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**Study of the function and diversity of the microbial
community in plant phyllosphere and rhizosphere and
interactions with the environment**

A thesis submitted by

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To my parents C. Katsoulas and E. Maurantza

“The most beautiful sea
hasn't been crossed yet...”
Nâzım Hikmet (1902-1963)

Abstract

Phyllosphere and rhizosphere are plant-associated micro-habitats that are known to support diverse microbial communities whose structure is mediated by plants. We aimed to disentangle the mechanisms shaping the microbial communities in the phyllosphere and the soil root zone and identify their response to agricultural practices like soil organic amendment and pesticide application. The focus was on plants indigenous to Mediterranean ecosystems, some of them producing essential oils which are known to exert antimicrobial activities, and also cultivated plants.

We initially explored the factors shaping the microbial community of the phyllosphere in plants native to semi-arid Mediterranean ecosystems using q-PCR and amplicon sequencing approaches. We collected leaves at two largely contrasting seasons (summer and winter) from 8 perennial plants with varying attributes, that belong to different functional groups: (i) woody sclerophyllous evergreen, semi-deciduous and non woody shrubs (ii) aromatic and non-aromatic. We determined the abundance of bacteria, Crenarchaea, fungi, *Alternaria* and *Cladosporium* (main airborne fungi) via q-PCR, and the structure of the epiphytic bacterial, archaeal and fungal community *via* amplicon sequencing. We observed strong seasonal effects but no clear plant-host effects on microbial abundance: bacteria showing higher abundance in the winter, and all others in the summer. Plant-host and season were equal determinants of the composition of the bacterial and fungal communities, whereas the archaeal community showed plant-host driven patterns. Plant habit exhibited a stronger filtering effect on the epiphytic microbial communities compared to the aromatic plant nature which affected only the fungal community. The bacterial community was dominated by *Chloroflexi* and α -proteobacteria in the summer and winter respectively, with OTUs of *Sphingomonas*, *Rhizobia* and *Methylobacterium* favored in the winter. The archaeal community was dominated by the Soil Crenarchaeotic Group (SCG) and Aenigmarchaeota. The fungal community is mostly comprised of Ascomycota with *Capnodiales*, *Pleosporaceae* and *Dothioraceae* being key members whose abundance varied by plant host and season.

We extended our study on aromatic plants by exploring their use, as soil amendments. We employed a pot study to examine the impact of peppermint (*Menta piperita*), spearmint (*Menta spicata*) and rosemary (*Rosemarinus officinalis*), in comparison with an organic fertilizer, on the dynamics of key bacterial taxa, Crenarchaea, fungi and functional microbial groups like ammonia-oxidizing bacteria (AOB) and

archaea (AOA), sulfur-oxidizing bacteria (SOB) and *catA*-, *pcaH*-carrying bacteria involved in C cycling. We further explored possible interactions between soil amendments and the presence of tomato plant. Soil amendment with peppermint, spearmint and the organic fertilizer increased the abundance of proteobacteria and fungi, in contrast to rosemary, characterized by essential oils with a different chemical profile compared to mints, which benefited these copiotrophic microbial groups only in the presence of tomato plants. We further explored this complex interaction via amplicon sequencing analysis of bacteria, archaea and fungi. This verified the key role of rosemary soil amendment in shaping the bacterial, archaeal and fungal community and its beneficial role in the abundance of proteobacteria. Multivariate analysis identified OTUs belonging to Actinobacteria, mostly associated with undisturbed soil systems (i.e. *Blastococcus*, *Rubrobacter*, *Solirubrobacter*, *Agromyces*) that were negatively affected by rosemary amendment. On the contrary we observed a striking dominance of the cellulose-decomposing basidiomycetes *Minimedusa* in soils amended with rosemary. The known antibiotic properties of this fungus might explain the negative effects of rosemary soil amendment on *Nectriaceae* also observed.

We finally explored the potential impact of pesticides, as external perturbation factor, on the abundance and diversity of the microbial communities on plant leaves and the soil root zone. We tested the hypothesis that these two habitats support distinct microbial communities but exhibit a similar response (accelerated biodegradation or toxicity) to their repeated exposure to the biodegradable fungicide iprodione. Pepper plants received four repeated foliage or soil applications of iprodione which accelerated its degradation in soil ($DT_{50_1st}=1.23$ and $DT_{50_4th} = 0.48$ days) and on plant leaves ($DT_{50_1st} >365$ and $DT_{50_4th} = 5.95$ days). The composition of the epiphytic and soil bacterial and fungal communities, determined by amplicon sequencing, were significantly altered by iprodione. The archaeal epiphytic and soil communities responded differently; the former showed no response to iprodione. Three closely related iprodione-degrading *Paenarthrobacter* strains were isolated from soil and phyllosphere. They hydrolyzed iprodione to 3,5-dichloraniline (3,5-DCA) via the formation of 3,5-dichlorophenyl-carboxiamide and 3,5-dichlorophenylurea-acetate, a pathway shared by other soil-derived arthrobacters implying a phylogenetic specialization in iprodione biotransformation.

Overall, we showed that phyllosphere is a habitat colonized by diverse bacteria and fungi, while archaea are less abundant and diverse. The epiphytic microbial

community in Mediterranean plants, is shaped by plant-host and seasonality. The use of aromatic plants as soil amendment was found to stimulate copiotrophic microorganisms and microorganisms allelopathic against soil-borne plant pathogens. Finally, we showed that the epiphytic microbiome, responds to pesticide applications, with some microbes became acclimated to degrade pesticides. This thesis has reported the first epiphytic bacterium, a *Paenarthrobacter* strain, that could degrade iprodione and also suggested an uncommon specialization of *Arthrobacter* in the degradation of this fungicide.

Περίληψη

Η φυλλόσφαιρα και η ριζόσφαιρα είναι μικρο-ενδιαιτήματα του φυτού που είναι γνωστό ότι φέρουν ποικιλόμορφες μικροβιακές κοινότητες, η δομή των οποίων εξαρτάται από το φυτό. Σκοπός μας ήταν η διερεύνηση των μηχανισμών που διαμορφώνουν τη μικροβιακή κοινότητα της φυλλόσφαιρας και του εδάφους που βρίσκεται περιμετρικά της ρίζας, και η ταυτοποίηση της απόκρισης τους σε καλλιεργητικές πρακτικές, όπως η χρήση εδαφοβελτιωτικών και οι εφαρμογές γεωργικών φαρμάκων. Εστίασαμε σε γηγενή φυτά του Μεσογειακού συστήματος, μερικά εκ των οποίων παράγουν αιθέρια έλαια που είναι γνωστά για τις αντιμικροβιακές τους ιδιότητες, και επίσης σε καλλιεργούμενα φυτά.

Αρχικά διερευνήσαμε τους παράγοντες που διαμορφώνουν τη μικροβιακή κοινότητα της φυλλόσφαιρας γηγενών φυτών του ημιάνυδρου Μεσογειακού οικοσυστήματος, χρησιμοποιώντας προσεγγίσεις με q-PCR και amplicon sequencing. Συλλέξαμε φύλλα στις δύο εποχές με τις μεγαλύτερες αντιθέσεις (καλοκαίρι και χειμώνα) από 8 πολυετή φυτά με διαφορετικά χαρακτηριστικά που ανήκουν σε διάφορες λειτουργικές ομάδες: (i) αειθαλή ξυλώδη σκληρόφυλλα, φρύγανα και πόες, (ii) αρωματικά και μη-αρωματικά. Καθορίσαμε την αφθονία των βακτηρίων, Κρεναρχαίων, μυκήτων *Alternaria* και *Cladosporium* (κύριοι αερομεταφερόμενοι μύκητες) μέσω q-PCR, και τη δομή της επιφυτικής κοινότητας των βακτηρίων, αρχαίων και μυκήτων μέσω amplicon sequencing. Παρατηρήσαμε έντονη επίδραση της εποχής, αλλά όχι ξεκάθαρη επίδραση του φυτού-ξενιστή στη μικροβιακή αφθονία: η βακτηριακή αφθονία ήταν υψηλότερη τον χειμώνα, και η αφθονία όλων των υπόλοιπων μικροοργανισμών ήταν αυξημένη το καλοκαίρι.

Φυτό-ξενιστής και εποχή είχαν ίση επίδραση στη σύσταση της μικροβιακής κοινότητας των βακτηρίων και μυκήτων, ενώ η κοινότητα των αρχαίων καθορίζεται από το φυτό-ξενιστή. Το φυτικό είδος αποτελεί σημαντικότερο παράγοντα διαμόρφωσης των επιφυτικών μικροβιακών κοινοτήτων, σε σχέση με την αρωματική φύση των φυτών, η οποία επηρεάζει μόνο τη μυκητιακή κοινότητα. Η βακτηριακή κοινότητα κυριαρχείται από *Chloroflexi* και α-Πρωτεοβακτήρια το καλοκαίρι και χειμώνα αντίστοιχα, με τα OTUs των *Sphingomonas*, *Rhizobia* και *Methylobacterium* να ευνοούνται τον χειμώνα. Η κοινότητα των αρχαίων κυριαρχείται από Soil Crenarchaeotic Group (SCG) και *Aenigmarchaeota*. Η μυκητιακή κοινότητα αποτελείται κυρίως από *Ascomycota* με *Capnodiales*, *Pleosporaceae* και *Dothioraceae*, να είναι τα μέλη-κλειδιά των οποίων η αφθονία διαφοροποιείται αναλόγως του φυτού-ξενιστή και της εποχής.

Επεκτείναμε τη μελέτη μας και στα αρωματικά φυτά, εξερευνώντας τη χρήση τους ως εδαφοβελτιωτικά. Πραγματοποιήσαμε ένα πείραμα με γλάστρες, ώστε να μελετήσουμε την επίδραση της μέντας (*Menta piperita*), του δυόσμου (*Menta spicata*) και του δενδρολίβανου (*Rosemarinus officinalis*), σε σύγκριση με ένα οργανικό εδαφοβελτιωτικό, στη δυναμική των κύριων βακτηριακών taxa, Κρεναρχαίων, μυκήτων και άλλων λειτουργικών μικροβιακών ομάδων, όπως τα αμμώνια-οξειδωτικά βακτήρια (AOB) και αρχαία (AOA), τα θείο-οξειδωτικά βακτήρια (SOB) και τα *catA*-, *pcaH*-φέροντα βακτήρια, τα οποία εμπλέκονται στον κύκλο του άνθρακα. Επιπλέον διερευνήσαμε πιθανές αλληλεπιδράσεις μεταξύ εδαφοβελτιωτικών και παρουσίας του φυτού τομάτας. Η βελτίωση του εδάφους με μέντα, δυόσμο και οργανικό εδαφοβελτιωτικό, αύξησε την αφθονία των Πρωτεοβακτηρίων και μυκήτων. Αντίθετα το

δενδρολίβανο, το οποίο έχει χαρακτηριστικό αιθέριο έλαιο με διαφορετική σύσταση σε σχέση με τις μέντες έδρασε ευεργετικά στις κοπιωτροφικές αυτές μικροβιακές ομάδες, μόνο κατά την παρουσία του φυτού της τομάτας. Επιπροσθέτως εξερευνήσαμε τις πολύπλοκες αλληλεπιδράσεις μέσω ανάλυσης amplicon sequencing στις μικροβιακές κοινότητες των βακτηρίων, αρχαίων και μυκήτων. Αυτό επιβεβαίωσε τον ρόλο κλειδί του δενδρολίβανου ως εδαφοβελτιωτικού στη σύσταση της βακτηριακής και μυκητιακής κοινότητας καθώς και αυτής των αρχαίων, αλλά και τον ευεργετικό του ρόλο στην αφθονία των Πρωτεοβακτηρίων. Η εφαρμογή του δενδρολίβανου επηρέασε αρνητικά OTUs που ανήκουν στα Ακτινοβακτήρια, τα οποία σχετίζονται κυρίως με ανέγγιχτα εδαφικά συστήματα (π.χ. *Blastococcus*, *Rubrobacter*, *Solirubrobacter*, *Agromyces*), όπως προέκυψε από την πολυπαραγοντική ανάλυση. Αντίθετα παρατηρήθηκε μία εντυπωσιακή κυριαρχία των διασπαστών κυτταρίνης βασιδιομυκήτων *Minimedusa* σε εδάφη εμπλουτισμένα με δενδρολίβανο. Οι γνωστές αντιμικροβιακές ιδιότητες του μύκητα αυτού ίσως να εξηγούν την αρνητική επιρροή του δενδρολίβανου ως εδαφοβελτιωτικού, που παρατηρήθηκε στα *Nectriaceae*.

Τέλος, εξερευνήσαμε την πιθανή επίδραση των γεωργικών φαρμάκων, ως εξωτερικού παράγοντα διαταραχής της αφθονίας και ποικιλότητας των μικροβιακών κοινοτήτων στα φύλλα του φυτού και στο έδαφος στην περιοχή της ρίζας. Εξετάσαμε την υπόθεση ότι αυτά τα δύο ενδιαιτήματα ενώ φέρουν διαφορετικές μικροβιακές κοινότητες, αντιδρούν το ίδιο (επιταχυνόμενη βιοδιάσπαση ή τοξικότητα) στην επαναλαμβανόμενη έκθεση τους στο βιοδιασπώμενο μυκητοκτόνο iprodione. Σε φυτά πιπεριάς εφαρμόστηκαν τέσσερις επαναλαμβανόμενες φυλλικές ή εδαφικές εφαρμογές του iprodione, διαδικασία που επιτάχυνε τη διάσπαση στο έδαφος ($DT_{50_1st}=1.23$ και $DT_{50_4th} = 0.48$ ημέρες) και στα φύλλα ($DT_{50_1st}>365$ και $DT_{50_4th} = 5.95$ ημέρες). Η σύσταση της επιφυτικής και εδαφικής βακτηριακής και μυκητιακής κοινότητας, η οποία καθορίστηκε με amplicon sequencing, άλλαξε στατιστικώς σημαντικά από το iprodione. Η επιφυτική και εδαφική κοινότητα των αρχαίων αντέδρασε διαφορετικά: με το πρώτο να μην αντιδρά στο iprodione. Τρία συγγενικά στελέχη *Paenarthrobacter* που διασπούν το iprodione, απομονώθηκαν από έδαφος και φύλλα. Υδρολύουν το iprodione σε 3,5-dichloraniline (3,5-DCA) μέσω σχηματισμού 3,5-dichlorophenyl-carboxiamide και 3,5-dichlorophenylurea-acetate, ένα μονοπάτι που μοιράζονται με άλλα arthrobacters που έχουν απομονωθεί από το έδαφος, προτείνοντας μία φυλογενετική ειδίκευση στη βιομετασχηματισμό του iprodione.

Συνολικά, αποδείξαμε πως η φυλλόσφαιρα είναι ένα ενδιάστημα που αποικίζεται από διαφορετικά βακτήρια και μύκητες, ενώ η αφθονία και ποικιλομορφία των αρχαίων είναι μειωμένη. Η επιφυτική μικροβιακή κοινότητα των Μεσογειακών φυτών διαμορφώνεται από το φυτό-ξενιστή και την εποχή. Η εφαρμογή αρωματικών φυτών ως εδαφοβελτιωτικού βρέθηκε να διεγείρει τους κοπιωτροφικούς μικροοργανισμούς και αλληλοπαθητικούς εναντίων εδαφογενών φυτοπαθογόνων. Τέλος, αποδείξαμε ότι το επιφυτικό μικροβίωμα, αντιδρά στις εφαρμογές γεωργικών φαρμάκων, με μερικά μικρόβια να εγκλιματίζονται στο να διασπούν γεωργικά φάρμακα. Αυτή η διδακτορική διατριβή ανέφερε το πρώτο επιφυτικό βακτήριο, ένα στέλεχος *Paenarthrobacter*, το οποίο είναι ικανό να διασπά το iprodione και επίσης προτείνει μία μη-κοινή ειδίκευση των *Arthrobacter* στη διάσπαση αυτού του μυκητοκτόνου.

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Chapter 1

General introduction

1. General introduction

Plant and microbes live closely together with the symbiotic association between arbuscular mycorrhizal fungi and plants been the most ancient (Selosse and Le Tacon 1998). The evolution and application of novel methodological tools in microbial ecology revealed that the different plant parts (roots, stems, leaves) support an enormous diversity of microorganisms, eukaryotic and prokaryotic (Bringel and Couée 2015; Lundberg et al. 2012; Manter et al. 2010; Vandenkoornhuysen et al. 2002). The identification of the different micro-habitats of microorganisms on plants and the diversity of microorganisms occupying these micro-sites, led to the introduction of new terms to describe this intimate association: ‘holobiont’ and ‘hologenome’. The former was coined by Zilber-Rosenberg and Rosenberg (2008) to describe the multi-cellular plant host and its associated microbiota as a functional entity in which co-evolutionary selection between the host and the microorganisms likely occurs. In accordance the hologenome refers to the genome pool of the holobiont comprising the genome of the plant host and the genomes of the microorganisms colonizing its different compartments (Rosenberg and Zilber-Rosenberg 2016). In this context Vandenkoornhuysen et al. (2015) argued that the functional traits of the plant microbiome should be included in plant holobiont, where some key plant host functions are outsourced to the associated microbiota. In addition, the plant microbiota, due to their genetic plasticity, offer an auxiliary but really effective mechanism for the plants to rapidly adapt to environmental conditions and inevitably, biologically evolve.

1.1. Phyllosphere

1.1.1. Phyllosphere as a microbial habitat

In the mid-50s the term 'phyllosphere' was first suggested by Last (1955) who stated that, "...as with roots and the 'rhizosphere', leaves have a 'phyllosphere', with a characteristic microflora that may contain many species". This term was further evolved by Ruinen (1961) which suggested that "The external surface of the leaf, as an environment for microorganisms, can be termed 'phyllosphere' by analogy with the 'rhizosphere' of roots". In present days, the term phyllosphere is referred to "the leaf surface (phyllosphere) as a habitat that features two intimately connected but very different compartments, i.e. the leaf surface landscape (phylloplane) and the surface waterscape (phylloplane). Phyllosphere includes all the cuticle-attached microbes in addition to those that are present in the waterscape" (Doan and Leveau 2015). Phyllosphere, in contrast to rhizosphere can be considered, for several plant species, an ephemeral habitat considering the life cycle of annual plants or leaf senescence and fall of perennial deciduous plants (Vorholt 2012).

The size of the plant phyllosphere at the global scale has been estimated to reach *ca.* 1 million km² (upper and lower leaf surface) (Lindow and Brandl 2003; Vorholt 2012) being the habitat of approximately 10⁶-10⁷ bacteria cells/cm². This sums up to an estimated 10²⁶ bacteria on the plant phyllosphere globally. Considering these numbers, we could presume that the epiphytic bacteria will have a major role in global ecosystem functioning and nutrients cycling (Lindow and Brandl 2003). Bacteria colonize plant phyllosphere along with other microorganisms such as fungi and archaea, which are also encountered in phyllosphere at a lower population (Vorholt 2012), although a good estimate of their global population is still missing.

1.1.2. Characteristics of the phyllosphere

Phyllosphere is a harsh oligotrophic environment characterized by low nutrients availability and highly heterogenic distribution of nutrients and water (Vorholt 2012). The extreme environmental conditions such as wind, UV radiation, rain and high and low temperatures determine the composition of the epiphytic microbial community along with the ability of microorganisms to compete under conditions of poor nutrient supply. This very characteristic, affects their interactions with other microbes as well as with the host (Doan and Leveau 2015) and makes phyllosphere a hotspot for microorganisms, such as prokaryotes (Bacteria and Archaea), eukaryotes (fungi, oomycetes and nematodes), and viruses (Koskella 2013; Lindow and Brandl 2003; Vorholt 2012).

In general, microbial survival on plant phyllosphere is based on the capacity of epiphytic microorganisms to develop mechanisms to cope with the extreme oligotrophic and adverse environmental conditions prevailing in this plant micro-habitat. Such mechanisms include (i) the capacity of epiphytic microorganisms to produce pigments to cope with UV radiation exposure (Sundin 2002) (ii) the production of chemical warfare agents by epiphytic bacteria to compete for nutrients and space (Helfrich et al. 2018) (iii) the production of extracellular polysaccharides (Gal et al. 2003) and biosurfactants (Schreiber et al. 2005) to facilitate their attachment on leaf surfaces and to get protected by desiccation (iv) the production of indole acetic acid (IAA) which facilitates plant nutrient leakage and microbial survival (Brandl et al. 2001), (v) the enhanced capacity of epiphytic bacteria (i.e. *Spingomonas*) to acquire substrates (sugars, amino acids, acetates) through their wide repertoire of porins and ABC transporters (Knief et al. 2012) and to utilize C1 (methanol) and organosulfonic compounds (i.e. *Methanobacterium* sp.) (Müller et al. 2016).

1.1.3. Leaf morphological and physiological features affect microbial colonization of the phyllosphere

Phyllosphere topography can be pictured as a jungle, with a harsh ground formed by epicuticular wax crystals where veins would be grooves, stomata and hydathodes being cracks and craters, while trichomes and fungi would appear as trees and vines (Figure 1) (Vacher et al. 2016). The cuticle wax composition is affecting the microbial composition on the phyllosphere (Bodenhause et al. 2014; Reisberg et al. 2013). Leaf stomata and hydathodes are microbial hotspots (Esser et al. 2015; Hirano and Upper 2000; Peredo and Simmons 2018; Remus-Emsermann and Schlechter 2018; Saldaña et al. 2011), where microbes gather to exploit water and nutrients exudated. An example of such a bacteria is *Methanobacterium extorquens*, that has been found to flourish in the microsites around stomata where methanol is released (Abanda-Nkpwatt et al. 2006). Regarding epiphytic glands, they are heavily colonized by microorganisms equipped with mechanisms that enables them to cope with oxidative stress (Karamanoli et al. 2012). One of many examples, is *Pseudomonas syringae* which colonizes the base of glandular trichomes and forms small colonies on grooves between epidermal cells, to have access to carbon-containing compounds exudated by glands (Monier and Lindow 2004). Another such example is *Pseudomonas citronellolis* which is equipped with genes encoding enzymes for the degradation of long chained alkanes and terpenes released by the glands of plants (Remus-Emsermann et al. 2016).

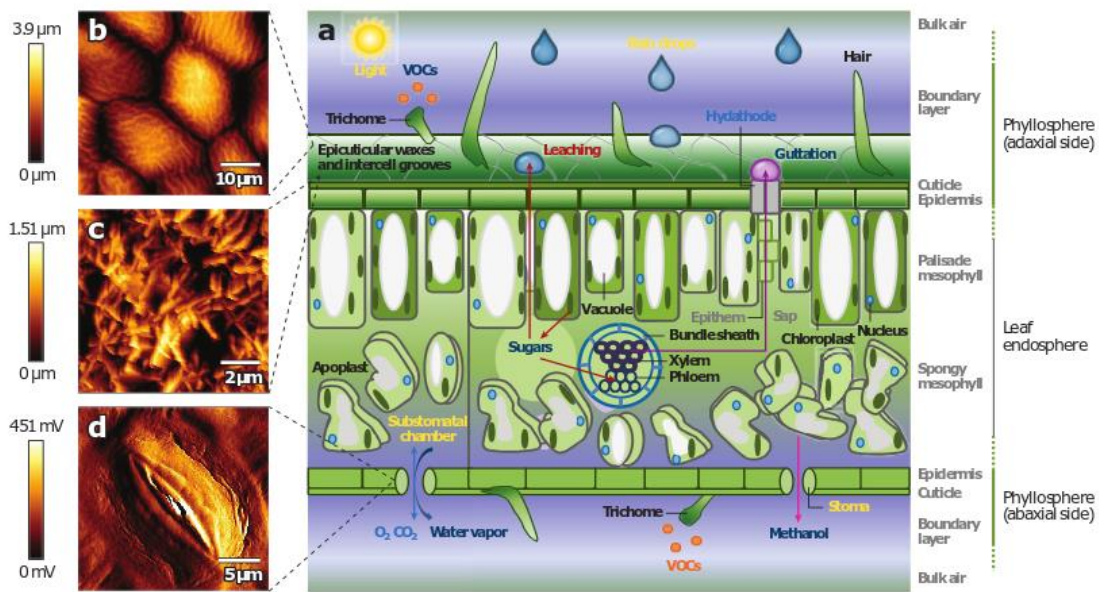


Figure 1. Illustration of some structural and functional attributes of the phyllosphere. (a) The leaf cross section diagram shows the flow of several metabolites used as nutrients by phyllosphere microorganisms. These microorganisms use the sugars and inorganic nutrients exported to the leaf surface through leaching (Van Der Wal and Leveau 2011) and guttation (Singh 2014) and can also use volatile organic compounds (VOCs) emitted by the leaf, such as methanol (Knief et al. 2012). Phyllosphere microorganisms are in contact with the waxy layer covering the leaf, the cuticle, on both the upper (adaxial) and the lower (abaxial) leaf surface. Stomata and hydathodes are major points of entry for microorganisms into internal leaf tissues. (b–d) These attributes of the phyllosphere habitat are revealed by atomic force microscopy: (b) cuticular striae on the upper leaf surface of grapevine (*Vitis vinifera* cv. Zweigelt), (c) wax rosettes on the upper leaf surface of pedunculate oak (*Quercus robur*), and (d) a stoma on the lower leaf surface of grapevine (*V. vinifera* cv. Zweigelt). Schematic representation of leaf surface (adopted by Vacher et al. (2016)).

1.1.4. Phyllosphere: who is there and why?

We do know that phyllosphere is a habitat of microorganisms, but how did they end up there? The microbial community of phyllosphere is assembled via transfer from other plant compartments (rhizosphere and endophytic community), from other environmental compartments (air and soil) or transferred vertically from the maternal plant (seed) (Lemanceau et al. 2017). Considering that plant phyllosphere constitutes the interface between plant and air, the latter is expected to constitute a main deposit of microbes colonizing the plant phyllosphere (Whipps et al. 2008). On the other hand plant-associated microbial communities have been found to be compositionally nested from the ground up suggesting that soil represents an important source of plant-surface microbiomes (Amend et al. 2019). In this frame first Copeland et al. (2015) in canola, bean and soybean and then Grady et al. (2019) in miscanthus and switchgrass observed a clear bacterial succession on the phyllosphere with leaf bacterial community resembling the soil bacterial community at the start of the growing season but gradually becoming enriched with epiphytic bacteria by the end of the season suggesting a strong plant filtering effect along the growing season.

Epiphytic Bacteria: The bacterial community of the phyllosphere is mainly composed of Proteobacteria, with α -Proteobacteria (e.g. *Rhizobiales*, *Methylobacterium*, *Sphingomonas*), being the most abundant, followed by γ -Proteobacteria (e.g. *Pseudomonas*) (Bodenhausen et al. 2013; Delmotte et al. 2009; Kembel et al. 2014; Knief et al. 2012; Redford et al. 2010; Redford and Fierer 2009; Ren et al. 2014; Ruiz-Pérez et al. 2016). Actinobacteria, β -Proteobacteria and Bacteroidetes have been also reported as common dwellers of the plant phyllosphere (Aydogan et al. 2018; Jackson and Denney 2011; Schlaeppi et al. 2014; Thapa and Prasanna 2018).

The bacteria of plant phyllosphere can serve various ecological and functional roles: (i) methylophilic bacteria are dominant members of the epiphytic bacterial community being able to transform C1 compounds like methanol released by plant surface processes and hence promoting global C cycling (Sy *et al.*, 2005; Abanda-Nkpwatt *et al.*, 2006; Delmotte *et al.*, 2009; Knief *et al.*, 2012; Iguchi *et al.*, 2015; Madhaiyan *et al.*, 2015; Trotsenko *et al.*, 2001; Fedorov *et al.*, 2011) (ii) diazotrophic bacteria have been found in the phyllosphere (Ali *et al.* 2012; Freiberg 1998; Fürnkranz *et al.* 2008; Rico *et al.* 2014; Ruinen 1965). They carried iron-molybdenum nitrogenase systems leading them able to fix atmospheric N₂, (iii) bacteria able to degrade organic pollutants reside on the plant phyllosphere (Ning *et al.* 2012; Sangthong *et al.* 2016; Scheublin *et al.* 2014) (iv) some epiphytic bacteria and archaea produce IAA and promote plant growth (Brandl *et al.* 2001; Taffner *et al.* 2019) (v) several of the epiphytic microbes are plant (i.e. *Pseudomonas*, *Erwinia*, *Septoria*, *Erysiphe*, *Cladosporium*) and human pathogens (i.e. *Salmonella*, *Enterobacteriaceae*) compromising plant health and food safety (Cernava *et al.* 2019; Moulas *et al.* 2013; Ottesen *et al.* 2015; Ramos 2004; Sapkota *et al.* 2015).

Epiphytic Fungi: The fungal community of the phyllosphere is dominated by Ascomycetes (Coince *et al.* 2014; Cordier *et al.* 2012a; Jumpponen *et al.* 2010; Jumpponen and Jones 2009a; Kembel and Mueller 2014; Perez *et al.* 2009) with the most common classes being Sordariomycetes, Dothideomycetes and Eurotiomycetes (Fonseca-García *et al.* 2016; Fort *et al.* 2016; Horton *et al.* 2014; Martirosyan *et al.* 2016), and the most abundant genera being *Aureobasidium*, *Cladosporium* and *Alternaria*. Basidiomycetes are less abundant on the plant phyllosphere with Tremellomycetes and Agaricomycetes being the most abundant classes, while members of the genera *Cryptococcus* are the most

common basidiomycetes on the plant phyllosphere (Cordier et al. 2012a; Jumpponen et al. 2010; Jumpponen and Jones 2009b; Ottesen et al. 2013).

The majority of epiphytic fungi are saprotrophs, biotrophic pathogens or lichens (Jumpponen and Jones, 2009b). Epiphytic fungi can act as (i) protectors of their host plant from other phytopathogens using various direct and indirect mechanisms (Arnold and Lutzoni 2007; Saikkonen 2007). Such an example is *Aureobasidium pullulans*, which is among the most abundant fungal species in the phyllosphere (Cordier et al. 2012b, 2012a; Fort et al. 2016; He et al. 2012; Magan and Baxter 1996) and has the capacity to antagonize plant pathogenic microbes (Castoria et al. 2001; Wachowska and Głowacka 2014; Zhang et al. 2010), (ii) as decomposers of plant exudates (Migahed and Nofel, 2001; Jumpponen and Jones, 2009b; Yang *et al.*, 2016) and (iii) as primary (exhibiting plant-specific traits like *Zymoseptoria tritici*) or secondary plant pathogens (being more general colonizers like *Cladosporium* and *Alternaria*) (Sapkota et al. 2015).

Epiphytic archaea: Archaea are less abundant on the plant phyllosphere, compared to bacteria and fungi (Vorholt 2012). Recent metagenomic analysis suggested that epiphytic archaea could participate in important ecosystem functions like N assimilation, CO₂ fixation, auxin biosynthesis, DNA repair and oxidative response (Taffner et al., 2018). Follow up studies by the same group reinforced the versatile metabolic potential of epiphytic archaea which carried genes for FMN, FAD and glycogen degradation plus an operative glyoxylate cycle which is used most probably as an adaptation mechanism in order to be able to use C1 compounds instead of sugars, which are not that abundant on the plant phyllosphere (Taffner et al. 2019). Little is known about the composition of the archaeal epiphytic community. The few studies available suggest a dominance of

Euryarchaeota (i.e. *Methanomicrobia*, *Halobacteria*, *Thermoprotei*) and Thaumarchaeota (i.e. *Candidatus Nitrosocosmicus* and *Nitrososphaera*) (Knief et al. 2012; Ruiz-Pérez et al. 2016; Taffner et al. 2018, 2019).

1.1.5. Factors shaping the epiphytic microbial community

1.1.5.1. Plant genotype

The structure of the epiphytic microbial community differs among different plant genotypes (Balint-Kurti et al. 2010; Bálint et al. 2013; Bodenhausen et al. 2014; Cordier et al. 2012b; Horton et al. 2014; Hunter et al. 2010, 2015; Mason et al. 2015; Wagner et al. 2016) and different plant species (Inácio et al. 2010; Kembel and Mueller 2014; Kembel et al. 2014; Kim et al. 2012; Lambais et al. 2014; Redford et al. 2010; Sapkota et al. 2015). All the above studies have identified plant host as the major determinant of the composition of the microbial communities colonizing the phyllosphere (Ruppel *et al.*, 2008; Kembel and Mueller, 2014; Kembel *et al.*, 2014; Laforest-Lapointe *et al.*, 2016a, 2016b; Martirosyan *et al.*, 2016). However, even within the same plant or the same plant species, there are structural variations in the phyllospheric microbial community which are shaped according to leaf age (Yadav *et al.*, 2011; Wagner *et al.*, 2016), the position of the leaves in the canopy (Cordier *et al.*, 2012a), and the leaf status and health (i.e. water content, infestations by pests and fungi/bacteria) (Yadav *et al.*, 2005). The recruitment of the microbial community by plants seems to be evolutionary more complex than previously thought, with higher bacterial diversities linked to higher plant productivity (Laforest-Lapointe et al. 2017). Hence it is now believed that plants recruit microorganisms in line with the biological features of the different microorganisms i.e.

plant growth promoting or antibiosis (Vorholt 2012), defense system against insects (Mason *et al.* 2016) and their own ecological strategies (Laforest-Lapointe *et al.* 2017).

Several studies have pointed to the morphological and chemical features of each plant as key determinants of the composition of the epiphytic microbial communities. For example Kembel *et al.* (2014) studied the composition of the epiphytic bacterial community in 57 tree species co-localized in a tropical forest and identified wood density, growth and mortality rates, leaf mass per area, leaf thickness and leaf N and P concentration as leaf traits that showed a significant correlation with the microbial composition of the phyllosphere. Similarly, Laforest-Lapointe *et al.* (2016a) observed leaf N content, leaf mass per area and wood density as the most important factors shaping the epiphytic bacteria community in five forest plant species. Hunter *et al.* (2015) identified leaf surface wax and leaf surface hydrophobicity as the key leaf traits affecting the composition of the fungal community in 26 different lettuce cultivars. Yadav *et al.* (2005) identified P leaf content, water content and thickness of the adaxial epidermis as the best explanatory variables for the size of the epiphytic bacterial community on the phyllosphere of nine native plants of a semi-arid Mediterranean ecosystem in Greece. A range of recent studies using plant mutants which exhibit altered leaf physiology and morphology further reinforced the role of leaf traits on the phyllospheric microbial community composition. For example, Bodenhausen *et al.* (2014) showed that *Arabidopsis thaliana* mutants *lacs2* and *pcc1*, that exhibited altered cuticle formation, showed altered epiphytic bacterial composition and increased bacterial abundance. Similarly, Ritpitakphong *et al.* (2016) showed that *A. thaliana* mutants *bdg* and *lacs2.3* characterized by thinner cuticle leaf phenotypes supported different bacterial communities dominated by *Pseudomonas* and *Rhizobium* compared to wild type plants where *Burkholderia* dominated.

1.1.5.2. Plants Biogeography

Microorganisms on the phyllosphere are exposed to local environmental conditions such as wind, UV radiation, high or low temperatures and rain events. Hence it is expected that the microclimate of a region would affect the composition of the epiphytic microbial communities. Several studies have looked among other factors on the impact of plant location on the epiphytic microbial assemblage process and tried to identify distance-decay relationships within certain microbial groups. Such studies have looked into the phyllosphere microbial composition in plants from the same or different species collected from geographically distant regions. In most of these studies biogeography seemed to be a less important factor than plant species (Copeland et al. 2015; Laforest-Lapointe et al. 2016b; Qian et al. 2018; Sapkota et al. 2015). However other studies have identified a strong endemism exhibited by epiphytic microbes. For example, Agler et al. (2016) identified plant location as the stronger determinant (explaining 25-35% of the variation) on the epiphytic bacterial, fungal and oomycetal communities on *A. thaliana* cultivars. Coleman-Derr et al. (2016) monitored the composition of the epiphytic bacterial and fungal community in agave plant cultivars collected from distant geographical areas. They showed that the major factor driving the assembly of the epiphytic fungal community is the geographic origin of the host, contrasting with bacterial assemblages that are primarily sculpted by the plant-host. These results pose for higher endemism of fungi compared to bacterial populations, most probably driven by climatic and dispersal constraints (Bonito et al. 2014). Beyond fungi, Methylobacteria, considered ubiquitous epiphytic dwellers, also exhibited strong endemism colonizing equally well different plant species in the same site, with plant location being the most significant determinant of the presence in the plant phyllosphere (Knief et al. 2010).

1.1.5.3. Seasonality

The epiphytic microbial community structure is seasonally dynamic. Seasonal effects on the composition of the epiphytic microbial communities have been reported for bacteria (Agler et al. 2016; Copeland et al. 2015; Peñuelas et al. 2012; Rastogi et al. 2012; Redford and Fierer 2009) and fungi (Jumpponen *et al.*, 2010; Cordier *et al.*, 2012b; Peñuelas *et al.*, 2012; Gomes *et al.*, 2018), whereas little is known about the seasonal patterns of archaea on the plant phyllosphere. Season alteration imposes drastic changes on the moisture, temperature and UV radiation levels which are expected to induce reciprocal changes on the structure of the epiphytic microbial community (Beattie 2011; Corrigan and Oelbermann 2010; Joung et al. 2017). Laforest-Lapointe et al. (2016a) looked at the seasonality of plant phyllosphere and identified limited variation on microbial composition along time, a result most probably attributed to the short study duration (90 days). In contrast other studies have identified strong seasonal effects on the composition of the epiphytic microbial communities (Copeland et al. 2015; Gomes et al. 2018; Redford et al. 2010). It is anticipated that studies following the dynamics and diversity of the epiphytic microbial communities in regions with strong seasonal variations regarding climatic conditions (i.e. Mediterranean basin) will magnify potential seasonal effects. Regarding seasonal abundance patterns of epiphytic microbes, bacteria show increasing abundance during winter (Copeland et al. 2015; Thompson et al. 1993), whereas fungi have a higher population during the summer period (Jumpponen and Jones 2014; Osono and Mori 2005; Peñuelas et al. 2012). However the diversity of both fungal and bacterial epiphytic communities seems to increase during the summer (Peñuelas et al. 2012; Rastogi et al. 2012). In contrast we know little about the seasonal response of epiphytic archaea both at the abundance and diversity level.

1.1.5.4. Microbe-microbe interactions on the plant phyllosphere

Beyond the interactions of epiphytic microorganisms with the plant host, which have a strong effect on the composition of the phyllospheric microbial community, microbe-microbe interactions could also exert strong structural effects on this plant micro-habitat. In most of the studies looking at the contribution of different factors like plant genotype, season and location on the epiphytic microbial community composition these factors explain usually not more than 40-50% of the variation (Agler et al. 2016; Grady et al. 2019; Laforest-Lapointe et al. 2016a; Redford et al. 2010). Hence the rest could be associated with other explanatory factors such as microbe-microbe interactions. In a pioneering study Agler et al. (2016) suggested that hub microorganisms on the plant phyllosphere act as replicators of the interactions of biotic and abiotic factors affecting the microbiome and mediate strong changes in the composition of the epiphytic microbiome. They identified the oomycete *Albugo* and the basidiomycetes *Dioszegia* as such hub microorganisms which were negatively correlated with the abundance of several microbial taxa but themselves are strongly affected by plant host and season respectively. Such complex interactions are expected to be the focus of future studies shedding more light on the assemblage mechanisms of the epiphytic microbial communities.

1.1.6. Phyllosphere microbial communities in a Mediterranean ecosystem

Mediterranean semi-arid ecosystems are characterized by alternation of cold and wet with hot and dry seasons expected to endure a strong selection on the plant microbiome (Yadav et al. 2008). The plant community of these ecosystems is dominated by non-woody shrubs and woody evergreen sclerophyllous or seasonally dimorphic plants, with several of them

being aromatic (Yadav et al. 2005). Previous studies in such ecosystems showed that non-woody shrubs like *Calamintha nepeta* and *Melissa officinalis* supported higher epiphytic bacterial populations compared to woody plants native to the same ecosystem (Yadav et al. 2004, 2005, 2008). In this context Yadav et al. (2005) observed significant positive correlations between bacterial abundance and the water and P content of leaves and the trichome density. Whereas they reported a negative correlation between bacterial abundance and total phenolics, leaf thickness, mesophyll and abaxial epidermis thickness. Contrasting results have been obtained from different studies when correlating bacterial abundance with essential oil presence and concentrations. For example, Yadav et al. (2005, 2008) reported a higher abundance of bacteria and higher functional diversity (determined by ECOPLATES) in aromatic vs non aromatic plants. In contrast, Karamanoli et al. (2000) showed that the abundance of epiphytic bacteria in four aromatic plants depended on the antibacterial activity of their essential oils with lavender (*Lavandula aungustifolia*), characterized by the lower levels of essential oils, having the higher bacterial population, compared to the other three plants (Greek oregano, Greek sage and rosemary), having lower epiphytic bacterial population and higher concentrations of more active essential oils. In a more extensive study, Karamanoli et al. (2005) studied 19 native and cultivated plants and showed that all plants rich in secondary metabolites harbored low epiphytic bacterial populations, in line with the high antimicrobial activity of the constituents of essential oils (Kadoglidou et al. 2011; Sivropoulou et al. 1997). In contrast to all the above studies Yadav et al. (2004) did not observe a significant correlation between the essential oil concentration of plants and epiphytic bacterial abundance. More recent studies by Vokou *et al.*, (2012) showed, via culture-independent analysis (denaturing gradient gel electrophoresis, DGGE), clear structural differences in the epiphytic bacterial community between sclerophyllic

evergreen plants (plus *Myrtus communis*) and aromatic Lamiaceae plants. Overall these studies suggest strong and complex interactions between the native plants of such semi-arid Mediterranean ecosystems and the bacteria colonizing their leaves. One key gap in our current knowledge about the epiphytic communities in such ecosystems concerns the factors shaping the epiphytic community of other key microbial communities, like fungi and archaea.

1.2. Rhizosphere as a microbial habitat

The soil zone, named rhizosphere for the first time by Hiltner (1904), is a thin layer of soil that surrounds the root. It is the home of a plethora of microorganisms. The rhizosphere, can support up to 10^{11} microbial cells per gram of root (Egamberdieva et al. 2008), composed of 10^3 to 10^6 different bacterial species (Gans et al. 2005; Mendes et al. 2011; Torsvik and Øvreås 2002; Tringe et al. 2005). Despite the adjacency of rhizosphere to soil, the richness of rhizospheric bacterial community is usually lower compared to the bacterial richness in the surrounding bulk (Bulgarelli et al. 2012) but often lower than the bacterial diversity of the phyllosphere (Fonseca-García et al. 2016; Yang et al. 2016).

1.2.1. Rhizosphere characteristics as a micro-habitat

Microorganisms colonize the rhizosphere to get access and exploit the energy-rich plant exudates. Plants tend to channel up to 50% of their photosynthates into the rhizosphere, which are then released as root exudates (Bais et al. 2006). This results in an abundant microbial population colonizing this root zone compared to the surrounding bulk soil, a phenomenon known as ‘rhizosphere effect’ (Figure 2) (Cheng 2009). Based on the content of root exudates, plant rhizosphere is dominated by heterotrophic microbes which could

exploit the C-rich organic compounds of the rhizodeposits (Berendsen et al. 2012; Bonfante and Anca 2009; Lemanceau et al. 2017; Mendes et al. 2011). Root exudates are composed of ions, free oxygen and water, enzymes, mucilage (polymerized sugars), and a diverse array of carbon-containing primary (i.e. low molecular mass compounds like amino acids, sugars and organic acids) and secondary metabolites like antimicrobial compounds, nematicides and flavonoids (Hejl and Koster 2004; Marschner et al. 2011; Philippot et al. 2013). As a result, rhizosphere is a battlefield, that microorganisms fight to acquire plant-derived nutrients (Raaijmakers et al. 2009).

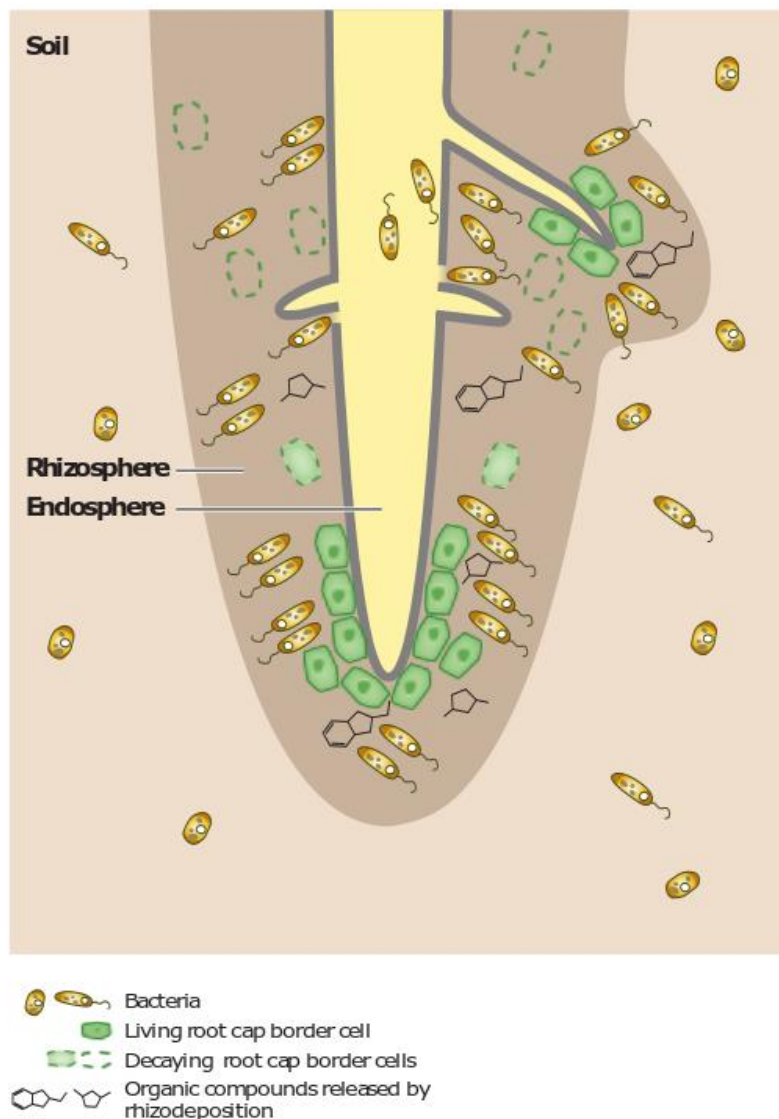


Figure 2 Schematic representation of niche differentiation at the root-soil interface, adopted by Bulgarelli et al. (2013). From outside to inside, the habitats are the soil, rhizosphere, and endosphere. Rhizodeposits generated from root cap border cells and the rhizodermis provoke a shift in the soil biome. Cellular disjunction of the root surface during lateral root emergence provides a potential entry gate for the rhizosphere microbiota into the root interior.

1.2.2. The microbial community of the rhizosphere; Who is there and why?

Rhizosphere is a multi-microbial habitat where bacteria, fungi, oomycetes, viruses and archaea coexist (Bonkowski et al. 2009; Buée et al. 2009; Meeting 1992; Raaijmakers et al. 2009), being attracted by the nutrient resources that the plant is exuding through its roots. Important processes that are supported by the rhizospheric microbial community includes pathogenesis, plant protection, antibiosis and geochemical cycling of minerals (Kent and Triplett 2002). Beyond all these the rhizosphere microbiome has a strong effect on plant health by helping plants to cope with abiotic stresses (i.e. drought or salinity) (Pérez-Jaramillo et al. 2016; Zancarini, Lépinay, et al. 2013) and stimulate growth through enhanced acquisition of nutrients (Mendes et al. 2011; Pieterse et al. 2014).

Regarding its composition the bacterial community in the rhizosphere is dominated by Proteobacteria, followed by Bacteroidetes, Acidobacteria and Firmicutes (Buée et al. 2009; DeAngelis et al. 2009; Fierer et al. 2009; Gomes et al. 2001; Mendes et al. 2011; Peiffer et al. 2013; Sharma et al. 2005; Uroz et al. 2010). It encompasses pathogens and their beneficial counterparts, plant growth promoters and symbionts. Undoubtedly, the best-known symbiosis between plant roots and bacteria is this of nitrogen-fixing bacteria of the family of Rhizobiaceae and legume plants. Other bacteria

may also contribute to biogeochemical cycling in the rhizosphere and soil like (i) ammonia-oxidizing bacteria belonging to the β -proteobacterial genera *Nitrosomonas* and *Nitrospira*, which convert ammonium to nitrate through the intermediate production of hydroxylamine (Hawkes et al. 2002; Leininger et al. 2006); (ii) methane-oxidizing bacteria which are able to convert methane produced by methanogens in soil anaerobic niches (Fierer et al. 2007) and (iii) denitrifying bacteria which convert nitrates to N_2 . In fact, denitrification is highly promoted in the rhizosphere due to the high concentration of available C that favor denitrifying bacteria (Philippot et al. 2009), and the high consumption of oxygen by plants leading to the establishment of anaerobiosis in rhizosphere micro-sites favoring denitrification (Henry et al. 2008).

The fungal community in the rhizosphere is composed mainly of Ascomycetes, Basidiomycetes and Glomeromycetes, the latter encompassing the obligatory symbiotic arbuscular mycorrhizal fungi. Saprotrophic fungi in the rhizosphere (Berg et al. 2005; de Boer et al. 2008; Viebahn et al. 2005; Zachow et al. 2008) could be yeasts and filamentous fungi with representatives of all major terrestrial phyla (Ascomycota and Basidiomycota) and subphyla like Mucoromycotina (Berg et al. 2005; Renker et al. 2004; Vujanovic et al. 2007). Rhizosphere is the habitat of (i) plant-beneficial, symbiotic or non-symbiotic, fungi which promote plant vigor and protect plants from infestations by other microorganisms. This group contains endo- and ectomycorrhizal fungi and non-symbiotic beneficial fungi (ii) mycoparasitic fungi (Mendes et al. 2013) and (iii) pathogenic fungi (i.e. *Fusarium oxysporum*, *Verticillium dahliae*) and oomycetes (i.e. *Pythium* sp., *Peronospora* sp., *Phytophthora* sp.) that compromise plant health (Mendes et al. 2011; Weller et al. 2002; Buée et al. 2009; Raaijmakers et al. 2009). The contribution of fungi in these processes has been shown to affect the composition of terrestrial plant community (Bell et al. 2006) and ecosystem productivity (Van der Heijden et al. 1998; Maherali and Klironomos 2007).

Archaea have been detected at appreciable abundance in the soil rhizosphere. They are key players in soil nitrification (Leininger et al. 2006), methane production (Erkel et al. 2006) and other nutrient cycling processes. Studies examining the role and abundance of ammonia-oxidizing archaea in rhizospheric soil have provided contradictory results. Some studies noted a predominance of ammonia-oxidizing archaea over bacteria in the plant rhizosphere (Chen et al. 2008; Hussain et al. 2011; Kleineidam et al. 2011), while others indicated the reverse (Glaser et al. 2010; Trias et al. 2012; Wei et al. 2011). Thion et al. (2016) studied the abundance of ammonia oxidizing archaea vs bacteria in the rhizosphere vs bulk soil of 20 plants and showed that ammonia-oxidizing archaea showed increasing abundance in the rhizosphere of plants with high N demands (vs bulk soil), but no rhizosphere effect under conservative plants. This was attributed to the higher affinity of ammonia-oxidizing archaea for NH_3 and hence their stimulation over their bacterial counterparts under low ammonium fertilization (Lehtovirta-Morley et al. 2016; Prosser and Nicol 2012). Regarding the composition of the archaeal rhizospheric community, Crenarchaeota are the most abundant taxon in the rhizosphere (Bintrim et al. 1997; Borneman and Triplett 1997; Ochsenreiter et al. 2003), followed by methanogenic archaea, which prevail in anaerobic soil conditions like in rice paddy fields (Conrad 2007; Conrad et al. 2008; Nouchi et al. 1990; Ramakrishnan et al. 2001).

1.2.3. Factors shaping the microbial community of the rhizosphere

Although rhizosphere is considered a more stable habitat than phyllosphere, quite many factors can affect the composition of its associated microbial community. Environmental factors (Bonito et al. 2014; Schreiter et al. 2014; Shakya et al. 2013) and seasonal fluctuations (Dunfield and Germida 2003; Van Overbeek and Van Elsas 2008) can impose strong filtering effects on the assemblage of the rhizospheric microbial community. Soil

is considered the biological pool that supplies microorganisms to the rhizosphere. Several studies have suggested that the soil microbial community determines to a large extent the rhizospheric microbial community, while plant has a secondary structural effect (Bulgarelli et al. 2012; Garbeva et al. 2008; Lundberg et al. 2012; Schläeppli et al. 2014; Shakyia et al. 2013). Soil physicochemical properties such as pH, texture, organic matter content, micro-aggregate stability and the availability of nutrients, have a confounding effect on the composition of the rhizosphere microbial community (Berg and Smalla 2009; Bulgarelli et al. 2012, 2013, 2015; Duffy et al. 1997; Dumbrell et al. 2010; Hamel et al. 2005; Hoper et al. 1995; Lacey and Wilson 2001; Lareen et al. 2016; Mendes et al. 2013; Philippot et al. 2013; Rasmussen et al. 2002; Rotenberg et al. 2005; Toljander et al. 2008). For example soil pH was identified as the most significant factor affecting the abundance of certain bacterial taxa (i.e. Acidobacteria, Verrucomicrobia) and functional microbial guilds (i.e. ammonia-oxidizing bacteria) in the soil and rhizosphere (Prosser and Nicol 2008; Da Rocha et al. 2013; Thion et al. 2016). In support of this, many studies have revealed that soil properties have a strong influence on the composition of bacterial and mycorrhizal communities in rhizosphere (Andrew et al. 2012; Inceoğlu et al. 2012; De Ridder-Duine et al. 2005; Santos-González et al. 2011).

Beyond soil effects on the rhizospheric microbiota, plants themselves could drive the colonization of their rhizosphere. Indeed several previous studies have reported significant differences in the abundance and composition of the soil microbial community in the rhizosphere of different plant genotypes (Andreote et al. 2009; Inceoğlu et al. 2010; Lundberg et al. 2012; Van Overbeek and Van Elsas 2008; Pérez-Jaramillo et al. 2016; Zancarini, Mougel, et al. 2013) or plant species (Grayston et al. 1998; Latour et al. 1996; Miethling et al. 2000; Pivato et al. 2009; Smalla et al. 2001). Chemical and morphological attributes of the plant root like their architecture (Satbhai et al. 2015) and their exudates

composition (Badri et al. 2012; van Dam and Bouwmeester 2016; Mendes et al. 2014; Pérez-Jaramillo et al. 2016; Vandenkoornhuysen et al. 2015) can impose strong filtering effects on the composition of the microbial community. In addition plant growth stage (Herschkovitz et al. 2005; Lerner et al. 2006; Van Overbeek and Van Elsas 2008) and the root zone (Baudoin et al. 2002; Liljeroth et al. 1991; Marschner et al. 2011; Yang et al. 2000) also affect the composition of the rhizospheric microbial community. This is not surprising if we consider that these factors are interlinked with the size, morphology and architecture of plant roots and affect the chemical composition of root exudates.

Beyond the taxonomic diversity of rhizospheric microbial communities, plants seem to modulate their rhizosphere microbiota by recruiting microorganisms with potential beneficial attributes (Philippot et al. 2013). Such microorganisms can exhibit phenotypes which promote seed germination, seedling vigor, plant growth and development, improved capacity to acquire nutrients, protection from pests and diseases, and overall enhanced productivity and mycorrhization (Berg and Smalla 2009; Chaparro et al. 2013; De-la-Peña et al. 2010; Mendes et al. 2011, 2013; Mougel et al. 2006).

1.3. Effects of agricultural practices on the epiphytic and rhizospheric microbial communities

Beyond the interactions of plant with its surrounding environment, farming practices seriously affect the composition of the microbial community in plant-associated compartments (phyllosphere and rhizosphere) and soil. Among those, application of agrochemicals (pesticides and fertilizers) and organic amendments can reshape the microbial communities in these compartments (Vacher et al. 2016; Walter et al. 2007). Initial studies using first generation molecular tools and phospholipid fatty acid analysis

showed strong structural differences in the rhizosphere and soils under organic and conventional cultivation regimes (Esperschütz et al. 2007; Hartmann et al. 2006; Widmer et al. 2006). Hartmann et al. (2015) explored the composition of the soil microbial community under long-term organic and conventional farming and detected distinct microbial composition with organic farming soils and rhizosphere dominated by microbial groups involved in the decomposition of organic compounds found in composts and manures. In a more recent study Li et al. (2019) observed profound differences in the β -diversity of the rhizospheric bacterial community under organic cultivation compared to integrated and conventional cultivation schemes which were also associated with increased suppressiveness towards *Phytophthora capsici*. Karlsson et al. (2017) showed a higher fungal richness in wheat leaves from organic farms compared to conventionally cultivated wheat farms in the same region.

Pesticides constitute an integral part of modern agriculture. Upon their application in soil, rhizosphere or on plant foliage they interact with indigenous microorganisms with the outcome ranging from toxicity to microorganisms not being able to cope with pesticide exposure, to microbial acclimation leading to energy-derived microbial degradation of pesticides (Karpouzas et al. 2016). To date several studies have explored the interactions of pesticides with the soil and rhizospheric microbial community (Gallego et al. 2019; Itoh et al. 2014; Karas et al. 2018; Storck et al. 2018), while less are known about the pesticides effects on the epiphytic microbial community (Gu et al. 2010; Ottesen et al. 2015; Perazzolli et al. 2014).

Organic soil amendment is another practice which is commonly used in low-input and conventional agriculture to improve soil fertility, porosity and structure. In this context organic amendments of variable forms and composition ranging from plant residues to biochar and animal manures have been used for this purpose. Such practices

impose strong changes in the diversity and function of the soil and rhizospheric microbial communities depending on the composition of the organic amendments applied (Francioli et al. 2016; Herrmann et al. 2019; Lehmann et al. 2011; Rieke et al. 2018). Most of these studies have proposed that the changes induced on the soil microbiota, could be largely explained by changes in the physicochemical characteristics of the soils amended with pH and organic carbon identified as the key explanatory variables.

1.3.1. Pesticides application

1.3.1.1. Pesticides microbial toxicity (negative interaction)

Phyllosphere is a habitat which is more directly exposed to environmental changes compared to soil and rhizosphere. However, regarding pesticide exposure things are different. Phyllosphere receives direct application of foliage applied pesticides, while soil and rhizosphere are exposed to pesticides through drenching with soil applied pesticides, and indirectly through runoff from leaves of the excess of sprayed pesticides. To date several studies have focused on the toxicity of pesticides on the epiphytic microbial community with results not being conclusive, largely varying based on the plant host and the pesticide studied. Andrews and Kenerley (1978); Glenn et al. (2015); Zhou et al. (2011) reported negative effects of pesticides on epiphytic microorganisms, while others observed beneficial or no effects (Walter et al. 2007; Moulas et al. 2013; Jensen et al. 2013; Perazzolli et al. 2014). For example, Zhang et al. (2008; 2009) showed that cypermethrin application induced significant alterations in the composition of the epiphytic bacterial community in cucumber and pepper plants reflected in a significant increase in the total bacterial abundance and especially of gram negative bacteria, while other bacteria were negatively affected. Similarly, Gu et al. (2010) showed that the

application of the fungicide enostroburin induced significant changes in the composition of the epiphytic bacterial community with *Pantoea* phylotypes benefiting and *Pseudomonas* being negatively affected. More recent studies using amplicon sequencing analysis revealed a remarkable resilience of the epiphytic microbial community to pesticides exposure (Ottesen et al. 2013; Perazzolli et al. 2014; Sapkota et al. 2015).

The impact of pesticides on soil and rhizosphere microbial communities have attracted much more attention compared to phyllosphere, however the results are also inconclusive. This probably stems from variable experimental protocols used and variation in the soil and pesticide properties which have a profound effect on pesticide dissipation and hence on the extent of exposure (Karpouzas et al. 2014b). Numerous studies have observed significant pesticide effects on the diversity and function of the soil bacterial community (Bruck 2009; Nettles et al. 2016; Pusenkova et al. 2016). For example Karpouzas et al. (2014a), following a tiered lab to field experimental approach, showed that the herbicide nicosulfuron induced at low soil concentrations ($0.25\text{-}1\ \mu\text{g g}^{-1}$) significant reductions in the abundance of Gram negative (β -proteobacteria, planctomycetes), Gram positive bacteria (actinobacteria) and fungi. In a similar study Karas et al. (2018) observed an inhibitory effect of chlorpyrifos and tebuconazole on the abundance of ammonia-oxidizing bacteria and archaea which recovered by the end of the study, and a stable reduction in the abundance of sulfur-oxidizing bacteria. Other studies did not observe significant effects of pesticides on the soil and rhizospheric microbiota (Wang et al. 2004; Lupwayi et al. 2009; Miñambres et al. 2010; Nettles et al. 2016; Ju et al. 2017; Storck et al. 2018). Regarding soil and rhizospheric fungi, their response to pesticide exposure has been the focus of a limited number of studies with the results indicating negative effects on their diversity and biomass, with a magnification of the

negative effects when fungicide application is involved (Bending et al. 2007; Cappelletti et al. 2016; Howell et al. 2014).

Several studies have showed that often it is not the pesticide parent compounds imposing the negative effects on the soil and rhizospheric microbial communities but transformation products that exhibit higher toxicity (Karas et al. 2018; Papadopoulou et al. 2016; Zhang et al. 2016). In such a study Vasileiadis et al. (2018) observed a significant effect on the bacterial and the fungal community by the application of iprodione which were eventually associated with the formation and accumulation of 3,5-dichloroaniline, a major transformation product of iprodione. Similarly, Karas et al. (2018) showed a significant negative correlation between the two demethylated transformation products of isoproturon and the activity of P-cycling enzymes and aminopeptidase.

Significant attention has been given to the effects of pesticides on the function of key microbial groups which are sensitive to pesticide exposure while at the same time they carry out key microbial functions, like ammonia-oxidizing microorganisms and arbuscular mycorrhizal fungi. The former are responsible for the rate-limiting step in nitrification, the oxidation of ammonia to nitrite through the intermediate formation of hydroxylamine (Prosser and Nicol 2008). The latter are obligate symbionts in most terrestrial plants increasing plant uptake of phosphorus, water and other nutrients sources (Kiers et al. 2011). Ammonia-oxidizing microorganisms have been proposed as ideal microbial indicators to assess the soil microbial toxicity of pesticides (Karpouzas et al. 2016) due to the existence of advanced and standardized tools to assess their abundance, function and diversity, their sensitivity to external perturbations (Wessén and Hallin 2011) and their key role in ecosystem functioning (Prosser and Nicol 2008). Previous studies have indicated that exposure of soil and rhizosphere to various non-fungicide pesticides like glyphosate (Feld et al. 2015), simazine, (Hernández et al. 2011), mesotrione (Crouzet

et al. 2009), ethoxyquin (Papadopoulou et al. 2016) and fungicides like iprodione (Vasileiadis et al. 2018) induced transitory or permanent inhibition of ammonia-oxidizing microorganisms. Similar studies with arbuscular mycorrhizal fungi have also suggested adverse effects in their capacity to colonize plant hosts (Ipsilantis et al. 2012; Karpouzas et al. 2014b) and facilitate P uptake by plants (Zocco et al. 2011). Most if not all of the studies, except those focusing on arbuscular mycorrhizal fungi, have been performed in the absence of plants whose presence might have increased the resilience of the soil microbial community. Newman et al. (2016) studied the impact of glyphosate in the rhizosphere of corn and soybean samples (not comparatively to bulk soil) and observed a significant increase of Proteobacteria upon glyphosate exposure and a complementary decrease in Acidobacteria. Similarly, Singh et al. (2015a; 2015b) reported strong adverse effects of chlorpyrifos, cypermethrin and azadirachtin on the diversity of rhizospheric bacteria and fungi and also on the abundance of microorganisms involved in the different steps of N cycle including nitrifiers, denitrifiers and nitrogen-fixing bacteria.

Despite the numerous reports on the response of bacteria and fungi to pesticides we know very little about the impact of pesticide compounds on phyllosphere and rhizospheric/soil archaea. The only relevant information is coming from studies looking at the impact of pesticides on archaea belonging to key functional groups like ammonia-oxidizers and methanogens. For example, ammonia-oxidizing archaea seem to be impaired by the application of pesticides like ethoxyquin, iprodione, glyphosate (Feld et al. 2015; Papadopoulou et al. 2016; Vasileiadis et al. 2018). Beyond these functional archaeal groups, only Howell et al. (2014) showed that the fungicide azoxystrobin did not impose any effects on the archaea community in soil.

1.3.1.2. Pesticides enhanced microbial degradation (beneficial interaction)

Repeated soil applications of certain pesticide groups like organophosphates (Singh and Walker 2006), carbamates (Karpouzas et al 1999), phenoxyalkanoics (Smith and Lafond 1990), phenylureas (Cox et al. 1996) and triazines (Krutz et al. 2010) could increase the population of microorganisms that carry or have evolved specialized catabolic enzymes for the rapid transformation of these pesticides (Baelum et al. 2006; Rousidou et al. 2017). This phenomenon has been termed ‘enhanced biodegradation’ and under conducive edaphoclimatic conditions could jeopardize the biological efficacy of pesticides (Suett et al. 1987). Soil exhibiting enhanced biodegradation of certain pesticides has been used for the isolation of pesticide – degrading bacteria. These bacteria could be exploited in bioengineering and bioaugmentation applications to maintain environmental quality. Examples of such bacteria include *Variovorax* and Sphingomonads that degrade phenylurea herbicides (Dejonghe et al. 2003; Yan et al. 2016), Arthrobacters that degrade iprodione (Athiel et al. 1995; Campos et al. 2015), Sphingomonads and *Pseudomonas* degrading carbamates (Nguyen et al. 2014; Rousidou et al. 2016) and triazines (De Souza et al. 1995). Following studies identified the genes and enzymes that were responsible for the degradation of these pesticides. Yan et al. (2018) identified a hydrolase CehA and a monooxygenase CfdC that are responsible for the transformation of carbofuran by a *Sphingomonas* strain. Perruchon et al. (2017) identified a monooxygenase (OppA) as responsible for the first step in the transformation of *ortho*-phenylphenol by a *Sphingomonas haloaromaticamans* strain and disentangled the genetic network of the bacterium involved in the complete transformation of the fungicide via a multi-omics approach. Similarly, Gu et al. (2013) identified *pdmAB*, encoding a N-demethylase, as the key gene controlling the first step in the transformation of the herbicide isoproturon from a *Sphingomonas* strain.

In contrast to soil and rhizosphere, the potential of the microbial community of the phyllosphere to degrade pesticides in an enhanced rate is largely unknown. In the only relevant study to date Ning et al. (2012) isolated bacteria from the phyllosphere of rape plants repeatedly treated with the organophosphorus insecticide dichlorvos which were able to rapidly transform this pesticide. The arsenal of catabolic genes and enzymes carried by epiphytic catabolic bacteria, their possible divergence from the catabolic genetic traits carried by their soil and rhizosphere counterparts is yet a black box.

1.3.2. Effects of soil amendments on the microbial community

Soils intensively cultivated are often deficient in soil nutrients. In addition soils in the Mediterranean region are rather poor in organic matter. Fertilization of agricultural soils with synthetic and organic fertilizers could avert these deficiencies and maintain a good nutrient status ensuring high crops productivity. The application of fertilizers depending on their composition are expected to alter soil physicochemical properties to a certain extent (i.e. pH, organic matter content, N content etc) and this is often reflected in the size and composition of the soil and rhizospheric microbial communities.

In a meta-analysis paper Geisseler and Scow (2014) showed that long term mineral fertilization increased microbial biomass by over 15% and this increase was a function of a parallel increase in the organic carbon content. When the effects of inorganic fertilizers on the soil microbial activity were explored, both positive and negative effects were reported (Gianfreda and Ruggiero 2006; Guo et al. 2011; Nannipieri et al. 2012). Compared to inorganic fertilizers, the application of organic amendments induced significant alterations in the soil and rhizospheric microbial communities, and increased soil microbial biomass (Esperschütz et al. 2007; Lentendu et al. 2014; Marschner et al.

2004 Lazcano et al. 2013) and microbial activity (Hoitink and Boehm 1999; Hu and Gru 1999; Malik et al. 2013; Peacock et al. 2001). This is probably a reflection of the oligotrophic nature of soil, with microorganisms responding rapidly to the release of fresh organic matter which could be easily assimilated and used for microbial growth (Demoling et al. 2007). In support of this Spyrou et al. (2009) showed that soil amendment with pulverized fruits of the *Melia azedarach*, which contain a mixture of limonoids with biocidal properties, induced significant increases in the soil heterotrophic bacteria and fungi and masked potential inhibitory effects of the bioactive compounds contained in this material. Soil amendment with various organic materials like composted plant residues are also effective in suppressing diseases caused by fungal pathogens (Diab et al. 2003; Veeken et al. 2005).

Mediterranean semi-arid ecosystems support phyto-communities rich in aromatic plants (Celiktas et al. 2007; Pintore et al. 2002; Vokou and Liotiri 1999). These produce essential oils characterized by high *in vitro* antimicrobial activity against plant pathogenic fungi, bacteria (Iskan et al. 2002; Karamanoli et al. 2000; Pintore et al. 2002; Santoyo et al. 2005; Soylu et al. 2010; Vokou et al. 2003) and also high allelopathic activity against weeds commonly found in Mediterranean agricultural soils like *Amaranthus retroflexus*, *Echinochloa crus-gali*, *Portulaca oleracea* (Argyropoulos et al. 2008; Vokou 1992, 1999). The antimicrobial properties of the essential oils have been associated with the presence of isoprenoid volatile compounds (Karamanoli et al. 2000; Daferera et al. 2003; Kalemba and Kunicka, 2003) like carvone, menthol and isomethone in the essential oils of *M. spicata* and *Metha piperita* (Kadoglidou et al. 2011; Karamanoli et al. 2018), α -pinene, cineol, camphor, borneol in the essential oil of *R. officinalis* (Cobellis et al. 2015; Karamanoli et al. 2000, 2018), thymol and carvacrol in the essential oil of *Origanum*

vulgare subsp. *hirtum* (origano) and 1,8-cineol and camphor in *Salvia fruticosa* (sage) (Karamanoli et al. 2000).

These properties of aromatic plants combined with their high abundance in the Mediterranean region gave birth to the idea of using their litter as organic soil amendment serving a dual purpose: enriching the nutrient poor soils of the Mediterranean region with fresh organic carbon and suppress soil-borne plant pathogens and weeds through the release of bioactive compounds present in the essential oils. Chalkos *et al.*, (2010) first tested the impact of soil amendment with composted aromatic plant residues from *M. spicata* (spearmint) and *S. fruticosa* (sage) on plant growth and soil microbiota. They observed significant increases in the population of bacteria and fungi, inhibition of weed emergence and a positive effect on the growth of tomato plants with the most prominent growth promotion effect seen with spearmint. Follow up studies by Kadoglidou et al. (2014) showed that soil amended, this time with dried plant residues of spearmint, had increased soil bacterial population and higher growth and vigor of tomato plants compared to sage-amended samples where no beneficial or inhibitory effect was observed. Cavaliere and Caporali (2010) tested the essential oils of cinnamon (*Cinnamomum zeylanicum* L.), lavender (*Lavandula* spp.) and peppermint (*Mentha x piperita* L.) on seed germination of 7 Mediterranean weed species (*Amaranthus retroflexus* L., *Solanum nigrum* L., *Portulaca oleracea* L., *Chenopodium album* L., *Sinapis arvensis* L., *Lolium* spp. and *Vicia sativa*) and showed high anti-germinating activity against all weeds at concentration levels of 1.8-5.4 mg L⁻¹ under controlled conditions, whereas higher concentrations of essential oils were required (345.6 mg L⁻¹) for effective inhibition of weed germination in greenhouse tests.

1.4. Objectives of the thesis

In the frame of this thesis we tried to identify the main environmental and genetic factors shaping microbial community in the phyllosphere of selected indigenous plants of the Mediterranean landscape using amplicon sequencing of the 16S rRNA gene and the ITS genomic region for the prokaryotic and fungal communities respectively. We assumed that plant host and season will have a confounding effect on the assemblage of the epiphytic microbial community, while extra plant host attributes like plant habit and their capacity to produce essential oils with antimicrobial properties (aromatic plants) could be an extra filtering mechanism in shaping epiphytic microbial communities in such ecosystems. Following up on the use of aromatic plants, we explored the impact of their utilization as soil amendments (dried plant residues) on the abundance and diversity of functional microbial groups (i.e. ammonia-oxidizing microorganisms, sulfur-oxidizing bacteria etc) and the whole bacterial, fungal and archaeal community using q-PCR and amplicon sequencing approaches respectively. Our hypothesis was that aromatic plant biomass will affect soil microbial communities, in the presence or absence of plant roots, through essential oils release and fresh biomass addition. Finally, we studied the response of the epiphytic microbial community of a cultivated crop (pepper) on the repeated exposure to a biodegradable fungicide (ie. Iprodione) comparatively to the microbial community in the soil root zone. We hypothesized that both communities will respond in the same way to pesticide exposure resulting either in enhanced biodegradation of the applied chemical or in toxicity to certain members of the microbial community (i.e. fungi). To address this hypothesis we employed a pot experiment where (a) we monitored the degradation of the pesticide applications in the two habitats with chromatographic analysis to determine if repeated applications induced enhanced biodegradation of the pesticide in both compartments; (ii) we determined the microbial succession in the two habitats along

the repeated pesticide application scheme using amplicon sequencing analysis and (iii) we isolated iprodione-degrading bacteria from both habitats and explored their taxonomic and metabolic association. To summarize the key objectives of this thesis were

1. to identify the effects of factors like season and plant host (and plant habit or aromatic character) on the abundance and composition of the epiphytic microbial community in native plants of a semi-arid Mediterranean ecosystem.
2. to investigate the potential impact of aromatic plants, native to the Mediterranean ecosystem, used as soil amendments on the soil microbial community in the presence or absence of roots from cultivated plants.
3. to determine the comparative response of the microbial community on the plant phyllosphere and on the soil plant root zone to successive pesticide applications and further to identify common cues associated either with microbial toxicity or microbial acclimation to pesticide biodegradation

1.5. References

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Chapter 2

Season or Plant species: Which factor shapes the epiphytic bacterial, archaeal and fungal community in a typical semi-arid Mediterranean ecosystem?

The work presented in Chapter 2 is included in the following article:

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2.1. Introduction

Phyllosphere constitutes an enormous habitat for microorganisms, with an estimated size of up to $4 \times 10^8 \text{ km}^2$ (Morris et al. 2002), making it sufficiently large to have major effects on global nutrient cycling (Delmotte et al. 2009). It is highly oligotrophic, with heterogenic nutrient and water availability where microorganisms are challenged by extreme environmental conditions like UV irradiation and temperature (Müller and Ruppel 2014; Rastogi et al. 2012). Despite that, phyllosphere is occupied by a highly diverse microbial community (Cernava et al. 2019; Kembel et al. 2014) whose members have evolved specific functional traits ensuring fitness in this hostile environment (Delmotte et al. 2009; Müller et al. 2016). In this context Helfrich et al. (2018) recently showed that epiphytic bacteria are champions in the biosynthesis of a wealth of novel biocidal natural products.

Plants employ filtering mechanisms to shape their epiphytic microbiota (Vorholt 2012). This in turn affects plant traits by mediating plant responses to biotic and abiotic stress (Ritpitakphong et al. 2016), biosynthesizing plant auxins (Taffner et al. 2019), degrading organic pollutants (Scheublin et al. 2014) and C1 compounds (Delmotte et al. 2009). Vandenkoornhuysen et al. (2015) argued that the functional traits of the plant microbiome should be included in an extended plant phenotype called plant holobiont, where some key plant-host functions are outsourced to the rhizospheric, endophytic or epiphytic microbiota. In this frame, Laforest-Lapointe et al. (2017) showed that plant community productivity is positively related to epiphytic bacterial diversity reinforcing the importance of the interplay between plant and phyllospheric microbes on ecosystem functioning.

Several studies have looked into the composition of the epiphytic microbial community. Bacteria are the main dwellers of the plant phyllosphere, followed by fungi,

while archaea are less abundant (Vorholt 2012) but encompass important functional traits like N assimilation, C fixation, auxin biosynthesis and oxidative response (Taffner et al. 2019). The bacterial community on the phyllosphere is dominated by Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria (Bodenhausen et al. 2014; Durand et al. 2018; Kembel and Mueller 2014; Knief et al. 2012; Peredo and Simmons 2018; Rastogi et al. 2012), the fungal community by Ascomycotes and Basidiomycetes (Coince et al. 2014; Jumpponen et al. 2010; Toju et al. 2018; Yao et al. 2019), while the archaeal community is less explored and the few studies available showed a dominance of Euryarchaeota (i.e. *Methanomicrobia*) and Thaumarchaeota (i.e. *Candidatus Nitrosocosmicus* and *Nitrososphaera*) (Knief et al. 2012; Ruiz-Pérez et al. 2016; Taffner et al. 2018, 2019).

Plant phyllospheric microbial communities are shaped following deterministic mechanisms with soil and air being the main reservoirs of microbial inocula (Grady et al. 2019; Wehking et al. 2018). Most studies have identified plant genotype as the main determinant of the composition of the bacterial and fungal epiphytic community (Bodenhausen et al. 2014; Laforest-Lapointe et al. 2016a; Sapkota et al. 2015) and have attributed this to plant leaf functional traits (i.e. leaf thickness, leaf surface wax, trichomes density) (Hunter et al. 2015; Kembel and Mueller 2014; Laforest-Lapointe et al. 2016b). Geographical location (i.e. local climatic conditions) has been identified as a secondary contributor to the variation in the composition of the epiphytic microbial communities (Agler et al. 2016; Knief et al. 2010) with distance-decay relationships being important for fungi but not for bacterial communities (Coleman-Derr et al. 2016). Epiphytic microbial communities are also seasonally diverse with climatic conditions having a profound impact on the bacterial and fungal diversity (Copeland et al. 2015; Gomes et al. 2018; Jackson and Denney 2011).

Mediterranean semi-arid ecosystems constitute unique environments characterized by alternation of cold and wet with hot and dry seasons expected to endure a strong selection on the plant microbiome. They are dominated by non-woody shrubs and woody evergreen sclerophyllous or seasonally dimorphic plants, with several of them being aromatic. Previous studies in such ecosystems showed that non-woody shrubs supported higher epiphytic bacterial populations and aromatic plants showed higher bacterial abundance, richness and metabolic diversity (Yadav et al. 2004, 2005, 2008). Following studies by Vokou et al. (2012) showed clear structural differences in the epiphytic bacterial community between sclerophyllic evergreen plants (plus *Myrtus communis*) and aromatic Lamiaceae plants. All the above studies used culture-dependent or low-resolution culture-independent methods (i.e. DGGE - cloning), which, unlike amplicon next generation sequencing methods, fail to provide an in-depth analysis of the bacterial epiphytic diversity (Müller and Ruppel 2014). Furthermore, little is known about the composition of the epiphytic fungal and archaeal communities in such Mediterranean ecosystems and the factors shaping epiphytic microbiomes.

In this context we tested the hypothesis that plant host and/or season are the key factors shaping the epiphytic bacterial, archaeal and fungal communities of plants in a semi-arid Mediterranean ecosystem. To verify this hypothesis we determined, via q-PCR and amplicon sequencing analysis respectively, the abundance and the composition of the epiphytic community of bacteria, archaea and fungi at two distinct seasons (summer vs winter) in eight perennial plants characterized by different ecophysiological and functional traits (woody sclerophyllous, woody semi-deciduous, non-woody, aromatic and non-aromatic etc.), all located in the same semi-arid Mediterranean ecosystem, hence exposed to the same climatic conditions and microbial inocula.

2.2. Materials and Methods

2.2.1. Sampling site, plant species and seasonality

The study site is a semi-arid Mediterranean ecosystem located in Sithonia peninsula (location Armenistis, Halkidiki, 40°9' N, 23°54' E), Northern Greece (Supplementary Figure S1). The climate of the region is characterized by rather mild and wet winters and hot and dry summers (Yadav et al. 2004). We studied the phyllosphere microbial community in eight indigenous and co-existing perennial plant species. The sampled plants species could be categorized according to their plant traits as (a) woody evergreen sclerophyllous like *Arbutus unedo* L., *Myrtus communis* L., *Phyllirea latifolia* L., *Pistacia lentiscus* L., *Quercus coccifera* L., (b) woody, low, drought semi-deciduous like *Cistus incanus* L. and *Lavandula stoechas* L., and (c) non-woody like *M. officinalis* L., found in less arid sites of the studied ecosystem. Plants can be further categorized as aromatic like *M. communis*, *L. stoechas*, *P. lentiscus*, *C. incanus* and *M. officinalis* and non-aromatic like the rest of the plants studied (Yadav et al. 2004).

Samples were collected in July 2013 (summer season) and January 2014 (winter season). For each sample three individual plants were randomly selected and 5 mature, well-developed and healthy leaves per individual plant were collected. Leaves were immediately placed in sterile plastic bags and transported on ice boxes in the laboratory where they were stored at -20°C until further processed.

2.2.2. DNA extraction

DNA extraction from plant phyllosphere was performed as described by Moulas et al. (2013) with slight modifications. Briefly, 1-3 g of intact fresh leaves were immersed in sterilized ddH₂O, in sterilized centrifuge tubes and were subjected to sonication for 7 min

to detach epiphytic microbial cells from the leaf surface. The leaves were removed, with forceps and the content of the tubes was centrifuged for 15 min at 15000xg. The supernatant was discarded, and the microbial pellet collected was used for DNA extraction with the PowerSoil® DNA isolation kit (MoBio Laboratories, Inc., West Carlsbad, CA, USA). The integrity of the extracted DNA was checked via agarose gel (0.8%) electrophoresis and it was quantified using a Qubit fluorometer with a Quant-iT HS double-stranded DNA (dsDNA) assay kit (Invitrogen, USA).

2.2.3. q-PCR analysis of the abundance of epiphytic microbial groups

We determined the abundance of total bacteria, Crenarchaea, fungi and of specific fungal genera like *Cladosporium* and *Alternaria*, known to be major constituents of the airborne fungal inoculum in urban, rural and semi-arid ecosystems (Grinn-Gofroń et al. 2019) via q-PCR. The abundance of total bacteria and Crenarchaea was determined using primers Eub338 (Muyzer et al. 1993) - Eub518 (Øvreås and Torsvik 1998) and 771f-957R (Ochsenreiter et al. 2003) respectively, amplifying the 16S rRNA gene. The abundance of total fungi was determined using primers ITS3F and ITS4R (White et al. 1990) that amplify the ITS2 region. The abundance of fungi belonging to the genus *Cladosporium* was determined with primers Clado-SYBR-PF-Clado-SYBR-PR (Zeng et al. 2006) that amplify a part of the mt SSU rDNA gene of most fungi belonging to this genus including *C. cladosporioides*, *C. sphaerospermum*, *C. cucumerinum*, *C. oxysporum*, *C. elatum* and *C. herbarum*. Finally the abundance of fungi of the genus *Alternaria* were determined with the primer pair of Dir1ITSSAlt - Inv1ITSAIt (Pavon et al. 2011) that amplify the ITS gene of most plant-associated *Alternaria* sp. including *A. alternata*, *A. arborescens*, *A. infectoria*, *A. solani*, *A. tenuissima* etc. The sequences of the primers used, and q-PCR

conditions are listed on Supplementary Table S1. Q-PCR reactions were carried out in a Stratagene Mx3005P Real-Time PCR System, in a total volume of 10 μ L containing 5 μ L of the KAPATaq SYBR Green® PCR master mix (Kapa Biosystems, Wilmington, Massachusetts, USA), 250 ng of BSA, 10 ng of soil DNA and 2 μ M of each primer. The abundance of each microbial group was determined with the use of standard curves constructed using serial dilutions of linearized plasmids containing the studied genes. Q-PCR efficiency in all cases ranged between 91 and 103%.

2.2.4. Amplicon sequencing analysis of the phyllosphere microbial community

The effects of plant host and season on the structure of the epiphytic community of bacteria, archaea and fungi were determined with amplicon sequencing of the 16S rRNA and ITS respectively via HiSeq Illumina Rapid Mode 2x250 bp paired-end in the DNA Sequencing Center of the Brigham Young University, USA. Bacterial and archaeal 16S rRNA were amplified with primers 515f-806r (Caporaso et al. 2012; Walters et al. 2015) following the protocol of the Earth Microbiome Project (Caporaso et al. 2018). The amplification of ITS was done with primers ITS7-ITS4 (Ihrmark et al. 2012; White et al. 1990) following the protocol described by Ihrmark et al. (2012). For all PCR amplification the Q5® High-Fidelity DNA Polymerase (NEB, Ipswich, Massachusetts, USA) was used. All samples were initially amplified (28 amplification cycles) using the domain-specific primers mentioned above, followed by a PCR (7 amplification cycles) using the same primers carrying indexes for meta-barcoding of samples. Primers sequences and PCR conditions used in amplicon sequencing analysis are presented in Supplementary Tables S2 and S3 respectively.

Removal of PCR and sequencing artifacts, OTU matrix generation and taxonomic sequence classification were performed as follows. The raw sequence data were demultiplexed with Flexbar v3.0 (Dodt et al. 2012) and they were quality controlled with Trimmomatic v0.32 (Bolger et al. 2014). The resulting high quality read pairs were assembled to the amplicon of their origin in cases overlaps occurred with FLASH v1.2.8 (Magoc and Salzberg 2011) using the default parameters amended to allow a maximum overlap of 250 bp and no mismatches between read-pairs. The remaining tasks were carried out with the IOTUs v1.58 perl wrapper (Hildebrand et al. 2014). OTU calling at 97% identities was performed with the UPARSE v10.0.240 software (Edgar 2013). Chimeric sequences were identified with the UCHIME v4.2 software (Edgar et al. 2011) using the RDP Gold database vMicrobiomeutil-r20110519 for bacteria and the UNITE ITS2 v985.20150311 reference database (Nilsson et al. 2015) for fungi, while sequence classification was performed with Lambda v0.9.1 (Hauswedell et al. 2014) against the Silva v128 small ribosomal subunit database (Yilmaz et al. 2014) for bacteria and the UNITE ITS v7_99_20150302 database (Kõljalg et al. 2013) for fungi.

2.2.5. Bioinformatic and statistical analysis of data

All statistical analysis was performed with the R v3.5.2 software (R Core Team 2017). Q-PCR data were subjected to two-way ANOVA, Tuckey HSD and Kruskal-Wallis post-hoc tests after Nemenyi, with agricolae v1.3-1 (De Mendiburu 2019) and PMCMR (Pohlert 2016) package respectively, to determine the effects of plant host x season and plant type (aromatic vs non aromatic / evergreen woody / semi-deciduous woody / non-woody) x season on the abundance of bacteria, fungi and Crenarchaea.

The OTU matrices of bacteria, archaea and fungi obtained by amplicon sequencing were used to assess the impact of season and plant species on the α - and β -diversity of epiphytic microorganisms. Alpha-diversity indices like Richness (S), Inverse Simpson (Jost 2006), Shannon, and Pielou's evenness (Pielou 1975) were calculated, using the *vegan* package v2.5-3 (Oksanen et al. 2018). The data obtained were subjected to two-way ANOVA, as described above for the q-PCR data, to determine the effect of season x plant host. Regarding effects on the β -diversity, differential abundance (DA) tests were employed to identify taxa and OTUs responsive to the two main factors (season and plant host) using the Fisher's exact test as implemented in the *EdgeR* package v3.24.3 (Robinson et al. 2010) for P-values of 0.05 (adjusted according to the Benjamini-Hochberg algorithm (Benjamini and Hochberg 1995)). The impact of season and plant host on the structure of the significantly affected member sub-communities (as determined by the DA tests) of bacteria, archaea and fungi was assessed via canonical analysis as follows. Detrended Correspondence Analysis (DCA) was performed and, depending on the first axis length value, Canonical Correspondence Analysis (CCA) was preferred over Redundancy Analysis (RDA), if this value was higher than 3 standard deviations (SD) and *vice versa* according to a previously suggested strategy (Lepš and Šmilauer 2003). DCA first axis values greater than 3 SD imply overall unimodal responses of community member abundances against the environmental gradients (rendering the chi squared distances of CCA more suitable) as opposed to lower values which imply overall linear responses to environmental gradients (rendering the Euclidean distances of RDA more suitable). Multivariate statistical analyses were performed using the packages *Vegan* v2.4-4 (Oksanen et al. 2018), *Entpart* v1.4-7 (Marcon and Hérault 2015) and *EdgeR* v3.24.3 (Robinson et al. 2010), the latter for DA tests, of the R v3.5.2 software (R Core Team

2017). The data were submitted to Sequence Read Archive (SRA) of NCBI with bioproject accession number PRJNA531404.

2.3. Results

2.3.1. Effects of plant-host and season on the abundance of epiphytic

microorganisms

The sampling season had a significant main effect ($p < 0.05$) on the epiphytic bacterial abundance, which was significantly higher in the winter, whereas no significant effect ($p > 0.05$) of plant-host was observed (Fig. 1a). Within each plant significant seasonal effects on the bacterial abundance were observed for *M. communis*, *A. unedo* and *M. officinalis*. Regarding Crenarchaea (Fig. 1b) and fungi (Fig. 1c), season was the sole factor that induced significant main effects ($p < 0.001$) with higher abundance observed in the summer. Within each plant significant seasonal variations were evident for the Crenarcheal abundance in *L. stoechas* and *M. officinalis* and for the fungal abundance in *C. incanus*, *A. unedo*, *M. communis*, *Q. coccifera*. In line with the overall fungal community, *Alternaria* showed significantly higher abundance ($p < 0.01$) in the summer, while the plant host and its interactions with season were not significant ($p > 0.05$) (Fig 1d). Significant seasonal variations within plants were evident for *C. incanus*, *P. lentiscus*, *Q. coccifera*. Similarly, *Cladosporium* showed significantly higher abundance in the summer ($p < 0.001$), with most plants supporting higher *Cladosporium* numbers in the summer vs winter (Fig. 1e). Plant host had a significant main effect ($p < 0.01$) on *Cladosporium* abundance with *P. latifolia*, showing significantly higher abundance in the summer compared to *M. officinalis*, which showed the lowest Cladosporia abundance.

We further explored potential differences in the abundance of epiphytic microorganisms based on their aromatic character (aromatic vs non-aromatic) or plant habit (woody evergreen, woody semi-deciduous, non-woody) (Supplementary Figure S2). There were no significant differences ($p>0.05$) in the abundance of the studied microbial groups between aromatic and non-aromatic plants with the sole exception of *Cladosporium* sp. where significantly higher abundance was evident in the non-aromatic plants at both seasons. Plant habit did not significantly affect ($p>0.05$) the abundance of bacteria, total fungi and *Alternaria* but had a significant effect on the abundance of Crenarchaea and *Cladosporium*. Hence a significantly higher abundance ($p<0.05$) of Crenarchaea was evident in *M. officinalis* (non-woody) compared to evergreen and semi-deciduous woody plants. Conversely, a significantly lower abundance ($p<0.05$) of *Cladosporium* was evident in *M. officinalis* compared to evergreen plants.

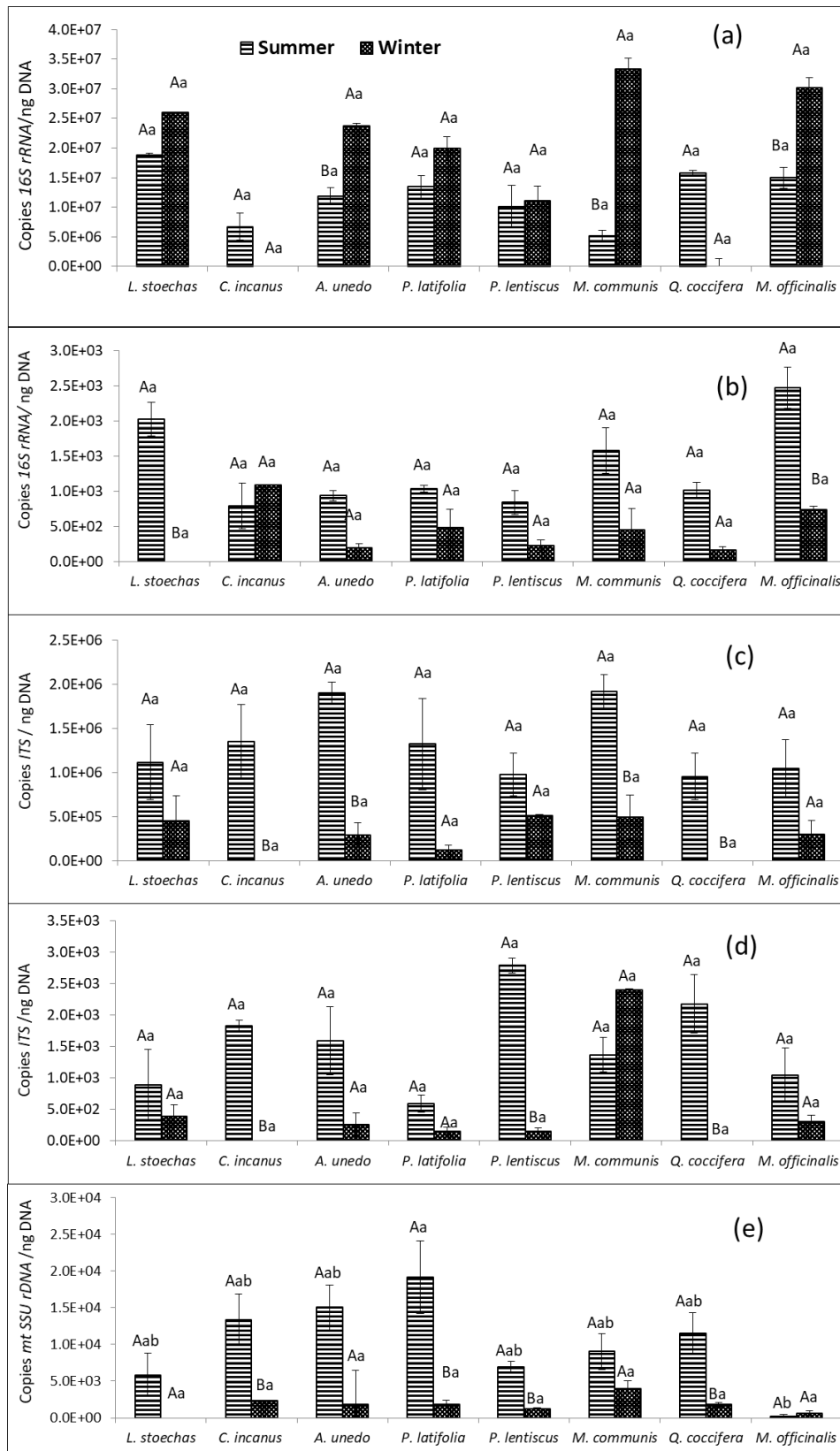


Figure 1. The abundance of bacteria (a), Crenarchaea (b), total fungi (c), *Alternaria* sp. (d) and *Cladosporium* sp. (e) in the phyllosphere of eight plants native to a semi-arid Mediterranean ecosystem. Capital letters above bars indicate significant differences

between seasons in each studied plant, while lower case letters indicate significant differences between plants within each studied season. Each bar is the mean of three biological replicates \pm the standard deviation.

2.3.2. Effects of plant-host/season on the epiphytic microbial diversity

Our sequencing effort provided adequate coverage of the microbial diversity on the plant phyllosphere as suggested by (a) the Good's coverage estimates (Supplementary Table S4) which had values of 0.98-1.00 for bacteria, 0.92-1.00 for archaea and 0.99-1.00 for fungi and (b) rarefaction curves which reached a plateau for all studied microbial domains (Supplementary Fig. S3).

2.3.2.1. Effects on the α -diversity of the epiphytic microbial community

Significant seasonal effects on the α -diversity of bacteria were evident in Shannon index and Pielou's evenness ($p < 0.05$) in *L. stoechas*, *P. latifolia*, *M. communis* and *Q. coccifera* and in Simpson index in *M. communis* and *L. stoechas* ($p < 0.01$) with consistently higher values in the winter (Supplementary Fig. S4). Within season analysis revealed that in the summer (i) *M. communis* showed significantly lower Simpson index values ($p < 0.05$) compared to most other plants, (ii) *P. lentiscus* showed significantly higher values ($p < 0.05$) of Richness compared to *L. stoechas*. Regarding archaea, we observed significant seasonal effects ($p < 0.01$) for Simpson and Pielou's evenness indices in *P. latifolia* with higher values observed in winter. Similarly, *P. latifolia* showed significantly lower values ($p < 0.05$) of Shannon, Simpson and Pielou's evenness indices compared to most other plant hosts in the summer (Supplementary Fig. S4). Regarding fungi, significantly higher values ($p < 0.05$) for Shannon and Simpson indices were evident in the

winter vs summer in *L. stoechas*, *M. communis*, *Q. coccifera* and in all plants except *P. lentiscus* and *C. incanus* for Pielou's evenness (Supplementary Fig. S4). Similarly, *C. incanus* showed significantly higher values ($p < 0.05$) of Simpson and Pielou's evenness compared *L. stoechas* in the summer.

2.3.2.2. Effects on the β -diversity of the epiphytic microbial community

The bacterial community was dominated by α -proteobacteria and KD4-96 (*Chloroflexi*), whose abundance on the phyllosphere of all plants (except *Q. coccifera*) showed a compensatory pattern in the different seasons; α -proteobacteria were dominant in the winter, while KD4-96 dominated in the summer (Fig. 2a). Other bacterial taxa which were detected consistently in the phyllosphere of the studied plants included Actinobacteria, γ -proteobacteria, Cytophagia, Bacilli and β -proteobacteria. It is worth noting that *Q. coccifera* was the sole plant whose epiphytic bacterial community was seasonally stable with α -proteobacteria dominating in both seasons and δ -proteobacteria being present at high relative abundance only in the phyllosphere of this particular plant (Supplementary Fig. S5). The epiphytic archaeal community was dominated by the Soil Crenarchaeotic Group (SCG) and Aenigmarchaeota. The former dominated the phyllosphere of *C. incanus*, *L. stoechas* and *M. communis* and the latter the phyllosphere of *A. unedo*, *P. latifolia* and *P. lentiscus* (Fig. 2b). In contrast to the bacterial community, we observed a strong seasonal variation in the archaeal community only for *Q. coccifera* with SCG and Aenigmarchaeota dominating in the summer and *Methanomicrobia* taking over in the winter (Supplementary Fig. S5). The epiphytic fungal community was dominated by Ascomycetes (orders *Capnodiales*, *Pleosporales* and *Dothideales*) and Basidiomycetes (orders *Tremellales*, *Agaricales* and *Rusullales*). A clear seasonal shift in the fungal community was evident in all plants with uncultured Ascomycota prevailing in the

summer. However, their abundance was significantly reduced in the winter when *Capnodiales* and *Pleosporales* showed increasing relative abundance (Fig 2c). Regarding basidiomycetes, *Russulales* and *Agaricales* showed compensatory patterns in the two seasons, with the former being more abundant in the summer and replaced by *Agaricales* in the winter (Fig. 2c, Supplementary Figure S5).

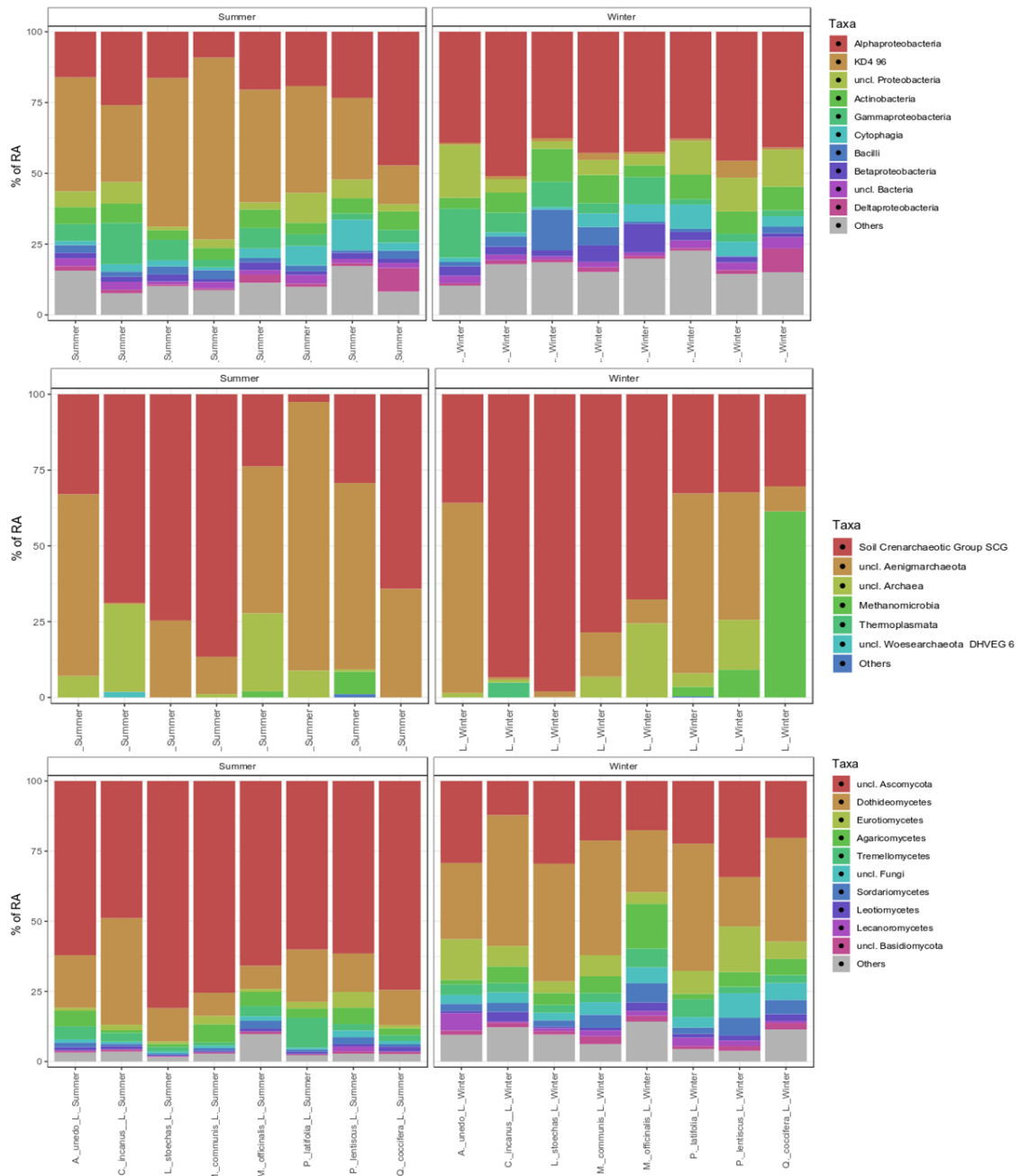


Figure 2. Stacked barplots showing the relative abundance of the main bacterial, archaeal and fungal taxa in the phyllosphere of the studied plants during summer and winter. Values are means of three biological replicates separately analyzed.

Multivariate statistical tests identified significant effects of plant host and season on the β -diversity of bacteria, archaea and fungi. RDA showed that both plant host and

season exerted significant effects ($p < 0.001$) on the bacterial and fungal community explaining 37.6% (20.7 and 16.9% attributed to plant host and season respectively) (Fig. 3a) and 53.6% (28.4 and 25.2% attributed to season and plant host respectively) respectively (Fig. 3c). Regarding archaea CCA revealed that although both main factors had a significant effect ($p < 0.001$) on the composition of the epiphytic archaeal community, plant host exerted a much stronger effect (19.3% of the variance) compared to season (3.7% of the variance) (Fig. 3b).

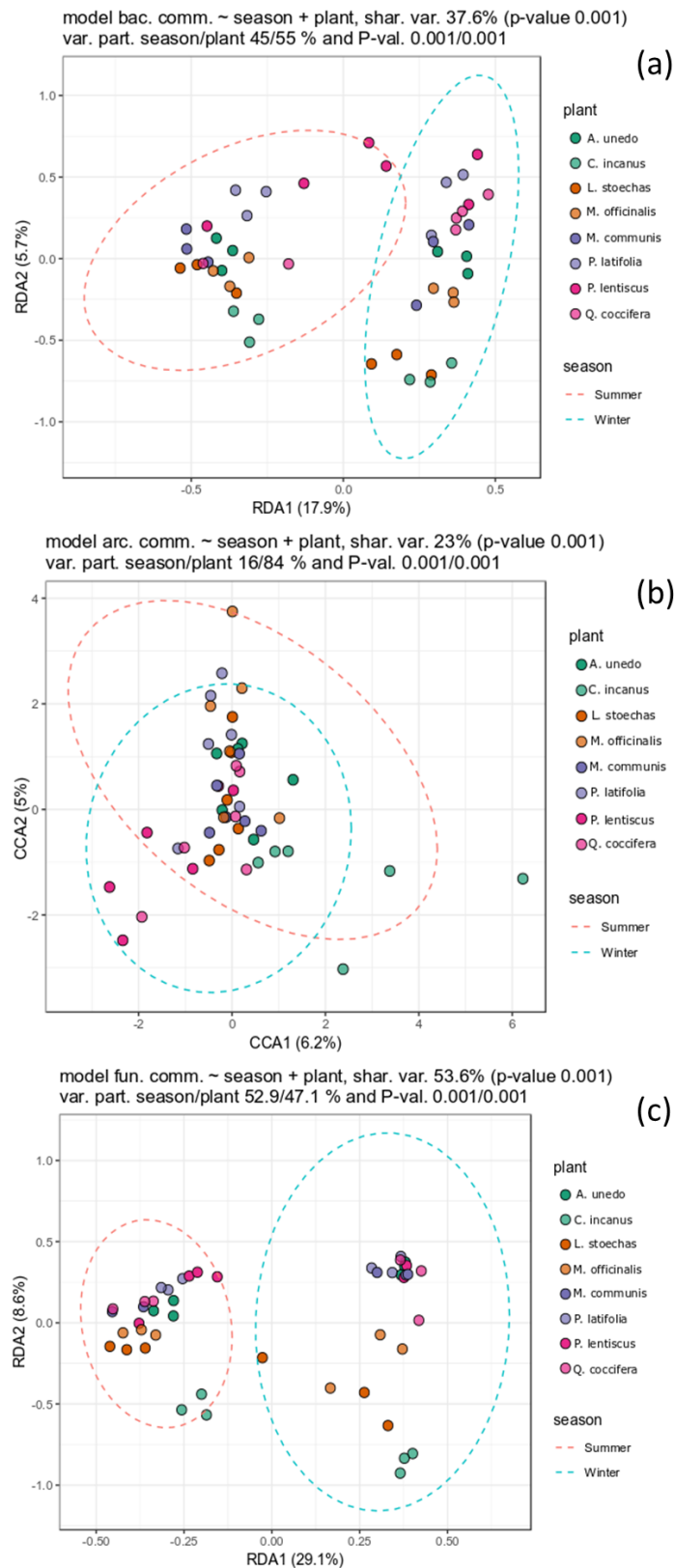


Figure 3. Canonical Correspondence Analysis (CCA) or redundancy analysis (RDA) (depending on the outcome of the first axis or detrended correspondence analysis) of the

bacterial (a), archaeal (b) and fungal (c) epiphytic community. The tested model was that of the microbial community structure being a function of the season and plant host, with the coefficient of determination providing the model shared variance and the p-value indicating the null hypothesis probability (i.e. no effect).

We further explored how epiphytic microbial communities are shaped according to plant habit (evergreen, semi-deciduous, non-woody plants) or their aromatic nature (aromatic vs non-aromatic plants). CCA analysis revealed a clear and significant ($p < 0.001$, ANOVA analysis) separation (i) only of the epiphytic fungal community between aromatic and non-aromatic plants along CCA2 (Fig. 4c), and (ii) of the bacterial, archaeal and fungal communities between evergreen, semi-deciduous and non-woody plants along CCA2 (Fig. 5). Apart from plant type effects, in all cases sampling season showed a consistent and significant effect ($p < 0.001$, ANOVA) on the composition of the bacterial, archaeal and fungal communities (Fig. 4 and 5).

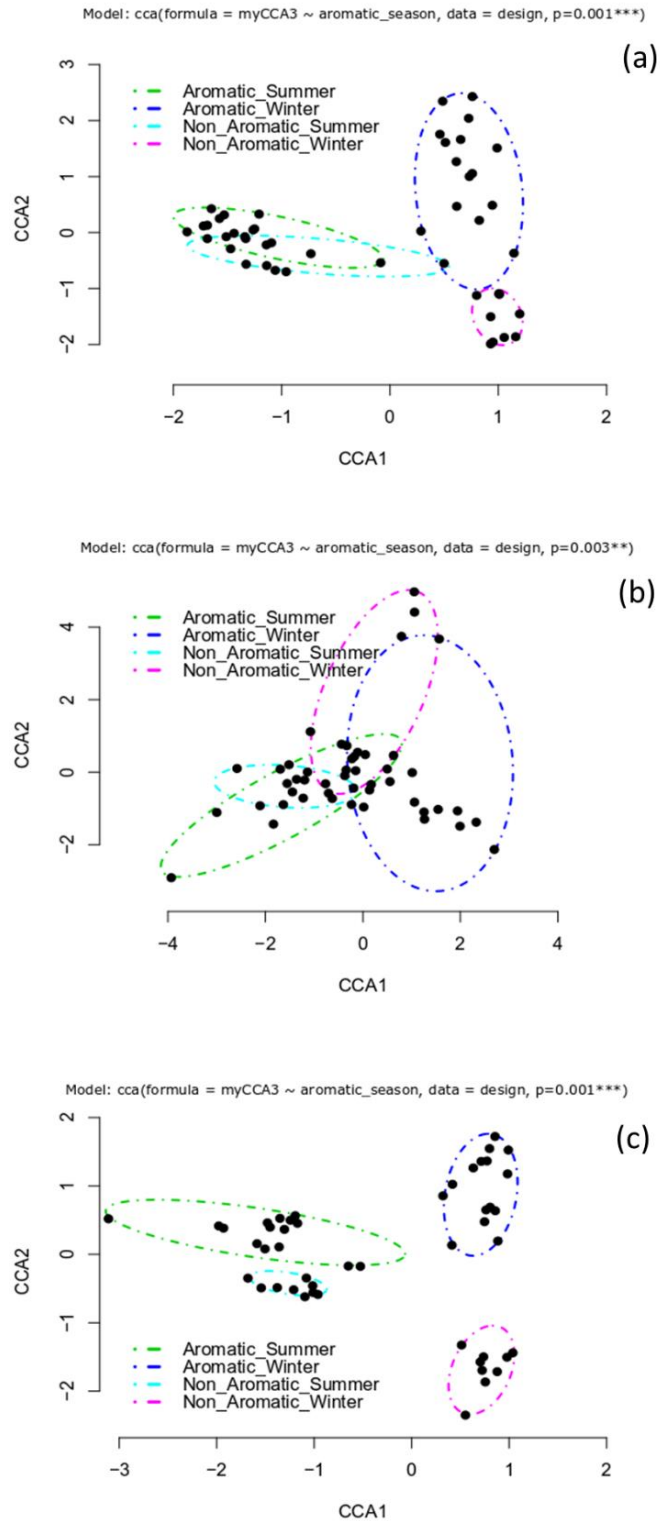


Figure 4. Canonical Correspondence Analysis (CCA) of the bacterial (a), archaeal (b) and fungal (c) community in aromatic and non aromatic plants during summer and winter.

Aromatic plants: *M. communis*, *L. stoechas*, *P. lentiscus*, *C. incanus* and *M. officinalis*;
 Non-aromatic plants: *A. unedo*, *Q. coccifera* and *P. latifolia*.

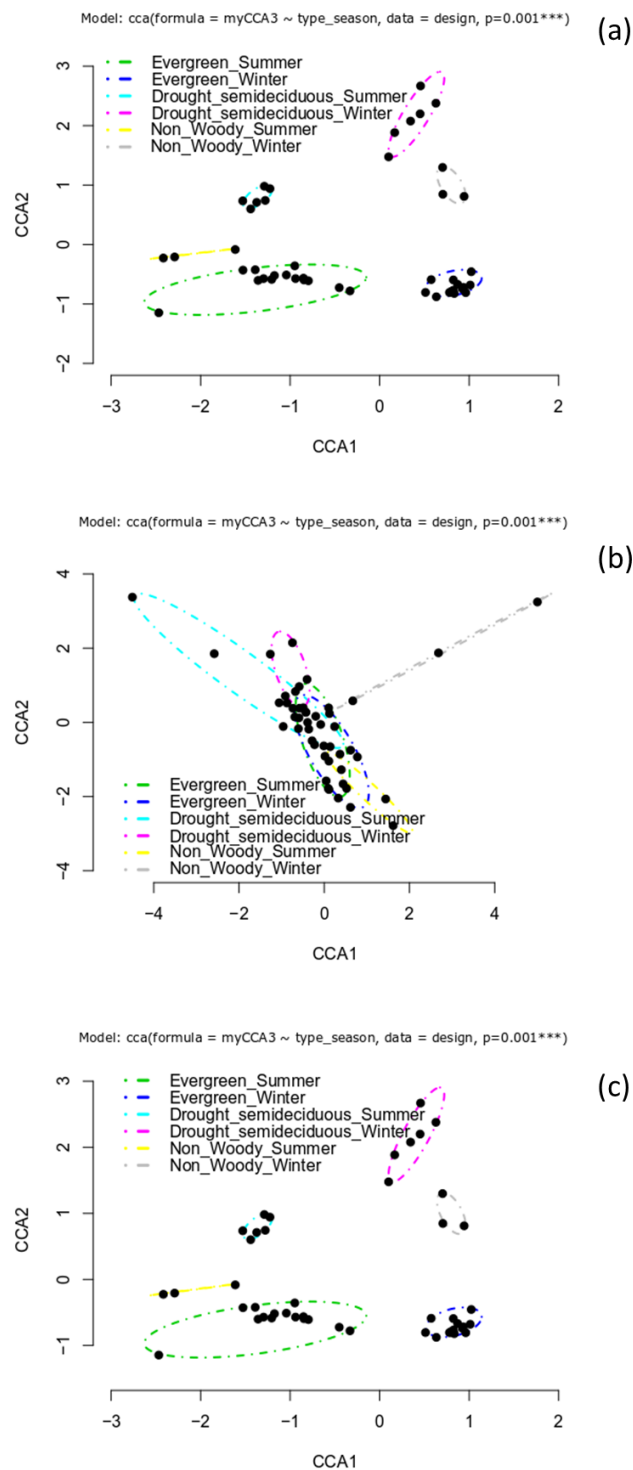


Figure 5. Canonical Correspondence Analysis (CCA) of the bacterial (a), archaeal (b) and fungal (c) community in woody evergreen, woody semi-deciduous and non-woody plants

during summer and winter. Woody evergreen plants: *A. unedo*, *M. communis*, *P. latifolia*, *P. lentiscus*, *Q. coccifera* (b) woody, semi-deciduous plants: *C. incanus* and *L. stoechas*; Non-woody: *M. officinalis*

Non-Metric Multidimensional Scaling (NMDS) analysis identified key OTUs, which were associated with particular plant - season combinations (Fig. 6). Furthermore, a heatmap presents the relative abundance of OTUs with relative abundance higher than 2% in the different plants in the two seasons (Supplementary Fig. S6). OTU 1 belonging to KD4-96 *Chloroflexi* (family *Anaerolinaceae*) became dominant in all studied plants in the summer. Several other bacterial OTUs were associated with certain plants in the summer season like (i) OTU49 belonging to proteobacteria which was associated with *C. incanus* (ii) OTU22 and OTU24, both belonging to *Afipia* sp., which were associated with *Q. coccifera* (Fig. 6a and Supplementary Fig. S6a). Conversely, several OTUs were associated with certain plant hosts in the winter like: (i) OTUs 11 and 14 belonging to *Sphingomonas* associated with the phyllosphere of *C. incanus*, *M. officinalis* and *A. unedo* (ii) OTUs 26 and 27 belonging to *Rhizobiales* associated with *C. incanus* and *L. stoechas* (iii) OTUs 38, 41, 80 and OTU 19, all belonging to *Methylobacterium*, associated with *M. officinalis* and *Q. coccifera* respectively (Fig. 6a, Supplementary Fig. S6). Regarding archaea OTUs 1621 and 1546, belonging to *Aenigmarcheota*, were associated with *P. lentiscus* regardless of the season and OTUs 1887 and 1987 assigned to *Methanomicrobia* were associated with *Q. coccifera* in the winter (Fig 6b, Supplementary Fig. 6b). NMDS analysis of the fungal community revealed that the relative abundance of OTUs 9, 26 and 28, belonging to the order *Capnodiales*, were favored in the winter samples collected from *C. incanus* (Fig. 6c). In contrast during summer OTUs 1 and 2 belonging to Ascomycota were dominant in all plants studied (Fig. 6c). Furthermore OTUs 24 and 29 belonging to the family *Venturiaceae* were associated with *P. latifolia* in the winter, while OTU 21

belonging to *Pezizomycetes*, was closely associated with *M. officinalis* regardless of the season (Supplementary Fig. 6).

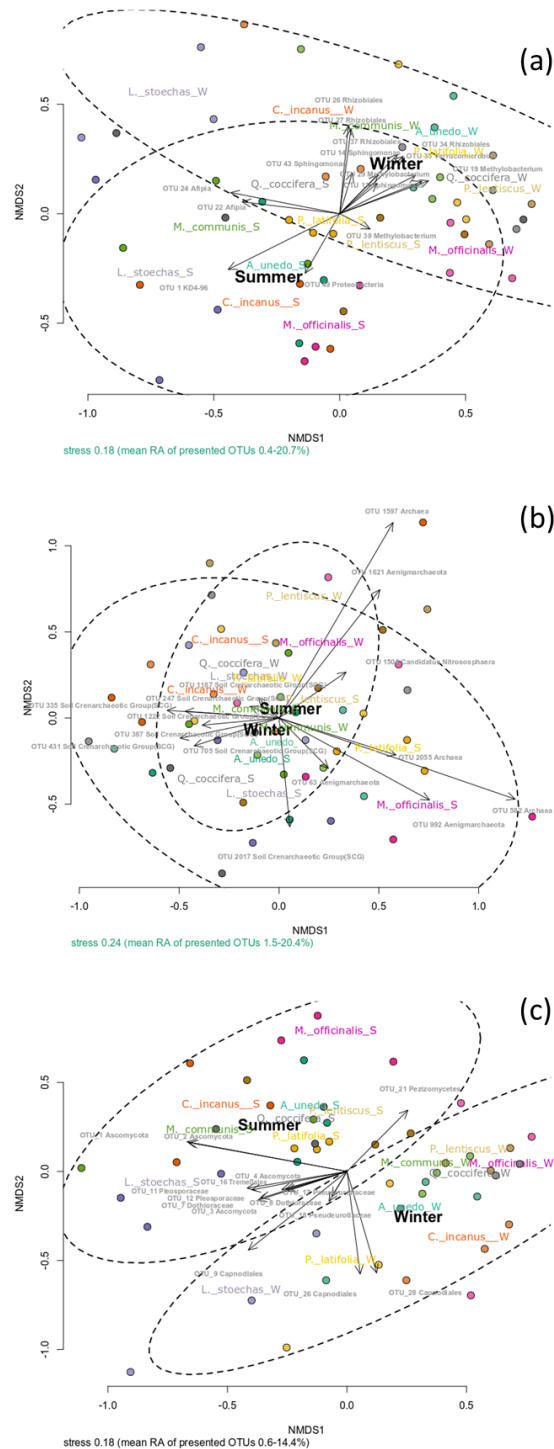


Figure 6. Non-metric Multi Dimensional Scaling (NMDS) analysis of the bacterial (a), archaeal (b) and fungal (c) microbial communities. The tested model was that of the

community structure being a function of the season (S for summer, W for winter) and the plant host, with the coefficient of determination providing the model shared variance and the p-value indicating the null hypothesis probability (i.e. no effect). Arrows indicate the OTU gradients among samples as linearly regressed to the sample scores (i.e. OTUs are more abundant in the samples of their arrow directions).

2.4. Discussion

We studied the effects of season and plant host on the abundance and diversity of bacteria, archaea and fungi colonizing the phyllosphere of a range of perennial plants native to a typical semi-arid Mediterranean ecosystem. Season appeared as the stronger determinant of the abundance of both prokaryotic and eukaryotic microbial groups studied. Epiphytic bacterial abundance was higher in winter, in line with several previous studies (Maignien et al. 2014; Peñuelas et al. 2012; Rastogi et al. 2012). This result could be attributed to the higher water content of the plant leaves during winter which exert a strong positive effect on bacterial abundance in semi-arid ecosystems like the one studied (Yadav et al. 2005). In the same ecosystem Yadav et al. (2004) showed, via plate counting, higher bacterial counts on plant leaves during the winter season. On the contrary Crenarchaea, fungi, *Alternaria* and *Cladosporium* exhibited a reversed seasonal pattern with higher abundance in the summer. Inácio et al. (2002) also found, in a similar semi-arid ecosystem in Portugal composed of *Quercus*, *Cistus* and *P. lentiscus*, that epiphytic fungal populations increased gradually from March to November. Similarly, Osono and Mori (2005) noted a gradual increase in fungal abundance on the phyllosphere of *Fagus crenata* as the growing season progressed. The seasonal effect on the abundance of Crenarchaea is reported for the first time and could be attributed to ecophysiological traits of this microbial group, which enable them to flourish under extreme conditions (Reed et al. 2013), or exploitation of new niches which were previously occupied by bacteria. Crenarchaeal abundance was

folds lower compared to the bacterial abundance. Similarly Taffner et al. (2019) studied the archaeome in phyllosphere, rhizosphere and bulk soil and reported that the former was the habitat with the lowest functional hits. Furthermore, Thapa et al. (2017) reported a folds lower abundance of archaea in the phyllosphere of different rice cultivars.

We did not observe a strong and consistent plant host effect on the abundance of the epiphytic microbial communities. We further explored possible effects of plant traits like the aromatic nature and plant habit on epiphytic microbial abundance. The aromatic nature of plants did not affect the abundance of bacteria, crenarchaea and fungi, except *Cladosporia* which showed a significantly lower abundance in aromatic plants. This is not surprising considering the well documented toxicity of essential oils produced by aromatic plants like *M. officinalis* (Menezes et al. 2015, 2016) and *M. communis* (Kordali et al. 2016) on *Cladosporium* sp. On the other hand, plant habit had a more clear effect on microbial abundance with enriched Crenarchaea in *M. officinalis* (non-woody plant) compared to woody plants, in contrast to *Cladosporia* which showed the opposite response (enriched in woody plants). This is probably a function of the different structural and chemical features of the leaves in evergreen woody and non-woody shrubs; the former characterized by thick leaves and mesophyll, low water and phosphorus contents, and absence of trichomes compared to the latter which are characterized by high nitrogen, phosphorus, and water contents (Yadav et al. 2005). Overall, our results contrast findings of Yadav et al. (2005) who reported in the same ecosystem, using a plate counting approach, a significantly higher bacterial abundance in aromatic plants and in non-woody shrubs. This discrepancy could be attributed to the different methodological approaches used in the two studies (q-PCR vs plate counting) and slight but significant differences in the plants studied (*Calamintha nepeta* studied by Yadav et al. (2004, 2005, 2008) is a non-woody shrub which was the second most colonized plant).

The epiphytic bacterial community was dominated by α -proteobacteria, especially of the orders *Rhizobiales* and *Sphingomonadales*, in line with several previous studies (Delmotte et al. 2009; Fierer et al. 2011; Grady et al. 2019; Kembel and Mueller 2014; Laforest-Lapointe et al. 2016b; Redford et al. 2010; Toju et al. 2018). The dominance of bacteria of these orders, i.e. *Methylobacterium* sp. and *Sphingomonas* sp., has been associated with key functional attributes like methanol consumption, capacity for anoxygenic aerobic photosynthesis, utilization of organosulfonic compound, assimilation of amino acids and dicarboxylates, increasing presence of porins for rapid transportation of sugars and other carbon sources (Delmotte et al. 2009; Knief et al. 2012; Müller et al. 2016), all favoring their epiphytic fitness. An interesting feature of the epiphytic bacterial community was the strong seasonal pattern of the *Chloroflexi*, belonging to the family *Anaerolineaceae*, which are common dwellers of the phyllosphere (Copeland et al. 2015; Knief et al. 2012; Ottesen et al. 2016). This family encompasses obligate anaerobic bacteria known to degrade low molecular weight alkanes (Liang et al. 2015; Savage et al. 2010), which are major components of the plant epicuticular waxes (Aragón et al. 2017). The presence of anaerobic bacteria on the phyllosphere is not uncommon (Gargallo-Garriga et al. 2016) and is probably associated with prevalence of oxygen limiting conditions on micro-sites of the spatial heterogenous leaf surface supporting the degradation of alkane components of epicuticular waxes.

The epiphytic fungal community in the studied ecosystem was dominated by Ascomycetes and Basidiomycetes at a lower frequency (Jumpponen et al. 2010; Perazzolli et al. 2014). Uncultured Ascomycota dominated the plant phyllosphere in the summer, but they were displaced partially by members of the orders *Capnodiales* and *Pleosporales* in the winter. These orders encompass fungi belonging to *Cladosporium* and *Alternaria*. *Aureobasidium* (order *Dothideales*) was also abundant on the phyllosphere of

all plants at both seasons. Members of these genera are typical epiphytic fungi colonizing the phyllosphere of all plants (Hunter et al. 2015; Jumpponen et al. 2010; Sapkota et al. 2015).

The archaeal epiphytic community on native plants of the semi-arid Mediterranean ecosystem were colonized by SCG and *Aenigmarchaeota*, both showing plant host specificity. The former has been reported as dominant in the phyllosphere of *Eruca sativa* (Taffner et al. 2019), while the presence of members of the new lineage of *Aenigmarchaeota* on the plant phyllosphere is reported for the first time. SCG dominated the phyllosphere of *C. incanus*, *L. stoechas* and *M. communis*, whereas the *Aenigmarchaeota* dominated *A. unedus*, *P. latifolia*, *P. lentiscus*. The first plant group encompass semi-deciduous seasonally dimorphic (*C. incanus*, *L. stoechas*) and aromatic plants (*M. communis*, *L. stoechas*), whereas the second includes sclerophyllous evergreen plants whose leaves exhibit different chemical and structural attributes (Yadav et al. 2005) that might select diverse archaeal phyllospheric communities.

A unique feature of the archaeal epiphytic community was the dominance of *Methanomicrobia* on the phyllosphere of *Q. coccifera* in the winter. *Methanomicrobia* encompass anaerobic methanogenic archaea which could survive under a wide range of environmental conditions (Taubner et al. 2015) including plant phyllosphere, where they have been detected before (Knief et al. 2012; Taffner et al. 2018). Beyond the unique assembly of the archaeal community, *Q. coccifera* supported an equally unique assembly of bacteria which was largely stable across seasons and characterized by the consistent presence of δ -proteobacteria. This proteobacterial class encompass anaerobic sulfate- (*Desulfovibrio*), sulfur- (*Desulfuromonas*) (Devereux et al. 1990) and iron-reducing bacteria (i.e *Anaeromixobacter*), aerobic nitrite oxidizers (i.e. *Nitrospira*) (Koch et al. 2015) and predatory bacteria (*Bdellovibrio* and *Mixococcus*) (Im et al. 2018; Reichenbach

1999). The presence of δ -proteobacteria on the phyllosphere has been reported before (Bragina et al. 2012; Redford et al. 2010; Sagaram et al. 2009). Members of the genus *Bdellovibrio* and the order *Myxococcales* participated at low relative abundance (<1%) in the core microbiome of Quebec temperate forest (Laforest-Lapointe et al. 2016b), while Miura et al. (2019) monitored the presence of *Bdellovibrio* and *Anaeromixobacter* on the leaves of conventionally cultivated vines. The concurrent presence of *Methanomicrobia* and δ -proteobacteria suggest the prevalence of anaerobic microsites on the phyllosphere of *Q. coccifera* where these microorganisms could thrive, as also suggested by Taffner et al. (2018). Among the plants studied *Q. coccifera* is characterized by high thickness of leaves and mesophyll, limited availability of nutrients and water, high phenolics content and absence of trichomes (Yadav et al. 2005). These features might promote the development of micro-anaerobic conditions on its phyllosphere favouring the proliferation of methanogenic archaea and anaerobic δ -proteobacteria.

Multivariate statistical analysis showed that plant genotype and season had an equivalent contribution in shaping the epiphytic bacterial and fungal community. The strong filtering effect of plant species on the epiphytic bacterial (Laforest-Lapointe et al. 2017a, 2017b; Redford et al. 2010; Wassermann et al. 2017) and fungal communities (Qian et al. 2018; Sapkota et al. 2015; Yao et al. 2019) is well documented and has been attributed to different ecological strategies, functional and chemical traits of the plant hosts (Kembel and Mueller 2014; Laforest-Lapointe et al. 2016b). Bacterial and fungal diversity on plant phyllosphere are dynamic in time although clear seasonal patterns are less well studied. For example Agler et al. (2016) and Laforest-Lapointe et al. (2016b) identified sampling time as the less significant variable in shaping the epiphytic bacterial and fungal community in *Arabidopsis* and in five plants in a temperate forest respectively. These studies looked at the temporal dynamics of the epiphytic communities at a rather

short scale (90 d), in contrast to our study which determined seasonal effects on two distinct seasons with clearly contrasting climatic conditions, hence expected to magnify possible effects on the epiphytic bacterial and fungal communities. Similarly, Gomes et al. (2018) observed a strong seasonal effect on the composition of the epiphytic fungal community on olives (Spring vs Autumn), and Jackson and Denney (2011) reported distinct seasonal patterns on the epiphytic bacterial community of *Magnolia grandiflora* plants with August samples carrying the most diverse community.

When plant traits were explored as a further mechanism shaping epiphytic microbial communities in this Mediterranean ecosystem, we observed that the plants aromatic nature exerted a significant effect only on the epiphytic fungal community. The potential effects of essential oils produced by aromatic plants on epiphytic fungi might be a plausible explanation for these results. Essential oils are known to exert high *in vitro* toxicity to bacteria and fungi (Hammer et al. 1999; Kadoglidou et al. 2011), although *in situ* their antimicrobial activity is less pronounced. Previous studies have suggested that essential oils could shift microbial balance in soil from fungi to bacteria (Vokou et al. 1984), in line with the selective effect of aromatic plants on the composition of the epiphytic fungal community. However further studies, this time focusing on the phyllosphere of aromatic plants are required to shed light into the filtering mechanism on epiphytic fungi. On the other hand, plant habit exerted a strong effect on the composition of all microbial communities grouping to evergreen, semi-deciduous and non-woody plants. Our findings are in agreement with Vokou et al. (2012) who observed, in the same ecosystem using DGGE analysis, a grouping of the epiphytic bacterial communities based on plant habit but not according to their aromatic character.

We further identified bacteria and fungi associated with certain plant hosts and seasons. *Methylobacteria*, *Rhizobiales* and *Sphingomonas*, all constituting typical

epiphytic bacteria (Delmotte et al. 2009; Grady et al. 2019; Knief et al. 2012; Ryffel et al. 2015) were negatively associated with the phyllosphere of woody semi-deciduous (*C. incanus*, *L. stoechas*), *M. officinalis* and *Q. coccifera* in the summer. In line with our findings Aydogan et al. (2018) observed a reduction in the abundance of *Sphingomonas* and *Rhizobium* on the phyllosphere of the herbaceous plant *Gallium album* upon exposure to warming conditions simulating a climate change scenario. Regarding fungi, we noticed an enrichment of *Capnodiales* in *C. incanus* during the winter season. *Capnodiales* encompass typical epiphytic fungi like *Cladosporium* sp., *Toxicocladosporium* sp. which exhibit tolerance to environmental conditions commonly encountered on the plant phyllosphere like high solar irradiation, osmotic stress and fluctuating water availability (Egidi et al. 2014), hence their epiphytic fitness (Qian et al. 2018). Gomes et al. (2018) also reported a strong seasonal pattern in the composition of epiphytic fungi on olive leaves with *Davidiellaceae* (i.e. *Cladosporium*) dominating in the spring.

In contrast to the the bacterial and fungal communities, plant host was the main determinant of the archaeal epiphytic community with season having a much weaker effect. Little is known regarding the factors shaping the archaeal epiphytic community. In a pioneering study Taffner et al. (2019) suggested that archaea are habitat-specific colonizers with their communities differing between phyllosphere, rhizosphere and bare soil, whereas the effect of plant-host has not been studied in a consistent manner. We provide first evidence that the archaeal epiphytic community is driven strongly by the plant host and less by seasonal variation in the studied Mediterranean ecosystem.

2.5. Conclusions

Semi-arid Mediterranean ecosystems support a unique plant community encompassing woody evergreen and semi-deciduous shrubs and non woody species with variable chemical and functional attributes, which are exposed to contrasting climatic conditions (summer vs winter). We report here that the native plants on these ecosystems support diverse bacterial, fungal and archaeal communities on their phyllosphere whose abundance vary seasonally, and their composition is shaped by both the plant host and the season, with the exception of archaea whose epiphytic community showed strong plant host patterns. Plant habit was a stronger determinant of the composition of the epiphytic microbial communities compared to plants aromatic character. *Q. coccifera* was the sole plant that exhibited strong filtering effects supporting a quite distinct bacterial and archaeal community with limited seasonal fluctuations for the former and large seasonal variations in the latter microbial domain. Our study provides the first comprehensive and in-depth analysis of the factors shaping the epiphytic prokaryotic and fungal communities in a semi-arid Mediterranean ecosystem.

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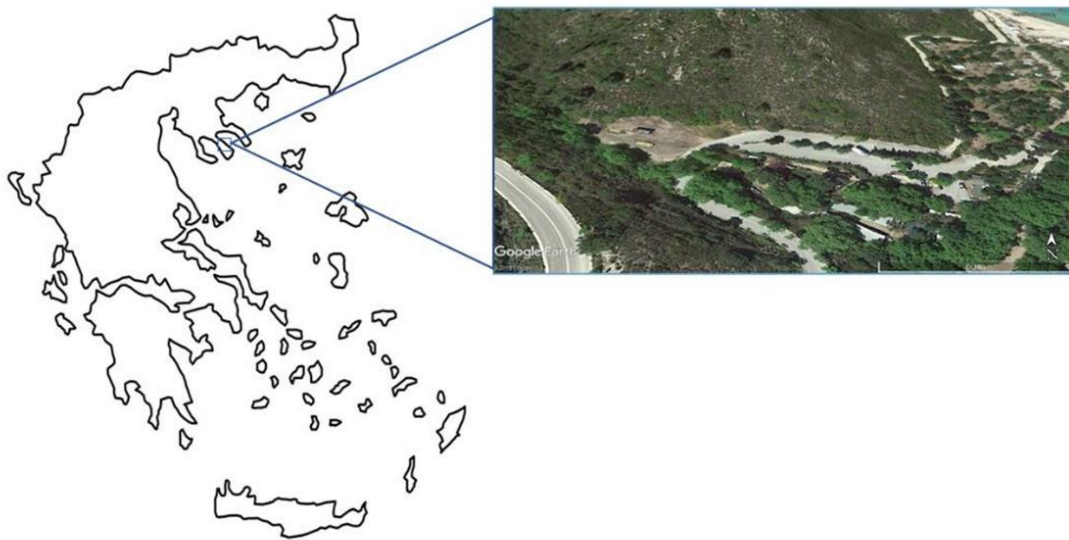
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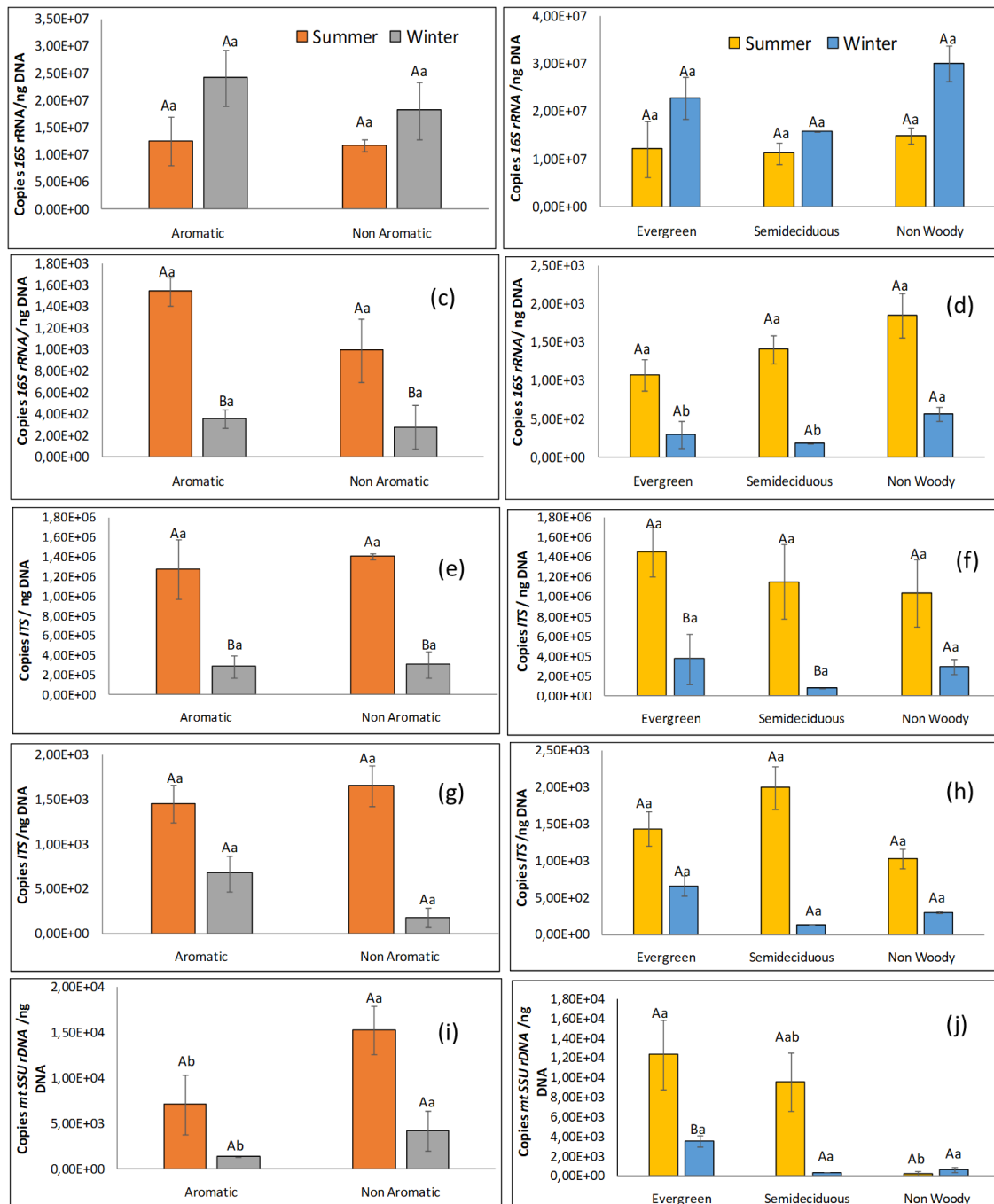
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2.7. Supplementary Data

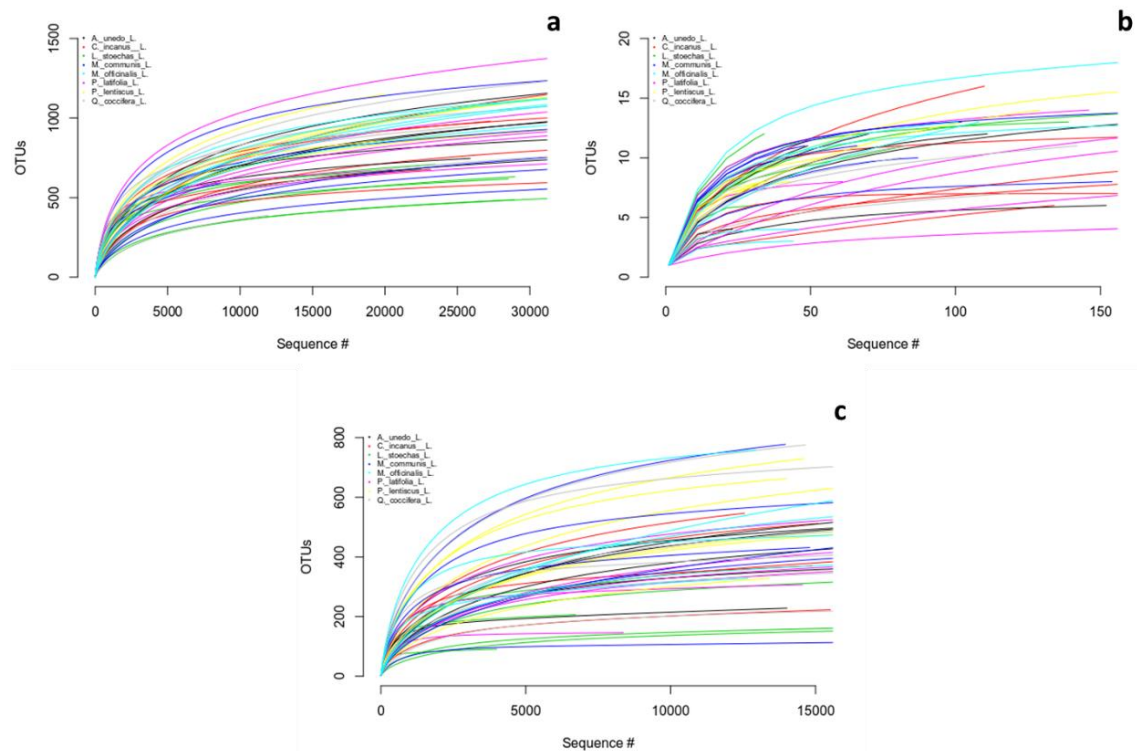
Chapter 2 - Season or Plant species: Which factor shapes the epiphytic bacterial, archaeal and fungal community in a typical semi-arid Mediterranean ecosystem?



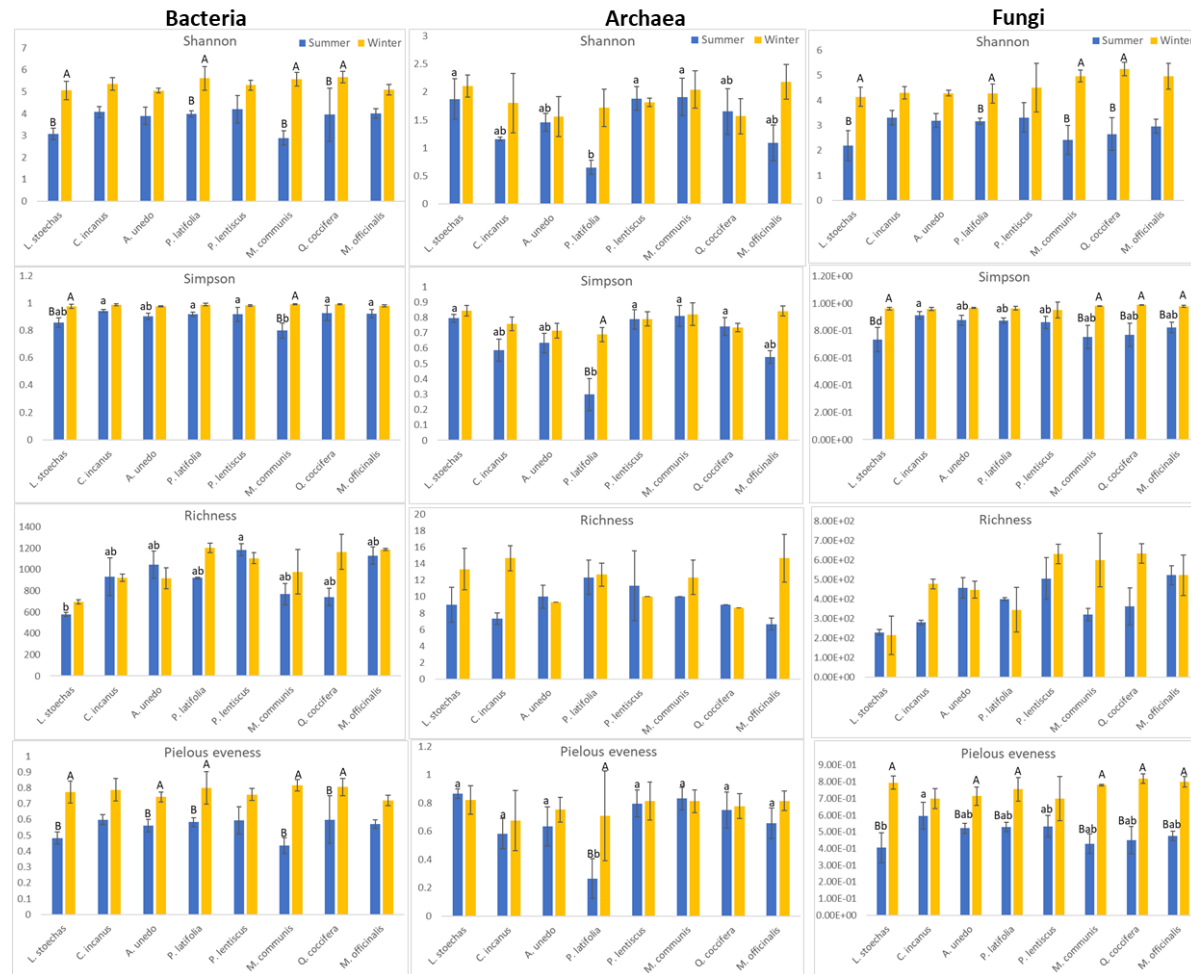
Supplementary Fig. S1. The location and a view of the studied ecosystem



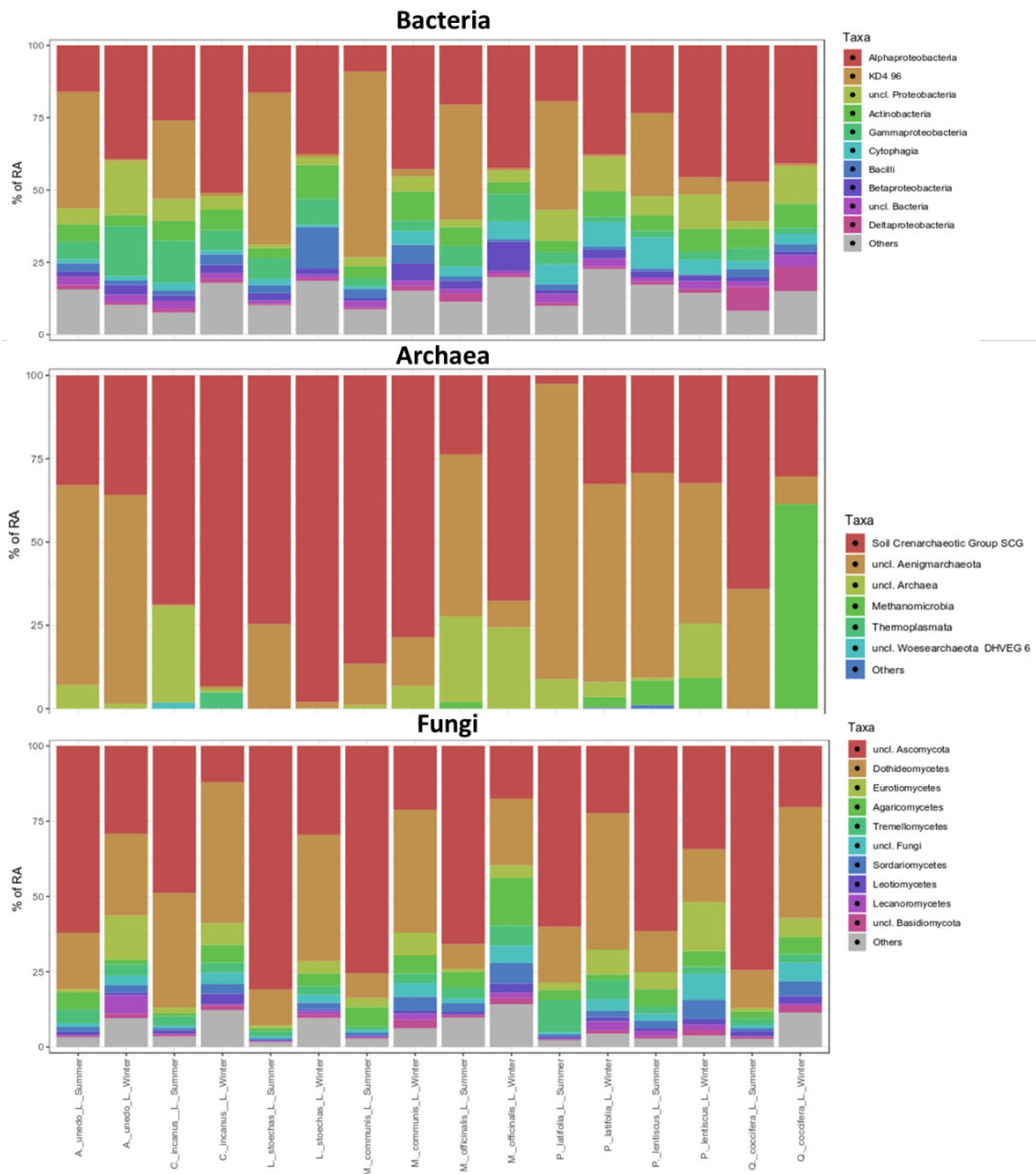
Supplementary Fig. S2. The abundance of epiphytic bacteria (a and b), Crenarchaea (c and d), total fungi (e and f), *Alternaria* sp. (g and h) and *Cladosporium* sp. (i and j) in the studied plants grouped according to their aromatic character (aromatic vs non-aromatic) and plant habit (evergreen woody, semi-deciduous woody, non woody). Capital letters above bars indicate significant differences between seasons in each studied plant group, while lower case letters indicate significant differences between plant groups within each season. Each bar is the mean of three replicates \pm the standard deviation.



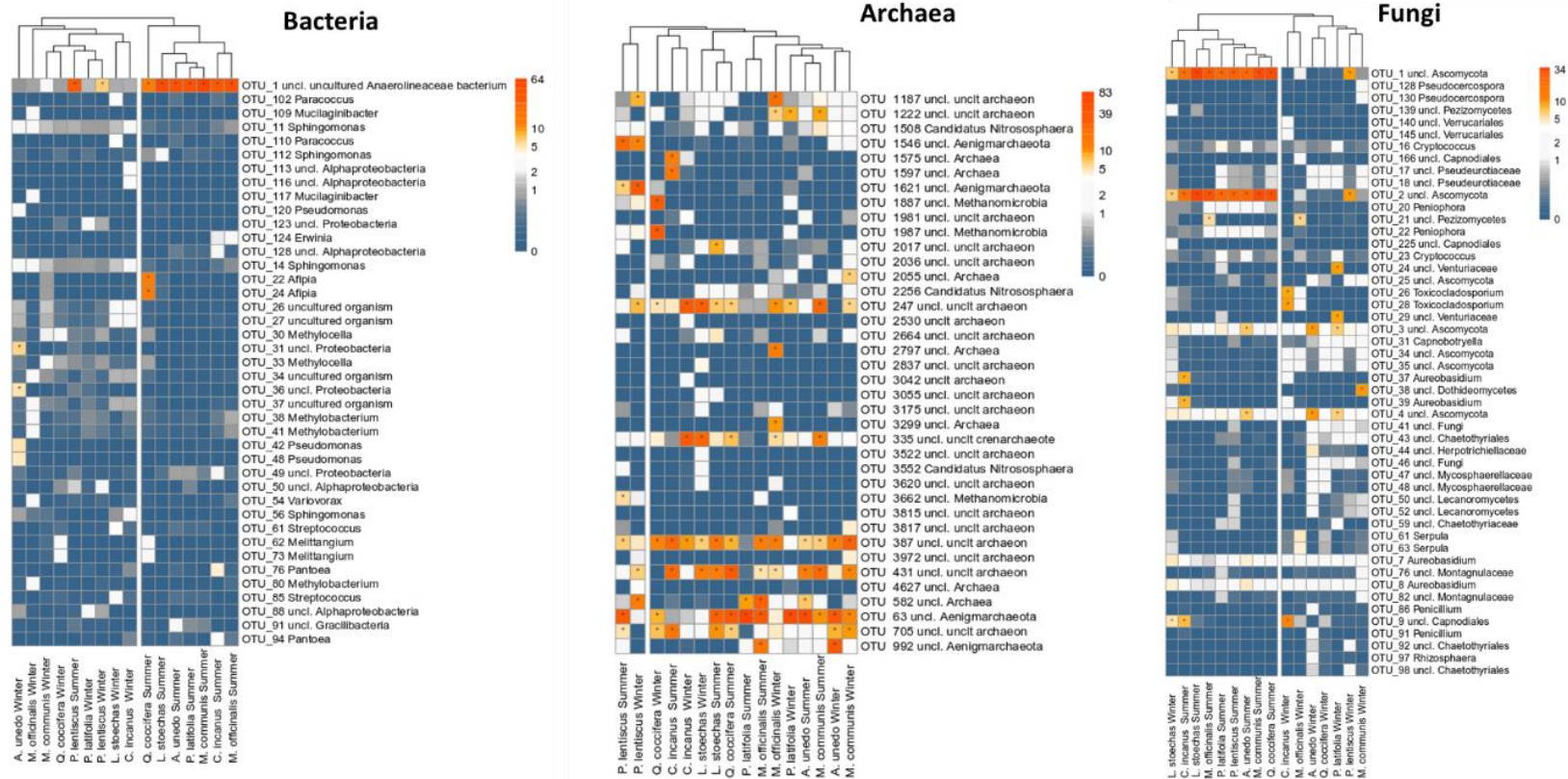
Supplementary Fig. S3. Rarefaction curves denoting the diversity coverage obtained by our sequencing effort for the bacterial (a), archaeal (b.) and fungal (c.) epiphytic community



Supplementary Fig. S4. The impact of season and plant-host on the α -diversity indices Shannon, Simpson, Richness and Pielou's evenness calculated for bacteria, archaea and fungi in the phyllosphere of the studied plants. Significant seasonal effects within each plant are denoted with capital letters, whereas significant plant host effect within each season are denoted with lower case letters (level of significance <0.05).



Supplementary Fig. S5. Stacked barplots showing the relative abundance (RA) of the main bacterial, archaeal and fungal taxa in the phyllosphere of each studied plant in summer and winter.



Supplementary Figure S6. Heatmaps presenting the dominant bacterial, archaeal and fungal OTUs (relative abundance (RA) >2%) and their association with plant hosts in the different seasons. The data are clustered in log10 scale and the legend scaling represents the percentage of RA. Asterisks indicate OTUs that showed higher than 5% RA.

Supplementary Table S1. Primers and conditions used for q-PCR determination of the abundance of bacteria, Crenarchaea, fungi, *Cladosporium* and *Alternaria*.

^a In all cases a melting curve of 95°C for 1 min, 60°C for 30 sec and a final step of 95°C for 30 sec was implemented to evaluate the specificity of the product formed.

Microbial Group	Primers	Gene target	Amplicon size (bp)	Primers sequences (5'-3')	Thermocycling conditions^a
Bacteria	338f-518r	16S rRNA	180	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	Initial denaturation at 95°C for 3 min, denaturation at 95°C for 15 sec, and annealing at 62°C for 20 sec (35 cycles)
Crenarchaea	771f-957R	16S rRNA	186	ACGGTGAGGGATGAAAGCT CGGCGTTGACTCCATTG	Initial denaturation at 95°C for 3 min, denaturation at 95°C for 3 sec, annealing at 55°C for 30 sec and an 120xtension at 72°C for 11 sec (35 cycles)
Fungi	ITS3F-ITS4R	ITS	336	GCATCGATGAAGAACGCAGC TCCTCCGCTTATTGATATGC	Initial denaturation at 95°C for 3 min, denaturation at 95°C for 3 sec, annealing at 53°C for 20 sec and

					an 121xtension at 72°C for 11 sec (36 cycles)
<i>Cladosporium</i>	Clado-SYBR-PF – Clado-SYBR- PR	mt SSU rRNA	110	TACTCCAATGGTTCTAATATTTTCCTCTC GGGTACTCAGACAGTATTTCTAGCCT	Initial denaturation at 95°C for 3 min, denaturation at 95°C for 15 sec, annealing at 68°C for 30 sec and an 121xtension at 72°C for 11 sec (40 cycles)
<i>Alternaria</i>	Dir1ITSAlt- Inv1ITSAlt	ITS	370	CGACTTGTGCTGCGCTC TGTCTTTTGCCTACTTCTTGTTTCCT	Initial denaturation at 95°C for 3 min, denaturation at 95°C for 10 sec, annealing at 60°C for 1 min (35 cycles)

Supplementary Table S2. The primers used in the current study. B000X-515f and FI000X-ITS4r are indexed primers used in the second amplification step which are composed of the sequence of the universal primers 515f (bacteria, archaea) and ITS4r (fungi) (bold), the indexes used for samples barcoding (underlined) and a TT sequence at the 5' end of each primer.

Primers	Sequence (5'-3')	Gene target	Fragment Length (bp)	Reference
Bacteria and Archaea				
515f	GTGYCAGCMGCCGCGGTAA	16S rRNA	290	Caporaso <i>et al.</i> , (2012)
806r	GGACTACNVGGGTWTCTAAT			Walters <i>et al.</i> , (2015)
B0001-515f	<u>TTCTTCTTCGT</u> GTGYCAGCMGCCGCGGTAA			This study
B0002-515f	<u>TTCTCAATGGT</u> GTGYCAGCMGCCGCGGTAA			
B0003-515f	<u>TTCAGTTCAGT</u> GTGYCAGCMGCCGCGGTAA			
B0004-515f	<u>TTCGAATCAGT</u> GTGYCAGCMGCCGCGGTAA			
B0005-515f	<u>TTGTCAGGT</u> GTGYCAGCMGCCGCGGTAA			
B0006-515f	<u>TTGAAGTTCGT</u> GTGYCAGCMGCCGCGGTAA			
B0007-515f	<u>TTGCAACAAGT</u> GTGYCAGCMGCCGCGGTAA			
B0008-515f	<u>TTGGACGACGT</u> GTGYCAGCMGCCGCGGTAA			
B0009-515f	<u>TTCTTCAAGGT</u> GTGYCAGCMGCCGCGGTAA			
B0010-515f	<u>TTCTCAGAAGT</u> GTGYCAGCMGCCGCGGTAA			
B0011-515f	<u>TTCAGTAAGGT</u> GTGYCAGCMGCCGCGGTAA			
B0012-515f	<u>TTCGACAATGT</u> GTGYCAGCMGCCGCGGTAA			
B0013-515f	<u>TTGTCGATAGT</u> GTGYCAGCMGCCGCGGTAA			
B0014-515f	<u>TTGAAGGAAGT</u> GTGYCAGCMGCCGCGGTAA			
B0015-515f	<u>TTGCAGTATGT</u> GTGYCAGCMGCCGCGGTAA			
B0016-515f	<u>TATATCAGGGT</u> GTGYCAGCMGCCGCGGTAA			
B0017-515f	<u>TTCTTGTCAGT</u> GTGYCAGCMGCCGCGGTAA			
B0018-515f	<u>TTCATATGGGT</u> GTGYCAGCMGCCGCGGTAA			
B0019-515f	<u>TTCAGACTTGT</u> GTGYCAGCMGCCGCGGTAA			
B0020-515f	<u>TTCGAGCACGT</u> GTGYCAGCMGCCGCGGTAA			

B0021-515f TTGTGTATCGTGTGYCAGCMGCCGCGGTAA
B0022-515f TTGACTATGGTGTGYCAGCMGCCGCGGTAA
B0023-515f TTGCCTAGTGTGTGYCAGCMGCCGCGGTAA
B0024-515f TATATCGTCGTGTGYCAGCMGCCGCGGTAA
B0025-515f TTCTTGAGTGTGTGYCAGCMGCCGCGGTAA
B0026-515f TTCATAGTCGTGTGYCAGCMGCCGCGGTAA
B0027-515f TTCAGAGGAGTGTGYCAGCMGCCGCGGTAA
B0028-515f TTGTTTCAGAGTGTGYCAGCMGCCGCGGTAA
B0029-515f TTGTGTGAAGTGTGYCAGCMGCCGCGGTAA
B0030-515f TTGACGTGAGTGTGYCAGCMGCCGCGGTAA
B0031-515f TTGCCTCACGTGTGYCAGCMGCCGCGGTAA
B0032-515f TATATGCACGTGTGYCAGCMGCCGCGGTAA
B0033-515f TTCTTGGACGTGTGYCAGCMGCCGCGGTAA
B0034-515f TTCATCACAGTGTGYCAGCMGCCGCGGTAA
B0035-515f TTCAGCAGTGTGTGYCAGCMGCCGCGGTAA
B0036-515f TTGTTTCGTTGTGTGYCAGCMGCCGCGGTAA
B0037-515f TTGTGACTAGTGTGYCAGCMGCCGCGGTAA
B0038-515f TTGACGAATGTGTGYCAGCMGCCGCGGTAA
B0039-515f TTGCCAATCGTGTGYCAGCMGCCGCGGTAA
B0040-515f TATAACGAGGTGTGYCAGCMGCCGCGGTAA
B0041-515f TTCTATAGGGTGTGYCAGCMGCCGCGGTAA
B0042-515f TTCATCGATGTGTGYCAGCMGCCGCGGTAA
B0043-515f TTCAGCCAAGTGTGYCAGCMGCCGCGGTAA
B0044-515f TTGTTGTAGGTGTGYCAGCMGCCGCGGTAA
B0045-515f TTGTGCAATGTGTGYCAGCMGCCGCGGTAA
B0046-515f TTGAGTTGGGTGTGYCAGCMGCCGCGGTAA
B0047-515f TTGCCAGAGGTGTGYCAGCMGCCGCGGTAA
B0048-515f TATAAGTGGGTGTGYCAGCMGCCGCGGTAA

B0049-515f TTCTATCTCGTGTGYCAGCMGCCGCGGTAA

Fungi

ITS7f	GTGARTCATCGAATCTTTG	ITS	310	Ihrmark <i>et al.</i> , (2012)
ITS4r	TCCTCCGCTTATTGATATGC			White <i>et al.</i> , (1990)
FI0001-ITS4r	TTAACCTTGGATCCTCCGCTTATTGATATGC			This study
FI0002-ITS4r	TTAACCGAAGATCCTCCGCTTATTGATATGC			
FI0003-ITS4r	TTAACGACAGATCCTCCGCTTATTGATATGC			
FI0004-ITS4r	TTACTTACGGATCCTCCGCTTATTGATATGC			
FI0005-ITS4r	TTACTTGTTCGATCCTCCGCTTATTGATATGC			
FI0006-ITS4r	TTACTAGAGGATCCTCCGCTTATTGATATGC			
FI0007-ITS4r	TTACTCTGAGATCCTCCGCTTATTGATATGC			
FI0008-ITS4r	TTACTCCTTGATCCTCCGCTTATTGATATGC			
FI0009-ITS4r	TTACTGGCAGATCCTCCGCTTATTGATATGC			
FI0010-ITS4r	TTACATTGCGATCCTCCGCTTATTGATATGC			
FI0011-ITS4r	TTACAGTAGGATCCTCCGCTTATTGATATGC			
FI0012-ITS4r	TTACAGGTTGATCCTCCGCTTATTGATATGC			
FI0013-ITS4r	TTACCTAACGATCCTCCGCTTATTGATATGC			
FI0014-ITS4r	TTACCTCTAGATCCTCCGCTTATTGATATGC			
FI0015-ITS4r	TTACCTGGTGATCCTCCGCTTATTGATATGC			
FI0016-ITS4r	TTACCATCGGATCCTCCGCTTATTGATATGC			
FI0017-ITS4r	TTACCGTTCGATCCTCCGCTTATTGATATGC			
FI0018-ITS4r	TTACGTCAGGATCCTCCGCTTATTGATATGC			
FI0019-ITS4r	TTACGATACGATCCTCCGCTTATTGATATGC			
FI0020-ITS4r	TTACGACCAGATCCTCCGCTTATTGATATGC			
FI0021-ITS4r	TTACGCCGCGATCCTCCGCTTATTGATATGC			
FI0022-ITS4r	TTACGCGTAGATCCTCCGCTTATTGATATGC			
FI0023-ITS4r	TTAGTCTGGATCCTCCGCTTATTGATATGC			

FI0024-ITS4r TTAGTTGGAGATCCTCCGCTTATTGATATGC
FI0025-ITS4r TTAGTAACCGATCCTCCGCTTATTGATATGC
FI0026-ITS4r TTAGTACGTGATCCTCCGCTTATTGATATGC
FI0027-ITS4r TTAGATCCTGATCCTCCGCTTATTGATATGC
FI0028-ITS4r TTAGATGAGGATCCTCCGCTTATTGATATGC
FI0029-ITS4r TTAGACTACGATCCTCCGCTTATTGATATGC
FI0030-ITS4r TTAGACATGGATCCTCCGCTTATTGATATGC
FI0031-ITS4r TTAGAGTCAGATCCTCCGCTTATTGATATGC
FI0032-ITS4r TTAGCAGATGATCCTCCGCTTATTGATATGC
FI0033-ITS4r TTAGCCTGTGATCCTCCGCTTATTGATATGC
FI0034-ITS4r TTAGGTACAGATCCTCCGCTTATTGATATGC
FI0035-ITS4r TTAGGCGCCGATCCTCCGCTTATTGATATGC
FI0036-ITS4r TTCTTATGGGATCCTCCGCTTATTGATATGC
FI0037-ITS4r TTCTTACTCGATCCTCCGCTTATTGATATGC
FI0038-ITS4r TTCTTAGCAGATCCTCCGCTTATTGATATGC
FI0039-ITS4r TTCTTCAGTGATCCTCCGCTTATTGATATGC
FI0040-ITS4r TTCTTCGACGATCCTCCGCTTATTGATATGC
FI0041-ITS4r TTCTTGAAGGATCCTCCGCTTATTGATATGC
FI0042-ITS4r TTCTTGGTTGATCCTCCGCTTATTGATATGC
FI0043-ITS4r TTCTATTCCGATCCTCCGCTTATTGATATGC
FI0044-ITS4r TTCTATAGGGATCCTCCGCTTATTGATATGC
FI0045-ITS4r TTCTAACAGGATCCTCCGCTTATTGATATGC
FI0046-ITS4r TTCTACCGAGATCCTCCGCTTATTGATATGC
FI0047-ITS4r TTCTAGTTGGATCCTCCGCTTATTGATATGC
FI0048-ITS4r TTCTAGCCTGATCCTCCGCTTATTGATATGC
FI0049-ITS4r TTCTAGGAAGATCCTCCGCTTATTGATATGC

Supplementary Table S3. PCR reagents and thermocycling conditions used for amplicon sequencing analysis.

PCR reaction			
Reagents	Volume (μl)	Concentrations	Comments
Primer F	1	0.5 μ M	
Primer R	1	0.5 μ M	
BSA	0.4	0.4 μ g/ μ l	Added only in the first amplification step
Polymerase Q5 (2x MasterMix)	10	1x	
ddH ₂ O	5.6		
DNA	2	0.2 ng/ μ l	
Total	20		
PCR conditions			
Step	Temperature ($^{\circ}$C)	Time	Number of Cycles
Initial Denaturation	98	30 sec	
Denaturation	98	10 sec	
Annealing	50 for bacteria/ 55 for fungi	30 sec	28 in the first amplification step / 7 in the second amplification step
Extension	72	30 sec	
Final extension	72	10 min	

Supplementary Table S4. Goods coverage estimation for each sample analysed via HiSeq Illumina next generation sequencing.

Samples	Bacteria	Archaea	Fungi
<i>Lavandula stoechas</i> Summer_Rep1	0.99	1.00	1.00
<i>Lavandula stoechas</i> Summer_Rep2	1.00	0.90	1.00
<i>Lavandula stoechas</i> Summer_Rep3	1.00	0.94	1.00
<i>Lavandula stoechas</i> Winter_Rep1	1.00	1.00	1.00
<i>Lavandula stoechas</i> Winter_Rep2	0.99	0.95	0.99
<i>Lavandula stoechas</i> Winter_Rep3	1.00	0.99	1.00
<i>Cistus incanus</i> Summer_Rep1	1.00	1.00	1.00
<i>Cistus incanus</i> Summer_Rep2	0.99	0.98	1.00
<i>Cistus incanus</i> Summer_Rep3	0.99	0.99	1.00
<i>Cistus incanus</i> Winter_Rep1	0.99	1.00	0.99
<i>Cistus incanus</i> Winter_Rep2	0.99	1.00	0.99
<i>Cistus incanus</i> Winter_Rep3	0.99	0.95	1.00
<i>Arbutus unedo</i> Summer_Rep1	0.99	0.98	0.99
<i>Arbutus unedo</i> Summer_Rep2	0.99	0.99	0.99
<i>Arbutus unedo</i> Summer_Rep3	1.00	0.92	1.00
<i>Arbutus unedo</i> Winter_Rep1	1.00	0.96	1.00
<i>Arbutus unedo</i> Winter_Rep2	1.00	1.00	1.00
<i>Arbutus unedo</i> Winter_Rep3	0.99	0.98	1.00
<i>Phyllirea latifolia</i> Summer_Rep1	0.99	1.00	1.00
<i>Phyllirea latifolia</i> Summer_Rep2	1.00	0.99	1.00
<i>Phyllirea latifolia</i> Summer_Rep3	1.00	1.00	0.99
<i>Phyllirea latifolia</i> Winter_Rep1	0.99	0.99	1.00
<i>Phyllirea latifolia</i> Winter_Rep2	0.99	0.98	1.00
<i>Phyllirea latifolia</i> Winter_Rep3	1.00	0.99	1.00
<i>Pistacia lentiscus</i> Summer_Rep1	0.99	0.94	0.99
<i>Pistacia lentiscus</i> Summer_Rep2	1.00	0.96	0.99
<i>Pistacia lentiscus</i> Summer_Rep3	0.99	0.98	0.99
<i>Pistacia lentiscus</i> Winter_Rep1	0.98	0.97	0.99
<i>Pistacia lentiscus</i> Winter_Rep2	0.99	0.97	0.99
<i>Pistacia lentiscus</i> Winter_Rep3	0.99	0.98	0.99
<i>Myrtus communis</i> Summer_Rep1	1.00	0.99	1.00
<i>Myrtus communis</i> Summer_Rep2	1.00	0.97	1.00
<i>Myrtus communis</i> Summer_Rep3	1.00	0.96	0.99
<i>Myrtus communis</i> Winter_Rep1	0.99	0.99	0.99
<i>Myrtus communis</i> Winter_Rep1	0.98	0.99	0.99
<i>Myrtus communis</i> Winter_Rep1	1.00	0.99	1.00
<i>Quercus coccifera</i> Summer_Rep1	1.00	0.98	0.99

<i>Quercus coccifera</i> Summer_Rep2	0.99	0.98	1.00
<i>Quercus coccifera</i> Summer_Rep3	0.99	0.96	1.00
<i>Quercus coccifera</i> Winter_Rep1	0.99	1.00	0.99
<i>Quercus coccifera</i> Winter_Rep2	0.99	0.98	1.00
<i>Quercus coccifera</i> Winter_Rep3	0.99	0.94	0.99
<i>Melissa officinalis</i> Summer_Rep1	0.99	0.94	0.99
<i>Melissa officinalis</i> Summer_Rep2	0.99	1.00	1.00
<i>Melissa officinalis</i> Summer_Rep3	0.99	1.00	0.99
<i>Melissa officinalis</i> Winter_Rep1	0.99	0.98	0.99
<i>Melissa officinalis</i> Winter_Rep2	0.99	0.99	1.00
<i>Melissa officinalis</i> Winter_Rep3	0.99	1.00	1.00

Chapter 3

The impact of soil amendment with peppermint, spearmint and rosemary on the abundance and diversity of the soil microbiota

The work presented in Chapter 3 is included in the following article:

Katsoula A., Vasileiadis S., Karamanoli K., Vokou D., Karpouzas D.G. (2019). The impact of soil amendment with peppermint, spearmint and rosemary on the abundance and diversity of the soil microbiota. To be submitted.

3.1. Introduction

Application of organic amendments derived from various sources, including crop residues (Nguyen et al. 2016), biosolids (Paramashivam et al. 2017; Sánchez-Monedero et al. 2004) and agro-industrial wastes (Negassa et al. 2011), is a common strategy to improve soil productivity, especially in organic farming where synthetic fertilizers cannot be used. This practice increases soil organic matter and nutrient concentrations and under certain conditions could augment the capacity of soils to suppress plant pathogens (Diab et al. 2003; Veeken et al. 2005) and weeds (Efthimiadou et al. 2012). In addition to all these, soil organic amendments impose strong alterations on the soil microbiota leading to significant increases in microbial biomass and activity (Peacock et al. 2001; Hu and Gru 1999; Malik et al. 2013; Lazcano et al. 2013), attributed to the release of copious amounts of easily assimilated C sources in soil (Demoling et al. 2007).

Previous studies have proposed the use of residues of aromatic plants indigenous to the Mediterranean region as soil amendments to increase soil fertility and control soil-borne plant pathogens (Chalkos et al. 2010) and weeds (Cavalieri and Caporali 2010). The latter is attributed to the capacity of these plants to produce essential oils exhibiting high biological activity against several soil plant pathogenic fungi, bacteria (Iscan et al. 2002; Soylu et al. 2010), and weeds (Argyropoulos et al. 2008). Essential oils are a blend of low-molecular weight isoprenoid compounds with oxygenated molecules exhibiting higher anti-germinating and anti-microbial activity compared to hydrocarbons (Vokou et al. 2003). Despite that, both stimulatory and inhibitory effect on soil microbial respiration and soil microbial biomass were evident when essential oils or their individual constituents were applied in soils (Miñambres et al. 2010; Vokou et al. 1984, 2002; Vokou and Liotiri 1999). Similarly, amendment of soil with

plant residues of various aromatic plants resulted in contrasting effects depending on the aromatic plant used (Chalkos et al. 2010; Hassiotis and Dina 2010; Kadoglidou et al. 2014).

Rosemarinus officinalis (rosemary), *Mentha spicata* (spearmint) and *Mentha piperita* (peppermint) are all members of the Lamiaceae family, indigenous aromatic plants in Mediterranean ecosystems, which are rich in essential oils. These exert a multifaceted bioactivity stemming from their highly diverse chemical composition (Karamanoli et al. 2018). The main constituents of the essential oil of spearmint and peppermint are carvone and menthol (Kadoglidou et al. 2011; Radaelli et al. 2016), while 1,8-cineol and camphor are the major components of the essential oil of rosemary (Karamanoli et al. 2000, 2018; Radaelli et al. 2016). The essential oil of spearmint exhibits antifungal activity (Adam et al. 1998; Kadoglidou et al. 2011) and the essential oil of rosemary has been shown to exert strong antimicrobial (Gachkar et al. 2007; Pintore et al. 2002), bacteriostatic (Karamanoli et al. 2000) and antifungal activity (Santoyo et al. 2005). Little is known regarding the effects of soil amendment with leaf litter of these aromatic plants on the soil microbiota. Initially Chalkos et al. (2010) reported a beneficial effect of soil amendment with composted residues of spearmint on the bacterial and fungal biomass and on tomato plants growth and similar results were observed by Kadoglidou et al. (2014), this time using non composted plant material.

The main aim of this study was to explore the effects of soil amendment with residues of spearmint, peppermint and rosemary, selected based on the different chemical composition of their essential oils, on the soil microbial community using q-PCR. We determined the abundance of important bacterial taxa, crenarchaea, fungi and key functional microbial groups like ammonia-oxidizing microorganisms (AOM), sulfur-oxidizing bacteria (SOB) and bacteria involved in the catabolism of biogenic and xenobiotic aromatic compounds in soil. In this

context we further explored the hypothesis that the presence of tomato plant roots will offset, through the supply of C- and N-rich root exudates (Broeckling et al. 2008; Steinauer et al. 2016), any effects of essential oil constituents on the soil microbial community. Based on the initial findings we further explored the impact of rosemary plant residues, in the presence or absence of tomato plants, on the diversity of bacteria, archaea and fungi using amplicon sequencing analysis of the 16S rRNA and ITS respectively.

3.2. Materials and Methods

3.2.1. Plants, Soil and Soil Amendments

Peppermint, spearmint and rosemary plants were purchased from a commercial supplier. For peppermint and spearmint, the whole aboveground biomass was used, whereas for rosemary only the leafy upper part of the shoots. Plant material was cut into small pieces, air-dried in the dark to a moisture content of 5–7%, and stored in the dark at 12 °C until use. A commercially available organic fertilizer produced by decomposed organic matter (Bio-Humus) was used in the pot experiment. The physicochemical characteristics of the soil amendments used are shown in Table 1.

Table 1. The physicochemical properties of the soil amendments used in the study

Soil Amendments	Organic Matter (%)	Total N (%)	C/N	P (mg g ⁻¹)	K (µg g ⁻¹)	Mg (µg g ⁻¹)
Organic amendment	41.5±0.25	1.05±0.01	22.96±0.66	127.6±7.8	10.8±0.3	12.9±0.6
Spearmint	31.5±0.5	0.31±0.02	58.38±2.98	52.9±0.6	22.9±0.5	24.4±0.2

Peppermint	33.5±0.5	0.31±0.03	61.78±0.43	65.0±0.5	23.5±1.1	26.6±1.1
Rosemary	13.5±0.5	0.08±0.01	104.49±4.66	39.4±0.5	10.0±0.2	11.8±0.6

The soil used for the pot experiment was a silty clay loam (32% clay, 56% silt, 12% sand), with pH 7.8 and an organic matter content of 3.1%, obtained from a field left in fallow for a 10-year period. A detailed analysis of its physicochemical properties is given by Kadoglidou et al. (2014) (Supplementary Table S1).

3.2.2. Pot experiment

A pot experiment was employed in March 2014 to assess the effects of soil amendment with residues of different aromatic plants on the soil microbial community. Sixty plastic pots (2 kg) were separated into five groups of 12 pots each. The first three sets of pots were filled with a mixture of soil with 4% (w/w) of residues of peppermint, spearmint and rosemary respectively. The fourth set of pots was filled with a mixture of soil with a portion of a commercial organic fertilizer which equals to the N and P offered by the aromatic plant residues. This treatment served as a comparative treatment to the plant residue amendment treatments but without essential oils. The final 12 pots were filled with soil without any amendment to serve as non-amended controls. Six pots from each treatment were seeded with 10-15 tomato seeds (*Solanum lycopersicum*, cv EZ NOAM) which were thinned to four upon emergence. All pots were watered daily, and they were maintained at ambient temperature (16-24°C) and 45-60% humidity. To summarize there were 5 soil amendment treatments (spearmint (Ms), peppermint (Mp), rosemary (Ro), organic fertilizer (A) and non-amended (C), by two sampling times (30

and 60 days), by two planting treatments (tomato or no tomato plants), by three replicates per combination. Thirty days later the three seeded pots and three non-seeded pots per treatment were harvested (plants were removed) and the soil of the pots was homogenized and used for DNA extraction and downstream measurements as described below. From the six remaining pots per treatment, three were seeded again with tomato as described before and the other three were left unseeded and incubated as described above. Thirty days later (60 days from the start of the experiment) all pots were harvested, and the soil was used for DNA extraction and downstream activities as described below.

3.2.3. Soil DNA extraction

Upon collection soil samples were homogenized and immediately stored at -20°C until processed for DNA extraction. This was performed from 0.5 g of soil (dry weight) with the PowerSoil® DNA isolation kit (MoBio Laboratories, Inc., West Carlsbad, CA, USA) according to the manufacturer's instruction. The integrity of the extracted DNA was checked via agarose gel (0.8%) electrophoresis and it was quantified using a Qubit fluorometer with a Quant-iT HS double-stranded DNA (dsDNA) assay kit (Invitrogen, USA).

3.2.4. Determination of the abundance of selected microbial groups via q-PCR

We determined the abundance of key soil microbial taxa including total bacteria, α -, β -, γ -Proteobacteria, Actinobacteria, Firmicutes, Crenarchaea, and total fungi via q-PCR. In all cases the 16S rRNA was used a target gene for the quantification of bacterial and Crenarchaeal

abundance, while the ITS region was used for the determination of fungal abundance. The primers and thermocycling conditions used are given in Supplementary Table S2.

We further determined the abundance of key functional microbial groups in soil having a key role in N, C and S cycling. In this respect we determined the abundance of the *amoA* gene of ammonia-oxidizing bacteria (AOB) and archaea (AOA), the *soxB* gene of sulfur-oxidizing bacteria (SOB) and the *pcaH* and *catA* genes encoding protocatechuate dioxygenase and 1,2-catechol dioxygenase respectively, involved in the degradation of biogenic aromatic molecules in soil. All primers and thermocycling conditions used are shown in Supplementary Table S3.

All q-PCR measurements were performed in a Stratagene Mx3005P Real-Time PCR System. Reactions volume was 10 μ L containing 5 μ L of the KAPATaq SYBR Green® PCR master mix (Kapa Biosystems, Wilmington, Massachusetts, USA), 0.2 μ l of each primer (except 0.1 μ l for AOM and 0.5 μ l for fungi and *catA*) (20 μ M), 0.2 μ l of BSA (400 μ M) and 1 μ l of soil DNA (0.2-10 ng depending on the target group) and sterilized ddH₂O to the final volume. The abundance of each microbial group was determined with the use of standard curves constructed using serial dilutions of linearized plasmids containing the studied genes. PCR efficiency in all cases ranged between 82 and 102%.

3.2.5. Amplicon sequencing analysis of the soil microbial community

The effects of rosemary soil amendment, in the presence or absence of tomato plants, on the community of bacteria, archaea and fungi was determined by amplicon sequencing analysis of the 16S rRNA and ITS respectively via HiSeq Illumina Rapid Mode 2x250 bp paired-end in

the DNA Sequencing Center Department of Biology of the Brigham Young University, USA. Bacterial and Archaeal 16S rRNA were amplified with primers 515f-806r (290 bp) (Caporaso et al. 2012; Walters et al. 2015) following the protocol of the Earth Microbiome Project (Caporaso et al. 2018). The amplification of ITS was done with primers ITS7-ITS4 (310 bp) (White et al. 1990; Ihrmark et al. 2012) following the protocol described by Ihrmark et al. (2012). All samples were initially amplified (28 amplification cycles) using the domain-specific primers mentioned above, followed by a semi-nested PCR (7 amplification cycles) using primers carrying indexes for meta-barcoding of samples. The PCR reaction volume was 20 μl composed of 1 μl of each primer (0.5 μM), 0.4 μl of BSA (400 $\text{ng } \mu\text{l}^{-1}$) only in the first PCR step, 10 μl of Q5® High-Fidelity DNA Polymerase master mix (NEB, Ipswich, Massachusetts, USA), 2 μl of template DNA (0.2 $\text{ng } \mu\text{l}^{-1}$) and 5.6 μl of sterilized ddH₂O. Primers sequences and PCR conditions are presented in Supplementary Table S4.

Removal of PCR and sequencing artifacts, OTU matrix generation and taxonomic sequence classification were performed as follows. The raw sequence data were demultiplexed to their samples of origin with Flexbar v3.0 (Dodt et al. 2012) and they were quality controlled with Trimmomatic v0.32 (Bolger et al. 2014). The resulting high quality read pairs were assembled to the amplicon of their origin with FLASH v1.2.8 (Magoc and Salzberg 2011) using the default parameters amended to allow a maximum overlap of 250 bp and no mismatches between read-pairs. The remaining tasks were carried out with the IOTUs v1.58 perl wrapper (Hildebrand et al. 2014). OTU calling at 97% identities was performed with the UPARSE v10.0.240 software (Edgar 2013). Chimeric sequences were identified with the UCHIME v4.2 software (Edgar et al. 2011) using the RDP Gold database vMicrobiomeutil-r20110519 for bacteria and the UNITE ITS2 v985.20150311 reference database (Nilsson et al. 2015) for

fungi, while sequence classification was performed with Lambda v0.9.1 (Hauswedell et al. 2014) against the Silva v128 small ribosomal subunit database (Yilmaz et al. 2014) for bacteria and the UNITE ITS v7_99_20150302 database (Kõljalg et al. 2013) for fungi.

3.2.6. Statistical analysis

3.2.6.1. Statistical analysis of q-PCR and relative abundance data

The q-PCR and relative abundance data (as derived by the amplicon sequencing analysis) were analyzed by MANOVA to determine the effects of soil amendment, plant and sampling time. Depending on the outcome of the MANOVA, we further focused on the interactions of soil amendment with plant and/or soil amendment with sampling time, where significant differences were identified by ANOVA and posthoc tests. All analyses were performed with the SPSS Statistics 21 software (IBM corporation, New York, U.S.)

3.2.6.2. Statistical analysis of microbial diversity data

The OTU matrices of bacteria, archaea and fungi were used to assess the impact of rosemary soil amendment, in the presence or not of tomato plants, on the α - and β -diversity. The effects on the α -diversity were determined via calculation of diversity indices like richness (S), Fisher Alpha, Inverse Simpson (Jost 2006), Shannon, and Pielou's evenness (Pielou 1975). The data obtained from soil, were subjected separately to two-way ANOVA and post-hoc tests to determine the impact of soil treatment, tomato plant and sampling time on the α -diversity of bacteria, archaea and fungi.

We further assessed the effects of rosemary soil amendment in the presence or absence of tomato plants on the β -diversity of bacteria, fungi and archaea. Hence, differential abundance (DA) tests performed with the Fisher's exact test as implemented in the EdgeR package v3.24.3 (Robinson et al. 2010) for P-values of 0.05 as adjusted according to the Benjamini-Hochberg algorithm (Benjamini and Hochberg 1995) were employed to identify taxa and OTUs responsive to the soil different soil treatments. The impact of soil amendment, tomato plant and sampling time on the structures of the significantly affected member sub-communities (as determined by the DA tests) of bacteria, archaea and fungi was assessed via canonical analysis as follows. Detrended Correspondence Analysis (DCA) was performed and, depending on the first axis length value, Canonical Correspondence Analysis (CCA) was preferred over Redundancy Analysis (RDA), if this value was higher than 3 standard deviations (SD) according to a previously suggested strategy (Lepš and Šmilauer 2003). DCA first axis values greater than 3 SD imply overall unimodal responses of community member abundances against the environmental gradients (rendering the chi squared distances of CCA more suitable) as opposed to lower values which imply overall linear responses to environmental gradients (rendering the Euclidean distances of RDA more suitable). All the statistical processing of amplicon sequencing data was performed with the R v3.5.2 software (R Core Team 2017) and the packages Vegan and ggplot2. The data were submitted to Sequence Read Archive (SRA) of NCBI with bioproject accession No PRJNA556152.

3.3. Results

3.3.1. Effects of the different soil amendment on the abundance of key microbial taxa

MANOVA showed that tomato plant and soil amendment treatments and their interactions imposed significant main effects ($p < 0.001$) on the abundance of α -proteobacteria, while significant interactions between these two factors were also observed ($p < 0.001$) (Supplementary Table S5). Regardless of sampling time and in the absence of tomato plants, peppermint, spearmint and the organic fertilizer induced a significant increase in the abundance of α -proteobacteria compared to the non-amended soil (Fig 1a). This pattern was differentiated in the presence of tomato plants where soil amendment with rosemary induced a significant increase in the abundance of α -proteobacteria ($p < 0.05$) compared to the unamended samples. Time-wise, the significant stimulatory effect of peppermint on the abundance of α -proteobacteria at 30 days ($p < 0.05$) was not maintained at 60 days where rosemary seemed to impose a significant increase in the abundance of α -proteobacteria always compared to the non-amended controls (Supplementary Fig. S1a).

Regarding β -proteobacteria, MANOVA showed that all main factors (plant, soil amendment, time) ($p < 0.001$) and their interactions ($p < 0.05$) had a significant effect on their abundance (Supplementary Table S5). Regardless of time, in the absence of tomato plants soil amendment with peppermint and spearmint significantly increased the abundance of β -proteobacteria compared to the control. Similarly, to α -proteobacteria, this pattern changed in the presence of tomato plants where soil amendment with peppermint, and rosemary, showed a significantly higher abundance of β -proteobacteria compared to the control (Fig. 1b). Furthermore, we observed a significantly higher abundance of β -proteobacteria ($p < 0.05$) in the rosemary-amended samples in the presence of tomato compared to the samples with no tomato

plants. (Fig. 1b). Regarding temporal changes, the significant increase in the abundance of β -proteobacteria in the peppermint and rosemary amended samples at 30 days was still visible and extended to spearmint amended samples at 60 days (Supplementary Fig. S1b).

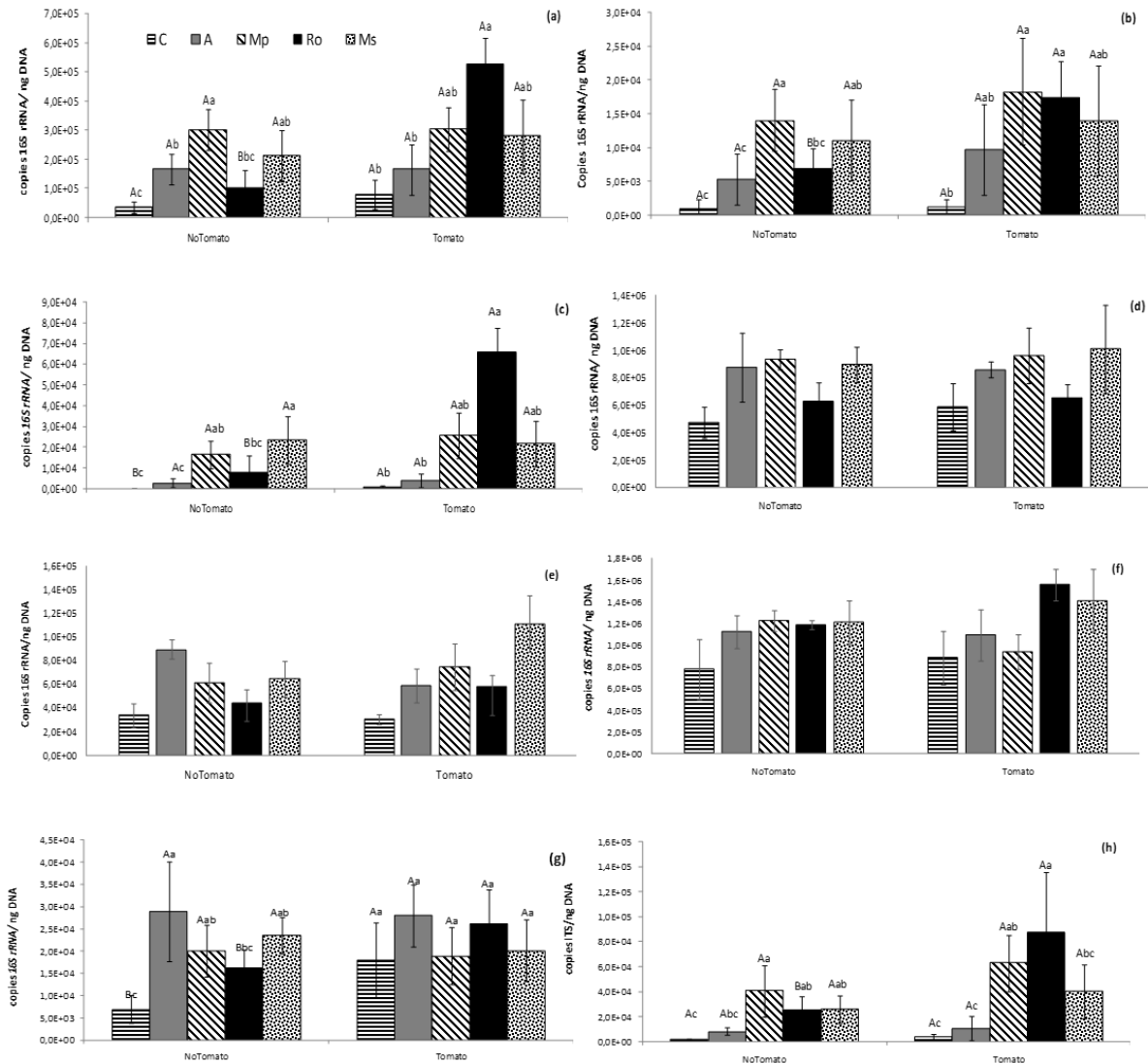


Figure 1. The abundance of α -proteobacteria (a), β -proteobacteria (b), γ -proteobacteria (c), actinobacteria (d), firmicutes (e), total bacteria (f), Crenarchaea (g) and total fungi (h) in soil samples amended with an organic fertilizer (A) or plant residues of peppermint (Mp), rosemary (Ro), spearmint (Ms) and in non-amended plants. Each value is the mean of three replicates with error bars representing the standard deviation of the mean. Within plant treatment bars designated by the same lower-case letter are not significantly different ($p < 0.05$). Whereas

within each amendment treatment bars designated by the same capital letter are not significantly different ($p < 0.05$).

Regarding γ -proteobacteria, MANOVA showed that plant and soil amendment had a significant main effect ($p < 0.001$) on their abundance with all main factors' interactions being also significant ($p < 0.05$) (Supplementary Table S5). We noticed that in the absence of tomato plants, and regardless of the sampling time, spearmint and peppermint induced a significant increase in the abundance of γ -proteobacteria (Fig. 1c). In contrast, in the presence of tomato plants we observed a significant increase in the abundance of γ -proteobacteria in the rosemary-amended samples compared to the non-amended samples and the samples amended with the organic fertilizer, but also compared to the corresponding rosemary amended samples in the absence of tomato (Fig 1c). Regarding temporal patterns, we observed an increase in the abundance of γ -proteobacteria in the samples amended with the three aromatic plants compared to the control and the samples amended with the commercial organic fertilizer (Supplementary Fig. 1c)

Soil amendment ($p < 0.001$) and sampling time ($p < 0.05$) were the only main factors that induced significant effects on the abundance of actinobacteria, while significant interactions were evident only between plant x time ($p < 0.05$) (Supplementary Table S5). Regarding soil amendment main effect, we noticed a significantly higher abundance of actinobacteria in the spearmint, peppermint and organic fertilizer amended samples compared to the unamended samples and the rosemary amended samples (Fig 1d). Significantly higher abundance of actinobacteria ($p < 0.05$) was evident at peppermint and spearmint amended samples at 30 days (Supplementary Fig. S1d). This stimulatory effect extended to the organic fertilizer amended

samples at 60 days. Regarding firmicutes, significant main effects of soil amendment ($p < 0.05$) and time ($p < 0.05$) on the abundance of firmicutes were observed, whereas there were no significant interactions between main factors (Fig. 1e)

We further looked in the abundance of total bacteria, crenarchaea and fungi. Significant main effects on the abundance of total bacteria were induced only by soil amendment ($p < 0.001$), while significant interactions ($p < 0.05$) were only observed between plant and time (Supplementary Table S5). Regardless of time no significant effects of soil amendment on the abundance of total bacteria in the presence or absence of tomato plants were observed (Fig. 1f). On the temporal basis, rosemary soil amendment induced a significant ($p < 0.05$) increase in the abundance of total bacteria compared to peppermint and the non-amended samples, whereas this effect did not persist at day 60 (Supplementary Fig. S1f).

Regarding Crenarchaea soil amendment was the sole main factor that had a significant effect ($p < 0.001$) on their abundance, while significant interactions between plant and soil amendment ($p < 0.05$) and between soil amendment and time ($p < 0.05$) were observed (Supplementary Table S5). Regardless of sampling time and in the absence of tomato plants, we observed a significant increase in the abundance of Crenarchaea in soils amended with peppermint, spearmint and the organic fertilizer compared to the non-amended soil (Fig. 1g). This effect was cancelled in the presence of tomato plants and no significant differences in the abundance of Crenarchaea between the different soil treatments were observed. Instead we noted a significant increase in the abundance of Crenarchaea in the rosemary amended samples and the non-amended samples in the presence of tomato plants vs non planted samples (Fig 1g). When the interactions between time and treatment were explored, we observed a significantly higher abundance of Crenarchaea in samples amended with the organic fertilizer

compared to the peppermint amended and the non-amended samples only at 30 days (Supplementary Fig. 1g).

Regarding fungi, all main factors and their interactions had a significant effect on their abundance except the interaction between treatment x time (Supplementary Table S5). Regardless of time and in the absence of plants soil amendment with peppermint, spearmint and rosemary showed a higher fungal abundance compared to the non-amended samples (Fig 1h). In contrast in the presence of tomato plants only rosemary and peppermint amended samples supported a significantly higher abundance of fungi compared to the non-amended controls. When the interactions between time and treatment were investigated, the amendment of soil with all three aromatic plants induced a significant increase ($p < 0.05$) in the abundance of soil fungi at 30 days, a stimulatory effect which persisted only in the rosemary amended samples at 60 days (Supplementary Fig. 1h).

3.3.2. Effects of the different soil amendments on the abundance of key functional microbial groups

Soil amendment was the sole main factor than induced significant effects on the abundance of AOA ($p < 0.001$), while significant interactions between plant and soil amendment ($p < 0.05$) and between soil amendment and sampling time ($p < 0.05$) were observed (Supplementary Table S5). In the absence of tomato plants soil amendment with the organic fertilizer significantly increased the abundance of AOA compared to the non-amended samples and the samples amended with spearmint, whereas in the presence of tomato plants the stimulatory effect of the organic fertilizer on the abundance of AOA was extended to rosemary-amended samples which

also showed significantly higher abundance of AOA compared to the non-amended samples (Fig. 2a). The significant positive effect of the organic fertilizer and rosemary compared to the non-amended samples was observed at 30 days and remained significant only in the samples amended with the organic fertilizer at 60 days (Supplementary Fig. 2a). Regarding AOB, we observed a significant main effect only of time ($p < 0.001$) with higher AOB abundance observed at 30 compared to 60 days (Supplementary Table S5). The abundance of SOB was significantly affected by soil amendment ($p < 0.001$), time ($p < 0.001$) and their interactions ($p < 0.05$). Regardless of time and plant presence, soil amendment with peppermint, spearmint and the organic fertilizer showed a significantly higher abundance of SOB compare to the non-amended samples, which did not differ from the rosemary amended. We observed a significant increase in the abundance of SOB with time in the samples amended with spearmint, peppermint and the organic fertilizer but not in soils amended with rosemary which were not significantly different from the control at both times (Supplementary Fig. S2c).

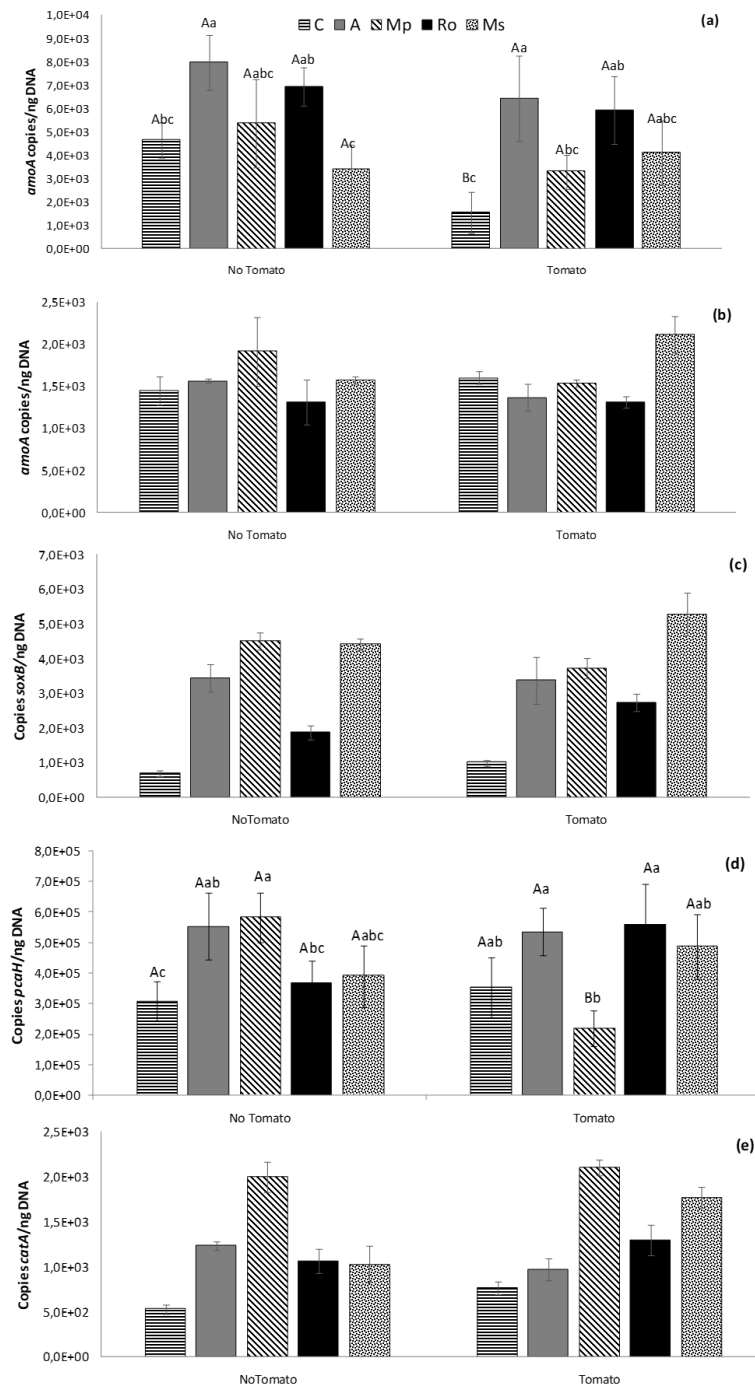


Figure 2. The abundance of ammonia-oxidizing archaea (AOA) (a), ammonia-oxidizing bacteria (AOB) (b), sulfur-oxidizing bacteria (SOB) (c) and of bacteria carrying genes *pcaH* (d) and *catA* (e) involved in the degradation of aromatic molecules in soil samples amended with an organic fertilizer (A) or plant residues of peppermint (Mp), rosemary (Ro), spearmint (Ms) and in non-amended plants (C). Each value is the mean of three replicates with error bars representing the standard deviation of the mean. Within plant treatment bars designated by the

same lower-case letter are not significantly different ($p < 0.05$). Whereas within each amendment treatment bars designated by the same capital letter are not significantly different ($p < 0.05$).

The abundance of *pcaH* was significantly affected by soil amendment ($p < 0.05$), while significant interactions between plant and soil amendment ($p < 0.001$) were also observed (Supplementary Table S5). In the absence of tomato plants, we observed a significantly higher abundance of *pcaH* in the samples amended with peppermint and the organic fertilizer compared to the non-amended samples, while the peppermint amended samples showed significantly higher abundance compared to the rosemary amended samples (Fig. 2d). This was reversed in the presence of tomato plants where the peppermint amended samples showed a significantly lower abundance compared to the corresponding samples in the absence of tomato plants but also to the samples amended with the organic fertilizer and rosemary (Fig 2d). Regardless of the presence of tomato plants, we observed a significant decrease in the abundance of *pcaH* in the rosemary amended samples from 30 to 60 days (Supplementary Fig. S2d). Finally, soil amendment ($p < 0.001$) and time ($p < 0.001$) induced significant main effects on the abundance of *catA* genes but no significant interactions between the main factors were observed (Supplementary Table S5, Fig. 2e). When temporal changes in the abundance of *catA* genes were explored, we observed a significantly higher abundance of *catA* in the peppermint and spearmint samples compared to the non-amended samples, a result which was maintained at 60 days only for peppermint (Supplementary Fig. S2e).

3.3.3. Effects of rosemary soil amendment, in the presence or absence of tomato plants, on the composition of the soil microbial community

Based on the abundance data, which indicated a clear beneficial effect of tomato plants on several of the microbial groups studied (proteobacteria, fungi, Crenarchaea) in the rosemary-amended samples, we further explored this interaction at the microbial diversity level using amplicon sequencing. An overall view of the composition of the soil microbial community and changes occurring at the order or class level in the relative abundance of bacteria, archaea and fungi is given in Figure 3. The soil bacterial community is composed of α -Proteobacteria, Actinobacteria, with classes *Thermoleophilia* and *Rubrobacteria* dominating, γ -proteobacteria, bacilli and β -proteobacteria (Fig. 3a). α -proteobacteria showed a significantly higher relative abundance ($p < 0.05$) in the rosemary-amended samples at both sampling days showing a compensatory effect with Actinobacteria of the classes *Rubrobacteria* and *Thermoleophilia*, both significantly decreasing ($p < 0.05$) in the rosemary amended samples. On the contrary, β - and γ - proteobacteria are favored ($p < 0.05$) in the soils amended with rosemary, the latter only at 60 days. Bacilli showed significantly higher abundance ($p < 0.05$) in the non-amended samples in the absence of tomato plants at 60 days respectively, whereas Sphingobacteria showed a significantly higher abundance ($p < 0.01$) in the rosemary-amended samples at 60 days regardless of the presence of tomato plant. The archaeal soil community was dominated by members of the Soil Crenarchaeotic Group (SCG), while *Thermoplasmata* appear only in the non-amended samples in the absence of tomato plants (Fig. 3b).

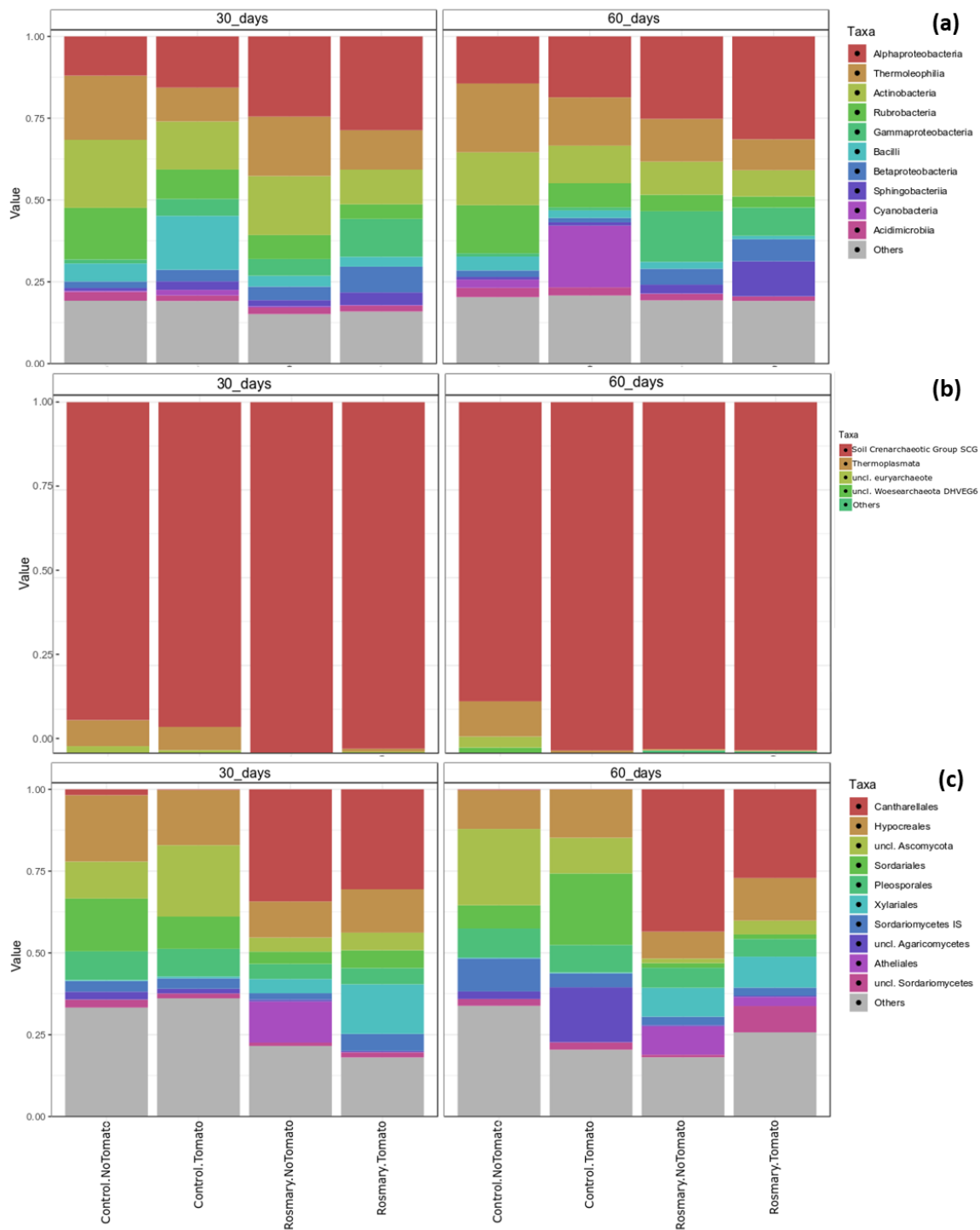


Figure 3. Stacked barplots showing the relative abundance of the main classes of bacteria (a), archaea (b) orders of fungi (c) in soil samples planted or not with tomatoes, amended with

rosemary plant residues or non-amended and collected at 30 and 60 days. Values are the mean of three biological replicates processed and analyzed separately.

The fungal community is dominated by Ascomycota belonging to *Sordariomycetes* (orders *Hypocreales*, *Sordariales*, *Xylariales*) and *Dothideomycetes* (order *Pleosporales*), while Basidiomycota become main members of the community in the samples treated with rosemary due to the dominance of *Cantharellales* and *Atheliales* (only in the absence of tomato plants), both belonging to *Agaricomycetes* (Fig. 3c). This significant increase ($p < 0.01$) in the abundance of *Cantharellales* in the rosemary amended samples was accompanied by (i) a significant decrease ($p < 0.01$) in the relative abundance of *Sordariales* and (ii) a significant increase ($p < 0.05$) in the relative abundance of *Xylariales*.

3.3.4. Effects of rosemary soil amendment, in the presence or absence of tomato plants, on the α - and β -diversity of soil bacteria, archaea and fungi

Overall our sequencing effort provided adequate coverage of the microbial diversity on all samples analyzed as suggested by the rarefaction curves which reached a plateau for all studied microbial domains (Supplementary Fig. S4). The OTU matrix obtained was used to calculate different α -diversity indices for the treatments employed. Overall we did not observe significant effects of the different factors (tomato, soil amendment and interactions) on the α -diversity of bacteria and archaea with the only exception being the significantly higher values of Fishers alpha in the presence of tomato between rosemary-amended and non-amended samples at 60 days (Supplementary Fig. S4). In contrast, the different treatments had a larger overall effect on the α -diversity of fungi with significantly lower values of Simpsons and

Shannon indices in the rosemary-amended samples in the absence of tomato compared with the non-amended samples at 60 days. Similarly, a significant lower value of Pielou's evenness were observed in the rosemary amended samples regardless of the presence of tomato plants compared to the non-amended samples in the absence of tomato at 60 days (Supplementary Figure S5).

Regarding the effects of the main factors on the β -diversity of the different microbial communities, RDA revealed that soil amendment ($p < 0.001$) and sampling time ($p < 0.05$) had a significant effect on the bacterial community explaining 63.1 and 19.6% of the variance respectively (Fig. 4a). Regarding archaea, RDA showed that only soil amendment ($p < 0.01$) had a significant effect on their community composition explaining 52.8% of the variance. Finally, CCA analysis of the fungal community revealed a significant effect of soil amendment ($p < 0.001$) and time ($p < 0.05$) explaining 48.6 and 27.7% of the variance respectively. The presence of plant did not have a significant effect in the composition of any of the microbial domains studied ($p > 0.05$).

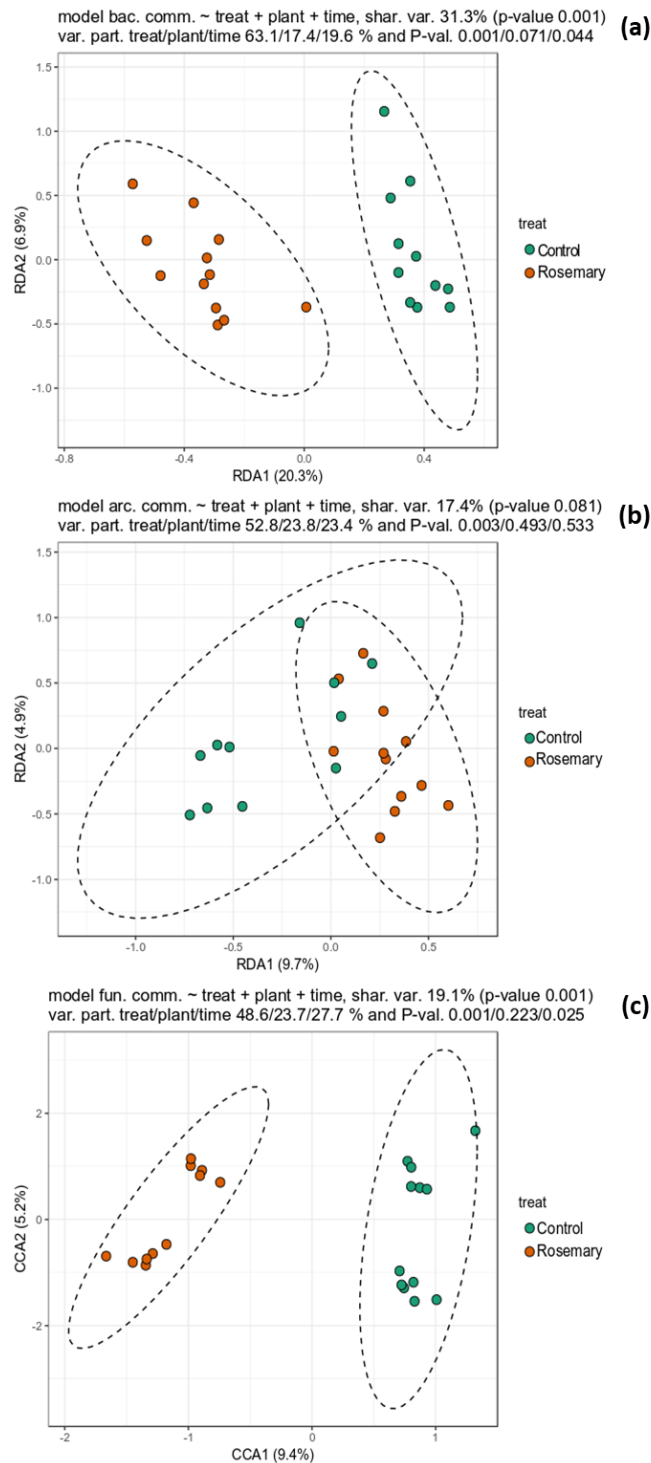


Figure 4. Canonical Correspondence Analysis (CCA) or redundancy analysis (RDA) (depending on the outcome of the first axis or detrended correspondence analysis) of the bacterial (a), archaeal (b) and fungal (c) community in soil. The tested model was that of the

community structure (bacterial/fungal/archaeal) being a function of the soil treatment, plant presence and sampling time, with the coefficient of determination providing the model shared variance and the p-value indicating the null hypothesis probability (i.e. no effect).

Further Non Metric Multidimensional Scaling (NMDS) analysis of the bacterial community revealed that OTUs belonging to *Rubrobacter* (OTUs 3 and 20), *Solirubrobacter* (OTUs 4 and 13), *Agromyces* (OTU 11), *Blastococcus* (OTU 7) and *Microcoleus* (OTUs 6 and 8) were thriving in the non-amended soil samples, the latter particularly at 60 days in the presence of tomato plants. Whereas OTU 24 belonging to *Xanthomonas* was favored in the rosemary amended soil samples (Fig. 5a, Supplementary Figure S5). NMDS analysis for archaea did not reveal a clear association between soil treatments and OTUs of SCG and *Candidatus Nitrososphaera* (Fig. 5b). Regarding fungi, we identified OTUs belonging to Ascomycota (OTUs 1 and 2), *Agaricomycetes* (OTUs 36 and 40) and *Nectriaceae* (OTUs 13 and 14) that are favored in the non-amended soil samples. In contrast OTUs belonging to *Cantharellales* (genus *Minimedusa*, OTUs 5 and 6) and *Atheliaceae* (genus *Athelia*, OTUs 27 and 33) were found to thrive in the soil amended with rosemary (Fig. 5c, Supplementary Fig. S5).

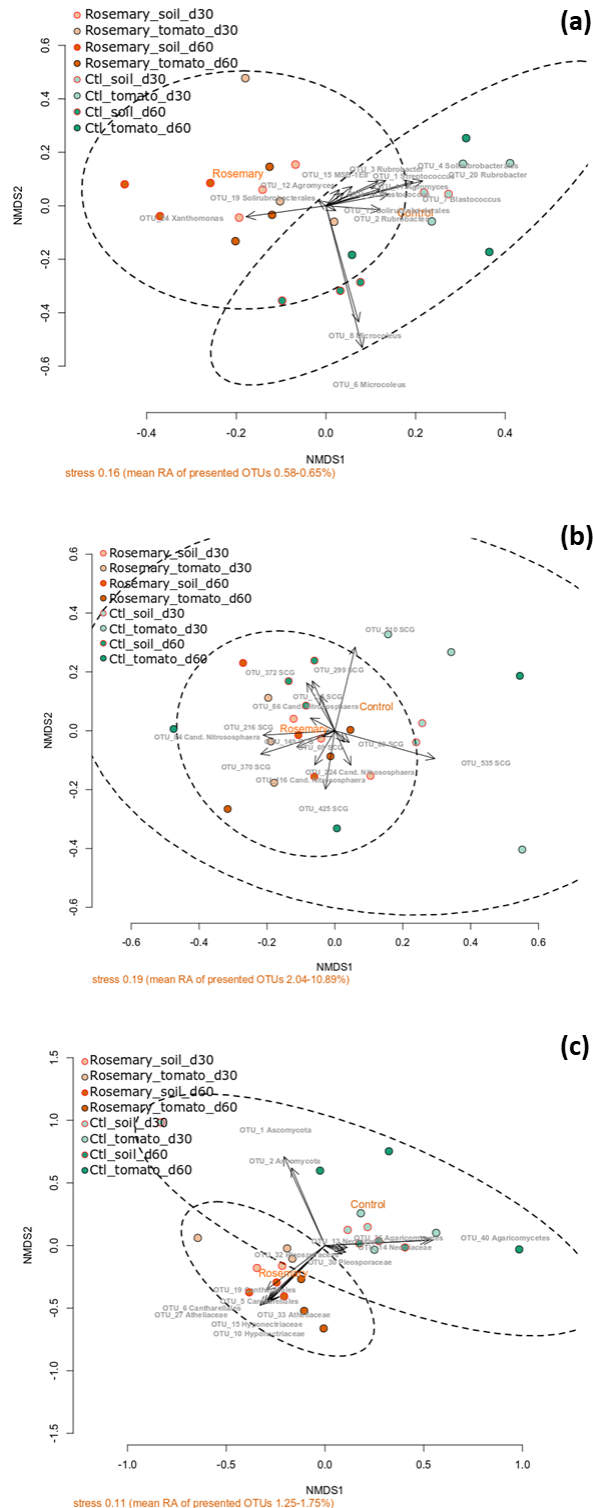


Figure 5. Non-metric Multi-Dimensional Scaling (NMDS) analysis of the bacterial (a), archaeal (b) and fungal (c) soil microbial communities. The tested model was that of the community structure (bacterial/fungal/archaeal) being a function of the soil treatment, plant

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presence and sampling time, with the coefficient of determination providing the model shared variance and the p-value indicating the null hypothesis probability (i.e. no effect). Arrows indicate the OTU gradients among samples as linearly regressed to the sample scores (i.e. OTUs are more abundant in the samples of their arrow directions).

3.4. Discussion

Soil amendment with aromatic plants, native to the Mediterranean region, could be a useful mean to enrich the poor soils of the Mediterranean basin with fresh organic carbon and at the same time impose a potential suppressive effect on soil plant pathogens through the release of the bioactive constituents of essential oils produced by these aromatic plants (Kadoglidou et al. 2014). In our study the amendment of soil with dried plant residues of such aromatic plants induced strong effects on the abundance of the different soil microbial groups with the direction of the effects depending largely on the aromatic plants used and the presence of tomato plant. Soil amendment with peppermint, and spearmint induced significant increases in the abundance of α -, β - and γ -proteobacteria in the absence of tomato plants. This is in accordance with the copiotrophic nature of members of these bacterial sub-classes which grow rapidly in carbon-rich environments (Fierer et al. 2007; Francioli et al. 2016). Whereas, no equivalent increases in the abundance of proteobacteria were evident in the samples amended with the organic fertilizer suggesting that the growth of proteobacteria upon soil amendment with mints could not be entirely attributed to the addition of large amounts of fresh and decomposable organic matter. Other constituents of the aromatic plants like mono-terpenes, exerting antimicrobial activities (Daferera et al. 2003; Iscan et al. 2002; Kadoglidou et al. 2011), might contribute to the proliferation of proteobacteria, in line with the involvement of members of these classes in the biodegradation of carvone, menthol (Marmulla and Harder 2014) and cineol

(Hawkes et al. 2002) in soil. Previous studies have also demonstrated a stimulatory effect of spearmint soil amendment on microbial abundance using plate counting approaches (Chalkos et al. 2010; Kadoglidou et al. 2014), which fail to differentiate between different microbial classes and provide a partial picture of the soil microbial abundance (Yarza et al. 2014). This is the first study to report on the effects of soil amendment with aromatic plants on individual microbial classes using advanced molecular tools.

In contrast to peppermint and spearmint, soil amendment with rosemary did not induce a significant increase in the abundance of α -, β -, γ -proteobacteria in the absence of tomato plants. Rosemary produces essential oils with different composition compared to peppermint and spearmint (cineol, camphor, α - and β -pinene are its major components) (Jiang et al. 2011), a feature that might dictate the different response of the microbial community to rosemary amendment. A further support to the differential effects of rosemary soil amendment on microbial abundance is provided by parallel measurements of the concentrations of the components of the essential oils in the soil samples amended with the different plant material (Karamanoli et al. 2018). The major components of the rosemary essential oils (i.e. cineol, camphor, α - and β -pinene) showed little if any degradation in the 60 days of the study. Whereas the components of the essential oils of peppermint (menthol, isomenthone) and spearmint (carvone, menthol, isomenthone) were degraded to levels up to 90% by the end of the study. The rapid degradation of the components of the essential oils of mints in soil explains the significant increases in the abundance of β -proteobacteria, Crenarchaea, AOA and SOB at 60 days. In accordance we did observe a reverse pattern in rosemary-amended samples, composed of persistent monoterpenoids, with significantly lower abundance of Actinobacteria, AOB and *pcaH*-carrying bacteria at 60 days.

The response of the soil microbial community to rosemary amendment drastically changed in the presence of tomato plants which appear to stimulate a significant increase in the abundance of α -, β -, γ -proteobacteria and fungi. This beneficial interaction of tomato plants with rosemary soil amendment was evident despite a phytotoxicity effect that tomato plantlets suffered in the rosemary-amended soil during the course of the study of (Technical Report ARISTEIA II project ESEPMINENT). It is widely known that plants could exert a strong filtering effect in their root zone through production of a cocktail of compounds including sugars, amino acids, low molecular weight organic acids and polysaccharides, collectively called rhizodeposits, which favor the proliferation of copiotrophic microorganisms (Philippot et al. 2013). We speculate that the plant, most probably through its root exudates, provides extra carbon sources to support the growth and proliferation of proteobacteria and fungi, hence counterbalancing potential inhibitory effects driven by the rosemary plant material essential oil to the soil microbiota.

We also examined the impact of different soil amendments on key functional microbial groups involved in C, N and S cycling. Soil amendment induced variable responses by the AOA which were stimulated by the organic fertilizer and rosemary, characterized by the highest and the lowest total N content amongst the materials studied, respectively (Table 1). On the other hand, we did not observe any clear effect of the different treatments on the abundance of AOB. The positive effect of certain soil amendments on AOA and not on AOB could be attributed to the proposed mixotrophic nature of the former (Qin et al. 2014). Previous studies have also reported variable response of ammonia-oxidizing microorganisms to soil amendments depending on the nature of the material added to the soil (Chen et al. 2008; Xue et al. 2016). Wessén et al. (2010) showed that AOA were more responsive to the different soil

amendments and increased in abundance upon straw amendment in soil, compared to AOB whose abundance was little affected by the different treatments. Similarly, AOA were benefited numerically by the addition of labile organic carbon in the form of glucose in soil, whereas AOB were not responsive (Wang et al. 2015). The response of AOA and AOB to the different treatments cannot explain the significantly lower concentration of nitrates in the soil amended with rosemary compared to all the other treatments (Appendix Figure I). This discrepancy might be associated with the limitations of DNA-based approaches to enumerate AOA and AOB compared to RNA-based approaches which have been proven more sensitive in identifying inhibitory effects of stressors to AOM (Papadopoulou et al. 2016).

Soil amendment with spearmint, peppermint and the organic fertilizer stimulated SOB compared to rosemary and the non-amended samples at both sampling dates. This could be explained by the release of readily oxidizable sulfur substrates by the specific soil amendments, although these data are not available in the current study. Little is known about the response of SOB to soil organic amendments, although they appear sensitive to abiotic stressors like pesticides (Karas et al. 2018), endocrine disrupting substances (Van Ginkel et al. 2010) and metals (Oh et al. 2011). We further assessed the effect of soil organic amendments on the abundance of bacteria carrying *pcaH* and *catA* genes encoding key enzymes of the β -ketoacid pathway being a major route of biogenic and xenobiotic aromatic compounds in soil (Harwood and Parales 1996). These genes are widespread in soil bacteria (El Azhari et al. 2008) and have been proposed as indicators of the genetic potential of the soil microbiota for organic C decomposition and C cycling (El Azhari et al. 2010, 2012). We observed contrasting patterns in the response of *pcaH* and *catA*-carrying bacteria. The former were stimulated by soil amendment with organic fertilizer and peppermint in the absence of tomato plants, while

no response of *catA*-carrying bacteria was observed. Amendment of soil with fresh organic matter releases large amounts of biogenic phenolic compounds (Marwati et al. 2003) which triggered the proliferation of bacteria carrying *pcaH* involved in the degradation of such compounds. In line with our findings, previous studies have observed significant increases in the abundance of *pcaH*-carrying bacteria in soil amended for a period of 19 years with sewage sludge (El Azhari et al. 2012) and pesticides like fenhexamide (Borzi et al. 2007). The lack of similar response by *catA*-carrying bacteria might be a function of the type of biogenic aromatic compounds released in soil by the amendments.

The beneficial effects of rosemary soil amendment on the abundance of proteobacteria and fungi in the presence of tomato plants, led us to further explore these complex interactions using amplicon sequencing analysis. Soil amendment was the factor having the strongest effect on the composition of all the studied microbial domains with time effects being marginally significant for bacteria and fungi. This time we did not observe a significant effect of plant on the composition of the microbial community, in contrast to its effect in the abundance of proteobacteria and fungi in rosemary-amended samples. This is not surprising considering that the q-PCR data focus on the abundance of certain microbial taxa compared to amplicon sequencing which gives a broad picture of potential effects which might obscure effects of plants on specific microbial groups. Similarly, with the abundance data we observed a significant increase in the abundance of α -, β - and γ -proteobacteria upon soil amendment with rosemary with OTUs belonging to *Xanthomonas* driving this effect for γ -proteobacteria. Members of the genus *Xanthomonas* are known as degraders of xenobiotic organic compounds like pesticides (Rayu et al. 2017) and antibiotics (Thelusmond et al. 2016), hence their involvement in the degradation of aromatic compounds and monoterpenes released upon soil

amendment with rosemary cannot be ruled out. On the other hand, we observed a significant decrease in the relative abundance of Actinobacteria driven by OTUs belonging to *Solirubrobacter*, *Rubrobacter*, *Agromyces* and *Blastococcus*. Members of these genera are ubiquitous in non-disturbed and pristine soils (Castro et al. 2019; Lee et al. 2011; Liao et al. 2019), while their functional and ecological role in soil seems to be associated with the degradation of xylose (i.e. *Agromyces*) (Pepe-Ranney et al. 2016) and of biogenic organic compounds (i.e. *Blastococcus*) (Wang et al. 2018).

Regarding fungal community, soil amendment with rosemary induced a striking beneficial effect on OTUs of the order *Cantharellales*, assigned to the genus *Minimedusa*. Members of *Minimedusa* are known as prolific early colonizers and decomposers of cellulose in soil (Pinzari et al. 2014), which explains their predominance in soil upon addition of cellulosic material like rosemary plant residues. In addition they are known to possess allelopathic properties exerting strong antifungal activity against *Fusarium oxysporum* f.sp. *narcissi* (Beale and Pitt 1995), a property that might be associated with their high dominance in the rosemary amended soils and the negative effect on the abundance of OTUs belonging to the family *Nectriaceae* where *F. oxysporum* belongs.

3.5. Conclusions

Soil amendment with biomass of peppermint, spearmint and rosemary, aromatic plants indigenous in the Mediterranean region, imposed variable effects on the abundance of key microbial taxa. Peppermint and spearmint had a beneficial effect on copiotrophic proteobacteria and fungi, compared to rosemary which showed the same stimulatory effect to

the same copiotrophic microorganisms only in the presence of tomato plants suggesting complex interactions between rosemary, soil microbiota and plant roots. Further investigation of these complex interactions via amplicon sequencing analysis revealed that soil amendment with rosemary was the key determinant of the composition of the bacterial, archaeal and fungal community. Rosemary soil amendment exerted negative effects on Actinobacteria mostly associated with non-disturbed soil ecosystems (i.e. *Blastococcus*, *Rubrobacter*, *Agromyces*, *Solirubrobacter*) and stimulated cellulose-degrading basidiomycetes (i.e. *Minimedusa*) with potential antifungal properties, a feature which will be explored in follow up studies aiming to identify the mechanisms and components of these microbiota responses to rosemary soil amendment.

3.6. References

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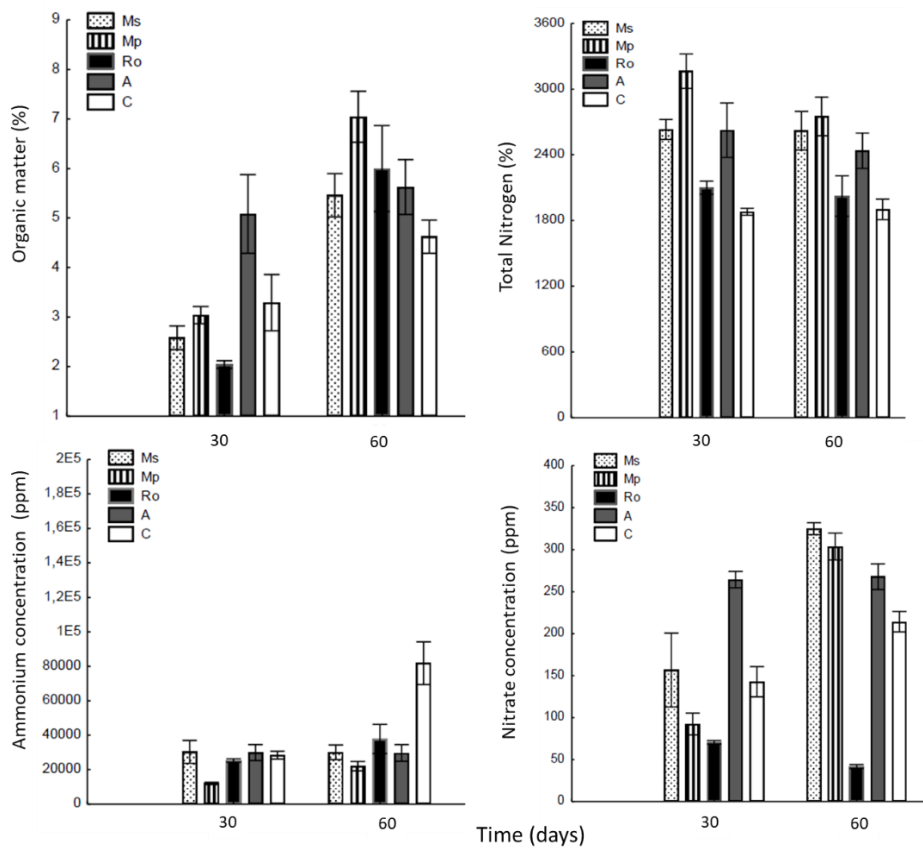
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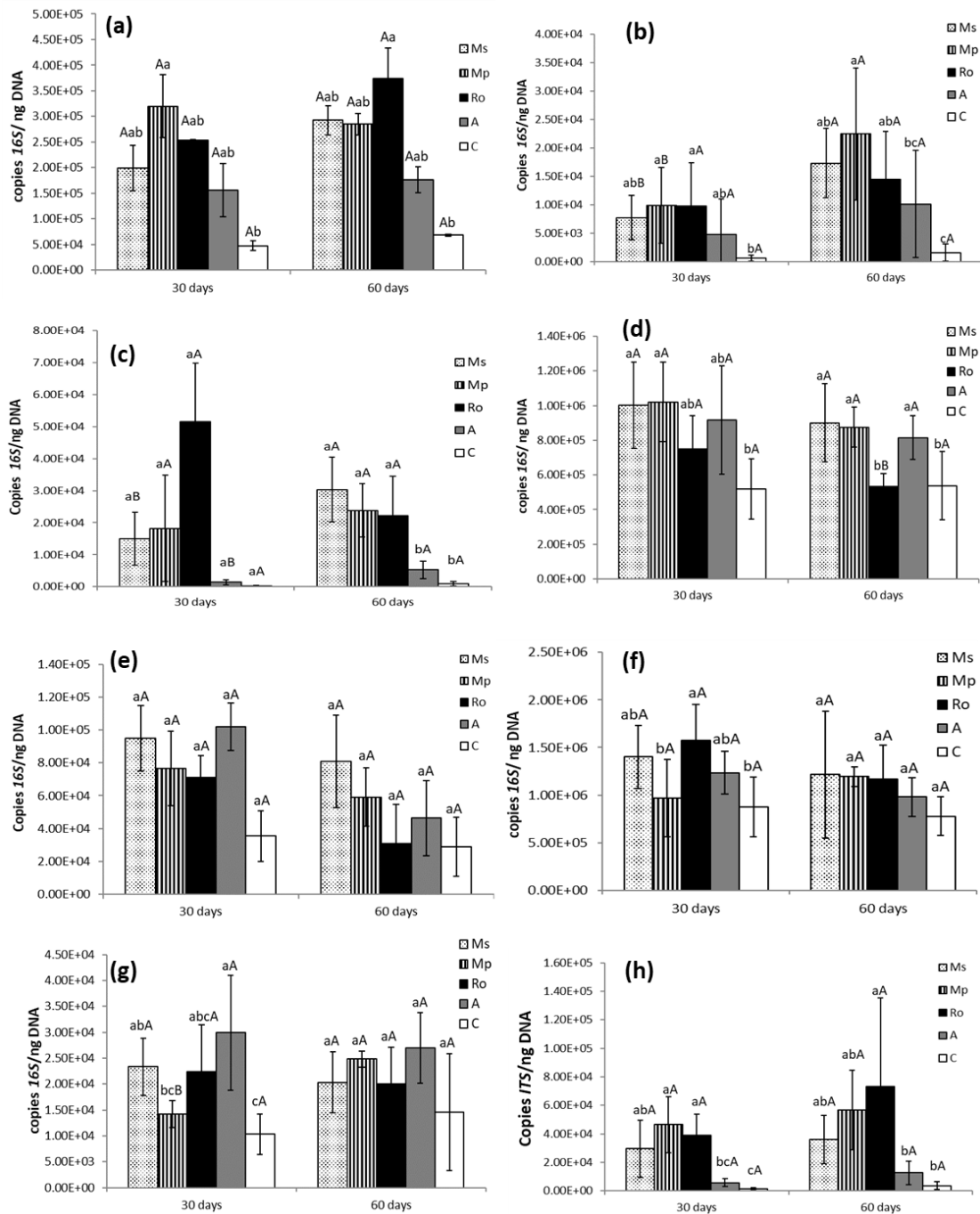
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3.7. Supplementary Data

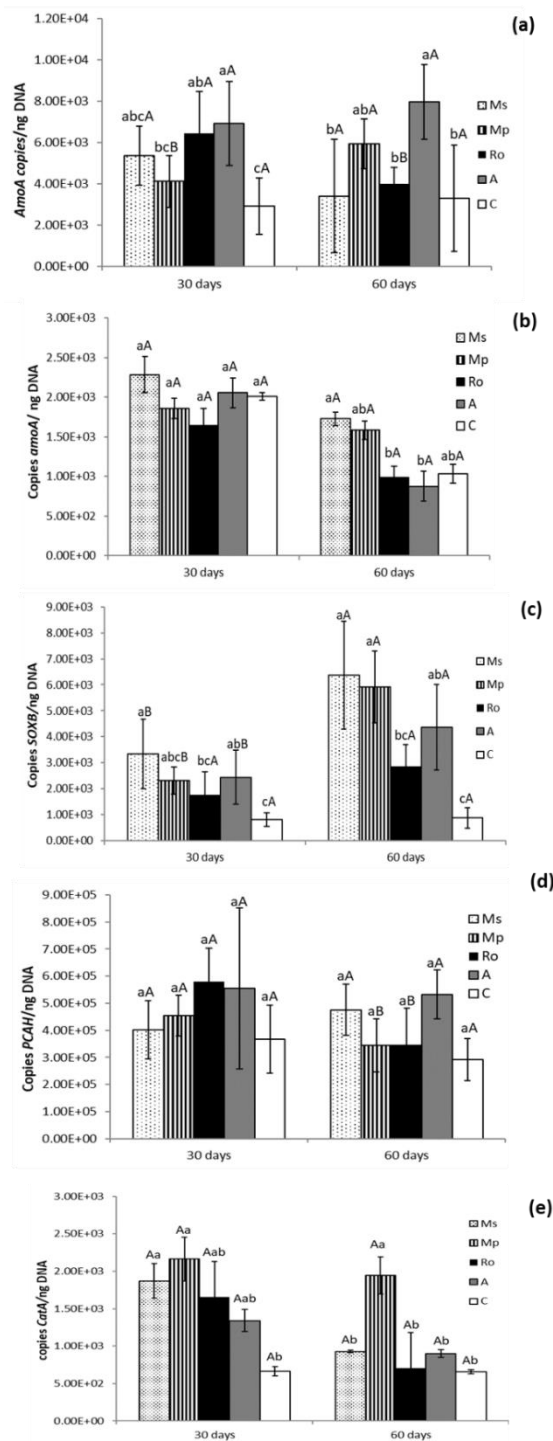
Chapter 3- The impact of soil amendment with peppermint, spearmint and rosemary on the abundance and diversity of the soil microbiota



Appendix Figure I. The organic matter content (a), total N (b), concentration of ammonium (c) and nitrates (d) in the soil samples amended with organic fertilizer (A), spearmint (Ms), peppermint (Mp), rosemary (Ro) and in unamended samples (C). Error bars represent the standard deviation of the mean of three replicates. Graphs were adopted from the final report of the ARISTEIA II project ESEPMINENT. Organic matter was determined with the wet oxidation method, total N with the Kjeldhal method and the concentrations of ammonium and nitrates according with Bremner (1960).

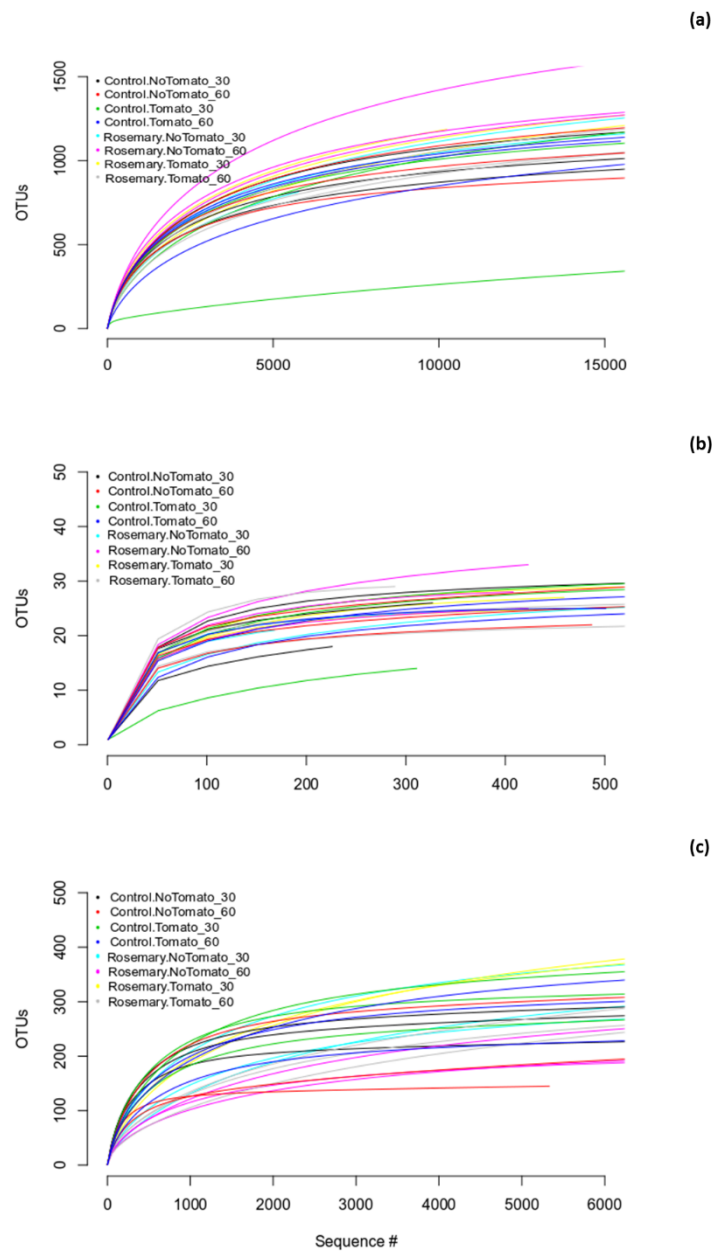


Supplementary Figure S1. The abundance of α -proteobacteria (a), β -proteobacteria (b), γ -proteobacteria (c), actinobacteria (d), firmicutes (e), total bacteria (f), Crenarchaea (g) and total fungi (h) in soil samples amended with an organic fertilizer (A) or plant residues of peppermint (Mp), rosemary (Ro), spearmint (Ms) and in non-amended plants. Each value is the mean of three replicates with error bars representing the standard deviation of the mean. Within time bars designated by the same lower-case letter are not significantly different ($p < 0.05$). Whereas within each amendment treatment bars designated by the same capital letter are not significantly different ($p < 0.05$).

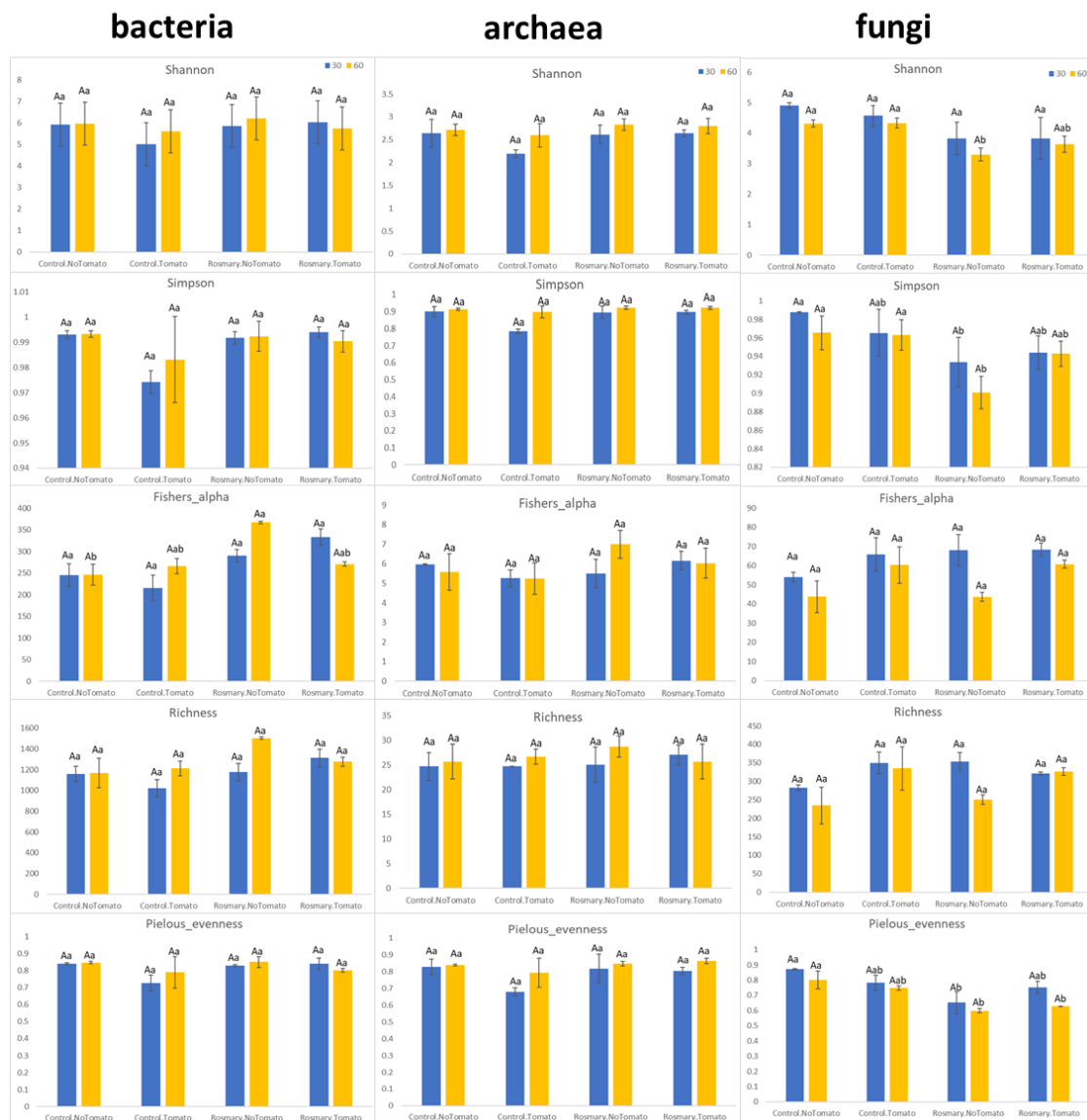


Supplementary Figure S2. The abundance of ammonia-oxidizing archaea (AOA) (a), ammonia-oxidizing bacteria (AOB) (b), sulfur-oxidizing bacteria (c) and of bacteria carrying genes *pcaH* (d) and *catA* (e) involved in the degradation of aromatic molecules in soil samples amended with an organic fertilizer (A) or plant residues of peppermint (Mp), rosemary (Ro), spearmint (Ms) and in non-amended plants (C). Each value is the mean of three replicates with error bars representing the standard deviation of the mean. Within time bars designated by the same lower-case letter are not significantly different

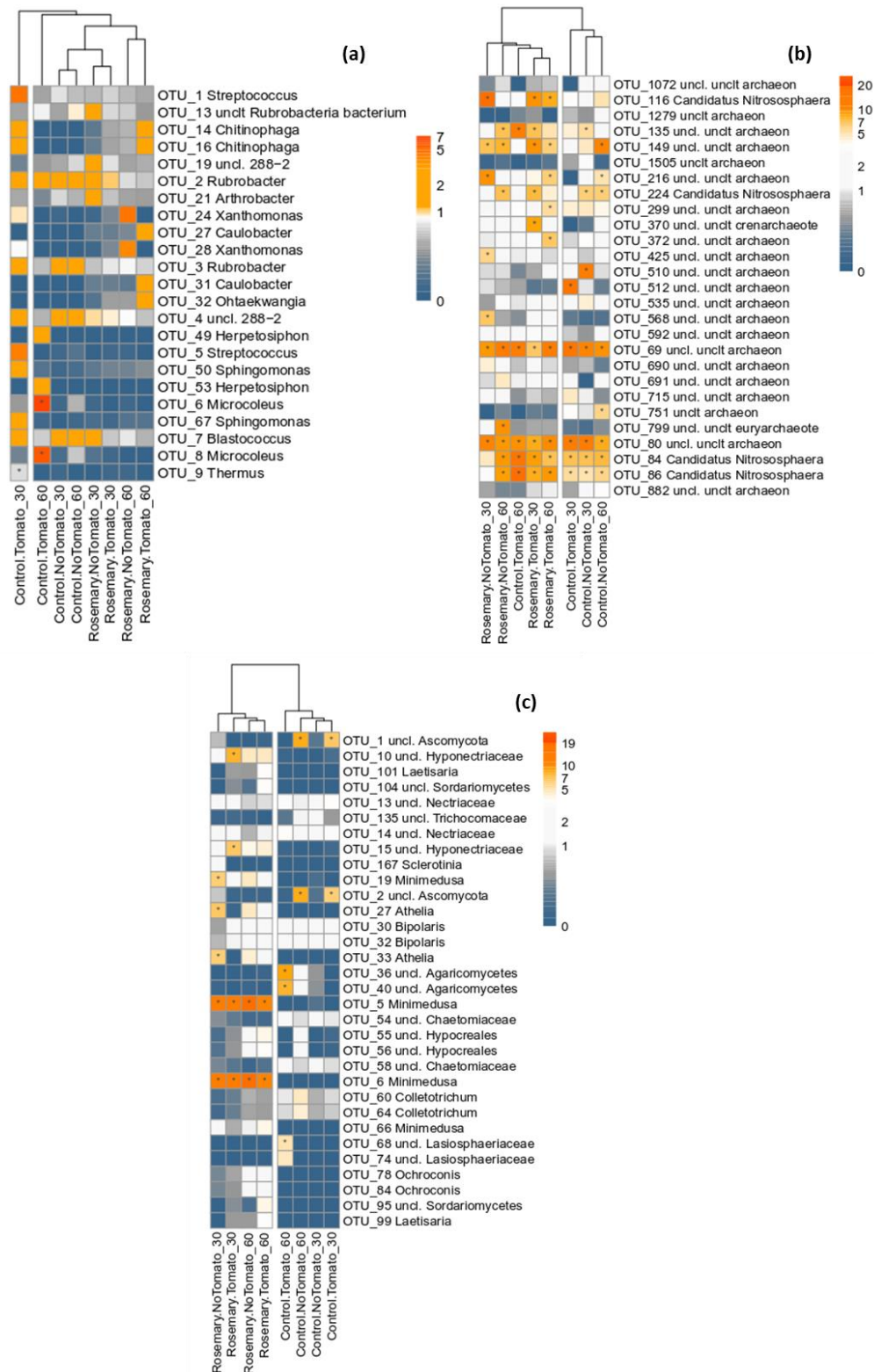
($p < 0.05$). Whereas within each amendment treatment bars designated by the same capital letter are not significantly different ($p < 0.05$).



Supplementary Figure S3. Rarefaction curves denoting the diversity coverage obtained by our sequencing effort for the bacterial (a), archaeal (b) and fungal (c) communities in the different samples analyzed.



Supplementary Figure S4. The α -diversity indices Shannon, Simpson, Fisher alpha, Richness and Pielous evenness calculated for bacteria, archaea and fungi in the non-amended (control) or rosemary-amended samples (Rosemary) in the presence (Tomato) or absence (No Tomato) of tomato plants and collected at 30 (blue) and 60 days (yellow). Values are the mean of three replicates + the standard deviation of the mean. Within each treatment bars designated with the same capital letter are not significantly different at the 5% level, whereas within each sampling time bars designated by the same lower-case letter are not significantly different at the 5% level.



Supplementary Figure S5. Heatmaps presenting the dominant bacterial (a), archaeal (b) and fungal (c) OTUs (relative abundance (RA) >2%) and their association with plant hosts in the different seasons. The data are clustered in log10 scale and the legend scaling represents the percentage of RA. Asterisks indicate OTUs that showed higher than 5% RA.

Supplementary Table S1. Physicochemical properties of the soil used in the pot experiment.

Soil physicochemical properties	
pH	7,82
Electrical conductivity (Ec)	1,80 mmhos/cm
CaCO ₃	1,73 g/100g of soil
Organic matter	3,12 %
Fe	14,91 ppm
Zn	2,75 ppm
Mn	36,31 ppm
Cu	3,13 ppm
N _{total}	1886 ppm
P	10567 ppm
NO ₃ ⁻	162 ppm
NH ₄ ⁺	29890 ppm
K ⁺	187 ppm
Mg ⁺	550 ppm
Ca ²⁺	>2000 ppm
C:N	6,77

Supplementary Table S2. Primers and thermocycling conditions used for the determination of the abundance of key microbial taxa.

Microbial group	Gene target	Primers	Primer sequences (5' to 3')	Thermocycling conditions	References
Total bacteria	16S rRNA	Eub338	ACTCCTACGGGAGGCAGCAG	Initial denaturation 95°C for 3 min; 35 cycles at 95°C for 3 sec, 62°C for 20 sec; melting curve at 95°C for 1 min, 60°C for 30 sec and 95°C for 30 sec	Ovreas & Torsvik 1998 Muyzer et al., 1993
		Eub_518	ATTCCGCGGCTGCTGG		
α -Proteobacteria	16S rRNA	Eub338	ACTCCTACGGGAGGCAGCAG	Initial denaturation 95°C for 3 min; 40 cycles at 95°C for 3 sec, 60°C for 20 sec, 72°C for 11 sec; melting curve at 55°C for 5 sec and 95°C for 1 sec	Ovreas & Torsvik 1998 Mühling et al. 2008
		Alf684R	TACGAATTTYACCTCTACA		
β -Proteobacteria	16S rRNA	Eub338	ACTCCTACGGGAGGCAGCAG	Initial denaturation 95°C for 3 min; 40 cycles at 95°C for 3 sec, 63°C for 20 sec, 72°C for 11 sec; melting curve at 55°C for 5 sec and 95°C for 1 sec	Ovreas & Torsvik 1998 Mühling. et al. 2009
		Beta682r	ACCATTTCACTGCTACACG		
γ -Proteobacteria	16S rRNA	Gamma 359f	CMATGCCGCGTGTGTGAA	Initial denaturation 95°C for 3 min; 35 cycles at 95°C for 3 sec, 56°C for 20 sec, 72°C for 11 sec; melting curve at 55°C for 5 sec and 95°C for 1 sec	Mühling. et al. 2008
		Gamma 871r	ACTCCCCAGGCGGTCDACTTA		
Actinobacteria	16S rRNA	Actino_235	CGCGGCCTATCAGCTTGTTG	Initial denaturation 95°C for 3 min; 35 cycles at 95°C for 3 sec, 60°C for 20 sec, 72°C for 11 sec; melting curve at 55°C for 5 sec and 95°C for 1 sec	Fierer. et al. 2005 Muyzer et al., 1993
		Eub_518	ATTCCGCGGCTGCTGG		
Firmicutes	16S rRNA	Lgc_353	GCAGTAGGGAATCTTCCG		Meier, et al. 1999

		Eub_518	ATTCCGCGGCTGCTGG	Initial denaturation 95°C for 3 min; 35 cycles at 95°C for 3 sec, 60°C for 20 sec, 72°C for 11 sec; melting curve at 65°C for 30 sec and 95°C for 30 sec	Muyzer et al., 1993
Crenarchaea	16S rRNA	771f	ACGGTGAGGGATGAAAGCT	Initial denaturation 95°C for 3 min; 35 cycles at 95°C for 3 sec, 53°C for 30 sec, 72°C for 11 sec; melting curve at 55°C for 5 sec and 95°C for 1 sec	Ochsenreiter <i>et al.</i> , 2003
		957R	CGGCGTTGACTCCAATTG		
		Eub_518	ATTCCGCGGCTGCTGG		Muyzer et al., 1993
Fungi	ITS	ITS3F	GCATCGATGAAGAACGCAGC	Initial denaturation 95°C for 3 min; 35 cycles at 95°C for 3 sec, 53°C for 20 sec, 72°C for 11 sec; melting curve at 95°C for 1 min, 65°C for 30 sec and 95°C for 30 sec	White <i>et al.</i> , 1990
		ITS4R	TCCTCCGCTTATTGATATGC		

Supplementary Table S3. Primers and thermocycling conditions used for the determination of the abundance of key functional microbial groups via q-PCR.

Microbial group	Gene target	Primers	Primer sequences (5' to 3')	Thermocycling conditions	References
Ammonia-oxidizing archaea	<i>amoA</i>	Arch-amoAF Arch-amoAR	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	Initial denaturation 95°C for 3 min; 40 cycles at 95°C for 15 sec, 53°C for 30 sec, 72°C for 45 sec; melting curve at 55°C for 5 sec and 95°C for 1 sec	Francis <i>et al.</i> , 2005
Ammonia-oxidizing bacteria	<i>amoA</i>	amoA-1F amoA-2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	Initial denaturation 95°C for 3 min; 45 cycles at 95°C for 15 sec, 57°C for 30 sec, 72°C for 45 sec; melting curve at 55°C for 5 sec and 95°C for 1 sec	Rotthauwe <i>et al.</i> , 1997
Sulfur-oxidizing bacteria	<i>soxB</i>	SoxB_710F SoxB_1184R	ATCGGYCAGGCYTTYCCS MAVGTGCCGTTGAARTTGC	Initial denaturation 95°C for 3 min; 40 cycles at 95°C for 5 sec, 55°C for 10 sec, 72°C for 30 sec; melting curve at 55°C for 5 sec and 95°C for 1 sec	Tourna <i>et al.</i> , 2014
Protocatechuate dioxygenase	<i>pcaH</i>	PCAHf PCAHR	GAGRTSTGGCARGCSAAY CCGYSSAGCACGATGTC	Initial denaturation 95°C for 3 min; 6 cycles at 95°C for 15 sec, 60°C for 30 sec (0.5°C increase per cycle), 72°C for 30 sec, 80°C for 15 sec and 30 cycles at 95°C for 15 sec, 57°C for 30 sec, 72°C for 30 sec and 80°C for 15 sec; melting curve at 55°C for 5 sec and 95°C for 1 sec	El Azhari <i>et al.</i> , 2008
		<i>catA</i>	CATAf	ACVCCVCGHACCATYGAAGG	

1,2-catechol dioxygenase	CATAr	CGSGTNGCAWANGCAAAGT	Initial denaturation 95°C for 3 min; 8 cycles at 95°C for 15 sec, 62°C for 30 sec (0.5°C increase per cycle), 72°C for 45 sec and 30 cycles at 95°C for 15 sec, 58°C for 30 sec and 72°C for 45 sec; melting curve at 55°C for 5 sec and 95°C for 1 sec
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Supplementary Table S4. Primers used for the amplicon sequencing analysis of the 16S rRNA gene of bacteria and archaea and the ITS region of fungi. B000X-515f and FI000X-ITS4r are indexed primers used in the second amplification step which are composed of the sequence of the universal primers 515f (bacteria, archaea) and ITS4r (fungi) (bold), the indexes used for samples barcoding (underlined) and a TT sequence at the 5' end of each primer.

Primers	Sequence (5'-3')	Thermocycling conditions	Reference
Bacteria and archaea			
515f	GTGYCAGCMGCCGCGGTAA	Initial denaturation 98°C for 30 sec, 28/7 cycles (first PCR step/second PCR step) at 98°C for 10 sec, 50°C for 30 sec and 72°C for 30 sec, final extension 72°C for 10 min	Caporaso <i>et al.</i> , (2012)
806r	GGACTACNVGGGTWTCTAAT		Walters <i>et al.</i> , (2015)
B0001-515f	TT <u>CTTCTTCGT</u> GTGYCAGCMGCCGCGGTAA		This study
B0002-515f	TT <u>CTCAATGGT</u> GTGYCAGCMGCCGCGGTAA		
B0003-515f	TTCAGTTCAGTGTGYCAGCMGCCGCGGTAA		
B0004-515f	TTCGAATCAGTGTGYCAGCMGCCGCGGTAA		
B0005-515f	TTGTCAGGTGTGTGYCAGCMGCCGCGGTAA		
B0006-515f	TTGAAGTTCGTGTGYCAGCMGCCGCGGTAA		
B0007-515f	TTGCAACAAGTGTGYCAGCMGCCGCGGTAA		
B0008-515f	TTGGACGACGTGTGYCAGCMGCCGCGGTAA		
B0009-515f	TTCTTCAAGGTGTGYCAGCMGCCGCGGTAA		
B0010-515f	TTCTCAGAAGTGTGYCAGCMGCCGCGGTAA		
B0011-515f	TTCAGTAAGGTGTGYCAGCMGCCGCGGTAA		
B0012-515f	TTCGACAATGTGTGYCAGCMGCCGCGGTAA		
B0013-515f	TTGTCGATAGTGTGYCAGCMGCCGCGGTAA		
B0014-515f	TTGAAGGAAGTGTGYCAGCMGCCGCGGTAA		
B0015-515f	TTGCAGTATGTGTGYCAGCMGCCGCGGTAA		
B0016-515f	TATATCAGGGTGTGYCAGCMGCCGCGGTAA		
B0017-515f	TT <u>CTTGTCAGT</u> GTGYCAGCMGCCGCGGTAA		

B0018-515f TTCATATGGGTGTGYCAGCMGCCGCGGTAA
 B0019-515f TTCAGACTTGTGTGYCAGCMGCCGCGGTAA
 B0020-515f TTCGAGCACGTGTGYCAGCMGCCGCGGTAA
 B0021-515f TTGTGTATCGTGTGYCAGCMGCCGCGGTAA
 B0022-515f TTGACTATGGTGTGYCAGCMGCCGCGGTAA
 B0023-515f TTGCCTAGTGTGTGYCAGCMGCCGCGGTAA
 B0024-515f TATATCGTTCGTGTGYCAGCMGCCGCGGTAA
 B0025-515f TTCTTGAGTGTGTGYCAGCMGCCGCGGTAA

Fungi

ITS7f GTGARTCATCGAATCTTTG
 ITS4r TCCTCCGCTTATTGATATGC
 FI0001-ITS4r TTATTACCGGATCCTCCGCTTATTGATATGC
 FI0002-ITS4r TTATTAGGCGATCCTCCGCTTATTGATATGC
 FI0003-ITS4r TTATTCTCCGATCCTCCGCTTATTGATATGC
 FI0004-ITS4r TTATTCGTGGATCCTCCGCTTATTGATATGC
 FI0005-ITS4r TTATTGCGAGATCCTCCGCTTATTGATATGC
 FI0006-ITS4r TTATACTGGGATCCTCCGCTTATTGATATGC
 FI0007-ITS4r TTATACCTCGATCCTCCGCTTATTGATATGC
 FI0008-ITS4r TTATACGCAGATCCTCCGCTTATTGATATGC
 FI0009-ITS4r TTATAGACCGATCCTCCGCTTATTGATATGC
 FI0010-ITS4r TTATGTTCGGATCCTCCGCTTATTGATATGC
 FI0011-ITS4r TTATGTGACGATCCTCCGCTTATTGATATGC
 FI0012-ITS4r TTATGAAGGGATCCTCCGCTTATTGATATGC
 FI0013-ITS4r TTATGAGCTGATCCTCCGCTTATTGATATGC
 FI0014-ITS4r TTATGCCATGATCCTCCGCTTATTGATATGC

Initial denaturation 98°C for 30 sec, 28/7 cycles (first PCR step/second PCR step) at 98°C for 10 sec, 55°C for 30 sec and 72°C for 30 sec, final extension 72°C for 10 min

Ihrmark *et al.*, (2012)
 White *et al.*, (1990)
 This study

FI0015-ITS4r TTATGGTGTGATCCTCCGCTTATTGATATGC
FI0016-ITS4r TTAATTCGCGATCCTCCGCTTATTGATATGC
FI0017-ITS4r TTAATCCAGGATCCTCCGCTTATTGATATGC
FI0018-ITS4r TTAATCGGTGATCCTCCGCTTATTGATATGC
FI0019-ITS4r TTAATGTGGGATCCTCCGCTTATTGATATGC
FI0020-ITS4r TTAATGCCTGATCCTCCGCTTATTGATATGC
FI0021-ITS4r TTAATGGACGATCCTCCGCTTATTGATATGC
FI0022-ITS4r TTAACTTCCGATCCTCCGCTTATTGATATGC
FI0023-ITS4r TTAACTAGGGATCCTCCGCTTATTGATATGC
FI0024-ITS4r TTAACAGTCGATCCTCCGCTTATTGATATGC
FI0025-ITS4r TTCATCTTCGATCCTCCGCTTATTGATATGC

Supplementary Table S5. The results of the MANOVA of the q-PCR data. Statistically significant differences are shown in bold letters (p<0.05).

Source	df	Mean Square	F	Probability
α-proteobacteria				
Plant	1	1.75E+11	9.68	0.001
Soil amendment	4	1.36E+11	7.54	0.001
Time	1	2.88E+10	1.60	0.210
Plant * Soil amendment	4	9.75E+10	5.40	0.001
Plant * Time	1	3.07E+09	0.17	0.680
Soil amendment * Time	4	1.17E+10	0.65	0.630
Plant * Soil amendment * Time	4	1.53E+10	0.85	0.500
β-proteobacteria				
Plant	1	2.43E+08	10.08	0.001
Soil amendment	4	5.28E+08	21.91	0.001
Time	1	5.26E+08	21.83	0.001
Plant * Soil amendment	4	9.00E+07	3.74	0.010
Plant * Time	1	1.14E+08	4.75	0.040
Soil amendment * Time	4	6.60E+07	2.74	0.040
Plant * Soil amendment * Time	4	9.91E+07	4.11	0.010
γ-proteobacteria				
Plant	1	2.69E+09	10.08	0.001
Soil amendment	4	2.71E+09	10.14	0.001
Time	1	9.63E+06	0.04	0.850
Plant * Soil amendment	4	1.90E+09	7.12	0.001

Plant * Time	1	1.38E+09	5.18	0.030
Soil amendment * Time	4	8.60E+08	3.22	0.020
Plant * Soil amendment * Time	4	1.01E+09	3.79	0.010
Actinobacteria				
Plant	1	4.10E+10	1.09	0.304
Soil amendment	4	4.43E+11	11.73	0.000
Time	1	1.80E+11	4.78	0.035
Plant * Soil amendment	4	1.04E+10	0.28	0.892
Plant * Time	1	1.98E+11	5.26	0.027
Soil amendment * Time	4	2.18E+10	0.58	0.680
Plant * Soil amendment * Time	4	5.50E+10	1.46	0.233
Firmicutes				
Plant	1	1.01E+09	0.60	0.440
Soil amendment	4	5.59E+09	3.33	0.020
Time	1	1.08E+10	6.43	0.020
Plant * Soil amendment	4	2.40E+09	1.43	0.240
Plant * Time	1	2.38E+09	1.42	0.240
Soil amendment * Time	4	1.24E+09	0.74	0.570
Plant * Soil amendment * Time	4	2.00E+09	1.19	0.330
Total bacteria				
Plant	1	7.39E+10	0.64	0.430
Soil amendment	4	5.48E+11	4.73	0.001
Time	1	3.12E+11	2.69	0.110
Plant * Soil amendment	4	1.86E+11	1.61	0.190

Plant * Time	1	4.88E+11	4.21	0.050
Soil amendment * Time	4	1.66E+11	1.43	0.240
Plant * Soil amendment * Time	4	6.49E+10	0.56	0.690
Crenarchaea				
Plant	1	1.42E+08	3.63	0.060
Soil amendment	4	3.94E+08	10.09	0.001
Time	1	2.56E+07	0.65	0.420
Plant * Soil amendment	4	1.39E+08	3.56	0.010
Plant * Time	1	1.18E+08	3.02	0.090
Soil amendment * Time	4	1.09E+08	2.78	0.040
Plant * Soil amendment * Time	4	5.54E+07	1.42	0.250
Total fungi				
Plant	1	6.37E+09	33.94	0.001
Soil amendment	4	7.03E+09	37.46	0.001
Time	1	2.16E+09	11.52	0.001
Plant * Soil amendment	4	1.83E+09	9.77	0.001
Plant * Time	1	4.29E+09	22.86	0.001
Soil amendment * Time	4	4.90E+08	2.61	0.050
Plant * Soil amendment * Time	4	1.08E+09	5.78	0.001
Ammonia - oxidizing archaea				
Plant	1	3.84E+06	1.55	0.220
Soil amendment	4	3.01E+07	12.11	0.001
Time	1	7.74E+05	0.31	0.580
Plant * Soil amendment	4	9.29E+06	3.74	0.010
Plant * Time	1	7.00E+06	2.82	0.100

Soil amendment * Time	4	1.06E+07	4.25	0.010
Plant * Soil amendment * Time	4	4.93E+06	1.99	0.120

Ammonia - oxidizing bacteria

Plant	1	1.20E+05	0.29	0.600
Soil amendment	4	8.63E+05	2.05	0.110
Time	1	7.99E+06	18.98	0.001
Plant * Soil amendment	4	6.96E+05	1.65	0.180
Plant * Time	1	8.54E+05	2.03	0.160
Soil amendment * Time	4	3.81E+05	0.90	0.470
Plant * Soil amendment * Time	4	6.46E+05	1.53	0.210

Sulfur-oxidizing bacteria

Plant	1	8.61E+05	0.60	0.440
Soil amendment	4	2.98E+07	20.77	0.001
Time	1	5.66E+07	39.48	0.001
Plant * Soil amendment	4	1.44E+06	1.00	0.420
Plant * Time	1	3.55E+05	0.25	0.620
Soil amendment * Time	4	6.17E+06	4.30	0.010
Plant * Soil amendment * Time	4	1.10E+06	0.77	0.550

***pcaH*-carrying microorganisms**

Plant	1	1.24E+09	0.06	0.810
Soil amendment	4	7.54E+10	3.40	0.020
Time	1	8.09E+10	3.65	0.060
Plant * Soil amendment	4	1.35E+11	6.09	0.001
Plant * Time	1	1.06E+09	0.05	0.830
Soil amendment * Time	4	3.87E+10	1.74	0.160

Plant * Soil amendment * Time	4	1.43E+10	0.64	0.630
<i>catA</i>-carrying microorganisms				
Plant	1	7.95E+05	3.53	0.070
Soil amendment	4	3.08E+06	13.70	0.001
Time	1	3.95E+06	17.56	0.001
Plant * Soil amendment	4	3.59E+05	1.59	0.190
Plant * Time	1	5.13E+05	2.28	0.140
Soil amendment * Time	4	5.41E+05	2.40	0.070
Plant * Soil amendment * Time	4	5.01E+05	2.23	0.080

Chapter 4

The response of soil and phyllosphere microbial communities to repeated application of the fungicide iprodione: Accelerated biodegradation or toxicity?

The work presented in Chapter 4 is included in the following article:

Katsoula A., Vasileiadis S., Sapountzi M., Karpouzas D.G. (2019). The response of soil and phyllosphere microbial communities to repeated application of the fungicide iprodione: Accelerated biodegradation or toxicity? *FEMS Microbial Ecology*. under review

4.1. Introduction

Microorganisms are highly responsive to environmental stress conditions. Pesticides applied either in soil or on plant foliage constitute potential environmental stressors for the microbial communities colonizing these habitats. Several studies have explored the responses of the soil microbial communities to pesticides (Gallego et al. 2019; Itoh et al. 2014; Karas et al. 2018). Repeated applications of pesticides, a common practice in modern agriculture, lead to the accumulation of pesticide residues in soil, when the indigenous microbial community has limited capacity to degrade the given compound, with potential negative effects on the soil microbial community. In such an example nicosulfuron at concentration levels of 0.25 - 1 $\mu\text{g g}^{-1}$ imposed significant reductions in the abundance of key microbial groups (β -proteobacteria, planctomycetes, actinobacteria), on the activity of C- and P-cycling (Karpouzas et al. 2014a), and reduced significantly the colonization levels and diversity of endomycorrhizal fungi in maize plants (Karpouzas et al., 2014b). In contrast, repeated soil application of certain pesticide groups like organophosphates (Singh and Walker 2006), carbamates (Karpouzas et al 1999), and triazines (Krutz et al. 2010) could lead to the proliferation of a small fraction of the soil microbial community which carries specialized catabolic enzymes used for the growth-linked degradation of these pesticides (Itoh et al. 2014; Rousidou et al 2017). This phenomenon has been termed accelerated biodegradation and under conducive edaphoclimatic conditions could jeopardize the biological efficacy of pesticides (Suett et al. 1987).

In contrast to our good knowledge of the interactions of pesticides with the soil microbial community, we are only just starting to explore the interaction of pesticides with microbial communities in other relevant habitats like plants (Perazzolli et al. 2014)

and insects (Cheng et al. 2017). The phyllosphere is a micro-ecosystem where microorganisms are exposed to various environmental constraints (i.e. UV, desiccation, limited nutrients) and potential stressors like pesticides (Vorholt 2012). Zhang et al.(2009a and 2009b) and Gu et al. (2010) first studied the potential effects of synthetic pesticides on the epiphytic microbial community, using PLFAs and molecular fingerprinting, and observed significant but transient effects. Subsequent studies using amplicon sequencing reported a remarkable resilience of the epiphytic microbial community to pesticides (Ottesen et al. 2015; Perazzolli et al. 2014). In contrast, very little is known about the potential of the epiphytic microbial community for accelerated biodegradation of pesticides or the identity of the microorganisms responsible for biodegradation and their relevant degradative genes. In the only relevant study to date, Ning et al (2012) isolated epiphytic bacteria from rape plants systematically treated with dichlorvos which were able to degrade this organophosphorus insecticide, although the establishment of accelerated biodegradation of dichlorvos on the plant phyllosphere was not explored. The capacity of the epiphytic microbial community to rapidly degrade foliage-applied pesticides can be beneficial from the environmental and human health perspective, while in its extreme, it could threaten the biological efficacy of foliage-acting pesticides, an aspect largely overlooked.

Iprodione is a fungicide used via foliage application or soil drenching for the control of a range of plant pathogenic fungi (Grabke et al. 2014). It has been identified as potential carcinogen (USEPA 1998) and endocrine disrupting substance (Blystone et al. 2007). Soil pH is the main factor affecting its dissipation in soil with higher degradation rates observed in alkaline soils (Walker 1987). Biodegradation is the main dissipation process of iprodione in soil. Repeated applications of iprodione in soil are

known to result in the establishment of accelerated biodegradation (Martin et al. 1990; Mitchell and Cain 1996) and loss of its biological efficacy (Entwistle 1986). Studies in soils exhibiting accelerated biodegradation of the fungicide led to the isolation of iprodione-degrading bacteria (Athiel et al. 1995; Campos et al. 2015; Yang et al. 2018) which hydrolyzed iprodione to 3,5-dichloroaniline (3,5-DCA) *via* the formation of two transient metabolic products; 3,5-dichlorophenyl-carboxiamide (metabolite I) and 3,5-dichlorophenylurea-acetate (metabolite II). On the contrary, soil application of iprodione has been also shown to induce negative effects on the abundance of soil bacteria (Zhang et al., 2017c), on the abundance and α -diversity of soil fungi (Zhang et al., 2017a) and on microbial activity and processes involved in N cycling (Zhang et al., 2017b; Zhang et al. 2018). Recently Vasileiadis et al. (2018) showed that 3,5-DCA and not iprodione was responsible for the inhibitory effects observed on the ammonia-oxidizing microorganisms in soil and the general microbial activity. Still, we are missing the information about the potential response, toxicity or accelerated biodegradation, of the epiphytic microbial community to iprodione exposure.

In this study, we explored the hypothesis that phyllosphere and the soil root zone support largely different microbial communities, however we expect them to exhibit a similar response to their repeated exposure to a biodegradable pesticide like iprodione. This response could span from accelerated degradation by a fraction of the microbial community, to toxicity on members of the microbial community. In this context, a pot experiment with pepper plants repeatedly treated with iprodione, either at the foliage or through soil drenching (chosen plant and pesticide application modes are relevant to iprodione commercial use), was undertaken. Potential accelerated biodegradation of iprodione was evaluated through determination of its degradation at

each application in the soil root zone and on pepper leaves, while the overall response of the bacterial, archaeal and fungal community on plant leaves and on the soil root zone was determined via 16S rRNA and ITS amplicon sequencing respectively. Bacteria able to degrade iprodione were isolated via enrichment cultures from both soil and plant leaves and the transformation pathway of iprodione was determined to explore the presence of habitat-specific catabolic traits in iprodione-degrading bacteria isolated from the phyllosphere and the soil root zone.

4.2. Material and Methods

4.2.1. Chemicals and soil

The commercial formulation of iprodione (Rovral® 50%WP), kindly provided by BASF Hellas, was used for the treatment of pepper plants and soil. Iprodione and 3,5-DCA analytical standards (Pestanal®, purity >97%) were purchased by Sigma-Aldrich (St. Louis, Missouri, USA), while 3,5-dichlorophenyl-carboxiamide (metabolite I) and 3,5-dichlorophenylurea-acetate (metabolite II), two intermediate transformation products of iprodione, were synthesized as described before (Campos et al. 2017). All analytical standards were used for the preparation of stock solutions in methanol (1000 mg L⁻¹). The soil used was collected from a fallow agricultural field of the Hellenic Agricultural Organization-Demeter in Larissa, Greece (39°63'27"N, 22°36'74"E), with no history of pesticide application for the last 15 years. Soil samples were collected from the top 20 cm according to a protocol of the International Organization for Standardization for collection and handling of samples (ISO 10381-2:2002). Upon

collection, the soil was homogenized, sieved (2-mm pore size), and stored at 4°C until used. The soil was clay loam with a pH of 7.55 and an organic carbon content of 1.05%.

4.2.2. Pot experiment set-up

Sixty-two pepper plants (*Capsicum annuum* var. *annuum* (*florinis*)) at the 3-4 leaf stage were transplanted into 5-L pots filled up with *ca.* 6 kg of soil wet weight. Plants were left to grow at ambient conditions (open air, below a net for protection against extreme weather conditions) in the pots (May to July 2017) until flowering, when applications of iprodione were implemented to simulate a realistic application timing of the fungicide. During this period, the plants were watered every day, adjusting the soil moisture content to 50% of its water holding capacity. The first 12 planted pots were treated via soil drenching with 50 ml of an aqueous solution of iprodione (100 mg L⁻¹) aiming to a soil concentration of 1.5 µg g⁻¹, assuming diffusion of the pesticide, applied at the recommended dose rate, to 5 cm depth and a soil bulk density of 1.5 kg L⁻¹. The plants in the second set of 30 pots were sprayed individually with 25 ml of an aqueous solution of iprodione (1500 mg L⁻¹). This dose was selected based on the recommended rate of 300 mg a.i. per 100 L of spraying liquid applied in 40000 plants per ha. The soil or the foliage of the pepper plants in the remaining 20 pots (2 x 10 pots) were treated with 50 or 25ml of water without iprodione respectively to serve as untreated controls. The same application scheme was repeated four times at 30-day intervals. Immediately after each pesticide application and at regular intervals thereafter triplicate soil (with a cork borer from the root zone in each pot) and leaf samples (each replicate sample was composed of five leaves per plant) were collected from relevant pots and stored at -20°C for analysis of iprodione and 3,5-DCA residues. Similarly, triplicate soil and leaf samples collected at 0 (only on the first application event), 10 and 30 days after each

pesticide application were processed for DNA extraction as described below. The leaves collected were well-developed and healthy, of the same size and maturity level, located in the upper part of canopy to minimize the risk of soil transfer.

4.2.3. Pesticides residue analysis

Iprodione and its transformation products were extracted from soil as described by Campos et al. (2015). Briefly, 5g of soil were extracted with 10 ml of acetonitrile by shaking at 200rpm for 1h at 25°C. The extract was centrifuged at 10,000 rpm for 10 min and the supernatant was collected and filtered through 0.22 µm PTFE membrane syringe filters (Whatman) before being directly analyzed by HPLC-PDA as described by Campos et al. (2017). The same procedure was followed for the extraction of iprodione and 3,5-DCA from leaves with the only difference that an extra sonication step of 5 min was employed prior to shaking. Fortification tests with pepper leaves at three concentration levels (7.5, 75, and 750 mg L⁻¹) gave recoveries of 92.9, 96.7 and 92.8% respectively for iprodione, 74.2, 85.7 and 80.1% for metabolite I, 100.3, 98.7 and 86.2% for metabolite II and 79.5, 100.2 and 94.9% for 3,5-DCA.

4.2.4. DNA extraction from soil and epiphytic microbial biomass

Soil DNA extraction was performed from 0.5 g of soil (dry weight) with the PowerSoil® DNA isolation kit (MoBio Laboratories, Inc., West Carlsbad, CA, USA). DNA extraction from leaves was performed as described by Moulas et al., (2013) with slight modifications. Briefly, 6 g of intact fresh leaves were immersed in sterilized ddH₂O in sterile centrifuge tubes and were subjected to sonication for 7 min to detach

epiphytic microbial cells from the leaf surface. The leaves were removed with forceps and the content of the tubes was centrifuged for 15 min at 15000xg. The supernatant was discarded, and the microbial pellet collected was used for DNA extraction with the PowerSoil® DNA isolation kit.

4.2.5. Amplicon sequencing analysis of the soil and epiphytic microbial community

The composition of the community of bacteria, archaea and fungi in soil and on plant leaves were determined with amplicon sequencing of the 16S rRNA gene and the ITS region via HiSeq Illumina Rapid Mode 2x250 bp paired-end reads (Illumina Inc., San Diego, CA, USA) in the DNA Sequencing Center Department of Biology of the Brigham Young University (GSC-BYU, Provo, UT, US). Bacterial and archaeal 16S rRNA genes were amplified with the primer set 515f-806r (Caporaso et al. 2012; Walters et al. 2015) following the protocol of the Earth Microbiome Project (Caporaso et al. 2018). The amplification of ITS was performed with the primers ITS7-ITS4 (Ihrmark et al. 2012; White et al. 1990) following the protocol described by Ihrmark et al.(2012). For all PCR amplifications, the Q5® High-Fidelity DNA Polymerase (NEB, Ipswich, Massachusetts, USA) was used. All samples were initially amplified (28 amplification cycles) using the domain-specific primers mentioned above, followed by a PCR (7 amplification cycles) using primers carrying sample associated indexes for performing the multiplex sequencing. Primers and PCR conditions are listed in Supplementary Tables S1 and S2 respectively.

The raw sequence data were demultiplexed to their samples of origin with Flexbar v3.0 (Dodt et al. 2012), the reads were quality trimmed with Trimmomatic v0.32 (Bolger et al 2014) using the default parameters for paired-end reads and filtering the Illumina adapter collection at the sequence edge. The resulting read pairs were assembled to the amplicon of their origin in cases overlaps occurred with FLASH v1.2.8 (Magoc and Salzberg 2011) using the default parameters to allow a maximum overlap of 250 bp and no mismatches between read-pairs. The remaining tasks were carried out with the IOTUs v1.58 perl wrapper (Hildebrand et al. 2014). OTU calling at 97% identities was performed with the UPARSE v10.0.240 software (Edgar et al. 2013). Chimeric sequences were identified with the UCHIME v4.2 software (Edgar 2011) using the RDP Gold database vMicrobiomeutil-r20110519 for bacteria and the UNITE ITS2 v985.20150311 reference database (Nilsson et al. 2015) for fungi. Sequence classification was performed with Lambda v0.9.1 (Hauswedell et al. 2014) against the Silva v128 small ribosomal subunit database (Yilmaz et al 2014) for bacteria and the UNITE ITS v7_99_20150302 database (Kõljalg et al. 2013) for fungi, while misclassified sequences were removed from downstream analysis.

4.2.6. Isolation and characterization of iprodione-degrading bacteria

4.2.6.1. Enrichment cultures and isolation

At 30 days after the fourth application of iprodione, soil and leaf samples from the iprodione-treated pots were collected. Three 50-g soil samples were collected from the plant root zone of each pot and they were composited to a single uniform soil sample which was used as starting inoculum in soil enrichments. Similarly, three to five intact

and fully developed leaves were collected from each of the pots that had received repeated iprodione applications and they were bulked up in one sample. The isolation of iprodione-degrading bacteria from soil and leaf samples was done by enrichment cultures in mineral salts medium (MSM), MSM supplemented with NH_4Cl (MSMN) or sodium citrate (MSM+SC) amended with iprodione (10 mg L^{-1}). In those media iprodione constituted the sole C and N, C or N source respectively. Growth media were prepared as previously described (Campos et al 2015; Perruchon et al. 2015) and they were supplemented with iprodione by addition of appropriate amounts of a sterile DMSO stock solution ($10,000 \text{ mg L}^{-1}$). In all cases the DMSO percentage in the growth media did not exceed 0.2%, which according to preliminary tests did not have any effects on the growth and degrading capacity of bacteria.

For the isolation of iprodione-degrading bacteria from plant leaves, 5 g of pepper leaves were placed in a centrifuge tube fully immersed in TE buffer and 0.01% Tween 80. The samples were vortexed for 30 sec, agitated for 15 min in an orbital shaker at 200 rpm and placed in an ultrasonic bath for 3 min. They were then vortexed (30 sec) and shaken for 5 min before centrifugation at $8000\times g$ for 7 min. The supernatant was discarded, and the microbial pellet was redissolved in 2 ml of sterilized ddH₂O which was used as inoculum for the enrichment cultures.

Enrichment cultures in the three selective media were inoculated with 0.5 ml of the epiphytic microbial pellet or 0.5 g of soil. Triplicate flasks for each selective media were inoculated, while duplicate non-inoculated flasks per medium were not inoculated to measure the abiotic degradation of iprodione. All flasks were placed in an orbital shaker at 180 rpm at 25°C . Immediately after inoculation and at regular intervals

thereafter, aliquots (0.5 ml) were collected from the enrichment cultures to determine the transformation of iprodione. At the point where more than 50% degradation of iprodione occurred, aliquots of the enrichment culture (0.5 ml) were used to inoculate fresh enrichment cultures of the corresponding medium. This enrichment procedure was repeated three more times and at the point where more than 50% degradation of the fourth iprodione application had occurred serial dilutions were prepared and spread on iprodione-amended (20 mg L⁻¹) MSM, MSMN and MSM+SC agar plates prepared as described by Karpouzas and Walker (2000). The plates were incubated for 4-5 days at 25°C and 120 well-separated colonies (20 per medium x matrix combination) were picked up and tested for their degrading ability in the corresponding liquid medium. Cultures exhibiting >50% iprodione degradation in 6 days were considered as positive and they were plated on LB and the respective selective media agar plates to check purity. The bacteria that appeared as pure in plates went through another cycle of single colony testing of their degradation capacity before processed for DNA extraction.

4.2.6.2. Identification of iprodione-degrading bacteria

DNA extraction from the bacterial isolates was performed with the NucleoSpin® Tissue kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). The primer pair 8f-1512r, which amplifies the near full size of the 16S rRNA gene (1504 bp) (Felske et al. 1997), was used for the identification of the isolated bacteria as described previously (Perruchon et al. 2015). The near full length 16S rRNA sequence was subjected to phylogenetic analysis as described by Campos et al. (2015) and the phylogenetic tree was prepared using Seaview4 (Gouy et al. 2010). The 16S rRNA

sequences of the iprodione - degrading strains were deposited in the GenBank under the accession numbers MK386866 to MK386885.

4.2.6.3. Characterization of the transformation pathway of iprodione by the isolated bacteria

Triplicate flasks of MSM amended with iprodione (10 mg L^{-1}) were inoculated with fresh cultures of the selected isolated bacterial strains grown to the late logarithmic phase ($\text{OD}_{600} = 0.1$ corresponding to *ca.* 2×10^7 cells ml^{-1}). Triplicate non-inoculated flasks for each medium were also prepared as abiotic controls. All samples were incubated on a shaking platform at 25°C . The degradation of iprodione and the formation of metabolite I, metabolite II and 3,5-DCA were measured immediately after inoculation and at regular intervals thereafter by HPLC-PDA as described by Campos et al. (2017).

In parallel we determined the proliferation of the two *Paenarthrobacter* strains along the degradation of iprodione via q-PCR. So 2 ml aliquots were removed from each bacterial culture (triplicates per bacterium) at 4, 8, 12, 24, 36, 48, 69, 72, 93 and 117 h. Samples were centrifuged at 11,000 rpm for 2 min and the bacterial pellet was used for DNA extraction with the Nucleospin Tissue kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) using the manufacturers' protocol for gram positive bacteria. The extracted DNA was quantified by the Qubit dsDNA BR assay kit using a Qubit® 2.0 Fluorometer (Life Technologies). A set of primers Paen_F (5'-ACATGAACCGGAAAGACCTG-3') and Paen_R (3'-TGGGATTAGCTCCACCTCAC-5') was specifically designed to amplify a 292 bp fragment of the 16S rRNA gene

of the *Paenarthrobacter* strains. Primers were designed with the online software Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) and their specificity was first checked *in silico* with the online tool Primer-BLAST (<http://ncbi.nlm.nih.gov/tools/primer-blast/>) and then *in vitro*. qPCR was performed in a CFX connect Real Time (Bio-Rad, Hercules, USA) system in 10 μ L reaction volumes containing 1 \times KAPA SYBR[®]FAST qPCR Master Mix (2 \times) Universal (KAPA BIOSYSTEMS, Boston, USA), 0.4 μ M of each primer and 0.1 ng DNA. The thermocycling program used was as follows: 3 min at 95 $^{\circ}$ C; 40 cycles of 10 s at 95 $^{\circ}$ C, 30 s at 58 $^{\circ}$ C, 10 s at 72 $^{\circ}$ C; and followed by melting curve analysis to check the specificity of the products. The copy numbers of the 16S rRNA gene were determined via external standard curves as described by Rousidou et al. (2013). qPCR efficiencies were 98.9% with $r^2 = 0.994$.

4.2.7. Data analysis

4.2.7.1. Pesticides degradation kinetics

The dissipation data of iprodione were fitted to four kinetic models as suggested by the FOCUS working group (FOCUS 2006): the single first order (SFO) exponential decay model and three biphasic models (hockey-stick, first order multi-compartment and double first order in parallel). The goodness of fit was assessed using a χ^2 test (<15%, for $\alpha=0.05$), visual inspection, and the distribution of residuals. All kinetics analysis were performed on the R software with the mkin package. Significant differences (level of significance 5%) between the degradation rates (k_{deg}) of the repeated applications of iprodione in soil and on plant leaves were determined with the student's t-test. The

confidence intervals of the degradation rates obtained by fitting the kinetic models to the degradation data were converted to standard deviations using the formula:

$$SD = \sqrt{N} \times (\text{Upper limit} - \text{Lower limit}) / 3.92 \text{ (eq.1)}$$

where N = sample size and 3.92 is the standard error for a 95% confidence interval.

4.2.7.2. Statistical analysis of microbial diversity data

The OTU matrices of bacteria, archaea and fungi were used to assess the impact of iprodione and 3,5-DCA on the α - and β -diversity. The impact of iprodione on the α -diversity was determined via calculation of the diversity indices richness (*S*), Fisher Alpha, Inverse Simpson, Shannon (Jost 2006) and Pielou's evenness (Pielou 1975). The data per habitat were subjected to two-way ANOVA and post-hoc tests to determine the impact of iprodione and time (main factors) on the α -diversity of bacteria, archaea and fungi and also on the relative abundance of the major bacterial, archaeal and fungal taxa. Moreover, differential abundance (DA) tests for identifying taxa and OTUs that were responsive to iprodione treatment were performed using the Fisher's exact test for P-values of 0.05 as adjusted according to the Benjamini-Hochberg algorithm (Benjamini and Hochberg 1995).

The impact of iprodione on the structure of the communities of bacteria, archaea and fungi in phyllosphere and soil was assessed by multivariate analysis. To enhance the statistical test sensitivity, only the differentially abundant OTUs were used for downstream multivariate tests that provided the variance portion of these subcommunities that coincided with the experimental treatments. Detrended

Correspondence Analysis (DCA) was performed and, Canonical Correspondence Analysis (CCA) was preferred over Redundancy Analysis (RDA) if the first axis value was higher than 3 standard deviations, in accordance to a previously suggested strategy (Lepš and Šmilauer 2003). DCA first axis values greater than 3 standard deviations imply overall unimodal responses of community member abundances against the environmental gradients (rendering the chi squared distances of CCA more suitable) as opposed to lower values which imply overall linear responses to environmental gradients (rendering the Euclidean distances of RDA more suitable). Spearman's correlation tests between the measured concentrations of iprodione and 3,5-DCA in soil and on plant leaves and the sequence counts of bacterial, fungal genera and archaeal classes were carried out to assess possible effects of either the parent compound or 3,5-DCA on the microbial community members. All statistical analyses were performed with the R v3.5.2 software (R Core Team 2017) using the packages Vegan v2.4-4 (Oksanen et al. 2018), Entopart v1.4-7 (Marcon and Herault 2015) and EdgeR v3.24.3 (Robinson et al. 2010), the latter for DA tests. The data were submitted to Sequence Read Archive of NCBI with bioproject accession number PRJNA513949.

4.3. Results

4.3.1. Degradation of iprodione in soil and phyllosphere

The degradation patterns of iprodione in soil and on plant leaves are presented in Figure 1. In all cases the degradation of iprodione was best described by single first order (SFO) kinetics (Table 1). Iprodione showed a rapid degradation in soil observed even from the first application ($DT_{50}=1.24$ days) (Table 1). Its degradation rate remained

constant in the second ($DT_{50} = 1.23$ d) and third ($DT_{50} = 1.14$ d) applications and it was significantly ($p < 0.05$) accelerated at the fourth application ($DT_{50} = 0.45$ d). On plant leaves, no degradation of iprodione was observed during the 30 d after the first application of iprodione (DT_{50} extrapolated > 365 days). However, a significant increase ($p < 0.05$) in its degradation was evident in the second, third and fourth application with DT_{50} values of 15.1, 11.5 and 5.95 days, respectively (Table 1). The soil degradation of iprodione was accompanied by the transient formation of 3,5-DCA (Figure 1a). In contrast, on pepper leaves no 3,5-DCA or any of the other transformation products considered (metabolites I and II) were detected during iprodione degradation (Figure 1b).

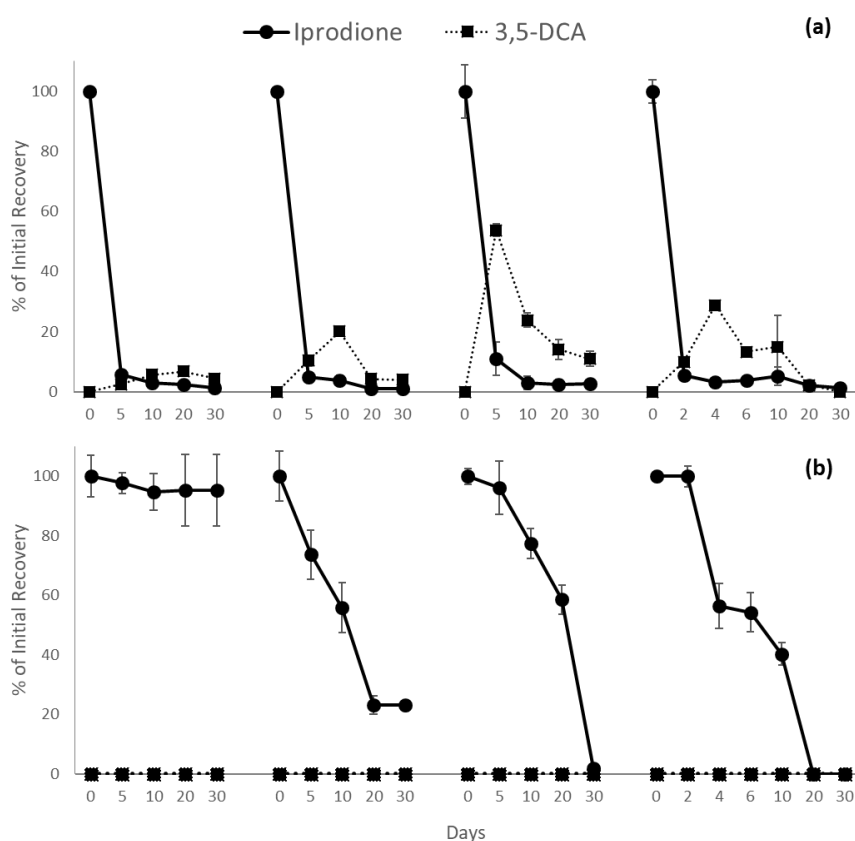


Figure 1. The degradation patterns of the four successive applications of iprodione (●) and the formation and dissipation of its main metabolic product 3,5 dichloroaniline

(3,5-DCA) (■) in soil (a) and plant leaves (b). Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

Table 1. The kinetic parameters describing the degradation of iprodione in soil and leaves of pepper plants calculated by fitting the data to the single first order (SFO) kinetics model.

Habitat	Application number	DT ₅₀ (days)	χ ² (%)
Soil	1	1.24	6.7
	2	1.23	7.1
	3	1.14	7.7
	4	0.45	14.3
Leaves	1	>365	1.3
	2	15.1	7.2
	3	11.5	20.6
	4	5.95	14.3

4.3.2. The impact of iprodione on the microbial community

4.3.2.1. The composition of the soil and epiphytic microbial community

In total 1,596,046 quality sequences for bacteria and archaea (9,959 - 59,201 and 10,132 – 45,180 sequences per sample in soil and leaves respectively) and 1,200,925 for fungi (9,137-29,835 and 8,344-35,455 sequences per sample in soil and leaves respectively) were obtained. These were assigned to 4,872 OTUs for bacteria and archaea, and to

4,560 OTUs for fungi. Rarefaction curves reached a plateau in all samples suggesting that our sequencing effort adequately covered the diversity of epiphytic and soil bacteria, archaea and fungi (Supplementary Figure S2). This is further supported by the Good's coverage (Good, 1953) values for bacteria, archaea and fungi which were $98.3 \pm 0.0\%$, $92.6 \pm 0.1\%$ and $99.7 \pm 0.0\%$ respectively (Supplementary Table S3).

Soil and plant leaves supported distinct bacterial, fungal and archaeal communities (Figure 2). The epiphytic bacterial community was dominated by *Proteobacteria* (mostly γ -, α - and β -*Proteobacteria*), which constituted on average more than 50% of the total bacterial community, followed by Actinobacteria (mostly of the class of *Rubrobacter*) and Bacilli (Figure 2a). In contrast, the bacterial community in the soil root zone showed a more even composition with high abundance of Actinobacteria (*Rubrobacteria*, *Thermoleophilia*), followed by Proteobacteria (γ - and α -*Proteobacteria*) and Bacilli (Figure 2a). The Soil Crenarchaeotic Group (SCG) prevailed in the phyllosphere at the earlier sampling dates, while *Aenigmarchaeota*, *Eyryarchaeota* and *Bathyarchaeota* were abundant only sporadically and their relative abundance did not follow a temporal or treatment trend (Figure 2b). The archaeal community in the soil root zone was dominated by SCG, while *Thermoplasmata* were detected at low abundances throughout the experimental duration in all samples (Figure 2b). The epiphytic fungal community was dominated by Ascomycetes (mainly *Dothideomycetes*, *Sordariomycetes*, *Microbotryomycetes*), and *Basidiomycetes* (mostly *Tremellomycetes*) were detected at a lower abundance (Figure 2c). *Ascomycetes* (*Sordariomycetes*, *Dothideomycetes*, *Eurotiomycetes*, *Pezizomycetes*) also prevailed in the soil root zone, while *Basidiomycetes* (*Agaricomycetes*, *Tremellomycetes*) were less abundant (Figure 2c). Significant temporal patterns on the relative abundance of certain

bacterial, archaeal and fungal taxa were observed only in the phyllosphere regardless of iprodione-treatment: (i) γ - and α -*Proteobacteria* were displacing each other in the bacterial community during the experimental duration; the former showed significant increases ($p < 0.05$) in their relative abundance after the 2nd and 4th applications and significant decreases ($p < 0.05$) after the 1st and 3rd applications, compared to α -*Proteobacteria* that showed the exact opposite patterns, (ii) the relative abundance of *Tremellomycetes* and *Microbotryomycetes* increased with time ($p < 0.001$) and (iii) the relative abundance of SCG decreased with time ($p < 0.05$) (Figure 2).

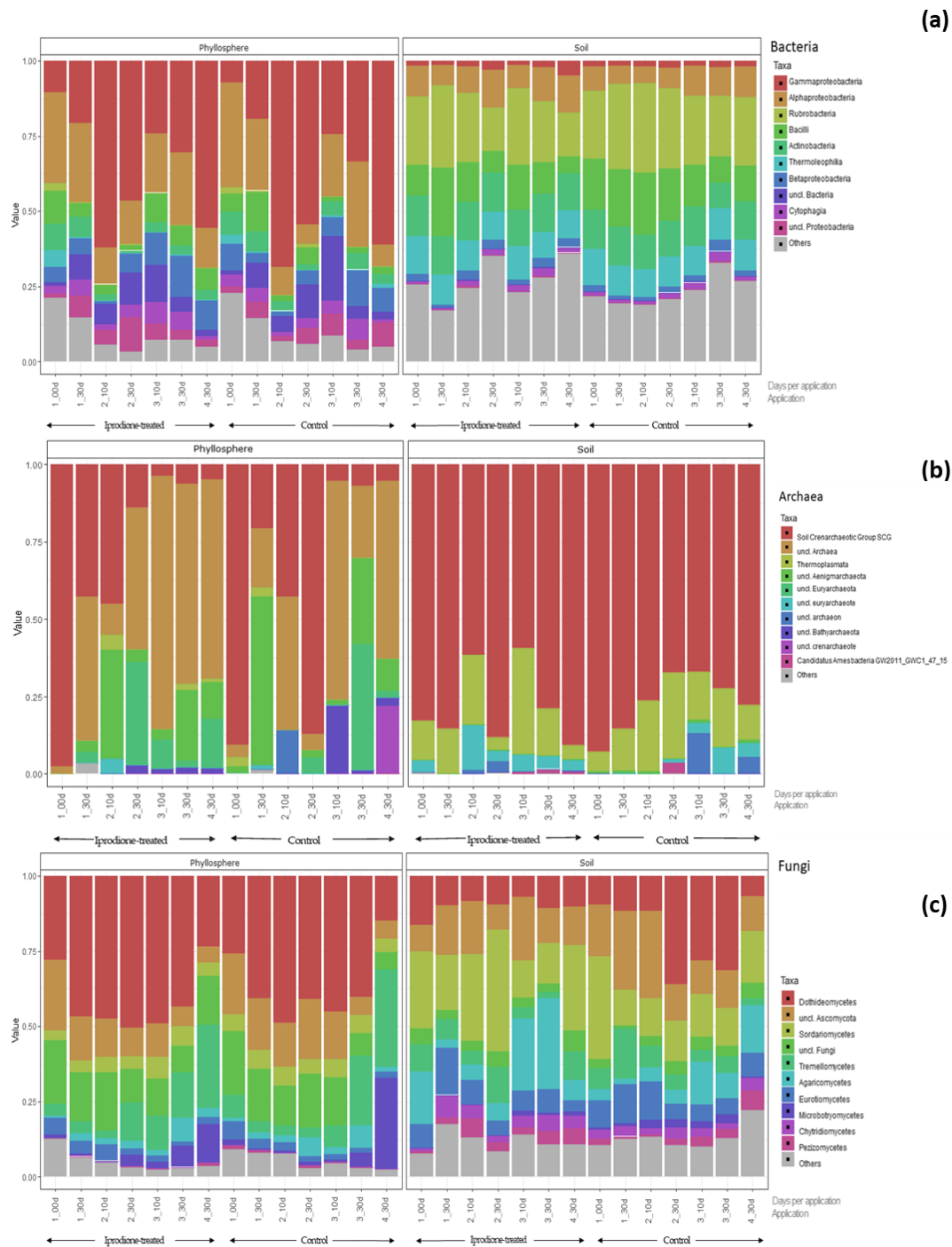


Figure 2. The relative abundance of the major classes of bacteria (a), archaea (b) and fungi (c) in the phyllosphere and the soil root zone of pepper plants repeatedly treated or not treated (control) with iprodione. The values presented at each time is the average of three biological replicates and error bars represent the standard deviation of the mean.

4.3.2.2. Effects of iprodione on the diversity of the microbial community

Iprodione did not induce significant effects on the α -diversity of bacteria, archaea and fungi in the soil root zone as shown by the different diversity indices (Supplementary Figure S1). Whereas, foliage applications of iprodione induced significant effects on the α -diversity of fungi (increase of Simpson index $p < 0.05$) and archaea (increase of Pielou's evenness index, $p < 0.05$) (Supplementary Figure S1).

CCA or RDA explored the effect of iprodione on the β -diversity in the two studied habitats and identified OTUs that increased in relative abundance in the presence or in the absence of iprodione exposure. The fungicide had a significant ($p < 0.001$) treatment-wise effect on the structures of bacterial community members in both habitats (Figure 3a and 3b). In the soil root zone, OTUs belonging to α -*Proteobacteria* (*Rhizobium*, *Rubellimicrobium*, *Microvirga*, *Altererythrobacter*), *Gemmatimonadetes* (*Longimicrobium*), *Chloroflexi* and *Blastococcus* increased in relative abundance in the samples treated with iprodione (Figure 3a). In the phyllosphere, OTUs belonging to *Bacteroidetes* (*Mucilaginibacter*, *Spirosoma*) *Enterococcus*, and *Entomoplasmateles* showed increasing abundance in the iprodione-treated samples, whereas OTUs belonging to *Actinobacteria* (*Corynebacterium*, *Arthrobacter*, *Pseudonocardia*), *Staphylococcus* and *Escherichia-Shigella* showed increased abundance in the phyllosphere of plants not treated with iprodione (Figure 3b). When the impact of iprodione on the β -diversity of archaea was investigated, RDA (Figures 3c) and CCA (Figures 3d), revealed a significant effect ($p < 0.01$) only in the soil root zone, where several OTUs (135, 751, 1042) affiliated to *Candidatus* Nitrososphaera showed increased abundance in the non-treated samples (Figure 3c). Iprodione induced significant treatment-wise changes ($p < 0.001$) in the β -diversity of

fungi in both studied habitats (Figure 3e and 3f). OTUs belonging to *Dothideomycetes* (*Lasiochaeriales*), *Sordariomycetes* (*Fusarium*, *Nectriaceae*, *Clonostachys*), Basidiomycetes (*Entoloma*), and *Chytridiomycetes* (*Spizellomyces*) increased in relative abundance in the soil root zone samples treated with iprodione, in contrast to OTUs belonging to *Cladosporium* and *Aureobasidium* which showed increased abundance in the samples not treated with iprodione (Figure 3e). In the phyllosphere, we observed OTUs belonging to *Saccharomycetes*, *Sordariomycetes* (*Nigrospora*), *Mucorales*, *Chytridiomycetes* (*Rhizophlyctidales*) and *Basidiomycetes* (*Puccinia*) that showed increased abundance in iprodione-treated plants, compared to OTUs belonging to *Agaricomycetes* (*Hypholoma*, *Parasola*) and *Taphrinomycetes* (*Taphrina*) that flourished in the samples not treated with iprodione (Figure 3f).

Further Spearman's correlation tests identified significant correlations between iprodione and 3,5-DCA concentrations and the abundance of bacterial and fungal genera, and archaeal classes, obtained from amplicon sequencing (Supplementary Figure S3). Hence, 3,5-DCA concentrations in the soil root zone were negatively correlated with *Sphingomonas* and positively correlated with *Thermoplasmatales*. Iprodione concentrations on the phyllosphere were positively correlated with fungi belonging to the genera of *Coniosporium*, *Chalastospora*, *Alternaria* and negatively correlated with fungi of the genera *Sordaria*, *Rhodotorula* and *Bensingtonia*.

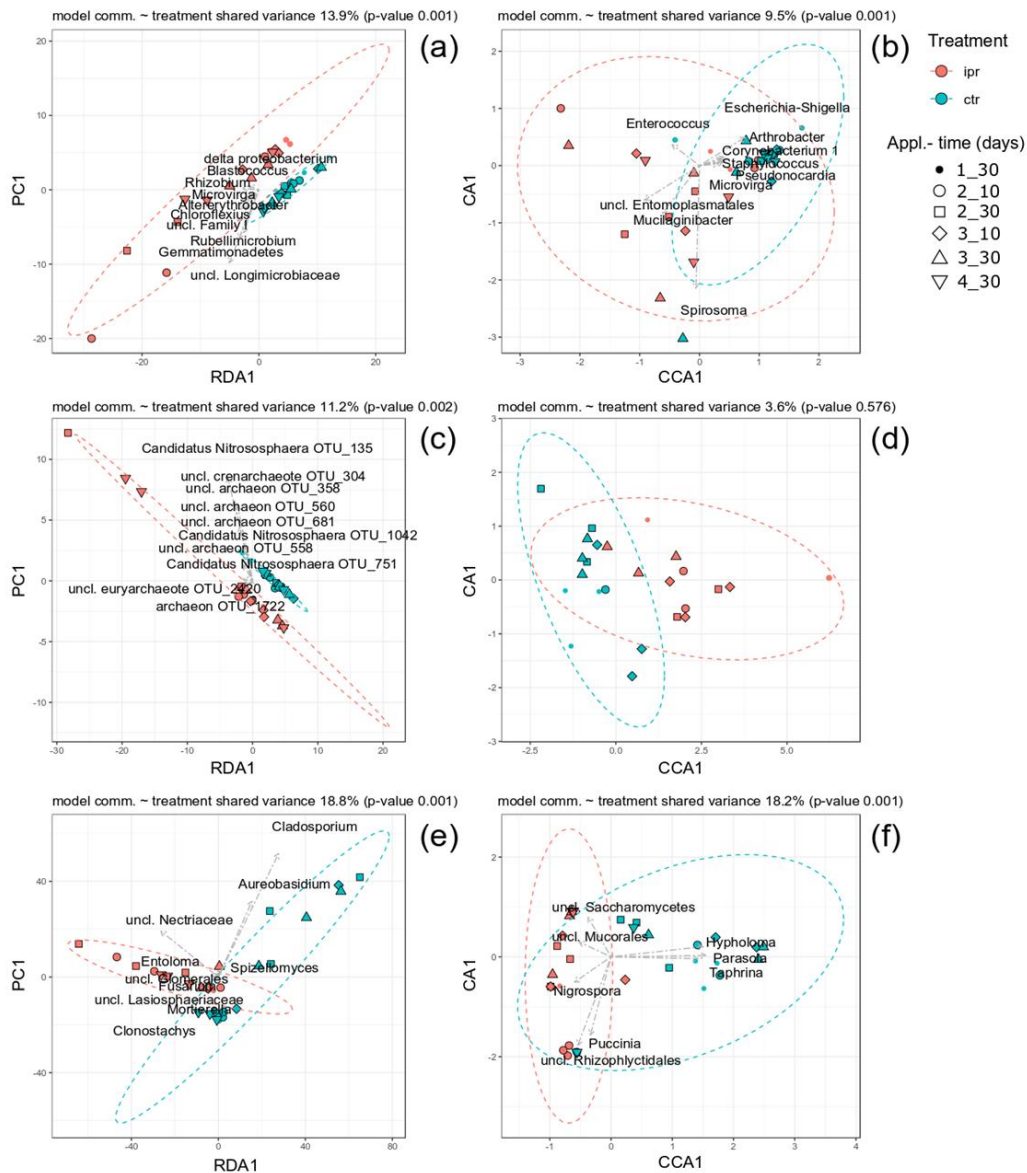


Figure 3. Multivariate analysis (Canonical Correspondence Analysis – CCA – or redundancy analysis – RDA – depending on the outcome of the first axis or detrended correspondence analysis) of the bacterial (a, b), archaeal (c, d) and fungal (e, f) OTU matrix in the soil root zone (a, c, e) and in the phyllosphere (b, d, f). The tested model was that of the community structure (bacterial/fungal/archaeal) being a function of the iprodione application with the coefficient of determination providing the model shared variance and the p-value indicating the null hypothesis probability (i.e. no effect). Arrows indicate the OTU gradients among samples as linearly regressed to the sample

scores (i.e. OTUs are more abundant in the samples of their arrow directions). Due to the fact that the tested parameter is one, only a single axis (X-axis) is canonical (contains the constrained variance) and the second axis (Y-axis) is that of the first principal component or the first correspondence analysis axis.

4.3.3. Isolation of iprodione-degrading bacteria from soil and phyllosphere

The transformation of iprodione in the enrichment cultures inoculated with soil and epiphytic microbial pellet was rapid in all media, while a slower degradation of iprodione was observed in the non-inoculated samples throughout the enrichment cultures (Supplementary Fig. S4). From the 120 colonies screened for iprodione degradation in the corresponding media, two colonies (TA1 and TA2), obtained from the MSM + iprodione soil enrichment cultures, and three colonies obtained from the MSM + iprodione (LP1, LP8) and MSM + SC (LP13) leaf enrichment cultures achieved more than 90% degradation in 6 days, compared to each medium control. Further sub-culturing and purification tests resulted in the isolation of three pure cultures named TA1.6, TA1.8 and LP13.7 which were composed of the same colony morphotype. Phylogenetic analysis based on the sequences of the 16S rRNA gene showed that all three isolates were closely associated and belonged to the genus *Paenarthrobacter* with highest match to a *P. nitroguajacolicus* strain (>99%) (Figure 4). Sequencing alignment of the full length 16S rRNA gene showed that the leaf isolate LP13.7 differed by the soil isolates TA1.6 and TA1.8 in 1 and 2 bp respectively, while the two soil isolates showed variation in 3 nucleotides.

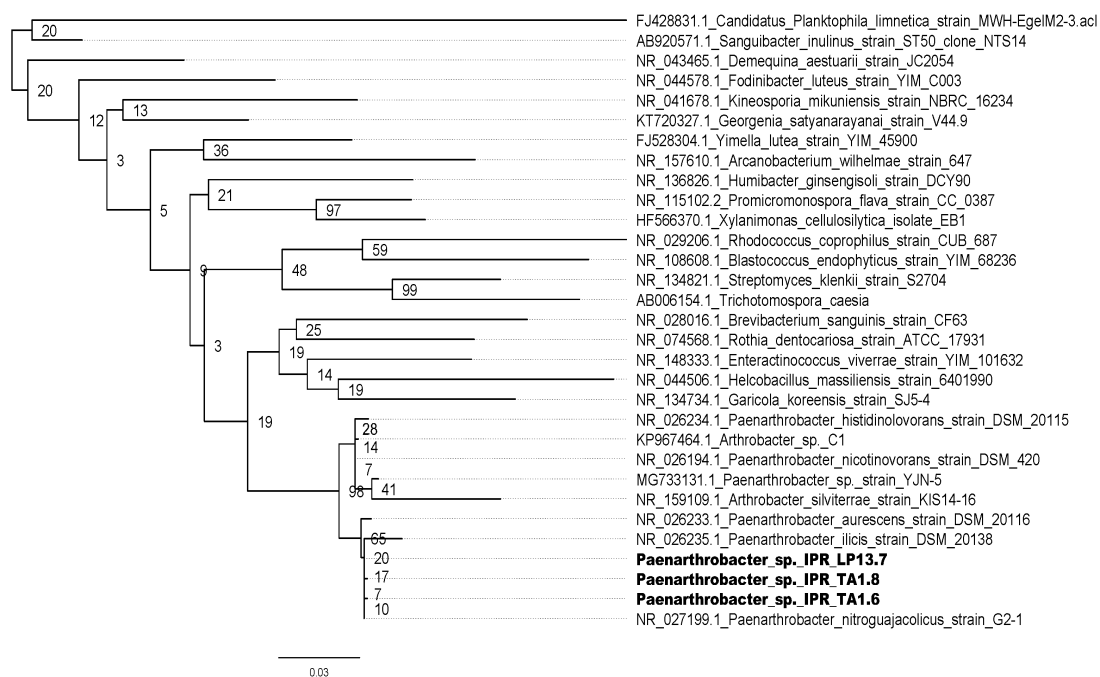


Figure 4. Phylogenetic analysis of the iprodione-degrading isolates TA1.6, TA1.8 and LP13.7 based on the complete 16S rRNA gene sequence. 1000 bootstrap replicates were run with PhyML following the GTRGAMMAI (General Time Reversible with GAMMA rate heterogeneity and considering Invariable sites) model. The bootstrap support is expressed in a scale from 0 to 100. The NCBI accession numbers of each bacterium are indicated.

4.3.4. Transformation of iprodione by the isolated bacteria

TA1.8 (soil-derived) transformed iprodione within 69 h with a DT_{50} of 19.8 h, as calculated by fitting the SFO kinetic model to the degradation data. The transformation of iprodione was accompanied by the transient formation of 60 and 40 nmol ml^{-1} of metabolites I and II respectively at 48 h. These were further transformed to 3,5-DCA which showed a peak concentration at 69 h and partially degraded thereafter (Figure 5a). A similar transformation pattern was evident for LP13.7 (phyllosphere-derived) where the rapid degradation of iprodione ($DT_{50} = 15.2$ h) was accompanied by the

transient formation of metabolite I and metabolite II, the latter at concentrations exceeding the 100 nmol ml⁻¹ (Figure 5b). Metabolite II was further transformed to 3,5-DCA which peaked between 36 and 48 d and degraded partially until the end of the study. Q-PCR analysis revealed that the rapid degradation of iprodione was accompanied by the proliferation of both bacteria strains from 36 h to maximum abundance at 117 h (Supplementary Fig. S5).

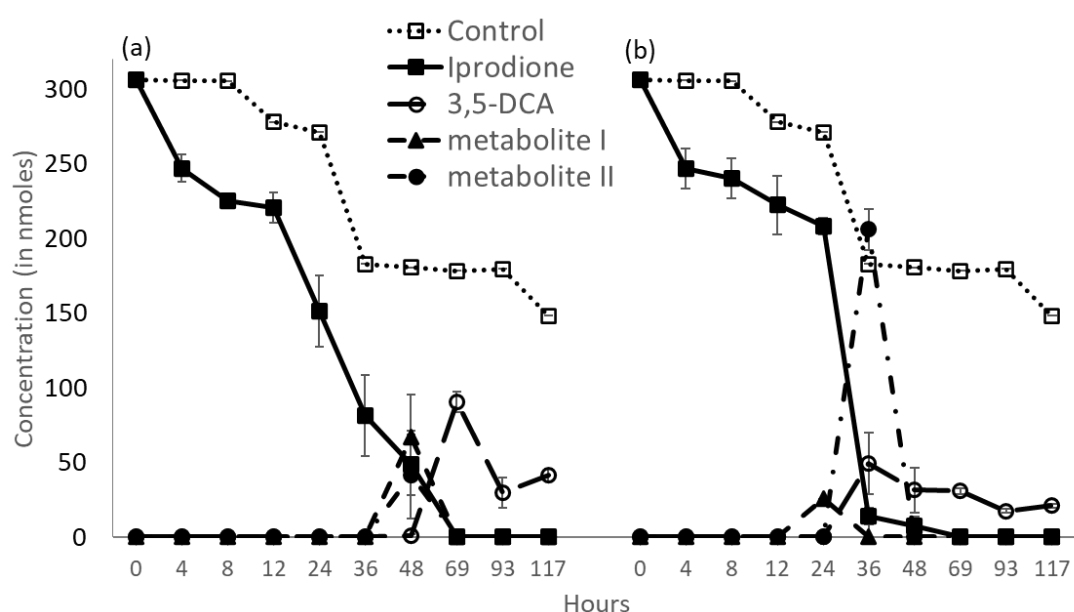


Figure 5. The degradation of iprodione (■) and the formation and degradation of metabolite I (▲), metabolite II (●) and 3,5-dichloraniline (3,5-DCA) (○) by isolates TA1.8 (a) and LP13.7 (b) in MSM. The degradation of iprodione in non-inoculated controls is also presented (□, dashed line). Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

4.4. Discussion

Repeated applications of iprodione in the soil root zone and on plant leaves accelerated the degradation of iprodione in both habitats. Previous studies have also reported an

accelerated degradation of iprodione in soils repeatedly treated with the fungicide in the laboratory and in soils from fields with history of fungicide exposure (Martin et al. 1990; Mercadier et al. 1996; Walker 1987). However, accelerated degradation of pesticides on the plant phyllosphere has not been reported before. The documented vulnerability of iprodione to accelerated biodegradation in soil coupled with the degradation pattern observed in the phyllosphere of pepper plants suggest that the epiphytic microbial community is equally capable to degrade iprodione in an accelerated mode.

Amplicon sequencing analysis showed that phyllosphere samples and samples collected from the soil root zone samples supported distinct microbial communities, in accordance with previous studies in rice (Knief et al. 2012), populus (Cregger et al. 2018) and the evergreen shrub *Scaevola taccada* (Amend et al. 2019). The epiphytic bacterial community was dominated by *Sphingomonadales*, *Methylobacteriaceae* and *Pseudomonadaceae*, in line with specific functional traits of members of these groups which support their epiphytic fitness like the efficient intracellular uptake of sugars (*Sphingomonas*), the assimilation of methanol released on plant phyllosphere (*Methylobacteriaceae*) and the motility to access nutrients (*Pseudomonas*), (Delmotte et al. 2009; Knief et al., 2012; Ryffel et al., 2016). Actinobacteria was the most abundant taxon in soil, as reported previously (Papadopoulou et al. 2018). The epiphytic and soil fungal communities were dominated by Ascomycetes and Basidiomycetes with different classes prevailing in the two habitats. *Dothideomycetes*, and *Tremellomycetes* prevailed on plant leaves most likely due to their capacity to thrive in extreme environments (Bálint et al. 2015; Yang et al. 2016; Gdanetz and Trail 2017), and the capacity of members of *Tremellomycetes* (i.e. *Cryptococcus*, *Dioszegia*) to tolerate

extreme temperatures and acquire and utilize nutrients in harsh environments (Wang et al., 2016). *Sordariomycetes* and *Agaricomycetes* dominated in soil in line with their capacity to efficiently exploit nutrients available in the root zone (Hussain et al. 2011; Simoes et al. 2015; Wang et al. 2018). The SCG was dominant in the soil archaeal community followed by *Thermoplasmata*, in accordance with previous reports (Chroňáková et al. 2015; Vasileiadis et al. 2013). In contrast the epiphytic archaeal community was more diverse and comprised of the SCG, *Aenigmarcheota* and *Eyryarcheota*. Previous studies have suggested that archaea are under-represented in the phyllosphere (Knief et al. 2012; Müller et al. 2015), hence their epiphytic communities have not been extensively explored. Recently Taffner et al. (2019) verified the epiphytic dominance of the SCG and *Eyryarcheota* on the phyllosphere of *Eruca sativa*, however the factors shaping epiphytic communities of archaea remain unknown.

The composition of the bacterial and fungal epiphytic and soil communities was significantly altered by the application of iprodione, in contrast to archaea whose β -diversity was significantly altered by iprodione only in soil. Recent studies using amplicon sequencing showed that iprodione, either repeatedly applied in soil (Zhang et al. 2017b; Zhang et al. 2017c) or used at increasing dose rates (Vasileiadis et al. 2018) induced significant changes on the β -diversity of soil bacteria and fungi. Additionally, our study provides first evidence for the response of the epiphytic microbial communities, including archaea, to pesticides exposure.

We further identified OTUs which increased in relative abundance in the presence or absence of iprodione. Hence iprodione application favored epiphytic microorganisms which (i) are involved in biomass decomposition like

Mucilaginibacter (Pankratov et al. 2007), *Saccharomycetes*, *Mucorales*, *Rhizophlyctidales* (Letcher et al. 2008; Hoffmann et al. 2013) (ii) are potential human pathogens like the lactic acid bacterium *Enterococcus* (Lebreton et al., 2013), often observed in plant phyllosphere (Vokou et al., 2012), and plant pathogens like *Nigrospora* (Wang et al. 2017) and *Puccinia* (Abbasi et al. 2005), (iii) are insect symbionts like *Entomoplasmatales* (Kautz et al. 2013), which could act as pathogens or exhibit mutualistic and manipulative effect on their host (Funaro et al., 2011). The stimulation of plant pathogenic fungi belonging to *Nigrospora* and *Puccinia*, that are within the spectrum of activity of iprodione (Mueller et al., 2005), might be associated with its accelerated biodegradation on plant leaves compromising its biological efficacy. In contrast in the absence of iprodione we observed increased abundance of OTUs assigned to (i) potential human and plant pathogens like *Staphylococcus*, *Escherichia-Shigella* and *Corynebacterium*, *Taphrina*, respectively (Chattaway et al. 2017; Oliveira et al. 2017; Richardson et al. 2018; Tsai et al. 2014) and (ii) organic matter decomposers like *Parasola* and *Hypholoma* (Nagy et al. 2009). In soil, iprodione treatment favored OTUs of α -*Proteobacteria* belonging to *Rhizobiales*, *Erythrobacteraceae*, *Methylobacteraceae*, in line with findings by Zhang et al., (2017c) who also reported an increase in the abundance of OTUs belonging to the same α -Proteobacterial taxa in soil after four repeated applications of iprodione. This could be attributed to their involvement in growth-linked degradation of iprodione or more likely to their capacity to tolerate iprodione and occupy soil niches liberated from competition upon toxicity of iprodione or grow on cell constituents released from dead microbial cells intoxicated by iprodione, in line with the copiotrophic lifestyle of α -proteobacterial classes (Fierer et al., 2012). Iprodione also favored fungal OTUs which

(i) are involved in cellulose decomposition like *Spizellomyces* (Letcher et al. 2008) (ii) are mycoparasitic like *Clonostachys* (Salamone et al. 2018) and (iii) belong to taxa rich in plant pathogens like *Fusarium* and *Nectriaceae* (Lombard et al. 2015), in line with the limited fungicidal activity of iprodione against Fusaria (Smiley and Craven, 1979). In contrast, in the absence of iprodione exposure fungal OTUs associated with saprotrophic fungi like *Cladosporium* (Bensch et al. 2012) and *Aureobasidium* (Zalar et al. 2008) were favored. An observation worth noting was the high abundance of OTUs belonging to *Candidatus Nitrososphaera*, an ubiquitous soil ammonia-oxidizing crenarchaeon (Tourna et al. 2011), in the untreated soil samples denoting a potential toxicity of iprodione. Similarly, Vasileiadis et al., (2018) demonstrated via amplicon sequencing analysis a significant negative correlation between iprodione soil concentrations and the abundance of OTUs belonging to the lineage *Nitrososphaerales* which encompass the OTUs of *Candidatus Nitrososphaera* identified in our study. Overall the application of iprodione significantly affected, positively or negatively, the abundance of OTUs assigned to microbial groups with important role for the homeostasis of the plant - soil ecosystem, which should be reconsidered in the context of the one-health system approach (Destoumieux-Garzon et al., 2018).

Apart from pesticide-driven effects, we observed clear succession in the abundance of certain bacterial and fungal taxa in the phyllosphere but not in soil. This is not surprising considering that compared to soil the leaf surface is directly exposed to extreme air temperatures (high or low), UV radiation, wind and precipitation which drastically affect the composition of the epiphytic community (Copeland et al. 2015; Hamonts et al. 2018). An observation worth noting is the compensatory relationship between α - and γ -*Proteobacteria* in the phyllosphere, regardless of iprodione treatment,

where one replaces the other along the experimental period. Similar observations were reported in the phyllosphere of perennial biofuel crops and were attributed to nutrient availability regulated by the plant development stage (Grady et al. 2019).

Enrichment cultures from soil and phyllosphere samples repeatedly treated with iprodione resulted in the isolation of phylogenetically close but not identical *Paenarthrobacter* strains from soil and plant phyllosphere. The genus *Paenarthrobacter* was recently formed by the reassignment of strains belonging to the *Arthrobacter aurescens* subgroup (Busse and Busse 2016). It comprises bacteria characterized by high catabolic versatility like the atrazine-, nicotine- and 4-nitroguaiacol-degrading strains *P. aurescens* TC1 (Mongodin et al. 2006), *P. nicotinovorans* pAO1 (Mihășan et al. 2018) and *P. nitroguaiacolicus* (Kotoučková et al. 2004), respectively, showing also remarkable fitness in soil (Mongodin et al. 2006) and plant phyllosphere (Scheublin and Leveau 2013; Scheublin et al. 2014). Our strains clustered together with two other iprodione-degrading strains; the recently isolated iprodione-degrading strain *Paenarthrobacter* YJN-5 (Yang et al. 2018) and *Arthrobacter* sp. strain C1 isolated from a grassland soil in Chile (Campos et al. 2015).

The bacteria isolated hydrolyzed iprodione to 3,5-DCA with the intermediate formation of metabolites I and II. This transformation pathway is shared among bacteria isolated from soils (Athiel et al. 1995; Campos et al. 2017; Yang et al. 2018) but it is reported for the first time in bacteria isolated from the plant phyllosphere. The capacity of the isolated bacterium, from the phyllosphere, to transform iprodione to 3,5-DCA contradicts to the lack of detection of this metabolite on plant leaves despite the accelerated degradation of iprodione. This could be most probably attributed to the

rapid photo-degradation and volatilization of 3,5-DCA on leaves surface once formed (Papantoni et al. 1995; Othmen and Boule 1999). The presence of extremely efficient 3,5-DCA-degrading epiphytic microorganisms, which rapidly degrade 3,5-DCA, is unlikely considering the remarkable recalcitrance of 3,5-DCA, the most recalcitrant amongst DCA isomers, to microbial degradation (Yao et al., 2011). The consistent presence of iprodione-catabolic traits in *Arthrobacter*-like bacteria isolated from distant geographic areas suggests a potential phylogenetic specialization of this bacterial genus in the degradation of iprodione which is not common in the bacterial world. This is further supported by the isolation of phylogenetically related iprodione-degrading *Paenarthrobacter* strains from soil and plant phyllosphere in our study. The mechanism driving this potential specialization of *Arthrobacter*-like bacteria to iprodione biodegradation would be further explored using comparative genomics.

No OTUs matching the 16S rRNA of our iprodione-degrading isolate were found in the amplicon sequences of the soil and epiphytic bacterial community. Correlation testing showed significant positive correlations between 3,5-DCA and iprodione concentration in soil and plant leaves with the abundance of bacterial, archaeal and fungal genera which have never been reported (i.e. *Thermoplasmata*, *Coniosporium*) or scarcely reported (*Micromonospora*, *Alternaria*) as pesticide degraders (Lipok et al, 2003, Fuentes et al., 2010). The positive correlation between *Alternaria* OTUs and iprodione concentrations might be attributed to resistance mechanisms since *Alternaria* plant pathogens are within the spectrum of fungicidal activity of iprodione (Mukherjee et al., 2003) and resistance to iprodione is ubiquitous amongst *Alternaria* strains (McPhee 1980; Ma et al., 2004). Although our sequencing effort provided a good coverage of the bacterial diversity in soil and phyllosphere

samples it cannot be excluded that the isolated bacterium remained at low abundance throughout the pot study due to the limited growth supported by the *in situ* concentrations of iprodione in the soil and phyllosphere environment studied. In support of this, Gallego et al. (2019) showed that pesticide-degrading bacteria constitute a particularly small fraction of the total bacterial community whose response to repeated pesticide exposure is not often detectable with DNA-based amplicon sequencing approaches and become visible only when RNA-based amplicon sequencing targeting the active fraction of the bacterial community are used.

4.5. Conclusions

Overall, repeated soil and foliage applications of iprodione induced compositional alterations in the soil and the epiphytic bacterial and fungal community. On the one hand it affected, negatively or positively, microorganisms with critical functional roles for the homeostasis of the plant-soil system. On the other hand it resulted in the accelerated biodegradation of iprodione, a result not previously reported in plant foliage and whose consequences for the (i) agricultural practice (i.e. loss of pesticide efficacy towards plant pathogens) (ii) environmental quality and (iii) consumers health (pesticides-free environment and products) could be important. Closely related iprodione-degrading bacteria of the genus *Paenarthrobacter* were isolated from soil and plant leaves repeatedly treated with iprodione, adding to the list of soil arthrobacters degrading iprodione and implying a possible phylogenetic specialization in the degradation of this compound. Further studies will aim to (i) disentangle the mechanism driving the development of pesticide accelerated biodegradation in the plant

phyllosphere and (ii) explore the arsenal of genes carried by the isolated bacteria with a putative role in the transformation of iprodione using comparative genomics and transcriptomics.

4.6. References

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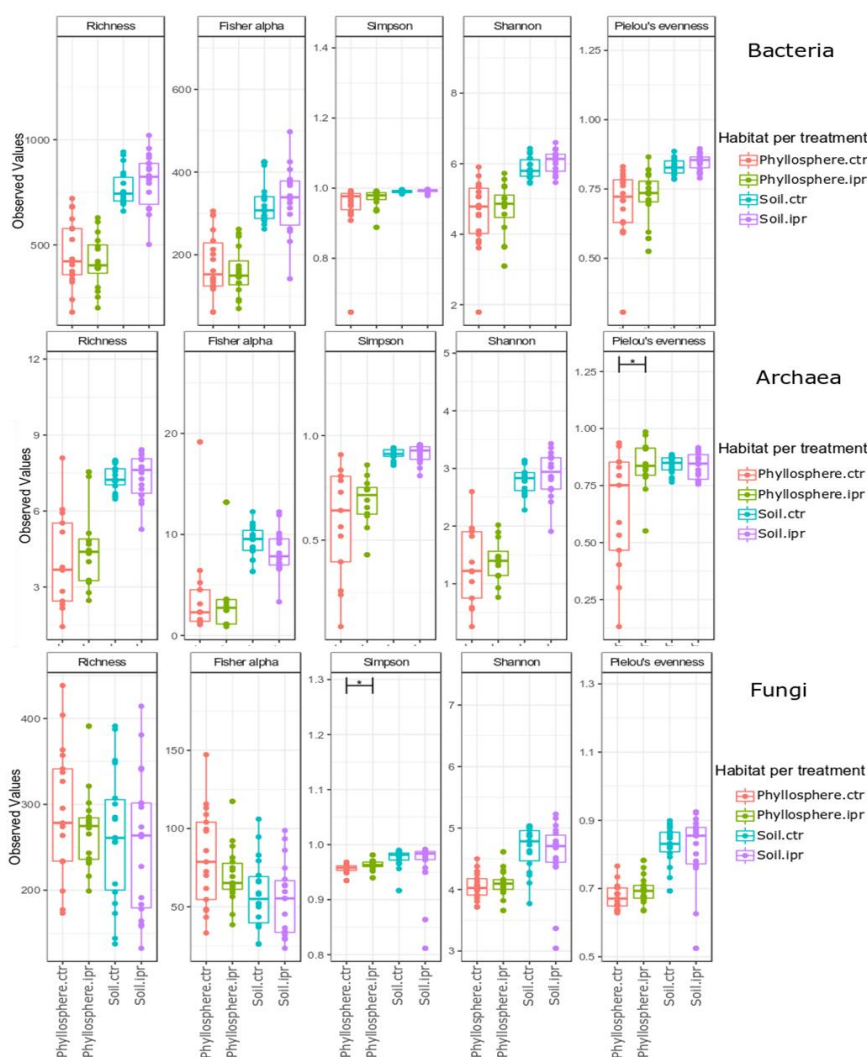
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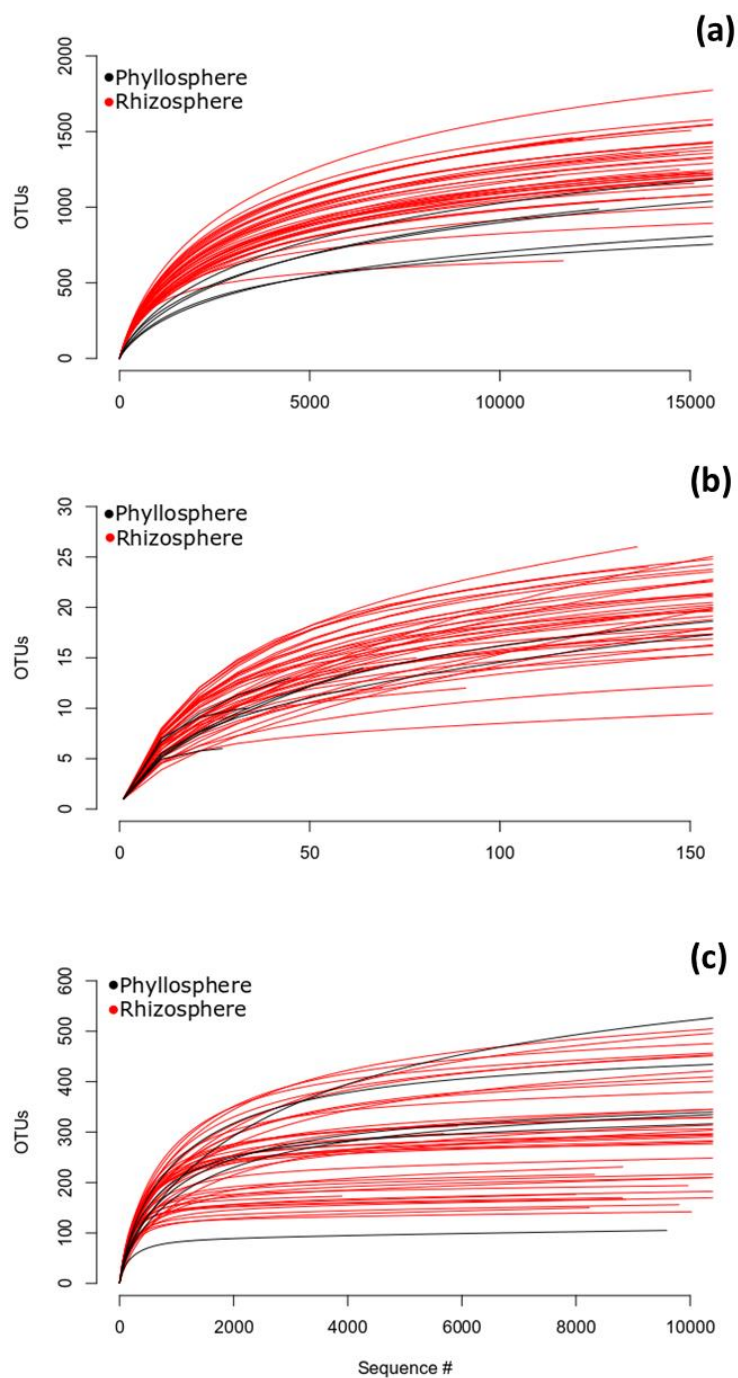
4.7. Supplementary Data

Chapter 4 - The response of soil and phyllosphere microbial communities to repeated application of the fungicide iprodione: Accelerated biodegradation or toxicity?

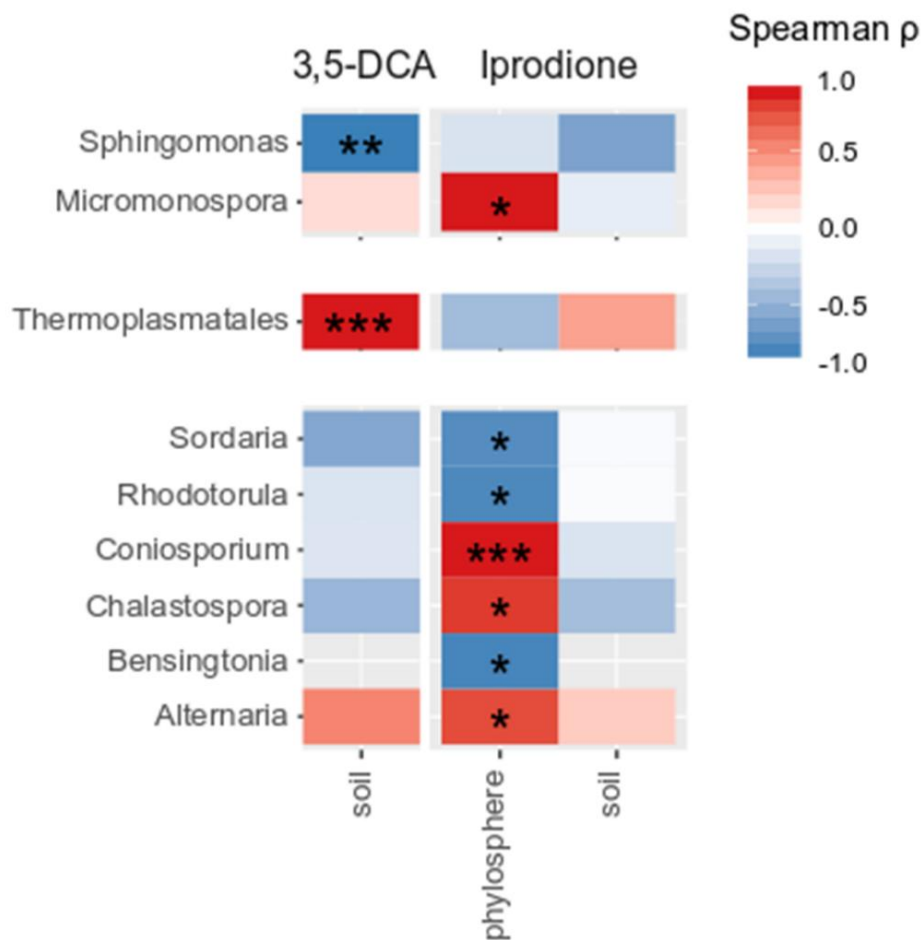


Supplementary Figure S1. The impact of iprodione on the α -diversity indices Richness, Fisher alpha, Simpson, Shannon and Pielou's evenness of bacteria, archaea and fungi in the phyllosphere of pepper plants and in soil. Significant differences between control (ctr) and iprodione-treated samples (Ipr) within each habitat are

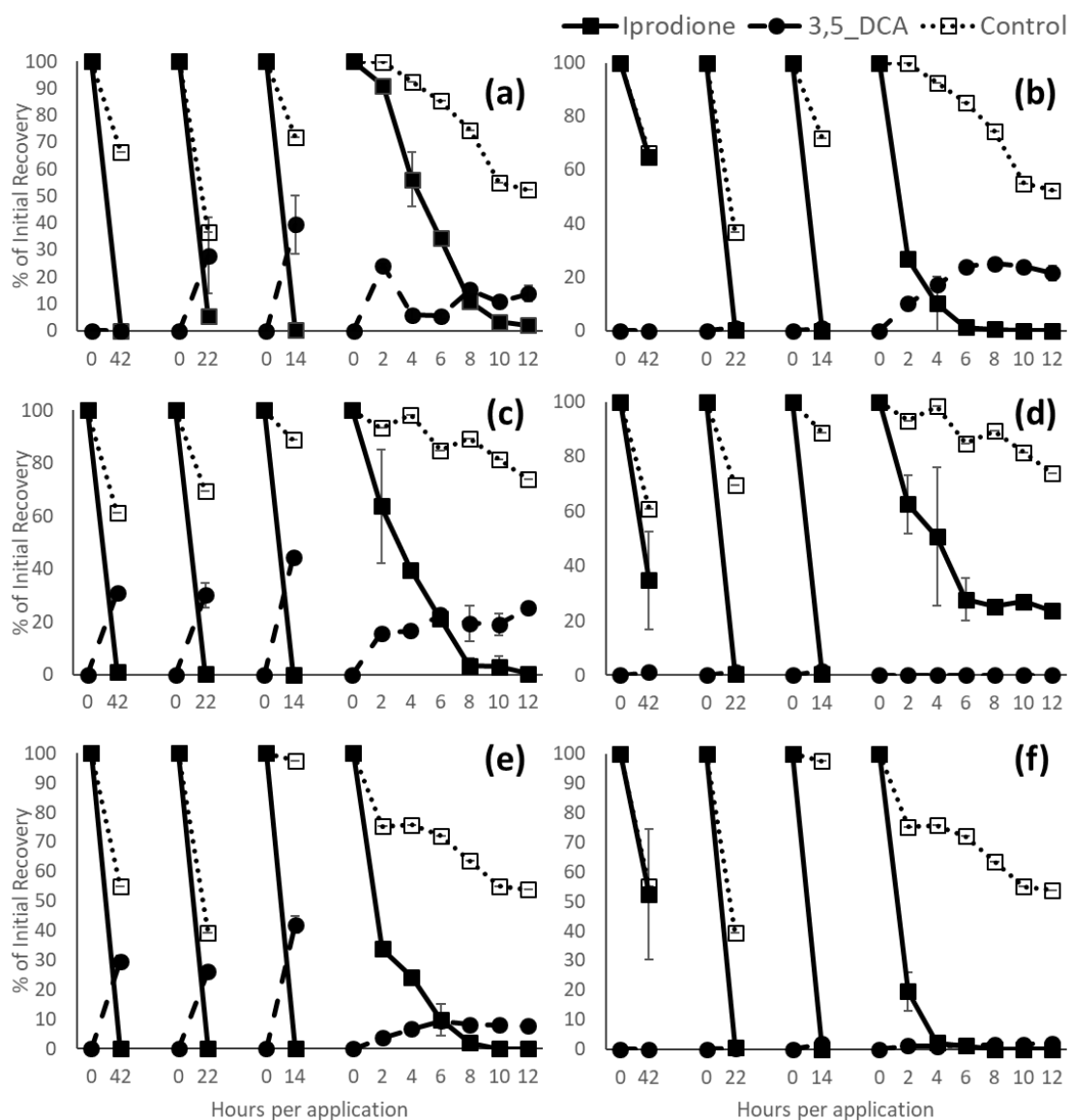
denoted with asterisks (* level of significance 0.05).



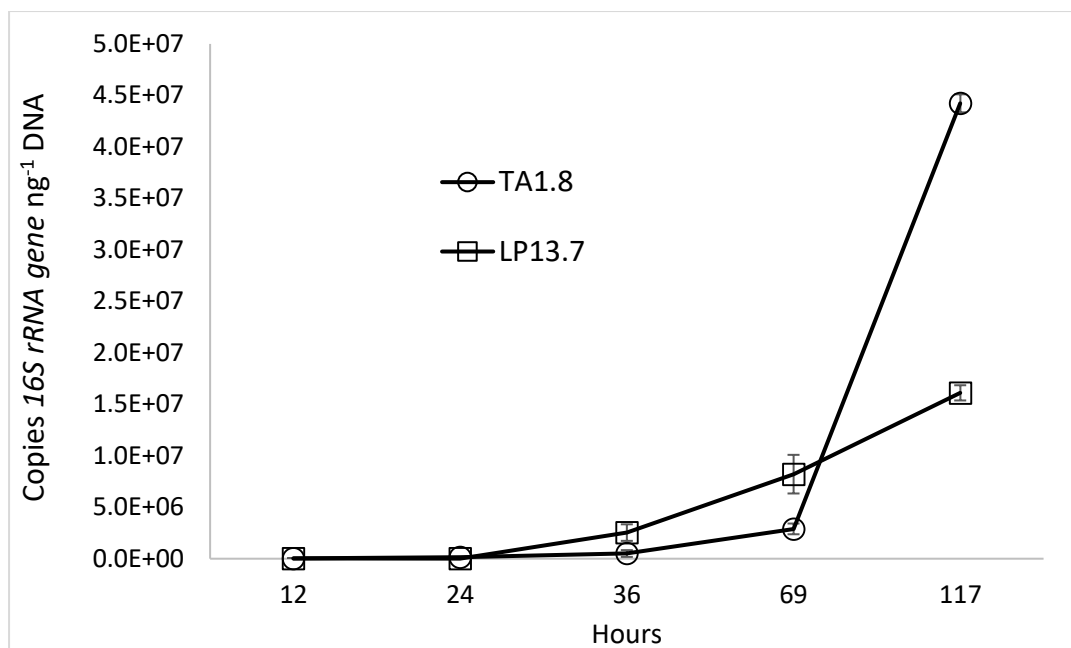
Supplementary Fig. S2. Rarefaction curves denoting the diversity coverage obtained by our sequencing effort for the bacterial (a), archaeal (b) and fungal (c) epiphytic and soil community.



Supplementary Figure S3. Heatmap of Spearman's correlation between the measured concentrations of iprodione and 3,5-dichloraniline (3,5-DCA) in soil and phyllosphere and bacterial and fungal genera and archaeal classes. The significance level of the different concentrations are designated with asterisks (*, **, *** correspond to significance levels of 0.05, 0.01 and 0.001 respectively).



Supplementary Figure S4. The degradation of iprodione (■) and the formation of 3,5-dichloroaniline (3,5-DCA)(●) in enrichment cultures in MSM (a, b), MSMN (c, d) and MSM+SC (e, f) supplemented with iprodione which were inoculated with soil or epiphytic biomass collected from pots repeatedly treated with iprodione. The transformation of iprodione and the formation of 3,5-DCA in non-inoculated cultures of the corresponding media was also determined (dashed line, empty symbols). Each value is the mean of three replicates \pm the standard deviation of the mean.



Supplementary Figure S5. The proliferation of *Paenarthrobacter* sp. strains TA1.8 (○) and LP13.7 (■) during degradation of iprodione as determined by q-PCR analysis of their 16S rRNA gene. Each value is the mean of three replicates with error bars representing the standard deviation of the mean.

Supplementary Table S1. The primers used in the current study. B000X-515f and FI000X-ITS4r are indexed primers used in the second amplification step which are composed of the sequence of the universal primers 515f (bacteria, archaea) and ITS4r (fungi) (**bold**), the indexes used for samples barcoding (underlined) and a TT sequence at the 5' end of each primer.

Primers	Sequence (5'-3')	Gene target	Fragment Length (bp)	Reference
Bacteria and Archaea				
515f	GTGYCAGCMGCCGCGGTAA	16S rRNA	290	Caporaso <i>et al.</i> , (2012)
806r	GGACTACNVGGGTWTCTAAT			Walters <i>et al.</i> , (2015)
B0001-515f	TT <u>CTTCTTCGT</u> GTGYCAGCMGCCGCGGTAA			This study
B0002-515f	TT <u>CTTCAAGGT</u> GTGYCAGCMGCCGCGGTAA			
B0003-515f	TT <u>CTTGTCAGT</u> GTGYCAGCMGCCGCGGTAA			
B0004-515f	TT <u>CTTGAGTGT</u> GTGYCAGCMGCCGCGGTAA			
B0005-515f	TT <u>CTTGACGT</u> GTGYCAGCMGCCGCGGTAA			
B0006-515f	TT <u>CTATAGGGT</u> GTGYCAGCMGCCGCGGTAA			
B0007-515f	TT <u>CTATCTCGT</u> GTGYCAGCMGCCGCGGTAA			
B0008-515f	TT <u>CTATGCAGT</u> GTGYCAGCMGCCGCGGTAA			
B0009-515f	TT <u>CTAACAGGT</u> GTGYCAGCMGCCGCGGTAA			
B0010-515f	TT <u>CTAGTTGGT</u> GTGYCAGCMGCCGCGGTAA			
B0011-515f	TT <u>CTCTTGTGT</u> GTGYCAGCMGCCGCGGTAA			
B0012-515f	TT <u>CTCTAACGT</u> GTGYCAGCMGCCGCGGTAA			
B0013-515f	TT <u>CTCAATGGT</u> GTGYCAGCMGCCGCGGTAA			
B0014-515f	TT <u>CTCAGAAGT</u> GTGYCAGCMGCCGCGGTAA			
B0015-515f	TT <u>CATATGGGT</u> GTGYCAGCMGCCGCGGTAA			
B0016-515f	TT <u>CATAGTCGT</u> GTGYCAGCMGCCGCGGTAA			
B0017-515f	TT <u>CATCACAGT</u> GTGYCAGCMGCCGCGGTAA			

B0018-515f TTCATCGATGTGYCAGCMGCCGCGGTAA
B0019-515f TTCAATCGTGTGYCAGCMGCCGCGGTAA
B0020-515f TTCAATGACGTGTGYCAGCMGCCGCGGTAA
B0021-515f TTCAACTAGGTGTGYCAGCMGCCGCGGTAA
B0022-515f TTCAACATCGTGTGYCAGCMGCCGCGGTAA
B0023-515f TTCAAGAGAGTGTGYCAGCMGCCGCGGTAA
B0024-515f TTCAAGGTTGTGTGYCAGCMGCCGCGGTAA
B0025-515f TTCAGTTCAGTGTGYCAGCMGCCGCGGTAA
B0026-515f TTCAGTAAGGTGTGYCAGCMGCCGCGGTAA
B0027-515f TTCAGACTTGTGTGYCAGCMGCCGCGGTAA
B0028-515f TTCAGAGGAGTGTGYCAGCMGCCGCGGTAA
B0029-515f TTCAGCAGTGTGTGYCAGCMGCCGCGGTAA
B0030-515f TTCAGCCAAGTGTGYCAGCMGCCGCGGTAA
B0031-515f TTCAGGTATGTGTGYCAGCMGCCGCGGTAA
B0032-515f TTCGTTCTAGTGTGYCAGCMGCCGCGGTAA
B0033-515f TTCGTTGGTGTGTGYCAGCMGCCGCGGTAA
B0034-515f TTCGTAGAGGTGTGYCAGCMGCCGCGGTAA
B0035-515f TTCGTGATCGTGTGYCAGCMGCCGCGGTAA
B0036-515f TTCGATGTGGTGTGYCAGCMGCCGCGGTAA
B0037-515f TTCGAATCAGTGTGYCAGCMGCCGCGGTAA
B0038-515f TTCGACAATGTGTGYCAGCMGCCGCGGTAA
B0039-515f TTCGAGCACGTGTGYCAGCMGCCGCGGTAA
B0040-515f TTGTTTCAGAGTGTGYCAGCMGCCGCGGTAA
B0041-515f TTGTTTCGTTGTGTGYCAGCMGCCGCGGTAA
B0042-515f TTGTTGTAGGTGTGYCAGCMGCCGCGGTAA

B0043-515f TTGTATCGAGTGTGYCAGCMGCCGCGGTAA
B0044-515f TTGTAATGGGTGTGYCAGCMGCCGCGGTAA
B0045-515f TTGTAAGTCGTGTGYCAGCMGCCGCGGTAA
B0046-515f TTGTAGAACGTGTGYCAGCMGCCGCGGTAA
B0047-515f TTGTCTTCAGTGTGYCAGCMGCCGCGGTAA
B0048-515f TTGTCTCTTGTGTGYCAGCMGCCGCGGTAA
B0049-515f TTGTCAGGTGTGTGYCAGCMGCCGCGGTAA
B0050-515f TTGTCGATAGTGTGYCAGCMGCCGCGGTAA
B0051-515f TTGTGTATCGTGTGYCAGCMGCCGCGGTAA
B0052-515f TTGTGTGAAGTGTGYCAGCMGCCGCGGTAA
B0053-515f TTGTGACTAGTGTGYCAGCMGCCGCGGTAA
B0054-515f TTGTGCAATGTGTGYCAGCMGCCGCGGTAA
B0055-515f TTGTGGTGTGTGTGYCAGCMGCCGCGGTAA
B0056-515f TTGATAGCAGTGTGYCAGCMGCCGCGGTAA
B0057-515f TTGATCTTGGTGTGYCAGCMGCCGCGGTAA
B0058-515f TTGATCAACGTGTGYCAGCMGCCGCGGTAA
B0059-515f TTGATGAGGGTGTGYCAGCMGCCGCGGTAA
B0060-515f TTGAACTCAGTGTGYCAGCMGCCGCGGTAA
B0061-515f TTGAAGTTCGTGTGYCAGCMGCCGCGGTAA
B0062-515f TTGAAGGAAGTGTGYCAGCMGCCGCGGTAA
B0063-515f TTGACTATGGTGTGYCAGCMGCCGCGGTAA
B0064-515f TTGACGTGAGTGTGYCAGCMGCCGCGGTAA
B0065-515f TTGACGAATGTGTGYCAGCMGCCGCGGTAA
B0066-515f TTGAGTTGGGTGTGYCAGCMGCCGCGGTAA
B0067-515f TTGAGTCATGTGTGYCAGCMGCCGCGGTAA

B0068-515f TTGAGAGTGGTGTGYCAGCMGCCGCGGTAA
 B0069-515f TTGAGCCTCGTGTGYCAGCMGCCGCGGTAA
 B0070-515f TTGAGGACAGTGTGYCAGCMGCCGCGGTAA
 B0071-515f TTGCATAAGGTGTGYCAGCMGCCGCGGTAA
 B0072-515f TTGCATGTTGTGTGYCAGCMGCCGCGGTAA
 B0073-515f TTGCAACAAGTGTGYCAGCMGCCGCGGTAA
 B0074-515f TTGCAGTATGTGTGYCAGCMGCCGCGGTAA
 B0075-515f TTGCCTAGTGTGTGYCAGCMGCCGCGGTAA
 B0076-515f TTGCCTCACGTGTGYCAGCMGCCGCGGTAA
 B0077-515f TTGCCAATCGTGTGYCAGCMGCCGCGGTAA
 B0078-515f TTGCCAGAGGTGTGYCAGCMGCCGCGGTAA
 B0079-515f TTGGTTGTCGTGTGYCAGCMGCCGCGGTAA
 B0080-515f TTGGTATGAGTGTGYCAGCMGCCGCGGTAA
 B0081-515f TTGGTCTATGTGTGYCAGCMGCCGCGGTAA
 B0082-515f TTGGTGCCAGTGTGYCAGCMGCCGCGGTAA
 B0083-515f TTGGAACTTGTGTGYCAGCMGCCGCGGTAA
 B0084-515f TTGGACATAGTGTGYCAGCMGCCGCGGTAA

Fungi

ITS7f	GTGARTCATCGAATCTTTG	ITS	310	Ihrmark <i>et al.</i> , (2012)
ITS4r	TCCTCCGCTTATTGATATGC			White <i>et al.</i> , (1990)
FI0001-ITS4r	TTATTACCGGATCCTCCGCTTATTGATATGC			This study
FI0002-ITS4r	TTATTAGGCGATCCTCCGCTTATTGATATGC			
FI0003-ITS4r	TTATTCTCCGATCCTCCGCTTATTGATATGC			
FI0004-ITS4r	TTATTTCGTGGATCCTCCGCTTATTGATATGC			
FI0005-ITS4r	TTATTGCGAGATCCTCCGCTTATTGATATGC			

FI0006-ITS4r TTTATACTGGGATCCTCCGCTTATTGATATGC
FI0007-ITS4r TTATACCTCGATCCTCCGCTTATTGATATGC
FI0008-ITS4r TTATACGCAGATCCTCCGCTTATTGATATGC
FI0009-ITS4r TTATAGACCGATCCTCCGCTTATTGATATGC
FI0010-ITS4r TTATGTTCCGGATCCTCCGCTTATTGATATGC
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FI0013-ITS4r TTATGAGCTGATCCTCCGCTTATTGATATGC
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FI0018-ITS4r TTAATCGGTGATCCTCCGCTTATTGATATGC
FI0019-ITS4r TTAATGTGGGATCCTCCGCTTATTGATATGC
FI0020-ITS4r TTAATGCCTGATCCTCCGCTTATTGATATGC
FI0021-ITS4r TTAATGGACGATCCTCCGCTTATTGATATGC
FI0022-ITS4r TTAACTTCCGATCCTCCGCTTATTGATATGC
FI0023-ITS4r TTAACTAGGGATCCTCCGCTTATTGATATGC
FI0024-ITS4r TTAACAGTCGATCCTCCGCTTATTGATATGC
FI0025-ITS4r TTAACCTTGGATCCTCCGCTTATTGATATGC
FI0026-ITS4r TTAACCGAAGATCCTCCGCTTATTGATATGC
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FI0028-ITS4r TTACTTACGGATCCTCCGCTTATTGATATGC
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FI0030-ITS4r TTACTAGAGGATCCTCCGCTTATTGATATGC
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FI0032-ITS4r TTACTCCTTGATTCCTCCGCTTATTGATATGC
FI0033-ITS4r TTACTGGCAGATCCTCCGCTTATTGATATGC
FI0034-ITS4r TTACATTGCGATCCTCCGCTTATTGATATGC
FI0035-ITS4r TTACAGTAGGATCCTCCGCTTATTGATATGC
FI0036-ITS4r TTACAGGTTGATCCTCCGCTTATTGATATGC
FI0037-ITS4r TTACCTAACGATCCTCCGCTTATTGATATGC
FI0038-ITS4r TTACCTCTAGATCCTCCGCTTATTGATATGC
FI0039-ITS4r TTACCTGGTGATCCTCCGCTTATTGATATGC
FI0040-ITS4r TTACCATCGGATCCTCCGCTTATTGATATGC
FI0041-ITS4r TTACCGTTCGATCCTCCGCTTATTGATATGC
FI0042-ITS4r TTACGTCAGGATCCTCCGCTTATTGATATGC
FI0043-ITS4r TTACGATACGATCCTCCGCTTATTGATATGC
FI0044-ITS4r TTACGACCAGATCCTCCGCTTATTGATATGC
FI0045-ITS4r TTACGCCGCGATCCTCCGCTTATTGATATGC
FI0046-ITS4r TTACGCGTAGATCCTCCGCTTATTGATATGC
FI0047-ITS4r TTAGTTCTGGATCCTCCGCTTATTGATATGC
FI0048-ITS4r TTAGTTGGAGATCCTCCGCTTATTGATATGC
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FI0050-ITS4r TTAGTACGTGATCCTCCGCTTATTGATATGC
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FI0052-ITS4r TTAGATGAGGATCCTCCGCTTATTGATATGC
FI0053-ITS4r TTAGACTACGATCCTCCGCTTATTGATATGC
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FI0055-ITS4r TTAGAGTCAGATCCTCCGCTTATTGATATGC
FI0056-ITS4r TTAGCAGATGATCCTCCGCTTATTGATATGC
FI0057-ITS4r TTAGCCTGTGATCCTCCGCTTATTGATATGC

FI0058-ITS4r TTAGGTACAGATCCTCCGCTTATTGATATGC
FI0059-ITS4r TTAGGCGCCGATCCTCCGCTTATTGATATGC
FI0060-ITS4r TTCTTATGGGATCCTCCGCTTATTGATATGC
FI0061-ITS4r TTCTTACTCGATCCTCCGCTTATTGATATGC
FI0062-ITS4r TTCTTAGCAGATCCTCCGCTTATTGATATGC
FI0063-ITS4r TTCTTCAGTGATCCTCCGCTTATTGATATGC
FI0064-ITS4r TTCTTCGACGATCCTCCGCTTATTGATATGC
FI0065-ITS4r TTCTTGAAGGATCCTCCGCTTATTGATATGC
FI0066-ITS4r TTCTTGTTGATCCTCCGCTTATTGATATGC
FI0067-ITS4r TTCTATTCCGATCCTCCGCTTATTGATATGC
FI0068-ITS4r TTCTATAGGGATCCTCCGCTTATTGATATGC
FI0069-ITS4r TTCTAACAGGATCCTCCGCTTATTGATATGC
FI0070-ITS4r TTCTACCGAGATCCTCCGCTTATTGATATGC
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FI0072-ITS4r TTCTAGCCTGATCCTCCGCTTATTGATATGC
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FI0074-ITS4r TTCTCTTAGGATCCTCCGCTTATTGATATGC
FI0075-ITS4r TTCTCTACAGATCCTCCGCTTATTGATATGC
FI0076-ITS4r TTCTCTCTTGATCCTCCGCTTATTGATATGC
FI0077-ITS4r TTCTCTGGCGATCCTCCGCTTATTGATATGC
FI0078-ITS4r TTCTCCATCGATCCTCCGCTTATTGATATGC
FI0079-ITS4r TTCTCCGCTGATCCTCCGCTTATTGATATGC
FI0080-ITS4r TTCTCGTGAGATCCTCCGCTTATTGATATGC
FI0081-ITS4r TTCTGTGTAGATCCTCCGCTTATTGATATGC
FI0082-ITS4r TTCTGAACCGATCCTCCGCTTATTGATATGC
FI0083-ITS4r TTCTGACGTGATCCTCCGCTTATTGATATGC

FI0084-ITS4r TTCTGCTCAGATCCTCCGCTTATTGATATGC

Supplementary Table S2. PCR reagents and thermocycling conditions used for amplicon sequencing analysis.

PCR reaction			
Reagents	Volume (µl)	Concentrations	Comments
Primer F	1	0.5 µM	
Primer R	1	0.5 µM	
BSA	0.4	0.4 µg/µl	Added only in the first amplification step
Polymerase Q5 (2x MasterMix)	10	1x	
ddH ₂ O	5.6		
DNA	2	0.2 ng/µl	
Total	20		

PCR conditions			
Step	Temperature (°C)	Time	Number of Cycles
Initial Denaturation	98	30 sec	
Denaturation	98	10 sec	28 in the first amplification step / 7 in the second amplification step

Annealing	50 for bacteria/ 55 for fungi	30 sec
Extention	72	30 sec
Final extention	72	10 min

Supplementary Table S3. Sequence depth of the samples analyzed based on Good's Coverage estimation. Each value is the mean value of three biological replicates± the standard deviation.

Samples			Good's coverage (%)		
Habitat	Treatment	Application No_days post application	Bacteria	Archaea	Fungi
Soil	Iprodione	1_00d	0.99±0.00	0.99±0.00	1.00±0.00
		1_30d	0.99±0.00	0.99±0.00	1.00±0.00
		2_10d	0.99±0.00	0.99±0.01	1.00±0.00
		2_30d	0.99±0.01	0.99±0.00	1.00±0.00
		3_10d	0.99±0.00	0.99±0.01	1.00±0.00
		3_30d	0.98±0.00	0.98±0.01	1.00±0.00
		4_30d	0.98±0.01	0.98±0.02	1.00±0.00
	Control	1_00d	0.99±0.00	1.00±0.00	1.00±0.00
		1_30d	0.98±0.01	0.98±0.01	1.00±0.00
		2_10d	0.97±0.01	0.96±0.03	1.00±0.00
		2_30d	0.99±0.00	0.98±0.01	1.00±0.00
		3_10d	0.96±0.03	0.97±0.01	0.99±0.00
		3_30d	0.98±0.01	0.93±0.08	1.00±0.00

		4_30d	0.98±0.00	0.97±0.01	1.00±0.00
Phyllosphere	Iprodione	1_00d	0.97±0.01	0.93±0.10	1.00±0.00
		1_30d	0.98±0.00	0.67±0.14	1.00±0.00
		2_10d	0.99±0.01	0.76±0.13	1.00±0.00
		2_30d	0.98±0.00	0.96±0.09	0.99±0.01
		3_10d	0.99±0.00	0.95±0.04	0.99±0.00
		3_30d	0.99±0.00	0.92±0.09	0.99±0.01
		4_30d	0.99±0.01	0.96±0.01	0.99±0.00
		Control	1_00d	0.97±0.01	0.95±0.01
	1_30d	0.98±0.01	0.89±0.05	0.99±0.01	
	2_10d	0.99±0.00	0.77±0.21	1.00±0.00	
	2_30d	0.99±0.00	0.64±0.08	0.99±0.00	
	3_10d	0.97±0.01	0.92±0.11	0.99±0.00	
	3_30d	0.99±0.00	0.92±0.11	1.00±0.00	
	4_30d	0.99±0.00	0.99±0.01	1.00±0.00	

Chapter 5

General Discussion and Future Perspectives

5.1. General Discussion

Phyllosphere and rhizosphere are plant-associated micro-habitats that are known to support diverse microbial communities whose structure is mediated by plants (Philippot et al. 2013; Vorholt 2012). We aimed to disentangle the mechanisms shaping microbial communities in the phyllosphere and the soil root zone and identify their response to agricultural practices like soil organic amendment and pesticide application. In this frame the focus was on plants indigenous to Mediterranean ecosystems, with some of them producing essential oils which are known to exert antimicrobial activities, while effects on cultivated plants were also explored.

We initially explored the factors shaping the microbial community of the phyllosphere in plants native to semi-arid Mediterranean ecosystems using q-PCR and amplicon sequencing approaches. We observed strong season effects on microbial abundance which varied according to the microbial domain studied; Crenarchaea, fungi, *Alternaria* and *Cladosporia* flourished in the summer as also reported before (Inácio et al. 2002; Osono and Mori 2005), in contrast to bacteria which showed increasing abundance in the winter (Maignien et al. 2014; Peñuelas et al. 2012; Rastogi et al. 2012; Yadav et al. 2004). Bacterial communities were dominated by typical epiphytic α -Proteobacteria (i.e. *Methylobacterium*, *Rhizobium* and *Sphingomonas*) (Aydogan et al. 2018; Delmotte et al. 2009; Grady et al. 2019; Knief et al. 2012; Ryffel et al. 2015) and *Chloroflexi* (*Anaerolinaceae*), (Copeland et al. 2015; Knief et al. 2012; Ottesen et al. 2016) which exhibited clear seasonal patterns; α -proteobacteria were favored in the winter and *Chloroflexi* in the summer. We extended our monitoring to epiphytic fungi and archaea, for which nothing is known regarding their ecological role and community composition in such plant communities. The fungal community was

dominated by Ascomycetes (Jumpponen et al. 2010; Perazzolli et al., 2014), *Capnodiales*, *Pleosporaceae* and *Dothioraceae* being key members whose abundance varied by plant host and season. We report that the archaeal epiphytic community of these plants is dominated by members of the Soil Crenarchaeotic Group (SCG), previously reported by Taffner et al. (2019) as main members of the community, and Aenigmarchaeota, reported for the first time as dwellers of the plant phyllosphere. The structure of the bacterial and fungal epiphytic communities were shaped by both season and plant-host, unlike the archaeal community whose composition was host-plant driven, as Taffner et al. (2019) previously reported. Further analysis on the plant features that determine the epiphytic microbial community showed that plant habit had a stronger effect on the assemblage of the epiphytic microbial communities compared to the aromatic nature which was a main determinant only of the fungal community. The strong seasonal effect on the abundance and diversity of epiphytic bacteria and fungi in plants of typical semi-arid Mediterranean ecosystems could be alarming considering the key role of the epiphytic microbiome in the ecological strategies and productivity of the plants (Laforest-Lapointe et al. 2017). These effects are expected to be further magnified in the coming years under climate change, especially in such semi-arid ecosystems exposed to rather extreme seasonal variations.

From the studied plants, *Quercus coccifera* showed unique features in the assemblage of its epiphytic microbiome. The bacterial community was stable across season, whereas its archaeal community showed strong seasonal variations in contrast to the patterns observed in the other studied plants. In addition, the epiphytic microbiome of *Q. coccifera* encompassed unique members like δ -Proteobacteria, previously reported in the plants of the same order as *Quercus* (Bragina et al. 2012;

Laforest-Lapointe et al. 2016; Miura et al. 2019; Redford et al. 2010; Sagaram et al. 2009) and *Methanomicrobia* (Taffner et al. 2018). *Q. coccifera* leaves are characterized by high thickness and thick mesophyll, limited availability of nutrients and water, high phenolics content and absence of trichomes (Yadav et al. 2005), features that might promote the establishment of micro-anaerobic conditions on its phyllosphere occupied by methanogenic archaea and anaerobic δ -proteobacteria.

We extended our study on aromatic plants by exploring their use, as soil amendment in the frame of implementation of sustainable agricultural practices in the Mediterranean region where these plants are native. We hypothesized that soil amendment with residues of aromatic plants like rosemary, spearmint and peppermint will impose strong alterations on the microbial community at the soil/rhizosphere interface driven by the release of the bioactive constituents of the essential oils of these plants (Kadoglidou et al. 2014). We determined these effects on different microbial groups, some of them having a key functional role in biogeochemical cycling, using q-PCR in comparison to the effects imposed by a commercial organic fertilizer and non-amended samples. In addition, we tested all these effects in the presence or absence of tomato plants, reinforcing the role of plant roots on shaping microbial communities in the root zone. We observed that soil amendment with peppermint, spearmint and the organic fertilizer stimulated the abundance of all proteobacteria and fungi regardless of the presence of tomato plants, in line with the copiotrophic character of these microbial groups (Fierer et al. 2007; Francioli et al. 2016). In contrast soil amendment with rosemary stimulated these copiotrophic groups only in the presence of tomato plants. The different effects of rosemary, compared to mints, on the soil microbiome is most probably associated with the different monoterpenoid components of the essential oils

of the different aromatic plants released in the soil upon incorporation of their residues (Karamanoli et al. 2018). The stimulation of copiotrophic microorganisms in the soil root zone upon amendment with rosemary plant residues only in the presence of tomato plants is most probably associated with the beneficial effect of rhizodeposits, known to favor copiotrophs (Philippot et al. 2013), which might avert any negative effects imposed by the components of the essential oils of rosemary. We further noticed variable response of different functional microbial groups to soil amendment. N-cycling microbial groups like AOA were stimulated by the amendment of soil with rosemary and the organic fertilizer, while AOB were not responsive. The differential response of the two ammonia-oxidizing microbial groups was most probably associated with the mixotrophic nature of AOA (Qin et al. 2014). S-cycling microbial groups like SOB were stimulated by soil amendment with mints and the organic fertilizer, a response most probably driven by the release of organosulfur containing substrates upon soil amendment, a hypothesis which remains to be tested. Little is known about the response of SOB to soil amendment with fresh organic matter and our study offers new insights on this area. Regarding microbial groups involved in C-cycling and specifically in the catabolism of aromatic compounds, we observed different responses of *pcaH*- and *catA*-carrying bacteria with the former stimulated by peppermint and organic fertilizer soil addition, while the latter showed no response. The differential response patterns of these two microbial groups could be related to the variable composition of the materials incorporated in soil.

All the above data suggested complex interactions between plant roots, components of the plant residues of rosemary and the soil microbiome which were further pursued via amplicon sequencing analysis. We noticed that rosemary soil

amendment was the stronger determinant of the structure of the bacterial and fungal community followed by time, whereas the presence of plant had no effect on the microbial composition. We observed a stimulation of α -, β - and γ -Proteobacteria with members of the genus *Xanthomonas* driving this effect for γ -Proteobacteria, in line with members of this group being capable of degrading xenobiotic organic compounds like pesticides (Rayu et al. 2017) and antibiotics (Thelusmond et al. 2016), making them candidate monoterpenes degraders. Whereas, Actinobacteria of the genera *Solirubrobacter*, *Rubrobacter*, *Agromyces* and *Blastococcus*, known to thrive in non-disturbed and pristine soils (Castro et al. 2019; Lee et al. 2011; Liao et al. 2019) were negatively affected by rosemary soil amendment. However, the most striking effect of rosemary soil amendment was the dominance of basidiomycetes of the order *Cantharellales*, genus *Minimedusa*, being favored by the release of fresh cellulosic material in the soil root zone. These fungi are known as early colonizers of cellulosic materials having allelopathic activity on *Fusarium* phytopathogens (Beale and Pitt 1995), hence the reduced abundance of *Fusarium oxysporium* f. sp. *narcissi* in the rosemary-amended soils. The stimulatory effect of rosemary plant residues soil incorporation on allelopathic basidiomycetes like *Minimedusa* could be exploited in soil amendment strategies to enhance the suppressiveness of agricultural soils to soil-borne plant pathogens.

We finally tested how another potential perturbation factor like pesticides could affect the epiphytic microbial community in comparison with the microbial community of the soil root zone. Our hypothesis was that repeated applications of a biodegradable fungicide like iprodione would impose similar responses to the microbial communities in the two plant-associated habitats which could span from microbial acclimation

towards enhanced biodegradation of the pesticide, to toxicity on members of the soil microbial community which could not readily metabolize the fungicide. Repeated application of iprodione on the foliage and via soil drenching of pepper plants, resulted in an accelerated degradation of the pesticide both in soil and on the phyllosphere. The vulnerability of iprodione to enhanced biodegradation is well-documented (Martin et al. 1990; Mercadier et al. 1996; Walker 1987), however it is the first time that this is demonstrated on the plant phyllosphere with potential consequences for the (i) agricultural practice (i.e. loss of pesticide efficacy towards plant pathogens) (ii) environmental quality and (iii) consumers health (pesticides-free environment and products), which should be further considered.

Amplicon sequencing analysis of the microbiome in the soil root zone and in the phyllosphere along this repeated application scheme showed that the bacterial and fungal communities were responsive to iprodione application at both studied habitats, whereas the archaeal community was affected only in soil root zone. Several members of the epiphytic and rhizospheric microbial community were found to be positively or negatively affected by iprodione including the ubiquitous in soil AOA *Candidatus Nitrososphaera*, plant and human pathogens and organic matter decomposers which might affect the homeostasis of the plant-soil system and should be reconsidered in the frame of the one-health approach.

Following up the enhancement of the degradation of iprodione on plant phyllosphere and in the soil root zone, we isolated from both habitats bacteria able to degrade iprodione. Interestingly all three isolates, two from soil and one epiphytic, were identified based on 16S rRNA sequencing as *Paenarthrobacter* strains clustering

together with other iprodione-degrading strains, like *Paenarthrobacter* YJN-5 (Yang et al. 2018) and *Arthrobacter* sp. strain C1 (Campos et al. 2015) previously isolated from distant geographical areas. Our bacteria degraded iprodione to 3.5-DCA, with the intermediate formation of metabolite I and II, a pathway shared with other soil iprodione – degrading bacteria (Athiel et al. 1995; Campos et al. 2017; Yang et al. 2018) but reported for the first time in epiphytic bacteria. All these results highlight a consistent presence of iprodione-catabolic traits in *Arthrobacter*-like bacteria isolated from distant geographic areas suggesting a potential phylogenetic specialization of this bacterial genus in the degradation of iprodione which is not common in the bacterial world.

Overall, we showed that plant phyllosphere is a habitat colonized by diverse bacteria and fungi, while archaea are less abundant and diverse. The epiphytic microbial community in plants native to a semi-arid Mediterranean ecosystem, including typical aromatic plants, was shaped by a variety of factors with plant-host and seasonality being strong determinants. The use of aromatic plants as soil amendment stimulated copiotrophic microorganisms found in the soil plant root zone and cellulose-degraders with allelopathic activities against soil-borne plant pathogens, an observation worth pursuing further. Finally, we showed that beyond native Mediterranean plants, the epiphytic microbiome of cultivated plants like pepper responds to external perturbations like pesticide applications with some of its members being affected negatively or positively, while others became acclimated to degrade pesticides with the same efficiency as their counterparts in the soil root zone. On top of all the above, this thesis reported the first epiphytic bacterium, a *Paenarthrobacter* strain, that could

degrade iprodione and suggested an uncommon specialization of Arthrobacters in the degradation of this fungicide.

5.2. Future perspectives

From our findings new scientific questions have emerged which we expect to pursue in the future like:

1. Finding the chemical and morphological plant traits that shape the epiphytic microbial communities in such semi-arid Mediterranean ecosystems, with particular attention given to *Q. coccifera* which appears to support a unique microbial assemblage on its leaves.
2. Further looking into the underling mechanism driving the beneficial outcome of the interaction of plant roots and rosemary soil amendment on the microbial community, by looking at reciprocal effects on plant primary metabolites and the persistence of essential oil components in the tomato rhizosphere.
3. Unraveling the genetic features that ensure the epiphytic and soil fitness and survival of the iprodione-degrading *Paenarthrobacter* sp. strains isolated from the two compartments via comparative genomic analysis.
4. Discovering the network of genes involved in the degradation of iprodione by the *Paenarthrobacter* strains and the evolutionary mechanisms of this phenotype (i.e. plasmid encoded or not, mechanisms of acquisition etc.) using transcriptomic/proteomic analysis.
5. Utilizing iprodione-degrading bacteria in bioremediation processes i.e. in the treatment of wastewaters from fruit packaging plants or the treatment of fruits

sprayed with iprodione to safeguard environmental quality and consumers safety respectively.

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- Katsoula A., Karamanoli K, Monokrousos N, Vokou D, Karpouzas DG, Which factors drive the composition of the microbial community in the phyllosphere of the plants of a Mediterranean ecosystem? 8th Congress of the Hellenic Ecological Society, Thessaloniki, Greece (20-23/11/2016)
- Katsoula A., Karagkiozi E, Karamanoli K, Vokou D, Karpouzas DG, The diversity of phyllospheric Crenarchaea and fungi in a Mediterranean ecosystem: the impact of season and plant hosts. 7th Conference of Mikrobiokosmos, 10 years of Microbial communities in Action, Athens, Greece (7-9/04/2017).
- Katsoula A., Vasileiadis S., Karpouzas D.G., Rhizosphere and phyllosphere response to repeated application of the fungicide iprodione: Selection for biodegradation or toxicity? 17th International Society of Microbial Ecology (ISME), Leipzig, Germany (12-17/08/2018).
- Katsoula A., Vasileiadis S., Sapountzi M., Karpouzas D.G., The response of the soil and phyllosphere microbial community to repeated application of the fungicide iprodione: Selection for biodegradation or toxicity? 8th Conference of Mikrobiokosmos, Microbial Communities as Growth Engines from Greece, Patra, Greece (18-20/04/2019).
- Papazlatani V.C., Perucchon C., Katsoula A., Lagos S., Papadopoulou E.S., Vasileiadis S., Karas P.A. and Karpouzas G.D., Isolating bacteria able to rapidly degrade fungicides used in fruit packaging industry: Tailored made inocula for the treatment of relevant agro-industrial effluents. 8th Conference of Mikrobiokosmos, Microbial Communities as Growth Engines from Greece, Patra, Greece (18-20/04/2019).
- Mitsagga C., Giavasis I., Katsoula A., Vasileiadis S., Karpouzas D. and Papadopoulou K., Characterization, Identification and Physiological Studies of a Pigment-producing Tentative Pseudomonas spp. with Antifungal Properties. 8th Conference of Mikrobiokosmos, Microbial Communities as Growth Engines from Greece, Patra, Greece (18-20/04/2019).

SEMINARS ATTENDANCE:

24/09/2015: Writing & Publication of Scientific Papers, University of Thessaly, Volos

19-23/09/2016: Attendance to Summer School of “Pesticides and Soil Microbes in the Era of Omics: Technological Advances and New Challenges for the Industry and the Academia”, organized within the frame of LOVE TO HATE project (IAPP Marie Curie Action FP7) of University of Thessaly, in collaboration with Patras University, INRA – Dijon-France, ENOVEO cp και AEIFORIA srl., Paou Monastery, Magnissia, Greece.

- 14-15/06/2017: Short course on meta-omics and their application on environmental microbiology. Organized by American Society of Microbiology, Paou Monastery, Magnissia, Greece.
- 24-28/06/2018: 1st Summer School of Mikrobiokosmos 2018, “The role of microbiome in ecosystem functioning, food security, human health and environmental protection”, Paou Monastery, Magnissia, Greece.
- 18/04/2019: Pre-Conference Hands-on Workshop: Analysis of microbial NGS data, organized in the context of the 8^{ou} Conference of Mikrobiokosmos, Microbial Communities as Growth Engines from Greece, Patra, Greece (18-20/04/2019).

SOCIAL CONTRIBUTION:

- 2019/2014: Volunteering to the project Researcher’s Night. A European volunteering project, that aims to inform the public about a researcher’s work.
- 2015/2016: Volunteering in the project “Microbes go kindergarten”. A volunteering project, visiting kindergarten schools to educate students about a researcher’s job.
- 2018: Volunteering to the project Thessaly Science Festival. A festival that presents local scientific work and explains science to public.