AN ABSTRACT OF THE DISSERTATION OF

Guilherme M. Chaer for the degree of Doctor of Philosophy in Soil Science presented on March 21, 2008.
Title: Response of Soil Microbial Communities to Physical and Chemical Disturbances: Implications for Soil Quality and Land Use Sustainability

Abstract approved:

Peter J. Bottomley

The objectives of this thesis were to evaluate the responses of soil microbial communities to physical and chemical disturbances, and associate these responses with soil functional stability and changes in soil quality. The first study consisted of application of heat shocks (HS) to soils with contrasting land use history to evaluate differences in the stability of soil enzymes (laccase, cellulase and fluorescein diacetate hydrolysis) and microbial community composition as determined by phospholipid fatty acid (PLFA) analysis. The conversion of land use from forest to agriculture resulted in a new microbial community that was less functionally stable. Loss of stability was indicated by the reduced resilience of laccase and cellulase activities in the agricultural soil, which suggested a less diverse community of microorganisms capable of producing these enzymes. The second study examined changes in microbial community composition and diversity that occurred across a gradient of soil disturbance. Disturbances were simulated by tillage events applied at different intensities to a 12-year-old fallow area. These treatments caused degradation of several soil physico-chemical properties, and alterations in microbial

structure based on PLFA and terminal restriction fragment length polymorphism (T-RFLP) analyses, and in metabolic potential based on community level physiological profiles (CLPPs). Multivariate ordination of soil properties revealed the formation of a linear gradient of soil degradation that was significantly correlated with CLPPs, but not with T-RFLP and PLFA profiles. Nevertheless, changes observed in microbial community structure were significantly associated with decreases in soil organic C and field hydraulic conductivity. The third study demonstrated that undisturbed forest soils from western Oregon express an equilibrium between soil organic matter and biochemical properties. A model fitted through multiple regression analysis showed that phosphatase activity and microbial biomass were able to explain 97% of the soil organic C in these soils. This equilibrium was disrupted when a soil from an old-growth site was submitted to chemical stresses (Cu addition or pH alteration) and physical disturbances (wet-dry or freeze-thaw cycles). The magnitude of this disruption was consistently expressed by the ratio between soil C predicted by the model (C_p), and soil C that was measured (C_m). This ratio is proposed as biochemically-based index of soil quality.

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Response of Soil Microbial Communities to Physical and Chemical Disturbances: Implications for Soil Quality and Land Use Sustainability

by
Guilherme M. Chaer

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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CONTRIBUTION OF AUTHORS

Dr. Marcelo F. Fernandes assisted in study design, laboratory analyses and data interpretation in Chapters 2 and 3. Dr. Fernandes also coordinated the field work of Chapter 3. Both Dr. Peter J. Bottomley and Dr. David D. Myrold assisted in manuscript preparation and data interpretation.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Response of Soil Microbial Communities to Physical and Chemical Disturbances: Implications for Soil Quality and Land Use Sustainability

General Introduction

Chapter 1

Guilherme M. Chaer

Soil degradation, microbial diversity and functional stability

About 1780 Mha of the world's soils have been degraded in some way with conversions of forests and grasslands to agriculture causing dramatic effects on physical and chemical properties (Davidson and Ackerman, 1993; Oldeman, 1994; Swift et al., 1998). In many soils, these effects are seen as losses of soil carbon and degradation of soil structure, both of which are difficult to reverse (Solomon et al., 2002; Qafoku et al., 2003). Soil microbial communities are also highly sensitive to changes in the soil environment, which often lead to alterations in community composition, and to declines in microbial biomass and activity (Borneman and Triplett, 1997; Islam and Weil, 2000; Waldrop et al., 2000; Bossio et al., 2005). In addition to these effects, there are several reports that disturbances caused by land use change and cultivation also result in decline in microbial biodiversity (Degens et al., 2000; Buckley and Schmidt, 2001; Torsvik et al., 2002; Gomez et al., 2004; Nsabimana et al., 2004).

A question that has been raised by soil ecologists regarding the decline in soil microbial diversity is whether it will affect the stability of the resulting communities to continue performing their ecological functions. One body of opinion holds that species richness is not an issue, because the diversity of the soil microbial genebank is generally so high that microorganisms can always play their full part in ecosystem functions (Andrén and Balandreau, 1999). Indeed, the employment of molecular techniques has shown that the number of microbial species in soil is much higher than shown by studies based on cultivation techniques. For example, 1 g of forest

soil can contain as many as 4000 different genetically distinct units ("species") (Torsvik et al., 1990), and some 350 and 8000 distinct units can be found in sandy soil and organic soils, respectively (Øvreås and Torsvik, 1998). The molecular evidence is supported by direct enumeration of cells from the soil matrix by epifluorescence microscopy, which generally give 100-1000 times more cells than the number obtained by plate counting (Johnsen et al., 2001).

Conversely, it has been speculated that high biodiversity may be vitally important in structurally diverse ecosystems such as soil because it may promote stability of this environment (Grime, 1997; van Bruggen and Semenov, 2000). Because soil is an environment subject to fluctuations, establishment of an unfavorable environmental condition can result in the inhibition of some populations that perform essential functions. In highly diverse communities, however, there is a higher probability of the occurrence of quiescent microorganisms that could perform the same functional role but having different exigencies regarding physical, chemical and biological factors (van Bruggen and Semenov, 2000). However, the central question is how widely distributed are specific ecological functions among the soil microbial population. In fact, some functions are more general and performed by a greater number of species than others. For example, one could expect that many soil microbial species are capable of degrading cellulose due to its abundance and importance as source of carbon and energy in soil environments. In contrast, fewer species are expected to degrade specific anthropogenic-made compounds such as pesticides. This idea has been corroborated by studies demonstrating that the effect

of stress or disturbance on the functional stability of soils depends on the level of specificity of the function. For example, broad scale functions such as decomposition of organic matter are generally not affected by declines in microbial diversity, whereas more specialized niche functions such as nitrification, denitrification, methane oxidation, and mineralization of xenobiotics decrease as biodiversity decreases (Griffiths et al., 2000; Girvan et al., 2005). A few studies have also shown that cultivated soils that have undergone losses of soil organic matter showed reduced catabolic diversity (Degens et al., 2001) or functional stability (Griffiths et al., 2001) compared to paired undisturbed sites when submitted to further physical and chemical stresses simulated in laboratory.

Linking soil degradation with changes in microbial communities

Although several studies have shown the effects of land use change and agriculture practices on the composition and diversity of soil microbial communities (Lupwayi et al., 1998; Gomez et al., 2000; Buckley and Schmidt, 2001; Gomez et al., 2004; Nsabimana et al., 2004), the link between degradation of soil physical and chemical properties and changes in microbial diversity and composition have not been properly established. This is mainly because the observational nature of those studies were not based on replicated field trials, or because the effects of changes in soil quality on microbial communities were confounded by non-edaphic factors such as vegetation composition. For instance, the soils being compared differed in vegetation types, diversity of plants, or had been exposed to different histories of

chemical applications in the form of fertilizers, pesticides, and herbicides (Degens et al., 2000; Gomez et al., 2000; Yan et al., 2000; Gomez et al., 2004; Gros et al., 2004; Nsabimana et al., 2004; Bossio et al., 2005). Considering that these factors can have a major influence on soil microbial diversity and composition (Smalla et al., 2001; Berg et al., 2002; Kowalchuk et al., 2002; Rousseaux et al., 2003; Grayston et al., 2004; Hackl et al., 2004), it is difficult to ascertain whether changes observed were due to soil disturbances, or to differences in management history and vegetation composition.

Soil quality measurements based on biological and biochemical soil properties

Given the high sensitivity of soil microbial communities and their activity in soil to disturbances, biochemical and microbiological soil characteristics have been widely proposed as indicators of soil quality (Dick et al., 1996; Riffaldi et al., 2002; Miralles et al., 2007). Conversely, the use of these properties as indicators has been increasingly criticized because they are vulnerable to temporal and spatial variation (Nannipieri, 1994; Murphy et al., 1998; Corre et al., 2002; Chen et al., 2003; Gil-Sotres et al., 2005). This fact prevents the definition of reference values for these properties which are necessary to assess the magnitude of soil degradation relative to its previous state. In addition, soil biological/biochemical properties show complex and contradictory behaviors when soils were disturbed or polluted by different types of contaminants (Trasar-Cepeda et al., 2000; Gianfreda et al., 2005).

A previous study suggested that the organic matter content of undisturbed native soils under climax vegetation is in equilibrium with biological and biochemical properties (Trasar-Cepeda et al., 1998). In addition, this equilibrium was shown to be robust to natural spatial and temporal variation in those soil properties. A model fitted through multiple regression analyses expressed such equilibrium in an equation able to explain 97% of the total soil N based on measurements of microbial biomass C, N mineralization capacity, and activities of phosphatase, β-glucosidase and urease. Follow up studies demonstrated that the ratio between the total N calculated from the model (Nc) and that measured by Kjeldahl digestion (Nk) consistently predicted the intensity of soil degradation or disturbance in soils affected by management or different sources of pollution (Leiros et al., 1999; Trasar-Cepeda et al., 2000). Despite these promising results, the lack of similar studies undertaken in other soils raises the question of whether other undisturbed soils express similar equilibrium between soil organic matter and biological/biochemical properties, and whether the disruption of this equilibrium can be used as a consistent index of soil quality.

Thesis objectives

The objectives of this thesis were to evaluate the responses of soil microbial communities to physical and chemical disturbances, and associate these responses with soil functional stability and changes in indicators of soil quality. These objectives were reached in three distinct studies described in chapters 2, 3, and 4.

Chapter 2 describes an experiment carried out under laboratory conditions where soil samples collected from a tropical forest and an adjacent agricultural site that had been cultivated for 14 years were compared in regard to their functional stability after being submitted to heat shocks of 40, 50, 60 and 70^{o}C for 15 minutes. It was hypothesized that the long-term disturbances caused by soil cultivation and the resulting negative impact on soil properties would culminate in lower functional stability of the associated microbial communities. Functional stability was estimated by periodical measurements of the activities of three soil enzymes (hydrolysis of fluorescein diacetate, cellulase and laccase) in parallel with changes in soil microbial community composition using PLFA profiling. This experiment constituted the first study to compare the functional stability of forest soils that were brought into long-term cultivation with their uncultivated controls.

Chapter 3 describes an experiment to generate a gradient of induced soil degradation (GRIND) through application of an increasing number of tillage events to a 12-year-old fallow area in Northeastern Brazil. In contrast to previous studies, GRIND provided appropriate control of non-edaphic variables to allow the establishment of unbiased associations between the degradation of soil physical and chemical properties with microbial diversity and composition. Two months after the disturbances, changes in microbial community structure were evaluated using terminal restriction fragment polymorphism (T-RFLP) and phospholipid fatty acid (PLFA) analyses, and changes in physiological potential were evaluated measuring community level physiological profiles (CLPP) with Biolog EcoPlates^{TM}.

Chapter 4 describes a study to test the hypothesis that an equilibrium exists between soil organic matter and biological/biochemical properties in undisturbed soils from the H.J. Andrews Experimental Forest (HJA) situated in the Western Cascades of Oregon. Soil samples from the organic and mineral horizons of four old-growth and five second-growth sites were collected during the Spring/2005 and Fall/2006. Several biological and biochemical soil variables analyzed from these soils were subjected to multiple regression analysis and fit to a predictive model for soil organic C. In analogy with the Nc/Nk ratio proposed by Trasar-Cepeda et al., (1998), the validity of the ratio between soil C predicted by the model (C_p), and soil C measured (C_m) was evaluated as a soil quality index. This was accomplished by accessing changes of C_p/C_m in an undisturbed soil from HJA submitted either to chemical stresses (Cu and pH), or to physical disturbances (freeze-thaw and wet-dry cycles).

References

Andrén, O., Balandreau, J., 1999. Biodiversity and soil functioning - from black box to can of worms? Applied Soil Ecology 13, 105-108.

Berg, G., Roskot, N., Steidle, A., Eberl, L., Zock, A., Smalla, K., 2002. Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different Verticillium host plants. Applied and Environmental Microbiology 68, 3328-3338.

Borneman, J., Triplett, E.W., 1997. Molecular microbial diversity in soils from eastern Amazonia: Evidence for unusual microorganisms and microbial population shifts associated with deforestation. Applied and Environmental Microbiology 63, 2647-2653.

Bossio, D.A., Girvan, M.S., Verchot, L., Bullimore, J., Borelli, T., Albrecht, A., Scow, K.M., Ball, A.S., Pretty, J.N., Osborn, A.M., 2005. Soil microbial community response to land use change in an agricultural landscape of Western Kenya. Microbial Ecology 49, 50-62.

Buckley, D.H., Schmidt, T.M., 2001. The structure of microbial communities in soil and the lasting impact of cultivation. Microbial Ecology 42, 11-21.

Chen, C.R., Xu, Z.H., Blumfield, T.J., Hughes, J.M., 2003. Soil microbial biomass during the early establishment of hoop pine plantation: seasonal variation and impacts of site preparation. Forest Ecology and Management 186, 213-225.

Corre, M.D., Schnabel, R.R., Stout, W.L., 2002. Spatial and seasonal variation of gross nitrogen transformations and microbial biomass in a Northeastern US grassland. Soil Biology & Biochemistry 34, 445-457.

Davidson, E.A., Ackerman, I.L., 1993. Changes in soil carbon inventories following cultivation of previously untilled soils. Biogeochemistry 20, 161-193.

Degens, B.P., Schipper, L.A., Sparling, G.P., Duncan, L.C., 2001. Is the microbial community in a soil with reduced catabolic diversity less resistant to stress or disturbance? Soil Biology & Biochemistry 33, 1143-1153.

Degens, B.P., Schipper, L.A., Sparling, G.P., Vojvodic-Vukovic, M., 2000. Decreases in organic C reserves in soils can reduce the catabolic diversity of soil microbial communities. Soil Biology & Biochemistry 32, 189-196.

Dick, R.P., Breakwell, D.P., Turco, R.F., 1996. Soil enzyme activity and biodiversity measurements as integrative microbiological indicators. In: Doran, J.W., Jones, A.J. (Eds.), Methods for assessing soil quality. SSSA, Madison, pp. 247-271.

Gianfreda, L., Rao, M.A., Piotrowska, A., Palumbo, G., Colombo, C., 2005. Soil enzyme activities as affected by anthropogenic alterations: intensive agricultural practices and organic pollution. Science of the Total Environment 341 265- 279.

Gil-Sotres, F., Trasar-Cepeda, C., Leiros, M.C., Seoane, S., 2005. Different approaches to evaluating soil quality using biochemical properties. Soil Biology & Biochemistry 37, 877-887.

Girvan, M.S., Campbell, C.D., Killham, K., Prosser, J.I., Glover, L.A., 2005. Bacterial diversity promotes community stability and functional resilience after perturbation. Environmental Microbiology 7, 301-313.

Gomez, E., Bisaro, V., Conti, M., 2000. Potential C-source utilization patterns of bacterial communities as influenced by clearing and land use in a vertic soil of Argentina. Applied Soil Ecology 15, 273-281.

Gomez, E.D., Garland, J.L., Roberts, M.S., 2004. Microbial structural diversity estimated by dilution-extinction of phenotypic traits and T-RFLP analysis along a land-use intensification gradient. FEMS Microbiology Ecology 49, 253-259.

Grayston, S.J., Campbell, C.D., Bardgett, R.D., Mawdsley, J.L., Clegg, C.D., Ritz, K., Griffiths, B.S., Rodwell, J.S., Edwards, S.J., Davies, W.J., Elston, D.J., Millard, P., 2004. Assessing shifts in microbial community structure across a range of grasslands of differing management intensity using CLPP, PLFA and community DNA techniques. Applied Soil Ecology 25, 63-84.

Griffiths, B.S., Bonkowski, M., Roy, J., Ritz, K., 2001. Functional stability, substrate utilisation and biological indicators of soils following environmental impacts. Applied Soil Ecology 16, 49-61.

Griffiths, B.S., Ritz, K., Bardgett, R.D., Cook, R., Christensen, S., Ekelund, F., Sørensen, S.J., Bååth, E., Bloem, J., de Ruiter, P.C., Dolfing, J., Nicolardot, B., 2000. Ecosystem response of pasture soil communities to fumigation-induced microbial diversity reductions: an examination of the biodiversity-ecosystem function relationship. Oikos 90, 279-294.

Grime, J.P., 1997. Biodiversity and ecosystem function: the debate deepens. Science 277, 1260-1261.

Gros, R., Monrozier, L.J., Bartoli, F., Chotte, J.L., Faivre, P., 2004. Relationships between soil physico-chemical properties and microbial activity along a restoration chronosequence of alpine grasslands following ski run construction. Applied Soil Ecology 27, 7-22.

Hackl, E., Zechmeister-Boltenstern, S., Bodrossy, L., Sessitsch, A., 2004. Comparison of diversities and compositions of bacterial populations inhabiting natural forest soils. Applied and Environmental Microbiology 70, 5057-5065.

Islam, K.R., Weil, R.R., 2000. Land use effects on soil quality in a tropical forest ecosystem of Bangladesh. Agriculture, Ecosystems & Environment 79, 9-16.

Johnsen, K., Jacobsen, C.S., Torsvik, V., Sorensen, J., 2001. Pesticide effects on bacterial diversity in agricultural soils - a review. Biology and Fertility of Soils 33, 443-453.

Kowalchuk, G.A., Buma, D.S., de Boer, W., Klinkhamer, P.G.L., van Veen, J.A., 2002. Effects of above-ground plant species composition and diversity on the diversity of soil-borne microorganisms. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 81, 509-520.

Leiros, M.C., Trasar-Cepeda, C., Garcia-Fernandez, F., Gil-Sotres, F., 1999. Defining the validity of a biochemical index of soil quality. Biology and Fertility of Soils 30, 140-146.

Lupwayi, N.Z., Clayton, G.W., Rice, W.A., 1998. Soil microbial diversity and community structure under wheat as influenced by tillage and crop rotation. Soil Biology & Biochemistry 30, 1733-1741.

Miralles, I., Ortega, R., Sánchez-Marañón, M., Leirós, M.C., Trasar-Cepeda, C., Gil-Sotres, F., 2007. Biochemical properties of range and forest soils in Mediterranean mountain environments. Biology and Fertility of Soils 43, 721-729.

Murphy, D.V., Fillery, I.R.P., Sparling, G.P., 1998. Seasonal fluctuations in gross N mineralisation, ammonium consumption, and microbial biomass in a Western Australian soil under different land uses. Australian Journal of Agricultural Research 49, 523-535.

Nannipieri, P., 1994. The potential use of soil enzymes as indicators of productivity, sustainability and pollution. In: Pankhurst, C.E., Double, B.M., Gupta, V.V.S.R., Grace, P.R. (Eds.), Soil Biota: Managment in Sustainable Farming Systems. CSIRO, Melbourne, pp. 238-244.

Nsabimana, D., Haynes, R.J., Wallis, F.M., 2004. Size, activity and catabolic diversity of the soil microbial biomass as affected by land use. Applied Soil Ecology 26, 81-92.

Oldeman, L.R., 1994. The global extent of soil degradation. In: Greenland, D.J., Szabolcs, I. (Eds.), Soil resilience and sustainable land use. CAB International, Wallingford, UK, pp. 99-118.

Øvreås, L., Torsvik, V., 1998. Microbial diversity and community structure in two different agricultural soil communities. Microbial Ecology 36, 303-315.

Qafoku, N.P., Van Ranst, E., Noble, A., Baert, G., 2003. Mineralogy and chemistry of variable charge soils. In: Lal, R. (Ed.), Encyclopedia of Soil Science. Marcel Dekker, New York.

Riffaldi, R., Saviozzi, A., Levi-Minzi, R., Cardelli, R., 2002. Biochemical properties of a Mediterranean soil as affected by long-term crop management systems. Soil & Tillage Research 67, 109-114.

Rousseaux, S., Hartmann, A., Rouard, N., Soulas, G., 2003. A simplified procedure for terminal restriction fragment length polymorphism analysis of the soil bacterial community to study the effects of pesticides on the soil microflora using 4,6-dinitroorthocresol as a test case. Biology and Fertility of Soils 37, 250-254.

Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., Roskot, N., Heuer, H., Berg, G., 2001. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: Plant-dependent enrichment and seasonal shifts revealed. Applied and Environmental Microbiology 67, 4742-4751.

Solomon, D., Lehmann, J., Zech, W., Fritzsche, F., Tekalign, M., 2002. Soil organic matter composition in the subhumid Ethiopian highlands as influenced by deforestation and agricultural management. Soil Science Society of America Journal 66, 68-82.

Swift, M.J., Kjoller, A., Ekschmitt, K., Smith, P., Loiseau, P., Brussaard, L., Andren, O., Couteaux, M.M., Briones, M., 1998. Global change, soil biodiversity, and nitrogen cycling in terrestrial ecosystems: three case studies. Global Change Biology 4, 729-743.

Torsvik, V., Goksøyr, J., Sorheim, R., Salte, K., 1990. Comparison of phenotypic diversity and DNA heterogeneity in population of soil bacteria. Applied and Environmental Microbiology 56, 776-781.

Torsvik, V., Ovreas, L., Thingstad, T.F., 2002. Prokaryotic diversity - Magnitude, dynamics, and controlling factors. Science 296, 1064-1066.

Trasar-Cepeda, C., Leiros, C., Gil-Sotres, F., Seoane, S., 1998. Towards a biochemical quality index for soils: An expression relating several biological and biochemical properties. Biology and Fertility of Soils 26, 100-106.

Trasar-Cepeda, C., Leiros, M.C., Seoane, S., Gil-Sotres, F., 2000. Limitations of soil enzymes as indicators of soil pollution. Soil Biology & Biochemistry 32, 1867-1875.

van Bruggen, A.H.C., Semenov, A.M., 2000. In search of biological indicators for soil health and disease suppression. Applied Soil Ecology 15, 13-24.

Waldrop, M.P., Balser, T.C., Firestone, M.K., 2000. Linking microbial community composition to function in a tropical soil. Soil Biology & Biochemistry 32, 1837-1846.

Yan, F., McBratney, A.B., Copeland, L., 2000. Functional substrate biodiversity of cultivated and uncultivated A horizons of vertisols in NW New South Wales. Geoderma 96, 321-343.

Land use impact on the stability of soil microbial community composition and enzyme activities to heat shock

Chapter 2

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Abstract

In this study, we investigated how changes in land use affect the response of soil microbial community composition and enzyme activities (resistance and resilience) to a heat shock disturbance (HS). Mineral soil samples from a tropical forest (FST) and an adjacent agricultural site (AGR) that had been cultivated for 14 years were analyzed for changes in physical, chemical and biological properties. In addition, these soils were incubated at 40, 50, 60 or 70^{o}C for a period of 15 min. Three and 30 days after the HS, fluorescein diacetate (FDA) hydrolysis, cellulase and laccase activities, and PLFA-based microbial community composition were measured. The AGR soil had a different microbial community composition, and lower microbial biomass, enzyme activity, total and particulate soil organic carbon, aggregate stability, and aggregate weighed mean diameter compared to the FST soil. Heat shock treatments affected differently the enzymatic activity and the microbial composition of the two soils. FDA hydrolysis activity was less affected by HS in the FST soil (more resistance), and cellulase and laccase activities recovered more rapidly from HS (more resilience) in FST relative to AGR. In the AGR soil, laccase activity did not show resilience to any HS level up to 30 days after the disturbance. Within each soil type, the microbial community composition did not differ between HS and control samples at day 3. However, at day 30, FST samples treated at 60 and 70^{o}C contained a microbial community significantly different from the control and with lower biomass regardless of high enzyme resilience. Our results suggest lower functional stability in AGR soil relative to its FST counterpart. Lower resilience of

laccase and cellulase activities in the AGR soil could be the result of a less diverse community of microorganisms capable of producing these enzymes.

Keywords

Disturbance; Soil degradation; Functional stability; Microbial diversity; Microbial community; PLFA; Laccase; Cellulase; Fluorescein diacetate hydrolysis

Introduction

The removal of natural vegetation followed by cultivation can cause severe changes to physical, chemical, and biological soil properties. These changes are often associated with reduction of soil organic matter, deterioration of soil structure, and decreases in microbial biomass and activity (Islam and Weil, 2000; Waldrop et al., 2000; Solomon et al., 2002a; Solomon et al., 2002b; Dinesh et al., 2004; Bossio et al., 2005; Nourbakhsh, 2007). Many studies have also shown that deforestation and soil cultivation alter soil microbial community structure (Borneman and Triplett, 1997; Nusslein and Tiedje, 1999; Waldrop et al., 2000; Bossio et al., 2005), and may lead to reduction in microbial biodiversity (Degens et al., 2000; Buckley and Schmidt, 2001).

It is uncertain how deterioration of soil properties and changes in microbial communities affect the functional stability of soils. Stability comprises both resistance, i.e. the ability to withstand a perturbation or stress, and resilience, i.e. the time required to recover to pre-perturbation levels (Griffiths et al., 2000). It has been hypothesized that soil functional stability is primarily generated by the inherent

functional redundancy present in the microbial diversity (Griffiths et al., 2000; van Bruggen and Semenov, 2000; Degens et al., 2001; Griffiths et al., 2004). This hypothesis is referred to as the ‘‘insurance hypothesis of biodiversity’’ (Naeem and Li, 1997; Loreau, 2000), and originally was proposed for macroorganisms (MacArthur, 1955; Ehrlich and Ehrlich, 1981).

Few studies have tried to validate the insurance hypothesis in soil environments (Griffiths et al., 2000; Griffiths et al., 2001b; Girvan et al., 2005). Overall, the data indicate that the effect of stress or disturbance on the functional stability of soils depends on the level of specificity of the function. For example, broad scale functions such as decomposition of organic matter are generally not affected by microbial diversity, whereas more specialized niche functions such as nitrification, denitrification, methane oxidation, and mineralization of xenobiotics decrease as biodiversity decreases (Griffiths et al., 2000; Girvan et al., 2005).

Studies investigating the effects of soil cultivation on functional stability are scarce. Degens et al. (2001) showed that an arable soil with low catabolic diversity and organic C was less resistant to stress (low pH, high salinity, heavy metal contamination) or disturbance (wet-dry or freeze-thaw cycles) than a similar soil type under pasture, where catabolic diversity and organic C were higher. In another study, Griffiths et al. (2001) found greater functional stability (short-term decomposition of plant residues) to copper and heat stresses in a grassland soil and an organically managed agricultural soil, than in an intensively managed soil.

To date we are unaware of any studies that compared functional stability of forest soils that were brought into long-term cultivation with their uncultivated controls. In this work, our objective was to evaluate how deforestation, followed by long-term cultivation, affected the stability of the microbial communities and their functions when this soil was subjected to heat shock disturbances. Transient heat shocks were applied over a range of temperatures (40 to 70^{o}C) to soil samples from a natural forest site and from an adjacent agricultural site that had been brought into cultivation with row crops for 14 years. We measured the functional stability of a "broad-scale" soil enzyme (hydrolysis of fluorescein diacetate) and two "narrow niche" soil enzymes (cellulase and laccase) in parallel with changes in soil microbial community composition using PLFA profiling. We hypothesized that long-term disturbance caused by soil cultivation and the resulting negative impacts on soil quality would culminate in lower functional stability of the associated microbial communities.

Materials and methods

Soils

The study soils represent fine-loamy, kaolinitic, isohyperthermic Typic Fragiudults collected from a Brazilian Atlantic Forest fragment (FST) and from an adjacent agricultural field (AGR) that were less than 200 m apart. Both sites are located in the Umbaúba Experimental Station of the Embrapa Coastal Tablelands in Sergipe State, Northeastern Brazil (11^{o} 16' S, 37^{o} 26' W, 105 m altitude; 1350 mm of

annual rainfall; 24.3^{o}C mean daily temperature). The FST is about 700 x 300 m in dimension and has been isolated from anthropogenic intervention for more than 50 years. The site is occupied mainly by mid-successional tree species and shrubs. During the 14 years preceding the soil sampling, AGR was in continuous cultivation of single cropped corn (*Zea mays*) under conventional tillage (one moldboard plowing and two diskings every year) from 1991 through 2000, and cultivated with intercropped corn and pigeon pea (*Cajanus cajan*) under zero-tillage from 2001 through 2004.

At each site, forty random soil cores were collected from 0 to 5 cm depth and pooled to form one composite sample. Samples were passed through a 4-mm sieve, air dried, and stored at 4^{o}C. Soil pH was measured in a slurry of soil and water (1:2.5). Total organic carbon (TOC) was quantified by ignition in a LECO WR-12 C auto-analyzer (LECO Corp., St. Joseph, Missouri). A 25-g soil subsample was used to obtain particulate organic C by a procedure adapted from Cambardella and Elliot (1992). The soil was dispersed in 100 ml of sodium metahexaphosphate (5 g l^{-1} pH 9), because it improved dispersion of colloids in this low-pH soil. Aggregates were disrupted by agitation of soil suspensions in a rotatory shaker (250 rpm) for 8 h. The slurry was passed through a 0.106-mm sieve, the portion retained was washed with excess distilled water, and dried at 60^{o}C for 24 h. The TOC in the dried fraction was determined as described above.

Extractable P (Mehlich-1) was determined following the procedure of Braga and Defelipo (1974). Water stable aggregates (WSA) were determined according to

Nimmo and Perkins (2002) using a single-sieve wet-sieving apparatus with a 0.106-mm sieve and NaOH (2 g l^{-1}) as dispersing agent. The weighed mean diameter of soil aggregates (Kemper and Rosenau, 1986) was determined after sorting the aggregates by dry sieving (Mendes et al., 1999). Soil texture was determined by the Bouyoucos densimeter method with hexametaphosphate as the dispersing agent (Bouyoucos, 1927).

Heat shock treatments

Soil water content was adjusted to 60% of the water holding capacity (WHC), and each soil type was divided into 40 portions, each equivalent to 11 g (wet weight), and placed into 25-ml flasks. This provided 80 experimental units for 4 replicates of 5 disturbance treatments, and two dates of evaluation for each soil. Samples were pre-incubated for 7 days at 25^{o}C in a dark room before treatment application. HS treatments were carried out after the pre-incubation period by exposing the FST and AGR soils to 40, 50, 60 or 70^{o}C (plus a control, kept at 25^{o}C). For each soil type, 8-flask subsets were partially immersed in a heated water bath. The total period of immersion equaled the sum of the time for the soil temperature to reach 95% of the nominal temperature of the treatment (e.g., for the water bath set at 40^{o}C, the soil sample took 3 minutes to reach 38^{o}C) (*time A*) and a constant period of 15 min (*time B*). *Time A* was previously determined in a parallel set of flasks using a thermometer with its bulb buried in the soil, and ranged from 3 to 5 min irrespective of the temperature treatment and soil type. After the HS treatments, the flasks were cooled

to room temperature and incubated at 25°C. Flasks were covered with plastic film pierced with a needle to minimize the water loss during the incubation. Every 3 days the soil water content was adjusted to its initial content (60% WHC).

Determination of biological properties

Soil respiration rates were monitored at 0.7, 1.2, 1.7 and 3 days after HS. Flasks were capped with a rubber septum and the air in the overhead space sampled from four replicates of each HS treatment. CO_2 was determined using a gas chromatograph equipped with a thermal conductivity detector (Carle Series 100 AGC, Loveland, CO).

The effect of HS on the microbial community structure was measured by analyzing the phospholipid fatty acid patterns (PLFA) at 3 and 30 days after HS treatments. The first sampling date (day 3) corresponded to the time taken for respiration to reestablish at a stable rate, after the initial flush of CO_2 induced by the HS. We assumed that the PLFA content of cells killed by the HS would have been turned over during the initial CO_2 flush and that PLFA profiles represent the composition of the resistant viable microbial biomass that survived the HS. After homogenization, 3 g of soil were sampled from each of four replicates per treatment. Extraction procedures and reagents for the PLFA analysis were similar to those described by Butler et al. (2003).

The summed mol% values of FAMEs reported as typical of fungi, Gram-negative (GN) bacteria, Gram-positive (GP) bacteria, actinomycetes (ACT) and

arbuscular mycorrhizal fungi (AMF) were used as signatures for these microbial groups (Olsson et al., 1995; Frostegård and Bååth, 1996; White et al., 1997; Zelles, 1999). The amount of FAME 16:0 (pmol g^{-1} soil) was used as an estimate of microbial biomass (Zelles et al., 1992).

Three soil enzymes were analyzed in the soil remaining in the flasks. Hydrolysis of fluorescein diacetate (FDA) was determined by incubating soil samples for 2 h (37^{o}C) and determining fluorescein released according to Schnürer and Rosswall (1982), and modified by Dick et al. (1996). Laccase was extracted from soil samples according to Lang et al. (1997) and its activity measured by the oxidation of ABTS (2,2'-azino-di(3-ethylbenzothiazoline-6-sulfonic acid)) (Niku-Paavola et al., 1990). Activity of cellulase was determined by incubating 1 g of moist soil at 50^{o}C, for 24 h with 10 ml of 2 M acetate buffer (pH 5.5) containing carboxymethyl cellulose sodium salt (0.7% w/v) and the reducing sugars released were determined with alkaline potassium hexacyanoferrate (III) at 690 nm (Schinner and von Mersi, 1990). Analytical triplicates were used for each soil sample. A mixture of soil and buffer, without substrate, was prepared as a blank for each sample. Results were calculated as μg glucose released g^{-1} soil h^{-1}.

Data Analysis

Patterns of community composition represented by the relative abundance of microbially-derived PLFA were graphically displayed using non-metric multidimensional scaling (NMS) (Kruskal, 1964; Mather, 1976) and Sørensen distance (Bray and Curtis, 1957). NMS was performed using PC-ORD statistical

package, V5 beta (B. McCune and M.J. Mefford, PC-ORD for Windows: multivariate analysis of ecological data, MjM Software, Gleneden Beach, OR) and a matrix that consisted of 80 soil samples (5 HS treatments x 2 soil types x 2 sampling periods x 4 replicates) and 38 FAMEs. The 38 FAMEs only included peaks in the region between tetradecanoic methyl ester (14:0) and arachidonic acid (20:4ω6c). FAMEs that contributed less than 5% of the fatty acids of all samples were excluded from the data matrix.

To simplify the ordination display, average NMS scores for each treatment were plotted. Statistical differences in the microbial community composition were analyzed by sum of squares multivariate regression tree (SS-MRT) models (De'ath, 2002). The scores from the 2 NMS axes were used as response variables; soil type, HS and sampling date were used as explanatory variables. A series of 20, 10-fold cross-validations was run to choose the modal tree size with minimum error rate (Breiman et al., 1984; De'ath and Fabricius, 2000). Because these trees were generally too large to interpret, the 1-SE rule was used, and the best tree was considered to be the smallest tree with an estimated error within one standard error of the minimum (Breiman et al., 1984). A library of SS-URT routines (T. Therneau, unpublished data), extended by the inclusion of additional C routines to fit multivariate regression trees (De'ath, 2002), was used in S-Plus 6.0 (Insightful Corp.) for the SS-MRT analysis.

Separate NMS ordinations were performed with PLFA data from each soil type to characterize changes in microbial communities using overlay gradients of

microbial groups. This allowed us to evaluate the relative changes in microbial composition due to treatments without interference by the original microbial composition of each soil type.

The values of enzymatic activity, soil respiration, and PLFA 16:0-based microbial biomass for all HS treated soils were transformed according to the following equation, in order to express the percent of change relative to the respective control in each sampling period:

$$\%\ change = \left[\left(\frac{T_i}{C_i}\right) - 1\right] \times 100$$

where T_i is the variable value in the treated soil sample at sampling time i, and C_i is the average of the values obtained for the four control replicates of each sampling time i. The transformed values were used to evaluate the stability (resistance and resilience) of the two soils to the HS treatments using the linear regression procedure (S-Plus) with soil type and sampling time as categorical variables and HS temperature as quantitative variable. When the relationships between the variables and HS levels were not linear, a three way ANOVA was used instead.

Results

Characterization of the forest and agricultural soils

Several physical and chemical properties differed between the forest (FST) and agricultural (AGR) soils. Fourteen years of cultivation had decreased total organic carbon (-37%), particulate organic C (-39%), and water stable aggregates (-13%) in the AGR soil relative to the FST soil (Table 2.1). Although the AGR soil

had a lower pH and smaller aggregate mean diameter (-18%) than FST soil, these differences were only marginally significant ($p < 0.10$).

Among the biological properties, the AGR soil expressed lower respiration (-40%), microbial biomass (FAME 16:0 equivalent) (-41%), and activities of laccase (-44%), cellulase (-20%) and hydrolysis of fluorescein diacetate (FDA) (-18%) (Table 2.1). The two soils also contained different microbial community structures based on PLFA profiles. Accordingly, the microbial community structure of the FST soil was richer in the PLFA marker for arbuscular mycorrhizal fungi (16:1ω5c) and Gram-negative bacteria (18:1ω7c; 17:0cy; 19:0cy), whereas the AGR soil was richer in actinomycetes (16:0 10-Me; 17:0 10-Me; 18:0 10-Me) and fungi (18:2ω6c). There were no differences in the relative abundance of Gram-positive bacteria, or in the ratio of fungi:bacteria determined from the abundance of PLFA markers.

Soil respiration after HS

Heat shock (HS) treatments affected short-term respiration rates in both soils (Figure 2.1). Sixteen hours after the heat treatment, FST treated at 50, 60 and 70^{o}C showed significant increases in respiration above the unheated control soil of about 50, 230, and 430%, respectively. These same temperature treatments increased the respiration rates in AGR by 51, 145, and 536%, respectively, relative to controls incubated at 25^{o}C. The respiration rates of soils treated at 40^{o}C did not differ from controls 16 h after HS, but the rate was 32 % higher in AGR 28 h after HS. Despite

the elevated flushes of CO_2 from HS treated soils, 3 d after HS the respiration rates of all treated soils were equal to or less than the controls (Figure 2.1).

Stability of soil enzymes to HS

To compare the functional stability (resistance and resilience) of AGR and FST soils, we evaluated fluorescein diacetate hydrolysis, cellulase and laccase activities 3 and 30 days after the HS treatments. Resistance was evaluated as the percent of change in HS treated samples relative to the control at day 3. Although laccase and cellulase activities were equally resistant in both soils (Figure 2.2A, B), they differed in their degree of sensitivity to HS. For instance, the lower HS temperature (40^{o}C) caused decreases of 19% and 11% in cellulase and laccase activities, respectively. The activities of both enzymes declined at the same rate (11%) in response to further incremental increases of 10^{o}C in HS temperature up to 70^{o}C. By contrast, the FDA activity was more resistant in FST than in AGR soil. Although the activity in AGR was unaffected by 40^{o}C, this same treatment increased the activity in FST by 5% (p<0.05) (Figure 2.2C), and this relative difference persisted throughout the temperature range evaluated. Heat shock above 40^{o}C severely affected the FDA activities in both soils, which were reduced by 18% for each 10^{o}C of increments in temperature.

The functional resilience of the two soils was assessed by monitoring the shifts in enzyme activities toward the pre-HS condition that occurred between 3 and 30 days post-HS. Each enzyme had a unique resilience pattern, which depended on

soil type. The FDA activity from the FST and AGR soils showed similar resilience patterns, with the percent change between 3 to 30 days being equivalent regardless of temperature (Figure 2.2C). However, this resilience was only expressed in response to the two highest HS temperatures (60 and 70^{o}C). Laccase activity was resilient only in FST where the enzyme activity significantly shifted toward its pre-HS condition between 3 and 30 days (Figure 2.2A).

Cellulase was the most resilient of the three enzymes especially in FST (Figure 2.2B). Thirty days after HS, the cellulase activity in FST treated at 40^{o}C was only 6% lower ($p<0.05$) than the controls; a recovery of almost 70% of the relative decrease observed 3 days after HS. At higher HS temperatures, the recovery was even higher and inversely proportional to the loss of activity that occurred in response to HS. Cellulase resilience in AGR was expressed only in samples treated above 50^{o}C. The difference in the percentage of relative change between the two soils was equal to 13% and remained constant throughout the HS range.

Stability of microbial community to HS

Heat shock treatments affected microbial biomass (MB) to a similar degree in both soils (Figure 2.3). Although MB in soils treated at 40 and 50^{o}C were not significantly different from controls, samples treated at 60 and 70^{o}C decreased by 24% (SE = 7%). Thirty days after HS, MB in AGR remained at the same level found 3 days after disturbance, irrespective of HS level. In contrast, MB from FST was

drastically decreased by 76% (SE = 13%) relative to the control in samples treated at 60 and 70^{o}C.

The NMS ordination of sample units in the FAME space graphically illustrated the microbial community profiles at 3 and 30 days after the HS treatments (Figure 2.4). About 87% of the total variability in PLFA profiles was represented by a 2-D NMS plot (64% along axis 1, and 23% along axis 2). Statistical differences among treatments were defined by the SS-MRT diagram (Figure 2.5) which resulted in five groups (terminal nodes - TN) represented in the NMS plot by encircling treatments with similar microbial communities. Three days after HS, the structures of the microbial communities from both soils were not significantly altered by any of the HS treatments (Figure 2.4). However, changes were revealed in FST after 30 days of incubation, specially in samples treated at 60 and 70^{o}C. The overlay of microbial groups in the ordination of FST sample units revealed that changes in samples treated at 60 and 70^{o}C were mostly associated with decreases in arbuscular mycorrhizal fungi (AMF), fungi, and Gram-negative bacteria (Figure 2.6). The 30-day incubation also altered the microbial community in AGR, but no significant differences were observed between HS treated soils and the controls on the same sampling date. The overlay of microbial groups in the ordination of AGR sample units showed that, over the 30-day incubation period, there was a relative decrease in AMF, and, at a lower intensity, in fungi, Gram-positive and Gram-negative bacteria.

Discussion

Fourteen years of cultivation of the agricultural soil (AGR) caused degradation of several soil properties as indicated by decreases in total and particulate soil organic carbon, aggregate stability, aggregate weighed mean diameter, and microbial biomass and activity relative to the forest soil counterpart (FST) (Table 2.1). These results corroborate other studies that have demonstrated similar changes in soil properties following deforestation and soil cultivation (Islam and Weil, 2000; Solomon et al., 2002a; Solomon et al., 2002b; Bossio et al., 2005; Nourbakhsh, 2007). In addition, cultivation altered the soil microbial community composition (Table 2.1), a phenomenon that has also been reported in tropical soils after forest conversion to agriculture (Waldrop et al., 2000; Bossio et al., 2005) or to cultivated pastures (Borneman and Triplett, 1997; Nusslein and Tiedje, 1999). In the following sections we discuss how these changes in AGR soil properties altered the microbial community and its functional stability to the heat shock (HS) disturbances.

Effects of HS on soil microbial functional stability

Our results support the hypothesis of higher functional stability in the undisturbed FST compared with its AGR counterpart. This was indicated by the responses of the activities of three enzymes which were less affected (higher resistance) or recovered more quickly (higher resilience) in FST across the HS range. For instance, laccase activity in AGR did not show resilience to any HS level, whereas in FST there was a significant recovery (Figure 2.2A). In the case of

cellulase, both soils expressed resilience, but recovery was greater in FST (Figure 2.2B). The rate of return of cellulase activity was directly proportional to the magnitude of the HS and agrees with other studies that showed an induction of cellulase activity in response to different stress/disturbance factors (temperature, mineral salts, drying-rewetting, and combinations of these factors) in peat bogs (Nizovtseva and Semenov, 1995; Semenov and Nizovtseva, 1995).

In contrast to laccase and cellulase, FDA hydrolysis differed between soils in terms of its resistance to heat rather than its recovery rate. The higher resistance of FDA hydrolysis activity in FST might be associated with the high diversity of enzymes expressing this type of catalytic activity. It is also possible that thermal stability might reflect the protective effect of organic substances and the formation of organic clay-enzyme complexes (Kandeler, 1990; Huang et al., 2005). In contrast to FDA hydrolysis, cellulase and laccase activities were similarly reduced in the two soils in response to HS (Figure 2.2A,B). This fact may be due to differences between enzymes regarding their adsorption and complexation with soil colloids. For instance, it has been suggested that the major portion of exo- and endoglucanase activity is found in soil solution, or attached to the outer surfaces of cellulolytic microorganisms (Hope and Burns, 1987). Therefore, if the cellulase complex does not form organo-complexes, the higher SOC in FST may have had little or no effect on the stability of this enzyme. We have found no reports on the effect of soil organic colloids on the thermal stability of laccase. However, laccase adsorption to a

mineral colloid (aluminum hydroxide) had little effect on the thermal stability of this enzyme within the 40–60°C range (Ahn et al., 2007).

Although the resistance of enzymes to HS might depend on physico-chemical interactions with soil colloids, their resilience will depend on the survival or proliferation of microbial populations capable of producing new enzymes. The higher total SOC in FST does not seem to be the reason for the higher resilience observed in this soil. Besides possessing higher SOC, FST contained initially higher biomass and enzymatic activity (Table 2.1) which means that in order to show equal rates of resilience, FST must produce more enzymes in comparison with AGR. The ratio between microbial biomass and total SOC calculated from Table 2.1 showed only a small difference between soils (3.1 and 2.9 for FST and AGR, respectively), which suggests that the relative availability of resources to sustain microbial activity was similar among soils. This observation, in addition to the fact that laccase did not show any resilience in AGR, indicates that differences in resilience among the two soils might be more associated with differences in microbial diversity and composition than in resource availability.

It has been proposed that the higher the microbial diversity and its inherent functional redundancy, the faster the rate at which an ecosystem can return to a pre-stress state (van Bruggen and Semenov, 2000; Degens et al., 2001). Therefore, the absence of resilience of laccase activity and the lower resilience of cellulase activity in AGR compared to FST suggest that land use change caused a reduction in microbial diversity in this soil. This fact would also explain the similar resilience

pattern of FDA hydrolysis in both soils since it is used to assess the activity of a "broad-scale" group of enzymes including lipases, and esterases (Guilbault and Kramer, 1964; Green et al., 2006). In contrast, cellulase and laccase activities are "narrow niche" enzymes that represent more specific functions within the soil microbial community. Assuming an inverse relationship between the specificity of the enzyme and the number of microbial species able to produce them, it is less probable that an eventual decrease in microbial diversity in AGR would affect the resilience of FDA hydrolysis compared to laccase and cellulase. This idea is corroborated by several studies that show lower stability of narrow niche soil functions compared with broad scale functions when microbial diversity was reduced. For example, decreasing microbial diversity by progressive fumigation of soil did not affect or increase microbial growth and decomposition of plant residues but it did decrease more specific functions such as nitrification, denitrification, and methane oxidation (Griffiths et al., 2000). In another study, the addition of copper sulfate or benzene to soils with naturally differing levels of diversity (mineral and organo-mineral soils) did not affect the stability of a broad scale function (i.e. mineralization of ^{14}C-labelled wheat shoot), but the resilience of the narrow niche function (mineralization of ^{14}C-labelled 2,4-dichlorophenol) only persisted in the high diversity soil (Girvan et al., 2005).

Effects of HS on microbial community

Microbial community composition from soils assessed 3 d after HS did not show significant differences relative to controls (Figure 2.4), suggesting that the

microbial groups from each soil were equally sensitive to HS. After 30 days of incubation, the microbial community of AGR shifted toward a relatively more actinomycetes-enriched and Gram-positive-, Gram-negative-, fungi-, and arbuscular mycorrhizal fungi-depleted community (Figure 2.6). However, these changes were also observed in the control soils indicating that recovery incubation time, rather than HS per se, had the major influence on the microbial community composition of AGR. In contrast, microbial community composition of FST samples treated at 40 and 50^{o}C were quite stable showing no shift in composition due to HS treatment or incubation time (Figure 2.4). The same pattern was not observed in FST samples treated at 60 and 70^{o}C which had a significant shift in microbial composition between 3 and 30 days, mostly characterized by relative decreases in Gram-negative bacteria, fungi, and arbuscular mycorrhizal fungi (Figure 2.6). These differences suggest a threshold temperature between 50 and 60^{o}C above which representative microbial species from the community were eliminated due to their heat sensitivity, thus allowing other surviving species to establish a new community with a different structure. This is corroborated by the significant decrease in microbial biomass in FST samples treated at 60 and 70^{o}C, which reached levels only 20% of the controls at the end of the incubation period (Figure 2.3). Nevertheless, it is surprising that regardless of these changes, FST samples still showed resilience for all three enzymes that were generally higher than observed in AGR where a smaller decline of biomass was observed. This fact indicates that the biomass that persisted in FST samples treated at 60 and 70^{o}C was more active than the original biomass. This

increase in activity per unit of biomass was probably induced by an increase in availability of labile nutrients released from the dead biomass as well as by the relative reduction of the competition for resources. Wright et al. (1995) reported that the activity of bacterial populations grazed by *Colpoda steinii* was drastically increased relative to the ungrazed population, despite a significant decrease in bacterial biomass caused by grazing. The authors suggest that the activity of the large, ungrazed bacterial population was substrate-limited, and that the release of nutrients from the preyed cells resulted in a flush of activity by the bacteria that escaped predation. By analogy, we might suggest that nutrients released from cells killed by the HS would cause a similar flush in enzyme activity by the surviving microorganisms.

Conclusions

Change in land use from an undisturbed forest soil to long-term cultivation caused changes in soil properties and resulted in a new microbial community that was less functionally stable when the soil was disturbed by heat shocks. The reduced resilience of laccase and cellulase in AGR was probably the result of a limited diversity of microorganisms capable of producing these enzymes, rather than of the availability of resources. Moreover, the similar resilience of FDA hydrolysis activity in both FST and AGR as well as the lack of sensitivity of other broad-scale soil functions to stress/disturbance observed elsewhere corroborate the idea of a considerable functional redundancy of soil microorganisms. This is not surprising

when the enormous microbial diversity observed in soils is considered (Torsvik et al., 1990; Øvreås and Torsvik, 1998). Conversely, the sensitivity of narrow niche-functions observed here and in other previously stressed/disturbed soils suggests that specific functional parameters are more sensitive measurements for evaluating change in the ecological stability of soils. These results indicate that the consequences of species loss in stressed/disturbed soil environments may not be immediately visible, but may decrease the system stability to further disturbances, and make it more vulnerable to ecological failure.

Finally, it is important to point out that these results were obtained after using a single kind of disturbance (HS) to test the effect of previous soil use on its functional stability. Assuming that the ultimate consequence of any kind of disturbance or stress on soil microbial communities will be the elimination of sensitive species and adaptation of the remaining ones under a new structure, it might seem unlikely that different conclusions would be drawn if other kinds of disturbance factors were used. However, stress/disturbance-dependent responses have been recently demonstrated after evaluating the functional stability of heavy metal contaminated soils (Tobor-Kaplon et al., 2006). Therefore, other stress/disturbance factors should be investigated to compare the stability of forest vs. cultivated soils.

Acknowledgements

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Table 2.1 Physical, chemical and biological properties of soil samples (0-5 cm depth) collected under a tropical forest fragment and an adjacent no-till corn field in the Brazilian Coastal Tablelands. Values are means for 4 replicates ± standard deviation.

Soil variables	Forest site	Agricultural site
Physical		
Texture	sandy loam	sandy loam
Aggregate weighed mean diameter (mm)	0.88 ± 0.13	0.72 ± 0.10
Water stable aggregate (%)	96.7 ± 3.0	84.2 ± 4.0
Chemical		
pH (H_2O)	5.45 ± 0.17	5.19 ± 0.15
Total organic carbon (%)	1.64 ± 0.26	1.03 ± 0.06
Carbon from particulate organic matter (%)	0.31 ± 0.01	0.19 ± 0.03
Biological		
Soil respiration (μg C-CO_2 g^{-1} h^{-1})	0.25 ± 0.02	0.15 ± 0.01
Laccase activity (nmol ABTS azine cation g^{-1} min^{-1})	6.14 ± 1.20	3.41 ± 0.48
Cellulase activity (μg glucose g^{-1} h^{-1})	127.5 ± 13.9	102.9 ± 13.7
FDA (μg fluorescein g^{-1} h^{-1})	35.93 ± 1.67	29.57 ± 3.32
Microbial biomass (nmol FAME 16:0 g^{-1})	5.10 ± 0.59	3.03 ± 0.50
Ratio of fungi:bacteria markers	0.095 ± 0.005	0.097 ± 0.007
Gram positive bacteria (mol %)	28.94 ± 0.42	29.87 ± 2.50
Gram negative bacteria (mol %)	6.54 ± 0.09	4.24 ± 0.36
Actinomycetes (mol %)	8.99 ± 0.34	9.75 ± 0.36
Fungi (mol %)	1.04 ± 0.28	1.47 ± 0.28
Arbuscular mycorrhizal fungi (mol %)	3.19 ± 0.04	2.78 ± 0.15

Microbial group markers: Gram-positive (15:0i; 15:0a; 16:0i; 17:0i; 17:0a); Gram-negative (18:1ω7c; 17:0cy; 19:0cy); actinomycetes (16:0 10-Me; 17:0 10-Me; 18:0 10-Me); fungi (18:2ω6c); arbuscular mycorrhizal fungi (16:1ω5c).

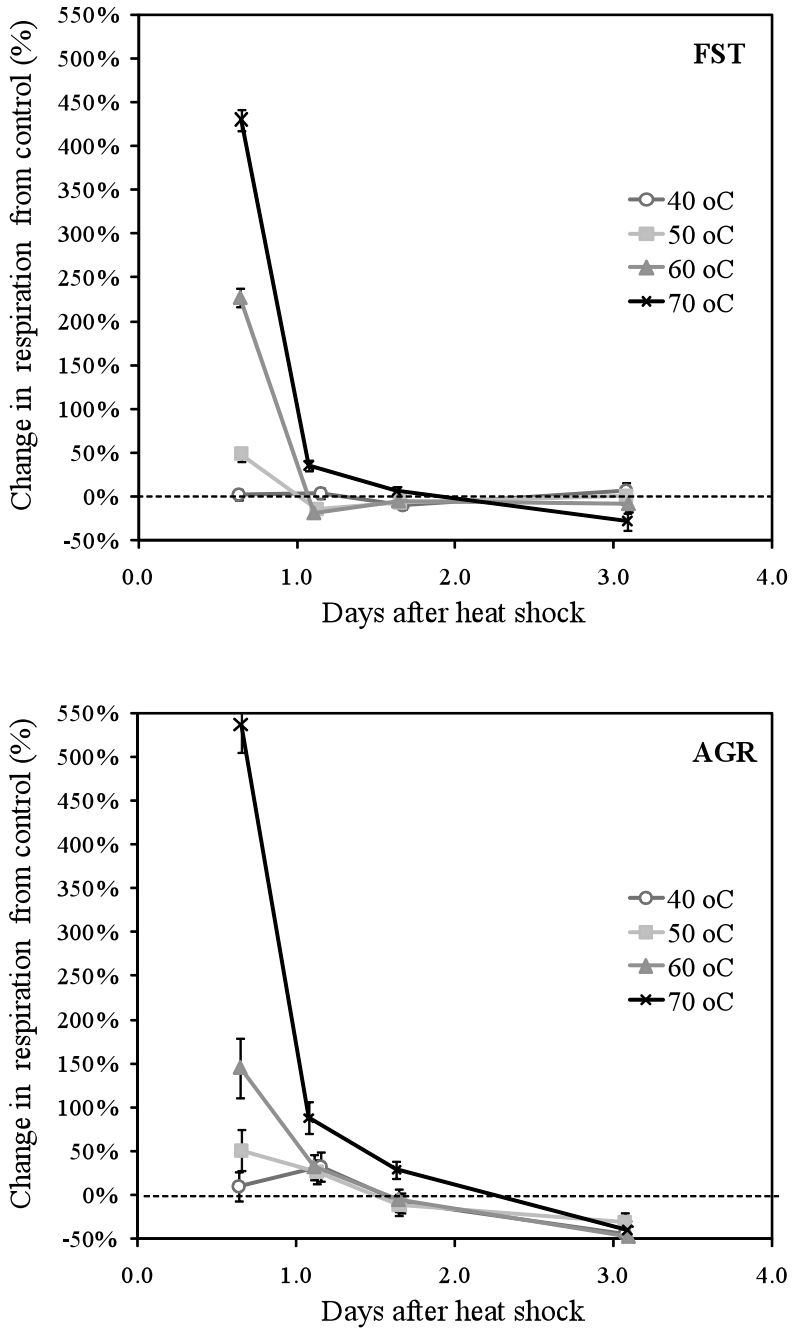

Figure 2.1 Relative changes in the rate of soil respiration up to 3 d after application of HS. Vertical bars indicate the standard error (n=4).

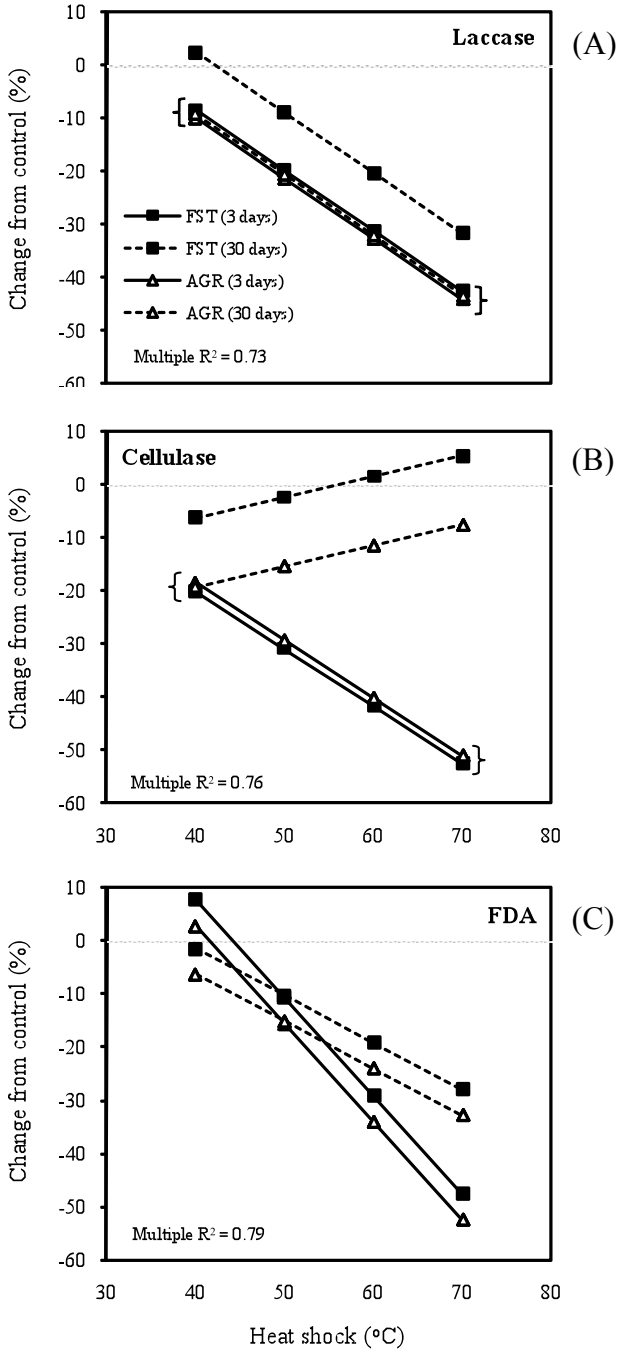

Figure 2.2 Fitted linear regression models for percent of change in soil enzymatic activities relative to controls along the gradient of heat shock (HS) temperatures. Forest soils (FST) are represented by filled squares and agriculture soils (AGR) by open triangles. Full and dashed lines correspond to samples taken at 3 and 30 days after HS, respectively. Regression lines within the same brackets did not differ at $p < 0.05$.

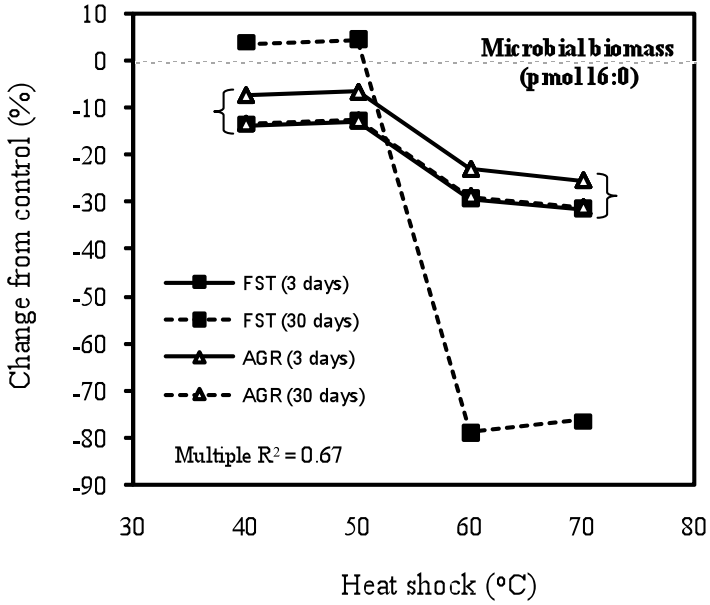

Figure 2.3 Percent of change relative to control soils for microbial biomass (pmol FAME 16:0) along the gradient of heat shock (HS) temperatures. Forest soils (FST) are represented by filled squares and agriculture soils (AGR) by opened triangles. Full and dashed lines correspond to samples taken at 3 and 30 days after HS, respectively. Lines within the same brackets did not differ at $p < 0.05$.

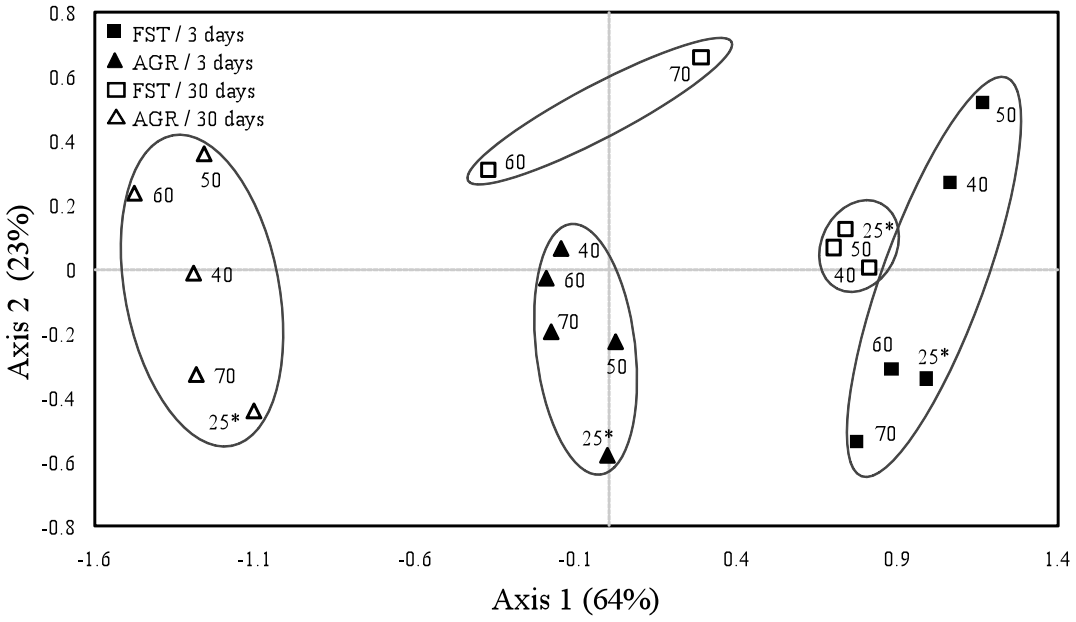

Figure 2.4 NMS ordination of PLFA profiles extracted from forest (FST) and agriculture (AGR) soils treated by heat shocks. Each observation plotted represents the average of four replicates. HS temperature (^{o}C) is indicated adjacent to graph markers. Samples within the same ellipse do not significantly differ in their PLFA profiles according with the multivariate regression tree diagram (Fig. 2.5).

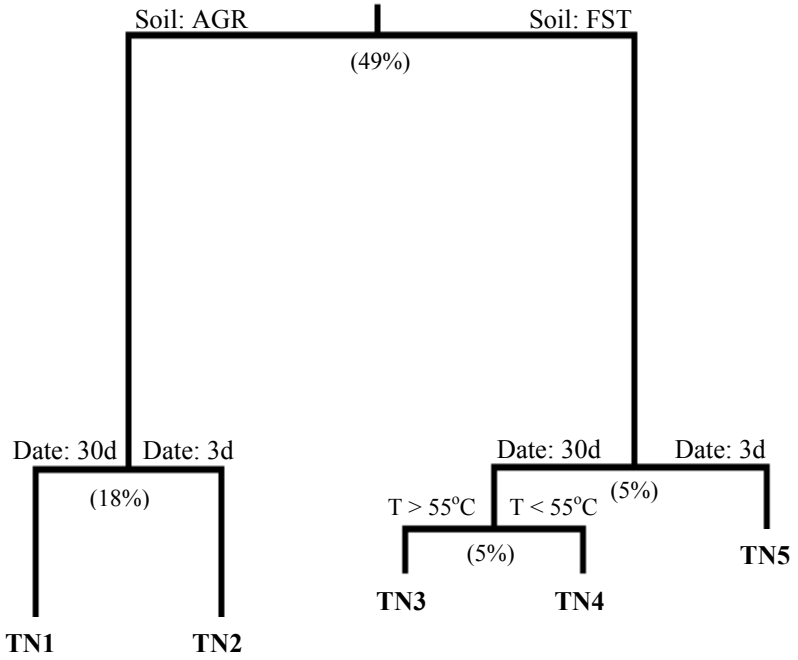

Figure 2.5 Multivariate regression tree (SS-MRT) diagram showing the separation of heat shock treated soils based on the first and second axes from the NMS ordination (Figure 2.4). The tree model is based on successive dichotomic partitions of treatments according to similarities between their fatty acid profiles. The length of the vertical branches is proportional to the fraction of the total variance explained by the respective partition (values in parentheses represent the percentage of variance explained in each split). Similar treatment groups are described above the tree branches after each partition. The diagram shows five terminal nodes (TN) which defined differences in microbial community composition in the heat shock treated soils.

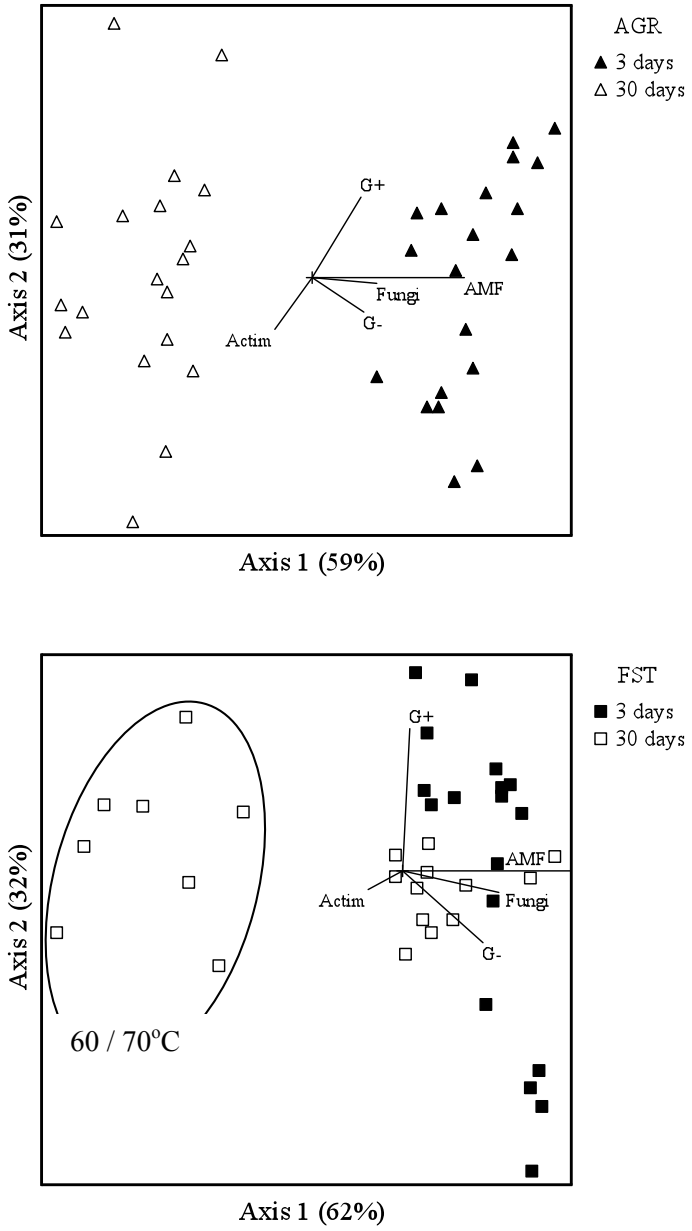

Figure 2.6 NMS ordination of PLFA profiles extracted separately for forest (FST) and agriculture (AGR) soils treated by heat shocks. Ordinations were rotated in order to maximize the correlation of axis 1 with sampling date. Major gradients of microbial groups were overlaid (G-, Gram-negative bacteria; G+, Gram-positive bacteria; AMF, arbuscular mycorrhizal fungi; ACT, actinomycetes).

References

Ahn, M., Zimmerman, A.R., Martínez, C.E., Archibald, D.D., Bollag, J., Dec, J., 2007. Characteristics of *Trametes villosa* laccase adsorbed on aluminum hydroxide. Enzyme and Microbial Technology 41, 141-148.

Borneman, J., Triplett, E.W., 1997. Molecular microbial diversity in soils from eastern Amazonia: Evidence for unusual microorganisms and microbial population shifts associated with deforestation. Applied and Environmental Microbiology 63, 2647-2653.

Bossio, D.A., Girvan, M.S., Verchot, L., Bullimore, J., Borelli, T., Albrecht, A., Scow, K.M., Ball, A.S., Pretty, J.N., Osborn, A.M., 2005. Soil microbial community response to land use change in an agricultural landscape of Western Kenya. Microbial Ecology 49, 50-62.

Bouyoucos, G.J., 1927. The hydrometer as a new and rapid method for determining the colloidal content of soils. Soil Science 23, 319-331.

Braga, J.M., Defelipo, B.V., 1974. Determinação espectrofotométrica de fósforo em extrato de solo e material vegetal. Revista Ceres 21, 73-85.

Bray, J.R., Curtis, J.T., 1957. An ordination of the upland forest communities in southern Wisconsin. Ecological Monographs 27, 325-349.

Breiman, L., Friedman, J.H., Olshen, R.A., Stone, C.G., 1984. Classification and regression trees. Chapman & Hall/CRC, Belmont, CA, USA, 368 pp.

Buckley, D.H., Schmidt, T.M., 2001. The structure of microbial communities in soil and the lasting impact of cultivation. Microbial Ecology 42, 11-21.

Butler, J.L., Williams, M.A., Bottomley, P.J., Myrold, D.D., 2003. Microbial community dynamics associated with rhizosphere carbon flow. Applied and Environmental Microbiology 69, 6793-6800.

Cambardella, C.A., Elliot, E.T., 1992. Particulate soil organic matter changes across a grassland cultivation sequence. Soil Science Society of America Journal 56, 777–783.

De'ath, G., 2002. Multivariate regression trees: a new technique for modeling species-environmental relationships. Ecology 83, 1105-1117.

De'ath, G., Fabricius, K.E., 2000. Classification and regression trees: a powerful yet simple technique for the analysis of complex ecological data. Ecology 81, 3178-3192.

Degens, B.P., Schipper, L.A., Sparling, G.P., Duncan, L.C., 2001. Is the microbial community in a soil with reduced catabolic diversity less resistant to stress or disturbance? Soil Biology & Biochemistry 33, 1143-1153.

Degens, B.P., Schipper, L.A., Sparling, G.P., Vojvodic-Vukovic, M., 2000. Decreases in organic C reserves in soils can reduce the catabolic diversity of soil microbial communities. Soil Biology & Biochemistry 32, 189-196.

Dick, R.P., Breakwell, D.P., Turco, R.F., 1996. Soil enzyme activity and biodiversity measurements as integrative microbiological indicators. In: Doran, J.W., Jones, A.J. (Eds.), Methods for assessing soil quality. SSSA, Madison, pp. 247-271.

Dinesh, R., Chaudhuri, S.G., Sheeja, T.E., 2004. Soil biochemical and microbial indices in wet tropical forests: Effects of deforestation and cultivation. Journal of Plant Nutrition and Soil Science 167, 24-32.

Ehrlich, P.R., Ehrlich, A.H., 1981. Extinction: the causes and consequences of the disappearance of species. Random House, New York.

Frostegård, Å., Bååth, E., 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biology and Fertility of Soils 22, 59-65.

Girvan, M.S., Campbell, C.D., Killham, K., Prosser, J.I., Glover, L.A., 2005. Bacterial diversity promotes community stability and functional resilience after perturbation. Environmental Microbiology 7, 301-313.

Green, V.S., Stott, D.E., Diack, M., 2006. Assay for fluorescein diacetate hydrolytic activity: Optimization for soil samples. Soil Biology & Biochemistry 38, 693-701.

Griffiths, B.S., Bonkowski, M., Roy, J., Ritz, K., 2001a. Functional stability, substrate utilisation and biological indicators of soils following environmental impacts. Applied Soil Ecology 16, 49-61.

Griffiths, B.S., Kuan, H.L., Ritz, K., Glover, L.A., McCaig, A.E., Fenwick, C., 2004. The relationship between microbial community structure and functional stability, tested experimentally in an upland pasture soil. Microbial Ecology 47, 104-113.

Griffiths, B.S., Ritz, K., Bardgett, R.D., Cook, R., Christensen, S., Ekelund, F., Sørensen, S.J., Bååth, E., Bloem, J., de Ruiter, P.C., Dolfing, J., Nicolardot, B., 2000. Ecosystem response of pasture soil communities to fumigation-

induced microbial diversity reductions: an examination of the biodiversity-ecosystem function relationship. Oikos 90, 279-294.

Griffiths, B.S., Ritz, K., Wheatley, R.E., Kuan, H.L., Boag, B., Christensen, S., 2001b. An examination of the biodiversity-ecosystem function relationship in arable soil microbial communities. Soil Biology & Biochemistry 33, 1713-1722.

Guilbault, G.G., Kramer, D.N., 1964. Fluorometric determination of lipase, acylase, alpha- and gamma-chymotrypsin and inhibitors of these enzymes. Analytical Chemistry 36, 409-412.

Hope, C.F.A., Burns, R.G., 1987. Activity, origins and location of cellulases in a silt loam soil Biology and Fertility of Soils 5, 164-170.

Huang, Q., Liang, W., Cai, P., 2005. Adsorption, desorption and activities of acid phosphatase on various colloidal particles from an Ultisol. Colloids and Surfaces B: Biointerfaces 45, 209-214.

Islam, K.R., Weil, R.R., 2000. Land use effects on soil quality in a tropical forest ecosystem of Bangladesh. Agriculture, Ecosystems & Environment 79, 9-16.

Kandeler, E., 1990. Characterization of free and adsorbed phosphatases in soils. Biology and Fertility of Soils 9, 199-202.

Kemper, W.D., Rosenau, R.C., 1986. Aggregate stability and size distribution. In: Klute, A. (Ed.), Methods of soil analysis. Part 1. ASA/SSSA, Madison, WI, pp. 425-441.

Kruskal, J.B., 1964. Nonmetric multidimensional scaling: a numerical method. Psychometrika 29, 115-129.

Lang, E., Eller, G., Zadrazil, F., 1997. Lignocellulose decomposition and production of ligninolytic enzymes during interaction of white rot fungi with soil microorganisms. Microbial Ecology 34, 1-10.

Loreau, M., 2000. Biodiversity and ecosystem functioning: recent theoretical advances. Oikos 91, 3-17.

MacArthur, R.H., 1955. Fluctuations of animal populations and a measure of community stability. Ecology 73, 1943-1967.

Mather, P.M., 1976. Computational methods of multivariate analysis in physical geography. J. Wiley & Sons, London, 532 pp.

Mendes, I.C., Bottomley, P.J., Dick, R.P., Bandick, A.K., 1999. Microbial biomass and activities in soil aggregates affected by winter cover crops. Soil Science Society of America Journal 63, 873-881.

Naeem, S., Li, S., 1997. Biodiversity enhances ecosystem reliability. Nature 390, 507-509.

Niku-Paavola, M.L., Raaska, L., Itavaara, M., 1990. Detection of white-rot fungi by a non-toxic stain. Mycological Research 94, 27-31.

Nimmo, J.R., Perkins, K.S., 2002. Aggregate stability and size distribution. In: Dane, J.H., Topp, G.C. (Eds.), Methods of soil analysis. Part 4, Physical methods. SSSA, Madison, pp. 317-328.

Nizovtseva, D.V., Semenov, A.M., 1995. Influence of moisture on cellulase activity of micro-organisms in upper peat. Microbiology (Reading) 64, 841-846.

Nourbakhsh, F., 2007. Decoupling of soil biological properties by deforestation. Agriculture, Ecosystems & Environment 121, 435-438.

Nusslein, K., Tiedje, J.M., 1999. Soil bacterial community shift correlated with change from forest to pasture vegetation in a tropical soil. Applied and Environmental Microbiology 65, 3622-3626.

Olsson, P.A., Bååth, E., Jakobsen, I., Söderström, B., 1995. The use of phospholipid and neutral lipid fatty acids to estimate biomass of arbuscular mycorrhizal fungi in soil. Mycological Research 99, 623-629.

Øvreås, L., Torsvik, V., 1998. Microbial diversity and community structure in two different agricultural soil communities. Microbial Ecology 36, 303-315.

Schinner, F., von Mersi, W., 1990. Xylanase-, CM-cellulase-, and invertase activity in soil: an improved method. Soil Biology & Biochemistry 22, 511-515.

Schnürer, J., Rosswall, T., 1982. Fluorescein diacetate hydrolysis as a measurement of total microbial activity in soil and litter. Applied and Environmental Microbiology 43, 1256-1261.

Semenov, A.M., Nizovtseva, D.V., 1995. Influence of temperature and mineral supply on cellulase activity and micromycete development in samples of peat from the upper bog. Microbiology 64, 97-103.

Solomon, D., Lehmann, J., Zech, W., Fritzsche, F., Tekalign, M., 2002a. Soil organic matter composition in the subhumid Ethiopian highlands as influenced by

deforestation and agricultural management. Soil Science Society of America Journal 66, 68-82.

Solomon, D., Tekalign, M., Zech, W., Fritzsche, F., Lehmann, J., 2002b. Soil organic matter dynamics in the subhumid agroecosystems of the Ethiopian highlands: evidence from natural 13C abundance and particle-size fractionation. Soil Science Society of America Journal 66, 969-978.

Tobor-Kaplon, M.A., Bloem, J., Römkens, P.F.A.M., Ruiter, P.C.d., 2006. Functional stability of microbial communities in contaminated soils near a zinc smelter (Budel, The Netherlands). Ecotoxicology 15, 187-197.

Torsvik, V., Goksøyr, J., Sorheim, R., Salte, K., 1990. Comparison of phenotypic diversity and DNA heterogeneity in population of soil bacteria. Applied and Environmental Microbiology 56, 776-781.

van Bruggen, A.H.C., Semenov, A.M., 2000. In search of biological indicators for soil health and disease suppression. Applied Soil Ecology 15, 13-24.

Waldrop, M.P., Balser, T.C., Firestone, M.K., 2000. Linking microbial community composition to function in a tropical soil. Soil Biology & Biochemistry 32, 1837-1846.

White, D.C., Pinkart, H.C., Ringelberg, D.B., 1997. Biomass measurements: biochemical approaches. In: Hurst, C.J., Knudsen, G.R. (Eds.), Manual of Environmental Microbiology. ASM Press, Washington, DC, pp. 91-101.

Wright, D.A., Killham, K., Glover, L.A., Prosser, J.I., 1995. Role of pore size location in determining bacterial activity during predation by protozoa in soil. Applied and Environmental Microbiology 61, 3537-3543.

Zelles, L., 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterization of microbial communities in soil: a review. Biology and Fertility of Soils 29, 111-129.

Zelles, L., Bai, Q.Y., Beck, T., Beese, F., 1992. Signature fatty acids in phospholipids and lipopolysaccharides as indicators of microbial biomass and community structure in agricultural soils. Soil Biology & Biochemistry 24, 317-323.

Shifts in microbial community composition and physiological profiles across a gradient of induced soil degradation (GRIND)

Chapter 3

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Abstract

A gradient of induced soil degradation (GRIND) was created in order to evaluate the relationship between changes in soil physical and chemical properties with microbial community composition. We used terminal restriction fragment polymorphism (T-RFLP) and phospholipid fatty acid (PLFA) analyses to evaluate changes in microbial community composition, and Biolog EcoPlates™ to evaluate changes in the community level physiological profiles (CLPPs). Plots arranged in a Latin Square design were disturbed through tillage events applied 0, 1, 2, 3 or 4 times during a three-month period to a 12-year fallow Ultisol in Northeastern Brazil. Sixty days after disturbances, plots were sampled at 0 to 20 cm depth to perform soil analyses. Disturbances negatively affected the following soil properties: available water, saturated field hydraulic conductivity, water stable aggregates, aggregate mean diameter, organic C, cation exchange capacity, and the PLFA-estimated microbial biomass. Multivariate ordination of these properties revealed the formation of a linear GRIND that was significantly correlated with CLPPs, but not with T-RFLP and PLFA profiles. Nevertheless, decreases in field hydraulic conductivity and losses of soil organic C in disturbed plots were significantly associated with changes in microbial profiles. Among the microbial profiling techniques, CLPP was the most sensitive and showed changes proportional to disturbance levels. Although T-RFLP profiles were also highly sensitive, the shifts in community composition were similar regardless of the disturbance level. Richness (*R*) and Shannon (*H'*) indexes, calculated from both the T-RFLP and CLPP data indicated that changes in these

profiles were associated with decreases in microbial genetic and functional diversity. Despite PLFA profiles being less able to detect microbial community shifts in response to disturbances, a linear decrease in the ratio of cyclopropyl (19:0cy) and its precursor fatty acid (18:1ω7c) corroborated the physiological changes observed in the CLPPs. Our results indicate that degradation of soil physical and chemical properties coincide with changes in microbial activity, community composition, and diversity. However, although the level of soil disturbance/degradation may not be reflected in the short-term by microbial composition, it is promptly reflected in changes in microbial physiology.

Keywords

Microbial diversity; Functional diversity; Soil tillage; Soil organic C; Soil physical properties; PLFA; T-RFLP; CLPP; BIOLOG

Introduction

The effects of land use change and agriculture practices on the composition and diversity of soil microbial communities have been demonstrated using different genetic and physiological techniques. Buckley and Schmidt (2001) showed that fields sharing a history of cultivation possessed different terminal restriction fragment length polymorphisms (T-RFLP) profiles and reduced diversity of rRNA genes associated with alpha-Proteobacteria, beta-Proteobacteria, and Actinobacteria compared to uncultivated fields. Microbial community structure also differed between cultivated and uncultivated soils based on phospholipid fatty acid (PLFA)

analysis (Bossio et al., 2005), and soils that were tilled (Lupwayi et al., 1998) or converted from native vegetation to agriculture (Gomez et al., 2000; Gomez et al., 2004; Nsabimana et al., 2004) presented different community level physiological profiles (CLPP), and reduced functional diversity.

Although these studies were focused on how soil use or management affect microbial communities, they failed to associate these effects with changes that occurred in soil physical and chemical properties. In most situations this type of association is impaired by the observational nature of these studies where the responses of microbial communities to treatments might be influenced by non-edaphic factors. For example, Yan et al. (2000) associated the reduced functional diversity of cultivated vertisols assessed by the CLPP method with reduced soil organic C compared to uncultivated vertisols. However, this difference could also be the result of other factors influencing soil microbial diversity, such as the history of chemical applications to cultivated fields in the form of fertilizer, insecticides, and herbicides, and higher plant diversity in the uncultivated sites. In another study, the evaluation of a grassland restoration chronosequence following ski run construction indicated that the high catabolic diversity of older restored soils was associated with improvements in soil physico-chemical properties (Gros et al., 2004). Nevertheless, the fact that the older restored soils also possessed higher plant diversity and biomass relative to the younger sites cannot be ignored as a cofounding factor because the composition of plant species and the quantity of carbon inputs have also been shown

to influence soil microbial diversity and composition (Smalla et al., 2001; Berg et al., 2002; Kowalchuk et al., 2002; Grayston et al., 2004; Hackl et al., 2004).

In this study, we designed an experiment to induce a gradient of soil degradation by applying tillage events at increasing frequencies to a previously undisturbed site. This gradient allowed us to follow the degradation of soil physical and chemical properties induced by the disturbance treatments with the concomitant changes in microbial diversity and composition. The control of non-edaphic confounding variables provided by the experimental conditions allowed these associations to be established without the biases usually associated with observational studies. Soil microbial community changes were evaluated using a multiple set of techniques that included PLFA and T-RFLP profiling to evaluate changes in structural composition, and the analysis of the CLPPs in Biolog EcoPlates^{TM} to evaluate physiological changes. We also compared the sensitivity of these techniques to indicate soil degradation, and evaluated how changes in the microbial community composition are associated with physiological changes.

Material and methods

Site descriptions and experimental design

The study site is located at the Umbaúba Experimental Station from Embrapa Coastal Tablelands in Sergipe State, Northeastern Brazil (11^{o} 16' S, 37^{o} 26' W, 105 m altitude; 1350 mm of annual rainfall; 24.3^{o}C mean daily temperature). The soil is a fine-loamy kaolinitic isohyperthermic Typic Fragiudults (Latossolo Amarelo,

according to Brazilian Soil Taxonomy). This area was deforested 30 years ago, cultivated with maize, cassava and cowpea for the following 16 years and then kept fallow for 12 years prior to this experiment. During the fallow period the area was mown every other year.

Experimental plots (36 x 12 m) were laid out in a Latin Square design with 5 treatments (disturbance levels) that consisted of the application of 1, 2, 3, or 4 tillage events, plus a no-till control (Table 1). Each tillage event consisted of two plowing operations (disk plow), each followed by two disking operations. These treatments were designed to establish a Gradient of Induced soil Degradation (GRIND) in a short period (4 to 5 months), based on the assumption that the extent of degradation of soil structure and decomposition of soil organic matter would increase with the number of tillage events. Treatments were applied from July to September of 2006 with a 2- to 5-week interval between events. The minimum interval between consecutive events was determined as the time necessary for the CO_2 flux in disturbed plots to decline and become equal to or less than the level observed in control plots (flux of soil CO_2 was measured twice a week using soda lime traps (Zibilske, 1994) installed in both the most recently tilled and control plots). After this period, tillage operations commenced as soon as soil moisture conditions were optimal. Fifteen days before the first tillage event, the vegetation of the experimental area (grasses and small shrubs) was desiccated with a glyphosate application. Vegetation that grew on the plots during the course of the experiment was mechanically mown monthly until November 2006.

Soil analyses

Soil samples were collected in November, 2006, 60 days after the last tillage event had been applied to all experimental plots (Table 3.1). Ten random soil cores were collected from 0 to 20 cm depth of each plot and pooled to form one composite sample. Samples were passed through a 4-mm mesh sieve, and air dried. Sub-samples were refrigerated at 4^{o}C for PLFA, and CLPP, and frozen at -20^{o}C for T-RFLP analysis. Cation exchange capacity (CEC) was calculated as the sum of H^{+} + Al^{3+} (extracted by 0.5 M calcium acetate buffer, pH 7.0) and bases (Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}) (Embrapa, 1997). Soil organic matter content was measured as the loss of weight on ignition after heating oven-dry soil samples at 430^{o}C for 24 h. A conversion factor of 1.724 was used to convert organic matter to soil organic carbon (SOC) (Nelson and Sommers, 1996). The stability of soil aggregates in water was determined by the wet sieving method using a Yoder apparatus (Yoder, 1936). Air dried aggregates (1 to 2 mm) were slowly rewet by capillarity and placed on the top of a 0.25-mm mesh sieve. The sieving was accomplished with a 4 cm stroke length at 42 cycles min^{-1} for 5 min. The mass of sand >0.25 mm was determined after dispersion of the fraction retained on the sieve in NaOH (2 g l^{-1}) (Kemper and Roseneau, 1986). Water stable aggregates (WSA) were expressed as the ratio between the masses of the soil fraction retained in the sieve (free of >0.25-mm sand) and the whole soil sample. Soil samples (< 4 mm) were used to determine the weighted mean diameter of soil aggregates (Kemper and Roseneau, 1986) after sorting the aggregates by wet sieving and using nested sieves with openings of 2, 1, 0.5, and 0.25 mm (Nimmo and Perkins, 2002). The Yoder

apparatus setting was the same as used for WSA. Available soil water and bulk density were determined in undisturbed soil core samples collected from the 7.5 to 12.5 cm depth. Available soil water, defined as water held between -10 and -1500 kPa, was measured using the pressure plate extractor method (Dane and Hopmans, 2002). Bulk density was determined by the core method (Grossman and Reinsch, 2002). Field-saturated soil hydraulic conductivity was determined using a double ring infiltrometer (Reynolds et al., 2002). Readings were taken every 10 minutes between 20 and 70 min after water addition to the rings. After the initial 20 min, all the plots had reached quasi-steady flow (equilibration time). The measurements were carried out in duplicate in each plot.

Community level physiological profiles

CLPPs were obtained with Biolog EcoPlates^{TM} (Biolog, Inc., Hayward, CA) as described by Sinsabaugh et al., (1999). Each plate was inoculated with 150 µl per well of soil sample suspension (1:100 dilution) and incubated at 25^{o}C. Each plate contains a triplicate of 31 substrate types allowing three measurements for each sample. The negative control corresponded to a well without any carbon source. Optical density at 596 nm was read at 24, 48, 72 and 96 h using a microplate reader BioTek PowerWave X 340 (BioTek Instruments, Inc., Winooski, VT). Individual absorbance values for each of the 31 single substrates were corrected by subtraction of the blank control value (raw difference; RD). Negative RD values were set to zero. To minimize effects of different inoculum densities, data were normalized by

dividing the RD values by their respective average well color development (AWCD) values (Garland and Mills, 1991).

Phospholipid fatty acid profiles

Extraction procedures and reagents for the PLFA analysis were similar to those described by Butler et al. (2003). The PLFA composition of extracts was analyzed using an Agilent 6890 gas chromatograph (Agilent Inc., Palo Alto, CA) equipped with an HP Ultra^{TM}-2 (5% phenyl-methylpolysiloxane) column and flame ionization detector. The identification of FAMEs was based on a comparison with chromatograms produced from a mixture of 37 FAMEs (FAME 37 47885-4; Supelco Inc., Bellefonte, PA), a mixture of 24 bacterial FAMEs (P-BAME 24 47080-U; Supelco), 16:0 10-Me, and 18:0 10-Me (Matreya, Pleasant Gap, Pa.), and 20:4ω6c (Supelco). A standard curve prepared from a solution of tridecanoic acid methyl ester (Supelco) was used to convert chromatographic areas into nmol of FAMEs.

The FAMEs analyzed only included those that formed peaks in the region between tetradecanoic methyl ester (14:0) and arachidonic acid (20:4ω6c). Uncommon FAMEs, defined as those with masses less than 4% of the mass of FAME 16:0, were excluded from the data matrix. Profile composition was expressed in mol% of PLFAs, after standardizing the absolute amounts of individual PLFAs by their totals within each sample.

FAMEs reported as biomarkers of fungi, Gram-negative (GN) bacteria, Gram-positive (GP) bacteria, actinomycetes (ACT) and arbuscular mycorrhizal fungi

(AMF) were used as signatures for these microbial groups (Olsson et al., 1995; Frostegård and Bååth, 1996; White et al., 1997; Zelles, 1999). The biomass of the FAME 16:0 (nmol g^{-1} soil) was used as an estimator of microbial biomass (Zelles et al., 1992), and the ratio of cyclopropyl (19:0cy) and its precursor fatty acid (18:1ω7c) as a stress indicator (Bossio and Scow, 1998; Bossio et al., 1998).

DNA extractions and amplification

From each plot one g samples of frozen soil (-20^{o}C) were extracted using a MoBio PowerSoil^{TM} DNA isolation kit (MoBio Laboratories, Carlsbad, CA), according to manufacturer's instructions except that a Bio101 FastPrep instrument was used to lyse cells (Bio 101, Carlsbad, CA). The MoBio bead beating tubes were shaken for 45 s on the FastPrep, and the DNA in extracts was quantified using a NanoDrop^{TM} ND-1000 UV-visible spectrophotometer (NanoDrop Technologies, Wilmington, DE) and subsequently diluted to 25 ng μl^{-1}.

The primers used for amplification of the bacterial 16S rRNA gene were: 8F (Edwards et al., 1989), and 907R (Muyzer et al., 1995). The PCR program was set as previously described (Hackl et al., 2004). The quality of PCR products was inspected by electrophoresis of 5 µl PCR product in 1% (w/v) agarose gels containing ethidium bromide (0.5 mg ml^{-1}). The PCR products were then purified using Qiagen DNA clean-up kit (Qiagen, Valencia, CA) to eliminate genomic DNA, excess primers, and unused nucleotides.

Terminal restriction fragment length polymorphism profiles

PCR products were digested using three restriction enzymes: CfoI, RsaI, and MspI (Promega Corp., Madison, WI). Digests were run according to manufacturer's specifications by incubating the restriction digest for 3 h at 37^{o}C, followed by heat inactivation at 65^{o}C for 15 min. Restricted samples (approximately 1 ng of amplified DNA equivalent) were submitted to Oregon State University Center for Genome Research and Biocomputing for analysis using a ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) and the GeneScan V3.5 software (Applied Biosystems). The baseline threshold for signal detection was set to 50 fluorescence intensity units. Electropherograms were transformed into numeric data of individual peak heights using the Genotyper V2.5 software (Applied Biosystems). To minimize the effects of different quantities of PCR products, data were normalized by dividing the fluorescence value of each T-RF by the total fluorescence of all T-RFs from the corresponding sample. After examining the electropherograms of negative controls, peaks contributing less than 1.5% of the total fluorescence in a single profile were excluded and the relative fluorescence recalculated, making the sum of each profile equal to 100% (Rich et al., 2003). Peaks were then aligned by base pairs and any peaks less than two base pairs apart were combined for final fragment identifications. The three enzyme profiles were combined to produce composite T-RFLP profiles.

Statistical Analyses

A regression approach applied to the 5 x 5 x 5 Latin Square was used to evaluate treatment effects on univariate data (S-PLUS® 8.0, Insightful Corp.). When

the relationship between soil variables and disturbance level was not linear, a two-sided multiple comparison with a control (MCC) test was used to evaluate treatment effects. Raw values of soil properties obtained for each experimental plot were subtracted from the respective row and column coefficients before graphic presentations. A similar correction was applied to the individual FAMEs, carbon sources or T-RFs before the multivariate analyses. Each community data set was ordinated using non-metric multidimensional scaling (NMS) (Kruskal, 1964a; Kruskal, 1964b) and Euclidean distance. Ordinations were performed using PC-ORD statistical package, V5 beta (B. McCune and M.J. Mefford, PC-ORD for Windows: multivariate analysis of ecological data, MjM Software, Gleneden Beach, OR). Resulting bidimensional ordinations were rotated in order to maximize the explained variance on axis 1. The resulting scores from axis 1 were then plotted against the disturbance levels (number of tillage events) to evaluate treatment effects on microbial community and physiological profiles. The relationships between changes in microbial community and soil physical, chemical, and biological properties were evaluated by applying the Pearson product-moment correlation coefficient between the axis 1 scores and each soil property. The PLFA-microbial groups, and CLPP-substrate categories (polymers, carbohydrates, carboxylic acids, amino acids, and amines/amides) were also correlated with their respective axis 1 scores.

NMS ordination was also performed using soil variables which included physical and chemical properties and the PLFA-based microbial biomass. Rotation of ordination axes and representation of axis 1 along the disturbance levels were

made as described for microbial community data. This procedure was used to determine the actual formation of GRIND and to associate it with changes in microbial profiles. Therefore, axis 1 scores from GRIND were correlated with axis 1 scores obtained from the CLPP, T-RFLP and PLFA ordinations.

Richness and diversity indexes were calculated based on the microbial community data sets using PC-ORD. Richness (*R*) was defined as the total number of FAMEs or T-RFs detected, or substrates utilized in Biolog plates for a given sample. The Shannon index (*H'*) was calculated according to the following equation: $H' = -\sum_i^S p_i \log p_i$, where p_i is the proportion represented by each FAME, T-RF, or catabolic activity relative to their totals. These indexes were analyzed statistically as described for soil properties.

Results

Effect of disturbance treatments on soil properties

The increasing levels of soil disturbance simulated by tillage events caused significant changes in most of the soil physical and chemical properties analyzed (Figure 3.1A-G). Soil available water, defined as the volume of water held by the soil between field capacity (-10 kPa) and wilting point (-1500 kPa), decreased linearly ($p<0.001$) from 0.27 m^3 m^{-3} in control plots to 0.19 m^3 m^{-3} in the most disturbed plots (Figure 3.1A). These changes resulted both from linear decreases in soil water retention capacity, and increases in wilting point across the disturbance gradient (data not shown). Field measurements of saturated hydraulic conductivity

were reduced from 4.3 cm h^{-1} in control plots to nearly zero in treated plots (Figure 3.1B). Disturbance also affected the percentage of water stable aggregates which decreased from 90% in control plots to 79-83% in plots submitted to more than two tillage events (Figure 3.1C). A similar pattern was observed for the aggregate mean diameter (Figure 3.1D). Although soil bulk density tended to increase at the highest disturbance levels, differences relative to the control were not statistically significant (Figure 3.1E). There was a small, but significant (p<0.05) decrease in soil organic C (SOC) from 24 g kg^{-1} in controls to about 22 g kg^{-1} in treated plots irrespective of disturbance level (Figure 1F). A similar trend was observed in the CEC measured at pH 7, which decreased from 5.2 to 4.7 $cmol_c$ kg^{-1} in the same set of treatments (p<0.10) (Figure 3.1G). Disturbance treatments affected the PLFA-based microbial biomass estimate (Figure 1H). Accordingly, the amount of 16:0 decreased from 2.2 nmol g^{-1} in undisturbed plots to 1.6 nmol g^{-1} in plots submitted to four tillage events.

Disturbance effects on soil microbial community

NMS ordination represented 94% of the total data variance of both the CLPP and T-RFLP profiles in two dimensional solutions. For the PLFA data, the total variance explained in two axes reached 98%. To simplify the visualization of treatment effects on the microbial community and physiological profiles, ordination axes were rotated to maximize the variance on axis 1 which was plotted against the disturbance levels (Figure 3.2). After rotation, axis 1 represented between 81 and 89% of the total variance in the three data sets. Results showed increasing changes in

the CLPPs with increasing disturbance level (Figure 3.2A). These changes were driven by shifts in the pattern of utilization of specific biochemical categories of C substrates. The greater the disturbance applied, the higher was the relative use of polymers and carboxylic acids as indicated by their positive correlation coefficients along axis 1 (r = 0.86 and 0.91, respectively) (Table 3.2). Conversely, there was a decrease in the relative use of amines/amides and carbohydrates with increasing disturbance (r = -0.86 and -0.68, respectively).

T-RFLP profiles were also significantly altered in treated plots, but changes were not proportional to the disturbance levels, as observed in the CLPPs (Figure 3.2B). A single tillage event resulted in a new genetic profile of the microbial community that was unaltered when further disturbances were applied to the soil.

PLFA profiles were less sensitive to disturbance treatments compared to T-RFLP and CLPP (Figure 3.2C). Significant differences relative to controls were only observed in plots submitted to the highest disturbance level (four tillage events). Correlation coefficients between PLFA markers from major microbial groups and scores from axis 1 (Table 3.3) demonstrated that these changes were associated with lower relative abundance of fungi, Gram-positive bacteria and actinomycetes in disturbed plots. Despite the apparent insensitivity of PLFA profiles to 1, 2 or 3 tillage events, the ratio 19:0cy/18:1ω7c presented a decreasing trend across all disturbance levels (Figure 3.3).

Changes in T-RFLP and CLPP in response to soil disturbance were associated with decreases in microbial genetic and functional diversity. This was

indicated by the negative correlation coefficient between either richness (*R*) or Shannon (*H'*) indexes and scores from axis 1 of the CLPP and T-RFLP ordinations (Table 3.4). There were no relationship between the diversity indexes and changes on the PLFA profiles.

Relationship between degradation of soil properties and microbial community

Soil physical and chemical properties and microbial biomass data (Figure 3.1) were submitted to multivariate ordination to evaluate the formation of the gradient of induced soil degradation (GRIND). The ordination resulted in two axes that explained 90% of total variance. The amount of variance maximized on axis 1 after rotation was equivalent to 76%. All soil properties, except bulk density, were negatively correlated with axis 1 ($p<0.01$). Plotting axis 1 scores against disturbance levels revealed the formation of a linear GRIND (Figure 3.4). This gradient was significantly correlated with CLPPs ($p<0.001$), but not with T-RFLP and PLFA profiles (Table 3.5). Nevertheless, correlations between all three microbial profiles and individual soil properties indicated a significant association with soil organic C and hydraulic conductivity (Table 3.5). The negative sign of the correlation coefficient indicated that changes in CLPP, T-RFLP and PLFA profiles in response to soil disturbance were associated with lower values of soil C and hydraulic conductivity ($p<0.05$). Although none of the other soil properties were correlated with the T-RFLP and PLFA profiles, correlations between CLPPs and microbial

biomass, CEC, available water, aggregate mean diameter and water stable aggregates were highly significant (p<0.001).

Discussion

GRIND characterization

Disturbance treatments simulated by tillage events were effective in forming a gradient of induced soil degradation (GRIND) during the 5-month period of the field experiment. GRIND was mostly characterized by the degradation of soil physical structure as a consequence of the intense physical disturbances. This degradation was expressed by sequential decreases of soil available water, water infiltration, aggregate mean diameter, and water stable aggregates (Figure 3.1A-D). In addition to physical changes, soil organic C was decreased in disturbed plots, irrespective of the number of tillage events (Figure 3.1F,G). Loss of soil organic matter in tilled soils has been widely reported in soils literature, but it is generally referred as a long-term effect of tillage (Elliott, 1986; Woods, 1989; Doran et al., 1998). It is likely that a large portion of the C lost in response to tillage in our study was derived from the decomposition of labile pools of organic matter. This suggestion is corroborated by the increase of up to 100% in CO_2 flux from recent tilled plots compared to controls (data not shown). This difference remained relatively constant for about 10 d indicating that it was caused by an increase in microbial respiration activity rather than by a short-lived physical process of soil degasification that has been observed in other soils (Jackson et al., 2003). Because tilled plots had lower microbial biomass (PLFA-estimate; Figure

3.1H), higher respiratory activity was also probably due to the decomposition of microbial cells killed by the disturbance. Furthermore, changes in more stable SOM pools should not be disregarded, especially in tropical, coarse-textured soils like the one evaluated in this study. Decreases in these stable SOM pools are likely to be the major reason for the decrease in soil cation exchange capacity (Figure 3.1G) because these stable pools are responsible for most of the CEC contributed to the soil by organic matter (Brady and Weil, 2002).

Response of soil microbial communities to GRIND

Although GRIND significantly affected soil microbial communities, the changes observed were dependent upon the profiling technique used. The changes observed in CLPPs were proportional to the disturbance level; however, this pattern was not followed by the community structure (PLFA and T-RFLP) (Figure 3.2A-C). For T-RFLP, profiles changed similarly in all tilled plots independent of the disturbance level. These results indicate that in spite of the similar microbial composition in disturbed plots, these communities differed in regard to their physiological status. According to CLPPs, physiological changes were associated with a relative decrease in the catabolism of amines/amides and carbohydrates, accompanied by an increase in the catabolism of polymers and carboxylic acids across GRIND (Table 3.2). Other soils submitted to disturbances have expressed contradictory results regarding substrate utilization in Biolog systems. For instance, metabolism of carbohydrates in Biolog EcoPlate^{TM} was decreased in soils under

conventional tillage compared with reduced tillage management (Diosma et al., 2006). Although this result partially corroborates ours, other cultivated soils did not differ in the metabolism of 30 different carbohydrates in Biolog GN™ microplates in comparison to uncultivated paired sites (Yan et al., 2000). Instead, the microbial community from the cultivated soils utilized less carboxylic acids, amino acids and amines/amides.

Changes in PLFA profiles in response to disturbances were less clear despite a significant change in plots submitted to the highest disturbance level (Figure 3.2C). Notwithstanding, there was a linear decrease in the ratio of cyclopropyl PLFAs (19:0cy) and its precursor (18:1ω7c) ($p<0.001$) (Figure 3.3), which corroborates the physiological changes observed in the CLPPs. This ratio has been referred as an indicator of the physiological status of Gram-negative bacterial communities (Bossio and Scow, 1998; Bossio et al., 1998). In these bacteria, the precursors are increasingly converted to cyclopropyl fatty acids as bacterial growth moves from logarithmic to stationary-phase growth in response to stresses such as C limitation. Therefore, the decrease of this ratio across the disturbance gradient might imply alleviation of a substrate limited condition. In more disturbed plots, substrate increase might be due to both a decrease in the surviving biomass, and larger supply of C and other nutrients from the dead microbes and organic residues made available upon mechanical disruption of soil aggregates.

GRIND also negatively affected the microbial genetic and functional diversity as indicated by the negative correlation between richness (*R*) and Shannon

(*H'*) indexes with T-RFLP and CLPP profiles (Table 3.4). Decreases in microbial functional diversity based on CLPP analyses have also been observed in several soils that have undergone disturbances such as changes in land use or intensive soil cultivation (Lupwayi et al., 1998; Degens et al., 2000; Gomez et al., 2000; Yan et al., 2000; Gomez et al., 2004; Nsabimana et al., 2004; Bossio et al., 2005; Diosma et al., 2006). Nonetheless, fewer DNA-based studies have demonstrated similar changes in microbial diversity (Buckley and Schmidt, 2001; Torsvik et al., 2002). Studies where both CLPP and T-RFLP analyses were used to compare soils under different land use (native vegetation, perennial or annual crops) revealed differences only in diversity indexes based on CLPPs (Gomez et al., 2004; Bossio et al., 2005).

With few exceptions (Lupwayi et al., 1998; Diosma et al., 2006), all studies cited above compared the microbial community from soils under different types of vegetation or diversity of plants. Considering that this factor can have a major influence on soil microbial diversity and composition (Smalla et al., 2001; Berg et al., 2002; Kowalchuk et al., 2002; Grayston et al., 2004; Hackl et al., 2004), it is difficult to ascertain whether changes observed were caused by soil disturbances, or by changes in vegetation composition. Conversely, we expect that influences of vegetation on microbial communities across GRIND were minimal because: (1) the whole study area was covered with homogeneous vegetation for 12 years previously to the experimental setting; and (2) the experimental plots were kept with minimal vegetation growth for most of the study by chemical desiccation and periodic mowing. Therefore, effects of GRIND on microbial diversity and composition

reported in this study are more likely to be directly associated with disturbances applied to the soil.

Similar unbiased relationships could be established between changes in microbial composition and degradation of soil properties. In this regard, changes in CLPPs relative to control soils were significantly associated with the degradation of most soil physical and chemical properties analyzed (Table 3.5). Moreover, microbial community composition based on T-RFLP and PLFA profiles were significantly associated with soil C and hydraulic conductivity. Some studies have also found significant correlations between microbial community composition and soil C in areas under different land use (Cookson et al., 2007), soil management (Yan et al., 2000; Grayston et al., 2004), or along a chronosequence of soil restoration (Gros et al., 2004). As discussed previously, these studies consisted of observational experiments where non-edaphic factors might have influenced the results. However, our results indicate that losses of soil organic C and degradation of soil structure might indeed be associated with changes in microbial community composition and decreases in diversity indexes observed in those studies.

Sensitivity of microbial community techniques

The three techniques used to evaluate microbial communities across GRIND clearly showed different sensitivities to disturbance. CLPP was the most sensitive method for detecting differences in disturbance levels and changes in soil properties (Figure 3.2A-C). Secondly, while T-RFLP profiling was more sensitive than PLFA composition in showing changes in microbial community composition, both

techniques failed to reveal changes in response to levels of soil degradation. Other studies have also shown that differences observed in microbial communities from different soils might depend on the profiling technique used (Widmer et al., 2001; Gomez et al., 2004; Grayston et al., 2004). For instance, microbial communities from improved, semi-improved, and unimproved grassland ecosystems from the UK were better differentiated using PLFA than CLPP or %G+C base distribution (Grayston et al., 2004). Widmer et al. (2001) showed that although RFLP, PLFA and CLPP analyses were able to differentiate three soils used in pesticide degradation studies, these methods did not reveal the same relative soils similarities based on cluster analysis.

The differences among the microbial profiling techniques observed in this and other studies are not surprising considering that each method focus on different aspects of soil microbiological characteristics. Therefore, the choice of one method over another might depend on the objectives of the study; however, the use of multiple methods is advantageous if they provide a broader understanding and reveal different aspects of changes in microbial communities.

Conclusions

This study aimed to: (1) generate a gradient of induced soil degradation (GRIND) through varying the number of tillage events; (2) study changes in microbial community composition and diversity across the GRIND; and (3) compare the sensitivity of different methods for assessing the response of microbial communities

to soil degradation. We have shown that GRIND resulted in an altered and less diverse microbial community, and that these changes were directly associated with losses of soil organic C and degradation of soil physical structure. However, the specific techniques used to assess microbial communities gave different information about these changes. Results from CLPP, T-RFLP and PLFA profiling techniques indicated that although the level of soil disturbance/degradation may not influence microbial community composition in the short-term, it is promptly expressed in the physiological status of the community.

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Table 3.1 Sequence of tillage events designed to induce the gradient of soil degradation. The check mark symbol indicates the dates when the tillage events were performed. Each tillage event consisted of two plowing operations, each of which was followed by two disking operations.

Disturbance level (tillage events)	Jun 27	Jul 19	Aug 7	Sep 15
0 (control)				
1				✓
2			✓	✓
3		✓	✓	✓
4	✓	✓	✓	✓

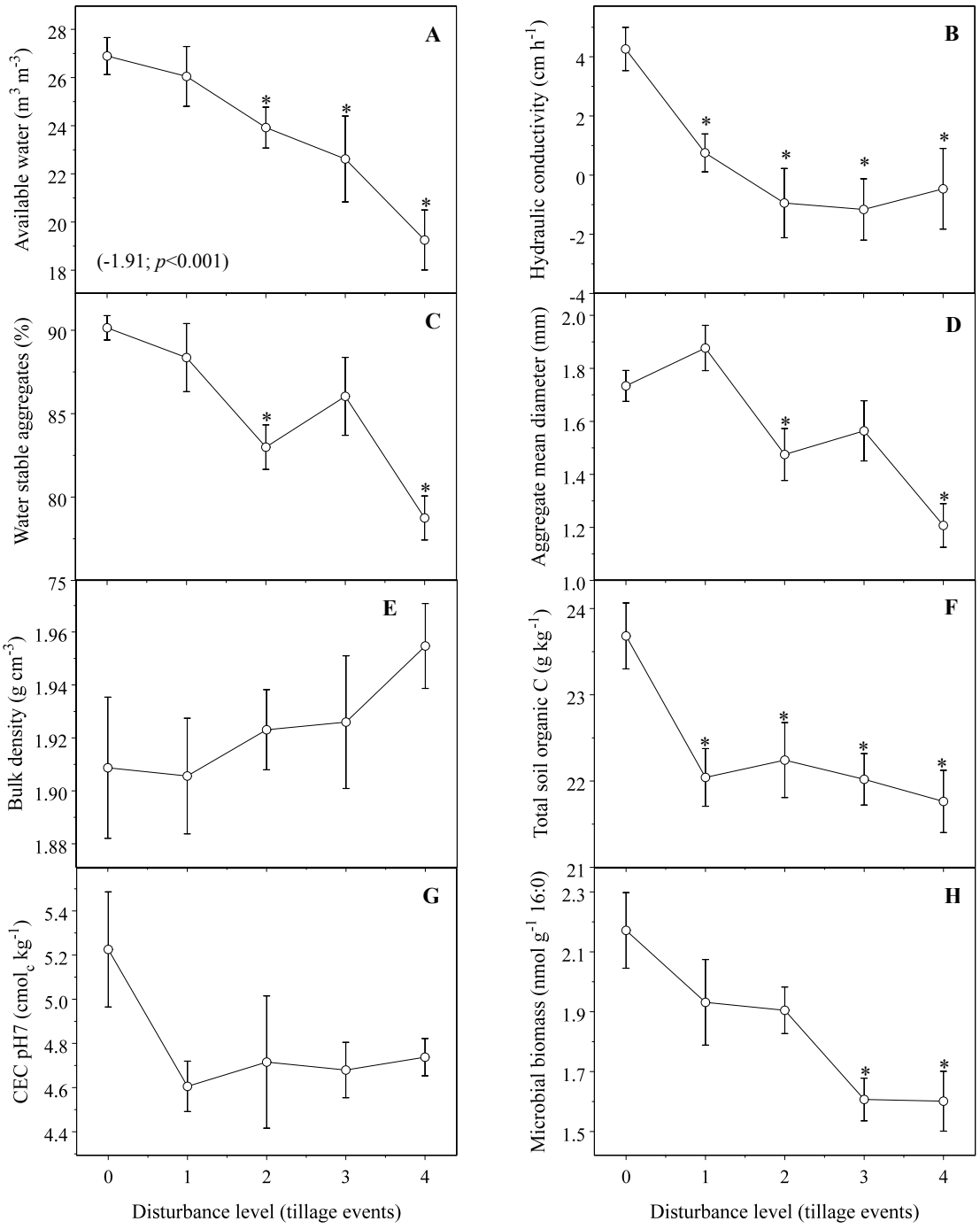

Figure 3.1 Changes in soil properties in response to the disturbance level applied to the soil as tillage events. Variables were adjusted to account for row and column effects associated with the Latin Square design (see *Statistical analysis* for details). Vertical bars correspond to the mean standard error (N=5). Asterisks indicate significant difference from controls (p<0.05). Values in parentheses indicate the slope and p-value of the linear regression.

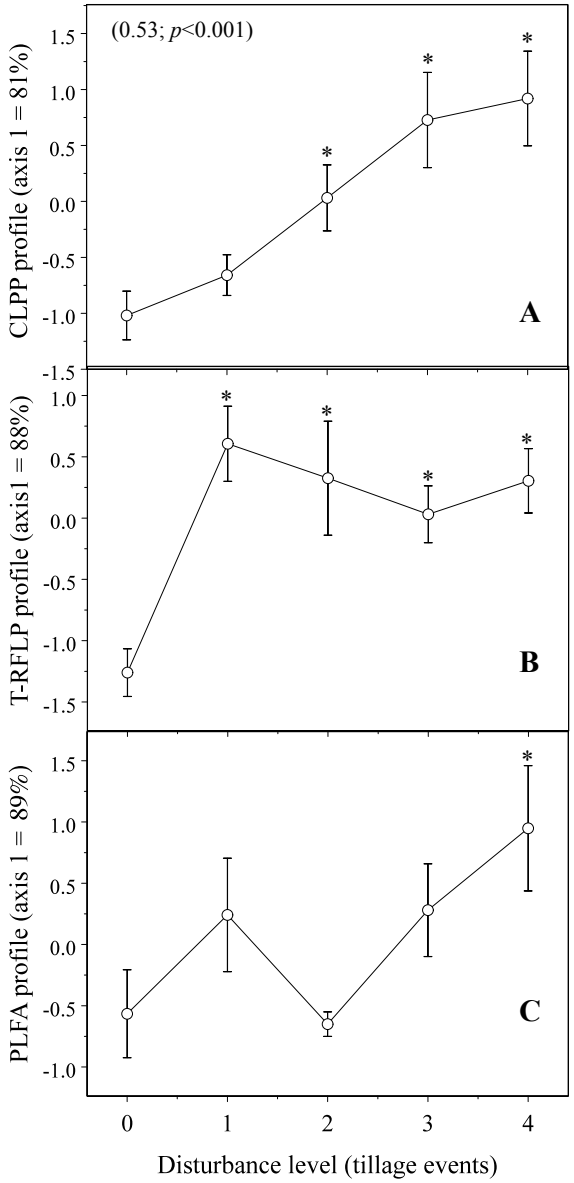

Figure 3.2 Changes in CLPP (A), T-RFLP (B), and PLFA (C) profiles in response to the disturbance level applied to the soil as tillage events. Microbial community and physiological profiles were represented by axis 1 from the NMS ordination. The fraction of the total variance accounted for by axis 1 is indicated in parentheses. Vertical bars correspond to the mean standard error (N=5). Asterisks indicate significant difference from controls (p<0.05). Values in parentheses indicate the slope and p-value of the linear regression.

Table 3.2 Correlation coefficients (r) between biochemical categories of C substrates and scores from axis 1 of the CLPP ordination (Figure 3A). Values in parentheses represent the number of substrates present in Biolog EcoPlate™ in each biochemical category

	CLPP
Carboxylic acids (10)	0.91***
Carbohydrates (9)	-0.68***
Polymers (4)	0.86***
Amino acids (6)	-0.08
Amines/amides (2)	-0.86***

C substrates from Biolog EcoPlates™ were classified according to Kohler et al., (2005).

***$p<0.001$.

Table 3.3 Correlation coefficients (r) between major microbial groups and scores from axis 1 of the PLFA ordination (Figure 3C)

	PLFA
Fungi	-0.64***
Gram-negative bacteria	0.26
Gram-positive bacteria	-0.58**
Actinomycetes	-0.75***
Arbuscular mycorrhizal fungi	0.08

Microbial group markers: Gram-positive (15:0i; 15:0a; 16:0i; 17:0i; 17:0a); Gram-negative (18:1ω7c; 17:0cy; 19:0cy); actinomycetes (16:0 10-Me; 17:0 10-Me; 18:0 10-Me); fungi (18:2ω6c); arbuscular mycorrhizal fungi (16:1ω5c).

***$p<0.001$; **$p<0.01$.

Table 3.4 Correlation coefficients (r) between richness (*R*) and Shannon index (*H'*), and scores from the NMS ordination (axis 1) representing changes in the CLPP, T-RFLP and PLFA profiles (Figure 3.3). Negative correlation coefficients indicate that changes in microbial profiles in response to soil disturbance were associated with a decrease of the diversity indexes

	CLPP	T-RFLP	PLFA
Richness (*R*)	-0.74***	-0.49**	0.07
Shannon index (*H'*)	-0.91***	-0.67***	0.07

***$p<0.001$; **$p<0.01$.

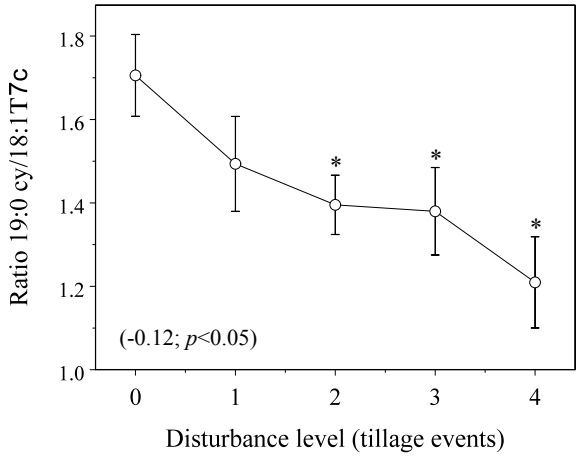

Figure 3.3 Changes in the ratio 19:0cy/18:1ω7c in response to the disturbance level applied to the soil as tillage events. Vertical bars correspond to the mean standard error (N=5). Asterisks indicate significant difference from control (p<0.05). Values in parentheses indicate the slope and p-value of the linear regression.

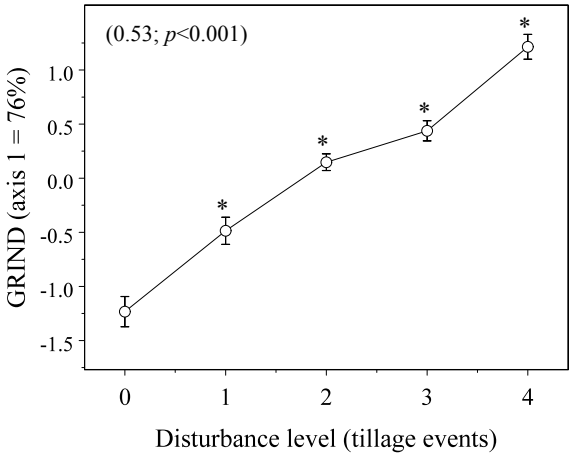

Figure 3.4 Gradient of induced soil degradation (GRIND) in relation to the disturbance level applied to the soil. GRIND was represented by the axis 1 of the NMS ordination of soil properties shown in Figure 1. The fraction of the total variance accounted for in axis 1 is indicated in parentheses. Vertical bars correspond to the mean standard error (N=5). Asterisks indicate significant difference from control (p<0.05). Values in parentheses indicate the slope and p-value of the linear regression.

Table 3.5 Correlation coefficients (r) between GRIND scores or individual soil properties, and scores representing changes in the CLPP, T-RFLP, and PLFA profiles (Figure 3.3). Negative correlation coefficients indicate that changes in microbial profiles in response to soil disturbance were associated with a decrease in the respective soil property

	CLPP	T-RFLP	PLFA
GRIND[†]	0.64***	0.13	0.14
Microbial biomass	-0.59***	-0.07	-0.13
Soil organic C	-0.62***	-0.65***	-0.42*
CEC	-0.15	-0.36	-0.19
Bulk density	0.38	0.27	0.13
Available water	-0.65***	-0.21	-0.26
Hydraulic conductivity	-0.60***	-0.46*	-0.49**
Aggregate mean diameter	-0.66***	-0.06	-0.09
Water stable aggregate	-0.64***	-0.10	-0.21

***$p<0.001$; **$p<0.01$; *$p<0.05$.

[†]Gradient of induced soil degradation (Figure 3.2)

5. References

Berg, G., Roskot, N., Steidle, A., Eberl, L., Zock, A., Smalla, K., 2002. Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different Verticillium host plants. Applied and Environmental Microbiology 68, 3328-3338.

Bossio, D.A., Girvan, M.S., Verchot, L., Bullimore, J., Borelli, T., Albrecht, A., Scow, K.M., Ball, A.S., Pretty, J.N., Osborn, A.M., 2005. Soil microbial community response to land use change in an agricultural landscape of Western Kenya. Microbial Ecology 49, 50-62.

Bossio, D.A., Scow, K.M., 1998. Impacts of carbon and flooding on soil microbial communities: Phospholipid fatty acid profiles and substrate utilization patterns. Microbial Ecology 35, 265-278.

Bossio, D.A., Scow, K.M., Gunapala, N., Graham, K.J., 1998. Determinants of soil microbial communities: Effects of agricultural management, season, and soil type on phospholipid fatty acid profiles. Microbial Ecology 36, 1-12.

Brady, N.C., Weil, R.R., 2002. The nature and properties of soils. Prentice Hall, Upper Saddle River, NJ, 960 pp.

Buckley, D.H., Schmidt, T.M., 2001. The structure of microbial communities in soil and the lasting impact of cultivation. Microbial Ecology 42, 11-21.

Butler, J.L., Williams, M.A., Bottomley, P.J., Myrold, D.D., 2003. Microbial community dynamics associated with rhizosphere carbon flow. Applied and Environmental Microbiology 69, 6793-6800.

Cookson, W.R., Osman, M., Marschner, P., Abaye, D.A., Clark, I., Murphy, D.V., Stockdale, E.A., Watson, C.A., 2007. Controls on soil nitrogen cycling and microbial community composition across land use and incubation temperature. Soil Biology & Biochemistry 39, 744-756.

Dane, J.H., Hopmans, J.W., 2002. Water retention and storage. In: Dane, J.H., Topp, G.C. (Eds.), Methods of soil analysis. Part 4, Physical methods. SSSA, Madison, pp. 688-690.

Degens, B.P., Schipper, L.A., Sparling, G.P., Vojvodic-Vukovic, M., 2000. Decreases in organic C reserves in soils can reduce the catabolic diversity of soil microbial communities. Soil Biology & Biochemistry 32, 189-196.

Diosma, G., Aulicino, M., Chidichimo, H., Balatti, P.A., 2006. Effect of tillage and N fertilization on microbial physiological profile of soils cultivated with wheat. Soil & Tillage Research 91, 236-243.

Doran, J.W., Elliott, E.T., Paustian, K., 1998. Soil microbial activity, nitrogen cycling, and long-term changes in organic carbon pools as related to fallow tillage management. Soil & Tillage Research 49, 3-18.

Edwards, U., Rogall, T., Blöcker, E.M., Böttger, E.C., 1989. Isolations and direct complete nucleotide determination of entire genes. Characterization of a gene coding 16S ribosomal RNA. Nucleic Acids Research 17, 7843-7852.

Elliott, E.T., 1986. Aggregate structure and carbon, nitrogen, and phosphorus in native and cultivated soils. Soil Science Society of America Journal 50, 627-633.

Embrapa, 1997. Manual de métodos de análise de solo. Embrapa CNPS, Rio de Janeiro, 212 pp.

Frostegård, Å., Bååth, E., 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biology and Fertility of Soils 22, 59-65.

Garland, J.L., Mills, A.L., 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-utilization. Applied and Environmental Microbiology 57, 2351-2359.

Gomez, E., Bisaro, V., Conti, M., 2000. Potential C-source utilization patterns of bacterial communities as influenced by clearing and land use in a vertic soil of Argentina. Applied Soil Ecology 15, 273-281.

Gomez, E.D., Garland, J.L., Roberts, M.S., 2004. Microbial structural diversity estimated by dilution-extinction of phenotypic traits and T-RFLP analysis along a land-use intensification gradient. FEMS Microbiology Ecology 49, 253-259.

Grayston, S.J., Campbell, C.D., Bardgett, R.D., Mawdsley, J.L., Clegg, C.D., Ritz, K., Griffiths, B.S., Rodwell, J.S., Edwards, S.J., Davies, W.J., Elston, D.J., Millard, P., 2004. Assessing shifts in microbial community structure across a range of grasslands of differing management intensity using CLPP, PLFA and community DNA techniques. Applied Soil Ecology 25, 63-84.

Gros, R., Monrozier, L.J., Bartoli, F., Chotte, J.L., Faivre, P., 2004. Relationships between soil physico-chemical properties and microbial activity along a

restoration chronosequence of alpine grasslands following ski run construction. Applied Soil Ecology 27, 7-22.

Grossman, R.B., Reinsch, T.G., 2002. Bulk density and linear extensibility. In: Dane, J.H., Topp, G.C. (Eds.), Methods of soil analysis. Part 4, Physical methods. SSSA, Madison, pp. 201-225.

Hackl, E., Zechmeister-Boltenstern, S., Bodrossy, L., Sessitsch, A., 2004. Comparison of diversities and compositions of bacterial populations inhabiting natural forest soils. Applied and Environmental Microbiology 70, 5057-5065.

Jackson, L.E., Calderon, F.J., Steenwerth, K.L., Scow, K.M., Rolston, D.E., 2003. Responses of soil microbial processes and community structure to tillage events and implications for soil quality. Geoderma 114, 305-317.

Kohler, F., Hamelin, J., Gillet, F., Gobat, J.M., Buttler, A., 2005. Soil microbial community changes in wooded mountain pastures due to simulated effects of cattle grazing. Plant and Soil 278, 327-340.

Kowalchuk, G.A., Buma, D.S., de Boer, W., Klinkhamer, P.G.L., van Veen, J.A., 2002. Effects of above-ground plant species composition and diversity on the diversity of soil-borne microorganisms. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 81, 509-520.

Kruskal, J.B., 1964a. Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. Psychometrika 29, 1-27.

Kruskal, J.B., 1964b. Nonmetric multidimensional scaling: a numerical method. Psychometrika 29, 115-129.

Lupwayi, N.Z., Clayton, G.W., Rice, W.A., 1998. Soil microbial diversity and community structure under wheat as influenced by tillage and crop rotation. Soil Biology & Biochemistry 30, 1733-1741.

Muyzer, G., Teske, G., Wirsen, C.O., Jannasch, H.W., 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Archives of Microbiology 164, 165-172.

Nelson, D.W., Sommers, L.E., 1996. Total carbon, organic carbon, and organic matter. In: Sparks, D.L. (Ed.), Methods of soil analysis. Part 3, Chemical methods. SSSA, Madison, WI, pp. 961-1010.

Nimmo, J.R., Perkins, K.S., 2002. Aggregate stability and size distribution. In: Dane, J.H., Topp, G.C. (Eds.), Methods of soil analysis. Part 4, Physical methods. SSSA, Madison, pp. 317-328.

Nsabimana, D., Haynes, R.J., Wallis, F.M., 2004. Size, activity and catabolic diversity of the soil microbial biomass as affected by land use. Applied Soil Ecology 26, 81-92.

Olsson, P.A., Bååth, E., Jakobsen, I., Söderström, B., 1995. The use of phospholipid and neutral lipid fatty acids to estimate biomass of arbuscular mycorrhizal fungi in soil. Mycological Research 99, 623-629.

Reynolds, W.D., Elrick, D.E., Youngs, E.G., 2002. Single-ring and double- or concentric-ring infiltrometers. In: Dane, J.H., Topp, G.C. (Eds.), Methods of soil analysis. Part 4, Physical methods. SSSA, Madison, pp. 821-826.

Rich, J.J., Heichen, R.S., Bottomley, P.J., Cromack Jr., K., Myrold, D.D., 2003. Community composition and functioning of denitrifying bacteria from adjacent meadow and forest soils. Applied and Environmental Microbiology 69, 5974-5982.

Sinsabaugh, R.L., Klug, M.J., Collins, H.P., Yeager, P.E., Peterson, S.O., 1999. Characterizing soil microbial communities. In: Robertson, G.P., Coleman, D.C., Bledsoe, C.S., Sollins, P. (Eds.), Standard soil methods for long-term ecological research. Oxford University Press, New York.

Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., Roskot, N., Heuer, H., Berg, G., 2001. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: Plant-dependent enrichment and seasonal shifts revealed. Applied and Environmental Microbiology 67, 4742-4751.

Torsvik, V., Ovreas, L., Thingstad, T.F., 2002. Prokaryotic diversity - Magnitude, dynamics, and controlling factors. Science 296, 1064-1066.

White, D.C., Pinkart, H.C., Ringelberg, D.B., 1997. Biomass measurements: biochemical approaches. In: Hurst, C.J., Knudsen, G.R. (Eds.), Manual of Environmental Microbiology. ASM Press, Washington, DC, pp. 91-101.

Widmer, F., Fliessbach, A., Laczko, E., Schulze-Aurich, J., Zeyer, J., 2001. Assessing soil biological characteristics: a comparison of bulk soil community DNA-, PLFA-, and Biolog (TM)-analyses. Soil Biology & Biochemistry 33, 1029-1036.

Woods, L., 1989. Active organic matter distribution in the surface 15 cm of undisturbed and cultivated soil. Biology and Fertility of Soils 8, 272-278.

Yan, F., McBratney, A.B., Copeland, L., 2000. Functional substrate biodiversity of cultivated and uncultivated A horizons of vertisols in NW New South Wales. Geoderma 96, 321-343.

Yoder, R.E., 1936. A direct method of aggregate analysis of soils and a study of the physical nature of erosion losses. Journal of American Society of Agronomy 28, 337-351.

Zelles, L., 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterization of microbial communities in soil: a review. Biology and Fertility of Soils 29, 111-129.

Zelles, L., Bai, Q.Y., Beck, T., Beese, F., 1992. Signature fatty acids in phospholipids and lipopolysaccharides as indicators of microbial biomass and community structure in agricultural soils. Soil Biology & Biochemistry 24, 317-323.

Zibilske, L.M., 1994. Carbon Mineralization. In: Weaver, R.W., Angle, J.S., Bottomley, P.S. (Eds.), Methods of soil analysis. Part 2, Microbiological and biochemical properties. SSSA, Madison, pp. 835-863.

Development and validation of a soil quality index based on the equilibrium between soil organic matter and biochemical properties in an undisturbed forest ecosystem

Chapter 4

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Abstract

Recent studies have suggested that the organic matter contents of undisturbed soils (under natural vegetation) are in equilibrium with biological and biochemical properties. Accordingly, such equilibrium should be disrupted when a soil is subjected to disturbance or stress. Measurement of this disruption can be expressed mathematically and used as a soil quality index. In this study, we evaluated these hypotheses in soils from the H.J. Andrews Experimental Forest in Oregon. Both O and A horizons were sampled from nine sites in Spring 2005 and Fall 2006. Soil samples were analyzed for enzyme activities (phosphatase, β-glucosidase, laccase, N-acetyl glucosaminidase, protease and urease), N-mineralization, respiration, microbial biomass C (MBC), and soil organic carbon (SOC) content. In addition, soil samples from one old-growth site were manipulated in the laboratory to simulate stresses (Cu addition or pH alteration) and disturbances (wet-dry or freeze-thaw cycles). The results showed variation in biological and biochemical soil properties that were closely correlated with the SOC. Multiple regression analysis of SOC against all properties showed that MBC and phosphatase activity explained 97% of the SOC variation among the sites. The model fit was independent of spatial and temporal variations because covariates such as site, stand age, sampling date, and soil horizon were not statistically significant. The application of stress/disturbance treatments inconsistently affected most of the individual biochemical properties. In contrast, the ratio between soil C predicted by the model (C_p), and soil C measured (C_m) was consistently reduced in soils submitted to at least one level of stress and

disturbance treatments. In addition, C_p/C_m was more affected in soils submitted to wet-dry cycles and Cu contamination than to freeze-thaw cycles or shifts in soil pH. Our results confirm previous evidence of a biochemical balance in high quality undisturbed soils, and that this balance is disrupted when the soil is submitted to disturbances or is under stress conditions. The reliability of the C_p/C_m ratio as a soil quality index is discussed in the text.

Keywords

Biochemical soil properties; Biochemical index; Soil enzymes; Soil stress; Soil disturbance; Oregon forest soils

Introduction

Biochemical soil properties have been widely used as indicators of soil quality because of their essential role in soil biology, ease of measurement, and sensitivity to environmental change compared to most physical and chemical properties (Dick et al., 1996; Riffaldi et al., 2002; Miralles et al., 2007). However, the use of these parameters as indicators of soil quality, both individually and combined, has been criticized due to the lack of reference values, their contradictory behaviors when a soil is degraded, and the seasonal and regional variations in expression levels (Nannipieri, 1994; Trasar-Cepeda et al., 2000; Spedding et al., 2004; Gil-Sotres et al., 2005).

Aiming to address these limitations, Trasar-Cepeda et al. (1998) proposed a new approach to determine a biochemical-based index of soil quality that dispenses

with the need for reference data, and that is independent of both seasonal and among-site variations in soil conditions. They showed that in undisturbed native soils under climax vegetation there is an equilibrium between soil organic matter content and biological activity. The equilibrium was expressed by an equation which defined total soil N content as a function of microbial biomass C, N mineralization capacity, and the activities of phosphatase, β-glucosidase and urease. Assuming that soils under climax vegetation are considered to be of high quality (Fedoroff, 1987; Doran et al., 1994), and that the equation was statistically very significant (R^2=0.97, p<0.001), the authors suggested that any disturbance or stress on the soil will modify this relationship in such a way that the total N calculated from the equation (Nc) will be lower or higher than the total soil N content measured by Kjeldahl digestion (Nk).

Further studies demonstrated the validity of the Nc/Nk ratio to indicate soil degradation or disturbance in soils affected by management, mining, or contaminated with organic effluents and with heavy metals (Leiros et al., 1999; Trasar-Cepeda et al., 2000). These studies showed that in all situations soil degradation was reflected by the Nc/Nk value, whereas none of the individual biochemical parameters responded consistently to the factors influencing soil quality. The Nc/Nk distinguished among biochemically balanced soils, soils in a transient state of high microbiological and biochemical activity (i.e. soils that received application of fertilizers or products that stimulate microbial activity) and truly degraded soils (Leiros et al., 1999). Finally, the approach distinguished between the effects of current pollutants versus prior soil degradation, and differentiated between pollutants

in regard to their ability to cause different degrees of soil degradation (Trasar-Cepeda et al., 2000).

Despite these promising results, similar studies have not been conducted in soils other than those where the original model was developed (Galicia, Spain). Therefore, it is uncertain whether other undisturbed high quality soils also express an equilibrium between soil organic matter and biological/biochemical properties, and whether it is disrupted when these soils are degraded. In this work, we aimed to evaluate these hypotheses in soils under forest vegetation in the Western Cascade Mountains of Oregon. Multiple regression analysis was used to define a model able to predict the soil C content based on soil biochemical and biological data obtained from a set of forest sites which had not been subjected to human intervention for at least 40 years. In analogy to the Nc/Nk ratio, we evaluated the validity of the ratio between soil C predicted by the model (C_p), and soil C measured (C_m) as a soil quality index. This was accomplished by accessing changes of C_p/C_m in an undisturbed soil submitted either to chemical stress (Cu and pH), or to physical disturbance (freeze-thaw and wet-dry cycles) treatments.

Materials and methods

This study was conducted with soils from the H.J. Andrews Experimental Forest (HJA) situated in the Western Cascades of Oregon (lat. 44°15´N, long. 122°10´W) in the 6,400 ha watershed of Lookout Creek, a tributary of Blue River and the McKenzie River. Mean annual temperature at the headquarters site of HJA is

8.7^{o}C (1973-2002) and annual precipitation during the same period is 2370 mm y^{-1}, mostly as rain (Brant et al., 2006). About 90% of the annual precipitation falls between October and April with the wettest month being December and peak drought conditions occurring in July (Franklin et al., 1990).

Soil samples were collected during late spring of 2005 and early fall of 2006 from four old growth and five second growth sites. The old growth and second growth sites contained trees that were more than 300 and approximately 40-50 years old, respectively (except the Red alder site replanted in 1987). Other site properties (elevation, soil texture, pH, and vegetation cover) are presented in Table 4.1. Composite soil samples from both the O and A horizons were collected from four positions at each site along the main slope gradient providing four pseudoreplications per site per season. The O horizon was sampled manually by collecting the organic material in the soil surface after removing the undecomposed litter. The A horizon was sampled in the 0 to 5 cm mineral soil layer using a trowel. No soil was collected from locations where ectomycorrhyzal mats were visually identified. After collection, samples were refrigerated, transported in a cooler, sieved (4 and 2 mm opening sieves for O and A horizons, respectively), and stored at 4^{o}C. All analyses were performed within one week of sampling.

Stress/disturbance treatments

Soil from one HJA old-growth site (site 37 – Table 4.1) was manipulated in the laboratory to simulate stress and disturbance. This soil was re-sampled in April

2007 by collecting a composite sample from the A horizon (0 to 5 cm layer). The soil was air dried, sieved through a 2-mm sieve, divided into 72 portions, each equivalent to 40 g of oven-dry soil, and placed into 150-ml flasks. This approach provided 64 experimental units for four treatments (with four levels of each treatment) and two sets of controls.

Stress treatments consisted of either changing pH or increasing soil Cu concentrations, and disturbance treatments consisted of wet-dry or freeze-thaw cycles. These treatments were applied according to (Degens et al., 2001) with some modifications. For pH stress, the current soil pH (5.5) was increased or decreased by one or two pH units (3.5, 4.5, 6.5, and 7.5) using dilute solutions of NaOH (0.5 M) or HCl (1 M), respectively. The amounts of NaOH and HCl necessary to attain each pH level were determined from pH-buffer curves. Different levels of Cu were attained by applying $CuSO_4$ at rates of 50, 200, 800 and 3200 μg Cu g^{-1} soil. Decreases in soil pH caused by $CuSO_4$ additions were corrected by adding solutions of NaOH to adjust the pH back to initial levels.

Disturbance simulated by wet-dry cycles consisted of air-drying with fan-forced air at 20-25^{o}C for 24 h followed by rapid rewetting to 60% of the water holding capacity (WHC) and incubation for 24 h in the dark. Soil samples were subject to one, two, four, or eight successive wet-dry cycles. A separate set of soils was subjected to one, two, four, or eight successive freeze-thaw cycles. Each freeze-thaw cycle consisted of freezing at -20^{o}C for 24 h followed by thawing and incubation at 25^{o}C and 60% WHC for 24 h.

Following application of both pH and Cu treatments, soils were incubated at 25^{o}C and 60% WHC for 4 weeks. For the two disturbance treatments, soils were incubated for 15 d counted from the last drying or freeze cycle (5 weeks total). This time interval was considered adequate to allow sufficient re-equilibration of the microbial communities after imposition of each disturbance or stress treatment and for decomposition of organic C released from microorganisms killed by the treatments (Degens et al., 2001). Each treatment was applied to four replicates. The two sets of controls (one for each group of stress and disturbance treatments) were continuously incubated at 25^{o}C and 60% WHC.

Soil analyses

Gravimetric water content was determined after drying samples at 54^{o}C for 36 h. pH was determined on a pH meter after equilibrating 10 g of wet soil for 60 min with 30 ml of deionized water. Soil organic matter content was measured as loss of weight on ignition after heating oven-dry soil samples at 430^{o}C for 24 h. A conversion factor of 1.724 was used to convert organic matter to soil organic carbon (SOC) (Nelson and Sommers, 1996).

Soil enzymes

Enzyme assays were performed by mixing 10 g of wet soil in 30 ml of deionized water and creating soil slurries. The resulting slurries were continuously stirred on a magnetic stir plate while 1 ml aliquots were dispensed into test tubes (2 replicates per sample per assay). Activities of β-glucosidase (β-GLC), phosphatase

(PME) and N-acetyl-glucosaminidase (NAG) were measured by conventional *p*-nitrophenyl-ester based assays (Tabatabai, 1994; Parham and Deng, 2000), with some modifications proposed by Caldwell et al. (1999). One ml aliquots of soil slurry were incubated with 1 ml of either 10 mM *p*-nitrophenyl-β-glucoside, 50 mM *p*-nitrophenyl-phosphate or 10 mM *p*-nitrophenol-N-acetylglucosamide at 30^{o}C. The PME activity was run without the conventional buffer (Tabatabai, 1994) to measure enzyme activity at soil pH (Caldwell et al., 1999). After 1 h, 0.5 ml 0.5 M $CaCl_2$ was added and reactions were terminated by adding either 2 ml of 0.1 M pH 12 tris(hydroxymethyl)aminomethane to the β-GLC assay or 2 ml of 0.5 M NaOH to the PME and NAG assays. Controls consisted of slurry without substrate and substrate without slurry. After centrifugation, supernatant *p*-nitrophenol (*p*-NP) concentrations were measured at 410 nm, and enzyme activities were calculated on a dry weight basis.

Phenol oxidase (PhOx) activity was measured spectrophotometrically by adding to each soil slurry 1 ml of 5 mM L-3,4-dihydroxyphenylalanine (DOPA) prepared in 50 mM acetate buffer pH 5 (Sinsabaugh et al., 1999). Controls were performed by adding 1 ml of acetate buffer to soil slurries. After 1 h of incubation at 30^{o}C, 1 ml of 0.6% sodium azide solution was added to stop the reaction. The suspensions were centrifuged and the activity was quantified in the supernatant by measuring absorbance at 460 nm. Calculation of the quantity of diqc (dihydroindole-quinone-carboxylate) released was based on a micromolar extinction coefficient of 1.6 (Sinsabaugh et al., 1999).

Protease (PRT) activity was determined as the rate of tyrosine equivalents released from casein substrate according to (Ladd and Butler, 1972) with the following modifications. One ml of soil slurry was added to 1 ml of caseinate (0.2 g l^{-1}) + sodium azide (0.1 g l^{-1}) solution and incubated at 30^{o}C. After 24 h the reaction was stopped with 1 ml of trichloroacetic acid. The concentration of tyrosine equivalents in 1 ml aliquots of supernatant was determined colorimetrically (578 nm) using Folin–Ciocalteau's phenol reagent, diluted threefold (Lowry et al., 1951). Tyrosine standards were treated in the same way as the samples. Control consisted of substrate solution mixed with 1 ml of dH_2O instead of soil slurry. Activity was expressed as µg of tyrosine equivalents released per gram of dry soil per hour.

Urease (URE) activity was measured by determination of ammonium released after the incubation of soil samples with a buffered urea solution for 2 h at 37^{o}C (Kandeler and Gerber, 1988). Ammonium was determined with an Astoria Pacific series 300 autoanalyzer (Astoria-Pacific, Inc., Clackamas, Oreg.).

Soil respiration and net N mineralization

Portions of soil (30 and 15 g for A and O horizons, respectively) were added to 500-ml mason jars and the water contents adjusted to 70% of water holding capacity. The jars were sealed with caps containing a rubber septum and incubated at 25^{o}C for 20 days. At 5-day intervals, the air in the overhead space was sampled from each jar and CO_2 determined by chromatography. After each reading the jars were left open for half an hour to ventilate and then re-sealed.

The rate of net N mineralization was determined by measuring the difference in inorganic nitrogen levels in soil samples before and after 20 days of incubation. The soil samples were the same as used to measure soil respiration. Extractions were performed on 5 g portions of wet soil with 15 ml of 2M KCl. Aliquots of the filtrate were analyzed for NH_4^+ and NO_3^- with an autoanalyzer. Nitrogen mineralization was expressed as μg of mineral N produced per gram of dry soil per day.

Microbial biomass C

Microbial biomass C (MBC) was determined by the chloroform fumigation extraction method, using 0.5 M K_2SO_4 as extractant (Vance et al., 1987). The organic C of extracts was estimated by combustion. The difference in C content between the fumigated and unfumigated extracts was converted to MBC by applying a factor (K_c) of 0.45 (Jenkinson, 1988). Results were expressed as mg of MBC per gram of dry soil. The metabolic quotient, qCO_2 (microbial respiration per unit of biomass), was calculated and expressed in μg CO_2-C mg^{-1} biomass C d^{-1} (Anderson and Domsch, 1985).

All chemical, biochemical and biological soil properties described above were analyzed in soil samples collected during spring 2005. Analyses of this data set revealed the best predictors of SOC (details in the "statistical analyses" section). Therefore, besides SOC, all samples collected afterwards were analyzed only for PME, β-GLU, PhOx, respiration and MBC. These included samples from fall/2006 and those subjected to stress and disturbance treatments.

Statistical analyses

Analytical results obtained from site pseudoreplicates within each sampling date were pooled before the statistical analysis.

Linear multiple regression was used to develop an equation (model) able to predict total soil carbon (SOC) based on the chemical and biochemical soil properties analyzed. All variables were subjected to natural logarithmic transformation (except N-MIN which included negative values) in order to assure homoscedasticity and linearity between the dependent and the explanatory variables. A preliminary model was selected using the spring 2005 data set which included a total of 10 explanatory variables. The Mallows' Cp Statistic (Mallows, 1973) was used to provide the best subset selection of predictors by examination of all possible regressions. The best model was considered that with the smallest number of parameters with acceptable "p-values", and a Cp value closest to the number of parameters. Alternative models were also selected after removing each one of the previously selected predictors from the data set. For example, if the variables "A" and "B" were selected using the original data set, two alternative models were defined using the same pool of original variables but excluded either "A" or "B". The selected predictors in both main and alternative models were the ones analyzed in soil samples collected in the fall 2006 and in the soils submitted to stress and disturbances.

A second data set which included both spring 2005 and fall 2006 samples was used to evaluate the consistency of the predictors previously selected and the

definition of a final model. The same model selection procedure described above was applied to the second data set. The robustness of the selected model to temporal and spatial variation was evaluated by testing the statistical significance of sampling date, site, stand age, soil horizon, and vegetation cover when these were added as covariates. Multicollinearity among the model predictors was evaluated by the variance inflation factor (VIF) (Marquard, 1970) and using 10 as cutoff value.

A two-sided multiple comparison with a control (MCC) test was used to evaluate the effects of stresses and disturbances on soil biochemical properties, and on the ratio between the SOC predicted by the model (C_p) and the SOC measured (C_m). All statistical analyses were performed using S-Plus 8.0 (Insightful Corp.).

Results

Variation in soil properties

Total soil organic carbon (SOC) measured among the sites ranged from 10 to 19% (wt/wt) in the 0 to 5 cm of the A horizon, and from 23 to 45% in the O horizon (Table 4.2). Variations in soil biochemical properties within soil horizon were generally higher than for SOC. For instance, the coefficient of variation (CV) for SOC in both soil horizons was about 19%, whereas the CV for N mineralization ranged from 97 to 429%, and for qCO_2 and soil enzymes, such as phosphatase, β-glucosidase, and protease from 30 to 40%. Variations between soil horizons were also higher for biochemical properties than for SOC. The O/A horizon ratio was 2.9

for SOC, and generally above 3.5 for soil enzymes and above nine for microbial biomass C (MBC) and soil respiration (Table 4.2).

Few differences in soil properties were observed when comparing old growth and second growth sites. For example, the former had about 30% more SOC, MBC, and phenol oxidase activity in the O horizon than the second growth sites ($p<0.05$). This same trend was observed for the activities of β-glucosidase and phosphatase, but at a lower statistical significance level ($p<0.10$).

Biochemical soil properties also presented high seasonal variation. In general, soil samples collected during the fall/2006 had lower microbial activity (respiration) compared to spring/2005; yet their microbial biomass values were either similar (O horizon) or higher (A horizon) than those observed during the Spring (Table 4.3). The highest seasonal variations in the A horizon occurred in MBC, qCO_2, and phosphatase activity, whereas the same was valid for soil respiration and qCO_2 in the O horizon.

Correlation between SOC and biochemical properties

Most of the biochemical properties were highly correlated with SOC (Table 4.4). The highest significant correlation coefficients were obtained for phosphatase activity (r = 0.978), MBC and soil respiration (both with r = 0.971) whereas the lowest one was for urease activity (r=0.758). Only N-mineralization and qCO_2 were not correlated with SOC.

Selection of SOC predictors

The analysis of the spring 2005 data set showed that phosphatase activity (PME) and microbial biomass C (MBC) were the most reliable SOC predictors based on the Mallow's Cp statistic analysis (Table 4.5). The equation fitted through linear multiple regression using these two variables was able to explain 98% of the SOC variation of the studied sites. PME and MBC were also selected as the best SOC predictors when data from both spring 2005 and fall 2006 were considered. In this case, the two variables were able to explain 97% of the SOC variation using the following equation:

(1) $\ln(\text{total C}) = 1.709 + 0.280 \ln(\text{PME}) + 0.285 \ln(\text{MBC})$

where total C is expressed as a percentage, PME in μmol of PNP g^{-1} h^{-1} and MBC in mg biomass-C g^{-1} of soil. This model was shown to be robust to seasonal and spatial variation in that none of the tested covariates (sampling date, site, stand age, soil horizon, and type of vegetation cover) were statistically significant when added to the model above.

Alternative sets of predictors were also evaluated after omitting either PME or MBC from the original data sets. For the spring 2005 data set, soil respiration and phenol oxidase activity were selected as substitutes for MBC and PME, respectively; for the clustered spring and fall data set, soil respiration was the most reliable predictor as substitute of either PME or MBC (Table 4.5). Nevertheless, all models fitted with the alternative data sets presented limitations associated with high

multicollinearity among predictors (VIF > 10), or spatial and temporal dependency as indicated by significant statistical *p*-values for some of the tested covariates.

Effect of stress and disturbance on biochemical soil properties

Stress and disturbance treatments simulated on soil samples from site 37 caused different effects on the biological and biochemical properties. The application of one, two, four or eight freeze-thaw cycles reduced significantly β-glucosidase activity and MBC (Table 4.6). However, changes in β-glucosidase were not proportional to the disturbance levels as occurred for MBC. Phosphatase, phenol oxidase, respiration, and qCO_2 activities did not respond to freeze and thaw cycles. When disturbances were applied as wet-dry cycles, MBC was significantly reduced by all treatment levels applied, whereas the activities of the three enzymes were only affected after four or more wet-dry cycles. This treatment did not cause significant change to soil respiration or qCO_2 (Table 4.6).

Although addition of Cu and modification of soil pH affected all five biochemical soil properties (Table 4.6), there was either no consistency in change in response to the stress or it was in the opposite direction from that observed after disturbance treatments. For example, soil respiration activity was increased by the two lowest Cu doses, and decreased by the two highest ones. Moreover, qCO_2, β-glucosidase and phenol oxidase activities tended to increase with the increase of Cu in the soil, whereas MBC and phosphatase activity decreased. Increase of soil pH from 5.5 to 6.5 or 7.5 also increased the phenol oxidase activity in addition to soil

respiration and MBC. However, a shift to pH 4.5 or 3.5 increased significantly the activities of all three soil enzymes, especially of phenol oxidase and β-glucosidase, and decreased soil respiration, MBC and qCO_2.

Changes in the C_p/C_m *ratio*

The ratio between soil C predicted by equation 1 and soil C actually measured (C_p/C_m) varied consistently with the levels of stress and disturbances. The C_p/C_m was close to unity in the two sets of controls, and tended to decrease with the increase of the stress or disturbance applied (Figure 4.1). In all disturbance and stress simulations there was no significant change in SOC relative to controls after the incubation period. Therefore, the decreases observed in C_p/C_m resulted exclusively from an underestimation of the predicted C (C_p).

Application of four and eight freeze-thaw cycles decrease significantly ($p<0.05$) C_p/C_m from 0.99 in the control to 0.93 and 0.92, respectively (Figure 4.1A). Wet-dry cycles caused a more drastic effect on soil, decreasing C_p/C_m from 0.93 after one freeze-thaw cycle to 0.85 after eight cycles (Figure 4.1B). Soils treated with Cu also showed significant decrease in C_p/C_m, but only in the two highest doses applied (800 or 3200 μg g^{-1} Cu). Notwithstanding, the application of the highest dose changed C_p/C_m to 0.68, the lowest value among all stress and disturbance simulations (Figure 4.1D). Increase in soil pH did not produce changes in C_p/C_m; though, a significant decrease in this ratio was observed when the soil pH was shifted to 3.5 (Figure 4.1C).

Discussion

The high degree of variability of soil biological and biochemical properties due to climate, season, geographical location, and pedogenetic factors has been cited as the main problem which severely limits their use as soil quality indicators (Gil-Sotres et al., 2005). Such high spatial and temporal variation was demonstrated in this and several other studies (Trasar-Cepeda et al., 1998; Leiros et al., 2000; Chen et al., 2003; Miralles et al., 2007). Notwithstanding, most biological and biochemical soil properties were highly correlated with soil organic C (SOC) despite differences in soil horizon, season, and sampled sites. Significant correlation coefficients between SOC and soil biochemical properties have been also described in other forest and agricultural soils (Perucci et al., 1997; Trasar-Cepeda et al., 1998; Leiros et al., 2000; Chodak et al., 2003; Miralles et al., 2007), but values as high as the ones found in this study have been rarely reported (e.g., Haynes (1999)).

In fact, SOC plays an important role in determining the size of the microbial biomass and the level of soil enzyme activity. Higher contents of SOC can support more biological activity because the energy and available nutrient status in soils are expected to be enhanced (Nourbakhsh, 2007). Our results demonstrated that such a relationship between SOC and biological and biochemical properties is in equilibrium in high quality soils (undisturbed native soils) as previously suggested by Trasar-Cepeda et al. (1998). This equilibrium was expressed in a spatially and temporally robust model that could explain 97% of the SOC variation in the HJA forest soils using only microbial biomass C (MBC) and phosphatase activity as

predictors (Table 4.5; Eq. 1). The adjustment of this model was as high as that obtained by Trasar-Cepeda et al. (1998) to express the total soil N of undisturbed soils in Galicia, Spain. Nevertheless, their model required five biological and biochemical properties as predictors which also included MBC and phosphatase activity. This fact confirms the close association of these two properties with SOC in undisturbed soils of different ecosystems.

Trasar-Cepeda et al. (1998) suggested that the equilibrium between total N and biochemical properties expressed in their model would be disrupted by variations in the biochemical quality of soils as a result of degradation processes. Subsequent studies showed that in agricultural and polluted soils the ratio between the calculated total soil N (Nc) using the equation previously defined, and the total N obtained by Kjeldahl method (Nk) was consistently altered relative to undisturbed soils (Leiros et al., 1999; Trasar-Cepeda et al., 2000; Miguéns et al., 2007). Therefore, the Nc/Nk ratio was proposed as an index of the biochemical quality of soils. By analogy, we tested the validity of the ratio between soil C predicted by the equation 1 (C_p), and soil C measured (C_m) to indicate the degradation caused by disturbances and stresses applied to one of the HJA forest soils. The ability of both C_p/C_m and individual biochemical properties to respond to these treatments is discussed in the following sections.

Response of individual soil properties to stresses and disturbances

A suitable soil quality indicator must be: (a) sensitive to as many degrading agents as possible; (b) show a consistent directional change in response to a given

contaminant, and (c) be able to reflect different levels of degradation (Elliott, 1997). Most of the biological and biochemical properties analyzed in soils submitted to stresses and disturbances failed in at least one of these requirements (Table 4.6). For instance, β-glucosidase, phosphatase, phenol oxidase, respiration and qCO_2 activities were insensitive or unable to differentiate the levels of freeze-thaw disturbances. Additionally, the decrease of qCO_2 with soil acidification was contrary to that trend observed in another study (Blagodatskaya and Anderson, 1999). Soil enzymes also expressed complex responses to the stress treatments. For example, β-glucosidase and phosphatase activities increased when soil pH was lowered, but they responded in opposite directions to addition of Cu with β-glucosidase being stimulated whereas phosphatase was inhibited. An increase of β-glucosidase activity in response to Cu was also observed in soils incubated in the laboratory with up to 10000 μg g^{-1} of Cu for 4 weeks (Leiros et al., 1999). In contrast, heavy metal contaminated sites (Cd, Zn, Cu and Pb) showed reduced activity of this enzyme compared to uncontaminated ones (Lee et al., 2002). Complex and inconsistent behavior of soil enzymes has also been described in soils polluted by different types of contaminants (Trasar-Cepeda et al., 2000; Gianfreda et al., 2005).

Microbial biomass C was the only variable that changed consistently in response to all stress and disturbance treatments. MBC was also considered to be the most reliable biochemical parameter for 41% of authors in a list of reviewed published papers where individual properties were studied as soil quality indicators (Gil-Sotres et al., 2005). Nevertheless, this review also describes a series of results

showing contradictory responses of MBC to soil management or contamination. In another study, MBC was not affected or slightly increased in a cropped and a pasture soils submitted to similar pH and Cu stresses or wet-dry and freeze-thaw disturbances (Degens et al., 2001). The validity of MBC as a reliable indicator of soil quality can be also questioned when its high spatial and seasonal variability in natural and agricultural soils are considered (Murphy et al., 1998; Corre et al., 2002; Chen et al., 2003). This fact prevents the definition of reference values for MBC that are necessary to access the magnitude of soil degradation relative to its previous state.

Validity and sensitivity of C_p/C_m as a soil quality index

The ratio C_p/C_m was consistently reduced by the increasing levels of stress and disturbances applied to the soils (Figure 4.1). In addition, C_p/C_m was able to discriminate which of the disturbance/stress treatments caused more soil degradation. For example, soils treated with wet-dry cycles and Cu seemed to be more affected than those treated with freeze-thaw cycles, and shifts in pH, respectively (Figure 4.1). Wet-dry cycles also caused greater declines in microbial catabolic evenness of a cropped soil compared to freeze-thaw cycles (Degens et al., 2001). However, this microbial parameter underwent a greater decline in response to soil acidification than to increase in Cu concentration up to 1000 μg Cu g^{-1} soil. The negligible alteration in C_p/C_m in response to increase in soil pH from 5.5 to up to 7.5 suggests that this treatment did not alter the soil biochemical balance. Such a response was not

surprising if the greater negative effects of soil acidification on a microbial community are considered relative to neutral conditions (Blagodatskaya and Anderson, 1999).

Considering our results, C_p/C_m can be considered a reliable biochemically-based soil quality index. In contrast to individual biological and biochemical soil properties, the C_p/C_m ratio provides a simple reference value against which the degrading effects of pollutants or management practices on soil quality can be readily accessed. Therefore, C_p/C_m will be 1 in high quality, undisturbed soils and will increase or decrease according to the intensity of soil degradation. Nevertheless, it should be considered that the equilibrium between biological and biochemical soil properties expressed by equation 1 might not be valid in other undisturbed soils, or these soils might better express this relationship with different biological and biochemical properties. Therefore, the use of C_p/C_m ratio as a soil quality index should be valid for the range of soils on which the equation was developed, or for other soils containing undisturbed fragments that were shown to have the same relationship expressed in equation 1. Furthermore, the 95% prediction interval for SOC calculated from the fit of equation 1 indicates that future values of C_p/C_m for the HJA undisturbed forest soils might range from 0.8 to 1.2. Thus, soils would only be considered significantly degraded at $p<0.05$ when C_p/C_m is outside this range of values. This certainly limits the C_p/C_m sensitivity as a soil quality index unless this ratio is known for a given soil prior to any kind of degradation process. For example, the effects of disturbances and stresses applied to the soil in this study were promptly

accessed by C_p/C_m because we could calculate the ratio for the non-treated control soils. Otherwise, only soils treated with the highest Cu dose, which had $C_p/C_m < 0.8$, would be considered degraded.

As a final point, it should be noted that the validity and consistency of the C_p/C_m response was tested under a limited range of stresses and disturbances simulated under laboratory conditions. It is likely that similar responses will occur in field conditions or as a result of other degrading agents. This was suggested in previous studies that showed changes in the Nc/Nk ratio in polluted and agriculturally-degraded soils (Leiros et al., 1999; Trasar-Cepeda et al., 2000; Miguéns et al., 2007). Nonetheless, further studies should evaluate the response of C_p/C_m to soil degradation under field conditions.

Conclusions

This study is the second report in the soil literature providing evidence for a biochemical balance in high quality undisturbed soils, which can be expressed by a simple mathematical model. In addition, our results confirm the hypothesis that such balance is disrupted when the soil is submitted to disturbances or is under stress conditions, as previously observed in other soils. Keeping in mind the considerations presented above, the use of the C_p/C_m ratio as a soil quality index is compelling because it provides a simple and straightforward interpretation of the status of soil degradation, and requires analysis of only a few soil properties.

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Table 4.1 Characterization of sampling sites at the H.J. Andrews Experimental Forest

Site	Elev.(m)	Texture	pH	Vegetation / management history
Old growth sites				
37	952	Silt loam	5.5	Douglas fir* (*Pseudotsuga menziesii*); vine maple (*Acer circinatum*)
41	976	Stoney loam	4.5	Douglas fir*; Pacific yew* (*Taxus brevifolia*); Pacific rhododendron (*Rhododendron macrophyllum*)
139	849	Stoney silty loam	5.2	Douglas fir*; western redcedar (*Thuja plicata*); western hemlock (*Tsuga heterophylla*)
170	784	Gravelly silty loam	4.5	Douglas fir*; western redcedar; vine maple
Second growth sites				
89	661	Clay loam	5.2	Douglas fir* / (clearcut 1954; burned, replanted 1957)
112	600	Gravelly silty clay loam	4.7	Douglas fir* / (clearcut 1959; burned; natural regeneration)
113	667	Silty clay loam	4.8	Douglas fir* / (clearcut 1959; burned; natural regeneration)
136	782	Gravelly silty loam	5.4	Douglas fir*; western redcedar / (clearcut 1954; buned; replanted 1967)
Alder	800	Loam	5.1	Red alder* (*Alnus rubra*) / (clearcut 1985; replanted to alder 1987)

* Dominant species in each site.
Data obtained from H.J. Andrews Experimental Forest data base (www.fsl.orst.edu/lter)

Table 4.2 Statistical descriptions of values of chemical and biochemical properties of the H.J. Andrews Experimental Forest soils (Table 4.1)

	A horizon (0-5 cm)				O horizon				O/A hor.†
	Min	Max	Mean	CV (%)	Min	Max	Mean	CV (%)	
Soil organic C (g kg^{-1})	98	192	124	18	232	448	354	19	2.9
β-glucosidase (μmol PNP g^{-1} h^{-1})	1.9	6.5	3.8	37	9.8	33	17	36	4.5
Phosphatase (μmol PNP g^{-1} h^{-1})	23	84	41	39	81	319	173	36	4.2
Glucosaminidase (μmol PNP g^{-1} h^{-1})	4.3	7.4	5.8	18	11	25	20	24	3.4
Laccase (mmol diqc g^{-1} h^{-1})	25	86	57	35	117	313	199	34	3.5
Urease (μg NH_4^+-N g^{-1} h^{-1})	50	86	67	17	72	260	176	38	2.6
Protease (μg Tyr g^{-1} h^{-1})	0.1	0.7	0.4	41	1.5	3.6	2.5	30	5.7
N mineralization (μg N g^{-1} d^{-1})	0.0	0.5	0.2	97	-0.8	0.5	-0.1	429	-0.6
Soil respiration (μg CO_2-C g^{-1} d^{-1})	23	54	35	26	115	513	346	35	9.9
Microbial biomass C (mg g^{-1})	0.3	0.9	0.5	26	2.3	6.9	4.8	24	9.3
qCO_2 (μg CO_2-C mg^{-1} biomass C d^{-1})	1.6	6.0	3.4	42	2.2	6.0	3.2	34	1.06

†Ratio between average values of O and A horizons

Table 4.3 Seasonal variation in biochemical soil properties expressed as a percentage of change that occurred in fall 2006 relative to spring 2005

	A horizon		O horizon	
	% change	CV (%)	% change	CV (%)
soil C	10	7	-5	3
β-glucosidase	-20	16	-16	13
Phosphatase	-28	23	5	4
Phenol oxidase	-10	7	-11	9
Respiration	-16	12	-34	29
Microbial biomass C	44	26	-2	1
qCO_2	-42	38	-37	32

Table 4.4 Correlation coefficients between soil organic carbon (SOC) and biological and biochemical soil properties

Soil property	SOC
Phosphatase	0.978*
Microbial biomass C	0.971*
Respiration	0.971*
N-acetyl glucosaminidase	0.942*
Phenol oxidase	0.935*
Protease	0.915*
β-glucosidase	0.894*
Urease	0.758*
N mineralization	-0.341
qCO_2	-0.064

*$p<0.001$

Table 4.5 Best set of prediction variables for SOC content defined by multiple regression analysis of the spring 2005 and spring 2005 + fall 2006 data sets

Data set	Selected variables	R^2	Cp[†]	VIF[‡]	df	Significant covariates
Spring	PME***; MBC**	0.979	7.6	8.9	13	-
Spring without MBC	PME**; RESP*	0.972	5.1	11.0	13	-
Spring without PME	MBC***; PhOx*	0.959	5.0	4.1	12	Stand age*
Spring+Fall (S+F)	PME***; MBC***	0.971	2.8	5.3	30	-
S+F without MBC	PME***; RESP***	0.950	7.0	6.7	30	Season***
S+F without PME	MBC***; RESP**	0.954	3.9	11.3	30	-

***p<0.001; **p<0.01; *p<0.05;
[†] Mallows' Cp Statistic (Mallows, 1973);
[‡] Variance inflation factor (Marquard, 1970).

Table 4.6 Effect of different disturbance and stress treatments on individual soil biological and biochemical properties. Control values are in bold. For units see Table 4.2.

	β-glucosidase	Phosphatase	Phenol oxidase	Respiration	MBC	qCO_2
Freeze-thaw cycles						
0	**1.99**	**33.4**	**0.13**	**19.1**	**0.36**	**0.53**
1	1.60*	35.6	0.13	15.5	0.33*	0.47
2	1.66*	35.1	0.13	16.4	0.32*	0.52
4	1.39*	31.6	0.12	16.3	0.30*	0.55
8	1.67*	34.3	0.13	15.9	0.27*	0.59
Wet-dry cycles						
0	**1.99**	**33.4**	**0.13**	**19.1**	**0.36**	**0.53**
1	1.90	31.3	0.12	17.8	0.30*	0.52
2	1.89	30.6	0.12	19.2	0.31*	0.61
4	1.62*	27.3*	0.11*	19.3	0.28*	0.68
8	1.54*	26.5*	0.10*	15.2	0.26*	0.58
$\mu g\ g^{-1}$ Cu added						
0	**1.61**	**30.3**	**0.11**	**14.7**	**0.41**	**0.36**
50	1.85	30.0	0.13*	16.9*	0.39	0.43
200	2.03	29.3	0.14*	16.2*	0.38*	0.43
800	1.74	22.9*	0.20*	13.5*	0.31*	0.44
3200	3.52*	14.0*	0.35*	13.3*	0.22*	0.61*
Adjusted pH						
7.5	1.39	27.8	0.20*	18.2*	0.46*	0.40
6.5	1.69	32.1	0.16*	16.6	0.42	0.40
5.5 (control)	**1.61**	**30.3**	**0.11**	**14.7**	**0.41**	**0.36**
4.5	1.81	31.1	0.18*	10.8*	0.37*	0.29
3.5	2.79*	34.1*	0.20*	7.1*	0.30*	0.24*

*values are statistically different from the control ($p<0.05$) within each soil property and stress/disturbance treatment.

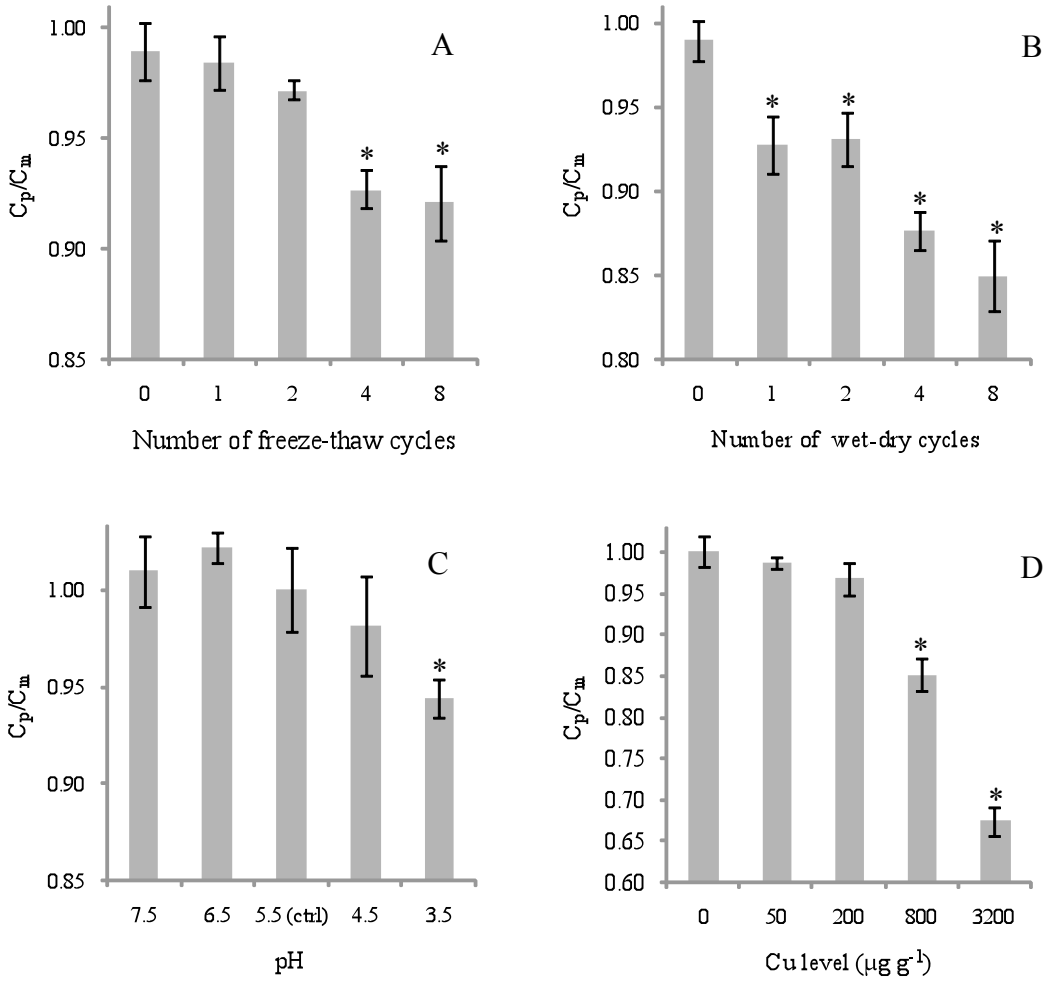

Figure 4.1 Changes in the ratio between the SOC predicted using equation 1 and SOC measured (C_p/C_m) in soils samples subjected to increasing levels of disturbance/stress imposed by freeze-thaw (A); by wet dry cycles (B); by changing soil pH (C); by Cu addition (D). Error bars indicate the standard error of means (4 replicates). Asterisks indicate significant difference from respective controls (p<0.05).

References

Anderson, T.H., Domsch, K.H., 1985. Determination of ecophysiological maintenance carbon requirements of soil microorganisms in a dormant state. Biology and Fertility of Soils 1, 81-89.

Blagodatskaya, E.V., Anderson, T.H., 1999. Adaptive responses of soil microbial communities under experimental acid stress in controlled laboratory studies. Applied Soil Ecology 11, 207-216.

Brant, J.B., Sulzman, E.W., Myrold, D.D., 2006. Microbial community utilization of added carbon substrates in response to long-term carbon input manipulation. Soil Biology & Biochemistry 38, 2219-2232.

Caldwell, B.A., Griffiths, R.P., Sollins, P., 1999. Soil enzyme response to vegetation disturbance in two lowland Costa Rican soils. Soil Biology & Biochemistry 31, 1603-1608.

Chen, C.R., Xu, Z.H., Blumfield, T.J., Hughes, J.M., 2003. Soil microbial biomass during the early establishment of hoop pine plantation: seasonal variation and impacts of site preparation. Forest Ecology and Management 186, 213-225.

Chodak, M., Khanna, P., Beese, F., 2003. Hot water extractable C and N in relation to microbiological properties of soils under beech forests. Biology and Fertility of Soils 39, 123-130.

Corre, M.D., Schnabel, R.R., Stout, W.L., 2002. Spatial and seasonal variation of gross nitrogen transformations and microbial biomass in a Northeastern US grassland. Soil Biology & Biochemistry 34, 445-457.

Degens, B.P., Schipper, L.A., Sparling, G.P., Duncan, L.C., 2001. Is the microbial community in a soil with reduced catabolic diversity less resistant to stress or disturbance? Soil Biology & Biochemistry 33, 1143-1153.

Dick, R.P., Breakwell, D.P., Turco, R.F., 1996. Soil enzyme activity and biodiversity measurements as integrative microbiological indicators. In: Doran, J.W., Jones, A.J. (Eds.), Methods for assessing soil quality. SSSA, Madison, pp. 247-271.

Doran, J.W., Sarrantonio, M., Jaure, R., 1994 Strategies to promote soil quality and health. In: Pankhurst, C.E., Doube, B.M., Gupta, V.V.S.R., Grace, P.R. (Eds.), Soil biota: management in sustainable farming systems. CSIRO, Melbourne, pp. 230-237.

Elliott, E.T., 1997. Rationale for developing bioindicators of soil health. In: Pankhurst, C.E., Doube, B.M., Gupta, V.V.S.R. (Eds.), Biological Indicators of Soil Health. CAB International, Wellington, pp. 49-78.

Fedoroff, N., 1987. The production potential of soils. Part 1. Sensitivity of principal soil types to the intensive agriculture of north-western Europe. In: Barth, E., L'Hermite, P. (Eds.), Scientific Basis for Soil Protection in the European Community. Elsevier, London, pp. 65-86.

Franklin, J.F., Bledsoe, C.S., Callahan, J.T., 1990. Contributions of the Long-Term Ecological Research Program. BioScience 40, 509-523.

Gianfreda, L., Rao, M.A., Piotrowska, A., Palumbo, G., Colombo, C., 2005. Soil enzyme activities as affected by anthropogenic alterations: intensive agricultural practices and organic pollution. Science of the Total Environment 341 265- 279.

Gil-Sotres, F., Trasar-Cepeda, C., Leiros, M.C., Seoane, S., 2005. Different approaches to evaluating soil quality using biochemical properties. Soil Biology & Biochemistry 37, 877-887.

Haynes, R.J., 1999. Size and activity of the soil microbial biomass under grass and arable management. Biology and Fertility of Soils 30, 210-216.

Jenkinson, D.S., 1988. The determination of microbial biomass carbon and nitrogen in soil. In: Wilson, J.R. (Ed.), Advances in nitrogen cycling in agricultural ecosystems. CAB Int, Wellingford, pp. 368-386.

Kandeler, E., Gerber, H., 1988. Short term assay of soil urease activity using colorimetric determination of ammonium. Biology and Fertility of Soils 6, 68-72.

Ladd, J.N., Butler, J.H.A., 1972. Short-term assays of soil proteolytic enzyme activities using proteins and peptide derivatives as substrates. Soil Biology & Biochemistry 4, 19-30.

Lee, I.S., Kim, O.K., Chang, Y.Y., Bae, B., Kim, H.H., Baek, K.H., 2002. Heavy metal concentrations and enzyme activities in soil from a contaminated Korean shooting range. Journal of Bioscience and Bioengineering 94, 406-411.

Leiros, M.C., Trasar-Cepeda, C., Garcia-Fernandez, F., Gil-Sotres, F., 1999. Defining the validity of a biochemical index of soil quality. Biology and Fertility of Soils 30, 140-146.

Leiros, M.C., Trasar-Cepeda, C., Seoane, S., Gil-Sotres, F., 2000. Biochemical properties of acid soils under climax vegetation (Atlantic oakwood) in an area of the European temperate-humid zone (Galicia, NW Spain): general parameters. Soil Biology & Biochemistry 32, 733-745.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193, 265-275.

Mallows, C.L., 1973. Some Comments on Cp. Technometrics 15, 661-675.

Marquard, D.W., 1970. Generalized Inverses, Ridge Regression, Biased Linear Eestimation, and Nonlinear Eestimation. Technometrics 12, 591-612.

Miguéns, T., Leirós, M.C., Gil-Sotres, F., Trasar-Cepeda, C., 2007. Biochemical properties of vineyard soils in Galicia, Spain. Science of the Total Environment 378, 218-222.

Miralles, I., Ortega, R., Sánchez-Marañón, M., Leirós, M.C., Trasar-Cepeda, C., Gil-Sotres, F., 2007. Biochemical properties of range and forest soils in Mediterranean mountain environments. Biology and Fertility of Soils 43, 721-729.

Murphy, D.V., Fillery, I.R.P., Sparling, G.P., 1998. Seasonal fluctuations in gross N mineralisation, ammonium consumption, and microbial biomass in a Western Australian soil under different land uses. Australian Journal of Agricultural Research 49, 523-535.

Nannipieri, P., 1994. The potential use of soil enzymes as indicators of productivity, sustainability and pollution. In: Pankhurst, C.E., Double, B.M., Gupta, V.V.S.R., Grace, P.R. (Eds.), Soil Biota: Managment in Sustainable Farming Systems. CSIRO, Melbourne, pp. 238-244.

Nelson, D.W., Sommers, L.E., 1996. Total carbon, organic carbon, and organic matter. In: Sparks, D.L. (Ed.), Methods of soil analysis. Part 3, Chemical methods. SSSA, Madison, WI, pp. 961-1010.

Nourbakhsh, F., 2007. Decoupling of soil biological properties by deforestation. Agriculture, Ecosystems & Environment 121, 435-438.

Nsabimana, D., Haynes, R.J., Wallis, F.M., 2004. Size, activity and catabolic diversity of the soil microbial biomass as affected by land use. Applied Soil Ecology 26, 81-92.

Parham, J.A., Deng, S.P., 2000. Detection, quantification and characterization of β-glucosaminidase activity in soil. Soil Biology & Biochemistry 32, 1183-1190.

Perucci, P., Bonciarelli, U., Santilocchi, R., Bianchi, A.A., 1997. Effect of rotation, nitrogen fertilization and management of crop residues on some chemical, microbiological and biochemical properties of soil. Biology and Fertility of Soils 24, 311-316.

Riffaldi, R., Saviozzi, A., Levi-Minzi, R., Cardelli, R., 2002. Biochemical properties of a Mediterranean soil as affected by long-term crop management systems. Soil & Tillage Research 67, 109-114.

Sinsabaugh, R.L., Klug, M.J., Collins, H.P., Yeager, P.E., Peterson, S.O., 1999. Characterizing soil microbial communities. In: Robertson, G.P., Coleman, D.C., Bledsoe, C.S., Sollins, P. (Eds.), Standard soil methods for long-term ecological research. Oxford University Press, New York.

Spedding, T.A., Hamel, C., Mehuys, G.R., Madramootoo, C.A., 2004. Soil microbial dynamics in maize-growing soil under different tillage and residue management systems. Soil Biology & Biochemistry 36, 499-512.

Tabatabai, M.A., 1994. Soil Enzymes. In: Weaver, R.W., Angle, J.S., Bottomley, P.J. (Eds.), Methods of soil analysis. Part 2, Microbiological and biochemical properties. SSSA, Madison, pp. 775-833.

Trasar-Cepeda, C., Leiros, C., Gil-Sotres, F., Seoane, S., 1998. Towards a biochemical quality index for soils: An expression relating several biological and biochemical properties. Biology and Fertility of Soils 26, 100-106.

Trasar-Cepeda, C., Leiros, M.C., Seoane, S., Gil-Sotres, F., 2000. Limitations of soil enzymes as indicators of soil pollution. Soil Biology & Biochemistry 32, 1867-1875.

Vance, E.D., Brookes, P.C., Jenkinson, D.S., 1987. An extraction method for measuring soil microbial biomass C. Soil Biology & Biochemistry 19, 703-707.

General Conclusions

Chapter 5

Guilherme M. Chaer

The objectives of this work were: (1) to evaluate how changes in land use affect the functional stability of soil microbial communities to disturbances simulated by heat shocks; (2) associate changes in soil microbial community composition and diversity with the degradation of soil physical and chemical properties across a disturbance gradient; and (3) define an index of soil quality based on a model able to measure the level of disruption of the equilibrium between soil organic matter and biochemical properties when the soil is submitted to physical and chemical disturbances.

In chapter two I demonstrated that the change in land use from forest to agriculture caused degradation of the soil physical and chemical properties and resulted in a microbial community less functionally stable when this soil was submitted to heat shock disturbances. However, microbial functional stability was differently expressed according to the enzymes evaluated in the forest and agricultural soils. The resilience of the broad scale activity of fluorescein diacetate hydrolysis, which encompasses the activity of enzyme pools common to most microorganisms (e.g., lipases, and esterases), did not differ between soils. Conversely, the resilience of cellulase and laccase activities, enzymes that are produced by specialized groups of microbial species, were less resilient in the agricultural than forest soil. Because both soils had a similar amount of resources for the microbial populations (organic matter content per unit of microbial biomass), I suggest that the reduced resilience of laccase and cellulase in the agricultural soil is the result of a less diverse community of microorganisms capable of producing these

enzymes. In conclusion, these results indicate that the consequence of species loss in stressed/disturbed soil environments may not be immediately visible due to considerable functional redundancy of soil microorganisms. However, these effects may decrease the stability of the system to deal with further disturbances, and make it more vulnerable to ecological failure, especially in functions fulfilled by specialized groups of microorganisms.

In chapter three, the application of increasing levels of mechanical disturbances (simulated by tillage events) to a soil under a long-term fallow, generated a gradient of induced soil degradation. This gradient allowed me to determine an unbiased relationship between degradation of soil physical and chemical properties and changes in microbial community composition and diversity. The results obtained by CLPP, T-RFLP and PLFA profiling techniques indicated that degradation of soil physical and chemical properties coincided with changes in microbial community composition, and with decreases in functional and genetic diversity. Nevertheless, these techniques gave different information about the response of microbial communities to the disturbances. In this regard, although the level of soil disturbance/degradation may not influence microbial community composition in the short-term, it is promptly expressed in the physiological potential of the community. Furthermore, the differences observed among the microbial profiling techniques indicated that the use of multiple methods is advantageous if they provide a broader understanding and reveal different aspects of changes in microbial communities.

In chapter four I investigated whether the equilibrium between soil organic matter and soil biological and biochemical properties observed in a previous study, could also exist in forest soils from western Oregon. Results showed that despite spatial and temporal variability, soil biological/biochemical properties were highly correlated with soil organic C. A regression model based on microbial biomass and phosphatase activity as predictor variables was able to explain 97% of the variation in soil organic C. This study also confirmed the hypothesis that the equilibrium between organic matter and biochemical soil properties in undisturbed soils is disrupted when the soil is submitted to disturbances or is under stress conditions. In this regard, the ratio between soil C predicted by the model (C_p), and soil C measured (C_m) effectively indicated the quantitative effects of chemical stresses (Cu and pH), and physical disturbances (freeze-thaw and wet-dry cycles). In conclusion, the use of the C_p/C_m ratio as a soil quality index is compelling because it provides a simple and straightforward interpretation of the status of soil degradation, and requires analysis of only a few soil properties.

Bibliography

Ahn, M., Zimmerman, A.R., Martínez, C.E., Archibald, D.D., Bollag, J., Dec, J., 2007. Characteristics of *Trametes villosa* laccase adsorbed on aluminum hydroxide. Enzyme and Microbial Technology 41, 141-148.

Anderson, T.H., Domsch, K.H., 1985. Determination of ecophysiological maintenance carbon requirements of soil microorganisms in a dormant state. Biology and Fertility of Soils 1, 81-89.

Andrén, O., Balandreau, J., 1999. Biodiversity and soil functioning - from black box to can of worms? Applied Soil Ecology 13, 105-108.

Berg, G., Roskot, N., Steidle, A., Eberl, L., Zock, A., Smalla, K., 2002. Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different Verticillium host plants. Applied and Environmental Microbiology 68, 3328-3338.

Blagodatskaya, E.V., Anderson, T.H., 1999. Adaptive responses of soil microbial communities under experimental acid stress in controlled laboratory studies. Applied Soil Ecology 11, 207-216.

Borneman, J., Triplett, E.W., 1997. Molecular microbial diversity in soils from eastern Amazonia: Evidence for unusual microorganisms and microbial population shifts associated with deforestation. Applied and Environmental Microbiology 63, 2647-2653.

Bossio, D.A., Girvan, M.S., Verchot, L., Bullimore, J., Borelli, T., Albrecht, A., Scow, K.M., Ball, A.S., Pretty, J.N., Osborn, A.M., 2005. Soil microbial community response to land use change in an agricultural landscape of Western Kenya. Microbial Ecology 49, 50-62.

Bossio, D.A., Scow, K.M., 1998. Impacts of carbon and flooding on soil microbial communities: Phospholipid fatty acid profiles and substrate utilization patterns. Microbial Ecology 35, 265-278.

Bossio, D.A., Scow, K.M., Gunapala, N., Graham, K.J., 1998. Determinants of soil microbial communities: Effects of agricultural management, season, and soil type on phospholipid fatty acid profiles. Microbial Ecology 36, 1-12.

Bouyoucos, G.J., 1927. The hydrometer as a new and rapid method for determining the colloidal content of soils. Soil Science 23, 319-331.

Brady, N.C., Weil, R.R., 2002. The nature and properties of soils. Prentice Hall, Upper Saddle River, NJ, 960 pp.

Braga, J.M., Defelipo, B.V., 1974. Determinação espectrofotométrica de fósforo em extrato de solo e material vegetal. Revista Ceres 21, 73-85.

Brant, J.B., Sulzman, E.W., Myrold, D.D., 2006. Microbial community utilization of added carbon substrates in response to long-term carbon input manipulation. Soil Biology & Biochemistry 38, 2219-2232.

Bray, J.R., Curtis, J.T., 1957. An ordination of the upland forest communities in southern Wisconsin. Ecological Monographs 27, 325-349.

Breiman, L., Friedman, J.H., Olshen, R.A., Stone, C.G., 1984. Classification and regression trees. Chapman & Hall/CRC, Belmont, CA, USA, 368 pp.

Buckley, D.H., Schmidt, T.M., 2001. The structure of microbial communities in soil and the lasting impact of cultivation. Microbial Ecology 42, 11-21.

Butler, J.L., Williams, M.A., Bottomley, P.J., Myrold, D.D., 2003. Microbial community dynamics associated with rhizosphere carbon flow. Applied and Environmental Microbiology 69, 6793-6800.

Caldwell, B.A., Griffiths, R.P., Sollins, P., 1999. Soil enzyme response to vegetation disturbance in two lowland Costa Rican soils. Soil Biology & Biochemistry 31, 1603-1608.

Chen, C.R., Xu, Z.H., Blumfield, T.J., Hughes, J.M., 2003. Soil microbial biomass during the early establishment of hoop pine plantation: seasonal variation and impacts of site preparation. Forest Ecology and Management 186, 213-225.

Chodak, M., Khanna, P., Beese, F., 2003. Hot water extractable C and N in relation to microbiological properties of soils under beech forests. Biology and Fertility of Soils 39, 123-130.

Cookson, W.R., Osman, M., Marschner, P., Abaye, D.A., Clark, I., Murphy, D.V., Stockdale, E.A., Watson, C.A., 2007. Controls on soil nitrogen cycling and microbial community composition across land use and incubation temperature. Soil Biology & Biochemistry 39, 744-756.

Corre, M.D., Schnabel, R.R., Stout, W.L., 2002. Spatial and seasonal variation of gross nitrogen transformations and microbial biomass in a Northeastern US grassland. Soil Biology & Biochemistry 34, 445-457.

Dane, J.H., Hopmans, J.W., 2002. Water retention and storage. In: Dane, J.H., Topp, G.C. (Eds.), Methods of soil analysis. Part 4, Physical methods. SSSA, Madison, pp. 688-690.

Davidson, E.A., Ackerman, I.L., 1993. Changes in soil carbon inventories following cultivation of previously untilled soils. Biogeochemistry 20, 161-193.

De'ath, G., 2002. Multivariate regression trees: a new technique for modeling species-environmental relationships. Ecology 83, 1105-1117.

De'ath, G., Fabricius, K.E., 2000. Classification and regression trees: a powerful yet simple technique for the analysis of complex ecological data. Ecology 81, 3178-3192.

Degens, B.P., Schipper, L.A., Sparling, G.P., Duncan, L.C., 2001. Is the microbial community in a soil with reduced catabolic diversity less resistant to stress or disturbance? Soil Biology & Biochemistry 33, 1143-1153.

Degens, B.P., Schipper, L.A., Sparling, G.P., Vojvodic-Vukovic, M., 2000. Decreases in organic C reserves in soils can reduce the catabolic diversity of soil microbial communities. Soil Biology & Biochemistry 32, 189-196.

Dick, R.P., Breakwell, D.P., Turco, R.F., 1996. Soil enzyme activity and biodiversity measurements as integrative microbiological indicators. In: Doran, J.W., Jones, A.J. (Eds.), Methods for assessing soil quality. SSSA, Madison, pp. 247-271.

Dinesh, R., Chaudhuri, S.G., Sheeja, T.E., 2004. Soil biochemical and microbial indices in wet tropical forests: Effects of deforestation and cultivation. Journal of Plant Nutrition and Soil Science 167, 24-32.

Diosma, G., Aulicino, M., Chidichimo, H., Balatti, P.A., 2006. Effect of tillage and N fertilization on microbial physiological profile of soils cultivated with wheat. Soil & Tillage Research 91, 236-243.

Doran, J.W., Elliott, E.T., Paustian, K., 1998. Soil microbial activity, nitrogen cycling, and long-term changes in organic carbon pools as related to fallow tillage management. Soil & Tillage Research 49, 3-18.

Doran, J.W., Sarrantonio, M., Jaure, R., 1994 Strategies to promote soil quality and health. In: Pankhurst, C.E., Doube, B.M., Gupta, V.V.S.R., Grace, P.R. (Eds.), Soil biota: management in sustainable farming systems. CSIRO, Melbourne, pp. 230-237.

Edwards, U., Rogall, T., Blöcker, E.M., Böttger, E.C., 1989. Isolations and direct complete nucleotide determination of entire genes. Characterization of a gene coding 16S ribosomal RNA. Nucleic Acids Research 17, 7843-7852.

Ehrlich, P.R., Ehrlich, A.H., 1981. Extinction: the causes and consequences of the disappearance of species. Random House, New York.

Elliott, E.T., 1986. Aggregate structure and carbon, nitrogen, and phosphorus in native and cultivated soils. Soil Science Society of America Journal 50, 627-633.

Elliott, E.T., 1997. Rationale for developing bioindicators of soil health. In: Pankhurst, C.E., Doube, B.M., Gupta, V.V.S.R. (Eds.), Biological Indicators of Soil Health. CAB International, Wellington, pp. 49-78.

Embrapa, 1997. Manual de métodos de análise de solo. Embrapa CNPS, Rio de Janeiro, 212 pp.

Fedoroff, N., 1987. The production potential of soils. Part 1. Sensitivity of principal soil types to the intensive agriculture of north-western Europe. In: Barth, E., L'Hermite, P. (Eds.), Scientific Basis for Soil Protection in the European Community. Elsevier, London, pp. 65-86.

Franklin, J.F., Bledsoe, C.S., Callahan, J.T., 1990. Contributions of the Long-Term Ecological Research Program. BioScience 40, 509-523.

Frostegård, Å., Bååth, E., 1996. The use of phospholipid fatty acid analysis to estimate bacterial and fungal biomass in soil. Biology and Fertility of Soils 22, 59-65.

Garland, J.L., Mills, A.L., 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-utilization. Applied and Environmental Microbiology 57, 2351-2359.

Gianfreda, L., Rao, M.A., Piotrowska, A., Palumbo, G., Colombo, C., 2005. Soil enzyme activities as affected by anthropogenic alterations: intensive agricultural practices and organic pollution. Science of the Total Environment 341 265- 279.

Gil-Sotres, F., Trasar-Cepeda, C., Leiros, M.C., Seoane, S., 2005. Different approaches to evaluating soil quality using biochemical properties. Soil Biology & Biochemistry 37, 877-887.

Girvan, M.S., Campbell, C.D., Killham, K., Prosser, J.I., Glover, L.A., 2005. Bacterial diversity promotes community stability and functional resilience after perturbation. Environmental Microbiology 7, 301-313.

Gomez, E., Bisaro, V., Conti, M., 2000. Potential C-source utilization patterns of bacterial communities as influenced by clearing and land use in a vertic soil of Argentina. Applied Soil Ecology 15, 273-281.

Gomez, E.D., Garland, J.L., Roberts, M.S., 2004. Microbial structural diversity estimated by dilution-extinction of phenotypic traits and T-RFLP analysis along a land-use intensification gradient. FEMS Microbiology Ecology 49, 253-259.

Grayston, S.J., Campbell, C.D., Bardgett, R.D., Mawdsley, J.L., Clegg, C.D., Ritz, K., Griffiths, B.S., Rodwell, J.S., Edwards, S.J., Davies, W.J., Elston, D.J., Millard, P., 2004. Assessing shifts in microbial community structure across a range of grasslands of differing management intensity using CLPP, PLFA and community DNA techniques. Applied Soil Ecology 25, 63-84.

Green, V.S., Stott, D.E., Diack, M., 2006. Assay for fluorescein diacetate hydrolytic activity: Optimization for soil samples. Soil Biology & Biochemistry 38, 693-701.

Griffiths, B.S., Bonkowski, M., Roy, J., Ritz, K., 2001a. Functional stability, substrate utilisation and biological indicators of soils following environmental impacts. Applied Soil Ecology 16, 49-61.

Griffiths, B.S., Kuan, H.L., Ritz, K., Glover, L.A., McCaig, A.E., Fenwick, C., 2004. The relationship between microbial community structure and functional stability, tested experimentally in an upland pasture soil. Microbial Ecology 47, 104-113.

Griffiths, B.S., Ritz, K., Bardgett, R.D., Cook, R., Christensen, S., Ekelund, F., Sørensen, S.J., Bååth, E., Bloem, J., de Ruiter, P.C., Dolfing, J., Nicolardot, B., 2000. Ecosystem response of pasture soil communities to fumigation-induced microbial diversity reductions: an examination of the biodiversity-ecosystem function relationship. Oikos 90, 279-294.

Griffiths, B.S., Ritz, K., Wheatley, R.E., Kuan, H.L., Boag, B., Christensen, S., 2001b. An examination of the biodiversity-ecosystem function relationship in arable soil microbial communities. Soil Biology & Biochemistry 33, 1713-1722.

Grime, J.P., 1997. Biodiversity and ecosystem function: the debate deepens. Science 277, 1260-1261.

Gros, R., Monrozier, L.J., Bartoli, F., Chotte, J.L., Faivre, P., 2004. Relationships between soil physico-chemical properties and microbial activity along a

restoration chronosequence of alpine grasslands following ski run construction. Applied Soil Ecology 27, 7-22.

Grossman, R.B., Reinsch, T.G., 2002. Bulk density and linear extensibility. In: Dane, J.H., Topp, G.C. (Eds.), Methods of soil analysis. Part 4, Physical methods. SSSA, Madison, pp. 201-225.

Guilbault, G.G., Kramer, D.N., 1964. Fluorometric determination of lipase, acylase, alpha- and gamma-chymotrypsin and inhibitors of these enzymes. Analytical Chemistry 36, 409-412.

Hackl, E., Zechmeister-Boltenstern, S., Bodrossy, L., Sessitsch, A., 2004. Comparison of diversities and compositions of bacterial populations inhabiting natural forest soils. Applied and Environmental Microbiology 70, 5057-5065.

Haynes, R.J., 1999. Size and activity of the soil microbial biomass under grass and arable management. Biology and Fertility of Soils 30, 210-216.

Hope, C.F.A., Burns, R.G., 1987. Activity, origins and location of cellulases in a silt loam soil Biology and Fertility of Soils 5, 164-170.

Huang, Q., Liang, W., Cai, P., 2005. Adsorption, desorption and activities of acid phosphatase on various colloidal particles from an Ultisol. Colloids and Surfaces B: Biointerfaces 45, 209-214.

Islam, K.R., Weil, R.R., 2000. Land use effects on soil quality in a tropical forest ecosystem of Bangladesh. Agriculture, Ecosystems & Environment 79, 9-16.

Jackson, L.E., Calderon, F.J., Steenwerth, K.L., Scow, K.M., Rolston, D.E., 2003. Responses of soil microbial processes and community structure to tillage events and implications for soil quality. Geoderma 114, 305-317.

Jenkinson, D.S., 1988. The determination of microbial biomass carbon and nitrogen in soil. In: Wilson, J.R. (Ed.), Advances in nitrogen cycling in agricultural ecosystems. CAB Int, Wellingford, pp. 368-386.

Johnsen, K., Jacobsen, C.S., Torsvik, V., Sorensen, J., 2001. Pesticide effects on bacterial diversity in agricultural soils - a review. Biology and Fertility of Soils 33, 443-453.

Kandeler, E., 1990. Characterization of free and adsorbed phosphatases in soils. Biology and Fertility of Soils 9, 199-202.

Kandeler, E., Gerber, H., 1988. Short term assay of soil urease activity using colorimetric determination of ammonium. Biology and Fertility of Soils 6, 68-72.

Kemper, W.D., Rosenau, R.C., 1986. Aggregate stability and size distribution. In: Klute, A. (Ed.), Methods of soil analysis. Part 1. ASA/SSSA, Madison, WI, pp. 425-441.

Kohler, F., Hamelin, J., Gillet, F., Gobat, J.M., Buttler, A., 2005. Soil microbial community changes in wooded mountain pastures due to simulated effects of cattle grazing. Plant and Soil 278, 327-340.

Kowalchuk, G.A., Buma, D.S., de Boer, W., Klinkhamer, P.G.L., van Veen, J.A., 2002. Effects of above-ground plant species composition and diversity on the diversity of soil-borne microorganisms. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 81, 509-520.

Kruskal, J.B., 1964a. Multidimensional scaling by optimizing goodness of fit to a nonmetric hypothesis. Psychometrika 29, 1-27.

Kruskal, J.B., 1964b. Nonmetric multidimensional scaling: a numerical method. Psychometrika 29, 115-129.

Ladd, J.N., Butler, J.H.A., 1972. Short-term assays of soil proteolytic enzyme activities using proteins and peptide derivatives as substrates. Soil Biology & Biochemistry 4, 19-30.

Lee, I.S., Kim, O.K., Chang, Y.Y., Bae, B., Kim, H.H., Baek, K.H., 2002. Heavy metal concentrations and enzyme activities in soil from a contaminated Korean shooting range. Journal of Bioscience and Bioengineering 94, 406-411.

Leiros, M.C., Trasar-Cepeda, C., Garcia-Fernandez, F., Gil-Sotres, F., 1999. Defining the validity of a biochemical index of soil quality. Biology and Fertility of Soils 30, 140-146.

Leiros, M.C., Trasar-Cepeda, C., Seoane, S., Gil-Sotres, F., 2000. Biochemical properties of acid soils under climax vegetation (Atlantic oakwood) in an area of the European temperate-humid zone (Galicia, NW Spain): general parameters. Soil Biology & Biochemistry 32, 733-745.

Loreau, M., 2000. Biodiversity and ecosystem functioning: recent theoretical advances. Oikos 91, 3-17.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193, 265-275.

Lupwayi, N.Z., Clayton, G.W., Rice, W.A., 1998. Soil microbial diversity and community structure under wheat as influenced by tillage and crop rotation. Soil Biology & Biochemistry 30, 1733-1741.

MacArthur, R.H., 1955. Fluctuations of animal populations and a measure of community stability. Ecology 73, 1943-1967.

Mallows, C.L., 1973. Some Comments on Cp. Technometrics 15, 661-675.

Marquard, D.W., 1970. Generalized Inverses, Ridge Regression, Biased Linear Eestimation, and Nonlinear Eestimation. Technometrics 12, 591-612.

Mather, P.M., 1976. Computational methods of multivariate analysis in physical geography. J. Wiley & Sons, London, 532 pp.

Mendes, I.C., Bottomley, P.J., Dick, R.P., Bandick, A.K., 1999. Microbial biomass and activities in soil aggregates affected by winter cover crops. Soil Science Society of America Journal 63, 873-881.

Miguéns, T., Leirós, M.C., Gil-Sotres, F., Trasar-Cepeda, C., 2007. Biochemical properties of vineyard soils in Galicia, Spain. Science of the Total Environment 378, 218-222.

Miralles, I., Ortega, R., Sánchez-Marañón, M., Leirós, M.C., Trasar-Cepeda, C., Gil-Sotres, F., 2007. Biochemical properties of range and forest soils in Mediterranean mountain environments. Biology and Fertility of Soils 43, 721-729.

Murphy, D.V., Fillery, I.R.P., Sparling, G.P., 1998. Seasonal fluctuations in gross N mineralisation, ammonium consumption, and microbial biomass in a Western Australian soil under different land uses. Australian Journal of Agricultural Research 49, 523-535.

Muyzer, G., Teske, G., Wirsen, C.O., Jannasch, H.W., 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Archives of Microbiology 164, 165-172.

Naeem, S., Li, S., 1997. Biodiversity enhances ecosystem reliability. Nature 390, 507-509.

Nannipieri, P., 1994. The potential use of soil enzymes as indicators of productivity, sustainability and pollution. In: Pankhurst, C.E., Double, B.M., Gupta, V.V.S.R., Grace, P.R. (Eds.), Soil Biota: Managment in Sustainable Farming Systems. CSIRO, Melbourne, pp. 238-244.

Nelson, D.W., Sommers, L.E., 1996. Total carbon, organic carbon, and organic matter. In: Sparks, D.L. (Ed.), Methods of soil analysis. Part 3, Chemical methods. SSSA, Madison, WI, pp. 961-1010.

Niku-Paavola, M.L., Raaska, L., Itavaara, M., 1990. Detection of white-rot fungi by a non-toxic stain. Mycological Research 94, 27-31.

Nimmo, J.R., Perkins, K.S., 2002. Aggregate stability and size distribution. In: Dane, J.H., Topp, G.C. (Eds.), Methods of soil analysis. Part 4, Physical methods. SSSA, Madison, pp. 317-328.

Nizovtseva, D.V., Semenov, A.M., 1995. Influence of moisture on cellulase activity of micro-organisms in upper peat. Microbiology (Reading) 64, 841-846.

Nourbakhsh, F., 2007. Decoupling of soil biological properties by deforestation. Agriculture, Ecosystems & Environment 121, 435-438.

Nsabimana, D., Haynes, R.J., Wallis, F.M., 2004. Size, activity and catabolic diversity of the soil microbial biomass as affected by land use. Applied Soil Ecology 26, 81-92.

Nusslein, K., Tiedje, J.M., 1999. Soil bacterial community shift correlated with change from forest to pasture vegetation in a tropical soil. Applied and Environmental Microbiology 65, 3622-3626.

Oldeman, L.R., 1994. The global extent of soil degradation. In: Greenland, D.J., Szabolcs, I. (Eds.), Soil resilience and sustainable land use. CAB International, Wallingford, UK, pp. 99-118.

Olsson, P.A., Bååth, E., Jakobsen, I., Söderström, B., 1995. The use of phospholipid and neutral lipid fatty acids to estimate biomass of arbuscular mycorrhizal fungi in soil. Mycological Research 99, 623-629.

Øvreås, L., Torsvik, V., 1998. Microbial diversity and community structure in two different agricultural soil communities. Microbial Ecology 36, 303-315.

Parham, J.A., Deng, S.P., 2000. Detection, quantification and characterization of β-glucosaminidase activity in soil. Soil Biology & Biochemistry 32, 1183-1190.

Perucci, P., Bonciarelli, U., Santilocchi, R., Bianchi, A.A., 1997. Effect of rotation, nitrogen fertilization and management of crop residues on some chemical, microbiological and biochemical properties of soil. Biology and Fertility of Soils 24, 311-316.

Qafoku, N.P., Van Ranst, E., Noble, A., Baert, G., 2003. Mineralogy and chemistry of variable charge soils. In: Lal, R. (Ed.), Encyclopedia of Soil Science. Marcel Dekker, New York.

Reynolds, W.D., Elrick, D.E., Youngs, E.G., 2002. Single-ring and double- or concentric-ring infiltrometers. In: Dane, J.H., Topp, G.C. (Eds.), Methods of soil analysis. Part 4, Physical methods. SSSA, Madison, pp. 821-826.

Rich, J.J., Heichen, R.S., Bottomley, P.J., Cromack Jr., K., Myrold, D.D., 2003. Community composition and functioning of denitrifying bacteria from adjacent meadow and forest soils. Applied and Environmental Microbiology 69, 5974-5982.

Riffaldi, R., Saviozzi, A., Levi-Minzi, R., Cardelli, R., 2002. Biochemical properties of a Mediterranean soil as affected by long-term crop management systems. Soil & Tillage Research 67, 109-114.

Rousseaux, S., Hartmann, A., Rouard, N., Soulas, G., 2003. A simplified procedure for terminal restriction fragment length polymorphism analysis of the soil bacterial community to study the effects of pesticides on the soil microflora using 4,6-dinitroorthocresol as a test case. Biology and Fertility of Soils 37, 250-254.

Schinner, F., von Mersi, W., 1990. Xylanase-, CM-cellulase-, and invertase activity in soil: an improved method. Soil Biology & Biochemistry 22, 511-515.

Schnürer, J., Rosswall, T., 1982. Fluorescein diacetate hydrolysis as a measurement of total microbial activity in soil and litter. Applied and Environmental Microbiology 43, 1256-1261.

Semenov, A.M., Nizovtseva, D.V., 1995. Influence of temperature and mineral supply on cellulase activity and micromycete development in samples of peat from the upper bog. Microbiology 64, 97-103.

Sinsabaugh, R.L., Klug, M.J., Collins, H.P., Yeager, P.E., Peterson, S.O., 1999. Characterizing soil microbial communities. In: Robertson, G.P., Coleman, D.C., Bledsoe, C.S., Sollins, P. (Eds.), Standard soil methods for long-term ecological research. Oxford University Press, New York.

Smalla, K., Wieland, G., Buchner, A., Zock, A., Parzy, J., Kaiser, S., Roskot, N., Heuer, H., Berg, G., 2001. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: Plant-dependent enrichment and seasonal shifts revealed. Applied and Environmental Microbiology 67, 4742-4751.

Solomon, D., Lehmann, J., Zech, W., Fritzsche, F., Tekalign, M., 2002a. Soil organic matter composition in the subhumid Ethiopian highlands as influenced by deforestation and agricultural management. Soil Science Society of America Journal 66, 68-82.

Solomon, D., Tekalign, M., Zech, W., Fritzsche, F., Lehmann, J., 2002b. Soil organic matter dynamics in the subhumid agroecosystems of the Ethiopian highlands: evidence from natural 13C abundance and particle-size fractionation. Soil Science Society of America Journal 66, 969-978.

Spedding, T.A., Hamel, C., Mehuys, G.R., Madramootoo, C.A., 2004. Soil microbial dynamics in maize-growing soil under different tillage and residue management systems. Soil Biology & Biochemistry 36, 499-512.

Swift, M.J., Kjoller, A., Ekschmitt, K., Smith, P., Loiseau, P., Brussaard, L., Andren, O., Couteaux, M.M., Briones, M., 1998. Global change, soil biodiversity, and nitrogen cycling in terrestrial ecosystems: three case studies. Global Change Biology 4, 729-743.

Tabatabai, M.A., 1994. Soil Enzymes. In: Weaver, R.W., Angle, J.S., Bottomley, P.J. (Eds.), Methods of soil analysis. Part 2, Microbiological and biochemical properties. SSSA, Madison, pp. 775-833.

Tobor-Kaplon, M.A., Bloem, J., Römkens, P.F.A.M., Ruiter, P.C.d., 2006. Functional stability of microbial communities in contaminated soils near a zinc smelter (Budel, The Netherlands). Ecotoxicology 15, 187-197.

Torsvik, V., Goksøyr, J., Sorheim, R., Salte, K., 1990. Comparison of phenotypic diversity and DNA heterogeneity in population of soil bacteria. Applied and Environmental Microbiology 56, 776-781.

Torsvik, V., Ovreas, L., Thingstad, T.F., 2002. Prokaryotic diversity - Magnitude, dynamics, and controlling factors. Science 296, 1064-1066.

Trasar-Cepeda, C., Leiros, C., Gil-Sotres, F., Seoane, S., 1998. Towards a biochemical quality index for soils: An expression relating several biological and biochemical properties. Biology and Fertility of Soils 26, 100-106.

Trasar-Cepeda, C., Leiros, M.C., Seoane, S., Gil-Sotres, F., 2000. Limitations of soil enzymes as indicators of soil pollution. Soil Biology & Biochemistry 32, 1867-1875.

van Bruggen, A.H.C., Semenov, A.M., 2000. In search of biological indicators for soil health and disease suppression. Applied Soil Ecology 15, 13-24.

Vance, E.D., Brookes, P.C., Jenkinson, D.S., 1987. An extraction method for measuring soil microbial biomass C. Soil Biology & Biochemistry 19, 703-707.

Waldrop, M.P., Balser, T.C., Firestone, M.K., 2000. Linking microbial community composition to function in a tropical soil. Soil Biology & Biochemistry 32, 1837-1846.

White, D.C., Pinkart, H.C., Ringelberg, D.B., 1997. Biomass measurements: biochemical approaches. In: Hurst, C.J., Knudsen, G.R. (Eds.), Manual of Environmental Microbiology. ASM Press, Washington, DC, pp. 91-101.

Widmer, F., Fliessbach, A., Laczko, E., Schulze-Aurich, J., Zeyer, J., 2001. Assessing soil biological characteristics: a comparison of bulk soil community DNA-, PLFA-, and Biolog (TM)-analyses. Soil Biology & Biochemistry 33, 1029-1036.

Woods, L., 1989. Active organic matter distribution in the surface 15 cm of undisturbed and cultivated soil. Biology and Fertility of Soils 8, 272-278.

Yan, F., McBratney, A.B., Copeland, L., 2000. Functional substrate biodiversity of cultivated and uncultivated A horizons of vertisols in NW New South Wales. Geoderma 96, 321-343.

Zelles, L., 1999. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterization of microbial communities in soil: a review. Biology and Fertility of Soils 29, 111-129.

Zelles, L., Bai, Q.Y., Beck, T., Beese, F., 1992. Signature fatty acids in phospholipids and lipopolysaccharides as indicators of microbial biomass and community structure in agricultural soils. Soil Biology & Biochemistry 24, 317-323.

Zibilske, L.M., 1994. Carbon Mineralization. In: Weaver, R.W., Angle, J.S., Bottomley, P.S. (Eds.), Methods of soil analysis. Part 2, Microbiological and biochemical properties. SSSA, Madison, pp. 835-863.