Microbial diversity in archived agricultural soils

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The past as a guide to the future

J. Dolfing
A. Vos
J. Bloem
P.J. Kuikman

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ABSTRACT

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Bacterial diversity and bacterially mediated processes are considered key to soil ecosystem functioning through decomposition and mineralization. However, there is a lack of understanding as to how activity and diversity of prokaryotic communities respond to changes in the environment. At present this issue is mostly addressed by real-time monitoring of long term field experiments, which is costly and slow. Using modern molecular methods we re-analyzed soil samples of up to 50 years old that have been stored in the Alterra soil archive TAGA. We showed that it is indeed possible to detect bacterial fingerprints in those samples and that fingerprints from different samples can be distinctly different, for example between fields that have or have not received organic amendments. These results are a promising first step towards unlocking the microbial information present in archived soil samples. This will help to assess the (likelihood of) changes in soil microbial diversity in response to environmental change (climate change) and human interference (fertilization) and to establish a reference condition and situation. This may further enable coupling to more functional assessments of soil functioning in standardized decomposition essays with the stored samples with known differences in diversity.

Keywords: Soil; Microbial diversity; DGGE; Soil archive; TAGA; Manure; Soil organic matter; Soil management; Bacterial diversity; Heino; Pr168; Pr65.

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Preface

The present project was carried as part of Alterra's Strategic Research Program (SEO). One of the objectives of this program is to strengthen the expertise of Alterra through exploring innovations through research and improving understanding in today's scientific challenges and so better support our clients. The objectives of the research that is presented here were:

- to find out it is possible with modern molecular techniques to detect "microbial fingerprints" of samples stored in Alterra's soil archive TAGA, and if so,
- whether a comparison between samples with a different history would yield meaningful differences, as this would provide a way to study microbial diversity in archived soils, and thus would greatly increase the value of TAGA.

Key words: microbial diversity; biodiversity, soil microbiology; soil archive; DGGE; denaturing gradient gel electrophoresis; TAGA; Alterra; manure; soil organic matter.

Summary

Bacterial diversity and bacterially mediated processes are considered key to soil ecosystem functioning through decomposition and mineralization of organic material. Biodiversity, i.e. biological diversity, is presently receiving much attention from both researchers and policy makers. The recognition of the importance of biodiversity stems from the recognition that an increasing number of species becomes extinct, the objective to protect species and importance given to diversity for a healthy functioning of ecosystems. It is important to be aware that the benefits of biodiversity are produced by populations of organisms in communities living in a specific abiotic environment. However, there is a lack of understanding as to how the activity and diversity of prokaryotic communities respond to long term changes in the environment. We do know that soil biodiversity is changing fairly slowly despite rapid changes in rhizospheres on an annual basis. This would require long term experimental trials to relate changes as they occur to environmental pressures. At present, this issue is mostly addressed by real time monitoring of long term field experiments. Such experimentation is costly and requires long term commitment of researchers and funding and years and years pass before results may provide hints for human interference. At that stage interference may be more costly or conditions may not be reversible anymore. We therefore sought a more rapid approach, and asked whether existing soil archives with historic soil materials from long-term field trials in the past could be useful in identifying effects of environmental parameters on microbial diversity.

Alterra has a soil archive (TAGA) with actual air-dried soil samples that are up to 120 years old. The objective of the present research was to find out if 16S-based PCR-DGGE analysis of soil samples archived in TAGA would provide an indication of microbial diversity. DGGE, which stands for denaturing gradient gel electrophoresis, is a technique that is frequently used to detect changes in microbial diversity. One of the characteristics of PCR-DGGE is that it overcomes the necessity to cultivate organisms before characterizing them. Thus the method should be ideally suited to detect bacteria in stored soil samples as it avoids the necessity to "wake them up from hibernation".

Our research shows that it is indeed possible to obtain DGGE profiles from samples that have been stored for more than 50 years. Drying of the soil may a have affected the composition of the community that can be detected by PCR-DGGE, but exploratory experiments gave no indication for a change in the number of bands that was detected. Nevertheless, further work is needed to evaluate exactly how drying and grinding of the soil affects the PCR-DGGE profile that subsequently can be retrieved.

Another encouraging result of the present research is that our data-set shows distinct differences between profiles from soils that have received manure (organic amendments) and soils that have not received such amendments. Apparently

environmental factors that were active 50 years ago can still be detected in those samples.

The present results should be regarded as a first step towards unlocking the microbial information present in archived soil samples. More specific methods (including sequencing of PCR amplified DNA from those samples) will undoubtedly provide more specific information on the type of organisms present in those samples.

1 Introduction

Bacterial diversity and bacterially mediated processes are considered key to soil ecosystem functioning through decomposition and mineralization of organic material. Biodiversity, i.e. biological diversity, is presently receiving much attention from both researchers and policy makers (EU Soil Strategy). Current policies in agriculture include set-aside, extensification and restoration management and optimizing the application of ecological principles (in Dutch "ecologisering"). The recognition of the importance of biodiversity stems from the recognition that an increasing number of species becomes extinct, the objective to protect species and importance given to diversity for a healthy functioning of ecosystems. It is important to be aware that the benefits of biodiversity are produced by populations of organisms in communities living in a specific abiotic environment.

By signing the Convention on Biological Diversity, as formulated in 1992 in Rio de Janeiro at an international conference under auspices of the United Nations, the Netherlands has taken the responsibility to protect biodiversity existing within its borders. The implementation of policies to achieve this goal is still in its infancy. Biodiversity in soils is extremely large. Indeed soil has been labeled "poor man's tropical rain forest".

In spite of the crucial role that soil communities play in the global elemental cyles, there is a lack of understanding as to how the activity and diversity of prokaryotic communities respond to changes in the environment. We do know that soil biodiversity is changing fairly slowly despite rapid changes in rhizospheres on an annual basis (Smalla et al. 2001). This would require long term experimental trials to relate changes as they occur to environmental pressures. At present, this issue is mostly addressed by real time monitoring of long term field experiments. Such experimentation is costly and requires long term commitment of researchers and funding and years and years pass before results may provide hints for human interference. We therefore sought a more rapid approach for assessing the rate of change in soil microbial diversity, and asked whether existing soil archives with historic soil materials from long-term field trials in the past could be useful in identifying effects of environmental parameters on microbial diversity.

Alterra as many other research institutes has a soils archive (TAGA) with samples that are up to 120 years old. The objective of the present research was to find out if 16S-based PCR-DGGE analysis of soil samples archived in TAGA would provide an indication of microbial diversity. DGGE is a technique that is frequently used to detect changes in microbial diversity (Smit et al. 2001). One of the characteristics of PCR-DGGE is that it overcomes the necessity to cultivate organisms before characterizing them. Thus the method should be ideally suited to detect bacteria in stored soil samples as it avoids the necessity to "wake them up from hibernation".

1.1 Rationale for the use of bulk soils to study biodiversity

Soil microorganisms carry out essential processes in soil such as decomposition and mineralization of organic matter. Only a fraction (max 20%) of the total microbial population in soil is culturable and can be studied by traditional cultivation methods. Even in the rhizosphere, only part of the microbial population is culturable. Yet, the rhizosphere and the deposition of substrates here, is the major driving force for any changes in the soil microbial diversity. Rhizospheres are only a fraction of the total soil environment (10% at the most) but foster the larger part of soil activity (at least 90%). The rhizosphere is more an actual use of that potential and may very well have a lower biodiversity than the bulk soil. In this sense, rhizospheres reflect the present, whereas bulk soils, non-rhizosphere, reflect the past and contain accumulated information over time. It is the bulk soil that will reveal the true microbial potential and show whether losses of biodiversity and function have occurred over time.

1.2 Objectives of the current study

The objective of the current study was to answer the following questions:

1. Can we obtain DGGE profiles from soil samples stored in TAGA, and if so
2. Are DGGE profiles obtained from a series of similar soils similar and series from dissimilar soils dissimilar, i.e. are patterns internally consistent?

2 Experimental approach

The assessment of soil microbial diversity should ideally be based on methodologies that are non-selective and can thus be best supported by non-cultivation methods. One such method is DGGE (Denaturing Gradient Gel Electrophoresis). Critical steps in this methodology concern the extraction of DNA or RNA from soil and the amplificability of the extracted nucleic acids. Results so far are promising and the method was successfully used to indicate and monitor minor changes in microbial diversity in contaminated soils and in soils with heavy organic amendments (Bloem and Breure, 2003; Dilly et al. 2004).

2.1 DGGE analysis of bacterial soil communities

Denaturing gradient gel electrophoresis of PCR[1]-amplified fragments of genes encoding for 16S rRNA (Muyzer et al. 1993) is a widely used method to evaluate microbial diversity in soils (Torsvik and Ørser, 2002). The method is routinely performed in our laboratory (Griffiths et al. 2001; Bloem and Breure, 2003; Dilly et al. 2004). One of the advantages of using PCR-DGGE is that the organisms have not to be cultivated before they can be analyzed: A disadvantage is that DGGE is not specific, and not necessarily quantitative, but as a first screening method DGGE is still the method of choice. In the present research PCR was used to amplify the variable V3 region of 16S rRNA gene sequences of eubacterial DNA from archived soil samples (see below). Materials and methods of PCR amplification and DGGE analysis as performed in our laboratory have been published before (Dilly et al. 2004).

2.2 Analysis of DGGE community profiles

Quantitative analysis of DGGE community profiles is routinely done with densitometric methods. Images of gels were made using a UV 300 transilluminator (Fotodyne, Hartland, USA)) and an Image Point Cooled CCD video camera (Photometrics LTD., Tucson, USA)). The video images were acquired with Leica QWin image processing and analysis software (Leica, Cambridge, UK) and stored as TIFF files. Banding patterns were analyzed using GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium). Background intensity was subtracted (10%), DNA bands were identified interactively and the position and mass (intensity) of each band was measured. The data were used for principal component analysis (PCA) in GelCompar to evaluate differences between the DNA profiles. Both quantitative and qualitative PCA (for the latter the presence but not the intensity of the bands) was performed. For qualitative PCR only the presence of the bands was taken into account, for quantitative PCR also the intensity of the bands was taken

[1] PCR: polymerase chain reaction.

into consideration. Dendrograms were constructed in GelCompar on the basis of intensity of the bands.

2.3 Selection of samples from TAGA

TAGA is an archive of soil, crop and manure samples of the former research Institute of Soil Fertility in Haren and its predecessor the National Agricultural Research Service in Groningen (Ehlert et al. 2002). The archive contains about 250 000 soil samples from experiments performed in the period 1879-1998, plus data and information on the experiments from which the samples were taken. The source of the samples is diverse. Most of the samples are from fields experiments, some of which lasted for 50 to 80 years.

Access to TAGA and the information contained in TAGA is not (yet) computerized. The samples all have a unique code (number) and are stored in numerical (chronological) order. The challenge is to find the description of the experiment from which the samples under consideration were taken, or, vice versa, to find samples from the experiment of choice. The codes of the samples have to be found by manually searching old ledgers. Ledgers are filed in boxes. Each box contains the files of one or a few distinct experiments. The experiments all have a unique code. Limited information on these experiments has recently been transferred to a digital database, but searching TAGA in its present format is still rather time-consuming (de Willigen et al. 2001).

For the current project four series of samples were selected. Selection criteria included:

- The age and duration of the original experiment: the older and longer the better;
- The age of the samples: the older the better;
- The objectives of the original experiment (effects of manure/organic amendments);
- The possibility to sample the site again;
- The soil type; both sandy soils and clay should be included in the analyses.

3 Exploratory experiments

To be stored in TAGA soil samples are dried for two days at 40 ^{0}C and then ground. This procedure may change the soil microbial community. The microbial community may (also) undergo changes during storage. Therefor a series of exploratory experiments was done to evaluate (i) how drying and grinding of soils affects bacterial community fingerprints as revealed by DGGE, (ii) how these fingerprints change during storage, (iii) whether the original community fingerprints may be restored by rewetting dried and ground soils, and (iv) whether DGGE analysis of archived soils gives reproducible bacterial community fingerprints. The results of these experiments (Kuikman et al. 1999) are to be published elsewhere (Naumova et al. in preparation). Briefly, the outcome of these experiments was that drying and grinding of soils results in a dramatic decrease in the numbers and biomass of the bacteria present in the soil. The effect of drying and grinding on the DGGE profiles was less clear, and appeared to be different in different soils. The major conclusion from this exploratory work was that it was indeed possible to detect DGGE fingerprints in archived soils from TAGA and that different patterns were observed for different soils. This encouraging result led us to focus in subsequent work on analyzing a series of soil samples from TAGA to see whether different patterns would be observed in samples that had a different history. From our exploratory work we know that these profiles are most likely not identical to profiles that would have been obtained from fresh samples, but the fact remains that differences between different samples are interesting and may give clues on the effect of certain treatments or changes in environmental variables on the microbial diversity of the soils under study.

4 Sample selection

Samples from four experiments were used in the present project. The oldest sample was from 1932, i.e. more than 70 years old. Most of the samples were form the 1940s and 1950s (experiments Pr 65 and Pr 168) and 1960s and 1970s (experiment Pr168). Table 1 gives the TAGA codes and a brief description of the experiments from which samples were analyzed. Tables A1 to A5 in Appendix 1 give the TAGA codes of the samples. The tables are organized per experiment from which the samples were taken. Experiment Pr 109 was permanent grass on clay. All other experiments had a crop rotation on sand. The samples were chosen in such a way that effects of variety of treatments (with and without manure) and crops (oats, rye, potatoes) could be evaluated over longer periods of time (up to 24 years).

Table 1. TAGA experiments from which samples were analyzed

TAGA code	Period	Soil type	Experimental variables
Pr 65	1927-1957	sand	manure, compost, N, P, K
Pr 109	1930-1951	heavy clay	manure/no manure plus fertilizers
Pr 168	1940-1975	sand	manure
Pr 927	1946-1965	sand (dalgrond)	straw at various N-gifts

5 DGGE profiles

In this chapter the results of a series of DGGE analyses of PCR-amplified DNA from samples stored in TAGA will be presented. The emphasis is on the effects of manure on the DGGE profiles. Other effects that will be presented are the effects of crop type and the effects of soil type. It should be kept in mind (i) that all samples were from the bulk soil, and not specifically from the rhizosphere, and (ii) that all samples were from one time point per year (after the harvest of the crop), and results may have been different when the samples had been taken at a wetter or drier point in time.

5.1 DGGE profiles from a sandy soil. Effects of manure

Pr 168 is a well described and documented field experiment with farmyard manure on a humic sandy soil in Heino (Wisselink, 1961; Kortleven 1963). The experiment was started in 1940 with the lay-out of 10 field plots receiving ten different treatments. The objective was to test the effect of stable manure and of non-leguminous secondary crops [harvested as well as ploughed in] on the fertility of an old arable sandy soil. The topsoil is a typical "esgrond", i.e. a soil which in the pre-fertilizer era received regularly heather-sod compost from sheep-barns. For the present project two series of samples were analyzed. One series was from a field plot that had received only chemical fertilizer, no animal manure. The other series was from a field plot that had received animal manure at a rate of 70 ton per 3 years. The samples were from the 1950s to 1970s, taken in the fall after cultivation of rye. The results of the DGGE analysis of both series of samples are presented in Figure 1.

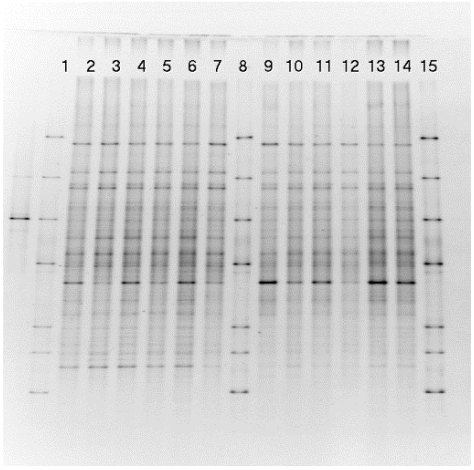

Figure 1. DGGE analysis of samples from experiment Pr168. Lanes 1, 8 and 15 are markers. Lanes 2, 4, 6, 9, 11 and 13 are with manure, lanes 3, 5, 7, 10, and 12 are with inorganic fertilizer (for details see Table 4a)

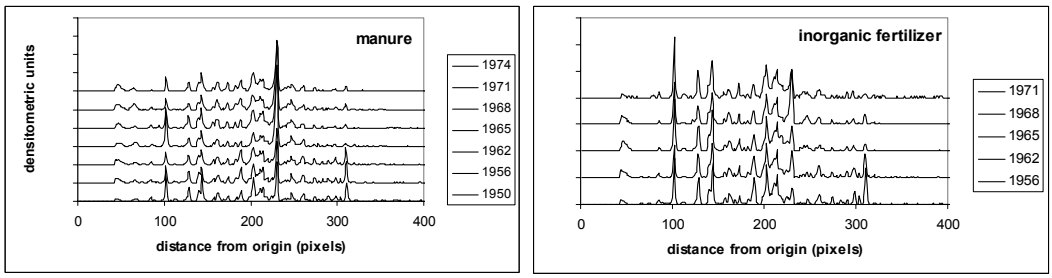

Figure 2. Densitometric profiling of the DGGE gels of samples from Pr168 from plots that had received manure (left panel) or inorganic manure (right panel) since 1940

With the naked eye it is not easy to see differences between the individual samples. This may be taken to suggest that there is a large, stable population of microorganisms present in the soils and that the effect of adding manure was relatively minor. Densitometric profiling of the data however shows that differences are apparent between the DGGE profiles of the two series (Fig. 2). A more detailed impression of differences between profiles from samples taken in the same year but from different treatments is given in Figure 3, while Figure 4 shows a comparison between two samples from the same treatment but from different years.

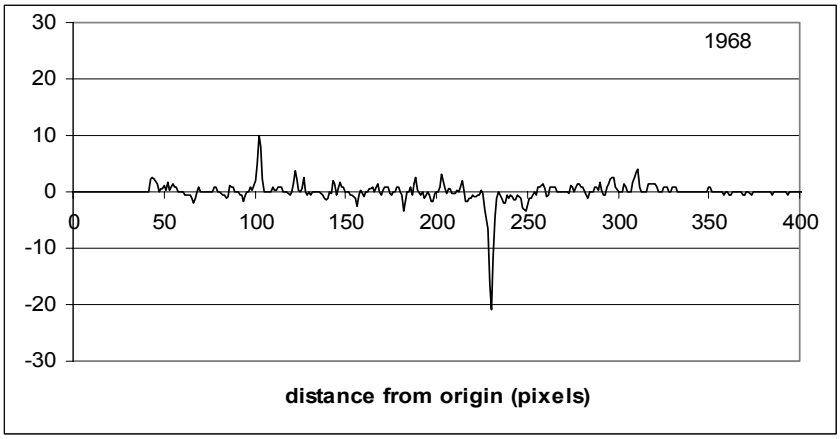

Figure 3. Density difference between DGGE profiles from samples taken in 1968 from field plots that had received manure or inorganic fertilizer since 1940

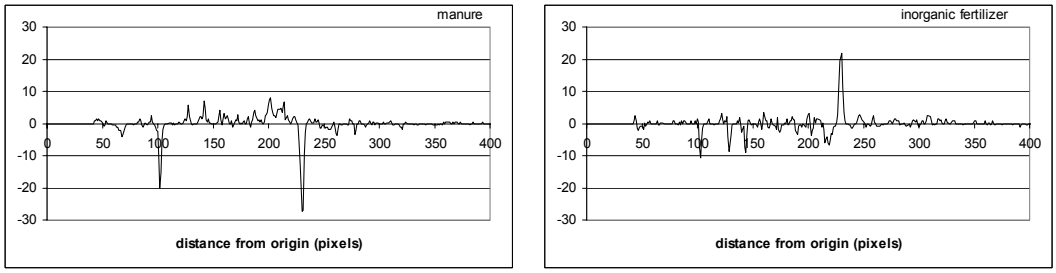

Figure 4. Density difference between DGGE profiles from samples taken in 1965 or 1968. Left panel: samples from plots that had received manure since 1940. Right panel: samples from plots that had received inorganic fertilizer since 1940

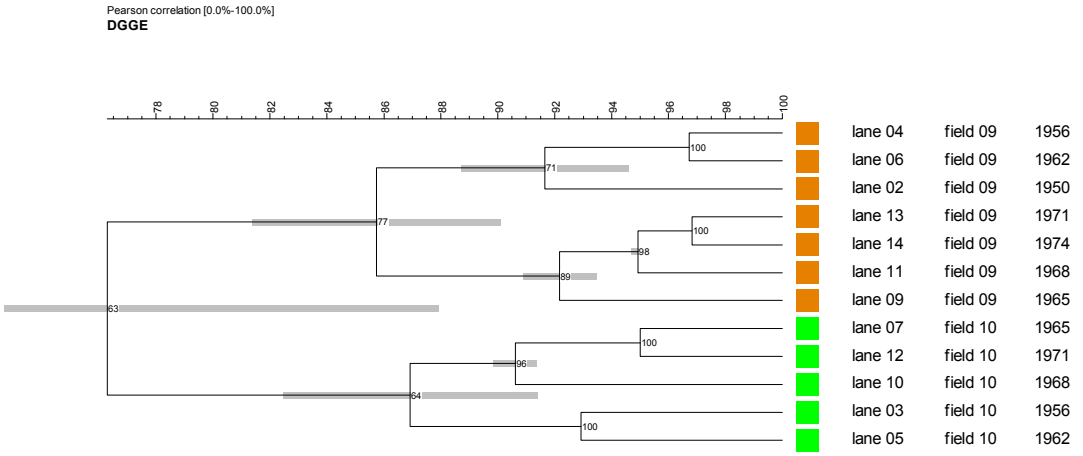

Figure 5. Dendrogram of DGGE patterns from samples of field plots that had received animal manure (brown squares) or inorganic fertilizer (green squares) since 1940

A dendrogram based on a comprehensive comparison of all the results (Fig. 5) shows that the samples within one treatment series are more related to each other than to the samples from the other treatment. **Thus DGGE analysis of the two series of samples has revealed consistent differences between them: apparently it is possible to detect a effect of a treatment (in this case the use of manure versus the use of inorganic fertilizer) on the microbial community profile in samples that had been stored in TAGA for between 25 and 50 years.** Cluster analysis (Figure 6) confirms that the samples of the soils that had received no manure formed a distinct group.

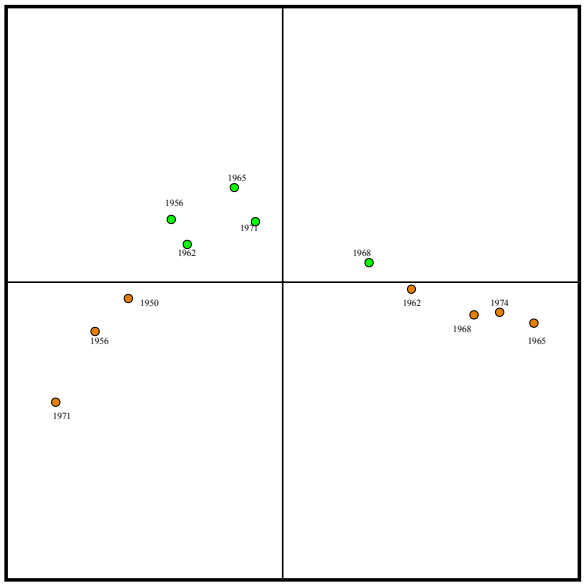

Figure 6. Quantitative principle component analysis of DGGE profiles from field plots that had received animal manure (brown dots) or inorganic fertilizer (green dots) since 1940

5.2 Effect of crop and secondary crops

In the above analysis the effect of manure versus chemical fertilizer for series of samples that had been taken after cultivation of rye was evaluated. In a subsequent analysis more variables were introduced, viz. (i) three different crops rather than rye only, and (ii) a third treatment, viz. a secondary crop. Again, analysis of the DGGE gels with the naked eye revealed no clear-cut differences between the various profiles (Fig. 7).

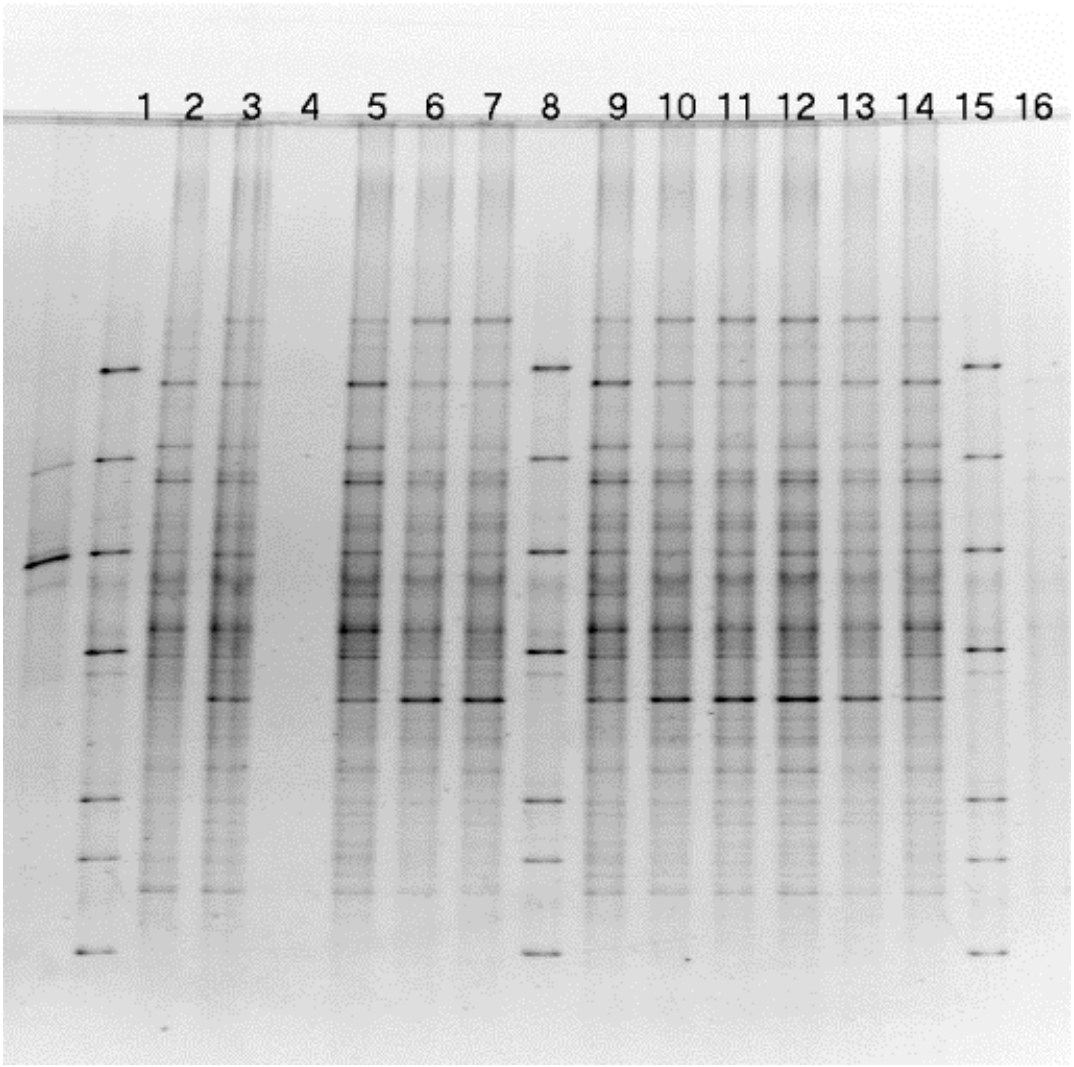

Figure 7. DGGE analysis of samples from experiment Pr168 (for details see table 4b; lanes 1, 8 and 15: markers)

A dendrogram of the data after densitometric profiling however revealed distinct clusters (Figure 8). Much to our surprise however the clustering was not along the lines observed in the previous experiment, or between different crops, or years. The culprits for this unexpected (see above) result were the samples from the soils on which potatoes had been cultivated. If we leave these samples out of the dendrogram the result resembles the result described above. This suggests that the effects of oats

and rye on the soil microflora are very similar. The effect of potatoes is not equivocal, and the potato samples do not form a cluster by itself.

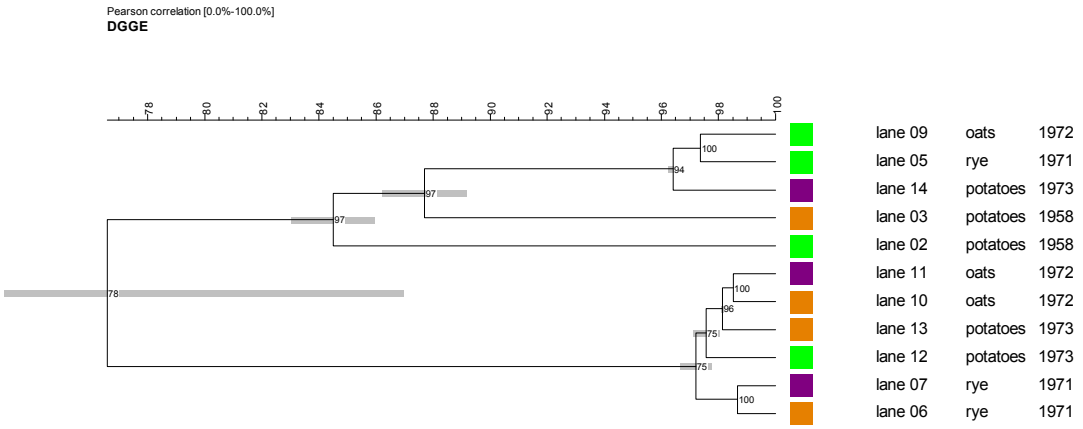

Figure 8. Dendrogram of DGGE patterns from samples of field plots that had received animal manure (brown), inorganic fertilizer (green), or animal manure plus a secondary crop (purple). The plots had been sampled in the year indicated after the harvest of the indicated crop

5.3 Replicates

In the two analytical series described above no replicates were included. We had elected to direct all analytical energy (read: space on the DGGE gels) towards analyzing as many samples as possible in short time. In a subsequent experiment however two sets of two replicates were included to get an impression of the variability of the analytical procedure. Replicates in this case were PCR-DGGE replicates, i.e. DGGE was performed on duplicate PCRs of the same DNA extracts. Densitometric profiling of the DGGE banding patterns thus obtained revealed similarities of 98.5 and 95.7 % respectively for the two sets of duplicates.

5.4 Effect of soil type

The above replicates were part of an analytical series in which the DGGE profiles of two sets of soils were compared on the same gel (Fig. 9). One soil was a clay soil, the other one was a sandy soil. With the naked eye there were clear-cut differences between the two sets of banding patterns from the different soils. A dendrogram of the profiles indeed gave a distinct clustering per type of soil (Figure 10). Principal component analysis also indicated that the samples of the two soils formed two separate clusters (Fig. 11).

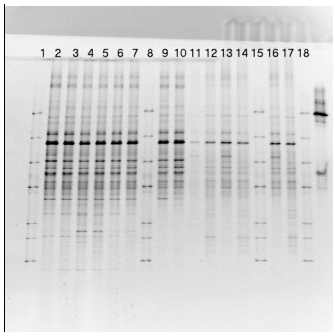

Figure 9. DGGE analysis of samples from a sandy soil and a clay soil

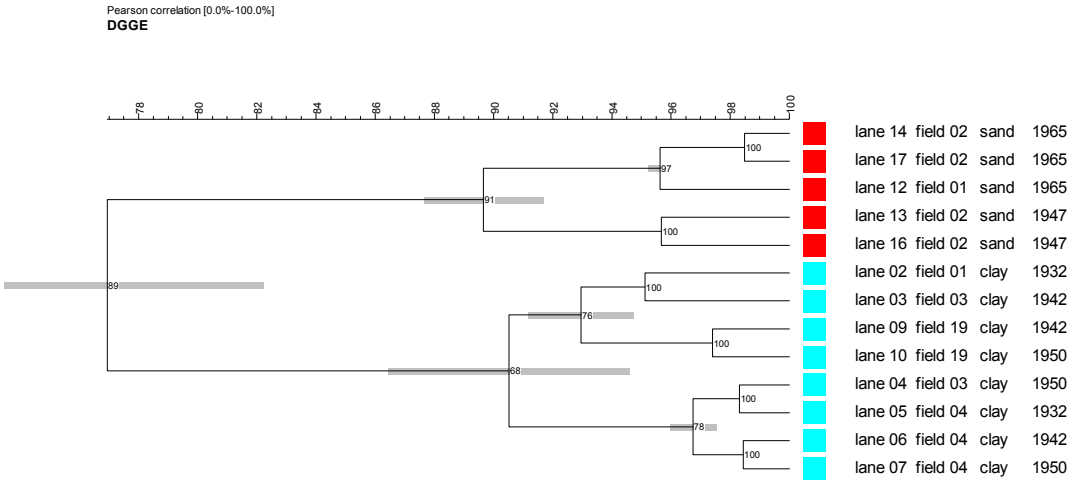

Figure 10. Dendrogram of DGGE patterns from samples of field plots on a sandy soil (red) and a clay soil (blue)

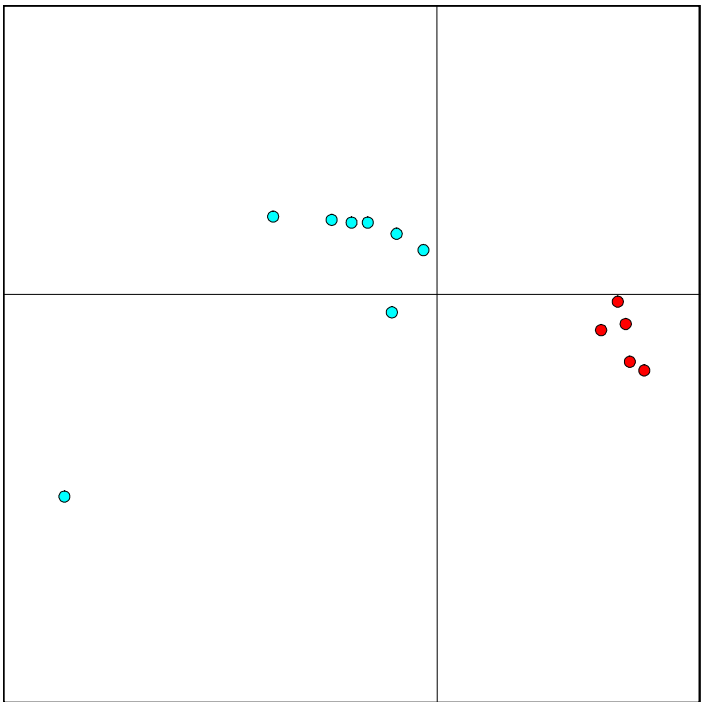

Figure 11. Quantitative principle component analysis of DGGE profiles from a sandy soil (red dots) and a clay soil (blue dots)

Among the samples from the sandy soil those taken in 1947 were somewhat different from those taken in 1965 (Fig. 10). In the series from the clay soil there were distinct differences between some of the samples, but a distinct pattern could not be distinguished.

5.5 Effect of manure and compost on a sandy soil

In a last screening a series of soil samples from experiment Pr 65 was analyzed. This experiment was started in 1927. It concerns an arable field on sand. The ten treatments applied to this soil are given in Table 2. Thirty samples from three different years (1948, 1954 and 1955) were analyzed. Because of the high number of samples for gel analysis the series had to be split in two sub-series. The sub-series were run on different gels, with one of the samples being run on each of these gels as a sort of internal standard. Following the results described above for the samples from soils that had or had not seen animal manure for many years, our hypothesis for the screening of the samples from experiment Pr 65 was that the samples from treatments 1 and 2 and possibly 5 would be clearly separated from the other samples that had not received organic amendments. The dendrogram presented in Figure 12 shows that this was indeed largely the case. With only one exception (six out of seven) the samples that had not received organic amendments clustered apart from the samples that had received organic amendments. All these samples had been run on the same gel to make a proper comparison possible. On the second gel 14 more samples from plots that had not received organic amendments were analyzed, plus

one sample from a plot that had received manure. Again this sample was clearly separated from the samples that had not received manure (see Appendix). Within the series of samples that had not received organic amendments some large differences were observed between some groups of samples. The logic behind this grouping is presently not clear. The year of sampling (or the type of crop) cannot be distinguished as a major factor, and neither can the type of fertilizer regime be distinguished as a clear factor.

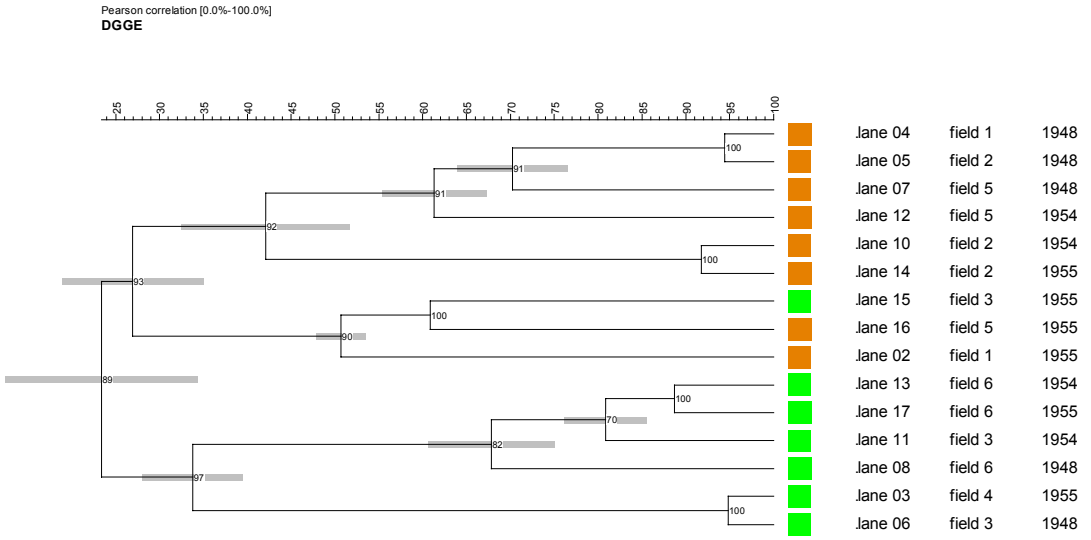

Figure 12. Dendrogram of DGGE gels from samples of field plots that had received organic amendments (brown) or inorganic fertilizer only (green)

6 Discussion

We have shown that it is possible to obtain DGGE profiles from samples that have been stored for more than 50 years. This is very encouraging as it opens up new avenues for studying the effects of gradual environmental changes on changes in the microbial diversity in soils. Despite that we were able to obtain DGGE profiles from these soil samples, we can not exclude that the population before drying has indeed been affected and changed as a result of drying and storage. In the testing we have done, drying of fresh samples was included. From this we anticipate minor changes during drying and storage. On the other hand, this drying may have benefits as well: by drying one may very well exclude the dominant groups of transient microorganisms present in the rhizosphere at the moment of sampling. Further work is needed to evaluate exactly how drying and grinding of the soil affects the PCR-DGGE profile that subsequently can be retrieved.

Another encouraging result of the present research is that our data-set shows distinct differences between profiles from soils that have received manure (organic amendments) from soils that have not received such amendments. Environmental factors indeed influence the soil microbial diversity to such an extent that these changes can still be quantified 50 years later. These changes are therefore highly likely to stay and certainly not transitory. Per se it is not unexpected that a long-term practice of giving organic supplements affects the composition of the soil microbial diversity in the soil. Indeed one of the objectives of the long term experiments from which these sample series were taken was to evaluate the effect of the long term gift of especially animal manure on the organic matter content of the soil, and one of the conclusions was that such gifts result in the accumulation of organic matter in the soil. Organic matter is known to affect the microbial composition of the soil, either directly by serving as a slowly degradable substrate, or indirectly by affecting the environmental conditions (water content, O_2 diffusivity etc.) of the soil. For the present research we used universal primers for the PCR procedure. In future work it will be interesting to use more group specific primers to evaluate the effect of environmental and management conditions on specific groups of bacteria or archea in the soil.

Our results give some indication of the rate with which long term changes in soil microbial diversity may occur. Experiment Pr 168 was started in 1940 and the first samples analyzed were from 1956. In those samples the treatment effect was already clearly visible. Thus within 16 years the flora had responded. Experiment Pr 65 had started in 1927 and the first samples analyzed were from 1948. Here effects were again clearly visible, i.e. effects were visible within 21 years. Obviously one of the challenges for future work will be to find and analyze time series on the effect of organic amendments where samples are also available for the years immediately following the start of the experiment. Presently searching time series in TAGA is time consuming and not very user friendly. It would be a major improvement to have

a searchable database on which samples from which experiments are available in TAGA.

The strategy employed in the present research was rather opportunistic. Originally the idea was to select a few old time series from a sandy and a clay soil that had or had not received manure for a series of years, and that were used to grow various crops in rotation or were under grass. Finding such series proved to be relatively time consuming. A subsequent setback was that the older experiments were run without replication. Thus strictly speaking it is not possible to conclude from e.g. the results of experiment Pr 168 that the observed effect is caused by the treatment imposed, especially (but not only) since no samples were available from time zero to check (proof) that at time zero the material was identical. In the present research this obstacle was at least partially neutralized by using parallel treatments that were almost identical. Furthermore the trend was observed in samples from various independent experiments.

TAGA gives access to approximately 250,000 soil samples from a range of experiments, soil types and locations and land use. Especially land use changes may be interesting to check for changes in microbial diversity and probing whether these changes are reversible.

7 Conclusions

We have shown in a series of experiments that it is possible to obtain DGGE banding patterns as an indicator of microbial diversity from soil samples that have been stored for more than 50 years. This opens up the possibility to re-analyze soils that have been stored for sequential influence of environmental changes. Such analyses may provide an answer as to the degree of vulnerability of soil functioning and microbial diversity to gradual human induced environmental changes such as climate change and agricultural intensification. The banding patterns observed in our analyses appear indeed to be influenced by the history of the soil sample analyzed. Thus it is possible to obtain information from archived soil samples on the history of the soil sample and hence on the effect of environmental parameters on the soil microflora at the time the sample was taken. If microbial diversity would change swiftly in response to warmer and drier summers (climate change) from year to year, this might be observed with DGGE. However, if such changes occur more slowly or not at all they may remain invisible. Further research is needed to assess the effect of storage (including drying and grinding of the sample) on the DGGE banding pattern obtained from those samples. The present results should be regarded as a first step towards unlocking the microbial information present in archived soil samples. More specific methods (including sequencing of PCR amplified DNA from those samples) will undoubtedly provide more specific information on the type of organisms present in those samples.

8 Recommendations

The present project was carried as part of Alterra's Strategic Research Program (SEO). One of the objectives of this program was to strengthen Alterra's market position by exploring innovative areas of research. The present research obviously fits within this scope. The results have increased the value of TAGA as a source of information on long term trends in the quality of agricultural soils, and they have opened avenues for scientific research on microbial diversity in soils. To fully cash in on TAGA's potential and to explore these scientific avenues more research is needed. The recommendations to achieve these objectives include:

- Apply PCR-DGGE on TAGA with other primers (e.g. for nitrifiers, archea, fungi) to assess additional microbial functions;
- Perform additional research on the effects of drying and grinding on soil samples as stored in TAGA to identify any further artifacts;
- Apply PCR-DGGE on series of TAGA samples with replicate treatments;
- Perform other microbiological tests on TAGA samples (e.g. Biolog™) or measures of biological activity with specific soil relevant substrates to assess any changes in potential activity ;
- Make TAGA information more accessible.

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Appendix 1 Samples analyzed in the present study

Table A1. Samples analyzed from Pr 65. The samples were run on gel D974.

Code	DGGE lane	Treatment	Field code	Year of sampling
A162489	2	manure +P	1	1955; oats
A162492	3	NPK	4	1955; oats
A7631	4	manure +P	1	1948; potatoes
A7632	5	manure + P	2	1948; potatoes
A7633	6	NPK	3	1948; potatoes
A7635	7	compost + P	5	1948; potatoes
A7636	8	no additions	6	1948; potatoes
A147409	10	manure + P	2	1954; oats
A147410	11	NPK	3	1954; oats
A147412	12	compost + P	5	1954; oats
A147413	13	no additions	6	1954; oats
A162490	14	manure + P	2	1955; oats
A162491	15	NPK	3	1955; oats
A162493	16	compost + P	5	1955; oats
A162494	17	no additions	6	1955; oats

Table A2. Samples analyzed from Pr 65 continued. The samples were run on gel D980.

Code	DGGE lane	Treatment	Field code	Year of sampling
A162489	2	manure +P	1	1955; oats
A162492	3	NPK	4	1955; oats
A7634	4	NPK	4	1948; potatoes
A7637	5	NPK	7	1948; potatoes
A7638	6	PK	8	1948; potatoes
A7639	7	NK	9	1948; potatoes
A7640	8	NP	10	1948; potatoes
A147414	10	NPK	7	1954; oats
A147415	11	PK	8	1954; oats
A147416	12	NK	9	1954; oats
A147417	13	NP	10	1954; oats
A162495	14	NPK	7	1955; oats
A162496	15	PK	8	1955; oats
A162497	16	NK	9	1955; oats
A162498	17	NP	10	1955; oats

Table A3. Samples analyzed from Pr 109. The samples were run on gel D963, i.e. on the same gel as the samples from Pr 927 described in Table A6.

Code	DGGE lane	Treatment	Field code	Year of sampling
32246	2	no manure, ammonium	1	1932
245697	3	manure*, saltpeter	3	1942
A65771	4	manure*, saltpeter	3	1950
32240	5	no manure, no nitrogen	4	1932
245698	6	manure*, no nitrogen	4	1942
A65772	7	manure*, no nitrogen	4	1950
245713	9	no manure, ammonium	19	1942
A65787	10	no manure, ammonium	19	1950

*: manure was given in 1934 and 1939.

Table A4. Samples analyzed from experiment Pr 168 after cultivation of rye. Analysis was on gel D967.

Code	DGGE lane	Treatment	Field code	Year of sampling
72664	2	Manure	9	1950
181502	3	N, P, K	10	1956
181501	4	Manure	9	1956
282501	5	N, P, K	10	1962
282500	6	Manure	9	1962
327196	7	N, P, K	10	1965
327195	9	Manure	9	1965
353748	10	N, P, K	10	1968
353747	11	Manure	9	1968
377339	12	N, P, K	10	1971
377338	13	Manure	9	1971
403718	14	Manure	9	1974

Table A5. Samples analyzed from experiment Pr 168. Analysis was on gel D959.

Code	DGGE lane	Treatment	Field code	Year of sampling
A215512	2	no manure or secondary crop	10	1958; potatoes
A215511	3	manure	9	1958; potatoes
A377339	5	no manure or secondary crop	10	1971; rye
A377338	6	manure	9	1971; rye
A377330	7	manure plus secondary crop	1	1971; rye
A387896	9	no manure or secondary crop	10	1972; oats
A387895	10	manure	9	1972; oats
A387887	11	manure plus secondary crop	1	1972; oats
A398698	12	no manure or secondary crop	10	1973; potatoes
A398697	13	manure	9	1973; potatoes
A398689	14	manure plus secondary crop	1	1973; potatoes

Table A6. Samples analyzed from Pr 927. The samples were run on gel D963, i.e. on the same gel as the samples from Pr 109 described in Table A3.

Code	DGGE lane	Treatment	Field code	Year of sampling
A314681	12	straw, low N	1	1964; oats
479930	13	no straw, no N	2	1947; beans
A314682	14	no straw, no N	2	1964; oats
479930	16*	no straw, no N	2	1947; beans
A314682	17*	no straw, no N	2	1964; oats

*: samples analyzed on lanes 16 and 17 were replicates of samples analyzed on lanes 13 and 14 respectively.

Appendix 2 DGGE profiles of soil samples analyzed in the present study

Code	*experiment*
D967	Pr168: effect of animal manure
D959	Pr168: effect of animal manure and secondary crops
D963	Pr109 and Pr 927: effect of soil type; similarity of replicates
D974	Pr65: effect of organic amendments
D980	Pr65: effects of organic amendments (continued)

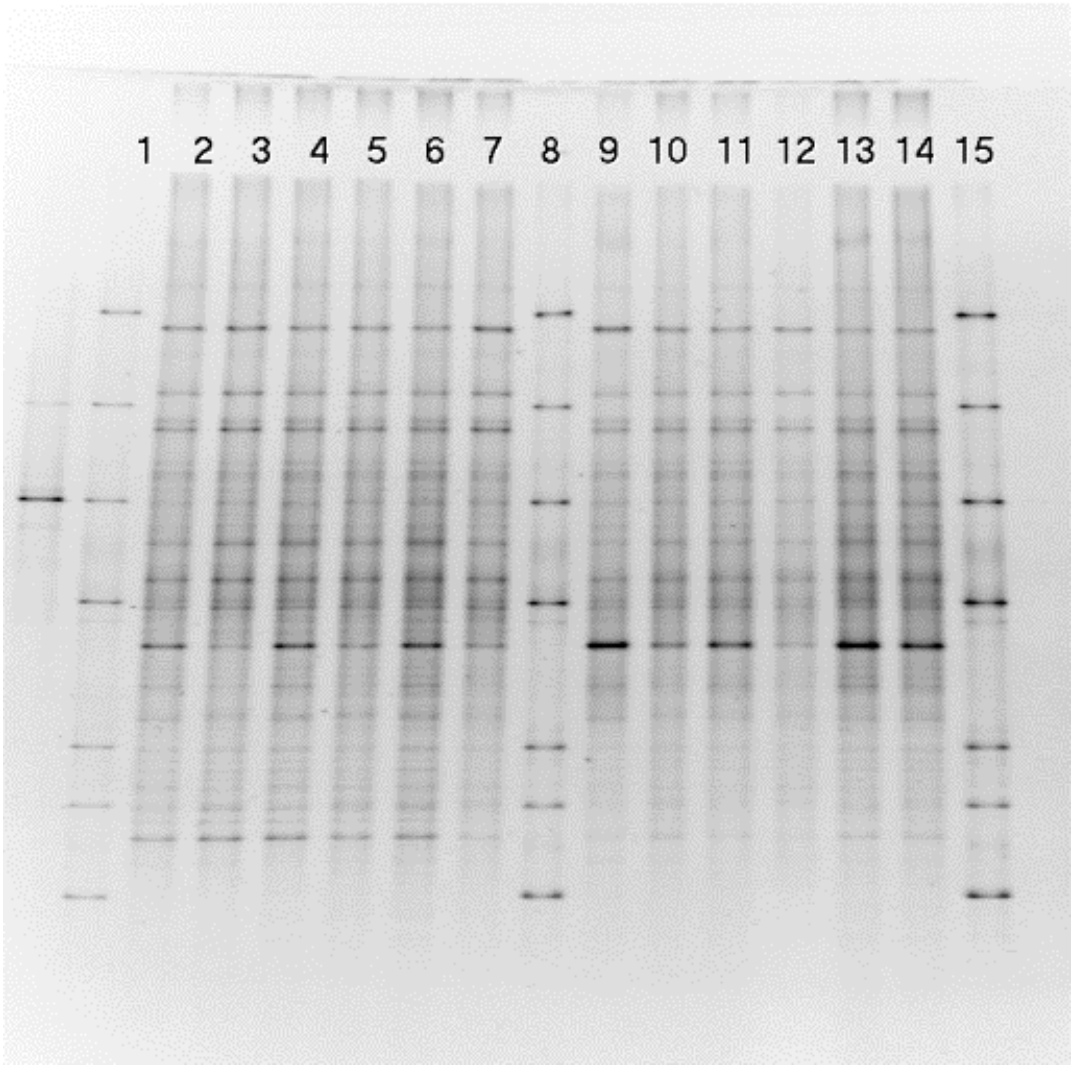


Figure A1. DGGE profile D967

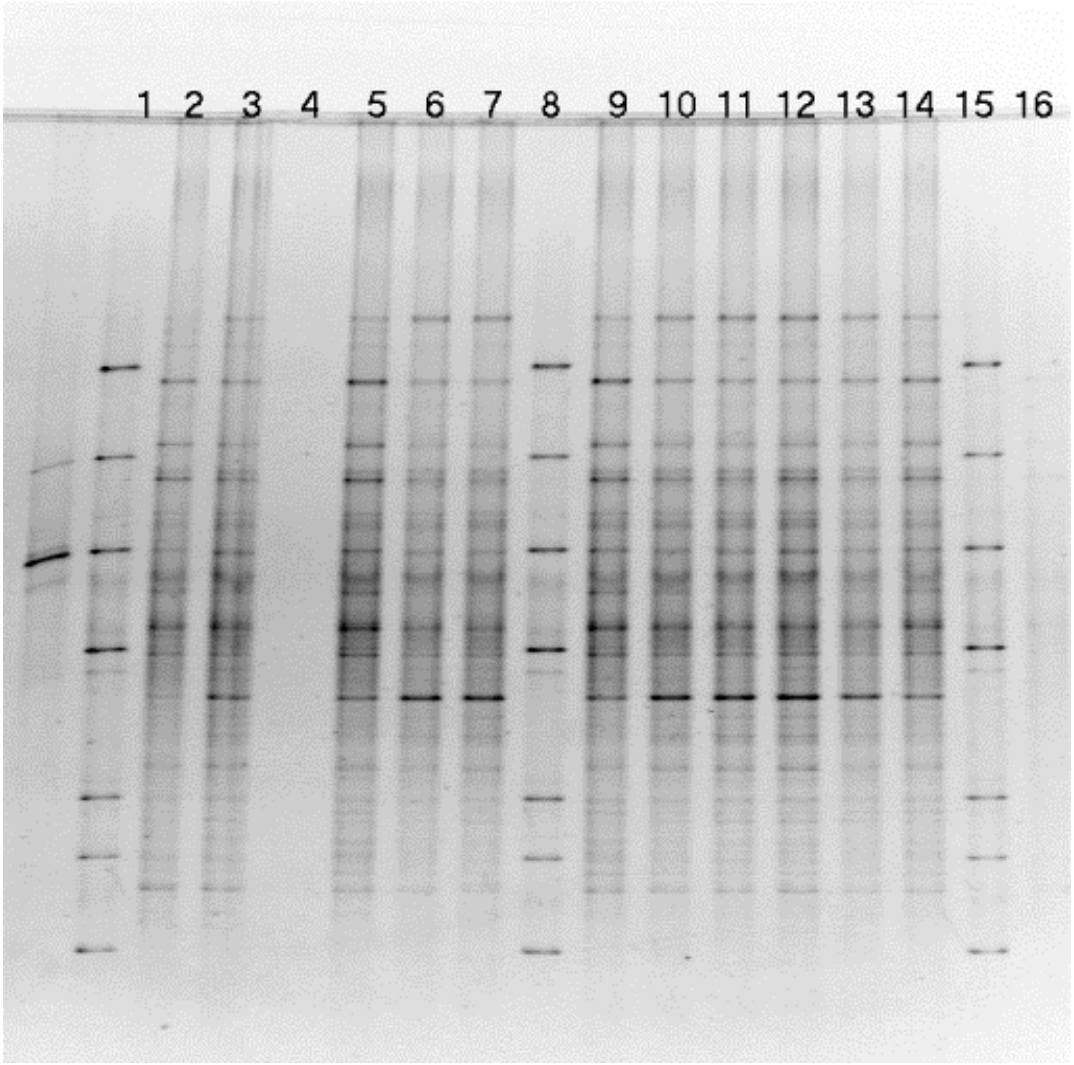


Figure A2. DGGE profile D959

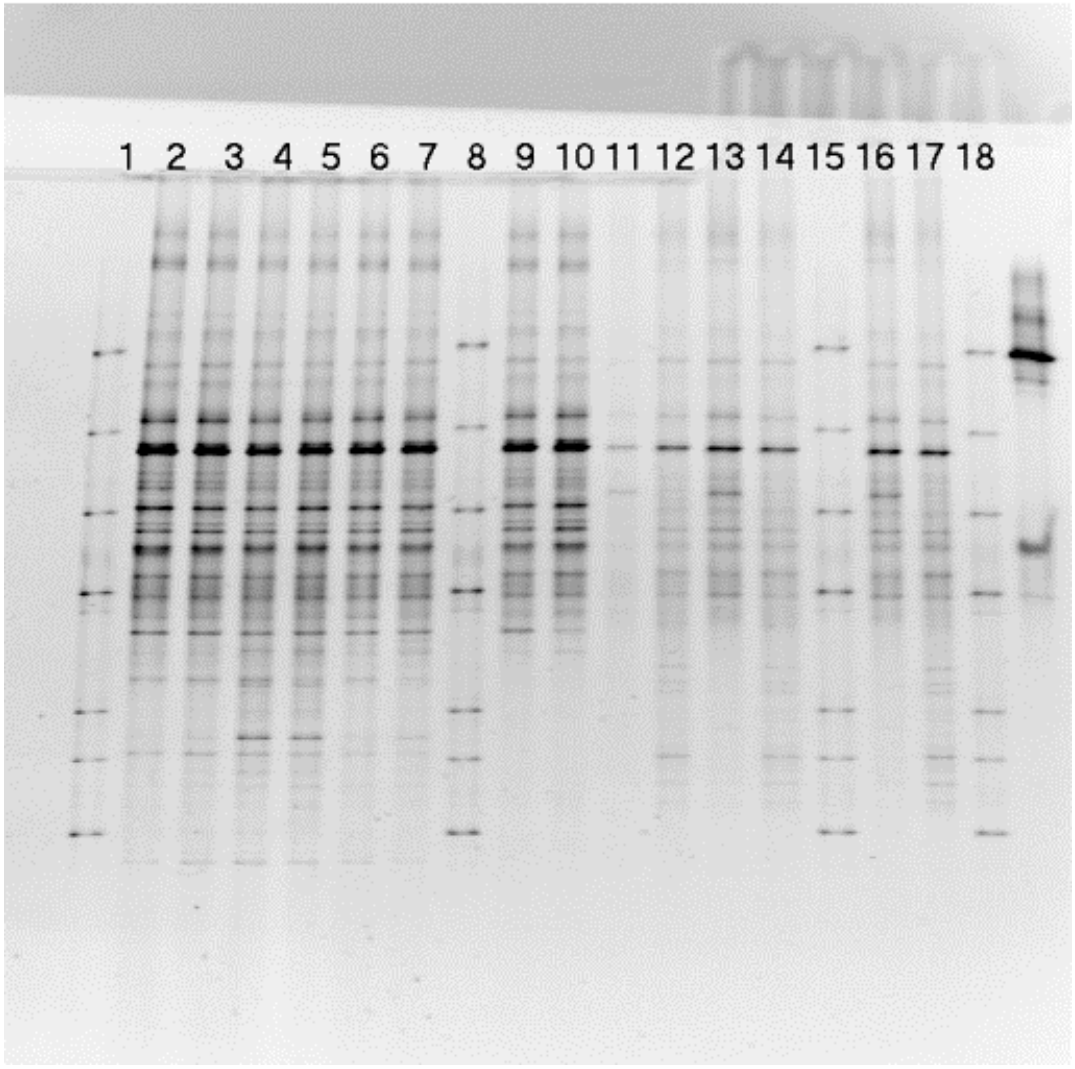


Figure A3. DGGE profile D963

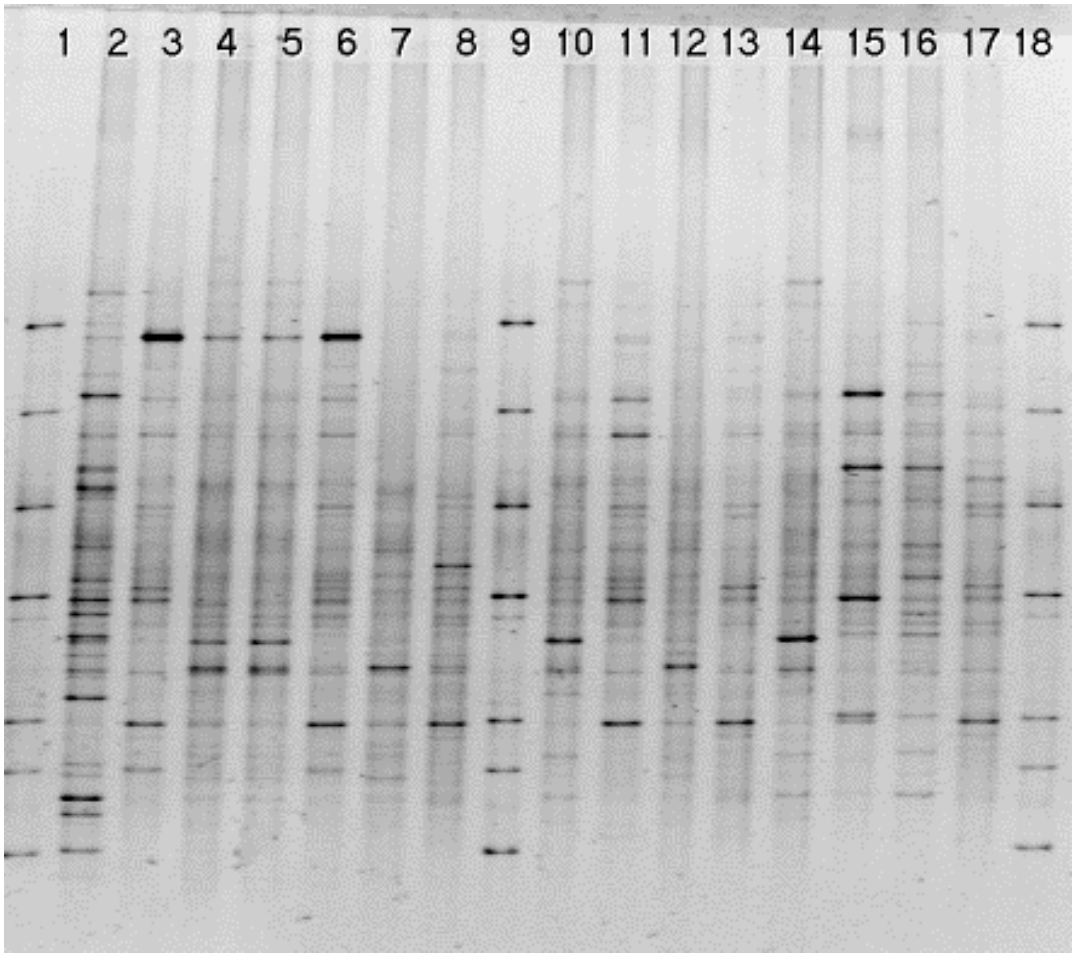


Figure A4. DGGE profile D974

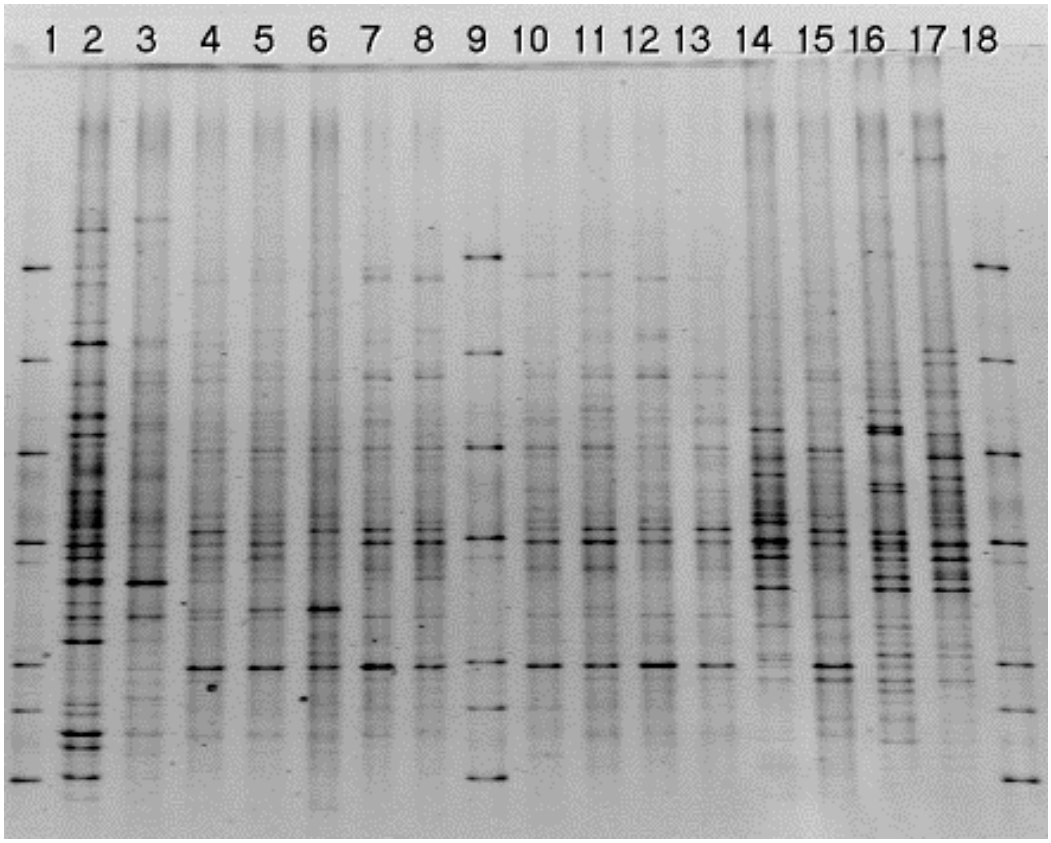
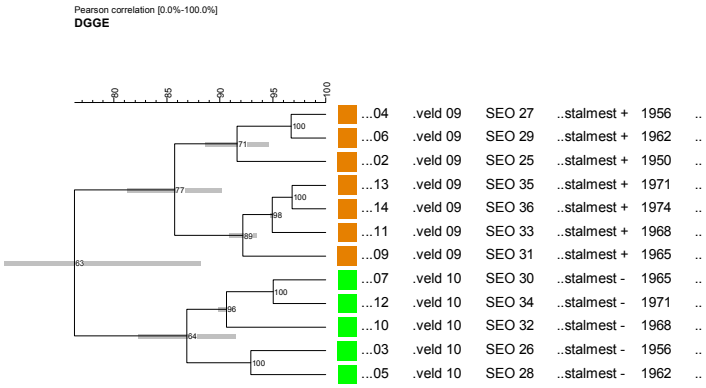
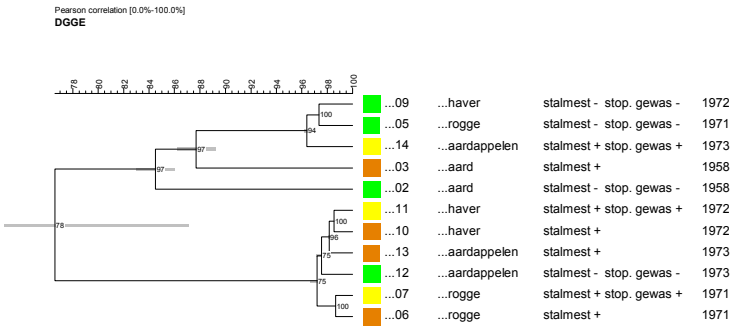
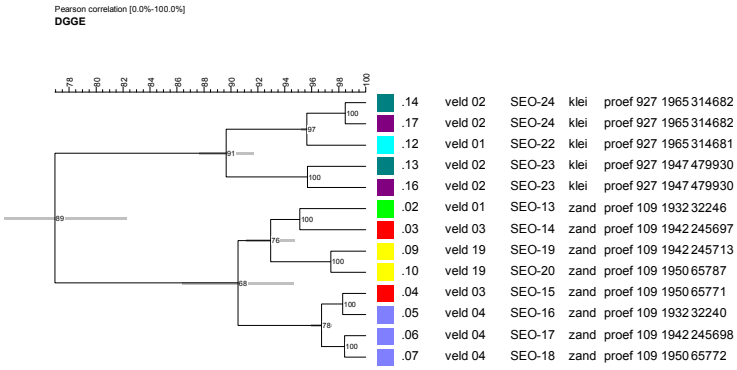


Figure A5. DGGE profile D980

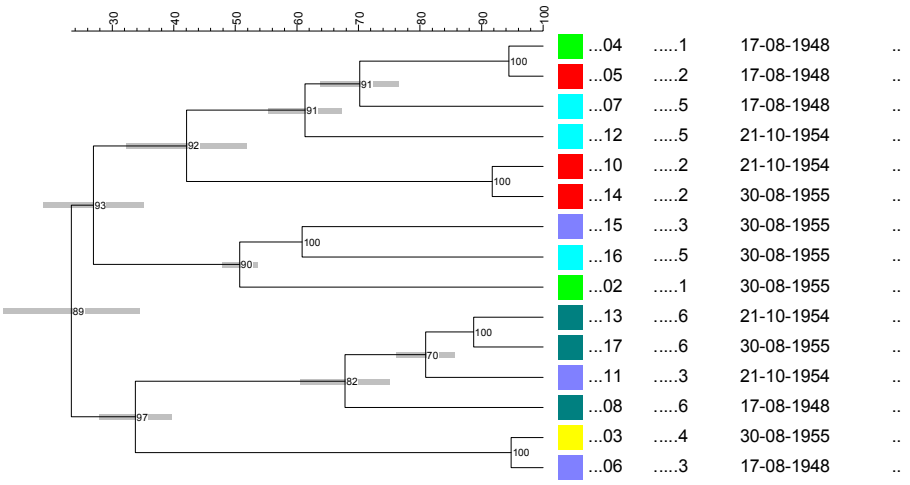
Appendix 3 Dendrograms of the DGGE gels presented in Appendix 2



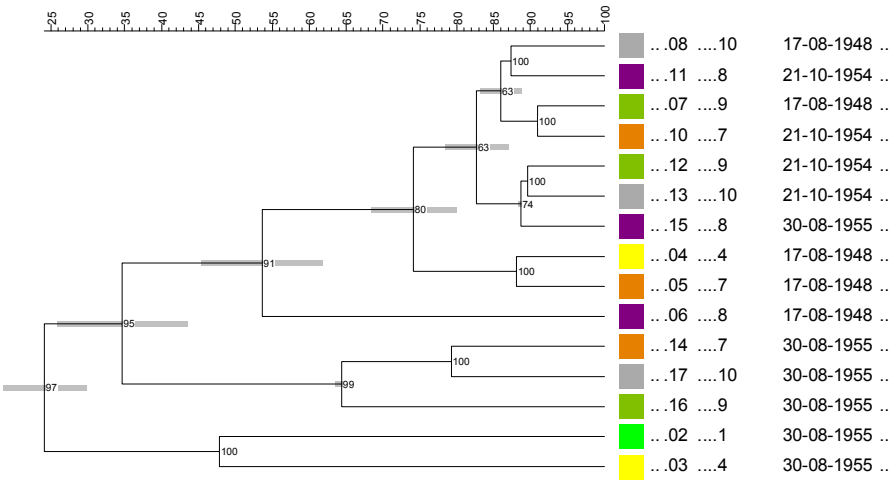




Pearson correlation [0.0%-100.0%]
DGGE



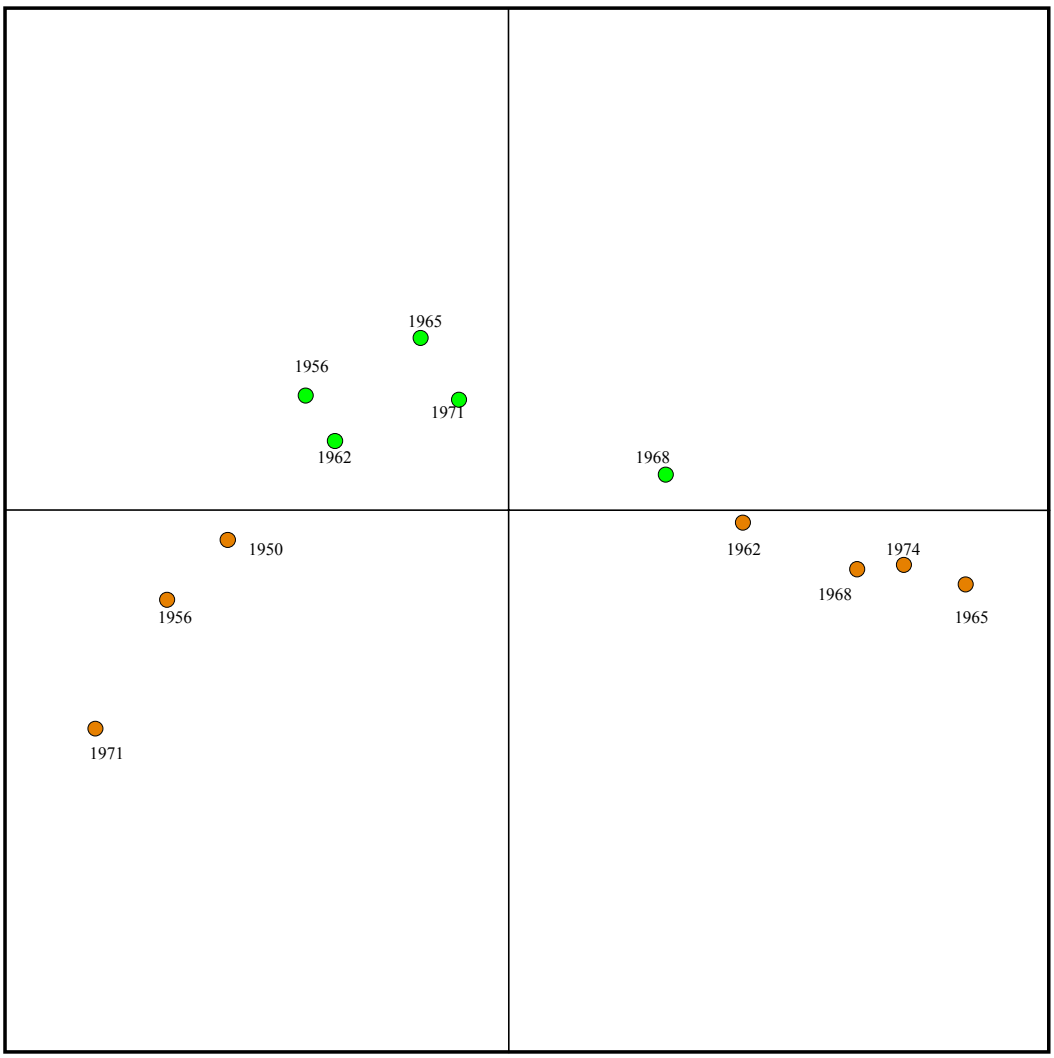
Pearson correlation [0.0%-100.0%]
DGGE



Appendix 4 Quantitative Principle Component Analysis of the gels presented in Appendix 2

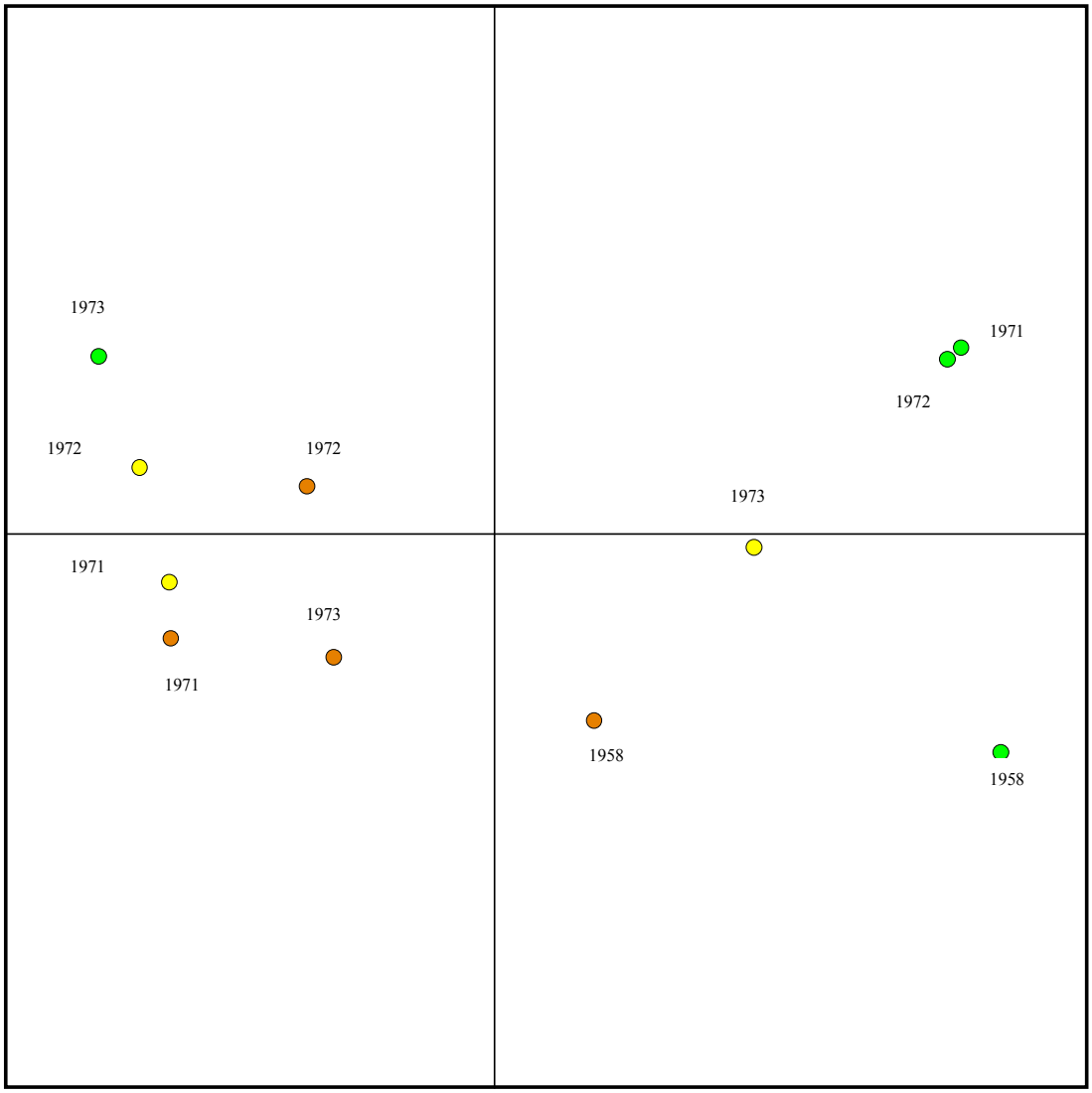
D967

Stable manure - green
Stable manure + brown



D959

Stable manure –	secondary crop –	green
Stable manure +	secondary crop +	yellow
Stable manure +	secondary crop –	brown



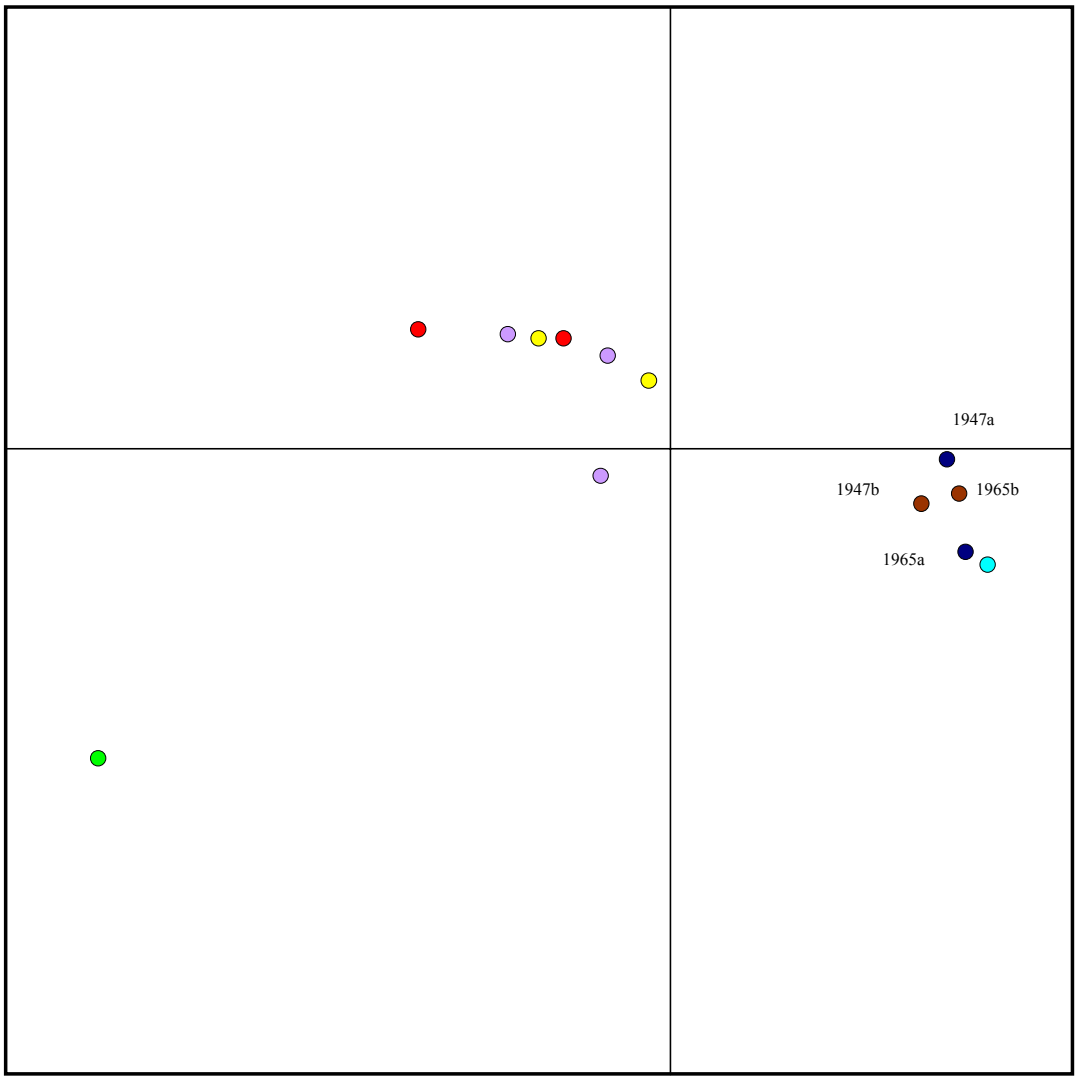
D963

Pr109

Location 1	light green	(1x)
Location 3	red	(2x)
Location 4	lila	(3x)
Location 19	yellow	(2x)

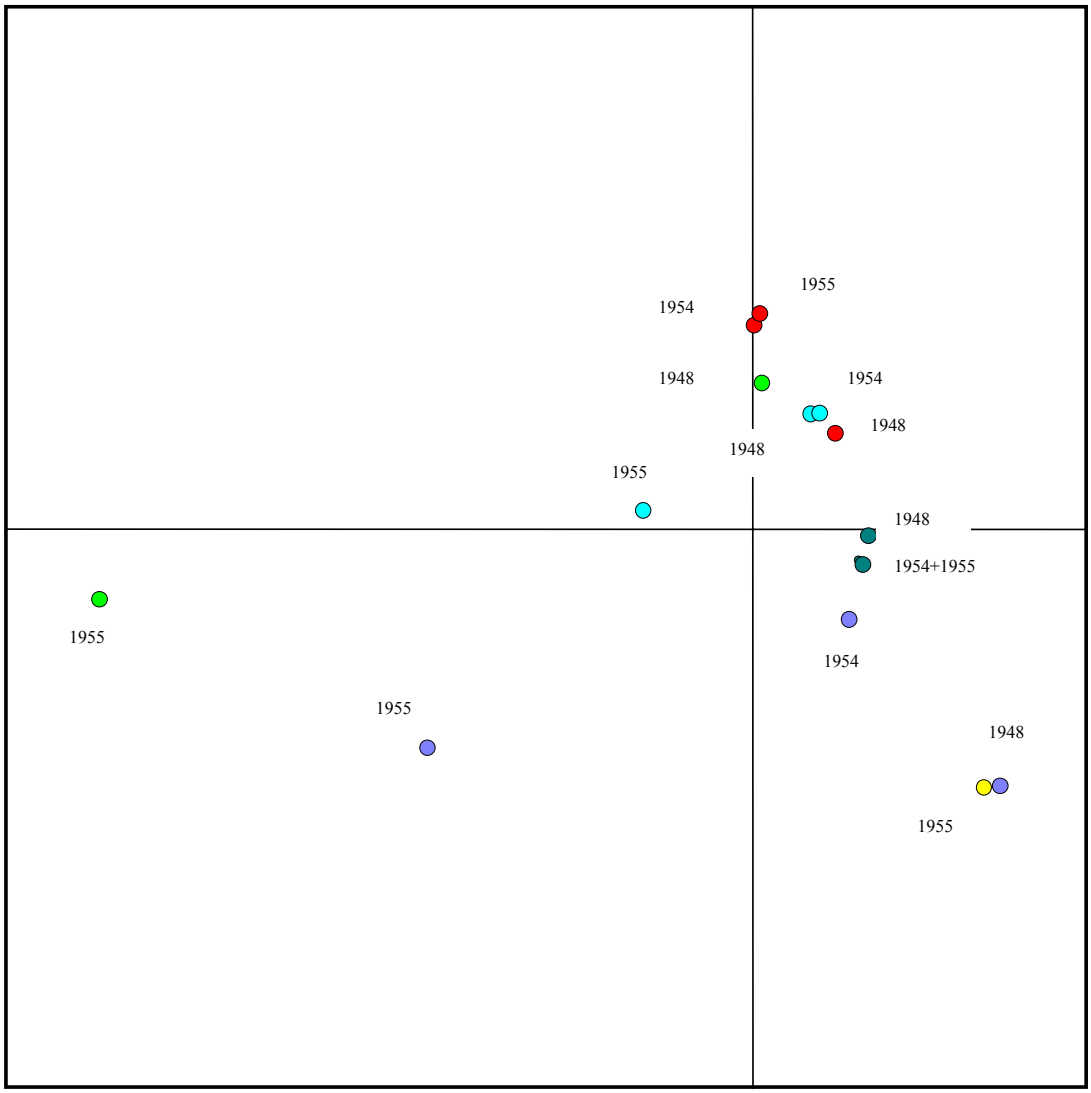
Pr927

Location 1	light blue	(1x)
Location 2	dark blue	(2x) 1947a en 1965a
Location 2	brown (replicates of dark blue)	(2x) 1947b en 1965b



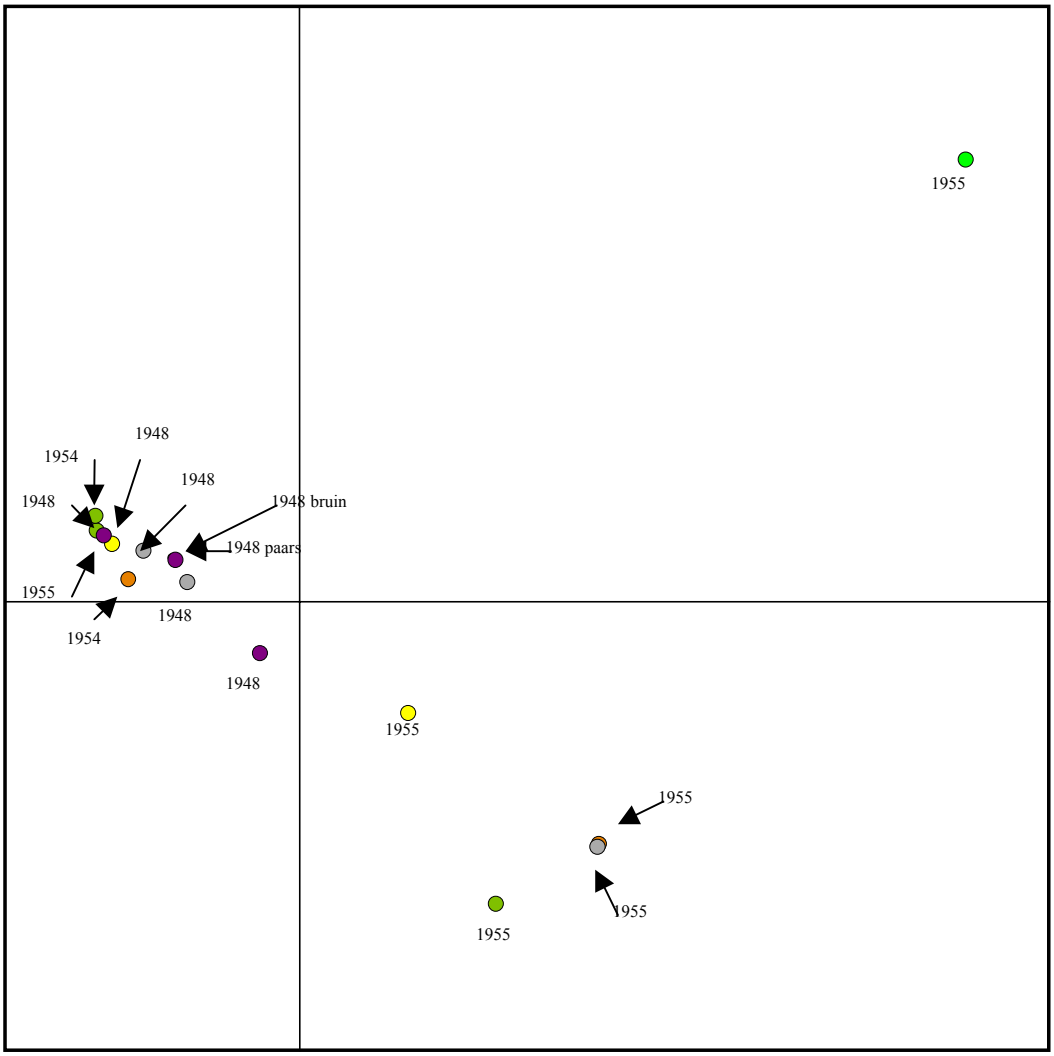
D974

Treatment 1	light green
Treatment 2	red
Treatment 3	lila
Treatment 4	yellow
Treatment 5	light blue
Treatment 6	dark blue



D980

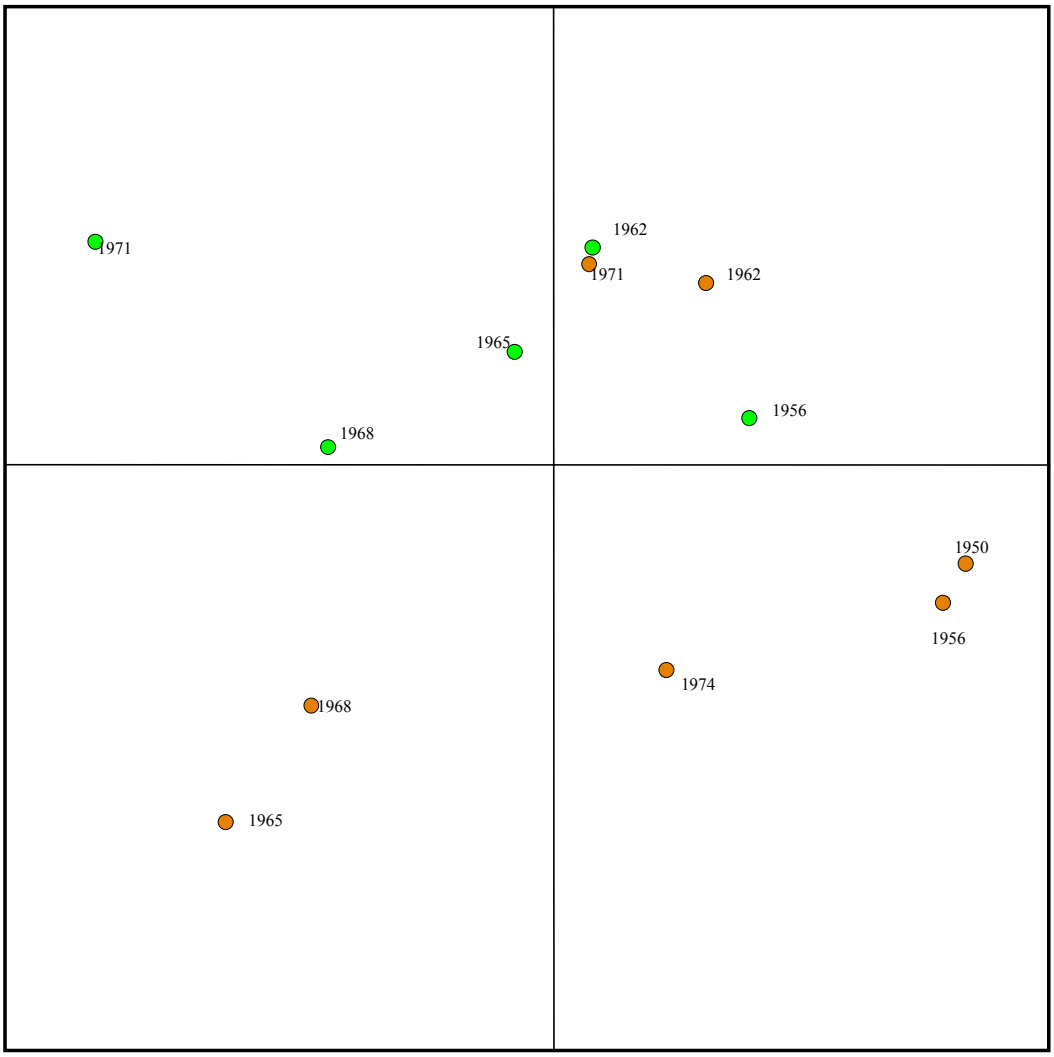
Treatment 1	light green
Treatment 4	yellow
Treatment 7	brown
Treatment 8	dark purple
Treatment 9	dark green
Treatment 10	grey



Appendix 5 Qualitative Principle Component Analysis of the gels presented in Appendix 1

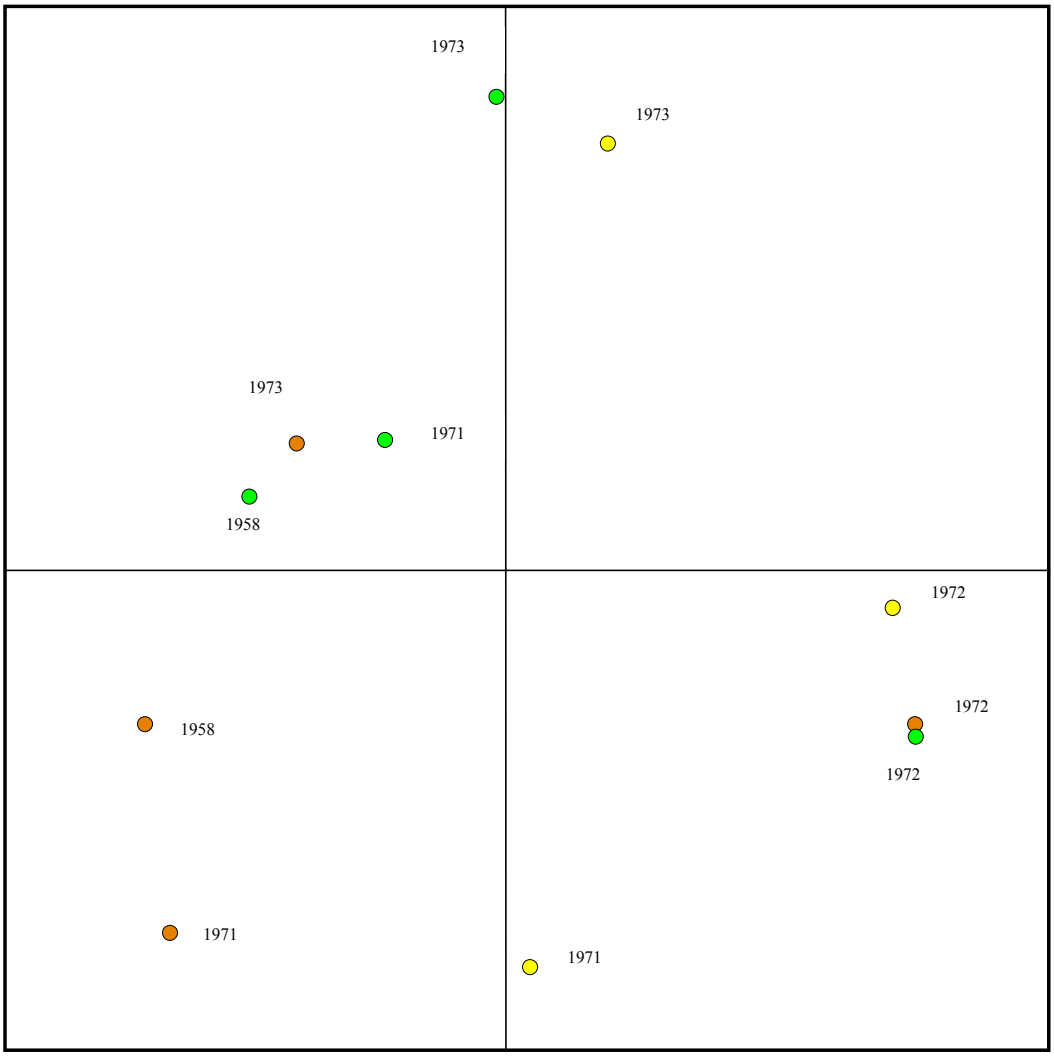
D967

Stable manure - green
Stable manure + brown



D959

Stable manure –	secondary crop –	green
Stable manure +	secondary crop +	yellow
Stable manure +	secondary crop –	brown



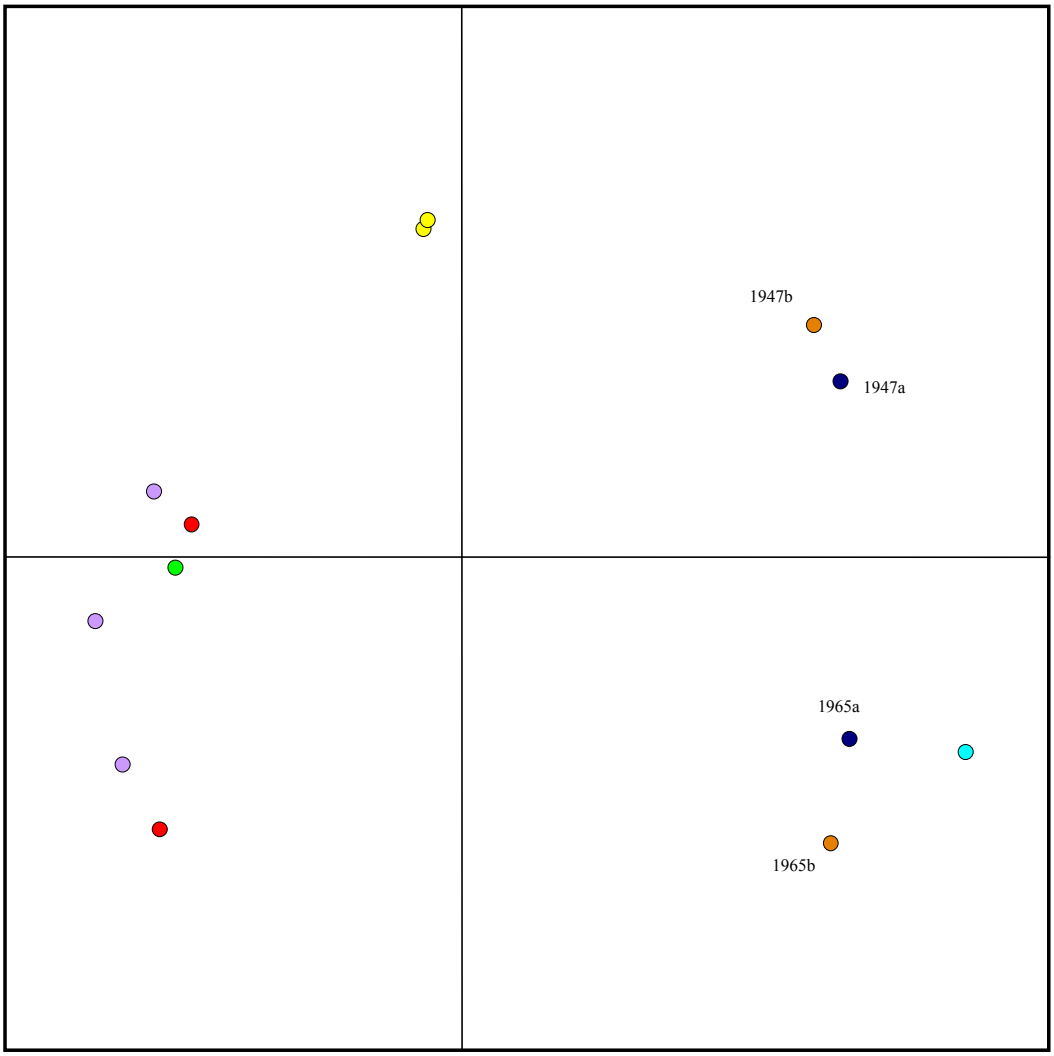
D963

Pr109

Location 1	light green	(1x)
Location 3	red	(2x)
Location 4	lila	(3x)
Location 19	yellow	(2x)

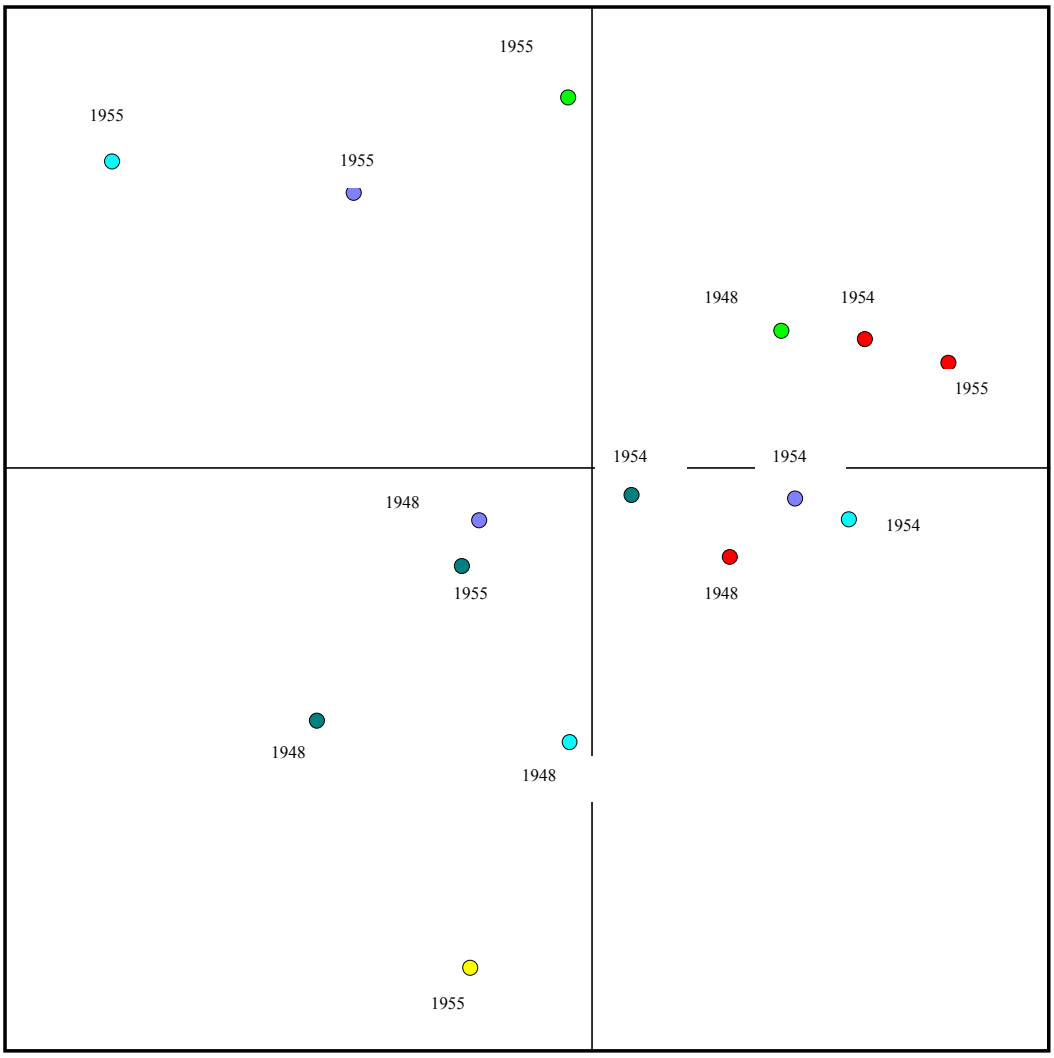
Pr927

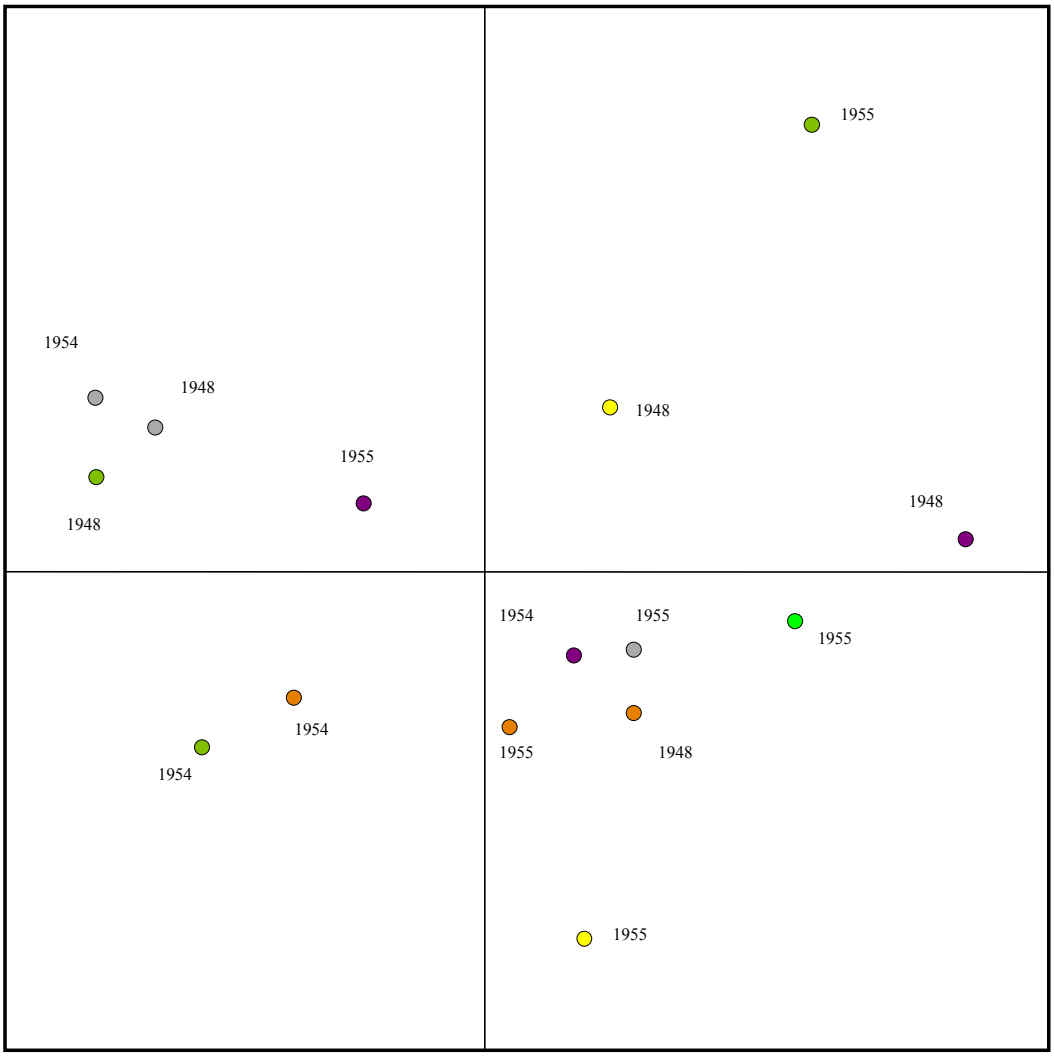
Location 1	light blue	(1x)
Location 2	dark blue	(2x) 1947a en 1965a
Location 2	brown (replicates of dark blue)	(2x) 1947b en 1965b



D974

Treatment 1	light green
Treatment 2	red
Treatment 3	lila
Treatment 4	yellow
Treatment 5	light blue
Treatment 6	dark blue



D980

Treatment 1	light green
Treatment 4	yellow
Treatment 7	brown
Treatment 8	dark purple
Treatment 9	dark green
Treatment 10	grey