# Belowground fungi are key sentinels in forest soils vulnerable to pentachlorophenol pollution: a mechanistic study in *Quercus suber* forests

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Dissertation presented to obtain the Ph.D. degree in Biochemistry

Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa

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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 in *Instituto de Tecnologia Química e Biológica António Xavier*, *Universidade Nova de Lisboa*, between January 2009 and December 2017.

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*Carolina, Gonçalo, Mãe e Pai*

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This study is the beginning of my personal development in a scientific career and a journey, full of good and some “not so good” moments.

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**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. The above statement has inspired the working hypothesis of this doctoral thesis – active sources of pentachlorophenol (PCP) pollution are reaching the soils of emblematic cork oak forests (Chapter II and III), hence altering the functioning of the belowground mycobiota (Chapter III and IV) that act as buffer against the disturbance caused by the biocide (Chapter III and IV).

This working hypothesis assumed that cork oak forests located in North Africa were probably contaminated with PCP since the cork of the oak trees was frequently reported to be contaminated with pentachloroanisole (PCA), likely formed due to microbial conversion of PCP. Besides, it also recognised the potential of PCP as model for unravelling the contribution of the soil mycobiota in the fate of pollution, especially in virtue of PCP ability for long range atmospheric transport, favourable partition into the soil layer and moderate abiotic resistance. Regardless, PCP was only classified as a banned POP in 2015. Finally, it assumes that fungi are key colonisers in cork work forest soils. These concepts have been translated into the project "*Preventive and remediation strategies for continuous elimination of PCPs from forest soil"* (S*f*p-NATO 981674), in collaboration with partners from Tunisia, among others, granting us access to cork oak forests located in Tabarka (N.W. Tunisia) (Chapter III).

In this doctoral thesis, **Chapter I** comprises a comprehensive state-of-the-art review of PCP history of use, since its discovery to its recognition as a banned POP, emphasising aspects on its toxicity, environmental dispersion and microbial degradation. In addition, it covers general aspects of environmental pollution and microbial diversity, especially in soil, as well as

of environmental proteomics – one of the techniques used in the last studies enclosed in this thesis (Chapter IV).

In **Chapter II**, a comprehensive description of the development of a suitable and robust method for identifying PCP in soil samples is presented. This method has been used as the basis for extracting PCP from soils collected in Tabarka forests.

**Chapter III** comprises the demonstration, for the first time, that the soils of cork oak forests in the Tabarka region were contaminated with significant levels of PCP (similar to levels in locations where PCP is currently used). This allowed us to advance further in our initial working hypothesis, answering if the soil mycobiota may act as a buffer against the disturbance caused by the biocide (Chapter III and IV). We have focussed on the cultivable fungal community (seventy-seven strains were isolated and taxonomically classified) and on the PCP-derived metabolome (*i.e.* PCP degradation intermediates and sub-products) as an experimental gold-standard to describe the PCP degradation pathway used by the community. The majority of the fungal strains within the community could significantly degrade PCP, further emphasising fungi widespread capacity to degrade the biocide. The degradation of PCP proceeds through three branches: the hydroquinone, the catechol and the resorcinol. The last constitutes a remarkable discovery since it has never been before linked to PCP degradation. Compounds derived from the resorcinol branch could be then identified in community assays as well as on the soils. This has challenged us to move forward in our working hypothesis – PCP pollution alters the functioning of the belowground mycobiota (Chapter IV).

In **Chapter IV**, our strategy has focussed on the activity of the whole community instead of its individual members during PCP mitigation, which we have analysed using essentially proteomics (mycelial) and community

level physiological profiles. The acquired data (which will be complemented also with metagenomics-based assays, not herein enclosed) revealed that PCP induced major stress responses. Additionally, along PCP depletion the functioning at the community level showed the capacity to slowly recover, regardless key functional alterations persisted even after major PCP depletion.

**Chapter V** presents a comprehensive discussion of all the attained data identifying also both, yet unanswered aspects and new questions raised by the data. Moreover, it covers in brief other side-studies already published focussing the optimisation and the development of the methods herein used (*e.g.* soil sampling strategy, harmonisation of the methods used for the characterisation of the soil samples and proteomic workflow in filamentous fungi), as well as on-going studies on specific strains capable to antagonise the growth of the other members within the community. Collectively, our data reinforced that 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. Data reinforced the wide-ranging principle of global and dispersed environmental pollution by PCP. The contamination of Tunisian *Quercus suber* forests with PCP, at levels similar to those found prevalent where PCP is currently used raises serious concerns. Above all, our data reinforced that the belowground fungal communities can ensure a short lifetime and the rapid mineralisation of chlorinated phenols, in opposition to bacterial counterparts which usually yield highly toxic and recalcitrant non-chlorinated or chlorinated phenol derivatives. Nonetheless, we demonstrated that PCP (even at very low levels) alters the functioning of the soil mycobiota. It remains to be understood how this in turn is affecting the multi-functionality of the entire ecosystem.

**Sumário**

Os poluentes orgânicos persistentes (POPs) constituem uma ameaça silenciosa, contaminando a atmosfera e os solos à escala global; todavia não dispomos ainda de conhecimento fundamental sobre a sua ocorrência, fontes primárias e dispersão. Estes factos “inspiraram” a hipótese de trabalho da presente tese de doutoramento - fontes activas de poluição de pentaclorofenol (PCP) contaminam o solo de florestas de sobreiro (Capítulo II e III), alterando o funcionamento do micobiota no solo (Capítulo III e IV), que potencialmente actua como “silenciador” do distúrbio causado pelo poluente (Capítulo III e IV).

Esta hipótese assume que florestas de sobreiro do Norte de África estão contaminadas com PCP, uma vez que contaminação de cortiça com pentacloroanisole (PCA) foi verificada frequentemente - provavelmente formado devido à conversão microbiana de PCP. O PCP tem a capacidade de transporte atmosférico de longo alcance, partição favorável para a camada do solo e moderada resistência abiótica, tendo assim potencial como modelo no estudo da influência do micobiota do solo na mitigação de poluição. Por fim, podemos assumir que os fungos são colonizadores-chave em solos de florestas de sobreiro. Estes conceitos fizeram parte do projecto "*Estratégias preventivas e de remediação para a eliminação contínua de PCPs de solos florestais"* (S*f*p-NATO 981674), em colaboração, entre outros, com parceiros da Tunísia que nos concederam acesso às florestas de sobreiro localizadas em Tabarka (NW Tunísia) (Capítulo III).

O **Capítulo I** desta tese compreende uma revisão bibliográfica abrangente da história do uso de PCP, desde a sua descoberta ao seu reconhecimento como um POP (apenas em 2015), enfatizando aspectos sobre a sua toxicidade, dispersão ambiental e degradação microbiana. Além disso, compreende aspectos gerais sobre poluição ambiental e diversidade microbiana, especialmente no solo, bem como a análise proteómica – uma

das técnicas utilizadas nos últimos estudos incluídos nesta tese (Capítulo IV).

No **Capítulo II**, é apresentada a descrição do desenvolvimento de um método robusto para a identificação e a quantificação de PCP em amostras de solo. Este método foi usado subsequentemente para a extracção de PCP de amostras de solo das florestas de Tabarka.

No **Capítulo III** é feita, pela primeira vez, a demonstração de que os solos das florestas de sobreiro na região de Tabarka estão contaminados com níveis consideráveis de PCP (semelhante a níveis descritos para locais onde o PCP é ainda usado). Ao validar a hipótese de trabalho inicial, torna-se possível estudar a influência do micobiota do solo no silenciamento do distúrbio causado pelo biocida (Capítulo III e IV). Para descrever a via de degradação de PCP, o trabalho experimental centrou-se na comunidade de fungos cultiváveis (setenta e sete estirpes foram isoladas e classificadas taxonomicamente) e na análise do metaboloma derivado do tóxico (ou seja, intermediários e subprodutos da degradação de PCP). A maioria dos isolados fúngicos conseguiu degradar o PCP em níveis significativos sendo possível identificar três vias para a sua degradação: a via da hidroquinona, a do catecol e a do resorcinol. A descoberta da última via é de realçar, uma vez que nunca tinha sido relacionada com a degradação de PCP. Os compostos abrangidos na via do resorcinol, foram também identificados em ensaios de degradação do PCP com a comunidade fúngica e nas amostras de solo. Estes resultados permitem avançar para o estudo do impacto do PCP no funcionamento do micobioma do solo (capítulo IV).

O **Capítulo IV** concentrou-se no estudo da actividade da comunidade durante a degradação de PCP como um todo, usando técnicas de proteómica (do micélio) e de análise de perfis fisiológicos “CLPP – community level physiological profile”. Os dados recolhidos (complementados por análises de metagenómica não incluídos nesta tese)

mostraram que o PCP induz respostas significativas a stress. Os dados demonstraram ainda que apesar da comunidade fúngica recuperar parte do seu "normal" funcionamento ao longo do decaimento do poluente, algumas funções críticas mantêm-se alteradas mesmo para níveis de PCP residuais.

No **capítulo V** é apresentada uma discussão integrada dos dados obtidos e das principais questões ainda por responder ou novas. Este capítulo incluiu referência a outros trabalhos já publicados que incidem na optimização e desenvolvimento de métodos utilizados ao longo da tese (*ex.* estratégia de amostragem de solo, métodos de caracterização de amostras de solo e protéomica de fungos filamentosos) assim como estudos em curso sobre o antagonismo entre diferentes estirpes presentes na comunidade. No seu conjunto, os dados obtidos demonstram que o estudo do comportamento de comunidades fúngicas pode modelar (*i.e.* revelar) *per se,* as capacidades funcionais do micobiota, possibilitando a identificação de novos marcadores para a avaliação de poluição ambiental e de isolados capazes de degradar eficientemente o poluente. Os dados reforçam ainda a ideia generalizada que a poluição ambiental com PCP é global. A contaminação das florestas tunisinas de *Quercus suber* com PCP em níveis comparáveis aos que se encontram em locais em que o PCP é ainda utilizado, cria preocupações sérias. De salientar que, a comunidade fúngica do solo aqui estudada mostrou ser capaz de diminuir o tempo de vida de compostos fenólicos clorados no solo, assegurando a sua rápida mineralização; em oposição à degradação bacteriana que resulta na produção de compostos fenólicos, clorados ou não clorados, recalcitrantes e altamente tóxicos. Em suma, demonstrámos que o PCP (mesmo em baixa concentração) altera o funcionamento do micobioma do solo; no entanto falta ainda compreender como esta alteração afecta a multi-funcionalidade do ecossistema como um todo.

## Members of the jury

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| *President* | Doctor Cecília M. Arraiano, Full Professor at Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, ITQB NOVA |
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**Thesis publications**

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, chapter of book “Fungal Bioremediation: Fundamentals and Applications”, CRC Press, 2018. [#]equally contributing authors listed alphabetically

**A. Varela**[#], C. Martins[#] 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

I. McLellan, A. Hursthouse, C. Morrison, **A. Varela**, C. Silva Pereira, Development of a robust chromatographic method for the detection of chlorophenols in cork oak forest soils. Environmental Monitoring and Assessment. 2014. 186: 1281-1293.

**A. Varela**[#], C. Martins[#], O. Núñez, I. Martins, J.A.M.P. Houbraken, T.M. Martins, M.C. Leitão, I. McLellan, W. Vetter, 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

**A. Varela**, C. Martins, C. Leclerq, O. Núñez, J. Renaut, E. Moyano and C. Silva Pereira, Proteomic and functional response of a fungal community during PCP depletion. *Part of a manuscript in preparation.*

**Additional publications (mentioned in the discussion)**

F.J. Deive, A. Rodríguez, **A. Varela**, C. Rodrígues, M.C. Leitão, J.A.M.P. Houbraken, A.B. Pereiro, M.A. Longo, M.A. Sanromán, J.M.S.S. Esperança, R.A. Samson, L.P.N. Rebelo, C. Silva Pereira, Impact of ionic liquids on extreme microbial biotypes from soil. Green Chemistry. 2011, 3: 687-696 doi: 10.1039/C0GC00369G.

I. McLellan, A. Hursthouse, **A. Varela** and C. Silva Pereira, Geochemical approach to assessing human impacts in Cork Oak forest soils of the MED region. Journal of Geochemical Exploration. 2013; 132: 34-40. doi: 10.1016/j.gexplo.2013.04.005.

I. McLellan, **A. Varela**, M. Blahgen, M.D. Fumi, A. Hassen, N. Hechminet, A. Jaouani, A. Khessairi, K. Lyamlouli, H.I. Ouzari, V. Mazzoleni, E. Novelli, A. Pintus, C. Rodrigues, P. Angelo Ruiu, C. Silva Pereira and A. Hursthouse, Harmonisation of physical and chemical methods for soil management in Cork Oak forests - Lessons from collaborative investigations. 2013. African Journal of Environmental Science and Technology. doi: 10.5897/AJEST12.101

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I. Martins, **A. Varela**, L.M.T. Frija, M.A.S. Estevão, S. Planchon, J. Renaut, C.A.M. Afonso and C. Silva Pereira, Proteomic Insights on the Metabolism of *Penicillium janczewskii* during the Biotransformation of the Plant Terpenoid Labdanolic Acid, Frontiers in Bioengineering and Biotechnology 2017, 5: 45. doi: 10.3389/fbioe.2017.00045.

**List of acronyms**

| | |
|---|---|
| **ACN** | Acetonitrile |
| **ANOVA** | Analysis of variance |
| **BSA** | Bovine serum albumin |
| **C branch** | Catechol branch |
| **CHAPS** | 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate |
| **CHCA** | α-Cyano-4-hydroxycinnamic acid |
| **DAD** | Diode array detector |
| **DG18** | Dichloran glycerol agar base |
| **DNA** | Deoxyribonucleic acid |
| **DTT** | Dithiothreitol |
| **$EC_{50}$** | Half maximal effective concentration |
| **ESI** | Electrospray ionization |
| **HQ branch** | Hydroquinone branch |
| **HPLC** | High performance liquid chromatography |
| **HRMS** | High resolution mass spectrometry |
| **$K_{o/w}$** | octanol/water partition coefficient |
| **LC** | Liquid chromatography |
| **MALDI** | Matrix-assisted laser desorption/ionization |
| **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 |
| **OSPAR Convention** | Convention for the Protection of the Marine Environment of the North-East Atlantic |

| | |
|---|---|
| **PCA** | Pentachloroanisole |
| **PCP** | Pentachlorophenol |
| **PCPL** | Pentachlorophenyl laurate |
| **PCR** | Polymerase chain reaction |
| **PDA** | Photodiode array detector |
| ***q*RT-PCR** | Quantitative real-time polymerase chain reaction |
| **R branch** | Resorcinol branch |
| **ROS** | Reactive oxygen species |
| $t_R$ | Retention time |
| **SIP** | Stable Isotope Probable |
| **SDS** | Sodium dodecyl sulphate |
| **SDS-PAGE** | Sodium dodecyl sulfate - polyacrylamide gel electrophoresis |
| **SOC** | Soil organic carbon |
| **SOM** | Soil organic matter |
| **TCA** | Tricarboxylic acid |
| **TIC** | Total ion chromatogram |
| **TOF** | Time-of-flight |
| **UHPLC** | Ultra-high performance liquid chromatography |
| **UV** | Ultraviolet light |

# CHAPTER I

***The Environment knows no boundaries.***

***What goes up into the environment goes around the world.***

The Introduction integrates three sections: **i)** one submitted *peer-reviewed* Book Chapter entitled: *A learning journey on toxicoproteomics: the neglected role of filamentous fungi in the environmental mitigation of pentachlorophenol*, comprising details of proteomics, filamentous fungi and pentachlorophenol (PCP); **ii)** one Review in Current Opinion in Microbiology, entitled *A three-act play: pentachlorophenol threats to the cork oak forest soils mycobiome*, integrating further details on PCP pollution; and **iii)** one subsection on the dispersion of pollutants in soil; an important topic which was lacking in the previous sub-sections.

Accordingly, this chapter integrates the following preprint and reprint:

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, 2018. [#] equally contributing authors listed alphabetically

**A. Varela**[#], C. Martins[#] and C. Silva Pereira. A three-act play: pentachlorophenol threats to the cork oak forest soils mycobiome, Current Opinion in Microbiology, 2017. 142-149. doi: 10.1016/j.mib.2017.06.007. Epub 2017 Jul 10. [#] equally contributing authors

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

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## Introduction

Proteomics stands for the "protein complement of the expressed genome by an organism in a particular biological state [1]. 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 [2]. 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" [3]. 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 [3] and to understand microbial networks in an effective way [4]. 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" [5].

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. A historical perspective of the use and hazards of pentachlorophenol (PCP) – our archetypal man-made halogenated pollutant; is also enclosed. 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.

## 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 [6]. 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) [7, 8]. It has been shown that LRAT can be responsible for ca. 70-90% of the POPs levels found in EU countries [7, 9].

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 [6]. They are detected all over the planet,

including remote locations where no significant local reservoirs prevail [10]. 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 [11]. This phenomenon justifies the detection of POPs in the Arctic environment and in the corpses of local animals and people (especially in fatty tissues [12]); 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 [13]. 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 [8]. 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 [13].

**Pentachlorophenol: from a reputable pesticide to an obsolete chemical**

Pentachlorophenol, which was first described in 1841 [14], started to be commercialised in 1936 essentially as pesticide, antifungal and antimicrobial due to its *“high degree of effectiveness in biological control, combined with its desirable physical properties”* [15] (Fig. I.1). PCP (as many other pesticides) was initially seen as a useful chemical to solve severe contamination problems in agriculture and industry [16]. Soon the perception of safe use of pesticides conflicted with scientific knowledge on their poisoning effects. Until 1940, applications and properties of PCP were often reported (*e.g.* [15, 17]), whereas its toxicity was analysed only twice, specifically in rabbits [18] and in humans (skin irritation) [19]. During the following decades, new reports on PCP toxicity came to light [20, 21], including the first report on poisoning in humans [22]. Its mechanism of action was also disclosed, *viz.* uncouples mitochondrial oxidative

phosphorylation [23] (Fig. I.1). Throughout the 60's, PCP poisoning effects in humans were more often reported, associated to direct industrial handling [24] or use of daily products washed with PCP based products, such as diapers [25]. Progresses in analytical techniques allowed higher accuracy in the detection of PCP, either in human tissues/fluids or environmental samples [24, 26].

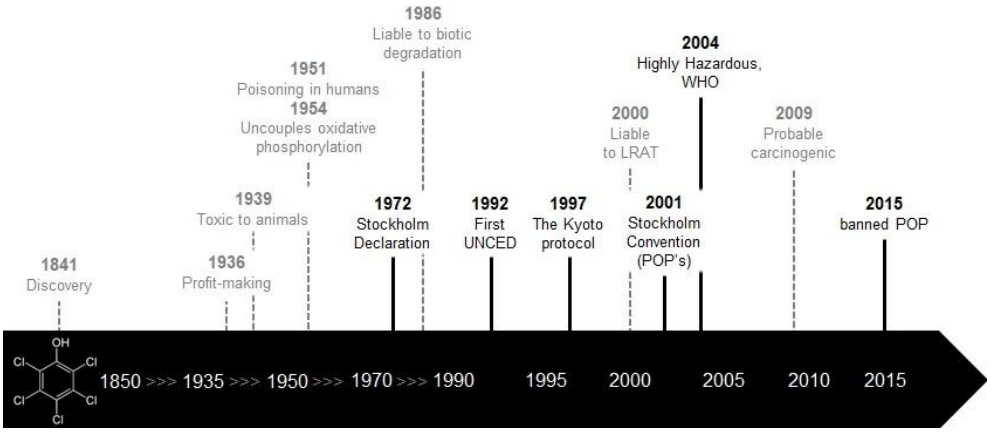


**Fig. I.1 -** Schematic representation of the historical path of pentachlorophenol (PCP), since its discovery to its classification as banned persistent organic pollutant (POP). Continuous and dashed lines mark key landmarks on PCP liability and scientific discoveries, respectively.

The Stockholm Declaration was adopted by the United Nations (UN) in 1972, acknowledging the right to a healthy environment [27], and giving rise to the first restrictions on PCP production and uses [28]; finally the era on the liability of PCP commences (Fig. I.1). Not surprisingly also PCP biotic degradation started to be scrutinised [29, 30]. The recognition that human activities were affecting seriously the balance of the Earth's ecosystems - UN Conference on Environment and Development (UNCED, Rio de Janeiro, 1992) [31] - boosted the importance of environmental pollution; naturally PCP was not an exception [32, 33]. In the Stockholm Convention on POPs (May, 2001), contrariwise PCP was not classified as POP and was left out from the original list of the "Dirty Dozen" [7]. Only in 2015, PCP was

recognised as a banned POP [10], obviously supported by numerous scientific evidences on its far-reaching hazards [34-36].

The global dispersion of PCP is a consequence of its long history of use, persistence and transboundary nature [34, 35, 37], as well as its current usage in many locations worldwide, *e.g.* in China [38], and its formation during the degradation of more volatile pollutants, *e.g.* pentachlorobenzene and hexachlorobenzene [39, 40]. Nowadays, PCP is detected in human fluids and tissues, worldwide, due to exposure in both indoor and outdoor environments (*e.g.* [41, 42]). One of the main reasons behind the unwillingness of classifying PCP as a POP was its low vapour pressure (mostly advertised in the early years of usage [15]) initially understood as a poor ability for undergoing LRAT. Controversy, in the last decade, PCP capability to undergo long-range transport has been well established [10, 35], similar to that reported to other POPs.

Since the 90's, scientific and anecdotal evidence of contamination of the bark of *Quercus suber* (cork oak) with PCP and its derivatives exists [43, 44]. These forests cover *ca.* 1.5 million ha in Europe and 700 thousand ha in North Africa [45]; presently a source of income for thousands of people, especially due to its most added-value product, the cork bottle-stoppers. The cork taint defect in bottled-wines (responsible for major losses in the cork industry in the 90s') is largely associated with the presence of chloroanisoles [43]. Their most direct precursors are PCP, 2,4,6-trichlorophenol (2,4,6-TCP) and 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP), originating through microbial *O*-methylation the corresponding anisole. Chloroanisoles are still occasionally identified in cork and its derivatives, regardless of current industrial best practice designed to eradicate any chlorinated phenol and/or anisole from processed cork. The cork bark behaves as a sampler, accumulating both gaseous and particulate pollutants [46] yet PCP partition to the soil is likely to be significant (estimated to be as high as 95% [35]). This has inspired us to seek for the prevalence of active sources of PCP pollution in soils from Tunisian cork oak forests (Tabarka, N.W. Tunisia).

Notably, soils collected along 100 $km^2$ within these forests contained PCP levels ranging from 2 to 30 $\mu g \cdot Kg^{-1}$ [47], similar to levels detected at locations now using PCP as antiparasitic [38]. The atmospheric deposition of PCP (or its precursors) is likely contributing for the prevalence of the biocide in these forests; its half-life in air and environment has been estimated to be 7.4 days and 1.5 months, respectively, with the potential to be transported over 1500 to 3000 km [35]. The contribution of regional forest management practices relying still on PCP usage cannot be disregarded, especially since the Tunisian legislation on PCP is not very prohibitive [48]. PCP was also found prevalent in soils of European cork oak forests under qualified management (unpublished data) where the most probable source is the atmospheric deposition of the biocide. It remains poorly understood 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 [48-50].

**Microbial decay of critical pollutants: fungi as main protagonists**

All the polluted Earth ecosystems are colonised by microorganism that display matchless capabilities to degrade and/or transform a wide diversity of POPs [51]. Soil is likely the most critical environmental compartment when accounting for the reception, transference and dispersion of pollution, particularly through microbial biotic processes. Many of these processes are potentially mediated by fungi in virtue of their high abundance (nearly ¾ of the soil microbial biomass), broad enzymatic capacities, extensive hyphae reach and high surface-to-cell ratio [48, 52].

Fungi are a highly diverse and heterogeneous Kingdom of organisms and most likely the second most common on Earth. They are widely distributed across all biomes (in mutualistic, pathogenic and commensal relationships) and can grow in adverse conditions of low nutrient availability, low water activity and low pH. Up to date, over 120 000 fungi species are described, yet 2.2 to 3.8 million species have been estimated to exist [53].

Fungi (heterotrophs) act as the major recyclers of organic matter, secreting enzymes that ensure degradation of complex and recalcitrant natural polymers such as keratin, chitin and lignin. Taken as an example, plant litter decomposition is initially accomplished mostly by Ascomycota which are largely replaced at latter stages and at lower soil depths by Basidiomycota [54, 55]. The soil mycobiota ensures even the degradation of lignin; its amount decreases in lower soil horizons reaching vestigial levels in the stable clay fractions [56]. Lignin degradation mainly involves lignin-modifying class II heme peroxidases (*viz.* lignin peroxidase – LiP, and manganese peroxidase - MnP), of which the origin has been linked to an ancestor of wood white rot Basidiomycota [57]. Other types of fungal peroxidases have been identified, namely unspecific or aromatic peroxygenases, dye peroxidase, and haloperoxidase, which are capable to undertake diverse reactions, *e.g.* aromatic peroxygenation, double-bond epoxidation and hydroxylation of aliphatic compounds, or to transform compounds poorly accepted by other peroxidases (*e.g.* textile dyes) [58, 59]. The ligninolytic mechanisms used by Ascomycota are less understood, notwithstanding they are efficient wood degraders in high moisture conditions [60], possibly assuming a major role in aquatic habitats where Basidiomycota are rare [48].

Fungi can also use non-enzymatic Fenton chemistry to generate highly reactive hydroxyl radicals that mediate depolymerization of cellulose, as well as degradation of some pollutants [48]. In addition, fungi own numerous and diverse cytochrome P450 enzymes (nearly 150 in some genomes) that are involved in secondary metabolism and detoxification of xenobiotics [61].

The non-specificity and versatility of fungal degradative enzymes has been the source of inspiration for research on the degradation of aromatic and aliphatic xenobiotics by fungi [62]. A parallel between the ligninolytic metabolism in fungi and the degradation of pollutants has been initially considered; well-illustrated in a first report on POPs oxidation by

*Phanerochaete chrysosporium* (Basidiomycota) [63]. Nonetheless, in many subsequent studies, the capability of non-ligninolytic Ascomycota and Zygomycota to degrade diverse pollutants become apparent [48, 62].

Currently, there are no doubts that fungi from every phylum can effectively degrade an extraordinary diversity of structurally dissimilar xenobiotics, including dichlorodiphenyltrichloroethane (DDT), - polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), benzene/toluene/ethyl benzene/xylene (BTEX) and dioxins among others [48, 62, 64] as well as complex mixtures *e.g.* creosotes or kerosene [65, 66]. It is also known that the high tolerance to xenobiotics in fungi is associated to pleiotropic drug resistance mechanisms [67].

## 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) [68, 69]. Gel dependent methods, which have been the work-horse of proteomics [69], 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 [70]. 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 [68, 71], 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 [72]. 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 [68, 71].

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 [72]. 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 [69]. 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 [73, 74].

A 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 [5]. Nonetheless standard methods for extracting proteins from distinct fungal cellular compartments have been established whenever cultures are grown axenically [2]. 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 [75]. 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 [76]; 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 bias recovery [74-76].

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 > 800 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* [77]. In the following years, gel dependent proteomics has been extensively applied to investigate the response of fungi to either xenobiotics [78-81], antifungal drugs [82] or various stresses [83-85], fungal secondary metabolism [86], development [87] and pathogenesis [88, 89], and interactions of fungi with bacteria [90], plants [91] or insects [92]. 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 [93]. Presently, gel independent proteomics is providing means for in-depth characterization of membrane [94], mitochondrial [95] and extracellular [96] sub-proteomes. Moreover, it is supporting discovery in fungi biotyping [97, 98] and interaction with a host [99].

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 [100]. This has inspired further studies on metaproteomes using either gel dependent methods, *e.g.* soil rhizosphere [101] (Table I.1), or gel independent ones, *e.g.* biofilm formation [102, 103] and litter decomposition [104] (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 [105]. 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 [73, 106]. For example, in the rhizosphere fungal proteins account for 12-30 % of the total identified proteins [101, 107] (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.**<br>Bacterial proteins >> fungal proteins (linked to high pH).<br>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. | [108] |
| 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.**<br>The most abundant $^{15}$N-utilizer are *Rhizobiales*.<br>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. | [109] |
| Dryland soils, predesertic. marshes and forests | LC-MS/MS | **Microbial functioning of the soil community in response to the C load.**<br>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. | [110] |
| Permafrost (30–35 cm) and soil (65–75 cm) | LC-MS/MS | **Microbial functioning in permafrost and adjacent soil layer.**<br>Fungi and fungal processes are poorly represented in the permafrost active microbial population. Fungal proteins (genes and transcripts) corresponded only to a small fraction in the analysed samples. | [111] |

| Sample | Methods | Research question and major findings | Work |
|---|---|---|---|
| 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). | [112] |
| 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. | [113] |
| 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 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. | [76] |
| 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. | [75] |

| Sample | Methods | Research question and major findings | Work |
|---|---|---|---|
| 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. | [104] |
| Rhizospheric soil Monocultures (1-2 years) | 2DE | **Functioning of rizhospheric microbial communities (monoculture).** 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. | [101] |
| 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). | [107] |

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

## 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 [81, 114-116] and Zygomycota [79, 117], covering xenobiotic degradation yields from 60 % (1 day) [81] to ~99 % (3 days) [79], 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 [118] (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 [119]. 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 [120] (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)<br>Amino acid metabolism<br>Cell wall remodelling (chitin deacetylase)<br>Detoxification of ROS (peroxiredoxin, nuclease C1) | [117] |
| Alachlor | *Paecilomyces marquandii (A)* | 2DE | Carbohydrate & Energy metabolism (malate dehydrogenase, enolase)<br>Detoxification of ROS (SOD, catalase)<br>Stress (HSP70)<br>Xenobiotic transformation (nitrilase) | [116] |
| 4-*n*-Nonylphenol | *Metarhizium robertsii (A)* | 2DE | Carbohydrate & Energy metabolism (malate dehydrogenase, pyruvate dehydrogenase)<br>Detoxification of ROS Species (peroxiredoxin, SOD) | [81] |
| Pentachlorophenol | *Mucor plumbeus (Z)* | 2DE | Carbohydrate & Energy metabolism (enolase, glyceraldehyde 3P dehydrogenase)<br>Cell wall remodelling (chitin deacetylase)<br>Detoxification of ROS (cytochrome c peroxidase, thiamine biosynthesis)<br>Stress (HSP70)<br>Xenobiotic transformation (alcohol dehydrogenase) | [79] |
| Gossypol | *Aspergillus niger (A)* | 2DE | Carbohydrate & Energy metabolism (malate dehydrogenase, citrate synthase)<br>Detoxification of ROS (thiamine biosynthesis)<br>Stress (HEX1) | [115] |
| Anthracene | *Fusarium solani (A)* | BN-PAGE | Xenobiotic transformation (Laccase) | [114] |

*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. | [120] |
| 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. | [121] |
| 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. | [122] |
| 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. | [123] |
| 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. | [124] |

| Sample | Methods | Major findings | Work |
|---|---|---|---|
| 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. | [125] |
| Groundwater and microcosms contaminated with **2,4-D** | 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 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. | [118] |

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

## Lessons from a learning journey on toxicoproteomics

*Past – 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) [33]. These intermediates were further dechlorinated through a reductive dehalogenase system involving a glutathione conjugate reductase that ultimately leads to full mineralisation [126]. Subsequent studies demonstrated the potential of numerous Ascomycota and Zygomycota to degrade PCP, *e.g. Trichoderma harzianum* [127], *Penicillium camemberti* [128], and several strains isolated by us either from cork slabs [43, 129] or from PCP contaminated forest soils [47]. TeCHQ was also formed during the degradation of PCP by non-lignolytic Zygomycota, namely *Mucor ramosissimusis* [130] and *M. plumbeus* (our study) [129], 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 [43, 131] has inspired our opening studies on the PCP degradation capacity of fungal strains capable to completely perforate the cork cell walls [132]. The cork cell wall comprises an inner layer of suberin [133], 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 [131] , including PCP, namely *P. glandicola*, *P. janczewski*, *T. longibrachiatum*, *Chrysonilia sitophila* and *M. plumbeus* [134]. The PCP degradation pathway of *M. plumbeus*, which could deplete virtually all the PCP in medium, was disclosed using a metabolomics-based study [129]. 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* [79] (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 [116] or 4-*n*-nonylphenol [81] 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 [79]. Instead, alachlor increased a cyanide hydratase (nitrilase) in *Paecilomyces marquandii*, which is involved in the regular cyanoamino acid metabolism [116]. 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 [81]. 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* [135] - impacts *Penicillium jancjewskii* metabolism during its stereo-selective hydroxylation to 3β-hydroxy-labdanolic acid [136]. 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 [137]. 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 [138]. 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 [138]. 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 [32, 139, 140]. Using (toxico)metabolomics we further unravelled the uniqueness of the biochemical reactions used by filamentous fungi for the degradation of chlorophenols, specifically in *Aspergillus nidulans* [141]. 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 (see below).

*Present: knowledge gaps on the catabolism of aromatic compounds by fungi*

The aerobic catabolism of aromatic hydrocarbons in fungi occurs ultimately via catechol, protocatechuate, hydroxyquinol, homogentisate or gentisate, which are channelled to central pathways (Fig. I.2) [142, 143]. Transcriptomic-based studies have ensured discovery of the homogentisate pathway (involved *e.g.* in the phenylalanine metabolism) in *P. chrysogenum* [144], and of both the gentisate and the hydroxyquinol variant of the 3-oxoadipate pathway in *Candida parapsilosis* [145]. Recently, we have also disclosed the 3-oxoadipate pathway in *A. nidulans* by relying essentially on 2DE and gene expression assays [146]. In our study, benzoate and salicylate were used as upper precursors for the protocatechuate branch and catechol branch of the 3-oxoadipate pathway, respectively (Fig. I.2). Instead, benzoate or vanillin, have been used as precursors of the hydroxyquinol variant of the 3-oxoadipate pathway in *P. chrysosporium* (Fig. I.2) [147-149].

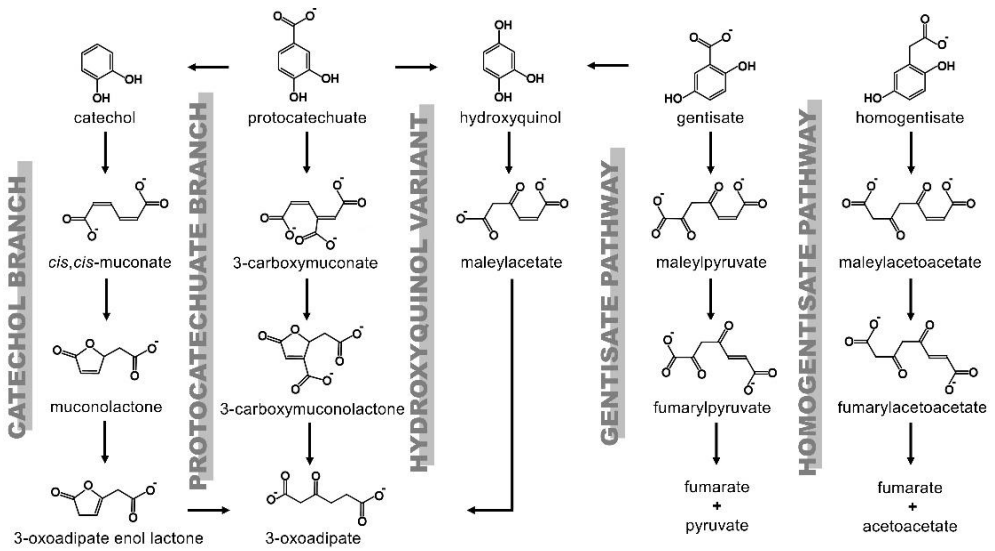


**Fig. I.2 -** Schematic representation of the catabolism of aromatics in fungi; the pathways used for each central intermediate are shown.

Up-accumulation of arylaldehyde and arylalcohol dehydrogenases, glutathione S-transferase, and flavonol reductase/cinnamoyl-CoA reductase, was observed in response to both compounds in *P. chrysosporium* [147, 148]. The first dehydrogenases possibly convert vanillin to vanillate or benzaldehyde to benzoate. In addition, a metabolic switch from the glyoxylate cycle to the TCA was observed, likely to increase the pool of succinyl-CoA required as co-substrate by 3-oxoadipate CoA-transferase [147, 148]. Two P450s were found only to increase in *P. chrysosporium* exposed to benzoate [148]: PcCYP1f and CYP63A1. The first was previously proposed as a functional benzoate 4-monooxygenase and is highly homologous to P450s of the CYP53 family hypothetically displaying similar activity [150-152], whereas the second, a putative 4-hydroxybenzoate 2-monooxygenase [148] is also found expressed in ligninolytic, nutrient limited, conditions [153]. Two flavin-containing monooxygenases (PcFMO1 and PcFMO2) were expressed in response to vanillin in *P. chrysosporium* [149]. PcFMO1 displayed activity against monocyclic phenols *e.g.* phenol, HQ and 4-CP, and is homologous to other phenol 2-monooxygenases [146, 149, 154]. PcFMO2 was not yet functionally characterized as it did not showed activity against the tested monocyclic phenols including vanillin, vanillyl alcohol, or vanillate [149], Vanillin addition increased an extradiol homogentisate dioxygenase family protein of 53 kDa [147], yet a previously biochemically characterized intradiol hydroxyquinol 1,2-dioxygenase has only 45 kDa [142]. A maleylacetate reductase - required in the hydroxyquinol variant of the 3-oxoadipate pathway – remains to be identified, though different alcohol dehydrogenases increased upon addition of either benzoate or vanillin [147, 148].

*Aspergillus nidulans* and other Ascomycota have both the protocatechuate and catechol branches of the 3-oxoadipate pathway, whereas *P. chrysosporium* (and many other) have the hydroxyquinol variant of the 3-oxoadipate pathway or only have one of the branches [143, 155-157].

Benzoate and salicylate as sole carbon and energy sources are directly channelled to the TCA cycle as sources of acetyl-CoA and succinate, consequently they have up-regulated gluconeogenesis and pentose phosphate pathway in *A. nidulans* [146]. The aromatics led to the differential up-accumulation of several short chain dehydrogenase/reductase family proteins and stress response proteins [146], similar to that seen in *P. chrysosporium* [148]. The benzoate catabolism in *A. nidulans* is mediated by five specific enzymes (Fig. I.2) of which only the benzoate 4-monooxygenase (BzuA) has been previously described [158]. In fungi, the intermediate 3-carboxymuconolactone is converted by a single enzyme to 3-oxoadipate (Fig. I.2), in opposition of formation of 4-carboxymuconolactone and a two steps conversion in bacteria [143, 146].

The salicylate catabolism involves eight specific enzymes (Fig. I.2) and has two catabolic shunts: salicylate is converted to catechol directly or in two steps; *cis*,*cis*-muconate is converted to muconolactone either directly or indirectly through its isomer *cis*,*trans*-muconate [146]. The genes codifying for muconate cycloisomerase and muconolactone isomerase were also identified for the first time in *A. nidulans* [146]. These genes are present in a catechol gene cluster in several fungi but not in *A. nidulans*, as subsequently verified in *C. albicans* and other phylogenetically related species [157]. The muconolactone isomerase has no known specific sequence domains and homologous proteins were only found in Dykaria fungi, mostly Ascomycota, indicating the uniqueness of the catechol branch in fungi. Of foremost importance is the functional redundancy of the salicylate and catechol branches owing fungi different tools to achieve degradation of an aromatic hydrocarbon [146].

The *A. nidulans* mutant carrying deletion of the 3-oxoadipic enol-lactone hydrolase gene, showed a phenotype able to utilise salicylate as carbon source during growth in solid media [146]. The hypothesised activation of the hydroxyquinol variant of the 3-oxoadipate pathway, is now refuted since the expression levels of hydroxyquinol 1,2-dioxygenase and maleylacetate reductase genes were unaltered in the mutant *(unpublished results)*. Additional unpublished observations support that a redundant and constitutive mechanism may exist for the degradation of either muconolactone or 3-oxoadipate enol-lactone, possibly related to the degradation pathway of the lactone protoanemonin previously reported for *A. nidulans* [141]. Further knowledge on the degradation of aromatic compounds in fungi (and their complex regulatory mechanisms) is essential to support further developments in the degradation of xenobiotics by fungi.

*Future: frontier studies on the degradation of PCP by belowground fungi*

The impact of PCP pollution in the functioning of belowground fungal communities is poorly understood. PCP (a)biotic transformation processes may led to formation of degradation intermediates that are usually more toxic to both bacteria and fungi than PCP, for example 2,3,4,5-TeCP or 3,4,5-TCP [159]. The fungal belowground community responds rapidly to PCP pollution during composting with diversity shifts that are apparently associated with PCP removal [36]. Ascomycota strains isolated from soils, namely *Byssochlamys nivea* and *Scopulariopsis brumptii*, were also demonstrated to degrade efficiently PCP; interesting more efficiently when co-cultivated due to synergist effects [140].

As detailed in **Chapter III**, we have recently disclosed the PCP degradation pathway used by fungal communities from soils chronically affected by the biocide, through integration of the PCP-derived metabolomes (LC-ESI-HRMS) of each fungal strain (grown axenically) within the community (Fig. I.3) [47]. The cultivable belowground community comprised seventy-seven isolates covering thirty-three species, with a clear dominance by *Penicillium* species [47]. 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 highlighting fungi widespread capacity to degrade the biocide. Most of the fungal PCP-degradation intermediates identified by us, have been observed before in pure cultures of fungi [160] or bacteria [161]. The exceptions consist of compounds never linked before to PCP degradation, namely those in the resorcinol branch and the tetrachloroguaiacol (TeC-G) isomers (Fig. I.3). In more detail, PCP initial dehalogenation to TeCP may involve its reductive dechlorination, either via biotic or abiotic steps (yielding the *meta* and the *para*/*ortho* isomers, respectively) or its peroxidative dechlorination (forming transient benzoquinone immediately followed by $H^+$ mediated reductions) [33, 47, 162]. Both reactions are consistent with the subsequent formation of TeCHQ and tetrachlorocatechol (TeCC), as well as tetrachlororesorcinol (TeCR). The toxicity of the TeCBQ is extremely high, however *Sphingobium chlorophenolicum* is able to circumvent its toxicity, using a TeCBQ reductase that catalyses its reduction to TeCHQ [163]. These tetrachlorinated PCP derivatives, can undergo successive biotic reductive dechlorinations through the resorcinol (R), hydroquinone (HQ) or catechol (C) branches (Fig. I.3), which intersect through additional hydroxylation of their derivatives, yielding the corresponding trihydroxybenzenes (THB) [47, 162].

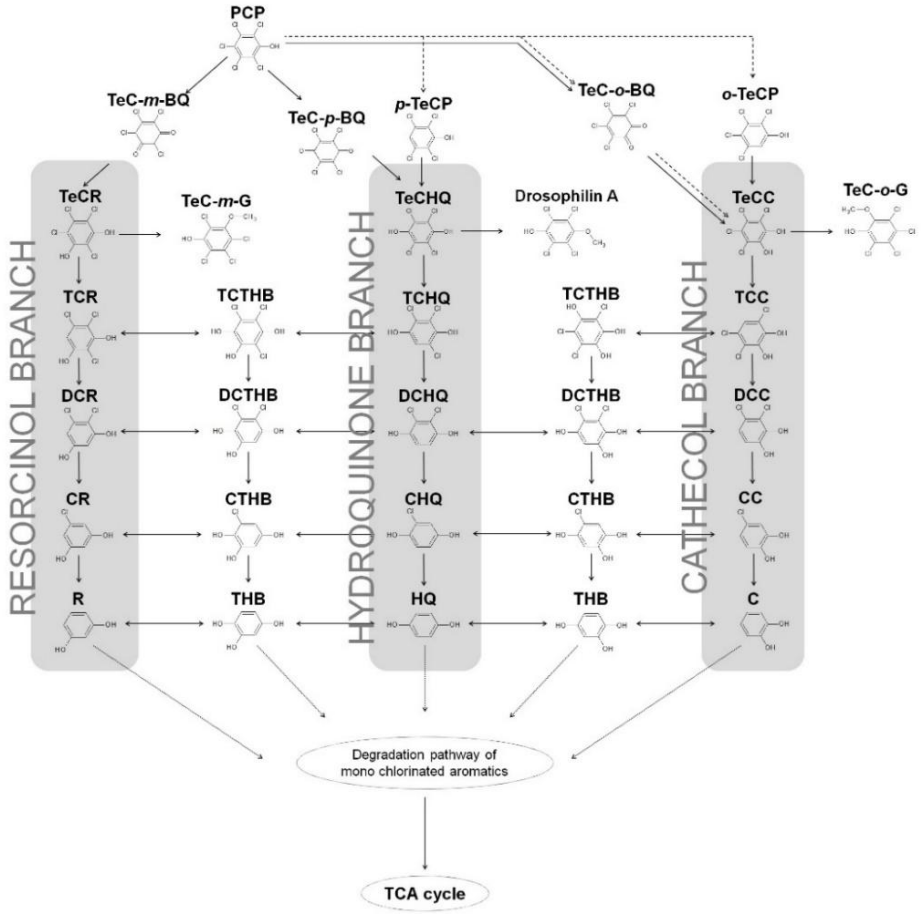


**Fig. I.3 -** Schematic representation of the three branches of the degradation pathway of pentachlorophenol by filamentous fungi, either grown axenically or as a community (submerged cultivation). Continuous and dashed lines mark biotic and abiotic steps, respectively. Conjugated intermediates (apart from those involving tetra-chlorinated species) are not shown in the pathway for matters of simplicity. Adapted from ([47], full reprint in **Chapter III**).

As often reported, fungi can also form several conjugated compounds, *e.g.* through the *O*-methylation and/or sulphation of PCP and/or its derivatives, *e.g.* sulphate trichlorometoxyphenol (S-TCMP) [47, 129]. In particular, the *O*-methylation of chlorophenols leads to formation of chloroanisoles as above mentioned (*e.g.* [164]). The PCP degradation pathway observed in community-based cultures perfectly matched that

inferred by integration of the pathways observed in the axenic cultures, regardless of much higher diversity of PCP-derivatives in the last [47]. 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 [16].

The PCP-derivatives so far identified may be also used as footprint of PCP environmental pollution, some of which, *e.g.* DCTHB and TeC-G (Fig. I.3), can directly link the mitigation of the biocide with fungal activity [47]. Once forest soils of high geochemical homogeneity may display very dissimilar fungal diversity (both taxonomic and functional) [165], community-based proteomic studies may provide complementary means to reveal key functional trends during the decay of the pollutant. Our initial observations on the degradation of PCP by fungal communities isolated from chronically stressed forest soils, show that most alterations are related to stress responsive proteins and carbohydrate metabolism; the major up- and down-proteins so far identified are depicted in the Chapter IV Tables IV.1 and IV.2. Metagenomics is now being explored to support the assignment of specific fungi species to specific functions.

## Conclusions

The comprehension of *in situ* microbial interactions and their response to environmental changes has been recognized as a major challenge for science [166]. 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.

**Acknowledgements**

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## Brief introduction to the complexity of the soil habitat

### Summary

This sub-section will cover some of the aspects that were left out from the previous section. In particular, it will focus multiple fundamental aspects of the soil habitat, comprising its major features, colonisers and functions and empathising agroforestry soils pollution and mycobiota.

### Keywords

Soil, cork oak forests, pentachlorophenol pollution, soil mycobiota

In the last decades, the rapid expansion of industry has been leading to environmental contamination and degradation, affecting human health and bio-diversity integrity worldwide. Human appropriation of natural resources, increasing land-use changes, chemical pollution and modification of climate, among other factors, are increasingly affecting both the phylogenetic and the functional diversity of most ecosystems, ultimately damaging the entire Earth's ecosystem [167].


In the context of environmental pollution, soil is the largest and most active environmental compartment [168].

In agroforestry environments, soil is regarded as a very diverse and invaluable resource (*e.g.* for plant productivity) yet also a very fragile one, being largely affected by atmospheric factors and pollution as well as management uses among other. Soil behaves as the main receptor (*e.g.* agroforestry additives, air pollution deposition), transfer (*e.g.* migration to ground water, root-uptake) and decay (absorption, microbial

transformation) vector of pollutants. Soil is an incredible and complex habitat; the central organizing entity in terrestrial ecosystems that contributes to link the multiple interactions of belowground biodiversity [169]. The soil-forming features (interconnected) are five: parent material, climate, biota, topography and age [170]. Each of these factors contributes differentially to the formation of distinct soil horizons. The degree of similarity (or dissimilarity) in a defined soil horizon, is used to categorize distinct soils; a critical classification for subsequent soil management decisions. The soil organic matter (SOM) is a keystone component of the ecosystem determining soil structure, hence erosivity, especially by retaining nutrients and water. Importantly, SOM acts as the energy store that drives many of the soil-based processes, particularly those that involve the soil microbiota. A well-charted phenomenon is the decline in SOM as a result of the appropriation of natural ecosystems for agricultural uses [171].

The importance of soil covers food, life, protection and home, yet regardless that life on Earth depends strictly on the soil quality and on its sustainable use, often soil is misused and considered an inert and dirty entity. However, soil assures several ecological functions such as biomass support, filter and reservoir of genetic variability and nutrient cycling as well as regulates hydrological cycles to control water quality and quantity. In fact, soil quality is essential to provide raw materials, reduce the risk of floods and preserve our history in terms of archaeological patrimony.

Soil quality is essential to provide raw materials, preserve our history and reduce the risk of floods. The soil ecosystem is home to over one fourth of all living species on Earth; hosting the most diverse microbial community [167, 172]. The biota under a footprint is extremely diverse, comprising a diversity of plants, animals and microbes that are linked together in complex ecological interactions [173].

The soil mycobiota is the major player when accounting the decomposition of plant organic matter into nutrients; essential to sustain animals and plants. The soil mycobiota that plays a central role as a soil-

forming factor especially in the upper soil horizon, is largely influenced by environmental conditions *in situ*, *e.g.* pH, temperature, moisture, nutrient availability and oxygen concentration [174].

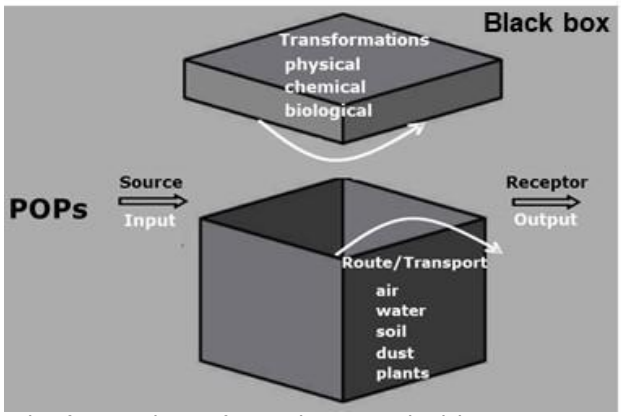
In soils, fungi play a very distinct function compared to bacteria; accordingly the microbial communities and the associated food webs might be dominated by either group, which may change during ecological succession and in response to anthropogenic disturbance, *e.g.* intensive farming [52]. Fungal-dominated food webs promote slow and highly conservative cycling of nutrients. In general, they dominate less disturbed ecosystems, late successional sites, often with acid soils that are of high organic matter content and low resource quality. Fast- and slow-growing plant species produce large amounts of high quality (*i.e.* N-rich) litter and low-quality phenolic-rich litter, respectively, accordingly promoting fast or slow nutrient cycling. By other words, fungi are likely to dominate the microbiota in forest soils.

Our understanding of the soil mycobiota (diversity and functioning) is slowly but steadily progressing [54, 175], however compared with pathogens and/or symbionts, fungi that are, at first glance, symptomless, remain largely overlooked. Such poorly investigated groups are an untapped reservoir that contributes to soil functional biodiversity, and may be important in promoting plant health and productivity, as well as in the mitigation of atmospherically dispersed pollutants. In polluted soils, PCP generally leads to a decrease in $CO_2$ respiration and to a reduction in the microbial species heterogeneity [176]. PCP reduces the resistance of the decomposer community and the efficiency of natural composting [47] and may lead to bioaccumulation and plant root-uptake [176]. The question that remains to be addressed is how important is the contribution of the mycobiota to the fate and mineralisation of PCP in forest soils. To address this, a mechanistic analysis of the degradation of PCP by the soil mycobiota, integrating information on the diversity of both, sub-products and species, is yet lacking.

*"Soil habitats probably contain the greatest microbial diversity of all environments on Earth"* [177]. To better understand soil microbial biodiversity and dynamics, soil sciences need to become a more interdisciplinary field of study, integrating a broad range of distinct expertise, from biology, chemistry and physics to geosciences [178, 179]. The soil microbes influence the continuous supply of important ecosystem processes (*e.g.* primary nutrients, soil formation, and nitrogen and carbon cycling) and final ecosystem services (*e.g.* provision of water, food and feed). The unseen world that exists in soil comprises a huge portion of microbial diversity [173]. Obviously, the transformation of pollutants reaching the soil compartment by endogenous microbes is a major fate component of environmental pollution. The multi-functionality of soils may be severely affected by losses of the integrity of microbial diversity [52, 180]. These questions are at the centre of the present thesis: to rely on a multidisciplinary approach to unravel the impact of an atmospherically relevant pollutant - PCP - in the functioning of the soil mycobiota.

The implications of catchment/management activities on the pollution status of agroforestry areas and the potential tensions between forest users and preservation of soil and water quality resources are critical. To ensure the development of sustainable forest management tools, improved monitoring of the implication of current practices becomes mandatory. This is particularly true facing long-time [181] EU discussions on the Soil Framework Directive, the principles of which have been translated into national Soil Strategies in many member states [182]. The Pesticides (914/414/EC) and Water (2000/60/EC) Framework Directives altered the Community water policy; however, the use of biocides in Europe is still the highest worldwide (http://www.loc.gov/law/foreign-news/article/european-union-new-regulation-on-biocides/). The soil quality is profoundly affected by agroforestry practices being frequently contaminated with *e.g.* pesticides and wood preservatives [178, 183]. Typically, agroforestry ecosystems receive man-made chemicals that lead to soil, water and air contamination, that

together with food, are the major routes/paths of exposure of humans to POPs. However, agricultural pollution has often been considered, but wood forests, commonly accepted as non-polluted environments, yet receiving high quantities of pesticides and wood preservatives, remain largely neglected [44].




Role of contaminants fate and transport in risk assessment (adapted from CARACAS, Ed. Ferguson, C., et al., 1998)

**Fig. I.4 -** Schematic view of POPs natural decay. Soil is the largest and most active compartment in the environmental decay of POPs, contributing to their dispersion through fate and transformation reactions, as well as transport to other environmental compartments.

The dynamic integration of chemical uptake and metabolism during exposure [184] is controlled by the soil's physiochemical properties (*viz.* pH [185], organic matter content [186] and concentration effects and kinetic factors [187]). Soil is an extremely heterogeneous system, therefore a given concentration in a small soil sample does not disclose the bioavailable fraction to a specific organism; also the same amount of a chemical is not equally bioavailable in distinct soil samples [184, 188]. The fate and transport of any chemical in the natural environment depends on its dissociation constant (Ka) and its partition coefficient (inferred usually in an octanol-water system, Kow). In chlorophenols, the dissociation constant increases (*i.e.* its pKa, logKa decreases) with an increasing number of chlorine atoms in the molecule [34]. On the other hand, the Kow strongly increases with the number of chlorine atoms, and the water solubility (hydrophilicity) inversely decreases. Chlorophenols can strongly adsorb to sediments because of their high lipophilicity, reducing bioavailability [34, 51].

Consequently, their bioavailability is greater in sandy sediments than in those with a high organic content [44, 51]. The variability of PCP toxicity over time results from the formation and destruction of toxic transformation products [189, 190]. Generalised restrictions on the sale and use of PCP have severely limited its agricultural use (detailed in Fig. I.4). Surprisingly, as extensively discussed above, such severe and broaden restrictions have not completely eliminated PCP from the environment, including soils. A wide range of data substantiates the global dimension of PCP contamination, reinforcing also its ability to undergo LRAT ***(see further details in the two other sub-section of Chapter I)***. Taken as an example, POPs found in the Arctic environment come from distant global sources via long-range atmospheric and oceanic transport from lower latitudes [12], *e.g.* chlorophenols, produced by microbial degradation of PCP [191], appear in sediments dated as 1940, shortly after PCP introduction. It was suggested that fresh applications of currently-use chlorinated pesticides are still adding to Arctic contamination [35, 192]. However, our knowledge on the distribution of several POPs, including PCP and other chlorophenols in Europe and worldwide, particularly in agroforestry soils, is still limited. In particular, the number of POPs monitoring sites is especially unsatisfactory in the south and east of Europe and a "multi-purpose" monitoring strategy, linking urban, rural and global sites is lacking.

The Mediterranean region exhibits significant data gaps, only Croatia, Egypt, Israel, Italy and Spain reported PCP studies [193]. Until recently, no information was available on the levels of PCP found prevalent in cork oak forests, regardless a high probability of contamination could be inferred from the recurrent contamination of cork with a PCP sub-product – PCA [44]. The following lines will cover in brief and introduction of the cork oak forests, comprising their most typical features.

## Mediterranean *Quercus suber* L. Forest Ecosystems

*Quercus suber* L., known as cork oak, is a slow-growing evergreen tree species native to western Mediterranean area. Cork oak woodlands cover *ca.* 1.5 million ha in Europe and 700 000 ha in North Africa [194-196], they are therefore of great significance and are well documented historically – highlighting their use as viable study locations. Their geographic range covers an area separated by 4000 km from Portugal - the largest Cork Oak forest area with > 700 000 ha (23% of total Portuguese forest and *ca.* one third of the world's cork oak forest area) - to Bulgaria, the smallest (1500 ha) and youngest area (introduced in 1954).

*Quercus suber* L. can be grown either in mixed or pure stands achieving 14-16 meters height and a longevity of 250 - 350 years [194]. Yet, the age of 150 - 200 years is regarded as the limit for industrial practice. This species is characterized by the presence of a rough bark that can grow up to several centimetres thick. The outer part of this layer, the phellem, is known as cork and constitutes a natural barrier that protects the plant from the surrounding environment [197]. Cork main application is in the manufacture of stoppers for wine bottles; probably the best material that can effectively and safely close a bottle while allowing proper maturation of the wine [43].

Extensive human intervention in the cork oak forest lead to the creation of a specific agro-forestry landscape call the "Montado" (or Dehesa), supported by their high economic importance and historical value. These are usually described as multifunctional agro-silvo-pastoral systems, where agriculture/forestry and nature conservation are combined in a sustainable manner. Obviously, the archetypal tree species of the Montado belong to the genus *Quercus* (oak), usually cork (*Q. suber* L.) and holm (*Quercus ilex*), although other tree species may be also present such as beech and pine (depending on geographical location and elevation). The Natura 2000 network classifies the Montado (Habitat 631) and Cork Oak Forests (Habitat 9330) as "very important" for the conservation of biodiversity. They support one of the world's highest levels of forest biodiversity that includes endemic

plants (*e.g. Cistus* spp.), and animal species, including some reference species such as the Iberian Lynx, Imperial Eagle, Wildcat, Short-toed Eagle, the Booted Eagle and the Bonnelli's Eagle, Black Stork, the fighting bull, and the black Iberian pig [195]. The importance of these habitats arises from direct benefits derived from cork exploitation and/or parallel activities carried out under cork canopies (*e.g.* cropping, grazing). The value of the cork oak forests is not just limited to the abovementioned; in fact when well managed and valued, these forests protect soil from erosion and desertification, contribute to climate change adaptation and mitigation and act as a buffer against forest fires. Apart from the economic potential, they have high social importance, particularly in maintaining rural population.

The selection of Cork Oak forests as our model location for investigating how the atmospherically relevant PCP pollutant affects the functioning of the soil mycobiota is supported by several inter-related facts: **i)** the cork taint defect, that affects 2 to 7 % of the bottled wines [131], may be indirectly linked to the contamination of cork with chlorinated phenols; **ii)** Portugal is the major producer of cork stoppers; *i.e.* cork and cork products represent about 30 % of forest sector exports, and 3 % of the national exports [198]; **iii)** our understanding of the impact of PCP pollution in the cork oak forest ecosystem, especially in the soil mycobiota, is virtually lacking and finally, **iv)** anecdotal evidence of contamination of cork produced in Tunisian with both chlorinated phenols and anisoles granted us with a remote location for developing our study and testing our initial hypothesis: these ecosystems are contaminated with high levels of PCP.

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

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### 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.

## Setup

### *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 [199]. 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 [200]. 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 [201]. 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) [10]; this calls for renewed interest in its global distribution and impacts in Earth's ecosystems. 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 [14], PCP efficiency as pesticide and antimicrobial compound, started to be recognised. Carswell and Nason [15] 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 [202] (Fig. I.5). 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) [22]. 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 [41, 42], as well as new cases of PCP poisoning, some of which resulting in death [25]. Moreover, the capacity of PCP to affect the uncoupling of mitochondrial oxidative phosphorylation was sorted out [23].

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 [27]. Consequently, in the early 80s the first restrictions on the use and production of PCP were established in most developed countries [35]. During this decade, scientific publications on the microbial degradation of PCP started to come to light [29, 30]. 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 [31]. 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 (Fig. I.5), stimulated also by the biotechnological interest in both microbes and enzymes mediating modification of organohalogenates [203]. 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 [27].

Now, PCP is already considered a POP [10] and severe actions to eliminate its production and use should be soon implemented.

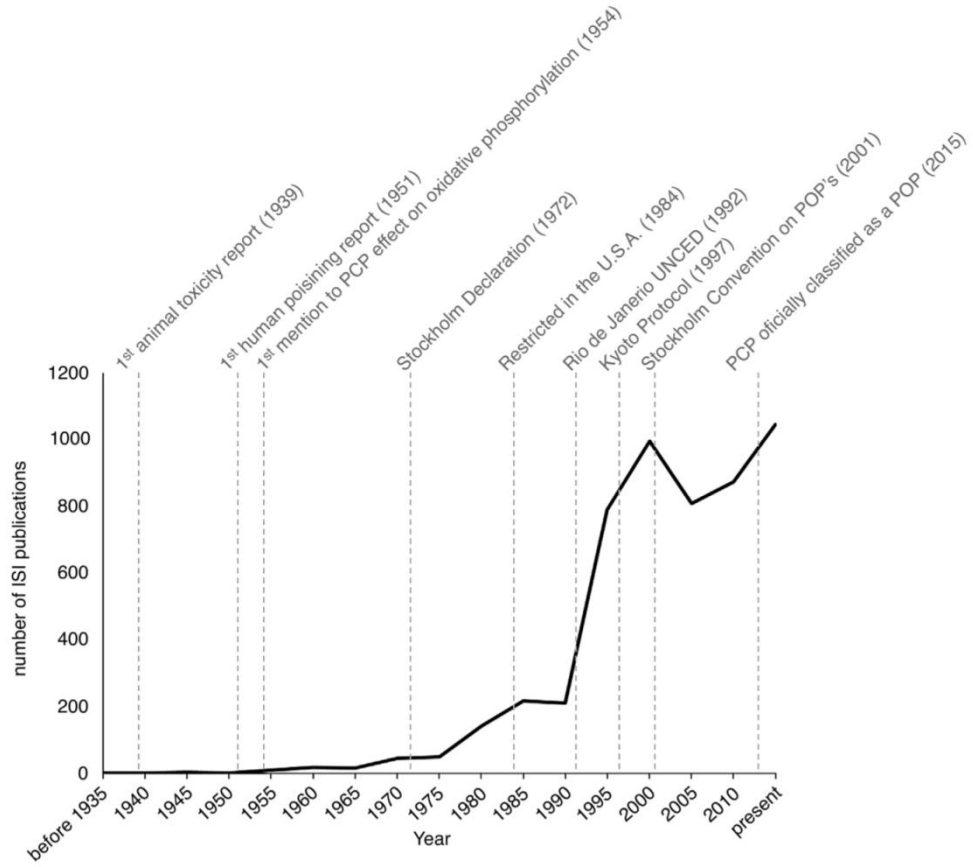


**Fig. I.5 -** Number of scientific publications on pentachlorophenol along time [source: ISI Web of Knowledge]. Papers published each 5-year were searched using the term 'pentachlorophenol' in the topic field, and plotted in a non-cumulative way. Important landmarks, either on environmental awareness 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.

## Confrontation

### *Potential active threats of PCP*

PCP is still used in many locations in the globe (*e.g.* in China [38]) and is also produced as a side-product during the degradation of more volatile biocides, for example, pentachlorobenzene [39] and hexachlorobenzene [40]. The long history of use, persistence and transboundary nature, has resulted in extensive environmental PCP contamination worldwide [34, 37]. Today, PCP is still globally detected in human fluids and tissues from exposure in both indoor and outdoor environments (*e.g.* [41, 42]). Data on its cytotoxicity are consistent with PCP acting as endocrine disruptor and carcinogen [204, 205]. 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 [131]. 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 [206], yet many fungi are weak anisole producers and the yield of anisole formation is also largely influenced by the availability of nutrients [131, 164]. 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 [43, 44], PCP impacts in *Quercus suber* forests remain largely overlooked. The bark behaves as a sampler accumulating both gaseous and particulate pollutants [207], but PCP partition to the soil is likely to be significant (estimated to be as high as 95% [35]). 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 [196] and supporting one of the highest levels of forest biodiversity [45]. 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 [48-50].

***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 [47] (full reprint in **Chapter III**). Soil samples collected along 113 $km^2$ contained detectable levels of PCP (Fig. I.6a), ranging between *ca.* 2 and 30 $\mu g \cdot Kg^{-1}$, often above levels detected at locations currently treated with the biocide [38]. 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 [35]. 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' [208].

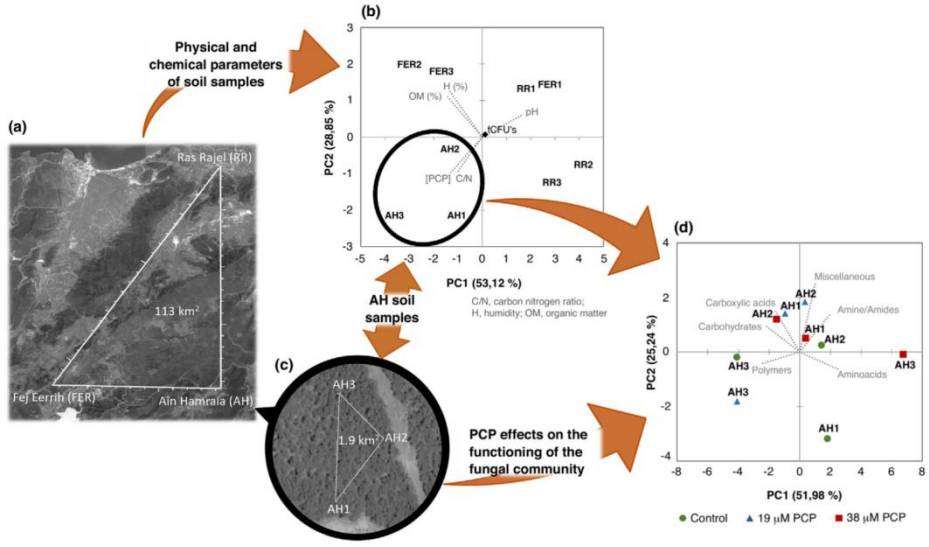


**Fig. I.6 -** Schematic representation of the dissimilarity degree of cork oak forest soils in N.W. Tunisia, comprising geographic location, geochemical features and the physiological profile of the colonising fungal communities. Adapted from [47]. Pentachlorophenol (PCP) was found prevalent in soils 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).**

## Resolution

### *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 [209, 210], for example, linked to increased degradation of organic matter and nutrient cycling and availability [49, 50, 199, 211]. The 'meta-profiling' of natural microbial communities may help disclosing the ecosystem' yellow-pages - 'who is there and who is doing

what' [201], revealing, for example, the structure, stability and/or function of real fungal communities in soil ecosystems [49, 54].

'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 [212], and to disclose the structure of the community that mediates polycyclic aromatic hydrocarbons degradation in the deep-water horizon [213, 214]. PCP has the potential to adversely affect the functional biodiversity in specific niches, for example, affecting soil invertebrates [215] and bacterial denitrification processes [216].

In particular, the fungal community responds rapidly to PCP pollution with diversity shifts that are apparently associated with PCP removal, for example, during composting [217] and in forest soils [47]. The widespread capacity of fungi to efficiently degrade PCP and other less chlorinated phenols has been established for long [30, 33, 47, 138, 160]. 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 [218]. Moreover, some PCP derivatives have been elucidated in pure cultures, either of fungi [33, 47, 129, 160, 219] or of bacteria [161, 163].

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 (Fig. I.6b,c), by integrating the PCP-derived metabolome of each cultivable member of this community (Fig. I.7) [47]. 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 [217] or bacteria [161]. 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) [33, 47, 162]. 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 [47]. 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 [47]. Not surprisingly, PCP prevalence in soil reduces biodiversity and induces specialization events in the colonising fungal community [47, 217]; for example, PCP was found to reduce the community functional dissimilarity (Fig. I.6d). Once forest soils of high geochemical homogeneity may display very dissimilar fungal diversity (both taxonomic and functional) [165], 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.

## Conclusion

Current studies on PCP are reinforcing wide-ranging principle of global and dispersed environmental pollution by this biocide [42]. 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 [47]. 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 [220]), is critically required, regardless of the capacity of the colonising fungal community to act as buffer against the disturbance caused by the biocide [47, 48, 218]. Fungal communities can ensure a short lifetime and the rapid mineralisation of chlorinated phenols [141], in opposition to bacterial counterparts which usually yield highly toxic and recalcitrant non-chlorinated or chlorinated phenol derivatives [160, 204, 219, 221]. 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 [218] as well as with other important soil colonisers such as the earthworms [222].

Nowadays numerous disparate chemicals are continuously discharged to the atmosphere under the influence of climate change that dramatically alters their global distribution and fate [9]. 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.

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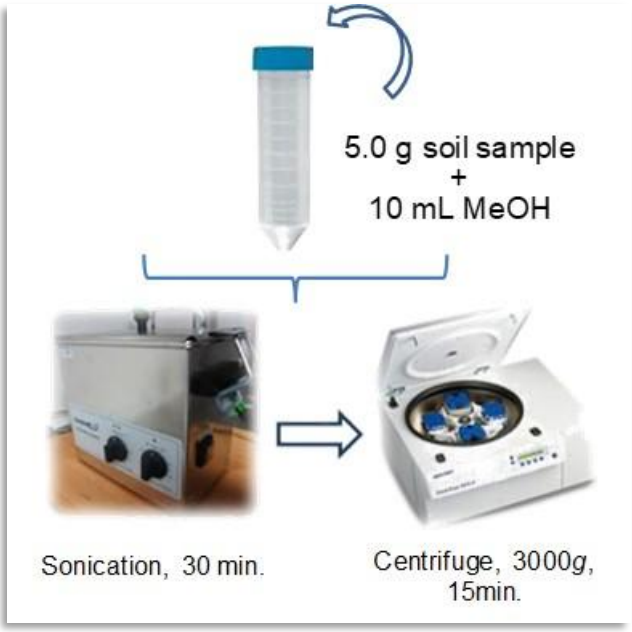
# CHAPTER II

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## Development of a robust chromatographic method for the detection of chlorophenols in cork oak forest soils

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### Abstract

A major concern for the cork and wine industry is 'cork taint' which is associated with chloroanisoles, the microbial degradation metabolites of chlorophenols. The use of chlorophenolic compounds as pesticides within cork forests was prohibited in 1993 in the European Union (EU) following the introduction of industry guidance. However, cork produced outside the EU is still thought to be affected and simple, robust methods for chlorophenol analysis are required for wider environmental assessment by industry and local environmental regulators. Soil samples were collected from three common use forests in Tunisia and from one privately owned forest in Sardinia, providing examples of varied management practice and degree of human intervention. These provided challenge samples for the optimisation of a HPLC-UV detection method. It produced recoveries consistently >75 % against a soil CRM (ERM-CC008) for pentachlorophenol. The optimised method, with ultraviolet (diode array) detection is able to separate and quantify 16 different chlorophenols at field concentrations greater than the limits of detection ranging from 6.5 to 191.3 $\mu g \cdot Kg^{-1}$ (dry weight). Application to a range of field samples demonstrated the absence of widespread contamination in forest soils at sites sampled in Sardinia and Tunisia.

**Keywords**

HPLC, Method development, *Quercus suber* L., cork oak, chlorophenols, NATO Science *for* Peace

## Introduction

Cork taint, a mouldy earthy aroma, is commonly associated with the presence of chloroansoles, in particular, 2,4,6-trichloroanisole, 2,3,4,6-tetrachloroanisole and pentachloroanisole [1] in bottled wine. Predominantly, an olfactory problem in its significance in the wine industry is such that it can affect up to 5 % of bottled wine [2]. In chemical terms, chloroanisoles are a microbial degradation product of the corresponding chlorophenol, *e.g.* 2,4,6-trichloroanisole is produced through the o-methylation of 2,4,6-trichlorophenol [3];[4]. Stringent quality control steps were introduced in C.E. Liège [5]; however, contamination of cork stoppers and wine can still occur [6].

It is known that persistent organic pollutants (POPs), such as organochlorine pesticides and polycyclic aromatic hydrocarbons, accumulate within cork oak bark due to their lipophilic properties and that any regional variation in pesticide presence is due to differing agricultural management practices [7]. A 2005 study found that if correct cork stopper manufacturing procedures are followed then POP levels in cork stoppers (< 60 $ng \cdot g^{-1}$ cork) are consistent with those in food [8]. This suggests that contamination of cork stoppers occurs at an early stage in the production process with pentachlorophenol (PCP) use within forests a probable cause. The International Code of Practice prohibits chlorophenol use within the cork stopper manufacturing process; however, the presence of chloroanisoles within cork stoppers indicates that the application of chlorophenol as a pesticide continues on a limited, regional basis [9, 10]. This regional and unknown variability requires robust and simple to apply analytical methods

for deployment in routine monitoring, which can help ensure the sustainable management of an important and threatened ecosystem.

The Kyoto Protocol calls for the “protection and enhancement of sinks and reservoirs of greenhouse gases”; and forest ecosystems, such as the cork oak (*Quercus suber* L.), are important carbon sinks to mitigate against climate change. These forests, situated along the coastlines of northern Africa and southern Europe, have been developed since the 19$^{th}$ century as a source of income and livelihood for a large local population [11]. The total value of cork products is €1.1 billion annually [12] with cork stoppers as the primary product. Approximately 11 % of the Tunisian population rely on forests as their primary source of income [13] and the North African forest regions are of major social as well as economic significance. However, organochlorine contaminants (including metabolites of chlorophenols) have previously been detected in North African river water, sediments and soil samples [14, 15], indicating widespread presence in the region. This observation has been supported anecdotally by issues of cork slab contamination reported by industrial processors in key locations in North Africa.

The work presented here is part of a wider NATO Science *for* Peace project involving the collaboration of five research facilities in Italy, Morocco, Portugal, Tunisia and the UK focussing on *in situ* remediation techniques to address the potential of chlorophenol contamination in *Q. suber* L. forests. The project aimed to develop common analytical methodologies so that a robust and defensible data set could be achieved and determine the contamination status of the forests. The soil extraction methodology [16] and the chromatography method [17] initially identified was based on common industry practice, but they encountered difficulties in obtaining reliable and efficient extraction and peak identification. In addition, methodologies reported in the wider literature commonly only identify a small number of chlorophenols (see Table II.1). Therefore, the aim of the work reported here was to develop **i)** a soil extraction technique with high

extraction efficiency and **ii)** a chromatographic method which has baseline separation of chlorophenols in soil extracts. This study is also aimed to provide an optimal sample preparation approach to support future deployment in collaborating laboratories and **iii)** to apply the method to soil samples collected from a number of cork oak forest sites, to establish the baseline chlorophenol status.

**Cork 0forest soils**

Soil samples were collected from three Tunisian *Q. suber* L. forests in February 2009 and from a Sardinian forest in June 2008 and March 2009 following the protocols of the International Sampling Standards [18]. Within each forest, three locations were chosen. From each location, a composite soil was prepared from five subsamples collected from the tip of the arms and the centre of a cross (each arm was 1 m in length). The samples were manually homogenised and coarsely sieved in the field to remove leaf litter and large pebbles before transportation to the local host laboratory as soon as possible for refrigeration, separation into aliquots of ~200 g prior to distribution by courier to the collaborating laboratories. Once samples were received at the laboratory, they were air-dried and sieved to < 2 mm for analysis. Samples collected were from surface (SF, 0–10 cm) and subsurface (SB, 10–20 cm) depths at each site (n=18). The sample locations are detailed in Table II.2.

**Table II.1 -** Analytical methods used to determine chlorophenols in soil

| GC/LC | Determinands | Extraction method | Extraction efficiency (%) | Internal/external standard | Detector | LOD/LOQ ($\mu g \cdot Kg^{-1}$) | Reference |
|---|---|---|---|---|---|---|---|
| GC | PCP | Soxhlet | * | External | ECD | * | [19] |
| GC | 2-MCP; 2,4-DCP; 2,4,6-TCP | Sonication | 96.80 ± 4.85; 107.80 ± 26.61; 91.53 ± 5.89 | External | AED | 0.54/1.82; 0.34/1.07; 0.08/0.27 | [20] |
| GC | PCP | Sonication | * | * | ECD | */5 | [21] |
| GC | 2-MCP; 2,4-DCP; 2,4,6-TCP; 2,3,4,6-TeCP; PCP | Soxhlet | * | 2,4,6-TBP | MS | * | [22] |
| GC | PCP | * | * | 2-MBP | ECD | 10 | [23] |
| GC | PCP | Sonication | 88-101 | * | ECD | 5 | [24] |
| GC | 2,4-DCP; 2,4,6-TCP; 2,3,4,6-TeCP; PCP | HS-SPME | 86.1; 88.6; 92.5; 89.2 | Ext Std | ECD | * | [25] |
| LC | 2,4-DCP; 2,4,6-TCP; PCP | * | * | * | DAD | * | [26] |
| LC | 2,4-dcp | * | * | * | uv | * | [27] |
| LC | 2,4-DCP; 2,4,5-TCP; PCP | * | * | Ext Std | DAD | * | [28] |
| LC | All chlorophenols | SPME | Varies | 3-MBP | APCI-MS | PCP: 103(LOD) | [29] |

ECD electron capture detector, AED atomic emission detector, MS mass spectrometer, DAD diode array detector, UV ultra-violet detector, APCI atmospheric pressure chemical ionisation; * Not listed within paper

**Table II.2** - Details of sample locations

| Sardinia station | | GPS coordinates North | GPS coordinates East | Alt. (m) | Tunisia sample | | GPS coordinates North | GPS coordinates East | Alt. (m) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 40°54′53.70″ | 009°07′52.30″ | 460 | AH | 1 | 36°46′47.50″ | 008°51′52.00″ | 547 |
| | 2 | 40°54′53.30″ | 009°07′52.10″ | 450 | | 2 | 36°46′49.20″ | 008°51′53.80″ | 538 |
| | 3 | 40°54′54.10″ | 009°54′54.10″ | 455 | | 3 | 36°46′50.40″ | 008°51′50.40″ | 548 |
| 2 | 1 | 40°54′56.10″ | 009°07′58.80″ | 442 | FER | 1 | 36°46′57.90″ | 008°43′47.20″ | 792 |
| | 2 | 40°54′55.60″ | 009°07′59.30″ | 445 | | 2 | 36°46′58.30″ | 008°43′49.60″ | 804 |
| | 3 | 40°54′55.30″ | 009°08′00.30″ | 421 | | 3 | 36°46′58.10″ | 008°43′52.70″ | 804 |
| 3 | 1 | 40°54′48.20″ | 009°08′00.50″ | 454 | RR | 1 | 36°57′14.30″ | 008°51′51.50″ | 61 |
| | 2 | 40°54′48.80″ | 009°08′00.60″ | 451 | | 2 | 36°57′16.20″ | 008°51′45.60″ | 56 |
| | 3 | 40°54′48.90″ | 009°08′01.50″ | 452 | | 3 | 36°57′15.20″ | 008°51′48.50″ | 48 |

AH - Aîn Hamraia; FER - Fej Errih; RR - Ras Rajel

## Experimental procedures

*Materials and equipment*

Methanol, acetone, hexane (HPLC grade, Rathburn Chemicals, Walkerburn, UK), cellulose extraction thimbles (25 × 100 mm, Whatman, Aldrich, UK), Waters Symmetry C18 column (150 × 3.9 mm, 5 µm; Sigma-Aldrich, UK), UHP water (Elgastat UHP system coupled to an Option 3 Water Purifier, Elgastat, UK), acetic acid (100 % extra pure, Riedel-deHaën, UK, or ACS reagent ≥ 99.7 %, Sigma-Aldrich). Standards of 2-monochlorophenol (99.9 %), 3-monochlorophenol (98.1 %), 4-monochlorophenol (99.9 %), 2,3-dichlorophenol (99.9 %), 2,4-dichlorophenol (99.4 %), 2,5-dichlorophenol (99.7 %), 2,6-dichlorophenol (99.9 %), 3,4-dichlorophenol (Primar), 3,5-dichlorophenol (99.8 %), 2,3,5-trichlorophenol (Primar), 2,4,5-trichlorophenol (99.6 %), 2,4,6-trichlorophenol (99.2 %), 2,3,4,5-tetrachlorophenol (98 ± 5 %), 2,3,4,6-tetrachlorophenol, pentachlorophenol (98 %), 2,4,6-tribromophenol (99 %; all from Sigma-Aldrich). Soil reference material (CRM): PCP in soil (2.04 ± 0.18 mg·Kg$^{-1}$, ERM-CC008, LGC-Promochem, UK). Chromatographic separations were carried out using the following: Thermo Separation Products P2000 LC

pump, with an AS1000 auto-sampler and a UV2000 UV/Vis detector (Thermo Fisher, UK); a Shimadzu Prominence LC-20AP pump with a SIL-20 AC auto-sampler, a CTO-20 AC column oven, a SPD-M20A diode array detector (DAD) and a DGU-20AS degasser (Shimadzu, UK).

*Soxhlet extraction*

A 15 g aliquot of soil was added to cellulose extraction thimbles and were Soxhlet extracted for 16 h using 500 mL hexane:acetone (4:1 v/v). Samples were reduced under rotary evaporation to approximately 10 mL and $NaSO_4$ added to dry the extract. The extract was centrifuged to remove all $NaSO_4$ and any other particulates, reduce to dryness and reconstituted in 1 mL methanol.

*Sonication extraction*

5 mL of 1 $mg \cdot L^{-1}$ 2,4,6-tribromophenol in methanol, was added to 2.5 g of soil, sonicated for 30, 45, 60 or 90 min and centrifuged at 3000*g* for 15 min. The supernatant was removed, and the procedure repeated (total extraction time of 60, 90, 120 or 180 min). Each supernatant was combined, reduced to dryness and reconstituted in 1 mL of methanol for analysis.

*Internal standard*

2,4,6-tribromophenol was selected as the internal standard as it was not a product expected to be found within cork forest soils and has a similar structure to the target analytes. It has previously been detected in wine [30], food [31] and the aquatic environment [32] but not to our knowledge in *Q. suber* L. forest soils.

*Initial chromatography conditions*

These were based on work previously reported for PCP in biodegradation studies [17]. Starting conditions were as follows: Mobile phase A: Acetonitrile; Mobile phase B: 0.1 % Phosphoric acid. Mobile phase gradient:

10 % A to 99 % A (30 min), 99 % A to 10 % A (2 min), hold at 10 % A for 13 min. Total run time was 45 min. Flow rate: 1.2 mL $min^{-1}$; Detector wavelength: 230 nm; Injection volume: 20 µL.

*Equations used in chromatographic identification*

A number of calculations are used to aid peak identification (i) the column dead time, $t_0$, (ii) the retention factor, *k*, (iii) the separation factor, α and (iv) the relative retention, $t_{RR}$.

(i) $t_0 = V_m/F$, where $V_m$ is the column dead volume (volume of mobile phase within column) and *F* is the flow rate (mL $min^{-1}$),

(ii) $k = (t_R-t_0)/t_0$, where $t_R$ is the retention time of the analyte,

(iii) $\alpha = k_2/k_1$, where $k_2$ and $k_1$ are *k* values for adjacent peaks, and

(iv) $t_{RR} = k_{IS}/k_{CP}$, where $k_{IS}$ is the *k* value of the internal standard, and $k_{CP}$ is the *k* value of the chlorophenol.

## Results and discussion

*Abbreviations*

The following abbreviations are used in the discussion section below: MCP, monochlorophenol; DCP, dichlorophenol; TCP, trichlorophenol; TeCP, tetrachlorophenol; PCP, pentachlorophenol; TBP, tribromphenol; CRM, certified reference material.

*Selection of the extraction method*

Although the Soxhlet extraction method was reproducible [as confirmed by the low relative standard deviation (SD)], our concern was the low extraction efficiency (32.01 ± 0.81 %) from the soil CRM. To determine the source of analyte loss, the hexane:acetone solvent mixture was spiked with a known concentration of PCP and the solvent analysed **i)** after the 16 h extraction process and **ii)** after the whole procedure had been completed. Only 28 % of PCP was recovered at the end of the whole procedure compared to 67 %

after the 16 h, indicating that the low extraction efficiency was caused by loses during the rotary evaporation stage.

Using the CRM, it was found that sonication for 60 min produced the highest extraction efficiency; the CRM was extracted in triplicate for 60 min; with an overall extraction efficiency of 75.9 ± 5.8 % (Table II.3).

**Table II.3** - Sonication extraction method efficiency of standard reference material (CRM ERM-CC008) with extraction time

| **Method development** | | **Optimised extraction time (60 min)** | | |
|---|---|---|---|---|
| **Extraction time (min)** | **Extraction Efficiency (%)** | **CRM replicate** | **Extraction efficiency (%)** | |
| 60 | 77.5 | | Replicate | Mean |
| 90 | 46.6 | A | 77.5 | 75.9 ± 5.8 |
| 120 | 53.1 | B | 80.7 | |
| 180 | 51.4 | C | 69.5 | |

*Optimisation of chromatographic separations*

The initial starting conditions were evaluated for suitability by analysing Soxhlet and sonicated extracts of cork forest soil samples. Peak identification was based on the *k* value (Soxhlet extraction) and the $t_{RR}$ value (sonication extraction). The initial evaluation highlighted potential difficulty with the identification of PCP. Within the forest soil samples and the CRM extracts, a peak was detected which had *k* and $t_{RR}$ values similar to the PCP in the standard solutions (see Fig. II.1 and Table II.4). The proximity of this peak to our target meant that a separation method should be able to resolve any co-eluting compounds. In forest soils, complex naturally occurring organic matter contains numerous co-extracting substances, recognised in hexane/acetone solvents and made this inappropriate for further application.

**Table II.4** - *k* and $t_{RR}$ values of potential PCP peak within the *Quercus suber* forest soil samples following analysis on HPLC-UV/Vis

| Sample | Soxhlet extract *k* Values* | Sonication extract $t_{RR}$ Values* |
|---|---|---|
| Standard solutions | 5.708 ± 0.160 | 0.914 ± 0.014 |
| Sample 1 | 5.775 ± 0.103 | 0.921 ± 0.004 |
| Sample 2 | 5.688 ± 0.122 | 0.897 ± 0.015 |
| Sample 3 | 5.769 ± 0.214 | 0.911 ± 0.025 |
| Sample 4 | 5.874 ± 0.298 | 0.915 ± 0.013 |
| Sample 5 | 5.787 ± 0.198 | 0.903 ± 0.008 |
| Sample 6 | 5.528 ± 0.177 | 0.922 ± 0.008 |
| | Samples n = 9; standard solutions n = 15 | Samples n = 3; standard solutions n = 22 |

* Values are mean ± $\Sigma$

Subsequent optimisation was therefore focused on modification of the retention time ($t_R$) of PCP in order to separate co-eluting compounds whilst simultaneously separating as many other chlorophenols as possible. Initial method development focussed on the separation of chlorophenols with a range of degree of chlorination, *i.e.* 4-MCP, 2,3-DCP, 2,4,6-TCP, 2,3,4,6-TeCP, PCP and the internal standard (2,4,6-TBP). Subsequent development focussed on the separation of chlorophenols with the same degree of chlorination, *i.e.* 2,3,5-TCP, 2,4,5-TCP and 2,4,6-TCP.

Method development was carried out on an HPLC system with a fixed wavelength UV/Vis detector: mobile phase A: 0.1 % acetic acid in methanol, mobile phase B: 0.1 % acetic acid in water, detector wavelength: 215 nm, injection volume: 20 µL, column: Waters Symmetry $C_{18}$(150 × 3.9 mm, 5 µm). The chromatographic run conditions were modified to optimise separation and details are summarised in Table II.5 and explained below.

**Table II.5 -** Chromatography optimisation conditions during method development

| Run | 1st Isocratic | 1st Gradient | 2nd Isocratic | 2nd Gradient | Final isocratic | Total run time (min) | Flow ($mL \cdot min^{-1}$) |
|---|---|---|---|---|---|---|---|
| A | 5 % MP A: 1.5 min | To 95 % MP A in 18 min | Hold for 1.5 min | To 5 % MP A in 5 min | Hold 5 min | 31 | 1.5 |
| B | 50 % MP A: 1.5 min | To 95 % MP A in 18 min | Hold for 1.5 min | To 50 % MP A in 5 min | Hold 5 min | 31 | 1.5 |
| C | 50 % MP A: 1.5 min | To 95 % MP A in 25 min | Hold for 1.5 min | To 50 % MP A in 5 min | Hold 5 min | 38 | 1.2 |
| D | 50 % MP A: 3.5 min | To 95 % MP A in 25 min | Hold for 1.5 min | To 50 % MP A in 5 min | Hold 5 min | 40 | 1.0 |

Mobile phase A: 0.1 % acetic acid in methanol; Mobile phase B: 0.1 % acetic acid in water; MP mobile phase

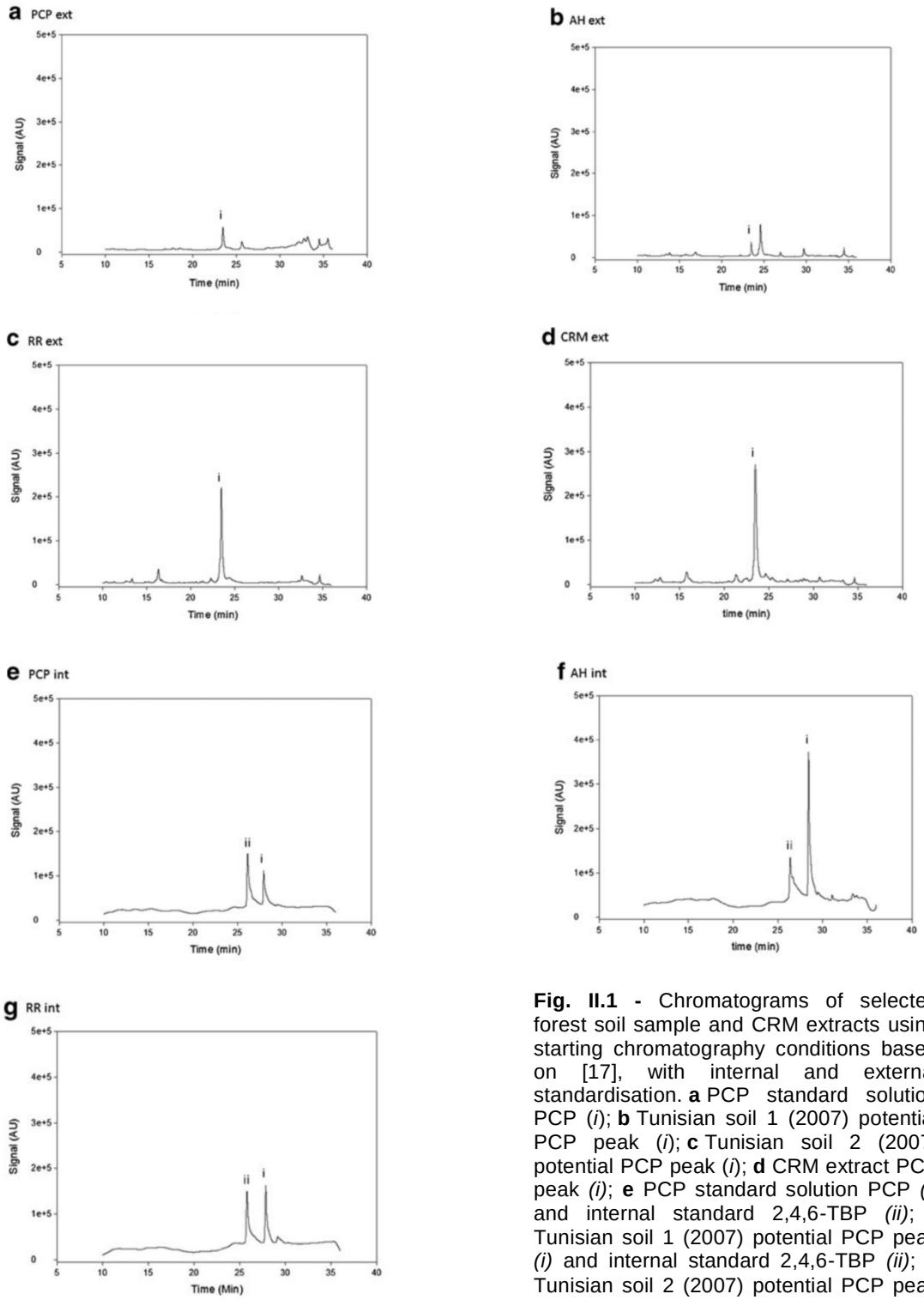


**Fig. II.1 -** Chromatograms of selected forest soil sample and CRM extracts using starting chromatography conditions based on [17], with internal and external standardisation. **a** PCP standard solution PCP (*i*); **b** Tunisian soil 1 (2007) potential PCP peak (*i*); **c** Tunisian soil 2 (2007) potential PCP peak (*i*); **d** CRM extract PCP peak *(i)*; **e** PCP standard solution PCP *(i)* and internal standard 2,4,6-TBP *(ii)*; **f** Tunisian soil 1 (2007) potential PCP peak *(i)* and internal standard 2,4,6-TBP *(ii)*; **g** Tunisian soil 2 (2007) potential PCP peak *(i)* and internal standard 2,4,6-TBP *(ii)*

Using conditions in Run A, the chlorophenols eluted within 8 min and although this is suitable to determine the degree of chlorination present (monochloro-, dichlorophenol etc.) it cannot be used to resolve the specific chlorophenol (see Table II.6). As 4-MCP did not elute until 14 min the initial concentration of Mobile Phase A was changed from 5 % to 50 % to promote earlier elution (Run B). The time for the first gradient ramp remained the same which caused an increase in separation across a lower gradient however the trichlorophenols and internal standard had similar retention times (Table II.7).

**Table II.6 -** $t_R$, *k* and α values of selected chlorophenols analysed using HPLC- UV/Vis (Run A)

| Chlorophenol | $t_R$ (min) | *k* Values | α Values |
|---|---|---|---|
| 4-MCP | 13.82 | 7.32 | |
| 2,3-DCP | 16.97 | 9.22 | 1.26 |
| 2,4,6-TCP | 19.78 | 10.91 | 1.18 |
| 2,3,4,6-TeCP | 20.72 | 11.47 | 1.05 |
| 2,4,6-TBP | 21.05 | 11.67 | 1.02 |
| PCP | 21.99 | 12.24 | 1.05 |

Values reported as mean ± σ (n = 3)

**Table II.7 -** $t_R$, *k* and α values of selected chlorophenol analysed using HPLC-UV/Vis (Run B)

| Chlorophenol | $t_R$ (min) | *k* Values | α Values |
|---|---|---|---|
| 3-MCP | 4.10 | 1.47 | |
| 2,4,6-TCP | 13.14 | 6.91 | 4.71 |
| 2,4,5-TCP | 13.37 | 7.05 | 1.02 |
| 2,4,6-TBP | 15.25 | 8.18 | 1.16 |
| 2,3,5-TCP | 15.29 | 8.21 | 1.00 |
| 2,3,4,6-TeCP | 17.46 | 9.51 | 1.16 |
| PCP | 19.78 | 10.91 | 1.15 |

Values reported as mean ± σ (n = 3)

For Run C, changes were made to the first gradient ramp, the length of time was increased from 19 to 25 min and flow rate was decreased to 1.2 mL·min$^{-1}$ to further separate chlorophenols in particular 2,3,5-TCP and 2,4,5-TCP (Table II.8). Due to the mobile phases used, a solvent front was present from 1.5 to 2.5 min. Consequently, for Run D the initial isocratic section was increased from 1.5 to 3.5 min to minimise the risk of a monochlorophenol eluting within the solvent front and the flow rate was reduced to 1.0 mL·min$^{-1}$ (Table II.9). This final optimised method allowed for the separation of 16 chlorophenols (Fig. II.2).

**Table II.8 -** $t_R$, *k* and α values of selected chlorophenols analysed using HPLC- UV/Vis (Run C)

| Chlorophenol | $t_R$ (min) | *k* Values | α Values |
|---|---|---|---|
| 2,4,6-TCP | 15.56 | 8.37 | |
| 2,3,5-TCP | 18.12 | 9.91 | 1.18 |
| 2,4,5-TCP | 18.21 | 9.96 | 1.01 |
| 2,3,4,6-TeCP | 18.23 | 9.98 | 1.00 |
| 2,4,6-TBP | 20.12 | 11.11 | 1.11 |
| PCP | 24.80 | 13.93 | 1.25 |

Values reported as mean ± σ (n = 3)

**Table II.9 -** *k*, α and $t_{RR}$ values of selected chlorophenols using HPLC- UV/Vis (Run D)

| Chlorophenol | *k* Values | α Values | $t_{RR}$ Values |
|---|---|---|---|
| 3-MCP | 2.31 ± 0.044 | – | 4.26 ± 0.013 |
| 3,5-DCP | 7.98 ± 0.101 | 3.45 ± 0.084 | 1.23 ± 0.033 |
| 2,4,5-TCP | 8.63 ± 0.091 | 1.08 ± 0.005 | 1.14 ± 0.025 |
| 2,4,6-TCP | 9.08 ± 0.114 | 1.05 ± 0.014 | 1.09 ± 0.009 |
| 2,3,5-TCP | 9.30 ± 0.145 | 1.03 ± 0.033 | 1.06 ± 0.006 |
| 2,4,6-TBP | 9.85 ± 0.193 | 1.06 ± 0.006 | – |
| 2,3,4,6-TeCP | 10.28 ± 0.174 | 1.04 ± 0.003 | 0.96 ± 0.002 |
| PCP | 11.42 ± 0.198 | 1.11 ± 0.001 | 0.86 ± 0.002 |

Values reported as mean ± σ (n = 3)

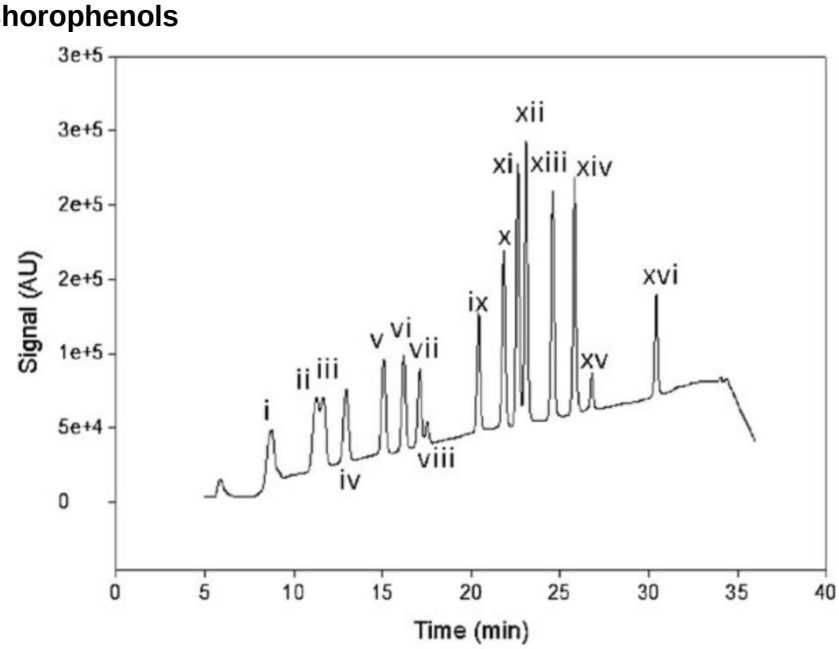


**Fig. II.2 -** Separation of 16 chlorophenols using HPLC-diode array detector (DAD), namely, 2-MCP (i); 4-MCP (ii); 3-MCP (iii); 2,6-DCP (iv); 2,3-DCP (v); 2,5-DCP (vi); 2,4-DCP (vii); 3,4-DCP (viii); 3,5-DCP (ix); 2,4,6-TCP (x); 2,4,5-TCP (xi); 2,3,5-TCP (xii); 2,4,6-TBP (xiii); 2,3,4,6-TeCP (xiv); 2,3,4,5-TeCP (xv); PCP (xvi). All concentrations are 10 $mg \cdot L^{-1}$ with the exception of PCP which is unquantified (present as a contaminant in 2,3,4,6-TeCP)

*Optimisation of LOD and quantitation*

The use of a DAD provides the opportunity to optimise detection sensitivity across mixtures of compounds. The stability of a UV detector affects the limits of detection [$LOD_{SOLN} = 3*(\sigma_{blank}/slope)$] and quantitation [$LO\ Q_{SOLN} = 10*(\sigma_{blank}/slope)$] [33, 34], which vary with background noise in the detector. Therefore LODs and LOQs were determined on a batch-by-batch basis using chlorophenol standard solutions to ensure that limits are achievable with the method during every run [35]. These standard equations were used to define limits “in soil” for the appropriate soil weight (in this case, 2.5 g, sonication, air-dried equivalent) in field sample analysis.

The signal was determined as: [maximum intensity within the peak detection time range]-[average intensity within the noise detection time range], *i.e*. [peak height intensity]-[average noise intensity]. No specific guidance is available for the time range which should be taken to calculate noise; given that the baseline is a gradient (Fig. II.2), a section of the baseline immediately before and after each peak was used to calculate the signal; this process takes into account for both long-term (*i.e.* the gradient) and short-term noise. Despite continued changes to the mobile phase gradients and the flow rate, no baseline separation could be achieved between 3-MCP and 4-MCP, and therefore these could not be used for quantitative analysis and the relevant LODs/LOQs could not be calculated.

A wide variation in the LODs/LOQs was observed (see Table II.10). The highest SD was obtained for 2,3,4,6-TeCP which was caused by one particular batch and was not observed for any other chlorophenol. The high SD for 2,3-DCP was not associated with one particular batch but by wide variation between all batches. The value of LOD determined for PCP is comparable to those reported for gas chromatography determination, *e.g.* 5 $\mu g \cdot Kg^{-1}$ [21] and 10 $\mu g \cdot Kg^{-1}$ [23]. The detection limits obtained, makes the method suitable for the determination of chlorophenols in forest soils as literature values for PCP in typical soils range from 15 to 642 $\mu g \cdot Kg^{-1}$ [22, 36, 37].

**Table II.10** – HPLC-UV (DAD) limits of detection and quantification (μg·Kg$^{-1}$ soil dry weight) for chlorophenols at the detector wavelength (λ) which gives the lowest value

| Chlorophenol | λ (nm) | LOD (μg·Kg$^{-1}$) | LOQ (μg·Kg$^{-1}$) |
|---|---|---|---|
| 2-MCP | 220 | 191.26 ± 78.78 | 637.53 ± 262.61 |
| 2,6-DCP | 210 | 32.43 ± 15.72 | 108.10 ± 52.41 |
| 2,3-DCP | 210 | 6.52 ± 5.72 | 21.73 ± 19.07 |
| 2,5-DCP | 225 | 27.05 ± 5.18 | 90.16 ± 17.26 |
| 2,4-DCP | 210 | 35.34 ± 18.39 | 117.80 ± 61.30 |
| 3,4-DCP | 210 | 172.35 ± 57.32 | 574.51 ± 192.73 |
| 3,5-DCP | 210 | 18.62 ± 6.39 | 62.06 ± 21.29 |
| 2,4,6-TCP | 210 | 14.52 ± 5.91 | 48.40 ± 19.72 |
| 2,4,5-TCP | 210 | 9.37 ± 3.79 | 31.24 ± 12.62 |
| 2,3,5-TCP | 210 | 10.45 ± 1.39 | 34.84 ± 4.62 |
| 2,3,4,6-TeCP | 210 | 24.60 ± 27.76 | 81.99 ± 92.53 |
| 2,3,4,5-TeCP | 215 | 36.13 ± 18.35 | 120.44 ± 61.16 |
| PCP | 215 | 11.40 ± 3.46 | 37.99 ± 11.55 |

Values reported as mean ± σ (n = 3)

*Analysis of Quercus suber L forest soils*

Soil samples, collected from Tunisian and Sardinian cork oak forests as described above, were extracted and analysed using the optimised sonication and chromatography method. A summary of the final conditions is given in Table II.11. Extracts were analysed immediately following extraction and were stored refrigerated for further analysis if needed. Peak identification was based on the $t_{RR}$ values of the calibration series. No chlorophenols were detected in either the Tunisian or the Sardinian samples (Fig. II.3).

**Table II.11 -** Summary of optimised extraction and chromatographic conditions for the determination of chlorophenols in *Quercus suber* forest soils

| | |
|---|---|
| Extraction | Soil (2.5 g) sonicated in 5 mL of methanol (containing 1 $mg \cdot L^{-1}$ 2,4,6-TBP) for 30 min, centrifuged for 10 min at 3000*g* and the supernatant removed; the procedure was then repeated. The supernatants were combined, reduced to dryness and reconstituted using 1 mL of methanol |
| HPLC | Mobile phase A: 0.1 % acetic acid in methanol; Mobile phase B: 0.1 % acetic acid in water. Mobile phase gradient: 50 % A for 3.5 min to 95 % A in 25 min, hold for 1.5 min, to 50 % A in 5 min, hold 5 min. Total run time = 40 min. Injection volume: 20 µL. Flow rate: 0.5 $mL \cdot min^{-1}$ |
| Column | Waters symmetry $C_{18}$(150 × 3.9 mm, 5 µm) |
| DAD wavelength | 210, 215, 220 and 230 nm |

A peak was detected in all forest soils and the soil CRM with a $t_R$ of 26.3 min (compared to 24.3 min for the internal standard and 30.4 min for PCP). To determine if this was the unresolved peak (URP) detected using the original chromatography method, comparison of the Tunisian peak samples revealed strong correlation ($r^2 = 0.892$). Compared to the other forest soil samples, the URP peak area in Sardinian Station 1 is smaller and is similar to the soil reference material (CRM: see Fig. II.3 for examples); forest fire swept through this area in 1983 which would have destroyed biomass input and surface organic matter. The ubiquity of the URP suggests that it is a common extractable soil constituent potentially derived from soil organic matter.

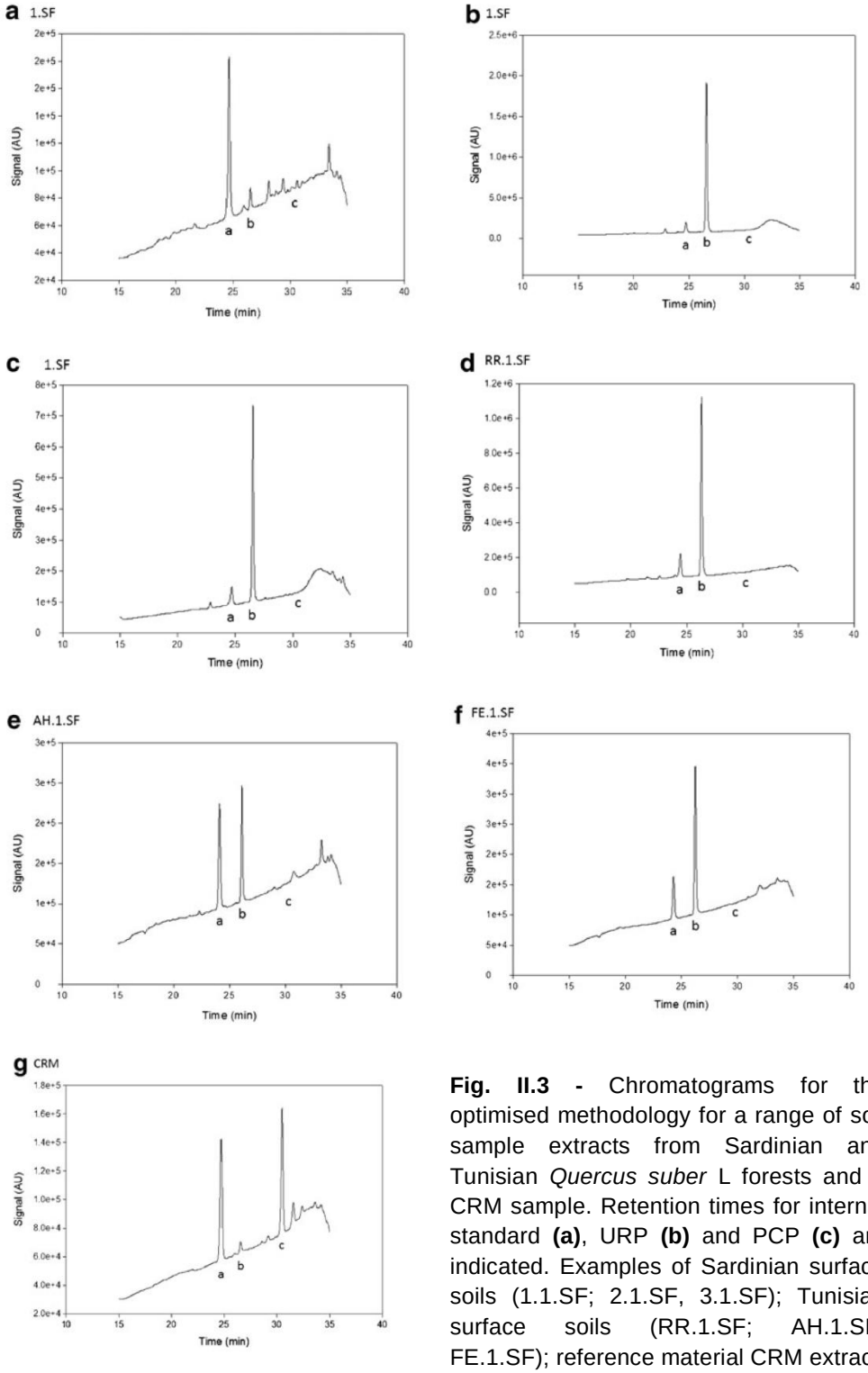


**Fig. II.3 -** Chromatograms for the optimised methodology for a range of soil sample extracts from Sardinian and Tunisian *Quercus suber* L forests and a CRM sample. Retention times for internal standard **(a)**, URP **(b)** and PCP **(c)** are indicated. Examples of Sardinian surface soils (1.1.SF; 2.1.SF, 3.1.SF); Tunisian surface soils (RR.1.SF; AH.1.SF; FE.1.SF); reference material CRM extract

## Conclusion

Stimulated by the high-level concerns regarding the potential for chlorophenol contamination at early stages in the cork production process, the NATO *SfP* project focused on this evaluation to provide robust and reproducible analytical tools. These were applied to cork oak forests subject to a varying degree human influence and management styles.

The initial extraction method (a 16 h Soxhlet extraction procedure) had low extraction efficiency and used 500 mL of solvent *per* extraction. Furthermore, the original HPLC method (which used phosphoric acid as a mobile phase) produced a peak which had a retention time similar to PCP. Both methods had previously been used in evaluating chlorophenols in the later stages of cork stoppers production and both have been shown to be unsuitable for use with a complex matrix such as soil.

The success of the sonication extraction method, with increased extraction efficiency, allows the method to be applied using different HPLC detectors, providing versatility for application in laboratories with a variety of instrumentation resources and application in partner laboratories returned > 70 % recovery based on this optimised method. The optimised chromatography conditions also modified the relative retention time of PCP, allowing the separation of PCP from an unknown peak. Literature values for typical levels of PCP and its chlorophenolic degradation products within *Quercus suber* L. forest soil are still to be quantified and this study provides a first systematic baseline assessment of two locations. Whilst literature data from other forest environments show PCP concentrations can range from 15 to 640 $\mu g \cdot Kg^{-1}$, the LOD obtained here of 6.5-191 $\mu g \cdot Kg^{-1}$ is at an appropriate sensitivity to be applied to situations where undefined source terms are interacting with pristine environments.

The confirmation of the absence of detectable chlorophenols levels in the FSC-accredited Sardinian forest is reassuring for management strategies being applied. In the case of the Tunisian forests, despite anecdotal evidence from the industry that chlorophenol contamination is

prevalent at some stage in cork production; the soil at these sites appears unaffected. This raises the possibility that chlorophenols, which are lipophilic, have been absorbed by cork bark at very specific points in the production process, resulting in the contamination in cork slabs and with low dispersion through soil matrix, and are relatively stable in these systems, making direct detection in environmental surveys difficult.

Cork ecosystems are in a precarious position. Damage to industrial production through contamination may reflect pressure on forest owners to exploit available resources, with a more short-term focus on income, increasing pressure on the inappropriate use of chemical agents. However, this ignores the longer-term feedback on the buoyancy of markets for a product with contamination concerns. The ecosystem is also acknowledge for its high biodiversity and support for subsistence communities, which can only be maintained through appropriate management practice. This must be supported by the use of robust analytical methods for monitoring at all stages in production with their development and application as identified in this study.

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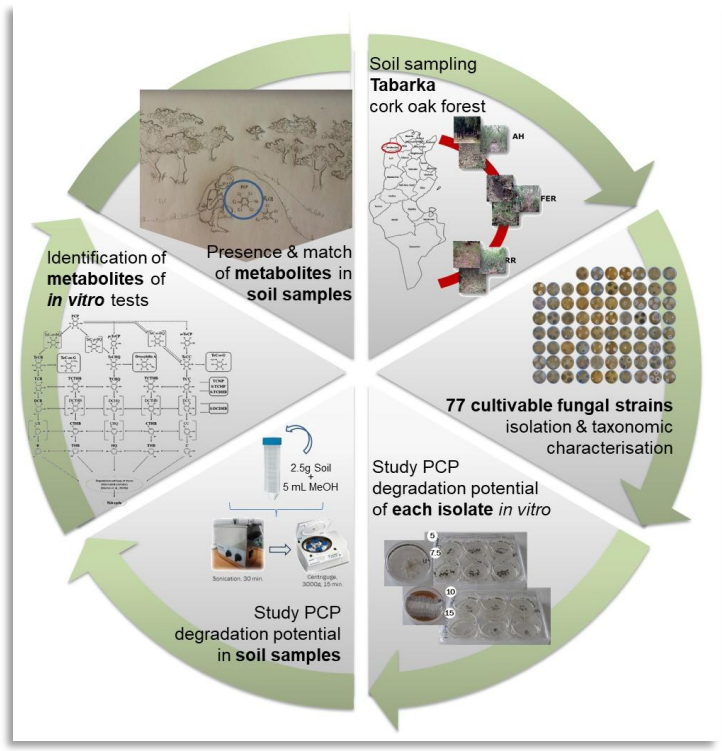
# CHAPTER III

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## 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 endoc0rine 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, 30]. 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 [31] and 2,3,5,6-tetrachloro-4-methoxyphenol (Dro A) was synthesized as described before [32].

*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 [33].

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 $2.04 \pm 0.18$ mg of PCP $Kg^{-1}$ (ERM-CC008, LGC-Promochem, Spain) [30].

*Composition of the cultivable fungal communities*

Fungi isolation and taxonomic identification were done as previously described [33]. 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 [34-37]. 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, 38]

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 [39].

*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 [38, 40]. 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 (Fig. III.1). 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 (Fig. SIII.1). 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 $\mu g \cdot Kg^{-1}$) or RR soils (1.7– 7.0 $\mu g \cdot Kg^{-1}$), 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) [41] verified that PCP degradation and oxidoreductase activity (Table III.1) were not correlated (Pearson correlation test $p$-value < 0.05).

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

| Strain | | PCP degradation yield (%) Starting concentration (µM) 19 | 28 | 38 | 56 | Oxidoreductase activity 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 ($AH_n$) 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 following strains were unable to germinate in the presence of PCP: *Absidia glauca* (DTO 099-B5); *Absidia pseudocylindrospora* (DTO 099-G6); *Absidia* sp. (DTO099-A1, DTO 099-C1); *Aspergillus fresenii* (DTO 099-F4, DTO 099-H2); *Aspergillus* sp. (*sect. Cremei*) (DTO 099-D8); *Aspergillus tubingensis* (DTO 099-F5); *Penicillium daleae* (DTO 099-E6); *Penicillium janczewskii* (DTO 099-H3, DTO 099-E5); *P.* (DTO 098-I8); *Penicillium restrictum* species complex (DTO 099-A4, DTO 099-C9, DTO 099-H4, DTO 099-F2); *Penicillium radiatolobatum* (DTO 099-E3); *Penicillium sanguifluum* (DTO 099-E9); *Phoma putaminum* (DTO 100-A5); *Trichoderma cf. virens* (DTO 099-B3); and *Zygorhynchus heterogamous* (DTO 099-B2). ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); RBBR, Remazol Brilliant Blue R.

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. 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 $AH_3$ (Fig. III.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.

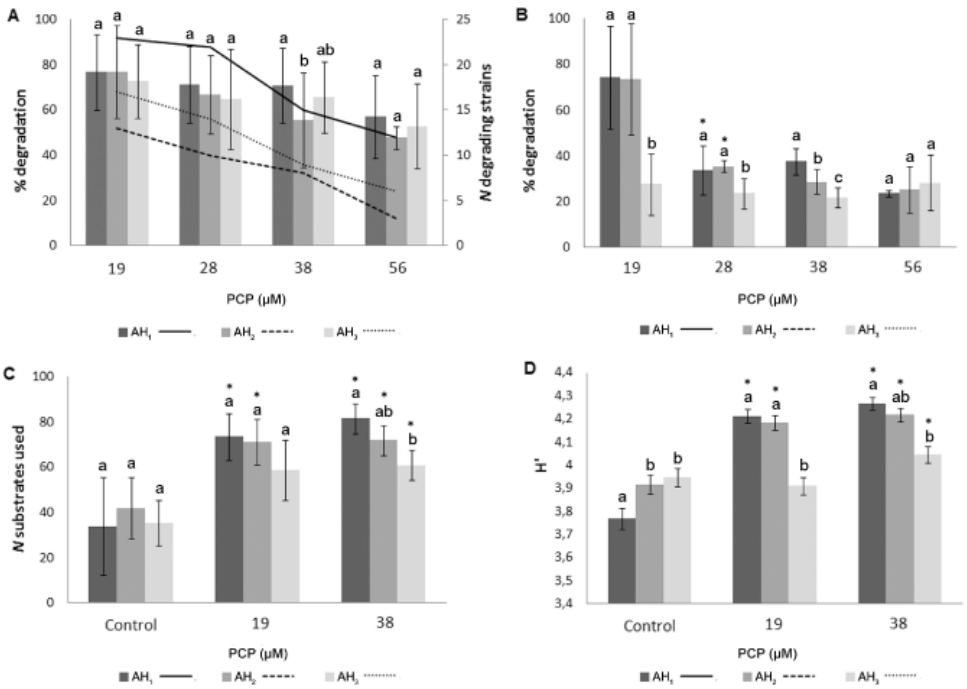


**Fig. III.1** - PCP-degrading capacity for axenic or community cultures (UPLC analyses), and physiological profile (Biolog plates SF-N2): (A) average PCP-degrading capacity of the strains (principal y axis) and number of PCP-degrading strains (secondary y axis, continuous, dashed and dotted lines for $AH_1$, $AH_2$ 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 14$^{th}$ day of incubation, PCP residual concentrations were analysed (Fig. III.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 (Fig. III.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 (Fig. III.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** | **Abr** | **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, 42] 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 [43], 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 [44].

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.* [45]. 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 [33, 46], 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 [47, 48]

or with comparable properties [49]. Ascomycota, particularly penicillia, typically dominate, as key decomposers, soils with a low abundance of lignin [50].

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) [47], 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 [36, 51]. 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 [52]; 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.* [53]). 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, 54]. 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 (Fig. III.1). Increasing PCP concentrations led to a continuous decrease in the number (hence biodiversity) of PCP-degrading strains (Table III.1 and Fig. III.1A), while simultaneously increasing both the catabolic richness and diversity of the community (Fig. III.1B and C). The latter has been often associated with specialization of microbial communities because of chronic exposure to pollutants [48, 55]. Regardless of this, the persistent strains demonstrated comparable average PCP decay levels in axenic (Fig. III.1A) and community (Fig. III.1B) cultures.

Overall, data indicated that community interactions hindered the capacity of the strains to remove PCP (Fig. III.1B). Ecological interactions are radically altered under *in vitro* conditions, generally favouring competition among strains and reducing the total fungal abundance [56, 57]. 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 (Fig. III.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 [58]. 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) [58] 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 [38]. 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 [32, 59]. Additional conjugates, namely after

*O*-methylation (TCMP), sulfation (S-TCDHB) or both (S-TCMP), were detected in some of the axenic cultures.

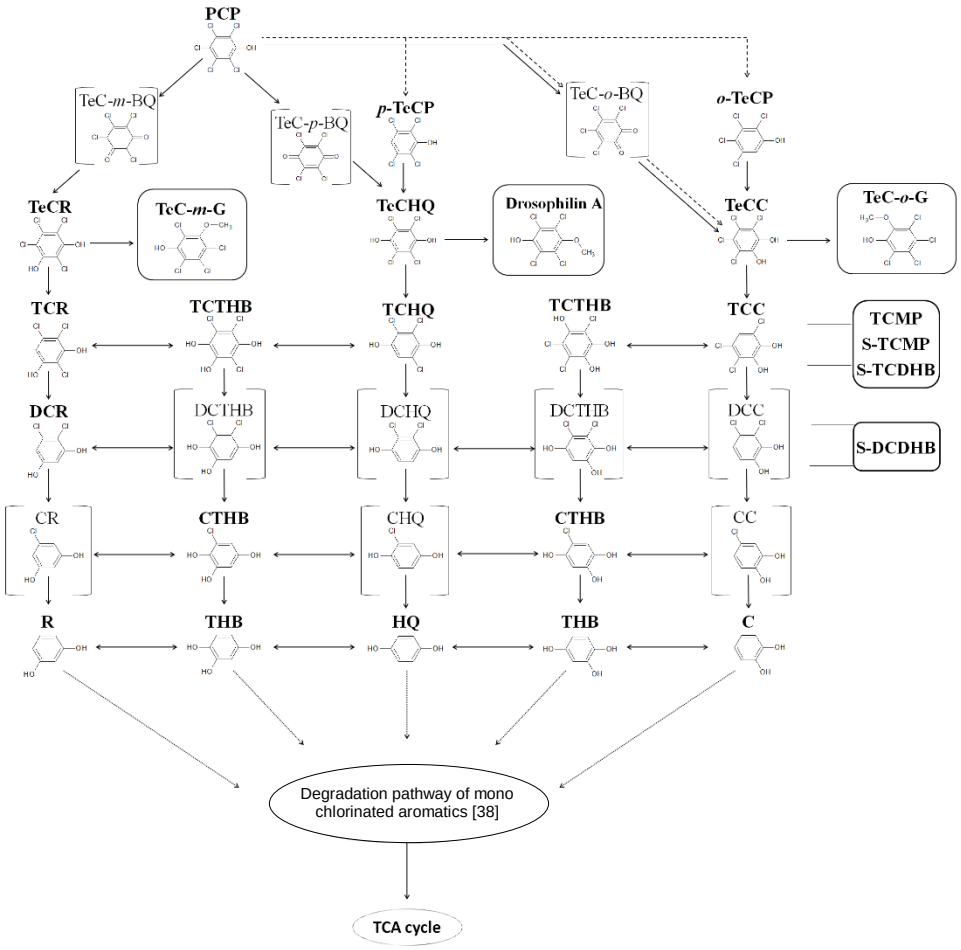


**Fig. III.2** - Proposed pathway of PCP degradation by filamentous fungi in soils, after integrating the metabolic data obtained in axenic and community experiments (UHPLC-ESI-HRMS analyses). Compounds in brackets are hypothetical intermediates. Full and dashed arrows stand for biotic and abiotic transformations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

Similar conjugated compounds have been reported in other eukaryotes, *e.g.* fish and daphnids [60, 61]. In particular, sulfate conjugates and sulfate di-conjugates of PCP degradation intermediates have been identified in other fungi [26, 62]. These reactions constitute a detoxification

mechanism that generally increases the solubility of the toxic compound facilitating its excretion from the cell [21, 63].

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, 64], 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 [38], 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 [65]. 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 [66] leading to the so-called 'circle of poison' [67]. 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 [68]. 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.

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# ANNEX

**Table SIII.1 –** 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 | H (%) | pH | O.M. (%) | 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 |

H – Humidity; O.M. – Organic Matter

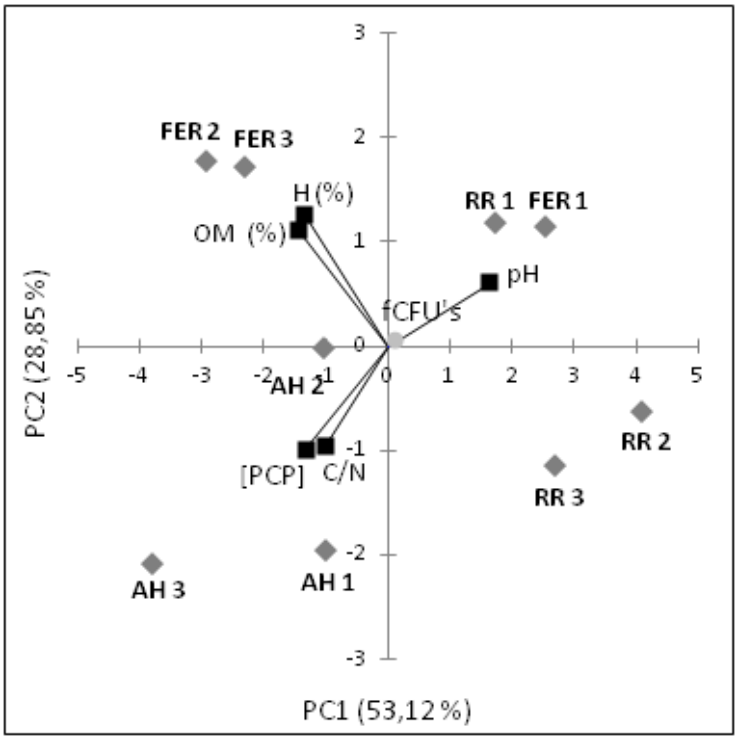


**Fig. SIII.1 -** Biplot containing principal component analysis (PCA) and multidimensional scaling (MDS) analyses comparing the observable quantitative variables measured in Aîn Hamraia (AH), Fej Errih (FER) and Ras Rajel (RR) soils.

**Table SIII.2 –** Taxonomic data of the 77 cultivable strains composing the fungal communities of $AH_n$ soils.

| Strain | Taxonomy<br>Taxa | GenBank no. | Locus | Lignolytic activity *in vivo*<br>Qualitative<br>ABTS | <br><br>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<br>Taxa | GenBank no. | Locus | Lignolytic activity *in vivo*<br>Qualitative<br>ABTS | <br><br>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 and 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 SIII.3 –** 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 |

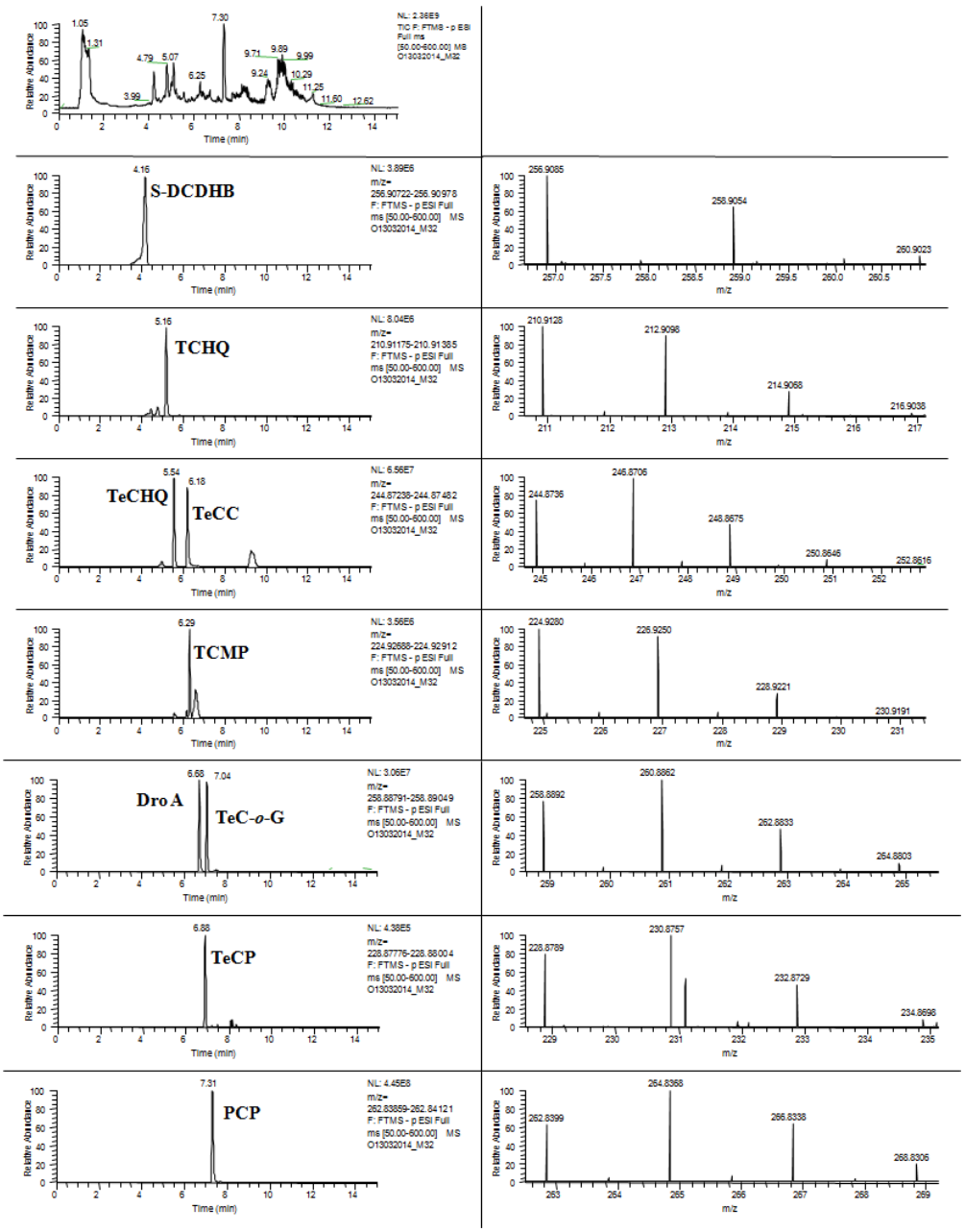


**Fig. SIII.2 –** Spectra and isotopic pattern obtained in one randomly selected sample, illustrating the data collected during the UHPLC-ESI-HRMS analysis.

# CHAPTER IV

This Chapter presents a part of an unpublished manuscript currently under preparation

Proteomic and functional response of a fungal community during PCP depletion

**Adélia Varela**, Celso Martins, Óscar Núñez, Céline Leclerq, Encarnación Moyano, Jenny Renaut, and Cristina Silva Pereira

# Proteomic and functional response of a fungal community during PCP depletion

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## Summary

In a previous study pentachlorophenol (PCP) was observed to be prevalent at high levels in the soils of cork oak forests located in Tunisia. Most of the cultivable fungal strains within the resultant belowground mycobiota were able to efficiently degrade or mineralise PCP. The degradation pathway was observed to comprise the hydroquinone, the catechol and the resorcinol branches. The last, never reported before, constitutes a signature of the degradation of PCP by fungi and was found active in the soils of these forests. In the present study we have analysed how PCP pollution impacts the functioning of the belowground metacommunity of fungi. In particular, the differential responses of the metacommunity of fungi during the mitigation of the biocide was analysed by proteomics, metabolomics and community level physiological profiles. The superior capacity of fungi to degrade PCP was made apparent by the formation of diverse metabolites derived from the three degradation branches of the PCP degradation pathway. Nonetheless, regardless of the great capacity of fungi to efficiently degrade PCP, the functional assays also revealed the activation of major stress responses and the alteration of the carbon and the nitrogen metabolisms. The functioning of

the metacommunity of fungi in the presence of PCP was altered compared to the control without PCP, even when most PCP was already degraded. New assays are on-going to correlate key functional changes taking place in the metacommunity of fungi during PCP mitigation with changes in taxonomic diversity.

## Introduction

Pentachlorophenol (PCP) is an organochlorine compound that has been extensively used worldwide; currently found as a pollutant in all Earth's ecosystems [1, 2]. Since 2015, PCP is considered a persistent organic compound (POP) [3], especially due to its ability to undergo long range atmospheric transport and to its intrinsic life-threatening toxicity [1, 2, 4]. It is well known that PCP can undergo biotransformation mediated by either fungi or bacteria [5, 6]. Previous studies have demonstrated that many fungal species are able to bio-transform PCP [7-9] as well as other chlorinated phenols [10, 11]. In particular, many Ascomycota strains were observed to be able to ensure the full mineralisation of chlorinated phenols [5, 8-10] in opposition to bacterial counterparts which usually yield highly toxic and recalcitrant non-chlorinated or chlorinated phenol derivatives [12].

In a previous study, we observed that soils from Tunisian *Quercus suber* forests were contaminated with PCP [8]. In particular, in Aîn Hamraia forest (Tabarka region) soils, the observed PCP levels - > 10 $\mu g \cdot Kg^{-1}$ dry weight soils - were similar to those found at locations where PCP is currently in usage [13]. This observation raises serious concerns as many derived agroforestry products are produced, including the outer cork bark that is used to manufacture stoppers that are commercialised globally [14]. We have previously disclosed the PCP degradation pathway of the fungal community and demonstrated that the pathway is active in the forest soils, where belowground fungi act as a buffer against PCP pollution [8]. The influence of PCP exposure to promote specialisation events in microbial

communities remains poorly investigated [15]. The aim of the present study is to understand how PCP impacts the proteome of the metacommunity of fungi along its decay, similar to that used to study the proteome of soil microbial communities exposed to diesel [16], toluene [17] or naphthalene [18]. The proteomic data were complemented with data on the community level physiological profiles (CLPP) and on the PCP-derived metabolome. This study supports that the metacommunity of fungi is able to efficiently degrade/mineralise the biocide while experiencing major stress responses and alterations in the carbon and the nitrogen metabolisms.

## Experimental procedures

### *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.

### *Fungal community*

Soil samples were collected in a Tunisian demarked cork oak forest called *Aîn Hamraia* (AH) in February 2009, as previously described [19] In brief, three locations were chosen, 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. In the present study, equal amounts of each composite sample were pooled and carefully homogenised. The fungal community was recovered from the pooled AH soil sample using a previously described methodology [8, 20]. Briefly, 20 mL of a 0.1 % (w/v) peptone solution supplemented with 0.1 % (v/v)chloramphenicol were added to each 2 g of soil, incubated during 60 min under gentle agitation but with the application of vacuum every 20 min to homogenize the mixture and remove excess moisture. The mixture was sieved by applying sequentially

sieves of lower pore size: 500 µm, 210 µm and 100 µm; and 1 mL aliquots of the filtrate were storage at -80º C until further use.

*$EC_{50}$ of PCP against the fungal community*

The concentration of PCP capable to reduce to half the growth of the fungal community, *i.e.* Half Maximal Effective Concentration ($EC_{50}$) was determined using cultures of 5 mL in 6-well plates (2 plates *per* replica). A mineral minimal media, contained, *per* litre of water, 1 g $K_2HPO_4$; 3 g $NaNO_3$; 10 mg $ZnSO_4 \cdot 7H_2O$; 5 mg $CuSO_4 \cdot 5H_2O$; 0.5 g $MgSO_4 \cdot 7H_2O$; 10 mg $FeSO_4 \cdot 7H_2O$ and 0.5 g KCl, containing 1 % w/v of glucose (MMG) was used [7, 21]. The pH of the media was adjusted to 7 with phosphoric acid. PCP was added from a concentrated stock to reach in medium the final concentration of 19, 38, 95, 190, 380 or 760 µM (3 biological replicas *per* concentration). The growth media were inoculated with aliquots of the fungal community (ratio of 10:1) and incubated at 27 ºC under gentle agitation (90 rpm). After 7 days, the 2 plates of each replica were pooled and agitated briefly. Then, 50 µL of each biological replica were spread onto MEA (3 technical replicas) and colonies counted daily during 5 days. The counts were then used to calculate the inhibition level of PCP compared to the negative controls (without PCP). Results were fitted in a logistic regression using the dose effect tool of XL-STAT software version 2009.1.02 (Addinsoft) to obtain the $EC_{50}$ value.

*Cultures of the metacommunity of fungi exposed or not to PCP*

To test the response of the metacommunity of fungi to degrade PCP, we applied a straightforward cultivation strategy comparable to that reported before for the establishment of metacommunities composed by several local bacterial communities [22]. Briefly, each biological replica comprised five 6-well plates (total of 30 wells), each well containing 5 mL of MMG supplemented or not with 38 µM of PCP (*i.e* the $EC_{50}$ concentration). The

growth media of each biological replica was inoculated with the fungal community (ratio of 10:1), then distributed into 30 culture-wells. Cultures were incubated at 27ºC, under gentle agitation (90 rpm) and sampled after 3, 5, 7 and 10 days (three biological replicas *per* day). The mycelial and the extracellular fractions were separated using vacuum filtration, the fresh mycelia weight annotated, and both fractions were conserved at -80ºC until further use.

*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 ($t_R$) = 5.9 min). The diversity of PCP-derived metabolites and sub-products was 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 [8]. MS data was processed by ExactFinderTM 2.0 software (Thermo-Fisher Scientific), applying a user target database list and validated, whenever possible, using standard compounds.

*Extraction of Mycelial proteins*

Mycelial proteins were extracted using a modified TCA/Acetone protocol [23]. Briefly, the frozen mycelia (in liquid nitrogen) was grinded using a mortar and pestle 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 homogenization and cell rupture, a TissueLyzer LT Adapter (Qiagen) was used, firstly 1g of glass beads (half of each size: 0.5 and 0.1 mm) were added, secondly two consecutive cycles of 5 min at top speed were applied.

Proteins were then precipitated for 1 h at 20 ºC in acetone containing 10% (v/v) trichloroacetic acid (TCA) and 40 mM of dithiothreitol (DTT) (1:10 w/v); the pellet was recovered by centrifugation at 10414*g* for 15 min, washed three times in 10 mL of acetone containing 40 mM of DTT, dried under a nitrogen flow and finally stored at -80º C until further analysis.

*Mass spectrometry analyses of mycelial protein*

The aliquots were recovered and 25 µg of protein (bovine serum albumin equivalents accordingly to the Bradford protocol) were loaded in a precast gel (Criterion TM XT precast 1D gel 4-12% Bis-Tris) and allow to migrate for 1 cm. The gel was stained with Instant Blue (Gentaur BVBA), sliced into 5 bands; (each cut into 1-2 mm cubes), first reduced, alkylated and destained, then digested by trypsin (Promega). The peptides were first desalted and finally fractionated by reverse phase separation in an Ultimate 3000 NanoLC System coupled to a LTQ-OrbiTrap Elite MS that was operated in data-dependent mode, automatically switching between MS and MS2, using XCalibur software. Data was processed in Mascot using Proteome Discoverer by searching against the SwissProt Fungi database (released on January 21$^{st}$, 2013). Protein identification was done using a set of pre-defined filters and a minimum confidence of 95 %. Full details in Table IV.S1 in electronic supplementary information.

*Analysis of the proteomic data*

Only the proteins that were present in the two biological replicates were considered for further analyses. The spectral counts of each mass were normalized and further analysed using the RStudio (version 1.0.153) Bioconductor package edgeR [24]. The use of R based packages for the analysis of MS based proteomic data has been previously suggested [25, 26]. In most proteomics studies, the normalised MS spectral counts are used to calculate the ratio of the difference between the final value and the initial

value, divided by the original value. Here we have used the normalised MS spectral counts as the means to calculate, with the help of generalized linear models, the differential expression of each identified protein.

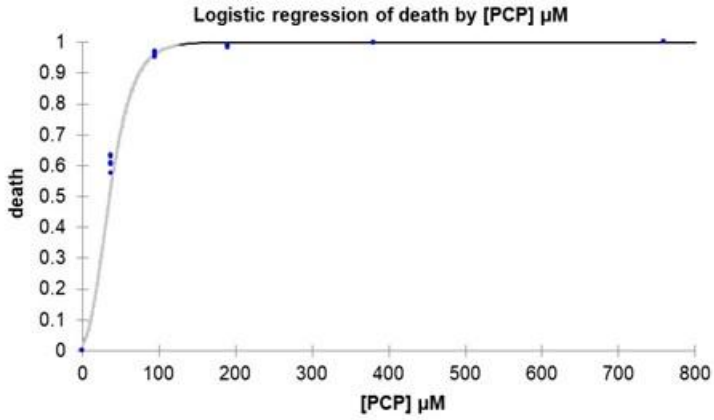
*Community level physiological profiles (CLPPs)*

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 above described, before testing in the Biolog FF plates. CLPPs at 27ºC were measured daily for 5 days monitoring the absorbance at 480 nm and 750 nm. Functional diversity (Shannon index, *H'*) and richness were calculated as previously described [27]. Each carbon and nitrogen source was classified in terms of functional category [28] and those where PCP exposure compare to the negative control led to significant differences were further analysed using informatics tools. The usage ratio of each substrate was normalized and an histogram constructed using XL-STAT software version 2014.5.03 (Addinsoft, France), to disclose which groups of substrates were differentially metabolised after three or ten days of exposure to PCP.

## Results and Discussion

*$EC_{50}$ value of PCP against the fungal community and PCP decay*

Herein the estimated $EC_{50}$ value of PCP against the fungal community was *ca.* 38 µM (Fig. IV.1). Therefore, this concentration of PCP was selected for all the remaining experiments.




| Probability (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 |
| 1.00 | 122.345 | 122.019 | 122.672 |

**Fig. IV.1** - $EC_{50}$ value of PCP against the fungal community.

Microbial communities are constituted by sub-communities, *i.e.* local communities, that often comprise the same dominant strains yet a distinct composition of the less abundant strains [22]. When using as inoculum a microbial community, the use of conventional biological replicas in single growth containers, may promote the establishment of particular local communities that do not cover the whole microbial diversity. In the present study, each biological replica pooled numerous growth containers (see materials and methods section). This might allow the random establishment of diverse local communities that when polled potentially reproduce more closely the heterogeneity of the whole myco-community, *i.e.* the metacommunity of fungi. Using this strategy, PCP at 38 µM ($EC_{50}$), was efficiently degraded by the metacommunity of fungi along the incubation

time: PCP decay values ranged from 1.3 % at the third day to 69.1 % at the tenth day (Fig. IV.2). The degradation level observed at the last time point – 69 % - was nearly the double of that observed when cultures were inoculated with the soil mycobiota of a defined $AH_n$ location (*i.e.* not the pooled AH soil) in conventional biological replicas.

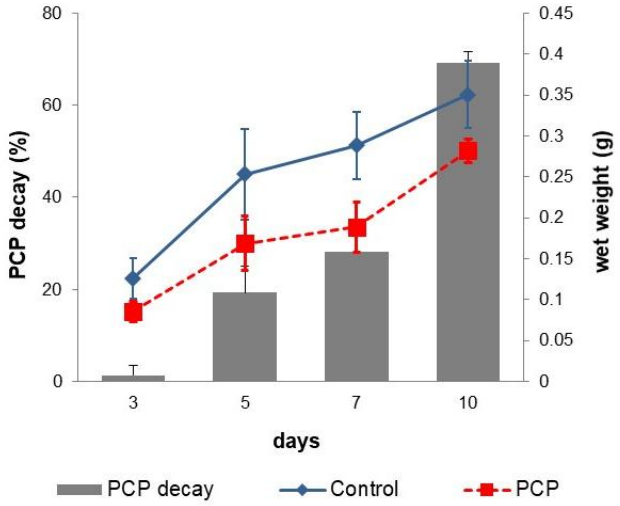



**Fig. IV.2** – PCP decay along the incubation time in cultures of the fungal community.

*Metabolomics*

To evaluate the course of PCP degradation by the metacommunity of fungi, PCP-derived metabolites were detected in the extracellular and the mycelial fractions collected after three, five, seven and ten days of incubation (Table. IV.S2 in supplementary information). In particular, herein the identification of metabolites which have been previously linked to the PCP degradation pathway of similar fungal communities was targeted [8].

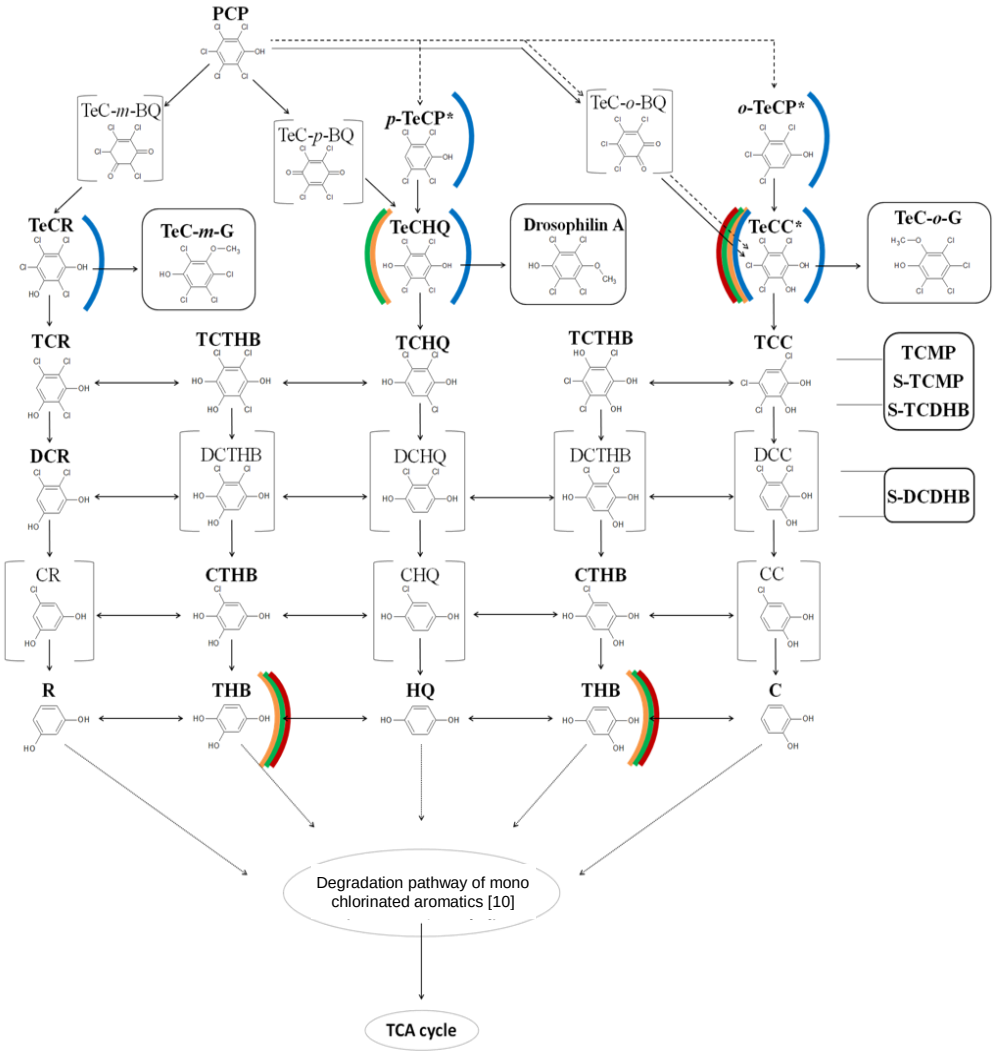



**Fig. IV.3** - Pentachlorophenol degradation pathway used by the metacommunity of fungi (adapted from [8]). PCP-degradation metabolites were detected by UHPLC-ESI-HRMS in cultures of the metacommunity of fungi along the degradation of 38 μM PCP. Mycelial and extracellular metabolites are marked at the left and right sides, respectively; the colours blue, yellow, green and red stand for three, five, seven and ten days of incubation, respectively. Compounds in brackets were not identified, hence hypothetical. Full and dashed arrows stand for biotic and abiotic transformations, respectively.

At the third day of incubation, metabolites derived from the three branches of the PCP degradation pathway, namely tetrachlororesorcinol (TeCR), tetrachlorohydroquinone (TeHQ) and tetrachlorocathecol (TeCC), were observed to accumulate extracellularly. The *para*- and *orto*-

tetrachlorophenol (TeCP) isomers and TeCR were detected in the extracellular fraction. This reinforces that the initial modification of PCP involves its reductive dechlorination, as previously described [7, 8, 29]. The accumulation of TeCHQ and TeCC in the mycelia was observed along the incubation, whereas the non-chlorinated trihydroxybenzene (THB) was detected only extracellularly for longer incubation periods than three days (Fig. IV.3). THB formation may account the contribution of all the branches of the PCP degradation pathway. The accumulation of TeCR in the mycelia was not observed (Fig. IV.3) likely because the R branch (entirely biotic) is less active than the HQ and the C branches that may involve abiotic steps in the modification of PCP.

*Mycelial proteomics*

The mycelial proteomes and the community level physiological profiles of the metacommunity of fungi exposed or not to PCP were analysed focussing on the first (three days) and the last (ten days) incubation time points. These times points were selected as they reflect both different PCP exposure levels and dissimilar signatures of PCP-derived metabolites (Figs. IV.2 and IV.3).

The impact of PCP in the mycelial proteome of the metacommunity of fungi compared to control conditions was significant with many identified proteins undergoing major up- or down-regulation when exposed to PCP. At each time point, the cumulative $\log_2$FC of the identified differential protein species by Gene Ontology (GO) functional category is presented (Fig. IV.S3 in supplementary information). The different colours stand for the taxonomic class of the organism which retrieved the best hit with the identified polypeptides. In general, PCP altered mostly fungal proteins matching identification in Eurotiomycetes, Saccharomycetes, Schizosaccharomycetes and Sordariomycetes, green, orange, blue and red, respectively.

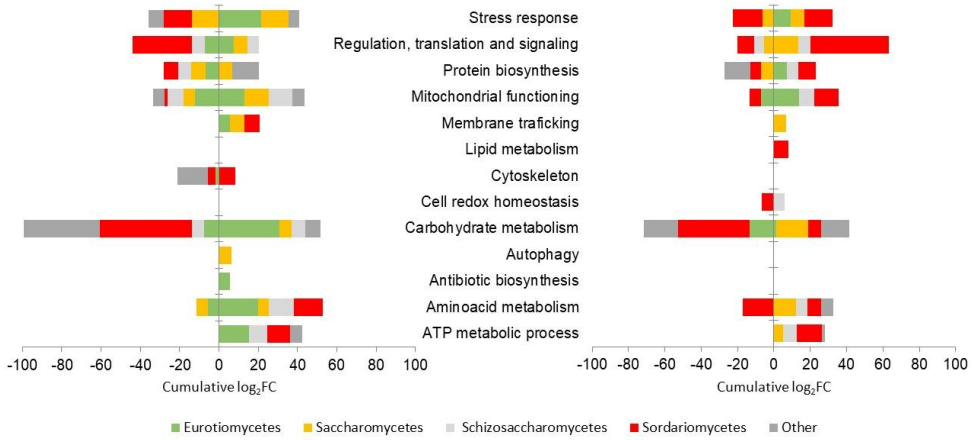



**Fig. IV.4** - Functional classification of mycelial proteins species identified in the metacommunity of fungi during exposure to PCP compared to control conditions, accordingly to Uniprot and Swissprot databases. Two time points were analysed: 3 days and 10 days, left and right panels. The horizontal axis represents the cumulative $log_2FC$ of proteins organized by their Gene Ontology (GO) functional categories (vertical axis). Colours stand for the taxonomic class of the organism which retrieved the best hit at the Uniprot database with the identified polypeptides.

PCP at a concentration near the $EC_{50}$ (third day of incubation) greatly increased proteins associated to Stress Response, Mitochondrial Functioning, Carbohydrate Metabolism, Amino Acid Metabolism, and ATP Metabolism. In all these up-regulated functional categories, most of the identified proteins were from Eurotiomycetes (especially in Carbohydrate Metabolism), followed by Saccharomycetes, Schizosaccharomycetes or Sordariomycetes. At the tenth day of incubation nearly 70% of the biocide was depleted from the media (Fig. IV.2); however the GO categories increased by PCP were similar in both time points even if their protein taxonomy differs: Sordariomycetes proteins dominate and many Eurotiomycetes proteins were lost at the last time point. After depletion of most PCP, the most affected GO categories - cumulative $log_2FC > 50$ - compared to control were Regulation, Translation and Signalling, especially in Sordariomycetes.

The most down-regulated GO category in the presence of PCP (indifferently of the time-point) was the Carbohydrate Metabolism, comprising proteins from Sordariomycetes and from other less abundant taxonomic classes. The other PCP down-regulated GO categories were similar to those up-regulated by PCP: Regulation, Translation and Signalling, Stress Response, and Mitochondrial Functioning. The taxonomic class that clearly dominated the PCP down-regulated events was Sordariomycetes; especially seen in the last time-point where the contribution of proteins from Schizosaccharomycetes, Eurotiomycetes and other less abundant taxonomic classes, was significantly reduced.

The major up- and down-regulated proteins at each time point are depicted in the Tables IV.1 and Table IV.2, respectively, naturally matching the GO categories which were found more dysregulated by PCP compared to control conditions. Regardless that the formation of PCP degradation metabolites by the metacommunity of fungi (Fig. IV.3) could potentially involve cytochrome P-450 monooxygenases, tyrosinases, reductive dehalogenases and transferases [11], in the present study none of the identified differential proteins could be linked to PCP degradation.

PCP is known to uncouple oxidative-phosphorylation in mitochondria [30], consequently to increase the need for energy production often leading to activation of tricarboxylic acid (TCA) cycle [31]. PCP exposure was observed to activate glycolysis through the up-regulation of many glycolytic enzymes in *Mucor plumbeus* [23]. In the present study, several glycolytic enzymes of the metacommunity of fungi were observed to be greatly affected by PCP presence at the first time point: aldehyde dehydrogenase increased; phosphoglycerate kinase and pyruvate kinase decreased; whereas enolases and glyceraldehyde-3-phosphate dehydrogenases were found both increased and decreased (Table IV.1). At the tenth day of incubation, when nearly 70% of PCP was depleted from the media, glycolysis was still greatly dysregulated compared to control conditions, seen in the major up- and down-regulation of glyceraldehyde-3-phosphate

dehydrogenases, and the major down-regulation of phosphoglycerate kinase and enolases (Table IV.2).

**Table IV.1** – Major up- and down-regulated proteins at the third day of growth of the metacommunity of fungi in media supplemented to PCP compared to negative control conditions

| Functional category | Accession | Protein name | $log_2FC$ |
|---|---|---|---|
| **Up-regulated** | | | |
| Amino Acid metabolism | Q12650 | Sulfate adenylyltransferase | **7,16** |
| ATP metabolic process | P22068 | ATP synthase subunit β | **9,36** |
| | P85446 | ATP synthase subunit β | **9,06** |
| Carbohydrate metabolic process | P41751 | Aldehyde dehydrogenase | **7,42** |
| | Q12560 | Enolase | **7,89** |
| Mitochondrial functioning | C8VG90 | Aconitate hydratase | **7,25** |
| Protein biosynthesis | Q00251 | Elongation factor 1-alpha | **7,84** |
| Regulation, translation andsignalling | B6H2I7 | 40S ribosomal protein S0 | **7,57** |
| Stress response | P08843 | Alcohol dehydrogenase | **8,50** |
| | G5EAZ2 | Thiamine thiazole synthase | **7,25** |
| **Down-regulated** | | | |
| Carbohydrate metabolic process | Q7RV85 | Enolase | **-8,55** |
| | Q6W3C0 | Enolase | **-8,15** |
| | Q00640 | Glyceraldehyde-3-phosphate dehydrogenase | **-8,58** |
| | P35143 | Glyceraldehyde-3-phosphate dehydrogenase | **-8,15** |
| | P41756 | Phosphoglycerate kinase | **-7,44** |
| | P31865 | Pyruvate kinase | **-8,04** |
| Cytoskeleton | Q6TCF2 | Actin | **-8,92** |
| Mitochondrial functioning | P24487 | ATP synthase subunit alpha, mitochondrial | **-8,15** |
| Regulation, translation and signalling | B0XWG9 | 40S ribosomal protein S0 | **-7,32** |
| Stress response | P78695 | 78 kDa glucose-regulated protein homolog | **-7,75** |
| | P41797 | Heat shock protein SSA1 | **-7,70** |

**Table IV.2** – Major up- and down-regulated proteins at the tenth day of growth of the metacommunity of fungi in media supplemented to PCP compared to negative control conditions

| Functional category | Accession | Protein name | $log_2FC$ |
|---|---|---|---|
| **Up-regulated** | | | |
| Amino Acid metabolism | P05694 | 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase | **6,43** |
| | Q9HFR6 | NADP-specific glutamate dehydrogenase | **6,57** |
| ATP metabolic process | P07038 | Plasma membrane ATPase | **8,45** |
| | P28876 | Plasma membrane ATPase 2 | **7,49** |
| Carbohydrate metabolic process | Q00640 | Glyceraldehyde-3-phosphate dehydrogenase | **8,22** |
| | P32636 | Glyceraldehyde-3-phosphate dehydrogenase 2 | **7,10** |
| Mitochondrial functioning | P24487 | ATP synthase subunit alpha, mitochondrial | **8,45** |
| | P51044 | Citrate synthase, mitochondrial | **7,49** |
| Protein biosynthesis | P34825 | Elongation factor 1-α | **8,30** |
| | Q9Y713 | Elongation factor 1-α | **7,10** |
| Regulation, translation and signalling | Q6FR65 | GTP-binding nuclear protein GSP1/Ran | **7,01** |
| | C7YTD6 | 40S ribosomal protein S1 | **7,19** |
| Stress response | Q5B2V1 | Heat shock 70 kDa protein | **9,20** |
| | P41797 | Heat shock protein SSA1 | **7,62** |
| | C7Z8P6 | Thiamine thiazole synthase | **7,49** |
| **Down-regulated** | | | |
| Amino Acid metabolism | P00369 | NADP-specific glutamate dehydrogenase | **-7,01** |
| Carbohydrate metabolic process | Q7RV85 | Enolase | **-9,67** |
| | P54118 | Glyceraldehyde-3-phosphate dehydrogenase | **-8,44** |
| | P87197 | Glyceraldehyde-3-phosphate dehydrogenase | **-7,92** |
| | Q9HGY7 | Glyceraldehyde-3-phosphatedehydrogenase | **-7,73** |
| | P14228 | Phosphoglycerate kinase | **-7,88** |
| Mitochondrial functioning | 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 signalling | Q01291 | 40S ribosomal protein S0 | **-7,83** |
| Stress response | J9N5G7 | Thiamine thiazole synthase | **-8,35** |
| | P23617 | Thiamine thiazole synthase | **-7,68** |

PCP primary toxic effect is to uncouple oxidative-phosphorylation in mitochondria, accordingly PCP exposure dysregulated the levels of several mitochondrial proteins in the proteome of the metacommunity of fungi compared to control conditions, *e.g.* aconitate hydratase as well as ATP synthase subunit alpha and a citrate synthase (Table IV.1 and IV.2). As expected, PCP induced stress responses, *e.g.* major up-regulation of Heat Shock proteins, namely P70 and SSA1, and of thiamine thiazole synthases, the last two proteins were found also greatly decreased. Heat shock proteins, which function essentially as molecular chaperones, assisting the correct folding of native and stress accumulated misfolded proteins, are usually induced under a variety of stress conditions [32, 33]. In particular, the HSP70 has been reported to be up-regulated by PCP exposure in *Mucor plumbeus* [23] and by alachlor in *Paecilomyces marquandii* [34]. The protein thiamine thiazole synthase has been linked to adaptation to various stress conditions and in DNA damage tolerance [35].

*Community level physiological profile (CLPP)*

As mentioned above the most affected functional category at both time points was the Carbohydrate Metabolism. To better understand how carbon metabolism in the metacommunity of fungi was affected by PCP we resorted to CLPPs analyses that specifically analyse its capacity to metabolise 95 different carbon sources. The CLPPs analysis showed that PCP altered neither the functional diversity (Shannon index, *H'*) nor the richness (*i.e.* number of substrates used) of the metacommunity of fungi compared to control conditions, *viz.* 4.36 (± 0.06) < *H'* < 4.34 (± 0.01); 92.33 (± 0.577) < richness < 91.67 (± 0.577). When each substrate class is individually considered in the CLPP analysis, the capacity of the metacommunity of fungi to use as carbon source the Carbohydrates was greatly hindered by PCP at both times points (Fig. IV.5). On the contrary, the usage of substrates in the groups of Amino-acids and Miscellaneous was

increased when the metacommunity of fungi was exposed to PCP compared to control. The sources responsible for the increased usage observed in the Miscellaneous group, are the N containing compounds adenosine, adenosine-5'monophosphate and uridine (Table IV.S4 in supplementary information). Together, the proteomics data and the carbon and nitrogen utilization profiles, support that PCP exposure impaired the capacity of the metacommunity of fungi to utilize carbohydrate substrates hence shifting to the use of substrates containing nitrogen.

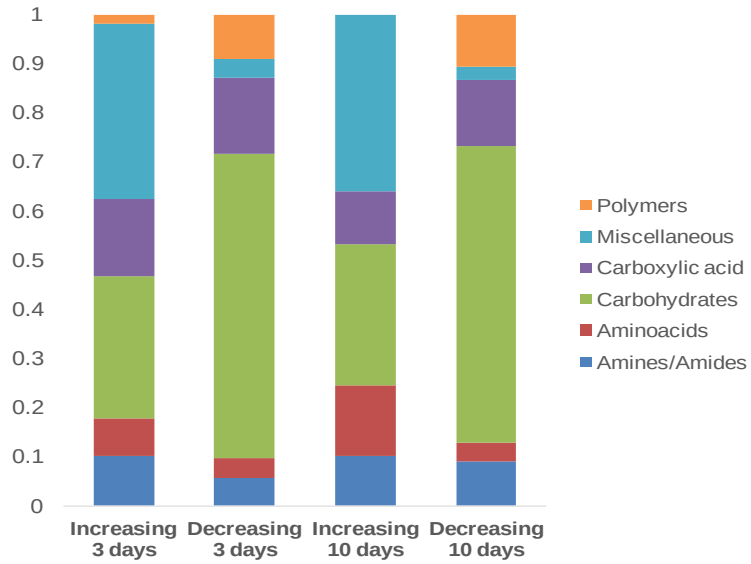



**Fig. IV.5** – Community level physiological profile (CLPP) demonstrating the capacity of the metacommunity of fungi to utilise 95 different substrates when exposed to PCP compared to negative control conditions. Decreased and increased utilization capacities are shown separately for the third day and tenth day of exposure to PCP.

## Conclusion

PCP greatly influenced the functioning of a metacommunity of fungi, altering along cultivation the levels of many mycelial proteins compared to control conditions. Most of the functional alterations that were detected at the first time point were still discerned when nearly 70% of the biocide was depleted from the media. One of the most striking observations is that the taxonomy of the identified proteins, either increased or decreased by PCP, changed

along the incubation time. This observation suggests that at different levels, PCP (together with a distinct signature of PCP-derived metabolites) affected different metabolic pathways in different taxa or altered the composition of the metacommunity. In the near future, it will be critical to address how the taxonomic diversity of the fungal metacommunity responds during PCP mitigation, especially to link the differential identified proteins with PCP degradation, detoxification and specialisation events.

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## CHAPTER V

**The final discussion contains data of the previous chapters as well as from the following publications of the author:**

F.J. Deive, A. Rodríguez, **A. Varela**, C. Rodrígues, M.C. Leitão, J.A.M.P. Houbraken, A.B. Pereiro, M.A. Longo, M.A. Sanromán, J.M.S.S. Esperança, R.A. Samson, L.P.N. Rebelo, C. Silva Pereira, Impact of ionic liquids on extreme microbial biotypes from soil. Green Chemistry. 2011, 3: 687-696 doi: 10.1039/C0GC00369G.

I. McLellan, A. Hursthouse, **A. Varela** and C. Silva Pereira, Geochemical approach to assessing human impacts in Cork Oak forest soils of the MED region. Journal of Geochemical Exploration. 2013; 132: 34-40. doi: 10.1016/j.gexplo.2013.04.005.

I. McLellan, **A. Varela**, M. Blahgen, M.D. Fumi, A. Hassen, N. Hechminet, A. Jaouani, A. Khessairi, K. Lyamlouli, H.I. Ouzari, V. Mazzoleni, E. Novelli, A. Pintus, C. Rodrigues, P. Angelo Ruiu, C. Silva Pereira and A. Hursthouse, Harmonisation of physical and chemical methods for soil management in Cork Oak forests - Lessons from collaborative investigations. 2013. African Journal of Environmental Science and Technology. doi: 10.5897/AJEST12.101

I. Martins, H. Garcia, **A. Varela**, S. Planchon, J. Renaut, L.P.N. Rebelo and C. Silva Pereira. Investigating *Aspergillus nidulans* secretome during colonisation of cork cell walls. Journal of Proteomics, 2014, 98: 175–188. doi: 10.1016/j.jprot.2013.11.023.

I. Martins, **A. Varela**, L.M.T. Frija, M.A.S. Estevão, S. Planchon, J. Renaut, C.A.M. Afonso and C. Silva Pereira, Proteomic Insights on the Metabolism of *Penicillium janczewskii* during the Biotransformation of the Plant Terpenoid Labdanolic Acid, Frontiers in Bioengineering and Biotechnology 2017, 5: 45. doi: 10.3389/fbioe.2017.00045.

The sustainability of Soils is fundamental to the functioning of all land ecosystems; however largely unnoticed despite of progressively threatened due to exposure to diverse man-made chemicals, globally. Cork oak forests - key habitats in the Mediterranean region - are of critical economic, social and ecologic value. As described in Chapter I, these managed forests are under increasing stress provoked by climate alterations and environmental pollution as well as variation in management practice. This doctoral thesis includes results from a NATO Science *for* Peace project that aimed to establish as proof-of-concept that cork oak forests were contaminated with pentachlorophenol (PCP) to disclose how the biocide impacts the forest ecosystem and to propose adequate mitigation solutions. The core studies were continuously supported by several side-studies focussing optimisation and development of the methods used to collect the forest soils [1] and to undertake their physicochemical characterisation [1, 2] while safeguarding the harmonisation of the selected methods between different research teams [3]. In addition, studies (see below) focussing analysis of complex fungal proteomes in single cultures of both reference and environmental strains have been undertaken [4, 5].

To discuss in an integrated way the results presented in the previous chapters and the abovementioned side-studies, I will organize this chapter into five topics as follows: *Soil sampling*; *Physicochemical analysis of soils*; *PCP contamination in soils*, *PCP degradation pathway of belowground fungi;* and *Analysis of soils' functioning*.

***Soil sampling.*** The soil samples were collected in three selected forest locations in the regions of Tabarka (Tunisia) and/or Sardinia (Italy) (Chapter II, III and IV). The soil sampling plan was tested and optimised first in a study that focussed the impact of ionic liquids – potential emergent pollutants – on extreme microbial biotypes from soil [1]. Sampling consisted of collecting five individual soil samples in quadrant of 1×1 $m^2$, using a 3 cm diameter gauge auger at a single depth: 0–20 cm; which were

then pooled. Each soil sample comprises the pool of a given quadrant. Using this sampling plan, reproducible chemical and physical characterisation data were attained. In this opening study, our goal was to identify, amongst extreme soil biotypes at locations of high salinity and high hydrocarbon load, microbial strains able to survive short or long-term exposure to the presence of selected ionic liquids [1]. The surviving microbial strains were isolated and taxonomically identified, and the ionic liquid degradation was analysed during cultivation. The continuing exposure of the microbial strains to petroleum hydrocarbons is likely to be the basis for their acquired resistance to some imidazolium salts. The higher capacity of fungi - relative to bacteria - to grow during exposure to the tested ionic liquids, became evident in this study [1]. This observation reinforced also the initial hypothesis of the thesis: *belowground fungi act as major players in the mitigation of pollutants prevalent in the soil matrix*. This study on ionic liquids was also important to learn and validate additional methodologies, including the isolation of cultivable fungi from the soils, the enumeration of their colony forming units (cfu's) and subsequently the taxonomic characterisation of the isolated fungal strains. I had never worked with fungi before the start of my doctoral studies, so the challenge of isolating fungi, characterise the morphological features of their colonies and produce spore suspensions for preservation of the isolates was significant. The taxonomic characterisation was undertaken mostly by collaborators in CBS, The Netherlands.

***Physicochemical analysis of soils.*** During the experimental course of the thesis, the soils sampled inside cork oak forests located in Tunisia and/or Sardinia were distributed among the five NATO S*f*P project partners. One of the first tasks of the project was to ensure that the physicochemical methods used to characterise the sampled soils support generation of reproducible datasets. My previous training in soil chemistry was extremely valuable to suggest the means to improve the harmonisation of methods.

In brief, each soil sample was initially characterised using standard methods, namely: humidity; pH (ISO 10390:1994); organic nitrogen; organic carbon (for wet and dry samples by the Walkley-Black method and the European Standard ISO 12879:2000, respectively); and particle size analysis (PSA). Taken as an example, the use of MilliQ water was applied to determine the pH of the sampled soils, reducing variability in the datasets generated by the different teams. High variability was also initially observed in the analysis of the organic carbon by the Walkley-Black method; associated to the use of phosphoric acid to improve the yield of hydrolysis of the organic matter by one of the partners. Extensive datasets on the physicochemical characterisation of the soil matrices (sampling operations in 2007, 2009 and 2010) were included in the final report of the Nato S*f*P project (data not show) to demonstrate that the rigours application of standardised methods support the generation of highly reproducible datasets across the different teams [3].

An additional side-study included the evaluation of geochemical methods to assess the potentially toxic element (PTE) input to field sites from *Quercus suber* forests located in the region of Tabarka, Tunisia [2]. The major conclusion of this study was that despite intensive human interaction and varied forest management practice, limited impact from PTE on cork oak forest soils was observed.

***PCP contamination in soils*.** As presented in the Chapter I, PCP was chosen as a model pollutant for our studies. We have foreseen that the soils of cork oak forests located in Tunisia were under a high probable risk of PCP pollution, because PCP and its degradation metabolites were found prevalent in the resultant cork (*personal communication by local industrials*). These forests also showed additional features that are of risk *e.g.* weak management, high vegetation density and lack of adequate forest sanitation [2]. At the time these studies were initiated, PCP was regarded only as a critical pollutant, yet recently it become recognised as persistent

organic pollutant (Chapter I, [6]). One of the initial challenges of the thesis was to ensure that PCP could be recovered from soils using a standard robust method. This is not trivial, especially as the soil matrix is highly complex and heterogeneous, where several pollutants potentially coexist, and is usually enriched in organic compounds that bind to PCP potentially hindering its extraction. In Chapter II, the development of a simple, robust method for chlorophenol analysis in soils is presented [7]. The method consists on a fast solvent extraction aid by sonication cycles, which leads to recoveries consistently >75 % against a certified reference soil for pentachlorophenol (CRM 141R, Community Bureau of Reference, ERM-CC008). The HPLC-UV optimised detection method, with ultraviolet (diode array) detection, is able to separate and quantify 16 different chlorophenols at field concentrations greater than the limits of detection ranging from 6.5 to 191.3 $\mu g \cdot Kg^{-1}$ (dry weight) [7]. In the specific case of PCP, the detection limit was defined as 11.4 $\mu g \cdot Kg^{-1}$ and the quantification limit as 38 $\mu g \cdot Kg^{-1}$. At this stage, the samples tested which originated either from Tunisia or Sardinia (Chapter II) were considered not polluted by PCP.

In Chapter III, the prevalence of PCP at levels from 1.7 to 29 $\mu g \cdot Kg^{-1}$ in soils which originated from Tunisia was demonstrated [8], opposing to that mentioned in our first study (Chapter II). In fact, soil contamination with PCP was detected at the three forest locations analysed in Tunisia yet Aîn Hamraia ($AH_n$) forest soils contained consistently the highest PCP levels (Chapter III, Table SIII.1, Table III.4). As above-mentioned, the levels detected in $AH_n$ soils – > 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 [9] but much lower than found in the vicinities of wood-mill and storage locations (*e.g.* [10]). The discrepancy observed in the two different datasets on PCP levels in soil samples which originated from the same location (Chapter II and Chapter III) may be associated to different factors. First, the soil samples analysed in Chapter II were processed by the UK team, where a mandatory quarantine of fifteen days at

-80°C is applied to any biological sample arriving in the country. Secondly, the soil samples were air-dried before PCP extraction, which can potentially volatise some of the biocide. On the contrary, the soil samples analysed in Chapter III – tests done in our lab – were kept always at 4°C in the dark and PCP was extracted directly from the humid soil samples. Furthermore, PCP detection/quantification in the ensuing extracts was done by UHPLC-ESI-HRMS and not by HPLC-UV. The detection threshold of the optimised HPLC-UV method (Chapter II) would support detection of PCP in the samples which originated from $AH_n$ and Fej Errih (FER) soils, but not from Ras Rajel (RR) soils (Table V.I). However, none of the samples which originated from Tunisia contain PCP levels which would allow their quantification by the HPLC-UV established method. One cannot disregard that the source of variability in the PCP contamination levels established in the different studies, may also reflect the natural variability of the sampled soils at the same location.

**Table V.I.** Prevalence of PCP and related compounds identified in soils which originated from cork oak forests located in Tabarka (Tunisia) or in Sardinia (Italy). The soil extracts were analysed by UHPLC-ESI-HRMS (see Chapter III for details).

| Compound | Abbrev. | Soil Samples | | | | | |
|---|---|---|---|---|---|---|---|
| | | $ST_1$ | $ST_2$ | $ST_3$ | AH | FER | RR |
| Dichlorotrihydroxybenzene | DCTHB | – | – | – | ✓ | – | – |
| Dichlorometoxyphenol | DCMP | – | – | ✓ | ✓ | – | – |
| Trichlorophenol | TCP | ✓ | – | ✓ | ✓ | ✓ | ✓ |
| Tetrachlorophenol | TeCP | – | – | – | ✓ | ✓ | – |
| Drosophilin A | Dro A | – | – | ✓ | – | ✓ | ✓ |
| Tetrachloro-*o*-guaicol | TeC-*o*-G | – | – | – | – | ✓ | – |
| Pentachlorophenol ($\mu g \cdot Kg^{-1}$) | PCP | 7-9 | 4-18 | 6-12 | 13-29 | 4-15 | 2-7 |
| Pentachlorobenzene | PeCB | – | – | – | ✓ | – | – |

ST, Forest Stations in Sardinia: GPS North [40°54′53′′] to [40°54′48′′]; AH, FER and RR, Forests in Tabarka: GPS North [36°46′47′′] to [36°57′15′′]

As discussed throughout Chapter III, the prevalence of PCP in the cork oak forest soils which originated from Tunisia may account for different contributory factors: i) Tunisian legislation is not very prohibitive [6]; ii) forest management practices in this region are, or have recently been, making use of this biocide or its precursors; and iii) deposition of PCP in the atmosphere [11, 12] (or of PCP precursors, such as pentachlorobenzene, PeCB, [13]). The deposition of PCP in the atmosphere is likely behind its prevalence also in soils which originated from Sardinia forests (Table V.I) which are under high-standard management practice and the severe European legislation on PCP. The demonstration of PCP contamination in Sardinian soils has not yet been published as this dataset is still lacking validation through new experimental trials and sampling campaigns. In conclusion, the high prevalence of PCP in Tunisian forests - associated to yet undefined active sources of PCP - reinforces the wide-ranging principle of PCP global and dispersed environmental pollution. Lastly, one cannot ignore the presence of PeCB in samples which originated from $AH_3$ soils (Chapter III, Table III.4), which can yield PCP either biotically or abiotically [13]; its presence suggests that multiple sources of soil contamination with PCP may coexist.

***PCP degradation pathway of belowground fungi.*** Fungi are key colonisers of soil; however their functional role as a community in the mitigation of soil pollution remains poorly investigated. To lead discovery, we first analyse the PCP degradation capacity of each of the cultivable strains within the belowground fungal community (Chapter III). As established in our study on the response of extreme soil microbial biotypes to ionic liquids [1], the fungal community was recovered without any enrichment from the soils, and the cultivable fungal strains were isolated using a straightforward approach (Chapter III). One of our early suppositions was the expectation of finding a high prevalence of Ascomycota fungi, especially of *Penicillium* spp. in the polluted forest soils, similar to that reported by others in previous studies [11, 14, 15]. In total, we isolated 77 strains that covered 33 species

groups, with *Penicillium* species (52 strains) as the dominant taxa (Chapter III, Table III.1). Most of these strains could degrade, to some extent, PCP (53 out of 77) when grown in a standard mineral liquid media containing distinct levels of PCP. To pursuit the hypothesis that the degradation pathway of PCP by the fungal community integrates the input of each of the composing strains, we have solved the ensuing PCP-derived metabolomes (by UHPLC-ESI-HRMS) of each strain (submerged cultures) focussing only on the extracellular derivatives. We have identified an array of degradation intermediates and by-products, including several resorcinol (R), hydroquinone (HQ), and catechol (C) derivatives, either chlorinated or not, some of which were yet uncharacterized, *e.g.* tetrachloroguaiacol isomers (Chapter III, Fig. III.2). The most remarkable discovery was the identification of the resorcinol branch which has never been reported before either in fungi or bacteria. The tetrachlorinated derivatives of PCP, specifically the tetrachlororesorcinol (TeCR) and tetrachloroguaiacol (TeCG) which cannot be linked to PCP degradation by bacteria, provided us with a unexpected tool for monitoring PCP degradation by fungi in other soils. Their presence in the soils which originated from Tunisia (and from Sardinia, Table V.1) supports a major participation of fungi in the degradation of PCP (Chapter III, Table III.4). In the specific case of $AH_n$ soils, we could only identify tetrachlorophenol (TeCP), trichlorophenol (TCP) and dichlorotrihydroxybenzene (DCTHB) that supports that the soil microbial community is actively degrading PCP, most likely involving fungi as well as bacteria once TCP implies bacterial degradation of PCP through reductive dichlorination [16], or, alternatively, an (in)direct soil contamination source for TCP.

Community-based cultures (*i.e.* the growth media was inoculated with the fungal community recovered from the soils) showed that the capacity of the $AH_n$ fungal communities to degrade the biocide was in average lower than observed in the axenic fungal cultures (Chapter III, Fig. III.1); notwithstanding similar diversity of PCP degradation intermediates was

observed (Chapter III, Table III.3). The identification of trihydroxybenzene (THB) in these cultures suggests that the used conditions supported the growth of strains that mineralize PCP. At a particular PCP concentration, differences in the diversity of cultivable fungal strains in the different $AH_n$ soils have not resulted into distinct PCP removal capacities, neither in different catabolic richness and diversity (Chapter III, Fig. III.1). As final remark, as PCP concentration increases, the number/diversity of PCP-degrading strains decreases whereas both the catabolic richness and diversity of the community increases (Chapter III, Fig. III.1). The last observation is suggestive that PCP induces specialization of the fungal community. Therefore, simulations using submerged cultures may provide us the means to unveil PCP-derived specialisation events of fungal communities.

***Analysis of soils' functioning.*** At this stage of our research, it become clear the prevalence of PCP in the forest soils which originated from Tunisia as well as the broad capacity of the fungal strains composing the cultivable community of $AH_n$ soils to degrade PCP. To analyse how PCP impacts the functioning of the belowground fungal community, the first challenge was to design a submerged cultivation system where PCP degradation by the community would equal that of the best axenic fungal degraders. Taken 38 µM PCP as an example ($EC_{50}$ value of PCP against the $AH_n$ fungal community, Chapter IV, Fig. IV.I), the average degrading capacity of the axenic fungal cultures (two weeks) was nearly 60% yet only 30 % in the community-based cultures (Chapter III, Fig. III.1A-B). As discussed in Chapter IV, microbial communities are constituted by sub-communities, *i.e.* local communities, that often comprise the same dominant strains yet a distinct composition of the less abundant strains [17]. Seeking the random establishment of diverse local communities, after inoculation of the growth media with the community inoculum, the seed culture was distributed through numerous growth containers, which were pooled at the end of the

experiment. Using this strategy - where the three $AH_n$ communities were also pooled to inoculate the seed culture – 38 µM PCP were efficiently degraded: PCP decay values ranged from 1.3 % to 69.1 % at the third and tenth day of incubation, respectively (Chapter IV, Fig. IV.2). This observation supports that a AH metacommunity of fungi was possibly formed. We have resorted to our gold-standard metabolomic approach to analyse the PCP degradation pathway of the metacommunity of fungi. The PCP derivatives detected along the incubation time, either extracellularly or in the mycelial fraction (Chapter IV, Fig. IV.3) included compounds of the three branches of the fungal PCP degradation pathway (Chapter III, Fig. III.2). Only one compound could be directly linked to the resorcinol branch, suggestive that this branch may be less represented in the metacommunity of fungi. Alternatively, the abiotic formation of tetrachlorinated derivatives of PCP might sustain higher demand of the downstream HQ and C branches compared to the R branch which is preceded only by biotic steps.

Next, to understand how PCP impacts the functioning of the metacommunity of fungi, an experimental proteomic workflow for analysing the mycelial proteome and/or the secretome (*i.e.* extracellular proteome) was defined. We have first implemented studies focussing the proteome of fungi grown axenically in media supplemented either with recalcitrant plant substrates (wood and cork) [4] or a plant terpenoid [5]. In both studies we have used differential two-dimensional gel electrophoresis (2DE). In the first study, we have identified the proteins up-regulated in the secretome of *Aspergillus nidulans* grown on cork compared to wood, aiming to find suberin degrading enzymes; suberin composes nearly 50 % of cork but is absent in wood. The study comprised proteomic assays, microscopic and spectroscopic evaluation of the colonised substrate and targeted MS analysis of lignin degradation compounds. Overall, the data showed that the fungus was able to form a complex network of hyphae around the cork cell walls, which enabled polysaccharides and lignin superficial degradation, but probably not of suberin. Degradation of polysaccharides and of lignin was

suggested by the identification of few polysaccharide degrading enzymes, and by identification of small aromatic compounds (*e.g.* cinnamic acid and veratrylaldehyde) and of several putative high molecular weight lignin degradation products, respectively. The differential 2DE analysis revealed that *A. nidulans* colonisation of cork and wood share a common set of enzymatic mechanisms.

This study allowed me to learn proteomic techniques and data analysis tools that I later used in the thesis (Chapter IV). We used a similar experimental 2DE approach (comprising mycelial and extracellular proteomes) to study the metabolism of *Penicillium janczewskii* during the biotransformation of labdanolic acid - terpenoid found abundantly in *Cistus ladanife*r [5]. In a previous study, our team demonstrated that this environmental fungal strain (which was isolated from cork slabs [18, 19]) was able to mediate a stereo-selective hydroxylation of labdanolic acid producing 3β-hydroxy-labdanolic acid with yields > 90 % [20]. The proteomic analysis provided a snap-shot view of major alterations provoked by the supplementation of the growth media with labdanolic acid compared to control conditions. Only one putative cytochrome P450 monooxygenase could be preliminary associated with the hydroxylation of labdanolic acid; observation of *in vivo* inhibition of the hydroxylation in the presence of a P450 inhibitor reinforces that this reaction involves a P450 enzyme.

In these initial studies we have established optimal protocols for the extraction of proteins from fungal cultures which contain many interfering phenolic compounds (from the mycelial or the plant substrates) [21]. Our prediction was that similar extraction protocols could be applied with minor modification to recover the proteome of cultures inoculated with AH metacommunity of fungi. In the second study, regardless that the genome of *P. janczewskii* was still unsequenced, a large fraction of the differential protein spots in either sub-proteome matched protein species annotated in *Penicillium* species already sequenced (*e.g. P. chysosporium, P. digitatum*

and *P. marneffei*) [22]. Most of the remaining protein identifications were matched against sequences from *Aspergillus* species. This supported that we could safely advance to analyse the differential proteomic response of the metacommunity of fungi irrespective that the genome of most of the composing strains is still unsequenced (Chapter IV).

When cultures of AH metacommunity of fungi were exposed to PCP the levels of mycelial proteins associated to functional categories related to Stress Response, Mitochondrial Functioning, Carbohydrate Metabolic Processes and Amino Acid Metabolism, among others, were significantly altered (either increased or decreased) (Chapter IV, Fig. IV.4). None of the differential mycelial proteins herein identified could be linked to PCP degradation. Furthermore, the major up-regulated and down-regulated proteins (Chapter IV, Tables IV.1 and IV.2) are, in general, comprised in the most affected functional categories. Importantly, the proteomic data made apparent that PCP impacts were still felt in the metacommunity of fungi after major PCP depletion. The most striking observation is that the taxonomy of the identified proteins, either increased or decreased by PCP, changed along the incubation time. As discussed in Chapter IV, this observation suggests that dissimilar PCP levels (and of the derived metabolites) impact differently fungi belonging to distinct taxa. One of the most affected functional categories was the Carbohydrate Metabolism, of which many more proteins were down-regulated than up-regulated at both time points. The Community Level Physiological Profile (CLPPs) analysis, showed that PCP altered neither functional diversity (Shannon index, *H'*) nor the richness (*i.e.* number of substrates used) of the AH metacommunity of fungi compared to control conditions. A similar behaviour was observed in the community-based assays, *i.e.* single cultivation container of each $AH_n$ community (Chapter III, Fig. III.1C and D). The CLPP data of the AH metacommunity of fungi was extremely heterogeneous when each substrate is individually considered (Chapter IV, Fig. IV.5), reinforcing that the effects of PCP prevail even after its major depletion. To fully understand how PCP is

impacting the AH metacommunity of fungi, more functional assays need to be undertaken, especially to reveal how PCP impacts its secretome and taxonomic diversity, crucial to correlate PCP with specialisation events.

**Future perspectives**

Logically the next set of actions will focus analyses of both the taxonomic diversity and the secretome of the metacommunity of fungi during PCP exposure compared to control conditions. Preliminary data showed that the species diversity of the metacommunity of fungi evolves differently when exposed to PCP (data not shown). One of the goals of these ongoing studies is to differentiate among the strains capable to survive PCP exposure, those which act as key PCP degraders, as well as strains which are lost under PCP exposure or that undergo critical phenotype alterations, both of which may potentially affect key ecosystem-services, *e.g.* water purification, erosion prevention, nutrient cycling, etc (reviewed in [23]).

Another aspect to explore in the near future, so far only preliminarily addressed, is to disclose interactions between the most efficient PCP degraders. We have screened preliminary the outcome of dual interactions of key fungal degraders within the cultivable community, and identify two strains, namely *Penicillium vagum* (DTO 099-F7) and *Penicillium restrictum* (DTO 099-E4), which were capable to greatly antagonise the growth of the remaining strains (Fig. V.1). The tests were done using a solid media cultivation system, which allows to pin-point antagonisms at distance and/or morphological alterations, possibly associated to production of diffusible compounds by the antagonising strain. This theme deserves further investigation to identify specific antibiosis compounds and evaluate how they influence PCP degradation.

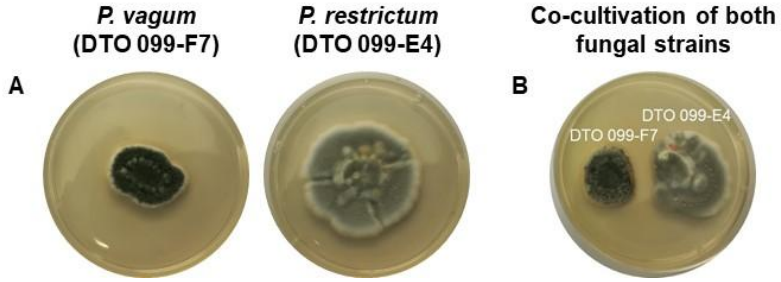


**Fig. V.1** – *Penicillium vagum* and *Penicillium restrictum* grown axenically (A) or in co-cultivation (B) in solid Malt Extract Agar medium during seven days.

Finally, one of my personal goals is to advance to cultivation systems that more closely mimic the soil environmental compartment by using soil microcosms. Initial steps were given to define conditions for handling and sterilising soils, to test the use of leaching columns, and to identify methods that allow to rapidly monitor the establishment of the metacommunity of fungi in the microcosm (ergosterol quantification, MTT assays, *etc*).

Collectively, the studies presented in the thesis have established important ground knowledge to analyse in the near future how the functioning of a metacommunity of fungi evolves during continuous exposure to PCP atmospheric deposition.

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