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# Sporulation and physiological profiles of bacterial communities of three Mediterranean soils affected by drying-rewetting or freezing-thawing cycles

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## ABSTRACT

In the global change context, the basal respiration (BR), the estimated number of bacterial spores (SP) and the community level physiological profiles (CLPPs) were investigated in three different Mediterranean soils following different hydric and thermic stress scenarios. The treatments consisted in an increasing number (1, 2, 4, and 7) of drying-rewetting (DRWc) or freezing-thawing cycles (FTc) at 20, 40 or -20 °C. The results highlighted that the different soils responded differently to the same treatment and that the three variables considered were weakly related one to each another. In almost all soils and modalities, the BR increased significantly during the first cycles before decreasing during the last. With regards to SP, it appeared that, for a given soil, the capacity of microbial communities to sporulate and/or germinate can be considerably more influenced by the temperature rather than by the hydric stress. Finally, the CLPPs literally collapsed with the treatment at 40 °C, irrespective of the soil considered. This suggested a progressive replacement of the catabolically diversified original bacterial communities by another showing lower functional diversities.

### Keywords:

Freezing-thawing  
Drying-rewetting  
Community level physiological profiles  
Soil respiration  
Mediterranean soil vulnerability  
Climate change

## 1. Introduction

Soil microbial communities are known to be sensitive to climate (Chemidlin Prevost-Boure et al., 2011), and it is globally admitted that under Mediterranean climate, the global change will lead to heat and drought waves that will durably affect the terrestrial ecosystems (IPCC, 2014), in part through increased vulnerability of microbial edaphic functions. If respiratory and enzymatic activities of soils subjected to climatic stresses are beginning to be well documented (e.g. Guénon and Gros, 2013; Daou et al., 2016; Gao et al., 2016), those regarding the effect of global change on the catabolic functional diversity of soil microorganisms are much rarer. Even more, data about climatic stress simulations on the sporulation abilities of soil microbial communities are still inexistent, despite the importance of sporulating microorganisms in soil

functioning. Indeed, among the affected microorganisms, the spore-forming bacteria are known to be particularly abundant in soils and it was demonstrated that their abundance and their specific richness could be influenced by hydric and thermic characteristics of their environment (Périssol et al., 1993; Brunel et al., 1994). Thus, the objectives of this study were to determine how different thermal (-20 °C, +20 °C or +40 °C) and/or hydric stress scenarios, i.e. drying-rewetting (DRWc) and freezing-thawing (FTc) cycles, would influence, negatively or positively, the basal respiration (BR), the estimated number of bacterial spores (SP) and the community level physiological profiles (CLPPs, Biolog Ecoplates™) of soil microorganisms. We aimed also to determine which climatic factor (DRWc/FTc or temperature) most influences the different microbial variables and if the responses observed are modulated by the origin and the pedological and pedoclimatic characteristics of the different Mediterranean soils investigated.

## 2. Materials and methods

Mesocosm experiments were conducted (Fig. 1) with three different Mediterranean surface soils (0–5 cm depth, five samples each) collected during February 2014 in the Bouches-du-Rhône,

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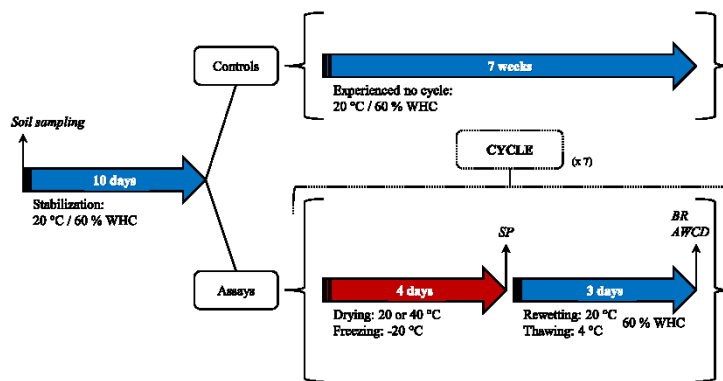


Fig. 1. Design of mesocosm experiments. Samples were incubated for stabilization at 20 °C and 60% WHC for 10 days prior to the experiments. Controls consisted in 5 replicates for each soil (3 soils  $\times$  5 samples; 15 controls in total) maintained at 20 °C and 60% WHC for 7 weeks. The different samples subjected to stress scenarios were obtained as follows: 3 soils  $\times$  4 cycle treatments (cycle1, cycle2, cycle4, cycle7)  $\times$  3 temperatures (–20 °C, 20 °C, 40 °C)  $\times$  5 replicates (180 samples in total). SP, the estimated number of bacterial spores was measured after the drying/freezing phase and BR, the basal respiration and AWCD, the average well color development (Biolog EcoPlates™) were measured after the rewetting/thawing phase. WHC, water holding capacity. 195 mesocosms in total.

France. A south oriented soil (S) (43°19'5" N, 5°40'2" E, 900 m altitude), a north oriented soil (N), opposite to the south oriented plot (43°19'29" N, 5°40'23" E, 700 m altitude), and a riparian forest soil (R) (43°31'11" N, 5°34'10" E, 420 m altitude). For each soil, samples were sieved at 2 mm, pooled into one composite sample, and the dry mass (DM) and the water holding capacity (WHC) were determined prior to the experiments. The textures of soils were classified as clay loam (S and N soils, Colluvic Rendzic Leptosols; IUSS, 2006) and silty clay loam (R soil, Calcic Stagnic Fluvisol; IUSS, 2006). The three soils showed an alkaline pH (7.6), and similar cation exchange capacity (22.8–24.8  $\text{cmol}^+ \text{kg}^{-1}$  DM) and total nitrogen content (4.4–5.0  $\text{g kg}^{-1}$  DM). The total organic carbon contents were 55.4, 64.7 and 82.4  $\text{g kg}^{-1}$  DM, respectively for S, R and N soils. Considering the climatic characteristics of the different plots, the S soil (transition between meso- and supra-Mediterranean bioclimatic stages) was, along the year, subjected to more drastic temperature and relative humidity variations than the R soil (medio-european influences). The pedoclimatic conditions of the N soil (supra-Mediterranean-subhumid bioclimatic stage) appeared as intermediary to the two other soils. See Appendix A for more details about floristic inventories of plots and physicochemical and pedoclimatic characteristics of soils (Daou et al., 2016). The BR was measured according to the technique of Anderson and Domsch (1978), using gas chromatography (Chrompack CHROM 3 – CP 9001, equipped with a Porapak™ column type, under a helium flux of 650  $\text{mL h}^{-1}$ ). The Biolog EcoPlates™ technique was used according to the protocol of Floch et al. (2011) to determine the metabolic potentialities (i.e. CLPPs) of aerobic heterochemoorganotroph communities of soils. After 72 h of growth in microplate, the average well color development (AWCD, i.e. average metabolic activity) was calculated according to the method of Garland and Mills (1991) and was used as indicator of the CLPPs (Preston-Mafham et al., 2002). Relative use of several classes of substrates (polymers, carbohydrates, carboxylic acids, amino acids, amines and miscellaneous) was also calculated according to Selmants et al. (2005). To achieve this, each class of substrate was calculated as corrected absorption values, i.e. divided by total absorption in the plate. The number of bacterial spores was

estimated by using the most probable number method (MPN) and the algorithm developed by Briones and Reichardt (1999). Briefly, 1 g of soil was suspended in 9 mL of 0.1% sterile sodium pyrophosphate solution, then agitated for 20 min at 50 rpm (i.e. 5/6 Hz) and submitted to decimal dilutions with a 0.85% sterile NaCl solution. The different dilutions were thereafter pasteurized at 80 °C for 10 min to eliminate non sporulated microorganisms and to preserve the bacterial spores (Travers et al., 1987). 20  $\mu\text{L}$  of these dilutions were inoculated in 96 wells microplates (Nunc™) filled with 180  $\mu\text{L}$  of nutrient broth medium (NB) at 20  $\text{g L}^{-1}$  (Biomar Diagnostics). For each dilution, eight repetitions were done and microplates were incubated at 30 °C during 3 days before reading and calculation of the MPN.

Concerning statistics, prior to analyses, the hypotheses of data normality (Shapiro-Wilk test) and variance homogeneity (Bartlett test) were checked. For each soil and temperature, repeated measures analyses of variance (rANOVA) were performed on each microbial variable to test the effect of cycle factor (within-subject factor; four levels: 1, 2, 4, and 7 cycles), at a significance level of  $p < 0.05$  ( $n = 5$ ). Thereafter, a *post hoc* test of pairwise multiple comparisons of Tukey (honestly significant difference, HSD) was used to verify whether the various levels of the tested factor were significantly different from each other, at a significance level of  $p < 0.05$ . For each soil, analyses of variance (ANOVA) were performed on each microbial variable of the last cycles to test the effect of temperature factor (between-subject factor; three levels: –20, 20, and 40 °C), at a significance level of  $p < 0.05$  ( $n = 5$ ). Thereafter, a *post hoc* test of comparisons with a control of Dunnett was used to verify whether the various levels of the tested factor were significantly lower (left-tailed test) or greater (right-tailed test) than the control, at a significance level of  $p < 0.05$ . All these analyses were conducted using XLSTAT software version 2009.3.02 (Addinsoft™, France).

### 3. Results and discussion

For all soils, results showed that the temperature, the cycles and their interaction had significant effects on BR (Table 1). Moreover,

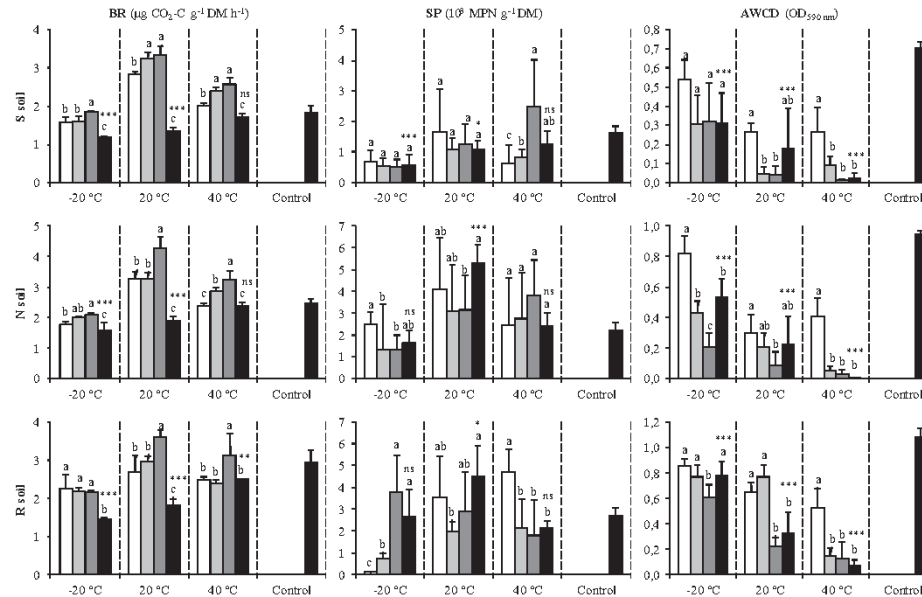
**Table 1**  
F-values of the repeated measures ANOVA for the temperature (Temp), cycle (Cycle), both DRWC and FTC) and the interaction Temp x Cycle factors on microbial variables of the three soils.

Soil	Factor	BR	SP	AWCD
S	Temp	367.5 ***	11.2 ***	32.1 ***
	Cycle	271.2 ***	1.6	12.4 ***
	Temp x Cycle	34.2 ***	3.2 **	0.8
N	Temp	263.3 ***	14.0 ***	68.0 ***
	Cycle	110.3 ***	1.0	38.0 ***
	Temp x Cycle	18.1 ***	2.1	5.3 ***
R	Temp	74.9 ***	28.9 ***	113.7 ***
	Cycle	61.0 ***	9.8 ***	29.5 ***
	Temp x Cycle	14.2 ***	22.1 ***	5.9 ***

S, soil from south slope; N, soil from north slope; R, soil from riparian forest; BR, basal respiration; SP, estimated number of bacterial spores; AWCD, average well color development (Biolog EcoPlates™); Cycle is the within-subject factor with four levels, 1, 2, 4, and 7 cycles; Temp is the between-subject factor with three levels, -20, 20, and 40 °C; \*, \*\*, \*\*\*, significant at  $p < 0.05, 0.01, 0.001$ .

BR increased significantly between the 1st and the 4th cycle, except for the R soil at -20 °C (Fig. 2). However, between the 4th and the 7th cycle, a systematic and significant decrease of BR was observed for all soils and temperatures. The increases observed during first cycles are consistent with numerous other studies (Kuz'yakov et al., 2000; Fierer and Schimel, 2003; Unger et al., 2010; Guénon and Gros, 2013). Indeed, the DRWC are known to induce a brutal raise

of BR called the 'priming effect'. Even though it is mentioned to be rapidly dropping down, in some studies it has been detected for up to 7 days after rewetting (Fierer and Schimel, 2003; Wang et al., 2003). The reasons of this effect are still discussed but two explanations are often outlined. On one hand, the DRWC can induce, by an osmotic shock, an immediate loss of microbial biomass due to the cell lysis, as proven by Guénon and Gros (2013) in Mediterranean soils. The surviving microorganisms would thereafter mineralize the organic carbon released from dead microbial biomass, resulting in an increased soil BR as observed by Fierer and Schimel (2003) after the rewetting of two Mediterranean soils. On the other hand, the second most often outlined explanation is that the observed pulse is due to the mineralization of osmoregulation related compounds, which are accumulated in cells to resist to osmotic/hydric stress. During rewetting, cells which can rapidly getting rid of these compounds increase the amount of easily degradable C sources in the soil and *ipso facto* the soil BR. Indeed, Warren (2014) showed that osmolyte quantities, which were 10 times higher in stressed soils compared to unstressed controls, rapidly dropped down to the control levels after rewetting. Finally, the brutal drop of respiration values observed in all mesocosms between the 4th and the 7th cycle could be relevant of a resistance threshold to DRWC beyond which the microbial biomass is significantly impacted whatever the soil and the incubation temperature. The same pattern was observed in the mesocosms submitted to FTC,



**Fig. 2.** Basal respiration (BR), estimated number of bacterial spores (SP), and average well color development (AWCD; Biolog EcoPlates™) of the three soils. Bars depict means ( $n = 5$ ) with standard deviations (in brackets). The soils experienced 7 drying-rewetting cycles (DRWC) at 20 or 40 °C, or 7 freezing-thawing cycles (FTC) at -20 °C. Controls were maintained at 20 °C, 60% WHC, and experienced no cycle. White bars, cycle 1; light gray bars, cycle 2; dark gray bars, cycle 4; black bars, cycle 7. For each graph, bars within a same temperature modality followed by the same letters do not differ significantly from each other at  $p < 0.05$ . For each graph, black bars (last cycle) followed by stars are significantly lower or greater than the control at  $p < 0.05$ . \*, \*\*, \*\*\*, significant at  $p < 0.05, 0.01, 0.001$ ; ns, non-significant. S, soil from south slope; N, soil from north slope; R, soil from riparian forest; DM, dry mass; MPN, most probable number; OD, optical density.

a result that corroborates previous studies which argued that such climatic events are comparable to DRW events since they also limit water availability to microbial cells and thus can result in similar effects on soil microbial respiration (Schimel et al., 2007). With regards to the bacterial spores, the cycles did not significantly influence their number in the S and N soils, only the temperature did. It was only in the R soil that sporulation was significantly impacted by the double stress and its interaction (Table 1). In this soil, two modalities were of particular interest, i.e.  $-20$  and  $40$  °C, since the observed responses were reversed. Actually, at  $-20$  °C an explosion of the number of spores along with an increasing number of Ftc was noticed, whereas the DRWc at  $40$  °C induced the opposite phenomenon (Fig. 2), probably because lysis of vegetative cells of spore-forming bacteria (SFB) occurred at high temperature. At  $20$  °C the number of spores of this soil was not significantly impacted by temperature, DRWc or their interaction. One is limited to speculations when it comes to explain these results. It appeared that the first Ftc destroyed most of the spores initially present in R soil. During the following incubation at  $4$  °C, and since spores formed at low temperature are known to be more cold tolerant than spores formed at higher temperatures (Nicholson et al., 2000; Carlin et al., 2010), the increase in the number of spores during the next Ftc of the soil R may be explained by the fact that the newly formed spores would be more tolerant to our treatment. Additionally, spores formed from the most psychrotrophic groups of SFB possess better germination abilities at low temperature (i.e.  $7$  °C) and are less cold sensitive (Carlin et al., 2006). These psychrotrophic groups possessing, by definition, better survival rates to our treatment than more cold sensitive groups, it would not be surprising if their abundance increased during the experiment and therefore the number of cold resistant spores. Finally, the low responses of the two other soils at  $-20$  °C could be due to a higher abundance of cold adapted communities. Indeed, according to Von Stetten et al. (1999) the abundance of psychrotrophic strains is correlated to climate (i.e. low in tropical soil and high in mountain soil). Since the S and N soils are submitted to colder temperatures, it would not be surprising if they also exhibit a higher abundance of more psychrotrophic strains.

Considering the CLPPs, both the DRWc (or Ftc) and the temperature had significant effects on the bacterial functional diversity (Table 1) and, when significant, they induced diversity losses (Fig. 2). However, correlations between the CLPPs and the other variables, as well as between the whole set of variables, were globally weak and ranged between  $-0.54$  and  $0.29$  (data not shown). This is not very surprising since these three variables do not concern the same microbial communities. Indeed, the BR concerns whole fungi and bacteria, the CLPPs only consider extractable bacteria capable of growing in Biolog EcoPlates™; while the SP only concerns the SFB. At  $-20$  °C, even if the CLPPs at the end of the experiment were generally lower than the controls, no or only weak significant effects of the consecutive Ftc were observed in the three different soils. Yergeau and Kowalchuk (2008) also obtained very low functional responses to frequent Ftc in soils regularly submitted to freezing events, even if their study was conducted on a different pedosystem (i.e. Antarctic soil).

However, as mentioned by these authors, and since the soils investigated during our study also freeze regularly during winter (37–46 times during 2014–15 winter), an explanation for the low responses in observed CLPPs would be that part of the soil microbial community may be adapted to freezing-thawing events.

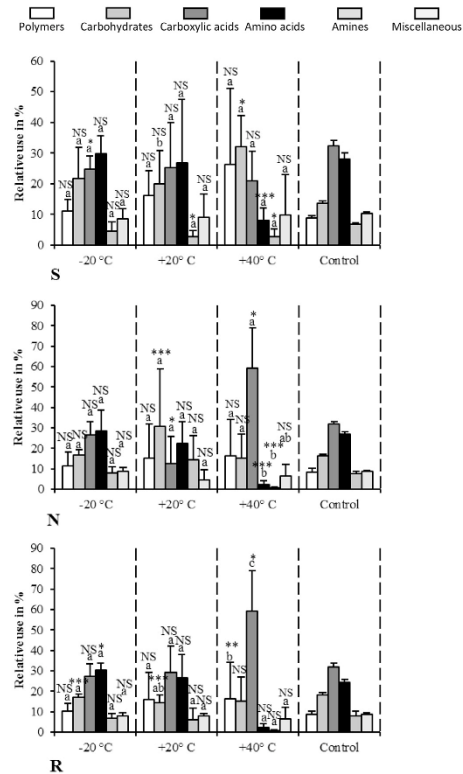
The experiments performed at  $40$  °C provided results especially marking in terms of functional vulnerability. Indeed, AWCD values, and thus the CLPPs, were literally collapsing between the 1st and the 7th cycle, with decreases of 87% in R soil, 90% in S soil and up to 98.4% in N soil (Fig. 2). As previously evidenced with correlation

calculations, these impressive collapses in microbial functional diversities were not related to variations in the number of spores nor in BR, which are known to reflect the physiological state and the maintenance requirement of soil microbial biomass (Blagodatskaya and Kuzyakov, 2013). Indeed, according to Anderson and Joergensen (1997), the BR is strongly correlated with total microbial biomass (i.e. bacteria and fungi). Thereby, explanations to this massive decrease in CLPPs under hot drastic climatic stress, cannot be supported exclusively on the basis of quantitative evolution of the microbial biomass. It is more likely though that, as mentioned by Guénon and Gros (2013), changes occurred in the composition of microbial communities and in their associated catabolic properties. Thus, it can be hypothesized that following intense stresses, occurred a progressive replacement of the original community, functionally diversified, by another less functionally diversified. However, a methodological bias of the Biolog technic cannot also be discarded. Indeed, this method only allows for extractable microbial fraction. In soils, there might be a large part of the microbial community that is not extractable, but still plays a major ecological role. The CLPPs were done on the extractable part of the community, and it may be difficult to make a general conclusion based on this method on microbial functional diversity. To assert this point of view, it would be interesting in further studies to explore, using molecular methods, if the collapse of functional diversity can be related to a loss of specific diversity of microbial communities.

At a finer scale, the relative use at the end of the experiment (cycle 7) of the different substrates as carbon sources was also investigated as suggested by Selmants et al. (2005). The relative uses varied during the experiment, especially at  $+40$  °C (Fig. 3). At  $-20$  °C, the freezing-thawing treatment did not induce any significant difference between the cycle 7 and the control except for the carboxylic acid in S soil and the carbohydrates and amino acids in R soil. The same applied to a lesser extent at  $+20$  °C. Indeed, in the N soil, no significant effect was detected except for the carbohydrates. In S and R soils, the carbohydrate relative use varied between the cycle 7 and the control. Also, at this temperature the relative use of amines varied. Finally, the most interesting results were observed at  $+40$  °C. At this temperature in the different soils, carbohydrate, amino acid and amine relative uses varied significantly in terms of decreases or increases. Finally, as also observed with the raw AWCD values, results of these relative uses seem also to indicate a possible shift in microbial functional populations.

#### 4. Conclusions

Results obtained during our different stress scenarios corroborated in most cases our hypothesis which was that hydric and thermic stress influence significantly the microbial variables considered. When significant, the effects observed on these variables, were mainly negative, and the temperature effect was always stronger than the hydric cycle effect (DRWc and Ftc) as well as the interaction effect of both (Table 1). Another result was that the different variables were weakly related when considering their evolution in the different mesocosms. Indeed, with regards to the basal respiration (BR), this variable showed during the first cycles a well-known “priming effect”, followed by a decrease of this activity during the last cycles; a dynamic pattern that was observed in all mesocosms (soils and temperature) and that was not correlated with any other variable. Considering the bacterial spores (SP), it appeared that their number was considerably more influenced by both soil origin and temperature than by hydric stress. Finally, the hydric stress performed at  $40$  °C evidenced an impressive vulnerability of microbial functions, since, at this temperature CLPPs were literally collapsing during the consecutive DRWc. In future researches, it would be interesting to investigate, at a finer scale, the



**Fig. 3.** Relative use (%), of the different substrates as carbon sources by extractable bacteria grown for 72 h in Biolog EcoPlates™. Bars depict means ( $n = 5$ ) with standard deviations (in brackets). The soils experienced 7 drying-rewetting cycles (DRWc) at 20 or 40 °C, or 7 freezing-thawing cycles (FTc) at -20 °C. Controls were maintained at 20 °C, 60% WHC, and experienced no cycle. White bars, polymers; light gray bars, carbohydrates; dark gray bars, carboxylic acids; black bars, amino acids; very light gray, amines; very pale gray, miscellaneous. For each graph, bars within a same temperature modality followed by different letters differ significantly from each other at  $p < 0.05$ . Missing letters indicate non-significant differences. For each soil, the last cycle was compared to control and stars indicate significantly lower or greater values than the control at  $p < 0.05$ , \*, \*\*, \*\*\*, significant at  $p < 0.05$ , 0.01, 0.001; NS, non-significant. S, soil from south slope; N, soil from north slope; R, soil from riparian forest.

relationships between these variables and more especially the influence of environmental factors on the capacity of microbial populations to sporulate and/or germinate. Indeed, this constitutes a microbial life trait still poorly explored in soil science despite its obvious importance in the regulation of soil biogeochemical processes, especially C cycle in the global change context.

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.soilbio.2017.06.008>.

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