Reference values for soil microbial communities in temperate forest ecosystems

1. Introduction

Biodiversity enhances ecosystem stability and productivity. This assumption has been broadly verified for plant communities, thanks to the vast body of evidence from more than 200 years of studies (Maron et al., 2018). Compared to plant ecology, microbial ecology is still lacking demonstrations of these relationships although it is widely recognized that microorganisms perform a crucial role in many ecosystem functions. One of the key ecological process in wich soil microbes are involved is litter decomposition. Litter decomposition is a highly complex process that involves a number of physical, chemical and biological factors; however, there is little information about the litter decomposition rate and the role of microbial diversity in different ecosystems. However, Maron et al. 2018 found that a decrease in microbial diversity affected (i) the decomposition of soil organic matter, thereby reducing global CO2 emission by up to 40%, and (ii) shaped the source of CO2 emission toward preferential decomposition of most degradable C sources. Colombo et al. (2016), Gartzia-Bengoetxea et al. (2009) and many others reported that forest management practices influenced soil bacterial communities. Colombro et al. (2016) found a positive linear relationship between bacterial richness and processes involved in nutrient cycling. Therefore, it seems that microbial diversity is critical when the impact of climate change and forest management on soil microbial diversity and ecosystem functioning is considered in the framework of sustainable forest management.

As we are living a situation with many possibilities, synergies, trade-offs and uncertainties in the forest sector, indicators can help to avoid unwanted impacts, and support successful and sustainable forest-based sector development. They can be used to inform policy makers, synthesize complex matters and act as tools for decision support (Wolfslehner et a., 2016). However, an indicator is only valuable if its values can be interpreted. In a simplistic approach, reference values for a given indicator could be either the conditions of a native soil, or of a soil with maximum production and/or environmental performance (Doran & Parkin, 1994). The old-growth stands are the most natural forest habitats available, and therefore, they are a valuable element of comparison, so they can be considered as "reference stands" for each type of forest. Old-growth forests help us to evaluate human impact on forest ecosystems and to understand the potential and limitations of silvicultural practices that imitate natural processes (close-to-nature forestry)(Bauhus et al., 2009). The study of old-growth stands can provide criteria to guide

forestry towards the achievement of sustainable forest management as they provide baselines for the delivery of ecosystem services under unmanaged conditions, including carbon stocks and sequestration, water purification or soil biodiversity.

According to Frelich and Reich (2003), old-growth forests can be subdivided in 'primary old-growth', being old-growth forests whose dynamics are driven exclusively by natural processes while human impacts are absent, and 'secondary old-growth', being previously managed forests that have developed old-growth features after decades of (intentional or non-intentional) non-intervention (Piovesan et al., 2008, Ziaco et al., 2012). In Western Europe, patches of primary old-growth forests are scarce (Sabatini et al., 2018), but still some secondary old-growth forests can be found for European common species such as *Pinus sylvestris*, *Fagus sylvatica* and *Quercus robur*.

Research on soil microbes can be categorized as structural diversity, functional diversity and genetic diversity studies, and these include cultivation based and cultivation independent methods. Microbial community structure is defined as the number and relative abundance of soil microbial populations. Most of the soil microorganisms can not be characterized by cultivation techniques. Therefore, a culture-independent approach is used to determine soil microbial community composition by phospholipid fatty acids analysis (PLFA) of microbial membranes (Frostegard et al., 1991; Baath, E. & Anderson, 2003). Unlike DNA, which can be present in living or dead cells, phospholipids are only present in living soil microbes. Phospholipid fatty acids (PLFAs) are the main structural component of the phospholipid molecule and can serve as useful biomarkers to determine the microbial types and abundance in the soil (e.g. branched chain fatty acids originate from Gram-positive bacteria). PLFA analysis is a useful technique for estimation of microbial total biomass and to observe changes in soil microbial community as phospholipid fatty acid (PLFA) analysis can provide a real-time snapshot of the microbial community structure.

The main aim of this study was to obtain a real-time snapshot of the microbial community structure during a litter decomposition experiment in a normal year in forests dominated by Scots pine, European beech and pedunculate oak in atlantic and submediterranean climate.

2. Materials and methods

Study area

The study was carried out in Artikutza and Montoria (Figure 1), both sites located in Basque Country, North-East Spain. The most important difference between two study areas is the climate, which is Atlantic in Artikutza (mean annual temperature of 16.5˚C and mean annual precipitations of 2527 mm) and Sub-Mediterranean in Montoria (mean annual temperature of 10.5 ˚C and mean annual precipitations of 653 mm). In the Table 1 there are the main features of the two locations.

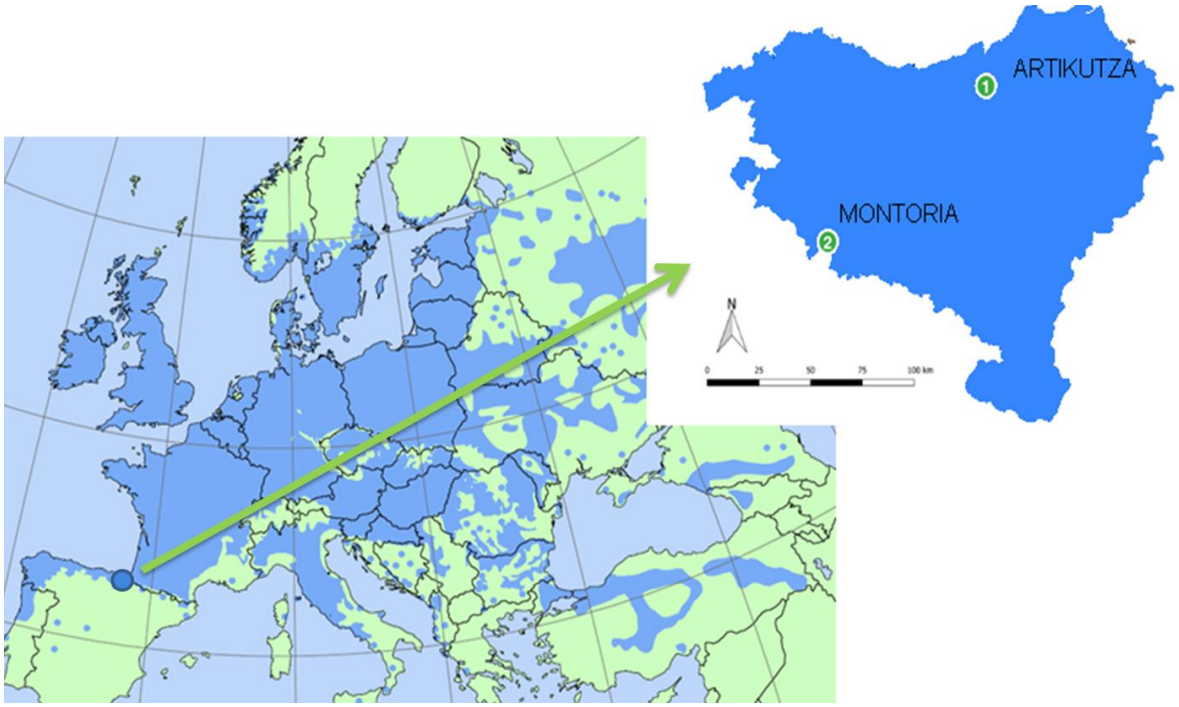


Figure 1. The location of study sites: 1) Artikutza (in North Basque Country) and 2) Montoria (in South Basque Country).

Table 1. The main values of sand (%), silt (%), clay (%), pH and SOC (%) of each study sites.

	Artikutza	Montoria
Soil texture	Sandy clay loam	Sandy loam
Sand(%)	52.6	57.7
Silt (%)	26.3	33.4
Clay(%)	21.1	8.8
pH	3.9	4.4
SOC (%)	13.4	9.4

We selected three different stands in each study site, one dominated by oak (*Quercus robur*), another by beech (*Fagus sylvatica*) and third one by Scots pine (*Pinus sylvestris*). The age of forests in

both sites is around 100 years. In each stand, we established 4 plots consisted of three individual trees standing in a triangle.

Sampling

Circular litter traps with a diameter of 50 cm were installed in each plot to litter collection. Naturally senesced Q. robur, P. sylvestris and F. sylvatica leaves were collected from litter traps in each study site between late October and December 2016. All litter was oven dried to constant weight for 48 h at 70˚C and stored by litter type at room temperature before the experiment.

The decomposition of litter was studied by litterbags method. We used fiberglass litterbags with dimensions of approximately 15 cm x 11 cm and 1 mm mesh size. Litterbags were filled with 12 g of each dried leaves types. On the forest soil surface buried in the litter layer, leaves of 3 different tree species from Artikutza (4 pseudoreplicate per litter type and per sampling time) were placed in Artikutza study sites and we did the same with Montoria leaves. Thus, the experiment involved 2 different treatments per tree specie (A site litter and M site litter). In total, 120 litterbags were tested (4 replicate x 3 litter type x 2 study site x5 sampling time).

Four replicate litterbag of each type were sequentially collected and transported to the laboratory on February (30 days), April (90 days), August (210 days), October (270 days) and December (330 days) to study microbial community structure over time.

When sampled, each litterbag was sealed in a plastic bag and immediately brought to the laboratory for analyses. First, we carefully removed soil particles and living plant parts that adhered to the surface and the sample was freeze dried, ground and was used to microbial analysis.

Analysis (PLFA)

The PLFA pattern was determined using Sherlock PLFA analysis following a high throughput PLFA extraction protocol as described by Buyer & Sasser (2012). Aproximatly 2 gr of freeze dried litter was use for the analysis. Bligh-Dyer extractant (4ml) containing internal standar (IS), 1,2-dinonadecanoyl-sn-glycero-3phosphocholine (19:0 PC, Avanti Polar Lipid sp/n850367) was used to lipid extraction. To lipid separation by solid phase extraction (SPE) 96-well SPE plate containing 50 mg of silica per well (Phenomenex. Torrance, CA, USA) was used. Then, the phospholipids were tranesterified to fatty acid methil sterase, extrated and analysed by gas chromatography.

The analysis was performed by gas chromatography using an Agilent 6890N gas chromatograph (GC) equipped with Agilent Ultra 2, 25 m x0.2 mmx 0.33 µm film thickness (MIDI p/n Column G) columnand Software MIDI Sherlock Software v.6.2B with PLFA Package. Initial oven temperature was190◦C ramping by 10◦C/min to 250◦C follow by 3◦C/min to 310◦C. The inyection temperature was 250◦C and hydrogen was use as a carrier gas (1:30 split ratio; 1.3 ml/min constant flow rate). Finally, The Sherlock PLFA Tool software was used to identify individual fatty acids. Fatty acids were summed into biomarker groups: eukaryotes, polyunsaturated fatty acids (Zelles, 1999); eubacteria, 15:0, 17:0 cyclo, 19:0 cyclo, 15:1 iso, 17:1 iso, 17:1 anteiso (Frostegård and Bååth, 1996); Gram positive bacteria, iso and anteiso saturated branched fatty acids (Zelles, 1999); Gram negative bacteria, monounsaturated fatty acids and cyclopropyl 17:0 and 19:0 (Zelles, 1999); actinobacteria, 10-methyl fatty acids (Zelles, 1999); fungi, 18:2 ω6 cis (Frostegård and Bååth, 1996); and protozoa, 20:3 and 20:4 fatty acids (Ringelberg et al., 1997). These biomarkers are not entirely specific for their taxonomic groups and therefore must be interpreted cautiously (Zelles, 1997). 54 fattyacids were identified in the samples. Total biomass was calculated adding the biomass of all the phospholipids identified. Shannon Weaver diversity index and species eveness were also calculated.

Statistics

Repeated-measures ANOVA over time were used to evaluate effects of climate on soil microbial community. Paired t-test was used to study seasonal changes on soil microbial community at the study sites.

3. Results and discussion

Pressures on soil organisms are well known. An ever increasing global population, and increased demand for food and fibre lead to intensified agriculture, greater use of fertilisers and pesticides as well as monocultures. Unsustainable agricultural practices, climate change, soil erosion and loss of aboveground diversity all negatively affect organisms that live in soil (JRC, 2016). As the ability of soil microorganisms to perform biogeochemical processes is critical for sustaining forest productivity, , we need to better understand the life beneath our feet in order to generate reference values to compare with and assure sustainable forest management. In our study, we intended to understand the effect of climate in order to be able to evaluate the impact of any climate change in soil microbial community. Overall, the microbial biomass (total PLFA) and bacterial, fungal, and selected other PLFAs showed similar temporal patterns in both sites, atlantic and sub-mediterramean sites (Fig. 2 and Fig. 3). However, differences were oberserved between sites and among different tree species. In oak stands, microbial biomass was higher in Montoria (sub-mediterramean) than in Artikutza (atlantic) while the inverse was evident for pine stands. Microbial biomass did not show any difference between sites at beech stands (Fig. 2). There was a significant increase in microbial biomass from april to december in pine stands at both sites that could coincide with the increase in soil temperature, available C, and plant activity (Kara et al., 2014) but in oak stands, there was no observed any seasonality in the atlantic site and lower microbial biomass was observed in the sub-mediterranean site during spring-summer period. (Fig. 2).

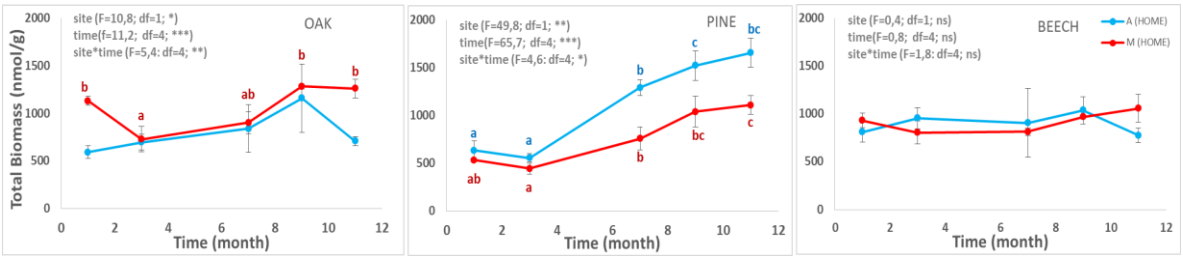
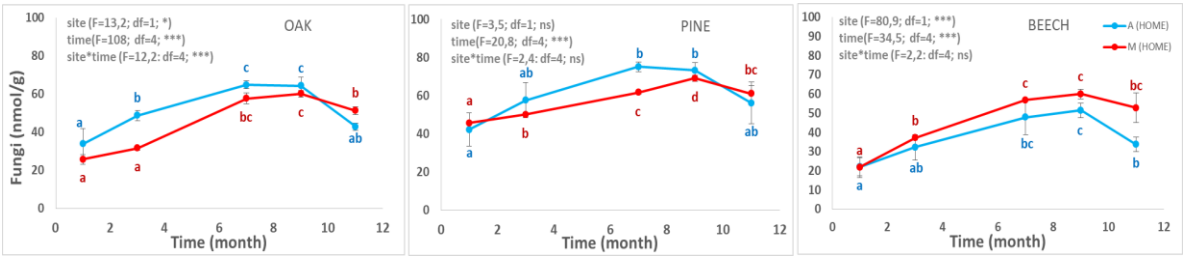


Figure 2. Microbial biomass during the litter decomposition experiment (February, April, August, October and December) at each study site and tree specie. during the litter decomposition experiment (February, April, August, October and December) at each study site and tree specie. Means and standard deviations are shown. Results of repeated measures ANOVA are also shown (ns: no significant; * p<0.05; ** p>0.01; *** p>0.001). Letters indicated significant differences between seasons (Blue=artikutza; red=Montoria).

In the three species and both sites, we observed successive changes in community composition, with a significant increase in fungal abundance and a significant decrease in Gram-negative bacteria as litter decomposition proceeded (Fig. 3). Fungal biomass increased until october and then decreased

significantly in most cases and Gram-negative bacteria decreased until october than then increased generally. Although both fungi and bacteria have the capacity to decompose litter components (Stursova´ et al., 2012), the traditional view has emphasized the role of fungi in the decomposition of plant biomass due to their adaptations, including filamentous growth, the ability to translocate nutrients and the possession of an efficient enzymatic apparatus (de Boer et al. 2005; Eichlerova´ et al. 2015). This ability might give them the advantage to develop on sites receiving predominantly poor quality litter inputs (Bray et al., 2012) that are characteristics of early stage decomposing leaf litter, even more in monospecific stands like ours. It has to be pointed out that fungal:bacterial ratio was always above 1, reflecting that the dominance of fungi in both sites and in all forest types (Fig. 4).

The effect of climate on fungal abundance was observed in broadleaved species but with different pattern depending on the tree species. In oak stands, fungal abundance was higher in the atlantic site and lower in the sub-mediterranean site (Fig. 3), in pine stands no significant differences were observed between the two sites. Gram-positive bacteria were sensitive to climate in all tree species and the concentration was lower always in Montoria, the sub-mediterranean site. Gram-positive bacteria are usually identified as K-strategists and Gram-negative soil bacteria as r-strategists. The r-strategists exhibit high growth rates and consume soil labile carbon, while K-strategists present slower growth rates and are likely to outcompete r-strategists in conditions of low nutrient availability due to their higher substrate affinities (Fierer, Bradford and Jackson 2007). Numerous studies suggest that K-strategist microorganisms are more resistant to global change induced disturbances (de Vries and Shade 2013; Bischoff et al. 2016; Villa et al. 2016; Zhang et al. 2016). Our results suggest that decomposing litters in Montoria, the sub-mediterranean climate, might be dominated by r-strategist while in the atlantic site K-strategists are more abundant (Fig 4). This is in accordance with Gram+/Gram- ratio which was was also sensitive to climate and always higher in Artikutza, the atlantic site (Fig 4). Gram+/Gram- bacteria ratio has been proposed as a proxy of the prevalence of K-strategists in the microbial community and are thus expected to be positively related to microbial community resistance (de Vries and Shade 2013).



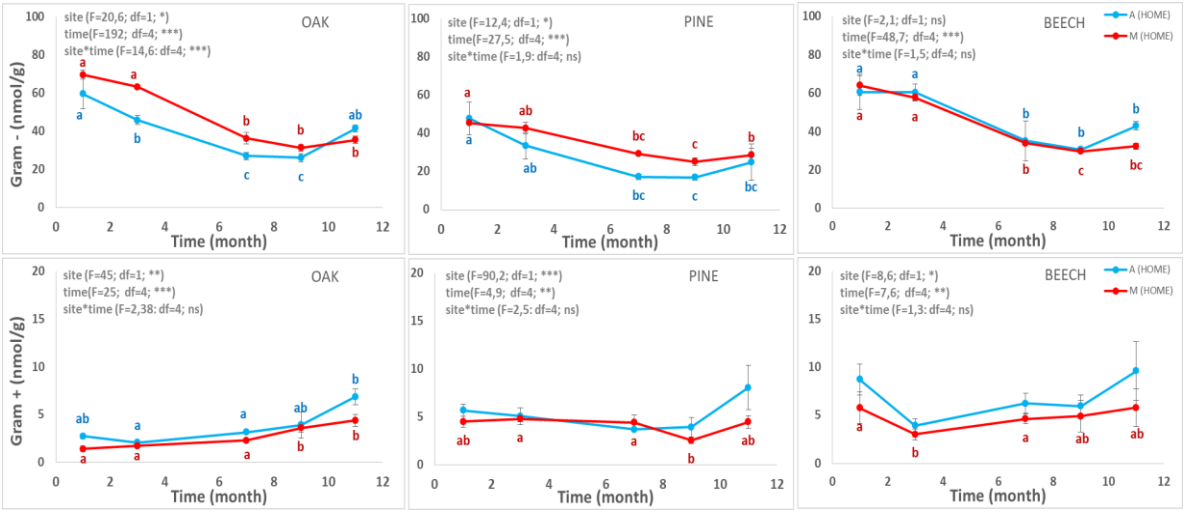


Figure 3. PLFA concentrations for each microbial group defined as in Table 2 (fungi, gram-negative and gram-posotive) during the litter decomposition experiment (February, April, August, October and December) at each study site and tree specie. Means and standard deviations are shown. Results of repeated measures ANOVA are also shown (ns: no significant; * p<0.05; ** p>0.01; *** p>0.001). Letters indicated significant differences between seasons (Blue=artikutza; red=Montoria).

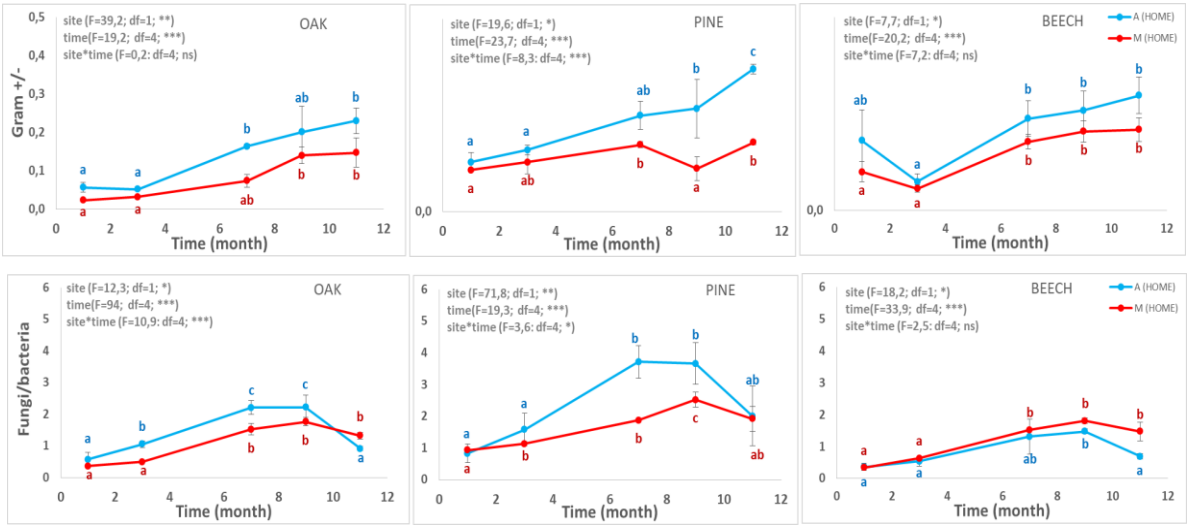


Figure 4. Gram+/gram- and bacteria/fungi ratios during the litter decomposition experiment (February, April, August, October and December) at each study site and tree specie. Means and standard deviations are shown. Results of repeated measures ANOVA are also shown (ns: no significant; * p<0.05; ** p>0.01; *** p>0.001). Letters indicated significant differences between seasons (Blue=artikutza; red=Montoria).

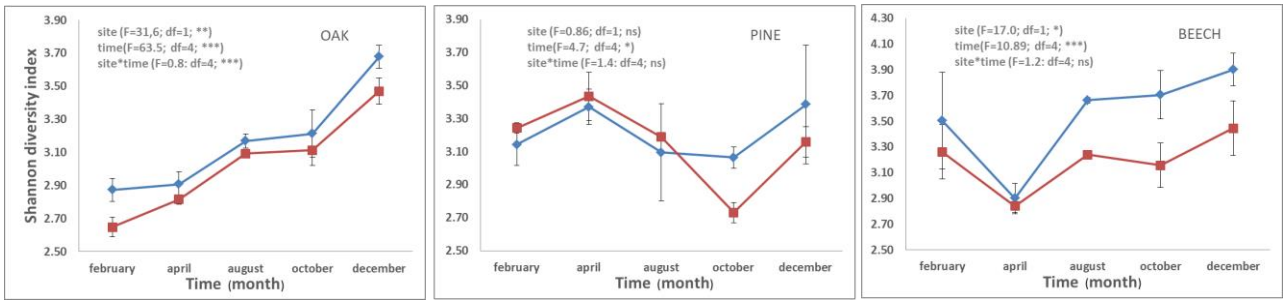


Figure 5. Shannon Weaver diversity indexes during the litter decomposition experiment (February, April, August, October and December) at each study site and tree specie. Means and standard deviations are shown. Results of repeated measures ANOVA are also shown (ns: no significant; * p<0.05; ** p>0.01; *** p>0.001). Letters indicated significant differences between seasons (Blue=artikutza; red=Montoria).

Shannon diversity index was sensitive to climate just for broadleaves. Pine stands showed no significant differences in PLFA diveristy between atlantic and sub-mediterranean sites (Fig. 5). However, oak and beech stands showed higher diversity indexes in the atlantic site than in the sub-mediterranean site. Our results suggestes that Shannon diversity index could be useful for broadleaves but not for conifers. Species eveness was also evaluated as biodiversity index and the same results were obtained (data not shown). The observed differences in specific biomarkers ratios such as Gram+/Gram- bacteria ratio and fungal/bacterial ratio were not reflected in diversity indexes. More research would be need to determine the best indicators to evaluate soil microbial biodiversity in forest litter.

4. Conclusions and research gaps

Our results suggested that seasonality has to be considered when evaluating soil microbial community structure and soil biodiversity and that each forest species needs its own reference values for soil microbial community structure and biodiversity. Significant differences were observed in Gram+/Gram- bacteria ratio and fungal/bacterial ratio between atlantic and sub-mediterranean sites suggesting that climate has a significant effect on microbial diversity and that climate has a significantly different effect on different tree species. In addition, it has to be pointed out that typically used diversity indexes might not be very useful when evaluating forest floor microbial diversity.

6. References

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