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Infra-red spectroscopy reveals chemical interactions driving water availability for enzyme activities in litters of typical Mediterranean tree species

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ABSTRACT

In Mediterranean ecosystems, water is one of the main drivers of the microbial activities that support organic matter turnover in soils or litters. In addition to drought stress, coastal areas are subject to osmotic stress linked to sea spray exposure. Here we explored i) how water availability, characterized by water activity a_w, is impacted by adding NaCl to litter, ii) the chemical interactions between water, NaCl and the litter matrix and iii) whether microbial activities (using lipase as a model) are affected under these conditions. Litters of two vegetal species typical of the Mediterranean area (*Quercus pubescens* and *Pinus halepensis*) were subjected to FTIR-ATR (Fourier-Transformed Infra-Red – Attenuated Total Reflectance) spectroscopy for chemical characterization. *Q. pubescens* and *P. halepensis* litters were characterized by cutin and aromatics respectively. Sorption isotherms were identical for both species litters; when NaCl was added, a shift in isotherm shape was observed at a_w ranging from 0.75 to 1. FTIR also discriminated samples with and without added NaCl and revealed that cellulose is probably the polymer in interactions with ions. Very interestingly, no differences were found between lipase hydrolytic activities with and without added NaCl: salt addition had no effect on these activities.

1. Introduction

Mediterranean ecosystems are known to be subject to severe climate constraints, mainly intense summer drought. Predicted climate change is expected to aggravate these conditions (Giorgi and Lionello, 2008). The question is whether saline environments, such as coastal zones, may be more constrained and thus potentially weakened under the threat of climate change. Water availability is indeed further hampered in the soils and litters of coastal areas by an additional stress specific to these environments, i.e. osmotic stress via sea spray exposure. Thus, water is the main environmental factor influencing organic matter decomposition in Mediterranean ecosystems (Coûteaux et al., 1995).

Litter decomposition is a vital process that ensures nutrient turnover. Microbial communities (both bacteria and fungi) play a crucial role in organic matter recycling, through the various extracellular enzymes they produce. The quantity of water available strongly influences the structure and composition of microbial communities and their metabolism (including extracellular enzyme production). At molecular level, water availability also affects the diffusion of substrates and the catalysis of hydrolytic enzyme reactions. In most studies, water is characterized as moisture, but this measurement is not informative enough when there is a low quantity of water, as observed in soils or litters from arid areas. A useful alternative parameter to precisely determine water availability under such conditions is water activity, a_w, the ratio of vapor pressure of a material to vapor pressure of pure water. This can provide information about the quantity of 'free' water molecules available for biological processes. Our previous study, (Farnet et al., 2013), first described the pronounced effect this parameter has on the balance of certain enzyme reactions (hydrolysis/synthesis), and the consequences for carbon mineralization or storage. Certain enzymes such as lipases are indeed able to catalyze either hydrolysis or synthesis according to water availability, since water is a substrate of the reaction (Goujard et al., 2009; Farnet et al., 2010). Thus, under the particular conditions linked to arid climates, water activity can be

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considered as a useful parameter to clearly understand enzyme activity dynamics.

The present study aimed to explore how additional matric stresses, such as osmotic stress, may alter hydric conditions in litters and thus microbial functioning. A further aim was to clarify the molecular mechanisms underlying water availability in litter, to determine whether certain vegetal species may favor water retention depending on their chemical signature. To do so, FTIR-ATR (Fourier-Transformed Infra-Red – Attenuated Total Reflectance) spectra were used to precisely describe water and NaCl adsorption onto plant polymers, and thus to determine how the chemical composition of litters from different vegetal species may modify their water availability. FTIR spectroscopy is well recognized for its ability to characterize the chemical structure of plant samples by identifying key compounds from different parts of the plant, such as polysaccharides (Kačuráková et al., 2000, lignin (Boeriu et al., 2004), and cuticular waxes (Dubis et al., 1999). The review of Heredia-Guerrero et al. (2014) summarized the main applications of Infrared and Raman spectroscopies to characterize plant cuticle and its components (cutin, waxes, polysaccharides and phenolics) in terms of assignment of the functional groups present in the cuticular matrix, interaction and macromolecular arrangement. Infrared spectra of litters can be very complex, because their macromolecular organic composition generates complex interactions with surrounding compounds or between their functional groups. The review of Fan et al. (2012) described the use of FTIR to examine the formation of inter- and intra-molecular hydrogen bonds in celluloses, investigating the effects of deterioration (crystallinity vs amorphous structure) and the change in chemical composition of major (cellulose, hemicellulose and lignin) and minor (pectin and waxes) constituents after decomposition.

We also determined sorption isotherms to describe the relationship between humidity and water activity, at different NaCl concentrations and without added NaCl, for litters of two typical Mediterranean plant species: *Pinus halepensis* and *Quercus pubescens*. The main environmental constraint in Mediterranean ecosystems is summer drought (Castro et al.,2008; Sofo et al., 2008), but coastal zones also experience windy conditions, higher temperatures, and additional stresses, such as osmotic stress due to sea spray exposure, which can further impact litter functioning (Qasemian et al., 2014). Hydrolytic lipase activities were measured in litters both with and without added NaCl, and at different a_w. These activities offer a particularly reliable way to investigate the effect of water availability on enzyme catalysis, since this enzyme reaction is performed in apolar organic solvents and therefore water quantity can easily be controlled. While many studies have examined extracellular enzymes involved in litter transformation in inland areas under a Mediterranean climate (Fioretto et al., 2009; Papa et al., 2008), few have investigated whether the enzymatic reaction may be affected by the salts present in coastal zones.

2. Materials and methods

2.1. Litter sampling

Litters of *Quercus pubescens* (QP) and *Pinus halepensis* (PH) were collected in the form of two composite samples for each species from three independent sites (La Quille, Le Coucou, Le Castellas) in the Massif de la Trévaresse (Bouches du Rhône, France, 43°37′7.31″, 5°27′41.56″) in February 2015. At each site, three sampling plots of 500 m^2 were determined, and horizon F was collected for both litters. All the experiments described below were performed for both QP and PH litters.

2.2. Water activity and humidity

Litter samples were reduced to powder using a bullet blender Retsch MM40 (Fisher Scientific, France). Water activities of the litter powders, as well as temperature, were measured with a HygroPalm 23-AW-A (Rotronic AG, Bassersdorf, Switzerland) portable analyzer equipped with a WP-40 sample holder. Water activity, a_w, was measured in an incubator at 25 °C using 4 g of litter (fresh weight). Litters were first hydrated to reach 80% of Water Holding Capacity, by adding 110 mL of water to 50 g of litter dry weight to reach $a_w = 1$. Then, to obtain different a_w values, litters were gently dried at room temperature (around 22 °C). To test the effect of NaCl, litters were similarly hydrated with solutions of NaCl (Sigma Aldricht) at different concentrations (35, 50, 75 and 100 $g.L^{-1}$) to reach 80% of Water Holding Capacity, in order to avoid percolation and optimize NaCl adsorption onto litter. 110 mL of NaCl solution were added to 50 g of litter dry weight and this protocol was used for each NaCl concentration. Then, a_w measurements were performed as described above.

Humidity was measured after 48 h of incubation at 100 °C using 1 g of fresh litter and was expressed as percentage of water in fresh litter. The experiment was performed three times for each sample.

2.3. Lipase hydrolytic activities

Enzyme activities were measured from litters for different a_w values as described by Farnet et al. (2010). This assay was adapted for litters from the method of Pencreac'h and Baratti (1996) using methyl *t*-butyl ether as the organic solvent of the reaction mixture. The mixture was composed of: 10 mM of *p*-nitrophenyl laurate solubilized in 2 mL of methyl *t*-butyl ether and 1 g of litter. The reaction mixture was incubated at 30 °C under magnetic stirring at 500 rpm for 2 h. Then, 500 μL of the organic phase were added to 4 mL of 0.1 M NaOH and after a brief shaking, *p*-nitrophenol was measured at 412 nm in the aqueous phase with Spectrophotometer Biomate (Bioblock Scientific). The same protocol was used with litters supplemented with solutions of NaCl at different concentrations: 35, 50, 75 and 100 $g.L^{-1}$.

To test for any abiotic reactions, litters were autoclaved and *p*-nitrophenol release was checked.

Three replicates were performed for each experiment. Activities were expressed as μmoles of *p*-nitrophenol released/hour/g of dry weight. A calibration curve of *p*-nitrophenol in methyl *t*-butyl ether was performed with and without litter under the same experimental conditions.

All the chemical compounds of Rectapur quality were purchased from Sigma Aldrich and methyl *t*-butyl ether was used as purchased, without further purification.

2.4. Chemical characterization of litters by FTIR-ATR (Fourier-Transformed Infra-Red – Attenuated Total Reflectance)

Litter powders were directly deposited onto a Specac's Golden Gate™ ATR Accessory of a Thermo Nicolet IS10 spectrometer equipped with a Mercury Cadmium Telluride (MCT) detector, an Ever-Glo source and a KBr/Ge beam-splitter. Spectra were acquired between 4000 and 650 cm^{-1}, with a 4 cm^{-1} nominal resolution. For each spectrum, 100 scans were co-added. A background spectrum in air (under the same acquisition conditions as those used for the samples) was acquired before each acquisition. The ATR crystal was carefully cleaned with ethanol to remove any residual traces of the previous sample. Three spectra were recorded for each sample. OMNIC 8.1 (Thermo Nicolet) was used to record FTIR-ATR spectra. The Unscrambler version 10.3 from Computer Aided Modeling software (CAMO, Trondheim, Norway) was used to perform data