

# Role of Humo-Enzyme Complexes in Restoring of Soil Ecosystems

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**Abstract** Conventional chemical (total organic carbon, total nitrogen, C/N ratio), biochemical (total and extracellular  $\beta$ -glucosidase, ATP, dehydrogenase) and unconventional IEF parameters (active humic carbon, humic-bound  $\beta$ -glucosidase activity), were used in the monitoring of the changes in biochemical properties caused by organic amendment practices. Two soil ecosystems characterized by a gradient of different grass covers, i.e. (1) a natural Catena (control) and (2) a managed Catena altered by amending practices were selected in a semi arid zone of the Mediterranean (Murcia region, Spain). Both natural and managed Catenas showed activation of carbon cycle which gradually shifted vegetal carbon toward the humic substance formation and accumulation of active humic- $\beta$ -glucosidase complexes. The model of study and the correlation among the selected parameters, has permitted to discriminate even little differences in soil biochemical properties and, on the basis of these properties, to rank the soil ecosystems in a decreasing order of quality: forest > shrub > bare. There was a narrow correlation between amount of humic carbon forms and humic-associated enzyme-activity, demonstrating that a sort of humification in situ was steadily taking place even after years and likely sustained by plant root exudates. The combination of UF and IEF resulted very efficient in the characterization of humic-enzyme complexes and biochemical processes which drive the humic substances formation, storage and activity in soils.

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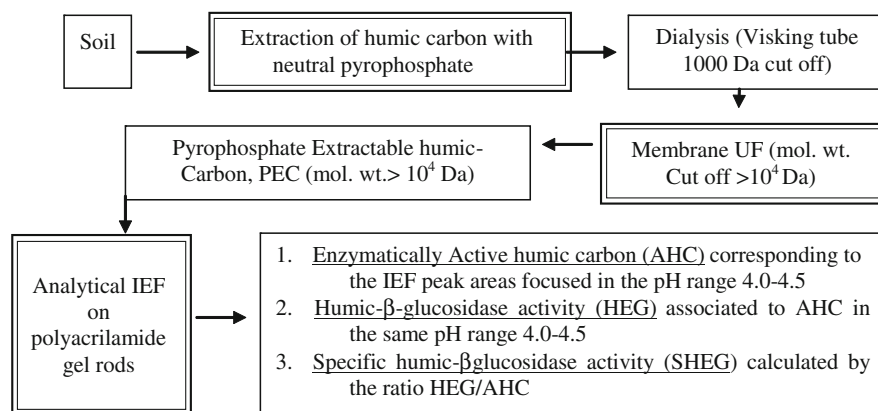
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## 1 Introduction

Native soil systems are characterised by an equilibrium between the main physical, chemical, and biochemical processes taking place in function of environmental conditions. The alteration of this equilibrium by natural or anthropogenic activity, may cause instability and stress (Doran and Parkin 1994). Usually microbial biomass and activity can be considered good indicators (bioindicators) of soil biological status, however, microorganisms are increasingly criticized because they are vulnerable to temporal and spatial variation (Chen et al. 2003; Gil-Sotres et al. 2005). For instance, dehydrogenase activity and ATP, and enzymes activities belonging to the microbial cells are likely changeable to transitory fluctuations in response to recent management and climatic effects, thus they are not reliable marker to assess of soil degradation (Gil-Sotres et al. 2005).

The extracellular biochemical activities stabilized out of the microbial cells, result suitable in assessing significant changes caused on soil ecosystems by external pressures. The extracellular enzymes, complexed with or entrapped within soil clays and humic matter (humo-enzyme complexes), are relatively stable and can persist for extended periods, thereby providing a long-term perspectives in indicating the history of the soil and not just a snapshot of the time of soil sampling (Lähdesmäki and Piispanen 1992; Ceccanti et al. 2008). Their importance arises from the fact that they can represent a reservoir of biochemical energy and nutrients capable to reactivate the ecosystem functionality even in heavy stressed situations, thus representing the necessary conditions for soil resilience (Ceccanti and Masciandaro 2003; Benítez et al. 2004). Since they have been found in a great variety of natural and managed soils humic-enzyme complexes they are supposed to constitute structural components of soil organic matter that significantly contribute to the empowering the biological barrier at the protection of the final and irreversible soil degradation (Klein et al. 1985; Ceccanti and Masciandaro 2003). The biochemical techniques to purify and characterize active humic-enzyme complexes were initially proposed by Ceccanti et al. (1978) and Ceccanti and Masciandaro (2003), revised by Ceccanti et al. (2008) and they are based on three steps: (1) sodium pyrophosphate extraction (pH 7.1) of humic matter, (2) purification by membrane cut off (mol wt > 10<sup>4</sup> Da) ultrafiltration (UF) of the organic extracts previously dialysed on Visking tube (1,000 Da), followed by (3) fractionation and characterization through analytical isoelectric focussing technique (IEF) (Fig. 1). IEF is an electrophoretic technique, which has been used with the purpose of the in-depth investigation of humic matter and humic-bound enzymes extracted from soils (Ciavatta and Govi 1993; Ceccanti et al. 1989) or other organic materials (Canali et al. 1998; Benítez et al. 2000). IEF is based on the separation of different humic substances on the basis of their isoelectric point (*pI*), that is, according to their net surface electric charges. Since the IEF is a non-denaturing technique, the humic-enzyme activity on the focussed bands can easily be detected.

The objective of this work was to compare the evolution of chemical and biochemical markers of soil quality in two soil ecosystems characterized by



**Fig. 1** Schematic flow-sheet of humic-enzyme complexes preparation and purification

(1) naturally altered soil caused by different plant cover (natural Catena) and (2) soil under a gradual practice of amelioration through organic amendment (managed Catena). For this purpose traditional parameters related to soil agro-chemical fertility and unconventional parameters related to the amount of the enzymatically active humic carbon have been proposed.

## 2 Materials and Methods

### 2.1 Case Studies

The sites selected for the study are located in the province of Murcia, Spain. The climate of the region is semi-arid Mediterranean with a mean annual rainfall of 300 mm, about 75% of which falls in April and October. One characteristic of the rainfall is its irregularity; it is infrequent but usually it is intense and gives rise to serious episodes of soil erosion. The mean annual temperature is 17°C and mean potential evapotranspiration reaches 800 mm year<sup>-1</sup>.

### 2.2 Managed Catena

An experimental field (sandy clay loam soil, USDA classification) was split into plots in which 16 years ago was added a single dose of fresh easily degradable municipal organic waste (MOW) in such dose as to increase the soil organic matter by 0.5, 1.0, 1.5 and 2.0%; MOW fraction (dry matter 55%; water 45%, ashes 22.4%, organic matter 32.6; pH 6.5; electric conductivity 4.2 dS m<sup>-1</sup>) was

incorporated into the top 15 cm of soil using a rotovator. The aim was to restore biochemical and microbial properties and to contrast the erosion through a stimulation of spontaneous establishment of grass cover. One plot was used as control (C). The plots were checked for three years: following organic amendments a 60–70% plant coverage developed and persisted throughout the experiment until today, while 20–30% plant coverage was found in the control soil. After 16 years from the treatment the same vegetated plots have been sampled in order to assess and investigate the changes occurred in consequence of the vegetal cover establishment.

### **2.3 *Natural Catena***

A site characterized by three different (sandy loam, USDA classification) gradual degradation states, related to different natural plant cover establishment (natural Catena) has been sampled in Santomera area (1) a natural soil with a vegetation of *Pinus halepensis* (50–60% vegetation cover) (forest); (2) a partially degraded soil with a 20–30% of vegetation cover (autochthonous xerophytic shrub) (shrub); and (3) a bare soil with only a 5–10% of vegetation cover (bare).

### **2.4 *Soil Sampling***

Three samples were taken from each sites: each sample consisted of eight subsamples taken from the top 15 cm of soil. The subsamples were mixed, homogenised, sieved (2 mm) and stored dry at room temperature until laboratory analysis.

### **2.5 *Chemical and Biochemical Parameters***

Electrical conductivity (EC) and pH were measured in 1:10 (w:v) aqueous solution. Total organic carbon (TOC) and total nitrogen (TN) were determined by dry combustion with a RC-412 multiphase carbon and a FP-528 protein/nitrogen determinator, respectively (LECO Corporation). Pyrophosphate-extractable carbon  $> 10^4$  Da (PEC  $> 10^4$ ) was extracted at 37°C for 24 h under shaking at 200 oscillation  $\text{min}^{-1}$ , using  $\text{Na}_2\text{P}_4\text{O}_7$  (0.1 M, pH 7.1) as extractant in a 1:10 w:v ratio following the Ceccanti et al. (2008) method. Then, pyrophosphate extract was filtered on a 0.22  $\mu\text{m}$  Millipore membrane and passed through an ultrafiltration AMICON PM10 cut-off membrane to obtain fractions  $>10,000$  and  $<10,000$  Da. The C content of PEC  $> 10^4$  Da was determined by dichromate oxidation (Yeomans and Bremner 1988). ATP was extracted from soil using the Webster et al. (1984) procedure and determined by the firefly luciferin–luciferase enzyme assay as described by Ciardi and Nannipieri (1990).

Dehydrogenase activity (DH-ase) was measured using 0.4% 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-tetrazolium chloride (INT) as substrate; idonitrotetrazolium formazan (INTF) produced in the reduction of INT was measured with a spectrophotometer at 490 nm (Masciandaro et al. 2000).

The  $\beta$ -glucosidase activity was determined on 1 g of air-dried soil (total  $\beta$ -glucosidase activity, TG) or 1 ml of soil pyrophosphate extract fraction  $> 10^4$  Da (extracellular  $\beta$ -glucosidase activity, EG), using 0.05 M *p*-nitrophenyl- $\beta$ -D-glucanopyranoside (PNG) as substrate. The *p*-nitrophenol (PNP) produced by both hydrolases was extracted and determined spectrophotometrically at 398 nm (Masciandaro and Ceccanti 1999).

## 2.6 Humic Matter Extraction

Pyrophosphate-extractable carbon (PEC) was extracted at 37°C for 24 h under shaking, using  $\text{Na}_2\text{P}_4\text{O}_7$  (0.1 M, pH 7.1) as extractant in a 1:10 w:v ratio (Ceccanti et al. 1978). Then, the suspension was centrifuged and filtered through a 0.22  $\mu\text{m}$  Millipore membrane to remove bacterial cells.

*Dialysis:* PEC was dialysed against distilled water using 1,000 MW Visking tube (1,000 Da), until reaching electrical conductivity values of less than 0.5 dS  $\text{m}^{-1}$ .

*Membrane ultrafiltration (UF):* The dialyses extract was recovered and concentrated by an Amicon PM-10 diaflomembrane (molecular cut-off 10,000 Da) under  $\text{N}_2$  atmosphere (1.5 bar) (Ceccanti et al. 1989). The concentration was carried out at 4°C, maintaining the sample constantly under stirring, until a final volume of about 10 ml. The fraction with molecular weight  $> 10^4$  Da (PEC  $> 10^4$ ) was recovered and used for further analysis.

## 2.7 Isoelectric Focussing (LEF)

IEF was carried out in cylindrical gel rods (0.5  $\times$  8 cm) containing polyacrylamide gel (5% w:v) and carrier ampholines in the pH range 4–6 (Bio-Rad Laboratories, Richmond, California, USA) at a final concentration of 2% (Ceccanti et al. 1989). TEMED (N,N,N',N'-Tetramethyl-1,2-diaminomethane) and ammoniumperoxy-disulfat were also added in gel solution at 0.03%. An aliquot of 100  $\mu\text{l}$  of organic material (PEC  $> 10^4$  Da) at 4.4% of glycerine was applied at the top of the gel rod (cathode). A little amount of glycerine at 2.2% was put on the sample to avoid interference and mixing with the cathodic solution (NaOH 0.02 N); 0.01 M  $\text{H}_3\text{PO}_4$  was used for the anodic cell. A pre-run of one hour at the same current intensity and voltage used for the samples run was carried out for each gel tube (1.5 mA for each tube, 100–800 Volt); subsequently the samples run was carried out for 2 h or more until a stable IEF banding was reached.

The electrophoretic bands were scanned by a Bio-Rad GS 800 densitometer, obtaining a typical IEF densitogram for each soil investigated. Gel pH was measured at 0.5 cm intervals with an Orion microprocessor (model 901, Orion research) connected to a microelectrode gel-pHiler (Bio-Rad Laboratories, Richmond, California, USA).

## ***2.8 Specific Selected Parameters***

### **2.8.1 Active Humic Carbon (AHC)**

The IEF peaks area was determined for each soil IEF densitogram, assuming as 100% the area under the entire IEF profiles (representative of the total loaded carbon). The peaks area focused in the pH range 4.0–4.5 was calculated and expressed as  $\text{mg C kg}_{\text{ds}}^{-1}$ ; this was labelled as active humic carbon (AHC).

### **2.8.2 $\beta$ -glucosidase Activity in Humic Bands (HEG)**

To analyse  $\beta$ -glucosidase activity of the humic bands obtained by IEF, the gel was gently removed from the inside of the glass tubes. The bands were cut, pre-washed for 1 h with 2 ml 0.1 M phosphate buffer, pH 6.4, at 37°C. Pre-washing removes the carrier ampholytes, salts and other impurities from the gel, without freeing the gel-trapped humic matter (Ceccanti et al. 1989). After removal of buffer, 2 ml of fresh 0.1 M phosphate buffer, pH 6.4 and 0.5 ml 0.05 M *p*-nitrophenyl- $\beta$ -D-glucopyranoside (PNG) were added in order to assay the enzyme activity. Incubation was carried out at 37°C under shaking for 17 h.

### **2.8.3 Specific Humic- $\beta$ -glucosidase Activity (SHEG)**

The activity was calculated by the ratio HEG/AHC and was expressed as  $\text{mg PNP g C}^{-1} \text{ h}^{-1}$ .

## ***2.9 Statistical Analysis***

All results are the means of three replicates. Differences among the soils were tested by analysis of variance (ANOVA). The means were compared by using least significant differences calculated at  $P < 0.05$  (Fisher's Test) (STATISTICA 6.0 software). A correlation matrix ( $P < 0.01$ ,  $P < 0.05$ ) of the data, for each site, was also calculated in order to determine the relationship between the parameters. Stepwise linear forward regression analysis was carried out. The stepwise

**Table 1** Chemical parameters of soil

Soil samples (%)	pH	EC	TOC	TN	C/N
0.0	7.82 ± 0.04 <sup>a</sup>	642 ± 46 <sup>d</sup>	13.9 ± 1.6 <sup>e</sup>	0.95 ± 0.11 <sup>e</sup>	14.6
0.5	7.67 ± 0.06 <sup>b</sup>	927 ± 35 <sup>c</sup>	23.4 ± 0.9 <sup>d</sup>	1.92 ± 0.06 <sup>c</sup>	12.2
1.0	7.39 ± 0.12 <sup>c</sup>	2,051 ± 60 <sup>b</sup>	27.2 ± 1.4 <sup>c</sup>	1.60 ± 0.18 <sup>d</sup>	17.0
1.5	7.45 ± 0.08 <sup>c</sup>	2,666 ± 115 <sup>a</sup>	33.7 ± 1.2 <sup>b</sup>	2.47 ± 0.13 <sup>a</sup>	13.6
2.0	7.50 ± 0.10 <sup>c</sup>	2,713 ± 88 <sup>a</sup>	38.8 ± 1.3 <sup>a</sup>	2.21 ± 0.10 <sup>b</sup>	17.6

Different letters indicate statistically different values ( $P < 0.05$ ). *EC* electrical conductivity (Ds m<sup>-1</sup>), *TOC* total organic carbon (mg C g<sup>-1</sup>), *TN* total nitrogen (mg N g<sup>-1</sup>)

regression analysis was used to quantify the relation between two and more co-variables and an outcome variable (Feinstein 1996). Stepwise selection indicates covariables with a statistically significant effect, simultaneously adjusting for the other covariables in the regression model.

### 3 Results

#### 3.1 Managed Catena

The content of total organic carbon (TOC) increased significantly with organic matter addition (Tables 1 and 2), as expected. Not expected was, instead, the persistence of such an increase during almost two decades as it has been found in our soils after 16 years. This demonstrated that the effect of a single application of organic matter, stimulated the growth and maintenance of a vegetation cover which still persisted until now. Similarly the pyrophosphate extractable carbon (PEC > 10<sup>4</sup>) and the  $\beta$ -glucosidase activity detected in the whole soil (TG) showed increasing values going from 0 to 2% treatments, these parameters resulted, in fact, significantly correlated at  $P < 0.01$  (TOC-PEC > 10<sup>4</sup>  $r = 0.99$ ; TOC-TG  $r = 0.99$ ; PEC > 10<sup>4</sup>-TG  $r = 0.98$ ) (Tables 2 and 4). This result suggested that carbon cycle persisted active and capable to transform most of added organic carbon into a more stable form (PEC > 10<sup>4</sup>); this pathways was probably sustained by plant root exudates released by the persistent plant coverage.

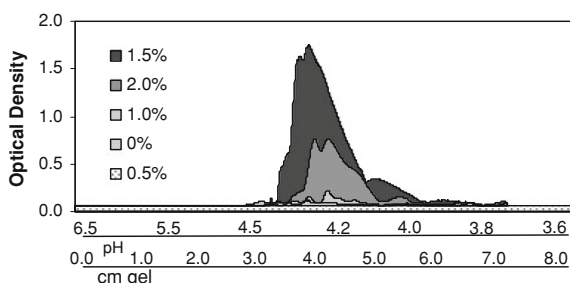
The DH-ase activity and ATP were correlated ( $r = 0.99$ ,  $P < 0.01$ ) and generally increased with the increase of organic amendment (Table 2). In general, the biochemical parameters showed a peak at 1.5% of MOW, meaning that some inhibition can be occurred at the highest (2.0%) MOW concentration (Table 2). The 1.5% treatment showed a maximum value of total nitrogen and, as a consequence, a minimum of C/N ratio (Table 1).

The relation between soil humic substances and associated extracellular enzyme activities was evidenced also by IEF results. Figure 2 and Table 3 showed an increase of humic carbon (AHC) and associated  $\beta$ -glucosidase activity (HEG)

**Table 2** Biochemical parameters of soil

Soil samples (%)	PEC > 10 <sup>4</sup>	TG	EG	DH-ase	ATP
0.0	522 ± 10 <sup>d</sup>	70 ± 6 <sup>c</sup>	3.6 ± 0.2 <sup>c</sup>	1.76 ± 0.09 <sup>d</sup>	25.0 ± 4 <sup>c</sup>
0.5	977 ± 22 <sup>c</sup>	206 ± 1 <sup>d</sup>	6.9 ± 0.5 <sup>c</sup>	1.72 ± 0.1 <sup>d</sup>	27.8 ± 3 <sup>c</sup>
1.0	1,070 ± 38 <sup>b</sup>	346 ± 13 <sup>c</sup>	20.4 ± 1.6 <sup>b</sup>	3.60 ± 0.22 <sup>c</sup>	62.2 ± 8 <sup>b</sup>
1.5	1,485 ± 75 <sup>a</sup>	407 ± 25 <sup>b</sup>	30.7 ± 2.4 <sup>a</sup>	5.07 ± 0.41 <sup>a</sup>	110 ± 7 <sup>a</sup>
2.0	1,501 ± 103 <sup>a</sup>	544 ± 43 <sup>a</sup>	21.8 ± 1.3 <sup>b</sup>	4.81 ± 0.33 <sup>a</sup>	106 ± 9 <sup>a</sup>

Different letters indicate statistically different values ( $P < 0.05$ ). *PEC* > 10<sup>4</sup> pyrophosphate extractable carbon fraction > 10<sup>4</sup> Da (mg C kg<sup>-1</sup>), *TG* total  $\beta$ -glucosidase activity (mg PNP kg<sup>-1</sup> h<sup>-1</sup>), *EG* extracellular  $\beta$ -glucosidase activity (mg PNP kg<sup>-1</sup> h<sup>-1</sup>) *DH-ase* dehydrogenase activity (mg INTF kg<sup>-1</sup> h<sup>-1</sup>), *ATP* (ng g<sup>-1</sup>)

**Fig. 2** IEF densitograms of humic matter (PEC > 10<sup>4</sup> Da) extracted from each soil

with the increase of amendments with the exception that the values in 1.5% treatment were higher than in 2.0%. The low humic-bound  $\beta$ -glucosidase activity (HEG) in 2.0% amendment was also evident in the specific humic- $\beta$ -glucosidase activity (SHEG) found in 2.0% treatment.

### 3.2 Natural Catena

Total organic carbon (TOC) and pyrophosphate extractable carbon (PEC > 10<sup>4</sup>), as expected, resulted higher in the forest site (Table 5), while not statistically significant differences were found in bare and shrub soils indicating that the vegetal cover in shrub site is not sufficient to preserve soil organic matter content (Table 7). All biochemical parameters resulted markedly higher in the forest site, generally showing values 2–3 fold those of the shrub and bare soils or even much higher (15-fold) as found for EG (Table 5).

Among these biochemical parameters, only dehydrogenase (DH-ase) and the extracellular  $\beta$ -glucosidase (EG) resulted able to discriminate the soils in the following order: forest > shrub > bare (Table 5). In the Fig. 3 are reported the densitograms tracing the profiles of the humic carbon PEC > 10<sup>4</sup> after IEF; in terms of PEC > 10<sup>4</sup>, the forest site showed two broad peaks corresponding to averagely 5 times the height of peaks in shrub and bare soils. Similarly the



**Table 3** Amount of enzymatically active humic carbon (AHC) and associated extracellular  $\beta$ -glucosidase activity (HEG) focussed in the pH range 4.0–4.5

Soil samples organic matter (%)	AHC (mg C kg <sup>-1</sup> )	HEG (mg PNP kg <sup>-1</sup> h <sup>-1</sup> )	SHEG (mg PNP g C <sup>-1</sup> h <sup>-1</sup> )
0.0	101 ± 6 <sup>c</sup>	1.01 ± 0.09 <sup>c</sup>	9.96
0.5	183 ± 11 <sup>d</sup>	1.20 ± 0.06 <sup>c</sup>	6.56
1.0	256 ± 15 <sup>c</sup>	2.11 ± 0.12 <sup>b</sup>	8.25
1.5	596 ± 45 <sup>a</sup>	7.27 ± 0.22 <sup>a</sup>	12.20
2.0	384 ± 26 <sup>b</sup>	1.31 ± 0.05 <sup>d</sup>	3.41

Different letters indicate statistically different values ( $P < 0.05$ ). *AHC* active humic carbon calculated from the IEF peak areas focused in the pH range 4.0–4.5, *HEG* humic-bound  $\beta$ -glucosidase activity pH 4.0–4.5, *SHEG* specific humic- $\beta$ -glucosidase activity: HEG/AHC

associated  $\beta$ -glucosidase activity (HEG) focussed in the 4–4.5 pH range (Fig. 3, Table 6) resulted particularly higher in the forest soil suggesting a higher metabolic efficiency in this ecosystem. The same trend was observed for the specific humic- $\beta$ -glucosidase activity (SHEG) (Table 6). These parameters ranked the soil in the following order of degradation: bare > shrub > forest, confirming their contribute and reliability to explain the dynamics of the chemical and biological processes taking place in soil.

## 4 Discussion

The addition of organic matter in the restoration of arid soils provoked an increase of total organic carbon, demonstrating that the effect of a single application of organic matter 16 years ago, is still manifested in the accumulation and persistence of  $\beta$ -glucosidase humic complexes. This was probably due to the growth and maintenance of grass cover with time that notoriously has a double effect: (1) provide a source of slow degradable ligno-cellulosic material in the surface and (2) a release of an easily bioavailable carbon source constituted by root exudates (Manns et al. 2007; Fuentes et al. 2009). Both surface and underground carbon sources might have induced microbial synthesis of active  $\beta$ -glucosidase enzyme (García et al. 1997; Ros et al. 2003) which act in the last step of cellulose degradation, promoting a flow of easily metabolizable carbon from the plants to the soil, where part of which was transformed into stable humic substance capable to bind the extracellular enzymes. In our soils this mechanisms was generally confirmed by the evolution of the analytical results and especially by the narrow correlation found among the chemical carbon forms and biochemical parameters (Table 4).

The DH-ase and ATP are here confirmed as good indicator of soil microbial activity that although they are notably sensible and changeable in relation to minor change of soil conditions, they allow us to follow the activation of carbon cycle and turnover in arid soil environments. A high significant correlation was

**Table 4** Correlation matrix between chemical and biochemical parameters of soil samples of Natural Catena

	pH	EC	TOC	TN	C/N	PEC	TG	EG	DH-ase	ATP	AHC	HEG	SHEG
pH	+1.00	-0.99*	-0.93	-0.99**	-0.99**	-0.87	-0.90	-0.99*	-0.99	-0.88	-0.91+	-0.99	-0.92
EC		+1.00	+0.96	+0.99*	+0.99*	+0.91	+0.93	+0.99**	+0.99**	+0.91	+0.94	+1.00	+0.89
TOC			+1.00	+0.93	+0.93	+0.99	+0.99	+0.95	+0.96	+0.99	+0.99*	+0.98	+0.72
TN				+1.00	+0.99**	+0.87	+0.90	+0.99*	+0.99	+0.88	+0.91	+0.99	+0.92
C/N					+1.00	+0.87	+0.90	+0.99*	+0.99	+0.88	+0.90	+0.99	+0.92
PEC						+1.00	0.99*	+0.89	+0.91	+0.99**	+0.99*	+0.94	+0.61
TG							+1.00	+0.92	+0.93	+0.99*	+0.99*	+0.96	+0.65
EG								+1.00	+0.99*	+0.90	+0.92	+0.99	+0.90
DH									+1.00	+0.91	+0.94	+0.99	+0.89
ATP										+1.00	+0.99*	+0.94	+0.62
AHC											+1.00	+0.96	+0.67
HEG												+1.00	+0.84

Coefficient correlation values significantly different at \* $P < 0.05$  and \*\*  $P < 0.01$

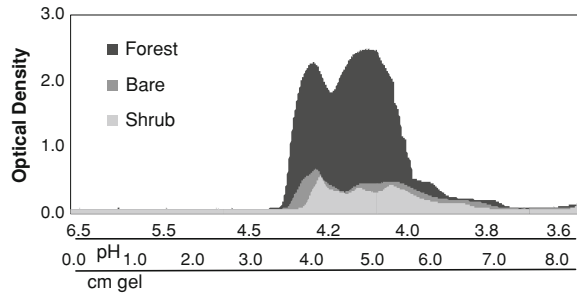
*EC* electrical conductivity, *TOC* total organic carbon, *TN* total nitrogen, *PEC*  $> 10^4$  pyrophosphate extractable carbon fraction  $> 10^4$  Da, *TG* total  $\beta$ -glucosidase activity, *EG* extracellular  $\beta$ -glucosidase activity, *DH-ase* dehydrogenase activity; *AHC* active humic carbon calculated from the IEF peak areas focused in the pH range 4.0–4.5, *HEG* humic-bound  $\beta$ -glucosidase activity pH 4.0–4.1; *SHEG* Specific humic- $\beta$ -glucosidase activity: HEG/AHC

**Table 5** Chemical and biochemical parameters of soil samples

Soil samples	pH	EC	TOC	TN	C/N	PEC $> 10^4$	TG	EG	DH-ase	ATP
Bare	7.90 $\pm$ 0.05 <sup>a</sup>	248 $\pm$ 8 <sup>c</sup>	10.6 $\pm$ 0.9 <sup>c</sup>	0.64 $\pm$ 0.04 <sup>c</sup>	14.6	1,100 $\pm$ 70 <sup>b</sup>	552 $\pm$ 44 <sup>b</sup>	7.02 $\pm$ 0.61 <sup>c</sup>	6.21 $\pm$ 0.5 <sup>c</sup>	444 $\pm$ 16 <sup>b</sup>
Shrub	7.74 $\pm$ 0.08 <sup>b</sup>	312 $\pm$ 19 <sup>b</sup>	13.8 $\pm$ 1.4 <sup>b</sup>	1.55 $\pm$ 0.09 <sup>b</sup>	15.6	1,150 $\pm$ 120 <sup>b</sup>	608 $\pm$ 36 <sup>b</sup>	25.7 $\pm$ 2.1 <sup>b</sup>	9.48 $\pm$ 0.9 <sup>b</sup>	469 $\pm$ 22 <sup>b</sup>
Forest	7.59 $\pm$ 0.04 <sup>c</sup>	415 $\pm$ 22 <sup>a</sup>	46.0 $\pm$ 2.5 <sup>a</sup>	3.62 $\pm$ 0.21 <sup>a</sup>	16.6	5,050 $\pm$ 240 <sup>a</sup>	1,603 $\pm$ 111 <sup>a</sup>	107 $\pm$ 5 <sup>a</sup>	16.2 $\pm$ 0.6 <sup>a</sup>	1,590 $\pm$ 43 <sup>a</sup>

Legend: see Tables 1 and 2

**Fig. 3** IEF densitograms of humic matter (PEC > 10<sup>4</sup> Da) extracted from each soil



**Table 6** Amount of enzymatically active humic carbon (AHC) and associated extracellular  $\beta$ -glucosidase activity (HEG) focussed in the pH range 4.0–4.5

Soil samples	AHC (mg C kg <sup>-1</sup> )	HEG (mg PNP kg <sup>-1</sup> h <sup>-1</sup> )	SHEG (mg PNP gC <sup>-1</sup> h <sup>-1</sup> )
Bare	273 ± 16 <sup>c</sup>	1.22 ± 0.10 <sup>c</sup>	4.48
Shrub	331 ± 20 <sup>b</sup>	3.21 ± 0.06 <sup>b</sup>	9.68
Forest	1,574 ± 47 <sup>a</sup>	16.9 ± 1.8 <sup>a</sup>	10.7

Legend: see Table 3

also found between PEC > 10<sup>4</sup> and the activity of the crude pyrophosphate extract (EG) (Table 4), confirming the capability of the humic substance to protect and accumulate active free enzymes. This finding is very important because allows us to recover the biochemical fertility and soil functionality through the application of a source of degradable organic matter which promote a sort of humification in situ (Ceccanti and Masciandaro 2003; Doni et al. 2009) capable of permanently accumulate humic carbon and restore biological properties which are fundamental factors to resist soil degradation and desertification. The evolution of these biochemical processes have generally been found in various managed Catenas, whose dynamics were investigated through conventional and biochemical parameters (Gil-Sotres et al. 1992; Ceccanti et al. 1994; Ros et al. 2003), and was also confirmed in a natural Catena. Moreover, the managed Catena showed negative not significant correlations between the specific activity measured in the active humic-enzyme complex after IEF (SHEG) and almost all parameters considered, while in the natural Catena SHEG showed a weak positive correlation with the other parameters that could change into significant correlation with the time, as usually found. If confirmed in other similar managed and natural Catenas, this result could be considered a reference threshold for assessing the re-naturalization of a stressed soil ecosystem. Both in the time (Gil-Sotres et al. 1992) and in the space (Ceccanti et al. 1994), we are capable to reconstruct and control a Catena in order to drive a degraded soil ecosystem to an acceptable soil functionality. The analytical UF and IEF techniques have resulted very promising in discriminating between positive and inhibitory effects of organic matter addition such as it occurred at 1.5 and 2%,

**Table 7** Correlation matrix between chemical and biochemical parameters of soil samples of managed Catena

	pH	EC	TOC	TN	C/N	PEC	TG	EG	DH-ase	ATP	AHC	HEG	SHEG
pH	1.00	0.92*	-0.83	-0.70	-0.46	-0.83	-0.89*	-0.95*	-0.85	-0.83	-0.82	-0.64	+0.10
EC		1.00	0.94*	0.79	0.50	0.92*	0.95*	0.99**	0.97**	0.98**	0.94*	0.64	-0.26
TOC			1.00	0.92*	0.34	0.99**	0.99**	0.92*	0.86	0.90*	0.92*	0.51	-0.45
TN				1.00	-0.05	0.96**	0.89*	0.81	0.69	0.75	0.90*	0.59	-0.29
C/N					1.00	0.22	0.39	0.40	0.55	0.50	0.18	-0.13	-0.46
PEC						1.00	0.98**	0.92*	0.84	0.88*	0.95*	0.59	-0.36
TG							1.00	0.94*	0.86	0.88*	0.90*	0.50	-0.43
EG								1.00	0.95*	0.95*	0.95*	0.73	-0.13
DH									1.00	0.99**	0.9*	0.67	-0.17
ATP										1.00	0.94*	0.67	-0.22
AHC											1.00	0.79	-0.10
HEG												1.00	+0.53

Coefficient correlation values significantly different at \* $P < 0.05$  and \*\* $P < 0.01$ 

Legend: see Table 4

respectively, or in ranking the soil subsystems of a natural catena in a decreasing order of quality: forest > shrub > bare.

In order to explain more clearly the factors controlling carbon metabolism and humification process in the two Catenas, stepwise linear regression analysis was performed.

Active humic carbon (AHC) was selected as the more suitable dependent variable. The study of the stable organic C fraction (AHC) is relevant since it determines the potential for soil resilience and resistance of stressed soils, particularly in extreme environments (Ceccanti and Masciandaro 2003).

$$\text{AHC} = +0.330 \text{ HEG} + 1.07 \text{ TG} + 0.431 \text{ PEC} - 0.25 \text{ C/N}$$
$$R^2 = 0.998; \quad p = 0.0393$$

The active humic carbon (AHC), isolated through UF and IEF, clearly represents a significant fraction of the whole soil  $\beta$ -glucosidase activity (TG) and, in addition, supports the finding of previous studies (Doni et al. 2009; Masciandaro and Ceccanti 1999) that most of hydrolases (including  $\beta$ -glucosidase) in soil are associated with stable humic complexes. The C/N ratio is confirmed here as a factor controlling microbial metabolism shifting most of metabolized organic carbon and biochemical energy of the soil ecosystem towards the formation and accumulation of active humic-enzyme complexes.

## 5 Conclusions

- Stressed soil can be recovered through induced or spontaneous vegetal cover able to promote a carbon flow from the plants into the soil where part of which was transformed into stable humic substance capable to bind the extracellular enzymes.
- The use of UF and IEF in combination with common methodologies and other biological parameters, resulted very efficient in the characterization of humic-enzyme complexes aimed to better understand those biochemical processes which drive the humic substances formation, storage and activity in soils.
- The empowering of separative-purification methodologies and the use of IEF at preparative scale could provide in the future sufficient amount of humic-enzyme complexes to be further investigated through molecular structural analysis such as pyrolysis and the more recent powerful biomolecular technique.

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