

3.1 INTRODUCTION

Hydrogen is a good growth substrate for a large diversity of bacteria. For example, H_2-oxidizing bacteria grow with H_2, O_2 and CO_2 as sole energy and carbon substrates (Aragno and Schlegel, 1992). However, it occurs at higher concentrations of hydrogen than those in the ambient atmosphere. Thus, H_2-oxidizing bacteria are able to scavenge H_2 whenever it reaches high concentrations, e.g. at N-fixing root nodules with *Bacteroides* lacking uptake hydrogenase activity (HUP^-).

Since the HUP^- nodules release hydrogen gas to rhizosphere, hydrogen-oxidizing bacteria should be found near rhizosphere. Indeed, La Favre *et al.* (1983) showed that the H_2 uptake rates and microbial mass decreased with distance from the nodules as H_2 uptake rates. Stein *et al.* (2005) showed that when soil was treated with H_2, CO_2 fixation increased corresponded with an increase in microbial activity and biomass. Using FISH analysis and DNA staining, they found that bacterial community structure changed. H_2 treatment stimulated β -and γ -subclasses of *Proteobacteria* and *Cytophaga-Flavobacterium-Bacteroides* phylum.

In our lab, three genera of H_2-oxidizing bacteria have been isolated from H_2-treated soil or soil adjacent to HUP^- nodules. And some of them have been tested for capability of promoting plant growth. The results showed that they indeed can promote the plant growth (Maimaiti *et al.*, 2007).

To better understand the effect of hydrogen on rhizobacteria communities, more effective methods should be utilized to analyze the variation of the community structure induced by hydrogen metabolism. Methodology such as fingerprinting technique and clone library has been referred above. Some former work has been done by combination of two methods (Liu *et al.*, 1997; Moeseneder *et al.*, 2001; Hayashi *et al.*, 2003). T-RFLP is a power tool to rapidly monitor the change of the microbial community. And clone library can collect more details of the each individual in a sample. Two methods can be supplementary to each other in the microbial communities analysis.

3.2 MATERIAL AND METHODS

3.2.1 Preparation of Soil

Sandy loam soil collected from the Plumdale Facility, NSAC (Nova Scotia Agricultural College) Experimental Farm, Truro, Nova Scotia one year ago, was mixed with fine sand (2:1 v/v). The mixed soil was filled into twelve 6inches-diametric pots and four 60ml syringes.

3.2.2 Treatment of Soil

We had 4 groups of treatment in greenhouse, each one contains three replicates. Seeds of soybeans (Glycine max, cv. RR Drako) and barleys were surface sterilized with bleach and 70% ethanol. Then the seeds were planted in the tray with promix for germination. Soybean seedlings were inoculated with JM47 (HUP^-) (derived from the parent strain USDA DES 110 with a Tn5 mutation in the 33-kDa subunit of the hydrogenase gene) (Hom *et al.*, 1988; Kim and Maier, 1990), JM (HUP^+) (derived from USDA 110 and

expresses hydrogenase activity under derepression conditions in the presence of Ni) (Graham *et al.*, 1984; Stults *et al.*, 1984; Fu and Mairer, 1991). The aim for this is to form the HUP^- and HUP^+ nodules. Non-inoculated soybean plants were used as control group. The barleys and soybean seedlings were transplanted into separated pots in greenhouse.

Lab treated soils were separated evenly into 2 groups labeled as H and A, and then treated by the same flow rate of H_2 in air (1000ppm) and air in lab (Fig. 2). The uptake of H_2 and CO_2 were monitored every day by Bryan Flynn.

3.2.3 Measurement of Soil H_2 Uptake Rates

The hydrogen uptake capacity of each soil sample was calculated by the difference of the hydrogen concentrations before and after through soil samples, which were measured by a H_2 sensor (Model S121, Quibit Systems Inc) using the Data Logger program as described by Dong and Layzell (2001). The hydrogen sensor is a ceramic tube equipped semi-conductor device. The sensor and a 10K resistor are the main components of a circuit supplied with a five voltage DC. The resistance of the semiconductor changes with the concentration of H_2 which varies with its combustion with O_2 in the gas flux. The voltage on the 10K resistor changes with the variation of the resistance of the semi-conductor and was recorded by the Data Logger Program as the computer analysis system (Dong and Layzell, 2001) (Fig. 3). Thus the concentration of hydrogen in the gas flux can be calculated by comparing the voltage value monitored by the program with a standard curve of H_2 concentration versus voltage on the 10K resistor

Figure 1: Flow chart for investigation of soil bacterial community structure by combination of T-RFLP and clone library methods.

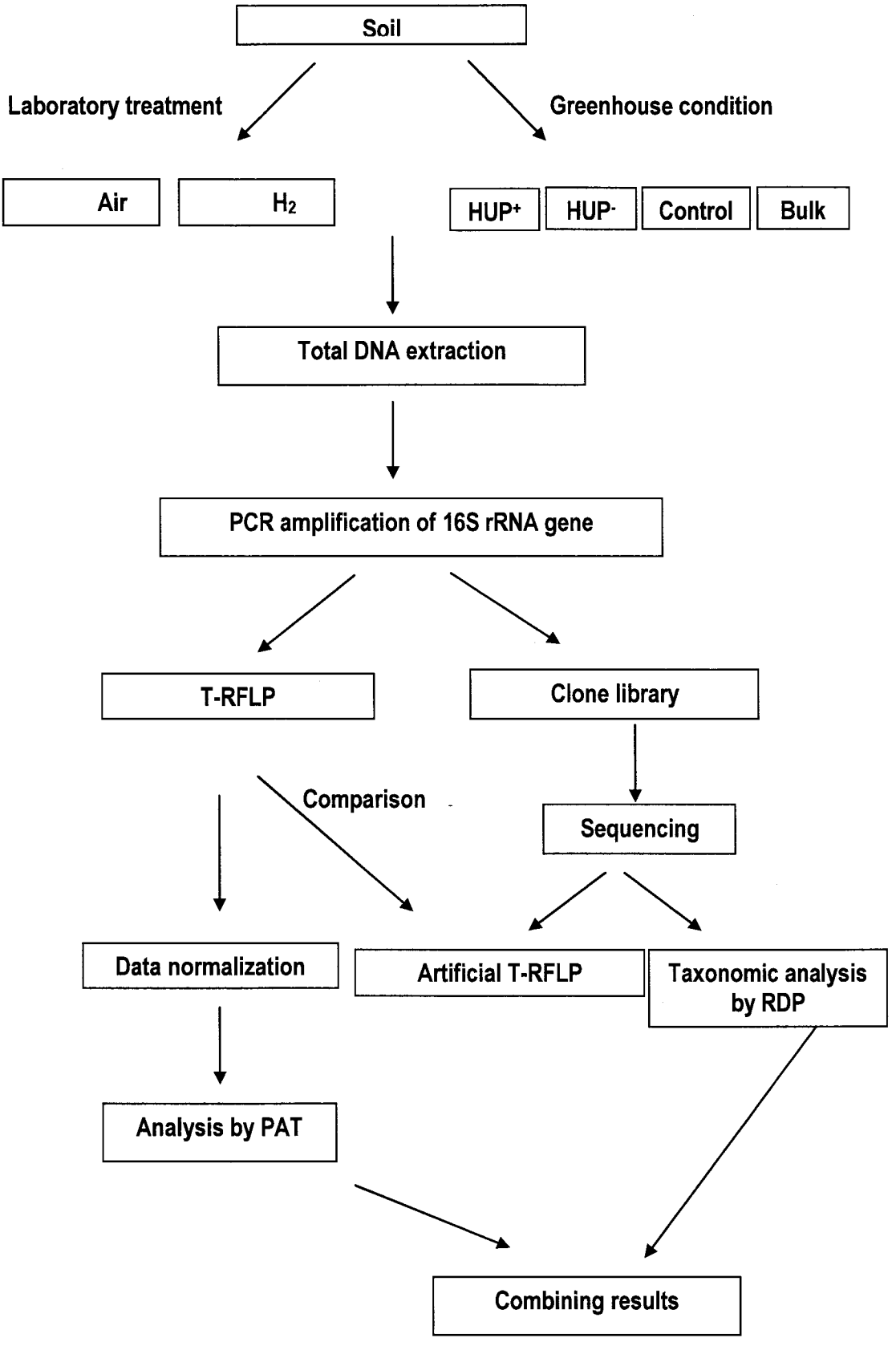

Soil
Laboratory treatment
Greenhouse condition
Air
H_2
HUP+
HUP-
Control
Bulk
Total DNA extraction
PCR amplification of 16S rRNA gene
T-RFLP
Clone library
Comparison
Sequencing
Data normalization
Artificial T-RFLP
Taxonomic analysis by RDP
Analysis by PAT
Combining results

Figure 2: A simplified diagram of the H_2 treatment system (Dong and Layzell, 2001).

The hydrogen gas is generated by the first flask being equipped with a regulated power supply to provide a direct electric current. The second flask acts as a control (air treatment). Air is provided at a stable rate to both flasks. For hydrogen gas treatment, the hydrogen enriched gas stream (V1) was connected with the soil column before venting to the atmosphere at (V2). For air treatment, the air (V3) was connected with the soil column before venting to the atmosphere (V4).

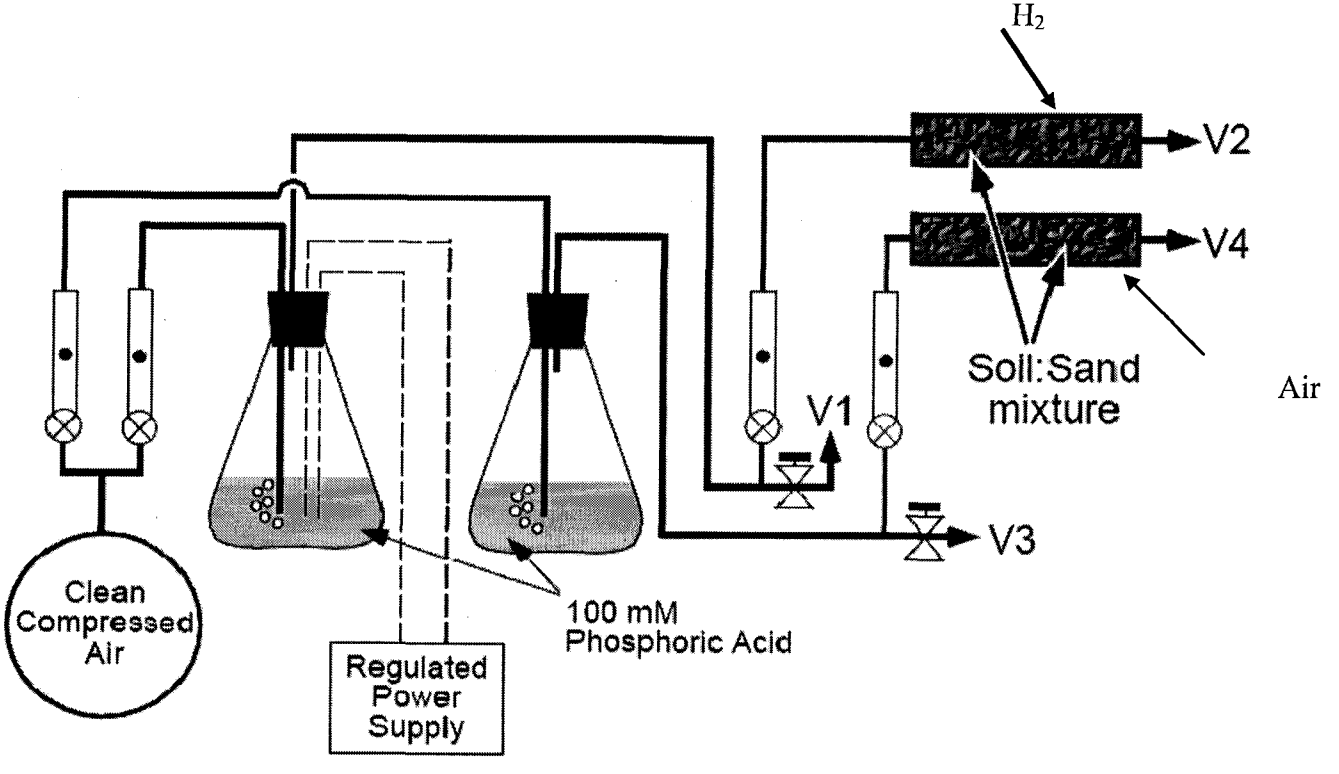
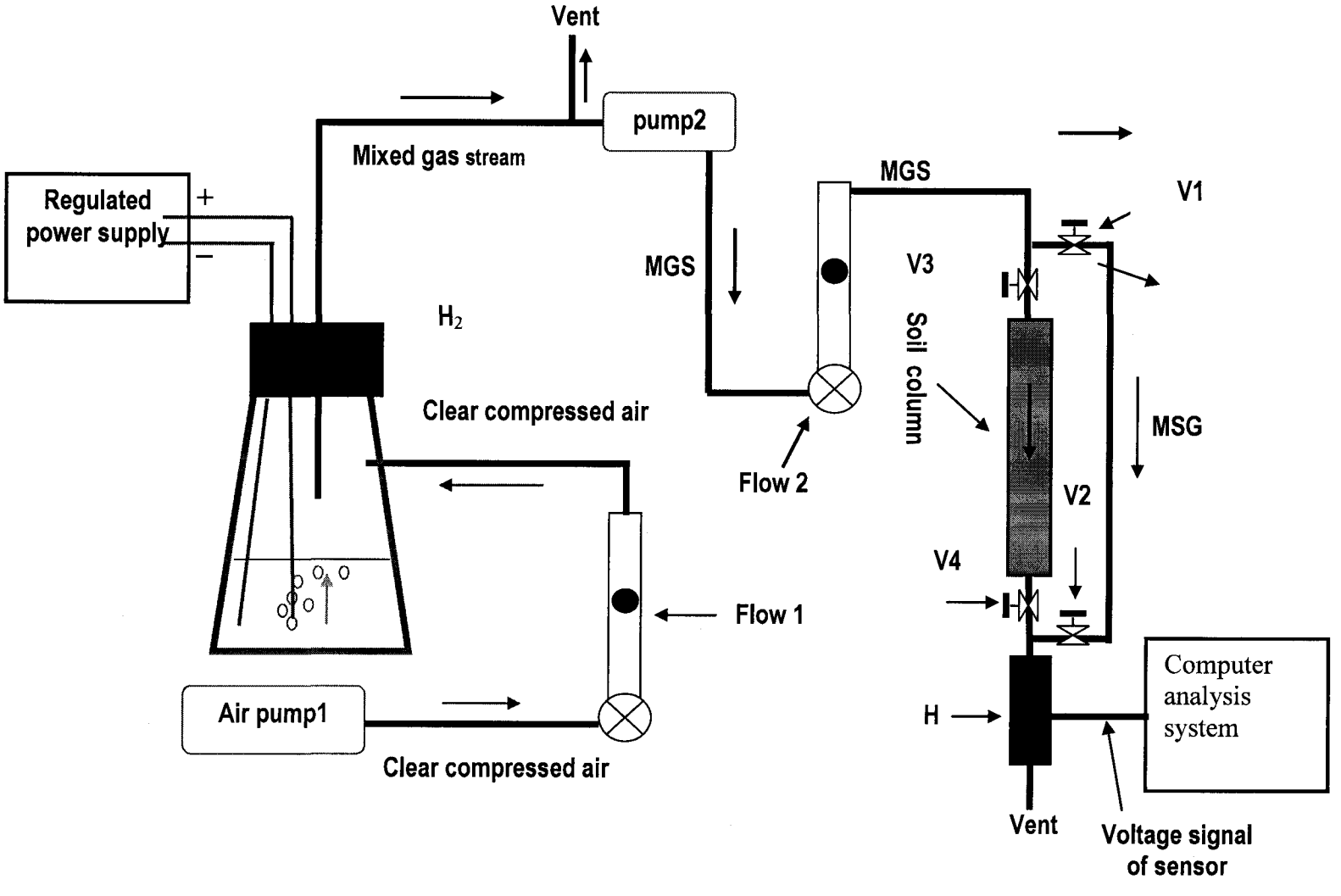

H_2
V2
V4
Soil:Sand mixture
Air
V1
V3
Clean Compressed Air
100 mM Phosphoric Acid
Regulated Power Supply

Figure 3: A simplified diagram of the hydrogen uptake capacity measurement system (He, 2008).

The hydrogen gas is generated in the flask equipped with a regulated power supply to provide a direct electric current. Air is provided at a stable flow rate by both pumps and combined with hydrogen gas to make a mixed gas stream before passing the soil column or hydrogen sensor. Valves 1, 2, 3 and 4 are operated to make the sensor determine the concentration of hydrogen in the mixed gas stream before and after passing the soil column. MGS=mixed gas stream.


Vent
pump2
Mixed gas stream
Regulated power supply
+
−
MGS
MGS
V1
V3
Soil column
H_2
Clear compressed air
Flow 2
MSG
V2
V4
Flow 1
Air pump1
Clear compressed air
H
Computer analysis system
Vent
Voltage signal of sensor

3.2.3.1 Standard Curve of Voltage vs. Hydrogen Concentration (ppm)

The amount of electrolytic hydrogen (Z: μmol/min) in the flask (Figure 2) was calculated using the following equation:

Z (μmol/min) = (3.00*104*C *Cu) / Av----------1

C (Coulomb Constant): 6.24*1018 ($A^{-}1$);

Cu (current of electrolysis): mA;

Av (Avogadro Constant): 6.02 *1023 ($mol^{-}1$).

From Equation 1, the following equation was derived to calculate the concentration of electrolytic hydrogen in the mixed gas stream (H: ppm):

H (ppm) = [1.00*103*Z *GC*(273.15^{+}T)]/(273.15*FR1)----------2

Z (amount of electrolytic hydrogen per minute): μmol/min;

GC (gas constant): 22.41 L/mol at 0 °C and 1 atmosphere pressure;

T (temperature): °C; FR1 (Flow Rate One): ml/min.

A series of mixed gas streams with gradient hydrogen concentration (from 0.55ppm to 147ppm) were generated by regulating the current of electrolysis and Flow Rate One. Then V1 and V2 were opened and V3 and V4 were closed to allow the mixed gas stream to pass the hydrogen sensor directly. Finally, voltage across the 10K resistor in hydrogen sensor was recorded by the computer analysis system when the mixed gas stream with known hydrogen concentration passed the hydrogen sensor (Fig. 3). Based

on Matlab, a standard curve of voltage versus hydrogen concentration (ppm) was fitted as an exponential function: ppm (H_2) = a*e $^{(b*v)}$ [v: voltage, e=2.718282].

3.2.3.2 Measurement of Samples

Firstly, the concentration of electrolytic hydrogen in the mixed gas flux before passing through the soil column ([H]in: v) was determined by passing the mixed gas stream by the hydrogen sensor directly (turning on V1 & V2 and turning off V3 & V4). Then, the concentration of electrolytic hydrogen in the mixed gas stream after passing the soil column ([H]out: v) was measured when V3 & V4 were turned on and V1 & V2 were closed (Fig. 3)

3.2.4 Collection of Soil Samples

Six different soil samples came from lab treatment and greenhouse. The soil lump in the pots was opened very carefully. The soil layer adjacent to nodules within 5mm was collected into 1.5ml sterile microcentrifuge tubes.

After one month, samples of laboratory treatment were sub packed into 2.0ml sterile microcentrifuge tubes and then frozen in liquid nitrogen immediately, stored at -80°C until use

3.2.5 Extraction of Soil Total DNA

The total DNA of 0.5 gram soil for each sample was extracted by using FastDNA® SPIN kit for soil following the protocols: 0.5 gram of soil sample was added to a Lysing Matrix E tube with 978µl Sodium Phosphate Buffer and 122µl MT Buffer;

After vortexing for 1 minute at highest speed; the tube was centrifuged at 14000×g for 13 minutes to pellet debris. Supernatant was transferred to a clean 2.0ml microcentrifuge tube with 250µl PPS (Protein Precipitation Solution) and mixed by shaking the tube by hand 10 times; after centrifuge at 14000×g for 5 minutes, supernatant was removed to a clean 15ml tube. Binding Matrix suspension was resuspended and 1ml was added to supernatant in 15ml tube; after inverting by hand for 2 minutes to allow binding of DNA then tube was placed in a rack for 3 minutes to allow settling of silica matrix. 500µl of supernatant was removed and discarded carefully to avoid settled Binding Matrix. Binding Matrix in the remaining amount of supernatant was resuspended, approximately 600µl of the mixture was transferred to a SPIN™ Filter and centrifuged at 14000×g for 1 minute; the catch tube was emptied and filled with the remaining mixture to the SPIN™ After filtration and centrifuge as before, the catch tube was emptied again. 500µl prepared SEWS-M was added and the pellet was gently resuspended using the force of the liquid from the pipette tip. After centrifuge at 14000×g for 1 minute, the catch tube was emptied and replaced back; Without any addition of liquid, the tube was centrifuged a second time at 14000 for 2 minutes to dry the matrix of residual wash solution. The catch tube was discarded and replaced with a new, clean catch tube and air dry the SPIN™ Filter for 5 minutes at room temperature. Binding Matrix was gently resuspended in 80µl of DES (DNase/Pyrogen-Free Water) and incubated for 5 minutes at 55°C in a water bath to increase yield; the centrifuge at 14000×g for 1 minute brought eluted DNA into the clean catch tube, SPIN filter was discarded and DNA was stored at -20°C until use.

3.2.6 Amplification of 16S rRNA Gene Fragments

Partial 16S rRNA gene (527bp) from all soil samples were amplified with a pair of bacterial universal primers: BSF8/20(5'-AGAGTTTGATCCTGGCTCAG-3') and BSR534/18 (5'-ATTACCGCGGCTGCTGGC-3'). The difference of PCR between T-RFLP and clone library is the forward primer for T-RFLP was labeled with fluorescence (6-FAM).

3.2.6.1 PCR Condition

Amplification was carried out with a Thermal Cycler (Applied Biosystem, Foster City, CA).

The reaction mixture of 25µl contained: 0.8µM of each primer for 16S rRNA, 2.5µl of 10× Buffer, 2.5µl of 2mM dNTP, 2µl of $MgSO_4$, 0.2 µl of 5U/ µl Taq enzyme (UBI Life Sciences Ltd, SK, Canada), 0.5µl of DNA template corresponding to 15 to 20 ng of total DNA, and Sigma® water to complete the 25µl volume.

The conditions for PCR were 3 minutes at 95°C for denaturation, 40 cycles of 30s at 95°C, 45s at 55°C for annealing, 45s at 72°C for extension, and a final cycle, 10 minutes at 72°C. Multiple PCR reactions were pooled together to minimize PCR random bias. PCR products were purified with the QIAquick® PCR purification kit (QUAGEN Inc. Mississauga, ON).

3.2.7 Generation of TRF Profiles

Four restriction endonucleases, *Bst*UI, *Hinf*I *Hae*III and *Msp*I (New England Biolabs, Ltd., Pickering, On, CA) were used to obtain four separate TRF profiles for each

sample. The apostrophe shows the cleavage site: *Bst*UI (CG'CG), *Hae*III (GG'CC), *Hinf*I (G'ANTC), and *Msp*I (C'CGG)

The digestion reaction of 50μl contained: 5μl 10×2# Buffer, 10μl purified PCR products, 20U one of 4 restriction enzymes, and water to complete the 50μl volume. All the reactions were performed at 37°C in the water bath for 4 hours except *Bst*UI ones, which were incubated at 60℃.After digestion, the products were purified with QIAquick® nucleotide removal kit (QUAGEN Inc. Mississauga, ON). Samples were sent to University core DNA services in University of Calgary. 6-FAM labeled terminal restriction fragments between 50 to 500bp were separated and recorded by model ABI3730 DNA sequencer.

TRF profiles were generated by software, GeneMarker V-1.4 (SoftGenetics LLC, USA). The information in the profiles are included: length of fragments (the apex position of each peak on a base pair scale relative to a DNA size ladder, GeneScan 500 LIZ Size Standard, Applied Biosystem, Foster City, CA), height of peaks and area of peaks.

3.2.7.1 Standardization of TRF Profiles

All profiles were standardized firstly by the application of the variable percentage threshold method (Osborne *et al.*, 2006). A unique percentage threshold value of each profile was generated by using a divisor to divide the total area of each profile from the same data set (digested by the same restriction enzyme). For each profile, peaks which

contribute less than its unique percentage threshold value were considered as noise peaks and removed from the profile.

The optimal divisors for each data set were obtained followed Zhang Y.'s protocols using TRFLPdemo, a Matlab based program designed by Luo F. (Master student in Computer Science Department, Saint Mary's University and Zhang Y.

3.2.8 Phylogenetic analysis using PAT

The normalized TRF profiles were compiled into 3 files separated by restriction enzymes *Bst*UI, *Msp*I and *Hae*III. As described in 2.2, the data sets were automatically analysis by PAT.

3.2.9 Construction of Clone Libraries for 16S rRNA Gene

Four libraries, from H_2-treated soil, air-treated soil, soil adjacent to HUP^+ nodules and soil adjacent to HUP^- nodules were constructed.

3.2.9.1 Cloning

3.2.9.1.1 Ligation

PCR products were inserted into the pGEM®-T Easy Vectors. The reaction was set up within a 10μl system, which contained: 5μl 2×Rapid Ligation Buffer, 1μl pGEM®-T Easy Vectors (50ng), 3μl insert DNA and 1μl T4 DNA Ligase (3 Weiss units/μl). The mixture was incubated at 4℃ for overnight.

3.2.9.1.2 Transformation

The ligation products were transformed into *E.coli* JM109 cell strain according to the protocols: Mixture of 3μl of PCR products and 40μl of JM109 competent cells in one microcentrifuge tube was set on ice for 20 minutes. Heat-shock was conducted at 42°C for 45s followed by a cold treatment on ice for 2 minutes. Then 400μl pre-warmed SOC medium was added to the tube and incubated at 37°C for 1.5 hours with shaking (225rpm). Finally 100μl broth was transformed onto LB/ampicillin (100μg/ml)/IPTG/X-Gal plate. Plates were incubated overnight at 37°C.

3.2.9.1.3 Inserts Checking

White (positive) colonies on the plates were randomly picked and inoculated on the new plates, some were ordered and labeled. Some of them were randomly chosen to amplify by PCR, and then loaded on the 1% agarose gel. After 30 minutes at 100 volts, the gel was stained with ethidium bromide and checked for inserts.

3.2.9.2 Plasmid DNA Extraction

For each library, 350 colonies were randomly picked. Each colony was dipped with one sterilized toothpick and inoculated in test tube with 2ml LB broth. Tubes were incubated at 37°C for 16 hours with shaking (225rpm).

The cells were harvested by centrifugation at 6000rpm for 3 minutes. The medium was removed before the pellets were resuspended in 250μl of Resuspension Solution (GeneJET™ Plasmid Miniprep Kit, Fermentas Inc., Burlington, ON, CA) by vortexing. 250μl Lysis Solution was added and mixed thoroughly by inverting the tube 4-

6 times until the solution becomes viscous and slightly clear. Then 350µl of the Neutralization Solution was added and mixed immediately and thoroughly by inverting several times. The cloudy solution was centrifuged for 5 minutes at 13000×g to pellet debris. The supernatant was transferred to supplied GeneJET^{TM} spin column by decanting. The spin was centrifuged for 1 minute at 13000×g. The flow-through was discarded and the column was placed back into the same catch tube. 500µl of the Wash Solution was added to the column and then centrifuged for 1 minute at 13000×g and emptied the catch tube again. Without any liquid, the column was centrifuged for an additional 1 minute to remove residual wash solution. Added 50µl of the Elution Buffer to the center of column, incubated for 2 minutes at room temperature and then centrifuged for 2 minutes at 13000×g. The pure plasmid DNA was stored at -20°C.

3.2.9.3 Sequencing

The plasmid DNA was sequenced by Macrogen Inc. (South Korea) with ABI 3700 sequencer. The sequencing reaction was performed with the forward universal primer M13F-pUC (5'-GTTTTCCCAGTCACGAC-3').

3.2.9.4 Sequences Analysis

3.2.9.4.1 Standardization of Sequences

The sequences of 4 libraries obtained were imported to 4 separate Microsoft Word files first. Then they were searched for 16s rRNA gene primers (BSR and BSR) manually using "replace" function of Microsoft Word and highlighted with different colors. If necessary, chimeric sequences (no target genes) were ignored in further analysis. All the

sequences in each library were set to fasta format (">" followed by sample ID, sequence of the sample started in the next line.) for analysis in RDP-II website.

3.2.9.4.2 Taxonomic Assignment

Four text files in fasta format (generated from standard word files), including 4 libraries sequences separately were submitted to the website of RDP-II for taxonomic assignment. "Seq Match" tool was utilized for phylogenetic analysis of 4 libraries to access the composition of communities. Air-treated and H_2-treated libraries, HUP^+ and HUP^- libraries were compared with bacterial diversity and richness by "Lib Comparison" tool of the website respectively.

3.2.9.4.3 TRFs generated from clone libraries

Counting the length of terminal fragments (from the first nucleotide of forward primer, BSR8/20 to the closest cutting site of each restriction enzyme) manually, the TRFs data obtained from all the positive sequences of each sample were listed in 4 different Microsoft Excel worksheets. Occasionally, it occurred that the entire sequence didn't have any cutting site of any enzyme. In that case, the data were omitted.

3.3 Result

3.3.1 Generation of TRF profiles

All the PCR products were checked using 1% agarose gel stained by ethidium bromide. The sharp and bright bands around 500bp found among all the samples as predicted.

3.3.1.1 Normalization of TRF profiles

The optimal divisor for each data set was obtained by running TRFLPdemo program (Table 3). The curves of number of TRFs remaining vs. the total area on original profiles resulting from those optimal divisors became horizontal lines after fitted as power function, R square of which almost equaled to zero. After that, the unique percentage threshold of each profile was calculated by total area dividing divisor. The percentage of intensity in total area of any TRFs lower than the threshold should be discarded from data set

3.3.2 Phylogenetic analysis result

3.3.2.1 T-RFLP analysis

PAT analysis (Table 3) showed that the dominant bacterial groups in both air and H_2-treated soil bacterial communities were *Proteobacteria*, *Actinobacteria* and *Firmicutes*. The main differences between two communities were the richness of *Proteobacteria*, *Firmicutes* and Bacteroidetes. In *Proteobacteria*, *Gammaproteobacteria* and *Betaproteobacteria* contributed most to the increase. After H_2 treatment *Alphaproteobacteria* decreased. *Bacilli* and *Clostridia* decreased and increased respectively dramatically in *Firmicutes*. *Sphingobacteria* and *Flavobacteria* decreased a little bit in *Bacteroidetes* after H_2 treatment.

Alphaproteobacteria increased a lot. *Beta-* and *Gamma-Proteobacteria* increased as well. In *Firmicutes*, *Bacilli* and *Clostridia* both decrease dramatically. *Flavobacteria* and *Sphingobacteria* in *Bacteroidetes* increased and decreased respectively.

Table 2: Optimal divisors for T-RFLP data sets and R squares of power curves resulting from best divisor generated by TRFLPdemo.

RE	R square	Best number	Best divisor
*Bst*UI	7.39178e-006	31	1.92833e^{+}008
*Hae*III	1.57033-006	25	2.89315e^{+}008
*Hinf*I	4.92501e-005	28	1.80413e^{+}008
*Msp*I	1.52962e-004	33	9.05921e^{+}007

Table 3: Results of PAT analysis of T-RFLP profiles generated from air-, H_2-treated soil, bulk soil, control soil, soil adjacent to HUP^+ and HUP^- nodules

	Air		H_2		Bulk		Control		HUP^+		HUP^-	
	abundance	%	abundance	%	abundance	%	abundance	%	abundance	%	abundance	%
Actinobacteria	4766	21.6	4793	10.2	1995	16.4	5451	22.9	834	10.9	816	11.0
Bacteroidetes	632	2.9	935	2.0	821	6.7	599	2.5	242	3.2	239	3.2
Firmicutes	3969	18.0	6093	12.9	1625	13.4	1530	6.4	1588	20.7	734	9.9
Gammaproteobacteria	1660	7.5	6210	13.2	1204	9.9	1391	5.9	609	7.9	631	8.5
Betaproteobacteria	500	2.3	6291	13.3	461	3.8	403	1.7	402	5.2	483	6.5
Alphaproteobacteria	2098	9.5	2320	4.9	1315	10.8	2026	8.5	868	11.3	1308	17.6
Bacili	3420	15.5	4084	8.7	1381	11.3	1030	4.3	1034	13.5	585	7.9
Flavobacteria	246	1.1	230	0.5	92	0.8	130	0.5	26	0.3	65	0.9
Sphingobacteria	386	1.7	698	1.5	659	5.4	450	1.9	206	2.7	184	2.5
Clostridia	461	2.1	1991	4.2	134	1.1	388	1.6	501	6.5	117	1.6
Total	22069		47186		12172		23756		7670		7428	

There was no significant difference of the species richness of bacterial communities among samples. Nine distinct phylums were found from the samples analyzed.

3.3.2.2 Clone library analysis

Four separate clone libraries were constructed from the 16S rRNA gene fragments amplified from air-, H_2-treated soil in lab, soil adjacent to HUP^- and HUP^- nodules with bacterial universal primer pairs, respectively. From the bacterial clone libraries, around 350 clones were randomly picked from each sample with more than 1400 in total and sequenced. Phylogenetic analysis revealed that most of them were affiliated with Bacteria. Due to the large number of clones, screening every one by re-PCR and checking insert is not practicable. The results showed that about 15% of clones didn't have the expected insert. Chimeric sequences were excluded from further analysis.

The remaining clones from 4 clone libraries could be assigned to 13 distinct phylogenetic groups (Table 4 and 7). Most clones were affiliated with *Proteobacteria*, followed by Bacteroidetes and *Actinobacteria*. Clones related to *TM7*, *Nitrospira*, *Cyanobacteria*, *Chloroflexi*, *Verrucomicrobia*, *Planctomycetes* and *Actinobacteria* were rare.

The distribution of 16S rDNA sequences among major taxonomic groups, *Proteobacteria* and Bacteroidetes were shown in Table 5 and 8. In *Proteobacteria*, α, β and γ subdivision are the majority. After treatment of H_2, γ subdivision is the most group with most significant increase. The difference between HUP^- and HUP^+ nodules soil

communities are that α, β and γ subdivision have higher composition in HUP^- nodule soil community. For *Bacteroidetes*, *Sphingobacteria* increase dramatically after H_2 treatment. When comparing HUP^+ and HUP^- communities, besides *Sphingobacteria*, *Flavobacteria* also contribute the increasing, not a lot, only 0.3%.

Comparing Tables 4 and 7, the differences between laboratory treatment and greenhouse condition can be found. Three more phylums, *TM7*, *Nitrospira* and *Planctomycetes* were detected in laboratory treatment. Only one new phylum, *Actinobacteria* were found in greenhouse clone libraries. In *Proteobacteria*, *Gamma*-subclass both increased in lab and greenhouse comparison. However, *more Alpha*- and *Beta*-subgroups were obtained in HUP^- than in HUP^+ libraries. They decreased when the soil was treated by H_2.

For other minor groups, it's hard to tell whether the data reveal the real situation. Only couples of clones selected from the community by chance may cover parts of the group. For example, *TM7* clones picked from H_2-treated soil community were affirmed that they are exactly the same sequence by aligning them. And all of them were selected in one time of experiment. The limitation of clone library, under sampling appeared here. However, screening of clone libraries provided accurate sequence information and revealed the main variety of bacterial community structure after H_2 treatment and difference between HUP^+ and HUP^- nodules soil ecosystems.

3.3.2.2.1 Species richness of clone libraries

Rarefaction curves are used to estimate taxonomic diversity (species richness) in ecological research (Raup, 1975). The rarefaction curves (Fig. 4 and 5) were generated *in*

silico from 4 libraries. In Fig. 4, the increase of different TRFs in air-treated soil library (pale blue) was faster than that of H_2-treated soil library (lavender) after 50 clones. Clone library from air-treated soil showed higher richness in TRFs than that of the H_2-treated. Nevertheless, the library of air-treated soil seemed to begin level off earlier than that of, the H_2-treated still went up. It's hard to predict whether the H_2's curve would exceed the air's finally. For HUP^+ and HUP^- libraries, they arose in the nearly same rate and were likely to reach the flat phase of the curves. Fig. 5(a) and (c) presented the TRFs richness of HUP^+ nodules soil was a little higher than HUP^-'s. Since many TRFs were shared by more than one phylogenetic group, the accurate number of species was not equal to the number of different TRFs.

3.3.2.3 Comparison of TRFs generated from clone library and T -RFLP

As the Figs. 6 to 21 show, for most of them, the size and diversity of TRFs are matched between the results of clone library and T-RFLP. In Fig. 6 as an example, the distributions of TRFs between two profiles were similar (The peaks with the same sizes were labeled with arrows). However, the heights of TRFs in two profiles were different. It can be explained by the limitation of sample size. T-RFLP profiles showed the whole community which represent billions of individuals, but artificial ones only consisted of 350 cells.

Abundance of different 16S rRNA gene estimated from T-RFLP profiles have been described in 3.3.2.1. The partial results of the T-RFLP analysis affirmed the clone abundance observed in the clone libraries. The 16S rRNA genes of *Proteobacteria* have the highest abundance in 4 libraries and T-RFLP analysis. But T-RFLP profiles analysis

Table 4: Diversity and abundance of phylotypes in air- and H_2-treated soil clone libraries. Phylogenetic comparison results of air- and H_2-treated clone libraries snapshot from website "RDP". Confident threshold=80%

Air	%	phylum	%	H_2
	0.0	TM7	0.3	
	0.5	Nitrospira	0.0	
	0.3	Cyanobacteria	0.0	
	0.5	Planctomycetes	0.0	
	0.3	Verrucomicrobia	0.0	
	0.5	Gemmatimonadetes	2.1	
	0.0	Chloroflexi	0.6	
	1.8	Actinobacteria	0.9	
	2.5	Bacteroidetes	5.7	
	29.9	Proteobacteria	27.6	
	41.6	unclassified Bacteria	50.2	

(* = significantly different at 0.01)

Table 5: Relative abundance (percent) of major different phylogenetic groups in air- and H_2-treated soil clone libraries, *Protebacteria* (a) and *Bacteroidetes* (b).

(a) *Proteobacteria*

Air	%	class	%	H_2
	1.2	Gammaproteobacteria *	7.8	
	14.1	Alphaproteobacteria *	7.2	
	5.1	Betaproteobacteria	6.9	
	0.0	Deltaproteobacteria	0.3	
	5.6	unclassified Proteobacteria	5.4	

(* = significantly different at 0.01)

(b) *Bacteroidetes*

Air	%	class	%	H_2
	1.8	Sphingobacteria	5.1	
	0.9	unclassified Bacteroidetes	0.6	

(* = significantly different at 0.01)

Table 6: Details of phylogenetic comparison results of air- and H_2-treated soil bacterial communities snapshot from website "RDP"

The order follows descending "significance" of every taxonomic level, rather than "rank".

Rank	Name	Air	H_2	Significance
class	Gammaproteobacteria	4	26	2.57E-5
family	Xanthomonadaceae	2	17	3.32E-4
order	Xanthomonadales	2	17	3.32E-4
	unclassified Xanthomonadaceae	0	10	NA
family	Crenotrichaceae	0	9	1.73E-3
genus	Terrimonas	0	9	1.73E-3
domain	Bacteria	267	291	1.74E-3
	unclassified Root	74	42	NA
order	Rhizobiales	25	8	3.08E-3
class	Alphaproteobacteria	48	24	3.86E-3
	unclassified Rhizobiales	17	5	NA
class	Sphingobacteria	6	17	1.68E-2
order	Sphingobacteriales	6	17	1.68E-2
	unclassified Bacteria	142	167	NA
	unclassified Gammaproteobacteria	2	9	NA
	unclassified Betaproteobacteria	22	10	NA
phylum	Bacteroidetes	9	19	4.66E-2
subclass	Actinobacteridae	6	1	7.57E-2
order	Actinomycetales	6	1	7.57E-2
	unclassified Alphaproteobacteria	19	10	NA
family	Gemmatimonadaceae	2	7	1.01E-1
order	Gemmatimonadales	2	7	1.01E-1
phylum	Gemmatimonadetes	2	7	1.01E-1
genus	Gemmatimonas	2	7	1.01E-1
genus	Xylella	2	7	1.01E-1
family	Comamonadaceae	0	3	1.19E-1
	unclassified Actinomycetales	5	1	NA
	unclassified Sphingobacteriales	1	4	NA
genus	Oligotropha	4	1	2.3E-1
phylum	Chloroflexi	0	2	2.41E-1
genus	Polaromonas	0	2	2.41E-1
	unclassified Actinobacteria	0	2	NA
family	Bradyrhizobiaceae	7	3	2.42E-1

phylum	Nitrospira	2	0	2.59E-1
family	Nitrospiraceae	2	0	2.59E-1
order	Nitrospirales	2	0	2.59E-1
family	Planctomycetaceae	2	0	2.59E-1
class	Planctomycetacia	2	0	2.59E-1
order	Planctomycetales	2	0	2.59E-1
phylum	Planctomycetes	2	0	2.59E-1
genus	Sporocytophaga	2	0	2.59E-1
class	Betaproteobacteria	31	23	2.98E-1
order	Burkholderiales	9	13	3.58E-1
phylum	Actinobacteria	6	3	3.64E-1
genus	Sphingosinicella	2	4	4.34E-1
	unclassified Flexibacteraceae	2	4	NA
class	Anaerolineae	0	1	4.88E-1
genus	Archangium	0	1	4.88E-1
family	Caldilineacea	0	1	4.88E-1
subclass	Caldilineae	0	1	4.88E-1
order	Caldilineales	0	1	4.88E-1
order	Chloroflexales	0	1	4.88E-1
class	Chloroflexi	0	1	4.88E-1
family	Cystobacteraceae	0	1	4.88E-1
suborder	Cystobacterineae	0	1	4.88E-1
class	Deltaproteobacteria	0	1	4.88E-1
genus	Levilinea	0	1	4.88E-1
genus	Methylibium	0	1	4.88E-1
order	Myxococcales	0	1	4.88E-1
phylum	TM7	0	1	4.88E-1
genus	TM7_genera_incertae_sedis	0	1	4.88E-1
genus	Variovorax	0	1	4.88E-1
	unclassified Chloroflexales	0	1	NA
family	Chloroplast	1	0	5.12E-1
suborder	Corynebacterineae	1	0	5.12E-1
phylum	Cyanobacteria	1	0	5.12E-1
family	Gordoniaceae	1	0	5.12E-1
family	Hyphomicrobiaceae	1	0	5.12E-1

genus	Isosphaera	1	0	5.12E-1
genus	Niastella	1	0	5.12E-1
genus	Pirellula	1	0	5.12E-1
phylum	Verrucomicrobia	1	0	5.12E-1
class	Verrucomicrobiae	1	0	5.12E-1
order	Verrucomicrobiales	1	0	5.12E-1
	unclassified Chloroplast	1	0	NA
	unclassified Gordoniaceae	1	0	NA
	unclassified Hyphomicrobiaceae	1	0	NA
	unclassified Incertae sedis 5	1	0	NA
	unclassified Verrucomicrobiales	1	0	NA
phylum	Proteobacteria	102	92	5.16E-1
family	Sphingomonadaceae	4	6	5.22E-1
order	Sphingomonadales	4	6	5.22E-1
	unclassified Bacteroidetes	3	2	NA
	unclassified Bradyrhizobiaceae	3	2	NA
	unclassified Burkholderiales	8	9	NA
family	Flexibacteraceae	5	4	7.83E-1
	unclassified Proteobacteria	19	18	NA
	unclassified Sphingomonadaceae	2	2	NA
family	Incertae sedis 5	1	1	9.82E-1
no rank	Root	341	333	1E0

Table 7: Diversity and abundance of phylotypes in HUP^+ and HUP^- soil clone libraries. Confident threshold=80%

HUP^+	%	phylum	%	HUP^-
	0.0	Cyanobacteria	0.3	
	0.0	Chloroflexi	0.6	
	1.4	Verrucomicrobia	0.3	
	2.0	Actinobacteria	4.4	
	0.3	Acidobacteria	0.3	
	2.3	Gemmatimonadetes	2.4	
	3.7	Bacteroidetes	5.0	
	0.3	Firmicutes	0.0	
	28.6	Proteobacteria *	53.8	
	43.4	unclassified Bacteria	25.3	

(* = significantly different at 0.01)

Table 8: Relative abundance (percent) of major different phylogenetic groups in HUP$^+$ and HUP$^-$ nodules soil clone libraries, *Protebacteria* (a) and *Bacteroidetes* (b).

(a) *Proteobacteria*

HUP+	%	class	%	HUP-
	5.1	Betaproteobacteria *	11.5	
	1.4	Gammaproteobacteria *	13.5	
	0.3	Deltaproteobacteria	0.0	
	16.6	Alphaproteobacteria	24.1	
	5.1	unclassified Proteobacteria	4.7	

(* = significantly different at 0.01)

(b) *Bacteroidetes*

HUP+	%	class	%	HUP-
	2.3	Sphingobacteria	3.8	
	0.0	Flavobacteria	0.3	
	1.4	unclassified Bacteroidetes	0.9	

(* = significantly different at 0.01)

Table 9: Details of phylogenetic comparison results of HUP^+ and HUP^- nodules soil bacterial communities snapshot from website "RDP"

The order follows descending "significance" of every taxonomic level, rather than "rank"

Rank	Name	HUP^+	HUP^-	Significance
phylum	Proteobacteria	100	183	2.1E-11
family	Xanthomonadaceae	0	32	1.44E-10
order	Xanthomonadales	0	32	1.44E-10
class	Gammaproteobacteria	5	46	6.93E-10
	unclassified Xanthomonadaceae	0	26	NA
	unclassified Bacteria	152	86	NA
domain	Bacteria	287	314	6.34E-5
	unclassified Root	63	26	NA
class	Betaproteobacteria	18	39	2.52E-3
	unclassified Alphaproteobacteria	13	31	NA
class	Alphaproteobacteria	58	82	1.39E-2
	unclassified Betaproteobacteria	6	17	NA
subclass	Actinobacteridae	3	12	1.83E-2
order	Actinomycetales	3	12	1.83E-2
family	Nocardioidaceae	0	5	2.86E-2
suborder	Propionibacterineae	0	5	2.86E-2
order	Burkholderiales	10	21	3.57E-2
genus	Xylella	0	4	5.81E-2
	unclassified Pseudomonadaceae	0	4	NA
phylum	Actinobacteria	7	15	7.19E-2
family	Flexibacteraceae	2	7	9.95E-2
family	Pseudomonadaceae	2	7	9.95E-2
order	Pseudomonadales	2	7	9.95E-2
	unclassified Burkholderiales	4	10	NA
	unclassified Actinomycetales	1	5	NA
genus	Duganella	0	3	1.18E-1
	unclassified Nocardioidaceae	0	3	NA
order	Rubrobacterales	3	0	1.32E-1
subclass	Rubrobacteridae	3	0	1.32E-1
suborder	Rubrobacterineae	3	0	1.32E-1
phylum	Verrucomicrobia	5	1	1.35E-1
class	Verrucomicrobiae	5	1	1.35E-1
order	Verrucomicrobiales	5	1	1.35E-1
family	Comamonadaceae	1	4	2.05E-1
family	Oxalobacteraceae	1	4	2.05E-1

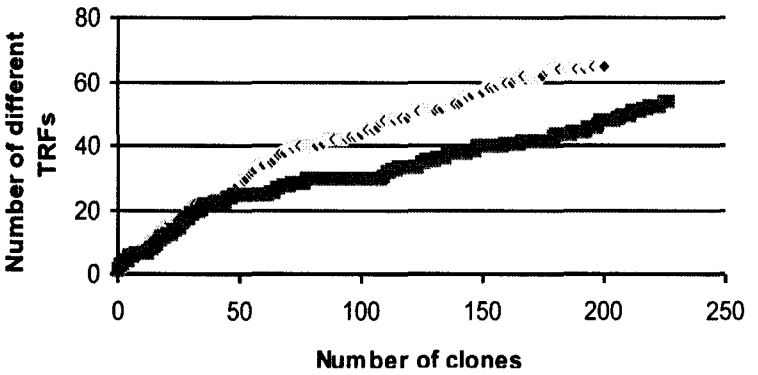
	unclassified Gammaproteobacteria	3	7	NA
genus	Sphingosinicella	4	1	2.33E-1
order	Rhizobiales	27	35	2.38E-1
class	Sphingobacteria	8	13	2.38E-1
order	Sphingobacteriales	8	13	2.38E-1
class	Anaerolineae	0	2	2.39E-1
family	Caldilineacea	0	2	2.39E-1
subclass	Caldilineae	0	2	2.39E-1
order	Caldilineales	0	2	2.39E-1
phylum	Chloroflexi	0	2	2.39E-1
family	Hyphomicrobiaceae	0	2	2.39E-1
genus	Marmoricola	0	2	2.39E-1
family	Micromonosporaceae	0	2	2.39E-1
suborder	Micromonosporineae	0	2	2.39E-1
genus	Polaromonas	0	2	2.39E-1
	unclassified Micromonosporaceae	0	2	NA
suborder	Micrococcineae	2	0	2.61E-1
family	Rubrobacteraceae	2	0	2.61E-1
	unclassified Methylophilaceae	2	0	NA
	unclassified Rubrobacteraceae	2	0	NA
	unclassified Flexibacteraceae	2	5	NA
	unclassified Rhizobiales	16	22	NA
	unclassified Actinobacteria	1	3	NA
	unclassified Verrucomicrobiales	3	1	NA
phylum	Bacteroidetes	13	17	4.07E-1
	unclassified Bradyrhizobiaceae	9	6	NA
genus	Agrobacterium	0	1	4.86E-1
genus	Agromonas	0	1	4.86E-1
genus	Bacillariophyta	0	1	4.86E-1
family	Chloroplast	0	1	4.86E-1
phylum	Cyanobacteria	0	1	4.86E-1
genus	Dyadobacter	0	1	4.86E-1
genus	Filomicrobium	0	1	4.86E-1
class	Flavobacteria	0	1	4.86E-1
family	Flavobacteriaceae	0	1	4.86E-1

order	Flavobacteriales	0	1	4.86E-1
genus	Hydrocarboniphaga	0	1	4.86E-1
genus	Levilinea	0	1	4.86E-1
genus	Methylophilus	0	1	4.86E-1
genus	Nevskia	0	1	4.86E-1
family	Phyllobacteriaceae	0	1	4.86E-1
genus	Sandaracinobacter	0	1	4.86E-1
genus	Sporocytophaga	0	1	4.86E-1
genus	Variovorax	0	1	4.86E-1
	unclassified Caldilineacea	0	1	NA
	unclassified Flavobacteriaceae	0	1	NA
	unclassified Hyphomicrobiaceae	0	1	NA
	unclassified Phyllobacteriaceae	0	1	NA
class	"Bacilli"	1	0	5.15E-1
order	Bacillales	1	0	5.15E-1
class	Deltaproteobacteria	1	0	5.15E-1
phylum	Firmicutes	1	0	5.15E-1
family	Intrasporangiaceae	1	0	5.15E-1
order	Myxococcales	1	0	5.15E-1
family	Planococcaceae	1	0	5.15E-1
family	Polyangiaceae	1	0	5.15E-1
genus	Polyangium	1	0	5.15E-1
genus	Sinorhizobium	1	0	5.15E-1
suborder	Sorangineae	1	0	5.15E-1
family	Subdivision 3	1	0	5.15E-1
genus	Subdivision 3_genera_incertae_sedis	1	0	5.15E-1
family	Verrucomicrobiaceae	1	0	5.15E-1
	unclassified Intrasporangiaceae	1	0	NA
	unclassified Micrococcineae	1	0	NA
	unclassified Planococcaceae	1	0	NA
	unclassified Rubrobacterineae	1	0	NA
	unclassified Verrucomicrobiaceae	1	0	NA
	unclassified Bacteroidetes	5	3	NA
genus	Oligotropha	1	2	6.03E-1
family	Methylophilaceae	2	1	6.47E-1

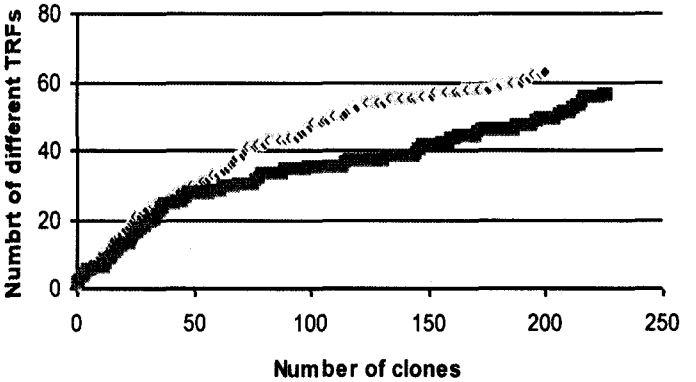
order	Methylophilales	2	1	6.47E-1
genus	Flavimonas	2	3	6.61E-1
	unclassified Sphingobacteriales	2	3	NA
	unclassified Incertae sedis 5	3	2	NA
family	Crenotrichaceae	4	3	7.58E-1
family	Incertae sedis 5	4	3	7.58E-1
genus	Terrimonas	4	3	7.58E-1
family	Sphingomonadaceae	18	16	7.87E-1
order	Sphingomonadales	18	16	7.87E-1
	unclassified Proteobacteria	18	16	NA
family	Bradyrhizobiaceae	10	9	8.65E-1
	unclassified Sphingomonadaceae	14	14	NA
family	Gemmatimonadaceae	8	8	9.52E-1
order	Gemmatimonadales	8	8	9.52E-1
phylum	Gemmatimonadetes	8	8	9.52E-1
genus	Gemmatimonas	8	8	9.52E-1
phylum	Acidobacteria	1	1	9.78E-1
family	Acidobacteriaceae	1	1	9.78E-1
order	Acidobacteriales	1	1	9.78E-1
genus	Gp4	1	1	9.78E-1
genus	Methylibium	1	1	9.78E-1
family	Rhizobiaceae	1	1	9.78E-1
	unclassified Comamonadaceae	1	1	NA
	unclassified Oxalobacteraceae	1	1	NA
no rank	Root	350	340	1E0

Figure 4: Rarefaction curves for H_2-treated and air-treated libraries. (a) TRFs generated from *Bst*UI (b) TRFs generated from *Hinf*I (c) TRFs generated from *Hae*III; (d) TRFs generated from *Msp*I

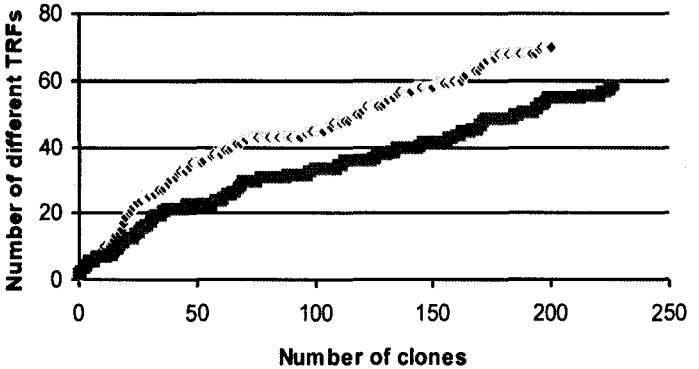
(a)


Number of different TRFs
0
20
40
60
80
0
50
100
150
200
250
Number of clones

(b)


Numbrt of different TRFs
0
20
40
60
80
0
50
100
150
200
250
Number of clones

(c)


Number of different TRFs
0
20
40
60
80
0
50
100
150
200
250
Number of clones

(d)

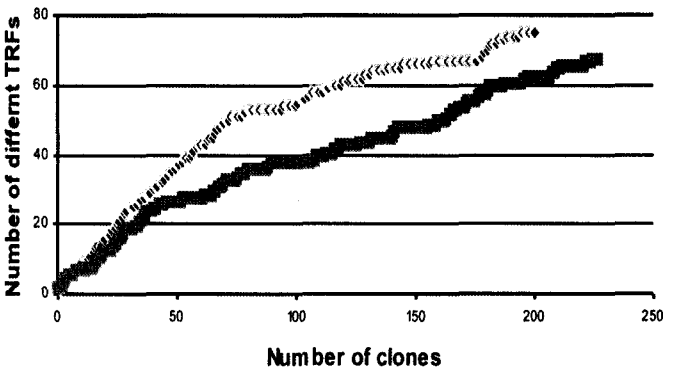
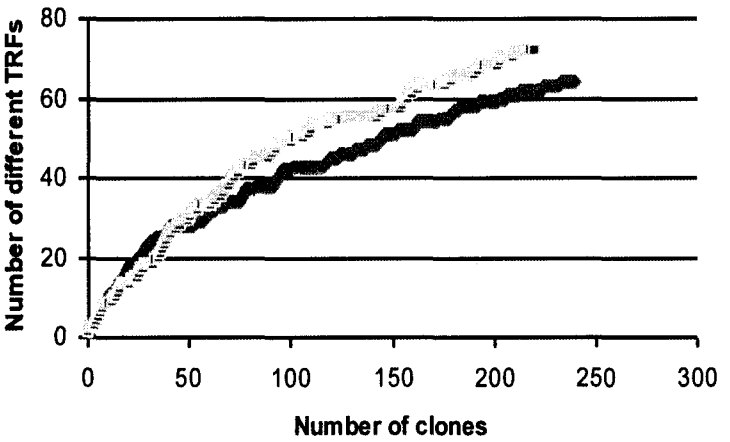
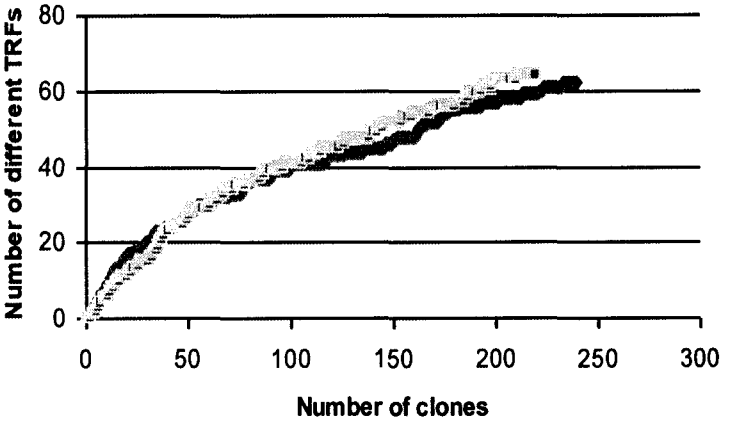

Number of differnt TRFs
0
20
40
60
80
0
50
100
150
200
250
Number of clones
air-treated
H2-treated

Figure 5: Rarefaction curves for HUP^+ and HUP^- libraries. (a) TRFs generated from *Bst*UI (b) TRFs generated from *Hinf*I (c) TRFs generated from *Hae*III; (d) TRFs generated from *Msp*I

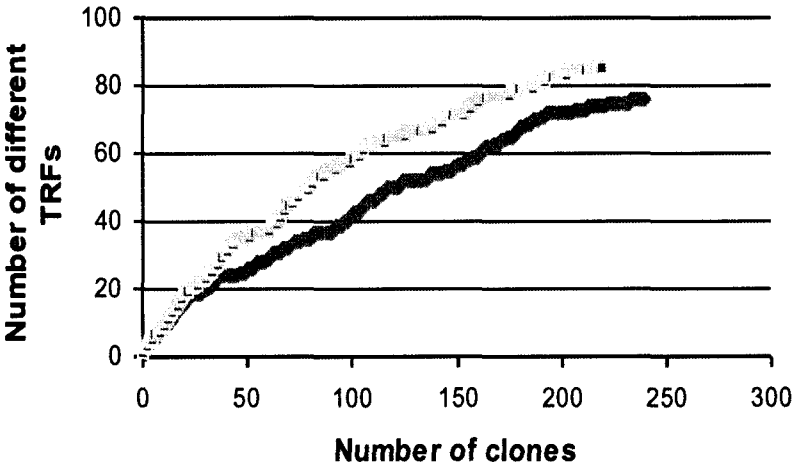
(a)


Number of different TRFs
0
20
40
60
80
0
50
100
150
200
250
300
Number of clones

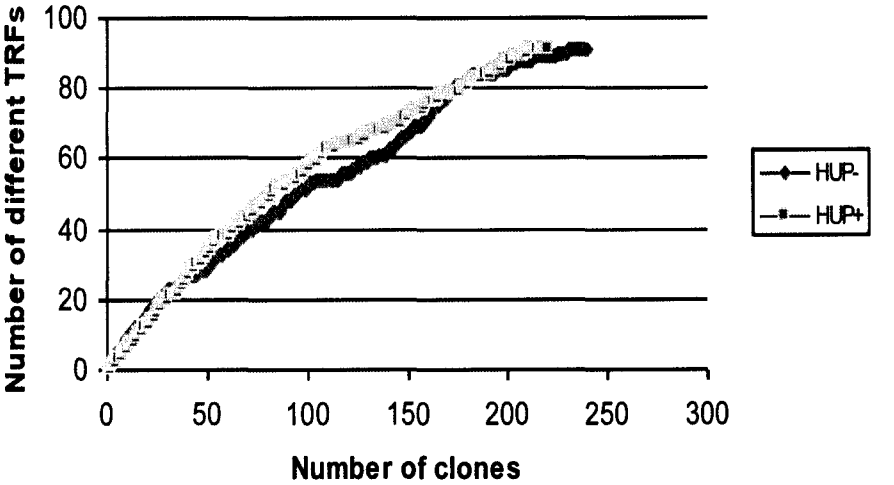
(b)


Number of different TRFs
0
20
40
60
80
0
50
100
150
200
250
300
Number of clones

(c)


Number of different TRFs
0
20
40
60
80
100
0
50
100
150
200
250
300
Number of clones

(d)


Number of different TRFs
0
20
40
60
80
100
0
50
100
150
200
250
300
Number of clones
HUP-
HUP+

of the *Firmicutes* were not supported by the clone libraries. In air- and H_2-treated soil clone libraries, there was even none of *Firmicutes*.

We detected numerous phylotpes from 16S rDNA libraries. Some of them didn't appear in T-RFLP analysis, like *Verrucomicrobia*, *Acidobacteria* and *Gammatimonadetes*. Similarly, *Thermomicrobia* and *Deferribacteres* were not screened in clone libraries, but showed in T-RFLP analysis. The result of majority groups of two methods were most matched, except *Frimicutes*. In addition, at least 25% sequences were not classified, especially in H_2-treated soil clone library; half of clones were unknown bacteria, which indicate that our knowledge of bacteria diversity in soils is still far from being exhausitive In Fig. 22, 23, 24 and 25, abundance of TRFs generated from control and bulk soils by 4 different restriction enzymes were compared. Results of PAT analysis showed that the abundance of control soil was richer than bulk, especially *Actinobacteria* and *Alphaproteobacteria*.

Figure 6: Abundance of TRFs generated from air-treated soil clone library (above) and T-RFLP (below) by restriction enzyme *Bst*UI

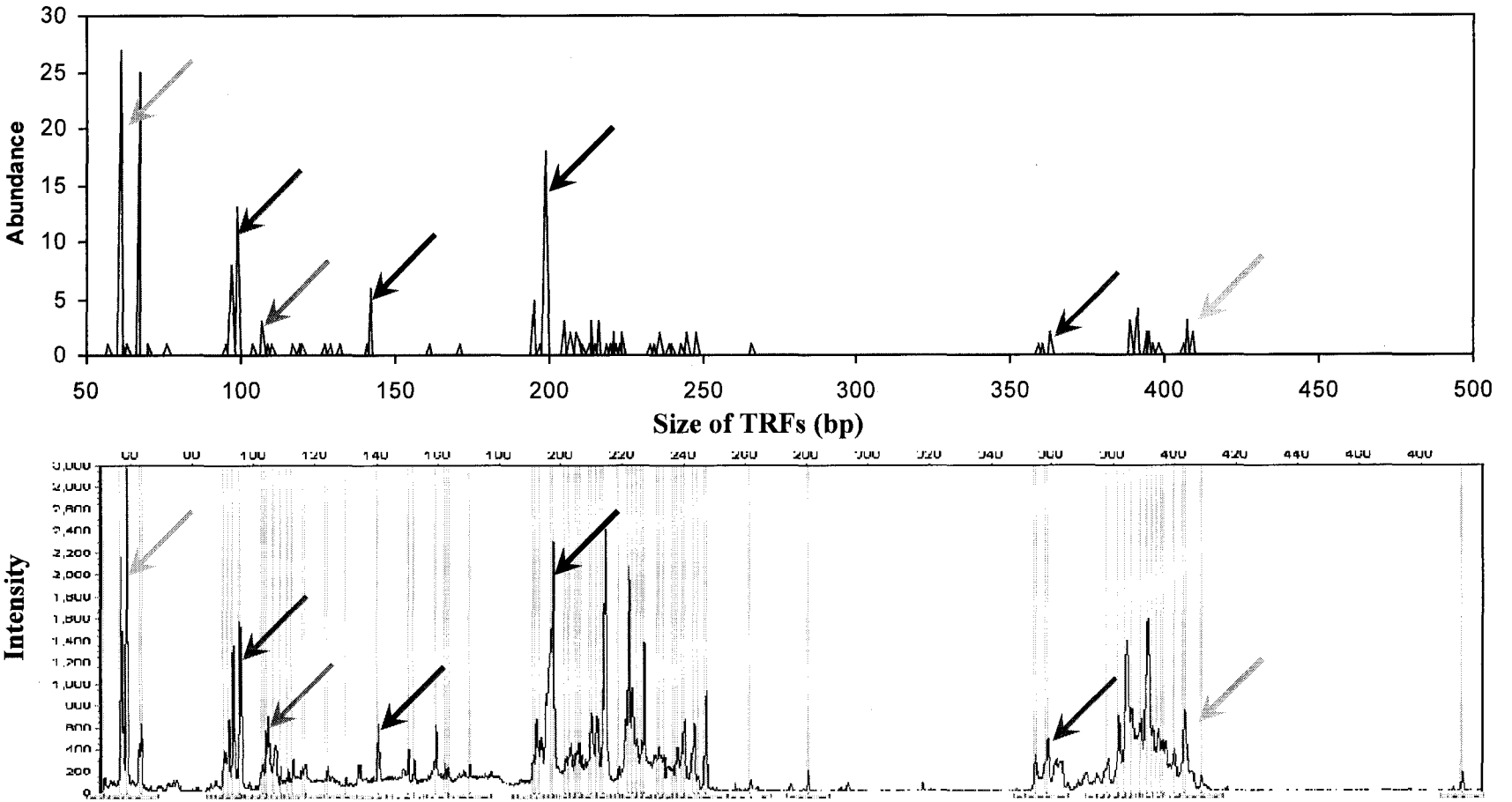

Abundance
30
25
20
15
10
5
0
50
100
150
200
250
300
350
400
450
500
Size of TRFs (bp)
Intensity

Figure 7: Abundance of TRFs generated from H_2-treated soil clone library (above) and T-RFLP (below) by restriction enzyme *Bst*UI

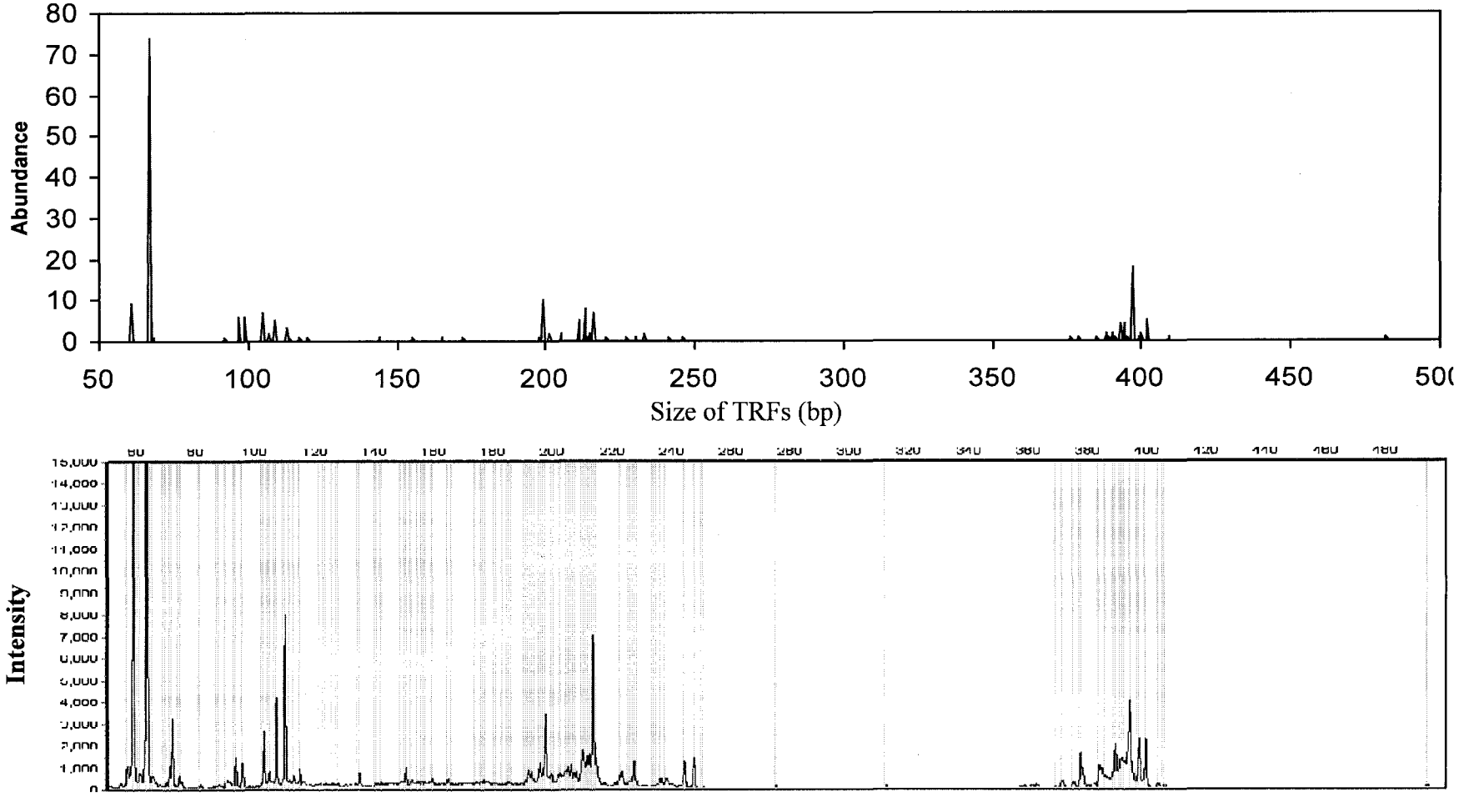

Abundance
80
70
60
50
40
30
20
10
0
50
100
150
200
250
300
350
400
450
Size of TRFs (bp)
Intensity

Figure 8: Abundance of TRFs generated from air-treated soil clone library (above) and T-RFLP (below) by restriction enzyme *Hinf*I

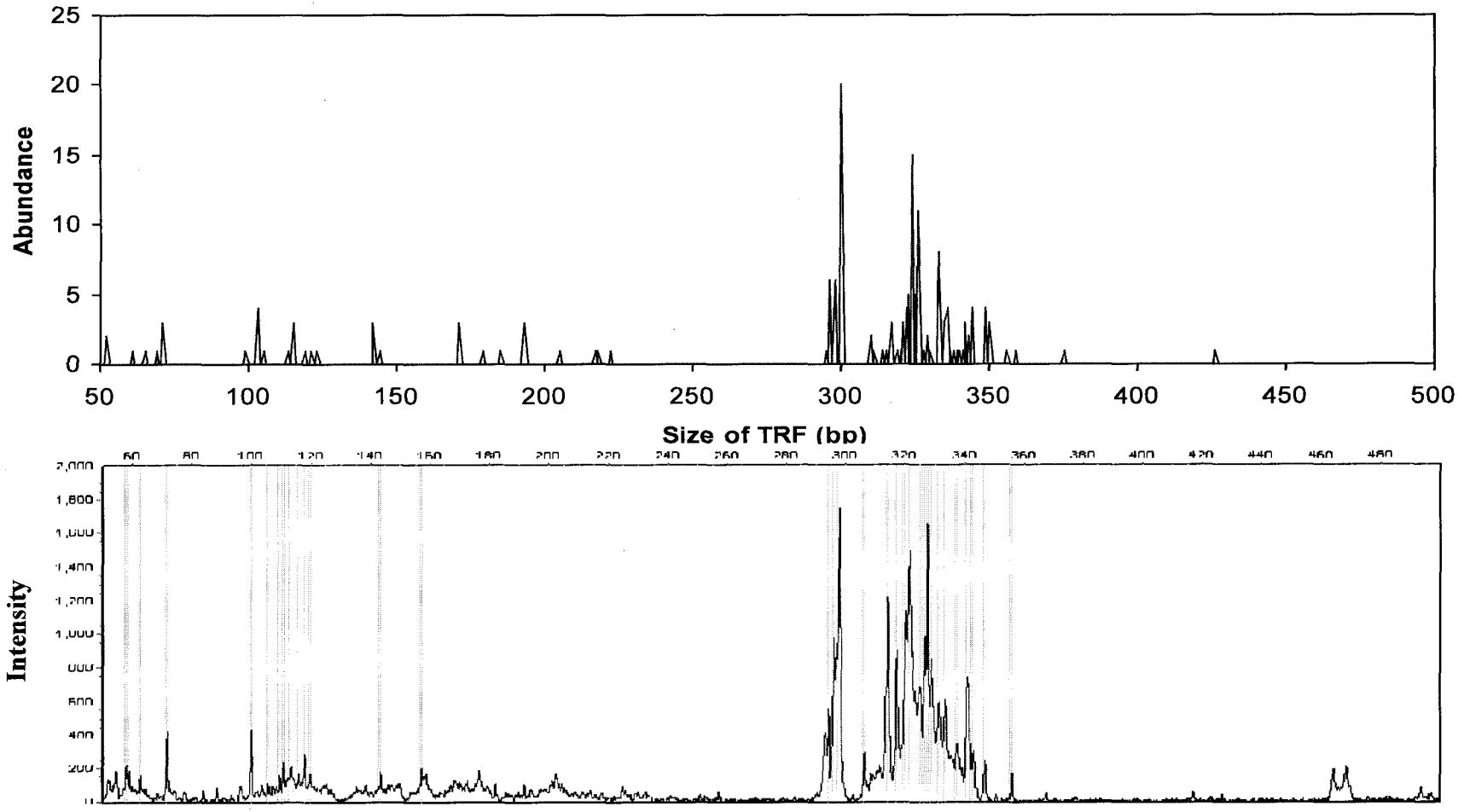
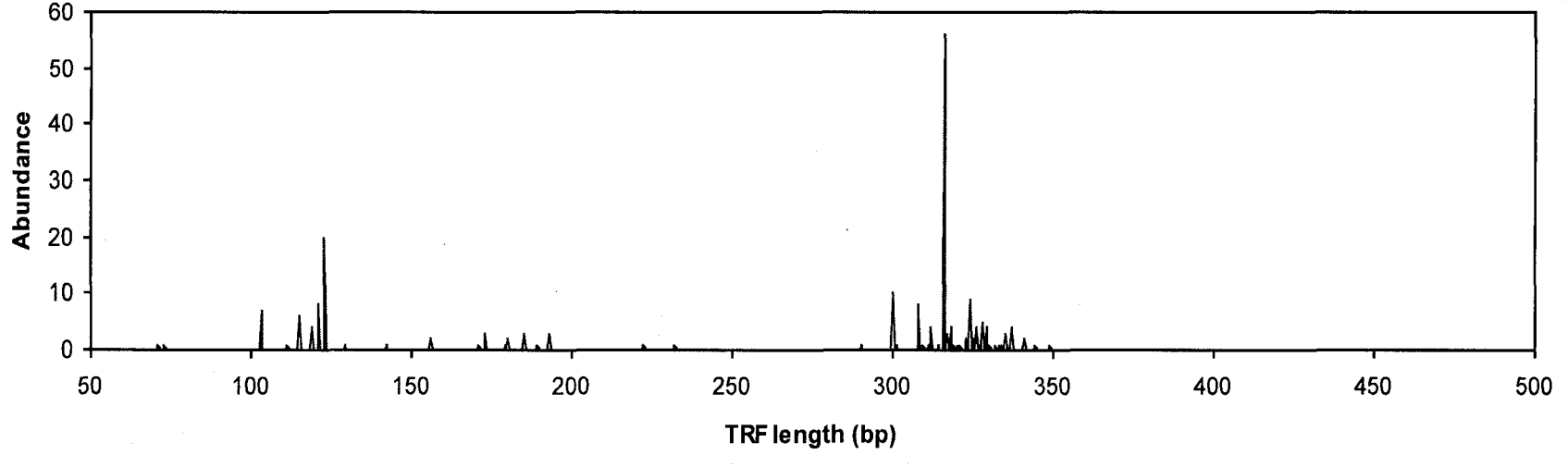

Abundance
25
20
15
10
5
0
50
100
150
200
250
300
350
400
450
500
Size of TRF (bp)
Intensity

Figure 9: Abundance of TRFs generated from H_2-treated soil clone library (above) and T-RFLP (below) by restriction enzyme *Hinf*I


Abundance
60
50
40
30
20
10
0
50
100
150
200
250
300
350
400
450
500
TRF length (bp)

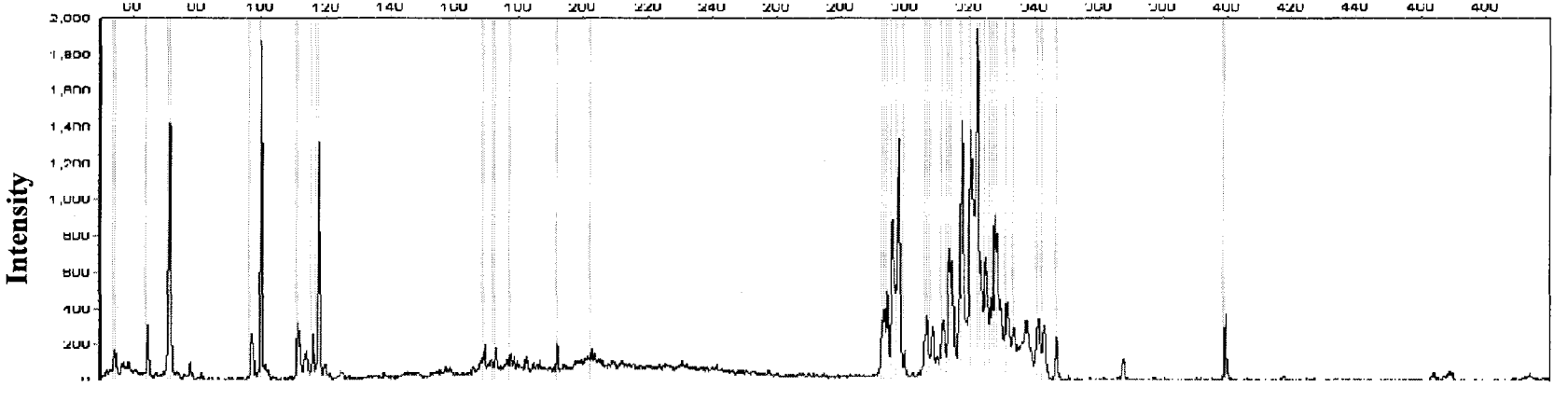

Intensity

Figure 10: Abundance of TRFs generated from air-treated soil clone library (above) and T-RFLP (below) by restriction enzyme *Hae*III

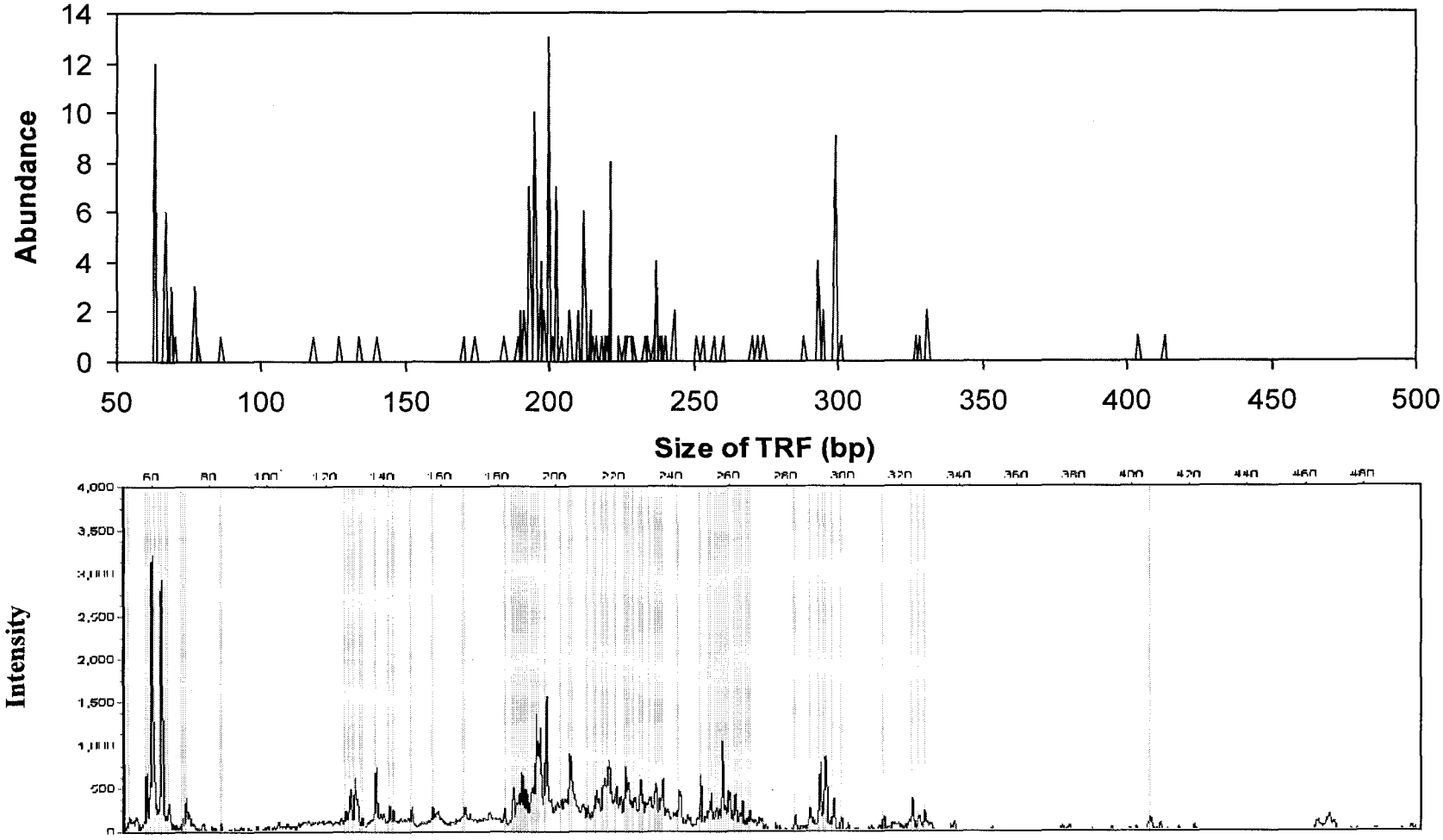

Abundance
14
12
10
8
6
4
2
0
50
100
150
200
250
300
350
400
450
500
Size of TRF (bp)
Intensity
4,000
3,500
2,500
2,000
1,500
500
0

Figure 11: Abundance of TRFs generated from H_2-treated soil clone library (above) and T-RFLP (below) by restriction enzyme *Hae*III

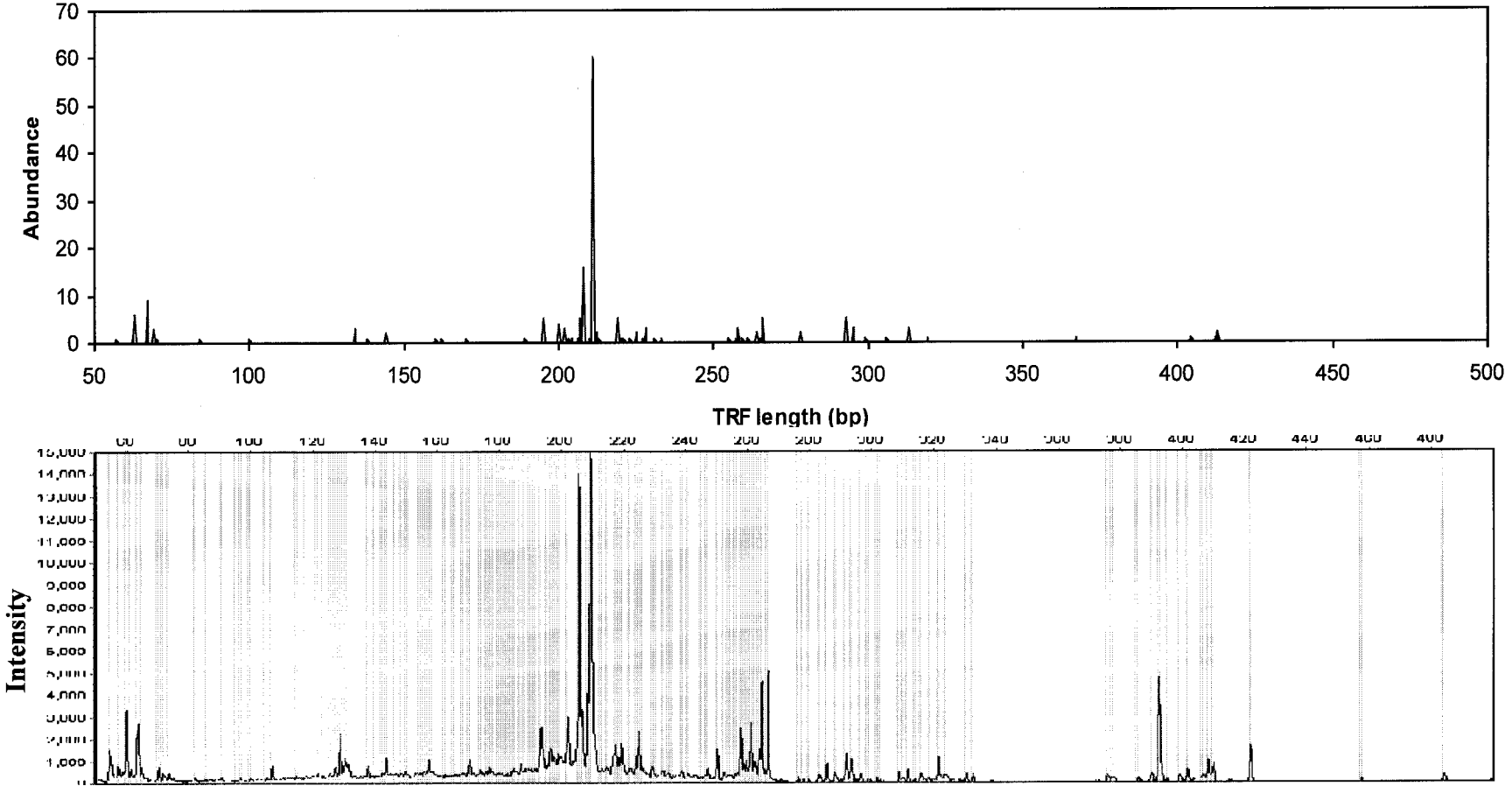
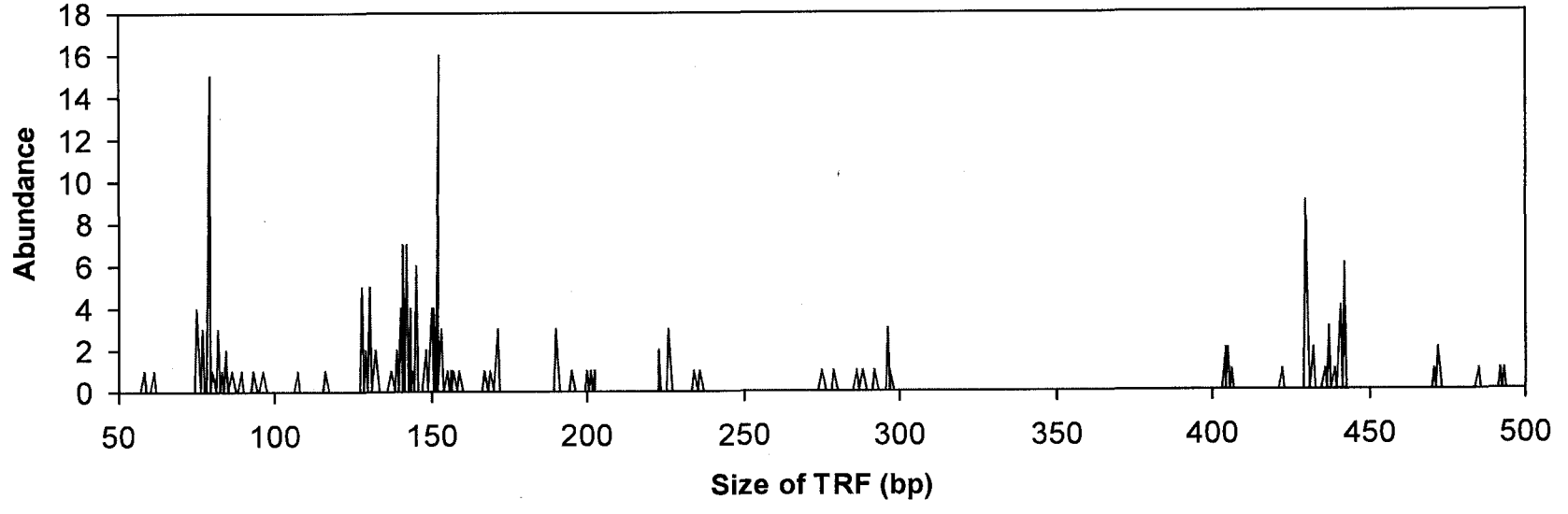

Abundance
70
60
50
40
30
20
10
0
50
100
150
200
250
300
350
400
450
500
TRF length (bp)
Intensity

Figure 12: Abundance of TRFs generated from air-treated soil clone library (above) and T-RFLP (below) by restriction enzyme *Msp*I


Abundance
18
16
14
12
10
8
6
4
2
0
50
100
150
200
250
300
350
400
450
500
Size of TRF (bp)

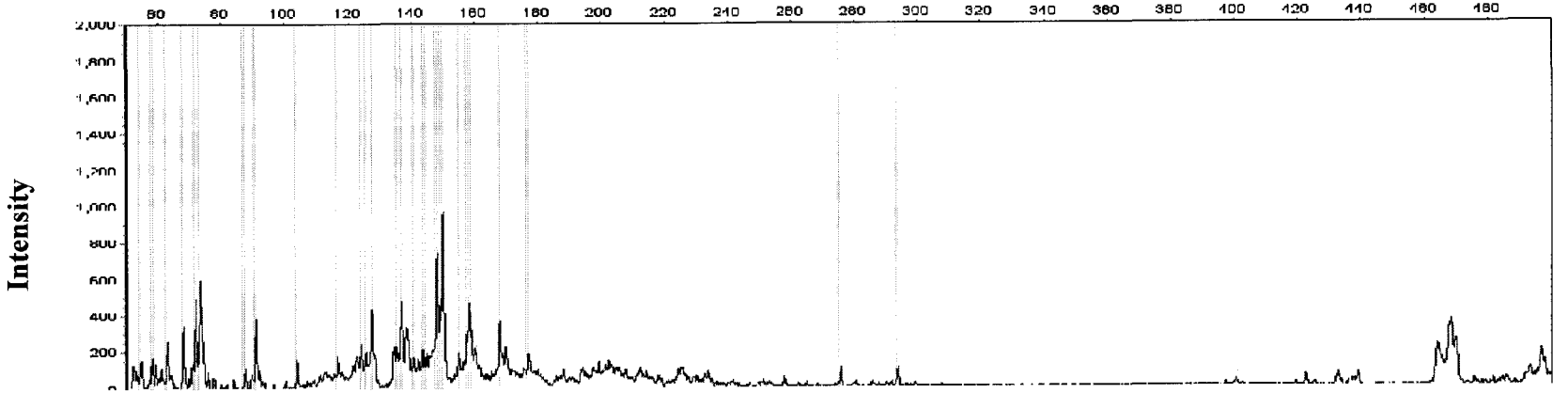
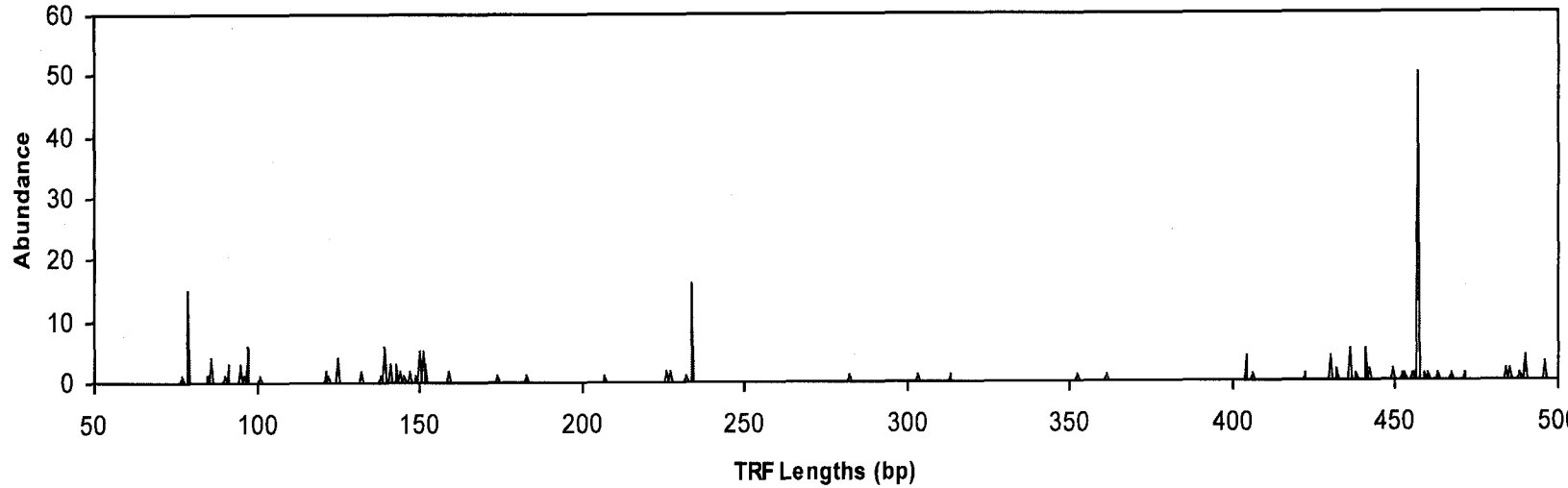

Intensity
80
60
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
460
480

Figure 13: Abundance of TRFs generated from H_2-treated soil clone library (above) and T-RFLP (below) by restriction enzyme *Msp*I


Abundance
60
50
40
30
20
10
0
50
100
150
200
250
300
350
400
450
500
TRF Lengths (bp)

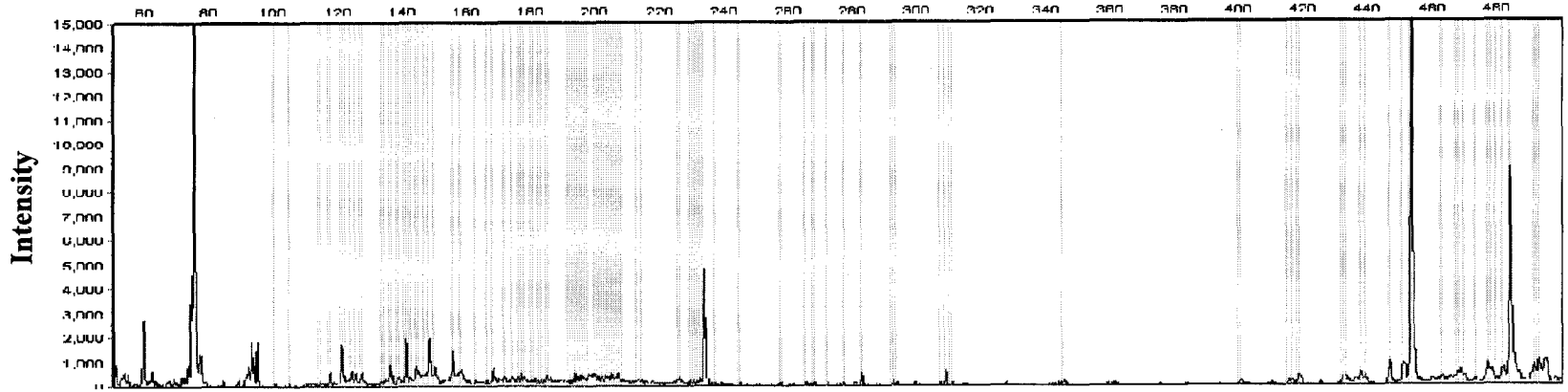

Intensity
15,000
14,000
13,000
11,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000

Figure 14: Abundance of TRFs generated from HUP^+ nodules soil clone library (above) and T-RFLP (below) by restriction enzyme *Bst*UI

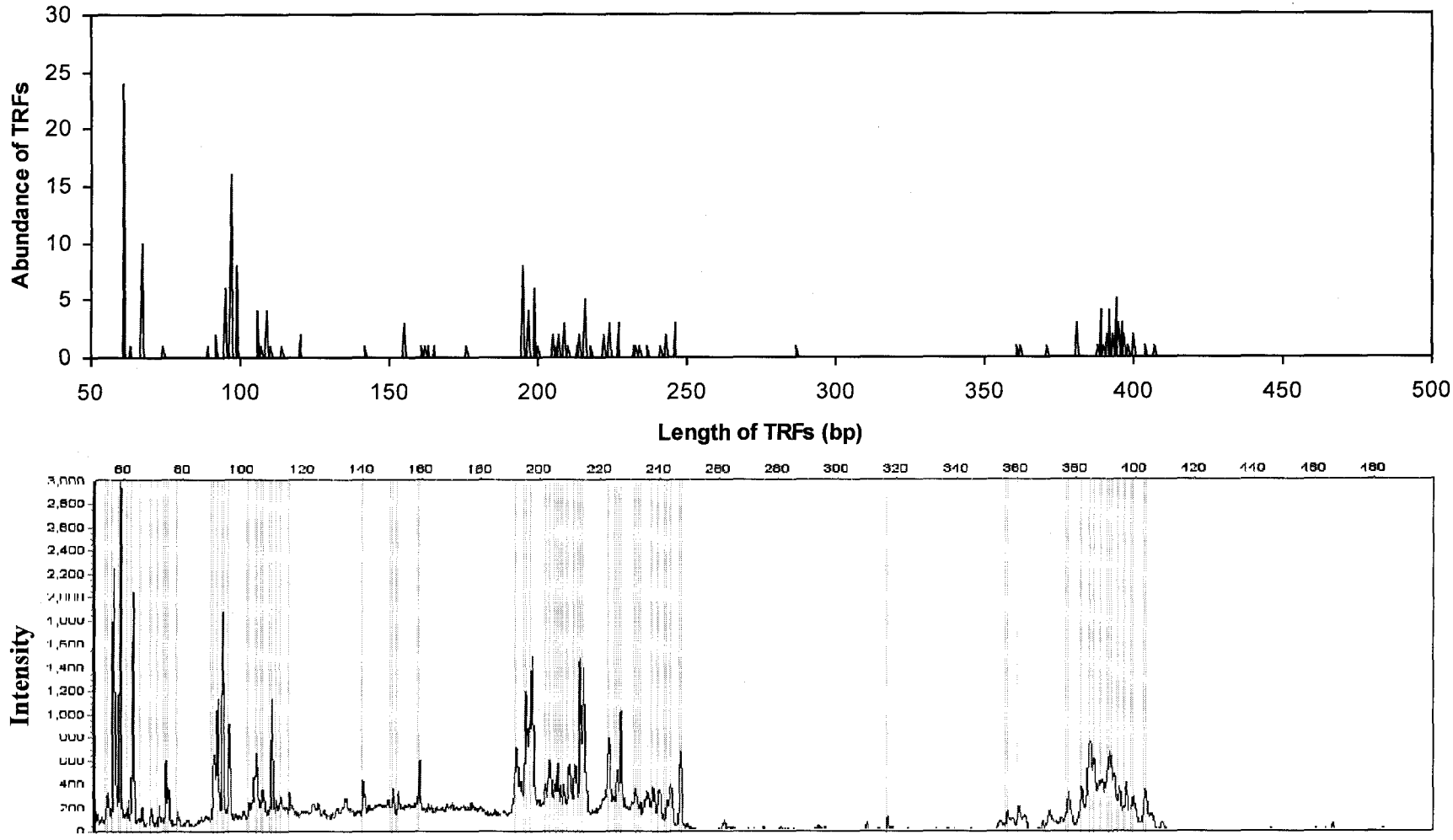

Abundance of TRFs
30
25
20
15
10
5
0
50
100
150
200
250
300
350
400
450
500
Length of TRFs (bp)
Intensity

Figure 15: Abundance of TRFs generated from HUP$^-$ nodules soil clone library (above) and T-RFLP (below) by restriction enzyme *Bst*UI

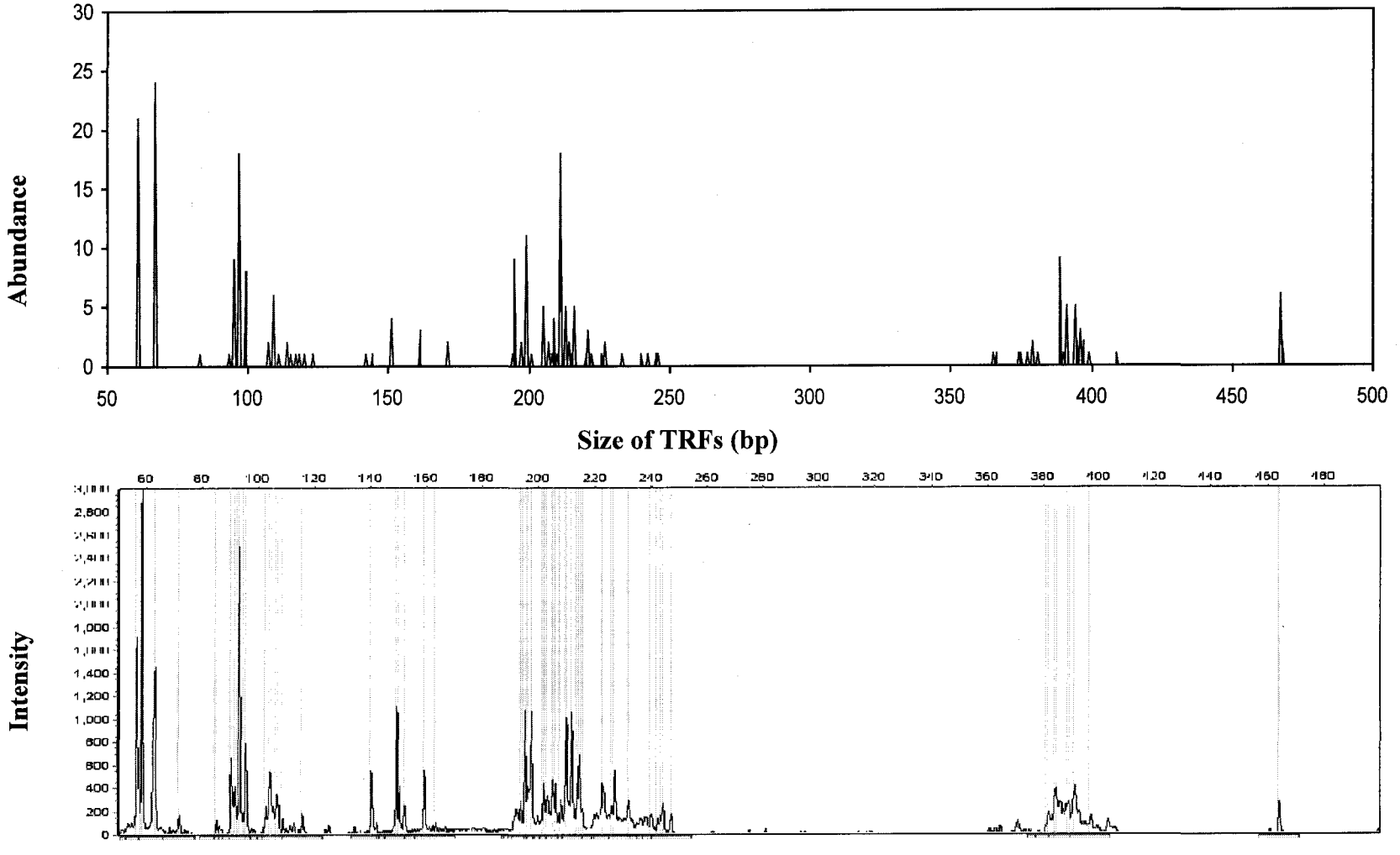
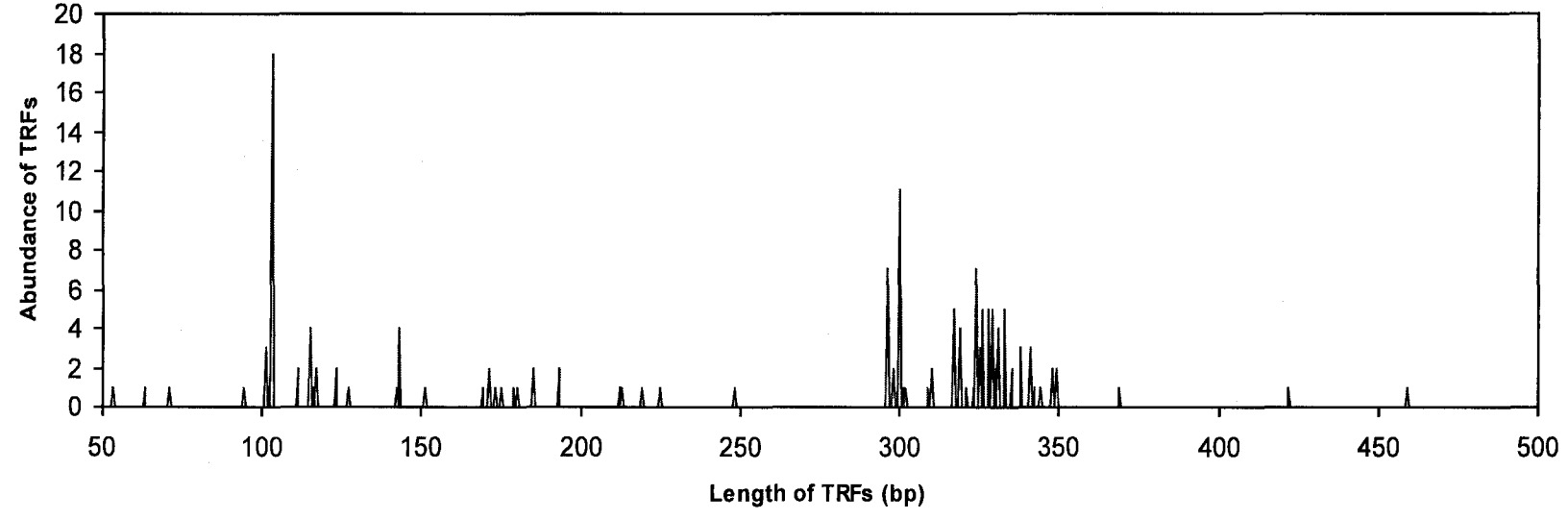

Abundance
30
25
20
15
10
5
0
50
100
150
200
250
300
350
400
450
500
Size of TRFs (bp)
Intensity
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
460
480
2,800
1,000
1,400
1,200
1,000
800
600
400
200
0

Figure 16: Abundance of TRFs generated from HUP^+ nodules soil clone library (above) and T-RFLP (below) by restriction enzyme *Hinf*I


Abundance of TRFs
20
18
16
14
12
10
8
6
4
2
0
50
100
150
200
250
300
350
400
450
500
Length of TRFs (bp)

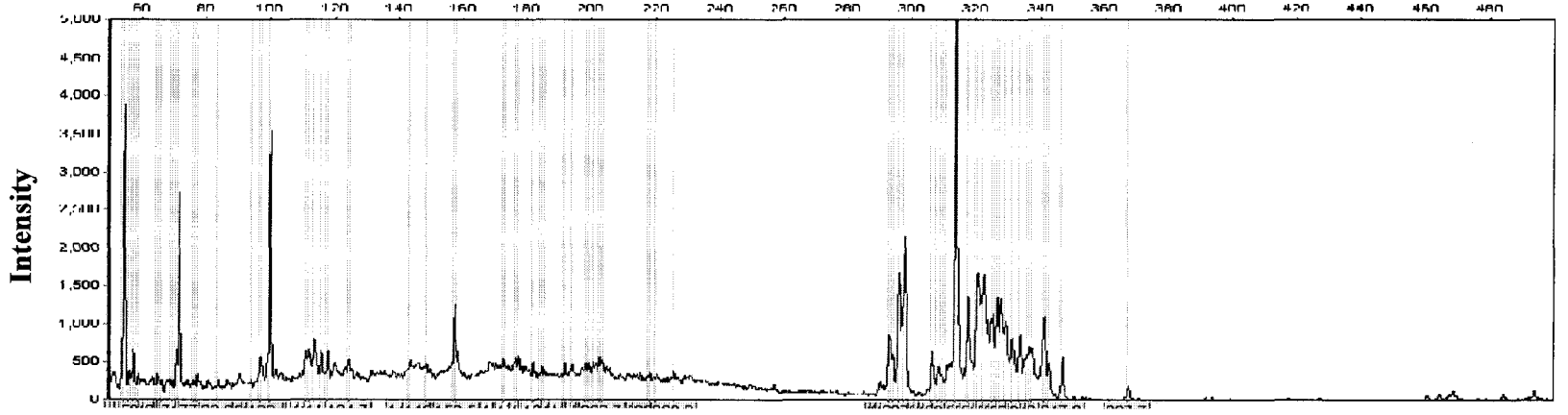

Intensity
4,500
4,000
3,000
2,000
1,500
1,000
500
0

Figure 17: Abundance of TRFs generated from HUP$^-$ nodules soil clone library (above) and T-RFLP (below) by restriction enzyme *Hinf*I

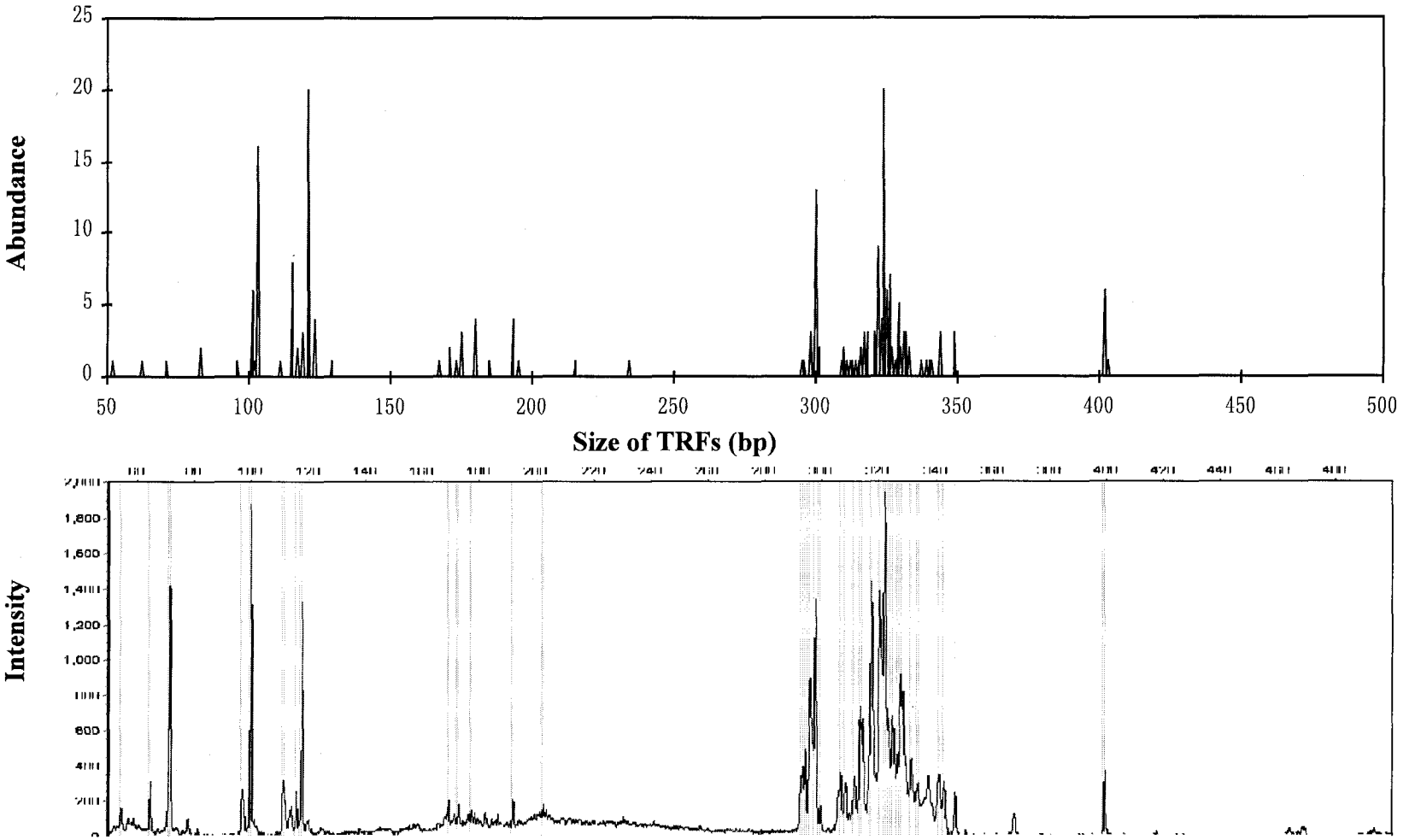
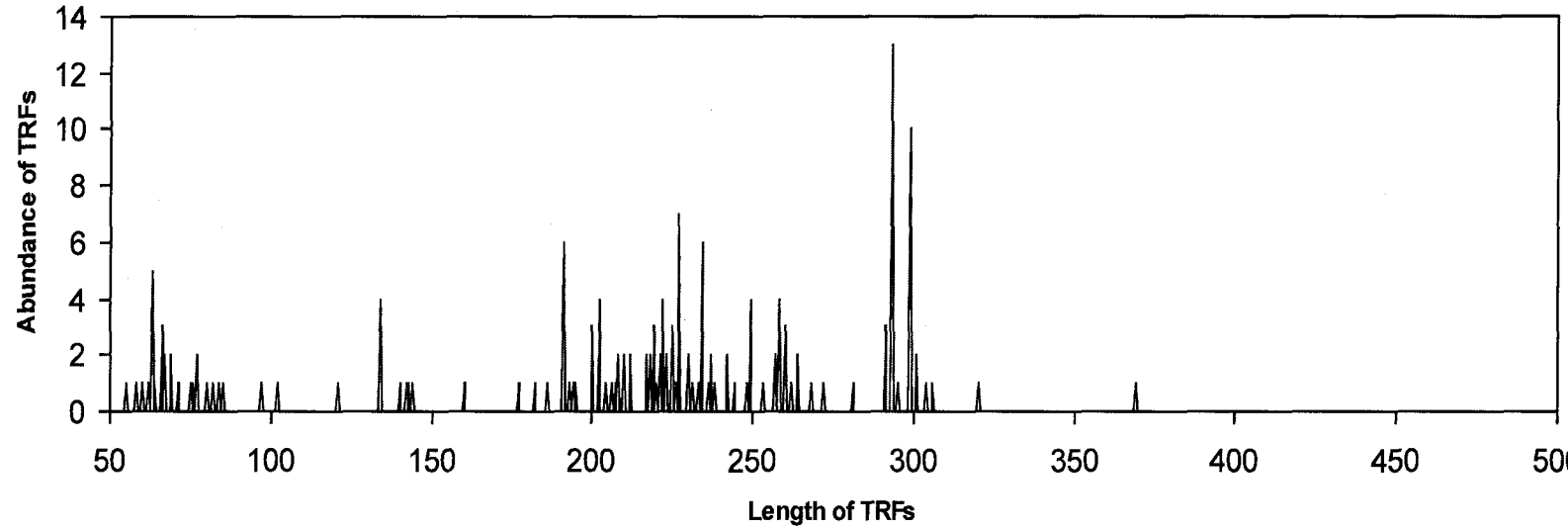

Abundance
25
20
15
10
5
0
50
100
150
200
250
300
350
400
450
500
Size of TRFs (bp)
Intensity
1,800
1,600
1,400
1,200
1,000
600

Figure 18: Abundance of TRFs generated from HUP^+ nodules soil clone library (above) and T-RFLP (below) by restriction enzyme *Hae*III


Abundance of TRFs
0
2
4
6
8
10
12
14
50
100
150
200
250
300
350
400
450
500
Length of TRFs

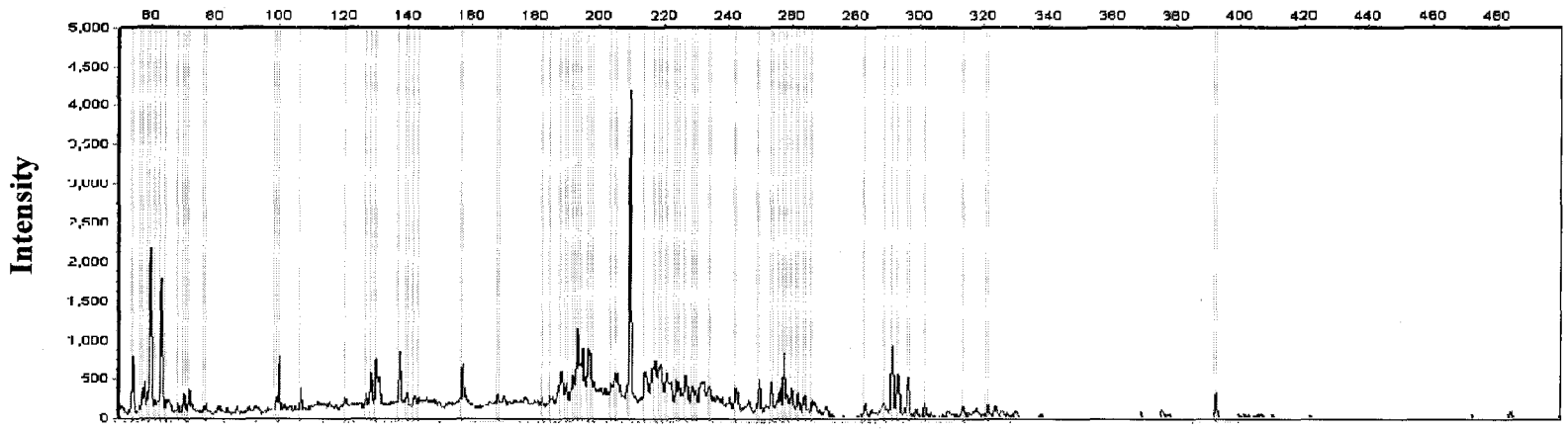
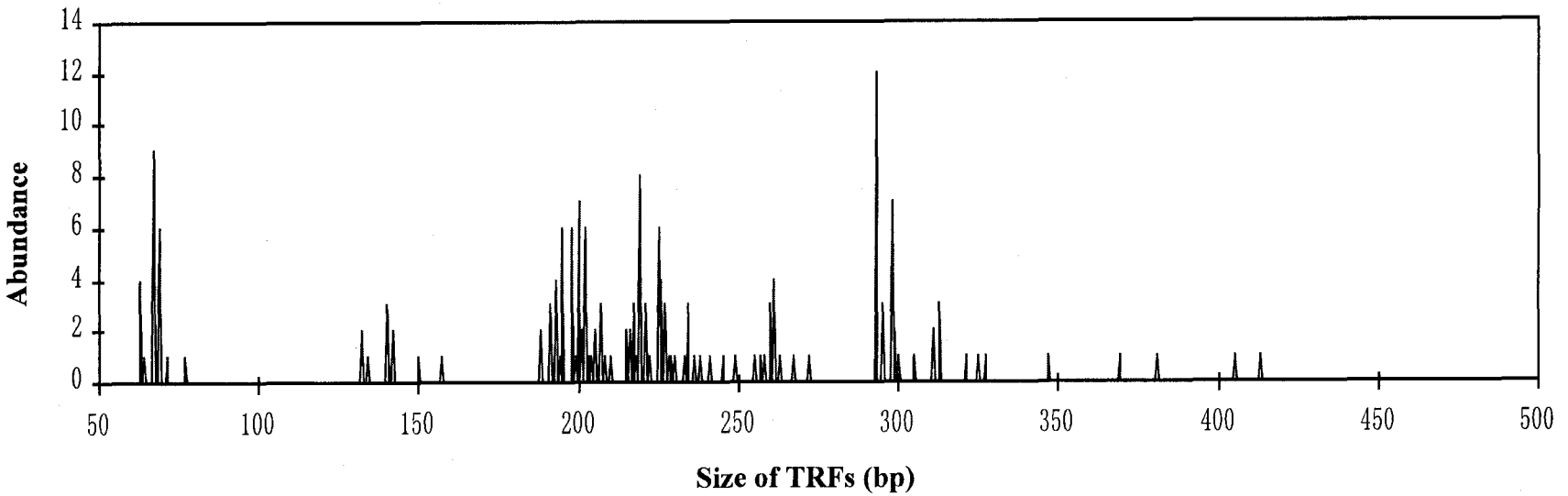

Intensity
0
500
1,000
1,500
2,000
2,500
3,000
3,500
4,000
4,500
5,000
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
460
480

Figure 19: Abundance of TRFs generated from HUP$^-$ nodules soil clone library (above) and T-RFLP (below) by restriction enzyme *Hae*III


Abundance
14
12
10
8
6
4
2
0
50
100
150
200
250
300
350
400
450
500
Size of TRFs (bp)

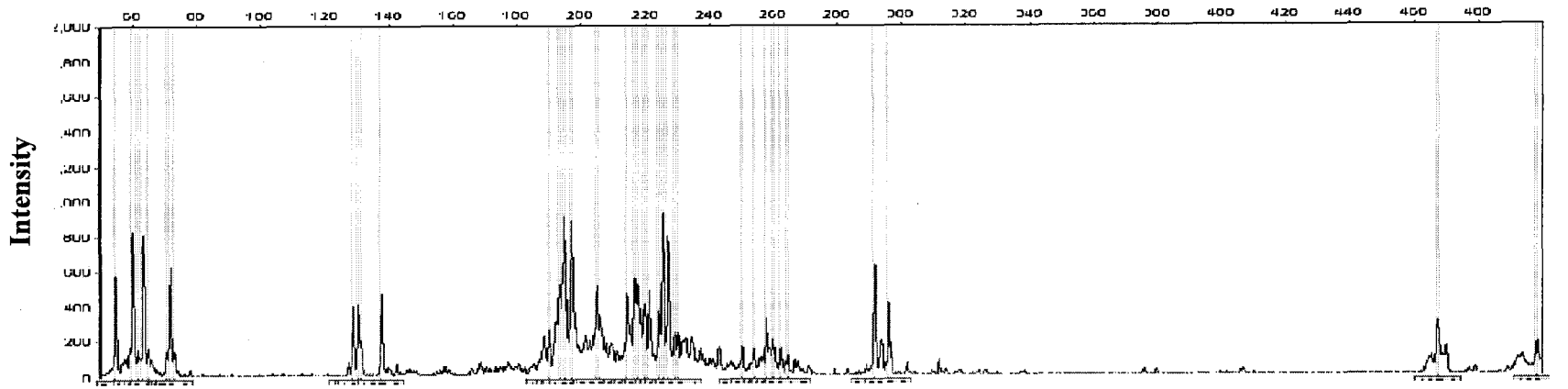
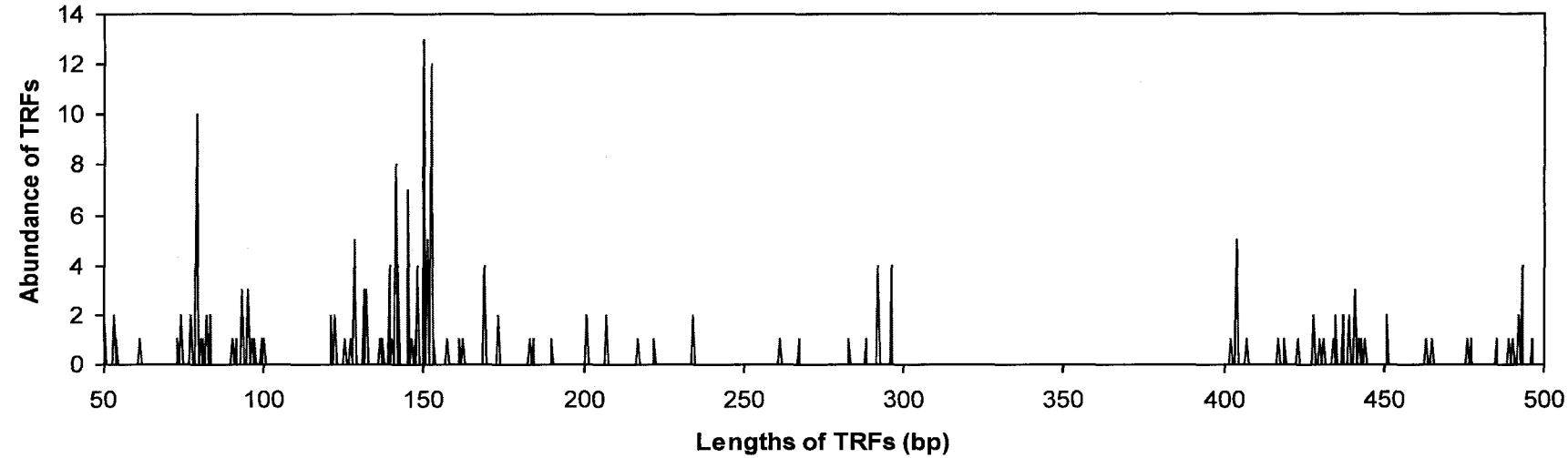

Intensity

Figure 20: Abundance of TRFs generated from HUP^+ nodules soil clone library (above) and T-RFLP (below) by restriction enzyme *Msp*I


Abundance of TRFs
14
12
10
8
6
4
2
0
50
100
150
200
250
300
350
400
450
500
Lengths of TRFs (bp)

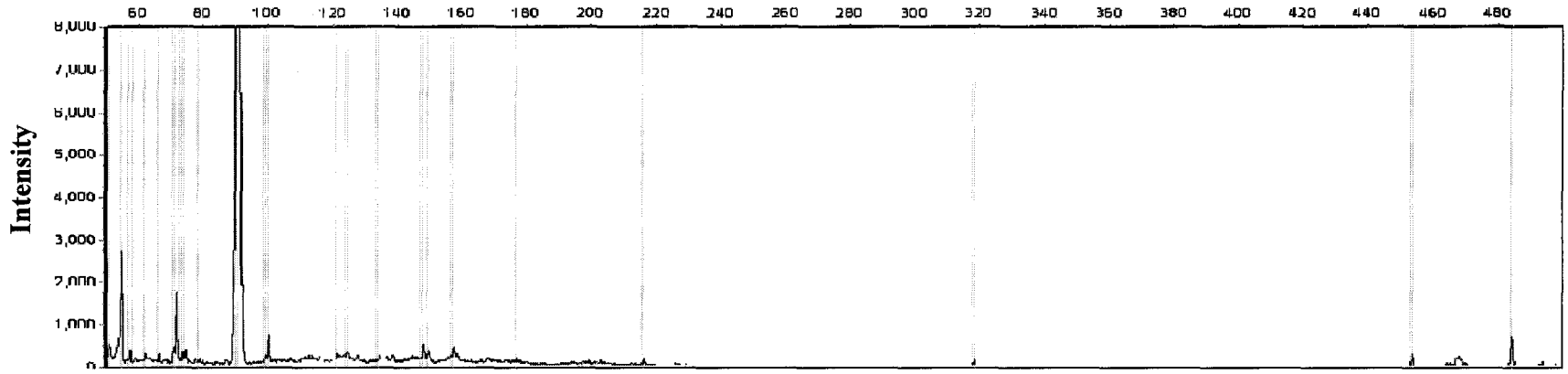

Intensity
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
460
480
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0

Figure 21: Abundance of TRFs generated from HUP$^-$ nodules soil clone library (above) and T-RFLP (below) by restriction enzyme *Msp*I

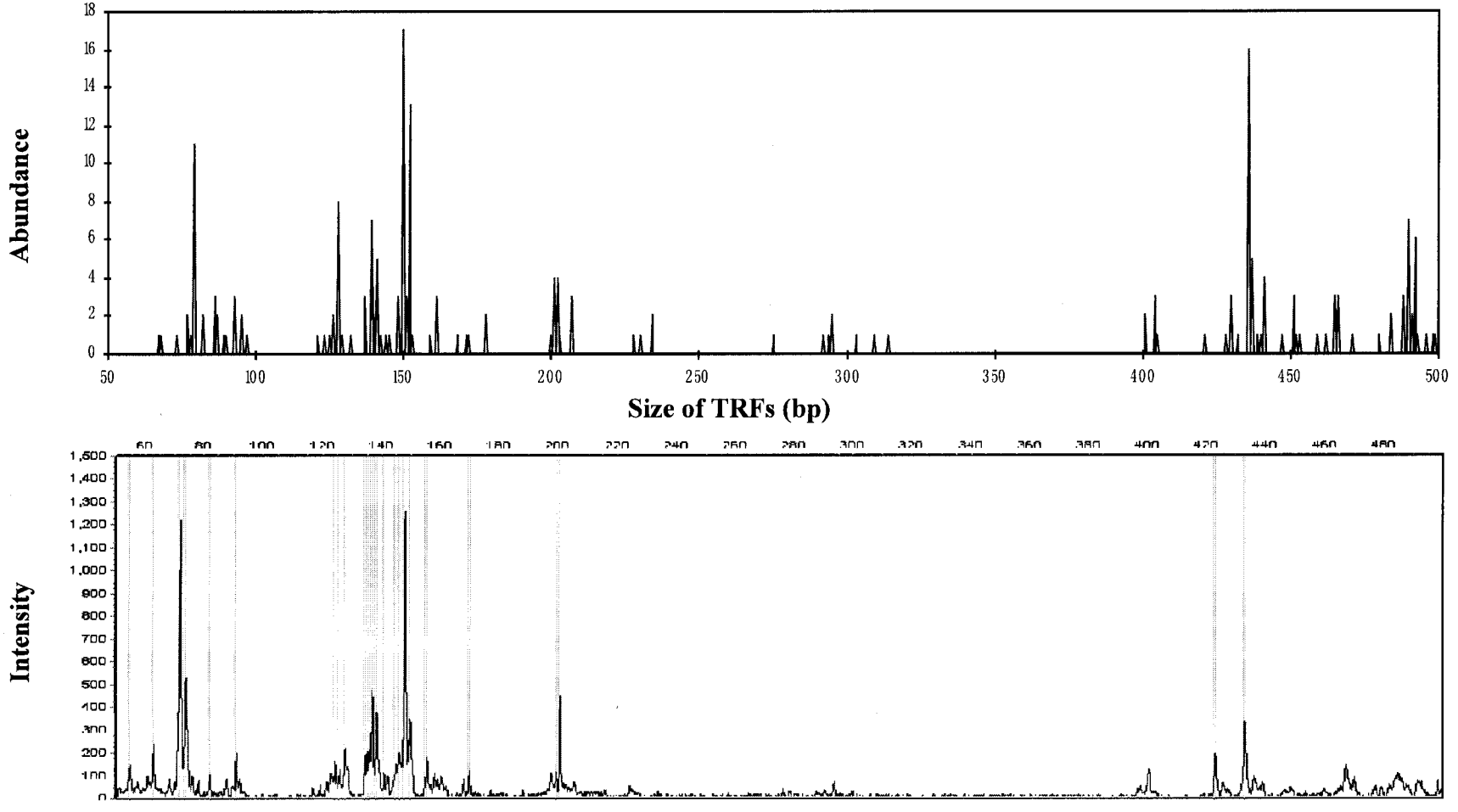
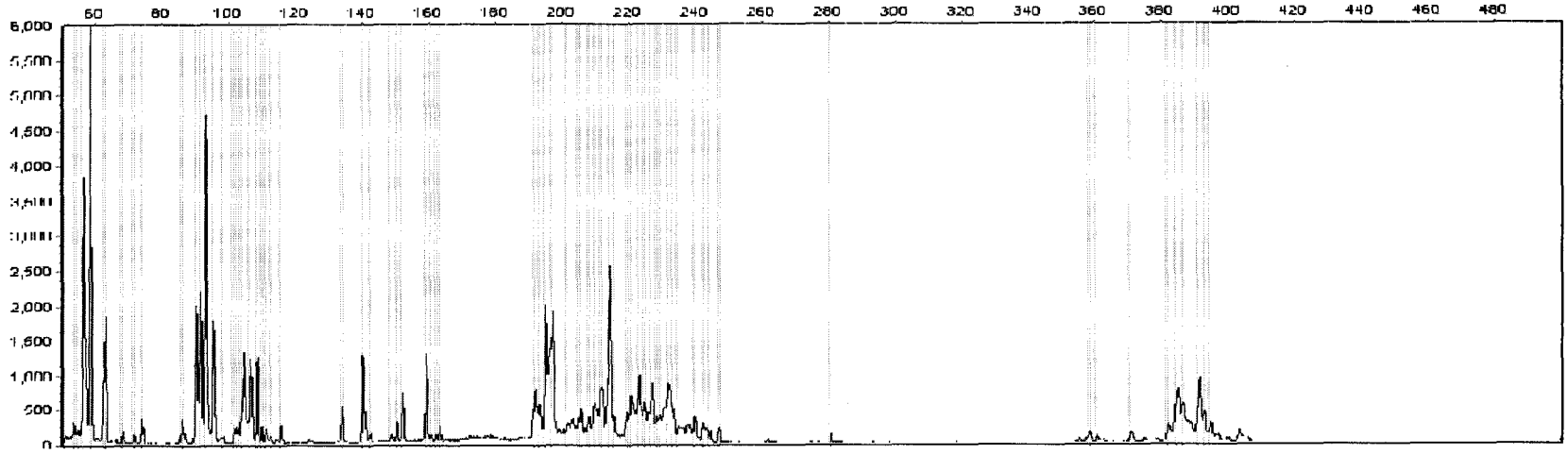

Abundance
Size of TRFs (bp)
Intensity

Figure 22: Abundance of TRFs generated from control soil (above) and bulk soil (below) by *BstU*I


60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460 480
6,000
5,500
5,000
4,500
4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
0

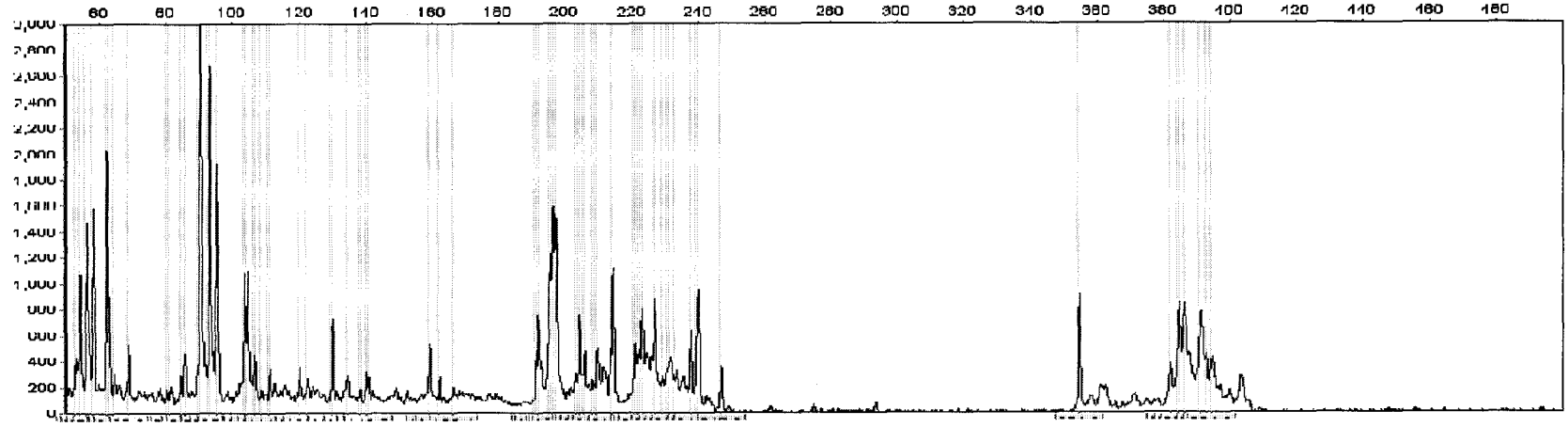
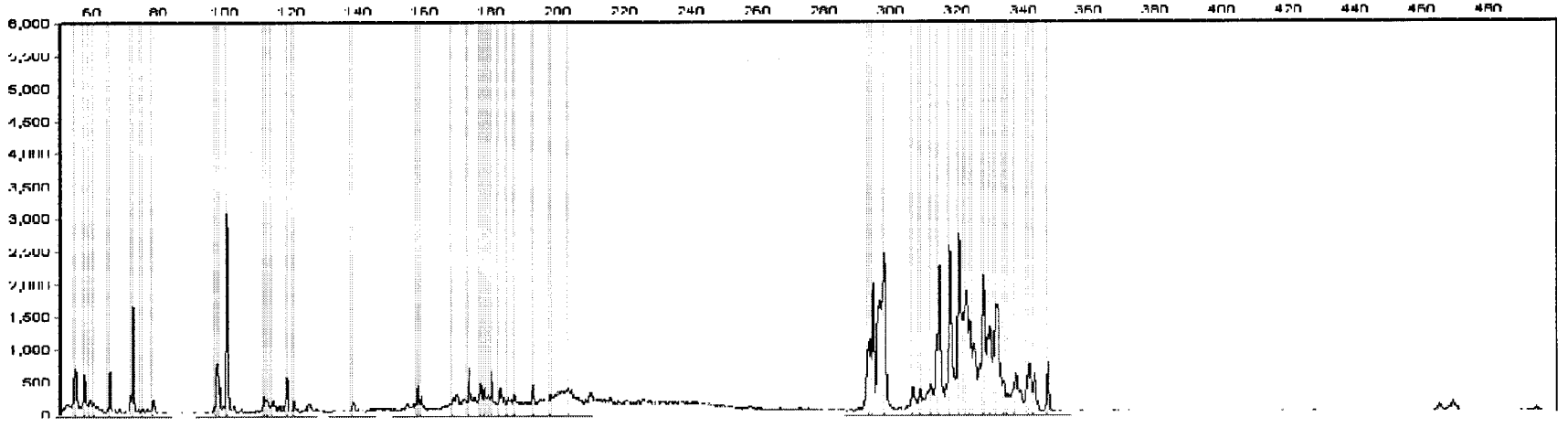

60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460 460
2,800
2,600
2,400
2,200
2,000
1,800
1,600
1,400
1,200
1,000
800
600
400
200
0

Figure 23: Abundance of TRFs generated from control soil (above) and bulk soil (below) by *Hin*fI


60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
460
480
6,000
5,500
5,000
4,500
4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
0

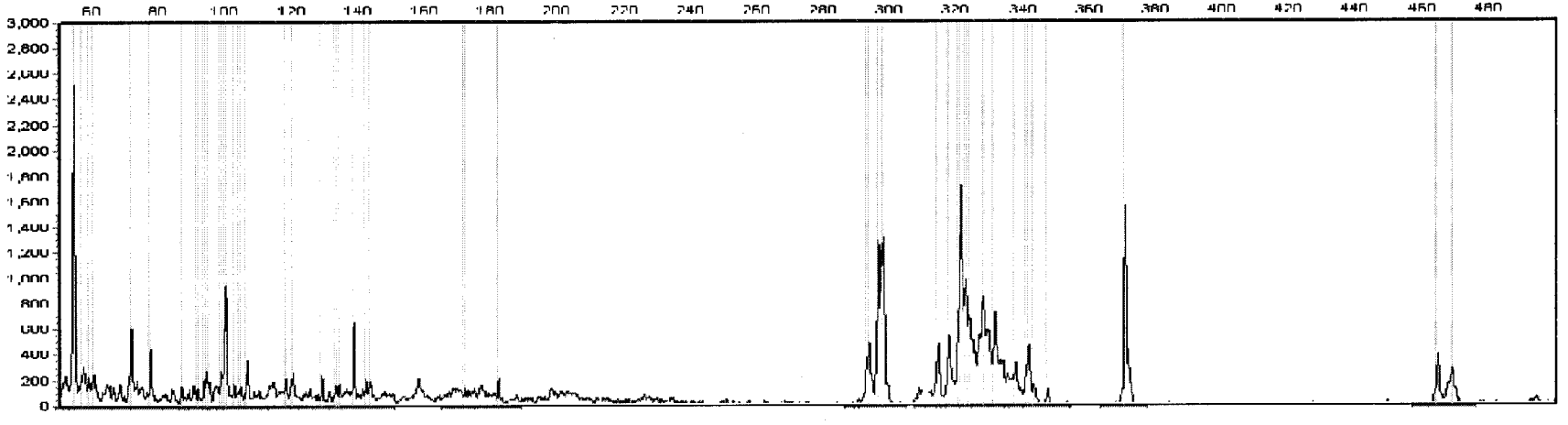
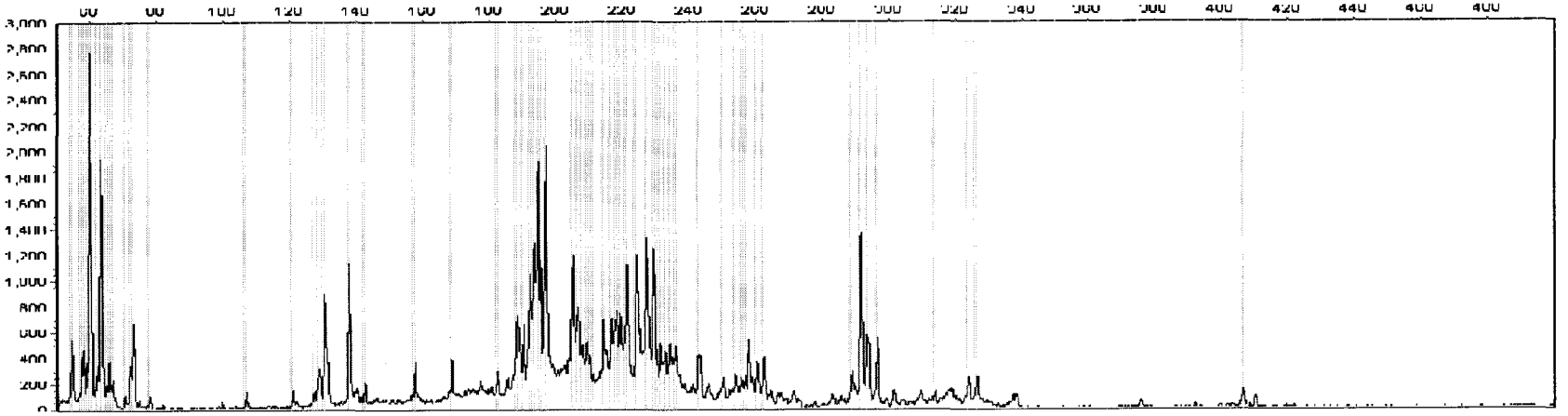

60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
460
480
3,000
2,800
2,600
2,400
2,200
2,000
1,800
1,600
1,400
1,200
1,000
800
600
400
200
0

Figure 24: Abundance of TRFs generated from control soil (above) and bulk soil (below) by *Hae*III



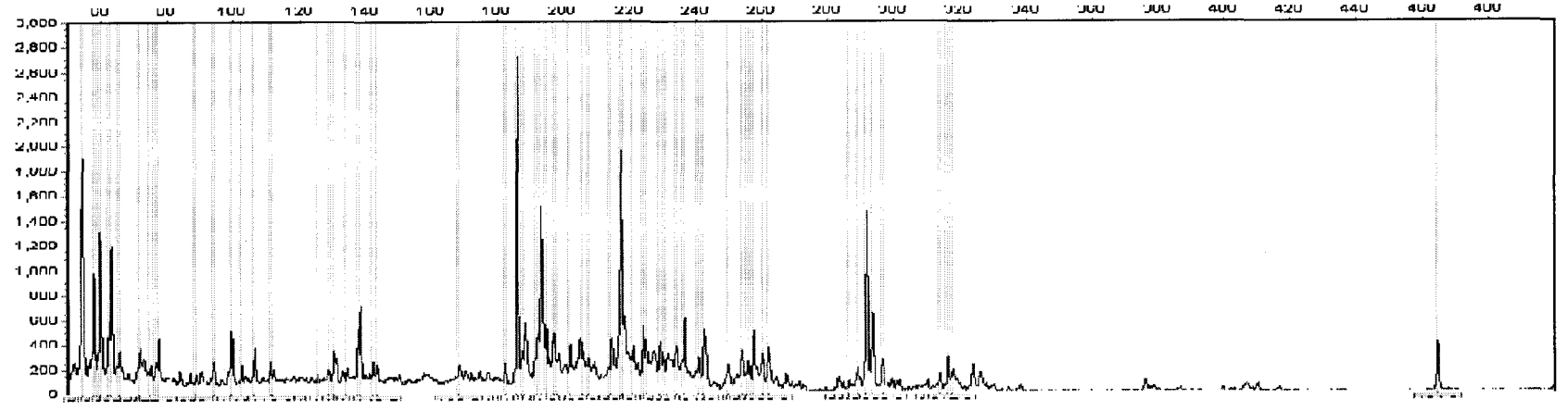
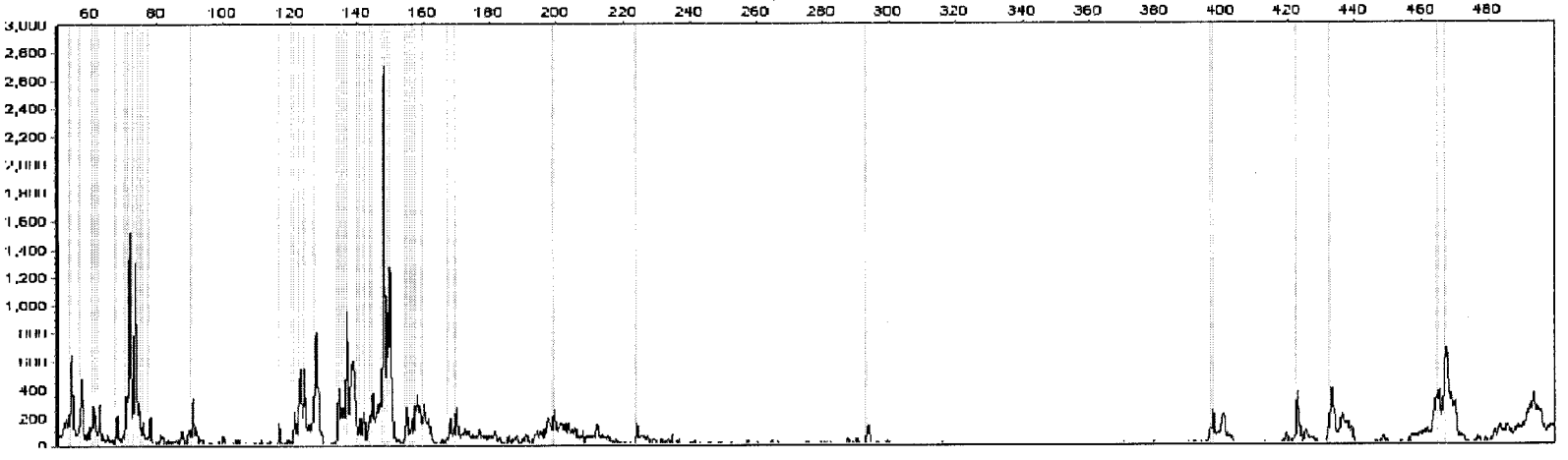
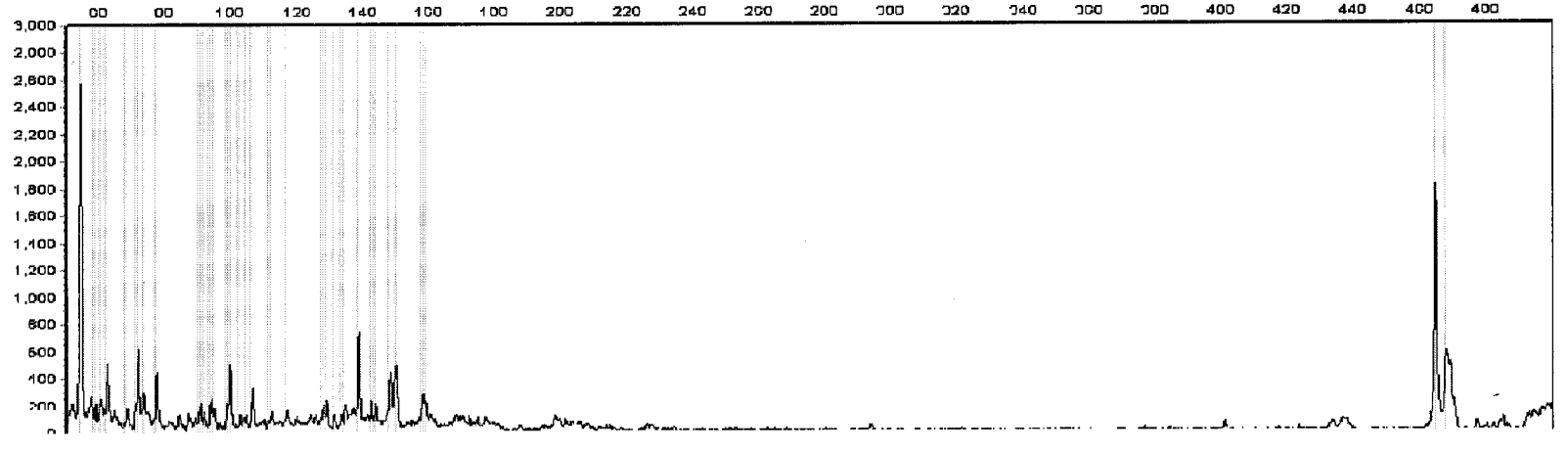


Figure 25: Abundance of TRFs generated from control soil (above) and bulk soil (below) by *Msp*I


60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460 480
3,000
2,800
2,600
2,400
2,200
1,600
1,400
1,200
1,000
400
200
0


60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460 480
3,000
2,000
2,800
2,400
2,200
2,000
1,800
1,600
1,400
1,200
1,000
800
600
400
200
0

3.4 Discussion

This is the first attempt to measure H_2 effect on the soil bacterial community structure using a combination of results from T-RFLP and clone libraries methods. The richness and relative evenness of microbial community reflect effect of H_2 that shape diversity with communities (Dunbar *et al.*, 2000). Acquiring of these values is very useful to assess treatment effects (Dunbar *et al.*, 2000).

3.4.1 T-RFLP and clone library

T-RFLP is a robust and reproducible method and has been used successfully for comparing microbial communities in different samples, like soil, waste water, intestinal samples (Wang *et al.*, 2004). It is currently one of the most rapid and powerful methods in microbial ecology to monitor the spatial and temporal changes in microbial community structure. Nevertheless, interpreting the data sets generated from complex communities is difficult (Wang *et al.*, 2004). And it is also hard to get the detailed taxons and information about their population change with confidence.

Clone libraries of 16S rDNA amplified from different samples have been applied to investigate the diversity of microorganisms in samples, which provide accurate sequences and relative abundance of individuals. However, generating libraries is an expensive and inefficient approach when comparison of complex and multiple communities is required (Dunbar *et al.*, 2000). Thus, it is not suitable for comparative analysis of a large number of samples (Wang *et al.*, 2004).

Application of T-RFLP to corroborate the results of gene clone libraries is an effective way to investigate and document differences in community structure with the necessary resolution. T-RFLP profiles reveal the whole community structure, though clone library only covers partial of the community. How many samples composing of library are suitable to represent the community is a question. Whether results of clone libraries can be used to show the detailed changes of the community with confidence is another question. Artificial T-RFLP profiles generated from clone library sequences is an attempt in this study to compare T-RFLP and clone library visually. According to the comparison results (3.3.2.3) of T-RFLP and artificial T-RFLP profiles generated from clone libraries, the similar patterns of TRFs distribution were found. It affirmed that combining results of two methods were reliable.

3.4.2 H_2 induced soil bacterial population changes

The apparent dominant groups from both methods results were *Proteobacteria*. *Gammaproteobacteria* subgroup and *Bacteroidetes* increased dramatically based on the analysis results.

Results between comparison of laboratory treatment and greenhouse condition were a little inconsistent. After treatment of H_2 in lab, it promoted *Gammaproteobacteria* and *Sphingobacteria* increasing mostly. However, between two communities of soil adjacent to HUP^- and HUP^+ nodules, except for those two groups, *Alpha-*, *Betaproteobacteria* and *Flavobacteria* increased as well. This difference can be explained by the existence of plants.

Compared with results of Zhang Y.'s work and this study, although the soils were different, the increased groups were similar. The population of *Gammaproteobacteria* and *Bacteroidetes* arose after H_2 treatment in common for both studies.

Liesack and Stackebrandt (1992) used clone libraries to examine non-cultivated bacteria in an Australian soil and identified a large percentage of clones related to the N-fixing bacteria in the α subclass of the Phylum *Proteobacteria.* Ueda *et al.* (1995) identified 16S rDNA sequences from soybean field soil with similarity to the high GC content subdivision of Gram-positive bacteria, green sulfur bacteria and *Proteobacteria.* Borneman *et al.* (1996) found 16S rDNA clones from *Proteobacteria*, the *Cytophaga-Flexibacter-Bacteroides* group, and low GC content Gram-positive bacteria in soil from an American clover-grass pasture. Using FISH analysis and DNA staining, they found that bacterial community structure changed. H_2 treatment stimulated β –and γ – subclasses of *Proteobacteria* and *Cytophaga-Flavobacterium-Bacteroides* phylum (Stein *et al.*, 2005). Compared with these previous studies, our work presents the similar results. It should be noticed that the collected soil samples are different among the studies. Some were collected from the top 10cm of field soil; some were taken from a zone 5 to 10cm below the surface of soybean or clover field. In addition, the treatment methods were different as well.

All above, the soils and treatment methods were different in these studies, but the selective pressure, H_2 is the same. In conclusion, H_2 promote population of *Gammaproteobacteria* and *Betaproteobacteria* in soil bacterial community.

3.4.3 Polymerase chain reaction (PCR) amplification bias

The PCR is widely used for the study of rRNA gene amplified from mixed microbial population. Amplification of bacterial 16S rDNA from extracted total DNA from soils give the most comprehensive and rapid ways of sampling. Both T-RFLP and clone library based on PCR amplification have to be concerned about the bias of PCR.

The frequency of different sequences in PCR-derived rRNA gene has sometimes been assumed to represent the relative abundance of different components of a microbial community. This cannot be claimed with any confidence, as the copy number of rRNA genes present within the genomes of different organisms can range from 1 to 14 (Head *et al.*, 1998). There is no obtained information about the relative efficiency of gene amplification in mixed-template PCR. Thus, you cannot assume the gene rations in the starting mixture relate to an abundance of amplicons of product.

In this study, to avoid the bias of PCR, 3 replicates for clone library and 10 replicates for T-RFLP were performed. Results show two sets of profiles matched, which means that replicate was useful to reduce the PCR bias.

Although a determination of the exact number of species is not feasible, the direction of DNA from soil and the amplification of rDNA genes provide a method of identifying many previously unknown microorganisms (Borneman, 1996).

4 Quantification of *nirK* gene encoding copper nitrite reductase in soils

4.1 INTRODUCTION

4.1.1 Nitrous Oxide (N_2O)

Nitrous oxide has been known for about 70 years as regular constituent of the atmosphere (Adel, 1939). But it was not considered as an important air constituent until 1970s. Scientists found that nitrous oxide in the troposphere can absorb terrestrial thermal radiation and thus contribute to greenhouse warming of the atmosphere (Crutzen, 1972). On a mass basis, N_2O is approximately 300 times more of potential impact than CO_2, and contribute about 6% of the total anthropogenic radiative forcing (IPCC, 2001; Davidson, 2009). Its lifetime is really long between 110 and 150 years (Hao *et al.*, 1987; Ko *et al.*, 1991). Nitrous oxide is also involved in the depletion of the ozone layer in the stratosphere which protects the biosphere form the harmful effect of solar ultra violet radiation (Crutzen, 1981). In the last few decades, the concentration of N_2O in the atmosphere has progressively increased at an annual rate of 0.2-0.3% as a result of human activities (Rasmusen and Khalil, 1986; Prinn *et al.*, 1990). About 70% of the anthropogenic N_2O increase is attributed to agriculture (Watson *et al.*, 1992).

Microbial production in soils is the dominant nitrous oxide source. Davidson (2009) showed that 2.0% of manure nitrogen and 2.5% of fertilizer nitrogen was converted to nitrous oxide between 1860 and 2005. In most agricultural soils, biogenic

formation of N_2O is enhanced by an increase in available mineral N which, in turn increases nitrification and denitrification.

Besides nitrifying and denitrifying bacteria, several microorganisms are able to produce N_2O. For example, it has been observed that N_2O is produced by certain fungi (Bollag and Tung, 1972; Burth and Ottow, 1983; Shoun *et al.*, 1992), by many nondenitrifying nitrate-reducing bacteria (Anderson and Levine, 1986; Smith and Zimmerman, 1981) and by some yeast during assimilatory nitrate reduction (Bleakley and Tiedje, 1982). There are also abiological processes such as chemodenitrification, which contribute very little N_2O to the evolution from soils (Bremner and Nelson, 1968).

4.1.2 Denitrification

Denitrification refers to the process of dissimilatory nitrate (NO_3^-) reduction that may finally produce dinitrogen (N_2) through a series of intermediate nitrogen oxide products. Denitrification generally proceeds through the following forms: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. The process is used for energy production when oxygen is limited, and return fixed nitrogen to the atmosphere from soil and water, thus completing the N cycle. Denitrification is influenced by temperature and pH, and occurs frequently when the soil becomes waterlogged or when anaerobic microsites happen in aerobic soils (Turner and Hummel, 1992).This removal of soluble nitrogen oxide from the biosphere is of great important in agriculture, where it can account for significant losses of nitrogen fertilizer form soil and also in wastewater treatment. The other important reason why denitrification received considerable interest is its leading N_2O emission, which is one of the three main greenhouse gases.

4.1.2.1 Denitrifier

Denitrification is carried out by respiratory denitrifiers that gain energy by coupling N-oxide reduction to electron transport phosphorylation (Tiedje, 1988). Denitrifiers are the denitrifying agent, which engage denitrification process. The difference between denitrifier and nitrate-respiring bacteria is nitrate-respiring bacteria do not reduce nitrite to gas (Prieme, 2002). Nitrate-respiring bacteria only reduce nitrate to nitrite ($NO_3^- \rightarrow NO_2^-$), while others are capable of further reduction to free nitrogen and are termed denitrifying bacteria or denitrifiers (Wang and Skipper, 2004). Denitrifying bacteria can be found in nearly all soils and survive under anaerobic conditions (Tiedje, 1988).

Although, only a small range of bacteria is capable of denitrification, the distribution of denitrifiers does not follow a distinct pattern. Nearly 130 species of bacteria and archaea belonging to more than 50 genera can denitrify (Zumft, 1992). *Pseudomonas, Bacillus, Paracoccus, Alcaligenes* and *Enterobacter* have been identified as dominant denitrifiers in soil (Weier and MacRae, 1992). In domain bacteria, most of them belong to various subclasses of the *Proteobacteria* (Zumft, 1997). It is reported that some symbiotic N-fixing bacteria have the ability to denitrify nitrate under anaerobic conditions and the ability is widespread among agricultural important species of *Rhizobium*, especially among free-living cells of *Bradyrhizobium* spp. (O'hara and Daniel, 1985).

4.1.3 H_2 effect on N_2O production

It has been observed that 1.4-1.9 folds higher rate of N_2O production in the corn field whose soil exposed to nodulated legume roots than soil exposed to non-nodulating legume roots and control bulk soil (unpublished by Dr. Cen in Queen's University). However, there was no significant difference between the HUP^+ and HUP^- symbioses. This result indicated the effect of soybean nodule development on soil N_2O emission was not only during planting soybeans, but also in subsequent year with corns (not legume).

When H_2-oxidizing bacteria isolates were introduced to bulk soil that had not seen legumes for at least 5 years, significant differences of N_2O emission between inoculate soil with isolates and control soil were found (unpublished). It was assumed the presence and activity of denitrification and nitrification genes associated with these H_2-oxidizing bacteria strains.

4.1.4 Copper Nitrite Reductase Gene (*nirK*)

Genes involved in denitrification pathways have been characterized and utilized to detect denitrifiers' populations in environmental samples. By PCR amplification of denitrification-related genes, some nonculturable soil denitrifiers were identified and found to belong to several phylogenetic clusters that were different from the culturable denitrifiers (Primem *et al.*, 2002; Gregory *et al.*, 2003). These genes include *narG* and *narH* encoding nitrate reductases that catalyze nitrate reduction (Petri and Imhoff 2000; Gregory *et al.*, 2003); *nirK* and *nirS* encoding nitrite reductases catalysing nitrite reduction (Braker *et al.*, 1998, 2000); *norB* encoding nitric oxide reductase (Braker and Tiedje, 2003); and *nosZ* encoding nitrous oxide reductase that catalyses the final step of

denitrification (Chan and Wheatcroft, 1993; Holloway *et al.*, 1996; Scale and Kerhof, 1998, 1999).

Among all the involved genes, *nirK* and *nirS* are the most well-studied genes. During denitrification, nitrite reductase is the key enzyme of this respiratory process since it catalyzes the reduction soluble nitrite into gas. Two types of this enzyme are found among denitrifiers. They are characterized by structure and prosthetic metal: a copper nitrite reductase encoded by the *nir*K gene and a cytochrome cd_1-nitrite reductase encoded by the *nir*S gene. No functional differences of these two enzymes have been reported so far (Kim *et al.*, 2006).

4.1.5 Real-Time PCR technique

Quantitative nucleic acid sequence analysis is very important in biological research and clinical diagnostics. Real-time PCR has been become a well-established procedure for quantification. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of a specific sequence in a DNA sample. Many methods have been described for the quantitative analysis of RNA and DNA. One approach is based on the binding of the fluorescent dye SYBR Green into the minor groove of helix DNA product in a sequence-independent way. The dye binds to all double-strand DNA in PCR, causing the fluorescence dye emitting. An increase in DNA product during PCR therefore leads to an increase in fluorescence intensity. Its power resides in the ability to detect the amount of PCR product at the end of each cycle.

Some detection systems, such as agarose gels, fluorescent labeling of PCR products and detection with laser-induced fluorescence using capillary electrophoresis or acrylamide gels, plate capture and sandwich probe hybridization, are used for quantitative PCR and RT-PCR analysis (Heid *et al.*, 1996).

A real-time PCR read-out is given as the number of PCR cycles (cycle threshold, Ct) necessary to reach a level of fluorescence. The advantages of using SYBR Green quantification over a 5'-nuclease assay with TaqMan™ probes are the relative simplicity and the reduced cost of SYBR Green compared to TaqMan™ probes.

4.1.5.1 Internal Control vs. Standard Curve

To obtain relative quantification, the unknown target PCR product is compared with the known PCR product (Heid *et al.*, 1996). To date, internal control genes are most frequently used to normalize the gene copies. This internal control- often referred to as a housekeeping gene- should not vary in the tissues or cells under investigation, or in response to experimental treatment (Vandesompele *et al.*, 2002). Common internal controls include β-actin and GAPDH mRNAs and 18S rRNA.

In the standard curve method, a standard curve is first constructed from a DNA of known concentration. This curve is then used as a reference standard for extrapolating quantitative information for DNA targets of unknown concentrations. It will help generate absolute copy number data.

For bacteria, internal control as 16S rRNA in well-study isolated strain and standard curve are usually used in the quantification research.

4.2 MATERIAL AND METHODS

4.2.1 Soil Treatment

Soil treatment was carried out by Dr. Yanping Cen. (Queen's University). Soil that had not seen legumes for more than 20 years (AAFC, Eastern Cereal & Oilseed Res Ctr, Ottawa) were mixed with silica sand (16 mesh) in a 4:1 (soil:sand, v:v) ratio to prevent clumping. Deionized water was added to the soil to reach a water content of 16% (w:w, H_2O:dry soil). About 2L of soil was placed in each of 12 plastic, 3.8 L containers, and each container was connected to a H_2 treatment system. In this system, fresh air from outside of the building drawn by 3 pumps (Model LR91926 Hagen, Montreal, QC Canada) was passed through 3 flasks of deionized water to provide humidification of the gas stream. Before humidification, 5% H_2 in N_2 (about 50mL min^{-1}) was added to 2 of the gas streams. The gas stream exiting each flask was provided to 4 of the soil containers. In this system, the high H_2 treatment soil (HH, 4 replicates) received H_2 at a rate of 200 μmolH_2 L^{-1}soil hr^{-1}, an exposure rate calculated to be representative of that measured in soil within a few centimeters of a legume nodule (Dong and Layzell, 2001). The medium H_2 treatment soil (MH – 4 replicates) received H_2 at a rate of 20 μmolH_2 L^{-1}soil hr^{-1}. The air treatment soil (Air) received H_2 at a rate of 0.1 μmolH_2 L^{-1}soil hr^{-1}. Every week, H_2 concentration in the inlet and outlet gas streams was measured by a H_2 sensor (Model S121, Qubit Systems Inc., Kingston, Canada) by drawing a portion of the inlet and outlet gas stream with a pump (Model 110, WISA GmbH, Wuppertal, Germany). The gas stream is dehumidified through a drying column (Mg perchlorate, 15 mL) before it reaches the H_2 sensor. The H_2 uptake rate of the soil was calculated from the flow rate

supplied to the soil chamber and the differences of H_2 concentrations between the inlet and outlet of the chamber gas streams. After 10, 56, 87, 108 days of H_2 or air exposures, containers were sealed over night, and the headspace air samples were draw using a syringe via a septa opening. N_2O levels in the samples were measured with a gas chromatograph (8610C Greenhouse gas GC system, SRI Instruments, Torrance, CA, USA) equipped with a 63Ni electron capture detector. The soil was delivered to Saint Mary's University and stored at -80℃ immediately until use.

4.2.2 Detection of key genes of denitrification pathway in isolates

Five pairs of primers (Table 10) were used to amplify *nirS, nirK, qnorB, cnorB* and *nosZ* genes in denitrification pathway in isolates.

PCR condition was as follow: 5 minutes at 95°C for denaturation, 40 cycles of 30s at 95°C, 45s for annealing, 45s at 72°C for extension, and a final cycle, 10 minutes at 72°C. 25μl PCR system included: 1mM per primer 2.5μl of 10× Buffer, 2.5μl of 2mM dNTP, 2μl of $MgSO_4$, 0.2μl of 5U/ μl Taq enzyme (UBI Life Sciences Ltd. SK, Canada)), a tiny bit of isolate colony as DNA template, and Sigma® water to complete the 25μl volume. The annealing temperature for 5 pairs of primers was 57°C for *nirS, nirK* and *nosZ*, 54°C for *qnorB*. The result was checked by 1% agarose gel with ethidium bromide stain.

4.2.3 Generating Standard curve

4.2.3.1 Total Soil DNA Extraction

Soil samples from Queen's University were frozen at -80°C immediately after receiving. Extraction of 1g soil DNA for each sample was performed with UltraClean™ DNA Purification Kit (MO BIO Laboratories Inc., Solana Beach, CA). For maximum yields, Alternative Protocol offered by MO BIO Labaratory Inc., was followed. The 0.5 gram soil was added to the 2ml Bead Solution tube provided and vortexed gently. 60µl Solution S1 and 200µl of Solution IRS (Inhibitor Removal Solution) were added into the tube and vortexed at maximum speed for 10 minutes. Following a centrifugation for 30s at 10000×g, the supernatant was transferred to a clean microcentrifuge tube. 250µl of Solution S2 was added to the tube and incubated for 5 minutes at 4°C after gently vortexing. Followed by 1 minute at 10000×g centrifuge, the clean supernatant was transferred to a new tube. 1.3ml of Solution S3 was added to the supernatant and mixed by vortexing. 700µl of the mixture was loaded onto a spin filter and centrifuged at 10000×g for 1 minute. The flow-through was discarded and the remaining supernatant was added to the spin filter. Repeat until all supernatant has passed through the spin filter. 300µl of Solution S4 was added to the spin filter and centrifuged for 30s at 10000×g. After the flow through was discarded, the spin filter was centrifuged again. 50µl of Elution Buffer was added to the center of the filter membrane and the harvested DNA was eluted from the filter into the flow through followed a centrifugation at 10000×g for 1 minute. After measure the concentration of the DNA samples by using Nanodrop® (Thermo Fisher Scientific, Wilmington, Delaware) store them at -20°C.

4.2.3.2 Amplification for Cloning

PCR condition was as follow: 3 minutes at 95°C for denaturation, 40 cycles of 30s at 95°C, 30s at 50°C for annealing, 30s at 72°C for extension, and a final cycle, 10 minutes at 72°C. Multiple PCR reactions were pooled together to increase final concentration. PCR products were purified with the QIAquick® PCR purification kit (QUAGEN Inc. Mississauga, ON). 25µl PCR system included: 1mM primers, nirK876 (5'-ATYGGCGGVAYGGCGA-3') and nirK1040 (5'-GCCTCGATCAGRTTRTGGTT-3'), 2.5µl of 10× Buffer, 2.5µl of 2mM dNTP, 2µl of $MgSO_4$, 0.2µl of 5U/ µl Taq enzyme (UBI Life Sciences Ltd. SK, Canada)), 0.5µl of DNA template corresponding to 15 to 20ng of total DNA, and Sigma® water to complete the 25µl volume.

4.2.3.3 Cloning

The amplicons were ligated into the pGEM®-T Easy Vectors with pGEM®-T Easy Vectors System (Promega Corporation, Madison, WI) followed by the same protocols above. The products were transformed to JM109 cells and screened for positive colonies.

Three plasmid DNA samples extracted from positive colonies were sent for sequencing. Sequencing results were identified using BLAST in GenBank, and checked manually with primers, size and enzyme cutting site.

4.2.3.4 Plasmid Linearization

Before real-time PCR, the plasmid DNA should be linearized. 1µg of each sample was digested by 60U SalI at 37°C for 2 hours. Afterward, the reaction was stopped by

using QIAquick® Gel Extraction Kit (QUAGEN Inc. Mississauga, ON). The products were checked by 1% agarose gel, ethidium bromide stain.

4.2.3.5 Generation of Standard Curve

Real-time PCR was carried out in ABI Prism 7000 (Applied Biosystem, Foster City, CA). The reaction system contained: 0.5mM primers, 1μl linearized plasmid DNA, 12.5μl of Power SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA). The condition of real-time PCR was similar with regular PCR. The differences were one additional step, 2 minutes at 50°C before denaturation, and after 10 minutes extension, there was one more association stage.

4.2.4 Quantification of *nirK* in Soil Samples

4.2.4.1 Detect Limitation

Dilute soil DNA samples into 1ng/μl first, and then do 10-fold dilution from 10^{-1} to 10^{-6}. Add 1μl of each dilution to the 96-well plate (Applied Biosystem, Foster City, CA) as tempelate, 0.5mM primers 12.5μl of Power SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA) and fill with Sigma® water to 25μl as reaction volume. The condition of real-time PCR is the same as construction of standard curve.

4.2.4.2 Quantification of *nirK* and inhibition effect

Eleven independent real-time PCR measurements were performed on triplicate DNA extraction for each soil. Inhibition effect was determined by adding 10^3 and 10^5 copies of the standard DNA to soil samples diluted below the detection limit.

4.3 RESULT

4.3.1 N_2O emission from soil samples

Soil treatment was carried out in.Queen's University. All the data and figures about the treatment were provided by Dr. Yanping Cen (Queen's University).

The N_2O emissions rates increased sharply for the first 56 days of soil exposed to either high (H) or medium (M) H_2 levels. While the N_2O emissions rate continue to increase from day 56 to 87 in soils exposed to M H_2 level, the N_2O emissions rate in soils exposed to H H_2 level reached its saturation value at day 56. After 87 days of H_2 exposure, the N_2O emission rates were similar for both H H_2 and M H_2 treatments. The air treated soil showed a constant low N_2O emission rates over the whole 108 days period (Fig. 26).

4.3.2 Detection of key genes of denitrification pathway in isolates

Five isolates, JM63, JM162, YP29, YP64 and YP73 were detected with 5 pair of primers for *nirS, nirK, qnorB, cnorB* and *nosZ* genes. The results showed that *qnorrB* gene was detected in JM162 and YP64; *nosZ* gene was contained in JM63, JM162 and YP64 (Table 10). The other 3 pairs of primers for *nirS, nirK* and *cnorB* were failed to be detected in our isolates.

4.3.3 Standard Curve

NirK gene, encoded nitrite reductase was amplified from soil samples and cloned. Three clones were picked for sequencing and confirm the insert by BLAST in GeneBank. The

plasmids with three different nirK genes were used to generate the real time PCR standard curve respectively.

4.3.3.1 Sequencing results of 3 plasmids with target genes

Three clones of *nirK* gene obtained by PCR with nirK876 and nirK1040 primers were sequenced. Following were three sequences. Primer nirK876 highlighted with red, primer nirK 1040 highlighted with yellow, complementary strand of nirK876 highlighted with blue, complementary strand of nirK1040 highlighted with green. Three target genes were found among the whole plasmid DNA sequences by searching the forward and reverse primers, nir876 and nir1040. The length of them was all 163 bp.

>N001
ATTGGCGGAATGGCGACTACGTGTGGGCGACGGGTAAGTTCCGCAACCCGCC
CGAGGTCGATCTCGAGACCTGGCACATCCCGGGCGGCTGCGCGGGGGCTGCG
ATGTACACCTTCCTACAGCCCGGCATCTACGCCTACGTGAACCACAATCTGAT
CGAGGC

>N002
GCCTCGATCAGGTTATGGTTGACATAGGCGTAGACGCCCGGCTGCAGGAAGG
TGTAGAGCGCCGCGCCCGCCGCGCCGCCGGGGATGAACCAGGTTTCGAGGTC
CACATCCGGCGCATTGTGGAACTTGCCGGTCTGCCAGACATAGTCGCCATGC
CGCCAAT

>N003
ATTGGCGGAATGGCGACCTGGTATGGCAAGGGGGCAAATTCAGCGATCCTCC
CATCACCAACCAGGAAACCTGGTTCATCCCGGGAGGTGCGGCGGGTGCGGCC
TTGTATGAATTCAAGCAGCCCGGACTGTATGTGTACCTCAACCAGAATCTGAT
CGAGGC

Based on the identification, we confirm that the fragments inserted into plasmids are all partial *nirK* gene (Table 11). Comparing each two of these three fragments using "Align" function in BLAST showed, there is no similarity among the three fragments.

Figure 26: N_2O emissions measured from soil exposed to H_2 at 200 $\mu molH_2\ L^{-1}soil\ h^{-1}$ (solid dot); 20 $\mu molH_2\ L^{-1}soil\ h^{-1}$ (open square) and 0.1 $\mu molH_2\ L^{-1}soil\ h^{-1}$ (open circle). Vertical bars indicate ±SE (n=4 reps). Provided by Dr. Yanping Cen (Queen's University)

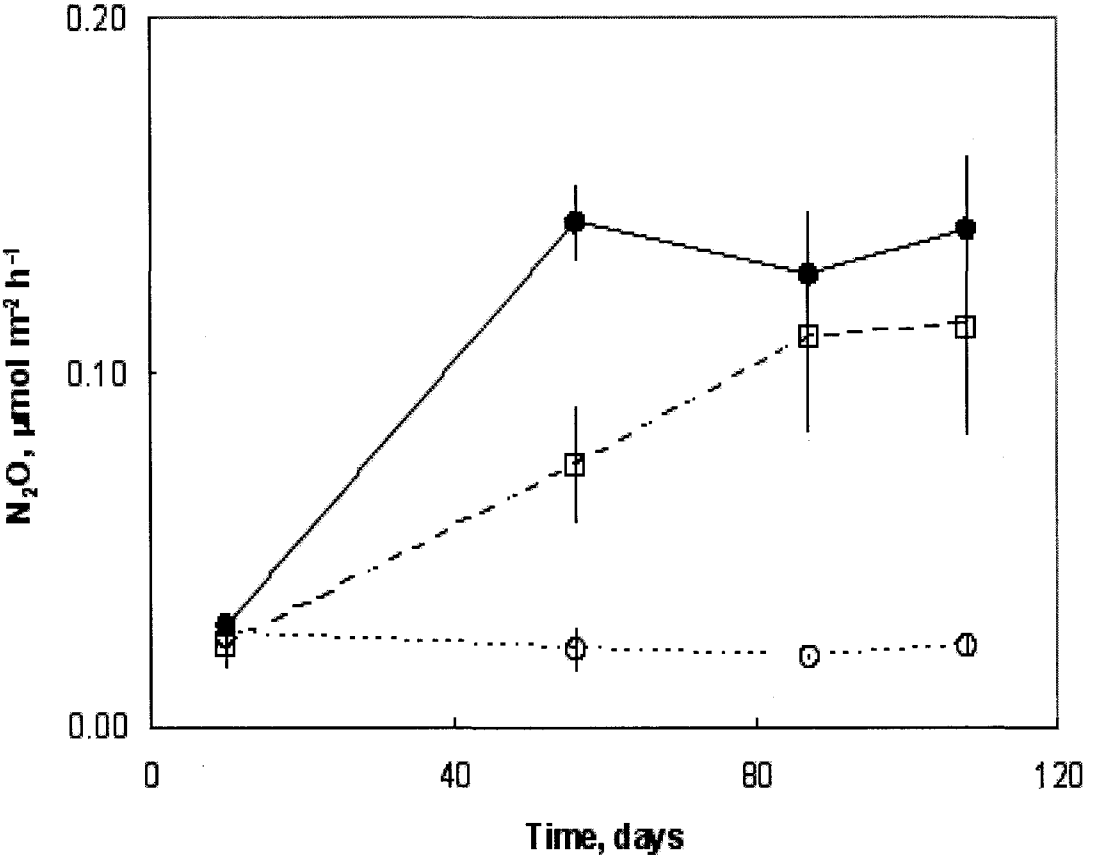

0.20
0.10
0.00
N_2O, µmol m^{-2} h^{-1}
0
40
80
120
Time, days

Table 10: Primer sequences used to amplify fragments from *nirS, nirK, qnorB, cnorB* and *nosZ* genes in the denitrification pathway.

Gene	Primer name	Sequence 5' to 3'	Length (bp)	Reference	Presence
nirS	cd3aF	GTSAACGTSAAGGARACSGG	425	Throback *et al.*, 2004	N/A
	R3cd	GASTTCGGRTGSGTCTTGA			
nirK	F1aCu	ATCATGGTSCTGCCGCG	472	Throback *et al.*, 2004	N/A
	R3Cu	GCCTCGATCAGRTTGTGGTT			
qnorB	qnorB2F	GGNCAYCARGGNTAYGA	637	Braker *et al.*, 2003	JM162, YP64
	qnorB7R	GGNGGRTTDATCADGAANCC			
cnorB	cnorB2F	GACAAGNNNTACTGGTGGT	389	Braker *et al.*, 2003	N/A
	cnorB6R	GAANCCCCANACNCCNGC			
nosZ	nosZ-F	CGYTGTTCMTCGACAGCCAG	453	Throback *et al.*, 2004	JM63, JM162, YP64
	nosZ1622R	CGCRASGGCAASAAGGTSCG			

In addition, none of cutting site (G↓TCGAC) of restriction enzyme Sal1 was found in three sequences. The aim for this is to make sure linear process did not destroy the inserted gene. After digestion with Sal1, the circular plasmids were linearized checked by electrophori in 1% agarose gel and ethidium bromide stain.

4.3.3.2 Performance of standard curve

Several standard curves were generated by 3 linear plasmids DNA with target genes. All standard curves share the similar slope with small shifts (Fig. 25). The detect limitation is 10^2 copies. At lower starting concentration, the detection of DNA templates was not stable with large Ct variations.

4.3.4 Quantification of *nirK* gene in soil samples

The very first real time PCR attempt on the soil samples *nirK* gene with standard curve (Fig. 28). Three samples, H H_2-, M H_2- and air-treated soil were detected. Two amounts of templates for each sample, 20ng and 0.2ng were performed. As a result, 0.2ng for H_2-treated soil DNA, 20ng for low H_2- and air-treated soil DNA were amplified successfully. Unfortunately, the result can not be replicated.

The inhibition was planed to be calculated by adding certain amount of known copies of gene under detect limitation. However, when the soil total DNA was diluted to 10^{-6} ng/μl, adding 1μl as template, the target gene still could be detected. Considering that when diluting DNA samples, the inhibitor was also diluted, the real-time PCR assay was performed with optimal DNA quantity as template. After several times of try,

optimal DNA quantity was determined between 0.1~1 ng. 0.5 ng DNA was used as template in this study.

Although the slope of standard curves was similar, they would shift up or down time to time. Thus, quantification of samples and standard curves were carried out together every time. Each sample was replicated for at least 3 times, and the results were calculated according to the standard curve performed at the same time. In most of situation, the copies of gene in samples were under the detect limitation and can not obtained by standard curve. Thus, the differences of Ct (cycle threshold) value among samples were used to compare three samples (Table 11).

Two-tailed test were performed to test whether there are significant differences between samples. Mean, standard deviation, standard error, t (mean divides standard error) were calculated (Table 12(b)) from delta values (Table 12(a)). In two-tailed test table, t value of P (probability) at 0.01 and 0.05 obtained were 3.169 and 2.228. The calculated t value of our data were all larger than 3.169. Thus, the significant differences exist between samples. In other words, the copies of *nirK* gene soil increased after H_2 treatment indeed.

The number of *nirK* target molecules for three samples were calculated according to the standard curve. The results were showed in Table 13. Higher *nirK* abundance was observed in the H H_2-treated soil than M H_2- and control treatment (Fig. 29). The *nirK* copies of 1 g H H_2-treated soil was 1.9 and 4.3 folds more than that of 1 g M H_2-treated and control soil, respectively.

Table 11: Information for identification of 3 target genes using BLAST to align in GenBank..

Sample	Accession	Max score	Total score	Query coverage	E value	Max ident.	Description
N001	DQ182164.1	198	198	93%	6e-48	90%	Uncultured bacterium clone Kasp6b putative nitrite reductase (nirK) gene, partial cds
N002	EU790856.1	241	241	98%	9e-61	93%	Uncultured bacterium clone HM 33 putative copper nitrite reductase (nirK) gene, partial cds
N003	DQ783304.1	285	285	100%	4e-74	98%	Uncultured bacterium clone T1R1_13-20cm_071 NirK (nirK) gene, partial cds

Figure 27: Calibration curve plotting log starting *nirK* copy numbers vs. threshold cycle.

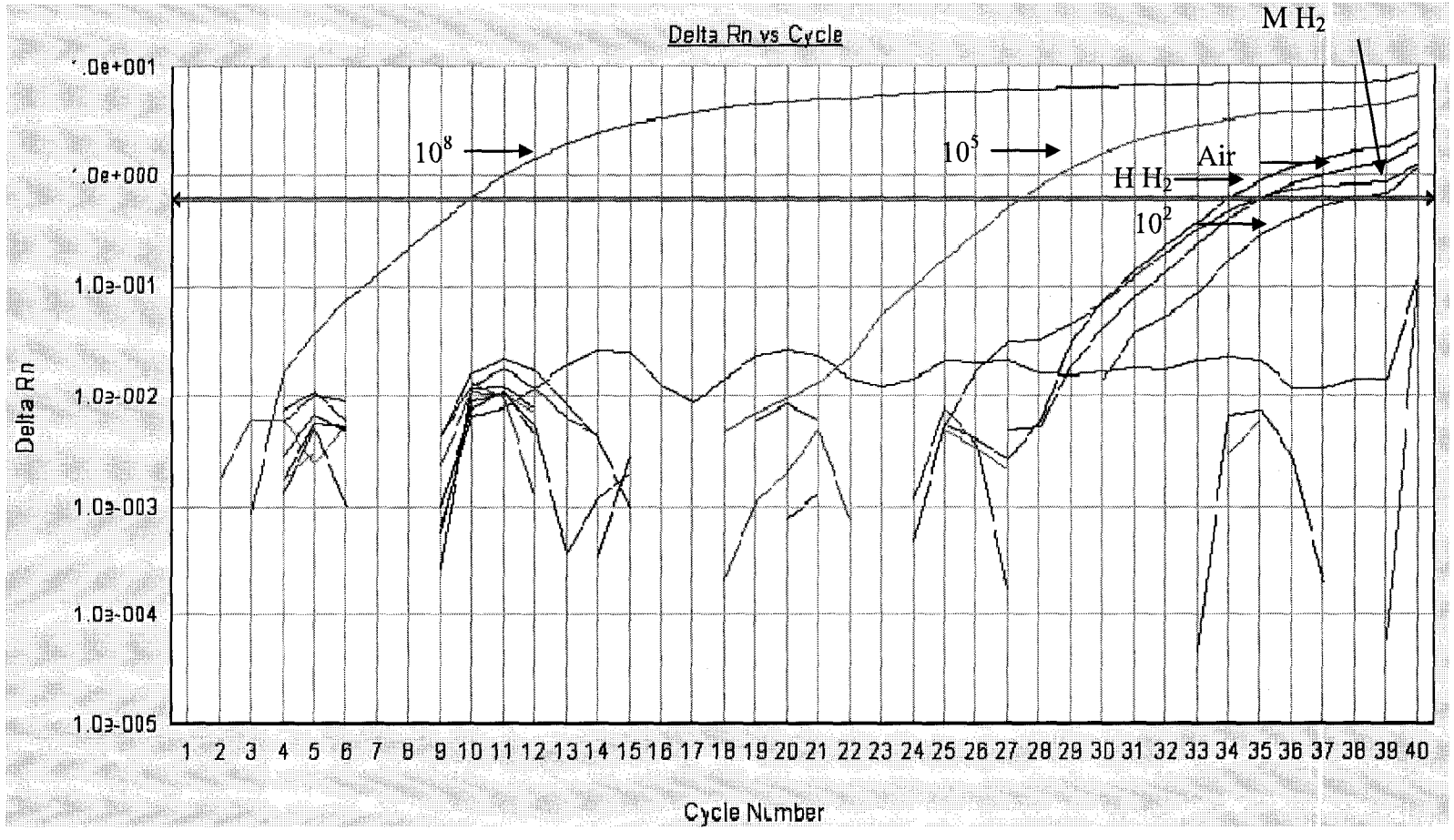

Delta Rn vs Cycle
M H2
1.0e+001
1.0e+000
1.0e-001
1.0e-002
1.0e-003
1.0e-004
1.0e-005
Delta Rn
10^8
10^5
Air
H H2
10^2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
Cycle Number

Figure 28: Calibration curve (delta Rn vs. cycle) of standard curve and samples snapshot from result of program 7000 SDS 1.2.3.

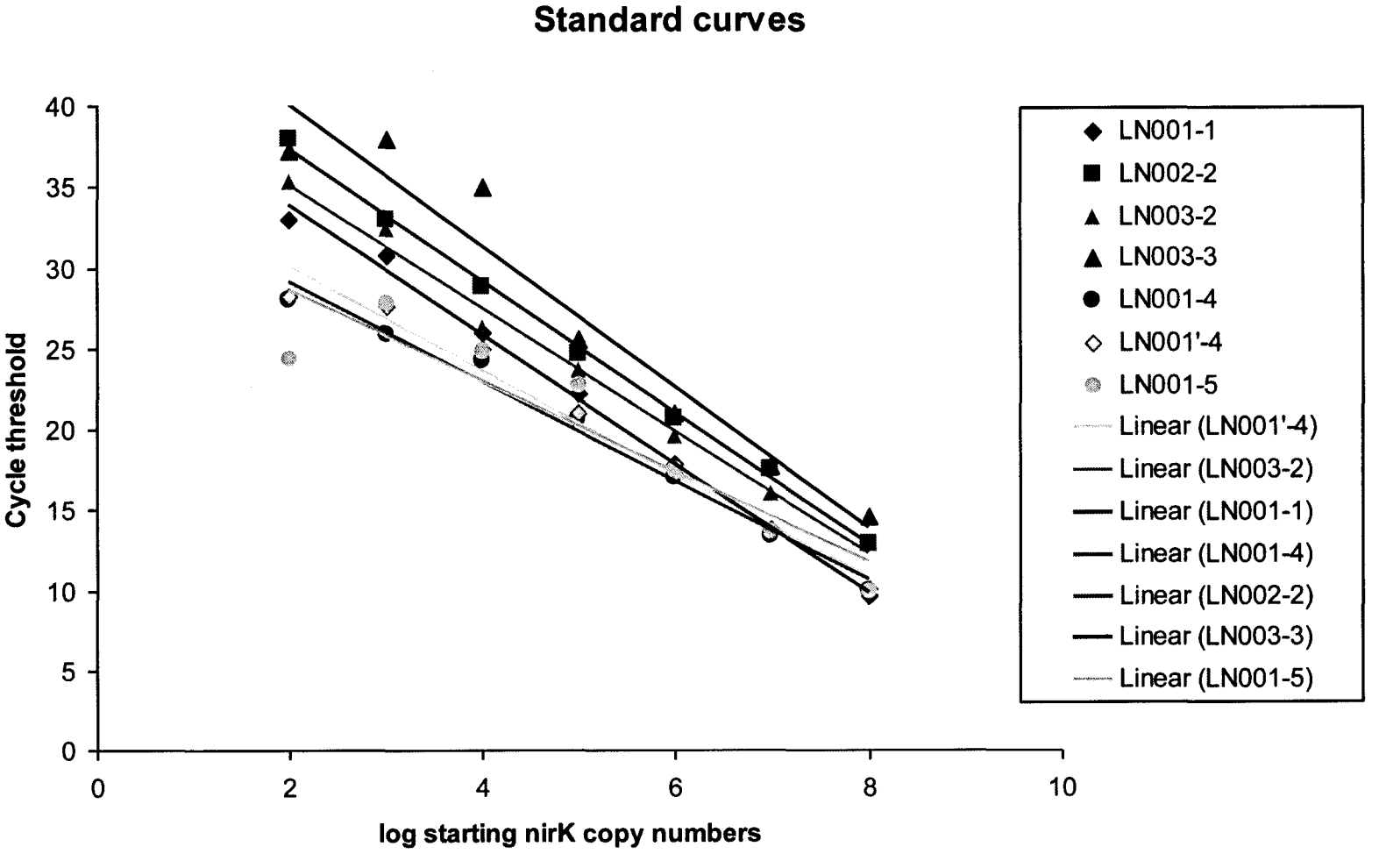

Standard curves
Cycle threshold
log starting nirK copy numbers
0
5
10
15
20
25
30
35
40
0
2
4
6
8
10
LN001-1
LN002-2
LN003-2
LN003-3
LN001-4
LN001'-4
LN001-5
Linear (LN001'-4)
Linear (LN003-2)
Linear (LN001-1)
Linear (LN001-4)
Linear (LN002-2)
Linear (LN003-3)
Linear (LN001-5)

Table 12: Raw data (cycle threshold) and statistical calculation for two-tailed test (a) raw data and delta of low H_2-H_2, air-H_2, air-low H_2;(b)statistical calculation for two-tailed test

(a)

	run 1	run 2	run 3	run 4	run 5	run 6	run 7	run 8	run 9	run 10	run 11
Ct (H H_2)	31.02	37.59	37.02	34.08	28.98	34.64	35.76	36.71	34.22	36.89	35.2
Ct (M H_2)	32.34	38.31	37.48	34.58	30.95	36.1	35.81	36.99	34.81	37.25	35.48
Ct (air)	36.94	38	38	36.39	31.22	38	38	37.72	37.25	38	35.7
Delta Ct (M H_2-H H_2)	1.32	0.72	0.46	0.5	1.97	1.46	0.05	0.28	0.59	0.36	0.28
Delta Ct (air-H H_2)	5.92	0.41	0.98	2.31	2.24	3.36	2.24	1.01	3.03	1.11	0.5
Delta (air-M H_2)	4.6	-0.31	0.52	1.81	0.27	1.9	2.19	0.73	2.44	0.75	0.22

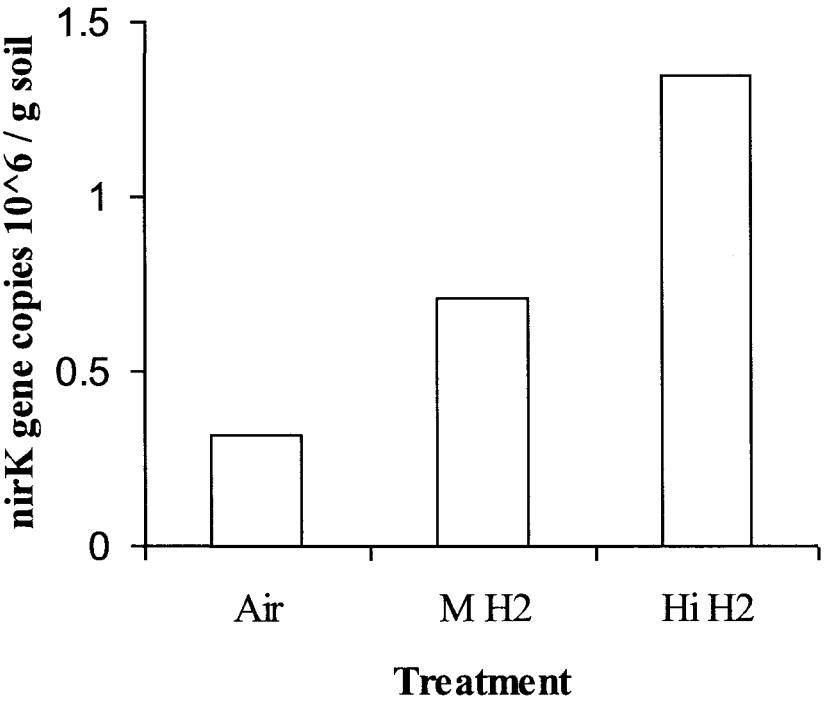
(b)

	Mean	St Deviation	St Error	t
Delta Ct (M H_2-H H_2)	0.726364	0.597349	0.180107	4.032947
Delta Ct (air-H H_2)	2.1009.91	1.611425	0.422071	4.324079
Delta Ct (air-M H_2)	1.374546	1.399853	0.485863	3.256665

Table 13: The average number of *nirK* gene copy in three soil samples

Sample	copies/ ng DNA	copies/ g soil
H H_2	443.29	1.35×10^6
M H_2	267.93	0.71×10^6
Air	130.34	0.32×10^6

Figure 29: The average number of *nirK* gene copy in three soil samples.


nirK gene copies 10^6 / g soil
1.5
1
0.5
0
Air
M H2
Hi H2
Treatment

4.4 DISCUSSION

Quantification of bacteria capable of denitrification is important for a better understanding of denitrifying activity and N_2O fluxes in the environment. In this study, a real-time PCR assay was carried out to quantify the denitrifiers using the *nirK* gene encoding the copper nitrite reductase. The results showed that the gene copies of soil after H_2 treatment increased significantly.

4.4.1 H_2 fertilization and N_2O emission

H_2 production by legume nodules coupled to the production of N_2O in legume soil was noticed by scientists these years. N_2O emission enhanced by H_2 was observed both in laboratory treatment and field study (unpublished, by Dr. Cen in Queen's University). Long term (weeks) exposure of soil to elevated concentration of H_2 (similar to that experienced by soil adjacent to legume nodules) induced a major increase in the emissions of N_2O. In field study, field soil collected adjacent to legume nodules also showed much higher rate of N_2O emission compared with soil collected further away from nodules.

H_2 is released to the soil and H_2-oxidizing bacteria uptake and oxidize it into water. The oxygen in soils is consumed; the soil becomes low oxygen concentration and moisture, which is the ideal condition for denitrification. These may be partially explained why H_2 promotes N_2O emission and denitrifiers. Some H_2-oxidizing bacteria have been isolated on the carbon-free medium and characterized to uptake H_2. Five genes in denitrification pathway were detected in some of these isolates (Table 10). The result

showed that most of them contain one or two denitrification genes, which indicated a connection between H_2 uptake and N_2O emission, and supported the result that the isolates inoculated into bulk soil enhanced N_2O emission from soil.

In this study, the number of key denitrification gene, *nirK* in different treatments were quantified. The results showed that more gene copies in H_2-treated soil than that of control soil, which provided evidence to that most H_2-oxidizing bacteria may contain denitrification gene or they are denitrifiers to produce N_2O. The results of Part 3 support this assumption. The dominant known denitrifiers are identified as *Pseudomonas, Bacillus, Paracoccus, Alcaligenes* and *Enterobacter* (Weier and MacRae, 1992). Except *Bacillus*, the others are all *Proteobacteria*. *Pseudomonas* and *Enterobacter* both belong to *Gammaproteobacteria*. *Paracoccus* and *Alcaligenes* belong to *Alpha-* and *Betaproteobacteria*, respectively. After H_2 treatment, *Gammaproteobacteria* increased dramatically. Additionally these three subgroups were found in soil adjacent to HUP^- nodules than that in HUP^+ nodules. However, more evidences should be provided to affirm this assumption.

4.4.2 Inhibition for amplification

Inhibition of PCR amplification is a big problem in gene quantification from soil samples. Humic acids or humic substances co-extracted with nucleic strongly inhibit DNA modifying enzymes (Porteous and Armstrong, 1991). When amplification of target gene cloned in plasmid, the inhibition effect wasn't observed. With dilution of DNA samples, inhibitors were diluted as well. Due to variable DNA concentrations, the same final concentration of DNA template induced the different times of dilution for inhibitors.

Thus, to reveal the real inhibition effect, dilution of DNA as few times as possible is recommended.

4.4.3 Why SYBR Green, not TaqMan?

Compared to the TaqMan™ probe detection, SYBR Green doesn't need to design additional probe which is unrealistic for the *nirK* gene due to its high polymorphism between the different taxonomic groups of denitrifiers (Philippot, 2002). Application of the *nirK* primers to environmental samples was performed using SYBR Green detection system (Stubner, 2002) and developed as a tool to quantify denitrifiers. In the meantime, SYBR Green is much cheaper and more practicable than TaqMan™.

4.4.4 Why only *nirK*?

There are several key enzymes in denitrification pathway, such as cytochrome nitrite reductase (*nirS*), nitric oxide reductase (*norB*) and n nitrous oxide reductase (*nosZ*). Five pairs of degenerate primers were used to amplify *nirS, nirK, qnorB,cnorB* and *nosZ* genes have been attempted to detect these target genes in soils. Only primers for *qnorB* and *nosZ* genes worked well and genes can be amplified successfully from our soil samples. Especially *nirS* gene, performs the same step in denitrification with *nirK* gene. Quantitation of *nirS* gene should be considered as well. Although the real-time and competitive PCR targeting *nirS* gene have been done by Gruntzig et al (2001) and Michotey et al (2000), respectively, the problem for real-time PCR is the primers for *nirS* only specific to *Pseudomonas stutzeri*. For *norB* and *nosZ*, the sequences in GenBank are limited, thus, it is hard to design universal primers to quantify the whole denitrifiers. So

far as known, the quantitative PCR for *nirK* is the most developed and widely used to quantify denitrifying bacteria.

4.4.5 Quantification at mRNA expression level

The detection of functional genes in the environment does not imply that the corresponding activity is present. Thus, detection of their mRNA is needed. There are a lot of successful studies to extract RNA from various soils (Hurt *et al.*, 2001), though amplification of mRNA from soils is still a challenge (Philippot and Hallin, 2005). Targeting the mRNAs for the denitrification enzymes will provide a shortcut to denitrification activity and monitoring of the active denitrifier, rather than mere indication of their presence. A comparison of the diversity of the denitrification genes amplified from DNA and mRNA will provide a means of distinguishing potential denitrifiers from those actually expressing their genes in the environment at the time of sampling (Philippot and Hallin, 2005).

5 GENERAL CONCLUSIONS

Our objectives in this study were to obtain an understanding of the hydrogen effect on soil bacterial community structure and N_2O production using culture-independent molecular techniques. In some reports, researches have utilized clone libraries or FISH to investigate the diversity and richness of bacterial phylotypes from soils adjacent to legumes or by H_2 treatment. The limitation of techniques, such as limited scale of libraries, sensitivity of probes for hybridization of FISH technique, should be concerned. Combination of results of clone library and T-RFLP can provide more accurate and detailed information about soil bacterial community structure. For N_2O production, there is few published literature for study about enhance of N_2O by H_2 with molecular evidence.

The results showed that dominant phylotypes *Gammaproteobacteria*, *Sphingobacteria* and *Flavobacteria* can be commonly detected by both analytical methods. And the common and differences of H_2 effect on soil bacterial community structure between laboratory treatment and greenhouse condition were presented as well.

The significant difference of *nirK* gene copies between H_2-treated and control (air-treated) soil was revealed in this study. It confirmed that H_2 indeed influenced the denitrification process in denitrifiers. However, more evidences are needed to support the conclusion. The other key genes in this process should be quantified and compared as well. And the direct proofs from expression level could be obtained by quantification of mRNA about these genes in the future.

Although it becomes much easier to investigate the soil microorganism ecosystem, the limitation of techniques affects the results. Our knowledge of the diversity and distribution of microorganisms in soils is rudimentary. Studies of soil microbial community undoubtedly will continue to reveal novel fields to expand our understanding of soil ecosystems.

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