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ON THE INVESTIGATION OF CHEMICAL PARAMETERS REFLECTING MICROBIAL ACTIVITY LINKED TO NUTRIENT AVAILABILITY IN FOREST SOIL

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"The soil is the great connector of lives, the source and destination of all. It is the healer and restorer and resurrector, by which disease passes into health, age into youth, death into life. Without proper care for it we can have no community, because without proper care for it we can have no life."

- Wendell E. Berry

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ABSTRACT

As agrarian society developed, the most fertile soils able to sustain the nutritional requirements needed for high crop yield were assigned to farming, while the more penurious soils were left to uphold the forest ecosystems. Some temperate forests are developed on acidic soils considered to be nutrient poor, as much of the inorganic nutrients are entrapped in poorly weatherable soil minerals and not easily accessed by plant roots. In an undisturbed ecosystem, the largest contribution of available nutrients comes from the recycling of organically bound nutrients via the decomposition of dead plant material. If biomass is removed, for instance with a more intensified exploitation of the forest ecosystems including whole tree harvesting, this source of nutrients is consequently decreased. The importance of soil mineral weathering as a source of nutrients, and especially that promoted by soil biota, is thereby emphasized.

This thesis addresses biotic parameters associated with mineral weathering. Different aspects of soil solution sampling strategies and analysis of different organic ligands as well as biomarkers for the estimation of fungal biomass were investigated. These chemical parameters were also evaluated as indicators of microbial activity in relation to mineral nutrient availability in soil.

With the assumption that the current nutrient status of a soil will affect the microbial interest of certain minerals as sources of inorganic nutrients, a mineral amendment trial was performed in a Swedish boreal forest soil. Overall, the amended soil presented good nutrient status, but with a possible shortage of iron. Due to this, it was hypothesized that the amended mineral with the highest iron content i.e. biotite would cause an elevation of microbial activity in its vicinity when compared to the bulk soil.

The level of microbial activity in the vicinity of the amended minerals was evaluated via quantification of organic acids and siderophores, as well as estimation of fungal biomass and enzymatic activity.

The highest microbial activity was measured for the O horizon of the investigated podzol, although nothing indicated an elevated association with the amended minerals. In the E horizon, however, elevation in microbial activity was observed in the vicinity of the biotite mineral when compared with bulk soil, although only a few of the investigated parameters differed significantly when evaluated separately.

To enable this study, a highly sensitive analytical method employing liquid chromatography and mass spectrometry was developed to quantify a number of hydroxamate siderophores. On-line pre-concentration enabled detection of these organic ligands in the pico-molar range – a necessity when analyzing natural samples.

Furthermore, an analytical method was developed for the estimation of fungal biomass via quantification of chitin-derived glucosamine, which also employed liquid chromatography and tandem mass spectrometry. Unlike currently available methods, the one presented in this thesis did not involve analyte derivatization, which resulted in high sample throughput while simultaneously avoiding complications involved with the additional derivatization procedure.

The distribution of a group of organic ligands known as *aromatic low molecular mass organic acids* was also studied in a boreal forest podzol soil. Different sampling and samples preparation techniques, namely tension-lysimeters, soil centrifugation and liquid-soil extraction, were compared when analyzing soil solution components. Significant differences in analyte amount and species type were found between these sampling techniques. Some of the differences could be accounted for by variation in soil composition at different depths of the investigated podzol, but others could be attributed to structural differences within the studied analyte group. This clearly illustrated the intricacy of sampling and analysis when working with a sample matrix as complex and diverse as soil.

As previously, liquid chromatography and mass spectrometry was used to quantify the analytes of interest. A highly sensitive analytical method was developed that was able to detect eleven aromatic low molecular mass organic acids in the nano-molar range. High selectivity was ensured by applying multiple reaction monitoring enabled by collision induced fragmentation of the analytes.

Keywords: boreal forest, electrospray ionization, fungal biomass, hydrophilic interaction liquid chromatography, hydroxamate siderophores, liquid chromatography-mass spectrometry, mineral amendment, aromatic acids, podzol soil

SAMMANDRAG

Historiskt sett har de mest näringsrika jordarna nyttjats inom jordbruket, medan de mer näringsfattiga övergetts till förmån för skogens utbredning. Att vissa skogsmarker anses vara *näringsfattiga* beror på att stora delar av de befintliga näringsämnena föreligger som olika mineral i marken och följaktligen svåråtkomliga för trädens rötter. Med ett mer vinstinriktat skogsbruk, med ökande råvaruuttag, ställs allt högre krav på näringstillförseln i skogsmarken. Att även kvistar och rötter förs bort leder till att allt mindre organiskt material, som kan brytas ner och återvända till näringskretsloppet, lämnas kvar. Detta innebär i sin tur att mer näring behöver tillföras utifrån eller frigöras från jorden genom vittring av olika mineral.

Många processer såsom vind, vatten och temperatur påverkar vittringens omfattning. Utöver det kan även växter och andra organismer som lever i marken ha en påverkan. Växter och svampar kan med sina rötter och hyfer mekaniskt sönderdela mineral. En viktig aktör i dessa sammanhang är den underjordiska symbios som många växter bildar med så kallade *mykorrhizasvampar*. Genom att infekterar växternas rötter kan dessa svampar med sina hyfer utforska jorden i större utsträckning än växterna själva kan. Detta medför att större mängder vatten och näringsämnen kan nås. Växter och mikroorganismer utsöndrar dessutom organiska föreningar som kemiskt kan vittra mineral genom att komplexbinda positiva metalljoner och därigenom göra dessa mer tillgängliga för växtupptag.

I denna avhandling behandlas provtagning och analys av dessa organiska föreningar med sikte på att överbrygga de svårigheter som följer av att föreningarna föreligger i låga halter och dessutom i en komplex miljö.

Vidare har fältförsök genomförts som visar att mikroorganismer tycks frodas i närheten av mineral som innehåller mycket av de näringsämnen som fattas i den befintliga jorden och att de då även utsöndrar mer av de föreningar som kan medföra ökad mineralvittring.

Ett flertal analytiska metoder har utvecklats för att möjliggöra analys av dessa kemiska komponenter. Den analytiska utrustning som använts är vätskekromatografi tillsammans med masspektrometri. Detta är en kraftfull analysmetod som kan detektera mycket låga halter av de eftersökta föreningarna och även med stor säkerhet urskilja dessa från övriga komponenter i provet.

TABLE OF CONTENT

LIST OF PAPERS

This thesis is mainly based on the following four papers, herein referred to by their Roman numerals:

Paper I **Evaluation of sampling and sample preparation procedures for the determination of aromatic acids and their distribution in a podzol soil using liquid chromatography-tandem mass spectrometry**
Madelen A. Olofsson, Sara H. Norström, Dan Bylund, Geoderma (2014) volume 232-234 page 373-380

Paper II **Effects of mineral amendment on soil chemistry parameters reflecting microbial activity in a Swedish boreal forest soil**
Madelen A. Olofsson, Dan Bylund, *Manuscript*

Paper III **Analysis of hydroxamate siderophores in soil solution using liquid chromatography with mass spectrometry and tandem mass spectrometry with on-line sample preconcentration**
Madelen A. Olofsson, Dan Bylund, Journal of Separation Science (in press), DOI 10.1002/jssc.201500509

Paper IV **Liquid chromatography with electrospray ionization and tandem mass spectrometry applied in the quantitative analysis of chitin-derived glucosamine for a rapid estimation of fungal biomass in soil**
Madelen A. Olofsson, Dan Bylund, *Submitted to the International Journal of Analytical Chemistry*

AUTHORS CONTRIBUTION

Paper I	All sampling and laboratory work together with S. Norström and all data analysis and writing in consultation with all other authors.
Paper II	Essentially all sampling and laboratory work. Data analysis and writing in consultation with D. Bylund.
Paper III	All laboratory work, data analysis and writing in consultation with D. Bylund.
Paper IV	All laboratory work, data analysis and writing in consultation with D. Bylund.

LIST OF ABBREVIATIONS

ACN	Acetonitrile
ANOVA	Analysis of variance
CCD	Central composite design
CID	Collision induced dissociation
DOC	Dissolved organic carbon
DOE	Design of experiments
EM	Ecto-mycorrhiza
ESI	Electrospray ionization
GlcN	Glucosamine
GalN	Galactosamine
GLM	General linear model
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
HS	Hydroxamate siderophores
LC	Liquid chromatography
LMMOAs	Low molecular mass organic acids
MA	Muramic acid
ManN	Mannosamine
MeOH	Methanol
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NP	Normal phase
PCA	Principal component analysis
RP	Reversed phase
SEM	Scanning electron microscopy
SIM	Selected ion monitoring
TOC	Total organic carbon

1. INTRODUCTION

1.1. General background and objective

Soil, the "skin of the earth" and the interface of our lithosphere, hydrosphere, atmosphere and biosphere is made up of minerals, organic matter, gases, liquids and an estimated one third of all living organisms. It is shaped by physical, chemical and biological processes which are influenced by climate, geography, parent material (bed rock) and its living inhabitants. The soil also stores, supplies and purifies water and functions as the medium for plant growth.

Since man first started utilizing the land for agricultural purposes some 10,000 year ago, huge progress has been made in rationalizing production and increasing yield. But despite increasingly refined methods, one balance point must be maintained for any sustainable harvest: the removal of nutrients cannot be exceeded by its input.

Normally, the largest source of nutrients within an ecosystem is derived from the internal circulation of decomposed organic material (Schlesinger 1991). Boreal forests soils are generally nutrient poor in comparison to agricultural soil, and the increasing practice of whole tree harvesting (the removal of twigs and stubs) means that the input of nutrients from the atmosphere (i.e. C, N and S) and from mineral weathering becomes more crucial for maintaining the soil nutrient pool (Figure 1).

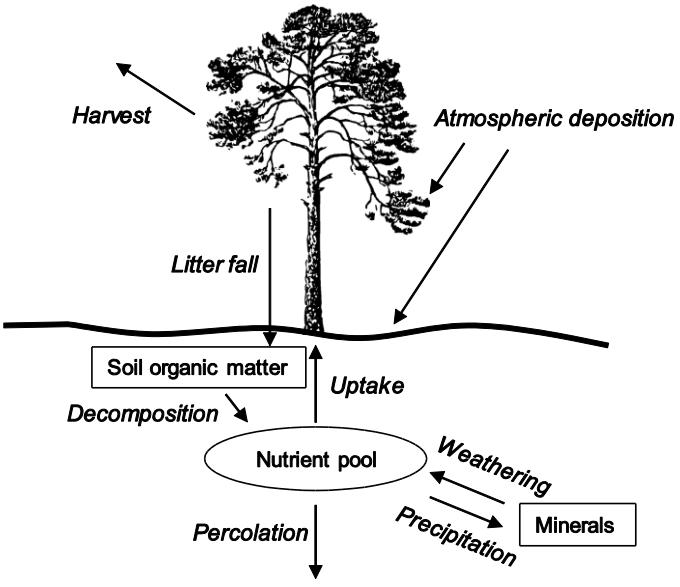


Figure 1. A simplified model illustrating input and output of nutrients in a forest ecosystem.

Mineral weathering is the result of both physical and chemical processes dependent on composition of the bed rock, climate, water availability as well as biota. The biota induces both mechanical and chemical weathering, the latter by releasing CO_2 from cell respiration and by the production and exudation of organic ligands. These ligands can in turn form complexes with metal ions both in solution and at mineral surfaces.

This thesis describes the development of analytical methods for the identification and quantification of some of these organic ligands, as well as quantification of chemical markers for the estimation of fungal biomass. It also presents results from field studies investigating the effects of mineral amendment on microbial activity, and how different sampling techniques influence the sample composition for a group of organic ligands. The purpose of these studies was to better understand the influence of biota on mineral weathering and nutrient availability in boreal forest soil.

1.2. Mineral nutrient release by weathering

Mineral nutrients are crucial to the fulfilment of a plants lifecycle, and these are categorized as macro- or micronutrients, reflecting the quantity in which they are required by the plant. Macronutrients such as S, N, and P are needed at higher concentrations as they are the building blocks for organic compounds such as proteins and nucleic acids (Marschner 1995). Micronutrients, on the other hand, are only needed at very low concentrations. The transition metals Fe, Cu and Mo are examples of micronutrients and these are mainly components of enzyme molecules as their ability to change valence number enables catalytic functions (Marschner 1995).

The contribution of mineral weathering to the available nutrient pool is generally small in comparison to the recycling of organically bound nutrients via decomposition (Schlesinger 1991). It is, however, still important for the compensation of nutrient losses (Smits et al. 2014) as sustainable forest production requires a balance between mineral nutrient loss from harvesting and leaching, and gain from weathering and deposition (Hagerberg et al. 2003).

Physical and chemical decomposition of rocks at the earth's surface releases P, K, Mg, and a number of other trace elements in a form directly available to the biota, thereby substantially contributing to soil fertility and ecosystem productivity.

Boreal forests are known to be naturally N limited (Tamm 1991) but its gain through fertilization and atmospheric deposition, may lead to other nutrients such as P, K and Mg to becoming limiting factors of production (Akselsson et al. 2008).

In order to fully understand the process of nutrient cycling in ecosystems, it is necessary to understand the role that organisms play in weathering (Hoffland et al. 2004).

1.3. Biotic effects on mineral weathering

Biotic weathering is defined as weathering promoted by organisms (the biome), in contrast to *abiotic* weathering. Biotic weathering is largely affected by plants which influence water dynamics, cycling of elements, cell respiration and the production of organic ligands (Lucas 2001). Cell respiration releases CO_2 into the soil, which in turn forms carbonic acid when dissolved in water, consequently decreasing soil pH, thereby promoting mineral dissolution. Organic ligands complex metal ions in solution as well as on mineral surfaces.

Cell respiration and production of organic ligands are also associated with fungi, both saprotrophic and mutualistic (i.e. mycorrhiza and lichens) (Hoffland et al. 2004) as well as bacteria residing in soil (Uroz et al. 2009; Uroz et al. 2011).

Saprotrophic fungi are self-supporting organisms that secure nutrients through decomposition of dead organic matter, while the mycorrhizal fungi live in co-existence with higher plants. The mycorrhiza symbiosis is a mutual and prosperous relationship whereby the photosynthesizing host plant provides the fungus with carbohydrates, and the fungi significantly enhances the plant's soil coverage, acting as the main pathway for input of water and nutrients from the soil. Mycorrhizal associations affect 90 % of all plant species (Robert et al. 1986) and have been present since the first land-living plants (Paul et al. 1989).

Ecto-mycorrhiza (EM) is the predominant type of mycorrhiza in boreal forests, and considered to be of great importance for mineral weathering and nutrient availably in these otherwise nutrient-poor soils (Landeweert et al. 2001; Wallander et al. 2004; Leake et al. 2008; Schöll et al. 2008; Smits et al. 2008).

There is a lack of consensus regarding the extent to which biotic weathering - especially that which is influenced by mycorrhiza - actually contributes to total mineral weathering (Finlay et al. 2009; Sverdrup 2009). Jongmans et al. (1997) reported of "tunneling" believed to be caused by saprotrophic and mycorrhiza fungi in feldspars and hornblendes in E horizons under boreal vegetation, suggesting direct fungal weathering through exudation of organic acids from the hyphal tip. The term "rock-eating" mycorrhiza was coined, and the direct weathering it caused was attributed great importance in total mineral weathering and podzolization processes (van Breemen et al. 2000a; van Breemen et al. 2000b).

These theories were strongly opposed by Sverdrup et al. (2002) who estimated the biotic contribution to total mineral weathering at a mere 2 %, not including reaction with carbonic acid and organic ligands in soil solution. A later investigation of the magnitude of the rock-eating mycorrhiza's impact on total weathering in feldspar (Smits et al. 2005) supported Sverdrup and colleagues' estimations, although other researchers maintain that biotic contribution is much higher and needs to be implemented in existing models for estimations concerning sustainable forest production (Leake et al. 2008; Smits et al. 2008; Finlay et al. 2009).

1.4. Organic ligands

Plants, fungi and bacteria produce organic compounds with chelating capacities, e.g. aliphatic and aromatic acids, amino acids, polyphenols and siderophores. (Tan 1986). These soil organic compounds, and in particular organic acids of both low and high molecular mass, are believed to be involved in mineral weathering when exuded to the surrounding soil (Stevenson et al. 1986). Complexation with organic or inorganic ligands enables trace metals, which would otherwise form insoluble precipitates to be present in soil solution in higher concentrations (Stevenson et al. 1986). Low molecular mass organic acids (LMMOAs) and siderophores are organic compounds with predominantly high metal complexing abilities. These two groups of organic ligands are presented more closely below.

1.4.1. Low molecular mass organic acids

Low molecular mass organic acids (LMMOAs) are produced in a number of metabolic processes in plants and microorganisms (Ryan et al. 2001), but are also formed as secondary metabolites in decomposition of organic matter (Pohlman et al. 1988). LMMOAs are introduced to the soil as exudates from plant roots, soil fungi and bacteria, and via forest floor leaching and rainfall and constitute up to 10 % of dissolved organic carbon in soil (Pohlman et al. 1988; Fox 1995; Marschner 1995; Uroz et al. 2011) .

The majority of the known LMMOAs hold a molecular weight less than 300 Da (Fox 1995), and consist of aliphatic acids, with one to three carboxylic acid groups, and aromatic acids, comprised of hydroxy and methoxy substituted benzoic or cinnamic acids (Strobel 2001) (Figure 2).

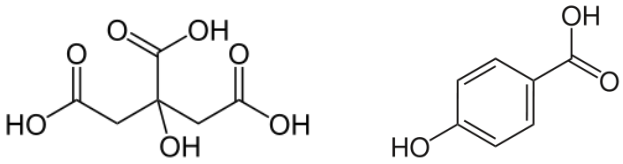


Figure 2. The aliphatic LMMOA citric acid (left), with three carboxylic acid groups and the aromatic LMMOA *p*-hydroxybenzoic acid (right).

The highest concentrations of LMMOAs are found in the upper, organic layer of forest soils, where they are also most rapidly decomposed (van Hees et al. 2000; van Hees et al. 2002). Furthermore, LMMOA concentration is significantly higher in close

vicinity of roots and fungal hyphae, i.e. the rhizosphere and mycorrhizosphere, when compared to the bulk soil (Ryan et al. 2001).

LMMOAs contributes to both proton- and ligand-based mineral weathering by decreasing soil solution pH, and by complex formation with metal ions in solution and on mineral surfaces (Drever et al. 1997).

The number and dissociation properties of the carboxylic groups, as well their arrangement relative to other acetic groups (carboxyl and hydroxyl) determines the LMMOA's number of negative charge and the stability of the ligand-metal complexes (Hue et al. 1986; Jones 1998).

With the ability to bind cations such as Al^{3+}, Fe^{3+}, and Ca^{2+}, LMMOAs do not only increase mobility of micro-nutrients such as Fe^{3+}, or P from Ca-P minerals, but also reduce the concentrations of plant-root toxic Al^{3+} in the rhizosphere (Jones et al. 1994; Jones 1998; van Hees et al. 2000; Ryan et al. 2001). These abilities to complex and translocate metal cations suggests that LMMOAs play a major role in the podzolization process (Vance et al. 1986; Marschner 1995; van Hees et al. 2000).

1.4.2. Hydroxamate siderophores

To circumvent low bioavailability of Fe in soil, bacteria, fungi and certain plants produce and exude Fe-specific chelating agents. These organic ligands are collectively named siderophores, Greek for *iron bearer* (Lankford et al. 1973).

With their high Fe^{3+} association constant (10^{12} to 10^{59}) (Neilands 1982), siderophores promote Fe solubilization from organic substrates and minerals, consequently increasing the Fe accessibility to plants and microorganisms (Matzanke 1991).

Even though these low molecular mass (300-1500 Da) ligands are found in nano-molar concentrations in soil solution (Powell et al. 1980; Holmström et al. 2005; Essén et al. 2006), they likely play a significant role in mineral weathering (Watteau et al. 1994; Reichard et al. 2007), by operating in synergy with less iron-specific organic ligands such as LMMOAs (Cheah et al. 2003; Reichard et al. 2007). By promoting soil mineral weathering they consequently also release macro nutrients such as P and Ca to the surrounding environment.

The siderophores molecular structure varies greatly, but their mutual feature is to form six-coordinated octahedral complexes with Fe^{3+}. This may also occurs for other trivalent metal ions such as Al^{3+}, Ga^{3+} and In^{3+} (Raymond et al. 1984).

Their categorization is based on the functional group acting as the electron donor in the Fe^{3+}-siderophore complex, and known subgroups are catecholates, phenolates, hydroxamates and mixed type (Matzanke 1991).

Hydroxamate siderophores (HS) are produced by both bacteria and fungi and form complexes with Fe^{3+} via three hydroxamate groups. Known hydroxamate

subgroups are ferrioxamines, ferrichromes and coprogens/fusigens and these all form 1:1 complexes with Fe^{3+} (Matzanke 1991) (Figure 3).

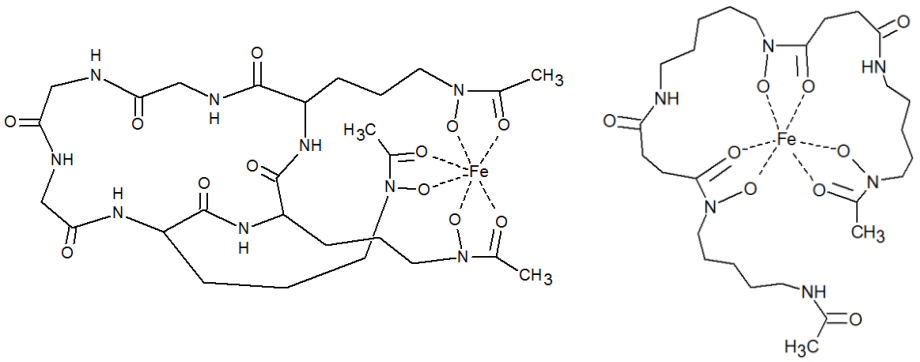


Figure 3. Examples of hydroxamate siderophores, ferrichrome (left), and ferrioxamine D_1 (right).

1.5. Soil solution sampling

The soil solution can be described as the "liquid phase" of soil and it consists of water with organic matter and dissolved gases and minerals. It participates in soil formation and mediates physical, chemical and biochemical reactions that, along with vegetation, climate and anthropogenic activity, define soil composition. It is the medium in which all soil processes communicate (Sverdrup 2009) and it enables the circulation of matter and plant nutrition in soil.

Soil solution can be categorized as percolating water, which moves downwards in the soil horizons due to gravitation; capillary water, which is retained by surface tensions in small pores; and hydroscopic water, which is adsorbed as a film onto soil particles (Figure 4).

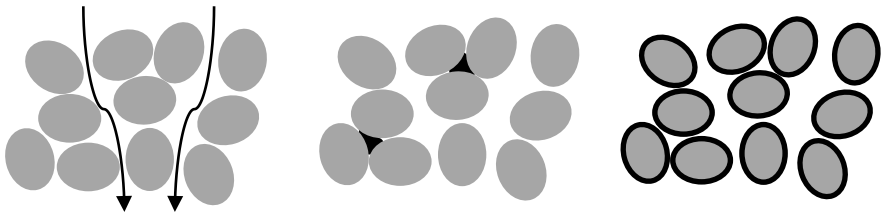


Figure 4. The three forms of soil water (from left to right): Percolating, capillary, and hydroscopic.

Soil solution analysis can provide more information than the analysis of whole soil (Wolt 1994), as it captures both the static and dynamic nature of soil in which chemicals are transferred and distributed between the solid, liquid, gaseous and biotic phases.

There are both laboratory and field methods for the sampling of soil solution. The use of various lysimeters is common in field sampling, as these can be installed and continuously emptied without further disruption of the soil, which make them ideal for long-term sampling at a specific site. Sampling with tension-free lysimeters may be the most natural method of collecting percolating soil water, as it disturbs soil solution composition less than tension lysimeters do (Gallet et al. 1999). Disadvantages of sampling with tension-free lysimeters are the time required, and that the soil needs to be saturated before water can be collected. Tension-lysimeters collect soil solution by an applied suction, thus also collecting capillary water (Haines et al. 1982).

Tension-lysimeter samples generally contain higher concentrations of the majority of solutes, when compared with samples acquired via zero-tension lysimeter, although this may vary for specific solutes (Haines et al. 1982; Marques et al. 1996; Watmough et al. 2013).

Soil centrifugation is a laboratory method for collecting soil solution, including soil capillary water. This generally results in higher concentrations of most solutes in comparison to solution sampled by lysimeters (Giesler et al. 1996; Gallet et al. 1999; Geibe et al. 2006). Centrifugation is performed on soil that has not been disturbed prior to sampling, which cannot be said of sampling via lysimeter (Giesler et al. 1996).

Soil centrifugation can be performed at low or high pressure (Wolt 1994), and the use of high-speed centrifugation increases the risk for cell damage and subsequent leakage of cell contents into the soil solution (Zabowski 1989; Nambu et al. 2005).

Liquid extraction of soil samples is practical when the level of moisture in soil samples varies (Strobel 2001). By extracting with water or buffer solutions, either alone or in combination with MeOH, analytes adsorbed via electrostatic interaction with clay particles or hydrophobic interactions with soil organic matter (SOM) can be obtain. Extraction using a high percentage of MeOH does however increase the risk of extracting living organisms via cell lysis (Lange et al. 2001; Faijes et al. 2007).

1.6. Estimating microbial biomass and activity

Soil microorganisms affect the formation and decomposition of soil organic matter and subsequently also the nutrient turnover and availability in soil. Microbial biomass is therefore an important indicator of soil fertility, and its composition and distribution can be used to study natural (e.g. seasonal) differences (Söderström

1979), as well as the effects of interventions such as tilling, clear-cutting and prescribed burning (Bååth et al. 1982; Bååth et al. 1995; Pietikäinen et al. 1995; Wagai et al. 1998).

A variety of approaches can be employed to estimate microbial biomass, including direct quantification or enumeration via histological analysis (Kästner et al. 1994; Kepner et al. 1994; Bölter et al. 2002). Direct quantification is, however, often tedious and may result in biased values as a result of differences in approach (Giovannetti et al. 1980; Kepner et al. 1994).

Total biomass (carbon) in soil can be estimated using techniques such as chloroform fumigation extraction (Vance et al. 1987), adenosine triphosphate (ATP) extraction (Tate et al. 1982) or substrate induced respiration (Anderson et al. 1978).

Microbial biomass can also be estimated through indirect quantification of biochemical markers. Phospholipid-derived fatty acids (PLFAs) are the main structural element in cellular membranes of all living organisms (archaea excepted). Due to the structural differences of PLFAs according to source, it is more or less a selective biomarker for different species of bacteria and fungi (Frostegård et al. 1993; Zelles 1999).

Ergosterol assays can also be applied to indirectly quantify fungal biomass, as this sterol is found almost exclusively in fungal membranes. As it is relatively unstable and degrades after fungal death, its quantification is considered an estimate of metabolically active fungi (Seitz et al. 1977; Salmanowicz et al. 1988).

Additional strategies for estimating fungal biomass include quantification of chitin-derived glucosamine (GlcN). Chitin is the naturally occurring polymer of N-acetyl-D-glucosamine and the structural building block of fungal cell walls (Parsons 1981) (Figure 5). This polymer is more resistant to degradation than ergosterol and phospholipids and are believed to have a recalcitrant portion of 10 to 15 % of the original biomass (Schreiner et al. 2014). Glucosamine is acquired via acid hydrolysis of chitin (Swift 1973; Ekblad et al. 1996).

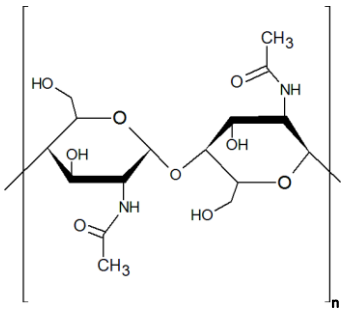


Figure 5. Chitin polymer consisting of N-acetylated-D-glucosamine.

An alternative to direct or indirect quantification of microorganisms/microbial presence is to monitor their activity via respiration, gene expression, or enzymatic activity. Numerous assays for the estimation of both specific and more general enzymatic reactions are available (Bandick et al. 1999; Taylor et al. 2002). An example is the broad-spectrum FDA assay. It monitors the enzymatic hydrolysis of 3', 6'-diacetylfluorescein (FDA) into fluorescein, promoted by both free and membrane-bound lipases, proteases and esterases (Schnürer et al. 1982; Adam et al. 2001; Green et al. 2006) (Figure 6).

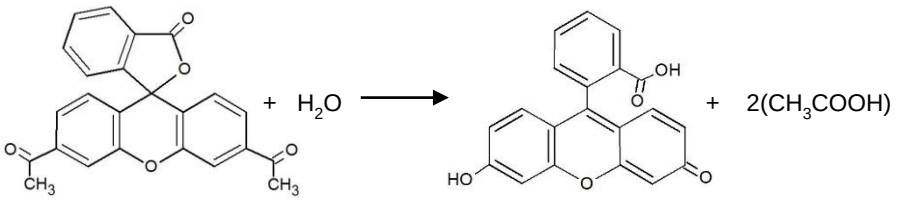


Figure 6. The enzymatically promoted hydrolysis reaction of 3', 6'-diacetylfluorescein (FDA) into yellow fluorescent fluorescein.

1.7. Statistical analysis

Studies of complex natural systems tend to generate large quantities of data with numerous variables. In such data sets it can be difficult to distinguish systematic variations from noise only by the use of basic univariate statistics. In these cases, the use of more advanced statistical tools able to handle multivariate data are beneficial.

Analysis of variance (ANOVA) is a powerful statistical tool for testing hypotheses, and through which variation in a data set can be separated and estimated to determine whether it derives from random error or from changes in control factors (Miller et al. 1988). ANOVA requires that the predictable values (factors) are categorical and that the response variables are continuous (Gotelli et al. 2004). If one factor is varied one-way ANOVA is applicable, and with two or more factors, two-way or multiple factor ANOVAs can be performed.

ANOVA can also be performed via a general linear model (GLM) approach. GLM comprise a collection of statistical methods with the common feature that they involve a model that can be calculated by least squares linear regression. In the case of ANOVA, this approach is especially useful when covariates are to be handled or when the underlying experimental design is unbalanced.

Principal component analysis (PCA) is a standard tool to reduce the dimensionality of large data matrices. In PCA, linear combinations of the original

variables are used to form principal components (PCs) describing as much of the variability in the data as possible. The degree to which an original variable contributes to the different PCs is established from its loadings, and a loading plot can thereby illustrate the presence of co-linearity between variables. The observations are then projected onto the PCs, resulting in score values. A subsequent score plot can thereby be used to illustrate how the different observations relate to each other. Creating a *bi-plot,* which combines both loading and score plots, allows for a single overview of correlations of variables and observations.

2. FIELD EXPERIMENTS

2.1. Site description

All soil sampling and field trials presented in this thesis were conducted at a site in Bispgården, Sweden (63°07'N, 16°70'E) at 258 meters above sea level (Figure 7). The site consists of a 50 ha catchment with naturally generated 80 to 90-year-old Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*), and slopes downward at a 2° angel towards the stream draining the catchment. This site has earlier been the subject of thorough investigations by Lundström and co-workers (Vestin et al. 2008a; Vestin et al. 2008b; Norström et al. 2010).

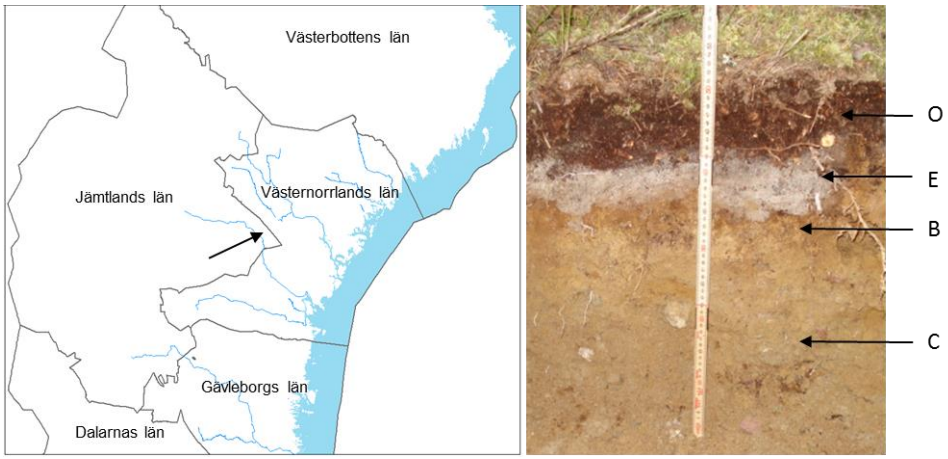


Figure 7. Location of the Bispgården site in central Sweden (left). The soil in the recharge area is characterized as podzol according to FAO (1990), with distinct organic (O), elluvial (E) and illuvial (B) horizons, as well as unaffected parent material (C) (right). (Map of central Sweden used with the curtesy of SGI and Lantmäteriet.)

The site is part of the boreal forest system also known as the taiga. It is the world's largest vegetation system, forming a circumpolar band around the northern hemisphere (Read et al. 2004), and its soil and biomass acts as a substantial global store of carbon (Smits et al. 2008).

The boreal forest type, largely consisting of coniferous trees like pine and spruce, growing in nutrient poor soils on a bedrock of granite and gneiss, is the most predominant in Sweden. The combination of vegetation and parent material conditions with a temperate climate, where precipitation exceeds evaporation, favors the development of podzol soils (Lundström et al. 2000a; Sauer et al. 2007;

DeAngelis 2008). Podzol soil has characteristic horizons, the most superficial of which is the organic horizon (O), a surface layer that is rich in more or less decomposed organic matter. Below O is the elluvial horizon (E), weathered and grayish in character due to the removal of aluminum and iron via leaching. When the leachates reach the underlying illuvial horizon (B), aluminum and iron precipitate giving this layer a dark reddish appearance. Below the B horizon is unaltered parent material (C) (Figure 7) (Lundström et al. 2000b).

2.2. Distribution of aromatic LMMOAs in a podzol, and the effects of sampling and sample preparation techniques

2.2.1. Experimental setup

Paper I presents a study investigating the distribution of eleven aromatic LMMOAs in a podzol soil. In addition, different sampling techniques and sample preparation procedures were compared in regard to obtained concentrations of free and weakly adsorbed aromatic LMMOAs.

Ten phenolic acids consisting of hydroxy and methoxy substituted benzoic and cinnamic acids were included in the study, as well as phtalic acid (Figure 8 and Table 1). They are jointly referred to as aromatic LMMOAs in this thesis.

Soil solution was sampled via tension-lysimeters, and soil centrifugation. Liquid-soil extraction was also investigated.

Tension-lysimeter samples were collected via lysimeters in the O, E and B horizons, three in each soil horizon. The soil used for laboratory sampling was collected from each of the three podzol horizons adjacent to the lysimeters. Soil centrifugation, including three replicates for each soil horizon, was performed at 14 000 rpm, which produced a relative centrifugation force of 19 800 × g. The replicates sampled from each horizon were pooled for the respective sampling techniques.

Liquid-soil extraction was performed at ambient temperature and in darkness on field moist soil using either 10 mM phosphate buffer (pH 7.2) or 1:1 (v/v) of the very same buffer and MeOH. Extraction was performed for five replicates for each extraction solution and soil horizon.

All samples were analyzed using the LC-ESI-MS/MS method also reported in Paper I. Soil moisture content was determined by differential weighing before and after drying so that concentrations could be reported in μM, allowing for comparison of the results from the different sampling and sample preparation approaches.

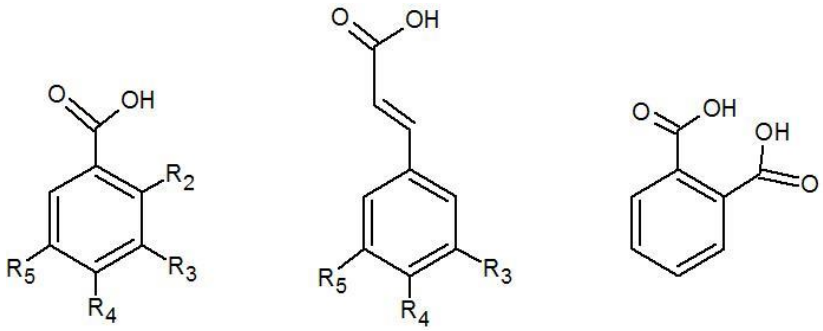


Figure 8. From left to right, substituted benzoic, substituted cinnamic and phthalic acid. Substituents are presented in Table 1.

Table 1. Substituents for ten benzoic and cinnamic acids (main structures in Figure 8).

Substituted benzoic acids		R_2	R_3	R_4	R_5
Salicylic acid	(Sal)	OH	H	H	H
p-Hydroxybenzoic acid	(*p*-Hyd)	H	H	OH	H
Protocatechuic acid	(Pro)	H	OH	OH	H
Vanillic acid	(Van)	H	OCH_3	OH	H
Syringic acid	(Syr)	H	OCH_3	OH	OCH_3
Gallic Acid	(Gal)	H	OH	OH	OH

Substituted cinnamic acids		R_3	R_4	R_5
p-Coumaric acid	(*p*-Cou)	OH	H	H
Ferulic acid	(Fer)	OCH_3	OH	H
Sinapic acid	(Sin)	OCH_3	OH	OCH_3
Caffeic acid	(Caf)	H	OH	OH

2.2.2. Results and discussion

Soil centrifugation and tension-lysimeters were used to investigate the recovery and distribution of free aromatic LMMOAs. In the case of weakly adsorbed aromatic LMMOAs, liquid extraction with two different extraction solutions was used.

It was anticipated that higher analyte concentrations would be obtained via soil centrifugation compared to sampling via lysimeters, in accordance with previous studies (Giesler et al. 1996; Gallet et al. 1999; Geibe et al. 2006).

Ali et al. (2011) used liquid soil extraction to investigate the yield of aliphatic LMMOAs in the O and E horizons of a podzol soil at the same study site as for this thesis. Equivalent extraction solutions were used in both Ali and colleagues' study as well as the present one i.e. 10 mM phosphate buffer (pH 7.2) and 1:1 (v/v) 10 mM phosphate buffer:MeOH.

Ali et al. (2011) found that buffer:MeOH extraction resulted in a higher yield of total aliphatic LMMOAs in the O horizon (80 times higher than when extracted with buffer only). The difference in extraction yield was not as obvious in the E horizon. Even though the total aliphatic LMMOA concentration was higher using buffer:MeOH extraction, it was less than doubled and several individual aliphatic LMMOAs had greater yields when extracted with pure buffer (Ali et al. 2011). Even though the structure of aromatic LMMOAs vary from that of the aliphatic acids, these findings due indicate possible trends.

Although alkaline extraction of phenolic acids and other phenolic compound is common (Whitehead 1964; Shindo et al. 1978; Hartley et al. 1979; Whitehead et al. 1983; Vance et al. 1986), a neutral extraction buffer was chosen for the present study, as highly alkaline treatments also result in the extraction of chemically-bound compounds (Whitehead et al. 1981). Treatments at very acidic or alkaline conditions might also jeopardize cell integrity (Lange et al. 2001) providing further cause for soil extraction at neutral pH.

PCA was used to illustrate the general patterns of sample type and analytes in this study. The bi-plot illustrated in Figure 9 is a combination of score (samples type) and loading (analytes) plots.

At first glance it appears as though lysimeter and centrifugation samples are clustered, suggesting correlation among these sample types. The buffer-extracted samples are also relatively grouped while the buffer:MeOH samples are more scattered, indicating a potential dependency on the sample matrix (i.e. soil horizon).

While the analytes are fairly spread out, trends can nonetheless be observed. The substituted cinnamic acids appear to correlate with each other and with buffer:MeOH extraction in the O and E horizons. Phthalic acid seems to correlate with buffer extraction, along with some of the substituted benzoic acids.

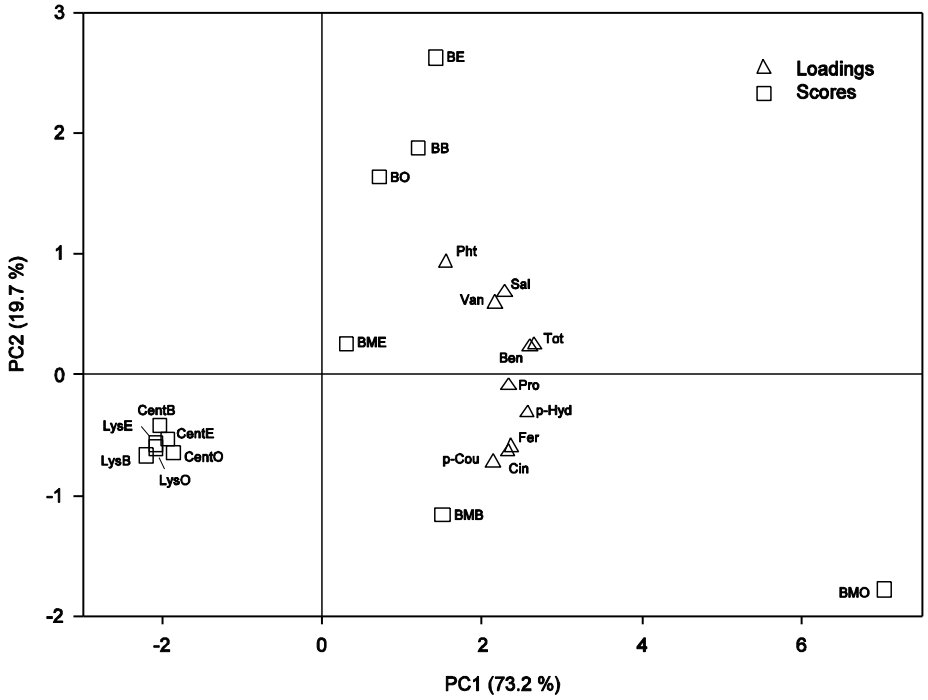


Figure 9. Bi-plot of the first two principal components from a PCA showing the pattern of correlation for sample types and selected analytes. Explained variances for PC1 and PC2 were 73.2 % and 19.7 % respectively. The abbreviations denotes sample type (B – extraction with pure buffer, BM – extraction with buffer:MeOH, Cent – centrifugation and Lys – lysimeter), soil horizon (O, E or B) and analytes (Tot – sum of all aromatic acids, Ben – sum of substituted benzoic acids and Cin – sum of substituted cinnamic acid). For denotation of abbreviations for individual analytes, see Table 1.

Aromatic LMMOAs quantified in tension-lysimeter and soil centrifugation samples for the three horizons are summarized in

Figure 10. Phthalic, vanillic and protocatechuic acid were found in lysimeter samples from the O and E horizons. Trace amounts, mostly of phthalic acid, were also identified in the B horizon. In addition to these three acids, p-hydroxybenzoic, p-coumaric and ferulic acid were positively quantified in the centrifugation samples. Phthalic acid deviates from the general trend of the aromatic acids as it rather increases in concentration with increasing soil depth, although not found in quantifiable amounts in the B horizon when sampled with lysimeter. No substituted cinnamic acids were found in the lysimeter samples, and total concentration of these acids were also lower compared to substituted benzoic acids throughout the soil profile, when sampled with centrifugation.

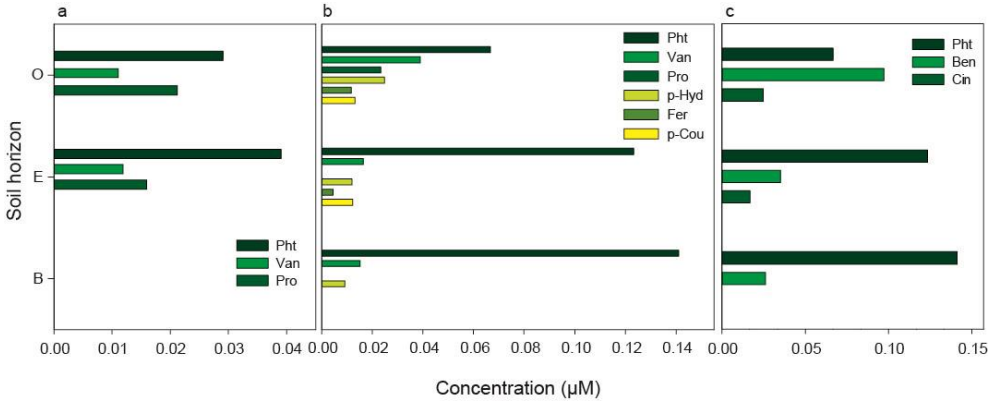


Figure 10. Individual aromatic LMMOAs obtained via tension-lysimeter (a). Individual (b) and sub-grouped (c) aromatic LMMOAs obtained via soil centrifugation.

Total concentration of aromatic LMMOAs and total concentrations of substituted benzoic and cinnamic acids as well as phthalic acid, quantified in liquid-soil extracted samples in the O, E and B horizons are presented in Figure 11.

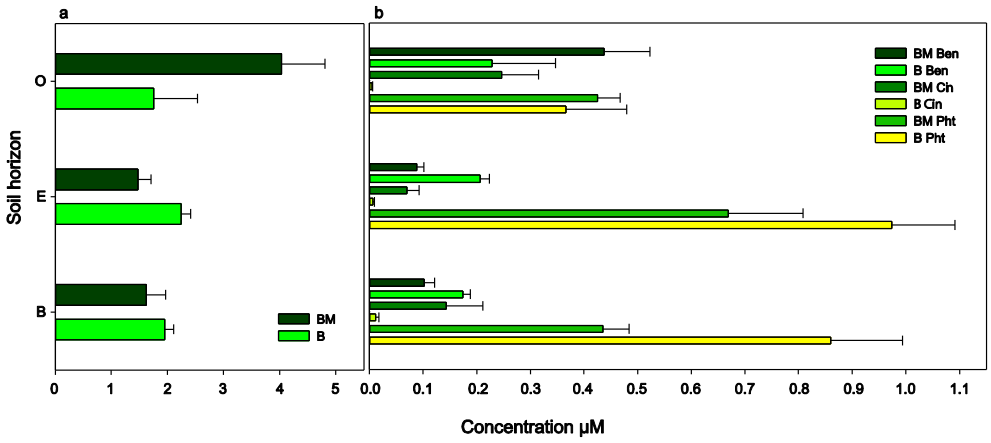


Figure 11. Total aromatic acid concentration (a), and total substituted benzoic (Ben); cinnamic (Cin); and phtalic acid (Pht), quantified in the O, E and B horizons in a podzol soil using liquid extraction with either pure buffer (B) or 50:50 v/v% MeOH:buffer (BM).

In addition to the aromatic acids quantified in the centrifugation samples, salicylic, sinapic, caffeic and low concentrations of syringic acid were found when extracting with buffer:MeOH. In the buffer extracted samples only salicylic and

sinapic acid were found in addition, and no quantifiable amounts of p-coumaric acid were present.

On average, the most abundant aromatic LMMOAs found when applying liquid extraction were (in order of decreasing amounts), vanillic, phtalic, p-hydroxybenzoic, ferulic, protocatechuic and salicylic acid.

Total aromatic LMMOA concentrations in the O, E and B horizons, obtained via tension-lysimeter, soil centrifugation and the two types of liquid extractions are presented in Table 2. By applying one-way ANOVAs and grouping methodology using Tukey's method for each separate soil horizon, significant differences between the sampling and extraction procedures could be derived (also included in Table 2).

Table 2. Total aromatic LMMOAs concentrations for each sample type and grouping information using multiple comparisons according to Tukey for each separate horizon.

	O horizon		E horizon		B horizon	
Sample type	*Conc.*[a]	*Group*[b]	*Conc.*[a]	*Group*[b]	*Conc.*[a]	*Group*[b]
Buffer:MeOH	4.04	A	1.48	B	1.62	A
Buffer	1.76	B	2.24	A	1.95	A
Centrifugation	0.19	B	0.18	C	0.17	B
Lysimeter	0.09	B	0.08	C	0.01	B

[a] Total aromatic acid concentrations are reported in μM

[b] Different letters indicate significant difference between sample type ($p<0.05$)

Significantly higher acid concentrations were obtained in the O horizon when applying liquid extraction with buffer:MeOH solution (Table 2). In contrast, liquid extraction using only buffer resulted in significantly higher concentrations in the E horizon. No significant difference between the two extraction procedures were noted for the B horizon, although a slightly higher total concertation was obtained using buffer extraction.

The different soil adsorption mechanisms were thought to offer a potential explanation for these results. Adsorption to SOM occurs via hydrophobic interactions, while adsorption to charged clay particles is promoted by electrostatic interactions. Logically, extraction solution with additions of MeOH should compete more effectively with the hydrophobic interactions in comparison to pure buffer. In the same manner, pure buffer should more effectively compete with the electrostatic interactions between the aromatic LMMOAs and the clay particles.

As SOM content is highest in the O horizon (Vestin et al. 2008a; Vestin et al. 2008b), extraction with buffer:MeOH should be the most efficient extraction procedure. In terms of electrostatic interaction, buffer should be more efficient when extracting the acids further down in the mineral soil.

Sub-grouping the aromatic LMMOAs into substituted benzoic and cinnamic and phtalic acids, revealed some contradictory trends (also seen in Figure 11b). The obtained analyte concentrations did not only vary with extraction procedure and soil horizon, but also with acid structure. To further investigate these differences, one-way ANOVAs with grouping methodology and the Tukey method were again performed for each soil horizon and separate subgroups.

Substituted benzoic and cinnamic acids were extracted more efficiently using buffer:MeOH in the O horizon, though not proven statistically significant in the case of substituted benzoic acid (Table 3). The extraction of substituted benzoic acids and phthalic acid coincided with the general trend, i.e. pure buffer resulted in significantly higher analyte concentrations in the E and B horizon, while significantly higher concentrations of substituted cinnamic acids were still obtained when applying buffer:MeOH extraction throughout the soil profile (Table 3).

Table 3. Grouping information for substituted benzoic (Ben) and cinnamic acids (Cin) as well as phthalic acid (Pht) by using multiple comparisons according to Tukey for each horizon. Different letters indicate significant difference between sample type ($p<0.05$)

	O horizon			E horizon			B horizon		
Sample type	*Ben*	*Cin*	*Pht*	*Ben*	*Cin*	*Pht*	*Ben*	*Cin*	*Pht*
Buffer:MeOH	A	A	A	B	A	B	B	A	B
Buffer	AB	B	A	A	B	A	A	B	A
Centrifugation	B	B	B	C	B	C	C	AB	BC
Lysimeter	B	B	B	C	B	C	C	AB	C

The complex behavior of the substituted cinnamic acids may be explained by the work of Cecchi et al. (2004), who studied sorption-desorption of both substituted benzoic and cinnamic acids in regard to soil properties. Cecchi and colleagues' found that phenolic acids had a high sorption percentage in soil with high organic content, but that K_{oc} (the sorption distribution coefficient to organic carbon) was more than twice as high for substituted cinnamic acids when compared to substituted benzoic acids.

This suggests that even though E and B horizons contain less SOM the substituted cinnamic acids are still adsorbed to organic matter via hydrophobic interactions and that buffer alone cannot extract them in the same extent as buffer:MeOH mixture.

Phthalic acid is plotted separately as it belongs to neither substituted benzoic nor cinnamic acids. Its behavior visibly differs from that of substituted benzoic and cinnamic acids, possibly due to phthalic acids' relatively low pK_{a1} and pK_{a2} (2.98 and

5.28 respectively). This characteristic, makes it more hydrophilic and downwardly mobile through the soil horizons, as well as more susceptible to buffer extraction.

In conclusion, soil centrifugation resulted in generally higher concentrations and a larger number of quantifiable aromatic LMMOAs compared with tension-lysimeter sampling. Buffer:MeOH extraction generally resulted in the highest concentrations in the O horizon, likely due to competition between MeOH and the acids' hydrophobic interactions with SOM. Substituted cinnamic acids were generally more effectively extracted with buffer:MeOH throughout the soil horizons, while substituted benzoic acids were more effectively extracted with pure buffer in the E and B horizons.

Lysimeter and centrifugation resulted in an approximately ten-fold reduction in aromatic acid concentrations compared to soil extraction, indicating the fractions of free and weakly adsorbed aromatic LMMOAs in the soil profile. An exception to this was p-coumaric acid which was found in centrifugation samples but not in those extracted with pure buffer.

Vanillic, phthalic and protocatechuic acid were obtained using all four methods and vanillic and phthalic were in general the most abundant ones independent of method, with the exception of lysimeter samples where vanillic and protocatechuic were most abundant.

It is evident that sampling and sample extraction procedure highly affected which species of aromatic LMMOA was found, and in what amounts they were obtained in this study.

2.3. Mineral amendment and its effect on microbial activity in a podzol

2.3.1. Experimental setup

The study referred to in Paper II describes a mineral amendment trial conducted at the Bispgården site, with the objective to investigate how minerals of varying composition affect microbial activity in their vicinity compared to the surrounding bulk soil. Both published and unpublished background data collected in connection with studies performed at the very same study site were used in the evaluation of the results from the present study (Vestin et al. 2008a; 2008b; Norström 2010).

The primary minerals, apatite ($Ca_{10}(PO_4)_6(OH,F)_2$) and biotite ($K(Mg, Fe)_3AlSi_3O_{10}(F,OH)_2$), were included in the amendment trial for their known impact on mineral nutrient availability in soil, as well as oligoclase ($CaAl_2Si2O_8$), a silicate mineral believed to be of less interest for microorganisms as a source of nutrients. The apatite and oligoclase minerals were cut into ~4 x 3 cm pieces and polished to a

smooth surface. Biotite was prepared by peeling off monolayers to reveal fresh surfaces (Figure 12).

Figure 12. Photos of the three amended minerals apatite, biotite and oligoclase (left), and the soil installation of mineral samples and tension-lysimeters in the O, E and B horizons of a podzol soil (right).

Duplicate minerals were inserted at soil layer transits beneath the O, E and B horizons, at on average 7, 14 and 34 cm depths from the soil surface. Fishing line was attached to the bottom of each mineral to enable their extraction at the end of the incubation time. In addition, tension-lysimeters were installed in the vicinity and at same depths as the minerals, to enable soil chemistry monitoring (Figure 12). The minerals were incubated from June 2009 until October 2013 and soil solution was collected via the tension-lysimeters at regular intervals during the frost free months (May to September). The pH of the collected soil solution samples was analyzed, as were dissolved organic carbon (DOC) and aliphatic LMMOAs according to Bylund et al. (2007). The samples were also screened for HS (Paper III), but no detectable amounts were found.

Results from the soil moisture samples were compared with historical data from the study site, and found to be consistent. This indicated that no notable changes had occurred regarding soil chemistry, allowing comparison with additional background data.

At the end of the soil incubation period, the minerals were extracted and soil from each mineral sample surface was collected. Bulk soil was also sampled at corresponding depths.

To investigate if the mineral amendment caused any differences in organic ligand production by microorganisms, soil samples were extracted with 1:1 (v/v) of 10 mM phosphate buffer (pH 7.2) and MeOH, and aliphatic LMMOAs and HS were quantified. Fungal biomass was estimated by extracting chitin-derived GlcN using the method described by Ekblad et al. (1996). Samples were then analyzed according to the method presented in Paper III. Enzyme activity, monitored via the enzymatic hydrolysis of FDA into fluorescein, was investigated spectrophotometrically using the method described by Green et al. (2006).

2.3.2. Results and discussion

N is considered to be the limiting factor for tree growth in northern forest ecosystems (Tamm 1991), but anthropogenic activities (e.g. burning of fossil fuels) increases N emission and consequently its deposition, which may render other nutrients such as P, K and Mg limiting (Schulze et al. 1989; Akselsson et al. 2008).

Mycorrhiza are known to improve the host plants' P uptake by covering a greater soil volume and thus decreasing the P diffusion distance (Bolan 1991), but also by improving plant-root P uptake from apatite mineral (Wallander et al. 1997; van Breemen et al. 2000a). Both laboratory and field studies show an increase in ectomycorrhizal production when applying apatite amendment in P-poor soils (Hagerberg et al. 2003; Leake et al. 2008; Berner et al. 2012).

The relatively rapidly weatherable biotite mineral is an important plant source of K. Wallander et al. (1999) reported that growth of pine seedlings was stimulated by biotite as a K source both with and without EM colonization, and that EM might produce citric acid to induce biotite weathering and K availability. When comparing different K sources, seedlings growing with biotite as the K source produced larger shoots containing more K, suggesting that biotite stimulated growth in other ways not entirely attributable to K (Wallander 2000b).

Foliar analysis is often applied to estimate a forest ecosystem's nutrient status, which can be used to determine if conditions for optimal forest growth and vitality are met. Linder (1995) previously determined the target nutrient ratios (in relation to N) in Norway spruce (*Picea abies*) needles. Ratios of P, K, Ca, Mg and Fe were compared with those obtained from analysis of spruce needles from the study site of the present mineral amendment trial. Calculated nutrient ratios in Bispgården were above target for all investigated nutrients except for Fe, which was less than half of the target ratio.

In addition, K, P, Ca and Mg ratios (in relation to N) were calculated for soil solution collected via centrifugation in May 2007, and compared with reported target ratios in nutrient solutions for spruce and pine seedlings (Ingestad 1979). The four mineral nutrients were above target for spruce and pine when comparing average values for the O, E and B horizons at the present site, although inspection

of separate soil horizons revealed suboptimal P ratios for both total and inorganic P in the mineral soil. This may suggest that P is a limited mineral nutrient in the area, though foliar content suggests that the trees are able to regulate this.

The estimated nutrient status of the study area suggests that apatite (as a source of P) and biotite (rich in Fe) are potential sources of required mineral nutrients. Comparison with elemental composition of the mineral soil (E and B horizons) performed in 2002 (Vestin et al. 2008a; 2008b) further emphasizes why apatite and biotite could be potential targets of biotic weathering (Table 4).

Table 4. Mineral nutrient concentrations (mg/g) of apatite, biotite and oligoclase calculated from atomic percentages obtained by energy dispersive X-ray spectroscopic analysis, and average value for the mineral soil in the study area.

Mineral	K	P	Ca	Mg	Fe
Apatite	-	173	348	-	5.3
Biotite	74	-	-	88	117
Oligoclase	4.2	-	32	-	3.0
Soil*	31	0.3	8.9	3.0	14

*Average concentration for the E and B soil horizons (Vestin et al. 2008a; 2008b)

Results from the soil analysis of the bulk and the mineral surfaces regarding organic ligand concentrations and fungal biomass and enzymatic activity are presented in Table 5. Oxalic and cis-aconitic acid were positively identified in the extracted samples, but could not be quantified safely due to co-elution with matrix components.

Only three HS (ferricrocin, ferrichrome and ferrioxamine B) were positively quantified in the extracted soil samples. Ferricrocin was found in all samples from the O and E horizon, while ferrichrome and ferrioxamine B were mostly found in O horizon and with no apparent variation among sample types. No HS were detected in the B horizon. HS obtained from the samples are reported as a sum (HS_{tot}).

Fungal biomass, enzymatic activity and organic ligand concentrations decreased with depth in the soil horizon. One-way ANOVAs were performed for each parameter, and a threshold for significant differences from the mean was established for p-values of less than 0.05. Enzymatic activity differed significantly between the O, E and B soil horizons, while fungal biomass and organic ligand concentrations only differed significantly for the O horizon, compared to E and B.

The same statistical analysis was performed within each soil horizon to compare differences between sample types (that is, soil extracted from the bulk and the surface of apatite, biotite and oligoclase).

Table 5. Average values of soil chitin-derived glucosamine, microbial activity, total hydroxamate siderophore (HS_{tot}) concentration, total and individual concentrations of low molecular mass organic acids (LMMOAs) quantified in soil from mineral surfaces and in bulk soil in the O, E and B horizons in a podzol soil.

Horizon	Sample	FB[a]	EA[b]	HS_{tot}[c]	LMMOA[d]														
					$LMMOA_{tot}$	Pyruvic	Lactic	Malonic	Maleic	Succinic	Citraconic	Glutaric	Malic	α-Ketoglutaric	Tartaric	Shikimic	Citric	iso-Citric	Fumaric	trans-Aconitic
O	Apatite	4.66	1.06	310.3	192.3	10.75	8.05	1.28	3.03	7.63	0.10	0.96	57.93	5.88	< LOD	20.41	37.81	2.74	35.56	0.14
	Biotite	5.13	0.89	254.1	265.3	13.38	9.25	1.41	< LOD	11.58	0.09	1.01	88.94	9.23	< LOD	7.29	80.95	4.84	37.15	0.15
	Oligoclase	7.34	1.77	211.3	281.6	8.80	13.61	1.23	< LOD	8.09	0.11	0.84	72.95	6.18	0.24	11.54	147.01	3.03	7.81	0.11
	Bulk	11.7	2.15	526.3	960.0	18.55	43.55	2.00	0.08	16.25	0.25	1.31	246.7	17.21	0.49	103.5	446.5	10.36	52.95	0.38
E	Apatite	0.46	0.50	6.97	10.8	0.81	< LOD	0.10	< LOD	0.63	0.01	0.10	2.93	0.36	< LOD	1.13	1.46	< LOD	3.32	< LOD
	Biotite	0.88	0.88	4.62	33.0	1.47	2.44	0.14	< LOD	1.15	0.02	0.13	6.45	0.69	< LOD	6.57	6.67	0.33	6.99	< LOD
	Oligoclase	0.57	0.63	4.74	4.20	0.88	< LOD	0.10	< LOD	0.55	< LOD	0.07	1.02	0.28	< LOD	< LOD	0.80	< LOD	0.48	0.01
	Bulk	0.31	0.43	10.04	6.90	0.69	1.10	0.15	< LOD	0.54	0.01	0.05	1.20	0.25	< LOD	1.13	0.68	< LOD	1.12	< LOD
B	Apatite	0.09	0.13	< LOD	2.13	0.26	< LOD	0.08	< LOD	0.18	< LOD	0.03	0.49	0.07	< LOD	0.80	0.09	< LOD	0.12	< LOD
	Biotite	0.08	0.14	< LOD	2.12	0.22	0.93	0.12	< LOD	0.35	0.02	0.03	0.31	0.05	< LOD	< LOD	0.08	< LOD	0.00	< LOD
	Oligoclase	0.11	0.17	< LOD	6.67	0.54	< LOD	0.10	0.01	0.51	< LOD	0.03	2.45	0.19	< LOD	2.27	0.23	< LOD	0.31	0.04
	Bulk	0.15	0.23	< LOD	4.63	0.29	2.04	0.13	< LOD	0.32	< LOD	0.05	0.72	0.07	< LOD	0.80	0.13	< LOD	0.08	< LOD

[a] Fungal biomass expressed as chitin-derived glucosamine (mmol/kg dry soil)

[b] Enzymatic activity expressed as formed fluorescein from the enzymatic hydrolysis of 3′, 6′-diacetylfluorescein (FDA) (mmol/kg dry soil)

[c] Total concentration of hydroxamate siderophores (nmol/kg dry soil)

[d] Total and individual LMMOAs concentration (μmol/kg dry soil)

In the O horizon, the highest values for fungal biomass, enzymatic activity and organic ligands were found in the bulk soil. Fungal biomass and enzymatic activity were significantly higher in bulk soil compared to apatite and biotite. There were no significant differences in organic ligand concentrations in the O horizon, presumably due to large variations within replicates.

The E horizon showed higher values of fungal biomass, enzymatic activity and LMMOAs (except trans-aconitic and malonic acid) in association with the biotite mineral, although not every parameter was significantly different in comparison to the other samples. Fungal biomass and enzymatic activity were significantly higher in the vicinity of biotite compared to the bulk soil and apatite mineral. For the LMMOAs, only citric acid was found in significantly higher concentration when associated with biotite, and then only in comparison to the bulk soil. HS_{tot} was higher in the bulk soil.

Only minor differences were found between the four sample types in the B horizon, though fungal biomass and enzymatic activity were slightly higher in the bulk soil and the concentration of some LMMOAs were slightly higher in the vicinity of oligoclase.

As the variation among sample replicates from the O horizon was large in both bulk soil as well as among mineral samples, it is difficult to draw any conclusions. These differences and the high values of microbial activity in the bulk soil might be explained by the O horizon's heterogenic nature and the difficulties associated with sampling bulk soil equivalent to those collected from the mineral surfaces. Sampling bulk soil only slightly closer to the soil surface would result in greater presence of organic matter and thus higher values of microbial activity. This suboptimal sampling may have been avoided in the thinner and more homogeneous E and B horizons.

The only distinct trend, albeit a very small one, was found in the E horizon, where higher values for fungal biomass, enzymatic activity and organic ligands associated with biotite in the E horizon suggested microbial interest towards this mineral. This trend could be visualized and further emphasized using PCA (Figure 13).

As K is not a limited nutrient in the area, Fe may account for this attraction. The fact that biotite weathers relatively rapidly and has a high Fe content may explain why HS were not higher in the vicinity of this mineral, as siderophore production is restricted to iron-limited conditions (Bagg et al. 1987).

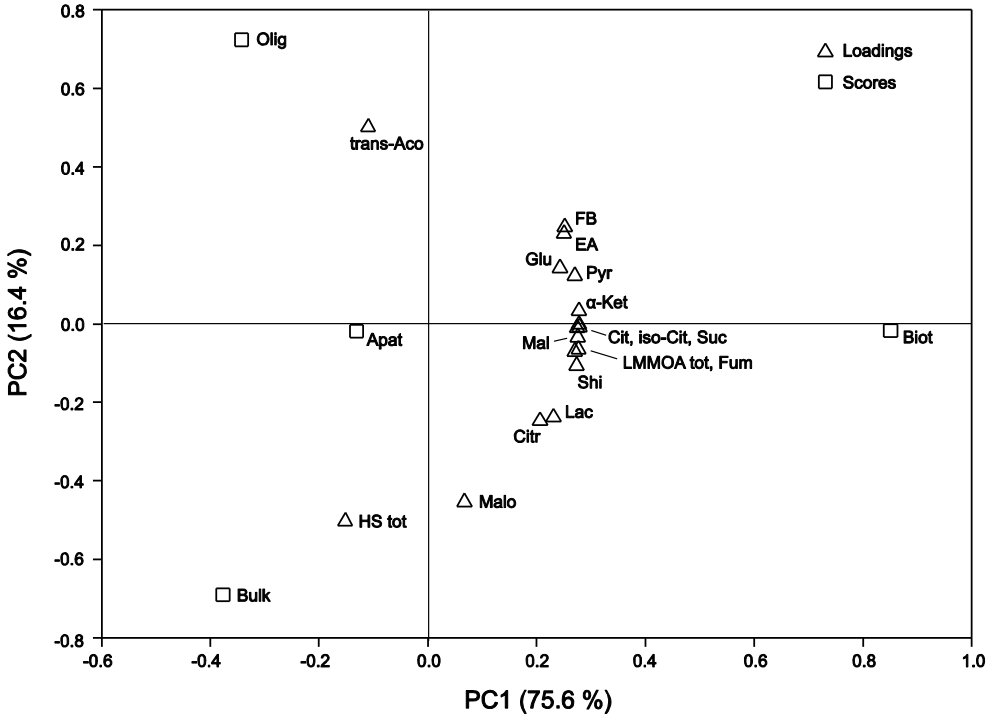


Figure 13. Bi-plot of the two first principal components from a PCA model of parameters reflecting microbial activity due to mineral amendment within the E horizon of a podzol soil. Explained variance for PC1 and PC2 were 75.6 and 16.4 %, respectively. Scores represent sample type and abbreviations denote; Olig - oligoclase, Apat - apatite, Biot - biotite and bulk – bulk soil. Loadings represent the analyzed parameters; FB - fungal biomass, EA - enzymatic activity, HS tot - total hydroxamate siderophores, LMMOA tot - total low molecular mass organic acids, Pyr - pyruvic, Lac - lactic, Malo - malonic, Suc - succinic, Citr - citraconic, Glu - glutaric, Mal - malic, α-Ket - α-ketoglutaric, Cit - citric, iso-Cit - iso-citric, Fum - fumaric and trans-Aco - trans-aconitic acid.

Several previous trials investigating EM growth or community structure in response to mineral amendment were restricted to the lower part of the O horizon (Hagerberg et al. 2003; Hedh et al. 2008; Berner et al. 2012), and thus disregarded the impact of EM species found exclusively in the E horizon (Rosling et al. 2003).

A number of reported studies investigating the effects on EM growth or community structure as a result of mineral or wood ash amendment employed mesh bags in their studies (Wallander et al. 2001; Hagerberg et al. 2002; Hagerberg et al. 2003; Korkama et al. 2007; Hedh et al. 2008; Wallander et al. 2008; Berner et al. 2012). These mesh bags are designed so that fungal hyphae can enter the bags, while

excluding plant roots and amending minerals were crushed and mixed with sand. The use of mesh bags simplifies the extraction of the amended samples compared to the approach used in the present study.

The chosen approach in this study was in order to, if possible, quantify biotic weathering visually. Mineral surfaces were thus investigated before and after the incubation using scanning electron microscopy (SEM). After the sample had been extraction it was clear that the minerals had been affected by weathering and that these effects decreased with soil horizon depth. However, it was impossible to distinguish biotic and abiotic weathering effects on the cut and polished minerals (apatite and oligoclase) (Figure 14). The biotite surfaces, on the other hand, revealed patterns which were presumably associated with fungal hypha progressing over the surfaces (Figure 15). As the weathering effects could not be compared between the different minerals these results were excluded from Paper II.

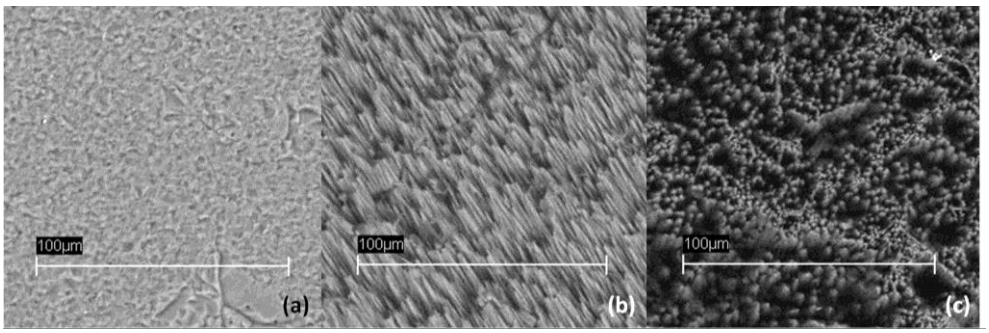


Figure 14. Apatite mineral surface investigated with SEM before (a) and after (b and c) incubation in the O-horizon in a podzol soil.

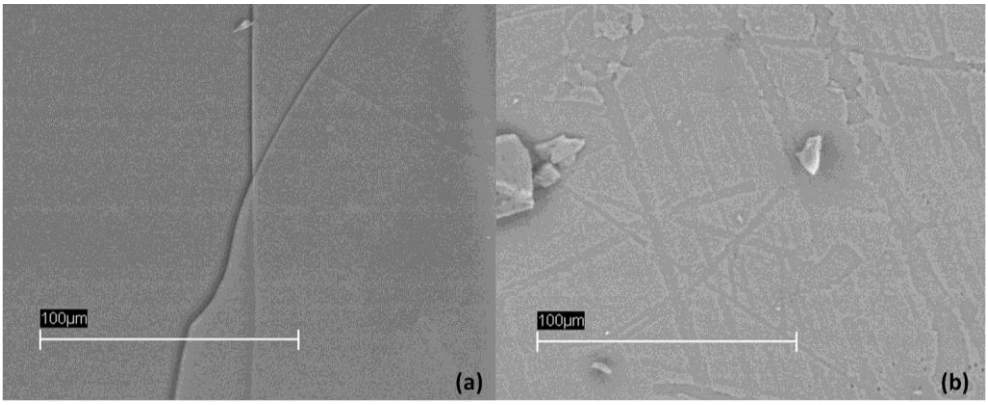


Figure 15. Biotite mineral surface investigated with SEM before (a) and after (b) incubation in the O-horizon in a podzol soil.

3. ANALYTICAL METHODS

3.1. Introduction to the instrumental setup

3.1.1. Liquid chromatography

Chromatography is the collective denomination for the techniques that separate compounds by their distribution between a stationary and a mobile phase. High performance liquid chromatography (HPLC; often simply referred to as liquid chromatography or LC), uses high pressure to force a liquid mobile phase through a column filled with the stationary phase. The mechanism of separation is the analytes difference in affinity to the stationary phase as a result of analyte properties, such as hydrophobicity, charge etc. (Harris 2003). LC is the preferred separation technique for non-volatile or thermically unstable analytes which are not compatible with gas chromatography. The LC system consists of a mobile phase delivery system, a sample injector, and the analytical column followed by a detector.

Different types of LC, classified according to separation mechanisms, include partition, adsorption, ion-exchange, and affinity chromatography (Skoog et al. 1998). Size- and molecular-exclusion mechanisms are referred to as chromatography, but do not comply with its definition. The LC stationary phase often consists of surface-modified spherical silica particles or polymeric resins.

Separation of analytes by adsorption to the polar, unaltered silica based stationary phase in combination with a nonpolar mobile phase is referred to as normal phase (NP) LC. Analytes are retained in the stationary phase with increasing polarity.

The opposite of NPLC is reversed-phase (RP) LC where bonded stationary phases are normally used. Typical RPLC stationary phases consist of hydrocarbon chains 8 or 18 carbon atoms in length (C8 and C18) covalently bound to silica spheres. These carbon chains form a hydrophobic layer, enabling separation of analytes via partitioning chromatography, and when combined with polar aqueous-organic solvent mobile phases, analytes are retained in the stationary phase with decreasing polarity.

Hydrophilic interaction liquid chromatography (HILIC) has similarities with both NPLC and RPLC. HILIC is used in the separation of polar analytes via hydrophilic partitioning or electrostatic interaction with polar or charged functional groups covalently bound to a silica-based stationary phase (Dejaegher et al. 2008). Hydrophilic partitioning is possible due to the layer of water that is retained on the hydrophilic stationary phase when using aqueous-organic solvent mobile phases with high organic solvent content (Greco et al. 2013).

Polar, charged (positively or negatively) or zwitter-ionic stationary phases are available for HILIC applications. Advantages of HILIC over NPLC include the aqueous-organic solvent mobile phase which enables solubility of polar and

hydrophilic analytes. In addition, the HILIC mobile phases are more compatible with mass spectrometry detection.

3.1.2. Mass spectrometry

The principle of mass spectrometry (MS) is the separation of ionized compounds by means of their mass to charge ratio (m/z). MS instruments achieve this through a variety of designs and solutions. Their communal mechanism is the application of electrical and/or magnetic fields to control analyte ions. Examples include *time-of-flight* (TOF) MS, which accelerates ions and separates them according to their velocity; *ion traps*, ions of specific m/z values are enriched; and *quadruple* MS, which only allows analytes with predetermined m/z values to pass through an electric field. The sensitivity and resolution of these MS instruments differ, and should be selected *a priori* according to analysis objectives.

A quadrupole MS instrument was employed in the studies described in this thesis. Quadrupole instruments possess both average sensitivity and resolution compared to the aforementioned techniques, and can be applied over a wide range of applications. Their distinguishing physical feature is four parallel metal rods through which direct and altering current is applied. Different combinations of the currents can be calibrated to only allow ions within narrow ranges of m/z to pass through and continue to the detector. This separation is performed at reduced pressure to avoid molecular collisions.

A single quadrupole instrument can measure analyte ions m/z between two end values (full scan mode), or by applying selected ion monitoring (SIM) only detecting analytes at specific m/z values.

Triple quadrupole instruments consist of two quadrupoles connected in series (Q1 and Q3) and separated by a collision cell (q2) (Figure 16) (Yost et al. 1978). Triple quadrupoles are applied in tandem mass spectrometry (MS/MS). The function of the two quadrupoles can be altered depending on the type of analysis required (de Hoffmann 1996). By disconnecting the collision cell and Q3, the instrument can be used as a single quadrupole to separate precursor ions (also referred to as parent ions) i.e. positively or negatively charged compounds as a whole. By employing the collision cell, analyte ions can be fragmented, via collision induced dissociation (CID). The derived product ions (also referred to daughter ions) can then be separated in Q3. By scanning Q3, a product ion spectrum is obtained, valuable in structural elucidation. Conversely, one can investigate precursor ions by specifying the fragment to be detected and conducting a full scan in Q1.

If both precursor and product ions are known for the analyte of interest, m/z values can be specified in both Q1 and Q3. This technique is called multiple reaction monitoring (MRM) and is the most selective and sensitive approach for separating

analytes using MS/MS (McLafferty 1981) (Figure 16). CID causes analyte collision by combining an inert collision gas with an applied collision energy.

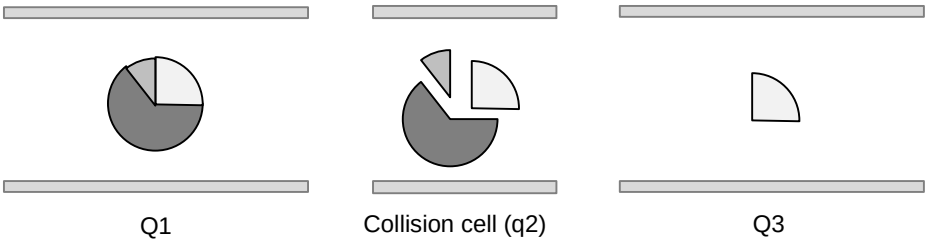


Figure 16. Schematic picture of a triple quadrupole illustrating the principals of multiple reaction monitoring (MRM) via collision induced dissociation.

3.1.3. Electrospray ionization

As MS instruments operate at reduced pressure and require gas phase ions for detection, an LC eluate cannot be introduced without modification. Electrospray ionization (ESI), one of several LC-MS interfaces developed, is a soft ionization technique used to transfer ions from solution into gas phase at atmospheric pressure. The technique was perfected by James Bennet Fenn and co-workers in the 1980s and their work made hyphenation of LC and MS using ESI possible (1984a; Yamashita et al. 1984b).

In ESI, the eluate is introduced via a capillary into a spray chamber (Figure 17). A potential difference is applied between the tip of the capillary and the oppositely positioned entrance to the MS. Depending on the direction of the electrical field, the applied potential induces either oxidation (positive mode) or reduction (negative mode) to occur within the spray capillary. The dominant reactions in aqueous solutions involves the following redox reactions of water:

$$6H_2O \rightarrow 4H_3O^+ + O_2 + 4e^- \quad \text{(oxidation)}$$

$$2H_2O + 2e^- \rightarrow 2OH^- + H_2 \quad \text{(reduction)}$$

Neutral bases or acids can react with these redox products to form positively or negatively charged ions via protonation or deprotonation. Depending on pH, these ions may also already be present in the solution. Furthermore, the analyte ions in ESI may be formed by non-covalent associations between neutral and charged species present in the solution, e.g. resulting in NH_4^+ or Na^+ adducts in positive mode.

Ions which are attracted towards the MS entrance due to their charge and the applied electrical field, are enriched at the tip of the capillary forming a so called Taylor cone. Disruption in the cone generates a spray of fine droplets carrying a net charge. Depending on eluate flow and composition, the spray formation can also be assisted by a nebulizer gas.

Traveling towards the MS entrance, solvents evaporate causing the droplets to shrink and consequently forcing ions of the same charge closer together. When the surface tension of the droplet is surpassed by the ions' repulsive forces, the droplets undergo a so called columbic explosion, converting the original droplet into several smaller ones (Figure 17). This process is repeated until the gas phase ions produced reach the entrance of the MS (Kebarle et al. 2009).

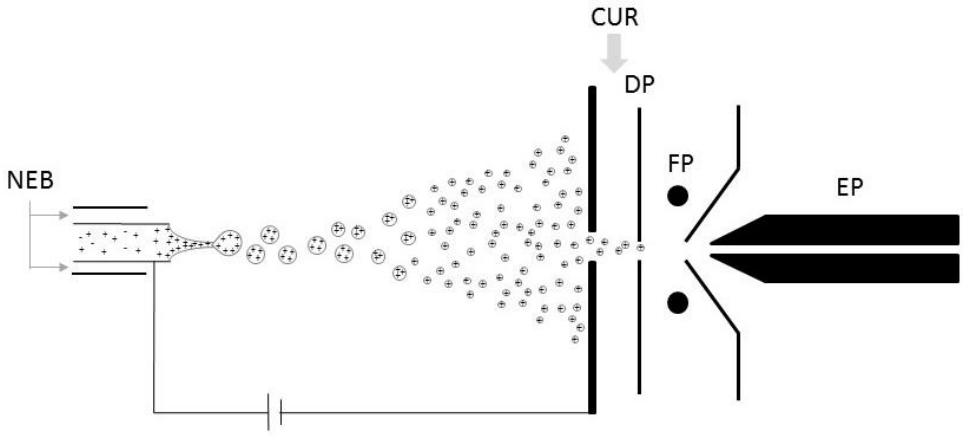
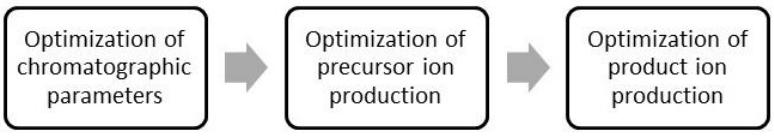


Figure 17. Schematic of the spray chamber illustrating the principle of electrospray ionization performed in positive mode. Nebulizer gas (NEB) and curtain gas (CUR) consitute gas flows. Potential settings include declustering, focusing and entrance potentials (DP, FP and EP).

3.2. Liquid chromatography mass spectrometry method development

Studies in Papers I, III and IV describe the development of quantitative analytical methods using LC-ESI-MS and LC-ESI-MS/MS. Analytical method performance is described in terms such as sensitivity, selectivity, and reproducibility, which all depend on the experimental conditions.

All parts of the LC-ESI-MS or MS/MS system need to be assessed both separately and as a whole in order to achieve optimal conditions and high performance. Moberg et al. (2006) describes the steps for optimizing LC-ESI-MS methods, which are summarized below.



Screening experiments are performed to establish important parameters prior to optimization of the three steps described above.

3.2.1. Optimization of chromatographic parameters

As MS is not merely a method of detection but also separation, samples could theoretically be introduced directly into the ESI-MS or MS/MS system as long as the m/z of the analytes differ. Even so, simultaneous introduction of analytes and sample matrix components could cause sensitivity loss due to ion suppression in the ESI (Annesley 2003). Therefore, analytes should preferably be retained and adequately separated on an analytical column.

When developing an analytical method, the LC stationary phase, eluent composition and flow, temperature and mode of elution (isocratic or gradient) are all important chromatographic parameters to consider (Harris 2003).

Analyte properties largely determine which LC stationary phase to select, which in turn restricts the choice of eluent. Once these basic components are identified, the eluent can be investigated in greater detail in order to achieve desirable analyte retention and separation.

Eluent pH is of great importance when separating ionizable analytes, and buffers are readily applied to ensure pH stability throughout the separation. With ESI-MS detection, choices of buffer and buffer concentrations are restricted as the eluent must be volatile and should not affect the analyte ionization negatively.

The eluent strength is a parameter describing the extent of interaction between the eluent and the stationary phase and thereby its ability to compete with the interaction taking place between the stationary phase and the analytes (Snyder et al. 1979). In the case of RPLC, the eluent strength is increased by the addition of a less polar constituent, i.e. an organic modifier. In HILIC separation, the opposite is applied. Both the choice and the amount of organic modifier affects eluent strength, e.g. ACN is less polar than MeOH, and is therefore the stronger eluent in RPLC.

When the eluent composition is kept constant throughout the LC separation, it is referred to as isocratic elution.

If adequate analyte separation is not achieved through isocratic elution or if elution time is unnecessarily long due to large differences in analyte retention, gradient elution is favorable, as it enables alteration of the mobile phase composition to increase the elution strength over time (Snyder et al. 1979; Skoog et al. 1998).

3.2.2. Optimization of ionization and mass spectrometry parameters

Eluent composition strongly affects analyte ionization and is therefore optimized after chromatographic parameters are established.

The applied eluent flow is determined by LC column dimension, stationary phase properties and eluent composition. Eluent flow entering the ESI should not exceed 100 μL/min as this would result in poor ionization; splitting the flow after the LC separation allows a flow reduction before continuing to the ESI.

The choice of ionization mode, i.e. positive or negative, is dependent on the analytes, and those able to both accept and donate protons can be ionized either way. If so, the ionization mode generating the highest sensitivity, in regard to the analytes' signal to noise ratio (S/N), should be chosen.

Suitable precursor ions are identified by MS scan in Q1. When using positive mode, detected ions often consist of the protonated analyte ($[M+H]^+$), though ionization can also occur as a result of association with other positively charged elemental or molecular ions, as described in section 3.1.3. For example, in the study presented in Paper III, the HS were analyzed in positive mode as protonated analytes, but to some extent they also formed Na^+ and NH_4^+ adducts ($[M+Na]^+$ and $[M+NH_4]^+$). Optimization of ESI parameters can alter adduct intensity to some extent.

A number of gas flows and potential settings influence the electrospray and the ionization of analytes. Nebulizer gas (NEB) and curtain gas (CUR) flow settings depend mostly on eluent flow and composition, so they are optimized for the method as a whole. In contrast, declustering, focusing, and entrance potentials (DP, FP and EP, respectively) are analyte-specific and can be altered throughout the analysis. These settings can be optimized via ramping experiments.

If ESI-MS/MS is used, collision cell settings i.e. collision gas (CAD) flow and collision energies (CE) are optimized to obtain the highest possible product ion signal. As changes in gas flow settings are sluggish, the established CAD setting is compromised and kept constant for one method. CE values are optimized from ramping experiments for each individual analyte.

3.2.3. Hyphenated sample pre-concentration

In the study described in Paper III, HS are quantified in soil solution. This requires low detection and quantification limits due to the low concentrations of HS reported in natural samples ((Holmström et al. 2005; Essén et al. 2006)). Sample pre-concentration is required to achieve this, either separate from the analysis or hyphenated with the LC-MS system. *On-line* sample pre-concentration has many advantages, including reduced risk of human error and less time required (Ali et al. 2008).

Pre-concentration on a separate column can be achieved by implementing a switch-valve setup. The valve is controlled via the instrumental software. In Paper III, on-line sample pre-concentration was performed by isocratically loading a large sample volume onto a pre-column. After a certain loading time the valve was switched and the enriched sample was eluted from the pre-column, onto the analytical column in opposite direction to the original flow (Figure 18). Pre-concentration also results in sample clean-up as matrix components not retained on the pre-column are eluted to waste before the sample is introduced onto the analytical column.

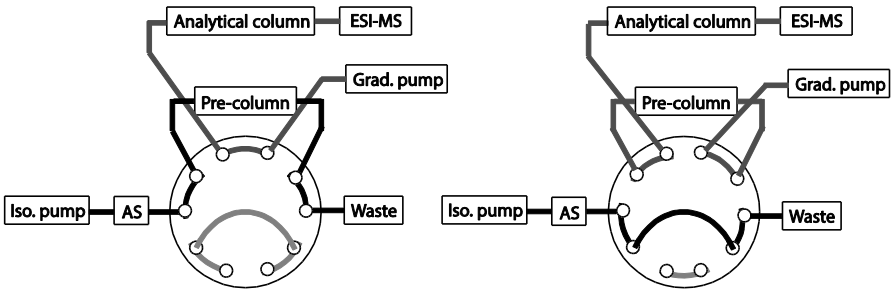


Figure 18. On-line sample pre-concentration via switch-valve setup. Sample loading onto pre-column (left) and sample elution from pre-column onto the analytical column (right).

3.2.4. Design of experiments

Design of experiments (DOE) can be applied in screening, optimization and robustness testing in order to solve specific problems (Eriksson 2008). Changing all significant experimental factors at the same time, according to a statistical experimental design, provides more information on the location of the optimum in comparison to varying factors individually. The latter is not only time consuming but can also result in an incorrect optimum if there are interactions between the factors. DOE experiments are normally preceded by screening tests to find the region for the experiment.

DOE was used in the study reported in Paper I for the purpose of optimization by applying response surface methodology. A central composite design (CCD) established the optimal elution parameters for the retention and separation of eleven aromatic acids. This experimental design enables the fit of quadratic models, and allows evaluation of both interactions and non-linear effects, with a minimum number of required runs. Initial screening tests were performed to establish factors of importance and within which ranges. By using response optimization, response

variables can be weighed in order of importance and a general solution to achieve the desired result is suggested.

The study reported in Paper IV investigated fragmentation patterns of two amino sugars, and a full factorial design was set up to verify whether found fragment proportions could be used to distinguish the two analytes. Examples of central composite and full factorial designs are presented in Figure 19.

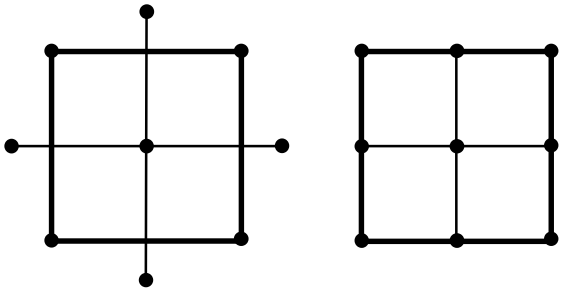


Figure 19. A central composite design with two factors (left) and a full factorial design with two factors and three levels (right). Each point represents an experiment.

3.3. Result and discussion

Papers I, III and IV include development of quantitative analytical methods using LC-MS and LC-MS/MS and the results are discussed individually below.

3.3.1. Aromatic low molecular mass organic acids

The study described in Paper I also included the development of an analytical method for the quantification of the eleven aromatic LMMOAs described in section 2.2.1. The included aromatic acids are presented in Figure 8 and Table 1.

The chromatographic retention and separation of the eleven analytes were performed on a C18 stationary phase, by applying gradient elution combining 0.01 % (v/v) formic acid and MeOH. Elution parameters including start MeOH percentage and gradient slope (% MeOH/min) were established by optimization using response surface methodology and CCD, described in 3.2.4.

Screening tests established model input values. The start MeOH percentage was varied between 10 and 20 % and the gradient slope between 1 and 6 % MeOH/min. Peak area and peak width at half peak height (PW50%) were considered the most important response variables. Optimal variable settings were illustrated as response surfaces using response optimization (Figure 20).

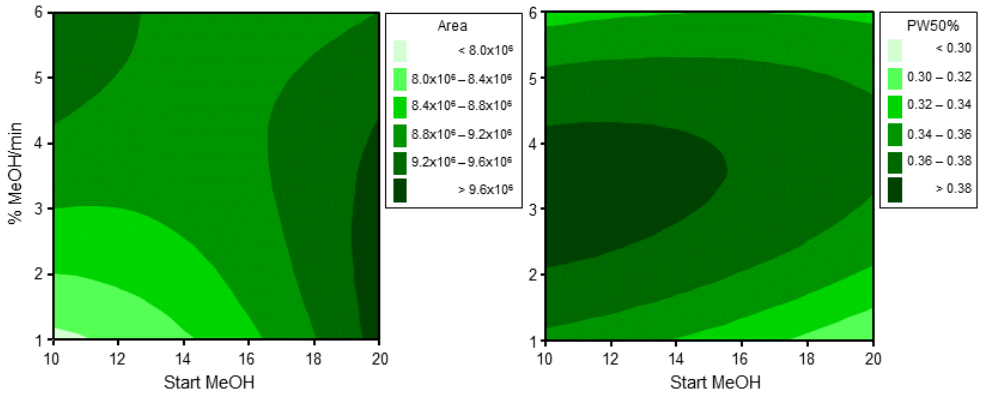


Figure 20. Response surfaces illustrating models on how start MeOH percentage and gradient slope (% MeOH/min) affects the peak area (left) and peak width at half height (PW50%) (right) for phtalic acid.

The CCD optimization was evaluated by fitting second order models to each response variable. High R^2 values were generally obtained, indicating that the majority of the variance in the data could be explained by the models. Lack-of-fit was determined by comparing the model's residuals with the pure error for the replicates, and was not significant ($p>0.05$) for the peak area models and for the majority of the peak width models, further indicating their validity.

A global optimum for start MeOH percentage and gradient slope could be defined by setting up a desirability function (Bourguignon et al. 1991). The optima differed depending on the inclusion and weighing of responses in the function. Sole consideration of PW50% and the global optima would suggest 10 % as start MeOH and an increase of 6 % of MeOH/min as a gradient slope; this is understandable as steep gradients often result in narrow analyte peaks due to reduced band broadening.

Since the lack-of-fit was significant for some of the PW50% responses, the final desirability function was comprised of analyte area responses and PW50% only for specific analytes predisposed to broad peaks. The general solution suggested was a start eluent composition of 20 % MeOH and 80 % 0.01 % (v/v) formic acid in MilliQ water with 5 % increase of the MeOH content per minute. These settings yielded satisfactory results upon experimental verification, in line with the model predictions for the analytes.

Baseline separation was not included in the model as MS/MS detection was applied. Hands-on evaluation was used to ensure separation of the two stereoisomers, salicylic and p-hydroxybenzoic acid, both of which also generate a 93 Th fragment.

All eleven aromatic LMMOAs eluted within eleven minutes when applying the settings described above (Figure 21). The gradient was run for twelve minutes and reached 80 % MeOH composition, before being restored to 20 %. Seven minutes of column conditioning was reserved prior to subsequent injection.

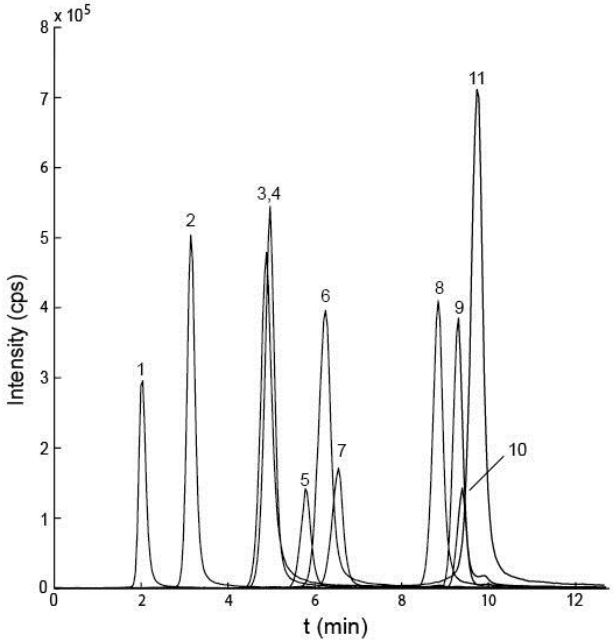


Figure 21. MRM chromatogram including the eleven aromatic LMMOAs included in the method. In order of elution: (1) gallic, (2) protocatechuic, (3) phthalic, (4) salicylic, (5) vanillic, (6) caffeic, (7) syringic, (8) p-coumaric, (9) ferulic, (10) sinapic and (11) p-hydroxybenzoic acid.

Ionization was performed in negative mode and ESI parameters, both communal and analyte-specific, were optimized by direct infusion of 5 μM solutions of each separate analyte. Gas settings were determined manually while potentials were determined from ramping experiments. In the same manner, analyte fragmentation via CID was investigated, which resulted in a MRM method for the quantification of the eleven aromatic LMMOAs. For specific ESI and collision cell settings, see Paper I.

The included aromatic acids produced $[M\text{-}H\text{-}COO]^-$ product ions, after the neutral loss of CO_2 (44 u), with the exception of vanillic, syringic, ferulic and sinapic acid. The common structural denominator for these four exceptions was that they all included one or two methoxy groups. By losing a methyl radical (15* u) they can form stable anionic radicals $[M\text{-}H\text{-}CH_3]^{*-}$ or $[M\text{-}H\text{-}COO\text{-}CH_3]^{*-}$ in accordance with the findings of Gruz et al. (2008) (Figure 22). This atypical CID behavior has also

been reported for other phenolic compounds such as methoxylated flavonoids (Justesen 2001). For the complete list of fragments included in the MRM method, see Paper I.

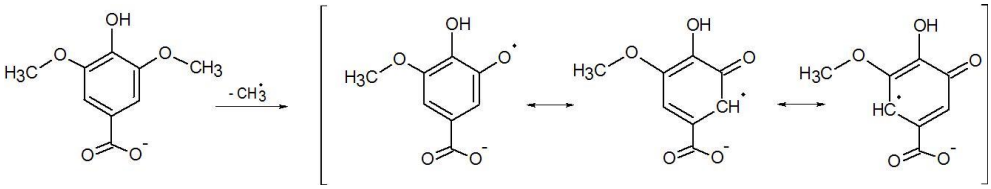


Figure 22. Syringic acid and the hypothesized formation of the resonance stabilized radical product ion of 182 Th.

Repeated injections of standard material was used to assess the linearity, sensitivity and precision of the method. LOD was defined as the analyte concentration resulting in a peak height equal to three times the noise level.

LOD values for the eleven aromatic acids included in this method ranged from 5 to 10 nM. See Paper I for a complete list of figures of merits for the presented LC-MS/MS method.

3.3.2. Hydroxamate siderophores

In Paper III the development of a quantitative and qualitative analytical method including thirteen hydroxamate siderophores (HS) of both bacterial and fungal origins is discusses. The included HS represent the three subgroups ferrichromes, ferrioxamines and coprogens/fusigens. Examples of the molecular structure of ferrichromes and ferrioxamines are illustrated in Figure 3 (Section 1.4.2.).

On-line sample pre-concentration using a switch valve setup was applied prior chromatographic separation, as described and illustrated in 3.2.3. In order to minimize analyte loss, it is imperative that these do not elute from the pre-column during sample-loading. Furthermore it is preferable that separation of the analytes are kept to a minimum as this may lead to losses in resolution. To minimize the risk of these events occurring, sample loading should be performed using an eluent with low elution strength (a polar mobile phase, in the case of RP). A pre-column supporting 100 % aqueous solution was thus chosen. RPLC retention and separation was performed using a Kinetex column with a C18 stationary phase.

As MS and MS/MS detection was employed, analyte baseline separation was not required with the exception of the two isobaric siderophores, ferrirubin (FRU) and ferrirhodin (FRH). In addition, it was assured that ferrioxamine E (FOE) and ferrioxamine D_1 (FOD_1) were chromatographically separated due to the risk of

isotopic overlap. The precursor ion of FOE has an m/z value of 654 Th. A 1 % abundance of ^{13}C and a 2 % abundance of ^{57}Fe resulted in the isotopic pattern illustrated in Figure 23 (left). The abundance of the precursor ion with an m/z value of 656 Th would result in a false positive detection of FOD_1 (m/z 656) if co-eluted (Figure 23, right).

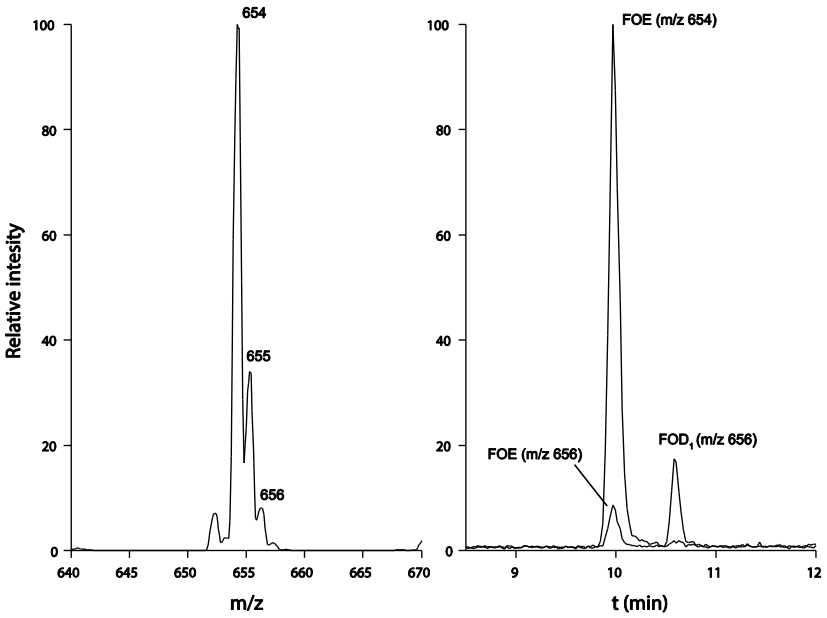


Figure 23. Mass spectrum (Q1) of ferrioxamine E (FOE) with relative isotope intensity (m/z 654, 655 and 656) (left) and SIM chromatogram of FOE and ferrioxamine D_1 (FOD_1) with individual concentrations of 4.9 nM and 5.3 nM, respectively (right).

In order to achieve sufficient retention and required separation of FRU/RFH and FOE/FOD_1, gradient elution was applied using a 12 mM ammonium formate buffer (pH 4) and ACN.

Optimization of chromatographic parameters resulted in the following procedure: 100 µL of the sample was concentrated on the pre-column using a loading solution consisting of 99 % ammonium formate buffer (pH 4) and 1 % MeOH, at a flow of 400 µL/min. After 2.5 minutes, the sample analytes were eluted in the opposite direction of the original flow, from the pre-column and onto the analytical column, after which data collection commenced. The mobile phase flow was 200 µL/min, applying gradient elution used two eluents: A consisting of 90 % ammonium formate buffer and 10 % ACN, and B consisting of 100 % ACN. The gradient elution was divided into two sections: for the first six minutes B increased

linearly from 0 to 10 %, followed by a linear increase of B to 35 % over five minutes. Finally, the columns were cleaned by raising the ACN content to 85 % prior reduction to the initial percentage. Both columns were conditioned for five minutes prior to subsequent injection, resulting in a total analysis time of 20.5 min. Before reaching the ESI interface the flow was split using a Valco tee union, resulting in a forward eluent flow of approximately 80 μL/min.

Analyte ionization was carried out in positive mode. Formed precursor ions included proton (H^+), ammonium (NH_4^+), and sodium (Na^+) adducts. Proton adducts ($[M+H]^+$) represented the most intense precursor ions for all included HS. NH_4^+ adducts can be expected when using an ammonium-based buffer, and these were most intense for the ferrichromes. NH_4^+ adduct intensity could be minimized through optimization of ESI parameters, especially by increasing the DP voltage. The presence of Na^+ adducts was rare and most likely due to impurities in solvents and glassware.

Additional investigations employing direct infusion of standard solutions revealed that FRH underwent partial up-front fragmentation, producing a precursor ion of m/z 899 Th (Figure 24).

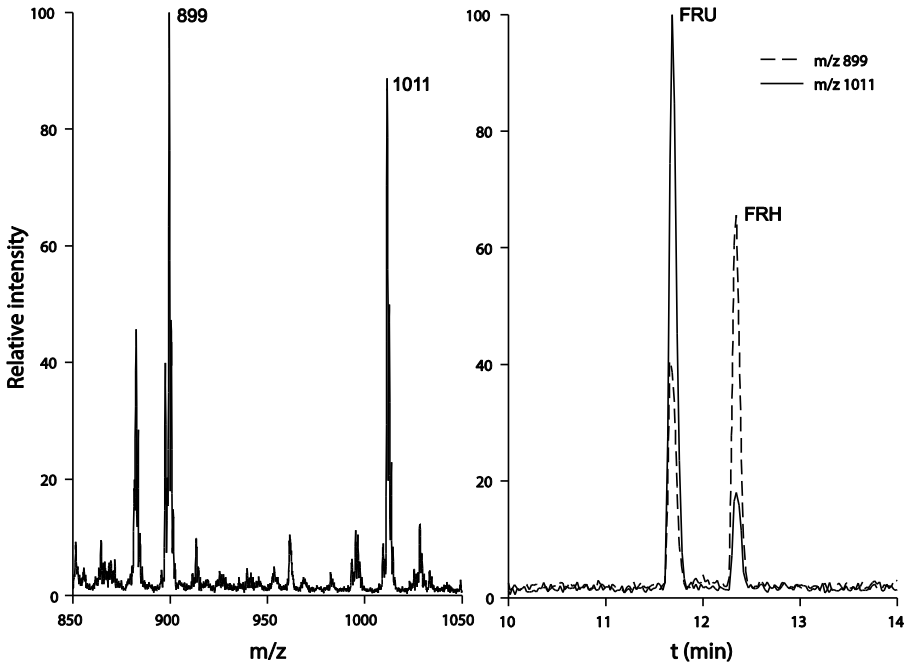


Figure 24. Mass spectrum of ferrirhodin (FRH) (left) and SIM chromatogram of ferrirubin (FRU) and FRH, analyzed at both 899 and 1011 Th (right).

Up-front fragmentation can occur in the ESI interface as a result of (in this case undesired) CID of accelerated ions colliding with gas molecules before entering the low pressure regions of the interface. Optimization of ESI settings for both the 899 precursor ion and the protonated analyte (m/z 1011) proved that the 899 precursor ion was the most intense, and therefore chosen for the quantitative SIM method. The mass difference for the two precursor ions was 112 u, suggesting fragmentation at one of the three hydroxamate groups.

Analyte fragmentation via CID was also investigated to enable structural identification. Using MS/MS detection, the LOD values were found to increase 2 to 9 times compared to MS. This is often the case when applying MRM, as 100 % of the precursor ion is never transferred to a single product ion.

When the included siderophores' fragmentation properties were investigated it became apparent that application of a relatively high CE was required in order to enable total demission of the precursor ions. Once that was achieved, a forest of fragment peaks appeared. As a result, a lower CE was applied to gain product ions of acceptable intensity, which also meant that a large part of the precursor ion remained intact (Figure 25). This observation corresponds with that of Essén et al. (2006) in regard to FCH, FCR and FCHRY, where the most intense signal actually corresponded to the intact precursor ion.

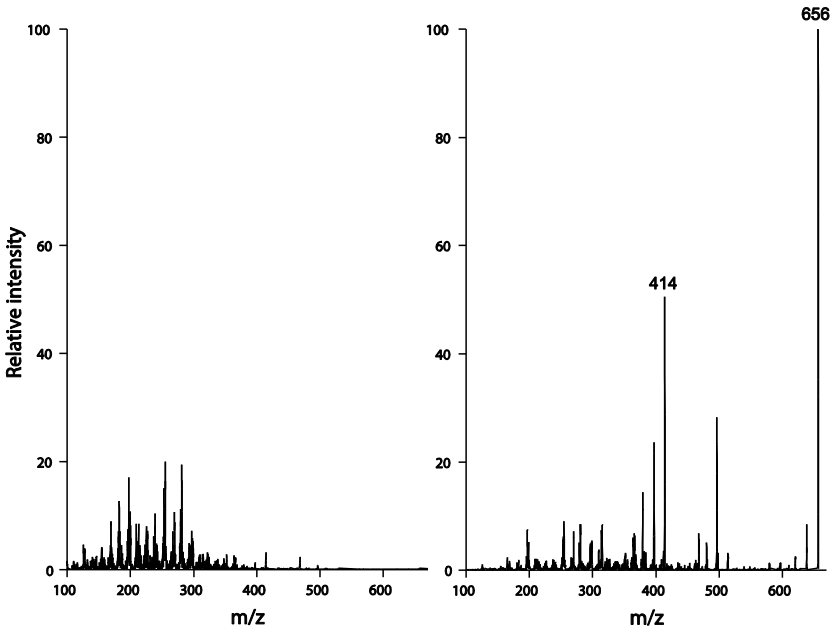


Figure 25. Product ion scan of ferrioxamine D_1 applying 63 eV (left) and 43 eV of collision energy (right) acquired via direct infusion at identical conditions except for collision energy value. Relative intensity is in regard to the most abundant product ion (m/z 656).

For all optimized ESI and collision cell parameters comprising the SIM and MRM method, see Paper III.

The fragmentation patterns of the majority of HS included in Paper III have been studied by Gledhill and co-workers (Gledhill 2001; Mawji et al. 2008), and Essén et al. (2006), and their findings correspond well with those of this study.

The fragmentation patterns of FOD_1 were examined in more detail, as these had not been reported earlier. FOD_1 closely resembles FOB apart from an additional acetyl group on FOD_1. Thorough investigation of the collision-activated fragmentation of desferrioxamines, with the formation of energetically favorable succinimide rings, has been reported by Feistner et al. (1995). Similar outcomes were assumed in the fragmentation of FOD_1, even though the present study investigated HS complexing Fe^{3+} (Paper III).

Retention time reproducibility, linearity, sensitivity, precision and recovery in typical sample matrices were investigated using the final method. All figures of merits can be found in Paper III.

To determine LOD, the sample concentration resulting in a peak height corresponding to three times the baseline noise level was determined from estimated S/N values. Standard solutions with concentrations less than five times that of the estimated LOD were then analyzed repeatedly and LOD was defined as three times the area standard deviation (SD). This approach proved useful, especially when determining LOD for the MRM method, as it produced very little noise.

The LOD values established for the SIM method imply possible analyte detection in the picomolar range for a majority of the included HS. Even though the LOD values were higher for the MRM method, that approach could prove to be beneficial when analyzing samples with complex matrices.

3.3.3. Chitin-derived glucosamine

The purpose of the study, reported in Paper IV, was to develop a simple and rapid approach to estimate fungal biomass via the quantification of chitin-derived glucosamine (GlcN). Available HPLC methods, developed for this purpose, are relatively complicated and time consuming as analyte-derivatization is applied to enable fluorescent detection and retention on RP stationary phases (Zelles 1988; Diaz et al. 1996; Ekblad et al. 1996; Appuhn et al. 2004; Indorf et al. 2011; Drigo et al. 2012). The derivatization step can either be performed automatically, which requires specialized auto-samplers, or manually which may involve human error as a factor.

The idea to develop a quantification method, avoiding derivatization, originated from the pharmaceutical field, where GlcN is analyzed in blood plasma and synovial fluid to investigate pharmacokinetics or study the physiological role of endogenous GlcN (Roda et al. 2006; Zhong et al. 2007; Pastorini et al. 2009). The mentioned

studies applied more polar stationary phases for the retention of GlcN and detection was performed using MS.

For the present study a zwitter-ionic column was chosen for the retention of GlcN. The stationary phase of this column consists of positively charged quaternary ammonium and negatively charged sulfonate groups (1:1) able to retain polar, positively or negatively charges analytes (Figure 26).

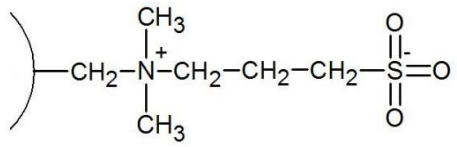


Figure 26. The ZIC-HILIC zwitter-ionic stationary phase contain permanently charged quaternary ammonium and sulfonate groups.

A large number of amino sugars are recognized in association with microorganisms, and the most commonly ones found in soil are GlcN, galactosamine (GalN), mannosamine (ManN) and muramic acid (MA) (Figure 27) (Amelung 2001). MA is exclusively found in bacteria as a component of peptidoglycan (Parsons 1981). Peptidoglycan also consists of GlcN, usually at a 1:1 ratio (Madigan 2005). By quantifying MA the bacterial contribution to total GlcN can be estimated. GalN is believed to be mostly of bacterial, while little is known about the origin of ManN (Amelung 2001).

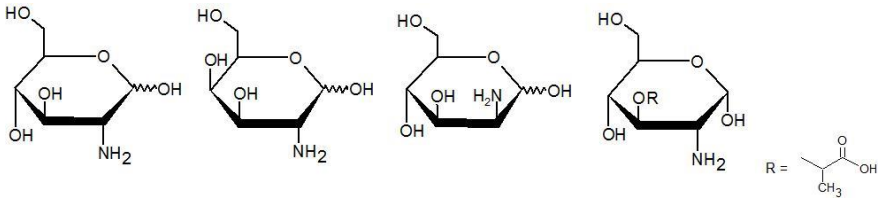


Figure 27. Structures of the four amino sugars most commonly found in soil. From left to right: glucosamine, galactosamine, mannosamine and muramic acid.

GlcN, GalN and ManN are isobaric compounds, hence they produce identical precursor ions when analyzed with MS. They also generate the same product ions when subjected to CID, why they are difficult to distinguish merely by the use of MS or MS/MS detection. In an attempt to avoid inaccurately high values in case of co-

quantification with these isobaric amino sugars, gradient elution was applied to chromatographically separate them. This undertaking proved to be difficult and even the α- and β-anomers of the analytes separated more readily than the isomers themselves.

When studying the fragmentation patterns of the three isobaric amino sugars more closely, differences in fragment intensities were observed between some analytes. GlcN and ManN produced nearly identical product ion spectra, but since the contribution of ManN to the total amino sugar content in soil is reported to be relatively low, up to 50 times less than GlcN in grass land (Amelung et al. 1999), its distinction was not considered to be critical in quantifying chitin-derived GlcN, and was therefore not considered further. Galactosamine, on the other hand, is believed to account for 30-50 % of the amino sugars content in soil (Joergensen et al. 2008).

Fragmentation patterns of GlcN and GalN, differed notably (Figure 28, left). One obvious divergence was that of the 84 Th fragment, which was produced in significantly greater proportion for GlcN compared to GalN (Figure 28, right).

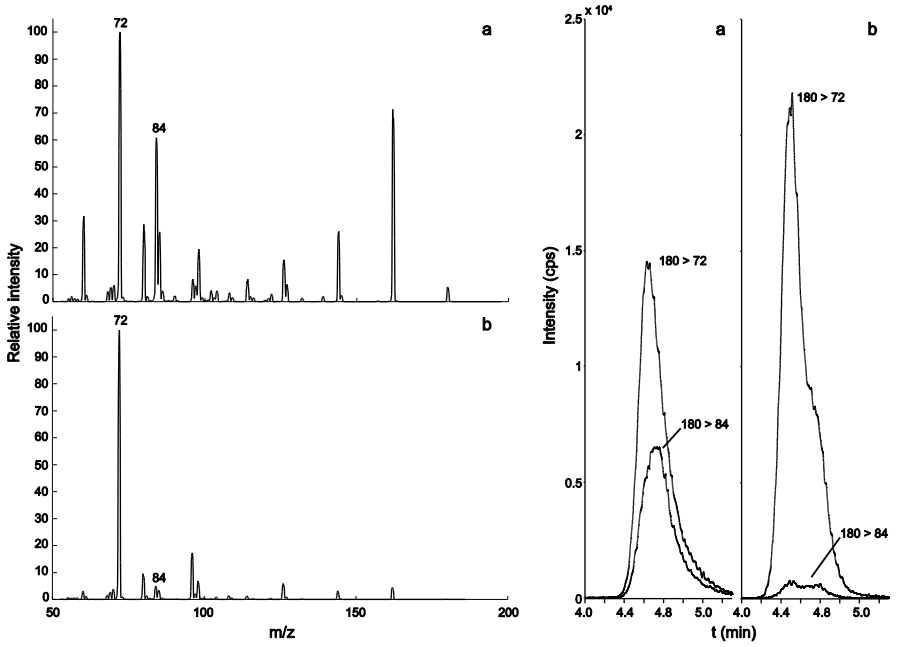


Figure 28. Positive ESI-MS/MS product ion spectra (left) and MRM chromatograms (right) of (a) glucosamine and (b) galactosamine, presenting the two m/z transitions investigated in the method (180 → 72 and 180 → 84). The mass spectra were produced through direct infusion of 100 µM reference solutions of each analyte with a collision energy of 25 eV, and MRM chromatograms via separate injections of GlcN and GalN reference solutions.

These findings suggested that the two isomers might be separable mathematically by analyzing both m/z transitions for an unknown sample peak. Initial tests were performed to verify that the ratios between the 72 and 84 Th fragments were indeed reproducible at different concentrations of GlcN and GalN. This also needed to be verified for different mixtures of the two analytes, at varying individual concentrations. To accomplish this, a full factorial design was set up using Minitab 16 (Minitab inc., State Collage, Pennsylvania). Three levels of analyte concentration (low, intermediate and high) were combined and analyzed in duplicate and in random order. The design can be found in Paper IV. Standard solutions were also included in the acquisition batch.

After completed analysis the areas of the integrated peaks corresponding to the two m/z transitions (180 → 72 and 180 → 84) were used as dependent variables in a regression analysis performed to investigate a potential relationship. The multiple linear regression models suggested for the two m/z transitions were significant with high R^2 values (97.6 and 98.2 % for the 72 and 84 Th fragments, respectively). The models also proved to be valid for the entire range of investigated concentrations, as no significant lack-of-fit was noted. The following linear relationships were found for the areas of the 72 and 84 Th fragments (A_{72} and A_{84}, respectively):

$$A_{72} = k_1 \times C_{GlcN} + k_2 \times C_{GalN} \quad \text{Equation 1}$$

$$A_{84} = k_3 \times C_{GlcN} + k_4 \times C_{GalN} \quad \text{Equation 2}$$

In Equation 1 and 2, $k_{1\text{-}4}$ represents the coefficients of slope specific for each combination of product ion and analyte.

When solving for the concentrations of GlcN and GalN (C_{GlcN} and C_{GalN}, respectively) in Equation 1 and 2, the following equations are derived:

$$C_{GlcN} = \frac{A_{72} - k_2 \times C_{GalN}}{k_1} \quad \text{Equation 3}$$

$$C_{GalN} = \left(A_{84} - \frac{k_3 \times A_{72}}{k_1}\right) / \left(k_4 - \frac{k_2 \times k_3}{k_1}\right) \quad \text{Equation 4}$$

By using Equation 3 and 4, it should be possible to calculate the individual concentrations of GlcN and GalN for an unknown sample by incorporating the areas of the two m/z transitions and the coefficients of slope from standard curves determined from individual standard solutions of GlcN and GalN. This was verified for the solutions used in the full factorial design by comparing the calculated

concentrations with the known values. Analyte peak areas and calculated concentrations are also reported in Paper IV. The calculated concentrations for GlcN and GalN were found to be slightly higher (10 and 2 % respectively), when compared to the known concentrations.

A number of soil samples prepared in accordance with Ekblad et al. (1996) were analyzed in duplicate and randomly and using the elaborated method. GlcN and GalN concentrations were calculated using Equation 3 and 4, and the pooled relative standard deviation for the sample duplicates were found to be 4, 3 and 5 % for MA, GlcN and GalN, respectively, indicating the level of reproducibility. Figure 29 illustrate one of these samples, which was collected from the O horizon of a podzol soil.

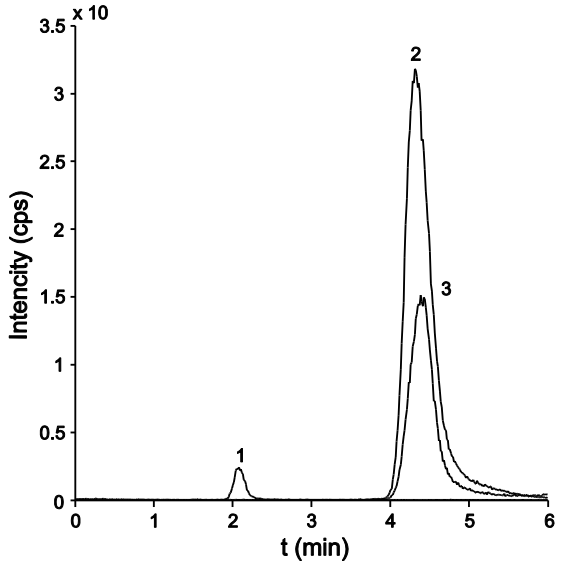


Figure 29. MRM chromatogram of an extracted soil sample with peaks representing m/z transitions (1) 252 → 126, (2) 180 → 74 and (3) 180 → 84. Peak areas correspond to 0.5 µM muramic acid, 15.3 µM glucosamine and 1.4 µM galactosamine.

Recovery tests were performed on spiked samples also extracted in accordance with Ekblad et al. (1996) and indicated somewhat low recovery percentages for GlcN and GalN. Analysis in full scan mode (both positive and negative mode) revealed co-eluting interferences corresponding to $[ACN+Na]^+$ and $[2ACN+Na]^+$. If accurately interpreted this Na^+ may be residues from the alkaline treatment, using NaOH, which is included in the described treatment. By excluding this step in the extraction

procedures, as it has been in numerous reported studies (Zhang et al. 1996; Kaiser et al. 2000; Appuhn et al. 2004; Bodé et al. 2009; Indorf et al. 2012), this interference may be avoided.

The final MRM method was investigated regarding detection limits, retention time reproducibility, linearity, sensitivity, precision and recovery for the included analytes (MA, GlcN and GalN). Overall satisfying performance was achieved implying the methods usefulness in the estimation of fungal biomass. In addition, the total analysis time was only six minutes, thereby enabling high sample throughput.

When this method was applied on hydrolyzed soil samples, low concentrations of GalN were estimated. This indicates that for the soil studied, GalN might be present in forms that are lost in the extraction procedure applied.

4. CONCLUSIONS AND FUTURE WORK

This thesis addresses different aspects of sampling, analysis and evaluation of chemical parameters linked to mineral nutrient availability and microbial activity in soil.

In the study performed to evaluate the distribution of free and weakly adsorbed aromatic LMMOAs in soil, it was found that obtained analyte concentrations varied considerably depending on the sampling strategy used. Liquid extraction resulted in the detection of several additional aromatic LMMOAs, and a total acid concentration many times higher than with sampling via lysimeters and centrifugation. The effectiveness of different extraction solutions was also found to be highly dependent on sample matrix i.e. SOM and clay content of the extracted soil, but also on analyte structure. This clearly demonstrates the intricacy of analyte sampling in the complex and heterogeneous sample matrix that is soil.

A quantitative analytical method using LC-ESI-MS/MS was developed to enable analysis of the analytes in question. Chromatographic parameters were optimized using DOE, and the selective and sensitive detection of the eleven aromatic LMMOAs was performed applying MRM via CID fragmentation, which resulted in detection limits in the nano-molar range.

The mineral amendment trial performed in the O, E and B horizons of a boreal forest podzol soil with estimated Fe shortage showed elevated microbial activity associated with the Fe-rich biotite mineral when compared to bulk soil in the E horizon. As established from estimations of fungal biomass and enzymatic activity as well as quantification of organic ligands, this was interpreted to be the result of an increased interest for biotite as a source of Fe.

To enable the analysis of HS in the mineral amendment trial, a qualitative and quantitative method was developed, where thirteen HS were analyzed using LC-ESI-MS and MS/MS. On-line pre-concentration enabled detection levels in the pico-molar range, which is necessary for quantifying HS in natural samples. Analyte fragmentation via CID allowed more selective identification of the HS in more complex sample matrices. In addition, an analytical method was developed for the quantification of chitin-derived GlcN using LC-ESI-MS/MS, to enable a rapid estimation of fungal biomass. Excessive samples preparation could be avoided by excluding analyte derivatization, consequently enabling high sample throughput.

LC-MS is a powerful tool for analyzing complex samples as it delivers high sensitivity and selectivity. This instrument setup is increasingly a part of the standard equipment in laboratories, enabling more sophisticated analyses. It is the author's hope that the analytical methods presented in this thesis may contribute to an increased use of this technique in the field of soil analysis, complementing the

traditional “bulk” measurement of soil components e.g. DOC, total amounts of N or P etc.

The analysis of soil and soil chemistry processes is highly complex involving a myriad of variables. It is difficult to identify the major mechanisms affecting soil processes, and comparison of the distribution and concentrations of organic compounds in soil from different field studies remains difficult, due to lack of standardized sampling and analysis methods. This emphasizes the need for research-based consensus on how these types of studies should be executed in the future.

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