Title: Revealing specialisation events induced by pollutants in belowground fungal communities

Dissertation presented to obtain the Ph.D. degree in Molecular Biosciences

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I declare that the work presented in this thesis, except where otherwise stated, is based on my own research. It was supervised by Professor Cristina Silva Pereira (ITQB NOVA). The work was mainly performed at *Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa,* between October 2015 and February 2020.

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“I love to travel, but I hate to arrive”

Albert Einstein

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Acknowledgements

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Summary

Chemical pollution has been in the centre of scientific discussion for decades, mainly due to toxicological motives. Pollution and climate change is leading to associated geological/geochemical and atmospheric alterations that negatively impact all ecosystems, including humans. To date our understanding of the environmental impact of pollution and climate change is still very limited. Among the ecological groups that are affected by pollution the “unseen “majority” are the microbial communities especially fungi, that dominate most habitats on Earth. Several studies exist focusing on the effects of organic pollutants towards microbes, especially considering their overall toxicity and degradation with emphasis on fungi due to their impressive catabolic capacities. However, how exposure to toxicants impacts the dynamics of microbial communities remains poorly understood.

In this thesis, we have used pentachlorophenol (PCP) as a model pollutant due to its high toxicity and potential for long range atmospheric transport. PCP simple structure is embedded in many other halogenated organic pollutants. PCP in particular has been extensively analysed and a number of studies have described the degradation pathways undertaken by filamentous fungi, even if most focused on Basidiomycota. The Basidiomycota phylum comprises fungi with impressive enzymatic arsenals, but their bioremediation efficiency in field studies has often revealed to be limited. A deep understanding of the dynamics of belowground fungi comprising all relevant phyla found to colonise soil, upon exposure to PCP, was missing.

Chapter I comprises an extensive literature review which integrates three distinct peer-reviewed published manuscripts (two journal articles and one book chapter). It covers in section 1 the current knowledge on the catabolism of simple aromatics in Dykarya fungi. Such fundamental understanding is paramount to establish knowledge foundations regarding the metabolic responses of fungi to halogenated aromatics. In section 2, an historical view of the usage of PCP and how its status changed from a

neglected pollutant to its inclusion in the list of Persistent Organic Pollutants (POP) is described. Additionally we shed light to our experimental approach by describing our sampling methodology and by comparing our results to the literature, and then discuss future challenges. Section 3 covers the state of knowledge in proteomics techniques, and how these techniques have been applied to study the protein level responses of microorganisms to toxicants. Finally, a literature review of high-throughput sequencing (HTS) is performed in section 4, showing how HTS technologies (namely amplicon sequencing) changed the paradigm of microbial ecology studies and specifically how HTS was used in the present thesis to help disclosing the dynamics of fungi during exposure to the toxicant.

In Chapter II (published journal article) we aimed at understanding how PCP affects the functional biodiversity of cork oak forest soils, particularly of fungi – key colonizers. The functional role of fungi as a community was still poorly understood, although a few pathways have been previously elucidated in pure cultures. This constituted the main challenges of Chapter II – to demonstrate, for the first time, that PCP is a major contaminant of forest soils and to elucidate fungi major roles during the mitigation of pollutants in forest soils. Circumstantial evidence existed that cork oak forests in N. W. Tunisia - economically critical managed forests - were likely contaminated with PCP, but the scientific evidence has previously been lacking. The presented data illustrate significant forest contamination through the detection of undefined active sources of PCP. By solving the taxonomic diversity and the PCP-derived metabolomes of both the cultivable fungi and the fungal community, we demonstrated that most strains (predominantly penicillia) participate in the pollutant biotic degradation. Fungal strains formed an array of degradation intermediates and by-products, including several hydroquinone, resorcinol and catechol derivatives, either chlorinated or not. The degradation pathway of the fungal community included uncharacterized derivatives, *e.g.* tetrachloroguaiacol isomers that can be regarded as markers to monitor PCP degradation in

other fungi dominated food webs. We have shown that fungi play a key role in PCP mineralization and its short lifetime in forest soils but raised the question if PCP pollution was leading to microbial specialization, losses in taxonomic diversity and shifts in functional biodiversity.

In Chapter III (published journal article) we implemented a culture-dependent metacommunity approach aiming to understand the alterations in the composition and functioning of the metacommunity provoked by exposure to PCP. We noticed that those alterations were not fully alleviated when most of the biocide was degraded. We have observed that PCP exposure lead to the loss of some of the less abundant taxa, and also caused shifts in the relative abundances of the dominant taxa compared to controls. A set of key PCP assimilators could be identified by using stable isotope probing followed by amplicon sequencing, which allow disclosing their different roles as early, late or stable assimilators of PCP. Moreover, the proteomic and physiological analyses showed that the carbon and nitrogen metabolisms were particularly affected. In this Chapter we have shown that this dysregulation is possibly linked to the higher pathogenic potential of the metacommunity following exposure to PCP, supported by the secretion of proteins related to pathogenicity and reduced susceptibility to a fungicide.

Our findings support the hypothesis that fungi pay a high functional cost during exposure to PCP pollution, regardless of their superior capacity to degrade the biocide. Specifically, this Chapter provides additional evidence for the silent risks of environmental pollution, particularly as it may favour the development of pathogenic trade-offs in fungi, which may impose serious threats to both animals and plant hosts.

Following the hypothesis raised in the third chapter, in Chapter IV (manuscript in preparation) we developed an innovative cultivation strategy aiming at collecting spores produced by the fungal community upon exposure to toxicants. The cultivations were performed in the presence of PCP or triclosan (TCS), and both surface and airborne spores were

collected. We have observed in the presence and absence of the toxicants a comparable number of spores as well as similar dispersion of spores' sizes. In addition, the spores' size adjustment to a normal distribution (Q-Q plots) showed that the patterns of size dispersion were comparable between surface and airborne fungal spores within each condition. The fungal spores were then inoculated on *Galleria mellonella* larvae, and strikingly, those produced by fungi exposed to pollutants were highly virulent, killing most larvae after only 24h, in opposition to the spores produced under control conditions (absence of pollutant). Though preliminary, the data contained in Chapter IV confirmed the hypothesis that pollutants can increase the virulence of fungi, as raised in the Chapter III.

Finally an integrated discussion is performed in Chapter V, underlining the main contributions of the work contained in this thesis to the field. A critical assessment of the work and its integration in the current state of research is also performed. Overall this work demonstrates the superior capacity of fungi as degraders of pollutants, yet disclosing that toxicants trigger important metabolic tradeoffs and selective pressure that are linked with increased virulence. These findings boost the urgency of fighting chemical pollution as novel and critical health concerns are raised.

Sumário

A poluição química está no centro da discussão científica há décadas, principalmente por motivos toxicológicos. A poluição e as alterações climáticas estão associadas a mudanças geológicas/geoquímicas e atmosféricas que afectam negativamente todos os ecossistemas, incluindo os seres humanos. Actualmente a nossa compreensão do impacto ambiental da poluição e das alterações climáticas é ainda muito limitada. Entre os grupos ecológicos que são afectados pela poluição, a "maioria invisível" são as comunidades microbianas, especialmente os fungos, que dominam a maioria dos habitats na Terra. Existem vários estudos com foco nos efeitos de poluentes orgânicos em microrganismos, considerando especialmente a sua toxicidade e degradação geral, dando ênfase aos fungos devido às suas impressionantes capacidades catabólicas. No entanto, a forma como a exposição a substâncias tóxicas afecta a dinâmica das comunidades microbianas permanece pouco estudada**.**

Nesta tese, utilizámos o pentaclorofenol (PCP) como poluente modelo, devido à sua alta toxicidade e potencial para transporte atmosférico de longo alcance. A estrutura simples do PCP está incorporada em muitos outros poluentes orgânicos halogenados. O PCP, em particular, foi extensivamente analisado e vários estudos incidiram sobre as vias de degradação realizadas por fungos filamentosos, ainda que mais focadas em Basidiomycota. O filo Basidiomycota compreende fungos com arsenais enzimáticos impressionantes, mas a sua eficiência de biorremediação em estudos de campo revelou-se muitas vezes limitada. Assim, continuava a faltar conhecimento profundo da dinâmica dos fungos subterrâneos após exposição ao PCP, considerando todos os filos relevantes que colonizam o solo.

O Capítulo I contém uma extensa revisão da literatura que integra três manuscritos publicados, revistos por pares (dois artigos de revista científica e um capítulo de livro). Neste capítulo, a secção 1 incide no conhecimento actual sobre o catabolismo de aromáticos simples em fungos

do domínio Dykarya. Um nível de conhecimento fundamental é central para o estabelecimento de bases sólidas relativamente às respostas metabólicas de fungos a aromáticos halogenados. Na secção 2 é descrita uma perspectiva histórica do uso de PCP e como o seu status mudou de poluente negligenciado para a sua inclusão na lista de Poluentes Orgânicos Persistentes (POP). Além disso, esclarecemos a nossa abordagem experimental, descrevendo a nossa metodologia de amostragem e comparamos os nossos resultados com a literatura para, em seguida, discutirmos desafios futuros. A secção 3 cobre o estado do conhecimento em técnicas de proteómica e de que forma essas técnicas foram aplicadas para estudar as respostas ao nível das proteínas em microorganismos expostos a substâncias tóxicas. Finalmente, uma revisão da literatura sobre a sequenciação de alto rendimento (HTS) é realizada na secção 4, mostrando como as tecnologias HTS (nomeadamente a sequenciação de amplicões) mudaram o paradigma dos estudos de ecologia microbiana e, especificamente, como o HTS foi usado na presente tese para ajudar a revelar a dinâmica dos fungos durante a exposição ao tóxico.

No capítulo II (artigo de revista científica) procuramos entender como o PCP afecta a biodiversidade funcional dos solos de floresta de sobreiro, principalmente dos seus colonizadores-chave: os fungos. O papel funcional dos fungos enquanto comunidade era ainda pouco compreendido, embora algumas vias de degradação de PCP tenham sido previamente elucidados em culturas puras. Isto constituiu os principais desafios do capítulo II - demonstrar, pela primeira vez, que o PCP é um dos principais contaminantes de solos florestais e elucidar o papel fundamental dos fungos na mitigação de poluentes nesses solos. Havia evidências circunstanciais de que as florestas de sobreiro na região noroeste da Tunísia - florestas de gestão economicamente crítica - provavelmente estavam contaminadas com PCP, no entanto as provas científicas estavam ainda em falta. Os dados por nós apresentados demonstram a existência de contaminação florestal significativa uma vez que a detecção de fontes activas de PCP foi

conseguida. Ao descrever a diversidade taxonómica e o metaboloma associado ao PCP, dos fungos cultiváveis e da comunidade fúngica, demonstramos que a maioria dos isolados (predominantemente *Penicilium*) participa da degradação biótica do poluente. Os isolados de fungos formaram uma série de intermediários e subprodutos de degradação, incluindo vários derivados de hidroquinona, resorcinol e catecol, clorados ou não. A via de degradação da comunidade fúngica incluía derivados não caracterizados, p.e. isómeros de tetracloroguaiacol que podem ser considerados marcadores para monitorizar a degradação de PCP noutros sistemas tróficos dominados por fungos. Mostramos que os fungos desempenham um papel fundamental na mineralização do PCP e na sua curta vida em solos florestais, mas levantamos a questão se a poluição de PCP estaaria causando especialização microbiana, perdas na diversidade taxonómica e mudanças na biodiversidade funcional.

No capítulo III (artigo de revista científica), implementamos uma abordagem de metacomunidade dependente da cultura, com o objetivo de compreender as alterações provocadas pela exposição ao PCP na composição e no funcionamento dessa metacomunidade. Notamos que essas alterações não foram totalmente aliviadas quando a maior parte do biocida foi degradado. Observamos que a exposição ao PCP leva à perda de alguns dos taxa menos abundantes e também causa mudanças nas abundâncias relativas dos taxa dominantes em comparação com os controlos. Um conjunto de assimiladores principais de PCP foi identificado usando marcação isotópica seguida de sequenciação de amplicões, o que permitiu discriminar os seus diferentes papéis enquanto assimiladores iniciais, tardios ou estáveis de PCP. Além disso, as análises proteómicas e fisiológicas mostraram que os metabolismos do carbono e nitrogénio foram particularmente afetados. Neste capítulo, mostramos que esse desiquilíbro está possivelmente ligado a um aumento do potencial patogénico da metacomunidade após a exposição ao PCP. Tais evidências são ainda

suportadas pela secreção de proteínas relacionadas a patogenicidade e pela redução da suscetibilidade da metacomunidade a um fungicida.

No seguimento da hipótese levantada no capítulo anterior, no Capítulo IV (artigo em preparação) desenvolvemos uma estratégia inovadora de cultura, com o objetivo de recolher os esporos produzidos pela comunidade fúngica após exposição a substâncias tóxicas. As culturas foram realizadas na presença de PCP ou Triclosan (TCS), e foram recolhidos os esporos da superfície das culturas e do ar. Observamos, tanto na presença como na ausência dos tóxicos, um número comparável de esporos, bem como uma dispersão semelhante relativamente aos seus tamanhos. Além disso, o ajuste dos tamanhos dos esporos a uma distribuição normal (gráficos Q-Q) mostrou que os seus padrões de dispersão eram comparáveis, para cada condição, entre esporos recolhidos da superfície das culturas e do ar. Os esporos foram depois inoculados em larvas de *Galleria mellonella* e aqueles que foram produzidos por fungos expostos a poluentes mostraram ser altamente virulentos, matando a maioria das larvas após apenas 24 horas, em contraste com os esporos produzidos em condições controlo (ausência de poluente). Embora preliminares, os dados contidos no capítulo IV confirmaram a hipótese levantada no capítulo III, de que os poluentes podem aumentar a virulência de fungos.

Por fim, é realizada uma discussão integrada no capítulo V, destacando as principais contribuições do trabalho contido nesta tese para a área de estudo. É também realizada uma avaliação crítica do trabalho e a sua integração no contexto atual da investigação na área. No geral, este trabalho demonstra a capacidade superior dos fungos enquanto agentes degradativos de poluentes, mas revela que os tóxicos desencadeiam importantes trocas metabólicas e pressões selectivas relacionadas com o aumento de virulência. Estas descobertas aumentam a urgência do combate à poluição química à medida que surgem novas e relevantes preocupações de saúde pública.

Thesis publications

T. Martins, **C. Martins** and C. Silva Pereira. “Multiple degrees of separation in the central pathways of the catabolism of aromatic compounds in fungi belonging to the Dikarya sub-Kingdom”. *Advances in Microbial Physiology*, 2019 (75).

C. Martins[#], A. Varela[#] and C. Silva Pereira. “A three-act play: pentachlorophenol threats to the cork oak forest soils mycobiome”. *Current Opinion in Microbiology*, 2017 (37), 142-149.

# equally contributing authors

C. Martins[#], I. Martins[#], T. Martins[#], A. Varela[#], and C. Silva Pereira. *“*A learning journey on toxico-proteomics: the neglected role of filamentous fungi in the environmental mitigation of pentachlorophenol”. Book chapter *Fungal Bioremediation: Fundamentals and Applications*, CRC Press, 2019.

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C. Martins[#], A. Varela[#], O. Núñez, I. Martins, J.A.M.P. Houbraken, T.M. Martins, M.C. Leitão, I. McLellan, V. W., M.T. Galceran, R.A. Samson, A. Hursthouse and C. Silva Pereira. “Understanding fungal functional biodiversity during the mitigation of environmentally dispersed pentachlorophenol in cork oak forest soils”. *Environmental Microbiology*. 2015 (17), 2922-2934.

[#] equally contributing authors

C. Martins, A. Varela, C.C. Leclercq, O. Núñez, T. Větrovský, J. Renaut, P. Baldrian and C. Silva Pereira. “Specialisation events of fungal metacommunities exposed to a persistent organic pollutant are suggestive of augmented pathogenic potential”. *Microbiome*, 2018, 6:208.

List of acronyms

2DE	Two-dimensional gel electrophoresis
ACN	Acetonitrile
ANOVA	Analysis of variance
BSA	Bovine serum albumine
C branch	Catechol branch
CEC	Contaminants of emergent concern
CFU	Colony forming units
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CTAB	Cetyl trimethylammonium bromide
DAD	Diode array detector
DGGE	Degrading gradient gel electrophoresis
DG18	Dichloran glycerol agar base
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC50	Half maximal effective concentration
ESI	Electrospray ionisation
GLM	Generalized linear models
HCA	Hierarchical cluster analysis
HQ branch	Hydroquinone branch
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
HTS	High throughput sequencing
ITS	Internal transcribed spacer
LC	Liquid chromatography

LRAT	Long range atmospheric transport
MALDI	Matrix-assisted laser desorption/ionization
MDS	Multidimensional scaling
MM	Minimal media
MS	Mass spectrometry
Na-PCP	Sodium salt of pentachlorophenol
NATO s*f*p	North Atlantic Treaty Organisation science *for* peace
NCBI	National Center for Biotechnology Information
NMDS	Non-metric multidimensional scaling
OTU	Operational taxonomic unit
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PCP	Pentachlorophenol
PCR	Polymerase chain reaction
PDA	Photodiode array detector
PLFA	Phospholipid fatty acid
POP	Persistent organic pollutant
R branch	Resorcinol branch
ROS	Reactive oxygen species
Rt	Retention time
SIP	Stable isotopic probing
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SOC	Soil organic carbon
SOM	Soil organic matter

T-RFLP	Terminal restriction fragment length polymorphism
TCA	Tricarboxylic acid
TCS	Triclosan
TGGE	Thermal gradient gel electrophoresis
TIC	Total ion chromatogram
UHPLC	Ultra-high performance liquid chromatography
UPLC	Ultra performance liquid chromatography
UN	United Nations
UV	Ultraviolet light

CHAPTER I

Chapter I contains four sections: **i)** one critical review in **Advances in Microbial Physiology**, entitled *Multiple degrees of separation in the central pathways of the catabolism of aromatic compounds in Dikarya fungi*; **ii)** one review in **Current Opinion in Microbiology**, entitled *A three-act play: pentachlorophenol threats to the cork oak forest soils mycobiome*; **iii)** selected excerpts of one *peer-reviewed* chapter in the book **Fungal Bioremediation: Fundamentals and Applications**, entitled *A learning journey on toxicoproteomics: the neglected role of filamentous fungi in the environmental mitigation of pentachlorophenol*; **iv)** literature review and critical analysis of the importance of high throughput sequencing based techniques for the study of microbial communities.

Accordingly, this chapter integrates the following published manuscripts:

T. Martins, **C. Martins** and C. Silva Pereira. “Multiple degrees of separation in the central pathways of the catabolism of aromatic compounds in fungi belonging to the Dikarya sub-Kingdom”. *Advances in Microbial Physiology*, 2019 (75) [1].

C. Martins[#], A. Varela[#] and C. Silva Pereira. *“*A three-act play: pentachlorophenol threats to the cork oak forest soils mycobiome”. *Current Opinion in Microbiology*, 2017 (37), 142-149 [2]. [#] equally contributing authors

C. Martins[#], I. Martins[#], T. Martins[#], A. Varela[#], and C. Silva Pereira. *“*A learning journey on toxicoproteomics: the neglected role of filamentous fungi in the environmental mitigation of pentachlorophenol”. Book chapter *Fungal Bioremediation: Fundamentals and Applications*, CRC Press, 2019 [3]. [#] equally contributing authors

Outline: The section **1** revises fundamental knowledge on the biology, ecology and biochemistry of fungi, with particular focus on the genes and enzymes involved in the catabolism of aromatics by Dikarya fungi. The understanding of the central pathways of the catabolism of aromatics by fungi provides foundations for a better perception of the molecular processes behind the degradation of aromatic pollutants.

1. Multiple degrees of separation in the central pathways of the catabolism of aromatic compounds in fungi belonging to the Dikarya sub-Kingdom

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1.1. Abstract

The diversity and abundance of aromatic compounds in nature is crucial for proper metabolism in all biological systems, and also impacts greatly the development of many industrial processes. Naturally, understanding their catabolism becomes fundamental for many scientific fields of research, from clinical and environmental to technological. The genetic basis of the central pathways for the catabolism of aromatic compounds in fungi, particularly of benzene derivatives, remains however poorly understood largely overlooking their significance. In some Dikarya species the genes of the central pathways are clustered in the genome, often in an array with peripheral pathway genes, even if the existence of a specific pathway does not necessarily mean that the composing genes are clustered. The current availability of many annotated fungal genomes in the postgenomic era creates conditions to reach a more holistic view of these processes through target analysis of the central pathways gene clusters. Inspired by this, we have critically analysed the established biochemical and genetic data on the

catabolism of aromatic compounds in Dikarya after dissecting the presence and distribution of central catabolic gene clusters (at times including also details on gene diversity, order and orientation) and of peripheral genes. Our methodological approach illustrates the multiple degrees of separation in these central pathways gene clusters across Dikarya. Surprisingly, they show a great degree of similarity irrespectively of the Dikarya division, emphasising that knowledge established on either phyla can guide the identification of clusters of comparable composition (in-cluster plus peripheral genes) in uncharacterised species.

1.2. Catabolism of aromatic compounds in Dikarya - introduction

Aromatic compounds are a large class of unsaturated chemical compounds that contain one or more planar rings of atoms covalently linked, forming resonance structures. They are intrinsic components of most classes of biological molecules, impacting central aspects of the biochemistry and biology of all living organisms. In addition, aromatic compounds own great and widespread industrial value, some of which constitute also a significant source of pollution. Consequently, the study of the catabolism of aromatic compounds particularly derivatives of benzene (object of this review) is of great importance, impacting a wide range of fields of science (*vide infra*).

Aromatic compounds are only second to polysaccharides in natural abundance [4, 5], however the central pathways for their catabolism have received considerable less attention. Naturally, many questions remain to be answered, some of which directly linked to human health, *e.g.* discovery of an additional enzymatic step in the catabolism of tryptophan that may impact immune response and neurodegenerative diseases (*i.e.* reassignment of the ALDH8A1 gene to the kynurenine pathway) [6]. In this respect, substantial knowledge on fungi is yet lacking since to date no systematic study on the central pathways for the catabolism of aromatic compounds across this very large and diverse group of organisms has been conducted, largely overlooking their major significance.

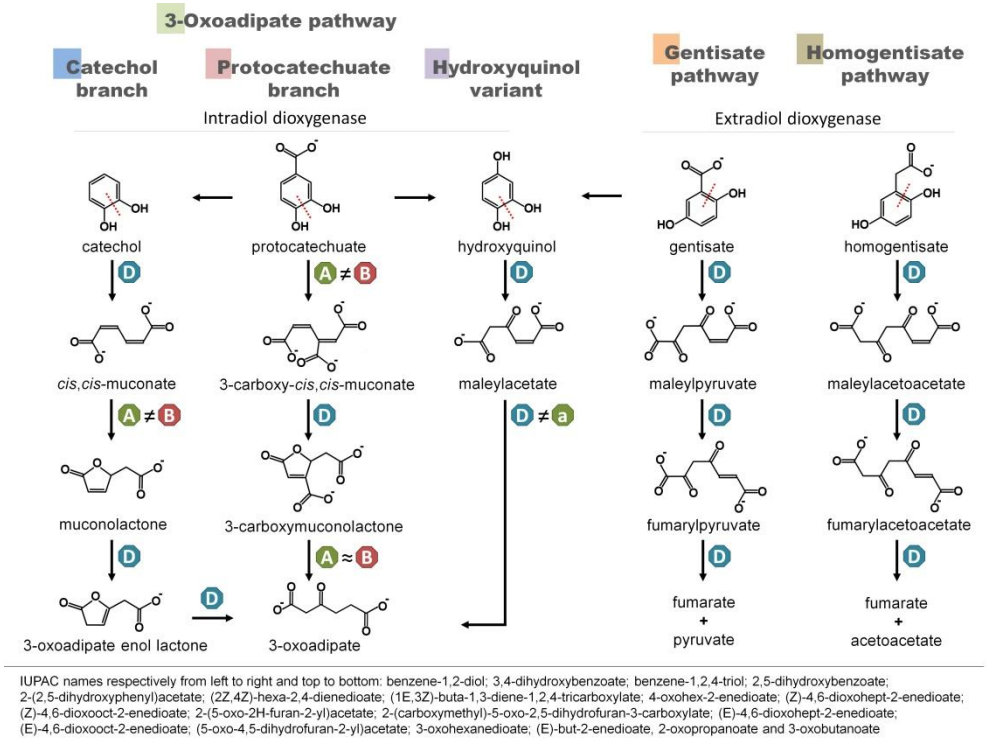
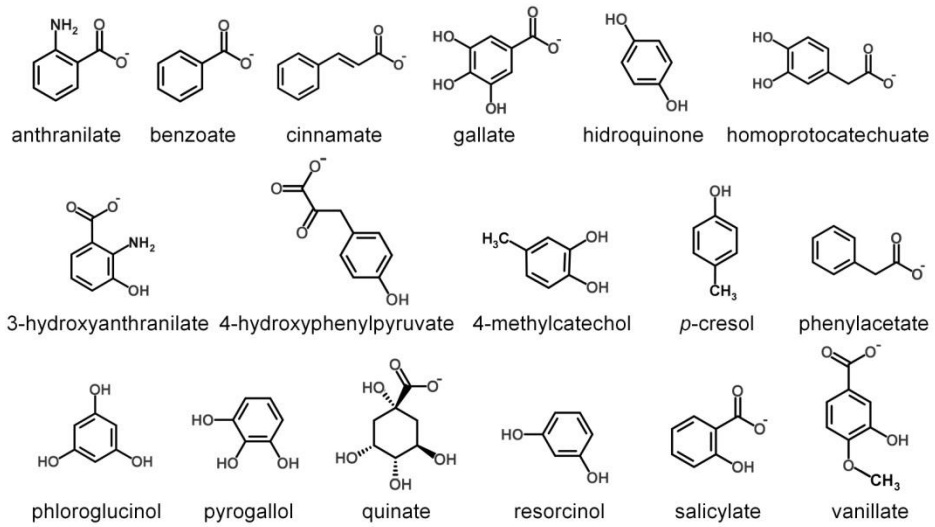


Figure I.1 - Schematic overview of the central pathways for the catabolism of aromatic compounds in Dikarya, showing the 3-Oxoadipate pathway that comprises the Catechol, Protocatechuate and Hydroxyquinol branches, and the Gentisate and the Homogentisate pathways. D, A, a, and B stands for Dikarya, Ascomycota, some Ascomycota, and Basidiomycota, respectively.

Fungi use several aerobic pathways for the catabolism of aromatic compounds, all of which show some degree of conservation to those existent in most living organisms. The catabolism of aromatic compounds is directly channelled to the tricarboxylic acid (TCA) cycle, through acetyl-CoA, pyruvate, succinate, fumarate and oxaloacetate, thus requiring gluconeogenesis. Their catabolism involves ring-cleavage by dioxygenases - **central pathways** (Figure I.1), a step that for many compounds (Figure I.2) is preceded by the activation of the aromatic ring usually through hydroxylation - **peripheral pathways**. Many pathways for the catabolism of aromatic compounds converge to a small number of intermediates that undergo *ortho* or *meta* ring-cleavage, respectively catalysed by intradiol (*i.e.*

between the two hydroxyl groups) and extradiol dioxygenases (*i.e.* adjacent to the hydroxyl group) (Figure I.1) [7, 8]. Naturally, ring-cleaving dioxygenases play a critical role in the catabolism of aromatic compounds and the diversity of pathways is in part determined by their presence. To date, five central intermediates pathways, namely catechol, protocatechuate, hydroxyquinol, gentisate and homogentisate, have been to some extent described in fungi (Figure I.1). The first three undergo *ortho* ring-cleavage and are ultimately converted to 3-oxoadipate, in the pathway with the same name, while the other two undergo *meta* ring-cleavage. To date the existence of specific fungal dioxygenases for gallate, hydroquinone, homoprotocatechuate and pyrogallol (Figure I.2), similar to those described for bacteria, has not been proven, regardless of few evidences suggesting that these compounds are channelled to central intermediate pathways [9-11].



IUPAC names respectively from left to right and top to bottom: 2-aminobenzoate, benzoate, (E)-3-phenylprop-2-enoate, 3,4,5-trihydroxybenzoate, benzene-1,4-diol, 2-(3,4-dihydroxyphenyl)acetate, 2-amino-3-hydroxybenzoate, 3-(4-hydroxyphenyl)-2-oxopropanoate, 4-methylbenzene-1,2-diol, 4-methylphenol, 2-phenylacetate, benzene-1,3,5-triol, benzene-1,2,3-triol, (1S,3R,4S,5R)-1,3,4,5-tetrahydroxycyclohexanecarboxylate, benzene-1,3-diol, 2-hydroxybenzoate and 4-hydroxy-3-methoxybenzoate

Figure I.2 - Chemical structures of representative compounds that are processed through peripheral pathways then channelled to the central pathways for the catabolism of aromatic compounds. Compounds are shown by alphabetic order of the common name.

It has been established that gallate and phloroglucinol catabolism (Figure I.2) may occur through pyrogallol that undergoes a intradiol ring-cleavage mediated by a catechol-like dioxygenase forming 2-hydroxymuconate [12-15], which is then channelled to a specific catabolic pathway, yet to be fully disclosed. The existence of a catabolic tryptophan/kynurenine pathway has been also suggested [16], regardless that its involvement in the formation of 2-hydroxymuconate, a few steps after *meta* ring-cleavage of 3-hydroxyanthranilate (Figure I.2), is yet to be proven. Moreover, in fungi it remains uncertain if there are pathways adapted or specific for methyl-substituted substrates such as methylcatechols [17].

1.3. Reviewing the catabolism of aromatic compounds in Dikarya in the postgenomic era

Presently we face a unique opportunity as many fungal genomes, especially from the Dikarya sub-kingdom, are increasingly being released at fast pace (*e.g.* the 1000 Fungal Genomes Project (http://1000.fungalgenomes.org). Naturally, this creates conditions to push discovery in the genetic basis of these central pathways as to reach a fairly holistic view of these processes in fungi – an idea that is at the heart, and has largely inspired, the present review. Accordingly, we critically analysed publicly available biochemical and genetic datasets (*ca.* 100 scientific publications) that were challenged with new information established through sequence homology searches of genes assigned to the central pathways against annotated genomes in Dikarya (MultiGeneBlast software) [18]. Precisely, herein we analysed the presence and distribution of catabolic gene clusters as well as their architecture, namely the constituting genes, their order and strand orientation. Representative examples within each of the central pathways are shown (Figure I.3-I.7), complemented by cladograms based on the ITS sequence similarity of the comprised species (PhyML analysis) [19], regardless that the high variability of the ITS region does not allow a precise topology. Using this methodological approach we have fingerprint new genes/clusters

belonging to the central pathways of the catabolism of aromatic compounds, notwithstanding that the existence of a pathway is independent of the presence of a cluster. Its potential is easily perceived by the proposed identification of a novel Basidiomycota protocatechuate 3,4-dioxygenase (*vide infra*).

Ultimately, our aim is to illustrate the existence of many common processes across the different divisions (and members) of Dikarya, which largely surpass their dissimilarities, often misread, and to provide potential clues on conservation and evolution events across the genomes of Dikarya. One important remark is that the number of annotated genomes in Ascomycota largely exceeds that of Basidiomycota, hence some aspects deserve to be brought up-to-date as more genomes are annotated. To further stress the significance of the catabolism of aromatics in Dikarya we will also revise, beforehand and in brief, how these pathways largely impact many aspects of the environment and human health as well as industry.

1.4. Ubiquity and significance of the catabolism of aromatic compounds in Dikarya

Dikarya are ubiquitous organisms; they colonise virtually all habitats on Earth, and are capable to survive in habitats with scarce food resources [20]. It is possible to find Dikarya members in terrestrial, aquatic and marine environments, as well as in some extreme locations such as the Arctic and Antarctic [20, 21]. As iconic examples, oligotrophic fungi (*i.e.* able to survive in nutrient poor environments) have been isolated and cultured from glass, where they survive by searching nutrients and carbon from the air [22, 23], whereas chemotrophic strains (*i.e.* survive on chemical nutrients alone) in aircraft fuel lines [24], deep oceans [25] and hypersaline environments [26]. Possibly the most remarkable example is the isolation of fungi - regarded as extremophiles - from the walls of the reactor room at Chernobyl after the nuclear explosion of 1986 [27].

Fungi (and not bacteria) often dominate in low carbon food webs, such as forest soils, constituting up to ¾ of the microbial biomass [28]. The role of fungi on agriculture is widely recognised, especially for the roles played by mycorrhizal symbionts in helping plants to absorb key nutrients [29]. Moreover, saprophytic fungi are also key drivers of ecological recycling, including the global carbon cycle and the cycling of additional organic and inorganic nutrients. Specifically, fungi contribute to the soil formation through rock dissolution and particle binding processes - mostly undertaken by lichens, saprotrophs and mycorrhizae; and generate fertility for primary production by the decomposition of organic residues and nutrient mineralisation processes - undertaken by saprotrophs [20, 28]. Fungi also contribute to defend plants against pathogens, with *Trichoderma* spp. as the most iconic example [30]. On the other hand, they help to define community structures by mediating plant-plant interactions, by serving as food sources for plants and animals, by controlling populations through pathogenic and poisonous species, and finally by performing carbon sequestration and storage [20].

One of the most important ecosystem services involving Dikarya is the decomposition of plant litter which comprises three phases: preferential decay of hemicelluloses and soluble compounds (early); cellulose degradation (intermediate); and lignin degradation (late) [31, 32]. These three phases are defined by rapid succession of fungal lineages, with the division of Ascomycota from Dikarya dominating the early stages of decomposition, followed by gradual increase of the Basidiomycota division possibly due to changes in the contents of litter nutrients and colonisation dynamics among other aspects [32]. The ability of Dikarya to degrade complex plant polyesters has also been demonstrated [33-35]. Elegant reports have been disclosing the distinct routes utilised by fungi to degrade the aromatic compounds present in the plant cell walls. Taken as an example, in the Basidiomycota *Phanerochaete chrysosporium* [33] the activation of the homogentisate pathway was observed during the

degradation of lignin, in particular the homogentisate 1,2-dioxygenase encoding gene (*hmg)* underwent major up-regulation.

Dikarya are also known for their ability to degrade a wide array of chemical pollutants, including dyes and azo-dyes in wastewaters [36], microplastics [37], and persistent organic pollutants such as polycyclic aromatic hydrocarbons [38] and chlorinated aromatic compounds [39]. The impressive versatility in terms of bioremediation potential of Dikarya can be partially attributed to their high surface to cell ratio, which constitutes an important advantage in complex matrices (*e.g.* soil) [28], and superior enzymatic capabilities as a result of the high abundance and diversity of enzymes encoded in their "genomic arsenal" [28]. Importantly, the degradation pathways of many organic pollutants ultimately converge towards the central pathways for the catabolism of aromatic compounds [19, 38-41]. Taken as examples, it has been demonstrated that the catechol branch and the hydroxyquinol variant of the 3-oxoadipate pathway participate in the degradation of chlorinated aromatic compounds [2, 19, 40, 41].

In addition, it has also been demonstrated that aromatic hydrocarbon degrading fungi are closely related to common human pathogens, establishing a physiological connection between hydrocarbon catabolism and certain patterns of mammalian infection [42]. These aspects are of critical importance since currently fungi kill *ca.* 2 million people annually, worldwide [43], especially immunosuppressed and immune-impaired individuals [43, 44]. The link between the catabolism of hydrocarbons by entomopathogenic fungi and their virulence mechanisms has been also demonstrated through disclosure that the cytochrome P450 CYP52 and CYP53 enzymes are involved in both processes [45]. We have also recently shown that the pathogenic potential of fungal communities increases upon exposure and degradation of an aromatic chlorinated compound, further supporting the existence of a link between catabolism of aromatic compounds and fungal pathogenicity [19]. Moreover, a link between the

capacity of a fungus to successfully thrive as phytopathogen and to degrade aromatic compounds has been established for long [46, 47]. These is yet a largely unexplored aspect, regardless that many Dikarya members are responsible for devastating host-specific diseases, *e.g. Magnaporthe oryzae* (wheat blast) and *Diplodia corticola* (cork oak decline), and broad-host-range diseases, *e.g. Ustilago* spp., and *Mycosphaerella* spp. [48] [49]. At times, the capacity of some fungal phytopathogens to infect the host plant has been directly correlated with their ability to catabolise plant defensive aromatic compounds [42]. For example, salicylate, which plays key roles as a trigger of the plant immune response, may be utilised by the invading phytopathogen further linking the catabolism of aromatic compounds with phytopathogenicity [47]. One emblematic example is the Ascomycota *Endoconidiophora polonica* that colonises *Ips typographus* - a bark beetle that attacks the Norway spruce [50]. The ability of the fungus to utilise the phenolic defence compounds produced by the spruce tree (initiated by catechol dioxygenase and processed through the 3-oxoadipate pathway) is necessary for the beetle to successfully invade the tree [50].

Finally, fungi have been also widely used as valuable tools in biotechnological and industrial processes [51], of which the best known are production of dairy, bread, beer and cheese by yeast and/or filamentous fungi belonging to the Dikarya sub-kingdom. Their versatility is also reflected on potential usage for many biotechnological processes ranging from synthesis of pharma compounds (*e.g.* lovastatin and cephalosporin) [52, 53] to production of organic acids (*e.g.* citrate, oxalate, succinate and gallate), often through heterologous expression [54, 55] [56] with applications in pharma [57], industry and agriculture [58]. Many of the abovementioned biotechnological processes are intimately linked with the central pathways for the catabolism of aromatic compounds, for example the production of organic acids [55], and the synthesis of penicillin G (phenylalanine metabolism) by *P. rubens* [59]. In addition, regardless of gallate potential for the manufacture of antioxidant and antibacterial ingredients, printing inks,

and food preservatives among other [51, 55, 60] its production by fungi, to date, is hindered since the peripheral enzymes remain to be discovered. Unsurprisingly, to disclose Dikarya potential impacts, it becomes essential to achieve a holistic comprehension of the central pathways for the catabolism of aromatic compounds.

1.5. Reviewing the central pathways for the catabolism of aromatic compounds in Dikarya

Catechol branch of the 3-oxoadipate pathway

The catechol branch is used for the catabolism of phenolic compounds that may contain carboxylic acid, methyl and amine groups or halogen derivatives [8, 40, 61]. This branch is complementary to the protocatechuate branch or to its hydroxyquinol variant or to both [10, 14, 61, 62]. It is also an important pathway for the catabolism of the aromatic amino acid tryptophan *via* anthranilate (Figure I.2) [63-66]. As with most of the other central pathways, the genetic basis of the catechol branch and of its respective peripheral pathways, has been for long poorly understood. In the peripheral pathways, salicylate 1-monooxygenase (decarboxylating), salicylate carboxy-lyase and 2,3-dihydroxybenzoate carboxy-lyase were described [67-71]. In addition, the catechol 1,2-dioxygenase and the muconate cycloisomerase of the central pathway were characterised in yeast, respectively the Ascomycota *Candida albicans* and the Basidiomycota *Cutaneotrichosporon cutaneum* [72, 73]. Recently, the catabolism of the model compound salicylate (Figure I.2) through the catechol branch was comprehensively characterised at the gene level in the Ascomycota *Aspergillus nidulans* [61]. Putative muconate cycloisomerase (AN3895), muconolactone isomerase (AN4061) and 3-oxoadipate enol-lactonase (AN4531) genes were for the first time described and their implication in the catechol branch was later confirmed in another Ascomycota species [10, 61].

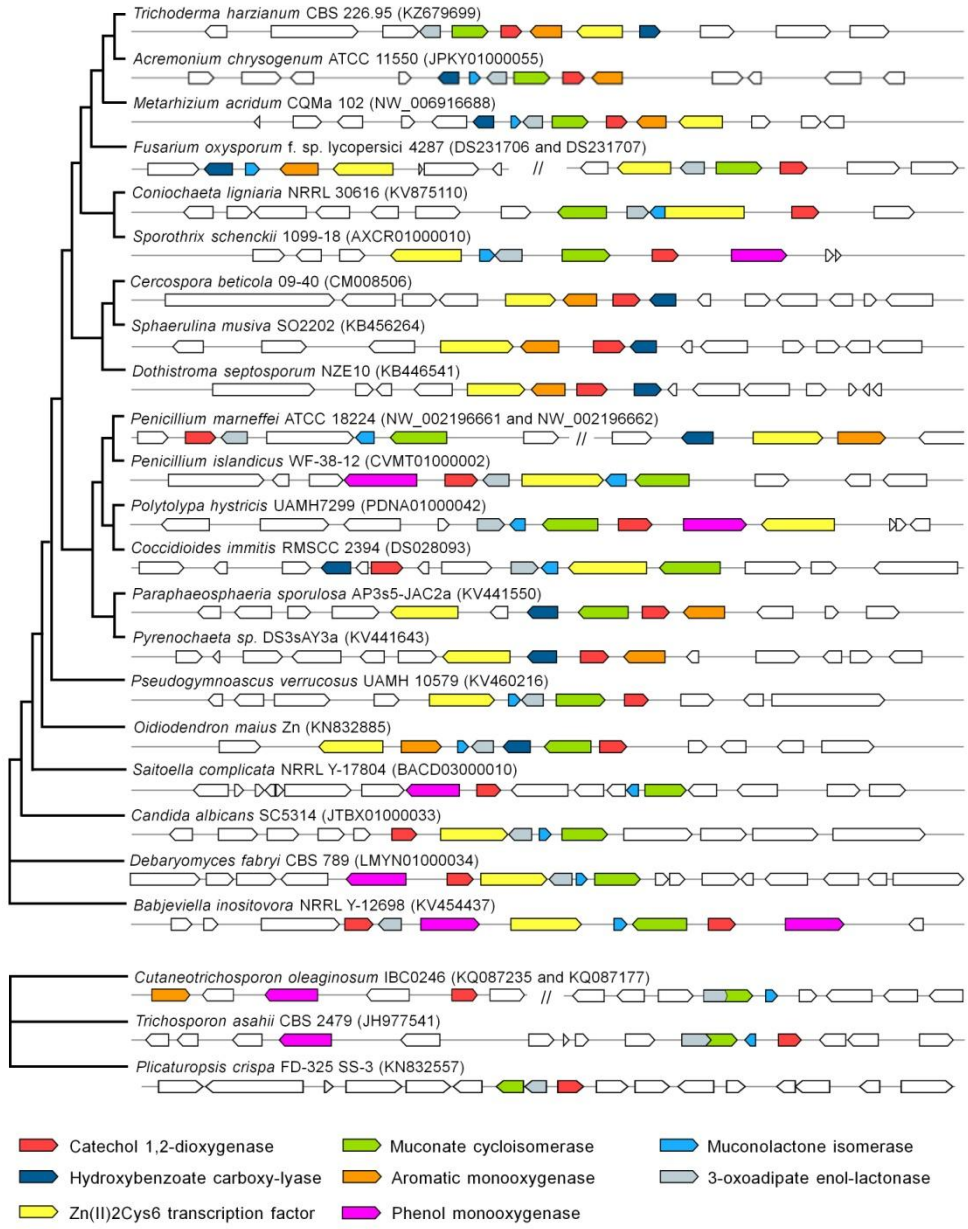


Figure I.3 - Cladograms based on the ITS sequence similarity (left) highlighting genes putatively coding in the catechol branch of the 3-oxoadipate pathway within annotated Dikarya genomes which were identified through sequence homology searches against known genes of the catechol cluster and of selected peripheral genes (MultiGeneBlast software). Genes are represented using a colour code. The cladograms for Ascomycota and Basidiomycota are shown individually (top and bottom, respectively). GenBank accession numbers are indicated between brackets after the species names.

Herein, analysis of the rarely present catechol gene cluster in Basidiomycota indicates that a muconolactone isomerase orthologue is also existent (represent in light-blue, Figure I.3). Divergence occurs in the identity of the muconate cycloisomerase that in Basidiomycota shares homology to 3-carboxy-*cis*,*cis*-muconate cyclase (protocatechuate branch), in part consistent with duplication events in numerous Basidiomycota genomes that hardly ever occurred in Ascomycota. The occurrence of a catechol *meta* ring-cleavage pathway in Basidiomycota has been made apparent [74]. Finally, some catechol 1,2-dioxygenases have been reported as highly active towards hydroxyquinol as substrate [75, 76], opening the hypothesis that the same dioxygenase participates in the two pathways in some species. To sum-up and as depicted in Figure I.3, in Dikarya the central catechol gene cluster comprises up to 4 specific genes (*n*= 3 to 4) that often includes also genes of the peripheral pathways, namely only a phenol monooxygenase (represented in pink, Figure I.3) or one salicylate monooxygenase family gene plus one 2,3-dihydroxybenzoate carboxy-lyase homolog gene (represented in orange and dark blue, respectively, Figure I.3).

Protocatechuate branch of the 3-oxoadipate pathway

In fungi the protocatechuate branch is used for the catabolism of numerous and diverse compounds that have a hydroxyl group in *para* and/or *meta* position, either inherently or acquired upon hydroxylation, including *inter alia* benzoates, cresols, cinnamates and quinate (Figure I.2) [8]. The great diversity of compounds that are channelled to the protocatechuate branch is well denoted in the variety of its peripheral pathways that have been described to date. The peripheral quinate pathway gene cluster (*n*= 7 to 8) was deeply characterised in *A. nidulans* and *Neurospora crassa* [77]. Moreover, the conserved benzoate 4-monoxygenase (CYP53), which belongs to the cytochrome P450 superfamily, has been characterised in several Ascomycota and Basidiomycota species [78-82], and both

hydroxylation and demethylation activity demonstrated to be largely influenced by the P450 reductase partner [81, 82]. In particular, the well characterised vanillyl-alcohol oxidase of the Ascomycota *Penicillium simplicissimum* is known to be active on a wide range of compounds with a 4-hydroxy benzyl group [83, 84]. Phenylacrylate (cinnamate) decarboxylases are widely represented in Ascomycota [85-88]. In Basidiomycota, the catabolism of phenylalanine occurs *via* the phenylpropanoid pathway with initial deamination by phenylalanine ammonia-lyase to cinnamate, which is then channelled to the protocatechuate branch of the 3-oxoadipate pathway [78, 89, 90]. Our group has recently disclosed at the gene level the catabolism of the model compound benzoate in *A. nidulans* [61]. We have specifically assigned in the protocatechuate branch of the 3-oxoadipate pathway the three genes encoding for protocatechuate 3,4-dioxygenase (AN8566), 3-carboxy-*cis*,*cis*-muconate cyclase (AN1151) and a putative hydrolase with decarboxylase activity (AN5232), all of which underwent differentially expression at both gene and protein levels during benzoate catabolism [61]. Previous to this study, only the 3-carboxy-*cis*,*cis*-muconate cyclase had been systematically characterised [91]. Interestingly, these three genes are not clustered in the *A. nidulans* genome. The protocatechuate gene cluster in Dikarya may have up to four genes, encoding for all the necessary enzymes; this includes an uncharacterised alpha/beta hydrolase (IPR000073) distantly related but of the same family as 3-oxoadipate enol-lactonase of the catechol branch (Figure I.4). In the most early studies, the conversion of 3-carboxymuconolactone to 3-oxoadipate in Ascomycota has been associated with a single enzyme displaying both hydrolase and decarboxylase activities [92]. In view of our recently identified gene with a decarboxylase domain (IPR003779) in *A. nidulans* (AN5232) [61] and the existence of a hydrolase encoding gene in the protocatechuate gene cluster of numerous species (light grey, Figure I.4), it is more likely that these two proteins participate in the conversion of 3-carboxymuconolactone in Ascomycota. In fact, the multifunctional enzyme previously characterised

[92] is most likely a hydrolase because the carboxylase activity may occur spontaneously under *in vitro* conditions and the experimental MW matches that predicted for aspergilli orthologues (*e.g.* An13g01940 and AN10520). Therefore, we believe that the AN5232 gene encodes for 3-carboxymuconolactone decarboxylase, also consistent with the experimental observation that this gene is essential for the catabolism of benzoate/protocatechuate [61]. Finally, the three genes for long reported as essential for the conversion of 3-carboxy-*cis*,*cis*-muconate to 3-oxoadipate, namely *pca*B chr. VIII, *pca*C chr. II and *pca*G chr. V [93] certainly correspond to 3-carboxy-*cis*,*cis*-muconate cyclase (AN1151), 3-carboxymuconolactone hydrolase (AN10520) and 3-carboxymuconolactone decarboxylase (AN5232), respectively. The predicted protocatechuate gene cluster of Basidiomycota (n= 4) (Figure I.4) comprises one gene that encodes for a novel protocatechuate 3,4-dioxygenase, one gene with both decarboxylase and hydrolase domains, one canonical cyclase gene, and finally one gene with unknown function - likely an isomerase - with no predicted domains. The first gene lacks the N-terminal catechol dioxygenase domain (IPR007535) and presents high overall sequence divergence compared to the ring-cleavage intradiol dioxygenases characterised in fungi to date. The second gene with both decarboxylase and hydrolase domains shows sequence homology to the 3-carboxymuconolactone decarboxylase (AN5232) and to the C-terminal of 3-carboxymuconolactone hydrolase (AN10520) of *A. nidulans* (represent in orange, Figure I.4). Finally, the last gene – likely an isomerase - is either specific for the Agaricomycetes class (yellow, Figure I.4) or with homology to the N-terminal of the Ascomycota hydrolase, *e.g.* AN10520 (dark blue, Figure I.4). Our interpretation is also in line with the biochemical characterisation of a protocatechuate 3,4-dioxygenase of the Basidiomycota *Pleurotus ostreatus* that shows unique characteristics including low substrate specificity [94], contrarily to that observed in the Ascomycota counterpart [95].

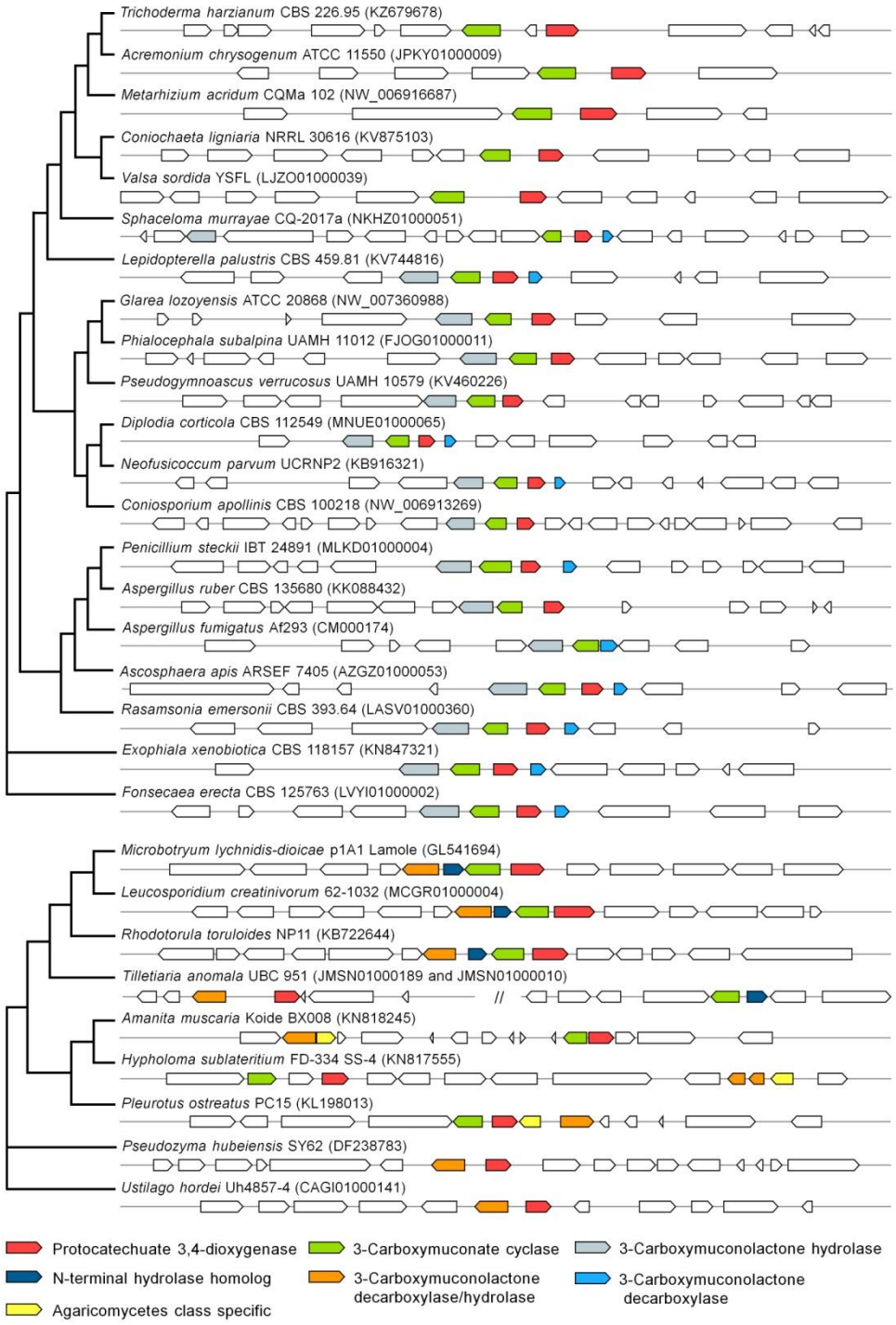


Figure I.4 - Cladograms based on the ITS sequence similarity (left) highlighting genes putatively coding in the protocatechuate branch of the 3-oxoadipate pathway within annotated Dikarya genomes which were identified through sequence

homology searches against known genes of the protocatechuate cluster (MultiGeneBlast software). Genes are represented using a colour code. The cladograms for Ascomycota and Basidiomycota are shown individually (top and bottom, respectively). GenBank accession numbers are indicated between brackets after the species names.

Interestingly, herein we observed that the protocatechuate gene cluster is present in the genome of *P. ostreatus* and also in several other Basidiomycota species that have been reported to use the protocatechuate branch [17, 89, 94] (Figure I.4). Remarkably, the architecture of the protocatechuate gene cluster in Ascomycota is largely conserved (*viz.* constituting genes, their order and strand orientation) even if in many genomes up to two of its genes have been relocated out of the cluster. In addition, peripheral pathway genes in an array with the cluster have not been reported to date (Figure I.4), differing from the other gene clusters herein revised. The same is not true for Basidiomycota that show limited protocatechuate gene cluster conservation within taxonomic classes (Figure I.4). These observations suggest that the protocatechuate gene cluster has evolved differently in the two divisions of the Dikarya, in Ascomycota possibly conserved from ancestral lineages.

Hydroxyquinol variant of the 3-oxoadipate pathway

The hydroxyquinol pathway is more ubiquitously distributed than the other central pathways in Dikarya, as verified by widespread hydroxyquinol 1,2-dioxygenase activity that results in maleylacetate formation [13, 17, 90, 96, 97]. The hydroxyquinol pathway can replace the protocatechuate branch and/or the gentisate pathway and may also function in parallel to them [98, 99]. Many aromatic compounds are channelled to this pathway through the action of peripheral enzymes such as 4-hydroxybenzoate 1-monooxygenase [decarboxylating; unknown protein sequence with broad substrate specificity towards many 4-hydroxybenzoate derivatives, *e.g.* vanillate (Figure I.2)], 1,4-benzoquinone reductase, benzoate 4-monoxygenase (*vide supra* the protocatechuate branch) and hydroquinone monooxygenase [100-104]. The

hydroxyquinol pathway is considerably short comprising only two specific enzymes: an intradiol ring-cleavage catechol-*like* dioxygenase and a maleylacetate reductase; both of which have been biochemically characterised in Dikarya [97, 98, 105-107]. Some species don´t have the canonical maleylacetate reductase gene which was conserved from bacterial counterparts, yet evidences of their ability to use the hydroxyquinol pathway have been established [10]. In Dikarya, this pathway seems specific for certain compounds, *e.g.* hydroquinone and resorcinol (Figure I.2) [108], whether or not other pathways are existent. Collectively, we observed that many species have the predicted hydroxyquinol gene cluster (*n*= 2) frequently in an array with peripheral genes, namely an aromatic monooxygenase or a 3-dehydroshikimate dehydratase (protocatechuate forming), the last often gathered with a quinate-*like* gene cluster (Figure I.5).

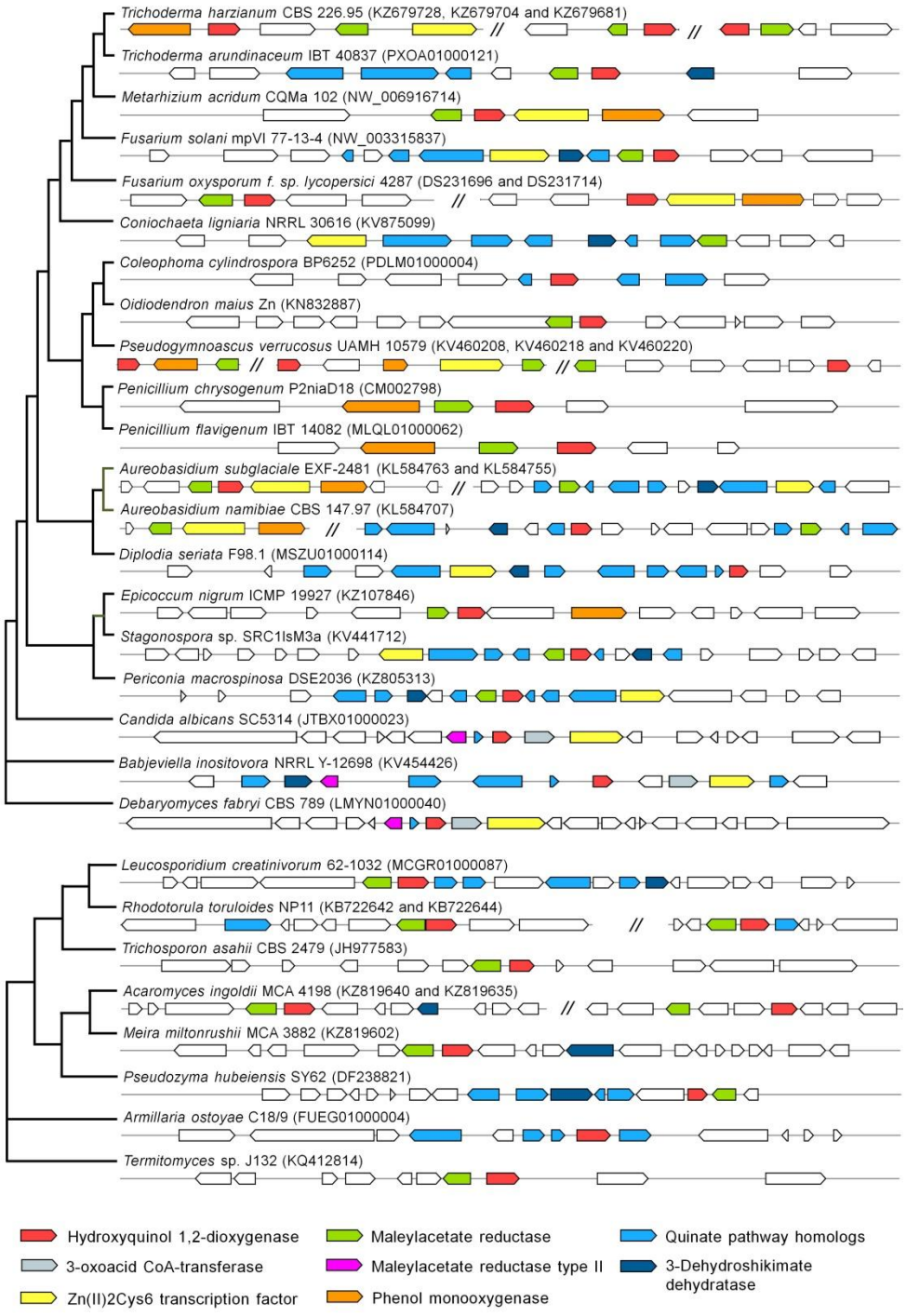


Figure I.5 - Cladograms based on the ITS sequence similarity (left) highlighting genes putatively coding in the hydroxyquinol variant of the 3-oxoadipate pathway within annotated Dikarya genomes which were identified through sequence

homology searches against known genes of the hydroxyquinol cluster and of selected peripheral genes (MultiGeneBlast software). Genes are represented using a colour code. The cladograms for Ascomycota and Basidiomycota are shown individually (top and bottom, respectively). GenBank accession numbers are indicated between brackets after the species names.

Gentisate pathway

The gentisate pathway for the catabolism of 3-hydroxycinnamates, 3-hydroxybenzoate and gentisate (2,5-dihydroxybenzoate) has been characterised in the yeast *Candida parapsilosis* [17, 98] [10]. The gentisate pathway composing genes are arranged in a cluster (*n*= 3): gentisate 1,2-dioxygenase (*gdx*), fumarylpyruvate hydrolase (*fph*) and the putative maleylpyruvate isomerase (*mpi*; glutathione-dependent formaldehyde-activating enzyme), sometimes in array with a peripheral 3-hydroxybenzoate 6-monooxygenase gene (*mnx*). This cluster is widely distributed in the Ascomycota division of Dikarya despite poorly conserved at the gene order and gene orientation levels (Figure I.6). Another gentisate-*like* gene cluster (*n*= 2) is widely distributed among Ascomycota that does not include *mpi* but often includes a putative stilbene dioxygenase and an aldehyde dehydrogenase [109]. The co-existence of the two clusters in several genomes of Ascomycota (Figure I.6) is suggestive that they may play distinct functions, one aspect that deserves more attention in the near future. In the genomes of Basidiomycota, we observed that the orthologue of *gdx* is rarely present but in some species a gentisate-*like* gene cluster comprising *gdx*, *fph* and *mnx* homologues is noted (Figure I.6). As depicted in Figure I.6, an unusually complex architecture was herein observed for the gentisate cluster (*n*= 3) in Ascomycota, which often congregates with a gentisate-*like* gene cluster (*n*= 2), as well as with some key peripheral genes, whereas in Basidiomycota the first was observed to be absent. Once more, the data are indicative that these gene clusters may have evolved differently in the two divisions of the Dikarya.

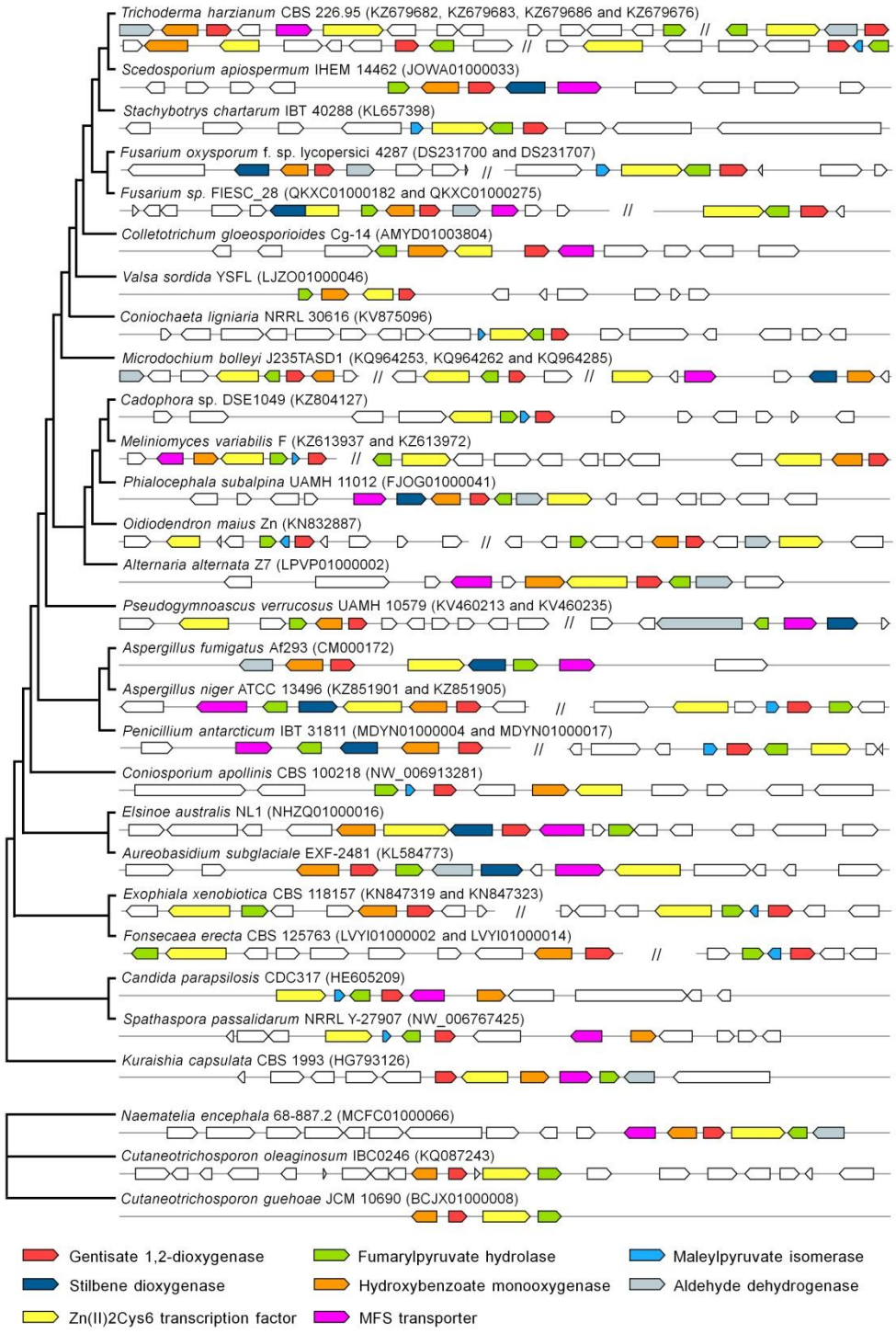


Figure I.6 - Cladograms based on the ITS sequence similarity (left) highlighting genes putatively coding in the gentisate pathway within annotated Dikarya genomes

which were identified through sequence homology searches against known genes of the gentisate cluster and of selected peripheral genes (MultiGeneBlast software). Genes are represented using a colour code. The cladograms for Ascomycota and Basidiomycota are shown individually (top and bottom, respectively). GenBank accession numbers are indicated between brackets after the species names.

Homogentisate pathway

The catabolism of the aromatic amino acids phenylalanine and tyrosine in Ascomycota occurs through the homogentisate pathway underlining a major dissimilarity to the Basidiomycota that mostly uses the phenylpropanoid pathway and the protocatechuate branch of the 3-oxoadipate pathway. The homogentisate pathway is better known in *A. nidulans* and *Penicillium rubens*, where the associated genes are organised in a cluster (*n*= 3): homogentisate 1,2-dioxygenase (*hmg*), maleylacetate isomerase (*mai*) and fumarylacetoacetate hydrolase (*fah*), which is associated with the peripheral 4-hydroxyphenylpyruvate dioxygenase genes (*hpd*A and *hpd*B*)* [59, 110] (Figure I.7). Amongst the Ascomycota, the homogentisate gene cluster shows to be particularly highly conserved in the class of Eurotiomycetes, where it also participates in the biosynthesis of the pyomelanin pigment [109]. On the contrary the 4-hydroxyphenylpyruvate dioxygenase encoding gene is seldom represented in the genomes of Basidiomycota, highlighting other dissimilarity between the two Dikarya divisions (Figure I.7). In addition, in Ascomycota the phenylalanine ammonia-lyase most likely functions as an alternative mechanism for use of nitrogen from phenylalanine, besides participating in secondary metabolism biosynthetic pathways [111, 112]. An upstream cytochrome P450 monooxygenases (*phac*) of the homogentisate pathway can mediate sequential reactions of hydroxylation and participate in the catabolism of phenylacetate and of its derivatives, possibly including homoprotocatechuate (3,4-dihydroxyphenylacetate) (Figure I.2) [11, 17, 59, 113, 114]. As a result, herein we denote the existence of a homogentisate-*like* gene cluster that typically comprises two genes: *hmg* and *fah* in an array with the peripheral *phac* gene, that differs from that previously established in *A. nidulans* and *P. rubens* [59, 110] (Figure I.7).

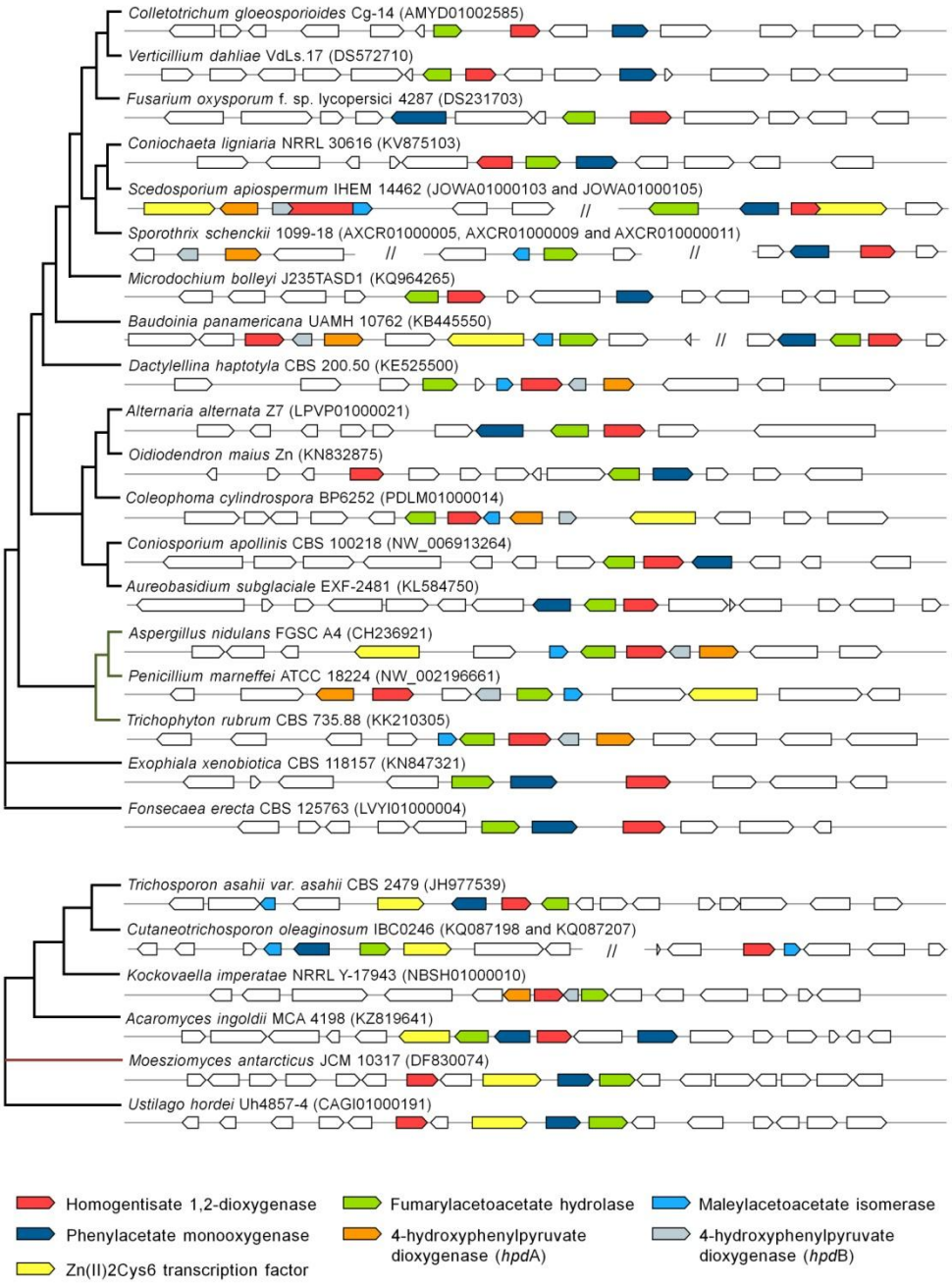


Figure I.7 - Cladograms based on the ITS sequence similarity (left) highlighting genes putatively coding in the homogentisate pathway within annotated Dikarya genomes which were identified through sequence homology searches against known genes of the homogentisate cluster and of selected peripheral genes (MultiGeneBlast software). Genes are represented using a colour code. The cladograms for Ascomycota and Basidiomycota are shown individually (top and

bottom, respectively). GenBank accession numbers are indicated between brackets after the species names.

The preferential substrate for ring-cleavage in the homogentisate-*like* gene cluster is possibly the 4-hydroxyhomogentisate (2,4,5-trihydroxyphenylacetate) with concomitant formation of oxalacetylacetoacetate, hence surpassing the need of isomerase activity. The *mai* gene orthologue is hardly present in the genomes of Basidiomycota (Figure I.7); besides once present it is included in a classical homogentisate pathway gene cluster as denoted in *Trichosporon* spp. and *Cutaneotrichosporon* spp. [115].

1.6. Conclusions and future perspectives

The catabolism of aromatic compounds in Dikarya is known to play essential roles in the environment, in human health and in industry, however most scientific analyses are hinder, in general, by the absence of a holistic comprehension of the genetics of these complex catabolic networks. Herein, we demonstrate that knowledge on the composition of central pathways gene clusters and of its peripheral genes in Dikarya is essential to evaluate critically well-known biochemical and genetic datasets and to push the discovery of yet uncharacterised genes. Pathway gene clusters for all the five central intermediates of the catabolism of aromatic compounds are found widespread in the Dikarya available genomes, most of which showing also peripheral pathway genes in an array with the cluster. When present, the architecture of the clusters, especially at the gene order and orientation levels, is frequently variable. One exception is the protocatechuate gene cluster in Ascomycota that is largely conserved (even if sometimes up to two of its genes are found out of the cluster), and lacks peripheral pathway genes in close proximity. This cluster in Basidiomycota shows limited conservation within taxonomic classes. The known structure of the gentisate and the homogentisate pathways gene clusters in Ascomycota (including specific peripheral genes) was found well represented in this phylum.

However, we also found, across Dikarya, a gentisate-*like* and a homogentisate-*like* gene clusters which differ from the classical arrangement (also in the peripheral genes diversity). This is of extreme relevance since the existence of these pathways still lacks substantial experimental evidence. The datasets herein critically revised support the establishment of an original methodological approach to walk back in the available literature, most of which was attained through classical biochemical and genetic methods, and to revise possible misconceptions. There should be no doubts that our review establishes a new rule of thumb in the genetics of the catabolism of aromatic compounds in fungi: no great divergence exists across the two divisions of Dikarya. Naturally, some of our interpretations should be confronted with searches on a large diversity of annotated fungal genomes – as they become available, even if we are confident that in general the same findings will be reached. Finally, most of the new genes proposed to participate in the central pathways (*in*-cluster and peripheral genes) require experimental demonstration. Nonetheless, their putative identifications will certainly speed the process of discovery that may in turn largely impact important developments in both fundamental and technological aspects on the catabolism of aromatics by eukaryotic organisms.

1.7. Acknowledgments

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Outline: The section **2** exposes an historical view of the research focussed on pentachlorophenol (PCP) since its discovery, covering its usage along decades and the current stage of the research. It also sheds light on our view and strategies to study PCP pollution in cork oak forests and how fungi are valuable tools to tackle this issue and also to understand the dynamics occurring in microbial communities.

2. A three-act play: pentachlorophenol threats to the cork oak forest soils mycobiome

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2.1. Summary

Atmospheric release of persistent organic pollutants (POPs) constitutes a silent threat through chronic contamination of soils at global scale; yet fundamental understanding of their occurrence, sources and fate is still largely lacking. Similar to a three-act play, this review comprises Setup, Confrontation and Resolution. The first emphasises the eighty years of the history of pentachlorophenol (PCP) usage, only recently classified as POP. The second focus on active sources of PCP pollution, including inside cork oak forests in N.W. Tunisia; a threat partially neutralised by the soil microbial diversity, especially fungi. As Resolution, the need for improved knowledge on the global distribution and impacts of PCP in soil microbial diversity as means to preserve the multi-functionality of terrestrial ecosystem is emphasised.

2.2. Setup

2.2.1. Introduction

Ecosystems worldwide are rapidly losing both phylogenetic and functional diversity as a result of human appropriation of natural resources, chemical

pollution and modification of climate, among others [116]. Such biotic impoverishment affects the continuous supply of basic (*e.g.* primary production, soil formation and nutrient recycling) and final ecosystem services (*e.g.* provision of water, food and feed). Environmental microbes still remain the unseen majority, notwithstanding growing evidence that losses in microbial diversity may severely impact the multi-functionality of the ecosystem [117]. Our current knowledge of the detrimental effects of chemical exposure on microbial diversity is still largely restricted to simple acute pollution scenarios; *viz.* local, driven by point sources that emit a restricted number of highly toxic compounds [118]. However, Earth's ecosystems are increasingly being confronted with continuous, low-dose contamination of mixtures of disparate chemicals, as direct consequence of their long-range atmospheric and/or oceanic transport around the globe. In fact, persistent organic pollutants (POPs) originating from remote and diffuse sources can potentially have subtle long-term effects on the microbial ecosystem structure, stability and function.

Pentachlorophenol (PCP) was one of the last chemicals to be added to the list of banned POPs — Corrigendum of the Treaty of the Stockholm Convention on POPs (May, 2015) [119]; this calls for renewed interest in its global distribution and impacts in Earth's ecosystems.

2.2.2. Historical view of the threats of PCP

The history of use of PCP, initiated in 1936, has just completed eighty years. One hundred years following its discovery by Erdmann in 1841 [120], PCP efficiency as pesticide and antimicrobial compound, started to be recognised. Carswell and Nason [121] sustained the added-value of PCP with scientific evidences of 'high degree of effectiveness in biological control, combined with its desirable physical properties'. These initial scientific testimonials on PCP effects were devoid of any precautionary principle. Until 1940, PCP adverse effects were reported only twice, the first concerning its toxicity in rabbits and the second its skin irritation properties in humans [122]

(Figure I.8). In the following decade the knowledge on PCP hazards was only marginally expanded, afterwards primary altered due to evidences of PCP poisoning, with lasting symptoms, in workers of an PCP manufacturing plant (Germany, 1951) [123]. Until the Stockholm Declaration (1972), the pace of research on the threats of PCP has steadily increased. Advances in environmental and analytical chemistry contributed continuously to disclosing the presence of PCP in a wide diversity of environmental or human samples [124, 125], as well as new cases of PCP poisoning, some of which resulting in death [126]. Moreover, the capacity of PCP to affect the uncoupling of mitochondrial oxidative phosphorylation was sorted out [127].

The right to a healthy environment, which is at the heart of the Stockholm Declaration (United Nations (UN) Conference on the Human Environment, 1972), has awakened concerns on the use of potentially harmful chemicals [128]. Consequently, in the early 80s the first restrictions on the use and production of PCP were established in most developed countries [129]. During this decade, scientific publications on the microbial degradation of PCP started to come to light [130, 131]. In the first Earth Summit (UN Conference on Environment and Development, Rio de Janeiro, 1992) the vast majority of the World's Nations acknowledged that human activities were resulting in the biotic impoverishment of the Earth's ecosystems [132]. Afterwards, the interest in environmental sciences developed dramatically, especially as to understand how biodiversity affects the functioning of ecosystems. Consequently, the scientific interest on PCP undertook a dramatic upboost (Figure I.8), stimulated also by the biotechnological interest in both microbes and enzymes mediating modification of organohalogenates [133]. The number of studies on PCP has declined after the Stockholm Convention on POPs (May, 2001), probably because PCP was not considered among the initial list of banned POPs - the dirty dozen [128].

Now, PCP is already considered a POP [119] and severe actions to eliminate its production and use should be soon implemented.

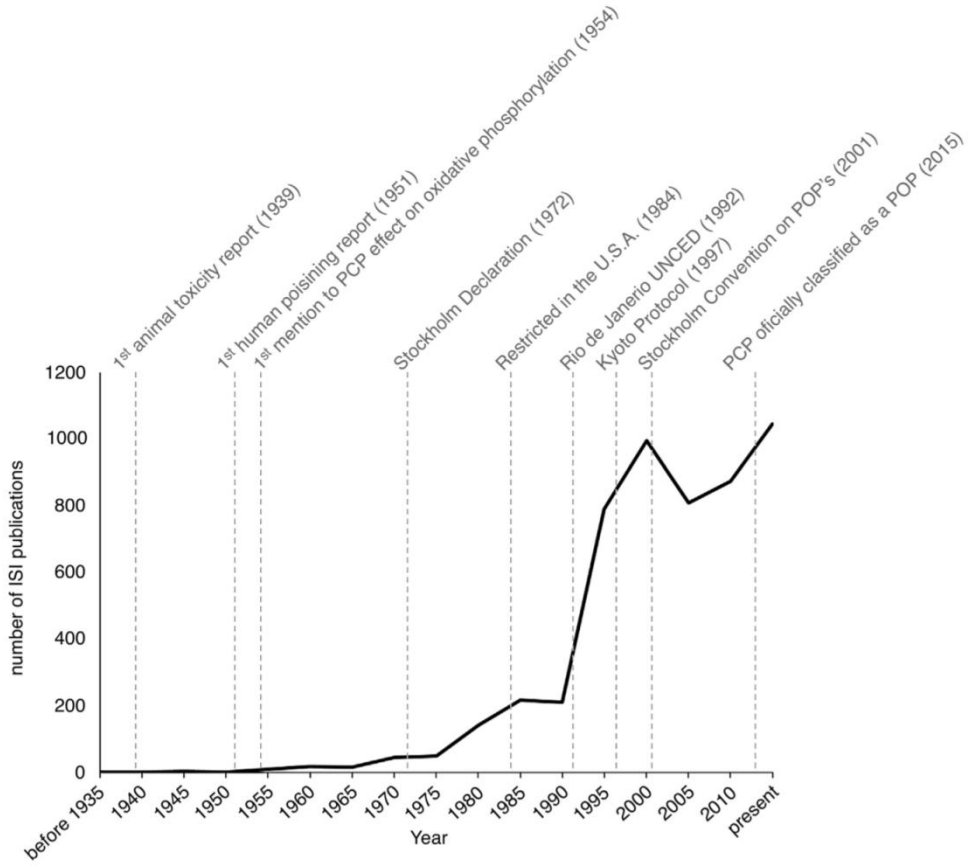

Figure I.8 - Number of scientific publications on pentachlorophenol along time [source: ISI Web of Knowledge]. Papers published each 5-year were searched using the term 'pentachlorophenol' in the topic field, and plotted in a non-cumulative way. Important landmarks, either on environmental awareness or PCP usage/hazards, are highlighted. The interest on pentachlorophenol (PCP) has grown continuously since the first reports of usage as biocide in 1936. The number of studies that have initially increased probably due to evidence of PCP poisoning, have escalated following major emblematic United Nations events on the Human Environment, especially after the first Earth Summit. This trend, which was temporary interrupted after the Stockholm Convention on POPs in 2001, was reestablished succeeding the classification of PCP as banned POP.

2.3. Confrontation

2.3.1. Potential active threats of PCP

PCP is still used in many locations in the globe (*e.g.* in China [134]) and is also produced as a side-product during the degradation of more volatile

biocides, for example, pentachlorobenzene [135] and hexachlorobenzene [136]. The long history of use, persistence and transboundary nature, has resulted in extensive environmental PCP contamination worldwide [137, 138]. Today, PCP is still globally detected in human fluids and tissues from exposure in both indoor and outdoor environments (*e.g.* [125, 139]). Data on its cytotoxicity are consistent with PCP acting as endocrine disruptor and carcinogen [140, 141]. The so-called cork taint defect in bottled-wines, which has been responsible for major losses in the cork industry (especially in the 90's), is largely associated with the presence of chloroanisoles [142]. Their most direct precursors are the chlorophenols, namely PCP, 2,4,6-trichlorophenol (TCP) and 2,3,4,6-tetrachlorophenol (TeCP), which can undergo microbial modification (*i.e. O*-methylation) to generate the corresponding anisole. For example, the capacity of *Trichoderma harzianum* isolated from an ascidian to degrade PCP yielding tetrachloroanisole has been recently reported [143], yet many fungi are weak anisole producers and the yield of anisole formation is also largely influenced by the availability of nutrients [142, 144]. Chloroanisoles are still occasionally identified in cork bottle-stoppers, regardless of current industrial best practice designed to eradicate any chlorinated phenol and/or anisole from processed cork.

Irrespective of both scientific and anecdotal evidence of contamination of the oak bark with PCP and its derivatives [142, 145], PCP impacts in *Quercus suber* forests remain largely overlooked. The bark behaves as a sampler accumulating both gaseous and particulate pollutants [146], but PCP partition to the soil is likely to be significant (estimated to be as high as 95% [129]). Mediterranean cork oak forests span many geographical and cultural boundaries, covering *ca.* 1.5 million ha in Europe and 700 thousand ha in North Africa [147] and supporting one of the highest levels of forest biodiversity [148]. The productivity of these forests is very sensitive to their management; presently a source of income for thousands of people, especially due to its most added value product, the cork bottle-stoppers. The likelihood of contamination of soils in cork oak forests with

PCP raises the question of how chronic exposure to this biocide is affecting the functional diversity of belowground microbes. In particular, of fungi that contribute greatly to preserve the functioning and ecological balance of forest soils [28, 149, 150].

2.3.2. Evidence of cork oak forest contamination with PCP

The existence of undefined active sources of PCP pollution in cork oak forests from the Tabarka district (N.W. Tunisia) has been recently established by us [39] (full reprint in **Chapter II**). Soil samples collected along 113 km^2 contained detectable levels of PCP (Figure I.9a), ranging between *ca.* 2 and 30 $\mu g \cdot Kg^{-1}$, often above levels detected at locations currently treated with the biocide [134]. The atmospheric deposition of PCP (or its precursors) is likely contributing for the prevalence of the biocide in the Tunisian cork oak forest; its half-life in air and environment has been estimated to be 7.4 days and 1.5 months, respectively, with transport distance of 1500 - 3000 km [129]. The contribution of regional forest management practices relying still on PCP usage cannot be fully disregarded, especially since the Tunisian legislation on PCP is not very prohibitive. Soon we may witness how the recognition of PCP as banned POP will or not alter such laissez-faire, especially as most forest products (*viz.* cork and agro-silvo-pastoral products, *e.g.* honey) are marketed both locally and globally, potentially feeding the 'circle of poison' [151].

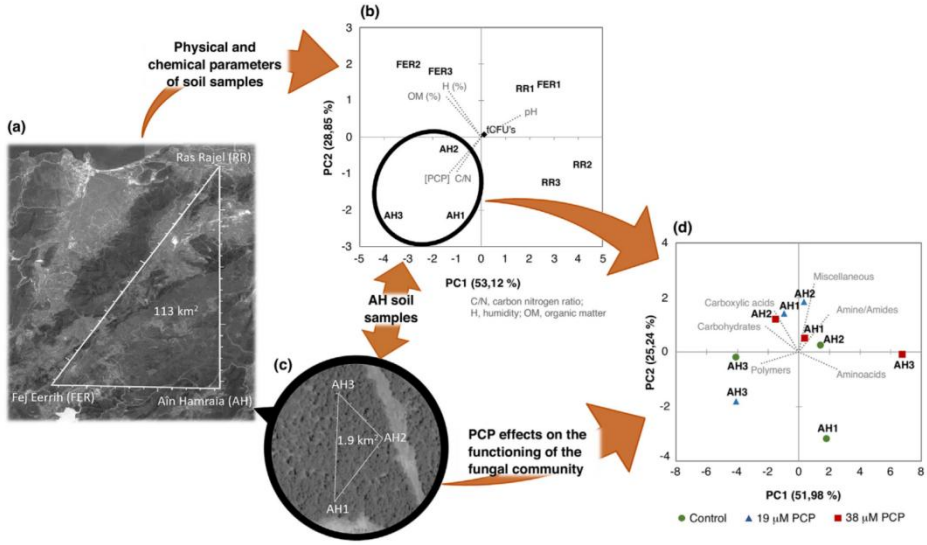

Figure I.9 - Schematic representation of the dissimilarity degree of cork oak forest soils in N.W. Tunisia, comprising geographic location, geochemical features and the physiological profile of the colonising fungal communities. Adapted from [39]. Pentachlorophenol (PCP) was found prevalent in soils of cork oak forests dispersed through 113 km^2 in N.W. Tunisia **(a)**. Principal component analysis showed that the close correlation of AH soils may be largely explained by the levels of contamination with PCP, higher in AH soils than in RR or FER soils **(b)**. The functional diversity (Shannon index, *H'*) of the fungal communities from AH forest soils **(c)** was significantly affected by PCP. Accordingly, the functional dissimilarity of communities grown in synthetic media supplemented with PCP decreased as depicted by closer spatial distribution in the principal component analysis compared to that of control conditions **(d).**

2.4. Resolution

2.4.1. The enduring resistance of belowground fungi against PCP

The precautionary principle of conserving biodiversity in Earth's ecosystems is based on the paradigm that the ecosystem structure dictates its functions and services. In particular, microbial diversity is known to drive multifunctionality in terrestrial ecosystems [152, 153], for example, linked to increased degradation of organic matter and nutrient cycling and availability [116, 149, 150, 154]. The 'meta-profiling' of natural microbial communities may help disclosing the ecosystem' yellow-pages - 'who is there and who is

doing what' [118], revealing, for example, the structure, stability and/or function of real fungal communities in soil ecosystems [32, 149].

'Meta-profiling' of artificial microbial communities (*viz.* enrichment or community-transplantation to microcosms) have allowed, for example, to link community diversity and evolution with productivity [155], and to disclose the structure of the community that mediates polycyclic aromatic hydrocarbons degradation in the deep-water horizon [156, 157]. PCP has the potential to adversely affect the functional biodiversity in specific niches, for example, affecting soil invertebrates [158] and bacterial denitrification processes [159].

In particular, the fungal community responds rapidly to PCP pollution with diversity shifts that are apparently associated with PCP removal, for example, during composting [160] and in forest soils [39]. The widespread capacity of fungi to efficiently degrade PCP and other less chlorinated phenols has been established for long [39-41, 130, 161]. Recently, for example, *Byssochlamys nivea* and *Scopulariopsis brumptii* strains isolated from soils were both shown to degrade efficiently PCP, and even more efficiently when co-cultivated due to synergist effects [162]. Moreover, some PCP derivatives have been elucidated in pure cultures, either of fungi [39, 41, 161, 163-165] or of bacteria [166, 167].

Accordingly, the PCP-derived metabolome (*i.e.* PCP degradation intermediates and sub-products) can be used as an experimental gold-standard. We have disclosed the putative PCP degradation pathway used by the fungal community colonising Tunisian cork oak forest soils chronically affected by the biocide (Figure I.9b,c), by integrating the PCP-derived metabolome of each cultivable member of this community (*vide infra*) [39]. Remarkably, fifty-three out of the seventy-seven fungal strains within the community, could significantly deplete PCP from the media (concentrations ranging from 19 to 56 µM), further emphasising fungi widespread capacity to degrade the biocide. Most of the fungal PCP-degradation products and intermediates identified by us, have been observed before in pure cultures of fungi [160] or bacteria [166]. The

remarkable exceptions consist of compounds never linked before to PCP degradation, namely those in the resorcinol branch and the tetrachloroguaiacol (TeC-G) isomers. In more detail, PCP initial dehalogenation to TeCP may involve its reductive dechlorination, either via biotic or abiotic steps (yielding likely the *meta* and the *para*/*ortho* isomers, respectively) or its peroxidative dechlorination (forming transient benzoquinones (BQ) immediately followed by H^+ mediated reductions) [39, 41, 163]. Both reactions are consistent with the subsequent formation by fungi of the so often reported tetrachlorohydroquinone (TeCHQ) and tetrachlorocatechol (TeCC), as well as tetrachlororesorcinol (TeCR), following the degradation pathway of PCP described above in this thesis.

In our study on cork oak forest soils, the diversity of PCP derivatives found in fungal community-based cultures (as well as in soils), were perfectly matched, regardless of much higher diversity in the first [39]. The metabolites formed by the community, including those internalised by the mycelia, further support the superior use of the C and HQ branches over the R branch by fungi. The PCP-derivatives so far identified may be also used as footprint of PCP environmental pollution, some of which, for example, DCTHB and TeC-G, can directly link the mitigation of the biocide with fungal activity [39]. Not surprisingly, PCP prevalence in soil reduces biodiversity and induces specialization events in the colonising fungal community [39, 160]; for example, PCP was found to reduce the community functional dissimilarity (Figure I.9d). Once forest soils of high geochemical homogeneity may display very dissimilar fungal diversity (both taxonomic and functional) [168], community-based studies per se offer means to reveal key community functional trends, identifying new markers for assessment of environmental pollution and highly efficient pollutant degrading strains/taxa.

2.5. Conclusion

Current studies on PCP are reinforcing wide-ranging principle of global and dispersed environmental pollution by this biocide [125]. The threat provoked by atmospheric pollutants is augmented when the multitude of degradation intermediates and sub-products is considered. Tunisian *Q. suber* forests contamination with PCP, at levels similar to those found prevalent where PCP is currently used, raises serious concerns [39]. Improved understanding of PCP occurrence in soils and of its sources, as well as a fundamental understanding of its fate (dramatically influenced by the soil type and organic matter content [169]), is critically required, regardless of the capacity of the colonising fungal community to act as buffer against the disturbance caused by the biocide [28, 39, 162]. Fungal communities can ensure a short lifetime and the rapid mineralisation of chlorinated phenols [40], in opposition to bacterial counterparts which usually yield highly toxic and recalcitrant non-chlorinated or chlorinated phenol derivatives [161, 170-172]. On the other hand, PCP chronic effects can reduce fungal diversity and induce specialization events. It remains to be seen exactly how affected is the community resilience, and if functional redundancy preserves key ecosystems services under chemical disturbance that decreases biodiversity. Another poorly understood aspect, is the existence of synergism and/or antagonism within members of the fungal community [162] as well as with other important soil colonisers such as the earthworms [173].

Nowadays numerous disparate chemicals are continuously discharged to the atmosphere under the influence of climate change that dramatically alters their global distribution and fate [174]. The paradigm that protecting ecosystem structure also protects its functions and services [38] justifies the precautionary principle of conserving microbial diversity. To build mechanistic understandings of what sustains the assembly of microbial communities, especially their diversity and stability when challenged by exogenous chemical perturbation, requires integration of multiple environmental disciplines. In the particular case of PCP, by providing

evidence of its prevalence in managed forests, challenges legislators to implement measures that may be consequential to economic activities hold by private forest stakeholders. The preservation of the multi-functionality of terrestrial ecosystems for future generations is dependent on our capacity to implement measures that protect soil microbial diversity from global pollution.

2.6. Acknowledgements

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Outline: The section **3** reviews the research conducted on the topic of metaproteomics and how this tool can be applied to study microbial responses to toxicants. To avoid excessive repetition some sections of the published peer-reviewed book chapter were removed, especially those reviewing the history of usage of PCP. Relevance is given to works focussing on proteomics studies that targeted proteomics responses to toxicants, including those using PCP as model compound.

3. A learning journey on toxicoproteomics: the neglected role of filamentous fungi in the environmental mitigation of pentachlorophenol

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3.1. Introduction

Proteomics stands for the "protein complement of the expressed genome by an organism in a particular biological state [175]. Proteins are key multifunctional operators in cells acting as essential structural components, catalysts and major regulatory elements. Therefore, proteomic-based tools can potentially unravel metabolic reactions and regulatory cascades taking place in an organism and/or cellular compartment at a specific condition, as well as their relative abundance, stability and post-translational modifications [176]. When "proteomics" is headed by "meta-", "environmental-" or "community-", it moves beyond that of a single species or cellular compartment to reach a microbial population: "the large-scale characterization of the entire protein complement of environmental microbiota at a given point in time" [177]. It goes without saying that as such, the contribution of uncultured microorganisms is also accounted for, offering

means to resolve major catalytic units of microbial populations [177] and to understand microbial networks in an effective way [178]. Scientific progresses are continuously increasing the resolution of proteomics and facilitating its integration in systems approaches for modelling complex phenomena. However, the major bottleneck in proteomics research is still that “our ability to generate (proteomic) data now outstrips our ability to analyse it” [179].

This chapter discloses our learning journey in toxicoproteomics, aiming to understand how pollution affects the functioning of belowground fungi, from a single species to communities. It covers also fundamentals of environmental pollution with emphasis on mitigation processes mediated by filamentous fungi. The grounds and major developments of proteomics are comprehensively analysed, including our toxicoproteomics-led studies in filamentous fungi. Our studies have contributed to highlight the widespread remedial potential of Ascomycota and Zygomycota, and to disclose central pathways for the catabolism of aromatics in a model fungus. Collectively our journey has been setting the knowledge foundations to move beyond that of a single fungal species; we are now undertaking complex metaproteomic analyses on the specialization of fungal communities during the mitigation of PCP, as will be detailed in the Chapter III of this thesis.

3.1.1 Environmental pollution is globally dispersed

The global dispersion of pollutants constitutes a critical and extremely alarming problem; especially as many undergo transport through middle and long range distances, from Persistent Organic Pollutants (POP’s), to microplastics and nanoparticles [180]. Numerous environmental processes are contributing to their global dispersion, yet the atmosphere plays the most central role in scattering pollution with limitless impacts on public health. Volatile compounds are generally regarded as the major contributors of atmospheric pollution, yet aggregation of compounds (including poor or non-volatile) to dust or particulate air matter ensures their Long Range

Atmospheric Transport (LRAT) [181, 182]. It has been shown that LRAT can be responsible for ca. 70-90% of the POPs levels found in EU countries [174, 181].

POPs persist for long in the environment, may reach regions far away from their application source and are dispersed throughout the environment as a result of natural processes [180]. They are detected all over the planet, including remote locations where no significant local reservoirs prevail [119]. Their global dispersion is known as the “Grasshopper Effect” or “Global Distillation” - a geochemical process that mediates the transport of chemicals from warmer to colder regions of the Earth [183]. This phenomenon justifies the detection of POPs in the Arctic environment and in the corpses of local animals and people (especially in fatty tissues [184]); compounds not used locally.

Reports on acute exposure to toxic pollutants (short-term exposure to a high concentration) are becoming increasingly scarce, possibly due to increase liability issues in most developed countries [185]. On the contrary, the continuous discharge of various toxic chemicals at (very) low doses is increasingly imposing a major (often inaudible) threat to public health and environmental stability [182]. The impact of both acute and chronic pollution has been widely investigated using model organisms and laboratory simulations as well as incidence reports and/or theoretical models (reviewed in [185].

3.1.2. Proteomics: matchless means for pushing discovery on fungal processes

Proteomic tools aim to separate, identify and quantify the polypeptides (*i.e.* protein species) present in a biological sample. Their identification by mass spectrometry (MS) may be physically detached from their electrophoretic separation (gel dependent) or coupled using liquid chromatography (LC) separation in tandem (gel independent) [186, 187]. Gel dependent methods, which have been the work-horse of proteomics [187],

usually use high resolution two-dimensional gel electrophoresis (2DE), *i.e.* isoelectric focussing (IEF) of polypeptides followed by electrophoretic orthogonal separation by molecular weight (MW) (*i.e.* sodium dodecyl sulfate-polyacrylamide gel electrophoresis, SDS-PAGE). Another example is the Blue-native (BN) PAGE that separates protein complexes according to their size/shape in a native run, subsequently splitting their individual components using SDS-PAGE [188]. Relevant polypeptides can then be excised from the gels and their identification retrieved using MS-based techniques. Progressively more powerful MS-based technologies are becoming available, pushing development of gel independent proteomics [186, 189], especially shotgun proteomics. In the last, proteins (that may previously undergo a SDS-PAGE separation) are submitted to proteolytic digestion, then the polypeptides analysed using LC-MS/MS.

Quantification of the polypeptides may be "label free" requiring calculation of the number of MS/MS spectra assigned to each peptide/protein or measuring the MS-signal intensity by direct integration of the chromatographic peak area of the peptide precursor ion [190]. Before the electrophoresis run, the polypeptides can be also covalently labelled (either *in vivo* or *in vitro*) to pairs of chemically identical molecules of different stable-isotope composition (containing ^{13}C, ^{15}N, ^{18}O or ^{36}S); heavy and light samples are then pooled and analysed together by MS for relative protein quantification with high accuracy [186, 189].

Currently, key improvements in MS instrumental resolution and cost reduction as well as the high availability of sequenced genomes, favour the use of gel independent methods in detriment of the gel dependent ones [190]. The lower amount of protein needed for a gel independent analysis compared to gel dependent is likely another key causal factor. Moreover, the last presents some limitations, such as weak-resolution of polypeptides with "extreme" IE and/or MW and highly hydrophobic, presence of highly abundant proteins masking the low copy number ones, and unprecise identification/quantification of polypeptides overlapping in the same

protein-spot [187]. Nonetheless, still today, one remarkable advantage of the gel based methods over the gel free ones is the possibility of *de novo* sequencing of individual intact proteins identified by quantitation of their levels in two or more biologically relevant states. This possibility is particularly relevant in metaproteomics of which the information derives largely from microorganisms with non-sequenced genomes [191, 192].

A comprehensive search in the web of knowledge (https://apps.webofknowledge.com) using 'Proteom*' and 'Fungi' as keywords revealed nearly 1500 publications (in July 2017) illustrating that proteomics has been widely used in research on fungi. Four to five times more studies are found when "Fungi" is replaced by either "Bacteria" or "Plant" possibly reflecting the size of the associated disciplines. Historically, proteomic analyses in fungi have been restrained by poor efficiency in protein extraction (especially due to the rigid cell wall) and reduced availability of sequenced genomes. Protein extraction/enrichment is generally hampered by their delicateness, existence of multiple isoforms and limited abundance of a diversity of polypeptides [179]. Nonetheless standard methods for extracting proteins from distinct fungal cellular compartments have been established whenever cultures are grown axenically [176]. When moving towards environmental samples, protein extraction faces unforeseen challenges, especially from soils where many interfering compounds are particularly abundant, *e.g.* humic colloids that strongly bind to proteins [193]. Soils display usually low abundance and high heterogeneity of proteins; taken as an example, 0.1-2 µg of protein *per* g of semiarid soil can be directly extracted, estimated to correspond to *ca.* 7 % of the total protein [194]; similar to levels typically achieved by us (unpublished results). Several methods have been described to overcome these constrains, yet no definitive standard protocol has been established, and some may led to biased recovery [192-194].

The limited availability of sequenced genomes from filamentous fungi has initially reduced the pace of proteomics research; the genome of

Neurospora crassa - the first filamentous fungus to be fully sequenced – was released in 2003, seven years after the completion of the yeast *Saccharomyces cerevisiae* genome. Luckily, this paradigm has been already altered; currently there are > 1000 fungal genomes sequenced. Moreover, "The 1000 Fungal Genomes Project" aims to sequence two species for every family-level clade of Fungi (http://1000.fungalgenomes.org), producing genomic data fully descriptive of the phylogenetic diversity of the Kingdom of Fungi.

The first 2DE study on a filamentous fungus was reported more than twenty five years ago: discovery of differentially accumulated polypeptides in distinct infection structures in the phytopathogenic fungus *Uromyces viciae-fabae* [195]. In the following years, gel dependent proteomics has been extensively applied to investigate the response of fungi to either xenobiotics [196-199], antifungal drugs [200] or various stresses [201-203], fungal secondary metabolism [204], development [205] and pathogenesis [206, 207], and interactions of fungi with bacteria [208], plants [209] or insects [210]. Taken as an example, extracellular multi-enzymatic complexes of hydrolytic enzymes that mediate the degradation of biomass in *Trichoderma harzianum* were disclosed using BN-PAGE [197]. Presently, gel independent proteomics is providing means for in-depth characterization of membrane [211], mitochondrial [212] and extracellular [213] sub-proteomes. Moreover, it is supporting discovery in fungi biotyping [214, 215] and interaction with a host [216].

Representative studies on the metaproteome of soils displaying very distinct geochemical properties are depicted in Table I.1, highlighting the major research questions and findings of each study. Metaproteomics (2DE) was first applied to unravel the functioning of a bacterial community during optimal phosphorus removal from sludge [217]. This has inspired further studies on metaproteomes using either gel dependent methods, *e.g.* soil rhizosphere [218] (Table I.1), or gel independent ones, *e.g.* biofilm formation [219, 220] and litter decomposition [221] (Table I.1). Taken as an

example, long-term deforestation was demonstrated to foster the diversity of belowground bacteria, particularly of *Cyanobacteria* mediating carbon-fixation processes [222]. Most metaproteomic studies on the functioning of the soil microbiota still highlight the role of bacteria in detriment of fungi, regardless that the contribution of fungal proteins may be significant [191, 223]. For example, in the rhizosphere fungal proteins account for 12-30 % of the total identified proteins [218, 224] (Table I.1).

Table I.1 - Selected metaproteomics studies on microbial populations associated to different soil habitats, in reverse chronological order. The central research question is marked in bold.

Sample	Methods	Research question and major findings	Work
Forest soils, (0–5 cm)	LC-MS/MS	**Microbial functioning of the soil community during short-term and long-term warming.** Bacterial proteins >> fungal proteins (linked to high pH). Soil warming altered taxonomic diversity: ↓ [Ascomycota / Basidiomycota] ratio, ↑ Actinobacteria and Cyanobacteria; Long-term soil warming: enhanced soil respiration, increased CO_2 efflux consequently altering the functioning of the community.	[225]
Soil with tobacco litter	Protein-SIP $^{15}NO_3^-$-labeled LC-MS/MS	**Analysis of the assimilation flux of plant-derived N by the soil microbiota.** The most abundant ^{15}N-utilizer are *Rhizobiales*. Short-term assimilation of N: dominance of bacteria over fungi; later stages of short-term ^{15}N-leaf litter degradation: *Saccharomycetales* and *Hypocreales* are more active. Fungi abundance increases with the decreasing of water soluble C.	[226]
Dryland soils, predesertic. marshes and forests	LC-MS/MS	**Microbial functioning of the soil community in response to the C load.** Ascomycota and Basidiomycota occupy different nutritional niches: Ascomycota expressed proteins in soils of moderate C content, while Basidiomycota protein levels were higher in soils with high DOC content.	[227]
Permafrost (30–35 cm)	LC-MS/MS	**Microbial functioning in permafrost and adjacent soil layer.** Fungi and fungal processes are poorly represented in the permafrost active	[228]

Sample	Methods	Research question and major findings	Work
and soil (65–75 cm)		microbial population. Fungal proteins (genes and transcripts) corresponded only to a small fraction in the analysed samples.	
10 years old amended soils with sewage sludge or compost	LC-MS/MS	**Functioning of the soil microbial community in soils undergoing different amendments.** Fungal proteins (of which 95% are Ascomycota) were 20-times < than bacterial ones, decreasing further in the amended soils. The phylogeny of the mycobiota is highly influenced by the amendment type, with the decrease and/or loss of some families (*e.g. Glomerellales* are virtually absent in soils undergoing amendment with sludge but increase for compost).	[222]
Cryosol[1], 0-5-cm	LC-MS/MS	**Functioning of the microbial community in cryosol.** Several methanotrophic proteins were identified, constituting the first evidence of active atmospheric CH_4-oxidizing bacteria in permafrost-affected cryosols, which may help to explain the atmospheric CH_4 uptake in the polar region.	[229]
Semiarid soils	LC-MS/MS	**Functioning of the microbial community in semiarid soils.** Proteomic data are consistent with an ecological adaptation for carbon and nitrogen fixation. The amount of protein extracted from soils is scarce and largely influence by the	[194]

Sample	Methods	Research question and major findings	Work
		extraction method which may be bias, *e.g.* Chourey and Singleton methods favour extraction of bacterial or fungal proteins with 1048 or 238 total identified proteins, respectively.	
Forest soils (0–10 cm) and commercial potting soil.	LC-MS/MS	**Functioning of the soil microbial community & comparison of 4 ≠ protein extraction protocols.** The amount of protein extracted from soil is scarce and largely influence by the extraction method and soil type; methods retrieved very few identical unique spectra (0.9% and 2.9% for potting soil and forest soil, respectively) and should be optimized for particular soil types and/or research questions.	[193]
Leaf litter from forests	1D-GE LC-MS/MS	**Functioning of the microbial community in leaf litter**. The season and litter nutrient content influence the community structure and function; yet Ascomycota dominated the litter decomposer community and cellulases are the most representative class of degradative enzymes. A total of 1724 unique protein clusters were identified.	[221]
Rhizospheric	2DE	**Functioning of rizhospheric microbial communities (monoculture).**	[218]

Sample	Methods	Research question and major findings	Work
soil **Monocultures (1-2 years)**		Proteomic data consistent with the existence of interaction between plants and microorganisms. The large majority of the identified proteins were derived from plants (75.73%), with only *ca.* 12% associated to either bacteria or fungi. Total of 103 protein spots were identified, categorized into 14 groups.	
Rhizospheric soil	2DE LC-MS/MS	**Functioning of rizhospheric microbial communities (crops).** Proteomic data consistent with the existence of interaction between plants and microorganisms. The large majority of the identified proteins (122 from 189 spots) were derived from plants (107), with 72 associated to either bacteria of fungi of which only 29 were associated to fungi (comprising functional classes of energy metabolism, protein metabolism, secondary metabolism, nucleotide metabolism and signal transduction).	[224]

[1]**Mineral soils formed in a permafrost environment; C, N and DOC stands for Carbon, nitrogen and Dissolved organic carbon.**

3.1.3. Toxicoproteomics on fungi, from single species to communities

Representative toxicoproteomics-based studies on the degradation of xenobiotics by filamentous fungi are depicted in Table I.2. Most studies focussed Ascomycota [199, 230-232] and Zygomycota [197, 233], covering xenobiotic degradation yields from 60 % (1 day) [199] to ~99 % (3 days) [197], regardless of major increase in stress responsive processes and proteins.

In Table I.3 selected metaproteomics studies on the influence/degradation of several xenobiotics in the functioning of microbial communities are shown, stressing their major observations. Taken as an example, the decay of 2,4-dichlorophenoxy acetic acid in soils and groundwater, was analysed using both 2DE and shot gun proteomics [234] (Table I.3). Data made apparent the involvement of bacterial enzymes belonging to the chlorobenzene degradation pathway via 3-chlorocatechol to 3-oxoadipate. Direct analysis of the soil microbiota led to discovery of a bacterial enoyl-CoA that possibly mediates the degradation of benzene in an anoxic aquifer [235]. Comparative shotgun proteomics was used instead to study the compost-assisted bioremediation of crude oils; efficient remediation was driven by *Sphingomonadales* and uncultured bacteria, with increased accumulation of catechol 2,3-dioxygenases, *cis*-dihydrodiol dehydrogenase and 2-hydroxymuconic semialdehyde dehydrogenase [236] (Table I.3).

Table I.2 - Overview of major functional categories of proteins identified in representative toxicoproteomics studies on different fungal systems grown axenically in media supplemented with a xenobiotic compound.

Xenobiotic	Fungus	Method	Major classes of protein species identified	Ref.
Tributyltin	*Cunninghamella echinulata (Z)*	LC-MS/MS	Carbohydrate & Energy metabolism (malate dehydrogenase, enolase and ATP synthase) Amino acid metabolism Cell wall remodelling (chitin deacetylase) Detoxification of ROS (peroxiredoxin, nuclease C1)	[233]
Alachlor	*Paecilomyces marquandii (A)*	2DE	Carbohydrate & Energy metabolism (malate dehydrogenase, enolase) Detoxification of ROS (SOD, catalase) Stress (HSP70) Xenobiotic transformation (nitrilase)	[232]
4-*n*-Nonylphenol	*Metarhizium robertsii (A)*	2DE	Carbohydrate & Energy metabolism (malate dehydrogenase, pyruvate dehydrogenase) Detoxification of ROS Species (peroxiredoxin, SOD)	[199]
Pentachlorophenol	*Mucor plumbeus (Z)*	2DE	Carbohydrate & Energy metabolism (enolase, glyceraldehyde 3P dehydrogenase) Cell wall remodelling (chitin deacetylase) Detoxification of ROS (cytochrome c peroxidase, thiamine biosynthesis) Stress (HSP70) Xenobiotic transformation (alcohol dehydrogenase)	[197]
Gossypol	*Aspergillus niger (A)*	2DE	Carbohydrate & Energy metabolism (malate dehydrogenase, citrate synthase) Detoxification of ROS (thiamine biosynthesis) Stress (HEX1)	[231]
Anthracene	*Fusarium solani (A)*	BN-PAGE	Xenobiotic transformation (Laccase)	[230]

A and *Z* stand for Ascomycota and Zygomycota, respectively; ROS stands for Reactive Oxygen Species.

Table I.3 - Selected metaproteomics studies on the influence of xenobiotics (in bold) in the functioning of the microbiota, in reverse chronological order. The studies cover culture-dependent methods, bed-reactors and microcosms.

Sample	Methods	Major findings	Work
Microcosms exposed to diesel **with or without compost**	SDS-PAGE	Diesel increases the abundance of proteobacterial proteins yet decreasing Rhizobiales proteins. Compost addition stimulated diesel degradation; the compost-assisted bioremediation was mainly driven by Sphingomonadales; the abundance of catechol 2,3-dioxygenases, *cis*-dihydrodiol dehydrogenase and 2-hydroxymuconic semialdehyde dehydrogenase increased. Several of the identified proteins (total of 2883) are involved in the biotransformation of byphenyls.	[236]
Bed reactor of a rhizospheric community during toluene **degradation**	LC-MS/MS	A total of 553 proteins were identified in day and night samples, of which 32 were differential. Burkholderiales proteins constituted 40% of the total, including catabolic enzymes involved in aerobic toluene degradation. The Rhizospheric community exhibited stable protein profiles during day and night; with a stable aerobic toluene turnover by Burkholderiales. PHA synthesis was upregulated in these bacteria during day, suggesting feeding on organic root exudates, while re-utilizing the stored carbon compounds during night via the glyoxylate cycle.	[237]
Bed reactor during toluene **biodegradation**	Protein-SIP ^{13}C-labbeled toluene	Burkholderiales proteins increased during toluene degradation; the microbiota apparently ensured the anaerobic toluene degradation *via* benzylsuccinate and benzoyl-CoA. Several proteins were involved in the metabolism of PHA, yet a correlation between toluene degradation and carbon storage could not be established.	[238]

Sample	Methods	Major findings	Work
Microcosms exposed to naphthalene **or** fluorene	Protein-SIP ^{13}C-labeled naphthalene or fluorene	The naphthalene degrading microbiota comprised essentially members of the orders Burkholderiales, Actinomycetales and Rhizobiales. The fluorene degrading community could not be disclosed. In total 847 proteins have been identified.	[239]
An enriched toluene **degrading community under submerged cultivation**	2DE LC-MS/MS	The proteins involved in anaerobic toluene activation, dissimilatory sulphate reduction, H_2 production/consumption and autotrophic C fixation were associated to Desulfobulbaceae. In total 202 proteins were identified (out of 236 protein spots) comprising the key enzymes for toluene degradation and sulfate reduction.	[240]
Microcosms contaminated or not with toluene	1-DE MALDI-TOF/TOF	No proteins known to be consistently involved in toluene degradation were identified. The identified glutamine synthetase, ABC transporters, extracellular solute-binding proteins, and outer membrane proteins, possibly play a role in the detoxification of toluene. In total 47 proteins were identified.	[241]
Groundwater and microcosms contaminated with	2DE 1DE, LC-MS/MS	The enzymes identified in groundwater reflected the metabolism of chlorobenzene that should represent a part of the functional metaproteome of this environment. The proteins extracted from the microcosms - autochthonous community established on 2,4-D – were similar to those of the community established after bio-stimulation (*i.e.* inoculation with a 2,4-D degrading bacterial community isolated from a contaminated aquifer). The optimised	[234]

Sample	Methods	Major findings	Work
2,4-D		protocol (allows the metaproteome analysis in soils and groundwater) led to identification of 29 proteins (out of 19 in 1DE bands) and 26 proteins (out of 50 in 2DE spots), including enzymes, such as chlorocatechol dioxygenases, likely participating in the degradation of the xenobiotic.	

2,4-D, 2,4-dichlorophenoxy acetic acid; PHA, polyhydroxyalkanoate; C, carbon

3.1.4. Lessons from a learning journey on toxicoproteomics

Deciphering how fungi mitigate PCP

The first study on the degradation of PCP by fungi was undertaken in *Phanerochaete chrysosporium.* PCP degradation yielded tetrachlorobenzoquinone (TeCBQ) and tetrachlorohydroquinone (TeCHQ) [41]. These intermediates were further dechlorinated through a reductive dehalogenase system involving a glutathione conjugate reductase that ultimately leads to full mineralisation [242]. Subsequent studies demonstrated the potential of numerous Ascomycota and Zygomycota to degrade PCP, *e.g. Trichoderma harzianum* [243], *Penicillium camemberti* [244], and several strains isolated by us either from cork slabs [165, 245] or from PCP contaminated forest soils [39]. TeCHQ was also formed during the degradation of PCP by non-lignolytic Zygomycota, namely *Mucor ramosissimusis* [246] and *M. plumbeus* (our study) [165], yet the degradation pathways differ as only in the first cytochrome activity was apparently involved.

The hypothesis that the prevalence of chloroanisoles in cork is linked to PCP pollution [142, 245] has inspired our opening studies on the PCP degradation capacity of fungal strains capable to completely perforate the cork cell walls [247]. The cork cell wall comprises an inner layer of suberin [248], hence its degradation suggests also a potential for the degradation of both aromatic and aliphatic polymers. Most strains found prevalent in cork were observed to efficiently degrade chlorophenols [142] , including PCP, namely *P. glandicola*, *P. janczewski*, *T. longibrachiatum*, *Chrysonilia sitophila* and *M. plumbeus* [249]. The PCP degradation pathway of *M. plumbeus*, which could deplete virtually all the PCP in medium, was disclosed using a metabolomics-based study [165]. The identified intermediates included tetra- and tri- CHQ and phase II-conjugated metabolites resulting from the conjugation of sulphate, glucose or ribose with PCP, TeCHQ and TCHQ; the sulphate glucose conjugates were reported for the first time in fungi.

These early observations inspired our first toxicoproteomics-based study (2DE): to disclose the molecular events associated with PCP exposure in *M. plumbeus* [197] (Table I.2). The identified differential polypeptides confirmed PCP capacity to uncouple oxidative-phosphorylation in mitochondria; PCP induced stress responses and led to alterations in cell wall architecture and cytoskeleton. However, the PCP degradation pathway in this fungus remained largely concealed, similar to that seen in subsequent 2DE studies on the degradation of alachlor [232] or 4-*n*-nonylphenol [199] by Ascomycota (Table I.2). In *M. plumbeus,* PCP exposure increased an alcohol dehydrogenase which may be involved in the last steps of the degradation of PCP [197]. Instead, alachlor increased a cyanide hydratase (nitrilase) in *Paecilomyces marquandii*, which is involved in the regular cyanoamino acid metabolism [232]. Nitrilase putative role in the direct metabolism of alachlor cannot be disregarded since functional redundancy is expected in the co-metabolic transformation of xenobiotics. 4-*n*-Nonylphenol metabolism in *Metarhizium robertsii* proceeds by consecutive oxidations of the alkyl chain with accumulation of 4-hydroxybenzaldehyde as major intermediate, followed by aromatic ring oxidation, presumably through the protocatechuate branch of the 3-oxoadipate pathway [199]. The differential proteome induced by 4-*n*-nonylphenol in this fungus, failed to disclose any enzyme directly involved in its metabolism (including those of the 3-oxoadipate pathway).

Recently, we have also used a 2DE differentially analysis to investigate how labdanolic acid - terpenoid found abundantly in *Cistus ladanifer* [250] - impacts *Penicillium jancjewskii* metabolism during its stereo-selective hydroxylation to 3β-hydroxy-labdanolic acid [251]. The plant terpenoid increased one putative P450 enzyme as well as stress responses, especially against oxidative stress (*e.g.* accumulation of superoxide dismutase) and apparently altered mitochondria functioning [252]. One P450 enzyme likely hydroxylates the terpenoid yet its unequivocal identification is yet to be attained. Disappointing results were also attained by us when using

a 2DE approach to disclose the degradation pathway of suberin in *Aspergillus nidulans* (cork media versus wood media), especially as suberin degradation was negligible compared to that of cork polysaccharides [253]. The successful identification of extracellular enzymes associated with suberin degradation (shot gun proteomics) required supplementation of the growth medium with suberin, though most of the pathway was revealed by transcriptomics [35]. The last study reinforces that one critical aspect for the success of any proteomic study is the experimental design.

Similar to fungi, numerous and diverse bacteria are also capable to degrade PCP, usually relying on successive reductive dechlorination reactions that yield non-chlorinated or chlorinated phenol derivatives that are often highly toxic and recalcitrant [171, 254, 255]. Using (toxico)metabolomics we further unravelled the uniqueness of the biochemical reactions used by filamentous fungi for the degradation of chlorophenols, specifically in *A. nidulans* [40]. Monochlorocatechols are recognised as key degradation intermediates of numerous chlorinated aromatic hydrocarbons, including monochlorophenols, yet their degradation in fungi was largely unknown. Two novel degradation paths were described in *A. nidulans*, namely for 4-chlorocatechol and 3-chlorocatechol, yielding 3-chlorodienelactone and catechol, respectively. Our results reinforced previous findings that enzymes mediating lactonisation of chloromuconates in fungi (*i.e.* 1,4-cycloisomerisation) differ from their bacterial counterparts (*i.e.* 3,6-cycloisomerisation). However, once more disappointing results were attained in a complementary 2DE analysis; the enzymes directly involved in the metabolism of the monochlorophenols could not be detected in the proteomic gels (unpublished data). To overpass these limitations, we decided to focus our toxicoproteomics-based studies on model aromatic compounds instead of chlorophenols, as detailed in the first section of this Chapter.

3.2. Conclusions

The comprehension of *in situ* microbial interactions and their response to environmental changes has been recognized as a major challenge for science [256]. Fungi own an impressive diverse array of mechanisms to tackle the stress imposed by xenobiotics, many of which are not yet properly characterized or remain unidentified. In this context, proteomic analyses may provide matchless means to discover new functional genes, proteins and metabolic pathways, which can be considered as functional bio-indicators for assessing the sustainability of ecosystems. We will further use proteomic-based tools (complemented by additional methods whenever necessary) for solving major knowledge omissions on the degradation of xenobiotics by filamentous fungi, including in the functioning of contaminated soils. The success of any experimental proteomic-based approach relies intrinsically in the experimental design (linked to the biological question under study), the protein extraction method and the downstream MS-based analysis. In every of the past studies (see examples listed in Tables I.1 to I.3), either in axenic fungal cultures or communities (enriched or naturally existing), several fungal proteins with unknown function were found to differentially accumulate upon exposure to a xenobiotic. The scientific community should periodically revisit and upgrade such old toxicoproteomics datasets to seek for new protein identifications, including some potentially involved in the degradation pathways of the targeted xenobiotics.

3.3. Acknowledgements

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Outline: The section **4** will briefly revise the evolution of molecular methods for the characterization of the composition of microbial communities. It will introduce concepts related with high throughput sequencing technologies, experimental design, data processing and analyses, as these were key tools in this thesis.

4. Next generation sequencing tools: a valuable asset to link functions to identities

Early cultivation independent methods: from coarse to fine tuning

The processes underlying the dynamics of microbial communities' functioning and composition remained locked inside the "black box" concept until recently [257]. For decades it was assumed that the identities of the major microbial drivers of ecological services would not be disclosed with accuracy in the near future [257]. In fact, several studies emerged as cutting edge for reaching identities of microbes at the family or even genus level, in a past not so far away [257, 258]. Among the most popular strategies used to study microbial communities' composition one must refer to guanine plus cytosine (G+C) analysis, which relied on the differences of G+C content that exist between prokaryotes (neglecting fungi), therefore achieving a coarse measure of resolution [257, 258]. The G+C content is independent from a polymerase chain reaction (PCR) result, eliminating the intrinsic bias associated with PCR (*e.g.* ineffective hybridization) [257, 259]. Other cultivation techniques were mostly PCR dependent, using universal, domain or specific primers for the amplification of rRNA [257]. Their main differences are on the methodologies used to separate the amplified fragments. Specifically, Degrading (and Thermal) Gradient Gel Electrophoresis (DGGE and TGGE) separated fragments based on their helix stabilities, in denaturing or thermal gradient gels [257, 260]. However they present a major constraint: lack of comparative sequence databases that could relate the melting temperatures (Tm) with taxonomic sequences [260, 261]. Similar limitations were presented by single strand conformation polymorphism (SSCP) analysis, which relied on the electrophoretic mobility differences of the amplification products, caused by changes in the conformational single-stranded products [257]. Due to the abovementioned limitations, terminal

restriction fragment length polymorphism (T-RFLP) and phospholipid fatty acid (PLFA) techniques were often used for microbial ecology studies in the late “pre-genomics” era [257], being still used nowadays. The first was manageable, though providing complex profiles, and the fact that it targeted (and measured) the terminal restriction fragment increased the chance that each visible band would represent a single ribotype [257]. Opposedly, in its parent technology (RFLP) each species could contribute to 4 or 5 distinct ribotypes at once. Though far from the currently used technologies, T-RFLP was able to provide quantitative data with a certain level of sensitivity. On the other hand PLFA analysis was mostly used to characterise shifts occurring in community structures upon exposure to some kind of stress [257, 262]. It relied on specific phospholipid markers for distinct taxonomic groups (*e.g.* the fungal marker 18:2x6,9c), but despite being sensitive for shifts in communities’ structures, it lacked taxonomic resolution to assign low level taxonomic identities (*e.g.* genus or species) [262]. Nevertheless PLFA studies include the majority of the few research works based on cultivation independent methodologies that consistently considered the key role of fungal communities in microbial ecology [262]. Interestingly, one can easily observe that, in the past, fungi were often overlooked by authors who referred solely to bacteria when mentioning “microbial communities”. This was probably due to the high difficulty in obtaining fungal DNA from environmental samples (particularly soil) with both quantity and purity enough to be analysed with the established methodologies [31]. Focusing on fungi, early cultivation independent molecular studies on their taxonomic identification revealed a diversity of nucleic acids that suggested the existence of uncultivable species which greatly differed from those that have been described so far [31]. The probable existence of unknown structures responsible for (also) unknown functions ultimately defied the established assumptions about microbial communities’ structure and ecology [263]. Moreover, studies with fungal isolates often reported their impressive metabolic and enzymatic capabilities (*vide supra*), leading mycologists to

wonder about the hidden potential of fungi [28, 263]. Therefore, when DNA sequencing evolved from single sequence analyses to parallel Sanger sequencing in the early 2000s [31], it became obvious that microbiologists would soon count with high throughput sequencing (HTS) tools capable to help disclosing the composition of the yet unseen majority that populates most ecosystems on Earth [264].

Second generation HTS technologies

As fast as parallel Sanger sequencing appeared, constituting a transient first generation of HTS technology, did pyrosequencing upsurge as the standard second generation HTS able to provide robust “high throughput” sequencing results [31]. The 454 pyrosequencing machine produced by Roche dominated the early boom of the “genomics era”. It produced reads up to 1000 bases, but the high costs associated with its operation, together with sequencing errors caused by insertions and/or deletions in homopolymer-rich DNA regions led it to be discontinued in 2016 [31, 265]. Similar problems associated with homopolymer-rich regions affected Ion Torrent, which was never a first choice for microbial ecologists worldwide [31]. On the other hand, Illumina technology quickly became the gold standard in terms of HTS for metataxonomics analyses. This technology produces high quality sequences - even if shorter in size comparatively to its direct competitors - which ultimately retrieve higher confidence in terms of taxonomic identification [31, 265, 266]. This is mostly due to the possibility of performing paired-end sequencing and the massive throughput it is capable to output (up to 20 gigabases in its less powerful machine, Illumina MiSeq). The length of the reads (up to 550 bases in MiSeq2) is just enough to sequence the full ITS2 sub-region, turning it the “go to” technology in terms of fungal metabarcoding [31, 266].

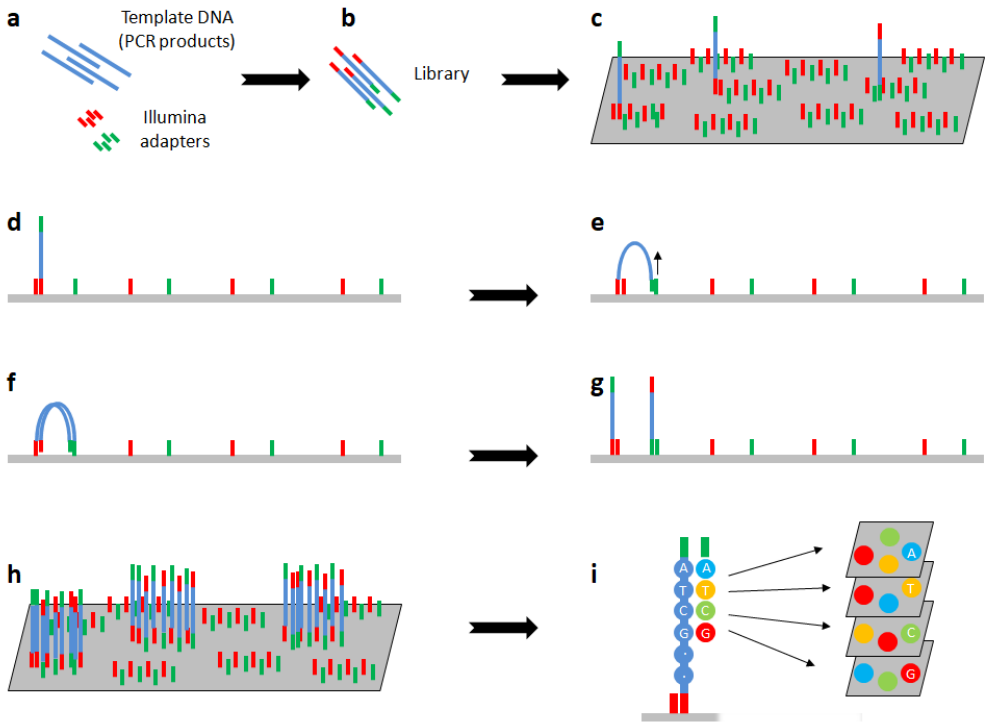

Figure I.10 - Schematic representation of the Illumina technology ranging from **a**) and **b**) the library construction, **c**) and **d**) the attachment to the flow cell, **e**) and **f**) the formation of bridge-like structures for paired-end sequencing, **g**) the detachment of one end, **h**) the formation of amplicon clusters and finally **i**) the luminous sequencing. This figure follows the scientific basis of the Illumina sequencing as published by the company, yet it has been designed specifically for this thesis by the author.

Specifically, amplicon sequencing using Illumina technology generally follows this workflow: upon extraction and purification of the DNA from the samples, a preliminary PCR is performed to amplify the region of interest (usually 16S for bacteria, ITS1 or ITS2, for eukaryotes). The amplicons are then purified and cut with transposases (*tag*mentation) at the same time that Illumina adapters are attached at the ends - used for subsequent amplification at the flow-cell. This step is called library construction (Figure I.10a and b). The library is then injected in the machine (*e.g.* Illumina MiSeq) where the inserted adapters will bind to complementary strands that are attached to the surface of the flow-cell (Figure I.10c and d). In the case of

paired-end sequencing, the amplicons form a bridge-like structure (Figure I.10e) and the forward and reverse strands are amplified in both directions, a step after which the fragments detach from one of the ends (Figure I.10g). This amplification is repeated *N* times (usually tens of thousands - called clonal amplification) and clusters of amplicons are formed at the surface of the flow cell (Figure I.10h). Then the clusters of amplicons are sequenced by synthesis, a method in which free fluorescently tagged nucleotides bind to its complementary nucleotide at the amplicon, emitting a coloured luminous signal that is captured by a sensor - each colour corresponding to one of the four nucleotides (Figure I.10i) [267]. These results are then processed by the machine, and a quality score is attributed to each nucleotide in the output file (FASTQ) taking into account the robustness of the sequencing. As each fragment of the initial sample is amplified and read thousands of times, this technology renders high quality, high-throughput data with very low error (0.1%, claimed by the company) [31, 267]. Nevertheless, a careful experimental and/or sampling design is mandatory to achieve reproducible results. Specifically it is important to guarantee that the design comprises both quantity and replicas enough to allow robust statistical analyses.

Experimental/sampling design

Regardless of the analytical power of Illumina technology - currently the gold standard in HTS, and one of the core techniques of the Chapter III of this thesis - microbial communities' compositions are highly variable even at nearby environmental locations [31]. Therefore, aiming at covering the microbial diversity of a certain location, an appropriate sampling strategy is required, especially when considering soils. In fact, the strategies commonly used by geochemists to study the physical and chemical features of soils are quite robust, and can be used for metataxonomics studies [2, 39, 268]. Such sampling methodologies have been used to collect the soil samples that were further analysed in this thesis, as detailed in the section 2 of the current Chapter, and in the Chapter II of this thesis.

In more detail, the common methodologies used for soil sampling usually comprise multiple sites of a given area of study (at least 3), in which each site is cross sampled (1 square meter). The ends and middle of the cross are collected (5 sub-samples) and pooled on site [2, 19, 39]. From a metataxonomics point of view this allows obtaining a comprehensive overview of the composition of microbial communities at a given area of study, and also allows discriminating between the sampling sites of that area. As different sites can have distinct geochemical features, so can the composition of the microbial communities be correlated with specific variables. Moreover, the DNA extractions must be performed in soil triplicates and three independent PCRs should be performed for each DNA sample, constituting technical replicates that are then pooled prior to sequencing. This strategy is widely recommended for fungal metataxonomics studies in which Illumina amplicon sequencing is used, since the produced results are quite robust, being reproducible in independent experiments (detailed in Chapter III) [19].

One important detail when considering amplicon sequencing is the choice of primers. It is crucial to take into account the size of the fragment to be amplified, the specificity and the coverage intended, as these features may influence the downstream processing and results in terms of taxonomic diversity. For fungal metataxonomics the ITS1 and ITS2 sub-regions are usually the target [31, 269]. The last currently appears as the most advantageous one as it includes lower variation in terms of length and more universal primer binding sites, which renders less taxonomic bias than ITS1. Specifically the gITS7/ITS4 pair of primers (targeting ITS2 subregion) has been recommended by most experts in fungal amplicon sequencing [31, 269].

Data analyses

Along with the impressive amount of datasets that result from environmental samplings and also from laboratory metacommunity experiments, rises a

major challenge regarding data processing and analysis [31, 270]. There have been several pipelines developed to assist users - who are not experts in bioinformatics - in the initial processing of the data. The most popular example is QIIME - a powerful pipeline that is mostly used to analyse bacterial 16S sequencing data - but SEED2 appears as a very complete (equally powerful) pipeline with an easy-to-operate graphical user interface, suitable for all kinds of amplicon sequencing analyses [31, 270]. Its main body is constituted by a versatile sequence editor that allows performing most simple operations (*e.g.* sequence editing, naming, cutting, generate reverse complementary, among others) while simultaneously integrating state-of-the-art bioinformatics tools [270]. Taking a quick tour through the data processing (FASTQ files) of amplicons of the ITS2 sub-region using SEED2, usually the first step is the joining of paired-end files, taking advantage of the Fastqjoin software [271] embedded in the pipeline. The joining rate is usually the first indicator of the quality of the data, as low joining percentages (below 80%) usually indicate problems in the sequencing step. If problems are detected in this step, one should submit the FASTQ files to quality check software (e.g. FastQC), obtain a full report of the quality checkpoints and contact the sequencing facility prior to advance with the data processing. After this preliminary quality check, the SEED2 pipeline recommends a quality trimming to discard sequences with average quality score below 30. Sequences that are too short (below 100 bp, for ITS2) or too long (longer than the limit size of the sequencing method), and that therefore may result from technical artefacts are also discarded [270]. After this initial trimming, a search for the primers used for ITS2 amplification is performed for both ends, considering the forward and the reverse complementary strands. This step acts as an additional trimming stage (as the sequences that do not contain the primers at the ends are discarded) and as means to discriminate between samples - in the event that tagged primers were used for multiplexing. Then, taking advantage of the ITSx tool [272, 273], the sequences recognized as ITS2 by the software are

fully extracted for subsequent clustering using UPARSE [274]. The last step also identifies and discards chimeric sequences and singletons, an additional quality checkpoint. At this stage it is possible to obtain a contingency table of number of reads per cluster. When working with multiple locations or experimental conditions it is also possible to generate a 2D contingency table to obtain the number of reads per cluster (at this stage called Operational Taxonomic Unit - OTU) at each sample/condition. After clustering one can opt by one of two strategies: either one finds the most abundant sequence of each cluster, or MAFFT [275] is used to generate consensus sequences, prior moving to the BLAST search. The first is faster and the results have proven to be reliable, the second is more robust as it allows more precise identifications, but it is quite time consuming and demanding from a computational standpoint. Therefore, when processing a very high number of sequences (e.g. above 500,000) the selection of the most abundant sequence for each OTU is more efficient. Afterwards, it is possible to move to a BLAST analysis in which SEED2 integrates the usage of remote or local databases. For fungi it is extremely efficient to use the local database UNITE (downloaded from https://unite.ut.ee/), which is constantly being updated. UNITE provides a fast search as it does not depend of network connection, being also dedicated to fungi which avoids useless matches which are time consuming.

Upon identification, the dataset available (xls format) is usually composed by hundreds of rows (number of OTUs) and quite a variable number of columns, as it depends on the number of samples/conditions. Nevertheless, the identification of the OTUs, the accession numbers, the best hit of the search, the E-value (significance level in a BLAST search), the similarity, the coverage, the query length and number of reads (per sample/condition), and the taxonomic classification at all taxa levels (from domain to species, occasionally sub-species) are always present. At this stage the processing phase has ended and the data analysis begins.

The most important recommendation prior to any further analysis is to perform a normalization followed by subsampling (by the number of reads in sequencing) of the data, to standardize the relative weights of each sample and of each OTU [31, 270]. Then the data is analysed using standard biostatistical tools aiming at describing properly the composition of the community at each sample/condition, always based on their relative abundances. The most widely used software for these analyses are XL-STAT, SPSS, R and PRIMER-E, the last more focussed on ecological description and testing. Independently of the used analyses it is important to keep in mind the nature of the data and its origin. To describe the composition of the community one can organize the data by taxonomic ranks and observe the differences (or similarities) between samples/conditions; perform multivariate analyses, such as hierarchical clustering (HCA), principal component (or coordinate) analyses (PCA or PCoA) or non-metric multidimensional scaling (NMDS) [276, 277]. As the data under analysis are quantitative but still taxonomic, Bray-Curtis should be used as the algorithm to generate distance matrices. Additionally one can also perform ecology oriented analyses such as permutational analysis of variances (PERMANOVA), to test the influence of factors (e.g. humidity, pH, carbon source, presence/concentration of toxicants) in the composition of each sample/condition [276, 278].

Interestingly, in cases of laboratorial experiments, some tools designed for analysing genetic differential expression can be also considered as the type of data are mathematically similar, perfectly fitting generalized linear models (GLM): specifically when the available data are composed by counts (set to whole numbers after normalisation and subsampling) of individual fragments (OTUs) at given test conditions compared to controls. Specifically, tools such as the Bioconductor based packages (R environment) *edgeR* or *DESeq2* - both based on GLM - can be used depending on the number of available replicas for each analysis [19], and help finding significant differences between conditions.

Limitations and biases

Regardless of its high power, HTS technologies are not devoid of some limitations and possible bias, the first being the DNA/RNA extraction methods. There is not one extraction protocol that can be considered flawless in terms of covering all microbial organisms [31, 279]. The composition of the cell walls greatly differs among fungi and bacteria, as do the complex matrices from where their nucleic acids are isolated [279]. Additionally, there are no perfect molecular markers, as discussed previously. The PCR setup is also a source of possible bias such as: variations in primers melting point, specificity and binding positions; types of polymerases and their required concentrations of $MgCl_2$ to achieve the perfect balance between efficiency and specificity of the reaction [31]. Regardless of the last, there is an intrinsic bias associated with PCR amplification for fungi metabarcoding, due the high variability in size of the ITS sub-regions, and the bias of most primers in favour of Dikarya fungi [31, 269]. Both, library preparation and sequencing steps, can also be sources of some bias, particularly caused by dissimilar A+T:G+C ratios, which should be between 30% and 70%, because amplicons with small size and regular A+T:G+C ratios are preferentially sequenced by the machines [31, 267]. During data processing, the loss of some rare fungal sequences during the extraction of ITS regions using ITSx can also happen [272, 273]. Some care should be taken in the detection of chimeric sequences (usually at low abundance) that may be formed either during PCR or library preparation and sequencing [31, 270]. The last may influence the clustering step, causing the formation of satellite OTUs - overestimating their number - which can be counteracted by the removal of rare OTUs (singletons) and by adopting dynamic similarity thresholds during the clustering step [31, 270].

Future perspectives in HTS

The upsurge of third-generation HTS platforms opens the door for even more robust and sensitive sequencing as a result of the higher quality and longer reads they produce. Particularly PacBio and Sequel technologies (Pacific Biosciences) are characterised by their long and high quality reads [280]. The first generates reads up to 30 kb with quality similar to that of classic Sanger sequencing; the second produces high quality sequences up to 3 kb in length [281], which turns it suitable to sequence medium length amplicons (*e.g.* the full ITS region) even if its output generates much less reads - *c.a.* 3.2 million read per run - than Illumina. The increasingly popular Oxford Nanopore MinION is, perhaps, the epitome of innovation in terms of HTS platforms. This machine is able to reach sequence lengths of 2.4 mb [31, 282], turning it suitable to sequence entire genomic scaffolds. It is an incredibly inexpensive (*c.a.* 1000€ each device) and flexible technology, ranging from high-throughput installations to portable devices that can be plugged directly to a laptop allowing real-time data analysis of a run. However it displays a quite high error rate (6% to 12%) [31, 282], constituting the major drawback for its definitive implantation as the current landmark technology.

The latest developments in terms of sequencing quality, but especially in terms of sequence lengths, anticipate exciting times for this field of research, but also intricate challenges in terms of data processing and analysis that must be overcome to extract the full potential of third generation HTS in the near future.

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CHAPTER II

Chapter II consists of the following published manuscript:

Celso Martins[#], Adélia Varela[#], Oscar Núñez, Isabel Martins, Jos A.M.P. Houbraken, Tiago M. Martins, M. Cristina Leitão, Iain McLellan, Walter Vetter, M. Teresa Galceran, Robert A. Samson, Andrew Hursthouse, and Cristina Silva Pereira. Environmental Microbiology, 2015, Volume 17, 2922–2934. (doi/10.1111/1462-2920.12837) [#]equally contributing authors

Outline: The following chapter presents a published work in its original form. However it is important to notice that, though PCP was not regarded as a persistent organic pollutant (POP) at the time the work was published, presently it is in the list of POPs, as mentioned in Chapter I. Nevertheless, this fact was fundamental for the scientific discussion of this paper and the global awareness that our work intended to raise at the time.

Understanding fungal functional biodiversity during the mitigation of environmentally dispersed pentachlorophenol in cork oak forest soils

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Abstract

Pentachlorophenol (PCP) is globally dispersed and contamination of soil with this biocide adversely affects its functional biodiversity, particularly of fungi – key colonizers. Their functional role as a community is poorly understood, although a few pathways have been already elucidated in pure cultures. This constitutes here our main challenge – elucidate how fungi influence the pollutant mitigation processes in forest soils. Circumstantial

evidence exists that cork oak forests in N. W. Tunisia – economically critical managed forests are likely to be contaminated with PCP, but the scientific evidence has previously been lacking. Our data illustrate significant forest contamination through the detection of undefined active sources of PCP. By solving the taxonomic diversity and the PCP-derived metabolomes of both the cultivable fungi and the fungal community, we demonstrate here that most strains (predominantly penicillia) participate in the pollutant biotic degradation. They form an array of degradation intermediates and by-products, including several hydroquinone, resorcinol and catechol derivatives, either chlorinated or not. The degradation pathway of the fungal community includes uncharacterized derivatives, *e.g.* tetrachloroguaiacol isomers. Our study highlights fungi key role in the mineralization and short lifetime of PCP in forest soils and provide novel tools to monitor its degradation in other fungi dominated food webs.

Introduction

Pentachlorophenol (PCP) is recognized as a critical pollutant worldwide, albeit not formally classified as a persistent organic pollutant at the date of publication of the research paper that served as basis for this chapter [1, 2]. Initially used as wood preservative in the 1930s [3], its application spread to numerous agricultural, industrial and domestic scenarios [4], and only in the 1980s it became severely restricted because of its high toxicity (*e.g.* probable endocrine disruptor and carcinogen in humans [5, 6]). By the end of 2008, PCP usage in Europe had virtually ceased [2], but is still increasing in some countries, *e.g.* in China [7]. PCP is also produced as a side product during the degradation of volatile herbicides and pesticides, *e.g.* pentachlorobenzene (PeCB, [8, 9]). The long history of use of PCP, together with its persistence (can be transported globally, via long-range atmospheric and oceanic transport [10]), has resulted in extensive environmental contamination worldwide [11]. Today, PCP is globally

detected in human fluids and tissues from exposure in both indoor and outdoor environments [12, 13].

Putative degradation products of PCP have been identified in Mediterranean oak forests/woodlands, particularly in the outer bark of *Quercus suber* L. (cork oak) [14, 15]. Its incidence and impact in such habitats has never been systematically examined despite the high ecological relevance of these landscapes [16]. They span many geographical and cultural boundaries, and their productivity (presently a vital source of income for thousands of people) is very sensitive to their management [14, 17].

PCP has the potential to adversely affect the functional biodiversity in both terrestrial and aquatic niches [3, 18], with estimated partitioning levels in soil of nearly 95% [19]. Fungi constitute up to 75% of the soil microbial biomass and play a key role in preserving the soil functioning and its ecological balance [20]. They are able to develop strategies to overcome numerous anthropogenic threats due primarily to their broad enzymatic capacities [21]. Numerous studies have demonstrated an increasing abundance and diversity of fungi in chronically stressed and polluted soils [22, 23]. Consequently, PCP degradation by fungi has been widely studied, but only a limited number of degradation pathways have been completely, or even partially, elucidated by analysing metabolites and proteins in pure cultures [21, 24-27]. Data available on bacterial communities indicate broad capacity to undertake successive reductive dechlorination reactions yielding non-chlorinated or chlorinated phenol derivatives, usually highly toxic and recalcitrant [25, 27, 28]. The pollution impact of PCP in the functioning of the soil fungal community, especially for diluted but chronic exposure, is poorly investigated, notwithstanding some elegant reports [4].

Here, we aim to address this knowledge gap by focussing on cork oak forest soils from the Jendouba region (N. W. Tunisia), which may be associated with widespread organochlorine contamination [15, 29]. The impact of PCP in the functional diversity of the soil's fungal community was investigated by analysing its PCP-derived metabolome and its physiological

profile. The role played by the fungal community in the biotic degradation of PCP will be thoroughly analysed, particularly by identifying the PCP degradation intermediates and by-products formed. We will discuss the putative sources of PCP contamination and the role of fungi in the pollutant mitigation processes, fighting the increasing threat from atmospherically derived pollutants.

Experimental procedures

Chemicals

If not explicitly stated otherwise, chemicals were of analytical grade and purchased from Sigma Aldrich. trans-Acetylacrylate (Alfa Aesar), MEA (HiMedia), DG18 agar (Oxoid) and triton X-100 (GE Healthcare) were also used. All LC and MS solvents, as well as those required in the fast-solvent extractions, were of the highest analytical grade. Chlorinated derivatives of resorcinol, hydroquinone and catechol were produced through an aqueous chlorination methodology [30] and 2,3,5,6-tetrachloro-4-methoxyphenol (Dro A) was synthesized as described before [31].

Collection and physicochemical characterization of soil samples

Soil samples were collected in three Tunisian demarked cork oak forests, namely AH, FER and RR in February 2009, as previously described [30]. In brief, three locations were chosen within each forest, and a composite sample was collected from five subsamples (0–20 cm), sieved to < 2 mm in the field and immediately conserved (dark, 4° C) until analysis. Total organic carbon content, total nitrogen content, pH, humidity and particle size analysis were performed using standard methodologies [32].

To evaluate the diversity of chlorinated compounds, as well as putative subproducts, in the soil samples, a fast-solvent extraction method was applied leading to PCP recovery of > 70 % from a certified reference material containing 2.04 ± 0.18 mg of PCP Kg^{-1} (ERM-CC008, LGC-Promochem, Spain) [29].

Composition of the cultivable fungal communities

Fungi isolation and taxonomic identification were done as previously described [32]. In brief, aliquots of peptone extracts of each sample (1:10 soil:peptone water, 0.1 % w/v, incubated for 1 h, 25 °C, 100 rpm) were spread onto solid media, namely MEA and DG18, both supplemented with 0.1 % (v/v) of chloramphenicol for inhibiting bacterial growth. The number of cfu's was monitored daily (27 °C, dark), in general, defined after 6/7 days of incubation since no new colonies could be detected afterwards. Each soil sample was analysed in triplicate.

Fungal colonies were isolated by transfer to fresh standard media, and isolates were then cultivated for 8 days on MEA and their preliminary taxonomic evaluation was done based on the colony morphology, either by macroscopic and/or microscopic analysis. DNA extraction was performed using the Ultra Clean Microbial DNA Isolation Kit (MoBio Laboratories). For the *Penicillium* isolates, a part of the β-tubulin (primers Bt2a and Bt2b) gene was amplified and sequenced and for the *Aspergillus* strains a part of the calmodulin (primers cmd5 and cmd6) gene was targeted. The Zygomycetes and *Cladosporium* strains were identified based on LSU (primers LR0R and LR5) and actin (primers Act-512F and Act-783R) sequences respectively. All other strains were characterized by ITS sequencing (primers V9G and LS266). Details on the PCR conditions, primers sequences and sequence assembly were as previously described at CBS-KNAW [33-36]. Sequence similarity searches were performed in public databases of GenBank (http://www.ncbi.nlm.nih.gov/) with blast (version 2.2.6) and in internal databases at the CBS-KNAW Fungal Biodiversity Centre (The Netherlands). Newly generated sequences were deposited in GenBank under accession numbers KM088815, KM088816, KM088817, KM088819, KM088820, KC695684, KC695685 and KC695686.

Biotic PCP degradation assays

The ability of each fungal strain to degrade PCP was tested using liquid cultures (3.5 mL). Cultures, initiated from spores collected from slants (MEA, 27 °C, dark, 7 days), were grown in a mineral minimal media [26, 37] containing 1 % w/v of glucose and either 19, 28, 38 or 56 µM of PCP (added after media sterilization from a 28.2 mM stock in ethanol) under controlled conditions (27 °C, in dark, 90 rpm.). After 14 days of incubation, mycelia were removed by centrifugation (3 min, 3000*g*), and the acidified supernatants (to pH 1-2 with phosphoric acid) were extracted with chloroform (1:1 v/v, twice). The extracts were air-dried, homogenized in 1 mL of methanol and conserved at −20 °C until further analysis. All assays were executed in triplicates, including controls.

To test the ability of colonizing fungal communities to degrade PCP, each AH_n soil sample was extracted using peptone water (see above) containing chloramphenicol (0.1 % v/v). Aliquots of these extracts (triplicates, including controls) were used to inoculate growth media containing PCP, which were incubated and processed as previously described. CLPP experiments were performed using SF-N2 Biolog plates (Biolog). Soil samples were homogenized in a peptone plus chloramphenicol solution, containing 0, 19 or 38 µM of PCP and incubated for 14 days (27 °C, in dark). After incubation, culture supernatants were used to inoculate the CLPP plates accordingly to the manufacturer instructions. Functional diversity (Shannon index, *H'*) and richness were calculated as previously described [38].

Analysis of PCP-derived metabolome

PCP concentration in the methanolic extracts was quantified using ultra-performance LC as previously described [26]. Chromatographic profiles were acquired at 212 nm, and PCP quantification limits were 0.38–56 µM [retention time (t_R) = 5.9 min]. The diversity of PCP-derived metabolites and subproducts was resolved using UHPLC-ESI-HRMS operated in negative

ESI mode using a Q-Exactive Orbitrap MS system (Thermo-Fisher Scientific) as previously described [37, 39]. MS data were processed by ExactFinder 2.0 software (Thermo-Fisher Scientific) by applying a user target database list and validated, whenever possible, using standard compounds.

Statistical analysis

The similarity/dissimilarity (*i.e.* Pearson correlation) of the observable quantitative variables measured in each soil sample was transformed into a biplot containing PCA and multidimensional scaling (Figure II.S1). Preliminary evaluation of the variance of the data used Bartlett's and Levene's tests. Pair-wise *t* - tests and Kruskal–Wallis comparisons were used to identify significant differences between the strains PCP degrading capacity, either in axenic or community cultivation, at each PCP concentration tested. All the analyses were performed using the XL-STAT software version 2009.1.02 (Addinsoft).

Results

Aîn Hamraia soils show strong association with PCP

The Jendouba oak forests (N. W. Tunisia) cover more than 491 km^2 (Ben Jamaa et al., 2006), including those in the Tabarka district, such as Aîn Hamraia (AH), Fej Errih (FER) and Ras Rajel (RR). Soil samples, collected at these locations, were preliminary categorized through analysis of physicochemical parameters (*e.g.* pH, humidity and carbon/nitrogen ratio), PCP contamination loads and number of fungal colony-forming units (cfu's) (Table SIII.1). The numbers of fungal cfu's were, in general, comparable in all soils, regardless of differences in their PCP levels (Table SIII.1). Through factor analysis [principal component analysis (PCA) of the quantitative variables], $AH_{1–3}$ soil samples were found to cluster, with PCP concentration exerting the most significant influence (Figure II.S1). The PCP levels in AH soils (13.2–28.8 $\mu g \cdot Kg^{-1}$) were higher than those detected either in

FER (4.4–14.8 μg·Kg^{-1}) or RR soils (1.7– 7.0 μg·Kg^{-1}), hence AH soils were selected for further study.

AH fungal community comprises 77 cultivable strains

We first characterized the cultivable fungal community at each AH sampling spot. In total, 77 isolates (full details in Table SIII.2) were generated, covering 33 species or species groups, with *Penicillium* species (52 strains) predominating the composition (Table SIII.3). Only six strains remain to be fully characterized (*e.g.* isolate DTO 099-G8).

Most fungal strains likely participate in PCP mitigation in soil

Our opening hypothesis is that the PCP-derived metabolome of the fungal community comprises compounds formed in the axenic cultures of its component strains. With this in mind, we have undertaken a functional analysis of the individual strains. Specifically, we first analysed their capacity to remove 19, 28, 38 or 56 μM of PCP (Table III.1). The PCP decay in the abiotic controls on the fourteenth day of incubation was *ca.* 9.5 %. Only 24 out of 77 strains failed to significantly remove PCP with decay levels similar to those found in the abiotic controls. Moreover, the majority of those was unable to germinate from spores at the lowest PCP concentration tested (Table SIII.2). Out of the 77 strains, 53 could remove PCP at the lowest concentration, whereas 21 could remove PCP at the highest concentration tested (Table III.1). Qualitative screening with Remazol Brilliant Blue R and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) [40] verified that PCP degradation and oxidoreductase activity (Table III.1) were not correlated (Pearson correlation test p-value < 0.05). Each sampling spot showed a unique soil biodiversity of cultivable fungal strains (Table III.1 and Table SIII.3). This raised the question if such differences can translate into distinct capacities to mitigate PCP contamination.

Table II.1 - Degradation yields for PCP of each degrading fungal strain at four starting concentrations

Strain		PCP degradation yield (%)				Oxidoreductase activity	
		Starting concentration (µM)					
		19	28	38	56	ABTS	RBBR
AH_1							
DTO 099-B4	*Aspergillus* sp. (sect. *Cremei*)	80.9	74.7	77.2	86.6	–	–
DTO 098-I4	*Aspergillus welwitschiae*	78.9	73.0	73.4	49.2	✓	
DTO 099-C5	*Cladosporium sphaerospermum*	89.9	85.9	–	–	✓	✓
DTO 098-I6	*Fusarium oxysporum* species complex	54.2	52.2	45.7	40.1	✓	–
DTO 099-A2	*Fusarium solani* species complex	55.0	51.2	–	–	✓	–
DTO 098-I9	*Penicillium brevicompactum*	78.1	47.3	–	–	–	–
DTO 099-C2	*Penicillium brevicompactum*	92.9	79.5	74.4	–	–	✓
DTO 099-A3	*Penicillium daleae*	71.1	65.3	–	–	✓	✓
DTO 099-B9	*Penicillium glabrum*	100	100	97.6	57.7	–	✓
DTO 099-A6	*Penicillium glabrum*	89.7	88.6	71.6	69.0	–	✓
DTO 099-A8	*Penicillium griseofulvum*	70.0	57.1	35.9	–	✓	✓
DTO 100-A4	*Penicillium janczewskii*	94.0	91.9	83.4	33.8	–	✓
DTO 099-C6	*Penicillium longicatenatum*	81.0	71.9	60.3	–	–	–
DTO 100-A6	*Penicillium radiatolobatum*	88.8	86.3	85.4	32.6	✓	✓
DTO 099-C4	*Penicillium radiatolobatum*	95.1	92.3	90.5	85.1	–	–
DTO 099-A5	*Penicillium restrictum* species complex	69.4	36.8	–	–	–	–
DTO 099-C3	*Penicillium restrictum* species complex	59.9	53.2	56.7	60.7	–	✓
DTO 099-B7	*Penicillium sizovae*	80.1	76.8	67.9	67.1	–	✓
DTO 099-B6	*Penicillium sumatrense*	80.4	75.6	–	–	–	–
DTO 099-C7	*Penicillium sumatrense*	51.2	56.5	8.9	–	–	✓
DTO 098-I7	*Penicillium vagum*	87.6	75.8	65.1	40.2	✓	✓
DTO 099-A7	*Penicillium vagum*	83.6	78.7	76.9	62.6	✓	✓
DTO 099-B1	*Phoma putaminum*	33.1	–	–	–	✓	✓
AH_2							
DTO 099-D1	*Penicillium brevicompactum*	86.3	80.0	57.3	–	–	–
DTO 099-D5	*Penicillium janczewskii*	84.4	57.3	53.3	44.6	–	✓
DTO 099-D2	*Penicillium murcianum*	98.7	96.5	69.1	53.6	–	✓
DTO 099-E1	*Penicillium murcianum*	82.6	47.0	37.3	–	–	✓
DTO 099-C8	*Penicillium radiatolobatum*	88.4	74.4	–	–	–	–
DTO 099-E8	*Penicillium radiatolobatum*	79.4	–	–	–	–	✓
DTO 099-D7	*Penicillium restrictum* species complex	58.5	51.5	–	–	–	–
DTO 099-D9	*Penicillium restrictum* species complex	66.7	38.1	–	–	–	–
DTO 099-E4	*Penicillium restrictum* species complex	62.4	67.0	85.2	45.2	–	–
DTO 099-D4	*Penicillium sanguifluum*	93.8	67.5	55.3	–	–	–
DTO 099-D6	*Penicillium vagum*	77.5	57.8	44.8	–	–	–
DTO 099-F1	*Penicillium vagum*	76.7	–	–	–	–	–
DTO 099-E2	*Penicillium yezoense*	88.1	81.6	42.7	–	–	–
AH_3							
DTO 099-F9	*Absidia* pseudocylindrospora	72.8	–	–	–	–	✓
DTO 099-G4	*Aspergillus novoparasiticus*	65.1	61.0	41.2	35.3	–	✓
DTO 099-H5	*Cladosporium phaenocomae*	63.5	44.1	8.3		✓	✓
DTO 099-G2	*Cladosporium ramotenellum*	48.8	–	–	–	✓	✓
DTO 099-G3	*Fusarium oxysporum* species complex	72.9	66.6	61.0	57.0	✓	–
DTO 099-F8	*Penicillium murcianum*	90.9	85.8	65.2	57.8	–	✓
DTO 099-H7	*Penicillium murcianum*	81.2	70.9	–	–	–	✓
DTO 099-G5	*Penicillium radiatolobatum*	82.9	81.4	81.4	76.5	–	✓
DTO 099-F6	*Penicillium restrictum species complex*	52.8	–	–	–	–	–
DTO 099-H1	*Penicillium shearii*	64.6	50.3	44.4	26.1	✓	✓
DTO 099-G9	*Penicillium shearii*	55.0	17.8	–	–	–	✓
DTO 099-G8	*Penicillium* sp. (sect. *Lanata-divaricata*)	84.8	82.0	74.4	–	–	✓
DTO 099-G7	*Penicillium vagum*	43.4	29.0	13.4	–	–	–
DTO 099-F7	*Penicillium vagum*	96.0	93.0	81.7	–	✓	✓
DTO 099-G1	*Penicillium vanoranjei*	89.0	76.9	1.6	–	✓	–
DTO 099-H6	*Penicillium vanoranjei*	84.4	76.9	75.8	64.1	✓	–
DTO 099-F3	*Penicillium vanoranjei*	89.6	73.8	–	–	✓	✓

Strains are ordered alphabetically within each Aîn Hamraia (AH) collection site (AH_1, AH_2 and AH_3) and DTO number (internal collection of the research group Applied and Industrial Mycology housed at CBS). Strains oxidoreductase activity (qualitative) is also shown. The strains unable to germinate in the presence of PCP are listed and highlighted in Table SII.2.

ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); RBBR, Remazol Brilliant Blue R.

To address this question, we analysed the variance of the PCP-degrading capacity of the strains found at each sampling spot. The number of PCP-degrading strains was nearly the double in AH_1 when compared with either AH_2 or AH_3 (Figure II.1a). Despite this remarkable difference, their average capacities to remove PCP were comparable, with a single exception: AH_1 and AH_2 averages significantly differ when exposed to 38 µM of PCP.

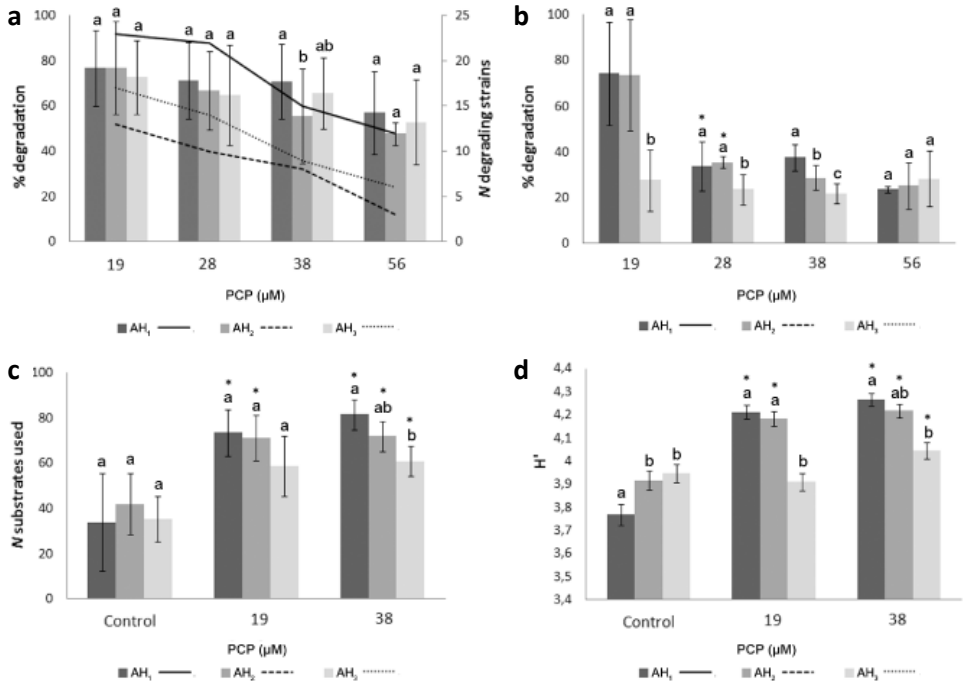

Figure II.1 - PCP-degrading capacity for axenic or community cultures (UPLC analyses), and physiological profile (Biolog plates SF-N2): (a) average PCP-degrading capacity of the strains (principal y axis) and number of PCP-degrading strains (secondary y axis, continuous, dashed and dotted lines for AH_1, AH_2 and AH_3 respectively); (b) average PCP-degrading capacity of the communities; (c) richness of catabolic processes (number of substrates used); and (d) diversity of catabolic processes (Shannon index *H'*). Different lowercase letters distinguish statistical differences between sites, assessed by Kruskal–Wallis and pair-wise *t*-test comparisons, when appropriate. Asterisks underline statistical differences in the functional analysis when comparing with the controls, tested by pair-wise *t*-tests.

Community assays were undertaken to complement the data obtained for the cultivable strains, specifically each soil fungal community was used to inoculate growth media containing PCP. At the 14th day of incubation, PCP residual concentrations were analysed (Figure II.1b). At each sampling spot, the capacity of the soil community to mitigate PCP contamination appears lower than that of the composing strains (average), except at the lowest PCP concentration where similar removal rates were observed (Figure II.1a and b). None of the communities showed meaningful variations in their removal capacity (p-value < 0.05) for PCP ≥ 28 µM. Only at an initial concentration of PCP = 38 µM did the three communities showed clear differentiation in their PCP removal capacity (pair-wise t-test).

Analyses of the community-level physiological profiles (CLPPs) showed that exposure to 19 or 38 µM of PCP, over 14 days, generally increased both richness (*i.e.* number of substrates used) and functional diversity (Shannon index, *H'*) of the communities (Figure II.1c and d). Nonetheless, in AH_3 both parameters significantly increased only when the community was exposed to an initial concentration of PCP = 38 µM. The catabolic potential of the communities under the control conditions showed similar richness, although AH_1 showed lower functional diversity than $AH_{2–3}$. Neither richness nor functional diversity clearly separates the three PCP fungal tolerant communities.

Comparable PCP-derived metabolomes in axenic and community-based cultures

PCP degradation intermediates and subproducts formed either by each strain (Table III.2) or community (Table III.3) were analysed by ultra-high-performance liquid chromatography-electrospray-high-resolution mass spectrometry (UHPLC-ESI-HRMS). The full MS data of the intermediates identified for each culture is available in the Table SIII.4. Most compounds identifications were confirmed using standards, some of which were

synthesized for that purpose (see Experimental procedures section for details).

Table III.2 - Full list of the compounds identified in the analysis of methanolic) extracts using UHPLC-ESI-HRMS, listed according to their retention times (t_R)

Compound	Abbreviation	Elemental composition	Theoretical mass	Retention time (min)	N_{axenic}	$N_{communities}$	N_{soils}	Abiotic control
Trihydroxybenzene	THB	$C_6H_6O_3$	125.0245	1.01–1.44	18	1	0	–
Hydroquinone	HQ	$C_6H_6O_2$	109.0295	1.75–1.85	4	0	0	–
Acetylacrylate	Ac	$C_5H_5O_3$	113.0246	1.73–1.91	0	0	2	–
Resorcinol	R	$C_6H_6O_2$	109.0295	2.85–2.92	2	0	0	–
Catechol	C	$C_6H_6O_2$	109.0295	3.58–3.60	4	0	0	–
cis-Dienelactone	DL	C6H4O4	139.0037	3.28–3.37	2	0	0	–
Chlorotrihydroxybenzene*	CTHB	$C_6H_5ClO_3$	158.9854	3.26–3.33	3	0	0	–
Dichlorotrihydroxybenzene*	DCTHB	$C_6H_4Cl_2O_3$	238.9520	3.77	0	0	1	–
Dichlororesorcinol	DCR	$C_6H_4Cl_2O_2$	176.9516	4.72	1	0	0	–
Dichlorodihydroxybenzene-sulfate conjugate*	S-DCDHB	$C_6H_4Cl_2O_5S$	256.9084	4.01–4.12	8	0	0	–
Trichlorodihydroxybenzene-sulfate conjugate*	S-TCDHB	$C_6H_3Cl_3O_5S$	290.8694	4.53–4.78	26	0	0	–
Trichlorohydroquinone	TCHQ	$C_6H_3Cl_3O_2$	210.9126	5.12–5.17	12	0	0	–
Trichlororesorcinol	TCR	$C_6H_3Cl_3O_2$	210.9126	5.39	1	0	0	–
Trichlorometoxyphenol-sulfate conjugate*	S-TCMP	$C_7H_5Cl_3O_5S$	304.8854	5.16–5.64	13	0	0	–
Trichlorocatecnol	TCC	$C_6H_3Cl_3O_2$	210.9126	5.81	1	0	0	–
Dichloromethoxyphenol*	DCMP	$C_7H_6Cl_2O_2$	190.9672	5.87	0	0	3	–
Trichloromethoxyphenol*	TCMP	$C_7H_5Cl_3O_2$	224.9282	6.29	1	0	0	–
Tetrachlorohydroquinone	TeCHQ	$C_6H_2Cl_4O_2$	244.8736	5.46–5.56	38	2	0	–
Tectrachlororesorcinol	TeCR	$C_6H_2Cl_4O_2$	244.8736	5.72–5.81	13	0	0	–
Tetrachlorocatechol	TeCC	$C_6H_2Cl_4O_2$	244.8736	6.12–6.20	53	3	0	✓
Trichlorophenol	TCP	$C_6H_3Cl_3O$	194.9177	6.45–6.48	0	0	5	–
Drosophilin A	Dro A	$C_7H_4Cl_4O_2$	258.8893	6.61–6.68	22	3	3	–
Tetrachloro-m-guaiacol	TeC-m-G	$C_7H_4Cl_4O_2$	258.8893	6.71–6.77	3	3	0	–
Tetrachloro-o-guaiacol	TeC-o-G	$C_7H_4Cl_4O_2$	258.8893	6.9–7.04	20	3	1	–
Tetrachlorophenol	TeCP	$C_6H_2Cl_4O$	228.8787	6.82–6.88	14	3	2	✓
Pentachlorophenol	PCP	C_6HCl_5O	262.8397	7.26–7.31	53	3	9	✓
Pentachlorobenzene*	PCB	C_6HCl_5	250.3371	8.19	0	0	1	–

The acronyms, molecular formulas and theoretical masses of the compounds are provided. The number of occurrences of each compound in axenic and community cultures, and in soils is also shown. *, putative compound identification. Pure standards were not available for identity validation.

Table III.3 - PCP-derived metabolome obtained in AH_n community cultures

Compound	Abbreviation	Community cultures		
		AH_1	AH_2	AH_3
Acetylacrylate	Ac	–	–	–
Trihydroxybenzene	THB	–	✓	–
Dichlorotrihydroxybenzene	DCTHB	–	–	–
Trichlorophenol	TCP	–	–	–
Tetrachlorohydroquinone	TeCHQ	✓	–	✓
Tetrachlorocathecol	TeCC	✓	✓	✓
Drosophilin A	Dro A	✓	✓	✓
Tetrachloro-*m*-guaiacol	TeC-*m*-G	✓	✓	✓
Tetrachloro-*o*-guaiacol	TeC-*o*-G	✓	✓	✓
Tetrachlorophenol	TeCP	✓	✓	✓
Pentachlorophenol	PCP	✓	✓	✓
Pentachlorobenzene	PeCB	–	–	–

The full list of PCP degradation intermediates identified (Table III.2) comprises different chlorinated derivatives of phenol (P), catechol (C) and hydroquinone (HQ). We also found different chlorinated derivatives of resorcinol (R) and *O*-methylated by-products of tetrachlorinated derivatives of C, R and HQ, namely the *ortho*, *meta* and *para* isomers of tetrachloroguaiacol (TeC-G), the latter known as drosophilin A (Dro A) (Tables III.2 and III.3). Additional *O*-methylated and sulfated by-products were detected. including the trichloromethoxyphenol-sulfate conjugate (S-TCMP) that involves both conjugation reactions (Table III.2). Inspection of the identified compounds revealed several non-chlorinated derivatives (*i.e.* tri- and di-hydroxybenzene isomers) (Tables III.2 and III.3); hence, some strains of the fungal soil community were capable of mineralizing PCP under the conditions tested.

Metabolites found in AH soils imply active pathways for PCP degradation

The PCP-derived metabolome of AH_n soils contained several chlorinated derivatives, namely tetrachlorophenol (TeCP), trichlorophenol (TCP) and dichlorotrihydroxybenzene (DCTHB) (Table III.4), as well as acetylacrylate (Ac). Traces of PeCB were also detected in AH_3 soils.

PCP-derived metabolome of FER_n and RR_n soil samples (Table III.4) reinforce the presence of chlorinated derivatives, including conjugate compounds [dichlorometoxyphenol (DCMP), Dro A and tetrachloro-*o*-guaiacol (TeC-*o*-G)].

Table III.4 - PCP and related compounds identified in the methanolic extracts of AH, FER and RR soils using UHPLC-ESI-HRMS

Compound	Abrev.	Soil Samples								
		AH_1	AH_2	AH_3	FER_1	FER_2	FER_3	RR_1	RR_2	RR_3
Pentachlorophenol (μg·Kg^{-1})	PCP	28.84	13.18	20.72	4.39	14.82	13.2	1.73	6.12	7.03
Acetylacrylate	Ac	–	✓	✓	–	–	–	–	–	–
Dichlorotrihydroxy-benzene	DCTHB	–	–	✓	–	–	–	–	–	–
Dichlorometoxy-phenol	DCMP	–	–	–	✓	–	–	✓	✓	–
Trichlorophenol	TCP	✓	✓	–	–	✓	✓	✓	–	–
Tetrachlorophenol	TeCP	✓	–	–	–	–	✓	–	–	–
Drosophilin A	Dro A	–	–	–	–	✓	–	–	✓	✓
Tetrachloro-*o*-guaiacol	TeC-*o*-G	–	–	–	✓	–	–	–	–	–
Pentachloro-benzene	PeCB	–	–	✓	–	–	–	–	–	–

PCP levels (μg·Kg^{-1}) are also indicated.

Discussion

PCP constitutes a public health and an environmental conservation concern worldwide. Global dispersion of PCP through long-range atmospheric transport via particulate matter in air *inter alia* [2, 11, 41] impacts on remote and unexpected locations provoking chronic effects [6]. In forest ecosystems, particularly in *Quercus suber* forests/woodlands, PCP impacts

are yet to be acknowledged, regardless of both scientific and anecdotal evidence of contamination of the oak bark with PCP and its derivatives [14, 15]. The bark behaves as a sampler, accumulating both gaseous and particulate pollutants [42], but PCP partition to the soil is likely to be significant [19]. This challenged us to evaluate PCP levels reaching soils by focusing on three cork oak forests located in the Tabarka district (Jendouba region in N. W. Tunisia). At the time of sampling, the forests contained several decaying oaks and both the vegetation and leaf litter were very dense (Table SIII.1). Compared with common practice in other managed forests, this identifies poor forest management, consistent with that reported previously [43].

PCP contamination was prevalent at all locations, but AH_n soils contained consistently the highest levels (Table SIII.1, Table III.4). Levels detected - > 10 $\mu g \cdot Kg^{-1}$ dry weight - are comparable with those reported for rural areas in China where PCP is currently used to fight the re-emergence of schistosomiasis [7] but much lower than found in the vicinities of wood-mill and storage locations (*e.g.* [44]. Tunisian legislation - prohibiting levels of PCP in soils of > 14000 $\mu g \cdot Kg^{-1}$ - is not aligned with the legislative restrictions in force in at least 26 countries around the world [2]. Our data suggest that forest management practices in this region are, or have recently been, making use of this biocide or its precursors. Atmospheric deposition may also be an important contributing factor [2, 11].

To shed light on this topic, we evaluated the capacity of soils to degrade the biocide focussing on fungi as its major colonizers. The numbers of fungal cfu's (Table SIII.1) found at each location were comparable with those typically found at similar forest habitats [32, 45], suggesting that levels of PCP in soil are not substantially decreasing the abundance of fungi (*i.e.* not directly correlated). The taxonomic diversity of the cultivable fungi colonizing AH_n soils (Table III.1, see full details in Tables SIII.2 and SIII.3) reasonably matches previous reports on soils from similar habitats [46, 47] or with comparable properties [48]. Ascomycota, particularly penicillia,

typically dominate, as key decomposers, soils with a low abundance of lignin [49].

The diversity of fungi identified in AH_n soils was lower (33 species, two phyla) than in Sardinian oak forest soils (Italy), which were analysed using metagenomic profiling tools (83 species, three phyla) [46], yet both studies reported a clear dominance of Ascomycota. In AH_n soils, *Penicillium* species (52 strains) predominated the composition, and three species (*P. vanoranjei*, *P. vagum*, *P. longicatenatum*) identified here have been recently reported as new species [35, 50]. Some of the remaining strains need to be fully characterized (six in total) and hide additional uncharacterized species (*e.g.* isolate DTO 099-G8). The fungal community was dominated by moderate xerophiles, namely penicillia and aspergilla [40]; accordingly, the number of fungal colonies growing in malt extract agar (MEA) and dichloran-glycerol (DG18) media were comparable, regardless of their divergent water activities (Table SIII.1).

In our study, the majority of the strains, 53 out of 77, were capable of degrading PCP under the conditions used even though this number was nearly halved when exposed to the highest PCP concentration (56 µM). Not surprisingly, PCP degradation and oxidoreductase activity (Table III.1) were not correlated (Pearson correlation test p-value < 0.05), which is consistent with previous reports (*e.g.* [51]). Strains belonging to the species *Fusarium oxysporum*, *Penicillium brevicompactum*, *P. glabrum*, *P. janczewskii*, *P. radiatolobatum*, *P. restrictum*, *P. murcianum*, *P. sizovae*, *P. vagum* and *P. vanoranjei* were able to degrade ≥ 50 % of the 56 µM of PCP in media (Table III.1). These data are consistent with the capacity of penicillia to utilize a wide variety of simple aromatic compounds [25, 27, 52]. Soils are composed of interconnected but distinctive microenvironments holding specific microbial colonizers and concentration/diversity of pollutants [20]. Accordingly, different strains of the same species showed distinctive PCP degrading capacities, *e.g. P. restrictum* strains of AH_2 and *P. radiatolobatum* strains of AH_1 or AH_2 (Table III.1).

At a particular PCP concentration, differences in the diversity of strains in AH_n soils were not, in general, translated into distinguishable features, namely PCP removal capacities and catabolic richness and diversity (Figure II.1). Increasing PCP concentrations led to a continuous decrease in the number (hence biodiversity) of PCP-degrading strains (Table III.1 and Figure II.1a), while simultaneously increasing both the catabolic richness and diversity of the community (Figure II.1b and c). The latter has been often associated with specialization of microbial communities because of chronic exposure to pollutants [47, 53]. Regardless of this, the persistent strains demonstrated comparable average PCP decay levels in axenic (Figure II.1a) and community (Figure II.1b) cultures.

Overall, data indicated that community interactions hindered the capacity of the strains to remove PCP (Figure II.1b). Ecological interactions are radically altered under *in vitro* conditions, generally favouring competition among strains and reducing the total fungal abundance [54, 55]. In the community cultivation, the low spore density *per* strain may differentially affect their capacity to germinate. Strong growth antagonisms between some of the most efficient degrading strains found in AH_1 and AH_3 soil samples were preliminarily observed (*i.e.* pair-wise cultivation in solid media, data not shown). As an example, within AH_3 community, *F. oxysporum* and *Cladosporium herbarum* inhibited the growth of *P. murcianum* and *P. radiatolobatum*, respectively.

Particular degradation intermediates and sub-products were match to the producing strain (Table III.2 and Table SIII.4), deconvoluting the PCP-derived metabolome formed by the cultivable community (Table III.3) and defining its PCP degradation pathway (Figure II.2). Data suggest multiple reaction steps in the initial modification of PCP, including its reductive dechlorination yielding TeCP isomers. Both *ortho* (2,3,4,5-TeCP) and *para* (2,3,5,6-TeCP) isomers can be formed abiotically in liquid media at neutral pH with the loss of chloride at the *ortho* position preferred, while the formation of the *meta* isomer (2,3,4,6- TeCP) has been considered unlikely

[56]. Regardless of using standards of the three TeCP isomers, their precise identity in cultures (or in the abiotic controls) remains inconclusive because of technical limitations. The degradation intermediates tectrachlororesorcinol (TeCR), tetrachlorohydroquinone (TeCHQ) and tetrachlorocatechol (TeCC) identified here may have been formed either through hydroxylation of the corresponding TeCP isomer or through peroxidative dechlorination of PCP (forming transient benzoquinones immediately followed by H^+ mediated reductions). TeCC and TeCP (most likely the *ortho* isomer) [56] were the only degradation products detected in the abiotic controls. This together with the lack of evidence for the biotic formation of *m*-TeCP is consistent with the idea that most likely the initial attack of PCP occurs through peroxidative dechlorination. The transient formation of TeCBQ would remain unseen in the negative ionization mode used here. After initial modification of PCP, either at *meta*, *para* or *ortho* position (respectively the resorcinol, hydroquinone or catechol branches), successive reductive dechlorination reactions occur. The HQ branch of PCP degradation pathway has been described previously in *Phanerochaete chrysosporium* [24] and others [26]. In *Aspergillus nidulans*, the catechol branch of the degradation pathway of monochlorophenols ensures its complete mineralization [37]. The identification of TCC implies that biotic transformation of TeCC occurred in some of the axenic cultures. The different branches intersect because of additional hydroxylation of R, HQ and C derivatives, either chlorinated or non-chlorinated, yielding the corresponding trihydroxybenzenes (THB).

The formation of TeC-G isomers, reported here for the first time in fungi exposed to PCP, occurs through phase II conjugation reactions, specifically *O*-methylation of the tetrachlorinated derivatives of PCP. The TeC-*p*-G isomer, *i.e.* Dro A, is a bactericidal compound, particularly active against Gram-positive bacteria, that has been previously identified in Basidiomycota strains [31, 57]. Additional conjugates, namely after *O*-methylation (TCMP), sulfation (S-TCDHB) or both (S-TCMP), were detected in some of the axenic cultures.

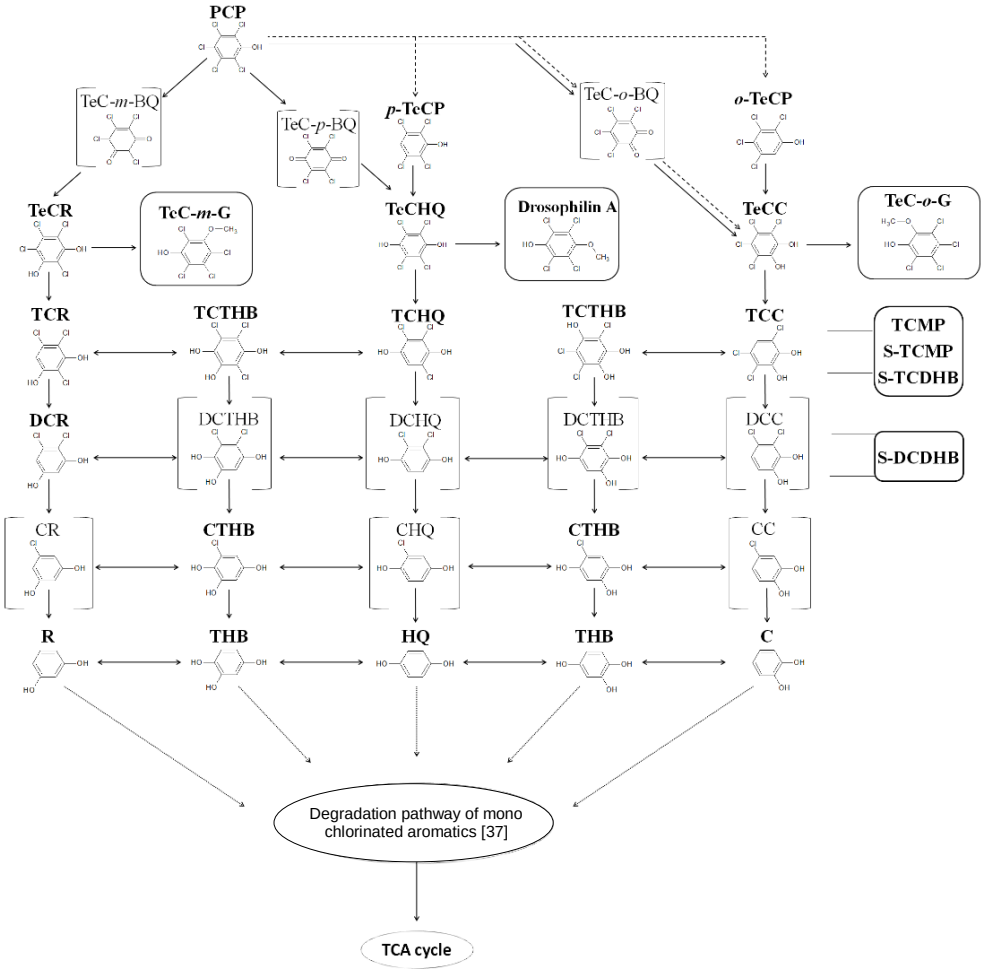

Figure II.2 - Proposed pathway of PCP degradation by filamentous fungi in soils, after integrating the metabolic data obtained in axenic and community experiments (UHPLC-ESI-HRMS analyses). Compounds in brackets are hypothetical intermediates. Full and dashed arrows stand for biotic and abiotic transformations respectively.

Similar conjugated compounds have been reported in other eukaryotes, *e.g.* fish and daphnids [58, 59]. In particular, sulfate conjugates and sulfate di-conjugates of PCP degradation intermediates have been identified in other fungi [26, 60]. These reactions constitute a detoxification

mechanism that generally increases the solubility of the toxic compound facilitating its excretion from the cell [21, 61].

Although the PCP-derived metabolome of the AH_n communities retrieved, in general, lower diversity of PCP degradation intermediates and by-products (Table III.3), they formed all the tetrachlorinated derivatives previously described, including the previously uncharacterized isomers of TeC-G. Only one non-chlorinated degradation intermediate – THB – was found to accumulate in these cultures implying that some strains within the community were capable of producing intermediates downstream of the tetrachlorinated derivatives, hence probably being capable of ensuring PCP mineralization.

The PCP degradation intermediates identified in AH_n soils, namely TeCP, TCP and DCTHB (Table III.4), constitute a valuable indicator that the soil microbial community is actively degrading PCP [25, 27]. Based on the data reported here, the last compound provides sufficient evidence that some of the degradation pathways occurring in soil involve fungal activity. The presence of TCP implies bacterial degradation of PCP through reductive dechlorination [25, 27, 62], or, alternatively, an (in)direct soil contamination source for TCP. In general, the low diversity of PCP-derived metabolites detected in AH_n soils (Table III.4) correlates with the low PCP levels measured in these samples. The origin of acetylacrylate (Ac), which was previously associated with fungal catabolism of aromatics [37], cannot be certainly attributed to PCP.

Finally, traces of PeCB were also detected in AH_3 soils (Table III.4). This compound can yield PCP either biotically or abiotically [63]. Its presence implies that multiple sources of soil contamination might be actively contributing to PCP occurrence in soil, increasing further the complexity of this problem. FER_n and RR_n soil metabolomes (Table III.4) revealed, in addition to TeCP and TCP, DCMP (*O*-methylation of DCDHB), Dro A and TeC-*o*-G, which may be associated with PCP degradation by fungi. Further studies and a more efficient monitoring at both regional and

global scale are necessary to fully elucidate the dynamics of PCP contamination in forest habitats.

This study reinforces wide-ranging principle of global and dispersed environmental pollution by PCP. The environmental dispersion of PCP into diverse degradation intermediates and subproducts is still poorly characterized. This compound cannot be considered as obsolete biocide, particularly since PCP levels in Tunisian *Q. suber* L. forests are similar to levels found prevalent where PCP is used [7]. Cork oak forests represent heterogeneous agro-silvo-pastoral systems where forest management coexist with other agro-practices (*e.g.* honey production). Forest products are marketed not only in the country, but also globally (*e.g.* Tunisian cork is largely exploited by foreign manufacturing industries [64] leading to the so-called 'circle of poison' [65]. PCP half-life has been estimated to be *ca.* 7.4 days in air and *ca.* 1.5 months in the environment, with transport distance of 1500 – 3000 km [2]. Based on these estimations, *e.g.* PCP emissions from regional sources in central European countries could reach the Jendouba region.

Overall, the data reported here is in agreement with the short lifetime of PCP in soil reported in recent studies [66]. Our data reinforce that the significance of the functional biodiversity surpasses that of the taxonomic biodiversity during PCP mitigation, notwithstanding both are intimately connected. From a purely ecological perspective, chronic exposure to low levels of pollutants may shift the microbial functional biodiversity of the soils, which in turn may affect the provision of ecosystem services. One hypothesis deserving further investigation is the occurrence of microbial specialization events because of competition/survival of highly degrading (adapted) fungal phenotypes. This study also revealed the existence of tetrachlorinated derivatives of PCP, namely TeCR and TeCG, so far uncharacterized in fungi. This provides us with unexpected tools for monitoring PCP degradation in other fungi dominated food webs. The

foundations to gather further knowledge about the enzymatic degradation pathways beyond that of a single species have also been established here.

In summary, the evidence reported here suggests that fungi play a key role in PCP mineralization and its short lifetime in forest soils, but many questions remain open*: e.g.* are chronic effects of PCP leading to microbial specialization, losses in taxonomic diversity and shifts in functional biodiversity? Is PCP a primary or a secondary contaminant? Answering these questions requires improved understanding of PCP occurrence in soil and of its sources, as well as a fundamental understanding of its fate.

Availability of data and material

The Electronic Supplementary Information word document is available in the Annex at the end of this chapter, containing more detailed tables and figures that support the figure panels at the main text. Electronic Supplementary Data file is available in the electronic version of this thesis. Electronic Supplementary data file ESI II provides mass spectrometry datasets (xls format) on the metabolomics of each of the isolates, and of the soil soil samples.

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ANNEX

This annex contains the Electronic Supplementary Material published along the main body of the research paper which composes Chapter II. This intends to allow better and easier understanding of the methodologies and analyses performed.

Table II.S1 – Characterisation of sampling sites and soil samples, including descriptors for the cork oak forest locations, physicochemical characterisation of the soils, PCP levels and the number of fungal colonies forming units (CFUs) in either MEA and DG18 (both containing chloramphenicol), expressed as CFU per g of fresh weight soil.

Location	Coordinates (GPS)		Vegetation	Litter	Humidity (%)	pH	Organic Matter (%)	C/N	[PCP] ($\mu g.Kg^{-1}$)	Fungal CFU's *per* g soil	
	Easting	Northing								MEA	DG18
AH1	008°51'52.00"	36°46'47.50"	sparse undergrowth (high bushes)	leaf litter	23.49	5.36	5.76	15.77	28.84	1.32E+04	5.35E+03
AH2	008°51'53.80"	36°46'49.20"	abundant undergrowth (high bushes)	dense layers of leaf litter	26.94	5.63	7.63	23.20	13.18	3.40E+04	4.20E+03
AH3	008°51'52.10"	36°46'50.40"	sparse undergrowth	dense layers of leaf litter	25.98	5.01	8.21	53.80	20.72	1.70E+04	7.48E+03
FER1	008°43'47.20"	36°46'57.90"	abundant undergrowth	leaf litter	26.35	7.40	6.52	15.58	4.39	0.68E+04	2.43E+03
FER2	008°43'49.60"	36°46'58.30"	sparse undergrowth	leaf litter	30.20	5.60	11.36	16.96	14.82	1.82E+04	8.00E+03
FER3	008°43'52.70"	36°46'58.10"	sparse undergrowth	leaf litter	31.80	5.70	9.69	16.72	13.20	2.46E+04	1.44E+04
RR1	008°51'51.50"	36°57'14.30"	sparse undergrowth	dense layers of leaf litter	26.05	6.60	7.17	15.02	1.73	2.44E+04	1.47E+04
RR2	008°51'45.60"	36°57'16.20"	abundant undergrowth	dense layers of leaf litter	19.35	7.40	5.15	17.52	6.12	2.54E+04	1.32E+04
RR3	008°51'48.50"	36°57'15.20"	abundant undergrowth	dense layers of leaf litter	19.75	6.10	4.92	16.07	7.03	2.64E+04	1.36E+04

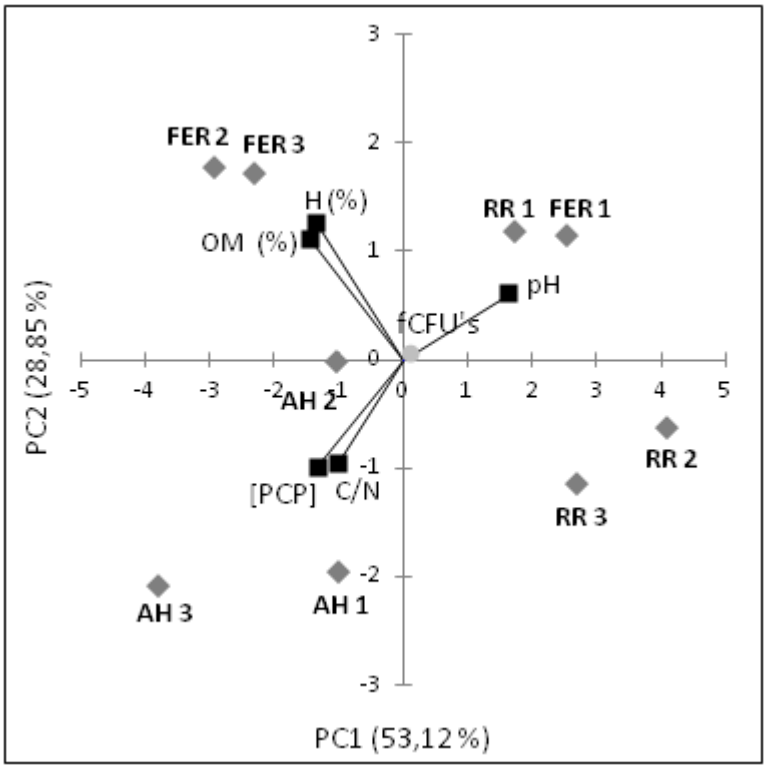

Figure II.S1 - Biplot containing principal component analysis (PCA) and multidimensional scaling (MDS) analyses comparing the observable quantitative variables measured in Aîn Hamraia (AH), Fej Errih (FER) and Ras Rajel (RR) soils.

Table II.S2 – Taxonomic data of the 77 cultivable strains composing the fungal communities of AH_n soils.

Strain	Taxonomy	GenBank no.	Locus	Lignolytic activity *in vivo*	
	Taxa			Qualitative	
				ABTS	RBBR
DTO 099-B5	*Absidia glauca*		LSU	-	-
DTO 099-A1	*Absidia* sp.		LSU	-	✓
DTO 099-C1	*Absidia* sp.		LSU	-	-
DTO 098-I5	*Aspergillus* sp. (sect. *Cremei*)		*CaM*	-	✓
DTO 099-B4	*Aspergillus* sp. (sect. *Cremei*)		*CaM*	-	-
DTO 098-I4	*Aspergillus welwitschiae*		*CaM*	-	-
DTO 099-C5	*Cladosporium sphaerospermum*		*Act*	✓	✓
DTO 098-I6	*Fusarium oxysporum* species complex		ITS	✓	-
DTO 099-A2	*Fusarium solani* species complex		ITS	✓	-
DTO 098-I9	*Penicillium brevicompactum*		*BenA*	-	-
DTO 099-D1	*Penicillium brevicompactum*		*BenA*	-	-
DTO 099-C2	*Penicillium brevicompactum*		*BenA*	-	✓
DTO 099-A3	*Penicillium daleae*		*BenA*	✓	✓
DTO 099-B9	*Penicillium glabrum*		*BenA*	-	✓
DTO 098-I8	*Penicillium glabrum*		*BenA*	-	-
DTO 099-A6	*Penicillium glabrum*		*BenA*	-	✓
DTO 099-A8	*Penicillium griseofulvum*		*BenA*	✓	✓
DTO 099-A9	*Penicillium janczewskii*		*BenA*	✓	✓
DTO 100-A4	*Penicillium janczewskii*		*BenA*	-	✓
DTO 099-C6	*Penicillium longicatenatum*	KM088816	*BenA*	-	-
DTO 100-A6	*Penicillium radiatolobatum*		*BenA*	✓	✓
DTO 099-C8	*Penicillium radiatolobatum*		*BenA*	-	-
DTO 099-C4	*Penicillium radiatolobatum*		*BenA*	-	-
DTO 099-A4	*Penicillium restrictum* species complex		*BenA*	-	✓
DTO 099-A5	*Penicillium restrictum* species complex		*BenA*	-	-
DTO 099-C9	*Penicillium restrictum* species complex		*BenA*	-	-
DTO 099-C3	*Penicillium restrictum* species complex		*BenA*	-	✓
DTO 099-B7	*Penicillium sizovae*		*BenA*	-	✓
DTO 098-I7	*Penicillium vagum*		*BenA*	✓	✓
DTO 099-A7	*Penicillium vagum*	KM088815	*BenA*	✓	✓
DTO 099-B6	*Penicillium sumatrense*		*BenA*	-	-
DTO 099-C7	*Penicillium sumatrense*		*BenA*	-	✓
DTO 099-B1	*Phoma putaminum*		ITS	✓	✓
DTO 100-A5	*Phoma putaminum*		ITS	✓	✓
DTO 099-B3	*Trichoderma* cf. *virens*		ITS	-	-
DTO 099-B2	*Zygorhynchus heterogamus*		LSU	-	-
DTO 099-D3	*Absidia pseudocylindrospora*		LSU	-	-
DTO 099-D8	*Aspergillus* sp. (sect. *Cremei*)		*CaM*	-	✓
DTO 099-E6	*Penicillium daleae*		*BenA*	-	-

Strain	Taxonomy Taxa	GenBank no.	Locus	Lignolytic activity *in vivo* Qualitative ABTS	 RBBR
DTO 099-D5	*Penicillium janczewskii*		*BenA*	-	✓
DTO 099-E5	*Penicillium janczewskii*		*BenA*	-	✓
DTO 099-D2	*Penicillium murcianum*		*BenA*	-	✓
DTO 099-E1	*Penicillium murcianum*		*BenA*	-	✓
DTO 099-E3	*Penicillium radiatolobatum*		*BenA*	-	✓
DTO 099-E8	*Penicillium radiatolobatum*		*BenA*	-	✓
DTO 099-D7	*Penicillium restrictum* species complex		*BenA*	-	-
DTO 099-D9	*Penicillium restrictum* species complex		*BenA*	-	-
DTO 099-E4	*Penicillium restrictum* species complex		*BenA*	-	-
DTO 099-F2	*Penicillium restrictum* species complex		*BenA*	-	-
DTO 099-D4	*Penicillium sanguifluum*		*BenA*	-	-
DTO 099-E9	*Penicillium sanguifluum*		*BenA*	-	-
DTO 099-D6	*Penicillium vagum*	KM088817	*BenA*	-	-
DTO 099-F1	*Penicillium vagum*		*BenA*	-	-
DTO 099-E2	*Penicillium yezoense*		*BenA*	-	-
DTO 099-G6	*Absidia pseudocylindrospora*		LSU	-	-
DTO 099-F9	*Absidia pseudocylindrospora*		LSU	-	✓
DTO 099-F4	*Aspergillus fresenii*		*CaM*	-	-
DTO 099-H2	*Aspergillus fresenii*		*CaM*	-	-
DTO 099-G4	*Aspergillus novoparasiticus*		*CaM*	-	✓
DTO 099-F5	*Aspergillus tubingensis*		*CaM*	-	-
DTO 099-H5	*Cladosporium phaenocomae*		*Act*	✓	✓
DTO 099-G2	*Cladosporium ramotenellum*		*Act*	✓	✓
DTO 099-G3	*Fusarium oxysporum* species complex		ITS	✓	-
DTO 099-H3	*Penicillium janczewskii*		*BenA*	✓	✓
DTO 099-F8	*Penicillium murcianum*		*BenA*	-	✓
DTO 099-H7	*Penicillium murcianum*		*BenA*	-	✓
DTO 099-G5	*Penicillium radiatolobatum*		*BenA*	-	✓
DTO 099-F6	*Penicillium restrictum* species complex		*BenA*	-	-
DTO 099-H4	*Penicillium restrictum* species complex		*BenA*	-	-
DTO 099-G9	*Penicillium shearii*		*BenA*	-	✓
DTO 099-H1	*Penicillium shearii*		*BenA*	✓	✓
DTO 099-G8	*Penicillium* sp. (sect. *Lanata-divaricata*)		*BenA*	-	✓
DTO 099-F7	*Penicillium vagum*	KM088819	*BenA*	✓	✓
DTO 099-G7	*Penicillium vagum*	KM088820	*BenA*	-	-
DTO 099-F3	*Penicillium vanoranjei*	KC695684	*BenA*	✓	✓
DTO 099-G1	*Penicillium vanoranjei*	KC695685	*BenA*	✓	-
DTO 099-H6	*Penicillium vanoranjei*	KC695686	*BenA*	✓	-

The strains written in red or blue were either those unable to germinate in the presence of PCP or that germinated but did not show PCP degradation levels above the obtained in the abiotic controls at any of the tested concentrations.

Table II.S3 – List of the cultivable fungal species identified in AH_n soils. The number of identified isolates *per* species (N), taxonomic data (A and Z stand for Ascomycota and Zygomycota, respectively) and lifestyle are also provided.

Isolated species	N	Phylum	Lifestyle
Absidia glauca	1	Z	saprobe
Absidia pseudocylindrospora	3	Z	saprobe
Absidia spp.	2	Z	n.a.
Aspergillus welwitschiae	1	A	saprobe
Aspergillus novoparasiticus	1	A	phytoparasite
Aspergillus sp. (sect. *Cremei*)	3	A	n.a.
Aspergillus fresenii	2	A	saprobe
Aspergillus tubingensis	1	A	saprobe
Cladosporium phaenocomae	1	A	saprobe
Cladosporium ramotenellum	1	A	saprobe
Cladosporium sphaerospermum	1	A	endophyte
Fusarium oxysporum species complex	2	A	phytoparasite
Fusarium solani species complex	1	A	saprobe
Penicillium brevicompactum	3	A	saprobe
Penicillium daleae	2	A	saprobe
Penicillium glabrum	3	A	saprobe
Penicillium griseofulvum	1	A	saprobe
Penicillium janczewskii	5	A	saprobe
Penicillium longicatenatum	1	A	saprobe
Penicillium murcianum	4	A	saprobe
Penicillium radiatolobatum	6	A	saprobe
Penicillium restrictum species complex	10	A	saprobe
Penicillium sanguifluum	2	A	saprobe
Penicillium shearii	2	A	saprobe
Penicillium sizovae	1	A	saprobe
Penicillium sp. (sect. *Lanata-divaricata*)	1	A	saprobe
Penicillium sumatrense	2	A	saprobe
Penicillium vagum	6	A	saprobe
Penicillium vanoranjei	3	A	saprobe
Penicillium yezoense	1	A	saprobe
Phoma putaminum	2	A	phytoparasite
Trichoderma cf. *virens*	1	A	endophyte
Zygorhynchus heterogamus	1	Z	saprobe

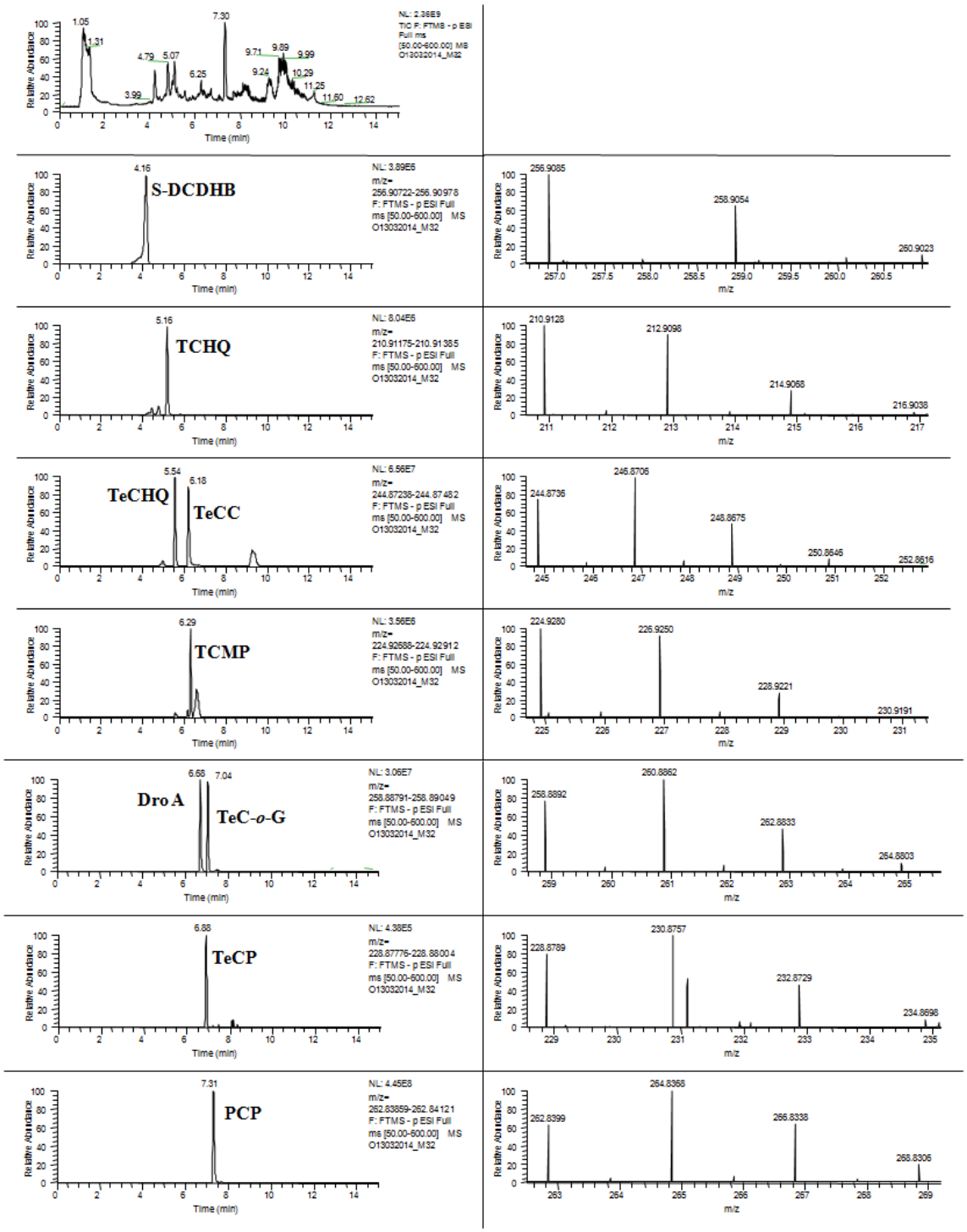

Figure II.S2 – Spectra and isotopic pattern obtained in one randomly selected sample, illustrating the data collected during the UHPLC-ESI-HRMS analysis.

CHAPTER III

Chapter III consists of the following published manuscript:

C. Martins, A. Varela, C.C. Leclercq, O. Núñez, T. Větrovský, J. Renaut, P. Baldrian and C. Silva Pereira. “Specialisation events of fungal metacommunities exposed to a persistent organic pollutant are suggestive of augmented pathogenic potential”. *Microbiome*, 2018, 6:208.

Outline: The following chapter presents a published work in its original form. It constitutes a landmark on the topic of the impact of pollutants towards fungal communities due to the innovative laboratory approach that was used in combination with state-of-the-art techniques usually applied to study microbial communities *in situ*. This allowed following the taxonomic and functional dynamics of a fungal metacommunity during the mineralisation of the archetypal pollutant PCP.

Specialisation events of fungal metacommunities exposed to a persistent organic pollutant are suggestive of augmented pathogenic potential

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Abstract

Background: The impacts of man-made chemicals, in particular of persistent organic pollutants, are multifactorial as they may affect the integrity of ecosystems, alter biodiversity and have undesirable effects on many organisms. We have previously demonstrated that the belowground mycobiota of forest soils acts as a buffer against the biocide pollutant pentachlorophenol. However the trade-offs made by mycobiota to mitigate this pollutant remain cryptic.

Results: Herein we demonstrate using a culture-dependent approach that exposure to pentachlorophenol led to alterations in the composition and functioning of the metacommunity, many of which were not fully alleviated when most of the biocide was degraded. Proteomic and physiological analyses showed that the carbon and nitrogen metabolisms were particularly affected. This dysregulation is possibly linked to the higher pathogenic potential of the metacommunity following exposure to the biocide, supported by the secretion of proteins related to pathogenicity and reduced susceptibility to a fungicide. Our findings provide additional evidence for the silent risks of environmental pollution, particularly as it may favour the development of pathogenic trade-offs in fungi, which may impose serious threats to animals and plant hosts.

Background

Chemical pollution constitutes a major threat to the sustainability of Earth's ecosystems; its impacts on biodiversity affect key ecosystem services, such as soil formation and nutrient recycling [1, 2]. Microbes – the unseen majority – are fundamental for the multi-functionality of ecosystems [3], yet progressively hindered by exposure to many disparate chemicals that are spread on a global scale. In particular, chronic exposure to Persistent Organic Pollutants (POPs) released either locally or remotely through long-range atmospheric/oceanic transport, is known to dramatically affect the structure, stability and function of microbial communities [4]. Pentachlorophenol (PCP) has a history of use dating back eighty years. Although it was regarded as mostly safe for the first few decades, PCP was eventually included in the Pesticide Action Network's Dirty Dozen list in 1998 and added to the Treaty of the Stockholm Convention list of banned POPs in 2015 [4], due to its far-reaching toxicity. Its long history of use, coupled with its persistence and ease of transboundary dispersal, has resulted in extensive environmental PCP contamination worldwide [5, 6]. Today, PCP is

still detected in human bodily fluids and tissues following exposure in indoor and/or outdoor environments around the world [4].

Recently we showed the existence of undefined active sources of PCP pollution in the Tabarka district (Tunisia), particularly in soils collected within cork oak forests [4, 7]. The soils were contaminated with PCP levels ranging from 13 to 28 $\mu g.Kg^{-1}$ of soil. The source and history of the pollution in these soils is unknown [4, 7]. Furthermore, we demonstrated that fungi isolated from these PCP-polluted forest soils can extensively degrade PCP, in theory acting as a buffer against PCP pollution in these habitats [7]. Due to their remarkable catabolic capacities, ubiquitous occurrence and lifestyle [8], saprotrophic fungi possess a peerless ability to degrade harmful chemicals, such as PCP [7, 9-11]. However, regardless of their ability to mitigate pollutants in soils, these activities raise several concerns: How are their communities affected by pollutants at the taxonomic and functional levels? Are there physiological costs underlying the trade-off between PCP degradation and survival?

To address these questions, we have relied on a culture-dependent approach to study the temporal response of a metacommunity of fungi to PCP exposure, uncovering the PCP-derived metabolome, physiological profile, metaproteome and metataxonomy (*i.e.* stable isotopic probing followed by amplicon sequencing). We show that when confronted with the half maximal effective concentration (EC_{50}) of PCP, the metacommunity degraded nearly 70% of the biocide in only ten days leading, in part, to its mineralisation. Furthermore, we show that PCP exposure altered the taxonomic diversity of the metacommunity, where the loss of some taxa was accompanied by the rise of key PCP-assimilators. It also influenced the proteome within the community; many of the affected proteins were associated with carbohydrate and nitrogen metabolisms. As a final point, PCP pollution was observed to induce functional shifts in the metacommunity suggestive of increased pathogenic potential, which in turn

may increase the dispersal of airborne opportunistic pathogens capable of affecting both animal and plant hosts.

Materials and Methods

Study design

Microbial communities consist of sub-communities that often contain the same dominant strains, yet contain a distinct composition of the less abundant strains [12]. Conventional culture-dependent assays may favour the development of only a subset of particular sub-communities. To establish a metacommunity of fungi comprising many distinct sub-communities, the community-based cultures were dispersed into many growth containers that were pooled at the end of the experiment, similar to the methodology applied for the establishment of metacommunities composed of several local bacterial communities [12]. Briefly, each biological replicate comprised five 6-well plates (total of 30 wells), each well containing 5 mL of growth medium with or without 38 µM of ^{13}C PCP (*i.e.* EC_{50}). The growth media of each biological replicate was mixed with the metacommunity inoculum (ratio of 10:1), then distributed into 30 culture-wells. Cultures were incubated at 30ºC, 90 rpm (triplicates of 30 wells *per* condition) and harvested at the third, fifth, seventh and tenth day of exposure (triplicates). Culture aliquots were used for the physiological profiling. The mycelial and the extracellular fractions were separated using vacuum filtration, the fresh mycelia weight was recorded, and both fractions conserved at -80ºC until further use. The extracellular fractions were used to evaluate the degradation of PCP (*viz.* PCP residual levels by liquid chromatography and PCP-derived metabolites by mass spectrometry) and the secretome. The intracellular fractions were used to study the community composition (amplicon sequencing), the mycelial proteome, and the intracellular PCP-derived metabolites (mass spectrometry). Complementary analyses included measures of the medium pH along cultivation as well as the effect of miconazole on the metabolic

activity (MTT reduction assay) of the metacommunity after ten days of exposure to PCP compared to control conditions (see Figure III.S3).

Chemicals

If not explicitly stated otherwise, chemicals were of analytical grade and purchased from Sigma-Aldrich. All Liquid Chromatography (LC) and Mass Spectrometry (MS) solvents were of the highest analytical grade.

Inoculum of the metacommunity of fungi

The inoculum of the metacommunity of fungi originated from soils sampled inside cork oak forests in Tunisia (E008°51'00.00 N36°46'00.00, Tabarka district, Tunisia) as previously described [7]. In brief, each soil sample is composed by soil collected in each quadrant defined by 1×1 m^2, using a 3 cm diameter gauge auger at a single depth: 0–20 cm, which was pooled and sieved (<2 mm). Herein, the three Aîn Hamraia forest soil samples corresponding to distinct forest locations (see Chapter II) were carefully combined before use (total soil volume of *ca.* 2 L). To recover the mycobiota a soil aliquot (15 g) was immersed (1:10, w/v) into a solution of 0.1% peptone (w/v) and 0.1% chloramphenicol (v/v) (60 min, soft agitation, vacuum cycle every 20 min), then sieved (pore sizes of 500 µm, 210 µm then 100 µm) and finally distributed into 1-mL aliquots that were stored at -80ºC, as established previously [7].

Half maximal Effective Concentration of PCP against the metacommunity of fungi

The half maximal Effective Concentration (EC_{50}) of PCP was determined using 5-mL cultures (6-well plates; 2 plates *per* replicate). Growth media (1 % w/v of glucose in a mineral minimal media [10, 13], MMG) containing 19, 38, 95, 190, 380 or 760 µM of PCP were mixed with the metacommunity inoculum (ratio of 10:1), incubated at 30ºC, 90 rpm for seven days (triplicates, including negative controls). Following incubation, 50 µL from

each biological replicate (pool of twelve wells) were spread onto MEA and the number of colony forming units (CFUs) monitored daily during 5 days and compared to that of the negative controls (triplicates). To obtain the EC_{50} value, results were adjusted to a logistic regression using the dose effect tool of XL-STAT software version 2009.1.02 (Addinsoft).

Chemical analyses

PCP was quantified using ultra-performance liquid chromatography (UPLC) as previously described [7]. Chromatographic profiles were acquired at 212 nm and PCP quantification limits were 0.38–56 µM (retention time of 5.9 min). The diversity of PCP-derived metabolites and sub-products in both the extra- and intra-cellular compartments at the third, fifth, seventh or tenth day of exposure to PCP, were resolved using Ultra High Performance Liquid Chromatography – Electrospray – High Resolution Mass Spectrometry (UHPLC-ESI-HRMS) operated in negative ESI mode using a Q-Exactive Orbitrap MS system (Thermo-Fisher Scientific) as previously described [7, 13, 14]. MS data were processed by ExactFinder™ 2.0 software (Thermo-Fisher Scientific), applying a user target database list and validated, whenever possible, using standard compounds.

Carbon and nitrogen metabolism

The ability of the metacommunity of fungi to use specific carbon and nitrogen sources was analysed using Biolog FF plates following the manufacturers' guidelines. The cultures were grown in MMG with or without 38 µM of PCP during 3 or 10 days, as described above, before testing. The plates were incubated at 30ºC and the absorbance of each plate at 490 nm and 750 nm was measured daily for 5 days. Functional diversity (Shannon index, H') and richness were calculated as previously described [15]. Carbon and nitrogen sources were grouped by category [16]. To reveal functional categories affected on the third or tenth day of PCP exposure compared to controls, the ratios of the increase or decrease of use of each substrate were

normalised, and a histogram constructed using XL-STAT software version 2014.5.03 (Addinsoft, France).

Metataxonomics of the metacommunity-based cultures

The metacommunity diversity on the third and tenth day of cultivation, both in the presence and absence of the stable isotope ^{13}C-labelled PCP, was analysed, as well as that of the inoculum, *i.e.* the metacommunity directly recovered from soils that originated from the cork oak forests. ^{13}C-labelled PCP was used to mark OTUs capable of PCP assimilation (see below *Isopycnic ultracentrifugation*).

DNA extraction

The frozen mycelia were macerated using a pestle and mortar, then further ground with the aid of an extraction buffer (50 mM of NaH_2PO_4, 50 mM NaCl, 500 mM Tris-HCl, 5% SDS, pH 8; 600 µL *per* culture) and glass beads (1 g, equal amounts of 0.5 and 0.1 mm beads) using a TissueLyzer LT Adapter (Qiagen, Germany), for 5 min at top speed. Afterwards, the sample was mixed with a half volume of each: phenol and chloroform containing isoamyl alcohol (24:1; hereafter defined as solution A); shaken for 2 minutes and centrifuged (5 min, 2,400*g*) to recover the upper supernatant (*i.e.* aqueous phase) which was re-extracted with an equal volume of solution A, and recovered as described before. To this mixture 1/3 volume of 6M NaCl and 1/10 volume of 10% of cetyl trimethylammonium bromide (CTAB) in 0.7M of NaCl were added, and the mixture was incubated for 30 min at 65ºC. After cooling to room temperature, an equal volume of solution A was added, shaken and centrifuged (20 min, 1,400*g*) to recover the supernatant. Finally, DNA was precipitated in 2/3 volume of isopropanol and 1/10 volume of acetate solution (3M) during 20 min at room temperature, and recovered by centrifugation (20 min, 6,800*g*). The DNA pellet was washed with 200 µL of ethanol (70%), recovered by centrifugation as before, air driedfor 60 min, eluted in 50 µL of TE buffer (Qiagen, Germany) and finally stored at -20ºC.

Prior to use, the DNA samples were cleaned using the GeneClean Turbo kit for 100-300 kb fragments (MP Biomedicals, USA) following the manufacturer instructions.

Isopycnic ultracentrifugation

Isopycnic ultracentrifugation was used to separate the "heavy" (*i.e.* that incorporated ^{13}C) and the "light" DNA fractions, both of which were used to generate amplicon sequencing data. The separation of the ^{13}C-labelled DNA from the unlabelled DNA was carried out following an established protocol with some modifications [17]. Specifically, the DNA samples were re-suspended in 10 mM ethylenediaminetetraacetic acid (EDTA, final volume of 4 mL), then mixed with 4.7 g of cesium chloride (CsCl) and 10 µL of RedSafe (Chembio Diagnostics, USA) and transferred to 4.7 mL OptiSeal tubes (Beckman Coulter, USA) and centrifuged in an Beckman Optima Max XP, equipped with a TLA-110 rotor, for 40 h, 311,438*g*, *k*-factor = 21.2, with no break. The light and heavy DNA bands were visualized under a fluorescent light (514 nm) and were recovered by piercing the tube with a syringe. DNA was extracted using a 2:1 butanol/NaCl solution (v/v, saturated NaCl), washed with ethanol, then eluted in milliQ water and stored at -20ºC.

Illumina sequencing

For the analysis of fungal community composition, the ITS2 region of fungal rDNA was PCR-amplified in a GeneAmp PCR system 2720 (Applied Biosystems) using barcoded gITS7 and ITS4 primers (gITS7, 5'- GTG ART CAT CGA RTC TTT G-3'; ITS4, 5'- TCC TCC GCT TAT TGA TAT GC-3') [18] in technical triplicates, including quality controls, as previously described [19]. The quality of the PCR products was monitored using gel electrophoresis. The technical replicates were pooled and sequenced on an Illumina MiSeq system. NGS analysis was performed by the Gene Expression Unit at Instituto Gulbenkian de Ciência (Oeiras, Portugal).

Amplicon sequencing data analysis

The amplicon sequencing data were processed using the pipeline SEED 2.1 [20]. Briefly, paired-end reads were joined using FASTQ-join [21]. The ITS2 region was extracted using ITSx1.0.11 [22] before processing. Chimeras were identified using USEARCH 8.1.1861 and deleted. Sequences were clustered using UPARSE implemented within USEARCH [23] at a 97% similarity level. The most abundant sequences were selected for each cluster, and the closest hits were identified using BLASTn against GenBank. Singletons were discarded. The cladograms based on the ITS2 sequence similarity of the identified OTUs were generated using PhyML to illustrate the diversity of the taxonomy within the metacommunity, regardless that the high variability of the ITS2 region does not allow a precise topology. The cladograms were then visualized and edited graphically using FigTree 1.4.3.

Metaproteomics of the metacommunity-based cultures

Extraction of mycelial proteins

Mycelial proteins were extracted using a modified Trichloroacetic acid (TCA)/Acetone protocol [24]. Briefly, the frozen mycelia (in liquid nitrogen) were ground using a pestle and mortar and homogenized in extraction buffer: 50 mM of Tris-HCl at pH 7.5, 200 mM NaCl, 5 mM EDTA, 0.5% Triton X-100 and EDTA-free EASYpack protease inhibitors (Roche, Switzerland). To facilitate homogenisation and cell rupture, a TissueLyzer LT Adapter (Qiagen, Germany) was used, first 1 g of glass beads (half of each size: 0.5 and 0.1 mm) were added and then two consecutive cycles of 5 min at top speed were applied. Proteins were precipitated in acetone containing 10% (v/v) trichloroacetic acid (TCA) and 40 mM of dithiothreitol (DTT) (1:10 w/v) for 1 h at -20ºC; the pellet recovered by centrifugation at 10,400*g* for 15 min, washed three times in 10 mL of acetone containing 40 mM of DTT, finally dried under a nitrogen flow and stored at -80ºC until further analysis.

Extracellular protein

The extracellular culture fractions were first concentrated *ca.* 30 fold using Vivaspin Turbo 15 ultra-filtration systems (Sartorius, Germany). The concentrated samples were mixed with 200 mL of a boiling SDS solution (2% SDS, 40 mM of Tris-base and 60 mM of DTT) and shaken at 99ºC and 350 rpm for 5 min in a Thermomixer (Eppendorf, Germany), then maintained at -20ºC overnight. Finally, proteins were precipitated in acetone with 60 mM DTT for 1 h at -20ºC, and recovered by centrifugation (4ºC, 15 min, 21,630*g*), washed five times with acetone containing 60 mM DTT, finally dried under nitrogen flow and stored at -80ºC until further analysis.

Mass spectrometry analyses of protein extracts

Proteins were recovered from the culture filtrates using denaturing precipitation conditions [9]. 10 µg of protein (Quantification performed with RC DC™ protein assay kit, Bio-Rad) was loaded on to a precast gel (Criterion ™ XT precast 1D gel 4-12% Bis-Tris, Bio-Rad) and separated using a short migration. The gel was stained with Instant Blue (Gentaur BVBA, Kampenhout, Belgium), sliced into bands. Proteins were first reduced, then alkylated and de-stained and finally digested using trypsin (sequencing mass grade, Promega). Peptides were extracted, dried and stored at -20 °C until LC-MS analysis. Peptides were analysed with a nano-HPLC system (NanoLC-2D, Eksigent, Sciex) coupled to a Triple TOF 5600+ mass spectrometer (Sciex, Darmstadt, Germany) operated on positive ESI mode with a Nanospray III source. In detail, after desalting and enrichment on C18 pre-column (C18 PepMap™, 5 µm, 5 mm x 300 µm, Thermo scientific), peptides were separated with a C18 reverse phase column (C18 PepMap™ 100, 3 µm, 100 Å, 75 µm × 15 cm, Thermo scientific) using a linear binary gradient (A: 0.1% formic acid; B: 80% acetonitrile, 0.1% formic acid) at a flow rate of 300 nl.min^{-}1. Peptides were eluted from 5% to 55% solvent B over 45 min. Solvent B was then increased to 100% to wash the column before re-equilibrating for 25 min prior to the next injection. The 20

most intense precursors were selected for fragmentation. The CID spectra were processed with Mascot (version 2.4.2) using Mascot Daemon interface (version 2.4.2, Matrix Science, London, UK) by searching against the SwissProt Fungi (31527 sequences) database released on May 2015 and the *Emericella nidulans* (36,970 sequences; 18,794,350 residues) database released on the 13 November 2015. Only the proteins identified with a significance MASCOT-calculated threshold *p-value*<0.05 and at least two significant peptides *per* proteins were accepted.

Statistical analyses

Amplicon sequencing

The amplicon sequencing data attained without any isotopic separation was first treated to assess the overall relative abundances between conditions (incubation with PCP and controls, at the third and tenth day). Descriptive statistics of the OTUs relative abundance and a Jaccard based Hierarchical Cluster Analysis of their diversity (presence *versus* absence) were performed using XL-STAT software version 2014.5.03 (Addinsoft, France). The histogram analysis used as weights the normalized number of reads of each OTU (relative abundance) *per* sample (SIP separation not considered), sub-sampled for the depth of the Illumina MiSeq run (100000 reads).

To study the differential relative abundance of each specific OTU in the light and heavy DNA fractions, we estimated the probability of major fold changes (FC) between the two conditions (negative binomial distribution) as follows: the OTU counts were normalized within each sample and sub-sampled as mentioned above, set to integers and then analysed using the RStudio (version 1.0.153) Bioconductor package DESeq2 [25]. Those presenting differential abundance (normalized counts bigger than 100 reads) between fractions or between the fractions and the controls were classified as ^{13}C assimilators. Non-metric multidimensional scaling (NMDS) was used to visualize treatment effects. To build the NMDS, the normalized and sub-sampled counts were standardized within each OTU and then a Bray-Curtis

resemblance matrix was built and plotted using a minimum stress of 0.01, using PRIMER 6.1.13 (PRIMER-E, Ltd). The assimilation category of each OTU was used as label.

To test differences at the pH measurements and MTT assays (comparing values obtained after incubation with PCP and controls) Student's two sample *t*-tests were performed after Cohen's D test (to assess the power of variance comparisons, $f > 0.04$) using the tool of XL-STAT software version 2014.5.03 (Addinsoft, France).

Metaproteomics

Only the proteins that were present in at least two out of three biological replicates were considered for further analyses. The relative quantification of the proteins has been calculated using the normalized spectral abundance factor (NSAF) [26]. The spectral counts of each mass were normalized and further analysed using the RStudio (version 1.0.153) Bioconductor package edger [27] following generalized linear models [28, 29]. This approach was used to analyse both the mycelial and the extracellular metaproteome. The cumulative $\log_2$FC for each functional category was plotted using Microsoft Excel, discriminating the contributions of the distinct taxonomic classes.

Results and Discussion

The trade-off between PCP degradation and physiological profile

When exposed to 38 µM PCP - the estimated EC_{50} (Figure III.S1) - the metacommunity of fungi ensured the rapid decay of the biocide: PCP decay values ranged from 1.3±2.0% to 69.1±2.4% at the third and tenth day of exposure, respectively (Figure III.1a). Initial modification of PCP by the metacommunity involved its reductive dechlorination, of which the resulting products – tetrachlorophenol isomers (TeCP) – were channelled into the three branches of the PCP degradation pathway: Resorcinol, Hydroquinone and Catechol (Figure III.1b), similar to those reported previously [7, 10, 13]. In fact, on the third day of exposure, tetrachlororesorcinol (TeCR),

tetrachlorohydroquinone (TeHQ) and tetrachlorocatechol (TeCC), as well as TeCP, were detected extracellularly (Figure III.1b). Only two compounds were found intracellularly, namely TeCC (throughout the entire incubation period) and TeCHQ (only in the middle of the exposure period, on days 5 and 7). The absence of internalised TeCR (Figure III.1b) suggests that the Resorcinol branch advances at a slower pace than the others, possibly because TeCR formation is preceded exclusively by biotic steps. Trihydroxybenzene (THB) was detected extracellularly at all time subsequent to day 3 (Figure III.1b); its formation may be linked to either branch of the PCP degradation pathway. Detection of THB, together with detection of maleylacetate and 3-oxoadipate (Table III.S1, dataset ESI III.1) directly link the degradation of aromatics to the tricarboxylic acid cycle [13], leading to PCP mineralisation.

To preliminarily uncover the functional costs of PCP degradation within the metacommunity, we analysed the Community Level Physiological Profiles (CLPP) at the first and last time points of PCP exposure (Figure III.1a). The biocide did not significantly alter either the functional diversity of the metacommunity (Shannon index, *H'*) or the number of used substrates compared to control conditions. At the final time point, these values ranged from *H'*= 4.34 (+/- 0.06) to 4.41 (+/- 0.02) and $N_{substrates}$ = 91.67 (+/- 0.79) to 94.33 (+/- 0.58) in the metacommunities exposed or not exposed to PCP, respectively. However, PCP effects on the utilisation of individual substrates were obvious: there was a 60% decrease in the utilisation of carbohydrates and 13% decrease in the utilisation of carboxylic acids; a 35% increase in the utilisation of nitrogen-containing substrates (grouped as miscellaneous) and 14% increase in the utilisation of amino-acids (Figure III.1c, Table III.S2, dataset ESI III.2).

Scoring PCP assimilators within the metacommunity of fungi confronted with PCP

The observed trade-off between PCP degradation and the community physiological profile may have resulted from shifts in the composition of the metacommunity (culture-dependent approach, see Methods for further details).

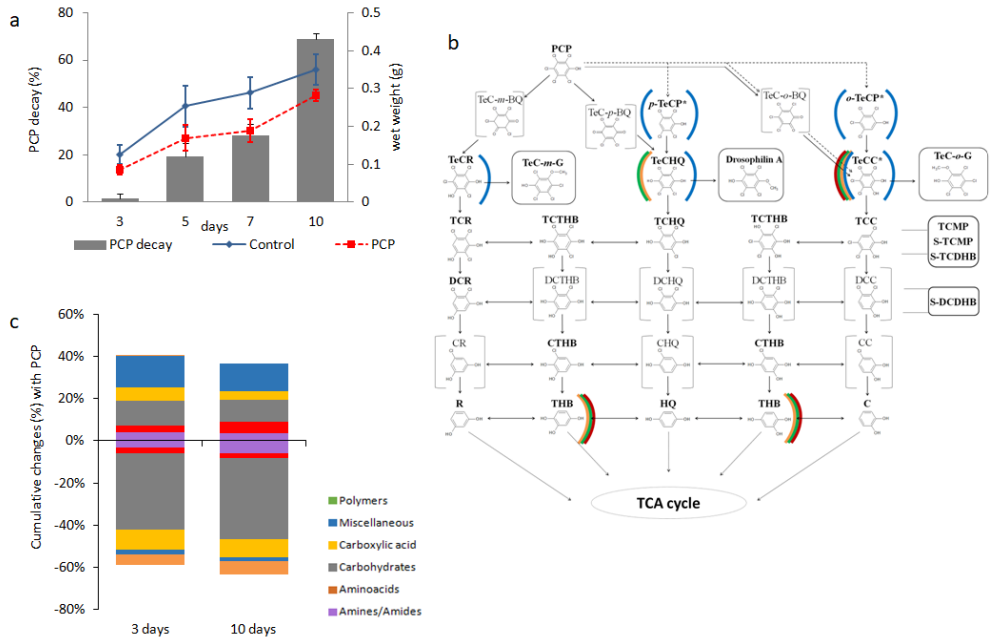


Fig III.1 - Trade-off between PCP degradation and physiological profile of a metacommunity of fungi: **(a)** Percentage of PCP degradation throughout the incubation time of the metacommunity exposed to PCP (grey bars). Also shown are the growth curve (fresh weight, FW) for the PCP exposed metacommunity (red line) and the control (blue line). **(b)** PCP degradation pathway of the metacommunity disclosing the diversity of intra (left brackets) and extracellular (right brackets) PCP-derivatives at the third (blue), fifth (orange), seventh (green) and tenth (red) day of exposure to the biocide. The names of the molecules are depicted as acronyms as follows: TeC (tetrachloro), TC (trichloro), DC (dichloro), P (phenol), BQ (benzoquinone) R (resorcinol), HQ (hydroquinone), C (catechol) and THB (trihydroxybenzene). Additionally, molecules inside boxes indicate conjugations, such as methylations (M). The ortho (*o*), para (*p*) and meta (*m*) isomers are also discriminated whenever needed. Asterisks mark compounds which were identified also at the abiotic control. **(c)** Utilisation profile of carbon and nitrogen sources by the metacommunity exposed to PCP compared to control conditions (Biolog FF microplates) revealing the cumulative differential utilisation of each substrate category.

A total of 398,591 amplicon sequences belonging to fungi were identified using Illumina MiSeq (following trimming based upon quality and size). To achieve a complete fingerprint of the taxonomic diversity, the operational taxonomic units (OTUs) were considered irrespective of relative abundance (excluding singletons) [19, 20]. The inoculum comprised 499 OTUs classified into 36 different orders at distinct relative abundances (Figure III.2).

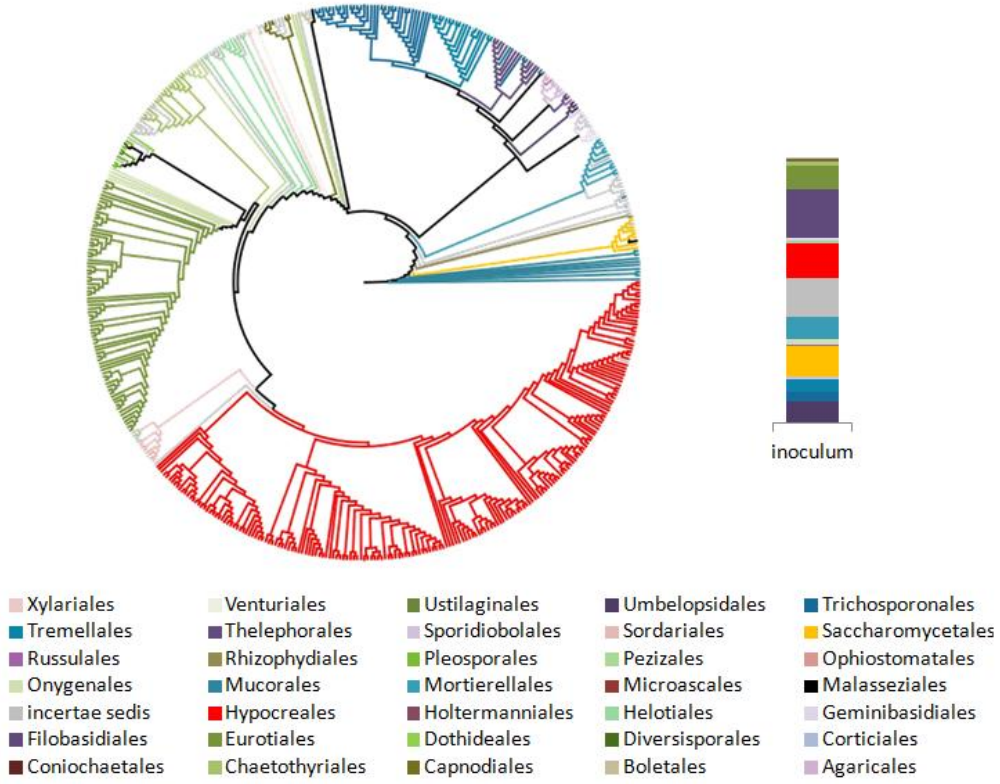


Fig III.2 - Taxonomic diversity of the metacommunity of fungi in the inoculum. Cladogram based on the ITS2 sequence similarity illustrating the diversity of OTUs identified in the metacommunity inoculum by amplicon sequencing (left) and their relative abundance *per* taxonomic order, comprising also unknown fungi (*incertae sedis*), using the normalised read counts, sub-sampled for the sequencing depth of the Illumina MiSeq run (100000 reads) (right).

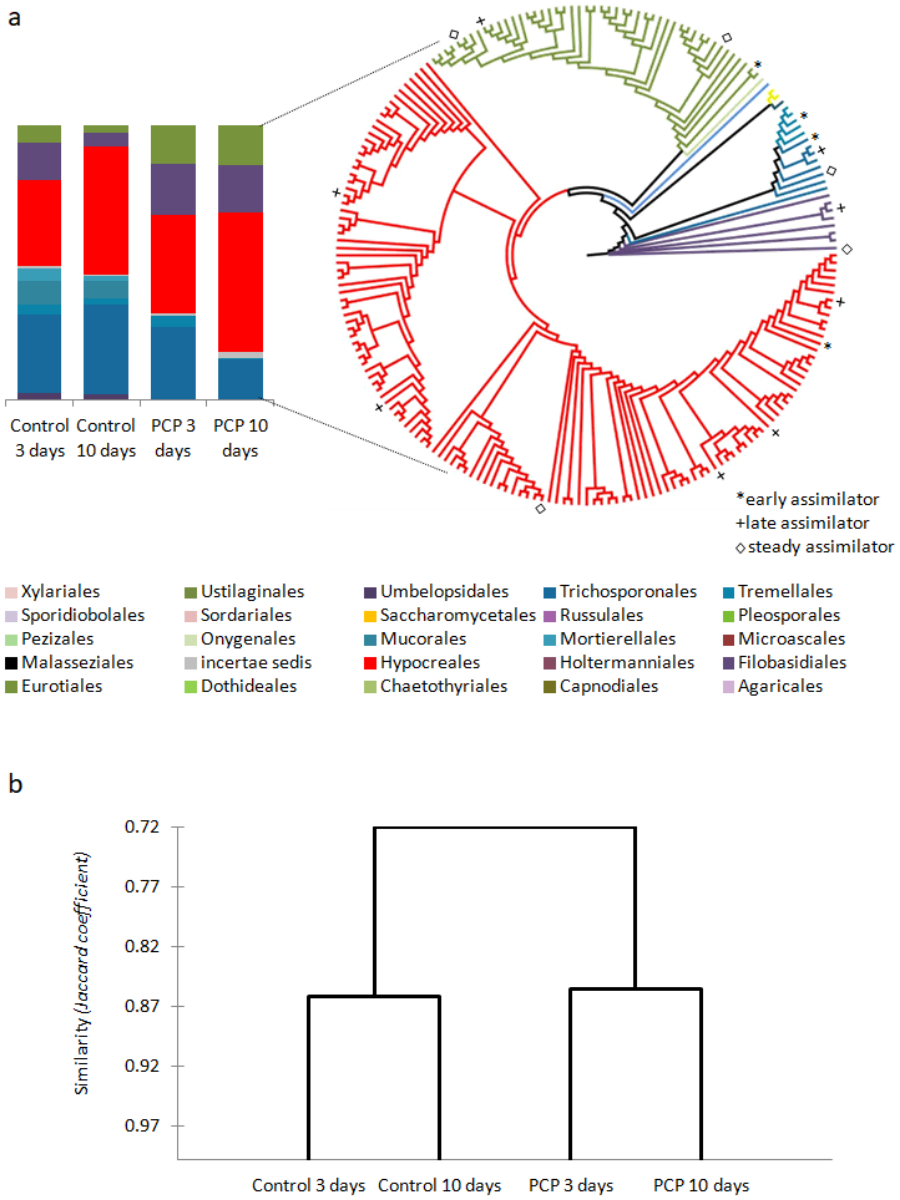
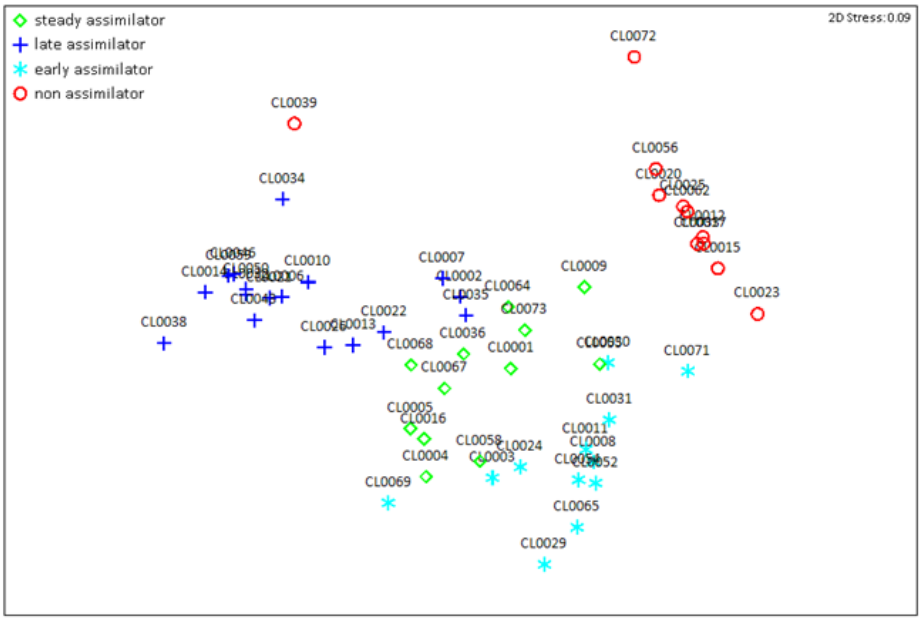


Fig III.3 - Shifts in the taxonomic diversity of the metacommunity of fungi exposed to PCP. **(a)** Relative abundances of the identified taxonomic orders in the metacommunity of fungi exposed to PCP on the third and tenth day of incubation, as well as in the corresponding controls (left). The cladogram based on the ITS2 sequence similarity of the metacommunity of fungi on the tenth day of PCP exposure is shown as an example (right), where the marked OTUs correspond to major PCP degraders (see below, Figure III.4). **(b)** Jaccard-based hierarchical cluster analysis of the taxa diversity of the metacommunity confronted or not with PCP

After cultivation for three or ten days, the number of OTUs varied from 228 (21 orders) to 215 (12 orders), and from 178 (16 orders) to 163 (14 orders) in the presence and absence of PCP, respectively (Figure III.3a, dataset ESI III.3). Cultivation led to the loss of some fungal orders, regardless of PCP levels and occurred even in its absence. In particular, one abundant taxonomic group, Saccharomycetales, which exhibited low OTU sequence diversity, were nearly entirely lost during cultivation (Figure III.2).

Seventy-seven strains were previously isolated by us from the same soil used here as the source of the metacommunity inoculum [7]. As expected, the sequences of the internal transcribed spacer (ITS) region of 34 of these fungal strains matched a few of the OTUs obtained in the present study (defined by their accession number in the CBS-KNAW culture collection, dataset ESI III.3). PCP exposure dramatically altered the share of the most abundant orders: Eurotiales, Filobasidiales and Hypocreales all increased compared to control conditions, while Trichosporonales decreased (Figure III.3a, dataset ESI III.2). Less abundant taxa that were largely conserved in the control were lost during exposure to the biocide. In particular, PCP decreased the relative abundances of Mortierellales, Mucorales, Umbelopsidales and Tremellales, all of which, except for Tremellales, were absent by the tenth day of exposure (Figure III.3a). PCP affected the taxonomic diversity, *i.e.* presence *versus* absence, of the metacommunity (Figure III.3b). To identify the OTUs capable of PCP assimilation, the metacommunity cultures were exposed to stable isotope ^{13}C-labelled PCP (Tables III.S3 and S4, dataset ESI III.3). The major ^{13}C assimilators were matched to 39 specific OTUs (total reads > 100; Figure III.4a), of which only 17 were particularly abundant (total reads > 1,000; Figure III.4b) largely explaining the multivariate partitioning of data (Figure 4a).

a



b

OTU	ID	Similarity (%) NCBI	Assimilation of ^{13}C PCP
CL0001	KY037850_*Vanrija albida*	100	steady assimilator
CL0002	DTO 099-G3_*Fusarium oxysporum* species complex	100	late assimilator
CL0003	MG725820_*Trichoderma* sp.	100	early assimilator
CL0004	KY104335_*Naganishia randhawae*	99.5	steady assimilator
CL0005	DTO 099-F7_*Penicillium vagum*	100	steady assimilator
CL0006	HG008760_*Trichoderma* sp.	100	late assimilator
CL0007	KY037851_*Solicoccozyma phenolicus*	100	late assimilator
CL0008	KX302067_*Trichosporon* sp.	100	early assimilator
CL0009	AB737864_*Trichoderma hamatum*	100	steady assimilator
CL0010	KU987904_*Acremonium* sp.	99.4	late assimilator
CL0011	KY320605_*Saitozyma podzolica*	100	early assimilator
CL0013	MG711821_*incertae sedis*	100	late assimilator
CL0014	KY315586_*Talaromyces verruculosus*	100	late assimilator
CL0016	DTO 099-E4_*Penicillium restrictum* species complex	100	steady assimilator
CL0021	KY101694_*Apiotrichum vadense*	100	late assimilator
CL0022	KY687957_*Trichoderma linzhiense*	100	late assimilator
CL0024	DTO 100-A6_*Penicillium radiatolobatum*	100	early assimilator

FigureIII.4 - Scoring PCP assimilators within the metacommunity of fungi confronted with PCP: **(a)** OTUs corresponding to early-, steady-, late- and non- assimilators by the spatial ordination of the normalised OTUs upon multidimensional scaling (MDS) of the constructed Bray-Curtis similarity matrix. **(b)** The 17 OTUs identified as the most abundant ^{13}C-labelled assimilators. The OTUs capable of assimilating ^{13}C-labelled were separated in the heavy DNA fraction by isopycnic ultracentrifugation. Major alterations in the abundance of the normalised OTUs were identified using the R-based package *DEseq2.*

Furthermore, their incorporation of ^{13}C-labelled DNA throughout the exposure period suggests distinct roles in the mineralisation of PCP, either as early (^{e}), steady (^{s}) or late (^{l}) assimilators. In this way, the major ^{13}C assimilators with regard to the number of OTUs *per* order were: Hypocreales (1^{s}, 3^{l} and 3^{e}), Eurotiales (2^{s}, 1^{l} and 1^{e}), Trichosporonales (1^{s}, 1^{l}), Filobasidiales (1^{s}, 1^{l}) and Tremellales (2^{e}) (Figure III.4, Table III.S4, flagged in Figure III.3a). Notably, the major assimilators also constituted the dominant taxonomic groups within the metacommunity during cultivation either in the presence (*ca.* 97%) or absence of PCP (*ca.* 81% and 86%, on the third and tenth day, respectively). In addition these taxonomic groups constituted *ca.* 23% of the total reads in the metacommunity inoculum, suggesting that the biocide has been influencing the mycobiota composition *in situ*.

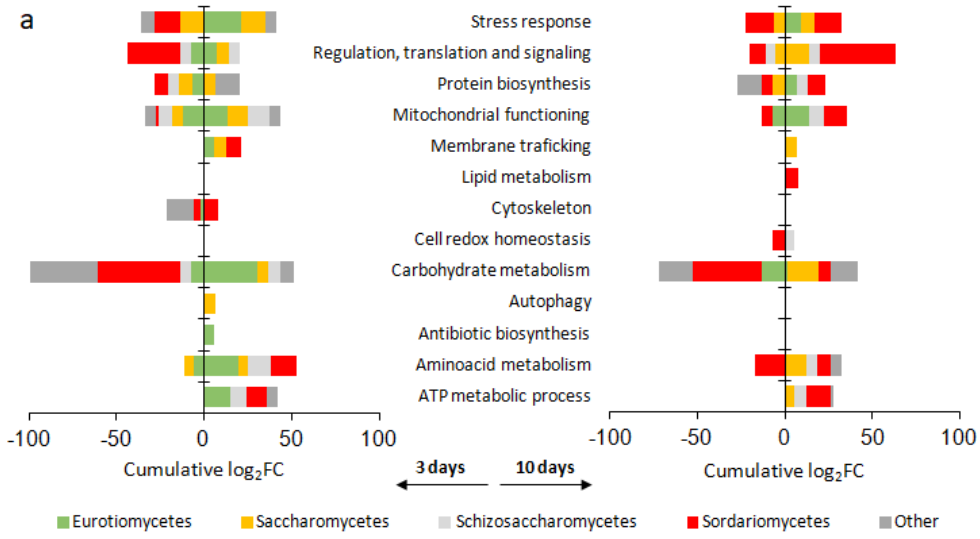
Unveiling the proteome responses of the metacommunity of fungi confronted with PCP

In addition to its effects on the metacommunity composition, PCP also greatly affected the utilisation profiles of carbon- and nitrogen-containing substrates. To verify if such alterations can be detected at the proteome level, changes in the levels of mycelial and extracellular proteins (*i.e.* secretome) of the metacommunity throughout the PCP exposure (relative to control conditions) were identified. The numbers of mycelial proteins with altered levels (relative to controls; referred to as differential proteins) were 94 and 74 on the third and tenth days of exposure to PCP, respectively. Of these, only 22 proteins were common to both sets. In the secretome these numbers were 10 and 15, with only 5 common to both time points (datasets ESI III.4 and ESI III.5).

The snapshot of the effects of PCP on the mycelial metaproteome at each time point is represented by the cumulative fold change (*i.e.* $\log_2$FC) of the differential proteins grouped according to Gene Ontology (GO) functional categories (Figure III.5a, Table III.S5). PCP affected many functional

categories, of which the most affected ($log_2FC \approx |50|$) on the third day were Carbohydrate Metabolism; Stress Response; Mitochondrial Functioning; Amino-acid Metabolism; and ATP Metabolism (Figure III.5a). On the tenth day the most affected were Carbohydrate Metabolism; Regulation, Translation and Signalling (Figure III.5a).

a



b

Accession	Protein	Function	3 days (log2FC)	10 days (log2FC)	SignalP	FSKB
A4QUT2*	Catalase-peroxidase 2	Stress response	7.22	-8.47	Yes	Yes
A6N6J0*	Endochitinase 46	Antagonistic activity	7.17	5.82	Yes	Yes
B0XXF8*	Endo-1,3-beta-glucanase	Sporulation	7.05	7.32	Yes	Yes
C8VG90	Aconitate hydratase	Carbohydrate metabolism	6.62		No	No
J9MJK9	Transaldolase	Carbohydrate metabolism		8.58	No	No
O13639	Adenosylhomocysteinase	Aminoacid metabolism		-7.02	No	Yes
P17729*	GAPDH1	Carbohydrate metabolism	8.19	6.18	No	Yes
P17730*	GAPDH2	Carbohydrate metabolism	6.85		No	Yes
P35049*	Trypsin	Aminoacid metabolism	7.95		No	Yes
P42882	HMP-P synthase	Aminoacid metabolism		6.71	No	Yes
Q00616*	Cytochrome P450 55A2	Stress response		6.33	No	Yes
Q03420*	Alkaline proteinase	Antagonistic activity	7.22	6.33	Yes	Yes
Q10318	Dihydroxy-acid dehydratase	Aminoacid metabolism		-6.61	No	Yes
Q4WLN1	Aconitate hydratase	Carbohydrate metabolism		-8.47	No	Yes
Q4WP12	PPIase B	Protein biosynthesis	6.34		No	Yes
Q7S986	GPI	Carbohydrate metabolism		7.32	No	Yes
Q8TFJ2	GAPDH	Carbohydrate metabolism		-7.02	No	Yes
Q96VJ7	NADP-GDH	Aminoacid metabolism	7.88		No	Yes
Q9HDT3	Enolase	Carbohydrate metabolism		-7.60	No	Yes
Q9HGU3	NADP-GDH	Aminoacid metabolism		-8.33	No	Yes

Figure III.5 - Snapshot of the alterations induced by PCP exposure in the proteome of the metacommunity of fungi compared to control conditions. **(a)** Changes in the levels of proteins identified in the mycelial proteome at each time point clustered *per* functional category (cumulative fold change, log_2FC) parsed into the assigned taxonomies (best match at the Uniprot database). **(b)** Changes in the

levels of proteins identified in the secretome, highlighting proteins possibly associated with fungal pathogenic and/or allergenic potentials (*). The accession number (best match in the Uniprot database), short name and functional category, fold change (log_2FC), signal peptide (SignalP) and presence (or not) at the Fungal Secretome Knowledge Base (FSKB), are indicated. The differential proteins were selected among the identified polypeptides using the R-based package *edgeR*

The most striking difference observed was the major down regulation of Carbohydrate Metabolism in the presence of PCP throughout the entire exposure period. Metataxonomics discloses the identities at the known taxonomic levels, whereas metaproteomics depends on the best hit of protein sequences available in databases, which is biased towards the best-studied taxa. This may explain the identification of many Saccharomycetes related differential proteins (*viz.* Saccharomycetales), of which diversity and abundance were minor factors in the metacommunity; it is possible that these proteins are actually associated with other yet overlooked Ascomycota. Despite this limitation, the majority of the differential proteins were assigned to model fungi related to the dominant taxa observed here (Figure III.3a), which also matched the taxa of the major ^{13}C-labelled PCP assimilators (Figure III.4), either Sordariomycetes (*viz.* Hypocreales) or Eurotiomycetes (*viz.* Eurotiales) (Figure III.5a). Sordariomycetes become the prominent group at both taxon and protein levels following 10 days of PCP exposure. No proteins related to Basidiomycota were identified, regardless of the fact that Trichosporonales, Filobasidiales and Tremellales, were abundant orders in the metacommunity exposed to PCP. On the other hand, many of the proteins exhibiting altered levels were associated with the Schizosaccharomycetes class, which was absent in the metacommunity. The assignment of proteins to a model fungus belonging to this class, does not take into consideration its high phylogenetic proximity to Basidomycota [30].

To investigate the biological significance of PCP effects on the mycelial metaproteome, we scrutinised the differential proteins exhibiting the highest log_2FC. Unsurprisingly, the changes in the levels of many glycolytic

enzymes were found to be amongst the largest changes observed ($|7.4| \leq \log_2 FC \leq |9.7|$), either when PCP levels were close to the EC_{50} [aldehyde dehydrogenase; phosphoglycerate kinase; pyruvate kinase; enolases; GAPDH (glyceraldehyde-3-phosphate dehydrogenases)] or three fold lower [GAPDHs; phosphoglycerate kinase; enolases] (Table III.S5, dataset ESI III.5). The levels of aldehyde and alcohol dehydrogenases greatly increased upon PCP exposure (P41751 and P08843, $\log_2 FC = 8.5$ and 7.42). These enzymes have been previously linked to the degradation of many aromatic hydrocarbons, *e.g.* naphthalene [31]. The involvement of these enzymes in the degradation of PCP (Figure III.1b, Table III.S6) is therefore a possibility. Many other enzyme classes have been linked to PCP degradation, *e.g.* cytochrome P-450 monooxygenases, tyrosinases, reductive dehalogenases and transferases [8]; none of which exhibited increased levels in the mycelial metaproteome following PCP exposure (dataset ESI III.5). Finally, the ability of the biocide to uncouple oxidative-phosphorylation in mitochondria [4] may be linked to the major dysregulation of some mitochondrial proteins [*viz.* aconitate hydratase, ATP synthase subunit alpha; citrate synthase; ($|7.1| \leq \log_2 FC \leq |8.5|$] (Table III.S5, dataset ESI III.5). The levels of several mycelial proteins associated with the Stress Response functional category were also greatly increased by exposure to PCP [*viz.* Heat shock protein (HSP) 70 kDa; HSP SSA1; thiamine thiazole synthases; ($|7.5| \leq \log_2 FC \leq |9.2|$] (Table III.S5). Increase levels of HSP, which act as molecular chaperones, assisting the correct folding of native and stress accumulated misfolded proteins, were reported in *Mucor plumbeus* exposed to PCP [9]. Thiamine thiazole synthase levels increased during adaptation to various stress conditions and are possibly involved in DNA damage tolerance [32].

Regardless of the fact that only a small number of proteins were identified in the differential extracellular metaproteome, it adds important details to the snapshot analysis of the biocide effects (Figure III.5b). Incorporation of data from the extracellular metaproteome adds Sporulation

and Antagonistic Activity to the list of affected functional categories (at both time points sampled post PCP exposure). PCP also affected extracellular proteins involved in Carbohydrate Metabolism, Amino-acid Metabolism, Protein Biosynthesis and Stress Response; a similar pattern to that observed in the mycelia (Figure III.5b). Amongst the extracellular proteins showing increased levels following 10 days of PCP exposure we found cytochrome P450 (CYP55A2, $\log_2$FC=6.3, Figure III.5b). Cytochrome P450 may play a role in the degradation pathway of PCP [8].

The burden of PCP augments the pathogenic potential of the metacommunity of fungi

When confronted with PCP, the metacommunity adjusted in order to degrade it, leading ultimately to its mineralisation (Figure III.1-3). This was achieved via a trade-off resulting in the impairment of many functional categories (Figure III.5) and the increased use of varied nitrogen-containing substrates (Figure III.1c). The ability of fungi to resort to varied nitrogen sources, either to bypass nitrogen starvation or any other conditional metabolic limitation is critical for the establishment of pathogenicity and considered as a virulence trigger. Taken as examples, the deletion of the gene encoding GAT1, which impaired nitrogen utilisation in *Candida albicans*, lowered its virulence in murine models [33]; whereas deletion of AreA/Nit2 gene encoding the transcription factor that controls the expression of genes involved in the transport/catabolism of nitrogen, was demonstrated to severely weaken phytopathogenicity in *e.g. Magnaporthe grisea*, and *Fusarium oxysporum* [34].

In line with the hypothesis that PCP may trigger pathogenicity in the metacommunity of fungi, we observed here that nearly half (8 out of 20) of the extracellular proteins that were increased at either sampled time point following PCP exposure, have been associated with pathogenic or allergenic potentials, namely GAPDH [35], trypsin [36], catalase-peroxidase [37], alkaline proteinase [38], endochitinase [39]; endo-1,3-β-glucanase [40] and

cytochrome P450 55A2 (CYP55A2) (Figure III.5b, marked with an asterisk). In addition, throughout incubation in the presence of PCP, the metacommunity greatly increased the pH of the medium, which was unaltered in the controls (Figure III.S2). The ability of fungi to change the surrounding pH and to grow at alkaline conditions has also been recognized as a key pathogenicity marker [41]. Finally, the metacommunity grown in media without PCP, after acute treatment with miconazole showed negligible metabolic activity ($absorbance_{(570nm)} = 0.07 \pm 0.02$), as expected (Figure III.S3). On the contrary, and remarkably, following only ten days of PCP exposure, the capacity of the metacommunity to bypass the effect of the fungicide increased dramatically ($absorbance_{(570nm)} = 0.3 \pm 0.07$), with a concurrent increase in pathogenic potential [42].

Conclusions

Our results support the hypothesis that fungi pay a high functional cost during exposure to PCP pollution, regardless of superior capacity to degrade the biocide. In a culture-dependent set-up, PCP affected the overall diversity of the metacommunity, in particular it reduced the diversity of the less abundant taxa and promoted also the growth of the most abundant taxa, most of which were capable of assimilating, to some extent, the biocide. Carbohydrate Metabolism was critically hindered throughout the entire exposure time, despite the fact that PCP levels were progressively reduced. The metacommunity of fungi circumvented the impacts of the biocide by utilising a variety of nitrogen-containing substrates, which potentially functioned as a virulence trigger. Essentially, PCP exposure greatly reduced the overall susceptibility of the fungal metacommunity to a fungicide and elicited the secretion of proteins that have been found to be associated with pathogenesis.

Atmospheric release of POPs constitutes a silent threat through the chronic contamination of soils on a global scale; yet a fundamental understanding of their impacts is still mostly lacking. The findings of our

study extend far beyond the specific issue of PCP pollution. PCP can be considered an archetypal POP since its simple structure – a halogenated aromatic – is found in many POPs that are potentially degraded through biotic pathways that converge on the same pathways used in PCP degradation [8, 11]. Our approach does not intend to directly simulate the natural setting, but it did allow capturing key specialisation events of the metacommunity exposed to the biocide at multiple functional and taxonomic levels; this despite the study's limits: the loss of some uncultivable taxa and assignment of most proteins sequences to well-known taxa. In this study, the taxa identified as dominant throughout PCP exposure were among the most abundant in the initial soil sample. One hypothesis is that the metacommunity of fungi, which originated from PCP polluted soils, has been long-suffering the impacts of PCP at both functional and diversity levels. One critical question to be addressed in the near future is how the metacommunity of fungi evolves under chronic PCP exposure conditions, especially as a further imbalance of nitrogen utilisation, with a consequential rise of opportunistic fungal pathogens, should not be ignored. The depletion of nitrogen sources from soils potentially impacts the continuous supply of ecosystem services (*e.g.* soil fertility and water retention capacity). Therefore our experimental observations indirectly interrogate if the atmospheric deposition of PCP in cork oak forests may be behind the deterioration observed in these agro-forestry landscapes. Surely, the importance of the link between pollution with PCP (or with other POPs containing similar structural units) and the increase of pathogenic potential in fungi goes way beyond matters of forest sustainability. Annually, fungi kill nearly 2 million people, worldwide [43]. The inhalation of fungal spores, even of non-pathogenic fungi, can lead to devastating invasive infections in vulnerable immunocompromised/suppressed patients of all ages. The stimulation of increased fungal pathogenicity due to POP exposure is not something that can be ignored.

Availability of data and material

The Electronic Supplementary Information word document is available in the Annex at the end of this chapter, containing more detailed tables and figures that support the Figure panels at the main text. Electronic Supplementary Data files are available in the electronic version of this thesis. Electronic Supplementary data file ESI III.1 provides mass spectrometry datasets (xls format) on the metabolomics of the metacommunity. Electronic Supplementary data file ESI III.2 contains Biolog FF datasets (xls format): normalised datasets of the absorbance of each substrate, disclosing alterations upon exposure to PCP compared to control conditions. Electronic Supplementary data file ESI III.3 provides the amplicon sequencing raw count data, including description of the identified OTUs and discrimination of OTUs as 13C-labelled PCP assimilators. Electronic Supplementary data file ESI III.4 shows the mass spectrometry datasets on the proteomes of the metacommunity. Electronic Supplementary data file ESI III.5 lists all the mycelial proteins that underwent alterations after exposure to PCP compared to control conditions.

The amplicon sequencing data has been deposited in the Sequence Read Archive (NCBI) with the submission code SRP145967. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [44] partner repository with the dataset identifier PXD009798 and 10.6019/PXD009798.

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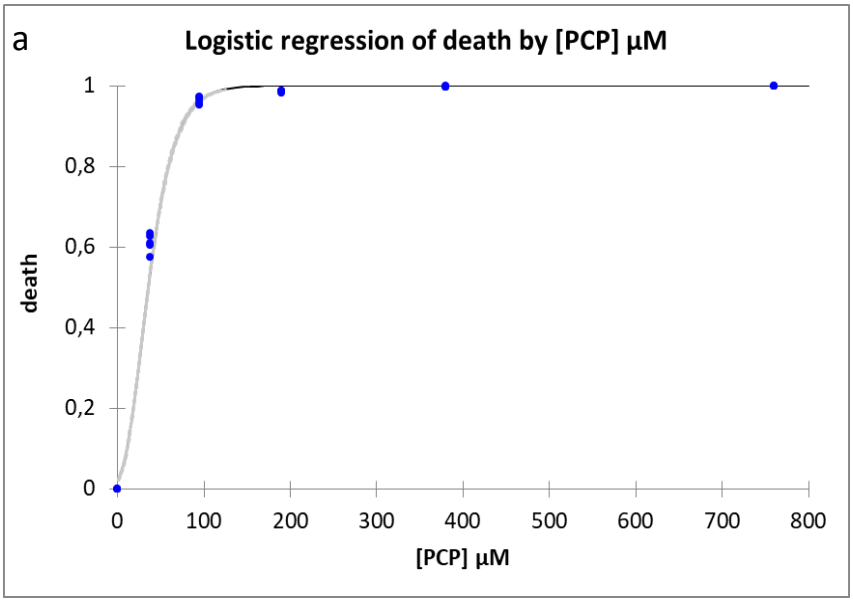
ANNEX

This annex contains the Electronic Supplementary Material published along the main body of the research paper which composes Chapter II. This intends to allow better and easier understanding of the methodologies and analyses performed.

a



b

EC	[PCP] µM	Lower bound 95%	Upper bound 95%
0.01	-3.147	-3.282	-3.012
0.05	5.660	5.548	5.772
0.10	11.049	10.949	11.149
0.20	18.385	18.297	18.472
0.30	24.329	24.248	24.410
0.40	29.921	29.842	30.001
0.50	35.637	35.555	35.720
0.60	41.888	41.798	41.979
0.70	49.245	49.140	49.350
0.80	58.851	58.722	58.980
0.90	74.220	74.048	74.393
0.95	88.962	88.745	89.181
0.99	122.345	122.019	122.672

Figure III.S1 - Half maximal Effective Concentration of PCP against the metacommunity of fungi. **(a)** The logistic regression of death (CFU counts) in community-based cultures exposed to PCP, fitted using Gompertz distribution is displayed. **(b)** The model prediction for the toxicity levels of the chemical is shown. The defined EC_{50} value was estimated as 35.637 µM, accordingly for simplicity in further experiments 38 µM PCP (= 10 $mg.L^{-1}$) was used.

Table III.S1 - PCP-derived metabolites identified in both mycelial and extracellular culture fractions, as well as in the abiotic controls. Full information is available in the Supplementary dataset ESI2.

Compound	Mycelia				Extracellular				Abiotic Control		
	3 days	5 days	7 days	10 days	3 days	5 days	7 days	10 days	1	2	3
Trihydroxybenzene					✓	✓	✓	✓			
Maleylacetate		✓	✓				✓	✓			
3-oxoadipate		✓					✓	✓			
Tetrachlorohydroquinone		✓	✓		✓						
Tetrachlororesorcinol					✓						
Tetrachlorocathecol	✓	✓	✓	✓	✓				✓	✓	✓
Tetrachlorophenol	✓				✓				✓	✓	✓
Pentachlorophenol	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

Table III.S2 - Utilisation profile of substrates by the metacommunity along the PCP exposure time, displaying the most increased (left) and decreased (right) sources after 3 and 10 days compared to controls. Full list is available in the Supplementary dataset ESI3.

	Functional category	C/N source	increase (%)	C/N source	decrease (%)
3 days	Amines/Amides	2-Amino Ethanol	36.73	Glucuronamide	52.24
	Aminoacids	Glycyl-L-Glutamic acid	32.30		
	Carbohydrates	Maltitol	45.95	D-Arabinose	72.27
		L-Sorbose	37.20	L-Fucose	70.49
				N-Acetyl-D-Galactosamine	70.44
				Lactulose	69.35
				α-D-Lactose	63.68
				Sedoheptulosan	54.45
				β-Methyl-D-Galactoside	48.62
	Carboxylic acid	N-Acetyl-L glutamic acid	55.02	D-Galacturonic acid	85.21
	Miscellaneous	Adenosine-5'Monophosphate	80.39	D-Lactic Acid Methyl Ester	46.42
		Uridine	80.17		
		Bromosuccinic acid	41.34		
		Salicin	38.25		
	Polymers			β-Cyclodextrin	100.00
10 days	Amines/Amides	Alaninamide	56.74	Glucuronamide	100.00
				D-Glucosamine	42.90
	Aminoacids	L-Phenylalanine	45.14		
	Carbohydrates	N-Acetyl-D-Galactosamine	62.37	N-Acetyl-D Mannosamine	100.00
		D-Xylose	30.69	D-Tagatose	94.94
				L-Arabinose	89.68
				Lactulose	81.65
				D-Arabinose	70.53
				Sedoheptulosan	53.19
				α-D-Lactose	47.48
				L-Fucose	45.22
				m-Inositol	41.48
				D-Cellobiose	37.00
				α-Methyl-D-Glucoside	36.89
	Carboxylic acid	D-Glucuronic Acid	36.18	D-Galacturonic acid	80.06
		N-Acetyl-L glutamic acid	34.25	γ-Hydroxy-butyric acid	44.32
	Miscellaneous	Adenosine	86.13	Succinic Acid Mono-Methyl Ester	44.09
		Uridine	62.40		
		Bromosuccinic acid	36.09		
	Polymers			α-Cyclodextrin	100.00

Table III.S3 - Results of the DESeq2 analysis (R based package) regarding the differential abundance (log_2FC) of OTU's at the third and the tenth day of exposure to PCP compared to controls, using raw and normalized data. To identify the OTU's able to assimilate ^{13}C PCP we have run comparisons of the controls with either the light or heavy DNA fractions, and between both light and heavy DNA fractions at two time points, as well as of each fraction between time points. No differential abundant OTU's were attained between the light and heavy DNA fractions, possibly because ^{13}C was not homogeneously assimilated by all the fungal cells.

		Control vs. Light 3 days		**Control vs Heavy 3 days**		**Control vs. Light 10 days**		**Control vs Heavy 10 days**		**Light 3 days vs Light 10 days**		**Heavy 3 days vs Heavy 10 days**	
		log_2FC	*p*-value	log_2FC	*p*-value	log_2FC	*p*-value	log_2FC	*p*-value	log_2FC	*p*-value	log_2FC	*p*-value
Normalized Counts	CL0003											1.54	2E-02
	CL0004			2.79	5E-02								
	CL0006					3.34	4E-02	3.21	5E-02	-3.31	4E-04	-3.48	8E-07
	CL0007									-2.98	6E-03	-2.32	7E-03
	CL0008					-7.67	5E-05	-7.51	1E-04	9.73	2E-12	9.39	3E-15
	CL0009	-2.40	4E-02	-2.13	9E-03								
	CL0010									-4.36	2E-04	-4.10	3E-04
	CL0011									4.95	5E-06	4.31	5E-05
	CL0012	-10.44	3E-09	-10.07	1E-09								
	CL0015	-7.08	4E-05	-6.15	1E-04								
	CL0020							-8.56	5E-05				

Table III.S4 - The relative abundances of sequences of the OTUs able to assimilate ^{13}C-labelled PCP (counts normalized and set to integers); OTUs with relative abundance above 100 counts, are listed. The 17 OTU's presenting over 1,000 normalized counts (bolded) constitute over 97% of the total abundance of the metacommunity exposed to PCP. The abundance variations in the heavy DNA fraction at the third and the tenth day of exposure were used to classify the OTU's regarding their timing in the assimilation of ^{13}C PCP: "early" assimilators, OTU's that present high abundance in the heavy fraction at early incubation with PCP, decreasing at the tenth day; "late" assimilators, OTU's that are very abundant in the heavy fraction at later incubation with PCP, but are less abundant at the third day; and "steady" assimilators, OTU's that are very abundant in the heavy fraction at both incubation times. Full information on the OTU's, raw counts and the list of all OTU's with abundance above 100 normalized counts (assimilators and non assimilators) is available in Supplementary dataset ESI4.

OTU	ID	Similarity (%) NCBI	Control 3 days	PCP Light 3 days	PCP Heavy 3 days	Control 10 days	PCP Light 10 days	PCP Heavy 10 days	Assimilation category
CL0001	**KY037850_*Vanrija albida***	100	23085	19199	18628	28140	15016	13731	**steady assimilator**
CL0002	**DTO 099-G3_*Fusarium oxysporum* species complex**	100	9818	7749	7353	26369	16012	16546	**late assimilator**
CL0003	**MG725820_*Trichoderma* sp.**	100	7186	27317	20927	9831	7115	7247	**early assimilator**
CL0004	**KY104335_*Naganishia randhawae***	99.5	4059	14712	19972	981	9267	10071	**steady assimilator**
CL0005	**DTO 099-F7_*Penicillium vagum***	100	5439	13506	10962	1966	11727	10483	**steady assimilator**
CL0006	**HG008760_*Trichoderma* sp.**	100	1619	1833	1556	1576	19807	18834	**late assimilator**
CL0007	**KY037851_*Solicoccozyma phenolicus***	100	9468	807	1500	4188	7264	7483	**late assimilator**
CL0008	**KX302067_*Trichosporon* sp.**	100	5059	6562	8034	4695	5	12	**early assimilator**
CL0009	**AB737864_*Trichoderma hamatum***	100	9599	1072	1418	4355	1237	1876	**steady assimilator**
CL0010	**KU987904_*Acremonium* sp.**	99.4	481	197	246	1362	4051	5560	**late assimilator**
CL0011	**KY320605_*Saitozyma podzolica***	100	3713	3140	4945	2248	107	200	**early assimilator**
CL0013	**MG711821_*incertae sedis***	100	625	737	602	376	2422	1788	**late assimilator**
CL0014	**KY315586_*Talaromyces verruculosus***	100	1	9	4	2	1783	2422	**late assimilator**
CL0016	**DTO 099-E4_*Penicillium restrictum* species complex**	100	374	821	1000	191	788	621	**steady assimilator**
CL0021	**KY101694_*Apiotrichum vadense***	100	68	31	58	20	661	668	**late assimilator**
CL0022	**KY687957_*Trichoderma linzhiense***	100	178	108	135	110	323	433	**late assimilator**
CL0024	**DTO 100-A6_*Penicillium radiatolobatum***	100	222	324	467	130	95	83	**early assimilator**
CL0026	KY687942_*Trichoderma hirsutum*	99.4	51	62	80	11	273	231	late assimilator
CL0029	KJ542213_*Thelonectria* sp.	100	47	252	383	54	0	1	early assimilator
CL0030	KX100356_*Fusarium* sp.	100	311	119	132	164	15	25	early assimilator

OTU	ID	Similarity (%) NCBI	Control 3 days	PCP Light 3 days	PCP Heavy 3 days	Control 10 days	PCP Light 10 days	PCP Heavy 10 days	Assimilation category
CL0031	KY105430_*Solicoccozyma aeria*	100	163	141	226	230	1	1	early assimilator
CL0032	AF414294_*Trichoderma hamatum*	95.9	5	12	18	2	223	225	late assimilator
CL0034	NR_152959_*Mucor moelleri f. californiensis*	100	0	3	6	13	214	51	late assimilator
CL0035	MG193751_*Trichoderma pubescens*	97.1	106	27	26	38	55	75	late assimilator
CL0036	DTO 099-F5_*Aspergillus tubingensis*	100	95	51	29	22	51	66	steady assimilator
CL0038	AB986458_*Oidiodendron* sp.	97.2	0	0	0	0	49	175	late assimilator
CL0043	*incertae sedis*		1	3	20	0	88	87	late assimilator
CL0046	*incertae sedis*_2		1	1	4	0	100	85	late assimilator
CL0050	LC177644_*Sagenomella striatispora*	100	1	5	4	2	78	72	late assimilator
CL0052	LC177652_*Sagenomella verticillata*	100	68	73	75	18	0	0	early assimilator
CL0054	DTO 099-G3_1_*Fusarium oxysporum* species complex	100	38	36	50	9	2	1	early assimilator
CL0055	MG722763_*Ustilago kamerunensis*	96.7	81	53	15	38	5	11	steady assimilator
CL0058	KY687942_1_*Trichoderma hirsutum*	99.4	28	35	45	2	19	15	steady assimilator
CL0059	KY101666_*Apiotrichum dulcitum*	100	0	0	1	2	53	52	late assimilator
CL0064	JX030261_*Tricholomataceae* sp.	99	22	7	17	38	19	15	steady assimilator
CL0065	*incertae sedis*_3		17	68	34	15	0	0	early assimilator
CL0067	LK052843_*incertae sedis*	98.6	26	12	23	2	20	20	steady assimilator
CL0068	KX302022_*Trichosporon akiyoshidainum*	100	9	14	15	15	29	19	steady assimilator
CL0069	DTO 099-E1_*Penicillium murcianum*	100	1	22	42	2	30	6	early assimilator
CL0071	KX911872_*Gongronella butleri*	99.4	85	7	23	6	0	0	early assimilator
CL0073	FJ265759_*Mycosphaerella* sp.	87.6	19	14	15	36	11	13	steady assimilator

Table III.S5 - Mycelial proteins with major fold changes in abundance (log_2FC) after PCP exposure for three and ten days compared to controls. Full data of the identified proteins is available in Supplementary dataset ESI5 and all fold changes are available in Supplementary dataset ESI6.

	Functional category	3 days			10 days		
		Accession	Protein name	log_2FC	Accession	Protein name	log_2FC
Up-regulated	*Aminoacid metabolism*	Q12650	Sulfate adenylyltransferase	**7.16**	P05694	5-methyltetrahydropteroyltriglutamate homocysteine methyltransferase	**6.43**
					Q9HFR6	NADP-specific glutamate dehydrogenase	**6.57**
	ATP metabolic process	P22068	ATP synthase subunit β	**9.36**	P07038	Plasma membrane ATPase	**8.45**
		P85446	ATP synthase subunit β	**9.06**	P28876	Plasma membrane ATPase 2	**7.49**
	Carbohydrate metabolic process	P41751	Aldehyde dehydrogenase	**7.42**	Q00640	Glyceraldehyde-3-phosphate dehydrogenase	**8.22**
		Q12560	Enolase	**7.89**	P32636	Glyceraldehyde-3-phosphate dehydrogenase 2	**7.10**
	Mitochondrial functioning	C8VG90	Aconitate hydratase	**7.25**	P24487	ATP synthase subunit alpha, mitochondrial	**8.45**
					P51044	Citrate synthase, mitochondrial	**7.49**
	Protein biosynthesis	Q00251	Elongation factor 1-alpha	**7.84**	P34825	Elongation factor 1-α	**8.30**
					Q9Y713	Elongation factor 1-α	**7.10**
	Regulation, translation and signaling	B6H2I7	40S ribosomal protein S0	**7.57**	Q6FR65	GTP-binding nuclear protein GSP1/Ran	**7.01**
					C7YTD6	40S ribosomal protein S1	**7.19**
	Stress response	P08843	Alcohol dehydrogenase	**8.50**	Q5B2V1	Heat shock 70 kDa protein	**9.20**
		G5EAZ2	Thiamine thiazole synthase	**7.25**	P41797	Heat shock protein SSA1	**7.62**
					C7Z8P6	Thiamine thiazole synthase	**7.49**

	Functional category	3 days			10 days		
		Accession	Protein name		Accession		Protein name
Down-regulated	*Aminoacid metabolism*				P00369	NADP-specific glutamate dehydrogenase	**-7.01**
	Carbohydrate metabolic process	Q7RV85	Enolase	**-8.55**	Q7RV85	Enolase	**-9.67**
		Q6W3C0	Enolase	**-8.15**	P54118	Glyceraldehyde-3-phosphate dehydrogenase	**-8.44**
		Q00640	Glyceraldehyde-3-phosphate dehydrogenase	**-8.58**	P87197	Glyceraldehyde-3-phosphate dehydrogenase	**-7.92**
		P35143	Glyceraldehyde-3-phosphate dehydrogenase	**-8.15**	Q9HGY7	Glyceraldehyde-3-phosphate dehydrogenase	**-7.73**
		P41756	Phosphoglycerate kinase	**-7.44**	P14228	Phosphoglycerate kinase	**-7.88**
		P31865	Pyruvate kinase	**-8.04**			
	Cytoskeleton	Q6TCF2	Actin	**-8.92**			
	Mitochondrial functioning	P24487	ATP synthase subunit alpha, mitochondrial	**-8.15**	O00098	Citrate synthase, mitochondrial	**-7.10**
	Protein biosynthesis				A4QVP2	ATP-dependent RNA helicase eIF4A	**-7.51**
					P23301	Eukaryotic translation initiation factor 5A-1	**-7.10**
	Regulation, translation and signaling	B0XWG9	40S ribosomal protein S0	**-7.32**	Q01291	40S ribosomal protein S0	**-7.83**
	Stress response	P78695	78 kDa glucose-regulated protein homolog	**-7.75**	J9N5G7	Thiamine thiazole synthase	**-8.35**
		P41797	Heat shock protein SSA1	**-7.70**	P23617	Thiamine thiazole synthase	**-7.68**

Table III.S6 - Extracellular proteins that showed altered levels following PCP exposure for three (top, light grey) and ten (bottom, darker grey) days. The log_2FC is displayed, as well as full information on the proteins. Entries that have been previously associated with pathogenesis, virulence or allergenic activity are marked with an *. Full data is available in Supplementary dataset ESI5.

	Accession	log_2FC	Order	Protein	Function	SignalP	FSKB
3 days	P17729*	8.19	Hypocreales	Glyceraldehyde-3-phosphate dehydrogenase 1	Carbohydrate metabolic process	No	Yes
	P35049*	7.95	Hypocreales	Trypsin	Aminoacid metabolism	No	Yes
	Q96VJ7	7.88	Hypocreales	NADP-specific glutamate dehydrogenase	Aminoacid metabolism	No	Yes
	A4QUT2*	7.22	Magnaporthales	Catalase-peroxidase 2	Stress response	Yes	Yes
	Q03420*	7.22	Sordariomycetes	Alkaline proteinase	Antagonistic or mycoparasitic activity	Yes	Yes
	A6N6J0*	7.17	Hypocreales	Endochitinase 46	Antagonistic or mycoparasitic activity	Yes	Yes
	B0XXF8*	7.05	Eurotiales	Glucan endo-1,3-beta-glucosidase	Sporulation	Yes	Yes
	P17730*	6.85	Hypocreales	Glyceraldehyde-3-phosphate dehydrogenase 2	Carbohydrate metabolic process	No	Yes
	C8VG90	6.62	Eurotiales	Aconitate hydratase	Carbohydrate metabolic process	No	No
	Q4WP12	6.34	Eurotiales	Peptidyl-prolyl cis-trans isomerase B	Protein biosynthesis	No	Yes
10 days	J9MJK9	8.58	Hypocreales	Transaldolase	Carbohydrate metabolic process	No	No
	B0XXF8*	7.32	Eurotiales	Probable glucan endo-1,3-beta-glucosidase	Sporulation	Yes	Yes
	Q7S986	7.32	Sordariales	Glucose-6-phosphate isomerase	Carbohydrate metabolic process	No	Yes
	P42882	6.71	Eurotiales	4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate	Aminoacid metabolism	No	Yes
	Q00616*	6.33	Hypocreales	Cytochrome P450 55A2	Stress response	No	Yes
	Q03420*	6.33	Hypocreales	Alkaline proteinase	Antagonistic or mycoparasitic activity	Yes	Yes
	P17729*	6.18	Hypocreales	Glyceraldehyde-3-phosphate dehydrogenase 1	Carbohydrate metabolic process	No	Yes
	A6N6J0*	5.82	Hypocreales	Endochitinase 46	Antagonistic or mycoparasitic activity	Yes	Yes
	A4QUT2*	-8.47	Magnaporthales	Catalase-peroxidase 2	Stress response	Yes	Yes
	Q4WLN1	-8.47	Eurotiales	Aconitate hydratase	Carbohydrate metabolic process	No	Yes
	Q9HGU3	-8.33	Sordariales	NADP-specific glutamate dehydrogenase	Aminoacid metabolism	No	Yes
	Q9HDT3	-7.60	Pleosporales	Enolase	Carbohydrate metabolic process	No	Yes
	O13639	-7.02	Schizosaccharomycetales	Adenosylhomocysteinase	Aminoacid metabolism	No	Yes
	Q8TFJ2	-7.02	Agaricales	Glyceraldehyde-3-phosphate dehydrogenase	Carbohydrate metabolic process	No	Yes
	Q10318	-6.61	Schizosaccharomycetales	Putative dihydroxy-acid dehydratase	Aminoacid metabolism	No	Yes

Complementary assays to assess pathogenic potential

Experimental details: Metacommunity-based cultures grown for ten days in medium with 38 µM of PCP, or in a control medium, were exposed or not to 0.1 $mg.mL^{-1}$ miconazole for 24h (30°C, 90 rpm). At the end of the acute treatment with the antifungal compound, the metabolic activity of the cultures was estimated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. Briefly, the cultures were treated with 1/10 volume of 5 $mg.mL^{-1}$ MTT in 1% NaCl (4h, 30°C, 90 rpm), then after removal of the medium, an equal volume of 0.1M HCl in isopropanol was added (4h, 30°C, 90 rpm). The supernatants' absorbance measured at 570 nm and 630 nm to remove noise in the background; Student's *t*-test and Kruskal-Wallis *H* test were used to infer (and confirm) significant differences between conditions (marked with different letters).

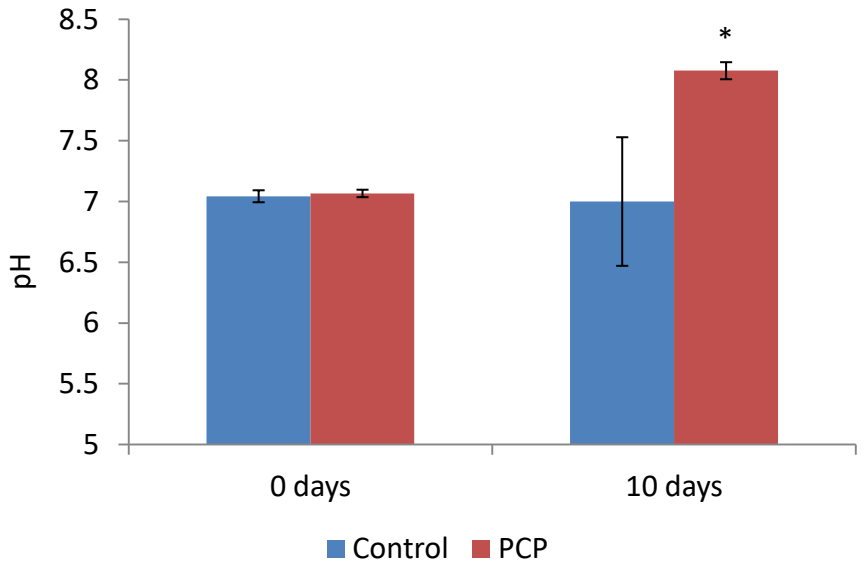


Figure III.S2 - The pH of the media of the metacommunity-based cultures exposed or unexposed to PCP was measured at the start and at the tenth day of incubation. The pH of the medium at the last incubation time point increased significantly (marked with *) only in the metacommunity cultures exposed to PCP.

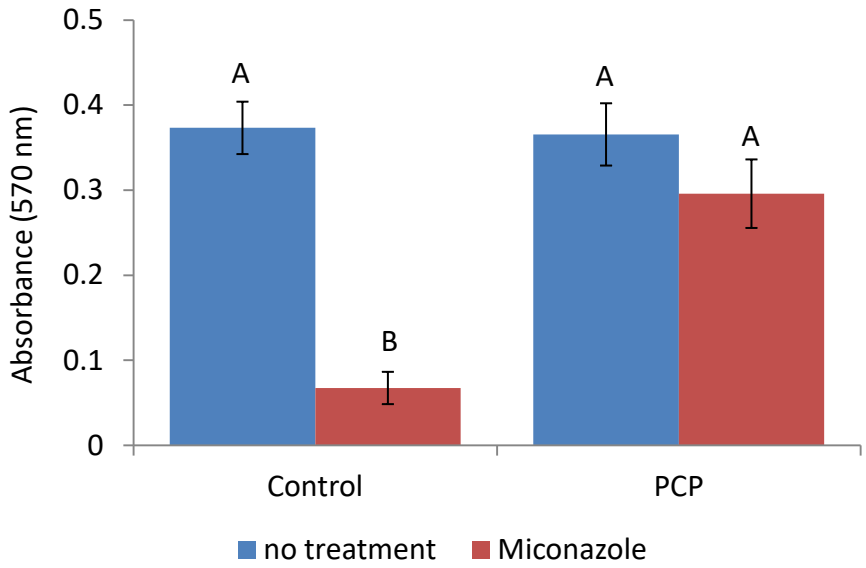


Figure III.S3 - Metabolic activity of metacommunity-based cultures exposed, or unexposed, to PCP during ten days, in the presence of miconazole (acute effect). The antifungal compound significantly reduced the metabolic activity of control cultures to a minimum, but not of the metacommunity exposed to PCP (the range of absorbance values to metabolic active cells is 0.2 – 0.7).

CHAPTER IV

This chapter contains preliminary unpublished data of a manuscript in preparation

Outline: The following chapter presents preliminary work which aims at demonstrating the potential of pollutants to trigger fungal virulence against animals. Herein we describe a custom made incubation apparatus which was designed to allow collection of airborne spores. In addition, the strategy used to test their impact *in vivo* focussing on the model *Galleria mellonella* is described. Though preliminary, this Chapter contains relevant data that will be used in a manuscript to be soon published.

Pollutants are drivers of increased virulence in fungi

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Introduction

Anthropogenic-related alterations in the environment have been extensively discussed in the past few years [1]. Among these alterations, climate change is the most publicized as it is believed to be a direct consequence of human activity, among which the contribution of chemical pollution is notorious [1, 2]. The shifts caused by climate change are widely publicized, and comprise geological/geochemical or atmospheric alterations that translate into severe consequences (natural disasters, global warming and drought) that impact either directly or indirectly human lives [2]. The 2018 Nobel Prize in Economics demonstrated that the impacts of climate change clearly transfer from the environment to the economy, adding impressive economic burdens to this issue [3]. One possible unseen consequence of such abrupt environmental changes caused by pollution is the rise of novel microbial pathogens [4]. In the previous Chapters of this thesis we demonstrated that

a metacommunity of fungi was able to effectively degrade the archetypal persistent organic pollutant (POP) pentachlorophenol [5], but this process elicited pathogenic tradeoffs, namely the dysregulation of the carbon and nitrogen metabolisms, the secretion of proteins associated with pathogenesis and a substantial decrease in the susceptibility of the metacommunity to a common fungicide [5]. The increased burden of fungal infections in humans, emergence of azole-resistant fungi (*e.g. Candida auris*) and cross-kingdom pathogens (phytopathogens that acquire capacity to infect animals) [6, 7] has been linked to many contributing factors, including climate change, but a direct link with the exposure to pollutants remains overlooked. Importantly, pollution has been demonstrated to contribute to the spread of antimicrobial resistant genes across some pathogenic bacteria [8]. Therefore, the hypothesis that pollutants in the environment might act as global drivers of acquired pathogenicity has gained momentum in the recent past. This is a timely topic, and its urgency is emphasised in the priorities of the United Nations "2030 Agenda for Sustainable Development", specifically addressing the Goals 3, 13 and 15. These goals consider that it is mandatory to take measures that promote healthy lives (Goal 3), take urgent action to combat and expose climate change and its impacts (Goal 13), and to help protecting, restoring and promoting the sustainable use of terrestrial ecosystems and sustainable management of forest (Goal 15). Moreover, a new group of emergent critical pollutants is now receiving great attention, as they are consistently found in wastewaters and agricultural and suburban soils due to their wide use as pharmacological and veterinary drugs [9]. These are classified as Contaminants of Emergent Concern (CECs), which include triclosan (TCS) that was recently banned as a pesticide in EU. TCS is also recalcitrant in soils [9, 10], where it may impose severe stresses to soil microbiota. Moreover its chemical structure comprises halogenated aromatics, similar to that of PCP. The aim of the present study is to analyse if both PCP and TCS can increase pathogenic potential as well as virulence in belowground fungi,

focussing on two banned pesticides: PCP and TCS. We designed a original strategy to assess if exposure to either toxicant is a driver of “rapid” acquired pathogenicity in microorganisms. This chapter contains data regarding the production and collection of airborne fungal spores upon exposure to the selected pollutants, the morphological features of the collected spores, the degradation rates of the toxicants and preliminary *in vivo* infection studies.

Materials and Methods

Chemicals

Unless stated otherwise, all chemicals were purchased to Merck (Germany) at the highest purity grade available.

Experimental design

We designed and 3D printed an incubation box with the capacity to hold constant air flow and to accommodate membrane filters to collect airborne spores (see detailed description below). An electric circuit was built to operate motors capable to spin fans at steady rotation. The goal was to move the air inside each box - avoiding external interference with the system - pushing the airborne fungal spores in the direction of the membrane filter. The entire system was named MC3000. The fungal community that was used for these experiments is the same of previous chapters (collected in Tunisian cork oak forest soils). The incubation was performed using minimal medium (described in chapters II and III) [5, 11] containing 1% glucose and 0.5% Phytagel as setting agent, during 10 days at 30 °C. The used concentrations of toxicants were 38 μM (10 $mg.L^{-1}$) of PCP and 172 μM (50 $mg.L^{-1}$) of TCS, defined as the EC50 values for the fungal community by using a CFU based strategy as described in the Chapter IV of this thesis [5]. The boxes were sealed with parafilm before covering the top with the lid.

MC3000

The incubation boxes of the MC3000 system were built in polylactic acid printing filament, using an Anet A6 3D printer. Three distinct parts compose the incubation boxes - collection box, lid and membrane adaptor (Figure IV.1a)

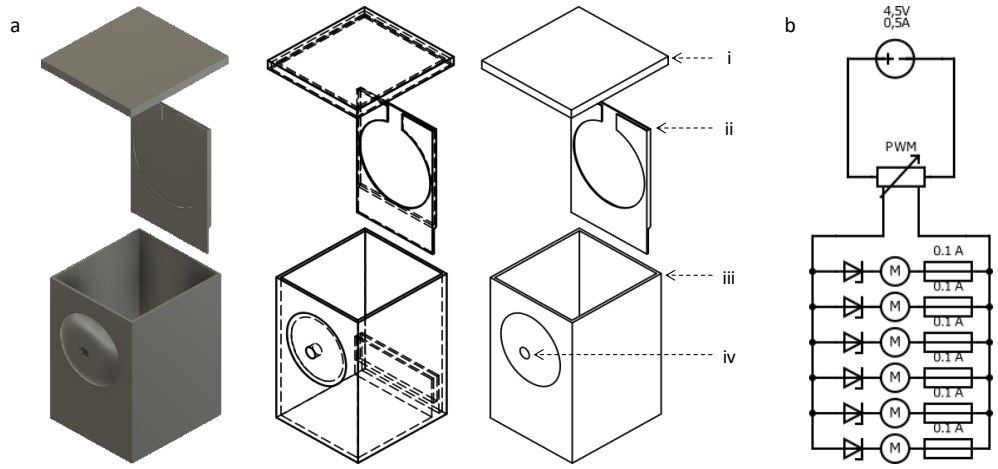

Figure IV.1 - MC3000 details: (a) three different perspectives of the MC3000 box. The components of the box are i) the lid, ii) the filter adapter, iii) the collection box which holds a hole for iv) the motor mount. (b) Schematic view of the electric circuit built to operate the spinning fans.

An electric circuit (Figure IV.1b) was built to operate the motors that spin fans at steady rotation. The circuit is powered by a DC adapter (4.5 V) and regulated with zener diodes (3.3 V). Fusing resistors (2 Watts) were used to protect the motors (3 V). This circuit allowed setting a customized intensity of air turbulence that was maintained steady in all experiments.

Organic extracts

To evaluate the decay of the pollutants after 10 days of incubation, the media (with fungal mycelia or without in the negative controls) were freeze dried and grinded using mortar and pestle, upon collection of fungal spores (see below). The grinded media were then extracted with HPLC grade methanol using an ultrasonic bath during 30 minutes. The samples were centrifuged at 13000*g* for 15 minutes at 4 °C, the supernatant recovered.

This step was repeated twice for every sample. The organic extracts were dried under a gentle air flow and stored until further analysis.

Chemical analyses

UPLC

The quantification of the toxics was performed by ultra-performance liquid chromatography as described previously [12]. Briefly, the chromatographic method consisted on a mobile phase, at a flow rate of 0.4 $mL.min^{-1}$, using a solution of 0.1% phosphoric acid (solvent A) and acetonitrile (solvent B), set as follows: a linear gradient of 10–95% B in 5.7 min, followed by 1.3 min to reach 100% B, 2.5 min to return to the initial conditions, and 2.5 min to re-equilibrate the column. The method was run in a Waters Acquity™ chromatograph, with a Photodiode Array Detector (PDA), temperature controlled autosampler, and column (Waters Corporation, USA). Data were acquired with the Empower 2 software, 2006 (Waters Corporation, USA). Injections of the extracts (20 μL) were made using a 50 μL loop, at partial-loop mode, with needle overfill. The separations were performed with an Acquity UPLC HSS C18 column (2.1 × 150 mm, 1.8 μm particle size) (Waters Corporation, USA), at a controlled temperature (35 °C). The chromatograms were acquired at 212 nm for PCP and 282 nm for TCS. Comparison with standards was used to identify PCP and TCS.

Collection of spores

The airborne fungal spores entrapped in the membrane filter were collected as follows: the membranes were carefully removed from the adaptor and immediately placed inside individual 50 mL falcon tubes. Then, a saline solution (0.9%) containing 0.1% of Tween 20 was added until covering the whole membrane. The tube was sealed, subjected to vigorous vortex cycles and then immersed into an ultrasonic bath (3x, 10 seconds each) to release the adherent spores. Additionally, 20 mL of saline solution containing 0.1% of Tween 20 was added to the surface of the cultures which were then

scraped with disposable cell scraper. The resulting suspension was passed through glass wool to remove the hyphal fragments. The spore's suspensions - from the filters and from the surface of the cultures - were centrifuged at 18000*g* for 20 minutes at 4 °C. The supernatants were discarded and the spore pellets were washed twice with saline solution. The spore suspensions were conserved in a glycerol containing (30%) solution at -80 °C until further use.

Optical microscopy: data acquisition and analysis

Aliquots of each sample (10 µL) were placed into a Neubauer chamber and the number of spores counted using a standard optical microscope at 400x magnification. The counts were registered and the cell densities calculated.

To study the sizes of the collected spores, 10 µL aliquots of each sample were stained using methylene blue to increase contrast; dilutions were performed whenever needed to improve cellular separation. The preparations were visualized using a DM5500 B microscope (Leica) at 630x (Leica HCX PL APO 63x, high resolution glycerol immersed objective) and images were captured with a DFC420C camera (Leica). At least 10 independent microscopic fields of each sample were captured for further processing. The images were then analysed with ImageJ (version 1.8.0_172) as follows: the size bar was used to set scale, the images converted to 8-bit format, black and white threshold used to remove noise, potential holes in the particles were digitally filled and the particle analysis command used. The results were trimmed (only areas above 5 μm^2 were considered) to remove remaining noise, registered and further analysed using XL-STAT (version 2014.5.03).

***In vivo* infection**

The infection studies were performed as previously described [13]. Briefly, aliquots of the stock spore suspensions were used to adjust the inoculum to reach 10^6 spores per larva at each tested condition. A micrometre was

adapted to control the volume of a microsyringe and inject 5 µl of fungal suspension (approx. 10^6 spores) into each larva via the hindmost left proleg, previously sanitized with 70% (v/v) ethanol. Following injection, larvae were placed in Petri dishes and stored in the dark at 30°C and 37°C. Blank larvae were injected with saline solution (pH 7·4). For each condition we used 10 larvae to follow the larval survival. Caterpillars were considered dead when they displayed no movement in response to touch.

Results and Discussion

Decay of the pollutants in solid media

The fungal community was able to promote the decay of both PCP and TCS. The decay level of PCP (70.8±3.6%) compared to abiotic controls (negligible decay) is comparable to that previously described by us using the same fungal community at a metacommunity experimental setup [5]. This observation suggests that the matrix formed by using the gelling agent can produce similar results in terms of degradation to those obtained using multi-well cultivation with the same minimal medium. The decay level of TCS (94.9±2.2%) is impressive, and underlines the superior capacity of this fungal community to degrade pollutants [5, 11, 14]. Interestingly, it has been described that the biotic transformation of TCS often yields the accumulation of dichlorophenol which is then metabolized through the hydroquinone branch [15], similar to what occurs during the fungal degradation of PCP, as described by us in Chapter II. This further emphasizes PCP as an archetypal pollutant, as its degradation pathway is central for many other more complex molecules containing halogenated aromatics including TCS.

Preliminary characterization of fungal spores: yields and size distributions

The strategy used to collect airborne fungal spores revealed to be successful, providing spores at the range of at least 10^7 in all the conditions. Illustrative micrographs of the spores' suspensions (diluted 100x) at each

condition can be observed in Figure IV.2, showing the high variability in spores size and shape.

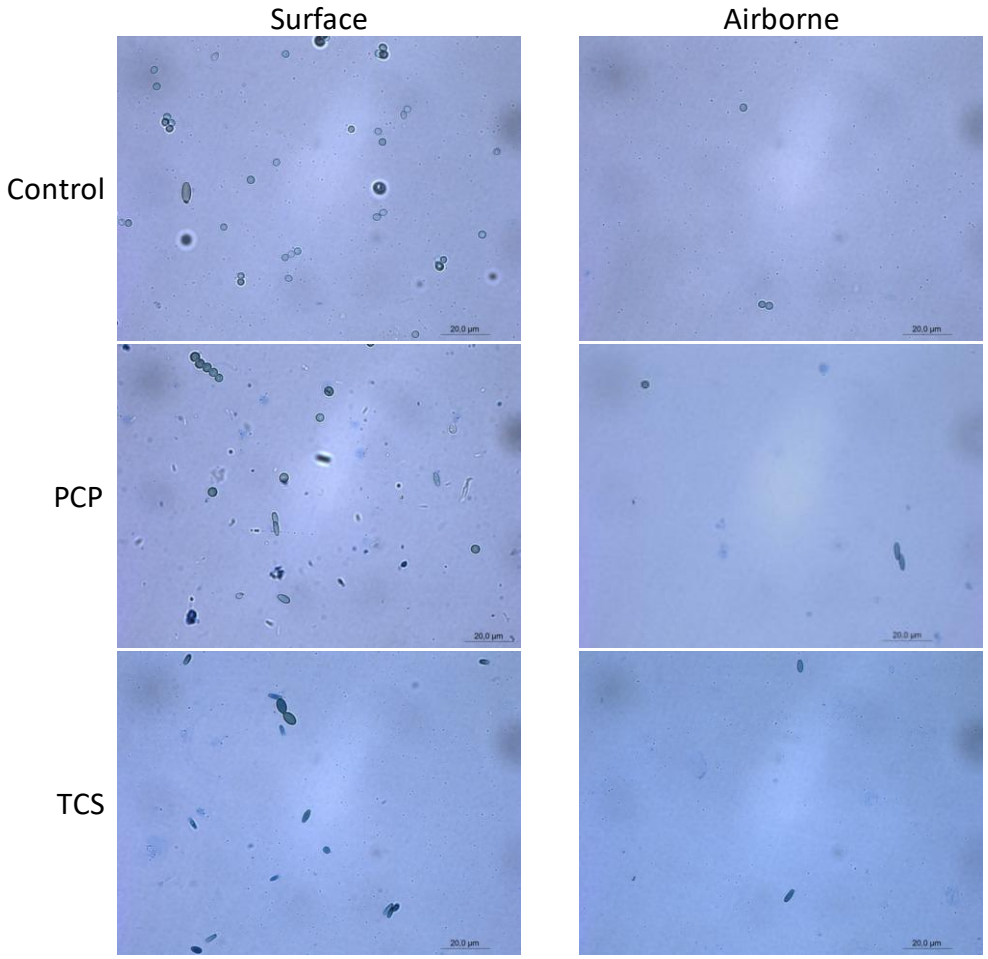

Figure IV.2 - The morphologies of the fungal spores collected at each experimental condition (Control, PCP and TCS) by surface scrapping and airborne filtration are depicted herein, revealing high variability in terms of size and shape.

The spores collected by gently scrapping the surface of the cultures provided higher spore yields, especially in the cultures containing the toxics, reaching a 10-fold higher yield (over 10^8 spores). The spore sizes (area, expressed in μm^2) measured for each condition are depicted in dot plots (Figure IV.3b-d) and clearly show the high variability of sizes that exist within the fungal community at each condition.

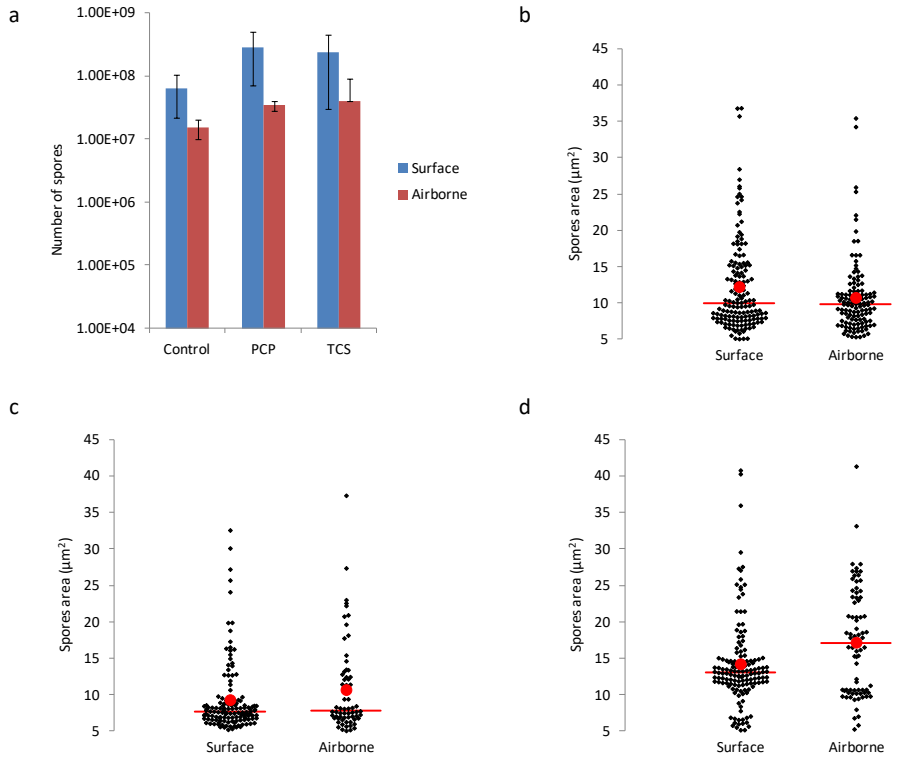

Figure IV.3 - The preliminary metrics of the collected spores includes **(a)** the yields of spores (Neubauer counts) and their sizes measured microscopically, depicting distinct distribution profiles between **(b)** control conditions, **(c)** exposure to PCP and **(d)** exposure to TCS. The red circle indicates the value of the average area while the red line indicates the median area.

Regardless that some differences in the average and median values of the spore sizes are detected between conditions, no direct effect can be inferred from this observation due to the high dispersal of the data. Moreover, the minimum and maximum values range similarly among all conditions. These measurements allowed assessing if dramatic differences would occur between airborne collected spores and surface collected spores. Therefore Q-Q plots were computed to compare the distribution of spore sizes with a normal distribution and graphically observe their adjustment (Figure IV.4).

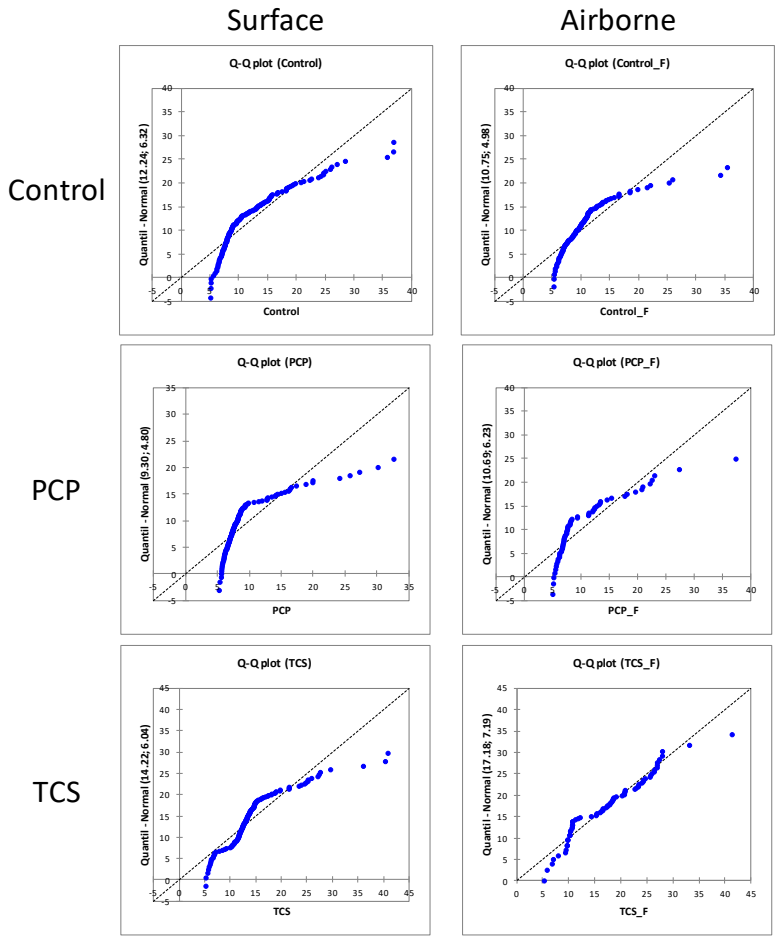

Figure IV.4 - Q-Q plots comparing the distributions of spore sizes (blue dots) with a normal distribution, at each condition and collection method.

As visible in the figure, no big differences are observed when comparing the surface spores with the airborne spores within each condition. Interestingly, this strategy allowed observing some differences between the distribution profiles at distinct conditions, better than those observed in the dot plots. Therefore we decided to pool both fractions of each condition before moving to *in vivo* infection assays, as high amounts of spores are required to achieve robustness and reproducibility in the experiments.

In vivo infection: first evidences of increased virulence triggered by pollutants

Galleria mellonella was the animal model selected to perform the *in vivo* infection assays with the spores collected in our experiments. The larvae of *G. mellonella* (greater wax moth), have been used as model to evaluate the virulence of microbial pathogens, specifically of fungal pathogens [13]. This model is extremely useful due to the strong correlation between the data it generates with data obtained in murine models [16]. The cellular and humoral innate immune system of insects is similar to the innate immune response of vertebrates, particularly as these responses are intervened by haemocytes, which are structurally and functionally similar to mammalian phagocytes [17-19]. Therefore, the examination of changes in haemocyte populations is used to assess immune responses to fungal infections [16]. Herein we assessed the survival rate of the larvae of *G. mellonella* as a preliminary indicator of virulence of the spores collected in our experiments. Our results revealed that the spores produced by the fungal communities upon exposure to toxicants are highly virulent. Figure IV.5 shows that the survival at control conditions was nearly total, with solely one dead larva after 24h of incubation at 37°C. On the other hand the survival at test conditions (spores collected upon exposure to pollutants) was nearly zero. In fact, only two larvae survived after 24h of being infected with the PCP-exposed fungi - one at each incubation temperature - and no larvae survived after 24h of being infected with TCS-exposed fungi. The observation of the larvae just a few minutes after the inoculation revealed prompt melanisation, a common feature that indicates the action of haemocytes whenever the insect is attacked by infectious agents [13, 17].

In Figure IV.5 it is possible to observe the dramatic differences in melanisation between larvae inoculated with spores collected at control conditions (no toxicants) and the completely melanised dead larvae inoculated with spores collected after exposure to toxicants. Importantly, no toxicants were detected in the inoculums of fungal spores (data not shown),

discarding any side effect coming from possible sorption of the chemicals to airborne fungal spores.

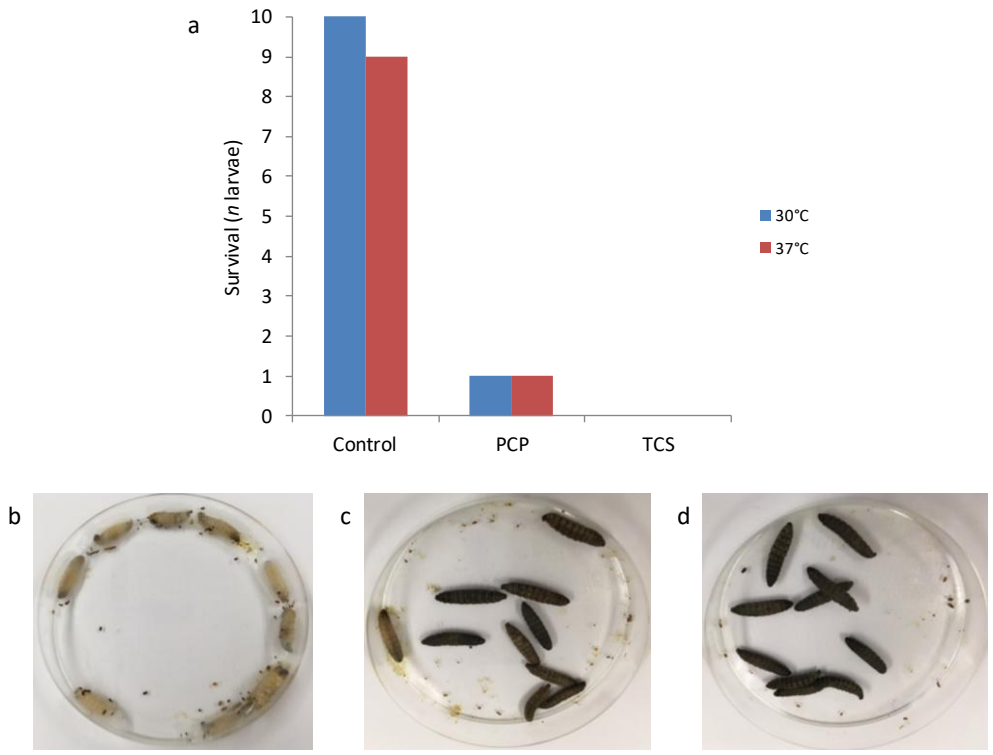

Figure IV.5 - Results of the *in vivo* infection test using *Galleria mellonella* as animal model shown by **a)** histogram of the survival rate at each condition (two temperatures of incubation) and pictures of the larvae aspect after 24 hours of incubation after being inoculated with 10^6 spores collected after 10 days **b)** in control conditions (no toxicant), **c)** exposed to PCP and **d)** exposed to TCS. The shown photographs refer to the larvae incubated at 30°C for illustrative purposes.

Combined, our results demonstrate that, despite the impressive capacity of fungal communities to degrade toxicants, important tradeoffs are occurring within the community that elicit the rise of virulence. In Chapter III, we detected shifts in the community composition and also at the physiological/metabolic levels by analysing the physiological profile and the proteome of the fungal metacommunity [5]. Both types of alterations are possibly linked, but the question whether such aggressive virulence *in vivo* is caused solely by selective pressure of the most pathogenic strains, or also

by massive metabolic dysregulation in the most abundant fungi, remains. Nevertheless, the last emerges as the most likely, as the shifts in the community composition caused by PCP (Chapter III) caused the loss of some of the less abundant taxa but did not alter dramatically the relative abundances of the most dominant operational taxonomic units [5]. Next steps will include a characterization of the pollutant-derived metabolome of the fungal community in the presented experimental setup; a full characterization of the virulence towards *G. mellonella* (inoculation with lower spore densities, haemocytes counts, fungal load in the larvae and histological characterization of the fungal infection) [13]; the isolation of the virulent fungal strains; study of the fungal community surviving inside the larvae and comparison with the airborne fungal community.

Regardless of the causes behind this phenomenon, the establishment of chemical pollutants as drivers of increased virulence in fungi has been demonstrated in this thesis. This adds concern to the problem of chemical pollution, especially when considering that novel classes of pollutants are constantly being detected by researchers and governmental organizations worldwide [1, 9, 14, 20-23]. Therefore, understanding the impact of pollutants towards fungal communities is paramount, as well as elucidating if pollution may cause fast adaptive evolution events in addition to selective pressure in favour of the most virulent strains. Answering these questions will stimulate further discussion among the scientific community regarding the risks of environmental pollution [1, 2, 4, 6, 14]. Higher visibility and risk awareness will enhance research focusing the development of tools to remediate polluted environments and the design of strategies to prevent chemical pollution in the future (*e.g.* development of alternatives to fossil fuels, decrease the consumption of meat, search for novel environmental friendly materials, etc), fostering the interest of valuable stakeholders and key decision makers. Only through deep understanding of the processes behind the increased virulence

of fungi caused by pollution, and by identifying specific molecular markers, can we design efficient tools to act at the environmental and clinical levels.

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CHAPTER V

Chapter V contains an integrated discussion of the contents of this thesis

Environmental pollution

From past to current state of research

The challenges posed by environmental pollution and climate change have been in the centre of scientific and political discussions for decades [1-5]. Global awareness regarding this issue has been increasing in the past few years, mainly due the impressive increase of natural disasters, polar ice melting, massive deforestation and biodiversity loss [2-4, 6].

Though divergent opinions arise in several social and political circles, the truth is that both, climate change and pollution have never been as discussed as nowadays. The pollutant we selected as model in this thesis, pentachlorophenol (PCP), is a good example on how the world is changing at a fast pace. PCP has been considered as highly harmful and with potential for long range atmospheric transport (LRAT) by scientists for decades [7-10]. However it has only achieved the official status of Persistent Organic Pollutant (POP) in late 2015, after the publication of the first research paper of this thesis (Chapter II) [11]. Extensive revision of its history of usage and toxicity reports is available in the Chapter I, but it is important to keep in mind that PCP was first synthesized in the XIX century and was used since the 30's of the last century [11]. This means that it took 85 years of usage, toxicity, transport and accumulation worldwide for PCP to be officially banned. The recent recognition of PCP as POP was accompanied by the inclusion of several other chemicals in the amendments to the Treaty of the Stockholm Convention [8, 12, 13]. The initial list of POPs (written in 2001) comprised only 12 chemicals and it took almost 15 years for the first amendments to be proposed [12, 13]. Since 2015 several updates and amendments have been proposed and accepted resulting in the introduction of new chemicals to the list, and several others are currently under evaluation.

Though important actions are being undertaken, mostly to mitigate any possible direct effects of pollutants towards humans, they still largely overlook the impact of pollutants in microbial ecosystems, which ultimately

will also exert an important indirect effect on humans. It is important that the scientific community succeed in demonstrating to the general public that the problem of pollution goes way beyond the toxicity of the parent compounds to humans, animals or plants. Pollutants are often biotically or abiotically degraded and/or transformed in the environment, forming a plethora of sub-products that are often unknown. The possibility that some sub-products can be equally or even more toxic than the parent compounds is frequently neglected. A mechanistic understanding of the formation of these pollutant sub-products and of their impacts towards microbial communities is vital to assess the real risks of chemical pollution to the planet and to our current life style.

From soil samples to laboratory experiments

Unboxing the black box of fungal remediation of pollutants

For several years the microbial processes occurring in the environment remained locked inside a “black-box”, especially in soil ecosystems. The high complexity underlying microbial consortia and their functioning was inflated by the complexity of soil matrices, as discussed in Chapter 1. However, our work demonstrates that by combining field sampling with laboratorial experiments through multidisciplinary techniques, it is possible to start unboxing the black box. The success of our approach started with the selection of the sampling methodology which is commonly used by geochemists to study the physical and chemical features of soils, being known for the robustness of its results. It allows pooling not only the physical and chemical traits contained at a certain location (key to assess pollution levels), but also a comprehensive snapshot of its microbial communities and functioning.

Our initial workflow (Chapters I and II) covered the physical and chemical characterization of the soil samples, the study of the levels of contamination of the soils *in situ*, the isolation of fungal strains, the characterization of the individual capacity of those strains to degrade PCP

and the study of their individual degradation products. To simplify this workflow we have used liquid media to conduct laboratorial experiments. This allowed us to assemble and propose a comprehensive PCP degradation pathway through the integration of more than 50 individual strains degradation pathways; which reveal an entire new degradation branch - the resorcinol branch – adding to those previously described - catechol and hydroquinone branches (Chapter II). Up to that point, most studies on the degradation of PCP considered each branch as an individual pathway, as they focused on single species. We have shown that fungal communities have the ability to integrate all of those individual pathways as branches of a larger pathway. The branches probably intersect along detoxification, likely following the most efficient branch, yet prone to quickly shift to other branch depending on the surrounding environment. Importantly, the identification of degradation products accumulating in soils which, up to now, can be exclusively attributed to the fungal degradation of PCP (*e.g.* dichlorometoxyphenol, drosophilin A or tetrachloro-*o*-guaiacol) constitutes the establishment of interesting molecular markers of past contamination (Chapter II). Additionally, we initiated the characterization of the physiological profiling of the fungal community upon exposure to PCP, even if our initial efforts were performed using peptone water as medium in a sludge-like setup. This approach allowed observing differences in the overall functional diversity (Shannon index) between PCP and control conditions, but the usage of a nitrogen rich medium (Chapter II) has possibly masked the massive dysregulation of the carbon and nitrogen metabolisms that we have observed afterwards when using a mineral medium instead (Chapter III).

In Chapter III we aimed at dissecting the functioning of the fungal community in the presence of PCP. Once more a liquid medium was used, but a metacommunity approach (multi-well setup) was applied, as previously described for bacteria. The metacommunity approach aimed at allowing the establishment of the distinct sub-communities that compose a

metacommunity, a parallel to what occurs in a soil matrix, but in a laboratorial context using liquid medium. This methodology permitted the extraction of proteins and DNA from a clean matrix, in opposition to what occurs when these molecules are extracted directly from soil samples. The drawbacks of extracting proteins and nucleic acids directly from soils include the risk of chemical contamination, inhibitors of enzymatic reactions (*e.g.* PCR), low availability due to sorption to organic debris (*e.g.* humic acids), among others. Having overcome these challenges, new ones arose namely the processing and analysis of the large and complex metataxonomics (amplicon sequencing) and metaproteomics datasets. The first required extensive training in Bioinformatics data processing and analysis, as well as the participation in parallel projects to allow consolidating the learned methodologies. In particular, the methodologies were tested in a work that aimed at studying the risks underlying the presence of fungal communities in silk-facing materials towards valuable ancient Chinese manuscripts, stored at the British library [14].

The challenge of analysing metaproteomics data was in part facilitated by previous critical reviews and opinion articles focusing proteomics data analysis [15, 16]. Those articles proposed the usage of generalized linear model (GLM) tools for the analysis of shotgun proteomics datasets, in opposition to direct fold-change calculation of individual proteins, as often performed. The direct calculation is prone to biases and false positives are likely to occur as protein expression is not an isolated process. Therefore, a parallel to transcriptomics data analysis can be done, as the type of data is similar (counts of fragments) and both biological processes are intimately connected. Keeping this in mind, adjustments to GLM were performed with success to compute the fold-change values for our proteomics datasets. The methodology proved to be robust, as it automatically discards proteins with very low yields of identification. To give a practical example a peptide that is detected with a count of one fragment in condition A and two fragments in condition B is often assumed to be

differentially accumulated in the dataset with the commonly used methodologies. However this peptide can be a technical artefact or even a fragment peptide from other protein, therefore it should not be considered for downstream analysis and discussion.

When considering the rationale behind Chapter III, the initial validation of the degradation capacity of the fungal community and the identification of PCP derived metabolites that matched our previously proposed degradation pathway is of high relevance. This consistency kicked off the subsequent more complex analyses that we performed. Namely, the identification of a set of 17 major assimilators of PCP was ground-breaking. The usage of stable isotopic probing followed by isopycnic ultracentrifugation to separate the fungi able to assimilate the carbon labelled PCP (^{13}C PCP) was a key step for this work. This discrimination allowed understanding that the differences in the relative abundances of the most dominant operational taxonomic units (OTUs) could be largely attributed to their capacity to use PCP (or its derivatives) as carbon source. Specifically, the major assimilators drive the taxonomic shifts in the community dynamics between control and PCP exposure , maintaining their influence regardless of PCP residual levels . These 17 assimilators are likely to be also the major players of the functional shifts observed in Chapter III, raising a direct link between the capacity to tolerate and degrade pollutants, and increased pathogenic potential. This possibility is reinforced by our metaproteomics data, as the taxonomy attributed to the best hits (database dependent) agree well with that of the metataxonomics analyses (Chapter III).

The physiological profiling data revealed to be an excellent complement to the metaproteomics data, as it validated and added strength to the obtained results. As discussed in Chapter III, the overall indices of functional diversity and abundance were not greatly altered, as they were in Chapter II, but massive alterations were observed when analysing the alterations of the consumption of each C or N source individually. This can be due the usage of distinct media when performing the physiological

profiling experiments, as previously mentioned in this Chapter. Moreover, the Biolog SF-N2 plates (used in Chapter 2) were discontinued and substituted by the FF plates. FF stands for “filamentous fungi”, and the C and N sources available are more adequate for studies with these organisms, in opposition to the SF-N2 plates which aimed at being used for fungi and filamentous bacteria. The taxonomic diversity retrieved in Chapter II, through the isolation of fungal strains is a small fraction of the multitude of OTUs obtained in Chapter III. The isolates comprised fungi belonging to the Ascomycota and Zygomycota phyla, possibly because our isolation methodologies favoured these two groups. Nevertheless Ascomycota fungi were indeed the dominating phylum in the soil samples, as observed in Chapter III, which is not surprising when considering other studies that characterised the fungal communities in forest soils [17-19]. In these types of soils fungi often dominate over bacteria, and specifically, Ascomycota fungi dominate and play key roles in basic ecosystems services, such as wood decay, litter decomposition and nutrient recycling, even if followed closely by Basidiomycota [17, 18]. Therefore, despite favouring a phylum of fungi (promptly assumed in Chapter II), our strategy allowed studying in detail a large set of isolates (77 in total) that were likely a good representation of the key providers of ecosystem services in the cork oak forest soils considered.

In summary, our experimental strategies fostered the learning of a wide array of technical and analytical skills, as well as of computational and analysis tools, which we hope to use in the near future, in the upcoming steps of this work.

Current and future challenges

Parallel studies using the established methodologies

The strategy behind our studies is consistent: we start by defining the stress level of the toxic/chemical agent of interest to the organism/community in study by using well-defined ecotoxicology methodologies. We establish different levels of effective concentration (EC) by fitting experimental data

(usually growth at several concentrations of the compound) to dose-response models (logit, probit Gompertz), and often opt to use the half maximal effective concentration (EC50). The main advantage of this strategy in opposition to the usage of percentages of minimal inhibitory concentrations (MICs), or levels available in the literature for other organisms, is that the stress level in use is adjusted to the specific dose response model of the organism/community. This is particularly important because the toxicity of chemicals towards organisms rarely follows a linear regression. This starting point has been used in an on-going study in which the ability of the model fungus *Aspergillus nidulans* to transform 5-hydroxymethylfurfural (HMF) and some of its derivatives. HMF is by industrials seen as promising alternative source of building blocks, as it is a side product in the paper industry that is prone to become an added value residue [20]. In this work we established the EC50 of each HMF derivative and then evaluated the capacity of *A. nidulans* to transform it using liquid chromatography and mass spectrometry, in an attempt to fill some gaps that still exist regarding the transformation products of HMF and its derivatives. Additionally, we now aim at disclosing the genes involved in the degradation pathways of these promising compounds in *A. nidulans*, taking it as a model for the widespread *Aspergillus* genus.

A closer parallel can be done with another on-going study in which lab scale microcosms composed by agricultural soils were spiked with a mix of contaminants of emergent concern (CECs) to study the taxonomic and functional dynamics occurring in response to the toxicants. The context behind this study is linked with the European Commission instructions for the use of reclaimed waste water (RWW) to irrigate agricultural soils [21]. However some toxicants remain in those waters after standard treatments, specifically CECs, which may therefore contaminate crops [22] worldwide due to the usage of RWWs. Though CECs are likely depleted from soils by microbial communities up to a certain level, little is known on the impact of RWWs usage continuous, especially in the contamination and functioning of

the irrigated agricultural soils. In parallel an electrochemical assisted technology is being developed, aiming at boosting the bioremediation capacity of microbial communities, preferentially without disturbing their natural balance and provision of ecosystem services. Therefore, metataxonomics and physiological profiling are being used to strengthen the understanding of the dynamics of microbial communities, in order tp help designing more efficient and safer bioremediation technologies.

Understanding how chemical pollution increases the virulence of fungi

The dissection of the functional alterations at the protein and physiological levels caused by PCP pointed towards a clear direction: the pollutant was inducing specialisation events that are suggestive of increased pathogenic potential (Chapter III). The shifts in the nitrogen and carbon metabolisms, together with the accumulation and secretion of proteins that have been previously associated with pathogenic events claimed for the execution of simple tests to strengthen or discard this hypothesis. Accordingly, we measured the pH of the medium and tested the effect of a common fungicide towards the fungal community in the presence (or not, for controls) of PCP exposure. The results pointed towards the same direction, as pH of the medium was clearly altered compared to the controls (biotic and abiotic) and the community became resistant to the antifungal upon exposure to PCP, but did not in the absence of the toxicant. These evidences raised new questions the first being if these molecular indicators of increased pathogenicity are translated in increased virulence *in vivo*. To address this question we designed the study described in Chapter IV. Though the collection of airborne fungal spores upon exposure to the toxicants constitutes a challenge, the microscopic assessment validated our strategy. In fact, upon overcoming this initial challenge, the remaining methodologies are well established in the literatures and in the research team, facilitating the implementation of this study. A screening on the pollutant derived metabolome (to confront with the literature) and further validation of the

infection studies are required. Specifically, we will lower the spore density to be inoculated onto the larvae to allow a follow-up along time, and complementary tests, such as fungal load, haemocytes counts and histological observations. However, despite their preliminary nature, our results suggest that in fact pollutants are drivers of increased fungal virulence.

Concluding remarks

This thesis was performed in a highly multidisciplinary environment which provided the development of valuable technical tools and data analyses methodologies that will be extremely useful in the future. It ranged from the handling of soil samples and basic mycology, to the application of complex analytical and molecular tools, reaching a high standard of quality in our data acquisition methods and subsequent analyses. We have achieved it by using a combination of relevant scientific influences and inspiration from high-level research, original approaches, and state-of-the-art technologies.

Taking a critical view on our work, some limitations must be referred: The fact that our laboratorial isolations and simulations favoured mostly Ascomycota, former Zygomycota (now divided in to Mucoromycota and Zoopagomycota) [23] and some Basidiomycota yeast can be seen as a limitation, as we were unable to perform experiments covering the full diversity of the soils' communities. A good example is the communities' composition in Chapter III which favoured the survival and growth of only a fraction of the whole inoculum. Still, we managed to cover nearly half of the total community at the third day and a third at the tenth day of the experiments. Therefore, regardless of this compositional limitation, it is still a remarkable achievement when considering liquid medium simulations. When considering the functional analyses, specifically metaproteomics, a major drawback is that it depends on the best hit of protein sequences available in databases, which is biased towards the best-studied taxa, as discussed by us in Chapter III. Though decent associations can be inferred, a deeper

knowledge on the roles that each strain is playing in the functioning of the community would be extremely valuable and perhaps novel tools may help disclosing this, such as the emerging pangenomics approach, which could, in the future be adapted to a panproteomics library. Another aspect that must be discussed is the residual toxicity upon exposure to the toxicants. Despite that we managed to measure the decay of the parent compound and identify several of its ensuing products, no evaluation of the residual toxicity was performed after our assays. This complementary test may help understanding if the increase in virulence that we observe is connected with a steady or even higher level of toxicity during the transformation of PCP (Chapters III and IV) and Triclosan (Chapter IV). This is a parameter that we will certainly measure in the near future, and that we suggest that others perform in their studies on the decay and transformation of chemical pollutants. Despite these limitations, we believe that the work contained herein pushed forward the understanding of the processes occurring in fungal communities upon exposure to chemical pollutants. Overall, we revealed that fungi are key drivers of ecosystems functioning, and that the disturbance of their dynamics can greatly impact both the environment and human health.

Science is made out of questions and, though we managed to answer a few, the ones that this thesis opens are certainly defying and worth to pursue: Is the impact of the pollutants similar to that imposed by antifungals in a clinical environment? Is the increased virulence occurring in most elements of the fungal community due to metabolic shifts, or is it occurring solely due to selective pressure of already virulent strains? Are there any connections between chemical pollution and the increase in fungal infections [24] and cross-Kingdom pathogenic events [25] that have been recently reported? Are any of these highly virulent strains clinically relevant?

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