

## **Hydrolase enzyme activities in a successional gradient of biological soil crusts in arid and semi-arid zones**

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### **ABSTRACT**

In arid and semi-arid regions, pioneer organisms form complex communities that penetrate the upper millimetres of the bare substrate, creating biological soil crusts (BSC). These thin crusts play a vital role in whole ecosystem functioning because they enrich bare surfaces with organic matter, initiate biogeochemical cycling of elements, modify hydrological cycles, etc., thus enabling the ground to be colonized by vascular plants. Various hydrolase enzymes involved in the carbon (cellulase,  $\beta$ -glucosidase and invertase activities), nitrogen (casein-protease and BAA- protease activities) and phosphorus (alkaline phosphomonoesterase activity) cycles were studied at three levels (crust, middle and deep layers) of three types of BSCs from the Tabernas Desert (SE Spain), representing an ecological gradient ranging from crusts predominated by cyanobacteria to crusts predominated by lichens (*Diploschistes diacapsis*, *Lepraria crassissima*). All enzyme activities were higher in all layers of all BSCs than in the bare substrate. The enzymes that hydrolyze low molecular weight substrates were more active than those that hydrolyze high molecular weight substrates (cellulase, casein-protease), highlighting the pioneering characteristics of the BSCs. The hydrolytic capacity developed in parallel to that of ecological succession, and the BSCs in which enzyme activity was highest were those under *Lepraria crassissima*. The enzyme activity per unit of total organic C was extremely high; the highest values occurred in the BSCs formed by cyanobacteria and the lowest in those formed by lichens, which

indicates the fundamental role that the primary colonizers (cyanobacteria) play in enriching the geological substrate with enzymes that enable degradation of organic remains and the establishment of more developed BSCs. The results of the study combine information on different enzyme activities and provide a clear vision of how biogeochemical cycles are established in BSCs, thus confirming the usefulness of enzyme assays as key tools for examining the relationship between biodiversity and ecosystem function in biological soil crusts.

*Key words:* Hydrolytic enzymes; Biochemical activity; Cyanobacteria; Lichens

## **1. Introduction**

Arid or semi-arid regions cover approximately one-third of the Earth's terrestrial surface. In these regions, the vegetation cover is incomplete and discontinuous as a result of combinations of adverse environmental factors (prolonged drought, high temperatures, high soil erosion rates, etc.). Nevertheless, highly specialized communities of organisms (algae, microfungi, cyanobacteria, liverworts, bryophytes and lichens) are able to adapt successfully to these adverse environmental conditions and colonize the bare disaggregated geological substrate (hereafter referred to as bare substrate). These organisms constitute complex communities that penetrate the upper millimetres of the geological substrate, forming thin biological soil crusts (BSCs) (Belnap and Lange, 2003).

The presence of BSCs is especially important in badlands systems (extremely dissected landscapes with scarce or no vegetation) under climates ranging from arid to sub-humid, where the crusts are a very frequent type of soil cover (Alexander et al., 1994). Soil is non-existent or incipient in these systems (Cantón et al., 2003), and it has been suggested that the growth and development of BSCs is crucial for geomorphological processes and preparation of the geological substrate for colonization by vascular plants (Lázaro et al., 2008). One example of such a system is the Spanish badlands, where the colonization of the geological substrate begins with growth of cyanobacteria, followed by establishment of pioneering crustose and squamulose lichens (Lázaro et al., 2008). This stage is followed by the replacement of pioneering lichen communities by complex communities (Malam Issa et al., 1999) and the establishment of vascular communities. Similar changes have been described in other parts of the world, where cyanobacterial BSCs are considered to represent the primary step in colonization and the crusts formed by lichens to represent the mature stage of BSC formation (Belnap and Lange, 2003; Lázaro et al., 2008).

BSCs carry out important functions in ecosystems (Belnap and Lange, 2003). Authors such as Bowker et al. (2010) have suggested that the main functions are as follows: a) fixation of C and N; b) participation in the control of stabilization/erosion processes by creating resistance to erosion and involvement in fixation of sediments; c) involvement in the hydrological cycle and in nutrient cycling by favouring availability of water and essential elements; and d) generation of indirect favourable effects derived

from the interspecific interactions between organisms. Each of these aspects has been the object of numerous studies in recent years (see Bowker et al, 2010 for a review). However, although much is known about the different functions of BSCs in ecosystems, very little is known about the biological and biochemical activity in BSCs and the geological substrate immediately below them, and even less is known about how this is affected by the successional stage of the BSC.

Measurement of enzyme activities is widely used to examine nutrient cycling processes in soil (Nannipieri et al. 1990; Tabatabai and Dick, 2002). Numerous enzymes are known to participate in the processes of organic matter decomposition and mineralization. However, few of these processes have been studied, largely because of a lack of suitable analytical methods resulting from the complexity of the reactions involved. The enzymes most commonly analyzed include various hydrolases involved in the C, N, P and S cycles, as well as some oxidoreductases. Analysis of soil hydrolases involved in C, N and P cycles may provide some insight into the metabolic capacity of the soil, so that the potential for transformation of specific sources of energy or nutrients can be assessed (Shaw and Burns, 2006).

Despite the importance of BSCs in favouring the establishment of biogeochemical cycles in bare substrate, few studies have analyzed enzyme activities in BSCs. Some of these studies have involved analysis of the activities of oxidative enzymes such as nitrogenase (Wu et al., 2009; Zhao et al., 2010), dehydrogenase (Bolton et al., 1993), peroxidase and phenol oxidase (Stursova et al., 2006, 2008). Other studies have focused on determining the activities of hydrolases such as urease (Bowker et al., 2011; Castillo-Monroy et al., 2011), arylsulphatase (Bolton et al., 1993) and  $\beta$ -glucosidase (Bolton et al., 1993; Bowker et al., 2011; Castillo-Monroy et al., 2011). As far as we know, the activities of several different hydrolytic enzymes that participate in the carbon, nitrogen and phosphorus cycles have only been analyzed in conjunction by Stursova et al. (2006). Although existing studies have considered different types of BSCs and have compared enzymatic activities (Collins et al., 2008), the developmental gradients of different types of BSC existing at a particular location have not been investigated, despite the great importance of this in understanding how the cycles of the main biological important elements are modified as the successional or developmental stages of BSCs are established.

The aim of the present study was to determine the activities of several hydrolases involved in the C, N and P cycles, in a successional gradient of BSCs in a badland ecosystem under a desert-subdesertic climate in the Tabernas Desert (Almería, SE Spain). The analyses were carried out immediately after a period of rainfall to provide a view of how different biogeochemical cycles are expressed in arid and semi-arid environments when biological activity is maximal. Moreover, unlike other studies that have only considered the upper part of the BSC in contact with the atmosphere (crust layer), different layers were analyzed (crust layer, middle layer, deep layer) in the present study, to determine how the geological substrate at different depths is affected by growth of the colonizing organisms. The starting hypothesis for the study was that

substitution of the autotrophic colonisers in BSCs by heterotrophic organisms produces an increase in hydrolase enzyme activity, which favours the availability of nutrients and thus guarantees the continuance of the heterotrophs.

## 2. Material and Methods

### 2.1. Site description

The study was conducted at an experimental site (El Cautivo) located in the Neogene–Quaternary Tabernas depression, in the province of Almería, SE Spain (Fig. 1). The basin is mainly filled with Neogene marine sediments, most of which are gypsum mudstones and calcaric sandstones. The climate is semi-arid thermo-Mediterranean, with dry and hot summers and mild temperatures throughout the rest of the year (the average annual temperature is 17.9 °C). The mean annual rainfall is 235 mm, most of which falls in winter. Most rainfall events are of low magnitude (less than 10% over 20 mm) and low intensity. The number of rainy days per year ranges from 32 to 69 (Cantón et al. 2001). The annual potential evapotranspiration is around 1500 mm, indicating a high water deficit. The main types of soil at the site are Epileptic and Endoleptic Leptosols, Calcaric Regosols and Eutric Gypsisols, according to the World Reference Base for Soil Resources (ISSS Working Group R.B., 1998). The soil texture is silty loam (mean percentages of sand, silt and clay under BSCs are 29±5, 59±6 and 12±4, respectively), the pH ranges from 6.7 to 8.7, and the calcium carbonate equivalent ranges from 11% to 30%, with a general increase in surface or intermediate horizons, associated with the presence of calcic horizons (Chamizo et al., 2010). The landscape of the study area comprises a mosaic of zones with vascular plants, BSCs and bare substrate. The zones predominated by vascular plants also have BSCs in the inter-plant spaces. The main perennial plants are dwarf shrubs such as *Helianthemum almeriense* Pau, *Hammada articulata* (Moq.) O. Bolós & Vigo, *Artemisia barrelieri* Besser, *Salsola genistoides* Poiret, *Euzomodendron bourgeanum* Coss., as well as some grasses (*Stipa tenacissima* L.) and annual herbaceous vegetation predominated by *Stipa capensis* Thunb. The BSCs also cover complete landforms at the site (Cantón et al., 2003), and overall they represent more than 45% of the surface cover. Together with for low intensity hunting, the site is often used as a location for films and television programs, due to its scenic character. Several studies have been carried out at the site to investigate aspects such as geomorphology, hydrology and erosion, and generally provide a very detailed description of the area (Cantón et al., 2001, 2003; Lázaro et al., 2008, amongst others).

### 2.2. Biological soil crust (BSC) types and field sampling.

On the basis of the stage of development of the BSC, Lázaro et al. (2008) identified the following successional stages at this site, using the predominant organism and crust colour as indicators: 1) brown BSC or cyanobacteria-dominated BSC (CYANO),

representing an early successional stage of BSC, which often includes diverse species such as *Nostoc* spp., a relatively high proportion of squamulose lichen species and *Microcoleus* spp. sheath material; 2) white lichen BSC, predominated by *Diploschistes diacapsis* (Ach.) Lumbsch (DIPLOS), which represents the second developmental stage, and 3) white lichen BSC, predominated by *Lepraria crassissima* (Hue.) Lettau (LEPRA), which represents the last development stage and includes diverse ecological associations with small lichen species such as *Collema* spp, *Placynthium nigrum* (Huds.) Gray and *Toninia sedifolia* (Scop.) Timdal.

Three composite samples of each type of the above-mentioned BSCs were obtained in January 2011 (i.e. during the rainy season; the total precipitation in the month prior to sampling was 61.50 mm, and the total precipitation for the three days immediately before sampling was 0.75 mm), by mixing 10-15 subsamples (each representative of an area about 0.1 ha), in the field. In all cases, BSC samples were collected from plant interspaces on hillslopes where vascular plants are scarce and disperse and the BSCs almost cover the entire landform. In the DIPLOS and LEPRA BSCs, the upper few centimetres of the surface (0-0.5 cm) were extracted (crust layer). Once the crust layer was isolated, samples were collected from two different depths (middle layer: 0.5-3 cm and deep layer: 3-5 cm). Only two layers were sampled in the CYANO BSCs because these crusts were less well developed: the upper 0-0.5 cm (crust layer) and the 0.5-3 cm immediately below the crust (deep layer) were sampled. The bare upper surface (0-0.5 cm) of the disaggregated geological substrate (bare substrate) was also sampled in surrounding areas (10-15 subsamples were also obtained as above).

The samples were transported to the laboratory in isothermal bags and were sieved (<4 mm) and stored at 4 °C until analysis of enzyme activities, in all cases within 15 days of collection. The moisture content was determined by gravimetry in an aliquot of field samples dried at 105 °C for 24 h. Another portion of each sample was air-dried before carrying out general analyses.

### 2.3. Analysis of general properties

The methods described by Guitián and Carballas (1976) were used to determine the following soil properties: pH in water (1:2.5, soil:water ratio), pH in 1 M KCl (1:2.5, soil:solution ratio), total organic carbon content (TOC content, by wet oxidation with potassium dichromate oxidation and previous elimination of carbonates), and total nitrogen content (TN content, by Kjeldahl digestion).

### 2.4. Analysis of enzyme activities

All of the enzymes analysed were hydrolases involved in carbon, nitrogen and phosphorus cycling and were selected for their role in key processes in the decomposition of organic remains.

The only P cycle enzyme analysed was alkaline phosphomonoesterase. This enzyme, which catalyzes the hydrolysis of phosphate monoesters to release

orthophosphate, is important for its fundamental role in phosphate nutrition of microorganisms and plants. Alkaline phosphomonoesterase activity was determined after incubation of the sample with 16 mM *p*-nitrophenyl phosphate as substrate, in Modified Universal Buffer (MUB) pH 11.0, at 37 °C. After 30 min, 0.5 M CaCl<sub>2</sub> was added and the *p*-nitrophenol released was extracted with 0.5 M NaOH (Tabatabai and Bremner, 1969). The enzymatic activity was quantified by reference to calibration curves corresponding to *p*-nitrophenol standards incubated with each sample, under the same conditions as for the samples; the activity is expressed as  $\mu\text{mol } p\text{-nitrophenol g}^{-1} \text{ h}^{-1}$ .

The C cycle hydrolases analysed were cellulase and  $\beta$ -glucosidase, which are both involved in different stages of degradation of cellulose to glucose, and invertase, which catalyzes the hydrolysis of sucrose to fructose and glucose. The  $\beta$ -glucosidase activity was determined as described for phosphomonoesterase activity, except that the substrate was 25 mM *p*-nitrophenyl- $\beta$ -D-glucopyranoside, the buffer (MUB) pH was 6.0, the incubation time was 1 h and the released *p*-nitrophenol was extracted with 0.1 M (Tris-hydroxymethyl-aminomethane)-NaOH (THAM-NaOH) of pH 12 (Eivazi and Tabatabai, 1988). In the same way as for alkaline phosphomonoesterase, the  $\beta$ -glucosidase activity was quantified by reference to calibration curves corresponding to *p*-nitrophenol standards incubated with each sample, under the same conditions as for the samples; the activity is expressed as  $\mu\text{mol } p\text{-nitrophenol g}^{-1} \text{ h}^{-1}$ .

Invertase activity was determined after incubating the sample with 35.06 mM sucrose, as substrate, for 3 h at 50 °C, with 2 M acetate buffer pH 5.5; the reducing sugars released were determined following the method of Schinner and von Mersi (1990). Carboxymethyl-cellulase (Cellulase) activity was determined in a similar way, except that the substrate was 0.7% carboxymethyl-cellulose and the incubation time was 24 h (Schinner and von Mersi, 1990). In both cases, the enzyme activities are expressed as  $\mu\text{mol glucose g}^{-1} \text{ h}^{-1}$ .

The N cycle enzymes analysed were protease-casein, which is considered to be an indicator of the capacity of a soil to degrade proteins (thus releasing polypeptides), and protease-BAA, which is considered to be an indicator of the capacity of a soil to hydrolyze relatively small peptides (thus releasing amino acids). The activity of benzoyl-argininamide hydrolysing protease (BAA-protease) was determined as described by Nannipieri et al. (1980). Briefly, BAA-protease activity was determined after incubation with 30 mM  $\alpha$ -N-benzoyl-L-argininamide (BAA), as substrate, for 1.5 h at 37 °C in 0.2 M phosphate buffer pH 7.0, and measuring the NH<sub>4</sub><sup>+</sup> released with an ammonium electrode; the enzyme activity is expressed as  $\mu\text{mol NH}_3 \text{ g}^{-1} \text{ h}^{-1}$ . The activity of casein hydrolysing protease was determined after incubation with 1% casein, as substrate, for 2 h at 50 °C in 0.05 M Tris-hydroxymethyl-aminomethane-HCl (TRIS-HCl) buffer pH 8.1, and the released amino acids were measured by the Folin-Ciocalteu colorimetric method described by Ladd and Butler (1972), modified by Nannipieri et al. (1979). Enzyme activity is expressed as  $\mu\text{mol tyrosine g}^{-1} \text{ h}^{-1}$ .

All determinations were performed in triplicate. The corresponding controls were carried out for each sample and enzyme activity by proceeding with the same analytical protocol described, but without the addition of the substrate at the moment of initiating the enzymatic reaction. For each sample the mean values of the three determinations (expressed on an oven-dried basis, 105 °C) were calculated.

### 2.5. Statistical analysis

Statistical analyses (means, deviations, Tukey tests of significance between means) were performed with Statistica 6.0 (StatSoft®) for Windows (StatSoft Inc., 2001). All individual *P*-values from statistical tests of significance between means are provided as supplementary data.

## 3. Results

### 3.1. General data

The pH (both in water and in KCl) of all three types of BSC was clearly lower than that of the bare substrate (Table 1). The difference was relatively small for the CYANO crust layer (the difference was 0.47 units for the pH in water), but rather large for the DIPLOS crust layer (1.44 units). The pH of the LEPRA crust layer was intermediate between the pH values of the other two crusts. In all cases the decrease in pH occurred gradually towards the surface, so that the pH of the deepest layer in each of the BSCs was not significantly different from that of the bare substrate (Table 1). Development of the BSCs affected the moisture content of the samples, which was always significantly higher, for all layers and types of BSCs sampled, than that of the bare substrate (Table 1).

The TOC content of all BSCs was significantly higher than that of the bare substrate; the TOC content was highest in the DIPLOS crust layer, followed by the LEPRA crust (Table 1). The TN content also increased as a result of the presence of the different BSCs, although unlike for the TOC content, the values of TN in the deepest layers were not significantly different from those in the bare substrate (Table 1). The CYANO crust layer had the lowest TN content and the upper layers of the DIPLOS and LEPRA crusts had similar TN contents (Table 1). The C/N ratio was higher in the upper and deeper layers than in the bare substrate, whereas in the crust layers the values for CYANO and DIPLOS were similar to those of the bare substrate and were much higher in LEPRA than in the bare substrate (Table 1).

### 3.2. Enzyme activities

All enzyme activities measured in the bare substrate were extremely low, sometimes almost zero, indicating that despite the relatively high content of TOC, there was almost no hydrolase-type biochemical activity (Table 2). The presence of the BSCs implied significant increases in all enzyme activities, although gradual decreases were generally

observed from the crust layer to the deep layer. However, in the deepest layers from which samples were taken the enzymatic activities were generally significantly higher than in the bare substrate (Table 2). The cellulase and casein-protease activities were an exception to this as the values in the deepest layer in all three types of BSCs were not significantly different from the values in the bare substrate. The  $\beta$ -glucosidase activity in CYANO was also an exception to this, as although the mean value obtained for the deepest layer was higher than that obtained for the bare substrate, the difference between the values was not significant because of large variations in the values. The invertase activity was also an exception to the general behaviour indicated above (Table 2), as in LEPRAs the values were higher in the middle layer ( $6.90 \mu\text{mol glucose g}^{-1} \text{h}^{-1}$ ) than in the corresponding crust layer ( $5.10 \mu\text{mol glucose g}^{-1} \text{h}^{-1}$ ).

In the crust layers, the values of the enzyme activities were generally higher in the LEPRAs crust than in the other two types of BSCs (although the differences were not always statistically significant), and in DIPLOS the values were intermediate between those in CYANO and LEPRAs (Table 2). Exceptions to this were observed in the cellulase activity, invertase and phosphomonoesterase activities. Thus, the cellulase activity was higher (127%) in the DIPLOS crust layer ( $0.28 \mu\text{mol glucose g}^{-1} \text{h}^{-1}$ ) than in the LEPRAs crust layer ( $0.22 \mu\text{mol glucose g}^{-1} \text{h}^{-1}$ ), and the invertase activity (Table 2), was highest in the CYANO crust layer ( $21.70 \mu\text{mol glucose g}^{-1} \text{h}^{-1}$ ), followed by the DIPLOS crust layer ( $12.30 \mu\text{mol glucose g}^{-1} \text{h}^{-1}$ ) and the LEPRAs crust layer ( $5.10 \mu\text{mol glucose g}^{-1} \text{h}^{-1}$ ). The value of the invertase activity in the CYANO crust layer represents 176% of the value in DIPLOS crust layer and 425% of the value in the LEPRAs crust layer. In regard to the phosphomonoesterase activity (Table 2), although the highest activity occurred in the LEPRAs crust layer ( $19.16 \mu\text{mol } p\text{-nitrophenol g}^{-1} \text{h}^{-1}$ ), higher activities also occurred in the CYANO crust layer ( $8.04 \mu\text{mol } p\text{-nitrophenol g}^{-1} \text{h}^{-1}$ ) than in the DIPLOS crust layer ( $6.61 \mu\text{mol } p\text{-nitrophenol g}^{-1} \text{h}^{-1}$ ). The value of this enzyme activity in the LEPRAs crust layer represented 238% of the value in the CYANO crust layer and 290% of the value in the DIPLOS crust layer. In regard to the nitrogen cycle enzymes, the BAA-protease activities were particularly high in the three types of BSCs, with values of 21.12, 31.18 and  $34.76 \mu\text{mol NH}_3 \text{ g}^{-1} \text{h}^{-1}$  in the CYANO, DIPLOS and LEPRAs crust layers, respectively (Table 2). In other words, the value of this enzyme activity in the LEPRAs crust layers represented 165% of the value in the CYANO crust layer and only 111% of the value in the DIPLOS crust layers.

### *3.3. Specific enzymatic activities (values of enzyme activities in relation to TOC content)*

The mean values of all specific enzymatic activities involved in C, N and P cycles were highest in the CYANO crust layer (Table 3), especially in the cases of BAA-protease ( $2611 \mu\text{mol NH}_3 \text{ g}^{-1} \text{TOC h}^{-1}$ ), phosphomonoesterase ( $1052 \mu\text{mol } p\text{-nitrophenol g}^{-1} \text{TOC h}^{-1}$ ) and invertase ( $2760 \mu\text{mol glucose g}^{-1} \text{TOC h}^{-1}$ ). By contrast, mean values of all the specific enzymatic activities, except for casein-protease and invertase, were

lowest in the DIPLOS BSCs (Table 3). The specific activity of BAA protease was approximately three times higher in the CYANO crust layer than in the DIPLOS and LEpra crust layers, whereas that of phosphomonoesterase was more than 6 times higher in the CYANO crust layer than in the DIPLOS crust layer, and the invertase activity was almost 21 times higher in the CYANO crust layer than in the LEpra crust layer.

In general, the specific activity of all of the enzymes tended to increase in the order deep layer < middle layer < crust layer. When this pattern was not followed, the maximum values obtained (independently of the layer in which they occurred) were not significantly different from those in the respective crust layer (Table 3).

#### 4. Discussion

The results show how the colonization of bare surface by BSCs formed by cyanobacteria and lichens clearly modifies the general properties of the bare substrate, although the changes are limited to the upper few centimetres of the substrate. The presence of the BSCs is related to surface acidification of bare substrate and fixation of carbon and nitrogen, along with a higher availability of water in the surface layers, as is often indicated in other studies (Eldridge and Tozer, 1997; Housman et al., 2006, amongst others). The observed acidification indicates the existence of a high degree of biological activity in the BSCs, probably caused by the acids excreted directly by living organisms and by the CO<sub>2</sub> generated during their metabolism. The higher availability of water in the upper layers of the BSCs suggests the start of aggregation process, simultaneous to the accumulation of carbon and nitrogen (Chamizo et al., 2012). The increase in TN content indicates the presence of organisms that fix atmospheric N<sub>2</sub>, whereas TOC may accumulate in the BSCs as a result of the excretion of organic products by the colonizing organisms (Mager and Thomas, 2011), and as a result of the presence of the remains of these organisms after their death (Thomas et al., 2011). The increase in TOC content in the crust and the middle layers of the BSCs was higher in the lichen-dominated BSCs than in the cyanobacteria-dominated BSCs, as previously reported (Housman et al., 2006). The TOC content of the bare substrate was relatively high (0.57 %), which suggests that carbon is fixed on these surfaces. However, the fact that the values of all enzyme activities were extremely low in the bare substrate (Table 3) suggests that this TOC is not the result of *in situ* biological processes. One possible explanation for this is accumulation of organic remains transported by the air (Throop and Archer, 2007). Incorporation of these remains into the bare substrate occurs as a result of physical fragmentation processes caused by heating-cooling or wetting-drying cycles or by photodecomposition (Austin, 2011). Another possible explanation is that the carbon is of geogenic origin, and is already present in the marine sediments that gave rise to the sedimentary rocks on which the crusts develop (Cantón et al., 2003).

On the other hand, it is also clear that the establishment of BSCs involves an increase in the activity of hydrolytic enzymes, relative to that in the bare substrate,

during periods of maximal biological activity induced by recent rain events. The enrichment of hydrolytic enzymes generated by the establishment of the BSCs suggests that the accumulated organic carbon will undergo degradation, although the increased TOC content in both the crust and middle layers indicates that the rate of fixation atmospheric C is higher than the rate of degradation. The hydrolytic action of the enzymes will guarantee the presence of readily metabolized low molecular weight substrates, which will be used by heterotrophs for nutrition and for other functions such as protection against desiccation (Mager and Thomas, 2011), thereby enabling occupation of the space by higher plants and favouring plant succession and the gradual transformation of the disaggregated geological substrate to an incipient soil (Malam Issa et al., 1999).

Cyanobacteria are the colonizers that least increase the activity of hydrolytic enzymes, whereas lichens of the genus *Lepraria* cause the greatest changes in both the activity of enzymes and the thickness of the substrate affected (Table 2). Ecologists consider that cyanobacteria are primary colonizers and that they are successively replaced or substituted by *Diploschistes diacapsis* and then by *Lepraria crassissima* (Lázaro et al., 2008). The results indicate that species succession is reflected by the enzyme activity in the substrate colonized by each species, which is consistent with the starting hypothesis of this study. However, different steps are observed during this process of increased enzyme activity, reflecting how the biogeochemical cycles of the main elements (C, N and P) are initiated in a substrate in which there was initially almost no biochemical activity. Thus, calculation of the increased enzyme activity, expressed as the ratio between the values of enzyme activity in the colonized substrate and the values in the bare substrate (enrichment ratio, ER) reveals that, for all colonizing organisms, the ER values were particularly high for BAA-protease (in both the crust and the middle layer, and even in the deep layer) (Fig. 2). This indicates that the colonizing organisms need simple readily metabolizable N compounds (such as amino acids, products of the reaction catalyzed by this enzyme) to guarantee their nutritional requirements for N. The higher ER values obtained for N cycle enzymes than for C cycle enzymes (see e.g. casein-protease compared with cellulase, and BAA-protease compared with  $\beta$ -glucosidase, for comparison of the enzymes from both cycles involved in the hydrolysis of complex and simple substrates, respectively) indicate the greater need for low molecular weight nitrogenous compounds than for small carbonaceous compounds (Fig. 2). In other words, this appears to reflect a scarcity of  $N_2$  fixing organisms in these BSCs (which would limit the availability of atmospheric  $N_2$ ), and a predominance of autotrophic organisms (as they do not require the presence of simple carbonaceous compounds, which they can synthesize themselves). The supposed scarcity of nitrogen fixing organisms, which would lead to low input of atmospheric  $N_2$  to the ecosystem, may explain the increase in the C/N ratio in the crust layers in the transition from CYANO to LEPR (Table 1) indicative of the accumulation of increasingly nitrogen-poor organic remains.

The relatively low value of the ER obtained for phosphomonoesterase (Fig. 2) suggests that hydrolysis of the phosphate esters present in the organic matter is not as highly favoured as hydrolysis of carbonaceous or nitrogenous compounds. The phosphate nutrition of microorganisms in BSCs may still depend on the solubilization (chemical or microbial) of the inorganic P forms present in the geological substrate. The data obtained support the classical view of the P cycle, i.e. that hydrolysis of the primary calcium phosphate (usually present in the geological substrate as apatite) predominates at the early stages of soil formation. The hydrolysis of primary calcium phosphates will be favoured by acidification at the surface, induced by the organisms forming the BSCs (Table 1), because the solubility of these phosphates increases as pH decreases. Nonetheless, the low ER values obtained for the phosphomonoesterase activity may indicate a protective mechanism in these ecosystems, as the scarce hydrolysis of the phosphate esters present in the organic remains will favour formation of an increasingly P-rich soil organic matter. Therefore, the TOC:TN:TP ratio (TP = total phosphorus) in the organic remains in the soil will be optimal and will favour the activity of decomposer organisms, as imbalances in the TOC:TN:TP ratio in organic remains may inhibit this activity (Walker and Syers, 1976).

Considering the enzymes that hydrolyze high molecular weight compounds in the C and N cycles (cellulase and casein-protease, respectively) and those that hydrolyze the final products of the hydrolysis sequence ( $\beta$ -glucosidase and BAA-protease, respectively), the activity followed the order CYANO < DIPLOS < LEPR (Table 2). The increase was much greater for the enzymes that hydrolyze low molecular weight substrates than for those that hydrolyze more complex substrates, and the higher activity of the enzymes that hydrolyze simple substrates confirms the pioneering nature of the organisms that form the BSCs. Pioneering organisms are usually *r* strategists with a particular capacity for degrading non complex substrates (Panikov, 1999), such as low molecular weight polypeptides and carbohydrates, substrates of BAA-protease and  $\beta$ -glucosidase, respectively. The behaviour of pioneering communities is therefore the opposite of that communities of mature or climax mesic ecosystems (*k* strategists), which usually have a particular capacity for hydrolyzing complex substrates, such as proteins and cellulose (substrates of casein-protease and cellulase, respectively). In mature mesic ecosystems, degradation of soil organic matter requires the action of a cascade of enzyme activities and an ample supply of enzymes that hydrolyze high molecular weight substrates (Sinsabaugh et al., 1994). The scarce synthesis of enzymes such as cellulase and casein-protease in BSCs may be due to a lack of specific substrates as the organic matter in the BSCs does not usually contain complex polymers (Bates and Garcia-Pichel, 2009). However, this may also be interpreted as a protective mechanism enabling the accumulation of complex organic substrates in the incipient soils. This organic matter, which generally does not decompose readily, is highly recalcitrant in environments containing carbonates (Duchaufour, 1970), and its gradual accumulation would contribute to the development of different essential soil functions, such as the development of soil structure, generation of microhabitats for

microorganisms, water retention, etc. (Sparling et al., 1992), which are typical functions of mature and much more highly developed systems than the crusts under study here.

Although the three types of BSCs under study are formed by pioneering species, the data on the specific enzyme activity (Table 3) provide more information about their role in colonizing land. In all crust layers, the values of specific enzyme activity were extremely high, much higher than those in the Ap horizons of soils under different climatic conditions (sub-arid, Mediterranean or Atlantic climates), under different types of vegetation cover (forest or crop), and with different organic carbon contents (Table 4). This is surprising as high values of specific activity have been related to intense stabilization of enzymes of soil colloids, particularly on humic colloids (Trasar-Cepeda et al., 2008a). However, in the BSCs under study, almost no extracellular enzyme activity would have been stabilized on humic colloids because of the almost total lack of humified organic matter in the crusts (Miralles et al., 2012), so that all of the enzyme activity determined must depend on active organisms. The high correlation usually observed between TOC content and abundance of microorganisms (Wardle, 1992; Ekenler and Tabatabai, 2003) suggests that high values of enzyme activity per unit of TOC may be considered almost equivalent to high values of enzyme activity per unit of microbial biomass. Therefore, the high values of specific activity suggest that the active microorganisms in the BSCs display a high enzymatic potential, which reflects a high ribosomal content, another typical feature of pioneer ecosystems (Panikov, 1999). The values of the specific enzyme activity were always higher in the CYANO crust layer and tended to decrease in the order DIPLOS > LEPRAs (Table 2). The higher ribosomal content of cyanobacteria than in the other two types of crusts is consistent with the fact that the cyanobacteria communities are primary colonizers (*proto-pioneers*). The *proto-pioneer* character (in regard to the enzyme activity) of the cyanobacteria communities can also be deduced from the invertase activities (Table 2), which are much higher in the crust layers of these communities than in the lichen communities, and confirm that *proto-pioneers* require large amounts of enzymes that are capable of hydrolyzing simple low molecular weight substrates (Panikov, 1999), such as sucrose. As ecological succession proceeds, the invertase activity decreases, also confirming that LEPRAs BSCs represent a more developed stage than DIPLOS BSCs (Lázaro et al., 2008).

However, despite the above, there is no simple explanation for the high invertase activity in the CYANO BSCs. The production of invertase implies that the environment surrounding the individuals that form the BSCs contains sucrose, which obviously must have been produced intracellularly by the autotrophic cyanobacteria and excreted to the exterior. The production and excretion of different types of polysaccharides represents, amongst other functions, a protective mechanism for the cyanobacteria against the drying generated by changes in the relative humidity of the air (Mager and Thomas, 2011; Chen et al., 2012), so that the presence of sucrose in these BSCs is perhaps not surprising, although the only sugars identified so far in BSCs dominated by cyanobacteria are monosaccharides such as glucose and galactose (Brüll et al., 2000). However, excretion of sucrose would not make sense as a protective measure against

the adverse environmental conditions, as high quantities of the enzyme that hydrolyzes sucrose are being synthesized at the same time. Nevertheless, the release by cyanobacteria of readily metabolizable monosaccharides (which could be produced both directly and by the hydrolysis of sucrose by invertase) has been interpreted as an advantage provided by autotrophic cyanobacteria growing at the surface of the BSC, to the cyanobacteria growing in deeper areas and which cannot synthesize carbohydrates because of the lack of light (Terauchi and Kondo, 2008).

However, the fact that the invertase activity is only high in the crust and is much lower in the deep layer (Table 2, Fig. 2) suggests another possible interpretation for the high invertase activity in CYANO BSCs, although this interpretation only would be applicable when the photobionts of the lichens were cyanobacteria. Cyanobacteria need to excrete simple carbohydrates to enable symbiosis with fungi and formation of a lichen (Mager and Thomas, 2011), and therefore the high contents of invertase in the cyanobacteria crusts may be related to the symbiotic relationship that must be established between cyanobacteria and fungi. The release of glucose and fructose via the hydrolysis of sucrose by invertase may facilitate the interaction between the fungal hyphae and the cyanobacteria, which will therefore form the lichen body. A similar mechanism (attraction of beneficial organisms) has been suggested to explain the release of N compounds by cyanobacteria (Belnap, 2002). In any case, further research is obviously required to obtain more information about the organic compounds excreted by cyanobacteria, the hydrolase activities, their variations during the successive climatic seasons, the interactions between cyanobacteria and fungi, and the changes that take place as lichens are formed, amongst others.

## 5. Conclusions

There is a clear correlation between the level of ecological succession and the characteristics of the substrate colonized by the BSCs. The development from cyanobacteria-dominated crusts to lichen-dominated crusts involves an increase in the organic C and total N contents and leads to higher activity of hydrolytic enzymes, except invertase, at both the surface and the deeper layers. In addition, the very high values of specific enzyme activity reflect the pioneering nature of these communities.

The BSCs, particularly those predominated by lichens, show high activities of N cycle enzymes that hydrolyze low molecular weight substrates. This may represent the gradual substitution of individuals that fix atmospheric N<sub>2</sub> by other autotrophs that do not fix N<sub>2</sub>.

The relatively low activities of C and P cycle enzymes in the BSCs must be related on one hand to the presence of autotrophic organisms capable of synthesizing simple carbonaceous molecules for use by fungi, and on the other hand, to the presence of apatite in the geological substrate, hydrolysis of which would provide sufficient phosphate to meet the inorganic P needs of these populations.

The extremely high invertase activity in the BSCs predominated by cyanobacteria may reflect the fact that the products resulting from sucrose hydrolysis will act to protect the cyanobacteria against desiccation, and may also reflect the need for monosaccharides to enable symbiosis between the fungi and cyanobacteria.

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## FIGURE CAPTIONS

Fig. 1. Map of the study area

Fig. 2. Mean values of the enrichment ratio (ER) for the enzyme activities (value for a particular layer/ value for the bare soil) in the different layers of the BSCs. Different capital letters indicate that the different layers under the same colonising organism are significantly different; different lower case letters indicate that similar layers for different individual colonizers are significantly different ( $P<0.01$ ).

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**Table 1.** General properties of the samples studied. Different capital letters indicate that the values in the different layers under the same colonizing organism are significantly different; different lower case letters indicate that similar layers for different individual colonizers are significantly different ( $P<0.01$ ). The bare substrate is compared with the deepest layer analyzed for each colonizing organism.

	pH water	pH KCl	Moisture %	TOC %	TN %	C/N
Bare substrate	8.15±0.06 $a$	7.65±0.05 $a$	9.48±0.0 $a$	0.57±0.03 $a$	0.10±0.00 $a$	6.0±0.3 $a$
CYANO						
Crust layer	A7.68±0.01 $a$	A6.93±0.04 $a$	A18.20±0.36 $a$	A0.86±0.35 $a$	A0.15±0.03 $a$	A5.5±1.2 $a$
Deep layer	B8.12±0.16 $a$	B7.44±0.12 $a$	B14.56±0.68 $b$	A0.83±0.33 $b$	B0.08±0.02 $a$	B9.5±1.4 $b$
DIPLOS						
Crust layer	A6.71±0.01 $b$	A6.02±0.01 $b$	A30.45±7.60 $b$	A4.28±0.96 $b$	A0.26±0.01 $b$	B6.2±3.0 $a$
Middle layer	B8.41±0.12 $a$	B7.58±0.12 $a$	B21.90±2.84 $a$	B1.17±0.19 $a$	B0.13±0.02 $a$	B9.4±2.5 $a$
Deep layer	C8.66±0.06 $b$	B7.71±0.10 $a$	B19.89±2.71 $c$	C0.79±0.07 $b$	C0.09±0.01 $a$	B8.5±0.6 $b$
LEPRA						
Crust layer	A6.99±0.08 $c$	A6.61±0.09 $c$	A28.40±1.75 $b$	A3.86±0.24 $b$	A0.27±0.03 $b$	A14.5±1.0 $b$
Middle layer	B8.26±0.03 $b$	B7.72±0.03 $b$	B20.27±1.42 $a$	B1.24±0.18 $a$	B0.13±0.02 $a$	B9.3±0.4 $a$
Deep layer	B8.27±0.15 $a$	B7.60±0.03 $a$	B18.79±0.63 $c$	C0.85±0.01 $b$	C0.09±0.00 $a$	B9.0±0.2 $b$

**Table 2.** Values of the enzyme activities in the different samples studied. Different capital letters indicate that the values in the different layers under the same colonizing organism are significantly different; different lower case letters indicate that similar layers for different individual colonizers are significantly different ( $P<0.01$ ). The bare substrate is compared with the deepest layer analyzed for each colonizing organism.

	Cellulase <sup>a</sup>	$\beta$ -glucosidase <sup>b</sup>	Invertase <sup>c</sup>	Casein-protease <sup>d</sup>	BAA-protease <sup>e</sup>	Phosphomooesterase <sup>b</sup>
Bare substrate	0.03±0.01a	0.02±0.00a	0.08±0.02a	0.11±0.02a	0.03±0.05a	0.26±0.05a
<b>CYANO</b>						
Crust layer	A0.13±0.08a	A1.03±0.28a	A21.70±3.60a	A0.84±0.15a	A21.12±5.56a	A8.04±1.20a
Deep layer	B0.04±0.01a	B0.21±0.20ac	B1.60±0.90b	B0.17±0.07a	B1.84±1.24b	B1.47±0.63b
<b>DIPLOS</b>						
Crust layer	A0.28±0.17a	A3.41±0.46b	A12.30±6.00b	A2.10±0.23b	A31.18±0.71b	A6.61±1.50a
Middle layer	B0.08±0.01a	B0.94±0.12a	B4.60±0.20a	B0.97±0.42a	B11.69±0.56a	B3.57±0.44a
Deep layer	C0.02±0.02a	B0.75±0.22b	C1.40±0.60b	B0.39±0.19a	C1.36±0.50b	C1.22±0.37b
<b>LEPRA</b>						
Crust layer	A0.22±0.06a	A4.05±1.25b	A5.10±1.00c	A2.97±0.23b	A34.76±3.43b	A19.16±2.35b
Middle layer	B0.06±0.01a	B0.80±0.23a	B6.90±0.30b	B0.90±0.18a	B12.06±4.02a	B4.25±0.58a
Deep layer	C0.02±0.02a	C0.41±0.05c	C2.00±0.70b	C0.21±0.13a	C1.51±0.26b	C1.27±0.12b

<sup>a</sup>  $\mu\text{mol glucose g}^{-1} \text{h}^{-1}$ ; <sup>b</sup>  $\mu\text{mol } p\text{-nitrophenol g}^{-1} \text{h}^{-1}$ ; <sup>c</sup>  $\mu\text{mol tyrosine g}^{-1} \text{h}^{-1}$ ; <sup>d</sup>  $\mu\text{mol NH}_3 \text{ g}^{-1} \text{h}^{-1}$ ;

**Table 3.** Values of specific enzyme activities in the different samples studied. Different capital letters indicate that the values in the different layers under the same colonizing organism are significantly different; different lower case letters indicate that similar layers for different individual colonizers are significantly different ( $P < 0.01$ ). The bare substrate is compared with the deepest layer for each colonizing organism.

	Cellulase/TOC <sup>a</sup>	$\beta$ -glucosidase/TOC <sup>b</sup>	Invertase/TOC <sup>c</sup>	Casein-protease/TOC <sup>d</sup>	BAA-protease/TOC <sup>e</sup>	Phosphomonoesterase/TOC <sup>b</sup>
Bare substrate	6±1a	3±0a	14±1a	19±2a	5±1a	45±3a
<b>CYANO</b>						
Crust layer	A14±3a	A128±22a	A2760±750a	A107±32a	A2611±467a	A1052±380a
Deep layer	B5±1ab	B22±12b	B197±32b	B22±11a	B214±62b	B180±10b
<b>DIPLOS</b>						
Crust layer	A6±3b	A84±24a	AB319±207b	A55±10a	A757±173b	AB166±67b
Middle layer	AB7±0a	A81±14a	B401±58a	A84±35a	A998±124a	B313±76a
Deep layer	A3±2b	A94±26c	A181±88b	A48±19a	B170±47b	A152±41c
<b>LEPRA</b>						
Crust layer	A6±1b	A104±26a	A133±35b	A77±2a	A900±32b	A499±33c
Middle layer	A5±0b	B64±11a	B560±87a	A72±7a	A955±182a	B343±17a
Deep layer	A3±2b	B48±7d	A241±88b	B24±15a	B178±28b	C150±12c

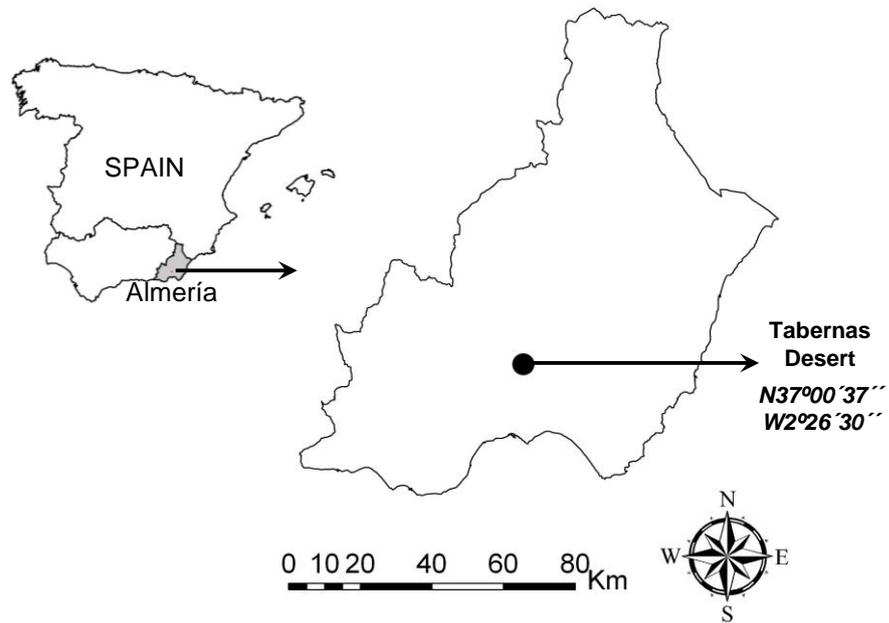
<sup>a</sup>  $\mu\text{mol glucose g}^{-1} \text{TOC h}^{-1}$ ; <sup>b</sup>  $\mu\text{mol } p\text{-nitrophenol g}^{-1} \text{TOC h}^{-1}$ ; <sup>c</sup>  $\mu\text{mol tyrosine g}^{-1} \text{TOC h}^{-1}$ ; <sup>d</sup>  $\mu\text{mol NH}_3 \text{g}^{-1} \text{TOC h}^{-1}$ ;

**Table 4.** Range of specific enzyme activities in the crust layers of the BSCs studied and in the Ap horizons of Spanish soils under different climatic conditions. In all cases the sets of soils chosen had the the lowest contents of organic matter and the enzyme activities were determined by the same method (same concentration of substrate, buffer, temperature and time of incubation).

	Crust layers	Sub-desertic soils *		Mediterranean agricultural soils <sup>#</sup>		Atlantic agricultural soils <sup>&amp;</sup>	
	(n=9)	Tabernas (n=6)	Sierras Béticas (n=9)	Murcia (n=40)	Alicante (n=18)	Vineyard (n=15)	Maize (n= 45)
Cellulase/TOC <sup>a</sup>	57-142	-----	-----	-----	-----	2-7	1-10
β-glucosidase/TOC <sup>b</sup>	84-128	23-113	25-82	8-149	66-110	37-157	8-49
Invertase/TOC <sup>a</sup>	133-2760	-----	-----	-----	-----	0-1	9-165
Casein-protease/TOC <sup>c</sup>	55-107	0-39	1-30	4-14	-----	3-29	6-63
BAA-protease/TOC <sup>d</sup>	757-2600	-----	-----	5-260	-----	195-618	49-533
Phosphomonoest./TOC <sup>b</sup>	166-1052	-----	-----	24-108	39-57	33-159	32-239

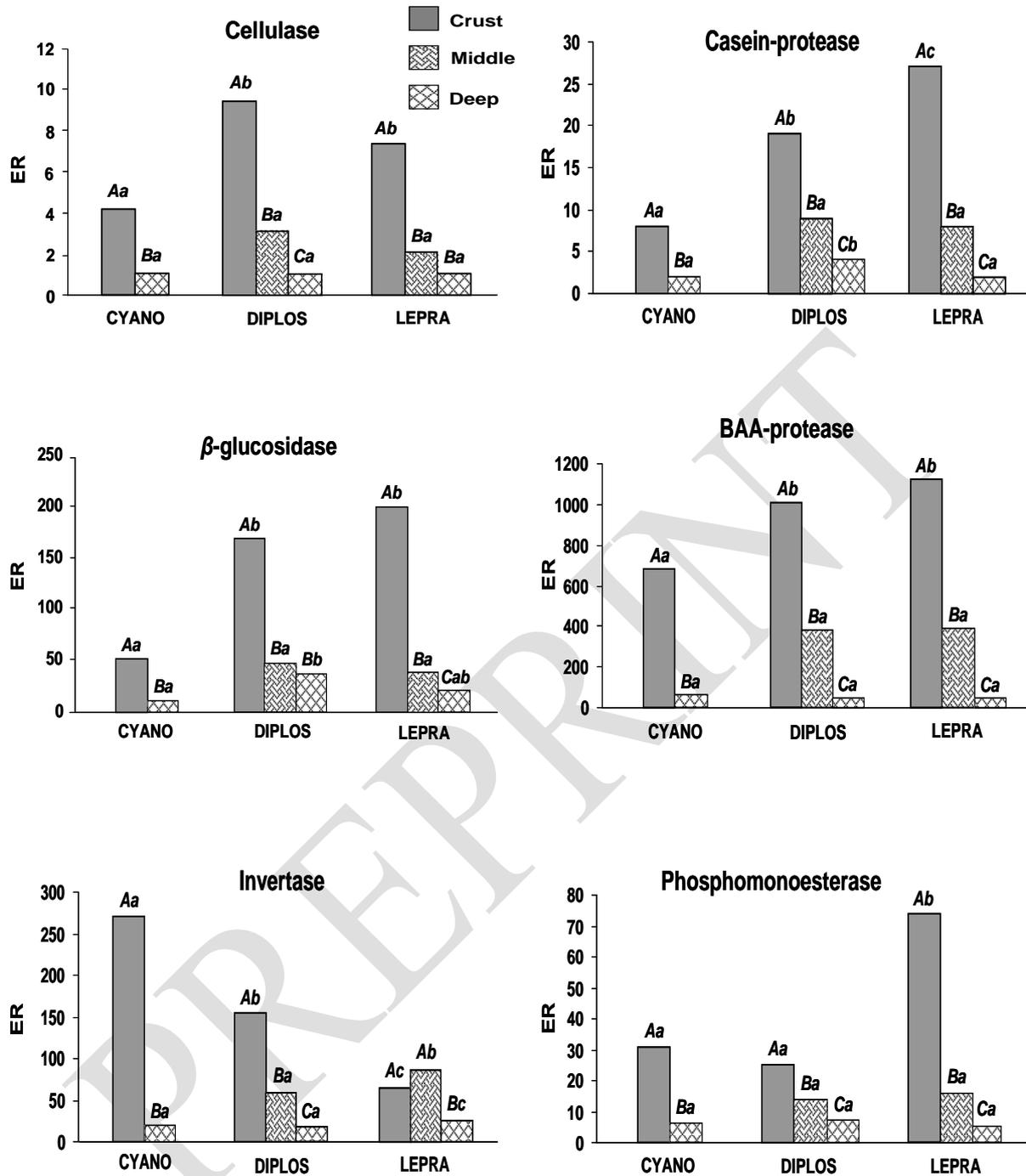
<sup>a</sup>μmol glucose g<sup>-1</sup> TOC h<sup>-1</sup>; <sup>b</sup> μmol *p*-nitrophenol g<sup>-1</sup> TOC h<sup>-1</sup>; <sup>c</sup> μmol tyrosine g<sup>-1</sup> TOC h<sup>-1</sup>; <sup>d</sup> μmol NH<sub>3</sub> g<sup>-1</sup> TOC h<sup>-1</sup>.

<sup>\*</sup>, soils from Almería, SE Spain (Tabernas Desert, Miralles et al., 2007a; Sierras Béticas, Miralles et al., 2007b); <sup>#</sup> soils from Murcia, SE Spain (Pascual, 1995) and Alicante, E Spain (Zornoza, 2006); <sup>&</sup>, soils from Galicia, NW Spain (vineyard soils, Miguéns et al., 2007; maize soils, Trasar-Cepeda et al., 2008b).



**Fig. 1.** Map of the study area

Fig. 1



**Fig. 2.** Mean values of the enrichment ratio (ER) for the enzyme activities (value for a particular layer/ value for the bare soil) in the different layers of the BSCs. Different capital letters indicate that the different layers under the same colonising organism are significantly different; different lower case letters indicate that similar layers for different individual colonizers are significantly different ( $P < 0.01$ ).