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Original article

Influence of seasons and land-use practices on soil microbial activity and metabolic diversity in the "Montado ecosystem"

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ABSTRACT

The "Montado ecosystem" is important both economically and ecologically; this ecosystem is dominated by cork and holm oak trees (*Quercus suber* L. and *Quercus rotundifolia* Lam. respectively) combined with a rotation of crops/fallow/pastures. Diverse management strategies, deviating from the sustainable use of the ecosystem, have been implemented, from which arise some extreme situations of over-use or abandonment. To evaluate the effects of different soil use and management, namely extensive cropping, intensive pasture and abandonment, in the activity of soil microorganisms, dehydrogenase, acid phosphatase, β-glucosidase and urease activities, N-mineralization and nitrification rates were measured in different land-use practices, in different seasons (winter, spring and autumn). Also, the potential metabolic diversity was evaluated by analysis of community-level physiological profiles (CLPPs). Seasonal effects were evident with maximum activity occurring in rainy seasons (winter and autumn) and lower substrate utilization in winter. Significant correlations between most microbial parameters and soil water content reflect this seasonal effect. Although showing mainly a seasonal change, microbial parameters were able to distinguish the abandoned area, with a general low activity and differential exponential rates in the use of several substrates, such as amino acids, miscellaneous and polymers, probably associated with changes in organic matter quality.

1. Introduction

The "Montado ecosystem" located in South Portugal is an agro-silvo pastoral system, dominated by cork and holm oak trees (*Quercus suber* L. and *Quercus rotundifolia* Lam.) combined with a rotation of crops/fallow/pastures. This ecosystem has an economic importance due to cork production, cattle raising and agriculture production [1] and is ecologically significant since it sustains a high biodiversity [2]. Several management strategies, deviating from the ancestral sustainable use of the land, have being implemented over the years, from which arise some extreme situations of over utilization or abandonment [1]. These land-use changes are leading to a modification of the ecosystem, reflected in soil quality degradation.

Soil microorganisms fulfil important ecosystem functions, intervening in soil biological processes, mainly in organic matter decomposition and nutrient cycling, thus maintaining soil quality. The diversity of functions performed by microbial communities is essential to maintain the soil multi-functionality [3]. These biological functions, mediated by enzymes, are associated with the microbial capacity to use several organic compounds as substrates. Therefore, enzymatic activity and measures of metabolic diversity represent the microbial activity, and are indicators of soil quality changes due to several natural and anthropogenic drivers, e.g., pollution, soil management practices and land-use changes [3–5]. Since microbial activity includes various metabolic processes, several enzyme activities involved in main biogeochemical cycles, such as C, N and P should be determined [6]. From the plethora of soil enzymes, dehydrogenase (DHA), acid phosphatase, β-glucosidase and urease activities have been widely used as response indicators of soil microbial communities to several environmental pressures. The activity of these enzymes has been found to be sensitive to seasonal and management effects in the Mediterranean area. Some studies, carried out in this region, demonstrated that the highest microbial activity occurred in spring [7]. In their study, Sardans and Peñuelas [8] found higher phosphatase, urease and β-glucosidase activities in spring when compared with autumn. Management effects were also observed with lower enzyme activity found in abandoned sites [9].

Nitrogen turnover parameters, like N-mineralization and nitrification rates, are important soil microbial processes contributing

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to soil fertility and plant growth, especially in Mediterranean soils that often suffer from nutrient deficiencies. N-Mineralization has been used to assess effects of management on soil quality [10]. Since only a small number of microorganisms have the capacity to oxidize ammonium into nitrate [11], the nitrification rate represents a sensible indicator of soil disturbance which may be used to assess ecosystem degradation susceptibility [12]. Despite this fact, estimations of these nitrogen turnover parameters are scarce for Mediterranean areas. Furthermore, the diversity of functions within a microbial community is an important parameter to understand their role in different environments [13] and to maintain the multiple functions of a soil [3]. Microbial metabolic diversity can be defined as the capacity of the microbial community to use several types of carbon sources as substrates and has been found to be very sensitive to environmental changes [14,15]. Carbon source utilization patterns were found to be sensitive to seasonal effects [16] and to distinguish soil microbial communities under different management practices [17–20].

This study aimed (i) to compare soil microbial activity and potential metabolic diversity from selected sites representing different land-use practices in the "Montado ecosystem", namely extensive cropping, intensive pasture and abandonment, (ii) to evaluate the seasonal variation of soil microbial parameters and (iii) to study the relations between microbial parameters and soil physico-chemical properties.

2. Materials and methods

2.1. Study site

This study was conducted in Alentejo, southern Portugal, in Herdade de Belver (Castro Verde) and Herdade Monte do Vento (Mértola). Three sampling areas, representing different land-use practices, were selected: AGRI (Herdade de Belver) is an agriculture area, last cultivated in 2001/2002 according to a rotation system of cereal crop/fallow with light grazing of gramineous plants (mainly *Agrostis pourretii* Willd.); PAST (Herdade Monte do Vento) is used as a pasture with a rotation system of intense grazing/fallow (where soil is turned over every 5 years to strength the pasture) and presenting some disperse *Q. suber* L. and *Q. rotundifolia* Lam. trees and some Compositae species (e.g. *Chamaemelum mixtum* All., *Leontodon taraxacoides* Mérat.); ABAN (Herdade Monte do Vento) was used in the past for agriculture and grazing and has been abandoned for about 30 years. The site is dominated by shrubs (e.g. *Cistus ladanifer* L., *Genista hirsuta* Vahl.) and some disperse *Q. suber* L. and *Q. rotundifolia* Lam. trees. Soil properties from the different areas are presented in Table 1.

2.2. Sampling procedures and soil physico-chemical characterization

Soil sampling occurred at three different seasons: winter 2003 (medium precipitation of 87 mm and an average temperature of 10.1 °C), spring 2004 (medium precipitation of 35 mm and an average temperature of 16.7 °C) and autumn 2004 (medium precipitation of 54 mm and an average temperature of 17.9 °C). A transect of approximately 100 m was made in each study site, and ten samples from the upper 10 cm layer were collected. Soil was kept in plastic bags and the Biolog method was performed as soon as possible (normally one day after sampling occurred). The remaining soil was sieved (5 mm), well homogenized and conserved at 4 °C until the remaining analyses were performed (within one month after sampling). Physico-chemical characterization was made by determining: soil water content (soil was dried at 105 °C during 12 h), soil organic matter content (soil was combusted at 500 °C during 6 h), soil pH (measured with KCl 1 M 1:6v/v), and soil ammonia and nitrate contents (extracted with a 0.0125 M calcium chloride solution and quantified photometrically at 660 nm and 210 nm, respectively [21,22]).

Table 1
Soil properties of the different land-use practices (AGRI – agriculture, PAST – pasture, ABAN abandoned) determined in one composite sample per land-use practice collected in winter. CEC – cation exchange capacity; WHC – water holding capacity.

Soil properties	AGRI	PAST	ABAN
pH (KCl)	4.39	4.97	4.55
Organic matter (%)	2.40	4.80	5.20
Phosphorus (μg/g)	69.0	66.0	12.0
Potassium (μg/g)	68.0	>200	138
Calcium (μg/g)	500	788	470
Magnesium (μg/g)	188	225	180
Sodium (μg/g)	60.0	45.0	25.0
Mineral nitrogen (μg/g)	18.0	147	77.0
Total nitrogen (%)	0.120	0.170	0.200
CEC (meq/100g)	8.48	13.4	11.8
WHC (%)	30.1	30.8	30.3
Sand (%)	42.8	69.2	62.9
Silt (%)	34.6	18.5	24.2
Clay (%)	22.6	12.3	13.0
Texture[a]	Silt loam	Sandy loam	Loam

[a] In agreement with Gomes and Antunes [54].

2.3. Microbial activity

Enzymatic activity was determined for all collected samples (ten samples per land-use practice at each sampling season). Dehydrogenase activity was measured following the method described by Öhlinger [23], adapted to a microplate reader. Briefly, 1 g of moist soil was suspended in 1 ml of triphenyltetrazolium chloride (TTC) solution (0.5%) and incubated at 40 °C for 24 h. After incubation the produced triphenyl formazan (TPF) was extracted with 5 ml of acetone. Each well of a 96-well plate was filled with 200 μl and the TPF measured photometrically at 546 nm in a microplate reader. Dehydrogenase activity was expressed as μg TFP g^{-1} d w h^{-1}. Acid phosphatase was measured following the method described by Margesin [24], adapted to a microplate reader. Briefly, 1 g of moist soil was suspended in 1 ml of buffered *p*-nitrophenyl phosphate solution (0.005 M) and incubated at 35 °C for 2 h. After incubation 4 ml of tris(hydroxymethyl)aminomethane buffer (0.1 M, pH 12) and 1 ml of calcium chloride solution (0.5 M) were added. A 1:1 dilution was made with distilled water. The produced *p*-nitrophenol (pNP) was measured photometrically at 405 nm by pipetting 200 μl to each well of a 96-well plate. Acid phosphatase activity was expressed as μg pNP g^{-1} d w h^{-1}. β-Glucosidase was determined following the method described by Tabatabai [25], adapted to a microplate reader. Briefly, 1 g of moist soil was suspended in 1 ml of *p*-nitrophenyl-β-D-glucoside (PNG) solution (0.05 M) and incubated at 37 °C for 1 h. After the incubation 1 ml of calcium chloride solution (0.5 M) and 4 ml of tris(hydroxymethyl)aminomethane (0.1 M) were added. Each well of a 96-well plate was filled with 200 μl and the produced *p*-nitrophenol (pNP) was measured photometrically at 405 nm. β-Glucosidase activity was expressed as μg pNP g^{-1} d w h^{-1}. Urease was determined following the method described by Kandeler and Gerber [26], adapted to a microplate reader. Briefly, 1 g of moist soil was suspended in 0.5 ml of urea solution (0.72 M) and incubated at 37 °C for 2 h. After incubation the ammonium (NH_4^+) produced was extracted with 6 ml of potassium chloride solution (2 M) and measured photometrically at 690 nm, by pipetting 200 μl to each well of a 96-well plate. Urease activity was expressed as μg NH_4^+–N g^{-1} d w h^{-1}.

Nitrogen cycle parameters were only determined for five samples (representative of the transect) per land-use practices at each sampling season. N-Mineralization was determined under aerobic conditions, following the method described by Kandeler [27], with some minor adjustments. Briefly, 2.5 g of moist soil, to which 0.75 ml of distilled water were added, was incubated at 25 °C during 28 days. After extraction with calcium chloride solution (0.0125 M), ammonium and nitrate contents were determined by spectrophotometry [21,22]. N-Mineralization was calculated as the difference between the inorganic N content (NH_4^+–N + NO_3^-–N) after and before incubation and expressed as μg N g d w d^{-1}. Nitrification was determined using an ammonium sulphate solution as substrate, following the method described by Kandeler [28], with some minor adjustments. Briefly, 250 μl of ammonium sulphate solution (0.0757 M) was added to 2.5 g of moist soil. Soil moisture was adjusted to 50–60% of the maximum water holding capacity. Samples were incubated at 25 °C during 3 weeks (control was stored at −20 °C). After extraction with calcium chloride solution (0.0125 M), ammonium and nitrate content were determined spectrophotometrically [21,22]. Nitrification was calculated as the amount of nitrogen released from the substrate solution per gram of dry matter and per day, hence it was expressed as percentage loss of initially added substrate (%/d).

2.4. Metabolic diversity

Microbial metabolic diversity was measured in four samples (representative of the transect) per land-use practices at each sampling season. Biolog Ecoplates® were inoculated with a soil suspension, prepared with 2 g of soil and 38 ml of a pyrophosphate solution (0.2%). Each plate well was inoculated with 140 μl of soil suspension. The oxidation process was monitored twice a day during 15 days, measuring colour formation with a microplate reader (Tecan Sunrise) at 590 nm. During that time the plates were incubated in the dark at 22 °C [29].

2.5. Data analysis

2.5.1. Soil physico-chemical properties

Soil physico-chemical properties from the different land-use practices along seasons were compared by a "between-within subjects repeated-measures ANOVA" followed by a Newman–Keuls test when significant differences among land-use practices and/or sampling seasons were found [30]. The relation between soil properties and the microbial parameters were evaluated using the Spearman rank coefficient [30]. Ammonium and nitrate were only correlated with N-cycle parameters (urease, N-mineralization and nitrification). Before the analysis, normality and homoscedasticity were verified using the Kolmogorov–Smirnov and Bartlett tests, respectively. Whenever these assumptions were not met, data was transformed accordingly [30]. Statistical analyses were performed with STATISTICA 7 (StatSoft, Tulsa, OK, USA).

2.5.2. Microbial activity

Soil microbial activity parameters from the different land-use practices along seasons were compared by a "between-within subjects repeated-measures ANOVA" followed by a Newman–Keuls test when significant differences among land-use practices and/or sampling seasons were found [30]. To evaluate significant differences among groups of samples from the different seasons and among different land-use practices at each sampling season, taking into account all microbial parameters measured, an Analysis of Similarity (ANOSIM), was performed [31]. In all cases similarity matrices were obtained using the Normalised Euclidean Distance metric after log transforming the microbial parameters values. These analyses were done with PRIMER 5 for Windows software (PRIMER-E Ltd. 2001). After detecting a significant influence of the sampling seasons and in order to represent the samples of the three land-use practices according to their microbial activity parameters, a Principal Component Analysis (PCA) was performed for each season individually, in Canoco for Windows software 4.5 (Ter Braak and Smilauer 2002).

2.5.3. Metabolic diversity

Absorbance values obtained with Biolog Ecoplates® were corrected for the colour intensity at time 0; each substrate value was then blanked against the control well. Using these values the average well colour development (AWCD) was calculated. The community-level physiological profile (CLPP) data was analysed estimating kinetic parameters by fitting the OD_{590} vs. time curve to a density dependent logistic growth equation [32]: $OD_{590} = K/(1 + e^{-R(T-S)})$, where K represents the asymptote or maximum degree of colour development (OD), R determines the exponential rate of OD change (h^{-1}), T is the time following inoculation of the microplates (h) and S is the time to reach the midpoint of the exponential phase of the curve, (when $K/2$) (h). These parameters provide information on different aspects of the carbon source utilization: the asymptote (K) is an estimate of the potential maximum amount of substrate used, the exponential rate parameter (R) provides information on how rapidly a carbon source is metabolized by a community once the density has reached the level at which colour production begins, and the time to reach the midpoint (S) supplies information about the initial inoculum density and the relative growth rates of the species able to use the carbon source in each well [13,32]. Kinetic parameters may be the most useful format for Biolog data, since they are weakly correlated with each other [13]. However, the influence of the inoculum density in the kinetic analysis must be considered, since an increase in density will decrease the lag time, increase the slope and the integral of colour development. One way to overcome this problem is to normalize data by dividing the kinetic parameter for the individual substrates by the mean value for the plate [33]. Kinetic parameters were estimated for the AWCD, to evaluate the season's effects and were correlated with soil physico-chemical properties by Spearman rank coefficient [30]. To obtain information about the carbon source utilization pattern, the 31 substrates were assigned into six guilds according to their chemical nature [14]: amines, amino acids, carbohydrates, carboxylic acids, miscellaneous and polymers. Kinetic parameters for each substrate were estimated and divided by the mean value of the plate (normalization). Afterwards, kinetic parameters for each guild were calculated as the average of the normalized individual kinetic parameters and were used to compare the microbial communities from the different land-use practices.

Kinetic parameters estimated, for AWCD and substrate guilds, from the different land-use practices along seasons were compared using repeated-measures ANOVA. Ordinations based on the samples similarity were obtained by a Non-metric Multidimensional Scaling (NMDS). Subsequently an ANOSIM was computed to determine the significant differences, either among sampling seasons (in the case of AWCD) or among land-use practices (in the case of substrate guilds). The underlined similarity matrix was obtained with Normalised Euclidean distance of the kinetic parameters values transformed as $\log(x + 1)$. When significant differences between land-use practices were found, an ordination based on the variables (substrate guilds kinetic parameters) similarity was obtained with NMDS, to compare substrate guild utilization pattern. The ANOVA and Spearman rank coefficient analyses were performed with STATISTICA 7; the NMDS and the ANOSIM were calculated with PRIMER 5 for Windows software.

Table 2
Soil physical-chemical properties of the different land-use practices (AGRI – agriculture, PAST – pasture, ABAN – abandoned) at the three sampling seasons. "1, 2" indicate significant differences ($p < 0.05$) among different sampling seasons within the same land-use practice; "a, b" indicate significant differences ($p < 0.05$) among different land-use practices within the same sampling season; $n = 10$.

Land-use practices	Seasons	Water content (%)	Organic matter (%)	pH	Ammonium (μg N/g dm)	Nitrate (μg N/g dm)
AGRI	Winter	$17.5 \pm 3.02^{(1)}$	4.14 ± 0.593	$4.56 \pm 0.130^{(1,b)}$	0.997 ± 0.522	$0.632 \pm 0.444^{(2)}$
	Spring	$8.29 \pm 3.25^{(2,a)}$	4.82 ± 0.913	$4.24 \pm 0.130^{(2,b)}$	0.574 ± 0.333	$0.141 \pm 0.084^{(2)}$
	Autumn	$15.9 \pm 1.49^{(1)}$	5.35 ± 0.324	$4.31 \pm 0.170^{(2)}$	$2.20 \pm 1.58^{(b)}$	$2.36 \pm 1.45^{(1)}$
PAST	Winter	$15.4 \pm 1.69^{(1)}$	5.16 ± 3.22	$5.01 \pm 0.220^{(1,a)}$	$1.21 \pm 0.707^{(2)}$	0.689 ± 0.601
	Spring	$3.56 \pm 1.93^{(2,b)}$	5.10 ± 1.02	$4.66 \pm 0.280^{(2,a)}$	$1.70 \pm 0.533^{(2)}$	0.090 ± 0.027
	Autumn	$15.5 \pm 2.77^{(1)}$	4.64 ± 0.774	$4.59 \pm 0.360^{(2)}$	$5.13 \pm 1.52^{(1,a)}$	1.70 ± 2.02
ABAN	Winter	$17.8 \pm 2.26^{(1)}$	4.63 ± 0.683	$4.80 \pm 0.240^{(1,a,b)}$	$0.889 \pm 0.418^{(2)}$	0.107 ± 0.042
	Spring	$1.91 \pm 1.54^{(2,b)}$	5.83 ± 1.22	$4.36 \pm 0.220^{(2,b)}$	$1.00 \pm 0.428^{(2)}$	0.063 ± 0.050
	Autumn	$15.8 \pm 2.70^{(1)}$	6.14 ± 0.732	$4.50 \pm 0.140^{(2)}$	$3.71 \pm 1.04^{(1,a)}$	0.392 ± 0.462

3. Results

3.1. Soil physico-chemical properties

Significant differences among sampling seasons and among land-use practices were found for all soil parameters except for organic matter content (Table 2). Differences among sampling seasons within each land-use practice were observed for water content with higher values in rainy seasons ($p < 0.05$) and soil pH with higher values in winter ($p < 0.05$). Seasonal variations were observed for ammonium and nitrate, with higher values in autumn. However, soil ammonium content was significantly different only within PAST and ABAN areas ($p < 0.05$), whereas nitrate showed significant variations within AGRI area ($p < 0.05$).

Differences among land-use practices were observed in the water content in spring, with significantly higher values at the AGRI area ($p < 0.05$) and in soil pH in winter and in spring, with higher values at the PAST area ($p < 0.05$). Ammonia also showed significant differences among land-use practices in autumn ($p < 0.05$), with the lowest value at the AGRI area (Table 2).

3.2. Soil microbial parameters: seasonal effects

Generally, microbial activity was highest in the rainy seasons (winter and/or autumn) (Fig. 1). Dehydrogenase activity of soils collected from all land-use practices increased along the sampling seasons, with the highest values being observed in autumn. In AGRI and PAST areas significant differences in DHA activity were observed among all seasons, whereas in the ABAN area only in autumn DHA activity was significantly higher than in winter and spring. Significant differences ($p < 0.05$) among seasons were obtained for acid phosphatase activity in all sampling areas, with the highest values being observed in autumn. In all land-use practices β-glucosidase was significantly lower at spring. For urease activity, significant differences among seasons ($p < 0.05$) were only found in ABAN, where the highest values occurred in autumn. In the case of N-mineralization rate, significant differences ($p < 0.05$) among seasons were only found in PAST where the highest values occurred in winter (Table 3). No significant differences among sampling seasons were found in nitrification rate due to the high variability found within each land-use practice. Even though, the highest values occurred in spring in all land-use areas; the lowest activity was found in autumn in AGRI and ABAN and in winter in PAST. Negative nitrification rates were obtained in rainy seasons (winter and/or autumn) in PAST and ABAN areas. The ANOSIM (taking into account all microbial parameters measured) revealed significant differences ($p < 0.05$) among seasons; pairwise test showed that the three sampling seasons were significantly different from each other ($p < 0.05$).

Considering the metabolic diversity (AWCD), lower levels of substrate utilization (lower *K* values) were observed in all land-use practices at winter, however significant differences between

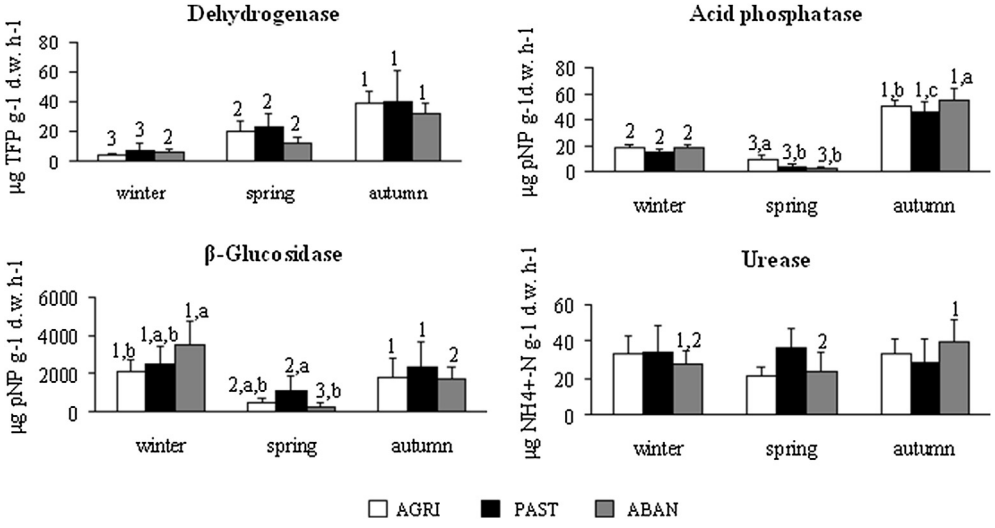

Fig. 1. Enzymatic activities (mean values and standard deviation) in the different land-use practices (AGRI – agriculture, PAST – pasture, ABAN – abandoned) at three sampling seasons. "1, 2, 3" indicate significant differences ($p < 0.05$) among different sampling seasons within the same land-use practice; "a, b, c" indicate significant differences ($p < 0.05$) among different land-use practices within the same season. $n = 10$.

Table 3
N-Mineralization and nitrification (mean values and standard deviation) in the different land-use practices (AGRI – agriculture, PAST – pasture, ABAN – abandoned) at the three sampling seasons. "1, 2" indicate significant differences ($p < 0.05$) among different sampling seasons within the same land-use practice; $n = 5$.

Land-use practices	Seasons	N-Mineralization (μg N/g dm/d)	Nitrification (%p/d)
AGRI	Winter	0.014 ± 0.041	4.01 ± 6.56
	Spring	0.012 ± 0.021	24.2 ± 25.2
	Autumn	0.061 ± 0.021	0.275 ± 0.459
PAST	Winter	0.123 ± 0.089$^{(1)}$	−0.293 ± 0.423
	Spring	−0.016 ± 0.045$^{(2)}$	5.39 ± 2.86
	Autumn	0.026 ± 0.054$^{(1,2)}$	−0.024 ± 0.133
ABAN	Winter	0.075 ± 0.035	0.059 ± 0.363
	Spring	0.002 ± 0.020	0.100 ± 0.240
	Autumn	0.095 ± 0.050	−0.357 ± 0.631

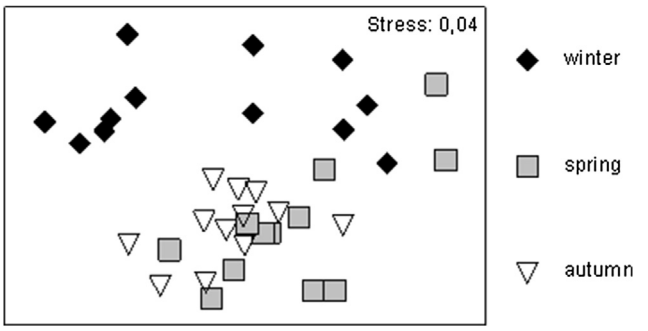

Fig. 2. Non-metric multidimensional scaling of average well colour development kinetic parameters along seasons.

seasons were only found in ABAN area ($p < 0.05$; Table 4). The minimum and maximum values of the other two kinetic parameters, exponential rate (R) and time to reach the midpoint of the curve exponential phase (S) occurred at different seasons and in different land-use practices. Significant differences among seasons were found in ABAN ($p < 0.05$) with higher and lower exponential rates occurring in winter and spring, respectively. Regarding the time to reach the midpoint (S) significant differences ($p < 0.05$; Table 4) among seasons were found in PAST (with higher S value in winter and the lowest in spring) and ABAN (with higher S value in spring and the lowest in autumn) areas. Results of the NMDS based on the kinetic parameters from the AWCD of the three sampling seasons showed a clear separation of winter samples (Fig. 2). The ANOSIM revealed significant differences ($p < 0.05$); pairwise tests results showed that the three sampling seasons were significantly different from each other ($p < 0.05$).

Generally, maximum and minimum substrate guild utilization values (K) occurred in the rainy seasons (winter and autumn) and were guild-dependent. Significant differences ($p < 0.05$) were found in the group of miscellaneous substrates that was less used in winter. At this sampling season, substrates of this guild were more used in PAST area ($p < 0.05$; Table A1). Concerning the exponential rate (R) significant differences ($p < 0.05$) among sampling seasons were found in AGRI and PAST areas, where the amino acids exponential rate was greater in autumn; significant differences were also found ($p < 0.05$) between winter and autumn in carboxylic acids exponential rate in PAST and ABAN areas. Analysis of the values corresponding to the time to reach the midpoint of the exponential phase (S) revealed significant differences between winter and the other seasons (spring and autumn) in all land-use practices, in the case of amines ($p < 0.05$). In the case of carbohydrates ($p < 0.05$) and miscellaneous guilds ($p < 0.05$), differences between seasons were only found in AGRI area (Table A3).

Table 4
Kinetic parameters (mean values and standard deviation) from the AWCD curve fitting. K represents the asymptote or maximum degree of colour development (OD), R represents the exponential rate of OD change, and S is the time to reach the midpoint of the exponential phase of the curve. "1, 2" indicate significant differences ($p < 0.05$) between different sampling seasons within the same land-use practice; "a, b" indicate significant differences ($p < 0.05$) between different land-use practices within the same sampling season; $n = 4$.

Land-use practices	Seasons	K	R	S
AGRI	Winter	0.450 ± 0.090$^{(b)}$	0.084 ± 0.030	61.4 ± 8.19
	Spring	0.919 ± 0.060	0.059 ± 0.000	61.3 ± 3.69
	Autumn	0.861 ± 0.120	0.065 ± 0.000	55.7 ± 3.24
PAST	Winter	0.794 ± 0.130$^{(a)}$	0.061 ± 0.010	73.2 ± 3.55 $^{(1)}$
	Spring	0.975 ± 0.100	0.073 ± 0.010	54.4 ± 2.95 $^{(2)}$
	Autumn	0.834 ± 0.080	0.073 ± 0.010	56.6 ± 3.64 $^{(2)}$
ABAN	Winter	0.451 ± 0.040 $^{(2,b)}$	0.084 ± 0.020 $^{(1)}$	59.1 ± 5.55 $^{(2)}$
	Spring	1.07 ± 0.250 $^{(1)}$	0.048 ± 0.010 $^{(2)}$	69.8 ± 10.9 $^{(1)}$
	Autumn	0.933 ± 0.150 $^{(1)}$	0.068 ± 0.010$^{(1,2)}$	57.2 ± 6.25 $^{(2)}$

3.3. Soil microbial parameters: land-use effects

The obtained results reveal an irregular response pattern among land-use practices within each season (Fig. 1). Significant differences among land-use practices were only obtained in the acid phosphatase ($p < 0.05$) and β-glucosidase activities ($p < 0.05$). Acid phosphatase activity in spring showed significant differences among AGRI area, with the highest enzyme activity, and the remaining land-use practices; in autumn all land-use practices were significantly different from each other. Significant differences in β-glucosidase activity occurred in winter between AGRI and ABAN areas, reaching the highest value in ABAN area. In spring the activity of this enzyme was significantly higher in PAST than in ABAN area. In the two indicator parameters of nitrogen transformation, again, the high variability obtained led to the inexistence of significant differences among land-use practices (Table 3). The ANOSIM performed at land-use level, considering each sampling season individually, revealed significant differences among land-use practices at both winter ($p < 0.05$) and spring ($p < 0.05$); pairwise tests showed that all land-use practices were significantly different from each other. In autumn no significant differences were obtained. The several Principal Component Analyses (PCA's) performed for each sampling season revealed a seasonal related response of the association between microbial parameters and the three land-use practices. The PCA biplot of winter showed along axis 1 (37.8% of total variance explained) the separation of the AGRI area, with higher values of acid phosphatase activity and nitrification rate, from the other two land-use practices. The PAST area showed, in general, higher values for all the other parameters, whereas the ABAN area revealed a comparable lower activity (Fig. 3). In spring the PCA biplot showed a clear separation of the ABAN area from the other two land-use practices along axis 1 (39.6% of total variance explained). While overall microbial activity is still maintained at low levels on the ABAN area, a differentiated increase in microbial activity is observed on both AGRI and PAST areas; axis 2 (28.3% of total variance explained) separated the AGRI area, more related with nitrification and N-mineralization rates, acid phosphatase and dehydrogenase activities, from the PAST area with higher values of β-glucosidase and urease activities (Fig. 3). The PCA biplot of autumn showed a different picture, with the ABAN area having higher values of N-mineralization rates, acid phosphatase and urease activities than the other two land-use practices (Fig. 3). However, in this case, no clear separation between land-use practices can be observed along axis 1 (40.0% of

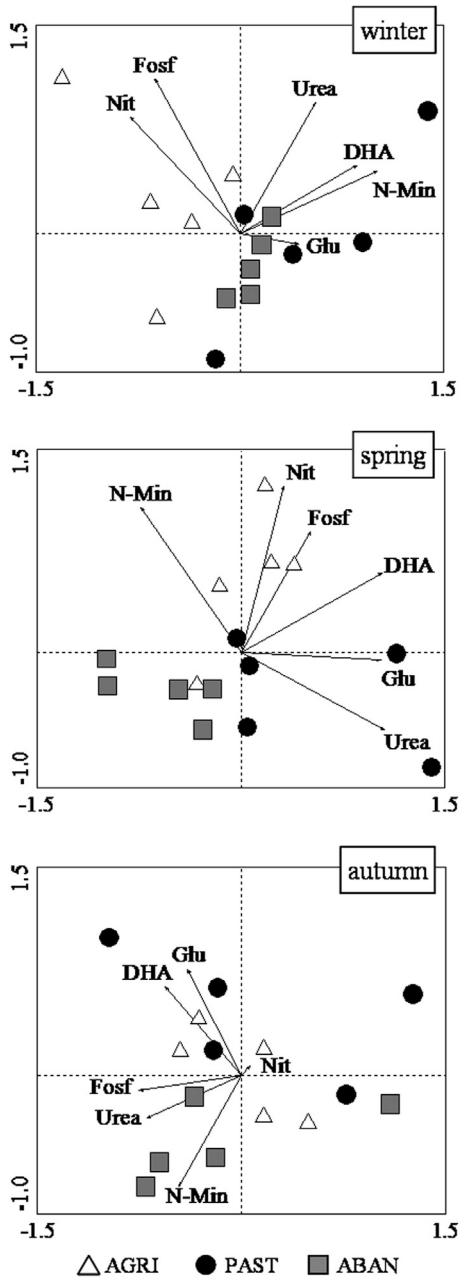

Fig. 3. Principal Components Analysis (PCA) biplots of microbial activity parameters measured at the three sampling seasons. Variables: DHA – dehydrogenase activity; Phos – acid phosphatase activity; Glu – β-glucosidase activity; Urea – urease activity; N-Min – N-mineralization rate; Nit – nitrification rate. $n = 5$.

total variance explained) nor axis 2 (24.4% of the total variance explained).

The ANOSIM performed with *K* values from each season did not show any significant differences between land-use practices. The maximum exponential rate (*R*) values of the different guilds were guild and land-use practice dependent (Table A2). Significant differences were found in autumn between ABAN and AGRI and PAST areas, in the case of amino acids ($p < 0.05$) and polymers ($p < 0.05$; Table A2). Results of the ANOSIM performed with *R* values from autumn showed that ABAN area was significantly different ($p < 0.05$) from AGRI and PAST areas. The NMDS performed with the exponential rate values from this season, showed that the guild utilization pattern differs mainly in amino acids, miscellaneous and polymers (Fig. 4). Results from the ANOSIM performed with *S*

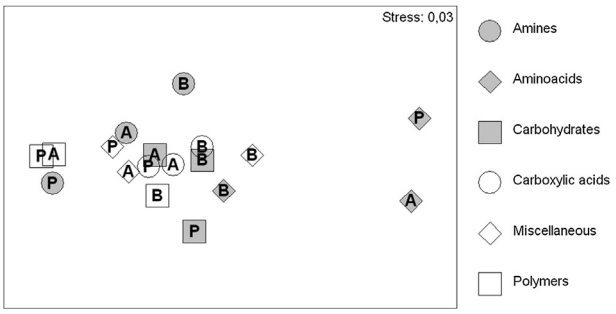

Fig. 4. Non-metric multidimensional scaling of guilds exponential rate values (*R*) in autumn (legend: A – agriculture; P – pasture; B – abandoned).

values from autumn, the only season revealing significant differences ($p < 0.05$) between land-use practices, showed that PAST area was significantly different from ABAN area. A NMDS performed at the substrate guild level, from this season, showed that the guild utilization pattern differs mainly in amines, carboxylic acids and polymers (Fig. 5).

3.4. Soil microbial parameters and soil physico-chemical properties

The activity of most enzymes and the N-mineralization rate showed significant positive correlations with soil water content. Dehydrogenase and urease activity were also significantly correlated with soil organic matter. This N-cycle enzyme showed, as expected, also positive correlations with ammonium and nitrate contents. On the contrary, nitrification rate was negatively correlated with soil ammonium content (Table 5). A negative significant correlation between maximum substrate used (*K*) and water content and a positive significant correlation with organic matter were found. The exponential rate (*R*) was positively correlated with pH (Table 5).

4. Discussion

Soil biological processes, such as organic matter decomposition and nutrient cycling are catalysed by enzymes. Thus, changes in enzyme abundance and or activity may affect soil ecosystem functioning. Enzyme activity is related to soil properties like moisture and organic matter content [34,35] and it is also influenced by management practices [36–39]. The results obtained in this study, although being able, in some circumstances, to show differences between land-use practices, reflected more the variations of microbial activity among seasons. Some studies have demonstrated that in Mediterranean ecosystems the highest enzymatic activities occurred in spring [7], when optimal conditions of temperature, water availability and litter quantity occurred [8]. However, the

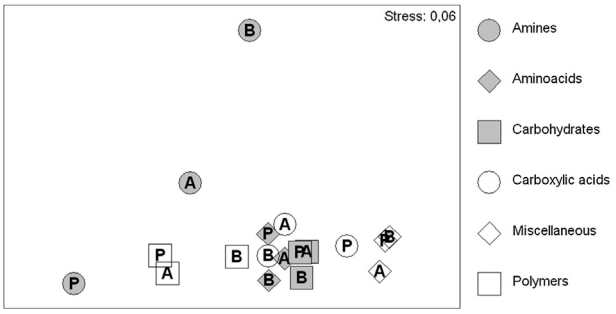

Fig. 5. Non-metric multidimensional scaling of guilds time to reach the midpoint values (*S*) in autumn (legend: A – agriculture; P – pasture; B – abandoned).

Table 5
Spearman rank correlations between microbial parameters and soil physico–chemical properties. *K* represents the asymptote or maximum degree of colour development (OD), *R* represents the exponential rate of OD change, and *S* is the time to reach the midpoint of the exponential phase of the curve. * Indicates significant correlations ($p < 0.05$); $n = 5$.

Microbial parameters	Water content	Organic matter	pH	Ammonium	Nitrate
Dehydrogenase	−0.155	0.514*	−0.275	ND	ND
Acid phosphatase	0.692*	0.167	−0.013	ND	ND
β-glucosidase	0.611*	−0.102	0.441*	ND	ND
Urease	0.425*	0.352*	0.377*	0.478*	0.349*
N-Mineralization	0.477*	0.224	0.174	0.149	0.289
Nitrification	−0.213	−0.055	−0.337*	−0.348*	−0.262
K	−0.592*	0.359*	−0.192	0.197	−0.125
R	0.270	−0.089	0.496*	0.209	0.290
S	−0.022	−0.219	−0.173	−0.321	−0.239

results obtained in this study showed a generally higher microbial activity (DHA, acid phosphatase, β-glucosidase and N-mineralization) in the rainy seasons (winter and/or autumn) in comparison to spring. The positive correlation of these parameters with soil water content suggests that this factor might be the major responsible of the microbial activity seasonal variation. Other studies performed in Mediterranean forest soils also attributed the increased microbial activity to higher water content [5,40]. The exception to this pattern occurred with the nitrification rate, which presented higher rate values in spring. Normally, maximum values of nitrification occur in spring due to optimal temperature, moisture conditions and higher substrate availability, the nitrification limiting factors [41]. In general, the nitrification values reported in literature for Mediterranean forest soils are very low [42], and this study was no exception. Microorganisms responsible for nitrification are very sensitive to low pH values. Consequently it has been proposed that, in acidic soils, nitrate production is due to the activity of autotrophic nitrifiers, located in microsites with relatively higher pH values [43,44]. The generally low nitrification rates obtained could be the result of a low availability of ammonium at the microsite level [45] and ammonium immobilization by soil microorganisms [46].

The variation of microbial activity amongst the different land-use practices did not follow a common pattern and its interpretation is not simple. When considering each microbial parameter individually, few significant differences were found between land-use practices. However, when microbial parameters were considered together (in a multivariate analysis) discrimination, mainly of the ABAN area with a general low activity, was obtained in winter and spring. These results are in accordance with Pascual et al. [9] who found lower enzymatic activities in abandoned Mediterranean sites, probably associated with the loss of plant cover and consequently with low levels of organic matter and nutrients.

The ability of microorganisms to metabolize a large variety of organic compounds is essential to several soil processes, such as nutrient cycling. Community-level physiological profiles based on the microbial ability to use sole carbon sources can be obtained with Biolog microplates®. This method is selective, since it only detects the activity of microorganisms capable of growing and metabolizing substrates in the microplate environment [14,32]. Therefore, it indicates only the potential functional diversity [3] and does not provide a direct view of microbial communities [33]. Kinetic parameters successfully discriminated microbial communities in several studies, such as in oil contaminated soil [32], compost maturity [47] and in Mediterranean forests [48]. In this study, however, the AWCD kinetic parameters, more than discriminating land-use practices, discriminated seasons. In general, substrate utilization levels (*K* values) were lower in samples from winter and higher in those collected in spring. This is in accordance with Papatheodorou et al. [16] who, when studying the effect of seasonal fluctuations on soil microbial functional diversity from Mediterranean grasslands, found a decreased in metabolic diversity in winter. In our study, this decrease could be related with the negative correlation between the *K* parameter and soil water content. Bossio and Scow [49] found a strong impact of soil water content in microbial metabolic diversity that caused a general decreased in the utilization of carbon substrates, such as carbohydrates and carboxylic acids, probably due to a reduction in the availability of suitable electron acceptors that lead to lower rates of organic matter decomposition. In winter the higher carbon source utilization levels occurred in the PAST area, probably related with organic matter improvement from grazing cattle dung and urine [50]; in spring the highest substrate consumption was found in samples from the ABAN area, with high organic matter content probably due to high vegetation density [51]. This shift could be attributed to changes in the quality of organic matter inputs [52].

AGRI and ABAN areas had a similar exponential rate pattern, with faster carbon source oxidation (high *R* values) in winter and slower carbon oxidation (low *R* values) in spring. Since the time to reach the midpoint (*S*) supplies information about the initial inoculum density and the relative growth rates of the species able to use the carbon source in each well [13], it can be considered that the AGRI and PAST areas in winter, and the ABAN area in spring had lower inoculum density and/or slower microbial growth. AGRI and ABAN areas, in autumn and PAST area in spring had higher inoculum density and/or faster microbial growth in the wells.

Substrate guild utilization pattern differences among land-use practices could be related with the ability of the microbial community to degrade specific categories of organic matter associated with differences in vegetation [14]. In this study, however, the kinetic parameters of the different substrate guilds revealed a similar carbon source pattern in the land-use practices under survey. In all seasons, land-use practices had a similar substrate guild consumption pattern, with amino acids as the most used and the miscellaneous guild as the least consumed. The polymers and the miscellaneous guilds had the lowest exponential rate and time to reach the midpoint, respectively.

Despite the general substrate use pattern similarity, in autumn, considering the exponential rate (*R*), a distinct carbon source pattern was found in the ABAN area, mainly caused by different exponential rates in the case of amino acids, miscellaneous and polymers. Also in autumn, the time to reach the midpoint (*S*) distinguished the ABAN area substrate use pattern from that of the PAST area, mainly caused by differences in amines, carboxylic acids and polymers. This discrimination of the ABAN area was consistent with the separation of less disturb from more disturbed soils obtained by Winding [20] in her study of the metabolic fingerprint from agricultural and forest soils. Contrary to other studies [47,48], the *K* parameter was not able to separate land-use practices; this may probably be an effect of the exhaustion of the carbon source in the wells [13] and may not represent a real carbon utilization pattern similarity.

In other studies, differences in soils due to the microorganisms ability to consume different substrate guilds, mainly amines, amino acids and carboxylic acids in different cropping systems [53] and amines, carbohydrates, carboxylic acids and polymers in different soil profiles from Mediterranean forests [48] were also found. However, this type of analysis should be done carefully, and should not be emphasized on particular substrates. Carbon sources present in Biolog Ecoplates®, despite being ecologically relevant, do not accurately represent the substrates present in soil. This means that a specific pattern of substrate consumption should not be directly related to *in situ* utilization, but be used for comparative purposes of the potential metabolic versatility [54].

In conclusion, seasonal variation was evident with maximum activity occurring in rainy seasons (winter and autumn) and with lower substrate utilization levels in winter. Significant correlations between almost all microbial parameters and soil water content reflect this seasonal effect. Within each season some microbial parameters were able to distinguish the different land-use practices, mainly separating the ABAN area from the other two land-use practices, with a general low activity and with different substrate exponential rates.

Acknowledgements

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Appendix A

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Table A.1
K values (mean values and standard deviation) of the different substrate guilds in the different land-use practices (AGRI – agriculture, PAST – pasture, ABAN – abandoned) at the three sampling seasons. "1, 2" indicate significant differences ($p < 0.05$) between different sampling seasons within the same land-use practice; "a, b" indicate significant differences ($p < 0.05$) between different land-use practices within the same season; $n = 4$.

Land-use practices	Seasons	Amines	Amino acids	Carbohydrates	Carboxylic acids	Miscellaneous	Polymers
AGRI	Winter	0.859 ± 0.352	1.30 ± 0.085	1.24 ± 0.188	0.872 ± 0.128	0.280 ± 0.073$^{(2,b)}$	1.04 ± 0.145
	Spring	0.666 ± 0.200	1.17 ± 0.081	1.10 ± 0.025	0.938 ± 0.025	0.794 ± 0.047$^{(1)}$	1.03± 0.107
	Autumn	1.01 ± 0.144	1.12 ± 0.026	1.02 ± 0.040	0.922 ± 0.042	0.779 ± 0.087 $^{(1)}$	1.12 ± 0.033
PAST	Winter	0.732 ± 0.256	1.32 ± 0.247	1.19 ± 0.036	0.897 ± 0.062	0.593 ± 0.073$^{(2,a)}$	0.865 ± 0.356
	Spring	1.03 ± 0.170	1.18 ± 0.062	1.08 ± 0.031	0.891 ± 0.053	0.798 ± 0.103$^{(1)}$	0.977 ± 0.130
	Autumn	1.09 ± 0.099	1.14 ± 0.042	1.07 ± 0.043	0.860 ± 0.049	0.866 ± 0.062$^{(1)}$	1.04 ± 0.122
ABAN	Winter	0.804 ± 0.287	1.24 ± 0.339	1.12 ± 0.261	1.06 ± 0.119	0.247 ± 0.085$^{(2,b)}$	0.972 ± 0.045
	Spring	0.504 ± 0.371	1.21 ± 0.087	1.15 ± 0.115	0.946 ± 0.070	0.696 ± 0.112$^{(1)}$	1.02 ± 0.112
	Autumn	0.852 ± 0.366	1.21 ± 0.050	1.06 ± 0.042	0.895 ± 0.029	0.805 ± 0.126$^{(1)}$	1.05 ± 0.041

Table A.2
R values (mean values and standard deviation) of the different substrate guilds in the different land-use practices (AGRI – agriculture, PAST – pasture, ABAN – abandoned) at the three sampling seasons. "1, 2" indicate significant differences ($p < 0.05$) between different sampling seasons within the same land-use practice; "a, b" indicate significant differences ($p < 0.05$) between different land-use practices within the same season; $n = 4$.

Land-use practices	Seasons	Amines	Amino acids	Carbohydrates	Carboxylic acids	Miscellaneous	Polymers
AGRI	Winter	1.85 ± 2.40	0.884 ± 0.230$^{(2)}$	0.990 ± 0.289	1.32 ± 0.240	0.542 ± 0.081	0.379 ± 0.132
	Spring	0.760 ± 0.111	1.23 ± 0.115$^{(2)}$	1.03 ± 0.177	1.08 ± 0.139	1.09 ± 0.271	0.472 ± 0.079
	Autumn	0.678 ± 0.193	2.12±0.574$^{(1,a)}$	0.786 ± 0.200	0.865 ± 0.159	0.667 ± 0.114	0.408 ± 0.064$^{(b)}$
PAST	Winter	1.23 ± 0.746	0.868 ± 0.121$^{(2)}$	1.02 ± 0.126	1.33 ± 0.164$^{(1)}$	0.769 ± 0.064	0.495 ± 0.118
	Spring	0.582 ± 0.140	1.54 ± 0.543$^{(2)}$	0.866 ± 0.119	1.11 ± 0.161$^{(1,2)}$	0.751 ± 0.295	0.570 ± 0.160
	Autumn	0.404 ± 0.079	2.22±0.409$^{(1,a)}$	0.961 ± 0.433	0.756 ± 0.092$^{(2)}$	0.633 ± 0.133	0.367 ± 0.079$^{(b)}$
ABAN	Winter	0.581 ± 0.243	0.689 ± 0.187	0.727 ± 0.347	1.59 ± 0.551$^{(1)}$	1.50 ± 2.32	0.456 ± 0.080
	Spring	0.549 ± 0.398	1.14 ± 0.239	0.975 ± 0.207	1.18 ± 0.271$^{(1,2)}$	1.19 ± 0.318	0.518 ± 0.089
	Autumn	0.909 ± 0.436	1.11 ± 0.210$^{(b)}$	0.995 ± 0.109	0.980 ± 0.106$^{(2)}$	1.20 ± 0.153	0.787 ± 0.175$^{(a)}$

Table A.3
S values (mean values and standard deviation) of the different substrate guilds in the different land-use practices (AGRI – agriculture, PAST – pasture, ABAN – abandoned) at the three sampling seasons. "1, 2" indicate significant differences ($p < 0.05$) between different sampling seasons within the same land-use practice; "a, b" indicate significant differences ($p < 0.05$) between different land-use practices within the same season; $n = 4$.

Land-use practices	Seasons	Amines	Amino acids	Carbohydrates	Carboxylic acids	Miscellaneous	Polymers
AGRI	Winter	0.762 ± 0.345$^{(2)}$	0.707 ± 0.244	1.39 ± 0.242$^{(1)}$	1.01 ± 0.084	0.455 ± 0.129$^{(2)}$	1.271 ± 0.206
	Spring	0.975 ± 0.410$^{(1)}$	1.09 ± 0.109	1.00 ± 0.132$^{(2)}$	0.914 ± 0.141	0.746 ± 0.050$^{(1)}$	1.26 ± 0.169
	Autumn	1.19 ± 0.300$^{(1)}$	0.983 ± 0.066	0.919 ± 0.050$^{(2)}$	0.978 ± 0.110	0.754 ± 0.075$^{(1)}$	1.31 ± 0.076
PAST	Winter	0.628 ± 0.248$^{(2)}$	1.21 ± 0.196	1.12 ± 0.173	0.966 ± 0.106	0.772 ± 0.120	0.912 ± 0.089
	Spring	1.52 ± 0.232$^{(1)}$	1.06 ± 0.049	0.928 ± 0.095	0.889 ± 0.055	0.791 ± 0.140	1.19 ± 0.109
	Autumn	1.58 ± 0.111$^{(1)}$	1.03 ± 0.093	0.947 ± 0.051	0.834 ± 0.056	0.740 ± 0.056	1.33 ± 0.046
ABAN	Winter	0.703 ± 0.258$^{(2)}$	0.748 ± 0.343	1.29 ± 0.227	1.11 ± 0.357	0.524 ± 0.160	1.12 ± 0.239
	Spring	1.02 ± 0.693$^{(1)}$	0.946 ± 0.106	1.02 ± 0.203	1.02 ± 0.159	0.694 ± 0.211	1.23 ± 0.275
	Autumn	1.16 ± 0.629$^{(1)}$	1.01 ± 0.150	0.954 ± 0.122	1.03 ± 0.054	0.741 ± 0.098	1.11 ± 0.045

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