Microbial Indicators of Fertility in Arable Land

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Abstract

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In this thesis eleven microbial variables were evaluated as indicators of soil fertility. The variables were derived from short-incubation tests based on respiration, denitrification, ammonium oxidation, and alkaline phosphatase activity. During the incubation, the formation rate of metabolic end-products or intermediates was recorded and the variables were derived by use of linear or non-linear regression. Soils from three sites of a long-term field experiment and from a short-term laboratory incubation experiment were used for the evaluation. The microbial variables were evaluated in relation to standard soil chemical variables and yields from a whole crop rotation cycle.
Substrate-induced respiration (SIR) and respiration rate under phosphorus limited conditions (Max-P) were indicators of labile organic material and available P, respectively. They were also the best yield predictors in unfertilised and nitrogen fertilised systems, respectively. SIR was shown to quantify both dormant and fully active microorganisms. Substrate amendment could transform the dormant biomass into an active stage. The division of SIR into active and dormant biomass fractions holds promise that the SIR assay can be of value as an indicator of the mineralisation dynamics at two different time scales. Moreover, the concept of active and dormant microbial biomass has implications on how to model the carbon fluxes in soil. Basal respiration was influenced by temporary substrate sources and rhizodeposits and was therefore not considered as a stable soil fertility indicator. Potential denitrification (PDA) and ammonium oxidation (PAO) was governed both by pH and substrate supply. The prediction models for PDA varied markedly between the sites and exhibited a relatively low predictive capacity, which renders PDA less suitable as a soil fertility indicator. However, PDA was satisfactorily predicted by a combination of PAO and SIR. When interpreting PAO, it should be regressed on pH to check for diverging soils that may be N limited. A combination of pH and organic matter characteristics was efficient for prediction of alkaline phosphatase activity (Alk-P), which implies that Alk-P is a pH-sensitive indicator of the microbial biomass.

Keywords: Arable land, fertility indicators, field experiments, microorganisms, physiological state, yield prediction.

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Appendix

Papers I-IV

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

I. Svensson K and Pell M. 2001. Soil microbial tests for discriminating between different cropping systems and fertiliser regimes. Biology and Fertility of Soils 33, 91-99.

II. Svensson K and Pell M. 2002. Relations between chemical and microbiological properties in soil after 40 years of different cropping strategies. Manuscript.

III. Svensson K, Pell M and Mattsson L. 2002. Microbial properties as yield indicators in 40-year-old field experiments. Manuscript.

IV. Stenström J, Svensson K and Johansson M. 2001. Reversible transition between active and dormant microbial states in soil. FEMS Microbiology Ecology 36, 93-104.

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Introduction

Soil as a resource – the microbial viewpoint

What is soil? Soil concerns all humans although it is appreciated and utilised quite differently depending on whom you ask. For the Greeks, soil was one of the four basic elements in nature. For the farmer, soil is both a companion and prerequisite for his life. For the environmentalist, soil can be a source of pollution, but also a filter that purifies the percolating water. For the medical researcher, soil is a source of contagious diseases like anthrax and tetanus. For kids, soil is an excellent building material. In a sustainable society, soil is the end station for source-separated organic waste. After the funeral ceremony, the soil becomes the end station for us all, six feet below ground, while for the microbe dwelling in the dark, the soil is a home. The soil has been a home for microbes for hundreds of millions of years. The soil provides microbes with food and the small pores that protect them from both drought and hungry soil animals. The general question addressed in this thesis is if the soil microbes, being an integrated part of the soil system, constitute a resource for us from an agricultural point of view. Or is it only the other way around, *i.e.* that we are a resource for the microbes when we pass away? To conceive why the research behind this thesis has been performed, three perspectives have to be considered: the global, the farmers and, thirdly, the historical perspective. They illustrate some of the problems agriculture will face in the future and why knowledge of soil microbiology can be important in this respect. The scope of the thesis is defined in the light of the three perspectives.

The global perspective

The world is not enough! In a shrinking world the motto of James Bond may soon be adopted by all people on planet Earth. Today, 6 billion people are fed on 1.5 billion ha of arable land and some 100 million tonnes of fish. The increase of resource acquisition has until now mainly been based on conquering of new and productive land. Virgin land to bring into cultivation is now becoming scarce (Hudson 1995). According to the demographic models, the world population will have increased to 9 billion people in 2050, but the resources will essentially be the same as today, if not less. The only strategy that remains to handle the 50% increase in the need for food in 50 years is to increase the hectare yield by 50%. However, this goal will be difficult to reach since mismanagement threatens soil productivity world-wide.

Erosion caused by wind and water sweeps away the soil and leaves naked bedrock or gully formations. The production capacity is irreversibly destroyed. There are many historical traces of erosional soil devastation, *e.g.* around the Mediterranean, in the Middle East and in China. Ruins of great cities (*e.g.* Petra in Jordan) bear witness of the richness once produced in such areas (Lowdermilk 1953). They also indicate the irreversible nature of erosional processes, since subsequent settlers have remained poor and unable to revive the green fields.

The production capacity of soil may also be lowered by various reversible processes. Mismanagement may lead to decreasing contents of plant nutrients and

organic matter in the soil, as well as acidification, compaction and pollution of the soil. Soil degradation not only makes seedbed preparation more difficult, but will also lower yield levels and crop quality. The declining trends are counteracted with mineral fertilisers, pesticides and new crop varieties bred to give high yields. These measures are, however, criticised as worsening the situation by eutrophication, poisoning and genetic impoverishment. Will knowledge of the microbial soil flora help us to reverse the declining trends encountered world-wide?

The world is not only shrinking, from the microbiologist's point of view it is also growing. Kluyver & van Niel (1956) estimated that half of the total living protoplasm is microbial. Later findings suggest that this estimation was much too conservative (Whitman *et al.* 1998). Instead, microbial cells probably constitute a dominating part of the whole biomass on planet Earth: Microorganisms are truly ubiquitous organisms capable of surviving nearly anywhere on our planet. Microbial cells have been detected as high up in the atmosphere as 77 km (Imshenetsky *et al.* 1978) and as deep as 5000 m down in Swedish bedrock (Szewzyk *et al.* 1994). In fact, it has been estimated that about 90% of all prokaryotic cells, or 3.7-6.2 $\times$ 10^{30} cells, can be found in the subsurface. Subsurface is here defined as terrestrial habitats below 8 m and marine sediments below 10 cm. Most of the remaining prokaryotic cells dwell in the ocean (0.12 $\times$ 10^{30} cells) and in the soil (0.26 $\times$ 10^{30}) (Whitman *et al.* 1998). In spite of their number, the biogeochemical role of deep-dwelling microorganisms is probably not significant (Brock & Madigan 1991), since the generation time for these organisms is estimated to be 1000 to 2000 years (Whitman *et al.* 1998). This leaves the microbes in the soil and oceans as the main biological agents to catalyse the global nutrient cycles. The biomass of prokaryotic cells in other habitats, *e.g.* the air, the gasto-intestinal tract, leaves, *etc.*, will not by far reach the size of the biomass encountered in the ocean and soil. The focus of this thesis is on microbial processes central to the turnover of carbon, nitrogen and phosphorus in soil. Since the microbial biomass in the soil is so dominating globally through its pure mass, quantification of the processes carried out by microorganisms may add to our understanding of their impact on the global biogeochemical cycles. However, the contribution of the thesis in this respect is mainly an increased understanding of the features that the methods used exhibit.

The farmer's perspective

In springtime a farmer walks out to his field. He takes a few soil crumbs and puts them in his palm, smells them and looks at them, and rolls them between his thumb and forefinger. Then, he looks out over the field. Finally, the decision is made. Now it is time to start sowing.

In everyday decision-making, the farmer gathers a lot of information and processes it on the basis of his earlier experience and theoretical knowledge. However, most of the potentially available information is overlooked (Slovic 2000). Probably without knowing it, the farmer had about one billion microorganisms in his hand, distributed on about ten thousand species. Due to

their life processes microbial cells can modify or even alter the soil environment in a beneficial or detrimental way for plant growth. Hence, the microbes might be decisive for the outcome of the efforts invested in the spring farming operations. If the farmer had been able to make use of information about the microbes in his soil, would he perform the farming operations according to his original plans? How would he have obtained the information? According to which models, frameworks or decision rules would he have made the interpretation of the information? It has been claimed that microbial variables integrate information on several soil properties (Stenberg 1998). Which information can the farmer replace by knowledge of microbial soil characteristics?

The scenario and questions above illustrate the applied perspective of this thesis. Researchers have stressed the benefits in obtaining information about microbial life both as a means of assessing fertility in order to increase yields, and to get indications of poisonous xenobiotics and negative environmental impacts in the soil plant system. However, in this thesis, focus is on the impact of traditional management practices on microbial processes.

The historical perspective

The field of Microbiology began to develop as a science in the middle of the 19th century. In France, Louis Pasteur demonstrated that abiogenesis or spontaneous generation did not exist. Some years later, the German scientist Robert Koch developed the germ theory of disease from his work on *Bacillus anthracis*. Soon a number of diseases were characterised microbiologically and the development of treatments for prevention and cure of contagious diseases began (Brock & Madigan 1991).

The first microbial investigations of soil were performed by medical men in the search for pathogenic microbes, and for some decades microbes were intimately linked with diseases, which were thought to be their only role. In the 1880s and 1890s Sergei Winogradsky and Martinus Beijerinck performed the first extensive systematic studies of microbial processes in soil. Their pioneering work and subsequent contributions by other researchers led to the insight that the activities of microbes are closely connected with soil fertility. Jacob Lipman (1911) placed emphasis on this connection in his book "Bacteria in relation to country life" where the expected benefits from soil microbes were outlined. He wrote "It will thus be seen that there is a direct relation between the crops on the soil and the bacteria in the soil" (p. 141). The new insights in the connection between microbial life and fertility influenced the way, the goals, and the strategies for the science of soil microbiology, and were formulated, *e.g.* in "Ziele und Wege der bakteriologischen Bodenforschung" (Löhnis 1912). In 1927 the book "Principles of Soil Microbiology" (Waksman 1927) was published in which the author had collected the known facts on soil microbiology that had accumulated during the previous 50 years, *i.e.* ever since the science of soil microbiology had started. The book contained about 2750 references, indicating the rapid expansion of the field at the beginning of the 20th century. It became the standard text in soil

microbiology for more than two decades. In his book, Waksman promoted the ideas that soil fertility and soil microbiology are closely connected.

One major obstacle for the breakthrough of microbial soil investigations as tools that could be used in common fertility diagnostics was the difficulty in developing good methods that produced unbiased and reproducible results. In the 1920s available methods could be categorised into three groups: (1) microscopic studies after staining the cells with an acid dye, *e.g.* rose bengal; (2) plate counts on different solidified media; and (3) measurement of microbial product formation or substrate consumption (Waksman 1927). These techniques are still in use, but have been refined in different ways. In 1922, Waksman cited a paper by Fischer (1909) where no relation had been found between performed bacteriological investigations and soil fertility. Waksman (1922) commented: "Were we to consider that same question today (*i.e.* in 1922), we should have to come to the same conclusion. All attempts to interpret crop production from a microbiological point of view have not been rich in consequences and some investigators, who have considered some of the methods carefully, such as bacterial numbers, ammonification, denitrification, etc, have come to the conclusion that they are practically worthless for the interpretation of soil fertility. The only exception to this statement may be found in some of the recent investigations on nitrification. This lack of definite information and the unreliability of the information that we do possess is due to a number of factors, chief among which is the lack of standard methods employed." Today, the lack of standardised and appropriate soil microbial methodology to assess the impact of management practices is still being a major concern, as emphasised by *e.g.* Jordan *et al.* (1995).

Concerning the methods, Waksman (1927) summarised the issue in one question: "In considering the soil microflora as a whole, can a group of methods be suggested, which would supply the information necessary for an understanding of the various processes carried on by microorganisms in the soil, so as to obtain an insight into the actual or potential fertility of the particular soil?" (p. 709). He subsequently stressed that the methods should be quantitative, both for the study of numbers of microorganisms and for measuring the physiological activities of the microorganisms.

Since those days, a large number of methods have been developed (Alef & Nannipieri 1995; Schinner *et al.* 1996; Weaver 1994) and used to describe soil conditions and processes. However, the question remains: which methods are the most suitable and how do we apply them?

Objectives

The aim of this thesis is to disclose the constraints, relations and potential usage of some recently developed microbial methods as indicators of soil fertility or soil quality. Such information is needed when interpreting microbial test results and thereby fully benefit from microbial soil testing. The methods are based on microbial product formation rates and are developed from the four microbial soil processes: respiration, denitrification, nitrification and alkaline phosphatase activity. In a 40-year-old long-term field experiment the microbial methods were

evaluated for their sensitivity to applications of different cropping systems, phosphorus and nitrogen fertiliser regimes, for their relationships to standard chemical soil variables, and for their capability to predict yield level during a crop sequence cycle.

In a special laboratory study the microbial biomass in soil was estimated by substrate-induced respiration (SIR), which has the potential to divide the biomass into an active and a dormant biomass. The dynamics of the transition between the active and dormant states are outlined and the implications for a more fine-tuned interpretation of microbial activity are discussed.

Soil fertility and soil quality

Definitions

There are several suggestions on how to define soil fertility. Waksman (1927) wrote "The measure of soil fertility is the crop itself" (p. 708). Nearly 70 years later, Persson & Otabbong (1994) defined soil fertility as "the long-term capacity of a soil to produce good yields of high quality on the basis of chemical, physical and biological quality factors" (p. 7). Furthermore, they discussed the concept of soil fertility thoroughly. They meant that the fertility components can be categorised into a biological, chemical and physical part. The components are continuously interacting with each other under the influence of climate, soil type and cultivation measures. The fertility can be improved, maintained or decreased, depending on the cultivation practices carried out. For example, the phosphorus status and base saturation of soils can be increased or lowered. However, some fertility factors cannot be modified regardless of cultivation management, *e.g.* soil type and topography. It is, moreover, motivated to distinguish between actual and potential soil fertility. Potential fertility is when all modifiable fertility factors are optimised. In this situation, the unmodifiable factors alone govern soil fertility. Persson & Otabbong (1994) concluded that yield level is an imprecise definition of soil fertility, due to the complexity of the soil and the soil processes. However, the terms "long-term capacity" and "high quality" render the definition itself imprecise. How long is long-term and what quality should be high? While awaiting the answers I have returned to the original definition proposed by Waksman.

During recent years the concept of soil quality has been discussed and developed. The intention with the soil quality concept is to widen the perspective beyond pure production goals, and the focus is sustainability on an (eco-)system level. Soil quality is therefore considered to be a broader concept compared with that of soil fertility, which is evident from the definition of soil quality as proposed by Doran & Parkin (1994): "The capacity of a soil to sustain biological productivity, maintain environmental quality, and promote plant and animal health". Gregorisch *et al.* (1994) simply stated that soil quality is "the degree of fitness of a soil for a specific use", while Karlen *et al.* (1998) defined it as "the capacity of soil to function" and further concluded that it is not possible to directly measure soil quality. Indicators of soil quality should be used instead. An indicator is defined as a pointing or directing device (Kennedy & Papendick 1995). By

pointing at certain circumstances or phenomena, a soil quality indicator will give an indication of the soil function or status. Doran & Parkin (1994) proposed that different indicators summarised in a weighted soil quality index should be used to describe soil quality. By use of different weights it is possible to adjust the index for, *e.g.* regional differences and socioeconomic concerns. A number of microbial soil characteristics have been proposed and evaluated for their sensitivity as soil quality indicators, *e.g.* microbial biomass (Boehm & Anderson 1997; Carter *et al.* 1999; Fauci & Dick 1994), enzyme activity (Badiane *et al.* 2001; Wick *et al.* 1998) and composition of the microbial flora (Jordan *et al.* 1995; Perkins & Kennedy 1996). Subsequently, the variables that for various reasons have been considered as the best ones have been included together with appropriate chemical and physical variables in so-called minimum data sets (MDS). Gregorich *et al.* (1994) discuss the use of MDS for assessing soil organic matter quality. However, several authors have stressed the difficulties with interpretation of microbial characteristics (Carter *et al.* 1999; Dalal 1998; Stenberg 1999), and with the adoption of the soil quality concept as such (Herrick 2000). Consequently, the great expectations linked with the microbial variables as indicators of soil quality and functioning still await to be fulfilled.

Significance of soil microorganisms to soil fertility

The microorganisms have a double role in relation to soil fertility. On one hand, the microorganisms are the agents that mineralise and liberate plant nutrients from the organic material. They can be viewed as the eye of the needle through which all organically-bound plant nutrients must pass in order to become plant-available (Brookes 1995). By understanding the nutritional needs of the microorganisms, the beneficial processes they perform can be used optimally and microbial competition with plants, *e.g.* for nitrogen, can be minimised.

On the other hand, the microorganisms can also be viewed as a collective observer of the soil environment. Since the microbes are in close contact with all three soil phases (solid, water and air), they can sensitively and rapidly probe responses to soil perturbations. The spectrum of possible responses is enormous but can be categorised into population changes (structure) and physiological changes (function). The time scale of the dynamics of these two categories is quite different and so is probably their value as fertility indicators. The physiological state and activity level of the population is expected to react much faster to an environmental change than the size and composition of the population. However, a population change is probably more fundamental and must therefore be regarded as more robust.

Methods and Experiments

A soil quality indicator must be sensitive to changes in soil management, soil perturbations, and inputs into the soil system (Gregorich *et al.* 1994). In addition, for routine analysis the assay of the indicator must be cheap, be possible to perform within a relatively short time and have a low experimental error (*i.e.* give reproducible results). In 1988 a research project was initiated in Sweden in which a number of microbial methods were elaborated to be used to test for the impact of pollutants and defined anthropogenic substances (Bengtsson & Torstensson 1988). The methods were published in 1993 (Torstensson 1993). Most of the methods are based on assaying the potential formation of microbial metabolites. They are quantitative and rely on some general microbial processes such as respiration, denitrification, nitrification, alkaline phosphatase and nitrogenase activity. The tests were subsequently used evaluate toxicity of pesticides (Pell *et al.* 1998) and silver (Johansson *et al.* 1998). Moreover, the tests have been used to assess effects on the soil ecosystem after application of sewage sludge (Johansson *et al.* 1999), their principal correlation patterns have been outlined (Stenberg *et al.* 1998), and they have been used as model components to predict yield under standardised laboratory conditions (Stenberg 1998). Stenberg (1999) concluded that the microbial test methods fulfilled the demands of efficient indicators. Therefore, this microbial test battery was chosen for the studies reported in this thesis.

The microbial test methods

The microbial tests included in the test battery were basal respiration (B-res), substrate-induced respiration (SIR), potential denitrification (PDA), potential ammonium oxidation (PAO) (viz. potential nitrification), and alkaline phosphatase activity (Alk-P). The tests quantify the microbial production rate of intermediates or end-products under conditions of standardised temperature and water potential. All tests, except basal respiration, are assayed with substrate excess. The product formed in the assay is sampled one or more times, depending on the assay, and plotted versus time (Fig. 1a-d). Kinetic models are fitted to the data by linear or non-linear regression.

Applied kinetic models

The rate of a microbial process (dp/dt) in the soil is determined by the amount (N) of microorganisms performing the process and their specific activity (q) (Stenström *et al.* 1998). Thus:

$$dp/dt = qN \qquad [1]$$

where dp/dt is the rate of product formation. In simple enzymatic reactions, qN is determined by K_m according to the Michaelis-Menten equation

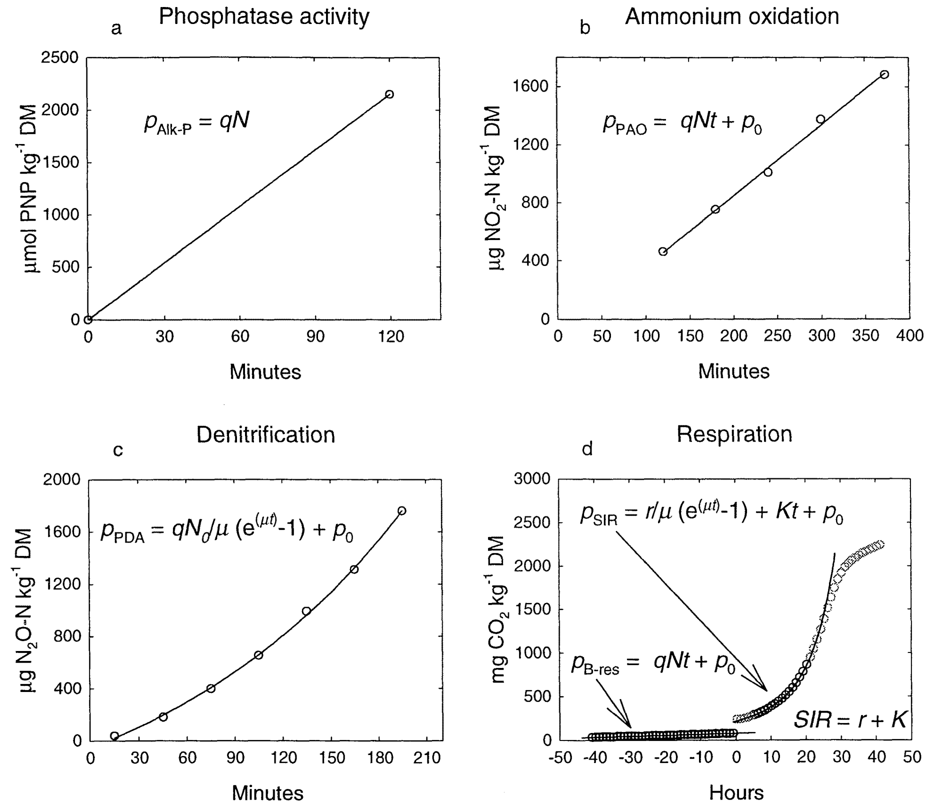

Fig 1. Illustration of the kinetic models applied for a) the alkaline phosphatase activity assay, b) the potential ammonium oxidation assay c) the potential denitrification activity assay, and d) the basal respiration and substrate-induced respiration assays.

$$qN = k(V_{max}S)/(K_m + S) \quad [2]$$

where V_{max} is the maximum rate when all enzymes are saturated with substrate, S the substrate concentration, K_m the substrate concentration that yields $1/2\,V_{max}$, and k is a proportionality constant. By setting $K_m << S$, qN becomes approximately equal to kV_{max}. Consequently, when the microbes are supplied with a surplus of substrate, q is the maximum rate of product formation per unit biomass. When the product formation is assayed with surplus of substrate and at a standardised temperature and water potential, N (amount of enzymes or cells) alone will be decisive for the outcome of the assay.

If the microbes do not grow during the incubation, the product formed will accumulate at a constant rate under substrate-saturated conditions. By integrating [1], the accumulation of the product formation then is described by

$$p = qNt + p_0 \qquad [3]$$

where p_0 is the amount of product at time zero. Microbial growth, *i.e.* exponential increase of N as a function of time, is described by

$$N = N_0 e^{\mu t} \qquad [4]$$

where N_0 is the amount of cells at time zero and μ is the specific growth rate. If the microbial population grows, the rate of product formation is given by combining [1] and [4], resulting in the equation

$$dp/dt = qN_0 e^{\mu t} \qquad [5]$$

Upon integration, the accumulation of the product then is described as

$$p = (qN_0/\mu)(e^{\mu t} - 1) + p_0 \qquad [6]$$

If one subgroup of the product-forming population grows and one does not grow, the product formation rate is the sum of [1] and [5]. For convenience, qN_0 for the growing population is denoted r and qN_0 for the non-growing population is denoted K, as adopted from Stenström *et al.* (1998) and paper **IV**. Then,

$$dp/dt = re^{\mu t} + K \qquad [7]$$

and the accumulation of product is described by

$$p = (r/\mu)(e^{\mu t} - 1) + Kt + p_0 \qquad [8]$$

The resulting product formation curves from the methods used to assay the processes in this thesis are shown in Fig. 1a-d. The background of the microbial processes described by the models is outlined below.

Alkaline phosphatase activity (Alk-P)

Commonly, more than 20% of the phosphorus in soil is organically bound (Oberson *et al.* 1996). Plant and microbial uptake of the organic P-component requires that it is mineralised by phosphatases to ortophosphate. The microbes in the soil produce a range of different phosphatases that catalyse the hydrolysis of the ester and anhydride bonds, which bind phosphate groups to various organic molecules. Phosphatases can be divided into phosphomonoesterases, phosphodiesterases, phosphotriesterases, polyphosphatases and enzymes acting on P-N bonds (Tabatabai & Page 1982). Phosphatases are induced predominantly under P-limited conditions (Schinner *et al.* 1996). They are known to occur abundantly in the periplasmic space of gram negative bacteria, but can also function as extracellular enzymes in the soil solution or be bound to clay or humus particles (Sjöqvist 1995).

The phosphomonoesterases (phosphatases in the following) hydrolyse monoester bonds, in *e.g.* phospholipids and sugar phosphates. They differ in substrate affinity and pH optimum. The acid phosphatases have a pH optimum around 6.5. They originate from both plants and microbes. The alkaline phosphatases are assayed at pH 9. They originate only from microorganisms (Chhonkar & Tarafdar 1981).

In papers **I**, **II**, and **III**, alkaline phosphatase activity was determined after a preincubation time of four weeks. During the preincubation the activity will approach a stable basic level, which is independent of variations induced by sample preparation (Sjöqvist 1995). In the assay, the substrate, p-nitrophenylphosphate (PNP-P), is hydrolysed to phosphate and p-nitrophenol (PNP) in a soil water slurry buffered to pH 9.0. The amount of the coloured product PNP (yellow) produced is measured spectophotometrically. Since PNP-P is an artificial substrate, it is assumed that no PNP exists in the soil at time zero. The additional assumption of a constant production rate results in a simple design of the assay with only one sampling point (Fig. 1a, Eq. [3]). The method is adopted from the assay originally suggested by Tabatabai & Bremner (1969).

Potential ammonium oxidation activity (PAO)

Ammonium oxidation is central for the nitrogen cycling in soil. Autotrophic nitrification is the major route for ammonium oxidation in soil, and is performed by a few species within the bacterial family *Nitrobacteriaceae*. The nitrate that is produced might subsequently be lost from the soil by leaching and denitrification. Nitrifying bacteria are thought to be highly sensitive to perturbations and disturbances, and their activity is therefore indicative of environmental stress factors, such as heavy metals and organic xenobiotics (Christensen *et al.* 2001; Pell *et al.* 1998).

Nitrification is a two-step process, in which the bacteria yield energy from the oxidation of ammonium or nitrite, whereas the carbon is derived from carbon dioxide. Thus, the bacteria are both chemolithotrophic and chemoautotrophic. The first step in the oxidation process, the conversion of ammonium to nitrite, is

predominantly performed by *Nitrosococcus*-like species in soil (Hermansson 2001). It is considered as the rate-limiting step. The second step, the oxidation of nitrite to nitrate, is performed by *Nitrobacter* spp and other genera.

In early days, nitrification was measured as the accumulation of nitrate in soil during an incubation period lasting somewhere between 10 to 30 days (Burgess 1918). The process rate was probably underestimated by this assay, since some of the nitrate could be immobilised or denitrified. Belser & Mays (1980) reported that chlorate blocks the nitrite oxidation and nitrite will therefore accumulate in the soil if chlorate is added to the medium. In the PAO assay the medium is composed of ammonium sulphate, chlorate and a P-buffer (pH 7.2), and the accumulated nitrite is measured spectrophotometrically. The generation time for ammonium oxidising bacteria is > 10 h. Since no significant growth occurs during the short incubation (6 h) the nitrite accumulates linearly (Fig. 1b, Eq. [3]). Traces of nitrite in the soil at time zero induce an error if the ammonium oxidation rate is estimated from a single measurement. Five samplings during a six-hour incubation make estimation of the potential ammonium oxidation rate highly accurate.

Potential denitrification activity (PDA)

Denitrification is a direct cause of gaseous losses of nitrogen from soil. Typically 20-30% of the mineral nitrogen may disappear from agricultural soils during a season (Firestone 1982). The nitrogen evolves primarily as dinitrogen gas (N_2), but incomplete reduction may produce significant amounts of nitric oxide (NO) or nitrous oxide (N_2O). The latter compound acts as a greenhouse gas (Isermann 1994) and is a natural catalyst of stratospheric ozone decay (Crutzen 1983). In the denitrification process, nitrate is reduced through a sequence of enzyme catalysed reductions where the nitrogenous oxides are used as terminal electron acceptors instead of oxygen gas. In contrast to nitrification, denitrifying bacteria can be found within most taxonomical groups of soil bacteria (Zumft 1992). Under aerobic conditions, denitrification is repressed since most denitrifying bacteria prefer oxygen as electron acceptor. When anaerobic conditions set in, *e.g.* when soil becomes waterlogged, denitrification is derepressed both at enzyme (Berks *et al.* 1995) and gene level (Bauer *et al.* 1999). The extent of energy conservation during denitrification is at a maximum of 70% of that during aerobic respiration (Stouthamer 1991). The process rate is not only regulated by redox conditions, but also by nitrate concentration, available carbon, pH, soil structure and size of the biomass (Simek & Hopkins 1999; Tiedje 1988).

The most common strategies used in studies of the denitrification process are to monitor nitrate consumption, or to use isotopic techniques or the acetylene inhibition method to estimate formation of the gaseous product (Tiedje 1988). Acetylene blocks the last step in the denitrification process, *i.e.* the nitrous oxide reductase, which results in the accumulation of nitrous oxide in the incubation vessel. This blocking effect was discovered in 1973 by Russian scientists when searching for extraterrestrial life forms (Payne 1991). Yoshinari & Knowles (1976) were among the first to use the effect of acetylene inhibition to study denitrifying bacteria in soil. Smith & Tiedje (1978; 1979) used the inhibitory effect to elaborate

an assay for the quantification of denitrification activity. The method used in this thesis is a modification of the method originally proposed by Smith & Tiedje, and has been described by Pell (1993) and Pell *et al.* (1996). In the modified method no chloramphenicol is used to inhibit protein synthesis and hence cell growth. Instead, a two-parameter non-linear model considering growth is applied (Fig. 1c, Eq. [6]). The parameters describe initial production rate (PDA) and specific growth rate (μ_{PDA}). During the assay, seven samplings of the headspace in the incubation vessels are performed. Time zero, which is crucial for correct parameter estimates, is defined as the moment for injection of acetylene.

Basal respiration (B-res) and Substrate induced respiration (SIR)

When organic matter in soil is mineralised to carbon dioxide, organically bound plant nutrients are released. The most available carbon fractions are degraded by fast-growing microorganisms, leaving the recalcitrant structures like lignin to slow-growing microbes. A flush of carbon dioxide is therefore induced when harvest residues, tops, *etc.* are ploughed into the soil. Not all carbon dioxide originates from microbial heterotrophic metabolism. Root respiration may contribute to a significant part of the carbon dioxide during the growing season (Buyanovsky *et al.* 1987). Also, in carbonate containing soils with a pH < 8.3, the carbonate is continuously dissolved into gaseous carbon dioxide. To avoid biased estimations of the microbial respiration rate, measurements should therefore be restricted to soils free from living roots and carbonate. Carbon dioxide can be measured directly as a gas or after entrapment in a hydroxide solution as a carbonate ion. Since it is easily measured using simple devices, the production rate was early used as an indication of fertility in soil, as reviewed by, *e.g.* Waksman & Starkey (1924).

In this thesis, the respiration rate was followed using a computerised respirometer (Nordgren 1988), where emitted carbon dioxide is absorbed in a hydroxide solution. The apparatus allows frequent measurements (1-2 measurements every hour) of the carbon dioxide evolution, which gives a high accuracy for estimations of the variables (Fig. 1d, Eq. [3] and [8]). Before the substrate-induced respiration (SIR) is initiated, the soil is incubated for ten days. At the start of the incubation, the respiration rate is usually elevated due to sieving, *etc.*, but declines successively to a stable level, the basal respiration (B-res), which is normally reached within a week (Martens 1995). In contrast to the other microbial variables, no substrate is added to the soil when the basal respiration is determined. Therefore, basal respiration should reflect intrinsic and available amounts of organic carbon.

The principle of the substrate-induced respiration (SIR) method was described early by, *e.g.* Waksman & co-workers (Waksman 1927; Waksman & Starkey 1924). They found that the microbial “decomposing power” of easily digestible carbon sources like dextrose and glucose was related to the carbon content and to the number of microorganisms as determined by plate counts. Fifty years later the concept of “decomposing power” was developed into a physiologically based quantitative method for biomass estimations by Anderson & Domsch (1978). They

showed that the microbial respiration rate increased to a certain level within 1 - 3 hours after mixing sufficient amounts of glucose into the soil. The elevated level of respiration, SIR, was found to be closely correlated to the microbial biomass estimated by the chloroform fumigation-incubation (CFI). Apart from CFI and chloroform fumigation-extraction (CFE), the SIR technique has become the most frequently used method for biomass determinations in soil, and has recurrently been reviewed for its advantages and disadvantages compared with the former methods (Brookes 1995; Jenkinson 1988; Martens 1995).

In the method applied in this thesis, SIR is induced by blending a mixture of glucose, ammonium sulphate, potassium phosphate and talcum into the soil. The resulting respiration curve is fitted to equation [8], which yields a quantitative estimation of the active (viz. growing) and dormant (viz. non-growing) microbial biomass. In addition, the specific growth rate for aerobic microorganisms is given and denoted μ_{res}. Time zero coincides with the substrate amendment. With few exceptions, the number of measured points included in the exponential phase exceeds 15, which should guarantee proper non-linear parameter estimations according to Robinson (1985).

Nutrient-limited respiration rate (Max-P and Max-N)

Stotzky & Norman (1961) showed that P and N might be limiting for the decomposition rate of glucose in soil. Strategies have subsequently been elaborated for using nutrient-limited respiration rates to determine microbially available amounts of P and N in soil (Demetz & Insam 1999; Nordgren 1992). In this thesis, the amounts of microbially available P (Max-P) and N (Max-N) were determined from the maximum respiration rate after amendment with SIR substrates, not containing P- and N-containing salts, respectively. The maximum respiration rate corresponds to the slope at the point of inflection of the cumulative data (Fig. 1d). No curve-fitting procedure was used when deriving these parameters, and they were not included in the original test battery published by Torstensson (1993).

The experiments

The Swedish Long-Term Fertility Experiment

In the nineteen fifties, ley production began to decrease and grain production increased in Sweden because of the need to rationalise agriculture. This trend induced concerns for the long-term fertility development of Swedish agricultural soils. Therefore, in 1957 six field experiments were started in Skåne (the southernmost county in Sweden) to study the effects of different cropping systems and fertiliser regimes (Fig. 2). The field experiments were designated the Swedish Long-Term Fertility Experiments (The SLTF experiments in the following). In 1963 and 1966 six additional experiments with principally the same design were established. Today, 10 of the originally 12 experimental sites remain.

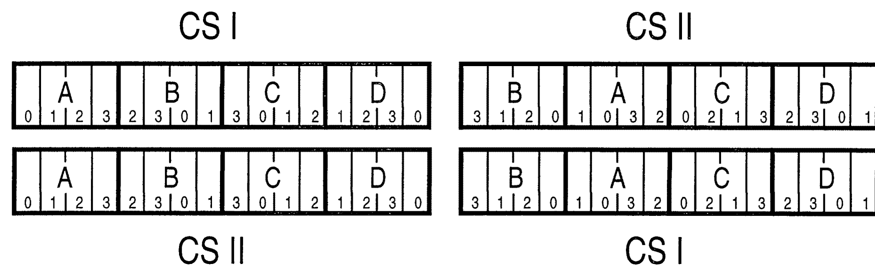

Fig 2. Design of the Swedish Long-Term Fertility Experiment. CS I and CS II denote cropping system with and without livestock, respectively. A, B, C and D indicate different PK fertiliser rates. A: no PK fertiliser, D: high rate of P and K. 0, 1, 2 and 3 indicates different N fertiliser rates. 0: no N fertiliser, 3: high fertiliser rate.

The three experimental sites in the county of Skåne, Orup, Örja and Ekebo, were chosen for the studies in papers **I**, **II** and **III** (Fig. 3). The basic characteristics of the soil at the three sites are summarised in Table 1. Orup and Ekebo are considered as less fertile sites, while Örja is regarded as highly fertile. In each experiment, two cropping systems (CS) are compared. CS I simulates cropping with livestock and CS II simulates cropping without livestock. The crop rotation in CS I encompasses winter wheat (*Triticum aestivum*), sugar beets (*Beta vulgaris*), barley (*Hordeum distichon*) undersown with clover (*Trifolium pratense*) and grass (*Festuca pratensis* and *Lolium perenne*), and one year of clover grass ley. The crop residues are removed in CS I, and once every fourth year, 20 tonnes of farmyard manure are applied between the winter wheat and sugarbeet crops. In CS II no farmyard manure is applied. Instead, the crop residues are left to be ploughed in and spring rape replaces the ley. Moreover, within each cropping system, four PK rates are applied and within each PK rate, four nitrogen rates are applied (Fig. 2). Hence, the experiments are split-split plot experiments with cropping system as main plot, PK rate as sub-plot and N rate as sub-sub-plot. For the studies in this thesis, plots representing both cropping systems, all PK rates and two of the four N rates (unfertilised designated zero N in the following, and 100 kg N ha^{-1} $year^{-1}$ designated N fertilised in the following) were sampled. The SLTF experiments have been presented in detail by, *e.g.* Carlgren & Mattsson (2001).

Table 1. *Some soil characteristics of the sites Orup, Örja and Ekebo*

Site	C_{org} (%)	N_{tot} (%)	C_{org}:N_{tot} ratio	Clay (%)	pH_{H2O}	$CaCO_3$ (%)
Orup	2.4	0.23	10.6	12	5.3	0
Örja	1.4	0.14	10.0	23	7.0	0.06
Ekebo	2.4	0.16	14.9	18	6.6	0

After Kirchmann & Eriksson (1993) and Kirchmann *et al.* (1999)

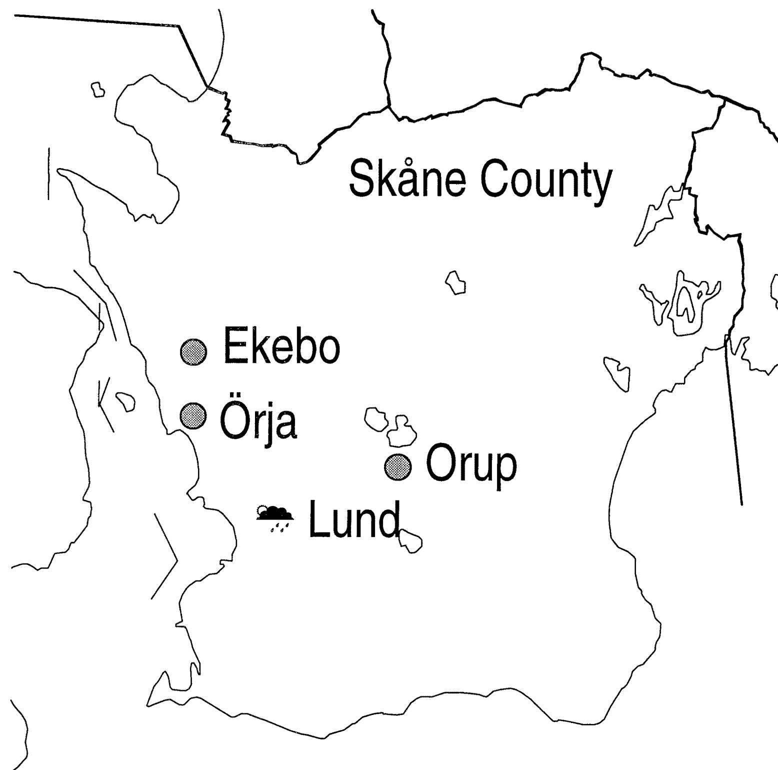

Fig 3. Location of the experimental sites Orup, Örja and Ekebo. The whether data presented in paper **III** were recorded in the city of Lund.

Applied statistical models in the SLTF experiments

Two different statistical models have been applied to test for significant differences between treatments in the SLTF experiment. Normally, in split-plot experiments, the statistics used to test for significant differences between the effect of treatments make use of the statistical interaction between main plot and block as an error term. A significant effect of the treatments applied in the sub-plots is tested using the model error as an error term. Consequently, it becomes more difficult to show significant effects of the main plot treatment compared with the sub-plot treatment. In the case of the SLTF experiments, the testing of treatment effects and differences between treatments is obscured due to a systematic localisation of the plots for PK and N treatments (Fig. 2). Cox (1951) and Seeger (1977) proposed that the treatment effect model should be complemented by a polynomial in, *e.g.* row and column position, to adjust for a systematic design. In the present studies, a polynomial describing the row and column position of each plot and interaction effects between row and column position was inserted into the ANOVA model for evaluation of treatment effects. Variation in data caused by underlying gradients in the experiment will be reduced by the polynomial and the

residual variation can be attributed to treatment effects or random effects. By including the polynomial in the model, the model is transformed from an ANOVA model to an ANCOVA model. Consequently, a general linear model procedure was applied.

If main plot effects are tested with main plot error, at the same time as the polynomial is included in the model, the test for significant treatment effects might be too conservative, since the polynomial occupies 15-20% of the available degrees of freedom in the model. The polynomial revokes the importance of the geographical plot position and thereby in part the split-split-plot design of the experiment, meaning that each plot can be assumed to have received its combination of treatments independently from the other plots. Therefore, it can be rational to test main plot error with model error as an error term, as done in paper **I.** On the other hand, there is a risk that the polynomial reduces the model error, so that the test promises too much if the main plots are tested against model error. In order to find a balance between the influence of the polynomial and the influence of the split-split-plot design, the procedure Mixed (SAS Institute Inc. 1999) was used in papers **II** and **III**, in which an analysis of the covariance structure determines the error term to be used. The treatmentwise mean values are given as least square means, which means that the mean value is adjusted for the polynomial and, hence, should represent an ideal value recorded in the geographic centre of the field experiment. Since the experiment is systematic, but not symmetric regarding the positions of the P and N fertiliser treatments, the mean values for treatment effects (Table 2 in **II** and **III**) might deviate from the arithmetic mean values.

Relations between variables in the SLTF experiment were outlined with simple correlations (**II** and **III**), multiple linear regression (**II**), partial regression (**II**, **III**) and principal components analysis (**I**). Principal components analysis is a multivariate technique, where the covariance or correlation matrix is used to calculate linear combinations (principal components or PCs) of the original data. The idea is that a large number of variables can be reduced to a few PCs representing the main variation in the data set.

The variables (microbial, chemical and yield variables) in this thesis can not be expected to be normally distributed, due to the fixed treatment factors in the field experiment, and the correlations should therefore be interpreted with more caution than the significance levels suggest. Subjectively, a significance level of 1% could probably be regarded as satisfactory.

The short-term incubation experiment

In paper **IV**, the transition between active and dormant microbial states in soil was studied as a function of substrate availability. Three soils were chosen for this purpose: One heavy clay soil (Ekhaga) and two sandy soils (Kungshamn and Ulleråker). Before the start of the incubation, soil portions of 25 g were weighed into plastic jars, and the water content was adjusted to 60% of WHC. Subsequently, the jars were sealed with plastic lids, grouped into 5 groups, and were left to pre-incubate at 20 ºC. In each jar, a small vessel containing potassium

hydroxide was placed to trap the carbon dioxide produced during the pre-incubation. In order to simulate a gradient in substrate availability, small amounts of glucose (0.18 to 1.68 g glucose kg^{-1} soil) were mixed into the soils at four different times during a pre-incubation. Three different amounts of glucose were used for each soil in order to induce first-order, zero-order, and growth-associated respiration kinetics. Glucose was mixed into the first group of jars 46 days before the SIR measurement. The glucose treatments for the second, third and fourth groups of jars were performed 27, 12 and 4 days before the SIR measurement, respectively. The fifth group was left as a control group. Hence, at the day for the SIR measurement, the incubation had produced a full factorial experiment with 3 soils × 4 incubation times × 3 glucose concentrations and sufficient controls.

Discussion

Each of the microbial variables tested and evaluated in this thesis performed a unique reaction pattern as a response to the treatments applied in the Swedish Long-Term Fertility (SLTF) experiment. This is evident from a principal components analysis of the microbial variables, where no variables superimposed each other (Figs 1-3 in paper **I**), although SIR, PDA, PAO and Alk-P formed a cluster at Örja. However, these variables were separated in PC 3. The variables are discussed separately in the following in order to stress the uniqueness of, and differences between, them. The main results are given in summary for each variable. Thereafter, the influencing factors and potential usage are discussed and recommendations concerning a proper interpretation of the microbial variables are given.

Basal respiration

- ✓ B-res discriminated between cropping systems (CS I > CS II).
- ✓ B-res was negatively correlated to pH and positively correlated to organic matter content.
- ✓ B-res was generally a weak predictor of yield, but at Örja, the total nitrogen yield in zero N plots is accurately predicted by basal respiration.

In the SLTF experiments the variation in basal respiration (B-res) was primarily an effect of the two cropping systems and only to a minor extent a result of the mineral N fertiliser rate (**I**). The P fertiliser rates did not affect B-res at all. A probable reason for the close connection of B-res to the cropping system was the higher carbon content in the cropping system with livestock, since the ley and farmyard manure had increased the organic carbon more than fertilising with mineral N (**II**). The B-res variable was also better correlated to the organic carbon than to total nitrogen at Orup and Örja. The soil at Ekebo contained a lot of charcoal (principally inert to biological degradation), which decreased the strength of the relationship between B-res and organic carbon. The B-res variable was

negatively correlated to pH. This correlation could also be referred back to an effect of the cropping systems, since cropping system with livestock had a lower pH.

According to several studies, basal respiration is closely and linearly related to the labile organic matter in soil (Alvarez *et al.* 1998; Friedel *et al.* 1996; Janzen *et al.* 1992). Often, basal respiration is also linearly related to total amounts of organic carbon (Insam 1990; Wirth 1999), but then organic carbon is expected to co-vary with a high content of readily metabolizable carbon (Haynes & Tregurtha 1999). Mineral N fertilisation is reported to increase carbon mineralisation (Lovell & Hatch 1998; Pankhurst *et al.* 1995). However, different crop rotations (Franzluebbers *et al.* 1995) and different grazing management practices on meadows (Bardgett & McAlister 1999) have a greater impact on basal respiration than N fertilisers. Basal respiration is also affected by the type of soil tillage, depending on the disruptive capability of the tillage device (Franzluebbers *et al.* 1999). Disruption of soil aggregates increases the exposure of physically protected carbon to microbial attack (Dexter *et al.* 1999). In spatial investigations, it was in some cases not possible to relate basal respiration to any measured soil variable (Robertson *et al.* 1997; Röver & Kaiser 1999), and P fertilisation generally has no effect on basal respiration (Bolan *et al.* 1996).

The findings referred to above suggest that temporary carbon sources like root exudates may affect B-res. The high values recorded for the B-res variable in CS I were probably promoted by both a high content of organic carbon in the soil, and by carbon originating from root exudates. In 1997, the year of soil sampling, there was an important difference between the cropping systems. The barley crop, which was harvested a few weeks before the sampling, was undersown with a clover-grass ley in the cropping system with livestock. The ley probably produced enough exudates to raise the respiration rate significantly above the rate in the system without an undersown ley (CS II). In the latter system, only stubble and chopped straw were available as a food base for the microbes. Cheng *et al.* (1996) have shown that the basal respiration decreases shortly after removal of roots from the soil, whereas the biomass measured as SIR remained constant. In the incubation experiment (**IV**), the basal respiration in the glucose-amended soils decreased as a function of time, but was still significantly higher than the respiration rate in the control soils after 46 days. Therefore, the effect of exudate input to soil can be expected to remain for a few weeks, meaning that the effect of a single year on the basal respiration rate may override the long-term trend. The difficulties to distinguish between short-term and long-term effects implies that B-res is not a proper indicator of long-term fertility or of a sustainable production level. This was evident in the SLTF experiments where B-res (**III**) was not as good when correlated to total nitrogen yield as to the respiration rate under glucose saturated conditions (viz. SIR).

Substrate-induced respiration

- ✓ SIR discriminated between cropping systems (CS I > CS II) and N fertiliser regimes (N fertilised > zero N). At the P-poor site Orup, SIR also discriminated between P fertiliser regimes (high P rate > low P rate > zero P).
- ✓ SIR was generally more strongly related to the content of organic matter compared with basal respiration. At Orup, SIR was also related to phosphorus concentrations in soil.
- ✓ SIR was a highly accurate predictor of grain yield and total nitrogen yield in the zero N plots. At Orup, SIR also predicted ley yield.

In the SLTF experiments, SIR reacted principally in the same manner as the basal respiration, however with some important differences (**I**, **II**). In this thesis, SIR was relatively strongly related to the organic matter in the soil. These relationships were induced by the cropping system and N fertiliser regimes at the P-rich sites Örja and Ekebo, while at the P-poor site Orup, the relation was also induced by P fertiliser. Probably, available plant nutrients governed the amount of plant residues returned to the soil in the SLTF experiments, which in turn determined the SIR level. As shown by, *e.g.* Alvarez & Alvarez (2000), the available amounts of plant residues may affect the glucose responsive biomass. Hence, if used as a soil quality indicator, SIR should be regarded as a long-term integrator of the carbon flow in the soil system, rather than as an indicator of a specific management practice.

Persson & Otabbong (1994) pointed out that the organic matter is an important determinant of production level and thus soil fertility. This is especially true in low-input systems where most of the plant nutrient supply originates from the mineralisation of organic matter. In the SLTF experiments, the organic carbon and total nitrogen correlated to yield level in systems where no mineral N fertiliser had been applied, *i.e.* the zero N plots (**III**). However, no variable could compete with SIR as the best predictor of yield level in the zero N plots. This was especially true for the total N yield during the whole crop cycle. The capacity of SIR to predict yield level has also been reported by, *e.g.* Insam *et al.* (1991).

As shown above, SIR can function as an indicator of the fertility in low input systems. The efforts to create sustainable farming systems will probably result in agricultural systems heavily dependent on organic fertilisers and consequently on the activity of the soil microorganisms. Such systems include ecological / organic farming and systems relying on urban organic waste products. In Sweden, the goal of ecological farming is to increase its share of the total production volume within the next eight years to 30% (Andersson 2001). To assess the production level in such systems, SIR might be a valuable tool. However, as a fertility indicator, SIR must be regarded as an indicator of the soil management history, and not as a causal agent. Insam *et al.* (1991) suggested that a relationship between SIR and crop yield should be expected since yield level is positively linked to C input to soil. When yield level is decoupled from C input the relationship expires (Carter *et al.* 1999). Waksman & Starkey (1924) commented upon this: “A soil composed of

little else than quartz with available elements essential to plant growth may support plants temporarily and still lack any abundant microbial flora. In such cases the microbiological activities and soil productivity are not correlated but the first may be better considered as forecasting the future possibilities of the soil." Hence, SIR should be informative on the basic fertility level even in N-fertilised systems in the sense that it will give an indication of the yield level if N fertilisation is omitted.

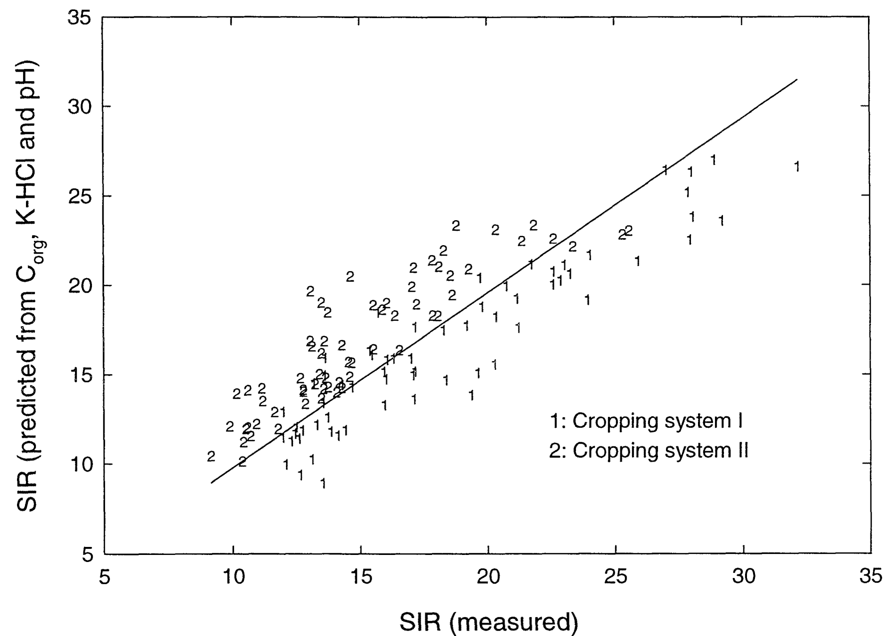

Fig 4. Measured and predicted SIR data (mg CO_2 kg^{-1} h^{-1}) in top-soil from eight sites belonging to the Swedish Long-Term Fertility Experiment. The prediction of SIR was made from an MLR model where organic carbon, pH and K-HCl were used as independent variables.

The size of the microbial biomass is not only dependent on management practices, but also on site specific factors such as soil texture, total organic carbon and precipitation (Gödde & Conrad 2000; Insam 1990; Weigand *et al.* 1995). In order to utilise the SIR variable as a general fertility indicator, a reference model must be developed that adjusts the SIR value according to the influence of site specific factors (Oberholzer & Höper 2001). In Fig. 4, SIR data from eight sites of the SLTF experiments are predicted by an MLR model with organic carbon, pH and potassium dissolvable in hydrochloric acid (K-HCl) as independent regressors. Only soils from N fertilised plots (100 kg N ha^{-1} yr^{-1}) are used in the model. The combination of chemical soil variables was calculated to be the best for describing SIR from a selection of eight different chemical soil variables. K-HCl has been shown to be strongly related to clay content (Wiklander 1976), and K-HCl can therefore be regarded as a substitute for the clay content in the model. Data for clay content was not available when the model was constructed. However, due to

the use of different PK fertiliser levels in the experiments, K-HCl and the clay content were probably not perfectly matched. In any case, the effect of the cropping system is not fully modelled, as seen from the separation of the cropping systems, CS I and CS II being located below and above the regression line, respectively, which implies that the carbon quality differs between the systems. Fig. 4 illustrates the potential of SIR to be an indicator of organic matter quality, while at the same time it demonstrates the practical application of the reference model idea. Soil samples located below the regression line have been gathered from plots managed to conserve soil fertility, whereas the samples located above the line originates from plots where soil fertility might be put at risk. In line with the model described here, Anderson & Domsch (1989) reported that SIR data was strongly related to organic matter in some differently managed farming systems, but the derived intercept of the regression line differed between the systems.

Direct measurements of organic carbon and clay content are rather expensive. Near infrared reflectance (NIR) technique has been shown to predict both carbon and clay content in soil (Janik *et al.* 1998; Stenberg 1997; Zhang *et al.* 1992). Hence, a cheap reference model might be produced and applied by use of NIR technique, making NIR and SIR measurements a powerful combination when assessing soil fertility.

Specific growth rate for aerobic microorganisms

- ✓ μ_{res} discriminated between cropping systems (CS II > CS I) and P fertiliser regimes (P fertilised > zero P).
- ✓ μ_{res} gave a fairly good prediction of yield in nitrogen fertilised plots.

The substrate amendment that induces the SIR measurement is followed by a period of approximately 24 hours characterised by exponential growth of a sub-fraction of the whole glucose responding biomass. The specific growth rate is derived as an additional parameter from the fitting procedure of the carbon dioxide production curve (Eq. [8]). The μ_{res} variable should be considered as an average value for all species-specific growth rates encountered in the soil. The μ_{res} variable was sensitive to cropping system and to P fertiliser rate at all sites, but totally indifferent to N fertiliser rate (**I**). The higher value in the cropping system without livestock (CS II), *i.e.* the less fertile system according to Fig. 4, might be an effect of a selection of oligotrophic microorganisms in these systems. Sato *et al.* (1995) reported that oligotrophic bacteria isolated from soil increase their growth rate more than eutrophic soil bacteria when supplied with glucose. Another factor that may affect the specific growth rate is pH. The pH value was generally higher in CS II. A low pH will result in a decreased bacterial:fungal ratio (Bolan *et al.* 1996) and difficulties to maintain a near-neutral pH in the cytoplasma (Madigan *et al.* 2000), both of these factors that will affect the growth rate. The positive response to P fertilisation might originate in an increased P storage in the cells as an effect of the P fertilisation. Both fungi (Wells & Boddy 1995) and bacteria (Khoshmanesh *et al.* 2002) are known to accumulate P during excess supply of P.

In some cases, the μ_{res} variable predicted yield in the N fertilised soils (**III**). The predictive capacity was closely associated with its relationships to P and K. The μ_{res} variable was curvilinearly related to plant-available P, as was the Max-P variable (see below). However, the scatter for the relation between μ_{res} and plant-available P was larger than for the relation between Max-P and plant-available P, partly due to the differences between the cropping system, and partly due to random scatter. Thus, the μ_{res} variable gives a very rough indication of available P in the soil and is not recommended to be used as a measurement of plant-available P. However, for the sugarbeet and rapeseed yields at Örja and barley yield at Orup in N fertilised plots, μ_{res} was the best predictor, probably due to its double sensitivity to both cropping system and P fertiliser rate.

Nutrient limited respiration rates

- ✓ Max-P discriminated between phosphorus fertiliser rates (P fertilised > zero P), whereas Max-N did not discriminate adequately between nitrogen fertiliser rates.
- ✓ Max-P was curvilinearly related to plant-available phosphorus (P-AL).
- ✓ Max-P performed just as well as the chemical P variables as a predictor of yield in nitrogen fertilised plots. Max-N was generally a poor yield predictor.

In the SLTF experiment Max-P was by far the best microbial discriminator of the different P fertilisation rates (**I**), and Max-P was also strongly related to plant available P as long as the P concentration was below 0.1 g kg^{-1} soil (**II**). Above this limit the glucose in the assay will become limiting for growth and consequently for respiration rate, rather than the P, causing a curvilinear relation (Fig. 5). As a yield level predictor, Max-P could be expected to perform better than the chemical variables, since yield level probably is limited by other factors than P in the heavily fertilised plots. However, in most cases, Max-P only performed on the level of the chemical variables (**III**).

The simplicity of the idea behind Max-P and Max-N could make it attractive as an alternative assay for determinations of available P and N in soil. If a respirometer is available, only a few cheap chemicals, like hydroxide, glucose and N and P salts, are necessary for the assay. However, the dependence of growth as a response to the glucose addition results in certain drawbacks for the method when applying it on soils with large variations in P and/or clay content. A proper determination of Max-P in soils where plant-available P exceeds 0.1 g kg^{-1} soil can be attained by increasing the amount of glucose added to the soil, but too much glucose will induce osmotic stress, which might impede the growth. In Fig. 5, Max-P values from yet another of the SLTF sites (Kungsängen, marked with ▼) are added to the graph. The low Max-P values can probably be explained by the fact that Kungsängen soil is a heavy clay soil. The clay may be limiting for the transport rate of glucose in the soil, and hence the growth rate, rather than the amount of glucose itself. Moreover, when the number of active/growing organisms is very low (see below), a substantial amount of the glucose will be consumed by

the non-growing microbes before the number of growing microbes have increased to become the dominating part of the microflora. All these limitations for growth-associated activity probably render P determinations made by bioassays like Max-P semi-quantitative.

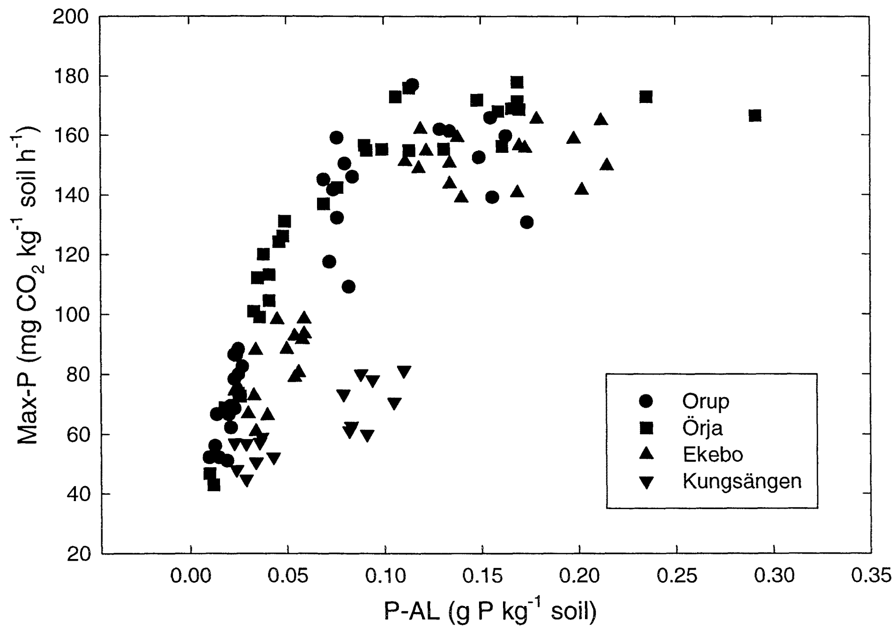

Fig 5. The relationship between plant-available P (P-AL) and Max-P is curvilinear, since the amount of glucose will be limiting for the respiration rate when the concentration of plant-available P exceeds 0.1 g kg^{-1} soil. In some cases, additional factors like a restricted transport rate or low pH may impede microbial growth, as exemplified by the Kungsängen soil (clay).

The Max-N assay showed that available N was limiting for microbial growth at all sites and in all treatment combinations (**I**). Max-N could not discriminate between the two N levels as well as Max-P could differentiate between the P levels. Max-N probably mirrored plant-available mineral N, which was not measured in the present investigations. Mineral soil N is mainly governed by short-term dynamics, and therefore Max-N can not be used as a long-term fertility indicator. The assay may be used as an alternative assay for analysing plant available N in connection with spring farming operations. However, the same drawbacks that were listed for the Max-P assay, also hold true for the Max-N assay. Aldén *et al.* (2001) reported that the rate of thymidine incorporation into bacterial cells is indicative of C, N or P limitations in the soil. The determination of thymidine incorporation is not dependent on carbon dioxide evolution and, hence, some of the limitations encountered for the Max-P and Max-N assays might be overcome by using the thymidine incorporation method for determination of limiting amounts of available N and P.

Active and dormant parts of the biomass

The glucose responsive biomass measured as SIR can be partitioned into a growing and a non-growing part according to equation [8]. In the SLTF experiment the growing part varied between 4-30% of the total SIR with a median value of 10%. However, the determination of SIR in the SLTF experiments involved two shortcomings that probably affected the result of the curve-fitting procedure, *i.e.* no determination of the salt effect (described in **IV**), and imprecise estimations of the time for glucose addition. These omissions did not affect the estimation of total SIR, although the partitioning of the biomass into growing and non-growing microorganisms became imprecise. However, the median value given above for the growing part is probably accurate due to the large number of samples (n = 96). The uncertainties in the estimates of the growing and non-growing microorganisms mentioned above were the reason for not evaluating the estimates of the sub-populations in the SLTF experiments. Instead, the dynamics of the transitions between the growing and non-growing states were outlined in a separate incubation experiment (**IV**) where three soils were incubated for four different times with three different amounts of glucose additions. Five main conclusions commented upon in the following text could be drawn from the incubation experiment.

✓ The microbial specific activity is regulated as being dormant or fully active.

Initially, a model assuming a lag phase was used to mathematically describe the SIR curves derived from the respirometer (Nordgren *et al.* 1988; Palmborg & Nordgren 1993). In 1995, Stenström & Stenberg (1995) described the kinetic model used in the present investigation. A full presentation of the theory behind the model is given by Stenström *et al.* (1998). The assumption in the model is that the biomass can be divided into a growing and a non-growing sub-population. The growing biomass is assumed to start to grow virtually at the time of glucose amendment, and therefore the lag phase was omitted from the model. In a starvation and reactivation experiment with *lux*-marked *Pseudomonas fluorescens* in soil (Van Dyke & Prosser 1998), the bacteria recovered their luminescence immediately after substrate addition, which supports the opinion that the lag phase is negligible in normal cases. Only when the soil was air-dried for two days was a lag phase detected, that lasted 5-10 hours. One of the most profound results in paper **IV** was that the growing biomass could be regarded as being fully active and the non-growing biomass as being dormant immediately before the SIR measurement. Therefore, the two sub-populations are called active and dormant in the following.

Independently of Stenström & Stenberg (1995), Russian scientists have published the same model (Blagodatsky *et al.* 2000; Panikov & Sizova 1996) to describe the kinetics of respiration curves after glucose amendments of soils. They give an alternative interpretation of the kinetic model. Instead of associating the two main parameters in the model with two functionally different sub-populations

of the biomass, the authors argued that the model parameters described two different groups of respiratory enzymes actually present in each cell. One group was considered to carry out energy-spilling oxidative reactions uncoupled from ATP generation (the dormant biomass in our interpretation), and the other to carry out productive substrate oxidation (the active biomass in our interpretation). However, in the SLTF experiment, 90% of the respiration rate was found associated with the former type of respiration, and it seems unlikely that 90% of the respiration in each cell is carried out in vain in the soil. The assumption that the model describes two different sub-populations is, in our opinion, a more plausible interpretation, especially when considering several other "postulated assumptions" presented by the Russians that underpinned their interpretation of the two sub-populations. According to them, each growing microorganism is composed of two main fractions, the amounts of which, by some mysterious mechanism, always must balance each other. Moreover, the equation was based on premises similar to those upon which SIR-data generally are analysed today, *e.g.* that all substrate-responsive microorganisms start to grow after the substrate addition and that growth is preceded by a more or less pronounced lag phase.

✓ The distribution between active and dormant microorganisms is determined by carbon availability and the history of the soil.

The supplementation of the soils with small amounts of glucose induced a transition of the dormant biomass into an active stage. The low and medium glucose amounts did not increase SIR. The largest amounts of active biomass were found in the soils that most recently (4 days before SIR) had received the highest rate of glucose, and the smallest amounts of active biomass, besides the controls, were found in soils treated with the lowest glucose rate at the start of the incubation (46 days before SIR). Marstorp & Witter (1999) reported that addition of small amounts of glucose to soil did not seem to increase dsDNA in the soil, which implies that growth of the microbial biomass was not induced by the glucose addition. The activation of the microbial biomass by different amendments or treatments has been reported by a number of authors. De Nobili *et al.* (2001) observed that the biomass was triggered into activation by trace amounts of low-molecular substrates. In a tillage experiment, Kandeler *et al.* (1999) observed higher enzyme activities in the reduced and minimum tillage treatments compared with conventional tillage, but the biomass, measured as SIR, was the same in all plots. Another example is provided by Cheng *et al.* (1996). They found that the basal respiration : SIR ratio increased in the rhizosphere as a function of soluble organic carbon in a sandy clay loam with a SIR value similar to that in our sandy soils. At a certain threshold value of the soluble organic carbon (0.1 mg C g^{-1} of soil dw) the basal respiration : SIR ratio reached 1. In our investigation, corresponding amounts of glucose (0.11 mg C g^{-1} of soil dw) induced zero order kinetics in the sandy soils, which in turn induced a complete transformation of the dormant microbes to an active stage. This suggests that exudates in the rhizosphere are capable of promoting a full transformation of the microbes closest to the root into active microorganisms, without necessarily increasing the biomass.

The respiration rate of the active biomass in the sandy control soils in our investigation was identical to the basal respiration rate. Probably, the basal respiration sets the lower limit to which the active fraction extrapolates during prolonged incubation times. However, in the heavy clay soil the respiration rate of the active biomass was eight times higher than the basal respiration. In this soil, the transition from an active to a dormant biomass was also much slower than in the two sandy soils. The clayey soil is regarded as a very fertile soil with a large amount of easily degradable organic material, which can support a large active biomass.

✓ The glucose-induced transition from dormant to active is very fast, but growth of active microorganisms is probably inhibited until all glucose-responsive microorganisms have been activated.

It has been shown in another experiment (unpublished) that the transition from the dormant to the active state occurred within one day. The fact that glucose-induced basal respiration directly corresponds to glucose-induced active biomass (Fig. 5 in paper **IV**) strongly suggests that the transition occurs just as fast as the change in respiration rate after addition of glucose, *i.e.* within two hours. Growth will then only occur when the whole biomass is transformed to the active stage. Therefore, the transition from the dormant to the active state can be considered as the first phase of the growth process. A complete transition to the active stage of the whole biomass had to occur before growth could take place. The complete growth inhibition that precedes growth suggests that control mechanisms are acting on the community level, *e.g.* competition (Hoitink & Boehm 1999; Metting 1992), fungistasis (Bruehl 1976), glucose-sensing (Surette & Bassler 1998) and quorum-sensing mechanisms (Greenberg 1999; Lazazzera 2000).

When the soil is amended with a large glucose amount, as the one used for the SIR measurement, the active biomass grows, but the transition from dormant to active is apparently inhibited. Obviously, the reaction of the biomass to a high glucose amount (SIR) is quite different from the reaction to a low one. It is known that substrate-limited conditions induce a high substrate uptake potential (Höfle 1983), which may render microbial cells sensitive to high substrate concentrations. Barja & Núñez (1999) speculated that membrane permeases, that allow substrate molecules to pass into the cytoplasma, may be blocked by an excess of extracellular sugars. Therefore, the cells are unable to maintain the intracellular substrate concentration, which induces an inhibitory effect on microbial growth.

✓ The back-transition from active to dormant is slow, with first-order half-lives in the range 12 – 70 days for the three soils studied.

In the treatments that received the largest glucose amount during incubation, SIR increased slightly, but soon declined to the SIR level of the untreated soil, where it

remained. Ritz *et al.* (1992) showed that microbial biomass formed from sugar amendments decreases to its normal value within 25 days, *i.e.* virtually the same time interval as in the incubation experiment. As long as SIR was elevated in the incubation experiment, the active biomass constituted 100% of SIR. Throughout the remaining time of the incubation, SIR persisted, but the active biomass continued to decline with first-order half-lives that were strongly dependent on the soil type. In a soil fertility perspective, the transition between the active and dormant biomasses has a very fast dynamics, and the distribution between them is probably inadequate as an indication of the fertility in a long-term perspective. Moreover, an assessment of the soil status from the active biomass : SIR ratio will not make sense without information on the half-life time. However, estimation of the half-life time is a tedious task when performed as done in paper **IV**, and there is a need to identify which soil factors that determine the half-life time in order to make accurate predictions. In the control soils, SIR ranked the soils in the same order as the first-order half-lives of the back transition, which may imply that the transition of the biomass has an inherent inertia that is proportional to the size of the biomass.

The glucose-induced basal respiration soon declined and within two days approached the uninduced basal respiration, while the glucose-induced active biomass declined much slower, as discussed previously. This discrepancy, together with the assumption that the biomass is regulated as being either dormant or fully active, would mean that the back-transition occurs as a two-step process. First, the active biomass is turned into a potentially active biomass due to the glucose shortage. Thereafter, the potentially active biomass is, gradually, transferred into the dormant state. Meanwhile, there is a third fraction of the active biomass, which performs the genuine basal respiration using the soil organic carbon. Since the active biomass seems to approach the basal respiration, the glucose-induced active biomass will eventually disappear through the transition from the active to the dormant state.

Kjelleberg *et al.* (1993) described how carbon starvation induces a highly ordered response that includes a number of physiological changes in the bacterial cell of a *Vibrio* strain. The process has similarities with the spore formation process. The result of the changes is that the cell is turned into an ultramicrocell. This cell can sustain prolonged periods of environmental stress, but also has the capacity to respond instantaneously to substrate additions by initialising growth. The dwarf cells in soil, described by, *e.g.* Bakken (1997), are probably identical to starved ultramicrocells. The dwarf cells constitute a majority of the number of cells in the soil, but their share of the total energy flux in soil is small. For *Vibrio*, it was found that the degradation of ribosomal RNA was a very slow process. The capacity of starved cells to recover appeared to coincide with the amount of ribosomal RNA (Kjelleberg *et al.* 1993). This suggests that the back-transition of the glucose-induced active biomass in the incubation experiment mirrors the degradation of ribosomal particles in the cells and, hence, the readiness for growth.

- ✓ The results suggest that the normal dynamics of the soil microbial biomass involves oscillation between active and dormant physiological states, while significant growth occurs only at substantial substrate amendments.

The amount of glucose needed for the complete transformation of the biomass from a dormant to an active stage in the clay soil (0.84 g kg^{-1} soil) corresponds to 25-50% of the total rhizodeposition during a whole year (Swinnen *et al.* 1995). The microbial affinity for glucose is generally high (Hopkins & Shiel 1996), making glucose a high quality substrate, which disappears within a few days of incubation in soil (Ladd *et al.* 1992; Sharabi & Bartha 1993). This suggests that the normal additions of carbonaceous material to the soil are not enough to induce the complete transformation apparently needed for the initiation of growth. A number of carbon flow models for the rhizosphere (Toal *et al.* 2000) assumes that the growth rate of bacteria in the soil is a function of the available carbon according to the Monod equation. Our data suggest that the carbon exuded by the roots is not enough to induce growth competence in the microflora, and consequently the growth rate parameter is irrelevant.

However, the conclusion is probably only relevant for low-molecular substrates that are similar to glucose. Glucose is a substrate that can be used by a large majority of the microbes in the soil (Hanson *et al.* 1999). Most of the carbonaceous material that is added to the soil is chemically complex and requires special pathways for degradation. Hence, degradation is performed by a succession of microbial populations, each specialised in perhaps only one or two steps of the mineralisation process. Since each sub-population only forms a minority of the whole biomass, the material added to the soil may very well provide for both the initiation and the continuation of growth for the microbes currently active. As mentioned above, growth inhibition during the glucose-induced transition of the dormant biomass to an active state probably involves chemical signalling on a community level. It is possible that such signalling will occur only when generally used low molecular substances are added, and therefore the inhibitory effect is expelled when the soil is supplied with complex substrates, or substrates that can be used by special groups only. The constitutively active biomass that performs the basal respiration can, therefore, alone capture the resource, and can provide for reinforcement by growth without being hampered by the prerequisite of growth induction. In addition, complex substrates are normally added to soil as relatively large particles (chopped straw, tops, *etc.*), which require growth for the sequestration of the material as a substrate.

Potential denitrification activity

- ✓ PDA provided no distinct discrimination between the treatments in the SLTF experiments.
- ✓ PDA was mainly related to total nitrogen (Orup and Örja) and total phosphorus (Orup), but was best described by combining total nitrogen with total phosphorus (Orup) or pH (Örja) in MLR models.

- ✓ PDA was generally a poor yield predictor, except for the rapeseed yield at Örja which was accurately predicted by the potential denitrification through a negative relationship.

Depending on the site, the treatments in the SLTF experiments induced differences in potential denitrification activity (PDA), pointing in opposite directions (**I**). For example, the activity was higher in CS II at Orup, whereas it was higher in CS I at Örja. The highly site-specific reactions of PDA suggest that the variable can not distinctly identify specific management practices, and in this respect the variable is not useful as a general indicator of soil quality.

PDA was closer related to total nitrogen than to organic carbon (**II**). According to Tiedje (1988), redox conditions and nitrate supply in soil are more important in regulating denitrification than is carbon supply. Together, these three factors are thought to be the major environmental regulators of denitrification in soil. The best MLR models for prediction of PDA using two chemical variables at Orup and Örja both contained total nitrogen (**II**). The second variable at Orup was total P, which can be explained by the high influence of P on plant growth at Orup and, consequently, the return of plant residues. The importance of labile carbon and substrate supply for the denitrification activity has attracted attention in other investigations (Katz *et al.* 1985; Koops *et al.* 1996), and can be simply explained by the size of the microbial biomass (Drury *et al.* 1998; Palma *et al.* 1997).

The second variable in the MLR model at Örja was pH (**II**). Other studies have shown the strong influence of pH on denitrification activity (Simek *et al.* 2000; Simek & Kalcik 1998). Regardless of former management history, the optimum pH for denitrification seems to be around 7 (Simek & Hopkins 1999), which may stem from the fact that denitrification is performed nearly exclusively by bacteria. In the SLTF experiment, PDA was related to pH, except for the soils that had not received any nitrogen (organic or inorganic) during the whole experimental period. The latter soils had low PDA values in spite of a high pH. Probably, the low biomass in these soils as indicated by the low SIR, made these soils fall out of the general trend for the dependence on pH. In fact, by replacing total N with SIR in the MLR model at Örja, the model (pH and SIR) performed even better, as seen by an increase in R^2 from 0.66 to 0.72. However, by replacing pH in this model with potential ammonium oxidation, the best two-variable model possible was obtained with an R^2 reaching 0.77. At least in clayey soils, denitrification therefore seems to depend on the size of the biomass as indicated by SIR and the supply of nitrate as indicated by the potential ammonium oxidation assay.

PDA can not be regarded as a yield predictor in the SLTF experiments, except for the rapeseed yield at Örja (**III**). The rapeseed was grown the year after soil sampling, so the negative correlation could be interpreted as a bacterially mediated withdrawal of nitrogen from the subsequent plants. However, PDA was closely related to organic carbon in this case ($r = 0.90$), and by means of a partial regression analysis it was shown that the organic carbon and PDA could explain the rapeseed yield level equally well. The carbon supplies the microbes with energy to perform both immobilisation and denitrification, both leading to a

depletion of easily available nitrogen. It may be important to identify which of these two processes that dominates when assessing, *e.g.* the effect of the preceding crop, since immobilised nitrogen may constitute a resource for the next crop, whereas denitrification permanently depletes the system of nitrogen. However, in this study it was not possible to distinguish between the two alternative mechanisms.

In the PDA assay seven head-space samples are withdrawn during a 3 h-incubation period. According to Robinson (1985), at least eight sampling points are recommended for accurate estimation of the parameters in two-parameter non-linear models. This indicates that the accuracy of the test may be increased if some more samplings are performed. However, since each data point is expensive in this test due to the dependence of a gas chromatograph, an extended sampling could only be recommended if the accuracy of the test will increase substantially.

Specific growth rate for denitrifying bacteria

- ✓ μ_{PDA} discriminated between cropping systems (CS II > CS I)
- ✓ μ_{PDA} predicted total nitrogen yield fairly well, but the predictive capacity could be explained as a pH effect

Like the specific growth rate of aerobic respiring organisms as determined by the SIR assay, the specific growth rate for denitrifying bacteria showed higher values in the cropping system without livestock (**I**). This was the only trend that was evident for all three sites. Since no statistical interactions were found between the cropping system factor and the mineral fertiliser rates, the μ_{PDA} variable might be useful for discriminating between different cropping systems. The higher pH in CS II could be one of the reasons for the higher growth rate in this cropping system. However, specific growth rate was rather poorly correlated to pH at Orup and Ekebo, and not at all at Örja. The inability of the μ_{PDA} variable to distinguish between P fertiliser rates, as could be done by the specific growth for the SIR assay (*i.e.* μ_{res}), may suggest that denitrifying bacteria do not accumulate and store excess amounts of P, if available in the environment. That is, however, not a realistic assumption, since denitrifying bacteria are found in most taxonomic groups (Zumft 1992), and P is known to accumulate in some bacteria (Khoshmanesh *et al.* 2002).

Commonly, chloramphenicol is added to the denitrification assay to inhibit growth, and therefore only few reports exist on the response of the μ_{PDA} variable to various environmental conditions. Johansson (1998) reported that the μ_{PDA} variable was highly sensitive to silver amendments, and that it was a ten-fold more sensitive than PDA to silver amendments. Stenberg (1998) showed that the variable was related to pH.

The μ_{PDA} variable repeatedly appeared as a highly significant predictor of yield level in the zero N plots (**III**). In accordance with the assumption above that pH controls the specific growth rate, pH accompanied the specific growth rate as a

predictive variable. Stenberg (1998) reported that the specific growth rate for denitrifying organisms was the best microbial predictor for N yield among a number of microbial variables.

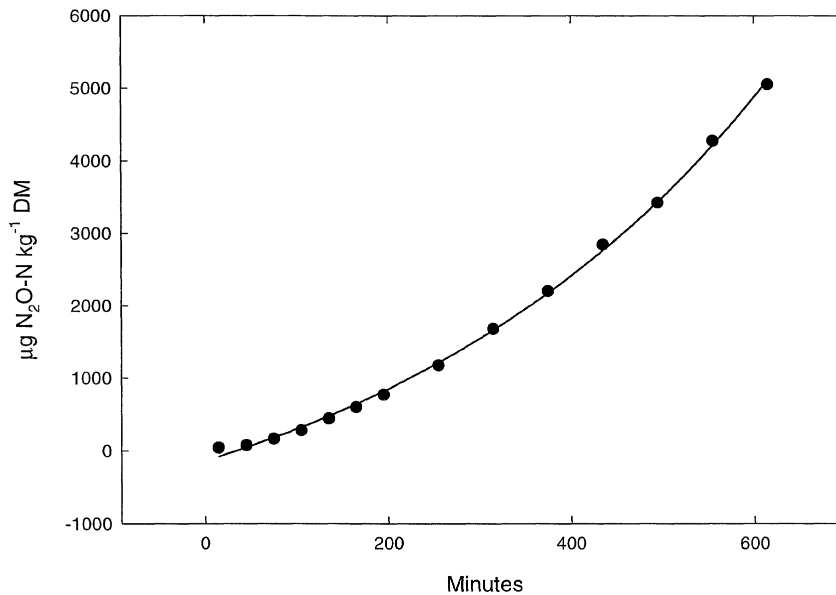

Fig 6. Production of nitrous oxide during a prolonged incubation. The soil originates from site Orup. Data were fitted to Eq. [6], which gave a highly accurate fit ($R^2 = 0.998$).

The applicability of the specific growth rate (μ_{PDA}) as an informative soil variable depends on its correct interpretation. In the denitrification assay used, the accumulation of nitrous oxide is followed after acetylene injection during a 3.5-hour incubation period. Smith & Tiedje (1979) interpreted the resulting curve of nitrous oxide accumulation as biphasic, phase II having a steeper slope than phase I. The curve entered phase II after typically 3-5 hours. A curve similar to theirs was reproduced in a special study using soil from the SLTF experiments where the sampling period was prolonged to 10 hours (Fig. 6). The curve is fitted to Eq [6]. Firestone & Tiedje (1979) showed that the first phase (1-3 hours) of the incubation was dominated by production of N_2 gas, and suggested that phase I reflects the pre-existing physiological state of the soil microflora. Therefore, the slope of phase I should vary with the aeration status of the soil. If correct, the specific growth rate as determined by the denitrification assay should not describe the growth rate, but rather the rate of reactivation of the denitrification enzyme complex during the switch from aerobic to anaerobic conditions at the start of the assay. In the bacterial cell, oxygen regulates both activity and synthesis of denitrifying enzymes. The activity of the enzymes is more sensitive to oxygen inhibition than their corresponding gene expression (Tiedje 1988). Such an explanation would be plausible in the light of the discriminating capacity for the cropping systems, which was only weakly related to the pH.

Klemedtsson (1986) and Petersen *et al.* (1996) have reported that a complete inhibition of the nitrous oxide reductase system can take up to three hours. Perhaps the specific growth rate neither reflects the specific growth rate, nor the rate of the onset of the reducing enzyme, but simply indicates the sensitivity of the enzymes to acetylene blockage. However, the biphasic curve produced without chloramphenicol reported by Smith & Tiedje (1979) was reanalysed by Pell *et al.* (1996). They showed that the whole curve (phase I + phase II) could be described by equation [6], and the bend of the curve should, therefore, be interpreted as a balanced growth without any lag phase. They achieved a curve fit similar to the fit in Fig. 6. Murray & Knowles (1999) evaluated a denitrification assay similar to that used both by Pell *et al.* (1996) and in the present thesis, and concluded that the underlying assumptions of the technique (balanced growth and constant enzyme activity during the whole assay) need to be verified for indigenous denitrifying organisms growing in soil slurries without growth inhibitors. They recommended that chloramphenicol dependent assays should be reconsidered. In any case, it is clear that the true nature of the specific growth rate of denitrifying bacteria in the assay must be elucidated before the μ_{PDA} variable can be used to assess the quality and fertility of soils.

Potential ammonium oxidation activity

- ✓ PAO discriminated mainly between the two N fertiliser rates. Zero N > N fertilised at Orup, and N fertilised > zero N at Örja and Ekebo.
- ✓ PAO was generally strongly related to pH. At Örja, the nitrogen-deprived plots diverged from the relationship between pH and PAO.
- ✓ PAO was a poor yield predictor.

As for denitrification, the treatments at the three sites of the SLTF experiment did not affect the potential ammonium oxidation (PAO) in a well-defined and uniform manner (**I**). A microbial variable will react in opposite ways to a single treatment factor when at least two soil factors affect the microbial variable. The factors that governed PAO in the SLTF experiments were identified as pH and substrate supply (ammonium) (**II**). At Orup, pH alone governed PAO. As a result of a higher pH in unfertilised soils and in the cropping system without livestock, PAO was higher in these soils. The strong influence of pH on ammonium oxidation has been known for many years (Waksman 1927), and the relationship has recently been illustrated by use of multivariate statistics (Gödde & Conrad 2000; Stenberg *et al.* 2000). However, in some cases, pH has been found inadequate to explain the ammonium oxidation potential. Stenberg (1998) reported that ammonium oxidation was related to the ammonium supply rather than to pH on a field where outdoor pigs had been kept two years earlier. The ammonium probably originated from the urine and excreta that the pigs had spread in a characteristic pig-behavioural pattern. The effect of ammonium supply on ammonium oxidation rate has also been shown by, *e.g.* Tabatabai *et al.* (1992), Kandeler & Eder (1993) and Zaman *et al.* (1999). At Örja, the soils that had not received any N either from

mineral fertiliser or from the clover-grass ley, fell below the regression line that described the relation between PAO and pH (Fig. 1 in paper **II**). In the diverging soils, a nitrogen depravation, rather than pH, limited PAO. An MLR model that included both pH and total N in the soil could best describe PAO at Örja, which indicates that pH and total N complement each other when explaining ammonium oxidation in soil (**II**).

PAO performed less well as a yield predictor, with two exceptions (**III**). At Orup, PAO was strongly and negatively related to both total N yield and wheat yield. However, this negative relationship could be fully explained by an effect of the high pH in the zero N soils at this site. Hence, ammonium oxidation is not useful when predicting yield level. The opposite conclusion was drawn in an early study by Burgess (1918) who found nitrification to be closely related to fertility level in Hawaiian soils. However, the nitrification rate was assayed as the nitrate accumulation during 30 days after supplementing the soils with different organic materials (dried blood, alfalfa meal, and fish scraps). Probably, the rate-limiting step in this case was the mineralisation of the organic materials, and thus the nitrate accumulation was not indicative of the nitrifying capacity of the soil.

The short-term incubation test of ammonium oxidation seems to be applicable for other purposes than as a fertility indicator. The test might be important when predicting the denitrifying capacity of the soil, as shown above for Örja. Other studies have shown that the ammonium oxidation may be useful to assess the impact of pesticides (Biederbeck *et al.* 1997; Pell *et al.* 1998) and heavy metals (Christensen *et al.* 2001) on the soil environment.

Alkaline phosphatase activity

- ✓ Alk-P discriminated between N fertiliser rates (N fertilised > zero N), and cropping system (CS II > CS I) at Ekebo, and P rate (P fertilised > zero P) at Orup.
- ✓ At Orup and Örja, 81 – 86% of the Alk-P variable could be explained by combining pH and the organic material in MLR models.
- ✓ Generally, Alk-P was a rather poor predictor of yield level.

As for denitrification and ammonium oxidation activities, no general treatment effect was found for the alkaline phosphatase activity (Alk-P) at the three sites of the SLTF experiment (**I**). Although the effect of nitrogen fertiliser was obvious at Ekebo, a strong interaction effect between N rate and cropping system restricted the use of Alk-P as a treatment discriminator at this site. The interaction was manifested as higher Alk-P values in CS I compared with CS II in the zero N plots, and higher values in CS II compared with CS I in the N fertilised plots. The stimulating effect of P fertiliser at Orup conflicts with the idea of alkaline phosphatase as an enzyme that is derepressed under P-limited conditions (Nannipieri *et al.* 1978). In spite of the seemingly weak and confounding effect of

the treatments, the Alk-P variable was highly attainable for modelling by a two-variable MLR model at Orup and Örja. In line with the results in this thesis, other authors have shown that alkaline phosphatase activity correlates to organic matter (Curci *et al.* 1997; Goyal *et al.* 1999; Jordan *et al.* 1995) and pH (Colvan *et al.* 2001; Sjöqvist 1995) , but only weakly or not at all to different P fractions (Beyer *et al.* 1992; Oberson *et al.* 1996; Wick *et al.* 1998). The relation to organic carbon probably originates in that a higher microbial biomass contains more enzymes, but also that organic soils have higher capacity to stabilise extracellular enzymes by electrostatic binding with humic substances (Sjöqvist 1995).

Stenberg (1998) suggested that alkaline phosphatase activity can be used interchangeably with SIR as a measurement of microbial biomass. Other authors have also found a relationship between alkaline phosphatase and microbial biomass (Beyer *et al.* 1992; Curci *et al.* 1997; Vekemans *et al.* 1989). SIR and Alk-P was analysed in five additional sites of the SLTF experiment. When taking all eight sites together, SIR was proved to be an outstanding predictor of Alk-P ($R^2 = 0.80$, $n = 128$). The second best predictor was pH ($R^2 = 0.55$). In the above analysis, the zero N plots were omitted. According to the MLR models from Orup and Örja (zero N plots included), the Alk-P is more related to the organic matter than to the microbial biomass (**II**). Probably, the relative importance of the microbial biomass as an enzyme producer in relation to the organic matter as an enzyme stabiliser differed depending on the treatments applied.

Generally, Alk-P was a rather poor predictor of yield level (**III**). In the zero N plots, the variable correlated to yield, but not as well as SIR. At Orup, Alk-P was the best predictor of ley yield in the N-fertilised plots. This was most probably an effect of the low yield level in P deprived plots and thereby a low return of organic material to the soil. There was no indication that alkaline phosphatase enzymes was engaged in scavenging P resources in P poor plot, and thereby compensating the plants for the low P status with an increased mineralisation of organic phosphorus. Perucci *et al.* (2000) found that microbial biomass decreases at the same time as alkaline phosphatase activity increases when high rates of herbicides were applied to soil. They speculated that alkaline phosphatase activity is induced as a stress response, *e.g.* under conditions unsuitable for growth. The relevance of this suggestion to soil microbiology and soil fertility is unknown at present. It seems, however, that alkaline phosphatase activity, as determined in the assay proposed by Tabatabai & Bremner (1969), does not relate to its putative function as a fertility factor in the phosphorus cycle.

Conclusions

Following evaluation of the microbial methods presented in this thesis, I have reverted to the question put by Selman Waksman 75 years ago. His intention was to identify a group of methods that would provide us with an insight into the actual or potential fertility of soil. The main result from this thesis is that microbial methods will only provide us with minor information about the causal agents of fertility in soil, although risk for nitrogen shortage due to, *e.g.* denitrification, can be identified. Instead, the value of microbial variables as indicators of the fertility

level or soil quality stems from a capacity of the microbial variables to describe current amounts of available carbon, nitrogen and/or phosphorus. In addition, pH generally have a strong influence on microbial variables linked to the N- and P-cycles, and resulting values of a measured variable may therefore originate from a combination of several factors. In order to be interpretable, reference models have to be built, which normalise the value for site-specific and treatment-specific factors, like clay content and organic carbon. However, knowledge of which models to apply, and how management practises should be adjusted according to derived normalised values is still lacking.

Regarding individual variables, SIR and Max-P are easily interpretable as indicators of labile organic material and available P, respectively. They are also the best yield predictors in unfertilised and nitrogen fertilised systems, respectively.

The division of SIR into an active and a dormant biomass fraction holds promise that the SIR assay may be of value as an indicator of the mineralisation dynamics at the soil in two different time scales. Moreover, the concept of active and dormant microbial biomass has implications on how to model the carbon fluxes in soil.

B-res is probably too much influenced by temporary substrate sources and rhizodeposits to be considered as a stable soil fertility or soil quality indicator.

The specific growth rates, μ_{res} and μ_{PDA}, were stimulated by a high pH, and μ_{res} was positively related to high P levels in soil. However, the true nature of these relations needs to be elucidated before these variables can be used as efficient indicators of soil fertility.

The prediction models for PDA varied markedly between the sites and exhibited a relatively low predictive capacity, which renders PDA less suitable as a soil quality indicator. However, by combining PAO and SIR, PDA seemed to be satisfactorily predicted.

Both pH and substrate supply governed the PAO variable. When interpreting PAO, it should be regressed on pH to check for diverging soils that may be N limited.

The single models for Alk-P also varied between the sites. A combination of pH and organic matter characteristics seemed to be efficient for prediction of the variable, which may imply that Alk-P is a pH-sensitive indicator of the biomass. Indeed, when all eight sites were considered, SIR predicted Alk-P quite accurately.

In the future, the purpose of different soil investigations will vary, and so will the need to utilise the tests evaluated in this thesis. Therefore, no general recommendations about which variables to choose can be made. Moreover, the value of the methods will increase as more information is gathered on their reactions to various management practices and soil perturbations.

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“The microbes will have the last word”

Louis Pasteur

“I am sure they will”

Kalle Svensson