Doctoral Thesis

Impact of storage related stress on microbial communities and pesticide degradation in soil

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IMPACT OF STORAGE RELATED STRESS
ON MICROBIAL COMMUNITIES AND PESTICIDE DEGRADATION
IN SOIL

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree of

DOCTOR OF SCIENCES

presented by

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2004
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SUMMARY

Ecological risk assessments for crop protection products (CPPs) require characterization of CPP degradation rates and pathways in different environmental compartments. For soils, degradation rates and pathways are determined using small-scale laboratory studies with natural soils under controlled conditions. OECD recommends using fresh soils for CPP degradation experiments, however, soil sampling in the field is not always possible throughout the year due to unfavorable conditions as for example frozen soils during wintertime. Therefore, it is often necessary to store the collected soils in the laboratory or greenhouse for periods of several months. It has been observed that soil storage can impose stress on microorganisms, inducing changes in microbiological soil characteristics (MSCs). These changes in turn may influence estimates of CPP degradation rates. Regular control of MSCs appears critical in order to allow for precise and repeatable results. Currently, there is a lack of sensitive tools to monitor MSCs in soils used in CPP degradation studies.

The goal of this thesis was to investigate the impact of storage related stress on soil microbial community structures and activities. Microbiological and molecular biological methods were combined with CPP degradation analyses and evaluated for their potential to detect changes in MSCs. The molecular approach was based on analyses of nucleic acids extracted directly from soil. In order to resolve the structure of different microbial groups, specific PCR amplification of 16S ribosomal rRNA genes and reverse transcribed 16S rRNA was performed and combined with restriction fragment length polymorphism (RFLP) and terminal restriction fragment length polymorphism (T-RFLP) analyses.

To provide a solid base for molecular biological analyses, DNA extraction from soil was optimized with respect to the parameters yield and fragment size using a bead beating method. DNA yields ranged between 90 and 540 μg g⁻¹ of soil [dry wt] for agricultural and forest soils of different texture and chemical characteristics. By multiple subsequent application of the whole procedure using harsh extraction conditions, the total DNA content of soil could be estimated.

In laboratory microcosm studies, soil samples were subjected to artificial freezing-thawing and drying-rewetting procedures to simulate stresses that can occur during soil storage. After these treatments, soils were re-equilibrated for several weeks under controlled conditions and compared to undisturbed reference soils. To characterize the microbial response, resistance and resilience of various MSCs was investigated after the stress and throughout the re-
equilibration period. Mineralization of the easily degradable insecticide methidathion appeared unaffected in frozen and thawed soils. After drying-rewetting of soil, degradation half-lives and mineralization rates of the fungicide metalaxyl-M and the insecticide lufenuron were increased by factors of 1.5 to 5.4. Bulk microbial activity measured as substrate-induced respiration revealed a reversible increase after both soil treatments. Microbial biomass determined by direct cell counts and DNA contents decreased by 22 to 24% in thawed and 24 to 51% in rewetted soils and was not restored within re-equilibration periods. Statistical analyses of T-RFLP profiles revealed that the community structure of the bacterial domain was significantly changed after both stress procedures and did not recover during re-equilibration of the soils. The archaeal domain was especially sensitive to freezing-thawing and drying-rewetting stress, as revealed by significant decreases in RFLP band intensities. Cloning and sequence analysis allowed the design of novel primers for specific detection of freezing stress sensitive and freezing stress resistant archaeal subgroups.

In conclusion, this thesis contributes to our understanding of the microbial stress response in soils. The combination of CPP degradation experiments, bulk microbial analyses and detailed community investigations allowed the critical evaluation of potential and limits of the used tools for the monitoring of MSCs in CPP degradation test soils.
ZUSAMMENFASSUNG


Diese Arbeit trägt zum Verständnis mikrobieller Stressreaktionen in Boden bei. Die Kombination von CPP-Abbauexperimenten mit mikrobiellen Summenparametern und detaillierten Populationsanalysen erlaubte eine kritische Beurteilung der Vor- und Nachteile der verwendeten Methoden, sowie deren Eignung für die Kontrolle von Böden, die für CPP-Abbauexperimente verwendet werden.
CHAPTER 1:

GENERAL INTRODUCTION
1 GENERAL INTRODUCTION

1.1 Environmental safety of crop protection products

Crop protection products (CPPs) represent a class of chemicals designed for the complete release on large areas in the environment. For example, in the United States agricultural crop protection, over $3.2 \times 10^7$ kg of the herbicide atrazine are sprayed each year (7). This is in contrast to other xenobiotic compounds that may only accidentally reach the environment during utilization, production or disposal. Therefore, environmental safety assessment of CPPs is particularly important. This is reflected by the fact that one third of the total average development costs of 200 million Swiss Franks for a CPP are spent on safety evaluations. A fundamental component of the safety assessment is to determine their fate and stability in the environmental compartments soil, water and air (93). Fate and stability of CPPs largely depend on complex interactions of environmental compartments and processes (115) (Fig. 1.1).

Fig. 1.1: Fate and effects of CPPs in different environmental compartments. The present thesis focuses on chemical and biological decomposition in the soil compartment. Reproduced from Novartis Crop Protection AG information bulletin (December 1998).
CHAPTER 1: GENERAL INTRODUCTION

To reduce complexity encountered in the environment and to control meteorological and system specific parameters, laboratory model studies need to be performed, where single processes that may influence CPP fate and stability are separately investigated and quantified (14, 43, 92, 115). CPP degradation may depend on biotic and abiotic processes. Microbially mediated degradation, however, is especially interesting, as it represents the only activity removing CPPs permanently from different environmental compartments by complete mineralization to harmless inorganic products (3, 115). There are two separate aspects to be considered for an integral description of CPP degradation. First, degradation pathways have to be analyzed including the identification of major metabolites and end products. Second, transformation rates of parent compound and major metabolites as well as mineralization rates have to be determined.

1.2 CPP degradation influenced by soil characteristics

The rate at which CPPs are degraded in soil is highly variable. The degradation half-life ($DT_{50}$) for different compounds can range from less than 2 days up to 4000 days (43, 106). In view of this wide range, considerable effort has been put into the identification of factors influencing CPP degradation rates. On the one hand, observed differences in $DT_{50}$ values are explained by chemical and structural characteristics of the respective compound. Aromatic rings are generally more resistant to biological transformation as compared to aliphatic structures (43). Also the partitioning between liquid and solid soil phase, described by the distribution coefficient $K_d$ (112), is strongly influenced by substance-specific parameters. Degradation of CPPs primarily occurs in the soluble fraction. Insoluble, sorbed or covalently bound molecules are less or not at all accessible to microbial uptake and degradation leading to delayed CPP degradation (15, 43, 98, 106, 115). On the other hand, various soil-specific factors play an important role in the degradation of CPPs. In order to classify these factors, differentiation between physical, chemical and biological soil characteristics is needed (62, 120) (Fig. 1.2). Among physical and chemical characteristics, particularly soil water potential (26, 42, 112, 137), temperature (26, 47, 137), pH (8, 77), organic matter (15), clay content, iron and aluminum oxides (43) and soil from different depth layers (47, 137) were found to significantly affect the stability of CPPs.
Fig. 1.2: Basic classification of soils according to physical, chemical and biological soil characteristics.

Besides abiotic chemical processes, such as hydrolysis, oxidation or photolysis, microbiological activities play a key role in degradation of CPPs in soil (125). Microorganisms are capable of degrading most CPPs; under natural conditions many degradation reactions are exclusively catalyzed by bacterial or fungal enzyme systems (3, 43). Despite their importance, microbiological soil characteristics were rarely correlated with CPP degradation rates. It was stated that microbiological populations constitute a source of unpredictable variation in the rates of degradation of pesticides in soil (43), suggesting correlations may not be possible. On the other hand, microbial biomass and respiration (as measured by substrate-induced respiration (SIR) and chloroform fumigation incubation (CFI)) were reported to be accurate predictors of CPP degradation in various soils (4, 5, 133). Based on these findings OECD requires the determination of microbial biomass by SIR or chloroform-fumigation extraction (CFE) along with the physical and chemical parameters texture, pH, cation exchange capacity, organic carbon content, bulk density and water holding capacity to characterize soils for CPP transformation studies (92). In addition, it is recommended to use soils with microbial biomass accounting for at least 1% of the total soil organic carbon content (91). In order to predict environmental concentrations of CPPs in soil, surface water and ground water, mathematical simulation models for CPP degradation have
been developed (e.g. (24)). Although these models include many of the above mentioned soil characteristics and processes, the complex and variable conditions in natural field soils often limit accurate quantitative descriptions (12). Nevertheless, computer models on CPP degradation have become essential tools for the regulatory risk assessment of CPPs in the environment.

1.3 Degradation time of CPPs in soil

Microbially mediated degradation of CPP parent compound and metabolites can be described at low concentrations according to equation 1),

$$\frac{-dC}{dt} = k \cdot C$$

where $C$ is the concentration of the test chemical and $k$ is the pseudo-first-order rate constant (117). Integration of equation 1) yields equation 2), where $C_0$ and $C(t)$ are the concentrations of the chemical at time 0 and time $t$.

$$C(t) = C_0 \cdot e^{-kt}$$

To determine the pseudo-first-order rate constant $k$, this function is fitted to the measured parameters $C(t)$ and $t$. Degradation half-lives ($DT_{50}$), defined as the time within which the concentration of the CPP parent or metabolites is reduced by 50%, can be calculated according to the equation 3).

$$DT_{50} = \frac{\ln 2}{k}$$

Initial CPP mineralization is assumed to follow zero-order rate reaction kinetics. Thus the rate of formation of CO$_2$ with respect to time is described by equation 4), where $k_{\text{min}}$ is the zero-order rate constant for the formation of CO$_2$.

$$\frac{d\text{CO}_2}{dt} = k_{\text{min}}$$

Integration of equation 4) yields equation 5), where CO$_2$ ($t$) is the concentration of CO$_2$ at time $t$.

$$\text{CO}_2 (t) = k_{\text{min}} \cdot t$$

If the zero-order rate constant is calculated in units of percent per day, the half-life can be estimated by equation 6) (117).

$$DT_{50} = \frac{50}{k_{\text{min}}}$$
1.4 Disturbance of soils during processing and storage

In contrast to physical and chemical characteristics, microbial parameters may be more easily affected by soil handling and storage (Fig. 1.3). Changes in microbiological soil characteristics may in turn decrease the CPP degradation potential. Therefore, different handling procedures have been proposed to preserve microbiological characteristics of soils after collection in the field. The first steps after soil sampling usually include mixing and sieving in order to facilitate gaseous exchange between soil particles and to maintain the aerobic nature of the soil (52). It has been reported that the concentration of the phospholipid fatty acid (PLFA) 18:2 ω6c, which is mainly of fungal origin, decreased after sieving (99). The authors of that study interpreted that filamentous fungi were susceptible to disruption by sieving, whereas single-celled organisms are not affected.

![Field](image1.png)
![Greenhouse](image2.png)
![Laboratory](image3.png)

Fig. 1.3: Soil handling procedures at Syngenta Crop Protection AG. Gartenacker soil is collected in the field (a) and subsequently transported to the greenhouse. There, the soil is homogenized, placed in boxes containing approx. 200 kg of soil and seeded with a clover-turfgrass mixture (b). Humidity (60%) and temperature (20°C) conditions are kept constant. For CPP degradation experiments, which are performed in laboratory microcosm experiments (c), subsamples are collected from stored greenhouse soil.

Storage of soil samples in the laboratory or greenhouse under controlled conditions is commonly applied, because it is simple and inexpensive and access to fresh soil may be seasonally limited. Storage parameters to be selected and controlled may primarily include soil temperature and moisture content and also presence or absence of a plant cover. Detected effects on microbial soil characteristics were generally more pronounced for storage at higher as compared to lower temperatures. Anderson (5) performed a series of storage experiments...
with varying temperature conditions at 2, 7, 12, 17, 22 and 27°C for 70 days and found that substrate-induced respiration (SIR) values were best maintained at the lowest temperature and decreased faster at increasing temperatures. Significant decreases in SIR and basal respiration of 40 to 80% were also reported in other studies (45, 75, 103) and have been attributed to a decline in active biomass due to decreasing amounts of available carbon during long-term incubations of soil (45, 104). Furthermore, it was observed that total soil PLFA concentration, an estimate of microbial biomass, declined at a faster rate at 25°C as compared to 10 or 4.5°C during 21 days of storage (99). Distinct changes in concentration of specific PLFAs were detected at 25°C, suggesting alterations in the structure of certain microbial populations. Microbial biomass carbon, measured with the chloroform fumigation extraction (CFE) method, decreased by 25% after 30 weeks of storage at 18°C in four grassland soils under different fertilizer regimes (75). This result was confirmed by Stenberg et al. (119), who found a 27% decline in CFE after 3 months storage at 2°C in twelve agricultural soils. In contrast, microbial biomass was reported to remain constant, when agricultural soils with different physical and chemical characteristics were stored for periods of 3 to 14 months (45, 103).

Plants provide soil microorganisms with nutrients and easily degradable organic carbon. Between 5 and 30% of the net fixed carbon is deposited in the rhizosphere soil (81), thereby promoting microbial growth and maintaining microbial biomass (27, 80). This plant-derived nutrient input may be utilized by planting soils for long-term storage in the greenhouse in order to avoid loss in microbial biomass or activity. It was shown that soils under wheat sustained higher total PLFA contents as compared to non-planted control soil (51). Different plant species, however, may also induce specific changes in microbial community structure (51). Currently no information is available on the consequences of microbial community structure changes by different plants for soil functions, as for example CPP degradation rates.

1.5 Resistance and resilience of soil ecosystems

Resistance and resilience represent concepts that were introduced to soil science to create a common theory that describes the reaction of soil to a range of impacts and disturbances (110). Resistance expresses the magnitude of change after a soil ecosystem is disturbed and resilience is defined as the ability of a soil to revert to its original structure and/or function after disturbance (Fig. 1.4). Resilience is characterized by the two aspects degree and rate of recovery after disturbance. However, in scientific literature there is very little specific
information available on resistance and resilience of biological soil components to stress and disturbance.

![Diagram showing resistance and resilience of soil characteristics after stress impact.](image)

**Fig. 1.4:** Resistance and resilience of soil characteristics after stress impact. Resistance can be determined by measuring the magnitude of change after a stress. Resilience is characterized by recovery time and degree of recovery.

One problem with the above definitions might be that they imply that soils are undisturbed systems in constant equilibrium. In reality, all soils are more or less frequently disturbed and changed by natural or anthropogenic processes on different time scales (9, 41, 56, 57, 128). Short- and medium-term disturbances include natural factors, e.g. rainfall, drought, fire, frost or bioturbation and anthropogenic activities such as crop and tree plantations, grazing management, tillage, irrigation, drainage, soil compaction, application of chemicals or contamination with industrial output. Long-term changes may occur through weathering, erosion, formation and decomposition of organic matter, release and fixation of nutrients, oxidation-reduction reactions or leaching and accumulation of compounds (40, 80). Resistance and resilience are consequently no inherent attributes of soils but have to be determined on relative scales by investigating different forms of stress and comparing the effects on physical, chemical and biological soil characteristics. When evaluating resistance of soil characteristics to storage related stress it may be a useful strategy to compare the observed effects to natural disturbances as regularly occurring in the field during the year due
to seasonal change or weather extremes. Another possibility is to relate the magnitude of the stress effects to changes, as expected to occur in different depth layers or at different sampling locations of a field site with the same soil type.

1.6 Detection and identification of CPP degrading microorganisms

Degradation of CPPs is performed by a wide range of microorganisms, including both bacteria (25, 42, 82, 100, 105, 108, 115) and fungi (11, 42, 95, 115, 131). Identification of CPP degrading microorganisms is generally achieved in a multi-step procedure. First, microbial communities capable of CPP degradation are selectively enriched in soil microcosms with the CPP as carbon, nitrogen or sulfur source or in liquid cultures amended with soil as inoculum (115, 122). Aliquots of the soil or liquid medium are then plated on agar plates containing the CPP to retrieve single colonies. Finally, single colonies are regrown and checked for CPP degradation activity. Although this procedure was successful for isolation and identification of many different CPP degrading microorganisms, a serious disadvantage is the need for culturing the organisms of interest. For example, it has been shown that linuron-degrading microorganisms affiliated with the genus *Variovorax* (ß-Proteobacteria) resisted the cultivation efforts (34). This finding raises the question, whether culture-dependent techniques allow retrieving the ecologically significant organisms or whether, by selective enrichment, naturally subordinate components of the community are isolated. Reasons for the inability to easily retrieve all organisms by simple culturing techniques may be due to the fact that certain CPPs are not used as nutrient source but are only degraded cometabolically together with structural analogues (3). It has also been shown that some CPP degraders depend on components supplied by association with other soil bacteria (116).

Alternative approaches to identify CPP degrading microorganisms may include the tracing of genes or gene products that encode for enzymes involved in the catabolic process of CPPs. Specific primers for the atrazine chloro- and aminohydrolase genes (28, 88) and 2,4-D dioxygenases (29, 54) were designed and successfully used to amplify gene fragments from complex soil microbial communities. A difficulty of this strategy might be that functional genes with conserved regions have to be selected in order to allow the design of general primers (136). Additionally, gene sequences not always contain sufficient information for proper phylogenetic identification of microorganisms. This problem might be overcome by construction of metagenomic libraries and subsequent screening for positive clones with gene specific probes (102). Analyses of flanking regions, ideally containing ribosomal RNA
sequences, may provide further information on the identity of a particular organism (70). Some key enzymes involved in the degradation of CPPs may be widespread among microorganisms and therefore are less suitable markers to track CPP degrading functions. It has been shown that several monoaromatic CPPs (e.g. atrazine, lindane, diuron or metalaxyl) were degraded by white rot fungi using ligninolytic peroxidases or cytochrome P450 monooxygenases (11). These enzymes, however, are not specific to CPP breakdown but are also frequently involved in the degradation of naturally occurring compounds as for example humic substances.

1.7 Naturally occurring and artificially applied stress and disturbance

Stress impact, as induced in this thesis by experimental freezing-thawing and drying-rewetting, can also naturally occur to soils. During winter and summer time, periods with hard frosts or without rainfall may create similar conditions in the upper few centimeters of a soil in the field as compared to the laboratory experiments (72, 128). There are, however, important differences between naturally occurring and the artificially applied stress impacts. In natural soils recovery of impaired microbial populations is facilitated by several factors. Through photosynthesis of plants, rhizosphere microorganisms are continuously supplied with readily assimilable organic substrates stimulating microbial growth and thus positively influencing resilience (80, 81). Microbial recolonization from deeper soil layers or transfer from conserved or distant places by wind and water transport or by animals is likely to occur. In contrast, the laboratory experiments performed during the course of this thesis represented strictly closed systems with respect to the processes discussed above. In the laboratory uniform stress impact was achieved by first sieving the soil to remove coarse particles such as small stones, fauna and plant debris and second by thorough freezing or by regularly turning the soil during drying and rewetting. In naturally impacted soils, protected sites may exist for example in plants, organic residues or aggregates and microorganisms may not be exposed to the stress as homogeneously as compared to the laboratory experiments.

1.8 How microorganisms cope with freeze-thaw and dry-rewet stress

Although from a physical point of view, freezing-thawing and drying-rewetting are different stress factors, their mode of action to the microbial cell is similar and consequently initiates similar cellular response and survival strategies (68, 134). When soil is cooled below –7°C, pore water will start to freeze (86), thereby increasing solute concentrations in the remaining liquid. Evaporation of pore water during drying also leads to an increased concentration of
soluble soil components as e.g. salts, organic acids or sugars. It is therefore assumed that microbial cells in either situation may experience hyper-osmotic shock by an increase in osmotic pressure of the surrounding soil solution (21, 48, 113). Without active counter measures cells would start to dehydrate, undergo plasmolysis and ultimately death. The opposite effect, referred to as hypo-osmotic shock, may be observed, when dry soil is rapidly rewetted, as naturally occurring during heavy rainfall events after drought periods (48, 65, 113). Microorganisms protect themselves against hyper- and hypo-osmotic stress by increasing cell wall and membrane stability, as it is the case in Archaea, fungi or gram-positive bacteria (21, 23, 48). Strategies that enhance osmoregulatory capabilities comprise the intracellular inclusion of salts (e.g. KCl) or low molecular weight compounds serving as compatible solutes (e.g. trehalose, proline, glycerol) to maintain enzyme function (71) and the synthesis of aquaporins for effective transport of water through the cell membrane (21, 61, 123, 134). It has been shown that the physiological state of a cell greatly influences the survival after stress impact. In general, dormant or stationary phase cells are more resistant to severe stresses as compared to active or exponentially growing cells (18, 66, 129, 134).

1.9 Parameters used for monitoring of microbiological soil characteristics

1.9.1 Microbial Biomass

Soil microbial biomass comprises the living component of soil organic matter, excluding soil animals and plant roots (27). Microbial biomass has been regarded as an important indicator of soil fertility as it constitutes a considerable pool of nutrients such as carbon, nitrogen, phosphorous and sulfur. By constant release of these elements from microbial biomass, plant growth is continuously supported. Soils that maintain high levels of microbial biomass are capable of not only storing more nutrients but also of cycling more nutrients (118). Microbial biomass may also be a sensitive and immediate indicator of disturbance or change, as induced by land use or agricultural practice, due to the fast turnover time ranging from 0.5 to 5 years (27).

Various techniques have been developed to measure microbial biomass parameters in soils (10, 27, 58, 83). Staining of microbial cells with fluorescent dyes (e.g. 4’, 6-diamidino-2’-phenylindole; DAPI) followed by direct microscopy allows quantification of cell numbers. Microscopy represents the only approach providing information on cell morphologies (13, 109, 138). Subsequent estimation of biomass, however, may be very laborious and requires
calculation of cell volumes and assumptions on the mean carbon content per cell volume (16, 109). The chloroform fumigation extraction (CFE) method aims to determine total soil microbial carbon. A soil sample is fumigated with chloroform to lyse microbial cells. Afterwards, released cell components are extracted together with other soil organic carbon using an aqueous solution of K$_2$SO$_4$ (130). Microbial biomass carbon is calculated by subtraction of the organic carbon fraction, obtained from extraction of an unfumigated control soil. The CFE method has also successfully been used to determine microbial nitrogen, phosphorous and sulfur (27). Potential sources of bias arise from differential extraction efficiencies of released carbon from soils with different clay and organic carbon contents (83).

With the substrate-induced respiration (SIR) method, O$_2$-consumption or CO$_2$-production is measured after adding an excess amount of an easily degradable carbon source, mostly glucose (6, 46). The maximum initial respiration rate is assumed to be proportional to the soil microbial biomass. It has been reported that SIR measures only the active component of the microbial biomass (6) and may vary in soils stored over longer periods (45, 75, 103) or with recent addition of organic carbon, as for example plant residues (83, 121). In these cases, SIR may not produce reliable estimates of microbial biomass and should therefore only be used as parameter of microbial activity. Phospholipid fatty acids (PLFAs) with chain lengths less than 20 carbon atoms are considered to be mainly of microbial origin (44). PLFAs are extracted from soil, purified and derivatized to the corresponding methyl esters, which can be separated and identified by gas chromatography and mass spectrometry. The total amount of PLFAs detected is used to estimate soil microbial biomass (37, 141). Some PLFAs are specific for certain defined microbial groups, as for example fungi or actinomycetes (139, 141). Analysis of these "signature PLFAs" therefore offers the opportunity to gain information on the microbial community structure.

### 1.9.2 Nucleic acids based methods

Microbial communities and their response to stress and disturbance have been traditionally studied at the whole community level, based on analyses of biomass, respiration or enzyme activities (35, 119, 140). Although whole microbial community analyses provide important information on bulk soil characteristics that are relevant for ecosystem processes, they may be insensitive to reveal changes in specific microbial groups or functions. Therefore, analysis techniques have to be applied that are able to resolve defined microbial populations at different phylogenetic levels in order to evaluate their presence, distribution and activity (22, 49).
The use of molecular biological techniques overcomes many limitations of traditional, culture-dependent microbiological procedures. Molecular markers, such as small-subunit (SSU) and large-subunit (LSU) ribosomal RNA or their encoding genes allow for a less biased detection and identification of soil microbial populations (97). Microbial soil communities are composed of highly diverse and specialized organisms (49). One gram of soil was estimated to harbour up to 10 billion microorganisms of possibly thousands of different bacterial species (126, 127). Techniques relying on sequence determination of ribosomal RNA are potentially capable of resolving the entire bacterial species richness in a particular soil sample (50). This procedure, however, requires considerable analysis effort and therefore may only be carried out for restricted sample numbers. Genetic fingerprinting methods are a more practicable alternative when assessing differences or changes in microbial community structure between different soil samples. With these approaches, common DNA regions are amplified by polymerase chain reaction (PCR) using group-specific primers. The different sequence types are separated in a gel by electrophoresis and are subsequently detected individually. Resulting signal patterns, i.e. genetic fingerprints, are representative of the microbial community detected. The main advantage of genetic fingerprinting is that it provides a rapid assessment of the microbial community structure in a single analysis, therefore allowing simultaneous processing and direct comparison of numerous samples in parallel. Disadvantages may include the limited phylogenetic information that can be inferred from genetic fingerprints and the lower differentiation between microbial types as compared to sequence analyses.

**Restriction fragment length polymorphism (RFLP)**

With the RFLP approach, an amplified sequence segment is subjected to restriction endonuclease digestion and the resulting fragments are resolved by size on agarose or polyacrylamid gels (1, 84, 87). This method is based on the principle that presence and location of restriction sites on a particular DNA segment vary among different organisms according to phylogenetic patterns (Fig. 1.5a). RFLP analysis usually yields highly reproducible results and does not require expensive equipment. In contrast to other fingerprinting techniques, as for example denaturing gradient gel electrophoresis (DGGE), a wide range of PCR product sizes is suitable for RFLP analysis. In highly diverse microbial communities, RFLP analysis of the domain bacteria often yields complex patterns that are difficult to resolve and analyze (124). In this case, the use of group specific primers on the
division, family or genus level may reduce complexity of the fingerprint and facilitates unambiguous detection of bands.

**a) RFLP**

- a
- b
- c

**PCR-products**

- a
- b
- c

**separation of restricted fragments by size**

- a
- c
- c
- b
- b
- b
- a
- a

**DNA double strand (detected fragments)**

**DNA double strand (undetected fragments)**

**|** enzymatic restriction site

**|** terminal fluorescence label

**b) T-RFLP**

- a
- b
- c

**PCR-products**

- a
- b
- c

**detection of fragments**

- a
- c
- c
- b
- b
- b
- a
- a

**Fig. 1.5: Principle of the RFLP (a) and T-RFLP technique (b).** In RFLP analysis, PCR products are cut with one or more restriction enzymes. Resulting DNA fragments are separated by electrophoresis on agarose or polyacrylamide gels and stained with fluorescent dyes (e.g. ethidium bromide, SybrGreen®) for visualization. In T-RFLP analysis, one PCR primer contains a fluorescent label. Separation of DNA fragments is achieved by capillary electrophoresis in a genetic analyzer. Only the labeled fragments are detected.

The RFLP technique has been successfully applied to analyze microbial population changes induced by various anthropogenic and natural factors in different ecosystems. In agricultural systems the influence of management and cultivation practices and of physical and chemical soil properties as well as the impact of heavy metal contamination was investigated (96, 114, 135). In forest environments the RFLP technique has been applied to compare leaf litter with top and deeper soil layers (136). Furthermore, the impact of clearcutting and burning of pristine soils has been investigated (55, 111). In the Mediterranean Sea, RFLP analysis was used to detect microbial community changes along transects from coastal to offshore water.
In these studies the RFLP technique was the key to address ecological questions, e.g. distribution, diversity, and behaviour of microorganisms in their natural or perturbed habitats.

**Terminal restriction fragment length polymorphism (T-RFLP)**

T-RFLP has been developed recently based on the RFLP technique (73). A primer labeled with a fluorescent dye is incorporated into DNA fragments during PCR amplification (Fig. 1.5b). Analysis of labeled terminal restriction fragments is performed by use of a capillary sequencer, which routinely enables separation of fragments with one base pair length difference. Only the terminal restriction fragments carrying the fluorescent label are automatically detected and quantified (Fig. 1.6). This procedure has several advantages over the basic RFLP technique. Signal intensities obtained from labeled fragments are proportional to the number of fragments passing the detector, allowing for a more accurate quantification as compared to traditional gel staining procedures. Additionally, each measured fragment represents one specific phylotype since only one restriction fragment from each amplified sequence is detected, whereas in RFLP analysis, multiple fragments may belong to the same organism (Fig. 1.5.a). T-RFLP proved to be superior to other fingerprinting methods, e.g. denaturing gradient gel electrophoresis (DGGE), in analysis of highly diverse microbial communities (20, 85, 124). On the other hand, in environments with low species richness or with many closely related organisms, T-RFLP may result in less resolution of distinct fragments.

The T-RFLP technique proved to be particularly useful for comparison of complex microbial communities in closely connected soil habitats. In grassland environments the composition of bacterial rhizosphere populations was shown to be specific for each investigated plant species (69) and in a potato field, T-RFLP profiles revealed temporal and spatial variations in bacterial community structure (79). Along a vertical oxygen gradient in flooded paddy soil, gradual changes in bacterial populations were observed on a fine spatial scale (76). Besides Bacteria, also fungi and Archaea were successfully investigated using T-RFLP analyses. Methanogenic representatives of the domain Archaea were analyzed in various rice soils from different geographical regions (101). Fungal community structure changes were studied across transects between natural and improved grassland soil (20). Furthermore, the influence of different nutrient amendments to a sandy soil and the effect of elevated CO₂ concentrations on an oak forest soil were investigated (67, 74). T-RFLP fingerprinting of genes that encode for enzymes involved in nutrient cycling may allow obtaining information on functional characteristics of microbial communities. The methyl-coenzyme M reductase α-subunit
(mcrA) gene served to investigate methanogenesis in rice soils (78, 101) and boreal fen (38).

The process of denitrification was studied by determining the genetic structure of nitrite reductase (nirS) and nitrous oxide reductase (nosZ) genes in marine sediments (19, 107).

Although very useful for analysis of fine-scaled differences in microbial community structure, it may not be possible to obtain reliable estimates of classical ecological population parameters, e.g. species diversity or evenness, based on T-RFLP profiles (32). Potential bias was reported to arise from terminal restriction fragment (TRF) length variability depending on differing purine content among investigated DNA sequences (59). Also the appearance of pseudo TRFs, attributed to single stranded DNA formation during PCR reaction, was observed (33).

**Fig. 1.6: Example of electropherogram resulting from T-RFLP analysis of a reference and a test soil sample.** Peaks (labeled 1 to 9) result from detection of single fluorescently labeled terminal restriction fragments. Each peak is characterized by relative intensity [ri] and relative migration units [rmu]. Ri is proportional to the fragment number passing the detector. Rmu correlates with the terminal restriction fragment size.

**Ribosomal RNA as indicator of microbial activity**

In numerous studies, ribosomal RNA genes (rDNA) were effectively used as molecular markers to investigate presence, structure and distribution of uncultured microorganisms in natural habitats (17, 30, 31). With rDNA based approaches, however, only restricted information on microbial activities can be gained. In order to overcome this limitation, analysis of ribosomal RNA (rRNA) may be more promising, as ribosomes, which contain
rRNA, are essential components for the synthesis of enzymes and structural proteins. It is assumed that metabolically active or growing cells have increased intracellular ribosome levels, whereas inactive or dormant cells contain fewer ribosomes. From pure culture studies with model microorganisms it is well established that promoter activity of rRNA genes is highly regulated and depends strongly on the nutrient status of a cell (2, 39, 60, 132). In experiments with organisms isolated from different environments, rRNA to rDNA ratios showed high correlations with specific growth rates (63, 64, 89). On the other hand, it was also reported that during special starvation conditions or during microbial growth at very low rates, ribosomes were maintained in excess over the apparent demand for protein synthesis (36, 53, 90). Therefore, cellular rRNA contents may not always be indicative of the metabolic state of a particular microorganism, especially under oligotrophic conditions. In experiments with isotopic tracers, the total RNA turnover rate of a rhizosphere soil microbial community was estimated to be more than 20% per day (94). This finding indicates that RNA of soil microorganisms represents a labile carbon pool with considerably shorter residence times as compared to other cell constituents, e.g. DNA or PLFA (94).

1.10 Objectives and outline of the thesis

During collection, preparation and storage of CPP degradation test soils, changes in microbial populations can occur and in turn lead to an undesired decrease in CPP degradation potential. It is an open question, which soil handling procedures and storage conditions should be selected in order to preserve best the indigenous microbial community and its functions. The methods currently recommended by OECD (92) for analyzing and controlling MSCs in stored soils are not capable of resolving changes in single microbial populations. Use of molecular biological tools, which allow for detailed analyses of microbial structures and activities, may help improving the existing strategies to monitor MSCs in CPP degradation test soils.

The overall objective of this thesis was to investigate microbial community changes induced by stress impact in Gartenacker soil, which is used as a standard in CPP degradation studies at Syngenta Crop Protection AG. Classical microbiological methods and novel molecular biological approaches were combined with chemical analyses of CPP degradation in order to evaluate the potential of the different methods to detect changes in MSCs in general and in the soil CPP degradation potential specifically.
This thesis is composed of a general introduction (chapter 1), three peer-reviewed articles (chapters 2 through 4), a section containing as yet unpublished data (chapter 5) and a general discussion (chapter 6).

In chapter 2, a protocol for the optimized extraction of DNA from soil is described. DNA provides a basis for many molecular biological analysis steps, as for example blotting, PCR amplification, genetic fingerprinting, cloning and sequencing. One goal was to determine the factors that affect quantity and quality of the extracted DNA. These investigations are important to avoid bias in subsequent microbial community analyses. Furthermore, a procedure to estimate total soil DNA content is presented. Soil DNA content can serve as a quantitative microbial parameter in soil analyses.

In chapter 3 and chapter 4, the effects of freezing-thawing and drying-rewetting on soil microbial communities and activities is described. All experiments were performed in small-scale laboratory studies under controlled conditions and served to simulate stress impact that can occur during soil storage in the laboratory or greenhouse. The microbial stress response was characterized by assessing resistance and resilience of MSCs following the stress events and during defined re-equilibration procedures. The usefulness of the different tools and strategies for the monitoring of MSCs was evaluated according to the following key questions:

- Which microbiological and molecular biological methods reveal changes in the soil CPP degradation potential?
- Should bulk microbial measurements or detailed analyses of single populations be preferred?
- Is the whole microbial community uniformly impacted by the stress events or are there stress resistant and sensitive microbial populations?
- Should abundant and diverse or rather small and less diverse microbial groups be monitored?
- Should 16 rDNA or 16S rRNA be used as nucleic acid markers?

In chapter 5, first a direct comparison of freezing-thawing and drying-rewetting stress is presented. The comparison is achieved by T-RFLP analysis of the bacterial community from both stress experiments. The purpose of these investigations was to find out, which stress
event induced stronger changes in bacterial community structure and whether the same components of the bacterial community responded to the two different stresses.

In chapter 6, a comprehensive discussion is provided including different aspects of the stress response of soil microbial communities and a review of the different tools and monitoring strategies applied in the course of this thesis.

1.11 References


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CHAPTER 2:

A STRATEGY FOR OPTIMIZING QUALITY AND QUANTITY OF DNA EXTRACTED FROM SOIL

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2 A STRATEGY FOR OPTIMIZING QUALITY AND QUANTITY OF DNA EXTRACTED FROM SOIL

2.1 Abstract

The efficiency of a bead beating method was studied in detail with regard to a variety of factors including beating time and speed, volume and temperature of the buffer, as well as amount and type of beads employed. The results presented here reveal that all of these parameters have a significant effect on yield and quality of DNA extracted from soils. Precise adjustment of extraction conditions allows for significantly higher yields of high quality DNA from soils than previously reported. We further evaluated the effect of the extraction conditions on the apparent soil microbial community structures, as observed by PCR and RFLP. Differences in the fingerprints of DNA extracted under different conditions suggest that results could be biased when using gentle extraction procedures. Based on multiple subsequent extractions using very harsh extraction conditions, we propose a protocol for the quantification of the total DNA content in soils. Extractions from six soils of different texture and chemical characteristics with selected bead beating protocols revealed that the quality (fragment size and purity) of the extracted DNA was generally very good, but also depended on the soil characteristics. While a single, general protocol for optimal DNA recovery from all soils can not be given, this study provides detailed guidelines on how to optimize the general method to obtain optimal DNA from individual soils.

2.2 Introduction

Molecular ecology relies heavily on methods for the direct extraction of DNA from environmental samples. Molecular methods for the analysis of gene pools using polymerase chain reaction (PCR) or cloning techniques rely on high quality nucleic acids as template as these techniques require pure, unfragmented DNA templates. Extraction of pure nucleic acids from soil samples has been a challenge because of the complex and heterogeneous nature of the soil matrix and the inhibition of biochemical reactions by coextracted substances such as humic acids [21, 33, 42]. The efficiency of the extraction is of equal importance. High DNA yields are important to obtain a low detection limit and to ensure the DNA sample is representative of the soil gene pool. This is increasingly important as molecular ecology focuses more on quantitative and activity-related analyses. Extraction methods failing to lyse
certain cell types or cells in protected soil habitats would introduce bias into the subsequent analyses

Within the last decade a number of direct extraction methods and purification protocols have been developed that yield sufficient amounts of pure DNA compatible with molecular analyses. Some of these techniques rely on chemical and/or enzymatic lysis [15, 22, 23, 38, 43]. However, the majority employ additional physical methods for cell disruption such as freeze-thaw cycles [7, 13, 19, 35, 39], freezing in liquid nitrogen followed by grinding [36] or bead beating [3, 4, 8-10, 13, 18, 19, 32, 40, 41].

The bead beating method is based on the physical disruption of cells by glass or ceramic beads under rapid agitation and the protection of the DNA by a stabilizing lysis buffer. The efficiency of cell disruption, but also the damage of DNA strands depends on the energy input during beating, as well as on the type and speed of the beads. Even under optimized conditions, bead beating results in more severe fragmentation of the DNA than chemical or freeze-thaw lysis. With respect to yield and total cell lysis, superior performance of bead beating methods compared to other protocols was shown [10, 18], especially where resistant cells like conidia or spores were targeted [8, 9, 13, 19, 20].

Routine applications that yield comparable results from different laboratories require stable, standardized protocols. The objectives of this study were to assess how various parameters of our bead beating procedure affect the quantity and quality of soil-derived DNA. In addition we focused on developing methods for quality control for the extraction procedure. We propose a method for the determination of the total extractable DNA contents of soils, allows for calculating the recovery of DNA from soil with a method optimized for quality of the extracted DNA, and we investigate the impact of the extraction method on the apparent microbial community.

2.3 Materials and methods

2.3.1 Soil sampling and storage

One agricultural and five forest soil samples were collected in August 1999 from sites in northern Switzerland and the upper Rhône valley in southern Switzerland. They represent a range of typical European soils with respect to parameters like pH, texture, and organic matter content (Table 2.1) [11, 25]. At each site, a block of soil was removed with a spade and the A horizons were separated and transported to the laboratory in plastic bags. All soils were passed through a 2.5 mm sieve and stored at 10°C. DNA extractions were performed after an
equilibration time of at least 3 weeks. While this method of storage allows for some change in the microbial communities over time it was undesirable to freeze soil samples because of the additional physical stress introduced by freezing and thawing.

Table 2.1: Chemical and physical characteristics of soils used for DNA extraction a).

<table>
<thead>
<tr>
<th>Soil</th>
<th>Texture</th>
<th>Clay (%)</th>
<th>Silt (%)</th>
<th>Sand (%)</th>
<th>Organic carbon (mg g⁻¹ [dry wt])</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gartenacker</td>
<td>loam</td>
<td>9</td>
<td>48</td>
<td>43</td>
<td>20</td>
<td>7.2</td>
</tr>
<tr>
<td>Abist</td>
<td>loam</td>
<td>19</td>
<td>32</td>
<td>49</td>
<td>42</td>
<td>5.9</td>
</tr>
<tr>
<td>Österliwald</td>
<td>clay loam</td>
<td>63</td>
<td>28</td>
<td>9</td>
<td>214</td>
<td>6.5</td>
</tr>
<tr>
<td>Rafz</td>
<td>silt loam</td>
<td>15</td>
<td>55</td>
<td>9</td>
<td>36</td>
<td>3.4</td>
</tr>
<tr>
<td>Steig</td>
<td>loam</td>
<td>18</td>
<td>31</td>
<td>51</td>
<td>70</td>
<td>4.5</td>
</tr>
<tr>
<td>Wmzlerboden</td>
<td>loamy sand</td>
<td>9</td>
<td>10</td>
<td>81</td>
<td>33</td>
<td>4.6</td>
</tr>
</tbody>
</table>

a) Data from [11, 25]
b) Gartenacker from the upper Rhône valley in southern Switzerland, all other soils from northern Switzerland

2.3.2 DNA extraction procedures

Extractions were performed with a modification of a buffer previously described for RNA extraction [6]. The buffer contains 0.2% hexadecyltrimethylammonium bromide (CTAB), 1 mM dithiotreitol (DTT), 0.2 M sodium phosphate buffer (pH 8), 0.1 M NaCl and 50 mM EDTA. Silica or ceramic beads (Table 2.2, type A, C, and D) or bead mixtures (Table 2.2, Type B and E) were weighed into sterile 2 ml microtubes, an amount of soil was added and the buffer was pipetted directly into the tube. The tubes were processed in the bead-beater (Fastprep FP120 bead-beater, Bio101 / Savant, Farmingdale, NY) which allowed simultaneous processing of up to twelve samples. The machine supports beating speeds (maximum speed of the tube during vertical movement) between 4.0 and 6.5 ms⁻¹ (in 0.5 ms⁻¹ increments), corresponding to approximately 4200 to 6800 rpm according to the manufacturer (FastPrep manual, Bio101/Savant).

After processing, the tubes were centrifuged at 16,000 × g for 5 min and an aliquot (0.8–1 ml, depending on the amount of beads, soil and buffer volume) of the supernatant fluid was transferred into a fresh sterile 2 ml microtube. The supernatant was subjected to a final extraction with phenol-chloroform [30] using 350 µl phenol (pH 8) and 350 µl CIA (chloroform/isoamyl alcohol; 24:1) and a second extraction with 700 µl CIA. 700 µl of the aqueous phase was incubated for 1 hour with 750 µl precipitation solution (20%
polyethyleneglycol 6000 and 2.5 M NaCl) at 37°C [28, 41]. DNA was pelleted by centrifugation at 16,000 x g for 30 min at room temperature. Pellets were washed once with 70% ethanol and resuspended in 300 μl TE (10 mM TRIS-HCl, 1 mM EDTA, pH 8).

DNA of extracts was visualized by subjecting 6 μl of the extract to agarose gel electrophoresis (2.5 V cm⁻¹ for 1 h) in a 1% agarose gel (Gibco/BRL, Life Technologies, Inc., Gaithersburg, MD) containing 0.5 μg ml⁻¹ ethidium bromide.

2.3.3 Experimental design

In a series of experiments with one soil ("Gartenacker") we determined the influence of bead beating time and speed, buffer volume and temperature, amount and type of beads on DNA yield and shearing (Table 2.2, exp. 1 to 4).

We also tested multiple extractions of the same "Gartenacker" soil sample to assess maximum DNA yield (Table 2.2, exp. 5 and 6). Experiments with repeated extractions of the same sample were conducted in the same manner as normal extractions, except that after extraction and centrifugation the removed volume of extract was replaced with an equal amount of fresh buffer. Tubes were vortexed briefly to resuspend soil pellets, then the extraction procedure was repeated. Based on the obtained results, we chose a method that was optimized to extract DNA of approximately 10kb size from "Gartenacker" with a high yield. This method was also applied to five additional soils (Table 2.2 exp. 7). Extractions using a lower and a higher bead beating speed were also performed for experiment 7.

2.3.4 Quantification of DNA

DNA in the soil extracts was quantified with PicoGreen [31]. Briefly, 1 μl of DNA extract, 2 μl of PicoGreen (Molecular Probes, Eugene, OR) and 397 μl of TE were mixed in a polypropylene tube, vortexed, and quantified for fluorescence in a luminescence spectrometer (Perkin Elmer LS 50 B, Rotkreuz, Switzerland.). DNA standards were prepared from bacteriophage λ DNA stocks (Appligene, Basel, Switzerland). DNA yield was calculated as μg total DNA in solution per g dry weight of extracted soil.
Table 2.2: Parameter settings used for bead beating extraction experiments.

<table>
<thead>
<tr>
<th>No.</th>
<th>Experiment</th>
<th>Beads (type a))</th>
<th>Beads weight (g)</th>
<th>Speed (ms⁻¹)</th>
<th>Time (s)</th>
<th>Buffer vol (ml)</th>
<th>Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FastPrep parameters</td>
<td>A 0.5</td>
<td>4, 5, 6</td>
<td>15, 30, 45</td>
<td>1.25, 1.35, 1.50, 1.75</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Bead types</td>
<td>A, B, C, D, E</td>
<td>0.5, 1.2, 0.7, 1.0, 1.7</td>
<td>6</td>
<td>15, 30, 45</td>
<td>1.35, 1.13, 1.19, 1.14, 1.38b)</td>
<td>25</td>
</tr>
<tr>
<td>3</td>
<td>Amount of beads</td>
<td>A 0.25, 0.50, 0.75, 1.0</td>
<td>5, 6</td>
<td>45</td>
<td>1.45, 1.35, 1.26, 1.18b)</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Temperature</td>
<td>A 0.5</td>
<td>6</td>
<td>45</td>
<td>1.35</td>
<td>0, 25, 50, 75</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Reextraction c)</td>
<td>A 0.5</td>
<td>5, 6</td>
<td>45</td>
<td>1.25, 1.35, 1.50</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Max. extraction d)</td>
<td>A 1</td>
<td>4, 5, 6, 6.5</td>
<td>45</td>
<td>1.18e</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Soil comparison</td>
<td>A 0.75</td>
<td>4, 5, 6</td>
<td>45</td>
<td>1.25</td>
<td>25</td>
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</tr>
</tbody>
</table>

a) A: standard 0.1 mm silica beads; B: Mixture of 0.1 and 1.4 mm silica beads and a single 4 mm silica sphere; C: 1.0 mm diameter silica beads; D: 1.4 mm ceramic beads; E: Single 6 mm ceramic sphere and garnet sand. (A by B. Braun Biotech; B-E by Bio101)

b) Volumes adjusted to same level as in tubes with 0.5 g soil, 0.5 g beads and 1.35 ml buffer

c) Four consecutive extractions, either bead beating each reextraction, or bead beating once, followed by resuspension only

d) Three consecutive extractions with repeated bead beating. 0.25 g soil were used in this experiment

e) Volume adjusted to have same fill height as in tubes with 0.5 g soil, 0.5 g beads and 1.25 ml buffer

2.3.5 Determination of DNA fragmentation

DNA extracts were diluted with sterile distilled water to a concentration of 15 µg DNA ml⁻¹. 6 µl of the diluted extract were subjected to electrophoresis (2.5 V cm⁻¹ for 1 h) in a 1% agarose gel. Gels were photographed, scanned, and analyzed with the public domain program NIH-image [24]. The fragment size distribution was determined using λ HindIII and a 1 kb ladder (Promega, Madison, WI) as molecular weight markers. DNA shearing was expressed as the size (kb) of the maximum of the fragment size distribution curve (MFSD).

2.3.6 PCR amplification

DNA extracts from three experimental settings representing intense (1.25 ml buffer, 45 s beating time, 6 ms⁻¹ beating speed), intermediate (1.35 ml, 45 s, 6 ms⁻¹) and gentle (1.35 ml, 45 s, 5 ms⁻¹) bead beating were diluted with TE to a DNA concentration of 20 µg ml⁻¹. The diluted extracts were used as templates for PCR amplification. The following phylogenetic groups were analyzed:


• Eukarya with uni-for (TACCGCGGCKGCTGGCA, modified from [12]) / EUK-rev (ATGATCCWKCYGCAGGTTCA, modified from [17]).

The PCR cocktails contained 2 mM MgCl₂, 0.2 μM of each primer, each deoxynucleoside (Boehringer Mannheim, Rotkreuz, Switzerland) at a concentration of 0.2 mM, 3 mg ml⁻¹ bovine serum albumin (SIGMA, Buchs, Switzerland), 1U of Taq polymerase (Amersham Switzerland, Zürich, Switzerland) and 1 x PCR reaction buffer (Amersham Switzerland) in a final reaction volume of 100 μl. One μl of DNA extract was added to each reaction. Heat-lysed pure culture suspensions or known positive soil extracts were used for positive controls, sterile distilled water replaced DNA in negative controls.

PCR amplification was carried out with a PTC–200 Peltier Thermal Cycler (MJ Research, Waltham, MA). After initial denaturation at 95°C for 5 min, samples were kept at hotstart conditions (80°C) while adding the Taq polymerase in a volume of 5 μl 1 x PCR reaction buffer.

Cycling conditions for EUB338-for / uni-b-rev and Arch915-for/uni-b-rev primers were the following: Denaturation at 95°C for 11 s and 92°C for 20 s, annealing at 63°C for 8 s and 65°C for 45 s, extension at 74°C for 8 s and 72°C for 45 s. For the other primer pairs conditions were changed as follows: The temperatures for the annealing were 58°C and 60°C for uni-for / EUK-rev and HGC1901 / 23s-unirev respectively and for HGC1901 / 23s-unirev annealing and extension time were 30 s instead of 45 s. A final 10 min extension step was performed and samples were kept at 4°C until being removed from the cycler.

2.3.7 RFLP fingerprinting

The PCR products were extracted once with equal volumes of chloroform. The supernatant was mixed with an equal volume of isopropanol, incubated for 1 h at -20°C and precipitated for 20 min at 16000 × g. Pellets were washed once with 70% ethanol and air dried. Twenty μl of restriction enzyme reaction mix, containing 1 x restriction enzyme buffer C (Promega) and 1 u HaelIII (Promega), were added to each pellet. Fragmentation was performed overnight at 37°C. Restriction fragments were visualized by gel electrophoresis with 3% MetaPhor agarose (FMC BioProducts, Rockland, ME) at 2.5 V cm⁻¹ for 2.5 h and ethidium bromide staining. As a molecular weight marker, a 1 kb ladder (Promega) was used. Band intensities were analyzed using the GelDoc 2000 system and the QuantityOne software (Bio-Rad Laboratories, Hercules, CA). Ratios of band intensities were subjected to pairwise t-tests.
Significant differences of band intensity ratios indicate probable changes in the composition of the analyzed microbial community.

### 2.3.8 PCR inhibition

DNA extracts from six different soils were tested for their suitability for PCR by performing an inhibition experiment based on the addition of soil extract to a PCR reaction targeting a sequence not present in soil [38]. Briefly, we used a standard 50 μl PCR reaction designed to amplify an insert of a cloned *Pseudomonas sp.* 16S rRNA gene (Accession Nr. AF006503) from approximately $3 \times 10^7$ copies of a pCR 2.1 vector (Invitrogen Co., San Diego, CA) with modified M13 primers [37]. These reactions were performed in the presence of different amounts of soil DNA extracts. PCR reaction conditions were as described above, except that 0.4 μM of each primer were used and the temperature program consisted of 25 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 62°C and extension for 40 s at 72°C. Control reactions containing only soil DNA but no plasmid were run to confirm the absence of target sequences in the soil DNA extracts.

The intensity of the resulting PCR product was quantified using gel electrophoresis and image analysis with the QuantityOne software (Bio-Rad Laboratories, Hercules, CA).

### 2.3.9 Data analyses

Errors are indicated as standard deviation (SD) of the mean of triplicate measurements. All statistical analyses and tests were performed with the SPSS software package (SPSS inc., Chicago, IL). Analysis of variance (ANOVA) was performed using the simple factorial ANOVA routine. Multiple regression was performed using stepwise variable inclusion. One-sided t-tests for pairwise comparison of band intensity ratios were calculated using Excel 98 (Microsoft Corporation, USA).

### 2.4 Results

#### 2.4.1 DNA yield and fragment size

The effects of bead beating time, speed and buffer volume on the quantity and fragment size of DNA extracted from "Gartenacker" soil were evaluated (Table 2.2, exp. 1). DNA yields increased with longer beating times, higher speeds and reduced extraction buffer volume (Fig. 2.1). The maximum DNA yield of $136 \pm 17 \mu g \ g^{-1} \ [soil \ dry \ wt]$ was obtained with 45 s beating time, a speed of 6 ms$^{-1}$ and a buffer volume of 1.25 ml. Using 15 s of beating at
4 m s\(^{-1}\) beating speed and 1.5 ml buffer volume resulted in the lowest observed yield of 13±1 \(\mu\)g g\(^{-1}\) [soil dry wt]. All other settings resulted in intermediate yields.

Fig. 2.1: DNA-yields from "Gartenacker" soil versus bead beating time. DNA yield was determined for beating speeds of 4 m s\(^{-1}\), 5 m s\(^{-1}\), and 6 m s\(^{-1}\). Each line represents a defined buffer volume: ◆ 1.25 ml; ■ 1.35 ml; ▲ 1.50 ml; ⚫ 1.75 ml. Each data point is mean ± SD (n=3).

The increasing slopes in Fig. 2.1 indicate that the effects of individual parameters on yield were cumulative. Multiple regression analyses indicated that all three parameters had a highly significant (p<0.001) effect on the DNA yield. ANOVA revealed that all treatments (speed,
time, and volume) had a significant (p<0.01) effect on DNA yield. Time by volume and speed by volume two-way effects were also highly significant (p<0.001), while time by speed did not contribute a significant effect.

To test if coextracted organic acids quenched the light emission from the PicoGreen-DNA complex, we analyzed one extract from "Gartenacker" soil (extracted at the following settings: 6 ms⁻¹, 45 s, 1.35 ml). One μl of bacteriophage λ standard DNA solutions containing 0 to 320 μg ml⁻¹ DNA, were added to tubes containing 9 μl DNA extract. Apparent DNA concentrations of these mixtures were quantified as described before. The difference of measured and calculated concentration values (data not shown) indicated only a low quenching effect of 2.2 μg ml⁻¹, 4% relative to the concentration in the extract of 53 μg ml⁻¹ DNA.

**Table 2.3: Maximum fragment size distribution (MFSD) of DNA extracted from "Gartenacker" soil samples during bead beating at different conditions.**

<table>
<thead>
<tr>
<th>Experimental setting (Time and speed)</th>
<th>Buffer vol (ml)</th>
<th>MFSD (kb) ( ^{a} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.25</td>
<td>1.35</td>
</tr>
<tr>
<td>45 s, 6 ms⁻¹</td>
<td>7.8±0.5</td>
<td>9.5±0.9</td>
</tr>
<tr>
<td>45 s, 5 ms⁻¹</td>
<td>8.6±0.8</td>
<td>12.1±1.0</td>
</tr>
<tr>
<td>30 s, 6 ms⁻¹</td>
<td>6.6±0.1</td>
<td>9.4±0.2</td>
</tr>
<tr>
<td>30 s, 5 ms⁻¹</td>
<td>7.6±0.3</td>
<td>13.6±0.2</td>
</tr>
</tbody>
</table>

\( ^{a} \) Mean ± SD (n=3)

We determined the fragmentation of the DNA for selected samples of the first series of experiments. Table 2.3 lists the MFSD (maximum of fragment size distribution) for a number of parameter combinations that, by visual inspection of the gel, had appeared as possible compromises between yield and shearing. The MFSD ranged between 7 and 20 kb. The triplicates showed little variation.

### 2.4.2 Influence of bead type and weight

Extractions with different beads were performed using 6 ms⁻¹ beating speed and a buffer volume individually adjusted to leave the same amount of headspace as in the previous experiment with 1.35 ml (Table 2.2, exp. 2). Only the bead mix B showed superior yield compared to standard 0.1 mm beads (A) for all beating times (Fig. 2.2a). However, the DNA
was also considerably sheared. For 15, 30 and 45 s MFSD were 8.0±0.4, 7.6±0.3, and 6.7±0.2 kb, respectively. The other beads (C, D, and E) did not perform better than 0.1 mm beads, showing lower yield and higher shearing (Fig. 2.2a and unpublished data). The bead-mix E (6 mm ceramic sphere with garnet sand) yielded the lowest amount of DNA (33±1.5 μg g⁻¹ [soil dry wt] after 45 s of beating).

In two separate experiments (Table 2.2, exp 3) we analyzed the effect of amounts of beads on DNA extraction efficiency. Increasing amount of beads at two different speed settings increased yield, but also increased DNA shearing (Fig. 2.2b). Since soil can contain very different amounts of sand grains, which could act as an additional 'lysing matrix' during beating, we tested extraction of two soils with different sand content ("Gartenacker": loam with 43% sand and "Österliwald": clay loam with 8% sand). The increase of yield relative to 0.5 g silica beads was comparable in both soils, although "Österliwald" yielded about 5 times more DNA than "Gartenacker". With 0.75 g of beads the relative yields were 148% in the "Gartenacker" soil and 138% in "Österliwald". With 1.00 g of soil relative yields were 235% in "Gartenacker" and 183% in "Österliwald", respectively. The soil with the higher sand content thus showed a more pronounced effect to added silica beads.

Fig. 2.2: a) DNA-yield from "Gartenacker" soil with different bead beating times and bead mixtures. O A: 0.5 g of 0.1 mm diameter silica beads. ▲ B: mixture of 0.1, 1.4 mm silica beads and a single 4 mm silica sphere (1.2 g / tube); ▼ C: 1.0 mm diameter silica spheres (0.7 g / tube); • D: 1.4 mm ceramic beads (1.0 g / tube); ■ E: Single 6 mm ceramic sphere and garnet sand (0.7 g / tube); b) DNA yield and DNA fragmentation as influenced by the amount of 0.1 mm diameter silica beads added to the same amount of soil. The fill height of the buffer was kept constant. The experiment was performed at two different beating speeds. Yield (O) and fragment size (●) at 5 m s⁻¹ beating speed. Yield (□) and fragment size (■) at 6 m s⁻¹ beating speed. Each data point is mean ± SD (n=3).
2.4.3 Influence of buffer temperature

Buffer and samples were adjusted to 0°C, 25°C, 50°C and 75°C (Table 2.2, exp. 4). Extraction at 0°C enhanced yield, but DNA was also more sheared. The extractions at 25°C and 50°C yielded DNA in similar quality and quantity. A buffer temperature of 75°C reduced DNA yield and shearing (Table 2.4).

Table 2.4: DNA yield and maximum fragment size distribution (MFSD) of DNA extracted from "Gartenacker" soil samples at different extraction temperatures

<table>
<thead>
<tr>
<th>Buffer temp.</th>
<th>DNA a) (μg g⁻¹ [soil dry wt])</th>
<th>MFSD a) (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0°C</td>
<td>62.0±5.4</td>
<td>7.4±0.7</td>
</tr>
<tr>
<td>25°C</td>
<td>50.2±2.0</td>
<td>7.7±0.3</td>
</tr>
<tr>
<td>50°C</td>
<td>53.4±6.3</td>
<td>8.8±0.3</td>
</tr>
<tr>
<td>75°C</td>
<td>37.2±3.8</td>
<td>12.7±2.8</td>
</tr>
</tbody>
</table>

a) Mean ± SD (n=3)

2.4.4 Repeated extraction

The previous experiments indicated that increasing yields by varying bead beating parameters increased shearing of DNA. One possible way to avoid this problem might be to repeatedly extract the same soil sample under mild conditions followed by pooling of extracted DNA. In order to test this approach "Gartenacker" soil was repeatedly extracted with bead beating. In a parallel experiment, samples were extracted once with bead beating and then repeatedly extracted with buffer but without bead beating (Table 2.2, exp. 5).

Very little DNA was extracted with the third and fourth extractions (Fig. 2.3a). The total amount of extractable DNA differed greatly for the applied bead beating parameters. With the gentlest extraction intensity (1.5 ml, 45 s, 5 ms⁻¹) 9±1 μg g⁻¹ [soil dry wt] were extracted after four consecutive extractions. The intermediate intensity (1.35 ml, 45 s, 6 ms⁻¹) yielded a total of 53±4 μg g⁻¹ [soil dry wt] while the harshest method (1.25 ml, 45s, 6 ms⁻¹) delivered 99±1 μg g⁻¹ [soil dry wt]. Consecutive extractions without bead beating yielded less total DNA (Fig. 2.3a).
Fig. 2.3: a) Cumulative DNA yield of successive extractions (I to IV) of the same "Gartenacker" soil sample using different bead beating methods. * indicates that bead beating was used. Above: Repeated extraction with repeated application of the full bead beating procedure. Below: Repeated extraction by extraction without bead beating by resuspending the soil pellet following a single application of the full bead beating process (extraction I). Extraction settings in both cases were 1.25 ml buffer, 45 s beating time, 6 ms$^{-1}$ beating speed (▲); 1.35 ml, 45 s, 6 ms$^{-1}$ (■); 1.5 ml, 45 s, 5 ms$^{-1}$ (●). b) DNA extracted from "Gartenacker" after 4 consecutive extractions using very harsh extraction conditions (0.25 g soil, 1 g beads, 1.18 ml buffer, beating for 45 s) in relation to bead beating speed. Each data point is mean ± SD (n=3).
For determination of maximum extractable DNA in "Gartenacker" soil 0.25 g of soil were extracted with 1 g of beads and a low fill volume (Table 2.2, exp. 6). Four consecutive extractions were performed with four different speed settings from 4 ms\(^{-1}\) to 6.5 ms\(^{-1}\).

DNA yield could be increased over that found in the previous reextraction experiment, but increasing the speed from 6 to 6.5 ms\(^{-1}\) did not further increase the total DNA extracted (Fig. 2.3b). In fact, the extraction at 6.5 ms\(^{-1}\) yielded slightly less DNA (126\(\pm\)6 \(\mu\)g g\(^{-1}\) [soil dry wt]) compared to the 6 ms\(^{-1}\) treatment (128\(\pm\)6 \(\mu\)g g\(^{-1}\) [soil dry wt]). The high extraction intensity caused severe shearing of the DNA obtained (data not shown).

2.4.5 Influence of extraction intensity on community fingerprints

DNA from soil extracts is used in many studies as template for PCR on bacterial genes and subsequent fingerprinting and/or cloning and sequencing. We diluted selected "Gartenacker" extracts obtained with different bead beating settings to the same DNA concentration and subjected them to PCR targeting 16S rRNA genes. The selected primers were specific for the domains Bacteria, Archaea, Eukarya and the bacterial group of high-GC Gram-positives.

The RFLP patterns obtained with different bead beating protocols were similar. However, the bacterial pattern showed that the ratio of intensity of several prominent bands (e.g. 315 / 285 bp, 480 / 285 bp, and 480 / 150 bp, among others) from the gentle extraction were significantly (p < 0.01) different from those from the intermediate and harsh extraction intensity (Fig. 2.4a).

![RFLP patterns](image)

Fig. 2.4: Agarose gels of HaeIII restriction fragment length polymorphisms (RFLP) of triplicate PCR reactions specific for 16S rRNA genes of a) bacteria and b) archaea. The same concentration of DNA extracted from "Gartenacker" was used in all PCR reactions for these patterns. Lanes are marked as follows: M: Marker; Gentle: extraction at 1.35 ml, 45 s, 5 m s\(^{-1}\); medium: extraction at 1.35 ml, 45 s, 6 m s\(^{-1}\); harsh: extraction at 1.25 ml, 45 s, 6 m s\(^{-1}\). The bands that were used to detect differences in the patterns and their approximate fragment size (bp) are indicated.
While the intermediate and harsh treatments were not significantly different for the 315 bp / 285 bp ratio, this was the case for the other combinations. Other band ratios did not change significantly, e.g. 285 / 185 bp. The archaeal patterns showed significant changes in band intensity ratios (360 / 220 bp and 360 / 175 bp) between the gentle and the intermediate extraction conditions (Fig. 2.4b). The ratio 360 / 140 bp did not change significantly. In no case were there significant differences of band ratios between the intermediate and the harsh extraction. The results of this analyses indicate that the composition of the DNA extracts derived with the various extraction protocols were significantly different with regard to the bacterial and archaeal 16S rRNA genes represented in them. Especially the low intensity protocol differed strongly from the other two. The RFLP patterns for the Eukarya and the high-GC Gram-positives (not shown) did not show any significant differences of band ratios between treatments.

2.4.6 Comparison of yield and shearing in 6 different soils

Our results indicated that extraction of 0.5 g soil with 0.75 g of beads, 1.25 ml buffer for 45 s at 5 ms$^{-1}$ beating speed was the protocol with the best yield that resulted in DNA with a fragment size greater than 10 kb [MFSD]. We applied this protocol together with a higher and a lower speed setting (Table 2.2, exp. 7) to "Gartenacker" and 5 other soils (Table 2.1), to test if fragmentation and PCR suitability of the extracted DNA would be comparable for a variety of different soils.

DNA of high quality was obtained from all of the soils. The highest yield was obtained from "Österliwald" (546±19 µg g$^{-1}$ [soil dry wt]), a 7 times higher DNA yield than from both "Rafz" and "Gartenacker". The fragment sizes ranged between 10.7±0.5 kb ("Rafz") and 6.5±0.1 kb ("Österliwald") at the 5 ms$^{-1}$ speed setting (Table 2.5).
### Table 2.5: DNA yield and maximum fragment size distribution (MFSD) of DNA from 6 soils at three different bead beating speeds.

<table>
<thead>
<tr>
<th>Soil</th>
<th>Speed (ms⁻¹)</th>
<th>DNA yield (μg g⁻¹ [soil dry wt])</th>
<th>MFSD (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gartenacker</td>
<td>4</td>
<td>30.7±1.5</td>
<td>12.2±0.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>45.7±0.8</td>
<td>10.2±0.5</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>106.3±3.0</td>
<td>6.0±0.1</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>78.1±2.8</td>
<td>9.4±0.2</td>
</tr>
<tr>
<td>Abist</td>
<td>5</td>
<td>128.7±1.1</td>
<td>7.7±0.6</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>197.1±10.3</td>
<td>5.6±0.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>207.1±3.6</td>
<td>8.2±0.4</td>
</tr>
<tr>
<td>Österliwald</td>
<td>5</td>
<td>317.2±8.3</td>
<td>6.5±0.1</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>546.0±18.7</td>
<td>4.9±0.2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>30.8±1.8</td>
<td>13.9±0.5</td>
</tr>
<tr>
<td>Rafz</td>
<td>5</td>
<td>44.8±4.7</td>
<td>10.7±0.5</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>138.2±9.8</td>
<td>7.4±0.6</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>44.4±3.7</td>
<td>9.7±0.6</td>
</tr>
<tr>
<td>Steig</td>
<td>5</td>
<td>70.7±1.3</td>
<td>8.1±0.6</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>131.3±7.7</td>
<td>6.0±0.6</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>44.3±2.0</td>
<td>11.8±0.3</td>
</tr>
<tr>
<td>Winzlerboden</td>
<td>5</td>
<td>57.2±2.3</td>
<td>9.5±1.2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>89.1±6.8</td>
<td>6.4±0.1</td>
</tr>
</tbody>
</table>

a) Details in Table 2.1
b) Mean ± SD (n=3)

### 2.4.7 PCR inhibition

Compared to a reference reaction, which did not contain soil extract, all soil extracts except "Rafz" maintained or even increased the sensitivity of the PCR when small volumes of extracts (1 or 2 μl) were added (Fig. 2.5). Five and 10 μl of soil extract increasingly inhibited PCR amplification. The addition of 5 μl "Abist" and "Österliwald" extracts reduced the PCR yield to 78 and 70%, respectively and 10 μl completely inhibited the reaction. 10 μl "Steig", "Gartenacker" and "Winzlerboden" extracts reduced the yield to between 74% and 79%. The soil extracts from "Rafz" strongly inhibited the PCR. 1 μl of "Rafz" extract reduced yield to 7% and no product was detected with higher extract additions.
Fig. 2.5: PCR amplification efficiencies and inhibition as influenced by the presence of soil DNA extracts. Band intensities were quantified from ethidium bromide stained agarose gels with a gel documentation system. Each data point represents one band. Values were plotted in relation to the amount of added soil DNA extracts from six different soils. The standard PCR reaction targeted a recombinant insert in a plasmid that was not detectable in soil extracts. The optical density (OD) of the standard reaction without added soil extract represented 100%. Additions of TE buffer were used as a control.

2.5 Discussion

2.5.1 Influence of bead beating parameters on DNA yield and fragment size

The published protocols for bead beating based DNA extractions from soil samples use different conditions. Beating times range from 30 s to 10 min [18, 19, 40] and bead to soil ratio also varies between about 10:1 (w/w) [19] to 1:1 [10]. The volume and the composition of the applied buffer vary greatly [4, 13, 18, 19, 40].

Recently, Miller et al. [18] provided data on the effect of homogenization time and speed on yield and fragmentation of DNA extracted from soil. Their study showed that yield and quality of the obtained DNA depended strongly on these parameters.

Our study confirmed these results and further showed that reducing the fill volume and increasing the beads to soil ratio affect yield as well.
An increased bead to soil ratio was found to increase yield with a small increase in DNA fragmentation. Since a surplus of beads also appears desirable from a theoretical point of view, in making the extractions of soils with different texture more comparable, a ratio of 0.75 g beads to 0.5 g soil appeared ideal. Higher amounts of beads are not desirable because the additional beads begin to limit the buffer volume that can be used.

Changing temperature and type of beads did not enhance the overall performance of the method since increased yield resulted in a large increase in shearing. Bead beating at ambient temperature [3, 5, 10, 18] thus seems a reasonable choice, but it is important to note that temperature is a factor that needs to be controlled. The unexpected decrease of yield and shearing at 75°C indicated that the lysing properties of the buffer had severely degraded under these conditions.

The slope of the curves (Fig. 2.1) suggests that it is possible to increase DNA yield further by applying even longer treatments or lower fill volumes. However, DNA in the high yield extracts was already sheared to an extent that was undesirable for PCR. In our experiments with "Gartenacker" soil, fragment size maxima varied between 6 and 20 kb [MFSD], which is in the range previously reported by others [14, 16, 18, 40, 41]. Our aim was to develop a method for "Gartenacker" with optimum yield DNA and a MFSD of at least 10 kb, which is about 10 times the size of a typical PCR product.

2.5.2 Stability of the protocol

For "Gartenacker" soil, a protocol using 0.75 g of regular 0.1 mm silica beads and 0.5 g of soil was the method with the best yield/fragmentation ratio for a MFSD greater 10 kb. This method was used for our experiment with different soils.

In this experiment DNA fragment size was inversely correlated (p<0.01) with DNA yield. ANOVA analyses revealed that if the effect of speed is eliminated as a linear covariate, soil had a significant effect on yield (p<0.01) and also on fragment size (p<0.05). This indicated that the developed method did not extract DNA of equal fragment size from the soils, and did not extract all soils with the same efficiency. Reasons for this could be different particle size distributions, apparent densities and water contents. In studies comparing very different soils, an individual optimization of the method, using the degree of fragmentation as a guideline, might therefore be necessary. This could be achieved e.g. by adjusting the amount of silica beads, which can be done with high accuracy.
The combination of buffer, phenol/chloroform extraction and precipitation with polyethylenglycol 6000 and NaCl was successful in producing PCR-suitable DNA from all non-acidic soils (pH>4.5) with a quick, simple and inexpensive protocol. As has been previously reported, the precipitation with polyethylenglycol was efficient and greatly reduced the co-precipitation of humic substances [28, 34, 41]. Only the strongly acidic forest soil "Rafz", which also yielded the most intensively colored extract, inhibited PCR in amounts normally used for PCR. For soils of this type, additional purification can be achieved using purification protocols based on polyvinylpolypyrrolidone (PVPP) and microconcentrators [38], PVPP and Sephadex spin-columns [10] or agarose gel purification [23], which have been demonstrated to be efficient in removing PCR-inhibiting components from soil DNA extracts.

2.5.3 Representative extraction

Results of our repeated extraction experiments showed that there was an amount of non-extractable DNA in soil, which depended on the applied extraction parameters. We speculate that a certain number of cells or sites (e.g. microaggregates) exist in soil, which cannot be lysed or disrupted at a given input of physical energy. This is in agreement with microscopical observations [19], and has been exploited e.g. in protocols to distinguish between soil extracted DNA from fungal mycelia and spores [3, 9]. It is likely that lysis resistant cells or protected microbial populations found in microaggregates are different from the bulk soil population [19]. The results obtained with RFLP fingerprinting at the domain-level (Fig. 2.4a and b), strongly suggest that the beating intensity is a source of bias in molecular studies of microbial communities. Since even the very general and low-resolution RFLP fingerprinting revealed significant differences between DNA extracted from one soil with different beating intensities, one might assume that more pronounced effects could be observed when studying bacterial subgroups that constitute the lysis resistant cells or spores. The high-GC Gram-positives however, which were tested because of their more resistant cell-walls, did not display differences in their patterns (data not shown).

We conclude that, especially if quantitative aspects are to be considered in a study, low intensity extraction methods should not be used in order to avoid this source of bias.

2.5.4 Total DNA content of soil

Microscopy and cell staining have been used to estimate the amount of lysed cells in the sample before and after DNA extraction procedures [10, 18, 19]. While this allows an
estimation of the lysis efficiency, there is currently no method available for the quantification of total DNA content in soil. When using repeated extractions under very harsh extraction conditions we were able to reach a maximum of extractable DNA (Fig. 2.3b). However, the DNA extracted under these conditions is highly fragmented and therefore not suitable for many molecular analyses.

The total extractable DNA content of the "Gartenacker" soil used in this experiment was approximately 130 µg g⁻¹ [soil dry wt]. The DNA recovery from "Gartenacker" in experiment 7 was calculated to be 24%, 36% and 83% for 4, 5, and 6 ms⁻¹ beating speed, respectively. Among the tested soils "Gartenacker" had a comparatively low DNA content. Still, this maximum yield is much higher than many previously recorded yields from agricultural and forest soil. Yields reported from a number of studies with direct lysis protocols ranged from 5 to 50 µg g⁻¹ [soil dry wt] [29]. Miller et al. (1999) extracted a maximum of 35±7 µg g⁻¹ [soil dry wt] from forest- and 7.9 µg g⁻¹ [soil dry wt] from agricultural soil. Cullen and Hirsch [10] reported a maximum yield of 27.1 µg g⁻¹ [soil dry wt] from a silty clay loam under agricultural use. This and other published data on DNA extraction yields [19, 23] indicated that common methods may extract a rather small portion of the total soil DNA, which might be problematic in view of the possible bias introduced by low intensity methods as discussed above.

During the last decade the development of molecular methods for the detection of specific marker genes, and the development of methods to isolate DNA from environmental sources have opened a window to a previously unknown diversity of microorganisms. In order to obtain comparable and quantitative data on these populations and their environmental functions, the methods need to be validated and standardized. With the approaches presented in this study a more precise characterization of soil DNA and hence a more reliable description of the microbial community can be obtained. We hope that the presented approaches to optimize extraction, estimate total soil DNA content and to evaluate quality of extracted DNA can contribute to establishing standardized protocols for soil molecular analyses.

2.6 Acknowledgements

This research was supported in part by COST action 831 "Biotechnology of soil: monitoring, conservation and remediation", by NOVARTIS Crop Protection AG and by Swiss National Science Foundation (Priority Programme Biotechnology) which is gratefully acknowledged.
2.7 References


Chapter 2: Optimizing Nucleic Acid Extraction from Soil


CHAPTER 3:

EFFECTS OF FREEZE-THAW STRESS DURING SOIL STORAGE ON MICROBIAL COMMUNITIES AND METHIDATHION DEGRADATION

Manuel Pesaro, Franco Widmer, Gilles Nicoller, Josef Zeyer

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3 Effects of Freeze-Thaw Stress during Soil Storage on Microbial Communities and Methidathion Degradation

3.1 Abstract

Prior to registration and commercialization, crop protection products (CPPs) need to be tested for their environmental impact and fate. For fate studies, laboratory microcosm experiments are performed with defined natural soils. Quality control of these soils represents a challenge, since it is often difficult to obtain fresh soil and soil storage may affect soil characteristics. According to guidelines of the Organization for Economic Cooperation and Development (OECD) frozen storage is recommended for periods exceeding three months. The present study was performed to investigate the influence of soil frozen storage on microbiological soil characteristics (MSCs) and CPP mineralization rates. For this purpose an agricultural soil was frozen for four days, thawed, and subsequently equilibrated and monitored during 42 days under controlled conditions. Substrate-induced respiration (SIR) rates increased shortly after thawing and returned to reference levels within ten days. Degradation of the CPP methidathion appeared unaffected in the soils that had been frozen. Conversely, we found that soil DNA contents and direct cell counts decreased by 24% and 22%, respectively, in the frozen stored soil. Impacts on soil microbial population structures were analyzed by use of domain-specific PCR of small subunit (SSU) ribosomal RNA (rRNA) genes for Eucarya, Bacteria, and Archaea in conjunction with restriction fragment length polymorphism (RFLP) analysis. Since the Archaea community appeared most sensitive to the freezing stress, cloning and sequencing was performed to identify marker genes for freezing stress sensitive and resistant groups. Novel specific PCR detections were developed for a freezing stress resistant and a freezing stress sensitive group. These new tools allowed monitoring and comparison of archaeal population shifts without using RFLP analysis. Over all, the molecular biological approach revealed that the initial MSCs were not restored after 42 days equilibration of the frozen soil, even though SIR rates and mineralization of one specific CPP appeared unaffected. Our results demonstrate that specific molecular tools can identify sensitive indicators of altered MSCs and may be applied for the description of stress effects that can occur during soil storage.
3.2 Introduction

Prior to registration of crop protection products (CPPs) their environmental safety must be assessed (13, 14). Besides ecotoxicity data, information on the stability and fate of CPPs and their transformation products in soil is of particular interest. These parameters are evaluated under controlled conditions with CPP transformation studies using various defined natural soils (25) that are usually subjected to a rigid examination with respect to their physical and chemical characteristics (5, 6, 21). Besides abiotic chemical processes, microbiological activities play a key role in the degradation of CPPs in soil. In contrast to physical and chemical characteristics, microbial parameters may be more easily affected by soil handling and storage. Therefore, removal from the field, aeration and disturbance of the structure during preparation, as well as storage conditions may influence soil microbial parameters and in turn also the results obtained from CPP degradation studies (2, 3). Controlling microbiological soil characteristics (MSCs) therefore may be helpful prior to use the soil for CPP transformation studies. So far, however, only limited information is available that documents the impact of storage conditions on CPP degradation and MSCs (39). Furthermore, sensitive tools for monitoring and controlling MSCs need to be developed and validated. The limited applicability and resolution power of existing analyses for routine control is reflected by the recommendation to use freshly collected soil (25). If storage in the laboratory is necessary, it has been recommended to store soils at 4°C for up to 3 months or at -20°C not exceeding 1 year (25). For soils used in CPP fate and ecological effects studies, OECD has suggested additional research on survival and activities of the soil microflora after frozen storage (25).

Several studies have been performed to evaluate the effect of soil freezing and thawing on MSCs, using various freezing temperatures (24, 45) and periods (39, 43). Consistently observed effects on soils include transiently enhanced basal respiration, often referred to as 'respiratory burst' (18, 32, 36, 38) and a decrease in colony counts (24, 33, 35, 36). Biomass determination based on substrate-induced respiration (SIR) and chloroform fumigation-extraction (CFE) revealed only small changes after conditioning for 9 days, even for frozen storage at -20°C of up to 13 months (39). On the other hand, total extractable phospholipid derived fatty acid (PLFA) contents were shown to decrease after storage at -20°C (33). Activity parameter analyses revealed diverse responses of microbial communities to freeze-thaw stress and appear difficult to interpret. For example, soil ATP content and heat output were found to decrease after freezing at -18 and -140°C (45), whereas nitrogen-
mineralization (39) and denitrification capacity (7) clearly increased after frozen storage at –20°C and –4°C, respectively. Despite significant changes of microbial biomass and activity parameters, no effects on CPP degradation kinetics have been reported so far (29, 33, 39).

Genetic fingerprinting techniques, e.g. restriction fragment length polymorphism (RFLP) analyses of 16S rRNA gene fragments (23) are useful for assessing differences of microbial population structures. The RFLP technique has been successfully applied to investigate microbial populations influenced by a number of different anthropogenic and natural factors including management and cultivation practices in agriculture and forestry, as well as physical and chemical properties of soils (9, 19, 27, 34, 40, 42).

The objectives of the present study were first to investigate the effects of one freezing and thawing cycle on soil microbial biomass. Secondly, soil microbiological population structures were examined by applying the PCR-RFLP technique. Our goal was to investigate whether certain soil microbiological groups are more susceptible to freeze-thaw stress than others and thus may serve as sensitive indicators of changes in MSCs. Thirdly, the influence of freezing and thawing on a model CPP degradation activity was determined in order to evaluate, if frozen storage of soil is an appropriate method to maintain its CPP degradation potential.

3.3 Materials and methods

3.3.1 Soil sampling and storage

An agricultural soil (calcareous Fluvisol; Table 1) was collected in July 1998 from a field site (Gartenacker) in the upper Rhône valley near Les Barges, Switzerland. At Syngenta Crop Protection AG this soil is used as reference soil in CPP degradation studies. For soil sampling the above ground vegetation was removed and approximately 400 kg of the upper 20 cm were collected. Soil was homogenized and stored in the greenhouse in boxes each containing approx. 200 kg of soil. A mixture of clover and turfgrass (Landis AG, Möhlin, Switzerland) was seeded as green cover and soil moisture was adjusted regularly. Temperature and relative atmospheric humidity in the greenhouse were kept constant at 20°C and 60%, respectively.
### Table 1: Chemical and physical characteristics of Gartenacker soil.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>Les Barges, VS, Switzerland</td>
</tr>
<tr>
<td>Classification (U.S. Soil Taxonomy)</td>
<td>silt loam</td>
</tr>
<tr>
<td>pH (KCl)</td>
<td>7.25</td>
</tr>
<tr>
<td>C$_{org}$ (%)</td>
<td>2.11</td>
</tr>
<tr>
<td>N$_{tot}$ (%)</td>
<td>0.23</td>
</tr>
<tr>
<td>CaCO$_3$ (%)</td>
<td>8.12</td>
</tr>
<tr>
<td>CEC mmolz$^{-1}$ 100 g$^{-1}$ soil [dry wt]</td>
<td>14.29</td>
</tr>
<tr>
<td>particle size:</td>
<td></td>
</tr>
<tr>
<td>Clay (&lt; 2 μm) (%)</td>
<td>7.21</td>
</tr>
<tr>
<td>Silt (2-50 μm) (%)</td>
<td>50.37</td>
</tr>
<tr>
<td>Sand (50 μm-2 mm) (%)</td>
<td>42.42</td>
</tr>
<tr>
<td>bulk density (g ml$^{-1}$)</td>
<td>1.14</td>
</tr>
<tr>
<td>Moisture content after greenhouse (%)</td>
<td>35</td>
</tr>
<tr>
<td>WHC (%)</td>
<td>66.8</td>
</tr>
</tbody>
</table>

$^a$ Percentages always refer to soil dry weight.

### 3.3.2 Experimental setup

The experimental setup corresponded to the one of a standard CPP degradation study (17). Briefly, soil samples were collected from the greenhouse and passed through a 2 mm sieve. Soil moisture was adjusted to 40% of the maximal water holding capacity (WHC). Samples of 300 g soil (for SIR, DNA, and cell counts) were filled in 1 l glass-flasks. For CPP degradation analysis 75 g of soil was placed into 300 ml Erlenmeyer-flasks. A specific sampling date was assigned to randomly selected flasks at the beginning of the experiment. The experimental design allowed for sampling of independent duplicate (CPP degradation) and triplicate flasks (other analyses). Pre-equilibration was carried out for three weeks in the dark at 20°C in a climate room under constant flow of water saturated air. The flasks in the climate room were sorted according to the assigned sampling dates. Twenty-four glass- and 4 Erlenmeyer-flasks were subjected to continuous freezing at −20°C for four days (test soil). After thawing (defined as t=0) they were again mounted to the controlled system. Twelve glass- and 4 Erlenmeyer-flasks remained in the climate chamber for undisturbed incubation (reference soil).
3.3.3 Sampling schedule

The first sampling was performed following the initial pre-equilibration phase before freezing of the test soil (day -5). Further samplings were performed 0, 1, 2, 3, 6, 10, 20, 42 days after thawing for the test soil. The reference soil samplings were performed after 10, 20, 42 days. The number of samplings was reduced, since no pronounced changes were expected in the reference soil during the course of this experiment. The day 0 sampling was performed 6 h after removing the soil from the freezer, as soon as the soil had reached ambient temperature. The test CPP methidathion was applied to duplicate Erlenmeyer-flasks (containing each 75 g of soil) at day 0 and day 10. Mineralization was determined 3, 7 and 13 days after application of the CPP.

3.3.4 Analyses of bulk microbial parameters

Substrate-induced respiration (SIR) measurements were used to determine maximal microbial respiration rates (4). Briefly, six portions of soil (corresponding to 75 g [dry wt]) were carefully mixed with 0.8 g of glucose-talcum mixtures to final concentrations of 0, 0.33, 1.33, 5.33, 8, and 10.66 mg glucose g⁻¹ soil [dry wt]. Oxygen-consumption rates were measured with a Voith-Sapromat B 12 (Voith Sulzer, Heidenheim, Germany) at 20°C according to the manufacturers’ recommendations. As maximal induced respiration rates, the saturated initial response (between 5 and 10 h) of three samples from individual flasks was used.

DNA-extraction was performed according to Bürgmann et al. (10) with slight modifications. Briefly, 500 mg of moist soil (triplicates from individual flasks), 500 mg of silica beads (0.10-0.11 mm diameter; B. Braun Biotech International, Melsungen, Germany) and 1 ml of extraction-buffer (11) (0.2% CTAB, 1 mM DTT, 0.2 M sodium phosphate buffer (pH 8), 0.1 M NaCl, 50 mM EDTA) were added into sterile, 2 ml reaction tubes (Vaudaux-Eppendorf AG, Basel, Switzerland). Bead beating was performed in a cell homogenizer (MSK Zellhomogenisator; B. Braun Biotech International) for 1 min at 4000 oscillations min⁻¹. Suspensions were centrifuged at 16000 × g and supernatants were extracted once with phenol/chloroform (1/1) and twice with chloroform. DNA was precipitated with polyethyleneglycol according to Widmer et al. (41). DNA was resuspended in TE-buffer (10 mM TRIS-HCl, 1 mM EDTA, pH 8) at 1 ml g⁻¹ [dry wt] of soil. DNA concentration was determined using a fluorometric assay (30). One μl of DNA solution was combined with 2 μl of PicoGreen® (Molecular Probes, Eugene, OR, USA) and 397 μl of TE-buffer. Fluorescence was quantified in a luminescence spectrometer (LS 50 B, Perkin Elmer, Rotkreuz,
Switzerland). DNA concentration standards were prepared from bacteriophage λ DNA (Appligene, Basel, Switzerland). DNA yield was presented as µg total DNA extracted per gram soil [dry wt].

Direct cell counts were determined in soil samples from triplicate flasks with 4’, 6-diamidino-2’-phenylindole (DAPI; Sigma, Buchs, Switzerland) according to standard protocols (44). Analyses were performed with a Zeiss Axioplan microscope (Carl Zeiss AG, Zurich, Switzerland) fitted for epifluorescence with a high-pressure mercury bulb (50 W) and filter set 02 (G365, Ft395, LP420) at 400 × magnification (Plan-Neofluar 40x/1.30 oil).

3.3.5 CPP degradation potential

Degradation of the insecticide methidathion (S-2,3-dihydro-5-methoxy-2-oxo-1,3,4-[2-14C]thiadiazol-3-ylmethyl O,O-dimethyl phosphorodithioate; Syngenta Crop Protection AG) was quantified based on 14CO₂ evolved from duplicate soil samples. Specific activity of the compound was 0.92 MBq mg⁻¹ with a radiochemical purity of 97.8%. At day 0 and 10, 75 µg (at 1.0 µg µl⁻¹) of acetone-dissolved methidathion was applied to 75 g of soil [dry wt]. For each replicate soil sample CO₂ was trapped in two sequential Vigreux-columns containing 50 ml 1 M NaOH each. 14CO₂ containing NaOH was replaced with fresh NaOH after each sampling. Radioactivity of the solution was simultaneously determined in duplicates for each Vigreux-column with a liquid scintillation counter 2500 CA (Packard Instruments Company Inc, Meriden, CT, USA). The extent of mineralization of the active compound was calculated as the percentage of radioactivity recovered (14CO₂) of total radioactivity applied (14C-methidathion).

No significant accumulation of metabolites was observed in earlier degradation studies using the same soil and CPP (G. Nicollier, unpublished). Mineralization of methidathion was assumed to follow first order reaction kinetics. Thus, the production of CO₂ is given by the equation

\[ C(t) = C^\infty \cdot (1-e^{-kt}) \]

where \( C(t) \) is the concentration of CO₂ at time \( t \), \( C^\infty \) the concentration of CO₂ at time \( \infty \), and \( k \) the first order rate constant for CO₂-evolution resulting from mineralization of methidathion. To determine \( k \) and \( C^\infty \), this function was fitted to the measured parameters \( C(t) \) and \( t \).

Remaining unmineralized radioactivity in soil was determined at the end of all degradation experiments. Triplicate soil samples of 0.8 g were air dried and homogenized in a disc mill. Soil samples were combusted at 900°C in an oxygen stream with copper oxide as catalyst
using a Robox II sample oxidizer (Zinssen Analytik, Frankfurt, Germany). $^{14}$CO$_2$ liberated from bound residues was absorbed in a vial containing 15 ml of Scintillation Oxysolve C-400 (Zinssen Analytik) absorption solution. Radioactivity present in the solution was measured by liquid scintillation counting (Tri-Carb 2500 TR, Packard Instruments Company Inc., Meriden, CT, USA). The percentage of total recoverable radioactivity was calculated as the sum of $^{14}$CO$_2$ trapped and $^{14}$CO$_2$ resulting from combustion.

### 3.3.6 rDNA analyses

PCR was performed as described by Widmer et al. (42) applying PCR primers for group-specific amplification of marker-genes (Table 2). PCR-reactions were performed in 100 µl for community RFLP analyses and in 50 µl for single clone analyses. Reactions contained 2 mM MgCl$_2$, 0.2 µM of each primer, 0.2 mM of each deoxynucleotide (Boehringer Mannheim, Rotkreuz, Switzerland), bovine serum albumin (3 mg ml$^{-1}$ for community PCR, 0.3 mg ml$^{-1}$ for single clone PCR), 1 U of Taq polymerase and 1 x PCR reaction buffer (Amersham, Zurich, Switzerland). PCR amplification was carried out with a PTC–200 equipped with heated lid (MJ Research, Waltham, MA, USA). A hot-start procedure (42) was used followed by standard cycling conditions (Table 2).

<table>
<thead>
<tr>
<th>target group</th>
<th>forward/reverse primer</th>
<th>position on 16/18S rRNA</th>
<th>Sequence (5'→3')$^a$ of group specific primers</th>
<th>PCR conditions: cycles, annealing temp.</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>Eub338/UNI-b-rev</td>
<td>338-355</td>
<td>ACTCCTACGGGAGGCAAC</td>
<td>30, 65°C</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td>UNI-b-rev</td>
<td>1390-1407</td>
<td>GACGGCGGTGTTGTRCAA</td>
<td></td>
<td>(9)</td>
</tr>
<tr>
<td>Eucarya</td>
<td>UNI-for/UNI-rev</td>
<td>522-536</td>
<td>TACCGCGCGCKGCTGGCA</td>
<td>38, 65°C</td>
<td>modified from (15)</td>
</tr>
<tr>
<td></td>
<td>Euk-rev</td>
<td>1516-1535</td>
<td>AGGAATTGGCGGGGGAGCAC</td>
<td></td>
<td>modified from (37)</td>
</tr>
<tr>
<td>Archaea</td>
<td>Arch915/UNI-rev</td>
<td>915-934</td>
<td>AGGAATTGGCGGGGAGCAC</td>
<td>35, 65°C</td>
<td>(1)</td>
</tr>
<tr>
<td>subgroup of Crenarchaeota</td>
<td>Cren1116A/UNI-b-rev</td>
<td>1116-1134</td>
<td>TACYGCTAGTGTGCTATGC</td>
<td>45, 58°C</td>
<td>this study</td>
</tr>
<tr>
<td>subgroup of Crenarchaeota</td>
<td>Cren1132B/UNI-b-rev</td>
<td>1132-1140</td>
<td>CATTACTCTCAGGAGTAGC</td>
<td>45, 55°C</td>
<td>this study</td>
</tr>
</tbody>
</table>

$^a$ R = A,G  K = G,T  Y = C,T  W = A,T  
$^b$ E. coli numbering

For RFLP fingerprinting, PCR-products were extracted with 100 µl chloroform. DNA was precipitated with one volume isopropanol for one hour at −20°C and pelleted for 20 min at 16000 × g. After ethanol (70%) washing, pellets were suspended in 20 µl restriction digest
mixture containing 2 U *HaeIII* and restriction enzyme buffer C (Promega, Madison, WI, USA) followed by overnight digestion at 37°C. Fragments were separated by electrophoresis in 3% MetaPhor agarose (FMC BioProducts, Rockland, ME, USA) at 2.5 V cm⁻¹ for 2.5 h and stained with ethidium bromide. Molecular weight marker was the 1 kb ladder (Promega). Gels were photographed using a Polaroid Shackman 7000 camera (Polaroid Ltd., St. Albans, UK).

Quantification and statistical analyses were performed on scanned images of gels containing independent triplicate samples. Each of the three lanes was analyzed separately with the plot profile tool of the public domain program NIH Image 1.62 (28). The pixel signal of single bands was quantified and integrated after background subtraction to determine total band intensities (40).

PCR-products were cloned into the pGEM®-T Easy vector without prior purification according to the manufacturers’ recommendations (Promega). For screening gene libraries, cells from colonies were resuspended in 50 μl PCR reaction mixtures and subjected to 30 PCR amplification cycles. Restriction analysis with *HaeIII* was used to determine the RFLP-pattern of each single clone. The abundance of clones affiliated to a certain RFLP-type was determined. Plasmid-DNA of representative RFLP-types was prepared with the QIAprep Spin Miniprep Kit (Qiagen AG, Basel, Switzerland). DNA sequences were determined using an ABI Prism® 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Theoretical RFLP analyses were performed with MacDNASIS Pro version 3.6 software (Hitachi Software Engineering America, Ltd., San Bruno, CA, USA). Sequences were screened for chimeras with the Chimera-Check routine from Ribosomal Database Project (RDP-II) online analyses (22). The 30 cloned sequences from this study were manually aligned using BioEdit (16) with 64 representative archaeal control sequences retrieved from RDP-II or GenBank. A phylogenetic tree was inferred by performing maximum likelihood analysis with the fastDNAml program (26). Binding sites for the primers Arch915 and UNI-b-rev were excluded from the analysis. A dendrogram was constructed with TreeExplorer version 2.12 from the MEGA2 software package (20) using *E. coli* as outgroup.

Primer target sites for distinct archaeal subgroups were deduced from the 16S rDNA sequence alignment that was established for phylogenetic analysis. To exclude exact matches with other known archaeal or bacterial sequences, the selected oligonucleotide sequences were checked with the small subunit (SSU) rRNA Probe Match tool available at RDP-II online analyses (22).
3.3.7 Statistical analyses

Data is presented as means with standard deviation (SD) derived from independent triplicate samples. Standard deviations of direct cell counts are based on counts of 20 microscopic fields among three independent sampling replicates. Two-sided T-tests for pairwise comparisons of means were calculated using Excel 98 (Microsoft Corporation, Redmond, WA, USA). Analysis of variance (ANOVA; Statistica v. 6; StatSoft, Tulsa, OK, USA) was applied for testing time×treatment interactions and temporal variations of microbial parameters. For these analyses all data sets from reference and test soils (days −5, 0, 10, 20, and 42) were analyzed and only significant findings are shown. CPP degradation data are presented as means of independent duplicate samples.

3.3.8 Nucleotide sequence accession numbers

Sequences generated in this study are deposited in the GenBank nucleotide library under accession numbers AF473876 to AF473905. Accession numbers for the new clones are shown in Figure 6.

3.4 Results

3.4.1 Analyses of bulk microbial parameters

SIR values of the reference soil were constant throughout the experiment (Fig. 1a). In contrast, SIR of the test soil increased significantly after thawing, reaching its maximum 6 hours after thawing on day 0 with 20.3 mg O₂ (h × 100 g)⁻¹ of soil [dry wt]. This corresponded to a 47% enhanced maximal respiration rate compared to the reference. SIR of the test soil subsequently decreased gradually. The respiration rate at day 0 significantly (***P < 0.001) exceeded the values at reference day −5, whereas the values at day 10, 20 and 42 were not significantly different from day −5 and the corresponding reference values. Significant time×treatment interaction (ANOVA ***P < 0.001) was found due to strong temporal changes of SIR values in the test soil.
Fig. 1. Effect of freezing-thawing and re-equilibration of soil on (a) substrate-induced respiration (SIR), (b) soil DNA content, and (c) direct cell counts. Reference soil sample (●) and test soil sample (◊) data are shown as mean ± S.D. of 3 replications for SIR and DNA and 20 measurements from 3 replications for cell counts. Day 0 designates the time point, when the frozen stored test soil was thawed.

The DNA content of the reference soil varied between 60.4 μg g⁻¹ (day 20) and 53.7 μg g⁻¹ of soil (day 42) and was significantly higher (**P <0.01) than the DNA content of the test soil at days 10, 20, and 42 (Fig. 1b). The DNA content of the test soil dropped after thawing from
57.5 ± 5.5 (day -5) to 45 ± 1.3 μg g⁻¹ of soil [dry wt] at day 0. This corresponds to a decrease of 21.7% (ns, P =0.05). Afterwards, first a further decrease to 40.7 μg until day 2 followed by a slight recovery to 45.2 μg until day 10 was observed (*P <0.05).

At day 0 direct cell counts of test samples were 23% lower (**P <0.01) than of the reference samples at day -5 (Fig. 1c). The difference between the two treatments amounted to 26 and 22% at days 10 and 20 (**P <0.001), respectively and was smallest at the end of the experiment (15%, *P <0.05), due to significantly decreased (*P <0.05) cell counts in the reference soil.

3.4.2 CPP degradation potential

For the methidathion application at day 0, 55.4% and 54.9% of the applied compound was mineralized after 13 days of incubation for reference and test soil, respectively (Fig. 2a). For the application at day 10, similar mineralization was observed with 56.0% and 55.2% of the applied compound after 13 days for the two treatments (Fig. 2b). For the application at day 0, first order mineralization rate constants amounted to 0.42 and 0.35 d⁻¹ for reference and test soil, respectively. Rate constants from the second application (day 10) were calculated as 0.31 (reference soil) and 0.28 d⁻¹ (test soil). For each application (day 0 and day 10), rate constants revealed smaller differences between test and reference soil as compared to the inter-replicate differences (data not shown). The total radioactivity recovered from soil ranged from 85 to 90% of the radioactivity applied.
Fig. 2. Effect of freezing-thawing and re-equilibration of soil on mineralization of the insecticide methidathion (a) for the application at day 0, and (b) for the application at day 10. Reference soil sample (●) and test soil sample (○) data indicate cumulative $^{14}$CO$_2$-evolution from soil samples. The full line indicates the best fit to the data assuming first order reaction kinetics. Data are shown as mean of two replications.

3.4.3 rDNA analyses of Eucarya, Bacteria and Archaea

The impact of the freeze-thaw stress on the eucaryal domain was detectable in the quantity of PCR-products obtained (Fig. 3). The signal decreased continuously from day 0 to 3 (~36% relative to reference day -5, *$P<0.05$). At day 10 the initial quantity was observed and remained close to the reference until day 42. The signal of the reference soil also revealed variability, however, no significant differences relative to the test soil were observed except for the ones described above. The *Hae*III RFLP-pattern of Eucarya PCR-products was only slightly influenced by the freeze-thaw procedure (Fig. 4a). The band migrating at 140 bp disappeared in freshly thawed soil between day 0 and day 2. It recovered at day 20 and 42 indicating regrowth of organisms represented by this band. The *Hae*III RFLP analyses of the
bacterial domain revealed no significant changes between reference and test soil samples (Fig. 4b). In contrast to Eucarya and Bacteria RFLP analyses the fingerprints derived from Archaea PCR-products revealed two distinct changes as response to freezing and thawing (Fig. 4c). The intensity of the prominent band migrating at 360 bp decreased at day 0 by 28% (**P < 0.01) and at day 1 by 52% (**P < 0.01) relative to the reference at day -5. From day 3 to 42 a steady increase in intensity of this band was observed. At day 42 the difference between reference and test samples was only 8% (ns, P = 0.93). Time x treatment interaction was highly significant (ANOVA ***P < 0.001), reflecting the recovery in intensity of this band. The intensity of the faint band migrating at 220 bp declined at day 0 to 10% of the reference value (**P < 0.001). From day 1 to 42 it was not detected in extracts originating from the test soil. Band intensities from reference soil samples showed no significant changes throughout the experiment.

Fig. 3. Effect of freezing-thawing and re-equilibration of soil on quantities of eucaryal 18S rDNA PCR-products. Reference soil sample (●) and test soil sample (○) data are shown as mean ± S.D. of 3 replications. Day 0 designates the time point, when the frozen stored test soil was thawed.
Fig. 4. Effect of freezing-thawing and re-equilibration of soil on HaeIII RFLP-patterns of (a) eucaryal, (b) bacterial, and (c) archaeal 16/18S rDNA fragments amplified from bulk soil DNA extracts. M: 1kb-marker; RFLP-patterns from reference soil samples (reference) and from test soil samples (test) are shown for representative sampling days. Each lane represents pooled triplicate samples from independent DNA-extractions and PCR-amplifications. The bands that revealed effects are marked with arrows and their approximate fragment size. Day 0 designates the time point, when the frozen stored test soil was thawed.

3.4.4 Cloning and characterization of archaeal rDNA fragments

In order to identify the components in the archaeal community responsible for the observed RFLP-pattern changes after freezing and thawing, PCR-products were cloned and characterized. For reference soils days -5 and 42 and for test soils days 3 and 42 were selected to construct archaeal SSU rDNA libraries. From each of the 4 libraries, 60 clones...
were analyzed by RFLP. From the 240 clones 11 (A-K) different RFLP-types were identified (Fig. 5).

![Diagram showing RFLP patterns](image)

**Fig. 5.** Calculated *Hae*III RFLP-patterns occurring among 240 cloned archaeal 16S rDNA fragments from reference (r) and test (t) soil (see also Table 3). M: 1kb-marker; RFLP-patterns were labeled A – K and were compared to the complex pattern derived from bulk soil DNA. Arrows mark bands that revealed effects. Fragment sizes (bp) are given on a logarithmic scale.

Four of them, named RFLP-types A, B, C and D represented between 94% and 98% of the patterns in each library and were isolated from at least three of the four libraries (Table 3). Restriction fragment sizes deriving from these patterns explained all detectable bands from the complex community fingerprints as shown in Figure 4c. The other seven RFLP-types were either unique (F, G, H, I, J, K) or detected only twice (type E) (Table 3) and thus were not considered in the quantitative differentiation of test and reference soils. Patterns C, E and D, H were candidates to account for the changing bands detected in the community fingerprints. RFLP-type B revealed the most pronounced changes in relation to freeze-thaw stress. In the two libraries derived from the reference soil it declined from 22% (13 clones) on day –5 to 13% (7 clones) on day 42 (Table 3). Conversely, in the two libraries of the test soil, type B was only found once (1.7%) in the library of day 42. RFLP-type A displayed the opposite trend (Table 3). In the reference libraries it accounted for 67% and 72% of the clones on day -5 and day 42, respectively. The two test libraries were enriched for type A, with relative amounts of 93% and 92% in the library of day 3 and day 42, respectively. Only 10 of the 240 clones screened represented artifacts and were not included in further analyses.
Table 3: HaeIII RFLP-types and frequencies observed in four clone libraries.

<table>
<thead>
<tr>
<th>RFLP-type</th>
<th>reference soil library</th>
<th>test soil library</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day -5</td>
<td>day 42</td>
</tr>
<tr>
<td>A</td>
<td>67.2 (39)</td>
<td>71.7 (38)</td>
</tr>
<tr>
<td>B</td>
<td>22.4 (13)</td>
<td>13.2 (7)</td>
</tr>
<tr>
<td>C</td>
<td>5.2 (3)</td>
<td>7.5 (4)</td>
</tr>
<tr>
<td>D</td>
<td>3.4 (2)</td>
<td>1.9 (1)</td>
</tr>
<tr>
<td>E</td>
<td>0</td>
<td>1.9 (1)</td>
</tr>
<tr>
<td>F</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G</td>
<td>0</td>
<td>1.9 (1)</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>I</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>J</td>
<td>0</td>
<td>1.9 (1)</td>
</tr>
<tr>
<td>K</td>
<td>1.7 (1)</td>
<td>0</td>
</tr>
<tr>
<td>sum</td>
<td>100 (58)</td>
<td>100 (53)</td>
</tr>
</tbody>
</table>

a) Eleven RFLP-types are labeled A-K
b) RFLP-type frequencies are indicated in percent for each library. The number of clones identified is given in parentheses.
c) Untreated reference soil
d) Test soil that was frozen and thawed

3.4.5 Phylogenetic analyses of archaeal clones

To resolve the phylogenetic affiliation of the eleven archaeal RFLP-types, 29 clones representing all RFLP-types (A: 9, B: 4, C: 6, D: 3, E-K: 1) were sequenced. Theoretically determined RFLP-types of the 29 sequences were consistent with the experimental data. A 16S rDNA phylogenetic tree was inferred including sequences from pure cultures (methanogenic and thermophile species) and uncultured environmental clones from various habitats available from public databases.
Fig. 6. Phylogenetic tree based on maximum likelihood calculation. Each of the 16S rDNA sequences is identified by its clone name, by its source, and by its sequence accession number. Sequences from this study are printed in bold. Clone names indicate the origin of the different clones (test soil: t; reference soil: r; sampling day: -5, 3, 42K, 42; clone number: 01-31; HaeIII RFLP-pattern: A – K). Cluster (I) defines the marine group of uncultured Crenarchaeota (group Ia), cluster (II) the terrestrial group of uncultured Crenarchaeota (group Ib), cluster (III) Methanobacteriales, cluster (IV) Methanosarcinales, and cluster (V) Methanomicrobiales. Shaded clusters mark the sequences used for primer design of freeze-thaw sensitive (CrenB) and non-sensitive (CrenA) archaeal subgroups. Cluster EuryC contains sequences of weakly associated Euryarchaeota. Numbers at unresolved clusters indicate the number of sequences included. The scale bar indicates the average substitution rate per nucleotide position.
The Archaea sequences derived from Gartenacker soil are diverse, but a distinct clustering of three major RFLP-types A, B, and D within the phylogenetic tree was found (Fig. 6). The RFLP-type A was characteristic for the terrestrial cluster (group I.1b) of nonthermophilic Crenarchaeota. Types B and D formed tight clusters within the marine lineage of nonthermophilic Crenarchaeota (group I.1a). RFLP-type C exhibited large sequence diversity. Of the 6 sequences clone t 3 11-C clustered together with group I.1b Crenarchaeota, clone r 42K 26-C with Methanobacteriales, clone r 5 06-C with Methanomicrobiales and 3 sequences grouped together in a yet unaffiliated euryarchaeal cluster termed EuryC (Fig. 6). The other RFLP-types E-K were associated with Methanosacinales (clone t 42 I 9-E, clone t 42 21-H) or with the same clusters as described above.

3.4.6 Selective detection of freeze-thaw sensitive archaeal subgroups

The phylogenetic information on freeze-thaw sensitive archaeal groups was further used to design group-specific PCR primers. Newly designed primers Cren1116A and Cren1132B amplified sequences from subgroups of the RFLP-types A (CrenA) and B (CrenB), respectively (Fig. 6). The new primers were evaluated in the RDP-II-database and used to amplify rDNA from reference and test soil DNA extracts. The results for Cren1132B confirmed the findings from single clone analysis. PCR-product intensities decreased strongly from day -5 to day 1 in the test soil (-76%, ***P <0.001) (Fig. 7a). Following this decrease, an increase was observed with a maximum at day 6 (62% of day -5). Afterwards, band-intensities remained approximately constant with values of 55% (day 10), 58% (day 20) and 42% (day 42) relative to corresponding reference values. Reference values were significantly higher than the corresponding test values (**P <0.01). The primer Cren1116A first revealed a similar response as Cren1132B. Band-intensities decreased from day -5 to day 1 by 21% (**P <0.01) and subsequently recovered until day 6. From day 10 to day 42 values were not significantly different for test and reference soils, thus contrasting the results obtained with the primer Cren1132B (Fig. 7b). Significant timetreatment interactions were detected for both primers (ANOVA **P <0.01). Separate analysis of temporal changes of the data from reference and test soils also revealed significant effects (*P <0.05). No trends or factors could be identified which would allow interpretation of the observed temporal effects.
3.5 Discussion

The purpose of this study was to evaluate the effects of freezing and thawing on the microbial populations of Gartenacker agricultural soil that is used as a standard soil at Syngenta AG to determine CPP degradation rates. It was also examined whether original MSCs can be reestablished by a defined equilibration procedure. Classical microbiological methods as well as novel molecular biological tools were applied for this investigation and combined with CPP degradation and substrate-induced respiration assays.

3.5.1 Microbial biomass

The measurements of DNA content and cell numbers in soil samples were used as an estimate for total soil microbial biomass. Both parameters were shown to decrease in the test soil that
was subjected to 4 days of frozen storage (Fig. 1b,c). Even after 42 days of re-equilibration time under controlled conditions, the DNA content was 33% and direct cell counts were 15% below the respective reference values and did not return to the values before freezing. Based on chloroform fumigation-extraction (CFE) measurements, biomass C has been shown to remain approximately constant after freezing soils for time periods of 1 day to 18 months (39, 43). Taking improved carbon extractability after freezing and thawing soils into account, a 41% loss of biomass was calculated by Winter et al. (43). The total extractable PLFAs of a soil stored at -20°C for 6 months decreased between 41% and 52% (33). This decrease was consistently observed 6 h as well as 20 d after thawing of frozen stored soils. Therefore, consistent with our findings, re-equilibration had no apparent positive effect on biomass recovery.

### 3.5.2 Microbial activity

The initial strong increase in SIR after thawing frozen stored soils is a commonly observed phenomenon (18, 32, 33, 36, 38). Nutrients and easily degradable carbon compounds are released from dead organisms as well as from soil organic matter and lead to a transient stimulation of the soil microbial metabolism (32, 36, 38). Our data on DAPI counts indicate that the enhanced respiration was not followed by proliferation of cells as had been observed with plate count experiments (24, 36). Ten days after thawing, the respiratory potential of the test soil had declined to the level of the reference and remained constant until the end of the experiment. This observation is in agreement with results from previous studies (33, 39). Even for long periods of frozen storage (6 to 13 months) SIR of various agricultural soils revealed only small differences compared to the control after 9 and 20 d re-equilibration (33, 39). In contrast to our DNA and direct cell count results, SIR-rates may lead to the conclusion that after a relatively short re-equilibration period, freeze-thaw stress on soil microbial communities is completely reversible. SIR detected only the active, glucose- and O₂-consuming fraction of the microbial community, while DNA and direct cell counts are not restricted to these groups. DNA and direct cell counts indicated a decrease in biomass, while SIR revealed an increase of the O₂-respiratory potential. For soils disturbed by frozen storage we therefore suggest to use SIR-rates exclusively as respiratory potential indicator and not to convert them into microbial carbon values.

### 3.5.3 CPP degradation

The amount of ¹⁴CO₂ originating from the mineralization of ¹⁴C-labeled methidathion was similar in test and reference soil after 13 days of incubation (Fig. 2). The differences of
calculated first order rate constants for CPP mineralization were small for the two treatments, considering the variations between duplicate samples. Freezing and thawing of Gartenacker soil did not significantly influence the mineralization of this particular CPP despite the fact that various microbial parameters revealed a clear impact of the treatment on the microbial community. In agreement with this finding, none of the studies published so far found freezing and thawing to adversely affect degradation of CPPs in soils (29, 33, 39). An explanation for this result might be that the affected microbial populations were not directly involved in the degradation of the tested CPPs. Frozen storage of soils appears a convenient method to maintain their CPP degradation potential, although, to our knowledge only three CPPs were evaluated so far: the herbicides atrazine (29) and linuron (39) and an unspecified insecticide (33). Therefore, the question remains of whether effects might be detectable with different soil and CPP combinations. Knowledge on occurrence and distribution of the CPP degrading microbial functions in soils is very limited. Some key enzymes may be common and widespread within the soil microbial community rendering their functions redundant. Other enzymes appear restricted to specialists (12) and their survival following stress events may be crucial for the degrading abilities of a soil.

3.5.4 PCR and RFLP analyses

The irreversible decrease in DNA contents and direct cell counts after freezing and thawing may also be accounted for by changes in bacterial populations not detected with the domain-level RFLP fingerprints (Fig. 4b). We therefore performed group-specific RFLP fingerprint analyses for the bacterial phyla Cytophaga-flavobacterium, high GC gram-positive Bacteria, \(\alpha\)-Proteobacteria, \(\delta\)-Proteobacteria, and Pseudomonas (data not shown). However, no changes in the genetic fingerprints were identified.

The pronounced decrease of the PCR-product quantity derived from eucaryal rDNA genes (Fig. 3) suggested that higher soil organisms, e.g., certain fungi or protozoa, were particularly sensitive to freeze-thaw stress. Eucaryal PCR-product quantity revealed a temporal correlation with the quantity of total DNA extracted from the test soil. Although no changes in genetic fingerprints were detected, this correlation suggested that eucaryal nucleic acids contributed to the changes in the DNA quantities extracted between day 0 and day 10.

3.5.5 Archaea affected by freeze-thaw stress

Analyses of the archaeal community composition from test and reference soil revealed the occurrence of freeze-thaw sensitive and resistant RFLP-types (Table 3). The strongest effects
were observed for type B Archaea. This sensitive group was affiliated to the marine group of Crenarchaeota (group I.1a; Fig. 6). The stress related intensity decrease of bands 360 bp and 220 bp in the domain level genetic fingerprints may be assigned primarily to RFLP-types C and D, respectively, since the other candidate types E (for band 360 bp) and H (for band 220 bp) were found less frequently. As compared to these sensitive types, the most abundant RFLP-type A that was related to the classical terrestrial cluster of Crenarchaeota (group I.1b) (8) appeared clearly more freeze-thaw stress resistant.

Two specific primers were designed for the archaeal clusters CrenA and CrenB and used to amplify target sequences from test and reference soil DNA (Fig. 7). This strategy allowed for a successful and independent validation of results obtained from domain level analyses. In addition, the use of more specific primers may reduce the risk of possible bias resulting from preferential amplification in complex sequence mixtures. The direct tracing of freeze-thaw susceptible indicators using specific PCR-primers has the main advantage that labor-intensive fingerprint or single clone analyses are not required. Therefore, specific probe design may represent a promising approach for the development of tools for routine monitoring of MSCs.

The potential of Archaea to serve as sensitive indicators for changes of MSCs is supported by previous observations that organisms from this domain readily respond to changes of soil properties. Clearcutting and burning of forest soils greatly affected the community structure of Archaea (19). The addition of sewage sludge contaminated with heavy metals caused a decrease in absolute numbers and also a shift in the structure of archaeal populations (31). Both studies identified sensitive organisms to be affiliated with terrestrial clusters of Crenarchaeota. Euryarchaeota and group I.1a Crenarchaeota, as revealed in the present study were not identified. Since the physiology of soil Archaea is largely unknown, it is also unknown which functions are affected by their disappearance. Archaea represented sensitive indicator organisms to freeze-thaw stress, however, appear not to be involved in the degradation of the CPP methidathion. Presently, it is not possible to relate changes in uncultured microbial populations, identified solely by their 16S rRNA sequences, to soil functions such as the degradation of a specific pesticide. However, critical treatments affecting MSCs can be identified and may help to standardize soil handling procedures and thereby reduce the risk of altering the CPP degradation potential of a soil.

Freezing is a standard method to store soil in the laboratory. Data presented in this study provide evidence that irreversible changes can occur to microbiological soil characteristics after freezing and thawing. The investigated activity measures appeared unaffected. For
investigation of specific microbial communities as for example Archaea, we recommend not to freeze soil samples prior to analyses.

3.6 Acknowledgements

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3.7 References


CHAPTER 4:

IMPACT OF SOIL DRYING-REWETTING STRESS ON
MICROBIAL COMMUNITIES AND ACTIVITIES AND ON
DEGRADATION OF TWO CROP PROTECTION PRODUCTS

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4 IMPACT OF SOIL DRYING-REWETTING STRESS ON MICROBIAL COMMUNITIES AND ACTIVITIES AND ON DEGRADATION OF TWO CROP PROTECTION PRODUCTS

4.1 Abstract

Prior to registration of crop protection products (CPPs) their persistence in soil has to be determined under defined conditions. For this purpose, soils are collected in the field and stored up to 3 months prior to the tests. During storage, stresses like drying may induce changes in microbiological soil characteristics (MSCs) and in turn may influence CPP degradation rates. We investigated the influence of soil storage related stress on resistance and resilience of different MSCs by assessing the impact of a single severe drying-rewetting cycle and by monitoring recovery from this event over 34 days. Degradation and mineralization rates of the fungicide metalaxyl-M and the insecticide lufenuron were delayed by a factor of 1.5 to 5.4 in the dried and rewetted soil as compared to an undisturbed reference. Microbial biomass, estimated by direct cell counts and soil DNA contents, decreased on the average by 51% and 24%, respectively. Bulk microbial activities measured as substrate-induced respiration and fluorescein diacetate hydrolysis increased after rewetting but recovered completely within 6 days of re-equilibration. Impact on Bacteria, Archaea, and Pseudomonas were investigated by PCR of 16S rRNA genes and reverse transcribed 16S rRNA followed by RFLP and T-RFLP fingerprinting. Statistical analyses of RFLP and T-RFLP profiles indicated that specific groups of the microbial community were sensitive to the stress. In addition, evaluation of rRNA genes versus rRNA as marker to monitor the stress response of microbial communities revealed overall similar sensitivities. We conclude that various structural and functional MSCs were not resistant to drying-rewetting stress and resilience depended strongly on the parameter investigated.

4.2 Introduction

Characterizing stability and fate of crop protection products (CPPs) and their transformation intermediates in soil represents an important component of their environmental safety assessment (3, 23). These parameters are evaluated in small-scale laboratory studies under different controlled conditions using defined natural soils (6, 36). For such CPP degradation studies, the use of field-fresh soils is preferred over stored soils (27). However, soil sampling in the field cannot be performed throughout the year due to seasonal or climatic conditions,
e.g. extensive drought periods in summer or frozen or snow-covered soil during winter. Therefore, soil storage under defined conditions becomes necessary and data describing the impact of storage on microbial soil characteristics (MSCs) needs to be provided (39, 49). For maintenance of biological CPP degradation potential of soils, indigenous microbial populations must be preserved. Yet, after removal from the field, microbial community structures and functions were shown to change when soils were stored frozen (39, 44) or stored at ambient temperature in the greenhouse (3). Soil moisture content is considered a critical factor for preserving MSCs. Drying-rewetting cycles may represent a stress event occurring to soils when stored in the greenhouse without regular watering and tight moisture control. Soil physical parameters, especially moisture content, also have a strong influence on CPP degradation and mineralization rates (11, 46) and have to be strictly controlled prior to CPP degradation studies (36).

The impact of drying and drying-rewetting of soil on microbial communities has been investigated in numerous studies. In most investigations, bulk microbiological parameters were analyzed, including microbial cell numbers (5, 47), biomass carbon (18, 35, 51), respiration (2, 34, 41, 47), enzyme activities (57), carbon and nitrogen mineralization (5, 18, 22) and substrate utilization (12, 18). Although useful for a general microbiological characterization of soils, analyses at the whole community level may be not sensitive enough to reveal changes in structures or activities of small subgroups. Many CPPs are degraded by specialized microorganisms that were shown to represent only small fractions of the entire microbial community (16).

Few studies exist that investigated the impact of soil drying-rewetting on CPP degradation in combination with MSCs (44, 49). In order to describe the impact of immediate stress and disturbance events on ecosystems the concepts of resistance and resilience were developed (40). Resistance is defined as the magnitude of change after a system is disturbed (40) and resilience expresses the capacity of a system to recover after disturbance (24, 45). Recovery consists of the two components rate and degree of recovery. Application of these concepts to investigate stress impact on soil microbial communities may impose difficulties, since appropriate microbiological characteristics have to be identified. Different microbial parameters may also display various degrees of resistance and resilience (45) and magnitudes of resistance can only be determined on a relative scale (2).

Recently developed genetic fingerprinting techniques offer the possibility to investigate the impact of soil perturbations on microbial community structure (29, 50). Due to its high
resolution power, terminal restriction fragment length polymorphism (T-RFLP) of 16S rRNA genes (was successfully applied to describe changes in microbial community structure in soil environments, which harbour high microbial species diversities (14, 31). In addition to the analyses of rRNA genes, reverse transcription (RT) and amplification of rRNA may be used to investigate metabolically more active microbial groups (13, 17, 26). Genetic fingerprints derived from rRNA can be directly compared to fingerprints obtained from rRNA gene analyses, thereby facilitating the identification of active components of the microbial community.

In the present study we investigated the impact of a single severe drying-rewetting cycle on MSCs and degradation rates of two CPPs in soil. Our goal was to identify suitable microbiological markers for monitoring changes that may occur during soil storage and to describe resistance and resilience of MSCs during a 34 days re-equilibration period after rewetting the dried soil. Bulk microbial biomass and activities were determined in order to analyze the impact of the stress event at the community level. To resolve the structure of microbial communities and specific groups, RFLP and T-RFLP fingerprinting of 16S rRNA genes was performed. Genetic fingerprints of reverse transcribed 16S rRNA served as a measure of active components of bacterial communities and specific groups.

4.3 Materials and methods

4.3.1 Soil sampling and pre-equilibration

The soil for the dry stress experiment was Gartenacker, a sandy loam grassland soil (39) that was harvested from a field site near Les Barges, Valais, Switzerland in April 2001. The soil is routinely used for CPP degradation tests at Syngenta Crop Protection AG. Approximately 200 kg soil (0 - 20 cm) was removed with a spade and put into open boxes for transportation to the laboratory. The soil was passed through a 2 mm sieve, and moisture content was adjusted to 40% maximum water-holding capacity (WHC). The fresh soil was distributed into 300 ml Erlenmeyer flasks (for CPP degradation analysis) and one-liter bottles (for other analyses) each containing 75 g and 500 g soil [dry wt], respectively. The soil was pre-equilibrated in a climate room for one week under controlled conditions (darkness, 20°C). During the entire experiment a constant flow of water saturated air was maintained in the bottles and soil moisture was maintained at 40% WHC.
4.3.2 Application of the drying-rewetting stress

After pre-equilibration in the climate chamber, a portion of the soil (test soil) was transferred from the bottles and Erlenmeyer flasks to a clean 200 cm × 100 cm metal tray, mixed and spread into a 3-4 cm thick layer. To induce a drying stress, the soil was air dried for 14 days in the climate chamber in the dark at 20°C. Once per day the soil was carefully turned to ensure homogeneous drying to a minimum water content of 1% WHC. The remaining portion of the equilibrated soil was kept undisturbed in the bottles and Erlenmeyer flasks and served as reference during the experiment. After 14 days under drying stress conditions, the water content of the test soil was slowly readjusted to the original water content of 40% WHC using sterile deionised water, which was dispersed with a gardening water sprayer equipped with a uniform-distribution delivery head. The soil was carefully mixed and divided again among Erlenmeyer flasks and one-liter bottles.

4.3.3 Sampling schedule

The first sampling for all analyses was performed following the initial pre-equilibration phase before drying of the test soil and was defined as day 0. Further samplings were performed at the end of the drying phase (day 14) and 2, 6 and 34 days (days 16, 20 and 48) after re-wetting of the test soil. The CPP metalaxyl-M was applied to reference and test soil samples at days 0, 20, 48 and days 20, 48, 48, respectively. The CPP lufenuron was applied at day 48 to reference and test soils. The experimental design allowed for sampling of independent duplicate (CPP degradation) and triplicate flasks (all other analyses).

4.3.4 CPP degradation

The CPP degradation experiment was carried out in an open gas-flow system according to OECD guidelines (36). The system consisted of a sequence of 6 interconnected flasks with continuous flow-through of water-saturated air. At the head position, a gas-washing bottle served to saturate incoming air with water. Two-300 ml Erlenmeyer flasks (at second and third position) contained 75 g of soil [dry wt] each, followed by one empty flask (at fourth position) and two flasks filled with 45 ml 2 N NaOH (at fifth and sixth position) to retain CO₂ resulting from mineralization of the CPPs.

CPP application was performed by adding [Phenyl-U-¹⁴C]metalaxyl-M (specific activity 2.08 MBq/mg) or [Dichlorophenyl-U-¹⁴C]lufenuron (specific activity 1.90 MBq/mg) to the soil to a final concentration of 0.3 mg kg⁻¹ of soil [dry wt]. This concentration corresponded to an application of 0.2 kg ha⁻¹.
One ml aliquots of the solution in each CO₂ collection flasks were transferred to scintillation vials and ¹⁴CO₂ was quantified by liquid scintillation counting (LSC; Packard 2500 TR, Meriden, CT). To extract the parent compounds and metabolites, soils were transferred to 500 ml glass centrifuge tubes and extracted by horizontal shaking for 1.5 hours at room temperature with 200 ml acetonitrile / water (4/1, v/v). Suspensions were centrifuged at 2000 x g for 20 min and the supernatant was collected in a 500 ml flask. This extraction procedure was repeated three times and supernatants were pooled. One ml aliquots of the pooled supernatants were used to quantify total extractable ¹⁴C by LSC. The pooled extracts were subsequently concentrated with a vacuum rotary evaporator at 30-40°C and 100 µl subjected to HPLC analyses (Shimadzu LC-10 AD series instrument; Shimadzu Corporation, Kyoto, Japan) using both ¹⁴C (Berthold Radiomonitor beta detector, Berthold AG, Wildbad, Germany) and UV (254 nm; Shimadzu SPD 6A) detectors. Two acetonitrile / water gradient conditions starting at 15/85 v/v and 50/50 v/v were used to separate metabolites of metalaxyl-M and lufenuron, respectively. HPLC was performed at 1.0 ml min⁻¹ through a C18-Nucleosil column (5 m, 250 mm x 4.6 mm; Macherey Nagel AG, Oensingen, Switzerland). For identification of compounds, four analytical reference standards were used (CGA 62826, CGA 67868, CGA 238277 and CGA 224443; Fig. 1).

The extracted soil was air-dried and ground to a fine powder with a planar mill (Fritsch GmbH, Idar-Oberstein, Germany). Bound ¹⁴C-residues (non-extractables) were combusted in an oxidizing oven (Harvey Biological Oxidizer OX500, R.J. Harvey Instrument, Hillsdale, NJ). ¹⁴CO₂ was collected in a 15 ml trap containing Scintillation Oxysolve C-400 absorption solution (Zinsser Analytik, Frankfurt, Germany) and was quantified by LSC.

Degradation of CPPs metalaxyl-M and lufenuron has been investigated previously in detail using different soils (Syngenta Crop Protection AG, unpublished). The degradation pathways outlined in Figure 1 were consistently observed. Metalaxyl-M is degraded in soil by cleavage of the methyl-ester group forming the metabolite CGA 62826 (Fig. 1a). A second metabolite (CGA 67868) is formed either directly from metalaxyl-M or from the metabolite CGA 62826 by N-dealkylation. This reaction only occurs in non-sterile soil and is therefore assumed to be exclusively mediated by microorganisms. Further metabolites are not detectable in significant amounts. End products of the metalaxyl-M degradation are non-extractables and CO₂. Degradation of lufenuron occurs by cleavage of the urea bond resulting in metabolite CGA 238277 (Fig. 1b). A second metabolite CGA 224443 is formed by release of the H₂N-CO-group. The degradation of lufenuron and formation of both metabolites is only detected in
non-sterile soil, indicating that microorganisms play a key role in the breakdown of this CPP. Significant accumulation of other metabolites than the ones described has not been observed. End products of the degradation process are non-extractables and CO$_2$.

Figure 1. Chemical structures and initial degradation steps of the two crop protection products (CPPs) metalaxyl-M and lufenuron. CGA 62826, CGA 67868 and CGA 238277, CGA 224443 designate the major metabolites for metalaxyl-M and lufenuron monitored in this study. Degradation end products were non-extractables and CO$_2$. The 6-fold $^{14}$C-labeled aromatic ring (*) and pseudo-first-order rate constants ($k_1$ - $k_3$) are indicated. Degradation steps observed exclusively in non-sterile soil are marked with dashed arrows.

4.3.5 Statistical analyses of CPP degradation data

Degradation of parent compounds and metabolites were assumed to follow pseudo-first-order reaction kinetics (48). Degradation rates ($k$) and half-lives ($\ln 2 / k; DT_{50}$) of metalaxyl-M and lufenuron and the metabolites CGA 62826 and CGA 238277 were calculated with the computer program ModelMaker (Cherwell Scientific Publishing Ltd, Oxford, UK). Linear correlations between measured data and curves fitted according to the pseudo-first-order model were determined with ModelMaker. Mineralization of [Phenyl-$^{14}$C]metalaxyl-M and [Dichlorophenyl-$^{14}$C]lufenuron was assumed to follow zero-order reaction kinetics (48). Mineralization rates were calculated from linear regression of $^{14}$CO$_2$-evolution curves using Excel 98 (Microsoft Corporation).
4.3.6 *Quantitative microbiological parameters*

For direct counts, cells were stained with the fluorescent dye 4′, 6-diamidino-2′-phenylindole (DAPI; Sigma, Buchs, Switzerland) according to the protocol of Zarda et al. (56). Substrate-induced respiration (SIR; (4)) was measured with the OxiTop Control System OC 110 (Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany). General microbial activity of soil microorganisms was estimated from hydrolysis of fluorescein diacetate (FDA) following the procedure described by Schnürer and Rosswall (43). Dried soils (day 14) had to be rewetted prior to measuring SIR and FDA hydrolysis.

4.3.7 *DNA- and RNA-extraction procedure*

DNA extraction was performed using a bead beating procedure following the protocol of Bürgmann et al. (8). DNA pellets were resuspended in TE-buffer (10 mM TRIS-HCl, 1 mM EDTA, pH 8) at 1 ml g⁻¹ [dry wt] of extracted soil. DNA concentration was determined using a fluorometric assay with PicoGreen® (Molecular Probes, Eugene, OR) according to Bürgmann et al. (8). DNA yield was presented as μg total DNA extracted per gram soil [dry wt]. RNA extraction was carried out according to the procedure described by Bürgmann et al. (9). RNA pellets were resuspended in a final volume of 30 μl RNase-free water and were stored at −80°C.

4.3.8 *Reverse transcription of 16S rRNA and PCR amplification*

Reverse transcription (RT) of bacterial and archaeal 16S rRNA was performed following the protocol of Bürgmann et al. (9) using avian myeloblastosis virus (AMV) reverse transcriptase (Amersham Biosciences Europe GmbH, Dübendorf, Switzerland). Primer UNI-b-rev (for sequence see Table 1) served as universal primer for RT. Controls without AMV reverse transcriptase were included to test for DNA traces in the RNA extracts.

Archaea- and *Pseudomonas*-specific PCR of 16S rRNA gene fragments was performed in a 100 μl volume according to Pesaro and Widmer (38) and Widmer et al. (54), respectively (Table 1). PCR analysis of 16S rRNA genes of the bacterial domain was done in a 50 μl volume using primers 27F (labeled with the fluorescent dye 6-carboxyfluorescein (FAM6) at the 5’ position) and 1378R (Microsynth, Balgach, Switzerland) (Table 1). Reactions contained 1.5 mM MgCl₂, 0.2 μM of each primer, 0.2 mM of each dNTP (Qiagen, Basel, Switzerland), 0.6 mg ml⁻¹ bovine serum albumin, 1 U of HotStar DNA polymerase (Qiagen), 1 × PCR reaction buffer (Qiagen), and 10 ng of soil DNA. The cycling conditions were as follows: one initial cycle of 15 minutes at 95°C to activate the HotStar DNA polymerase
followed by 35 cycles of denaturation at 94°C for 45 seconds, primer annealing at 48°C for 45 seconds and DNA synthesis at 72°C for 2 minutes. The reaction was completed by a DNA synthesis step of 5 minutes at 72°C.

Table 1: PCR primers and PCR conditions used.

<table>
<thead>
<tr>
<th>target group</th>
<th>PCR primers</th>
<th>Position on 16S rRNA</th>
<th>PCR primer sequence (5’→3’)</th>
<th>PCR conditions: cycles, annealing temp.</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>27F-(FAM)</td>
<td>8-27</td>
<td>AGAGTTTGATCMTGGCTCAG</td>
<td>35, 48°C</td>
<td>(31)</td>
</tr>
<tr>
<td></td>
<td>1378R</td>
<td>1378-1401</td>
<td>CGGTGTGTACAAGGCCCCCCGGGAAACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Archaea</td>
<td>Arct915for</td>
<td>915-934</td>
<td>AGGAATTGCGCCGGGGAGCAC</td>
<td>35, 65°C</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td>UNI-b-rev</td>
<td>1390-1407</td>
<td>GACGGGCGGTGAGTCRAA</td>
<td></td>
<td>(7)</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>Ps-for</td>
<td>292-311</td>
<td>GTCTGAGAGGATGATCGATG</td>
<td>40, 65°C</td>
<td>(54)</td>
</tr>
<tr>
<td></td>
<td>Ps-rev</td>
<td>1263-1280</td>
<td>TAGCTCCACCTCGGCG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\*a) \( R = A, G \) \quad M = A, C

4.3.9 RFLP analyses of Archaea and Pseudomonas

Products from Archaea- and Pseudomonas-specific PCR amplifications were subjected to restriction fragment length polymorphism (RFLP) analysis using restriction endonuclease \( HaeIII \) according to Widmer et al. (54). For restriction analysis of Archaea PCR products, 10 \( \mu \)l digestion product was separated by electrophoresis in 3% MetaPhor agarose (FMC BioProducts, Rockland, ME) at 2.5 \( V \ cm^{-1} \) for 2.5 h and stained with ethidium bromide. Pseudomonas PCR products were resolved on 12% polyacrylamide gels that were run for 4 h at 200 Volts and 35°C (DCode™ system, BioRad Laboratories, Hercules, CA). To assure high resolution of bands, volumes smaller than 5\( \mu \)l were loaded. Staining was performed with 50 ml SybrGreen® (1 : 5000 in TAE buffer; Molecular Probes) by shaking gels for 30 min in the dark.

4.3.10 T-RFLP analyses of Bacteria

Bacteria-specific amplification products were purified according to Widmer et al. (54) and subsequently digested overnight at 37°C in 20 \( \mu \)l restriction digest mixtures containing 3 U \( MspI \) (Promega, Madison, WI) and 1 \times restriction enzyme buffer B (Promega). For T-RFLP analysis 12 \( \mu \)l formamide and 0.2 \( \mu \)l of internal size standard ROX500™ (Applied Biosystems, Foster City, CA) were distributed in a 96 well plate and 2 \( \mu \)l of restriction digests were added to each well. DNA was denatured at 92°C for 2 min and chilled on ice for 5 min. Restriction fragments were separated on a genetic analyzer (ABI3100, Applied Biosystems)
equipped with an array of 16 × 36 cm capillaries filled with POP-4™ polymer (Applied Biosystems). Sizes of terminal restriction fragments (TRFs) were detected automatically relative to the internal standard using GenScan 3.1 software (Applied Biosystems). Conversion of peak signals into numeric data of fragment size and peak heights, was performed using the Genotyper 3.6 NT software (Applied Biosystems). If it was not possible to unambiguously determine the height of a specific peak (e.g. peak shoulder), it was omitted from analysis in all samples. The values of peak heights were recorded and compiled in a data matrix for statistical analysis.

4.3.11 **Statistical analyses of genetic fingerprinting data**

Intensities of all distinct bands were quantified from each RFLP pattern using the lane tool and trace quantity option of the Quantity One software (BioRad Laboratories, CA). Band intensities were expressed as percentage of the sum of all detected bands in a given lane. This standardization procedure allowed for objective comparison of relative band intensities across different lanes and gels. Two-sided T-tests for pairwise comparisons of means of band intensities obtained from three independent replicate samples were calculated using Excel 98 (Microsoft Corporation, Redmond, WA). TRF heights were standardized applying the same procedure as for RFLP band intensities. In addition, for multivariate analyses, heights of all TRFs belonging to the same length category were transformed to average 0 and standard deviation 1. This served to give each TRF the same relative weight in statistical analyses. These data were then used for hierarchical cluster analysis (CA) based on pairwise Euclidean distances and single linkage clustering and for principal component analysis (PCA) using the Statistica v. 6 software package (StatSoft, Tulsa, OK). Analysis of variance (ANOVA; Statistica v. 6; StatSoft) was used for testing treatment differences, time×treatment interactions and temporal variations of TRF heights. Evenness of T-RFLP profiles was calculated as the Shannon's evenness index of TRFs (33).

4.4 **Results**

4.4.1 **Degradation of metalaxyl-M and lufenuron**

In reference soil, mean half-lives for metalaxyl-M were 3.2 days for the application at day 0, 3.4 days for the application at day 20, and 3.6 days for the application at day 48 (Table 2). In the test soil metalaxyl-M degradation was delayed (Fig. 2) and half-lives increased to 16.6 days for the application at day 20 and to 11.9 days for the application at day 48 (Table 2). In general, large amounts of the metalaxyl-M metabolite CGA 62826 were
observed (Fig. 2). Mean half-lives of this metabolite were 11.0, 18.4 and 18.1 days for the three applications in the reference soil and 53.3 and 63.0 days for the two applications in the test soil (Table 2). Half-lives from duplicate analyses differed by less than 3% of the larger value. The metabolite CGA 67868 was detected in maximum quantities between 2.7% and 4.5% (reference soil) and between 1.1% and 2.9% (test soil) of the total radioactivity recovered (Fig. 2). At application day 20 the mineralization rate for metalaxyl-M was 0.8 % d$^{-1}$ in the reference soil and decreased to 0.2 % d$^{-1}$ in the test soil. At application day 48 the mineralization rate was 0.5 % d$^{-1}$ in the reference and 0.2 % d$^{-1}$ in the test soil. Mass balance calculations revealed that between 92.8 and 105% of the total $^{14}$C applied (as [Phenyl-U-$^{14}$C]metalaxyl-M) was recovered (as $^{14}$C-parent or $^{14}$C-degradation products). Correlation coefficients ($R^2$) of fitted curves to measured data from parent and metabolite degradation were larger than 0.98 for all applications.

Figure 2. Degradation of metalaxyl-M in reference soil (a) and the test soil (b) applied at day 48. Formation and decline of the two metabolites CGA 62826 and CGA 67868 as well as the formation of non-extractables (NE) and $^{14}$CO$_2$ are shown. Data points represent means of duplicate analyses.
The mean half-life of the lufenuron parent compound was 49.6 days for the application in reference soil at day 48, whereas in the test soil the half-life increased to 82.2 days (Table 2). Calculated mean half-lives of the lufenuron metabolite CGA 238277 were 20.4 days in the reference soil and 30.4 days in the test soil (Table 2). The differences between half-lives of replicates were smaller than 10% of the larger value for both the parent and the metabolite. In general, only slow accumulation and decline of the metabolite CGA 224443 was observed. Forty-nine days after CPP application, 16.2% and 15.3% of the recovered radioactivity had accumulated as the metabolite CGA 224443 in reference and test soil, respectively. After 120 days, this metabolite accounted for 4% and 10.5% of the recovered radioactivity for reference and test soil, respectively. Mineralization of lufenuron occurred with a rate of 0.09 % d\(^{-1}\) in the reference soil and decreased by a factor of 3 in the test soil. Mass balance calculations revealed that between 96.4 and 104.7% of the total \(^{14}\)C applied (as [Dichlorophenyl-U-\(^{14}\)C]lufenuron) was recovered (as \(^{14}\)C-parent or \(^{14}\)C-degradation products). Correlation coefficients (R\(^{2}\)) of fitted curves to measured data from parent and metabolite degradation were larger than 0.98.

**Table 2: Degradation of crop protection products (CPPs) metalaxyl-M and lufenuron in reference and test soil.**

<table>
<thead>
<tr>
<th>application</th>
<th>compound a)</th>
<th>half-life (^{b)}) [d]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>metalaxyl-M</td>
<td>lufenuron</td>
</tr>
<tr>
<td></td>
<td>reference</td>
<td>test</td>
</tr>
<tr>
<td>day 0</td>
<td>parent</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>CGA 62826 / CGA 238277</td>
<td>11.0</td>
</tr>
<tr>
<td>day 20</td>
<td>parent</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>CGA 62826 / CGA 238277</td>
<td>18.4</td>
</tr>
<tr>
<td>day 48</td>
<td>parent</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>CGA 62826 / CGA 238277</td>
<td>18.1</td>
</tr>
</tbody>
</table>

a) CPP metabolites (CGA 62826 from metalaxyl-M degradation and CGA 238277 from lufenuron degradation) as shown in Figure 1.

b) Degradation half-lives (DT\(_{50}\)) are calculated according to the equations DT\(_{50}\) (parent) = ln2 / (k\(_{1}\) + k\(_{2}\)) and DT\(_{50}\) (metabolite) = ln2 / k\(_{3}\), where k\(_{1}\), k\(_{2}\) and k\(_{3}\) are pseudo-first-order rate constants for parent compound and metabolite degradation as shown in Figure 1.
**4.4.2 Bulk microbiological parameters**

DNA yields from the test soil were approximately the same at the beginning (day 0) and at the end (day 14) of the drying phase (Fig. 3). After rewetting the soil on day 14, DNA contents dropped by 43% on day 16 (P <0.001). Between day 16 and day 48, DNA contents persisted at this reduced level and did not recover. DNA yields of the reference soil, which had remained in the climate room under controlled conditions, showed only small, non-significant fluctuations (P =0.05). DNA contents of the reference soil between day 16 and day 48 were significantly higher compared to corresponding test soil samples (P <0.001 at day 16; P <0.01 at day 20; P <0.05 at day 48).

![Figure 3. Effects of drying-rewetting and re-equilibration on (a) soil DNA content, (b) direct cell counts, (c) substrate induced respiration rates, and (d) fluorescein diacetate hydrolysis. Data from reference soil samples (open bars) and test soil samples (hatched bars) are presented as mean values with standard deviation of three independent replications.](image)

Direct cell counts as determined by DAPI staining yielded 29.5×10^7 cells per g soil [dry wt] at day 0 in the reference soil and stayed at constant levels throughout the experiment (Fig. 3). At day 14 (end of the drying phase), cell counts were 56% lower in the dried soil than in the
respective reference soil. At day 16, the difference between reference and test soil was 29% and further decreased to 25% (day 20) and 17% (day 48) (significant time×treatment interaction; P < 0.05). All differences between reference soil and test soil were highly significant (P < 0.001).

Substrate-induced respiration rates were increased by 20% at the end of the drying period (day 14) compared to the corresponding reference (P < 0.05; Fig. 3). After rewetting, first an increase by 38% at day 16 (P < 0.001) was observed followed by a decrease to reference soil levels at day 20. Respiration rates of the reference soil decreased gradually by 12% from day 0 until day 48 (P < 0.001; Fig. 3), but no significant differences between reference and test soil were detected except for the ones described above.

Hydrolysis of fluorescein diacetate showed some fluctuations in the reference soil, but no clear trend was identified over the course of the experiment (Fig. 3). The maximum value in the reference soil was measured at day 0 with 68.7 µg (h × g)^{-1} soil [dry wt], the minimum value, obtained at day 48, was 27% lower. In the test soil, a strong increase in FDA hydrolysis rates by 80% occurred at day 14 (P < 0.001). After rewetting, rates decreased again and no significant differences were detected between reference and test soil from day 16 until the end of the experiment at day 48.

4.4.3 RFLP analyses of Archaea

Comparison of 16S rRNA gene RFLP patterns from the domain Archaea revealed distinct changes between reference soil and test soil. These changes were associated with two bands migrating at 360 and 220 bp in the gel (Fig. 4a). The intensity of the band at 360 bp decreased to 60% in the dried soil at day 14 as compared to day 0. After rewetting, a further decrease to 41% was detected at day 16. Until day 48, intensities of this band remained constant and were significantly lower than the corresponding reference soil values (P < 0.01). The faint band migrating at 220 bp was only visible in the reference and at day 0 of the test soil samples. The Archaea RFLP pattern obtained after RT-PCR of 16S rRNA revealed similar changes as the pattern based on 16S rRNA gene analysis. In the dried soil (day 14), the intensity of the band at 360 bp declined to 54% of the initial value (Fig. 4b). Two days after rewetting (day 16), a marked intensity decrease to 23% relative to the day 0 value was observed. Band intensities then persisted at constant levels until day 48 and were significantly lower than the intensities detected in reference samples (P < 0.01). In contrast to the RFLP pattern based on rRNA
genes, the band at 220 bp was very faint in RFLP patterns obtained from rRNA analyses and could not be unambiguously quantified.

![RFLP patterns](image)

**Figure 4.** RFLP patterns obtained from 16S rRNA gene and 16S rRNA analyses of the domain Archaea (a) and (b)) and the genus *Pseudomonas* (c) and (d)) for reference and test soils. Bands described in the text are marked with arrowheads. Bands that show significant changes between reference and test soil are marked with closed arrowheads. For clear identification, fragment sizes (base pairs) of bands are indicated. Numbers at the bottom indicate sampling days. Only one of three replicates is presented. 1kb-marker (M) was used as DNA size standard.

4.4.4 RFLP analyses of Pseudomonas

RFLP patterns based on 16S rRNA genes of the genus *Pseudomonas* showed no detectable changes for the replications throughout the experiment (Fig. 4c). Band patterns derived from 16S rRNA, however, revealed several clear changes as response to drying and rewetting (Fig. 4d). At day 16, the intensity of the band located at 550 bp decreased to 66% of the value at day 0. At day 20 the signal intensity further declined to 51% and remained constant until day 48. In the reference soil, intensities of this band remained constant throughout the experiment. Differences in intensities of the band at 550 bp between reference and test soil were statistically significant (P <0.05). Further differences between reference and test soil
were associated with three bands located at 320, 225 and 125 bp. These bands were very faint and not consistently detected in the reference soil, but became dominant components of the patterns obtained from rewetted soil.

### 4.4.5 T-RFLP analyses of the domain Bacteria

Fifty-seven different terminal restriction fragments (TRFs) were identified in electropherograms from all rRNA gene and rRNA analyses. On the average, 43 and 44 TRFs were detected in rRNA gene fingerprints from reference and rewetted test soil, respectively (Table 3). In rRNA fingerprints, an average of 36 and 38 TRFs were identified. TRF numbers were not significantly different between reference and test soil samples of rRNA gene and rRNA analyses.

#### Table 3: TRF richness and evenness for analysis of the domain Bacteria in reference and test soil.

<table>
<thead>
<tr>
<th>marker</th>
<th>treatment</th>
<th>day 0</th>
<th>day 14</th>
<th>day 16</th>
<th>day 20</th>
<th>day 48</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRF richness rRNA genes</td>
<td>reference mean (N=3)</td>
<td>43.7</td>
<td>44.0</td>
<td>42.0</td>
<td>44.0</td>
<td>41.0</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.6</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>test mean (N=3)</td>
<td>41.3</td>
<td>43.7</td>
<td>45.0</td>
<td>42.7</td>
<td>43.3</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.6</td>
<td>1.5</td>
<td>0.6</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>rRNA reference mean (N=3)</td>
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<td>34.7</td>
<td>36.3</td>
<td>37.0</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>SD</td>
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<td>0.6</td>
<td>1.2</td>
<td>0.0</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>test mean (N=3)</td>
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<td>29.7</td>
<td>35.0</td>
<td>38.3</td>
<td>39.0</td>
</tr>
<tr>
<td></td>
<td>SD</td>
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<td>0.6</td>
<td>1.0</td>
<td>1.2</td>
<td>1.7</td>
</tr>
<tr>
<td>TRF evenness rRNA genes</td>
<td>reference mean (N=3)</td>
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<td>0.919</td>
<td>0.917</td>
<td>0.918</td>
<td>0.916</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.001</td>
<td>0.000</td>
<td>0.001</td>
<td>0.000</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>test mean (N=3)</td>
<td>0.923</td>
<td>0.918</td>
<td>0.889</td>
<td>0.900</td>
<td>0.906</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.002</td>
<td>0.009</td>
<td>0.004</td>
<td>0.002</td>
<td>0.005</td>
</tr>
<tr>
<td>rRNA reference mean (N=3)</td>
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<td>0.887</td>
<td>0.883</td>
<td>0.894</td>
<td>0.887</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.001</td>
<td>0.010</td>
<td>0.007</td>
<td>0.007</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>test mean (N=3)</td>
<td>0.901</td>
<td>0.896</td>
<td>0.868</td>
<td>0.866</td>
<td>0.875</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.006</td>
<td>0.001</td>
<td>0.010</td>
<td>0.005</td>
<td>0.012</td>
</tr>
</tbody>
</table>

SD: standard deviation

Cluster analysis (CA, data not shown) revealed a distinct clustering of soil treatments (reference, dried, rewetted) and nucleic acid type (rRNA genes, rRNA) (Fig. 5). Principal component analyses was entirely consistent with CA also revealing clear separation of reference from test soil and rRNA gene from rRNA based analyses (Fig. 5). The first two
principal components (PC 1 and PC 2) accounted for 65.4% of the variance. Soil treatments (reference, dried and rewetted) were mainly separated on PC 1, while nucleic acid types (rRNA genes and rRNA) were mainly separated on PC 2. Reference soil samples from rRNA gene and rRNA based analyses formed very tight clusters each containing the data points from the five sampling days. Samples from test soils were clearly separated from reference soil samples. For rRNA gene analyses of rewetted soil, three distinct subclusters containing the triplicates from days 16, 20 and 48 could be distinguished. For rRNA analyses of rewetted soil, two close subclusters from day 16 and from day 20 and day 48 were detected. The triplicate samples from rRNA gene and rRNA analyses of the dried soil were located between the respective reference and rewetted soil samples, however, closer to the reference data points. The two reference soil clusters from rRNA gene and rRNA analyses were clearly more distant from each other (mean relative distance on PC 2 = 8.95) compared to the respective clusters with the samples from rewetted soil (mean relative distance on PC 2 = 4.88) (P < 0.001).

Nine TRFs (21%) that were present in all reference rRNA gene analyses disappeared in all rRNA gene analyses of rewetted soil. On the other hand, 11 TRFs (25%) that were not detected in the reference rRNA gene analyses newly appeared in rRNA gene analyses of rewetted soil. Twenty-nine TRFs were common to T-RFLP profiles from reference and rewetted soil. In rRNA analyses of rewetted test soil, 6 TRFs (17%) were not detected as compared to the reference soil, whereas 10 TRFs (27%) not present in the reference soil appeared in rRNA analyses of rewetted soil. Twenty-seven TRFs were detected in both reference and rewetted soil samples. TRF evenness in fingerprints of rRNA gene analyses was on the average 0.919 in reference soil and decreased to 0.898 in rRNA gene analyses of rewetted soil (Table 3; P < 0.001). Evenness in rRNA derived fingerprints was 0.892 for reference soil samples and was reduced to 0.870 in the samples from rewetted soil (P < 0.001).

Analysis of variance revealed that 91% of all TRFs obtained from rRNA gene analyses showed significantly different peak heights between reference and rewetted soil samples (P < 0.05). Analogous calculations for rRNA based analyses resulted in a portion of 83% significantly different TRF heights (P < 0.05). In addition, time×treatment interactions were examined to assess the temporal changes that PCA had revealed after rewetting of the test soil. Considering the sampling days 16, 20 and 48, a fraction of 59% TRFs revealed significant time×treatment interactions for rRNA gene based analyses (P < 0.05). Thereof, 38% TRFs were at a significance level of P < 0.001. For rRNA based analyses, 40% of the
TRFs revealed significant time×treatment interactions (P < 0.05) and 9% were attributed to the significance level P < 0.001.

![Principal component analysis (PCA) of T-RFLP data obtained from 16S rRNA gene (●) and rRNA (◆) analyses of the domain Bacteria for reference and test soils. Each data point represents a single analysis from an independent sample. Circles around data points indicate the groups that formed clusters in hierarchical CA. Circle types indicate the different hierarchical clustering levels identified: bold: 3 main clusters at highest branching level; medium: subclusters at 2nd branching level; fine: subclusters at 3rd branching level. Numbers next to circles designate the sampling days included. Values in parentheses indicate the percentage of variance accounted for by the first two principal components PC 1 and PC 2.]

4.6 Discussion

4.6.1 Degradation of metalaxyl-M and lufenuron

Degradation and mineralization of the widely used CPPs metalaxyl-M and lufenuron were used as parameters to evaluate the practical importance of drying-rewetting stress in soil. In addition, they served as sensors to test specific catabolic processes in soil. The applied drying and rewetting procedure resulted in a strong decrease of the CPP degradation potential of the indigenous microbial soil communities (Fig. 2, Table 2). In agreement with these results, drying and rewetting of an agricultural soil caused a significant reduction of the degradation
and mineralization activity of the insecticide Carbofuran (46). Decomposition of plant material was found to be retarded in soil that had been dried for 7 days and subsequently rewetted (32). In contrast, no effects of soil drying and rewetting on mineralization of Atrazine and trichloroacetic acid were observed (42). These studies may not be directly comparable, since the compounds used for testing microbial degradation activity have different physical and chemical properties and may also be degraded by different microbial groups.

During our experiment, metalaxyl-M was applied to reference soil on days 0, 20 and 48 and to the soil on days 20 and 48. Observed half-lives for the reference soil suggested a tendency for decreasing CPP degradation potential over storage time. Comparing degradation of a thiocarbamate herbicide in a freshly collected soil to one stored for 90 days at 20°C and 40% WHC, Anderson (3) observed a 2.3-fold increase in parent half-lives in the stored soil. The phenomenon of slowly decreasing CPP degradation potential in stored soils was attributed to nutrient depletion in the absence of a plant cover and subsequent dormancy of the soil microorganisms (3). Apparently, these effects were less pronounced in the soil used in the present study. Reduced metalaxyl-M half-lives in the test soil at days 20 and 48 indicated that recovery of the CPP degradation potential to reference soil levels had not occurred until day 48.

Metalaxyl-M degradation and mineralization rates were decreased by factors of 3 to 4 in the test soil. For lufenuron the decrease was between 1.5 (metabolite degradation) and 3 (mineralization). In comparison, DNA content and microscopic cell counts declined only by factors of 1.3 and 2, respectively, suggesting that the delay in CPP degradation was not entirely explained by the decrease in microbial biomass. Due to cell death during drying-rewetting, specialized catabolic functions may have been lost, which were not or only partially replaced by the surviving microorganisms. Alternatively, soil conditions (e.g. nutrient availability) were altered by stress impact and in turn may have caused decreased activities of CPP degrading organisms.

4.6.2 Bulk microbiological biomass

Results obtained from DNA content and direct cell counts indicated a pronounced average decrease in microbial biomass by 51% and 24%, respectively, after soil drying and rewetting (Fig. 3). A similar trend for these two parameters was observed for the same soil (Gartenacker) when it was subjected to freezing and thawing stress (39). However, the magnitudes of these effects after drying and rewetting exceeded those after freezing and
thawing by a factor of 2.1 for DNA content and 1.1 for direct cell counts. This indicated that
drying and rewetting represented a considerably harsher stress as compared to freezing and
thawing. In the soil subjected to drying-rewetting stress, DNA content remained constant
during drying and dropped to 50% of the reference after rewetting. This change appears to
reflect that DNA of microorganisms that had been lyzed during the drying phase was not
degraded until the soil was rewetted. This moisture effect is in agreement with the finding that
DNA degradation of purified plasmid DNA was strongly delayed in a soil at 10% as
compared to 40% WHC (55). The lowest cell counts were obtained in the dry soil just before
rewetting (day 14). This observation may be explained by cell lysis during the drying phase
from day 0 to day 14 and possibly also by increased proportions of spores that may have
formed as an adaptation to decreasing water content. Due to decreased permeability for DAPI,
spores are less detectable by the cell fixation and staining procedure applied (20). Fungal
mycelial length as well as bacterial numbers, volumes and surface areas were shown to
decrease, when moist soils were air-dried (52) demonstrating microbial adaptation and
damage induced by decreasing soil moisture content.

4.6.3 Bulk microbiological activities

In contrast to CPP degradation activity, SIR and FDA hydrolysis were not significantly
different from the reference values after 6 and 34 days of re-equilibration (Fig. 3). It has been
shown that the number of active bacteria can increase after soil drying and rewetting (10).
Mamilov et al. (34) found that the ratio between SIR and microbial C (determined with the
chloroform fumigation-extraction method) increased by a factor of 1.3 after repeated soil
drying and rewetting. In our experiment, the activity per cell, as calculated by the ratio of SIR
and cell counts, increased by a factor of 1.2 after 6 and 34 days of re-equilibration. In
agreement with Mamilov et al. (34) we conclude that the microbial fraction responsive to the
addition of substrate had increased after rewetting. Our data further indicate that changes in
specific activities, e.g. CPP degradation, are not necessarily reflected by bulk microbial
activities.

4.6.4 Microbiological community structure and activity

T-RFLP analyses of rRNA genes from the domain Bacteria revealed a clear stepwise change
in the community structure from reference to dried and from dried to rewetted soil (Fig. 5).
These data agree with shifts in community composition that were observed after several
drying and rewetting cycles of an oak forest soil (19). When the same procedure was applied
to a grassland soil, comparable to the one used in the present study, no changes in bacterial
Community composition were induced as measured by T-RFLP analyses (19). It has been hypothesized that the moisture regime a soil is naturally exposed to may be responsible for the resistance of microbial communities to drying and rewetting stress (57). Annual mean precipitation in Les Barges (origin of Gartenacker soil) is approximately 1000 mm y\(^{-1}\). Microbial communities may be not as frequently exposed to severe drying and rewetting events as compared to the grassland soil in the study of Fierer et al. (19), which originated from a region with annual rainfall of only 500 mm y\(^{-1}\). The distinct groups formed by rRNA samples in PCA and CA analyses and the separation from the corresponding rRNA gene samples indicated that there was a significant difference between the pool of rRNA genes and the pool of rRNA present in soil (Fig. 4). This may reflect differences in the extraction and detection method applied but also the occurrence of inactive along with highly active bacterial groups at the same time in the soil (13, 17, 26). The distance between the respective PCA clusters of rRNA gene and rRNA samples decreased in the sequence from reference to dried to rewetted soil. This may be an indication that bacteria that survived the stress treatment may have become more uniformly active.

The use of rRNA analyses to characterize active microbial communities is based on the assumption that active or growing cells have increased intracellular rRNA levels, whereas inactive or dormant cells contain less rRNA. Kerkhof and Kemp (28) found clear, although non-linear correlations between 16S rRNA contents and growth rates and also rapid decrease of intracellular rRNA levels after cessation of in vitro growth for various pure cultures of marine Proteobacteria. They concluded that during steady-state conditions, species-specific growth rates may be determined by measuring cellular rRNA content. On the other hand, in pure culture experiments with a marine Vibrio strain, ribosome contents decreased only with a half-life of 79 h after carbon-starvation (21). These findings from RNA analyses of pure in vitro cultures indicate that active bacteria contain increased levels of rRNA. However, different rRNA stabilities were observed after cessation of growth. In rhizosphere microbial communities a total RNA turnover rate of 20% day\(^{-1}\) was calculated using an isotopic tracer approach (37). These results indicate that total RNA turnover in an undisturbed rhizosphere system may be relatively slow. Therefore, it can not be assumed that exclusively the active microbial community components are detected when analyzing rRNA.

Large portions of the detected TRF fragments completely disappeared from the reference soil samples, whereas many TRFs were exclusively found in samples from rewetted soil. This finding may reflect that some bacterial groups were strongly affected by the stress event,
While others may have profited from the changed conditions after drying and rewetting and became active or started to grow.

It may be surprising that average TRF richness did not change for both rRNA gene and rRNA analyses after rewetting the test soil (Table 3). Apparently, the decrease in biomass as indicated by DNA content and cell counts, was not accompanied by a decrease in detected bacterial subgroups. TRF evenness, however, was reduced after stress impact in rRNA gene as well as in rRNA based analyses, suggesting a change in dominance of certain TRFs (Table 3). However, it has been shown that due to detection biases genetic fingerprints do not accurately represent the entire microbial community richness (15, 30). Therefore, it is important to note that microbial community indices calculated from genetic fingerprints should be interpreted with caution. These indices should only be used for relative comparison among different samples and not as absolute characteristic of the investigated microbial community.

Certain groups from the domain Archaea appeared particularly sensitive to drying and rewetting as revealed by decreasing intensities of specific bands in rRNA gene and rRNA analyses (Fig. 4). Similar changes in Archaea RFLP fingerprints were observed when soil was frozen and thawed and these changes were confirmed with gene cloning and independent PCR detection with new specific primers (39). These consistent findings indicated that the affected archaeal groups may represent microorganisms that are not well adapted to rapid environmental changes. This characteristic may render these organisms suitable indicators for the monitoring of microbiological soil characteristics in stored soils where any changes in MSCs may not be desirable. Changes due to drying and rewetting within the genus *Pseudomonas* were more clearly detected with rRNA analyses. Three bands showed increased intensities in the rewetted soil, suggesting that certain *Pseudomonas* sp. were better adapted to the conditions after rewetting. One band revealed a decrease in intensity, indicating that organisms represented by this band may have been less successful to cope with the altered conditions in soil during and after the stress.

### 4.6.5 Resistance and resilience of microbiological parameters

All microbiological parameters determined indicated that MSCs of *Gartenacker* soil were not resistant to drying-rewetting stress. SIR and FDA hydrolysis suggested complete resilience of MSCs within less than one week after rewetting. This finding is in agreement with previous observations that bulk microbial activities are able to completely recover after transient stress impact. Based on measurements of the soil respiratory quotient (qCO₂), Ananyeva et al. (2)
found complete resilience of dried and rewetted soil within 7 days. After heat stress, short term decomposition of grass shoot residues revealed resilience within 13 days (25). In the present study, direct cell counts recovered partially, while CPP degradation, DNA content, and data on community structure and activity indicated that the MSCs were severely altered by the stress procedure and no substantial recovery occurred within 34 days of re-equilibration. Westergaard et al. (53) investigated the effects on microbial communities after a single application of the antibiotic tylosin to soil using plating experiments, substrate utilisation profiles and DGGE analyses. Similar to our results, molecular biological tools were most sensitive and detected permanent changes in microbial community structure even after disappearance of the antibiotic. T-RFLP analysis revealed significant time×treatment interactions due to temporal drifts in TRF heights in the rewetted soil. This analysis indicated that microbial structures and activities were still changing during the re-equilibration phase and thus a stable state was not yet reached after 34 days of re-equilibration.

4.6.6 Conclusions

Our results suggested that different MSCs were suitable to monitor the impact of drying-rewetting stress on soil. DNA content in particular may be used as a reliable and simple parameter to monitor bulk microbial changes. Bacteria, Pseudomonas and Archaea were comparably sensitive to drying-rewetting stress. T-RFLP analyses of Bacteria suggested that rRNA genes and rRNA reacted equally sensitive to drying-rewetting stress. Only for Pseudomonas, rRNA indicated stronger changes as compared to rRNA gene analyses. We therefore suggest a case by case selection of different microbial groups and marker molecules for monitoring of different stress impacts on MSCs.

4.7 Acknowledgements

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4.8 References


CHAPTER 5:

COMPARISON OF MICROBIAL COMMUNITY STRUCTURE

CHANGES INDUCED BY FREEZING-THAVING AND DRYING-REWETTING STRESS
Chapter 5: Comparison of Freezing-Thawing and Drying-Rewetting Stress

5 Comparison of Microbial Community Structure Changes Induced by Freezing-Thawing and Drying-Rewetting Stress

5.1 Introduction

Soil samples freshly collected in the field are often stored in the laboratory or greenhouse prior to scientific experiments or analyses. In order to maintain their field status with respect to chemical, physical and biological characteristics, soils should be processed and stored according to standard protocols (14). Soil storage conditions and handling procedures have to be carefully selected and controlled, since particularly microbiological characteristics were shown to react sensitive to soil sieving (10, 21) or changes in soil temperature or moisture content (17, 23, 24, 27). Freezing of soil represents a recommended and frequently applied method to preserve soil microbial structures and activities (20, 29). In contrast, soil drying should be avoided prior to biological analysis (14), but may accidentally occur in soils stored under aerated conditions in the greenhouse without regular watering.

Numerous studies have focused on short-term effects of freezing-thawing or drying-rewetting cycles on soil processes. It has been demonstrated that thawing of frozen and rewetting of dried soils results in a transient 1 – 4 days increase in carbon and nitrogen mineralization rates when compared to an unstressed control (2, 7, 9, 13). Additionally, the impact of freezing-thawing and drying-rewetting on microbial biomass and activities has been frequently investigated (15, 19, 26, 30, 32). In response to these stresses an average 10 – 60% loss of microbial biomass has been observed, but very few data exist that describe in detail the changes in microbial community structures and activities (5, 8).

The objectives of this study were to evaluate, which of the stress procedures, freezing-thawing and drying-rewetting, caused more pronounced changes in microbial community structure. Furthermore, we investigated, whether the same components of the microbial community responded to the two stresses. In order to answer these questions, terminal restriction fragment length polymorphism (T-RFLP) and statistical analysis of bacterial 16S rRNA genes was performed on DNA extracts, which were obtained from experiments described previously (chapters 3 & 4).
5.2 Material and Methods

5.2.1 Soil sampling and soil storage

The soil for the freezing-thawing experiment was collected in July 1998 from the Gartenacker field site and was subsequently stored in the greenhouse at 20°C under plant cover before the start of the experiment in February 1999. During storage in the greenhouse, soil moisture content was kept approximately constant at 40% water-holding capacity (WHC) by regular watering (chapter 3).

The soil for the study on drying-rewetting stress was sampled in April 2001 from the same field site and was immediately used for experiments without prior storage in the greenhouse (chapter 4).

5.2.2 Experimental setup

The experimental setups and the sampling schedules are described in detail in chapter 3 (freezing-thawing stress) and chapter 4 (drying-rewetting stress).

In the study on freezing-thawing stress, a portion of soil was subjected for four days to artificial freezing at −20°C in a cooling chamber (test soil). The soil was thawed and subsequently re-equilibrated for 42 days in a climate room at 20°C. In order to assess the impact of the freezing-thawing procedure, the test soil was compared to an undisturbed reference soil, which had been maintained in the climate room.

In the drying-rewetting experiment, a portion of soil was slowly air-dried during 14 days in a climate room at 20°C to a final water content of 1% WHC. Afterwards, the soil was rewetted to the initial water content of 40% WHC. Another portion of soil that had been maintained at constant water content of 40% WHC served as an undisturbed reference.

5.2.3 T-RFLP analyses

To allow for a direct comparison of freezing-thawing and drying-rewetting stress, DNA extracts from experiments described in chapters 3 and 4 were reanalyzed in a common sample set. Bacteria-specific PCR amplification of 16S rRNA gene fragments followed by T-RFLP analysis was performed according to the procedures described in chapter 4. Peak heights from individual terminal restriction fragments (TRFs) were determined and values from all analyzed samples were arranged in a two-dimensional data matrix.
5.2.4 Statistical analyses of T-RFLP data

In order to detect TRF height changes between reference and test soil samples, differences were determined between mean values of reference soil and test soil. From the freezing-thawing experiment, the sampling days 2, 10 and 20 and from the drying-rewetting experiment, the sampling days 2, 6 and 34 days were selected for this analysis. To directly relate the differences between the data sets of the two experiments, Spearman rank correlation was calculated using the Statistica v. 6 software package (StatSoft, Tulsa, OK). The data set including TRF height values of all sampling days was subjected to principal component analysis (PCA) and hierarchical cluster analysis (CA) using Statistica v. 6 (StatSoft).

5.3 Results

5.3.1 Basic description of the data set

The entire data set including both stress experiments consisted of 66 different terminal restriction fragments (TRFs). In the freezing-thawing experiment, the number of identified TRFs was 51, while in the drying-rewetting experiment, 66 TRFs were detected. As one measure for the magnitude of the stress impact, single TRFs were evaluated for presence and absence before and after the stress treatment. In the freezing-thawing experiment, 3 TRFs (7%) detected in reference soil samples completely disappeared from T-RFLP fingerprints from thawed soil samples. On the other hand, 5 TRFs (11%) were unique to T-RFLP analyses derived from thawed soil samples. In T-RFLP profiles from the drying-rewetting experiment, 8 TRFs (17%) completely disappeared from the rewetted soil samples, while 16 TRFs (30%) newly appeared in the rewetted soils.

The two stress events were compared in greater detail by determining the average differences in TRF heights between reference and test soil samples (Fig. 5.1). 49% of the TRFs changed in the same direction (i.e. common increase or decrease), while 29% of all TRFs revealed changes in opposite directions for the two stress experiments. 83% of all identified TRFs showed stronger absolute changes in the drying-rewetting experiment, whereas only 17% of all TRFs showed more pronounced changes in the freezing-thawing experiment.
Fig. 5.1: Relative mean differences in TRF heights between reference and test soil samples. For the freezing-thawing experiment (white bars) triplicate analyses of sampling days 2, 10 and 20 days were used. For the drying-rewetting experiment (black bars) triplicate analyses of the sampling days 2, 6 and 34 days were considered.
5.3.2 *Statistical analyses of the data set*

The question of whether the freezing-thawing and the drying-rewetting procedure induced changes in the same microbial community components was investigated by calculating Spearman rank correlation of TRF height differences. This analysis revealed that TRF height differences were significantly positively correlated between the two stresses ($R^2 = 0.42$; $P < 0.001$).

PCA revealed a close association of the reference soil samples from the freezing-thawing and the drying-rewetting experiment (Fig. 5.2).

![Principal component analysis (PCA) of T-RFLP data obtained from the drying-rewetting (O) and the freezing-thawing (◆) experiment. Data points are means of three independent replicates from one sampling day. Circles around groups were drawn according to clusters shown in Fig. 5.3. Values in parenthesis indicate the percentage of variance accounted for by the first two principal components PC 1 and PC 2.](image)
The samples from the dried soil as well as the frozen and thawed soil formed distinct groups and were mainly separated on PC 2 from corresponding reference samples. Data points from the dried and rewetted soil samples were mainly separated from those of dried soil and reference soil samples along PC 1.

The dendrogram obtained with CA confirmed the basic clustering of the different soil treatments observed by PCA (Fig. 5.3). The reference soil samples from both stress experiments were closely related; relative branch distances separating them were small. Samples subjected to freezing-thawing or drying were separated from corresponding reference soil samples and grouped each in distinct clusters. The cluster containing the data points from the dried and rewetted soil was clearly separated from all other analyzed samples, as revealed by larger branch distances.

5.4 Discussion

Different statistical analyses of T-RFLP data indicated that the magnitude of response of the microbial community was substantially stronger when Gartenacker soil was subjected to drying-rewetting as compared to freezing-thawing (Fig. 5.1 - 5.3). This is in agreement with the finding that the decrease in microbial biomass, estimated by soil DNA content and direct cell counts, was more pronounced after soil drying and rewetting (chapters 3 & 4). By measuring plate counts and CO\textsubscript{2}-evolution, Soulides and Allison (25) found drying-rewetting to have a significantly stronger impact on soil bacterial populations as compared to freezing-thawing. Zelles et al. (32) reported on strongly reduced soil ATP content and adenylate energy charge (AEC) in dried and rewetted soil, whereas the decrease in frozen and thawed soil was less pronounced.

The positive correlation of TRF height differences indicated that the bacterial community of Gartenacker soil responded in a similar way to freezing-thawing and drying-rewetting stress (Fig. 5.1). This finding can be explained by the fact that both stress procedures induce osmotic stress in microbial cells (16, 31). It may therefore be assumed that resistance or susceptibility to osmotic stress is one of the determining factors for the observed changes in microbial community structure.
Fig. 5.3: Cluster analysis (CA) of T-RFLP data obtained from the drying-rewetting (solid parenthesis) and the freezing-thawing (dotted parenthesis) experiment. Each branch is labeled with the sampling day and represents a single analysis from an independent sample. Day 0 analyses for the test soil (*) were performed before the start of the drying phase. Day 14 analyses for the test soil (**) were performed at the peak of the dry stress, immediately before rewetting.
Although significant biomass decreases were detected, the number of TRFs newly appearing after stress impact exceeded the number of disappearing TRFs, indicating both reduction and growth of different components of the microbial community. Growth of microorganisms may have occurred on nutrients that were released from cells and soil aggregates after thawing or rewetting (18). Ecological theory predicts that slow growing or specialized organisms (K-strategists) suffer, while fast growing, metabolically versatile organisms (r-strategists) profit from conditions following severe stress events (4, 22, 28). The validity of this concept for stress impact on microorganisms in soil habitats remains to be substantiated. This may be achieved by more quantitative detection of the changing microbial populations using e.g. slot blot analysis (3). However, testing for metabolic versatility of individual organisms is only feasible by cultivation dependent approaches.

It may be surprising that some TRFs revealed opposite trends after freezing-thawing and drying-rewetting stress. In all of these cases, TRFs increased after freezing-thawing and decreased at the same time after drying-rewetting (Fig. 5.1). This may suggest that these populations were specifically stimulated by the relatively moderate freezing-thawing stress, but on the other hand, were not able to tolerate the harsher drying-rewetting stress. A similar behaviour has been observed in plant communities under different disturbance regimes and is commonly referred to as hump-back response (12). In microbial community investigations, this phenomenon has rarely been described. Degens et al. (5) found that microbial catabolic diversity was increased at intermediate stress levels, introduced by 1 – 4 freezing-thawing and drying-rewetting cycles, but was significantly reduced after harsh stress impact, introduced by 8 cycles.

Between the two reference soil sample sets, only small differences in bacterial community structure were recorded. The observed differences may reflect the combined effects of soil storage in the greenhouse as well as of seasonal variations in the field. As compared to the two stress treatments, greenhouse storage and seasonal variations had only little influence on microbial community structures. Using denaturing gradient gel electrophoresis (DGGE) analysis, changes in bacterial populations of a grassland soils were detected (11). These changes were found to be smaller than the differences between the layers from 0 – 5 cm and 15 – 20 cm depth of the same soil.

In order to develop new specific monitoring tools for stress sensitive bacterial populations, a procedure comparable to the one for detection of a freezing-thawing susceptible archaeal subgroup (described in chapter 3) may be used. First, organisms that are associated with the
changing TRFs have to be phylogenetically affiliated by cloning and sequence analyses. Based on the retrieved sequences new probes for direct PCR or dot/slot blot analysis could be designed (1, 6). This strategy may be promising with regard to the development of novel tools for monitoring of stress and disturbance impacts on microbiological soil characteristics (MSC). The main improvement of these analyses as compared to bulk microbiological techniques is the possibility to identify and directly detect stress indicating microbial species or groups. This may lead to increased sensitivity for detection of community structure changes and may contribute to a better understanding of microbial stress response and community dynamics in soil.

5.5 References


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6 GENERAL DISCUSSION

6.1 Ribosomal RNA: A genetic marker for analyses of microbial communities

In recent years, enormous progress has been made in molecular biological methods to detect and analyze indigenous microbial populations in various ecosystems. This may be documented by the rapid increase in sequence data that is submitted to public databases. Currently, more than 73,000 small subunit (SSU) ribosomal RNA (rRNA) gene sequences are available through the ribosomal database project (RDP; (10)). The molecular biological approach has not only led to the discovery of numerous, previously unknown microorganisms (30), but also for the first time allowed to establish a consistent classification system for microorganisms that is based on genetic characteristics (2, 61, 90). Along with the retrieval of rRNA gene sequences from the environment, molecular biological detection and analysis techniques were improved for a more specific, rapid and precise assessment of microbial populations. Major improvements include recovery of high quantities of pure nucleic acids (29, 39, 42, 48, 49, 84), development of DNA/RNA hybridization (7, 45, 65) and PCR protocols (3, 50, 87, 88) using group-specific oligonucleotide probes and increasing sensitivity and resolution power of genetic fingerprinting techniques (41, 55, 56). In spite of the substantial technical progress made, only few of the new methods are currently included in protocols for the routine monitoring of microbiological soil characteristics (MSCs) (1, 18, 31). The reasons for the lack of standard molecular biological methods in soil analyses are often due to complex and laborious procedures and to experimental difficulties, which require experienced researchers to produce consistent results.

6.2 Resistance and resilience of soil microbial communities

CPP degradation in soil was found to be unaffected by freezing and thawing, however, was strongly reduced after drying and rewetting and not resilient during the 34 day re-equilibration period. The test CPP methidathion that was used in the freezing-thawing experiment was mineralized significantly faster as compared to metalaxyl-M and lufenuron, which were applied in the drying-rewetting study. It can not be ruled out that a decrease in CPP degradation potential would have been detected in the freezing-thawing stressed soil by testing a more persistent CPP.
Results from the freezing-thawing and the drying-rewetting experiments indicated that the investigated MSCs were not resistant to storage related stress. While similar findings were obtained in previous studies using cultivation dependent approaches or bulk microbial measurements (52, 72, 74, 77), effects on specific nucleic acid markers in combination with soil functions, such as the capacity to degrade CPPs, were not yet demonstrated. The changes in microbial community structure after stress impact were clearly more pronounced as compared to the differences between the two reference soils (chapter 5). This may indicate that microbial communities in the field were either subjected to relatively small disturbances or were more resilient after natural disturbance as compared to the harsh and defined stress applied in the laboratory experiments.

One objective of this thesis was to assess, whether specific stress sensitive microbial populations exist or if storage-related stress nonspecifically and uniformly impacts the whole soil microbial community. While this question has been addressed for soil macrofauna (63, 68) or selected bacterial isolates grown in pure culture (74), very few systematic investigations exist for natural soil microbial communities in this field. Notable exceptions are the studies of Sandaa et al. (69-71) on the impact of various concentrations of heavy metals on soil microbial communities. Organisms assigned to the α-subdivision of Proteobacteria were detected in increased amounts in soils with high metal amendments. In contrast, Archaea appeared to be most susceptible to heavy metals, as their numbers significantly decreased in contaminated soils.

Analyses of microbial community structure and activity using 16S rDNA and rRNA markers revealed that the resistance to stress of individual microbial populations was highly different. It therefore appears difficult to predict changes in soil functions due to stress impact without tools that allow microbial community analysis at the level of specific populations or even single organisms (34, 81). Resilience of MSCs depended on the parameter investigated and on the stress procedure applied. Substrate-induced respiration (SIR) and fluorescein diacetate hydrolysis revealed complete recovery, whereas cell counts, DNA content and specific nucleic acid based parameters indicated irreversible changes during the course of the experiments (chapters 3 & 4). The most pronounced changes in microbial community structure and DNA quantity had occurred already within one to two days after stress impact (Fig. 5.2 & 5.3). Compared to these initial changes, further changes until the end of the experiment were minor, suggesting a short equilibration time of microbial community characteristics.
6.3 Stress response of soil microbial communities under different climatic conditions

A high variability in the extent to which microbial biomass decreases after freezing-thawing and drying-rewetting has been reported in the literature. In some studies no significant decrease was found (14, 21, 40, 77), whereas in other studies, including the present one, up to 60% biomass loss was observed (36, 72, 83, 89). An explanation for this phenomenon might be that soils from different climatic regions have variable resistances to different stress impacts. Microbial communities in soils from northern areas (e.g. Scandinavia) are annually subjected to several freezing-thawing cycles and therefore may be less sensitive freezing-thawing stress (77). On the other hand, soils from a semiarid climate may be widely resistant to drying-rewetting stress (22, 83). Apart from the climatic influence, the water supply of a soil may be an important factor influencing the sensitivity of indigenous microorganisms to drying-rewetting stress. Zelles et al. (93) found that various activity and biomass measures were more strongly affected by drying of a peaty soil as compared to a sandy soil. They concluded that microbial populations in the peaty soil were not subjected to frequent fluctuations in moisture content and therefore they were not as well adapted to drying-rewetting stress compared to the populations in the sandy soil. Alpine tundra soils are particularly exposed to strong fluctuations in moisture content and temperature (40). Lipson et al. (40) observed that microbial communities in alpine dry meadow soils were highly frost and drought resistant. These findings suggest that selection and/or adaptation of microbial communities to climatic conditions occurs and consequently should be considered when interpreting and comparing results from different freezing-thawing or drying-rewetting experiments.

6.4 Evaluation of methods and parameters for monitoring of MSCs

6.4.1 Comparing rDNA with rRNA markers to evaluate stress impact

A thorough assessment of uncultivated microorganisms in natural habitats may include description of their presence, spatial distribution and community composition. Information on these parameters can be gained by analysis of genetic markers as for example the rRNA genes. In addition to structural characteristics, activities represent a key to understand the role of microbial populations in the environment. Ribosomal RNA has been proposed as marker for the detection of microbial activities (19, 26, 35, 54). In contrast to messenger RNA
(mRNA), which may be indicative of a specific function, rRNA may correlate with the overall metabolic state of a microbial cell (35, 54).

Currently, only few of studies are available that aimed at directly comparing structure and activity for diverse soil microbial communities. Differences of rDNA with rRNA pool complexity were evaluated in grassland, forest and flooded paddy soils by application of denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) analyses of Bacteria and Archaea (16, 19, 25, 26, 44, 57). In none of these studies, however, the question was addressed, whether rRNA analyses are more sensitive than rDNA analyses to detect stress impact or changes in soil characteristics. Results from the drying-rewetting experiment suggested that sensitivity of rDNA and rRNA were similar for the dominant components in the domain Bacteria (chapter 4). Higher sensitivity for either rRNA or rDNA was only detected, when smaller microbial groups as for example Pseudomonas or Archaea were investigated. A possible explanation, why the two markers revealed similar sensitivity for analyses of Bacteria may be that the carbon liberated due to soil drying-rewetting and freezing-thawing was not sufficient to allow for rapid and prolonged growth of the indigenous bacterial populations. Therefore, only a small increase in ribosome synthesis over rDNA copy number following stress impact may be expected and in turn also little differences in sensitivity between rRNA and rDNA.

After microbial growth or death, the increase or decrease of cellular rRNA may proceed more rapidly than of rDNA due to higher turnover rates of rRNA (59). However, in many populations the resulting difference between rRNA and rDNA pools may be only detected in a very short time interval after stress impact, possibly within less than a few hours. The limited temporal resolution of the microbial response after drying-rewetting stress did not allow for an assessment of this difference.

### 6.4.2 DNA content – a novel parameter to estimate soil microbial biomass

In order to ensure reproducible analyses of soil microbial communities by the application of molecular biological techniques, considerable effort has been made to develop efficient protocols for extraction and purification of high DNA quantities (29, 39, 42, 48, 49, 84). DNA is maintained in all living microorganisms and in contrast to other essential cell constituents (e.g. proteins), DNA is structurally more defined and exhibits uniform chemical characteristics (e.g. water solubility, reactivity). These properties may be favourable for successful DNA extraction from soil and for use of DNA to estimate the size of soil microbial communities.
Use of soil DNA contents has several advantages over other bulk microbial parameters to estimate microbial biomass such as cell numbers, substrate-induced respiration (SIR) or chloroform-fumigation extraction (CFE). First, DNA extraction and quantification is relatively fast and allows for simultaneous processing of up to 50 samples in parallel per person and day. The method proved to be also reliable as indicated by low standard deviations of DNA contents among replicate samples (chapters 2, 3 & 4). Second, for molecular biological studies including techniques such as PCR, genetic fingerprinting, cloning and sequence or DNA hybridization, nucleic acids extraction is a prerequisite (2, 66, 81). Quantification of obtained DNA requires little additional work and provides extra information on the microbial community under investigation. Third, a slightly expanded protocol allows for extraction and subsequent separation of RNA and DNA from the same soil sample (8). RNA subsequently may serve as marker for metabolic or activity related processes and can be directly compared to results obtained from DNA investigations (16, 20, 26).

Nevertheless, results from soil DNA content measurements need to be interpreted in relation to the classical parameters, microbial biomass carbon and cell numbers. Many cell count protocols include cell fixation and permeabilization steps that result in lysis or disruption of sensitive cell types. Therefore, only bacteria can reliably be counted whereas fungal and protozoa numbers may be underestimated. The soil DNA pool contains both bacterial and fungal DNA and extracts may represent DNA from all cell types, as well as spores (11, 39, 51). An important difference of DNA as compared to biomass carbon determination is that the DNA content of a cell is not influenced by actual cell size. Physiological processes (e.g. starvation) can lead to changes in biomass carbon, whereas DNA content remains constant. On the other hand, when comparing DNA content with cell numbers it must be considered that different microbial species can have different genome sizes (46). Depending on the species composition, two microbial communities with identical cell numbers may contain different amounts of DNA.

Limitations of the method presented in chapter 2 have to be considered, too. Only a portion of the DNA present in soil can be extracted when single DNA extractions with single bead beating are applied. To obtain total soil DNA contents, repeated extraction and repeated application of the full bead beating procedure were necessary and very harsh extraction conditions had to be selected. However, harsh treatment resulted in fragmented DNA not suitable for subsequent molecular biological analyses (chapter 2) (49).
6.4.3 Potential and limitations of the used genetic fingerprinting techniques

In general, genetic fingerprinting techniques proved to be useful for detecting subtle changes in microbial community structure and for identifying stress indicating microbial groups. However, sequence analyses of cloned archael 16S rDNA fragments indicated that community level RFLP patterns are not able to resolve sequences with high similarities and thus to display the entire microbial species richness (chapter 3) (62). Furthermore, in highly diverse microbial communities, as inhabiting grassland and forest soils (82), overlaying fragments can mask existing differences between samples (60, 81). Presumably, this has occurred in RFLP analyses using PCR products that were amplified with universal bacterial primers (chapter 3). For successful RFLP analyses, it may be necessary to select specific microbial subgroups and for a representative assessment of e.g. the bacterial domain, to perform several RFLP analysis of different subgroups in parallel. One of the advantages of RFLP may be the possibility to analyze PCR products with a high range of fragment lengths and to select or combine different restriction enzymes allowing for optimization of fragmentation pattern complexity (64). In contrast, for DGGE analysis, PCR product sizes should not exceed 300 – 500 bp in order to obtain distinct bands in genetic fingerprints (56, 91), thereby limiting the amount of sequence information that can be gained for phylogenetic inferences.

The T-RFLP technique may overcome some of the limitations of RFLP, as demonstrated with the reanalyzed data set of the freezing-thawing experiment (Fig. 5.2 & 5.3) (38, 81). Due to the high resolution power of T-RFLP, the structure of complex microbial communities (e.g. Bacteria, Fungi) can be assessed in a single analysis (6, 41, 43). On the other hand, T-RFLP may be not suitable for analysis of small microbial subgroups with limited genetic diversity. By measuring only the terminal restriction fragment of each amplified 16S rDNA gene, every detected fragment is representative of a single ribotype. Low sample amounts are sufficient for successful fragment detection, due to the high detection sensitivity of available genetic analyzers. Nevertheless, it remains an open question, whether T-RFLP profiles contain sufficient information to realistically estimate soil microbial diversity (17).

With both RFLP and T-RFLP, a direct cloning of detected fragments is not possible (38). Fragments of interest have to be identified by screening libraries of cloned PCR products. This procedure is more time-consuming than directly extracting bands from the gel matrix, as usually performed in DGGE analysis (55).
6.5  Recommendations for the selection of tools to monitor microbiological characteristics in soils for CPP degradation studies

6.5.1  OECD guidelines

OECD guidelines require a basic characterization of soils, prior to use for CPP degradation studies. Thereby, the chemical and physical parameters texture, pH, cation exchange capacity, organic carbon content, bulk density and water holding capacity have to be measured. For biological characterization, only determination of microbial biomass is recommended (58). The methods proposed for quantification of microbial biomass are substrate-induced respiration (SIR) and chloroform fumigation extraction (CFE).

6.5.2  Bulk microbial parameters

Data from the present thesis generally support the idea of measuring microbial biomass as a bulk parameter, but also suggest that not all methods to estimate microbial biomass produce consistent results. Particularly SIR appeared not to accurately reflect microbial biomass in disturbed soils (12, 47) and did not differentiate soils with different CPP degradation rates. Microscopic cell counts and DNA content were more sensitive and correlated also with CPP degradation rates in the drying-rewetting experiment (chapter 4). Using cell counts, many replicates have to be analyzed, which is labour-intensive. Therefore, microscopic cell counts may not be feasible for routine analyses of many soils in parallel. DNA content has the advantage that less replicates are required and many samples can be processed in parallel. Additionally, DNA extracts may be saved and used for more detailed molecular biological analyses. In contrast to SIR, the analysis procedures for direct cell counts and DNA content may be more complicated and - when performed by untrained personnel - may be more easily subject to experimental variation. Fluorescein diacetate hydrolysis (FDA) revealed results similar to SIR. This method therefore appears not suitable for the monitoring of soils used for CPP degradation experiments.

6.5.3  RFLP and T-RFLP analysis

RFLP proved to be a robust and reproducible method to detect changes in MSCs. Genetic fingerprints were highly reproducible could be compared across different gels and experiments. The analyzed microbial groups should be in a low to intermediate diversity range to yield genetic fingerprints that allow for unambiguous detection of distinct bands. A major advantage of RFLP as compared to other genetic fingerprinting techniques is that no expensive equipment is required.
T-RFLP is clearly superior to RFLP for investigation of complex microbial communities. Additionally, the method is highly automated and allows for analyses of many soil samples in parallel in short time periods. The use of equipment and software requires well-trained personnel. Purchase of equipment and software is expensive and maintenance is time consuming.

### 6.5.4 16S rDNA and rRNA analysis

When using 16S rRNA as genetic marker, mainly the metabolically active microorganisms are detected. Our results indicated that rRNA analyses may not be generally more sensitive than rDNA analyses, except for specific microbial groups as e.g. *Pseudomonas*. In our studies, the extraction and reverse transcription of rRNA were considerably more time-consuming than comparable rDNA analyses. We therefore conclude that analyses of rRNA did not provide a clear advantage for monitoring of microbiological soil characteristics. It has been speculated that rRNA reveals faster response and has greater amplitude than rDNA following changes of environmental conditions (16). The time period for which differences between the two genetic markers may be detected after stress impact may be rather short and therefore not relevant for the routine monitoring of storage related stress.

### 6.6 Conclusions

The parameters required by OECD to characterize MSCs in CPP degradation test soils are restricted to microbial biomass. The results of this thesis suggest that microbial biomass should be maintained for characterization of CPP degradation test soils. However, activity related methods, as for example SIR, should be replaced by methods measuring cell components, as for example DNA. Tools for microbial community analyses based on rDNA are superior to bulk soil microbial methods and have the potential to reveal changes in the soil CPP degradation potential. It is therefore recommended to expand the set of methods by molecular biological techniques. T-RFLP analysis may be particularly suitable, since single procedural steps can be standardized and documented and samples can be saved and reanalyzed easily. Nevertheless, before implementing this method for the routine monitoring of MSCs, testing in many different soils as well as investigating additional stress events in combination with CPP degradation tests is required.
6.7 Outlook

To improve the sensitivity and specificity of the methods used for monitoring of MSCs, more specific stress indicators may need to be identified based on genetic fingerprint analyses of different microbial groups. Design of new genetic probes based on retrieved rDNA sequences would provide the possibility for specific detection of the stress sensitive organisms. Furthermore, it would be promising to extend the molecular techniques also on fungal communities (6, 24, 32, 37, 43). These organisms are frequently neglected in soil analyses although they play an important role in the breakdown of many CPPs (4, 76, 85). Quantitative analyses may be applied by dot/slot blot analyses of rDNA or rRNA. An alternative may be the use of real-time PCR to quantify template concentrations in PCR reactions (27, 28, 78, 79, 86).

Detection of specific functions, as for example genes or gene products associated with specific CPP degradation steps may be an alternative to analysis of phylogenetic markers (13, 15, 33, 53). For a general characterization of MSCs this approach may be promising, if many genes involved in CPP degradation could be detected in a single analyses. This can be achieved by spotting template sequences on gene chips and subsequent hybridization with soil DNA or RNA extracts (5, 9, 15, 23, 75, 80, 92). Before such an analysis can be realized, more CPP degrading organisms have to be identified and gene sequences that are involved in CPP degradation have to be retrieved (67, 73).

6.8 References


Chapter 6: General Discussion


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