

# Assessment of soil microbial functional diversity: L ("Land" as lowercase) and use and soil properties affect CLPP-MicroResp and enzymes responses

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## 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™, 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.

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**Keywords:** Microbial processes; Shannon diversity index; Soil properties

## 1 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 ecosystem functions thus negatively impacting the provision of ecosystem services. [Adhikari and 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 microorganisms in terms of structure and/or activity is essential to maintain functioning of terrestrial ecosystems ([Pereira Silva 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 have 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).

## 2 Materials and methods

### 2.1 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).

**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 analysed, soil texture, total organic carbon (TOC), pH<sub>H2O</sub>, 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.

alt-text: Table 1

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 (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 (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 (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 (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	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.

(EC)	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 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 (2013)

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).

## 2.2 Soil sampling

All soils were sampled at 0–20 cm depth during the dry season (spring/summer), air dried, sieved at 2–mm 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.

## 2.3 Soil analyses and methodologies

The total organic carbon (TOC) was determined by combustion of sample in an oxygen-enriched atmosphere and detection and quantification of CO<sub>2</sub> evolved by infrared detector using the 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 (Wittmann 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 min 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<sub>2</sub> 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 595 nm. At the end the absorbance was normalised for any difference recorded at time zero and then converted to % CO<sub>2</sub> using the formula  $y = A + B/(1 + D \times Ai)$  where Ai is the normalised 6hr data. The formula is for a linear-to-linear (rectangular hyperbola) standard curve fit obtained by means of a calibration procedure taking into account the spectrophotometer used, the different soils and incubation conditions as reported in the MicroResp technical manual. In our experimental conditions the constants of the equation were: A: -1,62, B: -4,85 and D: -8,1. The % CO<sub>2</sub> was converted to μg C-CO<sub>2</sub> g<sup>-1</sup> h<sup>-1</sup> 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 diversity index (Kennedy and Smith, 1995) corresponding to the entropy concept defined by:  $H' = - \sum p_i \cdot \ln p_i$  (Shannon and Weaver, 1949; Spellerberg and Fedor, 2003), where p<sub>i</sub> 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-Loiñaz et al., 2008).

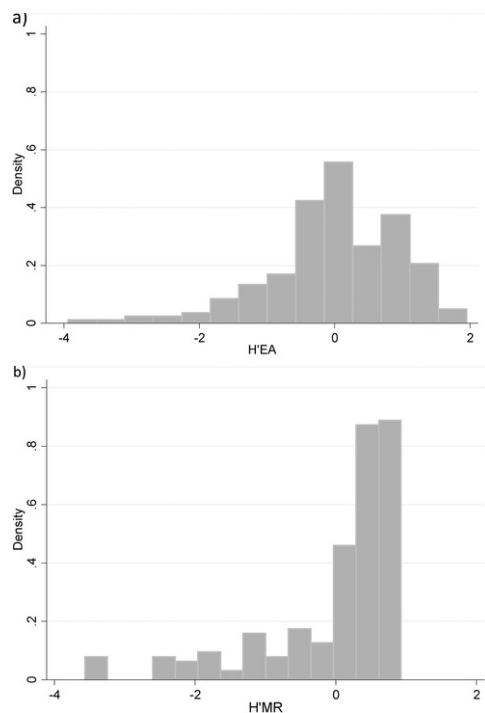
## 2.4 Statistical analyses

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 by transforming the original indexes into two new variables each one with mean equal to zero and variance equal to 1. As usual, the procedure was carried out by subtracting the variable mean (i.e. the mean value of H'EA and H'MR, respectively) from each observation and then dividing by its own standard deviation (each one obtained from the original indexes). As a property of standardized (z-scores) values, the standardized distributions maintain the same shape (i.e. in terms of skewness and kurtosis) of the two original distributions and the transformation does not change the location of any observation score relative to others in the distribution. 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. standardized H'EA and H'MR respectively, as shown in Fig. 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 were not 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).



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

alt-text: Fig. 2

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, soils were grouped into three categories in relation to TOC content: i) low: for TOC <1.5%; ii) medium TOC <1.5–3>; iii) high: for TOC ≥3%. Similarly, soils were grouped into three categories in relation to pH values: 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.

### 3 Results

Fig. 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 11,800 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  $\mu g CO_2 g^{-1} h^{-1}$ ). Agricultural soils show, for both methodological approaches, a smaller level of variability.

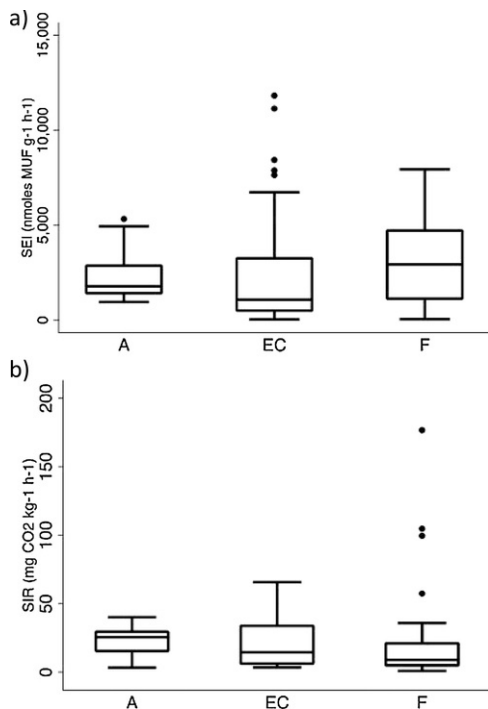


Fig. 1 Boxplot of microbial functional capacity measured by means of enzyme activities and CLPP-MicroResp. a) SEI (synthetic enzymatic index) and b) SIR (substrate induced respiration) distributions in the three soil categories (F, forest, A, agricultural and EC, extreme conditions soils).

alt-text: Fig. 1

Fig. 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 (expressed in terms of z-scores) 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.

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.

alt-text: Table 2

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

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).

**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

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  $\geq 7.4$ ).

**Table 4** Results of Chi-squared statistics  $\chi^2$  and  $p$ -values obtained with Kruskal-Wallis rank test on soil functional diversity (soil microbial functional diversity indices) (H'EA and H'MR) among the three land use categories ("among the three land use categories" should be deleted) within restricted classes of TOC (total organic carbon (TOC)) and pH. P values are reported in parentheses.

TOC values	H'EA	H'MR
Low: TOC < 1.5%	2.202 (0.333)	11.039 (0.004)
Medium: 1.5 $\leq$ TOC < 3%	7.640 (0.022)	6.272 (0.043)
High: TOC $\geq$ 3%	4.431 (0.035)	7.150 (0.007)
pH values (The columns of H'EA and H'MR for TOC and pH values should be aligned)		
pH < 6.5		11.843 (0.003)
6.5 $\leq$ pH < 7.4		13.867 (0.001)
pH $\geq$ 7.4		1.046 (0.306)
		8.971 (0.011)
		30.998 (0.000)
		2.517 (0.113)

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 analysis of 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.

**Table 5** Estimation results of (Relace the sentence "Estimation results of QRMs (0.25, 0.50, 0.75 quantiles)" with "Estimation results of quantile regression models (QRMs) between soil microbial functional diversity indices (H'EA and H'MR) and the selected variables (land use category: forest soils - F, agricultural soils - A, extreme conditions soils - EC; pH and total organic carbon ranges) at quantiles 0.25, 0.50, 0.75".) 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 $\leq$ TOC < 3	0.027	0.120		0.505	0.338	

High: TOC $\geq$ 3%	0.004	0.150		0.744	0.436	
pH (ref. pH < 6.5)						
6.5 $\leq$ pH < 7.4	-0.177	0.077	**	0.295	0.183	
pH $\geq$ 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 $\leq$ TOC < 3	-0.197	0.183		-0.043	0.097	
High: TOC $\geq$ 3%	-0.583	0.340		-0.031	0.271	
pH (ref. pH < 6.5)						
6.5 $\leq$ pH < 7.4	0.066	0.259		0.213	0.088	*
pH $\geq$ 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 $\leq$ TOC < 3	-0.001	0.142		0.008	0.072	
High: TOC $\geq$ 3%	-0.549	0.256	*	-0.070	0.136	
pH (ref. pH < 6.5)						
6.5 $\leq$ pH < 7.4	-0.008	0.233		0.079	0.073	
pH $\geq$ 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: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\* $p < 0.001$ .

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).

## 4 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 and Jackson, 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 potentials, 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) on pH was positive at 0.50, and 0.75 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 and Jackson (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  $\beta$ -diversity was observed at increasing pH in a spatial assessment of soil bacterial community profiles across Great Britain. Fierer and Jackson (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).

## 5 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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## 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.

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