Factors Affecting Rates of Change in Soil Bacterial Communities

Marie Pettersson

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List of papers

This thesis is based on the following papers, which will be referred to by their Roman numerals.

I Pietikäinen, J., Pettersson, M. & Bååth, E. Comparison of Temperature Effects on Soil Respiration and Bacterial and Fungal Growth Rates. Submitted.

II Pettersson, M. & Bååth, E. (2003) Temperature-Dependent Changes in the Soil Bacterial Community in Limed and Unlimed Soil. *FEMS Microbiology Ecology* 45: 13-21.

III Pettersson, M. & Bååth, E. (2003) The Rate of Change of a Soil Bacterial Community after Liming as a Function of Temperature. *Microbial Ecology* 46: 177-186.

IV Pettersson, M. & Bååth, E. Effects of the Properties of the Bacterial Community on pH Adaptation during Recolonisation of a Humus Soil. Submitted.

V Pettersson, M. & Bååth, E. Relationship between Structure and Function of Bacterial Communities during Recolonisation of a Humus Soil. Submitted.

VI Bååth, E., Pettersson, M. & Söderberg, K. H. (2001) Adaptation of a Rapid and Economical Microcentrifugation Method to Measure Thymidine and Leucine Incorporation by Soil Bacteria. *Soil Biology & Biochemistry* 33: 1571-1574.

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Abstract

The soil bacterial community is under the constant influence of its environment. Changing any of the factors affecting the bacterial community will induce a selection pressure which, with time, will change the community. In this work the effect of changing the temperature or pH on the soil bacterial community was studied.

Measurements using the [^{3}H]-thymidine incorporation technique showed that the soil bacterial community had an optimum temperature for growth and activity between 25 and 30°C. Compared to fungi, the soil bacterial community was more adapted to higher temperatures (above 30°C) and vice versa at low temperatures (below 10°C). Below optimum temperatures for growth the temperature dependency of the bacterial community was well modelled by the square root (Ratkowsky) model. When measuring instantaneous respiration at different temperatures an uncoupling was found between respiration rate and bacterial activities at temperatures above 30°C, indicating that respiration might not be an accurate activity measurement at higher temperatures.

Adaptation of the soil bacterial community to higher temperature occurred when increasing the temperature from 5 to 30°C, but later decreasing it from 30 to 5°C had a much smaller effect. Thus, the turnover rate of the bacterial community, which is lower at 5°C than at 30°C, affected the rate of adaptation. When increasing the temperature, the change in PLFA pattern was faster than the change in community temperature tolerance, indicating changes due to phenotypic plasticity.

The effect of changing the pH of the soil bacterial community has also been studied. The temperature influenced the rate of adaptation to higher pH of the soil bacterial community after liming from pH 4.9 to pH 7.5. The community tolerance to high pH increased fastest at the highest temperature (30°C) and slowest at the lowest temperature (5°C). The change in phospholipid fatty acids showed a similar pattern, although the changes were slower, indicating changes in species composition. Moreover, the rate of change of the bacterial community tolerance to pH was not correlated to the effect of temperature on bacterial activity, indicating that the rate of adaptation and the turnover of the bacterial community were not directly correlated.

The bacterial recolonisation after fumigation (used to kill 99% of the bacteria) was studied in limed and unlimed soil inoculated with high (6.1) or low pH (4.9) soil. The rate of recolonisation was found to be faster in the limed soil than in the unlimed one. The fastest recolonisation was found when the limed soil was inoculated with a high pH soil, indicating that the intrinsic properties of the soil bacterial community are important for the development of a community after a perturbative event. The bacterial community tolerance to high pH was also highest for this soil during the 78 days of the experiment, indicating that the effect of the inoculum was a long-lasting one. Studies of the development of community structure during recolonisation using PLFA and DGGE analysis revealed that changes in these measurements coincided with the change in function (community pH tolerance and activity) of the soil bacterial community.

1 Background

1.1 The soil bacterial community

When studying bacteria in soil the concept "community" is often used to denote all bacteria present. There are, however, several, slightly different definitions of a community, such as: "An association of interacting species living in a particular area" (Molles, 1999), "An equilibrial assemblage of organisms whose structure is heritable" (Collins, 2003) or just simply "the group of organisms being studied" (Wilbur, 1972). In this work I have used a definition similar to the last one: "The sum of all populations in the sample". This has the implication that "the community" includes all the species in a soil, but more accurately it can sometimes be defined as merely the bacteria studied with a specific technique, like the bacterial community able to grow on agar plates.

Soil is a complex habitat, inhabited by a large number of different organisms. Among these, bacteria and fungi are the most important since they are responsible for the vast bulk of decomposition, and also make up the largest part of the biomass in soil (Schimel, 2001). Many of the essential transformations in the nitrogen, sulphur, phosphorus and other element cycles are mediated by microbes. Bacteria are the most abundant microorganism group in soil and can attain concentrations of more than 10^8 cells per gram of soil (Sylvia *et al*., 1998), or 10^{11} per gram organic material, (Bååth, 1998). The number of different species, or genomes per gram of soil is not yet known, but estimates of as many as 10^4 to 10^6 have been presented (Sylvia *et al*., 1998). The supply of carbon is usually considered the limiting factor for growth of the bacterial community, even in soils high in organic matter. However, there are also studies suggesting that nitrogen and phosphorus might be limiting in some soils (Christensen *et al*., 1996; Duah-Yentumi *et al*., 1998; Schimel and Weintraub, 2003).

1.2 Environmental factors

There are a number of environmental factors that affect the bacterial community. Some of these factors are called *modulators* (Balser *et al*., 2001), in contrast to *resources* that the microbial community needs for growth (e.g. carbon, nitrogen). The difference between modulators and resources is that organisms actively compete for resources, while they can not compete for modulators. Examples of modulators are temperature, pH, water potential and salinity. Microbes can exhibit homeostasis in response to changes in modulators. If, for example, the external pH changes the internal pH will be maintained (Niedhardt *et al*., 1990). Similarly, microbes will change the composition of their membrane fatty acids after a change in temperature (Russel and Fukunaga, 1990), or alter their internal solute potential in response to changes in soil salinity (Niedhardt *et al*., 1990). However, it requires energy to maintain cell integrity after a modulator change and the resulting stress might affect the soil community. The altered selection pressure may eventually result in a change in the community composition towards a new community adapted to the new environmental conditions.

1.2.1 Temperature

Microorganisms can be found in a wide range of temperatures, from the cold of the Arctic to the near-boiling environments of geysers. Depending on the different temperature regimes, these bacteria have different temperature relationships with cardinal points (minimum, optimum, maximum) related to the environmental conditions.

Soil temperature greatly influences the rates of biological, chemical and physical processes in the soil. This is traditionally described by the Q_{10} relationship, i.e. the factor by which the activity increases when the temperature increases by 10°C. Within a limited range of temperatures the rates of biological and chemical processes usually increase two to three times for every 10°C increase in temperature. However, it is commonly reported that Q_{10} increases with decreasing temperature, from being about 2.5 at 20°C up to greater than 8 at 0°C (Kirschbaum, 1995; 2000). Since the turnover rate of the bacterial community is linked to temperature and increases until the optimum temperature is reached, one can assume that temperature will also affect other processes such as the adaptation rate of the bacterial community after a perturbation event.

1.2.2 pH

As one of the most important environmental factors, pH has a determining role in the type of microorganisms that predominate in different soils (Lynch and Hobbie, 1988; Matthies *et al*., 1997). Although pH does not normally vary much over time (Skyllberg, 1993), management practices such as liming to counteract acidification can induce rapid changes in the soil pH. Liming has frequently been reported to increase numbers of bacteria, measured using plate counts (e.g. Ivarson, 1977; Nodar *et al*., 1992; Shah *et al*., 1990), since the available carbon in acidic soils becomes susceptible to microbial attack when the pH is raised (Curtin *et al*., 1998; Persson *et al*., 1991; Shah *et al*., 1990). Increased soil microbial activity, measured as soil respiration rate, (Ivarson, 1977; Illmer and Schinner, 1991) and [^{3}H]-thymidine incorporation rate (Bååth and Arnebrant, 1994) are also commonly found after liming. Eventually, liming will affect the composition of the bacterial community and result in a community adapted to more alkaline conditions (Bååth *et al*., 1992; Bååth and Arnebrant, 1994).

1.3 Adaptation of the bacterial community

A soil bacterial community consists of many populations, each with a characteristic response curve to a particular environmental factor, indicating the community's physiological flexibility (Fig. 1). This intrinsic property of the microbial community is genotypically determined through the adaptation of the populations present to the selective pressure. In response to an environmental change the microbial community can change both its biomass and composition (Balser *et al*., 2001).

All microbes have a set of optimal environmental conditions under which their growth rate is maximal, the so-called growth optima (Atlas and Bartha, 1993; Brock and Madigan, 1991). If populations of the community are forced to operate near the limits of their growth range of an environmental factor their

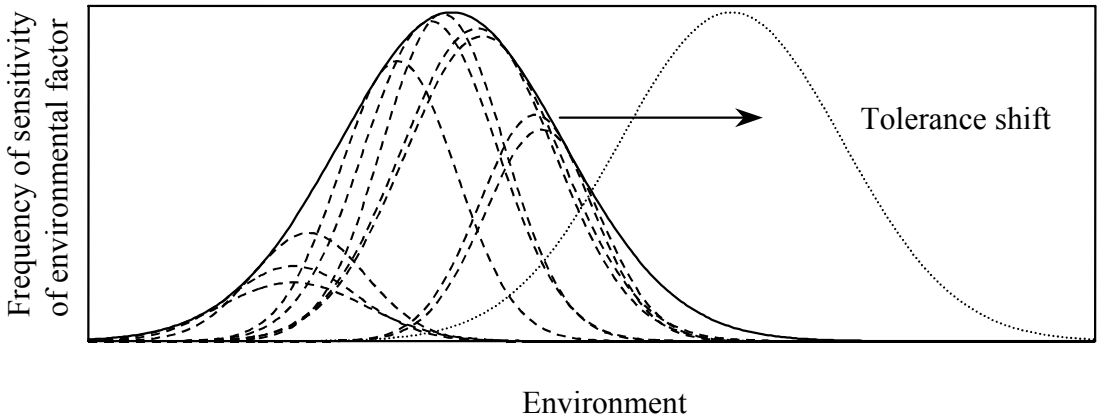

Fig. 1 The response (solid curve) of the soil bacterial community to an environmental factor is the sum of the responses of all subpopulations (dashed curves). This curve indicates the physiological flexibility of the whole bacterial community. If there is a change in an environmental factor there will be a shift in the tolerance response of the community to that factor leading to a bacterial community with a new response curve (dotted curve).

growth will decline and they will utilise substrates less efficiently (Anderson 1994; Anderson and Domsch, 1993). A change in the environment will therefore induce a selection pressure, which will gradually shift the growth optima of the community (Fig. 1). If the environment changes to a situation far from the initial optimum conditions for the bacterial community a shift will occur in the composition of the community. Organisms better suited to the new environmental conditions will grow and outcompete other organisms, or existing organisms will undergo evolutionary adaptation. Species of the microbial community can rapidly adapt even to momentary changes in the local environment (Finlay *et al*., 1997). The sensitivity to environmental change and disturbance differs between bacterial communities.

Davis and Shaw (2001) suggested that adaptive responses of vegetation to climate change occur at many levels, including phenotypic plasticity, genetic adaptation, succession and migration processes. The situation will be similar for the soil microbial community. If there is a drastic change in the environment, part of the community will die preparing for more tolerant species to take over, so-called "species replacement" (Lyon and Sagers, 1998) similar to succession. This type of tolerance shift has been reported by Díaz-Raviña and Bååth (1996) after heavy metal addition. If a strong selection pressure is exerted this can favour certain genotypes or mutants and thus introduce new genotypes in the community. A less dramatic change in the environment can lead to phenotypic changes in the population. In this work the term "*community adaptation*" is used to account for all these changes in the bacterial community after a perturbation event, since it is difficult to differentiate between the different mechanisms responsible for changes in the bacterial community.

There is some evidence that communities of different origin can differ in their adaptation and ability to function in a particular environment. For example, Hunt *et al*. (1988) found that a litter type decomposed faster when placed in its ecosystem of origin than in another type of ecosystem. Similarly, Cookson *et al*.

(1998) found that the soil community adapted to decompose added wheat straw performed this decomposition better than communities originating from soil without added straw. Thus, the response of a microbial community to a change in the environment can depend on the initial characteristics of the surrounding environmental factors.

Balser *et al*. (2001) proposed a conceptual model for the microbial response and development of adaptation after an environmental change (Fig. 2). The response was divided into four phases. Before any change in environmental

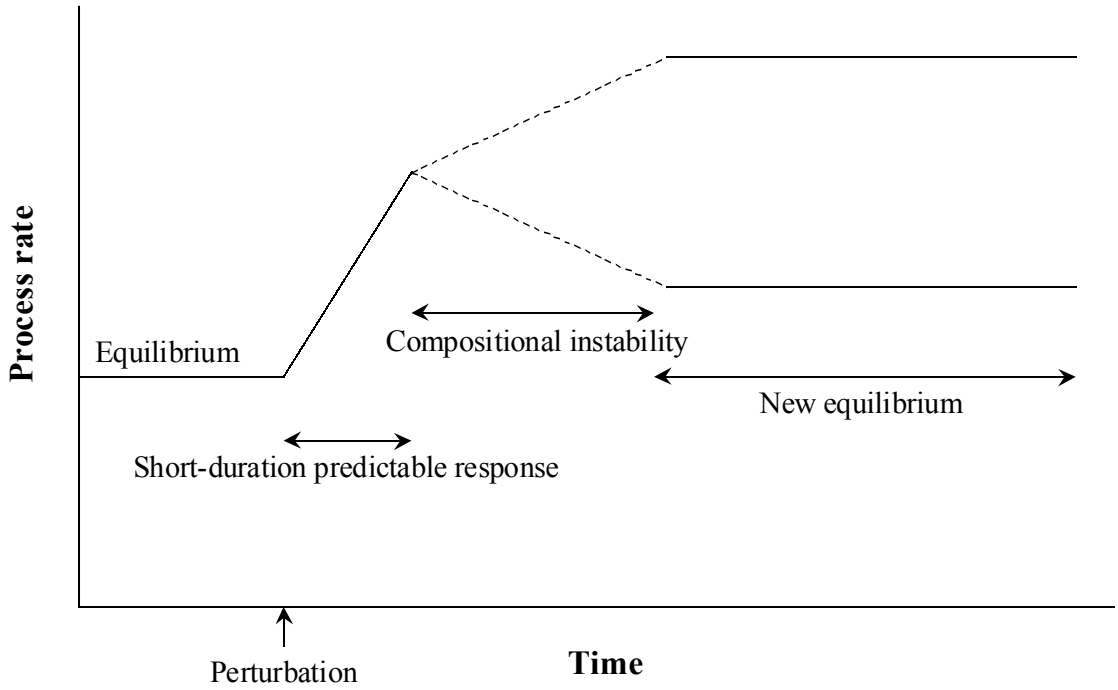

Fig. 2 Model of adaptation of the bacterial community after a perturbation (adapted after Balser *et al*., 2001). In a stable environment the bacterial community is in equilibrium, optimised to ambient environmental conditions. A perturbation will lead to a short period of predictable response. After a period of compositional instability, during which the community will adapt to the new conditions the bacterial community will reach a new equlibrium.

factors the microbial community is in equilibrium, well adapted to the ambient environmental conditions. If a drastic change in the environment occurs there will initially be a predictable response, such as after an increase in temperature where the response of the microbial community initially follows the square root model (Ratkowsky *et al*., 1982; 1983). Then there will be some structural changes in the community as it adapts to the new environmental conditions. During this adaptation some species will be outcompeted by others better suited to the new conditions. This period will be less predictable, since the extent and direction of the structural changes will be dependent on the composition of the initial community. Eventually, a new equilibrium will be reached with a community better optimised to the new environment. The composition of this community will, however, to a certain extent depend on the characteristics of the prevailing environment and the composition of the old community.

1.4 Methodology

After a perturbation event, the function, for example the activity of the bacterial community, can be altered. Furthermore, since there will be a change in selection pressure favouring species tolerant to the new conditions, the community tolerance of the soil bacterial community to the factor that has been changed will also be affected. This is similar to the PICT (pollution-induced community tolerance) concept which is based on the fact that organisms that survive in toxic environments do so because they are sufficiently tolerant to the concentration of the toxic chemicals in their surroundings (Blanck, 2002). Initially, this was used to quantify community tolerance of arsenate of algal species and measuring PICT, using inhibition of photosynthesis (Blanck and Wängberg, 1988). We have developed this concept further, studying intrinsic properties of the bacterial community such as community tolerance to pH and to temperature. The change in community tolerance after a perturbation event can be monitored with different techniques, for example, thymidine and leucine incorporation (Díaz-Raivña and Bååth, 2001), measurement of photosynthesis (Blanck and Dahl, 1998; Blanck and Wängberg, 1988), Biolog (Müller *et al*., 2001; Rutgers *et al*., 1998) and respiration (Gong *et al*., 2000).

An increase in tolerance can occur in different ways changing the structure of the community. These changes can be studied by analysing the composition of the phospholipid fatty acids (PLFAs) in the community. A change in the PLFA pattern of the community indicates that the community has been affected by the environmental change. However, this method can not determine actual species composition and can not clearly differentiate between phenotypical changes and changes in species composition. To obtain a measure of the changes in the species composition of the community, molecular methods, such as denaturing gradient gel electrophoresis (DGGE) should be applied. DGGE separates 16S rDNA fragments of the same length, but with different sequences, thereby indicating different bacterial species.

2 Aims of the study

Soil bacterial communities are under the constant control of the environment. When a change in the environment occurs, this will lead to a new selection pressure and, with time, a community adapted to the new conditions. We need a better understanding of how different factors influence the bacterial community. There is also a need to examine the rate of these responses to environmental changes and the factors that influence the rate of change. Furthermore, we also need to know how these responses are related to initial community composition. In this thesis this will be discussed together with related topics.

This study mainly concerns soil bacterial communities and their development. However, although the emphasis is on bacterial communities, fungi are also discussed in Paper I and some of the PLFAs included in other papers are/or can be of fungal origin.

This study addressed the following main questions:

- What is the response in activity of a soil bacterial community in relation to temperature? Is there a difference in response to different temperatures between the fungal and bacterial communities?
- What effects do different temperatures have on the development of temperature tolerance and community structure in the soil bacterial community?
- How is the rate of soil bacterial community pH adaptation affected by temperature and how does this affect the community structure?
- Do the intrinsic properties of a soil inoculum determine the soil bacterial community's recolonisation and adaptation rate after liming? Is the change in function of the community correlated to structural changes?

3 Methods

3.1 Activity measurements

3.1.1 Thymidine incorporation

The synthesis of new cell components, such as peptidoglycan (cell walls), membranes, protein, RNA and DNA, must occur before division of the bacterial cell. During balanced growth different macromolecules, including DNA, will be synthesised at the same rate as the increase in cell numbers. This means that the bacterial growth rate can be measured from the rate of DNA synthesis (Moriarty, 1984). DNA synthesis can be indicated by the rate at which DNA becomes labelled with a labelled precursor, like [^{3}H]-thymidine. This is used in the [^{3}H]-thymidine (TdR) incorporation technique (Thomas *et al*., 1974; Fuhrman and Azam, 1980; Moriarty, 1986). After uptake in the bacteria the labelled thymidine is phosphorylated by the enzyme thymidine kinase and further processed and inserted into DNA.

The TdR incorporation method is specific to actively growing heterotrophic bacteria, since fungi and other eukaryotic organisms generally do not contain thymidine kinase (Moriarty, 1986; Robarts and Zohary, 1993). Not all species of bacteria are, however, able to incorporate thymidine. Among these are the pseudomonas, which lack thymidine kinase (Robarts and Zohary, 1993), and some anaerobic bacteria and cyanobacteria, which are autotrophic.

There are several factors that can affect the estimates of bacterial growth rate and production using the TdR incorporation technique. Among these are non-specific labelling of macromolecules other than DNA and isotope dilution due to, for example, adsorption of [^{3}H]-TdR to humic and clay compounds in soil and sediment samples (Robarts and Zohary, 1993). These problems are, however, of minor importance when using the TdR incorporation values in a relative way, as in the present work to indicate community tolerance to different environmental factors. This is also the case when using the homogenisation-centrifugation technique to extract the bacteria from soil (Bååth, 1992). This does not release all bacteria, but only about 30% (Bakken, 1985). However, since the data was used as relative indicators, a less drastic method (shaking) to release bacteria was used (see below). This was faster and gave more reproducible results.

The TdR incorporation technique was originally used in aquatic habitats but has been adapted for use in soil. The technique of Bååth (1992) was used in the studies described in Papers III and IV. It was further adapted in a later study (Paper VI) using centrifugation instead of filtration to remove unincorporated TdR. The centrifugation technique was also originally used in aquatic habitats (Smith and Azam, 1992; Kirschner and Velimirov, 1999). The centrifugation technique has the advantage of being less costly and less laborious than the previous technique and was used in several of the studies (Papers I, II and V). Since this technique was central in my studies it is described in detail below.

The procedure of the centrifugation technique (Paper VI) was as follows. One gram of soil was shaken with 40 ml Milli Q water on a rotary shaker (200 rpm) for 15 min to liberate bacteria from the soil particles. The soil suspension was then centrifuged at 1000 x *g* for 10 min. After filtration through glass wool (to remove large soil particles floating on the water), 1.5 ml was put in an Eppendorf tube and 5 µl methyl[^{3}H]thymidine (926 GBq mmol^{-1}, Amersham) was added. Incubation was terminated after 2 h at 22°C by adding 75 µl cold 100% trichloroacetic acid (TCA). Non-incorporated thymidine was removed by repeated centrifugation (13 000 x *g*) and washing with 1.5 ml cold 5% TCA (to precipitate macromolecules) and 1.5 ml cold 80% ethanol (to remove thymidine bound to lipids). Then 0.2 ml of NaOH was added and the samples were incubated at 90°C for 60 min to solubilise the macromolecules. Finally, 1 ml of scintillation cocktail (Ultima Gold, Canberra-Packard) was added before scintillation counting.

An alternative method to the TdR incorporation technique is the [^{14}C]-leucine incorporation technique. This has been used for many years in aquatic systems to estimate bacterial growth rates (Kirchman *et al*., 1985; 1986; Kirchman and Hoch, 1988), and also more recently in soil (Söderberg *et al*., 2002; Söderberg and Bååth, 1998; Tibbles and Harris, 1996). The leucine incorporation technique is based on bacteria incorporating radiolabelled leucine during protein synthesis. The rate of protein synthesis is then used as an indicator of bacterial activity. However, changing pH appears to directly alter the availability of leucine added to the bacteria (Bååth, 1998), and since the TdR incorporation technique is more stable when using different pH buffers, this technique was employed throughout these studies.

3.1.2 Acetate-in-ergosterol measurement

Ergosterol is an important membrane lipid of fungal cells, and when the hyphae die, ergosterol is expected to decompose rapidly. Since ergosterol is a fungal-specific lipid sterol (Sylvia *et al*., 1998), quantification of ergosterol has become common when estimating fungal biomass (Nylund and Wallander, 1992; Montgomery *et al*., 2000).

The rate of synthesis of ergosterol can be used as an indicator of fungal growth rates in natural habitats. This technique has, for example, been used for estimation of fungal growth rates in soil (Bååth, 2001; Pennanen *et al*., 1998). The technique was originally described by Newell and Fallon (1991) to measure instantaneous fungal growth rates in dead plant material in aquatic habitats. It is based on the addition of [^{14}C]-acetate to a soil slurry and the subsequent uptake and incorporation of the labelled acetate into ergosterol by the fungi. After incubation with labelled acetate the ergosterol is extracted with KOH dissolved in methanol. The ergosterol is analysed using HPLC (high performance liquid chromatography), the peak containing ergosterol is collected in a vial and the amount of labelled acetate is measured with a scintillator.

3.1.3 Respiration

The total activity of a soil is usually estimated by respiration rate. This gives the respiration of all organisms in the soil including bacteria, fungi, animals and roots, although normally bacteria and fungi are assumed to contribute most to the respiration. Changes in soil respiration rate can therefore be used as an indicator of the soil's microbiological activity. The method used here to determine respiration is CO_2 evolution measured using gas chromatography.

3.2 Bacterial community tolerance measurements

The soil bacterial assemblage has a varying tolerance to different environmental conditions. Species present in a certain environment must be able to tolerate the current conditions to be able to thrive. Tolerance under specific conditions regarding temperature, pH, moisture, salinity, etc. depends on earlier exposure of the bacterial community. If the community is exposed to a certain condition the selection pressure of that condition will favour species tolerant to that environment.

Exposure of bacterial communities to toxicants in the environment is measured using pollution-induced community tolerance. PICT is an increase in community tolerance in response to a toxic effect on the community, in which more tolerant species replace less tolerant ones (Blanck, 2002). PICT can be defined as the average ability of community members to tolerate a toxicant. The bacterial community is sampled and its tolerance to a certain environmental factor is detected through short-term exposure to that factor. Furthermore, it is important to compare the shift in community tolerance with a baseline tolerance, i.e. a bacterial community not affected by the environmental stress, to obtain conclusive results (Blanck, 2002).

These studies of the impact of environmental factors on the soil bacterial community are similar to the PICT concept, but instead of measuring the effects of toxicants on the bacterial community the impact of environmental factors such as temperature and pH was measured. By exposing the bacterial community to different pH and temperatures, and measuring the activity (TdR incorporation) (Fig. 3), it is possible to obtain an indication of how the bacterial community responds to these environmental factors. In these studies the term "*bacterial community tolerance*" was used to account for the tolerance exhibited by the whole bacterial community.

To measure the bacterial community pH adaptation after liming, different pH buffers were added to the bacterial suspension before the addition of radioactive thymidine thereby providing a measure of the activity at specific pH values (Papers III, IV and V). To obtain a measure of bacterial community tolerance to pH one can use a total pH response curve (Fig. 1, Paper III). To obtain a simpler measure of the community tolerance, the logarithmic ratio between the TdR incorporation at pH 8.3 and pH 3.6 of the bacterial community was used (Papers III, IV and V). This simplified technique has been described by Bååth (1996), where similar results to those obtained using the whole pH response curve were found. Using two extreme pH values like pH 3.6 and 8.3 gives good separation in the tolerance pattern of the bacterial community.

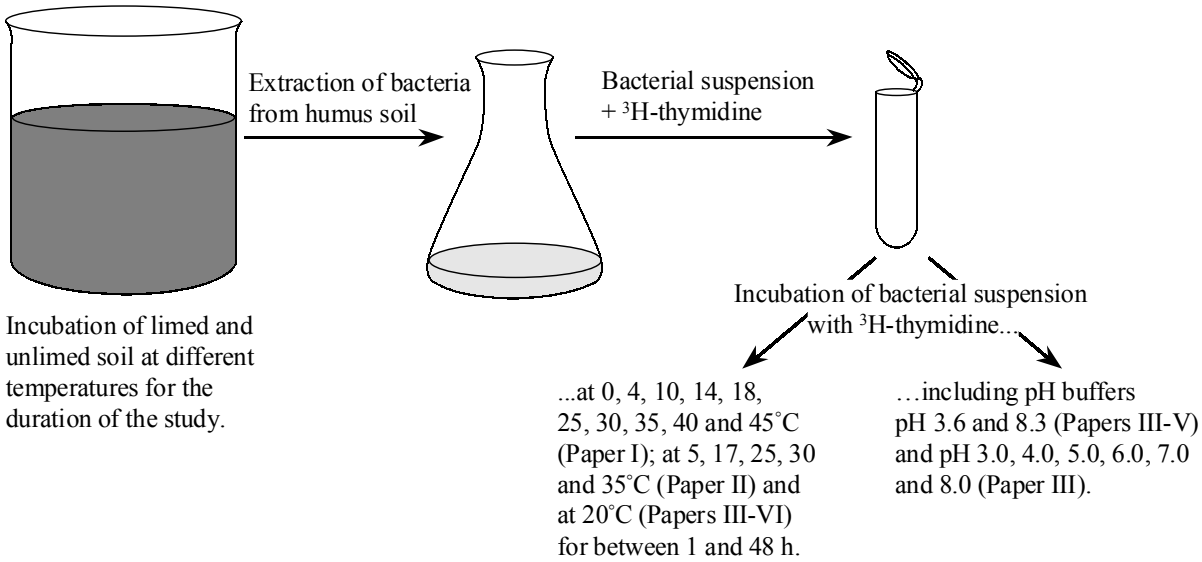

Fig. 3 Simplified overview of the bacterial community tolerance experiments in the different studies.

Similarly, when measuring the temperature tolerance of samples from various temperatures in the experiments, samples were incubated with radioactive thymidine at different temperatures to measure the difference in temperature response of different samples. To gain an indication of the temperature tolerance response curves at different temperatures were used, or simply a ratio between the community tolerance to a high and a low temperature.

3.3 Community structure

3.3.1 Phospholipid fatty acid analysis

Phospholipids are essential membrane components of all living cells. They are usually assumed to be rapidly degraded in soil and are not found in storage products (Zelles, 1999). Different subsets of the bacterial community have different PLFA compositions. PLFAs can therefore be used as indicators of different types of organisms. The composition of PLFAs has successfully been used for taxonomical purposes in studies on bacteria (Lechevalier and Lechevalier, 1988), and some fungal groups (Amano *et al.,* 1992). Changes in microbial community structure can be detected by changes in the PLFA pattern (White *et al*., 1979). For example, long, straight-chain fatty acids with several double-bonds are mainly characteristic of eukaryotes. Branched-chain fatty acids are largely found in gram-positive bacteria, while cyclopropyl fatty acids are common in some gram-negative strains. Methyl branching on the tenth carbon atom in the molecule is indicative of actinomycetes (Kroppenstedt, 1992). The PLFA 16:1ω5 has been used as an indicator of arbuscular mycorrhiza (Olsson, 1999). However, since most PLFAs are present in different concentrations in a wide range of microorganisms it is not possible to distinguish between different species in complex soil communities using only PLFA profiles. Nevertheless,

PLFA analysis is a good tool for studying the dynamics of soil microbial communities.

PLFA analysis is a common way of analysing the structure of microbial communities in soil. There have been a number of studies using this method to describe, for example, the prevailing soil community (Federle *et al*., 1986), or to describe changes in the community after a perturbation such as metal contamination (Frostegård *et al*., 1996), moisture stress during decomposition (Wilkinson *et al*., 2002) or changes during bioremediation of an oil spill (MacNaughton *et al*., 1999).

Two main extraction techniques are used to determine the community structure: determination of PLFAs or the detection of whole-cell fatty acid patterns (Zelles, 1999). For the latter, the commercially available microbial identification system MIDI is often used. The extracted fatty acid methyl esters (FAMEs) are then analysed using an automated GC procedure. In these studies the former method was used, since PLFAs are thought to more directly reflect the living biomass.

The procedure described by Frostegård *et al*. (1991), modified after Bligh and Dyer (1959), is as follows. Lipids are extracted directly from soil using a mixture of chloroform, methanol and citrate buffer. The extracted lipids are then fractionated into neutral lipids, glycolipids and polar lipids (mainly phospholipids). After mild alkaline methanolysis (transesterification) of the phospholipids, the PLFAs are transformed into free FAMEs. They are then separated and quantified on a gas chromatograph with a flame-ionisation detector.

To characterise the culturable portion of the bacterial community one can extract lipids for PLFA analysis from only cultivated bacteria (cfu-PLFA). To obtain a representative measure of the different species able to grow on agar plates, plates with between 100 and 200 colonies were chosen. Agar plates were flooded with citrate buffer and a portion of the bacterial suspension was collected for phospholipid extraction as described above.

3.3.2 Denaturing gradient gel electrophoresis

To be able to study changes in the bacterial community on a species level, molecular methods have to be applied, since PLFA analysis does not show exact changes in species composition. Today, there are a number of frequently used molecular genetic fingerprinting techniques such as DGGE, TGGE (temperature gradient gel electrophoresis), T-RFLP (terminal restriction fragment length polymorphism), and ARDRA (amplified ribosomal DNA restriction analysis). Among these techniques, DGGE is perhaps the most commonly used. The result of a DGGE analysis is a pattern of bands; the number of bands should correspond to the number of predominant members of the community (Fig. 4). The technique has frequently been used in different soil bacterial community studies after environmental perturbation events and for the analysis of bacterial succession (e.g. MacNaughton *et al*., 1999; Sekiguchi *et al*., 2002; Westergaard *et al*., 2001).

The basics of the technique when used as an indicator of the total bacterial community in microbial ecology was described by Muyzer *et al*. (1993). First, bacterial DNA is extracted from soil. There are numerous protocols describing

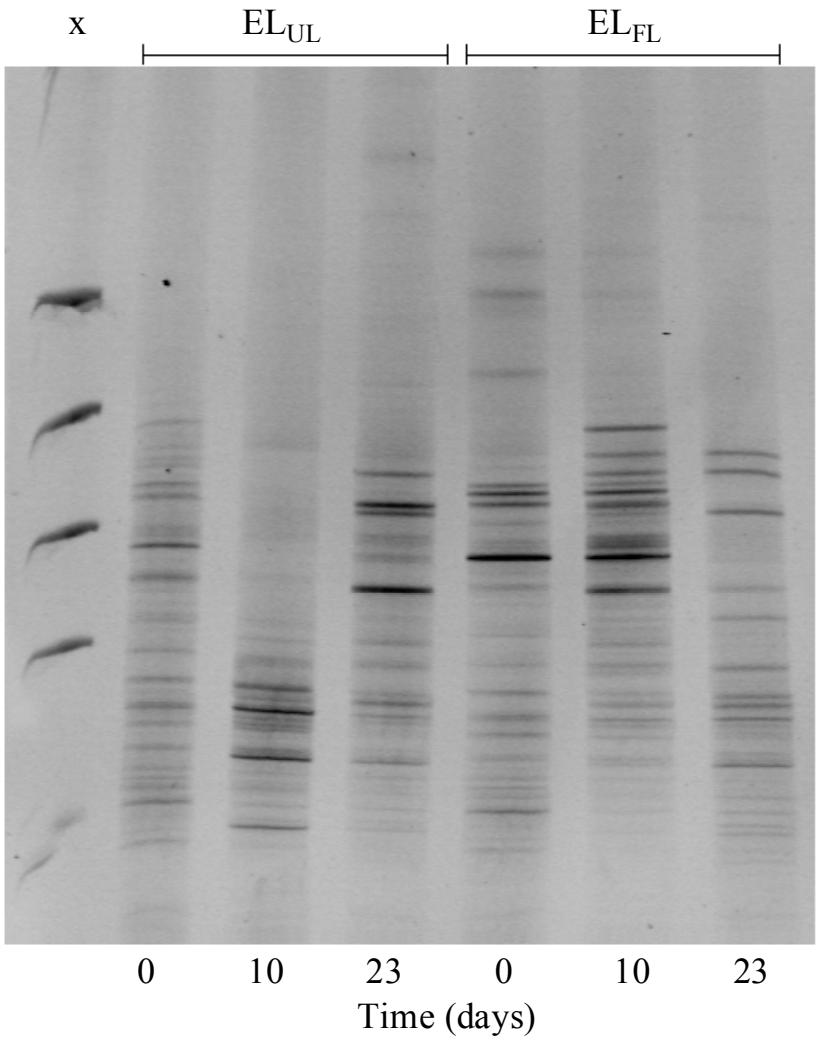

Fig. 4 Example of DGGE band patterns (from Paper V) for experimentally limed (EL) soil inoculated with high- (FL) or low- (UL) pH soil measured between 0 and 23 days from the start of the experiment. X is a molecular size marker.

this and commercial kits are now also available. For the PCR step, the variable V3 region of 16S rDNA was used, which is PCR amplified with primers to conserved regions of the 16S rRNA genes. DNA fragments of the same length, but with different sequences can then be separated. The DGGE separation is based on the decreased electrophoretic mobility of a partially melted double-stranded DNA molecule in a polyacrylamide gel containing a linear gradient of DNA denaturants. Attaching a GC clamp (30 to 50 nucleotides) to one side of the DNA fragment enhances the resolution of the analysis. After electrophoresis the gel is stained and photographed. In this study DGGE on both total bacteria in soil, and soil bacteria cultured on agar plates (cfu-DGGE) was used.

In spite of being a common method for studying the complex dynamics of soil bacterial communities, a number of problems are associated with the technique. Different methods of extracting bacterial DNA tend to give different DNA recoveries of the same community (Martin-Laurent *et al*., 2001), and the PCR reaction itself is a significant source of errors and biases. Only the most predominant species will probably be detected on the DGGE gel (Muyzer and

Smalla, 1998), or perhaps the ones most easily extracted and amplified. Furthermore, one species can give rise to more than one band on the gel, or, one band on the gel can represent more than one species. Thus, the interpretation of the band pattern and band intensity can be rather difficult. However, despite the above mentioned problems, the technique is still promising for community analysis.

3.4 Statistical analysis

The direct soil-PLFA composition and the cfu-PLFA composition were analysed using principal component analysis (PCA). Concentrations (expressed as mol% of the total amount of PLFAs) of the individual PLFAs were subjected to PCA after scaling each variable to unit variance. Similarly, for DGGE the band intensities (expressed as a percent of the total lane intensity) were subjected to principal coordinate (PCO) analysis (Paper V). PCO analysis was used instead of PCA analysis, since PCO is more suited for analysis when there are more variables (bands) than cases (samples), as in this case.

4 Factors affecting the bacterial community

4.1 Temperature

4.1.1 Modelling temperature relationships

A number of functions can be used to model the relationship between temperature and activity. Q_{10}, which is an exponential relationship, is commonly used. Another similar exponential function is the Arrhenius equation ($A = ae^{-E/(R(T+273.15))}$), where A is the rate of the activity at temperature T, R is the universal gas constant and E is the activation energy. If the activation energy is constant a plot of log activity against the inverse of the absolute temperature will result in a linear relationship. An overestimation of activity at low temperatures was found when using the Arrhenius equation and a straightforward Q_{10} relationship (Paper I). This is due to Q_{10} not being constant but increasing at lower temperatures (Kirschbaum, 1995; 2000).

The square root model ($A^{1/2} = b(T-T_{min})$) is a temperature function resulting in Q_{10} increasing at lower temperatures, which was proposed by Ratkowsky *et al.* (1982; 1983) for bacterial growth, where b is a slope parameter and T_{min} is the apparent minimum temperature for growth. Originally, this equation was used by Belehrádek (1926). Below the optimum temperature for growth all three activity measurements (respiration rate, thymidine incorporation rate and acetate-in-ergosterol incorporation rate) followed the square root model, i.e. a linear relation between the square root of the activity vs. temperature (Paper I). The Ratkowsky model has previously been found to adequately describe the bacterial activity in soil (Díaz-Raviña *et al.*, 1994) and water (Li and Dickie, 1987), and fungi activity in soil (Bååth, 2001). Moreover, it has been shown that the temperature dependency of soil respiration also follows this model (Lomander *et al.*, 1998; Persson *et al.*, 1999).

The temperature dependency of soil microorganisms has traditionally been studied by measuring the respiration rate (total activity). Lundegårdh (1927) already noted a strong correlation between soil respiration rate and temperature. Laboratory experiments have since then been performed in all types of soil habitats (see e.g. overview by Kirschbaum, 2000). Even for soils in cold climates, the instantaneous respiration rate often increases with temperature up to around 40°C or more (e.g. Anderson and Domsch, 1986; Chen *et al.*, 2000; Winkler *et al.*, 1996). Since this effect is observed after short incubation times, it can not depend on any substantial growth of thermophilic communities. Soil bacterial and fungal growth rates in cold climates usually have optimum temperatures below 30°C, with activity values decreasing at higher temperatures (Bååth, 2001). This was also found to be the case in the present studies (Papers I and II). In spite of this the respiration continued to increase above 30°C (Paper I). The reason for this might be an uncoupling between respiration rate and bacterial and fungal activities, which might be due to enzymes involved in respiration being less negatively affected at high temperatures than other enzymes.

4.1.2 Comparison of temperature relationships of bacteria and fungi

Microorganisms are the main group producing CO_2 during decomposition of organic material in soil. In a global perspective Kirschbaum (2000) suggested that warming would reduce organic carbon in soil by stimulating decomposition rates more than net primary production (NPP). A difference in temperature dependency of bacterial and fungal activity was observed (Paper I), where bacteria were less inhibited by higher temperatures and fungi less inhibited by low temperatures. This is in accordance with the finding that during winter and spring with snow coverage fungi dominated in high-altitude soils, whereas bacteria dominated during the snow-free conditions of summer (Ley and Schmidt, 2002). It is thus possible that changes in temperature, e.g. due to climate changes, may not only alter the balance between decomposition and NPP, but also the balance between these two groups of microorganisms. However, since the present study was on instantaneous activity, an altered temperature regime for a longer period of time may result in adaptation of the microbial community to the new temperature conditions (Paper II).

4.1.3 Temperature effects on PLFAs

Microorganisms exhibit phenotypic plasticity in response to temperature changes. One of the most well-known plasticities is in the lipid composition of the cell membranes. Sinensky (1974) argued that the temperature-dependent changes in lipid composition were a means of maintaining constant lipid fluidity, thereby optimising membrane function. For example, it is often found that bacteria that grow at low temperatures contain more unsaturated fatty acids than those, which grow at high temperatures (Russel and Fukunaga, 1990; Petersen and Klug, 1994). In this study (Paper II) the relative concentrations of the saturated PLFAs, i.e. 14:0, 15:0, 16:0, 17:0, 18:0 and 20:0, were positively affected by increasing the soil incubation temperature, while the relative concentrations of the unsaturated PLFAs, i.e. 16:1ω5, 16:1ω7c, 16:1ω9 and 18:1ω7c, were positively affected by low temperature. The concentration of the PLFAs 16:1ω7c and 18:1ω7c also decreased in a study by Petersen and Klug (1994) when the temperature of the soil was changed from 4.5 to 25°C. At higher temperatures longer fatty acids are formed (Russel and Fukunaga, 1990). This was also seen in Paper II, where the relative concentration of the PLFA *cy*19:0 increased more than the shorter PLFA *cy*17:0. The PLFA *cy*19:0 also increased after incubation at 25°C (Petersen and Klug, 1994).

4.2 Changing pH by liming

4.2.1 Effects on bacterial activity

Raising the pH in soil increases the amount of available carbon, since acidic soils contain potentially decomposable organic matter that becomes susceptible to microbial attack when the pH is raised (Curtin *et al*., 1998; Persson *et al*., 1991; Shah *et al*., 1990). Such a nutrient effect after liming was seen (Paper III) where the activity of the bacterial community increased several fold in the limed

samples, while only small changes in bacterial activity were found in the unlimed samples.

Fumigation of a soil has also been found to increase the amount of available carbon, since the dead bacteria after fumigation serve as a nutrient source for the living ones, thus increasing activity (Griffiths *et al*., 2000). In the studies described in Papers IV and V, where the soil was fumigated, peak activities were seen for the limed samples, but despite the extra carbon released as a result of fumigation, little increase in activity was seen in the unlimed samples. A possible explanation of this could be that the low pH in the unlimed soils inhibited the bacterial activity there.

The number of cfus revealed a similar trend to the bacterial activity (Paper V). This has been reported earlier in a study by Bååth (1994), where a correlation was seen between the TdR incorporation rate and the proportion of culturable bacteria. Söderberg and Bååth (1998) also found a correlation between the cell-specific TdR incorporation rate and the culturable fraction of the bacterial community. Thus, the number of cfus appeared to reflect the activity of the bacterial community.

4.2.2 Effects on bacterial community pH tolerance

Liming the soil from pH 4.9 to 7.5 (Papers II-IV) or from pH 4.0 to 7.6 (Paper V) changed the pH selection pressure of the bacterial community. After liming gradual adaptation of the bacterial community to the higher pH took place, and was still evident even about 100 days after liming in the first experiment (Paper III), while later experiments showed faster adaptation. However, as expected no differences over time in the pH tolerance of the community was seen in the high-pH (6.1) and low-pH (4.9) soils kept at constant pH showing the reliability of the TdR incorporation method. The large pH increase employed in the present studies (Papers II-V) was necessary in order to induce large changes in the bacterial community pH tolerance, which could be followed over time.

4.2.3 Effects on PLFA pattern

Earlier field studies have shown that increasing pH affects the structure of the soil microbial community (Frostegård *et al*., 1993; Bååth *et al*., 1995). Several PLFAs have been found to correlate to soil pH (Bååth *et al*., 1995; Bååth and Anderson, 2003). The changes in the PLFA pattern after liming in our laboratory experiments were similar to those observed in the earlier field studies on the effects after pH-increasing treatment (Papers III and V). Thus, the concentration (mol%) of the PLFAs *i*14:0, 16:1ω7c, 16:1ω9 and 10Me18:0 increased, both in the present laboratory study and in field studies (Bååth *et al*., 1992; Bååth *et al*., 1995; Frostegård *et al*., 1993), while decreasing relative amounts of the PLFAs 10Me16:0 and *cy*19:0 were found both in the laboratory and in the field.

The probable explanation of these changes in PLFA pattern (Paper III) after liming is increased growth of already high-alkaline-tolerant species at the expense of others together with phenotypical changes in the bacterial species present in the soil. Furthermore, the nutrient effect after liming probably also affected the development of different PLFAs (see below, Section 5.2).

One exception to the similarities between field and laboratory studies was the lack of effect on the PLFA 16:1ω5 in our laboratory studies (Paper III). In earlier studies this PLFA has been found to increase after liming and ash treatment (Bååth *et al*., 1992; Frostegård *et al*., 1993; Schutter and Fuhrmann, 2001), and increasing concentrations have been found at higher pH in a pH gradient (Bååth and Anderson, 2003). Thus, this PLFA usually has a strong relation to pH in the field. A possible explanation of our contradictory result is the absence of plant roots and root-associated microorganisms in our laboratory study. The PLFA 16:1ω5 has been used as an indicator of arbuscular mycorrhiza (AM) in soil (Olsson, 1999; Olsson *et al*., 1995), since these fungi are exceptional in that they contain high amounts of this PLFA, normally associated with gram-negative bacteria. Thus, the lack of increase in 16:1ω5 indicates that the increase of this PLFA in limed forests might depend on increased amounts of AM.

A similar discrepancy between laboratory and field studies was seen for the fungal indicator 18:2ω6,9, which decreased at 30°C (Paper III). Since a large amount of 18:2ω6,9 is probably produced by ectomycorrhizal fungi (Wallander *et al*., 2001), this discrepancy might also depend on the absence of plants. In the laboratory, the ectomycorrhiza will die without plants (carbon source). However, this PLFA did not decrease in soil at 20 or 5°C, which might be due to slower breakdown of this PLFA at lower temperatures.

Analysis of the PLFA pattern was performed on soil after fumigation (to reduce the bacterial community), liming and inoculation with fresh soil with high- (6.2) or low- pH (4.0) soil (Paper V). The soil PLFA analysis showed a shift in community structure for the limed soils, with the most prominent shift for the limed soil inoculated with high-pH soil. The PLFA composition of this soil became more and more similar to the composition of the high-pH inoculum soil. In this sample, the relative concentrations of PLFAs indicative of gram-negative bacteria increased, i.e. 16:1ω7c, 16:1ω5, *cy*17:0, 18:1ω7 and 19:1a, while the concentration of PLFAs indicating gram-positive bacteria, like *i*17:0, *a*17:0 and 10Me17:0, decreased. One reason for this might be that gram-negative bacteria are typical r-strategists that are able to grow fast when the nutrient supply is good, for example, after fumigation and liming. The gram-positive bacteria were perhaps outcompeted by the faster-growing gram-negative bacteria during the beginning of recolonisation.

The development of these PLFAs indicative of gram-positive and gram-negative bacteria (Paper V) was not the same as in the former liming study (Paper III). This may be due to the opportunity for fast-growing species, like the gram-negative bacteria, to fill empty niches after the reduction of bacteria following fumigation. For example, the PLFA 16:1ω5 might be indicative of a gram-negative bacterium in this study that proliferated under these conditions, while in the earlier study (Paper III) it could have been indicative of AM. Furthermore, the PLFA 16:1ω5 was also found on bacterial agar plates (fungal-inhibiting substance cycloheximid added) (Paper V), indicating that here it was of bacterial origin. Apparently further studies are needed on the origin of this PLFA.

The cfu-PLFA analysis also showed large difference between the limed and unlimed soils (Paper V). The differences between the unlimed and limed soils and

between the different inoculums were similar for the analysis of soil-PLFA and cfu-PLFA. In both cases the limed soils had high values along the PC1 axis in the PCA plot, while the unlimed soils had lower values. Moreover, the individual PLFAs affected, were to some extent, the same for soil-PLFA and cfu-PLFA. Thus, the small fraction of bacteria growing on plates reacted in a similar way to the whole soil bacterial community. One reason for this might be that the culturable bacteria are probably the largest, most active prokaryotes in a given sample (Ellis *et al*., 2003). Thus, the culturable bacteria may represent an ecologically relevant portion of the bacterial community.

4.2.4 Differences between changes in community pH tolerance and PLFA pattern

The changes in community pH tolerance after liming were faster than the change in PLFA pattern (Paper III) (Fig. 5a and b). This was especially evident for the soil incubated at 5°C where the PLFA pattern did not change at all during 82 days of incubation, while a clear increase in community tolerance to high pH was seen using the TdR incorporation technique. The reason for this may be that the PLFA technique measures changes in the fatty acid composition, which can take longer to detect, since the old fatty acids have to be broken down and a new composition appear before any changes will be observed. The TdR incorporation technique, on the other hand, measures instant activity so inactive and dead microorganisms will not be included.

4.2.5 Effects on DGGE pattern

The main difference in soil-DGGE pattern (Paper V) was between the limed and unlimed soils, where the limed soils had the highest values along the first principal coordinate. A change in DGGE pattern with time could be seen for both the limed and the unlimed fumigated soils. A significant difference was also seen between the limed soils, where the soil inoculated with high-pH soil had the highest PCO scores, while no difference was seen between the unlimed soils.

There was also a similar change in cfu-DGGE pattern, with significant differences between the limed soils inoculated with different inoculum, while no difference was found between the unlimed soils irrespective of inoculum origin. Half of the bands from the cfu-DGGE analysis agreed in position with the bands from the soil-DGGE, but these bands were differently affected by the different treatments in the soil and cfu-DGGE. Thus, despite the same position on the gel, they probably do not indicate the same bacterial species.

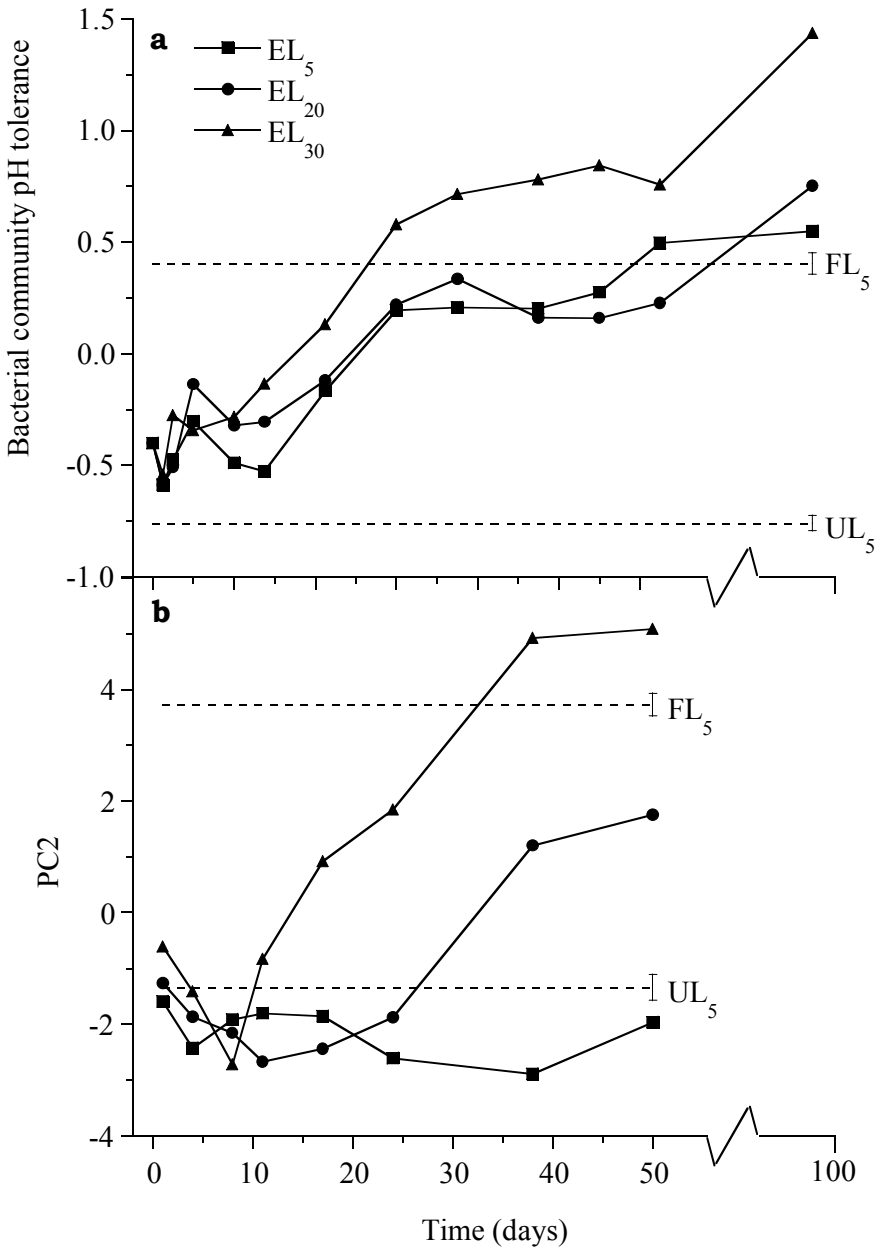

Fig. 5 a) The effect of experimental liming (from pH 4.9 to pH 7.5) on the bacterial community tolerance (from Paper III). Higher values indicate a bacterial community adapted to higher pH. b) The effect of experimental liming on the scores for the second principal component of the PLFA analysis plotted against time. The dotted lines show the mean values of the low-pH control (UL, pH 4.9) and high-pH control (FL, pH 6.1) soils. Samples were taken at the same time as the EL samples. However, since no changes over time were seen, mean values are reported. The error bars indicate the standard error. Abbreviations: UL = unlimed soil, FL = field limed soil and EL = experimentally limed soil. Indices indicate soil incubation temperature (5, 20 or 30°C).

5 Factors affecting the rate and extent of changes of the bacterial community

5.1 Temperature

Increasing the temperature increases the turnover of the bacterial community until the optimum temperature is reached. This implies that the rate of change after a perturbation would be faster at a higher temperature, since this will speed up the process of competition and take-over of a new community. This was also seen in the present work (Paper III) where the rate of community adaptation to higher pH after liming was significantly higher at 30°C than at 5°C. However, the rate of community adaptation was not proportional to the effect of temperature on activity and turnover. Thus, the rate of adaptation was, for example, not twice as fast at 30°C than at 20°C, which would be the case if the rate of community adaptation followed the square root model (Ratkowsky *et al*., 1982, 1983).

Another unexpected finding was that it appeared that temperature had a direct impact on the ability of the bacterial community to grow at a certain pH (Paper III). This affected the results of the bacterial community tolerance measurements, since both low-pH (UL) and high-pH control (FL) soils incubated at 5°C had a higher community pH tolerance than the corresponding soils incubated at 30°C. We do not know the reason for this, but it shows the importance of comparing samples incubated at the same temperatures.

In Paper II we concluded that the change in the PLFA pattern of the bacterial community after liming was fastest at the highest temperature. Furthermore, a decrease in the soil incubation temperature had a much smaller effect on the PLFA pattern than an increase, even when the temperature change was of the same magnitude. Thus, the turnover rate affected the rate of change of the PLFA pattern.

The study described in Paper I showed that bacteria and fungi had optimum temperatures for activity around 25-30°C, while at higher temperatures lower activities were found. The choice of the highest incubation temperature in a later study (Paper II) (30°C) was based on this, since 30°C is at or above the optimum temperature. One would expect an increased selection pressure for species tolerant to this high temperature, and thereby a change in community caused by temperature. Furthermore, since it is near the optimum temperature, changes would occur rapidly.

The bacterial community became more adapted to a high temperature following high-temperature treatment (30°C) (Paper II). This was also found in an earlier study (Ranneklev and Bååth, 2001) using the same methodology when temperatures were changed to those well outside the maximum temperature for growth; the temperature range in that study was thus quite extreme. The change in bacterial community after this extreme treatment can be compared with that after the fumigation treatment (Papers IV and V). In both cases most of the original community died and rapid recolonisation of species tolerant of the new conditions took place.

The study (Paper II) also showed that a temperature at or slightly above the optimum also affects the bacterial community temperature response. Decreasing the temperature had no effect on the temperature response of the soil bacterial community using the TdR incorporation technique. Thus, despite the change being of the same magnitude, the change in community tolerance was faster when increasing the temperature than when decreasing it. This is probably due to the much more rapid turnover rate at 30 than at 5°C.

Moreover, the temperature response measured with the TdR incorporation technique was slower than the change in PLFA pattern. One explanation of this could be that changes in the PLFA pattern caused by temperature were due to phenotypic plasticity, since such acclimation is assumed to occur faster than changes in species composition, which will affect temperature response measured with the TdR incorporation technique. In studies of the response of bacterial communities to pH increase (Paper III) and addition of heavy metals (Díaz-Raviña and Bååth, 1996; Frostegård *et al*., 1996), the opposite was found, i.e. changes in community tolerance were detectable before changes in the PLFA pattern. This is what we would expect after changes in species composition. This will lead to less rapid change in PLFA pattern, since the PLFAs of dead species have to be broken down, and new bacterial species appear before any change in PLFA pattern of the community can be seen.

5.2 Nutrients

Excess or limitation of nutrients will ultimately affect the bacterial community composition, since different species have different resource requirements. Thus, what is a limiting resource for one part of the bacterial community may not be for another. Different parts of the community will therefore be stimulated or depressed as the nutrients fluctuate.

It is well known that the availability of soil organic matter increases after liming and other pH-increasing treatments (Curtin *et al*., 1998; Persson *et al*., 1991; Shah *et al*., 1990). After liming the release of nutrients will induce a selective pressure favouring fast-growing, copiotrophic organisms. Thus, changes in the bacterial community may have two different causes after liming; i.e. the increased availability of soil organic matter or a change in pH. However, by using the community tolerance measurements we can ignore this confounding factor to a large extent, since this method gives a relative measure of the tolerance to a specific factor such as pH. Changes in the PLFA pattern will be more difficult to interpret, since one can not be certain which factor is affecting the outcome; the change in pH or increased nutrient availability.

Introducing nutrients (carbon) is a way of increasing the growth rate of the bacterial community, since soil microorganism growth usually is carbon limited. But an increased growth rate appeared not to be a guarantee for an increase in the rate of change of the bacterial community. The increased availability of carbon after liming and temperature increase, as factors increasing the activity were not equivalent. Thus, in one study (Paper II) the rate of change in PLFA pattern was highest for the samples incubated at 30°C, irrespective of whether they were limed or not, while the rate of change of limed samples incubated at lower

temperatures was slower, irrespective of pH conditions. Similarly, no difference in community temperature adaptation could be seen between limed and unlimed samples incubated at 30°C (Paper II). This may be because the nutrients present may represent a resource that is available to only parts of the bacterial community, while temperature is a modulator that affects the whole bacterial community. Thus, these factors affect the bacterial community in different ways, although the growth rate of the community increases in both cases.

5.3 Properties of the bacterial community

In the study described in Paper IV 99% of the bacterial activity was eliminated using chloroform fumigation. The removal of most of the soil microbial community affected the rate of the bacterial community's adaptation to pH after liming, which was faster when the indigenous community had been removed (Papers IV and V) than with the intact community (Paper III). Thus, the presence of a low-pH-adapted community in the unfumigated limed soil (Paper III) decreased the colonisation rate of a new high-pH-adapted community, even if it was not well adapted to the new environmental conditions.

The studies presented in Papers IV and V also showed the importance of inoculum origin in community pH adaptation after liming of soil from pH 4.9 to pH 7.5 (Paper IV) or from pH 4.0 to pH 7.6 (Paper V), where fumigation was used to reduce the original community. There was a difference both in community pH adaptation and activity depending on the origin of the inoculum (low- (4.9) or high-pH (6.1) soil) even after 78 days (Paper IV). The fumigated and limed soil inoculated with high-pH soil exhibited the highest bacterial community pH tolerance and initially the highest activity, while the fumigated and limed soil with a low-pH inoculum showed lower community pH tolerance and activity. The remaining difference in community pH tolerance and activity between the two limed soils even after 78 days could depend on all niches being colonised after initial recolonisation of the fumigated soil. The bacterial communities of the limed soils inoculated with the low-pH inoculum soils could thus withstand further changes due to the selective force of high-pH, although they were not optimally adapted, because of the selective advantage of the organisms being in place from the beginning. Thus, the initial community composition can be important when colonising fresh materials and/or recolonising after events such as fumigation, freezing or thawing.

Similar results with a long-lasting tolerance effect of an inoculum have also been found in a study on bacterial communities pre-exposed to different metals and re-inoculated in an unpolluted and sterilised soil (Díaz-Raviña and Bååth, 2001). In this study an effect of increased community tolerance to metals in the inoculum was observed, even after 12 months, in soils inoculated with metal-tolerant communities, emphasising the importance of the organisms being in place from the beginning. A parallel can also be drawn to the conceptual adaptation model of Balser *et al*. (2001) (Fig. 3). In this model the initial response is a fast change in process rate after a perturbation. After a period of instability, new equilibrium communities are formed on different levels of the process rate depending on the characteristics of the ambient environment (limed or unlimed

soil) and the composition of the antecedent community (low- or high-pH inoculum soil). In our case there were rapid changes in both the function (community pH tolerance and activity) and structure (PLFA and DGGE pattern) due to liming. After the initial response the communities of the limed and unlimed soils found new equlibria at different levels of function (community pH tolerance) (Fig. 6a). There were also differences in structure (PLFA and DGGE pattern) (Fig. 6b) depending on soil and inoculum origin.

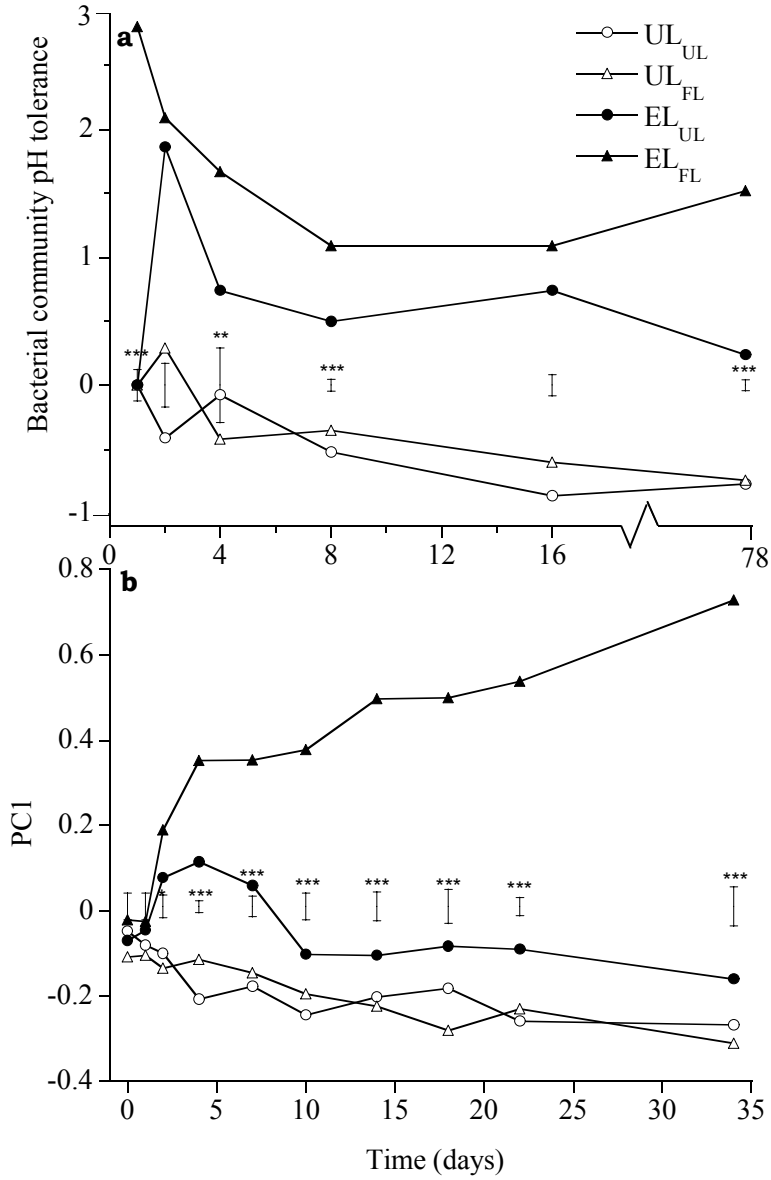

Fig. 6 The effect of inoculum origin on changes in the bacterial community after elimination of most of the community and liming. a) Development of bacterial community pH tolerance over time (from Paper IV). Higher values indicate a bacterial community adapted to higher pH. Bars indicate the standard error (n = 2) obtained from ANOVA at each separate time with significance levels for interaction between soil type and inoculum origin, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. The subscripts indicate the inoculum. b) Principal component analysis of the change in PLFA patterns of the soil bacterial communities over time in the soil samples (from Paper V). The changes in the soils of the first component (PC1) plotted against time. Bars indicate the standard error (n = 3) obtained from ANOVA at each separate time with significance levels for interaction between soil type and inoculum origin indicated. (Notation as described in Fig. 5.)

6 Structure and function in soil bacterial communities

A number of different studies have found connections between structure and function of soil microbial communities. For example, Kandeler *et al*. (2000) found that heavy metal pollution influenced both the structure and function of the microbial community in the bulk soil and in particle-size fractions. The structure was measured with PLFA and DGGE analysis and the function studied was enzyme activity. A long-term fertilizer experiment, on the other hand, revealed that the change in community structure (DGGE, PLFA) was not correlated to the function (enzyme activity) of the bacterial community (Marschner *et al*., 2003). It was claimed that this could be due to functional redundancy of soil microorganisms (i.e. one function can be carried out by a range of different microorganisms) and therefore changes in microbial community structure do not necessarily lead to changes in enzyme activity. Calderón *et al*. (2001) examined the short-term dynamics of nitrogen, microbial activity and PLFAs after tillage. They found that both the functions studied (respiration and denitrification) and the structure (PLFA profile) of the microbial community were affected. Thus, there appeared to be a relationship between microbial composition and ecosystem function.

Apart from the structure, the microbial diversity of the community might also be important in ecosystem functioning. Tilman *et al*. (1996) suggested that enhanced species diversity is beneficial to ecosystem function. Later, other authors have in contrast argued that the properties of an ecosystem depend more upon the functional abilities of particular species than on the number of species (Hooper and Vitousek, 1997; Tilman *et al*., 1997; Wardle *et al*., 1997). Finlay *et al*. (1997) claimed that microbial diversity is part of ecosystem function since reciprocal interactions between microbial activity and the physical-chemical environment create a continuous turnover of microbial niches that are always filled.

The studies of soil bacterial communities during recolonisation of a fumigated and limed or unlimed soil (Papers IV and V) revealed a connection between the developing structure and the function of the new community. The functions studied, i.e. the bacterial community tolerance to pH and the bacterial activity (TdR incorporation) coincided with the structural changes in the bacterial community measured with PLFA and DGGE analysis. For example, the limed soil inoculated with high-pH soil had the highest activity and community pH tolerance. The bacterial community of this sample also changed the most structurally (indicated by both the PLFA and DGGE techniques). Furthermore, the bacterial community in this sample became more and more similar to the high-pH inoculum source, as measured with PLFA analysis, showing that the community resembled a high-pH-adapted community. One conclusion that can be drawn is that the differences in pH tolerance between the unlimed and limed communities with different inoculum origin depend on different species composition and not just on phenotypic changes in the community.

Both the PLFA and the DGGE analysis (Paper V) revealed differences between the fumigated and limed soils depending on inoculum origin (high- or

low-pH soil), whereas only minor differences were seen depending on inoculum origin in the unlimed soils. The lack of difference between the unlimed soils might be due to bacteria surviving the fumigation in the unlimed soil outcompeting the bacteria of the high-pH inoculum, resulting in no difference between the two unlimed soils, irrespective of inoculum origin.

The intrinsic properties of the bacterial community in the limed soil with the high pH inoculum made it better able to take advantage of the carbon released after fumigation and liming compared with the limed soil inoculated with a low-pH inoculum. The bacterial community in the soil with the high-pH inoculum had the most suitable intrinsic properties to become further adapted to the higher pH environment after liming. Other studies have shown a similar pattern with bacterial communities functioning best in environments they have earlier adapted to (Cookson *et al*., 1998; Hunt *et al*., 1988).

7 Conclusions

- The soil bacterial community had an optimum temperature for growth between 25 and 30°C. The apparent, calculated minimum temperature was around -10°C and the maximum temperature approximately 45°C.
- The soil bacterial community was better adapted to higher temperatures (above 30°C) than fungi, and vice versa at lower temperatures (below 10°C).
- The temperature dependency for bacterial and fungal growth was well modelled by the square root model below the optimum temperature for growth.
- At temperatures above 30°C there was an uncoupling between respiration rate, and bacterial and fungal activities.
- Adaptation of the soil bacterial community to temperature occurred when increasing the temperature from 5 to 30°C, while decreasing it to 5°C had a much smaller effect. Thus, the turnover of bacteria affected the rate of change.
- The changes in PLFA pattern were faster than the changes in community temperature tolerance when altering the temperature regime. This indicated that the altered PLFA pattern was due to phenotypic changes in the cell membrane.
- The adaptation of the soil bacterial community to higher pH after liming was temperature dependent, with the fastest rate of change at the highest temperature (30°C).
- The rate of change of the soil bacterial community tolerance to pH was not correlated with the effect of temperature on activity (the square root model), indicating that the rate of adaptation and the turnover of the bacterial community were not directly correlated.
- The changes in community pH tolerance were faster than the changes in PLFA pattern, indicating that changes in PLFA pattern were due to species replacement.
- The rate of bacterial recolonisation and the community adaptation after liming in a soil were fastest when inoculating the soil with a community with suitable intrinsic properties for the new environment.
- There was a correlation between the structure and function of the soil bacterial community during recolonisation and adaptation after liming. Furthermore, the culturable portion of the soil bacterial community showed similar development to the whole bacterial community.

8 Populärvetenskaplig sammanfattning

Jorden i vår natur är en mycket komplex miljö som innehåller en mängd olika organismer. Bland dessa är bakterier och svampar av störst betydelse eftersom de står för en stor del av nedbrytningen av olika ämnen. Jordbakterierna utför t.ex. många av ämnesomvandlingarna av kväve, svavel och fosfor i marken. Nedbrytningen frigör näringsämnen som sedan kan utnyttjas av växterna.

Bakterierna i marken är mycket talrika. Man brukar uppskatta dem till ca 10^8 stycken per gram jord. Hur många arter det finns är ännu okänt, men man tror att det kanske kan vara så många som 10^4 till 10^6 i ett gram jord. Trots att det finns mängder med kol (föda) i jorden lever bakterierna den mesta tiden i ett tillstånd av svält. Detta beror på att mycket av kolet som finns är bundet svårnedbrytbara föreningar.

Kunskaper om hur bakteriesamhället i marken tillväxer och förändras under olika förutsättningar kan t.ex. vara av betydelse för förståelsen för vad som händer om medeltemperaturen på jorden förändras p.g.a. växthuseffekten. En ökad temperatur skulle kunna medföra att bakterierna tillväxte snabbare och genom sin andning återförde en större mängd kol till atmosfären, vilket i sin tur skulle kunna leda till ytterligare uppvärmning. Kalkning av sura marker är idag vanligt, men vad händer egentligen med bakteriesamhället och dess funktion efteråt? Kunskap om bakteriesamhällen i jorden kan också vara viktig vid marksanering och för förståelsen för hur tillgången av växtnäringsämnen i jorden regleras.

Det finns en mängd omvärldsfaktorer som kan påverka bakteriesamhället i marken. Exempel på sådana faktorer är temperatur, pH, vattenhalt i jorden, näringstillgång m.m. Ibland inträffar något som rubbar en eller flera av dessa faktorer, t.ex. en pH-höjning efter kalkning. Detta leder till olika typer av förändringar i bakteriesamhället som gör det bättre anpassat till de nya förutsättningarna. Det kan vara att vissa arter bättre rustade för den nya miljön gynnas, medan andra konkurreras ut, små förändringar av egenskaper hos de bakterier som finns i samhället, eller genomslag av en muterad bakterie med nya egenskaper, som ger den bättre förutsättningar att klara sig i den nya miljön än de andra. Kunskapen om hur och med vilken hastighet sådana förändringar sker är begränsad. Av intresse är också om förändringar i bakteriesamhället efter fluktuationer i omvärlden är kopplade till den ursprungliga sammansättningen av samhället, eller inte. Detta och en del relaterade frågor har jag undersökt i min avhandling.

De faktorer vars inverkan jag huvudsakligen studerade var temperatur och pH, samt betydelsen av den ursprungliga sammansättningen av bakteriesamhället för dess förmåga att anpassa sig till nya omvärldsfaktorer. Jag undersökte vad som händer med ett bakteriesamhälle om en omvärldsfaktor förändras, dels hur snabbt bakteriesamhället anpassar sig till de nya förhållandena och dels hur samhällets struktur (artsammansättning) förändras.

Temperaturen är en mycket viktig faktor eftersom den till stor del bestämmer bakteriernas tillväxthastighet. Ju högre temperatur, desto snabbare tillväxer bakterierna, tills deras optimala temperatur är nådd. Jag undersökte hur snabbt

bakteriesamhället i en humusjord anpassar sig till olika temperaturer, samt hur olika temperaturer påverkar anpassningshastigheten till ett högre pH efter att man kalkat den sura jorden. I den humusjord jag studerade växte bakteriesamhället snabbast mellan 25 och 30°C medan ca 45°C var den högsta temperatur det klarade av att växa vid. Det fanns en skillnad i temperaturanpassning mellan bakterier och svampar i marken, där bakterierna var mer anpassade till högre temperaturer (över 30°C) än svampar och vice versa vid låga temperaturer (under 10°C). När temperaturen i en humusjord höjdes från 5 till 30°C skedde en anpassning till den nya temperaturen, medan en senare sänkning från 30 till 5°C endast hade liten inverkan på bakteriesamhällets temperaturanpassning. Höjningen av temperaturen medförde både en ökad anpassning till den nya temperaturen och en förändring av strukturen i bakteriesamhället. De strukturella förändringarna var snabbare än temperaturanpassningen, vilket kan betyda att förändringarna skedde hos de bakterier som redan fanns där från början.

I ett annat försök kalkades en humusjord med ett lågt pH på 4,9 till ett högt pH på 7,5 och fick sedan stå i 5, 20 eller 30°C. Anpassningen till det högre pH-värdet var snabbast vid den högsta temperaturen (30°C) och långsammast vid den lägsta temperaturen (5°C). Anpassningen till det nya pH-värdet var snabbare än de strukturella förändringarna i bakteriesamhället, vilket kan bero på att det tar längre tid för artförändringar att synas eftersom de gamla bakterierna måste dö och brytas ner och nya växa till innan någon förändring syns. Jag upptäckte också att hastigheten med vilken bakteriesamhället anpassade sig till det högre pH-värdet inte var sammankopplad med temperaturens inverkan på bakteriernas aktivitet. Med andra ord; trots att aktiviteten hos bakterierna var sju gånger högre vid 20°C än vid 5°C var inte skillnaden i anpassningshastighet lika stor, d.v.s. anpassningshastigheten och omsättningshastigheten var inte direkt beroende av varandra.

Jag undersökte även hur ursprungssammansättningen av ett bakteriesamhälle i en humusjord påverkar dess förmåga att anpassa sig efter en höjning av pH-värdet. Först eliminerade jag större delen av bakterierna i jorden genom att behandla dem med kloroform. Därefter kalkades jorden (se ovan) och lite jord med lågt pH (4,9) eller högt pH (6,1) tillsattes som ymp. Sedan skedde en återkolonisering av bakterier i jordarna samtidigt som en anpassning till det högre pH-värdet efter kalkningen ägde rum. Denna anpassning avspeglade egenskaperna hos de bakterier som blivit inympade. Bakteriesamhället i jorden med hög-pH-ymp anpassade sig snabbt till det högre pH-värdet efter kalkningen. En anpassning skedde också i jorden med låg-pH-ymp. Men bakteriesamhället i den sistnämnda jorden var markant mindre anpassat till ett högt pH än bakteriesamhället i jorden med en hög-pH-ymp, även ca 80 dagar efter starten av försöket. Man kan därför säga att egenskaperna hos ursprungssammansättningen av bakteriesamhället bestämde hur bra samhället anpassade sig efter kalkningen. I denna studie kom jag även fram till att bakteriesamhällets anpassning till ett högt pH hängde samman med förändringar av strukturen, d.v.s. artsammansättningen i bakteriesamhället.

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Comparison of Temperature Effects on Soil Respiration and Bacterial and Fungal Growth Rates

JANNA PIETIKÄINEN[1,2], MARIE PETTERSSON[1] AND ERLAND BÅÅTH[1]

Department of Microbial Ecology, Ecology Building, Lund University, SE-223 62 Lund, Sweden[1], Department of Applied Chemistry and Microbiology, P.O. Box 56, University of Helsinki, FIN-00014 Helsinki, Finland[2]

ABSTRACT

We have measured the instantaneous total activity (respiration rate), bacterial activity (thymidine incorporation rate) and fungal activity (acetate-in-ergosterol incorporation rate) in soil at different temperatures (0 to 45°C). Two soils were compared: one an agricultural soil low in organic matter and with high pH, and the other a forest humus soil with high organic matter content and low pH. Fungal and bacterial activities had optimum temperatures around 25-30°C, while at higher temperatures lower values were found. This decrease was more drastic for fungi than for bacteria, resulting in an increase in the ratio of bacterial to fungal activity at higher temperatures. A tendency towards the opposite effect was observed at low temperatures, indicating that fungi were more adapted to low-temperature conditions than bacteria. The temperature dependence of all three activities was well modelled by the square root (Ratkowsky) model below the optimum temperature for fungal and bacterial growth. The respiration rate increased over almost the whole temperature range, showing the highest value at around 45°C. Thus, at temperatures above 30°C there was an uncoupling between the respiration rate and bacterial and fungal activity. At these high temperatures, the respiration rate closely followed the Arrhenius temperature relationship.

INTRODUCTION

Temperature, together with moisture content, is the most important environmental factor affecting microbial growth and activity in soils. To be able to understand fluctuations in microbial activity a reliable model for temperature dependency is therefore required. The importance of the temperature dependence of soil organisms has been further emphasized during recent years due to the global warming issue, since microorganisms are the main group producing CO_2 during decomposition of organic material in soil.

The temperature dependence of soil microorganisms is usually studied by measuring the respiration rate (total activity), and numerous field and laboratory experiments have been performed on all types of soil habitat (see, e.g (15, 16, 20)). Less is known about the temperature dependence of subsets of the soil microorganisms, such as bacteria and fungi. The temperature dependence of

bacterial activity (growth rate) has, however, been determined using the thymidine and leucine incorporation methods to estimate the activity of the soil bacterial community in two soils (7), and these techniques were later used to study the temperature dependence of the bacterial community in heated peat (31) and in soil incubated at different temperatures (29). The fungal activity (growth rate) can be estimated with the acetate-in-ergosterol incorporation technique, originally devised for aquatic habitats (22, 23). This technique was adapted to soil conditions by Bååth (3) and used to determine the temperature dependence of the soil fungal community in one soil.

The instantaneous soil respiration rate often increases with temperature up to around 40°C or more, even in soils from cold climates (e.g. (2, 5, 35, 38)). This is not due to the growth of thermophilic organisms at higher temperatures, as the effect is seen even when short incubations times are used, which would not allow for substantial growth of the thermophilic community (e.g. (2)). In contrast, soil bacterial and fungal activities in cold climates usually have optimum temperatures below 30°C, with activity values decreasing at higher temperatures (3, 29). The temperature dependence of pure culture isolates from temperate soils is usually also found to have an optimum temperature for growth below 30°C for fungi and bacteria (8, 34). One explanation of this discrepancy in optimum temperatures could, of course, be that different soils were studied using respiration techniques and the methods of determining fungal and bacterial activities. However, the uncoupling of respiration rate and microbial growth at higher temperatures can not be excluded, indicating the need for a direct comparison of these three different measures of activity.

It is often stated that fungi as a group are more adapted to low soil moisture conditions than bacteria (13, 26), and would therefore be more important in dry soil. Less is known about the effect of temperature. Persson *et al.* (28) found a difference in temperature dependence of respiration in a forest and an agricultural soil, in that a lower minimum temperature for respiration (T_{min}) was found in the former soil. One proposed explanation of this difference was a shift in the relative importance of fungi and bacteria as decomposers, i.e. that fungi are more important in the forest soil and are more active at low temperatures than bacteria (1, 28). However, no direct comparison has been made of the temperature dependence of soil fungal and bacterial communities.

The aim of the present study was to make such a comparison using two contrasting soil types from a temperate climate: one an agricultural soil with high pH and low organic matter content and the other a forest humus, with low pH and high organic matter content. Firstly, we wanted to compare the activity (growth rate) of fungi and bacteria at different temperatures to determine whether one of the groups of organisms appears to be favoured at certain temperatures. Secondly, we wanted to ascertain whether there was an uncoupling of microbial growth and respiration rate at high temperatures. Thirdly, we wanted to compare different ways of modelling the temperature relationship of total, bacterial and fungal activity.

MATERIALS AND METHODS

Soils. We used two different soils originating from southern Sweden. One was an agricultural soil with a dry weight of 88% of the wet weight, a pH of 7.8 and an organic matter content of 5%. The other was a humus soil (the A_{01}/A_{02} horizon) from a forest with mainly spruce, with a dry weight of 29% of the wet weight, a pH of 4.1 and an organic matter content of 82%. Both soils were collected in the spring of 2002, sieved (2.8 mm mesh size) and stored at 5°C until used in the experiment.

Total activity. The total activity was estimated as the respiration rate. Three grams of agricultural soil and 1 g of humus soil were put into 20 ml glass bottles. The samples were incubated at 0°C (water with ice kept in a 4°C cold room) and 4°C for 120 h, at 10°C for 72 h, at 14°C for 49 h, at 18°C and 25°C for 24 h, at 30°C for 8 h, at 35°C for 7 h, at 40°C for 6 h and at 45°C for 5 h. Three replicates were incubated at each temperature. The rate of CO_2 evolution was measured with a gas chromatograph.

Bacterial activity. Bacterial activity was estimated using the thymidine (TdR) incorporation technique on bacteria extracted from soil (4). The bacteria were extracted by putting 1 g of soil into a glass flask and adding 40 ml of Milli Q water. The samples were shaken on a rotary shaker (200 rpm) for 15 min and then centrifuged at 1000 x *g* for 10 min. The supernatant with the extracted bacteria was then used. 1.5 ml of each sample was put in an Eppendorf tube and the tubes were distributed between the different temperature regimes. After 15 min, 5 µl methyl[^{3}H]thymidine (926 GBq mmol^{-1}, Amersham) was added. Incubation was terminated by adding 75 µl cold 100% TCA. The incubation times were: 48 h at 0°C, 24 h at 4°C, 7 h at 10 and 14°C, 4 h at 18°C, 2 h at 25°C, 1 h at 30 and 35°C and 2 h at 40 and 45°C. Three replicates (bacteria extracted from 3 different soil samples) were used. Removal of excess non-incorporated TdR and subsequent determination of incorporated radioactivity were carried out as described by Bååth *et al.* (4).

Fungal activity. The fungal activity was estimated using the ^{14}C-acetate incorporation into ergosterol technique, modified for use in soil (3). One g of agricultural or 0.25 g of humus soil were put into small test-tubes with 1.5 ml distilled water, 0.05 ml ^{14}C-acetate solution ([1,2,-^{14}C]acetic acid, sodium salt, 2.07 GBq/mmol, Amersham, UK) and 0.45 ml 1 mM non-radioactive acetate. The tubes containing the soil slurry were incubated at 0°C for 72 h, at 4°C for 48 h, at 10°C and 14°C for 24 h, at 18°C and 25°C for 16 h, at 30°C for 8 h, at 35°C for 16 h and at 40°C for 8 h. A temperature of 45°C was not included in the fungal activity measurements, since it was assumed to be too high a temperature for fungal activity. Two replicates were used. One millilitre of 5% formalin was added to stop the incorporation of acetate. Zero time controls were made by adding formalin to samples before the labelled acetate to account for abiotic binding of acetate. Washing, extraction of ergosterol, measurement of ergosterol using HPLC, collecting of the ergosterol and the subsequent determination of

incorporated radioactivity on a liquid scintillator were performed as described by Bååth (3).

Calculations. The respiration rate was calculated as absolute values of CO_2 evolution h^{-1} g^{-1} organic matter. For fungal and bacterial activities the measured entities (DPM acetate incorporation into ergosterol per g of soil for fungi or DPM TdR incorporated into bacteria extracted from soil) have no direct meaning, but only give relative values. The incorporation data for the TdR and acetate-in-ergosterol technique were therefore normalised, setting the mean incorporation rate at 25°C to the value 1 for each soil.

Temperature functions. The data were modelled using two common functions to describe the temperature relationships. A square root relationship below optimum temperature has been proposed by Ratkowsky *et al.* (32, 33) for bacterial growth: $A^{1/2} = b(T-T_{min})$, where A is the "rate of the activity" (growth) at temperature T (°C), T_{min} is the apparent minimum temperature for growth, and b is a slope parameter without any direct biological meaning. A plot of the square root of the activity against temperature will result in a linear relationship. The Arrhenius equation, originally used for enzyme kinetics, is one of many exponential functions used in modelling temperature relationships: $A = ae^{-E/(R(T+273.15))}$, where a is a constant, R is the universal gas constant, and E is the activation energy. A plot of log activity against the inverse of the absolute temperature will result in a linear relationship if the activation energy is constant over the whole temperature interval. This function is very similar, over the temperature range used, to a simple exponential function, where a plot of log activity against temperature will result in a straight line if Q_{10} (the ratio of activity at two temperatures differing by 10°C) is constant. To model an activation energy or Q_{10} value that varies with temperature, further variables have to be introduced (15, 16, 20). The use of these models was, however, outside the scope of the present study.

RESULTS

Total activity. The respiration rate increased with increasing temperature over the whole temperature range in the agricultural soil (Fig. 1a) and up to 40°C in the humus soil (Fig. 1b). Thus, the maximum temperature for respiration (Table 1) was above these temperatures. The respiration rate at 45°C was around 120 times higher than at 0°C in the agricultural soil, while the corresponding value for the humus soil was 70 times.

The data showed good agreement with the square root (Ratkowsky) model up to 25°C, in that a linear relationship was found between temperature and the square root of the respiration rate (r^2 = 0.981 and 0.980, for the agricultural and humus soil, respectively) (Fig. 1c and d). The respiration rate was about 20 times higher at 25°C than at 0°C. Above 25°C, a steeper linear relationship between temperature and the square root of the respiration rate was observed for both soils. The use of the square root model resulted in calculated apparent minimum temperatures (T_{min}) of around -6°C for both soils (Table 1).

The respiration data were also plotted using the Arrhenius equation (Fig. 1e and f). In both cases very good linear relationships were found over the whole temperature range (r^2 = 0.992 and 0.993 for the agricultural and humus soil, respectively, not including the lowest temperatures in the regression). However, in both cases the respiration rates at low temperatures were overestimated by this function.

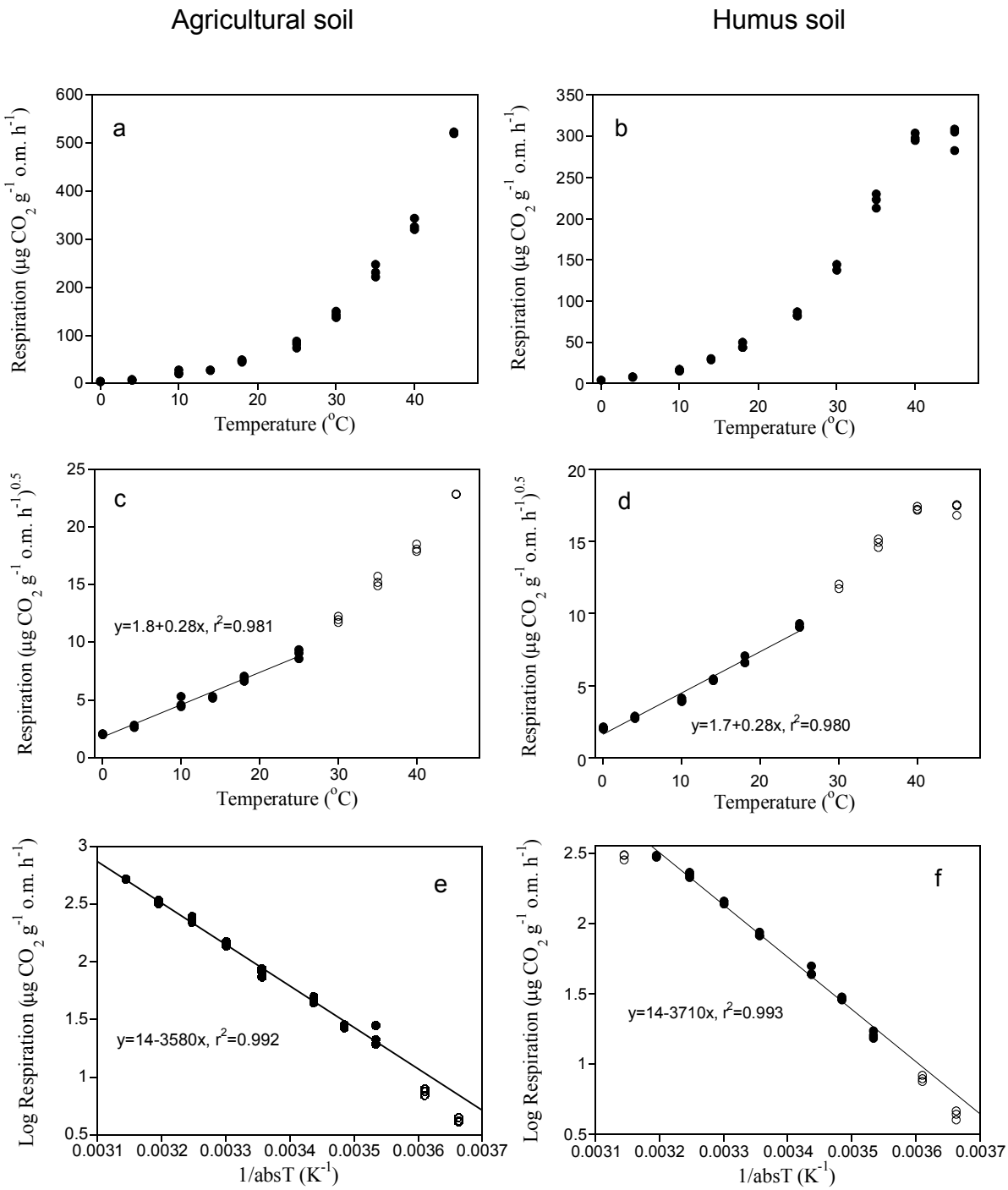

Fig. 1. Total activity (respiration rate) at different temperatures in an agricultural (a, c, e) and a forest humus soil (b, d, f). The data were plotted without transformation (a, b), with square root transformation (c, d; straight line follows the square root equation), and with logarithmic transformation against the inverse of the absolute temperature (e, f; straight line according to the Arrhenius equation). In c, d, e and f only data points with filled symbols were used in the calculation of the regression.

Table 1. Minimum, optimum and maximum temperatures for total (respiration rate), bacterial (thymidine incorporation rate) and fungal (acetate-in-ergosterol incorporation rate) activity in an agricultural and a forest humus soil. The apparent minimum temperature (T_{min}) was calculated from the square root equation, while the optimum and maximum temperatures were visually estimated from graphs.

	Soil	T_{min}	Optimum	Maximum
Respiration	Agricultural	-6.4	>45	>45
	Humus	-6.1	40-45	>45
Bacterial activity	Agricultural	-8.4	25-30	– 45
	Humus	-12.1	25-30	– 45
Fungal activity	Agricultural	-12.3	25-30	No data
	Humus	-17.5	25-30	– 40

Bacterial activity. Optimum temperatures for thymidine incorporation rates of the bacterial community were between 25 and 30°C for both soils (Table 1, Fig. 2a and b). The bacterial activities at the optimum temperatures were around 14 and 9 times above that at 0°C for the agricultural and humus soil, respectively. Above the optimum temperature the bacterial activity decreased, but some activity was observed even at 45°C, indicating that maximum the temperature for growth of the bacterial community was above this temperature.

The bacterial activity was well described by the square root function below the optimum temperature for growth in both soils (r^2 = 0.986 and 0.979 for the agricultural and humus soil, respectively; Fig. 2c and d). The calculated apparent minimum temperature for bacterial growth (T_{min}) was -8.4°C for the bacterial community from the agricultural soil and -12.1°C for that from the humus soil. Application of the Arrhenius equation resulted in overestimation of the bacterial activity at low temperatures (calculations not shown).

Fungal activity. It was more difficult to measure the fungal activity with the acetate-in-ergosterol technique than with the other two techniques. Thus, the variation in the data was higher. The optimum temperatures for fungal growth were found to be between 25 and 30°C for both soils (Table 1, Fig. 3a and b). At this temperature, values of fungal activities were around 10 times higher than at 0°C. Above this temperature the fungal activity decreased rapidly, resulting in a maximum temperature for growth of the fungal community of the forest humus soil at approx. 40°C (Fig. 3b, Table 1). In the agricultural soil high, but variable, values were found at this temperature (Fig. 3a) indicating that thermophilic fungi had started to grow during the incubation period (see Discussion). This data point was therefore not included in the evaluation.

The fungal activity below the optimum temperature for growth was well described by the square root function in both soils (r^2 = 0.883 and 0.920 for the agricultural and humus soil, respectively; Fig. 3c and d). The calculated apparent minimum temperature for fungal activity (T_{min}) was estimated to be -12.3 and -17.5°C for the fungal community from the agricultural and humus soil, respectively. Application of the Arrhenius function resulted in overestimation of the fungal activity at low temperatures (calculations not shown).

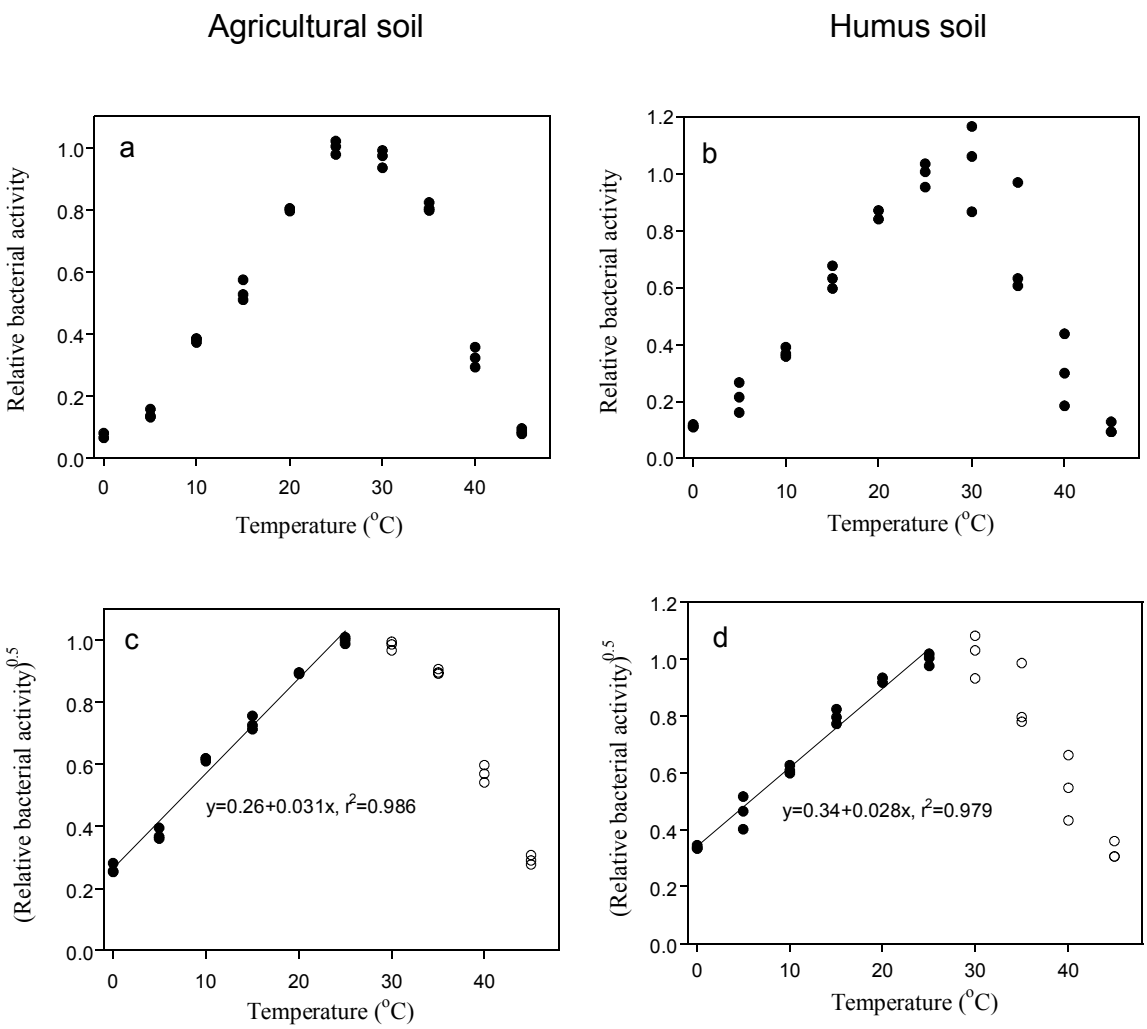

Fig. 2. Bacterial activity (thymidine incorporation rate) at different temperatures in an agricultural (a, c) and a forest humus soil (b, d). The data were plotted without transformation (a, b), and with square root transformation (c, d; straight line follows the square root equation). The data were normalised to 1 at 25°C. In c and d only data points with filled symbols were used in the calculation of the regression.

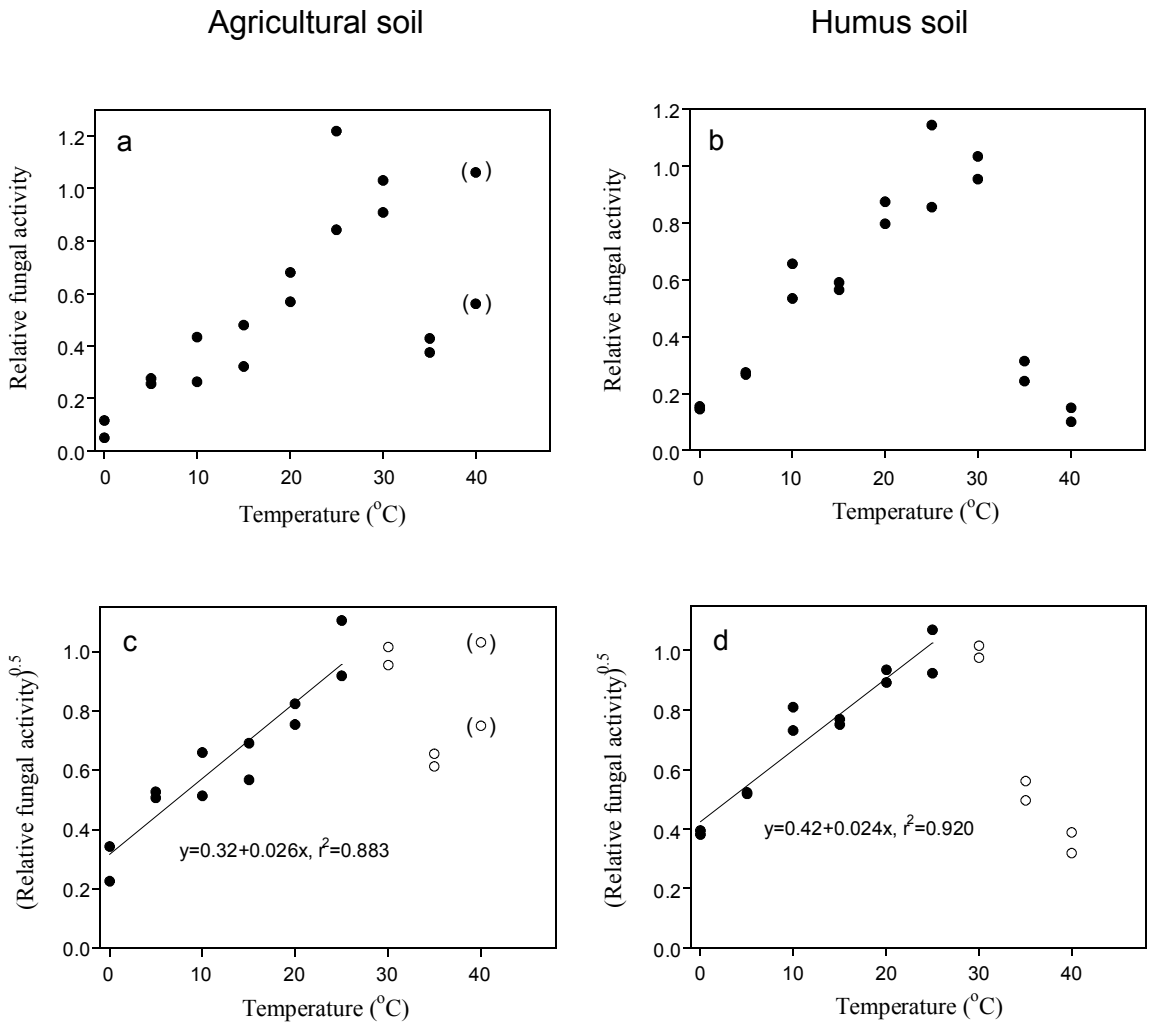

Fig. 3. Fungal activity (rate of acetate-in-ergosterol incorporation) at different temperatures in an agricultural (a, c) and a forest humus soil (b, d). The data were plotted without transformation (a, b), and with square root transformation (c, d; straight line follows the square root equation). The data were normalised to 1 at 25°C. In c and d only data points with filled symbols were used in the calculation of the regression.

Comparison of the temperature effect on bacterial and fungal activities. The activities at 25°C were set to one, when comparing the bacterial and fungal activities at different temperatures (Fig. 4). Thus, a ratio below one for the relative bacterial to fungal activity at a certain temperature indicates that bacterial activity was more negatively affected than fungi at this temperature, while a value above one indicates that fungal activity was more negatively affected. Both soils showed similar results. At 30°C and below, the ratio of bacterial activity to fungal activity did not differ much from one, although a tendency towards somewhat lower values was seen at the lowest temperatures, indicating that bacterial activity was slightly more negatively affected by low temperatures than fungi. At temperatures above 30°C high ratios were found, showing that fungal activity was more negatively affected by high temperatures than bacterial activity.

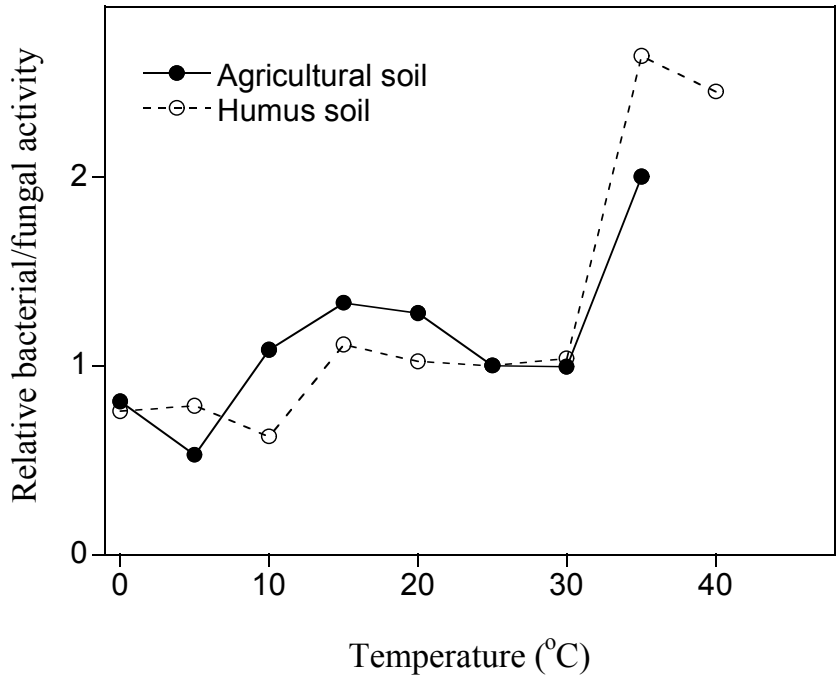

Fig. 4. The ratio of bacterial-to-fungal activity (rate of thymidine and acetate-in-ergosterol incorporation, respectively) at different temperatures in an agricultural and a forest humus soil. The data were normalised to 1 at 25°C.

DISCUSSION

The temperature dependence of fungal and bacterial activity differed in that the former group was less inhibited by low temperatures and the latter less inhibited by higher temperatures (Fig. 4). This can also be seen by the lower apparent T_{min} for fungal activity than that for bacterial activity (Table 1). The advantage of fungi at low temperatures is in accordance with the finding that fungi dominated in high-altitude soils during winter and spring, when the soil was covered with snow, whereas bacteria appeared to dominate during summer under snow-free conditions (17). The advantage of fungi at low temperatures may also explain the high amounts of fungal biomass found in forest soils during cold periods (36) and in oligotrophic peat sites (24).

One complicating factor is that the methodology used for determining the temperature dependence of growth and activity of a microbial community can affect the results. The use of leucine incorporation rate instead of thymidine incorporation rate resulted in higher values of the apparent T_{min} (a mean difference of 2.4°C between thymidine and leucine incorporation) in two soils (7), resulting in the ratio of leucine to thymidine incorporation decreasing with decreasing temperature. This was also found in peat over a large temperature interval (31) and in different aquatic habitats (37). The use of leucine instead of thymidine to determine the temperature dependency of the soil bacterial community will therefore result in a ratio of bacterial to fungal activity that is even lower at low temperatures than that shown in Fig. 4. Thus, the basic conclusion that fungal activity is less affected than bacterial activity at low temperatures is still valid, although caution must be exercised when stating the

extent of this difference.

All three activity measurements followed the square root (Ratkowsky) function (a straight line of the square root of the activity vs. temperature) below the optimum temperature for growth of the fungal and bacterial communities (Figs 1, 2 and 3). This has earlier been shown to be the case for bacterial activity in soil (7) and water (18), and for fungi in soil (3). The square root model has also been found to adequately describe the temperature dependence of soil respiration (7, 19, 28) and the total activity under anaerobic conditions (denitrification, (27)), and it has been used to model the decomposition of crop residues in soil (11, 14, 19). Thus, the square root function not only models the temperature dependency of bacterial growth in pure culture (32, 33), but also the growth and activity of the whole microbial community. The apparent T_{min} values determined in the present study (Table 1) were similar to those found earlier for bacteria (-6.3 to -10.8°C; (7)), fungi (-11°C; (3)) and respiration (-6.0°C; (28)).

Using the Arrhenius equation or a straightforward Q_{10} relationship resulted in overestimation of activity at low temperatures (Fig. 1e, f). This has been observed several times and is due to Q_{10} not being constant but increasing at lower temperatures (15, 16, 20). To adjust for the variation in Q_{10}, models with more variables have been used (12, 15, 16, 20, 25). This will result in temperature dependence being adequately modelled. However, the square root model, with an apparent T_{min} below 0°C, will also result in an increase in Q_{10} with a decrease in temperature (Fig. 1 in (19)). Using the equation proposed by Kirschbaum (16), based on several different studies of CO_2 efflux from soil and litter, and recalculating it on a square root basis results in an almost perfect straight line relationship between respiration rate and temperature ($r^2 = 0.998$ with a value of apparent T_{min} of -4.2°C). This does not mean that one should replace the equation used by Kirschbaum (16) with the square root function, for example, in modelling large-scale effects of changing climate on the carbon balance of soils. The square root function will be very dependent on fitting the correct T_{min} value at low temperatures, while the equation used by Kirschbaum (16), with more variables, will give more flexibility in providing the best possible fit to the data. However, if the square root model provides an adequate description of the instantaneous growth rate and activity of soil microorganisms, any deviation from this relationship may be interpreted as an indication that temperature does not only have a direct effect on a particular activity. This can be exemplified by the "uncoupled" respiration above 30°C, where little microbial growth occurred and where a breakpoint in the straight line between the square root of the respiration rate and temperature was found (Fig. 1c, d). In other situations temperature might have complex effects, resulting in a temperature dependence that does not follow the square root model. This may be the case with soil methane efflux, where temperature not only directly affects the growth of methanogenic bacteria and the rate of methane production, but also significantly affects the pathway of carbon flow, that is, the production of precursor molecules for methane production, and the rate of methane consumption by the methanotrophic community (6, 10).

The respiration rate above 30°C appeared not to be coupled to microbial growth, since the former increased at higher temperatures, while the latter decreased (compare Fig. 1 and Figs 2 and 3). This uncoupling could not be

detected without the simultaneous measurement of respiration rate and microbial activities. It is likely that many earlier studies on instantaneous respiration at high temperatures (2, 5, 35, 38) also included respiration not related to growth, although this could not be elucidated without the complementary measurements of bacterial and fungal growth. For example, replotting the data of Anderson and Domsch (2) according to the square root function revealed a similar situation to that found in the present study (Fig. 1c, d), with a breakpoint around 30°C. The reason for the uncoupling of soil respiration and microbial growth at higher temperatures may be explained by observations on soil sterilized by irradiation (30). Soil respiration continues for weeks after irradiation, as, despite the fact that the organisms are dead, the CO_2-producing enzymes are still functioning. Under such circumstances one would expect the CO_2 efflux to become equivalent to a first-order enzymatic reaction, which will follow the Arrhenius function closely. This was also the case at temperatures above 30°C (Fig. 1e, f).

The uncoupling of the respiration rate from microbial growth at high temperatures has two implications. First, including respiration rates at temperatures above the optimum temperature for growth of the microbial community will obscure the fact that the respiration rate follows the square root model, and instead the Arrhenius or an exponential function with constant Q_{10} may appear to be a better choice. Second, it appears unlikely that the respiration rate at these high temperatures (above the optimum for growth of the soil microbial community) will allow us to compare the temperature dependence of microbial communities living in different thermal environments. Thus, including measurements of bacterial and fungal activities makes it possible to decide which temperatures to include for respiration measurements when studying microbial community adaptation to changing temperatures.

We did not find any major differences in T_{min} between the two soils studied for any of the activity measurements, being below freezing in all cases (Table 1). This contradicts the findings of Persson *et al.* (28), that the respiration rate of an agricultural soil not only had a higher T_{min} than a forest soil, but also had a T_{min} above freezing point (+1.6°C). However, they acknowledged that their respiration measurements of the agricultural soil at low temperatures were uncertain, which might have severely affected the calculated T_{min} value. It therefore seems likely that T_{min} in temperate and arctic soils is below 0°C. However, one must bear in mind that T_{min} is an apparent value and can only be used to model activities under non-freezing conditions. For example, below freezing point the respiration rate will be affected by temperature in a different way from that above freezing, with Q_{10} values increasing abruptly (9, 21).

It is important to keep the incubation time at high temperatures to a minimum in order to avoid the growth of a thermophilic community when studying the temperature dependency of the original community. This was easy with the respiration technique (5 h at 45°C) and when measuring the bacterial activity (2 h at 45°C), while longer times were needed for the fungal activity measurements to achieve values above the background. The growth of thermophilic fungi during incubation was probably the explanation of the anomalously high values of the rate of acetate-in-ergosterol incorporation at 40°C in the agricultural soil (Fig. 3).

The main finding of the present study was that fungal and bacterial activities

were affected differently by temperature. It is thus possible that changes in temperature due, for example, to climate changes, may alter the balance between these two main groups of soil microorganisms. However, one must bear in mind that the present study was on instantaneous activity. Changes in temperature over a longer period of time may result in adaptation of the microbial community to the new conditions (29), probably through changes in species composition. This is a different situation from that studied here, and therefore the temperature dependence of the fungal and bacterial communities may be different.

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