Aix-Marseille UNIVERSITE

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DE CET OCEAN DE L'ETRE QUI SOURDIT DE L'INCONNU

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KHAYYAM

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Sommaire

Introduction générale

La décomposition de la matière organique est indispensable pour assurer la continuité de la vie sur la Terre, car l'ensemble de ces processus assurent le recyclage des éléments nutritifs à l'échelle de la biosphère. En effet, l'absorption de nutriments par les plantes se réalise majoritairement via des formes inorganiques ce qui rend cruciaux les processus biologiques de décomposition et de minéralisation de la matière organique morte. Parmi les acteurs de ces transformations, les microorganismes, ont une action prépondérante grâce aux multiples enzymes intra et extra -cellulaires qu'ils synthétisent. Ainsi, les mécanismes de décomposition de la matière organique sont directement dépendants des interactions entre les diverses populations constitutives des communautés microbiennes et entre ces différentes communautés et leur environnement abiotique et biotique (Fitter et al, 2005 ; Zimmer, 2002).

Face à une anthropisation de plus en plus forte des milieux naturels, de nombreuses études tentent de répondre aux questions liées à l'implication des mécanismes fonctionnels microbiens dans la résilience des écosystèmes soumis à diverses perturbations et ceci dans différents contextes écosystémiques. Ces perturbations intenses peuvent être de nature et d'origine très variées: pollutions chimiques (organiques et/ou minérales) des sols via les activités industrielles (Sorgi, 2007), destructions rapides et généralisées de l'ensemble de la biomasse végétale générées par des incendies (Certini, 2005). Certains facteurs d'importance majeure dans la capacité d'un écosystème à répondre à une perturbation ont ainsi été mis en évidence comme la diversité des communautés microbiennes et leur redondance qui contribue à la permanence des fonctions. Il en est de même des effets de la récurrence de la perturbation qui favoriserait dans certains cas l'adaptation des communautés microbiennes et ainsi la pérennisation de leurs fonctions (Zornoza et al*,* 2007). Toutefois, malgré la diversité des écosystèmes étudiés, le fonctionnement des milieux littoraux, interface complexe et hétérogène, est encore peu documenté.

Les écosystèmes littoraux méditerranéens sont soumis à de fortes contraintes environnementales naturelles: stress hydrique, sols à érodibilité élevée et pauvres en matière organique et en nutriments, litières sclérophylles et stress halins liés à l'exposition aux embruns. Les milieux littoraux subissent aussi une forte pression anthropique associée à une importante fréquentation touristique qui menace leur biodiversité. Les pollutions chimiques liées aux rejets atmosphériques en relation avec une urbanisation intense ainsi que ceux résultant des activités industrielles passées et actuelles (Asia et al, 2009 ; Floch, 2008; Mille et al, 2007) ou les impacts écologiques des activités de loisir et de tourisme sont quelques exemples des perturbations anthropiques qui sont susceptibles d'affecter les zones littorales et plus particulièrement en contexte peri-urbain. Parmi les divers polluants

chimiques, les Hydrocarbures Aromatiques Polycycliques (HAP) sont des composés largement disséminés dans l'environnement (Blanchard et al, 2007 ; Sorgi, 2007) présentant une grande toxicité pour les organismes vivants (Andreoni et Gianfreda, 2007). Outre des cas de pollution massive et brutale des côtes (marées noires), ces molécules affectent aussi les sols et les sédiments de manière chroniques, notamment via les précipitations, à proximité des grandes villes et des zones portuaires dotées d'infrastructures pétrolières, (Albinet, 2006 ; Johnson et Karlson, 2007). Ainsi, les zones littorales en périphérie de l'agglomération marseillaise présentent des niveaux de pollution importants susceptibles de générer des contaminations conséquentes et récurrentes d'espaces naturels patrimoniaux, reconnus pour leur richesse biologique et très attractifs aux plans écotouristiques et subissant ainsi une fréquentation touristique saisonnière importante.

Certaines enzymes extracellulaires en particulier les phénoloxydase (laccases), grâce à leurs propriétés oxydatives relativement peu spécifiques, peuvent contribuer à la biodégradation de diverses molécules aromatiques dont des composés polluants comme les HAPs. L'action potentielle des enzymes microbiennes sur ce type de polluants ubiquistes constitue donc un élément important pour l'évaluation du potentiel de décontamination des sols. Une revue bibliographique relative aux paramètres susceptibles d'intervenir dans le contrôle de l'activité laccase montre l'importance de la salinité qui, plus globalement, est considéré comme l'un des plus importants facteurs de structuration des milieux littoraux. L'inhibition des laccases en présence de concentrations faibles en ions chlorures a été rapportée dans de nombreuses études (Farnet et al, 2008 ; Jung et al, 2002; Kim et Nicell, 2006). D'autres études ont mis en évidence une diversité importante des gènes intervenant dans la synthèse des laccases produites par les champignons colonisant différents contextes environnementaux et dont en résulte une hétérogénéité importante de ces enzymes (Blackwood et al, 2007; Luis et al, 2004; Lyons et al, 2003). Ainsi, le fonctionnement des enzymes microbiennes, en particulier des laccases dans les sols et litières des écosystèmes littoraux présentant des salinités plus ou moins élevées nécessitait d'être mieux connu.

Le massif de Marseilleveyre situé en bordure sud de la ville de Marseille a été choisi comme site d'étude. Sa principale particularité réside dans sa localisation en zone littorale et en situation périurbaine. Il est ainsi soumis à des perturbations variées et intenses d'origine anthropique (pollution atmosphérique, piétinement, déchets, etc). La nature géologique de la roche mère est essentiellement des calcaires Urgoniens avec de nombreux faciès dolomitiques. Les précipitations annuelles, sont inférieures à celles reçues sur le bassin de Marseille (inférieures à 500 mm/an). La sécheresse estivale qui caractérise le climat méditerranéen, y est également plus prononcée. Associée aux embruns salés cette sécheresse conditionne la subsistance d'une végétation xérophile adaptée à contexte

géologique et climatique. Si les températures sont comparables en été à celles de la zone continentale elles sont moins rigoureuses en hiver à cause de l'influence maritime forte et de l'effet d'abri. Le sol y est quasi inexistant. Les falaises calcaires prolongées d'éboulis sont parcourues de très nombreuses failles et fissures dans lesquelles s'ancrent les racines des végétaux. (Eichinger et al, 1997). La sur-représentation dans les écosystèmes littoraux et continentaux de la région PACA de *Pinus halepensis*, le pin d'Alep, une espèce pionnière expansionniste en Méditerranée (Barbero et Quézel, 1989), nous a conduits à retenir cette espèce pour notre étude. Ses caractéristiques de croissance et de régénération expliquent en grande partie la rapide expansion de cette espèce en France méditerranéenne (Barbero et al, 1990 ; Thomas, 2005). En Provence calcaire les trois principales espèces arborées *Pinus halepensis*, *Quercus ilex* et *Quercus pubescens* appartiennent à des modèles théoriques d'occupation spatiale différents (Barbero et al, 1990). La plasticité écologique du conifère *P.halepensis* lui permet d'être présent sous des contraintes trophiques et des stress climatiques variés. De plus, la bonne survie des graines dans différents types de milieux et leur fort potentiel dispersif par le vent confèrent à cette espèce une grande capacité d'expansion.

Ce projet se décline selon trois approches majeures permettant de répondre séquentiellement aux objectifs suivants:

i) Etudier, en contexte littoral, le fonctionnement des communautés microbiennes présentes au sein de litières de pin d'Alep, une espèce végétale en expansion en Méditerranée pouvant, en particulier, coloniser aussi des espaces soumis à un stress halin via les apports des embruns.

ii) Evaluer l'impact d'un polluant ubiquiste de type HAPs (anthracène) sur le fonctionnement microbien des communautés présentes au sein de litière de pin d'Alep exposée aux embruns, en se focalisant sur les enzymes phénoloxydase exprimé *in situ*.

iii) Comparer les potentialités fonctionnelles des communautés microbiennes issues de litière de pin d'Alep en contexte littoral et continental et en présence d'anthracène.

Les travaux de recherches réalisées au cours de cette thèse seront présentés dans trois chapitres, dans lesquels seront exposés, en référence aux questionnements affichés, les résultats obtenus et ceci à travers différentes publications.

Le premier chapitre décrira des expérimentations à la fois *in situ* et *in vitro* pour décrire le fonctionnement des communautés microbiennes en contexte méditerranéen littoral. L'approche *in situ* réside dans un suivi spatio-temporel du fonctionnement des communautés microbiennes présentes au sein de litières de pin d'Alep soumises à

différentes expositions aux embruns et donc à priori à des salinités variées. En complément à ces expérimentations, des approches *in vitro* ont aussi été mises en œuvre en vue de réaliser une caractérisation des laccases produites par des souches fongiques isolées à partir de ces litières. Cette démarche 'pasteurienne' n'avait pas pour objectif de décrire de manière exhaustive les champignons producteurs de laccase mais de tester un certain nombre des isoformes produites *in vitro* afin de préciser notamment si ces enzymes étaient inhibées par les ions chlorures.

Le deuxième chapitre présente des expérimentations en mésocosme pour évaluer la toxicité d'un HAP modèle (anthracène), sur les activités enzymatiques des communautés microbiennes présentes au sein de litières de pin d'Alep recueillies en zone littorale. Dans ce chapitre nous nous sommes également focalisés sur les laccases exprimées e*x situ* lors ces incubations afin de préciser leur implication dans la transformation de l'anthracène et ainsi, d'évaluer leur rôle potentiel dans les processus de détoxification des milieux naturels littoraux.

Le troisième chapitre est une approche comparative des effets de l'anthracène sur les activités microbiennes de la litière de pin d'Alep en contexte littoral et continental. L'objectif de cette dernière partie de l'étude est de définir si en fonction du contexte environnemental, il existe des variations des réponses fonctionnelles des communautés microbiennes consécutivement à l'apport d'un polluant aromatique. En d'autre termes de vérifier si certains facteurs abiotiques (comme la salinité) concourent à des structurations qualitatives et/ou quantitatives différentes des communautés microbiennes induisant une réponse fonctionnelle dissemblable face à un toxique. En conclusion, une confrontation et une synthèse de l'ensemble des résultats obtenus au cours de ces recherches seront présentées. Ces observations et leur analyse critique introduiront, pour finir, de nouvelles perspectives et des problématiques complémentaires en vue d'étayer voire de généraliser les résultats actuellement acquits par les études dans ce projet.

Synthèse bibliographique

La décomposition et la minéralisation de la matière organique sont des processus importants pour la régulation du flux du carbone sur la Terre. Le climat, la composition chimique de la matière organique et la diversité et la quantité des microorganismes intervenant dans ces réactions sont autant de facteurs de contrôle de ces dynamiques (Coûteux et al, 1995). En effet, le recyclage de la matière organique se réalise par des processus écologiques complexes réalisés par les communautés microbiennes dont la structure et les activités sont contrôlées par la composition chimique et la disponibilité de la matière organique, et par les paramètres environnementaux (humidité, température, interactions biotiques….) (Berg et MacClaugherty, 2008; Moorhead et Sinsabaugh, 2006).

1. Processus de la transformation de la matière organique dans les litières

1.1. Structure chimique de la litière

La litière est constituée des débris végétaux (feuilles ou aiguilles, brindilles, racines, fruits et fleurs) trouvés sur le sol et en cours de décompositions (Toutain, 1987). Selon Satchell (1974) les litières présentent de nombreuse catégories de molécules chimiques dont les trois premières sont les plus abondantes: La cellulose, les hémicelluloses, la lignine, les sucres, les acides aminés libres et les acides gras, les constituants solubles dans l'alcool ou l'éther éthylique tels que les cires, les résines ou de nombreux pigments comme les caroténoïdes et les anthocyanines qui s'accumulent lors de la dégradation des feuilles et les protéines. La composition et l'abondance relative de ces composés sont variables d'une litière : certaines sont riches en nutriments et en carbone facilement assimilable (carbon labile principalement les polysaccharides) tandis que d'autres contiennent des concentrations elevés de composés organique récalcitrants à la dégradation comme la lignine (Gillon et al, 1994 ; Gessner et al, 2010).

Ainsi il est possible de classifier les molécules chimiques des litières selon leur biodégradabilité par les systèmes enzymatiques des microorganismes. La quantité relative de substrats aux structures facilement dégradables comme la cellulose ou les hémicelluloses par rapport aux autres composés comme la lignine présentant une structure polyphénolique plus stable, définit la qualité de la matière organique d'une litière.

- **La cellulose** est le composé biologique le plus abondant dans la nature. Constitutive de la paroi cellulaire des végétaux, elle est liée à d'autres polysaccharides ainsi qu'à la lignine. C'est un polymère linéaire composé de molécules de glucose liées entre elles par des liaisons 1,4-β-glucosidiques (Fig. 1). Selon son origine le nombre de molécules constitutives des chaînes de glucose peut varier de 1000 à 10000 unités. Cependant, il

existe très peu de variation de degré de polymérisation entre les celluloses d'espèces végétales différentes.

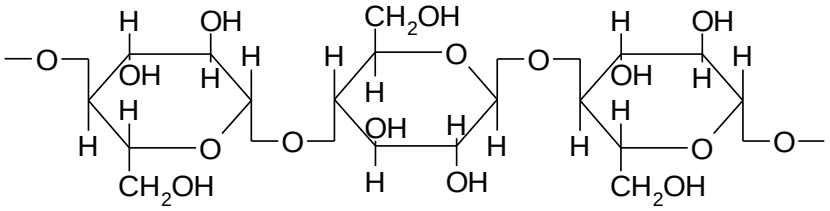


Figure 1. Structure de la cellulose

-**Les hémicelluloses** consituent le second polymère le plus abondant chez les végétaux. Ce sont des hétéropolysaccharides très ramifiés formées par des pentoses (D-xylose, L-arabinose), des hexoses (D-galactose, D-mannose, L-rammose, L-fructose) et des acides uroniques (acide D-glucuronique) (Fig. 2).

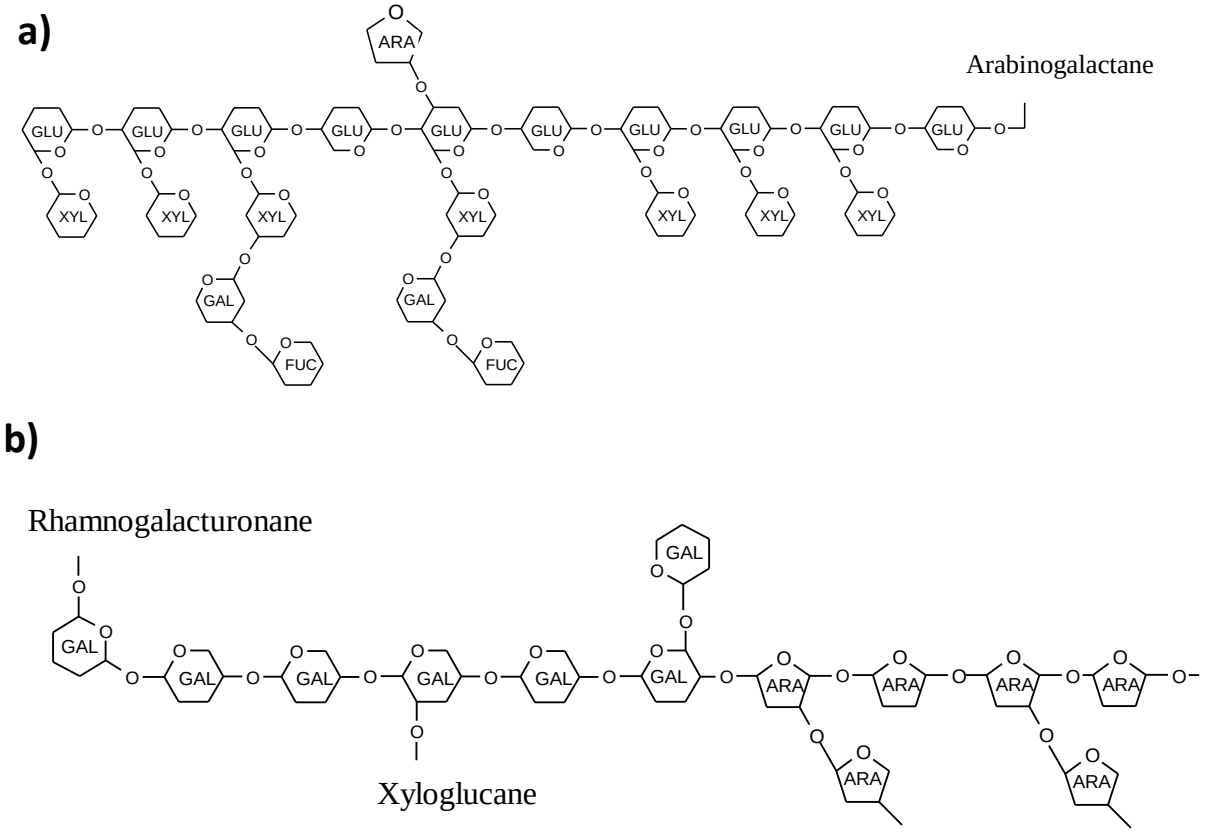


Figure 2. Structure d'hémicelluloses : a) arabinogalactane b) xyloglucane.

- **La lignine** est le troisième composé le plus abondant chez les plantes. Ce polymère aromatique est constitué de monomères à structure phénylpropanoïde comme l'alcool coumarilique (*p*-hydroxycinnamique), l'alcool conifférylique (4-hydroxy-3-métoxycinamique) et l'alcool sinapylique (4-hydroxy 3,5-diméthoxycinnamique). Ces unités sont liées entre elles par des liaisons arylglycérol-β-aryl éther (50% des liaisons), biphényle (liaisons carbone-carbone), et des liaisons phénylcoumaranes de nature plus complexe. Un modèle de structure linéaire a été récemment proposé par Banoub et Delmas, (2003) (Fig. 3). La proportion relative des trois alcools dépend de la plante et des tissus considérés.

La lignine est une macromolécule amorphe et insoluble dans l'eau. Dans la paroi cellulaire des végétaux, elle est associée aux hémicelluloses formant ainsi une matrice qui entoure la cellulose. La lignine confère donc, sa rigidité à la structure végétale. Elle joue également un rôle protecteur face aux attaques enzymatiques microbiennes (Higuchi, 1993). En effet la structure formée par ces trois composants est très résistante à la dégradation biologique car la lignine empêche l'accès des enzymes du type hydrolase aux composants polysaccharidiques (Reid, 1995).

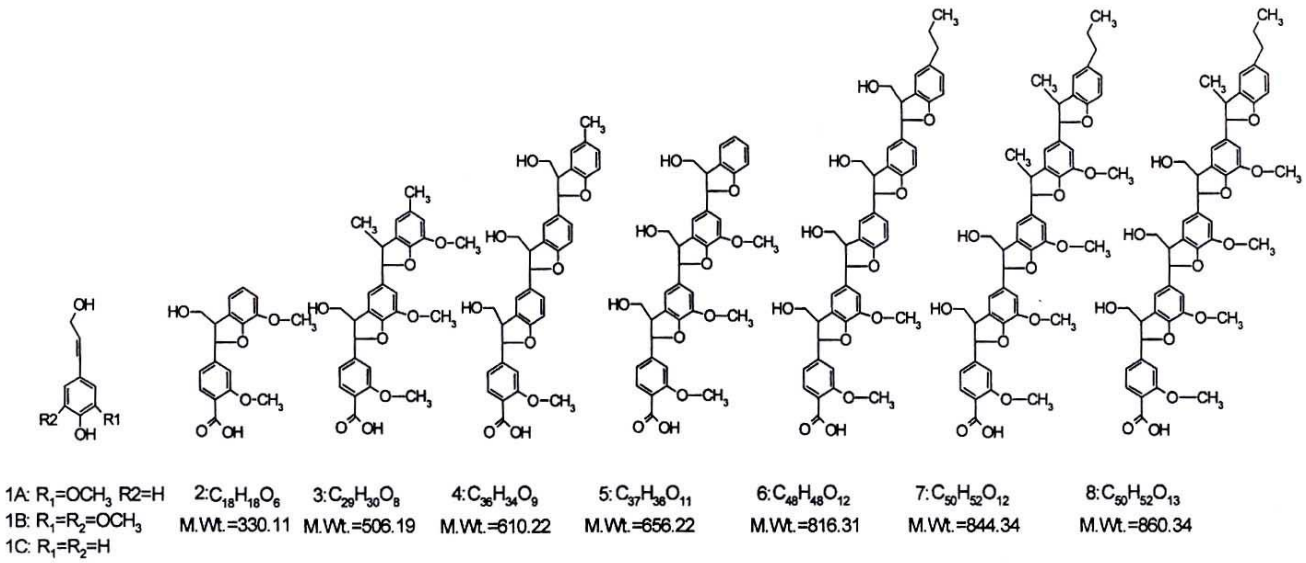


Figure 3. Structure de la lignine selon Banoub et Delmas (2003).

1.2. Les principaux modèles de dégradation des litières

Le recyclage de la matière organique est un processus fondamental de l'écosystème qui contrôle la croissance et la structure des communautés végétales. La composition des espèces végétale affecte également de manière significative le turnover de la matière organique par la qualité spécifique des litières mais également par les interactions plant-microorganismes dans la rhizosphère (Hättenschwiler et al, 2005).

Différents modèles ont été proposés afin de déterminer le rôle des facteurs biotiques et abiotiques du milieu influençant les processus de minéralisation des composés organiques (Berg, 2000 ; Johansson et al, 1995 ; Meentemeyer, 1978).

Berg et Staff (1980) ont proposé un modèle simple concernant la dégradation de litière de *Pinus silvestris* (Fig 4). Dans ce modèle, on peut distinguer deux phases principales de dégradations successives. Dans la phase précoce (I) le taux élevé des éléments nutritifs (azote, phosphore, soufre, etc) et de composés solubles ainsi que les facteurs climatiques, favorisent une dégradation rapide de la matière organique en particulier des composés non phénoliques et facilement assimilables par les microorganismes. Dans les phases tardives, le progrès de la dégradation est mesurable via l'évolution de la quantité de composés phénoliques dont la transformation est affectée négativement par la concentration de l'azote et positivement par la quantité de cellulose.

Dans ce modèle, la minéralisation de la matière organique est contrôlée par trois facteurs essentiels : le climat, la qualité de la litière, la nature et l'abondance des microorganismes décomposeurs. Ainsi le climat est le facteur dominant dans les milieux où les conditions climatiques sont drastiques (Couteux et al, 1995) alors que la qualité de la litière constitue le facteur dominant dans les milieux présentant des conditions climatiques plus favorable.

Toutefois, Moorhead et Sinsabaugh (2006) démontrent l'importance des microorganismes dans leur modèle GDM (guild-based decomposition model) dans lequel l'association entre différents groupes de microorganismes est mentionnée comme majeure. Les communautés microbiennes qui sont spécialisées dans la dégradation des composés récalcitrants - avec une croissance assez lente comme les champignons de la pourriture blanche - sont l'un des groupes considérés. En effet, l'efficacité de la décomposition est liée aux interactions compétitives entre différents groupes microbiens dont le résultat dépend notamment des capacités d'utilisation des substrats et des vitesses de croissance différentielles. En conséquence, ces interactions conduisent à un équilibre entre les populations microbiennes, dont la résultante sera une capacité de transformation de la matière organique plus ou moins efficace.

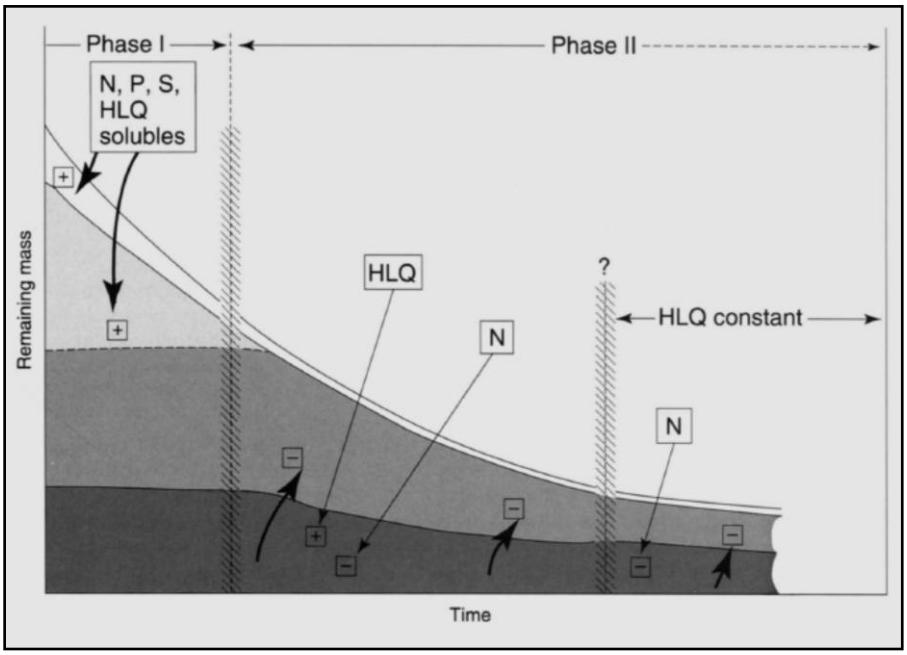


Figure 4. Modèle de transformation de la matière organique dans une litière de *Pinus sylvestris* extrait de Coûteux et al. 1995. Dans la première phase de décomposition (phase I), la concentration élevée des nutriments tels que l'azote (N), le phosphore (P) et le soufre (S), entraine une perte de mass accentué due à la dégradation des composés non lignifiées. La concentration élevée de composés facilement dégradables et solubles ainsi que la présence de cellulose influencent une perte de masse importante. Dans la phase tardive (phase II), où les matériaux lignifiés restent à décomposer, la dégradation de la litière dépend de la perte de lignine et est ainsi influencée négativement par la concentration de l'azote (N) et positivement par la présence de la cellulose. Les flèche noire indique les effets positifs et négatifs de la lignine sur la dégradation, (+) et (-). Les zones des plus claires au plus sombres représentent les composés solubles, les carbohydrates non lignifiés, les carbohydrate lignifiés et la lignine.

1.3. Rôle spécifique de communautés microbiennes dans la décomposition de la litière

Différents groupes d'organismes intervienne dans la décomposition de la litière. Les méso et micro faunes sont les premiers acteurs des processus de transformation des résidus végétaux et accélèrent les processus de décomposition par leur intervention dans la fragmentation et l'enfouissement de la matière organique. Ils contribuent aussi à la dissémination des bactéries et des champignons dans les différents horizons des litières et du sol (Berg et Laskowski, 2006).

En ce qui concerne les microorganismes, leur diversité dans la litière est supposée être extraordinairement élevée, mais sous estimée car en grande partie non identifiée.

Lors de la décomposition, le changement dans la qualité de la litière induit une succession des communautés microbiennes: les r-stratèges (les opportunistes) dominent au cours des premiers stades et sont plus tard remplacés par les k-stratèges (les communautés persistantes) en raison de la croissance des concentrations de substrat limitant. Le processus commence généralement avec la colonisation de la litière par des bactéries, les Ascomycètes et les Deutéromycètes (les champignons imparfaits), qui consomment les composés les moins récalcitrants (tel que la cellulose). La cellulose présente dans les tissus non lignifiés est alors attaquée par certains de ces organismes. Par la suite, la litière reste lignifiée est colonisé principalement par trois groupes de champignons saprophytiques: les champignons de la pourriture brune, molle et blanche, ces derniers étant les plus abondants (Eriksson et al, 1990 ; Fontain et al, 2003 ; 2004 ; Kubartová et al, 2009).

Les champignons de la pourriture brune, sont pour la plupart des basidiomycètes. Ils dégradent exclusivement la cellulose et les hemicelluloses laissant la lignine intacte (Kirk et Farrel, 1987). Certains ascomycètes et deuteromycètes comme *Aspergillus* et *Fusarium* appartiennent au groupe des champignons de la pourriture mole. Ils entraînent un ramollissement de la structure végétale accompagnée d'une perte significative de poids. L'action de ces microorganismes est plus limitée et superficielle (Blanchette, 1991). Les champignons de la pourriture blanche sont les seuls capables de produire tout l'arsenal enzymatique responsable de la transformation des composés phénoliques. (Hammel, 1997).

1.4. Les enzymes impliquées dans la décomposition de la litière

La transformation de la litière s'effectue grâce à la production de différentes enzymes produit par l'ensemble des communautés bactériennes et fongiques, ces derniers étant principalement responsables de la secrétion des enzymes extracellulaires:

Les cellulases : Elles correspondent à un ensemble d'enzymes qui réagissent de façon synergique pour dégrader la cellulose jusqu'à son monomère : le glucose. Trois groupes d'enzymes ont été décrits : les endoglucanases (E.C. 3.2.1.4) produisent des coupures au hasard dans le polymère; les cellobiohydrolases (E.C. 3.2.1.91) agissent sur les parties non réductrices de la cellulose en libérant le cellobiose comme produit unique de l'hydrolyse; et les β-glucosidases (E.C. 3.2.1.21) hydrolysent le cellobiose en glucose. Les deux derniers groupes d'enzymes sont inhibés par les sous-produits de leur métabolisme respectivement le cellobiose et le glucose pour les cellobiohydrolases et le glucose pour les β-glucosidases.

Les lipases : Elles appartiennent à la famille des estérases (glycérol ester hydrolase) qui catalysent l'hydrolyse des triglycérides en acides gras libres et du glycérol (Fig. 5). Les lipases catalysent l'estérification, la transestérification, l'acidolyse, l'alcoolyse et l'aminolyse

en plus de l'activité hydrolytique sur les triglycérides. Les substrats naturels des lipases sont les triacylglycérols (TAG) qui sont insolubles dans l'eau. La réaction se passe donc à l'interface entre une phase insoluble et la phase aqueuse dans laquelle se trouve l'enzyme. En absence d'eau cette réaction est réversible et les lipases sont capables de catalyser la formation d'ester en présence d'un acide gras et d'un alcool. Ces enzymes, produites aussi bien par les champignons que par les bactéries (Hasan et al, 2009) ont fait l'objet de nombreuses applications dans les domaines des biotechnologies (Salihu et al, 2012). Certains travaux se sont également intéressés au rôle de ces enzymes dans le sol et la litière en contexte de stress xériques (Farnet et al, 2010a ; Goujard et al, 2009) ou comme l'indicateur de l'activité microbienne dans les sols contaminés (Margesin et al, 1999, 2003).

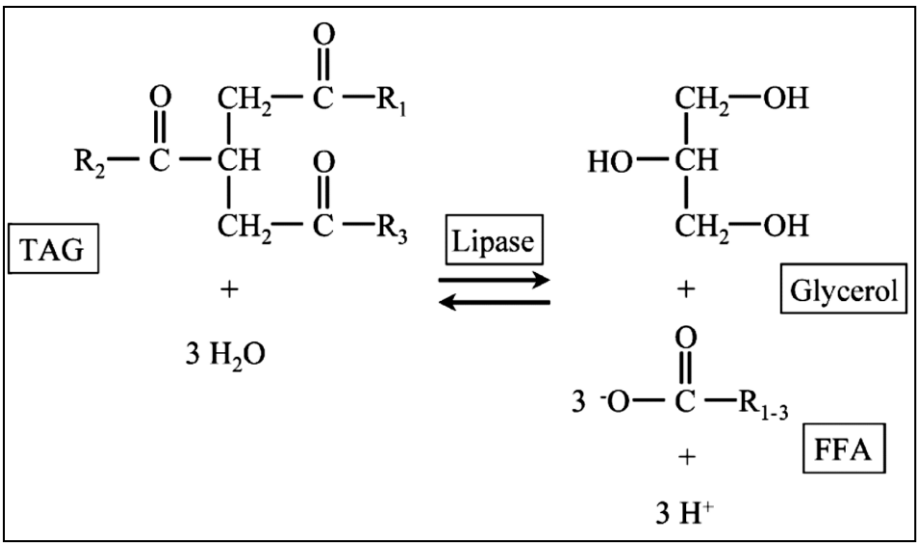


Figure 5. Réaction d'hydrolyse d'un triacylglycérol catalysée par les lipases. TAG, Triacylglycérol ; FFA, acides gras.

Les phosphatases : elles sont classées en différents groupes, y compris les phosphatemonoestérases, qui ont été largement étudiées dans le sol et la litière. Les phosphatases extracellulaires (alcaline ou acide) ont un rôle important dans la minéralisation du phosphate et hydrolysent les liaisons phosphomonoester, les phosphodiester, les acides nucléiques et les phospholipides en libérant le phosphate (Toor et al, 2003 ; Turner et al, 2002). Les facteurs environnementaux tels que le pH, la température ou la disponibilité du phosphore semble être les éléments les plus importants pour la production de ces enzymes par les microorganismes (à la fois les champignons et les bactéries). Certaines études mettent en évidence la régularisation de production des phosphatases comme étant

directement liées à la réserve de nutriments et la nécessité de minéralisation du phosphate (Olander et Vitousek, 2000). Ainsi de nombreuses études ont montré l'importance écologique et le rôle actif que jouent les champignons ecto-mycorhiziens en parallèle avec les autres communautés microbiennes dans la récupération du phosphore du sol notamment dans des périodes de carence (Courty et al, 2010ab).

Les phénoloxydases et les peroxydases: dans les sols et les litières, les enzymes impliquées dans l'oxydation des composés phénoliques peuvent être classées en deux groupes majeurs : les enzymes utilisant comme accepteur d'électrons l'oxygène (principalement les catéchol oxydases ou tyrosinases EC1.10.3.1 et les laccases EC 1.10.3.2, respectivement) et celles utilisant l'eau oxygénée (les manganèses peroxydases EC 1.11.1.13. et les lignines peroxydases EC 1.11.1.14). Ces métalloprotéines présentent des atomes de cuivre (laccases et tyrosinase) ou de fer (Mn- et lignine peroxydases) dans leur site actif et sont principalement d'origine fongique (Sinsabaugh, 2010). Elles sont en effet produites par des basidiomycètes (*Polyporus*, *Pleurotus*, *Agaricus*, *Pholiota…*), mais aussi des ascomycètes (*Neurospora*, *Podospora*, *Aspergillus…*), respectivement dénommés champignons des pourritures blanche ou molle.

Les manganèses peroxydases, peroxyde d'hydrogène oxydoréductases (Mn (II) : E.C. 1.11.1.13) ont été décrites pour la première fois par Kuwahara et al. (1984) dans des cultures de *Phanerochaete chrysosporium*. Leur rôle principal est l'oxydation de Mn^{2+} en Mn^{3+} en présence de H_2O_2. Ainsi, le Mn^{3+} généré est stabilisé par des chélateurs organiques pour pouvoir oxyder les substrats phénoliques (Wariishi et al, 1989).

Les lignines peroxydases *:* La diarylpropane: oxygène, peroxyde d'hydrogène oxydoréductase (E.C. 1.11.1.14) a été mise en évidence pour la première fois chez *Phanerochaete chrysosporium* (Tien et Kirk, 1983). C'est une glycoprotéine monomérique à noyau hémique qui catalyse des oxydations faisant intervenir un ou deux électrons. Elle participe dans les réactions de coupure de liaisons Cα-Cβ et alkyl-aryl, coupure de cycles aromatiques, oxydation Cα, deméthoxylation, déméthylation, hydroxylation et polymérisation (Heinzkill et Messner, 1997).

1.4.1. Les laccases

Parmi les enzymes extracellulaires microbiennes, les laccases jouent un rôle très important dans la dégradation de la lignine, le polymère le plus récalcitrant de la litière. Chez les champignons, les laccases concourent à la dégradation de la lignine tandis qu'elles sont impliquées dans la polymérisation de la lignine chez les cellules végétales (Bao et al, 1993 ; Eggert et al, 1995 ; Eggert et Eriksson, 1996). En effet, les laccases sont traditionnellement

considérées comme des enzymes fongiques. Toutefois, des gènes de « Laccase-like Multicopper Oxydases » (LMCO), enzymes fonctionnellement similaires, ont été décrits chez les bactéries et archaebactéries. Ces enzymes contiennent différents nombres et combinaisons d'atomes de cuivre (Baldrian, 2006 ; Badoei et al, 2006, Kellner et al, 2007ab, 2008; Li et al, 2008 ; McMahon et al, 2007 ; Mayende et al, 2006). Actuellement, les laccases sont très étudiées dans le domaine des biotechnologies étant données les réactions d'oxydation qu'elles catalysent, et leurs utilisations dans des processus de blanchissement de la pâte à papier, dans la bioconversion de composés xénobiotiques, dans la dégradation de colorants issus de l'industrie textile (Hou et al, 2004 ; Palmieri et al, 2005; Reid et Paice, 1994) ou dans les processus de bioremédiation (Borràs et al, 2010 ; Novotny et al, 2004) ont été abondamment décrites.

1.4.1.1. Structure des laccases

Les laccases (benzènediol : oxygène oxydoreductase [EC 1.10.3.2]) sont des glycoprotéines présentant de 10 à 20% de polysaccharides. Elles contiennent majoritairement 4 mais parfois 2 atomes de cuivre par molécule, lesquels sont classés en trois types différents en fonction de leurs propriétés spectrophotométriques, paramagnétiques et catalytiques (Baldrian, 2006 ; Reinhammar et Malmström, 1981 ; Thourston, 1994). Ces enzymes possèdent des poids moléculaires généralement compris entre 45 et 110 kDa, des températures optimales moyennes de 55°C et un pH optimum acide variant entre 4 et 5,5 (Baldrian, 2006). La production extracellulaire des laccases est constitutive ou inductible avec jusqu'à 8 isoenzymes différentes pouvant être produites par individu. Leur potentiel redox varie entre 450 à 800 mV, ce qui leur confère une capacité d'oxydation de nombreuses molécules.

1.4.1.2. Fonction des laccases

Comme indiqué précédemment, ces enzymes oxydent divers composés aromatiques comme les monophénols (guaiacol, 2,6-diméthoxyphénol) et diphénols (hydroquinone, syringaldazine [N,N'-bis(3,5-diméthoxy-4-hydroxybenzylidene hydrazine)]), les aminophénols, les polyamines dont les diamines (*p*-phénylène diamine).

L'oxydation de composés phénoliques par la laccase en présence d'oxygène moléculaire se traduit par la formation de radicaux. Ceci subissent une deuxième oxydation (considérée comme purement chimique) pour former des quinones ou pour engendrer des réactions de polymérisation (Thurston, 1994). Cette propriété peut sous-tendre les processus d'humification dans les milieux naturels (Sinsabaugh, 2010). Elle peut également faciliter l'élimination de composés phénoliques par précipitation et être mise à profit lors de traitement d'effluents riches en composés phénoliques (Ng, 2004).

Chez les champignons, les fonctions de ces enzymes sont multiples et leur intervention dans la morphogenèse a été déjà mise en évidence. Par exemple, chez *Aspergillus nidulans*, elle participe à la pigmentation de conidies et primordia (Law et Timberlake, 1980), au développement de fructifications chez *Agaricus bisporus* (Wood et Goodenough, 1977), et à la synthèse de pigments (phénoxazinone) qui confèrent la couleur rouge aux fructifications de *Pycnoporus cinnabarinus* (Eggert et Eriksson, 1996).

1.4.1.3. Induction et mécanismes de régulation de la transcription des laccases

Les laccases fongiques sont des enzymes qui peuvent être inductibles par la présence de molécules aromatiques dans le milieu de culture (Farnet et al, 2008 ; Gnanamani et al, 2006). Des acides phénoliques naturels comme l'acide benzoique, l'acide férulique ou l'acide vanillique ainsi que des composés aromatiques polluants comme les HAP ou les alkyl phénols ont été décrits comme modifiant l'expression qualitative des laccases (induction de nouvelles isoformes) ou quantitative (augmentation de la production d'isoforme constitutive) (Gnanamani et al, 2006 ; Terron et al, 2004 ; Ryan et al, 2007). Les processus de régulation de l'expression des gènes des laccases ont été très documentés notamment pour expliquer les mécanismes impliqués dans le développement de fructifications fongiques. L'implication de certains gènes de laccase dans la morphogenèse de champignons comme *Agaricus bisporus*, *Lentinula edodes*, *Volvariella volvacea* et *Pleurotus ostreatus* ont fait l'objet de nombreuses études (Chen et al, 2004 ; De Groot et al, 1998 ; Ohga et Royse, 2001 ; Sunagawa et Magae, 2005). Par exemple, chez *L. edodes*, les activités de laccases et cellulases sont régulées durant le développement du corps fructifère. En effet, le niveau de transcription de laccases est très important pendant le développement végétatif, tandis que le niveau de cellulases est plus important durant la formation des fructifications (Ohga et Royse, 2001). En revanche, dans le cas de *V. volvacea*, la laccase est fortement exprimée pendant la formation des fructifications (Chen et al, 2004). Par ailleurs, les études des gènes impliqués dans les réactions à la présence de pathogènes ou d'antagonistes de champignons cultivés ont également permis de montrer l'induction de l'expression de la transcription des gènes de laccases. Ainsi, des modifications de la transcription de gènes de laccases et tyrosinase chez *A. bisporus* par la présence de *Verticilium fungicola* ont été établies (Sarthou, 2004). Ces études ont été entreprises afin de mieux comprendre les mécanismes de résistance d'*A. bisporus* à la présence de son antagoniste. Ainsi il a été démontré que la transcription des gènes codant pour les laccases et les tyrosinases est induite lorsque *A. bisporus* est en confrontation avec *V. fungicola*. Velázquez et al. (2007) ont montré que certains gènes de laccases de *Pleurotus ostreatus*

étaient induits par la présence de moisissures antagonistes comme *Trichoderma* spp. Ceci suggère l'importance de ces enzymes dans les mécanismes de défense des champignons.

1.4.1.4. Application des laccases dans la transformation des composés aromatiques polluants

Les laccases sont largement utilisées dans différents domaines d'applications biotechnologiques du fait de leur capacité d'oxydation de composés aromatiques associée à une faible spécificité de substrats (Durán et al, 2000; Farnet el al, 2004). De plus, ces enzymes présentant des caractéristiques biochimiques particulières (thermostabilité importante, résistance aux solvants organiques…), elles constituent des outils catalytiques de choix en biotechnologie. Dernièrement, l'importance des problèmes de pollutions environnementales a orienté l'utilisation des laccases vers la dégradation des HAPs. Plusieurs études ont été consacrées à la capacité d'oxydation de nombreux HAPs par différents souches fongiques *in vitro* (Novotny et al, 2004 ; Steffen et al, 2002, 2007 ; Valentín et al, 2010). La dégradation des HAPs par les espèces fongiques comme *Pleurotus* spp. (Baldrian et al, 2000), *Irpex lacteus* (Cajthmal et al, 2002) ou *Cladosporium sphaerospermum,* espèce isolée de sites contaminés (Potin et al*,* 2004), sont quelques exemples. La majorité des études entreprises se sont attachées à tester les potentialités d'oxydation d'HAP variés par les laccases, à quantifier les rendements de transformation et à identifier, quand cela était possible les produits de l'oxydation (Cho et al, 2002 ; Collins et al, 1996 ; Dodor et al*,* 2004 ; Hu et al, 2007 ; Potin et al*,* 2004 ; Rodriguez et al, 2004). Steffen et al. (2007) dans son étude sur 18 espèces de basidiomycètes met en évidence l'importance des communautés fongiques surtout dans la dégradation des HAPs de hauts poids moléculaires et mentionne des capacités de transformation très variables selon les espèces fongiques. La transformation des HAPs en milieu naturel est majoritairement consacrée aux cas de pollutions massives des sols par les hydrocarbures (Andersson et al, 2003 ; Wu et al, 2008) et relativement peu d'études s'intéressent au potentiel de résistance de l'ensemble des communautés microbiennes vis-à-vis des HAPs. Etant donné le rôle des communautés fongiques dans les processus de biorémédiation de sols contaminés, il semble important de déterminer les communautés microbiennes potentiellement résistantes et impliqués dans la transformation de tels composés.

2. Effet de stress sur le fonctionnement microbien et la décomposition des litières

2.1. L'effet de stress caractéristiques du milieu méditerranéen

Le climat, facteur majeur influençant le fonctionnement des microorganismes décomposeurs, est particulièrement déterminant dans les écosystèmes méditerranéens présentant des conditions climatiques à l'origine d'un double stress (la sécheresse estivale et

le stress hivernal souvent inversement corrélés) (Quézel et Médail, 2003). La faible disponibilité en eau et les températures élevées en été sont considérés comme des facteurs importants affectant les activités biologiques des sols de ces milieux (Papa et al, 2008 ; Sabaté et al, 2002). Ainsi, différents études consacré à la minéralisation de la litière dans ces milieux a pu mettre en évidence le dynamique temporelle de nombreuse activités enzymatique dans les litières sclérophylles de ces milieux (Criquet et al, 2000, 2004 ; Fioretto et al, 2009 ; Sardans et Estiarte, 2008). Bien que la minéralisation de la litière dans les écosystèmes méditerranéens continentaux ait été particulièrement étudiée, les écosystèmes littoraux méditerranéens malgré leurs contraintes environnementales plus drastiques ont rarement fait l'objet de telles études.

2.1.1. Effet du stress hydrique sur les activités microbiennes

Le régime hydrique a été démontré comme déterminant dans la structure des communautés microbiennes des sols et ceci en conditions *in situ* et *ex situ* (Drenovsky et al, 2004 ; Frey et al, 1999 ; Zhang et Zak, 1998). En effet, lors d'un stress hydrique, la disponibilité des ressources en nutriments pour les microorganismes est modifiée. Lorsque les sols s'assèchent et que le potentiel hydrique augmente, les cellules accumulent des osmolytes pour réguler la pression osmotique (Harris, 1981). Ainsi, les bactéries utilisent généralement des composés aminés comme la proline, la glutamine et la glycine (Csonka, 1989), alors que les champignons utilisent des polyols comme le glycérol, l'érythrol, et le mannitol (Witteveen et Visser, 1995). De fait, le stress osmotique peut réduire les rendements de croissance c'est à dire la quantité de biomasse produite par gramme de carbone minéralisé, d'environ 90% (Killham et Firestone, 1984). Par ailleurs, des études réalisées sur les écosystèmes forestiers ont montré d'importantes diminutions des activités phénoloxydases et de la diversité des isoformes enzymatiques produites dans la litière, mais également une diminution de la biomasse fongique et bactérienne durant les périodes de sécheresses (Krivtsov et al, 2006 ; Di Nardo et al, 2004 ; Toberman et al, 2008ab).

2.1.2. Effet du stress halin sur les activités microbiennes

La salinité est un facteur influençant les activités des microorganismes particulièrement dans les zones arides et semi-arides. Ainsi, en zone littorale méditerranéenne, l'exposition à des variations de salinité, via les embruns ou directement par le contact avec l'eau de mer, constitue un stress supplémentaire notamment lors des sécheresses estivales. De même, la salinité peut influencer des processus microbiologiques en raison de la disponibilité restreinte de l'eau et de son influence sur la physiologie cellulaire et les processus du métabolisme (Sardinha et al, 2003 ; Toberman et al, 2008ab).

Il a été montré que la salinité au-dessus d'un niveau critique réduit l'activité enzymatique ainsi que la biomasse microbienne du sol (Liang et al, 2005; Tripathi et al, 2006, 2007). Plusieurs études ont été consacrées à l'effet de la salinité sur la composition ou la distribution des communautés microbiennes dans les zones côtières et/ou dans les sols où la salinité est un facteur dominant du milieu (Rousteau, 2004). Dans ce contexte, les auteurs se sont attachés à déterminer comment les teneurs en sel influencent la composition des communautés microbiennes et si ces communautés sont adaptées et donc fonctionnelles lorsqu'elles sont soumises à ce stress. Robinson et al. (2009) dans leur étude sur la distribution spatiale des communautés fongiques signalent l'importance des teneurs en sels sur la répartition des champignons au sein des sols côtiers. Rajaniemi et Allison, (2009) se sont intéressés aux écosystèmes dunaires et ont montré l'effet de la quantité de sources de carbone disponibles et du type de végétation dans la distribution des communautés microbiennes. Par ailleurs, ils ont mis en évidence l'abondance des bactéries gram négatives dans les zones plus proches de la mer (salinité élevée). Merilä et al. (2002) soulignent l'importance des facteurs environnementaux comme l'humidité dans la structure génétique des communautés microbiennes dans les zones côtières.

La recherche bibliographique met en évidence la faible quantité d'informations sur le fonctionnement microbien dans les sols ou les litières soumis au stress halin et dans les zones côtières. L'acquisition de ces informations reste cruciale tant dans la compréhension des processus fonctionnels de ces milieux fragilisés par des pressions environnementales naturelles majeures que pour l'élaboration de stratégies de restauration dans ces écosystèmes faisant partie des plus perturbés par une forte anthropisation.

2.2. Cas des perturbations d'origine anthropique

La contamination par les composés chimiques liés aux activités anthropiques sur l'ensemble des écosystèmes est en constante augmentation. Les composés à l'origine de ces contaminations sont de natures chimiques très diverses (détergents, pesticides, hydrocarbures aromatiques polycycliques (HAPs), molécules à effets ostrogéniques, métaux lourds...) mais présentent des caractéristiques communes de rémanence (liée à leur faible solubilité), d'ubiquité (contamination via l'atmosphère, les milieux aquifères…) et/ou de toxicité pour les organismes communément associés à une accumulation dans la chaîne trophique. La résistance et résilience d'un milieu varient en fonction notamment de la biodiversité de l'écosystème soumis à la perturbation. Ainsi, les fonctions microbiennes font partie des processus pouvant contribuer à une restauration de l'écosystème par transformation des composés polluants introduits. Selon la nature chimique des composés ainsi que la concentration présente de ces molécules, les microorganismes peuvent être plus ou moins sensibles ou efficaces à l'élimination des polluants. Les HAPs causent non

seulement des pollutions massives dans l'environnement mais, étant les polluants atmosphériques majeurs, ils sont aussi la cause des pollutions chroniques surtout à proximité des grandes agglomérations. Ainsi, ce constat soulève une question majeure : quel est le degré de résistance des communautés microbiennes à ce flux de polluants et est-ce ces communautés sont capables de procéder à l'élimination de ces polluants ? (Johnsen et al, 2006 ; Johnsen et Karlson, 2005, 2007). Dans les paragraphes suivants les pollutions chroniques atmosphériques seront décrites et l'importance des phénoloxydases comme les laccases dans les processus de détoxification va être détaillée**.**

2.2.1. Nature et origine des pollutions en littoral

La dégradation du milieu littoral par l'activité humaine correspond à une modification de l'équilibre naturel susceptible de perturber le fonctionnement naturel dans ces écosystèmes. Trois types de pollutions peuvent être distingués dans les zones littorales selon les origines des pollutions. En premier lieu, on peut évoquer les pollutions venant directement de la mer et qui sont apportées par les courants marins. Elles sont introduites au large, par les activités des transports maritimes et les exploitations des fonds marins (plates-formes pétrolières), ou proviennent d'autres zones côtières par l'action des courants littoraux. Une autre partie des pollutions littorales est d'origine continentale (tellurique). Parmi ces modes de contaminations, on distingue les rejets d'effluents en mer, les dépôts de déchets solides sur la côte, les apports par les fleuves et les eaux de ruissellement en secteur urbain et agricole (eaux pluviales). Ces contaminations peuvent être de nature microbiologique (effluents urbains, industries agro-alimentaires, abattoirs, élevages industriels…) ou chimique (rejets industriels, eaux de ruissellements contaminés). Par ailleurs, la pollution littorale peut avoir une origine accidentelle : collision et/ou échouage de navire, perte de conteneurs, accidents sur plate-forme de forage… Contrairement au type de pollution qui revêt un caractère chronique, les écosystèmes littoraux sont aussi gravement touchés par l'apport massif de polluants.

Selon leur nature chimique, les polluants peuvent suivre divers trajets dans un écosystème. Par exemple, les métaux lourds sont déposés surtout via les particules de poussière atmosphérique alors que les oxydes d'azote ou de soufre réagissent d'abord avec l'eau dans l'atmosphère avant d'être précipités sur le sol (phénomène des pluies acides). De manière générale les polluants atmosphériques contaminent l'écosystème soit par dépôts, soit par les précipitations (pluies et neiges) (Fig 6). Ainsi, le dépôt horizontal lié majoritairement aux nuages et brouillard est prédominant dans les zones littorales et montagneuses dû aux conditions climatiques particulières.

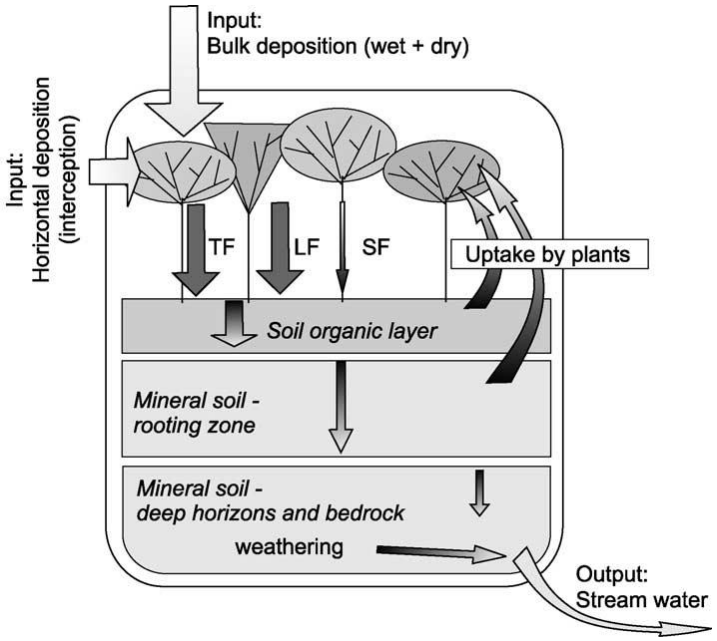


Figure 6. Les principaux fluxd'éléments chimiques dans les écosystèmes forestiers. TF, la chute directe; LF, chute de litière; SF, conduit par le tronc (Extrait de Berg et Laskowski 2006)

Ceci met en évidence un impact amplifié des pollutions dans les zones littorales étant données d'une part, les concentrations généralement plus élevées des polluants dans ces zones anthropisées et d'autre part, les conditions climatiques qui favorisent l'introduction des polluants dans le milieu. Ainsi, l'augmentation des molécules anthropiques dans l'atmosphère influence directement la composition chimique des tissus végétaux (phyllosphère, zone racinaire) ainsi que celle des litières et du sol ce qui pourrait engendrer une modification des processus de dégradation de la matière organique (Berg et Laskowski, 2006).

2.2.2. Origine et structure des Hydrocarbure Aromatique Polycycliques (HAPs)

Les HAPs proviennent principalement de la combustion incomplète de matières organiques. Les principales sources naturelles de HAPs dans l'environnement sont les incendies de forêt et les éruptions volcaniques. En ce qui concerne les sources anthropiques, la combustion incomplète de carbone fossile utilisée comme source d'énergie dans l'industrie, pour le chauffage, pour les moyens de transport, mais aussi les incinérations d'ordures sont quelques-unes des sources de diffusion atmosphérique des HAPs (Sorgi, 2007). Leur structure chimique comprend au moins deux cycles aromatiques accolés,

chacun étant composé de cinq ou six atomes de carbone. Les HAPs sont représentés par environ une centaine de substances qui diffèrent entre elles par le nombre de cycles et leur position respective (Fig 7). L'agence américaine de protection de l'environnement (USEPA) a établi une liste prioritaire comprenant 16 HAPs en raison de leur dangerosité (Samanta et al, 2002). La majorité de ces substances sont très persistantes dans les écosystèmes car ils sont peu dégradables à cause de leurs structures chimiques très récalcitrantes. Ainsi, l'étude du devenir des HAPs dans le compartiment édaphique est complexe du fait d'une multitude de facteurs intervenant : propriété du sol, durée de persistance, méthode de détermination de la biodisponibilité (Liste et Alexander, 2002). Ces composés sont le plus souvent liposolubles. Ces propriétés font que les HAPs tendent à s'adsorber sur les matières organiques ainsi qu'aux particules du sol ou des sédiments, ralentissant ainsi leur biodisponibilité et donc leur dégradation (Dean-Ross, 2005).

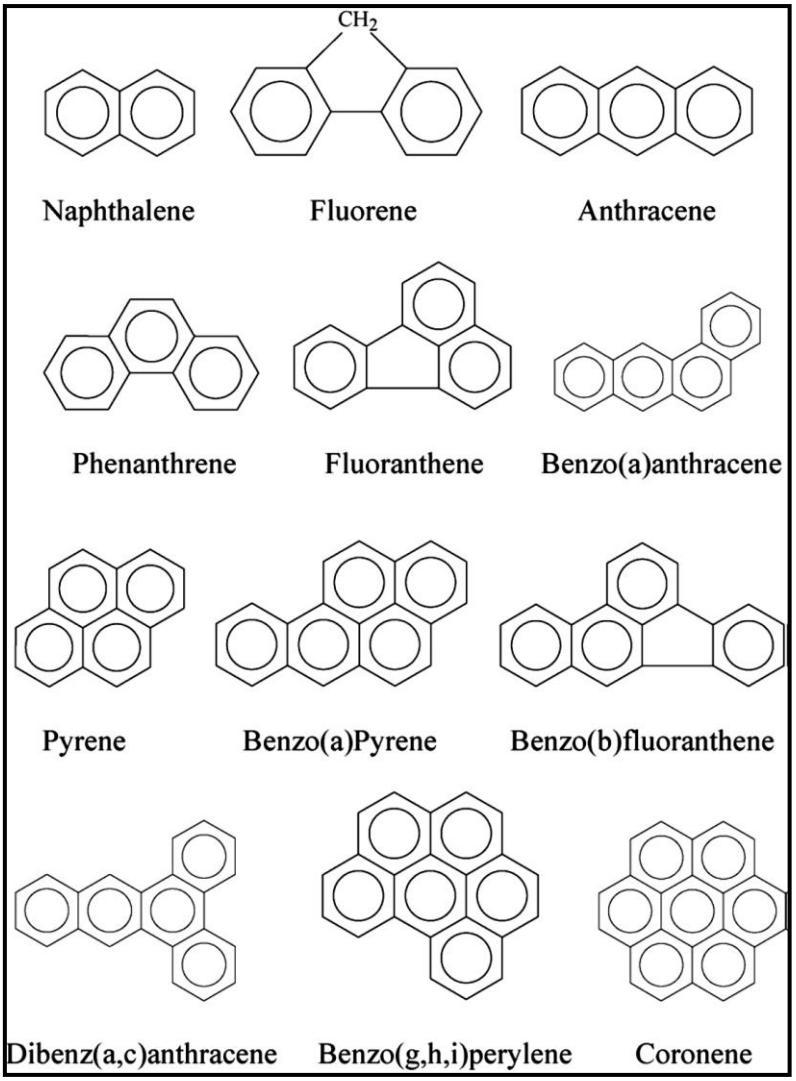


Figure 7. Structures chimiques de certains HAPs

2.2.3. Oxydation des HAPs par les microorganismes : exemple des phénoloxydases fongiques

Les microorganismes capables de dégrader les HAPs sont principalement les algues, les bactéries et les champignons (Haritash et Kaushik, 2009). Les études montrent aussi l'effet positif de la mésofaune comme les vers de terre, dans l'élimination des HAPs des sols contaminés même s'ils n'interviennent pas directement dans la biodégradation (Blakely et al, 2002 ; Coutiño-González et al, 2010). De manière générale les microorganismes transforment les composés organiques en métabolites moins complexes et ces processus peuvent conduire jusqu'à une minéralisation totale (H_2O, CO_2) en milieu oxique ou production de CH_4 en milieu anoxique. De plus, la biodégradation d'un polluant dépend des conditions environnementales : nombre et type de microorganismes, structures chimiques des composés à dégrader.

La dégradation d'HAP par les champignons lignolytiques grâce à la production des phénoloxydases a été intensivement étudiée au cours des quelques dernières années. La recherche sur les laccases, qui sont largement utilisées dans différents domaines d'applications biotechnologiques, a orienté l'utilisation de ces enzymes vers ce type de transformation. Plusieurs études ont été consacrées à la capacité d'oxydation de nombreux HAPs par différentes souches fongiques in vitro (Steffen et al, 2002). Pourtant, la transformation des HAPs en contexte naturel est relativement peu documentée où la diversité microbienne génétique et fonctionnelle est l'un des éléments fondamentaux pour le succès de la dégradation des composés polluants. Toutefois le rôle des communautés fongiques dans la bioremédiation de sols contaminés n'a été jusqu'à présent que très partiellement documentée (Andersson et al, 2003 ; Wu et al, 2008). Les analyses ne concernent alors que des rendements globaux de disparition de HAPs. Ceci peut trouver une explication notamment dans le fait que les études génétiques concernant les communautés fongiques dans les sols sont encore peu développées au profit des communautés bactériennes.

2.2.3.1. L'anthracène : une molécule modèle des HAPs

La dégradation de l'anthracène a fait l'objet de différentes études *in vitro* et la capacité des souches fongiques à dégrader cette molécule via des activités phénoloxydase ou peroxydase a été largement étudiée (Steffen et al, 2002, 2007). Cette molécule composée de trois cycles aromatiques linéaires (Fig.6) est très souvent mentionnée dans les contaminations causées par les HAPs. Il a été montré que les laccases peuvent être induites par les molécules aromatiques soit en produisant de nouvelles isoformes soit par l'augmentation quantitative d'une enzyme (Dodor et al, 2004; Edwards et al, 2002; Teng et

al, 2010). En outre, des études récentes indiquent que le potentiel d'ionisation des HAPs est un facteur majeur dans la dégradation de ces molécules par les enzymes fongiques (Farnet et al, 2009). Ainsi, les laccases sont de manière générale capable d'oxyder l'anthracène car le potentiel d'ionisation de cette molécule le permet. Cette molécule a donc été choisie pour estimer la capacité de transformation d'un HAP par les communautés microbiennes autochtones d'une litière dans les expérimentations au laboratoire.

Chapitre I

EFFET DE LA DISTANCE A LA MER SUR LES ACTIVITES DES COMMUNAUTES MICROBIENNES PRESENTES AU SEIN DE LITIERES DE PIN D'ALEP EN CONTEXTE LITTORAL MEDITERRANEEN

Ce chapitre est consacré à la présentation des résultats obtenus lors d'expérimentations *in situ* et *in vitro* afin d'étudier le fonctionnement des communautés microbiennes en contexte littoral. Il intègre 3 articles qui sont soumis à diverses revues à comité de lecture. Brièvement, le premier et le deuxième article traitent de l'effet de la distance à la mer sur le fonctionnement des communautés microbiennes selon une approche en contexte naturel et en recourant à la méthode des « litterbags ». A cet effet, un site d'étude a été choisi à proximité de l'agglomération marseillaise (massif de Marseilleveyre) et 2 aires se situant à des distances respectives de 10 m et de 300 m de la côte ont été retenues. Les litières de pin d'Alep issues de ces deux zones ont été introduites indépendamment dans des sacs de litière de maille de 1 mm et ont été soit réimplantées sur leur aire d'origine soit déplacées dans l'autre aire. Le fonctionnement des communautés microbiennes présentes au sein de ces litières a été suivi sur une période de 13 mois avec des prélèvements périodiques (après 2, 5 et 13 mois). Différents outils ont été mis en œuvre afin de caractériser l'évolution microbiologique des litières dans ces différentes conditions (activités enzymatiques, respirations basale et induite, diversité catabolique des microorganismes par CLPP) ainsi que leur degré de dégradation (mesures C/N, RMN ^{13}C). Des suivis de certains paramètres abiotiques comme la température, l'humidité, la conductivité et les concentrations en ions chlorures, ont été en outre réalisés au sein de ces deux aires pendant toute la durée des expérimentations. Dans le cadre du troisième article nous avons réalisé, par une approche *in vitro* et à partir de litière de pin d'Alep recueillie près du trait de côte - et donc particulièrement exposée aux influences marines - un isolement de souches fongiques productrices de laccases. La sensibilité de ces laccases aux ions chlorures ainsi que leurs éventuelles capacités de dégradation de différents HAPs ont été évaluées et ces résultats ont été confrontés aux données de la littérature. En outre, au cours de cette étude, trois nouvelles souches fongiques ont été identifiées.

Article 1: Spatio-temporal variations in microbial activities of a Pinus halepensis litter in a Mediterranean coastal environment, manuscrit

Article 2: Spatio-temporal variations in chemical and microbial composition of a Pinus halepensis litter estimated by ^{13}C NMR and CLPP, manuscrit

Article 3: Halotolerant laccases from Chaetomium sp., Xylogone sphaerospora and Coprinopsis sp. isolated from a Mediterranean coastal area, soumis à Fungal Biology

Article 1

Spatio-temporal variations in microbial activities of a *Pinus halepensis* litter in a Mediterranean coastal environment

1. Introduction

Litter decomposition is mainly controlled by three factors: climate, litter quality and the nature and abundance of the decomposing organisms (Coûteaux et al, 1995). For example, differences in litter chemical composition lead to variability in the type and relative abundance of microbial decomposers, and thus in their activity. Microorganisms are involved in organic matter decomposition through their extracellular enzyme production, which is directly linked to environmental conditions. Mediterranean forest litter decomposition in inland areas has been well documented, particularly with respect to litter chemical composition related to plant cover and/or vegetation succession and anthropogenic soil degradation due to chemical pollutants or fires (Fioretto et al, 2009; Francaviglia et al, 2004; Garcia et al, 2002; Gritti et al, 2006; Kurz et al, 2000; Papa et al, 2008). However, little is known about microbial communities involved in litter decomposition in coastal environments exposed to salinity stress caused by sea spray in addition to drastic climate conditions (Yuan et al, 2007). Salinity is actually a major factor influencing microbial community structure and functions and can strongly alter organic matter turnover, especially when combined with other stresses such as summer drought (Setia et al, 2011; Wichern et al, 2006). Furthermore, saline environments harbor great microbial diversity from both a taxonomic and phenotypic point of view (Ayuso et al, 2011; Tripathi et al, 2006, 2007; Zahran, 1997). Our objectives were thus to study, in a Mediterranean coastal environment, i) whether varying exposures to salinity, as a determinant environmental factor, may influence microbial activities and ii) whether microbial functions change with local (micro) environmental conditions, enabling microbial communities to adapt to salinity variations.

We selected two areas one of which was located close (10m) to the coast and one 300 m away. We hypothesized that microbial communities exposed to different salinities are functionally dissimilar. Various indicators of microbial activities, enzyme activities and microbial respiration were thus chosen to study microbial functioning. Litters of each area were transferred to the other area using litterbags in order to test the capacity of microbial communities to adapt to variations in salinity. Microbial activities and environmental parameters (temperature, humidity, conductivity and chloride ion concentrations) were monitored from both areas over a 13-month incubation.

2. Materials and Methods

2.1. Litter sampling

A composite sampling was performed in April 2009 from partially degraded litter (OL) of Aleppo pine (*P.s halepensis* L.) in two areas (200 m^2 each) on the French Mediterranean coast (Massif de Marseilleveyre, Marseille) (43° 12' 34'' N, 5° 21' 36'' E). The two areas were located 10 and 300 m from the coast, respectively named N (Near) and F (Far). Random litter samplings performed at each area were sieved (>2 mm mesh size) and homogenized before being placed in litterbags. Litterbags (20x25 cm) made of polypropylene (1 mm mesh size) were filled with 35-45 g (dry weight) of the litter from each area. Eighteen litterbags were then randomly placed in their area of origin (N or F) as control, while eighteen litterbags were transferred from one area to the other. Litters were then analyzed after 2, 5 and 13 months of in situ incubation, total of 12 litterbags thus being analyzed for each sampling time (6 controls and 6 with exchanged litters).

2.2. Environmental parameter measurement

Temperature, humidity, pH, electrical conductivity (EC) and Cl- concentration in litters from both areas were monitored throughout incubation. Mean daily temperature was measured using a USB temperature logger (VOLTCRAFT, 100T) placed 4 cm deep at both the N and F areas. Litter extract, 1:10 w/v litter/H2O, was used for other parameter measurements using a composite sampling from each area at least every 2 months. After a 30 min equilibrium time, pH was measured with Metrohm 744, electrical conductivity with DiST 3 (HANNA instruments) and chloride ion concentration with Cyberscan 510. Litter moisture was determined after drying (90°C) to a constant weight.

2.3. Basal and Substrate Induced Respiration

Microbial Basal Respiration (BR) and Substrate Induced Respiration (SIR) were measured on a 2 g sub-sample (dw) from each litterbag with real and standardized humidity at 60%. For BR, litter was placed in glass jars (117 mL), flushed with fresh air and closed by rubber septum before incubation at 25°C for 4 h. The concentration of the CO_2 produced was measured at the end of incubation by injecting one milliliter of the jar headspace gas into a gas chromatograph (Chrompack CHROM 3-CP 9001), equipped with a thermal conductivity detector and a fused silica capillary column (10 m x 0.53 mm). SIR was measured like BR, glucose being added (25 $mg.g^{-1}$ of litter dry weight) 90 min prior to a 90 min-incubation at 25°C. Microbial respiration was expressed as µg of C formed as CO_2 per gram of dry litter per hour. Metabolic Coefficient (qCO_2) was calculated as the ratio of basal respiration/microbial biomass according to Anderson and Domsch (1985).

2.4. Extracellular enzyme activities (EEA)

Laccase (LAC), cellulase (CEL), β-1,4-glucosidase (βGLU), acid phosphomonoesterases (PHA), alkaline phosphomonoesterases (PHB) and lipase (LIP) were quantified after 2-, 5- and 13-month incubations from a sub-sample of each litterbag. Except for lipase, which was estimated directly from 1 g of litter using *p*-nitrophenyl caprylate (10mM) as substrate according to Goujard et al. (2009), all the other activities were measured using a litter enzyme extract following a modified protocol from Criquet et al. (1999). Briefly, 10 g of litter was added to 200 ml $CaCl_2$, 2 H_2O (200 mM) and 0.1 % Tween 80, and then shaken for 1 h (500 rpm). The floating debris was removed, the extract was centrifuged (7 000 g for 20 min) and the supernatant was filtered (Whatman GF/C). The filtrate was concentrated by dialysis tubes (12-14 KDa-porosity) using Poly-Ethylene Glycol (PEG). Concentrated extract was obtained by adding 15 mL of 10 mM BisTris buffer (pH 6). The reaction mixtures for all the enzyme activities performed with the enzyme extract consisted of 300µl of enzyme extract with 700 µl of the corresponding buffer at 30 °C, except for cellulase activity which was incubated at 50 °C. Laccase activity was measured by monitoring the oxidation of syringaldazine to its quinone (ε^M = 65 000 M-1 cm-1) at 525 nm in acetate buffer (100 mM, pH 4.5). CM-cellulase (EC 3.2.1.4) activity was assayed using Carboxy Methyl Cellulose (CMC) at 0.7 % (w/v) as substrate with sodium acetate buffer (50 mM, pH 6). After a 4h-incubation, the glucose released was quantified according to Farnet et al. (2010). β-1,4-glucosidase activity was performed using p-nitrophenyl β-D-glucopyranoside (0.2 mM) with sodium acetate buffer (100 mM, pH 5.6). Acid and alkaline phosphomonoesterase were assayed using *p*-nitrophenyl phosphate monoester (0.2 mM) respectively in sodium acetate buffer (100 mM, pH 4.5) and in NaOH-Glycin buffer (100 mM, pH 9). *p*-nitrophenol was quantified at 412 nm after the addition of NaOH (0.5M). All analytical experiments were performed in triplicate for each litterbag. One unit (U) of enzyme activity is defined as one µmole of the reaction product formed per h and per g of litter dry weight (dw).

2.5. Statistical Analysis

A three-way ANOVA was performed taking into account litterbag location (N, F), area of litter origin (N, F) and time (June, September, May) as factors versus microbial respiration and enzyme activities as dependent variables (9 variables). Data were transformed as necessary to respect the normality and homogeneity of variance in parametric ANOVA. Because the time factor had a significant effect on almost all variables, the litterbags for each incubation time were further compared individually by a non-parametric comparison of Kruskal-Wallis. All measured microbial variables (9 variables) together with environmental parameters (EC, pH and humidity) measured in litterbags were subjected to Principal

Component Analysis (PCA). Pearson correlation between these variables was also tested. Statistica Vs 6 (StatSoft, Maison-Alfort, France) was used for statistical analysis and *p-value*<0.05 was considered as significant.

3. Results

3.1. Environmental Parameters

Variations in temperature, humidity, conductivity and chloride concentration are shown in Fig1 for both N and F areas. Similar annual variations in these parameters are observed for both areas. However, over the monitoring period, temperature was mostly higher in N than in F. Consequently, litter humidity was slightly higher in litter F than in litter N. Maximum and minimum humidity were respectively, 63% and 10%. Mean daily temperature varies between 2 and 28 over incubation. Conductivity measurements showed that, depending on when sampling occurred, differences between the two areas intensified, i.e. when humidity was low in both areas (in summer), conductivity was higher in area N. Differences in conductivity between the two areas were also linked to chloride ion concentration (Fig 1d). Variations in chloride concentration are similar in both areas, but higher values were found in N than in F. pH variations were also measured and were similar for both areas, but with a lower pH in N than in F (annual mean of 6.6±0.4 *vs* 6.0±0.4, respectively).

3.2. Enzyme activities

At t0, enzyme activities from the composite samples were generally higher in the Pinus halepensis litter from area F than in the litter from area N (data not shown). Fig. 2 shows enzyme activities both in litterbag controls (N/N or F/F) and in the litterbags exchanged between the two areas (litter N in area F, (N/F) or litter F in area N, (F/N)) over the 13-month incubation. According to the analysis of variance (table1) seasonal climate variations significantly affect enzyme activities, except for cellulase activities. The area of incubation also has an effect on all the enzyme activities (except lipase) independently of the other factors, i.e. litter origin or temporal variations. Of all the assayed enzymes in this study, only laccase was significantly correlated to all three tested factors. Cellulase, β-glucosidase, and acid phosphatase activities were dependent on litter origin, independently of the two other factors.

As observed in the litters at t0 for both areas, all enzymes except lipase were more expressed in litter F (litterbags F/F) than in litter N (litterbags N/N). When control and exchanged litterbags in area N (N/N and N/F) were compared, higher activities were generally found for N/F throughout incubations for both phosphatase activities and lipases. Placing litter F in area N (F/N) also affected most of the enzyme activities tested after 13

months (F/N < F/F), except for lipase activities (Fig. 2). Overall, independently of seasonal variations, enzyme activities in litterbags follow this trend; FF>FN/NF>NN.

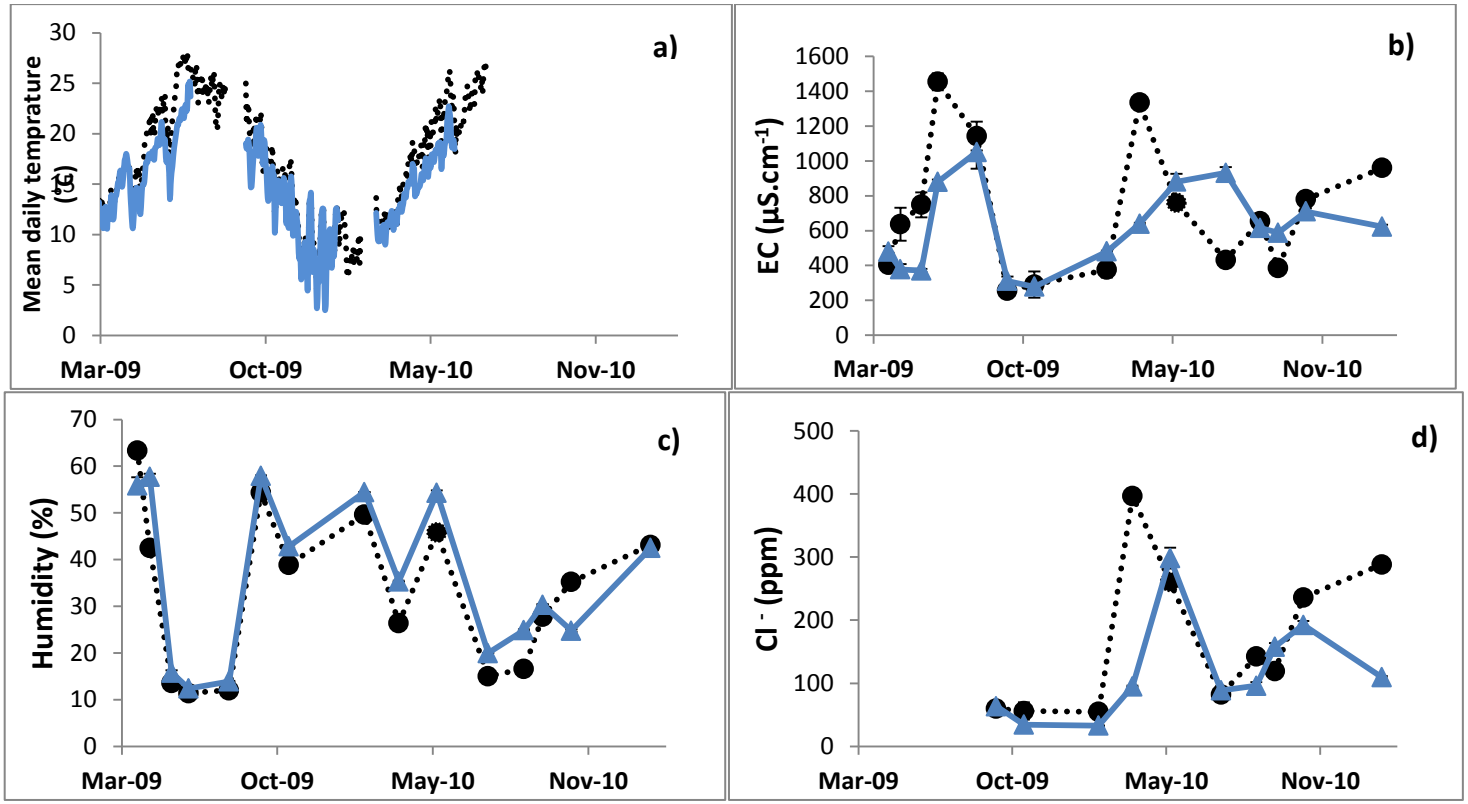


Figure 1. Temporal variations in temperature (a), electrical conductivity (b), humidity (c) and chloride concentration (d) measured in *Pinus halepensis* litters situated in areas N (. . . .) and F (____) located 10 and 300 m from the coast, respectively.

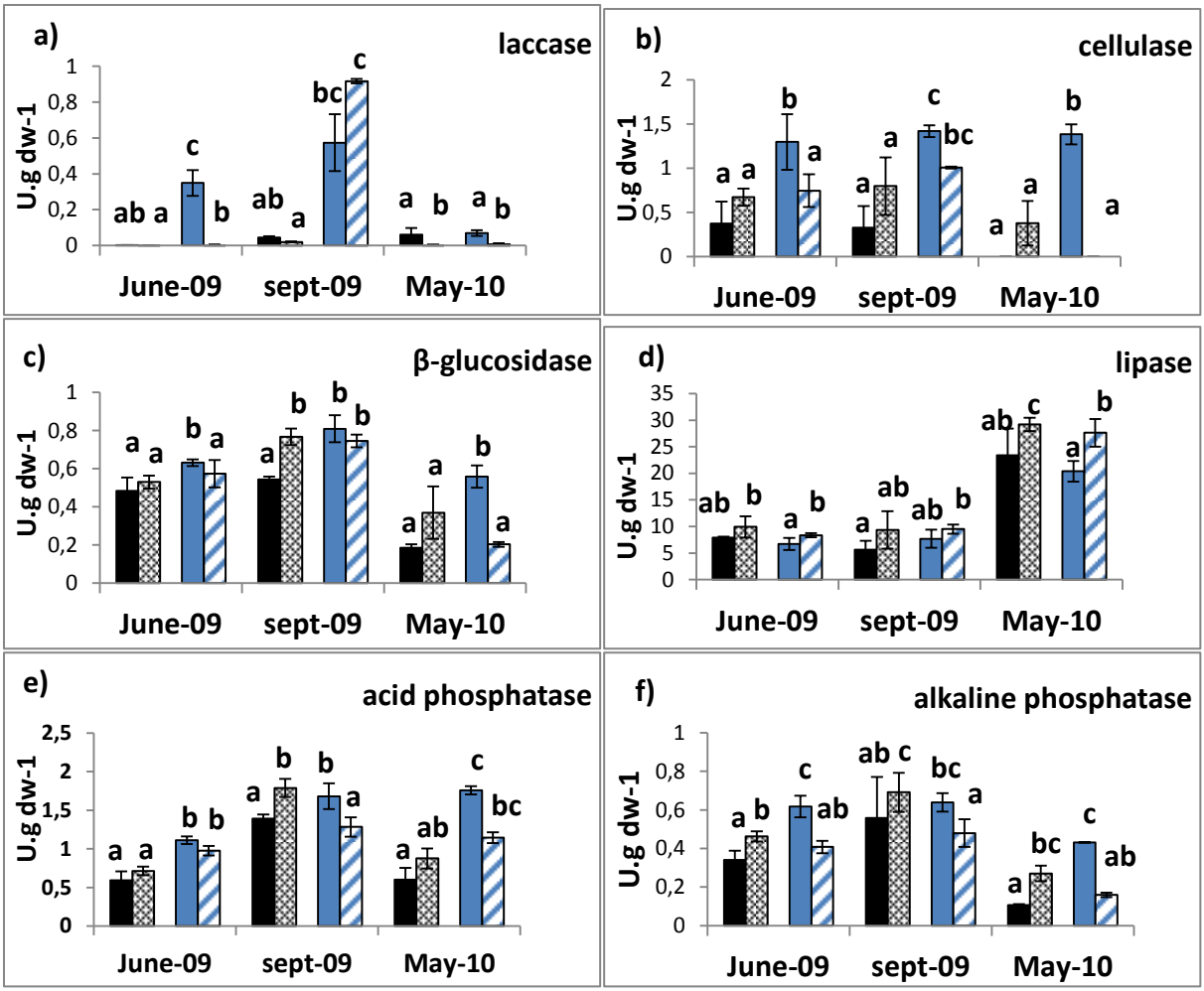


Figure 2. Temporal variations in microbial enzyme activities in *Pinus halepensis* litters measured after 2- (June), 5- (September), and 13- (May) month incubations in litterbags. Litterbags N/N ■ and N/F ▩ ; litters from area N placed in area N or F, respectively. Litterbags F/F ■ and F/N ▨: litters from area F placed in area F or N, respectively. The different letters show significant differences of means according to Kruskal-Wallis non-parametric comparison ($p<0.05$) between the litterbags tested separately for each incubation time (n=12). Error bars are SE (n=3).

3.3. Microbial respiration

Table 2 shows the results of factorial ANOVA test taking into account respiration variables: BR, SIR and qCO_2. Whatever the variables considered (BR, SIR or qCO_2) we observed a significant effect of time, independently of the two other factors. SIR and qCO_2 are also dependent on the origin of litters and BR on Litter and Time taken together (table 2). Fig 3 shows BR, SIR and qCO_2 levels in *P.halepensis* litter in litterbags over a 13-month incubation in areas N and F. Respiration was measured at standard humidity i.e. the maximum humidity detected in both areas over one year (60%, Fig 1b). Comparing BR of litters without standardizing humidity revealed great differences because of seasonal humidity variations especially in June-09 (data not shown). BR values after a 2-month

incubation (June 09) are similar whatever the litterbags (N/N, N/F, F/N and F/F), as shown in Fig. 3a. After a 13-month incubation, BR values are similar in litterbags N/F and F/F and in litterbags F/N and N/N, illustrating the importance of the area of incubation. In fact, after a 13-month incubation, respiration in area F is higher than in area N (FF litterbags *vs* NN litterbags). On the other hand, SIR data show that induced respirations depend on origin of litter after a 2- or 5-month incubation: SIR is similar for N/N and N/F and for F/F and F/N. In fact, while SIR is always higher in NN litterbags than in FF litterbags, BR follows the opposite trend, showing microbial biomass is greater in area N but less active. When respirations in the initial litters (before litter was placed in litterbags) were compared, we also found that N litter had a higher level of SIR and a lower level of BR than F litters (data not shown). Variations observed in qCO_2 can be ascribed either to litter origin (after 5-month incubation) or to area of incubation (after 13-month incubation). Thus, litter exchange seems to have a delayed effect on both BR and qCO_2 after a 13-month incubation (May-10).

3.4. Correlation between biological and environmental indicators

PCA was performed to reveal the correlation between different microbial indicators and environmental parameters in both areas. Fig 4 shows (a) the first two factors and (b) the first and third factors of this analysis. The first two factors account for 64% of the variance and mainly differentiate between samples according to incubation time, whatever the litter origin or location (2, 5 and 13M). The first factor (F1, 39%) is explained mainly by enzyme activities; βGLU, PHB, LAC, PHA, LIP, CEL, and EC by respectively 0.8, 0.9, 0.8, 0.7, 0.7, -0.7, 0.7 and 0.8 of correlation. The second factor explains 25% of variance and is highly correlated to BR, qCO_2 and humidity variables by respectively 0.9, 0.8 and -0.9 of correlation. Whatever the incubation time, we observed a differentiation between N and F areas according to F1, when the litters were located in their area of origin (N/N and F/F). After a 5-month incubation, the projection is explained by both F1 and F2 axes.

Fig 4b shows the scatter plot of the first and third factors. F3 is largely explained by the pH variable (0.9) and the projections are mainly explained by the origin of the litter (triangle *vs* diamond-shaped, Fig. 4b). This projection is also partly explained by the seasonal effect, as indicated by the arrows. After a 13-month incubation, only F/F is separated from the other points while N/N and N/F projections are close. That is, microbial activities from F litter transferred to area N have the same trend as local N litter after a 13-month incubation, while N litter transferred to area F does not follow the local trend.

Pearson correlation between enzyme activities and environmental measurements shows a significant negative correlation (*p-value*<0.05) among BR, laccase, phosphatase, β-

glucosidase activities and EC variations. However, lipase is highly positively correlated to EC ($p<0.0001$). BR and SIR are positively correlated to humidity variations ($p<0.0001$).

Table 1. Three-way ANOVA test on enzyme activities; LAC (laccase), CEL (cellulase), βGLU (β-glucosidase), LIP(lipase), PHA (acid phosphatase), PHB (alkaline phosphatase) according to different factors: Area (N and F), Litter (from N and F) and Time (June, September and May).

Variables	**LAC***		**CEL***		**βGLU***		**PHA***		**PHB**		**LIP***	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Area	13.2	<0.01	16.1	<0.001	61.8	<0.0001	21.1	<0.001	13.0	<0.01	0.1	ns
Time	343.7	<0.0001	2.4	ns	48.2	<0.0001	64.6	<0.0001	11.0	<0.001	18.1	<0.0001
Litter	507.5	<0.0001	12.1	<0.01	10.0	<0.01	27.5	<0.0001	1.1	ns	0.0	ns
Area x **Time**	37.6	<0.0001	3.3	ns	20.9	<0.0001	1.0	ns	0.2	ns	0.2	ns
Area x **Litter**	132.8	<0.0001	2.1	ns	1.8	ns	0.1	ns	0.4	ns	6.3	<0.05
Time x **Litter**	14.2	<0.001	0.7	ns	0.0	ns	15.6	<0.001	2.0	ns	1.4	ns
Area x **Time** x **Litter**	113	<0.0001	1	ns	1.9	ns	0.2	ns	0.1	ns	0.4	ns

*** log transformed, ns=non-significant ANOVA test, p=0.05.**

Table 2. Three-way ANOVA test on respiration variables; BR (Basal respiration), SIR (Substrate Induced Respiraion) and qCO_2 (Metabolic Quotient) according to different factors: Area (N and F), Litter (from N and F) and Time (June, September and May).

Variables	**BR***		**SIR***		**qCO_2**	
	F	*P*	*F*	*P*	*F*	*P*
Area	0.0	ns	1.0	ns	0.2	ns
Time	42.7	<0.0001	7.8	<0.01	9.1	<0.01
Litter	1.4	ns	4.6	<0.05	4.9	<0.5
Area x **Time**	1.6	ns	0.5	ns	1.7	ns
Area x **Litter**	0.0	ns	0.5	ns	0.3	ns
Time x **Litter**	3.5	<0.5	0.9	ns	2.8	ns
Area x **Time** x **litter**	0.8	ns	0.1	ns	1.1	ns

*** log transformed, ns=non-significant ANOVA test, p=0.05.**

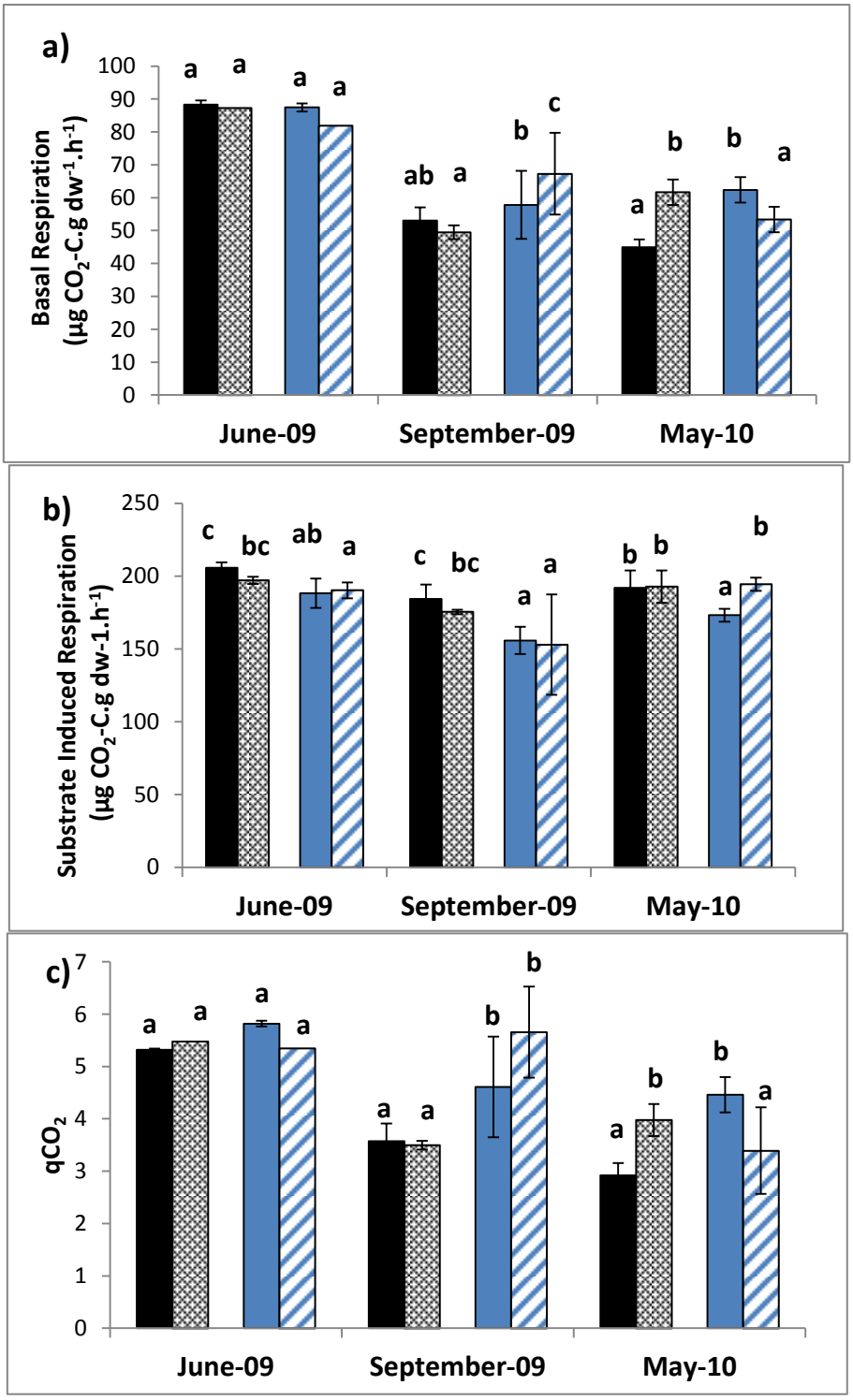


Figure 3. Basal Respiration (a), Substrate-Induced Respiration (b) and Metabolic Quotient (c) of microbial communities from the Pinus halepensis litter in litterbags measured at 60% humidity after 2- (June), 5- (September), and 13- (May) month incubations. Litterbags N/N ■ and N/F ▩; litters from area N placed in area N or F, respectively. Litterbags F/F ■ and F/N ▨ : litters from area F placed in area F or N, respectively. The different letters show significant differences of means according to Kruskal-Wallis non parametric comparison ($p<0.05$) between the litterbags tested separately for each incubation time. Error bars are SE (n=3).

4. Discussion

Climatic variations and litter chemical quality largely determine the efficiency of organic matter decomposition by microbial communities (Coûteaux et al, 1995, Kourtev et al, 2002; Ayres et al, 2009). Even microclimatic variations can affect microbial communities and consequently their functioning (Sommerkorn, 2008; Nannipieri et al, 2003). Here, we explored whether microclimatic conditions (i.e. differences in salinity exposure, humidity, temperature) caused a variation in microbial activities. It was important first to demonstrate that environmental conditions are actually different between the two areas selected. We observed that the areas studied which are close to each other and subject to the same annual climate conditions, can be differentiated with respect to microclimatic variations. For instance, during summer drought, salinity exposure, which is a major environmental structuring factor in coastal ecosystems, is more intense in litters situated at the coast than in those farther inland. Moreover, temperature and humidity are slightly different in each area, with a higher temperature and lower humidity in N.

Of the various microbial indicators, enzyme activities are particularly important because they provide information on the metabolic requirements of microbial communities and on available nutrients in litters (Caldwell et al, 2005, Sinsabaugh and Moorhead, 1994). The activities of various degrading enzymes decrease in a saline environment, because salinity particularly disturbs the tridimensional structure of proteins (Zahran, 1997). Different studies on salinity as a determinant factor influencing microbial activities have found that microbial biomass decreases (and consequently metabolic quotient increases) when salinity increases (Muhammad et al, 2008; Wichern et al, 2006; Egamberdieva et al, 2010). Rietz et al. (2003), who studied both enzyme activities and microbial biomass, proposed that an increase in salinity results in metabolically less efficient microbial communities.

Here our results show that microbial activities involved in *P.halepensis* litter decomposition are negatively influenced by salinity, since most of the enzyme activities produced in litter F decreased when the litter was placed in the N area, near the coast. The three-way ANOVA also demonstrates that Area and Time are the main factors affecting enzyme activities. This illustrates the importance of micro-climatic conditions in controlling the enzyme activities expressed in litter. Laccases are the only enzyme for which variations are explained by all three tested factors (considered alone or together, Table 1). These enzymes can be considered as markers of fungal diversity which, here, may vary depending on time, area and litter. For instance, Toberman et al. (2008a) showed that phenoloxidase activities strongly decreased during summer drought, which can be linked to a decrease in fungal diversity. Moreover, cellulase and β-glucosidase variations had nearly the same pattern as laccase: lignocellulolytic activities are known to be produced by fungal communities and thus laccase

and cellulase activities are supposed to follow similar temporal and spatial variations (Baldrian, 2006; Baldian and Valaskova, 2008).

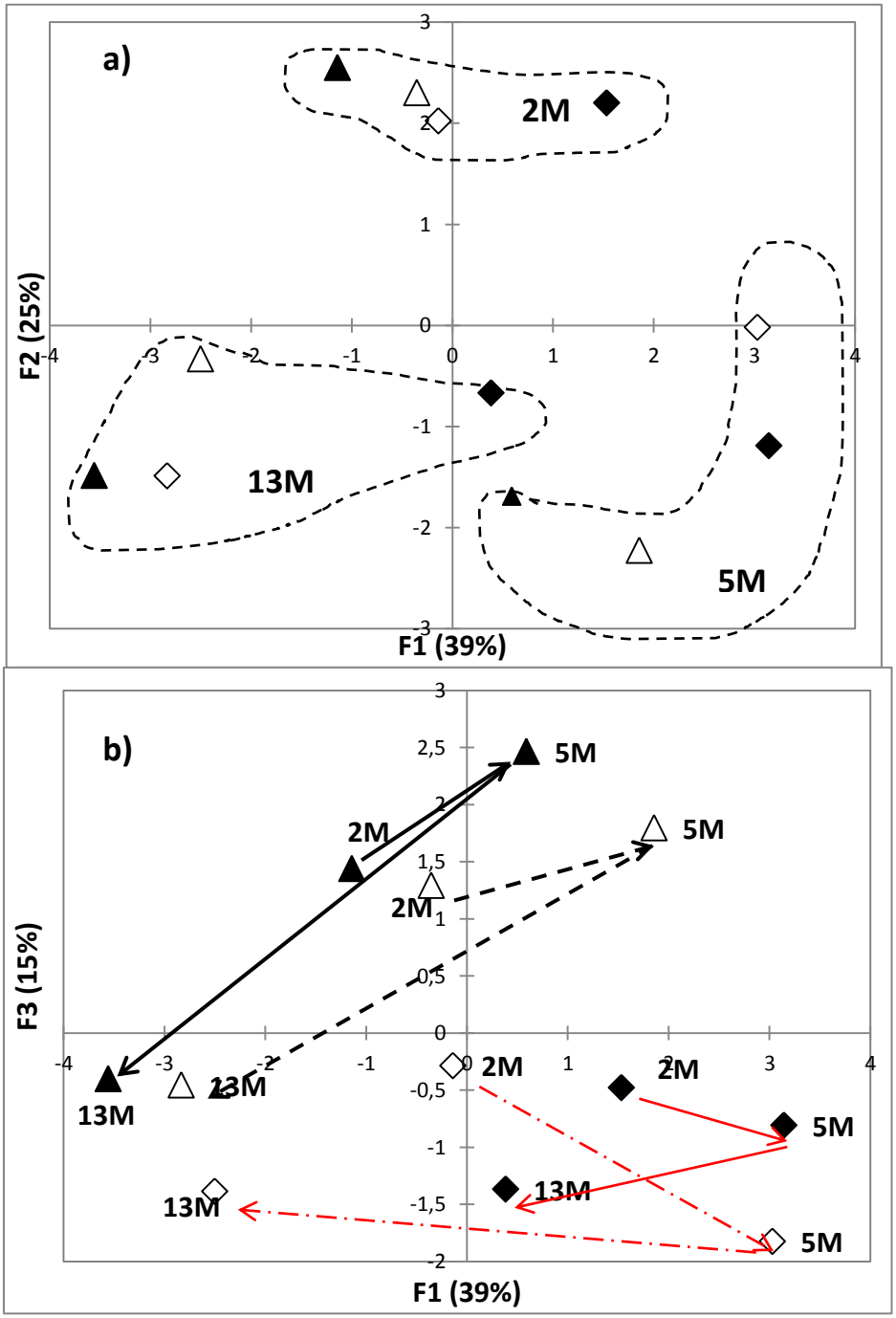


Figure 4. Principal Component Analysis (PCA) on microbial and environmental indicators measured in litterbags after 2-, 5- and 13- month incubations (M). a) Factor 1 and 2, b) factor 1 and 3 of PCA analysis accounted respectively for 64 and 54% of variance. Triangle corresponds to litter from N area placed in N area (black) and placed in F area (white). Diamond-shaped corresponds to litter from F area placed in F area (black) and placed in N area (white).

Here, we observed a greater expression of lignocellulolytic enzymes in area F over time and the negative correlation found between these enzymes and conductivity. However certain enzymes like lipases are positively affected by salinity. As suggested by Goujard et al. (2009), drastic environmental conditions such as limited water availability may not influence lipase activities, since this enzyme is known to work at hydrophobic interfaces. Thus, our results suggest that, while the seasonality remains the determinant factor most influencing microbial activities, salinity also affects certain microbial activities in a Mediterranean coastal climate.

Like other authors, we found microbial respiration to be highly dependent on humidity (Schimel et al, 1999; Zhu et al, 2008; Emel et al, 2008). Thus, in order to avoid variations due to different percentages of humidity in the samples, we compared microbial respiration at the highest *in situ* humidity measurement. The highest BR values were obtained, whatever the litterbags considered, in June 09, which corresponds to the summer drought period (Fig. 1b). Iovieno and Baath, (2008) in a study of soil rewetting have demonstrated that an immediate increase in microbial respiration is usually observed when rewetting the completely dried samples. Thus BR in June 09 seems not to be totally explained by *in situ* variations. The fact that the same level of BR was obtained for June for all litterbags can be explained by cell lysis, because of osmotic shock, which enhances the microbial respiration of the surviving microorganisms *via* nutrient release (Bottner, 1985; Turner et al, 2003).

Just as in previous studies comparing in its native environment and in a non-native environment, we also observed here that the microbial community is influenced by their new environmental conditions (Kourtev et al, 2002). Basal respiration indeed depends on where the litter was placed, and decreases in area N (N/N and F/N), in keeping with previous studies where the authors found that salinity has an impact on microbial respiration (Muhammad et al, 2008; Wichern et al, 2006). However, it is noteworthy that, microbial biomass (estimated by SIR) is not affected by salinity in our study. On the other hand, the metabolic quotient after the 13-month incubation is higher in F than in N. The potential of microbial activities in litter is therefore preserved, but its expression depends on local environmental conditions (for instance during spring or autumn when humidity increases).Thus, it appears that while local micro-variations in temperature, humidity and salinity do not affect the quantity of microorganisms, they influence its metabolic efficiency as characterized by qCO_2. Another key factor which may have influenced the metabolic efficiency of the microbial communities is the maturity of the litter inside the litterbags after the 13-month incubation.

Our results confirm that the seasonal alternation of humid and dry periods is a major factor influencing litter microbial activities in the Mediterranean area (Fioretto et al, 2007).

However, our findings here also suggest that variations in microbial metabolism are due to micro-climate arising from sea-spray exposure and local variations in temperature and humidity. Furthermore, we demonstrate the existence of these spatio-temporal variations in microbial activities at a small scale, since the areas under study were situated close to each other.

In litters from schlerophyllous plants such as *P. halepensis*, fungal communities play an important role in organic matter decomposition and their biodiversity strongly varies with litter type and habitat (Sinsabaugh et al, 2002). Certain authors claim out that there is no relationship between *in situ* salinity and salt-tolerant microorganism abundances, which suggests that soil salinity is not always a decisive factor for the structure of decomposer microorganisms (Rousk et al. 2011). Further studies need to be performed to investigate whether fungal communities are structured by salinity and whether certain salt-tolerant fungal populations are responsible for litter decomposition under these stressful conditions. Finally, many chemical pollutants can be found in sea sprays because of increasing anthropogenic pressures: their role on the microbial community structure in litter from coastal environments should also be investigated since these ecosystems, already stressed by climatic and salinity variations, are additionally threatened by anthropic pollution.

Article 2

Spatio-temporal variations in chemical and microbial composition of a *Pinus halepensis* litter estimated by ^{13}C NMR and CLPP

1. Introduction

The incidence of salinity on soil microbial activities has been largely studied as a major threat to agriculture especially in the arid and semiarid regions (Odeh and Onus, 2008; Peinemann et al, 2005; Tripathi et al, 2006, 2007). However, less attention has been paid on the effect of salinity on microbial activity and organic matter decomposition on the ecosystem function like coastal environments which include salinity as an environmental continuous constraint. Salinity is actually a major factor influencing microbial community structures and functions and can strongly alter organic matter turnover, especially when combined with other stresses such as summer drought (Setia et al, 2011; Wilchern et al, 2006). Furthermore, salinity greatly affects microbial diversity from both a taxonomic and phenotypic point of view (Ayuso et al, 2011; Tripathi et al, 2006, 2007; Zahran, 1997).

The Mediterranean coastal ecosystems have been extensively studied in ecological approaches on plant and animal communities, revealing a high biological diversity (Barea et al, 2011; Coll et al, 2010; Thomson et al, 2009). But less attention has yet been paid on microbial communities in these ecosystems especially their functional diversity as a major factor involved in organic matter decomposition. Here, using both chemical and biological approaches, we study through a small spatio-temporal survey the evolution of litter chemical variations and microbial catabolic diversity. We selected two areas one of which was located close (10m) to the coast and one 300 m far away. Using litterbags we transferred litters of one area to the other and samplings were performed after 2- , 5- and 13- month *in situ* incubation. Our main objective was to show how the increase or decrease in salinity intensity by transferring litters from their site of origin to another one may act on microbial functional diversity and consequently on organic matter decomposition of a *Pinus halepensis* litter, a pioneer plant species in these areas.

2. Materials and Methods

2.1. Litter sampling

A composite sampling was performed in April 2009 from partially degraded litter (OL) of Aleppo pine (*P. halepensis* L.) in two areas (200 m^2 each) on the French Mediterranean coast (Massif de Marseilleveyre, Marseille) (43° 12’ 34’’ N, 5° 21’ 36’’ E). The two areas were located at 10 and 300 m from the coast, respectively named N (Near) and F (Far). Random

litter samplings performed at each area were sieved (>2 mm mesh size) and homogenized before being placed in litterbags. Litterbags (20x25 cm) made of polypropylene (1 mm mesh size) were filled with 35-45 g (dry weight) of the litter from each area. Eighteen litterbags were then randomly placed in their area of origin (N or F) as control, while eighteen litterbags were transferred from one area to the other. Litters were then analyzed after 2, 5 and 13 months of *in situ* incubation, total of 12 litterbags thus being analyzed for each sampling time (6 controls and 6 with exchanged litters).

2.2. Litter chemical analysis

Litters in litterbags were characterized by solid-state ^{13}C CP/MAS NMR on a spectrophotometer Bruker DSX 400 MHz operating at 100.7 MHz. Samples (400 mg) were spun at 10 kHz at the magic angle. Contact times of 2 ms were applied with a pulse width of 2.8 µs and a recycle delay of 3 s. Chemical shift values were referenced to tetramethylsilane and calibrated to glycine carbonyl signal set at 176.03 ppm. The relative C distribution in ^{13}C NMR spectra was determined by integrating the signal intensity in different chemical shift defined using Dmfit 2003 (Massiot et al, 2002). Four common chemical shift regions were defined as alkyl C (0–45 ppm), O-alkyl (45-112 ppm, including di-O alkyl C), aromatic C (112–160 ppm, including phenolic C), and carboxyl C (160–185 ppm). Guaiacyl units, syringyl units, C1 anomeric, C2-C5 and methylen groups were considered individually at 148, 153, 105, 72-89 and 30 ppm, respectively. According to Baldock et al. (1997): Aromaticity ratio was calculated by division of aromatic C by the sum of all regions except carboxyl C.

Total Organic Carbon (TOC) and total Nitrogen were also measured as follows: dried sub-samples of initial litter and of each litterbag's litter were powdered in a ceramic mortar and analyzed by combustion in an Elemental Analyzer, FlashEA 1112, Thermofisher.

2.3. Community-Level Physiological Profiles (CLPP)

Catabolic profiles of microbial communities were assessed using Biolog EcoPlateTM (Biolog, California, USA). Briefly, 2 g of dry litter was added to 100 mL of 0.1 % sodium pyrophosphate sterile buffer in a 250 mL flask and shaken for 1 h (500 rpm). One extraction from litter of each litterbag was performed. The litter suspension was diluted and standardized at OD 595 nm = 0.02 with a sterile physiological solution (NaCl 0.85 %). 125 µL of microbial suspension were used to inoculate each well. The plates were incubated at 25 °C for 5 days and microbial development was followed by reading the absorbance at 590 nm. According to Garland (1997), absorbance value for each well was blanked against the control well and negative absorbance values were set to zero. The minimum OD for a positive well

was fixed at 0.25. The OD after 45h has been chosen according to exponential phase of growth curves for all plates for further comparison.

Overall rate of substrate utilization by microorganisms was measured by calculating the Average Well Color Development (AWCD) for each plate (n=3) at 45 h. Functional diversity assessed by Shannon's diversity index (H') was calculated using the equation: H'=-∑pi log pi where *pi* is defined as OD of each well /sum of OD of all wells.

2.4. Statistical Analysis

The significance of means was tested regarding litterbags location (N and F), litter origin areas (N and F) and incubation time (June, September, May) as factors using three ways ANOVA. Because factor of time has a significant effect on nearly all variables, for each incubation time a non-parametric comparison of kruskal-Wallis was done. Data were transformed as necessary to respect the normality and homogeneity of variance in parametric ANOVA. The optical absorbance of each well in ECO plate after 45h incubation was subjected to Principal Component Analysis (PCA), considering each substrate as a variable. The sum of relative intensity of each NMR region and individual unit were also subjected to PCA. Statistica Vs 6 (StatSoft, Maison-Alfort, France) was used for statistical analysis and p-value<0.05 was considered as significant.

3. Result and discussion

Firstly, we report the result of catabolic diversity of microbial communities able to use the Biolog substrates. Fig 1 shows the two calculated indexes regarding the optical density of utilization of all the substrates and their diversity, respectively presented as AWCD (Fig1a) and Shannon-weaver index of catabolic diversity, H' (Fig1b). The first important observation is the variation of AWCD through time. AWCD was initially the same regarding litter origins, and through time became identical according to litter locations. When comparing H' through time, we observe again the same tendency of variations. In fact, area N seems to structure metabolically less diversified microbial communities than that of F area. The result of ANOVA analysis considering these two variables (AWCD and H'), also confirms time as a highly significant factor on microbial catabolic diversity as defined by the indexes, F= 68.4 and 28.3, respectively for AWCD and H' ($p<0.0001$). The origin and location of litters had also a significant effect ($p<0.05$) but only on AWCD. Indeed, the origin of litter and their location do not show any significant effects on the diversity of the substrate used by microorganisms, but they affect the utilization intensity of substrates. Climate is known as one of the most important influencing factors on microbial activities and organic matter degradation (Berg and Laskowski, 2006). Salinity is also a drastic environmental factor which globally favors halo-

tolerant microorganisms and act as an additional environmental stress (Dendooven et al, 2010; Rasul et al, 2006; Rousk et al, 2011; Yuan et al, 2007). However, from these results, it seems that in saline environments like Mediterranean coastal areas, climatic variations combined with salinity strongly structure microbial catabolic profiles. Figure 2 shows the result of PCA analysis using optical densities of all the used substrates as variables. The same tendency of temporal variations is also noticeable on litterbag projections on factorial plot in this figure. Figure 2 indeed shows the first two factors of PCA accounting for more than 63% of the variance. Litterbag projections were grouped according to sampling time and this demonstrates again the influence of temporal variations on community structure. It is important to notice that though seasonality seems to be a major factor, organic matter decomposition in the litter bags may have also influenced microbial functional diversity. The substrates which mainly separate the litterbags by PC1 (49.8%) are: tween 40, glycogen, N-acetyl-D glucosamine, hydroxyl benzoic acid, arginine, asparagine, serine and phenylalanine (r^2>0.7 of correlation to PC1). PC2 is highly explained (r^2>0.7) by the utilization of two substrates: D-L-α-glycerol phosphate and xylose. Here, we observe the separation of sites N and F following PC2 (13.6% of variance) according to the litter origin i.e. litterbags NN and NF are close together after 2 month incubations but litterbags NN and FN are closer together after 13 month incubations. This confirms that though all the three tested factors do not have the same acuteness on microbial catabolic profiles, salinity has actually an impact on microbial functional diversity.

The chemical evolution of N and F litters in their original areas as well as in the areas of transfer is shown in Figure 3. Figures 3a and 3b present C/N ratio and an aromaticity ratio based on NMR spectra from the litterbags, respectively. It should be mentioned that N and F litters were analyzed before being introduced in litterbags and were chemically comparable (C/N 27±0.3 and 30±1.6 for N and F, respectively). Time as a factor considered alone or with litter origin, had significant effects on litter C/N variations ($p<0.0001$ or $p<0.01$, respectively). We obtained a decrease in C/N ratio over time which is mainly explained by a decrease in C content in all litterbags over time (data not shown). This especially shows that litter degradation has actively progressed over incubation inside the litterbags.

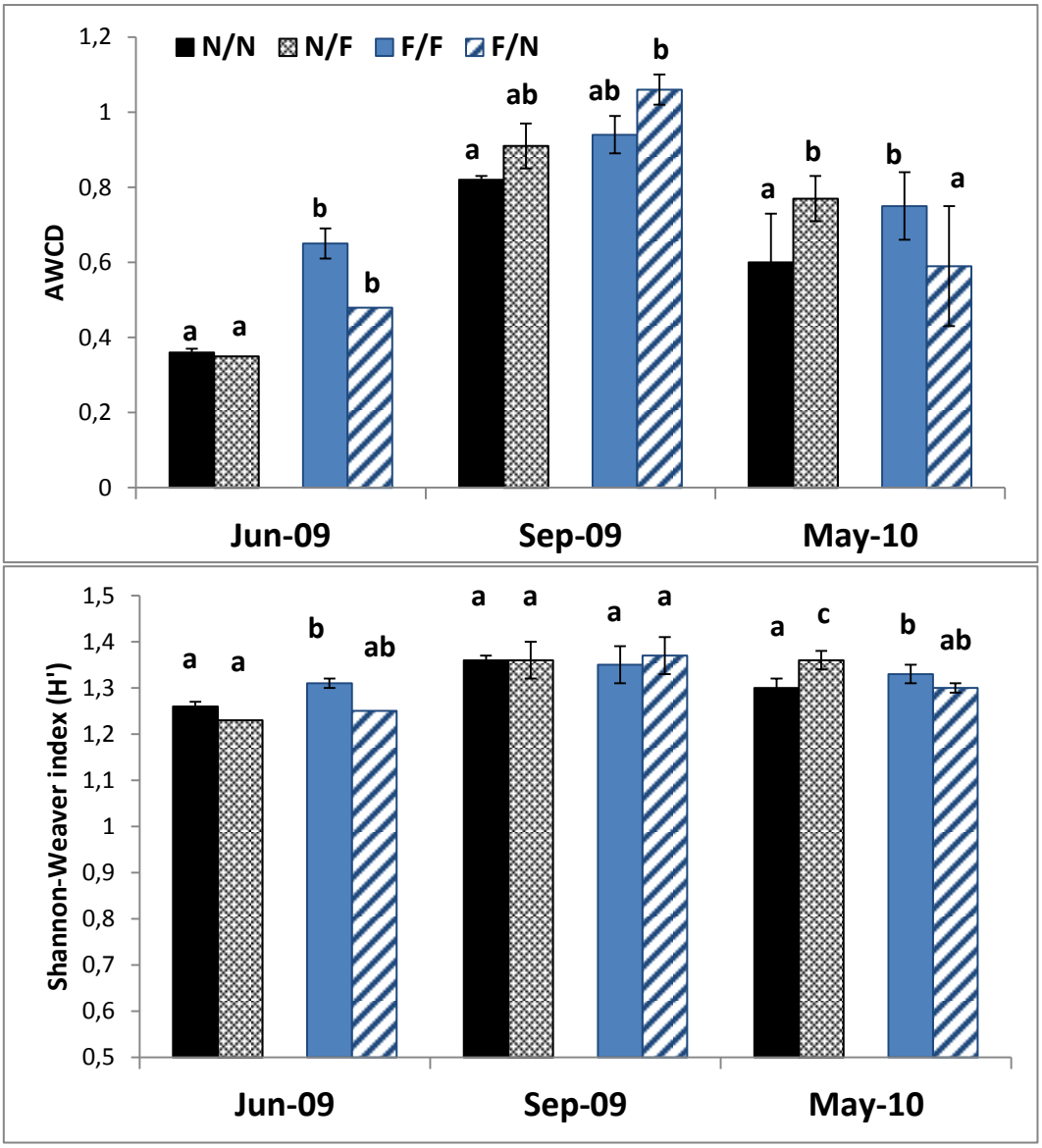


Figure 1. AWCD (a) and Shannon-Weaver index (H') (b) calculated on Eco plates after 45 H incubation. Different letters show the means of each incubation time which are significantly different ($p<0.05$) following kruskal-wallis non parametric comparison of means.

A significant difference between N and F litters after 13 months was also observed with a higher level of C/N in F than in N area. This result underlines that no changes in C/N were obtained when litters were transferred in a new location. Though the C/N ratio gives information about chemical variations, more precise data were obtained using a holistic approach of chemical characterization of organic matter via solid-state ^{13}C NMR: this technique precisely determines whether different chemical groups actually varied in organic matter.

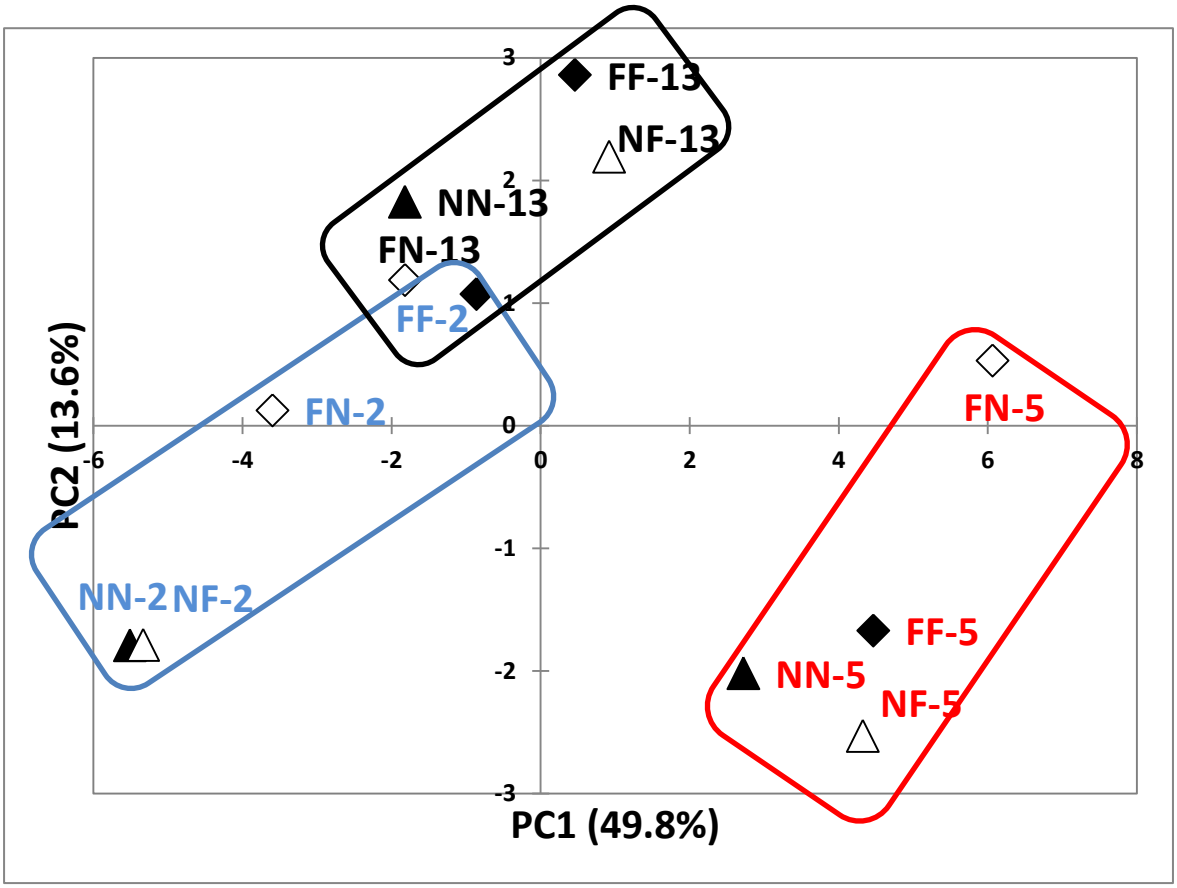


Figure 2. PCA on Eco plate substrate utilization profile after 45 H incubation of microbial communities of litterbags after 2- (bleu), 5- (red) and 13- (black) month *in situ* incubations. Triangle corresponds to litter from N area placed in N area (black) and placed in F area (white). Diamond-shaped corresponds to litter from F area placed in F area (black) and placed in N area (white). Each point represents the barycenter of three litterbags (n=3).

The aromaticity ratio was calculated as a global indicator of the degradation evolution over time. We mainly observed an increase in the aromatic groups in NN litterbags (Fig 3b) but though this ratio changed over time, it is not directly explained by litter origins or locations (ANOVA test was not significant). According to Preston et al, (1998), lignin content was estimated as following: [4.5*(phenolic C) + methoxyl C], and showed a decrease through time in all litterbags but any differences between N and F has been found (data not shown).

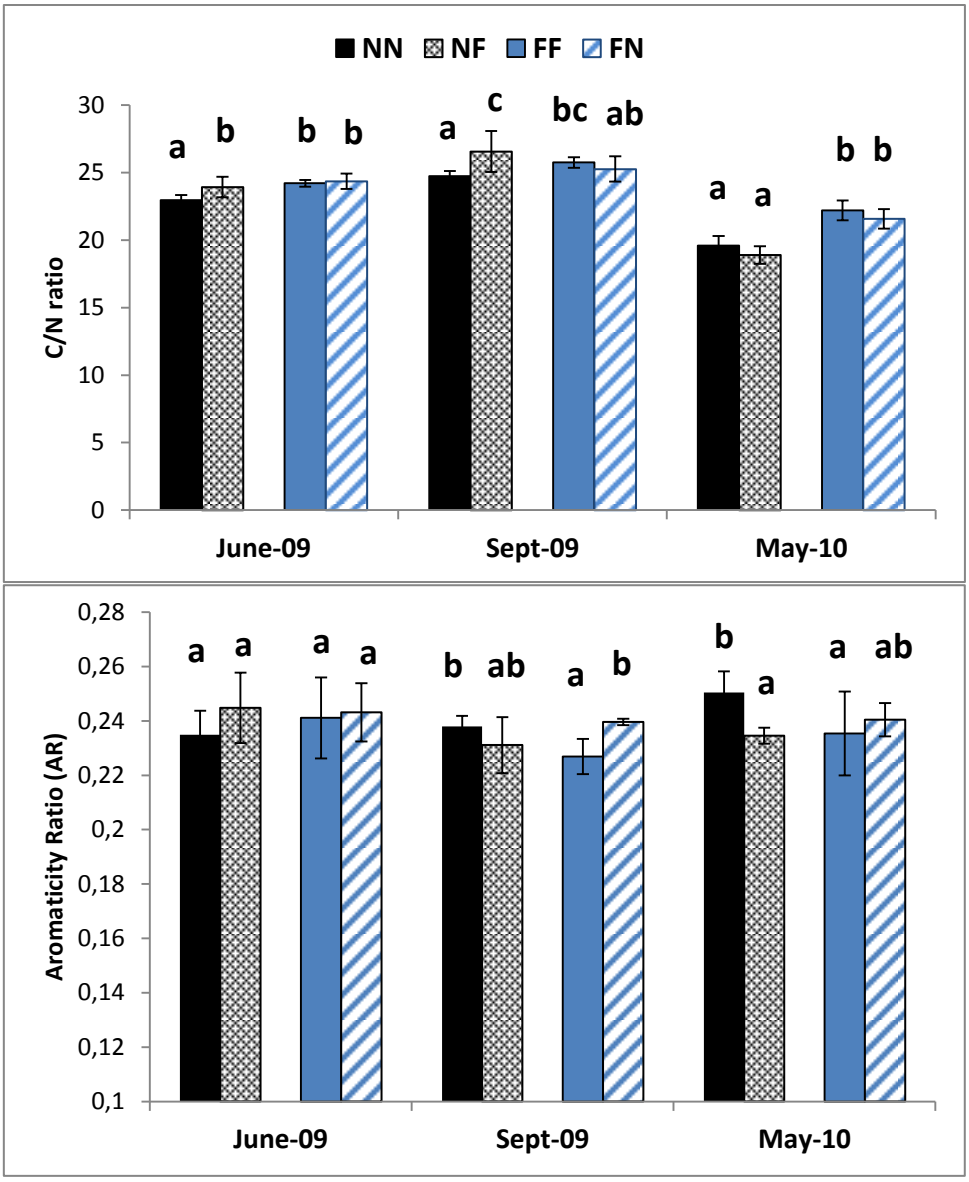


Figure 3. C/N ratio (a) and Aromaticity ratio (b) calculated on litterbags. Different letters show the means of each incubation time which are significantly different ($p<0.05$) following kruskal-wallis non parametric comparison of means.

In fact, ANOVA analysis of all chemical groups defined by ^{13}C NMR spectra showed that only time had a significant effect on chemical variations in litters, which is also corroborated by PCA analysis in Figure 4. This factorial plot is defined as follows: PC1 (32%) is highly positively correlated ($r^2>0.7$) to methylen, alkyl and C1 anomeric carbons and negatively correlated to aromatic and O-alkyl carbons, PC2 (23%) is highly positively correlated to carboxyl carbons and syringyl units. This figure shows the projection of litterbags sorted by incubation time.

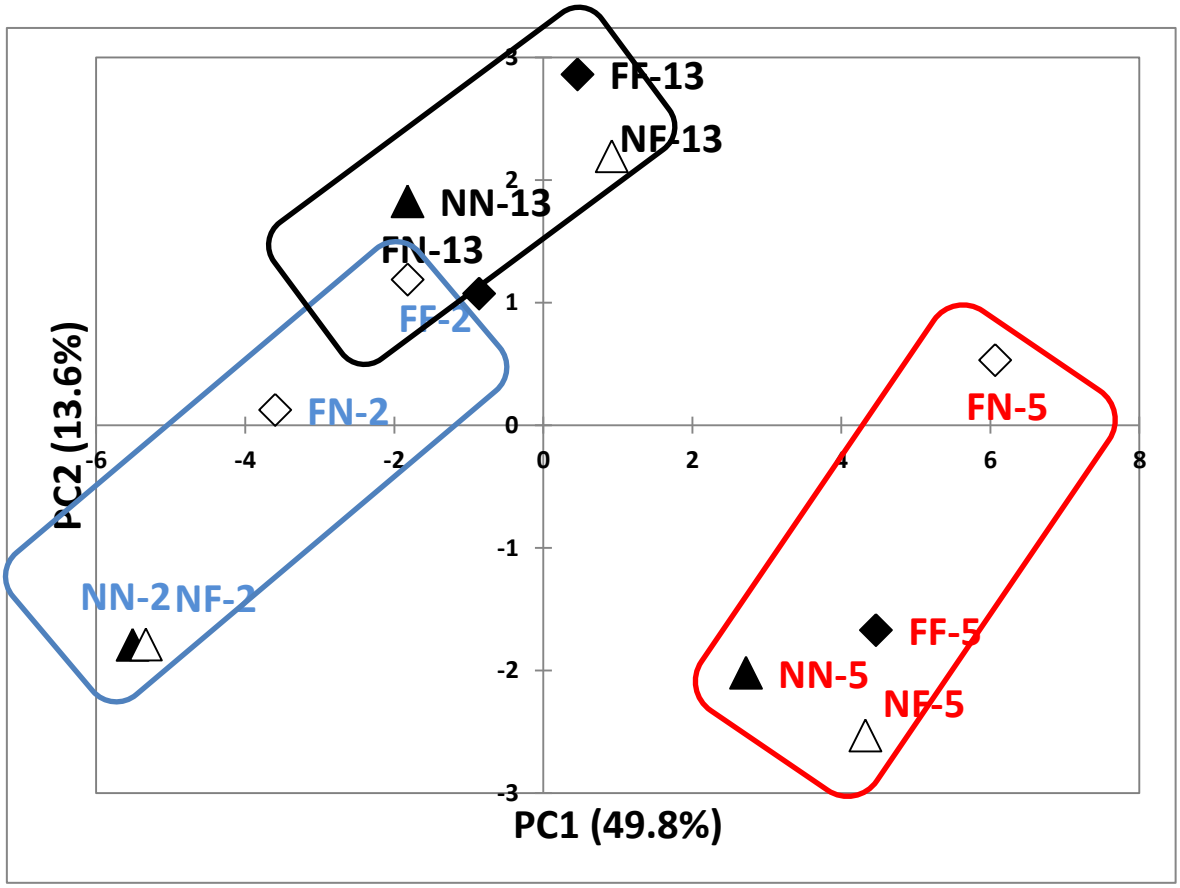


Figure 4. PCA on chemical groups as defined by ^{13}C NMR of litterbags after 2- (bleu), 5- (red) and 13- (black) month incubations. Triangle corresponds to litter from N area placed in N area (black) and placed in F area (white). Diamond-shaped corresponds to litter from F area placed in F area (black) and placed in N area (white). Each point represents the barycenter of three litterbags (n=3).

It also shows an increasing dissimilarity of litterbag chemical composition through time: the influence of the origin of litters on the projections is observable (FN is close to FF and NN is close to NF). This result reveals that chemical characteristics in transferred litterbags (FN or NF) strongly depend on the origin of litter. Moreover chemical characteristics of litter from N and F sites evolved differently after a 13-month incubation: we clearly observed a difference in maturity progress over time.

The microbial and chemical observations corroborate that salinity as an environmental factor has an impact on organic matter degradation and microbial community functional diversity though seasonality is obviously a major factor in such ecosystems. There are indeed certain differences between N and F areas in microbial and chemical composition, which underlines that litters located near to the sea, are subjected to a different micro-environmental context. These results are in accordance with our previous observations on enzyme production especially by fungal communities (article 1, chapter 1) in the same site of study. Thus,

though CLPP focuses on cultivable microbial communities, the results found here corroborate our previous findings using other microbiological indicators.

4. Conclusion

Mediterranean coastal environments are subjected to high environmental constraints especially summer drought combined with salinity. Both stresses can actually fasten microbial life to be metabolically active. In this study we showed that salinity partly affects microbial functional diversity at a small-scale and that climatic conditions may emphasize this effect especially during summer. Further studies should focus on the respective effects of salinity and drought on the microbial communities from coastal environments. A comparison between degradation of litter of the same plant species from coastal and inland areas should be performed to clearly show whether the impact of salt sprays structure microbial community and their function.

Article 3

Halotolerant laccases from *Chaetomium* sp., *Xylogone sphaerospora* and *Coprinopsis* sp. isolated from a Mediterranean coastal area

1. Introduction

White-rot fungi are known to be involved in the transformation of the recalcitrant fraction of organic matter in soils and litters i.e. natural aromatic compounds derived from lignin. These fungi transform phenols *via* phenoloxidases such as laccases, Mn-peroxidases or lignin-peroxidases (Sinsabaugh 2010). Under Mediterranean climate conditions, characterised by draught in summer, vegetal species such as *Quercus ilex* L. or *Pinus halepensis* L., are particularly sclerophyllous. In the mediterranean area, forest litters contain large quantities of various phenolic compounds which may favour the colonization by white-rot fungi. For instance, *Marasmius quercophilus* is a white-rot basidiomycete which extensively colonises evergreen-oak litter and produces several laccase isoforms (Farnet et al, 2009). These metalloenzymes (with several copper atoms in the active site) oxidize aromatic compounds while reducing oxygen to water. Since this reaction is not specific, laccases can also be involved in the transformation of aromatic pollutants such as PAHs (Majeau et al, 2010), pesticides or dyes (Jaouani et al, 2005; Rodríguez Couto et al, 2005). Indeed, laccases are used in various industrial applications such as pulp bleaching (Fillat et al, 2010) or treatment of industrial effluents which sometimes involving media with high osmotic pressure (Zeng et al, 2011). In a previous study, we tested the effects of various metal and halide salts on the laccases from *M. quercophilus* and we found that certain laccase isoforms were actually strongly inhibited by chloride ions (Farnet et al, 2009). This finding suggests that it would be useful to investigate how laccases function in natural environments such as coastal areas, where chloride ion concentrations are believed to be high. Because of the reaction they catalyse, these enzymes are indeed crucial both in recycling recalcitrant organic matter and in the detoxification of natural environments. Various organic pollutants can indeed greatly affect organic matter turn-over in soils or litters: for instance, PAHs can drastically alter microbial activities (Eom et al, 2007). These molecules have mutagenic and/or carcinogenic effects on living organisms, and are considered to be major environmental pollutants in peri-urban areas. Furthermore, these widespread pollutants are recalcitrant to biotransformation because of their aromatic and condensed structure leading to very low solubility (Muhit et al, 2008). Here, we isolated and identified three fungal strains producing laccases from a *Pinus halepensis* litter in a coastal Mediterranean area (Marseilleveyre, Bouches du Rhône, France) near the city of Marseille

(more than 850 000 inhabitants). Laccase activities were tested in the presence of chloride ions and we also examined their potential to transform various PAHs.

2. Materials and Methods

2.1. Isolation of fungal strains from a litter of *Pinus halepensis*

A composite sampling was performed from partially degraded litter (horizon L) of Aleppo pine (*P. halepensis* L.) located on the French Mediterranean coast (Massif de Marseilleveyre, Marseille).

About 1 g of litter was spread onto plates of a malt extract agar medium (MEA), (malt extract, (Bio Mérieux, France): 20 g L^{-1}, agar (Bio Mérieux, France): 20 g L^{-1}) complemented with guaiacol 0.5 g L^{-1} and supplied post autoclaving with chloramphenicol, (Sigma, France): 50 mg L^{-1}. This experiment was repeated 40 times. Fungal colonies which produced a purple halo were selected and used to inoculate a MEA medium to obtain pure mycelial cultures. Pure cultures on MEA medium were then used to prepare precultures.

2.2. Identification of the selected fungus by rDNA sequence analysis

The genomic DNA was extracted from the fungal cells using a Nucleon Phytopure extraction kit (Amesham Biosience) from lyophilised mycelium cultured on MEA medium. The absorbance ratio A260/A280 was determined to confirm the purity of the template DNA. Two flanking internal transcribed spacers (ITS1 and ITS2) were amplified using primers ITS1 (5'-TCCgTAggTgAACCTgCgg-3') and ALRO (5'-CATATgCTTAAgTTCAgCggg-3') (Collopy et al. 2001). The PCR mixture (25 µL) contained 5X PCR buffer (Promega, Madison, WI), 5 µL, dNTP mix (1.2 mM) (Eurobio, France), 3.5 µL, primer (10 µM), 2 µL of each, template DNA (50 ng μL^{-1}), 1 µL and GOTaq DNA polymerase 5 U (Promega M830B), 0.2 µL . The reagents were combined in 500 µL microtubes and placed in a Eppendorf Mastercycler (USA) programmed as follows: (a) initial denaturation at 95 °C for 5 min; (b) 35 cycles of denaturation at 95 °C for 1 min, annealing at 57 °C for 1 min and synthesis at 72 °C for 1 min; and (c) final extension at 72 °C for 4 min. Amplified DNA was separated on 1% agarose gel in (EUROMEDEX, France) 0.5 x Tris/Borate/EDTA (TBE) buffer for control. Sequencing was performed by Cogenics (Beckman Coulter genomics, United Kingdom). To compare the sequences of the amplified DNA with the sequences in GenBank, the basic local alignment tool (BLASTn) was used. The 666 bp (5.8S rDNA, ITS1, ITS2) sequences were submitted to GenBank under accession numbers JF681945, JF681946 and JF681944 respectively for CAL 1, CAL 2 and CAL 3.

2.3. Laccase production in liquid culture and optimum pH determination

For each strain, precultures were performed in 200 mL Erlenmeyer flasks with 50 mL of Malt extract liquid medium (MEL) containing malt extract (20 g L^{-1}) and $CuSO_4$ (5 g L^{-1}).

They were inoculated with a plug (1 cm diameter) from a fungal culture grown on MEA and incubated at 25 °C in darkness for four days. These precultures were used to inoculate 1 L-Erlenmeyer-flasks with 200 mL of MEL. Cultures were incubated at 25 °C for 5 days in darkness under shaking at 200 rpm (three replicates per strain).

Enzyme activity was measured by following the oxidation of syringaldazine [N,N'-bis-(3,5-dimethoxy-4-hydroxybenzylidene)hydrazine] to quinone (ε^{M} = 65 000 M^{-1} cm^{-1}) at 525 nm in acetate buffer 0.1 M, pH 4.0 on a Kontron Uvikon 860 spectrophotometer. One unit (U) of laccase activity is defined as the amount of enzyme that oxidizes 1 µmole of the substrate per minute. Culture sampling (two replicates) was performed during one week (each day) in order to determine the incubation time where the maximum laccase activity was obtained. The reaction mixture contained 500 µL of the medium, 10 µL of syringaldazine (0.6% in methanol) and 2.5 mL of acetate buffer (two replicates for each sampling). According to the best laccase production, cultures were filtered on a glass microfibre filter GF/D, 2.7 µm (Whatman, England). The filtered medium was concentrated using dialysis tubes (Cellu Sep, VWR, France) rated at 10 Molecular Weight (10 kD) cut-off. A total volume of 10 mL was obtained. Optimum pH was determined using 15 µL of the concentrated fraction with 10 µL of syringaldazine s.q.f. 1 mL of the adequate buffer. Acetate buffer 100 mM was used for the pH range from 4 to 5 and phosphate buffer 100 mM for the pH range from 5.7 to 8.

2.4. Polyacrylamide gel electrophoresis (PAGE) and Isoelectric Focusing (IEF)

Laccase isoform patterns were obtained from a PAGE carried out using 4% stacking gel and 10% separating gel at 220 V with the Mini-Protean II electrophoresis cell (Biorad). Laccase isoforms were stained using *p*-phenylenediamine 0.1% in acetate buffer 100 mM pH 4.5 at 30 °C under gentle shaking.

IEF was performed using the Biorad Rotofor preparative Cell. The samples were prepared as follow: 1 mL of Bio-Lyte 3/10 ampholytes (Biorad) was added to 10 mL of the enzyme extract and distilled water s.q.f. 50 mL. IEF was performed for 3 h at 200 V until the pH gradient become stable (voltage about 360 V). 2 mL-fractions were collected under vacuum and pH was immediately measured for each fraction. Laccase activity was also measured for each fraction using syringaldazine as a substrate as described above. For each strain, the fraction exhibiting the higher laccase activity was used to test the effect of chloride ions.

2.5. Effect of chloride ions and various solvents on laccase activities

For each strain, the IEF fraction with the highest laccase activity was used. Syringaldazine was used as substrate to determine the V_m and K_m, which were calculated using the Lineweaver-Burk transformation of the Michaelis-Menten equation. These kinetic

parameters were calculated with and without NaCl in the reaction mixture. The concentrations ranged from 12.5 to 75 µM for syringaldazine and two concentrations, 0.1 and 0.2 M, were used for NaCl in acetate buffer 0.1 M pH 4.5.

2.6. PAH detection by High Performance Liquid Chromatograph (HPLC)

The HPLC system was equipped with a C18 Reverse Phase column (Merck, 4.6 × 250 mm) in the following gradient system: solvent A, 90/10 (v/v) of 0.1% water-trifluoro acetic acid/acetonitrile, solvent B, 95/5 (v/v) of 0.1% water-trifluoro acetic acid/acetonitrile, gradient = 5 to 15 min, A 100% to B 100%; 15 to 25 min B 100% (flow rate 1 mL min $^{-1}$). The PAH transformation was realised using each enzyme extract from the three strains. The reaction mixture (5 mL) contained 250 mg L^{-1} PAH and 5 U of laccase in acetate buffer 0.1 M pH 4.5 and 10% acetone. The reaction mixtures were incubated at 30°C for 6h in the dark. An extraction with 5 mL of dichloromethane was performed for each reaction mixture prior to injection. The organic phase was evaporated and the products of extraction were dissolved in 1 ml of acetonitrile (30 µL injected). The PAHs used were: anthracene, 9-methylanthracene, pyrene, perylene and fluorene. All the PAHs were purchased from Sigma. *P*-hydroxybenzoic acid was used as reference in each injection. 9,10-anthraquinone was also injected in order to confirm the identification of the single oxidative product obtained after anthracene oxidation. Peaks were identified based on matching retention time and UV spectra at 254 nm with those of anthracene and anthraquinone standards.

3. Results and discussion

In this study we tested the effect of chloride ions on the laccases of three fungal strains isolated from a Mediterranean coastal environment. These phenoloxidase activities have previously been proved to be inhibited by chloride ions (Bonugli-Santos et al, 2010; Farnet et al, 2008). Our aim here was to demonstrate that areas naturally exposed to high osmotic pressure via sea sprays can be colonised by fungi producing laccases that remain active in the presence of such ions. Using ribosomal gene sequences, strains CAL 1 and CAL 3 were identified as ascomycetes, respectively *Chaetomium* sp. and *X. sphaerospora*. and strain CAL 2 was identified as a basidiomycete, *Coprinopsis* sp. CAL3 ribosomal gene sequences has 99% identity and only two gaps differences with the 450bp of the well characterised *X. sphaerospora* strain KACC 41222 (Kang et al, 2010). Laccase expression in the genus *Coprinopsis* has already been reported: Kilaru et al. (2006) described 17 laccase genes in the fungus Coprinopsis cinerea. Interestingly, we have recently found that peptides of laccases produced in litter from the same area can be assigned to laccases of Coprinopsis cinerea species (Qasemian et al, 2011). The identification of a species from this genus thus corroborates our previous findings and indicates that Coprinopsis species are involved in

laccase production in this type of natural environment. Laccase proteins and laccase genes (Lyons et al, 2003) have also been identified from ascomycetes such as *Penicillium chrysogenum* (Rodriguez et al, 1996) or Aspergillus nidulans (Scherer and Fischer, 2003). Moreover, laccases produced by *Chaetomium* sp. have been already reported for *Chaetomium thermophilium* (Chefetz et al, 1998) while, to our knowledge, this is the first report of laccase produced by *X. sphaerospora.*

First, we examined the laccase isoform diversity expressed by these strains using electrophoretic methods. PAGE revealed different profiles depending on the strains studied: two laccase isoforms (Rf = 0.41 and 0.5) for *Chaetomium* sp. strain, one isoform (Rf = 0.29) for *Coprinopsis* sp. strain, and one isoform (Rf = 0.22) for *X. sphaerospora* strain.

Preparative isoelectric focusing was also performed: for *Chaetomium* sp. strain, four isoforms with pHi 3.5, 4, 4.6 and 5.7 were found, for Coprinopsis sp. strain, two isoforms with pHi 3.2 and 3.5 and for *X. sphaerospora* strain, one isoform with pHi 4.3. As previously observed, isoelectric focusing discriminate better than PAGE when exploring protein diversity (Farnet et al, 2009). With this method, fractions containing laccases partially separated from the rest of the proteins of the extract were obtained. The highest activity was obtained for the isoform with pHi 4 for *Chaetomium* sp. strain and for the isoform with pHi 3.5 for *Coprinopsis* sp. strain and we used the fraction with the highest laccase activity for further biochemical investigations.

Figure 1 shows the optimum pH for laccase activity using syringaldazine as substrate, whatever the strain considered, optimum pH was 4.5 and we used this pH for all further experiments. This result corroborates previous studies: most laccases are active at low pH (Baldrian, 2006).

To define the effects of chloride ions on laccase activities, we used NaCl as representative of the principal salt believed to be spread via sea sprays on litters or soils in coastal environments. Furthermore, using NaCl should rule out interference with the cation on laccase activity: we have previously shown that certain metallic ions such as Cu^{2+} or Fe^{2+} react with enzymes leading to an apparent laccase activity increase (Farnet et al, 2008). Figure 2 shows the results obtained for NaCl concentrations ranging from 50 to 800 mM. Laccases from strain Chaetomium sp. strain were the most sensitive to chloride ions with 50% of the laccase activities remaining at 100 mM NaCl. With laccases from *Coprinopsis* sp. strain and *X. sphaerospora* strain, this value was obtained for 150 and 200 mM respectively. To clearly define the type of inhibition by chloride ions on the studied laccases, we measured laccase activities with various concentrations of substrate (syringaldazine) with 0.1 or 0.2 M

of NaCl in the reaction mixture using the Lineweaver-Burk representation. The results obtained are shown in Figure 3 and demonstrate that chloride ions act as competitive inhibitors: the Vm remains constant while Km decreases when the inhibitor is added. Km for syringaldazine was 57, 76.4 and 57 µM and Vm 0.99, 1.47 and 2.15 µmol min-1 mL -1 for *Chaetomium* sp. strain, *Coprinopsis* sp. strain and *X. sphaerospora* strain, respectively.

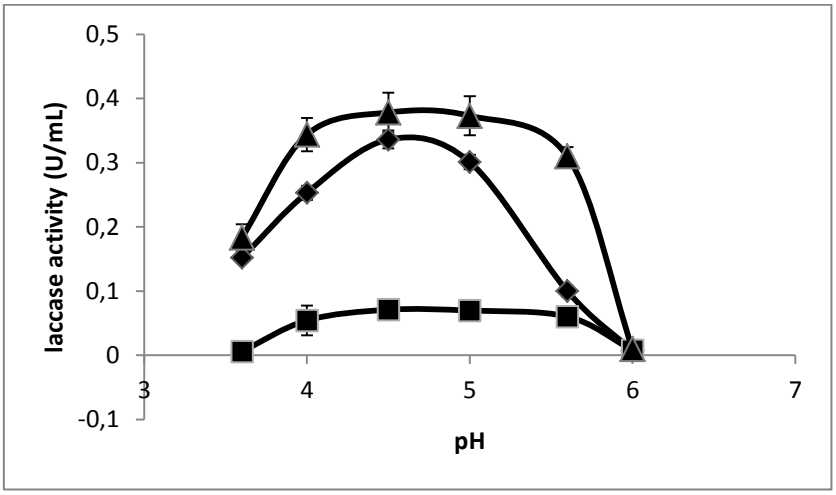


Figure 1. Determination of optimum pH for laccase activity using syringaldazine as substrate for isolates *Chaetomium* sp (■), *Coprinopsis* sp (♦) and *X sphaerospora* (▲)

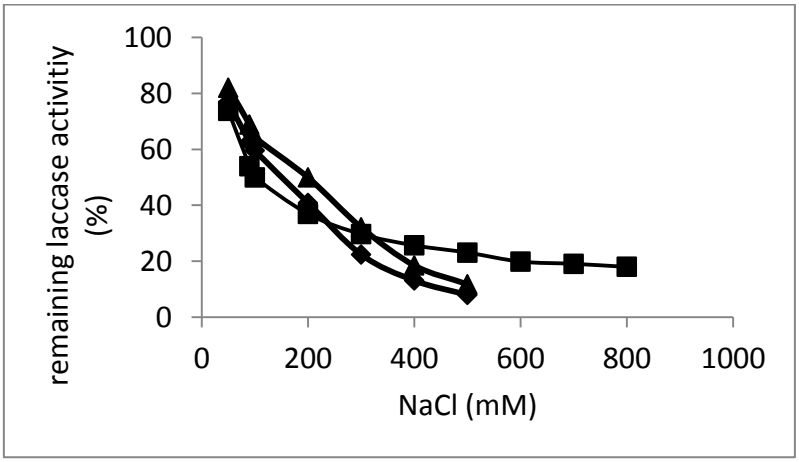


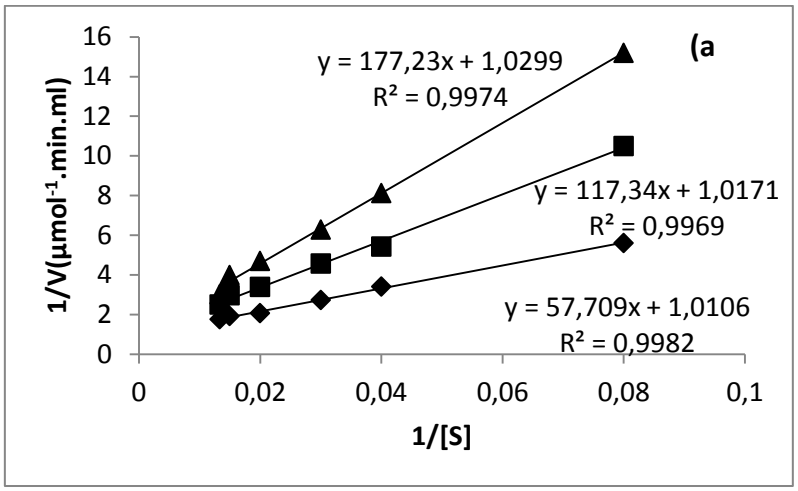
Figure 2. laccase activity Remaining (expressed as percentages of the activity without NaCl) with different concentrations of NaCl for isolates Chaetomium sp (■), Coprinopsis sp (♦) and Xsphaerospora (▲)

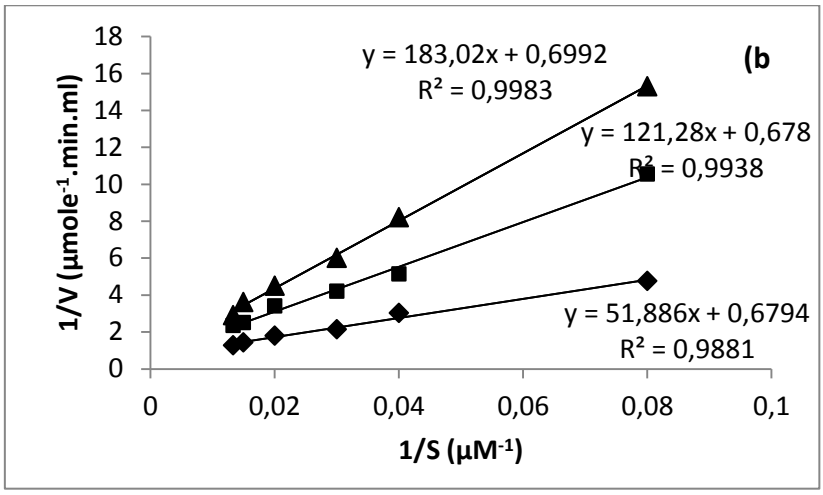
Various previous studies have described the inhibition of halide ions from an electrochemical point of view (Schneider et al, 1999; Xu et al, 1998): fluoride ions act as non-competitive inhibitors by blocking the intra-molecular electron transfer via binding to the T2/T3 copper cluster of laccase. On the other hand, chloride ions act as competitive inhibitors by blocking the access to the electron donor i.e. the aromatic compound to the copper of the T1 site.

Vaz-Dominguez et al. (2008) corroborated these results using a laccase electrode, showing the different modes of laccase inhibition by halide ions.

It is noteworthy that these fungal strains produce laccases which are inhibited to a lesser extent than those expressed by other fungi such as the white-rot basidiomycete Marasmius quercophilus (Farnet et al, 2008), whose laccases were strongly inhibited with 10 mM of NaCl (no residual activity for this concentration). Furthermore, Kim and Nicell (2006) found a two-fold decrease in the oxidation of bisphenol A by a laccase from Trametes versicolor with 50 mM of NaCl. Up to now, 'halotolerant' laccases have been found mainly in bacteria isolated from particular natural environments: Actinomycetes such as *Streptomyces psammoticus* (Niladevi et al, 2008) or marine bacteria such as *Marinomonas mediterranea* (Jimenez-Juarez et al, 2005). Laccases isolated from these bacteria show very high tolerance to chloride ions: enzyme activities remain at concentrations of NaCl up to 1 and 1.2 M respectively. It is noteworthy that both bacteria were isolated from natural media with high osmotic pressure: *M. mediterranea* is a marine bacteria and *S. psammoticus* was isolated from a mangrove swamp. In contrast, laccases from bacteria belonging to Rhizobiaceae such as Sinorhizobium meliloti (Rosconi et al, 2005) are strongly inhibited by halides (NaF or NaCl). Here, the laccases produced by three fungal species Chaetomium, X. sphaerospora and Coprinopsis were still active for 100 mM of NaCl. Two of them belong to Ascomycota: several studies have shown that this fungal class is frequently found in saline and hypersaline environment (Bergbauer and Newell 1992; Gunde-Cimerman et al. 2009; Raghukumar, 2004). Lyons et al. (2003) showed high diversity in laccase gene sequences in a study on salt marsh ascomycetes. Liers et al. (2007) biochemically described the laccases produced by an Ascomycete *Xylaria polymorpha* and found that 20% of the laccase activity remains with 1.2 M of NaCl. However other studies have also shown that a marine basidiomycete produces halotolerant laccases (D'souza et al, 2006). Thus it would be valuable to investigate the functional role of Basidiomycetes and Ascomycetes in phenolic compound oxidation in natural environments subjected to high osmotic pressure.

The effect of different solvents on laccase activities of the three strains was tested in order to choose the least inhibiting solvent, which would then be used to solubilize PAHs. Figure 4 shows that dioxane rapidly inhibits laccase activities for all the strains: no laccase activity remains with 10% of dioxane in the reaction medium. Solvent inhibition in decreasing order is as follows: dioxane, acetone, methanol and ethanol. We also found that laccase activities increase (more than 100% of the initial activity) for weak concentrations of ethanol and methanol (up to 10%). This is linked to the increase in syringaldazine solubility caused by solvents, as previously described (Farnet et al, 2008).





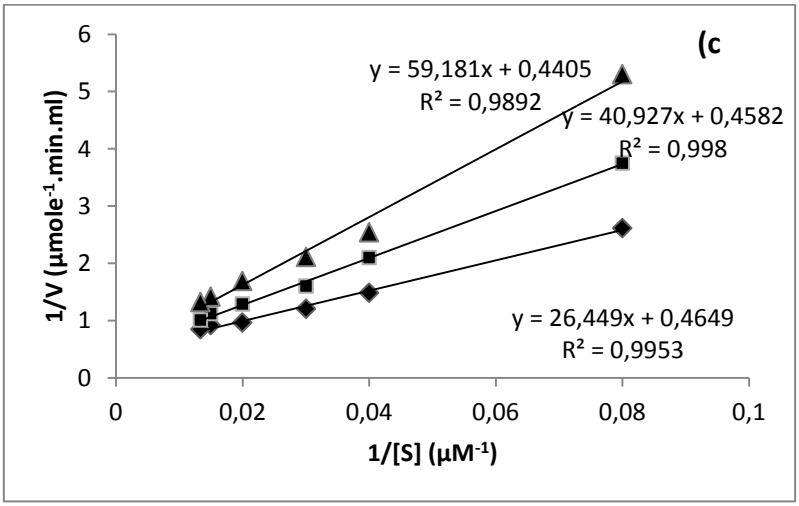


Figure 3. Determination of type of inhibition of chloride ions on laccase activity with syringaldazine as substrate Lineweaver-Burk transformations of Michaelis-Menten equation represented for isolates Chaetomium sp (a), Coprinopsis sp (b) and Xsphaerospora (c) with NaCl 01 M (■), 02 M (▲) or without NaCl (♦)

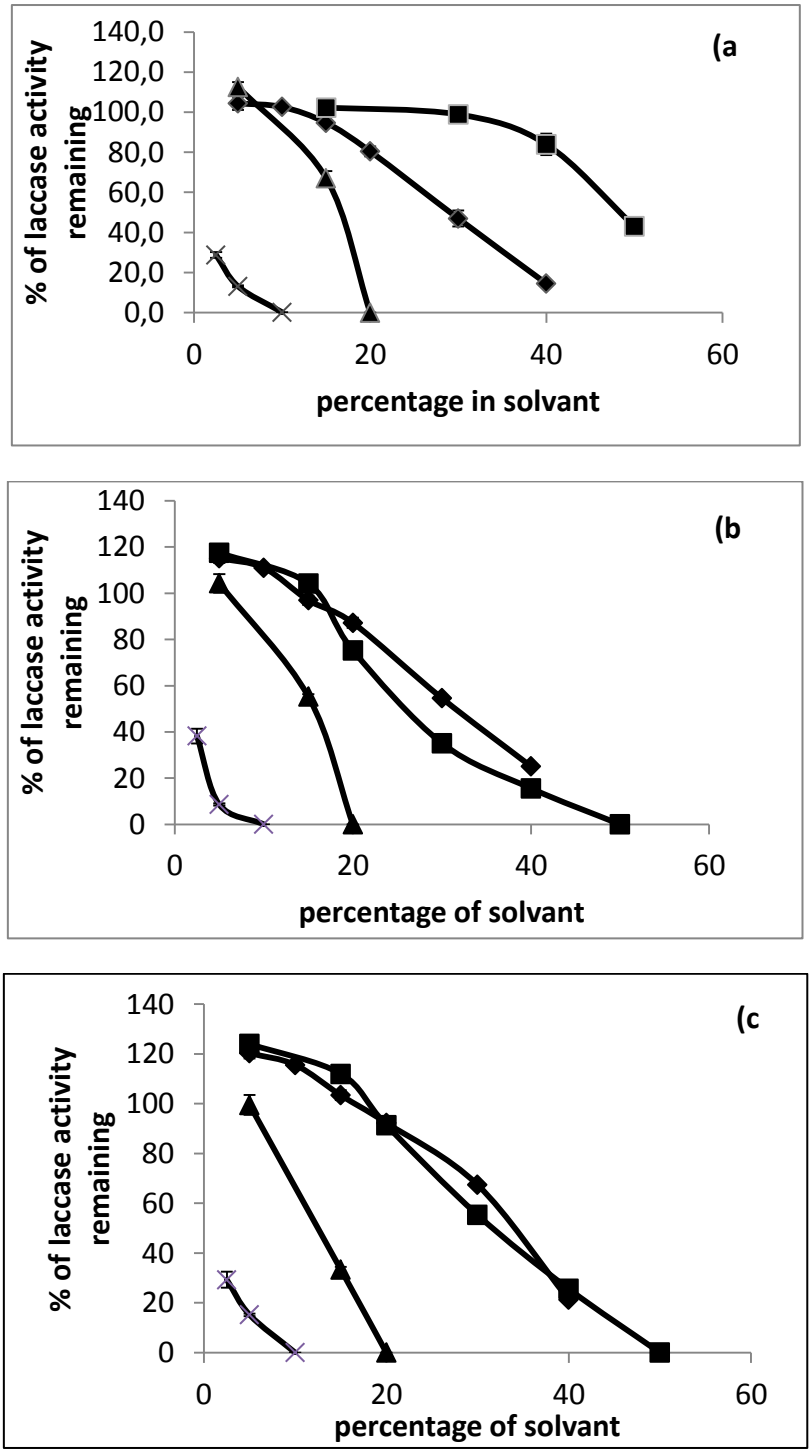


Figure 4. Effect of solvents on laccase activities of the three isolates Chaetomium sp (a), Coprinopsis sp (b), Xsphaerospora (c) expressed as percentages of activities remaining The solvents used were: dioxane (x), methanol (■), ethanol (♦), acetone (▲)

Since acetone did not drastically alter laccase activity and the studied PAHs can be dissolved in this solvent, we used 10% of acetone for the rest of our experiments in the reaction medium: about 90%, 80% and 70% of laccase activity remains for *Chaetomium* sp., *Coprinopsis* sp. and *X. sphaerospora* strains, respectively.

The potential for oxidation of PAHs by the three strains was studied using anthracene, 9-methylanthracene, pyrene, perylene and fluorene. These PAHs were chosen according to their Ionization Potential (IP), respectively 7.43, 7.5, 7.6, 7.76 and 7.96 eV.

A previous study on the phenoloxidases from the white-rot fungus Marasmius quercophilus (Farnet et al. 2009) showed that the ability of laccases to oxidize these molecules depended on their IP, and that molecules with IP over 8.1 eV were not oxidized. However the PAHs tested in this previous study were anthracene, naphthalene and phenanthrene with IP of 7.43, 8.1 and 8.19 eV, and our aim here was to define a more accurate threshold of IP starting from which the laccases under study oxidize aromatic compounds. For all the three strains, only anthracene was oxidized and this oxidation yielded 9,10-anthraquinone, according to the HPLC profile (data not shown). Here, our study reveals the threshold for PAH oxidation at 7.43 eV ionisation potential for theses laccases which is in agreement with previous studies (Dodor et al. 2004; Farnet et al. 2009; Hammel 1995; Kay-Shoemake and Watwood 1996). Furthermore, this finding clearly demonstrates that when certain laccases are produced in litters from coastal environments they are able to oxidize a toxic aromatic pollutant such as anthracene, which is known to be a widespread contaminant in these environments (Asia et al. 2009). Previous in vitro and in situ studies have shown that fungal phenoloxidases can be responsible for oxidation of PAHs (Canãs et al. 2007; Li et al. 2010). Laccases can thus play a major role in the detoxification of such natural ecosystems. Moreover, we have shown that high concentrations of chloride ions do not strongly affect the laccase activities of the fungal species isolated from coastal areas, suggesting that these enzymes are still active under these conditions associated with high osmotic pressure. This highlights the importance of this enzymatic system which is involved in the oxidation of both the recalcitrant fraction of soil organic matter and certain aromatic pollutants.

4. Conclusion

In conclusion, it appears that coastal environments, which are exposed to sea sprays, can be a potential source of fungal species producing laccases with higher resistance to chloride inhibition. Isolating new fungal strains producing laccases with such biochemical features holds great promise for biotechnological development in such fields.

Chapitre II

EFFET D'UN POLLUANT ORGANIQUE SUR LES ACTIVITES MICROBIENNES DE LA LITIERE DE PIN D'ALEP EN CONDITIONS CONTROLEES DE LABORATOIRE

Dans ce chapitre seront abordés 1) l'impact d'un HAP modèle, l'anthracène, sur le fonctionnement des communautés microbiennes d'une litière de pin d'Alep prélevée dans le même site d'étude de Marseilleveyre et 2) les capacités de transformation de ce polluant aromatique par ces communautés microbiennes. L'ensemble de ces études expérimentales ont été réalisées en microcosmes en conditions contrôlées de laboratoire (température, humidité) après une contamination de la litière par de l'anthracène (0.8 mg par g de litière sèche) préalablement solubilisé pour en faciliter la dispersion et l'accessibilité au sein de la litière. La litière a été recueillie sur la zone à proximité immédiate du littoral comme précédemment évoqué (cf. Chapitre 1, site de Marseilleveyre). Les incubations ont été réalisées pendant 1 et 3 mois dans des conditions d'humidité et de température considérées comme les plus favorables à la croissance des communautés microbiennes (60% WHC, 25°C). Différents indicateurs chimiques et microbiologiques ont été utilisés: RMN du ^{13}C et analyse C/N pour la caractérisation chimique de la matière organique et activités enzymatiques. Pour le volet microbiologique les activités enzymatiques impliquées dans les cycles biogéochimiques du carbone (cellulases, β-glucosidases, et lipases), du phosphore (phosphatases), ont été estimées ainsi que plus globalement, la diversité des fonctions cataboliques (par CLPP). Le recours à l'ensemble de ces outils est de nature à permettre une analyse comparative de la structuration fonctionnelle des communautés microbiennes en présence ou en absence de pollution par de l'anthracène. Dans le contexte de ces études, les laccases ont fait l'objet d'analyses plus spécifiques. Ainsi, outre la mesure de cette activité enzymatique, une caractérisation des laccases produites a été réalisée : après une électrophorèse mono-dimensionnelle, les différentes isoformes ont été séparées puis séquencées par spectrométrie de masse (LC/MS/MS). Par cette stratégie il a été ainsi possible de caractériser les laccases susceptibles d'intervenir dans les processus de détoxification de composés aromatiques polluants comme les HAPs et d'estimer le rendement de dissipation de l'anthracène par les microorganismes autochtones et naturellement présents au sein des litières méditerranéennes littorales.

Article 1: Identification of various laccases induced by anthracene and contribution to its degradation in a Mediterranean coastal pine litter. Chemosphere 84 (2011) 1321–1328.

Article 2*: *Effects of anthracene on microbial activities and organic matter decomposition in a Pinus halepensis litter from a Mediterranean coastal area. Soil Biology and Biochemistry 46 (2012)148-154.

Article 1

Identification of various laccases induced by anthracene and contribution to its degradation in a Mediterranean coastal pine litter

1 .Introduction

Due to the constant increase in environmental pollution, there is a growing interest in the microbial degradation of polycyclic aromatic hydrocarbons (PAHs). These molecules, considered major environmental pollutants (Johnsen et al, 2007; Sorgi, 2007; Wick, 2007), are persistent and toxic organic pollutants which have mutagenic and/or carcinogenic effects on living organisms (Sorgi, 2007). Because the Mediterranean ecosystem is subject to various natural and anthropogenic pressures e.g., Mediterranean climate with severe summer drought, urban pollution (De Nicola et al, 2005), studying the role of microbial communities in degrading environmental pollutants offers valuable insights into the resilience potential of Mediterranean ecosystems.

The massif of Marseilleveyre (Bouches du Rhône, France) is located in a peri-urban area subjected to strong anthropogenic pollution (industrial activities, transports, incinerators…) from the city of Marseille (about 850,000 inhabitants). In this study, we examine the potential of microbial communities from a *Pinus halepensis* litter, collected in this area, to transform anthracene, a molecule commonly found in PAH pollution. *P. halepensis* is a pioneer tree species in Mediterranean coastal regions (Quézel and Médail, 2003). Its litter contains high proportion of phenolic compounds, thus potentially favoring the development of lignin-degrading microorganisms, especially white-rot fungi (Valentín et al, 2010). They are known to produce phenoloxidases (Sinsabaugh, 2010; Baldrian, 2006): lignin peroxidase (LiP) (Hammel et al, 1986), manganese peroxidase (MnP) (Sack et al, 1997) and laccase (Collins et al, 1996). Previous studies have shown that laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) produced by white-rot fungi isolated from these particular ecosystems (Farnet et al, 2009, 2011) were able to transform various aromatic pollutants (Majeau et al, 2010; Oria-de-Rueda et al, 2010; Pozdnykova et al, 2006; Potin et al, 2004). It therefore seems likely that this enzyme has major potential for anthracene transformation in Mediterranean coastal litters.

Our objectives were i) to demonstrate the capacity of autochthonous microbial communities to transform anthracene under semi-natural conditions, ii) to clarify the potential role of phenoloxidases in this process and iii) to potentially identify the laccase produced under these conditions via protein sequencing.

Mesocosms with *P. halepensis* litter were subject to anthracene inputs: anthracene disappearance was monitored over a 3-month incubation period and different phenoloxidase activities (LiP, MnP and Laccase) were monitored throughout incubation. Particular attention was paid to laccase isoform production, since these enzymes are known to be induced by various aromatic compounds (Farnet et al, 1999; Teng et al, 2010).

2. Materials and methods

2.1.Litter sampling

A composite sampling was performed in November 2009 from partially degraded litter (horizon L) of Aleppo pine (*P. halepensis* L.) located on the French Mediterranean coast (Massif de Marseilleveyre, Marseille) (43° 12' 34'' N, 5° 21' 36'' E). Five samplings were randomly performed in a 100 m^2 area. The litter was sieved at 2 mm mesh size, homogenized and stored overnight at 4°C before being used in mesocosms.

2.2. Mesocosm preparation

Litter was placed in 2.6 L glass jars (25*20*7 cm) containing 50 g of litter (dry weight, DW) per mesocosm, covered with pierced lids to favor oxygen diffusion. Anthracene mesocosms (ANT) were prepared as follows: 40 mg of anthracene was dissolved in dichloromethane and added to a sub-sample of litter (10 g DW of litter) in order to minimize the harmful side-effects of solvent. After dichloromethane evaporation, the subsample was mixed with the litter of the mesocosm (40 g DW). Three different control mesocosms were also performed: a control mesocosm (C) i.e. litter without any treatment, a solvent control mesocosm (S-C) containing a sub-sample of litter treated with solvent alone and an abiotic control (A-C) using sterilized litter. Litter was sterilized 3 times by successive autoclaving at 120 °C for 20 min. This latter experiment was performed to assess the potential chemical oxidation of anthracene or its adsorption to litter particles. Each mesocosm type (C, S-C, A-C, ANT) was realized in triplicate (n=3) and incubated at 25°C in the dark both for 1- and for 3-months. Thus, for each incubation time, a total of 12 mesocosms were realized. Humidity of samples was maintained constant over incubation at 60% of the WHCmax.

2.3. Anthracene extraction and quantification with High Performance Liquid Chromatography (HPLC)

Anthracene was extracted from 20 g of litter from each mesocosm after 1- and 3- month incubation, using 200 mL of dichloromethane as solvent. The mixture was shaken twice at 500 rpm for 20 min. The extract was filtered and the solvent was then evaporated using a roto-vap chamber (Heidolf) and anthracene was dissolved in acetone. Associated debris were reduced via filtering and rinsed with acetone to a final volume of 100 mL.

Anthracene was quantified with HPLC (Waters) equipped with a C18 Reverse Phase column (Merck, 4.6×250 mm). The following gradient system was used: solvent A, water/trifluoroacetic acid 0.1%/acetonitrile 90/10 v/v, solvent B water/trifluoro acetic acid 0.1%/acetonitrile 95/5 v/v, gradient=5 to 15 min, A 100% to B 100%; 15 to 25 min B 100% (flow rate 1 mL min^{-1}). A calibration curve of anthracene was performed using 1, 2, 3, 4 and 5 nmoles of the compound. Peaks were identified based on matching retention time at 254 nm and UV spectra with those of anthracene and 9, 10-anthraquinone standards.

2.4. Enzyme activities

Proteins were extracted from mesocosms following a protocol modified from Criquet et al. (1999). Briefly, 10 g of litter was added to 200 ml $CaCl_2.2H_2O$ (200 mM) and 0.1% Tween 80, and then shaken for 1 h (500 rpm). The floating debris were removed, the extract was centrifuged (3,000 g for 20 min) and the supernatant was filtered (Whatman GF/C). The filtrate was concentrated by dialysis membrane (12-14KDa) using poly-ethylene glycol (PEG) and proteins were solubilized by adding 15 mL of BisTris buffer (20 mM, pH 6). Laccase activity was measured according to Harkin et al. (1973) by monitoring the oxidation of syringaldazine [N,N'-bis-(3,5-dimethoxy-4-hydroxybenzylidene)hydrazine] to quinone (ε^M = 65 000 M^{-1} cm^{-1}) at 525 nm. Both Lignin peroxidase (LiP) and Mn peroxidase (MnP) activities were quantified using 2,7diaminofluorene 75 µM as substrate with H_2O_2 and with both H_2O_2 and $MnSO_4$ (75µM) respectively (Farnet et al, 2011). Absorption was monitored at 600 nm (ε^M = 10 228 M^{-1} cm^{-1}). LiP activity and MnP activities were respectively calculated as follows: Activity with H2O2 - Activity without H_2O_2 and Activity with H_2O_2 and $MnSO_4$ - Activity with H_2O_2 - Activity without H_2O_2. The reaction mixture consisted of 300µL of enzyme extract with 700µL of acetate buffer 100 mM, pH 4.5 incubated in 30 °C. One unit (U) of enzyme activity is defined as the amount of enzyme that oxidizes one µmole of substrate per h. The significance of differences in enzyme activities between the treatment was tested by nonparametric multiple comparison of Kruskal-Wallis. Each replicate was considered as independent sample and compared over time. XLSTAT Vs7.1 (Addinsoft, France) was used for statistical analysis and P-value<0.05 was considered as significant.

2.5. Protein electrophoresis

The protein extracts obtained from mesocosms were subjected to polyacrylamide gel electrophoresis (PAGE), carried out according to Laemmli (1970). A Mini-Protean II electrophoresis cell (Bio-Rad) was used with 4% stacking gel and 10% separating gel at 220V. The protein extracts from each mesocosm condition (triplicate) were loaded onto the gel using 200 ng of proteins. The gels were soaked in sodium acetate buffer 100 mM, pH 4.5 and stained to reveal a laccase activity using p-phenylenediamine as a substrate at 0.1% (w/v). For protein sequencing, the triplicates from anthracene mesocosms were pooled and

concentrated at 4000 g for 10 min using a MACROSEP tube with a membrane of 10 KDa cut off (Pall Corporation, Life Science, USA). The concentrated samples were loaded twice into two separated lanes (12% separating gel). After migration, the gel was divided into two halves: one gel was stained using the standard Coomassie blue protocol and the other using laccase activities as previously described. The protein bands matching those stained by laccase activity were cut and subjected to sequencing.

2.6. Protein sequencing by Mass spectrometry (LC/MS/MS)

Sequence information from protein bands separated by 1D gel electrophoresis was obtained by mass spectrometry. The Coomassie blue stained bands were excised with a scalpel blade and cut into small blocks. The gel blocks were subjected to automatic trypsin hydrolysis using a Freedom EVO100 (Tecan) digestion robot. Basically, gels were rinsed with water and acetonitrile then reduced with DTT (Sigma), and alkylated with iodoacetamide (Sigma) and incubated overnight at 37 °C in a microtube with 12.5ng/µl of trypsin (Sequencing grade, Roche) in 25 mM NH4HCO3 as previously described (Shevchenko et al, 1996). The tryptic fragments were extracted, dried, reconstituted with 0.1% formic acid and sonicated for 10 min.

Tryptic peptides were sequenced by nano-LC-MS/MS (CapLC coupled to a Q-TOF-Ultima equipped with a nano-ESI source; Waters Micromass) in the data-dependent acquisition mode (four most intense ions per cycle, 1s MS scan, 10s maximum MSMS scan, and 60s dynamic exclusion). Only doubly and triply charged ions were selected as precursors over a mass range of 400-1300 m/z. The collision energy was selected depending on the precursor ion mass and charge and the Argon pressure in the collision cell was set at around 5 to 5.5 10-5 Torr. The source temperature and pressure were respectively 80 °C and 2.8 Torr and the capillary voltage was 3.2 kV. The mass spectrometer was calibrated using the fragmentation spectrum at 28 eV of Glu-fibrinopeptide (Sigma) at 100 fmol/ml in 50/50 water/ACN 0.1% formic acid. The protein digests were loaded on a precolumn (NanoEaseTM180mm i.d x 23.5mm, AtlantisTM dC18, Waters) and desalted with 0.1% formic acid for 5 min at 25ml/min on line with a C18 column (AtlantisTM dC18 75mm i.d x 150 mm NanoEaseTM, 3mm, 100 Á Waters). Peptides were eluted on a 5-60% linear gradient with water/acetonitrile 98/2 (v/v) containing 0.1 % formic acid in buffer A and water/acetonitrile 20/80 (v/v) containing 0.1% formic acid in buffer B in 90 min at a flow rate of 180-200 nl/min. The column outlet was connected via a fused silica capillary (20mm ID) to the nano-LC probe (Waters Micromass) and using another fused silica capillary as emitter (10mm ID).

Data were processed using ProteinLynx Global server 2.2 (PLGS 2.2, background noise subtraction 35%, smooth 3/2 Savitzky Golay and fast deisotoping) and matched to the NCBI non-redundant protein database (all taxa, version 20100411 containing 10820686 sequences) and to the GenBank ESTs database (EST_others , others_xyzzy containing 56245770 sequences). The search engine used was Mascot version 2.2 (Matrix Science, London, http://www.matrixscience.com) with the following search parameters: trypsin specificity, one missed cleavage, variable carbamidomethyl cysteine and oxidation of Met, and 0.1 Da mass tolerance on both precursor and fragment ions. De novo sequences were blasted against non-redundant database (nrdb95) using MS-BLAST software (http://dove.embl-heidelberg.de/Blast2/msblast.html). Only hits with significant high score peptide (HSP) according to Shevchenko et al. (2001) were taken into account. Briefly, sequence homology was considered as significant when the sum of HSPs of n peptides was equal to or higher than the threshold of the table from Shevchenko et al. (2001) for n reported HSPs.

3. Results

In examining anthracene degradation yields, our first concern was to distinguish between abiotic and biotic phenomena. The extraction tests performed immediately after anthracene was added to the litter yielded of 92% ± 7%. The abiotic controls (A-C) with sterile litter incubated with anthracene for 1 and 3 month(s) showed extraction yields of 57% ± 3%. The yield decrease may be due to anthracene evaporation or adsorption on litter, which mainly occurred during the first month of incubation since no differences in yields were observed between 1 and 3-month incubation periods. Yields from the microbiologically active mesocosms (the ANT mesocosms) showed no biotic degradation after 1 month but 28% ± 5% anthracene degradation after 3 months. We also identified 9,10-anthraquinone as the product of anthracene oxidation in the HPLC elution profile (data not shown) after a 3- month incubation.

Laccase activities were measured in each mesocosm (C, S-C, ANT) after 1- and 3-month incubations (Table 1). Whatever the mesocosms considered, a slight decrease in laccase activities was observed after a 1-month incubation. In control mesocosms, no significant variation in laccase activities was observed over time between 1- and 3-month incubations whereas laccase activities in ANT mesocosms or S-C mesocosms dramatically increased at 3-month incubation. Indeed, after this incubation time, laccase activities were 170- and 800- fold higher in mesocoms S-C and ANT respectively than those in mesocosms C. Laccase activities in mesocosms S-C were enhanced to a lesser extent than those in mesocosms with added anthracene (ANT). Furthermore, whatever the experimental

conditions, neither lignin-peroxidase activities nor Mn-peroxidase activities were detected (data not shown). This result seems to show that laccases are the principal phenoloxidases involved in aromatic compound degradation in such litters.

Table 1. Laccase activities (U. g dw-1) of *P. halepensis* litter after 1- and 3-month incubations in control C, Solvent Control S-C, mesocosm with 40 mg of anthracene ANT. For each mesocosm at each time, values followed by different letters differ significantly according to Kruskal-Walis test and Dunn procedure for multiple comparisons of means ($p<0.05$).

	Laccase activity (U. g dw^{-1})		
Mesocosms	**t_0**	**1-month**	**3-months**
C	0.027 ± 0.006^{cd}	0.004 ± 0.001^{ab}	0.008 ± 0.004^{bc}
S-C		0.002 ± 0.001^{a}	1.36 ± 0.73^{d}
ANT		0.004 ± 0.000^{ab}	6.68 ± 0.99^{d}

Table 2. Relative mobility (Rf) of laccase isoforms obtained from Native PAGE in different mesocosms of Pinus halepensis litter: control C, Solvent control S-C, mesocosm with 40 mg of anthracene ANT.

	Initial	**1-month incubation**			**3-month incubation**		
	C	**C**	**S-C**	**ANT**	**C**	**S-C**	**ANT**
Rf1	0.19						
Rf2						0.23	
Rf3	0.33						
Rf4							0.37
Rf5						0.40	
Rf6			0.42	0.42	0.42		
Rf7						0.47	
Rf8	0.51				0.51		0.51
Rf9		0.55					
Rf10		0.59	0.59	0.59			0.59
Rf11							
Rf12							0.66
Rf13							0.70
Rf14							0.87
Rf15						0.89	0.89
Rf16						0.96	0.96

Laccase isoform patterns found with PAGE corroborate the results obtained with laccase activity monitoring. We indeed observed an increase in both the number of bands of laccase isoforms and their staining intensity depending on the experimental conditions, corresponding to laccase activity variations. Laccase patterns from control mesocosms showed four bands over time which were weakly stained with Rf 0.55, 0.59 after a 1-month and with Rf 0.42, 0.51 after a 3-month incubation (Table 2). In mesocosms S-C, after the 1-month incubation, two bands with Rf 0.42 and 0.59 were observed while after the 3-month incubation, five different bands were found (Rf 0.23, 0.40, 0.47, 0.89 and 0.96) and both bands with Rf 0.89 and with 0.96 were strongly stained. These two bands are probably linked to the high laccase activity measured after this incubation period in mesocosms S-C (Table 1). The greatest number of bands, out of all the conditions, was obtained in ANT mesocosms after a 3-month incubation: eight strongly stained isoforms were detected with Rf 0.37, 0.51, 0.59, 0.66, 0.70, 0.87, 0.89 and 0.96. Isoforms with Rf 0.66, 0.70 and 0.87 were observed only under these conditions. Figure 1 shows a representative nano-HPLC/tandem mass spectrometry analysis of identified peptides. Twelve precursor ions with monoisotopic masses ranging from 428.58 to 1009.55 were found from MS/MS spectrum. Each MS/MS

spectrum was matched against the NCBI non-redundant protein sequence data base. Since no significant hit was obtained (except for contaminant proteins, e.g trypsin and keratins, data not shown), automatic *de novo* sequencing was performed. Table 3 lists the different sequences of peptides obtained after *de novo* sequencing for each monoisotopic mass (m/z). For instance, the precursor ion with m/z 1009.55 was assigned to only one sequence while the precursor ion with m/z 596.00 was assigned to 3 different peptide sequences.

MS BLAST homology searches in nrdb95 showed that all these peptides can be assigned to laccase proteins. Table 4 presents the different possible homology matches from MS BLAST which assigned the peptides sequenced to laccases from various fungal species. Among 24 different matches we found that these fungal laccase peptides correspond to genes *lac 1, 2, 4, 5, A, C* and *D*, *lcc, lcc 1, 2, 3, 4* and 7, *pox 1, 2* and *3*, and *bap.* These laccase genes belong to various species from Agaricales and Polyporales orders, all of which are basidiomycota and are either lignocolous fungi such as *Trametes* spp or humicolous fungi such as *Volvariella volvacea.*

Table 3. Characteristics of peptides from ANT mesocosms of *Pinus halepensis* litter obtained from HPLC MS/MS and *de novo* sequencing by Proteinlynx Global software.

Peptide abbreviation	Sequences obtained by *de novo* sequencing	Monoisotopic mass (m/z)	Charge state
P_1	(-)NNSSLDLLNANVAPDGFTR(-)	1009,55	2+
P_{2-1} P_{2-2} P_{2-3}	(-)ANPNPSSRAEFAQLGAR(-) (-)ANPNPSSRAEFAAGLGAR(-) (-)ANPNPTQTAEFAQLGAR(-)	596,00	3+
P_3	(-)VVDTGLFTGQR(-)	596,81	2+
P_{4-1} P_{4-2} P_{4-3}	(-)VVLEELLQLMCR(-) (-)VVLEEQLLLMCR(-) (-)VVLEQELLLMCR(-)	428,58	3+
P_{5-1} P_{5-2}	(-)LTATTALTAR(-) (-)LTATTLTAAR(-)	509,77	2+
P_{6-1} P_{6-2} P_{6-3} P_{6-4}	(-)FTANDVTSTANSTVGDTK(-) (-)TFANDVTSTANSTVGDTK(-) (-)FTANDVTSTANSVTGDTK(-) (-)TFANDVTSTANSVTGDTK(-)	914,92	2+
P_7	(-)TLTWNYLNSPR(-)	682,84	2+
P_8	(-)GNASPTPGKCKALVALVGAATPQR(-)	788,74	3+
P_{9-1} P_{9-2} P_{9-3} P_{9-4}	(-)FNLLGFAVGKNLLDLDTR(-) (-)FNLLGFAVKGNLLDLDTR(-) (-)FNLLGFAVGKGGLLDLDTR(-) (-)FNLLGFAVKGGGLLDLDTR(-)	1003,53	2+
P_{10}	(-)ANPNVGPSGFEKLNTLGLR(-)	992,53	2+
P_{11}	(-)HLYDVDDK(-)	502,76	2+
P_{12}	(-)DPTTVDVPGVR(-)	564,33	2+

4. Discussion

In the present study, the percentages of biodegradation of anthracene showed that a high proportion of this organic pollutant (11 mg in mesocosm ANT) was quite rapidly oxidized (within 3 months) by authochtonous microbial communities placed under favorable incubation conditions. Furthermore, an increase in laccase activities was obtained after a 3-month incubation, suggesting that they were strongly induced by anthracene. Steffen et al. (2007) have shown that fungal activities considerably enhanced anthracene transformation in mesocosms. This increase in laccase activities is corroborated by the increased number of laccase isoforms on PAGE in mesocoms with added anthracene. Here, PAGE patterns clearly showed new isoforms when anthracene was added to the mesocosms (mainly

isoforms with Rf 0.66, 0.70 and 0.87). Laccase activities are known to be induced under various conditions: addition of phenolic compounds (Terrón et al, 2004; Verdín et al, 2006), addition of $CuSO_4$ in the culture (Farnet et al, 1999, Fonesca et al, 2010) or antagonism between microorganisms (Velázquez-Cedeño et al., 2007).

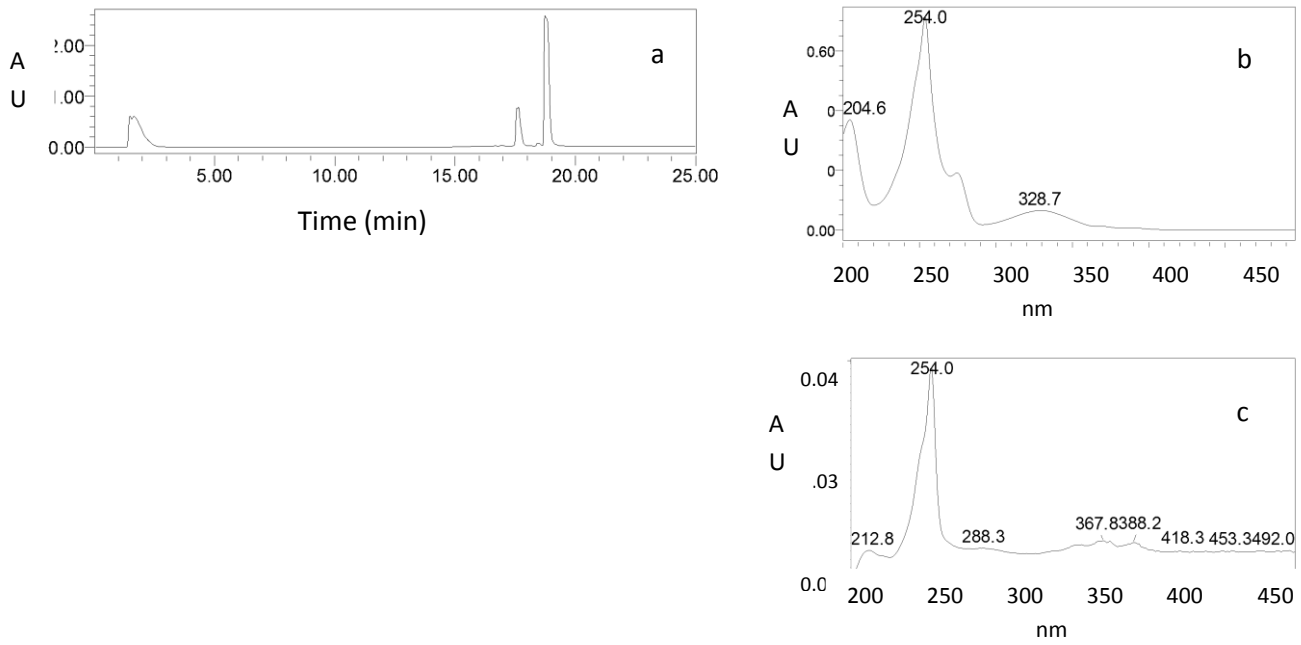


Figure1. Elution profile (a) at 254 nm of an extract of litter from ANT mesocosm after solubilization in acetone with UV scan of 9,10-anthraquinone (b) and anthracene (c).

Recent studies have also shown that anthracene is responsible for laccase induction in certain fungal species (Farnet et al, 2009). Induction of laccase can lead to the production of new isoforms of these enzymes and/or enhance the production of constitutive isoforms leading to an increase in laccase activity (Dodor et al, 2004; Edwards et al, 2002; Teng et al, 2010). Thus, our results revealed that the laccases produced by fungal communities in the *P. halepensis* litter can be induced by anthracene. The fact that 9, 10-anthraquinone was also identified as a product of oxidation of anthracene and that no significant peroxidase activities (LiP and MnP) were detected, suggests that anthraquinone production was essentially due to high laccase activities. The laccases expressed in these litters can thus transform a toxic pollutant, evidence of the major role these enzymes play in both ecosystem functioning and detoxification. This result is in agreement with previous studies which have also identified this

product of oxidation in soil mesocosms (Coutiño-González et al, 2010) or in liquid cultures of white-rot fungi (Cañas et al, 2007; Farnet et al, 2009). However, the quantity of 9,10-anthraquinone detected in our study is low in relation to the anthracene degradation yields. This result suggests that 9,10-anthraquinone, a more polar molecule than anthracene, is rapidly further transformed by other microbial enzymes. This finding is of importance since it demonstrates that laccase activities probably enhance aromatic pollutant degradation by authochtonous microbial communities in litter via a first step of oxidation.

It is noteworthy that laccase activities in the S-C mesocosms were higher than those in the C mesocosms. The S-C mesocosms were intended to reveal the potential effects on bacterial and fungal communities of residual dichloromethane, which was used as the solvent of anthracene. Although the solvent did evaporate after being added to sub-samples, a certain quantity of this molecule may still have been adsorbed on the litter. Moreover, even when no solvent remained on the litter, it may have chemically reacted with the litter compounds before evaporation. In other words, possible non-target side-effects of solvents used to solubilize apolar pollutants need to be considered in such experiments. Brinch et al. (2002) have emphasized the harmful side-effects of solvents on indigenous microbial consortia. Here, no toxic effects were observed on laccase activity: they were higher in S-C mesocosms than in C mesocosms, which may mean that dichloromethane extracts from the litter apolar molecules such as phenolic acids or tannins, which may induce laccase activities. Moreover, the laccase isoform pattern obtained from the S-C mesocosms showed strongly stained bands, which confirms the high level of laccase activities obtained. The strong laccase activities may be also related to the phenolic compounds released by dichloromethane: it has been suggested that these molecules may act as mediators for fungal laccase activities during litter degradation (Calcaterra et al, 2008; Cañas and Camarero, 2010).

While proteomics approaches have an essential role to play in determining changes in microbial function expression under environmental influences (Bastida et al, 2009; Chauhan and Jain, 2010), little work has been done on them. To our knowledge this is the first study focusing on laccase sequencing from proteins produced in litter. The transcript-level gene expression of laccases in forest soils is well documented and reveals both great gene diversity and variations in gene expression (Kellner et al, 2009, 2010; Luis et al, 2004). However proteomics studies could link the community genetic structure and the functions actually expressed in ecosystems (Kim et al, 2007; Urich et al, 2008).

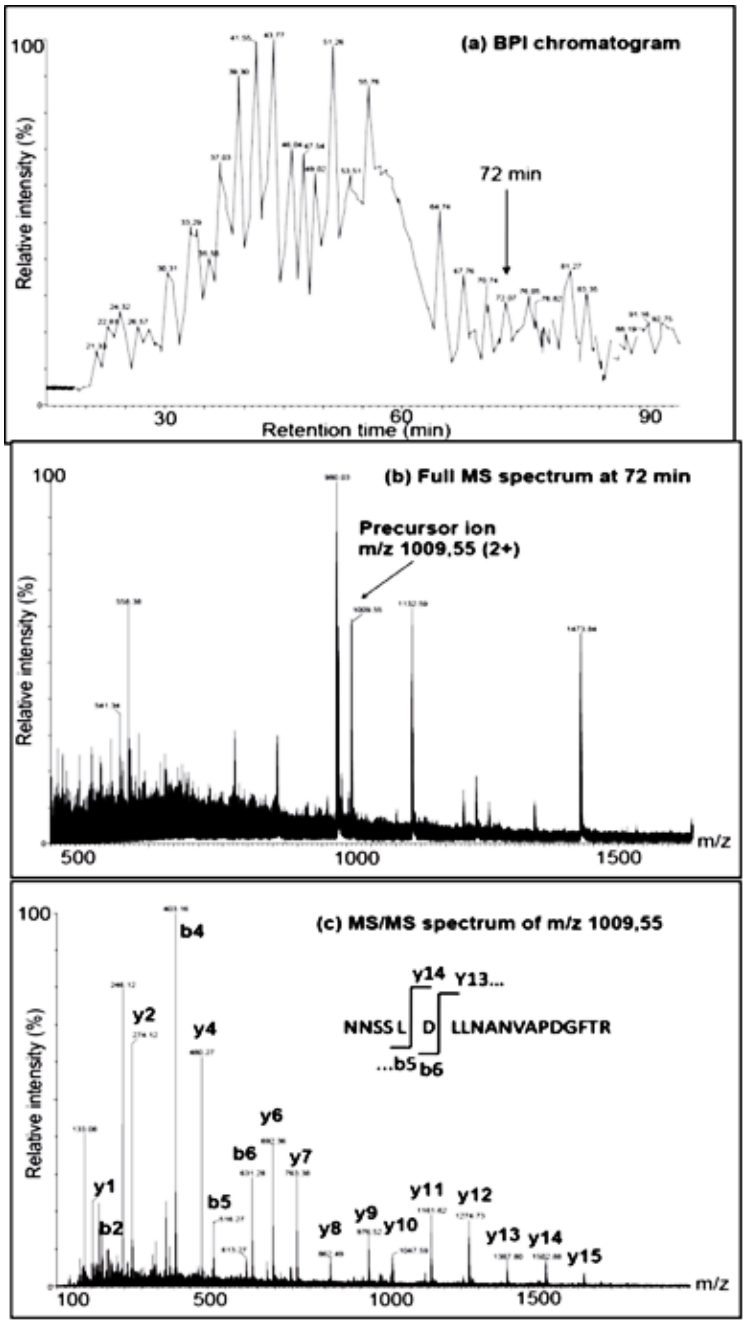


Figure 2. Example of nano-HPLC/tandem MS analysis of peptides obtained from mesocosms of Pinus halepensis litter: (a) Base Peak Intensity (BPI) chromatogram of nano-HPLC/tandem MS of the tryptic digest gel. (b) Full scan spectrum at the retention time of 72 min. (c) MS/MS spectrum of 1009,55 m/z, which identified the peptide NNSSLDLLNANVAPDGFTR.

Here we examined laccase expression in litter via direct protein sequencing of the enzymes produced by the whole microbial community and we identify several laccase sequences. It is noteworthy that laccase induction via anthracene made laccase sequencing possible since high laccase activities, up to 800 times higher than in control, was obtained under these experimental conditions.

MS BLAST searching shows that the peptides found in the mesocosms with added anthracen, match laccases from different fungal genera: *Coprinopsis* sp., *Pleurotu*s spp., *Trametes* spp., *Volvariella* spp, *Rigidoporus* spp, *Agaricus* sp. and *Panus* sp. (Table 4). However, while matching allowed the peptides found to be identified as laccases, these results have no ecological relevance. In fact, none of these fungi is considered endemic to French Mediterranean coastal habitats. For instance, *Volvariella volvacea* (Chang and Steinkraus, 1982) is known to be isolated to Asia and *Rigidoporus microporus* is a pan-tropical species (Corner, 1995). However, among all the different sequences found, it is notable that a sequence was assigned to *Basidiomycete C30*. This basidiomycete has been isolated from an evergreen oak litter in the Mediterranean area (Tagger et al, 1998) and has been identified as *Trametes* sp. (Klonowska et al, 2003). Our results are probably affected by a lack of data in the available protein database, which strongly limits assignment to a fungal genus. The full range of fungal species from the Mediterranean area is poorly documented (Laganà et al, 1999), especially those colonizing *P. halepensis* litter in coastal areas. Our study provides information about laccase peptide sequences found in *P. halepensis* litters. These peptide sequences could be useful for further studies focusing on functional genomics for instance to design primers for laccase gene amplification.

These experiments under laboratory conditions should be completed by setting further in situ experiments in order to demonstrate this transformation by microbial communities in natural areas. To better clarify the role of the indigenous microbial community in chronic pollution (Andreoni and Gianfreda, 2007; Canet et al, 2001; Wang et al, 2010) further work should be done on the effect of other recalcitrant molecules which are commonly detected in PAH contamination. Future studies should also investigate whether this detoxification potential is also expressed in inland areas, and whether other litter enzymes are affected by the presence of PAHs.

Table 4: Identification of *de novo* sequenced peptides from mesocosms of *Pinus halepensis* litter by MS BLAST from nrdb95 protein database. Matching was considered as significant by considering the sum of HSPs as described by Shevchenko et al. (2001).

Accession number	Protein	Gene	Microorganism	Query peptides	High Scoring Pairs
sptrembl|Q6A1A1|Q6A1A1	Laccase (EC 1.10.3.2)	*lac1*	*Pleurotus sapidus*	p_1, p_{10}, $p_{6\text{-}2}$, $p_{6\text{-}4}$, p_{12}, p_3, p_{11}	66, 49, 41, 41, 40, 35, 35
sptrembl|Q7Z8S6|Q7Z8S6	Laccase 1 (EC 1.10.3.2)	*lac1*	*Pleurotus sajor-caju*	p_1, p_{10}, $p_{6\text{-}2}$, $p_{6\text{-}4}$, p_3, p_{11}	71, 51, 45, 45, 35, 35
sptrembl|Q69AX8|Q69AX8	Laccase 1 (EC 1.10.3.2)	*lcc1*	*Coprinopsis cinerea* strain AmutBmut	p_1, p_3, $p_{2\text{-}3}$, $p_{6\text{-}2}$, $p_{6\text{-}4}$, $p_{4\text{-}1}$	75, 43, 43, 38, 38, 34
swiss|Q12729|LAC1_PLEOS	Laccase 1 precursor (EC 1.10.3.2)	*pox1*	*Pleurotus ostreatus*	p_1, p_{10}, $p_{6\text{-}2}$, $p_{6\text{-}4}$, p_{11}, p_3	66, 45, 41, 41, 40, 35
sptrembl|Q6UNT7|Q6UNT7	Laccase (EC 1.10.3.2)	*lcc*	*Rigidoporus microporus*	p_1, p_7, p_8, p_3, p_{11}	70, 42, 41, 35, 35
sptrembl|Q5MBH3|Q5MBH3_9APHY	Laccase A	*lacA*	*Trametes* sp. 420	p_1, p_{11}, $p_{5\text{-}2}$, p_3, $p_{4\text{-}1}$	70, 46, 37, 35, 34
sptrembl|Q69FW9|Q69FW9	Laccase 5	*lac5*	*Volvariella volvacea*	p_1, p_{10}, p_3, p_{11}	66, 59, 35, 35
sptrembl|Q5MBH1|Q5MBH1_9APHY	Laccase C (EC 1.10.3.2)	l*acC*	*Trametes* sp. 420	p_1, p_{11}, $p_{5\text{-}1}$, p_3	74, 46, 35, 35
sptrembl|Q69FX0|Q69FX0	Laccase 3	*lac3*	*Volvariella volvacea*	p_1, p_{11}, p_{10}, p_3	58, 50, 47, 35
sptrembl|Q6VMC1|Q6VMC1	Laccase 3 (EC 1.10.3.2)	*lcc3*	*Coprinopsis cinerea* strain AmutBmut	p_1, p_{10}, p_{11}, p_3	65, 43, 41, 35
sptrembl|Q7Z8S3|Q7Z8S3	Laccase 4 (EC 1.10.3.2)	*lac4*	*Pleurotus sajor-caju*	p_1, p_{10}, $p_{6\text{-}2}$, $p_{6\text{-}4}$, p_3	59, 49, 41, 41, 35

Accession number	Protein	Gene	Microorganism	Query peptides	High Scoring Pairs
swiss|Q12739|LAC2_PLEOS	Laccase 2 precursor (EC 1.10.3.2)	*pox2*	*P.ostreatus*	p_1, p_{10}, $p_{6\text{-}2}$, $p_{6\text{-}4}$, p_3	58, 49, 41, 41, 35
sptrembl|Q6VMC0|Q6VMC0	Laccase 4 (EC 1.10.3.2)	*lcc4*	*Coprinopsis cinerea* strain AmutBmut	p_{11}, p_1, p_3, $p_{4\text{-}1}$	55, 50, 43, 34
sptrembl|Q6VMB7|Q6VMB7	Laccase 7 (EC 1.10.3.2)	*lcc7*	*Coprinopsis cinerea* strain AmutBmut	p_1, p_3, p_{11}, $p_{4\text{-}1}$	58, 43, 41, 34
sptrembl|Q716A2|Q716A2	Laccase	*pox1*	*Trametes* sp. I-62	p_1, p_{11}, p_{10}, $p_{6\text{-}1}$, $p_{6\text{-}2}$	58, 41, 39, 34, 34
sptrembl|O13422|O13422	Phenoloxidase (EC 1.10.3.2)	*pox3*	Basidiomycete CECT 20197	p_1, p_{11}, p_3, $p_{4\text{-}1}$	63, 40, 35, 34
swiss|Q12541|LAC1_AGABI	Laccase I precursor (EC 1.10.3.2)	*lcc1*	*Agaricus bisporus*	p_{11}, p_{12}, p_1	55, 54, 53
swiss|Q12542|LAC2_AGABI	Laccase II precursor (EC 1.10.3.2)	l*cc2*	*Agaricus bisporus*	p_{11}, p_{12}, p_1	55, 54, 53
sptrembl|Q6RYA5|Q6RYA5	Laccase (EC 1.10.3.2)	AY450406	*Flammulina velutipes*	p_1, p_{11}, p_{10}	68, 46, 37
sptrembl|Q8NID5|Q8NID5	Laccase 2 (EC 1.10.3.2)	*lac2*	Basidiomycete C30	p_1, p_{11}, $p_{6\text{-}1}$, $p_{6\text{-}2}$	70, 46, 34, 34
sptrembl|Q5MP11|Q5MP11_9AGAR	Laccase precursor (EC 1.10.3.2)	*pel3*	*Pleurotus eryngii*	p_1, p_{10}, p_3	58, 57, 35
sptrembl|Q5MBH0|Q5MBH0_9APHY	Laccase D (EC 1.10.3.2)	*lacD*	*Trametes* sp. 420	p_1, p_{11}, $p_{4\text{-}1}$	69, 46, 34
sptrembl|Q5MBH7|Q5MBH7_9APHY	Laccase A (EC 1.10.3.2)	*lacA*	*Panus rudis*	p_1, p_{11}	73, 46

Article 2

Effects of anthracene on microbial activities and organic matter decomposition in a *Pinus halepensis* litter from a Mediterranean coastal area

1. Introduction

Polycyclic Aromatic Hydrocarbons (PAH) are widespread contaminants and their effects on the environment via massive pollution have received particular attention (Johnsen and Karlson, 2007; Sorgi, 2007). They are also associated to chronic pollution especially near urban areas where anthropic activities are intense (industrial activities, combustion of fossil carbon resources, transports, refineries). Recent studies have indeed demonstrated that these pollutant concentrations are especially high near urban areas (Lammel et al, 2010; Yang et al, 2010). Chronic exposure to these pollutants via atmospheric deposition can dramatically alter natural environments because of their mutagenic and carcinogenic effects on living organisms (Lammel et al, 2010; Schwarz et al, 2011).

The Mediterranean coastal environment is particularly weakened because of its strong urbanization and high touristic attraction. These ecosystems are also subjected to various natural environmental stresses under Mediterranean climate: summer drought, limestone with low organic matter and sclerophyllous litters. Furthermore, soils and litters from coastal areas are subjected to osmotic stress and pollutant deposition via sea sprays. Recent studies have shown that these environmental constraints combined to increased human pressures make the Mediterranean coastal ecosystems vulnerable (Francaviglia et al, 2004; Gritti et al, 2006; Tzatzanis et al, 2003). Several studies have focused on the occurrence and distribution of different pollutants in Mediterranean coastal areas (Asia et al, 2009; Mille et al, 2007). However, the effects of PAH on autochthonous microbial communities of soils in such ecosystems remain very poorly known.

Soil microorganisms are actively involved in the bio-chemical processes linked to organic matter mineralization (Falkowski et al, 2008; Schimel and Weintrub, 2003). Studies on variations in enzyme activities and microbial diversity during litter decomposition have already provided much information on the dynamics of decay and functional microbial succession in Mediterranean ecosystems (Duarte et al, 2010; Fioretto et al, 2000; Papa et al, 2008). The potential of fungal communities is especially of great interest due to their ability to produce lignolytic extracellular enzymes which play a particularly important role in detoxification processes of natural environments (D'Annibale et al, 2006).

In this study we aim to test the effects of anthracene, an ubiquitous pollutant, on the indigenous microbial communities of a Pinus halepensis litter from a Mediterranean urban coastal area. In a previous study, we have shown that indigenous microbial communities were able to oxidize anthracene via a high laccase production in semi-natural conditions (Qasemian et al, 2011). In order to assess the effect of such pollutant on the function of the whole indigenous microbial communities, including bacteria and fungi, mesocosms were set up with a Pinus halepensis litter from a peri urban coastal area (Marseille, France). Our main objectives were thus to show how anthracene added to the litter modifies both microbial functional diversity and litter decomposition, using certain microbial activities and 13C NMR to chemically characterize organic matter.

2. Materials and Methods

2.1. Litter sampling

A composite sampling was performed in November 2009 from partially degraded litter (horizon L) of Aleppo pine (*P. halepensis* L.) located on the French Mediterranean coast (Massif de Marseilleveyre, Marseille) (43° 12' 34'' N, 5° 21' 36'' E). Five samplings were randomly performed in a 100 m^2 area. The litter was sieved at 2 mm mesh size, homogenized and stored overnight at 4°C before being used in mesocosms.

2.2. Mesocosm preparation

The litter was placed in 2.6 L glass jars (25*20*7 cm) containing 50 g of litter (dry weight, DW) per mesocosm, covered with pierced lids to favor oxygen diffusion. Anthracene mesocosms (ANT) were prepared as follows: 40 mg of anthracene was dissolved in dichloromethane and added to a sub-sample of litter (10 g DW of litter) in order to minimize the harmful side-effects of solvent. After dichloromethane evaporation, the sub-sample was mixed with the litter of the mesocosm (40 g DW). Two different control mesocosms were also performed: a control mesocosm (C) i.e. litter without any treatment, a solvent control mesocosm (S-C) containing a sub-sample of litter treated with solvent alone. Each mesocosm type (C, S-C, ANT) was realized in triplicate (n=3) and incubated at 25°C in the dark both for 1 and for 3 month(s). Thus, for each incubation time, a total of 9 mesocosms were realized. Humidity of samples was maintained constant over incubation at 60 % of the WHC max.

2.3. Physico-chemical analysis

Litter were preliminary characterized for moisture, pH, electrical conductivity (EC). Total Organic Carbon (TOC) and total Nitrogen were measured as follows: oven dried sub-samples of initial litter and of each mesocosm's litter were powdered in a ceramic mortar and analyzed by combustion in an Elemental Analyzer, FlashEA 1112, Thermofisher. PAHs

concentration in the initial litter was determined after extraction by dichloromethane according to Qasemian et al. (2011). PAHs were quantified by HPLC (VARIAN Prostar 230, UK) equipped with fluorescence detector and a reversed phase column C18 (Varian, ChromSpher, UK) using a mobile phase of water and acetonitrile mixture.

2.4. Extracellular enzyme activities (EEA)

Cellulase (CEL), β-1,4-glucosidase (βGLU), Acid phosphomonoesterases (PHA) and Lipase (LIP) were quantified after 1 and 3-month incubations. Except for Lipase which was estimated directly from 1 g of litter using p-nitrophenyl laurate at 10mM as substrate according to Farnet et al. (2010a), all the other activities were measured using an enzyme extract from litter following a modified protocol from Criquet et al. (1999). Briefly, 10 g of dry litter was added to 200 ml $CaCl_2.2H_2O$ (200 mM) and 0.1 % Tween 80, and then shaken for 1 h (500 rpm). The floating debris were removed, the extract was centrifuged (7,000 g for 20 min) and the supernatant was filtered (Whatman GF/C). The filtrate was concentrated by dialysis tubes (12-14 KDa-porosity) using Poly-Ethylene Glycol (PEG). Concentrated extract was obtained by adding 15 mL of 10 mM BisTris buffer (pH 6). CM-cellulase (EC 3.2.1.4) activity was assayed using Carboxy Methyl Cellulose (CMC) at 0.7 % (w/v) as substrate with sodium acetate buffer (50 mM, pH 6). After incubation, released sugars were quantified according to Farnet et al. (2010b). β-1,4-glucosidase activity was performed using p-nitrophenyl β-D-glucopyranoside (0.2 mM) with sodium acetate buffer (100 mM, pH 5.6) and acid phosphomonoesterase using p-nitrophenyl phosphate monoester (0.2 mM) in sodium acetate buffer (100 mM, pH 4.5). The reaction mixtures for all the enzyme activities performed with the enzyme extract consisted of 300µl of enzyme extract with 700 µl of the corresponding buffer at 30 °C, except for cellulase activity (incubation at 50 °C). All the experiments were performed in triplicate for each mesocosm. One unit (U) of enzyme activity is defined as one µmole of substrate hydrolyzed per h and per g of litter dry weight (DW).

2.5. Community-level physiological profiles (CLPP)

Catabolic profiles of microbial communities were assessed using two Biolog plates: EcoPlateTM and FF MicroPlate™ (Biolog, California, USA). Briefly, 2 g of dry litter was added to 100 mL of 0.1 % sodium pyrophosphate sterile buffer in a 250 mL flask and shaken for 1 h (500 rpm). One extraction from litter of each mesocosm was performed. The litter suspension was diluted and standardized at OD 595 nm = 0.03 with a sterile physiological solution (NaCl 0.85 %). In order to limit bacterial growth in FF MicroPlate gentamycine and streptomycine were added to the diluted suspension respectively at 50 and 100 µg per mL, one hour before inoculating (Buyer et al, 2001). 125 µL of microbial suspension were used to inoculate each well. The plates were incubated at 25 °C for 5 days and microbial development was followed by reading the absorbance at 590 and 490 nm, respectively for

EcoPlate and FF Plate. According to Garland (1997), absorbance value for each well was blanked against the control well and negative absorbance values were set to zero. The minimum OD for a positive well was fixed at 0.25 for ECO and 0.15 for FF plates. The OD after 35h and 70h for ECO and FF plates have been chosen respectively, according to exponential phase of growth curves for all plates for further comparison.

Overall rate of substrate utilization by microorganisms was measured by calculating the Average Well Color Development (AWCD) for each plate at 35 h and 70 h for ECO and FF plates, respectively. Functional diversity assessed by Shannon's diversity index (H') was calculated using the equation: $H'=-\sum pi \log pi$ where pi is defined as OD of each well /sum of OD of all wells.

2.6. Solid-state 13C CP/MAS NMR

Litter from each mesocosm was characterized by solid-state ^{13}C CP/MAS NMR on a spectrophotometer Bruker DSX 400 MHz operating at 100.7 MHz. Samples (400 mg) were spun at 10 kHz at the magic angle. Contact times of 2 ms were applied with a pulse width of 2.8 µs and a recycle delay of 3 s. Chemical shift values were referenced to tetramethylsilane and calibrated to glycine carbonyl signal set at 176.03 ppm. The relative C distribution in ^{13}C NMR spectra was determined by integrating the signal intensity in different chemical shift regions with an integration routine supplied with Dmfit 2003 as software (Massiot et al, 2002). Seven common chemical shift regions were defined according to Mathers et al. (2003): alkyl C (0–45 ppm), methoxyl C (45–60 ppm), O-alkyl C(60-92 ppm), di-O-alkyl C (92–112 ppm), aromatic C (112–142 ppm), phenolic C (142–160 ppm) and carboxyl C (160–185 ppm). Some chemical signals were considered individually as follows: signals of anomeric C1 of the glycosidic bonds at 105 nm, C3 and C5 of syringyl units at 153 and C3 and C4 of guaiacyl units at 148 ppm. Two decomposition indexes have been calculated according to Baldock et al. (1997): (alkyl C/O-alkyl C) as the Humification Ratio (HR) and an Aromaticity Ratio (AR) by dividing the sum of aromatic C and phenolic C to the sum of all regions except carboxyl C.

2.7. Statistical Analysis

The significance of differences of each assay (enzyme activities, Biolog, NMR regions) was searched by nonparametric multiple comparison of Kruskal-Wallis ANOVA. The differences between samples were compared in each incubation time between different treatments (C, SC and ANT) and also for each treatment between two incubation times (1 and 3 month(s)). The optical absorbance of each well in ECO plate (after 35h incubation) and FF Plate (after 70h incubation) were subjected to Principal Component Analysis (PCA), considering each substrate as a variable. Regarding FF Plate, any substrate with OD<0.8 for any sample was not taken into account for PCA analysis. Specific relationships between

microbial activities and 13C NMR chemical groups were tested using Pearson's correlation coefficients. Statistica Vs 6 (StatSoft, Maison-Alfort, France) was used for statistical analysis and *p-value*<0.05 was considered as significant.

3. Results

3.1. Litter physico-chemical characteristics:

Table 1 describes the physico-chemical characteristics of the litter used for mesocoms set up. In order to clearly state the contamination of the studied area, total PAHs were quantified and 143 µg of total PAHs per Kg of dry litter were found with phenanthrene as the main PAH (80.78 µg per Kg of litter dry weight). Based on this PAH quantification, different ratios described by Simcik et al. (1999) were calculated and the values obtained show that the origin of this pollution is both pyrogenic and petrogenic. Ratios R1 to R3 are related to pollution caused by, for instance, car emissions or petroleum refineries while ratios R6 to R7 are linked to petrogenic PAHs.

3.2. Enzyme activities in mesocosms

Figure 1 shows cellulase (a), β glucosidase (b) acid phosphatase (c) and lipase (d) activities after 1- and 3-month incubations in ANT, SC and C mesocosms. After 1 month no significant differences in cellulase activities were observed except between SC and C mesocosms: cellulase activities were indeed higher in C mesocosms (Fig.1a). After a 3-month incubation, anthracene had a significant positive effect on cellulase activities in ANT mesocosms compared to both controls C and SC. Moreover, cellulase activities were higher in SC mesocosms than in C mesocosms. Whatever the mesocosm considered, β-glucosidase activity levels were similar after a 1-month incubation (Fig. 1b). As for cellulase activities, a significant increase in β-glucosidase activities was obtained after 3 months in ANT and S-C mesocosms (Fig.1b). Acid phosphatase was higher in C mesocosm than SC or ANT mesocosm after a 1-month incubation but after a 3-month incubation a significant increase was observed in ANT mesocosms compared to both controls (Fig.1c). Lipase activities drastically decreased in ANT mesocosms between 1- and 3-month incubations (Fig.1d). A significant difference was observed between C and SC mesocosms for each incubation time: lipase activities were lower in SC mesocosms. Thus, enzyme activities in ANT mesocosms strongly differed from those obtained from control mesocosms after a 3-month incubation: while lipase activity decreased, cellulase, β glucosidase and acid phosphatase activities strongly increased.

Table 1. Physico-chemical characteristics of the litter of *Pinus halepensis* studied and quantification of total PAH contamination, n.d: not detected.

WHC (g water 100 g^{-1} dry litter)	76
Moisture (%)	39
pH (H2O)	6.4
EC (µS cm^{-1})	290
TOC (%)	25
Total N (%)	0.9
C:N	24.8
PAHs (µg.kg^{-1} dry litter)	
Naphtalene (Naph)	n.d
Acenaphtene (ANaph)	21.2
Fluorene (Flu)	3.3
Phenanthrene (Phe)	80.8
Anthracene (Ant)	1.6
Fluoranthene (FL)	12.6
Pyrene (PY)	6.7
Benzo[a]anthracene (BzA)	6.2
Chrysene (Chr)	2.1
Benzo[b]fluoranthene (BzaFL)	3.4
Benzo[k]fluoranthene (BzkFL)	0.8
Benzo[a]pyrene (BaP)	4.0
Dibenzo[a,h]anthracene (DBzA)	n.d
Benzo[g,h,i]perylene (BPer)	n.d
Indeno (1,2,3,*c,d*) pyrene (IndP)	n.d
Sum PAHs	142.6
R1= FL/PY	1.87
R2= FL/(FL+PY)	0.65
R3= BzA/Chr	3
R4= Ant/(Ant+Phe)	0.02
R5= Phe/Ant	50.2

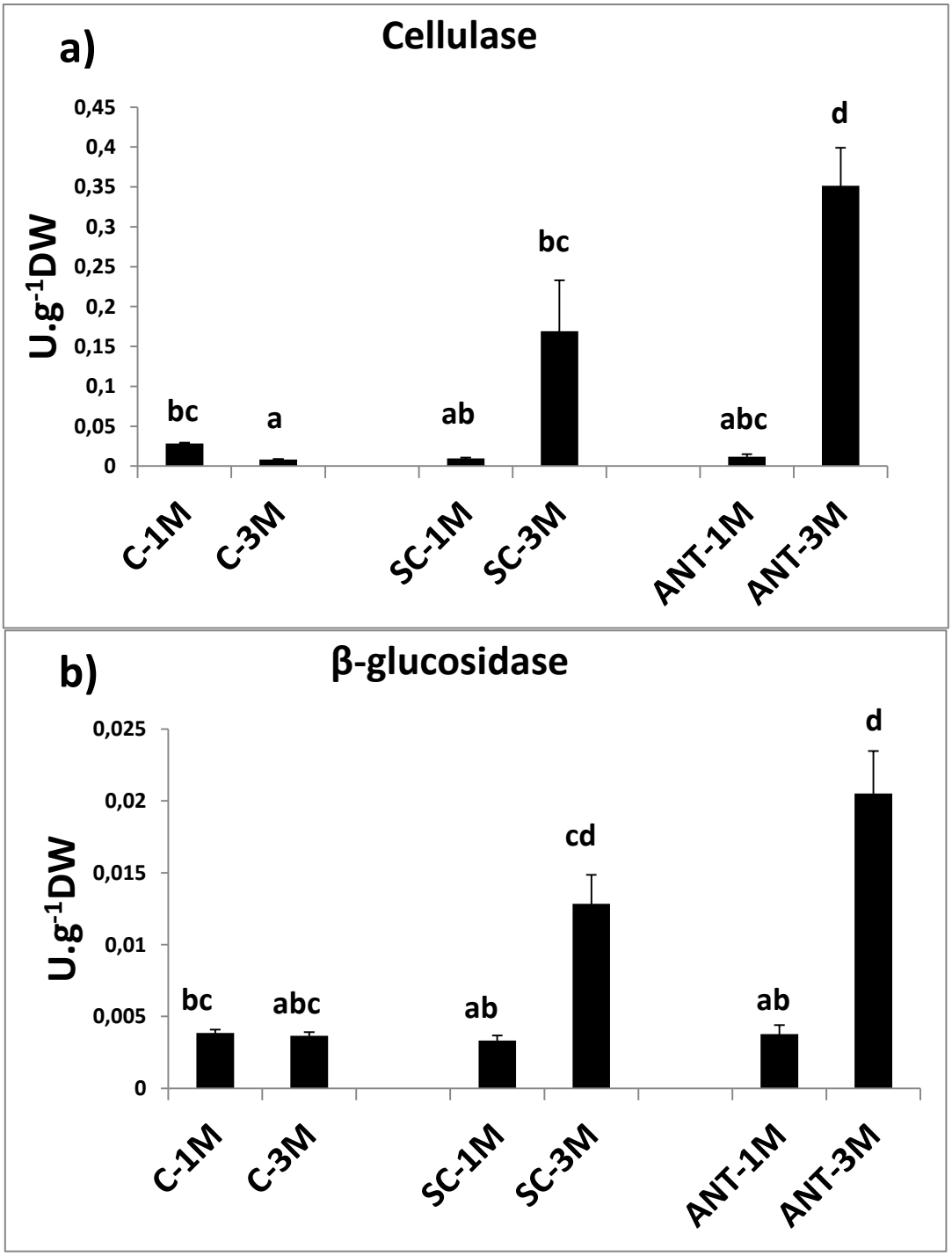


Figure 1. Cellulase (a), β-glucosidase (b), acid phosphatase (c) and lipase (d) activities (U. g DW-1) of Pinus halepensis litter in control mesocosms (C) , solvent control mesocosms (SC), anthracene mesocosms (ANT) after 1- and 3-month incubations (1M and 3M). Values followed by different letters differ significantly according to Kruskal-Walis test and Dunn procedure for multiple comparisons of means ($p<0.05$).

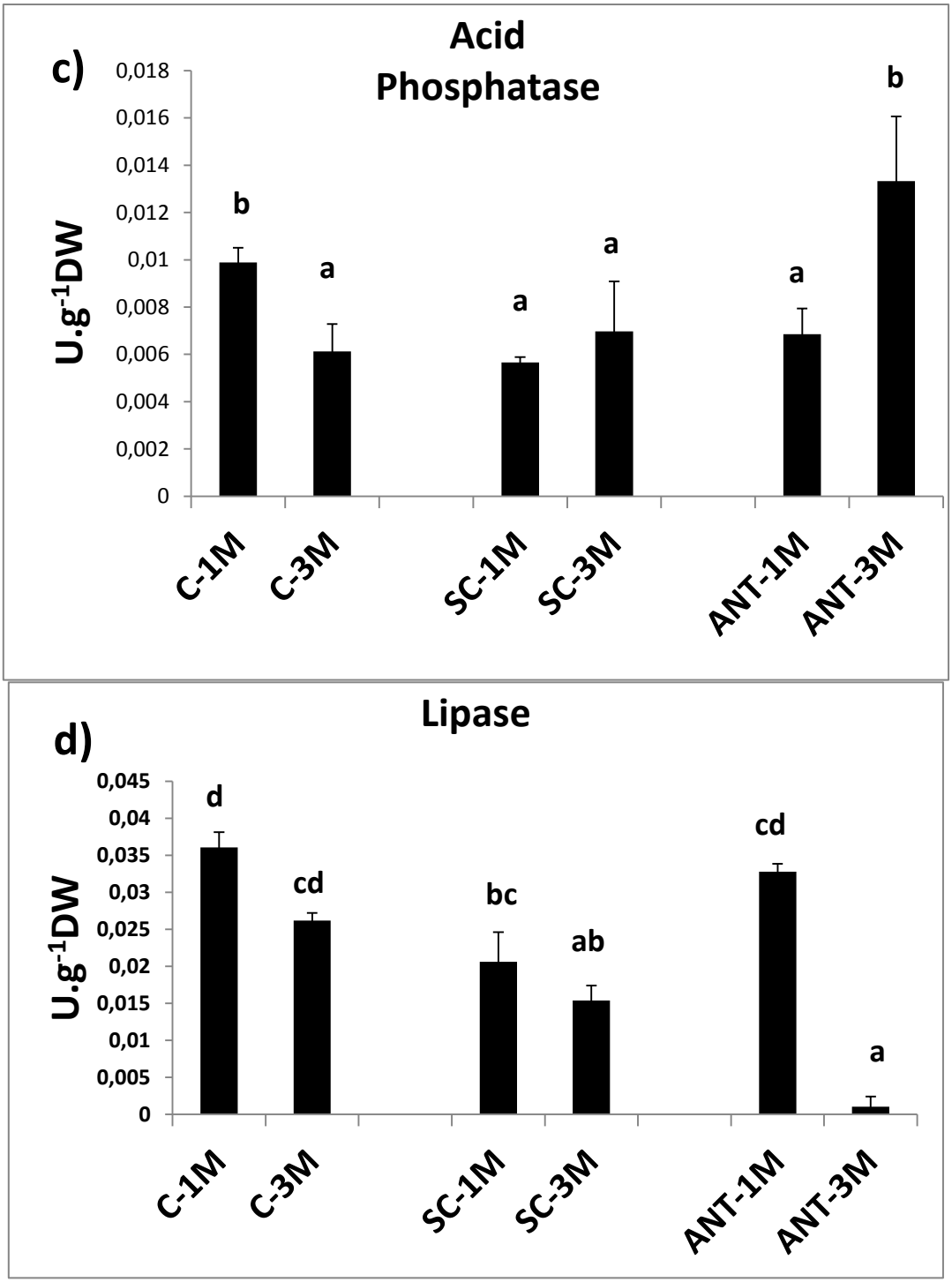


Figure 1. continued.

3.3. Catabolic diversity of bacterial and fungal communities

Average Well Color Development (AWCD) and Shannon-Weaver index of biodiversity (H') calculated from ECO and FF plates are shown in table 2. The effect of incubation time can be observed for both AWCD and H' from ECO and FF plates except for AWCD (ECO) in

ANT mesocosm. AWCD and H' indeed decreased between the two incubation times whatever the mesocosm considered except for AWCD (ECO) in ANT mesocosm (no significant decrease).

Anthracene inputs had no effects on AWCD (FF) and H' (FF): their values decrease to a similar extent whatever the experimental conditions. On the other hand, AWCD (ECO) and H' (ECO) from ANT and SC mesocosms similarly differed from those of C mescosms. These differences were strong after a 3-month incubation where AWCD (ECO) was 0.28, 0.48 and 0.53 for C, SC and ANT mesocosms, respectively. H' (ECO) values revealed that ANT and SC mesocosms (1.18 and 1.23 respectively) had a higher biodiversity than C mesocosms (1.09) after 3 months. Thus, after a 3-month incubation, each mesocosm is significantly less diversified and less active (excepted ANT mesocoms).

A multi-correlation analysis was performed from a matrix of OD values obtained from carbon utilization profiles of bacterial and fungal communities using respectively ECO and FF plates (Figure 2). The two first principal components (PC1 and PC2) were chosen and both accounted for 71% and 84% of variance for ECO and FF plates, respectively.

In Fig.2a (ECO plate), the projections of the mesocosms after a 1-month incubation were located together (C-1M, SC-1M and ANT-1M). Anthracene effects clearly appeared after a 3-month incubation. Indeed, ANT mesocosms were discriminated from both controls regarding PC1 (49%) and PC2 (22%). SC mesocosm was located between ANT and C mesocosms after a 3-month incubation. In Fig. 2b (FF plate) the 1-month incubated mesocosms were completely separated from the 3-month incubated mesocosms by PC1 (79%). After 1 month, anthracene effect was observed since ANT mesocosm projection was separated from both controls by PC2 (5%). Furthermore, for a 3-month incubation time, C, SC and ANT mesocosms were separated by PC2. Anthracene inputs seem to have an immediate and moderate effect on the functional diversity of fungal communities (PC2 explains only 5% of the variance). Incubation time seems indeed to be the major factor which influences the catabolic diversity of fungal communities under these experimental conditions.

3.4. Chemical characterization of litter by 13C CPMAS NMR and C/N ratio.

The solid-state 13C NMR spectra of litter in mesocosms varied depending on the treatment and the incubation time (Table 3). In ANT mesocosms, the Humification Ratio (HR) and the relative intensity of alkyl C, di-O-alkyl C and carboxyl C significantly increased while the Aromaticity Ratio (AR) and the relative intensity of O-alkyl C and aromatic C significantly decreased between 1- and 3-month incubations. ANT mesocosms differed from both controls: after 1 month di-O-alkyl C signal was weaker, after 3 months, aromatic C was weaker thought alkyl-C was higher. Methoxyl C relative intensity significantly increased only

in C mesocosms between the 2 incubation times. Alkyl C significantly varied over time under each experimental condition.

C/N decreased after 1 month from 24.8 (table 1) to 20.2, 18.8 and 18.4 respectively in ANT, SC and C mesocosms. A significant difference was observed between ANT mesocosms and both controls ($p<0.05$). However, after 3 months there was no significant difference between C/N ratio of mesocosms and they were respectively 17.8, 18.6 and 19.8 in ANT, SC and C mesocosms. After a 3-month incubation, total N content was greater in all mesocosms than in the initial litter.

Plate	Index	C		SC		ANT	
		1M	3M	1M	3M	1M	3M
ECO plate	AWCD	0.53±0.03 b	0.28±0.04 a	0.61±0.03 c	0.48±0.07 b	0.58±0.03 bc	0.53±0.1 bc
	H'	1.26±0.01 b	1.09±0.00 a	1.30±0.01 c	1.23±0.03 b	1.30±0.01 c	1.18±0.02 b
FF plate	AWCD	0.36±0.02 bc	0.13±0.04 a	0.46±0.03 c	0.14±0.03 a	0.47±0.03 c	0.18±0.03 ab
	H'	1.66±0.02 b	1.57±0.08 a	1.74±0.09 b	1.52±0.05 a	1.70±0.02 b	1.58±0.04 a

Table 2. Average Well Color Development (AWCD) and Shannon-Weaver index (H') calculated from ECO plate and FF plate and obtained from control mesocosms (C), solvent control mesocosms (SC) and anthracene mesocosms (ANT) after 1- and 3-month incubations.

3.5. Correlation between microbial activities and chemical characteristics of litter in mesocosms

Pearson's correlation between different chemical characteristics (13C NMR signals and C/N) and microbial activities (cellulase, β-glucosidase, acid phosphatase, lipase, including laccase from Qasemian et al, 2011) is shown in table 4.

A very significant negative correlation ($p<0.001$) was found between phenolic C and cellulase, β-glucosidase and laccase activities. More precisely, a negative correlation was found between these enzymes and C3-C4 of guaiacyl units at 147 ppm. As a corollary, AR was also negatively correlated with cellulase, β-glucosidase and laccase activities. Anomeric-C1 signal from glycosidic bonds was also negatively correlated with cellulase, laccase and acid phosphatase activities ($p<0.05$). On the other hand, positive correlations were found between carboxyl C signal and cellulase, β-glucosidase and laccase activities. Finally, O-alkyl C signal was positively correlated with lipase activities.

4. Discussion

The effect of PAHs on soil microbial communities together with the capacity of biodegradation of these pollutants is of great concern because of their recalcitrance and ubiquitous occurrence. In this study, we aimed to show how certain enzyme activities of autochthonous microbial communities and functional diversity, partially assessed via CLPP, are affected by the presence of such pollutant under semi-natural conditions. PAH quantification shows that the studied sampling site is representative of urban areas contaminated by both pyrogenic and petrogenic PAHs (Wilcke, 2007). Here, the variations in enzyme activities surprisingly show that anthracene has no immediate toxic effect (after 1 month) on microbial community functions though high concentrations were added. This finding demonstrates that the microbial activities probably still function when subjected to lower pollutant concentrations observed in contaminated environments. However, chronic pollution usually involved several toxic molecules which lead to more complex conditions. After a 3-month incubation, anthracene inputs have various effects depending on the enzyme activities. A strong increase in cellulolytic enzymes (cellulase, β-glucosidase) and acid phosphatase activities was detected in the presence of anthracene. In our previous study (Qasemian et al, 2011), we have shown that laccase activities were strongly induced by anthracene. These increases can directly be linked to a more intense fungal development which generally leads to higher amounts of extracellular enzymes (Burke et al, 2011; Sanchez, 2009). Previous studies have also shown that a considerable amount of anthracene can be degraded by fungal phenoloxidases showing a non-toxic effect of anthracene on

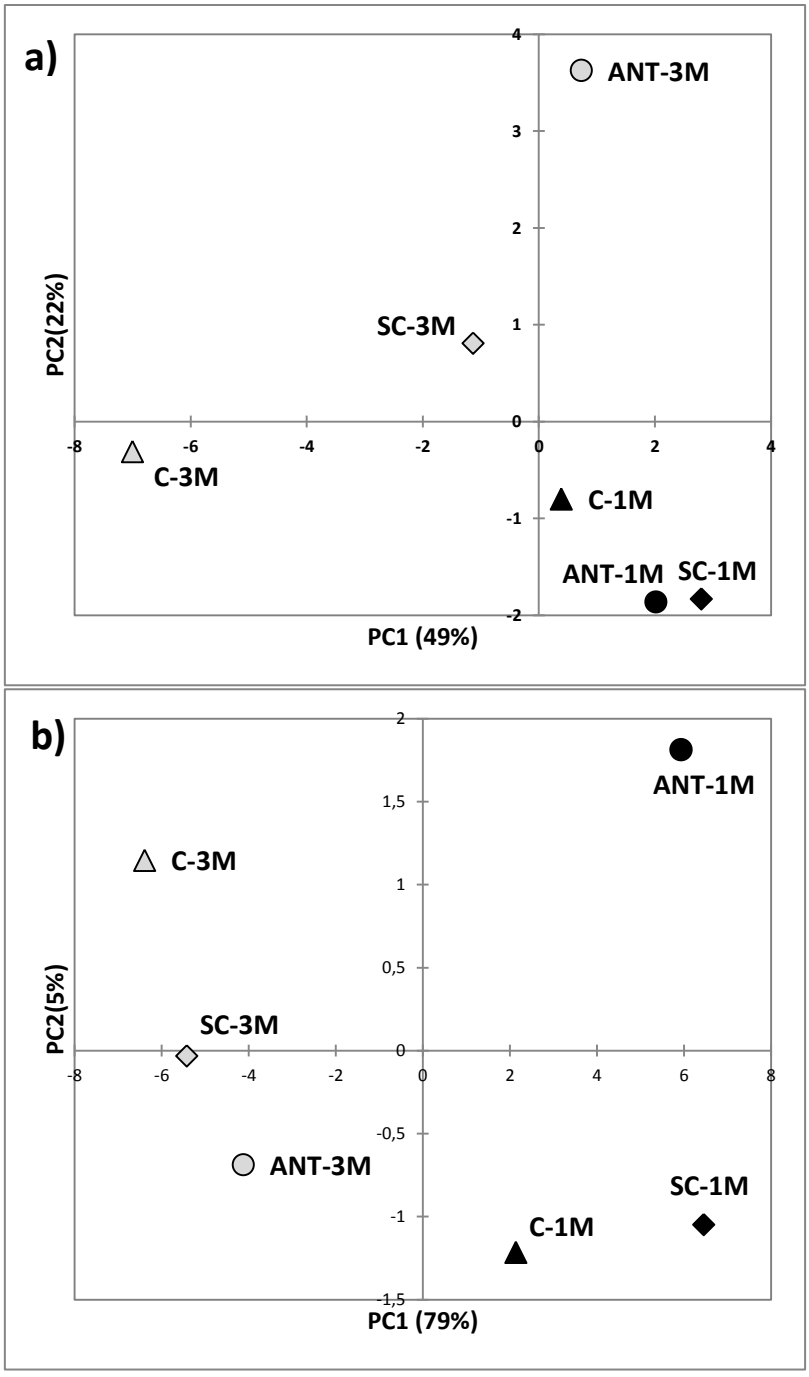


Figure 2. Principal Component Analysis (PCA) of carbon utilization profiles of (a) bacterial catabolic profile using ECO plate (b) fungal catabolic profile using FF plate. Triangle: control mesocosms (C), square: solvent control mesocosms (SC) and round: anthracene mesocosms (ANT) after 1- (white) and 3-month (black) incubations. Each point represents the barycenter of three replicates.

fungal communities (Chupungars et al, 2009; Coutiño-González et al, 2010; Potin et al, 2004). Fungi and especially white-rot fungi are highly resistant to aromatic pollutants since the enzymes expressed may contribute to their resistance by the degradation of toxic compounds (Cameron et al, 2000; Gianfreda and Rao, 2004). Thus, here, a higher

production of extracellular carbon and phosphorus-acquiring enzymes in the presence of anthracene seems to be due to saprotrophic communities which resist to this pollutant.

Of all the enzymes detected, lipases were the sole enzymes whose activities decreased in the presence of anthracene in the mesocosms. Lipases are secreted by microorganisms to hydrolyze esters of fatty acids and alcohols (Farnet et al, 2010a; Cooper and Morgan, 1981; Margesin et al, 2002). In this study, microbial communities producing lipases were probably affected by the significant development of lignocellulolytic fungi in ANT mesocosms after 3 months. Moreover, our results are in consistence with previous studies which also found a negative correlation between lipase and β-glucosidase activities in PAH contaminated-soils (Margesin et al, 1999; Riffaldi et al, 2006). These authors suggested that these measurements may be considered as useful parameters to indicate PAH degradation trends in soils: lipase activities decreased when PAH were actively bio-transformed. The functional response of indigenous microbial communities can only be partially assessed via CLPP since this method focused on easily cultivable microorganisms. Nevertheless, this part of our study clearly showed that fungal communities were weakly influenced by anthracene compared to bacterial communities after a 3-month incubation. It is in concordance with previous studies which have shown that fungal communities are more resistant than bacterial communities to PAH input in soils (Chávez-Gómez et al, 2003; Ding et al, 2011). Furthermore, in ANT mesocosms while H' decreased, AWCD remained constant between 1- and 3-month incubations for Eco plates. This result shows that, in the presence of anthracene, the bacterial communities are more specialized and oxidize more intensively the substrates under these laboratory conditions. The H' and AWCD values in SC mesocosms also suggest an important side-effect of solvent on bacterial communities (Brinch et al, 2002) but not on fungal communities. The functional diversity (H') was mainly affected by the incubation time which is not in concordance with previous studies which have shown a higher significant microbial diversity or resistance in polluted soils using CLPP (Derry et al, 1998; Niklinska et al, 2006).

The influence of anthracene on organic matter transformation was monitored by 13C CP/MAS NMR which is largely used in soil organic matter characterization as a robust method (Kögel-Knabner, 2002; Mathers et al, 2007). Different ratios such as aromaticity and humification ratios are useful to monitor litter degradation (Baldock et al, 1997; Wang et al, 2004). Here, an increase in the transformation of lignocellulosic material, linked to O-alkyl C and aromatic C signal decrease is found in the presence of anthracene. The decreases in both guaiacyl units and anomeric C confirm that, in the presence of anthracene, a strong transformation of lignocellulosic material occurs and this transformation is correlated with a high production of lignocellulolytic enzymes.

Table 3. Relative intensity of chemical functions from ^{13}C NMR data in control mesocosms (C), solvent control mesocosms (SC) and anthracene mesocosms (ANT) after 1- and 3-month incubations. Values followed by different letters (a-c) differ significantly according to Kruskal-Walis test and Dunn procedure for multiple comparisons of means ($p<0.05$).

Time	Treatment	Alkyl C (0-45 ppm)	Methoxyl C (45-60 ppm)	O-Alkyl C (60-92 ppm)	di-O-Alkyl C (92-112 ppm)	Aromatic C (112-160 ppm)	Carboxyl C (160-185 ppm)	Aromaticity Ratio (AR)	Humification Ratio (HR)
1 month	**ANT**	27.2±0.1^{a}	7.4±0.1^{ab}	32.5±0.2^{b}	6.2±0.1^{a}	21.7±0.3^{b}	6.4±0.0^{b}	0.252±0.002^{b}	0.73±0.01^{a}
	SC	28.7±0.19^{b}	6.2±0.9^{ab}	32.0±0.8^{b}	6.6±0.2^{b}	21.7±0.4^{b}	6.0±0.2^{a}	0.254±0.004^{bc}	0.82±0.05^{b}
	C	25.8 ±0.7^{a}	7.1±0^{a}	32.1 ±0.5^{b}	6.7 ±0.2^{b}	22.4±0.0^{c}	6.4±0.0^{b}	0.259±0.001^{c}	0.68±0.03^{a}
3 month	**ANT**	28.9±0.6^{b}	8.3±0.9^{b}	30.7±0.8^{a}	6.6±0.4^{b}	20.0±0.7^{a}	6.9±0.3^{c}	0.236±0.007^{a}	0.80±0.04^{b}
	SC	26.7±0.8^{a}	8.1±0.7^{b}	30.8±0.6^{a}	6.5±0.0^{b}	21.8±0.4^{b}	7±0.3^{c}	0.257±0.006^{bc}	0.74±0.02^{a}
	C	27.0±1.8^{a}	8.0±2.0^{b}	32.4±1.0^{b}	6.3±0.7^{ab}	21.4±0.14^{b}	6.5±0.3^{bc}	0.250±0.002^{b}	0.73±0.09^{a}

Table 4. Correlation coefficients (r values) between chemical functions from 13C NMR and C/N ratio of each mesocosm with biological indicators: CEL (cellulase), βGLU (β-glucosidase), LAC (laccase), PHA (Acid phosphatase), LIP (lipase). [1] Data from Qasemian et al, 2011, Significant correlations are marked with * <0.05, **<0.01 and ***<0.001.

Chemical shift (ppm)	Assignment	CEL	βGLU	LAC[1]	PHA	LIP
0-45 [a]	Alkyl C	0.29	0.32	0.33	-0.12	-0.49
45-60	Methoxyl C	-0.07	-0.02	-0.04	-0.03	-0.05
60-92[b]	O-Alkyl C	-0.17	-0.29	-0.17	-0.03	**0.64****
92-112[c]	di-O-Alkyl C	0.23	0.18	0.21	0.29	-0.09
105	Anomeric C-1	**-0.52***	-0.42	**-0.59***	**-0.63****	0.23
112-142[d]	Aromatic C	-0.20	-0.28	-0.33	-0.24	0.27
142-160[e]	Phenolic C	**-0.82*****	**-0.86*****	**-0.76****	-0.41	0.46
147	Phenolic C(C3-C4 of guiacyl units)	**-0.77*****	**-0.78*****	**-0.67****	-0.45	0.27
153	Phenolic C(C3-C5 of syringyl units)	0.21	0.18	0.13	0.19	0.12
160-185	carboxyl C	**0.70****	**0.76****	**0.60***	0.36	-0.36
(d+e)/(a+b+c+d+e)	Aromaticity Ratio (AR)	**-0.68****	**-0.73****	**-0.72****	-0.47	0.46
a/(b+c)	Humification Ratio (HR)	0.13	0.18	0.16	-0.21	-0.39
-	C/N	0.33	0.27	0.28	0.25	0.11

A high negative correlation between lignocellulolytic enzymes and guaiacyl units, which are known as free phenolic units of lignin in pine litters, corroborated this result (Lorenz et al., 2004).

The mineralization of litter in ANT mesocosms after 3 months is also confirmed when considering Humification Ratio (HR): this ratio decreases only in ANT mesocosms after 3 months and is significantly higher whatever the other conditions. A decrease in O-alkyl C relative intensity is considered as a marker of rapid mineralization of labile components (Skjemstad et al, 1997). This is also in agreement with Mathers et al. (2007) who found that HR was correlated with strong litter decomposition. The positive correlation between carboxyl C and the lignocellulolytic enzymes seems to confirm that a high level of mineralization occurs when anthracene was added to the mesocosms: the higher intensity of carboxyl C signal may be linked to the production of organic acids during organic matter decomposition. Thus, the addition of anthracene onto the litter seems to have several effects : i) it favors fungal development since fungal communities are less sensitive than the bacterial communities to this pollutant, ii) fungal lignocellulolytic activities increase and thus the mineralization of litter is strongly enhanced.

5. Conclusions

The vulnerability of Mediterranean coastal areas due to natural pressures together with intense anthropic activities is of great concerns. Here, we found a high resistance of microbial communities with enhanced lignocellulolytic activities which suggest a resistance of fungal communities to this pollutant. This study is a preliminary approach to show the response of microbial activities to an aromatic contaminant under semi-natural conditions. In further studies, these results should be compared with the functional response of microbial communities from litters of inland areas and of pristine coastal areas. This would enable us to determine which factor (coastal environment or pollutant exposure) actually explain the high potential of resistance of these communities.

Chapitre III

ETUDE COMPARATIVE DE L'EFFET DE L'ANTHRACENE SUR LES ACTIVITES MICROBIENNES DE LITIERES DE PIN D'ALEP ISSUES D'ENVIRONNEMENTS LITTORAUX OU CONTINENTAUX

Suite aux résultats obtenus précédemment relatifs à l'effet des embruns et donc à l'influence de la salinité sur les communautés microbiennes au sein de la litière de pin d'Alep en contexte naturel (litter bag, chapitre I)) et à leurs potentiels de dégradation de l'anthracène en mésocosmes (chapitre II), nous avons cherché à déterminer si l'origine littorale de cette litière était un facteur déterminant des réponses observées. Selon notre hypothèse, des communautés microbiennes adaptées à subir des stress importants (salinité variable et temporairement élevée, phase d'intense dessiccation, matière organique récalcitrante issue de végétation à caractère xérique), seraient plus résistantes à d'autres stress (pollution organique dans notre cas). A cet effet, les communautés microbiennes de litières issues de zones littorale et continentale ont été comparées.

Les expériences en mésocosmes ont ainsi été menées en conditions contrôlées en prenant en compte des litières de pin d'Alep issues de deux sites méditerranéens l'un littoral (Massif de Marseilleveyre) et l'autre continental (Massif de la Trevaresse, Bouches du Rhône). Comme pour les expérimentations précédentes l'anthracène a été apporté aux litières au cours d'une contamination unique et son impact sur les communautés microbiennes et leurs activités a été estimée après 3 mois d'incubation. En particulier les activités laccases, cellulases, β-glucosidases et phosphatases et la respiration microbienne (basale et induite) ont été quantifiées. En parallèle l'évolution qualitative de la matière organique initialement présente au sein des litières a été évaluée par RMN du ^{13}C et C/N.

Article: Does anthracene affect microbial activities and organic matter decomposition? A comparative study in Pinus halepensis litters from Mediterranean coastal and inland areas, soumis à Chemosphere

Article

Does anthracene affect microbial activities and organic matter decomposition? A comparative study in *Pinus halepensis* litters from Mediterranean coastal and inland areas

1. Introduction

Large quantities of pyrogenic Polycyclic Aromatic Hydrocarbons (PAHs) are continuously released into the atmosphere as by-products of incomplete combustion via leaking automobile fuel, incineration of waste and industrial exhaust systems (Fernández-Luqueño et al, 2011; Johnsen and Karlson, 2007; Sorgi, 2007). Complex recalcitrant organic molecules, PAHs are recognized as an environmental contamination problem of worldwide magnitude (Andreoni and Gianfreda, 2007; Marusenko et al. 2011). During the past few years, particular attention has been paid to chronic pollution through PAH deposits on vegetation and in soils especially in peri- urban areas (Johnsen et al, 2005; Johnsen and Karlson 2007). Studies have examined accumulations of PAHs on leaves and needles of plants, showing that vegetation plays an important role in the transfer of PAHs from air to soil especially by direct deposition from plants to topsoil (Berg and Laskowski, 2006; Holoubek et al, 2000; Howsam et al, 2001; Yutthammo et al, 2010).

Mediterranean ecosystems are characterized by specific pedo-climatic conditions (summer drought, calcareous soils…) and are additionally subjected to intense anthropic activities caused by increasing urbanization. Several studies also point to the presence of PAHs as chronic anthropic pollutants on French Mediterranean coastal areas (Mille et al, 2007; Asia et al, 2009) which are also subjected to salinity. While the ways in which microorganisms mineralize organic contaminants have been extensively documented in studies on bioremediation technologies (D'Annibale et al, 2006; Gianfreda and Rao, 2004; Novotný et al, 2004; Wu et al, 2008), little is known about how far autochthonous microbial communities can withstand PAHs and whether they may be involved in degrading theses pollutants. In our previous study, we showed that anthracene, a model PAH, artificially added in laboratory experiments (Qasemian et al, 2011, 2012) has no toxic effects on microbial activities expressed by whole microbial communities in Pinus halepensis litter from a coastal Mediterranean site. It therefore appeared useful to compare these results obtained from litters in coastal environments with the responses of microbial communities from inland areas.

Microbial community composition is known to depend greatly on the chemical composition of organic matter, thus differing according to plant chemical composition (Berg and MacClaugherty, 2008; Nazarreno Saparrat et al, 2008; Sariyildiz et al, 2005). Indeed, microorganisms associated with the same plant species in the same climatic context are supposed to be functionally equivalent and on the whole to harbor the same degrader communities (Ayres et al, 2009; Costa et al, 2006; Strickland et al, 2009). Considering that microbial degradation depends on the chemical composition of litter and on the degradation ability of indigenous microorganisms influenced by environmental factors, we hypothesized that, depending on the origin of the litters, anthracene might have differing effects on microbial communities from different Pinus halepensis litters under the same laboratory conditions.

Here, we compare the effect of anthracene on microbial communities from Pinus halepensis litter originating from both coastal and inland Mediterranean sites. Pinus halepensis litters were collected and anthracene, as a model PAH, was artificially added to the litter in the laboratory. The effect of this pollutant on whole microbial communities was monitored through extracellular enzyme measurement and microbial respiration combined with 13C Nuclear Magnetic Resonance (NMR), in order to precisely determine the chemical variations in the litters from both sites.

2. Materials and methods

2.1. Litter sampling

A composite sampling was performed in November 2010 from partially degraded litter of Aleppo pine (Pinus halepensis L.) from two sites under Mediterranean climate, one from a coastal area (massif de Marseilleveyre, 43° 12’ 34’’ N, 5° 21’ 36’’ E) and one from inland (massif de la Trevaresse, 43° 36' 88'' N, 5° 27' 72'' E), hereafter respectively referred to as COS and INL. The litters were sieved at 2 mm mesh size, homogenized and stored overnight at 4°C before being used in microcosms.

Total Organic Carbon (TOC) and total Nitrogen were measured as follows: oven-dried sub-samples of initial litter and of each microcosm litter were powdered in a ceramic mortar and analyzed by combustion in an Elemental Analyzer, FlashEA 1112, Thermofisher.

2.2. Microcosm preparation

The litter from each site was placed in 2.6 L glass jars (25*20*7 cm) containing 50 g of litter (dry weight, DW) per microcosm, covered with pierced lids to favor oxygen diffusion. Anthracene microcosms (ANT) were prepared as follows: 40 mg of anthracene was dissolved in dichloromethane and added to a sub-sample of litter (10 g DW of litter) in order to minimize the harmful side-effects of solvent. After dichloromethane evaporation, the sub-sample was mixed with the litter of the microcosm (40 g DW). Two different control microcosms were also performed: a control microcosm (C) i.e. litter without any treatment, and a solvent control microcosm (S-C) containing a sub-sample of litter treated with solvent alone. Each microcosm type (C, S-C, ANT) was realized in four replicates (n=4) and incubated at 25°C in the dark for 3 months. Thus, a total of 12 microcosms were prepared for each site. Humidity of the samples was maintained constant over incubation at 60 % of the WHC max by adding sterilized water to the microcosms.

2.3. Extracellular Enzyme Activities (EEA)*:*

Laccase, cellulase, β-1,4-glucosidase and acid phosphomonoesterases were quantified both in initial litters and after the 3-month incubation. All the enzyme activities were measured using an enzyme extract from litter according to a modified protocol from Criquet et al. (1999). Briefly, 10 g of litter was added to 200 ml CaCl2, 2 H2O (200 mM) and 0.1 % Tween 80, and then shaken for 1 h (500 rpm). The floating debris was removed, the extract was centrifuged (7 000 g for 20 min) and the supernatant was filtered (Whatman GF/C). The filtrate was concentrated by dialysis tubes (12-14 KDa-porosity) using Poly-Ethylene Glycol (PEG). Concentrated extract was obtained by adding 15 mL of 10 mM BisTris buffer (pH 6). The reaction mixtures for all the enzyme activities performed with the enzyme extract consisted of 300µl of enzyme extract with 700 µl of the corresponding buffer at 30 °C, except for cellulase activity, which was incubated at 50 °C. Laccase activity was measured by monitoring the oxidation of syringaldazine to its quinone (ε^{M} = 65 000 M^{-1} cm^{-1}) at 525 nm in acetate buffer (100 mM, pH 4.5). CM-cellulase (EC 3.2.1.4) activity was assayed using Carboxy Methyl Cellulose (CMC) at 0.7 % (w/v) as substrate with sodium acetate buffer (50 mM, pH 6). After a 4h-incubation, the glucose released was quantified according to Farnet et al. (2010). β-1,4-glucosidase activity was performed using *p*-nitrophenyl β-D-glucopyranoside (0.2 mM) with sodium acetate buffer (100 mM, pH 5.6). Acid phosphomonoesterases were assayed using *p*-nitrophenyl phosphate monoester (0.2 mM) in sodium acetate buffer (100 mM, pH 4.5). *p*-nitrophenol was quantified at 412 nm after the addition of NaOH (0.5M). All analytical experiments were performed in triplicate for each microcosm. One unit (U) of enzyme activity is defined as one µmole of the reaction product formed per h and per g of litter dry weight ($U.gdw^{-1}$).

Enzyme extracts were further subjected to gel electrophoresis according to Qasemian et al (2011) in order to compare the phenoloxidase bands, as revealed by activity, under each treatment.

2.4. Basal and Induced Respiration

2 g of litter of each microcosm was placed in glass jars (117 ml), flushed with fresh air and closed by rubber septum before incubation at 25°C for 4 h. The concentration of the CO2 produced was measured at the end of incubation by injecting one milliliter of the jar headspace gas into a gas chromatograph (Chrompack CHROM 3-CP 9001), equipped with a thermal conductivity detector and a fused silica capillary column (10 m x 0.53 mm). Glucose was added (25 mg.g-1 of litter dry weight) 90 min prior to a 90 min-incubation at 25°C for induced respiration. Microbial respiration was expressed as µg of C formed as CO2 per gram of dry litter per hour.

2.5. Solid-state ^{13}C CP/MAS NMR

Both the initial litters and the litters in each incubated microcosm were characterized by solid-state 13C CP/MAS NMR on a spectrophotometer Bruker DSX 400 MHz operating at 100.7 MHz. Samples (400 mg) were spun at 10 kHz at the magic angle. Contact times of 2 ms were applied with a pulse width of 2.8 µs and a recycle delay of 3 s. Chemical shift values were referenced to tetramethylsilane and calibrated to glycine carbonyl signal set at 176.03 ppm. The relative C distribution in 13C NMR spectra was determined by integrating the signal intensity in different chemical shift defined using Dmfit 2003 (Massiot et al, 2002).

Seven common chemical shift regions were defined according to Mathers et al. (2003): alkyl C (0–45 ppm), methoxyl C (45–60 ppm), O-alkyl C(60-92 ppm), di-O-alkyl C (92–112 ppm), aromatic C (112–142 ppm), phenolic C (142–160 ppm) and carboxyl C (160–185 ppm). Guaiacyl units were considered individually at 148 ppm. Two decomposition indexes were calculated according to Baldock et al. (1997): (alkyl C/O-alkyl C) was taken as the humification ratio, and an aromaticity ratio was calculated by dividing the sum of aromatic C and phenolic C by the sum of all regions except carboxyl Cical group (10 variables).

2.6. Statistical Analysis

Two-way ANOVA was performed taking into account litter location (COS, INL) and treatment (C, SC and ANT) followed by a Fisher's LSD post-hoc test. Data were transformed as necessary to respect the normality and homogeneity of variance. The significance of microbial respiration mean differences was tested following non-parametric comparison of Kruskal-Wallis. Pearson correlation was tested between enzyme activities and chemical groups defined by 13C NMR and C/N ratio (n=24). Principal Component Analysis (PCA) was performed on 13C NMR considering all peaks of NMR spectra as variables (23 variables). A

second PCA was performed considering only the variables most correlated to factorial plot (r2>0.5) having at least one peak in each chemical group (10 variables).

3. Results

3.1. Comparison of enzyme activities and microbial respiration

Enzyme activities measured in both litters (COS and INL) before incubation show laccase and cellulase activities at similar values in both litters with β-glucosidase and phosphatase activities higher in COS litter than in INL litter (Fig.1). After a 3-month incubation, the results of means comparison using ANOVA on enzyme activities in microcosms are shown in Table 1. The factor litter origin significantly affects all enzyme activities, especially laccase activities and the factor microcosm treatment, either alone or with litter origin, shows a significant effect, respectively on laccases and cellulases. Fig.1 shows the mean of each enzyme in the different microcosms C, SC and ANT of both COS and INL litters. For all the enzymes measured in this study we observed a higher level in COS microcosms after incubation than before, and a higher level than in INL microcosms. Furthermore, the highest levels of both laccase and cellulase activities were observed in the ANT microcosms of COS and cellulase activities significantly differed from controls (SC and C, Fig.1b) only for ANT microcosms. In INL microcosms, treatment had no effect on enzyme activities, i.e. C, SC and ANT had the same level of enzymes after a 3-month incubation. In COS microcosms, phosphatase activities were significantly lower in SC than in C and ANT microcosms.

Compared to their initial level, laccase activities strongly increased in COS microcosms up to 1000 fold. Gel electrophoresis of the enzyme extract stained by enzyme activity revealed three low molecular weight bands whatever the microcosms, but these bands were strongly stained in the ANT microcosms (shown as supplementary information). On the other hand, in INL microcosms, laccase activities were too low for bands stained by activity to be observed, even in the ANT microcosms.

Basal and induced respirations were estimated in order to show the impact of anthracene on global activity of microorganisms in microcosms. The values obtained for each litter before incubation were higher in COS litters than those in INL litters: 205 ± 4 vs 152 ± 7 for basal respiration and 35 ± 2 vs 28 ± 1 for induced respiration. After a 3-month incubation, while basal respiration values differ greatly between coastal and inland litters, the microcosms treated with anthracene show no effect (Fig 2a). Nor does the treatment affect induced respiration, although adding glucose induced respiration of microbial communities in INL litter but not in COS litter (Fig 2b).

Figure 1. Mean of laccase, cellulase, β-glucosidase and acid phosphatase activities measured on Pinus halepensis litters in microcosms after a 3-month incubation. COS litters; without pattern, INL litters; with pattern. ANT, SC and C correspond respectively to microcosms with anthracene, solvent control and control microcosms without treatment. Letters define the homogenous groups according to multiple comparison of means. Error bars are standard deviation (n=4). Initial activities in COS and INL litters expressed as U. gdw-1 were respectively: laccase (0.005 ± 0.002 vs 0.002± 0.001), cellulase (0.035 ± 0.023 vs 0.086 ± 0.024), β-glucosidase (0.183 ± 0.006 vs 0.068 ± 0.015) and acid phosphatase (0.569 ± 0.022 vs 0.406 ±0.056).

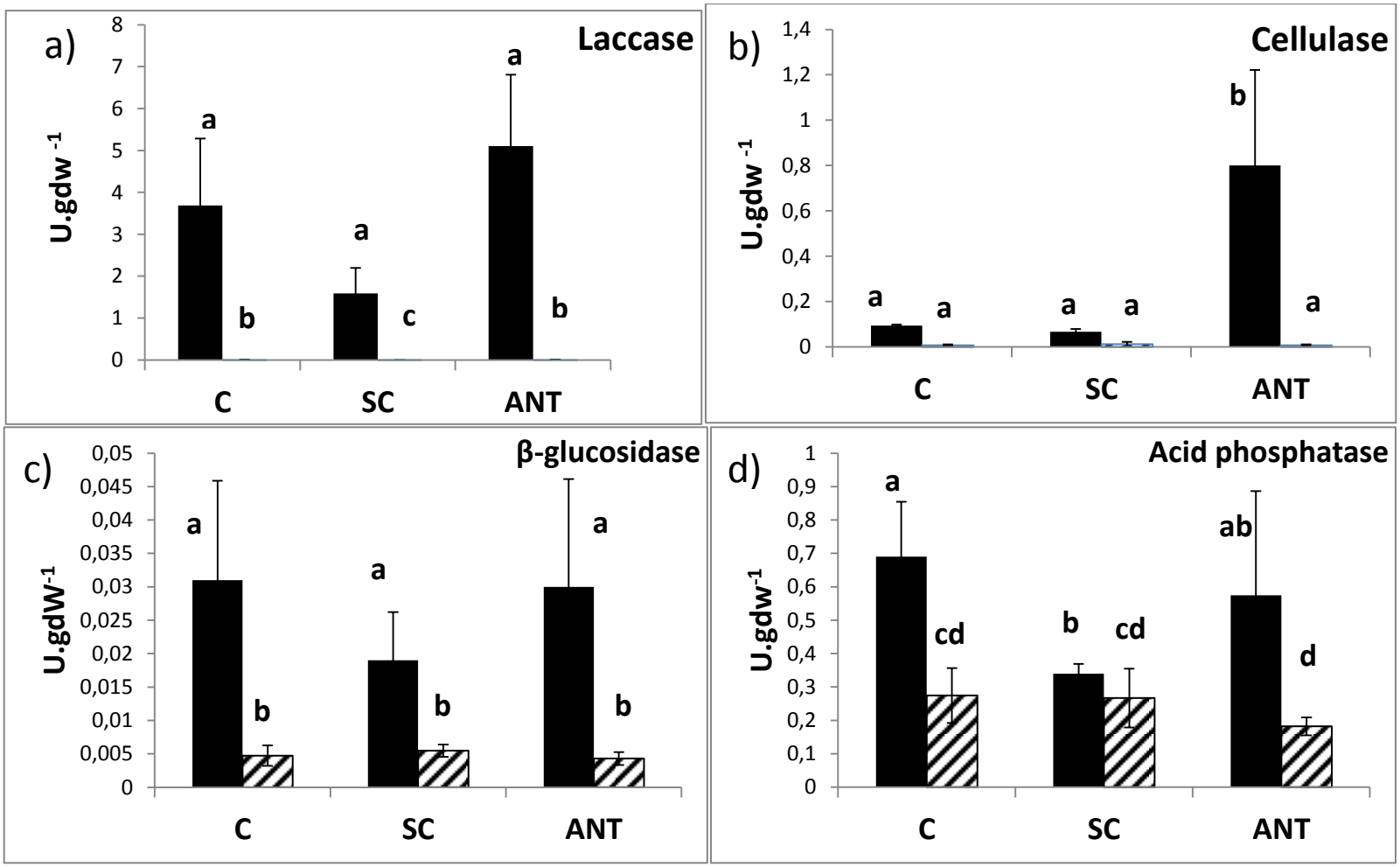
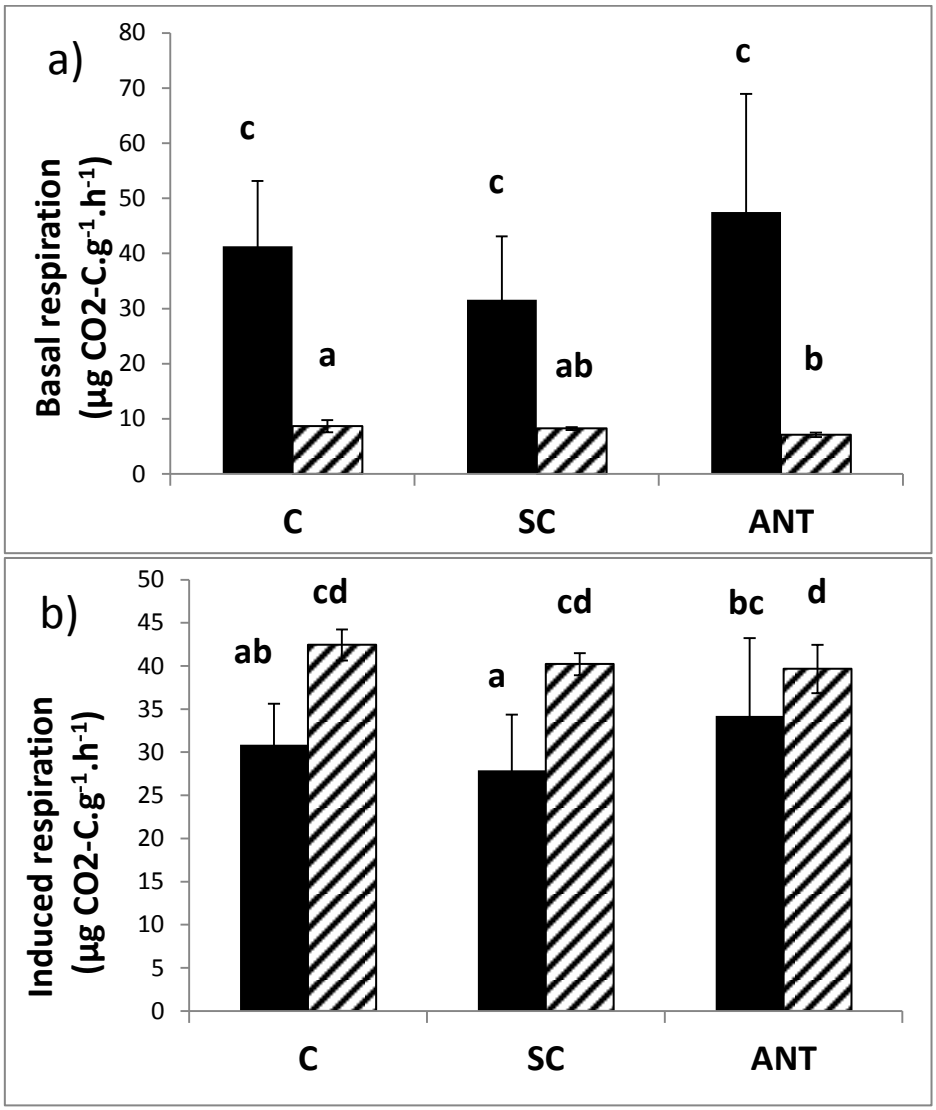


Figure 2. Basal (a) and Induced (b) respiration measured on Pinus halepensis litters in microcosms after a 3-month incubation. COS litters; without pattern, INL litters; with pattern. ANT, SC and C correspond respectively to microcosms with anthracene, solvent control and control microcosms without treatment. Letters define the homogenous groups according to multiple comparison of means. Error bars are standard deviation (n=4). Initial respiration in COS and INL litters expressed as µg CO2 -C.g-1.h-1 were respectively: 205 ± 4 vs 152 ± 7 for basal respiration and 35 ± 2 vs 28 ± 1 for induced respiration.



3.2. Comparison of chemical composition

Litter chemical characteristics were determined in initial COS and INL litters as well as in microcosms after incubation using 13C NMR and C/N ratio. C/N ratio was not significantly different in COS (21.6±0.4) and INL (22.6±0.6) litters before incubation. However, after incubation, the C/N ratios of COS and INL litters were significantly different, except in SC microcosms (shown as supplementary information), treatment did not show any effect.

Comparison of the percentages of different chemical groups defined with 13C NMR spectra showed that the initial litters were chemically different: the percentages of O-alkyl and di-O-alkyl C were higher in INL litter than in COS litter : 39.2 ± 0.9 vs 36.9 ± 0.4 and 23.1 ± 1.2 vs 27.1 ± 0.5, respectively. Table 2 shows the result of mean comparison of chemical groups using ANOVA and considering litter origin and/or treatment as factors. We observed that litter origin (COS or INL) has a significant effect on the variations in certain chemical groups in litters in microcosms after a 3-month incubation: O-alkyl and di-O-alkyl C, which already differed between COS and INL at t0, and alkyl C and guaiacyl units. C/N and humification ratio also differed between COS and INL. Treatment considered alone or with litter origin has a significant effect respectively on means of guaiacyl units and carboxyl C.

Figure 3 shows the means of chemical groups in C, SC and ANT microcosms of COS and INL, which were significantly different according to Table 2. We observed that COS litter had a higher Alkyl C signal and humification ratio than INL litter, whatever the microcosms. However, O-alkyl C, di-O-alkyl C and guaiacyl units were always higher in INL than in COS litter. Treatments (ANT microcosms) lowered the percentage of guaiacyl units and raised the percentage of Carboxyl C in COS.

To define the correlations between all the chemical groups, a PCA was performed considering all the peaks (23 peaks) of the NMR spectra (Fig. 4a). COS (circles) and INL (triangles) were clearly separated by PC1 (22.7%) with a negative and positive correlation, respectively. However, PC2 (16.6%) mainly separated the microcosms according to their treatment. For INL litter, microcosms treated with anthracene (ANT) were close to the initial litter, while controls (C and SC) were separated by PC2. On the other hand, for COS litter, C, SC and ANT microcosms were separated according to PC2. To better distinguish the position of each microcosm, a second PCA was subsequently performed, selecting the best correlated variables (peaks) belonging to each chemical group (Fig. 4b). PC1 and PC2 together accounted for 66% of the variance in this PCA. COS litter was separated from INL litter by a negative correlation with di-O-alkyl and O-alkyl compounds and by a positive correlation to alkyl C. On the other hand, PC2 was mainly explained by a positive correlation with carboxyl and aromatic carbons.

3.3. Correlation between chemical composition and enzyme activities

Pearson's correlation between different chemical characteristics (13C NMR signals and C/N) and enzyme activities (laccase, cellulase, β-glucosidase and acid phosphatase) is shown in table 3. Positive correlations were found between all the tested enzymes activities and alkyl C and humification ratio; in particular, the correlation between laccases and alkyl C

was highly significant ($p<0.001$). Except for β-glucosidase activity, we observed negative significant correlations between all the enzyme activities, and di-O-alkyl C, guaiacyl units, and C/N ratio in microcosms. We obtained a significant positive correlation between cellulase activities and carboxyl C and a significant negative correlation between phenolic C and laccase activities ($p<0.05$).

Table 1. Two-way ANOVA on enzyme activities as dependent variables measured on Pinus halepensis litters in microcosms after a 3-month incubation. Factors are: litter origin (COS, INL), mecososm treatment (ANT, SC and C).

Variables		Litter	Treatment	Litter X Treatment
Laccase	F	436.24	3.89	0.77
	p	**<0.0001**	**<0.05**	ns
Cellulase	F	20.22	2.22	3.56
	p	**<0.001**	ns	**<0.05**
β-glucosidase	F	50.88	1.08	1.83
	p	**<0.0001**	ns	ns
Acid Phosphatase	F	26.95	1.80	0.84
	p	**<0.0001**	ns	ns

4. Discussion

Here, we tested whether the microbial communities of litter from the same plant species but from different sites (coastal versus inland areas), under favorable conditions (temperature and humidity) in laboratory, are affected in the same way by anthracene. Chemical characterization of both litters at t0 revealed that the INL litter is composed of more labile carbon than that of COS litter as shown in Figure 4b. Moreover, microbial activities of litters from COS and INL were different before testing the effect of anthracene in microcosms. Both microbial and chemical differences were more intensified after incubation under laboratory conditions.

The decrease in C/N ratio over incubation (via an increase in total N of all microcosms) confirms that our experimental conditions conducted to microbial growth in microcosms especially in COS and to a less extent in INL litters. However, the overall activity of microbial communities, here estimated by respiration, slows down in microcosms comparing to their natural level in the environment. As well, a dramatic reduction on microbial induced respiration is found in all microcosms, usually considered as an indicator of microbial biomass (Anderson and Domsch, 2010). This result showed that, laboratory incubation conditions had a drastic effect on the whole microbial activities and biomass production.

Table 2. Two-way ANOVA on different 13C NMR signals corresponding to different chemical groups detected on Pinus halepensis litters in microcosms after a 3-month incubation. Factors are: litter origin (COS, INL), microcosm treatment (ANT, SC and C).

Variables		Litter	Treatment	Litter X Treatment
Alkyl C*	F	150.9	1.5	0
	p	**<0.0001**	ns	ns
Methoxyl C	F	0	0.93	2.48
	p	ns	ns	ns
O-Alkyl C*	F	28.9	1.3	1.9
	p	**<0.0001**	ns	ns
di-O-Alkyl C	F	16.561	1.28	2.14
	p	**<0.001**	ns	ns
Aromatic C	F	0.51	2.55	1.03
	p	ns	ns	ns
Phenolic C	F	0.58	3.30	0.89
	p	ns	ns	ns
Phenolic C (C3-C4 of guaiacyl units)	F	13.27	7.41	1.41
	p	**<0.01**	**<0.01**	ns
Carboxyl C	F	0.07	1.42	3.64
	p	ns	ns	**<0.05**
Aromaticity Ratio*	F	0.96	1.78	0.66
	p	ns	ns	ns
Humification Ratio	F	162.8	2.4	0.06
	p	**<0.0001**	ns	ns
C/N*	F	22.03	0.98	0.29
	p	**<0.0001**	ns	ns

Table 3. Pearson correlation between chemical groups defined from 13C NMR spectra and enzyme activities measured on Pinus halepensis litters in micrcosms (n=24).Significant correlations are marked with * p<0.05, ** p<0,01 and *** p<0,001.

Chemical characteristics	Laccase	Cellulase	β-glucosidase	Acid phosphatase
Alkyl C	**0.77*** **	**0.49***	**0.59****	**0.71****
Methoxyl C	-0.32	-0.37	0.12	-0.34
O-Alkyl C	-0.42	-0.15	-0.45	-0.41
di-O-Alkyl C	**-0.70****	**-0.67****	-0.31	**-0.55***
Aromatic C	0.06	0.32	-0.28	-0.03
Phenolic C	**-0.55***	-0.46	-0.40	-0.47
Phenolic C (C3-C4 of Guiacyl units)	**-0.73****	**-0.72****	-0.04	**-0.53***
Carboxyl C	0.46	**0.50***	-0.03	0.43
Aromaticity Ratio	-0.15	0.14	-0.42	-0.22
Humification Ratio	**0.77*** **	**0.52***	**0.59***	**0.69****
C/N	**-0.53****	-0.35	-0.25	**-0.64****

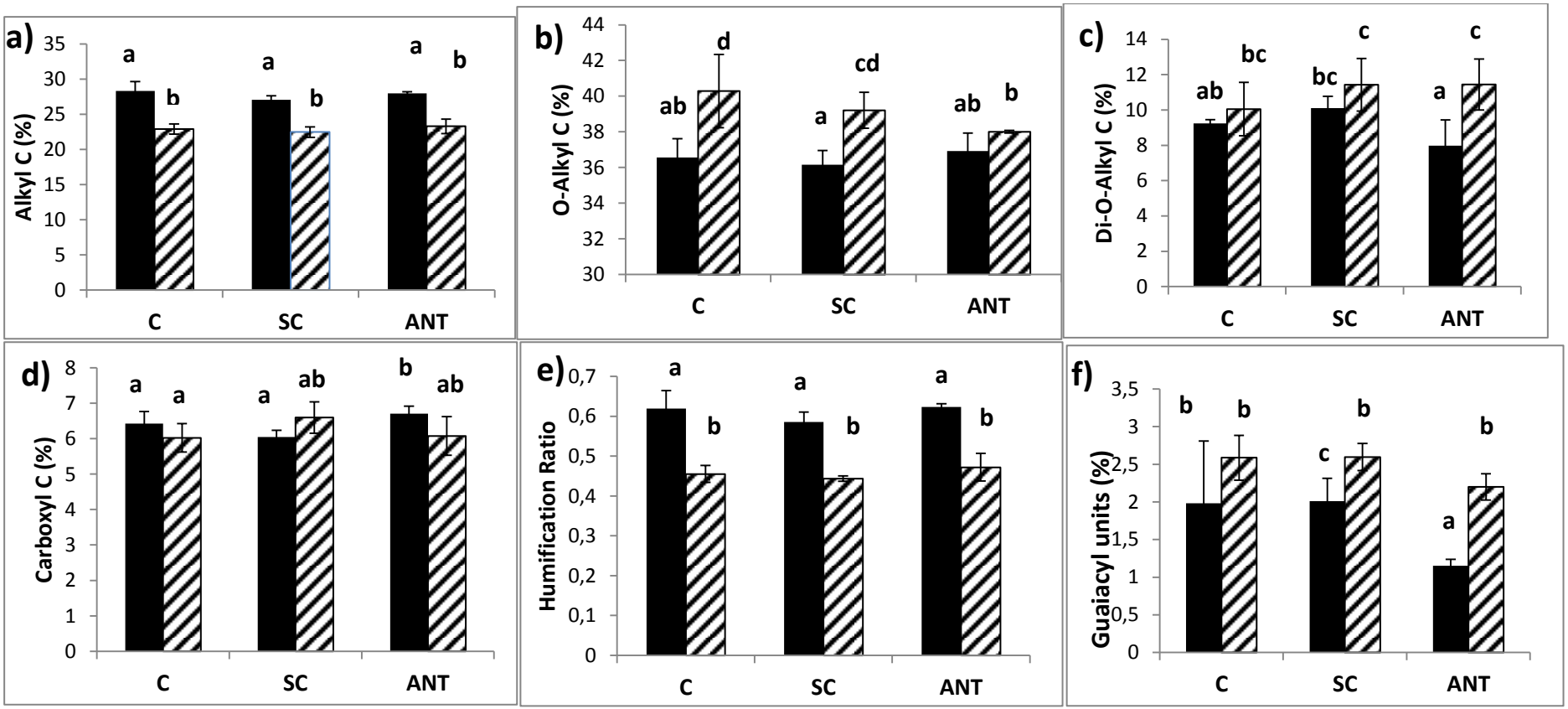


Figure3. Mean of relative percentage of different chemical groups based on 13C NMR spectrum, measured on Pinus halepensis litters in microcosms after a 3-month incubation. COS litters; without pattern, INL litters; with pattern. ANT, SC and C correspond respectively to microcosms with anthracene, solvent control and control microcosms without treatment. Letters define the homogenous groups according to multiple comparison of means. Error bars are standard deviation (n=4). Percentage of different chemical groups measured on initial COS and INL litters were, respectively: alkyl C (27.1 ± 0.5 vs 23.1 ± 1.2), O-alkyl (36.9 ± 0.4 vs 39.2 ± 0.9), di-O-alkyl (10.0± 0.1 vs 11.6 ± 0.4), carboxyl (5.8 ± 0.4 vs 6.1 ± 0.1), humification ratio (0.58 ± 0.02 vs 0.45 ± 0.02) and guiacyl units (2.6 ± 0.5 vs 2.7 ± 0.3).

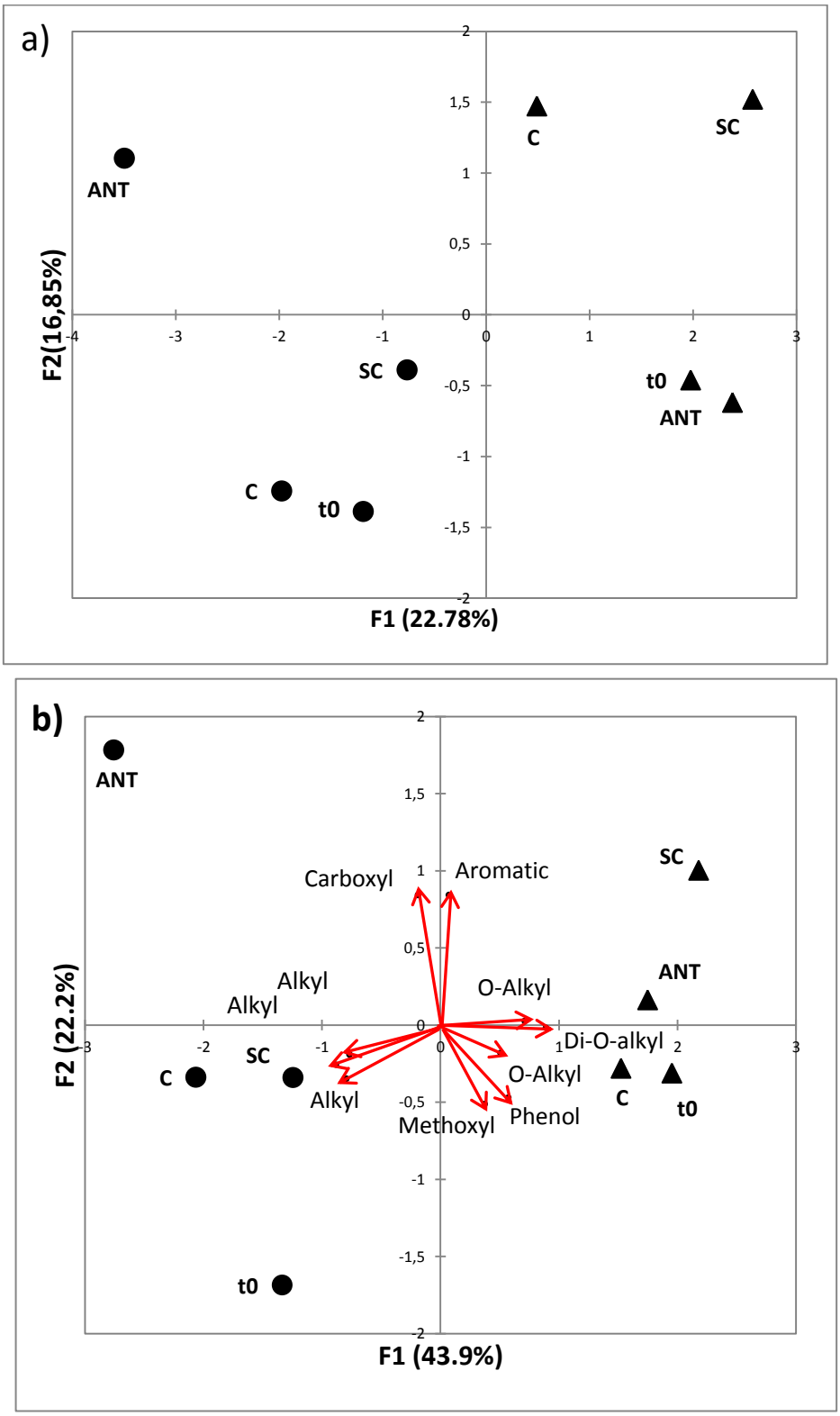


Figure 4. PCA analysis considering a) all defined peaks (n=23) on 13C NMR spectrum as variables b) the most correlated variables of (a), as representative of different chemical groups. COS and INL microcosms are presented as circles and triangles, respectively.

Enzyme activities in litter, produced by both fungal and bacterial communities, are commonly used as indicators of functional diversity of microbial communities (Burk et al, 2011; Wallenstein and Weintraub, 2008). Here we used specific enzyme detection and microbial respiration to assess the effect of our model PAH, anthracene, on microbial functioning. Interestingly, we found that microbial communities from coastal litter are more active with respect to enzyme activities and respiration in transforming available substrates in the litter. Basal respiration was higher in COS litter after incubation, while, when glucose was added as a carbon source, the respiration level was nearly the same for both litters. This highlights that, after 3 month incubation, microbial communities in COS are more active than those in INL for the same biomass amount in both microcosms. This also suggests that the INL litter communities were carbon-limited compared to those in COS.

Moreover, regarding enzyme activities, we detected a concomitant increase in both laccase and cellulase activities in COS litter, whereas the same lignocellulolytic activities were detected in initial litters of both sites. These increases can be directly linked to more intense fungal development, which generally leads to greater quantities of extracellular enzymes (Burke et al., 2011; Sánchez 2009). Thus, under our laboratory conditions, the development of certain microbial communities was favored depending on the origins of litters. Furthermore, the responses of the microbial communities in COS litters are strongly influenced by the presence of an aromatic pollutant. This suggests that the environmental conditions in coastal areas faster microbial communities with a special potential for recalcitrant compound decomposition. This result could have implications for detoxification processes especially in such environment that is known to be greatly impacted by anthropogenic pollution.

Natural recalcitrant molecules i.e. lignin, or anthropogenic compounds such as PAHs, can sometimes be used as carbon and/or energy sources for degrading microorganisms during detoxification (Andreoni and Gianfreda, 2007). However, recalcitrant compounds generally do not serve as growth substrates for a single microorganism, but rather are thought to be oxidized in several steps by consortia of microbes (Perry et al, 1979). Laccases, phenoloxidases generally produced by fungal communities, are one of the most important groups of enzymes degrading a wide variety of recalcitrant molecules, and as such are of great interest in bioremediation processes for contaminated sites (Baldrian 2006, Haritash and Kaushik 2009; Sinsabaugh 2010). Our finding demonstrate that, under favorable conditions, high production of extracellular enzymes can be obtained concomitantly with fungal growth, showing that microbial communities from coastal ecosystems may be particularly adapted to transforming both natural and anthropogenic refractory molecules.

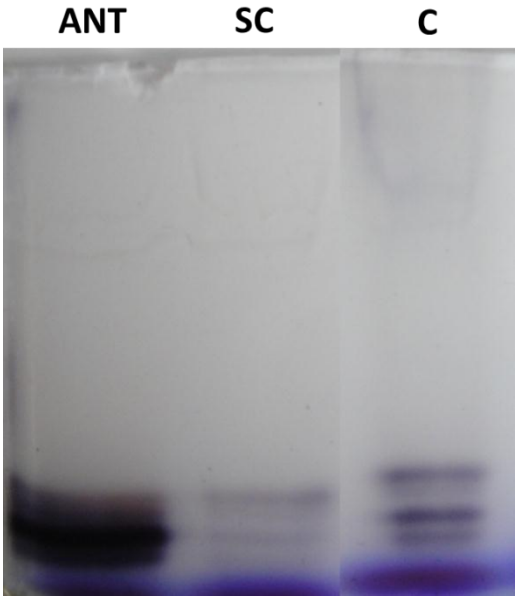
These results are corroborated by the chemical variations in the litter revealed by aromatic signals from NMR. The anthracene effect on chemical variations was especially correlated to the guaiacyl unit signal: after incubation in the presence of anthracene, this signal decreases in COS litter. High negative correlation between lignocellulolytic enzymes and guaiacyl units, which are known to be free phenolic units of lignin in pine litters, also corroborated this result (Lorenz et al., 2004). This finding shows that together with the origin of litter, anthracene is a factor influencing microbial activities.

On the basis of PCA of chemical composition of litters considered together with microbial activities, our results reveal that incubation slows down certain microbial activities in INL litter, while the same activities in COS litter seem to be intensified, especially in the presence of anthracene. Higher activities in COS litter may lead to high biomass production: the increase in alkyl C intensity in COS litters may be linked to higher microbial development correlated with microbial-produced carbon due to lipids of membrane (Mathers et al, 2007). This confirms previous findings (Fahy et al, 2005) showing that pollutants do not always affect microbial activities. Here we focused on the functioning of autochtonous microbial communities but further studies should investigate how PAH may influence the microbial community structure of litters from either coastal or inland areas.

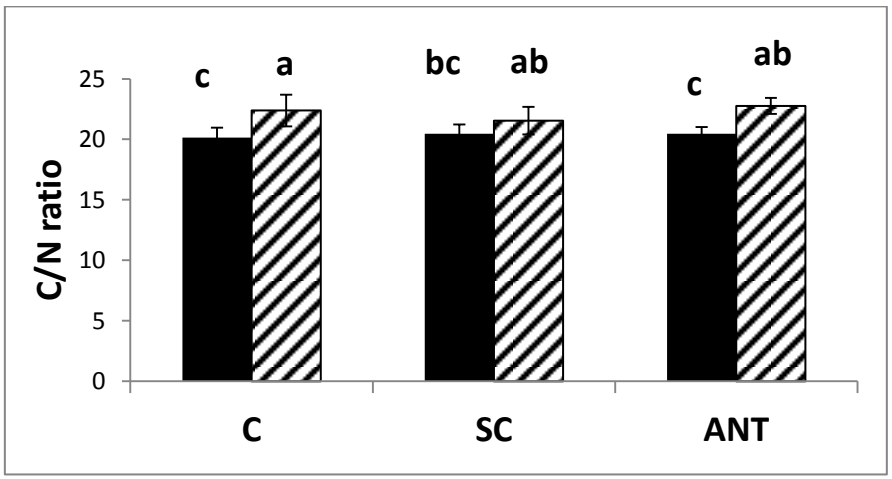
5. Conclusion

In this study, anthracene did not show any toxic effects on microbial activities of either coastal or inland litters, indicating that the influence of a pollutant on microbial activities and organic matter mineralization in litters from these ecosystems is not as high as was initially hypothesized. This finding is of major importance regarding contamination with aromatic pollutants, since it indicates that autochthonous microbial communities from coastal litters appear to better transform organic matter, including recalcitrant compounds. Thus environmental constraints from coastal areas appear to structure microbial communities in a different way compared with those in inland areas. More investigations should be performed through *in situ* studies in Mediterranean coastal environments to understand how multi-contaminations may structure microbial communities and influence their functions.

Supplementary information



Poly Acrylamide Gel Electrophoresis (PAGE) on enzyme extracts from COS mesocosms C, SC and ANT, respectively corresponding to controls without treatment, solvent control and anthracene mesocosms.



C/N ratio measured on *Pinus halepensis* litters in mesocosms after a 3-month incubation COS litters; without pattern, INL litters; with pattern. ANT, SC and C correspond respectively to mesocosms with anthracene, solvent control and control mesocosms without treatment. Letters define the homogenous groups according to multiple comparison of means. Error bars are standard deviation (n=4).

Synthèse générale

La complexité des interactions abiotique et biotique nécessite la combinaison de différentes stratégies et échelles afin de décrire les processus impliqués dans la dégradation de la matière organique morte, une étape jugée comme essentielle au fonctionnement durable des écosystèmes. La combinaison d'approches holistiques et analytiques multi-échelles ont ainsi été mises en œuvre dans le cadre de cette thèse afin de répondre à divers questionnements relatifs au fonctionnement des communautés microbiennes responsables de la minéralisation des litières au sein des écosystèmes littoraux méditerranéens. Ces questionnements visaient plus particulièrement à évaluer l'effet des stress halins sur le fonctionnement des communautés microbiennes autochtones des litières de pin d'Alep : une espèce pionnière, relativement ubiquiste et expansionniste. Compte tenu des spécificités propres de cette espèce ; résineux adaptés à des contextes plus ou moins xériques et donc composition chimique de sa biomasse particulière (faible biodégradabilité), des expérimentations ont été menées pour tester les éventuelles potentialités de décontamination de ces sous-systèmes écologiques vis-à-vis de polluants organiques via l'action de certaines activités enzymatiques naturellement exprimées ou induites *in situ*. En outre, nous avons cherché à établir si les communautés microbiennes présentes dans les litières de pin d'Alep en contexte littoral, et donc soumises à des stress halins, considérés à priori comme une contrainte environnementale supplémentaire, étaient plus robustes et/ou résilientes que celles colonisant les litières de la même espèce mais en domaine continental.

Une étude précurseur des communautés microbiennes dans les milieux littoraux méditerranéens péri-urbains

La zone littorale marseillaise et plus particulièrement les calanques de Marseille abritent un 'hot spot' de biodiversité, une biodiversité révélée par de nombreuses études aussi bien en écologie terrestre que marine (Chevaldonné et al, 2003 ; Duarte et al, 1999). Depuis de nombreuses années face aux menaces grandissantes qui sont susceptibles d'affecter ces milieux, les acteurs locaux et les gestionnaires ont pris conscience de la nécessité d'assurer par une meilleure protection de ces habitats naturels, la pérennité des espèces qui les colonisent dont certaines sont endémiques et strictement inféodées à ce type d'environnement. Les mesures de gestion et de sauvegarde nécessaires à mettre en œuvre ne pourront cependant être réellement pertinentes que si elles reposent sur une connaissance écologique approfondie des modes de fonctionnement et de structuration de ces écosystèmes. Alors que la biodiversité et l'écologie des communautés végétales et animales sont maintenant relativement bien documentées en région méditerranéenne (Barea et al, 2011; Coll et al, 2010; Thomson et al, 2009), les relations fonctionnelles entre sol, végétation et communautés microbiennes impliquées dans la minéralisation de la matière

organique morte et le recyclage des éléments nutritifs sont encore très parcellaires. Si les résultats obtenus dans le cadre de cette thèse relative au fonctionnement des communautés microbiennes impliquées dans la minéralisation des litières visent à combler ces lacunes, ils contribuent aussi à une description plus intégrative des modalités du fonctionnement écosystémique global de ces espaces à forte valeur patrimoniale. En complément de ces éléments d'information sur le fonctionnement de tels milieux, cette étude revêt aussi un intérêt supplémentaire lié à l'évaluation du potentiel de dégradation naturelle de molécules organiques polluantes et toxiques : les HAPs anthropogéniques.

Contraintes environnementales et vulnérabilité des écosystèmes littoraux méditerranéens à l'échelle microbienne

Le rôle primordial des facteurs environnementaux (édaphiques et bio-climatiques) dans la structuration et le fonctionnement microbien, et en conséquence, sur les modalités et les cinétiques des processus de décomposition de la matière organique dont ils sont les acteurs, a pu être mis en évidence dans le cadre de cette thèse en recourant à des approches intégratives *in situ* via les 'litter bags'. En particulier il a été possible de mettre en évidence au sein des litières de pin d'Alep et malgré leur relatif confinement, des évolutions des activités microbiennes fortement dépendantes du climat et selon les saisons définies par des critères de température et d'humidité. Les saisons, qui correspondent à l'un des facteurs majeurs de structuration et de variabilité des écosystèmes, influent donc aussi sur la diversité des fonctions assurées par les communautés microbiennes et plus encore, les intensités et les cinétiques des processus enzymatiques qui concourent collectivement au fonctionnement durable de l'ensemble de l'écosystème.

En outre, dans un contexte méditerranéen déjà contraint, la proximité du trait de côte et donc des influences marines (source d'apport de sels via les embruns) était supposée constituer une contrainte supplémentaire comparativement à des milieux continentaux de même nature (litière de pin d'Alep). Les résultats obtenus infirment cette hypothèse. En effet la variabilité des conditions halines, ne s'accompagnent pas au plan microbien de déséquilibres fonctionnels. Ce constat suggère que, par leur localisation et donc la permanence de leur exposition à ce facteur de perturbation, s'est opéré sur la durée une sélection des populations microbiennes ou des types d'enzymes produites par ces communautés microbiennes. Ainsi, les communautés microbiennes de ces milieux plus contraints apparaissent ainsi paradoxalement plus robustes avec une conservation de leurs fonctions et des services écosystémiques qu'elles procurent. En effet, la vulnérabilité de ces espaces, résulte des réactions et de l'adaptabilité individuelle de l'ensemble des populations constitutives de la biocénose dont les sensibilités et les optimums sont propres à chacune de

ces populations et sous-populations. Les pins d'Alep en tant qu'espèce expansionniste, ubiquiste et longive orientent le fonctionnement microbien pour des zones relativement étendues du littoral marseillais. Toutefois et malgré cette prépondérance, les étroites interactions existant entre sol-plante-microorganisme nécessitent de prendre aussi en considération les communautés microbiennes associées aux autres espèces végétales accompagnatrices comme par exemple l'astragale de Marseille et la sabline de Provence. La pérennité des fonctions microbiennes est ainsi assurée par la diversité des enzymes produites par l'ensemble de la communauté microbienne, dont la richesse conditionne alors en partie son potentiel d'adaptation à de nouvelles contraintes. Dans ce cadre, d'autres expérimentations de types comparatives pourraient être envisagées à diverses échelles pour analyser la contribution fonctionnelle respective des diverses composantes de la micro-biocénose. En outre l'impact d'un facteur abiotique isolé (ici la salinité), peut présenter des effets modérés mais devenir significatif quand il se trouve associé à d'autres facteurs climatiques de stress comme ceux induits par un allongement des périodes de sécheresse estivale. Dans le cadre de futurs travaux, il semble donc pertinent de tester les effets des pressions additionnelles liées aux changements des conditions hydro-climatiques sur les communautés microbiennes du sol en contexte littoral. D'autres types de contraintes comme les pollutions anthropiques peuvent également être envisagées. Si la robustesse des fonctions microbiennes a pu être mise en évidence, les communautés végétales pourraient cependant présenter une sensibilité plus marquée vis-à-vis des perturbations naturelles (variabilité des conditions salines) ou anthropiques (pollutions). Des modifications foliaires, de l'architecture, de la phénologie et de la productivité des végétaux sont des conséquences bien documentées des impacts et/ou des adaptations des végétaux soumis à des situations de stress et qui s'accompagnent de modifications qualitative et quantitative de leur matière organique produite et par voie de conséquence de leur litière. Ces contraintes affectant la végétation sont donc susceptibles d'agir indirectement sur la composition et le fonctionnement microbiens assurant le recyclage des éléments nutritifs. En retour ces évolutions des fonctions microbiennes contrôlant en particulier la disponibilité en éléments nutritifs peuvent soit amplifier, réduire ou prolonger les effets et les impacts sur la végétation de ces nouvelles contraintes. L'existence de ces effets directs et indirects à action immédiate ou différée impose pour la connaissance du fonctionnement global des écosystèmes de considérer les étroites relations de dépendance, de co-adaptation et de co-évolution existant entre les caractéristiques édaphiques des sols, la végétation qui les colonisent et les microorganismes qui en assurent, en aval la minéralisation et qui, en contrôle en amont la productivité.

La résistance microbienne aux polluants au sein des écosystèmes littoraux marseillais ; rôle des communautés fongiques

La présence croissante de polluants divers au sein des sols et des litières de la zone littorale marseillaise, liée à une intensification des activités anthropiques, suscite aujourd'hui de fortes inquiétudes compte tenu du danger que ces poly-contaminations peuvent représentées pour l'équilibre des écosystèmes naturels. Parmi les différentes sources de pollutions, nous nous sommes focalisés sur les pollutions chroniques. Moins spectaculaires que des pollutions de type accidentel, elles sont souvent négligées, alors que leurs impacts sur la durée peut tout aussi gravement nuire à l'intégrité fonctionnelle des écosystèmes, ne leur permettant plus d'assurer les services éco systémiques qu'ils sont susceptibles de nous procurer. Les Hydrocarbures Aromatiques Polycycliques (HAPs), résultant d'apports atmosphériques, correspondent à un ensemble de molécules toxiques dont la faible solubilité dans l'eau contribue à leur persistance dans l'environnement. Leurs dépôts durables à la surface de la végétation et des sols ont nécessairement des implications sur les activités microbiennes dont les fonctions sont de réaliser une minéralisation de la matière organique morte qui se trouve ainsi être plus ou moins contaminée. Des études récentes soulignent en effet le danger des dépôts cumulatifs d'HAP dans les milieux naturels à proximité de zones fortement urbanisées comme c'est le cas des espaces périphérique de la ville de Marseille (Johensen and Karlson 2009). Alors que les concentations en HAPs totaux ne permettent pas de considérer ce site péri-urbain comme véritablement pollué, certaines molécules peuvent néanmoins y être observées à des concentrations relativement importantes attestant de l'existence d'un flux de contaminants. Il est en outre important de signaler que les HAPs rejetés dans l'environnement peuvent subir de nombreux processus abiotiques conduisant à leur disparition plus ou moins totale, tels que la photo-oxydation ou l'oxydation par les réactions Fenton (Pontes et al, 2010). Ainsi selon l'endroit considéré, l'arrivée et la persistance de ces molécules toxiques est susceptible de présenter une forte variabilité avec des concentrations nécessairement pas représentatives des véritables taux d'exposition à ces polluants. Cette situation d'hétérogénéité spatio-temporelles des niveaux de contamination et des poly-contaminations couplée à la complexité des interactions abiotiques et biotiques propres aux écosystèmes d'interface entre continent et mer implique la nécessité de réaliser des suivis *in situ* reposant sur des approches intégrant des évaluations quantitatives et qualitatives des HAPs déposés et une analyse des fonctions microbiennes. Dans ce cadre en recourant à des expérimentations en laboratoire, il a été possible de démontrer clairement un fort potentiel des communautés microbiennes présentes au sein des litières de pin d'Alep en zone littorale pour la décontamination de ces molécules. En outre, on observe une résistance microbienne importante et des

performances fonctionnelles qui demeurent élevées en présence d'HAP, illustrant la richesse et l'adaptabilité des communautés microbiennes associées à la litière de pin d'Alep en zone littorale. La minéralisation enzymatique microbienne des HAPs constitue une voie de décontamination naturelle et contribue globalement à la restauration de la qualité de ces environnements. Au plan scientifique, ces résultats confirment le rôle des communautés fongiques productrices d'enzymes lignolytiques (telles que les laccases) en tant qu'acteurs clés du fonctionnement des litières. Il est important de noter également l'effet indirect de l'induction de l'expression des laccases consécutivement à l'apport expérimental d'HAP : il en résulte en effet, des performances accrues de minéralisation pour des litières peu biodégradables et dont les communautés microbiennes subissent de fréquentes situations de stress climatique et édaphique. La tolérance des communautés microbiennes acquise consécutivement à des expositions à des polluants (Pollution-Induced Community Tolerance, PICT) constitue aussi un processus important à prendre en considération (Schmitt-Jansen et al, 2004). Les réponses adaptatives de ces communautés sont basées sur des mécanismes physiologiques qui sous-tendent cette tolérance et la transformation du composé toxique par voie enzymatique fait partie de ces réponses. En particulier l'étude et la recherche des gènes impliqués dans la synthèse des enzymes dégradant des HAPs (PAH-catabolic genes) ou des molécules aromatiques présentes chez beaucoup d'espèce bactériennes (Lu et al, 2011), pourraient constituer des pistes prioritaires pour des recherches ultérieures.

Dans ce projet, nous avons pu mettre en évidence dans des conditions *ex situ*, la résistance et le fonctionnement des communautés microbiennes en présence d'anthracène, un HAP modèle dont il a été en outre possible de confirmer l'oxydation effective par des laccases fongiques. Ce constat démontre une diversité phénotypique importante des enzymes constitutives et induites au sein des litières de pin d'Alep littorale et ceci même en présence des concentrations élevées d'anthracène. Les études récemment réalisées sur les transcriptomes des enzymes lignolytiques (dont les laccases), ont aussi permis de mettre en évidence des modifications possibles de la composition des communautés fongiques (Kellner and Vandenbol, 2010). Cette forte diversité enzymatique fongique, non spécifique en terme de substrat, traduit notre manque de connaissance des modalités de réaction des communautés fongiques lorsqu'elles sont soumises à de nouveaux contextes environnementaux. A l'heure actuelle, des recherche plus actives devraient être entreprises pour relier les études de variabilité et résilience fonctionnelles à celles décrivant la diversité génétique des communautés fongiques (Bastida et al, 2009 ; Chauhan and Jain, 2010; Schneider and Riedel, 2010 ; Kellner et al, 2009). Une voie de recherche complémentaire pourrait reposer sur une généralisation des approches méta-protéomiques ciblant

spécifiquement certaines fonctions enzymatiques (comme cela a été partiellement mis en œuvre dans le cadre de cette thèse) en vue de répondre à divers questionnements complémentaires: (a) quelles enzymes sont responsables de la dégradation d'un contaminant et plus en amont quels sont les micro-organismes qui les produisent ? (b) quels sont les profils protéiques observables au cours de la dégradation des polluants et en particulier voit-on apparaitre de nouvelles isoformes induites aux propriétés nouvelles? (c) quels sont les microorganismes capables de survivre et de demeurer fonctionnellement actifs au sein des sols pollués ?

Par ailleurs, ces diversités génétique et enzymatique qui conduit à une pérennisation des fonctions peuvent aussi constituer des 'bioressources' potentiellement exploitables dans le cadre de nouvelles applications biotechnologiques (Rodríguez-Couto et Toca-Herrara, 2007). Le sol peut être considéré comme un 'hot spot' de diversité microbienne, diversité qui n'est pour l'instant que très partiellement explorée. Un vaste travail d'identification de nouvelles espèces microbiennes ainsi que de nouveaux gènes et fonctionnalités d'intérêts subsiste. Ceci peut correspondre aussi à une forme de valorisation de la stratégie que nous avons mises en œuvre par les laccases halotolérantes produites par de nouveaux isolats fongiques. Ces espèces fongiques capables d'oxyder les HAPs *in vitro* présentent comme d'autres espèces connues, des intérêts industriels potentiels en vue de contribuer à la biorémédiation des sols pollués par des molécules aromatiques (Steffen et al, 2007).

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Résumé

Les écosystèmes méditerranéens littoraux sont soumis à divers stress environnementaux naturels (stress hydrique et halin) et anthropiques susceptibles de s'intensifier dans les prochaines décennies. Dans ce contexte, le fonctionnement des communautés microbiennes - encore très peu étudiés dans de tels milieux - était important à préciser. L'effet du stress halin sur la transformation de la matière organique dans la litière de pin d'Alep issues des calanques de Marseille a été estimé ainsi que le potentiel de biodégradation d'un polluant chronique, l'anthracène, un Hydrocarbures Aromatiques Polycycliques. Différents approches *in situ*, *ex situ* et *in vitro* ont été utilisées en associant différentes méthodes afin de mesurer l'état fonctionnel du milieu (activités enzymatiques, respirométrie basale), la diversité fonctionnelle microbienne (Catabolic Level Physiological Profile), la biomasse microbienne et l'évolution chimique de la matière organique (RMN du solide du ^{13}C). En mésocosmes, les laccases, induites par la présence d'anthracène, ont contribué à son oxydation et ont été séquencées par LC/MS/MS afin de déterminer les espèces fongiques à l'origine de leur synthèse. Les résultats montrent que certaines activités enzymatiques du cycle du carbone sont peu affectées par la salinité et l'apport d'anthracène. Toutefois la diversité fonctionnelle des communautés autochtones de litière de pin d'Alep issues de ces environnements est modifiée à une échelle micro-locale par l'effet marin. Par ailleurs les réponses fonctionnelles face à l'apport d'anthracène des communautés microbiennes de litières de pin d'Alep en zone continentale sont différentes de celles des zones littorales avec notamment l'absence d'induction d'activités phénoloxydasiques.

Mots clés : RMN du solide ^{13}C, *Pinus halepensis*, stress halin, anthracène, laccases.

Abstract

Mediterranean coastal ecosystems are subjected to various natural and anthropogenic environmental pressures which are supposed to be enhanced because of climatic changes. Little is known about microbial community functioning in such ecosystems. Our site of study is located in the Calanques of Marseille, a hot spot of biodiversity. The effect of salinity (via sea spray exposure) on microbial communities and their ability to transform organic matter in an Aleppo pine litter have been studied as well as the potential of autochthonous microorganisms to transform anthracene used as a polycyclic aromatic hydrocarbon model. To do so, different approaches (*in situ*, *ex situ* and *in vitro* experimental design) were used and we combined various methods such as enzyme activities (laccase, cellulase, phosphatase, lipase), CLPP (Biolog ECO and FF plates), respirometry (basal and induced) and litter chemical characterization (solid-state ^{13}C NMR). Laccases were induced by anthracene in mesocosms and oxidized this compound (with anthraquinone as an intermediate). These enzymes were sequenced by LC/MS/MS to determine the fungal strains responsible for their production. We also found that enzyme activities were not strongly influenced by salinity or anthracene inputs. On the other hand, functional diversity was structured at a small-spatial scale. Moreover, functional responses of microbial communities from inland areas strongly differ from those of coastal areas regarding anthracene inputs since no laccase induction was observed in inland litter.

Key words: ^{13}C NMR, *Pinus halepensis*, salinity, anthracene, laccases.