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# Influence of the soil storage method on soil enzymatic activities in Mediterranean forest soils

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## Abstract

Soil storage method may alter enzymatic activity being storage conditions of the soil samples prior to analysis decisive for the results. Studies made on freshly collected soils are generally preferred. However it is always not possible due to practical reasons since for example sampling is often restricted to short period of the year or because a great quantity of microbiological analyses must be made on time and by few people. On this context, soil storage methods are needed, being cold at 4°C the most widely used although sometimes alternative storage methods are also utilized. The aim of this study is to evaluate the effect of two alternative storage methods of soil samples (freezing at –20°C and air drying conservation methods) in comparison to cold at 4°C on the enzymatic activities (dehydrogenase, phosphatase, β-glucosidase and urease soil enzymes). Samples of two forest ecosystems (pine and holm oak forest stand) were taken in two different season of the year (winter and spring 2009). Results showed that enzymatic activities differed when freezing or air drying conservation methods were used in comparison with cold soil samples. Generally, alternative soil storage methods presented lower enzymatic activity than cold at 4°C. However, these changes depend on season and sampling location.

**Key words**: soil enzyme; soil storage technique; soil conservation; enzymatic analysis.

## Resumen

**Influencia de los métodos de conservación en las actividades enzimáticas de suelos forestales mediterráneos**

El método de conservación del suelo utilizado puede alterar la actividad enzimática, siendo decisivas para los resultados, las condiciones de conservación previas a los análisis. Para los estudios es preferible realizar los análisis en muestras frescas y recién cogidas. Sin embargo, esto no es siempre posible por razones prácticas ya que las muestras se tienen que recoger en un corto periodo del año o porque los análisis los debe hacer poca gente y siempre en un determinado tiempo. En este contexto, se necesita un método de conservación, siendo el mantenimiento en el frigorífico a 4°C el método más usado, aunque existen otros métodos alternativos. El objetivo de este trabajo es el de evaluar el efecto de dos métodos alternativos de conservación (congelado a –20°C y secado a temperatura ambiente) sobre las actividades enzimáticas (deshidrogenasa, fosfatasa, β-glucosidasa y ureasa), en comparación con el mantenido en el frigorífico a 4°C. Las muestras se obtuvieron de dos zonas forestales diferentes (pinar y encinar) y en dos épocas distintas (primavera y verano de 2009). Los resultados mostraron diferencias en las actividades enzimáticas cuando se usó el congelado o secado al aire de las muestras de suelo en comparación con el mantenimiento en el frigorífico. De forma general, los métodos de conservación alternativos mostraron una menor actividad enzimática en las muestras de suelo analizadas. Sin embargo, estos cambios dependen de la época del año y la zona de muestreo.

**Palabras clave**: enzima del suelo; técnica de almacenamiento del suelo; método de conservación; análisis enzimáticos.

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## Introduction

Soil quality depends on a large number of physical, chemical, biological, microbiological and biochemical properties, the last two being the most sensitive since they respond rapidly to changes (Dick and Tabatabai, 1993; Trasar-Cepeda *et al.*, 1998; Ros *et al.*, 2003; Bastida *et al.*, 2008). Among the parameters related to the biochemical and microbiological state of the soil, particularly important are the indicators of the soil microbial activity, principally different enzymatic activities, both specifically related to the cycles of N, P, and C (urease, phosphatase, and β-glucosidase, respectively) and of a more general nature, such as dehydrogenase and respiration. These soil parameters are sensitive indicators of soil quality (Bastida *et al.,* 2008) and could have implications for the establishment of native plant communities and cover (Vance and Entry 2000). Several research works have focused on the measurement of enzyme activities and microbial biomass in forest soils (Caldwell, 2005; Lucas-Borja *et al.*, 2010a,b). On these studies, biochemical and microbiological parameters are calculated following an established procedure although nothing is argued in relation to how soil samples have been kept. The storage conditions prior to the analysis may be decisive for the results and there is a need for using satisfactory storage methods (Stenberg *et al.*, 1998). Different studies have showed that inappropriate storage conditions of the soil samples can adversely affect microbial communities, reducing their size and activity (Trabue *et al.*, 2006; Zornoza *et al.*, 2006). The reason seems to be related with the fact that a new environment (temperature, humidity, etc.) is created when soil samples are collected and stored (Mondini *et al.*, 2002; De Nobili *et al.*, 2006).

In all microbiological studies freshly collected soils from the field are preferred (Stenberg *et al.*, 1998) and parameters are measured as soon as possible after soil sampling. However, for practical reason this is not always possible and it is necessary to use a satisfactory soil storage technique. In many different studies, refrigeration at 4°C for a maximum of 3 month has been recommended when storage is required (ISO, 1993; OCDE, 1995), although biomass changes have been observed by different authors when this method is used (Ross *et al.*, 1980; Ross, 1991). For example, Ross (1991) found a 41% reduction of biomass after 14 months at 4°C, as well as a similar reduction in basal respiration rate. Moreover, there is usually a slow depletion of available substrate in soil samples refrigerated at 4°C, this generating a low microbial activity (Coxson and Parkinson, 1987). Thus, alternative soil storage methods need to be used, mainly freezing at –20°C and aeration or drying at high temperatures (Breitenbeck and Bremner, 1987; Stenberg *et al.*, 1998; Ross *et al.*, 1980), although little is known about the effect of these alternative methods in comparison with the normally used (refrigerated at 4°C).

With regards to frozen stored soils, Faccendini *et al.* (2003) showed that the soil storage by freezing at –20°C does not affect the biomass carbon. Others studies found that freezing at –20°C can change the size of bacterial populations, as well as their activities (Breitenbeck and Bremner, 1987; Stenberg *et al.*, 1998; Ross *et al.*, 1980). This technique may cause damage by forming intracellular ice crystals, which may kill sensitive organisms and cause a decrease in microbial activity (MacLeod and Calcott, 1976). However, structural changes in the soil sample due to the breakage of aggregates can result in a higher estimation of biomass and microbial activity. These two opposing effects may offset each other. Preincubation of the soil sample at optimum temperature and then a humidification of the sample to restore the population of microorganisms, are usually performed before the enzymatic analysis of frozen soil samples (Horwath and Paul, 1994).

Aeration or drying is a very common practice for soil storage although is it not recommended when making biochemical and microbiological studies since drying destroys an important part of the microbial population (Mondini *et al.*, 2002; De Nobili *et al.*, 2006). Moreover, air drying alters soil metabolism considerably being the decrease in soil microbial biomass much less marked in soils dried at 10°C, than those dried at 25°C (Shen *et al.*, 1987). No successful preservation of biological activity was achieved when soil was air dried, applying soil respiration as an index (Ahmed *et al.*, 1982). In the case of arid soils, probably the effect of air drying on the microbiological parameters may be minimal, so it could be a good soil storage system.

Different studies have been focused on the effect that soil storage techniques have on the soil enzyme activities, proving that conservation systems depend on the enzyme to be tested, the soil characteristics and even the type and amount of plant debris present in the soil (Pancholy and Rice, 1972; Zantua and Bremner, 1975a,b; Speir and Ross, 1975, 1981; Palma and Conti, 1990; Arias *et al.*, 1997; Brohon *et al.*, 1999). The most appropriate technique may depend on many factors, including soil precedence, parameters to be measure

and initial soil characteristics. Thus, information about good soil storage methods for Mediterranean forest soils is needed and it is necessary to test if alternative soil storage methods as freezing at –20°C or drying at room temperature may be used when refrigeration at 4°C is not possible. We asked: is it possible to use frozen or dried stored soil samples in microbiological soil studies developed in Mediterranean forest areas without alter enzymes activities in comparison with stored soil samples at 4°C? Our objective in the present investigation was to compare the effects on soil enzymatic activity when storing soils cold (4°C), when frozen (–20°C) or dried at room temperature (24°C). We have test the effect of these storage methods on different soil in enzyme activities (dehydrogenase, urease, phosphatase and β-glucosidase), which are very important in many soil research works and are specifically involved in the biochemical cycles of carbon, nitrogen and phosphorus. We hypothesized that frozen and air dried soil samples significantly affect soil enzyme activities in comparison with storing soils cold (4°C) although results may depend of soil type (collected under *Quercus* or *Pinus* forest stands) and season of the year when samples were collected (winter and spring).

## Material and methods

### Study area

The study was conducted in Almodóvar del Pinar (Central-eastern Spain) (Fig. 1). Two experimental forest areas were located at The Dehesa de Abajo forest (39° 38' 0" N; 1° 51' 10" W). The first one was burned in summer 2002 and mainly composed by holm oak (*Quercus ilex* L.) resprouts of about 7 years old whereas the second one was dominated by maritime pine (*Pinus pinaster* Ait.), which in some forest locations appeared mixed with holm oak. Shrub species composition in both experimental sites included rosemary (*Rosmarinus officinalis* L.), thyme (*Thymus vulgaris*), lavender (*Lavandula latifolia* L.), kermes oak (*Quercus coccifera* L.) and gorse (*Genista scorpius* L.). According to Allué (1990), the climate characteristics of the experimental area can be classified as Mediterranean climate with a mean annual temperature of 12.2°C and rainfall of 507 mm. Mean air temperatures usually reach 9.8°C in spring, 22.7°C in summer, 7.8°C in autumn and 4.3°C in winter whereas precipitation reach 166 mm in spring, 60 mm in summer, 180 mm in autumn and 101 mm in winter. The average elevation is 996 m a.s.l. and according to the Soil Atlas of Europe (2005) *Leptposol* is the typical soil of the studied area. The experimental area generally presents a very shallow soil over hard rock.

### Soil sampling and treatment

Soil samples for microbiological analyses were taken in winter and spring 2009 from the top 20 cm of soil in each experimental forest area. We focused our sampling on this horizon because a large portion of microbial activity occurs in these horizons, and the effects of tree species (through litter chemistry) should be strongest within it (as opposed to deeper horizons). 27 soil samples were randomly collected from the pine forest stand (Fig. 2) and 18 soil samples were ran-

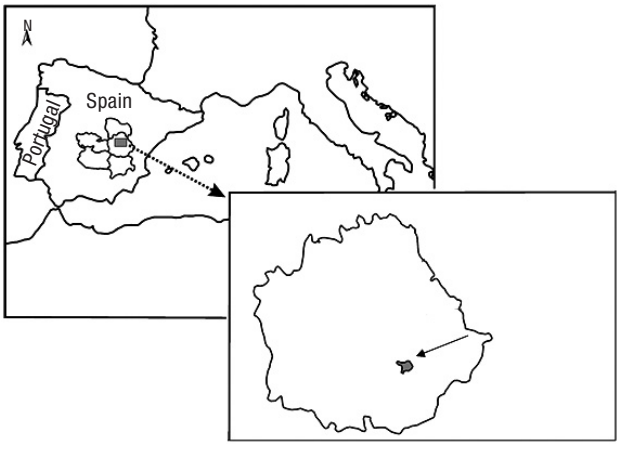


**Figure 1.** Geographical location of the study areas (shaded).

**Figure 2.** General view of the pine forest stand.

**Figure 3.** General view of the holm oak forest stand.

domly collected from the holm oak forest area (Fig. 3) in spring 2009. This field work was repeated again in winter 2009. Each sample was composed by six subsamples (200 g each), which were thoroughly mixed to obtain a composite sample. Following removal of plant remains, each composite sample was passed through a 2 mm and separated into three sections. Each section was moved to: group (1) Soil samples stored in a fridge at 4°C during one month (27 from pine forest areas and 18 from holm oak forest areas); group (2) soil samples frozen at –20°C in a freezer during one month (27 from pine forest areas and 18 from holm oak forest areas); group (3) soil samples air-dried at room temperature (24°C) during one month (27 from pine forest areas and 18 from holm oak forest areas). This procedure was repeated in winter and spring 2009. Finally and then to the storage period, 135 soil samples in winter and 135 soil samples in spring were taken to the laboratory and enzymatic analysis were made. The soil moisture of each sample was also measured before and after the soil storage period.

### Biochemical and microbiological parameters

Soil dehydrogenase activity was determined by using 1 g of soil, and the reduction of p-iodonitrotetrazolium chloride (INT) to p-iodonitrotetrazolium formazan was measured by a modification of the method reported by García *et al.* (1993). Soil dehydrogenase activity was expressed as μ-moles INTF $g^{-1}$ soil $h^{-1}$. Urease activity was determined by staining the ammonium released into the incubation solution at 37°C for two hours by Kandeler y Gerber method (1988), modified by Kandeler *et al.* (1999). Alkaline phosphatase and β-glucosidase activities were determined following the methods reported by Tabatabai and Bremmer (1969) and Tabatabai (1982), respectively, adding 2 mL of modified universal buffer (MUB) pH 11 and 0.5 mL of 0.025M pnitrophenyl phosphate (for phosphatase activity assay) or 2 mL of MUB pH 6 and 0.5 mL of 0.025M p-nitrophenyl β-D-glucopiranoside (for β-glucosidase activity assay) to 0.5 g of soil. The mixtures were then incubated at 37°C for 1 h, after which the enzymatic reactions were stopped by cooling on ice for 15 min. Then, 0.5 mL of 0.5M $CaCl_2$ and 2 mL of 0.5 M NaOH (for phosphatase) or 2 mL of 0.1M Tris-hydroxymethylaminomethane-sodium hydroxide (THAM-NaOH) pH 12 (for β-glucosidase) were added. In the control, the respective substrates were added before the addition of $CaCl_2$ and NaOH.

### Statistical analysis

Data were submitted to three-way ANOVA in which soil conservation (cold at 4°C as reference method, and freezing at –20°C and drying at room temperature as alternative methods), forest stand type (pine and holm oak forest stands), season of the year (winter and spring) and their interaction were selected as factors. All soil samples were collected in plots which can be considered as spatially independent. The post-hoc test applied was Fisher's least significant difference (LSD) method. A significance level of $P < 0.05$ was adopted throughout, unless otherwise stated. The software used for the statistical analysis was Statgraphics plus 6.0.

## Results

Soil moisture of each sample was significantly affected by the soil storage method (Table 1). The highest soil moisture reduction was showed for the air drying conservation method. In pine forest areas, the soil moisture reduction was about 70% and 83% in spring and winter respectively whereas in holm oak forest areas the soil moisture reduction was about 75% and 79% in spring and winter respectively when air drying conservation method was used. Soil moisture reduction was lower when any of the other soil storage methods were used (less than 30% in both pine and holm oak forest areas for cold at 4°C and freezing at –20°C).

**Table 1.** Mean percentage of soil samples moisture (standar error) for each experimental forest area and soil storage method before and after one month of soil storage

| | Spring 2009 | | Winter 2009 | |
|---|---|---|---|---|
| | **Before soil storage** | **After soil storage** | **Before soil storate** | **After soil storage** |
| *Cold at 4°C* | | | | |
| Pine forest | 14.34 (5.37) | 10.43 (3.33) | 17.94 (3.10) | 11.70 (5.37) |
| Holm oak forest | 10.44 (5.14) | 8.41 (5.14) | 21.18 (2.56) | 17.32 (4.99) |
| *Freezing at –20°C* | | | | |
| Pine forest | 14.34 (5.37) | 13.63 (5.14) | 17.94 (3.10) | 16.07 (3.42) |
| Holm oak forest | 10.44 (5.14) | 7.65 (4.53) | 21.18 (2.56) | 18.31 (2.49) |
| *Drying at room temperature (24°C)* | | | | |
| Pine forest | 14.34 (5.37) | 4.41 (1.89) | 17.94 (3.10) | 1.36 (0.25) |
| Holm oak forest | 10.44 (5.14) | 2.60 (0.89) | 21.18 (2.56) | 4.62 (1.62) |

According with the analysis of variance, studied factors (soil storage method, forest stand composition and season) significantly affected enzymatic activities (Table 2). In the case of the β-glucosidase enzyme, air drying conservation method significantly reduced the enzymatic activity in winter and spring whereas freezing at –20°C only decreases enzymatic activity in spring (Fig. 4). With respect to the interaction of forest stand composition with season, winter soil samples always presented higher β-glucosidase activity than spring soil samples being the difference between winter and spring season greater for holm oak samples than for pine soil samples (Fig. 5). Phosphatase and β-glucosidase enzyme activities presented a similar behavior with respect to the interaction soil storage method and season (Fig. 4 and 6). Dried soil storage samples showed the lowest soil enzymatic activity in both holm oak and pine forest areas. Significant differences between cold and frozen soil samples were only showed for pine forest areas (Fig. 7). Soil conservation method and forest stand composition was the only second order interaction for the urease enzyme (Table 2). For this enzyme, season was a not significant factor. Moreover, frozen soil samples presented higher enzymatic activity (only significant in spring) than cold soil samples and air dried soil samples tend to show lower enzymatic activity than cold soil samples (only significant in winter) (Fig. 8). In relation to dehydrogenase activity, alternative soil storage methods (air dried and freezing at –20°C) significantly reduced the enzymatic activity being these reductions more pronounced in winter than in spring (Fig. 9). Moreover, air dried and frozen soil samples always presented a lower dehydrogenase activity than cold soil samples in both pine and holm oak soil samples (Fig. 10).

## Discussion

Enzymes play a fundamental role in the cycles of important elements such as nitrogen (ureases and proteases), phosphorus (phosphatases) and carbon (β-glu-

**Table 2.** Results of the three-way ANOVA for the studied factors

| Factors | D.F. | Dehydrogenase activity | | Phosphatase activity | | β-glucosidase activity | | Ucrease activity | |
|---|---|---|---|---|---|---|---|---|---|
| | | **F-ratio** | ***P*-value** | **F-ratio** | ***P*-value** | **F-ratio** | ***P*-value** | **F-ratio** | ***P*-value** |
| MC | 2 | 58.16 | < 0.001 | 53.55 | < 0.001 | 28.48 | < 0.001 | 21.44 | < 0.001 |
| S | 1 | 103.85 | < 0.001 | 0.64 | ns | 44.63 | < 0.001 | 0.02 | ns |
| VT | 1 | 9.58 | < 0.001 | 207.17 | < 0.001 | 85.76 | < 0.001 | 9.89 | < 0.01 |
| MC × S | 2 | 13.12 | < 0.001 | 6.15 | < 0.05 | 324.94 | < 0.01 | 2.31 | ns |
| MC × VT | 2 | 3.00 | < 0.05 | 7.13 | < 0.001 | 4.16 | ns | 14.59 | < 0.001 |
| VT × S | 1 | 0.13 | ns | 0.36 | ns | 247.39 | < 0.05 | 0.08 | ns |

MC: soil sotorage method. VT: forest stand composition. S: season.

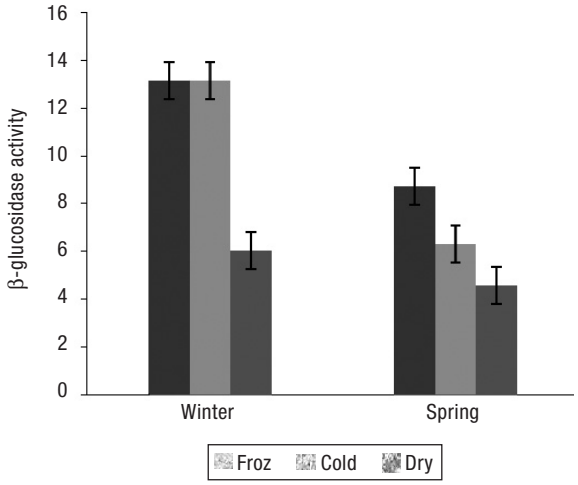


**Figure 4.** Mean values and standar error for β-glucosidase activity (μ-moles PNP $g^{-1}$ soil $h^{-1}$) with respect to soil storage method and season.

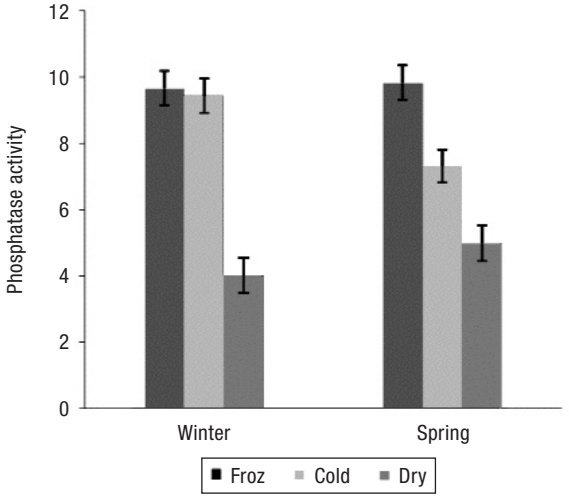


**Figure 6.** Mean values and standar error for phosphatase activity (μ-moles PNP $g^{-1}$ soil $h^{-1}$) with respect to soil storage method and season.

cosidases) (Ros *et al.*, 2004), carrying out hydrolysis reactions involved that transform complex organic compounds into simpler compounds (Bastida *et al.,* 2007). Many different research works have studied soil enzymatic activities with the aim to assess soil quality since soil degradative processes strongly influence soil enzymes (Ceccanti and García, 1994; Bastida *et al.*, 2007; Lucas-Borja *et al.*, 2010a,b). On this context, soil storage conditions used during the soil analysis may alter enzymatic activities and therefore research works conclusions and prescriptions. Our results show that the enzymatic activities analyzed generally reacted differently depending on the storage technique used. Moreover, these changes depended of season of the year when soil samples were collected and forest stand composition where soil samples were taken.

Enzymatic activities, both specifically related to the cycles of N, P, and C (urease, phosphatase, and β-glucosidase) presented a similar trend. Air dried soil samples always presented the lowest soil enzymatic activity suggesting that a low moisture percentage is related to a lower microbial activity rate. As it was showed in Table 1, air dried soil samples presented the highest soil moisture reduction (more than 70%), this affecting soil enzymatic activities. As Jeenkinson argued (1992), negative values of water potential in-

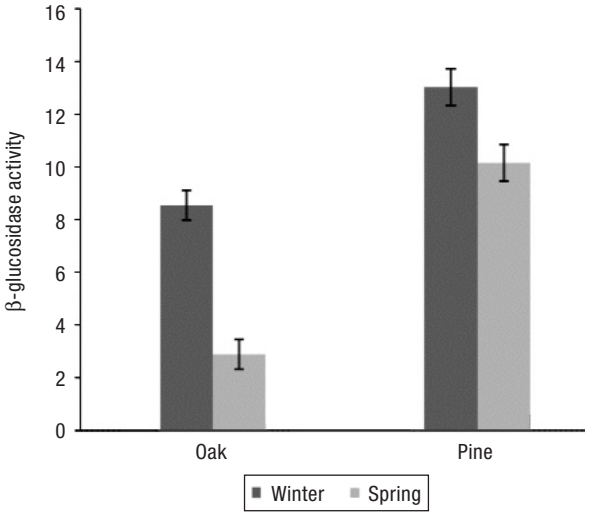


**Figure 5.** Mean values and standar error for β-glucosidase activity (μ-moles PNP $g^{-1}$ soil $h^{-1}$) with respect to season and forest stand composition.

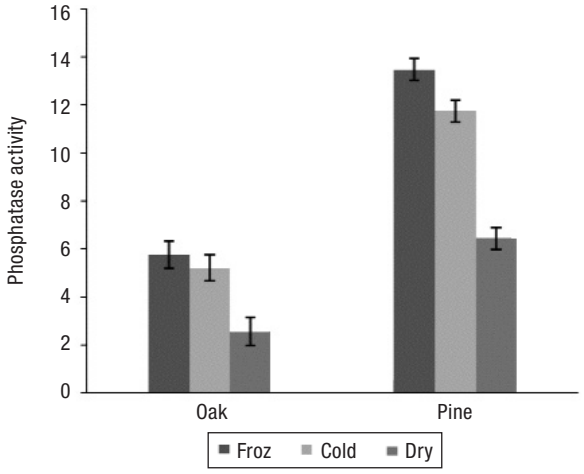


**Figure 7.** Mean values and standar error for phosphatase activity (μ-moles PNP $g^{-1}$ soil $h^{-1}$) with respect to soil storage method and forest stand composition.

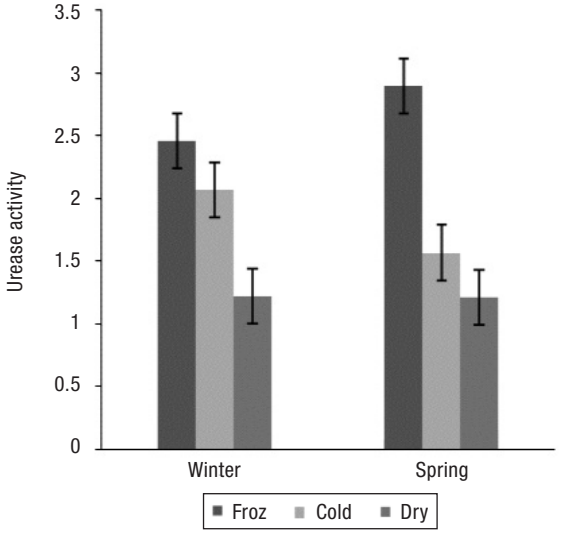


**Figure 8.** Mean values and standar error for urease activity (μ-moles N-$NH_3$ $g^{-1}$ soil $h^{-1}$) with respect to soil storage method and season.

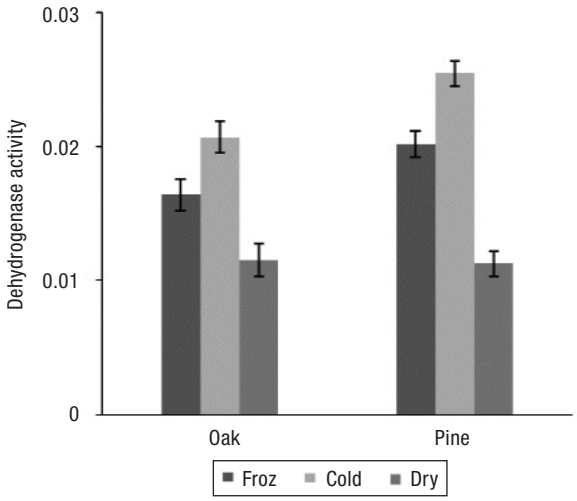


**Figure 10.** Mean values and standar error for dehidrogenase activity (μmoles INTF $g^{-1}$ soil $h^{-1}$) with respect to soil storage method and forest stand composition.

duce to lower enzymatic activity. With respect to dehydrogenase activity, the same circumstance was showed by Ross (1970). Therefore, given the high sensitivity of the enzymatic activities to soil moisture reduction during the soil storage period, soil samples should not be air dried before enzymatic analysis.

With respect to freezing and cold soil storage methods and for extracellular enzymes (urease, phosphatase, and β-glucosidase activities), generally frozen soil samples presented higher enzymatic activity although significant differences were only showed in spring. This may indicate that structural changes in the frozen soil samples due to the breakage of aggregates can result in a higher estimation of biomass and microbial activity if we compare with cold soil samples. Moreover, the soils in our study are not subjected annually to several freeze and thaw cycles. Microflora have not adapted to this stress factor and therefore less resistant to freezing than the microflora in soils where freeze and thaw cycles are so regular, such as the soils from Norway used by Rugbjerg and Helweg (1989). This may indicate that during freezing storage method the unadapted and most sensitive microorganism are killed and utilized as energy sources by the survivors, increasing the overall enzymatic activity of extracellular enzymes (Zelles *et al.*, 1991).

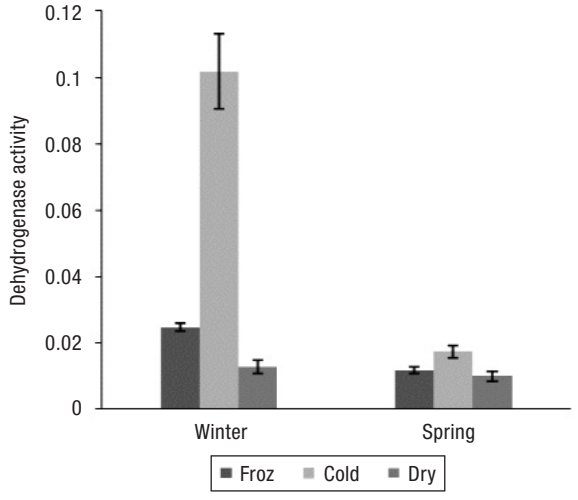


**Figure 9.** Mean values and standar error for dehidrogenase activity (μmoles INTF $g^{-1}$ soil $h^{-1}$) with respect to soil storage method and season.

Dehydrogenase activity is an enzymatic complex of an intracellular nature and it is widely used as a measure of general soil microbial activity (Nannipieri *et al.*, 1990). Results showed that dehydrogenase activity differed in spring and also in winter comparing cold and frozen soil samples and that alternative soil storage methods always reduce dehydrogenase activity (Fig. 9). Higher soil moisture of winter soil samples induced a higher dehydrogenase activity. Moreover, pine soil samples presented also higher soil moisture and thus, higher enzymatic activity. As Ross (1970) argued, dehydrogenase activity of a soil and its stability is affected by soil moisture. However, freezing soil storage method may introduce a negatively effect in spite of soil moisture, thus reducing dehydrogenase activity of frozen soil samples. Soil storage by freezing may cause damage by forming intracellular ice crystals, which

may kill sensitive organisms and cause a decrease in microbial activity of this intracellular enzyme (MacLeod and Calcott, 1976).

On the other hand, pine soil samples tend to positively affect enzymatic activity. This was expected since the experimental area found in the holm oak forest stand was burned in summer 2002. Forest fires severely alter soil conditions and organic matter content (Stephen *et al.*, 2005) diminishing soil enzyme activities. As Stephen *et al.* (2005) argued the direct effects of fire on the soil microflora occur via the lysing of microbial cells and the alteration of microbial reproductive capacity from soil heating.

## Conclusions

Methods of soil conservation affected distinctly to different enzymes. Moreover, the effectiveness of the soil conservation methods depends on the season. For extracellular enzymes (urease, phosphatase, and β-glucosidase activities), soil samples collected in winter presented similar enzymatic activities when freezing and cold soil storage methods were used. Thus, freezing or cold methods can be used for extracellular enzymes in soil sampled in winter. However, it cannot be said for spring season since freezing soil samples alter enzymatic activities, presenting higher values. Contrary to this, freezing soil storage method presents lower activity than the reference method (cold at 4°C) for dehydrogenase activity. Air drying always reduces enzymatic activities in relation to cold or freezing soil methods for both intracellular and extracellular enzymes. Thus, this method should be avoided in enzymatic analyses as soil moisture reduction is showed during the soil storage period, affecting enzymatic activities. Methods of soil conservation affect similarly to oak and pine samples and pine samples showed higher enzymatic activity than oak samples, which was some way expected due to the forest fire located at holm oak in the past years. Further work is clearly necessary to evaluate which of these changes is due to stress affects or to the conditions of soil storage and to assess differences between enzymatic activities analyzed in freshly collected and store soil samples.

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