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Development and applicability of an enzyme activity pattern measurement method

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Yhteenveto: Maaperän toiminnallinen monimuotoisuus
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Contents

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Helsinki, September 2012

Milja Vepsäläinen

List of original publications

This thesis is based on the following publications, which are referred to in the text by their roman numerals:

I **M. Vepsäläinen**. 2001. Poor enzyme recovery by extraction from soils. Soil Biology & Biochemistry 33: 1131–1135.

II **M. Vepsäläinen**, S. Kukkonen, M. Vestberg, H. Sirviö and R. M. Niemi. 2001. Application of soil enzyme activity test kit in a field experiment. Soil Biology & Biochemistry 33: 1665–1672.

III R. M. Niemi and **M. Vepsäläinen**. 2005. Stability of fluorogenic enzyme substrates and pH optima of enzyme activities in different Finnish soils. Journal of Microbiological Methods 60: 195–205.

IV **M. Vepsäläinen**, K. Erkomaa, S. Kukkonen, M. Vestberg, K. Wallenius and R. M. Niemi. 2004. The impact of crop plant cultivation and peat amendment on soil microbial activity and structure. Plant and Soil 264: 273–286.

V R. M. Niemi, **M. Vepsäläinen**, K. Erkomaa and H. Ilvesniemi. 2007. Microbial activity during summer in humus layers under *Pinus silvestris* and *Alnus incana*. Forest Ecology and Management 242: 314–323.

Author's contributions

I The experiment was planned and executed by Milja Vepsäläinen, and she wrote the article.

II The original idea of the enzyme activity pattern measurement was developed by Maarit Niemi. Maarit Niemi and Milja Vepsäläinen designed the experiment together based on previous joint work. Sanna Kukkonen and Mauritz Vestberg performed the field experiment and also organized sampling of soil. The detailed planning of the laboratory work, including methods selection, the essential part of measurements of enzyme activities, and analysis of results and basic statistical analysis were carried out by Milja Vepsäläinen. Advanced data analyses were carried out by Maarit Niemi and Hannu Sirviö, who created the ZymProfiler® clustering programs. All the authors contributed to the writing of the manuscript, Milja Vepsäläinen and Maarit Niemi sharing the main responsibility.

III The study was jointly planned by both authors. The laboratory experiment was in essence carried out by Milja Vepsäläinen. The writing of the manuscript was mainly carried out by Maarit Niemi, with a significant contribution by Milja Vepsäläinen.

IV The study was jointly planned by Milja Vepsäläinen and Maarit Niemi. Milja Vepsäläinen and Kaisa Wallenius organized sampling and experimental laboratory work and mainly carried out the measurements. Sanna Kukkonen and Mauritz Vestberg designed the field experiment, arranged the sampling and provided the site for investigation together with the background data. Kirsti Erkomaa was responsible for the PLFA analysis and its interpretation. The statistical

analysis was carried out by Maarit Niemi together with Milja Vepsäläinen and the results were interpreted jointly by these authors. Milja Vepsäläinen prepared the first draft of the article, followed by further editing by Maarit Niemi with contributions from the other authors.

V The idea for this study was generated by Maarit Niemi and Milja Vepsäläinen and Maarit Niemi arranged the co-operation between the authors. Milja Vepsäläinen took care of the sampling arrangements and detailed planning of the laboratory work, and she participated essentially in the laboratory work and analysed the results. Hannu Ilvesniemi provided the sampling site of his design, along with background data, and the sampling. Kirsti Erkomaa was responsible for the PLFA analysis and its interpretation. The results were interpreted and the manuscript was written jointly by all the authors, Maarit Niemi and Milja Vepsäläinen having the main responsibility.

Abbreviations

Ala AP	alanine aminopeptidase
AMC	aminomethylcoumarin
ATP	adenosine triphosphate
Da	Dalton
EC	Enzyme Commission
HPLC	high pressure liquid chromatography
K_m	Michaelis constant
Leu AP	leucine aminopeptidase
MUB	modified universal buffer
MUF	4-methylumbelliferyl
MUG	4-MUF ß-D-glucuronidase
N	nitrogen
NAGase	*N*-acetylglucosaminidase
NC-IUBMB	Nomenclature Committee of the International Union of Biochemistry and Molecular Biology
PDE	phosphodiesterase
pH_{KCl}	pH measured in potassium chloride solution
PME	phosphomonoesterase
SIR	substrate-induced respiration
SOM	soil organic matter
UPGMA	unweighted pair group method with arithmetic mean

Functional biodiversity in boreal soils

Enzyme activity pattern measurement method development and applicability studies

Milja Vepsäläinen

University of Helsinki, Faculty of Agriculture and Forestry, Department of Food and Environmental Sciences

Vepsäläinen, M. 2012. Functional biodiversity in boreal soils: Enzyme activity pattern measurement method development and applicability studies. Monographs of the Boreal Environment Research No. 41. 59 p.

Abstract

Soil microorganisms mediate central reactions of element cycles in a heterogenic environment characterized by discontinuity of energy, nutrients, and water together with sharp pH gradients. They are diverse in species, numerous in quantity and possess a multitude of functions. One gram of soil may contain $10x10^9$ microbial cells; for comparison, the Earth has only $7x10^9$ human inhabitants. Species richness, evenness and composition in soils is impossible to measure, and therefore a convenient means of characterising soil microorganisms is to measure the type and rate of reactions occurring.

The aim of this work was to develop a rapid, sensitive method to measure the activities of a set of soil enzymes simultaneously in a small scale. In the method, homogenized soil suspensions are investigated using fluorescent substrate analogues freeze-dried onto multiwall plates. It was shown that extraction of enzymes from soils produced inconsistent and unpredictable yields of the various activities and was therefore not applied as a pretreatment. Applicability of the method was evaluated by characterising soils treated with different agricultural practices, supporting a variety of crop plants and with fluctuating seasonal attributes. Bulk samples from experimental sites established both in agricultural and forest soils were utilized. Details of method development and of the effects of different treatments on enzyme activity pattern and on individual enzyme activities are discussed. The effects of eight crop plants, peat amendment and two consecutive sampling years yielded significant differences in soil extracellular enzyme activities. The effect of crop plants was most pronounced: eight of the measured ten activities yielded statistically significant differences in both years. The activities differed between years for six enzymes. The effect of peat was slight and was observed only two years after the addition. In another experiment, green or composted plant residues tended to enhance the activities of enzymes compared with chemical fertilizers, although the effect was not consistent. Forest soils usually yielded higher specific activities than field soils and the enzymes showed higher potential activities under alder than under pine. Temporal fluctuations of enzyme activities were also studied.

Cluster analysis was utilized for data analysis in order to combine all measured attributes and to reveal the differences in the entire pattern, even though the differences in individual enzyme level were not statistically significant and the enzyme activities often correlated with each other.

Due to the multitude of processes and functions, together with the wide taxonomic diversity in soils, method development in soil microbiology is still a major challenge. Interpretation of results usually requires a reference comparison. The method developed in the present study is proposed to be used as a sensitive measure of soil functional activity.

Keywords: Enzyme activity pattern, soil, microbes, functional activity, ZymProfiler, cluster analysis, method development, fluorescent substrates

Maaperän toiminnallinen monimuotoisuus
Entsyymiaktiivisuustestisarjan kehittäminen ja soveltaminen

Milja Vepsäläinen

University of Helsinki, Faculty of Agriculture and Forestry, Department of Food and Environmental Sciences

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Tiivistelmä (abstract in Finnish)

Mikrobit vastaavat alkuainekiertojen avainreaktioista heterogeenisessä, energianlähteitä, ravinteita ja vettä epätasaisesti sisältävässä ja jyrkkien pH-gradienttien muovaamassa maaympäristössä. Mikrobiyhteisöjen lajikirjo on runsas, solujen lukumäärä on valtaisa ja ne katalysoivat monentyyppisiä reaktioita. Gramma maata voi sisältää $10x10^9$ mikrobisolua. Vertailun vuoksi – koko maapallolla asukkaita on $7x10^9$. Lajien määrää, niiden runsaussuhteita ja lajirakennetta on mahdotonta määrittää, joten maaperän entsyymien välittämien reaktioiden laadun ja nopeuden mittaaminen on vaihtoehtoinen tapa mitata mikrobiston monimuotoisuutta.

Väitöskirjatyön tarkoituksena oli kehittää nopea ja herkkä pienen mittakaavan menetelmä usean maaperäentsyymin aktiivisuuden samanaikaiseen mittaamiseen. Menetelmässä hyödynnetään kuoppalevyille kylmäkuivattuja fluoresoivia substraattianalogeja ja mittaus tapahtuu homogenoidusta maalietteestä. Entsyymien uuttaminen maasta osoittautui saannoltaan epävarmaksi ja vaihtelevaksi menetelmäksi eikä sitä voitu soveltaa näytteiden esikäsittelyssä. Entsyymiaktiivisuustestisarjan käyttökelpoisuutta arvioitiin tekemällä mittauksia eri tavoin käsitellyiltä ja eri kasvilajeja kasvavilta koealoilta otetuista maanäytteistä. Lisäksi tutkittiin näytteenottoajankohdan vaikutusta entsyymiaktiivisuuksiin. Näytteet otettiin pelto- ja metsäalueilla sijaitsevilta tutkimusalueilta kokoomanäytteinä. Väitöskirjatyössä tarkastellaan yksityiskohtaisesti menetelmän kehittämistä ja erilaisten käsittelyjen vaikutusta entsyymiaktiivisuuksiin sekä koko analyysivalikoiman osalta että yksittäisen entsyymin tasolla.

Kahdeksan eri viljelykasvia ja turvelisäys aiheuttivat tilastollisesti merkittäviä muutoksia entsyymiaktiivisuuksiin kahtena perättäisenä näytteenottovuotena. Viljelykasvin vaikutus oli merkittävin, se havaittiin molempina vuosina kahdeksassa mitatussa entsyymissä kymmenestä. Näytteenottovuosi vaikutti kuuteen entsyymiaktiivisuuteen. Turpeen lisäyksen vaikutus oli havaittavissa ainoastaan kaksi vuotta lisäyksen jälkeen. Toisessa koejärjestelyssä viherlannoitus ja kompostoitujen kasvinosien lisääminen hieman nostivat entsyymiaktiivisuuksia verrattuna kemialliseen lannoitukseen, mutta vaikutus oli vaihtelevaa. Orgaanista ainetta kohden lasketut entsyymiaktiivisuudet olivat metsämaassa viljelymaata korkeammat ja lisäksi lepän vaikutusalueella koholla verrattuna mäntymetsään.

Tulosten käsittelyssä hyödynnettiin klusterianalyysiä, joka yhdistää kaikkien mitattujen muuttujien tiedot. Vaikka yksittäiset mittaukset eivät eroaisikaan toisistaan tilastollisesti merkittävällä tasolla ja vaikka tulosten välillä olisi korrelaatioita, klusterointi ryhmittelee näytteet.

Maaperämikrobiologisten mittareiden kehittäminen on edelleen haastavaa lajiston monimuotoisuuden ja sen välittämien toimintojen laajan kirjon vuoksi. Tulosten tulkinta vaatii yleensä vertailukelpoisen kontrollinäytteen. Tässä tutkimuksessa kehitettyä menetelmää voidaan hyödyntää herkkänä maaperän monimuotoisuuden mittarina.

Asiasanat: entsyymiaktiivisuus, testisarja, maaperä, mikrobisto, monimuotoisuus, ZymProfiler, klusterianalyysi, menetelmä, fluoresoiva substraatti

1 Introduction

1.1 Background

Four major challenges for mankind are to provide food, supply clean water, produce energy and sustain the health of future generations. Ensuring soil quality is essentially linked with all these challenges and it provides a sustainable basis for attaining the targets. The attention of policymakers can only be evoked after a consensus on the central attributes of quality has been reached by soil scientists. Soil conservation needs legally binding agreements which are based on solid scientific results.

Soil welfare is essentially involved in meeting all these challenges; citing Janke and Papendick (1994) *"for humankind, soil is the essence of life and health"*. Soils can be compared to a living system, with a complex structure consisting of solid, liquid and gaseous phases. The various aggregates and different fractions yield microniches with a multitude of conditions for growth and function. As a result of a wide array of simultaneous reactions, this tissue is in a constant non-equilibrium state (Ruggiero *et al.* 1996).

This work aims at developing a solid method for measuring soil welfare. A major role in soil functioning is played by soil enzymes, macromolecules catalysing chemical reactions in soils. The usefulness of the method developed is studied in various soil environments. The diversity of soils gives rise to major challenges throughout the process: planning, sampling, assay and interpretation.

1.2 Soil heterogeneity: a challenging framework for biological functions

Soil structure, mineral and organic compositions, vegetation, and physical conditions control soil microbiota and soil fauna. Soil microbial composition and diversity are of fundamental importance for the fertility of soils. The heterogenic and both spatially and temporally dynamic microbiota and its functioning are difficult to monitor for evaluation of the biological status of soil.

The reactions taking place in soils must be unravelled in order to understand element cycling, status in biota and possibly to combine the information on soil microbial diversity with functions occurring in soils. Effects of anthropogenic actions on these processes are of importance in preserving soil functions, diversity and ecosystem services. Homogenisation of e.g. European landscapes due to urbanisation, similar agricultural practices, technical means and choices in environmental planning may decrease the heterogeneity of soils, diminishing the number of different potential habitats for soil organisms (Turbé *et al.* 2010).

The solid phase of soils consists mainly of two key components: minerals and organic matter. Soil mineral composition and texture are affected by the rock composition and geological processes producing mineral soil. The grain size of mineral soil varies from clay fractions of <2 μm through silt and sand to gravel and rocks and the surface area and surface properties of mineral particles affect soil properties enormously. For example, illite, a low surface area clay mineral, has a surface area of 75–125 m^2/g whereas organic matter yields a surface area of 500–800 m^2/g (Burns 1978). Plant litter, animal faeces and other remains are the major precursors of soil organic matter (SOM). They are processed by soil fauna and especially microorganisms to produce nutrients taken up by microbiota, soil fauna and plants, and also humic matter, a rather stable organic component of soil. The amount of SOM determines greatly the size and composition of the microbial community in soils. There are different pools of SOM in soils, with different contributions to carbon metabolism of microorganisms (Farrar *et al.* 2012).

Soil structure provides the space and the surface for microorganisms and for extracellular enzymes and their substrates, mainly in particulate form. Microorganisms in surface soils are concentrated in areas of rhizosphere, faecal pellets, and plant and animal debris (Ladd *et al.* 1996, Marschner *et al.* 2012). In soil particles bacteria occur inside aggregates or in

micropores near the surface of aggregates. In environments rich in organic matter, bacteria may reside on the aggregate surface. The site for fungal hyphae is in the pores between the aggregates (Ladd *et al.* 1996). From a microbial point of view, soil is a structured, heterogeneous and discontinuous system, generally poor in nutrients and energy sources, with microorganisms living in discrete microhabitats (Stotzky 1997).

Due to the small organic and mineral particles with varying surface properties, sizes and three dimensional structures, the heterogenic nature of soils starts from the micrometer scale – the scale of microorganisms. Microniches provided by soils differ in e.g. pH, salt concentrations, nutrient concentrations, energy sources and availability of water, producing gradients with different conditions (Marshall 1976). Besides providing growth conditions, the niches provide habitats for microbes to occupy, surfaces to attach to, and shelter from predators. The different conditions and habitats produced by various interfaces enable incredible biodiversity of soil fauna, and the number of microbial cells per *gram* of soil can reach 10^{10} (Torsvik *et al.* 1990), higher than the human population on the Earth. However, not all microhabitats have suitable conditions for microbial life (Nannipieri *et al.* 2003), although microbes have the widest range of colonised habitats of all forms of life.

Besides obviously at microscale, the heterogeneity of soils expands to local scale with differing nutrients and litter quality, and further to the scale of ecosystem level where pH and organic matter content and land use and management determine the habitats provided (Turbé *et al.* 2010). The heterogeneity itself is difficult to assess due to many properties of soil having an influence on soil characteristics. Different soil characteristics have different scales for autocorrelation, which leads to multiple scales of heterogeneity (Berner *et al.* 2011). Nevertheless, it has been proposed that spatial variability of 50 % measured as coefficient of variation in e.g. a catchment area allows results to be interpreted for the catchment level (Šantrůčková *et al.* 2004). The various possibilities of interpretation must always be evaluated according to targets of the study.

The description of soils based on chemical and physical characteristics has been widely accepted, and a global classification system is available (IUSS Working Group WRB 2007). Although widely used to describe soils, this classification does not include soil biological characteristics, which are vital for understanding soil functioning. The biology and diversity of soils is still largely unknown, but it has been estimated that about one fourth of all living species are soil- or litter dwellers (Turbé *et al.* 2010). The living species in soils include e.g. mites, springtails, ants and earthworms, but the majority belong to Bacteria, Achaea and Fungi. The species concept of these organisms in soils is highly complex, since DNA is constantly being transferred from one species to another.

The disparity of soil heterogeneity and microbial ecological scale are a challenge for monitoring of soil microbiota and its functions. Sampling strategy must be carefully planned, measurement methods selected, and data must be interpreted with consideration (Nortcliff 2002).

1.3 Soil health and soil quality – what is behind the phrases?

1.3.1 Soil quality

In terms of quality, the productivity of agricultural soils is the most obvious association. The majority of human and domestic animal food literally originates from soil; cereals, vegetables, fruit, pulse, fodder, and it provides pasture for livestock. As well as a food producing system, soils act as filters for cleaning water and air, as a sink and source of carbon balancing the air content of CO_2, as a site for decomposing various pollutants and other organics and as the main matrix for nutrient recycling from debris to nutrient (Turbé *et al.* 2010). When well treated, a soil can act as a sink for pollutants to some degree, but misused soils may be a source of chemical and physical pollutants for air and water.

Soils in forestry use provide wood for various industries and for energy production, habitats for various macro- and microorganisms, and environments for recreational use and different activities – they are often equated with "nature".

In scientific research, soil quality is usually related to productivity of agricultural or forest soils. Urban soils of cities are excluded, although they provide a basis for buildings, roads, recreational use, etc, with soil physical structure as a main concern. Although some criteria of soil quality might have common relevance in all these areas of varying land use, the emphasis in this work is on biological measures of soil quality, mainly in agricultural and forestry areas.

Soil quality has been defined as *the capacity of soil to function, within land use and ecosystem boundaries, to sustain biological productivity, maintain environmental quality, and promote plant, animal, and human health* (Doran *et al.* 1994). Soil health, on the other hand, was encapsulated as *the ability of a soil to perform functions that are required for the biological components of an ecosystem within the constraints of local environmental factors* (Dick 1997). The definition *within ecosystem boundaries* or *local environmental factors* included in both the definitions is crucial, since not all soils can have similar demands even if they are characterized as *in good quality/health*. Depending on e.g. the amount of precipitation and filtration, soils can be weathered by natural processes, the amount of organic matter varies naturally and mineral composition ranges from fine to coarse, affecting e.g. the water holding capacity of soils. It is noteworthy that both definitions include functions.

Soil health and soil quality should, however, be distinguished as separate (but somewhat overlapping) concepts. Health is a somewhat narrower term, referring to soil condition. Quality, on the other hand, is defined largely by soil function or use and combines the biological, physical and chemical properties that provide various functions to an ecosystem (Dick 1997). The term soil quality is referred to in this text to describe the functionality and productivity and health-promoting capability of soils.

Until the term *soil quality* can be defined to some degree in numerical terms to gain common acceptance, it cannot be expected to be incorporated into mainstream thinking of soil use and regulations (Turbé *et al.* 2010). It should be borne in mind, however, that measuring the quality of air fit for breathing, or of water fit for drinking is considerably more straightforward than defining corresponding criteria for soil quality (Nortcliff 2002).

1.3.2 Biological indicators of soil quality

A quantitative definition for soil quality has been a scientific aim for decades but it is still out of reach. Indicators are one way of presenting and managing complex information in a simple and informative manner (Turbé *et al.* 2010). There is a constant need for a measure of soil response to different treatments, most urgently due to erosion, salinization and organic matter decrease, and also to define baseline conditions prevailing at possibly nondisturbed areas. The factor describing soil quality could be a single indicator, a set of indicators or possibly an index based on measured and modelled descriptors of soil.

There are several requirements which an inclusive (biological) indicator or index based on several indicators should meet, but different indicators can be used for specific needs. Presenting information in a compressed mode reduces the number of measurements and parameters needed to describe a complex situation and provides a decision aid to ease communication between parties. The indicator should respond in a sensitive, quantitative and predictable manner to e.g. soil perturbations, xenobiotics, crop plants, changes in biodiversity or in soil physical structure and to nutritional status. Furthermore, it should be insensitive to weather, or the effects should be well known. The indicator should be applicable for agro ecosystems, forest ecosystems and other natural ecosystems with widely different characteristics. The challenge is to find an appropriate balance between simplicity and completeness (Turbé *et al.* 2010) and it should be accepted that no biological indica-

tor can meet all the above-mentioned requirements. One challenge of defining soil quality indicator or index is the fact that soil processes occur over long periods of time, and a measurable shift in quality may be obvious only when a cumulative impact occurs (Nortcliff 2002), affecting the buffering capacity of soils (Dick 1997). Elliott *et al.* (1996) mapped several soil properties that characterize the biological and environmental quality of soils (Table 1).

Indicators responding to soil stress rapidly, referred to as "sensors", may sensitively warn us of soil degradation as compared to more slowly changing soil properties (Dick 1994). They are of importance, because it appears that procedures improving soil quality are difficult to measure during the first years of their application, due to the buffering capacity of soil. A comprehensive indicator should include soil productivity, environmental aspects and health of soil (Elliott *et al.* 1996) and should summarise the information on soil chemical, physical and biological components (Table 1). Is this a too challenging task bearing in mind the complex nature and huge heterogeneity of soils?

Soil organic matter (SOM) is the most used single property of soil quality and it reflects chemical, physical and biological properties of the soil matrix (Elliott *et al.* 1996, Nortcliff 2002). It is simple to measure and the measurement does not require special equipment, especially if loss on ignition is utilized as an estimate of SOM. The most important and the largest fraction of SOM in terms of soil productivity and quality is the readily decomposed fraction with rapid turnover rate. The rate, however, varies widely; from short periods of time to years and millennia (Allison and Jastrow 2006). However, total SOM is not a sensitive measure for change due to different pools and different types of organic matter included that respond variably in treatments (Farrar *et al.* 2012).

Probably the most important physical soil factor affecting soil processes directly and via its components is water. Water content is influenced by the water holding capacity of soils, it affects gas and solute diffusion and it influences soil strength. Soil water properties are closely linked to the biological activity of soils and it has even been suggested that soil water is one of the determining factors reflected in the functional activity of soils (Baldrian *et al.* 2010b). The level of the soil water table determines the start of the anaerobic zone; below the water table level the soil pores are filled with water, restricting gas exchange with air. Anaerobic processes often are slower than aerobic processes, and although some unique reactions occur many strictly aerobic reactions are missing. In relation to soil biological functions, sampling is usually carried out above the water table level in order to reveal the activity in the vadose zone. The results of soil biological measurements are often expressed per dry weight of soil in order to make the results more comparable and to reduce the effect of variation in water content, which is prone to impacts of rapid variation in different weather conditions.

Soil chemical factors are linked to availability of elements. Nitrogen is often the key element associated with several rate limiting processes, even though elementary N is abundant in the biosphere. Its cycling is closely associated with organic matter content and microbial biomass (Elliott *et al.* 1996). Toxic compounds in soil are readily measureable as total quantities of selected elements (e.g. heavy metals), but it is

Table 1. Characteristics included in soil quality. Modified from Elliott et al. 1996.

Soil property	Chemical	Physical	Biological
First order	pH Salinity Cation exchange capacity SOM Site-specific toxic compounds	Infiltration Available water Soil depth	Vegetative cover Microbial biomass Labile organic carbon Labile organic nitrogen
Second order		Water-stable aggregates Dispersible clay Bulk density	Key invertebrates Earthworms

difficult to determine the oxidation state or molecular composition, which greatly effects their toxicity, mobility and binding properties. The reactivity of other toxic compounds, such as pesticides, depends greatly on the soil properties SOM and clay and the binding of molecules to soil matrix.

Cell outer membranes are constituted mainly of phospholipid fatty acids (PLFAs) in Bacteria, Archaea and Eucarya. The molecules are rapidly metabolized during cell growth and are not detected in storage products of cells (Zelles and Alef 1995, Zelles 1999). Since organisms have different kinds of PLFAs, the community composition can to some extent be characterized by the nature and proportion of identified PLFAs. Furthermore, the total amount of PLFAs in soil can be used as an estimate of total microbial biomass, or subgroups of PLFAs can be used to describe the amount and type of either bacterial biomass (e.g. Kandeler *et al.* 2001, Kandeler *et al.* 2002, Baldrian *et al.* 2010a, Marschner *et al.* 2012) or fungal biomass (Frostegård and Bååth 1996). Ergosterol, the main sterol present in fungal membranes, is used as a biomarker for fungal growth and biomass (Zelles and Alef 1995).

Community level physiological profiles (CLPPs) of soils utilize the addition of various simple organic substrates and detect the responses of the activity of the microbial community. Measuring the growth response of soil microbial communities to different organic substrates (Biolog™) has been used widely for 20 years (Garland and Mills 1991, Zak *et al.* 1994, Haack *et al.* 1995). The main criticism towards this method arises from the requirement of microbial growth and from the fact that the response of an extracted population is measured, both being possible sources of bias, but the method also shows low reproducibility and poor detection of fungal activity (Nannipieri *et al.* 2003). A method indirectly utilizing enzyme activities in soils is substrate-induced respiration (SIR), in which O_2 consumption or CO_2 production is measured to indicate the respiratory potential of soils. Chapman *et al.* (2007) introduced a streamline multiwell plate method for measuring SIR of multiple substrates simultaneously (MicroResp™). It requires a shorter incubation period (6 h) than Biolog™, and enables the use of radiolabelled substrates as well as detection of CO_2 produced via a color reaction. The response of the microbial community to supplemented substrates depended on the soil type in the study of Roberts and Jones (2012), in which SIR of Eutric Cambisol was enhanced and Dystric Cleysol was unaffected by chitin addition. Both the soils responded to additions of small sugar units, namely glucose, *N*-acetylglucosaminide and glucosamine.

In soil microbiology, DNA-based methods developed to detect the amount of taxonomic diversity and to reveal differences in the community composition have been used for decades (Torsvik and Øvreås 2002). Taxonomic diversity is of importance when soil quality is addressed, because in general it is assumed that a more diverse microbial community is linked to a better quality of soil. Methods detecting differences in polymerase chain reaction (PCR) multiplied community DNA have been used, e.g. denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE), yielding DNA products that can be sequenced and linked to the phylogeny of soil microbes (Muyzer 1999). Challenges of these methods arise from e.g. selectivity of PCR primers and reaction, detection of small bands on gels, and quantification and reproducibility of DNA extraction. Methods have been extended to detect mRNA of soils in order to reveal the sequences actually transcribed in the population and to provide methods targeting functional genes, such as the GeoChip (He *et al.* 2012), employing hybridization of DNA/RNA molecules on small scale glass slides and detection of hybridized products by image analysis. Although this method does not require PCR, several problems arise from e.g. the three dimensional structure of DNA/RNA molecules, the sensitivity of hybridization (reported to be 100 to 10 000 times lower than with PCR amplification) or uncertainties with quantification (Zhou and Thompson 2002). The functional gene arrays are a promising method for use in conjunction with enzyme assays to reveal the connection between the actual potential enzyme

activities and the gene pool coding for them. This can be achieved for a single species as well as for a microbial metagenome (Torsvik and Øvreås 2002).

The convenient DNA-based methods usually require an extraction process as a sample pretreatment. The DNA extraction process introduces bias into the results; only the genes extractable under the conditions applied can be multiplied by the PCR required in many of the methods. The isolation and purification techniques affect the bacterial community structure detected in the soil samples (Niemi *et al.* 2001). Furthermore, the connections between the number of transcripts or genes and the functions achieved are still unclear.

For indicating shifts and changes in soil environments, measurement of enzyme activities yields an overall picture of the effects of microbial activity on certain elemental cycles (Dick 1997). These activities do not only measure the perturbation but also highlight the effects of enzymes on soil biochemical properties and on ecosystem function (Naseby and Lynch 1997). In surveys of indicators, the importance of the measurement of microbial activity is rather neglected (Turbé *et al.* 2010). Ecologically, the activity of extracellular enzymes is of particular interest because they catalyse the breakdown of macromolecules – a rate limiting process – and create nutrients for plants and soil microbiota. However, it is often difficult to interpret the changes in activity measurements (Turbé *et al.* 2010), which emphasises the need for further research. One enzyme activity, the activity of dehydrogenase, has also been applied as an index of total microbial activity. Unfortunately, a consistent correlation between the number of microorganisms and the activity of dehydrogenase has not been detected (Gianfreda and Bollag 1996). It appears that different enzymes respond to different treatments, for example ß-glucosidase to agricultural practices (Bandick and Dick 1999) and arylsulphatase to the presence of trace elements (Dick 1997). In one study on agricultural soils, enzymes were observed to respond rapidly to treatments compared with some other biological measures (Bandick and Dick 1999)

Acosta-Martinez *et al.* (2003) characterized chemical, biological and physical properties of soils by measuring different soil characteristics. The soils were consistently grouped by soil type (fine sandy loam, sandy clay loam and loam) in a three dimensional plot of pH and organic C and total N contents, but results of ß-glucosidase, ß-glucosaminidase and arylsulphatase yielded a more scattered plot. This illustrates well the results of enzyme analysis, which are affected by a multitude of factors. The results are more difficult to interpret, but they plausibly reflect different treatments and soil properties more sensitively.

Genes encoding enzymes and their occurrence in the soil are detected by several methods, such as processing PCR-probes for different kinds of ß-glucosidase genes of soils, multiplying the target genes and quantifying the outcome (Cañizares *et al.* 2011). The corresponding activities were detected with a proteomic method in-gel. This is an example of combination of techniques to reveal the link between function and genetic diversity in order to develop methods for e.g. soil quality measurements.

There is no perfect method; all of them have benefits and disadvantages. The most important aspect for a scientist in choosing a method is to understand the drawbacks and limitations of the technique; the second is to celebrate the potentials. In order to study responses of soil environments to different treatments we chose the enzyme activity approach to detect the potential functional activity in soils and to develop the existing methodology in order to yield a more streamlined and reliable assay.

1.3.3 Functional and taxonomic diversity

Diversity in the classical sense is a combination of species richness, evenness and composition. For soil microbiological communities it is however impossible to detect these parameters for all microbial species present and therefore the data existing is an interpretation of the available experimental approaches (Griffiths *et al.* 1997).

In the taxonomic sense, the diversity of microorganisms is overwhelming. Taxonomic diversity of soil microorganisms relates to the genetic diversity detected in soils, which is enormous; thousands of different species can be found in one gram of soil and the number of individual organisms is estimated to be around 10^{10} (Torsvik and Øvreås 2002). Is it therefore true that every species has a separate role and is necessary for the ecosystem? At least the information boost of genetic diversity is difficult to handle and its significance is difficult to interpret. To further demonstrate the enormity of genetic diversity, Tiedje *et al.* (2001) isolated fluorescent pseudomonas from soils worldwide and found that their genotypes were peculiar to each sampled site, indicating spatial heterogeneity of a species.

Biodiversity essentially relates to soil functioning, but the mechanisms and effects of biodiversity on soil functions are still unclear (Andrén and Balandreau 1999, Turbé *et al.* 2010). Could the relevant biodiversity be described as the functions and the rate of functions carried out by microorganisms in any environment? Since only about 1–10 % of the microbes are easily cultivated, it is difficult to examine the physiological characteristics, i.e. functions *in situ*, of soil microbiota on the basis of studies on pure cultures. Furthermore, the traits exhibited in pure cultures may not apply in environmental conditions (Turbé *et al.* 2010). Basically, high biodiversity means many species present and low biodiversity means few species present in a given environment. However, it has been suggested that there are only a limited number of functions to be carried out in an ecosystem, and that species composition does not have a great effect on process rates (Andrén and Balandreau 1999), assuming that selected and adapted taxa are active in each environment. It is also accepted that more species present does not necessarily lead to more functions performed (Turbé *et al.* 2010). The number of functions in soils can be assumed to be less than the number of taxa present, because several organisms can perform the same functions (Turbé *et al.* 2010), which leads to an appealing solution in handling the diversity issue. Studying processes that are limited in number is in a sense more understandable than studying taxa with thousands of different genomes, many still undescribed. Studying functions may yield data that can more readily be linked to soil properties, but the knowledge concerning these connections is still partial. However, in order to yield functionality that can sustain changes in the environment, taxonomic diversity is essential to provide the resilience and stability necessary to ensure the functionality of soil systems in all conditions (Nannipieri *et al.* 2003, Turbé *et al.* 2010).

An enormous challenge for environmental microbiologists is to link the phylogenetic information and the information on functions performed in soils. Therefore the relations between genetic diversity and community structure, and between community structure and function, are central issues for soil microbiology (Nannipieri *et al.* 2003). Even though the relationship between genetic and functional diversity is still unclear, it is assumed that high genetic diversity enhances the stability, productivity and resilience of an ecosystem (Torsvik and Øvreås 2002, Nannipieri *et al.* 2003, Turbé *et al.* 2010), somewhat contrary to the opinion of Andrén and Balandreau (1999). Enzymes mediate all the significant functions of macromolecule breakdown and nutrient cycling in soils and other environments. Microorganisms are the foremost source of biocatalysis in soils (Tabatabai 1994) and they produce both intracellular and extracellular enzymes. The functions of soil are strongly linked to extracellular enzymes that carry out various reactions in the process of organic matter breakdown and release of nutrients.

In a recent study, Koukol and Baldrian (2012) identified fungal strains isolated from pine needles and detected their hydrolytic enzyme spectra and activities. They hypothesized that genetically distinct fungi would also differ in functional traits, but nearly all (11/12) of the isolates produced ß-glucosidase, which is necessary in cellulose degradation. The strains exhibited different activities of other enzymes involved in the cycling of C, N and P. Only one of the twelve strains isolated was capable of mineralising N (by aminopeptidase activ-

ity), which could be a strong selective factor under stress conditions. This shows that genetic and functional diversity are not at least systematically consistent, although the study included only 12 pure cultures of fungi. Four ectomycorrhizal strains and eight fungi from shrub, on the other hand, all showed species-dependent enzyme activity levels (Pritsch *et al.* 2004, Bordo *et al.* 2011).

To date, EU and its nations lack legislation or regulations for protecting soil biodiversity, even though it is a fundamental basis for the functioning of society. Fortunately, some action has been taken in the form of Agenda 21, a document from the United Nations Conference on Environment and Development, in 1992. This Agenda has been applied for above-ground biodiversity, which however has little effect on soil biodiversity (Turbé *et al.* 2010).

1.4 Enzymes in soils

1.4.1 Overview

Enzymes catalyse rate-limiting steps of organic matter decomposition in soils. They are produced mainly by microorganisms, but plant debris, root exudates and soil fauna contribute to a smaller extent. Complex macromolecules (e.g. cellulose, lignin, pectin, hemicellulose) are not directly incorporated into cells and they need to be degraded by extracellular enzymes in order to yield substrates small enough (ca. 600 Da) to be incorporated into cells (Arnosti 2011). The term "extracellular enzymes" in this context refers to enzymes capable of coming into contact with substrates that are not incorporated into cells, i.e. they catalyse reactions outside the organisms that synthesised them (Ruggiero *et al.* 1996). Degradation of complex organic molecules requires broad biochemical capability of a single organism or co-operation between several organisms, each with a specific capability.

Enzymes may be large molecules with a size of 50 000 daltons or more, too large to fit into the small pores of the particulate matter, or smaller molecules with easier access to substrates. Even for a single reaction type there may be a number of different enzymes in soils produced by one species. For example, Cañizares *et al.* (2011) detected three different sized (120, 300 and 669 kDa) proteins with ß-glucosidase activity produced by *P. putida*. The conditions prevailing in any soil cannot be optimal for accumulation of all enzymes. Therefore, soil pH, moisture, oxygen concentration and availability of nutrients to enzyme-producing organisms (Ruggiero *et al.* 1996) are the key factors of enzyme activity accumulation in soils. Free enzymes diffusing in soil solution may be short-lived but with high activity, whereas immobilized enzymes on clay minerals or in humic matter persist much longer but usually have lower activity due to conformational changes or engulfment of the active site (Lähdesmäki and Piispanen 1988, Wetzel 1991).

The kinetics of pure enzymes are frequently measured and the same measurements can also be applied in soil environments. The Michaelis constant (K_m) is perhaps the most fundamental parameter of enzyme kinetics, indicating the substrate concentration at which the reaction velocity reaches half the maximum velocity. According to Ruggiero *et al.* (1996), K_m values of soils do not necessarily measure the substrate affinity of the enzyme molecule but also reflect the clay minerals or organic matter present. The values measured in soils are usually at a higher level than that of the same enzyme in vitro, indicating e.g. difficulties of substrate diffusion to the enzyme or conformational changes of enzymes due to adhesion on soil compounds.

Reductively explained, macromolecule degradation depends on the availability and accessibility of substrate and the presence and activity of enzymes. Substrates of particulate form have limited surface area that to which enzymes can have access. For example, cellulose substrate can have various pore sizes, which affects the obtainable surface. The most important macromolecules in soils, and their degradation products entering cells of Procarya and Eucarya, are presented in Table 2. While in soil, the substrates may become encrusted with clay minerals and degradation products of the material itself (Ladd *et al.* 1996), forming microaggregates of soil matrix. It has been demonstrated using ^{14}C compounds that in-

creasing the clay content of soil slows down the mineralisation rate of both simple and complex organic substrates. Degradation of the primary substrates is not necessarily retarded by the clay minerals, but decomposition products of the early steps of decomposition pathways are accumulated (Ladd *et al.* 1996).

Plants, animals and microorganisms provide the soil environment with the macromolecules described in Table 2 in different amounts. These molecules can be sorbed by soil particles, degraded by microorganisms or be incorporated into SOM as a fraction of humus.

The most abundant biological component in soils, cellulose, is a linear polymer consisting of ß-D-glucose monomers linked by ß-1,4-glycosidic bonding and forming complex fibrils by hydrogen bonding. It is degraded in a stepwise manner involving three distinctive hydrolases: (1) exoglucanases releasing cellobiose (a sugar dimer) from the non-reducing ends of macromolecules, (2) endoglucanases randomly cleaving internal glycosidic bonds and (3) ß-glucosidases releasing sugar monomers from cellobiose or other oligosaccharides. Microbes produce a multitude of different cellulases having affinity towards cellulose molecules of different sizes and with different inhibition properties (Ladd *et al.* 1996). For example, cellulases produced by the actinomycete *C. lindemuthianum* are induced by cellulose (Acosta-Rodriguez *et al.* 2005).

Chitin, as a different example, is also abundant in soils, and it is a constituent of fungal cell walls, exoskeletons of insects and insect eggshells. It is also degraded by a multitude of enzymes, and Metcalfe *et al.* (2002) showed that the majority of bacterial chitinases originate from streptomycetes and actinobacteria. Chitinases are also frequently produced by fungi (Redlak *et al.* 2001). The breakdown of chitin macromolecules involves both endo- and exo-acting hydrolases, and Roberts and Jones (2012) showed that the bottleneck of this process is the breakdown of insoluble macromolecules. The breakdown products of chitin, *N*-acetylglucosamine and glucosamine, are utilized by different microbes at different rates. *N*-Acetylglucosamine is more rapidly assimilated by soil microbiota than glucosamine. The authors concluded that *N*-acetylglucosaminide is an important substrate/intermediate in soil C and N cycling. Plant roots were unable to

Table 2. Major organic polymers of microbial (M), plant (P) and animal (A) origin in soils. Modified from Ruggiero et al. 1996. The molecule entering cell differs for different micro-organisms.

Macromolecular substrate	Structure	Molecule entering cell
Cellulose (P, M)	ß-(1-4)-D linked glucan	Glucose, cellobiose
Hemicelluloses (P)	ß-(1-4)-D linked xylan Glucuronans Galacturonans	Xylose, xylobiose Glucuronic acid Galacturonic acid
Pectin (P, M)	Xyloglucan Galacturonans	Xylose Galacturonic acid
Starch (P, M)	α-(1-4) and α-(1-6) linked glucans	Glucose, maltose
Lignin (P)	Polymers of *p*-hydroxycinnamyl alcohols	Mono-lignols, di- and tri-lignols
Chitin (A, M)	ß-(1-4)-D linked *N*-acetylglucosamine	*N*-acetylglucosamine, chitobiose
Proteins and peptides (A, M, P)	Polymer of amino acids	Amino acids, short-chain peptides
Lipids (A, M, P)	Triglycerides, phospholipids	Glycerols, fatty acids
Peptidoglycan (M)	Polymers of *N*-acetylglucosamine and *N*-acetylmuramic acid	*N*-acetylglucosamine, *N*-acetylmuramic acid, short-chain peptides
Teichoic acid (M)	Polymers of polyol phosphates with saccharides and D-alanine	Glycerol, ribitol, mono- and disaccharides, alanine
Microbial exopolysaccharides (M)	Mannans, dextrans, levans, xanthans, pullulan, alginate	Mono- and disaccharides

take up glucosamine from soils (Roberts and Jones 2012).

Intracellular enzymes associated with cytoplasmic functions play a critical role in the life processes of microorganisms. They do not, however, integrally participate in element cycling in such a way as do extracellular or potentially extracellular enzymes, and are not of special interest in soil quality determination. They are of course needed in the production of new cells and some of them require nutrient input from extracellular enzymes capable of macromolecule breakdown.

1.4.2 Location of enzymes in soils

In soils, enzymes are located in living cells, in dead cells and tissues, in cell and tissue fragments, in solution as free enzymes and immobilized on clays or humic colloids (Gianfreda and Bollag 1996, Ladd *et al.* 1996). Free, extracellular enzymes are short-lived in soils and are rapidly degraded by proteases or inactivated by adverse conditions such as temperature or changes in the conformation of the active site. Immobilization, on the other hand, protects enzymes from degrading and denaturing agents but often causes decrease of activity.

With the exception of dehydrogenase, it is virtually impossible to distinguish between intra- and extracellular enzymes in soils, and attempts involving toluene addition or intensive ionizing radiation have not been successful. Dehydrogenase is solely intracellular and is associated with living, intact cells, its activity representing intracellular flux of electrons to O_2 by enzymes catalysing the transfer of hydrogen and electrons from one compound to another (Nannipieri *et al.* 1990). The intracellular nature of dehydrogenases can be demonstrated by soil fumigation with chloroform, which totally destroys the activity. These intracellular enzymes are involved in cellular activities of various kinds, but do not affect the activity of extracellular enzymes except in enzyme synthesis and perhaps in acquisition of the end products of extracellular enzymes which affects end product inhibition or induction.

A large proportion of the enzyme activity of soils is due to immobilized enzymes bound to organic material, clay minerals or organomineral complexes. An enzyme's isoelectric point, number of binding sites, solubility, concentration, and shape and size (Ladd *et al.* 1996) are the determining factors of bond formation and strength. The means of attachment on clay minerals are adsorption by cation exchange mechanisms, via hydrogen bonding, or with van der Waals forces, which are weak interactions between molecules (Marshall 1976, Burns 1978, Gianfreda and Bollag 1996). Hydrophobic, Coulombic interactions have also been proposed. Their intensity depends on the surface area and charge of clay minerals, and on the nature of saturating cations (Marshall 1976). In Finland, the clay minerals are most often illitic, with low cation exchange capacity.

As humic matter is formed, enzymes are entrapped into the network via copolymerization. They can interact with humus polymers by ion exchange or with covalent bonds (Dick 1997). The hydrophobic nature of enzymes may cause them to migrate to the surfaces of humic matter or clay minerals (Tabatabai and Fu 1992). This physical adsorption may concern several layers of molecules, depending on the molecular size. Chemical, more stable adsorption, involves more specific reactions (Marshall 1976). Macromolecules adsorbing on soil surfaces alter the wetting properties of surfaces and possibly improve the nutritional status near the surface. It may be that microorganisms benefit from location on the surface of humic colloid containing a multitude of enzymes, because they may not have the ability to produce certain enzymes and therefore benefit from other organisms capable of enzyme production.

The amount of organic matter in soils is correlated with the activities of enzymes, but the strength of correlation depends on the enzyme. In a survey combining data from several ecosystems, (Sinsabaugh *et al.* 2008) claimed that hydrolysing enzymes, e.g. ß-glucosidase, cellobiohydrolase, *N*-acetylglucosaminidase (NAGase) and acid/alkaline phosphatase, showed less variation between ecosystems than leucine aminopeptidase and oxidizing enzymes, e.g.

phenol oxidase and peroxidise. It was estimated that a stronger relationship between organic matter and hydrolytic enzyme activities exists than between organic matter and other enzyme activities.

Allison and Jastrow (2006) presented a model for multi-pool enzymatic activity in different microhabitats of fractionated organic and mineral matter. They claimed that enzymes stabilized on mineral particles show low activities in field conditions, which allows for long-lived (turnover rate 196 yrs) organic matter to coexist with high-potential enzymatic activity. In particulate organic matter the activities of the same enzymes are high, enabling rapid degradation of plant-derived, easily degradable organic matter with a turnover rate of 9 to 31 years. It has also been shown that humic and clay colloids are extremely important in preserving enzyme activities against environmental and physical-chemical changes such as freezing, thawing, drying, heating and changes in pH (Lähdesmäki and Piispanen 1992).

The enzyme activities tend to decrease in soils with increasing soil depth, corresponding to the parallel decrease of organic matter (Gianfreda and Bollag 1996), root mass, and proportion of small-sized aggregates (Wick *et al.* 2012). The intensity of each effect is enzyme-dependent (Freeman *et al.* 1995, Niemi *et al.* 2005a). Contrary to the general trend, Naseby and Lynch (1997) reported non-soil depth related rhizosphere activities of acid phosphatase. It was concluded that the independence of phosphatase from soil depth was due to the presence of roots, which are the predominant source of acid phosphatase. However, phosphatase activity has been proposed as an indicator of soil phosphorus state; inorganic phosphorus has an inverse effect on phosphatase production (Skujins 1978).

The litter layer receives the majority of the particulate organic material produced by plants and animals. It is the starting point of most of the macromolecule breakdown, but conditions including temperature and humidity vary rapidly affecting the degradation rate. The enzyme activities and overall microbial abundance are highly dependent on native litter quality (Lähdesmäki and Piispanen 1988, Weand *et al.* 2010), and also vary according to litter quality amended into soil (Bending *et al.* 2002, Chigineva *et al.* 2011, Bray *et al.* 2012). In the very active litter layer adapted fungal species inhabit needles either externally or internally. It has been shown that internal colonizers exhibit higher diversity of enzyme activities than external colonizers, but also that the enzyme activity pattern produced by one species may vary according to growth conditions (Leake and Read 1990, Koukol and Baldrian 2012). None of the enzyme activities measured explicitly differentiated between the external and internal needle colonizers. Of the twelve strains tested, of which seven were external needle colonizers, eleven showed ß-glucosidase activity. Phosphomonoesterase activity and cellobiohydrolase activity were detected in eight strains (Koukol and Baldrian 2012).

In addition to litter, rhizosphere exhibits high activities of enzymes, probably due to nutrients in root exudates (Kandeler *et al.* 2002, Marschner *et al.* 2012). Although the effect of roots and litter in soil extends to several millimetres of root or litter surface as measured by C incorporation from the source, the activities of enzymes are enhanced only at a distance of about 1 mm (Kandeler *et al.* 2002, Marschner *et al.* 2012). In other studies, the influenced zone of enzyme activities has been wider, up to 3 mm (Poll *et al.* 2006).

The activities of enzymes appear to depend on a multitude of factors, uncontrollable in field experiments. Furthermore, the heterogeneity of soil environment poses a challenge to both the sampling strategy and interpretation of the results. In a study utilizing field and grassland soils Lebrun *et al.* (2012) pointed out that grassland soils had more spatiotemporal variation at the scale of meters than cultivated soils. The activity level was also higher in grassland. It has also been speculated that in some cases the variation within ecosystems may be underestimated due to the low number of samples, because the coefficient of variation was found to correlate with the number of samples (Sinsabaugh *et al.* 2008). The sampling should be planned with extreme care and carried out in the

same way by all the samplers. The soil depth-related decrease in microbial diversity is strong and even small differences in sampling depths may affect the outcome of diversity studies (Eilers *et al.* 2012); it should also affect the level of enzyme activities. Horizontal heterogeneity should be taken into account at a level relevant to the hypotheses of the study (Wallenius *et al.* 2011). Temporal variation is of importance if comparisons are to be made between different sampling dates or places, since temporal changes in soil attributes can be consequential due to many factors, most obviously soil moisture (Baldrian *et al.* 2010b, Tiemann and Billings 2012). Fluctuations of soil enzyme activities throughout seasons have been studied by a multitude of research groups, but the factors affecting enzyme activity and temporal stability of spatiotemporal scales still need to be defined (Kandeler *et al.* 2011).

It is plausible that at least to some extent boreal soil enzymes exhibit characteristics of psychrophilic enzymes. This is due to the long cold period of the Finnish winter, when the uppermost soil matrix is frozen. The reactions causing degradation of litter macromolecules produce a vast amount of readily available nutrients, enabling a growth and activity boost immediately after snow melt. Psychrophilic enzymes possess increased molecular flexibility, leading to a looser structure of the active site (Aghajari *et al.* 1998, Gerday *et al.* 2000), and it is therefore expected that these enzymes would show less specific selectivity for substrates than other enzymes. Margesin *et al.* (2009) detected cold-adapted microbes and enzymes in soils close to the temperature conditions of Finnish soils.

Despite the exact location of soil enzymes, the potential enzyme activity measurements yield the sum of activities present in the sample of soil in question.

1.4.3 Factors affecting enzyme activity

There are several factors affecting the enzyme activity of soils, and great efforts have been made to unravel their influence and interactions, or even the direction of change. Due to heterogeneity of soils and the variety of methods used, it is difficult to generalize the cause-and-effect relationships between different treatments and activities of enzymes (Gianfreda and Bollag 1996). Even in aquatic environments the causality of enzyme activities is difficult to interpret (Chróst 1991, Münster and De Haan 1998, Arnosti 2011).

The conditions in soil vary according to season. Temperature changes markedly only in the uppermost layers of soil, but humidity is at its highest level after snow melt in the spring and during the rainy season in the fall in the boreal zone. Substrate boost is expected in the autumn, when leaves fall and plants prepare for winter. Another boost may occur in the spring, when snow melts and soil temperature increases (Gianfreda and Bollag 1996, Niemi *et al.* 2005a), but the activity of soil enzymes is persistent in soils at 0 °C under snow cover (Bárta *et al.* 2010). The enzymes usually respond to season but the level of variability depends on the enzyme analyzed (Rastin *et al.* 1988, Rastin *et al.* 1990). The seasonal effects depend on the soil layer analysed, the uppermost layers responding more sensitively to season than deeper soil layers (Rastin *et al.* 1990). Seasonal fluctuation obviously depends on the climate zone of the sampling site. Seasonal variation of ß-glucosidase has been observed to be lower than the variation between years (Bergstrom *et al.* 1998).

Soil moisture has a profound effect on many extracellular enzyme activities, and it varies due to season, climate zone and small scale terrain. In fact, the most commonly measured soil enzymes, hydrolases, require water in the reaction which they carry out. Since water is the carrier of molecules in soil as well as in other environments, its abundance has a pronounced effect on enzyme activities. This was studied by Alarcón-Gutiérrez *et al.* (2010) by drying-rewetting cycles of Mediterranean zone litter layer. They concluded that soil drying decreases enzyme activities due to desorption of enzymes, conformational changes and formation of complexes between enzymes and organic components in an abiotic way. Water is also the main component of the intracellular matrix and af-

fects enzyme synthesis and the distribution and activity of intracellular enzymes.

Soil enzymes are most abundant and active in the uppermost layers of nondisturbed soils (Naseby and Lynch 1997, Šantrůčková *et al.* 2004), possibly due to highest cell density and total biomass and the greatest amount of substrates originating from plant litter. In litter itself, the microbial consortium is different from soil microorganisms (Bray *et al.* 2012). In the beginning of litter decomposition, the properties of litter determine the decomposition rate. Labile substrates are first degraded, but as the decomposition proceeds the microbial consortium present defines the rate of decomposition.

In an environment with an abundance of easily assimilable nutrients accessible to individual cells, microbes do not need to produce enzymes for breakdown of complex molecules in abundance. When limited labile substrate resources exist the enzyme production for degrading complex molecules is enhanced, depending on the availability of C, N, and P ratios for biosynthesis. This was studied by Allison and Vitousek (2005) by adding simple and readily assimilable substrates alone or in combination with complex substrates into soil. The basal rate of enzyme activity was thought to increase from constitutive production of enzymes to maintain a basal level of activity. They also concluded that since a pool of stabilized enzymes with varying activities is always present in soils, the responses of all enzyme pools may not be regulated in short time scales. Therefore, short experiments may reflect the response of newly synthesised enzymes or the rate of enzyme production during the experiment (Allison and Vitousek 2005). It should also be noted that the activity of enzymes is affected not only by the nutritional needs of microbes but also by the capacity of the surrounding soil to adsorb the end products. Šantrůčková *et al.* (2004) showed that around ten times the amount of microbial P demand is produced by soil enzymes in a soil with high adsorption capacity and that less excess P is produced in soils with lower adsorption capacities.

Throughout ecosystems, and irrespective of calculating enzyme activities per dry weight or SOM, the soil pH was estimated as the predominant factor affecting potential extracellular enzyme activity (Sinsabaugh *et al.* 2008). NAGase was strongly and negatively correlated with soil pH; other hydrolytic enzymes involved in C metabolism were weakly negatively correlated and leucine-AP and oxidases positively correlated with soil pH. This is of interest since the pH of niches occupied by enzymes may differ from the bulk soil pH. It is also worth noting that pH affects soil functioning on different scales: it influences the plant community composition, soil weathering, nutrient availability etc. (Sinsabaugh *et al.* 2008). Contrary to the findings of Sinsabaugh *et al.* (2008), Štursová and Baldrian (2011) observed that the value of pH and the quantity of humic substances had no significant impacts on enzyme activities in grassland soils. In forest soils of the same study, pH and the quantity of humic matter (measured as absorbance at 465 nm) were dominant factors, but in grassland soils the molecular mass of humic compounds (absorbance ratio of 465 nm and 665 nm) and the content of calsium were significant for many of the enzymes studied. In all the soils studied, the content of organic matter was the most important factor affecting enzyme activities (Štursová and Baldrian 2011). Another significant factor in forest soils governing enzyme activities may be moisture (Baldrian *et al.* 2010b).

According to a thorough literature survey of Gianfreda and Bollag (1996), potential enzymatic activity appears to be enhanced by substrate additions in several experiments. This is in accordance with the assumption that substrate concentrtions are limiting in soil environments. Since N is often a limiting nutrient in forest soils, N mineralisation rate is of interest. NAGase activity has been shown to increase with decreasing C:N ratio (increasing relative N content) (Ekenler and Tabatabai 2003, Andersson *et al.* 2004) and it has been suggested as an indicator of N mineralisation rate. The addition of only C decreased but addition of C and N was shown to slightly increase the activities of invertase, xylanase and phenoloxidase or have no effect, depending on the litter type (Chigineva *et al.* 2011). By contrast, some au-

thors have connected increase in soil N status with decreased activities of hydrolytic enzymes (Weand *et al.* 2010) but this decrease depended on the cover plant. Tateno (1988) concluded that both cellulase and protease activities are enhanced by adding easily decomposable substrates. Arylsulphatase has been suggested to be linked directly to microbial activity, since its activity is increased with organic amendments that are not substrates for this enzyme (Naseby and Lynch 1997). The activity of arylsulphatase has been proposed to be mainly extracellular (Germida *et al.* 1992). Its activity is also significantly correlated with soil organic matter content, which may cause the enhancement of activity after organic matter addition (Tabatabai and Bremner 1970, Naseby and Lynch 1997). In general, the enzyme activities correlate with measures of microbial activity, i.e. C_{mic} and basal respiration (Andersson *et al.* 2004, Šantrůčková *et al.* 2004), indicating that enzymes are associated with active microbial biomass. It has been shown that enzyme activities correlate with organic matter content even if they are calculated as specific activities, i.e. by SOM (Wallenius *et al.* 2011). The enzyme activity profile yields a more versatile picture of the studied area than a general measure such as SOM or basal respiration.

In *Eucalyptus diversicolor* forest soils, no correlations between soil P fractions and phosphatase or phosphodiesterase activities were observed (Adams 1992). In a continent scale study, Sinsabaugh *et al.* (2008) concluded that in weathered soils the activity of phosphatases was higher than in less weathered soils, and they judged that P acquisition is more pronounced than N acquisition. It is easier to find N sources than P sources in the environment, because N is unlimitedly available in the atmosphere if adequate reaction pathways are available, whereas P is derived from mineral materials through weathering. This may also be the cause of inconsistency in results of enzyme activity studies between N addition and activities of enzymes (Sinsabaugh *et al.* 2008). For phosphatase activity, the addition of end product in the form of phosphates and fertilization diminishes the activity of enzyme (Trasar-Cepeda *et al.* 1991, Dick 1997), possibly indicating end product inhibition. The use of phosphorus fertilization may, therefore, prevent the use of PME as an indicator of soil quality.

The impact of heavy metals on enzyme activities has been widely studied and the results are somewhat contradictory. Some enzymes even require heavy metals such as zinc as cofactors. Enzymes have been reported to respond sensitively to xenobiotics and other harmful substances introduced to soil or sediment systems (Al-Khafaji and Tabatabai 1979, Wittmann *et al.* 2000, Sannino and Gianfreda 2001). Arylsulphatase, for example, does not require metal ions for functioning of the active site, and is inhibited by a vast range of trace elements. On the other hand, Lebrun *et al.* (2012) showed in a terrestrial model ecosystem study that copper had no effect on six soil enzyme activities, namely acid and alkaline PME, ß-glucosidase, NAGase, urease and dehydrogenase, at concentrations of 2 mg/kg or 200 mg/kg. They explained this by assuming that all the enzymes measured were commonly present in all microorganisms, and that inhibitory effects on some species or some types of enzymes were revoked by organisms or enzymes insensitive to copper. Based on the study of Koukol and Baldrian (2012), not all fungi contain the enzymes studied, which diminishes the weight of this assumption. The buffering capacity of soil and the presence of adsorbent molecules were not taken into account.

Management of agricultural and forest soils is practised to produce greater crop yields. Less disturbed soils, e.g. pasture, meadow or no-till cultivation, usually contain higher enzyme activities than soils under efficient management (e.g. Dick 1984, Gupta and Germida 1988, Katsalirou *et al.* 2010). Tilling, for example, leads to loss of soil microbial biomass and has a negative effect on soil enzyme activities (Gianfreda and Bollag 1996). Amending soils with organic residues usually enhances enzyme activity, and ß-glucosidase has been cited as consistently responding to management treatments (Bandick and Dick 1999). Addition of pesticides may either stimulate or inhibit the activity of enzymes depending on the enzyme, chemical compound

added and soil type (Sannino and Gianfreda 2001). Carreira *et al.* (2008) utilized potential enzyme activities in verification of the rehabilitation of polluted soil. These treatment effects have mainly been measured from bulk soil from a depth of about 10–20 cm.

It is appealing to use laboratory model systems or greenhouse experiments to measure environmental variables. They are readily controllable over a variety of conditions and the results are more reproducible than those of field tests. Actual field conditions pose a great problem for true replicate experimental units, interpretation of data and weighing the significance of uncontrolled variables such as weather.

1.4.4 Enzyme activity method development

Several soil enzyme assays have been developed to detect the total potential activity against a specific substrate since the middle of 20th century. Various research groups have developed their own assays with different pretreatments, substrates, and assay conditions. It is almost impossible to measure the activities of soil enzymes *in situ*, retaining the soil properties intact throughout the process. Therefore the investigator must choose, using the best evaluation available, the assay conditions which best define the parameters of potential enzyme activity being measured. The various methods used make the comparison of results from different groups difficult or even impossible (Gianfreda and Bollag 1996, Baldrian 2009). A unique aim of many recent development steps has been prevention of enzyme synthesis and microbial growth during the assay. Great effort has been directed to the distinction between intracellular and extracellular enzymes, but with only limited success.

Extraction of enzymes from soil samples would be the optimal pretreatment for enzyme assays to enable easier handling of samples, less hindrance in product detection, easy dispensing of the sample and less variation in results due to handling a more homogenous liquid rather than a suspension. Naseby and Lynch (1997) reported successful measurement of 10 enzymes from 2 g of soil by extraction using mainly *p*-nitrophenol-linked substrates. Tabatabai and Fu (1992) compiled methods for enzyme extraction ranging from harsh methods involving alkali extraction or sonication to milder methods such as shaking with different buffers. During long extraction procedures, growth inhibitors such as toluene have frequently been used, but these have drawbacks such as interference with carboxymethylcellulose substrate, reducing its viscosity (McClaugherty and Linkins 1990, Tabatabai and Fu 1992).

The choice of buffer has a pronounced impact on enzyme activity results. It has been reported that unbuffered assays yield lower potential activities (Gianfreda and Bollag 1996) than buffered ones. The use of buffer enables standardized conditions throughout assays and a better comparison between experiments. Both the pH and the ionic composition of the buffer in question affect the reaction. In soil, the microniches inhabited by enzymes differ in pH and the gradients in soil can be sharp. Nevertheless, unbuffered assays have been adopted by e.g. Wittmann *et al.* (2004).

The reaction temperature for soil enzyme assays can be chosen from a broad range, since the immobilized enzymes usually tolerate even high temperatures without denaturation. The reactions are faster at higher temperatures, and therefore the assay temperature also affects the incubation time needed: at lower temperature a longer incubation is needed for a detectable amount of end product to be developed. For the assays of extracellular enzymes, the incubation time should ensure a constant reaction rate and the utilization of only a small fraction of the available substrate in the assay used (Gianfreda and Bollag 1996). Growth of microorganisms and production of newly synthesized enzymes should be prevented in order to study the potential activity of the soil sampled. It is possible to use inhibitors, e.g. toluene, to avoid growth, but these substances may affect the reactions in other ways. Therefore, a relatively short incubation time is preferred.

A variety of substrates have been used in enzyme activity measurements, from natural or near-natural substrates such as crystalline cel-

lulose to small substrate proxies (Hoppe 1983, Chróst 1991, Münster and De Haan 1998, Arnosti 2011), typically a monomer linked to a fluorophore or a chromophore (Figure 1). The choice of substrate is of major importance: cleavage of natural substrates exhibits more closely the reactions occurring in soil, since both endo- and exocleavage is measured and the three-dimensional structure of a molecule plays a role in the enzyme-substrate complex formation. In these assays the decrease in substrate concentration or accumulation of end products can be detected. Both are insensitive ways of measuring reaction rates, because only relatively large amounts of substrate removal or end product formation can be detected in soil samples.

In artificial substrate molecules, chromophores or fluorophores are linked to monomers or dimers of the relevant macromolecule in order to represent the hydrolysis of macromolecules with subsequent release of derived coloured or fluorescent end product. They may also readily enter the periplasm of cells for hydrolysis (Martinez and Azam 1993, Arnosti 2011). These substrates, or substrate proxies, do not resemble the three dimensional structure of natural substrates and only the final reactions in degradation pathways can be measured. Usually the exocleavage of non-reducing ends of sugar macromolecules is detected but endo catalysis remains undetected. Fluorogenic substrates were first applied in water studies (Hoppe 1983, Chróst and Krambeck 1986), and Münster *et al.* (1989) developed this methodology further. In soil, the chromophore *p*-nitrophenol has been widely used (Alef and Nannipieri 1995, Schinner *et al.* 1996), and, subsequently the fluorophores methylumbelliferone and aminomethylcoumarin (AMC) derivatives have been increasingly applied.

Chromophores are less sensitive, and difficulties in spectrophotometric detection of turbid or dark samples occur compared with fluorophores. The fluorophores are more sensitive, but quenching caused by soil humic compounds interferes with the detection of end products (Münster *et al.* 1989, Freeman *et al.* 1995, Marx *et al.* 2001, Baldrian 2009). The quenching can be taken into account using suitable controls and blanks, usually sample and sampling time specifically (Freeman *et al.* 1995). In both cases, detection of released artificial molecules is achieved at low concentration even from soil samples. It should also be noted that humus and clay colloids of soils are essential in preserving the activities of enzymes against environmental changes (Lähdesmäki and Piispanen 1992).

Widely used substrate proxies should always be used with caution and care should

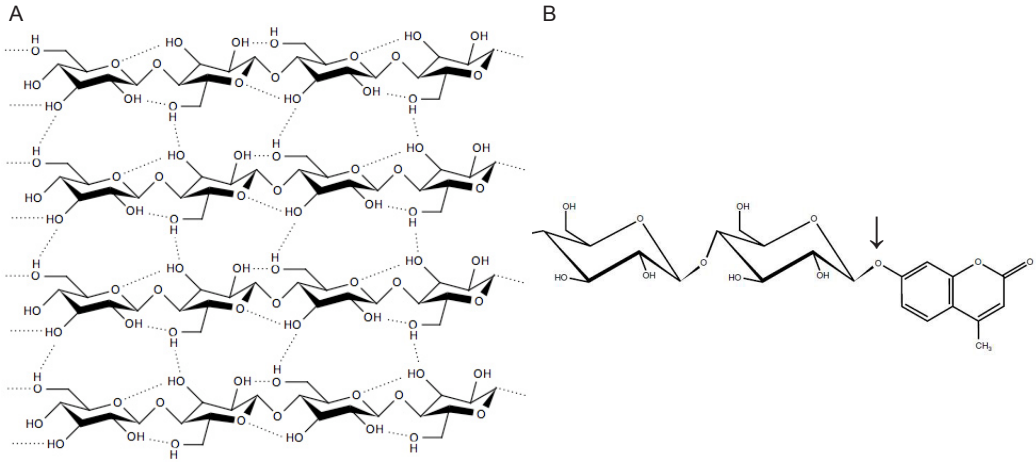

Figure 1. Schematic images of [A] a cellulose macromolecule, the naturally occurring substrate for cellulase (UC Davis BioWiki 2012) and [B] an artificial substrate for cellobiosidase, 4-methylumbelliferyl ß-D-cellobiopyranoside (Glycosynth Ltd 2012, p. 27) with the site of cleavage indicated with an arrow. Cellobiosidase is one of the enzymes carrying out the last steps of cellulose hydrolysis.

be taken in interpretation of the results: the results are for the breakdown of the specific artificial substrate and represent the catalytic activity of a group of enzymes capable of this particular exo-acting process, not necessarily of breakdown of the natural macromolecule. In marine environments, fluorescently labelled macromolecules have been used as substrates in order to achieve greater resemblance to natural substrates. Hydrolysis rates are detected by the decrease in substrate size (Arnosti 2011). This approach probably could not be adopted in soil environments because of the high background fluorescence of soil matrix and the difficulty of separating the substrate molecules from the matrix.

In soil, the substrate concentration generally inhibits the activities of enzymes, since it is not at the saturation level (Naseby and Lynch 1997). The naturally occurring substrates are usually insoluble compared to soluble, artificial substrates. Artificial substrates are provided in a homogenous form, whereas the natural substrates are often mixed with other molecules, i.e. cellulose is combined with highly resistant lignin.

The emission and excitation of widely used MUF and AMC substrates have emission and excitation maxima overlapping with organic material (Arnosti 2011). The excitation and emission wavelengths also have a great effect on the pH dependence of these molecules; Münster *et al.* (1989) reported that a 25 nm shift in emission wavelength (from 355 nm to 330 nm) reduced the pH dependence and actually yielded higher fluorescence counts with 330 nm excitation.

Methods based on fluorescent substrate derivatives for measurement of potential soil enzyme activities have been developed by several research groups and used with success in resolving different study aims. Freeman *et al.* (1995) used fluorogenic substrates for the first time for soils when studying peat with low activity. Their assay was conducted without pH control during incubation similarly to the microplate assay developed by Wittmann (2000) and further utilized by Kähkönen (2003). A multiwell plate method with fixed pH was also introduced (Marx *et al.* 2001), and this group measured the increase in fluorescence as a function of time to avoid the need for blank measurement. Quenching of fluorescence produced has been a challenge to be overcome, and e.g. autoclaved samples have been measured to obtain a blank measurement (Wittmann *et al.* 2000, Marx *et al.* 2001). A high pressure liquid chromatography (HPLC) method for detecting reduced amount of substrate has been used for simultaneous detection of multiple enzyme activities using fluorescent substrate derivatives (Freeman and Nevison 1999, Stemmer 2004). The substrate recovery rate from soils was not sufficient to yield a sensitive measurement (Stemmer 2004). It has also been suggested to extract the MUF end product formed with chloroform and to detect the amount formed with HPLC (Freeman 1997). A special multiwell plate method has been developed for example to detect enzyme activities of ectomycorrhizal root tips (Pritsch *et al.* 2004, Pritsch 2011).

There have been some efforts to measure in situ activities of enzymes, for example by Hirsch *et al.* (1998). They saturated filter papers with MUF-substrate solutions and pressed the papers against soil profiles. The fluorescence was detected in the laboratory under ultraviolet light. This was a fascinating attempt to reveal in situ activities, but the contact of substrate with enzymes in soils is restricted, and it is anticipated that several replicate measurements will be needed to reveal the heterogeneity of soils examined. Furthermore, proper *in situ* conditions would require natural substrates to be used and it may be that the exact sites of activity are masked by the diffusion of reaction products on the wet filter paper.

Very recently, Dick *et al.* (2011) proposed a method based on soil near infrared reflectance spectroscopy to develop a model to estimate the activity of the soil enzymes ß-glucosidase and ß-glucosaminidase together with estimates of organic carbon and amino sugar content of soils. In another study (De Cesare *et al.* 2011), an electronic nose was applied to model the activities of several enzymes. It is fascinating to see these new technologies emerging, but their usefulness must be carefully considered as

the relevance of the measurements modelled is hitherto unclear. In revealing activity at a scale of single cells, two and three dimensional measures have been applied to detect phosphatase activity (Díaz de Quijano *et al.* 2011).

The methods for soil enzyme analysis require development from two perspectives: methods with high sensitivity and ease of use are needed, together with a wider selection of enzymes assayed. From another point of view, the conditions of measurements should be comparable in order to yield equivalent results for comparison and analysis of large data sets for deeper understanding of soil enzymology, soil processes and microbial functions.

2 Aims of the study

2.1 Background

The state and quality of soil in Finland is traditionally measured through soil chemistry: pH, nutrient, and SOM concentrations, and concentrations of hazardous substances. The methods of soil biology, i.e. SIR, concentration of ATP, DNA-based methods or ecotoxicological studies, have only been applied by research scientists. The need for supplementing the widely standardized chemical analysis pattern with biological measures has been recognised, but the challenge of a suitable methodology has not permitted the widespread use of biological measures in soil state assessment. Several standardized soil microbiological tests have been available for some years, but interpretation of the results is rather demanding.

There is a need for an indicator of soil biological state enabling the interpretation of results in a practical way. Preferentially, the indicator should visualize the effects of changes of agricultural and forestry practices on soil state. The heterogeneity of soils locally, globally and temporally makes threshold values difficult to determine at any level. Our aim was therefore to develop an indicator for measurement of soil functions and their rate and to include a reference site within each experiment to detect the changes induced by soil treatments.

The method developed in this thesis is a measurement of potential enzyme activity patterns under chosen conditions. Suitable measurement parameters were studied, aiming at feasible laboratory work and sensitive enzyme activity measurements. They were not fully optimised for a single enzyme or soil. A set of standard measurement conditions was applied throughout the experiments to allow comparison of the results. This perspective does not require optimal conditions to be used. The substrates were chosen on the basis of availability and the macromolecules they relate to. The most common macromolecules cleaved hydrolytically were included.

Furthermore, the study sites and experimental areas were chosen to represent changes that should be detected in bulk soil. This was of importance, since one aim in the method development was a widely applicable method for routine use in soil quality measurement for the guidance of sustainable land use. The requirements of scientific use of enzyme activity measurements were also fully appreciated.

2.2 Specific aims

A To test the suitability of enzyme extraction as a pretreatment of soil samples for enzyme activity assays (I).

B To develop a method for enzyme analysis suitable for soil samples without separation of enzymes or reaction end-products (II). The requirements in mind were:
   (1) Sensitivity
   (2) Simultaneous measurement of several enzymes to provide an indicator of soil biological functionality

C Selection of different assay conditions to allow for a comprehensive method development (II, III, IV).
   (3) Recommendable buffer(s) for assay
   (4) Standardization
   (5) Comparing different multiwell plates for suitability in enzyme assays

(6) Rapidity of assay to exclude microbial growth
(7) Substrate concentrations
(8) Other conditions

D Testing the applicability and sensitivity of the enzyme activity assay developed in describing and differentiating samples from different seasons or with different treatments:
(9) Temporal variation of soil enzyme activity (V)
(10) Responses to different agricultural management strategies (II)
(11) Responses to cover plant species (IV)
(12) Responses to peat addition for increasing soil organic matter content (IV)
(13) Applicability in acidic forest soil environment (V).

3 Materials and methods

3.1 Experiments

Our aim was to develop a method applicable to field studies. Therefore all our sampling sites and experimental setups were field based and did not involve strictly controlled conditions such as for example in a greenhouse.

Briefly, the extraction efficiency of two commonly measured soil enzymes was assayed using several buffers from two soils with contrasting characteristics in the experiment published in Paper I. The enzyme activity pattern measurement was described in Paper II and applied in a field experiment detecting differences in soil microbiota in barley and rye fields receiving different levels of chemical fertilizers or different organic amendments. The enzyme pattern specific issues, especially stability of compounds and pH optima of the measured enzymes, were the main scope of Paper III. Organic matter amendment in fields of different crop plants for detecting the effects of peat and plant in bulk soil were observed in the experiment reported in Paper IV. Seasonal differences between spring and autumn sampling were observed in Paper II and between two consecutive years in Paper IV in agricultural soils. Paper V described an experiment monitoring enzyme activity fluctuations in two forest stands throughout a growing season. In addition, fluctuation of several other parameters was followed. Detailed descriptions of the experiments are given in the corresponding Papers I–V.

3.2 Soils sampled

The focus of sampling was to reveal changes in areas with similar backgrounds differing in soil treatments. The effect of treatment in the bulk soil was monitored to reveal changes in the scale of agricultural field or soil under different vegetation types. Samples were collected from bulk soil in order to limit extensive small scale spatial variation due to the rhizospheres of different plants, and to obtain a general view of the overall impacts of treatments. A large number of subsamples and a few composite samples were usually taken from replicate plots to enable sensitive statistical testing for the significance of treatments.

Sampling was carried out in the boreal zone from both field and forest sites. A soil sample from greenhouse was applied in the enzyme extraction study (Paper I). The soils sampled are briefly described in Table 3 and more detailed descriptions are given in the corresponding articles.

Forest soils were sampled at a depth of 5 cm of the litter and humus layer after removing mosses from the top of soil if necessary. In general two composite samples consisting of 20–40 typically 10 cm diameter cores were obtained from Harjavalta and Koverhar. In Hyytiälä a 2 cm wide sampling core was utilized and a composite sample consisted of 10 individual cores. Agricultural field soils were sampled from the depth of plough layer, 20 cm, with 2 cm diameter cores by taking 20 sub samples combined to form a composite sample.

All the samples were sieved, for field soils Ø 2 mm sieves and for forest soil Ø 4 mm sieves were used and all visible roots and soil fauna were removed prior to analysis. Field replicates

Table 3. Selected properties of soil and water samples utilized in the experiments of published articles and supplementary experiments.

Study aim / Paper	Site	Land use; vegetation	Sampling time; storage	LOI (%)	Sampling depth
Extraction efficiency of soil enzymes / I	Harjavalta	Forest soil; Scots pine	Stored at +4 °C for 11 weeks	65	Humic layer, 0–5 cm
	Greenhouse	Fine sand	Stored at +4 °C for 11 weeks	3.1	
Mineral and organic fertilization / II	Laukaa Elite plant station	Field; rye, full mineral fertilization	June and August 1999; +4 °C for 1 d	5.7	Plough layer, 0–20 cm
	Laukaa Elite plant station	Field; barley, full mineral fertilization	June and August 1999; +4 °C for 1 d	5.2	Plough layer, 0–20 cm
	Laukaa Elite plant station	Field; barley, half of recommended mineral fertilization	June and August 1999; +4 °C for 1 d	6.2	Plough layer, 0–20 cm
	Laukaa Elite plant station	Field; barley, green plant residues	June and August 1999; +4 °C for 1 d	5.5	Plough layer, 0–20 cm
	Laukaa Elite plant station	Field; rye, composted plant residues	June and August 1999; +4 °C for 1 d	6.1	Plough layer, 0–20 cm
	Laukaa Elite plant station	Field; barley, composted plant residues	June and August 1999; +4 °C for 1 d	5.6	Plough layer, 0–20 cm
	Koverhar	Forest; Scots pine	May and August 1999; +4 °C for 1 d		Humic layer, 0–5 cm
Substrate stability and pH optima of enzymes / III	Kotkaniemi	Field; oat	August 2000; -20 °C	6.6	Plough layer, 0–20 cm
	Laukaa Elite plant station	Field; strawberry		5.1	Plough layer, 0–20 cm
	Harjavalta	Forest; Scots pine, no pollution	September 2000; -20 °C	46	Humic layer, 0–5 cm
	Koverhar	Forest; Scots pine, pollution from Fe-steel works	August 2000; -20 °C	29	Humic layer, 0–5 cm
	Harjavalta	Forest; Scots pine, pollution from Cu-Ni-smelter	September 2000; -20 °C	78	Humic layer, 0–5 cm
	Viikinmäki sewage treatment plant	Active sludge	April 2001; analysed within 3 h after sampling	73	
Cover crop and peat manedment / IV	Laukaa Elite plant station	Field; strawberry, rye, timothy, turnip rape, buckwheat, onion, caraway or fiddleneck	August 2000 and August 2001; +4 °C for 1 d	11	Plough layer, 0–20 cm
	Laukaa Elite plant station	Field; strawberry, rye, timothy, turnip rape, buckwheat, onion, caraway or fiddleneck, peat amendment	August 2000 and August 2001; +4 °C for 1 d	12	Plough layer, 0–20 cm

Seasonal changes, litter type / V	Hyytiälä Forestry field station	Forest; Scots pine	In two week intervals between May and September in 2000; +4 ˚C for <1 d	30–85 depending on the sampling date	Humic layer, 0–4 cm
	Hyytiälä Forestry field station	Forest; alder	11 samplings between May and September in 2000; +4 ˚C for <1 d	75–94 depending on the sampling date	Humic layer, 0–4 cm
Substrate concentration	Harjavalta	Forest soil (H4b); pine	24.9.2001; stored at -20 ˚C	28	Humic layer; 0–5 cm
	Jokioinen	Agricultural soil; timothy	22.8.2001; stored at -20 ˚C	5.8	0–10 cm
	Helsinki, Hakuninmaa	Yard; grass	10.7.2002; analysed on the day of sampling	17	0–5 cm
	River Vantaa	-	3.7.2002; not stored	-	-

of the experiment described in Paper II were abandoned because storage of samples at +4 ˚C for a few days induced marked differences in enzyme activities. Only the analyses carried out immediately after sampling for one replicate field were applicable.

The sampling areas of this thesis are located in the boreal zone where the soil is frozen annually. The temperature of soil in deeper layers is low even during summer. It can be assumed that the enzymes measured exhibit the properties of psychrophilic enzymes, which have a high level of flexibility especially around the active site in order to provide faster reaction rates in cold environments (Joseph *et al.* 2008). The activity of psychrophilic enzymes is higher than that of mesophilic enzymes up to temperatures of 30 ˚C (Gerday *et al.* 2000).

3.3 Overview of the published field and laboratory experiments

A list of methods used is given in Table 3. Detailed descriptions of the methods used for previously published results are given in the corresponding papers and in references therein.

A selection of buffers was utilized in the enzyme extraction experiment (Paper I, Tables 1 and 2) and in further experiments 0.5 M sodium acetate (pH 5.5), 0.5 M Tris-acetate (pH 8.0, for alkaline PME) and modified universal buffer (MUB, pH between 4.0 and 8.0) were applied in enzyme activity measurements.

For statistical analyses, the data was logarithmically transformed when necessary to obtain normal distribution of results. Normalisation was based on the Wilk-Shapiro/Rankit Plot procedure. Enzyme activity data was standardized using averages of each variable in Paper II. Clustering analysis of enzyme activity profiles was performed using squared Euclidian distance as dissimilarity measure (Papers II, IV and V) or Gower's coefficient as similarity matrix (Paper V) and UPGMA (Paper II) or Ward's method (Papers IV and V) as the clustering method. Statistical analyses adopted in the experiments are presented in Table 4.

Table 4. Analysis methods in different soil studies and references to the original publications. PME=phosphomonoesterase, PDE=phosphodiesterase, AP=aminopeptidase, NAGase=N-acetylglucosaminidase.

Method	Brief description	Article	Reference
Sampling	Composite soil samples including 10 to 50 sub samples according to the site. Surface soil samples from 5 to 20 cm depth, core diameter 5 or 2 cm	I-V	
	Activated sludge from Viikki treatment plant taken to a sterile glass bottle	III	
Soil sample pretreatment	Sieving Ø 4 mm of fresh samples	I-V	
Sample storage before enzyme analysis	+4 °C	I	
	+4 °C max 1 d	II, IV, V	
	-20 °C	III	
Enzyme extraction	A modified method: 1 g soil + 5 ml buffer, shaken for 4 h at +30 °C, filtration	I	Mintz 1993; Ogunseitan 1993; Naseby and Lynch 1997
Colorimetric enzyme assays: Arylsulphatase PDE	Activity of enzymes directly from soils and soil extracts using *p*-nitrophenyl linked-artificial substrates	I	Naseby and Lynch 1997
Colorimetric enzyme assays: Xylanase	Formation of reducing sugars from xylan substrate measured spectrophotometrically	II	Schinner et al. 1996
Fluorometric enzyme assays (ZymProfiler®)	Simultaneous activity measurement from soil slurry on multiwall plates using fluorogenic MUF- and AMC-linked artificial substrates		First described in papers II and IV, applied in II-V
	α-Glucosidase, ß-Glucosidase, ß-Xylosidase, NAGase, Acid PME, PDE, Arylsulphatase, Leu AP, Ala AP	II-V	
	Alkaline PME	II, III	
	Cellobiosidase	III-V	
	Esterase, Lipase	II, III, V	
	Lys-Ala AP	II	
Dry weight	Loss of fresh mass at 105 °C	II-V	SFS-EN 12880:2000
Organic matter content	Loss of dry mass on ignition at 550 °C	I-V	SFS-EN 12879:2000
Soil moisture	Continuous measurement with time-domain reflectometer	V	
Humus and air temperature	Continuous measurement using thermocouples	V	
Soil pH	pH measurement in water	II-IV	
	pH measurement in 1 M KCl	II-IV	
	pH measurement in 0.01 M $CaCl_2$	V	ISO 10390:2005
Electrical conductivity	Measured from soil-water suspension (1:2.5 v/v)	IV	
CO_2 efflux from soil	Continuous measurement using automated chambers		Ilvesniemi and Pumppanen 1997
Inorganic nutrients	Soluble nutrients analysed in a commercial laboratory using ammonium acetate extraction at pH 4.65. P measured spectrophotometrically using molybden blue, Ca, K and Mg using ICP spectrophotometer.	IV	Vuorinen and Mäkitie 1955
ATP	A bioluminiometric method after trichloroacetic acid extraction of soil	II, IV	Vanhala and Ahtiainen 1994
Phospholipid fatty acid analysis	Blight Dyer extracted lipids separated with silica column. FAMEs derived after a mild methanolysis determined by gas chromatograph/flame ionisation detector. Chromatogram peak sum used as a measure of biomass; some peaks tentatively identified	IV, V	Frostegård et al. 1993, modifications described in Paper IV

Table 5. List of statistical methods applied.

Method	Program name	Article	Reference
Correlation	SPSS programs	II	
	Statistix for Windows	V	
Analysis of variation	Statistix for Windows	III, IV	
Clustering analysis	ZymProfiler™ programs	II, IV,V	Designed in Finnish Environment Institute for enzyme activity measurements and other data

3.4 Supplementary experiment: Substrate level confirmation

The sufficiency of substrate concentrations selected on the basis of preliminary tests and published literature for water samples (Hoppe 1983, Münster *et al.* 1989, extrapolated to soils) and for soils (Freeman *et al.* 1995) was further evaluated with several different soil samples and substrate concentrations. The aim was to check that the chosen concentration (500 µM) of different substrates was not inhibitory or substantially below the saturation level in different soil samples. A suitable concentration for each specific substrate applicable for a variety of soil types is an advisable feature of a test series, since it is not always feasible to optimize the substrate level for each sample. Additionally, one activated sludge sample was included in the trial. All test parameters are given in Table 6.

Two buffers were applied in the experiment for all the soil samples, namely 0.5 M Na-acetate at pH 5.5 (Paper II) and modified universal buffer (MUB) adjusted to pH 7.0 (Tabatabai 1994; Paper III). The solutions were prepared as described in Paper II but giving the desired substrate concentrations during the incubation and the substrate and standard solutions were freeze dried on multiwell plates. Lipase and esterase substrate solutions were not satisfactorily dissolved at high concentrations after adding the suspended sample solutions on multiwell plates.

Multiwell plates containing both the samples and the standards separately for each soil sample were incubated at + 30 ˚C.

The results were calculated on soil fresh weight basis.

The suitability of incubation time, 3 h, applied in all the previous experiments was evaluated based on the measurements of this experiment.

Table 6. Test parameters in the substrate concentration experiment. 4-MUF = 4-methylumbelliferyl; AMC = 7-amido-4-methylcoumarin

Substrate	Buffer	Incubation time (h)	Substrate conc (µM)
4-MUF sulphate	0.5 M Na-acetate, pH 5.5	0.5	0
4-MUF α-D-galactopyranoside	MUB, pH 7.0	1	10
4-MUF α-D-glucopyranoside		2	50
4-MUF ß-D-cellobiopyranoside		3	100
4-MUF ß-D-xylopyranoside		6	250
4-MUF ß-D-glucuronide		24	500
4-MUF acetate		48	750
4-MUF heptanoate			1 000
bis-(4-MUF) phosphate			2 500
4-MUF *N*-acetyl-ß-D-glucosaminide			5 000
4-MUF phosphate			
L-leucine AMC			
L-alanine AMC			

3.5 Nomenclature of enzymes assayed

Enzymes are specified according to the reactions catalysed using a hierarchical numerical system, in which a general activity mechanism is described on the first level and the mechanism and possible substrates are defined on the following three descriptive levels of increasing specificity. The levels are numbered to give an Enzyme Commission (EC) number; enzymes differing in structure but targeting the same bonds in a substrate have the same EC number.

This system is maintained by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB). On the broadest level, enzymes are divided into six groups: oxidoreductases (EC 1), transferases (EC 2), hydrolases (EC 3), lyases (EC 4), isomerases (EC 5) and ligases (EC 6). EC numbers, preferential enzyme names, reactions catalyzed and artificial substrates used in the experiments of this thesis are given in Table 7.

To be accurate, the enzyme analysis carried out for soil samples should be described as the activities of different enzymes capable of hydrolysing a specific artificial substrate. This would clearly separate the enzymes attacking natural substrates (e.g. cellulose) and artificial substrate proxies (e.g. 4-methylumbelliferone ß-D-cellobioside). The aim of using substrate proxies is, however, to illustrate the reactions naturally occurring in soils, and therefore the names referring to natural substrates are used.

According to different databases several enzymes may catalyze the cleavage of the same substrate. For example, both ß-xylosidase (EC 3.2.1.37) and β-*N*-acetylglucosaminylglycopeptide β-1,4-galactosyltransferase (EC 2.4.1.38) catalyse the hydrolysis of 4-MUF ß-D-xylopyranoside. The more apparent and descriptive names have been used in the text, in this case ß-xylosidase.

In any microbial consortia, there are several species and genera capable of carrying out any given reaction. The enzymes produced by these different organisms or are isozymes, and they catalyse the same reaction but have different kinetic properties, optimum conditions (e.g. pH) and peptide composition. Different isozymes may also be produced by a single organism in differing environmental conditions. In the experiments included in this thesis it was not significant to emphasize different kinds of isozymes present in a sample but merely the capability of a soil to perform a certain reaction. Nevertheless, it should always be borne in mind that the measured activities originate from different microbes or from plants and animals.

Authors often prefer to refer to more general and descriptive names of enzymes, and therefore names such as chitinase for *N*-acetyl-glucosaminidase (NAGase) and cellulase for cellobiosidase are used (chitinase was used in Papers II–V and cellulase in Paper IV). Nevertheless, the substrates used in many of the experiments in soil environment are monomers or possibly dimers connected to a fluorogenic (4-methylumbelliferyl) or chromogenic (*p*-nitrophenyl) derivative. The activities analysed using artificial substrates are solely exohydrolysis, i.e. the measured reactions occur only at the very end of macromolecules, releasing a terminal sugar or an aminopeptide monomer or dimer from macromolecule.

The enzymes chitinase and cellulase, for example, are complex molecules degrading large macromolecules in several steps in soils and other environments. The degradation of simple artificial molecules possibly describes the potential of one step in the pathway of e.g. cellulose degradation, and it should not be straightforwardly referred to as potential or actual rate of degradation of natural macromolecules, even though the aim of enzyme activity measurements is to describe the reactions of breakdown pathways of important macromolecules present in soil.

Table 7. EC numbers and enzyme names for the enzymes analysed in this thesis. The artificial substrates utilized are given together with the reaction description. Description of the reaction catalysed is obtained from NC-IUBMB internet pages, http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/.

EC number	Name	Biochemical cycle; Reaction catalysed	Substrate
EC 3.1.6.1	Arylsulphatase	Sulphur; A phenol sulphate + H_2O = a phenol + sulphate	4-MUF sulphate
EC 3.2.1.20	α-Glucosidase	Carbon; Hydrolysis of terminal, non-reducing (1→4)-linked α-D-glucose residues with release of α-D-glucose; A group of enzymes whose specificity is directed mainly towards the exohydrolysis of 1,4-α-glucosidic linkages, and that hydrolyse oligosaccharides rapidly, relative to polysaccharides, which are hydrolysed relatively slowly or not at all.	4-MUF α-D-glucopyranoside
EC 3.2.1.21	β-Glucosidase	Carbon; Hydrolysis of terminal, non-reducing β-D-glucosyl residues with release of β-D-glucose; Wide specificity for β-D-glucosides. Some examples also hydrolyse one or more of the following: β-D-galactosides, α-L-arabinosides, β-D-xylosides, β-D-fucosides.	4-MUF ß-D-glucopyranoside
EC 3.2.1.52	β-*N*-Acetylglucos-aminidase, NAGase	Carbon; Hydrolysis of terminal non-reducing *N*-acetyl-D-hexosamine residues in *N*-acetyl-β-D-hexosaminides; Acts on *N*-acetylglucosides and *N*-acetylgalactosides.	4-MUF *N*-acetyl-ß-D-glucosaminide
EC 3.2.1.91	Cellobiosidase	Carbon; Hydrolysis of (1→4)-β-D-glucosidic linkages in cellulose and cellotetraose, releasing cellobiose from the non-reducing ends of the chains	4-MUF ß-D-cellobioside
EC 3.2.1.37	β-Xylosidase	Carbon; Hydrolysis of (1→4)-β-D-xylans, to remove successive D-xylose residues from the non-reducing termini	4-MUF ß-D-xylopyranoside
EC 3.1.1.3	Lipase	Carbon; Triacylglycerol + H_2O = diacylglycerol + a carboxylate	4-MUF heptanoate
EC 3.1.1.6	Esterase	Carbon; Acetic ester + H_2O = an alcohol + acetate	4-MUF acetate
EC 3.1.4.1	Phospho-diesterase, PDE	Phosphorus; Hydrolytically removes 5'-nucleotides successively from the 3'-hydroxy termini of 3'-hydroxy-terminated oligonucleotides. Low activity towards polynucleotides.	bis-(4-MUF) phosphate
EC 3.1.3.1	Alkaline phosphatase, PME	Phosphorus; A phosphate monoester + H_2O = an alcohol + phosphate; Wide specificity. Also catalyses transphosphorylations.	4-MUF phosphate
EC 3.1.3.2	Acid phosphatase, PME	Phosphorus; A phosphate monoester + H_2O = an alcohol + phosphate; Wide specificity. Also catalyses transphosphorylations.	4-MUF phosphate
EC 3.4.11.1	Leucine aminopeptidase, Leu-AP	Nitrogen; Release of an N-terminal amino acid, Xaa ┼ Yaa-, in which Xaa is preferably Leu, but may be other amino acids including Pro although not Arg or Lys, and Yaa may be Pro. A zinc enzyme; activated by heavy metal ions.	L-leucine AMC
EC 3.4.11.2	L-alanine aminopeptidase, Ala-AP	Nitrogen; Release of an N-terminal amino acid, Xaa ┼ Yaa- from a peptide, amide or arylamide. Xaa is preferably Ala, but may be most amino acids including Pro (slow action). A zinc enzyme, not activated by heavy metal ions.	L-alanine AMC
	Lys-Ala aminopeptidase	Nitrogen; Release of an N-terminal dipeptide from a peptide.	Lysyl-alanine AMC

4 Results and discussion

4.1 Feasibility of enzyme extraction

Our aim was to develop a method for simultaneous assessment of several enzyme activities, and a uniform pretreatment method, extraction, yielding a homogenous liquid sample for assays was the first step on the way.

Extraction of enzymes has been carried out by number of investigators (McClaugherty and Linkins 1990, Wirth and Wolf 1992, Naseby and Lynch 1997) and at least about 20 enzymes have been extracted from soil environments including hydrolases, lyases, oxidoreductases, and transferases (Gianfreda and Bollag 1996). In general, the efficiency of extraction depends on the type of enzyme and of soil, extraction time, soil:extractant ratio, and buffer properties such as pH, concentration, and chemical composition. The most important factor affecting the mobility (extractability) of enzymes in soils has been reported to be soil pH (Štursová and Baldrian 2011). In our experiment, the enzyme activity yields from gentle extraction methods were compared to enzyme activities assayed directly from bulk soil. The more extreme extraction methods reported in e.g. Tabatabai and Fu (1992) were considered too harsh for proteins to remain active in solution.

Extraction buffers were amended with 1 mM EDTA, 1 mM dithiothreitol and 0.1 mM Pefabloc® SC (Lawson *et al.* 1982, Park *et al.* 1992, Tabatabai and Fu 1992, Ogunseitan 1993). EDTA was added for the metal chelating characteristic, for reduction of oxidation damage and to inhibit the action of metalloprotease activity. Dithiothreitol served to reduce oxidation damage and to prevent the formation of disulfide bonds inactivating the extracted proteins. Pefabloc® SC was used to inhibit the activity of serine proteases.

Arylsulphatase and phosphodiesterase (PDE) were chosen as model enzymes to be assayed because they play important roles in the sulphur and phosphorus cycles, respectively, and methodology for their detection was applicable and allowed for quantification of both bulk soil and soil extract. In a later study Štursová and Baldrian (2011) have shown these enzymes to be more difficult to extract compared with other hydrolytic enzymes such as α-glucosidase or aminopeptidases. They recovered 20–40 % of the activity of α-glucosidase and alanine and leucine AP, whereas the recoveries of arylsulphatase and phosphodiesterase were at the level of 0.2 % (mean values of different soil types).

The soils in the assay were derived from a greenhouse and from coniferous forest in order to exhibit as different characteristics of SOM content, mineral matter content, soil use and cover plant as possible. The extraction yields detected varied between 0.1 and 30 % of the activity of bulk soil. Since the extraction efficiency was not related to soil type, organic matter content or other soil properties in our experiment, a general efficiency rate could not be calculated.

Fornasier and Margon (2007) reported a greatly increased yield of extraction by adding bovine serum albumin and Triton X-100 to the samples. The extraction efficiency reached 13 % for acid phosphatase and 8 % for arylsulphatase and the extractants were light in colour. In general, the efficiency of extractions with salt solutions has been low and, as the extractant is mild, it removes only weakly bound proteins from samples (Tabatabai and Fu 1992). Although the extraction efficiency in the study of Fornasier and Margon was better than in our study, it was still rather poor. The enzyme extraction approach was rejected as unsuitable for evaluating the potential overall activities of enzymes in soil samples because it could not be related to the activity of bulk soil.

4.2 Methodological perspectives of multiple enzyme activity analysis

4.2.1 Stability of enzyme substrates and standard solutions

In order to prepare multiwell plates with substrates and standards preliminarily dispensed onto plates, we first attempted to freeze the

plates immediately after dispensing the solutions. This turned out to be unsuccessful, because the freezing points of some solvents used were below -20 ˚C and deep freezer capacity was not sufficient for multiple plate storage. Freeze drying was therefore applied to streamline the preparation of plates. In order to use fresh solutions, the stocks should have been made frequently, with intervals of three days according to recent studies by DeForest (2009). This would not have facilitated simultaneous analysis of large sets of soil samples.

The freeze dried plates were stored at -20 ˚C covered with lids until the day of analysis. To detect possible chemical conversion of molecules or other changes in fluorescence of standards, a sterile buffer was dispensed to each well of the multiwell plate containing the predried MUF and AMC standards.

At the immediate measurement after dispensing had taken place, the standards were not dissolved (Paper III, Fig. 1, open circles). This was seen as fluctuating fluorescence of AMC standards and low fluorescence of MUF-standards. After the freeze dried MUF and AMC standard solutions had been dissolved within 3 h of dispensing, they were stable over a wide pH range (Paper III, Fig. 1) for 24 h, the length of the experiment. The increase in fluorescence observed between the first measurement (0 h) and measurement at 3 h is due to dissolving of the standard chemicals. The fluorescence of standard solutions is pH-dependent with higher fluorescence at higher pH values (Münster *et al.* 1989). However, even at low pH values the fluorescence of the standard solutions was adequate to enable the measurement of standard curves without increasing pH for example by adding NaOH to the wells.

Depending on the sample, the fluorescence of standard solutions was fitted into a slope or a parabola to calculate the MUF or AMC concentrations of samples. The fluorescence readings obtained from a sample are highly dependent on e.g. pH, humic matter content and salt content, which must be taken into account in standardization of the experiments, i.e. using sample specific standards prepared in soil slurries.

The predried substrates were rather stable in MUB over the pH range of 4–8 and in Na-acetate at pH 5.5 (Paper III, Table 2). Only the substrates of esterase, lipase, PME and NAGase showed significant variation of results according to ANOVA analysis. For NAGase and PME substrates the disintegration took place only at particularly high (NAGase) or low pH (PME) and it was negligible in Na-acetate at pH 5.5 (Paper III, Fig. 2). However, 4-MUF acetate and 4-MUF heptanoate, substrates for esterase and lipase, respectively, disintegrated at a wide pH range during 24 h incubation (Paper III, Fig. 2). The disintegration of 4-MUF acetate and 4-MUF heptanoate was too intense to be corrected computationally, leading to exclusion of lipase and esterase from the test pattern in further studies. In assays of NAGase and PME the buffer pH must be considered carefully if Na-acetate buffer at pH 5.5 is not utilized.

4.2.2 pH optima of enzymes

Enzymes yield highest activities at their optimal conditions. One significant factor is the pH of reaction suspension, which potentially affects the enzyme itself, the substrate molecule and the soil binding sites for both enzymes and substrates. The pH also influences potential inhibitory substances in soils.

Buffers perform two functions in enzyme activity assays: they adjust the pH to a desired level and maintain the pH throughout the experiment (Burns 1978). In order to measure the pH optima of enzymes, the buffering capacity of the buffer used must be adequate to ensure stable pH conditions throughout the experiments. In non-published experiments it was confirmed that our buffers were adequate in this sense.

The forest soils in our experiment had pH_{KCl} values between 2.8 and 6.5 and the agricultural soils exhibited pH_{KCl} values of 4.5 and 5.6. The pH range of buffers was slightly higher, ranging from 4 to 8. The bulk soil pH plausibly differs from the pH of soil niches and the gradients in soil can be drastic (Marshall 1976). It is also assumed that attachment to soil surfaces (especially clays) affects the optimum pH of enzymes, because the attraction of negatively

charged clay surfaces attracts hydrogen ions, which lowers the effective pH at the particle surface (Marshall 1976).

The results of Paper III indicate clear tendencies of pH optima for different enzymes. Optimum activities were usually detected at low pH values, except for aminopeptidases with optimum pH at 6.5 or higher. For activated sludge the optimum activities were reached at higher pH values than for soil samples, indicating microbial consortia quite different from soil microbiota.

For enzymes involved in major carbohydrate degradation, *i.e.* cellobiosidase, ß-glucosidase, ß-xylosidase, α-glucosidase and NAGase, measurement in MUB at pH 4 or 4.5 yielded maximum activities. The pH optimum was widest in the forest soil sample with high pH contaminated by a Cu-Ni smelting plant, possibly reflecting impacts of heavy metal dissociation controlled by pH. It may also be that the microbes present before chalk fallout still had suitable niches for activity, and species adapted to chalk provided activity with another optimum pH, widening the overall optima of enzymes. In *Fragaria* soil with pH of 5.6 the optimum activity of all of these enzymes was at pH 4.5.

For arylsulphatase, PME and PDE maximum activities were detected in 0.5 M Na-acetate buffer at pH 5.5. In agricultural soils the optimum activity was reached in MUB at pH 6 to 6.5 as well, but for the other soils and for PDE and arylsulphatase the activities measured in MUB fell below the activities measured in Na-acetate.

For the enzymes leucine and alanine AP, NAGase and esterase, 0.5 M Na-acetate at pH 5.5 was not optimal, but the activities measured were at least 30 % of the maximum activity measured in MUB. The maximum activity for NAGase and esterase was reached at pH from 4 to 5.5, depending on the soil and enzyme. In general, the activity in Na-acetate was >50 % of maximum activity for all the enzymes except for esterase, deleted from test pattern due to instability.

PME activity measured in MUB was soil type dependent; the optimum activity was at pH 5 in forest soils with low pH, at 4 in Fe-contaminated forest soil with high pH (resulting from chalk emissions) and at 6–6.5 in agricultural soils. In *Eucalyptus diversicolor* forest soils both PME and PDE showed activity over a broad pH range, but the optima were at pH 4–5 depending on the soil (Adams 1992). Marked decrease was observed only above pH 8.

In conclusion, the optimum pH for different enzymes was usually enzyme, not soil dependent. An exception was PME showing optimum pH depending on the soil sampled (Paper III, Table 5). Even if the optimum activities had clear trends according to enzymes, there were slight differences between soil samples, typically 0.5 or 1 pH unit. This indicates that soils have different kinds of microbial consortia producing enzymes with different reaction optima but catalysing the same reactions, i.e. isozymes. Isozymes have e.g. differences in pH optima, K_m-values and affinity for substrates. Additionally, there may be other sources of enzymes in soils that differ by soil type.

To yield comparable results over a range of experimental setups, the enzyme activities should either (1) always be measured in the same buffer, in our case 0.5 M Na-acetate, pH 5.5, or (2) be measured in optimum pH determined separately for each soil sampled. Measurement in *in situ* pH would be an attractive option, but it is difficult to determine the pH of soil microsites relevant for enzymes due to small niches in soil with variable pH values and gradients between them (Marshall 1976). In Paper II we observed that although 0.5 M Na-acetate is not the optimum buffer for all the enzyme analyses, it yielded rather high activities for all the tested enzymes with the exception of esterase.

McClaugherty and Linkins (1990) sampled O1, O12, O2 and A horizons of forest floor and tested for optimal pH of activities of exo- and endocellulases, chitinase, laccase and peroxidase. They concluded that the optima for both bound and extractable enzymes were around pH 5. These results are in relatively good agreement with our results. In forest soils with pH adjustment carried out with NaOH or H_2SO_4 and measured unbuffered, the optima of enzyme activities fell generally slightly below the pH

optima detected in our study (Wittmann *et al.* 2004). It may be that in their study with unhomogenised samples the pH in soil niches did not reach the pH of suspension during 3–60 min incubation, but their soil samples also exhibited pH_{KCl} of 3.2, at the same level as our forest samples. The pH optima of enzymes associated with ectomycorrhizal roots were broad compared to those measured in soils, and NAGase and for some strains also ß-glucosidase and PME did not yield clear optima (Pritsch *et al.* 2004).

4.2.3 Evaluation of substrate concentration for different samples

Reaction rates of enzymes basically depend on enzyme concentration, activity of existing enzymes and substrate availability. The reaction rates are affected by pH and soil moisture at the microsite of substrate–enzyme interface (Ladd *et al.* 1996) and by the matrix surrounding the protein. In soil systems, reaction rates are usually limited by substrate availability. Excess substrate may also be inhibitory for the enzymatic reaction.

The reaction rate depends on the substrate concentration until all the reactive sites have been saturated with substrate molecules, which is the basis of Michaelis-Menten kinetics widely applied in enzymatic studies. The Michaelis-Menten constant, K_m is reached when substrate concentration enables enzyme reaction at half the maximum level. In order to be able to measure the potential rate of enzymatic reactions the substrate level must be adequate, and therefore we measured the reaction rates with several substrate concentrations, up to 5 000 µM. In the first experiments a concentration of 500 µM, selected on the basis of available literature (Hoppe 1983, Münster *et al.* 1989, Freeman *et al.* 1995) and a preliminary data set, was used for each substrate. The subsequent experiment with a range of substrate concentrations was carried out for more accurate evaluation.

It should be noted, however, that kinetic properties of soil enzymes differ from those of pure enzymes in solution, and that several enzymes catalyzing the same reaction are present in the samples.

The results are presented with representative figures of part of the data. For ß-glucosidase, the highest substrate concentration, 5 000 µM, inhibited the activity of the enzyme irrespective of the soil or the buffer in use (Fig. 2). In agricultural soil the inhibition was evident already at 2 500 µM, especially with Na-acetate (data not shown). For NAGase, on the other hand, substrate concentration of 750 µM already inhibited the activity of enzymes in forest soil (Fig. 3 A). The decrease of NAGase enzyme activities at a substrate concentration of 500 µM was pronounced in forest soils using MUB at pH 7.0 (data not shown).

The activity rate of ß-glucosidase increased with increasing incubation time after only 1 h of incubation, possibly indicating enzyme synthesis in samples. It may also indicate the dissolving of pre-dried substrate, altough this is unlikely since the concentration causing inhibition in soils did not change. Surprisingly, there was no difference in reaction rate between 24 h and 48 h of incubation in forest soils, and the increase in reaction rate was observed up to 3 h of incubation in freshly analysed grass soil. This phenomenon of possible enzyme synthesis was not observed with other enzymes, for which the reaction rates were approximately at the same level throughout the incubation times applied. An opposite phenomenon was seen especially with aminopeptidases; the reaction rate decreased with increasing incubation time. In addition to newly synthesized enzymes, the phenomenon of increasing reaction rate according to incubation time may be explained by the structure of soil: if the enzymes are located in capillaries or microniches of soil, the diffusion of substrate molecules to enzymes takes time.

V_{max} for ß-glucosidase was usually reached using a 4-MUF ß-D-glucopyranoside concentration of 250 µM in forest soil and grass soils (Fig. 2) as well as in agricultural soil (data not shown). With other enzymes, V_{max} was reached at substrate concentrations below 1 000 µM, usually between 250 µM and 750 µM. An exception was seen for alanine-AP, for which V_{max} values were only reached with a substrate

concentration of 2 500 µM. For arylsulphatase, V_{max} was not reached with the substrate concentrations used (data not shown).

The concentration of 500 µM was regarded as the most suitable for the majority of substrates. This concentration was usually not inhibitory to the enzymes and it provided adequate activity, although V_{max} was not reached for all the enzymes in all the soils tested. Higher concentrations often yielded inhibition at least in some of the samples or with one or other of the buffers. A higher substrate concentration (1 000 µM or 2 500 µM) for the assay of arylsulphatase, PME, PDE, ß-xylosidase and aminopeptidases would have been more optimal than 500 µM in most cases. However, concentrations of 2 500 µM and sometimes even of 1 000 µM yielded occasional inhibitory effects with some enzymes. Because it was not feasible to optimize the substrate concentration for all the soil samples, a compromise had to be made regarding substrate concentrations.

NAGase exhibited a clearly different behaviour, and substrate concentrations of 500 µM were inhibitory for this enzyme in forest soils. The results for forest soil in Na-acetate buffer and agricultural soil in MUB (pH 7.0) are given as an example in Figure 3. A lower concentration of 200 µM for the NAGase substrate 4-MUF *N*-acetyl-ß-D-glucosaminide was recommended for further studies.

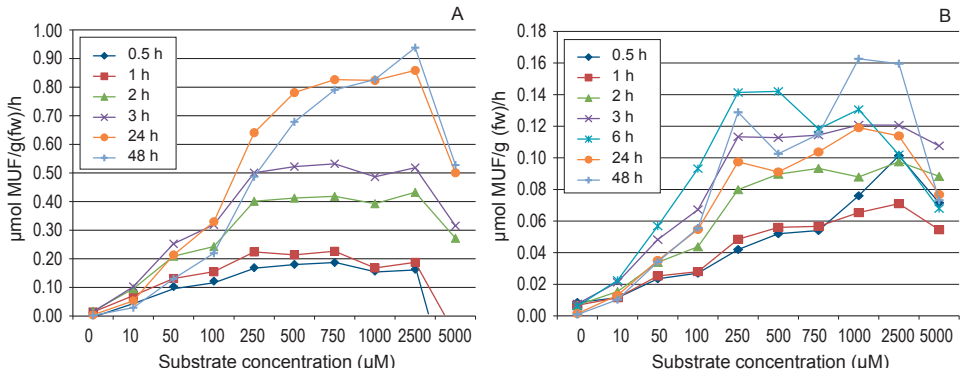

Figure 2. ß-Glucosidase activities in soils using different substrate concentrations and incubation times. A: Harjavalta forest soil in 0.5 M Na-acetate buffer at pH 5.5. B: Grass soil using different substrate concentrations in MUB at pH 7.0. The data from 6 h of incubation is omitted from A due to inconsistency of results throughout the forest soil data at this incubation time.

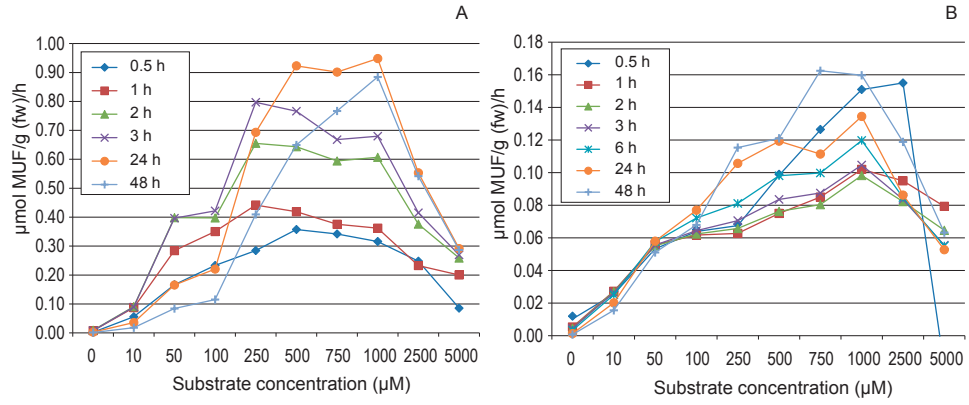

Figure 3. NAGase activities of soils using several substrate levels. A: forest soil in Na-acetate buffer at pH 5.5 and B: field soil with MUB at pH 7.0. The data from 6 h of incubation is omitted from A due to inconsistency of results throughout the forest soil data at this incubation time.

Our results show a similar trend with results obtained for peat soils when concentrations up to 1000 µM were used for the substrates of ß-glucosidase, phosphatase and arylsulphatase (Freeman *et al.* 1995). Although the optimum substrate concentration is soil dependent, it appeared that substrate analogues often inhibit the enzymes at concentrations at or below 1 000 µM. By contrast, Marx *et al.* (2001) did not detect inhibition at a substrate concentration of 900 µM for ß-glucosidase or phosphatase in grassland soil.

Despite possibly higher activity results for some enzymes achieved with substrate concentrations above 500 µM, this concentration was retained for most of the enzyme activity measurements in order to enable rapid dissolution of substrates, to enable the use of the same soil dilution (1:100) for all the enzymes and to facilitate comparison with previous data.

4.2.4 Measurement and incubation conditions

An incubation temperature of +30 ˚C was applied based on the existing literature with a temperature range for soil enzyme activity measurements between 20 and 37 ˚C (Tabatabai and Bremner 1969, Alef and Nannipieri 1995, Naseby *et al.* 1998, Andersson *et al.* 2004, Pritsch *et al.* 2004). This was a convenient temperature for laboratory routine because the plates, reagents etc were not temperated before the experiments. The buffers were used at refrigerator temperature of +4 ˚C and homogenization of soil samples with buffer was carried out on ice. The small volumes pipetted into multiwell plates rapidly reached the ambient temperature. In addition, the reagents were soluble at this temperature whereas at lower temperatures solubilisation of freeze-dried reagents would have been slower after sample addition. This temperature also supported the activity of psychrophilic as well as mesophilic enzymes (Gerday *et al.* 2000). At 30 ˚C, the enzyme reactions were rapid enough to yield differences detectable with the data handling procedure and in a practical sense to facilitate measurement of a large set of plates during a working day. The plates were incubated on a shaker to promote the dissolution of chemicals and contact of enzymes to substrates.

Incubation times between 0.5 and 48 h were tested using the same samples as used for investigating the optimum substrate concentration (Tables 3 and 6). For arylsulphatase and PME the reaction rate was not affected by incubation time, altough incubation times of 0.5 and 1 h yielded somewhat inconsistent results. The same was observed for NAGase with the lowest substrate concentrations (Fig 3b). Cellobiosidase yielded the most consistent results with incubation times between 1 h and 3 h, and for ß-xylosidase, α-glucosidase, PDE and α-galactopyranosidase only an incubation time of 0.5 h yielded higher activity rates compared to other incubation times. ß-Glucosidase and for some soils NAGase yielded the highest variation in activity rate between incubation times.

The dissolution of predried chemicals starts when samples are dispensed on the multiwell plates, but it is not an immediate process (Paper III, Figs. 1 and 2). An incubation time of 3 h was chosen for practical reasons to make laboratory work feasible, but also to ensure the dissolution of chemicals and sufficient contact time for enzymes with substrates after the dissolution. This incubation time was long enough for enzyme reactions to differentiate between samples (Papers II, IV and V). On the other hand, microbial growth is considered to be insignificant within this period, although the ß-glucosidase reaction rate increased during the incubation as shown in Fig. 2A. Shorter incubation time was used by Andersson *et al.* (2004), who measured chitinase (NAGase) activity of small samples after 1.5 h of incubation and by Wittman *et al.* (2004), who incubated soil samples in deionised water for 3–60 minutes. When using such a short incubation time, great care must be paid to accuracy of incubation time in order to avoid unnecessary distortion of results. A longer incubation period allows for more variation in incubation time without causing significant changes in results. Since the reaction rate is not always constant throughout the incubation (Figs. 2 and 3), the results were

not calculated on an hourly bases but per 3 h, the duration of the entire incubation.

It is usually beneficial to measure several samples simultaneously, and this requires long enough incubation times to be able to handle the samples. Based on the experiment reported in this thesis the samples should be incubated for at least 2 h and maximally for 6 h when predried chemicals on multiwell plates are used.

A number of investigators have chosen to measure enzyme activities from air-dried and sieved soil samples (Frankenberger and Tabatabai 1980, Bandick and Dick 1999, Aon and Colaneri 2001, Acosta-Martinez *et al.* 2003, Alarcón-Gutiérrez *et al.* 2010), even though these procedures have been shown to markedly affect enzyme activities (Adams 1992, Wallenius *et al.* 2010) and other soil indicators of biological activity (Zelles *et al.* 1991, Šimek and Šantrůčková 1999). It should be noted that Zelles *et al.* considered one week old samples as fresh in their comparison of changes in soil properties. According to DeForest (2009) storage at +4 ˚C and at -20 ˚C does not introduce significant changes in enzyme activities, which is contradictory to the findings of Stenberg *et al.* (1998) and Zelles *et al.* (1991) for biomass and different soil biological activity measurements. It has been shown that sample storage at -20 ˚C yields least distortion in results compared with storage of air-dried or refrigerated soil samples of different types (Zelles *et al.* 1991, Stenberg *et al.* 1998, Wallenius *et al.* 2010). The moisture content is reported to cause 'background noise' in the enzyme activity results (Alarcón-Gutiérrez *et al.* 2010). This should not be corrected by analyzing air-dried samples, but rather by calculating the results per dry weight. The effect of air drying is not similar in all the samples, nor for all the enzymes, and the magnitude of change is time-dependent (Wallenius *et al.* 2010), so this unpredictable variable would increase the uncertainty of the results and complicate their interpretation. Therefore, it is also not justified to compare results obtained from samples stored for variable time periods. Our method was routinely used for field moist samples analyzed within 24 h of sampling in order to minimize the effect of sample storage. The analyses of Paper III were carried out using frozen samples to enable simultaneous analysis of samples from different field experiments. Immediate analysis of soil samples is preferred, but often storage is required before the laborious laboratory analysis because a representative sampling yields numerous samples to be analysed. Freezing the homogenized soil samples in aliquots used in one enzyme assay is advisable for boreal soils. Samples stored in this way can be thawed rapidly by adding buffer to samples prior to homogenization.

In addition to enzyme activities and other soil biological parameters, storage has been reported to affect the fluorescence of soil matrix (Wallenius *et al.* 2010). The intensity of fluorescence of MUF standards was enhanced especially in soils with high humus content (forest O-horizon and compost). The main reason for this was attributed to water repellency caused by sample storage air dried or frozen at -20 ˚C. In the laboratory, the difficulty of wetting stored humus samples was observed during the analyses described in Paper III (unpublished data). The solution to the increased fluorescence bias was to carry out standardization with similarly stored soil samples.

Soil samples diluted to 1:100 yield a turbid slurry. The MUF- or AMC-linked substrates yielded concentrations of MUF or AMC fluorescent molecules which were detectable in this slurry. For PME and esterase activity in some samples, the 1:100 dilutions yielded such high activities that it was necessary to use 1:1000 dilutions of samples (Paper V). Since dilutions of 1:100 and 1:1000 did not yield comparable results (results not shown), the dilution of 1:1000 was generally avoided and the result from 1:100 dilution was used whenever possible.

The pH of measurements was set using buffers with adequate buffering capacity to ensure a constant pH throughout the entire incubation period. The composition of buffer influences the conditions in the reaction slurry and has a strong effect on potential enzyme activities, as observed in Paper III.

Even if a higher pH during measurement could have resulted in increased sensitivity due to higher fluorescence counts detected at

elevated pH with the same standard concentration (Paper III, Fig. 1; Chróst and Krambeck 1986), the results of Papers II, IV and V show that measurement in 0.5 M Na-acetate buffer at pH 5.5 could separate soil samples according to treatment in a sensitive manner. The homogenisation of samples in a single buffer for all the enzyme activities was applied in order to streamline the laboratory work and to limit random variation, and 0.5 M Na-acetate buffer was shown to be applicable to all the enzymes analysed (Paper III). The increase in fluorescence counts with increasing pH observed in Paper III was more pronounced in MUF solution than in AMC solution and the highest counts for AMC solution were detected in 0.5 M Na-acetate at pH 5.5. After the initial measurement immediately after adding the buffers, the fluorescence was stable throughout the incubation period of 24 h (Paper III, Fig. 1).

In Paper II, 2 M NaOH was added to the reaction mixture after incubation and prior to the measurement in order to increase the pH and to enhance the fluorescence of MUF in accordance with Chróst and Krambeck (1986). The measurements detecting AMC concentration were not amended with NaOH. The addition of NaOH prevented us from carrying out consecutive measurements with a multiwell plate to yield a time series. In non-published experiments it was detected that the fluorescence changed over time in NaOH-treated wells and the enzyme assays were sensitive enough to be measured without the increase in pH, and this procedure was applied in the experiments of Papers III, IV and V. Later on it has been shown by other research groups that addition of NaOH increases the fluorescence but that the increase is time dependent (DeForest 2009), and that care should be paid to the accurate reading time of multiwell plates when using NaOH addition; preferentially it should be avoided.

The multiwell enzyme assay allowed the reaction and measurement conditions to be adjusted to meet the scope of the study and to ensure constant measurement conditions throughout experimental setups. It should be noted that all the conditions described influence the results, which cannot be directly compared if the conditions are changed.

4.2.5 Some methodological aspects and sources of uncertainty

It has not been discussed in the literature that the type of multiwell plate markedly affects the behaviour and fluorescence of substrates and standard solutions. Black, clear and white multiwell plates, optical bottom plates and plates with different sorption capabilities were all tested for suitability of use (data not shown). The material of plates is of importance, and it was evident that the standard solutions of 4-MUF and AMC behaved differently on plastic plates. The multiwell plates most suitable for MUF-based substrates and standards yielded obscure results for AMC-based standards and substrates; the standards did not yield linear fluorescence responses. It was assumed that the coating of multiwell plates adsorbed the AMC molecules. Therefore, NUNC FluoroNunc™ 8x12 white PolySorp™ plates were used for MUF standards and MUF-linked substrates. These polystyrene plates have a coating that is moderately hydrophilic. For AMC standards and AMC-linked substrates, Labsystems® Cliniplate™ White, made of polystyrene without coating, was used.

The pH dependence of MUF fluorescence is also highly dependent on excitation and emission wavelengths (Münster *et al.* 1989) and it may be that the set used in our experiments was adequate to reveal the differences in a pH-independent manner. In a study of Münster *et al.* (1989) a filter set of λ^{exc}_{355} and λ^{em}_{455} yielded highly pH-dependent fluorescence, whereas a filter set of λ^{exc}_{330} and λ^{em}_{445} yielded much less pH-dependent fluorescence and fluorescence counts at low pH values were at a relatively high level. They also concluded that MUF fluorescence was strongly dependent on salt and organic matter composition. In our experiments a filter set of λ^{exc}_{355} and λ^{em}_{460} was used. Other filter sets were not tested because the laboratory equipment producer could not provide other relevant sets.

Sources of uncertainty arise from variation between original reagent batches, from preparing substrate and standard solutions, pipetting the solutions into multiwell plates and dispensing the samples. It was evident that some of the substrates, e.g. arylsulphatase substrate, differed from batch to batch in the amount of free MUF. Therefore, a single substrate batch was used throughout one experiment. In the first substrate batches the shelf life was not declared, but this was improved later on and the amount of free MUF decreased.

The reagents are costly, but larger volumes are more accurate to dispense. We prepared substrate and standard plates in large quantities at a time by freeze-drying the chemicals onto plates. This was for two reasons. Firstly, it enabled the measurement of several samples simultaneously by reducing the pipetting load on the day of analysis by the use of ready-made plates. Secondly, this procedure increased the accuracy when preparing larger volumes of solutions by weighing larger quantities at the same time. Separate, smaller batches of solutions would have yielded higher variation between the batches. Weighing of the substrate and standard compounds is a potential source of variation in preparing multiwell plates. The uncertainty of preparing the substrate and standard solutions can be diminished by using precision scales and preparing larger volumes of solutions; therefore a large batch size of freeze dried plates is beneficial. We adopted a precision scale with a sensitivity of 10 µg for the weighing of reagents.

Freeze drying of substrate and standard reagents necessitates small volumes to be dispensed, and the capacity of a single well in a multiwell plate is limited. Dispensing the solutions into multiwell plates with a multichannel automatic dispenser enhances the reproducibility of pipetting and differences between individuals are avoided. A multi channel dispenser with an accuracy of ±3 % at a volume of 5 µl and ±2 % at 20 µl was applied for dispensing.

Plate batches not freeze-dried simultaneously may exhibit different fluorescence counts. Therefore it is necessary to compare each batch in order to be able to combine results obtained with different batches. The shelf life of freeze-dried plates was found to be at least one year at -20 ˚C. Nevertheless, measurements carried out using fresh substrates and standards instead of pre-dried ones have been carried out by several research groups (Marx *et al.* 2001, Baldrian 2009).

Soil matrix itself is highly heterogeneous, as earlier pointed out. To evaluate the reliability of sampling, two replicate composite samples were taken from forest experimental sites. In field sites, replicate treatments indicated the heterogeneity of soils when one composite sample was taken from each replicate plot. Replicate samples were always analysed and standard curves were determined separately. Soil treatment and minor changes between experimental plots potentially (and in practice) affected the standardization slopes or curves and therefore sample specific standards were strictly applied as recommended by e.g. Münster *et al.* (1989). It may also be possible to cautiously combine information of standards at some level to reduce the work load of result handling.

The correspondence of replicate composite samples was good, with the exception of one experiment on agricultural soil, where one replicate out of three had accidentally received a different treatment than others. This unknown treatment was discovered in the analysis of enzyme activity results, when one field replicate clustered separately (Niemi *et al.* 2008). Furthermore, a delay in measurement requiring additional storage for 2 or more days at +4 ˚C yielded incomparable results for replicate samples in the experimental setup of one field experiment (Paper II).

Sieving of soils with simultaneous removal of large inorganic and organic particles was used to homogenize the samples. Agricultural soils were sieved through 2 mm sieves, but 4 mm sieves were used for forest soils. All the replicate samples from the field were analysed without replicate subsamples. Great care was paid to the weighing of the subsample (used quantitatively in the preparation of soil slurry in buffer), and the soil aliquots were collected throughout the original, sieved sample, to obtain a representative subsample.

Soil samples were mixed with buffer using an efficient and suitable household homogenizer, Bamix®. This device turned out to be more economical, efficient and easier to clean than homogenizers intended for laboratory use. Depending on the sample type, homogenization produced different kinds of challenges, although our homogenisation technique was sophisticated enough to yield slurries from different kinds of soils dispensable onto microwell plates with an automatic pipette. Clay samples were difficult to dispergate. Mineral samples of coarser texture were easier to homogenise, but the coarsest fraction settled very rapidly. Humus samples always included mineral matter that settled in the bottom of the soil suspension flasks, and were therefore excluded from the incubation. Humic particles did not fit into a regular pipette tip, and enlarged tip openings were used to avoid sorting of the sample.

Matrix effect, the variation in subsampling of a laboratory sample, is a major source of uncertainty with heterogeneous and particulate samples (ISO 29201:2012), such as soil. Expanding this view, the matrix effect was taken into account by using replicate field samples, but the samples were not subsampled repeatedly in the laboratory. The matrix effect of soil samples is dependent on the sample type, and in order to yield a good estimate and to reveal the heterogeneity, each sample should be analysed separately for this effect (Wallenius *et al.* 2011). The matrix effect of replicate measurements (subsamples of soil slurries) was diminished by homogenising the sample in buffer to make it more homogeneous and easier to dispense on the plates in three replicates (standards) or four replicates (wells with substrate or blank wells). In our experiments the variation between replicate enzyme activity wells was on average at the level of 10–12 % in both forest and agricultural soils.

Bearing in mind the challenges in interpreting data with different sampling strategies, we sampled the bulk soil at a depth of 5 or 20 cm using subsamples combined as a representative sample of the experimental area. It was assumed that our bulk soil sampling strategy would mask many differences but that a more specific sampling (e.g. from rhizosphere soil) could also benefit from the method developed. The strategy of composite samples consisting of a large number of individual subsamples was chosen to limit random variation, which increases the sensitivity of statistical tests in revealing treatment-related differences in the environment.

4.3 Applicability of the enzyme activity profiling method

4.3.1 General remarks

Extracellular enzyme activities in soils have different roles and they respond to soil treatments in different ways: the magnitude as well as the direction of response varies according to the enzyme and treatment. Our hypothesis was that measurement of a pattern of enzyme activities would reflect the changes in both microbial structure and especially in highly relevant microbial activities in a more sensitive way than analysis of single enzymes, total microbial biomass or other "sum" parameters of soil biota. DNA-based methods reveal the vast diversity of soils in a taxonomic sense, and these methods including extensive sequencing have seen many methodological advances in recent years. The description of soil functions and their diversity was seen as a similar methodological challenge to be addressed.

In our experiments, individual activities of enzymes were often correlated with each other (Paper II, Table 4) and treatment-associated differences for a single enzyme activity were often statistically significant (Paper IV, Table 3; Niemi *et al.* 2005a, Niemi *et al.* 2008). This is in accordance with the findings of other research groups. Acosta-Martínez *et al.* (2007) detected correlation between all the carbohydrate-degrading enzymes studied, i.e. α-galactosidase, ß-glucosidase and ß-glucosaminidase, and in another study (Acosta-Martinez *et al.* 2003) all the measured enzyme activities were correlated. Nevertheless, this is not always the case; it has also been reported that *N*-acetyl-glucosaminidase and cellobiosidase activities did not correlate in forest soils (Andersson *et al.* 2004),

and that the statistically significant correlation between different enzymes was either negative or positive depending on the sampling season or depth with the same enzyme pairs (Aon and Colaneri 2001). Neither the correlations nor the statistically significant differences diminished the need to analyse the pattern of activities in order to reveal the functional activities related to breakdown of different kinds of macromolecules. A larger data set made it possible to distinguish smaller differences in samples or in treatments by using the clustering method for weighted activity results.

4.3.2 Agricultural soils: vegetation

Plant cover of soils affects the functioning of soil systems by providing substrates in the form of leaf litter, root litter and root exudates. Roots also prepare soil matrix by root penetration and the zone of influence is plant species dependent (Niemi *et al.* 2005a). In agriculture, and partly in forestry, the plant type defines the treatments applied for soil, e.g. fertilization and tillage, thus affecting soil processes. In our studies, the effects of cover plant were measured in Papers II and IV in agricultural soils. Both the experimental setups were carried out in field scale.

The differences in enzyme activities of barley and rye fields were somewhat masked by the temporal changes, fertilization treatments and crop rotations in the study in which true replication in the field was not achieved (Paper II). The samples taken from 0–20 cm depth of bulk soil showed only tentative differences in enzyme activities according to cover crop, even though the enzyme activity data was combined in cluster analysis to reveal the pooled changes in the results of single enzymes. In another study, the influence of eight cover crops and their management practices on soil enzyme activities were studied (Paper IV, Table 1). The effect of crop was often significant, as three replicate field plots analysed made statistical testing possible. Cover crop had a significant effect on α-glucosidase, ß-xylosidase, ß-glucosidase, PDE, PME, NAGase, and leucine and alanine aminopeptidase activities on both sampling years. The effect was observed in cellobiosidase activity only in the second year of sampling.

Cluster analysis of soils with different cover crops with or without peat amendment (Paper IV, Fig.1) including enzyme activity, ATP and some chemical and physical data revealed groups based on different soil characteristics. Plots growing strawberry, timothy and caraway tended to group together, possibly due to lack of ploughing of the perennial crops. The activities of PME and PDE were low in these plots (Paper IV, Table 4). Acosta-Martínez *et al.* (2007) detected significant differences in activities of ß-glucosidase, ß-glucosaminidase, PME and arylsulphatase due to changes in land use (pasture, forest, agriculture), demonstrating that a set of enzymes can be used to reveal differences in sites with differing uses. Our results show that even minor changes in soil use, namely cover crop, can be detected using a pattern of enzymes even in bulk soil. It is assumed that the effects would be more pronounced with a sampling strategy concentrating on rhizosphere soil (Kandeler *et al.* 2002, Marschner *et al.* 2012). Furthermore, the addition of plant material affects soil enzyme activities according to the added plant type (Bending *et al.* 2002).

The levels of activities between samples of different cover plants with or without peat varied most for ß-glucosidase and PDE. The activity of ß-glucosidase in the least active treatment was 39 % and that of PDE was 17 % of the activity of the most active treatment when data from two consecutive sampling years were analysed separately. Radical fluctuation of PDE activities was due to high activities of peat-treated onion plots. The slightly higher P fertilization in onion plots compared to the other plots does not explain this phenomenon. For the other enzymes detected, the lowest activity level measured was at 50–60 % of the highest activity level measured, when esterase and lipase activities were considered to be unreliable based on chemical instability of the substrates. Aminopeptidase results yielded rather small differences between treatments and the lowest measured activity was almost 75 % of the highest result, possibly indicating a low level of resolution power (Paper IV, Table 4).

Difference between treatments should be higher than the variation within treatment for the detection of treatment-associated impacts. Relatively small differences and heterogeneous soil matrix necessitate sufficient replication in sampling in order to reveal differences of practical importance. By measuring several enzyme activities and by combining the information with cluster analysis, it was also possible to detect trends even if the individual variables did not yield statistically significant differences. The power of cluster analysis was revealed when all samples from one replicate plot clustered together, whereas the other two replicates were grouped in accordance with treatment (Niemi *et al.* 2008). It was later detected that the aberrant replicate plots had received heavy unintentional liming about 20 years previously.

4.3.3 Agricultural soils: fertilizer and organic matter additions

The loss of organic matter from agricultural soils is commonly restored by adding new organic material to the soil to improve its growth potential (Vestberg *et al.* 2009). When fresh litter is added, it must be modified by soil microorganisms to a more stable form of humus in order to yield long-term effects. Increase in organic matter content of soils is related to increase of several enzyme activities (Dick *et al.* 1988, Martens *et al.* 1992, Kandeler and Eder 1993, Klose *et al.* 1999, Pascual *et al.* 1999, Sinsabaugh *et al.* 2008). In agreement with the findings of other research groups, in our studies enzyme activities tended to be higher in soil treated with composted plant material or peat than in samples without organic amendment (Paper II; Fig. 1 and Paper IV, Table 4). The impact of addition of composted plant residues was still discernable in the soil enzyme activity pattern six years after the treatment when compared with soil receiving only chemical fertilization (Paper II).

Peat addition increased ß-xylosidase activity, and the effect on ß-glucosidase activity and PDE activity was dependent on the time elapsed after treatment (Paper IV). The effects were significant at the $P<0.01$ level or higher. Other enzymes did not react statistically significantly to the 3 cm peat amendment ploughed into the uppermost 0–20 cm of soil (Paper IV, Table 3 and Fig. 1). In addition to the few statistically significant changes in enzyme activities, peat addition increased soil moisture and the content of organic matter. The generally weak impact of peat addition during the duration of the experiment was reflected in cluster analysis results, where peat treatment did not create a specific cluster (Paper IV, Fig. 1). However, as seen in Table 4 of Paper IV, the means of all the enzyme activities studied were higher in peat amended plots than in unamended plots with the exception of lipase, although the differences were in most cases small.

Our results are in agreement with the findings of Kandeler *et al.* (1999) that small scale fractions of soil need a long period (>7 y) for changes in organic C content to appear. Pascual *et al.* (1999) also detected long lasting (>8 y) effects of municipal solid waste amendment on soil carbon content, that were effected by plants growing on the site.

4.3.4 Agricultural and forest soils: temporal changes

The conditions in soils change due to seasonal changes; e.g. moisture, temperature, litter input and root activity are all affected. These temporal changes have been shown to influence soil enzyme activities (Rogers and Tate III 2001, Wick *et al.* 2002, Courty *et al.* 2010, Lebrun *et al.* 2012). It is difficult to examine the determining factors of temporality for enzyme activities in field conditions, if such factors exist, because various different factors operate simultaneously in nature.

In an experiment detecting differences between chemically fertilized field soil and soils in a transitional stage to organic cultivation, sampling date was apparently the dominant factor affecting enzyme activity profile (Paper II, Fig. 1). The spring and autumn samples were separated into different clusters, with autumn samples characterized by generally low enzyme activities but high activities of aminopeptidases. The treatment effect was dispelled due to

lack of replicate treatments as described previously, thus preventing the use of statistical tests for the data. All three treatments were sampled simultaneously, but the analysis took place on a sequential dates. The storage effect on samples was predominant and only the results from the first replicate were valid (Paper II).

In Papers IV and V seasonal variation was further studied. Sampling in consecutive years on the same date yielded significant differences between years in nearly all the enzyme activities in the study of different cover crops and peat amendment on soil enzyme activities (Paper IV, Table 3). The activities were on a higher level in 2001, the latter year of sampling, which may indicate a stronger effect of peat after two years of addition. It has been shown that the effects of organic matter addition may need a considerable period of time before they are reflected in microbial consortia and activities of enzymes (Kandeler *et al.* 1999, Pascual *et al.* 1999, Pascual *et al.* 2000). The first year of sampling was rainy enough to complicate the maintenance of plots, which may also have affected the activities of enzymes.

It has been shown that the highest activities of forest soil reside in the litter layer (Andersson *et al.* 2004). Our sampling included the organic soil horizon with both litter layer and humus soil; only the mosses growing on top of soil were removed. The litter layer is more susceptible to weather and temperature changes, but precipitation readily reaches the humus layer as well at the depth of sampling, namely 4 cm. The temporal pattern was more frequently sampled under pine than under alder since the soil area affected solely by alder was smaller and prevented biweekly sampling throughout the growing period. Thus, temporal effects were clearer for soil under pine. Enzyme activities showed different temporal patterns under pine and alder (Paper V, Figs. 7 and 8); although the pattern of e.g. soil moisture was similar, only the level of moisture was higher in soil under pine (Paper V, Fig. 2). The effect of high activities in spring was evident under pine with the enzymes arylsulphatase, NAGase, ß-glucosidase, ß-xylosidase and α-glucosidase, indicating the activity boost possibly related to the litter input of the previous autumn and rewetting of soil after snowmelt. The activities tended to decrease after this boost and they slowly increased throughout the summer, independently of air temperature. A peak in activities of PME and all the enzymes involved in the carbon cycle was seen in late July, when soil moisture was high after the drought of midsummer (Paper V, Figs. 1 and 7). Accordingly, Andesson *et al.* (2004) observed a peak in NAGase and cellobiosidase activities in a beech forest in Sweden in July. The enzyme activities were not elevated in the spring under alder, with the exception of aminopeptidase which showed somewhat higher activities in May than during the rest of the growing season. The activities generally decreased in the beginning of July, which coincided with the dry season. The effect of drought was more pronounced on some enzymes (PME, NAGase, cellulase, and ß-glucosidase) than on others (PDE, aminopeptidases, ß-xylosidase, and α-glucosidase). Arylsulphatase showed a quite different behaviour throughout the sampling season. A peak in many of the enzyme activities under alder occurred in early September at the end of the growing season. This was evident for both enzymes involved in the phosphorus cycle and for enzymes degrading carbon compounds.

Andersson *et al.* (2004) detected a lower fluctuation of cellobiosidase than of NAGase activity in beech forest. In fact, they concluded that cellobiosidase activity did not fluctuate during the growing season except for one burst in July. They sampled the soil five times during the same period as our sampling took place, 17 and 10 times for pine and alder soil, respectively. In our experiment all the enzyme activities fluctuated and the difference between the lowest and highest activities was at least twofold. Using monthly sampling, Rastin *et al.* (1988) detected PME, PDE and ß-glucosidase activities of beech soil with maxima in April/May. The fluctuation of PDE was low during November–March and the fluctuation of ß-glucosidase was low during July–October. They calculated the results per dry weight. In spruce forest it was detected that seasonal fluctuation of enzyme activities was apparent in the uppermost O layer together

with the 0–5 cm layer but unclear at the depth of 5–20 cm (Rastin *et al.* 1990). Both PME and PDE fluctuated highly in the uppermost soil layer and ß-glucosidase peaked in November both in beech and in spruce soils (Rastin *et al.* 1988, Rastin *et al.* 1990). This might coincide with the activity peak observed in early September in our study (Paper V, Fig. 8), because the sampling in both sites coincides with autumn. Contrary to our finding that all the enzymes involved in the C cycle had a maximum in early September, Boerner *et al.* (2005) concluded that α-glucosidase activity did not vary seasonally in soils sampled in Ohio. However, their results show a statistically insignificant activity maximum in August when specific α-glucosidase activities of non-burned soil are reported. NAGase did not yield high activities in August and most of the assayed enzyme activities did not fluctuate during the growing season, contrary to our findings. It appears that the seasonal variation depends on several factors and a follow-up of enzyme activities through several growing seasons is needed. To link soil moisture and other soil parameters better with enzyme activities, it would be relevant to sample more frequently; the activity of soil microorganisms change over rather short time scales

4.3.5 Forest soils: vegetation

In forest soils with pine or alder as tree vegetation, the phospholipid fatty acid profile was slightly more efficient in separating the two soil types than the enzyme activity profile (Paper V, Figs 6 and 9). Overall, the enzyme activities were higher under alder than under pine, which is in accordance with the result of Andersson *et al.* (2004) that soil under a broadleaved tree exhibited higher activities than soil under a conifer. It has also been shown that enzyme activities in litter including vascular plants is higher than in litter including mosses (Straková *et al.* 2011). Soil under pine had low activities especially during the spring and summer (late May and in June and July).

The levels of NAGase and cellobiohydrolase activities in our experiment were somewhat higher (5 µmol MUF/g SOM/1 h and 1 µmol MUF/g SOM/1 h in pine soil) than the results reported by Andersson *et al.* (2004) with 0.3 µmol MUF/g org C/1 h and 0.02 µmol MUF/g org C/1 h in humus under spruce. However, they measured higher activities under beech than our results showed under either alder or pine. The differences may arise from differences in substrate concentrations, which were 10 µM in the experiment of Andersson *et al.* (2004) and other differences in enzyme analysis.

The composition of litter is characterized by the vegetation of the experimental site and humus is formed from litter through microbial processes. It has repeatedly been shown that litter is a crucial factor in defining the enzymatic or overall microbial activity in soils (Chemidlin Prevost-Boure *et al.* 2011, Bray *et al.* 2012), overriding the effects of e.g. fertilization or water table lowering (Weand *et al.* 2010, Straková *et al.* 2011).

4.4. Overview of applicability

The enzyme activity pattern measurement developed in our laboratory (registered with the now expired trademark for the European Union as ZymProfiler®) has been used in several experimental setups besides the ones included in this thesis. It has been sensitive enough to separate between treatments concerning e.g. soil management, cover crop effect, spatial heterogeneity of soil, and also for applications in pure cultures of fungi and for aquatic environments (Table 8). All the soil experiments have been carried out with bulk soil samples. It is advantageous to measure several activities simultaneously in order to increase the amount of data acquired and to reveal changes in soils more sensitively, even without statistically significant differences.

The activities of all the enzymes were at a detectable level in almost all the samples tested. As an example, a data set compiled from several experiments including different soil samples is given in Table 9.

The range of soil enzyme activity levels in different soils. Minima, maxima and medians are given as µmol MUF/g SOM/3 h or µmol AMC/g SOM/3 h.

All the enzymes measured have shown considerable differences between samples, but as can be seen in Table 9 the results are usually close to the minimum activity (the medians are closer to the minima than the maxima).

The applicability requires strict quality control throughout the experiment. The setup should be designed in such a way as to yield comparable samples for cluster analysis, or other statistical analysis since the activities must be studied as a basis of comparison. Homogenisation and other pretreatments of samples must be carried out cautiously. The weighing and pipetting of chemicals require accuracy to produce repeatable results and to diminish variation in results.

Using a constant substrate level for different samples diminishes the accuracy of meas-

Table 8. Experimental setups for enzyme activity pattern not included in this thesis. The sample type and aim of the study are given together with the references of the published results.

Sample	Aim	Reference
Agricultural soil	Impacts of organic and traditional cultivation, crop plant and peat addition on soil enzyme activity patterns	Vestberg et al. 2002 Niemi et al. 2008
Agricultural soil	Influence of crop plant, soil depth and season on enzyme activity pattern	Niemi et al. 2005a
Agricultural soil	Impact of fungicide and herbicides on soil enzyme activity pattern in potato cultivation	Niemi et al. 2009
Agricultural soil	Impact of different mulches on soil enzyme activities in organic strawberry cultivation	Niemi and Vepsäläinen 2004
Forest soil	Development of enzyme activity patterns in forest organic layer after transfer to a barren hill	Niemi et al. 2006
Agricultural and forest soil	Land use impact, level of variation and spatial structure of enzyme activity pattern	Wallenius et al. 2011
	Soil sample storage optimization	Wallenius et al. 2010
Pilot study using soil columns	Impacts of ethanol and acetate addition for the enhancement of denitrification in ground water formation: changes in soil microbiota	Martin et al. 2009
Litter	Impact of litter type, biodegradation period and peatland type on enzyme activity patterns	Straková et al. 2011
Fungal cultures	Comparison of enzyme activity patterns of fungal pure cultures	Heinonsalo et al. 2012
Aquatic environments	Comparison of enzyme activity patterns in waters in different eutrophic zones	Niemi et al. 2005b

Table 9. The range of soil enzyme activity levels in different soils. Minima, maxima and medians are given as µmol MUF/g SOM/3 h or µmol AMC/g SOM/3 h.

Enzyme	N	Minimum activity	Median activity	Maximum activity
Arylsulphatase	191	0.28	3.3	12
α-Glucosidase	196	0.18	2.3	7.4
ß-Glucosidase	196	1.9	13	39
NAGase	196	3.1	8.0	57
Cellobiosidase	184	<0.1	3.9	20
ß-Xylosidase	196	1.4	5.6	15
PDE	196	2.3	7.9	47
PME	196	19	67	440
Leu-AP	190	<0.1	6.3	15
Ala-AP	191	1.2	9.0	21

urement, but makes the laboratory work more feasible for large experiments with a multitude of samples by eliminating the work of pre-examining the optimal substrate level for each sample or sampling site. Use of the same dilution between samples was observed to be necessary in order to enable comparisons between experiments and sampling sites. Based on the results shown in this thesis and on some unpublished results, a concentration of 500 µM can be applied for boreal forest and agricultural soil samples for α-galactopyranosidase, α-glucosidase, arylsulphatase, 4-MUF ß-D-glucuronidase (MUG), PDE, cellobiosidase, ß-glucosidase, ß-xylosidase, PME, alanine-AP and leucine-AP. The concentration of 4-MUF *N*-acetyl-ß-D-glucosaminide, the substrate for NAGase, should be adjusted to 200 µM.

Soil heterogeneity is an intrinsic property of all soil studies and it is a continuous challenge in all steps of sampling design. Its effects are pronounced in small scale studies such as assays on multiwell plates, and it is a challenge to reveal the overall potential activity of enzymes with such a small reaction volume. As applied in our studies, the multiple enzyme assay yielded relatively low estimates of variation (Paper II, Fig. 1; Paper III, Table 6; Paper IV, Table 4). ß-Glucosidase yielded high variation in studies published in Papers IV and V, but not in the field soil of Paper II. Forest soils with humic matter difficult to homogenize produced the largest variation coefficients, whereas mineral soil usually showed lower variation. In further studies of our group an electronic dispenser was used in dispensing the standard and substrate solutions, which further diminished the variation coefficient.

In Papers II and IV the enzyme activity results were calculated on the basis of soil fresh weight and in the Paper V on the basis of SOM (defined as loss on ignition). Soil fresh weight reflects the actual situation in soil and differences in e.g. moisture are included in the enzyme activity results. If the effects of weather conditions or other factors affecting soil moisture are to be excluded from the data examination, calculations on the basis of soil dry weight are justified. Calculating the results on the basis of soil organic matter should exclude the effects of soil moisture and the amount of organic matter, which is of great importance since the enzyme activities are strongly related to soil organic matter content. The results *per* SOM reflect quality differences between SOM in different soils and perhaps more closely the activity differences of different microbial communities in soil. Furthermore, the nature of organic matter may be more reflected in the soil organic matter normalised results. Wallenius *et al.* (2011) reported the separation of different soils (field, meadow, and forest) according to enzyme activities calculated on the basis of both soil dry weight and soil organic matter and concluded that the results *per* SOM showed more variation between different soil types than did the results *per* soil dry weight. Based on our data, it may be advantageous to use results *per* dry weight if the comparison of samples is performed for one sampling date.

In the findings of Paper II many of the enzymes were intercorrelated, but NAGase activity did not correlate with other enzyme activities. Under pine and alder, all the enzymes involved in the C cycle (α- and ß-glucosidase, ß-xylosidase, cellobiosidase and NAGase) were intercorrelated (unpublished data). Leu- and ala-aminopeptidases correlated strongly with each other in both the above-mentioned experiments.

The method developed can be applied in various scales depending on the need for interpretations to be made (Nortcliff 2002); the quantity of soil that must be sampled is small due to the small size of multiwell plate wells. This enables sampling of e.g. rhizosphere soil, or thin soil layers, and still yields information on the variety of potential enzyme activities. Depending on the sampling system and on the study question, it is also applicable to medium and large scales. The heterogeneity scale of soils affecting enzyme activity must also be considered (Berner *et al.* 2011) and in order to describe a large area, sufficiently uniform quality of the soil should be confirmed or separate samples analyzed in order to be able to interpret the results in a reliable manner.

4.5 Future prospects

Microbiological characteristics of soil environment have been recognized to play a major role in the equilibrium present in, as well as in processes shaping the biosphere (Turbé *et al.* 2010). The research methods are in a stage of rapid development both in the fields of taxonomic and functional understanding of soil systems. However, due to the complexity, diversity, heterogeneity and obscurity of soil, more information of the reactions and species carrying out the reactions is needed. Therefore, there is a demand for methods such as the enzyme assay developed during this thesis. The enzymes assayed are all hydrolytic by nature. Oxidoreductases, transferases, lyases, isomerases, and ligases were not measured, and there are currently no or only a few methods available for the detection of these activities in soils. Furthermore, the reactions measured do not include actions of endo acting enzymes, which are of great importance in the breakdown of macromolecules. Therefore, soil science still requires method development to widen the spectrum of enzyme activities analysed in an easily applicable method.

In our data, different soil types were not included in one inspection but the effects were examined within a soil type (e.g. agricultural soil). In experiments with field soil, forest soil samples were only utilized as outliers in cluster analysis. By combining a larger data set, Štursová and Baldrian (2011) detected differences in the main factors affecting enzyme activities in grassland soils and in forest soils. pH and the amount of humic matter were the main factors affecting enzyme activities in forest soils, whereas Ca content and the size of humic compounds were the determining factors in grassland soils. This is of importance in the search for an activity index in different soils. It may be that a universal index applicable to different soils is not a practical possibility. By combining the data from our experiments, this kind of examination can be carried out to verify the results of Štursová and Baldrian (2011) and of other scientists and to possibly reveal new relationships.

More specific sampling strategies including strict rhizosphere soils (Kandeler *et al.* 2002, Pritsch *et al.* 2004) should be used in order to link the activities of different enzymes more specifically to soil functions. The need to describe the effects of temporal variation on enzyme activities has repeatedly been stressed (Chemidlin Prevost-Boure *et al.* 2011, Straková *et al.* 2011), together with the need for detecting biogeographical scales affecting different enzymes and their spatial patterns (Kandeler *et al.* 2011). The spatial scales differ both by the factor measured and by soil treatment (Katsalirou *et al.* 2010, Berner *et al.* 2011). This method, applicable for different scales, provides a convenient and rapid means to detect the microbial functions studied.

The enzyme activity pattern has been shown to differentiate sensitively between soil treatments. Addition of new substrates to the pattern is possible, and this would improve evaluation of the functional *diversity*. Currently, only ten enzymes are included in the pattern whereas the capacity of soil to process different molecules is much wider. To assess soil enzyme activities comprehensively, oxidative enzymes of specific interest e.g. those catalysing the breakdown of lignin, should be included in the investigation. This requires the use of different kinds of fluorescence-based artificial substrates aiming at the detection of oxidative enzymes (e.g. Amplex® Red products). The assay procedure and handling of the reagents is more difficult than for MUF- and AMC-linked reagents, but they can be used for soil samples to estimate oxidase and peroxidise activities (U. Münster, personal communication).

It is also important to combine information on functional diversity with that concerning genetic diversity in order to detect which enzyme activities change with changes in microbial community composition and how these changes are reflected in carbon cycling (Arnosti 2011). In a study combining enzyme activity measurements with genetic information on chitinase-producing microbes in soils, Metcalfe *et al.* (2002) reported that altough sewage sludge amendment generally induced the activity of chitinases, the diversity of different chitinases

was diminished. This underlines the need for different techniques to be used in environmental research, since it is not only the magnitude of reactions that are of interest, but also their diversity. Different isozymes introduce resilience to soil systems, allowing them to retain functionality even in changing conditions.

5 Conclusions

Substrate concentrations and other measurement conditions, such as buffers and incubation conditions, can be adjusted optimally for a specific experiment. However, the constant conditions applied over a wide variety of experiments yielded results revealing temporal, spatial and treatment-associated differences rather sensitively. This illustrates the potential of enzyme activity pattern as an indicator of soil biological quality.

Although soil enzyme activities are commonly correlated with each other, it is advisable in soil quality studies to measure a pattern of several enzyme activities simultaneously. This can reveal important cases with no correlation, which indicate essential changes in microbiota. It was also evident that the assay and its results should be analysed as an entity, and cluster analyses proved to be a sensitive and applicable means of handling the data obtained. Sensitivity of cluster analysis is always increased with increasing number of tests and samples analysed. A pattern of enzyme analysis always increases sensitivity compared with measurements of individual activities, and furthermore offers the possibility for statistical testing of impacts on individual enzyme activities. The need for several measures for classifying soils was also concluded by Tscherko *et al.* (2007). It may be unrealistic to anticipate that the potential of a single enzyme activity could represent the quality of a complex soil matrix. Dick (1997) stressed that the activity of one enzyme cannot be used as an indication of several different stress factors and recommended the use of an enzyme activity pattern. However, it has been suggested that for example arylsulphatase reacts especially sensitively to trace elements (Dick 1997). In accordance with our hypothesis of a need for several enzyme activities to detect significant changes in the environment Sinsabaugh *et al.* (2008) reported different ranges of variation and different distributions in relation to ecosystem variables of extracellular enzymes. By combining data from all the experiments of our group the enzyme pattern and the factors affecting individual enzymes can be further studied and evaluated. Other variables related to soil biology and chemistry must also be included in a descriptive soil quality indicator (Trasar-Cepeda *et al.* 2000).

The ZymProfiler® enzyme activity test pattern may not yield results that can be interpreted alone. This has also been concluded by other research groups on enzyme activity patterns (Carreira *et al.* 2008). A comparison is almost always needed in one sense or another and the pattern reveals changes or differences between samples. It is far beyond current knowledge to be able topresent reference values for soil enzyme activities indicating "high" or "poor" quality of soil, but fuzzy classification of soils has been proposed to yield a classification basis independent of reference values in one data set of 900 samples (Tscherko *et al.* 2007). A comparison also permits the use of suitable but not necessarily optional conditions in the measurement. Standardized conditions are necessary to enable a reliable comparison. This tool can be used to measure the functional diversity of soils to some extent – it yields a measure of changes in functional diversity of the selected enzymes active in the degradation of the most common macromolecules present in soils. In order to sensitively reveal the functional diversity in a larger scale, a wider repertoire of enzyme activities is needed.

Standardized methods for soil enzyme analysis have been called for (Gianfreda and Bollag 1996, Nortcliff 2002, Baldrian 2009, Turbé *et al.* 2010) in order to obtain comparable results between research groups. The methods used are typically published in journals or books with a methodological perspective, which is the appropriate route in scientific research. To yield a consensus on the methodology in detail is challenging even if the papers are peer reviewed and

the methodologies are tested with care, because they are often applied to local requirements. Bearing this in mind, the method developed has been worked into an ISO Technical specification (ISO/TS 22939:2010) describing the recommended details. The standardization process involving several comment steps of different parties of the ISO technical committee ISO/TC 190, Soil quality, subcommittee SC 4 on biological methods, has produced a technical specification with agreement between the member countries.

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