UNIVERSITÀ CATTOLICA DEL SACRO CUORE
Sede di Piacenza

Scuola di Dottorato per il Sistema Agro-alimentare

Doctoral School on the Agro-Food System

cycle XXIV

S.S.D: AGR/13 AGR/16

REFLECTIONS OF ECOSYSTEM SERVICES ON AGRICULTURAL-SOIL PHYLOGENETIC AND FUNCTIONAL DIVERSITY OF PROKARYOTES

Polymerase chain reaction (PCR) based approaches in prokaryotic ecology

Candidate: **Sotirios Vasileiadis**
Matr. n.: 3710441

Academic Year 2010/2011

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Tutor: Prof. Marco Trevisan

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Preface

The work described in the chapters of this document comprises the product of my 3-year PhD studies performed in the Institute of Agricultural and Environmental Chemistry of Universitá Cattolica del Sacro Cuore, Faculty of Agricultural Sciences of Piacenza. The research performed aimed in gaining insights concerning fundamental aspects of soil microbial ecology, and also in interpreting the responses of relatively well-defined microbial groups under frequently occurring human induced stresses. Two levels of resolution were selected for collectively achieving the experimental aims. The broad microbial responses were examined as part of a soil environment with various levels of human interference, while in a second approach, microbial groups of significance for ammonia oxidation (central pathway in the nitrogen cycle) were utilized as biomarkers for toxicity stresses in soil. The methodologies used involved state of the art analytical methods and development of bioinformatics and statistical background.

Overall, my involvement in these projects has been a joy and an excellent learning experience, regardless the difficulties faced.

The candidate Ph.D.

Sotirios Vasileiadis

Devoted to my parents and family

Summary

Soil is a complex environment comprising the basis for several ecosystem services, with many of them being connected to agricultural production. This complexity is reflected on the composition and functions of the hosted microbial life mainly responsible for the acquired services. Aim of the described studies was to explore microbial community responses to ecosystem services related human intervention in agricultural soils. Total prokaryotic diversity was studied in soils of common origin, which diverged in properties during the late 6-7 centuries due to different land use and management. For achieving this, related DNA markers were screened with high throughput sequencing. Cultivated environments had increased diversity compared to more natural soils. Factors potentially affecting the microbial community structure were: soil disturbance events; available nutrients; and microbial dormancy. In a second approach, ammonia oxidizing prokaryotes were used as biomarkers for studying stress effects caused by humidity and increased zinc concentrations and also the presence of organic pesticides in soil and litter respectively. In both referred cases the studied microbial guilds responded to the applied stresses showing strain or taxon level functional redundancy potentials, and tolerance variability. Overall, results show that human intervention is determining for the prokaryotic structure and functions in agricultural soils.

Keywords: soil, ecosystem services, prokaryotes, total diversity, ammonia monooxygenase

TABLE OF CONTENTS

Chapter 1

General introduction

Soil is a highly diverse matrix of crucial importance for agroecosystems, harboring a high diversity of life forms and a corresponding diversity of functions. Prokaryotic life dominates soil environments with cell numbers estimated to rich up to 10^9, with a maximum genotypic richness of up to 1 million per soil gram (Torsvik and Ovreas 2002; Gans *et al.* 2005; Schloss and Handelsman 2006). Comprising an immense pool of potential functions, Prokaryotes represent by far the largest P and N reservoirs among living organisms, while they are considered to be at least as significant as plants considering biological carbon content (Whitman *et al.* 1998). These are some of the facts indicating that human life and welfare heavily relies upon ecosystem processes performed by soil prokaryotic life, and therefore the increased interest about their ecology is more than fundamental (Chapin Iii *et al.* 2000; Coleman and Whitman 2005).

Paradigms of ecosystem services derived from soil prokaryotic activity

Several goods and services reflected in daily human life (like plant, animal production, bioremediation) are to a great extend outcomes of the prokaryotic interactions with their biotic and abiotic environments.

Ecosystem processes related to nutrient cycling are performed by broad or distinct microbial groups (Fierer *et al.* 2007). Prokaryotes with heterotrophic lifestyles contribute to decomposition of dead organic matter in soils, releasing encompassed nutrients and making possible the continuum of several biogeochemical cycles, with the carbon cycle (ensuring cycling of the element

comprising the backbone of all organic molecules) being a characteristic example (Gessner *et al.* 2010). Moreover, members of *Actinobacteria* known to be symbionts of earthworms, have been reported to contribute to phosphorous solubilization (Caroline C 1996). Autotrophs found in soils like the ammonia oxidizing *Bacteria* (AOB) and the more recently identified ammonia oxidizing *Archaea* (AOA - proposed to comprise an individual archaeal phylum, the *Thaumarchaeota*) have demonstrated their ability to obtain energy by the turnover of ammonia(um) to nitrite and use it for carbon fixation (Kowalchuk and Stephen 2001; Brochier-Armanet *et al.* 2008; Pratscher *et al.* 2011). Produced nitrites are further oxidized to nitrates by *Nitrobacter, Nitrospina* and the dominant of nitrite oxidation in various environments *Nitrospira* species, and a proportion the highly soluble nitrates is leached and extracted from of the soil ecosystem by underground water flow (Grundmann *et al.* 2001; Kowalchuk and Stephen 2001; Freitag *et al.* 2005; Knapp and Graham 2007; Lucker *et al.* 2010). In these systems nitrogen pool replenishing is merely achieved by nitrogen fixation performed by the root symbiotic rhizobacteria which comprise a major factor for the increase of plant growth and productivity (Jetten 2008) and therefore nutrient availability to the system.

Plant production is supported by prokaryotes in numerous other ways than the nutrient cycling discussed above, involving interactions with their biotic environment. Prokaryotes have demonstrated their abilities of communication and sensing their environment with sophisticated ways far beyond their basic immediate response to nutrients towards increasing their fitness (Faure *et al.* 2009). The nutrient-rich rhizosphere environments are hotspots of microbial

activity characterized by the presence of beneficial and harmful for plant growth microorganisms (Raaijmakers *et al.* 2009). Rhizodeposition of various allelochemicals serve as the plant-side line of defense against the large variety of microorganisms with "unknown intentions" (Bertin *et al.* 2003). This line of defense is circumvented by several Prokaryotes by exploiting plant hormonal signals that regulate these processes. Such cases have been identified for ethylene and 3-indolylacetic acid (IAA) manipulating free-living plant growth promoting rhizobacteria (PGPR) (Lucy *et al.* 2004; Leveau and Gerards 2008). Plant-growth is also facilitated by several bacteria indirectly, through pathogen control. The discovery of such bacteria was primed by observations in soils where the incidence of casual agents of several diseases has been proved but the disease was suppressed (Garbeva *et al.* 2004; Raaijmakers *et al.* 2009). This suppression of the disease was lost after soil sterilization, clearly showing that several biotic factors contribute to the phenomenon and was mainly attributed to nutrient competition, amensalism, microbial antagonism, parasitism and induced plant systemic resistance (ISR) (Garbeva *et al.* 2004).

Bioremediation is another one of the ecosystem services attributed to a great degree in prokaryotes. Persistent organic compounds with toxic activity like certain organophosphorous pesticides, have been demonstrated to be degraded to less- or non-toxic forms in enriched cultures of bacterial strains derived from distant taxa (Karpouzas *et al.* 2005; Singh and Walker 2006). Trace elements witch occur in increased, toxic, concentrations either naturally or due to human activity can become less bio-available, or even detoxified (like the case of mercury volatilization by the mercuric reductase enzyme encompassing

Bacteria) due to prokaryotic activity (Nies 1999; Bruins *et al.* 2000; Choudhury and Srivastava 2001).

Provided the above examples, it is important to identify and understand the immense diversity of prokaryotic functions and genotypes for sustainably managing agricultural soils (Welbaum *et al.* 2004; Kowalchuk *et al.* 2008).

Shifts in agricultural soil microbial communities caused by ecosystem services

Intensification of human activities in agroecosystems is altering soil physical-chemical properties environments and also the encompassed prokaryotic communities (Dick 1992). Tillage practices and seedbed preparation induce reduction of particulate organic matter, increase in respiration activity, pH alteration, water content and temperature shifts, increase of nitrogen mineralization and leaching of nitrates, reduction of trophic microsite diversity (Welbaum *et al.* 2004; Liu *et al.* 2006). Nutrient additions in organic or mineral forms have a priming affect on indigenous particular taxa able to exploit these nutrients at higher metabolic rates (Waldrop and Firestone 2004; Cleveland *et al.* 2007; Blagodatskaya and Kuzyakov 2008). Crop selection and rotation practices have been shown to contribute in altering microbial community composition (Welbaum *et al.* 2004; Barrios 2007). Finally pesticide applications have been shown to have effects on non-intended targets and to cause shifts in total bacterial community structure (Girvan *et al.* 2004).

Although land use and management effects on the soil microbial community have been long acknowledged (Garbeva *et al.* 2004), detailed information until

recently has been difficult to obtain and to a great extend is still missing. New tools described in the next section are expected to increase our understanding of human impact on microbial communities of agricultural soils.

Exploration beyond the genome

All the modes of prokaryotic activity mentioned in the ecosystem processes description above were elucidated up to genomic levels, mainly due to the major contribution to environmental microbiology of culture based approaches; or in other words, "existing observations and subsequent experimental validation" (Prosser *et al.* 2007). Microbial strain isolations started taking place centuries ago and since then microorganisms were cultivated in axenic assays or assays involving a few strains in attempt to elucidate their physiological and genomic aspects.

However, relatively recently developed molecular tools that have provided the ability to take snapshots of the information encompassed in complete genome collections occurring in natural environments, gave birth to metagenomics (Handelsman *et al.* 1998; Schloss and Handelsman 2006). A field, that encompassed and organized previous efforts for screening microbial functions and diversity. One of the major advantages of metagenomics, is the ability to look into existing genetic information as part of an interaction web rather than examining the potentials of a single genome in a controlled environment. Furthermore it became possible to study such information derived from the vast majority, something not possible or very laborious while using culture-based approaches.

Utilization of culture independent methods derived information from soil environments, indicated the magnitude of the identified microbial diversity was immense (Schloss and Handelsman 2006). This made clear that a joint effort by laboratories was necessary for achieving such a task and provided the foreground for generating consortia like the one referred as Terragenome project (Vogel *et al.* 2009b). Moreover, this is also indicative of the difficulties faced concerning one of the major aims of soil microbial ecology which is the formation of common theoretical grounds, a necessary task for understanding the extend of potential merits derived from the microbial world (Fierer *et al.* 2009; Vogel *et al.* 2009a).

Evolution of tools for microbial activity and diversity in environmental samples

Two major lines of research were generated in the emerging field metagenomics, the functional and the sequence screening of environmental DNA focusing to the main questions of "who is there?" and "what are they doing?" (Handelsman 2004). The basic idea described when the metagenomics term was coined, was related to the more demanding and interesting approach of functional screening of environmental DNA sequences. According to that, clone libraries that would in turn make storage of the environmental DNA encompassed information feasible were generated (Handelsman *et al.* 1998). Relatively large fragments of environmental DNA (with great potentials to encompass full operons or eukaryotic genes along with necessary transcription factors) would be inserted into suitable vectors (cosmids, fosmids, bacterial artificial chromosomes – BAC, yeast articifical chromosomes – YAC) and the constructs would be stored in

appropriate hosts in clone libraries. During functional screening and provided that conditions for heterologous gene expression of inserts are satisfied (e.g. necessary genetic information and transcription factors are present along with the corresponding to the encoded function stimulus), the gene is expressed, the response is measured and new functions are identified. Further studies are then carried out to reveal the involved mechanisms. The sequence-based approach frequently included the use of polymerase chain reaction (PCR) for targeting "homologue" genes throughout the full collection of environmental DNA. These would usually be assessed by a suitable technique that would reveal the diversity and richness of homologue genes.

Several methods have been proposed and tools developed since that period for increasing the throughput of both functional and sequence based screening. High throughput sequencing tools of the first decade of 2000, in cases of low in complexity environments or PCR product screening, have eliminated the laborious necessity of generating clone libraries (e.g. Sogin *et al.* 2006; Roesch *et al*. 2007 Dinsdale *et al.* 2008; Bartram *et al.* 2011). Efforts in functional screening throughput increase, lead to the development of trap vectors emitting fluorescent signal when the gene is expressed, which was combined with the throughput of detection of flow-cytometry (Uchiyama *et al.* 2005). A setup similar in terms of detection principle, was developed for quorum sensing or quenching related genes identification (Williamson *et al.* 2005). Furthermore, transcriptomics and proteomics tools were also applied on environmental samples towards more integrated approaches (Leveau 2007; Keller and Hettich 2009; Schneider and Riedel 2010).

Out of the wide variety of tools grouped under the defined field of metagenomics, the presented studies are oriented towards exploration of environmental samples using PCR based approaches in prokaryotic group targeted assays.

Outline of the thesis

Main objectives of this thesis were the assessment of the effect agricultural practices on total prokaryotic diversity, the development of a high-resolution methodology for this purpose and also the exploration of diversity of microbial groups performing a distinct function as soil quality indicators.

For achieving the development of a PCR based high-resolution methodology to be applied in total prokaryotic community screening, a theoretical approach was adopted as described in ***Chapter 2***. The ribosomal database project (RDP) database (currently one of the largest databases of small subunit – SSU – encoding gene sequences) was explored concerning potentials of usage of partial reads of the SSU encoding gene in combination with the resolution of millions of reads produced by Illumina sequencing technology, for assessing soil prokaryotic diversity with focus on bacterial community screening. This study was performed for two main reasons. Firstly, the immense prokaryotic diversity found in soils is not reflected on the human microbiome dominated databases used in similar previous studies and therefore soil derived parts of RDP database deserved to be examined as a distinct unity. Secondly, all previous studies have focused on the ability of the pyrosequencing technology having different specifications than Illumina sequencing technology. The focus on bacterial SSU sequences was selected on the basis of the far greater database support provided compared to the archaeal ones, that might introduce artifacts in the analysis.

In ***Chapter 3***, the knowledge generated in ***Chapter 2*** (according to contemporary to the experimental performance technology abilities and current knowledge) is used for exploring the ecological aspects of prokaryotic diversity, as a result of land use and management decisions of more than six centuries. An ideal sampling site was selected for this purpose, composed by soils derived from the beds of a former swamp that after drainage were turned into a collection of arable fields, minimally managed meadows and low land springs.

Chapter 4 comprises a literature-based exploration of effects of trace element stressors on the microbial community, the interpretation of this concerning risk assessment and the proposal of tools and tool combination for elucidating related phenomena. This chapter was part of a preparatory work for the following chapters.

Chapter 4 and ***Chapter 5*** are devoted on assessing the ecology of a distinct prokaryotic function based on responses to three types of stress. The selected function is ammonia(um) oxidation activity until recently known to be performed by a distinct bacterial group residing in the order of *Nitrosomonadales*. Late year discoveries indicated that potential homologues of genes coding for the subunits of the related protein (ammonia monooxigenase - AMO) are wide-spread among mesophylic *Crenarchaeota* (proposed as a new phylum, the *Thaumarchaeota*), some of which can grow on ammonia(um) as the sole energy source. Applied stresses aimed at looking into differential expression of genes related to AMO (for both *Bacteria* and *Archaea* where possible) or the 16S rDNA of the distinct bacterial as means of addressing differences in responses of these microbial groups. The three types of stresses were: a) the

common stress in soil environments of humidity shifts; b) a severe stress due to trace element concentrations (zinc) with direct effects on ammonia oxidizing microbial groups; c) an indirect stress (fungicides) aiming at other functional groups (saprophytic fungi) which in turn affect the nitrogen mineralization rates and therefore ammonia(um) availability. These experimental series were performed in microcosms and the environments tested were a low in organic carbon content maize field soil for (a,b) and a soil-litter interface for (c).

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Chapter 1

385

386

Title:

A theoretical approach for assessing practical aspects of soil bacterial diversity screening using Illumina technology.

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SV: original idea, analysis setup, data analysis, writing of the report

EP: discussion of data analysis and report

MA: discussion of data analysis and report

FC: discussion of data analysis and report

PSC: discussion of data analysis and report

TM: evaluation of original idea and discussion of data analysis and report

Chapter 2

Abstract

Out of the wide contemporary variety of tools for studying 16S rDNA bacterial diversity, Illumina technology appears to be the most prominent in fulfilling the necessary conditions for robust analysis 16S rDNA screening based for soil environments. However, there is an important limitation concerning the maximum sequence length screening abilities restricting studies in screening DNA stretches of single 16S rDNA hypervariable (V) regions. Aim of the present study was to assess effects of properties of four consecutive V regions (V3-6) on commonly applied analytical methodologies in bacterial ecology studies. Performance of each V region was assessed in respect to the full 16S rDNA stretch based on the non-redundant soil bacterial 16S rDNA sequence collection of the Ribosomal Database Project (RDP) database and also by generating a virtual dataset according to previous studies and RDP database. Results indicate that the overall most prominent V region for soil bacterial diversity studies was V3, although it was outperformed in some of the tests. V4 performed well in all tests but lacks highly conserved flanking sites that would allow high screening depths as confined by the length screening limitations of Illumina. V5 performed well in the non-redundant RDP database based analysis, but did not resemble as well the full-length 16S rDNA sequence results as the V3 and V4 did when the natural sequence frequency and occurrence approximation was considered at the virtual experiment. V6 had relatively low performances in all tests apart from the flanking sequence conservation analysis. Our results indicate that environment specific database exploration and theoretical assessment of the

41 experimental approach is strongly suggested in 16S rDNA based bacterial
42 diversity studies.

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Chapter 2

Introduction

Usage of the 16S rDNA gene as a bacterial diversity marker was a breakthrough for microbial ecology studies in the late 1980s (Woese 1987). Linkage of bacterial diversity with the properties of a single gene, detached related studies from the restricting cultivation based approaches and made the assessment of bacterial roles in natural environments possible. This advent was further exploited by expanding the application field of genomic tools with increased screening throughput abilities (Muyzer *et al.* 1993; Amann *et al.* 1995; Liu *et al.* 1997; Handelsman *et al.* 1998) to environmental DNA and RNA extracts or cell mixtures.

The 1990s methodologies along with the first applications of new generation high throughput screening of the 16S rDNA polymerase chain reaction (PCR) products, and particularly pyrosequencing in the first decade of 2000, revealed that bacterial diversity existing in few soil grams was far more immense than believed in the past (Schloss and Handelsman 2006; Roesch *et al.* 2007). This immense diversity coinciding with variability observed in soil environments, urged for the use of multiple replicates and increased number of 16S rDNA amplicons (~500.000 per soil gram) even for pyrosequencing technology abilities (Schloss and Handelsman 2006; Prosser 2010). Therefore, researchers turned their attention to the Illimina high throughput sequencing technology, having the required by soil environments multimillion partial 16S rDNA sequence reads screening abilities per sequencing run (Wu *et al.* 2010; Bartram *et al.* 2011). However, contemporary technology limitations restrict the screened sequence length to stretches of maxima of ~230 bp. This, according to 16S rDNA

properties related to bacterial classification in taxa or operational taxonomic units (OTUs), restricts the abilities to screening single hypervariable (V) regions of the gene.

Aim of the present study was to assess the usage Illumina sequencing for massive parallel screening of bacterial 16S rDNA diversity in soil environments based on information potentials of such short reads (single V region). 16S rDNA stretch for RDP database soil derived sequences was explored for conservation and potential primer designing sites were proposed. Afterwards, four consecutive 16S rDNA hypervariable (V) regions were analyzed; namely V3, V4, V5 and V6. These sequences were examined by means of properties related to contemporary Illumina technology limitations. Such were the V region length suitability, conserved sites, comparison of pairwise distances of sequences between their full length and their V region specific concatenated versions, taxonomy information loss of concatenated sequences compared to their full length versions. Finally, a virtual experiment based on sequences and outcomes of previously performed studies was used to identify expected differences between V regions according to 16S rDNA sequence frequencies.

Materials & Methods

Comparison of hypervariable regions and related properties

Datasets description. 42109 full or nearly full-length, soil-derived, ribosomal database project (RDP) database (Cole *et al.* 2009) bacterial 16S rDNA sequences comprised the core dataset used in the comparisons of the hypervariable regions with the complete sequence stretch of sequence reads. The *Escherichia coli* type

strain (ATCC 11775T) 16S rDNA sequence with Genebank accession number X80725 was added in all aligned datasets and was used as reference during analysis. For consistency with the widely accepted *E. coli* 16S rDNA nucleotide position numbering (Baker *et al.* 2003; Wang and Qian 2009), this sequence was aligned with the *E. coli* 16S rDNA sequence used in these studies (Genebank accession 1VS5_A) and nine gaps were introduced in the sequence beginning in all cases where numbering of positions is referred. Aligned sequences were concatenated in corresponding reference sequence positions 338-534 (V3), 515-700 (V4), 786-926 (V5), 1052-1193 (V6) for generating the desired hypervariable (V) region datasets. Concatenation positions were based on previously reported high coverage primer sites (Wang and Qian 2009).

Analysis of 16S rDNA conservation and V region lengths. Assessment of alignment based soil bacterial 16S rDNA sequences positional variability, was carried out by estimating the Shannon entropy (*H*') values per nucleic acid base position. Gap positions existing in the reference sequence were removed from all aligned sequences and the *H'* values were calculated. Based on these values, entropy plots were generated with plotted values per position being the result of the average *H'* value of 20 consecutive base positions. Moreover, a 90 % conservation cutoff value was applied for generating the consensus sequence of all soil 16S rDNA sequences using degeneracies according to the IUPAC annotation system and a 95 % cutoff for identifying highly conservative priming sites. Results of conserved sites were contrasted against the previous study of Wang and Qian (2009). *H'* calculations were carried out with the bio3d package (Grant *et al.* 2006) executed in R software (R_Development_Core_Team 2009),

while the nearly full 16S rDNA consensus sequence was calculated using the Mothur software (Schloss *et al.* 2009).

Finally, examined hypervariable regions were also tested for stretch length distribution across all soil-derived RDP database sequences, for assessing their potential usage according to Illumina limitations.

Length of V regions and also corresponding sequence distances and taxonomy comparisons between V region and FL datasets. Properties related to two major microbial diversity assessment approaches (OTU and taxonomy based) were examined in comparison to the respective properties of the full-length sequence variants. OTU and taxonomy based analyses were carried out using the Mothur software.

Using the complete linkage algorithm, distances between aligned sequences having the same identifiers were calculated and concomitantly compared for all V region datasets against the full-length sequences. Due to computational power limitations a subset of ~10,000 sequences per dataset (ones derived from agricultural and grassland soils) was used generating ~100,000,000 pairwise distances. Comparisons for 1,000,000 randomly selected distances per dataset corresponding to the same strain of origin, were used for performing Pearson correlation tests between each V region dataset and the full-read length variant. Taxonomy information differences throughout all datasets and the full-length sequence annotations were assessed using the naïve Bayesian classifier for 50 % confidence resulting from bootstrap analysis (Claesson *et al.* 2009), according to RDP taxonomy annotations that are consistent with Bergey's manual standards. Sequence classification depth for all taxonomical levels and also over- or under-

representations at phylum level for the hypervariable region datasets compared to the full-length sequences were reported. For the latter, taxa were divided in four categories according to the population of database in sequence numbers and also taking into account if or not sequences were classified. These categories were the highly populated (>1000 sequences for participating taxa), intermediate populated (above 100 sequences and up to 1000 per participating taxon), rare (less than 100 sequences) and the unclassified sequences containing group. The Shapiro normality test and the Levene's test of equality of variance were performed for assessing if conditions were met for performance of ANOVA for estimating significance of differences between the referred groups (excluding the unclassified sequences group and cases of phyla with miss-identified sequences in the V regions datasets not existing in the original full=length dataset). The Shapiro normality test showed that this condition was not met for all examined groups and the non-parametric Nemenyi-Damico-Wolfe-Dunn joint ranking test (for confidence intervals of 99 %) with Tukey test for pair-wise comparisons was applied using the Coin package (Hothorn *et al.* 2006) of R software.

Environmental sample analysis simulation

Datasets description. Nine datasets in total, derived from 16S rDNA bacterial soil diversity screening in previous studies using pyrosequencing, were used as templates for these analysis series. Major criteria for their selection were the range of sequence numbers per sample (26,000 to 54,000) and the read qualities. Studies and corresponding dataset or sequence accession numbers used were: Roesch *et al.* (2007) sequence accessions EF222481-EF248596, EF248597-

276844, EF308591-362836; Will *et al.* (2010) archive accessions SRR059809, SRR061002, SRR061009; Nacke *et al.* (2011) archive accessions SRR064358, SRR064370, SRR064374. Best matches of sequences derived from these files in the SILVA bacterial 16S rDNA reference alignment sequences (Pruesse *et al.* 2007) according to the NAST algorithm performance (DeSantis *et al.* 2006), were extracted and comprised the full read length replacements for each sequence in the nine-sample dataset used for concomitant analyses (referred as test dataset). The aligned sequence test dataset version was concatenated to the examined V regions (at positions as referred in V region analysis). Gap removal of the full length or concatenated sequences lead to the generation of 5 datasets containing: the full-length (FL), V3, V4, V5 and V6 variants of the test sequence dataset.

Data analysis. V region performance was assessed by means of Classification, operational taxonomic unit (OTU) and phylogenetic results for each of the V region dataset versions comparison with the FL dataset. For the classification based analysis (performed with the parameters described above) sequences of each dataset were classified and sample distances were calculated using the Bray-Curtis transformation for relative abundance matrices and the Jaccard transformation for presence absence matrices. The obtained pairwise distances were used as loadings for performing PCA analysis for corresponding sample distances between generated datasets. Using the same methods, sample distances generated by an OTU approach for OTU definition of 3 % sequence distances were used for OTU assessment differences between generated datasets (V-regions and FL) for relative abundance and presence absence matrices. The

phylogeny based analysis included calculations of dataset distances based on obtained sample distances per dataset as calculated by encompassed sequences evolutionary relationships. Initial step was the performance of the relaxed neighbor joining algorithm performed by the Clearcut application (Sheneman *et al.* 2006) for producing the phylogenetic tree which was concomitantly used for calculation of sample distances using weighted and unweighted Unifrac analysis (Lozupone and Knight 2005; Lozupone *et al.* 2007). Sample distances were used for generating one matrix for weighted distances and one for unweighted for all datasets and matrices were analyzed with PCA analysis.

Results

16S rDNA conservation and V region lengths. Sequence conservation was examined using the Shannon entropy values (*H'*), in order to assess conserved sites flanking the hypervariable regions that would be suitable for primer designing. Out of the four selected V regions, the ones showing higher variability were the V3 and V6, while the ones having greater V sequence lengths were the V3 and V4 (Fig. 1 and 2). Concerning the latter, stretches longer than 105 bp were identified as hypervariable for V3 and V4 while the corresponding value for V5 and V6 was a bit more than 27-35 bp. Conservation screening of nucleic acid bases that were common for at least 95 % of the examined sequences produced stretches with potential for being selected as priming sites (green background color in Fig. 2). Minimum amplicon lengths for the referred per primer coverage (or minimum 90 % per primer-set) were: 175 bp (348-533 *E. coli* numbering) with maximum 3 degeneracies per primer for 18 bp primers or 190 bp (341-531 *E. coli* numbering) without degeneracies per primer for V3; 282 bp (516-798 *E.*

coli numbering) with low primer degeneracies for V4; 108 bp (788-896 *E. coli* numbering) with low number of degeneracies per primer for V5; 137 bp (921-1068 *E. coli* numbering) with low number of per primer degeneracies for V6. When examined regardless the conservation of the various sites and based on previously indicated sites (Wang and Qian 2009), amplicon lengths were below 200 bp for more than 99.8 % of the amplicons for V3 and V4 and less than 150 bp for V5 and V6 (Fig. 3).

Sequence distances and taxonomy annotation of V region data. Effects of sequence length and V region variability patterns on obtained sequence distances was assessed by comparing distances of concatenated sequences to the V regions with their full length variants (Fig. 4). Correlation tests indicated and V region datasets performance with descending Pearson correlation (r) values in the order V4, V5, V6, V3. Overall trends were further assessed by linear model applications. Out of the four V regions slopes closer to 1 were observed for V4 (R^2 = 0.88) and V5 (R^2 = 0.82). V3 and V6 slopes had lower than one values and applied linear models did not describe as well the data-points like in the case of the V4, V5 (R^2 > 0.80). Linear model formulas indicate an over-estimation trend for V3 distances and a corresponding under-estimation for V5 and V6 for obtained distances between 0 and 10 %. The non-parametric locally weighted regression model analyses (LOWESS) showed that the applied linear regressions were approximately consistent to identified local trends, for full-length sequence distances of up to 15 % for all datasets, except V5, while V4-FL comparison consistency expanded to up to 20 %. The V5 dataset showed an underestimation trend according also to the locally weighted regression analysis. For FL sequence

distances of above 15 – 20 % all comparisons showed large deviations from both the linear models and the ideal linear correlation of FL and V region variant distances.

Classification depth testing, indicated that all V region datasets showed a similar under-representation of existing sequences throughout all taxa per taxonomical level, with V6 performing worse compared to the rest (Fig. 5). Phylum level taxonomical classification differences between the full-length sequences and the V region concatenated variants, was performed taking into account sequence numbers per phylum among soil-derived sequences of RDP database. Highly-populated phyla in the database, showed to be less affected by sequence concatenation according to the used parameters compared to phyla parted by 1000 sequences or less in the full-length dataset and the unclassified sequences (Table 1 and Fig. 6). Under-representation trends were observed for intermediate and low sequence numbers encompassing phyla, while over-representation by above 50 % was observed for the unclassified sequences. Highly populated phyla with differences more than 5 % of sequence content between the examined V regions and the corresponding full-length variants were the V4 and V6 that had underestimations for *Acidobacteria*. In the case of intermediate populated phyla, such differences existed for *Planctomycetes*, *Chloroflexi*, *Gemmatimonadetes* and *Nitrospira* that were under-represented for all V region datasets, wile the TM7 was under-represented only for V3 and V5 and *Verrucomicrobia* along with *Cyanobacteria* were under-represented for V6 In low populated phyla V3 and V5 had more (by one) bacterial phyla with smaller differences than 5 % compared to the full-length dataset, with *Chlamydiae* and

Fusobacteria having smaller differences for all V region datasets. Two cases were apparent where sequences were falsely identified as *Lentisphaere* (1 sequence for V4) and *Thermodesulfobacteria* (2 sequences for V6).

Simulated screening of soil samples using single V regions.

Published datasets were downloaded and used as templates for generating corresponding virtual samples. The latter were used for approximating differences between V region fragments and the full length sequences according to identified sequence taxonomical annotation, OTU and phylotype frequencies or presence that would be found in the template samples. Dataset topologies based on sample distances showed an overall better approximation of the FL dataset by the longer stretch V region datasets, V3 and V4 (Fig. 7). V3 showed a better clustering ability with the FL for both relative abundance and presence-absence taxonomical classification matrices compared to the rest, while V4 coincided close to FL for the relative abundance matrices only (Fig. 7 A). V3 and V4 performed better in the OTU approach as well compared to V5 and V6 for both relative abundance and presence-absence matrices of OTUs (Fig. 7 B). Sample distances according to weighted and unweighted Unifrac results indicated that when relative abundance of reads is estimated V4 ad V5 resided closer to the FL dataset (Fig. 7 C left). However, in the case that only sequence occurrence per sample was considered, sample distances for V4 and V3 resembled more the FL sample distances but did not reside as close as in the previously mentioned approaches comparing with V5 and V6 datasets (Fig. 7 C right). V5 and V6 datasets had an overall poor performance with V5 showing to

be slightly closer to the FL compared to V6 according at least at the horizontal axices where most of the variance is explained in all cases.

Discussion

16S rDNA diversity screening using technologies like Illumina producing multimillion sequence reads, is a very appealing method for elucidating ecology concepts in highly complex environmental samples like the ones derived from soil. However, as indicated in the present study, there are several issues that should be taken into account, having to do with contemporary technology abilities and screened environments.

Sequence conservation is an important factor for determining the potentials of screening depth of various taxa based on existing library. Our results (Fig. 2) differed from previous studies encompassing representative sequences of the total RDP database in the case of a few nucleotides in highly conserved areas. Although these areas were consistent for most of the conserved screened nucleic acid bases, there was an overall higher number of polymorphic sites compared to the previous extensive study of Wang and Qian (2009). A potential explanation to this observation has to do with the fact that RDP database deposited sequences, are dominated by human microbiome related bacteria. A simple keyword search (e.g. “human”, “soil”) shows that about 56 % of the ~1,000,000 16S rDNA sequences longer than 1200 bp deposited in RDP database are derived from human body related environments while less than 5 % of the sequences are derived from soil. The identified richness in these two environments in several studies is totally different compared to the corresponding diversity identified in the RPD database , with soil being by far more rich in estimated species numbers

306 (more than 5,000 different species were identified for the complete human
307 microbiome and more than 50,000 species are estimated to exist per soil gram
308 (Torsvik and Ovreas 2002; Gans *et al.* 2005; Tringe *et al.* 2005; Schloss and
309 Handelsman 2006; Huse *et al.* 2008)). Therefore it is important to consider the
310 particulars of the studied environment during experimental design. Moreover,
311 comparison of these differences indicates that identified differences between the
312 complete RDP database and the soil derived 16S rDNA sequences comprise a
313 reflection of existing differences in niches existing in the natural environments
314 where sequences were derived.

315 Interconnected to the previous discussion point is also the operational fragment
316 length for an Illumina technology application. Current Illumina technology
317 screening abilities using the latest available (v4) chemistry are maximized using
318 the Genome Analyzer IIx (GAIIx) and exploiting the paired-end reading ability
319 (obtaining reads from both sequence fragment ends). It has been demonstrated
320 that relatively good read quality results can be obtained for read-lengths of 125
321 nucleotides for each one of the two reads per fragment (with the second read
322 showing lower qualities at the error prone read ends) (Bartram *et al.* 2011).
323 Assembly of the paired-end reads per sequenced amplicon in previously
324 published studies required a minimum of 5-12 nucleotides of read overlap (Wu
325 *et al.* 2010; Bartram *et al.* 2011; Degnan and Ochman 2011), that reduces the
326 operational amplicon length to a maximum of 226 bp. Moreover, our screening
327 attempt of RDP sequences for potential tandem repeats that might interfere with
328 assembly at the overlapping regions, did not indicate that related problems
329 would exist by selecting the option of 10 nucleotides overlap (data not shown).

Therefore, the 226 bp of amplicon screening seems like an upper limit concerning length influence on screening abilities. However, multiplexing is the major objective of the technology application and this involves addition of barcode sequences, in at least one of the two primers used. Proposed multiplexing methods involve: a) primer indexing by addition of a few unique bases in the 5' end of one (or both) of the amplification primers plus a 2 bp linker sequence for reducing effects of barcode during environmental sample PCR amplification (Wu *et al.* 2010; Degnan and Ochman 2011); b) usage of primers during environmental PCR amplification with 5' extensions having the complete Illumina sequencing adapters plus an index sequence (Bartram *et al.* 2011), which allows a third sequence read (in paired-end reads usage) for identification of barcodes (similar philosophy to that of Illumina multiplexing kits (Meyer and Kircher 2010)). The second approach has the advantage that barcode index reading does not interfere with the operational read length (like in the first approach), but has the restriction of the number of samples that can be multiplexed (currently up to 96 error correcting barcodes – no such restriction exists for the first approach). For the case that the costs of screening are to be reduced dramatically, the first option allowing screening of more than 96 samples is the apparent choice. However, operational amplicon screening length is also reduced according to the number of barcode bases plus the linker sequence length. All these restrictions result in amplicon screening abilities of maximum length of 215 bp. This screening length was indicated to be enough for screening all V regions with less than 0.5 % information loss. However, 16S rDNA conservation around V4 indicated that for robust primers generation with high coverage such short length like the one tested here (based on Wang and Quian

2009) is difficult to be achieved for soil environmental samples or is possible for reference lengths longer than the ones allowed by Illumina specifications.

RDP database soil derived sequences were further analyzed for assessing representation of the tested full-length sequences concerning obtained distances and taxonomy annotations during sequence comparisons, when sequence parts belonging in the tested V-regions are used. Correlation tests of generated distances of sequences belonging to the same strains for the full length sequences and their V region variants, showed an overall superior performance for the V4 region dataset, followed by V5 for both the Pearson correlation values and the dispersal of points around the applied linear model. However, when examining more carefully V region datasets distances for 0 – 13 % corresponding FL dataset distances there appears to be a distance overestimation for V3 and an underestimation for V5 and V6. This indicates that concerning the tested V regions more per base variability is accumulated in the V3 region compared to the rest V regions and also the FL sequences at these sequence distances. Therefore higher resolution can be obtained at the referred OTU definitions (roughly corresponding at a species to family level).

When classification contrasting of the V region datasets was performed against the FL dataset it became clear that there is information loss along with the sequence size reduction particularly for the V6 dataset (Fig. 5). However, sequence classification was equal or above 70 % of the total reads and above 90 % of the FL classified sequences for the V3, V4 and V5 datasets even in the case of taxonomical level 5 (encompassing order, suborder and family level classifications) providing the opportunity for identifying well or relatively well

defined groups concerning global biogeochemistry of natural environments. According to phylum level analysis results, observed taxonomical information loss of V region datasets compared to the FL dataset was mainly derived from phyla having intermediate or low representatives in the reference database, resulting this way in increase of the unclassified bacterial sequences. V6 dataset in this analysis had more than twice the FL dataset unclassified sequences, while the rest V region datasets had approximately 1.5 times the unclassified FL sequences. The fact that not populated phyla were also under-represented during classification is partly due to the reference database composition. Low representation of taxa in the reference database affects the classification confidence and the probability of identification of partial sequence read (word) matches while searching for closest sequences with the naïve Bayesian classifier (Wang *et al.* 2007).

The performance of simulated analysis provided an approximation of the effect that sequence relative abundance and richness found in environmental soil samples would have on obtained results. Overall, it was shown that datasets of V-regions encompassing longer sequence stretches (V3 and V4) generated sample distances more similar to the ones produced by the FL dataset compared to V5 and V6. Such differences between the V3, V4 and the V5 dataset were not indicated in the previously performed analyses. That is potentially because multiple copies of sequences (or closely related sequences) with differences in performance of the V5 and V6 region compared to the full-length variants existed in the generated dataset, causing exacerbation phenomena.

Concluding remarks

Combination of Illumina sequencing technology with screening partial 16S rDNA sequence reads in environmental samples is a very powerful approach compared to existing methodologies. However, this combination has some limitations that are resulting from the sequence screening length. V3 region selection as the screened 16S rDNA stretch, did not perform as well in the case when the non redundant soil derived sequence dataset was screened, but had a superior performance with the examination of datasets where sequence frequencies approximated the ones found in soil environments. V4 had an overall good performance compared to the rest V regions, but is lacking comparable flanking sites conservation which would allow comparable screening depths. V5 has the screening abilities and had an overall good performance for the non redundant dataset, but apparently the information extracted by this region has differences for certain sequences compared to the full-length 16S rDNA sequence and this phenomenon is exacerbated due to sequence frequencies in soil environments. V6 was outperformed in all tests apart from that one of flanking sequence conservation.

Collectively, these results suggest that partial 16S rDNA reads corresponding to single V regions have flaws compared to the full length read, but there are some that appear to perform better for soil environments, like the V3 region sequence fragments. However, incorporation of database exploration during initial experimental setup stages is strongly suggested for strategy improvement towards experimental goals. This especially holds true during primer designing where the quality of produced data heavily relies. Careful selection of templates from the constantly growing database can improve primer-set collections for

427 selected environments. Potentials for further methodology improvements and
428 can be found in the usage of more than a single V region screening or even the
429 usage of multiple housekeeping genes (Roux *et al.* 2011). However, it must be
430 acknowledged that part of the power of the combination of bacterial 16S rDNA
431 screening with Illumina sequencing is relying on the extensive existing full or
432 nearly full gene length related databases, something lacking to that degree for
433 other genes.

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547 Table 1 Classification of the full-length sequences and their concatenated to the
548 examined V region variants. Taxa are ordered in a descending order according to
549 sequence abundance per taxon in RDP database and the various categories are
550 indicated by different background colors (blue: highly populated taxa; yellow:
551 unclassified; red: intermediate populated taxa; light-brown: low-participating or
552 rare events).

taxon	FL	V3	V4	V5	V6
Proteobacteria	15786	15875	15835	15678	15760
Firmicutes	7388	7209	7431	7619	7144
Actinobacteria	7373	7380	7292	7275	7353
Acidobacteria	4607	4421	4350	4418	3781
Bacteroidetes	1861	1870	1856	1836	1894
unclassified	1521	2318	2303	2307	3383
Planctomycetes	808	752	706	641	725
Verrucomicrobia	759	759	730	749	688
Chloroflexi	690	493	560	391	477
Gemmatimonadetes	446	226	304	392	124
Cyanobacteria	319	318	309	319	280
TM7	162	146	154	153	169
Nitrospira	114	96	95	106	101
Deinococcus-Thermus	97	91	89	98	58
OP10	59	40	19	33	41
WS3	45	36	19	22	9
Spirochaetes	12	13	7	13	12
Deferribacteres	11	9	9	6	8
BRC1	9	11	8	7	7
OD1	7	9	4	12	12
OP11	7	3	3	7	6
Tenericutes	7	7	6	6	6
Thermotogae	5	5	5	7	6
Chlamydiae	4	4	4	4	4
Chlorobi	4	3	3	3	3
Synergistetes	4	4	2	2	4
Aquificae	2	8	2	2	3
Fusobacteria	2	2	2	2	2
Lentisphaerae	0	1	0	0	0
Thermodesulfobacteria	0	0	0	0	2

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555 Figure captions:

556 Figure 1 Entropy plot of 42,109 soil derived 16S rDNA sequence alignment with
557 the hypervariable regions indicated as designated by Baker *et al.* (2003),
558 according to *E. coli* nucleotide numbering. Sequence area presented excludes
559 poorly supported areas of the beginning and the end of the sequences (due to
560 nearly full sequences), excluding this way the V9 region.

561 Figure 2 A) Nucleic acid base composition of the 16S rDNA consensus sequence
562 of the 41,109 RDP database soil derived sequences for 90 % conservation cutoff
563 value. Red background positions include hypervariable stretches as reported in
564 Baker *et al.* (2003) and expanded in the current study, while green background
565 positions are proposed primer designing sites by Wang and Quian (2009). The
566 IUPAC system was used for denoting per base variability (degeneracies) and
567 lower-case letters are used for nucleotide positions where gaps participated by
568 more than 10 % in the position throughout the sequence alignment. B)
569 Comparison of present study results for 95 % sequence conservation with the
570 ones provided by Wang and Quian (2009) for 90% sequence conservation. Letter
571 color coding referring to differences found on sequences of this study compared
572 to that of Wang and Quian (2009): red) increased variability; blue) altered
573 degeneracy without variability increase; green) reduced variability; grey)
574 Although presence of two nucleotides in that positions is implied according to
575 numbering provided by Wang and Quian (2009), these are missing in the
576 published table.

Figure 3 Fragment lengths between conserved sites including the examined hypervariable regions for all screened (41,109) sequences. Sequence fragments were plotted according to length ascending order.

Figure 4 Pearson correlation tests between corresponding sequence distances of the examined V regions and the full-length variants. All tests were significant (P<001). Test correlation index (r) values and linear models (presented with solid lines) used to describe overall trends are provided above and below each plot. Local relationships between corresponding sequence distances of the FL and the rest datasets are expressed with the non-parametric LOWESS (locally weighted regression and smoothing scatterplots) regression analysis plotting (dot-dashed lines), while the ideal y=x correlation is also plotted (dashed lines).

Figure 5 Classification depth comparisons among the FL and V region versions of the 42109 RDP soil derived bacterial 16S rDNA sequences.

Figure 6 Average values of over or under representation of phyla in the various V region datasets compared to the full-length sequences.

Figure 7 Taxonomy, OTU (3 % sequence distance) analysis and Unifrac results of performed simulation for soil environmental sample analysis. A) PCA results of matrix generated by sample distances based on classified sequences relative abundance (left) and presence absence (right) for the V regions and FL datasets. B) Similarly to A for OTU relative abundance (left) and presence absence (right). C) PCA results for matrices generated using the weighted (left - phylotype relative abundance based) and unweighted (right - phylotype occurrence based)

599 Unifrac analysis result distances between samples for the V regions and FL
600 datasets.

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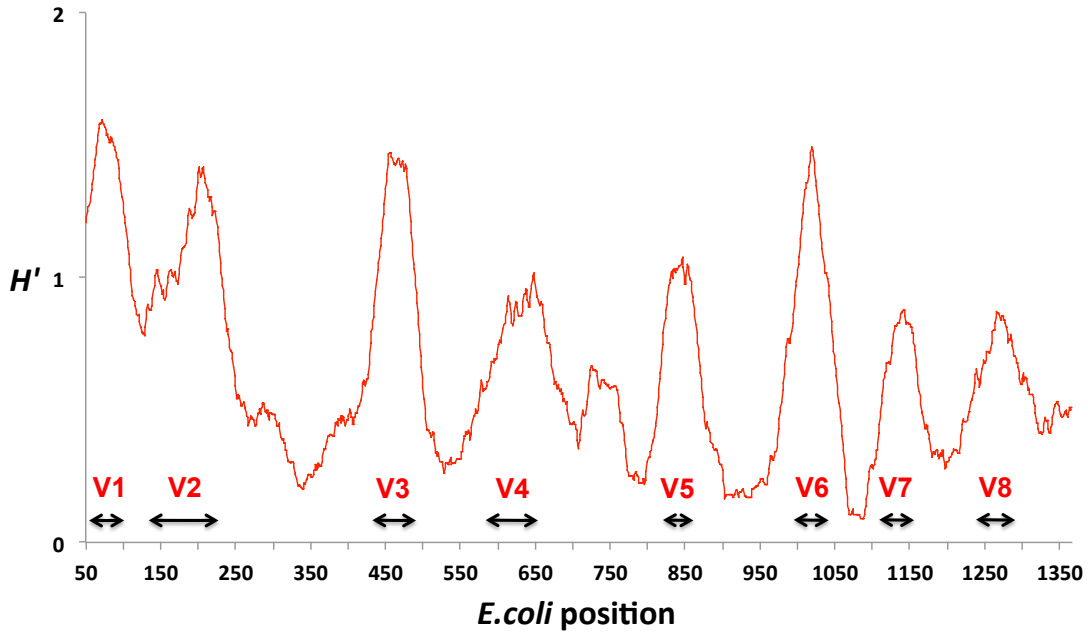
604 Figure 1



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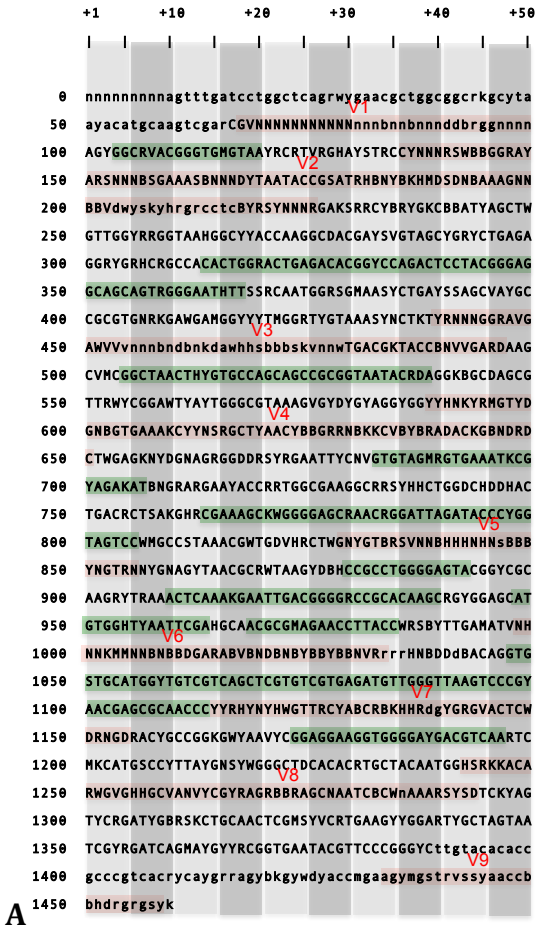
608 Figure 2



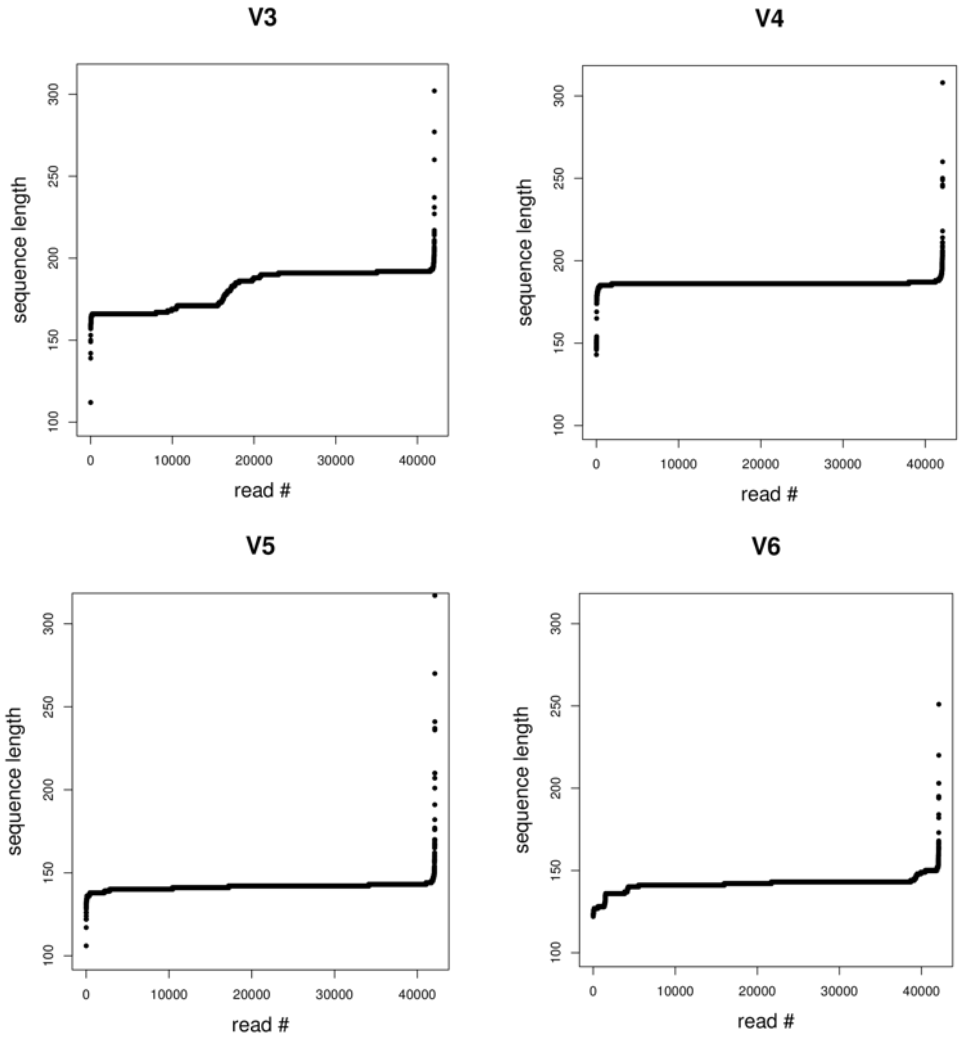
609 **A**

B

E.coli position	Sequence
104-120	GGCRVACGGGTGMGTAA
314-368	CAYTGGVACTGARACACGGBCCAGACTCCTACGGGAGGCAGCAGT VRGGAATHTT
505-539	GGCYAACTHYGTGCCAGCAGCCGCGGTAAKACRDR
683-707	GTGTAGMRGTGRAATKCGYAGAKAT
764-806	CGAAAGCNWGGGKAGCRAACRGGATTAGATACCCYGGTAGTCC
879-893	CCRCCTGGGRAGTAC
909-940	ACTCAAAKGAATTGACGGGGRCCGCACAAGC
949-964	ATGYGGHTYAATTCGA
969-985	ACGCGMARAACCTTACC
1048-1114	GTGSTGCATGGYTGTCGTCAGCTCGTGYCGTGAGRTGTTGGGTTAA GTCCCGYAACGAGCGCAACCC
1177-1197	GGAAGGYGGGGAYGACGTCAA

610

611 Figure 3



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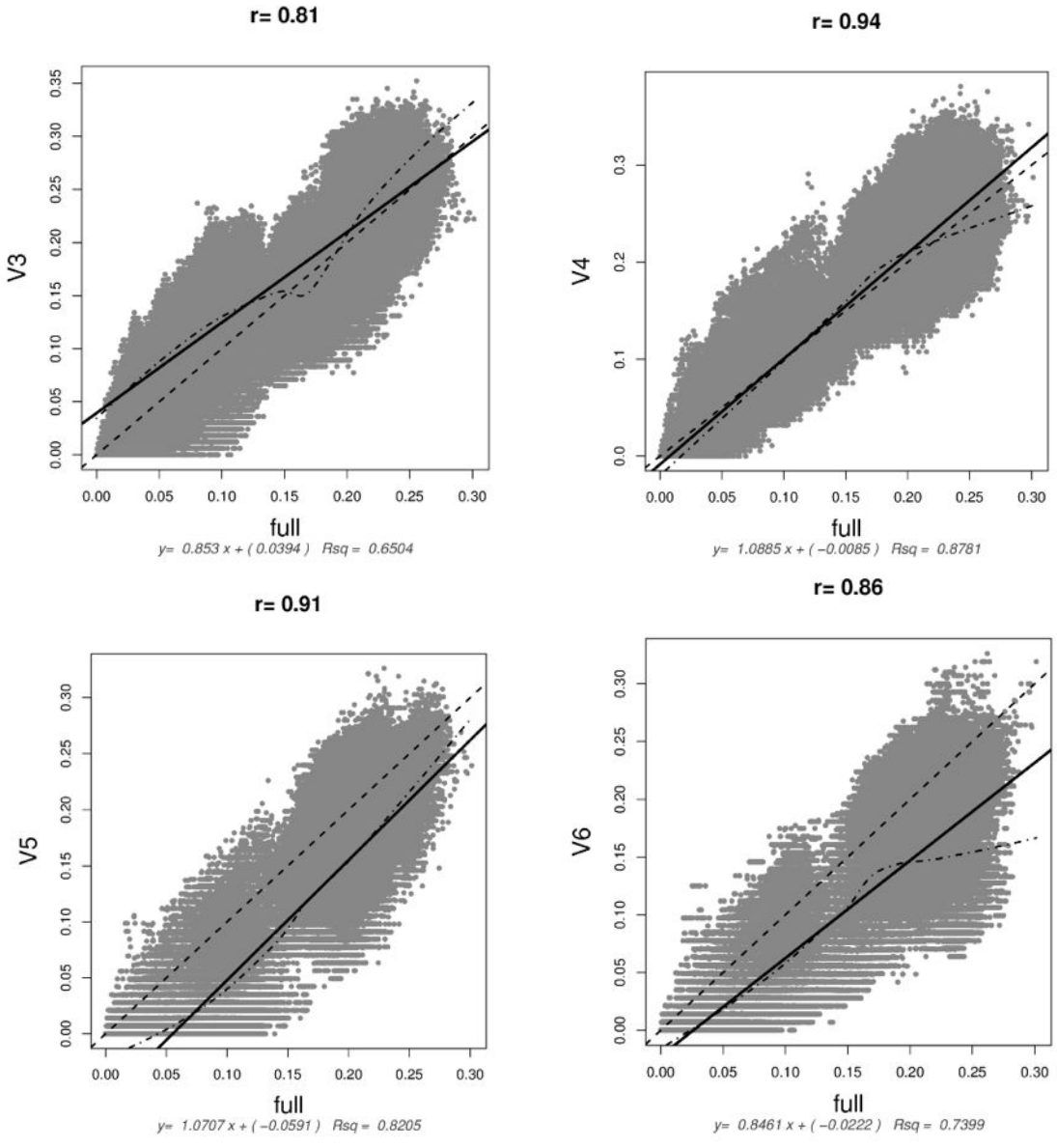
615 Figure 4



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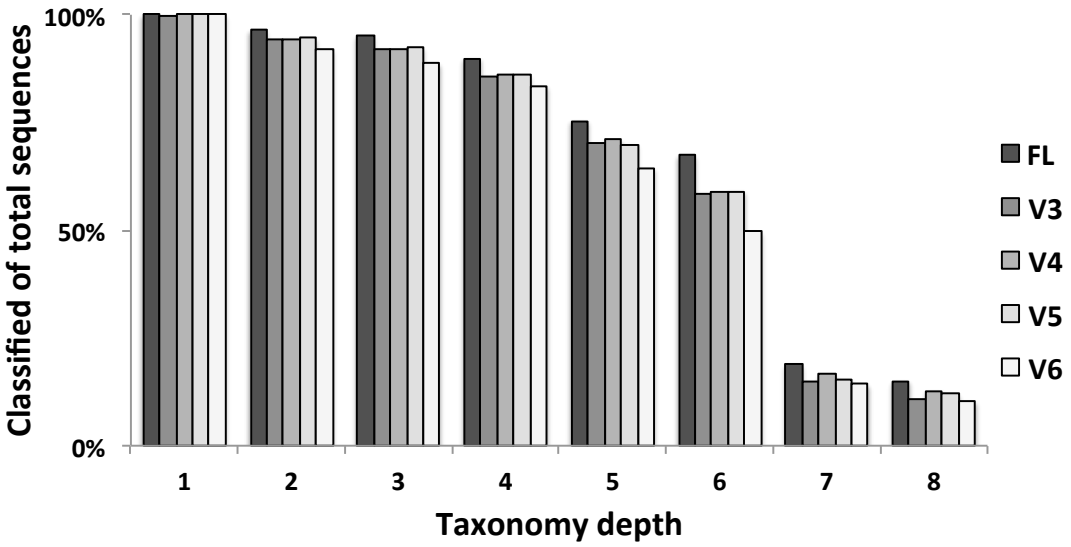
619 Figure 5



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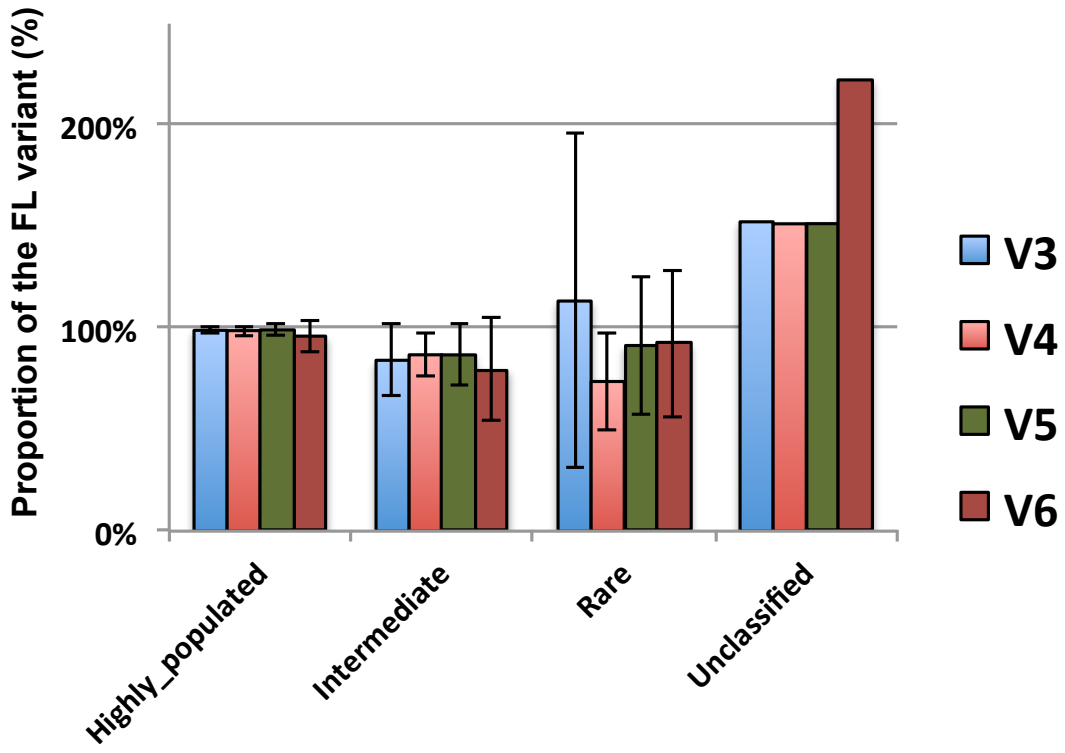
622 Figure 6



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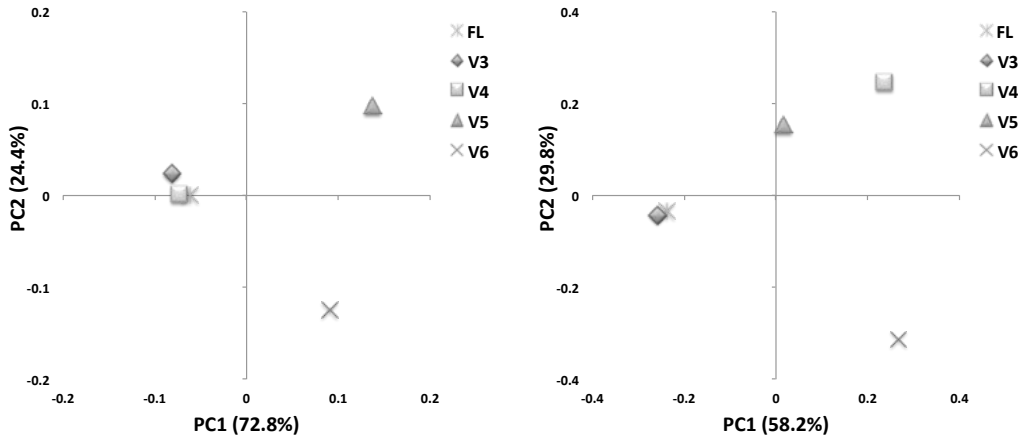
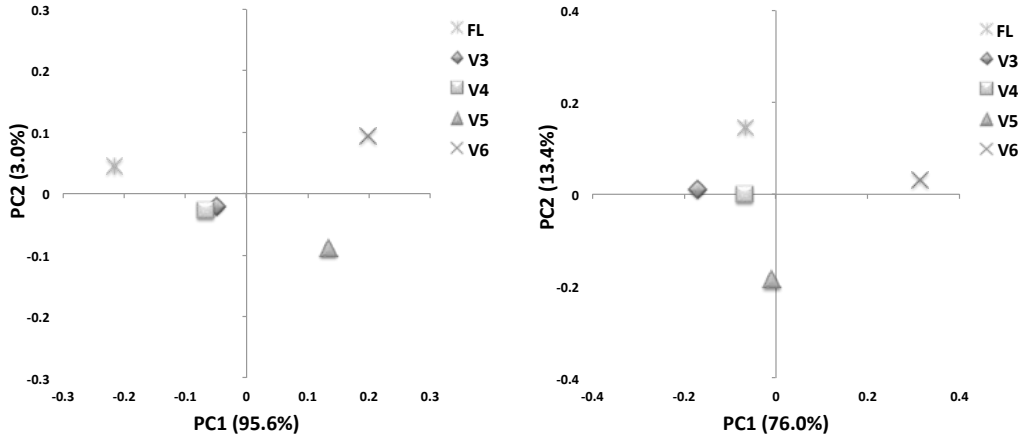
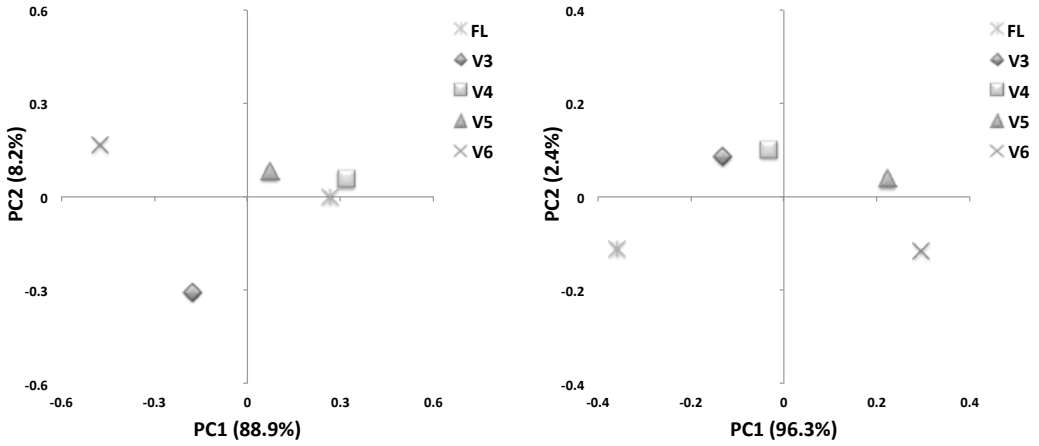
625 Figure 7



626 **A**



627 **B**



628 **C**

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Chapter 3

Title:

Soil prokaryotic diversity patterns of ecosystem services

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SV: laboratory methods setup, analysis setup, data analysis, writing of the report

EP: original idea, evaluation of methods, performance of laboratory work, discussion of data analysis and report

MA: evaluation of original idea and methods, discussion of data analysis and report

FC: evaluation of original idea and methods, discussion of data analysis and report

PSC: evaluation of original idea and methods, discussion of data analysis and report

TM: original idea, evaluation of methods and discussion of data analysis and report

Abstract

Several dominant ecosystem services are tightly associated with soil environments manipulated for agricultural production in expense of microbial functions. Focus of this study was to explore how acquired services are reflected on prokaryotic community structure and functional affiliations in agricultural environments. We screened communities using the 16S rDNA gene marker and the sequencing depth provided by Illumina technology in soils of common origin but with different use and management during the past 6 - 7 centuries. Major factors affecting community structure were the organic carbon quantitative and qualitative traits and also the pH. Human inputs also had an apparent effect on microbial community structure, not only by altering soil environmental conditions, but also through direct prokaryotic biomass carry-over. Taking into account year round disturbance events in the examined soils, higher diversity and evenness was observed in more disturbed soil environments. An appealing explanation to this phenomenon is provided by the intermediate disturbance hypothesis, depicting that non-deleterious (or intermediate) disturbances increase diversity in complex environments. Next to that, the less diverse and perturbed soils had higher measured organic carbon decomposition related activity, indicating that diversity and productivity (energy flow) do not necessarily coincide as opposed to previous beliefs.

Chapter 3

Introduction

Soil is a highly complex and important matrix considering encompassed bio-diversity and number of biological processes (Barrios 2007). Many of these processes, contribute to ecosystem services (e.g. nutrient cycling, soil erosion control and biological pest control) supporting human activity in either natural or managed environments. However, human input and related perturbations, derived from attempts to enhance ecosystem services, are altering soil qualitative traits close to or even beyond points at which natural attenuation mechanisms may lead to functional restoration (Shennan 2008).

Although land use and management attributed effects on the soil prokaryotic community structure have been long acknowledged, relevant detailed information is scarce (Garbeva *et al.* 2004). Moreover, even in cases of detailed outcomes, identified pattern interpretations focus towards dissimilarities rather than, useful for a fundamental microbial ecology theory build-up, unifying principles (Fierer *et al.* 2009). Scarcity of land use and management effect related information for soil microbes, can be partly explained due to past screening limitations of available methods for studying microbial communities using environmental samples. The small ribosomal subunit encoding gene usage as a single marker for assessing microbial evolutionary relationships (Woese 1987) and diversity (Muyzer *et al.* 1993) was a breakthrough towards increased screening resolution in the late 1980's and 1990's. However, uncovering the vast numbers of microbial occurrence in complex soil environments (Schloss and Handelsman 2006) was hampered by 1990's technological boundaries. Recent high throughput sequencing technologies have greatly overcome such problems

(Edwards *et al.* 2006; Sogin *et al.* 2006; Roesch *et al.* 2007; Lazarevic *et al.* 2009; Caporaso *et al.* 2010; Claesson *et al.* 2010; Gloor *et al.* 2010; Wu *et al.* 2010; Bartram *et al.* 2011), therefore allowing deeper investigation for filling the gaps of existing ecology theories. In respect to land use and management effects such are the ones examining the relations between microbial diversity and productivity (energy flow) in environmental samples.

Structural and functional diversity along with productivity have been roughly considered as convergent microbial community properties concerning responses to management practices inducing perturbation events in soil (Welbaum *et al.* 2004). In this consideration, soil homogenization, characteristic of agricultural soils, is expected to reduce the diversity of trophic microsites, microbial diversity and productivity. Two ecology approaches have been derived from plant ecology and also proposed for microbial communities, which are to an extend contradictory to this consideration. The first one is the intermediate disturbance hypothesis (IDH) originally formulated by Connell (1978) for modelling effects of non-severe stresses (e.g. not eliminating most populations) in highly diverse plant communities looking into diversity as a function of disturbance. According to the theory, non-severe stresses promote diversity by reducing the population sizes of the more dominating and competitive species of an environment. This theory was also found to be applicable in simple setups with single bacterial species populations laboratory experiments (Buckling *et al.* 2000). The second taking into account diversity as a function of availability of resources, proposes a hump-shaped relationship between diversity and productivity (Lynch *et al.* 2004). This model is based on the resource heterogeneity hypothesis (RHH)

suggesting that after reaching a pick, diversity decreases with increase of productivity due to reduction of resource heterogeneity and therefore number of niches. Prokaryotes, being key players in soil biogeochemistry (Prosser *et al.* 2007) and comprising most of soil biodiversity (Whitman *et al.* 1998), are ideal markers for assessing effects of ecosystem services on these microbial community properties.

Aim of the present study was to identify patters of prokaryotic diversity related to acquired ecosystem services as a result of long-term land use and management decisions. We exploited the sequencing depth provided by Illumina technology and took a snapshot of 16S rDNA soil prokaryotic diversity from soils of common origin but undergoing three different levels of human intervention. Historically, all soil environments studied here originated from the bed of a swamp owing its existence to underground water tension. Drainage, which took place 6 – 7 centuries ago, lead to the agricultural exploitation of the area located next to the river Po and generated the land use and management gradients whose soils were studied here (Kassen and Rainey 2004) (Fig S1). These gradients are: the low-land springs (locations where underground water-tension release takes place and starting points of above ground water networks ending in river Po); maintained meadow zones around the springs (receive occasional removal of above-ground plant biomass); and the surrounding maize fields (performance of year round agricultural treatments according to organic farming standards). Our results showed that both quantitative and qualitative environmental variables had apparent effects on microbial community structure

and they provided further support to the two above described models (IDH and RHH) for soil environments.

Materials and Methods

Soil sample physical-chemical-biochemical properties. Top-soil samples were collected in triplicates from three environments across a low-land spring area (Fonti della Gaverina 45°27'55.69'' North, 9°38'20.05'' East, elevation 97 m, Italy – sampling carried out in April 2010) land-use and management gradient next to river Po. The studied soil environments were derived from an organic farming maize field (silty clay loam texture), a minimally managed meadow (loamy texture) and the spring banks (referred as riparian, clay loam texture) (Fig. S1). The site sampled in this study was representative of the landscape gradient observed in low-land spring sites of the region. Namely, spring bank (referred as riparian hereafter), meadow and maize top-soil samples were collected at an overall distance within the range of 100 meters. Main human activity related site qualitative traits prior sampling time-point were: seedbed preparations, slurry applications and also sowing and weed removal for maize; no tillage and occasional harvesting of above-ground plant biomass encompassing grass and leguminous plants in meadow; water saturation of soil in riparian during the high precipitation season (late autumn to early spring), and abolishing of this effect due to spring water level drop as an outcome of reduced precipitation and increased water use for supporting agricultural activities.

Total organic carbon (TOC), total nitrogen (N), cation exchange capacity (CEC) and soil pH were determined using the standard methods recommended by SSSA

(Sparks 1996), while particle-size analysis was carried out using the pipette method (Day 1965). Three carbon fraction measurements (labile, moderately labile and recalcitrant) were based on the Walkley-Black method as modified elsewhere (2001) for obtaining the different fractions. β-glucosidase (EC 3.2.1.21) and acid phosphatase (EC 3.1.3.2) activities were determined by the p-nitrophenol method of Eivazi and Tabatabai (1988) and Margesin and Schinner (1994), with p-nitrophenyl-β-D-glucoside and p-nitrophenylphosphate as substrate respectively. Nitrate reductase was determined using KNO_3 as a substrate according to reference (Fu and Tabatabai 1989).

DNA isolation, PCR conditions, multiplexing and sequencing.

Top-soil (the upper 5 -10 cm) derived from each core was sieved through a 2 mm mesh, visible roots were removed and 500 mg were used to extract DNA. DNA extraction was performed using the FastDNA® SPIN kit for soil with a FastPrep® 24 instrument (MP Biomedicals, LLC, Solon, OH, USA) according to the manufacturer instructions. Extracts were quantified using the Quant-iT™ (HS dsDNA Assay and RNA Assay kits respectively, Invitrogen, Paisley, UK) in combination with the Qubit™ fluorometer (Invitrogen, Paisley, UK), while they were purity and shearing screened using a Biophotometer (Eppendorf, Hamburg, Germany) and 0.8 % agarose gel respectively. 2 ng of purified DNA extracts were used for the bacterial 16S amplifications while 20 ng was used for the respective archaeal. 50 µl reactions were performed according to the following PCR program: 94 °C for 5 minutes, 35 cycles X [94 °C for 30 seconds of denaturation; 50 °C (for bacterial primer-sets) and 60 ° C for 30 seconds of primer annealing for the bacterial and the archaeal 16S rDNA targeting primer-sets respectively

(Table S1); 72 °C for 30 seconds of elongation] and 72 °C for 10 min. 50 µl PCR reactions were performed using mixtures as follows: 1 X PCR buffer, 2.5 mM $MgCl_2$, 2.5 Units of AmpliTaq® Taq polymerase (Applied Biosystems, Foster City, CA, USA), 0.4 mM of each dNTP, 0.5 µM forward primer and 0.5 µM of reverse primer, 2 and 20 ng of template DNA for the case when bacterial and archaeal 16S rDNA targeting primers were used respectively. DNA extracts or PCR product were temporarily stored at – 20° C until further use.

PCR products were labelled with 6 bp indices (Table S2) according a previously published indexing method [37] and concomitantly pooled to a single sample. Sequencing of the PCR amplicon pool was performed with a HiSeq 2000 Illumina genome analyzer, using the paired-end reads module by Fasteris SA (Geneva, Switzerland) and the v4 chemistry.

Datasets preparation. Obtained single read sequences were separated by barcode and primer and low quality sequences were filtered out according to average per read Phred quality values (>25) and minimum per base quality (>5). This resulted in average Phred quality values above 30 and 95 % confidence minimum Phred quality of above 20 apart from the last base (Illumina reads are known to be low quality prone in the read-end (Caporaso *et al.* 2010)). Such bases were considered during concomitant analyses as sequencing artifacts and clustering of sequences differing by one base was performed prior further sequence analysis. Sequence and sequencing-run quality was assessed with the SolexaQA (Cox *et al.* 2010) and the FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) software.

Single reads prior any quality control were also used for performing paired-end read assemblies for the bv5m dataset. Parameters used were: sequence matching of at least the last 10 consecutive bases of each read with maximum 5 % of mismatch. Script used was the "mergepairs.py" provided as part of the Velvet software (Zerbino and Birney 2008) associated, Velvet assembly report suit (http://code.google.com/p/standardized-velvet-assembly-report/).

Data analysis workflow. Sequence data analysis and diversity indices calculations were performed using the Mothur platform (Schloss *et al.* 2009) v 1.16.0, while statistical tests were performed with the R software (R_Development_Core_Team 2009) using the BiodiversityR (Kindt and Coe 2005), R Commander (Rcmdr) (Fox 2010) and Coin (Hothorn *et al.* 2006) packages. Analyses performed reside in operational taxonomic unit (OTU) and taxonomy based approaches according to known strengths and weaknesses for each one of them [46]. Sequences differing by one nucleotide were clustered together, assuming this difference being a potential sequencing artifact.

Operational taxonomic unit (OTU) approach was used to describe diversity-based relations between samples and diversity drivers (in combination with quantitative environmental variables), according to genetic variation of analyzed sequences. Generated datasets were aligned against the ARB (http://www.arb-home.de/) aligned Silva 16S rDNA sequence reference databases for *Bacteria* and *Archaea* as curated by the Mothur development team, using the nearest alignment space termination algorithm (NAST) with the Needleman-Wunsch alignment method modification (DeSantis *et al.* 2006; Schloss 2009; Schloss 2010). Sequences were screened for miss-alignments and a cutoff minimal

alignment stretch length of 60 bp for single reads was applied. OTUs were formed post sequence distance calculation (Schloss 2010) using hierarchical clustering and the average linkage method (Sun *et al.* 2009; Schloss and Westcott 2011). OTU participation or presence per sample was used to calculate the Good's coverage estimate (Good 1953), the Shannon (*H'*) (Shannon 1948; Krebs 1989) diversity index, the Shannon based Equitability (H'/H_{max}) (Sheldon 1969) and the Chao1 (*S*) (Chao 1987) richness estimate (Table S6). Moreover, matrices were produced for the presence-absence and relative abundance of OTUs per sample for each dataset were used for statistical tests performed in R as shown further on.

The second approach involved classification of sequences microbial taxa for correlating known microbial attributes with qualitative environmental traits of samples and sample groups. Sequences were classified using the non aligned versions of the above mentioned reference databases and the naive Bayesian classifier with a bootstrap cutoff value of 50 % (Claesson *et al.* 2009). Classification was performed according to Bergey's manual taxonomy standards. Taxonomical assignment depths were calculated by subtracting the unclassified query sequence affiliation observations from the total query numbers. Phylum and class level analysis was performed for all generated presence-absence and relative abundance of the various taxa in samples per dataset as described further on. In the case of bv5m where classified sequences were more than 60 %, also an order level analysis was carried out.

Statistical tests. Analysis of variance of means (ANOVA) and Tukey's honestly significant difference (HSD) pair-wise comparison test ($\alpha < 0.05$) were

performed where mentioned in case the ANOVA assumptions were met. The ANOVA assumptions were tested using the Shapiro normality test and the Levene's test of equality of variance. In cases that ANOVA was not applicable, the Kruscal-Wallis non-parametric test for significant differences estimation and the Nemenyi-Damico-Wolfe-Dunn joint ranking test (for confidence intervals of 99 %) with Tukey test for pair-wise comparisons were performed.

Correlation of samples based on the normalized values of environmental factors and enzymatic assay measurement values was assessed via principal component analysis (PCA).

OTU or taxonomy approach generated relative abundance matrices were used in the following analyses: PCA on transformed distances with the Hellinger algorithm for sample distance estimation; canonical correspondence analysis (CCA) for taxonomy approach for correlating taxa with samples, while significance of the test was based on 1000 permutations; ANOSIM (Analysis Of Similarity) for OTU approach on the Bray-Curtis distance matrix in order to assess management type effects (test significance was based on 1000 permutations); mantel test for correlating environmental variables with diversity shifts in the OTU approach (1000 permutations).

The order level relative abundance matrix for bv5m as described in the Mothur analysis section, was used for CCA analysis for identification of potential associations of taxa with management types, environmental variables and measured biological activities, in a heuristic approach. All referred variables were plotted and based on constrained distances taxa mostly associated with each management type were extracted. This was carried out by assuming that

taxa within a range from a management type centroid, equal to 1/3 of the average distance between centroids (these distances are almost equal due to the constraining effect), were mostly associated to that management type (example is provided in Fig. S2). Out of the extracted taxa, the ones participating by 0.1 % or more were further studied as potentially significant contributors to the biogeochemistry of the management type samples in a considerable degree. Differences between management types were tested with ANOVA or non parametric tests if the ANOVA conditions were not met. Additionally, a similar approach was applied for the av5f dataset, encompassing the highest read amount among archaeal datasets. All taxon selection related parameters were the same except from the taxonomical level used (class instead of order) and the relative participation cutoff value (1 % instead of 0.1 %).

OTU approach generated presence-absence matrices were used for assessing environmental factor and environment quality influence on richness shifts by application of mantel tests and ANOSIM respectively. The referred analyses were performed as described for the relative abundance matrices processing except from the distance estimation method used during which the Jaccard algorithm was performed.

Results

Soil properties. Soil pH, total organic carbon (TOC) and its fractions, were the chemical properties showing statistically significant differences among management types (Table 1). Measured upper extremes for almost all properties along with the nitrogen content were derived either from meadow (highest TOC) or riparian samples (highest recalcitrant organic carbon – OC – and pH).

Exception was the labile OC proportion with observed highest value for the maize samples. Statistically significant differences were shown for the measured biochemical activities of β-glucosidase and acid phosphatase having the same trends between environments. Both of them had highest values in meadow followed by maize while riparian samples had the lowest values, with β-glucosidase being significantly different between the riparian and the other two environments, while for acid phosphatase differences were significant between all three tested soil environments. Nitrate reductase did not show significant differences following a descending trend with highest values for the riparian samples followed by meadow, while maize had the lowest values.

Correlation of soil chemical and biochemical properties with sample diversity shifts. Correlation of ranked unique OTU distances (OTUs formed by identical sequences) based on their abundance and presence with measured soil properties and microbial productivity (energy flow) was assessed for *Bacteria* and *Archaea*, with performance of Mantel tests.

Archaeal OTU occurrence and relative abundance showed lower correlation levels with environmental variable shifts compared to *Bacteria* (Table 2). Highest correlation was shown for TOC, labile OC and pH for *Bacteria* and pH and humidity for *Archaea*, when relative abundance was considered. In the case of OTU incidence, pH, TOC and soil humidity had highest correlation for *Bacteria*, while pH and humidity for *Archaea*.

Identified β-glucosidase differences correlated with richness and diversity for both *Bacteria* and *Archaea*, while acid phosphatase showed statistically significant correlations ($\alpha < 0.05$) only for shifts observed in *Bacteria* (Table 2).

Nitrate reductase activity having higher values in riparian samples, did not show a significant correlation with differences in either the bacterial or archaeal diversity and richness.

Management type effect on prokaryotic operational taxonomic unit abundance and incidence. Overall structural sample group differences were identified by the performed analysis of similarity (ANOSIM) on the operational taxonomic unit (OTU) relative abundance and presence-absence generated matrices. Unique OTU definitions were used in this analysis as explained in the previous section.

Differences of communities were indicated for both *Bacteria* and *Archaea* with *Archaea* showing lower dissimilarities between soil environment groups (Table S5). More distinct communities were indicated for both microbial domains for the relative abundance matrices compared to the presence-absence matrices in all cases that the test was significant. Exception to this trend was observed for the assembled reads dataset (bv5m) where higher between group dissimilarities were observed for the presence-absence data. Ranking of intra-group distances for relative abundance and presence-absence matrices, showed the following ascending order for *Bacteria*: maize, riparian, meadow. Similar diversity shift trends were observed for *Archaea*, but a reversed relation occurred for the OTU presence-absence analysis, with meadow samples having lower intra-group variability compared to maize and riparian.

Overall community structure differences between soil environments, was also estimated by ranking the diversity values obtained per sample within each dataset for the 3 % sequence distance OTU definition. Estimated richness and

329 diversity was overall higher for riparian samples (Fig. 2). Although meadow had
330 the second highest estimated richness, it had the lowest observed diversity
331 particularly for *Bacteria*. Shannon equitability values showed higher maize
332 evenness values followed by riparian with meadow being most uneven for
333 *Bacteria*. On the other hand, archeal datasets had highest evenness values for
334 riparian with maize coming second.

335 **Taxonomical classification of sequences.** Taxonomical associations were
336 utilized for pattern identification in relations of land use and management
337 qualitative traits with known microbial attributes. Analysis presented here was
338 performed for as deep as order or class level for bv5m (*Bacteria*) and av5f
339 (*Archaea*) datasets respectively. These datasets were selected since they
340 encompassed high sequence numbers, had the longest analyzed sequence
341 stretches (bv5m), and had the highest numbers of classified sequences (SI). For
342 further identifying taxa associated with examined soil environments a heuristic
343 CCA based approach (see *Materials and Methods*) was deployed.

344 *Proteobacteria* encompassed the highest participating taxa for all management
345 types among dominant bacterial phyla (Fig. 1 A) with α-*Proteobacteria* showing
346 major differences between soil environments (higher in meadow by ~ 3-5 %
347 from maize and riparian). The latter occurred mainly due to the high *Rhizobiales*
348 abundance in meadow compared to the other soil environments (Fig. 1 C).
349 *Verrucomicrobia* were also significantly higher in meadow compared to the rest
350 soils. *Firmicutes* were highly abundant in maize with *Lactobacillales* and
351 *Clostridiales* showing statistically significant differences while *Bacillales* had the
352 same trend (Fig. 1 B). Taxa associated to riparian samples were the

Planctomycetes, and the α-proteobacterial *Rhodobacterales* and also *Chlamydiales* (Fig. 1 C). Archaeal participation in all samples was dominated by *Crenarchaeota*. However, an increased incidence of the euryarchaeotal *Thermoplasmata* in maize compared to the rest environments was shown according to relative taxon abundance as indicated also in the PCA biplot (Fig. 1 A).

Discussion

Land use and management gradients examined here are representative of typical ones found in agricultural areas. The particularity of the present study is related to the common origin of soils allowing further investigation of long-term human impact in these environments. Human activity has altered the studied soils concerning both their chemical properties and their prokaryotic community structures. This is also reflected in the measured soil biochemical properties, indicative of microbial productivity (energy flow).

Diversity drivers and soil productivity. Chemical soil properties differed quantitatively but were also indicative of qualitative management traits and are factors with acknowledged major influence on the microbial structure. For example, although maize soils did not have the highest TOC content, the maize labile OC proportion was the highest. This is potentially associated with agricultural tillage practices known to reduce particulate organic matter size and proportion (Liu *et al.* 2006) and also the slurry amendments taking place in maize. Moreover, water induced OC erosion phenomena in riparian soil during the high precipitation period (Lal 2005) support the reduced observed OC content levels with most of it being recalcitrant. Collectively, OC fraction quantities and pH differences found here, although not always large in absolute

values, are factors known to regulate availability of microbial niches, diversity and activity (Sinsabaugh *et al.* 2008; Rousk *et al.* 2009; Kuramae *et al.* 2011; Lopez-Sangil *et al.* 2011). This was also indicated in the present study when correlation tests were performed with carbon and pH being dominant among soil chemical properties in influencing community structural shifts.

Measured biochemical activities were contrasted against prokaryotic community shifts. β-glucosidase, indicative of intense organic matter decomposition (Lynd *et al.* 2002), and acid phosphatase, commonly correlated with the energetically active rhizosphere environments (Nannipieri *et al.* 2003), had highest values in meadow. B-glucosidase was well correlated with both prokaryotic kingdoms structural shifts (Table 2) while acid phosphatase showed correlation only with *Bacteria*. Nitrate reductase however did not show any correlations with community structural shifts. That may be related to previous evidence showing that although nitrate reduction genes are well dispersed among microbial taxa found in soil environments (Philippot 2002), only a narrow set of these taxa are main contributors to the total activity (Wertz *et al.* 2009). Therefore, its effect on total community shifts might be difficult to identify in non-targeted community surveys like the one presented here.

Environment qualitative traits and microbial attributes. Management qualitative traits had a major influence on microbial community structure as indicated by known microbial attributes of identified taxa. High *Rhizobiales* incidence in meadow is consistent to a potentially leguminous species rhizosphere driven environment (Welbaum *et al.* 2004). Moreover, less inhibition of rhizobial growth should exist in meadow compared to maize where

agricultural practices induce increased ammonia presence (Patriarca *et al.* 2002), possibly contributing to the increased observed rhizobial incidence. Rhizosphere driven diversity in meadow compared to the rest soil environments was also indicated for other highly participating and often root environment associated taxa like *Actinobacteria* and *Verrucomicrobia* (Kielak *et al.* 2008; Strickland *et al.* 2009), with the latter showing statistically significant differences. Collectively, showing that meadow environments are quite much affected by roots and their exudates.

Firmicutes investigation revealed that often intestinal microbiome associated taxa (Wang *et al.* 2010) like *Lactobacillales*, *Clostridiales* and *Bacillales* were thriving in maize soils (Fig. 1 B) compared to the rest soil environments. A quite apparent explanation to this concerning their presence is associated with the slurry amendments taking place there. A similar effect was also apparent for previously characterized archaeal taxa. Although all soils were dominated by *Crenarchaeota*, increased incidence of the euryarchaeotal *Thermoplasmata* (characteristic of animal intestinal microbiomes) in maize compared to the rest environments (Fig. 1 A) could also be attributed to the maize soil slurry amendments (Cotta *et al.* 2003; Snell-Castro *et al.* 2005; Mao *et al.* 2011). Next to organic maize amendments, pronounced nitrogen mineralization taking place in agricultural soils due to culture practices (Liu *et al.* 2006), could be accounted for increased *Nitrosomonadales* abundance (Fig. 1 C).

Finally, taxa associated to riparian samples were the *Planctomycetes*, and the α-proteobacterial *Rhodobacterales* and also *Chlamydiales* (Fig. 1 C). All of them are known to encompass *Bacteria* thriving in freshwater environments, either as

425 free-living or as protozoan symbionts concerning *Chlamydiales* (Schlesner *et al.*
426 2004; Imhoff 2005; Corsaro *et al.* 2009; Fuerst and Sagulenko 2011).

427 **Soil Prokaryotes carry-over and colonization events.** Collectively, land use
428 and management had an apparent influence not only indirectly by altering the
429 soil properties *per se*, but also through direct introduction of microbial biomass.
430 For example carbon inputs are known to have priming effects on indigenous
431 communities (Waldrop and Firestone 2004; Cleveland *et al.* 2007; Blagodatskaya
432 and Kuzyakov 2008), yet the observed difference magnitudes imply that these
433 inputs may be responsible also for microbial carry-over. Applied method
434 screening depth was important for identifying input related patterns,
435 particularly concerning the slurry application. Previously Peu *et al.* (2006) have
436 attempted to assess the 16S rDNA dynamics of related taxa during the slurry
437 storage prior application and also post slurry incorporation in soils, using PCR-
438 single-strand-conformation-polymorphism (SSCP) analysis. Although the
439 patterns and clones produced in their study provided a robust analysis of the
440 community along the composting course, amended soil identification for related
441 taxa was not possible as opposed to the present study. This is quite likely due to
442 technique detection limit issues, resolved to a great degree in the present study
443 as indicated by the estimated diversity coverage (Table S4).

444 Land use related microbial carry-over effects might be more common in the
445 more dynamic (in terms of immigration events) micro-environments of the
446 riparian soil during the water saturation period. *Planctomycetes* and
447 *Rhodobacterales* incidence has been previously associated with both marine and
448 freshwater ecosystems (Crump *et al.* 1999; Buesing *et al.* 2009; Fuerst and

Sagulenko 2011) either as free living or embedded in biofilms. Taxonomical associations identified with riparian samples further support results of previous studies showing the high ability of these taxa to colonize freshwater related soils. A model previously proposed for describing microbial colonization of open ecosystems (marine algae colonization by *Bacteria*) was the competitive lottery model, which combines fitness of the colonizer together with the chance of its existence (Burke *et al.* 2011). In the present study due to higher examined system complexity and the not targeted assays it is difficult to assess. However, association of periodically water saturated soils with taxa known to encompass colonizers, relying on both planktonic and sessile stages (Fuerst and Sagulenko 2011) and also thrive in humid conditions, could partly support such a model.

Diversity as a function of management traits. Meadow being the least managed soil environment, had reduced prokaryotic diversity. Such effect for fallow periods in crop rotation has been interpreted as an outcome of resources depletion (Welbaum *et al.* 2004). However, measured enzymatic activities related to energy flow in the studied soil systems (β-glucosidase, acid phosphatase), show higher values in the meadow. This event along with the increased rhizosphere influence identified by taxonomical annotations for meadow, could support a declining diversity due to dominance of certain resources as proposed by the RHH (Lynch *et al.* 2004). Moreover, although disturbance is difficult to assess in field experiments (usually confounded with other factors affecting diversity and not easily quantified), observed diversity indices (Fig. 2) and the sampled environments disturbance history (Table 1) showed increased diversity dominancy in the most disturbed soils of this study.

473 This observation is consistent with Connell's IDH (1978) proposing that
474 perturbation events, when not deleterious (as in the present study for the
475 average community participating prokaryote), are reducing dominance and
476 promote community evenness.

477 Another important discussion point based on the outcomes of this study is
478 related to the measured estimated richness values. As shown in Fig. 2, both
479 *Bacteria* and *Archaea* have high estimated richness values in the less frequently
480 or less perturbed environments not always being consistent with the relative
481 abundance of OTUs. In the case of meadow as discussed above a relative
482 dominance of a few and more fit for the existing niches microbial populations,
483 might provide an explanation concerning observed equitability patterns in
484 respect to microbial productivity. This leads to a question concerning the
485 importance of the presence of the potentially less active "extra" richness in the
486 less or less frequently disturbed environments. An explanation to this
487 phenomenon was provided in a previous study showing that a significant
488 proportion of the observed richness is derived by dormant microbial forms
489 (Jones and Lennon 2010). Based on this explanation, the less frequently stressed
490 or less stressed soil environments studied may encompass members comprising
491 the diversity and functional reserves and not actively contributing individuals to
492 the identified soil phenotypic traits.

493 *Archaea* follow similar trends as *Bacteria* concerning diversity but no clear
494 differences between maize and meadow samples were identified. Thus indicating
495 the fundamental lifestyle differences between these two kingdoms in respect to
496 environmental qualitative traits identified in this study.

Concluding remarks

Findings of the present study provide evidence that macro-scale ecology encompasses theories with good potential for describing the microbial world, despite acknowledged substantial differences. Such differences are observed e.g. in the inverted pyramid of microbial dissimilatory food webs as opposed to higher organism trophic relations (McArthur 2006). Overall, major prokaryotic community shifts observed could be niche driven as indicated in several cases related to both quantitative and qualitative data. Soil homogenization and related perturbations increased diversity in accordance to Connell's IDH (1978). Reduced diversity observed in meadow could be attributed to the dominance of resources as previously proposed by the RHH (Lynch *et al.* 2004). Moreover, lower equitability and high richness in soils with high productivity support the possibility of increased richness due to dormancy phenomena which might act as reserves (Jones and Lennon 2010).

Collectively, our results show that diversity on its own cannot explain observed microbial activity in the examined highly complex natural soil environments. Thus pointing out that the important for ecosystem services soil quality concept (Liu *et al.* 2006), must be considered in respect with interaction of soil life with land use and management plans.

Acknowledgements

The authors are thankful for the critical evaluation of the manuscript by Dr Ioannes Stergiopoulos (Vanderbilt University, Department of Biological Sciences, Nashville, TN) and Drs Eiko E. Kuramae (Microbial Ecology group, Netherlands Institute of Ecology – NIOO-KNAW, Wageningnen, Netherlands). Moreover they would like to acknowledge the assistance during the study's preparatory stages by Dr Stefano Longo and the technical support by Giulia Maria Parisi and Pierluisa Fantini. This study was also supported by the Doctoral School on the Agro-Food System (Agrisystem) of the *Università Cattolica del Sacro Cuore* (Italy).

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795
796

797 Table 1 Soil properties and qualitative land use and management traits.

		Maize		Meadow		Riparian		
Management traits		**Slurry - Tillage**		**Plant biomass removal**		**seasonal soil saturation**		
Soil perturbation frequency		**Frequent**		**-**		**Not frequent**		
Texture		**Loam - Clay loam**		**Loam**		**Loam - Clay loam**		
Chemical properties		**AVG**	**SD**	**AVG**	**SD**	**AVG**	**SD**	**F statistic**
pH	***	(c) 6.4	±0.10	(b) 6.9	±0.00	(a) 7.16	±0.12	58.4
CEC (meq 100 g-1)		1.07	±0.18	1.79	±0.25	1.1	±0.71	2.5
Humidity (%)		14.57	±2.26	18.06	±2.28	24.31	±9.98	2
N (%)		0.17	±0.01	0.32	±0.03	0.2	±0.24	0.9
TOC (%)	***	(b) 1.43	±0.06	(a) 2.98	±0.26	(c) 0.74	±0.04	158
TOC/N		8.25	±0.08	9.34	±0.05	7.97	±5.57	N/A
labile OC proportion	***	(a) 0.74	±0.10	(a) 0.63	±0.01	(b) 0.37	±0.03	28.3
moderately labile OC proportion		0.05	±0.02	0.11	±0.07	0.12	±0.03	2.1
recalcitrant OC proportion	**	(b) 0.21	±0.08	(b) 0.26	±0.08	(a) 0.51	±0.04	15.6
Enzymatic activities		**AVG**	**SD**	**AVG**	**SD**	**AVG**	**SD**	**F statistic**
Nitrate reductase (µg N g-1 24 h-1)		59.43	±35.68	120.07	±156.35	159.2	±72.09	0.7
β-glucosidase (µg PNP g-1 h-1)	***	(a) 55.61	±10.35	(a) 66.99	±6.80	(b) 13.70	±5.59	38.4
Phosphatase (µg PNP g-1 h-1)	***	(b) 61.29	±0.73	(a) 170.07	±43.24	(c) 6.22	±2.98	33.3
		high	**intermediate**	**low**				
value								

ANOVA significance: '***' 0.001, '**' 0.01, '*' 0.05
Post-hoc pair-wise comparisons: Tukey HSD (α<0.05)
N/A: conditions were not met and non-parametric test was not significant

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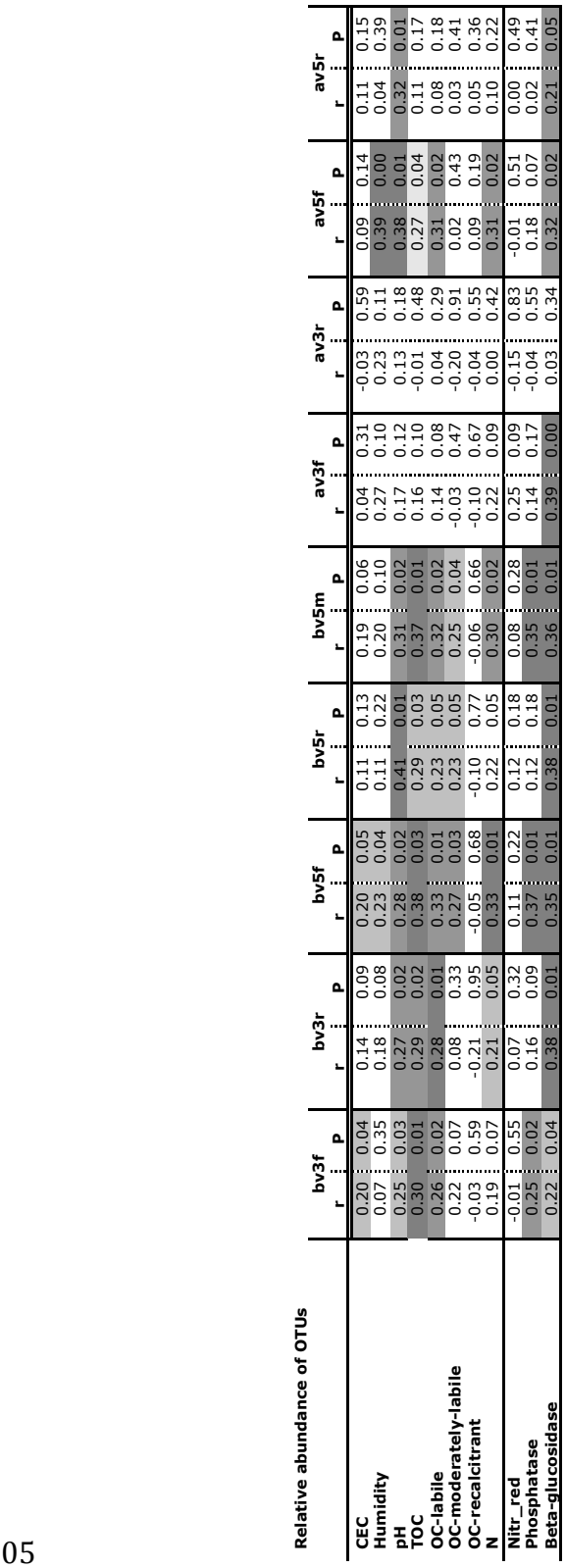
801

802 Table 1 Mantel correlation coefficient r values and test significance (1000
803 permutations) for generated OTU relative abundance (above) and presence-
804 absence (below) matrices for each dataset.



Relative abundance of OTUs

	bv3f		bv3r		bv5f		bv5r		bv5m		av3f		av3r		av5f		av5r	
	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P
CEC	0.20	0.04	0.14	0.09	0.20	0.05	0.11	0.13	0.19	0.06	0.04	0.31	-0.03	0.59	0.09	0.14	0.11	0.15
Humidity	0.07	0.35	0.18	0.08	0.23	0.04	0.11	0.22	0.20	0.10	0.27	0.10	0.23	0.11	0.39	0.00	0.04	0.39
pH	0.25	0.03	0.27	0.02	0.28	0.02	0.41	0.01	0.31	0.02	0.17	0.12	0.13	0.18	0.38	0.01	0.32	0.01
TOC	0.30	0.01	0.29	0.02	0.38	0.03	0.29	0.03	0.37	0.01	0.16	0.10	-0.01	0.48	0.27	0.04	0.11	0.17
OC-labile	0.26	0.02	0.28	0.01	0.33	0.01	0.23	0.05	0.32	0.02	0.14	0.08	0.04	0.29	0.31	0.02	0.08	0.18
OC-moderately-labile	0.22	0.07	0.08	0.33	0.27	0.03	0.23	0.05	0.25	0.04	-0.03	0.47	-0.20	0.91	0.02	0.43	0.03	0.41
OC-recalcitrant	-0.03	0.59	-0.21	0.95	-0.05	0.68	-0.10	0.77	-0.06	0.66	-0.10	0.67	-0.04	0.55	0.09	0.19	0.05	0.36
N	0.19	0.07	0.21	0.05	0.33	0.01	0.22	0.05	0.30	0.02	0.22	0.09	0.00	0.42	0.31	0.02	0.10	0.22
Nitr_red	-0.01	0.55	0.07	0.32	0.11	0.22	0.12	0.18	0.08	0.28	0.25	0.09	-0.15	0.83	-0.01	0.51	0.00	0.49
Phosphatase	0.25	0.02	0.16	0.09	0.37	0.01	0.12	0.18	0.35	0.01	0.14	0.17	-0.04	0.55	0.18	0.07	0.02	0.41
Beta-glucosidase	0.22	0.04	0.38	0.01	0.35	0.01	0.38	0.01	0.36	0.01	0.39	0.00	0.03	0.34	0.32	0.02	0.21	0.05

Presence-absence of OTUs

	bv3f		bv3r		bv5f		bv5r		bv5m		av3f		av3r		av5f		av5r	
	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P
CEC	-0.03	0.54	0.10	0.18	0.05	0.29	0.01	0.41	0.13	0.14	0.00	0.50	-0.06	0.69	0.00	0.44	0.01	0.42
Humidity	0.23	0.08	0.16	0.14	0.33	0.01	0.30	0.00	0.17	0.09	0.26	0.13	0.31	0.05	0.23	0.04	0.11	0.23
pH	0.09	0.25	0.22	0.04	0.25	0.04	0.32	0.02	0.36	0.01	0.09	0.27	0.16	0.10	0.37	0.01	0.27	0.04
TOC	0.21	0.05	0.23	0.04	0.30	0.03	0.20	0.06	0.30	0.02	0.07	0.26	0.07	0.26	0.11	0.13	0.03	0.35
OC-labile	0.18	0.07	0.20	0.05	0.23	0.04	0.15	0.10	0.23	0.05	0.06	0.25	0.11	0.15	0.06	0.25	0.00	0.47
OC-moderately-labile	0.14	0.18	0.15	0.16	0.18	0.07	0.22	0.02	0.14	0.13	-0.07	0.59	-0.14	0.80	0.09	0.28	0.04	0.39
OC-recalcitrant	0.34	0.01	-0.12	0.80	0.03	0.40	0.07	0.23	-0.15	0.87	-0.08	0.63	-0.01	0.46	-0.08	0.73	0.05	0.37
N	-0.01	0.53	0.11	0.19	0.22	0.05	0.12	0.13	0.26	0.05	0.06	0.25	-0.01	0.47	0.04	0.35	0.02	0.43
Nitr_red	-0.15	0.85	0.05	0.36	0.07	0.23	0.07	0.23	0.15	0.14	0.14	0.22	-0.11	0.77	0.02	0.44	-0.02	0.47
Phosphatase	0.18	0.10	0.13	0.17	0.31	0.01	0.19	0.07	0.30	0.02	0.02	0.40	0.04	0.36	0.03	0.38	-0.10	0.78
Beta-glucosidase	0.04	0.31	0.29	0.02	0.37	0.01	0.34	0.02	0.49	0.00	0.32	0.01	0.14	0.12	0.30	0.02	0.15	0.13

Empirical confidence

90% 95% 97.5% 99%

805

Figure captions

Fig. 1 Overview of bacterial and archaeal taxonomy according to selected datasets. ANOVA F-Statistic and P values are provided where applicable, while when this was not the case the non-parametric Nemenyi-Damico-Wolfe-Dunn (NDWD) joint ranking test (99 % confidence intervals) was performed as indicated in brackets.

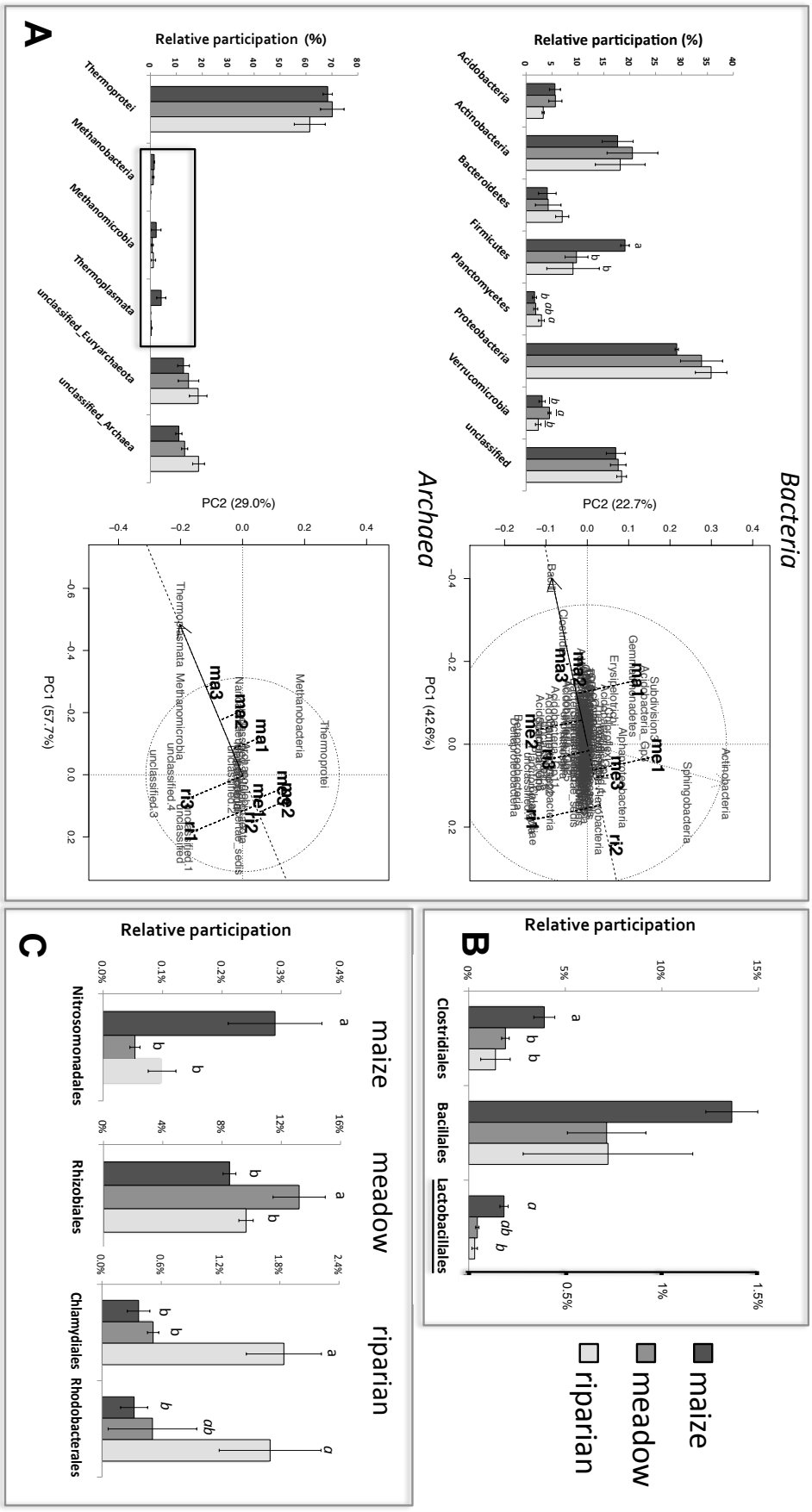
A. Column graphs show the relative participation in samples of dominant bacterial phyla and arhaeal classes (> 1% average relative participation). Significant differences were identified for *Firmicutes* (F-statistic = 9.058, P = 0.015), *Planctomycetes* (F-statistic = 7.344, P = 0.024) and *Verrucomicrobia* (F-statistic = 14.747, P = 0.004). In-frame archaeal classes are common in animal microbiomes. PCA taxon-sample biplots equilibrium circle and vector analysis, indicate taxa with major contribution to the observed variance and high taxon participation direction respectively.

B. Relative abundance of order level taxa related to slurry amendments. Bold formattedY axis refers to the *Lactobacillales* (underlined) relative abundance. Difference significance was indicated for *Clostridiales* (F-statistic = 17.459, P =0.003) and *Lactobacillales* (NDWD).

C. Mostly associated taxa to each sample group except from the animal microbiome taxa shown in B as identified with the CCA based heuristic approach (Methods). Significance of differences was indicated for *Nitrosomonadales* (F-statistic = 20.580 and P = 0.002), *Rhizobiales* (F-statistic = 15.417, P = 0.0043), *Rhodobacterales* (F-statistic = 10.346 and P = 0.011) and *Chlamydiales* (NDWD).

Fig. 2 Chao1 estimated richness, diversity (*H'*) and equitability (H'/H_{max}) comparison for land use and management types throughout all datasets for *Bacteria* and *Archaea* according to ranked values of samples per dataset.. The assembled reads dataset, although consistent, was excluded in order to avoid double counting of the particular hypervariable region. Significant differences are indicated by different letters, while F-statistic and P values for ANOVA were: Shannon-*Bacteria* (F = 13.377, P = $3{,}2\ 10^{-5}$), Shannon-*Archaea* (F = 11.424, P = $1.7\ 10^{-4}$), Equitability-*Bacteria* (F = 8.607, P = $7.3\ 10^{-4}$).

840 Figure 1



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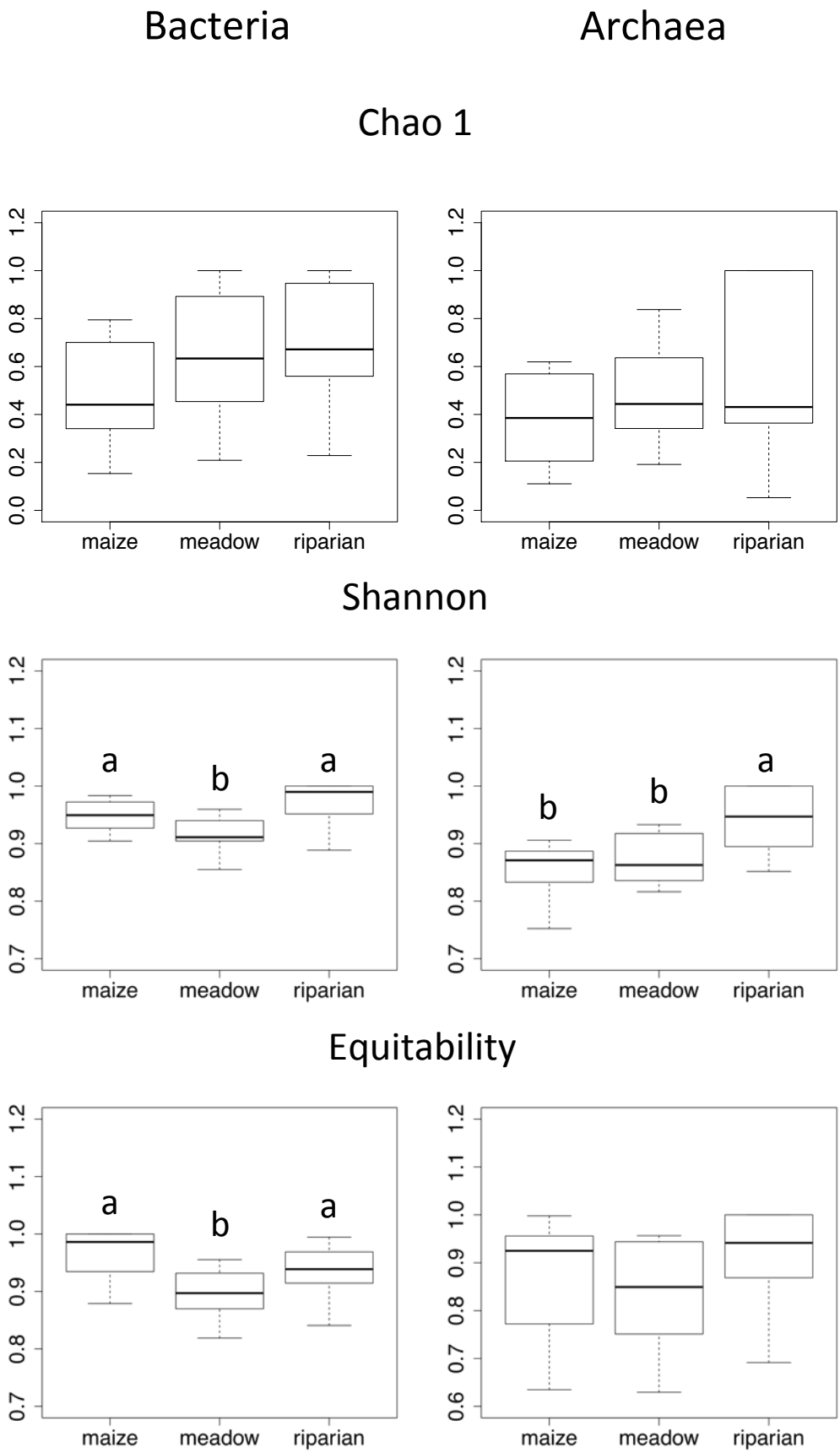
842 Figure 2



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Supplementary Information (SI)

A. Supplementary Results-Discussion

16S rDNA gene fragment generated datasets description.

Bacterial and Archaeal diversities were screened throughout samples using the 16S rDNA molecular marker. For this purpose, polymerase chain reaction (PCR) was performed for amplification of the V3 and V5 hypervariable regions directly on soil DNA extracts. Post PCR performance ligation of an index sequence per sample was selected for screening all samples in a single pool. Ligation efficiency, increased quality control stringency and computing power restrictions substantially reduced the reads output (Table S3). Out of the 131 million forward and reverse reads originally generated, 2.4 % of the single reads (of 70 bp length or longer, excluding the primer sequences) were analyzed for the single read datasets generation. Paired-end read assembly performed for one of the datasets on the other hand, resulted in a higher amount of reads passing the quality control step compared to the single read parental datasets. This was due to second read confirmation of low quality bases in the overlapping read-end, known to be low quality prone in Illumina technology (Caporaso *et al.* 2010). Despite the low percentage of usable sequences, reads provided high total microbial diversity coverage according to the Good's estimate as shown by the operational taxonomic unit (OTU) approach for 3 % distance OTU definition as shown in Table S5. In total nine datasets were generated and named after the targeted microbial group (b/Bacteria/ – a/Archaea/), V region (3 or 5), primer (f/forward/ – r/reverse/) and read assembly ("m" for merged) as shown in Table S4.

Dataset comparisons.

Generated datasets were assessed concerning several overall properties. Such were: the total read numbers per dataset; the diversity coverage provided by the read numbers using the Good's estimate in the OTU approach; the overall sequence classification depth (or percentage of classified sequences to known taxa at the various taxonomical levels) while using the taxonomic assignment based analysis method; and comparison of datasets concerning relative overrepresentation of taxonomical groups.

Concerning the sequence numbers analysed (Table S4) and sequence lengths the datasets bv5f, bv5r and bv5m were the most prominent for *Bacteria* while av5f is the one for *Archaea* as shown in Table 3. Coverage estimates (Table S5) of total diversity based on the experimental setup, showed all forward primer related sequences to provide more diversity coverage apart from the case of av3f for OTU definition of 3 % sequence distance. ANOVA and Tukey HSD post hoc analysis indicated two main groups in terms of achieved coverage of the total defined OTU diversity. The high coverage group of bv3f, bv5f, bv5r, bv5m, av5f, av5r and the lower coverage group of bv3r, av3f and av3r indicated by different letters in table 5. The V5 region was investigated more thoroughly than the V3 region, for both bacterial and archaeal datasets.

The datasets bv5f, bv5m and bv3f have had more classified sequences compared to the rest bacterial datasets, while av5f was shown to perform better for the archaeal datasets (Fig. S3).

As shown in Figs. S4 and S6, no taxonomical groups reside outside the equilibrium circle for both *Bacteria* and *Archaea* and therefore no significant

892 overrepresentations of taxa per dataset could be extrapolated. However, the
893 groups of *Actinobacteria* and unclassified *Bacteria* seem to be responsible for the
894 observed variance to a greater degree than the rest groups (Fig. S4), with the
895 bv3f encompassing more *Actinobacteria* than the rest datasets. Moreover,
896 unclassified *Bacteria* that are dominating the classification results of the bv5r
897 (>80 %) also show a respective correlation. For the case of Archea, av5f, one of
898 the datasets with the highest read numbers and classified sequences, has more
899 classified *Thermoprotei* and less unclassified sequences along with groups like
900 *Methanobacteria* (Fig. S5).

901 **Reference**

902 Caporaso, J. G. *et al.* Global patterns of 16S rRNA diversity at a depth of millions
903 of sequences per sample. *Proceedings of the National Academy of Sciences*,
904 doi:10.1073/pnas.1000080107 (2010).

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906

907

B. Supplementary tables

Table S2 Primer sequences used in the present study. References correspond to Materials section reference list.

Archaea	**Sequence**	**Targeted V Region**	**Reference**
A340F	CCCTACGGGGYGCASCAG		(Vetriani *et al.* 1999)
U529r	ACCGCGGCKGCTGGC	V3	(DasSarma and Fleischmann 1995)
A787f	ATTAGATACCCSBGTAGTCC		(Yu *et al.* 2005)
A927r	CCCGCCAATTCCTTTAAGTTTC	V5	(Jurgens *et al.* 1997)
Bacteria			
E343f	TACGGRAGGCAGCAG		(Wuyts *et al.* 2004; Liu *et al.* 2007)
E534r	ATTACCGCGGCTGCTGGC	V3	(Wuyts *et al.* 2004; Liu *et al.* 2007)
E786f	GATTAGATACCCTGGTAG		(Baker *et al.* 2003)
E926r	CCGTCAATTYYTTTRAGTTT	V5	(Wuyts *et al.* 2004; Liu *et al.* 2007)

Table S3 Adapters used in the present study for sample indexing of PCR products provided in reference (2008) of the Materials section. Bold characters indicate the *SrfI* enzyme restriction site, while the six bases at each end, were the sample index sequences.

Code name	Sequence	Sample
ma1	CAGAGA**GCCCGGGC**TCTCTG	maize1
ma2	CAGCTA**GCCCGGGC**TAGCTG	maize2
ma3	CAGTCA**GCCCGGGC**TGACTG	maize3
me1	CAGTGT**GCCCGGGC**ACACTG	meadow1
me2	CATACT**GCCCGGGC**AGTATG	meadow2
me3	CATATA**GCCCGGGC**TATATG	meadow3
ri1	CATCAT**GCCCGGGC**ATGATG	riparian1
ri2	CATCGA**GCCCGGGC**TCGATG	riparian2
ri3	CGACAT**GCCCGGGC**ATGTCG	riparian3

Table S4 Sequence reads passing the various filtering stages prior analysis and sequences analyzed. From the assembled reads only the bv5 showed adequate assembling (45 % using the Velvet-assembly-report python script) and therefore are mentioned here. For the datasets where the analysis was restricted in sequence numbers due to computing power abilities, the sequence numbers used as opposed to the sequence numbers passing quality are highlighted in yellow.

Stages	bv3f	bv3r	bv5f	bv5r	bv5m	av3f	av3r	av5f	av5r
Successful tagging	641711	814410	4580770	4884253	2468376	250973	315120	2178896	2092198
Quality check	233334	63385	1646114	1336416	2130585	86975	48675	263475	106450
Entered analysis	233334	63385	999999	1336416	870000	86975	48675	263475	106450
Used in analysis	228928	59937	999922	1145918	674422	82305	45165	262223	100054

Stages totals	Sequence #
Single reads passing tagging filter	15758331
Single reads passing quality check	3784824
Single reads used	2924452
Assembled reads available (bv5 only)	2130585
Assembled reads used (bv5 only)	674422

927 **Table S5 Good's coverage estimate of total diversity estimate percentages at a sequence**
928 **cluster distance of 3 % for all generated datasets. ANOVA with Tukey HSD pairwise**
929 **comparison was performed in order to assess within dataset management type**
930 **differences and also potential differences between datasets. Statistically significant**
931 **differences were indicated only for the between dataset comparisons (F-statistic = 26.678,**
932 **$P < 2.2 \times 10^{-16}$) with dataset groupings according to Tukey HSD as shown by the different**
933 **letters in brackets ($\alpha < 0.05$).**

	maize		meadow		riparian		total	
dataset	AVG	SD	AVG	SD	AVG	SD	AVG	SD
bv3f	82%	±4%	87%	±2%	88%	±3%	(a) 86%	±4%
bv3r	57%	±11%	65%	±4%	60%	±7%	(b) 61%	±8%
bv5f	93%	±2%	96%	±2%	94%	±2%	(a) 94%	±2%
bv5r	85%	±4%	90%	±3%	88%	±2%	(a) 88%	±4%
bv5m	89%	±1%	91%	±1%	89%	±1%	(a) 90%	±1%
av3f	72%	±7%	76%	±7%	59%	±32%	(b) 69%	±18%
av3r	67%	±4%	68%	±0%	66%	±2%	(b) 67%	±3%
av5f	89%	±4%	92%	±3%	89%	±4%	(a) 90%	±3%
av5r	82%	±6%	86%	±3%	83%	±5%	(a) 84%	±5%

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937 **Table S6 Analysis of similarity results R statistic and P values for OTU relative abundance**

938 **and presence-absence generated matrices.**

	Relative abundance		Presence-absence	
	R	P	R	P
bv3f	0.687	0.008	0.523	0.015
bv3r	0.704	0.005	0.638	0.007
bv5f	0.778	0.008	0.753	0.011
bv5r	0.794	0.003	0.753	0.011
bv5m	0.786	0.004	0.811	0.005
av3f	0.539	0.002	0.366	0.033
av3r	0.103	0.200	0.3	0.084
av5f	0.885	0.005	0.720	0.011
av5r	0.580	0.012	0.498	0.018

Differences due to treatment (R > 0.75)
Differences with some overlapping (0.25 < R < 0.75)
No or minor differences (R < 0.25)

939 Test not significant

940

941

C. Supplementary figures

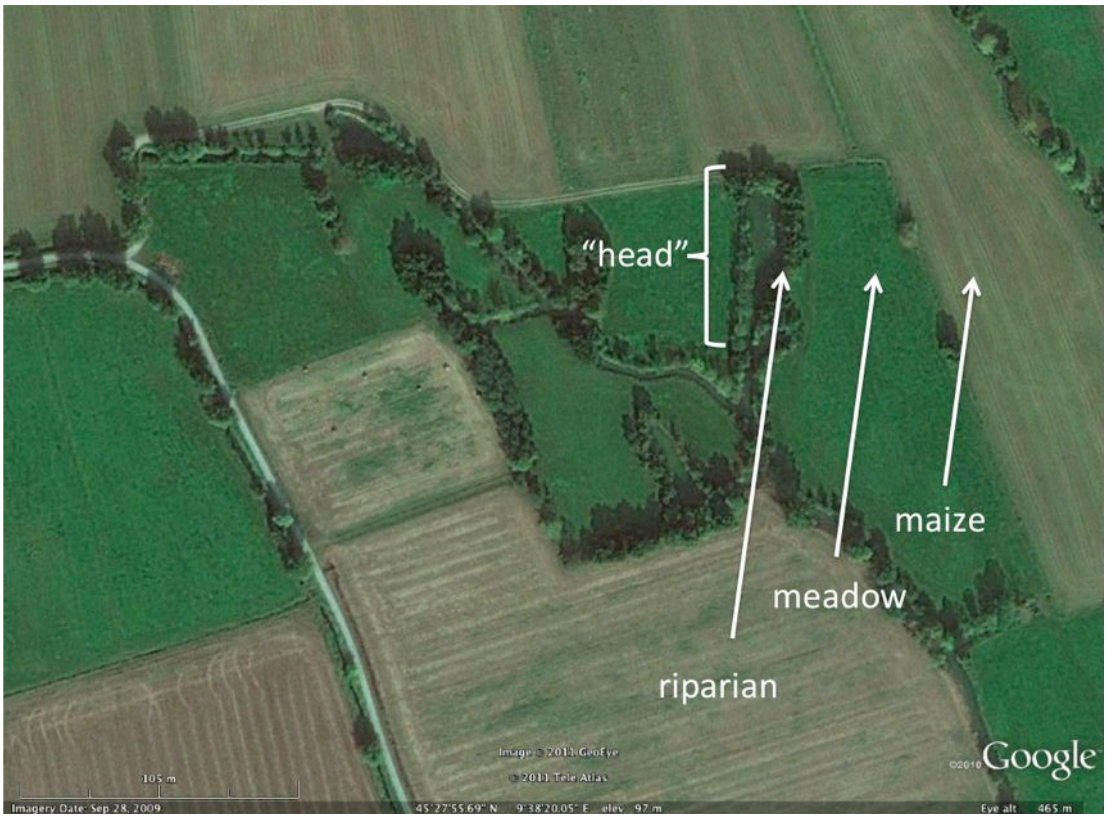


Figure S1 Satellite photograph of the Gaverina area lowland spring (Source: "Gaverina." 45°27'55.69'' North, 9°38'20.05'' East, Google Earth, September 28, 2009). Sampling terrains per management type are indicated, while samplings were performed in triplicates per soil environment.

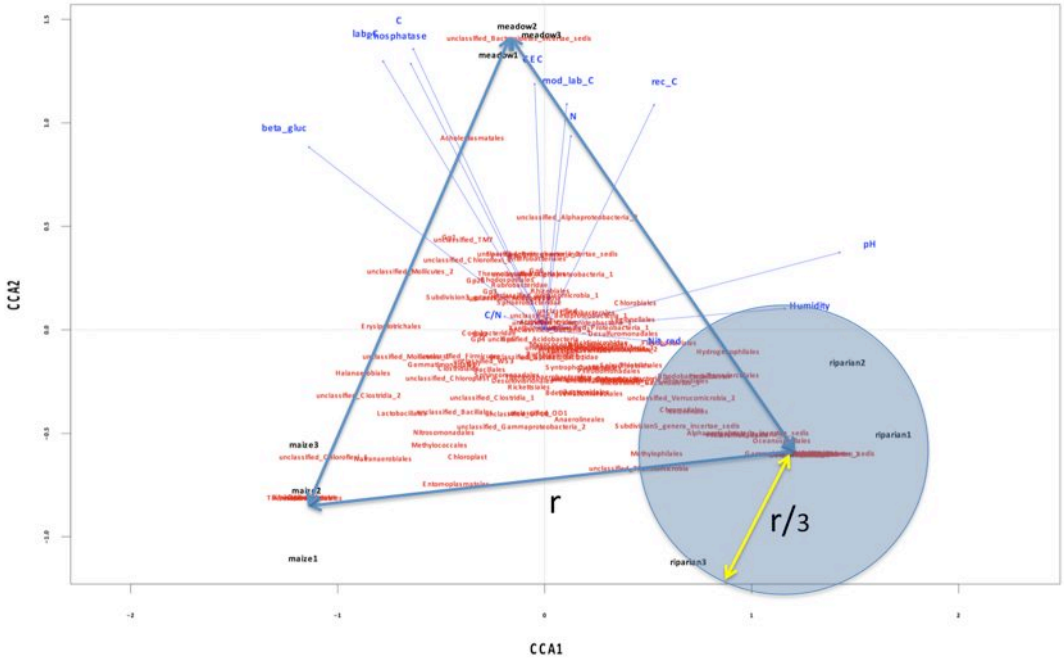
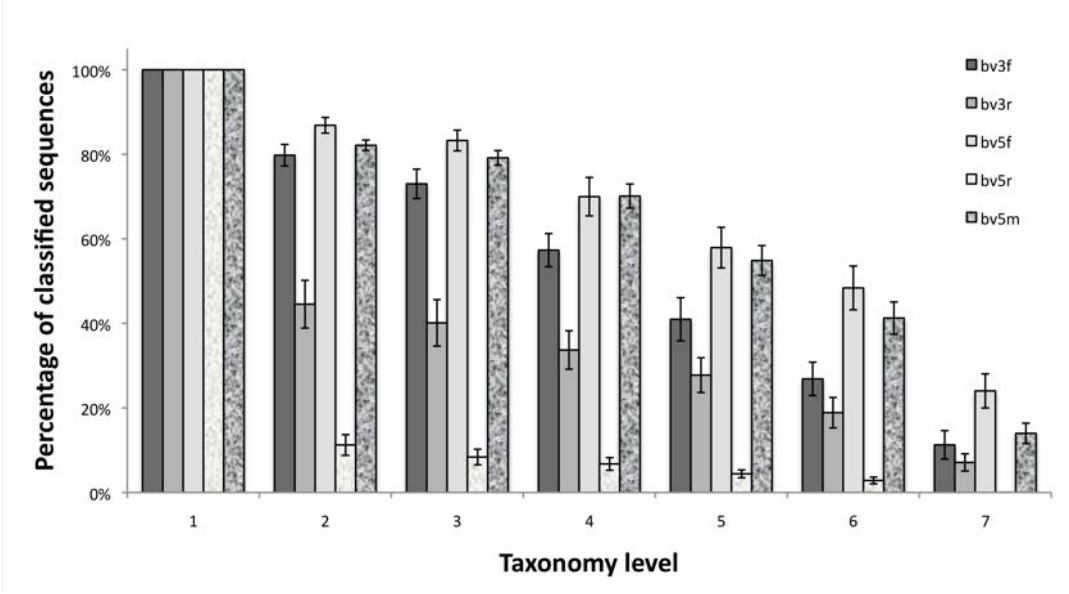
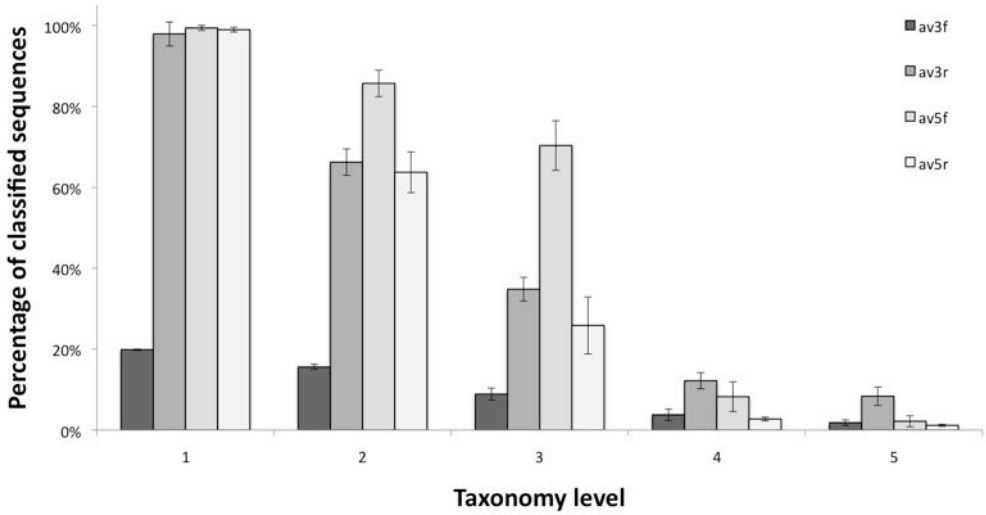


Figure S2 Example of the first step for identification of the mostly related taxa to management types for the bv5m order level dataset. Distances between management types were equal to r = 2.44 units. Environmental factor and enzymatic activity related arrows indicate the direction of value increase. Based on the heuristic approach, taxa residing within the r/3 distance (area boundaries indicated with the blue circle) were further investigated.

957

A.

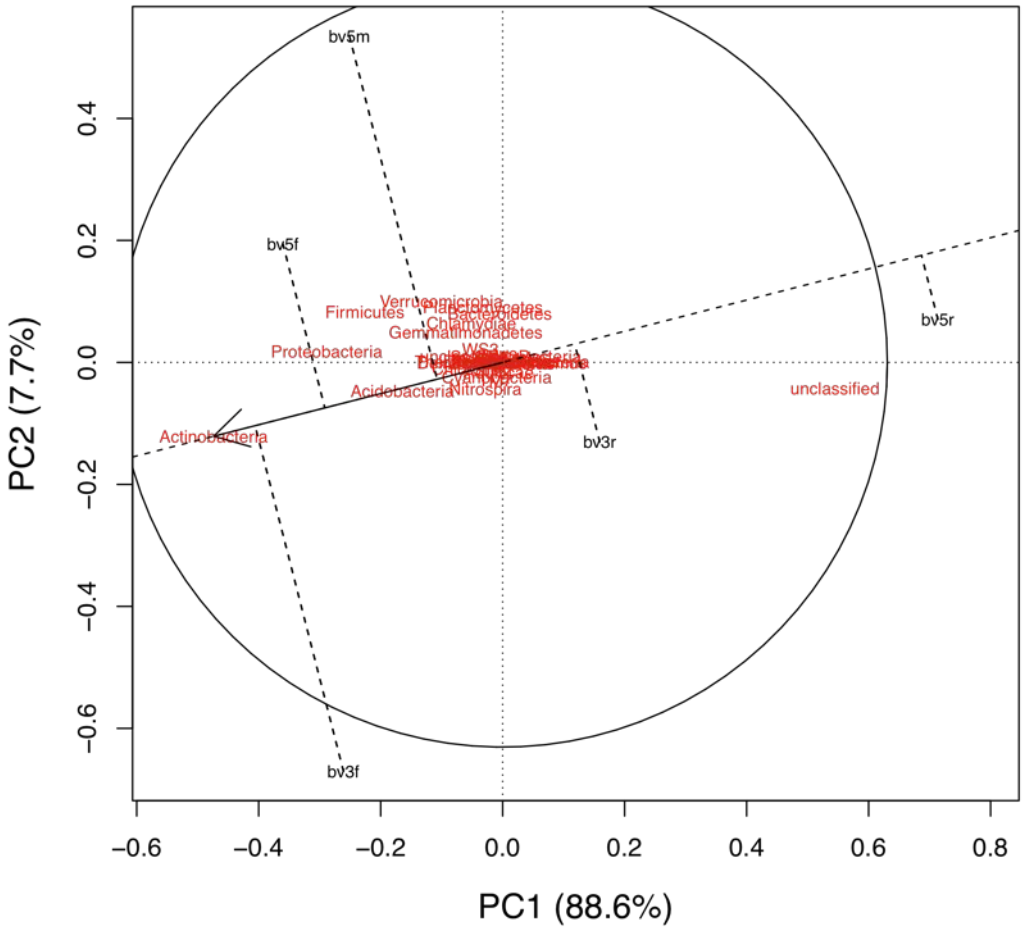




B.

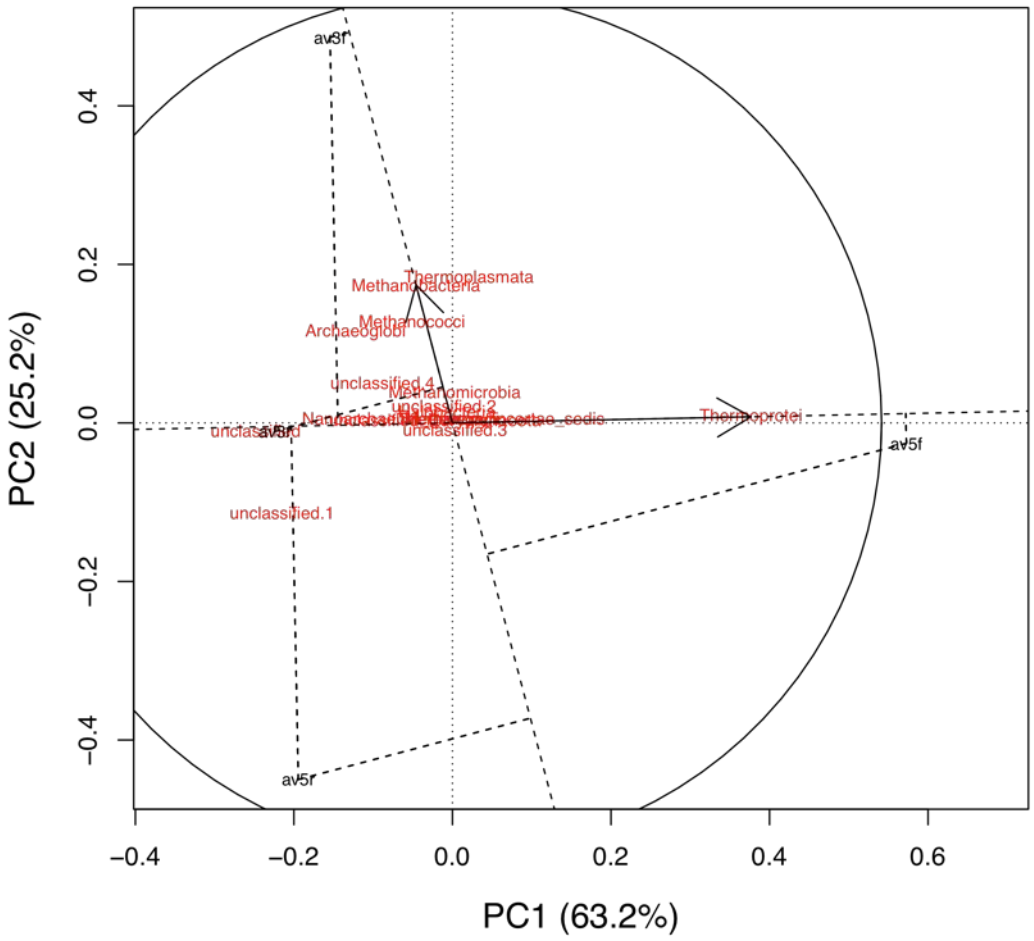
958

959 **Figure S3 Sequence classification depths (indicated by the percentage of classified**
960 **sequences per taxonomic level) for the six bacterial (A) and the four archaeal (B) datasets**
961 **according to the Silva database Systematics. Error bars represent the standard deviation.**



962

963 **Figure S4 PCA analysis for the sequence classification according to the dataset for the**
964 **bacterial 16S rDNA targeting primer-sets at a phylum level. Equilibrium circle analysis**
965 **was performed in order to assess any particular phylum contribution to the observed**
966 **variance among total datasets.**



967

968 **Figure S5 PCA analysis of the generated datasets in respect to the sequence abundances**

969 **according to taxonomic classification.**

970

971

972

Title:

Adaptation of soil microorganisms to trace element contamination: a review of mechanisms, methodologies and consequences for risk assessment and remediation.

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R.H: evaluation of original idea, writing of section 5 and discussion of the report

S.V: evaluation of origina idea, writing of section 4 and discussion of the report

D.C: evaluation of original idea and discussion of the report

T.M: original idea and discussion of the report

Chapter 4

Abstract

This review provides an updated and integrated view of the adaptation of soil microorganisms to elevated concentrations of trace elements. Starting with a summary of the occurrence of trace elements in soils and their effects on soil microorganisms, the scientific evidence underlying adaptation of microorganisms to trace elements from species to community level is discussed. Insights are given regarding the main physiological processes involved in the resistance of bacteria to toxic elements including the potential importance of horizontal gene transfer in the adaptation process. The review continues with a discussion of how new molecular and biotechnological techniques can enrich this field of study. Scientific evidence is utilized in constructing an illustration of microbial community responses with reference to ecological indicators during various adaptation stages, while the related effects on community biological functionality and resilience are discussed. We conclude with an evaluation of the importance of considering adaptation in risk assessment and possible remediation of trace element contaminated sites.

Key Words: contaminant, pollutant, metal, metalloid, tolerance, restoration, resilience, PICT, species evenness, species richness, soil function

Running title: Adaptation of soil microorganisms to trace elements

Index

Chapter 4

1. Introduction

Trace elements occur in nature, are also common contaminants, and when present in sufficient concentrations, are toxic to living organisms (Adriano, 1986). Negative effects of trace elements on microbial growth and survival have been known since the beginning of the last century (Lipman, 1914). However, it was only once large effects of emissions from smelters on surrounding ecosystems were observed in the 1960-70s that scientists started to realize how severely soil microorganisms and soil microbial processes can become disrupted by elevated concentrations of trace elements in soils (Giller et al., 1998). An increasing number of studies have thus been carried out in the last decades to deepen the understanding of the biological properties of soils, in terms of both structure and function, and to assess the effects of organic and inorganic pollutants on these properties (Bamborough & Cummings, 2009; Gelsomino et al., 2006; Rutgers, 2008; Zhang et al., 2009). In particular, many studies have demonstrated an inherent capacity of soil functions to withstand some inputs of toxic pollutants, confirming and sustaining the concept of soil as a "buffering" system (Doran et al., 1996). The key biological players in the buffering capacity of soil are microorganisms. Soil microbial populations, with their high degree of genetic malleability, can rapidly respond to changes in the soil environment and have evolved and are still evolving different ways to cope with the presence of toxic substances in soils.

Two primary means by which microorganisms can mitigate the toxic effects of pollutant exposure have been identified. The first is microbial-based transformation of contaminants to more (microbially) benign species, which includes, in the case of organic contaminants, degradation (Hussain et al., 2009),

and in the case of inorganic contaminants, chelation (Dimkpa et al., 2008), and in some cases (e.g. arsenic and mercury), methylation, which can lead to more volatile species and thus reduce the exposure of soil microorganisms (Cattani et al., 2008; Rinklebe et al., 2010), or detoxification through change of redox status (Borch et al., 2010). The second is a range of internal resistance processes, which can apply to both organic and inorganic contaminants. This review focuses on the relevance of these latter resistance processes to soil microbial survival in the presence of trace element contamination.

The terms resistance and tolerance are often used interchangeably in the literature and can be defined as "the ability of a community to maintain equilibrium conditions following exposure to a contaminant" (Clements & Rohr, 2009). These terms are distinguished from resilience which is defined as "the ability of a community to return to pre-disturbance conditions after a [stressor] is removed" (Clements & Rohr, 2009). Tolerance/resistance to contaminants can be conferred as a result of phenotypic changes at the individual level. These are physiological changes which do not result from genetic transformation but which occur through, for example, substrate induced alteration in levels of expression of pre-existing genes (Gruber & Gross, 2003; Haferburg et al., 2009; Moore et al., 2005; Nies, 2004). Such phenotypic changes are also termed "acclimation". Alternatively tolerance/resistance to contaminants can arise as a result of genetic changes at either the individual level (genotypic transformation as a result of selection pressure) or community level (proliferation of populations containing genes which confer tolerance/resistance, and decline of those which do not, as a consequence of selection pressure) or both. In the context of this review we use the term adaptation to refer to these latter processes of genotypic

transformation which enable soil microbial communities to restore ecological functions in affected environments (Bååth, 1996; DiazRavina & Bååth, 1996; Muller et al., 2001; Rusk et al., 2004). Restoration and recovery are used as synonyms referring only to functions.

2. Evidence of adaptation of soil microorganisms to trace elements

Even before anthropogenic emissions of trace elements began to threaten the ecology of many sites around the world, soil microorganisms had adapted to live in habitats with high concentrations of trace elements, as these can occur naturally. A well studied example is serpentine soils, containing high (up to thousands of mg kg^{-1}) concentrations of Ni, Cr and Fe (Pal et al., 2005). A range of multiple metal-resistance of resident microbes is a pre-requisite for the occupation of these ecological niches (Haferburg & Kothe, 2007).

Microbial adaptation to trace elements in agricultural soils is a different issue since a pre-requisite for sustaining plant productivity is a level of trace elements below plant toxicity thresholds and thus, generally, not toxic to microorganisms, though exceptions occur (Chaudri et al., 2008; Zhao et al., 2004). The situation however changed at the beginning of the 20^{th} century, as industrial and agricultural activities progressively contributed to an elevation of trace element concentrations in many soils (Renberg et al., 2000; Zaccone et al., 2007). Scientists started then to draw attention to the increasing presence and toxicity of these trace elements towards plants and microorganisms, and with these studies, the first evidence of microbial adaptation emerged. Many of the first studies were carried out at the population level, and were usually based on the isolation of microbial species from polluted environments. Ashida (1965)

was first to review the adaptation of fungi to metals, stating however that adaptation took place only infrequently in the field. At the end of the 1970's Carter & Hartman (cited in Bååth, 1989) found isolates of *Penicillium thomii* and *Fusarium oxysporum* in a polluted site that were more tolerant than isolates from non-polluted sites, and Doelman & Haanstra (1979) demonstrated increased soil bacterial tolerance to Pb following Pb exposure. On the other hand Arnebrant et al. (1987) found little evidence of acquired tolerance in different fungi isolated from a forest soil around a smelter. They concluded that the tolerant species which dominated in polluted soils were species that were already metal tolerant and could also be found in low frequencies in unpolluted sites. In the following years, a large body of scientific study has provided strong evidence supporting adaptation of soil microorganisms to trace elements, including both fungi and bacteria. Studies also started moving attention from the population to the community level (Angle & Chaney, 1991; Bååth, 1992; Hiroki, 1992; Kumar et al., 1998; Kunito et al., 1997; Margesin & Schinner, 1996; Saeki et al., 2002; Schmidt et al., 2009).

Until the mid 1990s, studies investigating the effects of contaminants on the composition, diversity and tolerance of soil microbial communities relied on culture dependent methods such as counting of colony forming units (CFUs) or growth on specific substrates (Klinger et al., 1992), and thus were biased towards cultivable soil organisms, whereas non-cultivable soil organisms are now known, on the basis of the results of several computational and high throughput molecular studies, to constitute by far the majority of soil organisms (Deutschbauer et al., 2006; Handelsman et al., 1998; Roesch et al., 2007; Schloss & Handelsman, 2005; Schloss & Handelsman, 2006). On the other hand, it has

also been argued (Ellis et al., 2003) that cultivable organisms may constitute the ecologically relevant genetic pool in soils and hence culture-dependent methods may be more appropriate than culture-independent methods for assessing effects of contaminants on soil biota. As discussed below, useful information regarding adaptation can be obtained from both types of methods. Information on the entire microbial gene pool is nevertheless important to allow identification of functions which could be subjected to selective pressure by contaminants.

A potential factor confounding tolerance studies *in situ*, is the chemical aging of metals in soils, i.e. the decrease of metal bioavailability in soils over time. Metal aging confounds assessment of microbial adaptation since the outcome (i.e. increase in microbial function over time) is the same for both of these processes (for discussion see Rusk et al. (2004) and Fait et al. (2006)). A method for assessing adaptation *in situ* which is simple to apply, eliminates interference effects of chemical aging for adaptation assessment, and does not rely on extraction and/or cultivation of microorganisms and hence can be applied to study adaptation in non-cultivable species was developed by Hamon et al. (2002). This method was used by Rusk et al. (2004) to investigate the adaptive response of soil biological nitrification to Zn and Pb. In this study soil microcosms were exposed to Zn, Pb, or to no metal ('control') and incubated for several months, and then sub-samples from the microcosms were mixed into the same soil, but which had been freshly spiked with increasing concentrations of metals and sterilized prior to being inoculated with the sub-sample. Results showed a significant increase in the nitrification EC_{50}, and hence metal tolerance of the nitrifiers, in the metal exposed populations in comparison to the control.

Interestingly, prior exposure to Pb resulted in adaptation of nitrification to increased Zn concentrations and *vice versa*. A following work (Fait et al., 2006) showed similar results for Ni and Cu, however acquisition of tolerance of the nitrifying community to Cu required significantly more time to occur than for the other metals.

Diaz-Ravina & Bååth (2001) extracted bacteria from soils, either uncontaminated or previously contaminated with high doses of Zn, Cu or Cd, and reinoculated them in a non-contaminated sterilised soil. The bacteria which had been pre-exposed to elevated concentrations of metals initially exhibited a higher metal tolerance than those which were not pre-exposed, but it was also found that the acquired tolerance was lost within the first week of reinoculation into the uncontaminated soil. The authors interpreted the rapid loss of tolerance from the community to suggest that metal tolerance must provide a strong selective disadvantage to microorganisms inhabiting an unpolluted environment.

3. Trace element stressors, horizontal gene transfer and adaptation of microbial communities

Metal resistance systems which contribute to microbial adaptation may have evolved shortly after the onset of prokaryotic life and are present in nearly all bacterial types (Ji & Silver, 1995). Compared to multicellular species, microorganisms have a higher surface/volume ratio, and are thus more exposed to the toxic effects of pollutants. Furthermore, with their widespread occurrence and mobility, they are usually the first to come in contact with trace elements in the soil matrix. To have any physiological or toxic effects, trace elements must

enter the cell. All bacterial cells possess uptake systems to acquire essential elements from their surrounding environment. There are two main groups of metal uptake systems: a group of fast, unspecific and constitutively expressed uptake systems, and slower ATP-linked specific systems usually activated by induction under starvation conditions (Nies, 1999). Since the first group of transporters is constitutively expressed, trace elements can be transported to the cytoplasm regardless of their toxicity. This "open gate" situation is considered the first reason of the toxicity of heavy metal ions (Nies et al., 1995).

Once inside the cell, metal cations, especially those with high atomic numbers, tend to bind to SH groups. Non-essential metal cations with similar properties to physiologically essential cations, may substitute for them in enzymes and in so doing, inhibit the activity of enzymes. Examples are Cd^{2+} with Zn^{2+}, or Ca^{2+}, Ni^{2+} and Co^{2+} with Fe^{2+}, or Zn^{2+} with Mg^{2+} (Nies, 1999). Another possible process, especially in gram negative bacteria, is the binding of the trace elements to glutathione, resulting in bisglutathionate complexes that tend to react with molecular oxygen to form oxidized bisglutathione (Kachur et al., 1998). This process is the basis of the oxidative stress often caused by toxic cations in microbial cells. Other toxic effects of trace elements are alterations in the conformational structure of nucleic acids and interference with oxidative phosphorylation (Bruins et al., 2000).

Given the processes outlined above, how can a microbial cell be (or become) resistant to the toxic effects of trace elements in high concentrations? Five different mechanisms, which can act separately or in different combinations, have been identified (Bruins et al., 2000): (i) exclusion by a permeability barrier, (ii) active transport out of the cell, (iii) intracellular

sequestration, (iv) extracellular sequestration, (v) enzymatic detoxification (Fig. 1).

Typical examples of resistance by exclusion of the element by a permeability barrier are reported in the case of copper and mercury. Resistant strains of *Pseudomonas syringae* are characterized by a 35-kb plasmid pPT23D containing an operon (*copABCD*) encoding four proteins: the plasmid confers resistance to the host strain by sequestering excess copper in the periplasm and in the outer membrane (Arnesano et al., 2003). One of the first steps of mercury resistance involves binding with the periplasmatic protein merP (Qian et al., 1998).

Active transporters out of the cells are considered to be the largest category of metal resistance systems. They can be plasmid or chromosomally encoded, and be non-ATPase or ATPase-linked (Bruins et al., 2000). The *arsA* gene is an example of an inducible gene encoding for an ATPase which is induced by arsenite and antimonite; it acts in the *ars* operon together with *arsB* (involved in moving arsenite across the inner membrane) and *arsC* (encoding an enzyme that converts arsenate to arsenite) in the plasmid R733 that confers arsenic resistance to *E. coli*. A similar example is found for cadmium with the *cadA* gene encoding for an efflux pump with a large homology to the ATPase that exports Cd^{2+} from the cell interior of *Staphylococcus aureus* (Oger et al., 2003)

Intracellular sequestration is a mechanism through which microbial cells accumulate metals within the cytoplasm, preventing at the same time metal exposure of essential cellular components. Typical examples are given by metallothionein and glutathione binding. In *Synechococcus* sp. two genes, *smtA* and *smtB*, confer resistance to cadmium and zinc: *smtA* encodes for a

253 metallothionein that binds to these two trace elements, while *smtB* acts as a
254 repressor control system of the activity of *smtA* (Morby et al., 1993). The binding
255 of heavy-metal cations in gram-negative bacteria to glutathione resulting in
256 bisglutathionato complexes is quite common (Nies, 1999). A limitation of this
257 strategy is that these complexes tend to react readily with molecular oxygen to
258 form oxidized bis-glutathione and hydrogen peroxide, which is a severe cause of
259 oxidative stress within cells (Wang et al., 2009).

260 The activity of the unicellular cyanobacterium *Synechococcus elongates*
261 towards uranium provides a good example of resistance achieved through
262 extracellular sequestration. The strain is able to bind uranium to extracellular
263 polysaccharides, and it has been proposed as a potential means for harvesting
264 uranium from aqueous environments (Acharya et al., 2009). Extracellular
265 polymeric substances made up of sugars, proteins, lipids and DNA similarly
266 allow the exclusion of cadmium in *Pseudomonas putida* biofilms (Ueshima et al.,
267 2008).

268 Mercury resistance probably gives the best example of enzymatic
269 detoxification of metals in microorganisms. The resistance is achieved by a
270 combination of activities encoded by the *mer* operon: binding (*merP*), transport
271 (*merC, merT*), lyase cleavage (*merB*), and detoxification through the reduction of
272 Hg^{2+} to Hg^{0} catalyzed by the mercury reductase *merA* (Nies, 1999). The
273 development of such a fine-tuned and organised resistance system may be
274 explained by the fact that mercury is one of the most toxic elements for bacteria,
275 with no known beneficial functions (Nies, 1999)

276 Global metabolic shifts occurring in an organism under stress are also
277 important for its survival and growth. An example is sigma (σ) factors which are

involved in the selection of genes to be transcribed in response to environmental stimuli (Gruber & Gross, 2003). Sigma factors are involved in a major mechanism mediating transcription regulation in prokaryotes. Their role in response to increased extracellular trace element concentration has been identified in *Ralstonia metallidurans* (Nies, 2004) and *Bacillus subtilis* (Moore et al. 2005). In these studies, transcription of sigma factors were demonstrated to activate amino acid biosynthesis and uptake pathways, processes which potentially support protein production necessary for metal resistance.

The selective pressure posed by the presence of trace elements at bioactive concentrations can lead to adaptation of the microbial community through different processes (Figure 2, Barkay et al., 1985): increase in the size and activity of already resistant populations, natural selection after random mutation, and horizontal transfer of resistance genes. The latter process is an appealing explanation of adaptation phenomena since it implies that the maintenance of trace element resistance related genetic elements, which imposes an energy cost, occurs in a significant proportion of the microbial pool only after toxicity takes place.

Rensing et al. (2002) described the process of horizontal transfer of resistance genes located on plasmids or transposons as an example of the "selfish gene" theory. This theory (Dawkins, 1976) views mobile elements such as plasmids and transposons as kind of genetic parasites, whose sometimes beneficial effects on the long-term evolution of prokaryotic hosts are coincidental. Thus, the resistance genes do not necessarily provide selective benefits to the individual itself, but enhance the fitness of the entire gene cluster, allowing it to invade novel ecological niches (Rensing et al., 2002).

303 The application of this theory has interesting consequences when we look
304 at resistance and adaptation not only at the gene or species level but also at the
305 community level. As stated by Doolittle (1999), prokaryotic operational genes,
306 such as resistance genes, can be seen as semi-autonomous agents within a global
307 super-organism: through the survival of "selfish" genes mediated by horizontal
308 transfer, the soil microbial community (i.e., the super-organism) is able to adapt
309 to and withstand the toxic effects of trace elements in high concentration. The
310 mobile genetic elements (MGE) responsible for such transmitted resistance do
311 not carry essential cell functional information which is located within
312 chromosomal DNA (e.g., ribosomal RNA encoding genes) (Frost et al., 2005).
313 Prokaryotic horizontal gene transfer occurs through transformation (free DNA
314 uptake), conjugation (genetic material exchange) and transduction (DNA
315 carryover by phages, prokaryotic viruses) (Frost et al., 2005). Although
316 transformation and transduction have been shown to occur in soils (Levy-Booth
317 et al., 2007), bacterial conjugation is thought more probable under selective
318 pressure (Massoudieh et al., 2007; Sorensen et al., 2005; Thomas & Nielsen,
319 2005).

320 The exact frequency of the horizontal gene transfer phenomenon in natural
321 environments is difficult to assess: for this reason not only phylogenetic but also
322 compositional methods have been proposed (Tamames & Moya, 2008).
323 Compositional methods are based on the assumption that signature traits
324 derived from intrinsic (genetic) and external (environmental) factors accompany
325 genetic elements (e.g. GC content and codon usage). Other difficulties related to
326 the functional or phylogenetic classification of trace element resistance
327 conferring genes, are the structural similarity and the existence of encoding

operons in both plasmids and chromosomes (Blanck, 2002). Structural similarity is partially responsible for the broad range of activity of the encoded proteins to trace elements (co-tolerance) (Blanck, 2002). Moreover, specificity of response to the targeted trace element is some times low. The *mer* operon is a characteristic example of this, having been shown to be responsive not only to the presence of Hg, but also to Zn and Cd, even though the mechanism related to Hg detoxification (volatilization after reduction to Hg^0) is not relevant to the respective Zn and Cd mechanisms (Barkay et al., 2003; Park & Ely, 2008a; Park & Ely, 2008b; Radniecki et al., 2009).

The case of mercury reduction has also been widely studied in recent years in the context of assessing the potential for horizontal gene transfer events. In vitro assays showed a positive correlation between conjugation events and donor metabolic activity, thus supporting the hypothesis of increased horizontal gene transfer under selective pressure (Johnsen & Kroer, 2007). Using a microcosm approach, Dronen et al. (1998) indicated occurrence of horizontal gene transfer within a period of 12 days in five out of eight different soils tested under Hg selective pressure. In the same study, the incidence of horizontal gene transfer was observed in only one of the eight soils when the Hg stress was absent. Strong indications of horizontal transfer of Hg resistance related genes in soil and other environments have been provided by comparison of abundance and diversity of bacterial genes and gene vectors related to Hg reduction (Mindlin et al., 2002; Oregaard & Sorensen, 2007; Smit et al., 1998; Tothova et al., 2006). A recent example involved the study of soil samples derived from various depths of both contaminated and reference sites (de Lipthay et al., 2008). Soil samples were used for the generation of microcosms that were treated with Hg.

The *merA* (mercuric reductase encoding gene) phylogeny was compared to isolated mercury resistance plasmid characterization data and bacterial 16S rDNA phylogeny in the soil community. The subsoil populations of previously non contaminated sites showed an increase in microbial diversity after the Hg amendment with a parallel increase of *merA* and one of its vectors (IncP-1), indicating a possible dispersal of *merA* related resistance among bacteria.

Similar effects on microbial community sharing of genetic elements have been demonstrated for other potentially toxic trace elements. Diversity studies on arsenate contaminated soils in India have demonstrated inconsistency between 16S rDNA based phylogeny and the *aoxB* (encoding the arsenite oxidase enzyme) and *arsB* (encoding the arsenite transporter protein) phylogeny (Cai et al., 2009) making the proposal of horizontal gene transfer a highly plausible explanation. A comparative genomic study of the widespread metal resistance model strain *Cupriavidus metallidurans* CH34 (categorized in the genera *Wautersia*, *Ralstonia*, *Alcaligenes*) was carried out focusing mainly on its broad metal resistance (Cd, Co, Pb, Cu, Hg, Ni, Cr) related pathways (von Rozycki & Nies, 2009). Results revealed evolutionary genetic shifts and pathway traces on both its chromosomes (particularly chromosome 2) and the hosted plasmids that could only be explained by assuming the occurrence of horizontal gene transfer phenomena. Finally, while studying a subsurface soil microbial community under extreme conditions (pH below 4.0, high trace element and radionuclide content – mainly U), Martinez et al. (2006) provided evidence for horizontal P_{IB}-type ATPase encoding gene dissemination among hosts belonging to different phyla.

4. Molecular tools with prospects for elucidating adaptive behaviour

At present, in order to demonstrate that a population has undergone adaptation, it is necessary to perform a functional assay, such as one of those described above (eg Diaz-Ravina & Bååth, 2001; Rusk et al., 2004), which compares the response of previously exposed and unexposed populations to additional contamination. These assays while technologically simple, are relatively laborious and further, do not reveal the mechanism underlying any adaptation, they can only reveal whether or not adaptation has occurred. Molecular tools show great promise in being able to unravel the mechanisms involved in adaptation at both the individual and community level. If molecular markers that are specific to adapted populations can be isolated, then development of rapid screening techniques to detect adaptation will also be feasible. The following section describes the range of molecular tools available and their current use and potential for use in investigating adaptation of soil microorganisms.

4.1 Bioreporters

Bioreporter technology can provide valuable insights into the responses of soil microbes to contaminants and other environmental factors (Ivask et al., 2009; Leveau & Lindow, 2002; Puglisi et al., 2008; Puglisi et al., 2009). The regulation of reporter genes such as GFP (green fluorescent protein) and luciferase encoding genes under the same promoters as genes of interest can provide "real time" information about gene function in relation to the environmental conditions tested (Leveau & Lindow, 2002).

Bioreporter technology can also be used to address questions related to population reproduction and viability, which are critical in advancing understanding of the adaptation process. An interesting example is that of *Erwinia herbicola* growth on plant leaf surfaces (Remus-Emsermann & Leveau,

2009). This bacterial strain encompassed a chromosomal transposon insertion that expressed stable GFP in a substrate dependent manner. In the absence of substrate, the quantity of GFP in the cells remained stable, and its amount in the descendants was proportional to its amount in the parental strains and thus could be used to identify different generational cohorts. Combination of screening (Epi-fluorescence microscopy and Flow Cytometry) and sorting techniques (Fluorescently Activated Cell Sorting-FACS) provided valuable information about the heterogeneity of strain growth on the plant leaf surface. With the caveat that the insertion of genetic material may affect the fitness of microorganisms, a similar approach could be used to monitor reproduction rates of trace element tolerant and sensitive cells in soils to provide indications about the influence of adaptation on reproductive fitness at the microbial strain level. In addition to this, use of FACS for specific cell recovery would allow comparative examination of possible genetic element changes (e.g. plasmid recovery and characterisation assays or total genome screening) and thus improve understanding of the importance of phenomena like genetic rearrangement or horizontal gene transfer in adaptation.

4.2 The meta-tools

Constraints related to the uncultivability of most soil microorganisms have concerned the scientific community for quite some time. One of the first techniques which aimed to overcome this issue is PLFA (Phospholipid Fatty Acid) analysis, where fingerprints for a microbial community can be obtained based on the membrane composition of its microbial constituents (Puglisi et al., 2005; White et al., 1979). Since then, the molecular tool-box for investigation of environmental samples has been enriched with several genomic techniques

which extend beyond a single genome or transcriptome, thus giving rise to a new discipline, termed "metagenomics" (Handelsman et al., 1998). *In situ* hybridization methods like FISH (Fluorescent *In Situ* Hybridization) have enabled total community studies combining both quantity and composition (Amann et al., 1995). PCR based methods such as quantitative PCR (qPCR), T-RFLP (Terminal Fragment Length Polymorphism), TGGE (Temperature Gradient Gel Electrophoresis), DGGE (Denaturing Gradient Gel Electrophoresis), to the more recent RISA (Ribosomal Intergenic Spacer Analysis), and ARISA (Automated Ribosomal Intergenic Spacer Analysis) (Anderson & Cairney, 2004; Borresen et al., 1988; Cherif et al., 2008; Winding et al., 2005) have also increased the rates of data generation. Besides these nucleic acid based techniques, proteomics tools have also recently been detached from culture based approaches and are now being applied in *in situ* microbial community investigations and single cell proteomic profiling (VerBerkmoes et al., 2009).

Some of these techniques have been successfully applied to elucidate the factors underlying community restoration. For example, the greater importance of bacterial ammonia oxidizers compared to archaeal ammonia oxidizers for nitrification restoration under Zn induced stress was demonstrated in a field study by Mertens et al. (2009). This was done through DGGE investigation of diversity indices related to the ammonia monooxygenase (*amoA*) encoding gene of ß-proteobacteria and crenarchaeota and by determining the absolute gene quantities per gram soil of the same gene for the respective groups via qPCR. In a following study (Ruyters et al., 2010a), it was demonstrated using the same techniques that the addition of ammonium accelerates the adaptation of bacterial nitrifying communities to Zn stress.

Recent techniques have allowed generation of large datasets for diversity indices and functions in environmental samples that are of major interest in adaptation studies. For example the PhyloChip and the GeoChip are tools based on microarray technology. These tools provide, respectively, information about total community composition and many known biogeochemical cycle related genes, and their set of probes are constantly being renewed as new genes of interest are identified (He et al., 2007; He et al., 2010; Yergeau et al., 2007; Yergeau et al., 2009).

However there are some disadvantages of the above methods which include their restriction to already identified sequences and methodological problems occurring with hybridization. Recent high throughput sequencing applications (Illumina, 454, SOLiD) have overcome these disadvantages, and have rapidly gained ground as prime choices for environmental studies (Angly et al., 2006; Caporaso et al., 2010; Claesson et al., 2010; Desnues et al., 2008; Dinsdale et al., 2008; Edwards et al., 2006; Frias-Lopez et al., 2008; Gloor et al., 2010; Guazzaroni et al., 2009; Krause et al., 2008; Lazarevic et al., 2009; Leininger et al., 2006; Petrosino et al., 2009; Roesch et al., 2007; Sogin et al., 2006; Wegley et al., 2007; Williamson et al., 2008; Wu et al., 2010; Yooseph et al., 2007). These methods allow screening of millions of phylogenetic markers, such as partial small ribosomal subunit (SSU) encoding gene fragments from environmental samples, and thus show great promise for detailed resolution and probing of microbial diversity and hence also any changes arising as a result of adaptation. Additionally, in the race for understanding microbial functions and roles in natural environments, consortia like the “TerraGenome” are being formed (Vogel et al., 2009a). With their main aim the so far un-achieved goal of

478 screening of the total metagenome contained in a few grams of soil and other
479 similarly complex environments, the Terragenome consortium will provide a
480 reference point for future studies. Although skeptics doubt the outcomes of such
481 effort (Baveye, 2009) these studies are expected to deepen knowledge of many
482 microbial ecology aspects (Vogel et al., 2009b), with trace element microbial
483 adaptation being one of them (Mengoni et al., 2009).

484 The above-mentioned molecular methods may identify genes of interest,
485 but the functions of such genes can only be characterized by reference to data
486 generated from culture based methods, computational predictions and
487 fundamental knowledge blueprinted in ecology related statistics (Johnson and
488 Omland, 2004; Ramette, 2007; Rodriguez-Brito et al., 2006). Hence frequently,
489 application of such methods generate more questions than they answer (Keller
490 and Hettich, 2009). To overcome this barrier, new fields based on traditional
491 proteomics are emerging such as community proteomics, metaproteomics, or in
492 the case of proteomics in combination with metagenomic tools, community
493 proteogenomics, (VerBerkmoes et al., 2009; Wilmes and Bond, 2004). Besides
494 their importance for evaluating microbial activity and related biogeochemical
495 pathways, for example in the underground acid mine drainage biofilm study of
496 Ram et al. (2005) or the cadmium study assessing differential protein expression
497 over time by Lacerda, et al. (2007), a comparison of environmental proteome
498 data to metagenomic and metatranscriptomic profiles (particularly in the less
499 complex extreme environments), can allow assignment of proteins to their
500 originating genes. An illustrative example is the proteogenomic analysis of green
501 sulfur bacteria derived from the O_2-H_2S interface of Ace Lake in Antarctica (Ng et
502 al., 2010). The majority of the genes identified by genomic data processing (1631

503 open reading frames (ORFs) corresponding to 1560 potential genes) were
504 assigned to the *Chlorobiaceae* family. The metaproteomics complement of the
505 study showed that 502 out of the 504 confidently identified proteins gave
506 positive matches to *Chlorobiaceae* assigned ORFs, thus providing a plethora of
507 information about their metabolism. At the same mine area studied by Ram et al.
508 (2005) (above), Goldtsman et al. (2009) studied various biofilms dominated by
509 *Leptospirillum* groups from areas with different drainage conditions. A
510 comparison of their metagenomic with metaproteomic results showed high
511 protein expression levels of unknown proteins that were unique to each biofilm
512 and which were hypothesized to be related to adaptation. Although still in its
513 infancy, technical advances in proteomics are likely to improve application of
514 proteomics to complex environments such as soils (Keller and Hettich, 2009).

515 Many microorganisms equipped with metal resistance genes are forced
516 by the energy burden accompanying these genes to restricted lifestyles when the
517 toxicant is absent (Bruins et al., 2000). Being overwhelmed by their more
518 dominant rivals in the struggle for survival, their study becomes a difficult task.
519 One of the most promising emerging fields for solving this problem is single cell
520 genomics. Single cell genomics is based on the concept of total genetic
521 information screening from a single to a few cells representing an individual
522 strain in the microbial community. FACS, microfluidics and micromanipulation
523 have been deployed in order to sort out enough cells of a single species to
524 provide the necessary genetic material for whole genome amplification (WGA)
525 and concomitant shotgun sequencing (Hutchison & Venter, 2006; Kalyuzhnaya et
526 al., 2006; Rajendhran & Gunasekaran, 2008). Although still in initial stages, this

method promises to overcome barriers related to obtaining sufficient genetic material for sequencing.

Functional screening of metagenomic libraries for cloned genes responsive to a certain stimulus (e.g., trace element stress) is time consuming. A relatively rapid alternative developed by Uchiyama et al. (2005) is a substrate-specific GFP-based high throughput technique for functional libraries screening. The first step of the method includes cloning of DNA (e.g. environmental) into an operon-trap gene expression vector and transformation into a suitable host maintained in liquid culture. The liquid culture is supplied with the substrate of interest and in the cases where clone transcription occurs, GFP is produced at the same time. This conditional GFP expression was used in order to identify and sort responsive clones to the substrate of interest via FACS. The proof-of-principle of the technique was provided by the authors when 58 benzoate and 4 naphthalene responsive clones out of an approximate total 152,000 clones derived from an aquatic environment, were identified and some characterized within only a few days. In a similar manner, trace element toxicity conditions could be utilized for example with metagenomic clone libraries of adapted soil communities in an attempt to identify new associated genes and pathways.

Each of the methods discussed above enable assessment of microbial diversity and function in a wide variety of environments, including complex environments such as soil. Ongoing technological advances which are resulting in rapidly increasing screening throughput and generation of databases with huge volumes of information can allow investigation of stressed environments as evolving entities in space and time. However until reliable molecular markers for adapted populations have been identified, molecular investigations of adaptation

552 will need to remain coupled to functional assays in order to confirm that the
553 communities under investigation have in fact adapted.
554
555 **5. Consequences of adaptation for risk assessment and possible**
556 **remediation of contaminated sites**
557 Evidence of the presence of community adaptation has been proposed as a tool
558 for use in environmental risk assessment of contaminants. This concept, known
559 as pollution induced community tolerance or PICT, was initiated by Blanck et al.
560 (1988) for exposure testing of aquatic biota and offers the possibility to
561 differentiate biological effects due contaminants from other confounding factors
562 such as pH or nutrient limitations (Blanck, 2002; Boivin et al., 2002). The
563 potential for use of the PICT concept in soil microbial ecotoxicological risk
564 assessment was soon recognised, and has resulted in ongoing research in this
565 area for over a decade (e.g. Diaz-Ravina et al., 2007; Siciliano & Roy, 1999; Van
566 Beelen et al., 2004). Briefly, a PICT assessment is conducted by determining
567 tolerance to a suspected toxicant of a community exposed to the suspected
568 toxicant and comparing whether the exposed community has developed a higher
569 degree of tolerance to the toxicant than a related community which has not been
570 exposed to the toxicant (Blanck et al., 1988). If the exposed community does
571 express a higher tolerance to the toxicant, then the toxicant is considered to have
572 exerted a selective pressure upon the community and thus caused a significant
573 biological disturbance in the ecosystem (Blanck et al., 1988). Although the PICT
574 concepts has a great importance in the soil ecotoxicological risk assessment,
575 some limitations have been identified. In order to discriminate which potential
576 toxicant is the causative agent of biological disturbance in sites polluted with a

577 mixture of contaminants, Blanck et al. (1988) discussed the prerequisite that the
578 selection pressure exerted by the toxicant be highly specific to that toxicant and
579 hence not result in co-tolerance to other toxicants. The issue of co-tolerance may
580 limit the reliability of PICT for assessing trace element contamination as it
581 appears that co-tolerance is a common feature in exposed populations. For
582 example Diaz-Ravina et al. (1994) found that exposure of soil microbes to Cu
583 increased the microbial community tolerance not only to Cu but also to Cd, Ni
584 and Zn. Similarly, Rusk et al. (2004) observed increased tolerance to Pb of
585 nitrifying bacteria previously exposed to Zn and *vice versa* and Philippot et al.
586 (2008) found that pre-exposure of soil microbes to Cu enhanced their Hg
587 tolerance. Co-tolerance induced by trace elements was also found not to be
588 limited to inorganic species as Berg et al. (2005) demonstrated increased
589 tolerance to a range of antibiotics of Cu tolerant soil bacteria. Furthermore, the
590 length of time for communities to adapt to the selective pressure posed by a
591 toxicant is variable and may be a significant confounding factor to PICT
592 assessment, particularly in more recently contaminated soils. While adaptation
593 in response to contaminant stress is often observed within days to a few weeks
594 of exposure (e.g., DiazRavina and Bååth, 1996; Rasmussen and Sorensen, 2001;
595 Fait et al., 2006) found no sign of increased tolerance to Cu of nitrifying bacteria
596 exposed to Cu for 15 months despite the added Cu having a large inhibitory
597 effect on nitrification. Likewise Blanck et al. (2009) demonstrated that years of
598 exposure to the anti-fouling algicide irgarol was required to induce increased
599 irgarol tolerance in a marine periphyton community. Performance of a PICT
600 assessment prior to adaptation having occurred will clearly erroneously mask
601 signs of community disturbance. Finally, the PICT concept was originally

developed for application to aquatic systems, which tend to be spatially more homogenous in terms of chemical characteristics than soils. Soils can vary significantly, and within only millimetres, in terms not only of their chemical but also physical properties and thus may host very different microbial communities within very close proximity to each other: the difference in microbial community structure between rhizosphere and bulk soil is an obvious example (Puglisi et al., 2009). In fact spatial variability in soil biological properties, if not similar to variability in soil physico-chemical properties, may be even higher (Girvan et al., 2005). This variability has implications for the choice of the un- or less-exposed 'reference community', i.e. the community against which an increase in tolerance is gauged, when PICT assessments are conducted in the field, as the reference community should aim to resemble the structure that the exposed community had prior to exposure because different communities may have different intrinsic tolerances to a suspected toxicant. This is particularly the case for PICT assessments of trace element contamination as background concentrations of trace elements can vary naturally by up to several orders of magnitude in soils (e.g. Hamon et al., 2004) and innate soil microbial tolerance to metals is variable but appears to be positively correlated to the natural soil background concentration (McLaughlin & Smolders, 2001). Large differences in soil pH, clay and organic matter content in soils subjected to PICT assessment by Boivin et al. (2006) may indicate very different initial soil microbial community composition in the different samples and hence account for the absence of a clear PICT response to metal contamination found in that study. A further potential complication for the selection of the reference community is recent evidence suggesting biological spatial variability at the micro-scale level. At this level the

627 exposure to metals can be locally influenced by strong adsorption on clays and
628 organic matter or entrapment in micropores (aging processes), while microbes
629 can be protected from exposure to toxic metals by micropores or biofilms. This is
630 exemplified by a study of Almas et al. (2005), who found after a fractionation by
631 sequential dispersion/density gradient centrifugation that in the same samples,
632 loosely attached cells exhibiting a strong PICT response can coexist with strongly
633 attached cells virtually unaffected in terms of metal tolerance. The results of this
634 study therefore also have implications for use of extractive methods in terms of
635 their ability to isolate a representative section of the whole community for
636 contaminant risk assessment.

637 Even when the above considerations are taken into account, there is
638 ongoing debate about the use of PICT in ecological risk assessment of
639 contaminants. The most significant criticism is that while PICT can reveal
640 community changes in response to a specific toxicant, the mere fact of a
641 community having undergone a change in response to contaminant exposure
642 does not *a priori* demonstrate an ecological risk. The debate can be summarised
643 by a single question, namely (Rusk et al. 2004), is adaptation (i.e. PICT) an
644 adverse ecological effect?

645 Two criteria important for soil protection in the face of trace element
646 contamination are that soils maintain their biological functions and their
647 biological resilience (Giller et al., 2009). Microbial adaptation to trace element
648 contamination will therefore be an adverse effect if it results either in the loss of
649 soil microbial function or the loss of soil microbial resilience or both. These
650 issues are discussed separately in the following two sections. In the last section,

651 the implications of adaptation for risk assessment and possible remediation of
652 contaminated sites are finally discussed.
653
654
655 *5.1 Adaptation in relation to soil biological function*
656 Studies looking at restoration of function following microbial adaptation to trace
657 elements are not numerous and most have focused on species involved in
658 nitrogen cycling, which is only one of the important functions performed by soil
659 microorganisms. However the majority of evidence to date gleaned from these
660 studies suggests that although exposure to toxic concentrations of trace
661 elements is initially detrimental to soil microbial functions, once organisms have
662 adapted, their functionality is restored (de Lipthay et al., 2008; Fait et al., 2006;
663 Mertens et al., 2006; Mertens et al., 2007; Mertens et al., 2009; Rusk et al., 2004).
664 In other words, the internal re-allocation of energy necessary to sustain metal
665 resistance mechanisms, such as those described above, does not result in a shut-
666 down of the environmental functions that the organisms perform. This could be
667 expected since the majority of functions performed by soil microorganisms are
668 the consequence of inherent metabolic processes that the microorganisms must
669 anyway exert in order to survive, whether they are adapted or not. However
670 research has revealed possibly one case where adaptation to high contamination
671 levels has apparently resulted in an important function not being maintained and
672 that is for rhizobia. Rhizobia species can exist as free living organisms in soils,
673 however they also perform an agronomically critical function in terms of fixing
674 nitrogen for leguminous plant species (Broos et al., 2004). This occurs through a
675 symbiotic association between the bacteria and the plants with the bacteria

676 nodulating plant roots and benefiting in turn from resources supplied by the
677 plant (Kiers et al., 2003). Giller et al. (1989) found that metal tolerant rhizobia
678 present in a soil contaminated with metals as a result of long-term sludge
679 applications were able to nodulate white clover but were ineffective in fixing
680 nitrogen for the plant host. In a following study, Hirsch et al. (1993) discovered
681 that the metal contamination had led to survival of only a single strain of
682 rhizobia in comparison to multiple strains inhabiting the neighbouring
683 uncontaminated soil. They also confirmed that the surviving strain could
684 nodulate white clover and also red clover, and was ineffective in fixing nitrogen
685 in both. However they found that the surviving strain was effective in fixing
686 nitrogen in subterranean clover whereas rhizobia from the adjacent
687 uncontaminated soil formed effective nodules in all clover types. While it could
688 be argued that a highly host specific strain of rhizobia was the only strain
689 present in the original community able to tolerate elevated metal concentrations,
690 it is theoretically possible that this is an example of adaptation constituting an
691 adverse effect on soil biological function. As mentioned above, rhizobia benefit
692 from the symbiotic association with the host plant, but they do not require this
693 symbiosis to survive. In the absence of the host plant, rhizobia do not fix
694 nitrogen. Hence in the contaminated soil, selection may have favoured allocation
695 of cellular resources away from structures underpinning nitrogen fixation
696 capability with at least some plant hosts in order to provide additional energy to
697 support metal resistance mechanisms essential for survival. However it should
698 be noted that in terms of the response of rhizobia to toxic concentrations of trace
699 elements, more commonly loss of effective rhizobia is found (e.g. Broos et al.,
700 2004; Chaudri et al., 2008)

In summary, we hypothesize that adaptation will rarely constitute an adverse effect in terms of maintenance of soil biological function because in most cases the soil biological function is the outcome of metabolic processes essential for organism/community survival irrespective of whether adaptation has occurred or not. However in cases where performance of the function enhances organism/community fitness but does not dictate organism/community survival, the strong selection pressure exerted by toxicants may result in diversion of energy resources away from performance of the function and towards resistance mechanisms enabling organism/community survival. In this latter case, adaptation would be an adverse effect on soil biological function.

5.2 Is adaptation an adverse effect in relation to soil biological resilience?

Soil biological resilience is defined as the ability of soil biological function to recover from a perturbation and is a key feature defining soil health (Griffiths et al., 2001) since ecosystems exist in a dynamic state subject to frequent and multiple perturbations, both natural and anthropogenic. Ecosystem resilience of above ground communities has been found to be positively correlated with species richness (i.e. number of species) and species evenness (i.e. the relative abundance with which each species is represented) and this is likely to also apply to soil microbial ecosystems, though research effort in this area is substantially under-represented in comparison to research exploring higher trophic levels (Botton et al., 2006; Degens et al., 2001; Nannipieri et al., 2003). Hence if adaptation results in a decrease of either richness or evenness in a soil microbial community, it could potentially constitute an adverse effect. A further consideration (Muller et al., 2002; Rusk et al., 2004; Tobor-Kaplon et al., 2006)

726 relates to the additional metabolic burden incurred by the need to express and
727 maintain resistance mechanisms (such as those discussed above) to alleviate
728 toxicity to the organism and whether this increases the vulnerability of adapted
729 populations to additional stressors since less of the total energy budget is
730 available to be used in tolerating the additional stressors. Each of these aspects is
731 discussed in more detail below.

732 The effect of contaminants on richness, as it pertains to soil microbial
733 genetic diversity, appears to be strongly related to the degree of contamination
734 to which the populations are exposed. A hump-backed response, where
735 increasing contaminant concentrations initially result in an increase in genetic
736 diversity up to a threshold, after which the soil responds to further increases in
737 contaminant concentrations with a decrease in genetic diversity, has been
738 reported in different studies (Giller et al., 2009). However in some studies it is
739 not clear whether the microbial populations under investigation are actually
740 adapted populations and thus whether results reflect the effect of adaptation on
741 genetic diversity, or whether they are populations still in the process of adapting
742 to the contamination. For example Zhang et al. (2009) found a hump-backed
743 distribution of genetic diversity in soil contaminated with increasing
744 concentrations of Cd immediately following amendment with Cd where it is
745 likely that adaptation had not occurred. In contrast in soil where populations had
746 presumably adapted to Cd as they had been exposed over 30 years to Cd
747 contamination, no effect of Cd on genetic diversity was observed (Zhang et al.,
748 2009). Studies demonstrating changes in genetic diversity in proven adapted
749 populations are fewer. Mertens et al. (2006) performed a comparative DGGE
750 analysis on nitrifying bacteria demonstrated to have adapted to Zn

751 contamination along a gradient in the field, and found structural differences in
752 community composition related to Zn exposure, but found no evidence for a
753 decrease in bacterial numbers at even the highest Zn concentration. In a
754 following work, Mertens et al. (2009) found that tolerance to added soil Zn in a
755 nitrifying community corresponded to a restoration of numbers and activity
756 (increased numbers of gene transcripts) of nitrifying bacteria in preference to
757 nitrifying archaea, with the number and activity of the latter immediately, and
758 for the whole duration of the 2 year experiment, reduced by the addition of Zn.
759 Genetic diversity is one indicator of the diversity of microbial communities,
760 however functional redundancy (i.e. different species performing the same
761 function) in soils is high. It has been argued that soils may not be as sensitive as
762 other ecosystems to decreases in genetic diversity due to this high functional
763 redundancy as well as to the enormous diversity and rapid turn-over rate of soil
764 microbial populations (Muller et al., 2002; Wertz et al., 2007), although this
765 hypothesis has been vigorously contested by Allison & Martiny (2008). A study
766 by Muller et al. (2002) assessed the effect of a gradient of Hg contamination,
767 which had occurred 14 years earlier in the field, on both the genetic and
768 functional diversity of the microbial community. The authors found that bacterial
769 genetic diversity was significantly decreased in response to Hg contamination,
770 however functional diversity (as determined by carbon substrate utilisation
771 profiles) was the same between the uncontaminated and contaminated samples.
772 Similar results were found by Rasmussen & Sorensen (2001) in a short-term
773 spiking experiment with Hg. In that study, adaptation to Hg contamination
774 occurred within 18 days following addition of Hg and after this time, while
775 genetic diversity in the contaminated soil remained significantly below that of

776 the uncontaminated control, functional diversity of the adapted population was
777 greater than that of the control. Davis et al. (2004) investigated functional
778 diversity in a soil contaminated in the field with a gradient of Zn. Adaptation to
779 Zn was demonstrated in populations from samples containing > 300 mg/kg Zn.
780 The authors reported a hump-backed response in terms of substrate utilization
781 pattern with increasing soil Zn and no evidence of a decrease in functional
782 diversity at even the highest concentrations of Zn (2000 mg kg^{-1}). In contrast,
783 Lock & Janssen (2005) using similar methodology to that of Davis et al. (2004)
784 found that the functional diversity of Zn adapted populations was significantly
785 lower than that in control populations in a survey of paired samples from 11
786 different soils contaminated in the field with Zn. Wenderoth et al. (2001)
787 measured microbial capability to degrade a suite of aromatic carbon compounds
788 and found a decrease in functional diversity corresponding to increased metal
789 content in long-term sewage sludge amended field plots from which the
790 microbes were derived. It is clear that further research is necessary to
791 understand the factors that result in either positive or negative effects of
792 adaptation on genetic and/or functional diversity to clarify under what
793 circumstances adaptation will be an ecologically adverse effect in terms of
794 decreasing diversity.

795 To date there are no studies examining species evenness in soil microbial
796 populations adapted to trace elements. However the relevance of this factor to
797 functional resilience of microbial populations in the face of trace element and
798 other stressors has been demonstrated. Degens et al. (2001) studied the effect on
799 catabolic evenness (i.e., the variability of substrate used by soil microorganisms
800 assessed by a respiration response method), in two soils with different initial

801 catabolic evenness quotients, of 5 stressors: increasing Cu, increasing salinity,
802 decreasing pH, wet-dry cycles, and freeze-thaw cycles. At high levels of stress
803 catabolic evenness in both soils decreased and the decrease was much more
804 marked in the soil with the low initial evenness quotient. Functional diversity in
805 the low initial evenness soil was also decreased following application of the
806 stressors in comparison to functional diversity in the high initial evenness soil.
807 Catabolic evenness also exhibited a hump-back pattern in response to salinity,
808 wet-dry cycles, freeze-thaw cycles and copper, particularly in the soil with low
809 initial catabolic evenness. From these results, the authors concluded that a
810 decreased catabolic evenness can reduce microbial community resilience:
811 further studies will be useful to understand the relationships between species
812 and functional evenness and thus expand these conclusions to species evenness.
813 Wittebolle et al. (2009) prepared microcosms containing mixtures of denitrifying
814 bacteria with equivalent species richness but which differed in initial evenness
815 and assessed functional resilience in the face of temperature or salt stress. They
816 found that functional resilience in the presence of salt stress depended strongly
817 on the initial evenness of the community with highly uneven communities less
818 functionally resilient to the salt stress. As yet it is not understood why species
819 evenness would increase the functional resilience of a microbial community
820 because if dominant species are more sensitive to a given stress, this should
821 provide an opportunity for the less dominant species to proliferate and occupy
822 the niche vacated by the dominant species. Further research into ecological
823 effects of contaminants should include both studies on the effect of contaminants
824 on species evenness and studies on the effect of adaptation to contaminants on
825 species evenness.

A range of studies have investigated the question of whether adaptation to trace element contamination increases the vulnerability of adapted populations to additional stressors, possibly as a consequence of an increased metabolic burden required to support resistance mechanisms, though effects due to decreased richness or evenness have generally not been ruled out. However evidence suggesting that trace element tolerance mechanisms do constitute a significant burden to their microbial hosts was elegantly demonstrated in a study by Diaz-Ravina and Bååth (2001) who found that metal adapted communities rapidly lost most of their acquired tolerance within one week of being inoculated into an uncontaminated soil. The majority of studies have found no evidence for a decrease in the resilience of trace element adapted communities to additional stressors. Thus for example Rusk et al. (2004) found no increased sensitivity to changes in soil pH or exposure to other metals of Zn or Pb adapted nitrifying bacteria, Mertens et al. (2007) found no increased sensitivity to pesticide addition, freeze-thaw or wet-dry cycles of Zn adapted nitrifying bacteria, and Philippot et al. (2008) found no increased sensitivity to Hg addition of Cu adapted denitrifying bacteria. Tobor-Kaplon et al. (2006), who used decrease in microbial respiration and bacterial growth rates as indicators of sensitivity, found metal adapted populations to be more sensitive than unadapted populations to salt and heat stress when assessed on the basis of respiration, but this was not the case when sensitivity was assessed on the basis of growth rate. Microbial respiration is notoriously variable and is often found to increase rather than decrease in systems under stress (Dahlin et al., 1997) so it may be that bacterial growth rates provide a more accurate picture of the sensitivity of adapted populations in this study. Indeed, as pointed out by Fait et

al. (2006), the presence of adapted but functioning communities in long-term field contaminated soils, which are by definition subjected to the multitude of environmental perturbations that occur in the field, indicates a high degree of resilience of adapted communities to additional stress. However it does seem theoretically likely that incurrence of the metabolic burden needed to sustain trace element resistance mechanisms would increase the vulnerability of adapted communities to other types of stressors which require expenditure of additional metabolic energy to support different types of resistance mechanisms. Hence given the potential consequences of failure of adapted communities to survive additional stress (i.e. catastrophic collapse of soil function), we recommend that further investigation be conducted in this area, extending the range of soil functions and stressors tested, and also examining vulnerability of adapted communities to multiple simultaneous stressors, particularly focusing on energy limited (e.g. carbon deficient) systems.

5.3 Adaptation in relation to risk assessment and remediation of trace element contaminated sites

A risk assessment of contaminated soil involves evaluation of the level of exposure to the contaminant and the effects on biota at that level of exposure in order to determine the hazard (Alexander, 2000). Chemical factors which affect the level of contaminant exposure include contaminant bioavailability and contaminant aging. Both of these factors can play a significant role in reducing the level of contaminant exposure and as such, have been considered important to include in any scientific approach to risk assessment (e.g., Chapman et al., 2010; Udovic and Lestan, 2010; Vasseur et al., 2008). Similarly, adaptation may

be a biological factor to consider when undertaking a risk assessment as theoretically (see discussion above) it should result in recovery of soil function and, as explored in this review, this appears to be borne out by experimental results. This means that the short-term toxicity testing laboratory protocols which are often used to assess risk, may significantly overestimate contaminant risks as the test organisms have typically not had sufficient time to adapt prior to the test being conducted. However a critical caveat before introducing adaptation as a risk mitigating feature in any risk assessment is the need to ensure that adaptation does not decrease community resilience. As discussed in detail above, evidence from long-term field studies tends to suggest that adapted soil microbial communities remain resilient to environmental stressors. However the focus of most of these studies has been on nitrifying populations and it is important to conclusively establish whether other key soil functions which may also undergo adaptation, such as denitrification (Ruyters et al., 2010b) also remain resilient. In addition, we hypothesize that there may be different adaptation thresholds depending on the selective pressure exerted to adapt, related to factors such as the inherent toxicity of a particular contaminant, or to its concentration. Hence for example, a greater metabolic burden may be carried by an organism/community that has needed to adapt to a highly toxic contaminant or a highly concentrated contaminant in comparison to one adapted to a less toxic or less concentrated contaminant and this greater burden may decrease the organism/community resilience. To our knowledge the effect of selection pressure on resilience of adapted communities has not yet been studied and this is an important area for future research, particularly in light of

the fact that different soil functions vary in their sensitivity to trace element toxicity (Coppolecchia et al., 2010).

Adapted populations of microorganisms may also have a role in bioremediation activities. An interesting example is represented by the utilization of organic acid-releasing fungi for the remediation of metal contaminated soils. Arwidsson et al. (2010) showed that fungal species such as *Aspergillus niger* and *Penicillium bilaiae* have a great potential to remediate metal contaminated soils through the release of organic acids, but their effectiveness is reduced by metal toxicity; utilization of adapted populations may overcome this problem. Similarly bacteria can also be used for the remediation of metal contaminated sites. The main process involved are metal reduction, precipitation or transformation (Valls and de Lorenzo, 2001). Alternatively, bacteria can also be used to improve the efficiency of phytoremediation strategies (Glick, 2010; Jiang et al., 2008), also referred as rhizoremediation. The identification and isolation by means of advanced molecular techniques of adapted strains from contaminated sites may thus represent an important means to improve the microbial-aided remediation of trace element contaminated sites. Another option is to identify and clone useful genes involved in adaptation processes and engineer these genes into bacterial strains or plants that will be used for bio- or phytoremediation to enable them to withstand metal toxicity in sites containing metals or metal/organic contaminant mixtures (Hassan et al., 2000).

6. Conclusions

Adaptation of soil microorganisms to trace elements has been demonstrated at both the species and community level. The phenotypical expression of adaptation is represented by the restoration of ecological functions following a contamination event. A range of advanced techniques such as bioreporters and culture-independent meta-tools are increasingly being employed to study the molecular mechanisms underlying adaptation. Further studies are necessary to improve understanding of the relationship between structural and functional responses to trace element stressors, the importance of functional redundancy in adaptation, and how metal-adapted soil populations will respond to further stresses to allow comprehensive risk assessment of metal contaminated sites. Our conclusions from the literature surveyed are that adaptation is unlikely to constitute an adverse effect in terms of ecological function, but could represent a problem in terms of biological or functional resilience. Adapted populations or identification and cloning of genes involved in adaptation may in future help to facilitate microbial-assisted bioremediation.

Acknowledgements

This study was carried out within the GEBEP project sponsored by Cariplo Foundation, Italy and was also supported by the Doctoral School on the Agro-Food System (Agrisystem) of the Università Cattolica del Sacro Cuore, Italy.

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Figure captions

Figure 1. Different mechanisms of microbial resistance to trace elements toxicity.

Figure 2. A conceptual model describing the adaptation of microbial communities to trace elements contamination

1578 Figure 1

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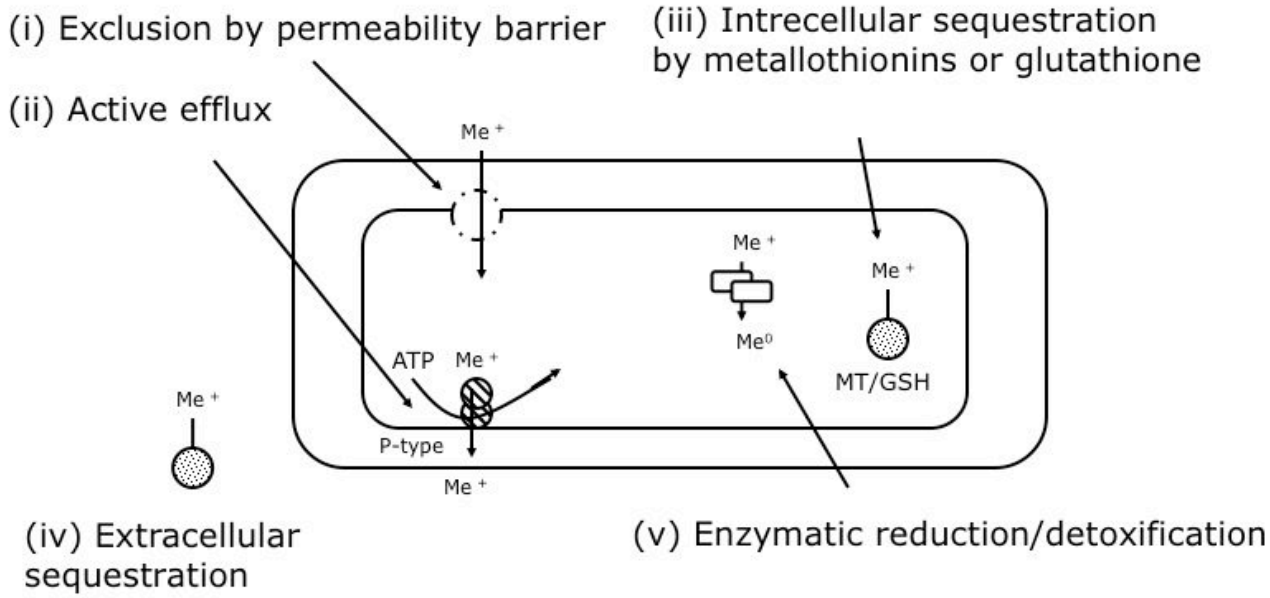

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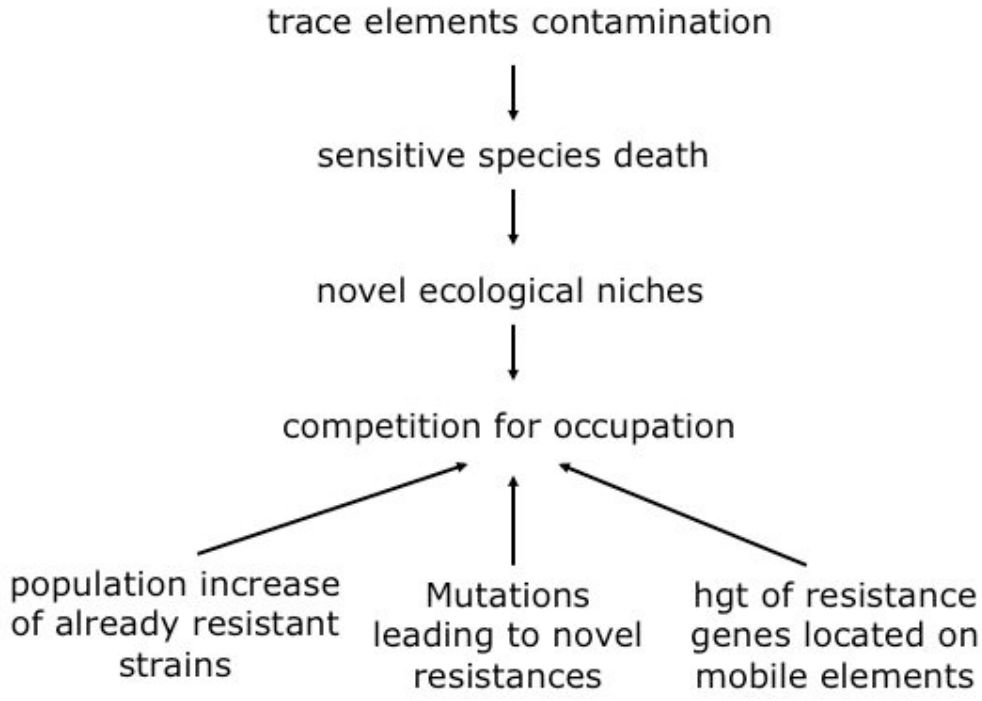
Figure 2


trace elements contamination
sensitive species death
novel ecological niches
competition for occupation
population increase of already resistant strains
Mutations leading to novel resistances
hgt of resistance genes located on mobile elements

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Title:

Response of ammonia oxidizing bacteria and archaea to acute zinc stress and different humidity regimes in soil

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F.M: results and report discussion

R.E.H: contribution to the original idea, experimental setup, results and report discussion

D.D: contribution to the original idea, experimental setup, results and report discussion

Chapter 5

T.M: contribution to the original idea, experimental setup, results and report discussion

Abstract

Ammonia oxidation, a rate-limiting step of nitrification, has been intensively studied for its sensitivity to various environmental shifts and stresses. However, acute stress effects on the occurrence and composition of ammonia oxidizing bacteria (AOB) and archaea (AOA) based on expression of related molecular markers in complex soil environments have been to a great extend overlooked. Particularly concerning transient but commonly occurring environmental factors like humidity. The present study investigates the response of AOB and AOA to humidity shifts and high Zn content in soil. *AmoA* gene copies and transcripts of AOB and AOA along with potential nitrification activity were measured in soil microcosms in response to humidity reduction. Humidity change from 87 to 50% of the water holding capacity caused a ~99% reduction of AOB but not of AOA *amoA* transcripts that did not change significantly. In a second approach Zn was spiked at concentrations ranging from 0 to 5000 mg kg^{-1} in soil. Zn content shifts due to spiking and leaching, the potential nitrification activity, the presence of *amoA* genes and transcripts (encoding the A subunit of ammonia monooxygenase) and the 16S rRNA genes of AOB were measured. AOB *amoA* transcripts responded more readily to low Zn contents and in a more specific manner than the respective AOA. Bacterial ammonia oxidizer 16S rRNA abundance shifted in accordance to the bacterial *amoA* abundance indicating an early response with potentially total cell activity loss along the Zn gradient. Differentiation of *amoA* responses among AOA and AOB enhances previous arguments about differences in occupied niches.

Introduction

Nitrification, a central pathway of the global nitrogen cycle (Kowalchuk and Stephen, 2001) and defined as the turnover of ammonia to nitrate, was used in ecotoxicology studies and proposed for risk assessment, due to its sensitivity to various stresses and environmental shifts (vanBeelen and Doelman 1997; Smolders *et al.* 2001; Rusk *et al.* 2004; Mertens *et al.* 2006; Mertens *et al.* 2007; Park and Ely 2008a; Park and Ely 2008b; Radniecki and Ely 2008; Radniecki *et al.* 2009; ISO 15685:2004).

Bacterial ammonia oxidizers (AOB) of the ß-proteobacterial class have been long considered to be solely responsible for ammonia oxidation (rate-limiting step of nitrification) in soil (Koops and Pommerening-Roser 2001; Kowalchuk and Stephen 2001; Prosser and Embley 2002). Their chemolithoautotrophic lifestyle is characterized by carbon fixation via the Calvin-Benson cycle (RubisCo mediated) while the necessary energy for performing this task is derived by the turnover of ammonia to nitrite (Voytek and Ward 1995). For tracking the presence and activity of AOB in soil, the *amoA* gene (encoding for A subunit of ammonia monooxygenase – AMO) has been used as molecular marker (Rotthauwe *et al.* 1997) together with β-proteobacterial ammonia oxidizer specific 16S rRNA gene targeting primers (Kowalchuk *et al.* 1997). Recently, isolation of crenarchaeal strains codifying an AMO encoding operon homologous to the AOB *amoA* and being able use ammonia for energy acquisition (Konneke *et al.* 2005; de la Torre *et al.* 2008; Hatzenpichler *et al.* 2008) changed the general perception of ammonia oxidation. Studies revealed the wide distribution of archaeal ammonia oxidizers like the ones belonging in mesophylic *Crenarchea* (AOA), which was recently proposed to comprise the new distinct phylum of *Thaumarchaeota*, in soil environments (Venter *et al.* 2004; Treusch *et al.* 2005; Leininger *et al.* 2006; Nicol and Schleper 2006; Francis *et al.*

2007; Brochier-Armanet *et al.* 2008; Erguder *et al.* 2009; Tourna *et al.* 2011). Despite being cosmopolitan, differences observed between AOA and the well-studied AOB raised debates about potential niche separation between the two groups (Hallam *et al.* 2006; Nicol *et al.* 2008; Erguder *et al.* 2009; Jia and Conrad 2009; Martens-Habbena *et al.* 2009; Schleper 2010).

Some of the observed differences were quite clear when naturally occurring or induced trace element stresses have been used in order to assess, among others, partition of ammonia oxidation between AOB and AOA or the adaptation of nitrification and ammonia oxidizers in soil environments (Mertens *et al.* 2009; Ruyters *et al.* 2010; Puglisi *et al.* 2011). However, the initial microbial responses of the total soil microbial community to such stresses or even commonly occurring environmental shifts (e.g. humidity shifts) have been greatly overlooked.

This study aimed at gaining insights about ammonia oxidation ecology of AOB and AOA in agricultural soil environments in respect to two types of environmental shifts: one commonly occurring change and one severe stress relevant to risk assessment studies. Total nitrification, *amoA* gene and transcript differences for both microbial groups and also the 16S rDNA presence and expression for AOB, were assessed for soil microcosms subjected to humidity shifts (commonly occurring environmental change) and increasing zinc concentrations (severe stress). Results indicate distinct responses between the studied groups in both cases, while distinct response to the zinc stress was indicated among the various AOB genotypes.

Materials and Methods

Experimental setup. Topsoil (0 – 15 cm depth) was collected from a maize-field located close to Alsenio (PC, Italy) in the end of October 2009. Total soil carbon and nitrogen and also soil texture was measured according to standard soil methods (Day 1965; Sparks 1996), while background trace element concentrations were determined via the *aqua regia* digestion method as described in Coppolecchia *et al.* (2011). Previous results on several biological activities and proposed models describing them for the same soil samples can be found in Coppolecchia *et al.* (2011).

Humidity effects on total nitrification activity, *amoA* and *amoA* transcripts per ammonia oxidizer microbial group were assessed for two water contents. Soil microcosms were water saturated and incubated at room temperature. Samples in triplicates were obtained one day (water content equal to 87 % of the water holding capacity – WHC) and four days (water content 50 % WHC) post leaching and stored in -20°C until analyzed as described further on.

AOB and AOA acute responses to zinc were examined by treating soil microcosms with increasing concentrations and assessing potential nitrification, *amoA* gene and transcript content and also *amoA* variant changes, for soil samples obtained after overnight incubation. Briefly, soil microcosms of 200 g each were spiked with $ZnCl_2$ to nominal Zn concentrations ranging from 0 to 5000 mg kg^{-1} (hereafter treatments will be referred at according to nominal zinc content). Cl^- effects were reduced by soil was leaching with ddH_2O (double distilled water). pH measurement was carried out prior and after leaching for assessing potential $ZnCl_2$ induced soil pH shifts. Post leaching pH drops above 0.5 units were observed only for high zing concentrations were measured microbial traits were below detection limits as presented further on. The soil microcosms were concomitantly incubated in open-air overnight at room

125 temperature and sampled the following day and samples were stored in -20°C for
126 downstream analyses.

127 **$CaCl_2$ 0.01 M extractable Zn (labile fraction).** Post leaching Zn extractable
128 fractions of soil samples were determined via the $CaCl_2$ (0.01 M) extraction method.
129 Briefly, 10g of soil were soaked in 100 ml of $CaCl_2$ 0.01 M and incubated at room
130 temperature in a rotary shaker at 30 rpm for 2 h. Extracts were obtained after
131 separation by centrifuging at 3000 × g for 10 min and were acidified with HNO_3. The
132 acidified extracts were stored at 4°C until ICP-OES (Inductively Coupled Plasma
133 Optical Emission Spectroscopy) analysis. The wavelength used for Zn determination
134 with ICP-OES was based on the related results of Bettinelli et al (2000).

135 **Potential nitrification assay.** Potential nitrification assay was performed according to
136 the Kandeler method (1995). In brief, 5 g of moist soil were incubated with 20 ml of
137 $(NH_4)_2SO_4$ 1 mM and 0.1 ml $NaClO_3$ 1.5 M in closed Erlenmeyer flasks for 5 hours at
138 37°C shaking at 100 rpm. As no incubation controls, flasks containing soil,
139 $(NH_4)_2SO_4$ and $NaClO_3$ were directly stored in -20°C until the next step. 5 ml of KCl
140 2M were added in all flasks, briefly mixed and the contents were filtered. 5 ml of
141 filtrates were mixed with 3 ml of NH_4Cl 0.19 M pH 8.5 solution and 2 ml of color
142 reagent (N-(1-naphtyl)-ethylenediamine hydrochloride with phosphoric acid solution)
143 and were incubated at room temperature for 15 minutes. A no filtrates control was
144 incubated with the color reagent as well. Samples and no incubation controls were
145 compared to the no filtrate controls at 520 nm absorbance.

146 **Nucleic acids isolation, quality control and quantification.** For the nucleotide
147 isolation the RNA – DNA co-isolation protocol of the MoBio PowerSoil™ RNA kit
148 (Carlsbad, CA, USA) when combined with the RNA PowerSoil™ DNA elution
149 accessory kit was followed with a slight modification. The modification referred to

the addition of EDTA pH 8.0 to a final concentration of 0.5 M right after the SDS containing reagent and prior vortexing for cell lysis enhancement.

DNA purity analysis was performed spectrophotometrically (260 nm/280 nm and 260 nm/230 nm ratios) while DNA and RNA extracts were also analyzed on 0.8% and 1% agarose gels respectively for shearing and degradation control. DNA and RNA were quantified using the Quant-iT™ (HS dsDNA Assay and RNA Assay kits respectively, Invitrogen, Paisley, UK) in combination with the Qubit™ fluorometer (Invitrogen, Paisley, UK).

General PCR conditions and RNA reverse transcription. Genes and transcripts studied were the β-proteobacterial and crenarchaeal *amoA* and also the β-proteobacterial 16S rDNA. Primer sets used for the respective genes amplification were the amoA1F/2R T (Rotthauwe *et al.* 1997), the CrenamoA 23f/616r (Tourna *et al.* 2008) and the CTO 189fABC/654r (Kowalchuk *et al.* 1997). PCR amplification was carried out in 50 µl reaction mixtures using the AmpliTaq® DNA polymerase with buffer I kit (Applied Biosystems, Foster City, CA, USA). 20 ng of template were added in each mixture containing 1× PCR buffer, 2.5 mM $MgCl_2$, 2.5 U Taq polymerase 0.4 mM dNTPs, 0.5 µM of each primer and ddH_2O. Conditions used were 94°C for 5 min, 35 cycles of 94°C for 30 sec denaturation, 54°C for 30 sec annealing, 72°C for 30 sec extension and a final extension step of 72°C for 10 min for the amoA1F/2R T and the CTO 189fABC/654r primer sets while for the CrenamoA 23f/616r primer set an extension step of 1 min instead of 30 sec was used.

Reverse transcription of RNA to cDNA was performed with the iScript™cDNA Synthesis kit of BioRad (BioRad Laboratories, Hercules, CA, USA) according to the manufacturer instructions.

Real Time quantitative PCR (qPCR) and standard curve generation. For the qPCR assays, the iQ™ SYBR® Green Supermix (BioRad Laboratories, Hercules, CA, USA) was used in 50 µl reactions. Absolute quantification was carried out in all cases based on standard curves as described further on. Transcript and gene copies were quantified using 10 ng (for cDNA) and 20 ng (for DNA) templates in a mixture containing 25 µl of Supermix and 0.5 µM of each primer and ddH_2O. PCR program followed was as referred in the general PCR conditions section for each primer set with differences for the initial enzyme activation stage time (95°C for 3 min instead of 5 min) and the cycles performed (50 instead of 35). A melting curve was performed right after each reaction set was finished for the evaluation of the specificity of the amplifications.

PCR products of environmental sample served as templates for the standard curve generation. Estimations of the average molecular weight of amplified fragments were based on primer BLAST against the non-redundant database of NCBI (National Center of Biotechnology Information, http://www.ncbi.nlm.nih.gov/) with kingdom level taxonomical restrictions and the BLAST results of the DGGE gel excised bands. Molecular weight values obtained demonstrated low dispersal among the arithmetic mean (data not shown) indicating good correlation among molecular weights and amplified fragments copy numbers. After these calculations the standard curve was generated using quantified PCR product in the range of 10 to 10^8 copies for all primer sets in order to assess detection limits and R^2 values.

DGGE analysis. PCR products obtained with the primer sets amoA 1F/amoA 2RT with forward primer containing the GC clamp in the 5' end previously published by Muyzer *et al.* (1993) and CrenamoA23f/616r were used for DGGE analysis. PCR conditions were the same as described above.

DGGE was carried out using a DCode Universal Mutation Detection System (BioRad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Denaturant contents of polyacrylamide gels used were 15% - 55% for the CrenamoA 23f/616r primer set and 50% - 65% of denaturant for the AmoA1F GC/2R T primer set. Gels were SYBR green stained and image analysis was carried out with the Cross Checker software (Buntjer 1999) for generating genotype presence absence (binary) matrices.

Statistical tests and analyses. Range weighted richness (Rr) values were estimated according to the Marzorati et al (2008) provided formula $Rr=(S^2 \times Dg)$ (S is the observed band richness per DGGE gel lane and Dg the gradient difference between the first and last band). Hierarchical clustering analysis was performed on the binary matrices generated by the DGGE gel images using the UPGMA (unweighted pair group comparison method with arithmetic means) algorithm and the Jaccard distance estimation. Principal Coordinates Analysis (PCoA) was also performed for the Jaccard algorithm transformed binary matrices. Vector analysis (R. H. G. Jongman and Tongeren 1995) after plotting of both treatments and the DGGE genotypes according to PCoA was used to assess potential genotype-treatment correlations. One-way ANOVA (analysis of variance of means) analysis and the Tukey HSD (honestly significant differences) test were performed for the potential nitrification, the Real Time PCR and the richness (S) and Rr data. Analysis of similarity (ANOSIM) and Mantel tests using 1000 permutations and the Jaccard distance transformation were performed to identify correlations between banding pattern shifts of the DGGE gels and treatments or measured variables respectively.

The R software (R_Development_Core_Team 2009) with the Biodiversity R related script packages were used for all statistical analyses (Kindt and Coe 2005).

Results

Effects of humidity shifts on nitrification, *amoA* genes and transcripts. For both the humidity contents tested the measured potential nitrification was not significantly different, indicating no significant differences for total enzyme numbers (figure 1 A). The same effect was observed for *amoA* gene copy numbers of both AOB and AOA (figure 1 B). Transcriptional results showed that for both microbial groups the measured *amoA* transcripts had a reducing trend but only for β-proteobacterial ammonia oxidizers this decrease was significant (99% drop of transcripts – figure 1 C).

Zinc effects on potential nitrification, AOA and AOB copy and transcript numbers.

Leaching did not significantly alter the total (applied plus background) [Zn] in soil for nominal concentrations up to 500 mg kg^{-1}, while a progressively increasing loss is observed for nominal [Zn] of 1000 mg kg^{-1} and above (data presented in Coppolecchia *et al*. 2011). Potential nitrification results indicate a Zn concentration dose dependent reduction of the enzymatic activity (figure 2 A). Zn concentration of 125 mg kg^{-1} nominal did not any significant sifts in the measured potential nitrification other than a reduction trend compared to the control. In the case of 250 mg kg^{-1} this reduction is becoming statistically significant while at 500 mg kg^{-1} or higher nominal Zn concentration the potential nitrification values approached zero. RNA copies followed similar trends to those of potential nitrification for all studied genes. Control treatment ([Zn] = 0 mg/kg) average *amoA* transcript numbers were one order of magnitude lower for AOA (315 transcrips) compared to the AOB (3211 transcripts) similarly to the soil moisture shift test. Spiking of 150 mg kg^{-1} Zn

concentration induced a decrease of transcription of ~ 60% or more compared to the controls, that further decreased down to non detectable levels at 1000 mg kg^{-1} nominal Zn concentration. The observed trend appeared to be more intense for the bacterial transcripts for both the functional gene and the small ribosomal subunit according to the average values and also the difference at [Zn] of 500 mg/kg which was significant compared to the control while for AOA *amoA* not. DNA derived data demonstrate no significant differences between treatments except from the β-proteobacterial *amoA* copy numbers which were greatly reduced under the nominal Zn concentration of 5000 mg kg^{-1} (figure 2 B).

Effect of Zn spiking on *amoA* richness and structure. *AmoA* gene and transcript composition shifts of the environmental samples in response to nominal Zn concentrations between 0 and 500 mg kg^{-1} were assessed with DGGE. DGGE *amoA* gene amplicon patterns showed no changes for the different treatments of the referred [Zn] range for both β-proteobacterial and crenarchaeal ammonia oxidizers (data not shown) and therefore are not further discussed.

AmoA transcriptional responses on the other hand varied between the two groups. Hierarchical clustering results showed formation of three major treatment related clusters for AOB but no treatment dependent relationship was indicated for AOA (figure 3 B and A respectively). Clusters formed for AOB *amoA* separated the samples into three treatment related groups: (i) the control group, (ii) the 125 and 250 mg/kg of applied [Zn] group and (iii) the 500 mg/kg applied [Zn] group. Range weighted richness values showed that *amoA* expression of both examined groups tended to decrease with increasing [Zn] (figure 3 C). This shift was more rapid for AOB with an average reduction of above 50 % for the lowest applied Zn dose, a

difference being significant also according to ANOVA. AOA on the other hand had a slight increase of average Rr value for 125 mg/kg of [Zn] followed by a decrease and a significantly lower Rr value for 500 mg/kg of applied [Zn]. Observed richness (*S*) followed the same trends with the Rr values (data not shown).

PCoA analysis of the DGGE *amoA* transcript patterns showed a treatment-wise topological relation of samples for AOB (figure 3 D), while no relation could be extrapolated for AOA (data not shown). The referred AOB patterns were further analyzed in order to assess potential associations of observed genotypes with samples or treatments. Vector analysis indicated 5 distinct groups of genotypes in relation to the applied treatments (an example of extrapolated correlation is shown for g12 in figure 3 D). Such were the ones associated with: (i) the 0 mg/kg [Zn] (g2, g5, g6, g7, g8), (ii) the 0, 125 and 250 mg/kg [Zn] (g9, g11 - with slightly higher correlation to 0 mg/kg of [Zn]), (iii) the 125 and 250 mg/kg (g1, g10), (iv) the 0 and 500 mg/kg [Zn] (g3, g4) and (v) the 500 mg/kg [Zn] (g12).

ANOSIM results showed two different responses between bacterial and archaeal *amoA* transcriptional profiles. Bacterial *amoA* transcriptional patterns had higher variability between treatments than within while the respective archaeal did not , as also indicated by R values and respective test significance (figure 4).

Mantel test results indicate low or no correlation of banding patterns of archaeal *amoA* transcripts while correlation is shown for bacterial *amoA* transcripts with all tested variables except from *amoA* transcripts.

Discussion

In the present study we studied the response differences between two prokaryotic groups known to carry the *amoA* homologue and be accounted for most ammonia

298 oxidation, the AOB and AOA, using a commonly occurring in nature environmental
299 shift and one severe stress.

300 **Soil humidity.** Significant humidity reductions in the soil environment were
301 previously shown to affect nitrification activity due to reduction of substrate
302 availability and triggering of physiological changes (e.g. downregulation of basic
303 metabolism and upregulation of stress related gene expression) for tolerating the
304 induced osmotic changes (Stark and Firestone 1995). Outcomes of this study did not
305 indicate that the applied humidity shift was severe enough to cause loss of enzymatic
306 activity as shown according to the potential nitrification results (figure 1 A). With
307 *amoA* gene presence maintained, *amoA* transcript number reduction for AOA and
308 AOB was in concordance to the soil humidity shifts. Thus, indicating that even
309 relatively mild environmental shifts are capable of inducing down-regulation of basic
310 metabolism gene expression and possibly causing the entrance of microbial cells to an
311 alarm stage. Significant differences were observed only for AOB *amoA* transcripts
312 and might be reflections of differences associated with the wider taxonomic
313 affiliations (higher extracellular osmolality tolerated by Archaea compared to Bacteria
314 as depicted by Martin *et al.* - 1999) or even differences concerning the importance of
315 the genes for basic metabolism between the two microbial groups for lineages
316 occurring in soil environments, as indicated elsewhere (Jia and Conrad 2009). The
317 latter might also be implied by the differences in *amoA* transcript numbers observed
318 between AOA and AOB in the high humidity tested level with AOB having one order
319 of magnitude more transcripts (a difference observed also in the control treatment of
320 the severe stress experiment performed in this study), as opposed to the counted gene
321 copies that reveal a nearly inverse relation.

322 **Zinc dose response.**

PN - *amoA* transcript and gene copy numbers. Microcosm water-leaching reduced effects of spiking on pH, previously shown to affect enzymatic activity, *amoA* presence and expression in soil (Kowalchuk and Stephen 2001; Nicol *et al.* 2008). Moreover, significant reduction of the $CaCl_2$ extractable Zn after leaching when compared to the total (background plus applied) prior leaching, was observed only in Zn concentrations above the ones resulting in near or below detection limit of measured parameters apart from the *amoA* gene copies.

Potential nitrification data indicate a decrease of the enzymatic activity in a Zn dose response manner, with most of it being abolished at nominal Zn concentrations of 500 mg kg^{-1} and above. All zinc treatments affected potential nitrification negatively as shown by trends and significance of differences (figure 1 A). This is consistent with previous observations of enzyme inhibition due to competition of zinc with copper for placement in the AMO active site (Radniecki and Ely 2008). Moreover, there is no confirmation of enzymatic functionality maintenance that could be attributed to a potential zinc role as discussed by Gilch *et al.* (2009) for any applied [Zn] in this study.

Quantitative analysis of expression of all screened genes showed similar trends but having a more rapid response compared to the measured nitrification activity (figure 2 A). Transcript numbers readily dropped for both the bacterial functional and the 16S rRNA markers at 125 mg/kg and 250 mg/kg of Zn treatment, showing a more rapid drop trend for the 16S rDNA marker. The 125 mg/kg [Zn] reduction although not statistically significant for both AOB and AOA, is more intense for all enumerated transcripts than measured potential nitrification. For 250 mg/kg [Zn] and above, both transcript numbers and enzymatic activity are reduced indicating total cell activity loss and possible fatality. This is quite apparent for AOB since both the *amoA*

transcripts and the 16S rRNA are dropping below detection limits for 500 mg/kg of [Zn] and above. These results are consistent with previous studies where down-regulation of the carbon fixation RubisCo marker was identified in *Nitrosococcus mobilis* and *Nitrosomonas europaea* under zinc stress (Park and Ely 2008; Radniecki and Ely 2008).

For DNA qPCR templates, [Zn] effects were observed only for AOB at 5000 mg/kg according to qPCR results (figure 2 B). Such reduction is consistent to extracellular DNA degradation, previously found to occur in some cases within 12 hours, revealing a potential cell death (Levy-Booth *et al.* 2007).

***AmoA* transcript patterns and correlations with treatment and variables.** Rr and richness (*S*) values of *amoA* transcripts followed similar to the *amoA* transcript qPCR trends for both AOA and AOB with the AOA slightly deviating with an increase for 125 [Zn] and AOB having significant drop for 125 mg/kg [Zn] and above. Such reduction could indicate a high selectivity of the applied stress. Nevertheless, DGGE patterns showed [Zn] dependent transcriptional profiles for AOB while this was not the case for AOA where results showed more random patterns according to clustering analysis (figure 3 A), while strong support to this outcome was provided by ANOSIM R values (figure 4). Furthermore, Mantel tests indicated a high correlation of AOB *amoA* transcript patterns with potential nitrification as opposed to the archaeal ones (Table 2). Showing this way the higher importance of AOB for potential nitrification rates in the examined agricultural soil. However, the soils examined here were soils with low organic matter content, receiving nitrogen in mineral forms, previously shown to favor AOB (Offre *et al.* 2009; Verhamme *et al.* 2011). Similar responses to the ones found in our study were observed also in the study of Mertens et al (2009) where no major differences of crenarchaeal *amoA* DGGE patterns obtained from

DNA templates could be connected to the applied Zn doses as opposed to the bacterial ones.

Mantel tests also showed a high correlation of Rr and *S* with DGGE patterns of AOB *amoA* transcripts compared to the AOA. Thus indicating a separation of AOB genotypes according to [Zn] tolerance as also indicated by the PCoA analysis results (figure 3 D), while less respective specificity could be extrapolated for AOA. Lack of specific sensitivity of the *amoA* homologue transcription in AOA to [Zn] along with the low may imply lower importance of this gene for the physiology of AOA found in the examined soils. This outcome is further supported by the correlation identified between AOB *amoA* transcript pattern shifts with respective *amoA* and 16S rDNA transcripts. These results are consistent to results of Xia *et al*. (2011) where AOB where found to be mainly responsible for ammonia oxidation in the tested agricultural soil, while not all AOA were active. The active AOA were affiliated to *Nitrososphaera gargensis* as identified by *amoA* sequence analysis combined with stable isotope probing for assessing carbon fixation by ammonia oxidizers. Moreover, although the constitutive expression of housekeeping genes like 16S rDNA particularly in natural environments is debated (Klappenbach *et al.* 2000; Smith and Osborn 2009), the identified correlation of bacterial *amoA* transcriptional patterns with the respective 16S rRNA measured copies, indicates certain reliability of the usage of this gene as an activity marker.

Concluding remarks

Recent discoveries related to nitrification have changed the perception of several related concepts in this research area and generated question marks. One step towards shedding light in nitrification partition among AOB and AOA in agricultural soils,

was taken with in present study by looking into effects caused by one common mild and one severe stress.

AOB were shown to be more affected by the humidity shift compared to AOA concerning transcription indicating a stress response. Such shift, associated to nutrient availability could signify the importance of limiting substrate concentrations for the respective microbial groups. Acute responses to Zn indicate AOB prevalence compared to AOA at Zn concentrations where both *amoA* transcriptional activity and potential nitrification rates have values above zero. At the same time, bacterial ammonia oxidizers appear to be more sensitive to the applied stress than the respective archaeal guild. The latter was demonstrated in high applied Zn doses in a DNA level and also according to the *amoA* transcriptional reduction trends observed for the above zero transcript values. Rapid reduction of the 16S rRNA for bacterial ammonia oxidizers in a similar fashion as with the *amoA* transcripts demonstrated a most probable viability loss rather than a recoverable state. Particular bacterial genotypes showed a strong correlation to certain applied Zn doses while poor connection was observed for archaeal ammonia oxidizers.

Collectively, differences observed in the transcriptional responses between AOB and AOA *amoA*, reflect potential differences in the importance of the encoded protein for basic metabolism between the referred groups. Therefore our results enhance previously stated opinions and study outcomes about potential niche differentiation.

Acknowledgements

The present study was carried out as part of the GEBEP project funded by the Caprilo Foundation (Italy) and was also supported by the Doctoral School on the Agro-Food System (Agrisystem) of the Università Cattolica del Sacro Cuore (Italy).

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Table 1 Basic properties (A) and trace element background concentration (B) of the soil used for generating the microcosms.

A

Total C (%)	Total N (%)	pH	WHC (%)	Particle Size Distribution		
				Clay (%)	Silt (%)	Sand (%)
0.81	0.28	8.2	34	24	42	33

B

Trace element	**Pb**	**Ni**	**Cu**	**Cd**	**Cr**	**Co**	**Zn**
mg/kg	18.2	38.6	19.6	0.1	67.9	13.7	84.9

610 Table 2 Mantel test r values and respective significance of bacterial and crenarchaeal
611 *amoA* transcriptional banding pattern shifts correlation with potential nitrification, Rr,
612 *S*, *amoA* transcript number and 16S rDNA transcript number changes (1000
613 permutations) under different [Zn] treatments. Empirical confidence of r provided by
614 means of different background shading per case as indicated below the table.
615

	Bacterial *amoA*		Crenarchaeal *amoA*	
	r	Sig	r	Sig
PN	0.389	0.002	0.001	0.429
Rr	0.407	0.002	0.174	0.126
S	0.528	0.001	0.221	0.093
amoA transcripts	0.233	0.027	0.129	0.076
16S rDNA transcripts	0.246	0.033		

	90%	95%	97.5%	99%
Empirical confidence of r:				

616

617

618 Figure captions

619 Figure 1 Soil humidity drop (from high = 87 % to low = 50 % of the WHC) effects on
620 measured potential nitrification rates (A) and also *amoA* copies (B) and transcripts (C)
621 of AOB and AOA. Significant differences due to change in humidity were observed
622 only for the β-proteobacterial ammonia oxidizer transcript numbers according to
623 performed ANOVA (indicated with the asterisk for $\alpha < 0.05$).

624

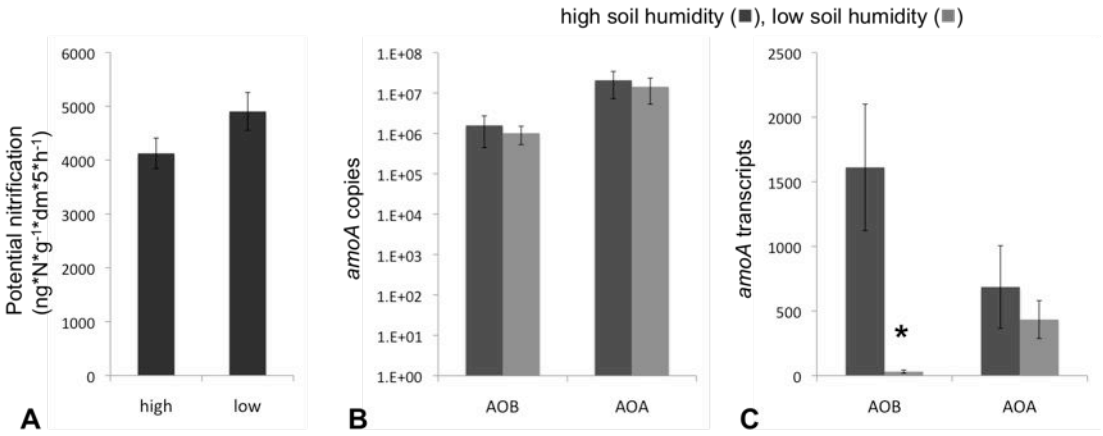
625 Figure 2 Potential nitrification (PN) results, AOA *amoA* transcripts, AOB *amoA*
626 transcripts and AOB 16S rRNA copies normalized to the average control (nominal
627 [Zn] = 0 mg/kg) values for Zn treatments range of 0 – 5000 mg/kg (A); *amoA* copy
628 numbers for AOA and AOB for the [Zn] range mentioned for A (B). Letters of
629 various formats in (A) indicate different groups according to Tukey HSD pairwise
630 comparisons performed for ANOVA per measurement type: normal letters for PN,
631 italics for AOA *amoA* transcripts, underlined for AOB *amoA* transcripts and
632 underlined italics for AOB 16S rRNA copies.

633

634 Figure 3 DGGE based transcriptional analysis results for AOA and AOB *amoA* of the
635 [Zn] treatment range of 0 – 500 mg/kg. AOA and AOB hierarchical clustering results
636 using UPGMA and the Jaccard coefficient along with the banding patterns are shown
637 in A and B respectively. Average Rr values for each treatment, normalized to the
638 average control value ([Zn] = 0 mg/kg) are shown in C (letters – normal for AOA and
639 italics for AOB – indicate statistically significant differences between treatments
640 according to ANOVA and Tukey HSD for $\alpha < 0.05$), while the genotype 12 (g12 - or
641 gel band number 12) correlation with the various treatments as indicated by vector
642 analysis of the PCoA plot, is shown in D. PCoA was performed on a Jaccard

643 transformed binary matrix and the explained variance by the first two dimensions was
644 88 % (48 % for Dim1 and 40 % for Dim2).
645
646 Figure 4 ANOSIM results of the treatment groups for bacterial (left) and crenarchaeal
647 (right) *amoA* banding patterns obtained by DGGE analysis of transcripts.
648

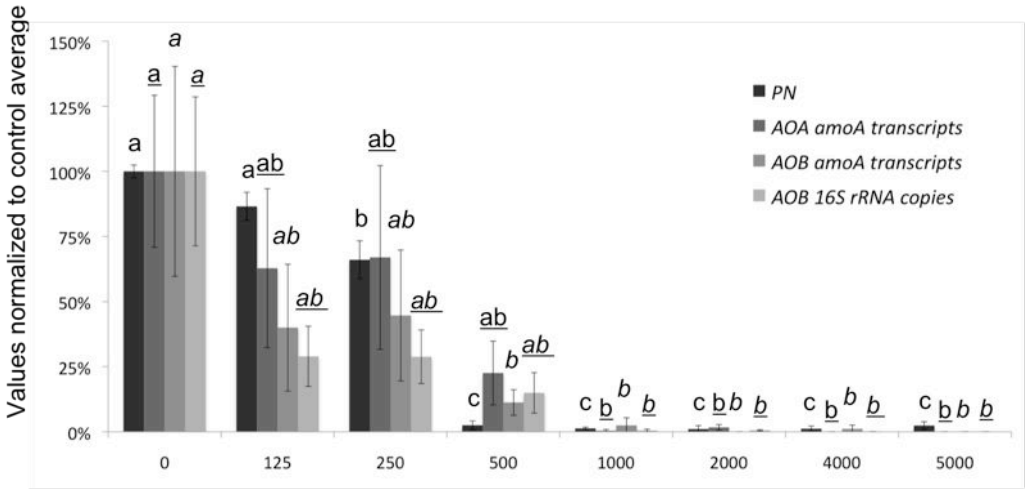
649 Figure 1



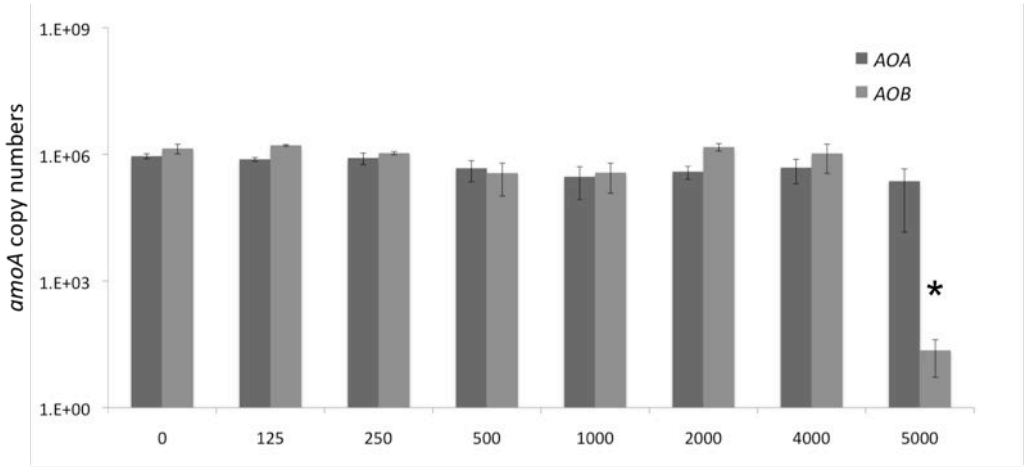
650

651

652 Figure 2



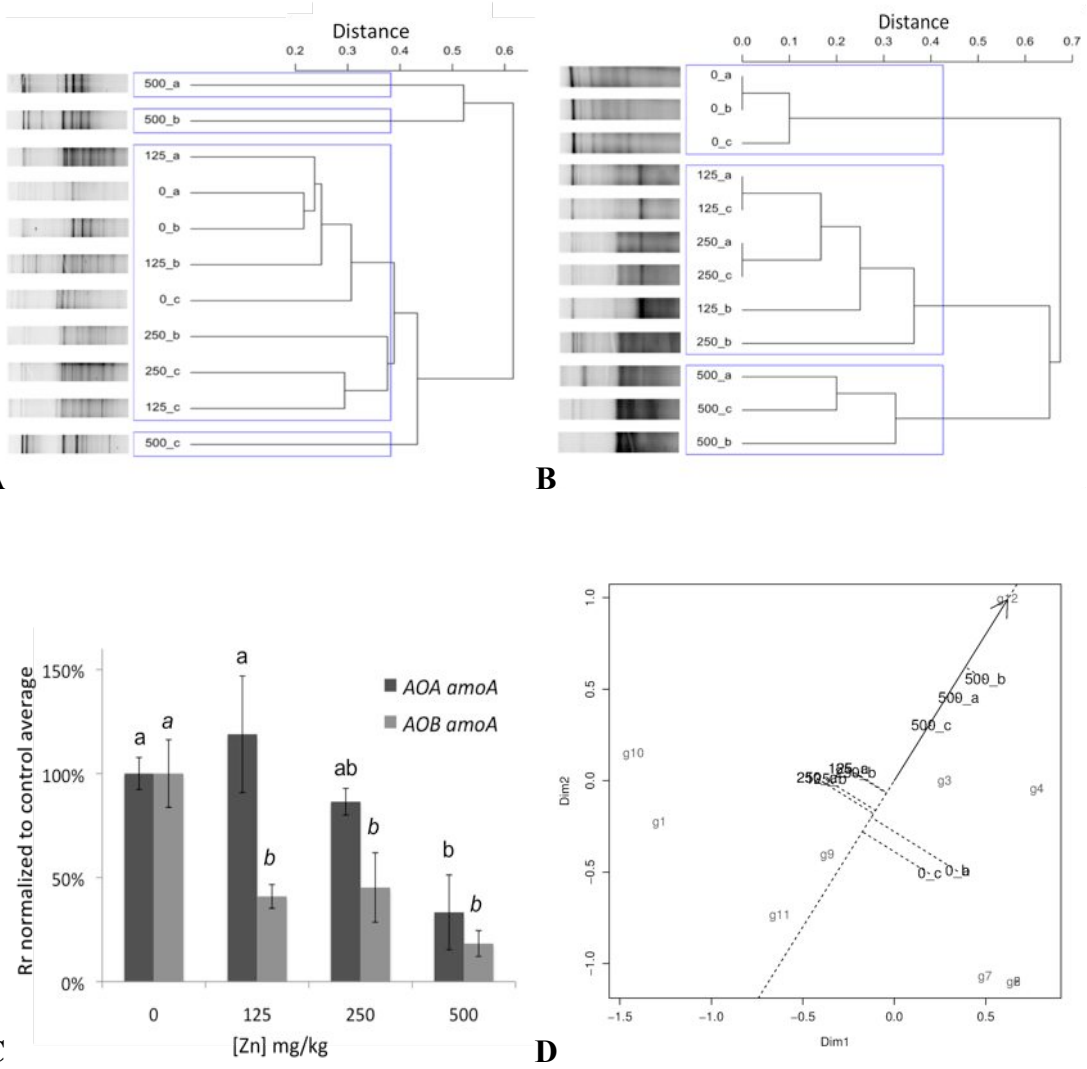
653 **A**



654 **B**

655

656 Figure 3

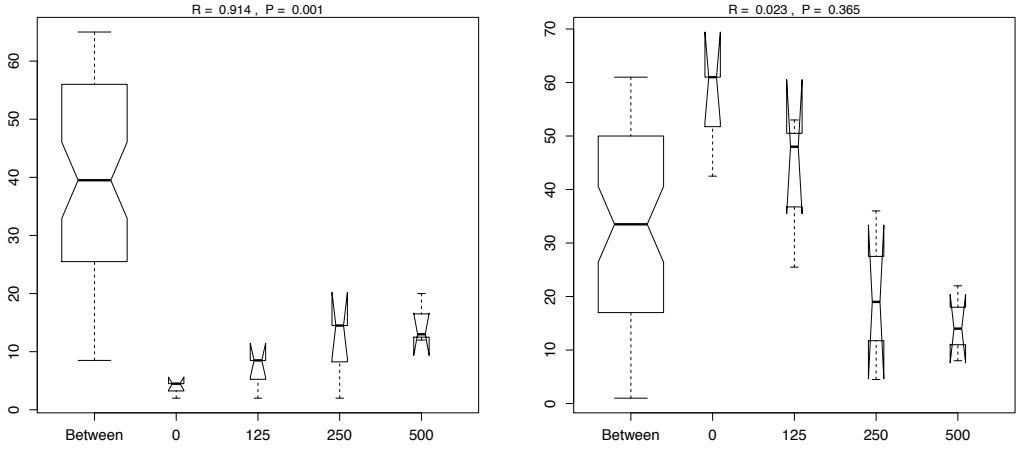


657 **A** **B**

658 **C** **D**

659

660 Figure 4



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Chapter 6

Title:

Fungicides impact on the diversity and function of non-target ammonia oxidizing microorganisms residing in a litter soil cover

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D.K: contribution in development and evaluation of original idea, discussion of results, writing of report

E.C: original idea, discussion of results and report

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Keywords: litter soil layer; ammonia oxidizing bacteria; ammonia oxidizing archaea; fungicides; nitrification; DGGE

Abstract - Litter soil cover constitutes a particularly important environmental compartment in sustainable viticulture serving as a habitat of complex microbial communities and having a key role in nutrient cycling and organic matter decomposition. Ammonia oxidizing bacteria (AOB) and archaea (AOA) are important microbial guilds in litter and soil and regulate the transformation of ammonia to nitrite. Our study aimed to assess the effect of two fungicides, penconazole and cyprodinil, on the function and diversity of total and active nitrifying microbial communities using a microcosm approach. Functional changes measured via potential nitrification and structural changes assessed via denaturating gradient gel electrophoresis (DGGE) were contrasted with pesticide dissipation in the litter layer. The latter was inversely correlated with potential nitrification which was temporarily inhibited at the initial sampling dates when high pesticide residues were still present in the litter. Pesticides induced substantial changes in the communities of both AOB and AOA were more visible in the RNA-based fingerprinting analysis. Potential nitrification patterns was less sensitive to pesticides and was restored faster than structural changes which persisted for longer. These results support the theory of the redundancy of microbial functions like nitrification, this time in a litter environment.

Introduction

Litter soil cover is a layer of mainly dead plant organic material present at the soil surface. It constitutes a transitional micro-environment between soil and plants that plays a key role in both natural and agricultural ecosystems. Litter is at the forefront of soil organic matter decomposition processes, and the major determinant of nutrient cycling in most terrestrial ecosystems (Meentemeyer 1978). Litter is also involved in other key ecological functions and services; it supports a complex and active microbial community, is a habitat of beneficial animals; improves soil structure, water-holding capacity and other physico-chemical properties; controls the bioavailability and degradation of pesticides; prevents erosion and controls the release of greenhouse gases from the soil ((PPR) 2010).

In natural ecosystems a litter layer is always present on the soil surface and it is influenced by natural processes, *in primis* plant species composition (Hobbie 1992). Whereas, in agricultural ecosystems the presence, type and activity of the litter layer is strongly influenced by agronomic practices like tillage, plant residues incorporation, pest control strategies and use of cover crops (Jacobs *et al.* 2011). The establishment of a litter soil cover is becoming an important agronomic practice in sustainable vineyard production systems, where vegetated strips and cover crops, along with an integrated use of pesticides, are applied to minimize pest infestation, improve soil properties and increase the quantity and quality of agricultural production (Danne *et al.* 2010).

In vineyard ecosystems it has been shown that a correct adoption of vegetated strips results in an increase of nitrogen (mainly ammonia and free amino acids) that is also very important for grapes quality (Hirschfelt 1998). In

soil and litter, ammonia-oxidizing bacteria (AOB) and archaea (AOA) regulate N cycling through the expression of ammonia monooxygenase, a heterodimer enzyme which is able to catalyze the conversion of ammonia to nitrite. Given their ecological importance, nitrifiers are among the most studied functional microbial guilds in soils, and molecular markers useful to assess their phylogenetic (16S rDNA) and functional diversity (*amoA*) have been developed and successfully applied in a number of studies (Kowalchuk *et al.* 1997; Prosser and Nicol 2008; Ruyters *et al.* 2010).

Among pesticides penconazole ((*RS*)-1-[2-(2,4-dichlorophenyl)pentyl]-1*H*-1,2,4-triazole) and cyprodinil (4-cyclopropyl-6-methyl-*N*-phenylpyrimidin-2-amine), are fungicides widely used in the vineyards of the Mediterranean region. Penconazole is a systemic triazole fungicide used for the control of powdery mildew, scab and other pathogenic ascomycetes, basidiomycetes and deuteromycetes. It acts by interfering with sterol biosynthesis (Gough *et al.* 2009). Cyprodinil is a systemic anilinopyrimidine fungicide which is used for the control of various fungal rots in vineyards. It acts through the inhibition of methionine biosynthesis (Cabras *et al.* 1997).

Several studies so far have investigated the effects of pesticides on the population and function of soil nitrifying microbes using enumeration techniques and biochemical tests (Wainwright and Pugh, 1973; Gaur and Mishra 1977; Gopal et al., 2007) which provide an incomplete view considering that only 1-5% of microorganisms could be cultivated in known growth media (Torvsik and Ovreas 2002). The introduction of novel molecular tools has significant advanced our knowledge of the ecology and function of nitrifying microorganisms. Even then, only little is still known regarding the impact of

pesticides on the diversity and function of ammonia-oxidizing microbes. Li et al., (2008) showed via PCR-DGGE that the herbicide acetochlor induced significant and persistent changes in the community of AOB at concentrations > 50 mg kg^{-1}. Similarly, Chang et al. (2001) showed via competitive PCR and DGGE that the application of an herbicide mixture at concentrations above 100 mg kg^{-1} significantly altered the structure and the numbers of AOB resulting in soil accumulation of NH_4. Recently, Hernández et al., (2011) demonstrated via DGGE that the herbicide simazine significantly impaired potential nitrification and altered the community structure of AOB. These studies have identified effects of herbicides on nitrifiers at application rates which were 5 to 10x higher than the recommended dose rate thus creating a rather unrealistic exposure scheme. Therefore there is a clear need for studies looking at the effects of pesticides on the diversity and function of AOA and AOB at realistic exposure schemes (recommended dose rates). Considering that all the above studies looked into the response of soil nitrifying communities, there is also a need for data regarding possible side-effects of pesticides on nitrifying microorganisms residing in the litter soil cover, a microbial habitat serving important functions.

The present work was thus employed with the aim to elucidate the impact of the fungicides penconazole and cyprodynil, on the structure and function of AOB and AOA in a litter soil cover. An integrated approach involving chemical analyses, nitrification measurements and DGGE fingerprinting was adopted to identify possible links between fungicides dissipation, potential nitrification activity and AOB/AOA diversity. In order to distinguish between the total and the active community of AOB and AOA, DGGE fingerprinting analyses were carried out at both DNA and RNA level.

Materials and Methods

Litter layer, soil and microcosms

Uncontaminated grass, vine branches and leaves were collected in July 2009 from an abandoned vineyard in Northern Italy (Oltrepò Pavese). The absence of fungicides residues for litter materials was verified by HPLC analyses. Upon collection, plant material was mechanically chopped to smaller particles (length ≤ 2 cm) and homogenized by a mixer. The final composition of the litter layer was ryegrass (*Lolium perenne*): vine branches and leaves 90:10 w:w. Litter had 21.0 and 1.1% total organic carbon and nitrogen content, respectively, and a C/N ratio of 19.1. The soil used in the current study was collected from the same site as plant materials. The soil was passed through a 2 mm mesh sieve and its moisture was maintained at 60% of its water holding capacity. The soil used was a sandy clay loam (29% clay, 27% silt, 43% sand), with 2.1% total organic carbon, 0.81% total nitrogen and a C/N ratio of 2.6.

Nine soil-litter microcosms were prepared. Each microcosm consisted of plastic boxes with a surface of 0.176 m^2 which were filled with 900 g of soil. On top of the soil 200 g of litter were added in order to obtain a litter layer of about 2 cm depth. The litter-soil microcosms were covered with a wet filter paper to equilibrate for a period of 3 weeks. During this period the litter layer was maintained moist by daily additions of water in the filter paper

Microcosm study set-up

The litter layer in the first three microcoms was treated with an aqueous solution of penconazole (Topas 100 g L^{-1}), while the other three microcosms

153 received a treatment with cyprodinil (Chorus 500 g kg^{-1}). Volumes of water used
154 and final concentrations of penconazole and cyprodinil reflected the rates
155 commonly used in the field. Final concentrations in the litter layers were
156 respectively 1.32 mg kg^{-1} for penconazole and 24.6 mg kg^{-1} for cyprodinil. The
157 last three litter microcosms received the same amount of water (375 mL kg^{-1} of
158 litter) without fungicides to be used as non-treated controls. All microcosms
159 were kept at constant moisture throughout the experiment by adding water
160 three times a week regular additions of water, and they were placed in a
161 phytotrone at 26 ºC and 65% air humidity. Immediately after pesticide
162 application (0 days) and 7, 21, 56 and 100 days later litter subsamples (25 g)
163 were carefully removed from each microcosm, avoiding the soil beneath, and
164 stored at -20 C° until processed for pesticide residue and molecular analyses.
165
166 *Pesticide residue analyses*
167 Pesticide extraction from litter samples was performed based on a previous
168 method proposed for soil (Sicbaldi *et al.* 1997). In brief, five grams of litter were
169 thawed at room temperature for 15 min. Six grams of diatom earth was added to
170 the sample and the mixture was homogenized. The mixture was placed in a
171 vertical column with glass-wool filtering plugs at each end, left at room
172 temperature for 10 minutes, washed with 50 ml dichloromethane and the
173 filtrates were collected. The washing step was repeated and the filtrates from
174 both washing steps were combined and dried in a rotary vacuum evaporator at
175 30 °C. Residues were re-suspended in 10 ml of dichloromethane and were
176 chromatographically analyzed. High Performance Liquid Chromatography
177 (HPLC) determination of penconazole and cyrpodinil, was performed as

178 described in detail by the method of Suciu and Capri (2009) for penconazole,
179 here extended also for cyprodinil. Fungicides were analyzed using an HPLC
180 Agilent HP1100 Series, equipped with a Phenomenex C18 110A column (100 mm
181 x 4.60 mm i.d., 3 μm) and a diode array detector. Elution was performed with a
182 linear gradient. Concentrations of penconazole and cyprodinil were quantified
183 from peak areas following linear interpolation of standards at increasing
184 concentrations between 0.02 and 10 mg L^{-1} for penconazole and 0.02 and 40 mg
185 L^{-1} for cyprodinil. The limit of detection for both analytes was 20 μg kg^{-1} of litter.
186 Recoveries for penconazole and cyprodinil with the given method were 94 and
187 97% respectively.
188
189 *Potential nitrification assay*
190 The potential nitrification in the litter samples was determined according to
191 Kandeler (1995) with a slight modification. In brief, 2 g of litter were placed in an
192 Erlenmeyr flask with 8 ml of 1 mM $(NH_4)_2SO_4$ and 0.04 ml 1.5 M $NaClO_3$. The
193 mixture was incubated for 5 h in a shaking incubator at 26°C at 100 rpm. As no-
194 incubation controls, flasks containing soil, $(NH_4)_2SO_4$ and $NaClO_3$ were directly
195 stored at -20°C until the next step. 2 ml of 2M KCl were added in all flasks. The
196 content was briefly mixed and filtered. An aliquot of the filtrates (2 ml) was
197 mixed with 1.2 ml of 0.19 M NH_4Cl solution, pH 8.5 and 0.8 ml of color reagent
198 (*N-(1-naphtyl)-ethylenediamine hydrochloride* in phosphoric acid solution) and
199 were incubated at room temperature for 15 min. 'Non-filtrated' controls
200 containing the color reagent were incubated as well. The absorbance of samples
201 and 'no–incubation' controls were compared to the non-filtrated controls at 520
202 nm.

DNA/RNA extraction and purification, reverse transcription and PCR-DGGE analysis.

DNA and RNA extraction from litter samples were performed with the PowerSoil™ DNA and PowerSoil™ RNA Isolation kits (MoBio Laboratories, Carlsbad, CA, USA), respectively with a slight modification for the RNA isolation protocol. The modification referred to the addition of EDTA at pH 8.0 to a final concentration of 0.5 M right after the SDS-containing reagent and prior to vortexing to enhance cell lysis.

DNA purity was checked spectrophotometrically (260/280 nm and 260/230 nm ratios), while DNA and RNA extracts were also checked for shearing and degradation by electrophoresis on 0.8% and 1% agarose gels respectively. DNA and RNA were quantified using the Quant-iT™ (HS dsDNA Assay and RNA Assay kits respectively, Invitrogen, Paisley, UK) in combination with the Qubit™ fluorometer (Invitrogen, Paisley, UK).

RNA was further purfied with the RNeasy® Mini Kit (Qiagen, Valencia, CA, USA) coupled with the on Column DNase digestion using the RNase free DNAse set (Qiagen, Valencia, CA, USA). Previous analyses conducted on selected samples (data not shown) showed negligible DNA residues after the DNAse treatment. Reverse transcription of RNA to cDNA was performed with the iScript™cDNA synthesis kit of BioRad (BioRad Laboratories, Hercules, CA, USA) according to the manufacturer instructions.

PCR-DGGE analyses at the DNA level were carried out on the β-proteobacterial *amoA* and the crenarchaeal *amoA* gene, while at transcript level (cDNA) the crenarchaeal *amoA* gene and the β-proteobacterial 16S rRNA were

used as microbial 'markers'. The respective primer sets used for PCR amplification were the following: amoA1F/2R T (Rotthauwe *et al.* 1997), the CrenamoA 23f/616r (Tourna *et al.* 2008) and the CTO 189fABC/654r (Kowalchuk *et al.* 1997). The amoA1F/2R T and the CTO189ABC/654r primer sets contained a 40 bp GC clamp at the 5' end of their forward primer as previously described by Muyzer et al. (1993). PCR amplification was carried out in 50 µl reaction mixtures using the AmpliTaq® DNA polymerase with buffer I kit (Applied Biosystems, Foster City, CA, USA). 20 ng of template were added in each mixture containing 1× PCR buffer, 2.5 mM $MgCl_2$, 2.5 U Taq polymerase, 0.4 mM dNTPs, 0.5 µM of each primer and ddH_2O. The thermocycling conditions used for the amplification of the *amoA* and the 16S rRNA gene of β-proteobacteria were 94°C for 5 min, 35 cycles of 94°C for 30 sec denaturation, 54°C for 30 sec annealing, 72°C for 30 sec extension and a final extension step of 72°C for 10 min, while an extension step of 1 min instead of 30 sec was used for the amplification of the *amoA* gene of Crenarcheota

DGGE was carried out using the INGENY phorU electrophoresis apparatus (Ingeny International BV, Goes, The Netherlands). Polyacrylamide gels (6%) in 1xTAE buffer (40 mM Tris base, 20 mM acetic acid and 1mM disodium EDTA, pH 8.2) were prepared with denaturating gradient ranging from 15% to 55% for AOA *amoA* gene , 50% to 65% o for the AOB *amoA* gene and 38% to 50% for the AOB 16S rRNA gene. The electrophoresis was run for 16 h at 60°C and 75 Volts. Gels were SYBR Green stained and image analysis was carried out with the Cross Checker software (Buntjer 1999) for generating genotype presence-absence (binary) matrices (Inc. 1995). DGGE analyses were carried out at all five

sampling dates (i.e., 0, 7, 21, 56 and 100) for cDNA fingerprints, but only at 0, 56 and 100 days for DNA fingerprints.

Statistical analyses

Chemical data on fungicide recovery and potential nitrification data were analyzed by ANOVA coupled to Tukey's test for comparison of means (PROC GLM) (Inc. 1995). Both time and fungicide effects were assessed on potential nitrification data, while on chemical data only the time effect was tested.

The binary data matrix obtained from DGGE profiling were subjected to multivariate statistical analysis in order to compare the effect of pesticide application and time on the structure of AOB and AOA communities at both DNA and RNA level. Thus Principal coordinate analysis (PCoA) with a Jaccard similarity matrix was applied to the binary dataset generated from the DGGE banding patterns to decrease dimensionality. Subsequently, the first six components of the PCoA were subjected to canonical variate analysis (CVA). This was done since the first two principal coordinates of the PCoA represented, in all cases, a low percentage of the overall variation of the (< 40%). This statistical approach has been used before in microbial ecology studies in order to identify subtle differences that other statistical approaches fail to discriminate (Mc Caig et al., 2001; Karpouzas et al., 2009). All statistical analyses were performed using Genstat 11.0v.

Results

Potential nitrification and fungicides recovery in litter

Potential nitrification remained constant throughout experimental duration in the control microcosms, whereas a time effect was found in the penconazole and cyprodinil microcosms where a significant increase was evident at day 56 compared to day 0, i.e., immediately after fungicides applications (Table 1). Regarding pesticide effects, both penconazole and cyprodinil significantly impaired potential nitrification shortly after application. Nitrification recovery was however fast, reaching values not statistically different from the control at day 7 for cyprodinil and at day 21 for penconazole.

The dissipation of penconazole and cyprodinil was determined and the data are presented as % of the initial recovery at day 0 comparatively to the potential nitrification (presented as% relative to the controls; values equal to 100 indicate the levels of potential nitrification in the controls) (Figure 1). Penconazole dissipation showed an initial lag phase for the first 21 days when no significant degradation was seen (Figure 1a). This period coincided with a significant inhibition of potential nitrification at levels around 50% of the controls. Dissipation of penconazole proceeded faster from day 21 onwards reaching to 22% of the initial dose at the final sampling date. This is again in accordance with the recovery of potential nitrification to levels similar with the control at day 56 onwards. Regarding cyprodinil, a faster dissipation was observed within the first week of incubation with its levels reaching 64.4% of the applied dose by day 7. Thereafter the degradation of cyprodinil was slow with *ca*. 42% of the initial dose being still present at the end of the experiment (Figure 1b). The rapid degradation of cyprodinil at the first 7 days coincided with the recovery of potential nitrification at levels similar to the control from day 7 onwards.

Effects of fungicides on nitrifiers microbial populations at DNA level

The impact of fungicides on the structure of the nitrifying microbial community was assessed via PCR-DGGE. Complex fingerprints for both microbial groups were obtained (data not shown) and multivariate statistical analysis was employed to assess the effects of pesticides and sampling time on their community structure. Canonical variate analysis of the fingerprints of the AOB community showed a clear separation of samples according to sampling time along CV1 with all samples collected at 0 clustering together and away from the respective samples collected at 56 and 100 days (Figure 2a). No separation according to pesticide treatment was observed overall and separately within each sampling time.

Canonical variate analysis of the DNA-based fingerprints of the AOA community showed that all samples collected at day 0, regardless of pesticide treatment, were clearly separated from the respective 100-d samples along CV1 and 2 (Figure 2b). Regarding pesticide treatments, samples treated with penconazole and cyprodynil were clearly separated from the control samples at 0 days and at 56 d, while pesticide-treated samples collected 100 days were not separated from the respective control samples.

Effects of fungicides on nitrifier microbial populations at RNA level

The effect of fungicides on the community of AOB and AOA in the litter layer was also studied at RNA level via PCR-DGGE. The crenarchaeal *amoA* gene was used as a functional marker for AOA, while the 16S rRNA gene was used for AOB. Repeated attempts to obtain transcripts of the *amoA* gene of AOB from the litter

samples were not successful and for this reason the 16S rRNA gene was used instead. Analyses were carried out at all five sampling times of the experiment (day 0, 7, 21, 56 and 100), to better correlate the functional diversity of nitrifiers at transcriptional level with potential nitrification and fungicide dissipation.

DGGE fingerprinting analysis of the different samples provided rather complex communities for both AOB and AOA (data not shown). CV analysis for AOB transcripts fingerprints showed that samples were clearly separated according to the sampling time along CV1 (Figure 3a). In particular, samples collected at 0 and 7 days clustered close and away from the samples collected at 56 and 100 days. Regarding the effects of pesticides, penconazole-treated samples were clearly separated from the control samples at days 21, 56 and 100. In contrast, the samples treated with cyprodinil were grouped away from the corresponding control samples only at day 100 (Figure 3a)

Regarding crenarchaeal *amoA* transcript DGGE fingerprints, CVA showed a clear time-dependent effect, where the samples obtained at day 0, regardless of pesticide treatment, clustered together and were separated along CV1 from the samples obtained at 56 and 100 days (Figure 3b). Regarding pesticide treatments, penconazole-treated samples were clearly separated from the controls samples at 7, 21 and 56 days. On the other hand, cyprodinil-treated samples were clearly separated from the control samples along CV1 at 7 and 56 days (Figure 3b).

Discussion

The present work assessed the potential impact of two fungicides commonly used in vineyards on the function and diversity of nitrifying microorganisms

residing on a litter soil layer. The latter has been identified as a micro-environment of growing importance and ecological significance (Hattenschwiler *et al.* 2005) in sustainable vineyard production (Jacometti *et al.* 2008). Nitrification was chosen as a functional endpoint because it is a key process in N cycling and the maintenance of soil fertility (Van Beelen and Doelman 1997), and a wide range of molecular markers are available for studying the ecology of the microbial guilds involved (Rotthauwe *et al.* 1997; Mertens *et al.* 2009) and it is considered as a reliable indicator of the ecotoxicological risk of chemical agents on soil microbial quality (ISO 1997) because of its sensitivity to a wide range of compounds including polycyclic aromatic hydrocarbons (Sverdrup *et al.* 2002) and polychlorinated biphenyls (Duöek 1995). In order to gain a complete picture of the mechanisms involved in possible inhibitory effects of pesticides, a multidisciplinary approach was followed including temporal measurements of pesticide dissipation, microbial function (potential nitrification), and the diversity of total and active nitrifying community.

The dissipation of the fungicides in the litter was moderate to slow with substantial amounts still present in the litter after 100 days. Although no studies have looked before the dissipation of these pesticides in litter, our data are in line with the dissipation of penconazole and cyprodinil in soils (Dec *et al.* 1997; Singh and Dureja 2009) and organic biomixtures (Fait *et al.* 2007; Coppola *et al.* 2011). The dissipation of the two fungicides was inversely related to potential nitrification. Thus immediately after pesticide application, when the higher concentrations were recovered, potential nitrification was significantly impaired (Table 1, Figure 1). The lack of dissipation of penconazole within the first 21 days coincided with a significantly reduced potential nitrification in the litter. On

376 the other hand, the initially rapid dissipation of cyprodinil in the first 7 days
377 resulted in a rapid recovery of potential nitrification to levels similar to the
378 control. Overall, the correlation between pesticide dissipation and potential
379 nitrification suggests that the inhibition of nitrification in litter is a transient
380 phenomenon which is clearly related to the persistence of the two fungicides.

381 The impact of fungicides on the diversity of the nitrifying microbial guilds
382 (AOB and AOA) was also studied. In order to distinguish between total and active
383 microbial communities, and to assess the transcriptional patterns possibly
384 involved in the response of nitrifiers to the fungicides, PCR-DGGE analyses were
385 conducted on both DNA and RNA extracted from the litter microcosms at
386 different sampling times. AOB and AOA communities were clearly affected by the
387 two fungicides, although differences between DNA and RNA data were evident.
388 Regarding AOB, DNA-based fingerprinting analysis showed that the application
389 of pesticide did not induce any significant alterations in their community
390 structure. Similarly Omirou et al., (2011) found by DNA-based fingerprinting
391 analysis that biofumigation and chemical fumigation did not substantially alter
392 the structure of the AOB community. In contrast, Li et al. (2008) used DNA-based
393 DGGE analysis to demonstrate that the herbicide acetochlor induced persistent
394 changes in the structure of the AOB community in soil at concentrations ≥50 mg
395 kg^{-1}. However, these pesticide levels are substantially higher than the
396 concentration expected in the topsoil after application of the recommended dose
397 of acetochlor. The results obtained by DNA-based fingerprinting analysis of AOB
398 in our study were not confirmed by RNA-based fingerprinting analysis. Thus the
399 application of penconazole induced significant alterations in their community
400 structure which became visible after the first 21 days and persisted until the end

of the study. In contrast, cyprodinil induced a late significant change in the community structure of AOB which appeared at the final sampling day (100 d).

AOA were more responsive to pesticide applications compared to AOB and this was evident at both DNA and RNA level. Indeed, DNA-based fingerprinting analysis showed that penconazole induced immediate changes in the structure of the AOA community which persisted for 56 days at both DNA and RNA level. In agreement with the AOB data mentioned above, cyprodinil appear to be milder in its effect on the community structure of AOA with significant structural changes observed only at 7 and 56 days,in the RNA-based fingerprints. The structure of the AOA community in the pesticide treated samples reverted to profiles similar to the controls by the end of the experiment (100 d), in contrast with the AOB community where pesticide-mediated structural changes persisted at the RNA level until the final sampling day. Our findings are not in agreement with recent reports by Hernandes et al. (2011) who showed that the herbicide simazine at concentrations 5x higher than the recommended dose induced substantial changes in the structure of the soil AOB community, whereas soil AOA were not affected. In a similar study, Crouzet et al., (2009) also showed that application of the herbicide mesotrione at 100x the recommended dose induced substantial changes in the AOB community while AOA were more resilient to structural perturbations. The difference in the sensitivity of AOA to pesticide exposure between our study and the other two cited studies could be related to the nature of the pesticides tested (herbicides vs fungicides), the artificially high exposure regime selected in the later studies and the difference in the matrices studied (soil vs litter) which might support nitrifying communities with different resilience in exogenous stressors.

Alternatively, the inclusion of an RNA-based fingerprinting analysis of the nitrifying microbial communities in the other two studies might have also identified possible effects for AOA which were ignored by DNA-based fingerprinting. Overall, our data indicate that penconazole application was more detrimental to both microbial guilds compared to cyprodinil whose effects were temporal. This is in agreement with the slower degradation of penconazole at the first 3 weeks after its application compared to cyprodinil.

RNA-based analysis increased resolution and identified structural changes which were not visible at DNA level. This was particularly evident for AOB where DNA analysis did not reveal any structural changes throughout the experimental duration for the fungicides tested, in contrast to RNA-based analysis which revealed a clear impact of penconazole and cyprodinil at selected days. In accordance with our findings, Mahmood and Prosser (2006) also reported that RNA-based DGGE fingerprinting indicated earlier, more reproducible and finer scale qualitative shifts in AOB communities than DNA-based analysis. Time-dependent changes in the structure of the AOB and AOA communities were clearly observed at both DNA and RNA level, with substantial changes being observed between 7 and 56 days after application in all cases.

The temporal patterns of pesticide-mediated structural changes induced in the community of AOB and identified via RNA-based analysis do not comply with the functional data. Indeed, the structural changes induced by penconazole were first observed at day 21 and were maintained until 100 days, compared to the inhibition of potential nitrification in the penconazole-treated samples which was limited to the first 21 days. Regarding cyprodinil the instantaneous inhibition of potential nitrificaton observed immediately after pesticide

451 application was not in compliance with the structural changes in the AOB
452 community which were observed 100 days later. Discrepancies between
453 potential nitrification and structural changes were observed also for AOA;
454 penconazole application induced changes in the structure of AOA community
455 which appeared immediately or 7 days later at DNA and RNA level respectively
456 and persisted for 56 days, compared to the temporal nature of the inhibition in
457 potential nitrification by penconazole. Similarly, cyprodinil-mediated changes in
458 the structure of the AOA community appeared at 0 and 7 days in the DNA- and
459 RNA-based profiles respectively, and persisted for 56 days. Our findings are in
460 line with previous results by Crouzet et al. (2009) who showed that application
461 of the herbicide mesotrione at 100x the recommended dose resulted in delayed
462 structural changes in the AOB community (42 days) compared to a temporal
463 inhibition of potential nitrification which lasted for only 6 days. Overall, our
464 results indicate that changes induced in the function of nitrifying microbial
465 communities appear concurrently (AOA) or earlier (AOB) than structural
466 changes but persist for longer than the functional changes. Since both fungicides
467 were still persisting in the litter at the end of the experiment, it can also be
468 hypothesized that the restoration of nitrification activity is the results of a
469 different modulation of expression (cDNA data) in similar communities (DNA
470 data) aimed at withstanding residual toxic effects of the fungicides. The earlier
471 restoration of nitrification compared to structural changes can also be related to
472 the well demonstrated redundancy of microbial communities involved in non
473 specialized microbial functions in disturbed environments (Allison and Martiny
474 2008).
475

Conclusions

The impact of two fungicides on the function and diversity of AOB and AOA in a litter soil cover was investigated in a microcosm study. Our findings indicate that the application of penconazole and cyprodinil at their recommended doses induced a temporal inhibition of nitrification whose restoration clearly coincided with the dissipation of the fungicides in the litter soil cover. Given the increasing use of vegetated strips in vineyards, and the common use of these two fungicides, this result should be taken into account in sustainable viticulture.

Fingerprinting analysis at both DNA and RNA level showed several interesting findings a) RNA-based analysis provided a more in-depth view of the impact of pesticides on microbial guilds and should be preferred in studies looking at the effects of stressors on soil microbial guilds on a temporal basis; b) Penconazole showed a more consistent impact on the structure of both microbial guilds; c) AOA community was more responsive to fungicides compared to AOB, and d) pesticide-mediated structural changes in the nitrifying microbial communities appear later (AOB) or coincided (AOA) with the initial but temporal inhibition of nitrification, however structural changes persisted for longer despite the restoration of nitrification. The latter finding provides evidence that the microbial redundancy of key microbial functions like nitrification is operative not only in soil but in litter stressed environments.

Acknowledgements

This study was carried out within the GEBEP project sponsored by Cariplo Foundation, Italy.

All authors contributed equally to the paper.

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597
598

599 Table 1. Potential nitrification (µg NO_2-N g^{-1} 5 h^{-1}) in litter microcosms treated or
600 not treated with fungicides 0, 7, 21, 56 and 100 days after application. Each value
601 is the mean of three replicates ± the standard deviation.

Table 1. Potential nitrification (µg NO_2-N g^{-1} 5 h^{-1}) in litter microcosms treated or not treated with fungicides 0, 7, 21, 56 and 100 days after application. Each value is the mean of three replicates ± the standard deviation.

days	0	7	21	56	100	*F* value
Control	1349.1 ± 358 aA	1553.2 ± 503 aA	1120.6 ± 300 aA	1425.1 ± 438 aA	1316.3 ± 466 aA	0.43 ^{ns}
Penconazole	637.8 ± 189 bB	553.9 ± 170 bB	687.4 ± 245 aB	1641.0 ± 437 aA	844.5 ± 414 aA	6.02 **
Cyprodinil	764.8 ± 119 bC	1467.6 ± 110 aAB	1260.4 ± 172 aBC	2035.6 ± 337 aA	1425.8 ± 353 aAB	10.61 **
F value	8.29 **	9.39 *	4.48 ^{ns}	1.74 ^{ns}	1.68 ^{ns}	

ANOVA significant differences are indicated by F values (*P < 0.05, **P < 0.01, ***P < 0.005, ^{ns} not significant) for comparisons between rows and columns. Data followed by the same lower case letter on each column or by the same capital letter on each row are not statistically different from each other (Tukey's test, P < 0.05).

602

603 Figure captions

604

605 Figure 1. Potential nitrification data, expressed as percentage relative to the
606 control (black squares), and fungicide recovery, expressed as percentage of the
607 applied dose (white squares), in penconazole (a) and cyprodinil (b)
608 contaminated litter microcosms.

609 Figure 2. Canonical variate analysis ordination of the DNA-based DGGE banding
610 patterns for the communities of AOB (a) and AOA (b) in triplicate litter samples
611 which were treated with the fungicides penconazole (●) and cyprodynil (▲) or
612 remained untreated (■) and collected immediately after (0 days; solid fill), 56
613 (no fill) and 100 days (grey fill) later. Circles around the treatments indicate 95%
614 confidence intervals

615 Figure 3. Canonical variate analysis ordination of the RNA-based DGGE banding
616 patterns for the communities of AOB (a) and AOA (b) in triplicate litter samples
617 which were treated with the fungicides penconazole (●) and cyprodynil (▲) or
618 remained untreated (■) and collected immediately after (0 days; black), 7
619 (blue), 21 (red), 56 (green) and 100 days (violet) later. Circles around the
620 treatments indicate 95% confidence intervals.

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626 Figure 1

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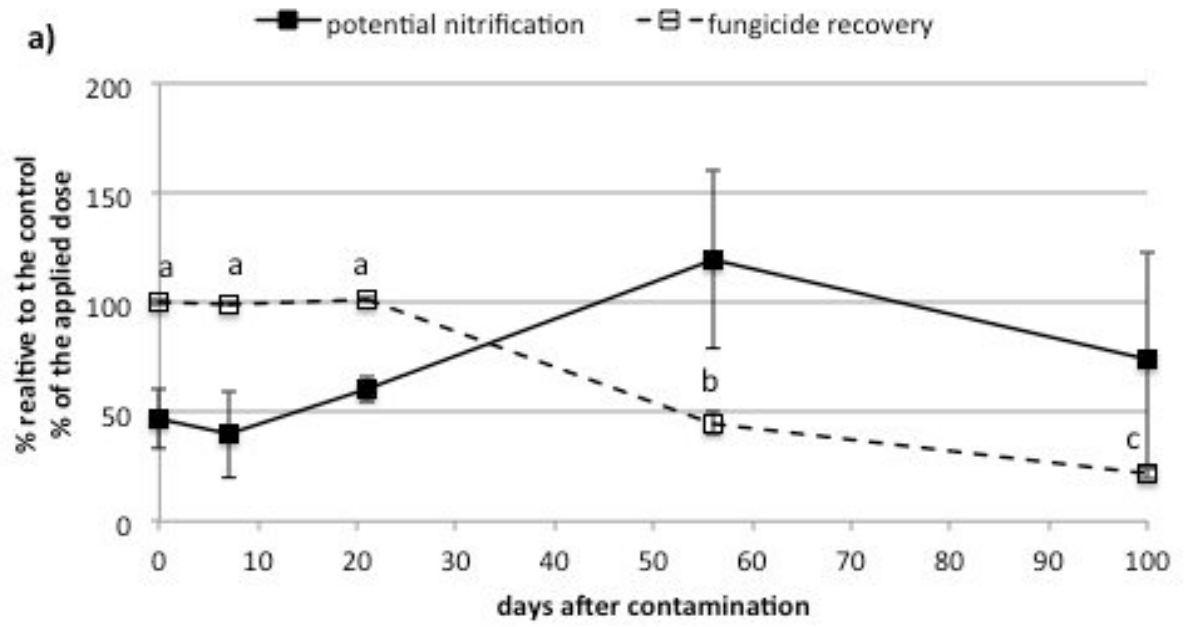

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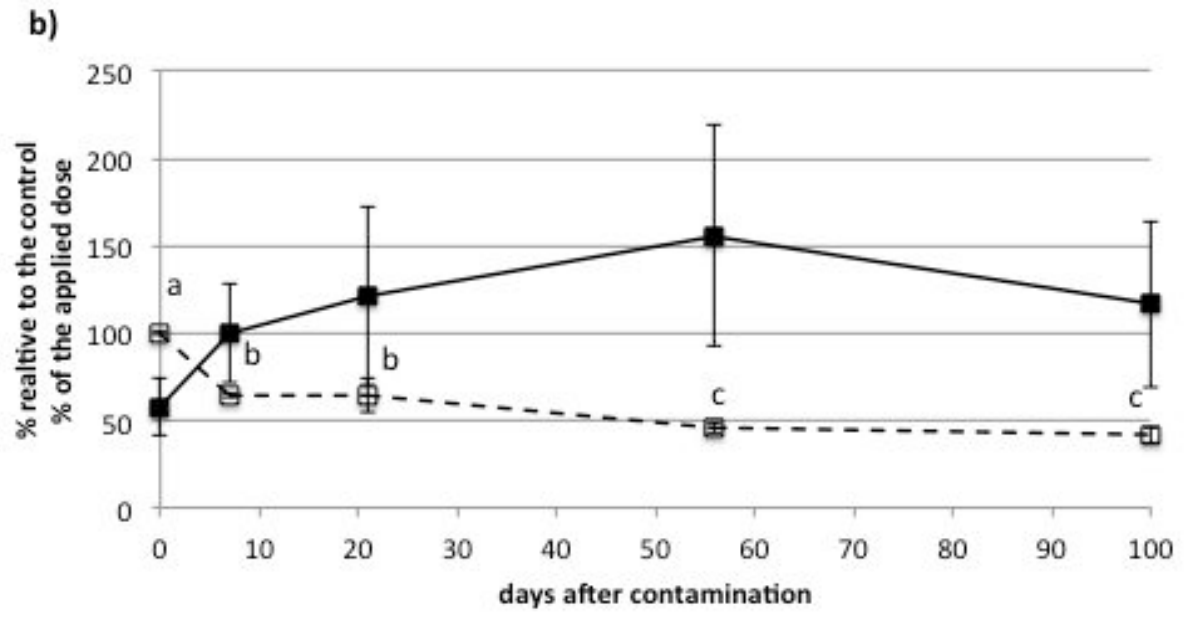

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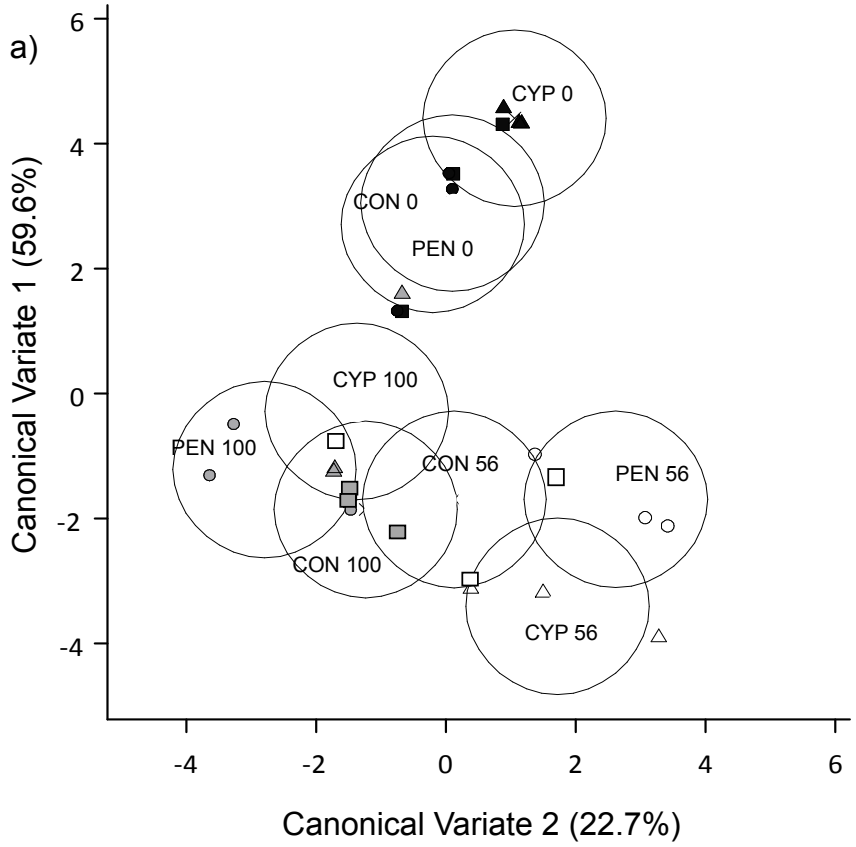
630 Figure 2



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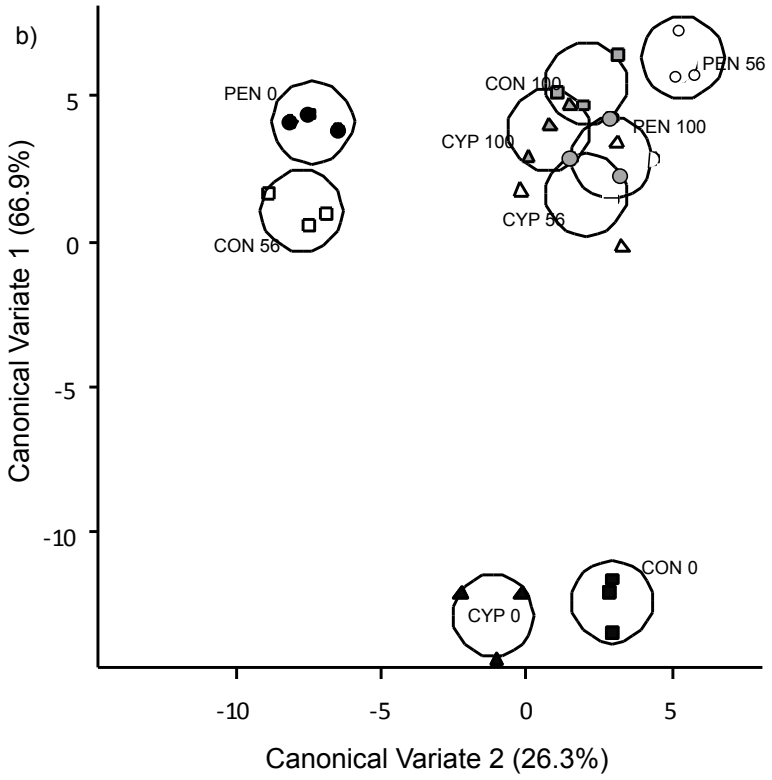

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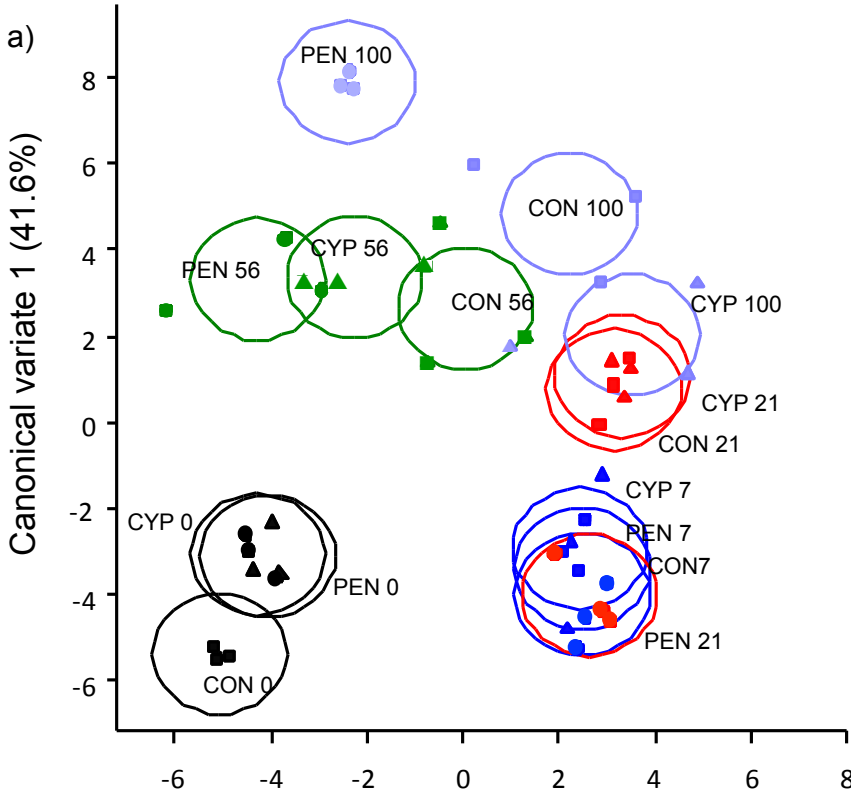
634 Figure 3



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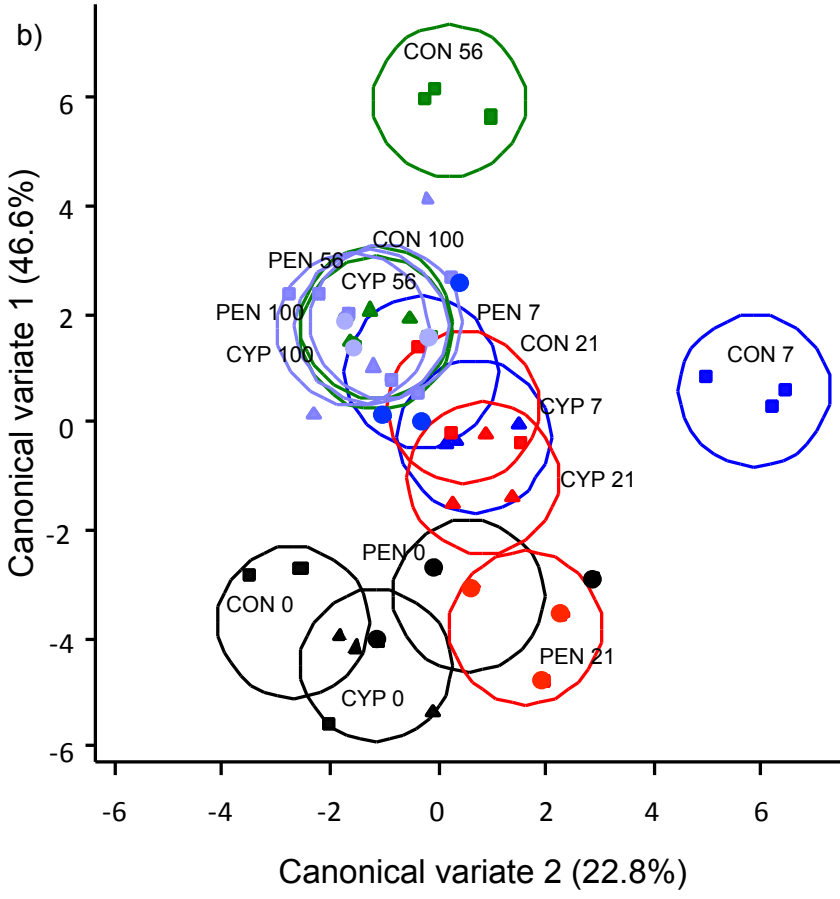

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General discussion on main study outcomes

Outcomes of the works included in the present thesis provided new insights concerning methodologies but above aspects of the ecology of the examined prokaryotic groups.

In ***Chapter 2*****,** an exploratory approach, useful for designing aspects of the experiment presented in ***Chapter 3*** was adopted. Unfortunately, not all outcomes of the exploratory study could be applied due to inexperience and limited access to materials and services. For example, access to sequencing services resulted in using the HiSeq 2000 technology (which restricts the screened fragment length to 100 bp) and along with the application of barcoding via ligation (Meyer *et al.* 2008, applied for avoiding potential PCR biases due to extended primer use) resulted in inability to perform assembly in most datasets and also obtain reduced in number and length useful sequence information. However, the study assisted to a great degree in gaining experience with data analysis and producing new insights about microbial ecology as shown in the results, discussion and conclusions of ***Chapter 3***.

Several skeptics doubt the utility of approaches like the one followed in ***Chapter 3*** (Baveye 2009). Our results showed that, besides being cost effective (Bartram *et al.* 2011) compared to e.g. multiple denaturant gradient gel electrophoresis (DGGE) runs, the obtained resolution is far superior than other considered up to now as high resolution technologies. Such example is the comparison of our study with the study of Peu *et al.* (2006), where using a PCR single strand conformation polymorphism (PCR-SSCP) approach, they failed to identify slurry encompassed microorganisms in soil right after slurry application. More insights

were apparent concerning the same microbial groups in our approach using Illumina technology. Differences identified in our experiment concerning slurry-associated taxa were clear between vicinal soils for both *Bacteria* and *Archaea* indicating that microbial carry-over through slurry application is quite probable. The implications of such technical issue have to do with falsely supporting a general perception that increase in the presence of some taxa after such organic amendments, is mainly related to organic carbon mediated priming of indigenous microorganisms (Blagodatskaya and Kuzyakov 2008) without taking into account potential carryover of fermenters via slurry incorporation.

Going beyond the methodological aspects, another important outcome of ***Chapter 3*** concerns the identified diversity differences between the studied vicinal more disturbed and less disturbed soils. These disturbances referred to the soil homogenizing seedbed preparations and cultivation approaches followed in the maize field soil as opposed to the stabile in soil structure with minimal human interference vicinal meadow soils. In several cases in the past, such an outcome was considered an artefact and was dealt with as such (Welbaum *et al.* 2004). However, when thinking from the perspective of the micro-scale ecology (Ranjard and Richaume 2001), disturbances like e.g. soil homogenization, result in dominance reduction and autochnonous microorganisms or dormant microbial forms have the opportunity to fill in generated gaps. Therefore although diversity in cultivated soils has been studied with various methods in the past this work demonstrates that there are still knowledge potentials that will allow a deeper understanding of microbial ecology.

In the second main part of the thesis, ***Chapter 4*** provided the opportunity to familiarize with stressors and potential strategies, experience used for the follow up work. In ***Chapter 5*** and ***Chapter 6*** where stress effects were examined for microbial ammonia(um) oxidizing *Bacteria* and *Archaea* (AOB and AOA), there was an interesting outcome regarding their ecology apart from the ones mentioned in the reports. Transcripts of AOB responded in a more consistent manner to potential nitrification measurements (PN) compared to AOA when soil used was poor in carbon. The opposite occurred when litter samples from soil litter interface were used. The increased transcriptional activity of AOA ammonia monoxigenase A subunit (*amoA*) encoding gene, found in high in organic content soil spots in other studies (Gärdenäs *et al.* 2011) along with our findings, further support a potential mixotrophy/heterotrophy by AOA.

Future perspectives

Collectively these results comprise a solid basis for follow-up research. In the experiment of ***Chapter 3***, RNA based analysis of 16S rDNA expression diversity will answer the far more elucidative question of "who is active?". This way "plasmatic" diversity due to potential microbial dormant forms (Jones and Lennon 2010) and thus prokaryotes contributing to the total community activity will be indicated. Furthermore, for a more complete characterization of microbial activity, metatranscriptomics profiles between samples can be compared. Past difficulties of rRNA interference with prokaryotic mRNA (lacking the polyA tail of *Eukaryotes*) have been greatly overcome (Stewart *et al.* 2010), and in combination with the constantly improving high throughput sequencing

technologies and sequence assembly algorithms (Namiki *et al.* 2011_*In_submission*), assembled meta-transcriptomes can be obtained.

Identifying lifestyle differences between AOB and AOA in complex soil environments is a complicated task. However, with the better characterization of the thaumarchaeotal group (Brochier-Armanet *et al.* 2008) taking currently place and designing of appropriate primers targeting 16S rDNA, it is possible to design experiments using soils which vary in organic matter content. Provided that it will be possible to design primers targeting the complete known thaumarchaotal group, the use of inhibitors of microbial growth and/or activity, will provide the desired answers.

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124

Europass Curriculum Vitae

Personal information

First name(s) / Surname(s)	**Sotirios Vasileiadis**
Address(es)	Via Christoforo Colombo 94, 29122 PC, Italy Iliados 2, 29100, Veroia, Greece
Telephone(s)	(Italy) Mobile: +393334321445 (Greece) Landline: +302331071102 Mobile: +306976833777
E-mail	vasiliad@gmail.com
Nationality	Greek
Date of birth	18th April 1977
Gender	male

Education

Dates	1st Nov 2008 – Currently (graduation date 24th Feb 2012)
Title of qualification **to be** awarded	**Doctorate (PhD)** in Agricultural and Environmental Chemistry Supported by living and fee expences coverage **scholarship (~45,000 € for 3 years / oral interview based)** by the Universitá Cattolica del Sacro Cuore PhD school (AGRISYSTEM)
Principal subjects/occupational skills covered	Doctorate Thesis title: "Reflections of ecosystem services on agricultural soil prokaryotic phylogenetic and functional diversity" **Scientific projects** participated(-ing) and time periods: a) Synthetic and Natural Agrochemical Compounds – ecological impacts on the soil system and effects on plant production (SNAC, Sep 2011 – currently); b) Gene expression in Bacteria Exposed to Pollutants for monitoring and remediation of contaminated sites (GEBEP, Nov 2008 – Mar 2011) Addressed research topics: total prokaryotic diversity, diversity of lignin and cellulose degrading fungal genes and exploration of related molecular markers, effects of abiotic stressors on the ammonia oxidizing prokaryotic comminity in soil
Name and type of organisation providing education and training	Universitá Cattolica del Sacro Cuore – Via Parmense 84, Piacenza, 29122 PC, Italy
Level in national or international classification	European Doctorate degree

Dates	1st Sep 2006 – 31st Aug 2008
Title of qualification awarded	**Master of Science** (MSc - Ing) in Organic Farming with specialization in Biocontrol Agents Supported with living and fee expences coverage **scholarship (~21,000 € for 2 years / written examination based)** by the Greek States Scholarship foundation
Principal subjects/occupational skills covered	MSc Thesis title: "Effects of *Pseudomonas fluorescens* strain SS101 and the cyclic lipopeptide massetolide A on plant growth"
Name and type of organisation providing education and training	Wageningen University – Forum building no. 102, Droeventaalsesteeg 2, 6708 PB, Wageningen, The Netherlands
Level in national or international classification	International Postgraduate degree

Dates	1st Sep 1996 – 31st March 2003
Title of qualification awarded	**Diploma** in Plant Production with specialization in Pomology, Horticulture and Viticulture
Principal subjects/occupational skills covered	Diploma thesis: "Sustainability in greenhouse production"
Name and type of organisation providing education and training	Aristotle University of Thesaloniki, Faculty of Agriculture – Aristotle University Campus, Building ID no 19, 54124, Thessaloniki, Greece
Level in national or international classification	International University Graduate (min 5-year diploma studies – ***MSc equivalent***)

Training

Academic

Dates	15th Aug 2009 – 17th Aug 2009
Type of training and "title"	Summer School "Bioinformatics and Comparative Genomics"
Principal subjects/occupational skills covered	General statistical concepts related to evolutionary models, sequence alignment and genome assembly, the "Hidden Markov Models", comparative genomics software tools, phylogeny reconstruction, codon models and positive selection in protein evolution, phylogenomics, protein function prediction
Name and type of organisation providing education and training	Organized by the Scottish Bioinformatics Forum and hosted in the University of Saint Andrews, St Andrews, Scotland

Non academic

Type of training: subject (duration-period, provider, location)	International standards: EN ISO 14001:2004 (5 days – May 2006, EUROCERT seminars, Thessaloniki Greece), EN ISO 9001:2000 (3 days – April 2006, PHYSIOLOGIKE ltd, Alexandria, Imathia, Greece), EN ISO 22000 (3 days – April 2006, EU.C.A.T. seminars, Thessaloniki, Greece) Software: Microsoft Windows and Microsoft Office (100 hours – October 2004, Prefecture of Imathia training project, Veroia, Greece)

Work experience

Dates	1st Dec 2011 – 23rd Dec 2011
Occupation or position held	Research project assistant
Main activities and responsibilities	Tasks carried out within the frame of the project entitled "Synthetic and Natural Agrochemical Compounds: ecological impacts on the soil system and effects on plant production (SNAC)": Protocol preparation for experimental setup, high throughput sequencing of the prokaryotic small ribosomal subunit gene marker data analysis protocol preparation, reporting on protocols. Porject supervised by researcher Edoardo Puglisi[1] (http://docenti.unicatt.it/ita/edoardo_puglisi/) and Professor Marco Trevisan[2] (http://istituti.unicatt.it/chimica_agraria_e_ambientale)
Name and address of employer	Departments of Microbiology[1] and Agricultural and Environmental Sciences[2] of the faculty of Agricultural Sciences of Universitá Cattolica del Sacro Cuore (Via Parmense 84, 29122, Piacenza, Italy)
Type of business or sector	**Academia - Research**

Dates	1st Aug 2007 – 30th Nov 2007
Occupation or position held	MSc Research internship – research assistant
Main activities and responsibilities	Exploration of potentials and protocol development for high throughput screening heterologous expression of the prokaryotic small ribosomal subunit in large insert metagenomic libraries using Fluorescent *In Situ* Hybridization (FISH). Porject supervised by the currently Assistant Professor of Plant Pathology of UC Davis Johan Leveau (California, USA, web page http://plantpathology.ucdavis.edu/faculty/leveau/)
Name and address of employer	Netherlands Institute of Ecology of the Royal Academy of Arts and Sciences (NIOO-KNAW), Droevendaalsesteeg 10, 6708 PB Wageningen, The Netherlands
Type of business or sector	**Academia - Research**

Dates	15th Oct 2005 – 4th Aug 2006
Occupation or position held	Organic Farming Inspector
Main activities and responsibilities	Assessment of compliance of farmers and traders of organic products with the related european and greek legislation for acquiring and maintaining the right to produce and trade organic products. Reporting to the greek Ministry of Agriculture.
Name and address of employer	PHYSIOLOGIKE ltd. (GR-BIO-02), Ethnikis Antistasis 66, 59300, Alexandreia (Imathia, Greece).
Type of business or sector	**Private Sector ltd. company (Organic Farming Certification Body)**

Dates	1st Jul 2005 – 1st Sep 2005
Occupation or position held	Plant production quality controler
Main activities and responsibilities	Quality assessment of industrial use (canned) peach production.
Name and address of employer	Visiting addr: AL.M.ME. Cooperative Trust, Kouloura, Imathia Postal addr: P.O. BOX 51, 59100, Veroia (Greece).
Type of business or sector	**Plant products industry (Cooperative trust)**

Dates	1st Oct 2004 – 31st May 2005
Occupation or position held	Estimator of damages caused to farming capital due to natural disasters
Main activities and responsibilities	*In situ* evaluation of capital loss and reporting to the national Greek Agricultural Insurances department of the Greek Ministry of Agriculture.
Name and address of employer	Greek Agricultural Insurances department of the Greek Ministry of Agriculture, Athens (Greece)
Type of business or sector	**Public Sector (Agricultural Insurances)**

Personal skills and competences

Languages

Mother tongue(s) **Greek**

Other language(s)

Self-assessment	Understanding				Speaking				Writing	
European level (*)	Listening		Reading		Spoken interaction		Spoken production			
English	C2	I have no difficulty in understanding any kind of spoken language, whether live or broadcast, even when delivered at fast native speed, provided. I have some time to get familiar with the accent.	C2	I can read with ease virtually all forms of the written language, including abstract, structurally or linguistically complex texts such as manuals, specialised articles and literary works.	C2	I can take part effortlessly in any conversation or discussion and have a good familiarity with idiomatic expressions and colloquialisms. I can express myself fluently and convey finer shades of meaning precisely. If I do have a problem I can backtrack and restructure around the difficulty so smoothly that other people are hardly aware of it.	C2	I can present a clear, smoothly-flowing description or argument in a style appropriate to the context and with an effective logical structure which helps the recipient to notice and remember significant points.	C2	I can write clear, smoothly-flowing text in an appropriate style. I can write complex letters, reports or articles which present a case with an effective logical structure which helps the recipient to notice and remember significant points. I can write summaries and reviews of professional or literary works.
Italian	A2	I can understand phrases and the highest frequency vocabulary related to areas of most immediate personal relevance (e.g. very basic personal and family information, shopping, local area, employment). I can catch the main point in short, clear, simple messages and announcements.	B1	I can understand texts that consist mainly of high frequency everyday or job-related language. I can understand the description of events, feelings and wishes in personal letters.	A2	I can communicate in simple and routine tasks requiring a simple and direct exchange of information on familiar topics and activities. I can handle very short social exchanges, even though I can't usually understand enough to keep the conversation going myself.	A2	I can use a series of phrases and sentences to describe in simple terms my family and other people, living conditions, my educational background and my present or most recent job.	A1	I can write a short, simple postcard, for example sending holiday greetings. I can fill in forms with personal details, for example entering my name, nationality and address on a hotel registration form.

(*) Common European Framework of Reference for Languages

Technical (scientific) skills

In vitro cultures of microorganisms and plants, microorganism isolation metthods, substrate induced enzymatic analyses, Fluorescent *In Situ* Hybridization (FISH), bright field / phase contrast / fluorescence microscopy methods, flow-cytometry parameters setup and data analysis, genomic and environmental DNA and RNA extraction and related quantitative-qualitative analyses methods, reverse transcription, cloning, PCR based analysis, quantitative real time PCR (qPCR), restriction digestion, DGGE, AFLP.

Computer skills

OS: Macintosh, Linux-GNOME, Windows
Statistics: SAS, R
Image: Image J, InkScape, Adobe Photoshop and Illustrator.
Scripting: Shell scripting
Bioinformatics: sequence manipulation and preliminary analysis (FASTX tools, GALAXY suite, EMBOSS), high thoughput sequencing population study suits (Mothur, CANGS, QIIME), primer designing tools (iCODEHOP, Primer Prospector, ecoPrimers-ecoPCR), alignment and evolutionary analysis software (e.g. ClustalW2, Muscle, Mafft, T-Coffee, RAxML, MrBayes), alignment viewing and evolutionary analyses GUI software (Jalview, MEGA 5, TOPALi, Bioedit), genome assembly tools (Velvet, Maq, ABySS), comparative genomics tools (ARTEMIS, MUMMER 3), T-RFLP data analysis (Ribosort R package) and protein prediction methods (BLAST, PSI-BLAST, reciprocal blast, inferential pattern based, motif based)

Publications

Articles/Review

1) Puglisi, E., R. E. Hamon, ***S. Vasileiadis***, D. Coppolecchia and M. Trevisan (2011). "Adaptation of soil microorganisms to trace element contamination: A review of mechanisms, methodologies and consequences for risk assessment and remediation." *Critical Reviews in Environmental Science and Technology* **In press**.

2) Coppolecchia, D., E. Puglisi, ***S. Vasileiadis***, N. Suciu, R. Hamon, G. Maria Beone and M. Trevisan (2011). "Relative sensitivity of different soil biological properties to zinc." *Soil Biology and Biochemistry* **43**: 1798-1807.

3) Moszczynska, A., ***S. Vasileiadis*** and M. Zanetti (2009). "Pesticide researchers face formidable challenges. Annual meeting report of the mediterranean group of pesticide research, piacenza, italy, 13 and 14 november 2008." *Trends in Analytical Chemistry* **28**: 135-140.

Oral presentation (presenter)

4) ***Vasileiadis, S.***, A. Balloi, F. Mapelli, D. Coppolecchia, E. Puglisi, D. Daffonchio, M. Trevisan and R. E. Hamon "Biochemical and molecular insights in the adaptation of soil microcosms to high zinc concentrations." *19th International Symposium in Environmental Biogeochemistry,* September 14-19, 2009, Hamburg, Germany.

Oral presentations (co-author)

5) Puglisi, E. and ***S. Vasileiadis*** "High-throughput sequencing approaches to elucidate prokaryotic diversity patterns." *International Conference on Soil Omics,* November 19-23 2011, Nanjing, China.

6) ***Vasileiadis, S.***, M. Arena, E. Puglisi, F. Cappa, P. S. Cocconcelli and M. Trevisan "Single hypervariable region usage for 16s rdna diversity screening of complex soil environments." *XXIX Convegno SICA,* September 21-23, 2011, Foggia, Italy.

7) Puglisi, E., ***S. Vasileiadis***, F. Cappa, M. Trevisan and P. S. Cocconcelli "Meta-genomic analysis of soil microbial communities in the “fontanili” (low-land springs) environments." *Soil Metagenomics 2010,* December 8-10, 2010, Branschweig, Germany

8) Puglisi, E., ***S. Vasileiadis***, C. Demiris, D. G. Karpouzas, E. Capri, P. S. Cocconcelli and M. Trevisan "Nitrifiers report on vineyard litter responses to fungicides." *Mediterranean Group of Pesticides Research (MGPR) 2010 Conference, Pesticides in the Mediterranean Area,* November 11-12, 2010, Catania, Italy.

9) ***Vasileiadis, S.***, A. Balloi, F. Mapelli, D. Coppolecchia, E. Puglisi, D. Daffonchio, M. Trevisan and R. E. Hamon "Short-term responses of ammonia oxidizers to increasing zn concentrations: A soil microcosm approach." *XXVIII Convegno Nazionale della Società Italiana di Chimica Agraria,* September 20-21, 2010, Piacenza, Italy.

10) Puglisi, E., ***S. Vasileiadis***, F. Cappa, Cocconcelli P. S. and M. Trevisan “Applicazione di techniche di sequenziamento, di nuova generazione per l’analisi metagenomica della biodiversitá del suolo." *XXVIII Convegno Nazionale della Società Italiana di Chimica Agraria,* September 20-21, 2010, Piacenza, Italy.

11) Coppolecchia, D., E. Puglisi, ***S. Vasileiadis***, N. A. Suciu, R. E. Hamon and M. Trevisan "Modelli dose-risposta per valutare l'ec50 di attivitá biologiche in un suolo contaminato con zinco." *XXVII Convegno Nazionale della Società Italiana di Chimica Agraria,* September 15-18, 2009, Matera, Italy.

12) Puglisi, E., D. Coppolecchia, A. Balloi, F. Mapelli, R. E. Hamon, ***S. Vasileiadis***, D. Daffonchio and M. Trevisan "Approfondimenti biochimici e molecolari dei mechanismi d'attamento del suolo ad alte concentrazioni di zinco." *XXVII Convegno Nazionale della Società Italiana di Chimica Agraria,* September 15-18, 2009, Matera, Italy.

13) Puglisi, E., R. E. Hamon, ***S. Vasileiadis***, D. Coppolecchia and M. Trevisan "Adaptation of soil microorganisms to trace element contamination: Mechanisms and consequences for risk assessment." *19th International Symposium in Environmental Biogeochemistry,* September 14-19 2009, Hamburg, Germany.

14) van de Mortel, J. E., ***S. Vasileiadis*** and J. M. Raaijmakers "Natural cyclic lipopeptide surfactants: Modes of action and effects on plant growth." *Xth Meeting of the Working Group: Biological control of fungal and bacterial plant pathogens,* September 9-12, 2008, Interlaken, Switzerland.

Posters

15) Arena, M., E. Puglisi***, S. Vasileiadis***, M. Zanetti, D. Spiewak, F. Cappa, P. S. Cocconcelli and M. Trevisan "Bioremediation of phenanthrene contaminated soil by pseudomonas veronii isolated from an alps glacier." *XXIX Convegno SICA,* September 21-23, 2011, Foggia, Italy.

16) Puglisi, E., ***S. Vasileiadis***, F. Cappa, M. Trevisan and P. S. Cocconcelli "Land-use management fingerprint on the soil microbial diversity. Fontanili: A case study." *FEMS Conference,* June 26-30, 2011, Geneve, Switzerland.

17) ***Vasileiadis, S.***, M. Arena, E. Puglisi, F. Cappa, M. Trevisan and P. S. Cocconcelli "V5 evaluation for single bacterial 16s rdna hypervariable region diversity based surveys of highly complex soil environments." *Bacterial Genomics and Ecology (BAGECO),* May 28 - June 2, 2011, Corfu, Greece.

18) ***Vasileiadis, S.***, A. Balloi, F. Mapelli, D. Coppolecchia, E. Puglisi, D. Daffonchio, M. Trevisan and R. E. Hamon "Acute responses of the soil ammonia oxidizers to zinc." *Structures and Processes of the Initial Ecosystem Development,* September 20-24, 2010, Cottbus, Germany.

19) ***Vasileiadis, S.***, E. Puglisi, D. G. Karpouzas, E. Capri, P. S. Cocconcelli and M. Trevisan "Structural and functional changes in nitrifying microbial communities during the degradation of fungicides in vineyard litter." *International Conference on Environmental Pollution and Clean Bio/Phytoremediation,* June 16-19, 2010, Pisa, Italy.

20) Puglisi, E., ***S. Vasileiadis***, D. Coppolecchia, R. E. Hamon and M. Trevisan "Correlating gene expression and enzymatic activities data: A case study of nitrification assessment in zinc contaminated soils." *FISV Conference,* September 23-25, 2009, Riva del Garda, Italy.

21) Coppolecchia, D., E. Puglisi, ***S. Vasileiadis***, N. A. Suciu, R. E. Hamon and M. Trevisan "Dose-reponse models to evaluate ecological doses (ec50) of biological activities in soils spiked with zinc." *International Symposium on Environmental Biogeochemistry (ISEB),* September 14-18, 2009, Hamburg, Germany.

Manuscripts

in preparation / under submission

1) ***Sotirios Vasileiadis***, Edoardo Puglisi, Maria C. Arena, Fabrizio Cappa, Pier S. Cocconcelli & Marco Trevisan. "Soil prokaryotic diversity patterns of ecosystem services."

2) Remy Hillekens, ***Sotirios Vasileiadis***, George A Kowalchuk, Eiko Kuramae. "Sequence based analysis of fungal degradation related molecular markers along a temporal gradient of organic matter decomposition in agricultural soils."

3) Edoardo Puglisi, ***Sotirios Vasileiadis***, Konstantinos Demiris , Daniela Bassi, Dimitrios Karpouzas, Ettore Capri, Pier S. Cocconcelli & Marco Trevisan. "Fungicides impact on the diversity and function of non-target ammonia oxidizing microorganisms residing in a litter soil cover."

4) ***Sotirios Vasileiadis***, Maria Arena, Edoardo Puglisi, Fabrizio Cappa, Marco Trevisan, Pier-Sandro Cocconcelli. "A theoretical approach for assessing practical aspects of soil bacterial diversity screening using Illumina technology."

5) ***Sotirios Vasileiadis***, Damiano Coppolecchia, Edoardo Puglisi, Annalisa Balloi, Francesca Mapelli, Rebecca E. Hamon, Daniele Daffonchio & Marco Trevisan. "Response of ammonia oxidizing bacteria and archaea to acute zinc stress and different humidity regimes in soil."

ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ
ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ
ΤΜΗΜΑ ΒΙΟΧΗΜΕΙΑΣ & ΒΙΟΤΕΧΝΟΛΟΓΙΑΣ

Tuesday, 13 December 2011

To:
PhD Coordinator
Prof. Romeo Astorri

With this letter I would like to comment, as an external expert, on the PhD Thesis of Mr. Sotirios Vasileiadis which was performed under the supervision of Prof. Marco Trevisan. I have through the thesis with great interest and I generally believe that is a well structure and written PhD Thesis report including all elements that a PhD Thesis should include: good theoretical knowledge of the subject (Chapters 1, 2 and 4) innovation and use of sound experimental approaches (Chapters 3, 5 and 6) and utilization of state-of-the-art techniques (Chapter 3).

Mr Vasileiadis appears to have well-established background knowledge of the complex mechanisms and interactions driving the structure and function of soil microbes. This has enabled him to succesfully plan experimental work, execute it using multidisciplinary approaches based on molecular biology, biochemistry, analytical chemistry, soil microbiology, analytical chemistry and finally interpret the results.

I generally believe that this thesis adds to our knowledge of microbial ecology and the effects of exogenous stressors on the function and the diversity of microbial communities. I have a few comments which I believe they should be corrected before the final and official submission of the thesis:

Chapter 1.

1. Page 2, L49: The old perception that *Nitrobacter* are the nitrite-oxidizing bacteria has been abolished since recent studies have indicated that in certain environments including terrestrial ones other bacterial genera like the *Nitrospira* are more ubiquitous and dominate over other nitrite oxidizers. So it is not correct to refer to just Nitrobacters…
2. Page 6, L135-137 and L149-151: Please rephrase they do not make sense these two sentences

Chapter 2. A very good example of application of bioinformatics as a tool for optimization of high-throughput sequencing techniques.

1. Page 12, L294-297: Does not make sense this sentence, please rephrase
2. Page 15, L359-363: I disagree that the sequence classification at taxonomical level 5. Please check and correct if needed
3. Figure 6. There is no title on y axis of this diagram. In general this diagram needs to be described better in the figure legend.

Chapter 3. A very good example of the application of newly introduced deep-sequencing techniques *in soil microbial ecology*

1. Page 2, L51-53: I am not sure that there are only a few studies in the literature looking the effects of land use and management of soil prokaryotic community structure. I think there are a lot of them using a wide variety of techniques

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2. Page 3, L79-82: I believe that a more thorough explanation of the main concept of the IDH theory is required. The way it is written at the moment (….looking at the diversity as a function of disturbance…) is not explanatory enough. In contrast the RHH theory is more thoroughly explained.
3. Page 6, L140: An explanation should be given on why acid instead of alkaline phosphatise was used as a marker since I noticed that the soils studied had a pH of netural to alkaline.
4. *Page 40, L862 and in other parts in the SI part: it should be Table S5 and not S4.* Similar mistakes in numbering of Tables and figures should be checked in the rest of the Supplementary information (e.g. Page 41, Lines 874, 876, 883, 887 etc)

Chapter 4. An excellent piece of review. I have no comments

Chapter 5. Well thought and executed study which stresses that the combined use of low resolution fingerprinting techniques, qPCR and functional measurements could provide a very convincing answer to the set experimental questions

1. Page 13, L316-322: I could not find the results about extractable Zn before and after leaching in the results section but I see them at the start of the discussion section. A mention in the results is needed
2. P14, L347-349: Why extracellular DNA degradation is not happening for AOA? Any explanation for this?

Chapter 6. The same comments made for Chapter 5 applies to Chapter 6 but with a different stressor this time. I have no comments

Chapter 7. Good concluding section.

1. Page 1, L21-25: Please avoid making comparisons about the resolution ability of two techniques based on different studies made on different soils under different conditions etc. It is incorrect.
2. Page 2, L34-36: A more specific statement should be made here regarding the differences in the diversity between adjacent disturbed and non-disturbed soils

Overall, I believe that this thesis is an excellent piece of work and it satisfies fully all the criteria of a well executed PhD study. Therefore I fully support the PhD candidacy of Mr Sotos Vasileiadis.

Yours Faithfully

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Italy

date December 15, 2011
our reference HvV/GG
your reference
subject Ph.D. thesis report of Sotirios Vasileiadis

Dear colleague,

The present document summarizes my overall conclusions concerning the Ph.D. thesis report of Sotirios Vasileiadis with title "Reflections of ecosystem services on agricultural soil prokaryotic phylogenetic and functional diversity". The thesis was performed under the overall supervision of Professor Marco Trevisan, director of the Institute of Agricultural and environmental Chemistry of the Faculty of Agricultural Sciences of Universitá Cattolica del Sacro Cuore.

I have read the thesis with great interest and, in general, I am impressed by its high quality! In particular the chapters 2 and 4 impressed me very much. Chapter 2 provides a highly relevant insight in the possibilities to extend the usage of the 16S rDNA molecule for the identification of microbial species using the most recently available sequencing methodology. It combines proper use of technological knowledge and mathematical approaches to assess the suitability of the different 16S rDNA regions for identification purposes. The review described in Chapter 4 provides a well-documented overview of the literature concerning the adaptation mechanisms of microbial cells and communities to stress caused by heavy metals. I was surprised by the use of the term "trace elements contamination" when dealing with heavy metal toxicity, but there is no reason to reject that terminology.

The other chapters deal with scientifically sound and relevant studies on the functional diversity of microbial communities in soil (Chapter3) and on the effects of pollutants, specifically heavy metals (Chapter 5) and fungicides (Chapter6) on the communities and activities of ammonium oxidizing microbes.

My suggestion for further improvement of the thesis and the individual chapters concerns issues related to the use (or better the lack) of clear and sharp definitions and statements. This holds in particular for Chapters 1 and 3. Examples of the complex and ambiguous statements used by the author are: "soon expected to become limiting for life phosphorus", " nitrates by the tightly associated to the better studied AOB in aggregate forms", "human activities plant production" (all at page 2),

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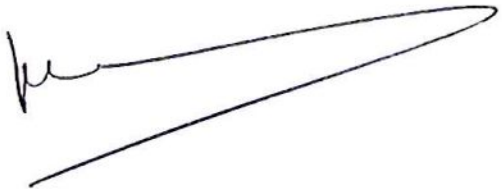
"all action taken concerning human activities" (page 4), "without even any necessity of knowledge priors" (page 6) . To my opinion this may be acceptable in a thesis but it will certainly be rejected for publishing in scientific journals.

In conclusion, the thesis provides sufficient evidence of the quality level of the candidate, which I regard to be sufficient to confer upon him the doctorate and to bestow on him the title of Doctor.



Dr J.A. van Veen,
Head of Department Microbial Ecology Netherlands Institute of Ecology, NIOO-KNAW
Professor of Microbial Ecology, Leiden University

Herewith I address my response and related changes performed according to the comments made by:

A) Assistant professor Dr Dimitrios G. Karpouzas

…

Chapter 1.

1. Page 2, L49: The old perception that *Nitrobacter* are the nitrite-oxidizing bacteria has been abolished since recent studies have indicated that in certain environments including terrestrial ones other bacterial genera like the *Nitrospira* are more ubiquitous and dominate over other nitrite oxidizers. So it is not correct to refer to just Nitrobacters…

   Done. The importance of *Nitrospira* and other taxonomical groups for nitrite oxidation has been underpinned by modification of the original statement and supported by referring to relevant literature.

2. Page 6, L135-137 and L149-151: Please rephrase they do not make sense these two sentences

   Done. The message of the sentence was divided into two small and clearer sentences.

Chapter 2. A very good example of application of bioinformatics as a tool for optimization of high-throughput sequencing techniques.

1. Page 12, L294-297: Does not make sense this sentence, please rephrase

   Done. The sentence was clarified and one more sentence was added to complete the comprehension gap.

2. Page 15, L359-363: I disagree that the sequence classification at taxonomical level 5. Please check and correct if needed

   The statement has been changed to “equal or above” instead of “above”, provided that the relative to the total classified sequence read percentages at level 5 were 70 % for V3, 71 % for V4 and 70 % for V5, while for the relative to the FL classified sequence reads the statement holds.

3. Figure 6. There is no title on y axis of this diagram. In general this diagram needs to be described better in the figure legend.

   Done.

Chapter 3. A very good example of the application of newly introduced deep-sequencing techniques in soil microbial ecology

1. Page 2, L51-53: I am not sure that there are only a few studies in the literature looking the effects of land use and management of soil prokaryotic community structure. I think there are a lot of them using a wide variety of techniques

   Indeed there is quite a lot of literature referring to land use and management. However, the methodologies followed do not provide as detailed outcomes as required by nowadays acknowledged existing soil diversity in terms of both presence-absence of genotypes and also relative abundance. The latter are well addressed in this study compared to previous studies and are the methodologically strong points of this work according to the authors' opinion. However, there is room for further improvement of the methodologies followed provided the experience obtained (e.g. in reducing PCR and other technical biases) in this study and they are going to be applied in future applications.

2. Page 3, L79-82: I believe that a more thorough explanation of the main concept of the IDH theory is required. The way it is written at the moment (….looking at the diversity as a function of disturbance…) is not explanatory enough. In contrast the RHH theory is more thoroughly explained.

   Done. An explanatory sentence was added to clarify the concept of the theory.

3. Page 6, L140: An explanation should be given on why acid instead of alkaline phosphatise was used as a marker since I noticed that the soils studied had a pH of netural to alkaline.

   This is a fair point made by the reviewer. However with pH values being close to neutral, expected differences between acid and alcaline phosphatase measurements in the performed correlation tests, might not be found or clear. This comment though is very helpful and will be considered in future experimental setups.

4. Page 40, L862 and in other parts in the SI part: it should be Table S5 and not S4. Similar mistakes in numbering of Tables and figures should be checked in the rest of the Supplementary information (e.g. Page 41, Lines 874, 876, 883, 887 etc)

   Done.

Chapter 4. An excellent piece of review. I have no comments

Chapter 5. Well thought and executed study which stresses that the combined use of low resolution fingerprinting techniques, qPCR and functional measurements could provide a very convincing answer to the set experimental questions

1. Page 13, L316-322: I could not find the results about extractable Zn before and after leaching in the results section but I see them at the start of the discussion section. A mention in the results is needed

   Done. A short result description has been added in the mentioned paragraph beginning, while the related reference (previously published study on the same soil taking place contemporarily and containing the full characterization of [Zn] in the soils examined) has been added in the results section and the materials and methods section.

2. P14, L347-349: Why extracellular DNA degradation is not happening for AOA? Any explanation for this?

   This might occur potentially due to lack of AOA death as a result of differences in anatomy and lifestyles. However no related viability test was carried out since it is quite difficult with contemporary means particularly for the examined groups.

Chapter 6. The same comments made for Chapter 5 applies to Chapter 6 but with a different stressor this time. I have no comments

Chapter 7. Good concluding section.

1. Page 1, L21-25: Please avoid making comparisons about the resolution ability of two techniques based on different studies made on different soils under different conditions etc. It is incorrect.

   Indeed the reviewer's comment is fair. For this reason this part was modified only towards emphasizing the superiority of the orders magnitude of analyzed sequence reads per sample by Illumina technology, instead of comparing the two technologies as failure to success.

2. Page 2, L34-36: A more specific statement should be made here regarding the differences in the diversity between adjacent disturbed and non-disturbed soils

   Done. An explanatory statement was added.

B) Professor Dr. Johannes A. van Veen

…
My suggestion for further improvement of the thesis and the individual chapters concerns the issues related to the use (or better the lack) of clear sharp definitions and statements. This holds in particular for Chapters 1 and 3. Examples of the complex and ambiguous statements used by the author are: "soon expected to become limiting for life phosphorous", "nitrates by the tightly associated to the better studied AOB in aggregate forms", "human activities plant production" (all at page 2), "all action taken concerning human activities" (page 4), "without even the necessity of knowledge priors" (page 6). To my opinion this may be acceptable in a thesis but it will certainly be rejected for publishing in scientific journals.
…

This is a fair comment and most probably it is attributed to my short experience with scientific writing in the English language. The expressions mentioned by the reviewer have either been altered and simplified or removed in case their contribution to the overall message was judged as reduced. However a more thorough revision of the thesis was not judged necessary, since it would require a much larger revision time-period and also according to the reviewer's opinion the expressions used are acceptable for the purpose served by the thesis.