Manuscript Details

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Title	ASSESSMENT OF SOIL MICROBIAL FUNCTIONAL DIVERSITY: LAND USE AND SOIL PROPERTIES AFFECT CLPP-MICRORESP AND ENZYMES RESPONSES
Article type	Research Paper

Abstract

The assessment of microbial functional diversity is an important indicator of soil quality. Different methodological approaches are currently used; among them are enzyme activities (EA) and CLPP (community level physiological profile) techniques (e.g. MicroRespTM, MR). The aims of the study were: i) to assess the efficacy of both methods in capturing differences among various land use categories when different levels of selected explanatory variables such as land use category, total organic carbon (TOC) and pH are considered, and ii) to explore, through a quantile regression approach, the possible relationships between each of the two methods with land use category, TOC and pH. The Shannon diversity index (H'), calculated from EA and MR data, was chosen as a synthetic index deriving from the same mathematical model. The quantile regression model (QRM), the Kruskal-Wallis and Spearman rank correlation tests were performed. Enzyme activities and MicroResp were reliable ecological indicators to assess soil microbial functional diversity. No correlation was found between the diversity indexes, H'EA and H'MR, it was therefore supposed that the two methods may target complementary components of microbial functional diversity. Both methods were effective in capturing differences among various land use categories, in particular H'MR in soils with low TOC content (<1.5%). Moreover, the QRM approach allowed a more detailed analysis along the distribution of the diversity indexes (H'EA and H'MR) indicating that H'EA was more dependent on the selected variables.

Keywords	Microbial processes; Shannon index; Soil properties.
Manuscript category	Soil microbial ecology

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Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:
We are able to provide some data upon request. However, since the work has been written analysing several different case studies, when these were not published, authorization needs to be asked

Viterbo October 30th, 2017

To the Editor of
Pedobiologia

Manuscript n. PEDOBI_2017_124

Dear Editor,

We wish to submit the revised article entitled "**ASSESSMENT OF SOIL MICROBIAL FUNCTIONAL DIVERSITY: LAND USE AND SOIL PROPERTIES AFFECT CLPP-MICRORESP AND ENZYMES RESPONSES"** modified following both yours and reviewers' comments.

The paper has been deeply revised in most of its sections in relation to the new results obtained after standardization of data asked by reviewer n. 1 and further amendments asked by reviewer #2.
Therefore also the discussion and conclusions were focused on the new aspects emerged.
For this reason we also agreed to change the title as you suggested**.**
We deeply thank you and the two anonymous reviewers for all the precious comments very useful to improve the whole paper.

We declare that we do not have conflicts of interest to disclose.
Please address all correspondence concerning this manuscript to me.
Thank you for your consideration of this manuscript.

Sincerely

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Comments from the editor and reviewers:

Editor

The paper has been deeply revised in most of its sections in relation to the new results obtained after standardization of data as asked by reviewer n. 1 and further amendments asked by reviewer #2. Therefore also the discussion and conclusions were focused on the new aspects emerged. For this reason we also agreed to change the title as you suggested into:

ASSESSMENT OF SOIL MICROBIAL FUNCTIONAL DIVERSITY: LAND USE AND SOIL PROPERTIES AFFECT CLPP-MICRORESP AND ENZYMES RESPONSES

-Reviewer 1

General comments

The authors perform a meta-analysis of functional diversity measurements (Shannon index) in a range of soils with different land uses and provide a useful comparison of two methodological approaches, which they suggest target different stages in the soil organic matter decomposition process. The values obtained for the index are greater than what I would expect and the calculation should be checked or explained. They apply Quantile Regression Modelling of the data, which they argue is useful for skewed distributions but more discussion is really needed on the meaning of the results. Some figures, or parts thereof, are duplicative and should be omitted. The authors' final conceptual model, while making a valid distinction between the two functional measurements, is rather over-simplified and some of the concepts are a bit strange. In places the English could be improved; some examples are given below but there are others which need some editing.

We thank reviewer 1 for the general comment. The manuscript has been deeply revised in many of its sections following his/her suggestions and those of reviewer #2.

Specific comments

L16 This first sentence is not actually a sentence; start with something like "Here we consider…".

Thanks! Actually there was a typing error and "as" was supposed to be "is".

L18 Replace "i.e." with "e.g.".

Done throughout the manuscript

L22 "and pH".

The sentence was changed

L52 I do not think this is entirely accurate. Catabolic processes continue all the time, generating maintenance energy – they become more apparent when energy for anabolic processes is lacking. Of course, certain types of stress may increase the demand for maintenance.

The sentence has been removed

L66 "pedogenic" is better than "pedogenetic".

Done

L92 Replace "cases" with "case".

Done

L93 Replace "On" with "For".

Done

L103 The period of reconditioning should be stated.

Done

L121 Macaulay Scientific Consulting Ltd has now been replaced by James Hutton Ltd.

Done

L129 It should be explained what the various symbols in the equation refer to and what were the values of the constants (or at least give a reference).

The conversion of absorbance to % CO_2 is a non linear relationship and the best fitted curve (regression analysis) is used to obtain the formula and parameters. A calibration procedure was performed taking into account the spectrophotometer used, different types of soils and incubation conditions. In our experimental conditions the constants of the following equation A+B/(1+ D x Ai) were: A:-1,62, B:-4,85 and D: -8,1. *(Campbell et al, 2003).* **See text.**

L135, 138 I have some difficulty in referring to the Shannon diversity index in terms of entropy. Admittedly Shannon's work on information theory had its roots in thermodynamics and there are parallels, but when applied to diversity within ecology it becomes a rather different concept. Increase in entropy is seen as negative (e.g. heat death of the universe), while increase in diversity is usually seen as something positive. In the context of the paper, I am not sure what "entropy of a system" and "microbial functions entropy" really mean (yes, I looked at Marinari et al. 2013 but it was not helpful and the reference Minasny et al. 2008 is really talking about the entropy in different mineralogies, with no biological component).

We agree that the increase of "entropy" of soil microbial functions means something positive, in any case, the term entropy can be also associated to the biodiversity in an ecological context as reported by Spellerberg and Fedor (2003). For this reason we added this reference in the text.

Moreover, the reference to Marinari et al. 2013 is related to the fact that microbial functions diversity is linked to the diversity of hydrolysable substrates. However, in that paper the concept of entropy was also related to pedogenesis that leads to a highest energy level when horizons differentiate and the spatial arrangement of soil particles (soil structure) becomes more defined.

L202 There are no lower outliers for SEI shown in Fig. 1 for any soil group. Also in these ranges, it is more conventional to go from low to high, e.g. 42 to 11821 (the latter should be written as 11800 as 11821 indicates unjustifiable accuracy).

We modified both the sentence concerning lower outliers and the order of values.

L206 Figure 2 and Table 2. I am having some difficulty in understanding the range of H' values. The maximum values, and even the mean values, exceed what I would calculate as the maximum possible. Assuming you have used the formula as given in L135, then the maximum value (total evenness) would be 2.708 for 15 'species' (carbon substrates) and 2.079 for 8 'species' (enzyme activities). The data in Table 2 is giving maximum values of 6.720 and 5.490, respectively. Or has the index been calculated in some other way?

We thank the reviewer for this observation which enabled us to identify some inaccuracies in index calculation. In the updated version of the paper we changed the values and reported descriptive synthesis values in Table 2.

LL214-216 I am not convinced that Figure 3 is required in addition to Figure 2. It is essentially the same data presented in two ways. Given that the same data is also summarised in Table 2, it does seem to be overkill. However, the contention that H'MR has greater variability is not sustained. To compare properly the two datasets should be standardized – normally by dividing by the mean. The interquartile distance then comes out as 0.24 and 0.25 for the H'EA and H'MR, respectively, – hardly a great difference.

We removed Figure 3 from the paper. In the revised version of the paper we used standardized measures for both indexes in order to deal with the issue of different ranges. This was specifically indicated in the M&M and Results section.

LL219-225 It is not necessary to repeat all the values given in Table 3 – omit the r_s values.

Done. Only p-values were left in the text.

L223 At this point there is a switch to sometimes using soil type in place of land use category; it is better to stick with the latter (See also Table 4).

Done throughout the whole manuscript

L233ff It is not clear (not being that familiar with quantile regression) what the significance of the constant is, particularly since it seems to be highly significant in all cases. I presume it is just the intercept on the regression but is it the same regardless of whether the regression is against TOC, pH or Soil type (Land Use)?

The interpretation of intercept in QRMs is quite similar to its interpretation in standard linear regression models (OLS) with the utmost importance to keep in mind a different intercept depending on the specific quantile that is being analysed.

L258ff It could be argued that because only 8 enzymes were assayed in comparison to 15 carbon substrates tested, that the enzyme approach in gauging diversity was necessarily more limited. Do you have a counter to this suggestion?

We agree that Shannon index is calculated using different numbers of enzymes and substrates (8 enzymes belonging to the 4 nutrients –C,N,P,S - biogeochemical cycles and 15 substrates representing four ecologically relevant categories of biomolecules – proteins, carbohydrates, organic acids and phenols). However, the aim of the study was to assess the efficacy of the two techniques, as they are generally used in the literature, to calculate a synthetic index aimed to capture differences among the different land uses when different levels of pH and TOC are considered. In particular we would like to stress that this study is a meta-analysis that has been conducted using data provided by previous researches performed. However, the comment is proper and we agree that to promote the use of both techniques in the same study, and to improve the interpretation of the obtained results, it should be suggested to select the same number of enzymes and substrates.

L280ff At the end of the day both enzyme activity (as assayed) and CLPP are both degradative, just that the former is one step back in the chain of events. One might have expected a greater degree of correlation in H' values. However, H' is only one way of expressing/summarising the data. What would have been the result had you looked at total activity (Figure 1) and made a comparison? Was this done or is it the subject of a separate study?

We thank the reviewer for this comment. The correlation between the functional capacity (total activity) measured according to the two methods (SEI and SIR reported in figure 1) showed a significant coefficient ($p<0.05$). We would like to stress that even if, the functional capacity measured by means of the two methods was correlated, the functional diversity was not. This means that, although the capability to perform functions by enzyme and respiration were positively related, their variability (diversity of functions) was not.

LL302-303. Is this statement true? Admittedly immobilized enzymes can have little impact on solid substrates such as cellulose and hemicellulose because of spatial separation, but once polymeric fragments are solubilised such enzymes can then come into contact with them. Their monomeric products then become available to the microbial community. Your tests with MUF substrates demonstrates that immobilized enzymes are still active against low molecular weight intermediates (if indeed this is the case).

Yes, the statement is true and we do not understand what the reviewer referred to. The sentence at lines 302-303 referred to the fact that, being enzymes also in the soil in the immobilized forms, these may not be directly expression of microbial activity, thus of microbial functional diversity. Immobilized enzymes represent a background biological activity giving resilience to soils under unfavourable conditions for microbial life. However, due to methodological limitations, we cannot subtract the immobilized enzymes contribution to the total activity measured in the laboratory with the current available methods.

LL311-315 There is not much discussion on the QRM results. Quite a lot of space is devoted to the methodology and results of the QRM so I was hoping for a bit more explanation as to what the ecological implications of the findings were.

We reduced the theoretical explanation of QRM consistently to what is required by Reviewer 2 as well. Moreover we added explanation concerning ecological implications of the results.

L315 Spelling of Zahlnina?

Corrected

Figure 3. The H'MR result does not need to be dashed – not done for other figures.

In accordance with your comments concerning Figure 3 (LL214-216) we removed this figure from the paper.

Figure 4. The two parts (figure and table) duplicate each other. The figure part should be omitted since all the information is in the table. It would probably aid clarity if numbers are given to three significant figures only (greater accuracy is unwarranted).

We deleted Figure 4; in the revised version of the paper only the table is reported, now table 4. Moreover, we considered three significant figures.

Table 1 L3 the abbreviation is "conv" not "con".

Done

Table 3 This table seems to be overly complicated. Why not 1X4 in place of 4X4, i.e. the four values in one row?

The table has been simplified as suggested

Table 4 TOC and Soil type are given as discrete variables whereas pH is given as a single (continuous?) variable, not as the ranges given in Figure 4 – this seems to be rather inconsistent. In actuality, for the purpose of these regressions, would it not be better to treat TOC as the continuous variable it is, rather than boxing it into these three categories?

We re-estimated QRM by considering pH in classes (as Table 5). The distinction of variables into classes enabled us to detect significant changes of the relationship with the dependent variables and within/across quantiles.

Reviewer 2

-

General Comments

Authors present an interesting study about the functional diversity of soil microbial activity in a wide range of soils submitted to different utilization. The variation of Shannon's Index of diversity for microbial functional diversity was studied by measuring the profiles both of different enzyme activities and micro-respiration of diverse C substrates. Also they determine the effect of pH and TOC of soil in this microbial functional diversity. Important conclusions are derived from the obtained results in this study. In general the manuscript is written in a good English language, although some specific parts of the text should be re-written in a clearly and simply way.

Specific comments

Abstract

L16. In this sentence, a verb is missing. Please re-write in a grammatically correct form.

The sentence was corrected, actually there was a typing error and "as" was supposed to be "is".

L22. Insert the conjunction "and" between the words "category" and "pH".

The sentence has been changed

L74. Correct publication year: 2002.

Done

Material and Methods

This section should be structured in several sub-sections, e.g.: Experimental design, soil sampling, soil analysis methodologies, statistical methods…

Done

L92. "Cases studies" should be write "case studies".

Done

What do you mean? Are these case studies treatments or conditions? Explain in a clearly way.

With the term "case study" we mean a particular investigation that included different treatments. For example: one of the 4 case studies included in the "forest soils category F" was related to different management practices in two adjacent soils, one under native forest and the other one under a recently coppiced forest. This was clarified in the text.

L95. Delete colon (:)

Deleted

L96. Include conjunction "and" between the words "afforestation" and "chronosequences".

Done

L98. Firstly, substitute conjunction "and" by comma and the include conjunction "and" between the words "tillage" and "natural".

Done

L100. Insert conjunction "and" between the words “paddies” and “highly”.

Done

L101. Explain better the case studies of EC category because only five case studies are distinguished.

There was a typing error. The case studies belonging the EC category are 6: three of them subjected to natural pedoclimatic conditions and the other three under heavy anthropic impact. It has been better specified in the text.

L103. You should specify conservation or store method of soil samples.

Conservation and treatment of soils from sampling to analyses has been detailed in the text.

L111. Why did you only analyze the acid-phosphatase activity? Alkaline phosphatase activity is a more important activity in alkaline soils.

We agree with this observation. However, in all case studies the same experimental set-up for enzymatic assays has been used and this allowed to perform this meta-analysis. Eight different enzymes on several soil samples were measured at the same time in the same microplate using a common buffer as that suggested by Marx et al. (2001) (NaAc pH 5,5). In this way the biondicator used responds to the requisites of providing fast results while processing a great amount of soils in a short time.

L129. If you include this equation you should explain what is every variable or parameter of it.

A calibration procedure was performed taking into account the spectrophotometer used, different types of soils and incubation conditions. In our experimental conditions the constants of the following equation A+B/(1+ D x Ai) were: A:-1,62, B:-4,85 and D: -8,1. *(Campbell et al, 2003).* **See text**

L131. Please explain SEI and SIR and how they are calculate or measured. Dumontet et al., 2001 is not a pertinent reference for specifically citation about Synthetic Enzymatic Index, because in this study this index is not introduced.

Explanation of calculation of SEI and SIR has been given in the M&M section. Dumontet et al., (2001) provided the suggestion of combining some enzyme activities leading to the same final product (e.g. pNP or MUF) as a synthetic index. However, we removed this citation since it is considered not pertinent.

L152. Specify these two distributions because Figure 1 represents SEI and SIR values and Figure 2 truly shows the distributions of H'EA and H'MR.

Modified. In the revised version of the paper we specified that this sentence refers to the two distributions illustrated in Figure 2.

L154. Re-write this sentence, clearly specifying the two explanatory variables.

The sentence has been clarified presenting the selected covariates in this study

L168-186. The explanation of the quantile regression model used in this study should be re-written in a clearly and simply way, without mathematical equations. Authors are not expected to write a statistical treatise in this part of the manuscript. Authors should explain what does it consist in and why do you use this particular regression?

This section has been re-written without mathematical equations and emphasizing the advantages of QRMs in soil analysis.

L192-197. In this paragraph, you should write statistical significance of the relationship. The correlation coefficient stablishes the relationship level between two variables.

We added the level of significance.

Results

L202. Delete “and lower”. Only upper outliers are shown in figure 1a

Modified

L201-204. Re-write this sentence in a clearly and simply way.

Modified, taking into consideration also suggestions by Reviewer 1.

L210. Delete “the null hypothesis of normal data is rejected for both distributions”. This part of the sentence is obvious and reiterative because it was previously stated that these two distributions were significantly different from normality.

Done

L217-218. Delete "lower" and "upper" because the outliers are shown under and above the whiskers.

We deleted box-plots taking also into consideration suggestions by Reviewer 1

L225. Authors should include p-values for these two cases.

Modified

L226. Delete "significantly" and after "distinguished" add "in different ways”.

Modified

L227. Substitute “However” by "Thus".

Modified

L239. Write "quantile" in singular.

Modified

L243. After "relationship", include "with the land use category A”.

Comments have been modified according to the new estimated models.

L245. “this land use category A”. Re-write such as: "the land use category A”

Comments have been modified according to the new estimated models.

L258-267. In this paragraph, authors should include an explanation how pH can affect microbial function diversity represented by H'MR.

Done

L324. Delete the preposition"at".

Done

LEGENDA:

Yellow marked text: modified text

Blu marked text: deleted text

Comments mark where new sentences have been added

EFFECTIVENESS OF ENZYME ACTIVITIES AND CLPP-MICRORESP AS INDICATORS OF MICROBIAL FUNCTIONAL DIVERSITY IN A WIDE RANGE OF SOILS

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Keywords: microbial processes, land-use, Shannon index, quantile regression model

Abstract

The assessment of microbial functional diversity, as an important indicator of soil quality. Different methodological approaches are currently used; among them are enzyme activities (EA) and CLPP (community level physiological profile) techniques (i.e. MicroResp™, MR). The aims of the study were: *i)* to assess the efficacy of both methods in capturing differences among various types of soils when different levels of selected explanatory variables such as Total Organic Carbon (TOC), land use and pH are considered, and *ii)* to explore, through a quantile regression approach, the possible relationships between each of the two methods with TOC, land use category, pH. TOC and pH were chosen as explanatory variables influencing microbial functional diversity. The Shannon diversity index (H'), calculated from EA and MR data, was chosen as a synthetic index deriving from the same mathematical model. The quantile regression model (QRM), the Kruskal-Wallis and Spearman rank correlation tests were performed. The QRM and Kruskal-Wallis tests evidenced that MicroResp technique generally provided a higher discrimination capacity within different land use categories, TOC and pH ranges (TOC <0.15-8.41%; pH <4.02-9.01>). Soil pH was found to be a key property, rather than TOC content, in differentiating microbial processes. H'EA and H'MR were not correlated but, when analysed separately, only agricultural soils showed a weak correlation ($P<0.1$) probably due to the fact that these soils features fall within the intermediate range of pH and TOC where both methods were found to be significantly sensitive. These results suggest that the two methodologies do not target the same microbial processes. We hypothesize that the two methodologies refer to sequential steps of microbial activity. In fact, pointing to complementary components of microbial functional diversity EA and MR provide a different ecological significance which may inform on the extent of dissipating energy pathways in the soil system.

Introduction

The links between ecosystem functioning and levels of soil biodiversity have been the focus of the recent scientific literature (Delgado-Baquerizo et al. 2016; Griffiths et al. 2016; Nannipieri et al. 2003). The first authors provided evidence that loss in microbial diversity will likely reduce multiple ecosystems functions thus negatively impacting the provision of ecosystem services. Adhikari & Hartemink (2016) claimed for new insights into soil microbial diversity and their role in soil functional variability. Since up to 80/90% of soil functions, from humification to mineralization, is microbially-mediated, the diversification of soil microrganisms in terms of structure and/or activity is essential to maintain functioning of terrestrial ecosystems (Pereira et al. 2013).

Microbial functional diversity is defined as “the sum of the ecological processes, and/or capacity to use different substrates developed by the organisms of a community” (Insam et al. 1989). Emmerling et al. (2002) and Wellington et al. (2003) report that if microbial genetic diversity assesses a latent diversity, which may not be expressed, functional diversity is related to the actual activities resulting from that potential so that "functional rather than taxonomic diversity may provide greater insight to microbial roles in ecosystems" (Zak et al. 1994). In fact, under stress or unfavourable conditions, microorganisms may switch from anabolic pathways to catabolic pathways (Anderson and Domsch 2010). In this case the soil turns into a dissipating energy system with enhanced energy demand.

Over the last 10 years, the scientific literature provided a great number of papers aimed to assess microbial functional diversity as an important ecological indicator to monitor and assess soil quality changes in different pedoclimatic conditions, land uses and human pressure levels (i.e. management practices)(Bardgett and van der Putten, 2014; Griffiths et al., 2016).

To measure the activity and diversity of the microbial community a number of methods can be applied, to cite few of the most common approaches: (i) catabolic activity investigated by Biolog™-plates (Garland and Mills, 1991; Rutgers et al. 2016), (ii) respiration of different substrates as investigated by the MicroResp™ method (Campbell et al. 2003; Chapman et al. 2007; Creamer et al. 2016) and (iii) enzyme activities (Nannipieri et al. 2012; Hendriksen et al. 2016).

Although all methodological approaches are reliable and sensitive, few studies aimed to understand their effectiveness to discriminate microbial functional diversity in relation to soil organic C and pH as the main properties being affected by land use and management practices, anthropic impact and other pedogenetic factors. To achieve this goal, a large number of case studies covering different types of soils is necessary. In this study, about 200 measurements of microbial functional diversity obtained over a broad spectrum of key soil properties and across different land uses and management, were selected. Furthermore, microbial functional diversity obtained through enzyme activities (EA) and CLPP-MicroResp (MR), was synthetically represented by the Shannon index (H') that transforms the obtained results to a comparable range of values deriving from the same mathematical model. The Shannon index is a comprehensive indicator of microbial species, individual numbers and evenness, or distribution of the enzyme activities and is influenced by richness of community species (Bending et al. 2004; Li et al., 2007).

The aim of the present study was therefore to: i) assess the efficacy of both methods in capturing differences among the different land use categories when different levels of pH and TOC are considered, *ii)* explore, through a quantile regression approach, the possible relationships between each of the two methods and selected explanatory variables (TOC, land use category, pH). Furthermore, the results of these analyses could help to assign an ecological significance to both methods in various environmental contexts and research issues.

Materials and methods

The results presented in this paper have been obtained performing additional statistical analyses on data collected in the Laboratory of Chemistry and Biochemistry, University of Tuscia, Viterbo, Italy during the last 6 years (2010-2016). Microbial functional diversity was measured, by means of enzyme activities and CLPP-MicroResp^{TM} technique, in a wide range of soils analysed within different research projects. Most of the sampling sites are located within the Mediterranean climatic area. Other climatic areas are the monsoon one for the Bangladesh case study, the temperate one for

96 Switzerland, oceanic for United Kingdom and boreal for Sweden. All soils represent a broad
97 spectrum of key soil properties across different land use categories, wide range of soil pH and soil
98 organic carbon content (TOC) (Table 1).
99 The soils were grouped into three main categories, including 15 cases studies, with the aim to
100 separate diverse land uses and/or specific conditions. On this purpose, three groups were identified: F
101 (forest soils, 4 case studies), A (agricultural soils, 5 case studies) and EC (extreme conditions, 6 case
102 studies). The case studies performed on forest soils (F) included different: management practices,
103 lithological substrates, afforestation, chronosequences. The soils under agricultural land use (A) were
104 characterized by different managements and/or agricultural practices such as: organic, biodynamic
105 and conventional cropping systems, tillage/no tillage, natural green cover/no cover. The third
106 category (EC) included soils with peculiar characteristics due to pedoclimatic conditions (saline
107 environments, natural arsenic contamination in rice paddies, highly calcareous soils) or heavy
108 anthropic impact (a multi-element contaminated dump, arsenic contaminated mine)(Table 1).
109
110 All soils were sampled at 0-20 cm depth during the dry season (spring/summer), air dried, sieved at 2
111 mesh and re-conditioned at 60% of their water holding capacity prior to biochemical analyses.
112 The total organic carbon (TOC) was determined by combustion by Shimadzu TOC VCSH analyzer
113 while soil pH was measured on sieved soil suspended in a solution of deionised water in 1:2.5 ratio
114 (w/v). The pH was measured in the supernatant with a pH meter (pH 211, Hanna Instruments).
115 A total of 196 values of microbial functional diversity, assessed by means of enzyme activities and
116 CLPP-MicroResp, were used for this study (Table 1). Enzymes were measured following Marx et al.
117 (2001) using fluorogenic methylumbelliferyl (MUF)-substrates. Soils were analysed for
118 cellobiohydrolase, β-1,4-glucosidase, α-1,4-glucosidase, β-N-acetyl-glucosaminidase, β-1,4-
119 xylosidase, acid-phosphatase, arylsulphatases and butyrate esterase which is considered a proxy of
120 endocellular activity (Wittman et al. 2004). The relative fluorogenic substrates, prepared with acetate
121 buffer 0.5 M pH 5.5, were: 4-MUF-β-D-cellobioside, 4-MUF-β-D-glucoside, 4-MUF-N-acetyl-β-

Commented [1]: Headings included

glucosaminide, 4-MUF-α-D-glucoside, 4-MUF-phosphate, 4-MUF-7-β-D-xyloside, 4-MUF-sulphate and 4-MUF-butyrate. Fluorescence (excitation 360 nm, emission 450 nm) was measured with an automatic fluorimetric plate-reader (Fluoroskan Ascent) and readings were performed after 0, 30, 60, 120 and 180 minutes of incubation at 30° C. The results were expressed as nmoles of product (MUF) of each enzymatic reaction released per g of soil per unit of time in relation to a standard curve prepared with increasing MUF concentrations and incubated at the same experimental conditions.

The community level physiological profile (CLPP) was determined using the MicroResp™ soil respiration system (MicroResp™, Macaulay Scientific Consulting Ltd, Aberdeen, UK) according to Campbell et al. (2003).

The 15 substrates selected in this study were: α-D-glucose, D-Galactose, D-fructose, L-arabinose, L-leucine, L-arginine, Glycine, L-aspartic acid, γ-amino-butyric and glutamic acid, three carboxylic acids: citric acid, oxalic acid and L-ascorbic acid, and two phenolic acids: vanillic and syringic acid. The emission of CO_2 by the microbial biomass was estimated using a colorimetric method (microplate spectrophotometer) before and after 6 h of incubation at 28 °C. The absorbance was read at 595nm. At the end the absorbance was normalised for any difference recorded at time zero and then converted to % CO_2 using the calibration curve y = A+B/(1+ D x Ai). The CO_2% was converted to µg C-CO_2 g^{-1} h^{-1} production rate using gas constant, T° C, headspace volume, soil dry weight (d.w.) and incubation time. The SEI (Synthetic Enzymatic Index, Dumontet et al, 2001) and SIR (Substrate Induced Respiration) for all soils within the three categories (F, A and EC) have been calculated as a synthetic measure of microbial functional capacity.

Microbial functional diversity was assessed calculating the Shannon-Weaver diversity index (Kennedy and Smith, 1995) corresponding to the entropy concept defined by: $H' = - \sum p_i * \ln p_i$ (Shannon and Weaver, 1949), where pi is the ratio of the activity of a particular enzyme to the sum of all enzymatic activities (H'EA) or the respiration rate of each single C-substrate for MicroResp™ (H'MR). Shannon diversity index is related to the entropy of a system and when applied as a measure of microbial functions entropy, may express the heterogeneity of soil organic substrates availability

Commented [2]: New sentence has been added as required

Commented [3]: New sentence added

Commented [4]: New ref added

and microbial processes (Marinari et al., 2013). Since the eight enzymes and the 15 substrates here tested did show activity in all the analysed samples, then, in this work, the diversity recorded reflects only the "evenness" or distribution of the enzyme activities or ability to use the different substrates (Bending et al., 2004; Rodríguez-Loinaz et al., 2008).

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The analysis of all collected data was carried out into three main steps. Firstly, descriptive analyses provided us with a clear picture of the distribution of the two indexes (H'EA and H'MR) as well as information about the shape of the two distributions. Moreover, rank correlation measures and test performed by using the Spearman correlation enabled us to evaluate if, and to what extent, the two methodological approaches (EA, enzyme activities and MR, MicroResp™) used to evaluate soil microbial functional diversity are related.

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The Kruskal-Wallis non-parametric test was used to test if and to what extent the two indexes distinguished the various land use categories in relation to TOC and pH ranges.

By considering the asymmetry of the two distributions (as shown in Figure 1 and Figure 2) as well as the results of the Shapiro-Wilk normality test, we analyzed the existence of association between each of the two measures and two explanatory variables, by estimating quantile regression models. Indeed, quantile regression offers the possibility to highlight how the effect of the selected covariates changes throughout the entire distribution of the dependent variable. To estimate the relationships (association) between the dependent variables and the set of selected covariates the classical OLS (Ordinary Least Squares) regressions can be applied. However, data obtained from experimental collection tend to be skewed so these models do not describe the "correct" relationships. Moreover, from the soil analysis perspective, it is interesting to understand what happens throughout the entire distribution of the two measures and at their extremes.

Quantile Regression Models (QRMs) are of special interest to studies characterized by skewed distributions. Indeed, these models allow for investigation of the potential different effect of a covariate on various quantiles in the conditional distribution, they are more robust to the presence of

outliers and can be consistent under weaker stochastic assumption than with least-squares estimation (Cameron and Trivedi, 2005). The application of these types of models to our analysis can help to understand if and to what extent the differences observed between the two measures can be attributed to the different effect played by the explanatory variables at the various quantiles. The QRM specifies the conditional quantile of the dependent variable y as a linear function of covariates (Koenker 2005):

$$Q_\theta\left(y_i \middle| \mathbf{x}_i\right) = \mathbf{x}_i'\boldsymbol{\beta}_\theta + \varepsilon_{i\theta} \quad (1)$$

where y_i (i=1,...,n) is the dependent variable represented, in turn, by, $\mathbf{x}_i$ is a sequence of k-vector of regressors, $\boldsymbol{\beta}_\theta$ is an unknown vector of regression parameters associated with the θ_{th} quantile and $\varepsilon_{i\theta}$ is an unknown error term. According to Koenker and Bassett (1978) who introduced QRMs the θ-th regression quantile, $0 < \theta < 1$, is defined as any solution to the minimization of the sum of absolute deviation residuals:

$$\min_{\beta \in R^k}\left\{\sum_{i:y_i \geq \mathbf{x}_i'\beta} \theta\left|y_i - \mathbf{x}_i'\boldsymbol{\beta}\right| + \sum_{i:y_i < \mathbf{x}_i'\beta} (1-\theta)\left|y_i - \mathbf{x}_i'\boldsymbol{\beta}\right|\right\} \quad (2)$$

which is solved by linear programming methods. When θ is continuously increased from 0 to 1, we obtain the entire conditional distribution of y conditional on $\mathbf{x}$. Starting from the general equation (1) and with the aim of identifying factors associated with values of the two measures estimated, two quantile regression models which assumed the dependent variable y_i (i=1,...,n) to be: (i) H'EA and (ii) H'MR respectively were estimated. In both models the k-dimensional vector $\mathbf{x}_i$ of covariates includes factors describing the land use category, pH and the level of TOC. Among the soil properties that mostly affect microbial biomass activity and diversity, TOC and pH were chosen as covariates to explain H' index variability (Fierer and Jackson, 2006; Constancias et al. 2015). In this

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196 study, the distribution of TOC values allowed to homogeneously group soils into three categories: i)
197 low: for TOC < 1.5 %; ii) medium TOC < 1.5 – 3>; iii) high: for TOC ≥3%. Similarly, pH values
198 allowed to group soils into three categories: i) <6.5 slightly acid – very strongly acid, ii) <6.5-7.4>
199 neutral, iii) >7.4 slightly alkaline – moderately alkaline. The grouping criteria were established with
200 the aim to obtain three groups with the same number of observations.
201 STATA software (STATA 13.2 edition) was used for statistical analyses. Three different levels of
202 significance were considered for the estimated coefficients and are reported in the model: a value of
203 $p<0.01$ (indicated in the tables of results with ***), emphasizing strong relationships between the
204 explicative variable of interest and the dependent variable; the value of $p<0.05$ (indicated in tables of
205 results with **) and finally a value of $p<0.10$ (indicated in the tables of results with *) emphasizing a
206 weak relationship between the variables.
207
208 **Results**
209 Figure 1 shows the functional capacity of soil microbial biomass calculated as the SEI and SIR for all
210 soils within the three categories (F, A and EC). Extreme conditions soils showed the highest level of
211 variability, including upper and lower outliers, for SEI (ranging from 11821 to 42 nmoles MUF g^{-1} h^{-1}),
212 while forest soils soils functional capacity was more variable for SIR (ranging from 177 to 0.9 μg
213 CO_2 g^{-1} h^{-1}). Agricultural soils show, for both methodological approaches, a smaller range of
214 variation and lower outliers.
215 Figure 2 shows the distribution of the two indexes values H'EA and H'MR, respectively, over the
216 196 values of microbial functional diversity.
217 The two distributions are positively skewed and leptokurtic - as emerged by the descriptive statistics
218 reported in Table 2 – and significantly different from normality as confirmed by the Shapiro-Wilk W
219 and Shapiro-Francia W′ test (the null hypothesis of normal data are rejected for both distributions, p-
220 value=0.000).

A similar level of overall variability (which also includes upper and lower outliers) characterizes the two distributions as described by the values of coefficient of variation (CV). On the other hand, by focusing on the box-plots in Figure 3, it should be noted that – while considering the different magnitude of the two indexes – the larger height of the rectangles highlights a greater level of variability in the middle part of the distribution (i.e. the central half of the sample) concerning H'MR index. The presence of values outside the whiskers (dots in Figure 3) identifies lower outliers for H'EA and upper outliers for H'MR index.

The Spearman rank correlation, verifying the similarity of the orderings of the data when ranked according to each of the measures, showed that the two measures are not related for measuring microbial functional diversity (r_s = - 0.0355; p-value = 0.6217) (Table 3).

However, by distinguishing rank correlation according to the land use category, we found a moderate level of concordance (r_s = 0.2213; p-value = 0.0656) when the two indexes refer to soil of type A. No correlation was found between the two measures for soil type EC and F (r_s = - 0.1410 and r_s = - 0.0579 respectively) (Table 3). According to the results of Kruskal-Wallis test, both H'EA and H'MR significantly distinguished the various soils when TOC or pH ranges were considered (Figure 4). However, H'MR showed a greater effectiveness than H'EA according to the p-values reported in Figure 3. In fact, while H'EA discriminated soils only for TOC values <1.5 - 3%> and pH values <6.5 - 7.4>, H'MR was significantly effective along all ranges for both soil properties.

The analysis of the potential relationships between each of the measures (H'EA and H'MR) and the two selected variables (TOC and pH) was carried out by referring to the quantile regression approach, which enabled us to analyse the effect of the covariates (TOC and pH) throughout the entire distribution as well as at the extremes. Table 4 shows the estimation results of regression models at quantiles 0.25, 0.50 and 0.75.

The total organic carbon content is not significantly associated neither with H'MR nor with H'EA, except for medium and high classes of TOC values at quantile 0.50 for H'EA (p-value<0.10). Conversely, pH values are negatively related to the H'EA measure in the lower part of the

247 distribution (i.e. for low values of the dependent variable H'EA) while a positive relationship was
248 observed in the highest quantiles of the distribution (i.e. for high values of the dependent variable
249 H'EA). On the other hand, pH values are positively related with H'MR in the middle part of the
250 distribution (quantile 0.50) only.
251 The land use category is an important factor distinguishing the values of the two measures. For H'EA
252 the relationship is positive and strongly significant in the lower part of the distribution (quantile 0.25)
253 while a negative relationship in the upper part (quantile 0.75) was found. A negative relationship
254 characterizes this land use category (A) and the H'MR measure in the first half part of the
255 distribution (quantiles 0.25 and 0.50) compared to the EC category representing the reference
256 category. Finally F soils category shows a positive relationship with H'MR only in the lower part of
257 the distribution (quantile 0.25)(Table 4).
258
259 **Discussion**
260 In this study, a large data set of 196 values of Shannon diversity index, calculated from data of
261 enzyme activities and CLPP-MicroResp techniques, was used. Griffiths et al. (2016) recently
262 included both techniques in a list of 18 potential, powerful indicators aimed to monitor soil
263 biodiversity and ecosystem function across Europe.
264 The first aim of this paper was to assess the relative sensitivity of each methodological approach in
265 capturing differences among the land use categories when different levels of pH and TOC are
266 considered.
267 The Kruskal-Wallis test showed that CLPP-MicroResp was a more powerful technique than enzyme
268 activities in highlighting differences among land use categories. Remarkably, while enzyme activities
269 were effective only within a certain range of TOC and pH values (<1.5-3%> and <6.5-7.4>,
270 respectively), MicroResp was able to discriminate soils along the whole range of TOC and pH
271 values, thus representing an effective tool for evaluating microbial functional diversity changes. This
272 result might be explained by the fact that differences in soil microbial catabolic evenness among

various land-uses are usually related to differences in organic C pools (Degens et al., 2000). Moreover, similar results were found by Creamer et al. (2009) reporting that the MSIR (multi substrate induced respiration) technique resulted in a much more distinct and relatively consistent pattern of separation between the tested soils with respect to enzyme activities.

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However, in relation to the lack of significant response of EA to pH variations we should keep in mind that enzymes determination requires NaAc buffer pH 5.5 as standardized in the protocol proposed by Marx et al. (2001). It is thus possible that the lower discriminant capacity of enzyme activities across a wide range of pH values may be ascribable to this methodological constraint. Nevertheless, since also TOC values did not affect significantly H'EA, except in the range <1.5-3.0%>, we can conclude that MicroResp showed a higher discrimination capacity among soil uses and managements.

In this study, no correlation was found between H'EA and H'MR over all the data collected. However, when looking at the correlation between H'EA and H'MR within the three categories of soils (A, F and EC) a weak relationship (significant at 10% level) emerges only in arable soils. We cannot exclude that this is due to the fact that agricultural soils features mainly fall within the intermediate range of pH and TOC values where both methods are sensitive.

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The general lack of correlation between enzymes and CLPP-MicroResp confirmed that the two techniques assess different steps of decomposition processes. Enzymatic hydrolysis focuses on the breakdown of complex organic polymers, which not necessarily leads to the complete mineralization of substrates but can also lead to anabolic pathways for biosynthetic processes, polymerization, condensation (i.e. humification, interaction with mineral colloids). Conversely, CLPP-MicroResp measures the complete mineralization of simple and complex organic compounds to CO_2, which represents the final step of decomposition process. Therefore, in our opinion, a comprehensive assessment of microbial functional diversity can be provided by the integration of both techniques. For this reason, they can be considered complementary components of microbial functional diversity providing a different ecological significance. In particular, in a comparison within soils we might

299 suppose that if H'EA:H'MR ratio decreases, the diversity of soil functions is completely oriented
300 towards mineralization of organic matter. In this case, it can be evidenced a dissipating energy
301 system; however this last hypothesis, aimed to assign a different ecological significance to the two
302 methods, needs to be further deepened and confirmed. Regarding the ecological significance of
303 increased catabolic diversity due to land use, it might be also supposed an amplified resistance of
304 microbial communities to stress or disturbance (Degens et al. 2001). Moreover, to further explain the
305 lack of correlation between the two methods, we should keep in mind that soil enzymes include the
306 contribution, considerable in most cases, of the immobilized fraction (humus-clay bound enzymes)
307 (Nannipieri et al. 2012). This fraction is considered a permanent bio-catalytic property of the soil, not
308 necessarily linked to the living biomass. Immobilized enzymes may represent soils background
309 hydrolytic potential, established and stabilized during time, and representing their resilient capacity
310 (Ceccanti et al. 2008). To date, no methods are available to distinguish between the extracellular
311 activity of stabilized enzymes from that of enzymes associated with active cells. Such separation is
312 important because only enzymes associated with active cells contribute to microbial activity. The
313 stabilized extracellular fraction is no more related to microbial metabolism and can persist in soil
314 under unfavourable conditions for soil microorganisms (Nannipieri et al. 2012).
315 Therefore, enzyme activities, and the functional diversity measures derived from using this
316 methodology, inform on the general soil biological functioning including not only the actual living
317 microbial activity but also the past biochemical activity still operating within soil matrix. Conversely,
318 CLPP-MicroResp has been considered a direct measurement of microbial communities' catabolic
319 profile providing an instant photograph of microbial physiology (Lagomarsino et al. 2007).
320 The QRM helped to understand if, and to what extent, the role of selected covariates (relevant soil
321 properties such as TOC and pH) change throughout the entire distribution of each dependent variable
322 (H'EA and H'MR). The QRM showed that both diversity indexes depended more on soil pH than on
323 total organic carbon content indicating soil reaction as the property mostly affecting microbial
324 diversity (Zahlnina et al. 2014).

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Microbial functional diversity expresses the capacity of microbial community to perform different processes and to metabolize diverse substrates. Soil pH variations can induce, more than the mere TOC content, significant changes within microbial biomass structure in terms of species and related functional patterns. Microbial biochemical processes are strictly dependent on pH values that control the majority of the reactions occurring in the soil. Fierer et al. (2006) and Lemanceau et al. (2015) reported soil pH as the best predictor of microbial diversity and richness affecting consequently microbial functions. However, the nature of this relationship is controversial. Griffiths et al. (2011) report that no decline in diversity was observed at increasing pH in a spatial assessment of soil bacterial community profiles across Great Britain. Fierer at et al. (2006), in a similar study performed across North and South America, showed a unimodal distribution of bacterial diversity, reaching possibly a plateau at near neutral pH.

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CONCLUSIONS AND FUTURE PERSPECTIVES

This study proved that CLPP-MicroResp technique provided a higher discrimination capacity, if compared to enzyme activities, as an ecological indicator to assess soil microbial functional diversity. In relation to soil chemical properties, pH was more relevant than TOC content in differentiating processes carried out by microorganisms. The diversity indexes obtained by the two methods, EA and MR, were not correlated; we hypothesize that they target complementary components of microbial functional diversity. This study could be improved in the future with the aim to verify if the two methodological approaches provide a different ecological significance informing on the extent of dissipating energy pathways in the soil system.

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Highlights

- ✓ Enzymes and MicroResp as reliable indicators to assess microbial functional diversity
- ✓ No correlation was found between the enzyme and MicroResp diversity indexes
- ✓ The two methods target complementary components of microbial functional diversity
- ✓ Both methods were effective to show differences among various land use categories
- ✓ Quantile regression model allowed analysis along the distribution diversity indexes

ASSESSMENT OF SOIL MICROBIAL FUNCTIONAL DIVERSITY: LAND USE AND SOIL PROPERTIES AFFECT CLPP-MICRORESP AND ENZYMES RESPONSES

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Keywords: microbial processes, Shannon index, Soil properties.

Abstract

The assessment of microbial functional diversity is an important indicator of soil quality. Different methodological approaches are currently used; among them are enzyme activities (EA) and CLPP (community level physiological profile) techniques (e.g. MicroResp^{TM}, MR). The aims of the study were: *i)* to assess the efficacy of both methods in capturing differences among various land use categories when different levels of selected explanatory variables such as land use category, total organic carbon (TOC) and pH are considered, and *ii)* to explore, through a quantile regression approach, the possible relationships between each of the two methods with land use category, TOC and pH. The Shannon diversity index (H'), calculated from EA and MR data, was chosen as a synthetic index deriving from the same mathematical model. The quantile regression model (QRM), the Kruskal-Wallis and Spearman rank correlation tests were performed.

Enzyme activities and MicroResp were reliable ecological indicators to assess soil microbial functional diversity. No correlation was found between the diversity indexes, H'EA and H'MR, it was therefore supposed that the two methods may target complementary components of microbial functional diversity. Both methods were effective in capturing differences among various land use categories, in particular H'MR in soils with low TOC content (<1.5%). Moreover, the QRM approach allowed a more detailed analysis along the distribution of the diversity indexes (H'EA and H'MR) indicating that H'EA was more dependent on the selected variables.

Introduction

The links between ecosystem functioning and levels of soil biodiversity have been the focus of the recent scientific literature (Delgado-Baquerizo et al., 2016; Creamer et al., 2016b; Griffiths et al., 2016; Nannipieri et al., 2003). The first authors provided evidence that loss in microbial diversity will likely reduce multiple ecosystems functions thus negatively impacting the provision of ecosystem services. Adhikari & Hartemink (2016) claimed for new insights into soil microbial diversity and their role in soil functional variability. Since up to 80/90% of soil functions, from humification to mineralization, is microbially-mediated, the diversification of soil microrganisms in terms of structure and/or activity is essential to maintain functioning of terrestrial ecosystems (Pereira et al., 2013).

Microbial functional diversity is defined as “the sum of the ecological processes, and/or capacity to use different substrates developed by the organisms of a community” (Insam et al., 1989). Emmerling et al. (2002) and Wellington et al. (2003) report that if microbial genetic diversity assesses a latent diversity, which may not be expressed, functional diversity is related to the actual activities resulting from that potential so that "functional rather than taxonomic diversity may provide greater insight to microbial roles in ecosystems" (Zak et al., 1994).

Over the last 10 years, the scientific literature provided a great number of papers aimed to assess microbial functional diversity as an important ecological indicator to monitor and assess soil quality changes in different pedoclimatic conditions, land uses and human pressure levels (e.g. management practices) (Bardgett and van der Putten, 2014; Griffiths et al., 2016).

To measure the activity and diversity of the microbial community a number of methods can be applied, to cite few of the most common approaches: (i) catabolic activity investigated by Biolog™-plates (Garland and Mills, 1991; Rutgers et al., 2016), (ii) respiration of different substrates as investigated by the MicroResp™ method (Campbell et al., 2003; Chapman et al., 2007; Creamer et al. 2016a) and (iii) enzyme activities (Nannipieri et al., 2012; Hendriksen et al., 2016).

Although all methodological approaches are reliable and sensitive, few studies aimed to understand their effectiveness to discriminate microbial functional diversity in relation to soil organic C and pH as the main properties being affected by land use and management practices, anthropic impact and other pedogenic factors. To achieve this goal, a large number of case studies covering different land use categories is necessary. In this study, about 200 measurements of microbial functional diversity obtained over a broad spectrum of key soil properties and across different land uses and management, were selected. Furthermore, microbial functional diversity obtained through enzyme activities (EA) and CLPP-MicroResp (MR), was synthetically represented by the Shannon index (H') that transforms the obtained results to a comparable range of values deriving from the same mathematical model. The Shannon index is a comprehensive indicator of microbial species, individual numbers and evenness, or distribution of the enzyme activities and is influenced by richness of community species (Bending et al., 2002; Li et al., 2007).

The aim of the present study was therefore to: i) assess the efficacy of both methods in capturing differences among the different land use categories when different levels of pH and TOC are considered, *ii)* explore, through a quantile regression approach, the possible relationships between each of the two methods and selected explanatory variables (TOC, land use category, pH).

Materials and methods

Experimental design, sites and soil categories

The results presented in this paper have been obtained performing additional statistical analyses on data collected in the Laboratory of Chemistry and Biochemistry, University of Tuscia, Viterbo, Italy during the last 6 years (2010-2016). Microbial functional diversity was measured, by means of enzyme activities and CLPP-MicroResp™ technique, in a wide range of soils analysed within different research projects. Most of the sampling sites are located within the Mediterranean climatic area. Other climatic areas are the monsoon one for the Bangladesh case study, the temperate one for Switzerland, oceanic for United Kingdom and boreal for Sweden. All soils represent a broad

spectrum of key soil properties across different land use categories, wide range of soil pH and soil organic carbon content (TOC) (Table 1).

The soils were related to 15 case studies, each one including different treatments, with the aim to separate diverse land uses and/or specific conditions. For this purpose, three groups were identified: F (forest soils, 4 case studies), A (agricultural soils, 5 case studies) and EC (extreme conditions, 6 case studies). The case studies related to forest soils (F) included different management practices, lithological substrates, afforestation and chronosequences. The soils under agricultural land use (A) were characterized by different managements and/or agricultural practices such as: organic, biodynamic, conventional cropping systems, tillage/no tillage and natural green cover/no cover. The third category (EC) included soils with peculiar characteristics due to either pedo-climatic conditions (saline environments, natural arsenic contamination in rice paddies and highly calcareous soils) or to heavy anthropic impact (thallium contamination, a multi-element contaminated dump, arsenic contaminated mine) (Table 1).

Soil sampling

All soils were sampled at 0-20 cm depth during the dry season (spring/summer), air dried, sieved at 2 mesh and preserved at room temperature., Then, prior to biochemical analyses, soil moisture content of air dried samples was adjusted to 60 % of their water holding capacity and soils were re-conditioned for 10 days .

Soil analyses and methodologies

The total organic carbon (TOC) was determined by combustion by Shimadzu TOC VCSH analyzer while soil pH was measured on sieved soil suspended in a solution of deionised water in 1:2.5 ratio (w/v). The pH was measured in the supernatant with a pH meter (pH 211, Hanna Instruments).

A total of 196 values of microbial functional diversity, assessed by means of enzyme activities and CLPP-MicroResp, were used for this study (Table 1). Enzymes were measured following Marx et al.

(2001) using fluorogenic methylumbelliferyl (MUF)-substrates. Soils were analysed for cellobiohydrolase, β-1,4-glucosidase, α-1,4-glucosidase, β-N-acetyl-glucosaminidase, β-1,4-xylosidase, acid-phosphatase, arylsulphatases and butyrate esterase which is considered a proxy of endocellular activity (Wittman et al. 2004). The relative fluorogenic substrates, prepared with acetate buffer 0.5 M pH 5.5, were: 4-MUF-β-D-cellobioside, 4-MUF-β-D-glucoside, 4-MUF-N-acetyl-β-glucosaminide, 4-MUF-α-D-glucoside, 4-MUF-phosphate, 4-MUF-7-β-D-xyloside, 4-MUF-sulphate and 4-MUF-butyrate. Fluorescence (excitation 360 nm, emission 450 nm) was measured with an automatic fluorimetric plate-reader (Fluoroskan Ascent) and readings were performed after 0, 30, 60, 120 and 180 minutes of incubation at 30° C. The results were expressed as nmoles of product (MUF) of each enzymatic reaction released per g of soil per unit of time in relation to a standard curve prepared with increasing MUF concentrations and incubated at the same experimental conditions.

The community level physiological profile (CLPP) was determined using the MicroResp™ soil respiration system (James Hutton Ltd, Aberdeen, UK) according to Campbell et al. (2003).

The 15 substrates used for MicroResp were: α-D-glucose, D-Galactose, D-fructose, L-arabinose, L-leucine, L-arginine, Glycine, L-aspartic acid, γ-amino-butyric and glutamic acid, three carboxylic acids: citric acid, oxalic acid and L-ascorbic acid, and two phenolic acids: vanillic and syringic acid. The emission of CO_2 by the microbial biomass was estimated using a colorimetric method (microplate spectrophotometer) before and after 6 h of incubation at 28 °C. The absorbance was read at 595nm. At the end the absorbance was normalised for any difference recorded at time zero and then converted to % CO_2 using the calibration curve y = A+B/(1+ D x Ai) (Campbell et al., 2003). The calibration procedure was performed taking into account the spectrophotometer used, the different soils and incubation conditions. In our experimental conditions the constants of the equation were: A:-1,62, B:-4,85 and D: -8,1. The CO_2% was converted to μg C-CO_2 g^{-1} h^{-1} production rate using gas constant, T °C, headspace volume, soil dry weight (d.w.) and incubation time. The SEI (Synthetic Enzymatic Index) and SIR (Substrate Induced Respiration) for all soils within the three categories (F, A and EC) have been calculated as synthetic measures of microbial functional

capacity. Both SEI and SIR represent the total microbial functional capacity expressed as sum of all enzymatic activities and of induced respiration of all substrates, respectively.

Microbial functional diversity was assessed calculating the Shannon-Weaver diversity index (Kennedy and Smith, 1995) corresponding to the entropy concept defined by: $H' = - \sum p_i * \ln p_i$ (Shannon and Weaver, 1949; Spellerberg and Fedor, 2003), where p_i is in turn: for H'EA, the ratio of the activity of a particular enzyme to the sum of all enzymatic activities while for H'MR it is the ratio of the respiration rate of each single C-substrate to the sum of all substrates. Shannon diversity index is related to the entropy of a system and when applied as a measure of microbial functions entropy, may express the heterogeneity of soil organic substrates availability and microbial processes (Marinari et al., 2013). Since the eight enzymes and the 15 substrates here tested did show activity in all the analysed samples, then, in this work, the diversity recorded reflects only the "evenness" or distribution of the enzyme activities or ability to use the different substrates (Bending et al., 2002; Rodríguez-Loinaz et al., 2008).

Statistical analyses

The analysis of all collected data was carried out into various steps. At first, with the aim to compare the two indexes, a standardization due to the existing differences in the range of H'EA and H'MR possible/admissible values was performed. As usual the new standardized indexes have mean equal to zero and variance equal to 1. It is worth noting that from now on, all the statistical analyses were carried out on the two standardized distributions.

The descriptive analyses provided a clear picture of the distribution of the two indexes (H'EA and H'MR) as well as information about the shape of the two distributions. Moreover, rank correlation measures and test performed by using the Spearman correlation enabled to evaluate if, and to what extent, the two methodological approaches (EA, enzyme activities and MR, MicroResp™) used to evaluate soil microbial functional diversity are related.

The Kruskal-Wallis non-parametric test was used to test if and to what extent the two indexes distinguished the various land use categories in relation to TOC and pH ranges.

By considering the asymmetry of the two distributions (e.g. H'EA and H'MR respectively, as shown in Figure 2) as well as the results of the Shapiro-Wilk normality test, we analysed the existence of association between each of the two measures and selected covariates by using Quantile Regression Models (QRMs). In fact, these types of regression models offer the possibility to highlight how the effect of the selected covariates, in this case TOC content, pH and land use category, changes throughout the entire distribution of the dependent variable. To estimate the relationships (in terms of association) between the dependent variables and the set of selected covariates, the classical OLS (Ordinary Least Squares) regressions could be applied. However, data obtained from experimental collection tend to be skewed so that these models could not be able to describe the "correct" relationships. Moreover, QRMs are more robust to the presence of outliers and can be consistent under weaker stochastic assumption than with least-squares estimation (Cameron and Trivedi, 2005; Koenker, 2005).

Referring to the soil context, the application of QRMs has important advantages. Firstly, QRMs can help to explore if the existing differences observed between the two measures can be attributed to different effects played by the explanatory variables at the various quantiles. Secondly, it can be interesting to understand what happens throughout the entire distribution of the two measures (H'EA and H'MR) and at their extremes.

We estimated two QRMs which assumed the dependent variable to be: (i) H'EA and (ii) H'MR respectively. In both models the set of covariates includes factors describing: the land use category (distinguished into Forest, Agricultural and Extreme soil Conditions), the levels of pH and TOC. Among the soil properties that mostly affect microbial biomass activity and diversity, TOC and pH were chosen as covariates to explain H' index variability (Creamer et al., 2016b; Fierer and Jackson, 2006; Constancias et al. 2015). In this study, the distribution of TOC values allowed to homogeneously group soils into three categories: i) low: for TOC < 1.5 %; ii) medium TOC < 1.5 –

3>; iii) high: for TOC ≥3%. Similarly, pH values allowed to group soils into three categories: i) <6.5 slightly acid – very strongly acid, ii) <6.5-7.4> neutral, iii) >7.4 slightly alkaline – moderately alkaline. STATA software (STATA 13.2 edition) was used for statistical analyses. Three distinct levels of significance were considered for the estimated coefficients and are reported in the model: a value of $p<0.001$ (indicated in the tables of results with ***), emphasizing strong relationships between the explicative variable of interest and the dependent variable significant at 0.1% level; the value of $p<0.01$ (indicated in tables of results with **) indicates a relationship significant at 1% level and finally a value of $p<0.05$ (indicated in the tables of results with *) emphasizing a relationship between the variables significant at 5% level.

Results

Figure 1 shows the functional capacity of soil microbial biomass calculated as the SEI and SIR for all soils within the three categories (F, A and EC). Soils characterized by extreme conditions showed the highest level of variability - including upper outliers - for SEI (ranging from 42 to 11800 nmoles MUF g^{-1} h^{-1}), while the functional capacity of forest soils showed a high level of dispersion for SIR (ranging from 0.9 to 177 μg CO_2 g^{-1} h^{-1}). Agricultural soils show, for both methodological approaches, a smaller level of variability.

Figure 2 shows the distribution of the two standardized indexes values H'EA and H'MR, respectively, over the 196 values of microbial functional diversity. The two distributions are positively skewed and leptokurtic - as emerged by the descriptive statistics reported in Table 2 – and significantly different from normality as confirmed by the Shapiro-Wilk W and Shapiro-Francia W′ test.

The Spearman rank correlation, verifying the similarity of the orderings of the data when ranked according to each of the measures, showed that the two measures are not related for measuring microbial functional diversity (p-value = 0.0987) (Table 3). However, by distinguishing rank

correlation according to the land use category, we found a moderate and significant level of inverse rank correlation (p-value = 0.0073) when the two indexes refer to soil of type A. No significant rank correlation was found between the two measures for soil type EC and F (p-value= 0.6534 and p-value= 0.8727 respectively) (Table 3).

According to the results of Kruskal-Wallis test, both H'EA and H'MR distinguished in different ways the various soils when TOC or pH ranges were considered (Table 4). Thus, according to the obtained p-values H'MR showed a slightly higher discriminatory potential than H'EA. H'MR, in fact, was significantly effective at low TOC ranges (<1.5%) where H'EA was not. On the other hand, both methods failed to discriminate in alkaline soils (pH values ≥7.4).

The analysis of the potential relationships between each of the measures (H'EA and H'MR) and the selected variables (land use, TOC and pH) was carried out by referring to the quantile regression model (QRM), which enabled to analyse the effect of the covariates throughout the entire distribution as well as at the extremes. Table 5 shows the estimation results of regression models at quantiles 0.25, 0.50 and 0.75.

Focusing on TOC content we only found a negative association at quantile 0.75 (p-value<0.05) between H'EA and high level of TOC (equal or greater than 3%). On the other hand, pH levels are negatively related to the H'EA measure in the lower part of the distribution (e.g at quantile 0.25 of the dependent variable H'EA, p value < 0.01) while a positive relationship was observed with high levels of pH in the highest quantile of the distribution (e.g. for high values of the dependent variable H'EA). Furthermore, a positive relationship was found between medium level of pH (values ranging between 6.5 and 7.4) and H'MR in the middle part of the distribution (quantile 0.50).

The land use category is a key factor distinguishing the values of the two measures. For H'EA the relationship is positive and strongly significant at quantiles 0.25 and 0.75 for land use category F (forest soils) while a negative relationship with agricultural land use category was observed at quantiles 0.50 and 0.75. At the same time, we observed positive and significant relationships between

the values of H'MR and agricultural land use category at all different quantiles throughout the entire distribution while forest soil only at 0.75 quantile (Table 5).

Discussion

In this study, a large data set of 196 values of Shannon diversity index, calculated from data of enzyme activities and CLPP-MicroResp techniques, was used. Griffiths et al. (2016) recently included both techniques in a list of 18 potential, powerful indicators aimed to monitor soil biodiversity and ecosystem function across Europe.

The first aim of this paper was to assess the relative sensitivity of each methodological approach in capturing differences among the land use categories when different levels of pH and TOC are considered.

The Kruskal-Wallis test showed that both methods were able to highlight differences among land use categories at almost all ranges of TOC and pH. However, while both of them failed to discriminate in alkaline soils (pH >7.4), only MicroResp was completely effective along the whole TOC gradient, including low TOC values (<1.5%). This result might point to MicroResp as a more powerful tool for evaluating microbial functional diversity, particularly in oligotrophic environments where the addition of easily available organic C sources (represented by the different substrates) may stimulate microbial respiration. Conversely, enzyme production is not similarly stimulated as it requires a higher energetic expense (Burns and Dick, 2002). In studies aimed to evaluate the effect of land use change on microbial functional diversity, the CLPP-MicroResp approach can be thus suggested as soil microbial catabolic evenness among various land-uses is usually related to differences in organic C pools (Degens et al., 2000). Creamer et al. (2009) also reported that the MSIR (multi substrate induced respiration) technique resulted in a much more distinct and relatively consistent pattern of separation between the tested soils with respect to enzyme activities. The lack of potential for both techniques to discriminate among different land uses in alkaline soils (pH >7.4) may be due to the

fact that the interrelationship between soil pH and microbial diversity may be lost (Fierer et al., 2006) or even decreased (Griffiths et al., 2011) at soil pH values higher than 7.

In this study, no correlation was found between H'EA and H'MR all over the data collected. Moreover, an opposite behaviour of the two indexes was found in agricultural soils where the significant ($p<0.01$) correlation coefficient was negative. This result confirms what was previously observed regarding oligotrophic environments characterized by lower organic matter content, such as agricultural soils. In fact, as reported by Lagomarsino et al. (2011), the microbial functional diversity determined by means of the enzymatic pattern is affected by land use showing an increase along a gradient of soil organic matter. In the same paper the authors reported an inverse relationship between microbial functional diversity and the catabolic response per unit of biomass expressed by the metabolic quotient (qCO_2).

The lack of correlation between H' by means of enzymes and CLPP-MicroResp suggests that the two techniques may assess sequential steps of decomposition processes, even if in this meta-analysis the product of most selected enzymatic reactions did not represent the substrates used to test CLPP-MicroResp. Enzymatic hydrolysis focuses on the breakdown of complex organic polymers, which not necessarily leads to the complete mineralization of substrates but can also lead to anabolic pathways for biosynthetic processes, polymerization, condensation (e.g. humification, interaction with mineral colloids). Conversely, CLPP-MicroResp measures the complete mineralization of simple and complex organic compounds to CO_2, which represents the final step of decomposition process. Therefore, in our opinion, a comprehensive assessment of microbial functional diversity can be provided by the integration of both techniques. For this reason, they can be considered complementary components of microbial functional diversity.

Moreover, to further explain the lack of correlation between the two methods, we should keep in mind that soil enzymes include the contribution, considerable in most cases, of the immobilized fraction (humus-clay bound enzymes) (Nannipieri et al., 2012). This fraction is considered a permanent bio-catalytic property of the soil, not necessarily linked to the living biomass.

Immobilized enzymes may represent soils background hydrolytic potential, established and stabilized during time, and representing their resilient capacity (Ceccanti et al., 2008). To date, no methods are available to distinguish between the extracellular activities of stabilized enzymes from that of enzymes associated with active cells. Such separation is important because only enzymes associated with active cells contribute to microbial activity. The stabilized extracellular fraction is no more related to microbial metabolism and can persist in soil under unfavourable conditions for soil microorganisms (Nannipieri et al., 2012). Therefore, enzyme activities, and the functional diversity measures derived from using this methodology, inform on the general soil biological functioning including not only the actual living microbial activity but also the past biochemical activity still operating within soil matrix. Conversely, CLPP-MicroResp has been considered a direct measurement of microbial communities' catabolic profile providing an instant photograph of microbial physiology (Lagomarsino et al., 2007).

The QRM helped to understand if, and to what extent, the role of selected covariates (land use, TOC and pH) changes throughout the entire distribution of each dependent variable (H'EA and H'MR). It is, in fact, known that microbial functions are largely dependent on organic substrates availability and soil reaction (Bardgett and van der Putten, 2017). The QRM was found to be an effective statistical approach to analyse microbial functional diversity response in relation to the selected covariates, particularly at the lowest (0.25) and highest (0.75) quantiles.

In this study QRM showed that both diversity indexes depended more on soil pH than on TOC content indicating soil reaction as the property mostly affecting microbial diversity (Zhalnina et al., 2014). In fact, only when TOC values were above 3% the H'EA was negatively affected suggesting that the increase of soil available organic compounds may cause a negative feedback on microbial hydrolytic reactions. On the contrary, it was more evident the relationship between pH and both indices. H'EA was negatively related to pH in the 0.25 quantile indicating that low levels of this index are more sensitive to soil pH variations (Griffiths et al., 2011). Conversely the dependence of both indexes (H' MR and H'EA) from pH was positive at 0.50, and 075 quantiles, respectively. Soil

pH variations can induce, more than the mere TOC content, significant changes within microbial biomass structure in terms of species and related functional patterns. Microbial biochemical processes are strictly dependent on pH values that control the majority of the reactions occurring in the soil. Fierer et al. (2006) and Lemanceau et al. (2015) reported soil pH as the best predictor of microbial diversity and richness affecting consequently microbial functions.

However, the nature of this relationship is controversial. Griffiths et al. (2011) report that a decline of β-diversity was observed at increasing pH in a spatial assessment of soil bacterial community profiles across Great Britain. Fierer et al. (2006), in a similar study performed across North and South America, showed a unimodal distribution of bacterial diversity, reaching possibly a plateau at near neutral pH.

Finally, the influence of the different land use categories was evident in some parts of the distribution for both indexes, especially at 0.75 quantile. In particular, the effect of forest soils was always positive, in most cases significant, for both indexes at all quantiles, confirming the strict relationship existing between the forest environment and soil microbial diversity (Creamer et al., 2016b).

CONCLUSIONS AND FUTURE PERSPECTIVES

This study demonstrates that both methods, enzyme activities and MicroResp, are reliable ecological indicators to assess soil microbial functional diversity. However, since no correlation was found between the diversity indexes H'EA and H'MR, it was hypothesized that the two methods may target complementary components of microbial functional diversity.

The results lead to the following conclusions: *i)* both methods were effective in capturing differences among various land use categories although MicroResp was more sensitive at low levels of soil organic matter, *ii)* the QRM approach allowed a more detailed analysis along the distribution of the diversity indexes (H'EA and H'MR) with H'EA showing a more significant dependence on the selected variables.

This study can lay the foundations to further studies aimed to assign an ecological significance to the assessment of microbial functional diversity.

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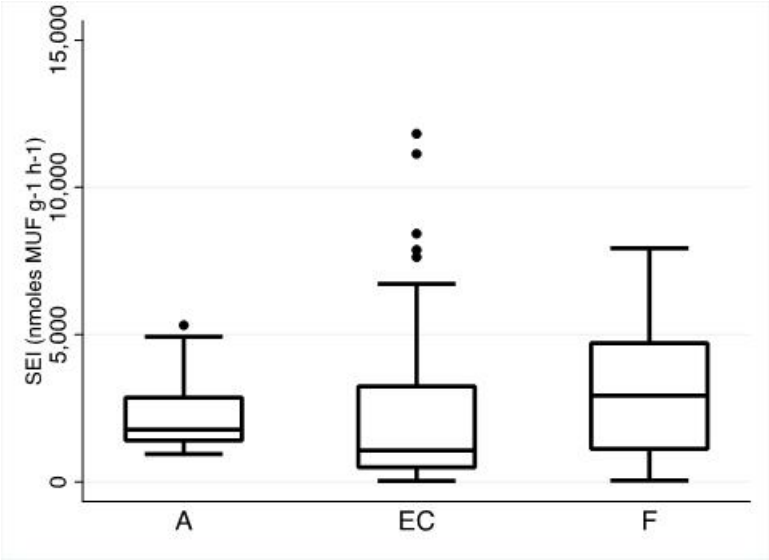
Figures

Figure 1: Boxplot of microbial functional capacity measured by means of enzyme activities and CLPP-MicroResp. a) SEI (synthetic enzymatic index) and b) SIR (substrate induced respiration) distributions in the three soil categories (F, forest, A, agricultural and EC, extreme conditions soils)

Figure 2: Distribution of the two standardized indexes: a) H'EA and b) H'MR

a)


SEI (nmoles MUF g-1 h-1)
0
5,000
10,000
15,000
A
EC
F

b)

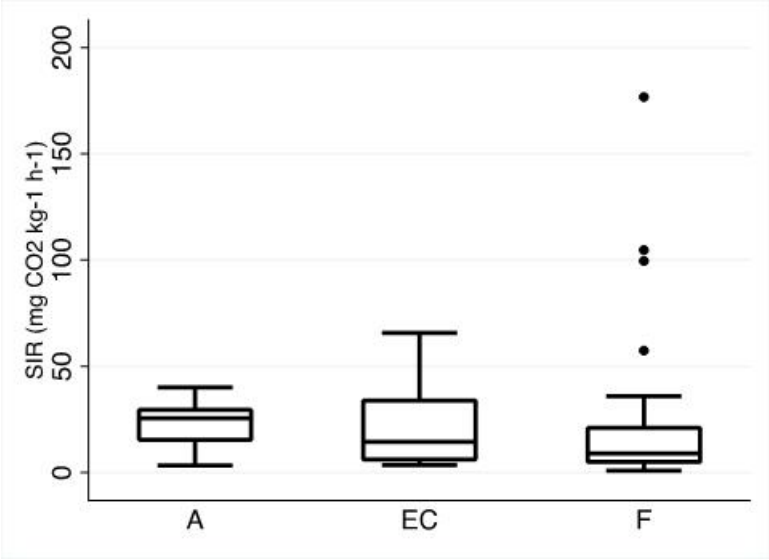
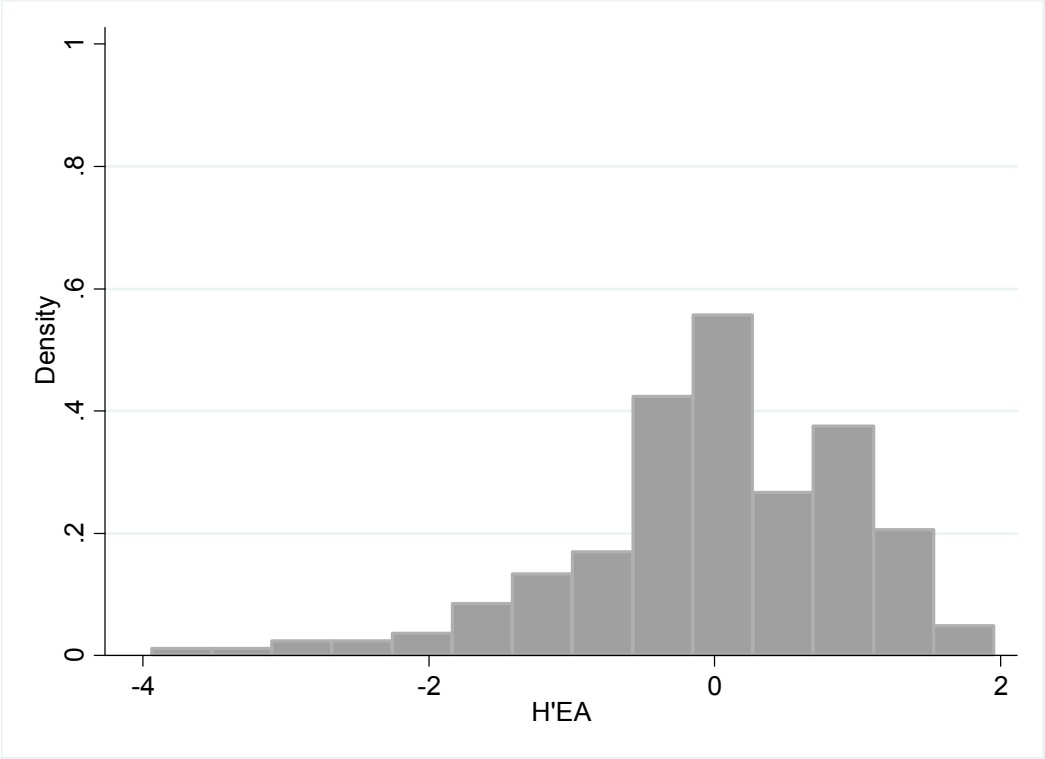

SIR (mg CO2 kg-1 h-1)
0
50
100
150
200
A
EC
F

Figure 1

a)



b)

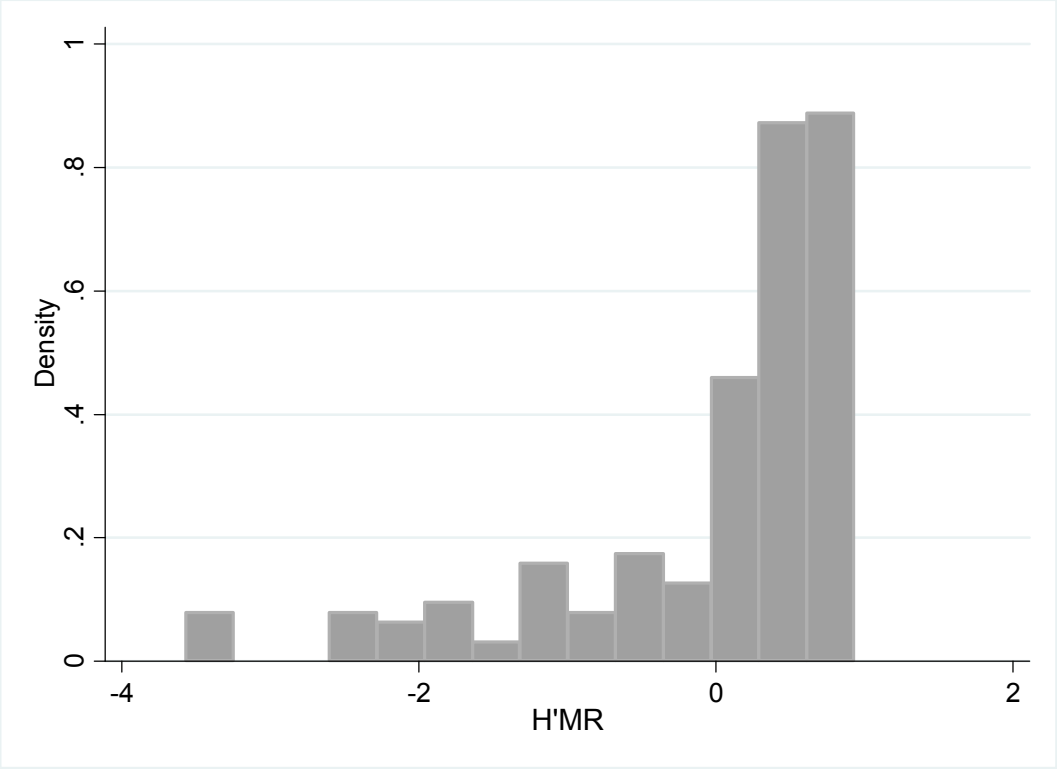


Figure 2

Table 1: Description of all data sources. All soils are grouped into three categories: F (Forest soils), A (agricultural soils) and EC (extreme conditions soils). For each case study the following data are reported: factors of variation analyzed, soil texture, total organic carbon (TOC), pH_{H2O}, standardized Shannon diversity index (H') measured by means of enzyme activities (H'EA) and MicroResp (H'MR), total number of samples, reference to data source. Org/conv/biodyn: organic, conventional or biodynamic management, n = number of samples. When reference was not available, a specific acknowledgement to research funds was added. Average data are reported with standard errors. n.p.=data not published.

Soil category	Factor of variation	Soil texture	TOC (%)	pH	H' EA	H' MR	n	Location	Reference or acknowledgement
Forest **(F)**	Management (coppiced/aged coppice)	Loam	5.9±0.5	6.4±0.1	0.61±0.01	1.11±0.01	12	*Central Italy – Umbria region*	*Pignataro et al., (2012)*
	Lithological substrate	Loam	4.2±0.7	5.8±0.2	0.78±0.02	1.07±0.04	10	*Central Italy – Lazio/Umbria region*	*Pignataro et al., (2011)*
	Afforestation (*Beech* and *Douglas*-fir)	Sandy-clay-loam, Loamy sand	3.1±0.8	5.6±0.2	0.64±0.01	1.12±0.01	8	*Central Italy – Emilia Romagna region*	*Papp R. (2016)*
	Chronosequence (*Douglas-fir*)	Sandy-loam, Loam	4.5±0.5	4.8±0.1	0.67±0.01	1.09±0.03	7	*Central Italy – Tuscany region*	*Papp R. (2016)*
Agricultural **(A)**	Management (org/conv)	Sandy-loam, Loam	1.5±0.0	7.2±0.0	0.59±0.01	1.14±0.00	30	*Central Italy, Lazio region*	*Brunetti P. (2014)*
	Management (tillage level)	Loam, Silt loam, Clay	1.9±0.2	7.2±0.3	0.55±0.02	1.08±0.01	12	*North Morocco, Central Italy, Switzerland, United Kingdom, Sweden*	*Papp R. (2016)*
	Vineyard (natural green cover/no cover)	Silty-loam	1.5±0.1	8.1±0.0	0.70±0.01	1.01±0.02	19	*Northern Italy, Piemonte region*	n.p.
	Management tomato crop (organic/conv)	Clay-loam	1.4±0.1	6.6±0.1	0.58±0.01	1.13±0.01	5	*Central Italy, Lazio region*	n.p.
	Vineyard (biodyn/conv)	Clay	1.3±0.1	6.7±0.2	0.59±0.01	0.97±0.09	4	*Central Italy, Lazio region*	n.p.
Extreme conditions **(EC)**	Thallium contamination	Loam	6.3±0.8	6.4±0.6	0.67±0.03	1.08±0.02	8	*Central Italy, Emilia Romagna region*	n.p.
	Arsenic contamination	Sandy-loam,Loam	2.7±0.3	5.7±0.2	0.61±0.01	1.02±0.03	14	*Northern Italy, Piemonte region*	*Stazi et al. (2017)*
	Highly calcareous, different plant cover	Sandy-loam	1.2±0.1	8.0±0.0	0.47±0.02	1.10±0.01	12	*Central Italy, Lazio region*	*Italian PRIN 20082FC352_002*
	Hydromorphous and subaqueous	Sandy	2.2±0.6	8.2±0.2	0.40±0.03	1.04±0.03	16	*Central Italy, Emilia Romagna region*	*Papp R. et al., (2015)*
	Waterlogged rice paddies and arsenic	Silty-loam, Clay-loam	1.2±0.1	7.3±0.1	0.69±0.02	0.84±0.03	20	*Bangladesh*	*Italian PRIN 2010JBNLJ7_006*
	Phytoremediation (heavy metals)	Clay-loam	1.4±0.0	7.9±0.2	0.76±0.01	1.10±0.00	19	*Central Italy, Tuscany region*	*Emili L. (2013)*

Table 2: Descriptive statistics of the two standardized measures. H'EA and H'MR: Shannon diversity index calculated by means of enzyme activities and MicroResp, respectively, over 196 soil samples.

Measure	Min	q0.25	q0.50	q.0.75	Max	Skewness	Kurtosis
H'EA	-3.940	-0.451	0.039	0.754	1.953	-0.851	4.279
H'MR	-3.567	-0.242	0.382	0.651	0.933	-1.746	5.506

Table 3: Spearman r_s values for both standardized indices H'EA and H'MR calculated for all data and within the three soil categories F, A and EC soils. ns: not significant, **p<0.01

	All data	(F)	(A)	(EC)
H'EA - H'MR	-0.1183 ns	-0.0273 ns	-0.3180**	-0.0482 ns

Table 4: Results of Chi-squared statistics X^2 and p-values obtained with Kruskal-Wallis rank test on soil functional diversity (H'EA and H'MR) among the three land use categories within restricted classes of TOC and pH. P values are reported in parentheses.

TOC values	**H'EA**	**H'MR**
Low: TOC<1.5%	2.202	11.039
	(0.333)	*(0.004)*
Medium: 1.5≤TOC<3%	7.640	6.272
	(0.022)	*(0.043)*
High: TOC ≥3%	4.431	7.150
	(0. 035)	*(0. 007)*
pH values		
pH<6.5	11.843	8.971
	(0.003)	*(0.011)*
6.5≤pH<7.4	13.867	30.998
	(0.001)	*(0.000)*
pH ≥7.4	1.046	2.517
	(0.306)	*(0.113)*

Table 5: Estimation results of QRMs (0.25, 0.50, 0.75 quantiles). SE= standard error, * Significant at 5%, ** 1% and *** 0.1% level.

	H'EA			**H'MR**		
	Coef.	*SE*	*Sign.*	*Coef*	*SE*	*Sign.*
Quantile 0.25						
TOC values (*ref. Low: <1.5%*)						
Medium: 1.5≤TOC<3	0.027	0.120		0.505	0.338	
High: TOC ≥3%	0.004	0.150		0.744	0.436	
pH (ref. pH<6.5)						
6.5≤pH<7.4	-0.177	0.077	**	0.295	0.183	
pH ≥7.4	-0.871	0.254	**	-0.471	0.343	
Land use category (*ref. EC*)						
F	0.462	0.160	**	0.821	0.417	
A	0.193	0.175		0.996	0.497	*
Constant	-0.487	0.125	***	-1.276	0.426	**
Quantile 0.50						
TOC values (*ref. Low: <1.5%*)						
Medium: 1.5≤TOC<3	-0.197	0.183		-0.043	0.097	
High: TOC ≥3%	-0.583	0.340		-0.0.31	0.271	
pH (ref. pH<6.5)						
6.5≤pH<7.4	0.066	0.259		0.213	0.088	*
pH ≥7.4	0.417	0.342		-0.060	0.176	
Land use category (*ref. EC*)						
F	0.415	0.305		0.418	0.230	
A	-0.377	0.180	*	0.259	0.114	*
Constant	0.260	0.307		0.171	0.120	
Quantile 0.75						
TOC values (*ref. Low: <1.5%*)						
Medium: 1.5≤TOC<3	-0.001	0.142		0.008	0.072	
High: TOC ≥3%	-0.549	0.256	*	-0.070	0.136	
pH (ref. pH<6.5)						
6.5≤pH<7.4	-0.008	0.233		0.079	0.073	
pH ≥7.4	0.527	0.235	*	-0.096	0.116	
Land use category (*ref. EC*)						
F	0.422	0.192	**	0.239	0.111	*
A	-0.703	0.136	***	0.160	0.080	*
Constant	0.804	0.246	**	3.212	0.414	**

Notes: * p<0.05; ** p<0.01;***p<0.001